Mechanism and role of tethering of active genes to nuclear pores in Saccharomyces cerevisiae

Dieppois, Guennaelle

UNIVERSITÉ DE GENÈVE
Département de biologie cellulaire

FACULTÉ DES SCIENCES
Docteur Françoise Stutz

Mechanism and Role of Tethering of Active Genes to Nuclear Pores in *Saccharomyces cerevisiae*

THÈSE

présentée à la Faculté des sciences de l'Université de Genève
pour obtenir le grade de Docteur ès sciences, mention biologie

par
Guennaëlle DIEPPOIS
de
Lyon (France)

Thèse N° 4098

GENÈVE
Atelier de reprographie ReproMail
2009

UNIVERSITÉ
DE GENÈVE

FACULTÉ DES SCIENCES

Doctorat ès sciences
mention biologie

Thèse de *Madame Guennaëlle DIEPPOIS*

intitulée :

" Mechanism and Role of the Tethering of Active Genes to the Nuclear Pores in *Saccharomyces cerevisiae* "

La Faculté des sciences, sur le préavis de Madame F. STUTZ, docteure et directrice de thèse (Département de biologie cellulaire), de Messieurs D. PICARD, professeur ordinaire (Département de biologie cellulaire) et B. PALANCADE, docteur (Université de Paris Diderot, Institut Jacques Monod, Paris, France) et de Madame A. TADDEI, docteure (Institut Curie, Compartimentation et dynamique des fonctions nucléaires, Paris, France), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

Genève, le 17 juin 2009

Thèse - 4098 -

Le Doyen, Jean-Marc TRISCONE

N.B.- La thèse doit porter la déclaration précédente et remplir les conditions énumérées dans les "Informations relatives aux thèses de doctorat à l'Université de Genève".

Nombre d'exemplaires à livrer par colis séparé à la Faculté : - 4 -

Acknowledgments

I would like to express my gratitude to all those who gave me the possibility to complete this thesis.

In the first place, I want to thank my supervisor Dr. Françoise Stutz for the opportunity she offered me to work in her lab, her stimulating suggestions and her support and encouragements during all the time of research and writing.

My sincere gratitude goes to Dr. Angela Taddei and Dr. Benoît Palancade, first for accepting to review my thesis as my external experts and more particularly for the fruitful advices they gave me to accomplish this work. I would like to thank Prof. Didier Picard for reviewing my thesis and to be member of the Jury for my defense.

I am grateful to all the former and present member of the Stutz lab with who I went through all these years, for all their valuable hints, interest and support. I want to thank Jurgi, for his enthousiam and Nahid, for her kindness, patience and professionalism. A special thank to Verena and Céline for their friendship, support, and all the fun we shared. I also want to thank Céline, Audrey and Barbara, for their interest and help in this work; it was a real pleasure to guide them. I want to thank Sylvie for listening to our little problems with interest and her involvement in her work. I want to thank, Nissrine, Elisa, Evelina and Elodie for what we shared during this last year and for forming a good “Girl” team.

I would like to thank all my colleagues from the BICEL department for their help and different nice personalities. Especially, I would like to give my big special thanks to Camille and Ashwin whose priceless friendship help me a lot during difficult times and for sharing all adventures.

I would further like to give many thanks to my family, my parents, my brother and sister for their unfailing support and trust which has always been an additional source of motivation.

Finally, my last thank goes to Peter, whose patient love enabled me to complete this work and for the harmony he brought into my life.

Résumé (in French)

Le noyau est l'organelle qui contient l'information génétique et qui de ce fait est à la base de la régulation d'une cellule eucaryote. L'organisation des chromosomes au sein du noyau n'est pas aléatoire et l'incidence fonctionnelle de cette organisation sur la chromatine est importante non seulement pour la réplication de l'information génétique et sa transmission, mais aussi pour le mode de réparation de l'ADN et l'expression du génome. Le noyau est délimité par une enveloppe nucléaire au sein de laquelle on trouve les pores nucléaires qui permettent la communication entre le noyau et le cytoplasme. Le pore nucléaire est un édifice multiprotéique complexe remarquablement bien conservé au cours de l'évolution de la cellule eucaryote et il joue de multiples rôles dans la régulation de la chromatine. Les nombreuses études réalisées sur ce sujet ont principalement été menées en utilisant la levure de boulanger (*Saccharomyces cerevisiae*) comme modèle. L'étude de cet organisme eucaryote unicellulaire, facile à manipuler génétiquement, montre que beaucoup des principes qui y sont découverts se vérifient dans des organismes plus complexes. Récemment, chez *Saccharomyces cerevisiae,* il a été découvert que de nombreux gènes régulés par des changements environnementaux sont mobiles et se déplacent vers les pores nucléaires quand ils sont induits. Au cours de cette étude nous avons cherché à élucider d'une part, les mécanismes qui régissent la localisation de ces gènes aux pores nucléaires lors de leur activation transcriptionelle, et d'autre part, l'importance physiologique de cet ancrage au cours de l'expression des gènes.

Alors que plusieurs études ont décrit que les processus d'activation des gènes sont à l'origine de la localisation des gènes aux pores, nous avons découvert que le recrutement du récepteur d'export des ARNs messagers, Mex67, sur la région codante des gènes au cours de l'élongation, est essentiel pour le maintien de l'ancrage des gènes au pore. De plus, nous avons montré que l'association de Mex67 avec la chromatine au cours de la transcription n'est pas dépendant de la production d'ARN, ce qui indique que Mex67 établit un lien direct entre la machinerie de transcription et le pore nucléaire. La mise en évidence de cet ancrage, qui intervient à un moment plus tardif que l'activation transcriptionelle a contribué à changer la vision du mécanisme de recrutement des gènes activés aux pores et à l'établissement d'un nouveau modèle reposant sur l'idée que les

gènes sont maintenus aux pores nucléaires par un processus impliquant de multiples liens dont l'importance varie selon le gène et/ou en fonction des étapes sous-jacentes à l'expression d'un gène.

Ce modèle a été confirmé par d'autres études qui ont mis en évidence des facteurs additionnels d'ancrage intervenant au cours de l'élongation, de la terminaison de la transcription et même après la transcription *via* des mécanismes dépendant de l'ARN. Notre travail a également contribué à mettre en évidence qu'un défaut dans l'assemblage des ARNs en particule exportables qui empêche la maturation de leur extremité 3' et leur relâchement du site de transcription, résulte en un blocage des gènes associés au niveau du pore. Ces résultats indiquent que le pore nucléaire est associé à chaque étape de la production d'un ARN messager mettant en évidence un lien étroit entre la synthèse et la maturation des ARNs messagers avec leur export dans le cytoplasme. Ces observations indiquent aussi un rôle du pore nucléaire dans la coordination et la surveillance de la biogenèse des ARNs messagers en particules exportables.

Dans la seconde partie de cette étude nous avons découvert une nouvelle fonction du pore nucléaire dans la régulation de l'activation transcriptionelle des gènes qui sont recrutés à la péripherie du noyau. Nous avons montré qu'une délétion des protéines Mlp, deux composants du pore nucléaire, affecte les cinétiques d'activation des gènes *GAL* qui sont recrutés aux pores lors de leur induction. Notamment, en absence des Mlps, les gènes *GAL* sont activés plus rapidement par le galactose lorsqu' ils ont été préalablement réprimés en présence de glucose alors qu'ils activés plus lentement lorsqu'ils n'ont pas été en présence de glucose. Ces résultats indiquent que le pore nucléaire est impliqué dans la régulation de deux mécanismes différents au cours de l'activation des gènes *GAL*, le processus d'activation en lui-même et la répression. Au cours de la première partie de cette étude nous avons aussi observé qu'une délétion des Mlps affecte le recrutement des gènes *GAL* aux pores. De ce fait, nous avons proposé que l'effet négatif d'une délétion des Mlps sur l'activation de la transcription pouvait être le reflet d'un rôle plus général du pore favorisant l'activation de la transcription. Comme les Mlps sont au contact de nombreux facteurs de transcription, ils pourraient aussi être directement impliqués dans la régulation transcriptionelle. De plus les Mlps sont impliqués dans la régulation de la spécificité de substrats de la SUMO-protéase Ulp1, en le contrôlant de sa localisation aux

pores. Nous avons montré que le rôle des Mlps dans la régulation de la répression transcriptionelle des gènes *GAL* était strictement liée à l'activité de Ulp1.

Nous avons finalement, identifié Tup1, un facteur impliqué dans la répression des gènes *GAL*, comme un substrat de Ulp1 et montré que la désumoylation de Tup1 a un effet négatif sur la répression en glucose. Le fait que Tup1 réside sur le promoteur des gènes *GAL* qui sont recrutés aux pores nucléaires et le fait que nous que nous l'ayons trouvé en association avec Mlp2, indique qu'il peut être naturellement amené à proximité de Ulp1 dans l'environnement du pore nucléaire lors de l'activation transcriptionelle. Nous avons proposé un modèle dans lequel les protéines Mlp facilitent la dérépression de gènes recrutés à la périphérie en régulant l'activité de répresseurs par leur désumoylation.

Notre étude à permis d'éclaircir le mécanisme de recrutement de gènes aux pores nucléaires et de l'inscrire dans un modèle plus global qui illustre une connexion fonctionnelle étroite entre le pore et toute les étapes de l'expression des gènes. Ce travail a aussi fait ressortir plusieurs aspects d'un rôle direct du pore nucléaire dans le contrôle de l'expression des gènes.

Summary

The nucleus is an important membrane bound organelle which characterizes all eukaryotic cells. It contains the genetic information thus rendering it central to the function of the cell by regulating the gene expression. Chromosome organization within the nucleus is not random and the functional incidence of this organisation is important for the replication of genetic information, its transmission as well as for DNA repair and the expression of this information. The nucleus is delimited by the nuclear envelope within which the nuclear pore complexes (NPCs) are embedded, ensuring the communication between the nucleus and the cytoplasm. The NPC is a multiprotein complex which is remarkably well conserved throughout the evolution and plays multiple roles in chromatin organization. Many studies related to this topic have been performed using the baker's yeast (*Saccharomyces cerevisiae*). *Saccharomyces cerevisiae* (*S. cerevisiae*) is a very good model system as it is easy to genetically manipulate and further shows a high degree of conservation with a lot of mechanisms present in higher eukaryotes. Recently in *S. cerevisiae*, it has been discovered that a number of inducible genes are mobiles and can move towards nuclear pores when activated. In the course of this study, we aimed at elucidating on one hand the mechanisms underlying gene to pore relocalization following transcription activation, and on other hand the physiological relevance of this process in gene expression.

Although several studies described that early activation events initiate gene to pore re-localization, we found that the loading of the mRNA export receptor Mex67 on gene coding regions during transcription elongation was also essential for the anchoring of genes to pores. Moreover, we showed that the association of Mex67 with chromatin is not dependent on mRNA production, indicating that Mex67 directly links transcription machinery to the nuclear pores. The occurrence of the gene anchoring during transcription at a later stage beyond activation contributed in adopting a new model explaining the gene to pore association. The model is based on the involvement of multiple tethers, the importance of which may vary from gene to gene and along the sequence of events underlying gene expression.

This view was confirmed by other studies describing additional anchoring factors during transcription elongation, termination and even after transcription through RNA dependent mechanisms. Importantly, all these factors are involved in RNA biogenesis and export. Moreover, this work has also contributed to reveal that mRNA packaging defects

preventing correct mRNA 3’ end formation and release from the transcription site further lead to the stalling of genes at the pores. All these data indicate that the nuclear pore is functionally linked to all stages of mRNA production and highlight a tight link between messenger RNA biogenesis and export. These observations also support a role for the nuclear pore in the coordination and surveillance of mRNA packaging into particles competent for export.

In the second part of this study, we identified at a novel function of NPC in the transcriptional activation of recruited genes. We showed that a deletion of Mlp proteins, two components of the nuclear pore, impair the activation kinetics of *GAL* genes relocate to the nuclear periphery when induced. Notably, we observed that in the absence of Mlps, *GAL* genes are activated more rapidly by galactose when they have been previously repressed by glucose; in contrast, they are activated more slowly when shifted to galactose from non repressive conditions. These results indicate that the NPC is involved in two distinct aspects of *GAL* genes regulation: i.e, the activation process per se as well as the repression in glucose. In the first part of this work we also observed that loss of Mlps decreases the recruitment rate of *GAL* genes to the pores. For this reason, we proposed that the negative effect of *MLP*s deletion on transcription activation may reflect a more general role of the NPC in favoring transcription activation. In addition, since the Mlps are interacting with various transcription factors, these proteins might also directly contribute to transcriptional regulation. Another role of the Mlp proteins is to restrict the substrate specificity of Ulp1, a key sumo-protease, by maintaining this enzyme at the nuclear pores. We provided evidence that the role of the Mlps in glucose repression is strictly due to Ulp1 activity. Furthermore, we identified the transcription co-repressor Tup1, involved in *GAL* gene repression, as a target of Ulp1 and showed that Tup1 desumoylation has a negative effect on glucose repression. Tup1 binds to *GAL* gene promoters, which are recruited to nuclear pores during activation. Notably we also identified a two hybrid interaction between Tup1 and Mlp2, indicating that Tup1 may be naturally brought into the vicinity of Ulp1 within the nuclear pore environment. On the whole, our observations lead us to propose that Mlp proteins, through the action of Ulp1, participate in transcription regulation at the nuclear periphery by promoting the desumoylation of transcription repressors.

More generally, our studies extend our understandings of the gene to pore interactions and allow building a more global model illustrating tight functional connections between the different steps of gene expression. This work also provides a molecular explanation for a role of the nuclear pore in controlling gene expression.

Table of contents

1 Introduction

The nucleus was the first intracellular structure discovered in eukaryotic cells and was originally described in 1802 by Franz Bauer. The organelle attached much attention because of its fascinating and essential role in storing and organizing genetic information, protecting genome integrity and ensuring accurate fertilization, DNA replication and inheritance. This compartmentalization in eukaryotic cells provides the opportunity of an additional protection and organization level of genetic information and a fine tuned regulation of both gene expression and mRNA processing that are not available in prokaryotic cells. The major function of the cell nucleus, including transcription and pre-mRNA processing, have been extensively studied, however, in contrast, very little is known about how these processes are integrated into the structural framework of the cell nucleus and how they are spatially and temporally coordinated within the three dimensional confines of the cell nucleus. Most of the nuclear volume is occupied by the genome and it has become clear first that the chromatin in the nucleus interior is organized in several functional landmarks, and second that these domains are highly dynamic structures. Recently, major efforts have been made to relate these subcompartmentalizations to nuclear function and particularly in maintaining genome integrity and regulating gene expression. In addition to its structural function and its role in the selective regulation of the considerable nucleo-cytoplasmic traffic, a major role of the nuclear periphery and more particularly of the nuclear pore complexes (NPC) in the regulation of various chromatin related processes, gene expression and mRNA biogenesis has been established. In the following introductory chapter, I will describe the structure and the proposed function of the nuclear periphery and its NPC microenvironments.

1.1 Structure of the nuclear envelope and nuclear pore complex

The nuclear envelope (NE) is composed of highly specialized membranes, the nuclear lamina when existing, and nuclear pore complexes. The NE membrane consists of two concentric membrane bilayers, the inner nuclear membrane (INM) and outer nuclear membrane (ONM). This double membrane is punctuated by nuclear pore complexes (NPCs), at which the INM and ONM fuse. The lumen between the bilayers, known as the perinuclear space, is contiguous with the lumen of the endoplasmic reticulum (ER). The INM and ONM are each intimately associated with distinct proteinaceous structures; these connections ensure the integrity of the nuclear environment and aid in coordinating cellular events. Specifically, the INM contacts the underlying nuclear lamina when present and/or chromatin, whereas the ONM is associated with both the cytoskeletal actin network and the centrosome.

The nuclear lamina consists of a meshwork of proteins called lamins that line the inner membrane of the metazoan NE. Although it can be seen how the different lamins have evolved from invertebrates to mammals (Erber et al., 1999), it remains unclear whether analogous proteins and structures exist in fungi and plants, and it is often assumed that such organisms do not have a lamina nuclear compartment. The lamina contributes to the size, the mechanical stability, and the shape of the nucleus (Lammerding et al., 2006). The major structural proteins of the lamina are the nuclear lamins, consisting of the A-type lamins (lamin A and lamin C) and the B-type lamins (lamin B1 and lamin B2) (Krohne et al., 2005). Little information is available on the structure and the assembly state of the lamins within nuclei, although it has been proposed that they can form filamentous structures (Aebi et al., 1986; Schermelleh et al., 2008) Targeted mutagenesis or naturally occurring mutation of the lamin A disrupts the localization of lamin A and B types in nuclei (Goldman et al., 1992), suggesting that they are part of the same structure or interdependent structures. Importantly, lamina structure has been shown as important for the organization of chromatin, DNA replication, and transcription (Dechat et al., 2008).

The NE houses the large macromolecular NPCs (Figure 1) that form the sole aqueous channels spanning the NE and serve as the gatekeepers of nucleo-cytoplasmic trafficking. The number of NPCs per cell varies greatly with cell size and activity (Gorlich and Kutay, 1999). Interestingly, a comprehensive ultrastructural study in yeast cells revealed that the distribution of NPCs in the NE is not equidistant but that they rather cluster at some regions of higher density (Winey et al., 1997). Although their mass increases from yeast (60 MDa) to vertebrates (120 MDa) (Cronshaw et al., 2002; Miller and Forbes, 2000; Rout et al., 2000), nuclear pores have evolutionary conserved function and architecture (Cronshaw et al., 2002; Macara, 2001; Stoffler et al., 1999; Yang et al., 1998) (Figure 1).

The NPC has an eightfold ring shaped radial symmetry structure perpendicular to the membrane and is asymmetric with respect to the plane of the nuclear envelope. The complex is composed of three main structures, a central core, the cytoplasmic fibrils and the nuclear basket (Rout and Aitchison, 2001). Proteomic characterization of the mammalian and yeast NPC has shown that it comprises multiple copies of about 30 distinct conserved proteins, called nucleoporins and distributed either symmetrically or asymmetrically between the nuclear and the cytoplasmic faces of the pore (Allen et al., 2000; Rout et al., 2000; Stoffler et al., 1999). These proteins have been assigned to three different groups based on the presence of primary sequence and structural motifs (Devos et al., 2006; Rout et al., 2000). The first

group is predicted to contain transmembrane α-helices that might help to anchor the NPC into the nuclear envelope; the second group is believed to form the structural scaffold of the NPC and consists of nucleoporins containing β-propeller and α-solenoid domains. Importantly, the third group contains nearly half of the nucleoporins and consists of proteins with conserved stretches of phenylalanine and glycine residues (FG). They localize inside the NPC, coating the central pore surface and serve as docking sites for nucleocytoplasmic transport receptors promoting the sliding of protein complexes through the NPC (Lim et al., 2006; Rout and Aitchison, 2001; Ryan and Wente, 2000).

Within the NPC, nucleoporins are organized in distinct coherent subcomplexes (Fabre and Hurt, 1997; Fahrenkrog and Aebi, 2003; Suntharalingam and Wente, 2003). One of the best characterized subcomplexes of the NPC is the Nup84 complex in yeast (Siniossoglou et al., 1996). This assembly is composed of seven subunits (Nup133p, Nup145p-C, Nup120p, Nup85p, Nup84p, Seh1p and Sec13p) and was shown to perform several essential roles, including functions in nuclear envelope organization, NPC biogenesis, mRNA export and recently in ensuring genome integrity (Palancade et al., 2007). The nuclear basket that protrudes into the interior of the nucleus contains another interesting subcomplex: Nup60-Mlp1-Mlp2. In budding yeast, Mlp1 and Mlp2 are two non essential globular myosin-like proteins that form large filamentous proteins anchored at the basket. These two proteins are also conserved throughout evolution as they have homologues in *Xenopus*, *Arabidospis Taliana*, *Drosophila* and Human (TPR) (Cordes et al., 1997; Strambio-de-Castillia et al., 1999; Zimowska et al., 1997). They homodimerize through interaction of their N-terminal coiled-coiled domain and are attached at the NPC nuclear basket through at least, the nucleoporin Nup60 (Bangs et al., 1998; Cordes et al., 1998; Feuerbach et al., 2002; Strambio-de-Castillia et al., 1999). Interestingly, Mlp proteins are asymmetrically distributed over the nuclear envelope preferentially located in regions abutted by chromatin while excluded from the NPCs adjacent to the nucleolus (Galy et al., 2004). The precise role of these large structures is not well known. Despite the fact that they don't play an evident role in mRNA export, they interact with mRNA export factors and mRNP components and thus have been proposed to function in docking or surveillance of mRNA complexes at the pore (Galy et al., 2004; Palancade et al., 2005; Strambio-de-Castillia et al., 1999; Vinciguerra et al., 2005). In addition, Mlp mutants are impaired in cell cycle and viability (Niepel et al., 2005; Zhao et al., 2004a), in asymmetric segregation of age during yeast budding (Shcheprova et al., 2008), in telomere length control (Hediger et al., 2002a) and are sensitive to DNA damage (Palancade

et al., 2007), suggesting a broader role of Mlp proteins and the NPC in regulating other important nuclear functions.

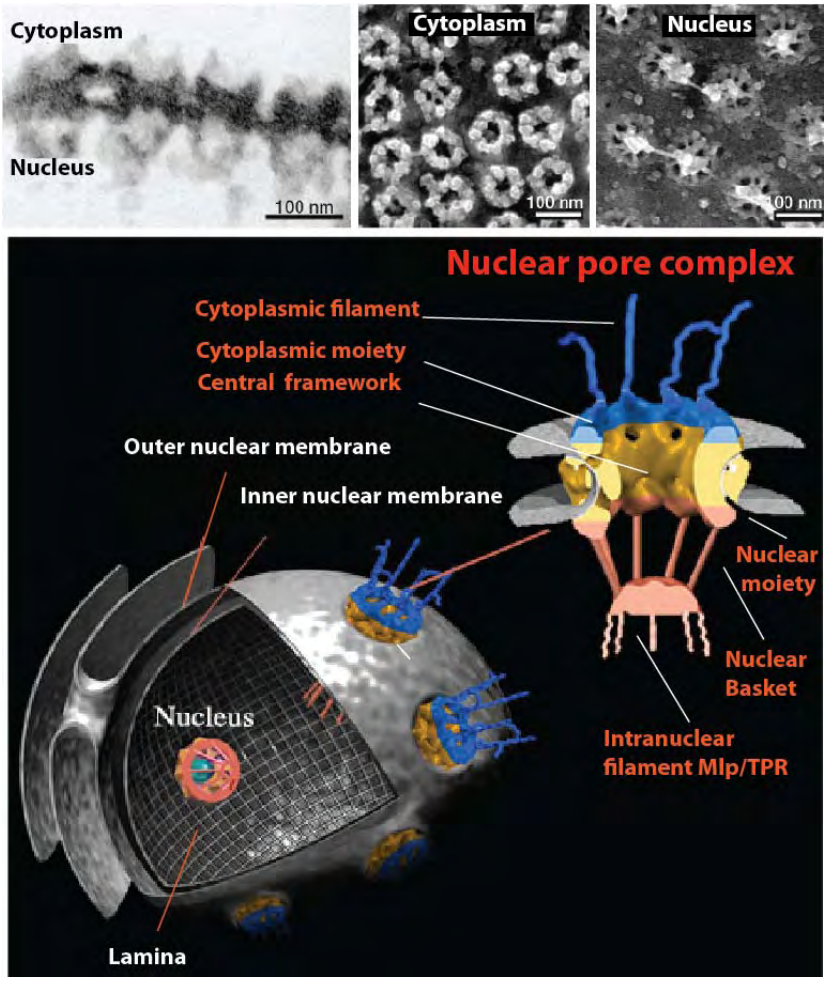


Figure1. The nuclear pore complex within the nuclear envelope. (Upper pannel) scanning elecron micrographs of a *Xenopus* oocyte envelope with from left to right: a side view of four nuclear pore complexes, the cytoplasmic face of the nuclear envelope, the nuclear face of the nuclear envelope. Pictures from (Fahrenkrog and Aebi, 2003). (Lower pannel) A shematic view of the nuclear envelope and the nuclear pore complex. Image adapted from D. Stoffler & U. Aebi M.E. Mueller-Institute for Structural Biology, Biozentrum University of Basel, Switzerland.

1.2 Nucleo-cytoplasmic transport

As the eukaryotic cell genome is encapsulated within a nucleus, the traffic that must pass through the nuclear envelope in order for the eukaryotic cell to function properly is considerable. Indeed, RNA and ribosomal subunits must be constantly transferred from the nucleus to the cytoplasm, whereas, genome packaging, gene expression and mRNA biogenesis proteins must be imported from the cytoplasm. In order for such a tremendous number of molecules to pass through the nuclear envelope in a timely manner, the NPC is highly efficient at selectively allowing the passage of materials to and from the nucleus. Although ions and solute metabolites, some intermediate macromolecules and hydrophobic small molecules can cross the NE and NPC by passive diffusion, the NE functions as a selective barrier and all macromolecular transport requires mediated, energy dependent, translocation along the NPC central axis. This active transport requires an appropriate signal: for example, proteins containing a nuclear localisation sequence (NLS) are imported, whereas those containing a nuclear export sequence (NES) are exported to the cytoplasm. Although there are several different nuclear trafficking pathways, they share a number of common features. Generally, substrates do not interact directly with NPCs, but instead are transported bound to soluble carrier transport receptors that mediate their interaction with the FG nucleoporins coating the NPC channel and which are then recycled back to the original compartment.

1.2.1 Nucleocytoplasmic transport mediated by karyopherins transport receptors.

The main class of transport receptors is designated as karyopherins or importin β-family members. This superfamilly comprises 14 members in yeast, more than 20 in higher eukaryotes, and mediate either nuclear import (also called importins) or nuclear export (also called exportins) by interacting directly, or sometimes via adaptators, with their cargoes and FG nucleoporins. Karyopherins associate with the specific localization signal sequence that provides selectivity to the transport process (NLS or NES) on their cargoes. They have been involved in the vast majority of nucleocytoplamic transport, including proteins and most classes of RNAs, transfer RNA (tRNA), ribosomal RNA (rRNA), small nucleolar RNA (snRNA) and microRNA (miRNA) (Bohnsack et al., 2004; Chook and Blobel, 2001; Gorlich et al., 1996; Gorlich and Kutay, 1999; Yi et al., 2003). The directionality of Karyopherin mediated transport is controlled by the small GTPase Ran. The binding of RanGTP to an import cargo–receptor complex lowers its affinity for the cargo, so that the cargo is released

into the nucleus. An export receptor, in turn, can only escort its cargo out of the nucleus in a complex with RanGTP. The Ran gradient in the cell in a compartimentalized manner is achieved by an asymmetric distribution of the Ran regulators, which determines the nucleotide-bound state of Ran: The Ran guanine-nucleotide exchange factor (RanGEF) is bound to chromatin in the nucleus and promotes the dissociation of GDP from Ran allowing the binding of GTP. Ran in the nucleus is therefore predominantly in its GTP-bound form. When RanGTP leaves the nucleus, the RanGTPase-activating protein (RanGAP) induces GTP hydrolysis by Ran at the cytoplasmic filaments of the NPC (Figure 2).

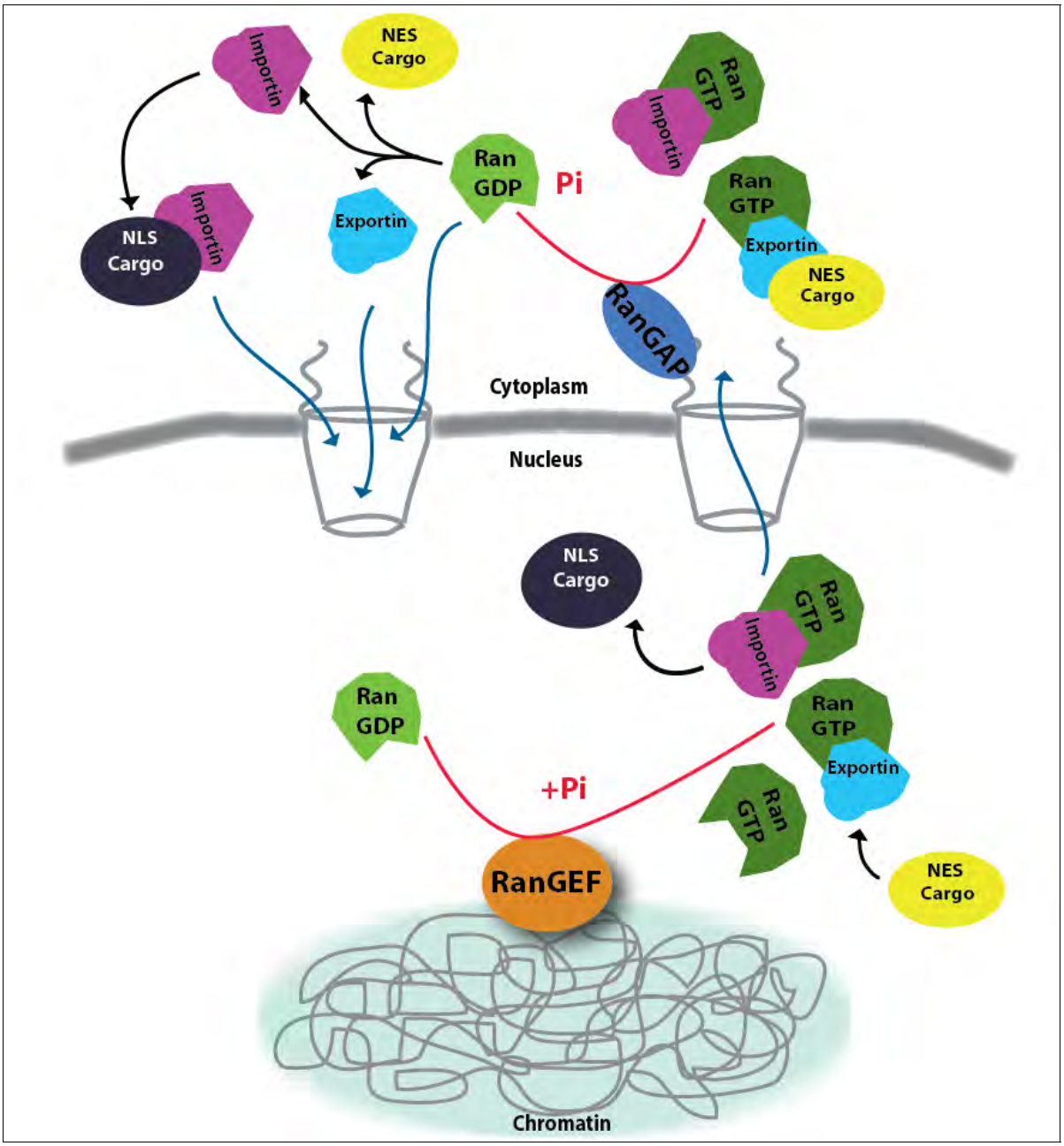


Figure 2. Scheme of import (left) and export (right) of proteins. In the case of import: in the cytoplasm, an importin binds to cargo molecules and mediates interaction with the NPC to translocate the complex into the nucleus. Once in the nucleus, RanGTP binds to importin and induces cargo release from the complex. The importin RanGTP complex is then recycled to the cytoplasm. In the cytoplasm, RanGTP is readily converted into RanGDP by the RanGAP bound to the nuclear pore complex cytoplasmic fibrils, triggering the release of the importin from Ran. RanGDP is then recycled back into the nucleus where it is converted to RanGTP by the RanGEF bound to chromatin. In the case of export: an exportin-RanGTP complex binds a cargo with an NES and the trimeric complex is then exported to the cytoplasm where RanGTP is converted to RanGDP triggering the dissociation of the complex. The exportin is then recycled back to the nucleus.

1.2.2 mRNA export

In eukaryotes, export of mRNA from the nucleus to the cytoplasm is a much more complex process than protein and other RNAs export. It requires an extensive processing of the precursor messenger RNA (pre-mRNA) generated by the transcription of a protein coding gene in order to render it competent for export through the NPC and subsequent translation into protein in the cytoplasm. Before being released to the cytoplasm the mRNA is subjected to three main processing events that include: the acquisition of a cap structure at the 5' end, splicing of introns within the body of the pre-mRNA, and the formation of a functional 3' end with the addition of a poly(A) tail after cleavage (Aguilera, 2005; Bentley, 2005; Jensen et al., 2003; Proudfoot and O'Sullivan, 2002; Saguez et al., 2005).

Furthermore, upon transcription and throughout their life time, pre-mRNAs and mRNAs are coated with numerous and distinct factors, forming ribonucleoprotein complexes (mRNPs). mRNP composition is highly dynamic and reflects the state of the mRNA during its biogenesis (Dreyfuss et al., 2002). The association of these proteins profoundly influences mRNA stability and coordinates the different mRNP biogenesis steps, through the sequential loading of factors that direct mRNA processing, export as well as downstream cytoplasmic events.

In addition, mRNP biogenesis is tightly connected to transcription. Coupling of mRNP formation to transcription occurs directly since numerous mRNP packaging factors are loaded on the nascent pre-mRNA following initial interactions with the transcription machinery. Indeed, the largest Polymerase II (RNA PolII) subunit contains a carboxyl-terminal domain (CTD) which is an unique feature of RNA PolII complexes and acts to specifically recruit mRNA processing and packaging activities to the nascent transcript (Dreyfuss et al., 2002; Proudfoot et al., 2002). This CTD consists of repeats of a heptad amino acid sequence (52 in mammals and 26 in yeast) containing two serine residues (Ser2 and Ser5) per repeat that undergo a cycle of extensive phosphorylation and dephosphorylation which is coordinated with the transcription cycle. Changes in phosphorylation patterns, as polymerase transcribes a gene, are thought to orchestrate the sequential association of the different sets of mRNA processing factors with the transcription machinery (Hirose and Manley, 2000; Proudfoot et al., 2002). This interplay between transcription, mRNP processing and packaging into export competent mRNP is likely to provide a control mechanism allowing the proper sequential coordination of all the mRNP biogenesis steps and the rapid detection of aberrant transcripts when mRNP formation is deficient or inefficient. Consistent with this view, mRNP assembly

is doubled by surveillance mechanisms responsible for the retention and the degradation of defective mRNPs in the nucleoplasm, ensuring that only correctly processed mRNAs are exported for further translation in the cytoplasm. In the following sections of this chapter, I will summarize (i) how the nascent mRNA is processed and packaged to become competent for export (ii) how all these processes are connected to transcription and export and (iii) what are the main quality control mechanisms ensuring that only properly processed mRNPs are translocated to the cytoplasm.

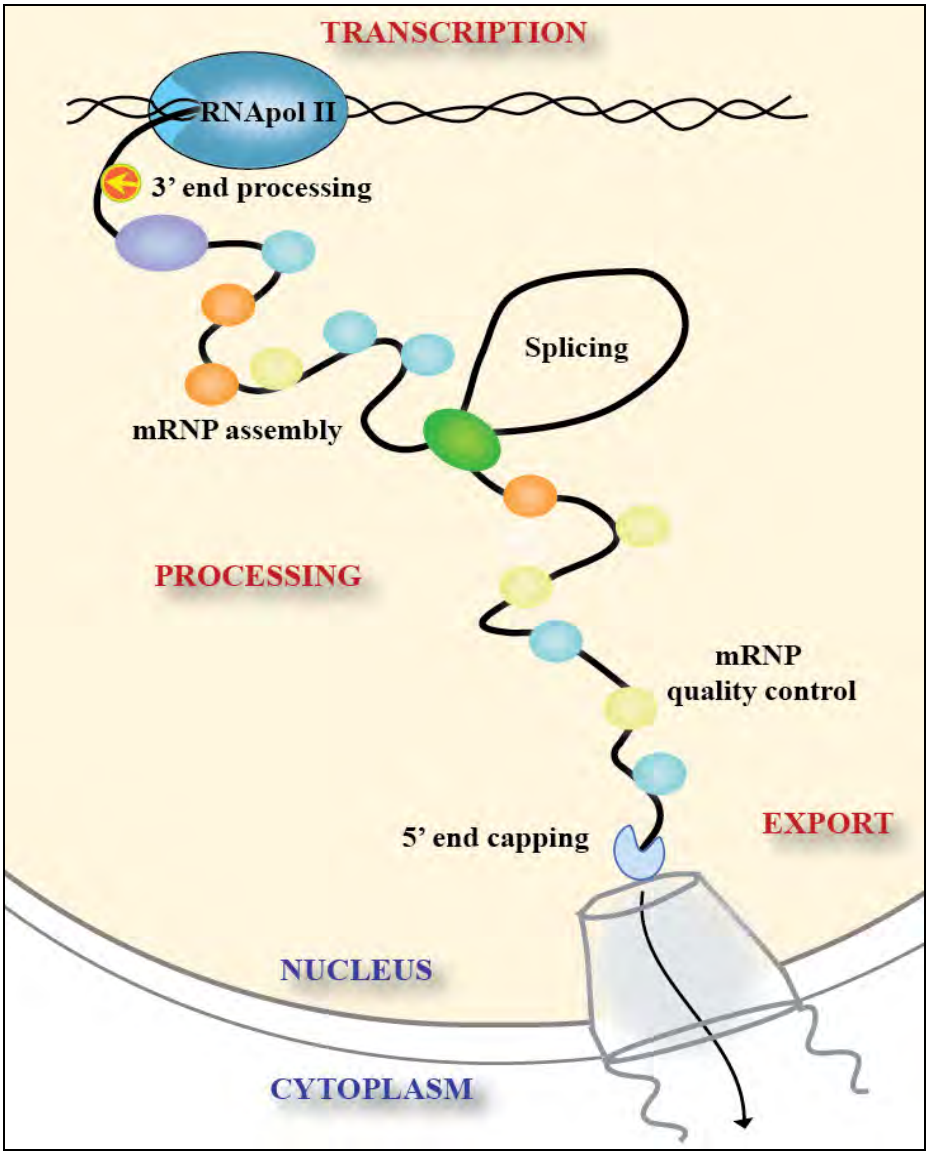


Figure 3. Simplified view of mRNP biogenesis and export. Adapted from (Aguilera, 2005).The nuclear mRNA processing steps of 5' end capping, splicing, mRNA assembly into an mRNP particle, 3' end cleavage and polyadenylation, mRNP surveillance are all coupled processes.

1.2.2.1 mRNP processing and assembly

Although specific RNA-binding proteins associate with discrete subsets of mRNAs sharing functional attributes, there are numerous general factors that are required for mRNP biogenesis steps and export (Hieronymus and Silver, 2003). In the following sections I will present the major mRNP biogenesis steps and factors.

1.2.2.1.1 5' capping

The first processing step occurs after the transcription of the first 22-25 nucleotides when nascent pre-mRNA is capped by methyl-guanylation at its 5' end. The capping reaction is conserved and carried out by two polypeptides in yeast, Cet1 and Ceg1 (human Hce1 or HCE) carrying triphosphatase and guanylyltransferase activities and by Abd1 (human Hcm1), the guanine-N7-methyltransferase. The capping apparatus is cotranscriptionally recruited via interaction with RNA PolII CTD and depends on Ser5 CTD phosphorylation (Bentley, 2005). Once the nascent pre-mRNA is capped, the nuclear cap binding complex (CBC), composed of the Cap binding proteins Cbp20 and Cbp80 (Izaurralde et al., 1994), is recruited on the monomethylated Cap co-transcriptionally (Visa et al., 1996). The CBC remains associated with the mRNP through all the nuclear biogenesis events. Upon export through the nuclear pore complex, the nuclear cap binding proteins are replaced by the cytoplasmic translation initiation factor eIF-4E (Proudfoot et al., 2002; Shatkin and Manley, 2000).

The primary role of the cap structure is to protect the growing pre-mRNA from degradation by 5' exonucleases (Beelman and Parker, 1995). However, mRNA stabilization is not the only consequence of the addition of the cap structure and it also has been involved in splicing and subsequent mRNP formation and processing (Cheng et al., 2006; Colot et al., 1996; Hosoda et al., 2005; Izaurralde et al., 1994; Lewis et al., 1996; Wong et al., 2007). Indeed, CBC facilitates splicing complex binding on the cap-proximal intron enhancing splicing efficiency (Colot et al., 1996; Kotovic et al., 2003; Lewis et al., 1996). In addition, the Cap structure has been shown to stimulate the rate of mRNP export (Hamm and Mattaj, 1990). However whether this is mediated by Cbp20 and Cbp80 is not clear since microinjection of anti Cbp20 in *Xenopus* oocyte did not detectably affect export (Jarmolowski et al., 1994). Moreover, in yeast, T7 transcripts that lack a Cap structure are still well exported in the cytoplasm, indicating that the Cap structure might not play a significant role in mRNA export (Dower and Rosbash, 2002).

More recently, Cbp20 and Cbp80 in human cells have been shown to play a major role in the export of spliced transcripts by directing the recruitment of mRNP components and export factors (Aly, TREX components) specifically on spliced transcripts (Cheng et al., 2006). By showing that the cap binding proteins do have a role in mRNA export only in the case of spliced mRNAs and not for non intron containing RNAs, this study reconciles the discrepancy of earlier data. Indeed, spliced mRNA was used in the original study (Hamm and Mattaj, 1990), while non intron containing transcripts were used in subsequent work (Dower and Rosbash, 2002; Jarmolowski et al., 1994).

Although the role of CBP in other steps of mRNP processing is still not well defined, it interacts physically with other mRNP assembly factors (THO complex, Npl3, (Shen et al., 2000; Uemura et al., 1996)) and is required for efficient 3' end formation, stabilization of polyadenylation complexes and the recognition and degradation of defective mRNA (Cooke and Alwine, 1996; Das et al., 2003; Das et al., 2000).

1.2.2.1.2 Splicing

Most of the eukaryotic pre-mRNAs contain introns interrupting the coding sequence and must be spliced. Splicing is carried out by an RNA protein complex, called the spliceosome which promotes the enzymatic removal of non coding introns and the ligation of the corresponding coding exons (Neugebauer, 2002; Proudfoot et al., 2002). It is now well established that splicing can occur during transcription (Gornemann et al., 2005; Kotovic et al., 2003; Lacadie and Rosbash, 2005; Lacadie et al., 2006; Listerman et al., 2006) and that a number of splicing factors interact with RNA PolII CTD in elongation complexes (Bird et al., 2004; Phatnani and Greenleaf, 2004; Ujvari and Luse, 2004).

Incompletely spliced pre-mRNAs are actively retained within the nucleus, indicating that splicing is a necessary step for the exit out of the nucleus (Casolari et al., 2004; Legrain and Rosbash, 1989). Furthermore, in *Xenopus*, it has been shown that export of spliced mRNA is more efficient than export of intronless mRNA (Luo and Reed, 1999), suggesting that splicing promotes export efficiency. Indeed, it was found that splicing contributes to the formation of mRNPs by catalyzing the deposition of a 335 kDa exon-junction protein complex (EJC) 20–24 nucleotides upstream of exon–exon junction sequences. It was proposed that the EJC contains the important export factor Yra1/Aly establishing a link between splicing and export (Kataoka et al., 2000; Le Hir et al., 2000; Zhou et al., 2000). The recent study from the Reed lab also proposes that both the EJC and CBP cooperate in

recruiting Yra1/Aly and other export factors (Cheng et al., 2006). Furthermore, the splicing machinery also contains factors that are involved in the formation of the mRNP and in the recruitment of the export receptor such as Sub2/UAP56 (Luo et al., 2001) SF2 and U2AF (Lai and Tarn, 2004; Zolotukhin et al., 2002). However, in yeast and even in vertebrates, many messages do not contain introns and are still efficiently exported, suggesting that other processing events may be important for the recruitment of the export machinery (Reed and Cheng, 2005). In addition, splicing also promotes cytoplasmic events as it enhances the association of the mRNP with polysomes in the cytoplasm (Nott et al., 2004).

1.2.2.1.3 3' end formation

In eukaryotes, formation of the mature 3′ end of a mRNA involves cleavage of the nascent transcript followed by polyadenylation. The mRNA sequence provides the signals that determine the site of polyadenylation. In mammals, these are the ubiquitous AAUAAA element located 20–30 nucleotides upstream of the cleavage site, and a GU-rich sequence immediately downstream of this sequence. In *Saccharomyces cerevisiae*, the *cis* sequences that determine 3' end processing are less well defined than in mammals and are identifiable by three redundant degenerate sequences that spread over 100 nucleotides of pre-mRNA sequences (Zhao et al., 1999): UA rich sequence located at variable distance upstream of the cleavage site (efficiency element) (Dichtl and Keller, 2001), A rich sequences generally found 20 nucleotides upstream of the cleavage site (positioning element), and the poly(A) addition site which is in most cases py(A)n (Heidmann et al., 1992; Russo et al., 1991).

These sequences are recognized by two key multisubunit complexes that are highly conserved from yeast to mammals (Proudfoot and O'Sullivan, 2002; Shatkin and Manley, 2000) (Figure 4): the Cleavage Factor I (CFI composed of CFIA and CFIB) and the Cleavage Polyadenylation Factor (CPF) in yeast; the Cleavage and Polyadenylation Stimulation Factor (CPSF), and the Cleavage Stimulation Factor (CstF which associates with the additional cleavage factors CF1 and CF2) in mammals. These two complexes set up a cleavage polyadenylation complex (CPC) that cleaves the RNA and adds a stretch of adenosine residues to the free 3' OH through the action of its poly(A) polymerase Pap1 subunit to generate the poly(A) tail (Proudfoot and O'Sullivan, 2002) and (Proudfoot, 2004).

Concomitantly with poly(A) tail addition by Pap1, the poly(A) binding proteins Pab1 and Nab2 are loaded onto the growing poly(A) tails and modulate their overall length. These proteins are involved in the regulation of the poly(A) tail length either by controlling its

synthesis via the Pap1 poly(A)polymerase or its trimming via the recruitment of deadenylases (Anderson et al., 1993; Brown and Sachs, 1998; Dunn et al., 2005; Hector et al., 2002). Trimming of the tail to its proper length is the last mRNA biogenesis step and may actively contribute to the release of mRNA from its transcription site (Dunn et al., 2005).

Strikingly, mutating poly(A) signals not only blocks mRNA polyadenylation but also disrupts the normal site of transcriptional termination by RNA polymerase II, causing nascent transcription to run through the gene's 3′ flanking region (Proudfoot and O'Sullivan, 2002). This indicates that, as with the other molecular mechanisms of gene expression, polyadenylation is tighly coupled with transcription (see below section 1.2.2.3). Indeed, ChIP analyses in yeast have demonstrated that polyadenylation factors mainly crosslink at the 3' end of genes around the polyadenylation sites (Kim et al., 2004). Furthermore deletion of the Ctk1 kinase which is responsible for Ser2 CTD phosphorylation strongly reduces this crosslink, indicating that Ser2 phosphorylated CTD contributes to the recruitment of these factors (Ahn et al., 2004). This is in agreement with structural studies that determined that CF1A in yeast specifically interacts with the Ser2 phosphorylated CTD via its component Pcf11 (Noble et al., 2005; Zhang et al., 2005).

However, there are several lines of evidence showing that the co-transcriptional recruitment of 3' end processing factors on RNApol II CTD is not an obligatory feature for cleavage-polyadenylation. An elegant demonstration of this was performed in a yeast strain that was engineered to express T7 bacteriophage RNA polymerase, which lacks a CTD and thus unable to co-transcriptionally recruit cleavage/polyadenylation components. This strain was used to transcribe a T7 promoter driven gene encoding pre-RNA containing a poly(A) signal (Dower and Rosbash, 2002). The mRNA transcribed from this gene was not subject to the usual 5′ 'capping', but cleavage-polyadenylation was normally detected. Consistently, another study showed that 3' end processing is only partially affected in cells lacking the CTD Ser2 kinase Ctk1 (Ahn et al., 2004). These data show that the 3'end processing machinery can be recruited to unprocessed transcripts, albeit less efficiently, in the absence of an interaction with the RNA PolII transcription machinery.

A proper 3' end processing appears to be the most crucial step for the acquisition of export competency. Indeed, mutations in both *cis* and *trans* 3' processing factors readily block mRNA export (Brodsky and Silver, 2000; Hammell et al., 2002; Hilleren et al., 2001). Indeed, transcripts produced with the T7 RNA polymerase, which lack capping but can still undergo

cleavage and polyadenylation are well exported (Dower and Rosbash, 2002), indicating that 3' end processing is both necessary and sufficient for export.

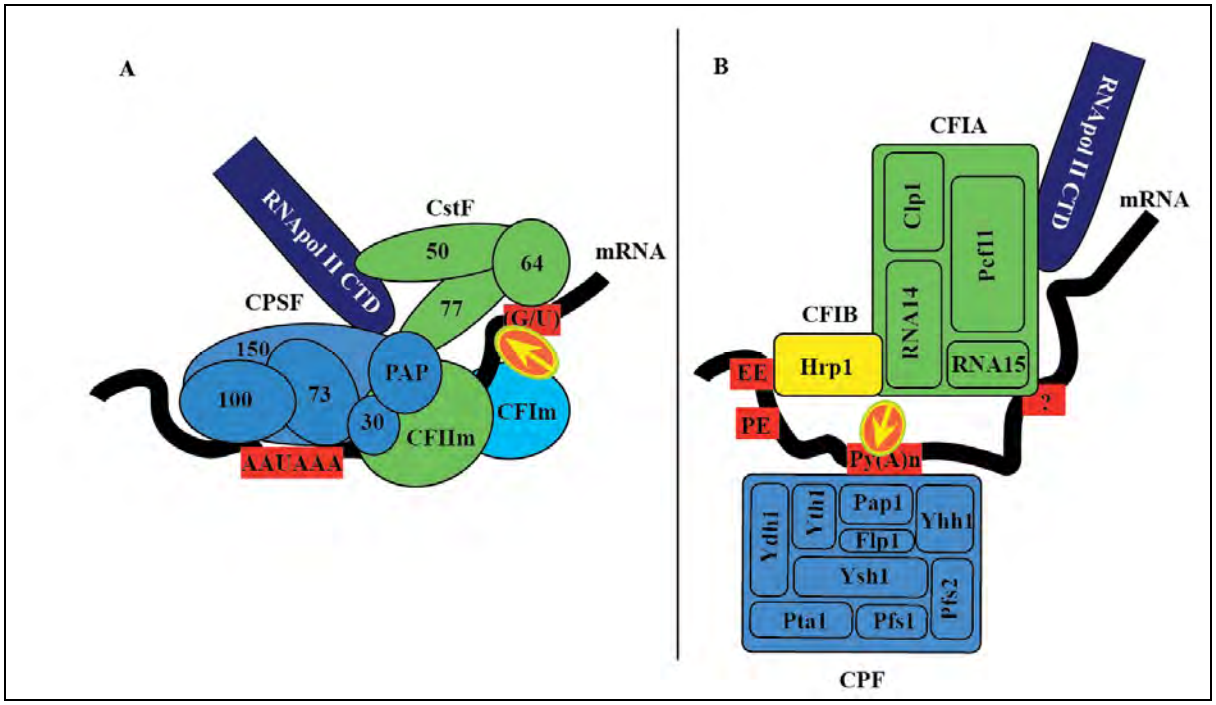


Figure 4. Comparison of cleavage-polyadenylation factors associated with poly(A) signals in mammals and budding yeast. Adapted from (Proudfoot and O'Sullivan, 2002). In mammals (A), the known factors involved are indicated, showing the subunit structures and molecular weights of CPSF and CstF. In yeast (B), two multi-subunit factors are involved with the subunit components indicated. For mammals and yeast, the positions of the different factors are intended to indicate (where known) the interactions between different factors and their subunits. The sequence elements that comprise the poly(A) signals are also indicated by Red rectangles, and the site of cleavage (and subsequent polyadenylation) is shown by an arrow. RNApolII CTD is also indicated in blue. Homologous factors between yeast and mammals are color matched. Known homologous subunits between yeast and human include: Pfs2p=$CstF_{50}$; Ysh1p=$CPSF_{73}$; Ydh1p=$CPSF_{100}$; Yth1p=$CPSF_{30}$; Yhh1p=$CPSF_{160}$; Pap1p=PAP; Rna14p=$CstF_{77}$; Rna15p=$CstF_{64}$.

1.2.2.1.4 mRNA exports factors

Most mRNA export factors were identified in screening for conditional mutants that accumulate poly(A)+ RNA in their nucleus at the restrictive temperature, indicating that they play a key role in the mRNA export pathway (Kadowaki et al., 1992). Mex67 in yeast and its mammalian homologue TAP have been described as the essential mRNA export receptors that mediate the translocation of the mature mRNP through the NPC from the nucleoplasm to the cytoplasm. It was originally thought that Mex67 binds directly to RNA (Segref et al., 1997), however subsequent analyses revealed that Mex67 affinity for RNA is weak implying that it requires adaptor proteins to bind to mRNP. Resolving how mRNPs are exported to the cytoplasm also necessitated to dissect the pathways leading to Mex67 recruitment on mRNPs.

1.2.2.1.4.1 Mex67

As previously described, nuclear export involved three key steps: generation of a cargo-carrier complex in the nucleus, translocation of the complex through the NPC channel and release of the cargo in the cytoplasm followed by recycling of the carrier (Stewart et al., 2001). Several studies in different model organisms have provided convincing lines of evidence that nuclear exit of mRNA is dependent on the essential protein Mex67 in yeast or its ortholog TAP/NXF1 in metazoans, indicating that they are evolutionary conserved export receptors for mRNA (Braun et al., 2001; Guzik et al., 2001; Herold et al., 2001; Hurt et al., 2000; Santos-Rosa et al., 1998; Segref et al., 1997; Strasser et al., 2000; Tan et al., 2000; Yoon et al., 2000; Zenklusen et al., 2001).

Mex67 and TAP indeed exhibit the characteristics of export receptors as they: (i) crosslink to poly(A) RNA *in vivo* (Katahira et al., 1999; Segref et al., 1997). As already mentioned it is likely that Mex67 does not recognize directly the mRNA but requires adaptors to associate to the mRNP (See the following sections); (ii) directly interact with FG nucleoporins at the NPC (Bachi et al., 2000; Katahira et al., 1999; Santos-Rosa et al., 1998; Segref et al., 1997; Strawn et al., 2001) and shuttle through the NPC (Bachi et al., 2000; Bear et al., 1999; Braun et al., 1999; Kang and Cullen, 1999; Katahira et al., 1999; Schmitt and Gerace, 2001); (iii) are directly required for mRNA export (Herold et al., 2001; Hurt et al., 2000; Santos-Rosa et al., 1998; Segref et al., 1997; Strasser et al., 2000; Zenklusen et al., 2001).

Mex67/TAP belongs to the family of nuclear export factors (NXF) conserved proteins (Izaurralde, 2002). It contains two main functional domains (Figure 5): the N-terminal domain

involved in mRNP binding through interaction with the adaptors proteins, and the C-terminal domain involved in shuttling and NPC binding (Bachi et al., 2000; Braun et al., 2001; Braun et al., 2002; Fribourg et al., 2001; Grant et al., 2002; Levesque et al., 2001; Suyama et al., 2000; Wiegand et al., 2002). Mex67/TAP form heterodimers with the small proteins, p15 and Mtr2, respectively, (Katahira et al., 1999; Santos-Rosa et al., 1998; Suyama et al., 2000) through interaction with their NTF2-like domain. p15 and Mtr2 are also essential for mRNA export (Herold et al., 2001; Wiegand et al., 2002) and are proposed to contribute to the proper folding of the C-terminal part of Mex67/TAP enhancing their ability to interact with FG nucleoporins.

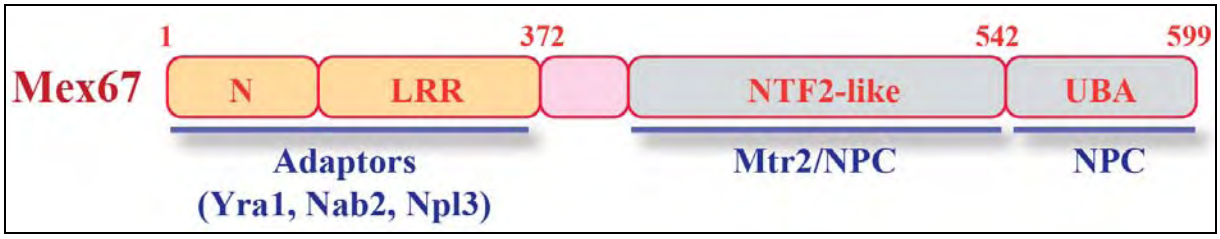


Figure 5. **Topological view of the nuclear export Mex67.** The N-terminal domain followed by a leucine rich region (LRR) domain constitutes the main mRNP binding site as it mediates the interaction with Yra1 and presumably other adaptor proteins. The NPC binding domain consists of the C-terminal part of the proteins. It is composed of the NTF2-like domain that interacts with Mtr2 and the UBA domain which interacts directly with nucleoporins.

1.2.2.1.4.2 Yra1

To date, Yra1 or REF in higher eukaryotes is the best characterized adaptor for Mex67/TAP. Yra1/REF factor is a member of the evolutionary conserved REF family (RNA Export Factor binding) and associates directly with mRNA (Strasser et al., 2000). mRNA export is blocked in a *yra1* mutant (Strasser et al., 2000; Stutz et al., 2000; Zenklusen et al., 2001), supporting a direct role for Yra1/REF in mRNA export. Indeed, Yra1 connects directly the mRNA to the export pathway as it interacts with the Mex67 nuclear export receptor both *in vivo* and *in vitro* (Strasser et al., 2000; Stutz et al., 2000; Zenklusen et al., 2001). Moreover, *yra1* mutants that inefficiently interact with Mex67 have significant lower amounts of Mex67 associated with their mRNPs (Zenklusen et al., 2001). These observations led to the proposal that Yra1/REF mediates the recruitment of Mex67/TAP on mature mRNPs and hence promotes their nuclear exit. However, Yra1 does not shuttle through the NPC, indicating that is has to be removed from the mRNP before export (Strasser et al., 2000; Stutz et al., 2000;

Zenklusen et al., 2001; Zhou et al., 2000). In addition, Yra1 only associates with a fraction of mRNPs (Hieronymus and Silver, 2003), indicating that other adaptors exist for Mex67 (See below Nab2 and Npl3).

Intriguingly, Yra1 is recruited to the transcribing gene early during transcription via an RNA independent mechanism (Abruzzi et al., 2004; Johnson et al., 2009). This observation suggests that packaging of the mRNP for export begins during synthesis of the pre-mRNA (Daneholt, 2001; Lei et al., 2001). Furthermore, this indicates that Yra1 associates first with the transcription machinery and is only transferred on mRNP at a later stage. The recruitment of Yra1 on the transcribing machinery and subsequently on the mRNP has been the object of further investigations. It has first been thought that Yra1 was recruited on the transcription machinery and transferred from the transcription machinery to the mRNP during transcription elongation through interactions with an ATP dependent RNA helicase called Sub2 and with a complex associated with the transcription elongation machinery, the THO complex (see below). However, a very recent study reported that Yra1 binds directly to Pcf11, the RNA PolII CTD-binding subunit of CF1A, and that this interaction mediates Yra1 recruitment on the transcription machinery. This last study proposes a mechanism for co-transcriptional assembly of the export competent mRNP in coordination with 3' end processing (Johnson et al., 2009) (See below for more details).

1.2.2.1.4.3 Sub2

Yeast Sub2 and its human homologue UAP56 belong to the superfamilly of RNA helicases and were first shown to be required for spliceosome assembly (Fleckner et al., 1997; Libri et al., 2001; Zhang and Green, 2001). However, further studies have also implicated Sub2 and its *Drosophila* homologue in mRNA export (Gatfield et al., 2001; Jensen et al., 2001a; Strasser and Hurt, 2001; Zenklusen et al., 2002). Conditional mutants of Sub2, indeed, display a marked phenotype in mRNA retention and strong genetic interactions with *yra1* mutants (Strasser and Hurt, 2001; Zenklusen et al., 2002). Subsequently, it has been shown that Sub2/UAP56 interacts with Yra1/REF (Luo et al., 2001; Strasser and Hurt, 2001) and determined that the binding of Sub2/UAP56 and Mex67/TAP to Yra1/REF are mutually exclusive (Hautbergue et al., 2008; Kohler and Hurt, 2007; Rodrigues et al., 2001; Strasser and Hurt, 2001; Stutz et al., 2000). Sub2 in yeast is recruited early during transcription to the growing mRNP through direct interaction with the Hpr1 subunit of the RNA PolII associated THO complex (Abruzzi et al., 2004; Strasser et al., 2002; Zenklusen et al., 2002). Because Sub2 binds Yra1 directly and is necessary for mRNA export, it was proposed that Sub2 is

primarily responsible for recruiting Yra1 to pol II transcription complexes and subsequently to the nascent mRNA (Strasser and Hurt, 2001; Zenklusen et al., 2002). Together these data suggested a model of the mRNP export pathway where Sub2/UAP56 recruits Yra1/REF to the nascent mRNP during transcription elongation and is then displaced by Mex67/TAP which then targets the mRNP to the NPC. Similar findings in Chironomus and human cells (Custodio et al., 2004; Kiesler et al., 2002) suggested that the Yra1/REF co-transcriptionnal recruitment by Sub2/UAP56 might be conserved. However, the direct role of Sub2 in Yra1 recruitment has recently been reevaluated revealing that Yra1 recruitment on the transcription machinery is independent on Sub2 and primarily dependent on the cleavage/polyadenylation complex factor Pcf11 (Johnson et al., 2009). This study also showed that the binding of Sub2 and Pcf11 on Yra1 is mutually exclusive (Johnson et al., 2009), suggesting a model where the interaction of Sub2 with Yra1 and the ATPase activity of Sub2 contribute to transferring Yra1 from Pcf11 to the nascent mRNA rather than recruiting Yra1 to the transcription machinery. Therefore, Sub2 might play a key role in remodeling the mRNP in coordination with 3' end formation events (See section 2.2.3).

1.2.2.1.4.4 THO and TREX complexes

Yra1/REF and Sub2/UAP56 interact both genetically and physically with components of the transcription elongation THO complex (Chavez et al., 2000; Piruat and Aguilera, 1998; Strasser et al., 2002). The THO complex is composed of Hpr1, Mft1, Thp2 and Tho2 subunits and is recruited to the active coding regions in a transcription dependent but RNase insensitive manner, suggesting that recruitment occurs via direct interaction with the transcription machinery (Abruzzi et al., 2004; Strasser et al., 2002; Zenklusen et al., 2002). A role of the THO complex in transcription elongation has been reported in several studies (Chavez et al., 2001; Huertas and Aguilera, 2003; Mason and Struhl, 2005), suggesting that the THO complex is part of the elongation machinery (see section 2.2.3). Moreover, it has been shown that the THO complex is released from the site of transcription before transcription termination occurs (Kim et al., 2004), suggesting that the loss of the complex is one event that determines termination.

Because the THO complex interacts with both RNA and DNA *in vitro,* and both the essential mRNA export factors Sub2 and Yra1 *in vivo* (Chavez et al., 2000; Piruat and Aguilera, 1998; Strasser et al., 2002), it has been proposed to play a role at the interface with transcription and mRNP metabolism. Notably, deletion of THO individual components results in the rapid accumulation of poly(A)+ transcripts within the nucleus (Libri et al., 2002;

Schneiter et al., 1999; Strasser et al., 2002), indicating that this complex plays an important role in mRNA export. In the light of these observations, the THO-Sub2-Yra1 multi protein complex was subsequently termed the TREX complex, proposed to couple transcription and export (Strasser et al., 2002). In situ hybridization (FISH) analysis with oligonucleotides directed against specific transcripts revealed that in THO complex mutants, mRNPs were retained at or near the site of transcription (Libri et al., 2002; Thomsen et al., 2003), suggesting that this complex plays a role in mediating correct mRNP assembly and release during transcription. Consistent with this view, some studies reported that deletion of THO complex components affects the recruitment of Sub2 and Yra1 to transcribing genes (Abruzzi et al., 2004; Zenklusen et al., 2002). As mentioned earlier, the initial model for Sub2 and Yra1 recruitment was that Sub2 is loaded onto the THO complex through interaction with its Hpr1 subunit (Zenklusen et al., 2002) and promotes Yra1 recruitment on the elongation machinery. This model has been revisited and the current view is that Sub2 does not recruit Yra1 on the elongation machinery but is required to recruit Yra1 from the transcription machinery to the mRNP at a later stage (Johnson et al., 2009). Whether the THO complex contributes to the co-transcriptional recruitment of Yra1 to the transcribing gene and how it facilitates the loading of Sub2 onto the nascent mRNA is still not clear. However, the THO complex by ensuring a proper co-transcriptional Sub2 recruitment might coordinate the efficient loading of Sub2 and subsequently Yra1 to nascent transcripts (See section 2.2.3). Both the RNA annealing activity of Yra1/REF and the helicase activity of Sub2/UAP56 might then facilitate the correct folding of mRNP complexes to recruit Mex67. Taken together, these observations suggest a central role for the THO complex in contributing to the mRNP assembly and ensure packaging of the mRNA into an exportable mRNP complex.

1.2.2.1.4.5 Nab2

The two poly(A) binding proteins Pab1 and Nab2 shuttle between the nucleus and the cytoplasm, however, Nab2 is essentially nuclear while Pab1 is more abundant in the cytoplasm where it plays important roles in translation (Sachs and Davis, 1990). Mutations in both Nab2 and Pab1 result in nuclear retention of the mRNP in the nucleus (Dunn et al., 2005; Hector et al., 2002), suggesting an important role of the poly(A) tail in mRNA export (see section 2.2.3). In the case of Pab1, the retention occurs at the transcription site and is dependent of a functional nuclear exosome, suggesting that this protein plays a role in mediating correct mRNP assembly. Importantly, Nab2 has been more directly implicated in mRNA export. Nab2 is an essential protein that interacts with the mRNP constituent Yra1

which is also required for export (Kashyap et al., 2005) and with the perinuclear protein Mlp1 (Green et al., 2003; Vinciguerra et al., 2005), indicating a role for Nab2 in addressing the mRNP to the NPC for export (Fasken and Corbett, 2005). Confirming this suggestion, Nahid Iglesias, a PhD sudent in our lab recently provided evidence that Nab2 can also interact with the essential nuclear export receptor Mex67 serving as an adaptor for its recruitment on the mRNP (N.Iglesias personal communication).

1.2.2.1.4.6 Npl3

Npl3 is the most abundant shuttling hnRNP in yeast (Wilson et al., 1994). It is recruited early to the nascent pre-mRNA in a transcription dependent manner, through direct interaction with RNA PolII, and remains bound to the mRNP during translocation (Lei et al., 2001; Windgassen et al., 2004). It contains two RNA recognition motifs (RRMs) and a serine-arginine rich (SR) domain in its C-terminal region. Mutations in these domains cause nuclear accumulation of polyadenylated mRNAs, indicating that this protein is involved in mRNA export (Lee et al., 1996; Singleton et al., 1995). Indeed, work from the Guthrie lab showed that Npl3 directly interacts with Mex67 and serves as an adaptor subunit to recruit Mex67 on mRNP (Gilbert and Guthrie, 2004). Importantly, the recognition of Mex67 by Npl3 requires Npl3 dephosphorylation by Glc7, a phosphatase associated with the cleavage/polyadenylation complex suggesting a role for Npl3 in the coordination of 3' end formation with Mex67 recruitment and subsequent mRNP export (See section 1.2.2.3).

1.2.2.2 Translocation of mRNP along the NPC channel

From all the previous findings, a model for translocation of mRNPs through the NPC has arisen: Mex67/TAP-Mtr2/p15 drive mRNPs through the pore by transient and sequential interactions with FG nucleoporins. Although, mRNA export is an energy dependent mechanism, the small GTPase Ran is not implicated in the mRNA export pathway. Instead, several studies have recently proposed a role for the essential DEAD-box RNA-dependent ATPase Dbp5 in the passage and the final release of the mRNP from Mex67-Mtr2 and the NPC at the cytoplasmic face of the NPC (Alcazar-Roman et al., 2006; Lund and Guthrie, 2005; Weirich et al., 2006) (Figure 6). Dbp5 is required for poly(A) mRNA export from the nucleus in both yeast and vertebrates (Schmitt et al., 1999; Snay-Hodge et al., 1998; Tseng et al., 1998) and is normally concentrated at the cytoplasmic filaments of the NPC by interacting with Gle1 and Nup159 (Strahm et al., 1999). The ATP-dependent RNA unwinding activity of Dbp5 and nuclear pore localization are essential for its biological function, suggesting that Dbp5 participates in mRNA export by remodeling the mRNP complexes during translocation

through and release from the NPC (Hodge et al., 1999; Schmitt et al., 1999; Snay-Hodge et al., 1998; Strahm et al., 1999). Evidences for a significant remodeling of the mRNPs upon the nuclear exit came from electron microscopic analyses of the giant balbiani ring (BR) mRNA of the insect *Chironomus tentans*. The BR transcript coated into a compact ring-shaped mRNP is gradually unfolded as it passes through the NPC and emerges extended on the cytoplasmic side (Daneholt, 2001). A recent study from the Wente lab provided additional evidence that Dbp5 controls export by triggering specific mRNA protein remodeling events at the NPC (Tran et al., 2007). In this case, Dbp5 was proposed to promote the dissociation of the shuttling poly(A)+ binding export protein Nab2 from mRNP complexes. This suggests that Dbp5 could act as a molecular motor to remodel and pull mRNPs into the cytoplasm using the energy delivered by ATP hydrolysis. This local interaction could generate directionality of mRNP export. Notably, a recent finding shows that Gle1 and phosphoinositide IP_6 dramatically stimulate Dbp5 ATPase activity *in vitro* (Alcazar-Roman et al., 2006; Weirich et al., 2006). This result could explain how Dbp5 activity is controlled spatially, so that disassembly of mRNPs does not occur at inappropriate locations. Gle1 and Nup159 may therefore function in providing a platform to which translocating mRNPs bind and then dissociate through the action of Dbp5. This model could explain how mRNAs are directionally exported and released from the export complex in the cytoplasm since RanGTP is not involved in mRNA export.

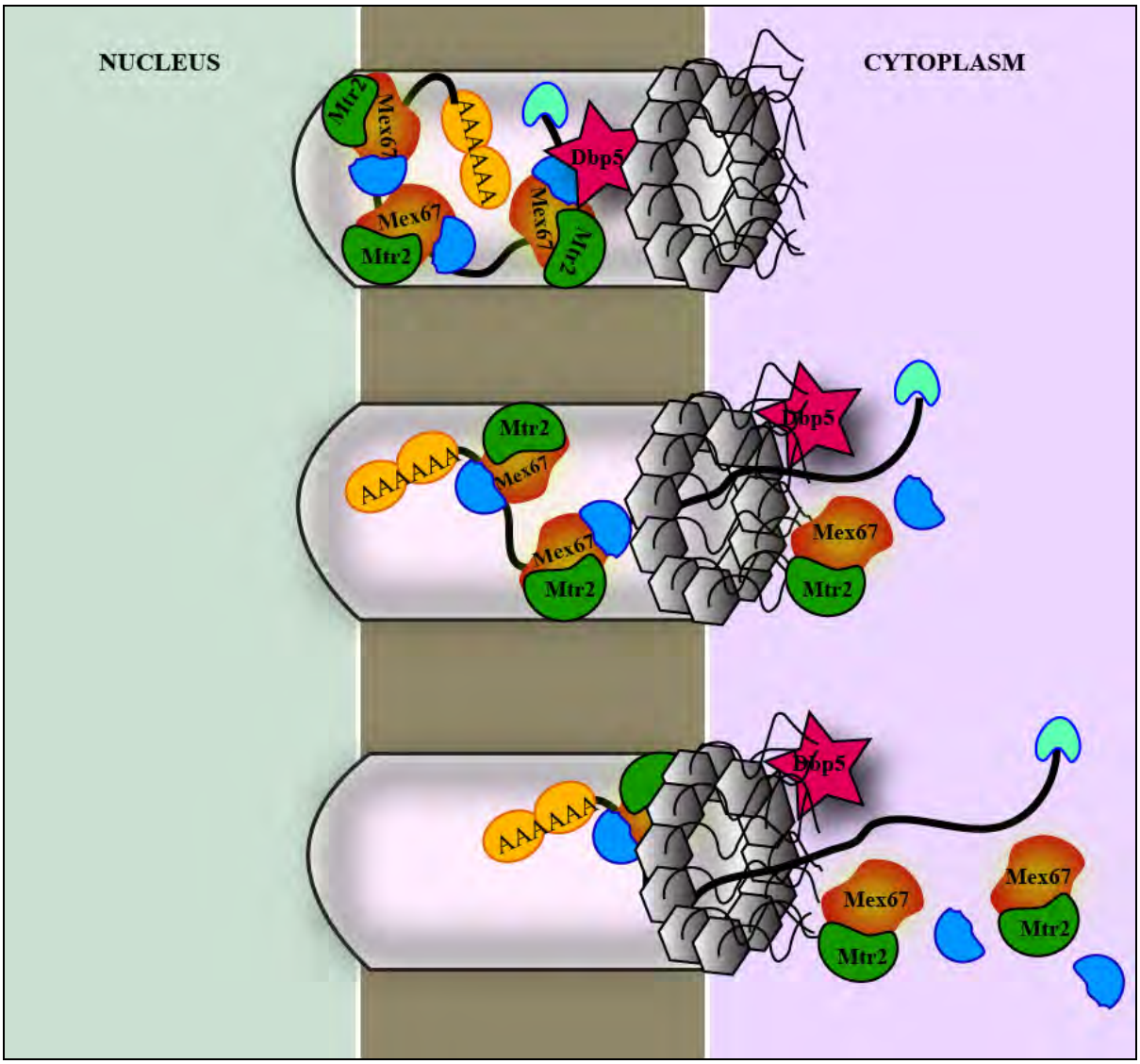


Figure 6. **Translocation of mRNP through the NPC.** Adapted from (Cole and Scarcelli, 2006). Mex67-Mtr2 interacts with the FG nucleoporins lining the NPC interior. Dbp5 binds to the mRNP within the pore and accompanies the mRNP out where it interacts with the cytoplasmic filaments proteins Gle1 and Nup159 and acts to remove bound proteins from the mRNP.

1.2.2.3 Crosstalk between transcription, processing and export

It is clear that all the mRNP processing and biogenesis events described above are interconnected with each other and coupled to the transcription and the NPC translocation processes. This extensive coupling is believed to ensure the proper coordination between all the steps as well as to allow the export machinery to distinguish immature pre-mRNA from fully processed mRNA.

1.2.2.3.1 Transcription, processing and early mRNP packaging are tighly connected

1.2.2.3.1.1 Co-trancriptional recruitment of mRNP processing and packaging factors is dictated by the transcription cycle.

The connection between transcription mRNA processing and packaging is direct since a growing number of processing factors are shown to interact directly with the CTD of the RNA PolII machinery. An important event in the transcription process is the phosphorylation of the CTD: CTD phosphorylation at serine 5 correlates with transcription initiation and early elongation events, whereas phosphorylation at serine 2 is mainly associated with active elongating complexes and late transcription events (Komarnitsky et al., 2000). The current paradigm for the recruitment of mRNA processing factors is that RNA PolII CTD acts as a loading platform and that different proteins bind to specific phosphorylated forms of the CTD. Consistent with this, ChIP experiments in yeast revealed that while the distribution of the capping enzyme is biased towards the 5’ end, the recruitment of cleavage polyadenylation factors and export factors on the transcribing gene is progressive with a small but significant quantity at the 5′ end which increases towards the 3′ end (Abruzzi et al., 2004; Kim et al., 2004; Licatalosi et al., 2002), opposite to the distribution of capping enzymes which is biased towards the 5′ end. The changing pattern of phosphorylation between the 5′ and 3′ ends of the gene may thus constitute a CTD code (Buratowski, 2003), that dictates a sequence of processing factor binding and release reactions as transcription proceeds.

In addition to CTD phosphorylation, the order of packaging and export factor binding to the nascent mRNA is also determined by an additional mechanism that involves the transcription elongation machinery associated THO complex. As previously described, the THO complex recruits the export factor Sub2 on the elongation machinery. Sub2 also interacts with Yra1 and both Sub2 and Yra1 co-purify with the THO in a complex called TREX. The TREX complex was proposed to couple transcription and export, as it is important for transcription elongation as well as for the co-transcriptional formation of an

export-competent mRNP. Because Sub2, Yra1 and THO co-purify in a complex, it was initially proposed that THO recruits Sub2 which recruits Yra1 and facilitates Sub2 and Yra1 loading onto the nascent mRNA. However, Sub2 is loaded early on the nascent mRNA whereas Yra1 recruitment is partially sensitive to RNAse (Abruzzi et al., 2004), suggesting that it is loaded at a later step during transcription elongation. A recent study shed light on how the THO complex could coordinate the loading of Sub2 and Yra1 from the transcription machinery to the nascent mRNA at different stage of transcription (Johnson et al., 2009) (See below). As previously proposed, Sub2 may be loaded on the mRNP early during transcription elongation through direct interaction with the THO complex. Yra1 instead may be recruited on the transcription machinery by other factors but transfered on the mRNA during transcription termination by a process mediated by the ATPase Sub2. One important role of the THO complex would thus be to control the sequential loading of export factors by promoting their transfer to the mRNP through the direct regulation of Sub2 recruitment on mRNA.

In agreement with the mRNP biogenesis being dependent on specific sequential steps during the transcription cycle, another study revealed that the use of transcription-defective mutants and transcription elongation drugs that slow down the transcription rate result in the suppression of several phenotypes associated with defective mRNA processing and export (Jensen et al., 2004). This further suggests that efficient mRNP assembly is influenced by the transcription kinetics, confirming the idea that the different processing events occur at specific transcription stages, presumably to ensure that they happen at the right time.

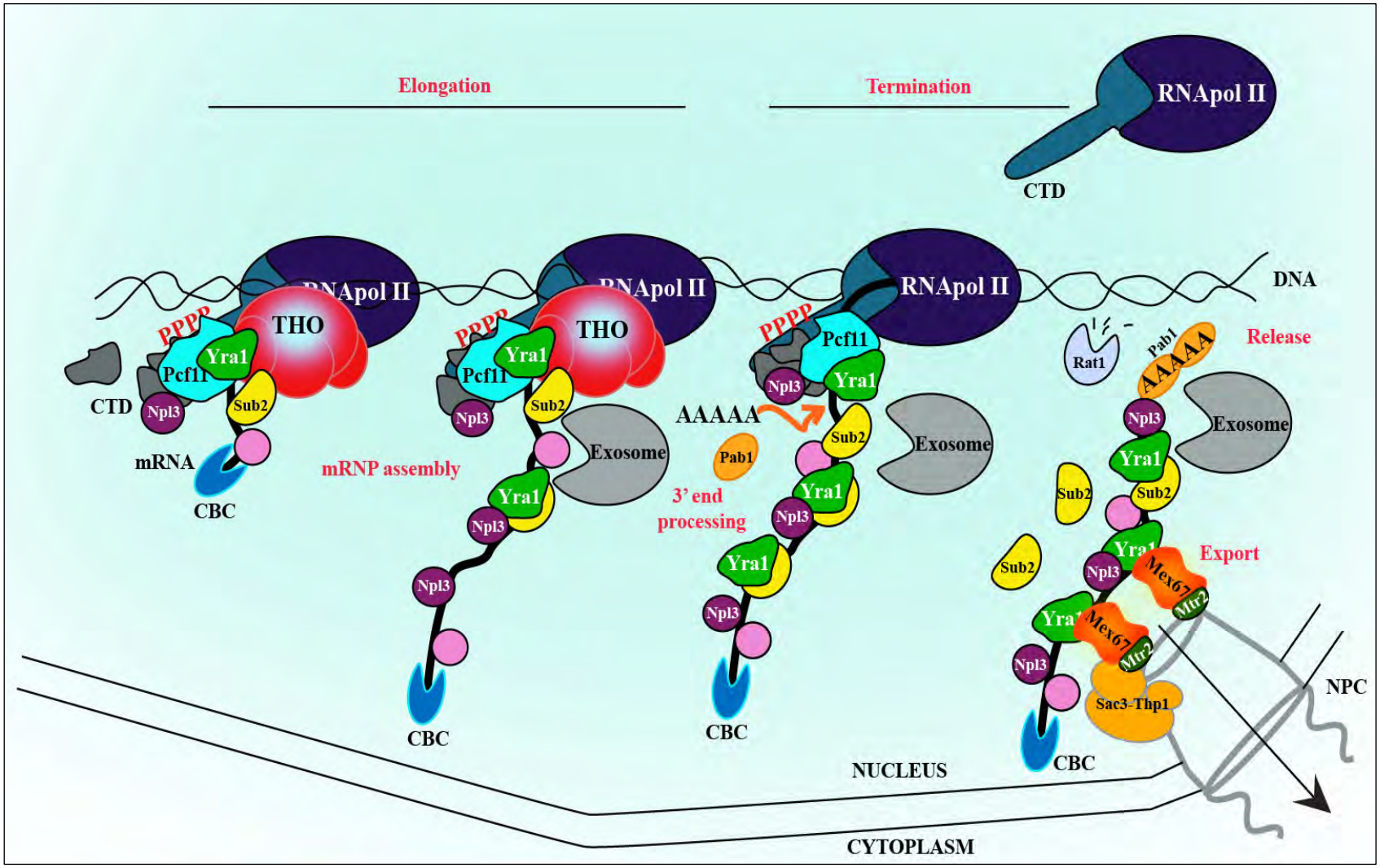


Figure 7. Simplified model of co-transcriptional mRNP assembly in budding yeast. Adapted from (Aguilera, 2005). During transcription elongation, the THO complex facilitates the loading of the Sub2 RNA dependent ATPase and the Pcf11 3' end processing factor recruits the export factor Yra1. Many other proteins associate with the RNA polII CTD and the growing mRNP, including, Npl3, Nab2 (not shown), components of the cleavage/polyadenylation complex and Pab1. The mRNP is processed by cleavage and polyadenylation followed by the mRNP release from the transcription machinery and export. The Mex67-Mtr2 nuclear export receptor binds to Yra1, displaces Sub2 and exports the mRNA through interaction with other mRNP binding factors such as Thp1-Sac3-Sus1 complex. All these events are under the surveillance of the exosome.

