Exploring the roles of Ccr4-Not complex in the regulating mitochondria biosynthesis

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EXPLORING THE ROLES OF CCR4-NOT COMPLEX IN THE REGULATING MITOCHONDRIA BIOSYNTHESIS

THÈSE

présentée aux Facultés de médecine et des sciences de l'Université de Genève
pour obtenir le grade de Docteur ès sciences en sciences de la vie,
mention Sciences biomédicales

par

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de

China

Thèse N° 244

GENÈVE

2023

ACKNOWLEDGEMENTS

As I begin the writing of my thesis, I would like to express my sincere gratitude to the individuals and organizations who have provided invaluable help and support throughout my Ph.D. journey. Without their contributions, this achievement would not have been possible.

I am especially grateful to Professor Martine Collart for her invaluable guidance and mentorship in my studies. Her expertise, and willingness to share her knowledge and techniques with me made a significant difference, especially given my limited background as a medical student. Beyond that, she also provided me opportunity for grant writing, coaching junior students and participating at an international conference. Looking back, I am immensely thankful for her support.

I would like to express my sincere appreciation to the members of my thesis committee. Professor Mafalda Escobar-Henriques, Professor Ramesh Pillai and Professor Guillaume Andrey, for accepting to be part of my thesis defense jury. I truly appreciate their willingness to dedicate their time and expertise in evaluating my research work conducted throughout my Ph.D.

I would also like to express my appreciation to all the members of Collart's lab, particularly Laetitica, who helped me at the beginning of my studies. She not only showed me techniques but also stood by me during the most challenging times. Moreover, she helped me adjust to life in Geneva, making the transition much smoother. I am grateful to Marta, Szabolcs, Olesya, George, Rachel, Luciana, Lena and Minghui, for providing me with enjoyable company during our leisure time.

Additionally, I would like to thank Professor Ildiko Szanto for her guidance and assistance during the preparation of my TAC and thesis writing. Her input and feedback were invaluable in shaping my research. I would like to thank Professor William Kelley, Professor Martina Valentini and Professor Joseph Curran for their insightful discussions during the lab meetings and Professor Dominique Belin for his encouragement throughout my research journey. I would like to extend my appreciation to the department's secretaries, Dylan and Hani, for

their support and assistance. Finally, I would like to thank the PhD school coordinator, Manel Essaidi, for all her help during my journey.

I would like to express my deepest gratitude to the China Scholarship Council for providing me with a four-year scholarship that allowed me to pursue my PhD at the University of Geneva. I would like to express my appreciation to my Chinese friends from diverse fields. Our time spent together on weekends and vacations has been truly remarkable. We had exciting hiking trips and memorable travels, creating cherished memories along the way. The friendship has not only enriched my personal life but also broadened my horizons through the exchange of experiences and perspectives from different backgrounds.

Lastly, I would like to reserve my deepest gratitude for my partner Jiangtao and our family. I thank my parents for providing me with a warm home and instilling in me a positive and resilient attitude towards life. Growing up in a material depletion and underdeveloped village in China, their influence has made me strong enough to face the challenges in work and life. I am also grateful to my partner for giving me unconditional trust, support, and companionship. Over the past four years, we have shared countless ups and downs together. We have experienced different cultures, bravely faced the challenges of life and work, grown together, understood and encouraged each other. They have become my greatest source of support in life, giving me the courage to fearlessly try new things.

Thank you all from the bottom of my heart.

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ABSTRACT

The objective of my PhD was to study the regulatory roles of the Ccr4-Not complex in mitochondrial protein synthesis using *Saccharomyces cerevisiae* as a research model organism.

In the first chapter, through a combination of gene editing approaches, Western blot analysis, qRT-PCR, Ribo-seq and single molecular fluorescence imaging techniques, I characterized an integrated co-translational quality control mechanism associated with mitochondria. We named it **MitoENCay,** which is an abbreviation representing the involved components: **E**gd1 and its ubiquitination by **N**ot4, **C**af130, **C**is1, Autophag**y** and No-Go-Dec**ay**. This mechanism maintains cellular homeostasis in response to overexpression of a mitochondrial matrix protein called Mmf1, expressed from a reporter construct, but does not influence overexpression of a mitochondrial inner membrane protein, Cox4. These two mitochondrial proteins are coded in the nucleus and have N-terminal cleavable mitochondrial targeting sequences (MTS) that are specific. Using biochemical and fluorescence imaging approaches, my findings revealed that Mmf1 is a co-translationally mitochondrial imported protein but not Cox4. I further explored the regulation of Mmf1 expression, which is repressed by its MTS and the co-translational machinery, in particular the nascent polypeptide-associated complex (NAC), the docking sites on mitochondria (Om14 or Sam37), and the mitochondrial receptor Tom20. *MMF1* mRNA, when overexpressed and targeted to mitochondria, activates an integrated quality control mechanism to manage Mmf1 mRNA levels and protein access. An intriguing finding was that pausing of ribosomes during translation is required for mitochondrial targeting of *MMF1* mRNA, creating a time window for mRNA targeting. Not4, a subunit of the Ccr4-Not complex, regulates ribosome pausing. In the absence of Not4, ribosome pausing was reduced which was determined by visualizing an increased in ribosome-protected mRNA fragments, especially after the main pausing site (at codons 92-95). These results are consistent with recent findings that Not5, another subunit of the Ccr4-Not complex, interacts with the E site of ribosomes, when the A site is occupied by a non-optimal codon, potentially maintaining the ribosome pausing. To further investigate, I optimized codons at the main pause site of *MMF1*, at codons 92 and 95. Codon optimality

can in principle lead to faster ribosome movement, and we observed an increase in Mmf1 protein, suggesting that ribosome pausing is required to repress *MMF1* expression. I also made new discoveries regarding the involvement of Caf130, a yeast-specific subunit of the Ccr4-Not complex. Previous studies have shown that NAC interacts with the Ccr4-Not complex through Caf130. NAC is the substrate of Not4, an E3 enzyme that can ubiquitinate its substrate proteins. My study has shown that the polyubiquitination of Egd1, a subunit of NAC, relies on the presence of Caf130. Specifically, the absence of Caf130 led to a partial reduction in its polyubiquitination. My study also provided the first evidence for the functional role of Egd1 ubiquitination as my results clearly shown an increased Mmf1 expression upon mutation of the ubiquitinated site of Egd1. However, it remains unclear whether Egd1 ubiquitination contributes to mitochondrial targeting or integrated quality control, or both. In this chapter, my thesis work sheds light on the mechanisms of regulation by the Ccr4-Not complex in the co-translational import of mitochondrial proteins and quality control.

In the second chapter of my thesis, I sought to explore the interaction between the Ccr4-Not complex and the mRNA-binding protein Puf3. Previous studies have indicated that mitochondrial mRNAs are major targets of Puf3, and its physical interaction with the Ccr4-Not complex contributes to mRNA degradation. Our preliminary data suggest that mRNA solubility is inversely regulated by Not1 and Not4, but the underlying mechanism remains unknown. Interestingly, a subset of mRNAs that become more soluble upon Not1 depletion and less soluble upon Not4 depletion overlap with Puf3 targets. We hypothesized that the Ccr4-Not complex might regulate the formation or dissolution of Puf3 condensates to fine-tune spatial expression of mRNA, thereby globally influencing mitochondrial protein synthesis. In this chapter, my current results demonstrated that the stability of the Puf3 protein is dependent on Not4 and Not5, and this under different conditions of cellular metabolism. Polysome profiling data indicated that Puf3 sedimentation in sucrose gradients is impaired in the absence of Not proteins. Puf3 was more present in heavy polysome fractions, and less in fractions where free mRNA sediments, 40S, 60S ribosomes and monosome fractions, suggesting that Puf3 stability is associated with its translation status. To continue my research, I generated *puf3Δ not5 Δ* double mutants and extracted total mRNAs, soluble mRNAs and

insoluble mRNAs from wild-type strains, *not4Δ, not5Δ, puf3Δ, not4Δ puf3Δ and not5Δ puf3Δ* to perform RNA-Seq to assess the solubility of RNA. 5'P-Seq was done in parallel, which is the sequencing of mRNA degradation intermediates, to assess the dynamics of the last translating ribosome. The data is currently being analyzed by our bioinformatician, Georges Allen. In addition, I created a Puf3-mScarlet reporter construct, which will allow to visualize the localization of Puf3 in strains where the Not proteins can also be visualized or in strains where the Not proteins are absent. Unfortunately, with time constraints, I was not able to fully follow the sequencing data analysis or use this reporter during my PhD.

Overall, the results of my thesis revealed new facets of the multivalent function of the Ccr4-Not complex in the interaction and regulation of various factors that contribute to mitochondrial biosynthesis and quality control.

RÉSUMÉ

L'objectif de mon doctorat était d'étudier les rôles régulateurs du complexe Ccr4-Not dans la synthèse des protéines mitochondriales en utilisant *Saccharomyces cerevisiae* comme organisme modèle de recherche.

Dans le premier chapitre, grâce à une combinaison d'approches d'édition génétique, d'analyse par Western blot, de qRT-PCR, de Ribo-seq et de techniques d'imagerie par fluorescence moléculaire, j'ai caractérisé un mécanisme de contrôle qualité co-traductionnel associé aux mitochondries. Nous l'avons nommé MitoENCay, qui est une abréviation représentant les composants impliqués : **E**gd1 et son ubiquitination par **N**ot4, les protéines **C**af130 et **C**is1, l'**A**utophagie et le No-Go-Deca**Y**. Ce mécanisme maintient l'homéostasie cellulaire en réponse à la surexpression d'une protéine de la matrice mitochondriale appelée Mmf1, exprimée à partir d'une construction rapporteur, mais n'influence pas la surexpression d'une autre protéine de la membrane interne mitochondriale, Cox4. Les gènes de ces deux protéines mitochondriales sont codés dans le noyau et les deux protéines possèdent des séquences spécifiques de ciblage à la mitochondrie, localisées dans leurs extrémités N-terminale. En utilisant des approches biochimiques et d'imagerie de fluorescence, mes découvertes ont révélé que Mmf1 est une protéine qui s'importe de manière co-traductionnelle à la mitochondrie. Ce mécanisme n'a pas lieu pour Cox4. J'ai exploré la régulation de l'expression de Mmf1, qui est réprimée par sa séquence de ciblage mitochondrial MTS (pour Mitochondrial Targeting Sequence), par la machinerie co-traductionnelle, le complexe associé au polypeptide naissant NAC (pour Nascent-polypeptide-associated complex), les sites d'amarrage sur les mitochondries Om14 ou Sam37 et le récepteur mitochondrial Tom20. L'ARNm de *MMF1*, lorsqu'il est exprimé en excès et ciblé sur les mitochondries, active un mécanisme de contrôle de qualité pour gérer les quantités d'ARNm et de la protéine Mmf1. Une découverte intrigante a été que la pause des ribosomes pendant la traduction est nécessaire pour le ciblage aux mitochondries de l'ARNm de *MMF1*, créant une fenêtre temporelle pour le ciblage de l'ARNm. Not4, une sous-unité du complexe Ccr4-Not, participerait à la régulation de cette pause du ribosome. En effet, il a été

visualisé que davantage de fragments d'ARNm protégés par le ribosome étaient détectés en l'absence de Not4 et ce en particulier après le site de pause principal (sur les codons 92-95). Ces résultats corrèlent avec des découvertes récentes selon lesquelles Not5, une autre sous-unité du complexe Ccr4-Not, interagit avec le site E des ribosomes lorsque le site A est occupé par un codon non optimal, maintenant potentiellement la pause du ribosome. Pour étudier davantage la pause des ribosomes, j'ai optimisé les codons sur le site de pause principal de *MMF1* : aux niveaux des codons 92 et 95. L'optimalité des codons peut en principe conduire à un mouvement plus rapide des ribosomes, et nous avons observé une augmentation de la quantité de la protéine Mmf1, ce qui suggère que la pause du ribosome est nécessaire pour réguler (réprimer) l'expression de *MMF1*. J'ai également fait de nouvelles découvertes concernant l'implication de Caf130, une sous-unité du complexe Ccr4-Not spécifique à la levure. Des études antérieures ont montré que NAC interagissait avec le complexe Ccr4-Not et ce *via* Caf130. NAC est un substrat de Not4, une enzyme E3 qui peut ubiquitiner ses protéines substrats. Mon étude a démontré que la poly-ubiquitination de Egd1, une sous-unité de NAC, est dépendante de Caf130, et que l'absence de Caf130 diminue partiellement la poly-ubiquitination de Egd1. Cela a fourni la première preuve du rôle fonctionnel de l'ubiquitination Egd1, qui avait été le premier substrat de Not4 découvert. Mes résultats ont clairement indiqué une augmentation de l'expression de Mmf1 lors de la mutation du site ubiquitiné sur Egd1. Cependant, il reste à préciser si l'ubiquitination de Egd1 contribue au ciblage des mitochondries, au contrôle de qualité intégré, ou aux deux. Dans ce chapitre, mon travail de thèse met en lumière les mécanismes de régulation par le complexe Ccr4-Not dans l'import co-traductionnel des protéines mitochondriales et le contrôle qualité.

Dans le deuxième chapitre de ma thèse, j'ai cherché à explorer l'interaction entre le complexe Ccr4-Not et la protéine de liaison à l'ARNm, Puf3. Des études antérieures ont indiqué que les ARNm mitochondriaux sont des cibles majeures de Puf3. L'interaction physique de Puf3 avec le complexe Ccr4-Not contribue par ailleurs à la dégradation de l'ARNm. Nos données préliminaires suggèrent que la solubilité de l'ARNm est inversement régulée par Not1 et Not4, le mécanisme sous-jacent restant inconnu. Fait intéressant, un sous-ensemble d'ARNm qui deviennent plus solubles lors de la déplétion de Not1 et moins solubles lors de la déplétion de Not4 est la catégorie des ARN ciblés par Puf3. Nous avons émis l'hypothèse que le

complexe Ccr4-Not pourrait réguler la formation ou la dissolution de condensats de Puf3 pour affiner l'expression localisée de l'ARNm, influençant globalement la synthèse des protéines mitochondriales. Dans ce chapitre, mes résultats ont démontré que la stabilité de la protéine Puf3 est dépendante de Not4 et Not5, et ceci dans différentes conditions de métabolisme cellulaire. Les données de profilage des polysomes ont indiqué que la sédimentation de Puf3 dans les gradients de sucrose est altérée en l'absence des protéines Not. Puf3 est davantage présent dans les fractions de polysomes lourds, et moins dans les fractions où sédimente l'ARNm libre : les ribosomes 40S, 60S et les fractions de monosomes, suggérant que la stabilité de Puf3 est associée à son statut de traduction. Pour approfondir mes recherches, j'ai généré des doubles mutants *puf3Δ not5 Δ* et extrait les ARNm totaux, les ARNm solubles et les ARNm insolubles des souches de type sauvage, *not4Δ, not5Δ, puf3Δ, not4Δ puf3Δ et not5Δ puf3Δ* pour réaliser un séquençage des ARN afin d'évaluer la solubilité de l'ARN. Des expériences de 5'P-Seq, qui est le séquençage d'intermédiaires de la dégradation de l'ARNm, ont également été réalisées en parallèle pour évaluer la dynamique des derniers ribosomes en cours de traduction. Les données sont actuellement analysées par notre bio-informaticien, Georges Allen. Enfin j'ai construit un rapporteur Puf3-mScarlet, qui permettra de visualiser la localisation de Puf3 dans des souches où on peut aussi visualiser les protéines Not, dans des conditions de type sauvage ou dans des conditions où les protéines Not sont absentes. Malheureusement, avec les contraintes de temps, je n'ai pas pu suivre complètement l'analyse des données de séquençage ni utiliser ce rapporteur pendant mon doctorat.

Dans l'ensemble, les résultats de ma thèse ont révélé de nouvelles facettes des fonctions multiples du complexe Ccr4-Not dans l'interaction et la régulation de divers facteurs qui contribuent à la biosynthèse mitochondriale et au contrôle de la qualité.

PREFACE

Mitochondria are essential organelles found in most eukaryotic cells. Mitochondria produce energy in the form of ATP through oxidative phosphorylation and play a role in cellular metabolism and signaling pathways. Mitochondrial dysfunction is responsible for multiple human diseases. Mitochondria are double-membraned structures that contain their own DNA and are thought to have originated from an ancient symbiotic relationship between a bacterium and a eukaryotic cell. Mitochondria DNA encodes only very few proteins : in *Saccharomyces cerevisiae* (also named budding yeast), it encodes 8 mitochondrial proteins, while in mammalian cells, mitochondrial DNA encodes 13 mitochondrial proteins. It is currently believed that there are over 1000 different proteins that make up the mitochondria, meaning that 99% of mitochondrial proteins are encoded by nuclear genes. Nuclear-encoded mitochondrial proteins are synthesized in the cytoplasm and can be transported and imported into mitochondria either post-translationally or co-translationally, assisted by RNA-binding proteins or nascent chains docked to the mitochondrial outer membrane.

There has been increasing evidence showing that a conserved and major regulator of mRNA metabolism in eukaryotic cells, the Ccr4-Not complex, participates in co-translational events, including co-translational assembly of proteins and co-translational quality control. This complex is the major interest of the Collart laboratory, whose recent results have indicated that nuclear-encoded mitochondrial mRNAs might be importantly regulated by Ccr4-Not in budding yeast. However, the underlying regulatory mechanism is unknown. Therefore, my doctoral research project has been to explore how the Ccr4-Not complex regulates mitochondrial protein synthesis.

I. INTRODUCTION

1. Mitochondria

1.1 Structure and function of mitochondria

Mitochondria are typically shaped like a long, thin cylinder or rod, although their shape can vary depending upon the specific cell type and the metabolic demands of the cell. They consist of four compartments: outer membrane, inner membrane, intermembrane space, and matrix [1-3] (**Figure 1)**.

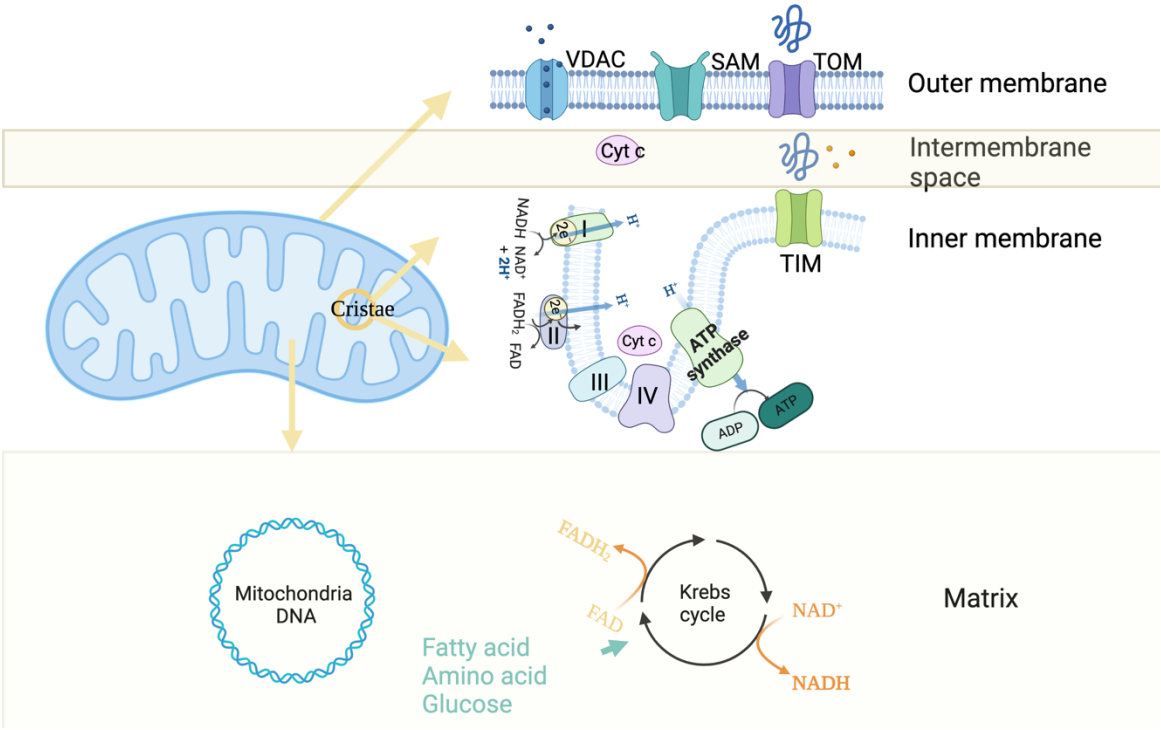


Figure 1: Overview of the structure and functions of mitochondria in budding yeast

The mitochondrial outer membrane contains the VDAC (Voltage-Dependent Anion Channel), TOM (Translocase of the Outer Membrane) and SAM (Sorting and Assembly Machinery) channels, which selectively allow the entry and exit of metabolic products and mitochondrial proteins. The mitochondrial inner membrane is composed of respiratory chain complexes and the ATP synthase, which generates energy as the cell's power source. The TIM (Translocase of the Inner Membrane) channel specifically selects proteins for entry and exit of the matrix. Soluble proteins in the intermembrane space of mitochondria, such as cytochrome C, regulate cellular apoptosis. The mitochondrial matrix contains mitochondrial DNA and various enzyme systems that participate in the metabolism of fatty acids, amino acids and glucose. The generated metabolites NADH and FADH2 serve as substrates for the electron transport chain.

- **Outer membrane**

The outer membrane is composed of a double layer of phospholipids. It is smooth and thin, enclosing the mitochondrion and serving as a protective barrier. This barrier allows the mitochondrion to maintain a unique internal environment that is essential for carrying out its metabolic functions. The outer membrane also contains numerous transport proteins, which play a critical role in regulating the flow of molecules into and out of the mitochondria [4, 5]. These transport proteins are, for instance, the voltage-dependent anion channel (VDAC), allowing for the passage of small molecules, such as pyruvate, ADP and ATP that are necessary for mitochondrial metabolism and apoptosis [6, 7]. Additionally, the translocase of the outer membrane, namely the TOM complex, enables the import of proteins that are synthesized outside the mitochondria to be transported into the organelle[8, 9]. Moreover, the outer membrane also contains specific proteins that are involved in protein import and sorting such as the mitochondrial Sorting and Assembly Machinery (SAM) complex[10, 11]. These mitochondrial outer membrane proteins closely interact and cooperate to precisely regulate the import and export of nutrients, energy and proteins into and out of mitochondria.

- **Inner membrane**

The inner membrane is mainly composed of a phospholipid bilayer, like the outer membrane. However, it also contains a variety of specialized proteins (enzymes) that are responsible for carrying out the electron transport chain and oxidative phosphorylation. These specific proteins form complexes, well known as the respiratory chain, to transfer electrons in order (either complex I or II to complex III and IV) from electron donors NADH or FADH2 to electron acceptors, generating a proton gradient across the inner mitochondrial membrane. The energy stored in this gradient is then used by ATP synthase to produce ATP, where ADP is converted to ATP[12, 13]. The inner membrane also is a highly folded membrane that forms numerous cristae (folds)[14]. These cristae increase the surface area of the membrane, allowing for more enzymes to be present in the same volume of space, which greatly increases the efficiency of ATP production. The inner membrane is also highly impermeable to most molecules, and it transports proteins from the Translocase of Inner Membrane

complex, namely the TIM complex, which is highly selective. Numerous transport proteins within the inner membrane regulate the transport of specific molecules across the membrane, such as ions, metabolites, and cofactors as well as the transport and sorting of matrix proteins[15-17].

- **Intermembrane space**

The intermembrane space is the space between the outer and inner membranes. It is relatively small and contains a small number of soluble proteins, such as cytochrome c, which is essential for the electron transport in the respiratory chain and cell apoptosis [18, 19].

- **Matrix**

The matrix is the innermost compartment of the mitochondrion, enclosed by the inner membrane. One of the key components of the matrix is mitochondrial DNA (mtDNA), a circular molecule of genetic material that encodes several essential proteins involved in oxidative phosphorylation. mtDNA is replicated and transcribed in the matrix, and mutations in mtDNA can lead to a variety of genetic disorders[20, 21]. The matrix also contains ribosomes, which are distinct from cytoplasmic ribosomes and are composed of both mitochondrial and nuclear-encoded proteins. Mutants of the mitochondrial ribosome are associated with various diseases as well[22-24]. In addition to mtDNA and ribosomes, the matrix contains a wide variety of soluble enzymes that are involved in the metabolism of nutrients. The citric acid cycle, also known as the Krebs cycle, takes place in the matrix and generates reducing equivalents in the form of NADH and FADH2, which are used to power oxidative phosphorylation. Fatty acid oxidation also occurs in the matrix and is an important source of energy for cells during periods of fasting or exercise. Amino acid metabolism, including the synthesis of non-essential amino acids and the degradation of nitrogen-containing compounds, also takes place in the matrix[25-27].

In summary, mitochondria are multi-functional organelles that play a crucial role in cellular metabolism and energy production. Besides participating in energy, metabolism and signal transduction, mitochondria also play an important role in cell death, calcium ion regulation,

lipid metabolism, and other biological processes. In short, mitochondria are indispensable organelles in cells, and their complex and organized structure and functions are essential for maintaining normal cellular function and survival[28].

1.2 Synthesis and assembly of mitochondria

As mentioned above, mitochondria are highly organized organelles composed of membrane systems, matrix, DNA, ribosomes, and proteins. These components interact and collaborate to carry out crucial functions. Therefore, studying the synthesis, assembly, structure, and function of mitochondrial proteins is crucial for a better understanding of cellular metabolism, the occurrence and treatment of mitochondrial disorders and other associated diseases. Because my thesis work has been using budding yeast as a model organism, from here on I will mostly focus on budding yeast unless specifically mentioned.

1.2.1 Mitochondrial DNAs encode proteins

The mitochondrial DNA in yeast encodes only eight proteins, but these proteins are crucial for the function of the mitochondria during respiration. The three subunits of the respiratory chain complexes (Cox1, Cox2, and Cox3) are involved in electron transfer reactions that ultimately produce ATP. The three subunits of ATP synthase (Atp6, Atp8, and Atp9) and apocytochrome b (Cob) are responsible for the synthesis of ATP from ADP, a process known as oxidative phosphorylation. A subunit of the mitochondrial ribosome (Var1) is essential for the translation of mitochondrial DNA-encoded genes into proteins. These proteins are synthesized by mitochondrial ribosomes, and the translation process is coupled with transcription. Defects in mtDNA-encoded protein synthesis or function can result in a variety of phenotypic effects such as respiratory defects, slow growth, and altered mitochondrial morphology[29, 30].

1.2.2 Nuclear DNAs encode mitochondrial proteins and five major import pathways

The majority of mitochondrial proteins are encoded by nuclear DNAs. Those genes are transcribed into RNA in the nucleus and transported to the cytoplasm for translation. During

transcription, RNA polymerase II transcribes the DNA into an RNA sequence, which is called pre-mRNA. Pre-mRNA needs to undergo a series of processing steps to become mature mRNA, including splicing, adding a 5' cap, and a 3' polyadenylation tail. After these processing steps are completed, the mature mRNA is transported to the cytoplasm. In the cytoplasm, mRNAs are translated by ribosomes into proteins [31]. These then need to pass through the mitochondrial membranes to reach their required locations. This process requires the assistance of a series of chaperones and transport proteins, which form complex transport channels on the outer and inner membranes of the mitochondria[32]. To date, five major protein import pathways have been identified, each of which is distinguished by a unique targeting signal on the mitochondrial precursor proteins [32-34] (**Figure 2)**.

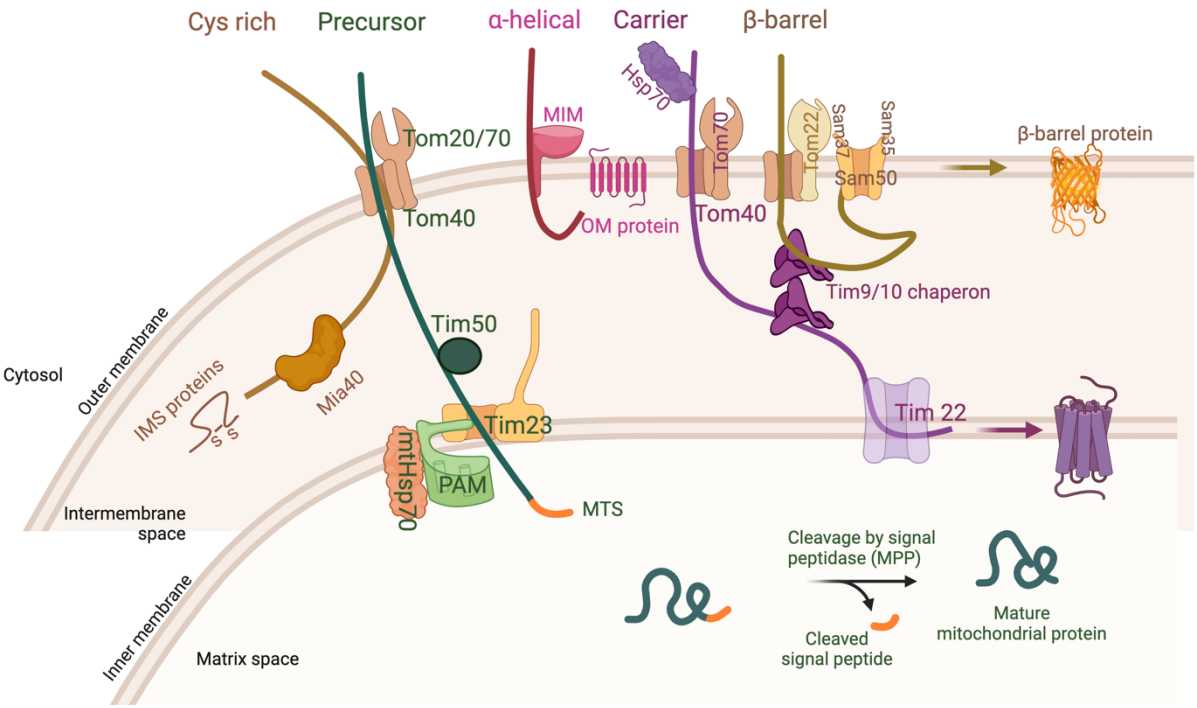


Figure 2: Five major pathways of mitochondrial protein import (picture adapted from reference[33])

Precursor pathway: Proteins with cleavable targeting sequences are recognized by receptors Tom20 or Tom70 and translocated across the outer and inner membranes via Tom40 and Tim23, and targeted to the matrix or inner membrane of mitochondria. *Carrier pathway*: Precursors of hydrophobic metabolite carriers, synthesized in the cytosol and bound to the cytosolic chaperone Hsp70, are recognized by the receptor Tom70 and cross the outer

membrane via Tom40. The small TIM chaperones deliver them to the carrier translocase of the inner membrane (Tim22) for final insertion into the inner membrane. *β-barrel pathway*: Precursors of β-barrel proteins are translocated through TOM to small Tim chaperones in the IMS, and then sorted and inserted into the outer membrane by the sorting and assembly machinery (SAM). *α-helical pathway*: OM proteins with α-helical transmembrane segments are imported and inserted into the OM by the mitochondrial import (MIM) complex. *MIA pathway*: Cysteine-rich proteins are imported into the intermembrane space (IMS) through TOM and proper folding and insertion of disulfide bonds (S-S) is mediated by the mitochondrial IMS import and assembly (MIA) system.

- **Precursor pathway**

The precursor pathway is the mechanism by which mitochondrial precursors are transported into the matrix and inner membrane. One key feature of mitochondrial precursors is their N terminal cleavable mitochondrial targeting sequence and formation of an amphipathic α-helix, which exhibits a positively charged face and a hydrophobic face. The elements are recognized by the receptors Tom20 or Tom70 of the translocase of the outer membrane. The precursors are then translocated across the mitochondrial outer membrane (MOM) via the channel-forming protein Tom40. The precursors are then further recognized by the receptor Tim50 of the translocase of the inner membrane, which transfers them to the channel-forming protein Tim23. The positively charged precursors are driven into the matrix by the precursor translocase-associated motor (PAM) with the help of the ATP-dependent mitochondrial heat-shock protein 70 (mtHsp70). The dimeric mitochondrial processing peptidase (MPP) removes the N terminal mitochondrial targeting sequence, and additional proteases can degrade the cleaved targeting sequence. The chaperonins Hsp60 and Hsp10 promote the formation of the mature matrix proteins devoid of the MTS.

- **Carrier pathway**

The carrier pathway into the inner membrane is a specialized import mechanism designed for multi-spanning inner membrane proteins that lack N-terminal cleavable presequences but possess internal targeting signals containing undefined hydrophobic elements. The import process initiates in the cytosol, where hydrophobic precursors interact with ATP-dependent heat-shock protein 70 (Hsp70) chaperones. These precursor-chaperone complexes are recognized by the receptor Tom70, located in the translocase of the outer membrane. Tom70 binds both the precursor and the chaperone, facilitating the transfer of precursors to the

Tom40 channel for translocation across the outer membrane. Once the precursors reach the Tom40 channel, a specific N-terminal segment of the Tom40 protein recruits small Tim chaperones, namely Tim9/10, from the intermembrane space (IMS) to the outer membrane. The presence of small Tim chaperones is crucial in preventing the aggregation of hydrophobic precursors. Subsequently, the small Tim9/10 chaperones guide the precursor to the translocase of the inner membrane, known as the TIM22 complex. The TIM22 complex utilizes the membrane potential (Δψ) to drive the efficient insertion of the multi-spanning proteins into the inner membrane.

- **β-barrel pathway**

The β-barrel pathway is used to transport β-barrel proteins, which are a small proportion of the mitochondrial proteins, but they are critical to form the metabolite channel porin. The precursors of β-barrel proteins are first imported through the translocase of the outer membrane. They then bind to small Tim9/10 chaperones, sharing components with the carrier pathway, and are subsequently inserted into the outer membrane with the help of the sorting and assembly machinery(SAM). To facilitate the efficient transfer of precursor proteins, a transient supercomplex is formed between the receptor Tom22 and the peripheral membrane protein Sam37, linking TOM and SAM. Folding of the β-barrel proteins take place at the Sam50-Sam35 site, followed by the release of the barrel into the lipid phase of the outer membrane.

- **MIA pathway**

The Mitochondrial Intermembrane Space Import and Assembly (MIA) machinery is responsible for importing and assembling intermembrane space proteins, which contain specific cysteine motifs. The precursors of these proteins are maintained in a reduced and unfolded state in the cytosol and are transported across the outer membrane through the Tom40 channel. Within the IMS, Mia40 acts as a receptor and binds to the precursors through hydrophobic interactions and temporary intermolecular disulfide bonds. The imported proteins are oxidized by Mia40, leading to the formation of intramolecular disulfide bonds. The MIA system not only supports the formation of IMS proteins but also some inner membrane and matrix proteins.

- **α- helical pathway**

This pathway is used by sorting signal for these proteins is typically present within the α-helical transmembrane segment(s) and is often accompanied by flanking positively charged amino acid residues. The process is facilitated by the MIM channel instead of Tom40 channel, and the receptor Tom70 assists in the import of multi-spanning proteins.

In summary, mitochondrial proteins targeted to different locations possess specific targeting signals and require different assistant factors and import channels.

The process of importing proteins into mitochondria after their synthesis in the cytoplasm is referred to as post-translation import. Additionally, a subset of mRNA molecules are transported to the proximity of mitochondria during translation for co-translation import of mitochondrial proteins. To ensure co-translational import, proper transfer of mRNA ribonucleoprotein particles (mRNPs) from the cytoplasm to the surface of mitochondria is required. This process involves the mRNA, mRNA-binding proteins and various molecular chaperones.

1.3 Transport of nuclear encoded mitochondrial mRNAs to the mitochondrial outer membrane.

In recent decades, multiple techniques have been developed to investigate mRNA localization, providing valuable insights into mRNA localized to mitochondria and the factors that mediate it. Early studies using in situ hybridization combined with electron microscopy discovered two nuclear-encoded mRNAs associated with the oxidative phosphorylation pathway in rat hepatocytes. The mRNA for the F1β subunit was observed to be clustered in close proximity to the mitochondria, indicating the possibility of co-translational import[35]. Subsequent research in yeast employed techniques such as differential centrifugation, filter arrays, and microarrays to identify mRNAs associated with the mitochondrial fraction, supporting the concept of co-translational import dependent on ribosomes[36-38]. *In vivo* tagging methodologies, involving the fusion of MS2 coat-protein binding sites to mRNA 3'-UTRs, further confirmed the localization of mRNAs（*ASH1*, *SRO7*, *PEX3* and *OXA1*） near mitochondria in living yeast cells [39]. Moreover, RNA fluorescent in situ hybridization (FISH) was employed to detect endogenous transcripts and provide additional evidence for the

association of mRNAs with the mitochondrial outer membrane[40]. Recent advancements in proximity-specific ribosome profiling have enabled the isolation and characterization of mRNAs translated by mitochondria-associated ribosomes. These studies have identified numerous mRNAs, particularly those encoding inner membrane proteins, that undergo localized translation near mitochondria, further supporting the existence of co-translational import[41].

Several factors have been identified in the regulation of localized translation near mitochondria. The mitochondria targeting sequence (MTS) was firstly characterized **(Figure 3A)**. Reacher attached an MTS to the N-terminus of EGFP and an ER targeting signal to its C-terminus. They found that the dual-signal EGFP exclusively localized to mitochondria in HeLa cells, indicating the co-translational import [42]. The interaction between the MTS and the mitochondrial outer membrane receptor Tom20 has become evident for many mRNA associations with mitochondria, occurring in a translation-dependent manner[43]. Although the deletion of Tom20 reduces mRNA association, it is important to note that mRNA localization is not completely abolished, indicating the involvement of additional factors.

The nascent-polypeptide associated complex (NAC, a heterodimer consisting of an alpha subunit Egd2 and a beta subunit, either Egd1 or Btt1) has emerged as an alternative factor involved in ribosome-bound mRNAs association with mitochondria[44]. *In vitro* studies have demonstrated that NAC enhances the binding of ribosome-bound nascent chain complexes (RNCs) to mitochondria, facilitating protein import[45]. Deletion of the mitochondrial outer membrane protein Om14, which interacts with NAC, leads to reduced NAC association with mitochondria and a decrease in mitochondria-RNC association. The interaction between NAC and Om14 provides a physical mechanism for the co-translational import model. Om14 interacts with another mitochondrial outer membrane protein, Om45[46]. NAC can alternatively dock on mitochondria via Sam37[21]. However, it remains unclear whether there is association between Om14 and Sam37.

Extensive research has established the significance of the 3’-UTR in mRNA localization to the mitochondrion, particularly facilitated by the RNA-binding protein Puf3. Deletion of Puf3

reduces mitochondrial localization of multiple mRNAs, and removing the Puf3 binding site in the 3'-UTR of *BCS1* reduces its mitochondrial localization[47]. These findings highlight the crucial role of Puf3 in mediating mRNA localization to the mitochondria through its interaction with the 3'-UTR. It has been proposed that transport of mRNAs to mitochondria may utilize the cellular cytoskeleton and specific motor proteins, however the specific mechanisms are not fully understood.

Additionally, studies in higher eukaryotes have identified the involvement of the mitochondria outer membrane kinase PINK1 in localization of mRNAs that encode proteins that are subunits of oxidative phosphorylation complexes **(Figure 3B).** These mRNAs are maintained in a translationally repressed state near mitochondria, and their repression is relieved upon association with Parkin, leading to the subsequent release of PUF family protein (Pum) [48]. The exact role of Pum in human mRNA localization is still unclear.

It worth to mention that the necessity for each pathway for mRNA localization at the mitochondria and for co-translational import differs amongst different mRNAs. Notably, deletion of either Puf3 or Tom20 has only a marginal effect on yeast growth under respiratory conditions, yet deletion of both proteins completely inhibits growth. This emphasizes the existence of redundant pathways for targeting mRNAs to mitochondria[43].

In summary, the targeting of mRNAs to mitochondria is a conserved mechanism from yeast to human that enables co-translational import. The MTS and the 3'- UTR of mRNAs serve as regulators *in cis*. Moreover, mRNA binding proteins such as PUM or Puf3 regulate these events *in trans*. The transport of the RNP can occur in a translation dependent or independent manner.

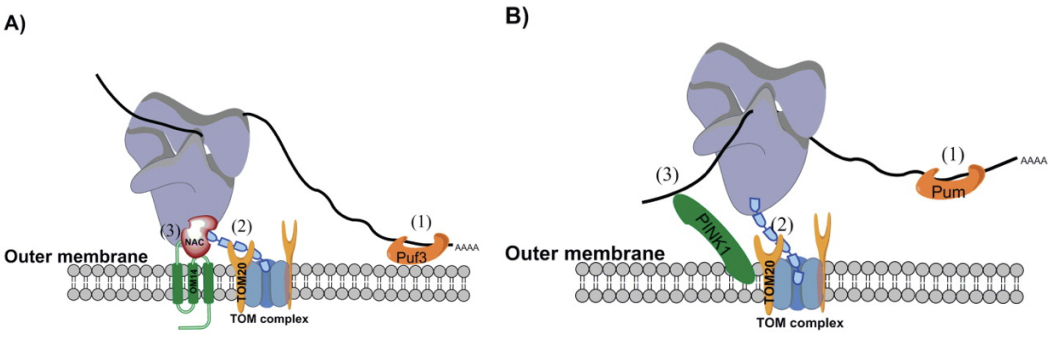


Figure 3: Localized translation near mitochondria in different organisms

The figure taken from [49]shows the mechanism of translation localized near mitochondria in different organisms. (A) In *S. cerevisiae*, Puf3 binds 3'-UTR of the mRNA, anchoring it near the mitochondrion independent of translation. Additionally, NAC interacts with the nascent chain, docking the RNC on the mitochondrion via Om14 and the MTS is recognized by Tom20, all this obviously dependent upon translation. (B) In *Drosophila* and *human*, PINK1 interacts with mRNAs and assists in their localization to the mitochondrial outer membrane, promoted by Tom20, where translation repression is maintained by PUM.