1.2.2.3.1.2 Bi-directional interplay between transcription and mRNP formation

Several experimental evidences support the idea of a bi-directional interplay between transcription and mRNP formation with mRNA processing events also influencing on transcription. First, capping enzymes regulate transcription through a network of interactions that influence early steps in transcription (Pei et al., 2003), stimulate early elongation (Kim et al., 2004; Schroeder et al., 2004), and inhibit re-initiation (Mandal et al., 2004; Myers et al., 2002)These observations raised the proposal that they may operate a checkpoint to ensure that the cap has been added before commitment to productive elongation of the transcript.

Second, the THO complex, that recruits the export factors on the transcription machinery, functions in transcription elongation and impacts on termination. Indeed, a study using a LacZ reporter gene fused to different promoter and 5' regions reported that transcription elongation but not initation was defective in mutants of the THO complex (Chavez et al., 2001). Furthermore, ChIP experiments determined that in THO mutants RNA polII displays lower processivity and elongation rate (Mason and Struhl, 2005). A mechanism that might account for the transcriptional defect in THO mutants has been proposed by the Aguilera laboratory (Huertas and Aguilera, 2003), suggesting that in mutants of the THO complex nascent mRNA folds back and hybridizes to single strand DNA: such DNA-RNA hybrids could affect the transcription cycles in preventing the next engaged RNA PolII to reach the 3' end of genes and result in a transcription elongation impairment. In addition, mutants of the THO complex components exhibit premature transcription termination and poorly polyadenylated pre-mRNA phenotypes (Saguez et al., 2008), indicating that the THO complex plays a key role in transcription termination. Other mRNP export factors that are recruited co-transcriptionally, such as components of the TREX complex, affect transcription. Indeed, for instance, Yra1 mutants lead to decreased transcription levels (Vinciguerra et al., 2005), suggesting either that these factors promote transcription or that defects in mRNP biogenesis negatively feedback on transcription.

Third, the termination of RNApolII transcription is coupled to mRNP 3' end formation. Indeed, mutation in the poly(A) signal sequences and in several of the cleavage and polyadenylation factors, including CFIA and CPF subunits cause inefficient transcription termination which results in a run through into the gene's 3′ flanking region (Dichtl et al., 2002; He et al., 2003; Sadowski et al., 2003; Zhang et al., 2005). Two models have been proposed to explain how mRNA 3' end processing could contribute to a proper transcription termination: the "allosteric" and the "torpedo" model (Bentley, 2005; Rosonina et al., 2006).

The “allosteric” model posits that transcription termination is caused by the destabilization or a conformational change of the mRNP polII elongation machinery after transcribing the poly(A) site. This change in mRNP processivity would be triggered by the cleavage/polyadenylation factors that are co-transcriptionally recruited, and may occur before the cleavage of the nascent transcript. The “torpedo” model proposes that it is the release of the transcript from the 3’ end processing machinery that triggers RNA PolII dissociation from the DNA template. The cleavage by the polyadenylation machinery at the poly(A) site would provide an entry site for 5’ to 3’ endonucleolytic attack of the 3’ RNA fragment still attached to the transcribing complex and this degradation activity, once it reaches the transcription machinery, would destabilize it. Supporting this view the 5’ to 3’ exonucleases Rat1 in yeast and Xrn2 in human were reported to be required for efficient termination (Kim et al., 2004; West et al., 2004). However, also consistent with the “allosteric” model, these enzymes are progressively recruited between the 5′ and 3′ ends of the gene like conventional cleavage/polyadenylation factors (Kim et al., 2004) and ChIP analyses revealed that the integrity of Rat1 was necessary for an efficient recruitment of Pcf11 and Rna15 polyadenylation factors on the transcribing machinery (Luo et al., 2006). Furthermore, evidence has also been provided that degradation of the 3’ end fragment after cleavage is not sufficient for termination (Luo et al., 2006) suggesting that the contribution of the 3’ end processing machinery in transcription termination could rather be the result of both models.

1.2.2.3.2 Export competency is linked to 3’ end formation

A proper 3’ end formation and polyadenylation is crucial for the acquisition of export competency since both *cis* and *trans* acting 3’ end processing mutants block mRNA export (Brodsky and Silver, 2000; Dower et al., 2004; Hammell et al., 2002; Huang and Carmichael, 1996; Libri et al., 2002). Reciprocally, mutants of some export proteins and mRNP biogenesis result in transcripts with improperly processed 3′ ends (Jensen et al., 2001b; Saguez et al., 2008)support the idea that 3′ end processing and export are linked in yeast and metazoans. The mechanisms by which this coupling occurs are poorly understood, however, two examples have been reported.

1.2.2.3.2.1 Yra1 recruitment on mRNP is directed by the 3’ end processing machinery

Consistent with a role for cleavage/polyadenylation in export, Lei and Silver initially showed that cotranscriptional Yra1 recruitment to yeast genes was inhibited in a mutant of the CFIA subunit RNA15 (Lei and Silver, 2002). Moreover, as mentioned above recent study

demonstrated that the co-transcriptional recruitment of Yra1 on active genes requires Pcf11, the CF1A subunit which interacts with the RNA polII CTD from an early stage of transcription. It was previously proposed that Sub2, loaded on the nascent mRNP via interaction with the THO complex, contributes to the recruitment of Yra1 to the transcribing gene during elongation. However, Pcf11 and Sub2 seem to bind the same domain on Yra1 suggesting a mutually exclusive interaction (Johnson et al., 2009). Reconciliating all these hypotheses and the involvement of the THO complex and Sub2 in 3' end processing a model has been proposed. Yra1 could be recruited on the transcription machinery through interaction with Pcf11 and possibly the THO complex and transferred to Sub2 on the mRNP progressively during transcription elongation and mainly during 3' end processing (Figure 7). This is in aggrement with the loading profile of Pcf11 on the transcription machinery which increases towards the gene 3' end. We can speculate that the ATPase activity of Sub2 might catalyse the transfer of Yra1 from Pcf11 to the nascent mRNP in conjunction with the recruitment of the other 3' end processing factors. Indeed, transfer of Yra1 may allow the 3' end processing factors to associate with Pcf11 and proceed with cleavage. These results suggest a mechanism for cotranscriptional assembly of the export competent mRNP and for coordinating export with 3′ end processing.

1.2.2.3.2.2 Npl3 recruitment of Mex67 is linked to 3' end processing events

As decribed previously, phosphorylated Npl3 is recruited to transcribing genes and Npl3 interacts with Mex67 and acts as an adaptor to recruit the export receptor on mRNP (Gilbert and Guthrie, 2004). The recognition of Mex67 by Npl3 requires Npl3 dephosphorylation by Glc7 a phosphatase associated with the cleavage/polyadenylation complex (CPC). Importantly, Glc7 induced Npl3 dephosphorylation also coincides with the dissociation of CPC from the mRNP, suggesting a role for Npl3 in coordinating 3' end formation with Mex67 recruitment. Accordingly, Npl3 has recently been proposed to compete with 3' end processing factor for binding to the nascent mRNA, protecting the transcript from premature termination (Bucheli and Buratowski, 2005).

1.2.2.3.3 Transcription and export

A number of factors implicated in mRNA export through pores have also been found in association with chromatin bound proteins and involved in transcription regulation, creating an unexpected link between the NPC associated mRNP export events and chromatin/transcription regulation.

1.2.2.3.3.1 Dbp5 could also function in mRNP biogenesis and transcription

As previously described, Dbp5 is mainly associated with the nuclear rim and plays a key role in mRNP translocation along the NPC channel. However, Dbp5 shuttles between the nucleus and the cytoplasm and homologue of Dbp5 is recruited to the mRNPs early during transcription in *Chironomus tentans* (Hodge et al., 1999; Strawn et al., 2001; Zhao et al., 2002). There is also evidence that Dbp5 associates with Yra1 containing complexes (Schmitt et al., 1999), suggesting a role for Dbp5 in earlier steps of mRNA biogenesis. Consistent with this observation, Dbp5 has been recently shown to interact with the early transcription factor TFIIH (Estruch and Cole, 2003) further suggesting that Dbp5 might function in transcription as well.

1.2.2.3.3.2 Sus1-Sac3-Thp1 complex links the NPC with transcription initiation

Another interesting complex located at the NPC has been identified that binds to mRNP components and is involved in mRNP export. This complex contains Sac3 and Thp1 (Fischer et al., 2002; Gallardo et al., 2003) and mutants of these two proteins have an mRNA export defect phenotype. Sac3 has also been found to associate with Sub2 and Mex67, and to genetically interact with Yra1 (Fischer et al., 2002; Lei et al., 2003; Strasser et al., 2002). Both Sac3 and Thp1 localize at the nuclear periphery through an interaction between Nup1 and Sac3, suggesting that the Thp1-Sac3 complex is involved in mRNA export with a primary role at the NPC. However, the Sac3-Thp1 complex was initially implicated in transcription elongation and genome stability (Gallardo and Aguilera, 2001). A third factor, Sus1 was also recently isolated as part of this complex (Rodriguez-Navarro et al., 2004). Sus1 has a similar location than Sac3 and Thp1, and *Δsus1* induces a similar poly(A)+ accumulation than loss of Sac3 and Thp1, indicating that it is required for proper mRNA export. Importanly, Sus1 is also part of the SAGA histone acetylase co-activator complex, binds to gene promoter regions and is required for efficient transcription. Furthermore, a fraction of Thp1 and Sac3 co-sediments with SAGA components, indicating the existence of a supercomplex. Although the functions exerted by Sus1 in transcription and export might be distinct and dependent on two

independent complexes, a new model emerged in the light of these findings, proposing that this supercomplex, called TREX2, may couple SAGA dependent gene transcription to mRNA export (Figure 8).

The interconnection between transcription, processing and export can also be a way to directly control the fate and the production of mRNP upon a defective or inefficient processing step, by directly blocking downstream events or inducing feedback on the upstream events.

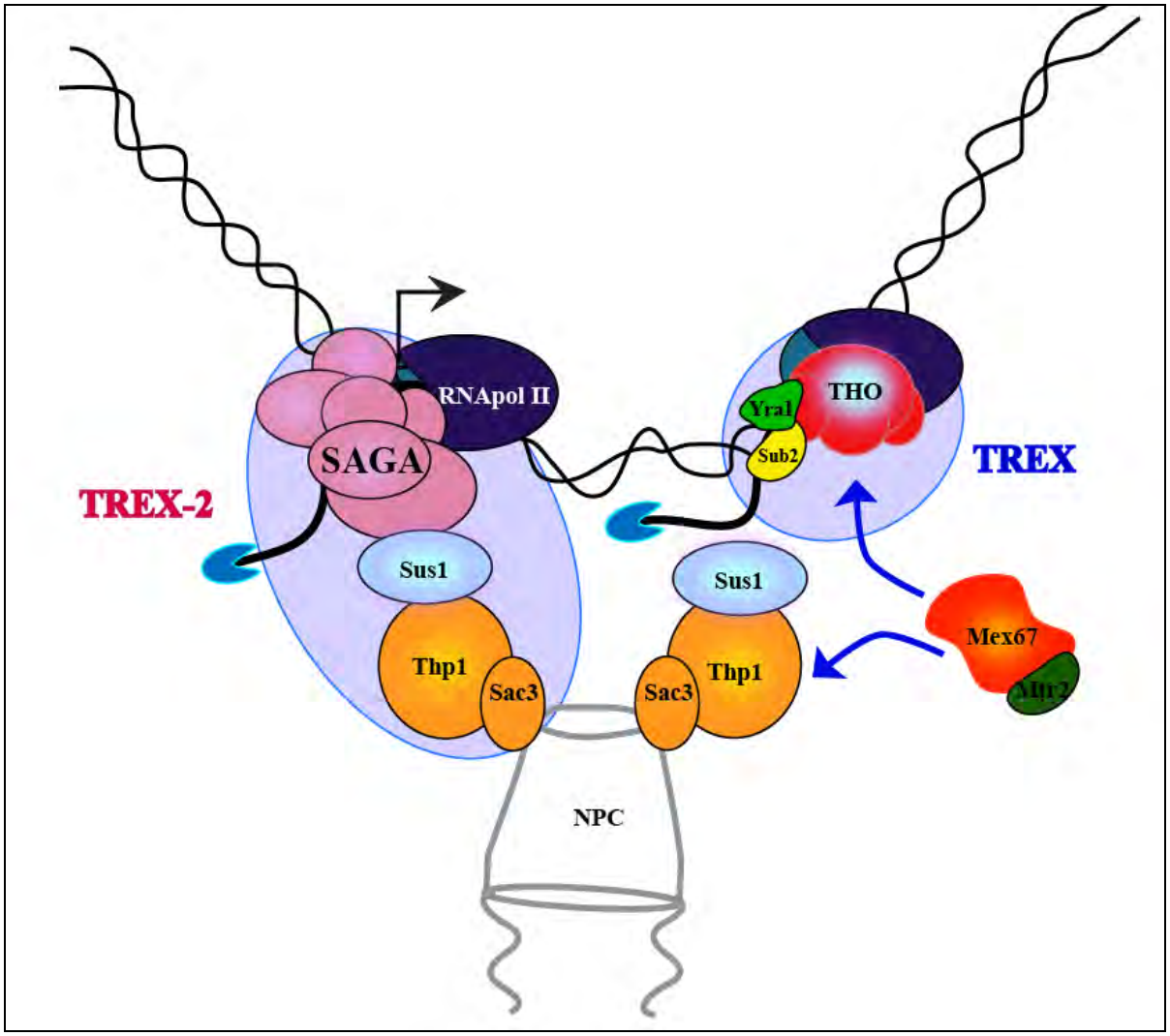


Figure 8. Model for transcription coupled export by the Sac3-Thp1 complex. Adapted from (Vinciguerra and Stutz, 2004). Sus1 connects the SAGA complex, involved in transcription initiation to Sac3-Thp1, a complex associated with the NPC. Sus1 may recruit Sac3-Thp1 to SAGA dependent transcripts. Alternatively, it may tether the SAGA dependent genes to the nuclear periphery. Sac3-Thp1 may also facilitate the docking of mRNP via interaction with both Mex67 and the nucleoporins. Of note, Sub2 also interacts with Sac3 (Fischer et al., 2002)(not shown).

1.2.2.4 Surveillance

RNA processing must be completed before mRNAs can be exported; however, the processing events generate a quantity of improperly processed transcripts that are retained within the nucleus. Concomitant to the mRNP processing and export pathways, the eukaryotic cells have evolved quality control mechanisms that prevent the export of suboptimal mRNPs and their translation (Sommer and Nehrbass, 2005). The nuclear exosome, a protein complex of several 3′–5′ exonucleases, is responsible for the retention and degradation of aberrant transcripts at sites of transcription (Saguez et al., 2005). Additional factors operate in quality control at the NPC, including the myosine-like poteins Mlp1 and Mlp2 and other associated proteins (Galy et al., 2004; Palancade et al., 2005; Vinciguerra et al., 2005).

1.2.2.4.1 Degradation by the nuclear exosome

The nuclear exosome is a conserved complex of several 3’ to 5’ exoribonucleases involved in RNA processing and degradation. In yeast it is distinguishable from its cytoplasmic counterpart by the exoribonucleas Rrp6. The nuclear exosome is critical for the monitoring and the degradation of several types of aberrant transcripts: unspliced pre-mRNAs (Bousquet-Antonelli et al., 2000), transcripts with abnormal 3’ ends or poly(A) tails (Burkard and Butler, 2000; Hilleren et al., 2001). In the absence of TREX components, nascent mRNAs are not correctly assembled into mRNPs, resulting in hybridization with the DNA template (R-loops formation) as well as defective polyadenylation (Garcia-Rubio et al., 2008; Huertas and Aguilera, 2003; Saguez et al., 2008). Rrp6 somehow “senses” aberrant mRNPs produced in TREX complex mutants and contributes to their accumulation and sequestration in foci near the transcription sites, presumably for further degradation (Libri et al. 2002). Consistent with this observation, nuclear exosome components were reported to be recruited to transcribing genes in drosophila (Andrulis et al., 2002a), and to interact with Yra1/REF both physically and genetically (Zenklusen et al., 2002). Together these observations strongly suggest the existence of a transcription-coupled surveillance mechanism by the exosome, ensuring that transcripts unfully or inefficiently processed are retained in foci at the transcription site for further processing and/or degradation.

1.2.2.4.2 Role of the NPC in an additional step of mRNP surveillance

In addition to the exosome, recent studies in yeast also point out a role for the NPC nuclear basket in monitoring mRNP quality control at the nuclear periphery, just before mRNP exit out of the nucleus.

One study showed that the loss of the nuclear basket Mlp proteins and their binding partner Nup60 leads to leakage of intron containing mRNA (Galy et al., 2004), indicating that the perinuclear Mlps implement a quality control step prior to export. In order to know more about the mechanism by which Mlp could prevent the export of faulty mRNA, Patrizia Vinciguerra, a Phd student in our lab, analysed the expression levels of an overexpressed reporter mRNA in the temperature sensitive mRNA export mutant, *GFP-yra1-8,* in presence or absence of the Mlps (Vinciguerra et al., 2005). These experiments revealed that the *GFP-yra1-8* mutant induces a significant decrease in mRNA production. Importantly, the loss of both Mlp proteins rescued both the termosensitive phenotype *GFP-yra1-8* mutant and the low mRNA level observed in *GFP-yra1-8* mutant, allowing a fraction of mRNA to reach the cytoplasm. In the light of these observations and other results, it was concluded that Mlp proteins are able to physically block and sequester faulty transcripts at the nuclear periphery and that this block may feedback on mRNA production by downregulating gene transcription. In aggrement with the view that Mlp proteins could physically associate with mRNP complexes at the nuclear periphery, interaction with a number of mRNP proteins including Yra1, Nab2, Mex67, Npl3, Sub2 and Cbp80 were found in interaction with the Mlp proteins (Vinciguerra et al., 2005). However, among these, only Nab2 interacted with Mlp1 or Mlp2 in an RNA independent manner, suggesting that Nab2 plays a key role in docking mRNP to the Mlp platform. These observations are consistent with the earlier proposition that Nab2 directly interacts with Mlp1 (Green et al., 2003). Furthermore, another recent study from the Corbett lab, determined that the N terminal domain of Nab2 interacts directly with the C terminal domain of Mlp1 (Fasken et al., 2008). In this study, cells expressing the Nab2 F73D mutant that cannot interact with Mlp1 exhibit nuclear accumulation of poly(A) RNA, providing *in vivo* evidence that Nab2 is important for targeting mRNAs to the nuclear pore for export. All these data suggest a model where the mRNA-binding protein Nab2 promotes mRNP docking to the Mlp gate. Mlps in turn would act as a selective filter that either promote nuclear export by guiding mRNAs competent for export for translocation through the NPC, or block nuclear export by retaining faultly mRNP. Consistent with the view of a tight interconnection between transcription, mRNP processing and export, the stalling of defective

mRNP at the Mlp gate may in certain cases negatively feeds back on transcription (Vinciguerra et al., 2005).

The analysis of the Mlp-dependent surveillance mechanism at the nuclear periphery has further been completed with a study by Palancade et *al*. that identified a previously uncharacterized open reading frame (renamed PML39), as an upstream effector of Mlps in the retention of faulty mRNP at the nuclear periphery (Palancade et al., 2005). Pml39 has been identified as genetically interacting with the NPC Nup84 complex in a synthetic lethal screen using a deletion of the *NUP133* gene. Strickingly, localization experiments of a Pml39-GFP fusion revealed that this protein localizes asymmetrically at the nuclear periphery with a pattern similar to what was previously reported for the Mlp proteins (Galy et al., 2004). Further localization experiments and a two hybrid screen established indeed that Pml39 is docked to a subset of nuclear pore complexes through interaction with Mlp1 and Mlp2. By performing epistatis analyses using a specific reporter gene to test the retention of unspliced mRNA, Palancade et *al.* also determined that Pml39 acts in the same pre-mRNA retention pathway than Mlp1 and Mlp2. Furthermore, consistent with this idea, overproduction of Pml39 resulted in the specific sequestration of intron containing mRNAs and Nab2 within discrete nuclear domains. These data suggest that Nab2/Mlps/Pml39 interactions mediate the retention of unspliced mRNP. Loss of Pml39 also recued the thermosensitive phenotype of the mRNA export factor mutants *GFP-yra1-8* and Δ*N-nab2*, indicating that, similar to a deletion of *MLP* genes (Vinciguerra et al., 2005), it can bypass the requirement for normal mRNP assembly factors. Thus, Pml39 is implicated in the retention of not only unspliced mRNA but also defective mRNP. Together, these data indicate that Pml39 is anchored at the nuclear pore basket through interaction with Mlps and is responsible for the function of Mlps in the nuclear retention of improperly formed mRNPs.

Another recent study from the Hurt lab, reported that the conserved RNA endonuclease protein Swt1 diplays strong genetic interactions with the THO/TREX and TREX-2 complexes as well as with Mlp1, Nup60, and the nuclear envelope protein Esc1, suggesting an implication of this protein in mRNP assembly and the perinuclear mRNP surveillance system (Skruzny et al., 2009). Indeed, deletion of *SWT1* enhances cytoplasmic leakage of splicing-defective pre-mRNAs in the absence of Mlp1 and Nup60. Consistent with Swt1 interacting with mRNA, overexpression of Swt1 causes strong nuclear poly(A)+ RNA accumulation. Analysis of the localization of Swt1 revealed that Swt1 is distributed throughout the nucleus and cytoplasm but becomes concentrated at NPCs in the *Δnup133* NPC clustering mutant.

Moreover, Swt1 lacking its endonuclease domain became concentrated in perinuclear spots even in cells with a normal complement of nucleoporins. These observations suggest that Swt1 endoribonuclease might be transiently recruited to NPCs to initiate the degradation of defective pre-mRNPs or mRNPs trapped at the nuclear periphery by Mlp/Pml39 in order to avoid their cytoplasmic export and translation (Skruzny et al., 2009).

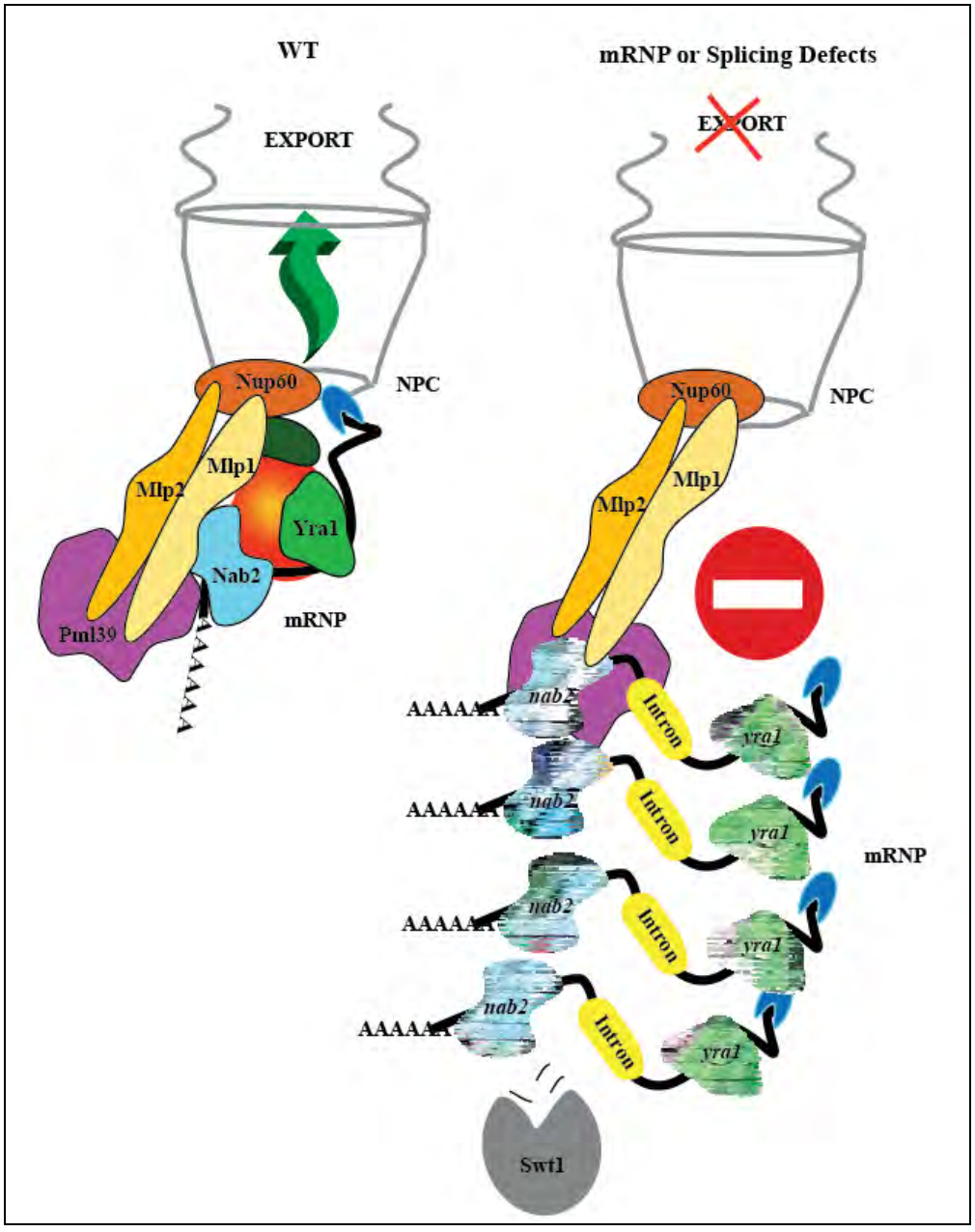


Figure 9. Model for the mRNP peripheral surveillance mechanism. In a wild type situation, Nab2 bound to the mRNP docks the mRNP to the Mlp gate which promotes nuclear export through the NPC channel. In presence of a splicing defect, in *nab2* and *yra1* mutants, and presumably upon an mRNP assembly defect, mRNP that dock to the Mlp gate are selectively trapped by Pml39, which retains them at the nuclear periphery, preventing their export. The endonuclease Swt1 may participate in the degradation of the accumulated defective mRNPs.

1.3 Nuclear periphery and chromatin organization

Chromatin is not randomly organized within the nucleus and experimental data have long revealed that chromatin fibers can be attached to the NE (Comings, 1968; Woollam et al., 1967), indicating that the NE plays a special role in the three dimensional organization of chromatin. Two types of chromatin can be discerned by electron microscopy analysis. A dark staining heterochromatin stays condensed throughout the interphase and is located in many cell types against the inner face of the NE and the nucleoli (Rae and Franke, 1972). A lighter staining euchromatin decondenses in interphase and is traditionally associated with transcriptional activity. Notably, the heterochromatin patches at the nuclear periphery are interrupted by the light staining of euchromatin at NPC locations. In the light of these observations, the "gene gating" hypothesis was proposed speculating that compact chromatin associates with the nuclear membrane while active transcribable chromatin associates with the nuclear pore, aiding in nuclear export of mRNA (Blobel, 1985).

The association of defined heterochromatin structures and silenced loci with the nuclear membrane subcompartments has clearly been established afterwards by several studies in most organisms (Guelen et al., 2008; Gotta and Gasser, 1996; Hochstrasser, 1987; Hochstrasser et al., 1986; Hochstrasser and Sedat, 1987; Kosak and Groudine, 2002; Mathog et al., 1984; Pickersgill et al., 2006; Walker et al., 1991). Furthermore, the presence at the nuclear periphery of DnaseI sensitive chromatin in vertebrate cells (Hutchison and Weintraub, 1985) was an early sign of the possibility that active chromatin also associates with the nuclear envelope. However, the confirmation of the existence of active chromatin at the nuclear periphery only recently emerged in budding yeast with the thrilling discovery that not only a set of highly active genes associates with NPC components (Casolari et al., 2004), but also that a number of these genes are specifically recruited to the nuclear periphery upon their transcriptional activation.

All together these data are in amazing agreement with the visionary "gene gating" hypothesis emitted by Blobel in 1985, and strongly indicate that the nuclear periphery is divided into at least two distinct functional parts that play a structural role in organizing chromatin: the nuclear membrane with its associated lamina and the NPC. Moreover, a growing number of studies recently proposed that these two different perinuclear environments contribute directly to the differential chromatin folding and to gene regulation.

1.3.1 Role of the nuclear periphery in facilitating silencing

Telomeres, the end of chromosomes, were the first genetic elements found to be localized at the nuclear periphery. They are in a peripheral location in *Drosophila*, *Trypanosoma*, plant and budding yeast (Gotta and Gasser, 1996; Hochstrasser et al., 1986; Mathog et al., 1984), but not in mammalian cells (Luderus et al., 1996; Vourc'h et al., 1993). In Human, the inactive X chromosome associates with the nuclear periphery (Walker et al., 1991). The mapping of genomic regions by microscopy on polytene-chromosome in *Drosophila* suggested that specific loci corresponding to "intercalary chromatin" associate with the nuclear envelope with high frequency (Hochstrasser, 1987; Hochstrasser and Sedat, 1987). This storage of presumably silenced loci at the nuclear periphery was further confirmed first in mammalian cells with the finding that silenced immunoglobulin genes are found at higher frequency at the nuclear periphery (Kosak and Groudine, 2002). Subsequently, in Drosophila and *Human* cells, the use of DamID high resolution molecular mapping revealed that a large number of specific sequences lacking active histone marks associate with B type lamins (Guelen et al., 2008; Pickersgill et al., 2006).

First evidences for an active role of the nuclear periphery in facilitating silencing and heterochromatin formation emerged with studies in the budding yeast *Saccharomyces cereviae.* This organism does not contain cytologically visible heterochromatin but heterochromatin-like structures are formed at telomeres and at silent loci such as the cryptic mating type loci *HML* and *HMR*. These heterochromatin domains are established and maintained through the coordination of *cis* acting elements or silencers and *trans* acting factors (Rusche et al., 2003). These *trans* factors include the telomere binding proteins Rap1, Abf1, Sir1 and Orc1 that in turn recruit the Sir2/Sir3/Sir4 complex which then spreads chromatin silencing along nearby genes. Andrulis et *al.* observed that mutation in the *HMR* locus silencer region results in a defective silencing of the locus that can be rescued by artificially anchoring the locus to the nuclear periphery, indicating that perinuclear localization is able to bypass the requirements of complete silencer sequences to establish transcriptionally silent chromatin domains (Andrulis et al., 1998). In budding yeast, silent telomeres are anchored to the nuclear membrane (Andrulis et al., 2002b; Gotta et al., 1996; Taddei and Gasser, 2004) through a least two redundant pathways (Gartenberg et al., 2004; Hediger et al., 2002b; Taddei and Gasser, 2004). One is mediated by the yKu heterodimer yKu70 and yKu80, which interacts directly with subtelomeric DNA or through association with the heterochromatin factor Sir4, and involves still unknown nuclear membrane proteins.

The second one is mediated by Sir4, which directly binds to the inner nuclear membrane protein Esc1, present on the nucleoplasmic surface primarily between pores (Hediger et al., 2002b; Taddei and Gasser, 2004). These anchoring pathways correlate strictly with chromatin repression since they involve direct interactions between the heterochromatin factor Sir4 with either Ku or Esc1 (Taddei and Gasser, 2004). Collectively these data suggest that crucial elements for gene silencing are concentrated at the nuclear membrane, which provides a localized structure for gene silencing to occur.

In higher eukaryotic cells, there are several indications that the nuclear lamina proteins play a major role in the organization and the formation of heterochromatin. It has been shown that lamins directly interact with chromatin (Belmont et al., 1993; Holmer and Worman, 2001; Luderus et al., 1992; Marshall, 2002; Paddy et al., 1990) and preferentially with silenced loci (Guelen et al., 2008; Makatsori et al., 2004; Pickersgill et al., 2006), presumably through direct interactions with core histones (Goldberg et al., 1999; Taniura et al., 1995). Interestingly, the lamin B receptor, a nuclear membrane protein that associates with lamin B, and the lamin associated LAP2β protein interact with the heterochromatic protein HP1 (Ye and Worman, 1996) and the histone deacetylase HDAC3 (Somech et al., 2005) respectively, two proteins that are specifically involved in the formation, the spreading and the maintenance of heterochromatin. All together these observations suggested that the nuclear lamina play a key role in chromatin repression. Confirming this view, mutations in human and mouse lamin A strongly disturb the nuclear envelope structure and result both in a displacement of heterochromatin from the nuclear periphery (Capell and Collins, 2006; Goldman et al., 2004; Sullivan et al., 1999) and reduced amounts of heterochromatin and associated H3K9 and H3K27 methylation (Lammerding et al., 2006; Shumaker et al., 2006). These observations suggest that formation or maintenance of heterochromatin requires the interactions of genomic loci with an intact and functional lamina. Finally, experiments aimed at testing whether localization to the nuclear lamina is a direct cause of transcriptional silencing have involved the tethering of a reporter gene to proteins normally associated with the lamina (Reddy et al., 2008). The results showed that targeting to the lamina results in silencing of the reporter gene and of nearby endogenous genes within a region of about 200 kb of flanking sequences on either side of the targeted sequence, confirming that the nuclear periphery environment actively contributes to the establishment of heterochromatin.

1.3.2 Insulator function of the nuclear periphery.

Insulators are regulatory elements that mark the boundaries of chromatin domains by limiting the range of action of enhancers and silencers (Burgess-Beusse et al., 2002). They are defined in most cases by *cis* elements called boundary elements and *trans* specific protein elements that bind to them. Boundary elements have been found in diverse organisms including mammals (Zhong and Krangel, 1997), chicken (Chung et al., 1993), flies (Kellum and Schedl, 1991) and yeast (Bi and Broach, 1999). Although the insulators vary greatly in their DNA sequences and protein compositions, they are thought to exert their boundary action by the establishment of structural chromatin layouts that physically delimit discrete and topologically independent chromatin domains. A number of studies in yeast, human and Drosophila revealed that insulators could organize chromatin fibers in at least two not mutually exclusive ways: the formation of chromatin loops and/or anchorage to topologically defined structures (Burgess-Beusse et al., 2002; Gerasimova et al., 2000; Ishii et al., 2002; Noma et al., 2006; Xu et al., 2004; Yusufzai and Felsenfeld, 2004; Yusufzai et al., 2004). Several studies proposed that the nuclear envelope serves as a substrate for attachment of chromatin by insulators and therefore plays a key role in regulating boundaries. Indeed, the *D. melanogaster* gypsy insulator often associates with the nuclear periphery (Gerasimova et al., 2000; Xu et al., 2004) and its perinuclear anchoring correlates with its insulator function. Consistent with this, in the yeast *Saccharomyces cerevisiae*, the direct artificial physical association of a partially silenced genomic mating-type locus *HML* to the NPC component Nup2 or to components interacting with the NPC such as the transport receptors Mex67, Cse1, Los1, was sufficient to block the spreading of the of heterochromatin and to turn on the silenced locus (Ishii et al., 2002).

The role of the NPC in displaying an insulator activity through direct or indirect interactions with chromatin might occur naturally in yeast. Indeed, in fission yeast the TFIIIC RNA PolII transcription factor has been shown to exhibit boundary activity (Donze and Kamakaka, 2001), and to localize with its associated loci at the nuclear periphery (Noma et al., 2006). It is likely that the chromatin TFIIIC complex is anchored at the periphery through interaction with the NPC, since the tRNA locus on which TFIIIC exerts an insulator function (Donze and Kamakaka, 2001) was also found to interact with the nucleoporin Nup2 (Schmid et al., 2006).

Other studies proposed that the boundary activity displayed by Nup2 may be mediated by the interaction of Nup2 with the Ran guanylyl-nucleotide exchange factor, Prp20p which

in turn might act with the histone variant H2AZ to delimit transcriptionally active promoter and alter nucleosome organization (Dilworth et al., 2005; Redon et al., 2002). These observations strongly indicate a structural and active role of the NE and more specifically the NPC in some chromatin remodelling activity to isolate the different chromatin domains.

1.3.3 Active genes at the nuclear pore complex

Consistent with the possibility of a tight coupling between transcription and export mediated by components associated with both the chromatin and the NPC (Rodriguez-Navarro et al., 2004), many active genes in yeast, have been found to physically interact with nucleoporins (Casolari et al., 2004). Moreover, the quantitative analysis of subnuclear localization of several inducible genes, including *INO1, HXK1, GAL* genes, *HSP104*, using a LacO/LacI tagging system revealed that these become preferentially positioned at the nuclear periphery when activated (Brickner, 2007; Brickner and Walter, 2004; Cabal et al., 2006; Casolari et al., 2005; Casolari et al., 2004; Taddei et al., 2006) and (this study). In the light of these observations, a role for the nuclear pore complex in chromatin activation and/or gene expression has been hypothesized. However, despite the considerable efforts that have been made to unravel the principles underlying this mechanism, the recruitment mechanism of genes at the NPC upon their transcriptional activation and its physiological relevance are still largely unknown and a subject of debate.

1.3.3.1 Recruitment mechanism

Where do genes relocalize at the NE? What step(s) in the activation/transcription processes initiate and are important for this movement? And finally what are the genetic elements and protein specific actors of this recruitment? In order to dissect the mechanisms by which inducible genes are dynamically relocalizing to the nuclear periphery depending on their transcription states, two main types of experiments have been performed: analysis of the general behavior of gene dynamics during transcription activation combined with the targeted or genome wide identification of the *cis* and *trans* factors involved. These studies produced numerous albeit controversial data. Nevertheless, clear principles have emerged and a putative model guiding the positioning of active genes to the nuclear periphery has arisen.

1.3.3.1.1 Where do the genes relocalize at the nuclear periphery?

The visual inspection of the trajectories of two inducibles genes (*GAL* and *HXK1* genes) over time revealed consistent and marked differences in the mobility between the repressed state and the actively transcribed state. Under repression the loci have a continuous diffuse

movement exploring a broad volume of nuclear interior; in deep contrast under activating conditions, the loci often have exclusive perinuclear localization with discrete trajectories along the NE. This confined motility of genes when activated indicates that they are spatially constrained at the nuclear periphery, seemingly by a non static type of peripheral tethering since sliding movements continue along the NE (Cabal et al., 2006; Taddei et al., 2006). The crosslink of active chromatin with components of the NPC (Casolari et al., 2004), suggests that active gene anchoring to the nuclear periphery reflects association with the NPC. This was confirmed by further microscopy experiments showing that the activated *HXK1* gene colocalizes with the NPC by using a mutant strain (Δ*nup133*) in which the nuclear pores cluster at one side of the nuclear rim (Taddei et al., 2006). Alltogether these findings indicate that the tethering of induced genes at the nuclear periphery might be mediated by transient or labile interactions with the NPC.

1.3.3.1.2 The NPC gene recruitment is not obligatory for transcription to occur but relies on transcription activation

Quantitative analysis of the NPC-recruitment of genes in activating conditions revealed that the NPC repositioning of activated genes greatly differs from gene to gene and is not uniform within a cell population, with in some cases peripheral confinement occurring only in half of the sampled cells (Brickner and Walter, 2004; Cabal et al., 2006; Casolari et al., 2004; Taddei et al., 2006). By performing RNA-fluorescence in situ hybridization (FISH) analyses on *GAL* gene transcripts, Cabal et *al.* observed that within a cell population, not all genes are being transcribed under the same activating conditions and that the transcribed genes are mostly found in peripheral positions (Cabal et al., 2006). These findings explained the inconsistency of inducible gene repositioning and demonstrated that the transcriptional state directly correlates with the position within the nucleus. In these experiments, some genes where also found to be transcribed and not associated with the nuclear periphery, indicating that NPC localization of inducible genes is not compulsory for their expression. Consistent with this observation, inducing *HXK1* through an alternative pathway using a LexA targeted VP16 activation domain, resulted in a loss of perinuclear anchoring although it did not affect the transcription process, indicating that stable NPC association is not an absolute requirement for transcription in yeast (Taddei et al., 2006). This last finding also led to the further suggestion that NPC localization of activated genes does not depend on the transcription process *per se*, but more likely depends on upstream specific activation events.

In order to explore this possibility, Schmid et al. designed a new method, called chromatin endogenous cleavage (ChEC), to map the genomic interaction sites of the nucleoporin Nup2 fused to micrococcal nuclease (Nup2-MN) *in vivo*. Confirming an interaction of the NPC with activated chromatin, this approach revealed that Nup2–MN cleavage occurs at the promoters of the *GAL* and *HXK1* genes upon activation with galactose. In agreement with the previous assumption that NPC gene localization depends on early activation steps and the mode of activation, Nup2 interaction at the *GAL* loci occurs close to the TATA box and requires the *GAL* gene activator Gal4 and their UAS and TATA box elements (Schmid et al., 2006). This study also showed that neither the SAGA activating complex nor active transcription are required for Nup2 interaction with promoters, indicating that NPC-gene interaction depends on very early activating events. However, the peripheral localization of the genes was never verified in this study, leaving open the possibility that the binding of Nup2 with chromatin could be due to non NPC associated Nup2. Consistent with this view, the contribution of Nup2 in the recruitment of activated genes to the NPC is a subject of controversy (Cabal et al., 2006). Nevertheless, in perfect agreement with the previous finding, the use of a thermo-sensitive mutant of RNA polymerase II shows that ongoing transcription is not required for either establishment or maintenance of *INO1* association with the nuclear periphery (Brickner, 2007). This set of data demonstrates that it is very likely that initiation of transcription rather than transcription *per se* is critical to promote gene anchoring to the NPC.

1.3.3.1.3 What factors are required for stable association of active genes with the NPC

In agreement with promoters and activation pathways being selective and essential for NPC recruitment of genes during activation, the galactose inducible genes require the components of the core SAGA complex Ada2, and the SAGA associated factor Sus1 for a detectable association to the NPC by microscopy (Cabal et al., 2006). In the light of these findings and the earlier observations that Nup2 interaction with galactose promoters does not require SAGA complex factors (Schmid et al., 2006), it can be suggested that if the initial association of these genes to the NPC is mediated by the specific transcription activators binding to promoters, it may be so weak and transient that it requires subsequent recruitment of general activator complexes, such as SAGA, to be stabilized and detectable by microscopy. This would mean that the association of active genes at the nuclear periphery may require more than one transcription related step to be stable. The study by Navarro-Rodriguez et *al.*

showing that Sus1 co-purifies with both the SAGA complex and the NPC associated Sac3-Thp1-Cdc31 export complex provided a molecular basis for one way through which the SAGA complex could stabilize NPC gene tethering (Rodriguez-Navarro et al., 2004). Consistent with this model, the export-complex component Sac3 and its pore ligand Nup1 are both needed for the stable relocalization process of *GAL* genes (Cabal et al., 2006). The important role of mRNP export factors together with the fact that the nuclear basket associated Mlp proteins interact with chromatin via an RNA dependent process (Casolari et al., 2005; Casolari et al., 2004), also suggest a role for Mlp proteins, RNA or other mRNP processing or export components in contributing to the dynamic tethering of activated gene at the NPC (this study). Thus, the stable association of yeast genes to the NPC could occur in several steps related to different transcription events. Consistent with this view, the *HXK1* gene requires its 3' UTR region for stable association at the NPC, suggesting that 3' end binding factors or 3' end formation are contributing to this process (Taddei et al., 2006). Collectively, these observations indicate that numerous sequential interactions may contribute to gene tethering, the individual importance of which may vary from gene to gene.

1.3.3.2 Physiological relevance

Very little information is available on the main physiological advantage for NPC gene gating. However, different lines of evidences allow speculating how this process could impact on gene expression.

The stable association of genes with the NPC is not an obligatory feature for the transcription process *per se* to occur (Cabal et al., 2006; Taddei et al., 2006). Nevertheless, this does not exclude the possibility that the NPC environment is involved in a transcription regulatory function. Indeed, three studies reported that artificial tethering of genes to the NPC renders their induction more efficient, suggesting that the NPC sublocalization confers maximal activation levels (Brickner and Walter, 2004; Menon, 2005; Taddei et al., 2006). There are several ways by which the NPC could positively influence gene expression. First, the insulator activity of the NPC nucleoporin Nup2 could allow the separation of silenced from active regions and the establishment of a more active domain in the NPC vicinity (Dilworth et al., 2005; Ishii et al., 2002). Second, the nuclear face of the NPC could specifically recruit transcription activator complexes. Illustrating this model, it has been shown that artificial tethering of genes to the Nup84 nuclear pore subcomplex activates their transcription at the periphery through the recruitment of the Rap1-Gcr1-Gcr2 co-activator complex (Menon, 2005). Reinforcing the view of a role of the NPC in activating Rap1

controlled promoters, genes under Rap1-Gcr1 regulatory control preferentially crosslink with NPC components (Casolari et al., 2004). Third, juxtaposition of promoter and terminator sequences resulting in gene looping has been proposed to facilitate transcription reinitiation thereby increasing transcription efficiency (Ansari and Hampsey, 2005; Proudfoot, 2004). As 3' UTR regions also seem to be important in stabilizing the gene NPC connection (Taddei et al., 2006), we can speculate that the NPC could contribute to formation of such transcriptional loop units.

Collectively, these observations suggest a model where the NPC provides a favorable environment to positively influence transcription (Blobel, 1985). An interesting view is that it could provide a multifunctional platform analogous to "transcription factories" described in higher eukaryotes where positive chromatin remodeling, transcription activators and mRNA processing and export factors concentrate, thereby promoting the overall gene expression efficieny. However most of these potential roles of the NPC environment in influencing transcription regulation are highly speculative and require further investigation in order to uncover the underlying mechanisms.

Since the diffusion coefficient of RNA molecules is such that the position of a gene with respect to the NPC should play little role in the export efficiency of its mRNA (Gorski et al., 2006), gene gating is unlikely to promote export efficiency by shortening the path between the sites of mRNA production and export. However, proper mRNP formation is the main rate limiting export event and is the resultant of strong interconnections between the transcription, mRNA processing and export steps (See Introduction Chapter 1. 2). The physical tethering of the transcription site to the NPC export site fits perfectly in this model and suggests that NPC gene gating plays a major role in promoting the coordination of mRNP biogenesis events with the control of the mRNP quality.

1.3.3.3 Conservation througout evolution

Several studies indicate that the nuclear envelope subnuclear compartment is also not exclusively repressive in other species, supporting the view that gene activation at the nuclear periphery may be conserved throughout evolution.

In mammalian cells, two studies reported that gene activation could occur at the nuclear periphery. First it was demonstrated that the IFN-gamma gene was constitutively associated with the nuclear periphery, even when primed for expression during T helper cell differentiation (Hewitt et al., 2004). Second, Ragozki et *al*. (Ragoczy et al., 2006) showed that

at the time of activation, the β-globin locus is localized at the nuclear periphery and only moves to the nuclear interior at a later stage.

The malaria parasite *Plasmodium falciparum* undergoes antigenic variation to evade host immune responses through switching expression of variant surface proteins encoded by the *var* gene family. It has been demonstrated that the *var* genes are constitutively located at the nuclear periphery independently of their transcription state. However, they localize at two distinct peripheral sites depending on their transcription state. This bipartite localization is essential for the establishment of repression as well as for the switch between repressive and activated states, presumably because activation involves repositioning into another peripheral location permissive for transcription (Duraisingh et al., 2005; Ralph et al., 2005).

In *Drosophila*, the male X chromosome associates with the nuclear periphery in part through interaction with the NPC components Nup153 and mTOR, homologous to the yeast Mlps. These interactions have been involved in the recruitment of the MSL complex required for the two-fold increase of X-linked genes transcription levels, a process known as dosage compensation (Mendjan et al., 2006).

1.4 Other Roles for the NE and the NPC in yeast

Increasing evidences indicate that the NE and the NPC also function in several aspects of genome stability and integrity, and even cellular integrity by participating in the cell cycle progression as well as in cell division and aging control. Although, most of these roles have been discovered in yeast, analogous mechanisms have also begun to emerge in other organisms.

1.4.1 Role of the Mlp proteins in Telomere length control

As previously described, yeast telomeres are tethered to the nuclear periphery. Thus, it is not surprising that telomere length maintenance involves factors that contribute to their localization. Indeed, the Yku complex, one of the components that is responsible for telomere anchoring to the periphery, has also been involved in telomere protection as Yku mutants exhibit short telomeres (Driller et al., 2000). However, although the NPC associated Mlp proteins are not involved in the anchorage of telomeres to the nuclear periphery, mlp mutants also have an effect on telomere length (Hediger et al., 2002a). Indeed, Hediger et *al*. found that in *mlp1/2* deficient strains telomeres are more extended than in wild type, indicating an unexpected role for these NPC associated proteins in impairing telomere elongation or promoting telomere degradation. This role is independent of the Yku complex, but is at least partially dependent on Tel1, a kinase implicated in both a checkpoint response and telomerase positive regulation (Mallory and Petes, 2000; Ritchie et al., 1999; Ritchie and Petes, 2000). One hypothesis is that Mlp proteins somehow inhibit the activity of Tel1. Notably, Rap1 and Rif1 binding also inhibits Tel1 in order to slow down telomerase when telomeres are too extended. The model proposed for Mlps mediated telomere length regulation is that the Mlp proteins may directly interact with either Tel1 or Rif1 to modulate their activity in telomere length control (Hediger et al., 2002a).

1.4.2 Role of the NPC in Double strand breaks (DSBs) repair

1.4.2.1 NPC and DNA repair in yeast

Although the role of the NPC in telomere anchoring has been debated, NPC componenents have recently been reported to contribute to telomere anchoring (Therizols et al., 2006). Therizols et *al* show that NPC components of the Nup84 core complex, Nup84, Nup145C, Nup120, and Nup133, serve to anchor telomeres at the nuclear periphery and even contribute to repression of the subtelomeric region. Therefore, Yku70 or Sir4 interactions with Esc1 may not be the only ways to anchor telomere at the periphery.

Interestingly, earlier work showed that repair efficiency of DSBs depends on the position of the DSBs on the chromosome (Ricchetti et al., 2003). In their work, Therizol et *al* tested wether these differences in DSB repair rate could be linked to telomere position by analyzing the survivals rates after induction of DSB in mutant of the Nup84 complexe, in which telomere are delocalized. They found that altering the integrity of the Nup84 complex decreases the efficiency of repair only when DSBs are generated in the subtelomeric region, suggesting a specific role for the Nup84 complex and telomere anchoring in subtelomeric DSB repair.

A role for the Nup84 complex and other NPC components in DNA repair had previously been suggested. First the loss of Mlp proteins and of components of the Nup84 complex increases cells sensitivity to DNA damaging agents (Chang et al., 2002; Galy et al., 2000; Hediger et al., 2002a; Kosova et al., 2000). Second, synthetic lethal screens revelead strong genetic interactions between components of the Nup84 complex (Nup133, Nup120 and Nup84) and all the components of the Rad52 DSB repair pathway, suggesting that they are functionally related. Consistent with this view, loss of Nup133 results in an increased number of spontaneous DNA repair foci containing Rad52, indicating that impairment of the Nup84 complex integrity results in the accumulation of unrepaired DNA damages (Loeillet et al., 2005).

A recent study proposed a molecular mechanism for a function of the NPC in DNA repair (Palancade et al., 2007). It revealed that in addition to the Nup84 complex, the Nup60/Mlp1/Mlp2 complex as well as the SUMO protease Ulp1 exhibit genetic interactions with members of the Rad52 repair pathway and are required to prevent DSB accumulation. These data suggest that the Nup84 complex, the Nup60/Mlp1/Mlp2 complex, and Ulp1 could be involved in a common DNA damage repair pathway through sumoylation-dependent processes. DSBs can be repair via two distinct mechanisms: homologous recombination (HR) which involves Rad52 and non homologous end joining (NHEJ) which mainly involve Yku70. Utilization of a specific reporter system allowing to distinguish between these two pathways {Karathanasis, 2002 #380, revealed that the nucleoporin and *ulp1* mutants do not affect the *RAD52*-dependent HR pathway, but rather impair the Yku70-dependent NHEJ pathway. Therefore, these observations indicate that the NPC and Ulp1 are specifically required for DSB repair through the NHEJ pathway.

Ulp1 in *Sacharomyces cerevisiae* is the main SUMO protease {Li, 1999 #1217} and is mainly found at the nuclear periphery as established by the analysis of Ulp1-GFP fusion by

microscopy (Li and Hochstrasser, 2003; Palancade et al., 2007). This peripheral localization requires the N-terminal domain of Ulp1 that contains binding sites for the karyopherins Kap121 and Kap60–Kap95 (Li and Hochstrasser, 2003; Panse et al., 2003) for proper targeting of Ulp1 to the nuclear envelope (Makhnevych et al., 2003; Panse et al., 2003). the nuclear basket protein complex Nup60/Mlp1/and Mlp2 (Palancade et al., 2007; Zhao et al., 2004a), the Nup84 complex (Palancade et al., 2007) and the nuclear envelope protein Esc1, that affects the nuclear basket assembly (Lewis et al., 2007), indicating that Ulp1 is mainly anchored at the NPC through a complex interplay between different tethers.

Importanly, loss of NPC-associated Ulp1 in nucleoporins mutants or deletion of its N terminal domain results in the release of Ulp1 from the nuclear periphery, as well as an increased proteasomal degradation of this SUMO-protease (Palancade et al., 2007; Zhao et al., 2004a). These mutants exibit complex alterations of the cellular sumoylation profiles (Lewis et al., 2007; Palancade et al., 2007; Zhao et al., 2004a) with both increases and decreases of the sumoylation levels of specific targets. Notably, Ulp1 is involved in two aspects of SUMO metabolism, i.e, the removal of SUMO from sumoylated targets and in an early step of SUMO processing. Therefore the altered sumoylation profiles observed may reflect that mislocalization of Ulp1 alters both its substrate specificity giving access to non natural substrates of Ulp1 for SUMO degradation (Lewis et al., 2007; Palancade et al., 2007; Zhao et al., 2004a) and SUMO availability resulting in a reduced level of sumoylation of some of the targets (Palancade et al., 2007). Several studies that attempted to define the pool of sumoylated proteins in *S. cerevisiae* (Hannich et al., 2005; Panse et al., 2004; Wykoff and O'Shea, 2005; Zhao et al., 2004b) showed that sumoylated proteins are involved in various important mainly nuclear processes, including DNA repair. Indeed, among the proteins identified, the NHEJ repair pathway protein Yku70 has been found as being a target for SUMO modifications both *in vitro* and *in vivo* (Zhao and Blobel, 2005).

Analyses of Yku70 sumoylation levels revealed that these are strongly reduced upon *NUP120* and *NUP60* deletion as well as in *ulp1* mutants lacking the N-terminal region, indicating that mislocalization of Ulp1 impairs Yku70 sumoylation and presumably activity (Palancade et al., 2007). In agreement with this, the restoration of Ulp1 level at the nuclear periphery in the delocalization mutants rescues the normal sumoylation profiles and the DNA repair related phenotypes. These results strongly suggest an important role for the NPC in the NHEJ DNA repair pathway through the regulation of the SUMO protease Ulp1 substrate specificity or stability. Importantly, other SUMO modified proteins involved in DNA repair

could be regulated by Ulp1. Indeed, the absence of Yku70 does not lead to Rad52 foci accumulation whereas delocalization of Ulp1 does, therefore indicating that the role of Ulp1 in DNA repair may be larger that only regulating Yku70 activity in the NHEJ pathway.

Reinforcing the idea of an important role of the NPC in DSB repair, a recent study reported the existence of another pathway mediated by Nup84 for DSB recovery (Nagai et al., 2008). It has been reported that two components of a heterodimer complex acting in DNA repair, the protein Slx5 which harbors SUMO recognition motifs, and the ubiquitin ligase Slx8, physically associate with Nup84. Furthermore, induced DSBs associate with the nuclear pore and their localization requires Nup84 (Nagai et al., 2008). Overall, these data support a model for a novel SUMO dependent DNA repair pathway, requiring Nup84 and Slx5/Slx8, which would specifically recruit DSBs to the NPC to resolve them.

Based on this study and the requirement of Nup84 for subtelomeric DSB repair, it has become clear that the NPC mediate additional repair pathways for DSBs that are specifically localized at the nuclear periphery. One can speculate that NPC mediated DSB repair mechanisms are dependent on specific signaling pathways that determine DSB spatial localization. What distinguish the peripheral DSBs from others and why do these DSBs need specific additional repair pathways is not yet clearly elucidated. In the case of subtelomeric regions, additional NPC mediated repair mechanisms may be required to compensate for the particularly inefficient recombination activity in these regions (Ricchetti et al., 2003). Since the telomere binding protein Rap1 inhibits NHEJ between telomere ends (Pardo and Marcand, 2005), repair inefficiency at subtelomeric regions is likely to be due to their chromatin structure. For other DSBs, Nagai et *al*, proposed that irreparable DSBs and collapsed forks are specifically recruited at the NPC. Indeed, stalled replication forks and DSBs reparable by HR do not relocalize at the nuclear periphery (Nagai et al., 2008). The current view is that sumoylated protein may accumulate at these DSBs requiring Slx5/Slx8 ubiquitylation of for proteasomal degradation at the NPC.

All together, these different studies established a clear role for the NPC in ensuring genome integrity. In one hand, NPC is involved in DNA repair by regulating Ulp1 activity and thus the activity of the different DNA repair pathways controlled by sumoylation. In another hand it displays important function in the DNA repair of DSBs that are specifically localized at the nuclear periphery (telomere, NPC recruited of DSBs). One common point between most if not all these NPC mediated repair mechanism is that they relies on SUMO metabolism. There are no experimental evidences for an implication of Ulp1 in the repair

pathways mediated specifically at the NPC. However, although not involve in the recruitment mechanism Ulp1 could still be implicated at later stage in the pathway.

1.4.2.2 NE and DNA repair in other organisms…

A role for the nuclear periphery in DNA damage repair has also been reported in other organisms indicating a conserved role for the NE in genome integrity throughout evolution.

In *Aspergillus Nidulans*, a mutation encoding a single amino acid substitution within the nucleoporin Nup98 causes a significant DNA damage sensitivity at 42°C, suggesting that NPC is involved in a new pathway of damage response (De Souza et al., 2006).

In higher eukaryotes, mutations affecting the processing of the nuclear lamin A result in increased sensitivity to DNA damaging agents, an elevated DNA damage response as well as a senescent phenotype. These observations underscore the role of the nuclear envelope in maintaining genomic stability and the interplay between nuclear architecture and the DNA damage response (Lees-Miller, 2006).

1.4.3 Other NPC functions mediated by Ulp1

Because SUMO modifications regulate a large number of proteins involved in different essential cellular process, the NPC might play a key role in other SUMO regulated processes by controlling the activity of Ulp1. Indeed, a role of the NPC in regulating various SUMO regulated functions, via Ulp1, has clearly been established.

1.4.3.1 Clonal lethality

The first study that established that the nuclear basket Nup60/Mlp1/Mlp2 complex anchors Ulp1 at the nuclear periphery also revealed a role for the NPC in ensuring cell viability via Ulp1 activity regulation (Zhao et al., 2004a). This work showed that deleting *MLPs*, *NUP60* or affecting sumoylating or desumoylating enzymes results in the formation of "nibbled" colonies leading to cell death, a phenotype described as clonal lethality. It further demonstrated that clonal lethality is due to an increased level of 2 micron circles, an extrachromosomal plasmid found naturally in *S. cerevisiae* (Volkert and Broach, 1986). Moreover, *NUP60* or *MLPs* deletion result in the delocalization of Ulp1 from the nuclear periphery, which leads to a drastic decrease of Ulp1 levels, and restoring Ulp1 levels in these strains, rescued the "nibbled" colony phenotype and the levels of 2 micron circles. These results indicate that clonal lethality is due to decreased levels of Ulp1 in these strains. Consistent with this, a role of Ulp1 in preventing 2 micron circles proliferation and "nibbled"

colony phenotype has also been reported by another study (Dobson et al., 2005). These observations indicate a role of the NPC in protecting cell viability by regulating 2 micron plasmid proliferation via stabilizing Ulp1 levels. Sumoylation may regulate 2 micron circle levels by different mechanisms. One involves the recombinase Flp1, an enzyme encoded by 2 micron plasmids and which is necessary for its replication: Flp1 increases 2 micron levels and modification by SUMO negatively regulates Flp1 levels (Chen et al., 2005). Other sumoylated proteins with possible roles in plasmid partitioning can also be involves in this process (Chen et al., 2005; Dobson et al., 2005).

1.4.3.2 Regulation of the septins during cell cycle

The septins make up a family of guanine-nucleotide binding proteins, some of which polymerize to form the septin ring at the bud neck of budding yeast. The septin ring appears before bud formation in G1 phase of the cell cycle and extends through the bud neck as the bud grows forming a double ring which later divides during cytokinesis, and persists in the mother and daughter until disassembling during G1 (Lew, 2003; Longtine and Bi, 2003). Of note, at least three septins (Cdc3, Cdc11, and Shs1) become sumoylated before the anaphase during mitosis and undergo desumoylation at cytokinesis (Johnson and Blobel, 1999; Takahashi et al., 1999). The function of this modification is not fully understood. One proposal is that it plays a role in the disassembly of the septin ring (Johnson and Blobel, 1999).

Interestingly, Septin desumoylation requires Ulp1 (Takahashi et al., 1999), indicate that Ulp1 might be released at some point from the NPC to gain access to the cytoplasmic septins. As described above, Ulp1 localization at the NPC is controlled by a complex mechanism involving different nucleoporin complexes and the shuttling karyopherins Kap121 and the Kap95p–Kap60p complex (Li and Hochstrasser, 2003; Panse et al., 2003). Importantly, defects in any of these karyopherin interactions lead to abnormalities in the cycle of septin sumoylation and desumoylation. These observations indicate that these karyopherins might regulate septin disassembly by controlling the transient released of Ulp1 from the NPC during cytokinesis (Makhnevych et al., 2007). More generally, these data suggest an important function of the NPC in regulating the timing of septin desumoylation.

The NPC through the regulation of Ulp1 activity might be involved in a much broader range of SUMO-regulated mechanisms. Notably, it has been established that the NPC environment promotes gene expression and several transcription factors or chromatin remodeling complex are modified by SUMO (Hannich et al., 2005; Panse et al., 2004;

Wykoff and O'Shea, 2005; Zhao et al., 2004b). On the light of these findings we can speculate for a role of the NPC in transcription regulation via Ulp1 (This study). Homologues of Ulp1 have been found in other organism, notably in *Drosophila* where Ulp1 is also associated with the NPC (Smith et al., 2004), suggesting that the regulation of Ulp1 through NPC tethering may be conserved throughout evolution.

1.4.4 Role of the NE in maintaining DNA repeat stability

Maintenance of genomic stability requires a mechanism to minimize homologous recombination between DNA repeats. One proposed mechanism ensuring repeat stability is the assembly of DNA into silent chromatin thought to limit the access to the recombination machinery (Moazed, 2001). Consistent with this view, the stability of the highly repetitive ribosomal DNA (rDNA) sequences requires the recruitment of the RENT (regulator of nucleolar silencing and telophase exit) complex containing Sir2 and Cohibin (mitotic monopolin proteins Lrs4 and Csm1) resulting in rDNA silencing (Bryk et al., 2002; Huang and Moazed, 2003; Moazed, 2001; Smith and Boeke, 1997). Recently, the NE has also been involved in this mechanism. Indeed, biochemical analysis revealed that Cohibin associates with two proteins of the inner nuclear membrane, Heh1 a nd Nur1, indicating a physical link between rDNA associated silencing complexes and the NE. Moreover, loss of these two INM proteins leads to an increase in rDNA recombination between sister chromatids, indicating that the NE environment promotes rDNA stabilization. Microscopy analyses showed that rDNA is separated from the bulk of DNA through its localization towards the nuclear periphery. It has also been shown that the absence of the two nuclear membrane proteins Hde1 and Nur1 or components of the RENT complex results in a disruption of this peripheral organization, indicating that these components act in a pathway that anchors rDNA loci at the nuclear periphery. In addition, these observations suggest that the maintenance at the nuclear periphery might be critical for rDNA silencing and stability. Indeed, artificial targeting of rDNA repeats to the INM results in the suppression of the instability observed in absence of components of the RENT complex. However, it did not restore totally rDNA silencing loss, indicating that NE tethering rather than silencing mainly prevents recombination within the rDNA locus. These findings established another role for the NE in the perinuclear localization of chromatin and showed that tethering to the NE via interactions between INM proteins and chromosomal proteins is required for genome stability. Interestingly, Heh1 and Nur1 INM protein homologues have also been implicated in chromosome organization in metazoans

(Reddy et al., 2008), indicating that this anchoring mechanism has been partially conserved throughout evolution.

1.4.5 The NPC functions in cell division by promoting spindle pole body assembly in yeast.

In yeast, the spindle pole bodies (SPBs) are the sole yeast microtubule-organizing centers and they are directly embedded within the NE throughout the cell cycle (Jaspersen and Winey, 2004). The SPBs play critical roles in forming the microtubules responsible for their separation and chromosome segregation as well as in ensuring that nuclear migration and division occur in concert with DNA replication and bud formation. Several observations suggested a functional relationship between SPBs, the NE, and NPCs (Chial et al., 1998; Iouk et al., 2002).

First, some components are shared between these structures such as Ndc1, a nuclear membrane protein which is required for proper SPB duplication (Winey et al., 1993) and colocalizes with both the SPBs and NPCs. In addition, proteins known to regulate spindle function are found to reside at the NPC. Indeed, Mad1 and Mad2, two proteins required for the execution of the spindle checkpoint, reside predominantly at the NPC throughout the cell cycle in association with a subcomplex of nucleoporins containing Nup53, Nup170, and Nup157. Upon activation of the spindle checkpoint, Mad1 becomes hyperphosphorylated in a Nup53 dependent manner and triggers the release of Mad2 from the NPC allowing its accumulation at kinetochores (Iouk et al., 2002).These events indicate a direct role for the NPC in regulating the spindle assembly and thus promoting cell cycle.

In addition, other biochemical analysis revealed a direct association between the NPC basket protein Mlp2 and SPB core components (Niepel et al., 2005). Since lack of Mlp2 displays delays and errors of cell division, it has been proposed that the association of the SPB core with Mlps through direct interaction with Mlp2 is required for a proper assembly of SPB components. Indeed, the absence of Mlp2 and Mlp1 leads to smaller SPBs, indicating that the Mlps are required for a proper incorporation of components into the SPBs (Niepel et al., 2005), thereby providing another mechanism by which the NPC promotes cell division.

Finally, a recent study pointed out novel genetic interactions between components of the SPB and components of the NPC. A screen searching for mutants synthetically lethal with loss of the nucleoporin Nup1 revealed an interaction with the components of the SPB Nud1. In addition to its function in the SPB, Nud1 acts in the mitotic exit network. Two other

proteins, Bub2 and Bfa1 that function in the mitotic exit network also display genetic interactions with Nup1 and colocalize with Nud1. Thus, these results indicate a novel functional connection between Nup1 and proteins comprising both the spindle pole body and early mitotic exit network (Harper et al., 2008).

All together these data show that the NE structures, the NPC and SPB, function in concert to ensure proper cell division process in yeast.

1.4.6 Role of the NPC in retaining ageing markers within mother cells during cell division.

S. cerevisiae multiplies through budding of a new daughter cell from the surface of its mother (Pruyne and Bretscher, 2000). Although mother cells age and have a determined fitness and lifespan, they are able to give birth to cells with a reset fitness and lifespan. This suggests that the factors defining the age of the mother remain confined to the mother cell during division. One of the factors that contribute to cell ageing is the accumulation of extrachromosomal ribosomal DNA circles (ERCs) in the mother cells over time (Murray and Szostak, 1983; Sinclair and Guarente, 1997). These observations indicate that the yeast nucleus divides asymmetrically protecting daughter cells from inheriting ERCs. The mechanism of this confinement has been recently uncovered and relies on the NE structures (Shcheprova et al., 2008). This work show using fluorescent loss in photobleaching, that during cell division NPC complexes are retained in the mother nucleus by a septin diffusion barrier located at the bud neck, and that pores in the buds results from *de novo* insertions of NPC. Moreover, it has been demonstrated that plasmid retention within the mother cell depends on the presence of the septin diffusion barrier as well as on the nuclear basket proteins Mlp1 and Mlp2, indicating that the barrier restricts plasmid diffusion by means of plasmid anchorage to NPCs. Loss of the diffusion barrier integrity results in ERCs sharing between mother and their buds, an extended lifespan for mother cells, and buds that fail to rejuvenate. These data underline the importance of the NE in controlling ageing and age segregation during mitosis. Finally, in mammalian cells, the contribution of lamin mutations to progeria also suggest a link between the NE and ageing (Navarro et al., 2006; Scaffidi and Misteli, 2006).

1.5 Aim of the study

Numerous studies have shed light on how the different steps of gene expression from transcription regulation to export and mRNA translation occur and revealed that all these events are functionally and even physically tighly interconnected. Importantly, the NPC appears as a key central multifunctional platform mediating this coupling.

When this work was initiated, our lab had identified a striking role of the NPC nuclear basket proteins Mlp1/2 in negatively feedbacking on transcription regulation in response to accumulation of defective mRNPs (Vinciguerra et al., 2005). At that time it was not obvious how Mlps could regulate transcription. This view became a more and more valid possibility since in parallel to these findings, increasing evidences for a physical tethering of active genes to the NPC emerged (Casolari et al., 2004; Rodriguez-Navarro et al., 2004). On the basis of microscopy experiments it was even demonstrated that a number of inducible genes were able to move towards the nuclear periphery when activated. At that time the mechanisms guiding gene movement were really poorly characterized. In light of our initial proposal that Mlp proteins contribute to mRNP quality control by physically docking mRNP, we speculated that this interaction and the interaction of other export factors associated with the NPC with the growing mRNP could mediate at least in part the interaction between the transcription site and the NPC.

The first objective of this thesis was to better characterize the process guiding inducible genes towards the NPC when activated. In particular, we wanted to test wether the growing mRNP could stabilize this connection, by investigating the importance of mRNA, mRNP and export factors in this mechanism. A second objective, directly derived from the first one, was to understand the physiological relevance of this process by investigating how the NPC environment could impact on gene expression efficiency.

2 Results

The results are presented in the two following sections:

SECTION 2.1: This section is presented in the form of a publication preceded by an overview including background, specific aims and summary of the results. It is followed by additional results which completed the study and which were partially published in the context of collaboration with the D. Libri laboratory.

SECTION 2.2: In this second section, I assembled my data in the form of a template article to be submitted for publication.

2.1 Cotranscriptional recruitment to the mRNA export receptor Mex67 contributes to nuclear pore anchoring of activated genes.

2.1.1 Background

The proposal of an interphase chromatin organization maintained by the attachment of the chromatin to the nuclear membrane has been first considered in 1968 (Comings, 1968). On this basis, several further observations led to the birth of the "gene gating" hypothesis (Blobel, 1985): compact chromatin associates with the nuclear lamina, while transcribable genes associate with the NPC to favor their expression. This innovative model was subsequently experimentally challenged with the extensive development of techniques and research in the chromatin field. However, experimental evidence confirming an interaction of active genes with the NPC only recently emerged in yeast. It all started with the striking discovery that artificial anchoring of a genomic silenced gene to the NPC blocks the spreading of heterochromatin and positively influences its expression (Ishii et al., 2002). Later on, Silver and colleagues provided direct indication for the association of a set of active genes with the NPC using NPC chromatin immunoprecipitation combined with microarrays (ChIP on chip) (Casolari et al., 2004). Moreover, another study from the Silver group revealed that genes involved in galactose metabolism or mating response relocated from the nuclear interior to the nuclear periphery upon transcriptional activation (Casolari et al., 2005; Casolari et al., 2004). Meanwhile, the Hurt laboratory identified Sus1 as a component of both, the SAGA complex and the mRNA export machinery associated with the NPC (Rodriguez-Navarro et al., 2004). This study reinforced the view of a tight coupling between transcription and RNA export (see Introduction) and provided the first genetic evidence for components that could physically link an active genomic locus to the NPC. Taken together, these studies indicated the existence of an amazing mechanism that dynamically recruits inducible genes to the nuclear periphery in the course of transcriptional activation.

These findings stimulated extensive research on the underlying recruitment mechanism of a number of specific inducible yeast gene (*INO1, HXK1, GAL1*) and produced numerous albeit controversial data (see Introduction and Discussion) (Abruzzi et al., 2006; Brickner and Walter, 2004; Cabal et al., 2006; Taddei et al., 2006). A variety of nuclear pore proteins, transport factors and transcription activators have been involved (among which Nup2, Nup1, Mlp1, Mlp2, Nup60, Sac3, Sus1, Ada2, Hac1) in this mechanism at several specific loci (Brickner and Walter, 2004; Cabal et al., 2006; Casolari et al., 2005; Casolari et al., 2004;

Drubin DA, 2006). However, the precise molecular basis for the targeting and the tethering of genes is still not elucidated.

The physiological relevance of this process remains even more mysterious. Two studies proposed that gene association with the nuclear periphery has a fine tuning transcription regulatory role (Brickner and Walter, 2004; Taddei et al., 2006). Consistent with this observation some components of the NPC have been proposed to play a direct role in transcription activation (Menon, 2005). In addition, a study carried out in our laboratory proposed that the NPC-associated Mlp proteins may contribute to transcriptional regulation in response to an export defect thereby linking transcription to mRNA processing surveillance and export (Vinciguerra et al., 2005).

2.1.2 Aim of the study

In this work, we have investigated the mechanism(s) by which genes anchor to the NPC upon transcription activation. A previous study performed in our group in collaboration with the Dargemont laboratory established that Mex67, the essential export receptor in *S.cerevisiae* which interacts directly with the nucleoporins lining the pore, was recruited to active genes during transcription elongation through interaction with a component of the THO complex (Gwizdek, 2006). This observation and the fact that a number of other factors required for mRNA export are recruited co-transcriptionally suggested that the physical link between the transcription site and the NPC could be mediated in part by the formation of the growing mRNP complex during transcription elongation. The questions we aimed at answering were:

(i) Are selected export factors and functionally related proteins required for gene anchoring?

(ii) Is mRNA production important in this process?

(iii) Does NPC gene anchoring affect transcription?

2.1.3 Summary of the results

A number of studies suggested a possible role for Mlp proteins in linking mRNA export to transcription (Vinciguerra et al., 2005), as well as in stabilizing the association of genes with the nuclear periphery via the nascent mRNPs (Casolari et al., 2005). We wanted to confirm the significance of these suggestions by directly investigating the functional role of Mlp proteins in the tethering of activated genes to the nuclear periphery. This was achieved by examining the repositioning of the *GAL10* cluster locus and the *HSP104* locus following induction by galactose and ethanol respectively in WT versus a Δ*mlp1* strain. Using a LacO/LacI-GFP gene detection system, we first showed that Mlp1 participates in tethering activated *GAL* and *HSP104* gene loci. Second, we used this approach to evaluate the role of the export receptor Mex67 in this process. The *mex67-5* mutant is a temperature sensitive mutant identified by the Hurt laboratory (Segref et al., 1997). It induces rapid dissociation of the *mex67-5* mutant protein from the nuclear periphery and a polyA mRNA export defect after a shift at 37°C. In the *mex67-5* mutant strain, activated *GAL10* and *HSP104* genes were not found in association with the nuclear periphery following heat shock and ethanol stress. Surprisingly, the *mex67-5* protein remained localized at the nuclear periphery under ethanol stress conditions but failed to be properly recruited to the activated *HSP104* gene. This observation indicated that transcription induced association of active genes to the NPC depends on the recruitment of Mex67 to the transcribing gene during transcription elongation. Importantly, the level of RNA polymerase II gene binding and mRNA production were not affected by the loss of peripheral localization, indicating that the expression of these genes is not dependent on stable NPC association.

We then decided to investigate the importance of mRNA production in gene to NPC tethering. We constructed strains containing various deletions of the *GAL2* gene and found that the protein coding region was not necessary for the repositioning of *GAL2* whereas the promoter regions (UAS and TATA elements) were required for transcription induced gene repositioning. By analyzing mRNA production in the strain lacking the coding region of *GAL2*, we found that truncated mRNA production was drastically affected, indicating that NPC-gene tethering is an RNA independent process. However, by performing ChIP we observed that both the wild type and the mutant Δ*gal2* genes recruited similar amounts of TBP and RNA polymerase II to their promoter, indicating comparable levels of transcription activation. These observations suggest that transcription activation, and not stable mRNP, is both necessary and sufficient for transcription-induced *GAL2* gene repositioning. Consistent with this finding, we also observed that Mex67 co-transcriptional recruitment on the activated

Δ*gal2* gene still occurs, confirming that it is not mediated by nascent RNA and does not require stable mRNP biogenesis. This observation is in perfect agreement with the earlier finding that Mex67 is recruited to transcribing genes via components of the transcription machinery such as Hpr1 (Gwizdek, 2006).

Based on these observations, we proposed a model in which Mex67 is recruited early during transcription *via* interaction with components of the transcription machinery through a process independent of the mRNP. Mex67 may be transferred to the mRNP at a later step *via* interaction with adaptors such as Yra1. The early recruitment of Mex67 could promote the association of activated genes with the nuclear periphery. Mlp proteins in turn could contribute to the stable association of activated genes by interactions with transcription activators, elongation factors as well as nascent mRNPs.

Whether transcription induced gene repositioning is the cause or the consequence of transcription activation has not been clearly addressed in this study since all the localization experiments have been performed under steady state transcription conditions and not during the activation process. However, we showed that at steady state, transcription does not require a stable association of the gene with the NPC. Possible views are that stable gene gating favors: (i) recruitment of mRNA processing and export factors, (ii) mRNP quality surveillance and (iii) mRNP export.

2.1.4 Article

Co-transcriptional recruitment of the mRNA export receptor Mex67p contributes to the nuclear pore anchoring of activated genes
G Dieppois*, N Iglesias*, F Stutz. * Equal contribution.
Mol Cell Biol. 26(21):7858-70. (Nov 2006)

MOLECULAR AND CELLULAR BIOLOGY, Nov. 2006, p. 7858–7870 Vol. 26, No. 21
0270-7306/06/$08.00+0 doi:10.1128/MCB.00870-06

Cotranscriptional Recruitment to the mRNA Export Receptor Mex67p Contributes to Nuclear Pore Anchoring of Activated Genes[▽]

Guennaelle Dieppois,† Nahid Iglesias,† and Françoise Stutz*

Department of Cell Biology, Sciences III, 30 Quai E. Ansermet, 1211 Geneva 4, Switzerland

Received 16 May 2006/Returned for modification 14 June 2006/Accepted 24 August 2006

Transcription activation of some *Saccharomyces cerevisiae* genes is paralleled by their repositioning to the nuclear periphery, but the mechanism underlying gene anchoring is poorly defined. We show that the nuclear pore complex-associated Mlp1p and the shuttling mRNA export receptor Mex67p contribute to the stable association of the activated *GAL10* and *HSP104* genes with the nuclear periphery. However, we find no obligatory link between gene positioning and gene expression. Furthermore, gene anchoring correlates with the cotranscriptional recruitment of Mex67p to transcribing genes. Notably, the association of Mex67p with chromatin is not mediated by RNA. Interestingly, a mutant *GAL2* gene lacking the coding region is still able to recruit Mex67p upon transcriptional activation and to relocate to the nuclear periphery. Together these data suggest that, at least for *GAL2*, nascent messenger ribonucleoprotein does not play a major role in gene anchoring and that the early recruitment of Mex67p contributes to gene repositioning by virtue of an RNA-independent process.

A growing number of recent studies point to a functional relationship between gene expression and nuclear organization of chromatin. Indeed, the nucleus is subdivided into spatially defined domains, and the nonrandom distribution of chromosomes within these domains is thought to regulate their transcriptional state (8, 25). Notably, the nuclear periphery has first been viewed as a transcriptionally repressive zone. In *Saccharomyces cerevisiae*, telomeres and mating-type loci, consisting of silent chromatin, concentrate in nuclear peripheral regions (16), and artificial tethering of nonsilenced DNA to the envelope induces its repression (2). In particular, the nuclear pore complex (NPC) has been implicated in perinuclear gene silencing and maintenance of gene expression states. Indeed, loss of a subset of NPC components, including the nuclear basket-anchored Mlp1p and Mlp2p, results in the activation of subtelomeric reporter genes (10, 12, 20). However, another study showed that artificial tethering of nuclear transport factors to a partially silenced mating-type locus allowed its expression. Thus, recruitment of genes to the nuclear periphery can also have important effects on their activation (24). Consistent with these observations, genome-wide analyses of NPC-bound loci identified preferential association of highly transcribed genes with the nuclear periphery. These studies also showed that transcriptional activation of genes induced by galactose or α-factor is accompanied by their relocation from the nuclear interior to the nuclear periphery (5, 6). *INO1* gene activation is also paralleled by its repositioning to the periphery, and this relocation contributes to optimal *INO1* gene expression (3).

Importantly, recent studies pointed to a direct physical link between Sus1p, a component of the SAGA histone deacetylase coactivator complex, and the Sac3-Thp1 complex, which is part of the mRNA export machinery associated with pores (31). These data together suggested that transcription regulators could control the recruitment of genes to the nuclear periphery, possibly linking gene repositioning to optimal activation. However, a strict and systematic dependence of gene expression on peripheral positioning has not been demonstrated. More generally, the molecular basis of transcription-induced gene repositioning is poorly understood and whether it is the cause or consequence of transcription activation is still unclear. Several observations indicated a possible role for the nascent messenger ribonucleoprotein (mRNP) in stabilizing the association of a gene with the nuclear periphery. First, mRNP components physically interact with the NPC-associated Mlp1p and Mlp2p proteins (11, 17, 43), and the results of chromatin immunoprecipitation (ChIP) experiments suggest that Mlp1p associates with transcribing genes in an RNA-dependent manner (5). These observations raised the possibility that Mlp proteins contribute to gene anchoring by interacting with nascent transcripts. Second, several mRNA export factors bind mRNA cotranscriptionally (28, 38, 45), consistent with a potential role for growing mRNPs in bridging active genes to the NPC. Moreover, we recently showed that the mRNA export receptor Mex67p, which promotes the translocation of mRNP complexes through the NPC (35), is also recruited cotranscriptionally (19). The association of Mex67p with transcribing genes and its ability to interact with various pore components raised the possibility that mRNP-bound Mex67p helps the anchoring of transcribing loci to the nuclear periphery.

To test the potential roles of Mlp1p and Mex67p in gene anchoring, we compared the localization of inducible genes in wild-type (WT) and Δ*mlp1* or *mex67-5* mutant cells (35). The results indicate that both Mlp1p and Mex67p are required for efficient anchoring of the galactose-inducible *GAL10* and stress-inducible *HSP104* genes; however, gene anchoring appears to be not essential for the transcription of these two

* Corresponding author. Mailing address: Department of Cell Biology, Sciences III, 30 Quai E. Ansermet, 1211 Geneva 4, Switzerland. Phone: 0041 22 379 67 29. Fax: 0041 22 379 6442. E-mail: Francoise.Stutz@cellbio.unige.ch.

† These authors contributed equally to this work.

▽ Published ahead of print on 5 September 2006.

TABLE 1. Yeast strains used in this study

Code	Name	Genotype	Reference or source
W303 background			
W303	Wild type	*MAT***a** *ade2 his3 leu2 trp1 ura3*	
FSY1982	*mex67-5*	*MAT***a** *ade2 his3 leu2 trp1 ura3 mex67-5* (integrated mutant)	26
FSY2395	MEX67-GFP	*MAT***a** *ade2 his3 leu2 trp1 ura3* MEX67-GFP-Kan^r	This study
FSY2455	*mex67-5*-GFP	*MAT***a** *ade2 his3 leu2 trp1 ura3 mex67-5*-GFP-Kan^r	This study
FSY1651	HA-Sub2	*MAT***a** *ade2 his3 leu2 trp1 ura3 sub2*::HIS3 <YCplac111-HA-SUB2>	45
GA1320 background			
GA1320	LacI-GFP Nup49-GFP	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-HIS3 Nup49-GFP	21
FSY2811	WT *GAL10*-LacO	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-HIS3 Nup49-GFP *GAL10*-LacO-*TRP1*	This study
FSY2812	WT *HSP104*-LacO	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-HIS3 Nup49-GFP *HSP104*-LacO-*TRP1*	This study
FSY2813	Δ*mlp1 GAL10*-LacO	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-HIS3 Nup49-GFP *GAL10*-LacO-*TRP1 mlp1*::Kan^r	This study
FSY2814	Δ*mlp1 HSP104*-LacO	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-HIS3 Nup49-GFP *HSP104*-LacO-*TRP1 mlp1*::Kan^r	This study
FSY2815	*mex67-5 GAL10*-LacO	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-HIS3 Nup49-GFP *GAL10*-LacO-*TRP1 mex67-5*	This study
FSY2816	*mex67-5 HSP104*-LacO	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-HIS3 Nup49-GFP *HSP104*-LacO-*TRP1 mex67-5*	This study
FSY2817	WT *GAL2*-LacO	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-HIS3 Nup49-GFP *GAL2*-LacO-*TRP1*	This study
FSY2818	Δ*gal2*-LacO	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-HIS3 Nup49-GFP Δ*gal2*-LacO-*TRP1*	This study
FSY3042	Δ*3′UTR*-LacO	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-HIS3 Nup49-GFP Δ*3′UTR*-LacO-*TRP1*	This study
FSY3041	Δ*gal2-3′UTR*-LacO	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-HIS3 Nup49-GFP Δ*gal2-3′UTR*-LacO-*TRP1*	This study
FSY2821	Δ*prom-gal2*-LacO	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-HIS3 Nup49-GFP Δ*prom-gal2*-LacO-*TRP1*	This study
FSY2822	Δ*UAS-3′UTR*-LacO	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-HIS3 Nup49-GFP Δ*UAS-3′UTR*-LacO-*TRP1*	This study

genes. Notably, loss of gene anchoring in the *mex67-5* mutant correlates with the inability of the *mex67-5* mutant protein to associate with the transcribing genes. Moreover, we find that transcription-induced NPC anchoring of the *GAL2* gene does not require the mRNA-coding region, suggesting that nascent mRNP may not be essential for bridging an interaction between an active gene and the NPC. These data and the observation that the cotranscriptional binding of Mex67p is RNA independent suggest that Mex67p may contribute to gene anchoring by interacting with activated chromatin rather than nascent RNA.

MATERIALS AND METHODS

Plasmid constructions. To insert LacO repeats downstream of the *GAL2*, *GAL10*, and *HSP104* genes, the 3′ untranslated region (3′UTR) region of each gene was cloned in front of the LacO repeats carried by the integrating plasmid pAFS52 (*TRP1*) (21) to generate pFS2912, pFS2913, and pFS3013, respectively. For directed insertion, these plasmids were linearized by cleavage of a unique restriction site within the 3′UTR and transformed into relevant strains. Wild-type Mlp1p was expressed from plasmid pFS2614 (pRS316 *LEU2* CEN), isolated in a synthetic lethal screen and carrying the *MLP1* gene as the only complete open reading frame (D. Zenklusen and F. Stutz, unpublished data).

Yeast strains. The yeast strains used in this study are listed in Table 1. The *mex67-5* strain contains an integrated mutant gene (26). Wild-type and *mex67-5* genes were genomically tagged with green fluorescent protein (GFP)-Kan^r by homologous recombination (29). GA1320-Δ*mlp1* and GA1320-*mex67-5* strains were obtained by crossing strain GA1320 (LacI-GFP-HIS3 Nup49-GFP) with the *mlp1*::Kan^r (S. Gasser lab) and *mex67-5* (26) strains. The *GAL10* and *HSP104* loci were subsequently tagged with LacO repeats in the GA1320, GA1320-Δ*mlp1*, and GA1320-*mex67-5* strains by transformation of linearized pFS2913 and pFS3013, respectively, followed by selection on Trp$^-$ plates. Insertions were confirmed by PCR on genomic DNA. The Δ*gal2* strain was obtained by transformation and homologous recombination of a PCR-generated *loxP-URA3* cassette (18) carrying ends complementary to the 5′ and 3′ ends of the *GAL2*-coding region in the WT GAL2-LacO strain (FSY2817). The forward and reverse primers used were GAL2-loxP-F1 (OFS1071) (5′AACACAAGAT TAACAT AATA AAAAAAATAA TTCTTTCATAC AGCTGAAGCT TCGTACGC 3′) and GAL2-loxP-R1 (OFS1072) (5′AAAATTAAGA GAGATGATGG AGCG TCTCAC TTCAAACGCAG CATAGGCCACT AGTGGATCTG3′), respectively. The Δ*gal2-3′UTR* and Δ*3′UTR* strains were constructed using the same strategy with the forward primers GAL2-loxP-F1 (OFS1071) and GAL2-3′UTR-loxP-F1 (OFS1113) (5′TTACAACATG ACGACAAACC GTGGTACAAG GCCATGCTAG AATAACAGCT GAAGCTTCGT ACGC3′), respectively, in combination with the reverse primer GAL2-3′UTR-loxP-R1 (OFS1104) (5′GT TAGCTCAG GAATTCAACT GGAAGAAAGT CCAGGCAAGT ACCTGA CGCA TAGGCCACTA GTGGATCTG3′). The Δ*prom-gal2* and Δ*UAS-3′UTR* strains were obtained similarly using the forward primers −200GAL2-loxP-F1 (OFS1103) (5′CAAACATTTC GCAGGCTAAA ATGTGGAGAT AGGATA AGTT TTGTAGCAGC TGAAGCTTCG TACGC3′) and −550GAL2-loxP-F1 (OFS1102) (5′CAAAAGGTAC TCAACGTCAA TTCGGAAAGC TTCCTT CCGG AATGGCCAGC TGAAGCTTCG TACGC3′), with the reverse primer GAL2-loxP-R1 (OFS1072) and GAL2-3′UTR-loxP-R1 (OFS1104), respectively. The *URA3* selective marker was subsequently excised by expression of Cre recombinase and selection of colonies on 5-fluoroorotic acid (18). Deletions were confirmed by PCR on genomic DNA.

Live fluorescence microscopy and quantification. Wild-type, *mex67-5*, or Δ*mlp1* strains bearing LacO repeats downstream of *GAL10*, *HSP104*, or *GAL2* and expressing integrated LacI-GFP repressor and Nup49-GFP fusions were grown and induced as indicated in figure legends. Live microscopy was performed as described previously (41). Briefly, a Zeiss Axioplan microscope was used to capture 21-image stacks of 95-nm step size. In the focal plane in which the GFP spot is brightest, its position was mapped to one of three concentric zones of equal surface by dividing the spot-to-pore distance by the nuclear

diameter. A gene was scored as being located at the nuclear periphery when it was present in the most peripheral zone, i.e., when the distance between the spot and the nuclear periphery was <0.184 of 1/2 nuclear diameter. A random distribution of the tagged gene would result in a 33% occurrence in each of the three zones. Values above 33% indicate enrichment in this particular zone. Statistical analysis used a proportion method to compare zone 1 percentages between two different samples. Significance was determined with a 95% confidence interval.

Northern blot analyses. Total RNA was extracted from the cultures used for gene localization using a hot phenol method, and 10 to 15 μg was fractionated on 1% agarose-formaldehyde gels or 8% polyacrylamide-urea gels. Agarose gels were transferred to Hybond membranes by vacuum blotting, whereas polyacrylamide gels were transferred by semidry blotting as described previously (40). Membranes were hybridized with randomly primed labeled PCR fragments using standard protocols. The *GAL1* and *HSP104* probes correspond to protein-coding regions. The *GAL2* and 3′UTR probes correspond to positions 920 to 1600 of the *GAL2* protein-coding region and to positions +123 to +550 of the *GAL2* 3′UTR, respectively. The signals were quantified with a Bio-Rad Instant Imager and normalized to those obtained with probes specific for 18S rRNA or actin mRNA hybridized to the same membranes.

Chromatin immunoprecipitations. For ChIP analysis of *HSP104*, strains were grown in yeast extract-peptone-dextrose (YEPD) rich medium to an optical density at 600 nm (OD_{600}) of 0.8 to 1, divided into three equal cultures for subsequent analysis in triplicate, treated with 10% ethanol for 30 min at 25°C, and cross-linked for 10 min with formaldehyde. For ChIP analysis of *GAL10*, strains were grown at 25°C in yeast extract-peptone (YEP) plus 2% raffinose to an OD_{600} of 0.7, divided into three equal cultures, induced with 2% galactose for 2.5 h, mixed with 1 volume of YEP plus 2% galactose at 25°C or 49°C and either kept at 25°C or incubated for 15 min at 37°C, and cross-linked for 10 min at the same temperatures. For ChIP analysis of *GAL2*, the relevant strains were precultured in YEP plus 2% raffinose as described above, divided into three equal cultures, induced with 2% galactose for 2.5 h, and cross-linked for 10 min. Cross-linking was reduced to 5 min when preparing ChIP extracts for RNase sensitivity tests. In all cases, glass bead extracts were sonicated so as to shear the chromatin down to 300- to 400-bp fragments. Sonicated extracts (1 mg protein in 1-ml final volume) were immunoprecipitated with polyclonal antibodies against Mex67p (gift from C. Dargemont), TATA binding protein (TBP) (gift from M. Collart,) or a monoclonal antibody against RNA polymerase II (PolII) C-terminal domain (8WG16 from Covance). Each immunoprecipitation was performed three times using three independent extracts. Immunoprecipitated DNA was quantified by real-time PCR as described earlier (45) using primer pairs described at http://www.unige.ch/sciences/biologie/bicel/stutz/Dieppois_MCB06_Suppl_Mat.pdf. To calculate the increase in signal in the different gene regions, the absolute values obtained by quantitative PCR were normalized to the values obtained with the nontranscribed intergenic region, arbitrarily set to 1. To evaluate the importance of RNA in ChIP, cross-linked and sonicated extracts, prepared from the galactose-induced HA-Sub2p strain FSY1651, were immunoprecipitated with antibodies against hemagglutinin (HA) (monoclonal 16B12; Covance), RNA PolII, or Mex67p. Immunoprecipitated extracts on beads were washed and resuspended in 1 ml buffer alone or containing 15 μl RNase Cocktail TM (500 U/ml RNase A and 20,000 U/ml RNase T_1; Ambion) and incubated for 30 min at room temperature. Beads were washed, and the DNA was purified and quantified as described above.

Western blotting. Total protein extracts were prepared from aliquots of cultures used for ChIP analysis prior to cross-linking, fractionated on sodium dodecyl sulfate-polyacrylamide gels, and examined by Western blotting with polyclonal antibodies specific for Mex67p (1:10,000) (a gift from C. Dargemont) and TBP (1:2,000) (a gift from M. Collart).

RESULTS

Mlp1p is required for efficient anchoring of the activated *GAL10* and *HSP104* genes to the NPC. The NPC-associated Mlp1p and related Mlp2p could be involved in stable association of active genes with the NPC, as they were proposed to contact chromatin and/or nascent transcripts, as well as to influence the expression of genes located at the nuclear periphery (5, 6, 9, 17, 43). To test whether Mlp1p contributes to gene anchoring, two inducible genes, *GAL10* and *HSP104* were localized in wild-type and Δ*mlp1* cells. *GAL10* is part of a

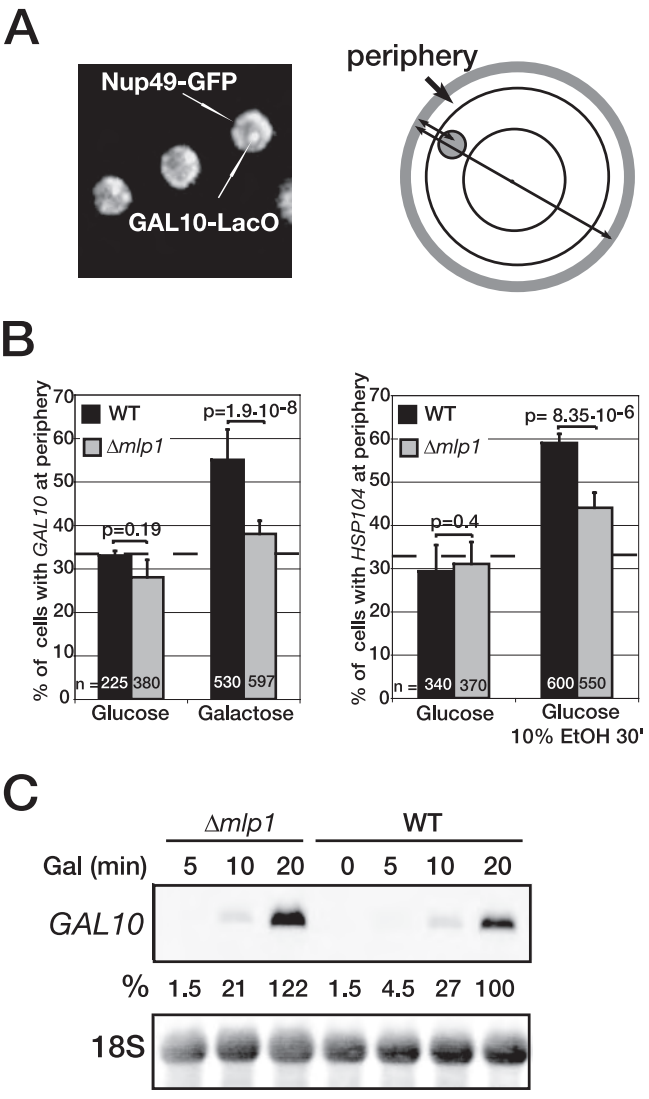


FIG. 1. Mlp1p is required for efficient transcription-induced *GAL10* and *HSP104* gene relocation. (A) Yeast cells with *GAL10* tagged with LacO repeats and expressing LacI-GFP and Nup49-GFP. Binding of LacI-GFP to LacO gives rise to the intense bright spot, whereas Nup49-GFP marks the nuclear periphery. A spot was scored as peripheral when located in the most peripheral 33% cross-sectional area of the nucleus (see Materials and Methods). (B) Localization of *GAL10* or *HSP104* tagged with LacO repeats in the wild-type (black bars) or Δ*mlp1* (gray bars) cells. To ensure isogenicity, WT and Δ*mlp1* strains were obtained by transforming the Δ*mlp1* strain with a plasmid expressing Mlp1p or an empty vector, respectively. *GAL10*-LacO cells were grown at 25°C in selective medium containing 2% raffinose to an OD_{600} of 0.5 and shifted to 2% glucose or 2% galactose for 2.5 h. *HSP104*-LacO cells were grown at 25°C in YEPD to an OD_{600} of 0.5 and examined before or after a 30-min treatment with 10% ethanol (10% EtOH 30′). A population of live cells was photographed with a fluorescence microscope. In each cell, the position of the *GAL10* or *HSP104* gene spot was defined with respect to the nuclear periphery. Graph bars represent the percentage of cells with the indicated tagged gene positioned at the periphery. Error bars were derived from three independent experiments; *n* is the total number of cells counted for each strain and condition. The broken line at 33% marks a random distribution; based on a proportional test, two distributions were considered as significantly different when $P < 0.05$. (C) Northern blot analysis of *GAL10* mRNA in Δ*mlp1* and WT cells after induction with galactose (Gal) at 25°C for the indicated times. The *GAL10* mRNA signal was normalized to 18S rRNA used as internal control and expressed as a percentage of the WT level, after 20 min in galactose at 25°C.

cluster of galactose-inducible genes that relocate to the nuclear periphery upon transcriptional activation (6), and *HSP104* transcription can be activated by heat stress or ethanol at 25°C (32). To locate the genes with respect to the nuclear periphery, LacO repeats were inserted 600 to 700 bp downstream of the *GAL10* and *HSP104* loci in wild-type or Δ*mlp1* strains that also expressed chromosomally encoded GFP-LacI repressor and GFP-Nup49 (Fig. 1A) (21). The *GAL10*-LacO strains were grown in glucose- or galactose-containing medium at 25°C followed by gene localization in living cells by fluorescence microscopy. Consistent with earlier observations (6), transcription activation of *GAL10* in wild-type cells promoted the repositioning of the *GAL10* locus, as the percentage of cells with the tagged gene at the periphery increased from 30% in glucose to 55% in galactose (Fig. 1B, left panel). Similarly, transcription activation of *HSP104* with ethanol in *HSP104*-LacO wild-type cells resulted in the repositioning of the locus to the nuclear periphery (from 30% to 60%) (Fig. 1B, right panel). Ethanol is unlikely to trigger general chromatin reorganization, as it had no effect on the localization of the *GAL10* locus (data not shown). Interestingly, for both conditions, the absence of Mlp1p resulted in a significant loss of peripheral localization for these two genes (from 55% to 38% for *GAL10* and from 60% to 44% for *HSP104*) (Fig. 1B, left and right panels). Northern blot analyses showed that neither *GAL10* nor *HSP104* mRNA levels were substantially affected in the absence of Mlp1p (Fig. 1C and data not shown). These results indicate first that Mlp1p contributes to transcription-induced *GAL10* and *HSP104* gene anchoring and second that *GAL10* and *HSP104* gene expression does not require stable association with the NPC.

Mex67p is required for transcription-induced anchoring of *GAL* genes. A similar approach was taken to investigate a contribution of the mRNA export receptor Mex67p in peripheral gene anchoring by comparing the wild-type strain and the *mex67-5* strain. The *mex67-5* strain is a temperature-sensitive mutant with a single amino acid substitution in the NTF2-like domain interacting with Mtr2p. At 37°C, the *mex67-5* mutant protein dissociates from the nuclear periphery, and the cells exhibit a very rapid poly(A)$^+$ RNA export defect (35). *GAL10*-LacO-tagged wild-type and *mex67-5* strains were grown in glucose- or galactose-containing medium at 25°C and either kept at 25°C or shifted to 37°C for 15 or 30 min prior to fluorescence microscopy analysis (Fig. 2A). In both wild-type and *mex67-5* cells at 25°C, the percentage of cells with *GAL10* at the periphery increased from 25% in glucose to more than 55% in galactose, indicating efficient transcription-induced *GAL10* gene anchoring at the permissive temperature. In contrast, a 15-min shift to 37°C strongly decreased peripheral *GAL10* localization in *mex67-5* cells compared to the wild type. After 30 min at 37°C, only 25% of *mex67-5* cells were scored positive, a value similar to that in glucose, indicating complete loss of *GAL10* gene association with the nuclear periphery. Comparable results were obtained with a LacO-tagged *GAL2* gene located on a different chromosome (see below; also data not shown).

To examine the relationship between gene localization and expression, *GAL10* transcripts were analyzed by Northern blotting. mRNA levels were somewhat lower in *mex67-5* cells than in wild-type cells at 25°C and decreased slightly (from 88% to 66% of the wild-type level) in the mutant shifted to 37°C for 30 min (Fig. 2B). ChIP experiments revealed comparable amounts of TBP associated with the *GAL10* promoter in wild-type and *mex67-5* cells at 25°C or 37°C, and the association of RNA PolII with the coding region was slightly reduced in *mex67-5* cells after 30 min at 37°C (Fig. 2C). The lower *GAL10* mRNA levels in *mex67-5* cells at 37°C could be due, at least in part, to reduced transcription but also to mRNA instability as a result of the mRNA export block (see Discussion). In any case, the loss of *GAL10* from the periphery was more pronounced and occurred faster than the decrease in mRNA levels. These observations indicate that association with the nuclear periphery is not absolutely required for *GAL10* gene transcription. They also suggest that mRNA production is not sufficient for gene anchoring and that Mex67p is required for stable association with the nuclear periphery.

To further examine the role of Mex67p in gene anchoring, ChIP was used to compare the association of Mex67p with the *GAL10* gene in wild-type and *mex67-5* mutant cells. Wild-type Mex67p was detected in association with *GAL10* when cells were grown in galactose but not in glucose, confirming that cotranscriptional binding of Mex67p correlates with active transcription (Fig. 2C) (19). Mex67p was detected at very low levels at the promoter, clearly increased at the 5′ end, reached a maximum at the middle of the gene, and declined towards the 3′end and 3′UTR. This profile indicates that Mex67p recruitment starts at an early phase of transcription. The *mex67-5* mutant protein showed a similar binding profile at 25°C. At 37°C, however, while association of wild-type Mex67p with *GAL10* was even more efficient, the recruitment of *mex67-5* was strongly inhibited. Western blotting of total cell extracts confirmed that wild-type and *mex67-5* proteins were present in similar amounts (Fig. 2D). These data suggest that the binding of Mex67p to *GAL10* contributes to the stable association of this transcribing gene with the nuclear periphery.

Mex67p is required for transcription-induced *HSP104* gene repositioning. To address the role of Mex67p in gene anchoring under different inducing conditions, *HSP104*-LacO was localized in wild-type and *mex67-5* cells before or after a 30-min treatment with 10% ethanol at 25°C (Fig. 3A). As shown above (Fig. 1B), the number of cells with *HSP104* at the nuclear periphery increased from 25% to nearly 60% after ethanol treatment in the wild-type cells. In contrast, *HSP104* relocation was completely abolished in *mex67-5* cells. To verify that ethanol has no indirect effects on gene positioning, the localization of the LacO-tagged *PHO84* gene was examined in these cells under different conditions described at http://www.unige.ch/sciences/biologie/bicel/stutz/Dieppois_MCB06_Suppl_Mat.pdf. The *PHO84* gene is located at 23 kb from the telomere of chromosome XIII. As a consequence of telomere anchoring, the LacO-tagged *PHO84* gene is detected at the nuclear periphery in 60% of wild-type cells independent of its transcriptional state. Importantly, shifting the LacO-tagged *mex67-5* strain to 37°C or exposing the cells to 10% ethanol for 30 min did not dissociate the *PHO84* locus from the periphery. Therefore, ethanol treatment of *mex67-5* cells does not induce general chromatin rearrangements.

Northern blot analysis confirmed that *HSP104* transcripts were induced in wild-type cells after a 30-min ethanol treat-

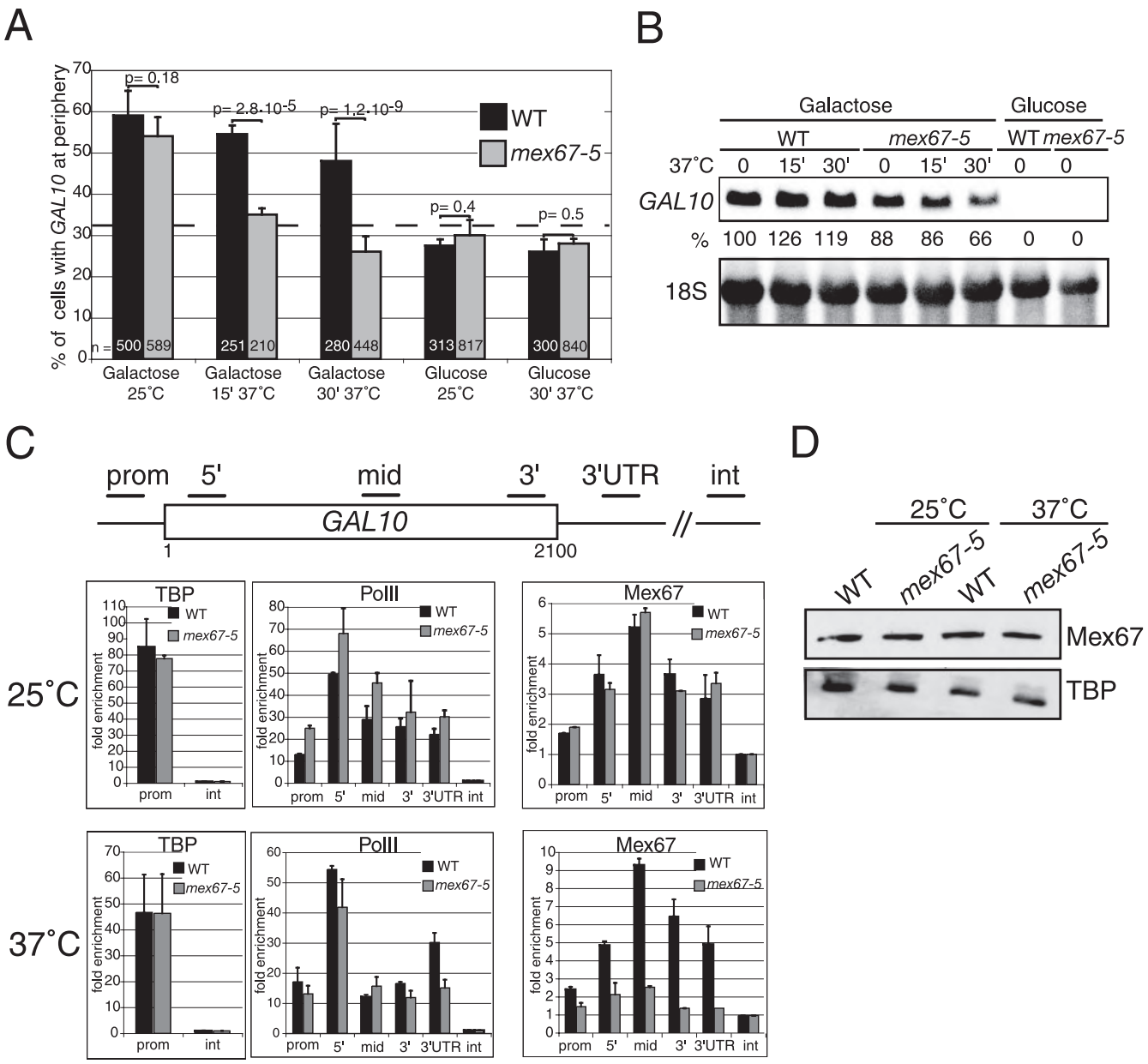


FIG. 2. Mex67p is required for transcription-induced *GAL10* gene relocation. (A) The *GAL10* gene tagged with LacO was localized in wild-type (black bars) and *mex67-5* (gray bars) cells also expressing LacI-GFP and Nup49-GFP. Cells were grown from an OD_{600} of 0.1 to 0.5 at 25°C in synthetic complete (SC) medium containing 2% raffinose and shifted to 2% glucose or 2% galactose for 2 h. Cells were then either kept at 25°C or shifted to 37°C for 15 min (15′) or 30 min (30′). *GAL10* gene localization was defined as described in the legend to Fig. 1. (B) Northern blot analysis of *GAL10* mRNA in wild-type and *mex67-5* cells grown as described above for panel A and shifted to 37°C for the indicated times. The *GAL10* mRNA signal was normalized to 18S rRNA and expressed as a percentage of the wild-type value before the shift to 37°C. (C) Association of TATA binding protein, RNA polymerase II, and Mex67p with galactose-induced *GAL10* in wild-type and *mex67-5* cells at 25°C and 37°C. Cross-linked and sonicated extracts were immunoprecipitated with antibodies against TBP, PolII, or Mex67p. Coprecipitating DNA was amplified by real-time PCR with primers specific for the *GAL10* promoter (prom), 5′, middle (mid), 3′, 3′UTR, and a nontranscribed intergenic region (int), as indicated. The relative enrichment of the *GAL10* gene segments in each ChIP was expressed as the increase with respect to the nontranscribed intergenic region value, arbitrarily set to 1. Values correspond to the means of three independent experiments. Bars correspond to standard deviations. (D) Western blot analysis of Mex67p levels in wild-type and *mex67-5* extracts from cells used for ChIP. TBP was used as an internal loading control.

ment at 25°C and reached even higher levels in *mex67-5* cells. For comparison, heat stress induced accumulation of even more *HSP104* mRNAs in both wild-type and *mex67-5* cells (Fig. 3B) (23). ChIP analysis of wild-type and *mex67-5* extracts revealed similar amounts of TBP and RNA PolII associated with the *HSP104* promoter and coding regions upon ethanol induction at 25°C (Fig. 3C), indicating comparable transcription rates in the two strains. The higher *HSP104* mRNA levels in the mutant may therefore result from different mRNA turnover rates. These observations

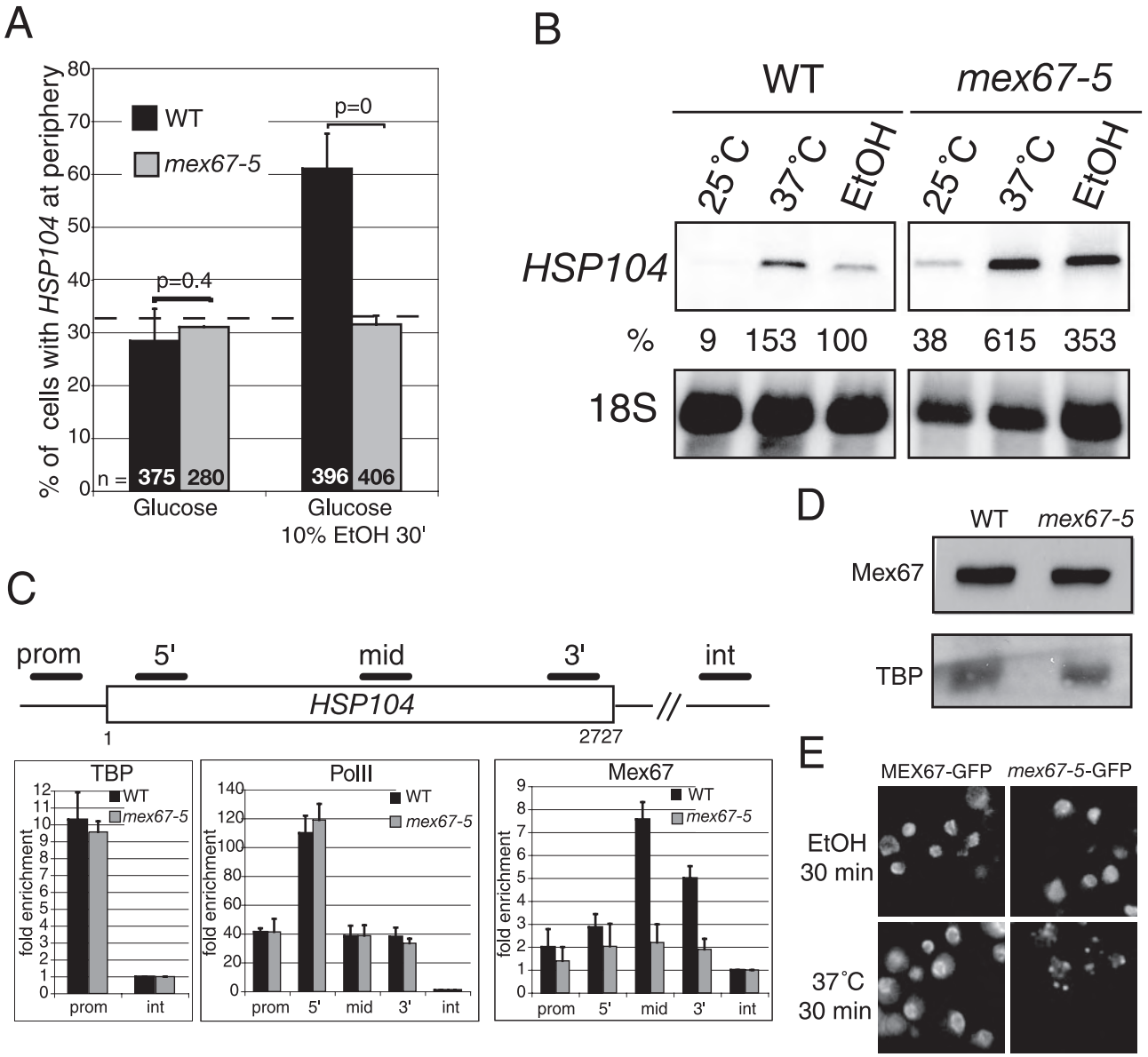


FIG. 3. Mex67p is required for transcription-induced *HSP104* gene relocation. (A) The *HSP104* gene tagged with LacO was localized in wild-type (black bars) and *mex67-5* (gray bars) cells expressing LacI-GFP and Nup49-GFP. Cells were grown in YEPD at 25°C and scored before and after a 30-min treatment with 10% ethanol (10% EtOH 30′) as described in the legend to Fig. 1. (B) Northern blot of *HSP104* mRNAs in wild-type and *mex67-5* strains grown at 25°C, shifted to 37°C, or treated with 10% ethanol for 30 min. *HSP104* mRNA levels were normalized to 18S rRNA values and expressed as a percentage of the value for the wild type incubated with ethanol for 30 min at 25°C. (C) ChIP analysis of TBP, RNA PolII, and Mex67p association with *HSP104*. Extracts were prepared from wild-type or *mex67-5* cells induced with 10% ethanol for 30 min at 25°C and immunoprecipitated with antibodies against TBP, RNA PolII, or Mex67p as indicated. Coprecipitating DNA was amplified with primers specific for *HSP104* promoter (prom), 5′, middle (mid), 3′, and a nontranscribed intergenic region (int). The relative enrichment of the *HSP104* gene segments was calculated as described in the legend to Fig. 2C. Values correspond to the means of three independent experiments. (D) Western blot analysis of Mex67p and TBP levels in wild-type and *mex67-5* extracts from cells used for ChIP. (E) Genomically tagged MEX67-GFP and *mex67-5*-GFP strains were grown in YEPD at 25°C, incubated with 10% ethanol, or shifted to 37°C for 30 min and immediately examined with a fluorescence microscope.

indicate that efficient *HSP104* gene transcription does not require peripheral localization.

The role of Mex67p in *HSP104* gene anchoring was further examined by comparing the association of wild-type and mutant *mex67-5* with the *HSP104* gene by ChIP. These experiments showed that wild-type Mex67p was efficiently recruited to *HSP104* after ethanol induction at 25°C, starting at the 5′ end, reaching a maximum in the middle of the gene, and decreasing towards the 3′ end (Fig. 3C). In contrast, *mex67-5* was barely detectable, despite comparable Mex67p and *mex67-5* protein levels in these cells (Fig. 3D). Notably, ethanol stress at 25°C does not affect *mex67-5*-GFP localization, in contrast to heat stress, which rapidly dissociates *mex67-5*-GFP from the nuclear periphery (Fig. 3E) (35). Ethanol stress therefore af-

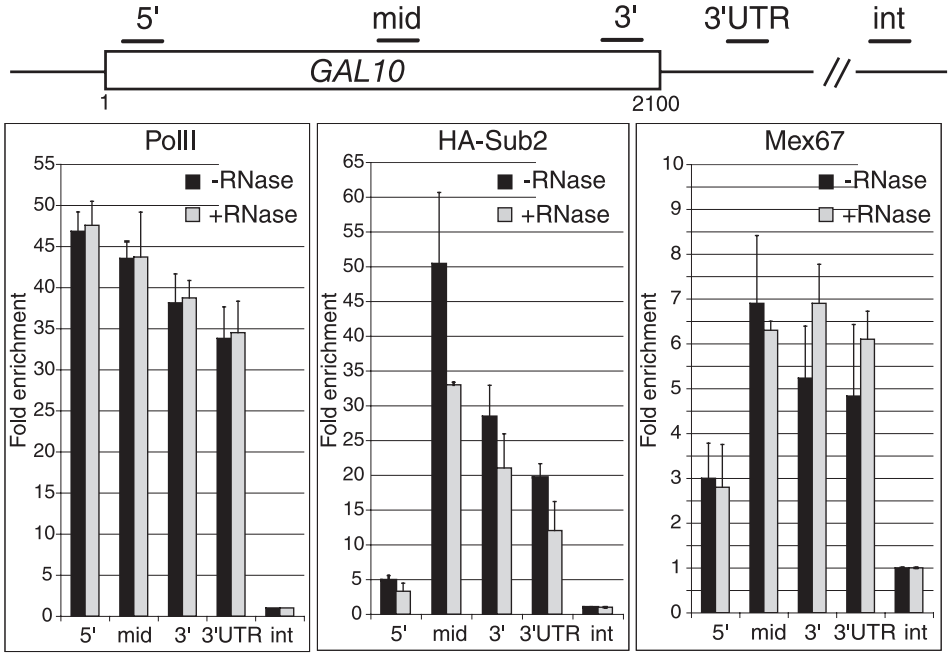


FIG. 4. The cotranscriptional recruitment of Mex67p is RNA independent. Strain FSY1651 expressing HA-tagged Sub2p was induced for 2 hours with 2% galactose. Cross-linked and sonicated extracts were immunoprecipitated with antibodies against HA, RNA PolII, or Mex67p. Coprecipitating DNA was purified from beads before or after RNase treatment and quantified by real-time PCR with primers specific for *GAL10* as described in the legend to Fig. 2C. Values correspond to the means of three independent immunoprecipitation experiments.

fects more severely the binding of *mex67-5* to the *HSP104* gene than to the NPC. Thus, the lack of *mex67-5* cotranscriptional recruitment in ethanol is not due to the dissociation of the mutant protein from the NPC but to its inability to associate with the transcribing gene. As for *GAL10*, these observations suggest that Mex67p binding to *HSP104* contributes to the stable association of this transcribing gene with the nuclear periphery.

Cotranscriptional recruitment of Mex67p is RNA independent. To determine whether the recruitment of Mex67p to transcribing genes is mediated by nascent mRNP, the association of Mex67p with *GAL10* was examined before or after RNase treatment (Fig. 4). RNA PolII and Sub2p were used as negative and positive controls, as the cotranscriptional recruitment of these proteins is RNA independent and dependent, respectively (1). Extracts prepared from a galactose-induced strain expressing HA-Sub2p were immunoprecipitated with antibodies against RNA PolII, HA, or Mex67p. Consistent with earlier studies (1), quantification of coprecipitating DNA showed that the association of RNA PolII along the *GAL10* gene was not affected by RNase treatment, whereas the binding of Sub2p was substantially reduced. Importantly, the association of Mex67p was insensitive to RNase, indicating that the early recruitment of Mex67p to transcribing genes is primarily mediated by adaptors associated with the transcription machinery.

***GAL2* gene anchoring does not require the mRNA-coding region.** The RNase-insensitive early recruitment of Mex67p suggested that Mex67p might contribute to gene anchoring in a process independent of nascent mRNP. To test the importance of the mRNA-coding region or other *cis*-acting sequences in gene anchoring more directly, repositioning of the galactose-inducible *GAL2* gene was examined in strains containing the wild-type gene or various deletions of this gene. Similar experiments could not be performed with *GAL10* or *HSP104*, as *GAL10* is part of a cluster of galactose-inducible genes, and microarray analyses report that *HSP104* lies in a region containing several ethanol-inducible genes (13). In contrast, *GAL2* is the only galactose-inducible gene within more than 100 kb on the left arm of chromosome XII (13). Cre-LoxP recombination (18) was used to generate five strains with increasing *GAL2* deletions on the chromosome (Fig. 5A). The first mutant strain lacks the *GAL2* protein-coding region (Δ*gal2*), the second lacks only the 3′UTR (Δ*3′UTR*), the third lacks the mRNA-coding and 3′UTR sequences (Δ*gal2-3′UTR*), the fourth lacks the promoter region encompassing the TATA box and the protein-coding region (Δ*prom-gal2*), and the fifth lacks the whole *GAL2* gene unit, including the upstream activation sequence (UAS), promoter region, protein-coding, and 3′UTR sequences (Δ*UAS-3′UTR*) (22). The *GAL2* locus was tagged with LacO repeats in wild-type and mutant strains, and its position was examined in cells grown in glucose or galactose (Fig. 5B). In the wild-type *GAL2* strain, the number of cells with the tagged gene at the periphery increased from 25% in glucose to nearly 45% in galactose. In contrast, no galactose-induced repositioning was observed in the Δ*UAS-3′UTR* mutant, confirming that the relocation observed in wild-type cells is exclusively due to *GAL2* gene activation. Surprisingly, deletion of the protein-coding region (Δ*gal2*) or 3′UTR (Δ*3′UTR*) or both (Δ*gal2-3′UTR*) did not substantially affect *GAL2* gene anchoring (locus repositioning from 25% in glucose to more than 40% in galactose), indicating that the protein-coding and 3′UTR regions are not essential for *GAL2* gene anchoring. In contrast, repositioning was completely lost in the Δ*prom-gal2*

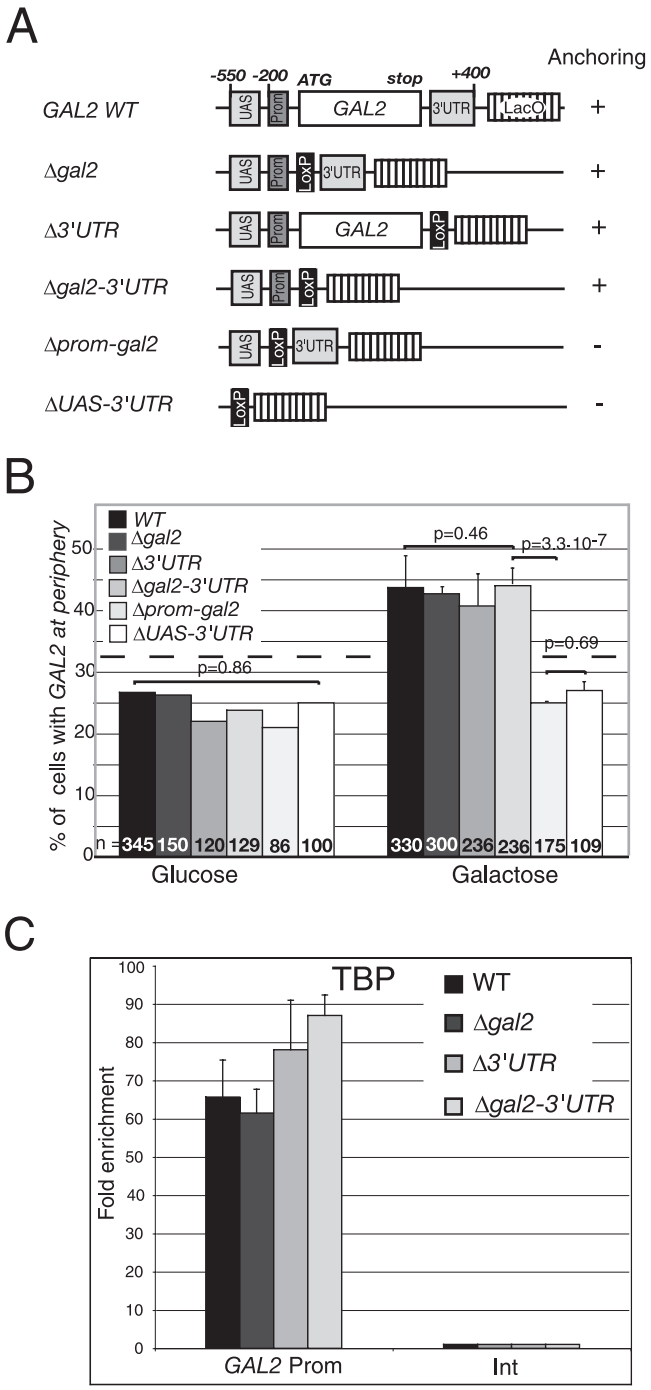


FIG. 5. The promoter, but not the protein-coding region or 3′UTR, is required for transcription-induced *GAL2* gene anchoring. (A) Diagram of *GAL2* genomic deletions. The positions −200 and −550 are relative to *GAL2* ATG. The UAS lies between −350 and −550, the TATA box lies between −100 and −200, and transcription initiation was mapped at −97 (22). The deleted 3′UTR lies between the *GAL2* stop codon and position +400 relative to the stop codon. The LoxP sequence is 106 bp long and results from the Cre-LoxP recombination procedure. (B) Localization of the *GAL2* locus tagged with LacO repeats in wild-type, Δ*gal2*, Δ*3′UTR*, Δ*gal2-3′UTR*, Δ*prom-gal2*, and Δ*UAS-3′UTR* cells grown in YEP medium containing 2% glucose or 2% galactose to an OD_{600} of 0.5. (C) ChIP analysis of TBP binding at the *GAL2* promoter (position −181 to −106 encompassing the TATA box) in the wild type and the indicated mutant strains induced for 2 h with 2% galactose. The relative enrichment of the *GAL2* promoter segment in each ChIP was expressed as the enrichment with respect to the nontranscribed intergenic region (Int) value, set to 1. Values are the means of three independent experiments.

cells. These experiments indicate that the promoter region containing the TATA box is required and that the UAS alone is not sufficient for the association of *GAL2* with the nuclear periphery.

To determine whether the repositioning of truncated *GAL2* genes correlates with transcription activation, ChIP was used to compare TBP binding to the *GAL2* promoter in the wild-type locus and in the Δ*gal2*, Δ*3′UTR*, and Δ*gal2-3′UTR* mutant loci following galactose induction (Fig. 5C). Interestingly, TBP is recruited to similar levels to the *GAL2* promoter region encompassing the TATA box in all four strains, indicating that *GAL2* transcription activation is not affected in the absence of the mRNA-coding regions. These results together with the gene localizations suggest that the mRNA-coding region is not essential for *GAL2* gene anchoring, and that transcription activation and TBP recruitment are both necessary and sufficient for transcription-induced *GAL2* gene repositioning.

Stable mRNP biogenesis is not required for early recruitment of Mex67p. To define the nature and amounts of transcripts encoded by the *GAL2* locus in the absence of the protein-coding region or the 3′UTR, total RNA from galactose-induced wild-type, Δ*gal2*, Δ*3′UTR*, and Δ*prom-gal2* strains was analyzed by Northern blotting with probes spanning either the protein-coding region (probe *GAL2*) or the 3′UTR (probe 3′UTR) (Fig. 6A). The *GAL2* probe detects *GAL2* transcripts encoded in the wild type and the mutant lacking the 3′UTR, whereas the 3′UTR probe detects transcripts encoded in the wild type and the Δ*gal2* mutant. Neither probe should generate a signal in the mutant lacking the promoter and coding region. The *GAL2* probe detected no transcript in the Δ*gal2* strain and showed that in the absence of the 3′UTR, *GAL2* transcripts were expressed at roughly 20% of wild-type *GAL2* mRNA levels (Fig. 6A, left panel), indicating that correct 3′-end formation contributes to optimal *GAL2* mRNA levels. Notably, the 3′UTR probe detected a low-abundance 450-base-long transcript in strain Δ*gal2* (Fig. 6A, right panel, lane 2). Since the 5′ end of the *GAL2* mRNA has been mapped to position −97 upstream of the ATG codon, and the LoxP-derived sequence is 106 bp (18, 22), the 3′end of this short 450-base transcript is predicted to lie around position +250 of the 3′UTR. Northern blot quantification as well as reverse transcription combined with real-time PCR (see http://www.unige.ch/sciences/biologie/bicel/stutz/Dieppois_MCB06_Suppl_Mat.pdf) indicate that this small transcript accumulates to roughly 10% of wild-type *GAL2* mRNA levels. The low level of expression of this short transcript despite wild-type levels of TBP recruitment at the promoter (Fig. 5C) suggests that this small RNA is very unstable.

The efficient repositioning of the Δ*gal2* locus despite the production of very limited amounts of RNA suggests that mRNP does not play a major role in transcription-induced gene repositioning. Furthermore, the RNase-insensitive recruitment of Mex67p (Fig. 4) raises the possibility that Mex67p contributes to gene anchoring in the absence of stable mRNP

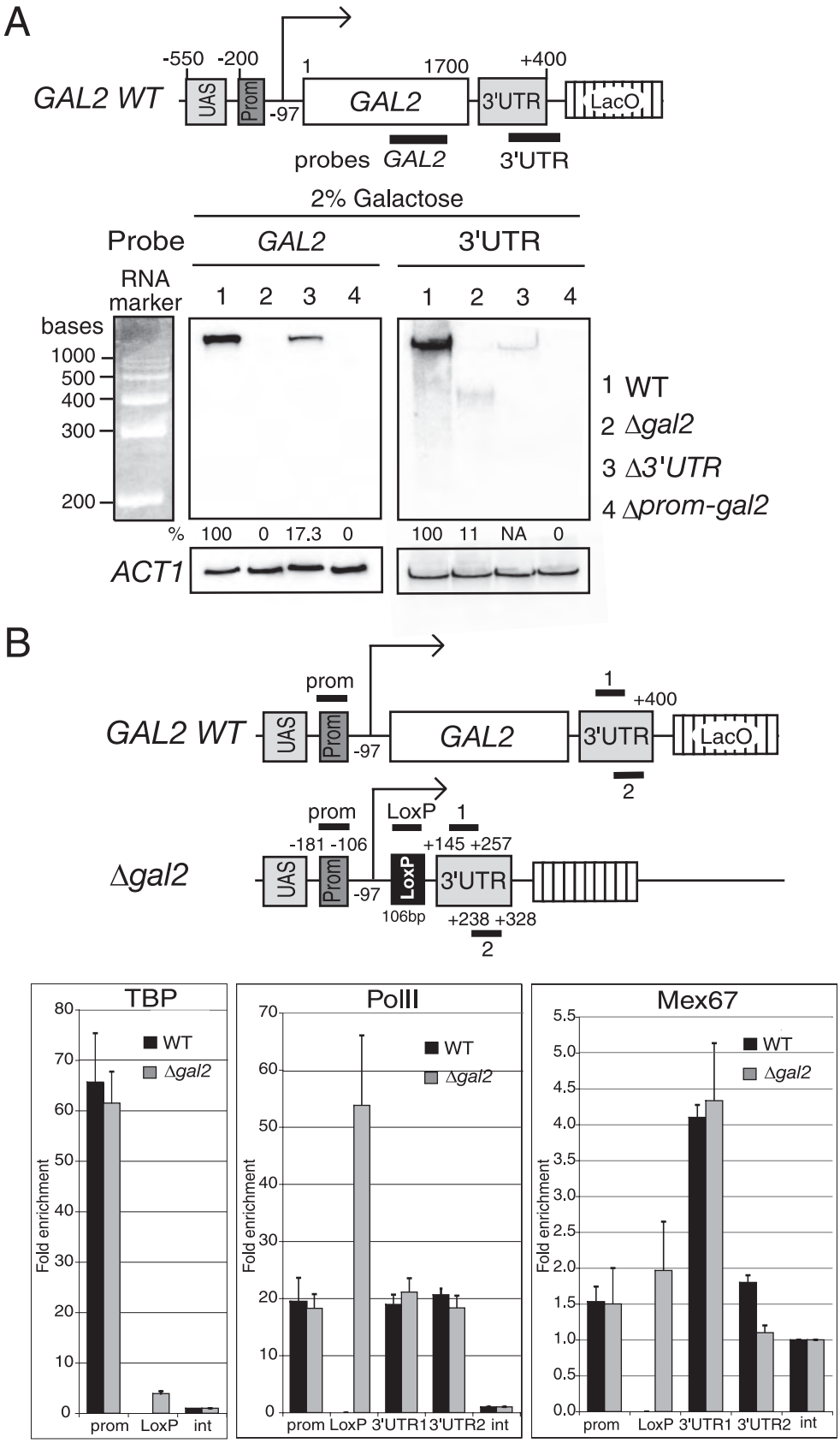


FIG. 6. The Δ*gal2* mutant gene encodes a short unstable transcript but recruits wild-type levels of RNA PolII and Mex67p. (A) Northern blot analysis of total RNA from the wild type (lane 1) and indicated mutant strains (lanes 2 to 4) with probes diagrammed at the top and spanning

formation. To define whether Mex67p is recruited to the Δ*gal2* gene, ChIP was used to compare the recruitment of TBP, RNA PolII, and Mex67p on the wild-type and Δ*gal2* genes after galactose induction (Fig. 6B). Four primer pairs were used to examine the association of these proteins with chromatin: one corresponds to the *GAL2* promoter region, another to the LoxP sequence present only in the Δ*gal2* mutant, and two others are specific for the 3′untranslated region and amplify gene segments from +145 to +257 (3′UTR1) and +238 to +328 (3′UTR2) (Fig. 6B, top). As shown in Fig. 5C, similar levels of TBP were recruited to the *GAL2* promoter in the two strains. TBP was barely detectable at the LoxP site located less than 200 bp downstream from the promoter region in Δ*gal2*. The very low level of TBP at this site demonstrates the high resolution of these ChIP analyses and is consistent with efficient shearing of chromatin in the extracts. RNA PolII levels were comparable within the promoter and 3′UTR regions of the two strains. The RNA PolII signal was higher at the LoxP site in the Δ*gal2* strain, consistent with the increased levels of RNA polymerase at the 5′ end of *GAL10* (Fig. 2C). Finally, Mex67p was detected at very low levels at the *GAL2* promoter in both strains, increased at the LoxP site, and reached a fourfold enrichment in the 3′UTR1 region of both strains, before dropping in the 3′UTR2 region. Notably, the size of the short transcript produced in Δ*gal2* cells predicts that 3′-end formation occurs somewhere between the 3′UTR1 and 3′UTR2 regions (Fig. 6A). These results suggest that 3′-end processing may coincide with the loss of Mex67p from the chromatin. More importantly, the association of Mex67p with the Δ*gal2* locus strengthens the view that early recruitment of Mex67p depends on the transcription machinery rather than mRNP formation and that Mex67 may act as a *trans*-acting factor in NPC gene anchoring by virtue of an RNA-independent process.

DISCUSSION

Live imaging of GFP-tagged yeast cells has revealed that genomic loci constantly move within the nucleus and frequently ricochet off the nuclear membrane (24, 30). Transcription-induced gene anchoring is likely to result from the stabilization of these transient contacts by specific interactions between the activated gene and NPC components. The aim of this study was to test the potential role of the nascent mRNP complex in *GAL2*, *GAL10*, and *HSP104* gene anchoring by examining gene repositioning in both *cis*- and *trans*-acting mutants.

***GAL2* gene anchoring does not require the mRNA-coding region.** Our analyses of *GAL2* gene anchoring in strains lacking various portions of the *GAL2* gene show that the protein-coding and 3′UTR regions are dispensable, while the UAS and promoter region encompassing the TATA box are necessary for *GAL2* association with the nuclear periphery (Fig. 5A and B). The results of ChIP experiments confirmed that similar amounts of TBP and RNA PolII associate with the *GAL2* promoter of wild-type, Δ*gal2*, Δ*3′UTR*, and Δ*gal2-3′UTR* cells, indicating that transcription activation and initiation occur efficiently in the absence of the protein-coding and 3′UTR regions (Fig. 5C and 6B; also data not shown). Despite efficient TBP recruitment, *GAL2* transcripts encoded by the Δ*3′UTR* mutant were poorly expressed (20% of wild-type levels), probably due to message instability as a result of improper 3′-end processing. Notably, the short 450-base transcript encoded by the Δ*gal2* mutant was present at even lower levels (10% of wild-type levels). Thus, the poor expression of this short RNA together with the efficient transcription-induced repositioning of the Δ*gal2* gene suggests that RNA is unlikely to act as a major determinant in *GAL2* gene anchoring. However, the possibility that the *GAL2* mRNA encoded in the wild-type strain contributes to the maintenance of the induced gene at the periphery cannot be excluded. This possibility could be tested by comparing the dissociation kinetics of wild-type *GAL2*, Δ*gal2*, or Δ*3′UTR* loci from the nuclear envelope after transcriptional shut-off.

So far, our data on *GAL2* support the view that transcription activation but not mRNA production plays a major role in NPC anchoring of this gene. Accordingly, physical interactions have been identified between Sus1p, a component of the SAGA coactivator complex recruited upstream of galactose-inducible genes, and components of the mRNA export machinery associated with pores (31). Moreover, a recent study showed that the promoter regions of numerous active genes, including the *GAL* genes, physically interact with the NPC component Nup2p. Notably, these interactions are independent of transcription, suggesting that early activating events might be sufficient for connecting a gene to the NPC (33, 34). The results of our gene localization experiments on *GAL2* deletion strains are consistent with the results of these biochemical studies, but this may not be the case for all genes. Indeed, a recent study shows that efficient repositioning of the *HXK1* gene requires the 3′UTR (42). One possibility is that gene-to-pore interactions involve a number of partially redundant interactions, which may occur simultaneously or sequentially during the tethering process. Thus, the contribution of

the *GAL2* protein-coding region (*GAL2* probe [left panel]) or the *GAL2* 3′UTR region (3′UTR probe [right panel]). For quantification, the *GAL2* RNA signals were normalized to endogenous actin mRNA levels. The 3′UTR probe weakly hybridized to the *GAL2* mRNA produced in the Δ*3′UTR* strain, as both the probe and the transcript produced by this mutant extend beyond the 3′UTR-deleted region. This band was not quantified, as it is only partially complementary to the probe. NA, not applicable. (B) ChIP analyses of TBP, RNA PolII and Mex67p on wild-type *GAL2* and the mutant Δ*gal2* gene. Extracts from galactose-induced cultures were immunoprecipitated with antibodies against TBP, PolII, and Mex67p. Coprecipitating DNA segments (diagrammed as short bars on top) were quantified by real-time PCR with primers specific for the *GAL2* promoter (positions −181 to −106), LoxP (106 bp derived from Cre-Lox recombination), 3′UTR1 (positions +145 to +257) and 3′UTR2 (positions +238 to +328). The 3′UTR was numbered positively starting from the *GAL2* stop codon, and 3′ end formation is predicted to occur around +250 (A). The relative enrichment of DNA segments in each ChIP was expressed as the enrichment with respect to the nontranscribed intergenic (int) value, set to 1. Values are means of three independent experiments.

the mRNP to gene anchoring may depend on the strength of other, i.e., transcription linked, interactions.

How do Mex67p and Mlp1p contribute to gene anchoring? To identify *trans*-acting factors implicated in gene anchoring and following up on the initial hypothesis that anchoring may be mediated by factors interacting with nascent mRNPs, we found that both Mex67p and Mlp1p contribute to *GAL10* and *HSP104* gene anchoring (Fig. 1B, 2A, and 3A). Indeed, the activated *GAL10* gene rapidly dissociated from the periphery in *mex67-5* cells shifted to 37°C. Similarly, ethanol-induced *HSP104* gene anchoring observed in wild-type cells at 25°C was abolished in *mex67-5* cells at this temperature. Importantly, the loss of gene anchoring was paralleled by the loss of cotranscriptional binding of the *mex67-5* protein to ethanol-induced *HSP104*, a condition under which the *mex67-5* protein remains at the nuclear periphery (Fig. 3C and E). These observations indicate that maintenance of the *mex67-5* protein at the periphery is not sufficient for gene anchoring and that productive interaction of Mex67p with the gene is required. Although we cannot exclude the possibility that gene movement is restricted in a *mex67-5* mutant heated to 37°C or exposed to ethanol or distinguish whether Mex67p cotranscriptional recruitment is the cause or consequence of peripheral gene association, the results of both the *GAL10* and *HSP104* experiments support the view that Mex67p contributes to NPC anchoring by physical association with activated genes.

Our ChIP analyses show that association of Mex67p with *GAL10* and *HSP104* is clearly detectable at the 5′ end, reaches a maximum in the middle part, and decreases at the 3′ end of these genes (Fig. 2C and 3C). Importantly, the interaction of Mex67p with the *GAL10* gene is not sensitive to RNase treatment, indicating that the early recruitment of Mex67p to transcribing genes is not mediated by RNA (Fig. 4). Thus, Mex67p is likely to be recruited via adaptors associated with the transcription machinery. Despite the good resolution of our ChIP experiments, the Mex67p signal detected at the promoter is too weak to conclude that Mex67p already binds in this region. The ChIP profile more likely indicates that Mex67p becomes associated with activated genes at a very early step of transcription, possibly within the transition from initiation to elongation.

Notably, Mex67p is efficiently recruited to the Δ*gal2* gene, which does not produce a stable mRNP (Fig. 6A and B). This observation further supports the view that the early recruitment of Mex67p is not mediated by RNA, but by an adaptor(s) associated with the transcription machinery. Mex67p is probably transferred to the mRNA at a later step, an event not easily detected by ChIP. Notably, analysis of Mex67p recruitment on the wild-type *GAL2* and Δ*gal2* genes indicated a drop in Mex67p binding around position +250 within the 3′UTR. Interestingly, this site corresponds to the region within which the 450-base transcript encoded by the Δ*gal2* gene is predicted to end (Fig. 6A and B). This observation suggests that transfer of Mex67p from chromatin to mRNA may coincide with 3′-end processing. This view is consistent with an earlier study proposing that binding of Mex67p to mRNA is coupled to 3′-end processing and release of the mRNP from the transcribing gene (15).

The finding that early recruitment of Mex67p is RNA independent raises the question of the nature of the adaptor(s) mediating the association of Mex67p to active genes. One candidate is Npl3p, an hnRNP protein interacting with Mex67p and recruited to active genes by the RNA PolII complex at an early step of transcription (15, 28). Another is the hnRNP-like protein Yra1p, which interacts with the N-terminal domain of Mex67p (37, 39, 44). The association of Yra1p with transcribing genes is largely RNase insensitive, suggesting that this mRNA export adaptor is first recruited via interaction with the transcription machinery and subsequently transferred to mRNA (1, 28, 45). Thus, Mex67p could be recruited via interaction of its N-terminal domain with Yra1p. However, the results of our recent studies indicate that the early recruitment of Mex67p largely depends on its C-terminal UBA domain (19). Interestingly, Hpr1p, a component of the THO complex implicated in transcription elongation and mRNA export (7, 38, 45), directly interacts with the Mex67p UBA domain, and this interaction facilitates the recruitment of Mex67p to the *GAL10* gene (19). Future studies will address whether early Mex67p recruitment is mediated via multiple, possibly sequential, adaptors associated with the transcription initiation and elongation machineries.

This work also shows that the NPC-attached Mlp1 protein contributes to efficient transcription-induced *GAL10* and *HSP104* gene anchoring (Fig. 1B). Genome-wide mapping of Mlp1p-bound DNA sequences suggested that interaction of Mlp1p with chromatin occurs according to different modes. Whereas the association of Mlp1p with induced genes is RNA dependent and biased towards the 3′ end, the binding to subtelomeric regions is largely RNA independent (5). Mlp1p has also been found in association with components of the SAGA coactivator and mediator complexes implicated in transcription activation (14). Thus, Mlp proteins may contribute to stable *GAL10* and *HSP104* gene tethering at an early step by interaction with chromatin-associated transcription regulators, or at a later step by interacting with nascent transcripts, or both. In contrast to our observations, a recent report identified no effect of Δ*mlp1* on gene anchoring (4). It is presently unclear whether this discrepancy results from strain background or experimental differences. Importantly, this recent study identified additional factors implicated in gene anchoring, including Ada2p and Sus1p, two components of the SAGA coactivator complex, as well as Nup1 and Sac3, two factors belonging to the mRNA export machinery associated with pores (4). Whether these factors, as well as Mex67p and Mlp1p, act in the same pathway and in a defined chronological order during the anchoring process are questions for the future. It is possible that both early transcription-linked and later potentially mRNP-dependent tethers contribute to gene anchoring. However, the relative importance of individual tethers may vary from gene to gene.

Relationship between gene expression and anchoring. Although peripheral localization has been proposed to optimize the expression of some inducible genes (3, 42), our analyses indicate that gene anchoring may not be a general requirement for gene expression. Indeed, whereas *HSP104* gene anchoring was strongly inhibited in the *mex67-5* mutant (Fig. 3A), *HSP104* mRNA levels were even higher in *mex67-5* cells than in the wild-type cells (Fig. 3B). ChIP analyses of TBP and RNA PolII indicated similar transcription rates for *HSP104* in wild-type and *mex67-5* cells exposed to ethanol, indicating that dissociation of *HSP104* from the periphery affects mRNA turnover rather than transcription rates (Fig. 3C). In contrast, a

shift of *mex67-5* cells to 37°C led to a slight decrease in *GAL10* mRNA levels and RNA PolII recruitment to this gene (Fig. 2B and C). Whether this decrease is due to indirect effects of *mex67-5* on transcription and/or a decrease in mRNA stability as a result of the mRNA export block is unclear. However, the slow and modest effect on mRNA levels compared to the rapid and strong effect on *GAL10* gene localization suggests that NPC anchoring does not influence the expression of this gene. This conclusion is consistent with the report by Cabal et al. (4) showing that the changes in *GAL* locus positioning, observed in strains lacking the SAGA components Ada2p or Sus1p or the pore-associated mRNA export factors Nup1 or Sac3, do not affect *GAL1* mRNA transcription levels. Our analyses with Δ*mlp1* cells led to similar conclusions. As this mutant presents no mRNA export defect (27, 36), the relationship between gene localization and expression could be examined without potential indirect effects of an export block. Neither *GAL10* nor *HSP104* levels were affected in Δ*mlp1* cells despite a clear effect on gene repositioning (Fig. 1B and C; also data not shown). At this time, it is unclear whether the increased expression of *INO1* and *HXK1*, observed when these genes were positioned at the periphery, reflects a natural regulatory pathway or results from their artificial tethering to the nuclear envelope (3, 42).

The functional significance of *GAL10* and *HSP104* gene anchoring is an open question, as gene repositioning appears to be the consequence rather than the cause of their transcriptional activity. Peripheral localization may be more important for the efficient expression of other types of genes under different physiological or inducing conditions. Another likely possibility is that gene anchoring contributes to gene expression efficiency by facilitating mRNA export through nuclear pores. Since Mlp proteins have been implicated in the nuclear retention of unprocessed pre-mRNAs (11), an interesting view is that gene anchoring also contributes to this late step of mRNA surveillance at the nuclear periphery.

ACKNOWLEDGMENTS

We are grateful to Susan Gasser, Catherine Dargemont, and Martine Collart for plasmids, strains, and antibodies. We also thank Sylvie Dersi and Céline Fickentscher for technical assistance and Thierry Laroche and Christophe Bauer for help in microscopy. We thank Michael Rosbash for discussions and Martine Collart, Catherine Dargemont, and Jurgi Camblong for critical comments on the manuscript.

This work was supported by the Swiss National Science Foundation (SNF grant 102235 to F.S.) and support from the SNF program "Frontiers in Genetics" to F.S.

REFERENCES

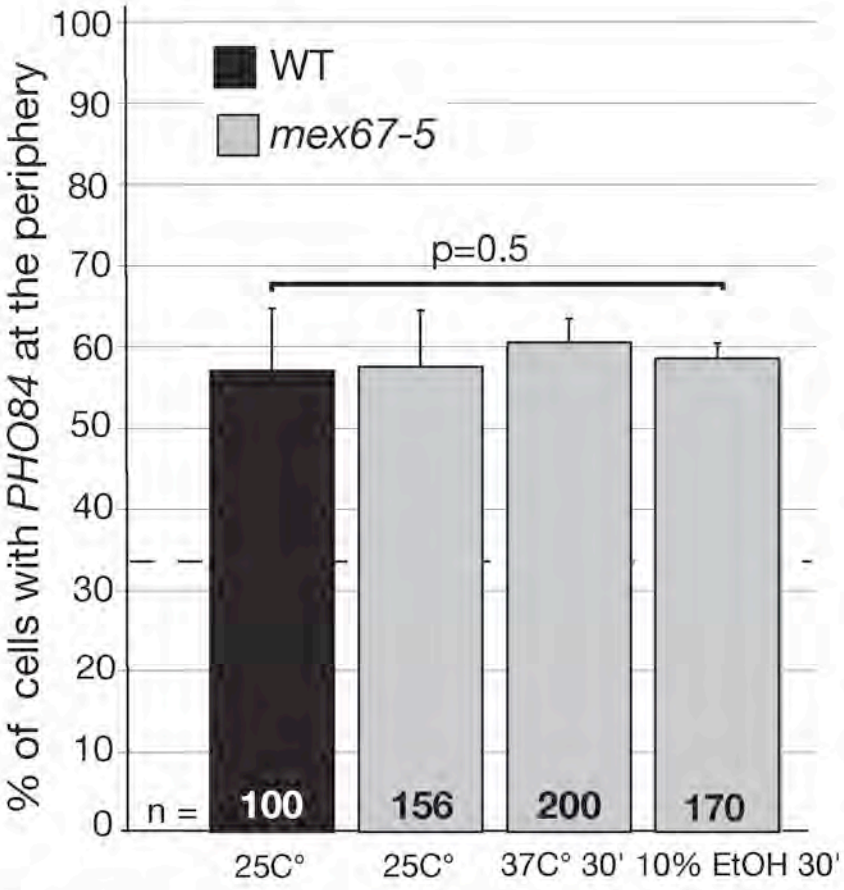
1. **Abruzzi, K. C., S. Lacadie, and M. Rosbash.** 2004. Biochemical analysis of TREX complex recruitment to intronless and intron-containing yeast genes. EMBO J. **23:**2620–2631.
2. **Andrulis, E. D., A. M. Neiman, D. C. Zappulla, and R. Sternglanz.** 1998. Perinuclear localization of chromatin facilitates transcriptional silencing. Nature **394:**592–595.
3. **Brickner, J. H., and P. Walter.** 2004. Gene recruitment of the activated INO1 locus to the nuclear membrane. PLoS Biol. **2:**e342.
4. **Cabal, G. G., A. Genovesio, S. Rodriguez-Navarro, C. Zimmer, O. Gadal, A. Lesne, H. Buc, F. Feuerbach-Fournier, J. C. Olivo-Marin, E. C. Hurt, and U. Nehrbass.** 2006. SAGA interacting factors confine subdiffusion of transcribed genes to the nuclear envelope. Nature **441:**770–773.
5. **Casolari, J. M., C. R. Brown, D. A. Drubin, O. J. Rando, and P. A. Silver.** 2005. Developmentally induced changes in transcriptional program alter spatial organization across chromosomes. Genes Dev. **19:**1188–1198.
6. **Casolari, J. M., C. R. Brown, S. Komili, J. West, H. Hieronymus, and P. A. Silver.** 2004. Genome-wide localization of the nuclear transport machinery couples transcriptional status and nuclear organization. Cell **117:**427–439.
7. **Chavez, S., T. Beilharz, A. G. Rondon, H. Erdjument-Bromage, P. Tempst, J. Q. Svejstrup, T. Lithgow, and A. Aguilera.** 2000. A protein complex containing Tho2, Hpr1, Mft1 and a novel protein, Thp2, connects transcription elongation with mitotic recombination in Saccharomyces cerevisiae. EMBO J. **19:**5824–5834.
8. **Cockell, M., and S. M. Gasser.** 1999. Nuclear compartments and gene regulation. Curr. Opin. Genet. Dev. **9:**199–205.
9. **Dilworth, D. J., A. J. Tackett, R. S. Rogers, E. C. Yi, R. H. Christmas, J. J. Smith, A. F. Siegel, B. T. Chait, R. W. Wozniak, and J. D. Aitchison.** 2005. The mobile nucleoporin Nup2p and chromatin-bound Prp20p function in endogenous NPC-mediated transcriptional control. J. Cell Biol. **171:**955–965.
10. **Feuerbach, F., V. Galy, E. Trelles-Sticken, M. Fromont-Racine, A. Jacquier, E. Gilson, J. C. Olivo-Marin, H. Scherthan, and U. Nehrbass.** 2002. Nuclear architecture and spatial positioning help establish transcriptional states of telomeres in yeast. Nat. Cell Biol. **4:**214–221.
11. **Galy, V., O. Gadal, M. Fromont-Racine, A. Romano, A. Jacquier, and U. Nehrbass.** 2004. Nuclear retention of unspliced mRNAs in yeast is mediated by perinuclear Mlp1. Cell **116:**63–73.
12. **Galy, V., J. C. Olivo-Marin, H. Scherthan, V. Doye, N. Rascalou, and U. Nehrbass.** 2000. Nuclear pore complexes in the organization of silent telomeric chromatin. Nature **403:**108–112.
13. **Gasch, A. P., and M. Werner-Washburne.** 2002. The genomics of yeast responses to environmental stress and starvation. Funct. Integr. Genomics **2:**181–192.
14. **Gavin, A. C., M. Bosche, R. Krause, P. Grandi, M. Marzioch, A. Bauer, J. Schultz, J. M. Rick, A. M. Michon, C. M. Cruciat, M. Remor, C. Hofert, M. Schelder, M. Brajenovic, H. Ruffner, A. Merino, K. Klein, M. Hudak, D. Dickson, T. Rudi, V. Gnau, A. Bauch, S. Bastuck, B. Huhse, C. Leutwein, M. A. Heurtier, R. R. Copley, A. Edelmann, E. Querfurth, V. Rybin, G. Drewes, M. Raida, T. Bouwmeester, P. Bork, B. Seraphin, B. Kuster, G. Neubauer, and G. Superti-Furga.** 2002. Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature **415:**141–147.
15. **Gilbert, W., and C. Guthrie.** 2004. The Glc7p nuclear phosphatase promotes mRNA export by facilitating association of Mex67p with mRNA. Mol. Cell **13:**201–212.
16. **Gotta, M., T. Laroche, A. Formenton, L. Maillet, H. Scherthan, and S. M. Gasser.** 1996. The clustering of telomeres and colocalization with Rap1, Sir3, and Sir4 proteins in wild-type Saccharomyces cerevisiae. J. Cell Biol. **134:**1349–1363.
17. **Green, D. M., C. P. Johnson, H. Hagan, and A. H. Corbett.** 2003. The C-terminal domain of myosin-like protein 1 (Mlp1p) is a docking site for heterogeneous nuclear ribonucleoproteins that are required for mRNA export. Proc. Natl. Acad. Sci. USA **100:**1010–1015.
18. **Gueldener, U., J. Heinisch, G. J. Koehler, D. Voss, and J. H. Hegemann.** 2002. A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. Nucleic Acids Res. **30:**e23.
19. **Gwizdek, C., I. Iglesias, M. S. Rodriguez, B. Ossareh-Nazari, M. Hobeika, G. Divita, F. Stutz, and C. Dargemont.** Proc. Natl. Acad. Sci. USA, in press.
20. **Hediger, F., and S. M. Gasser.** 2002. Nuclear organization and silencing: putting things in their place. Nat. Cell Biol. **4:**E53–E55.
21. **Heun, P., T. Laroche, K. Shimada, P. Furrer, and S. M. Gasser.** 2001. Chromosome dynamics in the yeast interphase nucleus. Science **294:**2181–2186.
22. **Huibregtse, J. M., P. D. Good, G. T. Marczynski, J. A. Jaehning, and D. R. Engelke.** 1993. Gal4 protein binding is required but not sufficient for derepression and induction of GAL2 expression. J. Biol. Chem. **268:**22219–22222.
23. **Hurt, E., K. Strasser, A. Segref, S. Bailer, N. Schlaich, C. Presutti, D. Tollervey, and R. Jansen.** 2000. Mex67p mediates nuclear export of a variety of RNA polymerase II transcripts. J. Biol. Chem. **275:**8361–8368.
24. **Ishii, K., G. Arib, C. Lin, G. Van Houwe, and U. K. Laemmli.** 2002. Chromatin boundaries in budding yeast: the nuclear pore connection. Cell **109:**551–562.
25. **Isogai, Y., and R. Tjian.** 2003. Targeting genes and transcription factors to segregated nuclear compartments. Curr. Opin. Cell Biol. **15:**296–303.
26. **Jimeno, S., A. G. Rondon, R. Luna, and A. Aguilera.** 2002. The yeast THO complex and mRNA export factors link RNA metabolism with transcription and genome instability. EMBO J. **21:**3526–3535.
27. **Kosova, B., N. Pante, C. Rollenhagen, A. Podtelejnikov, M. Mann, U. Aebi, and E. Hurt.** 2000. Mlp2p, a component of nuclear pore attached intranuclear filaments, associates with nic96p. J. Biol. Chem. **275:**343–350.
28. **Lei, E. P., H. Krebber, and P. A. Silver.** 2001. Messenger RNAs are recruited for nuclear export during transcription. Genes Dev. **15:**1771–1782.
29. **Longtine, M. S., A. McKenzie III, D. J. Demarini, N. G. Shah, A. Wach, A. Brachat, P. Philippsen, and J. R. Pringle.** 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast **14:**953–961.
30. **Marshall, W. F., A. Straight, J. F. Marko, J. Swedlow, A. Dernburg, A. Belmont, A. W. Murray, D. A. Agard, and J. W. Sedat.** 1997. Interphase

chromosomes undergo constrained diffusional motion in living cells. Curr. Biol. **7:**930–939.
31. **Rodriguez-Navarro, S., T. Fischer, M. J. Luo, O. Antunez, J. E. Perez-Ortin, R. Reed, and E. Hurt.** 2004. Sus1, a functional component of the SAGA histone acetylase complex and the nuclear pore-associated mRNA export machinery. Cell **116:**75–86.
32. **Sanchez, Y., J. Taulien, K. A. Borkovich, and S. Lindquist.** 1992. Hsp104 is required for tolerance to many forms of stress. EMBO J. **11:**2357–2364.
33. **Schmid, M., G. Arib, C. Laemmli, J. Nishikawa, T. Durussel, and U. K. Laemmli.** 2006. Nup-PI: the nucleopore-promoter interaction of genes in yeast. Mol. Cell **21:**379–391.
34. **Schmid, M., T. Durussel, and U. K. Laemmli.** 2004. ChIC and ChEC: genomic mapping of chromatin proteins. Mol. Cell **16:**147–157.
35. **Segref, A., K. Sharma, V. Doye, A. Hellwig, J. Huber, R. Luhrmann, and E. Hurt.** 1997. Mex67p, a novel factor for nuclear mRNA export, binds to both poly(A)+ RNA and nuclear pores. EMBO J. **16:**3256–3271.
36. **Strambio-de-Castillia, C., G. Blobel, and M. P. Rout.** 1999. Proteins connecting the nuclear pore complex with the nuclear interior. J. Cell Biol. **144:**839–855.
37. **Strasser, K., and E. Hurt.** 2000. Yra1p, a conserved nuclear RNA-binding protein, interacts directly with Mex67p and is required for mRNA export. EMBO J. **19:**410–420.
38. **Strasser, K., S. Masuda, P. Mason, J. Pfannstiel, M. Oppizzi, S. Rodriguez-Navarro, A. G. Rondon, A. Aguilera, K. Struhl, R. Reed, and E. Hurt.** 2002. TREX is a conserved complex coupling transcription with messenger RNA export. Nature **417:**304–308.
39. **Stutz, F., A. Bachi, T. Doerks, I. C. Braun, B. Seraphin, M. Wilm, P. Bork, and E. Izaurralde.** 2000. REF, an evolutionary conserved family of hnRNP-like proteins, interacts with TAP/Mex67p and participates in mRNA nuclear export. RNA **6:**638–650.
40. **Stutz, F., E. Gouilloud, and S. G. Clarkson.** 1989. Oocyte and somatic tyrosine tRNA genes in Xenopus laevis. Genes Dev. **3:**1190–1198.
41. **Taddei, A., M. R. Gartenberg, F. R. Neumann, F. Hediger, and S. M. Gasser.** 2005. Multiple pathways tether telomeres and silent chromatin at the nuclear periphery: functional implications for sir-mediated repression. Novartis Found. Symp. **264:**140–156, 156–165, 227–230.
42. **Taddei, A., G. Van Houwe, F. Hediger, V. Kalck, F. Cubizolles, H. Schober, and S. M. Gasser.** 2006. Nuclear pore association confers optimal expression levels for an inducible yeast gene. Nature **441:**774–778.
43. **Vinciguerra, P., N. Iglesias, J. Camblong, D. Zenklusen, and F. Stutz.** 2005. Perinuclear Mlp proteins downregulate gene expression in response to a defect in mRNA export. EMBO J. **24:**813–823.
44. **Zenklusen, D., P. Vinciguerra, Y. Strahm, and F. Stutz.** 2001. The yeast hnRNP-like proteins Yra1p and Yra2p participate in mRNA export through interaction with Mex67p. Mol. Cell. Biol. **21:**4219–4232.
45. **Zenklusen, D., P. Vinciguerra, J. C. Wyss, and F. Stutz.** 2002. Stable mRNP formation and export require cotranscriptional recruitment of the mRNA export factors Yra1p and Sub2p by Hpr1p. Mol. Cell. Biol. **22:**8241–8253.