- **The functional roles of mRNA localization at mitochondria.**

The localization of mRNAs at mitochondria has several physiological roles. The most obvious one is to enhance protein import into the organelle through co-translational import. This process is particularly advantageous for membrane proteins that contain hydrophobic domains, as it minimizes their chances of aggregating in the cytoplasmic environment[50, 51]. Moreover, localized translation near mitochondria is of importance for cytosolic processes as well, especially for proteins with dual localization[52]. Besides translation, mRNA localization to mitochondria may have other roles that do not involve translation, such as regulating mRNA and nascent peptide stability. For example, the Puf3 protein may mediate the degradation of specific mRNAs near mitochondria under anaerobic conditions via the Ccr4-Not complex[53, 54]. In addition, multiple quality control pathways remove aberrant polypeptides at the mitochondria outer membrane. These findings suggest novel means by which gene expression is spatially regulated and highlight the importance of mRNA localization to mitochondria in various cellular processes.

1.4 Mitochondria-associated quality control mechanisms

Due to the complex processes of mitochondrial mRNA transport and mitochondrial protein import, mitochondria are constantly exposed to stress. In budding yeast growing in glucose-rich medium, a shift in metabolism from fermentative to respiratory status occurs when the glucose is depleted, resulting in an increased demand for mitochondria[55]. To be able to quickly adapt to this shift, nuclear DNAs are continuously transcribed to produce mitochondrial mRNAs even during fermentation. These however, are translated according to the physiological needs and when the demand is low, the excess mRNAs are either degraded or stored in membrane-less compartments ready for a rapid translational response upon

need. Puf3 is a key factor for this regulation according to physiological demand[56, 57]. In addition, many quality control pathways are involved to cope with all stresses resulting from overproduction of mitochondrial precursors to maintain cellular protein homeostasis. The activation of specific quality control pathways depends upon the magnitude and type of stress and whether the stress occurs in the cytosol or/and at mitochondria. These pathways can be minor and involve the clearance of one or several specific proteins, or major, to removal proteins in bulk, for instance to eliminate partially damaged mitochondria through a mitochondria vesicular-driven pathway (MDV), mitophagy or autophagy. Some quality control mechanisms occur post-translationally while others are activated co-translationally. Specific factors activate distinct pathways, and some pathways share common factors (**Table 1),** indicating an intricate network of quality controls linking the cytosol and the mitochondria to ensure cellular homeostasis.

Table 1: Mitochondrial associated quality control pathways and involved key factors.

Pathway	Involved key Components	Key Stress condition
mPOS	Nog2 and Gis2	Impaired mitochondrial protein import machinery, defects in the processing and folding of precursor proteins, increased mitochondrial biogenesis
mitoUPS	ATFS1	Multi types of stress as mPOS
mitoCRP	Cis1 and Msp1	Impaired mitochondrial protein import, overload of proteins in import channel, mislocalized proteins
MAGIC	Hsp104	Protein aggregates and misfolding in cytoplasm
mitoTAD	Ubx2 and Cdc48	Non-stress conditions
mitoRQC	Vms1 and Cdc48	Ribosome stalling

MDV	PINK1/Parkin	Non-stress/mild oxidative stress
Mitophagy	Atg32	Starvation, aging, oxidative stress, mitochondrial dysfunction, signaling proteins
Autophagy	Atg proteins	Multi-stress, nutrient deprivation

1.4.1 Mitochondria associated post-translational quality control pathways

Mitochondrial precursor over-accumulation stress (mPOS) results from various stresses, such as an impaired mitochondrial protein import machinery, defects in the processing and folding of precursor proteins, or increased rates of mitochondrial biogenesis, and it results in the formation of aggregates and other toxic intermediates that can disrupt normal cellular processes (**Figure 4**, in yellow**)**. mPOS has been shown to upregulate the expression of two conserved proteins, Nog2 and Gis2. Nog2 is involved in the reconfiguration of ribosomes, while Gis2 is involved in cap-independent protein translation. However, the mechanisms have yet to be fully understood [58, 59].

The mitochondrial Unfolded Protein Response (UPR^{mt}) pathway has been described in multicellular eukaryote can be activated by multiple forms of mitochondrial dysfunction and promotes cell survival and mitochondrial recovery [60]. Activating Transcription Factor associated with Stress（ATFS-1）is a key factor for the UPR^{mt} pathway in mammalian cells. It is targeted to mitochondria in normal conditions via a mitochondrial targeting sequence, but is relocated to the nucleus under mitochondrial stress conditions via nuclear localization sequence (NLS). Thereby, it activates the mitochondrial unfolded protein response (**Figure 4,** in brown). As a transcription factor, it induces the expression of a variety of genes that contribute to the restoration of mitochondrial function and import efficiency, such as the upregulation of chaperones and proteases to maintain mitochondrial proteostasis[61-63].

Another study in budding yeast found that inhibiting mitochondrial protein import activates a surveillance mechanism called mitochondrial Compromised Protein Import Response

(mitoCPR), which improves mitochondrial import and protects the organelle during import stress (**Figure 4,** in green). The mitoCPR pathway induces the expression of Cis1, which associates with the mitochondrial translocase to reduce the accumulation of unprocessed precursor proteins. These precursor proteins are cleared through the action of the Cis1-interacting AAA+ ATPase Msp1 and the proteasome [64].

The Mitochondria As Guardian In Cytosol (MAGIC) pathway is another quality control mechanism that directs misfolded and damaged cytosolic proteins to mitochondria via the disaggregase Hsp104 for their degradation by mitochondrial proteases[65] (**Figure 4,** in blue).

The mitochondria Translocation-Associated Degradation (mitoTAD) pathway is a continuous quality control mechanism that functions to maintain mitochondrial and cellular homeostasis by selectively removing mislocated and misfolded proteins from the mitochondria (**Figure 4**, in pink). This pathway operates by recognizing these proteins through the ubiquitin-binding protein Ubx2, and extracting them from the import channel using the AAA+ ATPase Cdc48. The extracted proteins are then delivered to the proteasome for degradation, thereby ensuring that only properly folded and localized proteins are present in the mitochondria [66].

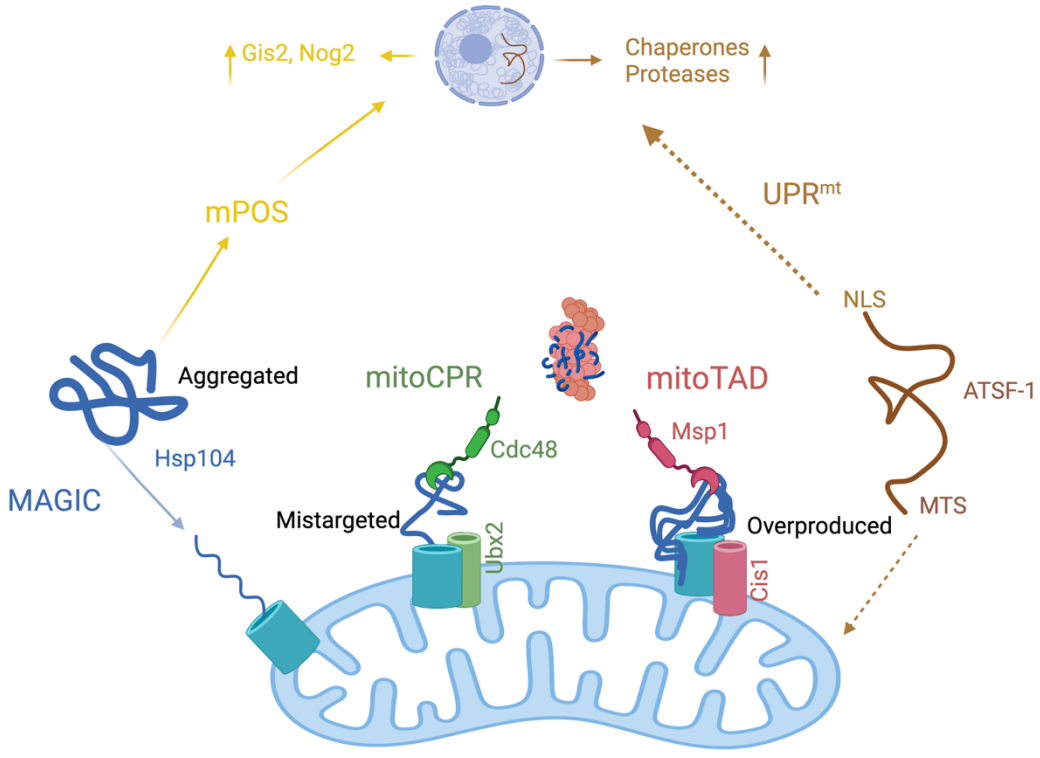


Figure 4: Mitochondria associated post-translational quality control pathways

Upregulation of Nog2 and Gis2 proteins, in response to multiple stresses, is called Mitochondrial Precursor Over-accumulation Stress (mPOS). The Mitochondrial Unfolded protein reponse occurs in response to stress, with ATFS-1 inducing the expression of molecular chaperones and import channels, a mechanism termed the Protein Response (UPRmt). The Mitochondrial Compromised Protein Import Response (mitoCPR) involves the upregulation of Cis1, which aids in the degradation of unimported proteins through interaction with Msp1 and the proteasome. The MAGIC pathway directs misfolded and damaged proteins to mitochondria using Hsp104 for mitochondrial degradation, serving as a quality control mechanism. The mitoTAD pathway ensures mitochondrial and cellular homeostasis by identifying and removing mislocated and misfolded proteins from mitochondria via Ubx2 and Cdc48, followed by degradation by the proteasome.

1.4.2 Mitochondrial associated co-translational quality control pathways

In addition to the post-translational quality controls, there are co-translation quality control pathways occurring during translation elongation to safeguard cellar protein hemostasis. In the cytoplasm, the ribosome-associated quality control (RQC) mechanism is responsible for dealing with ribosome pausing or stalling during translation. When such pausing occurs, the 80S ribosome dissociates, leaving the peptidyl-tRNA retained by the 60S subunit. These 60S RNCs are recognized by Ribosome-associated quality control (RQC) components, including Rqc2, which plays a role in recruiting the E3 ubiquitin ligase Ltn1. Ltn1 is responsible for ubiquitylating the nascent peptide by targeting its N-terminal lysines[67, 68]. After ubiquitination, the Cdc48 ATPase complex, along with Vms1 (a tRNA hydrolase), facilitates the extraction of the ubiquitinated proteins from the 60s [69-71]. These extracted proteins are then targeted to the proteasome for degradation (**Figure 5**, panel A). Rqc2 also recruits alanine- and threonine-charged tRNAs to the A site and directs the elongation of nascent chains (CAT-tail) independently of the mRNA template or 40S subunits, and the CAT- tail extension of the nascent chain can favor ubiquitination Ltn1 by exposing new lysine residues [72]. If the ubiquitination of stalled chains is impaired, the stalled polypeptides still dissociate from the 60S ribosome. Instead of being properly degraded, they form stable aggregates. The aggregation is dependent on the presence of CAT-tail[73-75]. For nuclear-encoded mitochondrial proteins, there are two distinct co-translational quality control pathways (**Figure 5**, panel B). In the Ltn1 pathway, the translocation of proteins into mitochondria is halted. Ltn1 recognizes and ubiquitylates the exposed N-terminal lysines of the proteins in transit. The Cdc48 ATPase complex mediates the subsequent extraction of ubiquitylated proteins from the ribosome, leading to their degradation by the proteasome as in the

cytosolic protein pathway. In the Vms1 pathway, the ribosome is pulled into close contact with the outer membrane of the mitochondria by the import machinery. This prevents Ltn1 from accessing the N-terminal lysines of the nuclear-encoded mitochondrial proteins for ubiquitylation. Instead, Vms1, which associates with the 60S ribosome at the outer membrane, plays a crucial role. It counteracts the CAT tailing process mediated by Rqc2[76]. By inhibiting CAT tailing, Vms1 facilitates the import of nascent chains into the mitochondrial matrix and avoids proteotoxic stress due to CAT-tailed aggregates inside mitochondria [77].

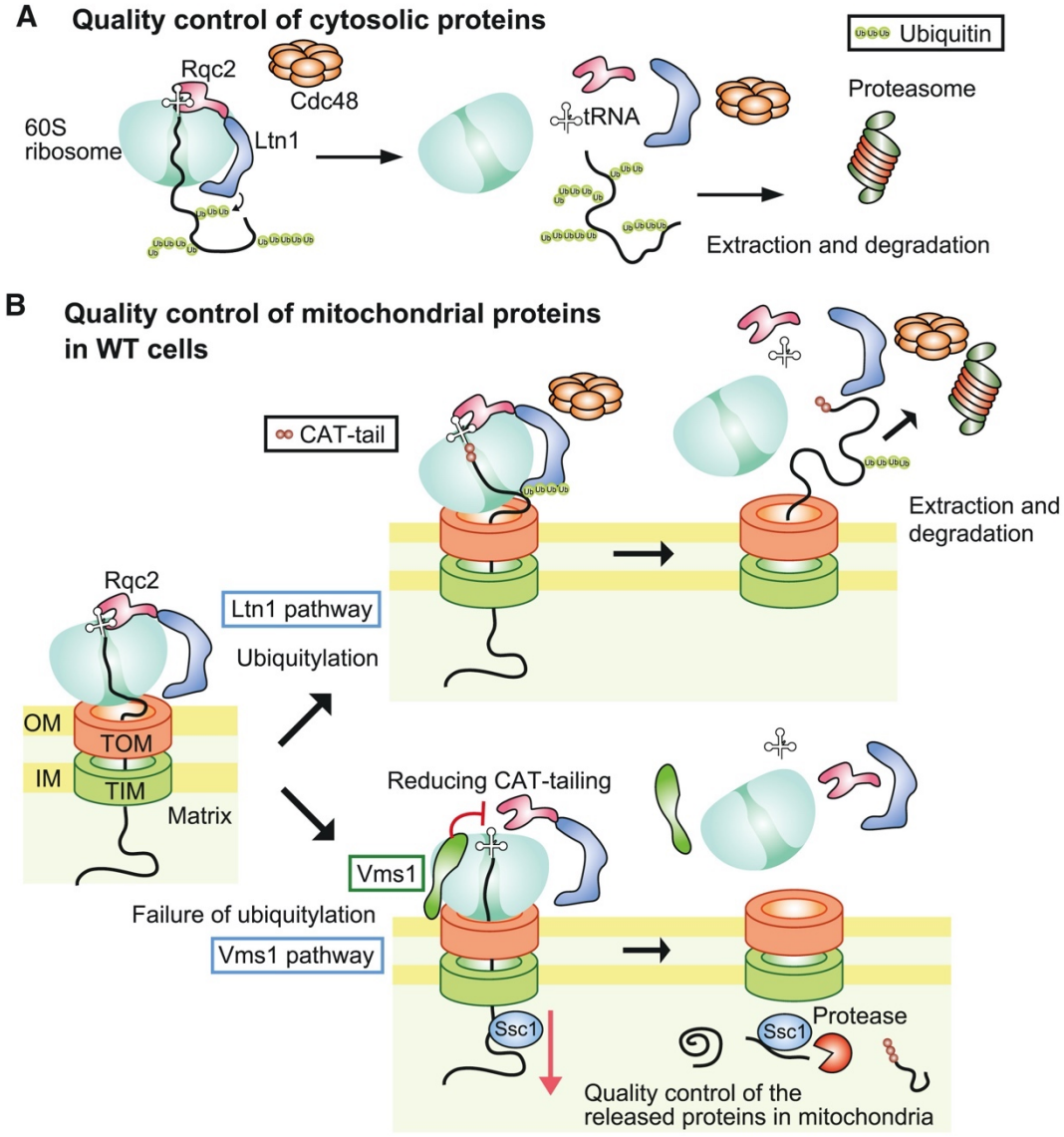


Figure 5: Co-translational quality control mechanisms for cytoplasmic proteins and mitochondrial proteins (Picture taken from reference[77])

The RQC machinery efficiently targets cytosolic proteins stalled on ribosomes for proteasomal degradation (Panel A). However, mitochondrial proteins with N-terminal targeting sequences may evade RQC by engaging the mitochondrial translocation machinery, leading to partial

import into mitochondria before triggering the RQC response. The proximity of the 60S ribosome to the TOM complex on the outer mitochondrial membrane limits access of the RQC machinery to the translocating chain. Ltn1 can ubiquitylate stalled chains prior to import, and some partially translocated and ubiquitylated chains may be extracted from the translocon and degraded by the proteasome. In the absence of functional Ltn1, Vms1 plays a crucial role in facilitating the import and degradation of accumulated proteins in the mitochondrial matrix by blocking CAT-tailing (Panel B).

A study from *Drosophila* shows that the translation of mitochondrial outer membrane-localized mRNAs undergoes a quality control mechanism that intersects with the turnover of damaged mitochondria and is regulated by the mitochondrial kinase PINK1. When mitochondrial damage occurs, the translation of complex I 30 kDa subunit (C-I30) mRNA on the MOM is stalled. This triggers the recruitment of co-translational quality control factors Pelo (Dom34 in yeast), ABCE1（Rli1 in yeast), and NOT4 to the mRNA-ribonucleoprotein complex. ABCE1 undergoes ubiquitination by NOT4, leading to the generation of a poly-ubiquitin signal. This signal attracts autophagy receptors to the MOM, initiating mitophagy, a process of selective degradation of damaged mitochondria[48, 78] (**Figure 6)**.

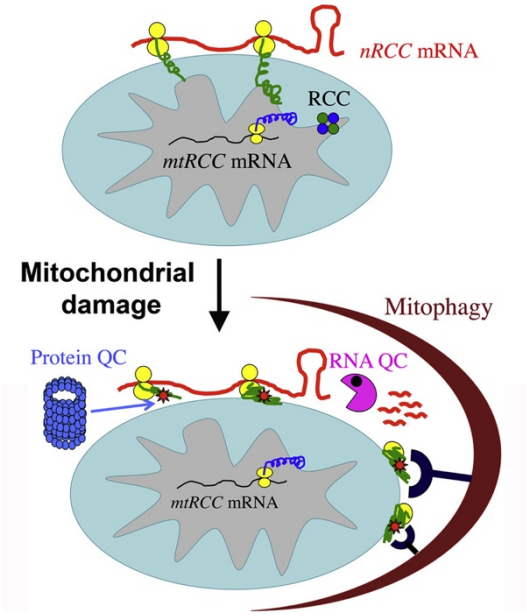


Figure 6: Ubiquitination of ABCE1 by NOT4 connects co-translational quality control with mitophagy (picture taken from reference[79])

Mitochondrial damage causes translation stalling of the complex-I 30 kDa subunit (C-I30) mRNA on the outer mitochondrial membrane. This leads to the recruitment of co-translational quality control factors Pelo, ABCE1, and NOT4 to the ribonucleoprotein complex.

NOT4 ubiquitinates ABCE1 in response to the damage, generating poly-ubiquitin signals. These signals attract autophagy receptors to the MOM, initiating the process of mitophagy.

1.4.3. Bulk quality control mechanisms

- **The mitochondria-derived vesicles (MDVs) pathway**

The mitochondria-derived vesicles (MDVs) pathway is generated under physiological conditions and mild oxidative stress to remove partial damaged mitochondrial components and restore cellular homeostasis. MDVs are small (70-150 nm in diameter) vesicles that transport oxidized and dysfunctional proteins and lipids from mitochondria to the lysosome for degradation in human cells. The generation of MDVs involves the budding of the mitochondrial outer membrane, and the selective cargo packaged into MDVs depends upon the specific stressors. MDV trafficking is mediated by the cytoskeleton and molecular motors, and MDVs have diverse biological functions, including quality control and organellar crosstalk [80-82].

- **Mitophagy**

Mitophagy is a crucial process for maintaining cell homeostasis and removes damaged or unnecessary mitochondria. It is a type of selective autophagy that occurs in parallel to other cellular processes, including mitochondrial biogenesis. The process involves several autophagy-related proteins (Atg) and their complexes. Mitophagy in yeast is triggered by various factors such as starvation, aging, oxidative stress, mitochondrial dysfunction, and signaling proteins. The process is initially activated by Atg32, a mitochondrial outer membrane protein, and regulated by several factors, including ubiquitination-deubiquitination processes, and it interacts with other types of autophagy[83, 84].

- **Autophagy**

Autophagy is a cellular process in which cells break down and recycle their own components. This process is essential for maintaining cellular homeostasis, by removing damaged or unwanted cellular materials and providing the cell with nutrients during periods of stress or nutrient deprivation. During autophagy, a portion of the cytoplasm or an organelle including

mitochondria is enclosed in a double-membrane vesicle called an autophagosome. The autophagosome then fuses with a lysosome or vacuole in budding yeast, forming an autolysosome, where the contents of the autophagosome are degraded by lysosomal enzymes. The breakdown products are then released into the cytoplasm, where they can be used to generate energy or build new cellular components[85]. As mentioned above, mitophagy is a type of selective autophagy. Therefore, it is not surprising that mitophagy shares a common molecular machinery with other forms of autophagy. For example, the autophagy-related genes encode proteins, which are required for the formation of autophagosomes that are also required for mitophagy[86]. In addition, some of the signaling pathways that regulate autophagy, such as the mTOR (mechanistic target of rapamycin) pathway, also regulate mitophagy[87, 88]. This suggests that there is a crosstalk between different quality control pathways.

Overall, these above mentioned mitochondrial quality control pathways act individually or cooperatively to maintain cellular homeostasis.

2. The Ccr4-Not complex

2.1 Structure of Ccr4-Not complex

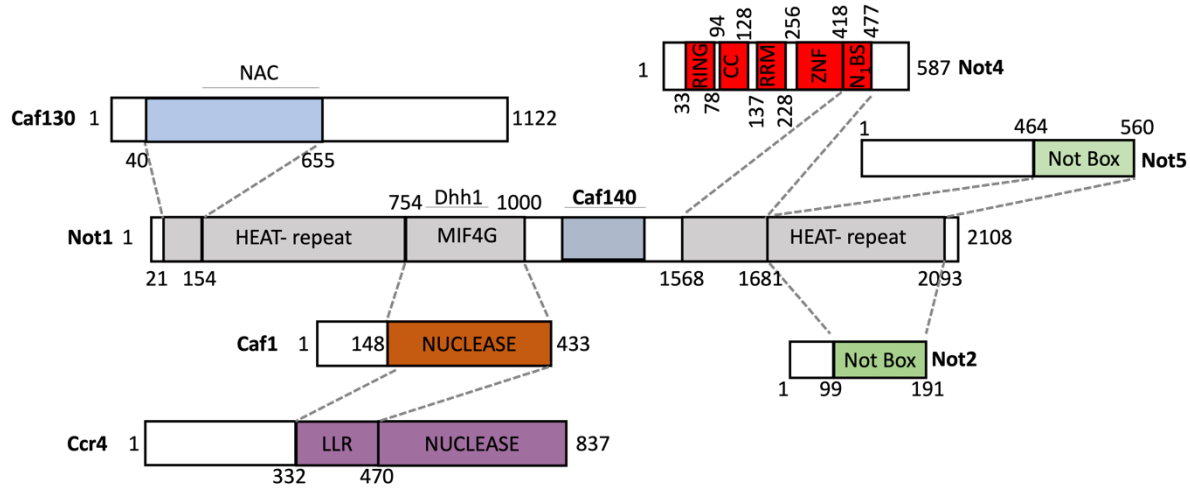
The Ccr4-Not complex is a highly conserved multiprotein complex that plays a crucial role in gene expression regulation in eukaryotic cells. In budding yeast, the complex consists of nine core subunits and is about 1 MDa. It includes the Not proteins (Not1, Not2, Not3, Not4, and Not5). The name "Not"（ Negative On TATA-less）was derived from a selection indicating their importance in repressing promoters lacking a canonical TATA box[89]. Ccr4 (Carbon Catabolite Repression), initially identified as a gene involved in regulating glucose-repressible genes, is also a component of the complex[90]. Additionally, the Caf proteins (Caf1, Caf40, and Caf130) are named for their association with Ccr4 (Ccr4-Associated Factor)[91, 92]. In addition, other proteins, including Caf4, Caf16, Dhh1, Btt1, and several other less well-characterized proteins, can also associate with the core of the Ccr4-Not complex, forming larger complexes with a size of 2MDa [92] [93, 94]. Across different species, there are variations in the components of the core complex **(Table 2).**

Table 2 Name of *CCR4-NOT* Genes in Yeast and Human, table adapted form reference[95]

Species / Core name	*Saccharomyces cerevisiae*	*Homo sapiens*
Not1	*NOT1/CDC39*	*CONT1*
Not2	*NOT2/CDC36*	*CNOT2*
Not3	*NOT3*	/
Not4	*NOT4/MOT2/ SIG1*	*CNOT4*
Not5	*NOT5*	*CNOT3*
Ccr4	*CCR4*	*CCR4a/CNOT6C* and *CCR4b/CNOT6L*
Caf1	*CAF1/POP2*	*Caf1a/CNOT7/CAF1* and *Caf1b/CNOT8/POP2/CALIF*
Caf130	*CAF130*	/
Caf40	*CAF40*	*CNOT9/Rcd1/CAF40/RQCD1*
Not10	/	*CNOT10*
Not11	/	*CNOT11*
TAB182	/	*TAB182*

Not1/CNOT1 is a scaffold protein, which provides a platform for docking of the other subunits (**Figure 7)**. Electron microscopy showed that Not1 has an L-shape architecture with two arms of approximately equal length, but one arm is shorter than the other (lower panel, **Figure 7)**. The N-terminal domain of Not1 adopts an elongated HEAT-repeat fold, and interacts with the yeast specific subunit Caf130, precisely between amino acids 21 to 154 of the Not1 protein (**Figure 7**). The mammalian specific subunits CNOT10 and CNOT11, which might be functional homologues of yeast Caf130, also dock onto the N-terminus of CNOT1[96, 97]. The central region of Not1 consists of a MIF4G (Middle domain of Initiation Factor 4G) domain followed by a helical domain. The MIF4G domain facilitates the docking of the deadenylases Caf1 and Ccr4[98]. It also interacts with the DEAD-box helicase Dhh1, a translational repressor and decapping activator [94, 99](**Figure 7**). Mammalian and fly CNOT9, orthologues of yeast Caf40, bind to a domain at the C-terminus of CNOT1, between the nuclease binding domain and the NOT module binding domain [100, 101] (**Figure 7**). The C-terminal domain of Not1, once again adopting an elongated HEAT-repeat fold, binds the Not2-Not5 heterodimer of the Not module[102, 103] and Not4 of the ubiquitination module[102] (upper panel, **Figure 7**).

A



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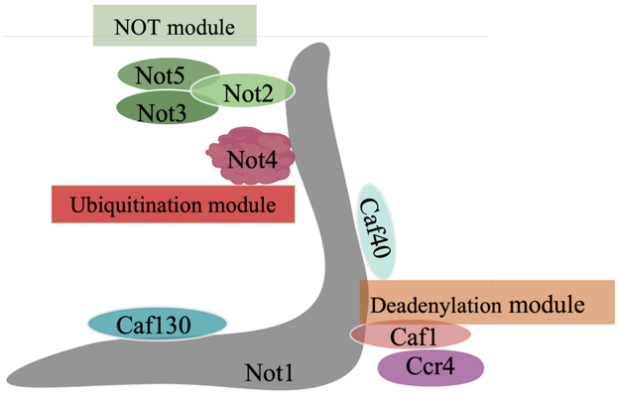

Figure 7. Linear and cartoon representations of the Ccr4-Not complex in budding yeast. Picture A was adapted from[95, 96] and picture B was adapted from[95]**.** The upper panel illustrates the protein domains of the core subunits of the Ccr4-Not complex. The interactions between these domains are indicated by dashed lines. The lower panel is a representation of the L-shape of the Ccr4-Not complex defined by electron microscopy [104] with the expected position of the core subunits on the Not1 scaffold. The subunits are organized into three different modules.

Not3 is homologous to Not5. Its exact location remains unclear. The current knowledge places it with Not2 and Not5, due to similar mutant phenotypes [104] (lower panel, **Figure 7)**. Not4, a RING E3 ligase, docks onto a C-terminal region of Not1 as well, positioned upstream of the site where a Not5-Not2 heterodimer docks[102] (lower panel, **Figure 7)**. The complex can be divided into three functional modules: the deadenylation module, the ubiquitination module and the NOT module (lower panel, **Figure 7)**.

- **The deadenylation module**

The deadenylation module consists of the Ccr4 and Caf1 catalytic subunits that posess deadenylase activity. Caf1, belonging to the DEDD-type nuclease superfamily, is named after the conserved catalytic residues (aspartate, Asp, D; glutamate, Glu, E) found in three sequence motifs[105, 106]. It possesses an RNase D characteristic of the DEDD superfamily, albeit with a suboptimal SEDQ signature sequence (serine, Ser, S; glutamine, Gln, Q)[107]. On the other hand, Ccr4 belongs to the Mg^{2+}-dependent exonuclease-endonuclease-phosphatase (EEP) superfamily[108]. It has a non-conserved N-terminal region rich in glutamate (Glu) and aspartate (Asp), connected to a central region containing five leucine-rich repeats (LRR) via a disordered amino acid linker[109, 110]. The C-terminal region of Ccr4 contains the deadenylase domain, which is typical of its superfamily and features conserved catalytic glutamate (Glu) and histidine (His) residues in the active site [111](upper panel, **Figure 7**). Caf1 functions as a tether, connecting Ccr4 to Not1. This tethering process involves two steps. Firstly, the deadenylase module of Caf1 is recognized by the central MIF4G domain of Not1. Secondly, Caf1 binds to the leucine-rich repeat (LRR) domain of Ccr4, thereby anchoring its nuclease domain (upper panel, **Figure 7**)[112]. It is important to note that Ccr4 does not directly interact with Not1, and its nuclease domain remains accessible. The interaction between Caf1 and Ccr4 is crucial for the stable association of Ccr4 with the complex, ensuring efficient deadenylation[109].

- **The ubiquitination module**

The ubiquitination module comprises Not4, which consists of various domains in its conserved N-terminal region. These domains include the RING domain (residues 33-78), a positively charged linker with coiled-coil propensity (CC, residues 94-128), an RNA recognition motif (RRM, residues 137-228), and a C3H1-type zinc finger domain (ZNF, residues 256-420) (upper panel, **Figure 7)[113]**. The E3 ligase activity of Not4 relies on its interaction with a specific E2 enzyme, such as Ubc4/5 in yeast or UbcH5B in humans[114, 115].

Furthermore, the unconserved C-terminal region of Not4 (residues 418-477), known as the Not1 binding domain (N1BS), forms an α-helix that interacts with residues 1568-1681 of Not1 in yeast. This interaction occurs within the HEAT repeats of Not1 and is independent of the

NOT module[102, 116]. In yeast Not4 is a confirmed subunit of the Ccr4-Not complex, however, it does not exhibit stable association with the complex in human and *Drosophila* cells[117, 118]. Nevertheless, the functional role of Not4 remains conserved, as the human CNOT4 can compensate for the deletion of Not4 in yeast [119].

- **The NOT module**

The NOT module is composed of the Not2, Not3, and Not5 subunits, which are closely associated with each other genetically and biochemically[103]. Specifically, residues 5-75 in Not2 and residues 346-464 in Not5, known as NOT box, wrap around the C-terminal HEAT repeat domain of Not1[103, 119] (upper panel, **Figure 7**). Not2, a 26 kDa protein, contains two functional regions: the C-terminal Not-box domain, essential for the conserved function of the Ccr4-Not complex, and the N-terminal domain, that can interact with other factors such as for instance Ada2 of the SAGA transcriptional co-activator[120]. Not5 consists of an N-terminal coiled-coil region that can interact with the ribosomal E-site, a low-complexity linker, and a C-terminal Not-box domain[120, 121]. The N-terminal domain of Not3 shares homology with the N-terminal domain of Not5. Loss of Not3 exhibits a relatively similar impact on growth compared to loss of the Not5 protein[122]. In human there is only one gene, CNOT3, that is the ortholog of yeast Not3 and Not5, which have probably originated from a gene duplication[123]. In human, CNOT2 is responsible for recruiting CNOT3 (Not5 in yeast) into the complex and maintaining the integrity of the complex[117]. In yeast, Not2 and Not5 have been reported to interact with components of the transcription machinery, including subunits of TFIID [124, 125] and as mentioned above SAGA[126].

The Ccr4-Not complex is involved in a wide range of cellular processes, including histone modification, transcription initiation and elongation, mRNA export, mRNA translation initiation, elongation, and mRNA degradation. In addition, protein ubiquitination is also mediated by the complex. The following is a detailed summary of the roles of the Ccr4-Not4 complex in each of these steps.

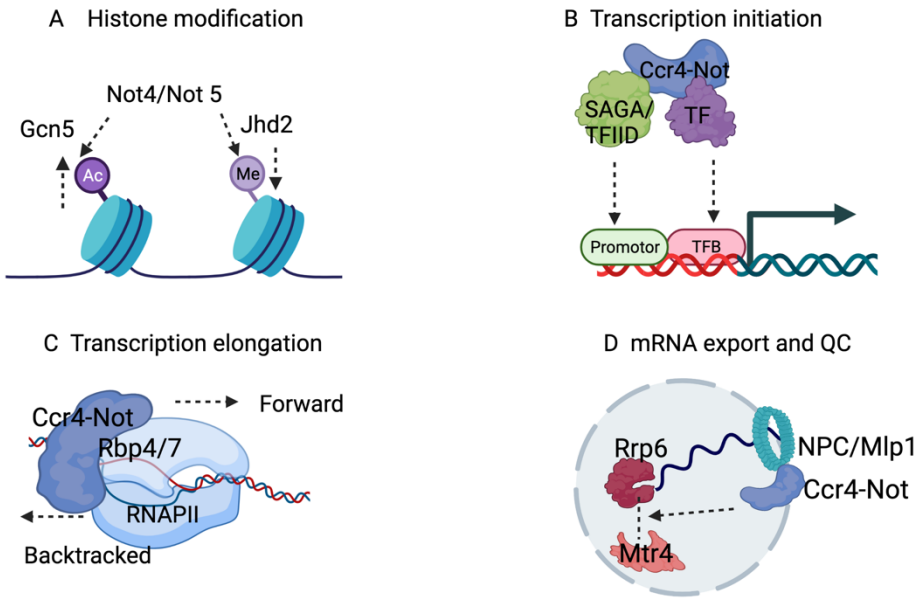

Figure 8. Nuclear roles of Ccr4-Not: from histone modifications to transcriptional control and mRNA export

The Ccr4-Not complex, specifically Not4 and Not5, regulates histone modifications, namely acetylation of histones H3 and H4 and histone H3 tri-methylation, impacting chromatin structure and transcription (Panel A). The complex impacts the relative presence of TFIID and SAGA transcription complexes at gene promoters to regulate transcription (Panel B). During transcription elongation, the complex helps RNA polymerase II (RNAPII) overcome transcriptional blocks and resume elongation by promoting realignment of the RNA transcript (Panel C). The Ccr4-Not complex also interacts with various components involved in mRNA export and nuclear quality control (Panel D).

2.2 Ccr4-Not's functions in the nucleus

Histone modification is regulated by Ccr4-Not (panel A, **Figure 8)**. The organization of DNA into chromatin fibers poses a challenge for transcription, and chromatin remodelers are needed for RNAPII to access the DNA. The post-translational modifications of histone tails play a crucial role in transcription by providing a landing platform for protein complexes, such as ATP-dependent chromatin remodeling complexes. The Ccr4-Not complex is involved in regulating two histone marks: histone H3 and H4 acetylation and histone H3 lysine trimethylation. In yeast cells lacking Not4 or Not5, histones H3 and H4 are globally hypoacetylated[127]. The histone acetyltransferase (HAT) Gcn5 acetylates histones within nucleosomes when associated with other proteins in HAT complexes. However, Gcn5 purified from cells lacking Not5 can acetylate free histones, but it fails to acetylate nucleosomes *in*

vitro. The Ccr4-Not complex also affects histone H3 lysine trimethylation, significantly reduced in cells lacking Not4 or Not5. This is likely because Not4 is responsible for the turnover of the Jhd2 demethylase that demethylates histone H3[128, 129] . Thus, the Ccr4-Not complex plays a crucial role in regulating histone modifications, which contribute to transcription regulation by providing a platform for protein complexes to access the DNA.

The inhibition or activation of transcription initiation by the Ccr4-Not complex is mediated by specific transcription factors or general transcription factors (panel B, **Figure 8)**. For example, Not1 interacts with the retinoic acid X receptor in a ligand-dependent manner, and Caf40 binds to transcriptional activators such as c-Myb and AP-1[130, 131]. Regarding general regulators, the presence of the Ccr4-Not complex is crucial for regulating the relative abundance of TFIID and SAGA at promoters[132, 133]. TFIID and SAGA are general transcription complexes that facilitate recruitment of the TATA binding protein to promoters. While TFIID is typically present in constitutive housekeeping genes, SAGA is predominantly found in highly inducible genes. Ccr4-Not subunit crosslinking is enriched in SAGA-dependent genes, and analysis of gene expression in *ccr4-not* mutants indicates that SAGA-dependent genes are more significantly affected. Numerous studies have reported genetic and physical interactions between Ccr4-Not subunits and TFIID or SAGA[132-134].

Transcription elongation is also affected by Ccr4-Not (panel C, **Figure 8**). Research conducted both *in vivo* and in *vitro* have demonstrated that the Ccr4-Not complex plays a role in transcription elongation. *In vitro*, it was found that the Ccr4-Not complex can directly interact with RNAPII transcription elongation complexes isolated and purified from yeast, and this interaction is dependent on the presence of the Rpb4/7 module of the polymerase[135]. *In vivo* evidence supporting the involvement of the Ccr4-Not complex in transcriptional elongation comes from an assay that observes the slower clearance of the last wave of RNAPII across a long gene in *ccr4-not* mutants. Ccr4-Not plays a direct role in transcription elongation by aiding in the rescue of backtracked RNAPII. However, it does not impact the transcription rate of un-arrested RNAPII [136]. Moreover, The Ccr4-Not complex enhances the recruitment of TFIIS (Transcription elongation Factor IIS) to backtracked RNAPII complexes, facilitating the cleavage of the displaced transcript within backtracked RNAPII by TFIIS. This process enables the resumption of elongation[137, 138].

The Ccr4-Not complex plays multiple roles in the process of mRNA export from the nucleus to the cytoplasm (panel D, **Figure 8**). It interacts with proteins involved in the maturation of the mRNA ribonucleoprotein particle (mRNP), such as the nuclear poly(A) binding protein Mlp1 of the inner basket of the nuclear pore complex (NPC)[139]. The Ccr4-Not complex also promotes proper interaction between the nuclear exosome subunit Rrp6 and its cofactor the RNA helicase Mtr4, which is involved in nuclear RNA quality control. Deletion of Ccr4-Not subunits can lead to the production of low quality RNAs that are monitored and repaired through the Rrp6-mediated nuclear quality control mechanism[140, 141]. Therefore, the Ccr4-Not complex plays an important role in both mRNA quality control and the process of export from the nucleus to the cytoplasm.

2.3 Ccr4-Not's functions in the cytoplasm

In the cytoplasm, Ccr4-Not has roles in mRNA translation initiation, elongation, and decay, as well as protein ubiquitination.

- **Translation initiation**

Translation initiation is the first step in protein synthesis. It involves the binding of the ribosome's small subunit 40S with the initiator methionyl-tRNA on the mRNA, followed by scanning of the 5' UTR of mRNA to identify the AUG start codon. In this process, the key player is the cap-binding complex eIF4F, which consists of the cap-binding protein eIF4E, the large regulatory scaffold eIF4G, and the DEAD-box RNA helicase eIF4A[142]. The eIF4F complex also interacts with the poly(A)-binding protein (PABP in human, Pab1 in yeast) through the eIF4G subunit. This circularization of the mRNA is believed to contribute to the regulation of translation initiation[143](**Figure 9)**. Therefore, disturbed circularization, either via deadenylation or decapping, can repress translation initiation. Studies in human have shown that the Ccr4-Not complex represses translation initiation in the context of miRNA-mediated repression through CNOT1 interaction with either of two DEAD-box RNA helicases: DDX6 (Dhh1 in yeast) and eIF4A2[99, 144, 145]. DDX6 is a translational repressor and plays a role in mRNA decapping. It is also a component of cytoplasmic mRNA degradation bodies (P bodies)[146, 147]. The interaction between CNOT1 and DDX6 promotes decapping. eIF4A2 is a paralog of eIF4A1 and is involved in miRNA-mediated repression. It associates with the Ccr4-

Not complex component CNOT7[144, 148]. Studies have suggested that the binding of eIF4A2 inhibits translation by directing initiation to upstream AUG start sites[149]. The exact mechanism by which DDX6 might function in this process remains to be further investigated. In budding yeast, Dhh1 associated with mRNAs can phase separate into liquid droplets that are dissolved by Not1 to regulate translation[150]. Interestingly, the absence of Not4 or Not5 in yeast cells leads to an accumulation of ribosomes at the start codon, suggesting the involvement of these proteins in translation initiation or at the transition from initiation to elongation[151]. Moreover, Not4 and Not5 associate with ribosomes at start, supporting this idea [121].

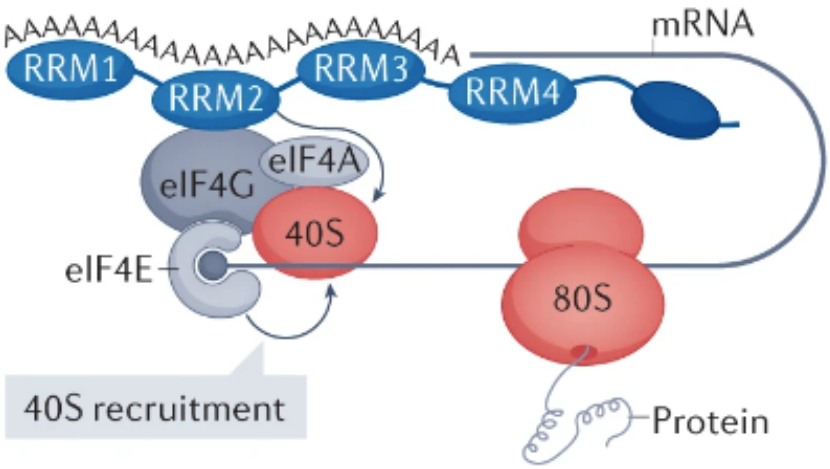

Figure 9. The circularization of mRNA model in eukaryotes. (Picture taken from reference[152])

The 5'-cap of mRNA is bound by translation initiation factor 4E (eIF4E). Simultaneously, eIF4G interacts with eIF4E, Poly(A)-binding protein, and the RNA helicase eIF4A. This complex formation facilitates the recruitment of the small ribosomal subunit (40S) to the mRNA, leading to the formation of an 80S ribosome on the start codon, which initiates translation.

- **Translation elongation**

Translation elongation is the process of protein synthesis where the ribosome reads the mRNA codons, adds amino acids to the growing polypeptide chain, and translocates along the mRNA template. It involves codon recognition, peptide bond formation, and translocation until all codons have been read. This process is influenced by factors like codon composition,

which varies for each mRNA transcript. Several events occur during translation elongation, including modifications and folding of the nascent protein, interactions with chaperones or partner proteins, and co-translational quality control responses. Growing evidence indicates that the speed of ribosome movement along the mRNA, referred to as translation elongation dynamics, is a significant regulator of these co-translational events. For instant, slowdown of translation elongation contributes to co-translational protein complex assembly, and mitochondrial or ER mRNA co-translational targeting. The development of advanced techniques such as polysome profiling, 5'-Seq, Cryo-EM, and Ribo-Seq enables us to investigate the conformation and speed of ribosome movement on mRNA. Several studies show that Ccr4-Not has roles in regulating co-translation events.

- **Co-translational assembly**

Most cellular proteins do not function in isolation but form protein complexes to carry out their cellular roles. The assembly of these complexes can occur after protein synthesis (post-translational) or during the process of translation (co-translational) (**Figure 10)**[153, 154]. While our understanding of complex assembly is still limited, recent studies have highlighted the importance of the Ccr4-Not complex, particularly the proteins Not1, Not4, and Not5, in this process. For example, in the case of RNAPII, the two largest subunits Rpb1 and Rpb2 initially form intermediate complexes in the cytoplasm with specific RNAPII subunits and dedicated chaperones[155]. The interaction between Rpb1 and its chaperone, R2TP-Hsp90, during translation is critical for the formation of soluble and functional intermediate complexes[156]. Without Not5, Rpb1 is prone to aggregation, resulting in the accumulation of the Rpb2 assembly intermediate complexes that lack Rpb1. Not5 is believed to prevent Rpb1 aggregation by facilitating the association of Not1 with *RPB1* mRNA, which is essential for recruiting the Rvb2 co-chaperone to the site of Rpb1 translation[156]. Another example is the SAGA histone acetyltransferase complex. Studies have demonstrated the significant role of co-translational assembly for SAGA in mammalian cells. In yeast, the mRNAs encoding different SAGA subunits, such as Ada2, Gcn5, and Spt20, co-localize. In the absence of Not5, the formation of Gcn5 complexes is impaired, leading to the accumulation of Ada2, Gcn5, and Spt20 in cytoplasmic speckles instead of their proper localization in the nucleus. The

association of Not1 with *ADA2* mRNA, facilitated by Not5, is crucial for the co-translational interaction and correct assembly of SAGA subunits[157]. Another example is the assembly of the proteasome, a complex composed of core and regulatory particles, that also relies on co-translational processes. Two subunits of the proteasome base, Rpt1 and Rpt2, undergo ribosome pausing during synthesis to allow co-localization of their mRNAs and co-translational association of nascent chains in Not1-containing assemblysomes (NCAs)[158]. The specific mechanisms underlying this process still require further exploration. Recent studies suggest that regulation of ribosome dynamics may be a possible mechanism.

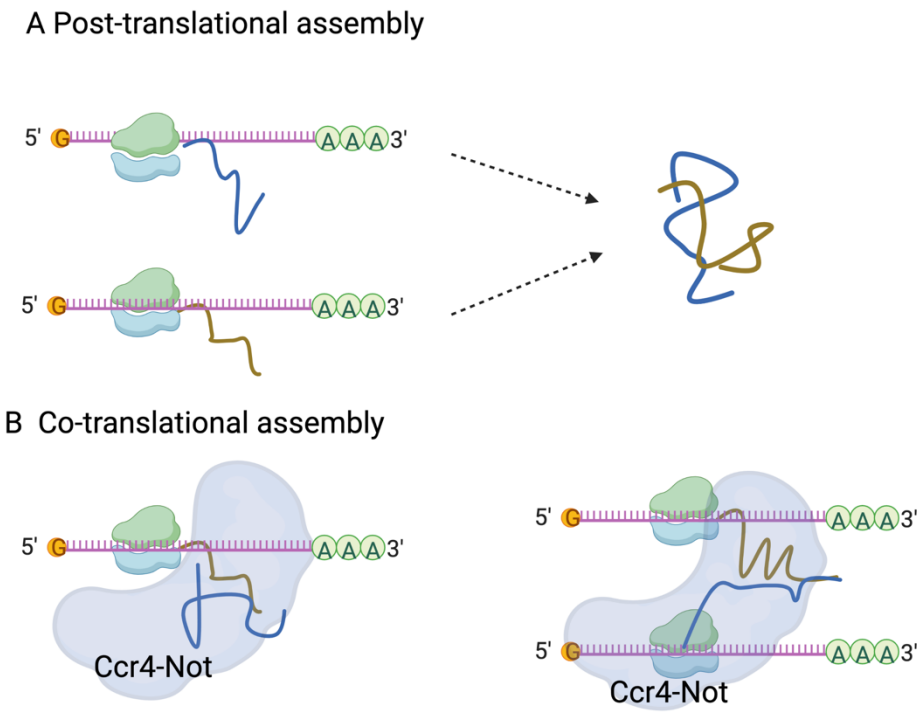

Figure 10. Protein assembly: post-translational vs co-translational modes

(A)In post-translational assembly, proteins are synthesized individually, and assembly occurs only after the synthesis of each protein is complete. (B) In co-translational assembly, nascent polypeptides fold or assemble with the assistance of chaperones (left panel) or co-localize with partner mRNAs during translation (right panel). This process is regulated by translation elongation dynamics, including ribosome pausing, and the recruitment of Not proteins to the mRNA, which facilitate the recruitment of chaperones or the co-localization of mRNAs encoding partner proteins.

- **mRNA translation elongation dynamics and mRNA solubility**

In order to ensure co-translational assembly, it is crucial for mRNAs and nascent chains to meet each other at the right time and location. Slowing down ribosome movement becomes necessary to allow sufficient time for mRNA or nascent chain interaction with its partners. Membrane-less condensation sites, such as Not1-containing assemblyosomes, have emerged as a safeguarding locations for co-translational assembly. Codon optimality plays a significant role in regulating ribosome movement, and recent studies suggest an involvement of Not proteins in regulation of dynamics according to codon-optimality. Not4 and Not5 bind to actively translating ribosomes, particularly when non-optimal codons are present in the ribosome A-site. Cryo-electron microscopy has confirmed the direct interaction between the N-terminal domain of Not5 and the E-site of post-translocation ribosomes in the presence of empty A site, a situation more likely to occur when non-optimal codons are in the in A site[121]. Furthermore, ribosome profiling data demonstrates an enrichment of non-optimal codons in the A-site of Not4-associated ribosomes, along with an increased abundance of ribosomes carrying non-optimal A-site codons in cells lacking Not5[121, 151]. These findings suggest a potential mechanism in which Not proteins might maintain ribosome pausing when encountering non-optimal codons at the A site, allowing for productive interactions of nascent chains with their partner. However, if the partner fails to arrive in a timely manner, the mRNAs may undergo co-translational decay.

The pattern of mRNA co-translational decay is hence also influenced by Not proteins. This can be detected through 5'P decay intermediates (5'P-Seq), which additionally provides insights into the last translating ribosome. Indeed, the 5’ to 3’ exonuclease Xrn1 follows the last translating ribosome and decay intermediates show a 3-nt periodicity. One advantage of this technique is its ability to capture mRNA translation dynamics for the entire cellular RNA pool, whereas ribosome profiling (Ribo-seq) only provides measures for the mRNA from the soluble pool. It should be noted that Ribo-seq data may not represent the complete picture of translation elongation dynamics since mRNAs can be insoluble, found in membrane-less condensates, attached to membranes (e.g., mitochondria or ER), or even aggregated. Recently, the Collart laboratory investigated mRNA elongation dynamics using both the total RNA pool and the soluble RNA pool. Their study revealed, via 5'P-Seq of soluble RNAs, an increase in A-site ribosome dwelling occupancy (RDOs) at non-optimal codons upon depletion

of Not1, while the opposite trend was observed upon depletion of Not4. In contrast, no codon-optimality related changes in A-site RDOs were detected for the total RNA pool when Not proteins were depleted[159]. This compelling evidence indicates that the primary impact of Not proteins may be modulating the solubility of specific mRNA categories, consequently influencing detectable translation elongation dynamics according to codon optimality in the soluble RNA pool.

- **mRNA decay**

Upon export from the nucleus to the cytoplasm, mRNA is protected by cap-binding proteins at the 5' end and poly(A)-binding proteins (Pab1) at the 3' end, ensuring stability and efficient translation. mRNA degradation can occur through post-translational decay or co-translational decay. Current knowledge is that the majority of mRNAs undergo post-translational decay.

Post-translational decay typically involves four steps. Deadenylation, mediated by deadenylase enzymes, leads to the shortening of the poly(A) tail. Pan2, Pan3, Ccr4, and Caf1 are key proteins involved in deadenylation, with Pan2 and Pan3 removing the terminal poly(A) tail and Caf1 and Ccr4 handling the rest[160, 161]. It is important to note that the genes encoding these proteins are not essential in yeast, suggesting functional redundancy. Deadenylase components can directly bind to the 3' end of mRNA or be recruited through RNA-binding proteins such as Puf3 or the miRNA-induced silencing complex (miRISC)[162, 163]. Following deadenylation, mRNA transcripts may undergo decapping or translation silencing. Eukaryotic mRNA decapping is initiated by the formation of a decapping complex comprising the Dcp1 and Dcp2 enzymes, which recognize mRNA cap structures[164]. Decapping can also occur independently of deadenylation. For example, Lsm1-7 proteins can associate with oligo-A or 3' uridylated tails, facilitating the recruitment of the decapping machinery[165, 166]. The decapping process is tightly regulated by activators and inhibitors, such as the enhancer Dhh1, which interacts with Not1 as mentioned earlier[99, 101]. Upon decapping, mRNA transcripts are degraded from the 5' end to the 3' end by the exonuclease Xrn1, as confirmed by cryo-electron microscopy[167-169]. Alternatively, post deadenylation, mRNA transcripts can be degraded from the 3' end to the 5' end by an exonuclease complex known as the exosome[170].

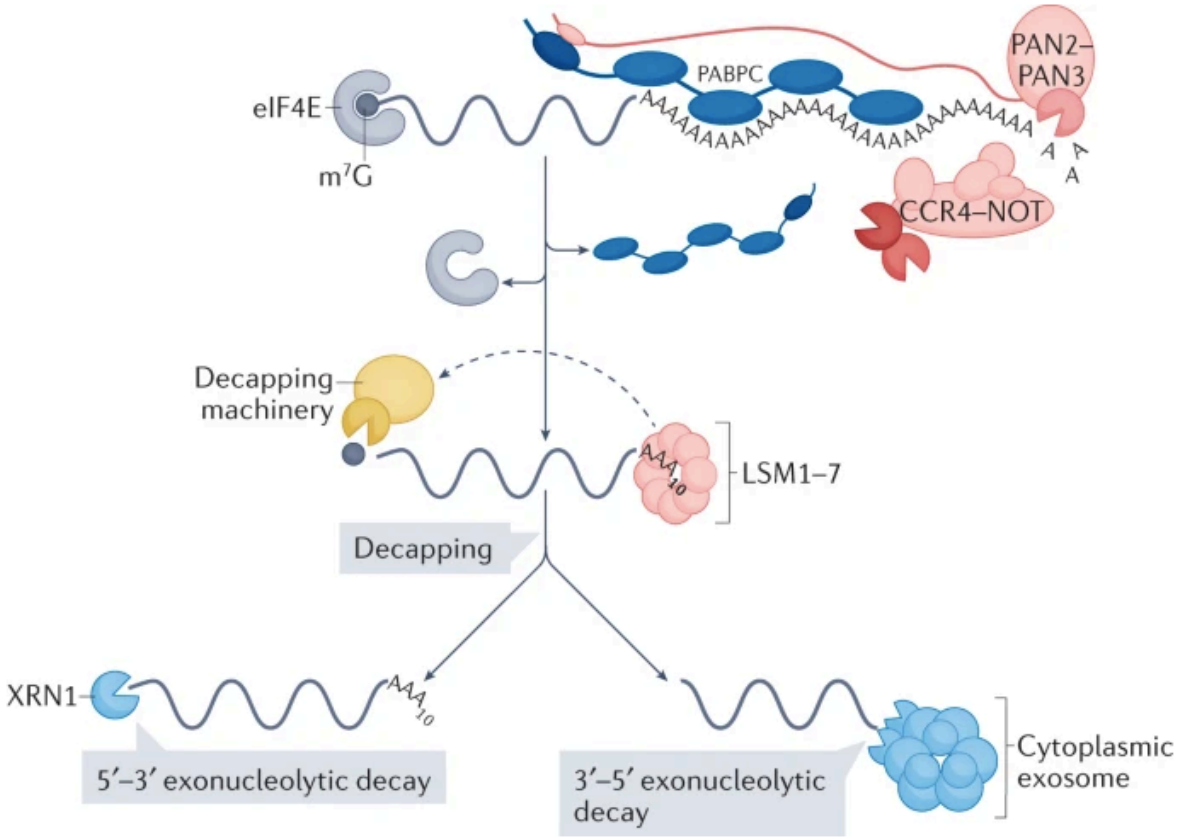

Figure 11. Eukaryotic mRNA deadenylation and decay (Picture taken from reference[152])

The deadenylation and decay of eukaryotic mRNA involves multiple steps. Firstly, the Pan2-Pan3 and/or Ccr4-Not deadenylase enzymes remove the poly(A) tail. This leads to a potential weakening of the association between the poly(A) binding protein (PABPC) and the eIF4E-5′-cap complex. Subsequently, the decapping machinery removes the 5′-cap structure. Alternatively, decapping can occur independently of deadenylation, facilitated by Lsm1-7 proteins that bind to oligo-A or 3′ uridylated tails. Following decapping, the mRNA is degraded either in the 5′-3′ direction by Xrn1 exonuclease or degradation can occur in the 3′-5′ direction by the cytoplasmic exosome whether decapping has occurred or not.

On top of mRNA post-translational degradation, a growing focus has emerged on co-translational mRNA decay. It used to be considered that ribosomes protect mRNA from degradation, but we now know that substantial levels of decay is co-translational. Moreover, co-translational mRNA degradation can be induced in specific conditions of quality control, such as no-go decay (NGD), nonsense-mediated mRNA decay (NMD), and non-stop decay (NSD) (**Figure 12)**.

NGD is triggered by when the ribosome stalls and this can be due to a variety of conditions some of which are for instance rare codons, mRNA secondary structures, amino acid starvation leading to a deficiency in charged tRNAs, tRNA deficiencies or oxidative stress. Long-lasting stalling may lead to ribosome collisions. The E3 ubiquitin ligase Hel2 can recognize the conformation of collided ribosomes that will trigger ribosome-associated quality control initiated by Hel2 ubiquitination of specific ribosomal proteins and recruitment of other factors, leading the degradation of an incomplete nascent chain. The mRNA is endonucleolytically cleaved near the site where the ribosome stalls. This cleavage produces two fragments of mRNA, termed 5' NGD and 3' NGD-mRNA intermediates. These mRNA fragments are then further degraded. The 3' NGD fragment is degraded by the exoribonuclease Xrn1. On the other hand, the 5' NGD fragment is degraded by the exosome. No-Go Decay can alternatively involve Not4 in specific conditions, such as if the usual pathway is non-functional, where Not4 first monoubiquitinates ribosomal protein eS7 (Rps7A) and this is followed by Hel2-mediated polyubiquitination, leading to the cleavage and decay of mRNAs[171, 172] (left panel, **Figure 12)**. Non-stop Decay identifies and degrades transcripts lacking a stop codon, and this is primarily carried out by the exosome complex. It is facilitated by Ski7 and involves removing the poly(A) tail of the transcript and degrading the main body [173] (middle panel, **Figure 12)**. Nonsense-Mediated Decay identifies and degrades mRNAs containing premature termination codons (PTC), preventing the synthesis of potentially harmful truncated proteins. The core NMD machinery, comprising of Upf1, Upf2, and Upf3, plays a critical role in this process. The degradation of mRNA in NMD is achieved through accelerated deadenylation and deadenylation-independent decapping, leading to 5'-3' digestion by Xrn1p and 3'-5' degradation by the exosome[174] (right panel, **Figure 12)**. Although these three mRNA degradation mechanisms arise from different causes, they share many common factors in the subsequent mRNA decay process, such as Xrn1 and exosome.

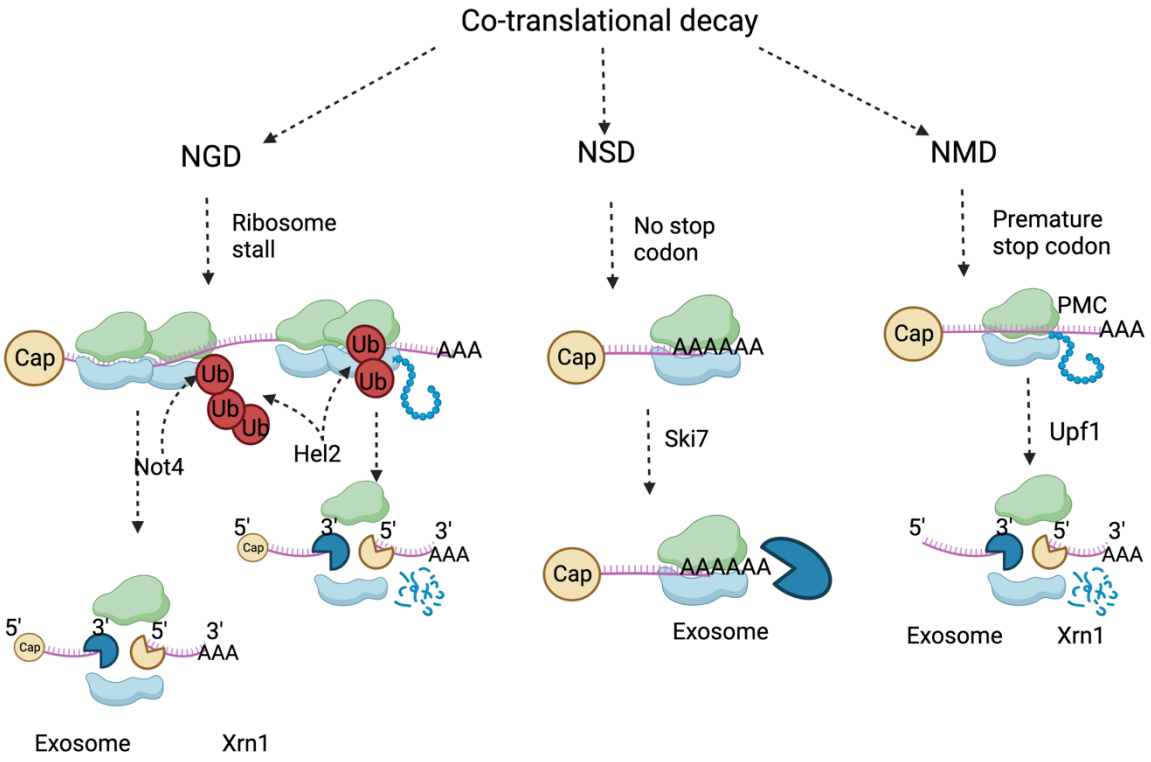

Figure 12. An overview of co-translational mRNA decay pathways

NGD is triggered by ribosome stalling, leading to ribosome-associated quality control and mRNA cleavage. NGD fragments are further degraded by Xrn1 and the exosome. Not4 is involved in NGD when Hel2-mediated NGD is non-functional. NSD identifies and degrades transcripts without a stop codon, primarily through the exosome. NMD identifies and degrades mRNAs with premature termination codons, involving Upf proteins and degradation via Xrn1 and the exosome.

As discussed above, the Ccr4-Not complex is a master regulator during the whole lifespan of mRNAs and consistently its' subunits are present in both the nucleus and cytoplasm, and they can interact with different factors and stages of gene expression. The complex can act independently at different steps along the gene expression pathway, or its' functions at different steps can be physically connected. Overall, the ability of the Ccr4-Not complex to interact with a wide range of proteins and complexes allows it to coordinate and fine-tune gene expression in response to a range of internal and external signals[175].

3. The functional connection between mitochondria and Ccr4-Not complex

As I mentioned above, there are functional connections between the mitochondria and the Ccr4-Not complex. Puf3 is involved in mitochondrial targeting of mRNAs, by interacting with mRNA 3'UTRs, but it also degrades such mRNAs by recruiting the Ccr4-Not complex, depending upon the metabolic state of yeast. During yeast fermentation, when an excess of mRNAs are generated, Puf3 can store a portion of the mRNAs to meet a future increased demand for mitochondria[56]. Another portion of the mRNAs may undergo degradation mediated by Puf3 and the Ccr4-Not complex[53]. On the other hand, NAC guides the nascent polypeptide to the mitochondria through its interaction with the mitochondria outer membranes, such as Om14 or Sam37, and NAC interacts with the Ccr4-Not complex via Caf130 and it is a target of Not4 ubiquitination[176-178]. mRNAs bound by NAC and Ccr4-Not are enriched for nuclear encoded mitochondrial mRNAs, indicating that the Ccr4-Not complex may regulate the mitochondrial mRNAs through its interaction with NAC[45, 175, 179].

Membrane-less condensation is considered to limit mRNA solubility, and the regulation of mitochondrial mRNA solubility can also involve the Ccr4-Not complex, as discussed earlier[159]. Research has demonstrated that the Ccr4-Not complex is capable of forming membrane-less condensates to modulate mRNA expression. For instance, Not1 has been found in P bodies where it regulates gene expression[150]. In addition, Not1-containing assemblysomes allow proteosome co-translational assembly[158]. Additionally, studies from the Collart lab have proposed that Not4 and Not5 proteins can dynamically regulate translation by promoting the formation and dissolution of condensates through the ubiquitination of ribosomal proteins Rps7a and Rli1 by Not4[151]. Interestingly, it has also been observed that Puf3 also can form condensates, which function as storage compartments for mitochondrial mRNAs. These stored mRNAs can be rapidly released for translation when cells shift to a respiratory status[56]. Furthermore, studies have revealed that Puf3 can recruit the Ccr4-Not complex, leading to mRNA decay[54]. However, it is still unclear whether Puf3 and the Ccr4-Not complex coexist within the same granule structure to jointly regulate mitochondrial mRNA expression.

II. AIMS OF MY THESIS PROJECTS

My research project was aimed at investigating how the Ccr4-Not complex regulates mitochondrial protein synthesis in budding yeast. In the first part of my thesis, I explored the interaction between Ccr4-Not and NAC, and their roles in regulating the fate of mitochondrial mRNAs produced in excess, specifically *MMF1*. In the second part of my thesis, I investigated the interaction between Puf3 and Ccr4-Not, and their relationship in the regulation of mitochondrial mRNAs. These studies will contribute to a better understanding of the crucial roles played by the Ccr4-Not complex in mitochondrial protein synthesis (**Figure 13)**.

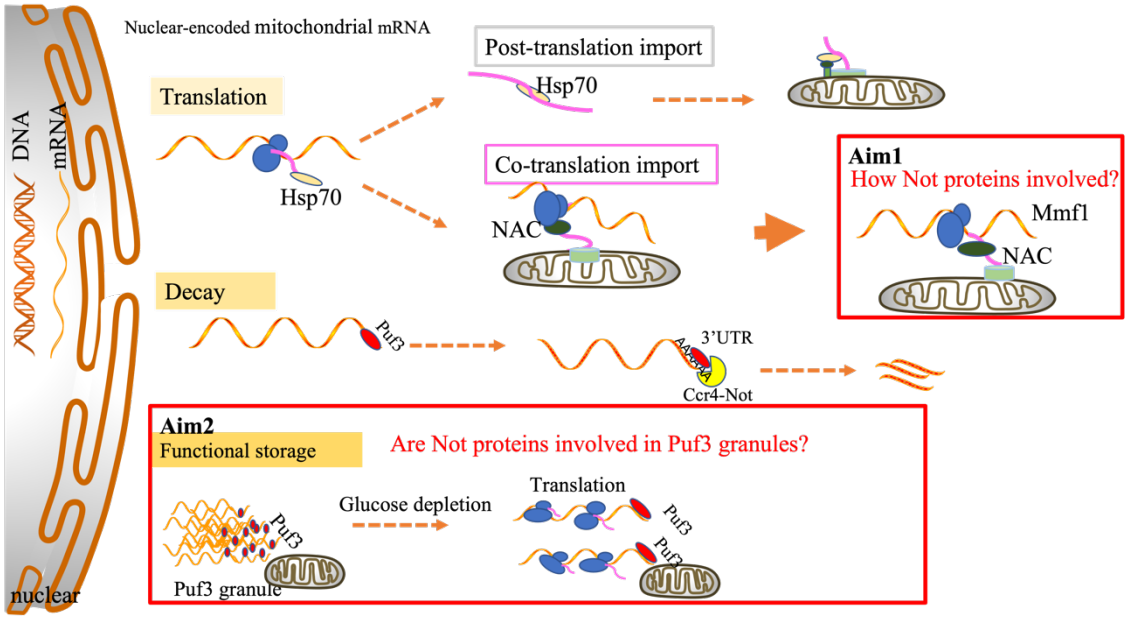

Figure 13. Aims of my thesis projects

Nuclear-encoded mitochondrial mRNAs can undergo post-translational or co-translational import into mitochondria, while others are targeted for decay by Puf3 or are inserted into protective Puf3 granules. In my thesis project, first I focused on studying the expression of the mitochondrial *MMF1* mRNA and investigated the involvement of Not proteins in co-translational events. Then I aimed to understand if Not proteins participate in Puf3 functions, namely in addition of mRNA decay also mRNA translation.

III. RESULTS

1. CHAPTER 1: Not4-dependent targeting of *MMF1* mRNA to mitochondria limits its expression via ribosome pausing, Egd1 ubiquitination, Caf130, No-Go-Decay and autophagy

This manuscript, authored by the following individuals, was published in the journal "Nucleic Acid Research" on April 24, 2023.

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Summary of chapter 1

In this study, we identified an integrated Ccr4-Not dependent mitochondrial co-translational quality control mechanism that eliminates overproduced nuclear-encoded mitochondrial *MMF1* mRNAs and precursors, avoiding proteotoxic stress. We have named this mechanism MitoENCay.

MitoENCay involves the participation of various key players, including mitochondrial outer membrane proteins Om14, Om45 and Cis1, Tom20, and Sam37. Additionally, the chaperone Egd1, Ccr4-Not subunits Not4 and Caf130, cytosolic disaggregase Hsp104, as well as the ribosome-associated quality control and No-Go Decay pathways together with autophagy, are also involved in this complex mechanism. One essential aspect of this mechanism is the requirement for ribosome pausing during translation of the *MMF1* mRNA, which provides sufficient time for proper mitochondrial targeting. The presence of the disaggregase ensures the dissolution of any aggregated nascent Mmf1, thereby guaranteeing effective targeting of ribosome-nascent chain complexes to the mitochondria. This docking is facilitated by the chaperone Egd1, the docking site Om14, and the mitochondrial targeting sequence of the nascent chain. Furthermore, Cis1 at the mitochondria acts as a sensor for an excess influx of precursor proteins. It is likely that increased ribosome pausing results in ribosome collision, triggering the activation of Ribosome-associated Quality Control and No-Go Decay pathways. Two ligases, Hel2 and Not4, are responsible for ubiquitinating proteins within the ribosome nascent chain complex. Subsequently, fission events lead to the generation of small vesicles containing fragmented mitochondria. Among these vesicles, those with heavily ubiquitinated ribosome nascent chain complexes are selectively targeted by autophagy and subsequently degraded within the vacuole. This comprehensive mechanism, involving multiple molecular components and intricate interactions, provides an integrated quality control system to ensure the proper level of production of mitochondrial proteins.

My contribution was extensive. I conducted the majority of the experiments and analyses, wrote the first draft of the manuscript and made the figures. The only experiments that were not mine are the ribosome profiling and its analysis.

Nucleic Acids Research, 2023 **1**
https://doi.org/10.1093/nar/gkad299

Not4-dependent targeting of *MMF1* mRNA to mitochondria limits its expression via ribosome pausing, Egd1 ubiquitination, Caf130, no-go-decay and autophagy

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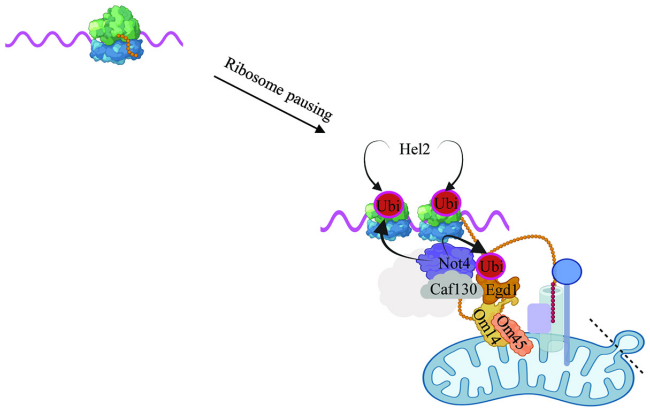
Received August 29, 2022; Editorial Decision March 31, 2023; Accepted April 11, 2023

ABSTRACT

The Ccr4–Not complex is a conserved multi protein complex with diverse roles in the mRNA life cycle. Recently we determined that the Not1 and Not4 subunits of Ccr4–Not inversely regulate mRNA solubility and thereby impact dynamics of co-translation events. One mRNA whose solubility is limited by Not4 is *MMF1* encoding a mitochondrial matrix protein. In this work we uncover a mechanism that limits *MMF1* overexpression and depends upon its co-translational targeting to the mitochondria. We have named this mechanism Mito-ENCay. This mechanism relies on Not4 promoting ribosome pausing during *MMF1* translation, and hence the co-translational docking of the *MMF1* mRNA to mitochondria via the mitochondrial targeting sequence of the Mmf1 nascent chain, the Egd1 chaperone, the Om14 mitochondrial outer membrane protein and the co-translational import machinery. Besides co-translational Mitochondrial targeting, Mito-ENCay depends upon Egd1 ubiquitination by Not4, the Caf130 subunit of the Ccr4–Not complex, the mitochondrial outer membrane protein Cis1, autophagy and no-go-decay.

GRAPHICAL ABSTRACT



INTRODUCTION

Mitochondria are essential organelles with functions in cellular metabolism and homeostasis. They are of central importance for cellular energetics and participate in signaling mechanisms that ensure survival or promote death of cells under stress (1,2). Disruption of mitochondrial function has been associated with a large variety of diseases (3,4). Mitochondria have a characteristic architecture, delimited by outer and inner membranes, with inner membrane invaginations called cristae where oxidative phosphorylation occurs. The inner most aqueous compartment is the matrix. More than 1000 proteins have been identified in yeast mitochondria and nuclear genes encode over 99% of these. Hence, mitochondrial precursor proteins are for the most part produced in the cytoplasm and must be targeted to the appropriate mitochondrial compartments by targeting signals. In some cases the mitochondrial mRNAs are targeted to the mitochondria where they are translated and proteins co-translationally imported ((5–8) and for review see (9)), while in other cases proteins are synthesized in the cytosol and must reach the mitochondria

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post-translationally. Little is known about how such proteins reach the mitochondria *in vivo* (10). Targeting of the mRNAs to the mitochondria can be mediated by RNA binding proteins associating with 3' untranslated regions (UTR) independently of translation, or by the mitochondrial targeting sequence of the nascent chains during translation. In budding yeast, the Puf3 RNA binding protein has important roles in targeting mitochondrial-specific mRNAs to the surface of mitochondria in respiratory conditions (6,7,11). For translation-dependent targeting, mitochondrial mRNAs can rely on the Nascent Polypeptide-associated Complex (NAC) chaperone, the Om14 or Sam37 mitochondrial outer membrane (MOM) proteins and Tom20 of the import machinery (8,12,13). The NAC chaperone is a heterodimer composed of alpha and beta subunits, respectively Egd2 and Egd1 or Btt1 in yeast, and it binds nascent peptides during translation (14,15). It is present in polysomes producing nuclear-encoded mitochondrial mRNAs (16,17). In all cases, the mitochondrial protein import machineries must take up the mitochondrial precursor proteins. These machineries are diverse and at least five major import pathways have been identified so far, each pathway characterized by a different machinery and different targeting signals (for review see (18)).

Important quality control (QC) systems respond to overexpressed mitochondrial precursors, to aberrant, mistargeted or stalled nascent proteins at the MOM, to a saturated or compromised import channel, but also to excessive aggregated proteins in the cytoplasm, that all collaborate to maintain cellular homeostasis (for review see (19)). For instance, nascent chains stalled on the ribosome and engaged with mitochondrial import channels are rescued by the ribosome-associated quality control (RQC) complex, comprised of the Ltn1 ubiquitin ligase, the ATPase Cdc48, Rqc1 and Rqc2. RQC assembles on the 60S ribosomes containing unreleased peptidyl-tRNA. Vms1, a tRNA hydrolase that releases the stalled polypeptide chains engaged by the RQC (20), antagonizes Rqc2 to prevent elongation of the nascent chain with carboxy-terminal alanyl/threonyl (CAT) tails. The Hel2 ubiquitin ligase is a subunit of the ribosome-associated quality control trigger complex (RQT) and is essential to trigger RQC (21). Another example is 'MitoCPR', a response that facilitates degradation in the cytosol of unimported mitochondrial precursor proteins accumulating at the mitochondrial translocase. It involves inducing expression of Cis1 at the translocase, that functions with the AAA^+ adenosine triphosphatase Msp1 and the proteasome (22). This improves mitochondrial import during import stress. 'MAD' is the response by which the components of the ubiquitin proteasome system (UPS) are recruited to the MOM to trigger degradation of proteins peripherally associated with the MOM, integral MOM proteins, mitochondrial intermembrane space proteins, and potentially also inner membrane or matrix proteins (23). An increase of mitochondrial precursor proteins in the cytosol triggers the 'UPR^{am}', leading to increased proteasome assembly by the enhanced activity of the proteasome assembly factors Irc25 and Poc4, that degrades excess proteins (24). Inversely, upon accumulation of high levels of aggregated proteins in the cytoplasm, Hsp104 helps to dissociate the aggregates. Thereby it contributes to MAGIC (Mitochondria As Guardian In Cytosol), a mechanism by which aggregation-prone proteins can enter via import channels the mitochondrial intermembrane space or matrix for degradation (25). All of these mechanisms are indicative of a major crosstalk between the cytoplasm and the mitochondrion to maintain protein homeostasis. In addition to these mechanisms, autophagy can sequester and remove unnecessary or dysfunctional components in bulk from the cytoplasm and mitophagy is the specific form of autophagy that serves to remove damaged mitochondria (for review, see (26)).

Ccr4–Not is a conserved, multi-subunit complex that plays multiple roles in the control of gene expression and mRNA metabolism. In yeast Ccr4–Not consists of 9 subunits: Ccr4, Caf1, Caf40, Caf130 and the five Not proteins (Not1, Not2, Not3, Not4 and Not5) (27–30). Our current knowledge about the functional roles of this complex is that its regulatory functions span the entire lifespan of mRNAs, from their synthesis to their decay. Moreover, it plays extensive roles in translation and protein turnover (31–33). Recent studies have uncovered key roles of the Not proteins in co-translational processes, such as co-translational assembly of proteins (32,34,35) and translation elongation dynamics (36). Not5 can associate with the E site of post-translocation ribosomes bearing an empty A site. This has been proposed to enable the Ccr4–Not complex to monitor the translating ribosome for mRNA turnover according to codon optimality (37). Consistently, depletion of Not5 changes A-site ribosome dwelling occupancies inversely to codon optimality (36). In addition, ubiquitination of Rps7A by Not4 can contribute to degradation of mRNAs by no-go-decay (NGD) in conditions where the RQC response is defective (38).

Recently, we noted that Not1 and Not4 depletions inversely modulated mRNA solubility thereby determining dynamics of co-translation events (39). Notably, mRNAs encoding mitochondrial proteins were enriched amongst mRNAs whose solubility was most extremely inversely regulated upon Not1 and Not4 depletion. In this context, it is interesting to note that the Ccr4–Not complex interacts with factors that contribute to targeting of mitochondrial mRNAs to the mitochondria: Egd1 is ubiquitinated by Not4 (40) and Puf3 recruits the Ccr4–Not complex to its target mRNAs for degradation (41–44). Moreover, mitochondrial mRNAs are enriched amongst mRNAs bound by Not1 in a Not5-dependent manner (33).

In our current study we uncover an integrated QC mechanism that limits levels of a mitochondrial mRNA co-translationally and mobilizes components of several of the QC systems linking cytoplasm and mitochondria described above, as well as Ccr4–Not subunits. We focused our attention on one nuclear-encoded mitochondrial mRNA, *MMF1*, more soluble upon Not4 depletion (39). *MMF1* encodes a mitochondrial matrix protein required for transamination of isoleucine and it couples amino acid metabolism to mitochondrial DNA maintenance (45). It forms a homotrimer proposed to interact with a trimer of Mam33 (46), a translational activator in yeast mitochondria (47). We determine that Not4 limits Mmf1 overexpression during fermentative growth by contributing to ribosome pausing and promoting the co-translational docking of its mRNA to mitochondria via the mitochondrial targeting sequence of

the Mmf1 nascent chain, Egd1 and the co-translational import machinery. Accumulation of excessive *MMF1* mRNA, Mmf1 precursor and mature Mmf1 protein is then avoided in a mechanism requiring Egd1 and Rps7 ubiquitination by Not4, Caf130, Cis1, RQC and NGD, Hsp104, as well as autophagy, a mechanism that we have called Mito-ENCay.

MATERIALS AND METHODS

Yeast strains and plasmids

The strains, oligos, plasmids and antibodies used in this study are listed in Supplementary Table S1. Yeast strains were grown in rich medium with 2% glucose (YPD) or in synthetic drop out medium selective for plasmid maintenance. For copper induction, cells were grown to exponential phase after dilution of an overnight culture to OD_{600} of 0.3 and a stock solution of 0.1 M $CuS0_4$ was added to a final concentration of 0.1 mM. To arrest protein synthesis a stock solution of cycloheximide (CHX) was added to a final concentration of 0.1 mg/ml in the growth medium.

The reporter plasmid expressing Mmf1 fused to Flag (pMAC1211) was constructed by cloning a PCR fragment amplified with oligos 935 and 936 and genomic DNA, digested by MfeI and Not1 in pE617 digested by EcoRI and Not1. The reporter plasmid expressing Mmf1 without the MTS (pMAC1327) was made using PCR with oligos 1028 and 1030, transformation of the PCR fragment with pE617 digested by EcoRI and Not1 into yeast, and plasmid rescue. The one with the Cox4 MTS (pMAC1328) was made similarly with oligos 1029 and 1030. The one expressing Cox4 (pMAC1200) was made similarly with oligos 691 and 692, except that the PCR fragment was digested by EcoRI and Not1. MS2 loops were added in the pMAC1211 and pMAC1327 plasmids by co-transforming into yeast the pMAC1211 and pMAC1327 plasmids digested with SacI and a PCR fragment obtained with oligos 1087 and 1088 and pE659, leading to pMAC1365 and pMAC1367. Constructs were verified by sequencing with oligo 1113. For both plasmids the *URA3* marker was swapped to the *HIS3* marker by transforming pE23 digested by SmaI and selection of His + Ura- colonies, followed by plasmid rescue leading to plasmids pMAC1430, and pMAC1431. For pMAC1211, the *URA3* marker was swapped to the *LEU2* marker by transforming pE24 digested by StuI and selection of the Leu + Ura- colonies followed by plasmid rescue leading to pMAC1342. The plasmid with altered codons around the ribosome-pause-site on *MMF1*, pMAC1425, and the plasmid with the *MMF1*-MTS replacement in *COX4*, pMAC1424, were created by a Q5 Site-Directed Mutagenesis Kit (NEB, E0554S) and oligo pairs 1301,1302 and 1297 with 1298. All plasmids were verified by sequencing. Plasmids encoding Egd1, Not4 and Rli1 derivatives have already been published (see Supplementary Table S1).