Suppl. TABLE I. Primers used for real-time PCR

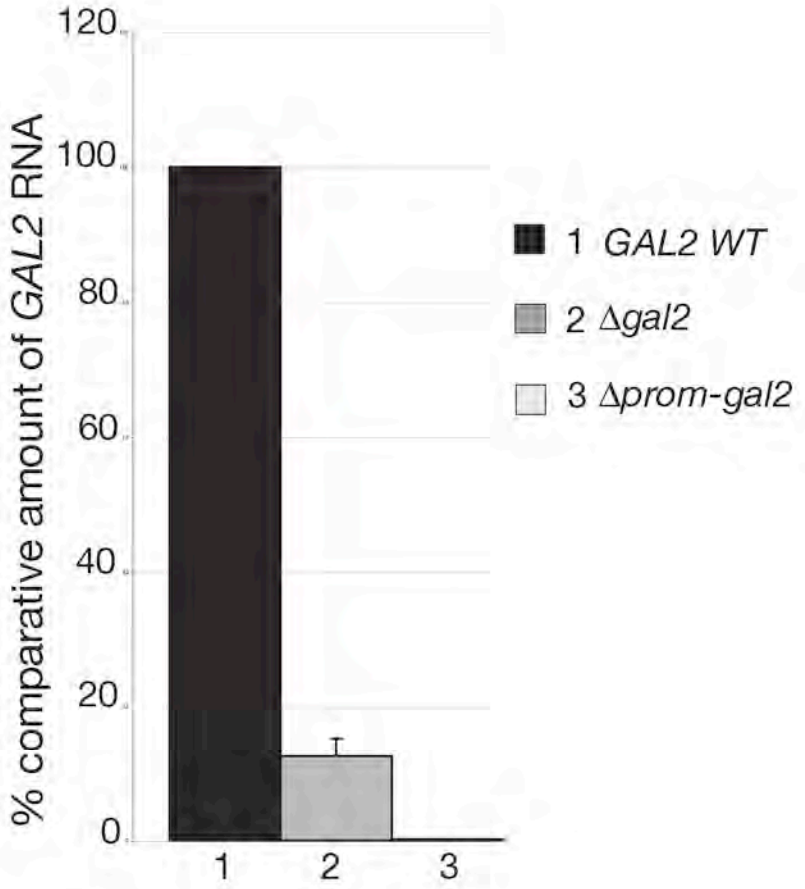
Intergenic F	OFS710	5'CGCATTACCAGA CGGAGATGT3'
Intergenic R	OFS711	5'CAAGCAAGCCTTGTGCATAAGA3'
GAL10 Prom F	OFS1059	5'TTTTAGGCTAAGATAATGGGGCTCTTTAC3'
GAL10 Prom R	OFS1060	5'TTACATGGCATTACCACCATATACATATCC3'
GAL10 5' F	OFS1061	5'CACTGTGGTAGAGCTAATTGAGAATGG3'
GAL10 5' R	OFS1062	5'AAGACCTCTAACCTGGCTACAGAATC3'
GAL10 M F	OFS1063	5'CCAGTTAAGGGGTGTCGAGGC3'
GAL10 M R	OFS1064	5'AAATTGGCAAACGTGGCTTGAAATC3'
GAL10 3' F	OFS1065	5'CAAGACAAGGTTTTGCAATTGAGCC3'
GAL10 3' R	OFS1066	5'CAATCTTGGACCCGTAAGTTTCACC3'
GAL10 3'UTR F	OFS1067	5'TGTAGGGACCGAATTGTTTACAAGTTC3'
GAL10 3'UTR R	OFS1068	5'GTTGCTACCGTCCATATCTTTCCATAG3'
HSP104 Prom F	OFS937	5'TTATATCACAGTAAAAGGCAAAGGGGC3'
HSP104 Prom R	OFS938	5'GGAACAAGTGACAAAGGAACGAATTATG3'
HSP104 5' F	OFS857	5'TTCTAGCTGCCTTCATTGAAACGC3'
HSP104 5' R	OFS858	5'TCTTGAAAAG ATCATAGTCGTAACGGC3'
HSP104 M F	OFS853	5'GCTGATTTAAGG TACTTCGCCATCC3'
HSP104 M R	OFS854	5'AGTTGGCACCAGCACGTGTC3'
HSP104 3' F	OFS880	5'TGAACAAA CTGGCACTAAGGATCTT3'
HSP104 3' R	OFS881	5'CATTCTTCAGCTTCCTCAGGAACA3'
GAL2 Prom F	OFS1096	5'TTTCGCAGGCTAAAATGTGGAGATAG3'
GAL2 Prom R	OFS1097	5'GAGCAATTCACAACACCAAATTTTCAATC3'
LoxP F	OFS1138	5'ACAACCCTTAATATAACTTCGTATAATGTATGC3'
LoxP R	OFS1139	5'GATGATGGAGCGTCTCACTTCAAAC3'
GAL2 3'UTR1 F	OFS1142	5'CTTACCTAAACCTATTATTTGTGTACA TATATCAGAG3'
GAL2 3'UTR1 R	OFS1143	5'CAAAGGATGGCAGAGCATGTTATCG3'
GAL2 3' UTR2 F	OFS1140	5'AAACGATAACATGCTCTGCCATCC3'
GAL2 3'UTR2 R	OFS1141	5'GTCTGGTGATGTGGTCCTTTAATAATTTC3'
GAL2 cDNA 3'UTR F	OFS1144	5'GATATCCTTACGATTATATAAATTCC3'
GAL2 cDNA 3'UTR R	OFS1145	5'TATATGTAATAATACTCTGATATATGTAC3'

Dieppois et al. Mol. Cell. Biol. 2006



Suppl. Fig 1. Localization of *PHO84* tagged with LacO repeats in wild-type (black bar) or *mex67-5* (grey bars). The two srains were grown in YEPD rich media to OD_{600}=0.5 and examined after a 30 min shift to 37C° or a 30 min treatment with 10% ethanol.

Dieppois et al. Mol. Cell. Biol. 2006



Suppl. Fig. 2. RT-PCR GAL2 transcript quantification in wild type (black bar), Δ*gal2* (grey bar) and Δ*prom-gal2* (light grey bar).
Total RNA was extracted from cells grown in YEP rich medium containing 2% galactose to OD_{600}= 0.5. *GAL2 and actin* mRNAs were reverse transcribed using the Quantitect custom assay kit (Qiagen GmbH) and reverse primers specific for the *GAL2 3'UTR* (OFS 1143: position +250) and the actin coding region (OFS736). cDNAs were quantified by real-time PCR using primers pairs OFS1144 and OFS1145 for *GAL2* and OFS1146 and OFS1147 for *ACT1* (Suppl. TABLE I), using SYBr green (BIO-RAD) as an indicator. *GAL2* and *ACT1* oligo pairs had the same amplification efficiency. The relative amounts of *GAL2* mRNA in the assayed strains were determined by using the ΔΔCt comparative method, in which the expression fold value was calculated with the equation $2^{-\Delta\Delta Ct}$.
The experiment was repeated three times and the *GAL2* RNA values in Δ*gal2* and Δ*prom-gal2* expressed as percent of those in WT.
Primers for the RT: OFS 1143 *GAL2* 5'-CAAAGGATGGCACATGTTATCG-3'
OFS 736 *ACT1* 5'- TACTCCGTCTGGATTGGTGGTT-3'

2.1.5 Additional results on the characterization of nuclear pore anchoring of activated genes via processing and export factors.

The previous findings lead to a conceptual change in the model of gene recruitment at the NPC during their transcription activation. It highlights that Mex67 in association with the transcription machinery contributes to an additional tether that links the transcribing genes to the NPC, possibly indicating a role for the NPC in transcription elongation and co-transcriptional mRNP assembly. In the following section I will present the results of additional investigations on the role of Mex67 in the tethering of the gene to the NPC and on the role of the NPC-gene recruitment. Various questions relative to this phenomenon will also be addressed.

2.1.5.1 The *mex67-5* mutant exhibits a defect in GAL gene transcription rate but not in transcription activation kinetics at the non permissive temperature.

As previously described, the loss of peripheral localization of activated *GAL10* in *mex67-5* mutants after a shift from 25°C to 37°C is followed by a drop in mRNA production and recruitment of polymerase II levels on *GAL10* (Dieppois et al., 2006). As the effect on transcription appears after the localization defect, we argued that the reduced expression was not a consequence of the loss of localization and that the transcription of *GAL* genes does not strictly require a peripheral localization of the gene. However, we performed these experiments in a situation where the *GAL* genes were already fully activated and did not investigate whether the lack of recruitment of the gene at the NPC may affect transcription activation. In order to address this question we examined the effects of the *mex67-5* mutation at the non permissive temperature upon activation of *GAL10* after a shift from glucose to galactose or raffinose to galactose. The results showed that in *mex67-5* cells at 37°C, *GAL10* mRNA levels were drastically decreased during activation compared to a wild type at 37°C (Figure 10). These results were not surprising since we already saw that the mutant *mex67-5* at 37°C exhibits a reduced transcription rate (Dieppois et al., 2006). However, the activation kinetics were not significantly different in *mex67-5* mutant compared to wild type at the non permissive temperature, indicating that transcription initiation *per se* is not disturbed in the *mex67-5* mutant at 37°C. These observations suggest either that gene recruitment to the NPC during the very early phase of the activation process does not require an intact Mex67, or that transcription initiation, albeit reduced, can still occur in the absence of NPC localization. I never observed a transient recruitment of *GAL* genes at the periphery during activation in a *mex67-5* mutant at the non permissive temperature. However, for technical reasons we could not discard this possibility. In any case, at the non permissive temperature, the *mex67-5*

mutant exhibits a transcription defect and we did not yet elucidate whether it is due to its inability to relocate genes to nuclear periphery or to its inability to be recruited to transcribing genes or a combination of both.

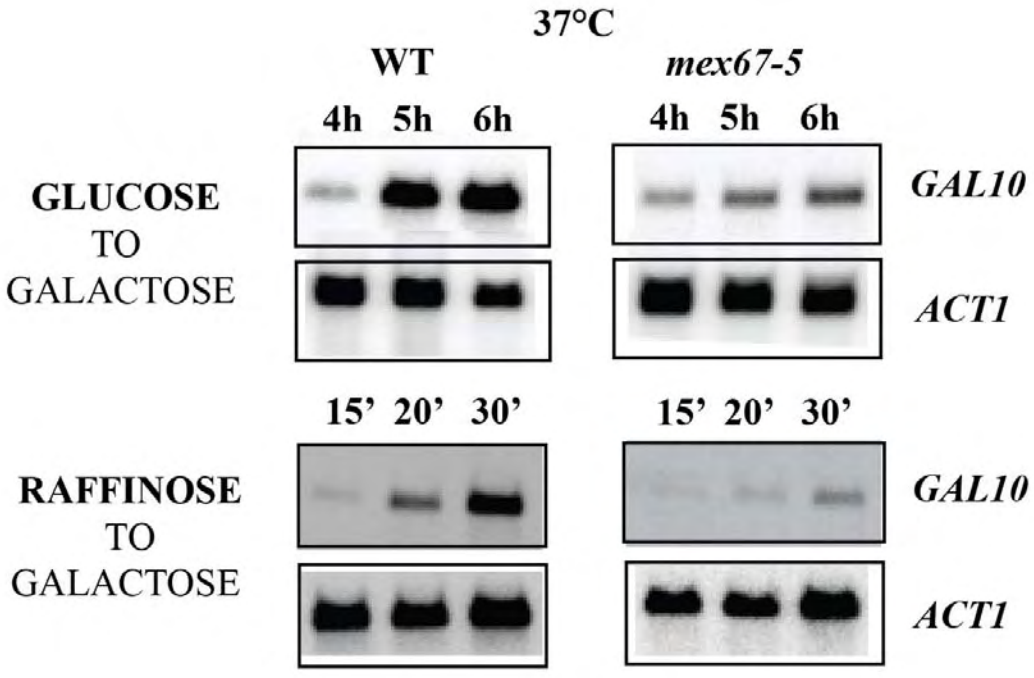


Figure 10. The activation kinetics of *GAL10* in WT versus *mex67-5* strains at 37°C are similar. Northern blot analysis of total mRNA extracted at different time points during galactose induction at 37°C of WT and *mex67-5* strains cells previously grown in glucose or raffinose.

2.1.5.2 The transcription defect in *mex67-5* is not due to the loss of GAL10 localization at the nuclear periphery.

To investigate the causal relationship between *GAL10* nuclear envelope association and its transcription rate, we examined the effect of artificially targeting *GAL10* to the nuclear envelope. This was accomplished by fusing the integral membrane proteins Yif1 to the LexA DNA-binding domain and overproducing the hybrid protein, causing it to accumulate in the nuclear membrane. This hybrid protein was expressed in a strain carrying a LexA binding site downstream of the *GAL10* gene. Artificial targeting of *GAL10* to the nuclear periphery via Yif1-LexA binding in a *mex67-5* strain in galactose at 37° rescued the localization defect in *mex67-5* at 37°C from 27% to 53% (Figure 11A). However, artificial tethering had no effect on *GAL10* mRNA levels in *mex67-5* at 25°C and 37°C as assayed by Northern blot analysis (Figure 11B). These results indicate that reduced *GAL10* transcription in *mex67-5* cells at 37°C is not due to the loss of envelope-gene anchoring. Another explanation could be that Yif1 being an integral membrane protein, it does not anchor *GAL10* close enough to the NPC, so that the NPC proximity effect might not be optimal in this experiment. Alternatively, the

lack of efficient mRNA production may be due to the lack of *mex67-5* association with the gene, which may reduce transcription and processing efficiencies.

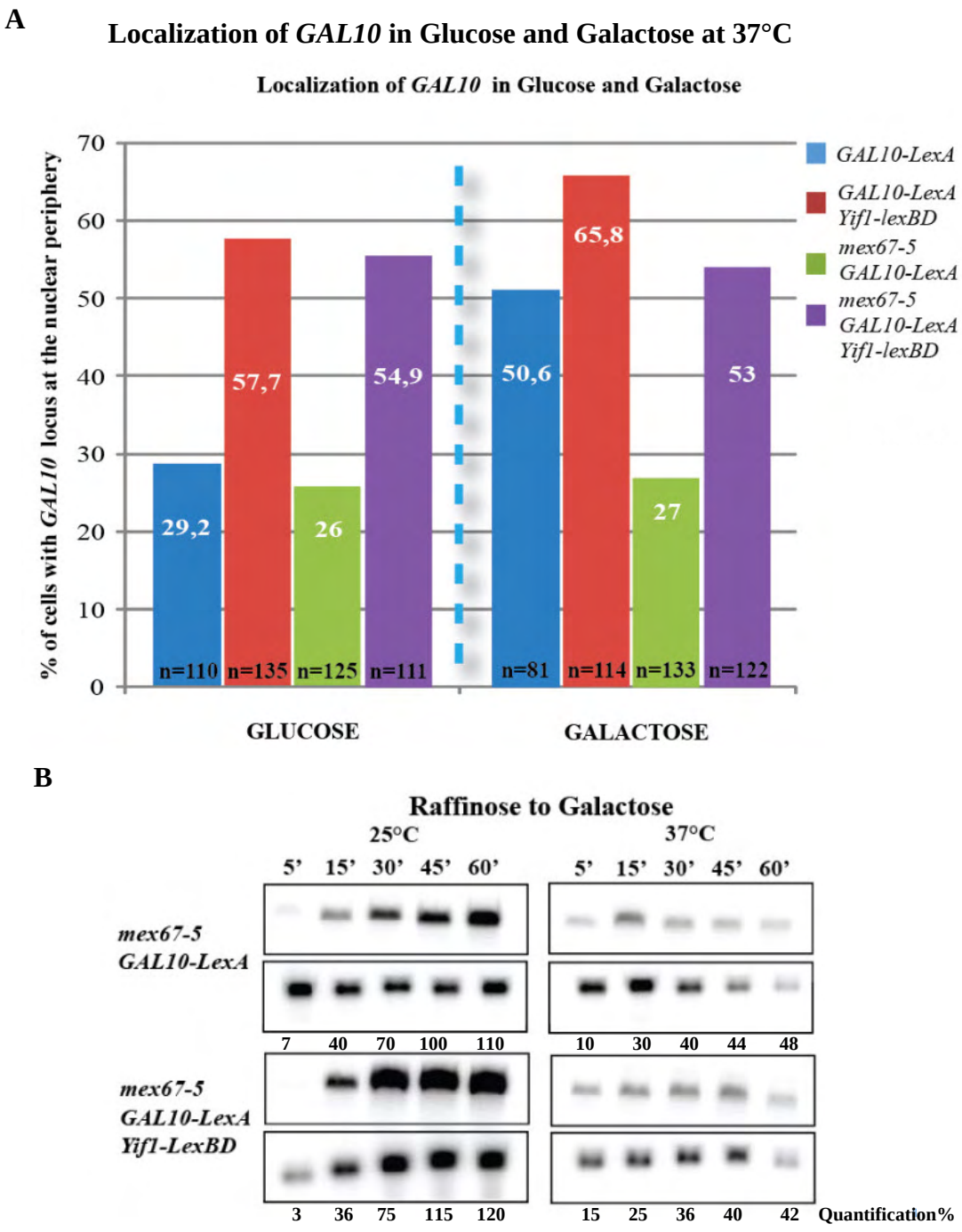


Figure 11. Artificial tethering of *GAL10* at the nuclear periphery does not rescue the low transcription rate observed in *mex67-5* at 37°C. (A) Localization assay of *GAL10*-LexA relative to the nuclear periphery. WT-*GAL10*-LexA, WT- *GAL10*-lexA + pYIF1-lexBD, *mex67-5-GAL10*-LexA, *mex67-5-GAL10*-LexA+ pYIF1-lexBD, were grown in glucose or galactose and shifted for one hour at 37°C. (B) Northern Blot analysis of the activation kinetics of *GAL10*-LexA in a *mex67-5* strain in presence or absence of pYIF1-lexBD, when shifted for the indicated time (min) from glucose to galactose at 25°C and 37°C.

2.1.5.3 The UBA domain of Mex67 is involved in the anchoring of *GAL10* to the NPC.

Data from our lab and the Dargemont lab described that the UBA domain of Mex67 interacts with ubiquitylated chromatin associated proteins and that this interaction contributes to the co-transcriptional recruitment of Mex67 (Gwizdek, 2006). The Dargemont lab with the participation of Nahid Iglesias in our lab also provided further mechanistic insights of how Mex67-UBA domain could selectively interact with ubiquitylated substrates (Hobeika 2007). Mex67-UBA domain is different from other UBA domain in the sense that it contains an additional helix 4 (H4, the last 10 amino acids of Mex67), and the deletion of H4 reduced the co-transcriptional recruitment of Mex67. Based on their data, they hypothesized that H4 may function as a switch that regulates the ubiquitin binding activity of the Mex67-UBA domain by promoting the recognition of specific substrates as well as preventing binding to non specific substrates. As the co-transcriptional recruitment of Mex67 is required for proper NPC localization of activated genes, we next decided to investigate the contribution of the Mex67 H4 in peripheral gene anchoring by comparing a wild type strain and a *mex67* mutant lacking the C terminal helix 4 (*mex67ΔH4*). After induction in galactose for 3h at 37C°, *GAL10* remained at the periphery in the wild type strain but dissociated from the periphery in the *mex67ΔH4* mutant (Figure 12A). When *GAL10* transcripts were analyzed by Northern blotting, we observed that mRNA levels were decreased in the *mex67ΔH4* mutant (Figure 12B). These observations confirmed that the recruitment of *GAL10* to the NPC requires the early recruitment of Mex67 to the transcription machinery in part *via* ubiquitylated proteins such as Hpr1, and that early recruitment of Mex67 is important for optimal gene expression *per se*.

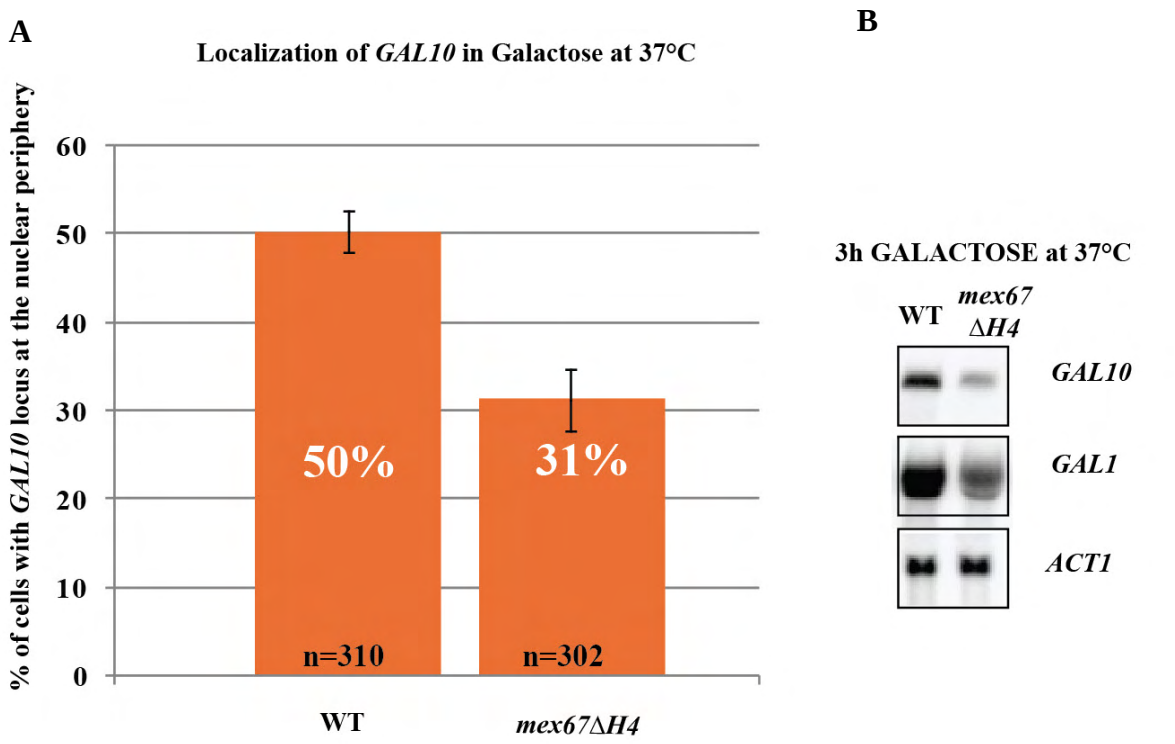


Figure 12. Analysis of the effect of a mutation in the UBA domain of Mex67 on the localization and the transcription of *GAL* genes at 37°C. (A) Localization assays of *GAL10* LacO relative to the nuclear periphery. WT and *mex67ΔH4* mutant strains were grown in galactose and shifted for 3 hours to 37°C. (B) Northern Blot analysis of *GAL10* and *GAL1* mRNA levels in total RNA extracted from WT and *mex67ΔH4* cells induced from raffinose to galactose during 3 hours at 37°C.

2.1.5.4 *GAL* gene retention at the nuclear periphery might be mediated by the mRNA processing machinery.

In order to clarify the relevance for a role of Hpr1 in gene-nuclear pore association we examined whether the localization of the heat shock gene *HSP104* was affected in a Δ*hpr1* deletion mutant. We decided to work with *HSP104* because it has been previously reported that mutations in THO components strongly affect mRNP formation and processing of this transcript. Indeed, THO mutants lead to a detectable accumulation and sequestration *HSP104* mRNAs within nuclear foci close to the site of transcription upon activation at 37°C (Thomsen et al., 2003, Zenklusen, 2002 #11, Jensen, 2001 #76). Surprisingly, we could not observe relocalization of *HSP104* when this gene was activated at 37°C in a wild type strain (Figure 13). Even more striking, in Δ*hpr1*, *HSP104* located at the nuclear periphery in more than 40% of the cells already at 25°C compared to 28% in a wild type strain. This enrichment did not increase significantly upon *HSP104* induction by the temperature shift at 37°C in Δ*hpr1*. When complemented by the wild type *HPR1* gene on a plasmid, the peripheral localization of *HSP104* in the *Δhpr1* strain dropped to 32%, indicating that the *Δhpr1* deletion is the real cause of the abnormal peripheral location of *HSP104* at 25°C. These data suggest that the recruitment of *HSP104* to the nuclear periphery upon its induction at 37°C might be so transient that we could not detect it in a wild type strain with a microscopy assay. However, in Δ*hpr1* the gene might somehow be retained at the nuclear periphery which rendered the NPC association of *HSP104* visible. As Mex67 recruitment through the transcription machinery seems to be necessary for proper gene anchoring to the periphery (Dieppois et al., 2006), these results are in contradiction with a role of Hpr1 as the only factor recruiting Mex67. However, they are perfectly consistent with another result from the Libri group and Nahid in our lab showing that in THO mutants and more specifically *Δhpr1* mutant, the recruitment of Mex67 increases (Rougemaille et al., 2008) (N. Iglesias and D. Libri personal communication). This increase is strongest at the 3' end of the gene and is likely due to the association of Mex67 with alternate adaptors that may accumulate at the 3' end of certain genes in the absence of Hpr1, such as components of the 3' end processing machinery. The unexpected recruitment of *HSP104* at the periphery at 25C° could be due to the retention of other genes affected by the Δ*hpr1* deletion in the vicinity of *HSP104* and expressed at 25°C. Indeed *HSP104* lies in a region containing other stress inducible genes (Gasch and Werner-Washburne, 2002). It may also reflect some *HSP104* expression leakage under our experimental conditions at 25°C.

As previously mentioned, THO mutants lead to defects in early mRNP assembly and the sequestration of newly synthetized transcripts within nuclear foci near the transcription site (Thomsen et al., 2003; Zenklusen et al.). An active player in the degradation of aberrant mRNPs produced in THO mutants is Rrp6, an exonuclease specific for the nuclear exosome (Bousquet-Antonelli et al., 2000; Burkard and Butler, 2000; Hilleren et al., 2001; Libri et al., 2002; Torchet et al., 2002; Zenklusen et al., 2002). The loss of Rrp6 in THO mutant strains releases transcripts from the dots suggesting that the exosome contributes to the retention of aberrant mRNPs in foci (Hilleren et al., 2001; Libri et al., 2002; Zenklusen et al., 2002). To test whether the mRNA accumulation at the transcription site in the *Δhpr1* mutant correlates with the abnormal localization phenotype of *HSP104*, we analysed the localization of *HSP104* in the double mutant *Δhpr1Δrrp6*. Importantly, deleting *RRP6* in a *Δhpr1* context led to the loss of *HSP104* association with the nuclear periphery. These observations suggest that *HSP104* localization at the nuclear peripery in the *Δhpr1* mutant is linked to defects in mRNP assembly. These results are in agreement with previous studies proposing that RNA processing is relevant to gene to pore tethering. First it was shown that both *GAL1* and *HXK1* tethering require their 3′ UTRs (Abruzzi et al., 2006; Taddei et al., 2006). This anchoring may reflect the existence of suboptimal 3' processing signals in these 3'UTRs. Second, work from the Rosbash lab showed that a Rrp6 sensitive posttranscriptional pool of *GAL* mRNPs is retained near the site of transcription after transcription repression, and contributes to posttranscriptional gene to pore tethering of *GAL* genes (Abruzzi et al., 2006, Vodala , 2008 #1211).

These data were published in collaboration with the Libri laboratory as part of a study by Rougemaille et *al.* which provides a molecular basis for the role of the THO complex in coordinating mRNP 3' processing with mRNP release from the transcription site and NPC-gene tethering (see below Section 2.1.5.5). The overall results indicate that in *tho/sub2* mutants, during a late phase of transcription, a DNA-binding complex containing 3' end processing factors accumulates that cannot be resolved for further mRNA processing and release. Interestingly, the stalling of this large protein complex that specifically sequesters the 3' regions of *HSP104* in *tho/sub2* can be suppressed by deletion of *RRP6* (Rougemaille et al., 2008), correlating with the loss of *HSP104* gene retention phenotype at the periphery. Mex67 is very likely involved in the coordination of transcription elongation with 3' end processing events and mRNA release through sequential interactions with components of the transcription machinery, the polyadenylation/termination machinery and the mRNPs, respectively (see General Discussion). Therefore, the stalling of the

polyadenylation/termination machinery and subsequent block of mRNA release and export may be the cause of Mex67 accumulation on transcribing genes in *tho* mutants, (Rougemaille et al., 2008)(N.Iglesias and D.Libri personal communication). Since Mex67 recruitment on transcribing genes promotes gene anchoring to the periphery (Dieppois et al., 2006), accumulation of Mex67 on the chromatin in the *tho* mutant might be responsible for the higher and/or persistent recruitment of *HSP104* at the nuclear periphery that we observe in *tho* mutants compared to wild type. Altogether, these observations strongly reinforce the idea of a central role of the THO complex and Mex67 in coordinating transcription elongation and mRNP maturation, and the existence of a NPC linked mechanism of mRNP surveillance (Vinciguerra et al., 2005).

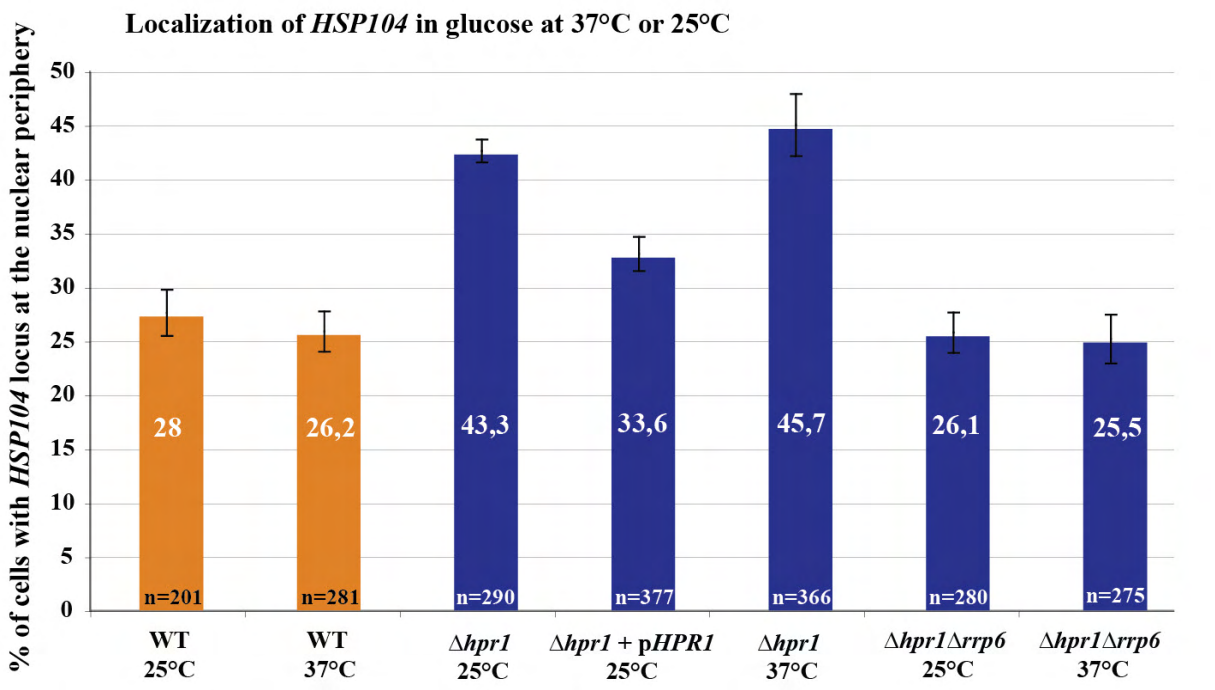


Figure 13. The increased localization of *HSP104* at the nuclear periphery in *Δhpr1* mutant is rescued by a loss of Rrp6. Localization assay of *HSP104*-LacO relative to the nuclear periphery in wt, *Δhpr1*, *Δhpr1Δrrp6* cells grown in rich medium at 25°C or shifted for 20 min at 37°C.

2.1.5.5 Article

THO/Sub2p functions to coordinate 3'-end processing with gene-nuclear pore association
M Rougemaille, G Dieppois, E Kisseleva-Romanova, R Kanth Gudipati, S Lemoine, C Blugeon, J Boulay, T Heick Jensen, F Stutz, F Devaux, D Libri.
Cell 135, 308-321,October 17, 2008

Summary In this study, the D. Libri lab provided an insight in the connection between mRNA 3' end processing and NPC-gene tethering by identifying a novel intermediate in the export pathway. They have shown that in *tho/sub2* mutants a large DNA-binding protein complex sequesters the 3' end of the *HSP104* heat shock gene and other THO target genes. Because formation of this complex leads to the depletion of these regions from chromatin extracts, they have called this phenomenon DCF for Differential Chromatin Fractionation. A modified chromatin extraction procedure allowed identification of the components of the complex responsible for DCF and revealed that it is mostly composed of polyadenylation factors. Moreover, formation of "heavy chromatin" was shown to depend on a functional 3' end processing machinery and on the nuclear exosome, suggesting that it is linked to defects in mRNP assembly. Intriguingly, persistent interactions between several genes and components of the Nuclear Pore Complex were also detected in the *tho* mutants, suggesting that formation of the DCF complex might somehow retain the genes at the NPC. The microscopy experiments carried out by our lab confirmed these biochemical data by showing that *HSP104* persists at the nuclear periphery in the *tho* mutant *Δhpr1* (see Section 2.1.5.4). and that this persistence correlates with DCF formation since it also depends on a functional nuclear exosome. These results suggest that during the late phases of transcription in *tho/sub2* mutants, a DNA-interacting complex containing polyadenylation factors and nucleoporins cannot be resolved for further mRNA processing/export. Chase experiments further demonstrated that such a complex reflects the accumulation of a stalled intermediate in the mRNP export pathway. Finally, microarray analyses of DNA trapped in "heavy chromatin" revealed the genome-wide occurrence of DCF, leading to the identification of roughly 400 novel target genes of the THO complex. Altogether, these data suggest that the THO complex and the ATPase Sub2p are involved in an mRNP remodeling event which occurs subsequently to mRNA 3' end processing and before mRNP release from the transcription site.

Cell

THO/Sub2p Functions to Coordinate 3′-End Processing with Gene-Nuclear Pore Association

Mathieu Rougemaille,[1,4,5] Guennaelle Dieppois,[2] Elena Kisseleva-Romanova,[1] Rajani Kanth Gudipati,[1,4,5] Sophie Lemoine,[3,4] Corinne Blugeon,[3,4] Jocelyne Boulay,[1,4,5] Torben Heick Jensen,[1,5] Françoise Stutz,[2] Frédéric Devaux,[3,4] and Domenico Libri[1,5,*]

[1]LEA Laboratory of Nuclear RNA metabolism, Centre de Génétique Moléculaire, Centre National de la Recherche Scientifique - UPR2167, 1, av de la Terrasse, 91190, Gif sur Yvette, France

[2]Department of Cell Biology, Sciences III, University of Geneva, 30, Quai E. Ansermet, 1211 Geneva 4, Switzerland

[3]Plate-forme transcriptome, IFR36, Ecole Normale Supérieure, 46 rue d'Ulm, 75006 Paris, France

[4]Laboratoire de Génétique Moléculaire, Ecole Normale Supérieure, Centre National de la Recherche Scientifique - UMR8541, 46 rue d'Ulm, 75006 Paris, France

[5]Centre for mRNP Biogenesis and Metabolism, Department of Molecular Biology, Aarhus University, C.F. Møllers Alle, Bldg. 130, DK-8000 Aarhus C., Denmark

*Correspondence: libri@cgm.cnrs-gif.fr

DOI 10.1016/j.cell.2008.08.005

SUMMARY

During transcription, proteins assemble sequentially with nascent RNA to generate a messenger ribonucleoprotein particle (mRNP). The THO complex and its associated Sub2p helicase are functionally implicated in both transcription and mRNP biogenesis but their precise function remains elusive. We show here that THO/Sub2p mutation leads to the accumulation of a stalled intermediate in mRNP biogenesis that contains nuclear pore components and polyadenylation factors in association with chromatin. Microarray analyses of genomic loci that are aberrantly docked to the nuclear pore in mutants allowed the identification of approximately 400 novel validated target genes that require THO /Sub2p for efficient expression. Our data strongly suggests that the THO complex/Sub2p function is required to coordinate events leading to the acquisition of export competence at a step that follows commitment to 3′-processing.

INTRODUCTION

The nascent RNA is wrapped in a ribonucleoprotein particle (mRNP or ncRNP) that contains factors required for processing and export. Critical steps in the acquisition of mRNP functional competence occur during transcription termination and mRNA 3′-end processing. These events are subject to quality control, which discards defective or incomplete molecules (Jensen et al., 2003). When RNA polymerase II (Pol II) transcribes through termination signals, the cleavage and polyadenylation complex (CPF/CF) assembles on the nascent RNA and promotes cleavage of the primary transcript and transcription termination.

Release of mRNP from the transcription template is a regulated process and many defects in mRNA processing or mRNP assembly result in the accumulation of defective molecules in nuclear foci associated to the site of transcription (Jensen et al., 2001a, 2001b; Libri et al., 2002; Thomsen et al., 2003). Such retention of defective mRNPs is believed to reflect the existence of a quality control mechanism that restricts aberrant molecules to the nucleus and degrades a large share of their RNA content (Hilleren et al., 2001; Jensen et al., 2001a; Libri et al., 2002). The nuclear exosome, a multimeric 3′-5′ exonuclease involved in several aspects of nuclear RNA metabolism (Butler, 2002; Houseley et al., 2006), has been shown to play an important role in this quality control step (Hilleren et al., 2001; Jensen et al., 2001a; Libri et al., 2002).

mRNP composition changes as it transits to the cytoplasm (Daneholt, 2001) but the remodeling steps that occur during and after mRNP release from chromatin are poorly understood. The RNA binding factors Npl3p and Yra1p are recruited early in the transcription process (Lei et al., 2001) and are believed to act as adaptors for Mex67p, an essential factor required for docking of the mRNP to the nuclear pore complex (NPC) (Santos-Rosa et al., 1998). The interaction of Mex67p with Npl3p has been shown to require dephosphorylation of Npl3p by the Glc7p phosphatase, possibly to favor a rearrangement step that is also required to release the mRNP from the 3′-end processing machinery (Gilbert and Guthrie, 2004). Another documented remodeling event occurs at the cytoplasmic face of the NPC and is operated by the Dbp5p helicase. This factor has been shown to promote the dissociation of Mex67p from the mRNP, releasing the latter into the cytoplasm (Lund and Guthrie, 2005).

Recent biochemistry and imaging studies in yeast converge toward the notion that gene expression is linked to (and possibly favored by) the physical proximity of the gene with the NPC and gene relocation to the nuclear periphery has been shown to occur upon transcriptional activation (Abruzzi et al., 2006; Brickner

et al., 2007; Brickner and Walter, 2004; Cabal et al., 2006; Casolari et al., 2004; Dieppois et al., 2006; Luthra et al., 2007; Schmid et al., 2006; Taddei et al., 2006). The molecular basis and the functional significance of such gene-NPC gating are still unclear. One model posits that physical interactions between transcription activators and NPC components favor or determine NPC-gene interaction, consistent with an RNA-independent mechanism (Cabal et al., 2006; Dieppois et al., 2006; Luthra et al., 2007; Schmid et al., 2006). Other reports, however, suggest a role for the mRNP in the process (Abruzzi et al., 2006; Taddei et al., 2006) and in some instances the association with the NPC persists even after transcriptional repression, presumably due to the slow release of the mRNP from the transcription region (Abruzzi et al., 2006).

The THO complex is required for the production of a functional mRNP (Libri et al., 2002; Schneiter et al., 1999; Strasser et al., 2002; Zenklusen et al., 2002). Deletion of individual THO complex subunits (Hpr1p, Mft1p, Thp2p or Tho2p) leads to retention of mRNAs in transcription site-associated foci and nuclear degradation by the exosome and its co-activator TRAMP (Libri et al., 2002; Rougemaille et al., 2007; Thomsen et al., 2003). The precise function of the THO complex in mRNP assembly is not understood. THO complex integrity is required for the recruitment of Sub2p (Zenklusen et al., 2002), a conserved DEcD putative helicase that functions in splicing and mRNA export (Jensen et al., 2001a; Kistler and Guthrie, 2001; Libri et al., 2001; Strasser and Hurt, 2001). However, the molecular role of Sub2p in mRNA export and whether the protein functions in mRNP remodeling is also not clear, Additional phenotypes described for THO complex mutants include transcription defects (Luna et al., 2005; Mason and Struhl, 2005; Rondon et al., 2003; Voynov et al., 2006) and genomic instability (Chavez et al., 2000). However conclusive evidence that the THO complex impacts directly transcription elongation and/or inherent RNA Pol II processivity has yet to be provided.

Here we show that upon transcription induction in THO/*sub2* mutants an atypical protein-DNA complex is formed in the 3′-end region of several genes. The high density of this complex results in the specific depletion of hundreds of genomic loci when chromatin extracts are prepared with conventional protocols. We show that this complex is a stalled intermediate in the mRNP biogenesis pathway that reflects the persistent association of transcribed DNA with the nuclear pore. Microarray analysis of DNA trapped in association with the NPC allowed the identification of roughly 400 genes displaying THO complex dependency. Our findings pinpoint the timing of action of the THO/Sub2p complex after commitment to 3′-end processing and define an essential function to coordinate events leading to efficient export.

RESULTS

Formation of "Heavy" Chromatin in THO Complex Mutants

To study mRNP composition in THO mutant backgrounds, we undertook ChIP experiments using several tagged candidate proteins. During these analyses, we noticed to our surprise that the DNA content of chromatin extracts prepared from wild-type (WT) versus THO mutant strains was not homogeneous. For instance, real time PCR (qPCR) analyses showed that the 3′-ends of the *HSP104* gene were 2–4 times less abundant in THO mutant compared to WT extracts (SN_{18k}, Figure 1A). In contrast, sequences deriving from the 5′ region of the gene, the *ACT1* gene or an intergenic region from chromosome IV (IG) were equally abundant (Figure S1A available online and data not shown). The missing sequences were sequestered in the pellet fraction of an 18000 g centrifugation step (P_{18k}) that is part of the current ChIP extracts preparation protocols (Figure 1A) and highly enriched in the *Δmft1* mutant relative to a WT strain (Figure 1B). This phenomenon depends on formaldehyde crosslinking of DNA to proteins (Figure S1B) and on the *HSP104* gene being actively transcribed (Figure 1B). qPCR analysis of the sequences enriched in the P_{18k} fraction indicates that the "heavy"chromatin fraction spans approximately 9 kb of genomic DNA, is highest around the stop codon of the *HSP104* gene and extends for more than 2 kb downstream where a second heat shock gene, *SSA2*, is transcribed convergently (Figure 1C). This gene is also misregulated in *Δmft1* cells as shown by RT-qPCR analysis (Figure S2A). By extension, we also found that other heat shock genes (*SSA3*, *SSA4*, and *HSP82*) were enriched in the "heavy" chromatin fraction of *Δmft1* extracts and displayed THO complex dependency for expression (Figure S2A; see also below).

Taken together our observations suggest that a protein-DNA complex accumulates in the 3′ region of several genes upon transcription induction in *Δmft1* cells and is sequestered in the pellet fraction of a standard chromatin preparation. We refer to this phenomenon as differential chromatin fractionation (DCF).

Heavy Chromatin Formation Is Specific for Mutants of the THO/Sub2p Mutants

To assess the specificity and the biological significance of this phenotype we analyzed chromatin extracts prepared from additional mutants of the mRNP biogenic pathway. Heavy chromatin formation was readily observed in other mutants of the THO complex (i.e., *Δhpr1* and *Δtho2*) and the associated factor Sub2p (i.e., *sub2-201*) but neither in mutants of the 3′-end processing machinery (i.e., *rna15-2, rna14-3, pcf11-13* and *pap1-1*), nor in mutants of other factors involved in mRNA export (i.e., *mex67-5*, *yra1-8-GFP, Δnup60*, *Δmlp1, Δmlp2*) (Figures S2B and 2D, and data not shown).

Expression of the *HSP104* and *SSA4* genes in THO/*sub2* mutants is associated with two major phenotypes: (1) retention of *HSP104* and *SSA4* RNAs in intranuclear foci connected to the site of transcription and (2) degradation of a large share of these transcripts by the nuclear exosome (Jensen et al., 2001a; Libri et al., 2002). Both phenotypes can be suppressed by mutation of Rad3p, a component of the TFIIH transcription initiation factor (Jensen et al., 2004) or by deletion of Rrp6p, a nuclear subunit of the RNA exosome (Jensen et al., 2001a; Libri et al., 2002). As shown in Figure S2B, formation of heavy chromatin in *Δmft1* cells was suppressed both by mutation of Rad3p and deletion of Rrp6p. Together these data indicate that sequestration of genes in protein-DNA complexes is functionally related to the mRNP export phenotype of THO/*sub2* mutants and likely reflects the existence of a specific function for this set of genes that is distinct from other processing and export factors.

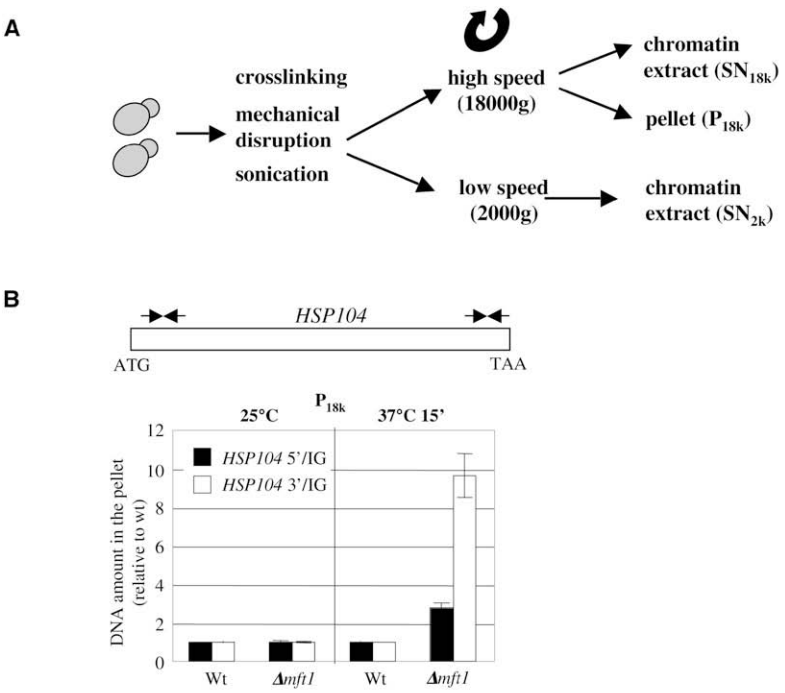


Figure 1. Differential Fractionation of the *HSP104* Locus in Chromatin Preparations from *Δmft1* and WT Cells

(A) Scheme of the chromatin extraction procedures. Above: procedure used in conventional chromatin immunoprecipitation experiments, including a 18000 g centrifugation step. SN_{18k} is the corresponding extract. Below: the modified procedure used in this work.

(B) Specific enrichment of *HSP104* 3′ ends in the 18,000 g pellet fraction (P_{18k}). This phenomenon depends on active transcription as it is not observed at 25°C when the *HSP104* gene is silent. The DNA extracted from the P_{18k} fraction was subjected to qPCR quantification. Values were normalized to the amount of IG DNA and expressed relative to the wild-type. Error bars indicate standard deviations.

(C) Analysis of the DNA content in P_{18k} throughout the *HSP104* genomic locus. QPCR analysis was conducted with primers pairs spanning several kilobases around the *HSP104* locus. Values are normalized to the amount of IG DNA as in (B) The genomic organization of the region is schematically indicated below the graph. Average of at least three independent experiments. Error bars indicate standard deviations.

Altered Pol II Occupancy and Nucleosome Density in the 3′-End of *HSP104* in THO Mutants

Formation of heavy chromatin might relate to the accumulation of a stalled intermediate due to defective THO/Sub2p function. Therefore, the characterization of this complex could provide important information on the function of these factors. ChIP analysis of proteins associated with the 3′-end of THO target genes is expected to be biased by the absence of the heavy chromatin fraction in conventional extracts prepared from mutant cells. However, since the chromatin fraction that is lost after high-speed centrifugation is instead present in low speed (2000 g) supernatants (SN_{2k}, Figures 1A and S1C). we used these for ChIP analyses to probe for candidate protein factors interacting with *HSP104* 3′-end DNA.

Pol II occupancy was first assessed using an antibody recognizing the N-terminus of its largest subunit Rpb1p (Y-80, Santa-Cruz). As shown in Figure 2A, a small increase in ChIP signal was observed in the 5′ region of the *HSP104* gene in *Δmft1, Δhpr1* and *sub2-201* mutants compared to the WT. Conversely, an approximately two-fold lower signal was detected in the 3′ region of the gene in these mutants. Similar results were obtained using antibodies recognizing the CTD of Rpb1p or the Rpb3p subunit of Pol II (data not shown).

Lower Pol II occupancy in the *HSP104* 3′-end might imply that fewer polymerase molecules attain this region of the gene because of *intrinsic* poor processivity in THO/*sub2* mutants. However, this would not be consistent with the observation that formation of heavy chromatin in mutants requires active transcription (Figure 1B). Because nucleosome density correlates inversely with the occurrence of transcription, less Pol II molecules reaching the termination region in mutants should be associated with higher nucleosome occupancy. We therefore probed nucleosome density by ChIP using an antibody recognizing total (i.e., regardless of its modification state) histone H3 (ab1791-100, Abcam) or a tagged version of histone H2B. As expected, nucleosome density markedly decreased in the 5′ region of the *HSP104* gene upon transcription activation (data not shown). Importantly, this decrease was similar for the WT and THO/*sub2* cells, indicating similar transcriptional activity in the region (Figure 2A; data not shown). In contrast, histones H3 and H2B occupancy in the 3′ region of the gene was significantly lower in *Δmft1*, *Δhpr1* and *sub2-201* mutants compared to the WT (Figures 2A and S3A),

indicating a significant decrease in nucleosome occupancy in spite of the reduced transcriptional activity in this region.

This result indicates that histones have been evicted in the 3′ end of the *HSP104* gene, suggesting that transcription has occurred in the region. The observation that nucleosome density is lower than expected suggests that the equilibrium between eviction and re-deposition has been altered in mutants. Importantly, this provides in vivo evidence for an abnormal chromatin structure, which presumably relates to the presence of the protein-DNA complex leading to the formation of heavy chromatin.

Increased Residency of the 3′-End Processing Complex and Mex67p at the Genomic Locus in THO Complex Mutants

Because formation of heavy chromatin is restricted to the 3′-end of the genes analyzed, we surmised that the recruitment of factors involved in mRNA 3′-end processing and export might be altered. Therefore, we first assessed recruitment of the mRNA export factors Npl3p and Mex67p in the *Δmft1* mutant. As both factors are recruited in a transcription-dependent manner (Dieppois et al., 2006; Lei et al., 2001), presumably via interaction with Pol II and/or the nascent RNA, we also normalized Npl3p and Mex67p ChIP signals to Rpb1p occupancy detected in the same experiments. As shown in Figure 2B, left panels, in the *Δmft1* mutant Npl3p was distributed similarly to Pol II, i.e., a small increase in the 5′- and a decrease in the 3′-region of the *HSP104* gene, implying no difference in Npl3p occupancy *per* transcription event. A different scenario was observed for Mex67p as a more marked increase was observed in the 5′ end and no significant decrease in the 3′ end of the *HSP104* gene (Figure 2B, right panels). When normalized to Pol II occupancy, Mex67p ChIP signals were overall higher in the mutant implying increased residency of this protein at the genomic locus.

We next analyzed recruitment of the 3′-end processing complex. To this end, we used strains containing TAP-tagged versions of Rna15p and Pcf11p, two components of the CPF/CF complex, required for both transcription termination and 3′-end formation. In a WT strain ChIP signals were detected in the *HSP104* 3′region and close to the poly(A)-addition site. Interestingly, Pcf11p and Rna15p signals were significantly higher in *Δmft1* and *Δhpr1* strains, which was even more pronounced *per* transcription event (i.e., when signals were normalized to Pol II occupancy, Figures 2C and S3B). Thus, in THO complex mutants the 3′-end processing complex is crosslinked more efficiently to the 3′-end of *HSP104* DNA, indicating a more favorable topology and/or an increased residence time of the complex in proximity to the DNA. This suggests that in THO mutants 3′-processing factors have failed to be released from the mature mRNP or the transcribing polymerase in association with the site of transcription.

Transcription Termination Is Required for the Occurrence of DCF in THO Mutants

Because formation of heavy chromatin does not occur within coding regions, we hypothesized that it might require transcription termination/polyadenylation events. Thus, thermosensitive alleles of the essential CPF/CF components Rna14p, Rna15p, Pcf11p and of the Pap1p poly(A) polymerase were coupled to the *MFT1* deletion by genetic crosses and the resulting double mutant strains were analyzed. At the non-permissive temperature the *rna14-3*, *rna15-2* and *pcf11-13* alleles are all defective for transcription termination, while the *pap1-1* allele is termination proficient but defective for polyadenylation (Figure S4 and data not shown). Interestingly, preventing transcription termination in a *Δmft1* background decreased considerably or completely abolished formation of heavy chromatin (Figure 2D), which instead persisted in the *Δmft1/pap1-1* mutant, indicating that assembly of the 3′-end processing complex and/or transcription termination is required. Importantly, these observations suggest that defective THO complex function affects mRNP formation/export at a step that follows commitment to 3′-end processing.

Association of Nuclear Pore Components with the *HSP104* Locus in THO Complex Mutants

Several reports have suggested that upon transcriptional activation several genes relocate to the nuclear periphery and associate with the NPC. We therefore assessed by ChIP the interaction of the *HSP104* locus with NPC components in the *mft1Δ* and *hpr1Δ* mutants using TAP-tagged versions of the Nup116p, Nup60p, Nup85p, Nup159p, Nsp1p and Gle1p. As similar results were obtained only data for the *Δmft1* mutants are reported here. We could not detect any significant formaldehyde crosslinking of these nucleoporins to *HSP104* DNA in the WT background. Strikingly, however, a robust ChIP signal, that was more prominent in the 3′-end of *HSP104*, was observed for all NPC components in the *Δmft1* strain (Figures 3A and S5A). Importantly, nucleoporins-*HSP104* DNA adducts were present in the heavy chromatin fraction as ChIP signals were strongly reduced when conventional extracts (SN_{18k}), deprived of this fraction, were used for immunoprecipitation experiments (Figure S5B). Consistently, nucleoporins were specifically enriched in the heavy chromatin fraction (i.e., the 18,000 g pellet, P_{18k}) as assessed by western blot analysis (Figure S5C).

DCF in THO mutants can be suppressed by deletion of Rrp6p (Figure S2B) or by mutation of 3′-end processing factors (Figure 2D). Consistent with the notion that nucleoporins crosslinking to the DNA is linked to formation of heavy chromatin, in both *Δmft1/Δrrp6* and *Δmft1/rna14-3* double mutants the Nup60p ChIP signal was reduced to background levels (Figure 3B).

Crosslinking of NPC components to the *HSP104* DNA in THO mutants might imply the proximity of the locus to the NPC. Therefore we tagged the *HSP104* locus with LacO repeats in WT or THO mutant strains that also express an integrated LacI-GFP repressor and a GFP-tagged Nup49p to mark the nuclear rim (Dieppois et al., 2006). In these conditions (20 min at 37°C) we did not observe a marked repositioning of the *HSP104* locus to the nuclear periphery in a WT strain (Figure 3C). In contrast, the *HSP104* gene localized to the more peripheral portion of the nucleus in 45.73% (standard deviation (SD) = 2.94, n = 366) of *hpr1Δ* cells versus 26.23% (SD = 1.91, n = 281) of WT cells, which is statistically significant (Figure 3C). Importantly, deleting the *RRP6* gene in this context led to loss of association with the nuclear periphery (25.53% of cells, SD = 2.3, n = 275), which parallels the loss of DCF and Nup60p ChIP signal described above.

From these experiments we conclude that in THO/sub2 mutants the 3′-end of the *HSP104* locus is trapped in a protein complex that contains NPC- and 3′-end processing complex-components.

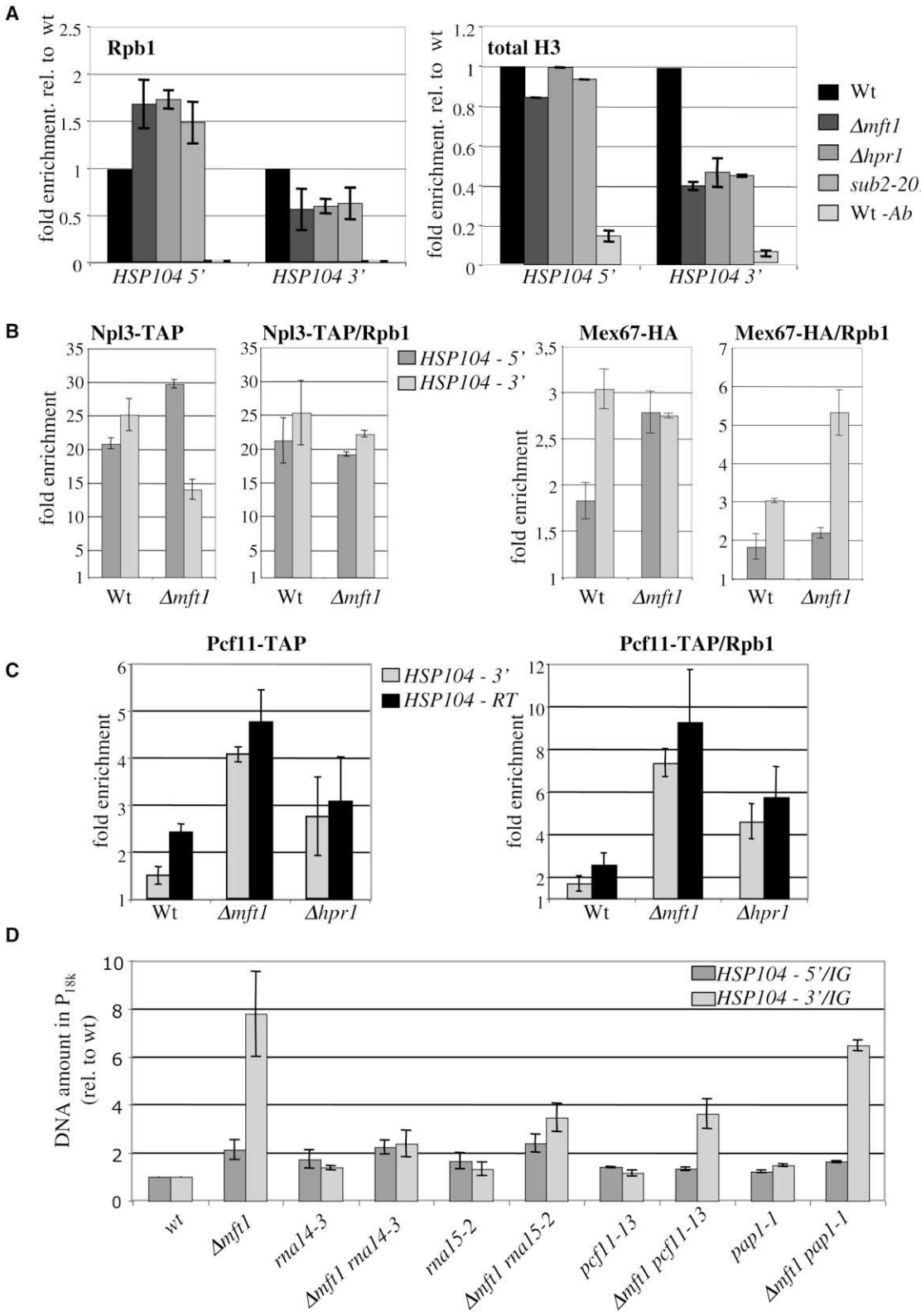


Figure 2. Chromatin Immunoprecipitation Analysis of Factors Recruitment to the *HSP104* Locus in THO-Sub2p Mutants
In all cases a low speed supernatant (SN_{2k}) was used.

Formation of this complex reflects the persistence of the gene at the nuclear periphery, most likely in association with the NPC.

Formation of Heavy Chromatin Reflects the Accumulation of a Stalled Intermediate in the mRNP Export Pathway

Formation of heavy chromatin might either be the consequence of the accumulation of a stalled intermediate or a dead-end complex in the mRNP export pathway. Only in the first scenario is a preformed complex expected to be resolved under conditions resuming THO/Sub2p function. To distinguish between these possibilities, we undertook chase experiments in a *sub2-201* mutant. The complex was allowed to form in the usual conditions (37°C, 15 min) before shifting back the temperature to 25°C to reactivate Sub2p function in the absence of *HSP104* transcription. As shown in Figure 4A, left panel, both DCF and Nup60-TAP crosslinking to the *HSP104* locus rapidly and synchronously disappeared during the chase period indicating that the complex accumulating in mutants can be resolved when conditions permissive for THO/Sub2p function are resumed. The fraction of *HSP104* RNA that escapes nuclear degradation in THO/Sub2p mutants is predominantly nuclear and does not enter the normal cytoplasmic turn-over pathway (Rougemaille et al., 2007, and data not shown). However, when Sub2p function was restored, the *HSP104* RNA levels decreased with similar kinetics as DCF, which might either indicate nuclear degradation or export to the cytoplasm and turn-over by the 5′ to 3′ Xrn1p-dependent pathway. Consistent with the latter hypothesis, *HSP104* RNAs were strongly stabilized in an *xrn1Δ/sub2-201* strain during the chase period (Figure 4B, right panel). Note that in similar conditions, but preventing de novo transcription of Sub2p with the general transcription inhibitor thiolutin, *HSP104* RNAs persist in the nucleus (Rougemaille et al., 2007, and data not shown), indicating that restoration of Sub2p function is crucial for the resolution of the stalled intermediate. Finally, and consistent with the existence of a normal intermediate in the mRNP assembly/export pathway that accumulates in THO/*sub2* mutants, we also detected a low but significant level of heavy chromatin in a WT strain (Figure 4B).

Several Genomic Loci Are Retained in Heavy Chromatin: Genome-Wide Analysis of THO/Sub2 Targets

Because genes found by educated guess in the heavy chromatin fraction are all dependent on THO complex function, we reasoned that analysis of the DNA trapped in the stalled complex might provide an unbiased approach to identify new THO/Sub2 complex targets. To this end, the DNA extracted from the chromatin pellet (P_{18k}) was used for hybridization to yeast ORF microarrays and signals were normalized to values obtained from total DNA contained in the low speed chromatin extract (SN_{2k}). The complete data set is available in the Supplemental Data. The normalized log2 ratio values follow a Gaussian distribution with a standard deviation of 0.33 in the WT and 0.62 in the *Δmft1* mutant (Figure 5A). Most features in the mutant (83%) have enrichment values comprised within two SD of the WT distribution (±0.6), indicating that DCF only occurs at a minority of genes. In the *Δmft1* mutant 398 features (8.3%) have enrichment values higher than 1 (i.e., higher than three times the SD of the WT distribution) against 69 (1.4%) in the WT. Remarkably, heat shock genes or genes that are induced upon a temperature shift to 37°C were very frequent among features with high enrichment values (43 in the first 100, Table S1). A similar enrichment was not observed in the WT, indicating that the selection of this class of genes is not the mere effect of experimental conditions (i.e., exposure to 37°C for 15 min). In a few cases (e.g., *SSA1, ACO1, HSP42, GSY2)* heat inducible genes were subject to DCF even in the WT strain although to a lesser extent, consistent with the notion that the intermediate complex also forms in WT cells.

We used t-profiler (Boorsma et al., 2005) to analyze the correlation between global enrichment, gene functional categories (GO) and transcription factor binding (Table S2). A note of caution here is that in several cases of clustered signals the primarily affected gene remains undetermined. With this caveat in mind, we found, in addition to heat shock genes, enrichment for genes involved in carbohydrate and alcohol metabolism, and genes encoding ATPases from the cell membrane (e.g., *PDR5*, *PMA1, YOR1, SNQ2*). Additionally, a GO search of the 398 most enriched genes in the mutant using the SGD GO Term finder (Dwight et al., 2002) identified an enrichment of Ty1 transposable elements ($p < 10^{-53}$). qPCR analysis largely validated the new THO complex targets identified (Figure 6). Moreover, increased ChIP signals for nucleoporins were confirmed for *SPT16*, *YOR1* and the assayed Ty1 elements (Figure 6D). Finally, RNA fluorescent in situ hybridization (FISH) analysis was performed with probes targeting *PDR5* and *YOR1* RNAs (Rougemaille et al., 2007 and Figure 6C) confirming in both cases that the relevant mRNPs are retained at or near their respective transcription sites. These results provide a genomewide

(A) Immunoprecipitations were conducted with an anti-Rpb1 antibody recognizing the N-terminal domain of the protein (left panel) or an antibody recognizing total histone H3 (right panel) in the indicated strains. QPCR analysis was performed with 5′- and 3′-specific *HSP104* primers with corrected amplification efficiency. Values are normalized to immunoprecipitation input and expressed relative to the wild-type.

(B) Npl3p and Mex67p occupancy at the *HSP104* locus in wild-type and *Δmft1*cells. Left: enrichment of *HSP104* DNA in Npl3-TAP immunoprecipitates was calculated as in A but expressed relative to the DNA from an intergenic region. A value of 1 indicates no specific enrichment. Panel Npl3-TAP/Rpb1: Npl3-TAP signals were corrected for the relative levels of RNA Pol II in the same experiments. Right panels: Mex67-HA occupancy was calculated as for Npl3-TAP, but normalization was performed relative to signals from a nontagged strain.

(C) Higher Pcf11p occupancy at the *HSP104* locus in *Δmft1* and *Δhpr1* strains relative to the wild-type. Analysis as in (B). Values expressed relative to a nontagged control and corrected for Pol II occupancy (right panel). A value of 1 indicates no enrichment as above. "RT" primers amplify a region of the *HSP104* gene that is located downstream of the poly(A) addition site.

(D) Formation of heavy chromatin depends on the occurrence of transcription termination. DCF was assessed as in Figure 1 by measuring the DNA content for the 5′ and 3′ ends of the *HSP104* gene in the high speed pellet (P_{18k}) of chromatin preparations from the strains indicated. Values are normalized as in Figure 1.

In all panels, values represent the average of at least three independent experiments, and error bars indicate standard deviations.

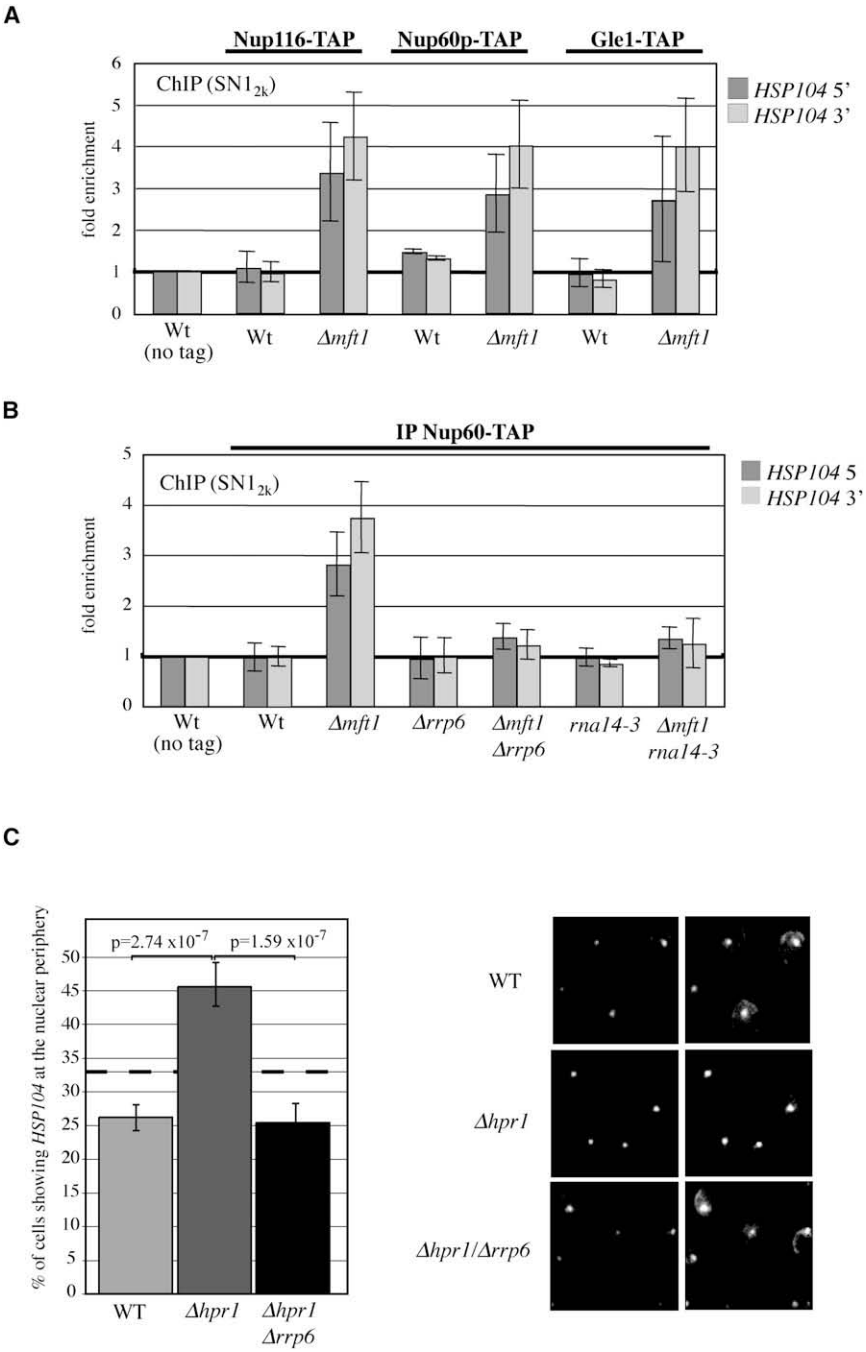


Figure 3. Altered Nuclear Localization of the *HSP104* Locus in *mft1Δ* Cells

(A) ChIP analysis of Nup60p, Nup116p and Gle1p occupancy in the WT and *Δmft1* background. A low speed supernatant (SN_{2k}) was used in all experiments. Values (output/input) were normalized to a non tagged control as in Figure 2. The no enrichment threshold (1) is indicated by a thick line.

(B) Crosslinking of Nup60p to the *HSP104* locus (upper panel) is dependent on the integrity of the nuclear exosome and the termination/polyadenylation complex. Analysis as in (A).

(C) Fluorescence microscopy detection of the GFP-tagged *HSP104* locus in WT, *Δhpr1* and *ΔhprΔ/rrp6* cells. The *HSP104* locus was visualized through the interaction of the LacI repressor-GFP fusion with LacO operators integrated in proximity of the gene. The nuclear rim is marked by Nup49-GFP. Representative fields are shown on the right of the histograms. P-values for the differences observed in the different strains are indicated above the histograms. Average of three independent experiments. Total number of cells counted: 366 for *hpr1Δ*, 281 for WT and 275 for *hpr1Δ/rrp6Δ* strains.

In all panels, error bars represent standard deviations.

landscape of genes requiring THO/Sub2p function for the resolution of an intermediate linking the genomic locus to the nuclear pore.

DISCUSSION

Despite considerable efforts, the mechanisms underlying the multiple phenotypes associated with dysfunction of the THO/Sub2p complex are still elusive. In this study we provide new insights into the THO/Sub2p-dependent pathways linking transcription and the acquisition of mRNP export competence.

THO Function and Transcription Site-Associated 3′-End Processing

After transcription termination and 3′-end processing the mRNP is released from the transcription site and the CPF/CF is released from the mRNP. Because formation of heavy chromatin in THO/*sub2* mutants depends on the integrity of the CPF/CF, we conclude that dysfunction of THO/Sub2p affects events that follow the commitment to transcription termination but precede mRNP release. In this view, increased residency of CPF/CF at the transcription site is interpreted as a release failure, presumably because of a persistent association of the polyadenylation complex with DNA-bound factors or the retained mRNP. This suggests that mutant cells have failed to undergo a necessary rearrangement step at the site of transcription, and it is tempting to speculate that this is due to insufficient function of the Sub2p helicase. Consistent with persistence of defective mRNPs in association with chromatin, we also detected increased ChIP signals for Mex67p. Surprisingly, Npl3p ChIP signals closely followed Pol II occupancy, suggesting either that this factor is lost from mRNPs that remain associated with the site of transcription or that crosslinking of Npl3p to the DNA occurs preferentially while this protein is associated with the transcribing polymerase.

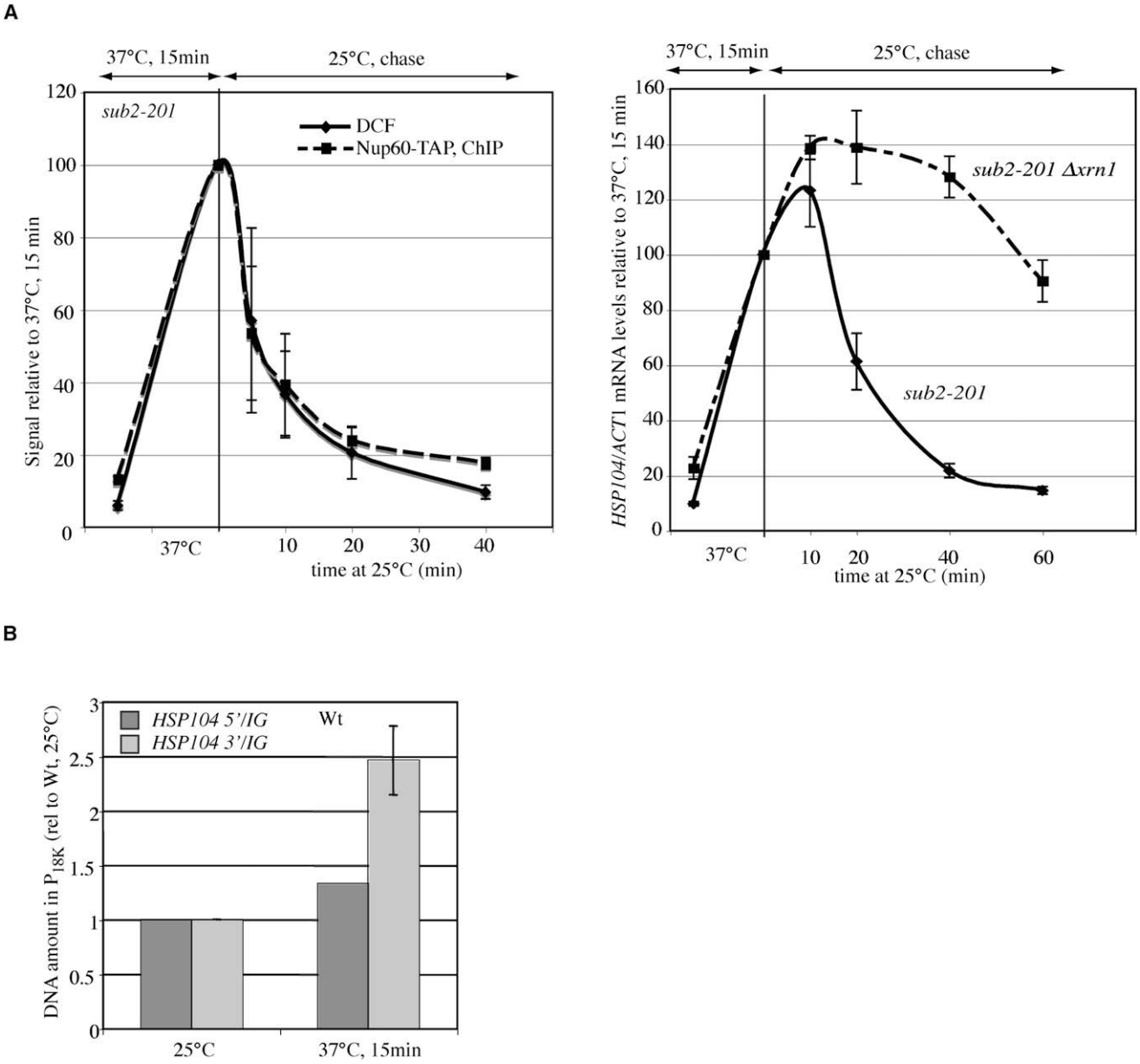


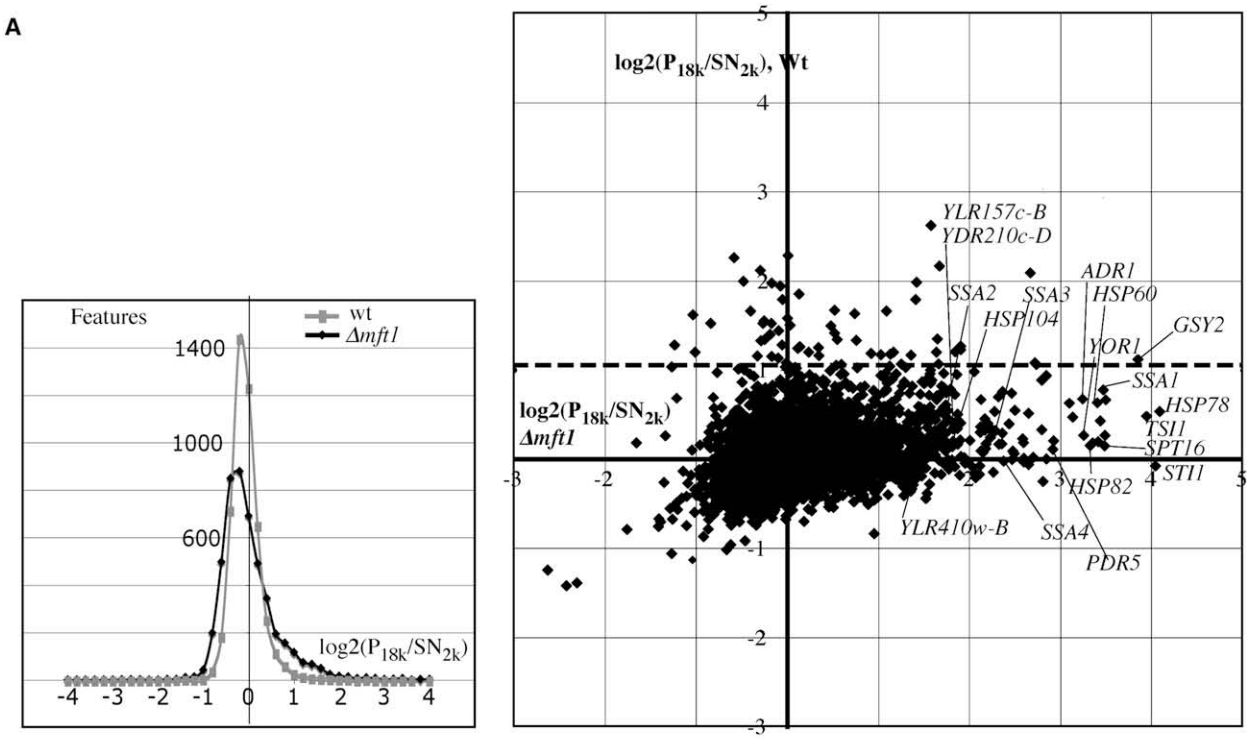
Figure 4. Chase Experiments in *sub2-201* and *sub2-201/xrn1Δ* Strains

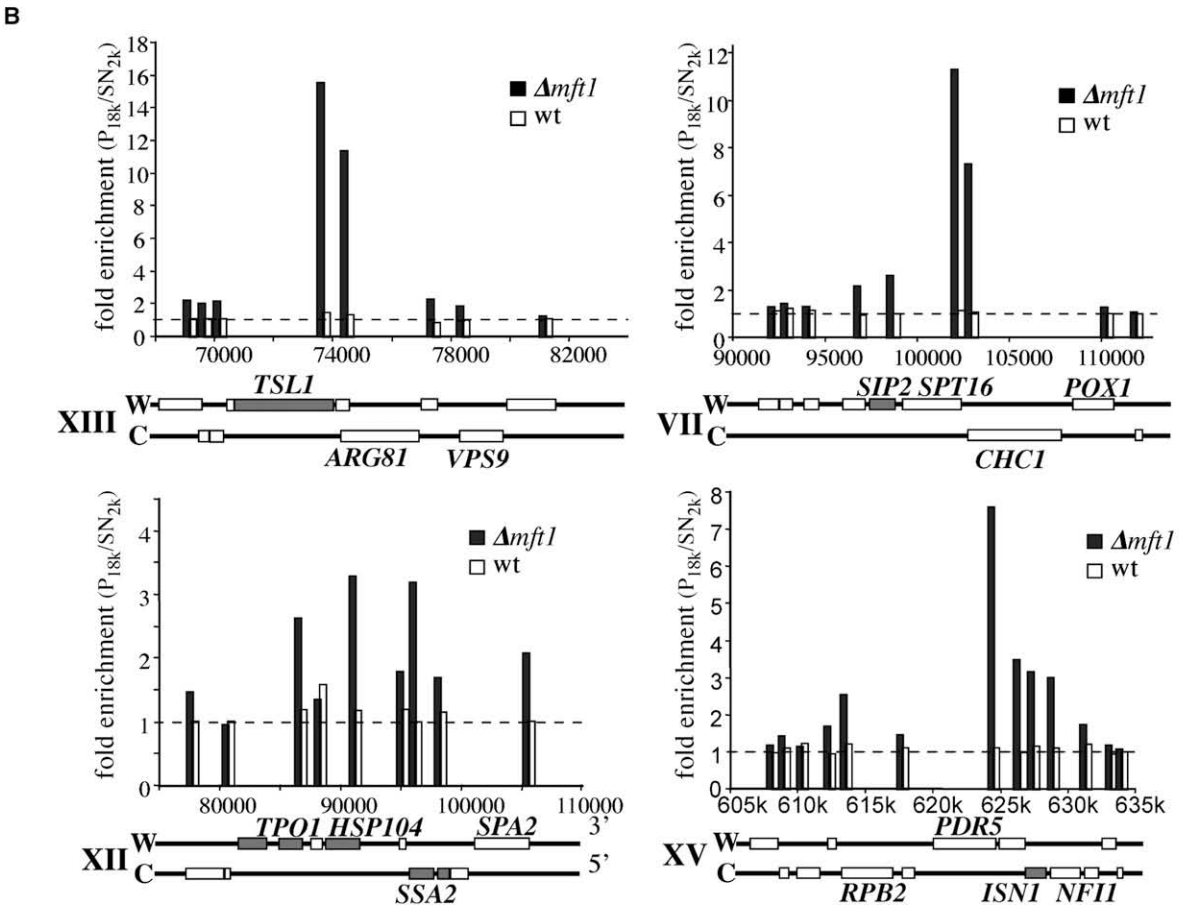
(A) Transcription of *HSP104* is activated for 15min at 37°C, which are non-permissive conditions for the *sub2-201* mutant. This leads to formation of the stalled complex and crosslinking of Nup60-TAP to the *HSP104* locus (left panel, vertical bar). Cells are then rapidly shifted to 25°C for the indicated times to restore Sub2p function in the absence of *HSP104* transcription. DCF levels and Nup60-TAP crosslinking to the *HSP104* locus (left panel, Nup60-TAP ChIP and DCF analysis) as well as *HSP104* RNA levels (right panel, RT-qPCR analysis) are analyzed during the chase period. Upon restoration of Sub2p function at 25°C, DCF and Nup60-TAP ChIP signal rapidly disappear. *HSP104* RNAs are rapidly exported and degraded in the cytoplasm as indicated by the strong stabilization observed when the major cytoplasmic turn-over pathway is also impaired in the *sub2-201/xrn1Δ* strain (right panel, compare plain and dashed line plots).

(B) Formation of heavy chromatin in WT cells. Analysis as in Figure 1 Values are normalized to an intergenic region (IG) and expressed relative to values observed at 25°C when the *HSP104* gene is silent. Note that in other experiments the low level of DCF in extracts from wild-type cells is masked by the normalization procedure employed. In all panels, error bars represent standard deviations.

Defective THO Complex/Sub2p Function Leads to Persistent Gene-NPC Association

Association of genes with the nuclear periphery is believed to be mediated by physical interactions between NPC components and transcription co-activators (e.g., the SAGA complex, Cabal et al., 2006; Luthra et al., 2007; Rodriguez-Navarro et al., 2004). The role of the mRNP in this process is still controversial (Abruzzi et al., 2006; Dieppois et al., 2006; Schmid et al., 2006; Taddei et al., 2006), but it is possible that gene-NPC association through the nascent mRNP is a redundant mechanism that


A
Features
wt
Δmft1
1400
1000
600
log2(P18k/SN2k)
-4 -3 -2 -1 0 1 2 3 4
log2(P18k/SN2k), Wt
log2(P18k/SN2k)
Δmft1
YLR157c-B
YDR210c-D
ADR1
SSA2
SSA3
HSP60
HSP104
GSY2
YOR1
SSA1
HSP78
TSI1
SPT16
STI1
HSP82
YLR410w-B
SSA4
PDR5


B
fold enrichment (P18k/SN2k)
Δmft1
wt
TSL1
XIII
ARG81
VPS9
SIP2 SPT16
POX1
VII
CHC1
TPO1 HSP104
SPA2
XII
SSA2
PDR5
XV
RPB2
ISN1 NFI1

overlaps an RNA-independent interaction of the NPC with the transcription initiation machinery. Thus, RNA-dependent NPC association might only be detected when the release of the mRNP from (or near) the transcription site is sufficiently slow (Abruzzi et al., 2006) and/or when transcription activators-dependent docking is less robust.

To our surprise we did not detect significant crosslinking of nucleoporins to the *HSP104* locus nor did we detect a nuclear periphery localization for this gene in a WT strain (Figures 3), perhaps because in these conditions *HSP104* transcription is rapidly shut-off and the residence time in proximity of the nucleopore is not sufficient for biochemical or microscopic detection. In contrast, we detected robust nucleoporin-DNA interactions in THO/*sub2* mutants (Figures 3A and S5A), which was paralleled by a nuclear peripheral positioning of the *HSP104* locus (Figure 3C). Although gene-NPC association detected by ChIP was not RNase sensitive (presumably because of redundant crosslinks in the complex, data not shown), NPC-*HSP104* ChIP signals were clearly dependent on functional 3′-end processing (Figure 3C) and were not sensitive to deletion of the SAGA component Sus1p (our unpublished results). We therefore favor the hypothesis that a different mechanism underlies retention of these loci at the NPC and we suggest that this relates to non-productive commitment to export of mRNPs that have not yet completed all previous processing steps (Figure 7).

Upon mutation of THO/*sub2*, *SSA4* and *HSP104* RNAs are confined at or near their sites of transcription (Jensen et al., 2004; Libri et al., 2002). Although gene-NPC association occurs concomitantly with transcription site retention in THO/*sub2* mutants (Figures 3C and 4A), the two phenomena are not necessarily coupled. For instance, in mutants of the 3′end processing machinery or in the mRNA export mutants *mex67-5* and *yra1-8-GFP*, retention of the mRNP at or near the site of transcription is not accompanied by gene relocation at the nuclear periphery or crosslinking to NPC components (as shown for the mex67-5 mutant, Dieppois et al., 2006). In these cases, the retained mRNP might not be competent for association with the NPC and detection of a nuclear dot by RNA-FISH analysis only reflects persistence at the transcription site.

Our results suggest (Figure 7) that both persistent association of the mRNP with the transcription site and gene-NPC association are required for the accumulation of the export intermediate and formation of heavy chromatin in THO/*sub2* mutants. This suggests that THO/Sub2 function is unique in the export pathway in that it allows mRNP translocation to the NPC before the acquisition of full competence for transcription site release.

The Impact of THO Complex Mutations on Transcription

Several reports have suggested a role for the THO complex in transcription elongation and mutation of its components has been reported to affect Pol II processivity (Luna et al., 2005; Mason and Struhl, 2005). In this study we indeed find that formation of heavy chromatin is associated with lower Pol II occupancy in the 3′-end of genes. However, our genome-wide and single gene based analyses converge on the notion that this is strongly dependent on commitment to 3′-end processing and not on the length of the gene being transcribed, which argues against the idea that defective THO/Sub2p function affects *inherent* Pol II processivity or elongation rate. Consistently, we did not detect a progressive decrease in transcriptional activity within the body of the *HSP104* gene by nuclear run on (NRO) assays (Rougemaille et al., 2007). Rather, NRO signals decreased abruptly with probes located in the 3′ untranslated region of the gene (Saguez et al., 2008) suggesting that Pol II progression might be impeded by steric hindrance due to the presence of heavy chromatin. Whether occurrence of DCF is also related to the formation of RNA-DNA hybrids that have been suggested to occur in THO complex mutants (Huertas and Aguilera, 2003) is presently unclear.

It is tempting to speculate that some of the transcription phenotypes of THO mutants might pertain to the existence of cryptic transcription termination sites that would provoke aberrant 3′-processing and consequently affect polymerase progression. The existence of cryptic polyadenylation was indeed reported for the bacterial *LacZ* gene, one of the most strongly affected THO targets (Cui and Denis, 2003). It is possible, however, that different mechanisms exist through which the THO complex directly or indirectly impacts transcription on a distinct set of genes as the ones studied in this report (see for instance Voynov et al., 2006).

Genome-Wide Analysis of THO Complex Function

Our genome-wide analyses show that heat shock genes and genes that are induced by exposure of yeast to 37°C are largely predominant among genes present in the heavy chromatin fraction in *Δmft1* cells. This is fully consistent with a genome-wide study of *Drosophila m.* THO complex targets (Rehwinkel et al., 2004). However, we also identified genes for which DCF cannot be explained by heat shock induction or proximity to a heat inducible gene, indicating that the function of the THO complex is not restricted to the heat-induced regulon. We also observed that the region of the *HSP104* locus is significantly displaced toward the nuclear periphery in *hpr1Δ* cells at 25°C in an Rrp6-dependent manner (data not shown), indicating that additional THO-dependent transcription units might drive the chromosomes to the NPC in THO mutants.

Figure 5. Genome-Wide DCF Distribution in WT and *Δmft1* Strains

(A) Left panel: distribution of DCF values (normalized log2 (P_{18k}/SN_{2k}), 0.2 increment classes) for the wild-type (gray line) and *Δmft1* (black line) strains. Right panel: dot plot of DCF values (normalized log2 (P_{18k}/SN_{2k}) for every feature in the wild-type (*y* axis) and *Δmft1* mutant (*x* axis) strains. A dashed line indicates the threshold of three standard deviations for the DCF distribution in the WT strain (0.99). The features that have been subjected to further experimental analyses are indicated.

(B) DCF occurrence in four genomic regions. The normalized P_{18k}/SN_{2k} ratio is indicated for the WT (white bars) and *Δmft1* (black bars) at each genomic position defined by the microarray probes. A value of 1 (dashed line) indicates no enrichment. A scheme of the genomic organization is indicated below each plot together with the corresponding genomic coordinates. Genes drawn in gray are upregulated upon heat shock (data from Gasch et al., 2000). W and C indicate, respectively, the Watson and Crick strand.

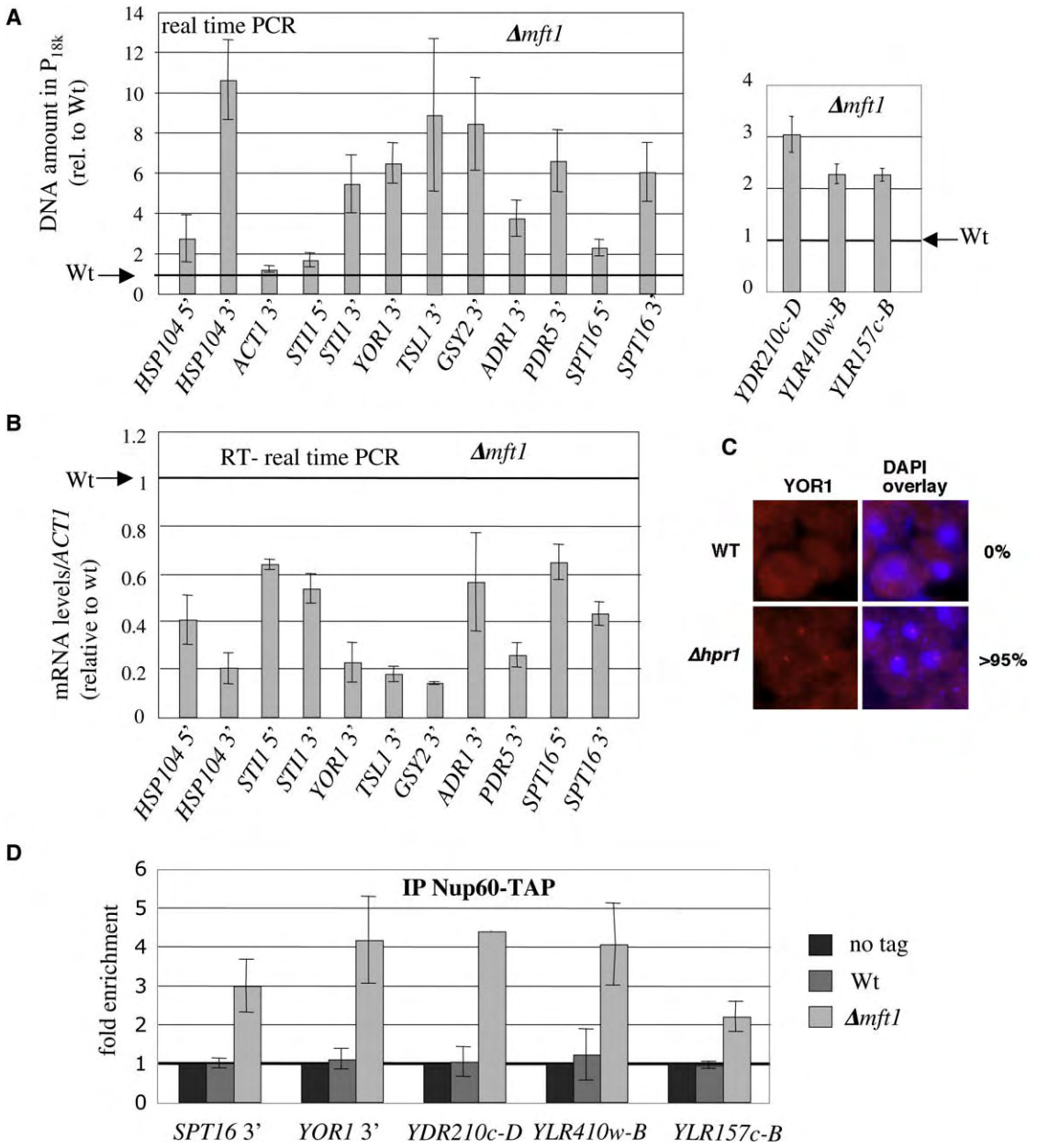


Figure 6. Analysis of New THO Complex Targets Identified by Genome-Wide DCF Studies

(A) qPCR analysis of DCF for the genomic loci indicated. In the case of *SPT16* and *STI1* two sets of primers have been used to amplify the 5′ and the 3′ end of the genes. Note the prevalence of DCF in the 3′ region of the two genes. *ACT1* and *HSP104* were analyzed in parallel as negative and positive controls respectively. *YDR210c-D*, *YLR410w-B*, and *YLR157c-B* belong to Ty elements and have been amplified using sequence specific primers. Normalizations as in Figure 2B. Average of at least three independent experiments. Error bars indicate standard deviations.

(B) mRNA levels in the *Δmft1* strain relative to the WT for the genes subjected to DCF analysis in (A). Signals were normalized to *ACT1* mRNA (that is not affected by mutation of the THO complex) and expressed relative to the WT signal. Average of at least three independent experiments. Error bars indicate standard deviations.

(C) FISH analysis of *YOR1* RNAs localization in a *Δhpr1* strain. The percentage of cells containing *YOR1* mRNPs localized in a nuclear, DAPI overlapping, focus is indicated (D). Chromatin immunoprecipitation analysis of nucleopore association (Nup60-TAP) for the indicated loci. Normalization as in Figure 4.

The accumulation of a stalled intermediate linking the NPC and the DNA might be one of the molecular reasons for the described genetic instability and hyper-recombination phenotypes in THO/*sub2* mutants. This could either be related to pausing of replication forks (Wellinger et al., 2006) at sites of heavy chromatin formation with ensuing higher frequency of double strand breaks or to an inherent sensitivity of these regions to DNA damage. In this perspective, the enrichment of Ty1 sequences in heavy chromatin is intriguing. Recombination between Ty sequences is a powerful source of genomic rearrangements that

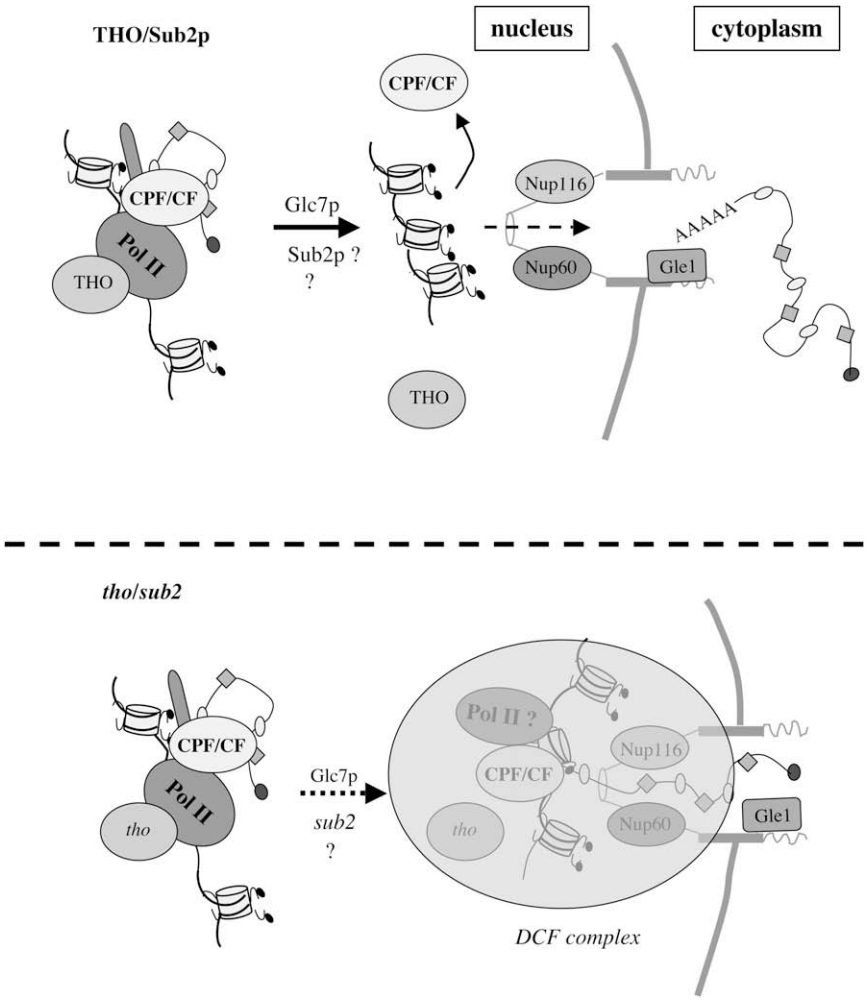


Figure 7. Model for the Molecular Origin of DCF

are stimulated under stress conditions (Mieczkowski et al., 2006).

Our results also have important technical bearings. ChIP is a powerful technique to analyze in vivo the association of protein factors and complexes with the DNA. The findings reported here suggest that in some instances the occurrence of multiple protein-DNA adducts in mutant or WT cells may lead to depletion of the factors of interest in the extract and introduce major biases in the outcome of immunoprecipitation assays.

EXPERIMENTAL PROCEDURES

Yeast Strains and Manipulations

Yeast strains used in this study are all derived from W303 (Table S1). Crosses were carried out using standard laboratory procedures. Chase experiments were performed by incubating cells at 37°C for 15 min to allow DCF build up in a *sub2-201* strain. Cells were then rapidly shifted back to 25°C by the addition of cold medium, which restores Sub2p function in the absence of additional *HSP104* transcription. Cells were collected at the indicated time points for DCF, ChIP and RNA analysis.

Chromatin Immunoprecipitation Experiments and DCF Analyses

Chromatin immunoprecipitation experiments were performed essentially as described (Jensen et al., 2004). A detailed protocol is described in supplementary methods. DCF analysis was performed by analyzing by qPCR the DNA content of the pellet obtained after the 18,000 g centrifugation step (P_{18k}).

Fluorescence Microscopy Detection of the HSP104 Locus

Live fluorescence microscopy and quantification was performed as described (Dieppois et al., 2006). Wild-type and *hpr1Δ* strains containing LacO repeats inserted 800 nt downstream of the *HSP104* and expressing integrated LacI-GFP repressor and Nup49-GFP fusions were shifted to 37°C for 20 min before scoring *HSP104* position relative to the nuclear periphery as previously described (Dieppois et al., 2006).

Microarray Analyses

DNA fragments obtained from P_{18k} fractions or low speed supernatants (SN_{2k}) for a total DNA control were labeled by random priming and direct incorporation of fluorescent nucleotides as described (Koszul et al., 2004). We used 0.75 to 3 μg of each sample, depending on the amount of DNA available, 12 μg of random hexamers (Invitrogen) and 50 units of Klenow (Biolabs) in each reaction. The final dNTP concentration was: 0.25 mM dATP, dGTP, dTTP, 0.05 mM dCTP and 0.09 mM Cy3- or Cy5-dCTP. A Yeast probe collection (70mers oligonucleotides, Operon), matching the coding region of most yeast ORF, was spotted in duplicates onto ultragaps slides (Corning) as described at www.transcriptome.ens.Fr. The labeled DNAs from either the P2 pellet or the SN1 supernatant were competitively hybridized to these DNA microarrays. The slides were scanned with a Genepix 4000B from Axon and the images analyzed using the Genepix Pro software. The DCF enrichments were normalized using the median per block method available in the Goulphar software (Lemoine et al., 2006). Each DCF enrichment value presented in this article is the result of at least two independent experiments, using dye swap.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, five figures, and four tables and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(08)01006-4.

ACKNOWLEDGMENTS

We thank S. Camier, A. Morillon, B. Séraphin, and T. Villa for critical reading of the manuscript. A. Aguilera, B. Dicht, F. Feuerbach-Fournier, H. Klein, and F. Lacroute for gifts of strains. This work was supported by the CNRS (D.L.), the ANR (programme CUTs, D.L.), the FRM (programme équipes 2006, D.L.), the EEC (programme EMIL, LSHC-CT-2004-503569, D.L.), the Danish National Research Foundation (D.L. and T.H.J.), the Novo Nordisk Foundation (T.H.J.). The plate-forme transcriptome, IFR36, is funded by the RNG. M.R., E.K.R., and R.K.G. were supported by fellowships from the French Ministry of Research, the A.R.C. and the Région Ile de France, respectively. F.S. and G.D. were supported by the Swiss National Science Foundation (grants 102235 and 116541) and the SNF program "Frontiers in Genetics." This

research was carried out within the scope of the Associated European Laboratory LEA Laboratory of Nuclear RNA Metabolism.

Received: May 27, 2007
Revised: March 26, 2008
Accepted: August 1, 2008
Published: October 16, 2008

REFERENCES

Abruzzi, K.C., Belostotsky, D.A., Chekanova, J.A., Dower, K., and Rosbash, M. (2006). 3′-end formation signals modulate the association of genes with the nuclear periphery as well as mRNP dot formation. EMBO J. *25*, 4253–4262.

Boorsma, A., Foat, B.C., Vis, D., Klis, F., and Bussemaker, H.J. (2005). T-profiler: scoring the activity of predefined groups of genes using gene expression data. Nucleic Acids Res. *33*, 592–595.

Brickner, D.G., Cajigas, I., Fondufe-Mittendorf, Y., Ahmed, S., Lee, P.C., Widom, J., and Brickner, J.H. (2007). H2A.Z-Mediated Localization of Genes at the Nuclear Periphery Confers Epigenetic Memory of Previous Transcriptional State. PLoS Biol. *5*, e81. 10.1371/journal.pbio.0050081.

Brickner, J.H., and Walter, P. (2004). Gene recruitment of the activated INO1 locus to the nuclear membrane. PLoS Biol. *2*, e342. 10.1371/journal.pbio.0020342.

Butler, J.S. (2002). The yin and yang of the exosome. Trends Cell Biol. *12*, 90–96.

Cabal, G.G., Genovesio, A., Rodriguez-Navarro, S., Zimmer, C., Gadal, O., Lesne, A., Buc, H., Feuerbach-Fournier, F., Olivo-Marin, J.C., Hurt, E.C., and Nehrbass, U. (2006). SAGA interacting factors confine sub-diffusion of transcribed genes to the nuclear envelope. Nature *441*, 770–773.

Casolari, J.M., Brown, C.R., Komili, S., West, J., Hieronymus, H., and Silver, P.A. (2004). Genome-wide localization of the nuclear transport machinery couples transcriptional status and nuclear organization. Cell *117*, 427–439.

Chavez, S., Beilharz, T., Rondon, A.G., Erdjument-Bromage, H., Tempst, P., Svejstrup, J.Q., Lithgow, T., and Aguilera, A. (2000). A protein complex containing Tho2, Hpr1, Mft1 and a novel protein, Thp2, connects transcription elongation with mitotic recombination in Saccharomyces cerevisiae. EMBO J. *19*, 5824–5834.

Cui, Y., and Denis, C.L. (2003). In vivo evidence that defects in the transcriptional elongation factors RPB2, TFIIS, and SPT5 enhance upstream poly(A) site utilization. Mol. Cell. Biol. *23*, 7887–7901.

Daneholt, B. (2001). Assembly and transport of a premessenger RNP particle. Proc. Natl. Acad. Sci. USA *98*, 7012–7017.

Dieppois, G., Iglesias, N., and Stutz, F. (2006). Cotranscriptional recruitment to the mRNA export receptor Mex67p contributes to nuclear pore anchoring of activated genes. Mol. Cell. Biol. *26*, 7858–7870.

Dwight, S.S., Harris, M.A., Dolinski, K., Ball, C.A., Binkley, G., Christie, K.R., Fisk, D.G., Issel-Tarver, L., Schroeder, M., Sherlock, G., et al. (2002). Saccharomyces Genome Database (SGD) provides secondary gene annotation using the Gene Ontology (GO). Nucleic Acids Res. *30*, 69–72.

Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G., Botstein, D., and Brown, P.O. (2000). Genomic expression programs in the response of yeast cells to environmental changes. Mol. Biol. Cell *11*, 4241–4257.

Gilbert, W., and Guthrie, C. (2004). The Glc7p nuclear phosphatase promotes mRNA export by facilitating association of Mex67p with mRNA. Mol. Cell *13*, 201–212.

Hilleren, P., McCarthy, T., Rosbash, M., Parker, R., and Jensen, T.H. (2001). Quality control of mRNA 3′-end processing is linked to the nuclear exosome. Nature *413*, 538–542.

Houseley, J., LaCava, J., and Tollervey, D. (2006). RNA-quality control by the exosome. Nat. Rev. Mol. Cell Biol. *7*, 529–539.

Huertas, P., and Aguilera, A. (2003). Cotranscriptionally formed DNA:RNA hybrids mediate transcription elongation impairment and transcription-associated recombination. Mol. Cell *12*, 711–721.

Jensen, T.H., Boulay, J., Olesen, J.R., Colin, J., Weyler, M., and Libri, D. (2004). Modulation of transcription affects mRNP quality. Mol. Cell *16*, 235–244.

Jensen, T.H., Boulay, J., Rosbash, M., and Libri, D. (2001a). The DECD box putative ATPase Sub2p is an early mRNA export factor. Curr. Biol. *11*, 1711–1715.

Jensen, T.H., Dower, K., Libri, D., and Rosbash, M. (2003). Early formation of mRNP: license for export or quality control? Mol. Cell *11*, 1129–1138.

Jensen, T.H., Patricio, K., McCarthy, T., and Rosbash, M. (2001b). A block to mRNA nuclear export in S. cerevisiae leads to hyperadenylation of transcripts that accumulate at the site of transcription. Mol. Cell *7*, 887–898.

Kistler, A.L., and Guthrie, C. (2001). Deletion of MUD2, the yeast homolog of U2AF65, can bypass the requirement for sub2, an essential spliceosomal ATPase. Genes Dev. *15*, 42–49.

Koszul, R., Caburet, S., Dujon, B., and Fischer, G. (2004). Eucaryotic genome evolution through the spontaneous duplication of large chromosomal segments. EMBO J. *23*, 234–243.

Lei, E.P., Krebber, H., and Silver, P.A. (2001). Messenger RNAs are recruited for nuclear export during transcription. Genes Dev. *15*, 1771–1782.

Lemoine, S., Combes, F., Servant, N., and Le Crom, S. (2006). Goulphar: rapid access and expertise for standard two-color microarray normalization methods. BMC Bioinformatics *7*, 467.

Libri, D., Dower, K., Boulay, J., Thomsen, R., Rosbash, M., and Jensen, T.H. (2002). Interactions between mRNA export commitment, 3′-end quality control, and nuclear degradation. Mol. Cell. Biol. *22*, 8254–8266.

Libri, D., Graziani, N., Saguez, C., and Boulay, J. (2001). Multiple roles for the yeast SUB2/yUAP56 gene in splicing. Genes Dev. *15*, 36–41.

Luna, R., Jimeno, S., Marin, M., Huertas, P., Garcia-Rubio, M., and Aguilera, A. (2005). Interdependence between transcription and mRNP processing and export, and its impact on genetic stability. Mol. Cell *18*, 711–722.

Lund, M.K., and Guthrie, C. (2005). The DEAD-box protein Dbp5p is required to dissociate Mex67p from exported mRNPs at the nuclear rim. Mol. Cell *20*, 645–651.

Luthra, R., Kerr, S.C., Harreman, M.T., Apponi, L.H., Fasken, M.B., Ramineni, S., Chaurasia, S., Valentini, S.R., and Corbett, A.H. (2007). Actively transcribed GAL genes can be physically linked to the nuclear pore by the SAGA chromatin modifying complex. J. Biol. Chem. *282*, 3042–3049.

Mason, P.B., and Struhl, K. (2005). Distinction and relationship between elongation rate and processivity of RNA polymerase II in vivo. Mol. Cell *17*, 831–840.

Mieczkowski, P.A., Lemoine, F.J., and Petes, T.D. (2006). Recombination between retrotransposons as a source of chromosome rearrangements in the yeast Saccharomyces cerevisiae. DNA Repair (Amst.) *5*, 1010–1020.

Rehwinkel, J., Herold, A., Gari, K., Kocher, T., Rode, M., Ciccarelli, F.L., Wilm, M., and Izaurralde, E. (2004). Genome-wide analysis of mRNAs regulated by the THO complex in Drosophila melanogaster. Nat. Struct. Mol. Biol. *11*, 558–566.

Rodriguez-Navarro, S., Fischer, T., Luo, M.J., Antunez, O., Brettschneider, S., Lechner, J., Perez-Ortin, J.E., Reed, R., and Hurt, E. (2004). Sus1, a functional component of the SAGA histone acetylase complex and the nuclear pore-associated mRNA export machinery. Cell *116*, 75–86.

Rondon, A.G., Jimeno, S., Garcia-Rubio, M., and Aguilera, A. (2003). Molecular evidence that the eukaryotic THO/TREX complex is required for efficient transcription elongation. J. Biol. Chem. *278*, 39037–39043.

Rougemaille, M., Gudipati, R.K., Olesen, J.R., Thomsen, R., Seraphin, B., Libri, D., and Jensen, T.H. (2007). Dissecting mechanisms of nuclear mRNA surveillance in THO/sub2 complex mutants. EMBO J. *9*, 2317–2326.

Saguez, C., Schmid, M., Olesen, J.R., Ghazy, M.A., Qu, X., Poulsen, M.B., Nasser, T., Moore, C., and Jensen, T.H. (2008). Nuclear mRNA surveillance

in THO/sub2 mutants is triggered by inefficient polyadenylation. Mol. Cell *31*, 91–103.

Santos-Rosa, H., Moreno, H., Simos, G., Segref, A., Fahrenkrog, B., Pante, N., and Hurt, E. (1998). Nuclear mRNA export requires complex formation between Mex67p and Mtr2p at the nuclear pores. Mol. Cell. Biol. *18*, 6826–6838.

Schmid, M., Arib, G., Laemmli, C., Nishikawa, J., Durussel, T., and Laemmli, U.K. (2006). Nup-PI: the nucleopore-promoter interaction of genes in yeast. Mol. Cell *21*, 379–391.

Schneiter, R., Guerra, C.E., Lampl, M., Gogg, G., Kohlwein, S.D., and Klein, H.L. (1999). The Saccharomyces cerevisiae hyperrecombination mutant hpr1Delta is synthetically lethal with two conditional alleles of the acetyl coenzyme A carboxylase gene and causes a defect in nuclear export of polyadenylated RNA. Mol. Cell. Biol. *19*, 3415–3422.

Strasser, K., and Hurt, E. (2001). Splicing factor Sub2p is required for nuclear mRNA export through its interaction with Yra1p. Nature *413*, 648–652.

Strasser, K., Masuda, S., Mason, P., Pfannstiel, J., Oppizzi, M., Rodriguez-Navarro, S., Rondon, A.G., Aguilera, A., Struhl, K., Reed, R., and Hurt, E. (2002). TREX is a conserved complex coupling transcription with messenger RNA export. Nature *417*, 304–308.

Taddei, A., Van Houwe, G., Hediger, F., Kalck, V., Cubizolles, F., Schober, H., and Gasser, S.M. (2006). Nuclear pore association confers optimal expression levels for an inducible yeast gene. Nature *441*, 774–778.

Thomsen, R., Libri, D., Boulay, J., Rosbash, M., and Jensen, T.H. (2003). Localization of nuclear retained mRNAs in Saccharomyces cerevisiae. RNA *9*, 1049–1057.

Voynov, V., Verstrepen, K.J., Jansen, A., Runner, V.M., Buratowski, S., and Fink, G.R. (2006). Genes with internal repeats require the THO complex for transcription. Proc. Natl. Acad. Sci. USA *103*, 14423–14428.

Wellinger, R.E., Prado, F., and Aguilera, A. (2006). Replication fork progression is impaired by transcription in hyperrecombinant yeast cells lacking a functional THO complex. Mol. Cell. Biol. *26*, 3327–3334.

Zenklusen, D., Vinciguerra, P., Wyss, J.C., and Stutz, F. (2002). Stable mRNP formation and export require cotranscriptional recruitment of the mRNA export factors Yra1p and Sub2p by Hpr1p. Mol. Cell. Biol. *22*, 8241–8253.

2.1.5.6 *GAL* genes peripheral localization persists after transcription shut-off

The above mentioned studies from the Rosbash lab (Abruzzi et al., 2006; Vodala 2008), as well as those from the Brickner lab (Brickner, 2007), showed that *GAL* genes remain at the nuclear periphery well after transcription has been fully repressed by glucose addition. However, while the study of the Rosbash group is consistent with a role of mRNP retention in foci in the tethering of a recently repressed gene at the nuclear periphery, the Brickner team observed that *GAL* genes remain tethered to the pore for generations, much longer than the persistence of the mRNP dot (Brickner, 2007). In addition, in the same study, the Brickner lab reported that modifications in chromatin itself might be required for posttranscriptional tethering of the *GAL1* and *INO1* loci to the pore. In order to clarify the relevance of these observations in our strains, we next tested how localization to the nuclear periphery changed after transcription repression. *GAL10* is repressed rapidly after the addition of glucose to the medium. mRNA levels decrease quickly and are no more detectable within 30 minutes (see Section 2.2.3.1 Figure 17B for mRNA stability of *GAL* genes). However *GAL10* remained localized to the nuclear periphery for more than 30 min after repression and returned progressively to the nuclear interior within about two hours (Figure 14). This persistent localization at the nuclear periphery confirmed that genes can be retained by another mechanism than on going transcription . However these results are more consistent with a role of the mRNP and processing machineries in the posttranscriptional tethering of *GAL* genes rather than a role of epigenetic modification on chromatin in this process.

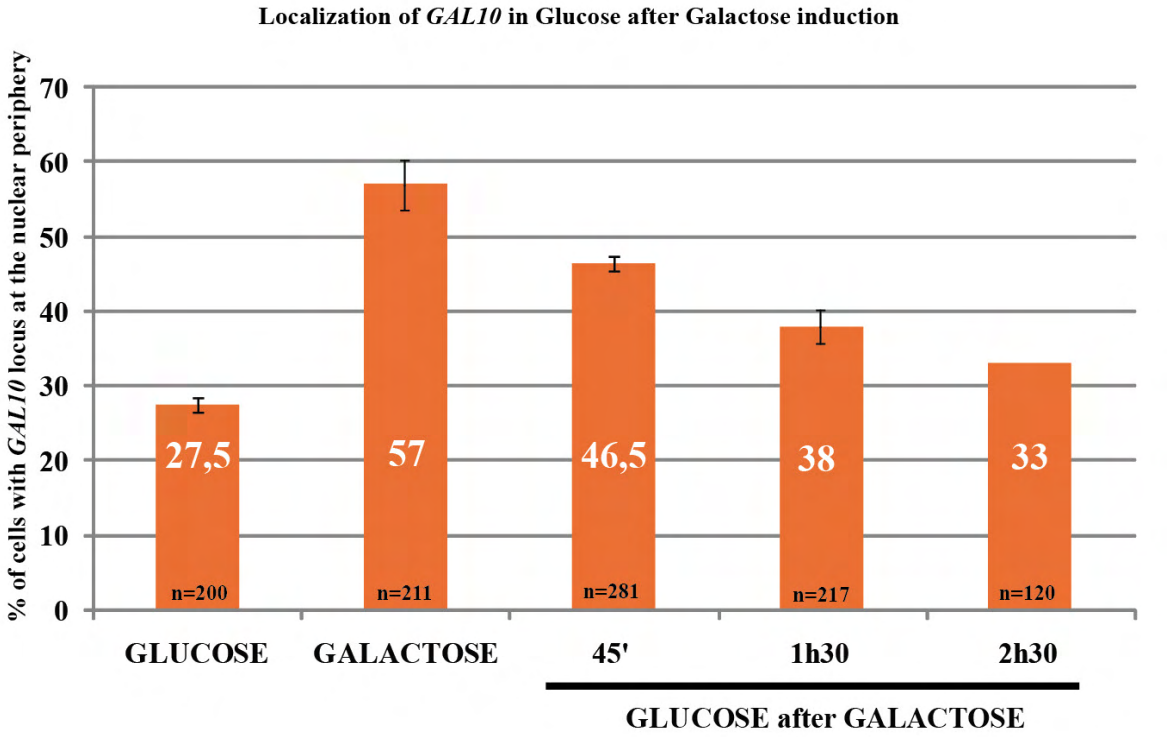


Figure 14. Activated *GAL10* gene tethering at the nuclear periphery disappears progressively following glucose repression. Localization assay of *GAL10*-LacO relative to the nuclear periphery. WT cells were grown in SC 2% glucose and galactose. Cells grown in galactose were then washed and shifted to glucose for 45', 1h30 and 2h30 before taking pictures.

2.1.5.7 *GAL10* and *GAL2* do not share the same nuclear territories upon activation.

GAL10 and *GAL2* both relocalize towards the nuclear periphery upon activation and are activated by the same upstream activating sequences and binding factors. This suggests that both genes are recruited to the NPC at the same time and possibly share the same transcription machinery (Osborne, 2004). We wanted to test this possibility by investigating the localization of *GAL10* and *GAL2* using the LacO/LacI GFP system. For this purpose, we used a diploid strain in which each locus was tagged with GFP on one chromosome and counted the number of cells containing two distinct dots relative to the total number of cells in glucose and in galactose (Figure 15B). More than 80% of the analysed cells showed two distinct fluorescent dots corresponding to *GAL10* and *GAL2* in glucose as well as in their activated state, in galactose. The localization of the loci relative to the nuclear periphery was also analysed in parallel and was consistent with what was previously reported for *GAL10* and *GAL2* separately (Figure 15A). Based on these results, *GAL10* and *GAL2* occupy distinct territories even when activated. Therefore, they are unlikely to be transcribed in the same

transcription factory. This result has been further confirmed in the recent study by Berger et *al.* which mapped the *GAL1* and *GAL2* gene territories (Berger, 2008).

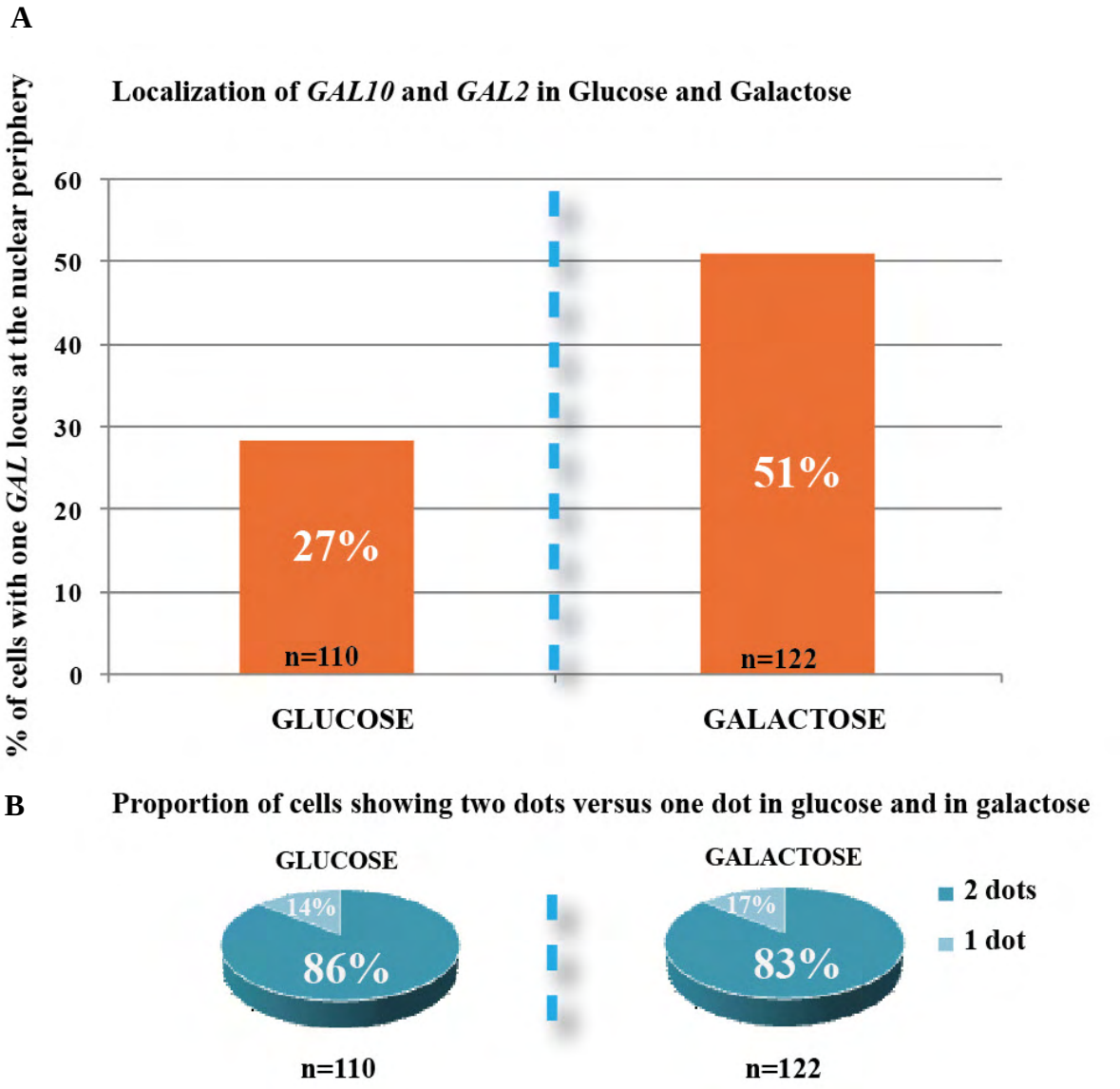


Figure 15. *GAL2* and *GAL10* occupy different nuclear spaces. (A) Localization assay of *GAL10* or *GAL2* dots relative to the nuclear periphery in glucose and in galactose. (B) Relative proportion of cells exhibiting 2 dots versus cells showing only one dot.

2.1.5.8 *GAL* gene transcriptional "memory"does not require peripheral localization

Transcriptional activation of *GAL* genes exhibit a "memory"of a preceding transcriptional state. Indeed, the kinetics of transcription activation of a "naïve" gene are slower than for a gene that was previously activated (Figure 16). This ability to reinduce *GAL1* with fast kinetics persists more than twelve hours and several rounds of cell division, suggesting that it might be epigenetically inherited. It was proposed that the physiological role of the gene to pore interaction during activation may be to promote the establishment of epigenetic modifications on *GAL* gene chromatin during the first activation (Brickner, 2007). These modifications, in turn, could favor rapid re-activation. Nup2 has been decribed as one of the nuclear pore components which is required for proper anchoring of *GAL* genes to the nuclear periphery (Brickner, 2007). In order to confirm these previous observations, we decided to generate a Δ*nup2* deletion in our strain background to test for *GAL10* localization and re-activation kinetics. Unfortunatly, this mutant strain failed to exhibit a localization defect in our genetic backgroung and its re-activation kinetics were also similar to wild type (data not shown).

To further test this hypothesis, we examined whether in a condition where gene recruitment is abolished during the first activation phase, cells conserved the ability to re-induce *GAL10* with faster kinetics. For this purpose, we used the *mex67-5* mutant and induced cells from glucose to galactose during 6h at 37°C. We then repressed the cells with glucose during twelve hours at 25°C and re-induced them with galactose at 25°C. Since we found that *Δmlp1* affected *GAL10* peripheral localization, we also examined in parallel *GAL10* re-activation kinetics in *Δmlp* mutants at 25°C. None of these mutants had a negative effect on the accelerated re-activation kinetics of *GAL10,* unless our Northern Blot assay was not sensitive enough to detect differences at very early stages of activation (Figure 16). These data suggest that the transcripional "memory" of *GAL* genes does not rely on NPC recruitment during activation.

As presiously described in Section 2.1.6.1, the *mex67-5* mutant exhibited a low transcription rate during the first activation at 37°C. When re-activated at 25°C, the activation kinetics were not modified, *GAL10* was re-induced within the same time frame than a wild type (Figure 16). However, the transcription level during this second induction at 25°C remained lower than in a wild type. This was surprising since in a *mex67-5* mutant at 25°C transcription is occuring normally during a first activation. This might indicate the existence of a "memory" mechanism of both, (i) previous activation and (ii) previous transcription rate

(see General Discussion). It could also mean that transcription in *mex67-5* cells that have been shifted to 37°C remains disturbed for more than twelve hours.

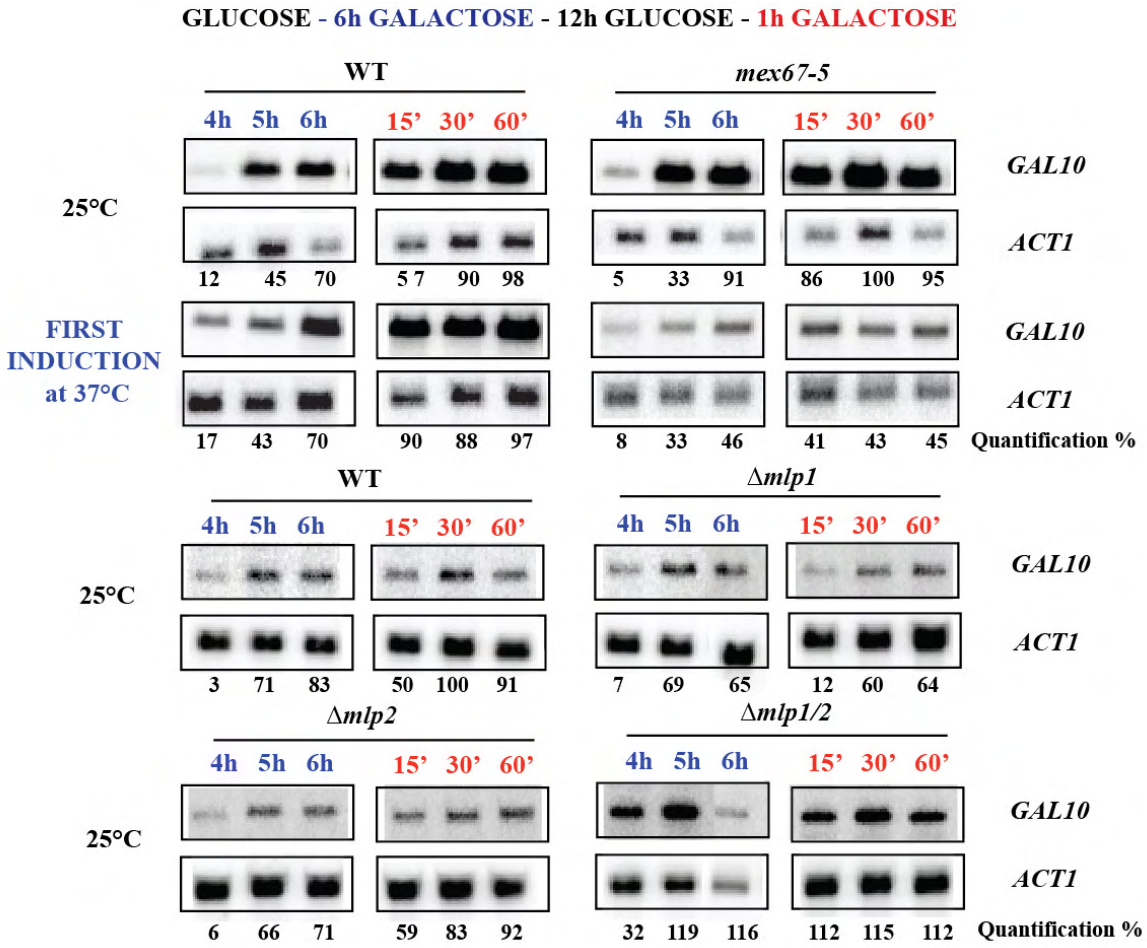


Figure 16. *mex67-5* and Δ*mlp* mutants do not affect the fast re-activation kinetics of the *GAL10* gene. "Memory" assay. WT, *mex67-5* mutant and *mlp* simple or double deletion mutants were assayed for re-activation after a first activation step in galactose at 25°C or 37°C followed by a 12h repression step in glucose and reactivation in galactose at 25°C. Total mRNA was extracted and analysed by Nothern blot at various time points during the first activation and the subsequent re-activation step.

2.1.6 Additional Materials and Methods

2.1.6.1 Plasmid and yeast strain constructions

Strains used in this study are summarized in Supplementary Table I**.**

Plasmid constructions. To insert LexA LacO repeats downstream of the *GAL10* gene, the 3' untranslated region (3'UTR) region of *GAL10* was cloned in front of the LacO repeats carried by the integrating plasmid pFN7 pAFS52LexAop (*TRP1*) (Frank Neumann, S. Gasser lab) to generate pFS2913. For directed insertion, these plasmids were linearized by cleavage of a unique restriction site within the 3'UTR and transformed into relevant strains. The plasmid pAT4-YIF1 (Angela Taddei, S. Gasser lab) carrying the membrane protein YIF1 fused to the LexA Binding Domain was subsequently transformed in these strains. Wild-type *HPR1* was expressed from plasmid pFS2733 (pRS316 *LEU2* CEN), isolated in a synthetic lethal screen and carrying the *HPR1* gene as the only complete open reading frame (D. Zenklusen and F. Stutz, unpublished data).

Yeast strains. All the strains were constructed in the W303 genetic background. *mex67ΔH4* was obtained by crossing strain GA-1320 with *mex67ΔH4* (FSY3113). *Δmlp2* and *Δmlp1/Δmlp2* strains were obtained by crossing strain GA1320 (Heun et al., 2001) with a *mlp1*::Kan^r *mlp2*::*URA* strain (S. Gasser lab). The *Δhpr1* and *Δhpr1Δrrp6* mutant strains were constructed by crossing strain GA1320 with a wild type W303 strain to lose the *HISp*-GFP-lacI-*HIS3* integrated at the at the *HIS3* locus of GA1320 and the resulting strain was transformed by linearized plasmid PGVH60 (PRS402-*HISp*-GFP-lacI-*ADE2*) followed selection on Ade- plates and confirmation of the insertion by PCR. It was then crossed with *hpr1*::*HIS rrp6*::Kan^r strain (FSY2224). The *GAL10* and *HSP104* loci were subsequently tagged with LacO repeats in the *mex67ΔH4*, *Δmlp2*, *Δmlp1/Δmlp2* strains and *Δhpr1*, *Δhpr1/Δrrp6* strains respectively by transformation of linearized pFS2913 (Dieppois et al., 2006) and pFS2914 (Dieppois et al., 2006) followed by selection on Trp$^-$ plates. Insertions were confirmed by PCR on genomic DNA. The diploid strain 2N *GAL10* LacO/*GAL2* LacO was obtained by mating GA1320-*GAL10* LacO (FSY2811) and *GA1320*-GAL2 LacO (FSY2817).

2.1.6.2 Media and Culture conditions

Different growth conditions were used:

In section 2.1.4.1 the wt and *mex67-5* mutant strains were exponentially grown ($OD_{600}\leq$ 0.8) in synthetic complete medium (SC) with glucose 2% or raffinose 2% as a carbon source. Cells grown in glucose were then washed with sterile water and resuspended in SC medium containing galactose 2% at 37°C during 6 hours. Cells grown in raffinose were shifted to 37°C during 30 min before addition of galactose 2% for 10, 20 or 30 min followed by RNA extraction. For the localization assays in section 2.1.5.2 and 2.1.5.6, cells were grown exponentially in SC glucose or galactose 2% overnight and analysed by microscopy as such or after a shift at 37°C. For the induction assay in section 2.1.5.2, cells were grown exponentially in raffinose and induced by addition of galactose 2% at 25°C or shifted to 37°C 30 min before addition of galactose 2%. In section 2.1.5.3 cells were grown exponentially in SC 2% raffinose at 25°C and shifted 3h in SC 2% galactose at 37°C. For the localization assays after glucose repression in section 2.1.5.4, cells were grown exponentially in SC 2% glucose or SC 2 % galactose. Cells grown in galactose were then washed and resuspended in SC medium containing glucose 2% for 45', 1h30 or 2h30. To perform the localization assay in section 2.1.4.5, cells were grown in glucose 2% rich medium (YEPD) at 25°C. The heat shock was done by shifting the cells to 37°C for 20 min. The memory assay of section 2.1.4.7 was carried out by exponentially growing cells in SC medium containing glucose 2%. Cells were then washed and resuspended in SC containing galactose 2% during 6 hours at 25°C or 37°C and repressed in SC medium containing glucose 2% during 12 hours at 25°C. After repression, cells were washed and reinduced in SC medium containing galactose 2% for 1 hour at 25°C.

2.1.6.3 Northern Blot analysis

Total RNA was extracted and fractionated on denaturing formaldehyde agarose gels and transferred to nylon membranes as described previously (Dieppois et al., 2006). For *GAL10* mRNA, membranes were hybridized with P^{32} randomly primed labeled PCR fragments corresponding to the protein coding region at 42°C in 50% Formamide, 5XSSC, 20% dextran sulfate, 1% SDS and 100µg/ml boiled salmon sperm DNA. *GAL1* mRNA were detected using a P^{32} labeled T7 single stranded riboprobe located within the coding region at 62°C in 50% Formamide, 7% SDS, 0.2 M NaCl, 80 mM sodium phosphate pH 7.4, 100µg/ml boiled salmon sperm DNA. In both cases, the membranes were washed with 0.5X SSC/0.1% SDS for 45 min at 62°C. The signals were quantified with a Bio-Rad Instant Imager and

normalized to those obtained with DNA double stranded probes specific for actin mRNA *ACT1* hybridized to the same membranes.

2.1.6.4 Live fluorescence microscopy

The localization assays were performed as previously decribed (Dieppois et al., 2006).

2.1.6.5 Supplementary Tables

code	name	genotype	reference
GA-1320	Wild type LacI-GFP-HIS3 Nup49-GFP	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-HIS3 Nup49-GFP	(Heun et al., 2001)
FSY2811	WT *GAL10*-LacO	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-HIS3 Nup49-GFP *GAL10*-LacO-*TRP1*	(Dieppois et al., 2006)
FSY2812	WT *HSP104*-LacO	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-HIS3 Nup49-GFP *HSP104*-LacO-*TRP*	(Dieppois et al., 2006)
FSY2813	*Δmlp1 GAL10*-LacO	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-HIS3 Nup49-GFP *GAL10*-LacO-*TRP1 mlp1*::Kan^r	(Dieppois et al., 2006)
FSY4084	*Δ mlp2 GAL10*-LacO	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-HIS3 Nup49-GFP *GAL10*-LacO-*TRP1 mlp2*::*URA*	(Dieppois et al., 2006)
FSY4085	*Δ mlp1/Δmlp2 GAL10*-LacO	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-HIS3 Nup49-GFP *GAL10*-LacO-*TRP1 mlp2*::*URA mlp1*::Kan^r	(Dieppois et al., 2006)
FSY2815	*mex67-5 GAL10*-LacO	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-HIS3 Nup49-GFP *GAL10*-LacO-*TRP1 mex67-5*	(Dieppois et al., 2006)
FSY4087	*Δ hpr1 HSP104*-LacO	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-*ADE2* Nup49-GFP *HSP104*-LacO-*TRP1 hpr1*::*HIS*	This study
FSY4088	*Δ hpr1Δrrp6 HSP104*-LacO	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-*ADE2* Nup49-GFP *HSP104*-LacO-*TRP1 hpr1*::*HIS rrp6*:: Kan^r	This study
FSY4091	WT *GAL10*-LexA LacO	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-HIS3 Nup49-GFP *GAL10*-LexA LacO-*TRP1*	This study
FSY4092	*mex67-5 GAL10*-LexA LacO	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-HIS3 Nup49-GFP *GAL10*-LexA LacO-*TRP1 mex67-5*	This study
	2N WT *GAL10*-LacO/*GAL2*-LacO	*MAT***a/α** *ade2/ade2 his3/his3 leu2/leu2 trp1/ trp1 ura3/ura3* LacI-GFP-HIS3 Nup49-GFP *GAL10*- LacO-*TRP1 GAL2*- LacO-*TRP1*	This study
FSY3113	*mex67ΔH4 GAL10*-LacO	*MAT***a** *ade2 his3 leu2 trp1 ura3* LacI-GFP-HIS3 Nup49-GFP *GAL10*-LexA LacO-*TRP1 mex67ΔH4*-3HA Kan^r	This study

Supplementary Table I. Strain Table.

2.2 The nuclear pore complex regulates *GAL* gene activation by controlling the sumoylation of transcription repressors.

G Dieppois, A Letourneau, A Groner, P Vinciguerra, F Stutz.

2.2.1 Abstract

Transcription activation of some yeast genes is paralleled by their repositioning to the NPC. This mechanism requires NPC constituents, transcription factors and components of the mRNA-processing and export machinery. Mlp proteins have been involved in this process and they are also known to restrict the substrate specificity of Ulp1, a key sumo-protease, by maintaining this enzyme at the NPC. In strains lacking Mlp proteins, Ulp1 is released from the NPC. Interestingly, the kinetics of *GAL* gene activation are increased in this strain. Tup1 and Ssn6 are general transcription repressors involved in the repression of *GAL* genes by glucose. We identified a two-hybrid interaction between Tup1/Ssn6 and the NPC-associated Mlp2 protein. Notably, Tup1 is sumoylated and our data indicate that its sumoylation state is regulated by Ulp1 and impacts on its repressor function. We propose that Mlp proteins, through the action of Ulp1, participate in transcription regulation at the nuclear periphery by desumoylating Tup1 and Ssn6.

2.2.2 Introduction

Although the nuclear periphery was long thought to be associated with gene repression (Andrulis et al., 1998; Galy et al., 2000; Pickersgill et al., 2006), there is now evidence that it is also linked to gene activation. Indeed, several recent studies have highlighted the existence of a dynamic coupling between the NPC and transcription by revealing that several genes relocate to the nuclear periphery upon transcriptional induction ((Brickner and Walter, 2004; Dieppois et al., 2006; Drubin DA, 2006; Taddei et al., 2006, Cabal, 2006 #545). However, the precise molecular basis of the gene repositioning mechanism is still a subject of debate and even more mysterious remains its physiological relevance.

Given the number of genes recruited to the NPC upon transcription activation (*GAL1-10, GAL2, HXK1, INO1, HSP104*, mating response genes), and the fact that they are all critical for rapid adaptation to environmental changes, it is likely that gene gating promotes optimal gene expression and provides an additional level of regulation for the expression of these genes. Consistent with this idea, several studies showed that artificially tethering *HXK1* and *INO1* genes to the nuclear envelope positively influences their expression (Brickner and Walter, 2004; Taddei et al., 2006) and even bypassed the requirement for a transcription

activation factor specific for *INO1* (Brickner and Walter, 2004). Subsequent work from the Brickner group showed that tethering of *INO1* increases the activation rate of this gene (Brickner, 2007). Furthermore, artificial tethering of reporter genes to nucleoporins of the Nup84 subcomplex promotes their expression as well, suggesting that the NPC environment is sufficient to activate transcription (Menon, 2005; Sarma et al., 2007). These studies collectively suggested that gene recruitment is coordinated with activation events and promotes transcription initiation. A proposed molecular mechanism for NPC favoring gene activation is that gene recruitment could primarily target Rap1 regulated genes (Casolari et al., 2004) and promote the loading of the Rap1-Gcr1/Gcr2 activating complex on chromatin (Menon, 2005). However, peripheral localization may promote transcription in several other ways and the functional role of NPC-gene anchoring in transcription is far from being elucidated and requires further analyses to better understand the coupling between chromatin dynamics and gene activation.

The Mlp proteins have been implicated in gene expression in several studies. First, Mlps were proposed to associate with activated chromatin, and nascent mRNPs (Luthra et al., 2007, Casolari, 2005 #508, Casolari, 2004 #490). Some years ago, we and others provided evidence that the NPC-anchored myosin like Mlp1/2 proteins participate in mRNP quality surveillance at the nuclear periphery by retaining unprocessed or defective mRNP complexes (Galy et al., 2004; Vinciguerra et al., 2005). Moreover, our data indicated that Mlp proteins may promote transcription downregulation in response to an mRNA export defect. Indeed, the low mRNA levels produced in a *yra1* mutant, which has an mRNP biogenesis and export defect, are rescued to nearly wild-type levels in the absence of Mlp proteins (Vinciguerra et al., 2005). Subsequently, we also found that Mlp1 contributes to the recruitment of activated genes to the nuclear periphery (Dieppois et al., 2006), confirming functional interactions of Mlp proteins with active chromatin.

Consistent with the possibility that the perinuclear Mlp proteins positively influence the transcription of recruited genes, the Mlp homologue in Drosophila, called Mtor, was shown to be required for the two fold up-regulation of the X-chromosome linked genes in males to achieve dosage compensation (Mendjan et al., 2006). In addition, Mlp1 has been directly linked to the Spt-Ada-Gcn5-acetyltransferase (SAGA) histone acetyltransferase co-activator complex through interactions with Sus1, Gcn5 and Ada2 and shown to associate with the *GAL* UAS, the upstream activating sequence that interacts with the SAGA complex (Lei et al., 2003; Luthra et al., 2007; Rodriguez-Navarro et al., 2004).

A crucial role of Mlps is to maintain a proper level of the SUMO-protease Ulp1 at the NPC (Zhao et al., 2004a). SUMO attachment to a protein modifies activity, distribution, and/or binding partners. The levels of sumoylated substrates reflect a highly dynamic balance between rates of SUMO conjugation and deconjugation. In yeast, SUMO conjugation requires a series of enzymes related to the ubiquitin E1, E2, E3 ligases and SUMO cleavage requires only two specialized proteases: a major essential form, Ulp1, and a minor non essential form, Ulp2 (Smt4) (Li, 1999; Li and Hochstrasser, 2000). Ulp1 is a dual-function protease that is also the primary SUMO precursor processing enzyme. Sumoylated proteins function in processes as diverse as cytokinesis, transcription, DNA repair, and chromosome segregation (Johnson, 2004; Melchior et al., 2003; Schwartz and Hochstrasser, 2003). Reflecting the importance of sumoylation in key cellular processes, many of the components of the SUMO pathway, including Ulp1, are essential for viability.

Ulp1 is primarily localized at the nuclear pore. The sequestration of Ulp1 at the nuclear periphery appears as a complex and highly regulated process involving the interaction of the Ulp1 N-terminal domain with the karyopherins Kap121 and Kap60-Kap95, the Nup84 complex, the Nup60-Mlp1-Mlp2 complex, as well as the nuclear envelope protein Esc1 (Lewis et al., 2007). Mislocalisation of Ulp1 in the nucleoplasm through mutation of its N-terminal NPC targeting domain as well as mutation or deletion of the nuclear periphery anchors (Kap60, Kap95 , Kap121, Nup84, Mlp proteins or Esc1) profoundly changes the overall sumoylation profiles and affects cell viability, suggesting that the localization of Ulp1 at the NPC is crucial for proper control of protein desumoylation (Li and Hochstrasser, 2003; Panse et al., 2003; Zhao et al., 2004a). Finding the targets desumoylated by Ulp1 appears therefore of primary importance to understand the role of the NPC and Mlp proteins in nuclear metabolism.

A number of recent studies have already uncovered functional connections between NPC dependent regulation of sumoylation and different aspects of nuclear metabolism, including DNA repair, DNA replication, mRNA quality control and nuclear organization (Lewis et al., 2007; Loeillet et al., 2005; Nagai et al., 2008; Palancade et al., 2007; Therizols et al., 2006; Zhao et al., 2004a). Surprisingly, despite the role of the NPC in gene expression and the identification of a great number of sumoylated transcription activators and repressors such as Tup1 and Ssn6, no connection between NPC-dependent sumoylation states and activation of NPC recruited genes have been described so far.

As *GAL* genes are well recruited to the NPC during their transcription activation, the *GAL* system is a good model to study the connection between the NPC and gene activation.

The *GAL* genes are required for the growth of yeast on galactose and they comprise structural (*GAL1*, *GAL10*, *GAL2* and *GAL7*) and regulatory (*GAL4*, *GAL80* and *GAL3*) genes. The structural *GAL* genes are regulated at the level of transcription in a carbon source-dependent manner. The induction of *GAL* genes by galactose requires the transcriptional activator Gal4 that operates through an upstream activating sequence (UAS_{GAL}) present in their promoter (Lohr et al., 1995). In the absence of galactose, when cells are grown in raffinose for instance, Gal4 is bound by Gal80 and unable to activate transcription. This is called a pre-induced or non repressed state since in response to galactose; Gal3 immediately inhibits Gal80 allowing Gal4 to rapidly activate the *GAL* genes transcription. However, the expression of *GAL* genes is rapidly shut off upon addition of glucose even when galactose is present. This phenomenon, known as glucose repression (Johnston, 1999) is mediated by Mig1 repressor protein (Frolova et al., 1999), which binds to regions upstream of the *GAL* genes and requires the activity of Ssn6 and Tup1 acting as Mig1 co-repressors (Treitel and Carlson, 1995; Williams and Trumbly, 1990). When glucose is replaced by galactose, transcription activation of *GAL* genes is a slow process as it requires the alleviation of repression prior to activation. In this study, we took advantage of these different regulatory pathways to dissect the mechanisms by which the NPC may regulate transcription.

Our results show that Mlp proteins have a positive effect both on transcription activation as well as on transcription repression of *GAL* genes. Interestingly, the effect on transcription repression, but not activation, is dependent on Ulp1, consistent with a role of Ulp1 in regulating the activity of repressors. Notably, we detected a two-hybrid interaction between Mlp2 and the Tup1 and Ssn6 transcription co-repressors, known to form a complex essential for glucose repression. Moreover we provide evidence that sumoylated Tup1 is a target of Ulp1 by showing that delocalization of Ulp1, either by deletion of the Ulp1 NPC-anchoring domain or by disrupting *MLPs,* leads to desumoylation of transcription repressors such as Tup1 in the nucleoplasm. Importantly this desumoylation is paralleled by loss of glucose repression and increased activation kinetics of *GAL* genes when shifting cells from glucose to galactose. Finally, we show that non-sumoylated *tup1* mutants, like *mlp* deletion mutants and *ulp1* mutants defective in NPC association, alleviate glucose repression. Thus, our data support the view that NPC-anchored Mlp proteins may positively affect transcription by regulating the sumoylation state of transcription repressors such as Tup1 through the action of Ulp1 when genes reposition to the nuclear periphery. Tup1 is not the only transcription factor that could be modified by Ulp1 at the nuclear periphery. Its partner Ssn6, or

components of the SAGA complex such as the sumoylated Gcn5 histone deacetylase (Sterner et al., 2006) are also good candidates and will be the objects of further investigations.

2.2.3 Results

2.2.3.1 Loss of Mlps has a negative effect both on gene activation and on transcription repression.

The NPC-associated Mlp1 and related Mlp2 proteins were proposed to contact chromatin and/or nascent transcripts (Casolari et al., 2005; Casolari et al., 2004; Galy et al., 2004; Green et al., 2003) and participate in the stable association of active genes with the NPC (Dieppois et al., 2006). In addition, they were shown to influence the expression of genes located at the nuclear periphery in response to export or processing defects (Vinciguerra et al., 2005). As a number of studies have indicated that active gene gating to the NPC promotes gene expression (Brickner, 2007; Brickner and Walter, 2004; Menon, 2005; Taddei et al., 2006), our aim was to test whether Mlps contribute to early *GAL* gene activation. For this purpose, we analysed the effect of the combined deletions of *MLP1* and *MLP2* (*Δmlp1/2*) on the activation kinetics of *GAL1* in two distinct conditions: (i) in the absence of glucose repression, by shifting cells from raffinose to galactose; in raffinose, *GAL* genes are in a pre-induced state allowing rapid activation in galactose; (ii) in the presence of glucose repression by shifting cells from glucose to galactose; in this case activation is a slow process that depends on the relief of the repressive state. We observed that *Δmlp1/2* has an effect on *GAL1* mRNA levels at very early steps of transcription activation in both conditions, indicating that Mlp proteins may play a direct role in regulating *GAL1* transcription activation kinetics (Figure 17A). At later stages, *GAL1* mRNA levels reached similar steady state levels in wild-type and *Δmlp1/2* cells (data not shown). Surprisingly however, loss of Mlps has opposite effects on *GAL1* activation kinetics depending on whether cells are pregrown in raffinose or in glucose (Figure 17A). Indeed, *Δmlp1/2* has a negative effect on the activation kinetics when cells are shifted from raffinose to galactose, and a positive effect when cells are shifted from glucose to galactose. As the only difference between the two conditions is glucose repression, which is absent in raffinose, the results indicate that Mlps have a dual role: a positive effect on transcription activation revealed when cells are shifted from raffinose to galactose, and an unexpected role in enhancing glucose repression.

Since Mlps associate with the nucleoplasmic side of the NPC through interaction with the nucleoporin Nup60 (Feuerbach et al., 2002), we also tested the effect of *Δnup60* on the *GAL1* activation kinetics. We observed that *Δnup60* exhibits similar and even enhanced effects on *GAL1* induction in the two conditions compared to *Δmlp1/2*, indicating that Nup60

may contribute to *GAL* gene regulation at the nuclear periphery by other means than just through the anchoring of Mlp.

Mlps have been involved in the retention and surveillance of malformed mRNPs (Galy et al., 2004; Vinciguerra et al., 2005). To address whether the differences in mRNA levels in *Δmlp1/2*could be due to a change in mRNA stability , *GAL1* mRNA levels were compared in WT and *Δmlp1/2* strains after a shift from galactose to glucose repressive conditions (Figure 17B). *GAL1* mRNA half-lives were identical in the WT and *Δmlp1/2* strains, confirming a primary effect of *Δmlp1/2* on transcription rather than mRNA stability.

Δmlp1/2 and *Δnup60* strains exhibit a growth defect and irregularly shaped colonies (Figure 17C). This "nibbled" phenotype reflects clonal lethality, which has been linked to an increased number of 2μ circles in a fraction of cells as well as to the altered stability of Ulp1 when its anchoring to the NPC is compromised (Dobson et al., 2005; Zhao et al., 2004a). Indeed, both of these phenotypes are suppressed in *Δmlp1/2* when Ulp1 levels are restored by overexpression from a high copy plasmid (Zhao et al., 2004a) or even by transformation of a centromeric plasmid in our strain background (*ULP1 CEN*) (Figure 17C, right panel).However, rescue of the *Δmlp1/2* growth defect by *ULP1 CEN* was not accompanied by restoration of normal *GAL1* activation kinetics. Indeed, we observed the same accelerated*GAL1* activation from glucose to galactose in *Δmlp1/2* cells transformed with a low copy *ULP1* expressing plasmid (*ULP1 CEN*) than with an empty vector (Fig. 18 C, left panel). These observations indicate that the enhanced *GAL1* activation in *Δmlp1/2* is not an indirect effect of clonal lethality. Notably, mild over-expression of *ULP1* on a centromeric plasmid in WT cells also induces a positive effect on *GAL1* activation kinetics from glucose to galactose similar to that observed in *Δmlp1/2* (Figure 18 C, left panel), further supporting that the effects of *Δmlp1/2* on transcription repression are likely to involve Ulp1. Northern blot analyses also showed that the effects of *Δmlp1/2* and mild over-expression of Ulp1 are additive (Figure 1C, compare lanes 5, 6, 8. 9, 11 and 12). Since *Δmlp1/2* affects Ulp1 stability as well as its localization (Zhao et al., 2004a) and (data not shown), one view is that Ulp1 over-expression in *Δmlp1/2* increases the amount of Ulp1 mislocalized to the nucleoplasm. Thus, if the effects of *Δmlp1/2* on transcription activation are really linked to Ulp1, they might be due to increased levels of Ulp1 in the nucleoplasm rather than to an overall decrease in the amount of this SUMO protease.

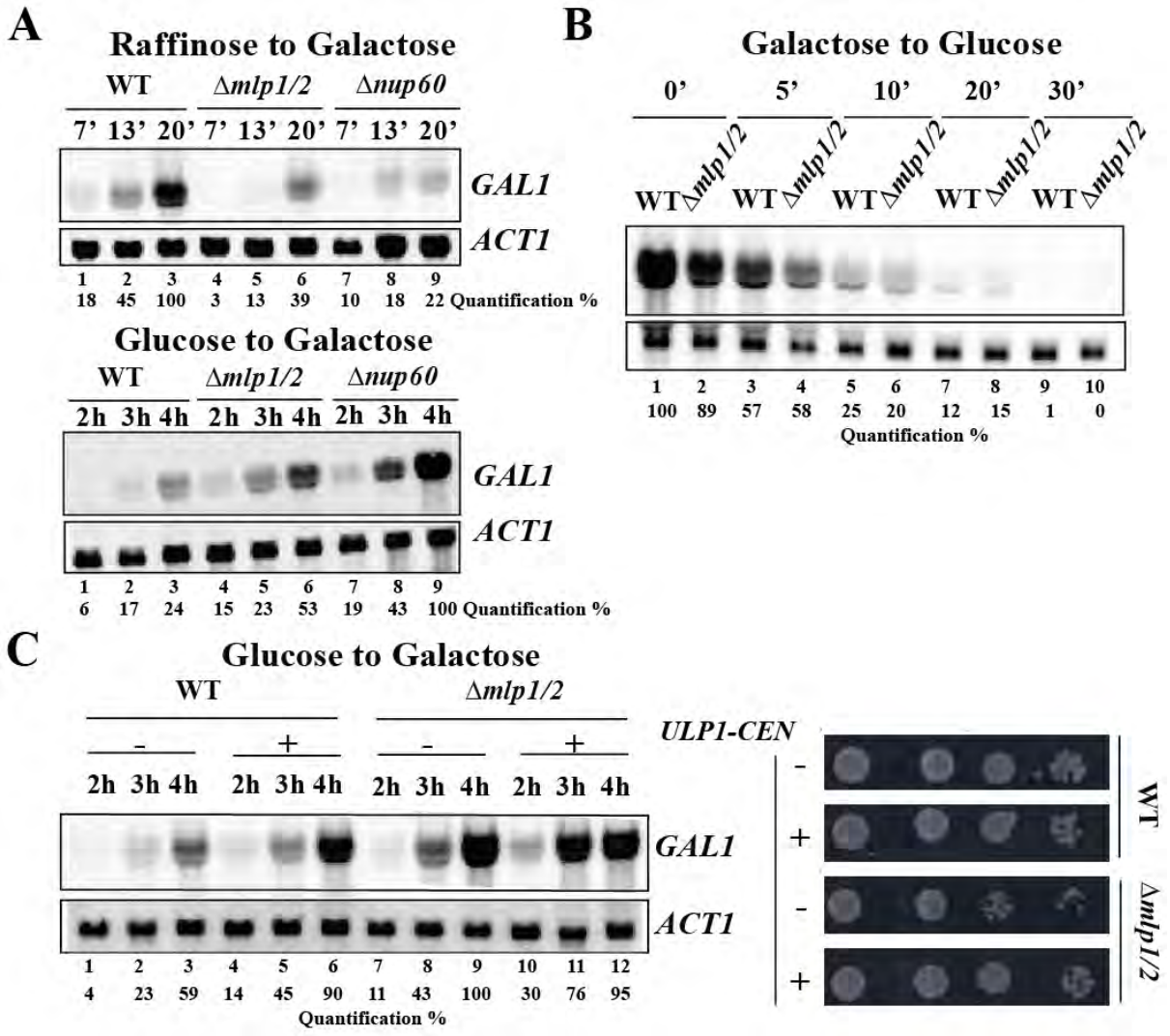


Figure 17. Mlps directly regulate both *GAL1* transcription activation and repression. (A) Northern Blot analysis of the activation kinetics of *GAL1* in WT, *Δmlp1/2*, *Δnup60* strains when shifted for the indicated time (hours or minutes) from glucose to galactose or from raffinose to galactose at 30°C. (B) *GAL1* mRNA stability assay. To follow and compare the rate of *GAL1* mRNA degradation in WT and *Δmlp1/2* strains, cells were shifted from activating conditions (galactose) to repressing conditions (glucose) for 5, 10, 20 or 30 minutes followed by Northern blot analysis . (C) WT and Δ*mlp1/2* cells were transformed by an empty vector (-) or a centrometric plamid expressing Ulp1 (+) and activated by shifting cells from glucose to galactose for the indicated time. The same transformed strains were assayed in parallel for growth by spot test.

2.2.3.2 Delocalization of Ulp1 from the NPC affects transcription repression of *GAL* genes

To further investigate a possible effect of Ulp1 delocalization on *GAL* gene activation, we used a *ulp1* mutant unable to interact with the nuclear periphery thus mimicking the effect of *Δmlp1/2* on Ulp1 localization, and analyzed its effects on *GAL1* transcription activation kinetics. For this purpose we constructed the *ulp1ΔN* strain expressing a Ulp1 protein lacking the portion of its N-terminal domain required for NPC anchoring (amino acids 172 to 340 Figure 18A). Replacing this internal deletion with an in-frame GFP sequence confirmed that *ulp1ΔN* was completely delocalized from the nuclear periphery (Figure 18B). Furthermore, Northern blot analysis showed that *ulp1ΔN* positively influences *GAL1* activation kinetics only when cells were activated starting from repressive conditions, i.e., by shifting cells from glucose to galactose. Indeed, *ulp1ΔN* had no effect on *GAL1* activation when shifting the cells form raffinose to galactose (Figure 18B). These results indicate that delocalization of Ulp1 may interfere with efficient glucose repression, resulting in accelerated *GAL1* activation. Importantly, correct Ulp1 localization is not required for the activation process *per se*. Surprisingly, the *ulp1ΔN* mutant did not exhibit any growth defect in spot test analysis, confirming that Ulp1 effects on transcription regulation are not due to clonal lethality (Figure 18C). This could be due to *ulp1ΔN* being more stable than wild type Ulp1 in *Δmlp1/2*, stable enough to prevent the clonal lethality phenotype. Consistent with this hypothesis, *ulp1ΔN* rescues the growth phenotype of *Δmlp1/2* (Figure 18C), possibly because *ulp1ΔN* accumulates to higher levels than wild-type Ulp1 in this mutant. These data further suggest that the effect of *ulp1ΔN* or *Δmlp1/2* in alleviating transcription repression primarily depends on the presence of mislocalized Ulp1 in the nucleoplasm rather than on a general decrease in Ulp1 levels.

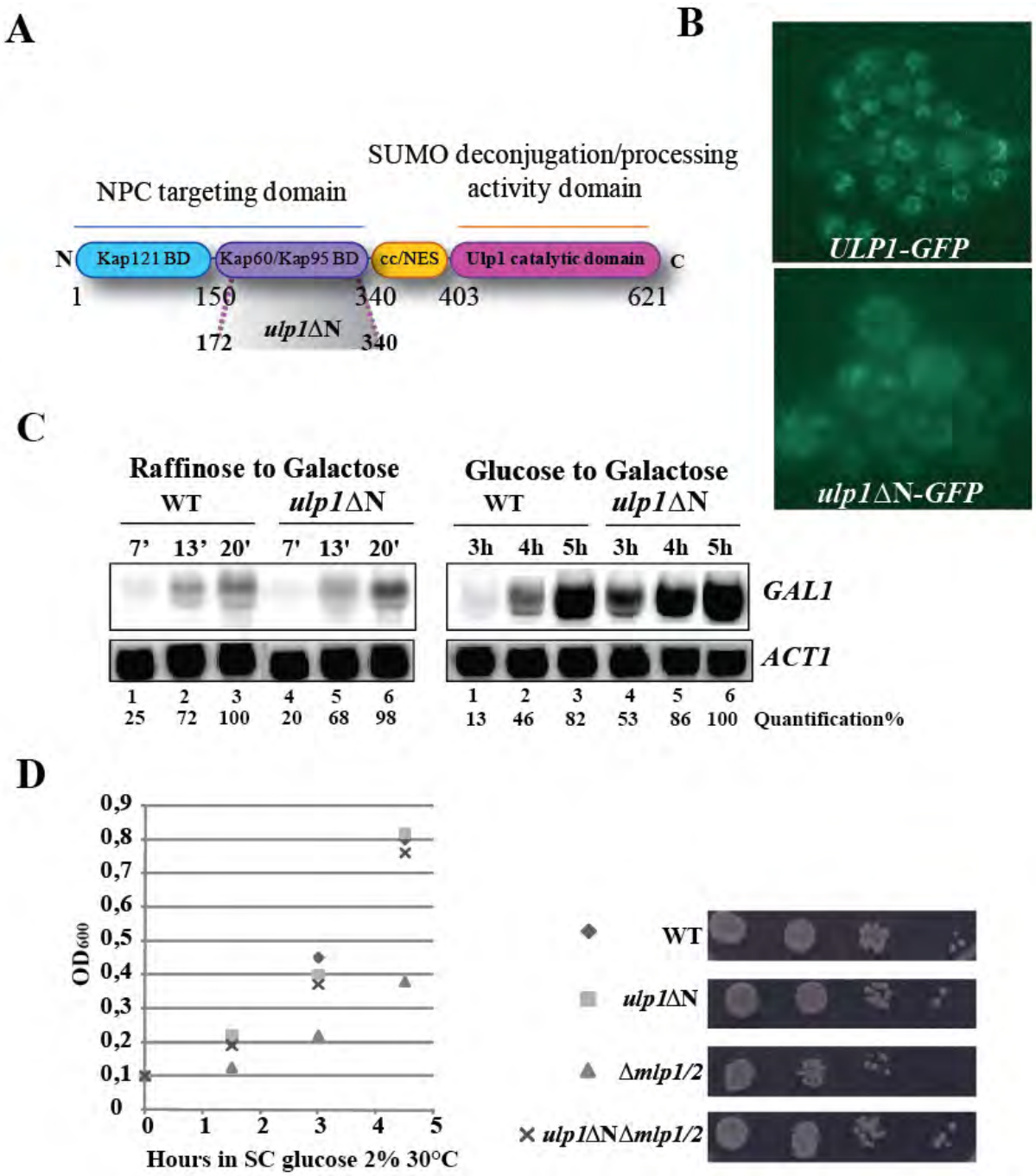


Figure 18. A *ulp1ΔN* mutant mimics the effects of *Δmlp1/2* on transcription repression without affecting cell viability. (A) Schematic representation of the Ulp1 domains and the deletion in the *ulp1ΔN* mutant. (B) Localization of GFP tagged Ulp1 and *ulp1ΔN* by fluorescent microscopy in live cells (C) Northern Blot analysis of the activation kinetics of *GAL1* in WT and *ulp1ΔN* strains when shifted for the indicated time (hours or minutes) from glucose to galactose or from raffinose to galactose at 30°C. (D) Growth assay of WT and *ulp1ΔN* cells in liquid culture and spot test on plates.