Protein ubiquitination assay

This method was done as previously described (40). A plasmid expressing 6His-tagged ubiquitin under the control of the inducible *CUP1* promoter was transformed into cells. The transformants were cultured in medium selective for plasmid maintenance in the presence of 0.1 mM $CuSO_4$. 100 OD_{600} of cells were harvested when they reached late exponential phase. Cell pellets were weighed and resuspended with G-buffer (100 mM sodium Pi, pH 8.0, 10 mM Tris–HCl, 6 M guanidium chloride, 5 mM imidazole, 0.1% Triton X-100) at 100 mg/ml. 0.6 ml of glass beads was added and cells were disrupted by bead beating for 15 min at room temperature (RT). Following centrifugation, 20 μl of the supernatant was taken as total extract (TE), and 700 μl of the supernatant was mixed with 30 μl of nickel-nitrilotriacetic acid-agarose (Ni-NTA, Qiagen) for 2 h at RT with mild rotation. U-buffer (100 mM sodium Pi, pH 6.8, 10 mM Tris–HCl, 8 M urea, 0.1% Triton X-100) was used to abundantly wash the Ni-NTA-agarose to which ubiquitinated proteins were bound. SB was added directly to the Ni-NTA with the ubiquitinated proteins for analysis by western bloting with relevant antibodies.

Confocal microscopy

Cells were grown in 2% glucose synthetic medium and harvested at an OD_{600} between 0.6 and 1.2. Two OD_{600} of cells were then spun at 3000 g for 5 min at RT, washed and fixed with 600 μl of 4% paraformaldehyde for 30 min at RT. Fixed cell pellets were resuspended in 200 μl of PBS, and 10 μl were evenly distributed on polysine coated slides. Nail polish was used to mount the coverslips, and images of the prepared slides were acquired using a stand confocal microscope (LSM800 Airyscan) with a 63× oil objective (NA = 1.4). Z-stacking was employed to acquire each image at fixed intervals of 0.23 μm. To determine the shortest distance between mRNAs and mitochondria, the Imaris software (version 9) was used, with its sport model generating the 3D model of mRNA and its surface model building the 3D model of mitochondria. Statistical analysis was performed using Prism9, with a two-tailed unpaired *t*-test, with Welch's correction. Each sample was assessed for >100 spots.

Protein extracts, SDS- or native PAGE and western blotting

Total protein extracts were prepared by incubating pelleted yeast cells in 0.1 M NaOH for 10 min at RT. After a quick spin in a microfuge, the cell pellet was resuspended in 2 X sample buffer (post-alkaline lysis). Samples were subjected to SDS-PAGE and western blotting according to standard procedures. For native gels (48), ready-made native 3–12% Bis–Tris gels were used (Invitrogen) according to instructions. Briefly, 20 OD_{600} of cells were harvested at exponential growth. Cells were disrupted by 0.2 ml glass beads in the presence of 0.4 ml lysis buffer (20 mM HEPES pH 7.5, 20 mM KCl, 10 mM $MgCl_2$, 1% Triton X-100, 1 mM DTT, 1 mM PMSF, supplemented with a cocktail of protease inhibitors (Roche)). The indicated amount of total protein extract was mixed with native sample buffer from Invitrogen. Following the electrophoresis (150 V, 3 h, 4°C) and transfer (40 W, 1 h, RT) to PVDF or nitrocellulose membranes, the blots were incubated with the indicated antibodies. To quantify expression of reporter proteins, after revelation of the western blots the captured images were imported into the Fiji software and converted to an 8-bit format. Background subtraction was performed before image analysis.

The processed images were measured, and the pixel values over the Flag signal were normalized over the pixel values for the Egd2 signal.

RNA preparation and analysis

RNA extraction and analysis was performed as previously described (33). Relative mRNA abundances were determined by RT-qPCR with the Pfaffl method (49). For normalization, we measured *EGD2* as an invariable control mRNA and calculated the ΔCT values. Oligos 687 and 999 were used for *MMF1* reporter mRNAs, 714 and 999 for *COX4* reporter mRNA and oligos 1000 with 1001 for *EGD2*.

Fractionation and mitochondria isolation

Mitochondrial fractionation was performed from 1 l of yeast cells as previously published (50) and simplified (http://www.jove.com/details.php?id=1417) with minor modifications (Tris–HCl rather than Tris/H_2SO_4 and addition of protease inhibitors). Briefly, cell pellets were washed with a buffer containing 100 mM Tris–HCl (pH 9.4) and 10 mM DTT. Spheroplasts were then generated using Zymolyase 100T (Biological, Z1004, US). A homogenization buffer consisting of 0.6 M sorbitol, 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM PMSF and 0.2% BSA was supplemented with a protease inhibitor cocktail (Roche). The cells were broken using a glass dounce homogenizer, and 20 μl of the lysate was collected as input. The remaining lysate was spun at 1500 g for 5 min at 4°C to remove cell debris and nuclei. The supernatant was then spun at 3000 g for 5 min at 4°C, and mitochondria were pelleted from this next supernatant at 12 000 g for 15 min at 4°C. The remaining supernatant fraction was collected as 'cytoplasm fraction'. The pellet was washed with SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM Mops, pH 7.2) and resuspended in SEM buffer for loading onto a 3-step sucrose gradient. After centrifugation at 134 000 g in a Beckman SW41 TI swinging-bucket rotor for 1 h at 2°C, the purified mitochondria were recovered from the 60% to 32% interface. The collected mitochondrial fraction was further pelleted by centrifugation at 10 000 g at 2°C and resuspended in a storage buffer (SEM buffer without sucrose). Input, cytoplasm and mitochondrial fractions were mixed with sample buffer for western blot analysis.

Ribosome profiling and bioinformatic analysis

Samples for ribosome profiling were prepared and analyzed previously (35) and the data was extracted to show ribosome footprints on *MMF1* and *COX4* mRNAs in wild type and *not4Δ*.

RNA-seq and solubility analyses

The data was generated and analyzed in (39). We extracted the data to show the change in solubility of *MMF1* and *COX4* upon Not1 and Not4 depletion.

RESULTS

Mmf1, but not Cox4, is co-translationally imported

To start dissecting how the Ccr4–Not complex regulates solubility of mRNAs to regulate co-translation events, we focused our attention on two mitochondrial mRNAs, *MMF1* and *COX4*, rendered more soluble upon Not4 depletion, but less soluble upon Not1 depletion (Supplementary Figure S1A) (39). Both mRNAs express mitochondrial precursor proteins with an N-terminal cleavable targeting sequence and assemble into multi-protein complexes. Cox4 is a component of the respiratory complex IV located in the mitochondrial inner membrane (51) whereas the Mmf1 trimer resides in the matrix (45).

To study the regulation of *MMF1* and *COX4* expression dependent upon their coding sequences, we used reporter constructs with the heterologous and inducible *CUP1* promoter and the heterologous *ADH1* 3'UTR in between which we cloned the *MMF1* and *COX4* coding sequences (CDS) fused to a C-terminal Flag tag (Figure 1A). We transformed the plasmids in wild type cells and tested expression of the reporter before and after induction with copper for 10 min. Before induction some mature Mmf1 was already detectable, due to some leakage of the *CUP1* promoter. Immediately after induction, levels of unprocessed and mostly mature Mmf1 were increased, whilst mostly unprocessed Cox4 was visible, with very low levels of mature protein (Figure 1B). This suggests that processing of induced Mmf1 might be faster than that of Cox4, compatible with the idea that the former but not the latter might be co-translationally processed and imported.

To look at this further, we transformed the two plasmids in strains defective for the mitochondrial co-translational import machinery (8), namely cells lacking the Egd1 chaperone or its receptor on the MOM, Om14 or finally the Tom20 receptor (see cartoon on Figure 1C). We also transformed the plasmids in cells defective for the cytoplasmic Hsp70 chaperones (called Ssa1-4 in yeast) reported to contribute to effective post-translational import of mitochondrial proteins (52). As before, we analyzed the expression from the reporter plasmids before and after a 10 min induction with copper. After induction, we noted elevated levels of the unprocessed Mmf1 protein in the mutants of the co-translational machinery but not in the *ssaΔ* mutant (Figure 1D, upper panels). The ratio of unprocessed to mature Mmf1 was also increased in all mutants relative to wild type, except for the *ssaΔ* mutant (Supplementary Figure S1B). In all strains, very little Cox4 was detectable before induction and after induction we noted mostly unprocessed Cox4, at levels similar in all strains (Figure 1D, lower panels). Since the *MMF1* and *COX4* reporters have identical 5' and 3' untranslated sequences, the difference in their regulation must depend upon the coding sequences. Hence, these results are compatible with a negative regulatory role of the co-translational import pathway for control of the expression of *MMF1*, but not *COX4*, coding sequences.

By performing an 18 h cycloheximide (CHX) chase after copper induction for some of the same strains tested above, and additionally cells lacking the Om14 partner Om45, we

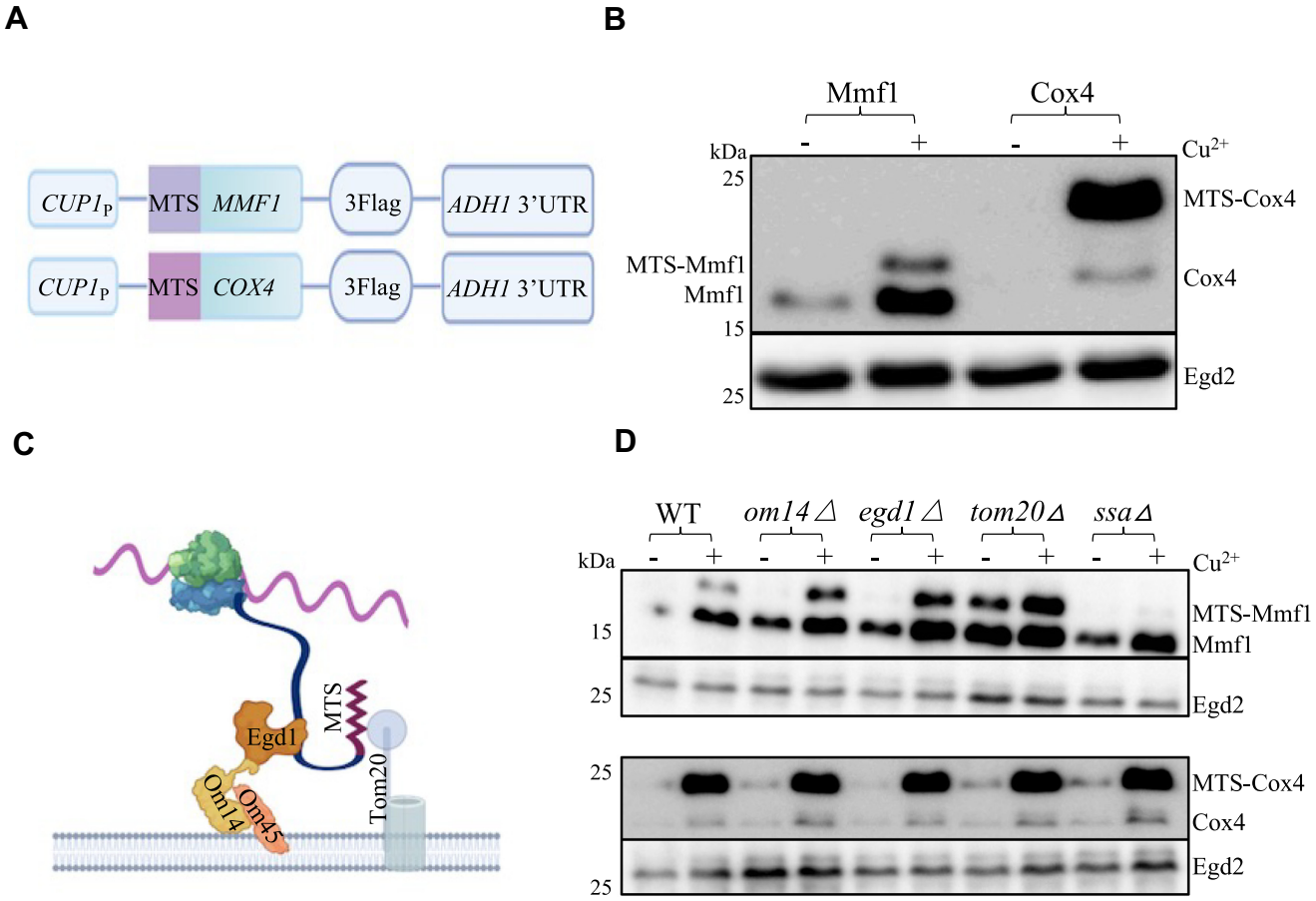

Figure 1. Mmf1 but not Cox4 is co-translationally imported and regulated. (**A**) Cartoon of the reporter constructs used in which coding sequences are fused to a C-terminal Flag tag, under the control of the *CUP1* inducible promoter. (**B**) Wild type cells (WT) transformed with the reporters and growing exponentially in medium selective for the plasmids were untreated (-) or treated (+) with 0.1 mM $CuS0_4$ (Cu^{2+}) for 10 min. Cells were collected for total protein analysis by western blotting with antibodies to Flag or with antibodies to Egd2 to control for protein loading. Precursor and mature Mmf1 and Cox4 are indicated respectively left and right of the blot. Molecular weight markers are indicated on the left. (**C**) Cartoon of the co-translational import machinery with the nascent chain exposed from the ribosome interacting with the Egd1 chaperone itself docking onto the Om14 MOM protein interacting with Om45, and the MTS of the nascent chain recognizing Tom20 to enable transfer of the nascent chain into the Tom channel. (**D**) Analysis of the reporters as in panel B in the indicated strains (upper panels Mmf1 and Egd2, lower panels Cox4 and Egd2).

noted again that after induction the Mmf1 precursor was overexpressed except in *ssaΔ* cells. Furthermore, Mmf1, whether precursor or mature, was relatively stable. Instead, the overexpressed Cox4 turned over rapidly and neither precursor nor much mature protein was detectable already by 2 h of CHX chase in all strains tested (Supplementary Figure S1C). We considered the possibility that the difference in stability of the Mmf1 and Cox4 reporter proteins could be related to the Cox4 precursor being cytoplasmic, while the Mmf1 precursor instead could be 'stuck' in the mitochondrial import machinery. We thus prepared mitochondria and cytoplasmic fractions from *egd1Δ* cells after copper induction that have high levels of both precursor and mature Mmf1 reporter protein. We followed on one hand the Mmf1 reporter protein with antibodies to Flag, and as a control for the fractionation procedure, we evaluated the presence of the mitochondrial Por1 protein (53) and the Hxk1 hexokinase (54) for the cytoplasmic fraction, with specific antibodies. The Mmf1 precursor was unstable in cell extracts, but nevertheless it was detected in the cytoplasmic fraction but not in the mitochondrial fraction, whereas like Por1, the mature Mmf1 was detectable exclusively in the mitochondrial fraction (Figure 2A).

Both Mmf1 and Cox4 have an N-terminal cleavable MTS sequence, but the amino acid composition of the MTS is very different (Figure 2B). We investigated the role of the mitochondrial targeting sequence (MTS) for regulation of Mmf1 expression, and the ability of the Cox4 MTS to replace the Mmf1 MTS. Mmf1 without its MTS or with the Cox4 MTS to replace its own MTS, was overexpressed (Figure 2C). These results indicate that the Mmf1 MTS is necessary to limit Mmf1 expression, and that the Cox4 and Mmf1 MTS are not functionally interchangeable for this function. We then replaced the MTS of Cox4 by that of Mmf1 and tested expression before and after copper induction. The replacement of the Cox4 MTS by that of Mmf1 totally repressed expression of Cox4 (Figure 2D). These results demonstrate that specifically the MTS of Mmf1 represses expression of the reporter protein to which it is fused.

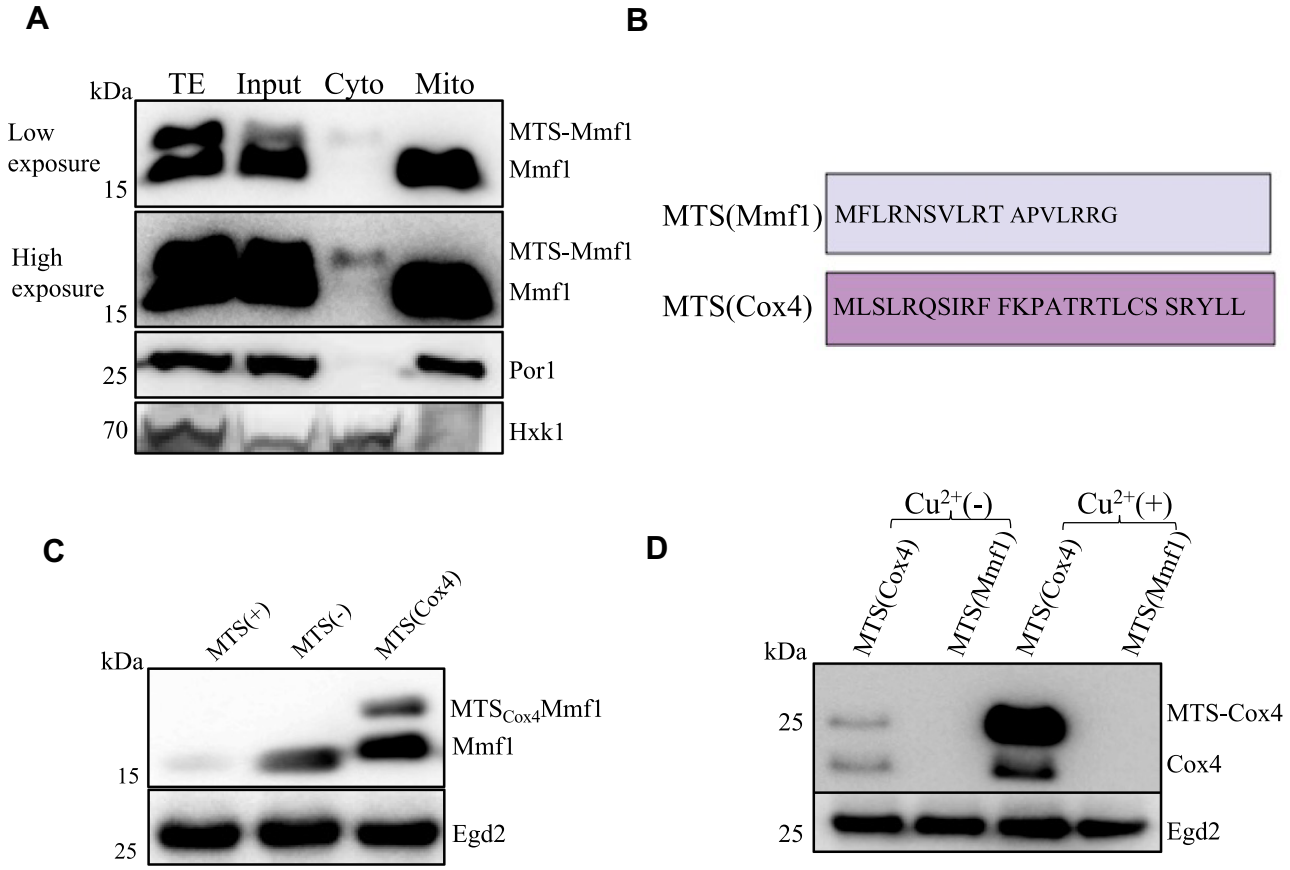

Figure 2. The *MMF1* MTS represses expression and can exert this effect on a heterologous gene. (**A**) *egd1Δ* cells expressing the Mmf1 reporter were lysed after a 10 min copper induction for a purification of mitochondria. Total extracts (TE), cell lysate (Input), cytosolic fraction (Cyto) or the mitochondrial fraction (Mito) were tested by western blotting for expression of Mmf1 with antibodies to Flag. A low and high exposure are shown. Antibodies to a mitochondrial protein Por1 or a cytosolic protein hexokinase (Hxk1) were used as a control for the fractionation procedure. (**B**) Amino acid sequence of the Mmf1 and Cox4 MTS sequences. (**C**) Expression of the *MMF1* reporter with MTS (left), without MTS (middle), or with the Cox4 MTS instead of its own MTS (right), in wild type cells growing exponentially analyzed by western blotting as in Figure 1B. (**D**) Expression of the *COX4* reporter with its MTS or with the *MMF1* MTS (as indicated) before and after copper induction analyzed as in panel (C).

Regulation of *MMF1* but not *COX4* expression requires Not4 and the MTS

We next tested expression of the Mmf1 and Cox4 reporters before and after copper induction in cells lacking Not4, because of Not4's role in the regulation of the solubility of the *MMF1* and *COX4* mRNAs mentioned above (39). The expression of the Mmf1 precursor and mature protein was much higher in *not4Δ* (Figure 3A, upper panels). In contrast the expression of Cox4 was mostly indistinguishable between the wild type and mutant (Figure 3A, lower panels). Since the overexpression of the Mmf1 reporter was already detectable in mutant cells without copper induction due to leakage of the *CUP1* promoter, we worked further without copper induction.

We tested which functional domains of Not4 were important for control of Mmf1 expression and transformed *not4Δ* cells carrying the reporters with plasmids encoding wild type or mutant Not4 derivatives, in particular Not4 mutants lacking their C-terminal Not1-interaction domain or the N-terminal RING domain (55). Only wild type Not4 showed complementation of the Mmf1 overexpression. Notably however, the complementation from the plasmid expressing wild type Not4 was only partial (Figure 3B), maybe because of the presence of an N-terminal tag, or because Not4 is expressed from an episome rather than from the genomic locus.

The Not proteins are known to be important for co-translational assembly of specific protein complexes (34,35). Mmf1 forms homotrimers proposed to assemble with Mam33 trimers (46). We thus questioned whether Mmf1 complexes were appropriately formed in cells lacking Not4 and analyzed extracts of wild type and mutant cells expressing endogenous tagged Mmf1 expressed from its endogenous locus on native gels. Mmf1 from all strains migrated with a size between 146 and 242 kDa, larger than expected for Mmf1 homotrimers. Hence, the same apparent Mmf1 complexes could be formed in wild type cells and cells lacking Not4. However, faster migrating Mmf1 complexes were additionally seen in cells lacking Not4 (Figure 3C). These faster migrating Mmf1 complexes likely reflect higher expression levels of Mmf1 compared to its partner proteins, though we cannot exclude that they indicate ineffective complex assembly in mutant cells if the partner proteins are not limiting.

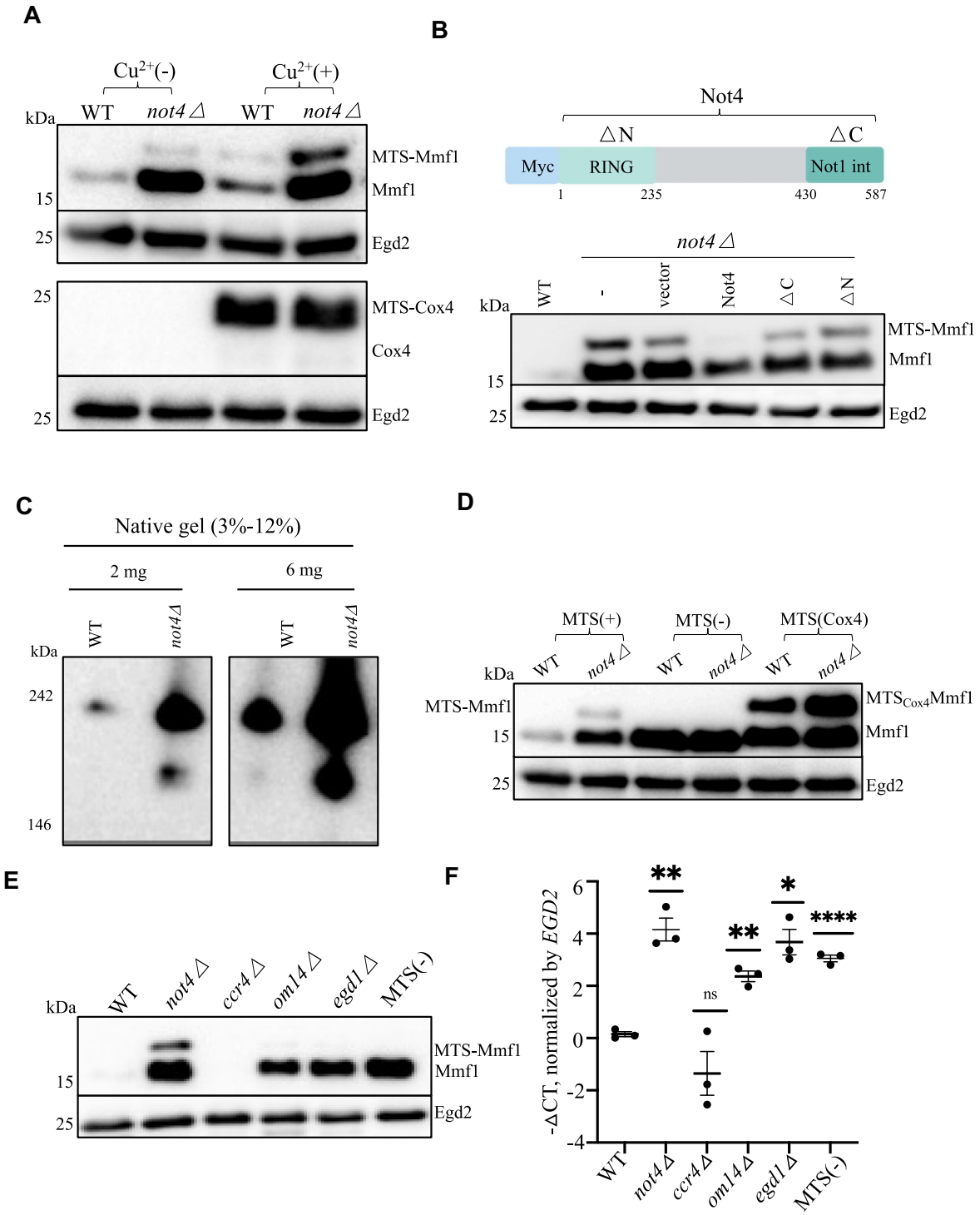

Figure 3. Overexpression of *MMF1* mRNA and protein in cells lacking Not4 or when Mmf1 lacks its MTS is epistatic. (**A**) Analysis of the reporters was evaluated in wild type and *not4Δ* cells as in Figure 1B. (**B**) Top: cartoon of the Myc6-Not4 coding sequence. The RING domain is located before amino acid 235 and the Not1-interaction domain is located after amino acid 430. Bottom: wild type cells (WT), *not4Δ* cells (–) or *not4Δ* cells transformed with plasmids expressing with an N-terminal Myc tag, wild type Not4, a derivative lacking the RING domain (ΔN) or a derivative lacking the Not1-interacting C-terminal domain (ΔC) and the *MMF1* reporter, were analyzed before copper induction as in panel A. (**C**) The indicated amounts of total soluble protein extract from wild type or *not4Δ* cells expressing TAP-tagged Mmf1 (the TAP tag has a calmodulin-binding entity and a Protein A entity) from its endogenous locus were analyzed by Native PAGE and western blotting with PAP antibodies. (**D**) Wild type and *not4Δ* cells were analyzed for expression of the *MMF1* reporter without the MTS or with the *COX4* MTS before copper induction as in panel A. (E and F) Wild type and the indicated mutant cells transformed with the *MMF1* reporter or wild type cells transformed with the *MMF1* reporter without the MTS as indicated were collected at the exponential growth phase without copper induction and analyzed by western blotting with antibodies to Flag (**E**) and by RT-qPCR (**F**). The *EGD2* protein and mRNA were used as a control for loading. The *MMF1* reporter mRNA levels were plotted to show means ± SD of –ΔCT values. The level of significant change, relative to WT is indicated with asterisks using a two-sided, Welch, unpaired *t*-test ($n = 3$).

We next questioned whether increased expression of Mmf1 due to the absence of Not4 and the MTS were additive. However, the expression of Mmf1 without its MTS or with the Cox4 MTS was not further increased in *not4Δ* (Figure 3D). Hence Not4 and the Mmf1 MTS are epistatic with regard to their regulation of the Mmf1 reporter.

As mentioned above, overexpressed Cox4 turns over rapidly, whilst Mmf1 is stable, leaving open the possibility that the overexpression of Mmf1 but not Cox4 in mutants could be explained by this differential protein turnover. In such a case, we would not expect a change in *MMF1* mRNA levels. However, when Mmf1 protein was overexpressed in the different mutants or when Mmf1 was expressed without its MTS (Figure 3E), the *MMF1* mRNA was also overexpressed (Figure 3F). Instead, the levels of the *COX4* reporter mRNA were unaffected in all mutants, except in *not4Δ* (Supplementary Figure S2A). We also noted a very striking elevation of the *MMF1* but not *COX4* reporter mRNA in the *tom20Δ* mutant (Supplementary Figure S2B). These results indicate that the MTS and co-translational import machinery have a negative regulatory effect on the mRNA of the *MMF1* reporter. Interestingly, not only the Cox4 reporter protein levels (Figure 2D) but also the *COX4* reporter mRNA levels were reduced by the replacement of the Cox4 MTS by the Mmf1 MTS (Supplementary Figure S2C). Thus, the negative regulatory effect of the *MMF1* MTS can be transferred to heterologous coding sequences. Interestingly, neither reporter mRNA was affected in cells lacking the Ccr4 deadenylase subunit of the Ccr4–Not complex (Figure 3F and Supplementary Figure S2A). On the other hand, both Cox4 and Mmf1 proteins were increased in mutants of the proteasome, but in this case the mRNA levels were not significantly changed (Supplementary Figure S2D and E).

Taken together, these results show that repression of the *MMF1* but not *COX4* reporter is exerted at the mRNA level, by the Mmf1 MTS and components of the co-translational import machinery as well as by Not4, but independent of the Ccr4 deadenylase. This supports a model in which repression of *MMF1* overexpression occurs co-translationally at the mitochondrial co-translational import machinery.

Ribosome pausing defined by codon context and Not4 regulate Mmf1 expression

Most of the results presented so far were obtained with a reporter that expresses Mmf1 from an episome above the background of the endogenous *MMF1* gene. We used our published Ribo-Seq data that provides information about endogenous *MMF1* and *COX4* regulation in wild type cells and in cells lacking Not4 (36). From these experiments, we note that both *MMF1* and *COX4* mRNAs are up-regulated in the absence of Not4 (Figure 4A and Supplementary Figure S3A). We additionally observe that *MMF1* mRNA is translated with important ribosome pausing at codons 93 and 94, whilst no such pausing is detectable for *COX4*. Moreover, the pausing on *MMF1* mRNA is less effective in the absence of Not4, since ribosome footprints increase more after the pause site than before the pause site, in *not4Δ* (Figure 4B). *MMF1* pause site codons 93 and 94 are amongst the 15 most optimal codons in budding yeast, whereas the following codon 95 (that will be in the ribosomal A site for ribosomes pausing with codon 94 in the P-site) is one of the 15 least optimal codons (56). Codon 92 preceding the pause site is neither particularly optimal nor non-optimal. We replaced codons 92 and 95 with optimal codons encoding the same amino acids in the *MMF1* reporter (Figure 4C). This codon change was sufficient to lead to overexpression of the Mmf1 reporter (Figure 4D). Hence, not only ribosome pausing and Not4, but also codon optimality, is contributing to limit Mmf1 overexpression.

Egd1 ubiquitination and Caf130 limit co-translationally *MMF1* expression

Both Egd1 and Not4 contribute to limit Mmf1 reporter overexpression. Egd1 is a substrate for the ubiquitin ligase activity of Not4 and ubiquitinated residues have been characterized (40,57). We thus tested expression of the Mmf1 reporter in wild type cells, or in *egd1Δ* cells transformed with either an empty vector, a vector expressing wild type Egd1, or a plasmid expressing the non-ubiquitinated $Egd1_{K29,30,R}$ derivative (57). The Mmf1 precursor was overexpressed in *egd1Δ* as expected, and this was complemented by wild type Egd1, but not by the non-ubiquitinated Egd1 (Figure 5A).

We recently determined that Not4 ubiquitination of Rps7A and overexpression of another target of Not4 ubiquitination, Rli1, wild type or with 16 mutated lysine codons, increased translation of a reporter with a stalling sequence (36). Because ribosome pausing appears relevant for the response that limits the overexpression of the *MMF1* reporter, we tested the impact of non-ubiquitinated Rps7A on expression of the *MMF1* reporter. Mmf1 was increased in the non-ubiquitinated Rps7A mutant (Figure 5B). Similarly, Rli1 overexpression increased the over-expression of the *MMF1* reporter (Figure 5C) but it had no effect on Cox4 expression (Supplementary Figure S3B).

We have observed using Not5 affinity purification that Egd1 co-purifies with the Ccr4–Not complex from wild type cells, but it does not co-purify with it from cells lacking Caf130, another subunit of the Ccr4–Not complex, as previously reported by others (58) and confirmed recently (59). The Mmf1 reporter was also overexpressed in cells lacking Caf130 (Figure 5D), whilst Cox4 was unaffected (Supplementary Figure S3C). Since Egd1 ubiquitination by Not4 is important to repress Mmf1 overexpression, and Caf130 is important for co-purification of Egd1 with the Ccr4–Not complex, we determined whether ubiquitination of Egd1 was impaired in cells lacking Caf130. We transformed a plasmid expressing His-tagged ubiquitin from the *CUP1* promoter in *caf130Δ* cells expressing HA-tagged Egd1. After induction with copper, we affinity purified ubiquitinated proteins on a nickel resin. Total proteins and affinity-purified proteins were analyzed by western blotting for Egd1 with antibodies to HA (Figure 5E). Egd1 ubiquitination was not abolished in cells lacking Caf130. However, in cells lacking Caf130 there was higher accumulation of lower molecular weight ubiquitinated forms and reduced accumulation of higher molecular weight ubiquitinated forms of Egd1, suggesting reduced turnover of ubiquitinated Egd1 in *caf130Δ*. The ubiquitination of Egd2,

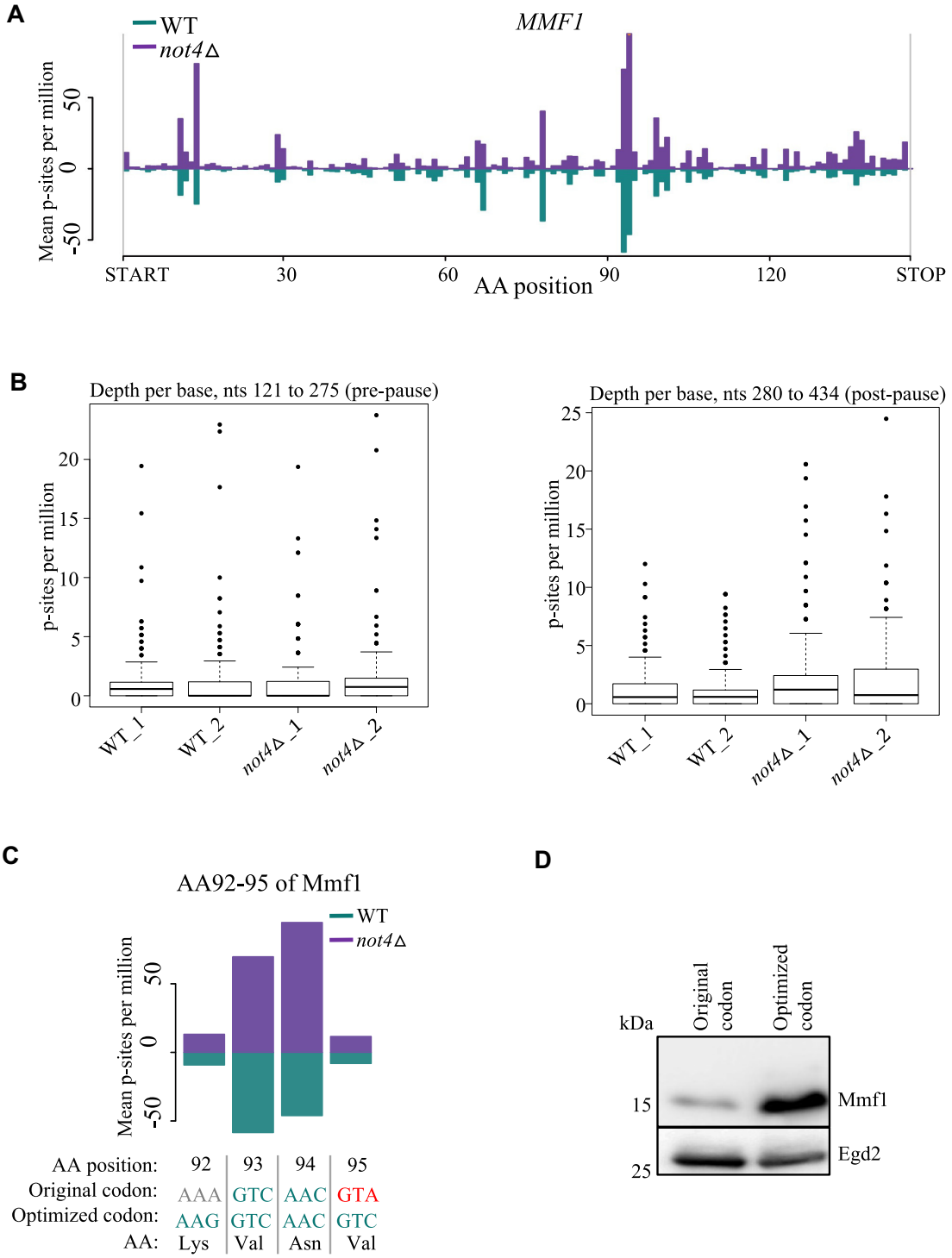

Figure 4. Translation dynamics according to codon optimality contributes to regulate Mmf1 expression. (**A**) Profiles of ribosome footprints (P-site depth plots) on *MMF1* with footprints in wild type cells in green and those in *not4Δ* cells in purple. The number of P-sites, per million genome-wide for each sample, covering each CDS codon with corresponding amino acid position indicated (AA position) is calculated, averaged for each condition and plotted. (**B**) Quantification of mRNA footprints in wild type and *not4Δ* cells for duplicate samples on equal segments of the mRNA before (left) and after (right) the apparent ribosome pausing site. Boxplots of P-sites per million for each base of the *MMF1* CDS in WT and *not4Δ* cells for the region between the large pause and the stop codon (nucleotides 280–434, right panel) and an equally-sized region just upstream of the pause (nucleotides 121–275, left panel). Only the region post-pause shows significant changes (DESeq2 p-value = 3.19e-5). (**C**) Visualization of the 4 codons at the *MMF1* ribosome pause site with encoded amino acids. Codons in blue are amongst the 15 most optimal and in red amongst the 15 most non-optimal yeast codons. The first line indicates the position, the second line indicates the codon in the wild type *MMF1* sequence and the third line indicates the mutations created to change codon optimality but not the encoded amino acid. (**D**) Expression of the *MMF1* reporter with the wild type sequence ('Original codon') or the codon-optimized sequence ('Optimized codon') around the pause site in cells growing exponentially was evaluated by western blotting with antibodies to Flag or with antibodies to Egd2 to control for protein loading.