2.2.3.3 The effect of *Δmlp1/2* in alleviating transcription repression is due to Ulp1 delocalization

Since loss of Mlps affects Ulp1 localization at the NPC and compromises transcription repression in a similar way than when Ulp1 is released from the NPC, we tested whether the role of Mlps in regulating *GAL1* glucose repression was directly linked to their role in Ulp1 localization. To address this possibility, we first performed an epistastis analysis by comparing *GAL1* induction kinetics from glucose to galactose in WT, *Δmlp1/2*, *ulp1ΔN* strains and a *Δmlp1/2 ulp1ΔN* strain. *GAL1* mRNA levels in *Δmlp1/2* and *ulp1ΔN* were almost identical and higher than in the WT strain, as previously described (Figure 19A). When combined in the same strain, the *Δmlp1/2* and *ulp1ΔN* mutations still exhibited similar *GAL1* activation kinetics as in the *Δmlp1/2* and *ulp1ΔN* simple mutants. These observations indicate that Ulp1 and Mlps are functionally linked and act in the same pathway to alleviate *GAL1* repression.

As both *Δmlp1/2* and *ulp1ΔN* mutations result in the dissociation of Ulp1 from the nuclear periphery and have the same effect on transcription repression, we next addressed more directly whether Ulp1 mislocalization was responsible for the *Δmlp1/2* phenotype in *GAL1* activation kinetics. For this purpose, we disrupted the chromosomal *ULP1* gene and covered the deletion with a plasmid encoding a fusion between the nuclear pore protein Nsp1 and the catalytic C-terminal domain of Ulp1 (*NSP1-ulp1C*) (Panse et al., 2003). Consequently, in cells expressing the plasmid, the catalytic domain of Ulp1 is artificially anchored at the nuclear periphery, whether in a WT or a *Δmlp1/2* background (Figure 19B). Importantly, artificial anchoring of the Ulp1 catalytic domain to the nuclear periphery rescued the effect of *Δmlp1/2* on *GAL1* repression, resulting in slower activation kinetics comparable to those observed in wild type cells (Figure 19B), thereby confirming the role of Ulp1 delocalization in the alleviated glucose repression observed in *Δmlp1/2*. These data indicate a direct role for Ulp1 SUMO protease activity in transcription repression regulation. Consistently, a thermosensitive *ulp1* mutant (*ulp1TS)* (Li, 1999) also rescued the *GAL1* repression defect of the *Δmlp1/2* mutant at the semi-permissive temperature of 30°C (Figure 19C), thus confirming a role for a functional Ulp1 in mediating lack of repression in this strain background.

Taken together these data suggest a model in which Ulp1 release from the NPC may negatively impact on the activity of sumoylated transcription repressors by inducing SUMO cleavage at the wrong time and place. Indeed, the sequestration of Ulp1 at the NPC by Mlp

proteins may ensure tighter repression when genes are inactive within the nucleoplasm, resulting in slow activation kinetics once they are turned on. Thus, the apparent negative role of Mlps on transcription activation probably reflects the positive effect of Ulp1 sequestration on transcription repression. However, the transcription-induced relocalization of activated genes brings them close to the nuclear periphery possibly favoring Ulp1-triggered desumoylation of chromatin-associated factors, thereby optimizing transcription kinetics. Therefore, the positive effect of Ulp1 sequestration by Mlps on transcription repression may convert into a positive effect on transcription activation of the subset of genes recruited to the nuclear periphery. We propose a model in which the recruitment of genes to the NPC upon activation brings the chromatin-associated sumoylated repressors in the vicinity of Ulp1 (Figure 19D). Repressor desumoylation will negatively regulate their activity at the nuclear periphery and facilitate gene expression in this location.

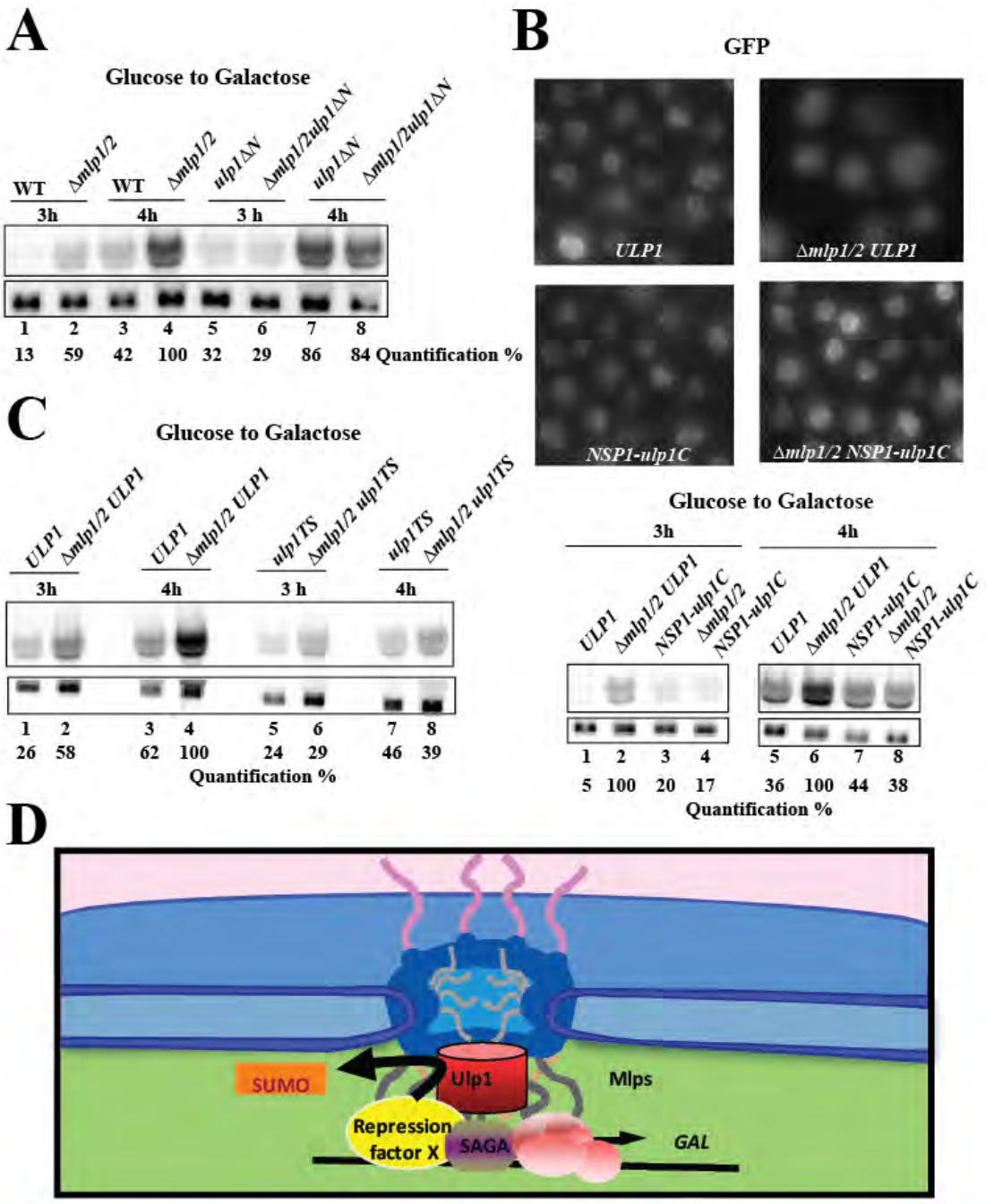


Figure 19. The *Δmlp1/2* negative effect on glucose repression is due to its effect on Ulp1 delocalization. (A) Epistasis analysis of *Δmlp1/2* and *ulp1ΔN* mutations. Northern blot analysis of the activation kinetics of *GAL1* in WT, *Δmlp1/2*, *ulp1ΔN* strains and in the double mutant *Δmlp1/2 ulp1ΔN* strain when shifted for the indicated time (hours) from glucose to galactose at 30°C. (B) Localization of GFP tagged Ulp1 expressed on a centromeric plamid in *Δulp1* and *Δmlp1/2Δulp1* strains and *NSP1-ulp1C*-GFP expressed on a centromeric plasmid in the same strains. Northern blot analysis of *GAL1* mRNA in these strains, when shifted for the indicated time (hours) from glucose to galactose at 30°C (C) Northern blot analysis of *GAL1* mRNA in a *Δulp1* strain complemented with *ULP1* on a centromeric plasmid and *Δulp1* with *ulp1TS* on a centromeric plasmid when shifted for the indicated time (hours) from glucose to galactose at 30°C (D) Model: Ulp1 regulates the sumoylation levels of repressors bound to promoters of NPC-recruited genes.

2.2.3.4 Mlp2 interacts with two sumoylated factors Tup1 and Ssn6 involved in *GAL* gene glucose repression

The validation of the proposed model requires the identification of the sumoylated repressors targeted by Ulp1 at the nuclear periphery. Some years ago, in order to investigate how Mlps regulate transcription in response to export defects (Vinciguerra et al., 2005), we performed a two-hybrid screen using the Mlp2 C-terminal portion as a bait. Interestingly, the results of this screen revealed that Mlp2 interacts with several factors implicated in transcription regulation or chromatin modification (Table 1). Among them, Tup1 and Ssn6 were of particular interest. Indeed, these two proteins are acting together as general co-repressors in glucose repression (Redd et al., 1997). Moreover, they were identified in several studies as sumoylated proteins (Hannich et al., 2005; Panse et al., 2004; Wykoff and O'Shea, 2005; Zhao et al., 2004b). Tup1 and Ssn6 primarily bind to highly induced stress and SAGA dependent genes, including the *GAL* genes. The binding of the Tup1/Ssn6 complex to chromatin during gene repression is mediated by several interactions and induces the recruitment of specific histone deacetylases resulting in chromatin compaction. The fact that Tup1 interacts with genes recruited to the NPC, that it is sumoylated and found in association with Mlps makes it a very good candidate to be a target of Ulp1 at the NPC.

HIS-	NAME	FUNCTION
14	YIL029C	Hypothetical ORF, 2H with Npa3 Cytoplasmic protein required for cell viability, identified by association with pre-ribosomal particles
17	MFALPHA2	alpha mating factor
17	YGR294W	Hypothetical ORF / association with transcription factor SUA7 (GRID)
21	YGR136W	LSB1, Protein containing an N-terminal SH3 domain; binds Las17p, which is a homolog of human protein involved in actin patch assembly and actin polymerization, interacts with Rsp5
21	YFL051C	Hypothetical ORF
10	CKS1	Subunit of the Cdc28 protein kinase, required for mitotic proteolysis, may also be involved in the proteolysis of the G1 cyclins
10	ALPHA1	Silenced copy of ALPHA1, encoding a transcriptional coactivator involved in the regulation of mating-type alpha-specific gene expression
10	HGH1	Protein of unknown function with similarity to human HMG1 and HMG2; localizes to the cytoplasm
10	CDC12	Component of the septin ring of the mother-bud neck that is required for cytokinesis
10	YHR151C	Hypothetical ORF, synthetic lethal with swf1, spore wall formation
10	CDC24	Guanine nucleotide exchange factor (GEF or GDP-release factor) for Cdc42p; required for polarity establishment and maintenance
14	SWC3	Protein of unknown function, component of the Swr1p complex that incorporates Htz1p into chromatin.
10	YLL056C	Protein of unknown function
21	YLR462W	Hypothetical ORF
10	IME2	Serine/threonine protein kinase involved in activation of meiosis
10	SSN6	General transcriptional co-repressor, acts together with Tup1p; also acts as part of a transcriptional co-activator complex that recruits the SWI/SNF and SAGA complexes to promoters
10	YPL077C	Hypothetical ORF
10	YHR073W	Member of an oxysterol-binding protein family with seven members in S. cerevisiae
14	PET494	Specific translational activator for the COX3 mRNA, mitochondria
14	SNZ2	Member of a stationary phase-induced gene family
21	ADP1	Shows homology to ATP-dependent permeases; Active transport ATPase
14	YOR186W	Hypothetical ORF, BIND reb1 RNA polymerase I enhancer binding protein; DNA binding protein which binds to genes transcribed by both RNA polymerase I and RNA polymerase II
10	GIP1	Meiosis-specific protein proposed to be a regulatory subunit of the protein phosphatase Glc7p
10	CDC7	DDK (Dbf4-dependent kinase) catalytic subunit required for firing origins and replication fork progression in mitosis through phosphorylation of Mcm2-7p complexes and Cdc45p
14	NRD1	RNA-binding protein that interacts with the C-terminal domain of the RNA polymerase II large subunit (Rpo21p), required for transcription termination and 3' end maturation of nonpolyadenylated RNAs, bind mlp1
17	YBR139W	hypothetical orf, vacuole
14	EFD1	Protein required for S phase progression and telomere homeostasis, forms an alternative replication factor C complex important for DNA replication and genome integrity
14	YDL148C	Nucleolar protein, forms a complex with Noc4p that mediates maturation and nuclear export of 40S ribosomal subunits
14	MSH5	Protein of the MutS family, forms a dimer with Msh4p that facilitates crossovers between homologs during meiosis
14	VPS8	involved in vacuolar protein sorting
14	RET1	Second-largest subunit of RNA polymerase III
21	LYS2	Alpha aminoadipate reductase
ADE-		
17	SET1	Histone methyltransferase, subunit of the COMPASS complex, which methylates histone H3 on lysine 4 and is required in transcriptional silencing near telomeres
10	TUP1	General repressor of transcription (with Cyc8p), mediates glucose repression
14	GAL4	DNA-binding transcription factor required for the activation of the GAL genes
14	TPS2	Trehalose-6-phosphate phosphatase

Table 1. Mlp2 C-terminal domain interacts with Tup1 and Ssn6 involved in glucose repression. List of the proteins that have been found as interacting with Mlp2 in a two hybrid screen using Mlp2 C-terminal domain as a bait and two reporter systems allowing identification of interactions between bait and prey fusion proteins by selection on HIS- and ADE-. In yellow, transcription activation factors; in pink, transcription repressors; in blue, chromatin remodeling factors.

2.2.3.5 Sumoylated Tup1 is a target of Ulp1

To directly test whether sumoylated Tup1 (SUMO-Tup1) is a target of Ulp1 at the nuclear periphery, we performed sumoylation assays on Tup1 in various mutants affecting Ulp1 localization as well as Ulp1 activity. Because SUMO attachment is a very versatile modification, it is usually difficult to estimate the sumoylated pool of a protein. In order to increase the rate of SUMO conjugation and more easily detect sumoylated Tup1, we took advantage of an inducible 2μ plasmid carrying a His6-tagged *SMT3* gene in order to over-express His6-tagged SUMO (His-SUMO) in the strains of interest. Over-expression of His-SUMO in WT cells lead to high levels of sumoylated Tup1 (His-SUMO-Tup1) in total protein extracts (Figure 20A). However, a basic level of endogenous sumoylated Tup1 (SUMO-Tup1) could be detected in WT cells even without forcing SUMO attachment by over-expression of His-SUMO, suggesting that Tup1 is a highly sumoylated protein in the nucleus. Importantly, in the *Δmlp1/2* strain, the level of His-SUMO-Tup1 as well as endogenous SUMO-Tup1 were dramatically affected and dropped under the detection level of our Western blot assay (Figure 20A). To confirm that Tup1 sumoylation is affected upon Ulp1 delocalization, we performed the same assay using the *ulp1ΔN* mutant (Figure 20B). Consistently, the level of sumoylated Tup1 was strongly decreased when Ulp1 is released from the nuclear periphery.

In agreement with our model, these results indicate that Ulp1 targets SUMO-Tup1 for desumoylation when it gets into the proximity of Tup1. As Tup1 localizes everywhere in the nucleus, the effect of Ulp1 on global Tup1 sumoylation levels becomes visible only when Ulp1 is free to reach all the fractions of Tup1 in the nucleoplasm, as in *Δmlp1/2* or *ulp1ΔN* strains for instance. To further address the relevance of this hypothesis, we analysed the sumoylation levels of Tup1 in the *ulp1TS* mutant, which is impaired in its SUMO-protease catalytic activity but has no localization defect since its N-terminal NPC anchoring domain is intact (Li, 1999). At the semi-permissive temperature of 30°C, we show that the level of endogenous SUMO-Tup1 significantly increases in *ulp1TS* compared to strains expressing wild type Ulp1 (Figure 20C). This confirms that Tup1 is a natural target for Ulp1 and that Ulp1 regulates the sumoylation of Tup1 even when confined to the nuclear periphery.

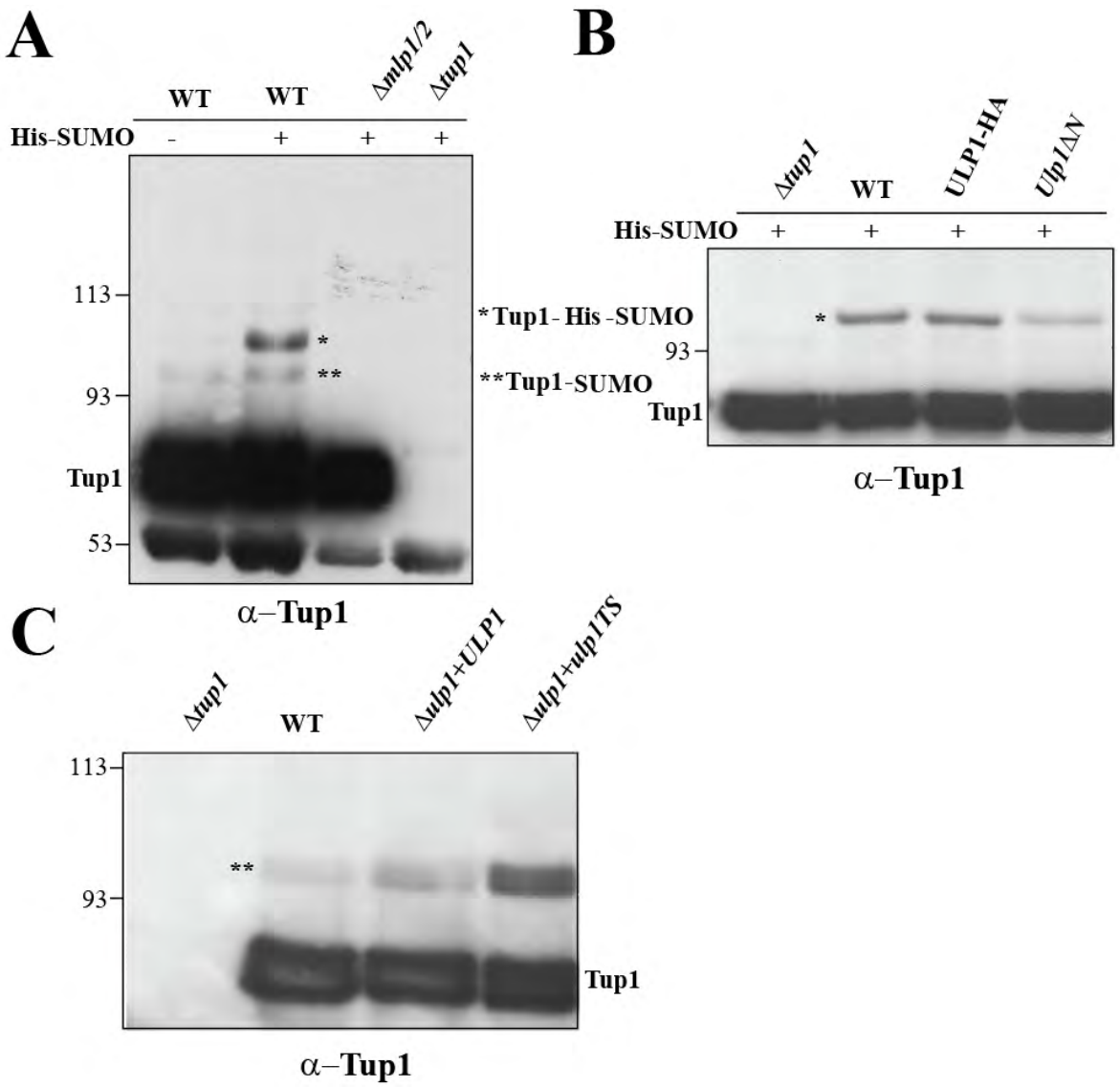


Figure 20. Sumoylated Tup1 is a target for Ulp1 SUMO-protease activity. (A) Sumoylation assay. WT, *Δmlp1/2*, *Δtup1* strains were transformed by a 2μ plasmid expressing His6-Smt3 from a galactose promoter or an empty vector. Cells were grown in galactose for 4 hours followed by total protein extraction and Western blot analysis using a anti-Tup1 antibody. (B) Sumoylation assay with WT, *ULP1-HA* and *ulp1ΔN*, *Δtup1* strains. The procedure was like in (A). (C) Tup1 Western blot analysis using total protein extracted from WT, *Δtup1*, *Δulp1* complemented by Ulp1 expressed on a centromeric plasmid and *Δulp1* with *ulp1TS* on a centromeric plasmid using anti-Tup1 antibody.

2.2.3.6 Tup1 desumoylation alleviates glucose repression

On one hand, we showed that Ulp1 release into the nucleoplasm weakens glucose repression resulting in faster activation kinetics when shifting cells to galactose. On the other hand, we identified the Tup1 co-repressor, associated with repressed *GAL* genes, as a target of Ulp1. Based on our model, we propose that Ulp1 SUMO protease activity at the nuclear periphery negatively regulates the repressor activity of the fraction of Tup1 bound to *GAL* genes when they relocate to the NPC upon activation. To test this hypothesis, we decided to investigate whether constitutive desumoylation of Tup1 affects its repressive function on *GAL* genes. To create a *TUP1* mutant that cannot be sumoylated, we replaced the lysines of the three major putative sumoylation sites of Tup1 (K81, K229, K270) by arginines using site-directed mutagenesis. Analysis of the resulting mutants (K81R, K229R, K270R) in a sumoylation assay confirmed K270 as being the major SUMO attachment site of Tup1 (Figure 21A) and (Wykoff and O'Shea, 2005). This mutant does not completely abolish the repression activity of Tup1 since *GAL* genes were still repressed by glucose in *tup1K270R* (data not shown). However, upon induction from glucose to galactose, the activation kinetics of *GAL1* were increased in the *tup1K270R* mutant compared to those observed in wild type cells (Figure 21B). Sumoylation may therefore alleviate Tup1 repressor function.

Unfortunately, for some still undefined reason, the effect of *tup1K270R* on *GAL1* activation is not always detectable. It may be due to the fact that Tup1 functions in a complex with Ssn6, another sumoylated protein. Possibly, desumoylation of only one of these factors might not be sufficient to relieve glucose repression rapidly each time.

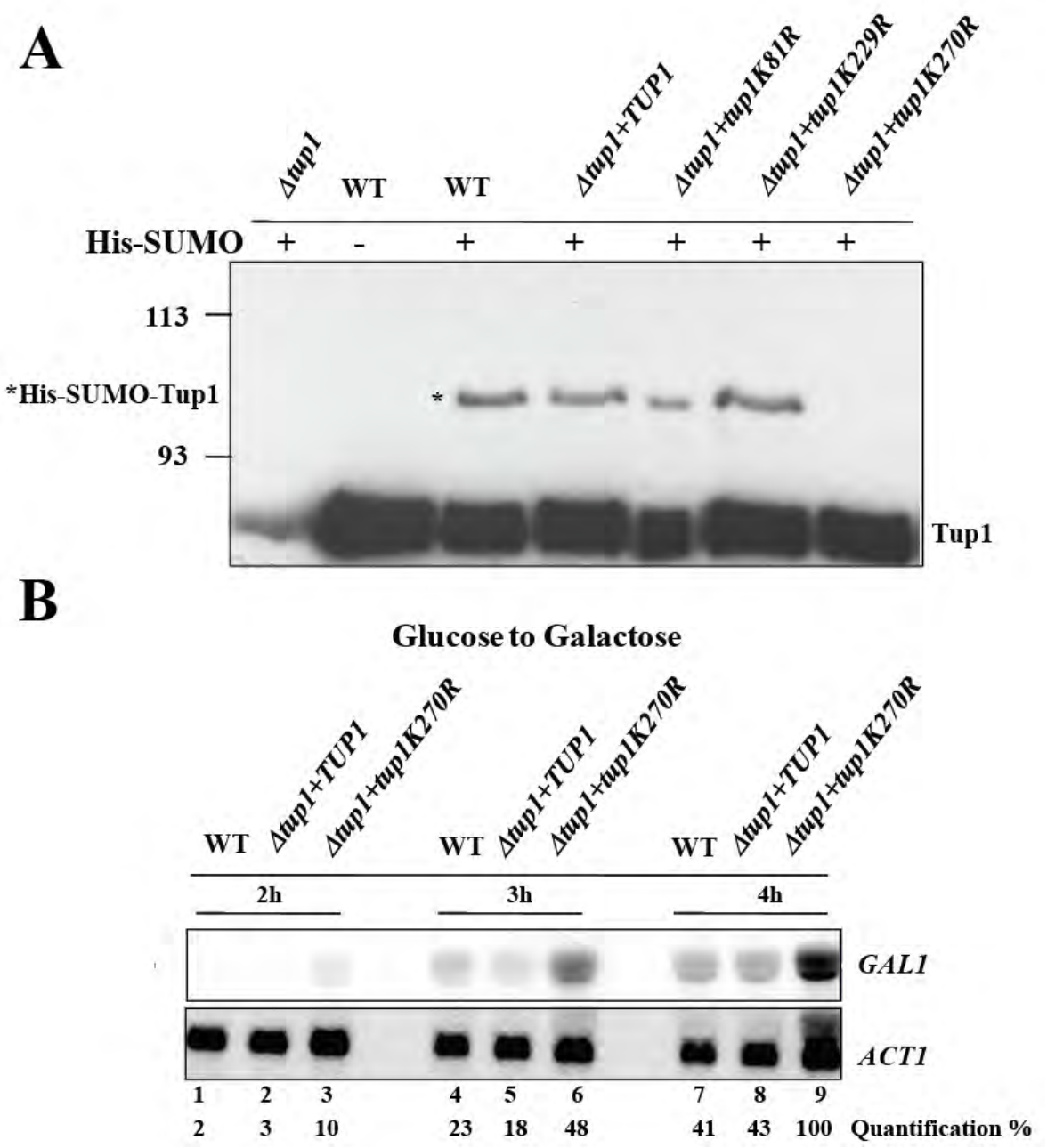


Figure 21. Constitutive desumoylation of Tup1 inibits glucose repression during *GAL* genes activation. (A) Sumoylation assay. WT, *Δtup1*, *Δtup1* complemented with *TUP1* on a centromeric plasmid and *Δtup1* strain carrying a plamid with the mutants *tup1K81R*, *tup1K229* and *tup1K270R* genes. The procedure was similar to the one in Figure 5 A and B. (B) Northern blot analysis of *GAL1* mRNA in WT, and *Δtup1* strains transformed with centromeric plasmids carrying wild-type *TUP1* or *tup1K270R*. Cells were activated from glucose to galactose for the indicated time (hours) at 30°C. *GAL1* mRNA levels were normalized to *ACT1*, used as internal loading control and expressed as percentage of the highest *GAL1* mRNA levels.

2.2.4 Discussion

2.2.4.1 Mlp proteins impact on the activity of transcription repression factors by regulating the substrate specificity of the Ulp1-SUMO protease.

Our analyses show that loss of Mlp proteins has opposite effecs on *GAL1* transcription activation depending on the state of the gene when expression is induced. Thus, *GAL1* activation kinetics are compromised when the *Δmlp1/2* cells are pre-grown in raffinose but enhanced when cells are pre-grown in glucose. These observations indicate that Mlp proteins have a positive effect on transcription activation when cells are shifted from the pre-induced to the induced state in galactose, as well as a positive effect on repression in cells grown in glucose, indicating that Mlps function both in activation and repression. In addition, our data demonstrate that the SUMO-protease Ulp1, which function is regulated through anchoring to the NPC via Mlp proteins, is involved in the Mlp-dependent repression of *GAL1* gene expression. Indeed, both loss of Mlps and a *ulp1* mutation, affecting its localization at the NPC, lead to similar enhanced *GAL1* mRNA levels when shifting the cells from glucose to galactose. Notably, combining the two mutations does not further increase *GAL1* mRNA levels, suggesting that Mlps and Ulp1 act in a common pathway of transcription repression regulation. Importantly, artificial tethering of Ulp1 to the NPC suppresses the enhanced *GAL1* activation kinetics observed in *Δmlp1/2* strains. Collectively, these results indicate that Ulp1 acts as a downstream effector of Mlp proteins in their transcription repression regulation function, suggesting that glucose repression is at least in part regulated by SUMO modification.

Since Ulp1 is required for SUMO processing and because the overall levels of Ulp1 are decreased in the *Δmlp1/2* mutant, the transcription defects observed in this strain could be caused either by reduced availability of conjugatable SUMO, or to facilitated access of free Ulp1 to intra-nuclear substrates. Indeed, even in low amounts, Ulp1 was shown to target the genuine substrates of its paralogue Ulp2 when delocalized from the nuclear periphery (Li and Hochstrasser, 2003). By modest over-expression of Ulp1,we were able to rescue the clonal lethality phenotype but not the transcription repression defect in *Δmlp1/2*, suggesting that the increased activation kinetics observed in this strain are more likely due to the mis-regulation of Ulp1 substrate specificity rather than to a lack of processed SUMO. Consistent with the view that Ulp1 SUMO protease activity alleviates transcription repression, SUMO conjugation to transcription factors has in most reported cases been reported to negatively affect transcription either by inhibiting activation or by stimulating transcription repression (Gill, 2005).

Taken together these data suggest a model in which delocalization of Ulp1 negatively impacts on the activity of sumoylated transcription repressors by inducing extensive SUMO cleavage. In natural conditions, the fact that transcription activation promotes gene relocalization, bringing them into the vicinity of Ulp1 at the nuclear periphery, leads us to propose that Ulp1-triggered desumoylation of chromatin-associated repressors may participate in optimizing transcription kinetics in this location. This model provides a molecular mechanism to explain the enhanced transcription activation kinetics of NPC-recruited genes (Menon, 2005; Taddei et al., 2006) (Figure 19, model).

In agreement with this proposal, we found that the sumoylated co-repressor Tup1, which binds to *GAL* gene promoters and acts in glucose repression, physically interacts with the NPC-associated Mlp2 protein and is regulated by Ulp1, indicating that it could be desumyolated in the NPC environment. Moreover, mutation of the sumoylated lysine negatively impacts on Tup1 repressor function in *GAL* gene transcription. However, Tup1 is probably not the only sumoylated target of Ulp1 regulating gene transcription. Indeed, many other transcription factors, including DNA binding factors, cofactors and chromatin remodeling activities (Hannich et al., 2005; Panse et al., 2004; Wykoff and O'Shea, 2005; Zhao et al., 2004b), have been identified as natural substrates for SUMO modification in yeast and represent Ulp1 potential targets. Notably, Ssn6, which acts in the same co-repressor complex as Tup1 and exhibits a similar two-hybrid interaction with Mlp2, is sumoylated and its repressor function may also be negatively regulated through Ulp1 dependent SUMO cleavage. Another interesting sumoylated substrate is Gcn5, the histone-modifying subunit of the SAGA complex involved in *GAL* gene activation (Sterner et al., 2006). The level of Gcn5 sumoylation substantially decreases when cells are shifted to galactose. In addition, crosslinking captures Gcn5 in association with both Mlp1 and the *GAL* gene promoter, suggesting that it could be a target for Ulp1 when *GAL* genes relocate to the NPC (Luthra et al., 2007). Finally, the fusion of SUMO to the N-terminus of Gcn5, to mimic constitutive sumoylation, results in reduced basal and activated transcription levels of the SAGA-dependent gene *TRP3*, indicating that Gcn5 sumoylation may have an inhibitory role in transcription regulation. However, the K-to-R mutation abolishing Gcn5 sumoylation led to no obvious effects on *GAL* gene transcription *in vivo*. Similarly, the non-sumoylated K270R Tup1 mutant strain did not systematically show an increase in *GAL1* transcription activation kinetics. Therefore, it is likely that Tup1 and Gcn5 represent only two of a whole set of proteins that become desumoylated by Ulp1 upon transcription activation and relocation to

the nuclear periphery. Interefering with the sumoylation of several factors may be required to observe a strong and reproducible effect on *GAL* gene activation.

2.2.4.2 Tup1 desumoylation might be required to counteract its own repression activity during transcription activation.

Once targeted to a gene through interaction with sequence-specific DNA binding repressors, Tup1 spreads through the promoter region and contributes to repress the downstream gene. Tup1-mediated repression is a complex process involving several distinct mechanisms. It involves both physical interactions between the Tup1 repression domain and specific RNA polymerase II mediator subunits (Han et al., 2001; Papamichos-Chronakis et al., 2000; Zhang and Reese, 2004), as well as interactions with histones and HDACs. In a Δ*tup1* mutant strain, glucose repression is drastically impaired and *GAL1* transcription activation occurs as rapidly as from a non repressed state (Supplementary Figure 1). However, in the *tup1* mutant unable to become sumoylated (*tup1K270R*), we showed that *GAL1* activation kinetics are faster than in a wild type context, but remain slower than in the absence of repression. Thus, Tup1 desumoylation does not abolish its repression activity in glucose, but is more likely to play a specific role in alleviating its repressor function during activation.

Importantly, a recent study described a new function of Tup1 in counteracting its negative effect during transcription activation (Gligoris et al., 2007). It is proposed that Tup1 triggers the deposition of the Htz1 histone variant at a particular nucleosome (+1) of Tup1 regulated promoters during the early phase of repression, and that in turn Htz1 incorporation antagonizes Tup1 negative effects on RNA Pol II mediator recruitment during transcription activation.

Based on our findings, one possibility could be that the Tup1-mediated incorporation of Htz1 is the function of Tup1 regulated by desumoylation at the NPC. Consistent with this hypothesis, we found that a component of the Swr1 complex involved in Hzt1 incorporation into chromatin, interacts interacts with the C-terminal domain of Mlp2 in our two hybrid screen. Of note, Ssnt6, the co-repressor partner of Tup1 is also sumoylated and participates in Htz1 incorporation at gene promoters (Gligoris et al., 2007). Overall the data suggest that the function of the Tup1/Ssn6 complex in Htz1 incorporation may depend on desumoylation by Ulp1 at the NPC.

2.2.4.3 NPC-gene gating promotes gene expression by facilitating repression relief as well as transcription activation.

Here, we propose a novel pathway by which the NPC could promote optimal activation of genes recruited to the NPC by relieving repression. We postulate that the tight control of Ulp1 localization at the NPC by Mlp proteins may participate in the fast relief of gene repression through the desumoylation of a pool of repressive factors associated with their promoter. Along the same lines, another study proposed that glucose-derepression was taking place at the nuclear periphery. Indeed, subunits of the Snf1 kinase complex accumulate at the NPC in the absence of glucose, resulting in the phosphorylation and subsequent export into the cytoplasm of the glucose repressor Mig1 associated with the promoters of NPC-recruited glucose-repressed genes (Sarma et al., 2007). In the light of these observations, we can draw a model in which the NPC environment promotes specific post-translational modifications such as desumoylation and phosphorylation that down-regulate the activity of NPC-recruited gene repressors.

Our data show that in addition to alleviating repression, loss of Mlp proteins also negatively influences transcription kinetics in the absence of repression, highlighting a positive role of the NPC in promoting gene activation process *per se*. Importantly, these effects are not due to Ulp1 delocalization, suggesting that NPC- mediated transcription activation is regulated by another process than sumoylation/desumoylation events. A direct role for the NPC in favoring gene activation has already been proposed in a study (Menon, 2005) reporting that the Nup84 nucleoporin complex mediates transcription activation of Rap1 controlled genes through the recruitment of the Rap1/Gcr1/Gcr2 activation assemblage (Deminoff and Santangelo, 2001; Shore, 1994). Consistent with a role of the NPC in the activation of Rap1-dependent genes, chromatin immunoprecipitation of NPC-associated genes results in a significant enrichment of those under Rap1/Gcr1/Gcr2 regulatory control (Casolari et al., 2004). The decreased transcription activation of the Rap1 regulated *GAL* genes in the absence of Mlps (Freeman et al., 1995) may corroborate this model as it may be a direct consequence of the reduced level of NPC-*GAL* gene recruitment in *Δmlp1* mutants that we reported in an earlier study (Dieppois et al., 2006).

Nevertheless, because loss of Mlp proteins does not completely abolish NPC-gene recruitment of activated *GAL* genes, the activation defect in *Δmlp1/2* could also reflect a direct positive role of Mlp proteins in transcription activation of NPC-recruited genes. Consistent with this view, Mlp1 interacts with *GAL* gene UAS sequences as well as with Gcn5, Ada2, Spt7, three members of the SAGA histone acetyltransferase core complex

(Balasubramanian et al., 2002; Grant et al., 1997; Sterner et al., 2002; Wu and Winston, 2002), suggesting that Mlp protein modulate the recruitment and/or the activity of SAGA complex components in the UAS of NPC- recruited genes. In addition, deletion of the co-repressor *TUP1,* which interacts with the Mlp2 C-terminal domain, severely reduces the levels of *GAL1* mRNA during activation in the absence of repression (Supplementary Figure 1), indicating a positive role for Tup1 in transcription activation of *GAL* genes. An inherent potential of the Ssn6-Tup1 co-repressor complex in transcriptional activation has already been been reported for other genes (Conlan et al., 1999), supporting a model in which specific signals may convert Ssn6-Tup1 complex into a co-activator on certain promoters. One view is that Mlp2 could also be involved in the modulation of this Ssn6-Tup1 co-activator function through direct interaction.

Taken together these data highlight a dual role of the NPC in promoting transcription activation, either by facilitating the relief of repression or by directly promoting activation. The confinement of specific transcription modulators at the NPC may thus provide an additional topological level of transcription regulation that relies on genome dynamics.

2.2.5 Materials and Methods

2.2.5.1 Plasmid and yeast strain constructions

All the strains and plasmids used in this study are listed in Supplementary Table I and II respectively.

Yeast strains All the strains used in this study were constructed in the W303 genetic background. The *Δnup60* strain (*nup60*:: Kan^r) was obtained by homologous recombination with a PCR fragment amplified from a *nup60*::Kan^r (Euroscarf.) genomic DNA template using oligonucleotides homologous to regions located +/- 500bp of the *NUP60* ORF as primers. The *ulp1ΔN* and *Δmlp1/2 ulp1ΔN* strains were generated by crossing a W303 wild type and *Δmlp1/2* strains (FSY1187) with a GFP-*yra1-8 ulpΔN-HA* strain (FSY2826). After tetrad analysis, the progenies containing *ulp1ΔN-HA* mutation were identified by western blot analysis using antibodies against the HA epitope.

The *ULP1-GFP* and *ulp1ΔN*-GFP *strains* were obtained by homologous recombination with PCR fragment containing a kanMX6 marker flanked by Lox recombination sites in a strain containing and additional *ULP1* allele on a plasmid. The oligos were designed to internally tag GFP after amino acid 340 and to tag GFP and delete amino acids 172 to 340 according to the method described in (Gauss et al., 2005). Expression of the Cre-recombinase under a gal-inducible promoter on a plasmid led to excision of the marker and to restoration of the Ulp1 reading frame.

The *Δulp1* strain (*ulp1*::*HIS3* + p*ULP1-TRP1*) was obtained with a PCR based homologous recombination method as described in (Longtine et al., 1998). This was performed in a diploid strain containing an additional *ULP1* allele on a *TRP1* plasmid (Ycplac22). After tetrad analysis the progenies containing the *Δulp1* deletion and the *ULP1* plasmid were identified by auxotrophie selection. Δ*mlp1/2 Δulp1* was obtained by deleting *MLP1* and *MLP2* by homologous recombination in *Δulp1*. The PCR fragment used to delete *MLP1* and *MLP2* were generated from a *Δmlp1/2* strain (*mlp1:URA3, mlp2:kan*(MX6) (FSY4085)) genomic DNA as template and oligos located +/- 500bp of *MLP1* and *MLP2* ORFs.

The Δ*tup1* strain (*tup1*::Kan^r) was generated by homologous recombination with a PCR fragment amplified from a *tup1*::Kan^r (Euroscarf) genomic DNA template using 20bp oligonucleotides located +/-500pb of the *TUP1* ORF as primers.

Plasmid constructions The plasmid p*LEU2-TUP1* (+*TUP1*) has been obtained by PCR amplification of the *TUP1* locus +/- 500bp with SalI containing primers followed by cloning into SalI-cut vector YCpLac111 (*LEU2/CEN*). p*LEU2-tup1*K81R, p*LEU2-tup1*K229R and p*LEU2-tup1*K270R were constructed by site directed mutagenesis using 3 PCR reactions. The overlapping PCR1 and PCR2 products were amplified from the *pLEU2-TUP1* plasmid using SalI containing oligos flanking the *TUP1* ORF +/- 500 bp with complemetary mutagenic oligos. The PCR1 and PCR2 products were then mixed and amplified with the two SalI external primers to generate PCR3, corresponding to the full length mutated gene, subsequently cloned into SalI-cut YCpLac111.

2.2.5.2 Northern Blot analysis

For all Northern blot analyses of *GAL1* activation kinetics, cells were exponentially grown at 30°C ($OD_{600} \leq 0.8$) in synthetic complete medium (SC) with glucose 2% or raffinose 2% as a carbon source. Cells grown in glucose 2% were then washed with sterile water and resuspended in SC medium containing galactose 2% at 30°C for 2 hours, 3 hours and 4 hours followed by RNA extraction. Cells grown in raffinose 2% were shifted to galactose 2% at 30°C for 7, 13 or 20 minutes followed by RNA extraction. For the *GAL1* mRNA stability analysis, cells were exponentially grown in SC medium containing galactose 2% before the addition of glucose 2% to the medium for 5, 10, 20 or 30 minutes followed by RNA extraction. Total RNA was extracted and fractionated on denaturing formaldehyde agarose gels and transferred to nylon membranes as described previously (Dieppois et al., 2006). *GAL1 transcripts* were detected using a ^{32}P-labeled T7 single stranded riboprobe located within the coding region at 62°C in 50% formamide, 7% SDS, 0.2 M NaCl, 80 mM sodium phosphate pH 7.4, 100µg/ml boiled salmon sperm DNA. For *ACT1* mRNA, membranes were hybridized with ^{32}P random primed labeled probes corresponding to the protein coding region at 42°C in 50% formamide, 5XSSC, 20% dextran sulfate, 1% SDS and 100µg/ml boiled salmon sperm DNA. In both cases, the membranes were washed with 0.5X SSC/0.1% SDS for 45 min at 62°C. The *GAL1* mRNA signals were quantified with a Bio-Rad Instant Imager and normalized to those obtained with double stranded DNA probes specific for actin mRNA (*ACT1)* hybridized to the same membranes.

2.2.5.3 Growth phenotypes

Yeast cultures grown to stationary phase were diluted to $1x10^7$ cells/ml and 10 fold dilutions were spotted on YEPD or selective plates and grown at 30°C for 3 days. Liquid

assays: yeast cultures were grown exponentially to OD_{600}=0,1 and OD_{600} was taken every 1h30 during 4h30.

2.2.5.4 Yeast two hybrid analysis

The bait plasmid was constructed by cloning the region corresponding to the last 180 amino acid of *MLP2* (*mlp2 C ter*) in the plasmid pOBD2 (Uetz et al., 2000). *mlp2-C ter* fragment was amplified by by PCR using the oligo, OFS947 and OFS633 and cloned into NccI and SacI restriction sites. The strain containing the bait was mated to the two hybrid library of 6000 ORFs expressed as prey fusion using 96 well plates as described in (Uetz et al., 2000).

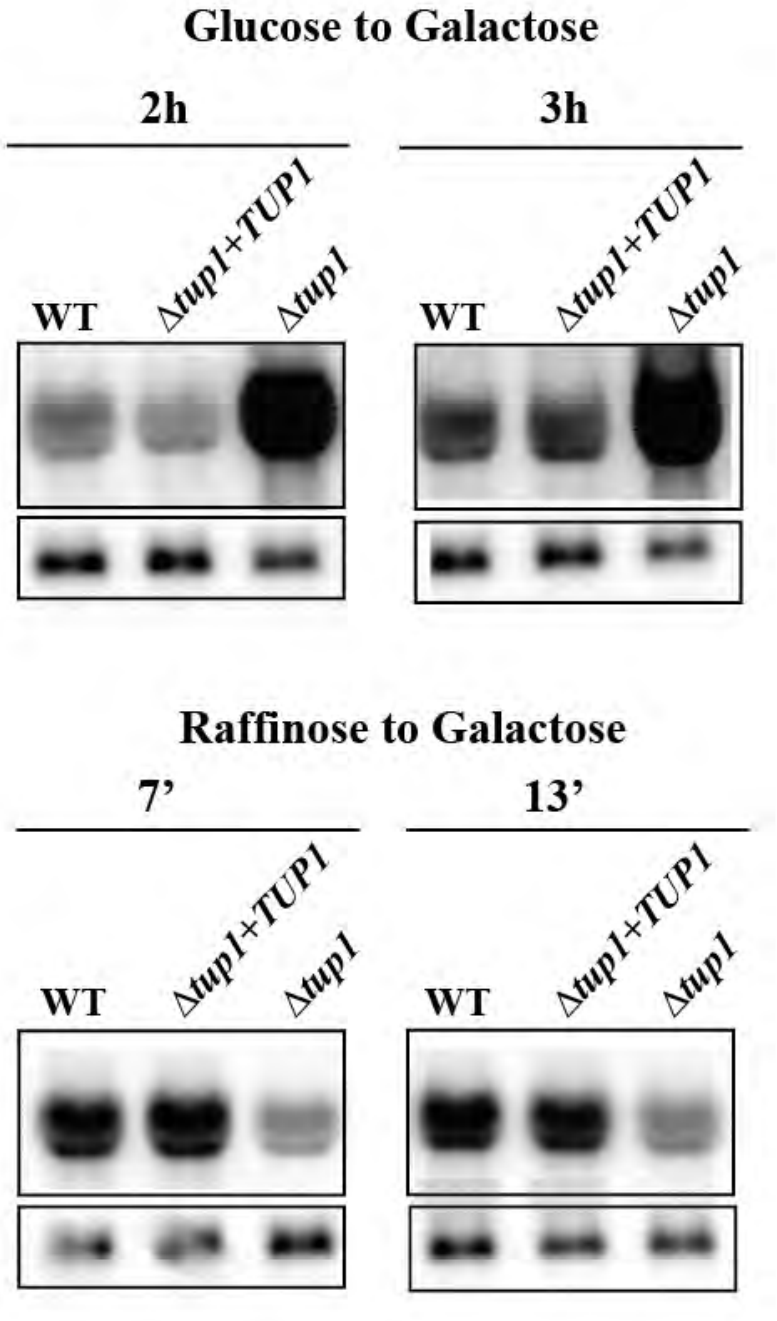
2.2.5.5 Sumoylation assay

Yeast strains were transformed with p*URA3*-Gal_{prom}-*SMT3*-His6 (His-SUMO) or YCpLac33 (*URA3/CEN*). The transformants were exponentially grown in 5ml selective medium containing raffinose 2% at 30°C and shifted in 5ml selective medium containing galactose 2% for 4 hours at 30°C. Cells were collected and resuspended at 1 OD_{600}/ml before total protein extraction.

2.2.5.6 Western blot

Total protein extraction and Western blotting were performed according to standard procedures. The anti-Tup1 antibody (a gift from C. Reese) was used at 1:20 000 dilution. The proteins were revealed with ECL Western Blotting Detection Reagents (Amersham).

2.2.6 Supplementary Data



Supplementary Figure 1. Tup1 plays a role in both glucose repression and transcriptional activation. Northern blot analysis of total RNA from WT, *Δtup1* complemented with *TUP1* on a centromeric plasmid and *Δtup1* strains when cells are shifted from glucose to galactose or raffinose to galactose for the indicated time (minutes and hours).

code	name	genotype	reference
W303	Wild type	*MAT***a** *ade2 his3 leu2 trp1 ura3*	
FSY1187	*Δmlp1/2*	*MAT***a** *ade2 his3 leu2 trp1 ura3 mlp1:HIS mlp2:HIS*	(Galy et al., 2000)
FSY3979	*Δnup60*	*MAT***a** *ade2 his3 leu2 trp1 ura3 nup60: Kan*^{r}	This study
FSY3873	*ulp1ΔN*	*MAT***a** *ade2 his3 leu2 trp1 ura3 ulp1Δ172-340-3HA.*	This study
FSY2824	*ulp1ΔN–GFP*	*MAT***a** *ade2 his3 leu2 trp1 ura3, yra1::LEU2* +p*YRA1-URA, ulp1Δ172-340-GFP*	This study
FSY2823	*ULP1-GFP*	*MAT***a** *ade2 his3 leu2 trp1 ura3, yra1::LEU2* +p*YRA1-URA, ULP1*:340-GFP	This study
FSY3872	*ULP1-HA*	*MAT***a** *ade2 his3 leu2 trp1 ura3, ULP1:340-3HA*	This study
FSY3987	*Δulp1*	*MAT***a** *ade2 his3 leu2 trp1 ura3 ulp1:HIS* + p*ULP1-URA*	This study
FSY3964	*Δmlp1/2 ulp1ΔN*	*MAT***a** *ade2 his3 leu2 trp1 ura3 mlp1:URA mlp2:HIS, ulp1Δ172-340-HA*.	This study
FSY4029	*Δmlp1/2 Δulp1*	*MAT***a** *ade2 his3 leu2 trp1 ura3 mlp1:URA mlp2:* kan^r *ulp1:HIS* + p*ULP1-TRP*	This study
FSY2553	*Δtup1*	*MAT***a** *ade2 his3 leu2 trp1 ura3 tup1*:kan^r	This study

Supplementary Table I. Strain table.

code	name	Backbone /Gene	reference
pFS3345	*ULP1-CEN*	Ycplac22/*ULP1*	(Li, 1999)
pFS3413	*NSP1-ulp1C*	*Pun100/NSP1-ulp1C-GFP*	(Panse et al., 2003)
pFS3052	*ULP1-GFP*	p*NOP1-ULP1-GFP-LEU*	(Palancade et al., 2007)
pFS3346	*Ulp1TS*	YCPlac22*/ulp1TS*	(Li, 1999)
pFS3246	*ULP1*	Ycplac111/*ULP1*	M. Hochstrasser (unpublished)
pFS3376	*TUP1*	Ycplac111/*TUP1*	This study
FSY4037	*tup1K81R*	Ycplac111/ *tup1K81R*	This study
FSY4038	*tup1K229R*	Ycplac111/ *tup1K229R*	This study
pFS3394	*tup1K270R*	Ycplac111/ *tup1K270R*	This study
pFS3011	*mlp2 Cter-bait*	pOBD2/*mlp2* (last 180 a.a)	This study

Supplementary Table II. Plasmid Table.

2.2.7 Ackowledgments

We thank M. Hochstrasser for *ULP1* and *ulp1TS* plasmid, H. Zhou for His-*SMT3* plasmid; V. Doye for *ULP1-GFP* plasmid; C. Reese for anti-Tup1 antibodies. We thank V. Panse for the *NSP1-ulp1C-GFP* plasmid construct and for valuable discussion and B. Palancade for fruitful discussions and comments. We are grateful to C. Fickensher and B. Pinheiro-Tonneau, the two technician apprentices involved in this study for their technical support.

3 General discussion

3.1 Connecting the transcription site to the nuclear pore: a process involving multiple tethers and involved in several regulations

Consistent with the growing evidences of tight interconnections between transcription, mRNP biogenesis and export steps, a number of inducible genes relocalize towards the nuclear periphery when activated (Casolari et al., 2004). It was demonstrated that the thethering of induced genes to the nuclear periphery was mediated through interactions with the NPC (Casolari et al., 2004; Taddei et al., 2006). However, analyses of the movement of these induced genes at the nuclear periphery indicated that the NPC association with activated genes might be mediated by transient or labile interactions since their sliding movements continue along the NE (Cabal et al., 2006). Our results and data from other labs revealed that the non static maintenance of genes to the NPC environement appears to involve multiple tethers, the importance of which may vary from gene to gene. I will discuss how all these interactions may fit into a model involving various steps of transcription and in turn how the NPC could promote and coordinate these events.

3.1.1 The initial recruitment of genes to the NPC is mediated by activation events

As described above in the Introduction part, the available data on NPC-gene recruitment support a model in which promoter and early transcription events are required for NPC gene recruitment (Akhtar and Gasser, 2007; Taddei, 2007). This model was in part based on the results of the Laemmli lab, which demonstrated that interaction of *GAL1* with the nucleoporin Nup2 requires the Gal4 activator but does not require the SAGA histone deacetylase co-activator complex and is not affected by inactivation of RNA polII using a thermosensitive *rpb1* mutant (Schmid et al., 2006). These observations indicated that the initial association of genes with the NPC is likely promoted by the early activating steps specific of each gene, upstream of TBP binding and transcription.

However, it was further established that the subsequent recruitment of more general activators, such as components of the SAGA complex, are necessary to make NPC-gene tethering detectable by microscopy suggesting that SAGA might immediately contribute to stabilize NPC-gene association during the activation process (Cabal et al., 2006). This

view is supported by data from a previous study, which provided a molecular insight of the physical link between genes and the NPC mediated by the SAGA complex (Rodriguez-Navarro et al., 2004). This study established that Sus1 is a component of both the SAGA complex and the mRNA export factor complex Sac3-Thp1 associated with the NPC, and that all these components co-purify in a supercomplex that was called TREX-2 (Rodriguez-Navarro et al., 2004). These data indicated that Sus1 may bridge SAGA and associated chromatin to the NPC. Consistent with this hypothesis, all TREX-2 complex components including Sus1, Thp1 and Sac3 as well as their NPC anchor, the nucleoporin Nup1, are primarily required for a detectable NPC-gene association by microscopy (Cabal et al., 2006).

More recent studies provided additional data on SAGA/Sac3-Thp1 (TREX-2) complex formation (Kohler et al., 2008; Pascual-Garcia et al., 2008). They revealed that the recruitment of Sus1 by SAGA is dependent on Sac3, and inversely that the recruitment of Sus1 on Sac3-Thp1 is dependent on SAGA formation, indicating that Sus1 recruitment to both complexes needs a tight coordination between SAGA assembly and its NPC localization (Kohler et al., 2008; Pascual-Garcia et al., 2008). These results reinforce the view that genes could reach the NPC before SAGA formation, and also suggest that SAGA and Sac3-Thp1 may interact prior to Sus1 loading. However, consistent with previous findings, loss of the Sgf73 SAGA subunit impairs TREX-2 integrity by affecting the recruitment of Sus1 on both the SAGA complex and Sac3-Thp1 and prevents NPC-gene gating (Kohler et al., 2008), confirming that Sus1 recruitment, and thus the formation of a functional TREX-2 complex, are required for a stable NPC-gene association. All together these data suggest that activation events direct gene relocalization to the NPC mainly in a two step mechanism: first, early activation events may determine initiation of gene localization to the NPC and second, the subsequent recruitment of SAGA may stabilize anchoring through TREX-2 formation at a minimum.

3.1.2 NPC-gene gating promotes transcription activation

A role of the NPC in promoting gene expression has been highlighted by several studies (Brickner and Walter, 2004; Menon, 2005; Sarma et al., 2007; Taddei et al., 2006) and (see Results Chapter 2.2). Indeed, artificial tethering of genes to the NPC promotes their expression (Brickner and Walter, 2004; Menon, 2005; Sarma et al., 2007; Taddei et al., 2006). Furthermore, we showed that loss of Mlps, which are required for an optimal NPC-gene recruitment, have a negative effect on transcription activation kinetics (See Results, Chapter 2.2), suggesting a direct role for the NPC in favoring gene activation. One mechanism by which the NPC might promote gene activation events was prososed to be mediated by the Nup84 nucleoporin complex. The emerging model is that Nup84 complex components bind to the Rap1/Gcr1/Gcr2 activation complex and facilitate its recruitment on the Rap1 controlled genes that relocate to the NPC early during transcription activation (Casolari et al., 2004; Menon, 2005). However, since we found that loss of Mlp proteins partially affect NPC-gene recruitment of activated *GAL* genes (Dieppois et al., 2006), we speculate that the two fold decrease in *GAL* gene activation kinetics we observed in the absence of Mlps may reflect a direct role for Mlp proteins in positively regulating transcription activation of NPC-recruited genes (see Results Chapter 2.2). In agreement with this view, Mlp proteins interact with gene promoter chromatin and a number of transcription regulators such as members of the SAGA complex (Luthra et al., 2007), suggesting that Mlp proteins may modulate the recruitment and/or the activity of SAGA components in the UAS of NPC-recruited genes.

Interestingly, our work indicates that the NPC may also function in regulating transcription repression (Results Chapter 2.2). In addition, we revealed that Mlp2 interacts with the general co-repressor proteins Tup1 and Ssn6, suggesting that the NPC may also function in regulating transcription repression. Indeed, our results point to a new role for the Mlps in promoting fast alleviation of transcription repression of NPC-recruited genes. We present evidence that Mlp proteins contribute to the regulation of *GAL* gene repression by confining Ulp1 SUMO-protease activity to the NPC environment, implying that Mlps may regulate the activity of transcriptional repressors at the nuclear periphery by promoting their desumoylation. Consistent with this model, we showed that one of the substrates, which sumoylation is regulated by Mlp proteins *via* Ulp1, is the co-repressor Tup1. Tup1 can be found at the NPC environment as it interacts

with Mlp2 in the two hybrid assay and associates with NPC-recruited gene promoters. Moreover, we found that desumoylation of Tup1 affects its activity, resulting in the relief of its repression activity in certain cases. Collectively, our data suggest a model in which the confinement of Ulp1 to the NPC environment might primarily alleviate the activity of those sumoylated repressors that are brought to the NPC on recruited gene promoters. Another study showed that upon activation in galactose, the phosphorylation and subsequent export in the cytoplasm of the glucose repressor Mig1, associated with the promoters of glucose-repressed genes, was also specifically mediated at the NPC, indicating a similar role for the NPC in promoting alleviation of transcriptional repression through posttranscriptional modification of repressors (Sarma et al., 2007).

Taken together these data highlight a dual role of the NPC in promoting transcription activation. On one hand, it may facilitate repression relief; on the other hand it may directly promote activation. The confinement of specific transcription modulators at the NPC may thus provide an additional topological level of transcriptional regulation which relies on genome dynamics.

3.1.3 Mex67 may contribute to NPC-gene association during transcription elongation

In contrast to the view that only transcription activation events contribute to NPC gene recruitment, the 3' UTR region of *HXK1* has been shown to be important for its anchoring, suggesting that 3' end formation might also be involved in this process (Taddei et al., 2006). Furthermore, the implication of the mRNA export factors Sac3 and Thp1 in this process, and the proposal that the NPC basket component Mlp1 interacts with chromatin through mRNA (Casolari et al., 2005), raised the possibility that physical interactions between the nascent mRNA and the export machinery associated with the pores also contribute to gene maintenance at the nuclear periphery.

Consistent with this view, the mRNA export receptor Mex67, which mediates mRNP translocation through direct interactions with the NPC, has been found to be recruited early to a variety of transcribing genes (*PMA1, HSP104, GAL10*) (Gwizdek, 2006). In the light of these observations, we speculated that Mex67 association with the growing mRNP could contribute to NPC-gene anchoring. Indeed, by using the *mex67-5* mutant under stress conditions that affect its co-transcriptional recruitment but not its interaction with the NPC, we found that Mex67 loading on transcribing genes is required

for stable association of activated *GAL* and *HSP104* genes with the pores (Dieppois et al., 2006). To address directly whether the growing mRNP contributes to NPC-gene anchoring, we examined the relocalization of a mutant *GAL2* gene lacking either the coding region alone or both the protein coding region and 3'UTR. We found that the UAS and the promoter regions, but not the coding region nor the 3' UTR, are required for *GAL2* gene repositioning. Importantly, in the absence of the protein coding region, the production of a short mRNA was still detectable but strongly impaired (10% of WT *GAL2* mRNA levels) suggesting that mRNP formation is unlikely to contribute to gene anchoring. Nonetheless, similar levels of TBP were recruited to the promoter of the WT and *Δgal2* genes indicating that, constistent with earlier findings, transcription activation rather than mRNP formation is required for NPC gene gating. In addition, we found similar levels of Mex67 recruited to *GAL2* and *Δgal2*, suggesting that the co-transcriptional recruitment of Mex67 does not depend on the production of a stable mRNA. The recruitment of Mex67 on transcribing genes in an RNA independent manner was further confirmed by ChIP experiments in presence or absence of RNAse. Therefore, our findings indicate that in addition to transcription activation events, the early co-transcriptional recruitment of Mex67 on the transcription machinery promotes gene interactions with the NPC (Dieppois et al., 2006).

A model for Mex67 co-transcriptional recruitment through direct interactions with the transcription machinery has been proposed (Gwizdek, 2006; Hobeika 2007). While Mex67 is recruited to mature mRNP through RNA binding adaptors that interact with its N-terminal domain, the recruitment of Mex67 to transcribing genes mainly involves the short C-terminal ubiquitin associated domain (UBA, last 57 amino acids). Data from our lab in collaboration with the Dargemont lab showed that the Mex67-UBA domain recognizes ubiquitylated proteins both *in vivo* and *in vitro,* and is specifically required for Mex67 co-transcriptional recruitment, indicating that Mex67 is recruited through an ineraction of its UBA domain with ubiquitylated proteins associated with the elongatind RNA PolII complex. In order to identify the ubiquitylated chromatin associated partner of Mex67, a two hybrid screen was performed using Mex67-UBA domain as a prey, which led to the identification of Hpr1 (Gwizdek, 2006), a component of the THO complex known to function in transcription elongation (Chavez et al., 2000; Piruat and Aguilera, 1998). It was further shown that Hpr1 ubiquitylation contributes to Mex67 co-

transcriptional recruitment (Gwizdek, 2006). Notably, Nahid Iglesias in our lab and the Libri lab, both observed an increased co-transcriptional recruitment of Mex67 in the *Δhpr1* mutant, indicating that Hpr1 is not the only binding partner recruiting Mex67 to the transcription machinery (Nahid personal communication and Rougemaille et al 2008). Accordingly, additional potentially ubiquitylated chromatin-associated factors have been identified as specific partners of the Mex67-UBA domain (C. Dargemont personal communication). One of them is Swd2 (Vitaliano-Prunier, 2008), a subunit of two functionally distinct complexes: the SET1 complex implicated in the methylation of the lysine 4 of histone H3 during transcription activation and CPF, the cleavage and polyadenylation factor involved in mRNA 3' end formation (Dichtl 2004). These data together suggest that Mex67 could be recruited at early steps of transcription *via* interaction with Swd2 in the SET1 complex, and coordinate subsequent steps in mRNA transcription and 3' end processing by sequential interaction with different ubiquitylated factors.

Mechanistic insights on how Mex67 selectively may bind its ubiquitylated substrates have been further provided. The proposed model is that substrates are selected by a two step mechanism: first, the C-terminal helix H4 (last 10 amino acids) of the Mex67-UBA domain acts as a switch that specifically recognizes Mex67 substrates and prevents the binding of non specific substrates; second the interaction of helix H4 with a specific substrate induces a conformational change allowing ineraction of the entire UBA domain with the ubiquitylated part of the selected substrates. Consistent with the model that Mex67 co-transcriptional recruitment is mediated by its UBA domain through specific interaction with chromatin associated proteins, removal of H4 results in a reduced Mex67 co-transcriptional recruitment (Hobeika 2007) as well as in an impaired NPC-gene recruitment (See Results Chapter 1). This last result also confirmed that the co-transcriptional recruitment of Mex67 participates in NPC-gene anchoring. The interaction of Mex67-UBA domain with components of the transcription machinery suggests that Mex67 mediates gene to pore interactions by interacting with the ongoing transcription machinery via its UBA domain, and with the NPC through its NTF2-like domain.

3.1.4 Role of Mex67 in coordinating transcription elongation events with export

The interactions of Mex67 with several factors involved in different transcription steps, such as components of the THO complex or Swd2, may suggest a role for Mex67 co-transcriptional recruitment in these processes as well. In agreement with this view, we found that the *mex67-5* mutant protein is not recruited to activated genes at the non permissive temperature and exhibits reduced mRNA levels (See Results Chapter 1), suggesting either that Mex67 promotes a proper transcription process or that the mRNA export block in *mex67-5* negatively feeds back on transcription. In addition, removal of H4, the last α helix of Mex67-UBA domain, proposed to mediate Mex67 recruitment by the transcription machinery, also led to impaired mRNA levels. Furthermore, analysis of *GAL* genes activation kinetics in the *mex67-5* mutant indicated that transcription elongation events rather than transcription initiation may be impaired in this mutant (Results Chapter 1). Since Mex67 recruitment on transcribing genes occurs during transcription elongation, these last observations are in agreement with the view that Mex67 is directly required for the process of transcription elongation.

Data from the Dargemont lab, in collaboration with Nahid in our lab, showed that the binding of Mex67 to ubiquitylated Hpr1 transiently protects Hpr1 from proteasomal degradation (Gwizdek, 2006). A role for Mex67 in transcription could therefore be, at least in part, to preserve the transcription elongation machinery integrity by protecting it from proteasomal degradation. Several indications for a role of Mex67 in transcription termination and 3' end processing have also recently been reported. First, like *tho* mutants, the *mex67-5* mutant accumulates *HSP104* mRNAs at the non permissive temperature in a dot. The formation of this dot, presumably located at the transcription site, depends, like in the *tho* mutants, on the nuclear exosome component Rrp6 (J. Camblong personal communication). These data suggest that a defect in Mex67 co-transcriptional recruitment leads to impaired mRNP co-transcriptional assembly or 3' end processing, that block the release of mRNP from the transcription site. Consistently, in addition to the observation that Mex67 interacts with the CPF subunit Swd2 (C. Dargemont personnal communication), our lab found that the cleavage/polyadenylation factor Pcf11 abnormally accumulates at the 3' end of the *HSP104* gene in the *mex67-5* mutant shifted to 37°C (data not shown). Moreover, the same *mex67-5* mutant also

displays cleavage and polyadenylation defects, either hyperadenylation or readthrough (Hilleren and Parker, 2001) and (T Jensen personal communication). Importantly, Mex67 interacts both genetically and directly with the mRNA binding and export protein Nab2 ((Green et al., 2002) and N. Iglesias personal communication), which is recruited co-transcriptionally (N. Iglesias unpublished data) and involved in mRNA polyA tail length control (Hector et al., 2002). It has been proposed that Mex67 loading on mRNP coincides with Npl3 dephosphorylation by Glc7 which occurs upon the dissociation of the cleavage/polyadenylation complex from the mRNP (Gilbert and Guthrie, 2004). These data indicate that Mex67 recruitment on mRNP occurs only once the transcripts are properly terminated and suggest that Mex67 loading is directly involved in the transition between 3' end processing and export. Altogether, these findings reveal a possible important function of Mex67 co-transcriptional recruitment in coordinating different transcription steps with 3' end processing and export.

As one function of Mex67 early co-transcriptional recruitment is to mediate NPC anchoring of active genes, the role of Mex67 in transcription elongation and mRNA processing could be an indirect consequence of the NPC-gene gating function. In order to address this issue, we artificially anchored *GAL* genes at the nuclear periphery of *mex67-5* strain and analysed their transcription levels (See Results Chapter 1). We found that restoring the peripheral localization of *GAL* genes in *mex67-5* did not rescue their mRNA levels, indicating that Mex67 may indeed play a direct role in transcription elongation and mRNP processing.

3.1.5 A new function for the Sus1-Sac3-Thp1 complex in stabilizing NPC-gene interactions during transcription elongation and in coordinating mRNP biogenesis events

3.1.5.1 The Sus1-Sac3-Thp1 complex may function with Mex67 in stabilizing NPC-gene association during transcription elongation.

It has been recently reported that Sus1, which is required for TREX-2 formation during transcription activation and NPC-gene anchoring, is loaded on the elongating transcription machinery through interaction with the phosphorylated RNA PolII CTD (Pascual-Garcia et al., 2008). In addition, Sus1 co-transcriptional recruitment on the elongation machinery depends on its interaction with the SAGA and Sac3-Thp1 complexes during transcription activation, suggesting that it is first recruited early *via* the TREX-2 complex and then transferred to the elongation machinery. Sus1 may still be attached to the Sac3-Thp1 NPC-associated complex when loaded onto the elongation machinery and, as Mex67, may contribute to NPC-gene association during transcription elongation. Sus1 and Mex67 may therefore act in concert to mediate gene to NPC interactions. It has been recently described that Sus1 co-purifies with the export factors Yra1 and Mex67 in a RNA PolII CTD phosphorylation dependent manner, suggesting that Sus1 contacts Mex67 and Yra1 over the coding region during transcription elongation. In addition, Sus1 participates in Mex67-NPC localization (Pascual-Garcia et al., 2008). Collectively these observations suggest that Sus1 and Mex67 may act in two partially redundant pathways to anchor genes at the NPC during transcription elongation.

3.1.5.2 The Sus1-Sac3-Thp1 complex is important for coordinating transcription, mRNP co-transcriptional processing and export.

Notably, absence of Sus1 also leads to reduced RNA PolII processivity (Pascual-Garcia et al., 2008), indicating that Sus1 may also play an active role in transcription elongation, and possibly in linking mRNA co-transcriptional processing and export. Indeed, loss of Sus1 results in the accumulation Poly(A)+ RNA in the nucleus (Rodriguez-Navarro et al., 2004). In addition, loss of the SAGA component Sgf73, which impairs loading of Sus1 on chromatin, results in mRNA retention within a dot at the transcription site (Kohler et al., 2008). These indications strongly suggest that, as for the THO complex and Mex67, Sus1 might contribute to promote mRNP release from the transcription site by coordinating mRNA co-transcriptional assembly, 3’ end processing

and export. Consistent with this hypothesis and the view that Sus1 remains associated with the NPC during transcription elongation, another recent study from the Aguilera lab (Gonzalez-Aguilera et al., 2008) demonstrated that mutations in any component of the Sus1-Sac3-Thp1 NPC-associated complex are suppressed by overexpression of the RNA helicase Sub2, which is thought to play a major role in triggering mRNP remodeling during 3' end processing (See Introduction and below). Furthermore, this study also noticed defects in transcription elongation as well as enhanced transcription-dependent hyper-recombination in mutants of all the components of this complex, and reported that these phenotypes were dependent on mRNA formation. In the light of these findings, integrity of the Sus1-Sac3-Thp1 NPC associated complex, rather than Sus1 alone, might be involved in ensuring substained transcription elongation and genetic stability during transcription. One view is that mutants of this complex accumulate improperly processed mRNAs that negatively feedback on transcription elongation and impair gene integrity. Collectively these informations suggest that the loading of Sus1 on chromatin and on Sac3-Thp1 during transcription activation is important for NPC gene gating and for the regulation of important processes underlying mRNP release from the transcription site.

Since Sus1 interacts with Yra1 and Mex67 during transcription, Sus1 could be, at least in part, involved in the co-transcriptional recruitment of Yra1 and Mex67 to the transcription machinery and /or the mRNP. It would therefore be interesting to test the co-transcriptional recruitment of these two export factors in the absence of Sus1 or other members of the TREX-2 complex.

All these findings suggest that the fraction of Sus1 associated with the NPC through Sac3-Thp1 mediates gene to NPC interactions during all transcription events. In turn NPC-gene gating might play an important role in the coordination of transcription steps as it may allow the loading of Sus1 on the SAGA and Sac3-Thp1 which are then involved in the regulation of the transcription events required for proper mRNP release and subsequent export.

3.1.6 NPC-gene anchoring during mRNP 3' end processing may act as a control mechanism preventing the export of improperly terminated mRNAs

3.1.6.1 3' end processing events may vary from gene to gene and participate in NPC-gene anchoring *via* an RNA dependent mechanism.

Consistent with a role of Sus1 and Mex67 in coordinating NPC-gene anchoring with transcription termination events, the 3' UTR sequence of the *HXK1* gene was shown to be involved in NPC-gene anchoring (Taddei et al., 2006). In contrast, we observed that the 3' UTR sequence of *GAL2* does not impair NPC gene anchoring (See Results Chapter 1), indicating that the importance of the 3'UTR signals in NPC-gene association may vary from gene to gene. Consistent with this idea, experimental evidences from the Rosbash lab indicate that different 3'UTR sequences differentially affect the retention of genes at the NPC after transcription repression (Abruzzi et al., 2006). In this study, 3' UTR sequences were shown to play a major role in the retention of the gene at the nuclear periphery after transcription, rather than during the active transcription process. It was shown that some reporters that contain a WT 3′ UTR, generating properly cleaved and adenylated RNAs such as the *GAL1* 3'UTR sequence, accumulate in nuclear dots whereas others exibit diffuse signals. The localization of mRNAs in dots for some genes indicates reduced diffusion compared to most nuclear RNAs, presumably due to a tether that restrains the RNA from moving away from its transcription site. Based on the correlation between the RNA dots stability and the retention of genes at the nuclear periphery after transcription shut off, a model has been proposed in which this mRNA dot acts as a transcription independent tether of the gene to the NPC (Abruzzi et al., 2006). In agreement with these views, deletion of *SUS1*, *SAC3*, and *THP1* leads to both the dissociation of the mRNA dots, produced by reporter genes having *GAL1* 3' UTR sequence, from the transcription site, and the loss of post-transcriptional retention of the loci at the nuclear rim (Chekanova et al., 2008). These observations suggest a role for the Sus1-Sac3-Thp1 complex in controlling the confinment of mRNP at the transcription site and extend the link between RNA dots and NPC-gene association. This last function of the Sus1-Sac3-Thp1 complex perfectly matches with its role in coordinating mRNP biogenesis, release and export (See previous section).

Although evidence suggests that transcription activation is the cause of gene to pore interactions, and that transcription is not required to establish gene-NPC localization, these studies suggest that the NPC-gene gating is also linked with mRNA biogenesis, processing and export. A model proposing multiple, partially redundant tethering mechanisms that work in concert to stabilize NPC-gene association would explain the relevance of the variety of factors involved in NPC-gene gating (Figure 22).

Along the same idea, the involvement of proteins such as the Sus1-Sac3-Thp1 complex or Mex67 in the stabilization of NPC-gene association could either be direct during their early loading at transcription activation and elongation, or indirect reflecting their role in coordinating mRNP assembly, 3' end processing and export, or more probably a combination of both.

3.1.6.2 NPC-gene retention may participate in surveillance and reflect the link between 3' end processing and export

The retention of mRNA in dots is usually reminiscent of inefficient 3' end processing in a process dependent on nuclear exosome (Hilleren et al., 2001; Libri et al., 2002; Zenklusen et al., 2002). Although, the formation of the WT *GAL1* mRNP dot is independent on the nuclear exosome component Rrp6, it has been recently further demonstrated that its stability is drastically decreased in nuclear exosome mutants, indicating the exosome may act directly to generate or stabilize dot RNA of certain WT genes (Vodala et al., 2008). As a mechanism for this, it has been proposed that the nuclear exosome inhibits poly(A) tail addition on certain transcripts, which may block the release of RNA from the transcription machinery and result in an increased retention of RNA in the dots. In addition, posttranscriptional *GAL1* gene-NPC tethering depends on two nuclear exosome components Rrp6 and Lpr1, indicating that the mRNAs that are sequestered at the transcription site by the nuclear exosome bridge the locus and the NPC (Vodala et al., 2008). All together these data suggest that some genes contain 3' UTR sequences that direct less efficient or more controlled 3' end processing events, delaying mRNP release and maintaining the gene at the nuclear pore. All these findings suggest that NPC-gene localization during 3' end processing may reflect an additional control mechanism preventing improperly terminated mRNA from being translocated through the NPC.

According to these observations, other dot forming mutants may display similar phenotypes because they affect cleavage or polyadenylation or because they present inappropriate mRNP substrate for this machinery. By investigating the role of THO/Sub2 complex in mRNP biogenesis, the Libri lab, in a study in which I participated, proposed molecular insights for the mechanism of retention of unprocessed mRNP at the transcription site in *tho* mutants (Rougemaille et al., 2008). Consistent with the previous hypothesis, they found that this retention occurs during 3' end processing in association with the NPC. ChIP experiments revealed that a large protein complex sequesters the 3' end DNA regions of several heat shock genes upon transcriptional induction in *tho* and *sub2* mutants. This complex contains Mex67 and factors such as Rna14 and Pcf11 that are required both for 3' end formation and transcription termination, indicating an increased residency of these factors in a complex bound close to the 3' end of these genes in *tho* mutants. This suggests that in THO/Sub2 mutants, 3' end processing and export factors have failed to be released from the mature mRNP or the transcribing polymerase, and are still associated with the transcription site. Interestingly, preventing transcription termination using *rna14*, *rna15* or *pcf11* mutants abolished the formation of the complex in *tho* mutants, which instead persisted using a *pap1* mutant that is termination proficient but defective for polyadenylation. These observations indicated that 3' end processing factors are required for the formation of the complex, suggesting that defects in THO complex block mRNP release and export at a step following 3' end processing complex formation. Together these results support a model, in which THO and Sub2 are involved in mRNP remodeling events triggering the release of mRNP from the transcription site. As one function of the THO complex might be to direct the co-transcriptional loading of Sub2 on the nascent mRNA, the formation of the 3' end complex and retention of mRNP at the transcription site in the *tho* mutants could be due to absence of Sub2 on the mRNP. Indeed, one view is that the ATP dependent helicase activity of Sub2 during 3' end processing promotes remodeling events that trigger the release of the mRNP from the transcription site.

Notably, nucleoporins were also crosslinked with the 3' end stalled complex observed in *tho* mutants, indicating that this process occurs at the NPC. Furthermore, localization experiments of the *HSP104* heat shock gene in the *Δhpr1* mutant of the THO complex confirmed that the gene, which is normally undetectable at the nuclear periphery, is maintained at the nuclear rim in *tho* mutants. The association of the *HSP104*

locus at the nuclear periphery upon a 3' end processing defect might reflect that this gene is transiently located at the NPC during its activation, but detectable at the nuclear periphery only when blocked there. This is consistent with the previous observation of gene maintenance at the NPC upon mRNA retention at the transcription site (Abruzzi et al., 2006; Vodala et al., 2008). In *tho* mutants, the loci might be tethered at the periphery though several redundant possible mechanisms involving Mex67, Sus1 and the bulk of mRNP. All these data highlight a crucial role of 3' end processing in giving the licence for export. As the coordination of mRNP release and export occur at the NPC for the studied genes, one can propose a role for the NPC in these processes.

3.1.7 Mlp proteins might be involved in the stabilization of NPC gene anchoring

In addition to Mex67, we found that Mlp1 is required for a stable association of the activated genes with the NPC (Dieppois et al., 2006). However, the participation of Mlp proteins in this mechanism is controversial (Cabal et al., 2006). Mlp proteins form large filamentous structures that are likely to contact various proteins involved in gene expression when genes relocate to the NPC. Indeed, it has been shown that Mlp proteins interact with *GAL* gene promoters through direct interaction with components of the SAGA complex (Luthra et al., 2007), and with the chromatin of activated genes via RNA dependent mechanisms (Casolari et al., 2005). Moreover, Mlp proteins interact directly with the RNA PolII mediator subunit Srb6 (Gavin et al., 2006; Gavin et al., 2002), with the co-transcriptionally recruited export factor Nab2 (N. Iglesias unpublished data, (Fasken et al., 2008; Green et al., 2003; Vinciguerra et al., 2005) and with the TREX-2 component Sac3 (Lei et al., 2003; Oeffinger et al., 2007). Mlp2 also associates via RNA with Yra1, Mex67, Sub2 and Cpb80 (Vinciguerra et al., 2005) that are loaded on nascent mRNA co-transcriptionally. Thus, the picture that emerges is that Mlp proteins might play a global role in stabilizing the gene–NPC association through interactions with all these factors during various gene expression steps (Figure 22). Although how Mlp proteins function in maintaining gene to NPC is not well defined, their role in gene activation and mRNP surveillance when genes are located in the NPC environment may be more tractable (See Results Chapter 2, (Vinciguerra et al., 2005)).

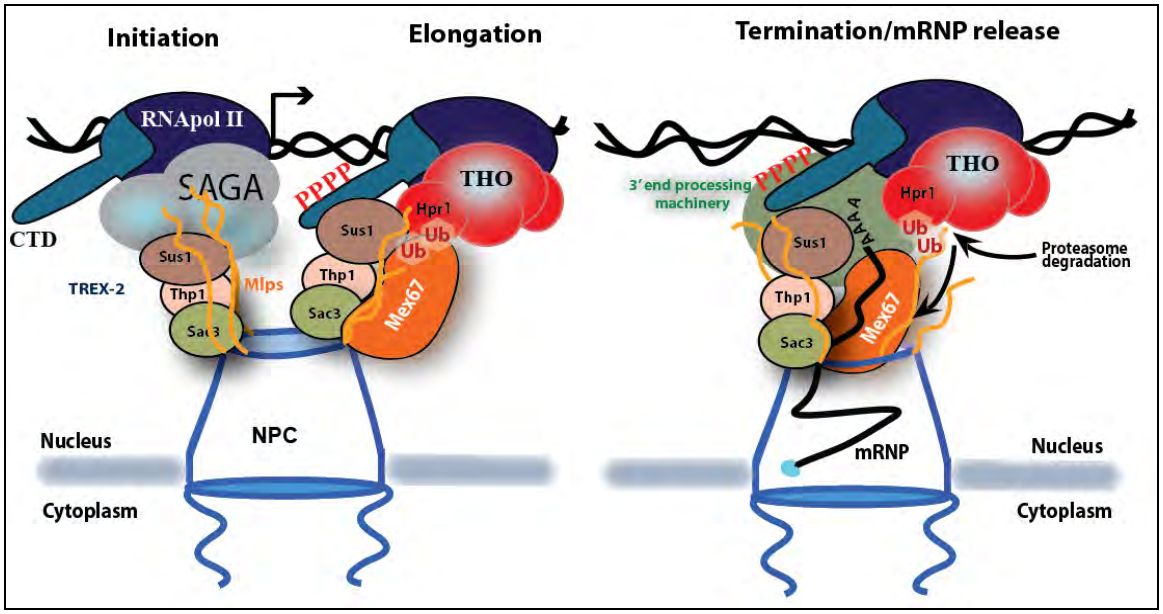


Figure 22. Model: Mex67 and Sus1 co-transcriptional recruitment on active genes contributes to their anchoring to nuclear pores. Based on our results and the above observations, we currently think that active gene association to the NPC is stabilized by TREX-2 during activation and by the early recruitment of Mex67 and Sus1 *via* the transcription machinery during transcription elongation and termination. Mex67 and Sus1 are recruited on the transcription machinery in a manner independent of the nascent mRNP and mediate interaction with the NPC. Sus1 is recruited *via* the RNA PolII CTD and Mex67 is recruited at least, in part, via interaction with ubiquitylated Hpr1. Sus1 and Mex67 coordinate 3' end processing events and may be recruited to the mRNP following 3' end processing. One other possible role of the interaction of Mex67 with ubiquitylated components of the elongation machinery could be to protect them from degradation during the course of transcription. At the end of each transcription round the release of Mex67 from the transcription machinery may allow proteasome degradation of some elongation factors, facilitating the recycling of the transcription machinery. Mlp1 could play a role in promoting and stabilizing the anchoring of activated genes to the NPC, possibly by interaction with components of the pre-initiation complex, as well as with transcription, mRNP processing and export factors.

3.2 *GAL* gene transcription "memory" and underlying mechanisms

3.2.1 Controversy about the role of NPC-gene gating in regulating GAL gene memory

Transcriptional induction of the yeast *GAL* genes exhibits "memory" of the preceding transcriptional state (Acar et al., 2005; Brickner, 2007; Kundu et al., 2007) and (Results Chapter 1). Specifically, the rate of transcriptional induction of a naïve gene is slower than for *GAL* genes that were previously transcribed. This ability to reinduce *GAL* genes with fast kinetics survives during several cell divisions up to twelve hours of repression (Results Chapter 1 and data not shown), (Zacharioudakis et al., 2007). A study from the Brickner lab (Brickner et al., 2007) correlated the *GAL* gene "memory" with their ability to remain associated with the NPC after transcription shut off. It revealed that *GAL* genes stay associated with the NPC after transcription during several rounds of replication and that disrupting this association using the *Δnup2* mutant abolishes *GAL* gene fast re-induction, suggesting that NPC recruitment establishes epigenetic modifications that confer transcriptional "memory". Indeed it was found that both *GAL* gene retention at the nuclear periphery and optimal re-activation require incorporation of the histone variant Htz1 at promoter nucleosomes. In the light of these findings, it was proposed that NPC-gene gating during transcription activation promotes Htz1 incorporation into promoter nucleosomes, which then retains the gene at the NPC and promotes fast re-activation through its own action on chromatin or through the maintenance of genes in the NPC optimal environment for transcription (Brickner, 2007). However, our results and results from other labs contradict some of these data that are still a subject of debate. Other mentioned above studies (Abruzzi et al., 2006; Vodala 2008) and our results (See Results Chapter 1), showed that *GAL* genes are indeed retained at the NPC after transcription shut off, but were not maintained more than 2-3 hours before going back to the center. Moreover, it has been shown that this retention is likely to be mediated by post-transcriptional mRNP (Abruzzi et al., 2006; Vodala 2008). In addition, the involvement of Nup2 in a stable *GAL* gene-NPC association is not consensual, as for instance Δ*nup2* deletion in our genetic background does not affect NPC-gene recruitment (data not shown). Finally, we showed that disrupting NPC-*GAL* gene localization by using a *mex67-5* mutant or using a deletion of *MLP1,* which in our

genetic background results in a reduced *GAL* gene-NPC localization, does not affect the ability of *GAL* genes to be re-induced faster after the first activation. All these data argue against the proposed mechanism of *GAL* genes transcription "memory" involving NPC-gene gating.

3.2.2 Proposed mechanisms for GAL gene transcription "memory"

The persistence of the *GAL* gene transcriptional "memory" phenotype through several rounds of cell divisions indicates that it might rely on the establishment during the first activation of an epigenetic state inherited by daughter cells. One other study investigated this hypothesis and found that transcriptional memory requires the ATP-dependent chromatin remodeling enzymes SWI/SNF complex, which is involved in chromatin opening (Kundu et al., 2007). Because loss of ISWI-like chromatin remodeling enzymes restores "memory" of *GAL1* in the absence of SWI/SNF, and because SWI/SNF and ISWI-like enzymes are thought to have opposing effects transcription, it has been proposed that the SWI/SNF complex chromatin remodeling activity antagonizes the activity of the ISWI-like enzymes to establish a chromatin structure at *GAL1* promoter permissive for rapid reinduction (Kundu et al., 2007). These data together with a role of Htz1 incorporation in promoting *GAL* gene "memory" indicate that chromatin-based mechanisms regulate the rate of *GAL* gene transcriptional reinduction after glucose repression.

Because the structural *GAL* genes (*GAL1*, *GAL7*, *GAL10*, *GAL2*) are regulated by multiple nested feedback loops, the role of Gal regulatory cytoplasmic determinants in the "memory" phenomenon has also been investigated. Galactose enters the cells through specific sugar transporter such as Gal2 and binds to the protein Gal3. The complex in turn binds and sequesters Gal80, the inhibitor that covers the Gal4 activating region. Once freed from Gal80, Gal4 activates transcription of various *GAL* genes including *GAL2*, *GAL3* and *GAL80* which results in positive (Gal2 and Gal3) and negative (Gal80) feedback loops. To pinpoint the network of interactions which could be responsible for the "memory" in *GAL* gene regulation, the three feedback loops were systematically interrupted in strains where *GAL1* gene promoter activity was monitored by fusion with a fluorescent reporter gene (Acar et al., 2005). It has been found that *GAL1* "remembers" whether it was previously exposed to high or low concentrations of galactose and that this

memory phenomenon involves the positive regulator Gal3, which expression is positively influenced by galactose concentration during the first round of induction. These observations suggested that the cytoplasmique inheritance of *GAL* regulatory factors were the principle determinant of *GAL* gene "memory" (Acar et al., 2005). Susbequently, the use of heterokaryons resulting from mating between cells that have undergone galactose induction followed by several divisions on glucose and "naïve" cells, confirmed that the memory ability was principally transmitted through the cytoplasm (Zacharioudakis et al., 2007). However in this last study, it was found that rapid reactivation of *GAL* gene transcription depends on a Gal3-like function of Gal1 protein (Bhat and Hopper, 1992; Platt and Reece, 1998; Platt et al., 2000; Zacharioudakis et al., 2007) instead of Gal3 itself. Gal3 display a stronger Gal80 inhibitor activity than Gal1 but is produced at a much lower level than Gal1 and thus, may be more quickly diluted out than Gal1 as the cells divide. One view to reconcile the two studies (Acar et al., 2005; Zacharioudakis et al., 2007) is that the predominant effects of Gal1 and Gal3 might be seen after different number of cell divisions. After a reduced number of cell divisions, Gal3 which displays a stronger Gal80 inhibitor activity than Gal1 might be predominantly required for the fast re-induction, whereas, after a bigger number of cell divisions Gal1 levels might be higher than Gal3 levels and therefore, Gal1 would be responsible for the *GAL* gene "memory" phenomenon.

The effect of chromatin modifiers and histone Htz1 deposition on *GAL* genes transcription memory could be indirect through their effect on the regulation of Gal1 and Gal3 expression levels during the first round of activation. Indeed, loss of Htz1 drastically slows down transcription activation of *GAL* genes (Gligoris et al., 2007) and (data not shown). However, SWI/SNF does not affect *GAL3* or *GAL1* expression significantly during the first round of galactose induction (Kundu et al., 2007) suggesting that the rates of transcriptional re-induction of *GAL* genes are controlled by both a cytoplasmic mechanism and this chromatin based mechanism.

4 Annexes

During the course of this PhD, I participated to one other research project which is not included in this thesis.

Anti-sense RNA stabilization induces transcriptional gene silencing *via* histone deacetylation in *S. cerevisiae*.

J Camblong, N Iglesias, C Fickentscher, G Dieppois, and F Stutz.

Cell. 2007 Nov 16;131(4):649-51.

Cell

Antisense RNA Stabilization Induces Transcriptional Gene Silencing via Histone Deacetylation in *S. cerevisiae*

Jurgi Camblong,[1] Nahid Iglesias,[1] Céline Fickentscher,[1] Guennaelle Dieppois,[1] and Françoise Stutz[1,*]
[1]Department of Cell Biology, Sciences III, University of Geneva, 30 Quai E. Ansermet, 1211 Geneva 4, Switzerland
*Correspondence: francoise.stutz@cellbio.unige.ch
DOI 10.1016/j.cell.2007.09.014

SUMMARY

Genome-wide studies in *S. cerevisiae* reveal that the transcriptome includes numerous antisense RNAs as well as intergenic transcripts regulated by the exosome component Rrp6. We observed that upon the loss of Rrp6 function, two *PHO84* antisense transcripts are stabilized, and *PHO84* gene transcription is repressed. Interestingly, the same phenotype is observed in wild-type cells during chronological aging. Epistasis and chromatin immunoprecipitation experiments indicate that the loss of Rrp6 function is paralleled by the recruitment of Hda1 histone deacetylase to *PHO84* and neighboring genes. However, histone deacetylation is restricted to *PHO84*, suggesting that Hda1 activity depends on antisense RNA. Accordingly, the knockdown of antisense production prevents *PHO84* gene repression, even in the absence of Rrp6. Together, our data indicate that the stabilization of antisense transcripts results in *PHO84* gene repression via a mechanism distinct from transcription interference and that the modulation of Rrp6 function contributes to gene regulation by inducing RNA-dependent epigenetic modifications.

INTRODUCTION

Recent studies in various organisms, from yeast to mammals, provide evidence that genomes are transcribed on a scale larger than previously assumed (Johnson et al., 2005). In the yeast *S. cerevisiae*, genome-wide studies reveal that many protein coding sequences also encode antisense transcripts and that a large fraction of intergenic regions are transcribed as cryptic unstable transcripts (CUTs) subjected to degradation by the nuclear exosome component Rrp6 (David et al., 2006; Davis and Ares, 2006; Wyers et al., 2005). The nuclear exosome is a complex of 3′ to 5′ exonucleases implicated in RNA processing and quality control (Houseley et al., 2006). Detailed studies show that the degradation of CUTs implicates TRAMP, a complex containing the poly(A) polymerase Trf4, which polyadenylates intergenic transcripts and promotes their degradation by facilitating the recruitment of Rrp6 (LaCava et al., 2005; Vanacova et al., 2005; Wyers et al., 2005).

Whether this widespread and unstable transcriptome just reflects nonspecific RNA polymerase II activity or participates in the control of gene expression is still unclear. In *S. cerevisiae*, the *SER3* and *IME4* genes are regulated by a mechanism of transcription interference. The *SER3* promoter encodes a short *SRG1* RNA whose transcription competes with the binding of activators to the downstream *SER3* promoter, thereby repressing *SER3* transcription (Martens et al., 2004). In the case of the *IME4* gene, antisense transcription interferes with sense transcription (Hongay et al., 2006). For both *IME4* and *SER3*, noncoding RNA transcription prevents gene expression via a *cis*-acting mechanism distinct from the RNAi-mediated gene silencing described in other eukaryotes.

In fission yeast and higher eukaryotes, short double-stranded interfering RNAs (siRNAs) have been implicated in transcriptional gene silencing (TGS), a process mediated by components of the RNA interference (RNAi) machinery. In *S. pombe*, TGS is promoted by the RNA-mediated cotranscriptional recruitment of the RNA-induced transcriptional silencing (RITS) complex. The subsequent association of chromatin-modifying activities including histone methyl transferases and histone deacetylases then results in heterochromatin formation and gene silencing (Almeida and Allshire, 2005; Moazed et al., 2006; Volpe et al., 2002).

In *S. cerevisiae,* components of the RNAi machinery are absent, and gene repression can be mediated by histone deacetylation. Histone acetylation and deacetylation occur through both targeted and global mechanisms. In the targeted mechanism, histone acetyltransferases (HATs) and histone deacetylases (HDACs) are recruited to promoters by specific transcriptional activators and repressors, respectively. In the global mechanism, HATs and HDACs modify histones through large chromatin domains, including coding sequences (Kurdistani and Grunstein, 2003). Yeast expresses at least three classes of HDACs. Hda1 is an evolutionary conserved class II HDAC

that functions in a complex with the Hda2 and Hda3 cofactors to deacetylate mainly histone H2B and H3 (Wu et al., 2001a; Wu et al., 2001b). In the absence of Hda1, intergenic regions close to promoters as well as Hda1-affected subtelomeric (HAST) domains become enriched in histone H3 acetylated at position Lys18 (H3K18), resulting in increased gene expression (Robyr et al., 2002).

In this study, we found that *PHO84* gene transcription is repressed in the absence of Rrp6, indicating a role for the exosome in efficient gene transcription. *PHO84* is also downregulated during aging in wild-type (WT) cells, suggesting a decrease in Rrp6 function over time. In both conditions, the loss of Rrp6 function leads to the stabilization of *PHO84* antisense transcripts and the subsequent inhibition of *PHO84* gene transcription. The data support that *PHO84* repression is not due to transcription interference but results from antisense RNA-induced histone deacetylation by the Hda1/2/3 complex.

RESULTS

The Progeny of Chronologically Aged Yeast Cells Inherits a *PHO84* Gene Silent State

While studying *PHO84* gene expression, we serendipitously discovered that *PHO84* messenger RNA (mRNA) levels progressively decrease during chronological aging. Yeast cells are chronologically aged by maintaining fresh cells for a certain number of days on plates at 4°C prior to 24 hr exponential growth in minimum medium at 25°C, RNA extraction, and northern blotting (Figures 1A and 1B). *PHO84* mRNA levels gradually decrease with time and are as much as 20-fold lower in the progeny of cells kept on plates for 10 days compared to those kept on plates for only 3 days (Figure 1C). The low levels of RNA polymerase II (RNA Pol II) bound to *PHO84* 5′ and 3′ ends in aged cells indicate that *PHO84* transcription is repressed over time (Figure 1D). Although the decrease in *PHO84* mRNA levels occurs faster in cells kept on plates at room temperature (data not shown), the slow and progressive decrease at 4°C allowed us to identify the order of events underlying the repression process (see below).

The analysis of the *PHO8* and *AAH1* genes showed that the repression is specific to *PHO84* under these conditions (Figure 2A). Indeed, *PHO8* and *AAH1* expression, regulated like *PHO84* by phosphate and glucose levels, respectively (Escusa et al., 2006; Ogawa et al., 2000), is unaffected in the progeny of older cells. Moreover, the expression of the two genes adjacent to *PHO84*, *GTR1* and *TUB3*, is unaltered, indicating that the effect on *PHO84* transcription is localized (Figures 1A and 2A).

To verify that *PHO84* gene repression is due to chronological aging, we maintained cells in stationary liquid cultures for 1–3 days before exponential growth and RNA analysis. The data confirmed that the progeny of cells maintained in stationary phase for longer time inherits a lower *PHO84* expression level (Figure 2B). Furthermore, the swapping of the media of young and old cell cultures did not invert *PHO84* mRNA levels, indicating that *PHO84* repression is not due to the secretion or depletion of some metabolite in the culture medium (Figure 2C). Finally, this low *PHO84* mRNA level is not due to acquisition of mutations during aging because the silent *PHO84* state can be reversed by the streaking of cells on a fresh YEPD plate (Figure 2D). Overall, these data suggest that if grown in minimum medium, the progeny of aged cells inherits a transcriptional silent state of the *PHO84* gene.

Interestingly, the decrease in *PHO84* expression with aging is paralleled by the appearance of a longer transcript (Figure 1C). This RNA is also detected with a probe specific for the short hypothetical open reading frame (ORF) *YML122C* upstream of *PHO84*, indicating that the long transcript spans both *YML122C* and *PHO84* sequences (Figures 1A and 1C). To address the potential role of this transcript in *PHO84* repression, we further characterized its origin and orientation. We generated sense and antisense SP6 riboprobes and found that not one but two antisense transcripts appear over time (Figure 1C). The longer one corresponds to a *PHO84-YML122* chimeric RNA, whereas the shorter one is similar in length to the *PHO84* sense mRNA (Figure 1A). 5′ RACE experiments identified several antisense transcript start sites located between 20 and 80 bp downstream of the *PHO84* stop codon (Figure S1A in the Supplemental Data available with this article online).

The Stabilization of *PHO84* Antisense Transcripts Is Necessary to Promote *PHO84* Gene Repression

The decrease in *PHO84* gene expression upon appearance of antisense transcripts raises the possibility that antisense RNAs participate in *PHO84* repression. To define whether antisense RNA accumulation is the cause or the consequence of *PHO84* gene repression, we attempted either to increase the amount of antisense RNAs without increasing their transcription or to abolish *PHO84* gene transcription. The first aim was achieved by the loss of the exosome component Rrp6, resulting in strong stabilization of the antisense transcripts and accelerated gene repression (Figure 3A). The comparison of sense and antisense *PHO84* transcripts in *Δrrp6* cells kept on plates for 3 or 6 days indicates that the accumulation of antisense transcripts precedes *PHO84* downregulation. Next, to confirm that the stabilization of *PHO84* antisense transcripts is not secondary to a decrease in sense transcription, we took advantage of the *Δhpr1* strain, in which we found that *PHO84* transcription initiation is almost totally abrogated. Hpr1, previously described as being required for transcription elongation (Chavez et al., 2000; Voynov et al., 2006), appears to participate as well in *PHO84* gene transcription initiation. Indeed, virtually no TATA-binding protein (TBP) or RNA Pol II is associated with the *PHO84* promoter and coding regions compared to the WT, and no sense RNAs can be detected (Figures 3B and 3C). The absence of *PHO84* transcription in *Δhpr1* cells is not accompanied by an increase in antisense levels (Figure 3C). Similarly, loss of the Pho4 transcription factor completely inhibits sense transcription without affecting

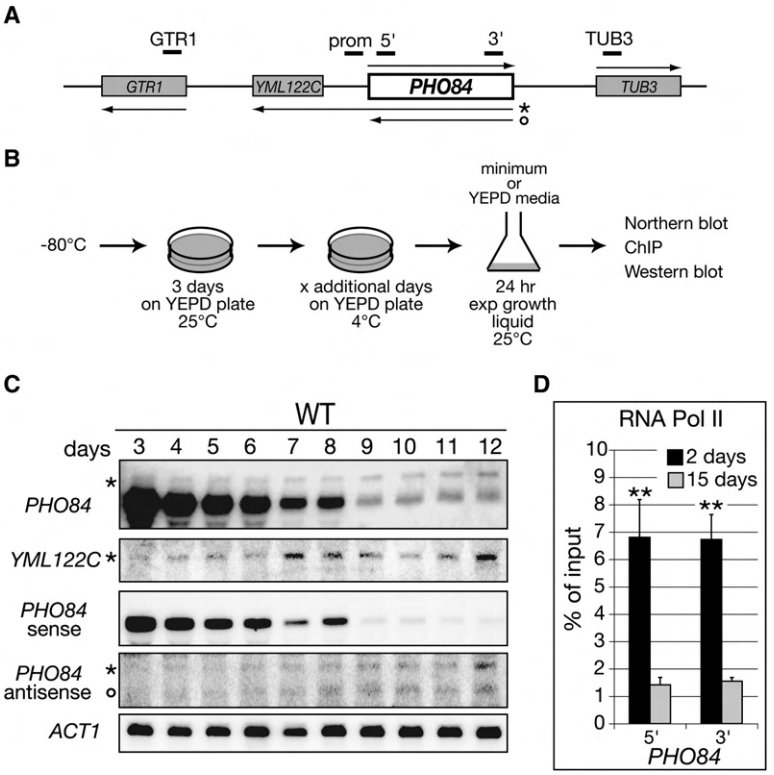


Figure 1. The *PHO84* Gene is Downregulated in the Progeny of Wild-Type Aging Cells

(A) Map of the *PHO84* genomic locus. Arrows indicate the transcription direction of the *PHO84*, *GTR1*, and *TUB3* genes. Arrows below *PHO84* represent the long *PHO84+YML122C* antisense RNA ("*") and the shorter *PHO84* antisense RNA ending before *YML122C* ("○"). The bars indicate the regions amplified by real-time PCR for ChIP analyses.

(B) Scheme of strain manipulation and culturing.

(C) Northern analysis of total RNA from WT (W303) cells kept on plates for the indicated number of days before 24 hr exponential growth in minimum medium. Northern blots were probed with random primed labeled probes specific for *PHO84* or *YML122C* and single-stranded RNA probes mapping to the 3′ end of *PHO84* and specific for sense or antisense *PHO84* transcripts, marked with "*" and "○" as in (A). *ACT1* mRNA was used as loading control.

(D) ChIP analysis of RNA Pol II along *PHO84* in old versus fresh WT (W303) cells. WT (W303) cells expressing HA-tagged Rrp6 were maintained on plate for 2 and 15 days (black and gray bars, respectively) and cultivated in minimum medium for 24 hr. Immunoprecipitated DNA was quantified by real-time PCR with *PHO84*-specific primers as shown in (A). The histogram represents the average enrichment ratios (with standard deviations) of the immunoprecipitated fractions relative to whole-cell extracts. Values were obtained from three independent extracts. Statistical analyses were done with the Student's t test ("*" indicates $p < 0.05$, "**" indicates $p < 0.01$, "***" indicates $p < 0.001$, and "****" indicates $p < 0.0001$; no asterisk indicates not significant).

antisense levels (Figure 3D), confirming that antisense transcript accumulation is not the consequence of a block in sense transcription. Furthermore, antisense RNAs strongly accumulate upon the loss of Rrp6 in both mutants, demonstrating that antisense amounts depend on Rrp6 function rather than the level of sense transcription (Figures 3C and 3D). Together, these data discard a simple transcription interference mechanism and favor a process in which the stabilization of antisense transcripts participates in the repression of *PHO84* transcription.

The establishment of low *PHO84* expression levels over time could either be due to a complex signaling cascade or specifically to antisense accumulation caused by a decrease in Rrp6 function. To address this question, we looked for a condition in which *PHO84* antisense transcripts do not appear over time, thus allowing us to test the effect of *RRP6* disruption alone. When cells are grown in a YEPD rich medium, *PHO84* antisense transcripts never accumulate and mRNA levels are not reduced in the progeny of old WT cells (Figure 4A). In contrast, *PHO84* mRNA levels are strongly reduced in *Δrrp6* cells under these conditions. Interestingly, although antisense transcripts are revealed in *Δrrp6* cells, the repression of *PHO84* still depends on the time mother cells spend on plates before inoculation, indicating that if antisense transcript accumulation is a primary event, additional time-dependent steps are required for the silencing of *PHO84* (Figures 3A and 4A). To confirm that this phenotype is not specific to W303, we performed the same experiment in the MCY829 background. The loss of Rrp6 similarly stabilizes antisense RNAs and results in *PHO84* repression, but these changes occur faster and are detected already after 2 days on plates in this strain (Figures 4B and 4C).

Because *PHO84* antisense transcripts are polyadenylated (Figure 4B), we tested whether the Trf4 poly(A) polymerase, implicated in the polyadenylation of Rrp6 targets (LaCava et al., 2005; Wyers et al., 2005), is involved in the degradation of *PHO84* antisense RNAs. The loss of Trf4 also results in increased *PHO84* antisense and decreased *PHO84* sense RNA levels (Figure 4D). Note that antisense transcript levels in *Δtrf4* are weaker than in *Δrrp6*, possibly reflecting the activity of the redundant Trf5 poly(A) polymerase (LaCava et al., 2005). Nonetheless, *PHO84* becomes repressed in *Δtrf4*, indicating that *PHO84* silencing can be established in the presence of limited amounts of antisense RNA. Thus, Trf4 is likely to restrict antisense transcript accumulation and to maintain *PHO84* sense mRNA production in cooperation with Rrp6. All together, these data provide additional evidence that the stabilization of *PHO84* antisense transcripts is a necessary event for the initiation of *PHO84* gene silencing.

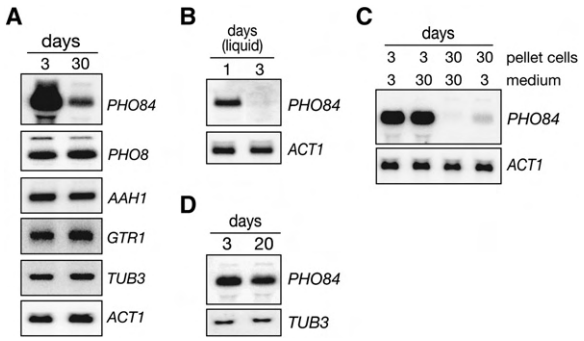


Figure 2. *PHO84* Gene Repression Is Specific and Localized

(A) Northern analysis of indicated mRNAs in WT (W303) cells kept on plate for 3 or 30 days before growth in minimum medium for 24 hr.

(B) WT (W303) cells were kept as stationary liquid cultures for 1 or 3 days at 25°C before exponential growth for 24 hr in minimum medium and RNA analysis.

(C) Low *PHO84* mRNA levels are not due to changes in the medium. WT (W303) cells kept on plates for 3 or 30 days were cultivated exponentially for 24 hr in minimum medium. Media were swapped, i.e., the supernatants from 3 or 30 days cells were added to the 3 or 30 days pellets. Cultures were grown for another 6 hr before RNA analysis.

(D) *PHO84* mRNA levels are restored when cells were streaked on new plates. WT (W303) cells kept on plates for 3 or 20 days were streaked on fresh YEPD plates for 24 hr before 24 hr exponential growth in minimum medium and RNA analysis. Hybridizations were with random primed labeled DNA probes. *ACT1* (A, B, and C) and *TUB3* (D) mRNAs served as loading controls.

Rrp6 Association with the *PHO84* Gene Is Regulated during Aging

The role of antisense RNA stabilization in *PHO84* repression in both *Δrrp6* and the progeny of old WT cells (Figures 1C, 3A, and 4A) prompted us to investigate the loss of Rrp6 function over time. Because Rrp6 interacts with chromatin (Andrulis et al., 2002; Hieronymus et al., 2004), we asked whether its association with the *PHO84* gene is affected in the progeny of aged cells. Indeed, we observed that although the global levels of hemagglutinin (HA)-tagged Rrp6 are unaffected during aging, the binding of HA-tagged Rrp6 over the *PHO84* locus decreases significantly in the progeny of 15- versus 2-day-old cells (Figures 5A and 5B). Thus, antisense stabilization and *PHO84* silencing over time could result from the weaker association of Rrp6 with the *PHO84* gene. To address whether aging might have a general effect on Rrp6 function, we examined other Rrp6 targets under these conditions. Rrp6 has been involved in Nab2 mRNA degradation (Roth et al., 2005), and Nab2 protein levels are increased in the *Δrrp6* strain (Figure 5C). However, Nab2 protein levels are not affected in the progeny of 15-day-old cells (Figure 5B), consistent with the absence of a significant decrease in the binding of Rrp6 to the *NAB2* gene under these conditions (Figure 5A). Similarly, no defect in the processing and accumulation of other Rrp6 substrates, including 7S and 5.8S ribosomal RNAS (rRNAs), as well as U24 and snR72, is detected in aged cells (Figure 5D).

Finally, the examination of *PHO84* sense and antisense RNAs in two earlier described *rrp6* mutants (Phillips and Butler, 2003) shows that the more severe *rrp6-3* mutant behaves like *Δrrp6*, with high antisense RNA levels and no detectable sense RNA, whereas the *rrp6-13* mutant shows a milder phenotype with lower antisense and higher sense RNA levels. These observations are consistent with a direct role of Rrp6 in *PHO84* gene regulation.

These data taken together suggest that during chronological aging, it is not the ability of Rrp6 to degrade its targets that seems to be affected, but rather its ability to access a subset of substrates within specific genomic regions. In addition, they support the view that Rrp6 degrades its targets when associated with chromatin and hence that these transcripts act in *cis* to repress *PHO84* transcription. Notably, Rrp6 also binds the neighboring *TUB3* and *GTR1* genes, and this association is similarly reduced in aged cells (Figure 5A). Yet, *TUB3* and *GTR1* RNA levels are not affected under these conditions (Figure 2A), presumably because these loci do not encode Rrp6-regulated antisense transcripts.

PHO84 Gene Silencing Requires Antisense Transcript Stabilization and the Histone Deacetylase Hda1/2/3 Complex

Our results indicate that antisense transcript stabilization represents an initial event in *PHO84* repression and that additional time-dependent steps are required for the establishment of *PHO84* gene silencing (Figures 1C, 3A, and 4A). The progressively lower *PHO84* mRNA levels observed in WT cells upon chronological aging could reflect the inheritance of epigenetic modifications. To address this possibility, we first tested whether *PHO84* remains repressed when cells are grown over more generations. When we inoculated old cells for 25 days in minimum medium and allowed them to proliferate exponentially for 96 hr instead of 24 hr, *PHO84* expression was not restored (Figure 6A). Remarkably, *PHO84* repression also occurs when fresh cells are grown for prolonged time under exponential conditions in minimum medium (Figure 6A). These observations suggest that over time, the cell population progressively acquires some epigenetic marks resulting in *PHO84* gene silencing.

Gene repression often correlates with histone deacetylation (Ekwall, 2005). To further investigate the mechanism by which *PHO84* becomes repressed, we examined *PHO84* mRNA levels in mutant strains lacking various histone deacetylases or their cofactors in conditions under which *PHO84* antisense transcripts are stabilized (Figure 6B). Strikingly, time-dependent *PHO84* repression does not occur in mutants lacking Hda1, Hda2, and Hda3, which belong to the same complex. *PHO84* repression is slightly compromised in the absence of Cpr1 but is not affected in the absence of other histone deacetylases including Rpd3, Hos1, Sir2, or Hst1 (Figure 6B and data not shown). These observations suggest that *PHO84* transcription repression in aging cells specifically requires the Hda1/2/3 histone deacetylase complex.

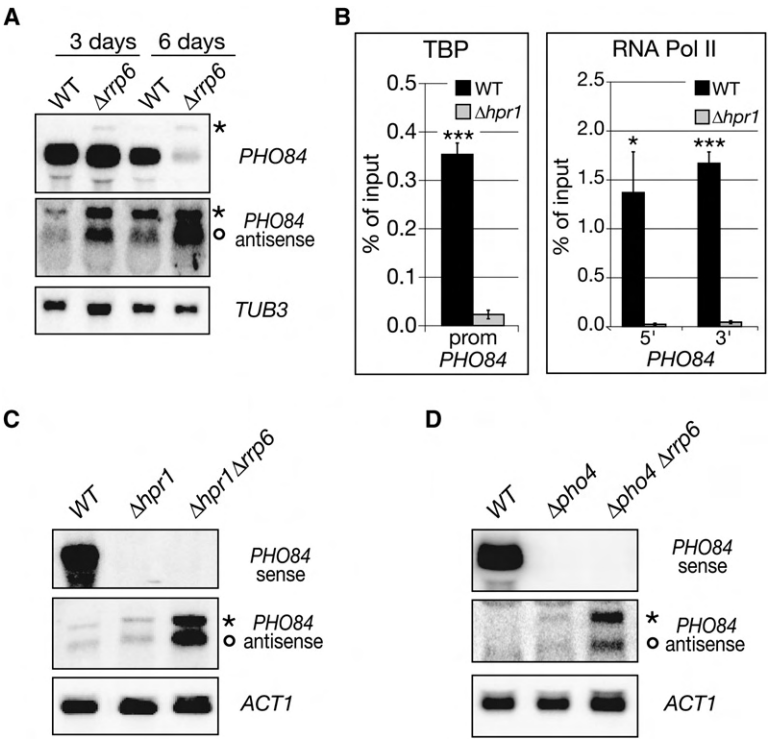


Figure 3. Increased Levels of *PHO84* Antisense Transcripts Result from the Absence of Rrp6 and Not from a Defect in *PHO84* Sense Transcription

(A) Northern analysis of total RNA from WT (W303) and *Δrrp6* strains kept on plates for 3 or 6 days before 24 hr exponential growth in minimum medium, with double-stranded probes specific for *PHO84* or *TUB3* used as loading control and an RNA probe specific for *PHO84* antisense transcripts mapping to the *PHO84* 3′ end.

(B) Loss of Hpr1 abolishes the association of TATA-binding protein (TBP) and RNA Pol II with the *PHO84* gene. Shown is a ChIP analysis of TBP and RNA Pol II in WT (W303) (black bars) and *Δhpr1* (gray bars) strains kept for 3 days on plates and exponentially grown in minimum medium for 24 hr. Immunoprecipitated DNA was amplified with *PHO84*-specific primers and quantified as described in Figures 1A and 1D. Values derive from three independent experiments.

(C) Total RNA was extracted from WT (W303), *Δhpr1*, and *Δhpr1Δrrp6* strains kept on plates for 3 days and grown in minimum medium. Northern blotting of *PHO84* mRNAs was performed as in Figure 1C.

(D) WT (W303), *Δpho4*, and *Δpho4Δrrp6* strains were grown in YEPD, and total RNA was analyzed as in (C).

To confirm that Hda1/2/3 is also required for *PHO84* gene silencing when antisense transcripts accumulate in the absence of Rrp6 (Figures 3 and 4), we examined *PHO84* mRNA levels in *Δrrp6*, *Δhda1*, and *Δhda2* simple mutants or in *Δrrp6Δhda1* and *Δrrp6Δhda2* double mutants (Figure 6C). *PHO84* is well expressed in the WT, *Δhda1*, and *Δhda2*, but repressed in *Δrrp6*. However, *PHO84* expression is rescued in *Δrrp6* by the disruption of *HDA1* or *HDA2* despite the accumulation of *PHO84* antisense transcripts in these double mutants, indicating that *Δhda1* and *Δhda2* are epistatic to *Δrrp6*. Together, these observations indicate that once antisense RNAs are stabilized, Hda1/2/3 is required for the repression of *PHO84*.

The absence of *PHO84* repression in the *Δrrp6Δhda1* and *Δrrp6Δhda2* double mutants (Figure 6C) suggests that the loss of Rrp6 and/or antisense transcript stabilization facilitates Hda1-dependent histone deacetylation and gene repression. To verify that the role of Hda1 is direct, we compared the binding of myc-tagged Hda1 to *PHO84* and the adjacent genes *TUB3* and *GTR1* in WT and *Δrrp6* strains (Figure 6D). Hda1-myc association with the promoter and coding regions of *PHO84* increases at least 5-fold in *Δrrp6*, and this increase extends into the neighboring *TUB3* and *GTR1* genes. Western analysis of Hda1-myc confirms that this increase is not due to higher global levels of Hda1 in *Δrrp6* (Figure 5C). Thus, antisense RNA stabilization in *Δrrp6* is paralleled by the increased recruitment of Hda1 to the *PHO84* locus.

PHO84 Antisense RNA Stabilization Leads to H3K18 Deacetylation at the *PHO84* Promoter

Next, to assess whether Hda1 recruitment results in histone deacetylation, we examined the level of histone H3 acetylation on lysine 18 (H3K18Ac). Remarkably, although H3 acetylation modestly decreases at the 5′ end of the *GTR1* and *TUB3* genes (1.3- to 1.5-fold), it is strongly reduced (7- to 8-fold) in the *PHO84* promoter and 5′ coding regions but not at the *PHO84* 3′ end (Figure 7A and see Discussion). Thus, histone deacetylation does not strictly correlate with Hda1 recruitment in *Δrrp6* but is restricted to a region where Hda1 and antisense RNA overlap. Finally, to define whether the repression in aged cells of *PHO84*, but not *GTR1* and *TUB3* (Figure 2A), could also result from targeted histone deacetylation, we compared H3K18Ac levels over this region in fresh and old WT cells (Figure 7B). Strikingly, the pattern of H3K18Ac in old cells was identical to that observed in *Δrrp6,* strongly suggesting that *PHO84* repression implicates the same mechanism in both situations (Figures 7A and 7B). These results confirm that *PHO84* repression by Hda1 is direct and suggest a new mechanism in *S. cerevisiae* in which targeted *PHO84* gene silencing depends on antisense RNA accumulation and RNA-induced histone deacetylation.

Finally, to confirm that antisense RNA indeed plays a direct role in *PHO84* gene silencing, we blocked its production by inserting a *HIS5* gene cassette within the *PHO84* coding region, such that the antisense transcripts terminate at the *HIS5* terminator (Figure 7C and Figure S2).

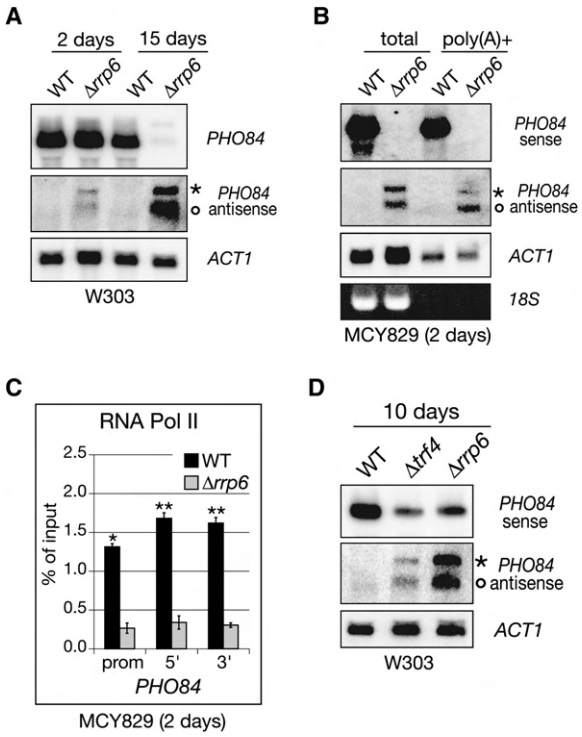


Figure 4. Loss of Rrp6 Induces *PHO84* Repression

(A) Northern analysis of total RNA from WT (W303) and *Δrrp6* strains kept for 2 or 15 days on plates and exponentially grown in YEPD for 24 hr, with the *PHO84*-specific double-stranded probe or the *PHO84* antisense-specific riboprobe mapping to the 3′ end of *PHO84*. *ACT1* served as a loading control.

(B) Total (10 μg) and poly(A)$^+$ RNA (500 ng) from WT (MCY829) and *Δrrp6* cells kept 2 days on plates and grown in YEPD were subjected to northern-blot analysis with *PHO84* sense- and antisense-specific riboprobes mapping to the 3′ end of *PHO84*. *ACT1* served as a loading control; 18S rRNA ethidium bromide staining confirmed efficient poly(A)$^+$ RNA purification.

(C) The loss of Rrp6 affects *PHO84* transcription. Shown is a ChIP analysis of RNA Pol II with extracts from strains described in (B). Immunoprecipitated DNA was quantified by real-time PCR with *PHO84*-specific primers shown in Figure 1A. Values derive from two independent experiments.

(D) *PHO84* mRNA levels are affected in *Δtrf4*. Total RNA from WT (W303), *Δtrf4*, and *Δrrp6* strains kept 10 days on plates and exponentially grown in YEPD for 24 hr were subjected to northern-blot analysis as in (B).

In control cells, antisense transcripts accumulate in the absence of Rrp6, and *PHO84* sense transcription is repressed. In contrast, in the *+HIS5* mutant strain, an antisense RNA of intermediate size is detected in *Δrrp6* with the 3′ but not the 5′- (*YML122C*) specific probe. This chimeric antisense RNA starts at the 3′ end of the *PHO84* gene and ends at the *HIS5* terminator. Importantly, the *PHO84* gene is not repressed in this mutant and produces a longer *PHO84-HIS5-PHO84* chimeric sense RNA both in the presence and absence of Rrp6 (Figure 7C and Figure S2). This result demonstrates that antisense RNA is directly implicated in the mechanism of *PHO84* transcriptional silencing and that *PHO84* gene repression is not an indirect effect of the absence of Rrp6.

DISCUSSION

A recent study has implicated *IME4* antisense transcripts in the downregulation of the sense *IME4* mRNA via a *cis*-acting transcription-interference mechanism (Hongay et al., 2006). Here, we find that stabilization, rather than the increased transcription of *PHO84* antisense transcripts, is implicated in *PHO84* repression. The data rule out a simple transcription-interference model and support a mechanism in which the accumulation of antisense RNAs leads to targeted histone deacetylation by Hda1 and the silencing of sense transcription (Figure 7D). Our observations further indicate that *PHO84* antisense RNAs are stabilized in aged cells and could therefore be regulated upon changes in physiological conditions. More generally, the data suggest that the modulation of Rrp6 activity in response to external cues might contribute to the establishment of new genetic programs by inducing RNA-dependent epigenetic modifications inherited by the progeny cell population.

Role of Trf4-Rrp6 Regulated RNAs in Targeted Gene Repression

Previous studies have implicated Trf4 and Rrp6 in the degradation of cryptic unstable transcripts, most of which map at intergenic regions (Davis and Ares, 2006; Wyers et al., 2005). Our results show that Trf4 and Rrp6 also degrade antisense transcripts encompassing an ORF (Figures 3 and 4). Importantly, we describe a new regulatory mechanism in which antisense RNA stabilization results in the repression of sense transcription (Figure 7D). In contrast to the mechanism of transcription interference proposed to regulate *IME4* gene expression (Hongay et al., 2006), the accumulation of *PHO84* antisense RNAs is not the consequence of a decrease in sense transcription. When sense transcription is blocked (*Δhpr1* or *Δpho4*), antisense RNAs are not increased compared to a normal (WT) condition (Figures 3C and 3D). Conversely, when sense RNA is transcribed (*Δhda1*), antisense RNA is still able to accumulate (*Δhda1Δrrp6*) (Figure 6C).

Although clearly detectable in *Δrrp6*, *PHO84* antisense transcripts are less abundant than sense mRNAs in WT cells (Figure 4B). Accordingly, the low RNA Pol II amount detected at the 3′ end of *PHO84* in *Δrrp6* confirms that the rate of antisense transcription is much lower than the rate of sense transcription observed under normal WT conditions (Figure 4C). These observations lead us to propose first that *PHO84* antisense transcription is constitutively low and independent of sense transcription and second that the increase in *PHO84* antisense transcript levels, which is key for *PHO84* repression, is primarily due to increased stability. The fact that antisense RNA accumulation is independent of sense transcription strengthens the view that antisense transcript stabilization is the cause rather than the consequence of *PHO84* gene

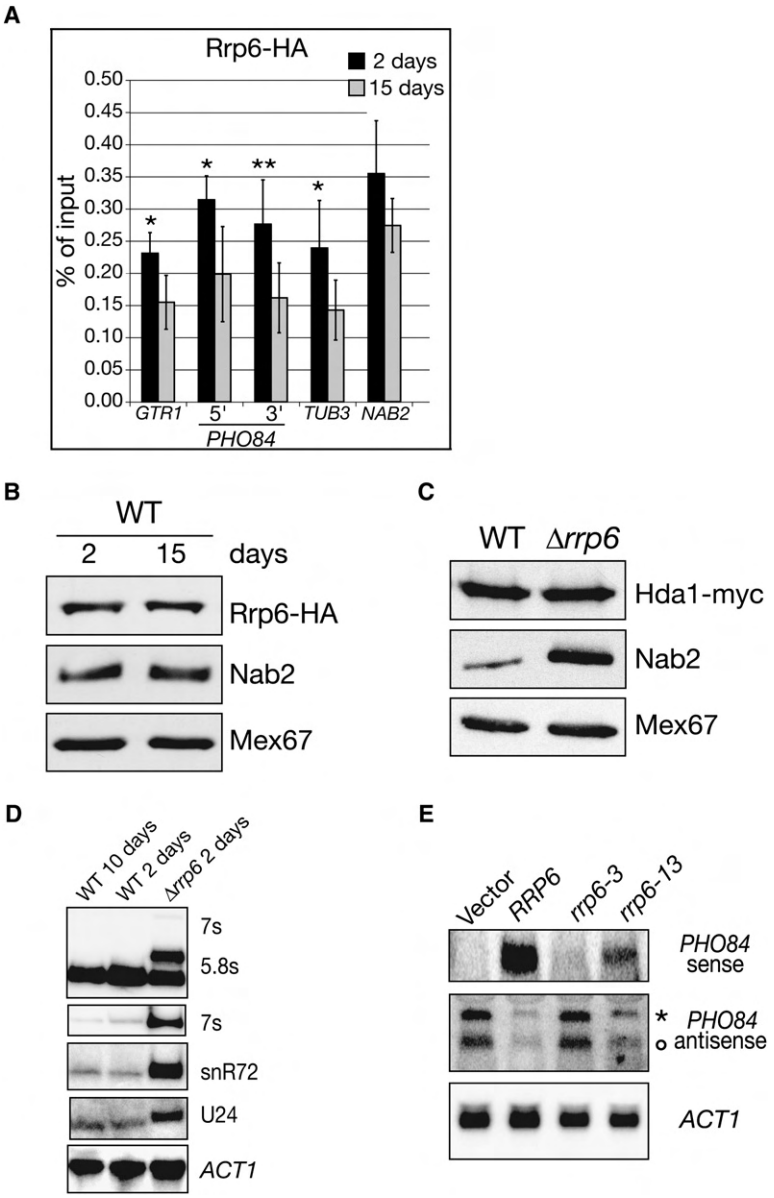


Figure 5. Rrp6 Dissociates from the *PHO84* Locus over Time

(A) ChIP extracts prepared from the Rrp6-HA strain grown on plates for 2 and 15 days before 24 hr exponential growth in minimum medium were immunoprecipitated with anti-HA and DNA quantified by real-time PCR with primers specific for *PHO84*, *GTR1*, *TUB3*, and *NAB2* as described in Figure 1A and Table S2. Values derive from three experiments. Corresponding northern blots are presented in Figure S1D.

(B) Fresh and old cells contain similar amounts of Rrp6-HA. Shown is a western-blot analysis of total protein extracts from strains grown as in (A) with antibodies against HA, Nab2, and Mex67 as a loading control.

(C) The absence of Rrp6 affects Nab2 but not Hda1-myc levels. Shown is a western analysis of total protein extracts from Hda1-myc (MCY829 WT and *Δrrp6*) strains kept on plate for 3 days before 24 hr exponential growth in YEPD medium with antibodies against myc, Nab2, or Mex67 to control for loading.

(D) Rrp6-dependent processing of 5.8S ribosomal RNA, snRN72, and U24 is not affected in aged cells. Total RNA was extracted from WT cells kept on plates for 2 or 10 days, or from *Δrrp6* kept on plates for 2 days, before cultivation for 24 hr in minimum medium, fractionated, and hybridized with oligonucleotide probes specific for the indicated Rrp6 targets.

(E) *PHO84* sense and antisense RNA levels in the *Δrrp6* strain transformed with empty vector or plasmids carrying wild-type *RRP6* or the mutant *rrp6-3* and *rrp6-13* genes. *ACT1* served as a loading control.

repression. However, we observed no strict correlation between antisense RNA levels and the extent of *PHO84* gene silencing. The data rather support that time, in combination with antisense RNA, even in low amounts, is key for the establishment of *PHO84* repression.

Initial attempts to knock down antisense transcripts by the generation of deletions, encompassing putative antisense promoter and mapped initiation sites, did not affect antisense RNA production (Figure S1B). As recently described for the *PHO5* gene (Uhler et al., 2007), it is likely that antisense production results from transcription initiation at multiple cryptic sites within the 3′ end and 3′ untranslated region (UTR) of the *PHO84* gene and depends on the chromatin state in this region rather than specific promoter elements. So far, the insertion of a gene cassette within the *PHO84* coding region has been the only way of blocking antisense RNA production. In this situation, antisense RNAs do not reach the *PHO84* 5′ end and *PHO84* does not become repressed even in the absence of Rrp6, demonstrating the direct role of antisense RNA accumulation in *PHO84* gene silencing (Figure 7C).

Our data further indicate that the stabilization of *PHO84* antisense transcripts correlates with a decrease in the association of Rrp6 with the *PHO84* gene (Figures 1C and 5A). This observation suggests that Rrp6 degrades *PHO84* antisense RNAs at their transcription site and acts in *cis* to promote *PHO84* repression. Consistently, the orthologs of Rrp6 in *Drosophila* and mammals are similarly proposed to act in association with chromatin (Andrulis et al., 2002; Ciaudo et al., 2006). In addition,

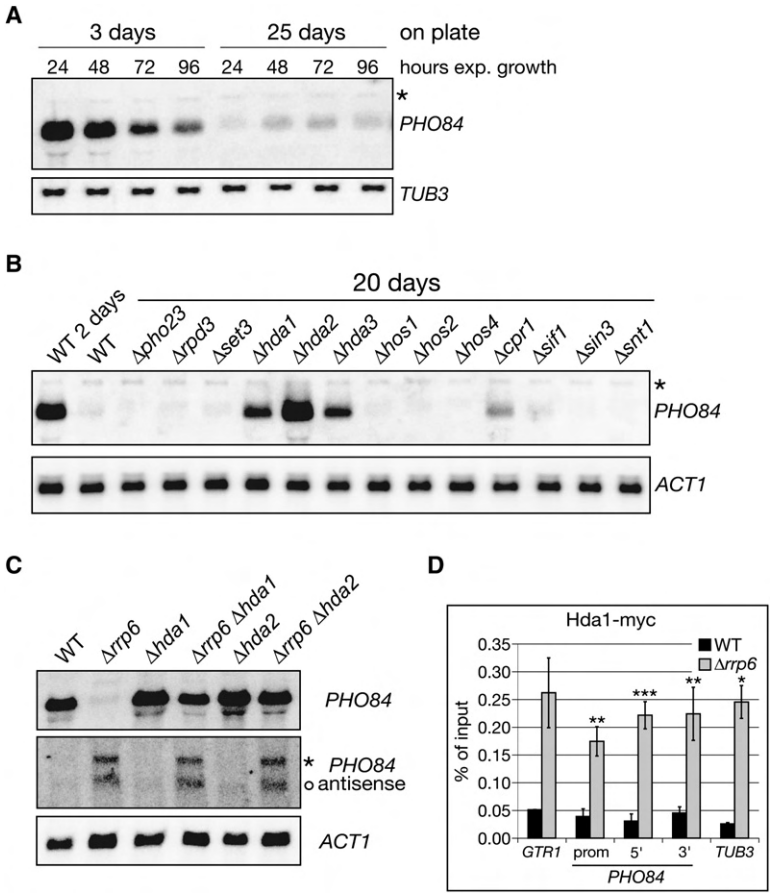


Figure 6. The Hda1/2/3 Histone Deacetylase Complex Mediates *PHO84* Gene Repression when Antisense RNAs Are Stabilized

(A) *PHO84* mRNA levels in WT (W303) cells kept on plates for 3 and 25 days before cultivation in minimum medium for the indicated number of hours under exponential growth conditions at 25°C. *PHO84* mRNA was detected with a random primed labeled probe. *TUB3* mRNA served as a loading control.

(B) *PHO84* mRNA levels in WT (BY4741) and indicated deletion strains, kept on plates for the indicated number of days and grown for 24 hr in minimum medium at 25°C, were detected as in (A). *ACT1* served as a loading control.

(C) *PHO84* gene repression in *Δrrp6* implicates Hda1/2/3. Northern analysis of *PHO84* mRNA in WT (W303) and the indicated mutant strains kept 15 days on plates and exponentially grown in YEPD for 24 hr with a double-stranded or an antisense-specific *PHO84* probe mapping to the 3′ end of *PHO84*.

(D) ChIP analysis of Hda1-myc over *PHO84* in WT (MCY829) (black bars) or *Δrrp6* (gray bars) cells. Strains kept 2 days on plates were grown in YEPD-rich medium, and crosslinked chromatin extracts were immunoprecipitated with antibodies against myc. Immunoprecipitated DNA was quantified with primers specific for *GTR1*, *TUB3*, and *PHO84* as described in Figures 1A and 1D. Values derive from three experiments, except those for *GTR1* and *TUB3,* which derive from two. WT and *Δrrp6* extracts used in (D) contain comparable levels of Hda1-myc (Figure 5C).

RNAs implicated in TGS in *S. pombe* also act in *cis* (Buhler et al., 2006). Our finding that Rrp6 is key for the regulation of *PHO84* antisense RNA accumulation and *PHO84* gene repression suggests that other Rrp6-regulated transcripts might be involved in the control of proximal genes. Thus, the widespread and unstable transcriptome might not just reflect nonspecific RNA polymerase activity but might contribute to genome plasticity and be required for yeast adaptation to various physiological processes.

Loss of Rrp6 and Antisense RNA Stabilization Stimulates Histone Deacetylation by Hda1

In *S. cerevisiae*, HDACs are required for transcriptional repression and heterochromatin formation. Proteins bound to specific upstream regulatory sequences have been proposed to mediate the targeting of HDACs to specific chromatin regions. In particular, Hda1 is recruited through Tup1, a general transcription repressor that regulates major physiological pathways (DeRisi et al., 1997; Green and Johnson, 2004; Wu et al., 2001b). However, genome-wide analyses demonstrate that Hda1 recruitment might not always depend on Tup1. Although Tup1 is required for Hda1-mediated deacetylation in the HAST regions, the association of Hda1 with intergenic regions (IGRs) is Tup1 independent, suggesting the existence of alternative targeting mechanisms (Robyr et al., 2002).

Accordingly, we found that the recruitment of Hda1 to *PHO84* is not dependent on Tup1 because the loss of Tup1 does not rescue *PHO84* gene transcription (Figure S1C). Instead, our data indicate that the recruitment of Hda1 to chromatin coincides with the absence of Rrp6 or its dissociation from chromatin in aged cells, as well as the stabilization of *PHO84* antisense RNAs (Figures 1C, 4A, 5A, and 6D). The trigger for Hda1 recruitment is unclear because its binding increases both at *PHO84* and the neighboring *TUB3* and *GTR1* genes. One possibility is that *PHO84* antisense RNA stimulates the initial binding of Hda1 to the *PHO84* gene region, facilitating its subsequent spreading to adjacent regions. In *S. pombe*, nascent transcripts bound by targeting complexes containing siRNAs have been proposed to serve as binding platforms for the recruitment of histone-modifying enzymes (Buhler et al., 2006; Motamedi et al., 2004). By analogy, in the absence of RNAi, antisense transcripts might form structures specifically recognized by modifying enzymes (Yang and Kuroda, 2007). An alternative possibility is that the presence of Rrp6 might antagonize the binding of Hda1 to chromatin. In this view, the greater association of Hda1 with *PHO84*, *TUB3*, and *GTR1* in *Δrrp6* or aged cells could be due to

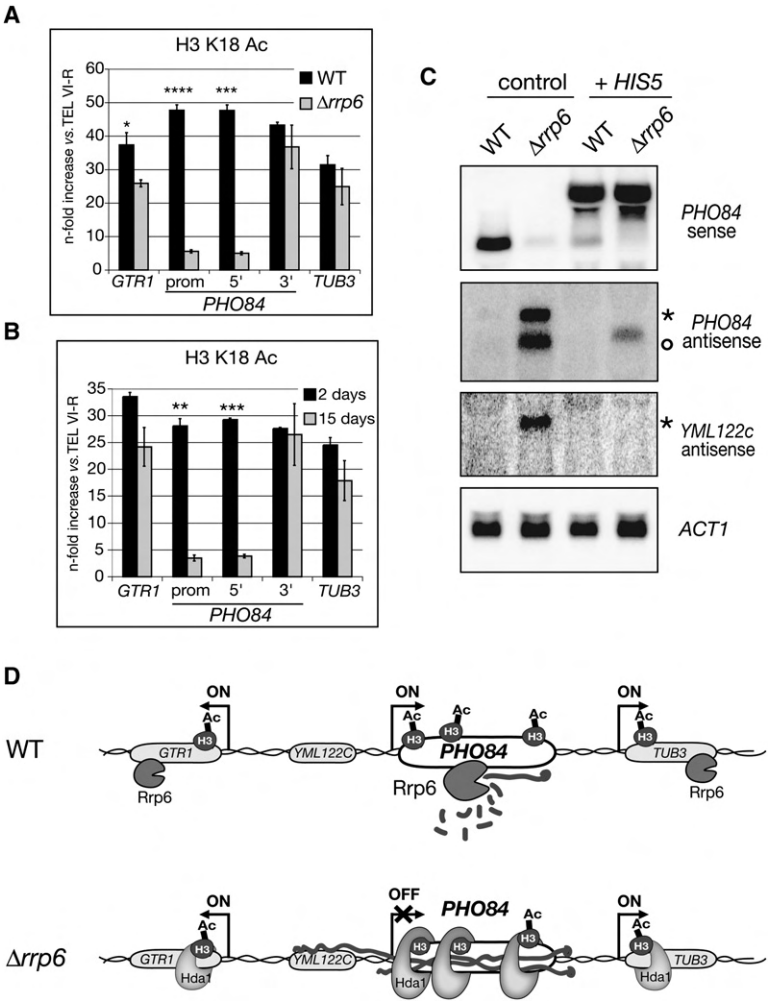


Figure 7. *PHO84* Antisense Transcript Stabilization Results in Targeted Histone Deacetylation

(A) ChIP analysis of histone H3K18 acetylation (H3K18Ac) over the *PHO84* locus in WT (MCY829) (black bars) or *Δrrp6* (gray bars) cells. The extracts described in Figure 6D were immunoprecipitated with antibodies against acetylated H3K18. Immunoprecipitated DNA was quantified with primers specific for *GTR1*, *TUB3*, and *PHO84*, as well as with primers specific for telomeric TEL VI-R sequences. The relative enrichment of the different gene segments was expressed as the n-fold increase with respect to TEL VI-R sequences. Statistical analyses were as in Figure 1D. Values derive from three independent experiments.

(B) ChIP analysis of H3K18 acetylation over *PHO84* in WT fresh (2 days) and aged (15 days) cells with the same chromatin extracts as in Figure 5A. DNA was quantified with the same primer pairs as in (A). Values derive from two independent experiments.

(C) Block of *PHO84* antisense RNA prevents *PHO84* gene repression in the presence and absence of Rrp6. Control WT (MCY829) and *Δrrp6* or mutant WT+*HIS5* and *Δrrp6*+*HIS5* strains were kept on plates for 2 days before exponential growth in YEPD for 24 hr. Total RNA was hybridized with riboprobes mapping to the 3′ end of *PHO84* and specific for *PHO84* sense and antisense RNAs or specific for the antisense RNA spanning the *YML122C* putative ORF.

(D) Model. *PHO84* antisense RNA stabilization is paralleled by Hda1 recruitment, histone deacetylation, and *PHO84* transcriptional repression. In WT cells, Rrp6 rapidly degrades *PHO84* antisense transcripts, allowing efficient *PHO84* gene transcription (top). In the absence of Rrp6 or in aging cells, in which Rrp6 dissociates from the gene, *PHO84* antisense transcripts are stabilized (bottom). This accumulation correlates with the recruitment of Hda1 to *PHO84* and adjacent loci. However, Hda1-induced histone deacetylation is restricted to regions encoding antisense RNA, resulting in specific *PHO84* gene repression. The following abbreviations are used: acetylated histone H3 (H3-Ac) and nonacetylated histone H3 (H3).

lack or lower levels of Rrp6 binding in these regions rather than to antisense RNA stabilization (Figures 5A and 6D).

Most importantly, H3K18 deacetylation is confined to the *PHO84* gene encoding antisense RNA, suggesting a role for these transcripts in Hda1 activity (Figure 7). The targeted deacetylation of *PHO84* is consistent with the silencing of *PHO84* and the maintenance of *TUB3* and *GTR1* expression in aged cells (Figures 1C and 2A). Notably, although antisense RNAs extend from the 3′ end to the promoter region of *PHO84*, histone deacetylation is restricted to the promoter and 5′ end but excluded from the 3′ end (Figures 7A and 7B). Thus, histone deacetylation by Hda1 might depend on the conjunction of antisense RNA and specific factors bound to the *PHO84* promoter and/or 5′ end. Such specific and localized deacetylation might be required for the maintenance of antisense transcription and *PHO84* gene silencing.

The proposed mechanism might not be restricted to *S. cerevisiae* but is likely to be more general and conserved in *S. pombe* and higher eukaryotes. Indeed, Clr3, the homolog of Hda1 in *S. pombe*, contributes to gene silencing in an RNAi-independent mechanism (Yamada et al., 2005). More recent data show that the exosome component Rrp6 also influences sense transcription by regulating antisense production at several euchromatic loci (Nicolas et al., 2007). Although a specific HDAC has not yet been implicated in this particular context, these recent findings on transcriptional gene silencing in *S. pombe* together with our observations in *S. cerevisiae* suggest the existence of an alternate RNA-mediated TGS mechanism independent of RNAi that might be evolutionarily conserved. Accordingly, mammalian X chromosome inactivation, which implicates the transcription of noncoding sense and antisense RNAs, also depends on Rrp6 (Ciaudo et al., 2006; Yang and Kuroda, 2007).

Overall, our data raise new questions: Is the stabilization of Rrp6 regulated RNAs a general mechanism for the targeting and activation of Hda1 throughout the genome; is

RNA-induced histone deacetylation mostly required for transcriptional repression and gene silencing; and finally, could such a mechanism be implicated in the recruitment and activation of HDACs other than Hda1? The comparison of genome-wide histone H3 acetylation in the presence or absence of Rrp6 should define the extent of such a regulatory mechanism.

Regulation of Rrp6 Participates in Gene Expression Reprogramming

Importantly, the *PHO84* repression mechanism we describe is not only observed in a *Δrrp6* strain but also occurs in WT cells and is conserved among the different *S. cerevisiae* tested backgrounds (W303, BY4741, and MCY829). We found that the occupancy of *PHO84* by Rrp6 depends on the aging status of the mother cells because the amount of Rrp6 bound to *PHO84* in the progeny of old mother cells is significantly lower than that in the progeny of fresh cells (Figure 5A). Furthermore, this decrease in the targeting of Rrp6 to *PHO84* correlates with the progressive stabilization of *PHO84* antisense transcripts, suggesting that the association of Rrp6 with chromatin is regulated under these conditions (Figures 1C and 5A). Notably, although *PHO84* antisense RNAs accumulate in the progeny of aged cells, other Rrp6 targets are not affected (Figure 5D). One possibility is that aging primarily impacts the stability of chromatin-associated Rrp6 targets such as the *PHO84* antisense RNAs.

Earlier studies proposed that exosome activity is regulated by carbon source (Bousquet-Antonelli et al., 2000). It is therefore likely that multiple parameters influence exosome function. So far, we have not been able to understand the physiological relevance of *PHO84* gene silencing in aging yeast cells, nor have we been able to connect Rrp6 to factors implicated in aging or to components of signaling cascades that might regulate its activity (Fabrizio et al., 2004). Nevertheless, the role of Rrp6 in response to the physiological changes described in this work reveals its potential importance in yeast adaptation to environmental variations. Together, these observations raise a novel gene regulatory alternative through the modulation of Rrp6 function. We speculate that by the differential stabilization of RNA targets and activation of HDACs on chromatin, the regulation of Rrp6 could be a key event in gene expression plasticity. The conservation of an analogous mechanism in mammalian cells might be of fundamental importance in cell differentiation and the maintenance of cell-type-specific gene expression programs.

EXPERIMENTAL PROCEDURES

Yeast Strains

Strains used in this study are described in Table S1. The experiments were performed in W303 but also in BY4147 and MCY289 backgrounds. Gene deletions were transferred from BY4147 to W303 background by the transformation of polymerase chain reaction (PCR) fragments generated with primers listed in Table S2. For the construction of the *PHO84+HIS5* strains, PCR fragments produced by the amplification of the *HIS5* gene cassette pUG27 (Gueldener et al., 2002) and containing sequences homologous to the *PHO84* coding region were transformed into *HDA1-myc* (WT and *Δrrp6*) strains. Colonies were selected on His-medium, and *HIS5* insertion verified by PCR.

Media and Culture Conditions

Yeast strains were streaked for 3 days on YEPD plates at 25°C and kept at 4°C for additional days. Liquid cultures were inoculated with cells taken from plates and grown at 25°C for 24 hr under exponential conditions (OD_{600} < 0.8) in YEPD-rich medium or synthetic complete (SC) minimum medium.

RNA Extraction, Polyadenylated RNA Purification, and Northern Blotting

Total RNA was extracted with the hot phenol procedure. Total and poly(A)$^+$ RNA, purified on oligodT Dynabeads, were fractionated on denaturing formaldehyde agarose gels and transferred to nylon membranes. Membranes were hybridized overnight at 42°C with ^{32}P random primed labeled probes in 50% formamide, 5x standard saline citrate (SSC), 20% dextran sulfate, 1% sodium dodecyl sulfate (SDS), and 100 μg/ml boiled salmon sperm DNA. Hybridization with ^{32}P-labeled SP6 riboprobes was in 50% formamide, 7% SDS, 0.2 M NaCl, 80 mM sodium phosphate (pH 7.4), and 100 μg/ml boiled salmon sperm DNA overnight at 62°C. All blots were washed with 0.5x SSC and 0.1% SDS for 1 hr at 62°C. Double-stranded probes were obtained by the random primed labeling of PCR fragments. Riboprobes were obtained by SP6 in vitro transcription of gene-specific PCR fragments containing an SP6 promoter. Primers are described in Table S2. Small RNAs were fractionated on 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nylon membranes with a semidry blotting machine. Membranes were pre- and hybridized with ^{32}P 5′ end labeled oligonucleotide probes OSB156 (7S rRNA), OSB157 (5.8S rRNA), OSB267 (snR72), and OSB138 (U24) in 25 mM KPO4 (pH 7.4), 5x SSC, 5x Denhardt's, and 50 μg/ml salmon sperm DNA overnight at 37°C and washed in 1x SSC and 0.1% SDS for 30 min at 42°C as described (Phillips and Butler, 2003).

Chromatin Immunoprecipitation

Chromatin immunoprecipitations (ChIPs) were performed essentially as described previously (Zenklusen et al., 2002). Yeast strains were grown to OD_{600} = 1 either in YEPD or SC minimum media at 25°C and crosslinked for 1 hr by the addition of formaldehyde to a final concentration of 1.2% (except in Figure 3, where cells were crosslinked only 10 min.). Crosslinked and sonicated chromatin extracts from ~10^8 cells were immunoprecipitated overnight in the presence of 50 μl of 50% protein G Sepharose (Amersham Pharmacia) with antibodies against the C-terminal domain of the RNA Pol II (Abcam 8WG16), the TATA-binding protein (gift from M. Collart), acetylated histone H3K18 (Abcam 1191), the HA epitope (Covance 16B12) for the Rrp6-HA tagged strain, and the myc epitope (Covance 9E10) for the Hda1-myc tagged strain. All immunoprecipitations were repeated at least twice with different chromatin extracts. Immunoprecipitated DNA was quantified by real-time PCR with the primer pairs shown in Table S2 and expressed as the percent of input DNA or n-fold increase over TELVI-R as indicated. Error bars correspond to standard deviations. Statistical analyses were done with the Student's t test ("*" indicates $p < 0.05$, "**" indicates $p < 0.01$, "***" indicates $p < 0.001$, and "****" indicates $p > 0.0001$; no asterisk indicates not significant).

Protein Extraction and Western Blotting

Total protein extracts were prepared from cultures used for ChIP analysis prior to crosslinking, fractionated on SDS-PAGE, and examined by western-blot analysis with antibodies specific for HA (16B12 from Covance), myc (9E10 from Covance), Nab2 (gift from A. Corbett), or Mex67 (gift from C. Dargemont).

Supplemental Data

Supplemental Data include two figures and two tables and can be found with this article online at http://www.cell.com/cgi/content/full/131/4/706/DC1/.

ACKNOWLEDGMENTS

We thank S. Butler, M. Grunstein, and D. Libri for material. We also thank S. Dersi and J. Dimitrova for technical help. We are grateful to M. Bühler, M. Collart, B. Daignon-Fornier, D. Libri, R. Loewith, A. Morillon, M.P. Peli-Gulli, D. Picard, B. Pinson, M. Rosbash, D. Shore, M. Strubin, P. Vinciguerra, and C. de Virgilio for discussion and/or critical reading of the manuscript. This work was supported by the Swiss National Science Foundation (grant no 3100-102235 to F.S.), the Swiss National Science Foundation program Frontiers in Genetics, the Leenaards Foundation, and the Canton de Genève.

Received: February 2, 2007
Revised: July 11, 2007
Accepted: September 6, 2007
Published: November 15, 2007

REFERENCES

Almeida, R., and Allshire, R.C. (2005). RNA silencing and genome regulation. Trends Cell Biol. *15*, 251–258.

Andrulis, E.D., Werner, J., Nazarian, A., Erdjument-Bromage, H., Tempst, P., and Lis, J.T. (2002). The RNA processing exosome is linked to elongating RNA polymerase II in Drosophila. Nature *420*, 837–841.

Bousquet-Antonelli, C., Presutti, C., and Tollervey, D. (2000). Identification of a regulated pathway for nuclear pre-mRNA turnover. Cell *102*, 765–775.

Buhler, M., Verdel, A., and Moazed, D. (2006). Tethering RITS to a nascent transcript initiates RNAi- and heterochromatin-dependent gene silencing. Cell *125*, 873–886.

Chavez, S., Beilharz, T., Rondon, A.G., Erdjument-Bromage, H., Tempst, P., Svejstrup, J.Q., Lithgow, T., and Aguilera, A. (2000). A protein complex containing Tho2, Hpr1, Mft1 and a novel protein, Thp2, connects transcription elongation with mitotic recombination in Saccharomyces cerevisiae. EMBO J. *19*, 5824–5834.

Ciaudo, C., Bourdet, A., Cohen-Tannoudji, M., Dietz, H.C., Rougeulle, C., and Avner, P. (2006). Nuclear mRNA degradation pathway(s) are implicated in Xist regulation and X chromosome inactivation. PLoS Genet *2*, e94.

David, L., Huber, W., Granovskaia, M., Toedling, J., Palm, C.J., Bofkin, L., Jones, T., Davis, R.W., and Steinmetz, L.M. (2006). A high-resolution map of transcription in the yeast genome. Proc. Natl. Acad. Sci. USA *103*, 5320–5325.

Davis, C.A., and Ares, M., Jr. (2006). Accumulation of unstable promoter-associated transcripts upon loss of the nuclear exosome subunit Rrp6p in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA *103*, 3262–3267.

DeRisi, J.L., Iyer, V.R., and Brown, P.O. (1997). Exploring the metabolic and genetic control of gene expression on a genomic scale. Science *278*, 680–686.

Ekwall, K. (2005). Genome-wide analysis of HDAC function. Trends Genet. *21*, 608–615.

Escusa, S., Camblong, J., Galan, J.M., Pinson, B., and Daignan-Fornier, B. (2006). Proteasome- and SCF-dependent degradation of yeast adenine deaminase upon transition from proliferation to quiescence requires a new F-box protein named Saf1p. Mol. Microbiol. *60*, 1014–1025.

Fabrizio, P., Pletcher, S.D., Minois, N., Vaupel, J.W., and Longo, V.D. (2004). Chronological aging-independent replicative life span regulation by Msn2/Msn4 and Sod2 in Saccharomyces cerevisiae. FEBS Lett. *557*, 136–142.

Green, S.R., and Johnson, A.D. (2004). Promoter-dependent roles for the Srb10 cyclin-dependent kinase and the Hda1 deacetylase in Tup1-mediated repression in Saccharomyces cerevisiae. Mol. Biol. Cell *15*, 4191–4202.

Gueldener, U., Heinisch, J., Koehler, G.J., Voss, D., and Hegemann, J.H. (2002). A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. Nucleic Acids Res. *30*, e23.

Hieronymus, H., Yu, M.C., and Silver, P.A. (2004). Genome-wide mRNA surveillance is coupled to mRNA export. Genes Dev. *18*, 2652–2662.

Hongay, C.F., Grisafi, P.L., Galitski, T., and Fink, G.R. (2006). Antisense transcription controls cell fate in Saccharomyces cerevisiae. Cell *127*, 735–745.

Houseley, J., LaCava, J., and Tollervey, D. (2006). RNA-quality control by the exosome. Nat. Rev. Mol. Cell Biol. *7*, 529–539.

Johnson, J.M., Edwards, S., Shoemaker, D., and Schadt, E.E. (2005). Dark matter in the genome: Evidence of widespread transcription detected by microarray tiling experiments. Trends Genet. *21*, 93–102.

Kurdistani, S.K., and Grunstein, M. (2003). Histone acetylation and deacetylation in yeast. Nat. Rev. Mol. Cell Biol. *4*, 276–284.

LaCava, J., Houseley, J., Saveanu, C., Petfalski, E., Thompson, E., Jacquier, A., and Tollervey, D. (2005). RNA degradation by the exosome is promoted by a nuclear polyadenylation complex. Cell *121*, 713–724.

Martens, J.A., Laprade, L., and Winston, F. (2004). Intergenic transcription is required to repress the Saccharomyces cerevisiae SER3 gene. Nature *429*, 571–574.

Moazed, D., Buhler, M., Buker, S.M., Colmenares, S.U., Gerace, E.L., Gerber, S.A., Hong, E.J., Motamedi, M.R., Verdel, A., Villen, J., et al. (2006). Studies on the mechanism of RNAi-dependent heterochromatin assembly. Cold Spring Harb. Symp. Quant. Biol. *71*, 461–471.

Motamedi, M.R., Verdel, A., Colmenares, S.U., Gerber, S.A., Gygi, S.P., and Moazed, D. (2004). Two RNAi complexes, RITS and RDRC, physically interact and localize to noncoding centromeric RNAs. Cell *119*, 789–802.

Nicolas, E., Yamada, T., Cam, H.P., Fitzgerald, P.C., Kobayashi, R., and Grewal, S.I. (2007). Distinct roles of HDAC complexes in promoter silencing, antisense suppression and DNA damage protection. Nat. Struct. Mol. Biol. *14*, 372–380.

Ogawa, N., DeRisi, J., and Brown, P.O. (2000). New components of a system for phosphate accumulation and polyphosphate metabolism in Saccharomyces cerevisiae revealed by genomic expression analysis. Mol. Biol. Cell *11*, 4309–4321.

Phillips, S., and Butler, J.S. (2003). Contribution of domain structure to the RNA 3′ end processing and degradation functions of the nuclear exosome subunit Rrp6p. RNA *9*, 1098–1107.

Robyr, D., Suka, Y., Xenarios, I., Kurdistani, S.K., Wang, A., Suka, N., and Grunstein, M. (2002). Microarray deacetylation maps determine genome-wide functions for yeast histone deacetylases. Cell *109*, 437–446.

Roth, K.M., Wolf, M.K., Rossi, M., and Butler, J.S. (2005). The nuclear exosome contributes to autogenous control of NAB2 mRNA levels. Mol. Cell. Biol. *25*, 1577–1585.

Uhler, J.P., Hertel, C., and Svejstrup, J.Q. (2007). A role for noncoding transcription in activation of the yeast PHO5 gene. Proc. Natl. Acad. Sci. USA *104*, 8011–8016.

Vanacova, S., Wolf, J., Martin, G., Blank, D., Dettwiler, S., Friedlein, A., Langen, H., Keith, G., and Keller, W. (2005). A new yeast poly(A)

polymerase complex involved in RNA quality control. PLoS Biol. *3*, e189.

Volpe, T.A., Kidner, C., Hall, I.M., Teng, G., Grewal, S.I., and Martienssen, R.A. (2002). Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science *297*, 1833–1837.

Voynov, V., Verstrepen, K.J., Jansen, A., Runner, V.M., Buratowski, S., and Fink, G.R. (2006). Genes with internal repeats require the THO complex for transcription. Proc. Natl. Acad. Sci. USA *103*, 14423–14428.

Wu, J., Carmen, A.A., Kobayashi, R., Suka, N., and Grunstein, M. (2001a). HDA2 and HDA3 are related proteins that interact with and are essential for the activity of the yeast histone deacetylase HDA1. Proc. Natl. Acad. Sci. USA *98*, 4391–4396.

Wu, J., Suka, N., Carlson, M., and Grunstein, M. (2001b). TUP1 utilizes histone H3/H2B-specific HDA1 deacetylase to repress gene activity in yeast. Mol. Cell *7*, 117–126.

Wyers, F., Rougemaille, M., Badis, G., Rousselle, J.C., Dufour, M.E., Boulay, J., Regnault, B., Devaux, F., Namane, A., Seraphin, B., et al. (2005). Cryptic pol II transcripts are degraded by a nuclear quality control pathway involving a new poly(A) polymerase. Cell *121*, 725–737.

Yamada, T., Fischle, W., Sugiyama, T., Allis, C.D., and Grewal, S.I. (2005). The nucleation and maintenance of heterochromatin by a histone deacetylase in fission yeast. Mol. Cell *20*, 173–185.

Yang, P.K., and Kuroda, M.I. (2007). Noncoding RNAs and intranuclear positioning in monoallelic gene expression. Cell *128*, 777–786.

Zenklusen, D., Vinciguerra, P., Wyss, J.C., and Stutz, F. (2002). Stable mRNP formation and export require cotranscriptional recruitment of the mRNA export factors Yra1p and Sub2p by Hpr1p. Mol. Cell. Biol. *22*, 8241–8253.

5 References

Abruzzi, K.C., Belostotsky, D.A., Chekanova, J.A., Dower, K., and Rosbash, M. (2006). 3'-end formation signals modulate the association of genes with the nuclear periphery as well as mRNP dot formation. Embo J.

Abruzzi, K.C., Lacadie, S., and Rosbash, M. (2004). Biochemical analysis of TREX complex recruitment to intronless and intron-containing yeast genes. Embo J *23*, 2620-2631.

Acar, M., Becskei, A., and van Oudenaarden, A. (2005). Enhancement of cellular memory by reducing stochastic transitions. Nature *435*, 228-232.

Aebi, U., Cohn, J., Buhle, L., and Gerace, L. (1986). The nuclear lamina is a meshwork of intermediate-type filaments. Nature *323*, 560-564.

Aguilera, A. (2005). Cotranscriptional mRNP assembly: from the DNA to the nuclear pore. Curr Opin Cell Biol *17*, 242-250.

Ahn, S.H., Kim, M., and Buratowski, S. (2004). Phosphorylation of serine 2 within the RNA polymerase II C-terminal domain couples transcription and 3' end processing. Mol Cell *13*, 67-76.

Akhtar, A., and Gasser, S.M. (2007). The nuclear envelope and transcriptional control. Nat Rev Genet *8*, 507-517.

Alcazar-Roman, A.R., Tran, E.J., Guo, S., and Wente, S.R. (2006). Inositol hexakisphosphate and Gle1 activate the DEAD-box protein Dbp5 for nuclear mRNA export. Nat Cell Biol *8*, 711-716.

Allen, T.D., Cronshaw, J.M., Bagley, S., Kiseleva, E., and Goldberg, M.W. (2000). The nuclear pore complex: mediator of translocation between nucleus and cytoplasm. J Cell Sci *113 (Pt 10)*, 1651-1659.

Anderson, J.T., Wilson, S.M., Datar, K.V., and Swanson, M.S. (1993). NAB2: a yeast nuclear polyadenylated RNA-binding protein essential for cell viability. Mol Cell Biol *13*, 2730-2741.

Andrulis, E.D., Neiman, A.M., Zappulla, D.C., and Sternglanz, R. (1998). Perinuclear localization of chromatin facilitates transcriptional silencing. Nature *394*, 592-595.

Andrulis, E.D., Werner, J., Nazarian, A., Erdjument-Bromage, H., Tempst, P., and Lis, J.T. (2002a). The RNA processing exosome is linked to elongating RNA polymerase II in Drosophila. Nature *420*, 837-841.

Andrulis, E.D., Zappulla, D.C., Ansari, A., Perrod, S., Laiosa, C.V., Gartenberg, M.R., and Sternglanz, R. (2002b). Esc1, a nuclear periphery protein required for Sir4-based plasmid anchoring and partitioning. Mol Cell Biol *22*, 8292-8301.

Ansari, A., and Hampsey, M. (2005). A role for the CPF 3'-end processing machinery in RNAP II-dependent gene looping. Genes Dev *19*, 2969-2978.

Bachi, A., Braun, I.C., Rodrigues, J.P., Pante, N., Ribbeck, K., von Kobbe, C., Kutay, U., Wilm, M., Gorlich, D., Carmo-Fonseca, M., *et al.* (2000). The C-terminal domain of TAP interacts with the nuclear pore complex and promotes export of specific CTE-bearing RNA substrates. Rna *6*, 136-158.

Balasubramanian, R., Pray-Grant, M.G., Selleck, W., Grant, P.A., and Tan, S. (2002). Role of the Ada2 and Ada3 transcriptional coactivators in histone acetylation. J Biol Chem *277*, 7989-7995.

Bangs, P., Burke, B., Powers, C., Craig, R., Purohit, A., and Doxsey, S. (1998). Functional analysis of Tpr: identification of nuclear pore complex association and nuclear localization domains and a role in mRNA export. J Cell Biol *143*, 1801-1812.

Bear, J., Tan, W., Zolotukhin, A.S., Tabernero, C., Hudson, E.A., and Felber, B.K. (1999). Identification of novel import and export signals of human TAP, the protein that binds to the constitutive transport element of the type D retrovirus mRNAs. Mol Cell Biol *19*, 6306-6317.