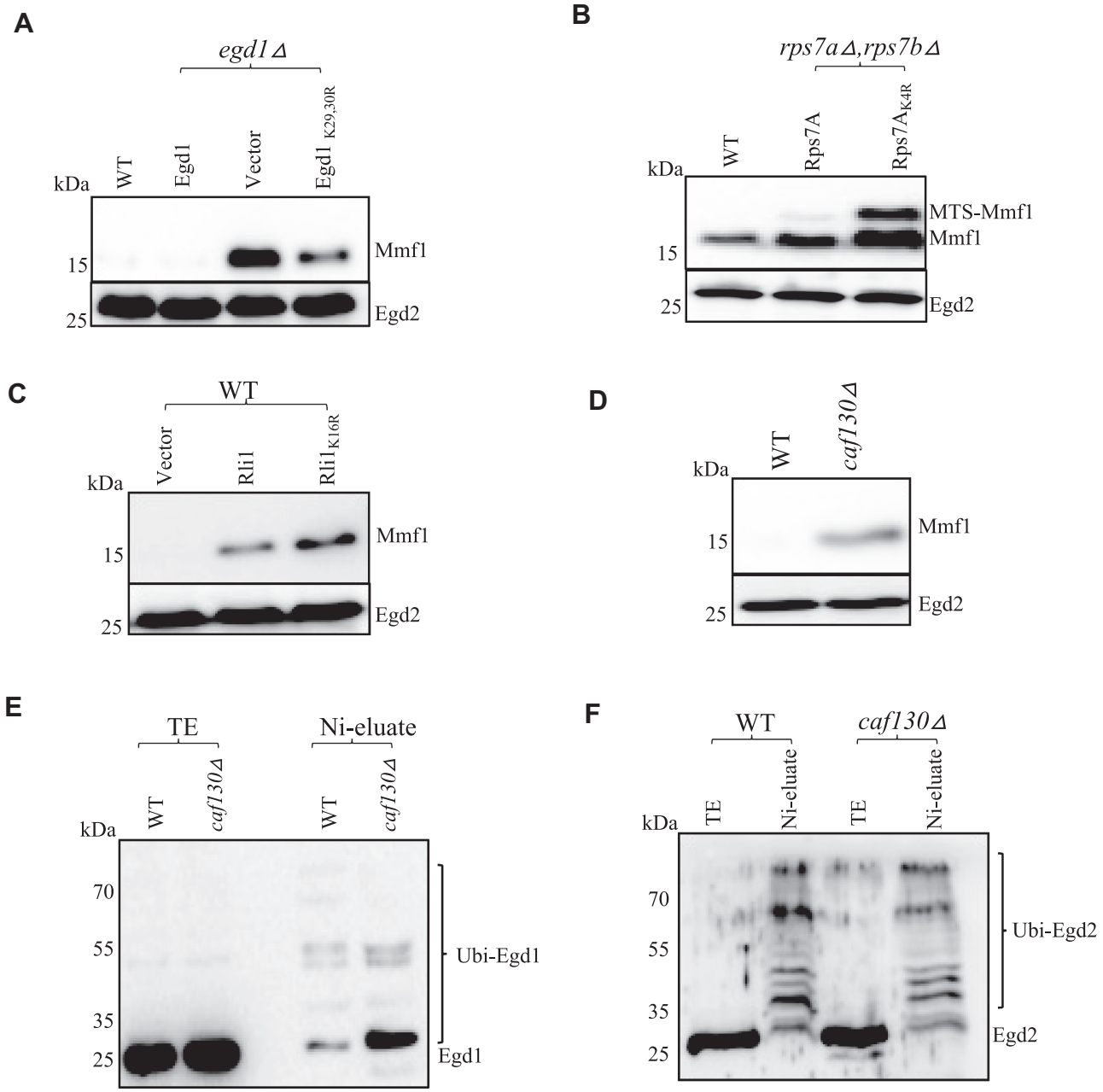

Figure 5. Ribosome-associated targets of Not4 ubiquitination, namely Egd1, Rps7A and Rli1, as well as Caf130, limit expression of the *MMF1* reporter. (**A**) Wild type (WT) or *egd1Δ* cells transformed with a plasmid expressing wild type HA-tagged Egd1 (Egd1), a control vector (vector) or a plasmid expressing an HA-tagged Egd1 derivative that does not get ubiquitinated ($Egd1_{K29,30R}$) growing exponentially were tested for expression of the *MMF1* reporter. (**B**) Expression of the *MMF1* reporter was evaluated in WT cells and in cells expressing wild type (Rps7A) or non-ubiquitinated ($Rps7A_{K4R}$) Rps7A from a plasmid to complement the deletion of genomic *RPS7A* and *RPS7B*. (**C**) Expression of the *MMF1* reporter in wild type cells transformed with a control plasmid or with a plasmid overexpressing Rli1 or a non-complementing Rli1 derivative with 16 lysine codons mutated to arginine was evaluated. (**D**) Wild type and *caf130Δ* cells growing exponentially were tested for expression of the *MMF1* reporter. In panels A–D the expression of the *MMF1* reporter was tested by western blotting with antibodies to Flag. Antibodies to Egd2 were used as loading control. (**E**, **F**) Wild type and *caf130Δ* cells expressing HA-tagged Egd1 from the endogenous *EGD1* locus and transformed with a plasmid 6His-tagged ubiquitin under the *CUP1* promoter were grown in the presence of 0.1 mM $CuS0_4$. Ubiquitinated proteins were purified by nickel affinity chromatography and the presence of Egd1 in the total extract (TE) and nickel eluate (Ni-eluate) was tested with respectively antibodies to HA (E) or the presence of Egd2 with antibodies to Egd2 (F).

the heterodimeric partner of Egd1 in the NAC complex, was not detectably affected (Figure 5F). Notably, Mmf1 was overexpressed to similar levels whether only Egd1 was deleted, versus if all NAC subunits (Egd1, the other β NAC subunit Btt1, and Egd2) were deleted (Supplementary Figure S4A). Sam37 has been proposed to cooperate with NAC to mediate early stages of mitochondrial protein import (13) and interestingly, the *MMF1* but not *COX4* reporter protein and mRNA, were overexpressed in cells lacking Sam37 (Supplementary Figure S4B and C).

RQC, as well as Cis1, Hsp104 and autophagy limit overexpression of Mmf1

As mentioned above, ribosome pausing appears relevant for limitation of *MMF1* overexpression. Long lasting ribosome pausing can cause ribosome collisions and induce the RQC response. We thus tested expression of the reporters in wild type cells and in cells lacking Hel2, a major effector of RQC, or lacking Vms1, the tRNA hydrolase that antagonizes Rqc2. Mmf1 was overexpressed in both mutants (Figure 6A). RQC is accompanied by NGD that involves both 5' to 3' and 3' to 5' mRNA degradation, for which Xrn1 and Ski2 contribute. *MMF1* was overexpressed in cells lacking either protein (Supplementary Figure S5A). *COX4* was not affected by the absence of *XRN1* but interestingly it was a little up-regulated in the absence of *SKI2*.

As mentioned above, many QC pathways exist to avoid accumulation of proteins that arrive at the mitochondria, either overexpressed precursor proteins, mistargeted proteins or misfolded and defective proteins. We tested the role played by components of these QC responses, starting with Cis1. Cis1 associates with the mitochondrial translocase and is known to be key to reduce the accumulation of mitochondrial precursor proteins with the Cis1-interacting AAA^+ adenosine triphosphatase that contributes to extract proteins from the outer membrane (MitoCPR) (22). Mmf1 was overexpressed in cells lacking Cis1 (Figure 6A), but not in cells lacking Msp1 (Supplementary Figure S5B). Thus, the regulation of Mmf1 overexpression involves Cis1 but by a mechanism distinct to 'MitoCPR'. Mmf1 was also overexpressed in cells lacking the Hsp104 disaggregase (Figure 6A). In all these cases, not only Mmf1 protein, but also *MMF1* mRNA, was overexpressed (Figure 6B). Cox4 expression was not affected in any of these mutants (Supplementary Figure S5C–E).

We also tested whether mitophagy that removes aged and damaged mitochondria (26) contributed to limit Mmf1 overexpression using a strain lacking Atg32, the receptor for mitophagy. However, Mmf1 levels were unaltered in *atg32Δ* (Supplementary Figure S5F). Mitophagy is a selective type of autophagy, so we tested whether autophagy contributed to limit Mmf1 overexpression, using cells lacking the Atg17 scaffold protein. Mmf1 was indeed overexpressed in cells lacking Atg17, both the protein (Figure 6C) and the mRNA (Figure 6D), though in this latter case it was only clearly visible after copper induction. Cox4 was unaffected in cells lacking Atg17 (Supplementary Figure S6A). Many other autophagy mutants were tested, with the same effect on Mmf1 but not Cox4 overexpression (Supplementary Figure S6B-C).

Expression of *MMF1* without its MTS was not increased in any of the mutants of these different QC pathways (Supplementary Figure S6D). Even in the case of cells lacking Hel2, expression of *MMF1* without its MTS was not increased, either at the protein (Figure 6E) and or at the mRNA (Figure 6F) level.

These results indicate that many QC pathways work together to limit *MMF1* mRNA, and hence synthesis and accumulation of the Mmf1 precursor, as long as the Mmf1 has its mitochondrial targeting sequence.

The Mmf1 MTS contributes to localize the *MMF1* mRNA to mitochondria

All of the results above raise the question of how Not4 and the Mmf1 MTS together with the co-translational import machinery repress overexpression of the *MMF1* reporter. Our observations are consistent with the possibility that the Mmf1 MTS together with Not4 might contribute to target the *MMF1* mRNA to the co-translational import machinery. To determine anchoring of the *MMF1* mRNA to the co-translational import machinery, we inserted new generation MS2 stem loops (sl) (60) into the 3'UTR of the *ADH1* terminator on the reporter carrying the *MMF1* ORF, with or without its MTS (Figure 7A). We transformed this reporter into cells expressing the MS2-stem loop binding protein (MCP) fused to 4 GFPs and expressing the matrix marker Su9-mCherry (61). The *MMF1* reporter mRNA was detectable with the bound MCP-4GFP fluorescent protein with (Figure 7B, upper left panel) and without MTS (second from the top left panel), and as expected was present at higher levels in the latter case. The mitochondria were clearly detectable via the Su9-mCherry fluorescence (second to left panels). Merging of the signals allowed us to evaluate the extent of co-localization. The experiment was performed before (upper 2 lines) and after (lower 2 lines) copper induction. Interestingly copper induction increased the amount of mRNA detectable for the *MMF1* reporter with MTS but not without MTS (when it is already overexpressed). Before copper induction, some co-localization was detectable for the *MMF1* with its MTS, but it did not appear statistically significantly different from the *MMF1* without its MTS. However, the level of these two different reporter mRNAs was very different before copper induction making it difficult to conclude. After copper induction, the co-localization of the *MMF1* mRNA with its MTS and mitochondria was significantly higher compared to that of the *MMF1* mRNA without its MTS (Figure 7C).

DISCUSSION

Targeting and pausing for quality control at the mitochondria outer membrane

In this work we show that budding yeast cells growing in glucose with limited need for mitochondria can mobilize an integrated QC response to avoid overexpression of the Mmf1 mitochondrial precursor induced from an episome. We have called this mechanism Mito-ENCay (Figure 8). According to our model, Mito-ENCay relies on the co-translational targeting of the *MMF1* mRNA to the MOM via the MTS of the Mmf1 nascent chain and its NAC-bound

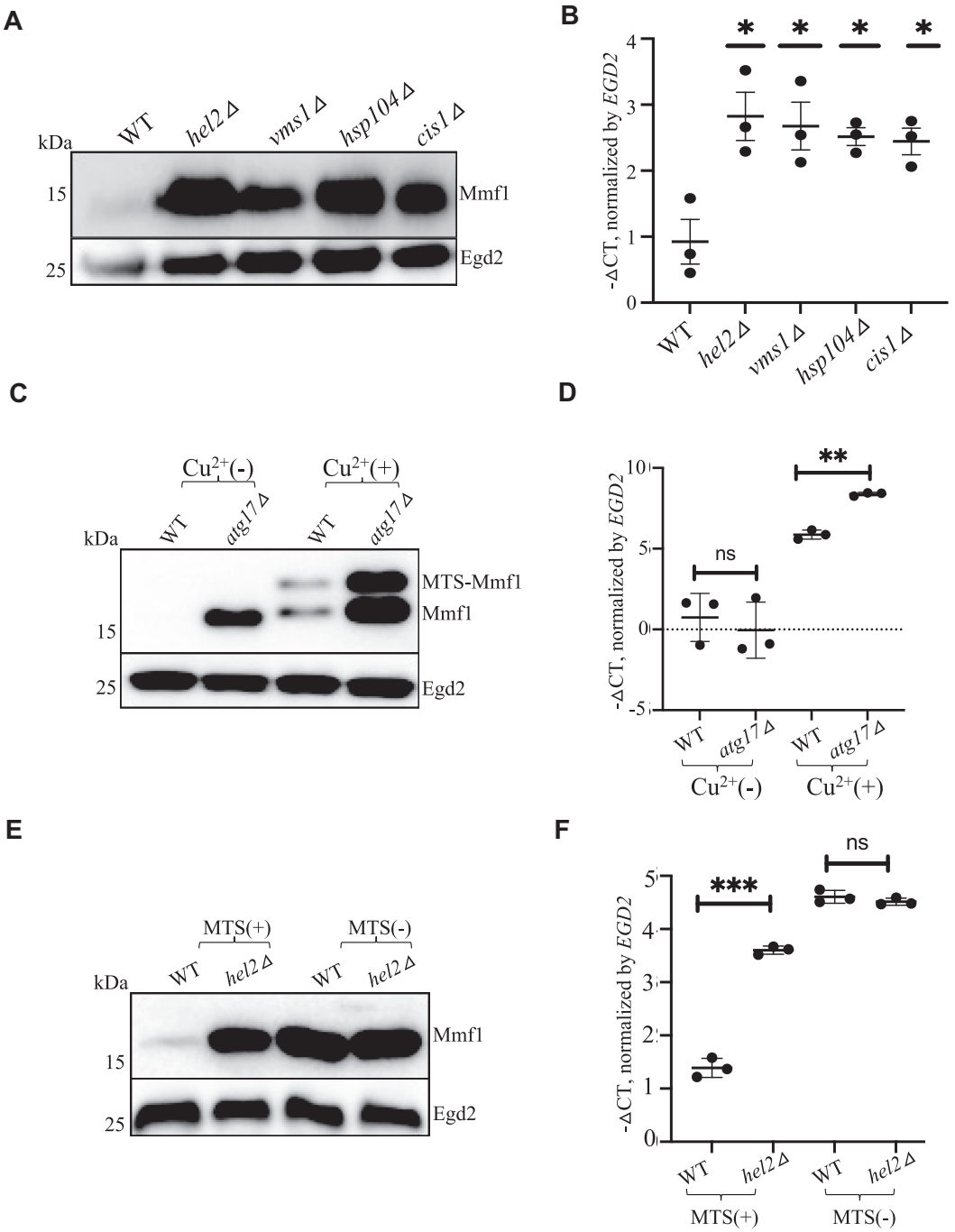

Figure 6. An integrated quality control response regulates expression of the *MMF1* reporter mRNA and protein. (**A**, **B**) Expression of the *MMF1* protein and mRNA was tested in wild type cells (WT) and in cells lacking *HEL2*, *VMS1*, *HSP104* or *CIS1* growing exponentially by western blotting with antibodies to Flag or with antibodies to Egd2 used as loading control (A) or by RT-qPCR (B). For the mRNA, the levels were normalized to *EGD2* and the results are expressed as – ΔCT values in the different strains relative to WT. The level of significant change, relative to WT is indicated with asterisks using a two-sided, Welch, unpaired *t*-test ($n = 3$). (**C**, **D**) Expression of the *MMF1* reporter was tested in WT and *atg17Δ* cells growing exponentially, before and after a 10 min copper induction for protein (C) and mRNA (D) levels. (**E**, **F**) Expression of the *MMF1* reporter with or without MTS was tested in WT or in cells lacking *HEL2* growing exponentially as in panels A and B, respectively.

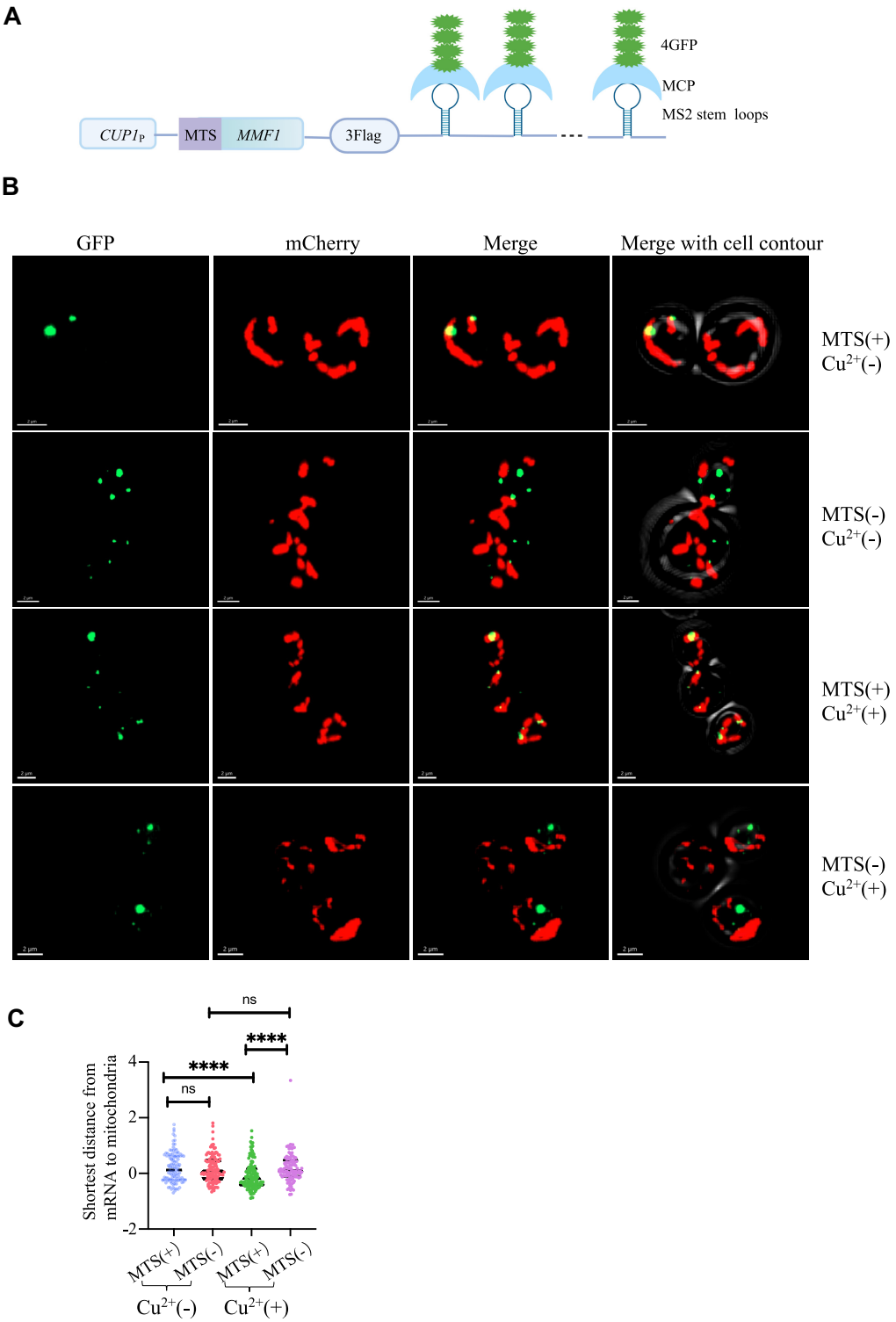

Figure 7. The Mmf1 MTS contributes to the localization of the *MMF1* mRNA to the mitochondria. (**A**) Cartoon of the *MMF1* reporter with inserted MS2 stem loops in the 3'UTR that can be recognized by MS2 binding protein (MCP) fused to 4 GFP. (**B**) Wild type cells with an integrated su9-mCherry reporter to follow mitochondria were transformed with the plasmid expressing the *MMF1* reporter with or without its MTS as indicated. Cells were grown to exponential phase, and induced (+) or not (–) with copper (Cu^{2+}) for 10 min, then were fixed and visualized at the confocal microscope to see GFP (left panels), Su9-mCherry (second to left panels), and the merged signal (second to right panels), with the contour of the cells indicated (far right panels). Representative images of 2–3 cells are shown. (**C**) To determine the distance between mRNAs and mitochondria and evaluate mRNA localization at the mitochondrion, the Imaris software (version 9) was used, with its sport model generating the 3D model of mRNA and its surface model building the 3D model of mitochondria. Statistical analysis was performed using Prism9, with a two-tailed unpaired t-test, with Welch's correction. Each sample was assessed for more than 100 spots.

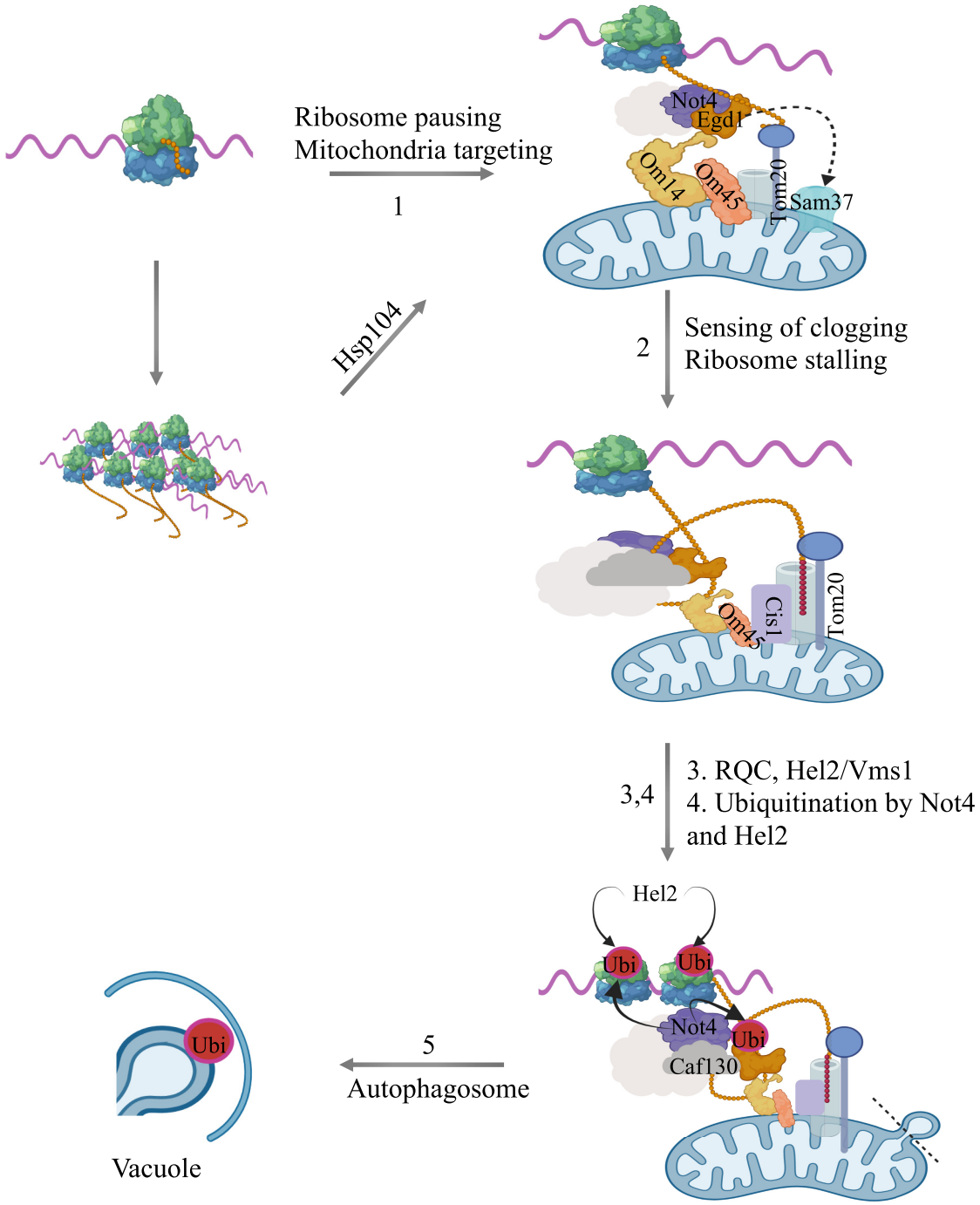

Figure 8. Model for limitation of translationally arrested mRNAs at the mitochondrial surface: Mito-ENCay. Overexpressed *MMF1* mRNA is targeted to the mitochondria via its nascent chain where its translation undergoes pausing, and both induction of the RQC/NGD and autophagy pathways reduce mRNA levels to limit protein synthesis and accumulation of Mmf1. This system relies on ribosome pausing and the co-translational targeting of the *MMF1* mRNA to the mitochondria via the Mmf1 nascent chain, the Egd1 chaperone, Om14, (alternatively Sam37), Om45 and Tom20 at the mitochondrial OM and Not4 of the Ccr4–Not complex (step 1). The Hsp104 disaggregase also plays a role, likely for targeting, if the nascent chain starts aggregating before targeting is ensured. Then, at the mitochondrial OM, ribosome pausing is increased if the import of the nascent chain is slowed down. Sensing is likely to be contributed to by Cis1, Om45 and Tom20 (step 2). Upon increased ribosome pausing, ribosome collisions will induce RQC and NGD initiated by ubiquitination by Hel2 (step 3), and result in degradation of the *MMF1* mRNA to limit new Mmf1 synthesis and accumulation. The limitation of *MMF1* mRNA is additionally provided by further ubiquitination of RNC-associated proteins by both Hel2 and Not4 (step 4), then autophagy, whereby vesicles of mitochondria fragments, rich in OM with docked RNCs and accumulated ubiquitinated proteins, are targets for autophagosome formation and targeting to the vacuole for degradation (step 5).

chaperone docking the RNC onto the MOM via its receptor Om14 or Sam37 (step 1). In addition, the Hsp104 disaggregase is important for Mito-ENCay, maybe by disaggregating nascent chains during translation to allow more efficient targeting of the *MMF1* RNC to the MOM (Figure 8, step 1).

Previous studies analyzing translation of nuclear-encoded mitochondrial mRNAs expressed at physiological levels from their endogenous loci in glucose did not detect *MMF1* as an mRNAs being translated at the MOM (5,62). The difference is that in this work we look at how the cell copes with additional *MMF1* mRNA expressed from an episome. It could be that co-translational MOM targeting occurs only when *MMF1* mRNA is in excess, or that it is only detected under these conditions. In this regard, our imaging of the *MMF1* mRNA showed that its detectable presence at the MOM was significantly increased when *MMF1* mRNA expression was increased (Figure 7). One explanation for this can be that under conditions of *MMF1* overexpression the presence of the RNC at the MOM is longer lasting. Indeed, we see that *MMF1* is translated with ribosome pausing. This pausing may enable co-translational targeting of the mRNA to the MOM or instead be the consequence of mRNA targeting to the MOM. Regardless, under low levels of expression, the nascent Mmf1 chain is likely to enter the import channel rapidly, resulting in a lift of ribosome pausing and the mRNA is rapidly released from the MOM at the end of translation. Instead, at high levels of the *MMF1* mRNA, one can imagine that the import channel becomes overwhelmed by the Mmf1 precursor and that ribosome pausing for *MMF1* RNCs arriving at the MOM will be sustained. Thereby, the mRNA has more chances to be detected at the MOM and ribosome collisions can occur resulting in RQC. It is interesting to note that accumulation of ribosome footprints 30 nucleotides and 60 nucleotides upstream of the major ribosome pause site on *MMF1* is already detectable under low levels of *MMF1* expression in glucose (Figure 4A). This suggests that ribosome collisions may already occur for *MMF1* expressed from its endogenous locus in glucose.

The *MMF1* MTS plays a key role in Mito-ENCay. Indeed, *MMF1* mRNA without MTS is not regulated by Mito-ENCay and it is overexpressed. The MTS could be essential for mitochondrial targeting, or the MTS might be important for ribosome pausing, in turn essential for targeting to the MOM. These two possibilities may not be independent. Indeed, ribosome pausing may occur because of the docking of the RNC onto the MOM, or ribosome pausing might give more chance to the RNC to be targeted to the MOM by slowing down translation. It has already been demonstrated that slowing down translation gives more chance for co-translational targeting to the MOM (5), and here we show that codon optimality around the ribosome pause site is important for Mito-ENCay, supporting the idea that translation elongation dynamics is an important factor. Deletion of the RQC machinery does not increase the already high levels of *MMF1* mRNA without an MTS. This indicates that there is no ribosome stalling or ribosome collisions for an *MMF1* mRNA without its MTS. This could be because the MTS directly affects ribosome pausing or because ribosome stalling occurs at the MOM. We favor this latter hypothesis. First, the docking site on the MOM for the chaperone that binds the nascent chain is important for Mito-ENCay supporting a role for targeting to the MOM in MitoENCay. Second, the MTS is only a few codons, and the major ribosome pause site on *MMF1* is nearly 90 codons after the MTS (Figure 4A). Finally, the MTS can work when fused to the *COX4* coding sequences instead of the Cox4 MTS. A model whereby this is because the MTS contributes to dock the RNC onto the MOM seems the most likely.

We determine that the Not4 subunit of the Ccr4–Not complex is important for Mito-ENCay in a manner epistatic to the MTS. Not4 is important for effective ribosome pausing when *MMF1* mRNA is produced from the endogenous locus in glucose and ubiquitination of its ribosome-associated targets, namely Rli1 and Rps7A that regulate ribosome pausing (36), contribute to Mito-ENCay. Like the MTS, Not4 might play a direct role in targeting, for instance via its interaction with NAC that binds the nascent chain, or it could play a role in translation elongation dynamics, enabling co-translational targeting of the *MMF1* RNC to the MOM. The fact that *MMF1* solubility is increased in the absence of Not4 is compatible with both models. However, we favor the latter one. Indeed, Not4-associated ribosomes are post-translocation ribosomes with an empty A site (37) more likely to occur when the codon in the A site is non-optimal, and the first codon after the *MMF1* ribosome pause site is a non-optimal codon that is important for Mito-ENCay. Furthermore, we have proposed that the binding of Not proteins to the translating ribosome may result in tethering of the RNC to condensates, in which translation elongation dynamics is different (36).

Another subunit of the Ccr4–Not complex, Caf130, is important for Mito-ENCay. Not much is known about the function of Caf130, but it is necessary for stable association of NAC with the complex ((58,59) and our own unpublished results). Hence it might contribute to recruit the Ccr4–Not complex to the NAC-associated nascent chain, or *vice-versa* recruit NAC to the Not4-associated RNC, or finally it may stabilize the complex of the RNC with NAC and Not4 (Figure 8, step 1). Caf130 might also contribute to subsequent steps of Mito-ENCay enabling effective ubiquitination of NAC (Figure 8, step 4).

Degradation of excess *MMF1* mRNA by Mito-ENCay implies not only that there is targeting of the *MMF1* mRNA to mitochondria during translation (Figure 8, step 1), but also that there is a mechanism that regulates ribosome pausing at the MOM (Figure 8, step 2). This raises the question of what determines the lift of ribosome pausing versus a stalling of translation. We observe that Cis1, already described to be important for detection of excess and/or aberrant precursor proteins at the MOM for 'Mito-CPR' (22), is needed for Mito-ENCay. For Mito-CPR it works together with the Msp1 ATPase to extract proteins from the MOM for degradation in the cytoplasm by the proteasome. Instead, for Mito-ENCay Msp1 is not relevant. Moreover, while we detect that some of the overexpressed Mmf1 and Cox4 is degraded in the cytoplasm by the proteasome, mutants of the proteasome have no impact on the level of the *MMF1* mRNA, unlike the *cis1Δ* mutant. Instead, Om45

that interacts with Om14, Sam37 and Tom20 responsible for recognition and initial import steps for all mitochondrially directed proteins, all contribute to Mito-ENCay (Figure 8, step 2). These factors are all candidates, with Cis1, for a role in regulation of ribosome pausing at the MOM according to the availability of the import channel.

If ribosome pausing at the MOM is sustained, then the risk for ribosome collisions increases and the RQC response will be induced. Consistently, the RQC response factors Hel2 and Vms1 are important for Mito-ENCay (Figure 8, step 3). It seems likely that the RQC can be rapidly overwhelmed and this can result in the induction of an additional QC response, namely autophagy (Figure 8, steps 4 and 5). Increased ubiquitination of RNC-associated proteins, probably by both Hel2 and Not4, appears to be a major contributor to this additional QC. Hel2 ubiquitinates ribosomal proteins in response to collided ribosomes (38,63), including Rps7A first mono-ubiquitinated by Not4, and it could be that Hel2 can similarly polyubiquitinate Egd1 and Rli1 after Not4 mono-ubiquitination in specific QC conditions. Protein ubiquitination is necessary in many types of selective autophagy as a mark for cargo recognition and a signal for process initiation by recruitment of specific autophagy adaptor proteins (reviewed in (64)). In addition, in a recent study a role for Not4 ubiquitination of Rli1 in the context of paused RNCs at the MOM for mitophagy in flies has been proposed (65). Ultimately fission and degradation of mitochondrial vesicles with highly ubiquitinated RNCs on their membrane will be degraded by autophagy (Figure 8, step 5), thereby preserving regions of the mitochondria without stalled RNCs. In this context it is interesting to note that Not4-dependent ubiquitination of Rps7A is important for *HAC1* translational up-regulation in response to ER stress, and the presence of the *HAC1* mRNA at the ER is necessary for this up-regulation (66). *HAC1* mRNA solubility, like the solubility of *MMF1*, increases upon Not4 depletion (39). Hence, it could be that Not4 contributes to ER targeting of the *HAC1* mRNA.

An important question is whether all mitochondrial mRNAs can be targets for Mito-ENCay when overexpressed, or whether this mechanism is specific for some mRNAs. Our results show that the *COX4* ORF is not a target for Mito-ENCay, but it can be repressed if the *MMF1* MTS replaces the *COX4* MTS. This suggests that maybe only mRNAs with specific N-terminal MTS sequences can be subject to Mito-ENCay. We noted that overexpressed Cox4 turns over very rapidly, and from this we can imagine that its overproduction does not endanger cellular proteostasis. So it could be that Mito-ENCay is a mechanism that has evolved to limit overexpression of mRNAs encoding proteins that are likely to aggregate and/or block the import channel. Intriguingly, solubility of *COX4* mRNA is also regulated by Not4, and overall ribosome footprints are increased on *COX4* mRNA in *not4Δ*. Moreover, *COX4* mRNA levels are also increased in *not4Δ*. It could be that solubility of *COX4* mRNA is regulated by Not condensates (35) that might also play a role in production of Cox4, for instance for effective interaction of nascent Cox4 with cytosolic chaperones or post-translational targeting of Cox4 to mitochondria. Furthermore, *COX4* regulation by the Not proteins might also depend upon 5' or 3'UTR sequences rather than on the coding sequence as was tested in this study.

DATA AVAILABILITY

No new data was generated in this work. The data analyzed was already deposited in public data bases.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We would like to thank Agnieszka Chacinska, Bertrand Daignan Fournier, Catherine Dargemont and Evelina Tutucci for plasmids, and the laboratories of Mafalda Escobar Henriques, Wolf Dieter, Lars Steimetz, Rachel Green, Marc Hochstrasser, Koji Okamoto, Sabine Rospert, Toshifumi Inada, Brian Zid and Shuh-ichi Nishikawa for strains. We thank Denis Martinvalet and Ravish Rashpa for preliminary observations, ideas and technical support, Laetitia Maillard and Szabolcs Zahoran for experimental support. We thank the Bioimaging platform of the Faculty of Medicine of the University of Geneva for technical help with microscopy and image processing and the BioRender software that was used to create the figures.

FUNDING

Swiss National Science Foundation [31003a_135794, 31003A_172999]; Novartis Foundation [15A043]; Ernst and Lucie Schmidheiny Foundation (to M.A.C.); China Scholarship Council [201907040077 to S.C.]; Swiss National Science Foundation. Funding for open access charge: Swiss National Science Foundation.
Conflict of interest statement. None declared.

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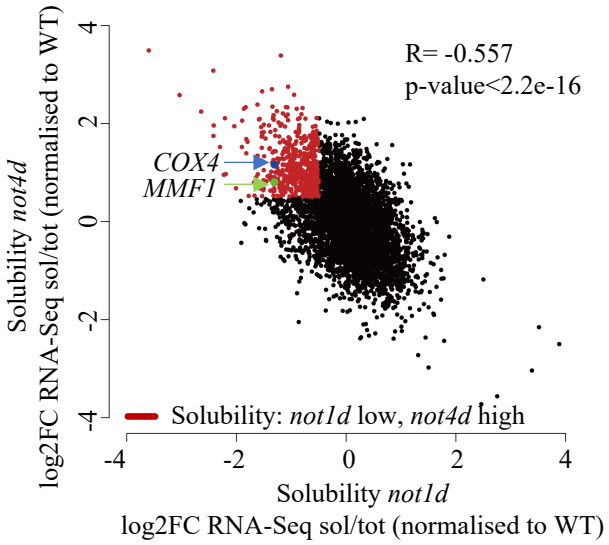
S1

A



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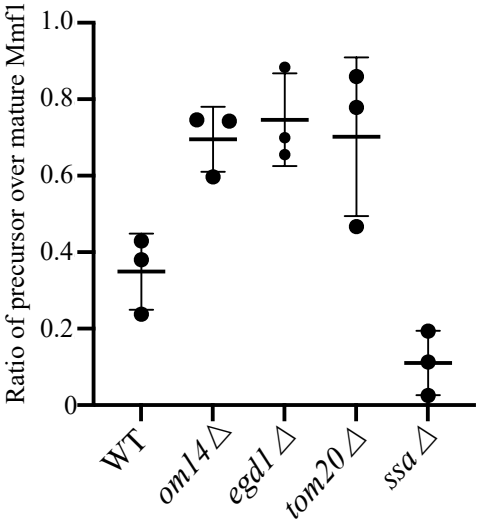

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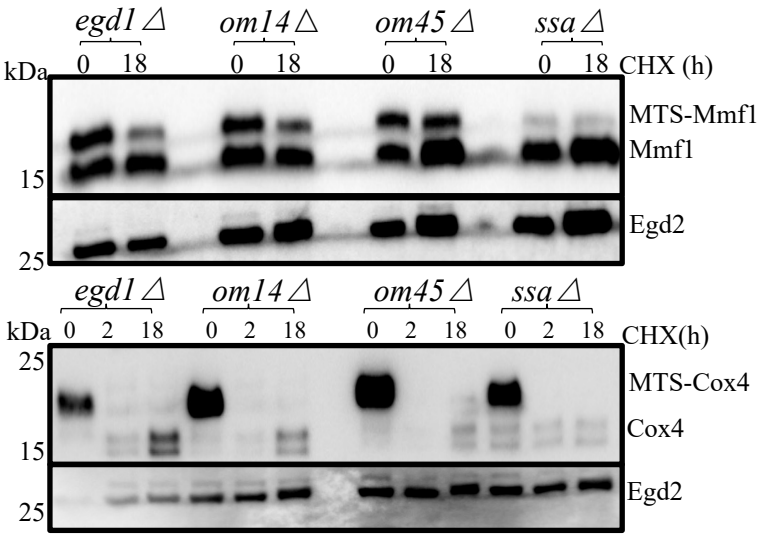


Figure S1. A. Indication of *MMF1* and *COX4* mRNAs on a scatterplot comparing changes in mRNA solubility before and after Not1 and Not4 depletion (43). mRNAs more soluble upon Not4 depletion but less upon Not1 depletion are indicated in red. B. Quantification of Mmf1 precursor relative to mature Mmf1 after copper induction for experiment in Figure 1D. C. Expression of the Mmf1 and Cox4 reporters in the indicated strains growing exponentially after a 10 min copper induction (0) followed by treatment with CHX for 2 or 18h as indicated, by western blotting with antibodies to Flag or Egd2 as loading control.

S2

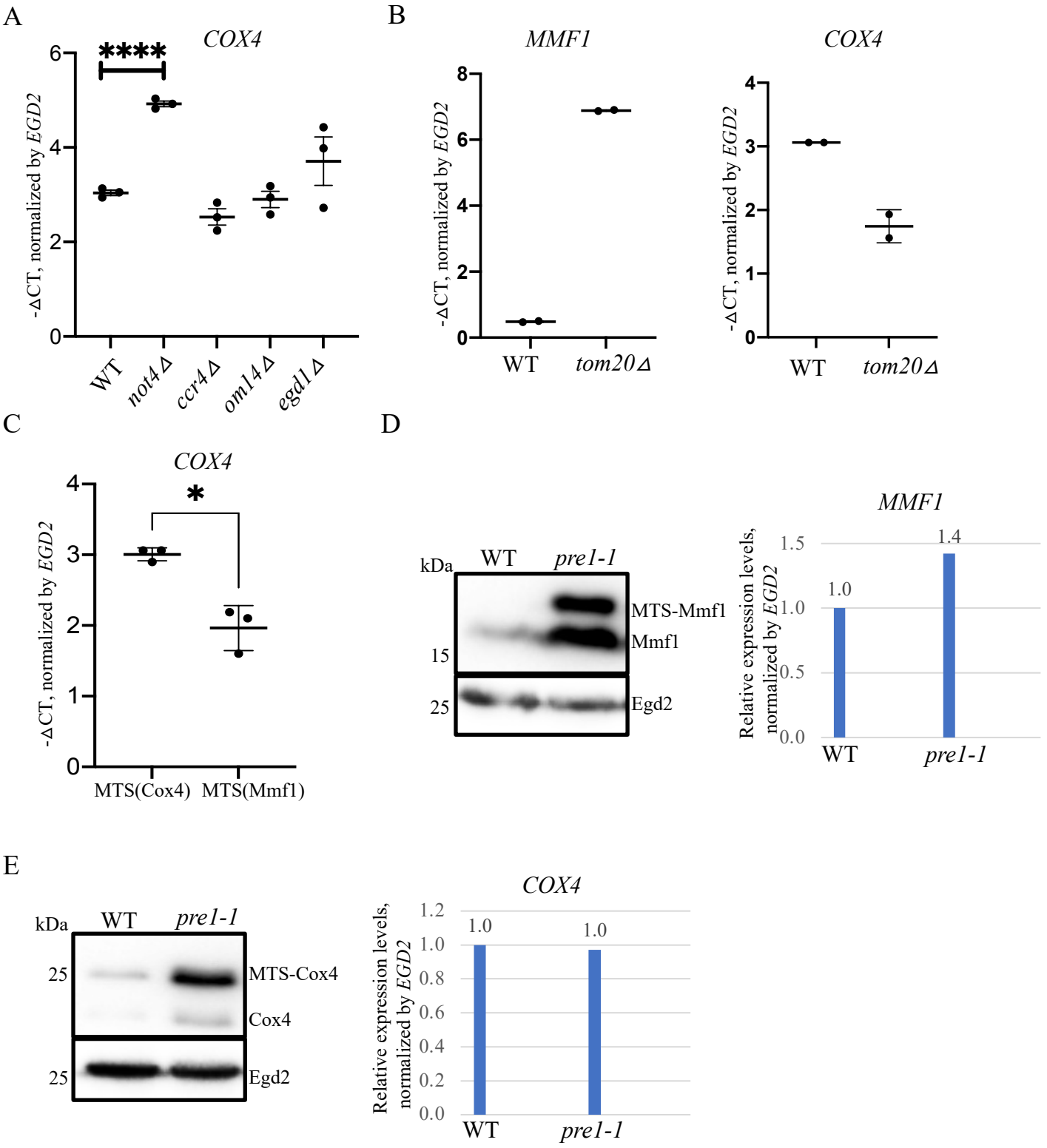


Figure S2. **A**. Analysis of *COX4* reporter mRNA levels by RT-qPCR in the indicated strains. The *EGD2* mRNA was used as a control for loading. The *COX4* reporter mRNA was plotted to show means +/- SD of – ΔCT values. The level of significant change, relative to WT is indicated with asterisks using a two-sided, Welch, unpaired t-test (n = 3). (**B**) Analysis of *MMF1* and *COX4* reporter mRNA in wild type and *tom20Δ* cells as indicated growing exponentially by RT-qPCR. Results are of biological duplicate experiments. (**C**) Analysis of *COX4* reporter mRNA levels corresponding to the experiment in **Figure 2D** by RT-qPCR as in panel A. (**D**) Analysis of the *MMF1* reporter in wild type cells or the proteasome *pre1-1* mutant growing exponentially, by western blotting with antibodies to Flag or to Egd2 for loading control (left panel) or by RT-qPCR as in panel B (right panel). **(E)** Analysis of the *COX4* reporter as for *MMF1* in panel **D**.

S3

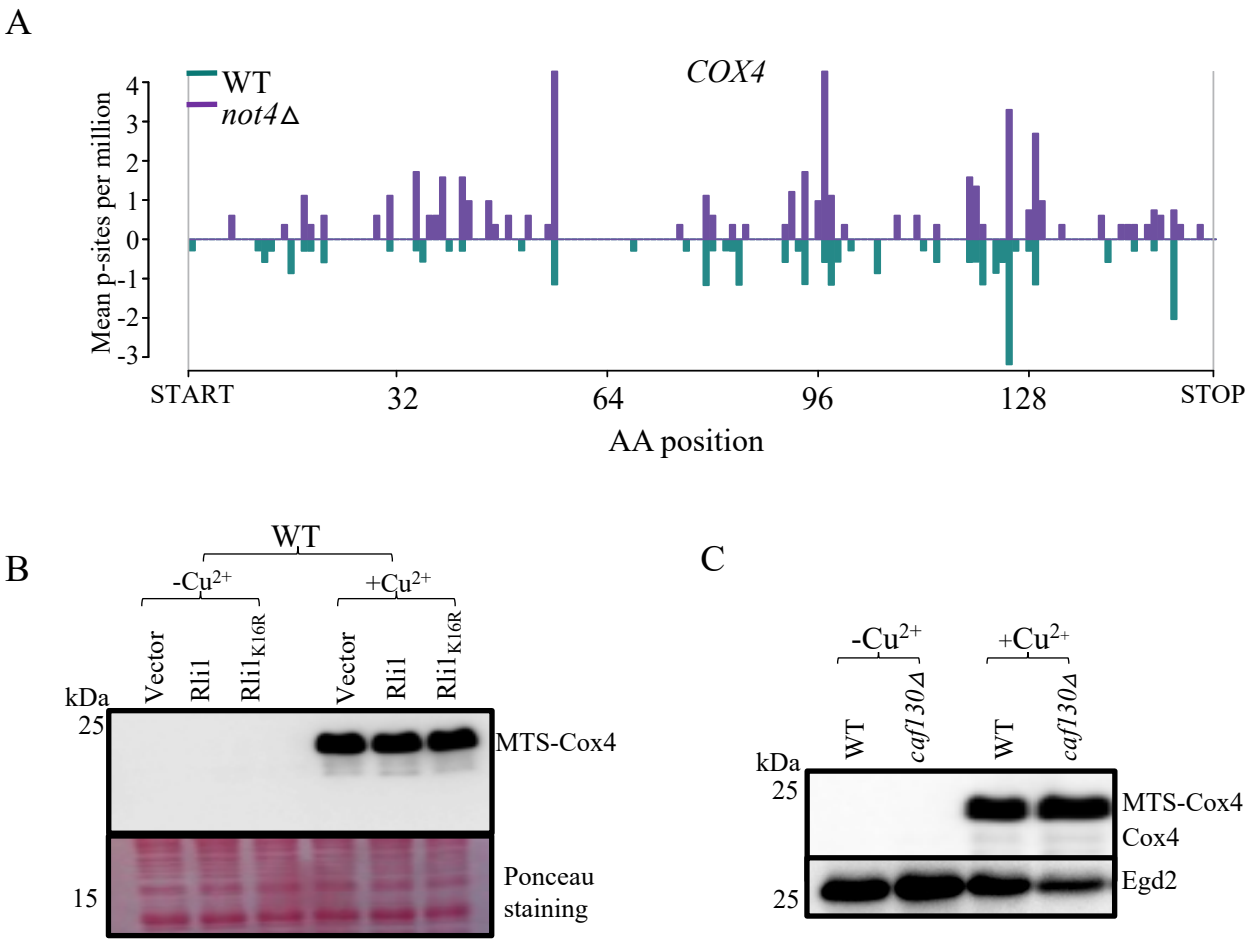
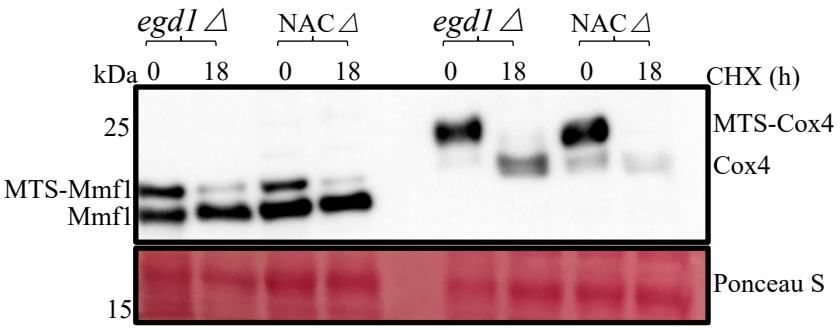


Figure S3. A. Profiles of ribosome footprints (P-site depth plots) on *COX4* with footprints in wild type cells in green and those in *not4*△ in purple. The number of P-sites, per million genome-wide for each sample, covering each CDS codon with corresponding amino acid position indicated (AA position) is calculated, averaged for each condition and plotted. **B**. Expression of the Cox4 reporter in wild type cells growing exponentially with or without overexpression of wild type or K16R-mutated Rli1, before and after copper induction, by western blotting with antibodies to Flag. The ponceau staining is shown as loading control. **C**. Expression of the Cox4 reporter in wild type cells or cells lacking Caf130 growing exponentially before and after copper induction is analyzed by western blotting with antibodies to Flag or to Egd2 for loading control.

A



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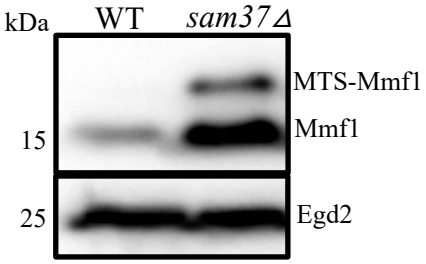

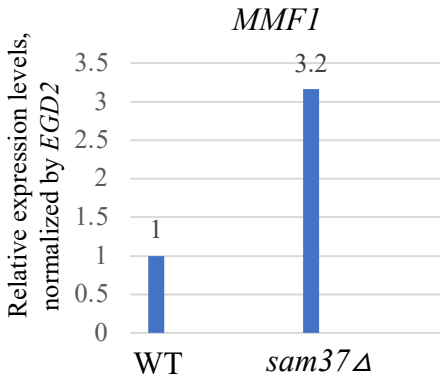

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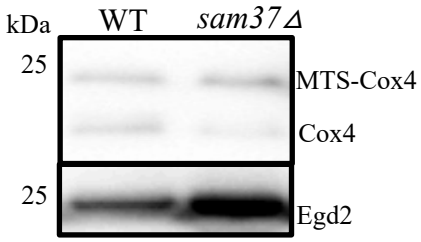


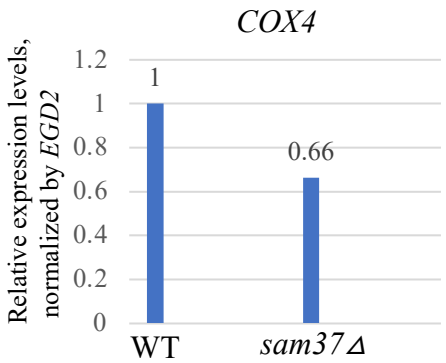


Figure S4. **A**. Expression of the Mmf1 and Cox4 reporters is compared in cells lacking Egd1 or all 3 NAC subunits and growing exponentially after a 10 min copper induction treated or not with CHX for 18 h, by western blotting with antibodies to Flag or to Egd2 for loading control. **B**. Expression of the *MMF1* reporter is compared in wild type cells and in cells lacking Sam37 for protein levels by western blotting with antibodies to Flag or to Egd2 for loading control (left) or for mRNA by RT-qPCR (right). Results are for a single experiment. **C**. The same experiment as in panel B was done to evaluate expression of the *COX4* reporter.

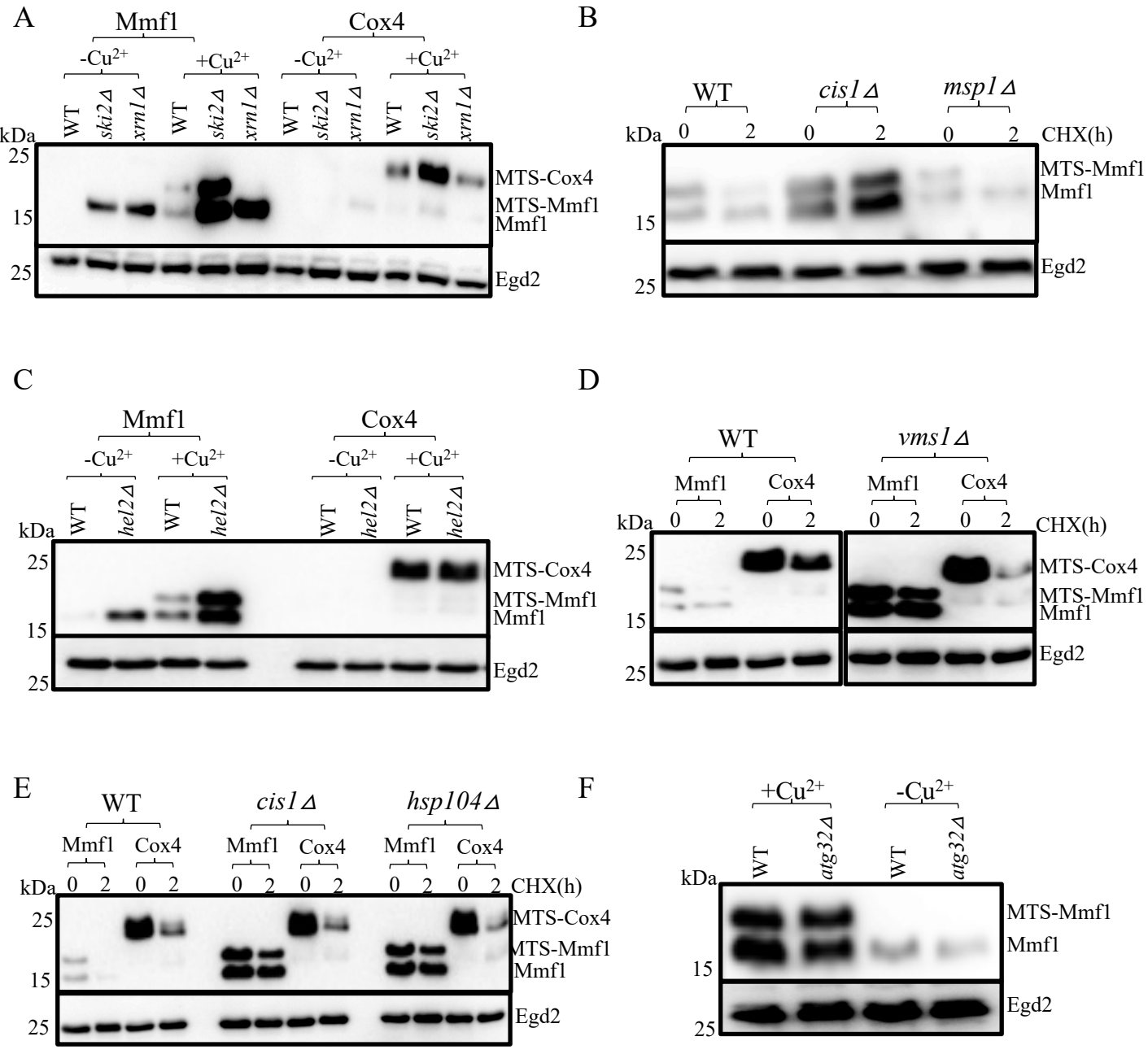
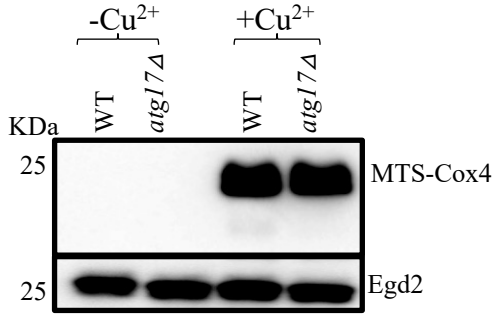


Figure S5. **A**. Expression of the Mmf1 and Cox4 reporters were tested in wild type cells, or in cells lacking Ski2 or Xrn1 before and after copper induction by western blotting with antibodies to Flag or to Egd2 for loading control. **B**. Expression of the Mmf1 reporter was compared in wild type cells, or in the *cis1Δ* or *msp1Δ* mutants after 10 min copper induction or 2 hours after adding CHX, by western blotting with antibodies to Flag or to Egd2 for loading control. **C**. Expression of the Mmf1 or Cox4 reporters were tested in wild type or *hel2Δ* cells before and after copper induction by western blotting with antibodies to Flag or to Egd2 for loading control. **D**. Expression of the Mmf1 or Cox4 reporters were tested in wild type or *vms1Δ* cells after a 10 min copper induction or 2 hours after adding CHX by western blotting with antibodies to Flag or to Egd2 for loading control. **E**. Expression of the Mmf1 and Cox4 reporters were tested in wild type cells, or in *cis1Δ* and *hsp104Δ* cells after a 10 min copper induction and 2 hours after adding CHX, by western blotting with antibodies to Flag or to Egd2 for loading control. **F.** Expression of the Mmf1 reporter was tested before and after copper induction in wild type cells or cells lacking Atg32 by western blotting with antibodies to Flag or to Egd2 for loading control.

A



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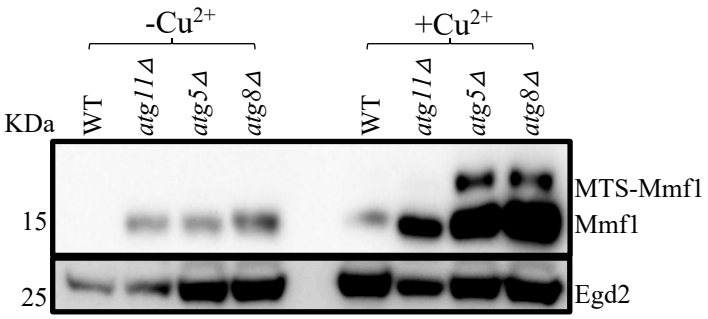

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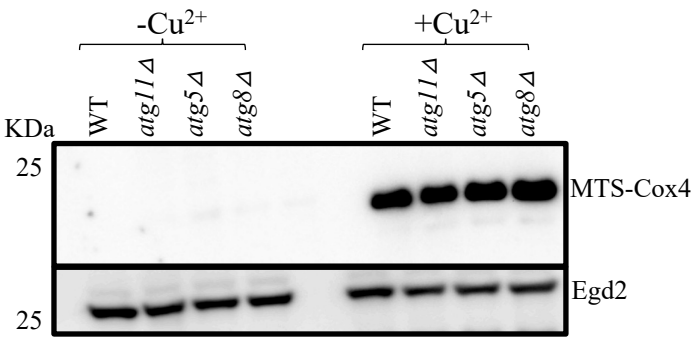

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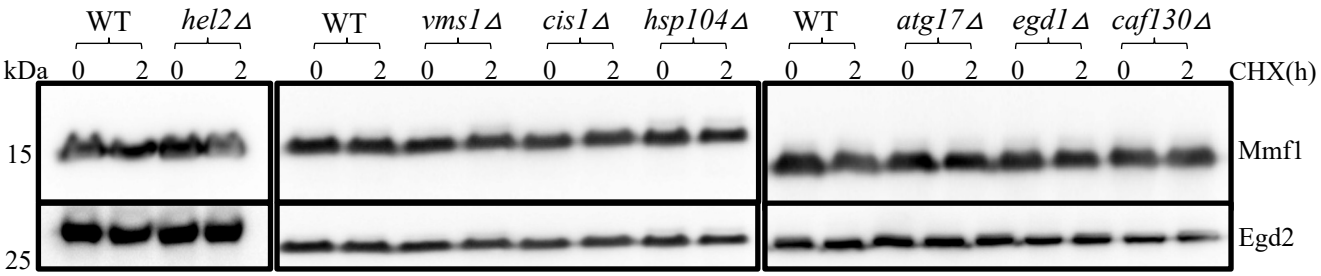
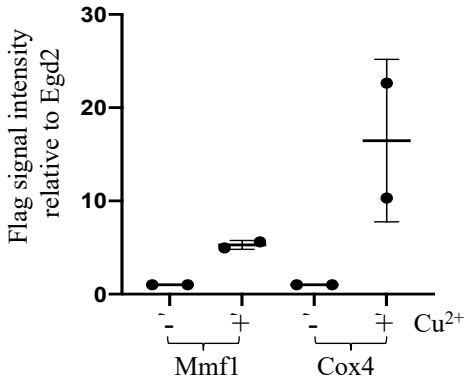


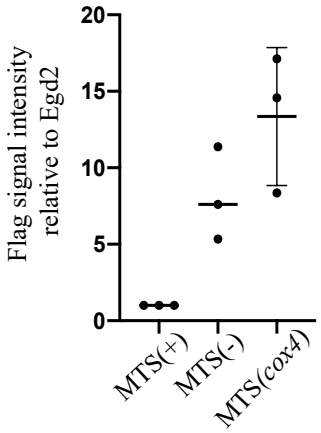
Figure S6. **A**. Expression of the Cox4 reporter was tested before and after copper induction in wild type cells or cells lacking Atg17 by western blotting with antibodies to Flag or to Egd2 for loading control. **B**. Expression of the Mmf1 reporter was tested in the indicated strains before and after copper induction by western blotting with antibodies to Flag or to Egd2 for loading control. **C**. Expression of the Cox4 reporter was tested as in panel B. **D**. Expression of the Mmf1 reporter without the MTS was tested in the indicated strains growing exponentially by western blotting with antibodies to Flag or to Egd2 for loading control.

S7

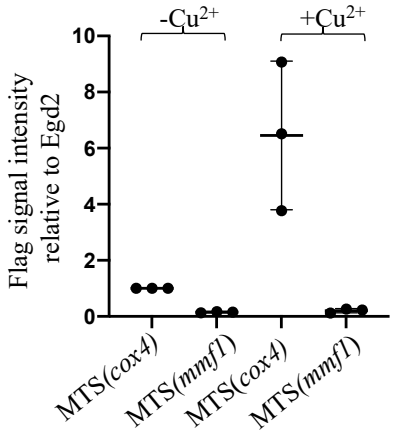
1B



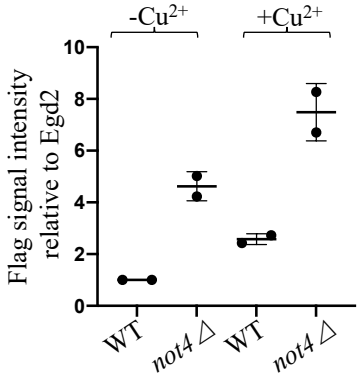
2C



2D



3A



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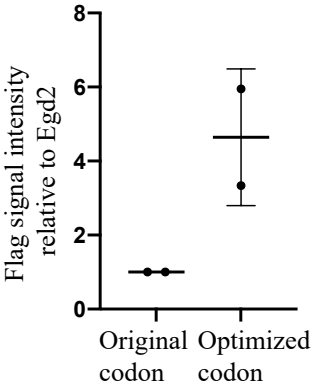
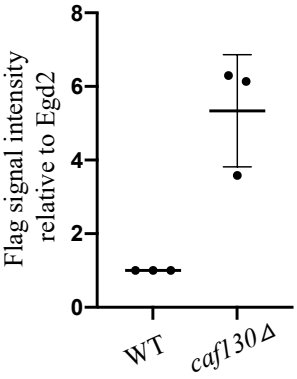


5D



6C

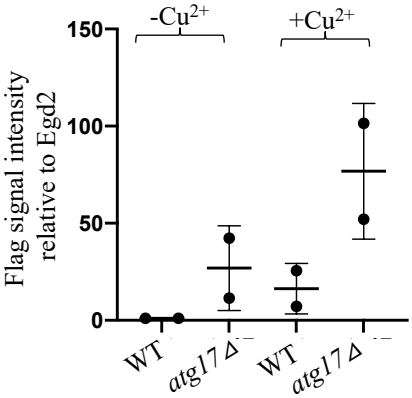


Figure S7. The relevant western blots shown in the main figures were performed at least in biological triplicates and for some of these quantification as indicated is shown (Figure 1B, 2C, 2D, 3A, 4D, 5D, 6C).

Internal number	Plasmids	Reference
pMAC594	*ADH1p-EGD1-HA-TRP1*	Panasenko et al., 2009
pMAC634	*ADH1p-egd1-K29,30R-HA-TRP1*	Panasenko et al., 2009
pMAC684	*NOT4p-Myc6-NOT4-ADH1t*	Panasenko and Collart, 2012
pMAC721	*NOT4p-Myc6-not4-1-430-ADH1t*	Panasenko and Collart, 2012
pMAC751	*NOT4p-Myc6-not4-235-587-Q560R*	Panasenko and Collart, 2012
pMAC1200	*CUP1p-COX4-Flag-ADH1t-URA3*	This work
pMAC1211	*CUP1p-MMF1-Flag-ADH1t-URA3*	This work
pMAC1321	*CUP1p-Flag-rli1-K16R-LEU2*	Pansenko et al., 2019
pMAC1322	*CUP1p-Flag-RLI1-LEU2*	Panasenko et al., 2019
pMAC1327	*CUP1p-MTS-less-MMF1-Flag-ADH1t-URA3*	This work
pMAC1328	*CUP1p-Cox4MTS-MMF1-Flag-ADH1t-URA3*	This work
pMAC1342	*CUP1p-MMF1-Flag-ADH1t-ura3::LEU2*	This work
pMAC1365	*CUP1p-MMF1-Flag-MS2bps-URA3*	This work
pMAC1367	*CUP1p-MTS-less-MMF1-Flag-MS2bps-URA3*	This work
pMAC1424	*CUP1p-Mmf1MTS-COX4-Flag-ADH1t-URA3*	This work
pMAC1425	*CUP1p-MMF1* $_{AAA92AAG,\ GTA95GTC}$*-Flag-ADH1t-URA3*	This work
pMAC1430	*CUP1p-MMF1-Flag-MS2bps-ura3::HIS3*	This work
pMAC1431	*CUP1p-MTS-less-MMF1-Flag-MS2bps-ura3::HIS3*	This work
pE23	*ura3::HIS3*	pUH7 Daignan Fournier lab, Cross 1997
pE24	*ura3::LEU2*	pUL9 Daignan Fournier lab, Cross 1997
pE116	*pRS315*	Euroscarf
pE298	*CUP1p-UBI-6His*	Dargemont lab
pE617	*CUP1p-COX12-Flag*	Agnes Chacinska lab
pE659	pET264-puc24xMS2V6-U-Var-LoxP_KanMX4_LoxP	Evelina Tutucci lab

Internal number	Oligo description	Sequence
687	MMF1 qPCR for	CAACGTATTCTTGGCTGACATG
691	Cox4 For EcoRI	AAAAAAGAATTCATGCTTTCACTACGTCAATCTATAAGATTTTTCAAGCC
692	Cox4 Rev Not1	ACTAGTGCGGCCGCGTGATGGTGGTCATCATTTGGAACACC
714	COX4 qPCR for	CCGTTCCAACAGACCTAGATCAAG
935	MMF1 Rv Not1	TTT TTT GCG GCCGCA TTC TTT TCA ACA GCG ATA ACT TCC
936	MMF1 MfeI Fw	AAA AAA CAA TTG ATG TTT TTA AGA AAT TCC GTT TTG AG
999	Flag R	CTTATCGTCGTCATCCTTGATATC
1000	EGD2 F	AAGACGTCGCTACCAAGTCC
1001	EGD2 R	GTCACCGGCATCAACTTCAC
1009	MMF1 without target sequence	CGAAATAGCAATTGATGATAACAACATTGACCCCGGTCAG
1028	MMF1 without target sequence	CTTGTTAGTGCAATATCATATAGAAGTCATCGAAATAG GAATTCATGATAACAACATTGACCCCGGTCAG
1029	MMF1 with COX4 target sequence	CTTGTTAGTGCAATATCATATAGAAGTCATCGAAATAG GAATTCATGCTTTCACTACGTCAATCTATAAGATTTTTCAAGCCAGCCACAAGAACTTTGTGTAGCTCTAGAATAACAACATTGACCCCGGT
1030	MMF1 reverse Not1	CTTATCGTCGTCATCCTTGTAATCCATCGATACTAGTGCGGCCGCATTCTTTTCAACAGCGATAACTTCC
1087	MMF1 F	TATCGCTGTTGAAAAGAATGCGGCCGCACTAGTATCGATGGATTACAAGGATGACGACGATAAGATCTGACCGCTCTAGAACTAGTGGAT
1088	MMF1 R	GTAGACAAGCCGACAACCTTGATTGGAGACTTGACCAAACCTCTGGCGAAGAATTGTTAATTAAGAGCTCATTAAGGGTTGTCGAGATCG
1113	MS2 R	GAGAAAGCAACCTGACCTACAGGA
1297	MTS-Mmf1-Cox4 F	ACAGCTCCAGTCTTGAGGAGGGGTATATATCTGCTTCAGCAAAAACC
1298	MTS-Mmf1-Cox4 R	TCTCAAAACGGAATTTCTTAAAAACATGAATTGATACAAGACAAGGA
1301	Change Mmf1 codons 92, 95 F	TTAGACAATATAGTCAAGGTCAACGTCTTCTTGGCTGACATG
1302	Change Mmf1 codons 92, 95 R	AGAAGAATTACTTTCTGCTAAGATATTCTTAACGTT

Internal Number	Strains	Genotype	Reference
1	WT	*MAT a gcn4Δ ura3-52 trp1Δ1 leu2::PET56 gal2*	KY803, Hope and Struhl, 1986
3415	WT	*MAT a his3 leu2 ura3 lys2*	Euroscarf
3417	*not4Δ*	*MAT a his3 leu2 ura3 lys2 not4::KanMX*	Euroscarf
3421	*caf130Δ*	*MAT a his3 leu2 ura3 lys2 caf130::KanMX*	Euroscarf
3422	*ccr4Δ*	*MAT a his3 leu2 ura3 lys2 ccr4::KANMX*	Euroscarf
3465	*vms1Δ*	*MAT a his3 leu2 ura3 lys2 vms1::KanMX*	Euroscarf
3612	*Egd1-HA3*	*MAT a gcn4Δ ura3-52 trp1Δ1 leu2::PET56 gal2 egd1::EGD1-3HA-KanMX4*	Derived from KY803, Panasenko et al., 2006
4555	*caf130Δ Egd1-HA3*	*MAT a ura3 leu2 his3 trp1 caf130::KanMX4 egd1::EGD1-HA3-KanMX4*	Euroscarf
8662	*atg17Δ*	*MAT a leu2Δ0 ura3Δ0 met15Δ0 his3Δ1 atg17::KanMX4*	Euroscarf
10005	*pre1-1*	*pre1-1*	From Mafalda Escobar-Henriques lab
10451	*ssaΔ*	*ura3-52 leu2-3,112 his3-11 lys2 trp1Δ1 ssa1-45 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2 prc1-1 (CPY*)*	From Wolf Dieter lab
10652	*NAC Δ*	*his3 trp1 leu2 ura3 egd1::URA3 egd2::ADE2 btt1::HIS3MX4*	From Sabine Rospert lab
11809	*hel2Δ*	*MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hel2::kanMX4*	Euroscarf
12303	*RPS7A-K4R*	*MAT a leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 rps7a::HISMX4 rps7b::NATMX4 pLEU2-rps7a-4KR-HA*	W303-1a and PCR from Toshi Inada pRS315 based plasmid (K72, K76, K83 and K84)
12304	*RPS7A*	*MAT a leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 rps7a::HISMX4 rps7b::NATMX4 pLEU2-RPS7A-HA*	W303-1a and PCR from Toshi Inada pRS315 based plasmid
12971	*atg32Δ*	*MAT a leu2Δ20 ura3Δ met15Δ his3Δ1 atg32::URA3*	KOY1380 (BY4741 background)
13549	*Mmf1-Taptag*	*MATa ade2 arg4 leu2,3112 trp1-289 ura3-52 mmf1::MMF1-Taptag-URA3*	Euroscarf
13551	*msp1Δ*	*MAT a his3 leu2 ura3 lys2 msp1::KANMX4*	Euroscarf
13701	*Mmf1-Taptag not4 Δ*	*MATa his3Δ leu2Δ lys2Δ0 ura3Δ not4::NATMX4 mmf1::MMF1-Taptag-URA3*	This work
13784	*egd1Δ*	*MAT a his3 leu2 ura3 egd1::KANMX4*	Euroscarf
13785	*hsp104Δ*	*MAT a his3 leu2 ura3 lys2 hsp104::KANMX4*	Euroscarf
13787	*om45Δ*	*MAT a his3 leu2 ura3 lys2 om45::KANMX4*	Euroscarf
13790	*om14Δ*	*MAT a his3 leu2 ura3 lys2 om14::KANMX4*	Euroscarf
13791	*cis1Δ*	*MAT a his3 leu2 ura3 lys2 cis1::KANMX4*	Euroscarf
13989	*tom20Δ*	*tom20::LEU2*	SNY1005, from Shuh-ichi Nishikawa, W303-1A background
14470	*atg11 Δ*	*MAT a his3 leu2 ura3 lys2 atg11::KanMX*	From Mafalda Escobar-Henriques lab
14471	*atg5 Δ*	*MAT a his3 leu2 ura3 lys2 atg5::KanMX*	From Mafalda Escobar-Henriques lab
14472	*atg8 Δ*	*MAT a his3 leu2 ura3 lys2 atg8::KanMX*	From Mafalda Escobar-Henriques lab
14486	*sam37 Δ*	*MAT a his3 leu2 ura3 lys2sam37::KanMX*	Euroscarf
14492	*skI2Δ*	*MAT a his3 leu2 ura3 lys2*	From Rachel Green lab
14493	*xrn1Δ*	*MAT a his3 leu2 ura3 lys2*	From Rachel Green lab
14612	Su9-mCherry	*MATa trp1::Su9-mCherry-TRP1 ade2 leu2 his3 ura3 can1 SUC2*	From Brian Zid lab ZY783
14613	su9-mCherry MCP-4GFP	*MATa trp1::Su9-mCherry-TRP1 ade2 leu2 his3 ura3::CYC1p-MCP-4GFP-URA3 can1 SUC2*	From Brian Zid lab ZY801

	Antibodies		Reference
Commercial	PAP（Peroxidase Anti Peroxidase complex）		Sigma （P1291）
Commercial	Flag	Monoclonal	Sigma （F3165）
Homemade	Por1	Polyclonal	From Yoav Arava lab
Homemade	Hxk1	Polyclonal	From Yoav Arava lab
Commercial	HA	Monoclonal	Sigma （H3663）
Homemade	Egd2	Polyclonal	Panasenko et al., 2009

References

Panasenko, O., Landrieux, E., Feuermann, M., Finka, A., Paquet, N. and Collart, M.A. (2006) The yeast Ccr4-Not complex controls ubiquitination of the nascent-associated polypeptide (NAC-EGD) complex. *J Biol Chem* , **281**, 31389-31398.

Panasenko, O.O., David, F.P. and Collart, M.A. (2009) Ribosome association and stability of the nascent polypeptide-associated complex is dependent upon its own ubiquitination. *Genetics* , **181**, 447-460.

Panasenko, O.O. and Collart, M.A. (2012) Presence of Not5 and ubiquitinated Rps7A in polysome fractions depends upon the Not4 E3 ligase. *Mol Microbiol* , **83**, 640-653.

Cross, F.R. (1997) 'Marker swap' plasmids: convenient tools for budding yeast molecular genetics. *Yeast* , **13**, 647-653.

Hope, I.A. and Struhl, K. (1986) Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. *Cell* , **46**, 885-894.

Annexes of unpublished data

Chapter 1 - Annexes 1

I not only studied the expression of Mmf1 produced from reporter constructs as described above, but I also studied the expression of C-terminally tagged Mmf1 from its endogenous locus in wild type and mutant cells, and this both in cells growing fermentatively in glucose where I also checked Mmf1 stability, or in cells switched to respiratory growth in glycerol/ethanol-containing medium.

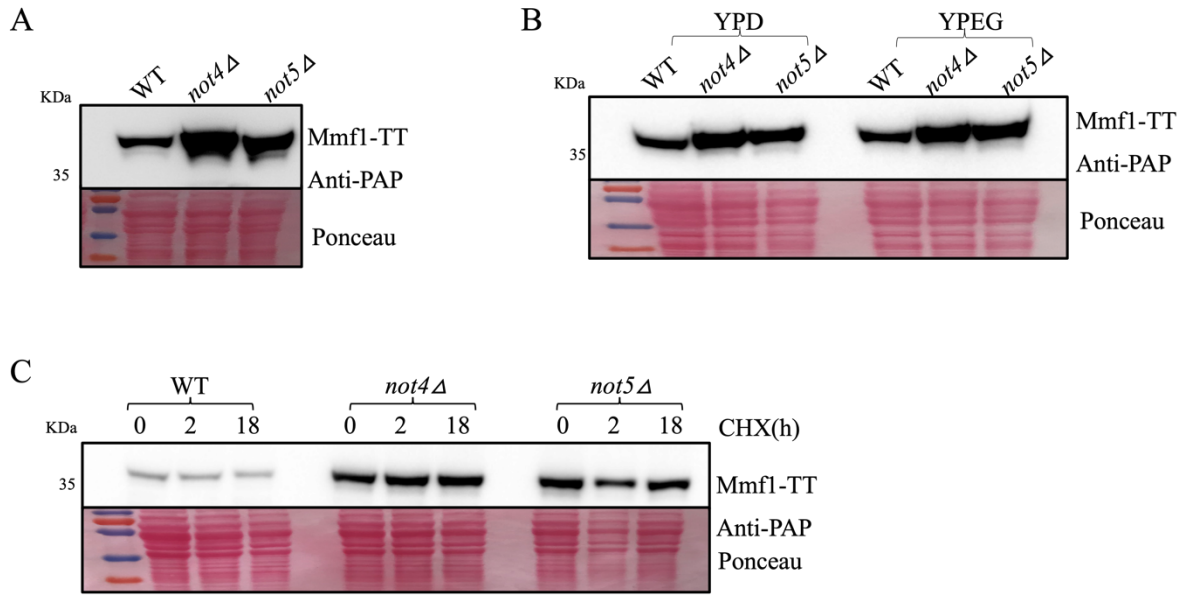


Figure 14. Analysis of Mmf1 expressed from its endogenous locus in *not* mutants and in different metabolic conditions

Panel A: Cells cultured in rich medium with glucose as the carbon source. Mmf1 was tagged with protein A (Mmf1-TT) at its endogenous locus in WT and *not* mutants. Total protein analysis was performed by western blotting with antibodies to Protein A (Anti-PAP). Ponceau staining was used as a loading control. Panel B: In addition to glucose rich medium (YPD), cells were also grown in YPEG medium, which contains glycerol and ethanol. This medium induces mitochondrial biogenesis for the utilization of non-fermentable carbon sources. The expression of Mmf1 was analyzed as in Panel A. Panel C: Analysis of the expression of endogenous Mmf1 as in panel A in the indicated strains during logarithmic growth (marked as 0). The cells were then treated with cycloheximide (CHX) at 100 µg/ml and incubated at 30°C for 2 or 18 hours, as indicated.

These experiments show that chromosomally encoded tagged Mmf1 is also over-expressed in *not* mutants in fermentation and respiration, and it is stable up to 18 hours after protein synthesis arrest. One caveat is that this is not exactly endogenous *MMF1* because the 3'UTR sequence is heterologous due to the tagging, and this may have an impact on regulation.

Chapter 1- Annexes 2

I imaged wild type and *not4Δ* strains expressing different mitochondrial proteins (Atp5 or Om45) tagged with GFP at their endogenous loci, by fluorescence microscopy.

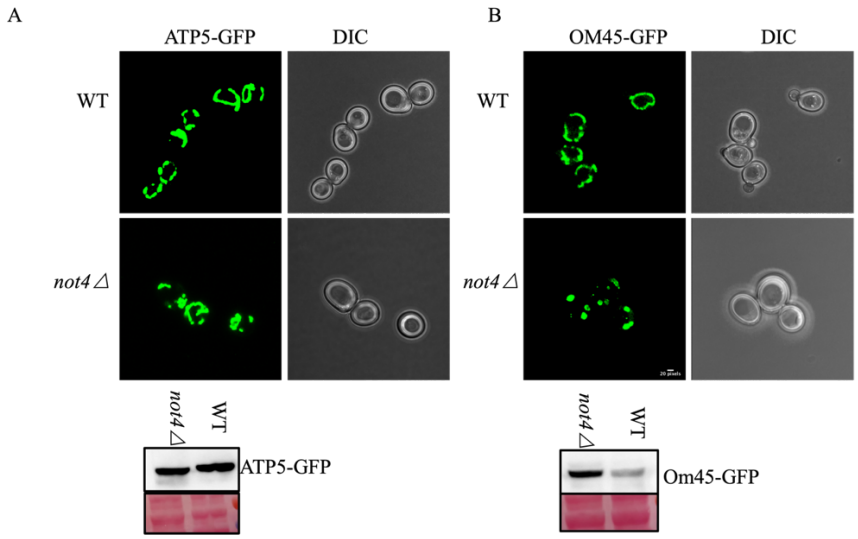


Figure 15. Expression and distribution of Om45-GFP but not Atp5-GFP is impacted by Not4

In this study, outer membrane protein Om45 and inner membrane protein Atp5 were fused with GFP in both wild-type (WT) and *not4Δ* strains. The expression and distribution of Atp5-GFP were visualized using fluorescence microscopy (left upper panel A), while the cell boundary was indicated using light microscopy (right upper panel A). Panel B: The expression and distribution of Om45-GFP were visualized as panel A. Furthermore, the expression of Atp5-GFP and Om45-GFP was also examined using western blotting with antibodies specifically targeting GFP (bottom panels A and B).

The data shows that in the absence of Not4, the expression level of Om45-GFP is higher compared to WT. Moreover and interestingly, Om45-GFP tends to form aggregated structures rather than being evenly distributed on mitochondria membranes. In contrast, the expression and distribution of Atp5-GFP seems to be independent of Not4.

Chapter 1- Annexes 3

I imaged WT cells with the Mmf1 reporter to see the how the cells cope with overexpressed protein.

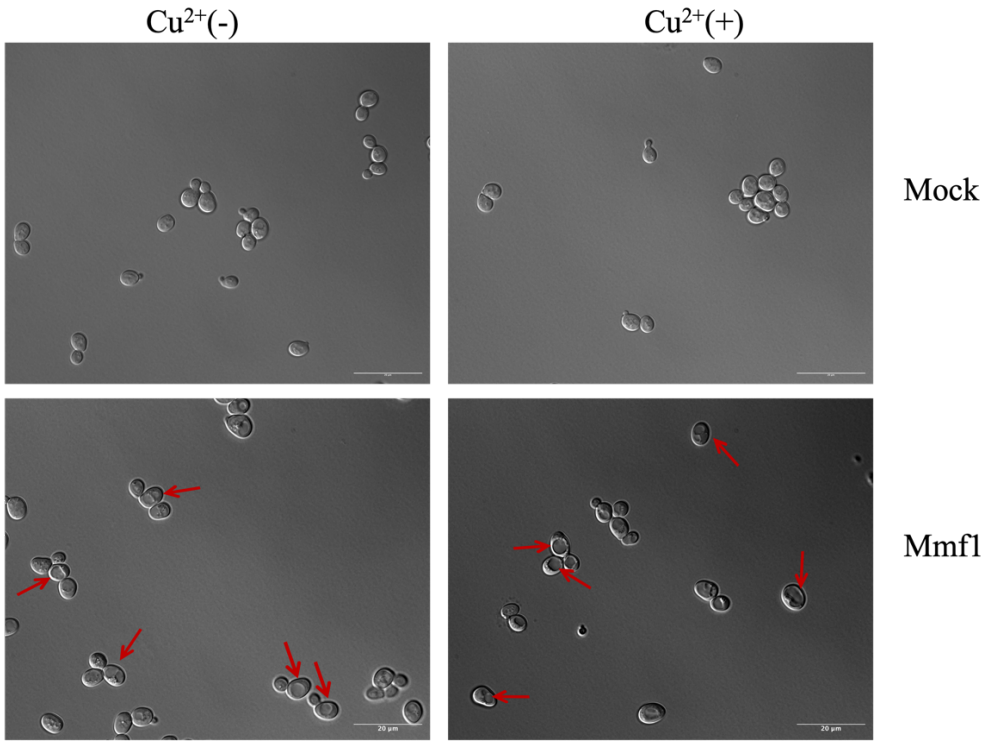


Figure 16. Overexpression of Mmf1 active autophagy

The WT cells were transformed with an empty plasmid containing the same selected nutritional marker (Mock) as the Mmf1 reporter. When the cells reached the logarithmic phase of growth, they were evaluated for morphology using a light microscope. Two conditions were examined: without copper induction (Cu^{2+} (-)) and with copper induction for 10 minutes (Cu^{2+} (+)). In the figure, the upper panel shows cells containing the mock reporter, while the bottom panel shows cells containing the Mmf1 reporter. The cells containing the Mmf1 reporter have a bigger vacuole (indicated by red arrow) compared to cells containing the mock reporter with or without copper induction.