Beelman, C.A., and Parker, R. (1995). Degradation of mRNA in eukaryotes. Cell *81*, 179-183.

Belmont, A.S., Zhai, Y., and Thilenius, A. (1993). Lamin B distribution and association with peripheral chromatin revealed by optical sectioning and electron microscopy tomography. J Cell Biol *123*, 1671-1685.

Bentley (2005). Rules of engagement: co-transcriptional recruitment of pre-mRNA processing factors. Curr Opin Cell Biol 2005 *17*, 251-256.

Berger, A., Cabal GG, Fabre E, Duong T, Buc H, Nehrbass U, Olivo-Marin JC, Gadal O, Zimmer C. (2008). High-resolution statistical mapping reveals gene territories in live yeast. Nat Methods *5*, 1031-1037.

Bhat, P.J., and Hopper, J.E. (1992). Overproduction of the GAL1 or GAL3 protein causes galactose-independent activation of the GAL4 protein: evidence for a new model of induction for the yeast GAL/MEL regulon. Mol Cell Biol *12*, 2701-2707.

Bi, X., and Broach, J.R. (1999). UASrpg can function as a heterochromatin boundary element in yeast. Genes Dev *13*, 1089-1101.

Bird, G., Zorio, D.A., and Bentley, D.L. (2004). RNA polymerase II carboxy-terminal domain phosphorylation is required for cotranscriptional pre-mRNA splicing and 3'-end formation. Mol Cell Biol *24*, 8963-8969.

Blobel, G. (1985). Gene gating: a hypothesis. Proc Natl Acad Sci U S A *82*, 8527-8529.

Bohnsack, M.T., Czaplinski, K., and Gorlich, D. (2004). Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. Rna *10*, 185-191.

Bousquet-Antonelli, C., Presutti, C., and Tollervey, D. (2000). Identification of a regulated pathway for nuclear pre-mRNA turnover. Cell *102*, 765-775.

Braun, I.C., Herold, A., Rode, M., Conti, E., and Izaurralde, E. (2001). Overexpression of TAP/p15 heterodimers bypasses nuclear retention and stimulates nuclear mRNA export. J Biol Chem *276*, 20536-20543.

Braun, I.C., Herold, A., Rode, M., and Izaurralde, E. (2002). Nuclear export of mRNA by TAP/NXF1 requires two nucleoporin-binding sites but not p15. Mol Cell Biol *22*, 5405-5418.

Braun, I.C., Rohrbach, E., Schmitt, C., and Izaurralde, E. (1999). TAP binds to the constitutive transport element (CTE) through a novel RNA-binding motif that is sufficient to promote CTE-dependent RNA export from the nucleus. Embo J *18*, 1953-1965.

Brickner, D., Cajigas I, Fondufe-Mittendorf Y, Ahmed S, Lee PC, Widom J, Brickner JH. (2007). H2A.Z-mediated localization of genes at the nuclear periphery confers epigenetic memory of previous transcriptional state. PLoS Biol *5*, e81.

Brickner, J.H., and Walter, P. (2004). Gene recruitment of the activated INO1 locus to the nuclear membrane. PLoS Biol *2*, e342.

Brodsky, A.S., and Silver, P.A. (2000). Pre-mRNA processing factors are required for nuclear export. Rna *6*, 1737-1749.

Brown, C.E., and Sachs, A.B. (1998). Poly(A) tail length control in Saccharomyces cerevisiae occurs by message-specific deadenylation. Mol Cell Biol *18*, 6548-6559.

Bryk, M., Briggs, S.D., Strahl, B.D., Curcio, M.J., Allis, C.D., and Winston, F. (2002). Evidence that Set1, a factor required for methylation of histone H3, regulates rDNA silencing in S. cerevisiae by a Sir2-independent mechanism. Curr Biol *12*, 165-170.

Bucheli, M.E., and Buratowski, S. (2005). Npl3 is an antagonist of mRNA 3' end formation by RNA polymerase II. Embo J *24*, 2150-2160.

Buratowski, S. (2003). The CTD code. Nat Struct Biol *10*, 679-680.

Burgess-Beusse, B., Farrell, C., Gaszner, M., Litt, M., Mutskov, V., Recillas-Targa, F., Simpson, M., West, A., and Felsenfeld, G. (2002). The insulation of genes from external enhancers and silencing chromatin. Proc Natl Acad Sci U S A *99 Suppl 4*, 16433-16437.

Burkard, K.T., and Butler, J.S. (2000). A nuclear 3'-5' exonuclease involved in mRNA degradation interacts with Poly(A) polymerase and the hnRNA protein Npl3p. Mol Cell Biol *20*, 604-616.

Cabal, G.G., Genovesio, A., Rodriguez-Navarro, S., Zimmer, C., Gadal, O., Lesne, A., Buc, H., Feuerbach-Fournier, F., Olivo-Marin, J.C., Hurt, E.C., *et al.* (2006). SAGA interacting factors confine sub-diffusion of transcribed genes to the nuclear envelope. Nature *441*, 770-773.

Capell, B.C., and Collins, F.S. (2006). Human laminopathies: nuclei gone genetically awry. Nat Rev Genet *7*, 940-952.

Casolari, J.M., Brown, C.R., Drubin, D.A., Rando, O.J., and Silver, P.A. (2005). Developmentally induced changes in transcriptional program alter spatial organization across chromosomes. Genes Dev *19*, 1188-1198.

Casolari, J.M., Brown, C.R., Komili, S., West, J., Hieronymus, H., and Silver, P.A. (2004). Genome-wide localization of the nuclear transport machinery couples transcriptional status and nuclear organization. Cell *117*, 427-439.

Chang, M., Bellaoui, M., Boone, C., and Brown, G.W. (2002). A genome-wide screen for methyl methanesulfonate-sensitive mutants reveals genes required for S phase progression in the presence of DNA damage. Proc Natl Acad Sci U S A *99*, 16934-16939.

Chavez, S., Beilharz, T., Rondon, A.G., Erdjument-Bromage, H., Tempst, P., Svejstrup, J.Q., Lithgow, T., and Aguilera, A. (2000). A protein complex containing Tho2, Hpr1, Mft1 and a novel protein, Thp2, connects transcription elongation with mitotic recombination in Saccharomyces cerevisiae. Embo J *19*, 5824-5834.

Chavez, S., Garcia-Rubio, M., Prado, F., and Aguilera, A. (2001). Hpr1 is preferentially required for transcription of either long or G+C-rich DNA sequences in Saccharomyces cerevisiae. Mol Cell Biol *21*, 7054-7064.

Chekanova, J.A., Abruzzi, K.C., Rosbash, M., and Belostotsky, D.A. (2008). Sus1, Sac3, and Thp1 mediate post-transcriptional tethering of active genes to the nuclear rim as well as to non-nascent mRNP. RNA *14*, 66-77.

Chen, X.L., Reindle, A., and Johnson, E.S. (2005). Misregulation of 2 microm circle copy number in a SUMO pathway mutant. Mol Cell Biol *25*, 4311-4320.

Cheng, H., Dufu, K., Lee, C.S., Hsu, J.L., Dias, A., and Reed, R. (2006). Human mRNA export machinery recruited to the 5' end of mRNA. Cell *127*, 1389-1400.

Chial, H.J., Rout, M.P., Giddings, T.H., and Winey, M. (1998). Saccharomyces cerevisiae Ndc1p is a shared component of nuclear pore complexes and spindle pole bodies. J Cell Biol *143*, 1789-1800.

Chook, Y.M., and Blobel, G. (2001). Karyopherins and nuclear import. Curr Opin Struct Biol *11*, 703-715.

Chung, J.H., Whiteley, M., and Felsenfeld, G. (1993). A 5' element of the chicken beta-globin domain serves as an insulator in human erythroid cells and protects against position effect in Drosophila. Cell *74*, 505-514.

Cole, C.N., and Scarcelli, J.J. (2006). Unravelling mRNA export. Nat Cell Biol *8*, 645-647.

Colot, H.V., Stutz, F., and Rosbash, M. (1996). The yeast splicing factor Mud13p is a commitment complex component and corresponds to CBP20, the small subunit of the nuclear cap-binding complex. Genes Dev *10*, 1699-1708.

Comings, D.E. (1968). The rationale for an ordered arrangement of chromatin in the interphase nucleus. Am J Hum Genet *20*, 440-460.

Conlan, R.S., Gounalaki, N., Hatzis, P., and Tzamarias, D. (1999). The Tup1-Cyc8 protein complex can shift from a transcriptional co-repressor to a transcriptional co-activator. J Biol Chem *274*, 205-210.

Cooke, C., and Alwine, J.C. (1996). The cap and the 3' splice site similarly affect polyadenylation efficiency. Mol Cell Biol *16*, 2579-2584.

Cordes, V.C., Hase, M.E., and Muller, L. (1998). Molecular segments of protein Tpr that confer nuclear targeting and association with the nuclear pore complex. Exp Cell Res *245*, 43-56.

Cordes, V.C., Reidenbach, S., Rackwitz, H.R., and Franke, W.W. (1997). Identification of protein p270/Tpr as a constitutive component of the nuclear pore complex-attached intranuclear filaments. J Cell Biol *136*, 515-529.

Cronshaw, J.M., Krutchinsky, A.N., Zhang, W., Chait, B.T., and Matunis, M.J. (2002). Proteomic analysis of the mammalian nuclear pore complex. J Cell Biol *158*, 915-927.

Custodio, N., Carvalho, C., Condado, I., Antoniou, M., Blencowe, B.J., and Carmo-Fonseca, M. (2004). In vivo recruitment of exon junction complex proteins to transcription sites in mammalian cell nuclei. RNA *10*, 622-633.

Daneholt, B. (2001). Assembly and transport of a premessenger RNP particle. Proc Natl Acad Sci U S A *98*, 7012-7017.

Das, B., Butler, J.S., and Sherman, F. (2003). Degradation of normal mRNA in the nucleus of Saccharomyces cerevisiae. Mol Cell Biol *23*, 5502-5515.

Das, B., Guo, Z., Russo, P., Chartrand, P., and Sherman, F. (2000). The role of nuclear cap binding protein Cbc1p of yeast in mRNA termination and degradation. Mol Cell Biol *20*, 2827-2838.

De Souza, C.P., Hashmi, S.B., Horn, K.P., and Osmani, S.A. (2006). A point mutation in the Aspergillus nidulans sonBNup98 nuclear pore complex gene causes conditional DNA damage sensitivity. Genetics *174*, 1881-1893.

Dechat, T., Pfleghaar, K., Sengupta, K., Shimi, T., Shumaker, D.K., Solimando, L., and Goldman, R.D. (2008). Nuclear lamins: major factors in the structural organization and function of the nucleus and chromatin. Genes Dev *22*, 832-853.

Deminoff, S.J., and Santangelo, G.M. (2001). Rap1p requires Gcr1p and Gcr2p homodimers to activate ribosomal protein and glycolytic genes, respectively. Genetics *158*, 133-143.

Devos, D., Dokudovskaya, S., Williams, R., Alber, F., Eswar, N., Chait, B.T., Rout, M.P., and Sali, A. (2006). Simple fold composition and modular architecture of the nuclear pore complex. Proc Natl Acad Sci U S A *103*, 2172-2177.

Dichtl , A.R., Keller W (2004). Functions for S. cerevisiae Swd2p in 3' end formation of specific mRNAs and snoRNAs and global histone 3 lysine 4 methylation. RNA *10*, 965-977.

Dichtl, B., Blank, D., Sadowski, M., Hubner, W., Weiser, S., and Keller, W. (2002). Yhh1p/Cft1p directly links poly(A) site recognition and RNA polymerase II transcription termination. EMBO J *21*, 4125-4135.

Dichtl, B., and Keller, W. (2001). Recognition of polyadenylation sites in yeast pre-mRNAs by cleavage and polyadenylation factor. EMBO J *20*, 3197-3209.

Dieppois, G., Iglesias, N., and Stutz, F. (2006). Co-transcriptional recruitment of the mRNA export receptor Mex67p contributes to nuclear pore anchoring of activated genes. Mol Cell Biol.

Dilworth, D.J., Tackett, A.J., Rogers, R.S., Yi, E.C., Christmas, R.H., Smith, J.J., Siegel, A.F., Chait, B.T., Wozniak, R.W., and Aitchison, J.D. (2005). The mobile nucleoporin Nup2p and chromatin-bound Prp20p function in endogenous NPC-mediated transcriptional control. J Cell Biol *171*, 955-965.

Dobson, M.J., Pickett, A.J., Velmurugan, S., Pinder, J.B., Barrett, L.A., Jayaram, M., and Chew, J.S. (2005). The 2 microm plasmid causes cell death in Saccharomyces cerevisiae with a mutation in Ulp1 protease. Mol Cell Biol *25*, 4299-4310.

Donze, D., and Kamakaka, R.T. (2001). RNA polymerase III and RNA polymerase II promoter complexes are heterochromatin barriers in Saccharomyces cerevisiae. EMBO J *20*, 520-531.

Dower, K., Kuperwasser, N., Merrikh, H., and Rosbash, M. (2004). A synthetic A tail rescues yeast nuclear accumulation of a ribozyme-terminated transcript. Rna *10*, 1888-1899.

Dower, K., and Rosbash, M. (2002). T7 RNA polymerase-directed transcripts are processed in yeast and link 3' end formation to mRNA nuclear export. Rna *8*, 686-697.

Dreyfuss, G., Kim, V.N., and Kataoka, N. (2002). Messenger-RNA-binding proteins and the messages they carry. Nat Rev Mol Cell Biol *3*, 195-205.

Driller, L., Wellinger, R.J., Larrivee, M., Kremmer, E., Jaklin, S., and Feldmann, H.M. (2000). A short C-terminal domain of Yku70p is essential for telomere maintenance. J Biol Chem *275*, 24921-24927.

Drubin DA, G.A., Silver PA (2006). Motion as a phenotype: the use of live-cell imaging and machine visual screening to characterize transcription-dependent chromosome. BMC Cell Biol *24*, 7:19.

Dunn, E.F., Hammell, C.M., Hodge, C.A., and Cole, C.N. (2005). Yeast poly(A)-binding protein, Pab1, and PAN, a poly(A) nuclease complex recruited by Pab1, connect mRNA biogenesis to export. Genes Dev *19*, 90-103.

Duraisingh, M.T., Voss, T.S., Marty, A.J., Duffy, M.F., Good, R.T., Thompson, J.K., Freitas-Junior, L.H., Scherf, A., Crabb, B.S., and Cowman, A.F. (2005). Heterochromatin silencing and locus repositioning linked to regulation of virulence genes in Plasmodium falciparum. Cell *121*, 13-24.

Erber, A., Riemer, D., Hofemeister, H., Bovenschulte, M., Stick, R., Panopoulou, G., Lehrach, H., and Weber, K. (1999). Characterization of the Hydra lamin and its gene: A molecular phylogeny of metazoan lamins. J Mol Evol *49*, 260-271.

Estruch, F., and Cole, C.N. (2003). An early function during transcription for the yeast mRNA export factor Dbp5p/Rat8p suggested by its genetic and physical interactions with transcription factor IIH components. Mol Biol Cell *14*, 1664-1676.

Fabre, E., and Hurt, E. (1997). Yeast genetics to dissect the nuclear pore complex and nucleocytoplasmic trafficking. Annu Rev Genet *31*, 277-313.

Fahrenkrog, B., and Aebi, U. (2003). The nuclear pore complex: nucleocytoplasmic transport and beyond. Nat Rev Mol Cell Biol *4*, 757-766.

Fasken, M.B., and Corbett, A.H. (2005). Process or perish: quality control in mRNA biogenesis. Nat Struct Mol Biol *12*, 482-488.

Fasken, M.B., Stewart, M., and Corbett, A.H. (2008). Functional significance of the interaction between the mRNA-binding protein, Nab2, and the nuclear pore-associated protein, Mlp1, in mRNA export. J Biol Chem *283*, 27130-27143.

Feuerbach, F., Galy, V., Trelles-Sticken, E., Fromont-Racine, M., Jacquier, A., Gilson, E., Olivo-Marin, J.C., Scherthan, H., and Nehrbass, U. (2002). Nuclear architecture and spatial positioning help establish transcriptional states of telomeres in yeast. Nat Cell Biol *4*, 214-221.

Fischer, T., Strasser, K., Racz, A., Rodriguez-Navarro, S., Oppizzi, M., Ihrig, P., Lechner, J., and Hurt, E. (2002). The mRNA export machinery requires the novel Sac3p-Thp1p complex to dock at the nucleoplasmic entrance of the nuclear pores. EMBO J *21*, 5843-5852.

Fleckner, J., Zhang, M., Valcarcel, J., and Green, M.R. (1997). U2AF65 recruits a novel human DEAD box protein required for the U2 snRNP-branchpoint interaction. Genes Dev *11*, 1864-1872.

Freeman, K., Gwadz, M., and Shore, D. (1995). Molecular and genetic analysis of the toxic effect of RAP1 overexpression in yeast. Genetics *141*, 1253-1262.

Fribourg, S., Braun, I.C., Izaurralde, E., and Conti, E. (2001). Structural basis for the recognition of a nucleoporin FG repeat by the NTF2-like domain of the TAP/p15 mRNA nuclear export factor. Mol Cell *8*, 645-656.

Frolova, E., Johnston, M., and Majors, J. (1999). Binding of the glucose-dependent Mig1p repressor to the GAL1 and GAL4 promoters in vivo: regulationby glucose and chromatin structure. Nucleic Acids Res *27*, 1350-1358.

Gallardo, M., and Aguilera, A. (2001). A new hyperrecombination mutation identifies a novel yeast gene, THP1, connecting transcription elongation with mitotic recombination. Genetics *157*, 79-89.

Gallardo, M., Luna, R., Erdjument-Bromage, H., Tempst, P., and Aguilera, A. (2003). Nab2p and the Thp1p-Sac3p complex functionally interact at the interface between transcription and mRNA metabolism. J Biol Chem *278*, 24225-24232.

Galy, V., Gadal, O., Fromont-Racine, M., Romano, A., Jacquier, A., and Nehrbass, U. (2004). Nuclear retention of unspliced mRNAs in yeast is mediated by perinuclear Mlp1. Cell *116*, 63-73.

Galy, V., Olivo-Marin, J.C., Scherthan, H., Doye, V., Rascalou, N., and Nehrbass, U. (2000). Nuclear pore complexes in the organization of silent telomeric chromatin. Nature *403*, 108-112.

Garcia-Rubio, M., Chavez, S., Huertas, P., Tous, C., Jimeno, S., Luna, R., and Aguilera, A. (2008). Different physiological relevance of yeast THO/TREX subunits in gene expression and genome integrity. Mol Genet Genomics *279*, 123-132.

Gartenberg, M.R., Neumann, F.R., Laroche, T., Blaszczyk, M., and Gasser, S.M. (2004). Sir-mediated repression can occur independently of chromosomal and subnuclear contexts. Cell *119*, 955-967.

Gasch, A.P., and Werner-Washburne, M. (2002). The genomics of yeast responses to environmental stress and starvation. Funct Integr Genomics *2*, 181-192.

Gatfield, D., Le Hir, H., Schmitt, C., Braun, I.C., Kocher, T., Wilm, M., and Izaurralde, E. (2001). The DExH/D box protein HEL/UAP56 is essential for mRNA nuclear export in Drosophila. Curr Biol *11*, 1716-1721.

Gauss, R., Trautwein, M., Sommer, T., and Spang, A. (2005). New modules for the repeated internal and N-terminal epitope tagging of genes in Saccharomyces cerevisiae. Yeast *22*, 1-12.

Gavin, A.C., Aloy, P., Grandi, P., Krause, R., Boesche, M., Marzioch, M., Rau, C., Jensen, L.J., Bastuck, S., Dumpelfeld, B., *et al.* (2006). Proteome survey reveals modularity of the yeast cell machinery. Nature *440*, 631-636.

Gavin, A.C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J.M., Michon, A.M., Cruciat, C.M., *et al.* (2002). Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature *415*, 141-147.

Gerasimova, T.I., Byrd, K., and Corces, V.G. (2000). A chromatin insulator determines the nuclear localization of DNA. Mol Cell *6*, 1025-1035.

Gilbert, W., and Guthrie, C. (2004). The Glc7p nuclear phosphatase promotes mRNA export by facilitating association of Mex67p with mRNA. Mol Cell *13*, 201-212.

Gill, G. (2005). Something about SUMO inhibits transcription. Curr Opin Genet Dev *15*, 536-541.

Gligoris, T., Thireos, G., and Tzamarias, D. (2007). The Tup1 corepressor directs Htz1 deposition at a specific promoter nucleosome marking the GAL1 gene for rapid activation. Mol Cell Biol *27*, 4198-4205.

Goldberg, M., Harel, A., and Gruenbaum, Y. (1999). The nuclear lamina: molecular organization and interaction with chromatin. Crit Rev Eukaryot Gene Expr *9*, 285-293.

Goldman, A.E., Moir, R.D., Montag-Lowy, M., Stewart, M., and Goldman, R.D. (1992). Pathway of incorporation of microinjected lamin A into the nuclear envelope. J Cell Biol *119*, 725-735.

Goldman, R.D., Shumaker, D.K., Erdos, M.R., Eriksson, M., Goldman, A.E., Gordon, L.B., Gruenbaum, Y., Khuon, S., Mendez, M., Varga, R., *et al.* (2004). Accumulation of mutant lamin A causes progressive changes in nuclear architecture in Hutchinson-Gilford progeria syndrome. Proc Natl Acad Sci U S A *101*, 8963-8968.

Gonzalez-Aguilera, C., Tous, C., Gomez-Gonzalez, B., Huertas, P., Luna, R., and Aguilera, A. (2008). The THP1-SAC3-SUS1-CDC31 complex works in transcription elongation-mRNA export preventing RNA-mediated genome instability. Mol Biol Cell *19*, 4310-4318.

Gorlich, D., Kraft, R., Kostka, S., Vogel, F., Hartmann, E., Laskey, R.A., Mattaj, I.W., and Izaurraide, E. (1996). Importin provides a link between nuclear protein import and U snRNA export. Cell *87*, 21-32.

Gorlich, D., and Kutay, U. (1999). Transport between the cell nucleus and the cytoplasm. Annu Rev Cell Dev Biol *15*, 607-660.

Gornemann, J., Kotovic, K.M., Hujer, K., and Neugebauer, K.M. (2005). Cotranscriptional spliceosome assembly occurs in a stepwise fashion and requires the cap binding complex. Mol Cell *19*, 53-63.

Gorski, S.A., Dundr, M., and Misteli, T. (2006). The road much traveled: trafficking in the cell nucleus. Curr Opin Cell Biol *18*, 284-290.

Gotta, M., Gasser, S.M. (1996). Nuclear organization and transcriptional silencing in yeast. Experientia. 1996 Dec 15;52(12):1136-47.

Gotta, M., Laroche, T., Formenton, A., Maillet, L., Scherthan, H., and Gasser, S.M. (1996). The clustering of telomeres and colocalization with Rap1, Sir3, and Sir4 proteins in wild-type Saccharomyces cerevisiae. J Cell Biol *134*, 1349-1363.

Grant, P.A., Duggan, L., Cote, J., Roberts, S.M., Brownell, J.E., Candau, R., Ohba, R., Owen-Hughes, T., Allis, C.D., Winston, F., *et al.* (1997). Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex. Genes Dev *11*, 1640-1650.

Grant, R.P., Hurt, E., Neuhaus, D., and Stewart, M. (2002). Structure of the C-terminal FG-nucleoporin binding domain of Tap/NXF1. Nat Struct Biol *9*, 247-251.

Green, D.M., Johnson, C.P., Hagan, H., and Corbett, A.H. (2003). The C-terminal domain of myosin-like protein 1 (Mlp1p) is a docking site for heterogeneous nuclear ribonucleoproteins that are required for mRNA export. Proc Natl Acad Sci U S A *100*, 1010-1015.

Green, D.M., Marfatia, K.A., Crafton, E.B., Zhang, X., Cheng, X., and Corbett, A.H. (2002). Nab2p is required for poly(A) RNA export in Saccharomyces cerevisiae and is regulated by arginine methylation via Hmt1p. J Biol Chem *277*, 7752-7760.

Guelen, L., Pagie, L., Brasset, E., Meuleman, W., Faza, M.B., Talhout, W., Eussen, B.H., de Klein, A., Wessels, L., de Laat, W., *et al.* (2008). Domain organization of human chromosomes revealed by mapping of nuclear lamina interactions. Nature *453*, 948-951.

Guzik, B.W., Levesque, L., Prasad, S., Bor, Y.C., Black, B.E., Paschal, B.M., Rekosh, D., and Hammarskjold, M.L. (2001). NXT1 (p15) is a crucial cellular cofactor in TAP-dependent export of intron-containing RNA in mammalian cells. Mol Cell Biol *21*, 2545-2554.

Gwizdek, C., Iglesias, I., Rodriguez, M.S., Ossareh-Nazari, B., Hobeika, M., Divita, G., Stutz, F. and Dargemont, C. (2006). Ubiquitin-Associated domain of Mex67 synchronizes recruitment of the mRNA export machinery with transription. Proc Natl Acad Sci U S A.

Hamm, J., and Mattaj, I.W. (1990). Monomethylated cap structures facilitate RNA export from the nucleus. Cell *63*, 109-118.

Hammell, C.M., Gross, S., Zenklusen, D., Heath, C.V., Stutz, F., Moore, C., and Cole, C.N. (2002). Coupling of termination, 3' processing, and mRNA export. Mol Cell Biol *22*, 6441-6457.

Han, S.J., Lee, J.S., Kang, J.S., and Kim, Y.J. (2001). Med9/Cse2 and Gal11 modules are required for transcriptional repression of distinct group of genes. J Biol Chem *276*, 37020-37026.

Hannich, J.T., Lewis, A., Kroetz, M.B., Li, S.J., Heide, H., Emili, A., and Hochstrasser, M. (2005). Defining the SUMO-modified proteome by multiple approaches in Saccharomyces cerevisiae. J Biol Chem *280*, 4102-4110.

Harper, N.C., Al-Greene, N.T., Basrai, M.A., and Belanger, K.D. (2008). Mutations affecting spindle pole body and mitotic exit network function are synthetically lethal with a deletion of the nucleoporin NUP1 in S. cerevisiae. Curr Genet *53*, 95-105.

Hautbergue, G.M., Hung, M.L., Golovanov, A.P., Lian, L.Y., and Wilson, S.A. (2008). Mutually exclusive interactions drive handover of mRNA from export adaptors to TAP. Proc Natl Acad Sci U S A *105*, 5154-5159.

He, X., Khan, A.U., Cheng, H., Pappas, D.L., Jr., Hampsey, M., and Moore, C.L. (2003). Functional interactions between the transcription and mRNA 3' end processing machineries mediated by Ssu72 and Sub1. Genes Dev *17*, 1030-1042.

Hector, R.E., Nykamp, K.R., Dheur, S., Anderson, J.T., Non, P.J., Urbinati, C.R., Wilson, S.M., Minvielle-Sebastia, L., and Swanson, M.S. (2002). Dual requirement for yeast hnRNP Nab2p in mRNA poly(A) tail length control and nuclear export. Embo J *21*, 1800-1810.

Hediger, F., Dubrana, K., and Gasser, S.M. (2002a). Myosin-like proteins 1 and 2 are not required for silencing or telomere anchoring, but act in the Tel1 pathway of telomere length control. J Struct Biol *140*, 79-91.

Hediger, F., Neumann, F.R., Van Houwe, G., Dubrana, K., and Gasser, S.M. (2002b). Live Imaging of Telomeres. yKu and Sir Proteins Define Redundant Telomere-Anchoring Pathways in Yeast. Curr Biol *12*, 2076-2089.

Heidmann, J., Poulsen, S., Arnbjerg, D., Kirkegaard, E., and Laurberg, L. (1992). Caries development after termination of a fluoride rinsing program. Community Dent Oral Epidemiol *20*, 118-121.

Herold, A., Klymenko, T., and Izaurralde, E. (2001). NXF1/p15 heterodimers are essential for mRNA nuclear export in Drosophila. RNA *7*, 1768-1780.

Heun, P., Laroche, T., Shimada, K., Furrer, P., and Gasser, S.M. (2001). Chromosome dynamics in the yeast interphase nucleus. Science *294*, 2181-2186.

Hewitt, S.L., High, F.A., Reiner, S.L., Fisher, A.G., and Merkenschlager, M. (2004). Nuclear repositioning marks the selective exclusion of lineage-inappropriate transcription factor loci during T helper cell differentiation. Eur J Immunol *34*, 3604-3613.

Hieronymus, H., and Silver, P.A. (2003). Genome-wide analysis of RNA-protein interactions illustrates specificity of the mRNA export machinery. Nat Genet *33*, 155-161.

Hilleren, P., McCarthy, T., Rosbash, M., Parker, R., and Jensen, T.H. (2001). Quality control of mRNA 3'-end processing is linked to the nuclear exosome. Nature *413*, 538-542.

Hilleren, P., and Parker, R. (2001). Defects in the mRNA export factors Rat7p, Gle1p, Mex67p, and Rat8p cause hyperadenylation during 3'-end formation of nascent transcripts. RNA *7*, 753-764.

Hirose, Y., and Manley, J.L. (2000). RNA polymerase II and the integration of nuclear events. Genes Dev *14*, 1415-1429.

Hobeika , B.C., Iglesias N, Gwizdek C, Neuhaus D, Stutz F, Stewart M, Divita G, Dargemont C. (2007). Coordination of Hpr1 and ubiquitin binding by the UBA domain of the mRNA export factor Mex67. Mol Biol Cell *18*, 2561-2568.

Hochstrasser, M. (1987). Chromosome structure in four wild-type polytene tissues of Drosophila melanogaster. The 87A and 87C heat shock loci are induced unequally in the midgut in a manner dependent on growth temperature. Chromosoma *95*, 197-208.

Hochstrasser, M., Mathog, D., Gruenbaum, Y., Saumweber, H., and Sedat, J.W. (1986). Spatial organization of chromosomes in the salivary gland nuclei of Drosophila melanogaster. J Cell Biol *102*, 112-123.

Hochstrasser, M., and Sedat, J.W. (1987). Three-dimensional organization of Drosophila melanogaster interphase nuclei. II. Chromosome spatial organization and gene regulation. J Cell Biol *104*, 1471-1483.

Hodge, C.A., Colot, H.V., Stafford, P., and Cole, C.N. (1999). Rat8p/Dbp5p is a shuttling transport factor that interacts with Rat7p/Nup159p and Gle1p and suppresses the mRNA export defect of xpo1-1 cells. EMBO J *18*, 5778-5788.

Holmer, L., and Worman, H.J. (2001). Inner nuclear membrane proteins: functions and targeting. Cell Mol Life Sci *58*, 1741-1747.

Hosoda, N., Kim, Y.K., Lejeune, F., and Maquat, L.E. (2005). CBP80 promotes interaction of Upf1 with Upf2 during nonsense-mediated mRNA decay in mammalian cells. Nat Struct Mol Biol *12*, 893-901.

Huang, J., and Moazed, D. (2003). Association of the RENT complex with nontranscribed and coding regions of rDNA and a regional requirement for the replication fork block protein Fob1 in rDNA silencing. Genes Dev *17*, 2162-2176.

Huang, Y., and Carmichael, G.C. (1996). Role of polyadenylation in nucleocytoplasmic transport of mRNA. Mol Cell Biol *16*, 1534-1542.

Huertas, P., and Aguilera, A. (2003). Cotranscriptionally formed DNA:RNA hybrids mediate transcription elongation impairment and transcription-associated recombination. Mol Cell *12*, 711-721.

Hurt, E., Strasser, K., Segref, A., Bailer, S., Schlaich, N., Presutti, C., Tollervey, D., and Jansen, R. (2000). Mex67p mediates nuclear export of a variety of RNA polymerase II transcripts. J Biol Chem *275*, 8361-8368.

Hutchison, N., and Weintraub, H. (1985). Localization of DNAase I-sensitive sequences to specific regions of interphase nuclei. Cell *43*, 471-482.

Iouk, T., Kerscher, O., Scott, R.J., Basrai, M.A., and Wozniak, R.W. (2002). The yeast nuclear pore complex functionally interacts with components of the spindle assembly checkpoint. J Cell Biol *159*, 807-819.

Ishii, K., Arib, G., Lin, C., Van Houwe, G., and Laemmli, U.K. (2002). Chromatin boundaries in budding yeast: the nuclear pore connection. Cell *109*, 551-562.

Izaurralde, E. (2002). Nuclear export of messenger RNA. Results Probl Cell Differ *35*, 133-150.

Izaurralde, E., Lewis, J., McGuigan, C., Jankowska, M., Darzynkiewicz, E., and Mattaj, I.W. (1994). A nuclear cap binding protein complex involved in pre-mRNA splicing. Cell *78*, 657-668.

Jarmolowski, A., Boelens, W.C., Izaurralde, E., and Mattaj, I.W. (1994). Nuclear export of different classes of RNA is mediated by specific factors. J Cell Biol *124*, 627-635.

Jaspersen, S.L., and Winey, M. (2004). The budding yeast spindle pole body: structure, duplication, and function. Annu Rev Cell Dev Biol *20*, 1-28.

Jensen, T.H., Boulay, J., Olesen, J.R., Colin, J., Weyler, M., and Libri, D. (2004). Modulation of transcription affects mRNP quality. Mol Cell *16*, 235-244.

Jensen, T.H., Boulay, J., Rosbash, M., and Libri, D. (2001a). The DECD box putative ATPase Sub2p is an early mRNA export factor. Curr Biol *11*, 1711-1715.

Jensen, T.H., Dower, K., Libri, D., and Rosbash, M. (2003). Early formation of mRNP: license for export or quality control? Mol Cell *11*, 1129-1138.

Jensen, T.H., Patricio, K., McCarthy, T., and Rosbash, M. (2001b). A block to mRNA nuclear export in S. cerevisiae leads to hyperadenylation of transcripts that accumulate at the site of transcription. Mol Cell *7*, 887-898.

Johnson, E. (2004). Protein modification by SUMO. Annu Rev Biochem *73*, 355-382.

Johnson, E.S., and Blobel, G. (1999). Cell cycle-regulated attachment of the ubiquitin-related protein SUMO to the yeast septins. J Cell Biol *147*, 981-994.

Johnson, S.A., Cubberley, G., and Bentley, D.L. (2009). Cotranscriptional recruitment of the mRNA export factor Yra1 by direct interaction with the 3' end processing factor Pcf11. Mol Cell *33*, 215-226.

Johnston, M. (1999). Feasting, fasting and fermenting. Glucose sensing in yeast and other cells. Trends Genet *15*, 29-33.

Kadowaki, T., Zhao, Y., and Tartakoff, A.M. (1992). A conditional yeast mutant deficient in mRNA transport from nucleus to cytoplasm. Proc Natl Acad Sci U S A *89*, 2312-2316.

Kang, Y., and Cullen, B.R. (1999). The human Tap protein is a nuclear mRNA export factor that contains novel RNA-binding and nucleocytoplasmic transport sequences. Genes Dev *13*, 1126-1139.

Kashyap, A.K., Schieltz, D., Yates, J., 3rd, and Kellogg, D.R. (2005). Biochemical and genetic characterization of Yra1p in budding yeast. Yeast *22*, 43-56.

Katahira, J., Strasser, K., Podtelejnikov, A., Mann, M., Jung, J.U., and Hurt, E. (1999). The Mex67p-mediated nuclear mRNA export pathway is conserved from yeast to human. Embo J *18*, 2593-2609.

Kataoka, N., Yong, J., Kim, V.N., Velazquez, F., Perkinson, R.A., Wang, F., and Dreyfuss, G. (2000). Pre-mRNA splicing imprints mRNA in the nucleus with a novel RNA-binding protein that persists in the cytoplasm. Mol Cell *6*, 673-682.

Kellum, R., and Schedl, P. (1991). A position-effect assay for boundaries of higher order chromosomal domains. Cell *64*, 941-950.

Kiesler, E., Miralles, F., and Visa, N. (2002). HEL/UAP56 binds cotranscriptionally to the Balbiani ring pre-mRNA in an intron-independent manner and accompanies the BR mRNP to the nuclear pore. Curr Biol *12*, 859-862.

Kim, M., Ahn, S.H., Krogan, N.J., Greenblatt, J.F., and Buratowski, S. (2004). Transitions in RNA polymerase II elongation complexes at the 3' ends of genes. Embo J *23*, 354-364.

Kohler, A., and Hurt, E. (2007). Exporting RNA from the nucleus to the cytoplasm. Nat Rev Mol Cell Biol *8*, 761-773.

Kohler, A., Schneider, M., Cabal, G.G., Nehrbass, U., and Hurt, E. (2008). Yeast Ataxin-7 links histone deubiquitination with gene gating and mRNA export. Nat Cell Biol *10*, 707-715.

Komarnitsky, P., Cho, E.J., and Buratowski, S. (2000). Different phosphorylated forms of RNA polymerase II and associated mRNA processing factors during transcription. Genes Dev *14*, 2452-2460.

Kosak, S.T., and Groudine, M. (2002). The undiscovered country: chromosome territories and the organization of transcription. Dev Cell *2*, 690-692.

Kosova, B., Pante, N., Rollenhagen, C., Podtelejnikov, A., Mann, M., Aebi, U., and Hurt, E. (2000). Mlp2p, a component of nuclear pore attached intranuclear filaments, associates with nic96p. J Biol Chem *275*, 343-350.

Kotovic, K.M., Lockshon, D., Boric, L., and Neugebauer, K.M. (2003). Cotranscriptional recruitment of the U1 snRNP to intron-containing genes in yeast. Mol Cell Biol *23*, 5768-5779.

Krohne, G., Benavente, R., Scheer, U., and Dabauvalle, M.C. (2005). The nuclear lamina in Heidelberg and Wurzburg: a personal view. Eur J Cell Biol *84*, 163-179.

Kundu, S., Horn, P.J., and Peterson, C.L. (2007). SWI/SNF is required for transcriptional memory at the yeast GAL gene cluster. Genes Dev *21*, 997-1004.

Lacadie, S.A., and Rosbash, M. (2005). Cotranscriptional spliceosome assembly dynamics and the role of U1 snRNA:5'ss base pairing in yeast. Mol Cell *19*, 65-75.

Lacadie, S.A., Tardiff, D.F., Kadener, S., and Rosbash, M. (2006). In vivo commitment to yeast cotranscriptional splicing is sensitive to transcription elongation mutants. Genes Dev *20*, 2055-2066.

Lai, M.C., and Tarn, W.Y. (2004). Hypophosphorylated ASF/SF2 binds TAP and is present in messenger ribonucleoproteins. J Biol Chem *279*, 31745-31749.

Lammerding, J., Fong, L.G., Ji, J.Y., Reue, K., Stewart, C.L., Young, S.G., and Lee, R.T. (2006). Lamins A and C but not lamin B1 regulate nuclear mechanics. J Biol Chem *281*, 25768-25780.

Le Hir, H., Izaurralde, E., Maquat, L.E., and Moore, M.J. (2000). The spliceosome deposits multiple proteins 20-24 nucleotides upstream of mRNA exon-exon junctions. Embo J *19*, 6860-6869.

Lee, M.S., Henry, M., and Silver, P.A. (1996). A protein that shuttles between the nucleus and the cytoplasm is an important mediator of RNA export. Genes Dev *10*, 1233-1246.

Lees-Miller, S.P. (2006). Dysfunction of lamin A triggers a DNA damage response and cellular senescence. DNA Repair (Amst) *5*, 286-289.

Legrain, P., and Rosbash, M. (1989). Some cis- and trans-acting mutants for splicing target pre-mRNA to the cytoplasm. Cell *57*, 573-583.

Lei, E.P., Krebber, H., and Silver, P.A. (2001). Messenger RNAs are recruited for nuclear export during transcription. Genes Dev *15*, 1771-1782.

Lei, E.P., and Silver, P.A. (2002). Intron status and 3'-end formation control cotranscriptional export of mRNA. Genes Dev *16*, 2761-2766.

Lei, E.P., Stern, C.A., Fahrenkrog, B., Krebber, H., Moy, T.I., Aebi, U., and Silver, P.A. (2003). Sac3 is an mRNA export factor that localizes to cytoplasmic fibrils of nuclear pore complex. Mol Biol Cell *14*, 836-847.

Levesque, L., Guzik, B., Guan, T., Coyle, J., Black, B.E., Rekosh, D., Hammarskjold, M.L., and Paschal, B.M. (2001). RNA export mediated by tap involves NXT1-dependent interactions with the nuclear pore complex. J Biol Chem *276*, 44953-44962.

Lew, D.J. (2003). The morphogenesis checkpoint: how yeast cells watch their figures. Curr Opin Cell Biol *15*, 648-653.

Lewis, A., Felberbaum, R., and Hochstrasser, M. (2007). A nuclear envelope protein linking nuclear pore basket assembly, SUMO protease regulation, and mRNA surveillance. J Cell Biol *178*, 813-827.

Lewis, J.D., Izaurralde, E., Jarmolowski, A., McGuigan, C., and Mattaj, I.W. (1996). A nuclear cap-binding complex facilitates association of U1 snRNP with the cap-proximal 5' splice site. Genes Dev *10*, 1683-1698.

Li, H.M. (1999). A new protease required for cell-cycle progression in yeast. Nature *398*, 246-251.

Li, S., and Hochstrasser, M. (2000). The yeast ULP2 (SMT4) gene encodes a novel protease specific for the ubiquitin-like Smt3 protein. Moll Cell Biol *20*, 1069-1081.

Li, S.J., and Hochstrasser, M. (2003). The Ulp1 SUMO isopeptidase: distinct domains required for viability, nuclear envelope localization, and substrate specificity. J Cell Biol *160*, 1069-1081.

Libri, D., Dower, K., Boulay, J., Thomsen, R., Rosbash, M., and Jensen, T.H. (2002). Interactions between mRNA export commitment, 3'-end quality control, and nuclear degradation. Mol Cell Biol *22*, 8254-8266.

Libri, D., Graziani, N., Saguez, C., and Boulay, J. (2001). Multiple roles for the yeast SUB2/yUAP56 gene in splicing. Genes Dev *15*, 36-41.

Licatalosi, D.D., Geiger, G., Minet, M., Schroeder, S., Cilli, K., McNeil, J.B., and Bentley, D.L. (2002). Functional interaction of yeast pre-mRNA 3' end processing factors with RNA polymerase II. Mol Cell *9*, 1101-1111.

Lim, R.Y., Aebi, U., and Stoffler, D. (2006). From the trap to the basket: getting to the bottom of the nuclear pore complex. Chromosoma *115*, 15-26.

Listerman, I., Sapra, A.K., and Neugebauer, K.M. (2006). Cotranscriptional coupling of splicing factor recruitment and precursor messenger RNA splicing in mammalian cells. Nat Struct Mol Biol *13*, 815-822.

Loeillet, S., Palancade, B., Cartron, M., Thierry, A., Richard, G., Dujon, B., Doye, V., and A., N. (2005). Genetic network interactions among replication, repair and nuclear pore deficiencies in yeast. DNA Repair (Amst) *4*, 459-468.

Lohr, D., Venkov, P., and Zlatanova, J. (1995). Transcriptional regulation in the yeast GAL gene family: a complex genetic network. Faseb J *9*, 777-787.

Longtine, M.S., and Bi, E. (2003). Regulation of septin organization and function in yeast. Trends Cell Biol *13*, 403-409.

Longtine, M.S., McKenzie, A., 3rd, Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast *14*, 953-961.

Luderus, M.E., de Graaf, A., Mattia, E., den Blaauwen, J.L., Grande, M.A., de Jong, L., and van Driel, R. (1992). Binding of matrix attachment regions to lamin B1. Cell *70*, 949-959.

Luderus, M.E., van Steensel, B., Chong, L., Sibon, O.C., Cremers, F.F., and de Lange, T. (1996). Structure, subnuclear distribution, and nuclear matrix association of the mammalian telomeric complex. J Cell Biol *135*, 867-881.

Lund, M.K., and Guthrie, C. (2005). The DEAD-box protein Dbp5p is required to dissociate Mex67p from exported mRNPs at the nuclear rim. Mol Cell *20*, 645-651.

Luo, M.J., and Reed, R. (1999). Splicing is required for rapid and efficient mRNA export in metazoans. Proc Natl Acad Sci U S A *96*, 14937-14942.

Luo, M.L., Zhou, Z., Magni, K., Christoforides, C., Rappsilber, J., Mann, M., and Reed, R. (2001). Pre-mRNA splicing and mRNA export linked by direct interactions between UAP56 and Aly. Nature *413*, 644-647.

Luo, W., Johnson, A.W., and Bentley, D.L. (2006). The role of Rat1 in coupling mRNA 3'-end processing to transcription termination: implications for a unified allosteric-torpedo model. Genes Dev *20*, 954-965.

Luthra, R., Kerr, S., Harreman, M., Apponi, L., Fasken, M., Ramineni, S., Chaurasia, S., Valentini, S., and Corbett, A. (2007). Actively transcribed GAL genes can be physically

linked to the nuclear pore by the SAGA chromatin modifying complex. J Biol Chem *282*, 3042-3049.

Macara, I.G. (2001). Transport into and out of the nucleus. Microbiol Mol Biol Rev *65*, 570-594, table of contents.

Makatsori, D., Kourmouli, N., Polioudaki, H., Shultz, L.D., McLean, K., Theodoropoulos, P.A., Singh, P.B., and Georgatos, S.D. (2004). The inner nuclear membrane protein lamin B receptor forms distinct microdomains and links epigenetically marked chromatin to the nuclear envelope. J Biol Chem *279*, 25567-25573.

Makhnevych, T., Lusk, C.P., Anderson, A.M., Aitchison, J.D., and Wozniak, R.W. (2003). Cell cycle regulated transport controlled by alterations in the nuclear pore complex. Cell *115*, 813-823.

Makhnevych, T., Ptak, C., Lusk, C.P., Aitchison, J.D., and Wozniak, R.W. (2007). The role of karyopherins in the regulated sumoylation of septins. J Cell Biol *177*, 39-49.

Mallory, J.C., and Petes, T.D. (2000). Protein kinase activity of Tel1p and Mec1p, two Saccharomyces cerevisiae proteins related to the human ATM protein kinase. Proc Natl Acad Sci U S A *97*, 13749-13754.

Mandal, S.S., Chu, C., Wada, T., Handa, H., Shatkin, A.J., and Reinberg, D. (2004). Functional interactions of RNA-capping enzyme with factors that positively and negatively regulate promoter escape by RNA polymerase II. Proc Natl Acad Sci U S A *101*, 7572-7577.

Marshall, W.F. (2002). Order and disorder in the nucleus. Curr Biol *12*, R185-192.

Mason, P.B., and Struhl, K. (2005). Distinction and relationship between elongation rate and processivity of RNA polymerase II in vivo. Mol Cell *17*, 831-840.

Mathog, D., Hochstrasser, M., Gruenbaum, Y., Saumweber, H., and Sedat, J. (1984). Characteristic folding pattern of polytene chromosomes in Drosophila salivary gland nuclei. Nature *308*, 414-421.

Melchior, F., Schergaut, M., and Pichler, A. (2003). SUMO: ligases, isopeptidases and nuclear pores. Trends Biochem Sci *28*, 612-618.

Mendjan, S., Taipale, M., Kind, J., Holz, H., Gebhardt, P., Schelder, M., Vermeulen, M., Buscaino, A., Duncan, K., Mueller, J., *et al.* (2006). Nuclear pore components are involved in the transcriptional regulation of dosage compensation in Drosophila. Mol Cell *21*, 811-823.

Menon, S., Pasula, Deminoff,Willis,Barbara,andrew,santangelo (2005). Reverse recruitment: the Nup84 nuclear pore subcomplex mediates Rap1/Gcr1/Gcr2 transcriptional activation. Proc Natl Acad Sci U S A *102*, 5749-5754.

Miller, B.R., and Forbes, D.J. (2000). Purification of the vertebrate nuclear pore complex by biochemical criteria. Traffic *1*, 941-951.

Moazed, D. (2001). Common themes in mechanisms of gene silencing. Mol Cell *8*, 489-498.

Murray, A.W., and Szostak, J.W. (1983). Pedigree analysis of plasmid segregation in yeast. Cell *34*, 961-970.

Myers, L.C., Lacomis, L., Erdjument-Bromage, H., and Tempst, P. (2002). The yeast capping enzyme represses RNA polymerase II transcription. Mol Cell *10*, 883-894.

Nagai, S., Dubrana, K., Tsai-Pflugfelder, M., Davidson, M.B., Roberts, T.M., Brown, G.W., Varela, E., Hediger, F., Gasser, S.M., and Krogan, N.J. (2008). Functional targeting of DNA damage to a nuclear pore-associated SUMO-dependent ubiquitin ligase. Science *322*, 597-602.

Navarro, C.L., Cau, P., and Levy, N. (2006). Molecular bases of progeroid syndromes. Hum Mol Genet *15 Spec No 2*, R151-161.

Neugebauer, K.M. (2002). On the importance of being co-transcriptional. J Cell Sci *115*, 3865-3871.

Niepel, M., Strambio-de-Castillia, C., Fasolo, J., Chait, B.T., and Rout, M.P. (2005). The nuclear pore complex-associated protein, Mlp2p, binds to the yeast spindle pole body and promotes its efficient assembly. J Cell Biol *170*, 225-235.

Noble, C.G., Hollingworth, D., Martin, S.R., Ennis-Adeniran, V., Smerdon, S.J., Kelly, G., Taylor, I.A., and Ramos, A. (2005). Key features of the interaction between Pcf11 CID and RNA polymerase II CTD. Nat Struct Mol Biol *12*, 144-151.

Noma, K., Cam, H.P., Maraia, R.J., and Grewal, S.I. (2006). A role for TFIIIC transcription factor complex in genome organization. Cell *125*, 859-872.

Nott, A., Le Hir, H., and Moore, M.J. (2004). Splicing enhances translation in mammalian cells: an additional function of the exon junction complex. Genes Dev *18*, 210-222.

Oeffinger, M., Wei, K.E., Rogers, R., DeGrasse, J.A., Chait, B.T., Aitchison, J.D., and Rout, M.P. (2007). Comprehensive analysis of diverse ribonucleoprotein complexes. Nat Methods *4*, 951-956.

Osborne, C., Chakalova L, Brown KE, Carter D, Horton A, Debrand E, Goyenechea B, Mitchell JA, Lopes S, Reik W, Fraser P. (2004). Active genes dynamically colocalize to shared sites of ongoing transcription. Nat Genet *36*, 1065-1071.

Paddy, M.R., Belmont, A.S., Saumweber, H., Agard, D.A., and Sedat, J.W. (1990). Interphase nuclear envelope lamins form a discontinuous network that interacts with only a fraction of the chromatin in the nuclear periphery. Cell *62*, 89-106.

Palancade, B., Liu, X., Garcia-Rubio, M., Aguilera, A., Zhao, X., and Doye, V. (2007). Nucleoporins prevent DNA damage accumulation by modulating Ulp1-dependent sumoylation processes. Mol Biol Cell *18*, 2912-2923.

Palancade, B., Zuccolo, M., Loeillet, S., Nicolas, A., and Doye, V. (2005). Pml39, a novel protein of the nuclear periphery required for nuclear retention of improper messenger ribonucleoparticles. Mol Biol Cell *16*, 5258-5268.

Panse, V.G., Hardeland, U., Werner, T., Kuster, B., and Hurt, E. (2004). A proteome-wide approach identifies sumoylated substrate proteins in yeast. J Biol Chem *279*, 41346-41351.

Panse, V.G., Kuster, B., Gerstberger, T., and Hurt, E. (2003). Unconventional tethering of Ulp1 to the transport channel of the nuclear pore complex by karyopherins. Nat Cell Biol *5*, 21-27.

Papamichos-Chronakis, M., Conlan, R.S., Gounalaki, N., Copf, T., and Tzamarias, D. (2000). Hrs1/Med3 is a Cyc8-Tup1 corepressor target in the RNA polymerase II holoenzyme. J Biol Chem *275*, 8397-8403.

Pardo, B., and Marcand, S. (2005). Rap1 prevents telomere fusions by nonhomologous end joining. EMBO J *24*, 3117-3127.

Pascual-Garcia, P., Govind, C.K., Queralt, E., Cuenca-Bono, B., Llopis, A., Chavez, S., Hinnebusch, A.G., and Rodriguez-Navarro, S. (2008). Sus1 is recruited to coding regions and functions during transcription elongation in association with SAGA and TREX2. Genes Dev *22*, 2811-2822.

Pei, Y., Schwer, B., and Shuman, S. (2003). Interactions between fission yeast Cdk9, its cyclin partner Pch1, and mRNA capping enzyme Pct1 suggest an elongation checkpoint for mRNA quality control. J Biol Chem *278*, 7180-7188.

Phatnani, H.P., and Greenleaf, A.L. (2004). Identifying phosphoCTD-associating proteins. Methods Mol Biol *257*, 17-28.

Pickersgill, H., Kalverda, B., de Wit, E., Talhout, W., Fornerod, M., and van Steensel, B. (2006). Characterization of the Drosophila melanogaster genome at the nuclear lamina. Nat Genet *38*, 1005-1014.

Piruat, J.I., and Aguilera, A. (1998). A novel yeast gene, THO2, is involved in RNA pol II transcription and provides new evidence for transcriptional elongation-associated recombination. Embo J *17*, 4859-4872.

Platt, A., and Reece, R.J. (1998). The yeast galactose genetic switch is mediated by the formation of a Gal4p-Gal80p-Gal3p complex. EMBO J *17*, 4086-4091.

Platt, A., Ross, H.C., Hankin, S., and Reece, R.J. (2000). The insertion of two amino acids into a transcriptional inducer converts it into a galactokinase. Proc Natl Acad Sci U S A *97*, 3154-3159.

Proudfoot (2004). New perspectives on connecting messenger RNA 3' end formation to transcription. Curr Opin Cell Biol *16*, 272-278.

Proudfoot, N., and O'Sullivan, J. (2002). Polyadenylation: A tail of two complexes. Curr Biol *12*, R855-857.

Proudfoot, N.J., Furger, A., and Dye, M.J. (2002). Integrating mRNA processing with transcription. Cell *108*, 501-512.

Pruyne, D., and Bretscher, A. (2000). Polarization of cell growth in yeast. J Cell Sci *113 (Pt 4)*, 571-585.

Rae, M.M., and Franke, W.W. (1972). The interphase distribution of satellite DNA-containing heterochromatin in mouse nuclei. Chromosoma *39*, 443-456.

Ragoczy, T., Bender, M.A., Telling, A., Byron, R., and Groudine, M. (2006). The locus control region is required for association of the murine beta-globin locus with engaged transcription factories during erythroid maturation. Genes Dev *20*, 1447-1457.

Ralph, S.A., Scheidig-Benatar, C., and Scherf, A. (2005). Antigenic variation in Plasmodium falciparum is associated with movement of var loci between subnuclear locations. Proc Natl Acad Sci U S A *102*, 5414-5419.

Redd, M.J., Arnaud, M.B., and Johnson, A.D. (1997). A complex composed of tup1 and ssn6 represses transcription in vitro. J Biol Chem *272*, 11193-11197.

Reddy, K.L., Zullo, J.M., Bertolino, E., and Singh, H. (2008). Transcriptional repression mediated by repositioning of genes to the nuclear lamina. Nature *452*, 243-247.

Redon, C., Pilch, D., Rogakou, E., Sedelnikova, O., Newrock, K., and Bonner, W. (2002). Histone H2A variants H2AX and H2AZ. Curr Opin Genet Dev *12*, 162-169.

Reed, R., and Cheng, H. (2005). TREX, SR proteins and export of mRNA. Curr Opin Cell Biol *17*, 269-273.

Ricchetti, M., Dujon, B., and Fairhead, C. (2003). Distance from the chromosome end determines the efficiency of double strand break repair in subtelomeres of haploid yeast. J Mol Biol *328*, 847-862.

Ritchie, K.B., Mallory, J.C., and Petes, T.D. (1999). Interactions of TLC1 (which encodes the RNA subunit of telomerase), TEL1, and MEC1 in regulating telomere length in the yeast Saccharomyces cerevisiae. Mol Cell Biol *19*, 6065-6075.

Ritchie, K.B., and Petes, T.D. (2000). The Mre11p/Rad50p/Xrs2p complex and the Tel1p function in a single pathway for telomere maintenance in yeast. Genetics *155*, 475-479.

Rodrigues, J.P., Rode, M., Gatfield, D., Blencowe, B.J., Carmo-Fonseca, M., and Izaurralde, E. (2001). REF proteins mediate the export of spliced and unspliced mRNAs from the nucleus. Proc Natl Acad Sci U S A *98*, 1030-1035.

Rodriguez-Navarro, S., Fischer, T., Luo, M.J., Antunez, O., Perez-Ortin, J.E., Reed, R., and Hurt, E. (2004). Sus1, a functional componenet of the SAGA histone acetylase complex and the nuclear pore-associated mRNA export machinery. Cell *116*, 75-86.

Rosonina, E., Kaneko, S., and Manley, J.L. (2006). Terminating the transcript: breaking up is hard to do. Genes Dev *20*, 1050-1056.

Rougemaille, M., Dieppois, G., Kisseleva-Romanova, E., Gudipati, R.K., Lemoine, S., Blugeon, C., Boulay, J., Jensen, T.H., Stutz, F., Devaux, F., *et al.* (2008). THO/Sub2p functions to coordinate 3'-end processing with gene-nuclear pore association. Cell *135*, 308-321.

Rout, M.P., and Aitchison, J.D. (2001). The nuclear pore complex as a transport machine. J Biol Chem *276*, 16593-16596.

Rout, M.P., Aitchison, J.D., Suprapto, A., Hjertaas, K., Zhao, Y., and Chait, B.T. (2000). The yeast nuclear pore complex: composition, architecture, and transport mechanism. J Cell Biol *148*, 635-651.

Rusche, L.N., Kirchmaier, A.L., and Rine, J. (2003). The establishment, inheritance, and function of silenced chromatin in Saccharomyces cerevisiae. Annu Rev Biochem *72*, 481-516.

Russo, P., Li, W.Z., Hampsey, D.M., Zaret, K.S., and Sherman, F. (1991). Distinct cis-acting signals enhance 3' endpoint formation of CYC1 mRNA in the yeast Saccharomyces cerevisiae. EMBO J *10*, 563-571.

Ryan, K.J., and Wente, S.R. (2000). The nuclear pore complex: a protein machine bridging the nucleus and cytoplasm. Curr Opin Cell Biol *12*, 361-371.

Sachs, A.B., and Davis, R.W. (1990). Translation initiation and ribosomal biogenesis: involvement of a putative rRNA helicase and RPL46. Science *247*, 1077-1079.

Sadowski, M., Dichtl, B., Hubner, W., and Keller, W. (2003). Independent functions of yeast Pcf11p in pre-mRNA 3' end processing and in transcription termination. EMBO J *22*, 2167-2177.

Saguez, C., Olesen, J.R., and Jensen, T.H. (2005). Formation of export-competent mRNP: escaping nuclear destruction. Curr Opin Cell Biol *17*, 287-293.

Saguez, C., Schmid, M., Olesen, J.R., Ghazy, M.A., Qu, X., Poulsen, M.B., Nasser, T., Moore, C., and Jensen, T.H. (2008). Nuclear mRNA surveillance in THO/sub2 mutants is triggered by inefficient polyadenylation. Mol Cell *31*, 91-103.

Santos-Rosa, H., Moreno, H., Simos, G., Segref, A., Fahrenkrog, B., Pante, N., and Hurt, E. (1998). Nuclear mRNA export requires complex formation between Mex67p and Mtr2p at the nuclear pores. Mol Cell Biol *18*, 6826-6838.

Sarma, N., Haley, T., Barbara, K., Buford, T., Willis, K., and Santangelo, G. (2007). Glucose-responsive regulators of gene expression in Saccharomyces cerevisiae function at the nuclear periphery via a reverse recruitment mechanism. Genetics *175*, 1127-1135.

Scaffidi, P., and Misteli, T. (2006). Lamin A-dependent nuclear defects in human aging. Science *312*, 1059-1063.

Schermelleh, L., Carlton, P.M., Haase, S., Shao, L., Winoto, L., Kner, P., Burke, B., Cardoso, M.C., Agard, D.A., Gustafsson, M.G., *et al.* (2008). Subdiffraction multicolor imaging of the nuclear periphery with 3D structured illumination microscopy. Science *320*, 1332-1336.

Schmid, M., Arib, G., Laemmli, C., Nishikawa, J., Durussel, T., and Laemmli, U.K. (2006). Nup-PI: The Nucleopore-Promoter Interaction of Genes in Yeast. Mol Cell *21*, 379-391.

Schmitt, C., von Kobbe, C., Bachi, A., Pante, N., Rodrigues, J.P., Boscheron, C., Rigaut, G., Wilm, M., Seraphin, B., Carmo-Fonseca, M., *et al.* (1999). Dbp5, a DEAD-box protein required for mRNA export, is recruited to the cytoplasmic fibrils of nuclear pore complex via a conserved interaction with CAN/Nup159p. Embo J *18*, 4332-4347.

Schmitt, I., and Gerace, L. (2001). In vitro analysis of nuclear transport mediated by the C-terminal shuttle domain of Tap. J Biol Chem *276*, 42355-42363.

Schneiter, R., Guerra, C.E., Lampl, M., Gogg, G., Kohlwein, S.D., and Klein, H.L. (1999). The Saccharomyces cerevisiae hyperrecombination mutant hpr1Delta is synthetically lethal with two conditional alleles of the acetyl coenzyme A carboxylase gene and causes a defect in nuclear export of polyadenylated RNA. Mol Cell Biol *19*, 3415-3422.

Schroeder, S.C., Zorio, D.A., Schwer, B., Shuman, S., and Bentley, D. (2004). A function of yeast mRNA cap methyltransferase, Abd1, in transcription by RNA polymerase II. Mol Cell *13*, 377-387.

Schwartz, D.C., and Hochstrasser, M. (2003). A superfamily of protein tags: ubiquitin, SUMO and related modifiers. Trends Biochem Sci *28*, 321-328.

Segref, A., Sharma, K., Doye, V., Hellwig, A., Huber, J., Luhrmann, R., and Hurt, E. (1997). Mex67p, a novel factor for nuclear mRNA export, binds to both poly(A)+ RNA and nuclear pores. Embo J *16*, 3256-3271.

Shatkin, A.J., and Manley, J.L. (2000). The ends of the affair: capping and polyadenylation. Nat Struct Biol *7*, 838-842.

Shcheprova, Z., Baldi, S., Frei, S.B., Gonnet, G., and Barral, Y. (2008). A mechanism for asymmetric segregation of age during yeast budding. Nature *454*, 728-734.

Shen, E.C., Stage-Zimmermann, T., Chui, P., and Silver, P.A. (2000). 7The yeast mRNA-binding protein Npl3p interacts with the cap-binding complex. J Biol Chem *275*, 23718-23724.

Shore, D. (1994). RAP1: a protean regulator in yeast. Trends Genet *10*, 408-412.

Shumaker, D.K., Dechat, T., Kohlmaier, A., Adam, S.A., Bozovsky, M.R., Erdos, M.R., Eriksson, M., Goldman, A.E., Khuon, S., Collins, F.S., *et al.* (2006). Mutant nuclear lamin A leads to progressive alterations of epigenetic control in premature aging. Proc Natl Acad Sci U S A *103*, 8703-8708.

Sinclair, D.A., and Guarente, L. (1997). Extrachromosomal rDNA circles--a cause of aging in yeast. Cell *91*, 1033-1042.

Singleton, D.R., Chen, S., Hitomi, M., Kumagai, C., and Tartakoff, A.M. (1995). A yeast protein that bidirectionally affects nucleocytoplasmic transport. J Cell Sci *108 (Pt 1)*, 265-272.

Siniossoglou, S., Wimmer, C., Rieger, M., Doye, V., Tekotte, H., Weise, C., Emig, S., Segref, A., and Hurt, E.C. (1996). A novel complex of nucleoporins, which includes Sec13p and a Sec13p homolog, is essential for normal nuclear pores. Cell *84*, 265-275.

Skruzny, M., Schneider, C., Racz, A., Weng, J., Tollervey, D., and Hurt, E. (2009). An endoribonuclease functionally linked to perinuclear mRNP quality control associates with the nuclear pore complexes. PLoS Biol *7*, e8.

Smith, J.S., and Boeke, J.D. (1997). An unusual form of transcriptional silencing in yeast ribosomal DNA. Genes Dev *11*, 241-254.

Smith, M., Bhaskar, V., Fernandez, J., and Courey, A.J. (2004). Drosophila Ulp1, a nuclear pore-associated SUMO protease, prevents accumulation of cytoplasmic SUMO conjugates. J Biol Chem *279*, 43805-43814.

Snay-Hodge, C.A., Colot, H.V., Goldstein, A.L., and Cole, C.N. (1998). Dbp5p/Rat8p is a yeast nuclear pore-associated DEAD-box protein essential for RNA export. Embo J *17*, 2663-2676.

Somech, R., Shaklai, S., Geller, O., Amariglio, N., Simon, A.J., Rechavi, G., and Gal-Yam, E.N. (2005). The nuclear-envelope protein and transcriptional repressor LAP2beta interacts with HDAC3 at the nuclear periphery, and induces histone H4 deacetylation. J Cell Sci *118*, 4017-4025.

Sommer, P., and Nehrbass, U. (2005). Quality control of messenger ribonucleoprotein particles in the nucleus and at the pore. Curr Opin Cell Biol *17*, 294-301.

Sterner, D.E., Nathan, D., Reindle, A., Johnson, E.S., and Berger, S.L. (2006). Sumoylation of the yeast Gcn5 protein. Biochemistry *45*, 1035-1042.

Sterner, D.E., Wang, X., Bloom, M.H., Simon, G.M., and Berger, S.L. (2002). The SANT domain of Ada2 is required for normal acetylation of histones by the yeast SAGA complex. J Biol Chem *277*, 8178-8186.

Stewart, M., Baker, R.P., Bayliss, R., Clayton, L., Grant, R.P., Littlewood, T., and Matsuura, Y. (2001). Molecular mechanism of translocation through nuclear pore complexes during nuclear protein import. FEBS Lett *498*, 145-149.

Stoffler, D., Fahrenkrog, B., and Aebi, U. (1999). The nuclear pore complex: from molecular architecture to functional dynamics. Curr Opin Cell Biol *11*, 391-401.

Strahm, Y., Fahrenkrog, B., Zenklusen, D., Rychner, E., Kantor, J., Rosbach, M., and Stutz, F. (1999). The RNA export factor Gle1p is located on the cytoplasmic fibrils of the NPC and physically interacts with the FG-nucleoporin Rip1p, the DEAD-box protein Rat8p/Dbp5p and a new protein Ymr 255p. Embo J *18*, 5761-5777.

Strambio-de-Castillia, C., Blobel, G., and Rout, M.P. (1999). Proteins connecting the nuclear pore complex with the nuclear interior. J Cell Biol *144*, 839-855.

Strasser, K., Bassler, J., and Hurt, E. (2000). Binding of the Mex67p/Mtr2p heterodimer to FXFG, GLFG, and FG repeat nucleoporins is essential for nuclear mRNA export. J Cell Biol *150*, 695-706.

Strasser, K., and Hurt, E. (2001). Splicing factor Sub2p is required for nuclear mRNA export through its interaction with Yra1p. Nature *413*, 648-652.

Strasser, K., Masuda, S., Mason, P., Pfannstiel, J., Oppizzi, M., Rodriguez-Navarro, S., Rondon, A.G., Aguilera, A., Struhl, K., Reed, R., *et al.* (2002). TREX is a conserved complex coupling transcription with messenger RNA export. Nature *417*, 304-308.

Strawn, L.A., Shen, T., and Wente, S.R. (2001). The GLFG regions of Nup116p and Nup100p serve as binding sites for both Kap95p and Mex67p at the nuclear pore complex. J Biol Chem *276*, 6445-6452.

Stutz, F., Bachi, A., Doerks, T., Braun, I.C., Seraphin, B., Wilm, M., Bork, P., and Izaurralde, E. (2000). REF, an evolutionary conserved family of hnRNP-like proteins, interacts with TAP/Mex67p and participates in mRNA nuclear export. RNA *6*, 638-650.

Sullivan, T., Escalante-Alcalde, D., Bhatt, H., Anver, M., Bhat, N., Nagashima, K., Stewart, C.L., and Burke, B. (1999). Loss of A-type lamin expression compromises nuclear envelope integrity leading to muscular dystrophy. J Cell Biol *147*, 913-920.

Suntharalingam, M., and Wente, S.R. (2003). Peering through the pore: nuclear pore complex structure, assembly, and function. Dev Cell *4*, 775-789.

Suyama, M., Doerks, T., Braun, I.C., Sattler, M., Izaurralde, E., and Bork, P. (2000). Prediction of structural domains of TAP reveals details of its interaction with p15 and nucleoporins. EMBO Rep *1*, 53-58.

Taddei, A. (2007). Active genes at the nuclear pore complex. Curr Opin Cell Biol *19*, 305-310.

Taddei, A., and Gasser, S.M. (2004). Multiple pathways for telomere tethering: functional implications of subnuclear position for heterochromatin formation. Biochim Biophys Acta *1677*, 120-128.

Taddei, A., Van Houwe, G., Hediger, F., Kalck, V., Cubizolles, F., Schober, H., and Gasser, S.M. (2006). Nuclear pore association confers optimal expression levels for an inducible yeast gene. Nature *441*, 774-778.

Takahashi, Y., Iwase, M., Konishi, M., Tanaka, M., Toh-e, A., and Kikuchi, Y. (1999). Smt3, a SUMO-1 homolog, is conjugated to Cdc3, a component of septin rings at the mother-bud neck in budding yeast. Biochem Biophys Res Commun *259*, 582-587.

Tan, W., Zolotukhin, A.S., Bear, J., Patenaude, D.J., and Felber, B.K. (2000). The mRNA export in Caenorhabditis elegans is mediated by Ce-NXF-1, an ortholog of human TAP/NXF and Saccharomyces cerevisiae Mex67p. Rna *6*, 1762-1772.

Taniura, H., Glass, C., and Gerace, L. (1995). A chromatin binding site in the tail domain of nuclear lamins that interacts with core histones. J Cell Biol *131*, 33-44.

Therizols, P., Fairhead, C., Cabal, G.G., Genovesio, A., Olivo-Marin, J.C., Dujon, B., and Fabre, E. (2006). Telomere tethering at the nuclear periphery is essential for efficient DNA double strand break repair in subtelomeric region. J Cell Biol *172*, 189-199.

Thomsen, R., Libri, D., Boulay, J., Rosbash, M., and Jensen, T.H. (2003). Localization of nuclear retained mRNAs in Saccharomyces cerevisiae. Rna *9*, 1049-1057.

Torchet, C., Bousquet-Antonelli, C., Milligan, L., Thompson, E., Kufel, J., and Tollervey, D. (2002). Processing of 3'-extended read-through transcripts by the exosome can generate functional mRNAs. Mol Cell *9*, 1285-1296.

Tran, E.J., Zhou, Y., Corbett, A.H., and Wente, S.R. (2007). The DEAD-box protein Dbp5 controls mRNA export by triggering specific RNA:protein remodeling events. Mol Cell *28*, 850-859.

Treitel, M.A., and Carlson, M. (1995). Repression by SSN6-TUP1 is directed by MIG1, a repressor/activator protein. Proc Natl Acad Sci U S A *92*, 3132-3136.

Tseng, S.S., Weaver, P.L., Liu, Y., Hitomi, M., Tartakoff, A.M., and Chang, T.H. (1998). Dbp5p, a cytosolic RNA helicase, is required for poly(A)+ RNA export. EMBO J *17*, 2651-2662.

Uemura, H., Pandit, S., Jigami, Y., and Sternglanz, R. (1996). Mutations in GCR3, a gene involved in the expression of glycolytic genes in Saccharomyces cerevisiae, suppress the temperature-sensitive growth of hpr1 mutants. Genetics *142*, 1095-1103.

Uetz, P., Giot, L., Cagney, G., Mansfield, T.A., Judson, R.S., Knight, J.R., Lockshon, D., Narayan, V., Srinivasan, M., Pochart, P., *et al.* (2000). A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae. Nature *403*, 623-627.

Ujvari, A., and Luse, D.S. (2004). Newly Initiated RNA encounters a factor involved in splicing immediately upon emerging from within RNA polymerase II. J Biol Chem *279*, 49773-49779.

Vinciguerra, P., Iglesias, N., Camblong, J., Zenklusen, D., and Stutz, F. (2005). Perinuclear Mlp proteins downregulate gene expression in response to a defect in mRNA export. Embo J *24*, 813-823.

Vinciguerra, P., and Stutz, F. (2004). mRNA export: an assembly line from genes to nuclear pores. Curr Opin Cell Biol *16*, 285-292.

Visa, N., Izaurralde, E., Ferreira, J., Daneholt, B., and Mattaj, I.W. (1996). A nuclear cap-binding complex binds Balbiani ring pre-mRNA cotranscriptionally and accompanies the ribonucleoprotein particle during nuclear export. J Cell Biol *133*, 5-14.

Vitaliano-Prunier, M., Hobeika, Géli, Gwizdek, Dargemont (2008). Ubiquitylation of the COMPASS component Swd2 links H2B ubiquitylation to H3K4 trimethylation. Nat Cell Biol *10*, 1365-1371.

Vodala , A.K., Rosbash M. (2008). The nuclear exosome and adenylation regulate posttranscriptional tethering of yeast GAL genes to the nuclear periphery. Mol Cell *31*, 104-113.

Volkert, F.C., and Broach, J.R. (1986). Site-specific recombination promotes plasmid amplification in yeast. Cell *46*, 541-550.

Vourc'h, C., Taruscio, D., Boyle, A.L., and Ward, D.C. (1993). Cell cycle-dependent distribution of telomeres, centromeres, and chromosome-specific subsatellite domains in the interphase nucleus of mouse lymphocytes. Exp Cell Res *205*, 142-151.

Walker, C.L., Cargile, C.B., Floy, K.M., Delannoy, M., and Migeon, B.R. (1991). The Barr body is a looped X chromosome formed by telomere association. Proc Natl Acad Sci U S A *88*, 6191-6195.

Weirich, C.S., Erzberger, J.P., Flick, J.S., Berger, J.M., Thorner, J., and Weis, K. (2006). Activation of the DExD/H-box protein Dbp5 by the nuclear-pore protein Gle1 and its coactivator InsP6 is required for mRNA export. Nat Cell Biol *8*, 668-676.

West, S., Gromak, N., and Proudfoot, N.J. (2004). Human 5' --> 3' exonuclease Xrn2 promotes transcription termination at co-transcriptional cleavage sites. Nature *432*, 522-525.

Wiegand, H.L., Coburn, G.A., Zeng, Y., Kang, Y., Bogerd, H.P., and Cullen, B.R. (2002). Formation of Tap/NXT1 heterodimers activates Tap-dependent nuclear mRNA export by enhancing recruitment to nuclear pore complexes. Mol Cell Biol *22*, 245-256.

Williams, F.E., and Trumbly, R.J. (1990). Characterization of TUP1, a mediator of glucose repression in Saccharomyces cerevisiae. Mol Cell Biol *10*, 6500-6511.

Wilson, S.M., Datar, K.V., Paddy, M.R., Swedlow, J.R., and Swanson, M.S. (1994). Characterization of nuclear polyadenylated RNA-binding proteins in Saccharomyces cerevisiae. J Cell Biol *127*, 1173-1184.

Windgassen, M., Sturm, D., Cajigas, I.J., Gonzalez, C.I., Seedorf, M., Bastians, H., and Krebber, H. (2004). Yeast shuttling SR proteins Npl3p, Gbp2p, and Hrb1p are part of the translating mRNPs, and Npl3p can function as a translational repressor. Mol Cell Biol *24*, 10479-10491.

Winey, M., Hoyt, M.A., Chan, C., Goetsch, L., Botstein, D., and Byers, B. (1993). NDC1: a nuclear periphery component required for yeast spindle pole body duplication. J Cell Biol *122*, 743-751.

Winey, M., Yarar, D., Giddings, T.H., Jr., and Mastronarde, D.N. (1997). Nuclear pore complex number and distribution throughout the Saccharomyces cerevisiae cell cycle by three-dimensional reconstruction from electron micrographs of nuclear envelopes. Mol Biol Cell *8*, 2119-2132.

Wong, C.M., Qiu, H., Hu, C., Dong, J., and Hinnebusch, A.G. (2007). Yeast cap binding complex impedes recruitment of cleavage factor IA to weak termination sites. Mol Cell Biol *27*, 6520-6531.

Woollam, D.H., Millen, J.W., and Ford, E.H. (1967). Points of attachment of pachytene chromosomes to the nuclear membrane in mouse spermatocytes. Nature *213*, 298-299.

Wu, P.Y., and Winston, F. (2002). Analysis of Spt7 function in the Saccharomyces cerevisiae SAGA coactivator complex. Mol Cell Biol *22*, 5367-5379.

Wykoff, D.D., and O'Shea, E.K. (2005). Identification of sumoylated proteins by systematic immunoprecipitation of the budding yeast proteome. Mol Cell Proteomics *4*, 73-83.

Xu, Q., Li, M., Adams, J., and Cai, H.N. (2004). Nuclear location of a chromatin insulator in Drosophila melanogaster. J Cell Sci *117*, 1025-1032.

Yang, Q., Rout, M.P., and Akey, C.W. (1998). Three-dimensional architecture of the isolated yeast nuclear pore complex: functional and evolutionary implications. Mol Cell *1*, 223-234.

Ye, Q., and Worman, H.J. (1996). Interaction between an integral protein of the nuclear envelope inner membrane and human chromodomain proteins homologous to Drosophila HP1. J Biol Chem *271*, 14653-14656.

Yi, R., Qin, Y., Macara, I.G., and Cullen, B.R. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev *17*, 3011-3016.

Yoon, J.H., Love, D.C., Guhathakurta, A., Hanover, J.A., and Dhar, R. (2000). Mex67p of Schizosaccharomyces pombe interacts with Rae1p in mediating mRNA export. Mol Cell Biol *20*, 8767-8782.

Yusufzai, T.M., and Felsenfeld, G. (2004). The 5'-HS4 chicken beta-globin insulator is a CTCF-dependent nuclear matrix-associated element. Proc Natl Acad Sci U S A *101*, 8620-8624.

Yusufzai, T.M., Tagami, H., Nakatani, Y., and Felsenfeld, G. (2004). CTCF tethers an insulator to subnuclear sites, suggesting shared insulator mechanisms across species. Mol Cell *13*, 291-298.

Zacharioudakis, I., Gligoris, T., and Tzamarias, D. (2007). A yeast catabolic enzyme controls transcriptional memory. Curr Biol *17*, 2041-2046.

Zenklusen, D., Vinciguerra, P., Strahm, Y., and Stutz, F. (2001). The yeast hnRNP-Like proteins Yra1p and Yra2p participate in mRNA export through interaction with Mex67p. Mol Cell Biol *21*, 4219-4232.

Zenklusen, D., Vinciguerra, P., Wyss, J.C., and Stutz, F. (2002). Stable mRNP formation and export require cotranscriptional recruitment of the mRNA export factors Yra1p and Sub2p by Hpr1p. Mol Cell Biol *22*, 8241-8253.

Zhang, M., and Green, M.R. (2001). Identification and characterization of yUAP/Sub2p, a yeast homolog of the essential human pre-mRNA splicing factor hUAP56. Genes Dev *15*, 30-35.

Zhang, Z., Fu, J., and Gilmour, D.S. (2005). CTD-dependent dismantling of the RNA polymerase II elongation complex by the pre-mRNA 3'-end processing factor, Pcf11. Genes Dev *19*, 1572-1580.

Zhang, Z., and Reese, J.C. (2004). Redundant mechanisms are used by Ssn6-Tup1 in repressing chromosomal gene transcription in Saccharomyces cerevisiae. J Biol Chem *279*, 39240-39250.

Zhao, J., Hyman, L., and Moore, C. (1999). Formation of mRNA 3' ends in eukaryotes: mechanism, regulation, and interrelationships with other steps in mRNA synthesis. Microbiol Mol Biol Rev *63*, 405-445.

Zhao, J., Jin, S.B., Bjorkroth, B., Wieslander, L., and Daneholt, B. (2002). The mRNA export factor Dbp5 is associated with Balbiani ring mRNP from gene to cytoplasm. Embo J *21*, 1177-1187.

Zhao, X., and Blobel, G. (2005). A SUMO ligase is part of a nuclear multiprotein complex that affects DNA repair and chromosomal organization. Proc Natl Acad Sci U S A *102*, 4777-4782.

Zhao, X., Wu, C.Y., and Blobel, G. (2004a). Mlp-dependent anchorage and stabilization of a desumoylating enzyme is required to prevent clonal lethality. J Cell Biol *167*, 605-611.

Zhao, Y., Kwon, S.W., Anselmo, A., Kaur, K., and White, M.A. (2004b). Broad spectrum identification of cellular small ubiquitin-related modifier (SUMO) substrate proteins. J Biol Chem *279*, 20999-21002.

Zhong, X.P., and Krangel, M.S. (1997). An enhancer-blocking element between alpha and delta gene segments within the human T cell receptor alpha/delta locus. Proc Natl Acad Sci U S A *94*, 5219-5224.

Zhou, Z., Luo, M.J., Straesser, K., Katahira, J., Hurt, E., and Reed, R. (2000). The protein Aly links pre-messenger-RNA splicing to nuclear export in metazoans. Nature *407*, 401-405.

Zimowska, G., Aris, J.P., and Paddy, M.R. (1997). A Drosophila Tpr protein homolog is localized both in the extrachromosomal channel network and to nuclear pore complexes. J Cell Sci *110 (Pt 8)*, 927-944.

Zolotukhin, A.S., Tan, W., Bear, J., Smulevitch, S., and Felber, B.K. (2002). U2AF participates in the binding of TAP (NXF1) to mRNA. J Biol Chem *277*, 3935-3942.