This data shown WT cells with the Mmf1 report have lager vacuole, indicated by red arrow, indicating autophagy. Upon the initial discovery of autophagy in yeast, an increase in vacuole size was observed during starvation and our work suggests that autophagy is induced to deal with excess Mmf1, hence this increase in vacuole size is compatible with our suggestion that autophagy is activated.

2. CHAPTER 2: Investigating the interplay of Ccr4-Not complex and Puf3 in mitochondrial biosynthesis regulation

Puf3 is an RNA-binding protein involved in regulating mitochondrial mRNA metabolism. It interacts with specific RNA motifs to bind to the 3' end of certain mitochondrial mRNAs and can localize some RNAs to the outer mitochondrial membrane. In yeast cells during fermentation, Puf3 recruits the Ccr4-Not complex to degrade specific mRNAs, while forming Puf3 granules to store certain mitochondrial mRNAs. However, during the respiratory growth phase, Puf3 is phosphorylated by the casein kinase I protein Hrr25, leading to the dissolution of Puf3 granules and the release of stored mRNAs. These mRNAs are then rapidly translated into mitochondrial proteins to meet the cell's increased demand for mitochondria[56, 180-183]. Different research laboratories have identified various Puf3-bound mRNAs, but their results have shown differences, possibly due to variations in cellular growth states. This suggests that the binding of Puf3 to RNA is dynamic[184-186]. It appears that Puf3 impacts expression of many genes not related to mitochondria, some which do not have obvious binding sites for Puf3[185]. Moreover, Puf3 appears to associate with mRNAs for which obvious Puf3 consensus are not detectable. A recent study has defined core Puf3 target mRNAs according to the quality of a detectable Puf3 binding site[184].

Recent work conducted by the Collart laboratory has revealed that cellular mRNAs exhibit diverse solubilities, and translation elongation dynamics differ between the insoluble and soluble mRNA pools. Specifically, ribosomes tend to dwell longer at non-optimal codons in the insoluble mRNA pool. Moreover, they showed that depleting Not1 and Not4 inversely modulated mRNA solubility genome-wide and that inverse changes in ribosome dwelling occupancy according to codon optimality upon Not1 and Not4 depletion was due to changes in solubility of specific groups of mRNAs.

An intriguing finding was that in fermentative growth, mRNAs that became less soluble upon Not4 depletion but more soluble upon Not1 depletion are enriched for core targets of Puf3 (**Figure 17**, green category). We were thus interested in determining if changes in Puf3 were playing any role in the changes in mRNA solubility upon depletion of the Not1 and Not4

proteins, potentially acting as a regulator of translation elongation dynamics and thereby impacting mitochondria biosynthesis.

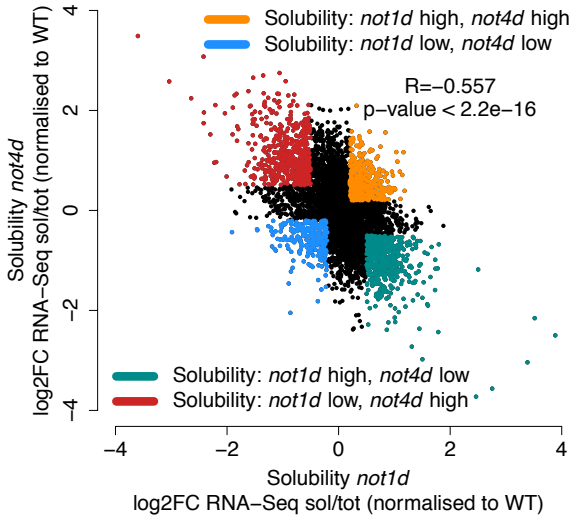


Figure 17. Scatterplot comparing changes in mRNA solubilities before and after Not1 and Not4 depletion (Picture take from [159])

We designed experiments to determine if there are interactions between the Ccr4-Not complex and Puf3 that could be relevant for dynamics of co-translation events. For this I decided to investigate expression of Puf3 and its sedimentation pattern in a sucrose gradient in wild type versus *not* mutants. I am also interested in its PTMs because the PTMs are relevant to regulate Puf3 condensates that regulate mRNA translation of its targets.

I also prepared total, soluble and insoluble RNA samples from wild type, single and double *puf3Δ/ notΔ* mutants for analysis by RNA-Seq to evaluate RNA solubility and for sequencing of decay intermediates by 5'P-seq to evaluate dynamics of the last translating ribosomes. Finally, I tried to evaluate the localization and possible co-localization of Puf3 and Not proteins in cells.

Annexes (unpublished data)

Chapter 2-1 Determine whether Not proteins regulate the expression, PTMs, and stability of Puf3, and investigate the metabolic dependence of this regulation.

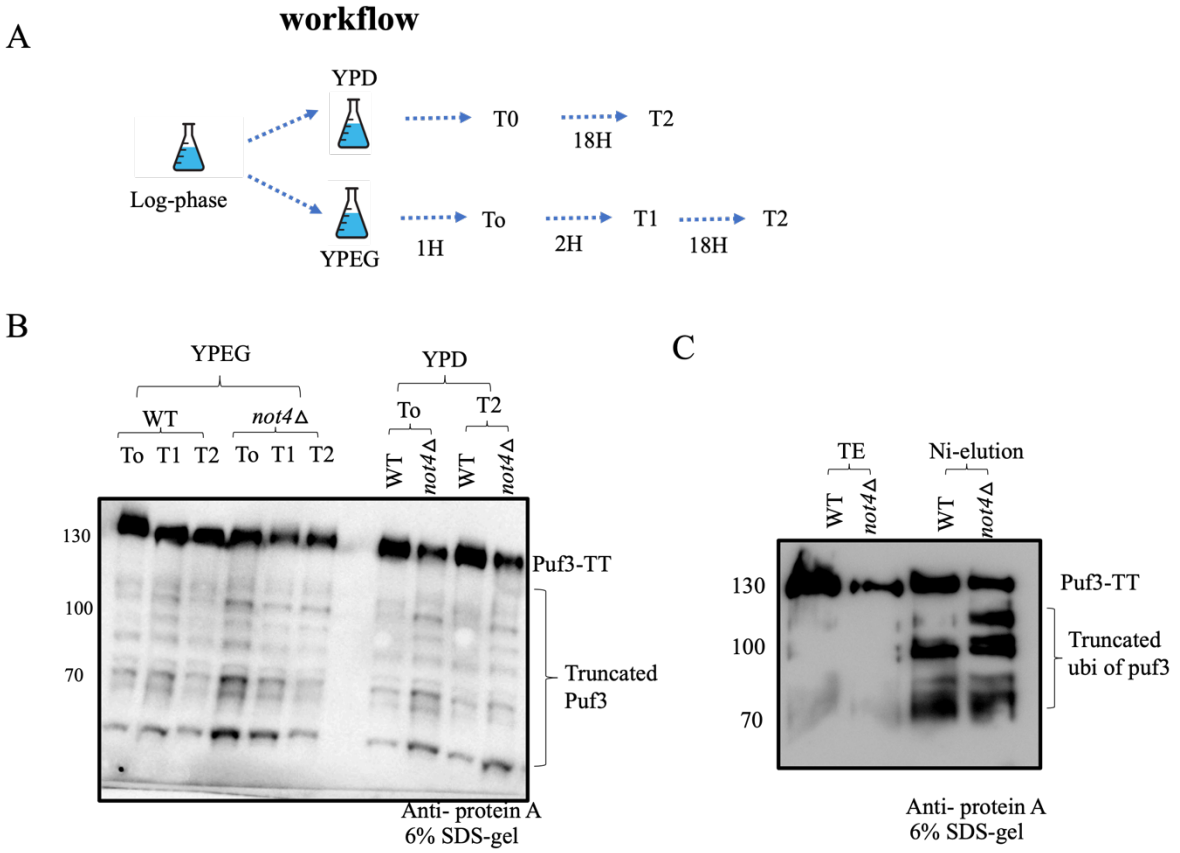


Figure 18. Puf3 expression levels appears to be independent of Not4 and metabolism but its integrity and modification status appears to be modified in the absence of Not4

(A)Cells expressing Puf3 with a tag for tandem affinity purification (TT) from its endogenous locus and promoter were collected after being grown in YPD (yeast extract peptone dextrose) early log phase during fermentation and after the diauxic shift to respiratory growth. In YPGE medium cells grow continuously using respiration. Cell pellets were collected at the indicated time points as described in panel A. The expression of Puf3 was detected using anti-Protein A antibody in panel (B). The same cells analyzed in panel A were transformed with a plasmid expressing His6-tagged ubiquitin under the control of the *CUP1* promoter. Cells were grown in the presence of copper and total extracts (TE) and ubiquitinated proteins purified on a nickel column (Ni-elution) were analyzed by western blotting in panel C.

The data reveals that the overall expression level of Puf3 appears similar between wild-type (WT) and *not4Δ* in both YPD (fermentative) and YPEG (respiratory) growth conditions. Notably, truncated forms of Puf3 are detected in all strains and growth conditions. However, there is clearly more detected truncated Puf3 in the absence of Not4 under both growth conditions. This suggests that Puf3 is degraded in cells prior to extract preparation, more in the absence of Not4, but at the same time the CHX chase experiment does not really indicate that Puf3 is

overall unstable. Hence there may be a minor pool of Puf3 in cells that is unstable, and this pool is more pronounced if Not4 is absent.

Additionally, an interesting observation was made regarding the smear band of Puf3 at the T0 time point specifically in the YPEG growth condition. This smear band is suspected to represent phosphorylated Puf3, as previous studies have shown that Puf3 undergoes phosphorylation mediated by the casein kinase I protein Hrr25 during respiratory conditions. However, in the absence of Not4, the smear band was not observed, suggesting that Not4 may contribute to Puf3 phosphorylation.

Finally, the ubiquitination assay data suggests that Puf3 is indeed ubiquitinated, but for degradation, as truncated forms of Puf3 are the forms that are enriched after nickel affinity. Consistently with the idea of some amount of Puf3 that is less stable in the absence of Not4 suggested in panel B, there is in particular one form of truncated ubiquitinated Puf3 that appears to be more abundant in the absence of Not4 (panel C). Notably, the ubiquitination of Puf3 for degradation is independent of Not4.

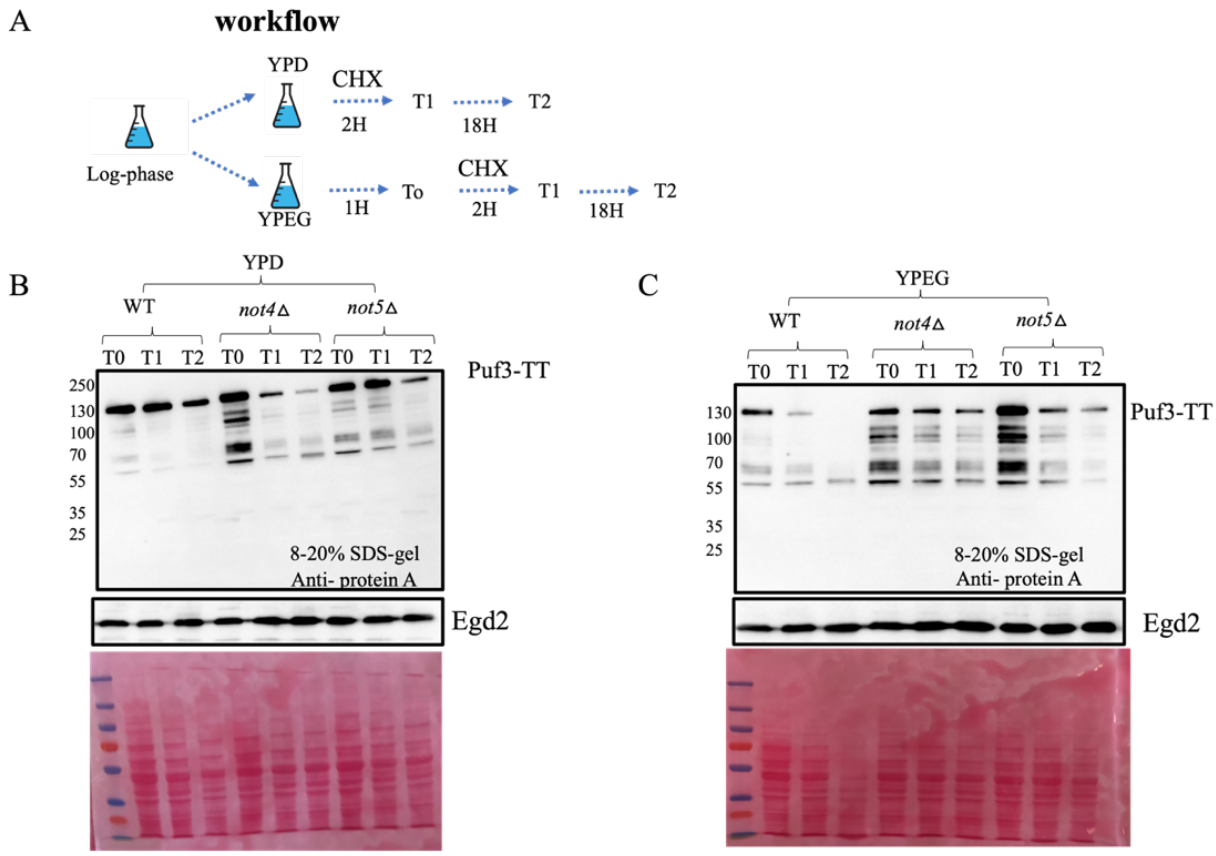


Figure 19. During fermentation, Puf3 has a longer lifespan that during respiration. Notably, during fermentation Puf3 has a shorter lifespan in *not* mutant strains compared to the wild type, while inversely during respiration, Puf3 exhibits a longer lifespan in the mutants.

(A). Description of the workflow for the experiment analyzed in panel B. Cells were grown in the indicated media, and during the log phase, cells were divided into two groups. One group was collected as T0 and treated with 100 μg/ml CHX to block translation, followed by collection at indicated time points (T1 and T2) in YPD medium. The other group was shifted to YPEG medium, and 1 hour later, cells were collected as T0 and treated with 100 μg/ml CHX, followed by collection at indicated time points (T1 and T2) in YPEG medium. (B) and (C). The expression of Puf3 with a tag for tandem affinity purification (TT) from its endogenous locus and promoter was detected using an anti-Protein A antibody in WT and Not4 or Not5-deleted strains grown in YPD or YPEG medium as indicated. The blots revealed with antibodies to Egd2 and the Ponceau stain were shown as loading control.

The results demonstrate again that there are increased truncated forms of Puf3 in the absence of Not proteins compared to the WT in both growth conditions. A very interesting finding is that the stability of Puf3 is different in different metabolic growth conditions and inversely regulated in wild type and in cells lacking Not4 or Not5. In wild type cells, during fermentation, Puf3 has a longer lifespan than during respiration, and during fermentation Puf3 has a shorter lifespan in *not* mutant strains compared to the wild type, while inversely during respiration, Puf3 exhibits a longer lifespan in the mutants. It will be interesting to further explore the ubiquitinated form of Puf3 in different growth conditions to confirm this by following ubiquitination of Puf3 responsible for its turnover. Importantly, the different phosphorylation evens of Puf3 that are associated with turnover will be important to investigate in wild type and mutants. Finally, it will be important to investigate how the presence of condensate pools of Puf3 is related to the turnover and phosphorylation changes of Puf3 (cause or consequence).

Because of this last point, we next focused on the sedimentation pattern of Puf3.

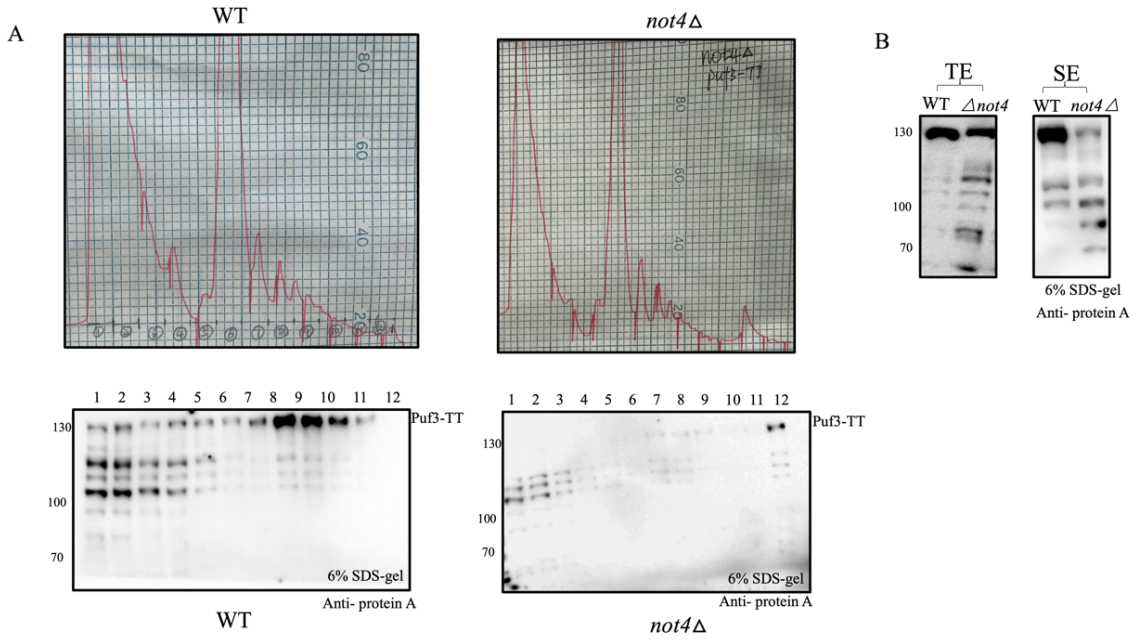


Figure 20. Puf3 is present in lower amounts in soluble extracts in *not4Δ*, but tends to be present in heavier fractions after sucrose gradient sedimentation

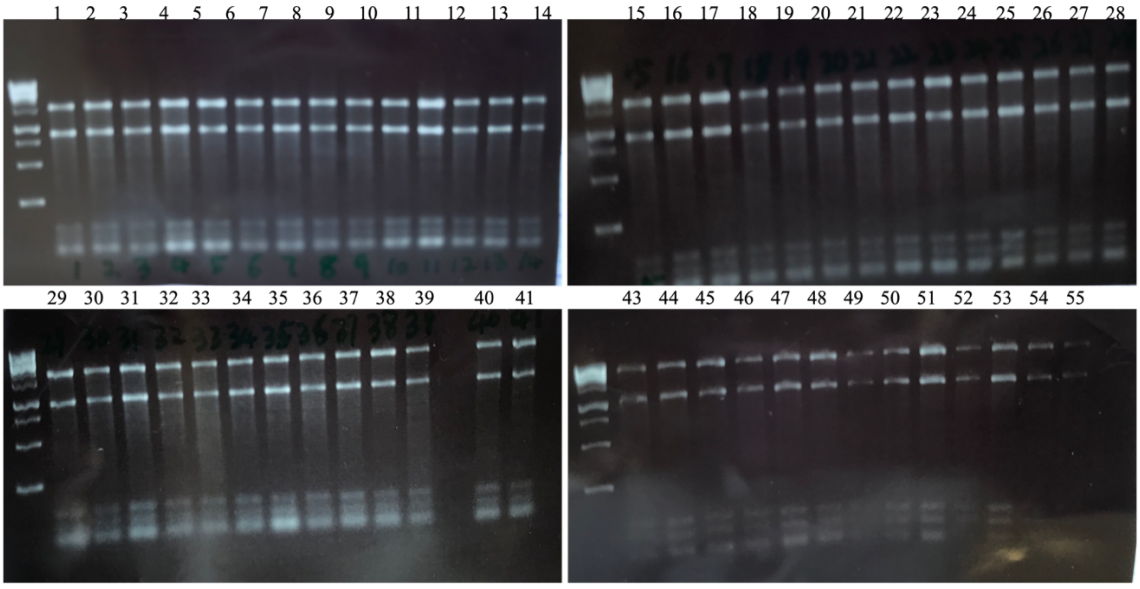
(A). Polysome profiles were obtained by sucrose gradient (10-50%) sedimentation from wild-type (WT) and Not4-deleted cells (*not4Δ).* The top panel shows on the y axis the A260 reading for the different fractions collected corresponding to the top (left) to bottom (right) of the gradient. The bottom panel shows the immunoblot analysis using anti-Protein A antibodies from all collected fractions shown in panel A. (B). Proteins extracted by post alkaline lysis (TE, left panel) and proteins extracted by bead beating and lysis buffer for the polysome profiling shown in panel A (SE, right panel) were analyzed by western blotting with anti-Protein A antibodies.

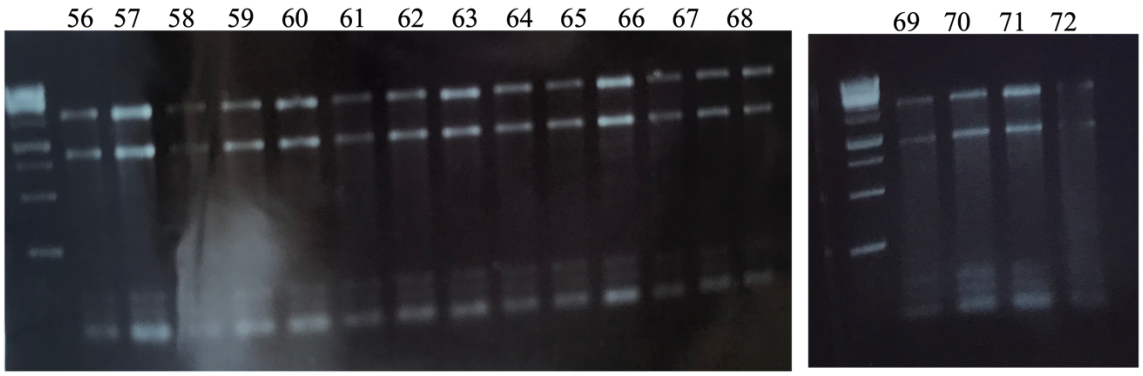
The abundance of Puf3 in the soluble extract from *not4Δ* was dramatically reduced compared to the other conditions, further supporting the idea that Not4 is important for Puf3 stability and further indicating that this is amplified upon cell lysis in extracts. In addition, more truncated Puf3 was detectable in the free mRNA or light polysome fractions in both WT and *not4Δ*, indicating that the stability of Puf3 may depend upon its translation status or presence in heavy condensates. What's more is that Puf3 was present in the heaviest fraction from the sucrose gradient in *not4Δ*, regardless of its decreased abundance is the soluble extract. This may represent the remaining condensate Puf3.

All of these exciting results need to be confirmed and consolidated.

Chapter 2-2. Investigate whether the Not proteins alter the solubility and translation of Puf3 target mRNAs and determine the role of Puf3 in the regulation of mRNAs by the Not proteins.

I generated double mutants of *not5* and *puf3* and extracted total, soluble, and insoluble mRNA from WT, *puf3Δ*, *not4Δ*, *puf3Δ not4Δ*, *not5Δ*, and *puf3Δ not5Δ*, strains in fermentation and respiratory conditions. I sent the 72 samples to our collaborators (laboratory of Vicent Pelechano) for RNA-seq and 5'P-Seq and the data is currently being analyzed by our bioinformatician Georges Allen. The concept behind extracting RNAs from the insoluble pool in indicated stains is based on the hypothesis that certain mRNAs targeted by Puf3 tend to associate with insoluble pools, possibly forming condensates or attaching to mitochondria. Additionally, we suspected that the solubility of these Puf3 target mRNAs could be regulated by Not proteins. For instance, Not proteins may participate in the formation or dissolution of Puf3 condensates, potentially competing or interacting with specific factors involved in Puf3 condensation. This competition or interaction could influence the dynamics of Puf3 condensation and subsequently impact the translation status of its target mRNAs. To gain insights into the behavior and regulation of Puf3 target mRNAs, we therefore plan to isolate RNAs from the different fractions. By studying the RNA content and translation status in the different pools, we aim to elucidate how Puf3-mediated condensation and interaction with Not proteins might affect the localization and translation of its target mRNAs.





Decode the numbers						
	YPD			YPEG		
	Total RNAs	soluble RNAs	insoluble RNAS	Total RNAs	soluble RNAs	insoluble RNAs
WT-1（3415）	1	2	3	4	5	6
WT-2（3415）	7	8	9	10	11	12
WT-3（3415）	13	14	15	16	17	18
puf3Δ-1（8058）	19	20	21	22	23	24
puf3Δ-2（8058）	2	26	27	28	29	30
puf3Δ-3（8058）	31	32	33	34	35	36
puf3Δ not4Δ-1（12455）	37	38	39	40	41	42
puf3Δ not4Δ-2（12455）	43	44	45	46	47	48
puf3Δ not4Δ-3（12455）	49	50	51	52	53	54
puf3Δ not5Δ-1（14146）	55	56	57	58	59	60
puf3Δ not5Δ-2（14146）	61	62	63	64	65	66
puf3Δ not5Δ-3（14146）	67	68	69	70	71	72

Figure 21. Quality of extracted RNAs assessed by visualization on a 2% agarose gel

Total RNA, soluble RNA, and insoluble RNAs were extracted from four different strains: wild type (3415), *puf3Δ* (8058), *not4Δ puf3Δ* double deleted strain (12455), and *not5Δ puf3Δ*

(14146). The quality of the extracted RNAs was assessed by running a 2% agarose gel. The table provides a detailed explanation of the RNA represented by the numbers in the picture.

The quality of the samples for both the 5'P-Seq and RNA-Seq has been validated. Unfortunately, I will not have enough time before the end of my PhD to follow up with this analysis and design experiments to validate what findings emerge.

Chapter 2-3. Investigate whether there is any colocalization of Not proteins with Puf3.

I have successfully created a Puf3-mScarlet reporter construct and validated it through sequencing (**Figure 22)**. The next step will be to transform this reporter construct into cells where endogenous Not proteins are tagged with GFP. This will enable us to visualize the location of Puf3 and the Not proteins using confocal microscopy. Unfortunately, I have not had the time to follow and use this reporter myself during my PhD.

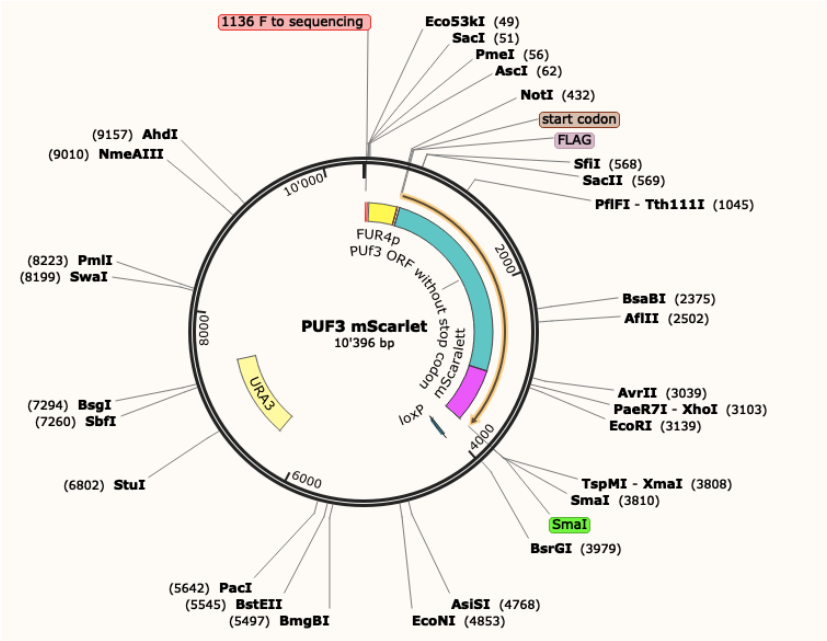


Figure 22. Circular map of Puf3-mScarlet report construction

The open reading frame of Puf3 in green was inserted into the report with heterologous promoter at the N-terminal in yellow and fused with mScarlet in purple to visualize the location by fluorescence microscopy.

3. Materials and methods for annexes

Yeast strains and plasmids

The strains, oligos, plasmids and antibodies used in this chapter2 are listed in **Table 3**.

Yeast strains were grown YPD medium (1% yeast extract, 2% peptone and 2% glucose). When cell reach to log phage they were then spun down, washed with water, and re-suspended in the same volume of YPGE (1% yeast extract, 2% peptone, 3% glycerol and 2% ethanol). Samples were collected at indicated times. To arrest protein synthesis a stock solution of cycloheximide (CHX) was added to a final concentration of 0.1 mg/ml in the growth medium.

The reporter plasmid, pMAC1421, was created by cloning a PCR fragment obtained from genomic DNA. The PCR fragment was generated using oligos 1285 and 1286 and then digested with EcoRI and Not1. The recipient vector, PMAC1377, was also digested with EcoRI and Not1. The digested PCR fragment was inserted into the digested PMAC1377 vector. To confirm the successful construction of the plasmid, sequencing was performed using oligos 1293, 1294, and 1295. To delete the *NOT5* genes in strain 8058, a homologous recombination approach was employed following standard procedures. Paired oligos Not5 5' and V4 were used to amplify the Nourseothricin N-acetyl transferase (NAT) cassette from the 5673 strain. The amplified NAT cassette was transformed into the 8058 strain to replace the *NOT5* genes via homologous recombination. The transformed strains were then validated using oligos F1 and R1 specific to the NAT cassette.

Protein extracts, SDS PAGE and Western blotting

Total protein extracts were prepared from yeast cells by incubating the pelleted cells in 0.1 M NaOH for 10 minutes at room temperature. After a quick spin in a microfuge, the cell pellet was resuspended in 2X sample buffer (post alkaline lysis). Soluble proteins were extracted by resuspending the cell pellet in a buffer containing 20 mM HEPES (pH 8.0), 50 mM KCl, 10 mM MgCl2, 1% Triton X-100, 1 mM DTT, 1 mM PMSF, and a protease inhibitor cocktail. The cells were then broken by adding 0.2 ml of glass beads to 0.4 ml of buffer and vertexing for 15 minutes at 4°C. After a brief centrifugation, the supernatant, containing the soluble proteins,

was collected. The collected sample was mixed with 2X sample buffer. The samples were then subjected to SDS-PAGE and western blotting using standard procedures.

RNA preparation

Total RNA extraction was performed following the previously described acid phenol method[175]. The procedure involved the extraction of soluble RNA in the presence of 400 µl of lysis buffer containing HEPES (20 mM), KCl (20 mM), MgCl2 (10 mM), Triton X-100 (1%), PMSF (1 mM), DTT (1 mM), and protease inhibitor (1X). To initiate the extraction, the cell pellet was resuspended with the addition of 200 µl glass beads and vortexed strongly for 15 minutes at 4 degrees. After a short centrifugation, the supernatant, containing the soluble RNA, was carefully transferred to a new Eppendorf tube and one volume of acid phenol was added, while the debris containing the insoluble RNA was then treated as for total RNA extraction by resuspension in TES buffer (10mM Tris-Hcl, 10mM EDTA,and 0.5% SDS) and additing one volume of acid phenol. The subsequent steps for handling the soluble, insoluble and totals RNA were the same.

Protein ubiquitination assay

A plasmid expressing 6His-tagged ubiquitin under the control of the inducible *CUP1* promoter was transformed into cells. The transformants were cultured in medium selective for plasmid maintenance in the presence of 0.1 mM $CuSO_4$. 100 OD_{600} of cells were harvested when they reached late exponential phase. Cell pellets were weighed and resuspended with G-buffer (100 mM sodium Pi, pH 8.0, 10 mM Tris-HCl, 6 M guanidium chloride, 5 mM imidazole, 0.1% Triton X-100) at 100 mg/ml. 0.6 ml of glass beads was added and cells were disrupted by bead beating for 15 min at room temperature. Following centrifugation, 20 µl of the supernatant was taken as total extract (TE), and 700 µl of the supernatant was mixed with 30 µl of nickel-nitrilotriacetic acid-agarose (Ni-NTA, Qiagen) for 2 h at RT with mild rotation. U-buffer (100 mM sodium Pi, pH 6.8, 10 mM Tris-HCl, 8 M urea, 0.1% Triton X-100) was used to abundantly wash the Ni-NTA-agarose to which ubiquitinated proteins were bound. SB was added directly to the Ni-NTA with the ubiquitinated proteins for analysis by western bloting with relevant antibodies.

Table 3. The plasmids, primers, strains, and antibodies used in the second chapter are listed.

Plasmid number	Description	Reference
pMAC1421	FUR4p-PUF3-mscarlett	Collart lab
pE298	CUP1 p-UBI-6His	Dargemont lab

primer number	Sequence	Reference
Not5 5’	CGACAATCCACCAATTCTAACTAC	Collart lab
Not5 V4	GGGGAAAGCCCTGATCATCG	Collart lab
NAR F1	TATTTTTTATTGATTGCATGAAACATCCGCTCATTCTGTCCAGCTGAAG CTTCGTACGC	Collart lab
NAR R1	GTAAATCACGATGAGAATTATATAAGTAAAAGGAAACTGTGCATAGG CCACTAGTGGATCTG	Collart lab
1293	ACAATAACTATCATACTCATCATACTGCGGC	This study
1294	AATAACAAGAATCATCCTGCAAATAACTCCAAC	This study
1295	GTCGAATACTCTAAACATAAATTTGCCTCCAAC	This study

strain number	Genotype	Reference
8057	*MATa ade2 arg4 leu2-3,112 trp1-289 ura3-52 puf3::PUF3-Taptag-URA3*	Euroscarf SC1249
3415	*MATα his3 leu2 ura3 lys2*	Euroscarf
8058	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 puf3::KanMX4*	Euroscarf Y11501 BY4742
12357	*MATa ade2 arg4 leu2-3,112 trp1-289 ura3-52 puf3::PUF3-Taptag-URA3 not4::NATMX4*	Collart lab
12358	*MATa ade2 arg4 leu2-3,112 trp1-289 ura3-52 puf3::PUF3-Taptag-URA3 not5::NATMX4*	Collart lab
14521	*MATa ade2 arg4 leu2-3,112 trp1-289 ura3-52 puf3::PUF3-Taptag-URA3 pCUP1p-UBI-6His-LEU2*	This study
14530	*MATa ade2 arg4 leu2-3,112 trp1-289 ura3-52 puf3::PUF3-Taptag-URA3 not4::NATMX4 pCUP1p-UBI-6His-LEU2*	This study
14146	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 puf3::KanMX4 not5::NATMX4*	This study
5673	*MATα leu2Δ20 ura3Δ met15Δ his3Δ1 not5::NATMX4 lys2 ARG4*	Collart lab
14544	*MATa ade2 arg4 leu2-3,112 trp1-289 ura3-52 puf3::PUF3-Taptag-URA3*	Collart lab
12455	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 puf3::KanMX4 not4::NATMX4*	Collart lab

Antibodies	Reference
PAP（Peroxidase Anti Peroxidase）	Sigma
Egd2	Panasenko et al., 2009

IV. DISCUSSION

In recent decades, significant progress has been made in understanding the localization of mRNAs near mitochondria and the regulatory factors involved. Localization of mRNAs to mitochondria can occur through translation-dependent mechanisms, involving various molecular components such as mitochondrial targeting sequences (MTS), chaperones (NAC), and mitochondrial outer membrane receptors (Tom20, Om14, Sam37). Alternatively, localization can also be achieved through translation-independent mechanisms, mediated by mRNA-binding proteins (Puf3). However, the precise molecular mechanisms underlying mRNA localization to mitochondria and its functional roles are still the subject of investigation.

My doctoral research investigates the involvement of the Ccr4-Not complex in the regulation of nuclear DNA-encoded mitochondrial mRNAs. It reveals that this role is related in part to its importance for the localization of mRNAs to the surface of mitochondria, expanding the current understanding on the mRNA localization and its role in regulation of gene expression. My research explores two distinct mechanisms. In Chapter 1, it focuses on translation-dependent pathways that involve the MTS, mitochondrial outer membrane receptors, and the nascent-polypeptide associated complex. In Chapter 2, it explores the interaction between the Ccr4-Not complex and a key mRNA-binding protein Puf3.

In chapter I, I describe our discovery of a complex and highly integrated mitochondrial co-translational quality control mechanism that we have named MitoENCay. This mechanism plays a role in preventing the overproduction of nuclear-encoded mitochondrial mRNAs and proteins. We discovered it studying specifically a *MMF1* reporter. A critical aspect of MitoENCay is the requirement for ribosome pausing during translation of *MMF1*, which provides sufficient time for proper mitochondrial targeting.

1. Co-translational mitochondrial targeting and import

To invest the translation-dependent pathway we employed reporter constructs, with a copper inducible promoter and a heterologous 3’ untranslated region from *ADH1* to avoid any possible impact of the *MMF1* 3’UTR that could provide translation-independent effects

or mask translation-dependent effects. We cloned different mitochondrial open reading frames fused to a flag tag to follow the proteins in this reporter construct. In our study we identified that the mitochondrial matrix protein Mmf1 undergoes co-translational import, whilst Cox4, a protein that ultimately locates in the inner membrane of mitochondria, does not follow the same mechanism. Both proteins are encoded by nuclear DNAs and with an N-terminal cleavable targeting sequence, but the targeting sequence is different. Mmf1 is a homotrimeric protein involved in maintenance of mitochondrial DNA stability and the transamination of isoleucine. Cox4 is subunit IV of cytochrome c oxidase, responsible for the transfer of electrons from cytochrome c to oxygen. We concluded that Mmf1 but not Cox4 is co-translationally imported based on several observations. First, the majority of overproduced Mmf1 observed after 10 minutes of copper induction is in its cleaved mature form, whereas the majority of overexpressed Cox4 at the same time point remains in its precursor form **(Figure 1B).** This suggests that Mmf1 is processed more rapidly than Cox4, indicating a co-translational import pathway that saves time during protein maturation. Second, we observed that in mutants lacking components of the co-translational import machinery, specifically the chaperone Egd1 (a subunit of NAC) and proteins involved in docking NAC at the outer membrane (Om14, Om45, or Sam37), less Mmf1 precursor is processed compared to the situation in wild type cells or in the absence of Ssa1, a subunit of Hsp70, the chaperone involved in post-translational import [48, 187] (**Figure 1D, S1C** and **S4B).** The decreased maturation of Mmf1 in mutants lacking components of the co-translational import machinery suggests a slowdown in Mmf1 import speed, supporting the notion that Mmf1 undergoes co-translational import. In contrast, the processed form of Cox4 did not show obvious differences in all mutants compared to the wild type, indicating that the transport of Cox4 is independent of the co-translational import machinery and may undergo post-translational import **(Figure 1D S1C** and **S4C**). Thirdly, ribosome profiling data revealed that *MMF1* mRNA exhibits obvious ribosome pausing at amino acid positions 93 and 94, indicating the occurrence of co-translational events during its translation elongation, likely associated with co-translational import. In contrast, Cox4 did not display any obvious ribosome pausing during translation, further supporting their different import mechanisms (**Figure 4A**). Fourth, imaging data provides clear evidence of colocalization between *MMF1* mRNA and mitochondria, revealing additional support for the notion that Mmf1 undergoes

co-translational import (**Figure 7B)**. It worth to notice that Mmf1 protein, whether expressed from endogenous locus or reporter construct, remains stable even after 18 hours of cycloheximide (CHX) treatment, which blocks translation and allows assessment of protein stability. Conversely, Cox4 is an unstable protein that is degraded within 2 hours of production. This instability probably explains the lack of detectable Cox4 before copper induction despite the fact that the copper promoter is somewhat leaky (**Figure S1C** and **Figure 14C).** In essence this observation might indicate that Mmf1 is more likely to accumulate and cause proteotoxic stress compared to Cox4 and it would be more important to have its production depend upon a mechanism that makes sure it gets to mitochondria to be imported, or if in excess gets degraded, compared to Cox4.

Curiously, previous genome-wide studies have shown that *MMF1* is not typically localized to the mitochondria under physiological conditions when expressed from its endogenous locus in glucose[37, 41]. However, our current study focused on investigating how cells respond to the presence of additional *MMF1* mRNA overexpressed from an episome. It is possible that co-translational targeting of *MMF1* mRNA to the mitochondria occurs only when there is an excess of *MMF1* mRNA, or more likely that this targeting is only detectable under these conditions. The transient nature of the interaction between *MMF1* mRNA and the mitochondrion might make it difficult to observe under normal physiological conditions.

Previous studies have provided evidence that proteins imported co-translationally tend to localize to the inner membrane of mitochondria, specifically components involved in the electron respiratory chain[48]. This observation is not surprising as these nuclear-encoded proteins require interaction with mitochondrial DNA-encoded proteins to form functional complexes. The co-translational import mechanism ensures the synchronized assembly of protein complexes during translation, promoting their proper integration into the inner membrane. Interestingly, Mmf1 has been proposed to form a protein complex, with one Mam33 trimer and two tandem Mmf1 trimers arranged in a head-to-tail fashion[188]. The binding interfaces between these trimers are predominantly driven by complementary electrostatic interactions, specifically facilitated by negatively and positively charged patches. These findings suggest that co-translational import might be involved in facilitating co-

translational assembly processes. This is particularly interestingly considering the previously discussed roles of Not proteins in co-translational assembly. We examined the native status of Mmf1 (**Figure 3C)**. Indeed, we observed the presence of faster migrating Mmf1 complexes in mutant cells. This observation may reflect either the absence of its partner protein Mam33 or ineffective complex assembly in the absence of Not4. This can in the future be further confirmed by conducting experiments in cells lacking Mam33 will indicate whether the slower migrating (heavier and larger) Mmf1 complexes are indeed complexes with Mam33. In addition, supplementation experiments by introducing additional Mam33 in the absence of Not4 will allow us to determine whether the faster migrating Mmf1 complexes can be rescued. If the rescue is not successful, it strongly suggests Mmf1 co-translational import may couple co-translation assembly in the control of Not proteins.

It is intriguing to note that genes encoding mRNAs targeted to mitochondria, besides forming protein complexes, can also be of ancient bacterial origin. Notably, genes such as *FUM1* and *MDH1* have been identified as ancient bacterial genes and similarly to *MMF1*, their expression is dependent on the NAC[179]. These observations raise the possibility that *MMF1* may also be an ancient bacterial gene.

At present we have no evidence for the co-translational import of Cox4, and previous studies have shown that a few other Cox subunit mRNAs are present in the cytoplasm[189, 190]. Further investigations will be needed to elucidate its specific import pathway.

2. The MTS contributes to *MMF1* mRNA mitochondria targeting and repression of its overexpression

The role of the mitochondrial targeting sequence (MTS) in mitochondrial protein targeting has been well-established in previous research. The MTS serves as a signal that directs nascent chains to the mitochondria. It has been shown that NAC associates with nascent chains at the exit tunnel of cytosolic ribosomes, facilitating their mitochondria targeting. The presence of NAC enables the segregation of nascent chains from the cytosol until they reach a length of approximately 30 amino acids, thereby preventing degradation and misfolding [191,

[192]. Recent research has provided additional insights by showing that NAC directly interacts with a positively charged amino acid cluster in the MTS of specific mitochondrial precursors, such as Oxa1 and Fum1[193]. Furthermore, upon NAC association with the nascent chain, this nascent chain can be docked to the mitochondria outer membrane via outer membrane proteins like Om14 or Sam37 and this in turn may facilitate the transfer of the nascent chain to the major receptor of the import channel, Tom20. Tom20, a 20-kDa subunit of the translocase of the outer mitochondrial membrane complex, acts as a crucial receptor for precursor proteins synthesized in the cytosol and targeted to the mitochondrial matrix and inner membrane. Its specific interaction with the N-terminal sequences of precursor enhances their efficient import into mitochondria. Structurally, the cytosolic domain of Tom20 adopts an α-helical conformation with a groove that accommodates the presequence peptide. Upon binding, the presequence peptide forms an amphiphilic helical structure where hydrophobic leucines are aligned on one side, facilitating their interaction with a hydrophobic patch in the Tom20 groove[194-197]. However, the precise mechanism by which NAC transfers the MTS to Tom20 has not yet been clarified.

In our study, we made several key observations regarding the role of the mitochondrial targeting sequence in the co-translational import and expression regulation of Mmf1. First, our data confirmed that the MTS of Mmf1 contributes to its mitochondrial targeting, as evidenced by a reduced localization of *MMF1* mRNAs to mitochondria in its absence in our imaging experiments **(Figure 7B** and **7C).** Furthermore, in the absence of the MTS, we did not detect Mmf1 in isolated mitochondrial fractions. Additionally, we made an intriguing discovery that there is an accompanying increase in both mRNA and protein levels when Mmf1 loses its MTS. This is consistent with increased Mmf1 in mutants of other co-translational machinery components, such as the chaperone Egd1, as well as docking sites Om14, Om45, Sam37, and Tom20. These findings provide strong evidence supporting that the co-translational import machinery and co-translation import itself are necessary to limit Mmf1 overexpression. This observation indicated that mRNA targeting to mitochondria co-translationally allows to repress the expression, potentially through mRNA instability. Previous studies have suggested a correlation between mRNA localization to mitochondria and mRNA instability. This was suggested to occur in a translation-independent and Puf3-

dependent manner. Our study supports the new notion that mRNA localization to mitochondria can be associated with mRNA instability and be translation dependent.

It is worth to note that the MTS of *COX4* promotes the expression of Mmf1, whereas the MTS of *MMF1* represses the expression of Cox4 **(Figure 2C** and **2D).** This highlights that the mechanism is MTS-specific, raising the intriguing question of which features of MTS determine mRNA stability. Recent advancements in bioinformatics have significantly advanced our understanding of MTS features. For example, a global analysis of the mitochondrial N-proteome has revealed that approximately 60% of mitochondrial proteins are synthesized with a cleavable MTS[37]. These MTS sequences exhibit variable lengths, typically ranging from 15 to 50 amino acid residues. One important characteristic of mitochondrial MTS sequences is the formation of an amphipathic α-helix, which contains a positively charged face and a hydrophobic face[198]. However, due to the eventual release and degradation of MTS during protein maturation, gaining a precise understanding of MTS structure remains challenging. Furthermore, it is worth exploring whether all MTS sequences are capable of targeting proteins to the import machinery. If so, then the differences between MTS sequences could lie in their ability to bind to Om14 or Sam37, or in their capacity to interact with NAC and Not4. It is possible that certain MTS sequences can induce ribosome stalling downstream, while others cannot. Additionally, the ability of an MTS sequence to bind NAC may facilitate the recruitment of Not4, enabling the association of Not5 with the translating ribosome and causing pausing at downstream sequences. However, all of these ideas still require further research and investigation.

Further research and technological advancements are required to comprehensively unravel the structural properties of MTS and gain more precise insights into their roles in protein targeting and mitochondrial function. Such knowledge will not only deepen our understanding of mitochondrial biology but also pave the way for the development of targeted approaches for studying and manipulating mitochondrial proteins in various cellular processes and diseases.

3. Ribosome pausing contributes to mRNA membrane targeting and repression of its overexpression

Ribosome pausing is a phenomenon where ribosomes temporarily halt or stack during mRNA translation. This phenomenon is observed in both eukaryotes and prokaryotes and has significant implications for gene expression and protein synthesis. The development of the advanced technique called ribosome profiling, which involves sequencing ribosome-protected fragments (RPFs) to map ribosome occupancy on mRNAs, has enhanced our understanding of the location and timing of ribosome pauses and their regulation. Ribosome pausing can be caused by various factors, including mRNA sequence and structure, tRNA availability, and the nascent peptide being synthesized. It is regulated by multiple factors and can have both beneficial and restrictive effects on translation. For example, eIF5A has been identified as a ribosomal pausing inhibitor that promotes the efficiency of translation initiation and elongation, possibly through with an involvement in the hypusine modification pathway[199, 200]. On the other hand, Not5 has been proposed as a ribosomal pausing promoter. Its N-terminus interacts with the E site of the ribosome when the A site is empty more likely to occur when it is occupied by a non-optimal codon, maybe contributing to maintain ribosome pausing[121]. Recently, our laboratory has proposed that the interaction between Not5 and the ribosome forms a membrane less condensate, which can exclude eIF5A and maintain ribosome pausing[151]. However, this mechanism requires further clarification and confirmation. The consequences of ribosome pausing has been considered for co-translational events such as protein folding, protein targeting, and protein interactions.

In our study, we observed significant ribosome pausing on the *MMF1* mRNA. This ribosome pausing could be influenced by two potential factors: codon optimality or/and the targeting of the mRNA to the mitochondria. It is plausible to consider that when the MTS of Mmf1 guides the mRNA to reach the surface of mitochondria, this may slow down the movement of ribosomes, leading to ribosome pausing, or vice versa. Ribosome pausing may provide time to reach the mitochondria before translation is completed. To investigate the influence of codon optimality, I performed codon usage optimization experiments. Based on the tRNA adaptation index analysis, which takes into account the efficiencies of various wobble

interactions, we focused on the major pausing site of *MMF1* spanning codons 92 to 95. Interestingly, we found that the middle two codons within this pausing site were optimal, while the last codon was non-optimal [201] (**Figure 4C).** This differs with the previously identified DP (Asp-Pro) codon pair, which contributes to pausing on *RPT1* mRNA for co-translational assembly of proteasome[202]. Obviously, ribosome pausing as a regulatory mechanism is not limited to a single mechanism. Different codon contexts can elicit varied outcomes, indicating the complexity of ribosome pausing regulation. It is also possible that the consequences of ribosome pausing are not solely determined by the different codons themselves, but rather by the nature of the mRNA and the protein it encodes.

Another interesting observation in our study is that the ribosome pausing on the *MMF1* mRNA is dependent upon Not4. We noticed a decrease in ribosome footprints specifically after the major pause site but not before, in the absence of Not4, indicating its involvement in maintaining ribosome pausing **(Figure 4A** and **4B).** This finding aligns with the concept of Not5, which interacts with the E site of the ribosome when the A site is a non-optimal codon and is in turn involved in maintaining ribosome pausing. This leads us to question why ribosome is paused on *MMF1* needed and what are the downstream events. A study enlightens us. This study suggests that CNOT1 is required for the proper targeting of mRNAs to the ER in human cells[203]. Depletion of CNOT1 leads to the accumulation of ER-targeted mRNAs in the cytoplasm instead of their delivery to the ER. Ribosome profiling analysis has revealed that ER-targeted mRNAs exhibit ribosome-protected fragments that cover the initial section of the mRNA, coinciding with the signal recognition motif (SRM). Investigations utilizing disome sequencing, which examines collided ribosomes, have shown an accumulation of disomes over SRMs. This finding suggests a potential mechanistic link between the Ccr4-Not complex involved in mRNA decay and ER targeting. When we consider these data collectively, it suggests a plausible model in which ribosome pausing on the *MMF1* mRNA contributes to its targeting to the mitochondria in a Not4-dependent manner. Collectively, it is plausible to consider that ribosome pausing may play a role in mRNA membrane targeting not only in mitochondria and the ER but also to other compartments such as the nucleus, vesicles, and peroxisomes. This process is likely regulated by the Ccr4-Not complex as discussed above. It worth and possible to investigate this hypothesis using

the technique called proximity-specific ribosome profiling in future work. In this approach, a specific membrane protein is fused to a biotin ligase called BirA, while cytosolic ribosomes contain a biotin target sequence called AviTag. By adding biotin to the growth medium, ribosomes near the membrane protein can be specifically tagged with biotin. These tagged ribosomes can then be isolated and their protected mRNAs can be identified through deep sequencing. By employing this approach, we can gain insights into ribosome-membrane interactions and identify the specific mRNAs that are targeted to different cellular membranes, and how this changes upon mutant Ccr4-Not function. This technique offers a powerful tool to study the role of ribosome pausing in mRNA membrane targeting and further investigate the regulatory mechanisms involving the Ccr4-Not complex.

Based upon our findings that ribosome pausing is necessary to repress Mmf1 overexpression, it is worth noting that ribosome pausing can also act as a signal for mRNA degradation and the degradation of truncated nascent chains[204]. In light of this, we propose that the ribosome pausing observed on the *MMF1* mRNA provides enough time for proper targeting to the mitochondria. Once targeted, excess levels of Mmf1 can be addressed by the existing mitochondrial-associated quality control pathway.

4. MMF1 mRNA mitochondrial targeting activates a mitochondria-associated integrated quality control mechanism

The results of our study strongly suggest the presence of a mitochondrial-associated quality control mechanism that regulates the levels of Mmf1 to avoid its overexpression. When *MMF1* mRNA targeting to the mitochondria is impaired, for example in the absence of MTS or components of the co-translational machinery, this leads to excessive accumulation of Mmf1. This suggests the involvement of a regulatory system handling the overproduction of Mmf1 at mitochondria. Ribosome-associated quality control and No-go Decay (NGD) pathways, caused by ribosome pausing and collisions, are involved in handling *MMF1* overexpression. One idea is that ribosome pausing might increase at the mitochondrion when *MMF1* is overexpressed, leading ribosome collisions. This has not been demonstrated but would be interesting to investigate by performing ribosome profiling comparing

overexpressed *MMF1* with or without the MTS or with or without an optimized codon at the pause site. One previous study has highlighted the sensing mechanism at mitochondria when overexpressed mitochondrial proteins clog the import channel. This mechanism involves the outer mitochondrial membrane protein Cis1, which detects the clogged channel and recruits the ATPase Msp1. Msp1 assists in extracting the blocked mitochondrial proteins from the channel, leading to their degradation in the cytoplasm by the proteasome. This process helps reduce the burden on mitochondria[64]. In our data, we investigated the role of Cis1 and the mutant proteasome in the context of Mmf1 overexpression. We observed that in the absence of Cis1 and mutant proteasome, Mmf1 accumulation occurred, while the absence of Msp1 did not significantly impact Mmf1 levels **(Figure 6A, 6B, S2D,** and **S5B**). These findings suggest that Cis1 may serve as a common sensor for overproduced mitochondrial proteins, with downstream factors being variable depending on the specific stress conditions.

The data presented in our study uncovers an integrated mitochondrial-associated quality control mechanism relying on the intricate interplay between different quality control mechanisms, suggesting that the cell employs multiple pathways to ensure the elimination of overproduced Mmf1. The stress magnitude in determining the mode of quality control response is evident, as shown by the more pronounced role of Atg17 under copper induction when overexpression of Mmf1 was greater. The molecular interplay across the different quality control pathways remains to be further investigated.

5. Ccr4-Not complex limits *MMF1* overexpression in a multi-layer process

Mitochondrial targeting

As discussed above, our findings shown that *MMF1* mRNA ribosome pausing and mitochondrial targeting are essential for activating the integrated mitochondrial-associated quality control pathway, which limits Mmf1 overexpression. Our data also clearly demonstrate the involvement of the Ccr4-Not complex in multiple layers of regulation, involving specifically at least Not4 and Caf130. By comparing the *MMF1* mRNA ribosome footprints between wild type and Not4-deleted strains, we noted that in the absence of Not4, there was less pausing on *MMF1*, and in Not4-deleted strains there was also less *MMF1*

reporter mRNA targeting to mitochondria and subsequent decay, leading to the accumulation of Mmf1. This suggests that Not4 is necessary to maintain *MMF1* mRNA ribosome pausing, which provides the necessary time for mRNA targeting to mitochondria. One potential mechanism underlying this phenomenon may involve the interaction between Not5 and ribosomes when there is a non-optimal A site codon that remains empty long enough such that the tRNA also leaves the E site. It is plausible to assume that ribosomes at the non-optimal A site serve as targets for Not5 binding to slow down translation sufficiently to promote co-translational targeting of the mRNA to the mitochondria. Additionally, the increased Mmf1 levels in ubiquitination mutants of Rps7A and Rli1 **(Figure 5B** and **5C**) support the notion that Not proteins contribute to Mmf1 mitochondria targeting through the formation of condensates. Indeed, our previous studies have suggested that Not4 regulates the ubiquitination of Rps7A and Rli1, and thereby regulates the formation of condensates [151]. To gain further insights into this hypothesis, additional experiments, such as confocal microscopy, are needed to examine the condensation of Not4 and its localization, including whether it colocalizes with mitochondria in particular in presence of overexpressed *MMF1* reporter. Moreover, investigating whether the formation of these condensates is further regulated by Not4-ubiquitinated Rps7A and Rli1 would provide valuable information.

Another exciting unpublished observation is that Not4 has an impact on the expression and distribution of Om45, the partner of Om14, a mitochondrial docking site for NAC. The absence of Not4 led to increased Om45 expression, and its distribution tended to aggregate on the mitochondria outer membrane rather than being evenly distributed as observed in the wild type. In contrast, the expression and distribution of Atp5, a mitochondrial inner membrane protein, was independent of Not4 (**Figure 15).** These findings provide another additional possibility that Not4 regulates the co-translational machinery, thereby impacting *MMF1* mRNA mitochondria targeting. In any event, the interplay between NAC and the Ccr4-Not complex highlights the intricate regulatory network involved in modulating Mmf1 mitochondria targeting and expression.

The roles of Not4's ubiquitination

Over the past years, several substrates of Not4 ubiquitination have been identified, including NAC, Rps7A, Jhd2, Yap1, Srb10, and Rli1/ABCE1. Interestingly, the substrates exhibited differences in their stability upon ubiquitination by Not4. Jhd2, Yap1, and Srb10 are destabilized. However, NAC, Rps7A, and Rli1 remain stable even after ubiquitination. It is important to mention that all of the stable Not4 substrates are associated with the ribosome. One of the key findings is the essential role of Not4-mediated ubiquitination of Rps7A for the presence of Not5 in polysomes, highlighting its contribution to translation elongation. Rps7A is primarily detected in its ubiquitinated form in the 80S or polysome fractions, suggesting that it is ubiquitinated when 40S subunits are assembled with 60S subunits during ribosome assembly or it is de-ubiquitinated in 40S ribosomes. It has been proposed that the de-ubiquitination of Rps7A by Otu2 is required for translation re-initiation. The ubiquitination of Rps7A by Not4 regulates ribosome stalling and monoubiquitinated Rps7A can be further polyubiquitinated by the Hel2 ligase for ribosome quality control. Moreover, in flies, CNOT4 ubiquitinates the ribosome release ATPase ABCE1 (Rli1 in yeast) upon mitochondrial damage, leading to mitophagy initiation. Similarly, in yeast, Rli1 is also ubiquitinated by Not4. Overexpression of Rli1 affects translation through an R12 stalling sequence, indicating the involvement of Not4-mediated ubiquitination in co-translational events such as ribosome pausing. These evidence clearly show that Not4-mediated ubiquitination plays a critical role in co-translational quality control processes. Our data further support this notion as we notice that more Mmf1 was detected in the ubiquitination mutants of Rps7A and Rli1 (**Figure 5B** and **5C)**. Importantly, our data also finally evidences a role for NAC ubiquitination by Not4, and this for the first time despite it being the first identified substrate of Not4. Indeed, our data clearly indicates that the ubiquitination of Egd1 by Not4 is necessary to limit *MMF1* expression **(Figure 5A)**. The other subunits of the NAC complex, NAC α-subunit Egd2 and β-subunit Btt1, were less crucial in the regulation of *MMF1* expression. Deletion of all the NAC subunits, including Egd1, did not lead to further accumulation of Mmf1(**Figure S4A).** This is quite intriguing, as previous studies found mitochondrial mRNAs mainly enriched upon Egd2-ribosome enrichment. This could suggest different functions for NAC subunits. Egd2 may mainly contribute to mRNA targeting, while Egd1 may have a major role on mRNA quality control. This notion can be supported by a recent study that highlights the role of Btt1 (also a NAC β-subunit) in recruiting the Ccr4-Not complex, mediated by the yeast specific subunit

Caf130, for the co-translational decay of *RPL4* mRNA when the availability of its dedicated chaperone was limited [205]. Our data shows that Mmf1 is overexpressed in the absence of Caf130, emphasizing the importance of Egd1 ubiquitination by Not4, which is Caf130-independent (**Figure 5D).** Moreover, ubiquitination assays revealed that while ubiquitination of Egd1 was not dependent on Caf130, heavy polyubiquitination did, indicating that Caf130 contributes to Egd1 turnover **(Figure 5E**). These findings indicate that Caf130 stabilizes the interaction between NAC and the Ccr4-Not complex, allowing Not4 to ubiquitinate Egd1 and thereby limit *MMF1* expression. Considering that ubiquitination plays a crucial role in mRNA and protein degradation through pathways such as RQC, NGD, mitophagy, and autophagy[171, 206, 207], it is plausible to assume the involvement of Not4 in integrated mitochondrial associated quality control, in addition or together with its role in co-translational quality control pathways described earlier [208, 209].

As we discussed above, for RQC, Not4 monoubiquitinates the ribosomal protein uS7, and Hel2 subsequently adds polyubiquitin chains to it[171]. In flies, Not4 ubiquitinates ABCE1 to activate mitophagy and remove stalling ribosomes at the surface of mitochondria[78]. These findings connect different research, and all involve ribosome pausing and the Ccr4-Not complex, in particular Not4. Our research provides a potential explanation for the diverse studies mentioned above. Not4 might dynamically contribute to form and dissociate condensates, probably contributing to cycles of continuous ubiquitination and deubiquitination of regulatory factors affecting ribosome pausing and elongation. This dynamic process regulates the dynamics of translation elongation in a codon-dependent manner, as Not5 interacts with E site of ribosomes with empty A sites. Under conditions when the equilibrium of condensates is challenged, the function of ubiquitination rapidly adapts to respond to the changing pressure. For instance, monoubiquitination of ribosomal-associated proteins by Not4 may regulate the formation of condensates. However, upon prolonged challenge like prolonged ribosome pausing, monoubiquitinated Not4 targets can be rapidly polyubiquitinated by Hel2, leading to the degradation of specific mRNAs and associated proteins.

In summary, our research has provided a framework for understanding the role of Not4 in regulating translation elongation dynamics through ubiquitination events, and possibly the

dynamic formation and dissolution of condensates modulated by the ubiquitination processes. We propose that the switch between monoubiquitination and polyubiquitination of ribosomal proteins in response to overexpression stress conditions allows for rapid modulation of condensation and targeted protein degradation.

In addition to investigating the roles of Not proteins in regulating mitochondrial biosynthesis in a translation-dependent manner, my research also explored their involvement in mRNA mitochondria targeting independent of translation. Specifically, I focused on the interaction between Not proteins and the mRNA binding protein Puf3 in the second chapter**.**

I wanted to know if Ccr4-Not regulates the fate of mitochondrial mRNAs by interplaying with Puf3. Our previous RNA-Seq and SILAC data suggested that the Not4 and Not5 proteins did not significantly regulate the levels of Puf3 mRNA and nascent protein[175]. However, my experimental data shows that the stability of the Puf3 protein is significantly reduced in *not* mutants, especially during the logarithmic growth phase **(Figure 19)**. Additionally, the post-translational modifications of Puf3 are different in the *not4* mutant **(Figure 18 B).** Further experiments are needed to confirm that it is the phosphorylation level of Puf3 that is altered in the *not4* mutant. Moreover, a ubiquitin assay shows that the degradation of Puf3 depends on its ubiquitination but that it is independent of Not4 **(Figure 18 C).** In addition, Puf3 tends to be present in very heavy fractions of a sucrose gradient in the absence of Not4 **(Figure 20).** This seems consistent with previous research, as non-phosphorylated Puf3 leads to more accumulation of Puf3 condensation[56]. However, it is puzzling why Puf3 is unstable in extracts in the absence of Not4. This could be due to the increased ubiquitination level of Puf3 caused by the lack of Not4. However, it is not clear who mediates the ubiquitination of Puf3, and more experiments are needed to explore this. It could also simply be that Puf3 is not protected by interaction with other proteins, for instance if Not proteins impact the proteins with which Puf3 can associate or simply the ability of Puf3 to form condensates.

To further investigate how Not proteins and Puf3 shape the distribution of mitochondrial mRNAs and translation dynamics, we performed 5'P-seq on total RNA, soluble RNA, and insoluble RNA extracted from both the wild type, single and double mutant strains. 5'P-seq is

a powerful tool used to study mRNA degradation intermediates, which is crucial for understanding 5'-3' co-translational mRNA decay and ribosome dynamics[210-212]. By comparing the distribution of mRNAs in the soluble and insoluble pools between the wild-type and mutant strains, we can gain further insights into whether Not proteins affect the dynamics of Puf3 granules and determine which mRNAs depend upon Puf3 for their solubility. Additionally, by comparing the distribution of ribosomes on mRNA 5' ends between the wild type and mutant strains, we can gain further understanding of how Puf3 and Not proteins regulate the dynamics of mRNA translation and their interactions. These data are currently undergoing further analysis using bioinformatics methods.

V. CONCLUSION AND PERSPECTIVE

During my Ph.D., I focused on understanding how the Ccr4-Not4 complex regulates mitochondrial biosynthesis. Through the use of reporter constructs, I uncovered an integrated mitochondrial associated quality control mechanism. This research provided evidence for the important role of the mitochondrial targeting sequence in gene expression, highlighting the significance of mRNA and protein localization in fine-tuning gene expression. This finding opens avenues for further investigation into the transport and sequestration of specific mRNA and proteins to their appropriate locations. Additionally, my research demonstrated that the ubiquitination of Egd1 by Not4 functionally regulates *MMF1* expression. However, further investigation is required to determine which steps in the process require Egd1 ubiquitination, mitochondria targeting, quality control, or both. Furthermore, my study established a connection between ribosome pausing, mitochondria targeting, and mitochondrial quality control, which are tightly regulated by the Ccr4-Not complex, and independent of deadenylation in budding yeast but dependent upon Caf130, a yeast-specific subunit of the Ccr4-Not complex.

There remain many open questions to further investigate. Some were mentioned above. In addition, for example, why does the cell develop an integrated quality control mechanism to remove overexpressed Mmf1, but not Cox4? What are the key players that mediate the crosstalk between different quality control pathways? Is it possible for this crosstalk to remodel the transcriptional level? It would also be interesting to investigate what is the anchor site on mitochondria for Puf3? What are the motors and transports for *MMF1* mRNA or Puf3 moving to mitochondria?

In conclusion, my research has provided insights into the regulation of mitochondrial biosynthesis through the Ccr4-Not4 complex and has revealed an integrated mechanism of mitochondrial-associated co-translational quality control. Beyond mitochondria, my findings also contribute to our understanding of the crucial role of mRNA and protein localization in cellular processes. Moreover, this study opens up new avenues for exploring how mRNA localization regulates gene expression and its implications in cellular homeostasis and mitochondrial function.

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VII APPENDIX

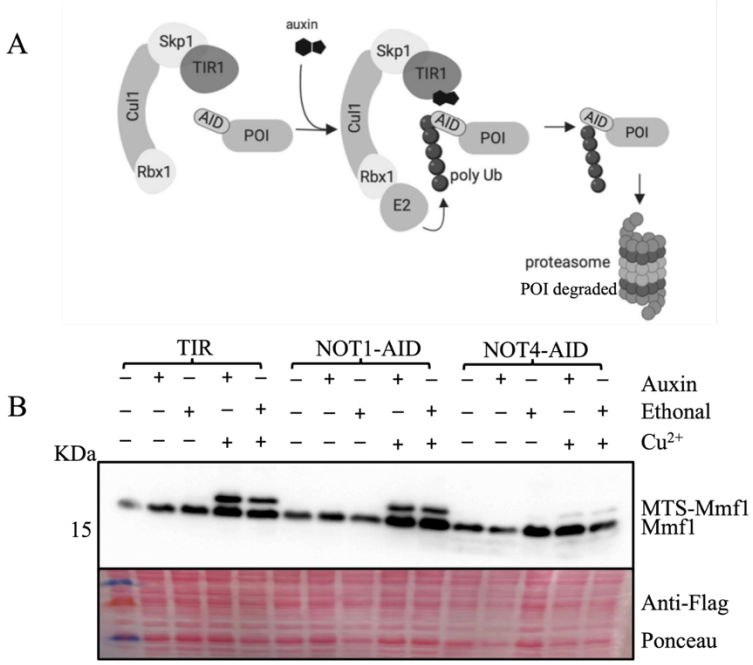

Figure 23. Expression of Mmf1 upon Not proteins depletion

Panel A: AID-Induced Protein Degradation System. The AID-induced protein degradation system enables rapid and specific degradation of a protein of interest (POI) in yeast cells. The system involves the formation of the SCF（Skp1-Cullin-F-box complex）-TIR1 complex, consisting of the TIR1 protein and endogenous components of the SCF complex. In the presence of auxin, the complex recognizes the POI through its degron sequence and facilitates ubiquitination. Ubiquitination tags the POI for degradation by the proteasome. Panel B: Endogenous URA locus was fused with the TIR sequence, or Not1 and Not4 were tagged with the TIR sequence as NOT1-TIR and NOT4-TIR, respectively. Cells were treated with or without auxin, which induces the depletion of Not1 or Not4. Ethanol was used as a control (as it dissolves auxin), while Copper (Cu^{2+}) was used to boost the expression of Mmf1. The expression of Mmf1 was analyzed by western blotting using antibodies specific to Flag. Ponceau stain was used as a loading control. There is no significate difference was observed in all three stains either before copper or after copper induction, indicating Not protein regulate Mmf1 expression via other players, in another word undirectedly.

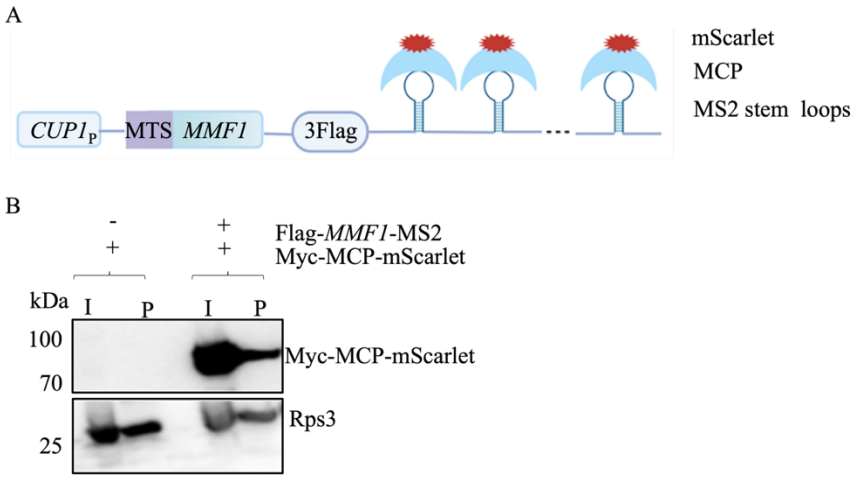

Figure 24. Creating and verifying an *MMF1*-MS2 system used to visualize *MMF1* mRNA

Panel A: Cartoon of the *MMF1* reporter with inserted MS2 stem loops in the 3'UTR that can be recognized by MS2 binding protein (MCP) fused to mScarlet. Panel B: Cells expressing Myc-MCP-mScarlet with or without the *MMF1* reporter with MS2 stem loops (MS2sl) were lysed and the total extract (I) was further sedimented on a 60% sucrose cushion. The ribosome-containing pellet (P) was collected and analyzed by western blotting for the presence of the Myc-MCP-mScarlet was detected by antibodies to Myc. The levels of Rps3 were analyzed as a control. The presence of Myc-MCP-mScarlet in the ribosome-containing fraction indicated the interaction of Myc-MCP-mScarlet and *MMF1-MS2* mRNA.

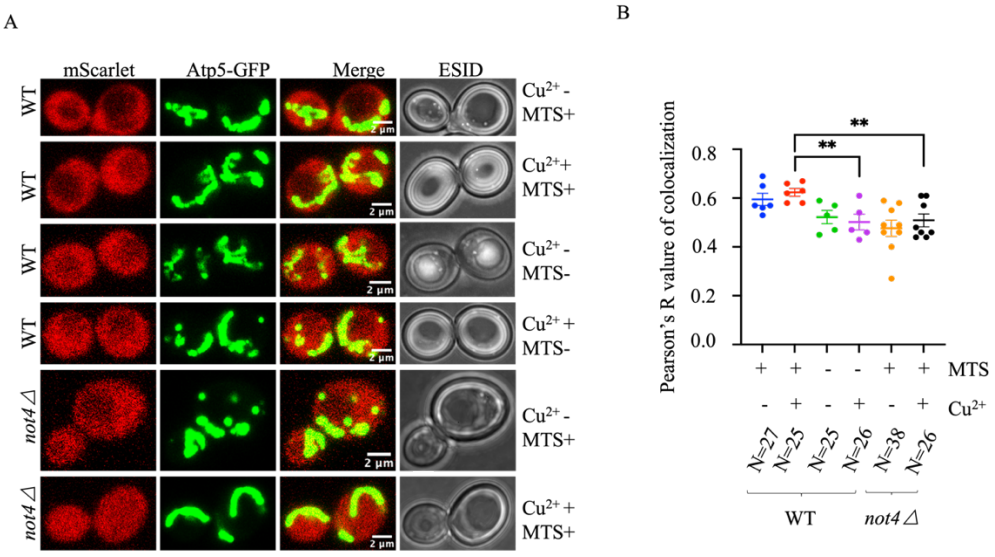

Figure 25. Colocalization of *MMF1* mRNA and mitochondria visualized by confocal microscopy.

Wild type or Not4 deleted cells as indicated expressing from its endogenous locus Atp5 fused to GFP, were transformed with the plasmid expressing MCP fused to mScarlet and the *MMF1-MS2* reporter with or without its MTS as indicated. Cells were grown to exponential phase and induced (+) or not (-) with copper ($^{Cu2+}$) for 10 min, then placed on agar-containing slides that were visualized at the confocal microscope to see mScarlet (left panels), Atp5-GFP (middle panels), and the merged signal (right panels), as well as the cells by phase contrast (far right panels). Representative images of 2 cells are shown. More than 25 cells were analyzed, and the co-localization of the green and red signals evaluated to provide a Pearson's R value for co-localization.

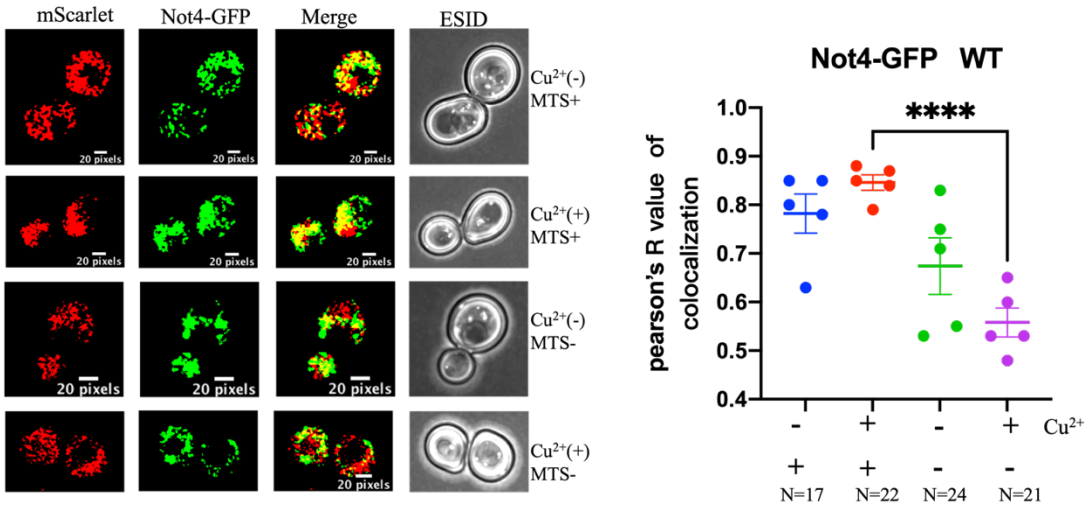

Figure 26. Colocalization of *MMF1* mRNA and Not4-GFP visualized by confocal microscopy

Cells expressing from its own locus Not4 fused to GFP, were transformed with the plasmid expressing MCP fused to mScarlet and the *MMF1-MS2* reporter with or without its MTS as indicated. Cells were grown to exponential phase, and induced (+) or not (-) with copper (Cu^{2+}) for 10 min, then placed on agar-containing slides that were visualized at the confocal microscope to see mScarlet (left panels), Not4-GFP (middle panels), and the merged signal (right panels), as well as the cells by phase contrast (far right panels). Representative images of 2 cells are shown. More than 15 cells were analyzed and the co-localization of the green and red signals evaluated to provide a Pearson's R value for co-localization.

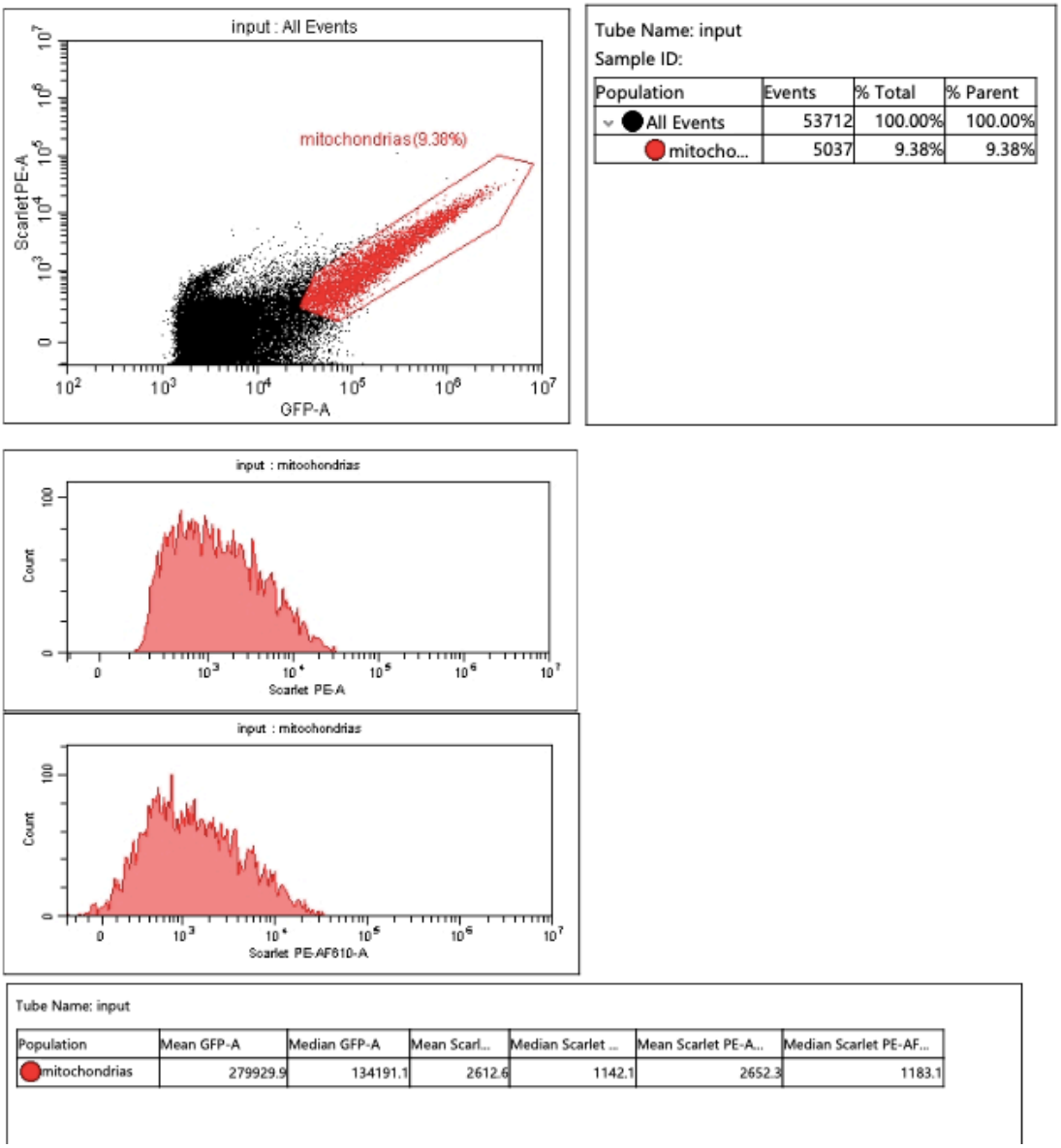

Figure 27. Colocalization of *MMF1* mRNA with a crude mitochondrial fraction analyzed by flow cytometry.

WT cells, with the Atp5-GFP fusion from its endogenous locus, were transformed with a plasmid expressing MCP fused to mScarlet and the *MMF1-MS2* reporter containing its mitochondrial targeting sequence (MTS). The cells were grown to the exponential phase and induced with copper ($Cu^{2+)}$ for 10 minutes. The cell pellets were collected for mitochondria isolation using a commercial kit. Flow cytometry analysis was performed to evaluate the presence of mScarlet in the Atp5-GFP fraction, representing mitochondria. Each dot in the plot represents one detected event, with the x-axis representing the intensity of GFP and the y-axis representing the intensity of mScarlet. The red dots indicate mScarlet present in the GFP fraction, which defines the mitochondria. The upper-left panel shows a representative plot, while the upper-right panel provides a quantification of the mitochondria events. For the middle panel, the x-axis represents the channel of mScarlet or PE 610 wavelength, and

the y-axis represents the counts of detected mitochondria events in each channel. This provides further visualization of the fluorescence distribution in the mitochondria. The bottom panel represents the quantification of GFP, mScarlet, and PE610 fluorescence intensity. It offers a quantitative analysis of the fluorescence signals in the respective channels. The presence of mScarlet in the Atp5-GFP fraction indicates the localization of *MMF1 mRNA* in the mitochondria.

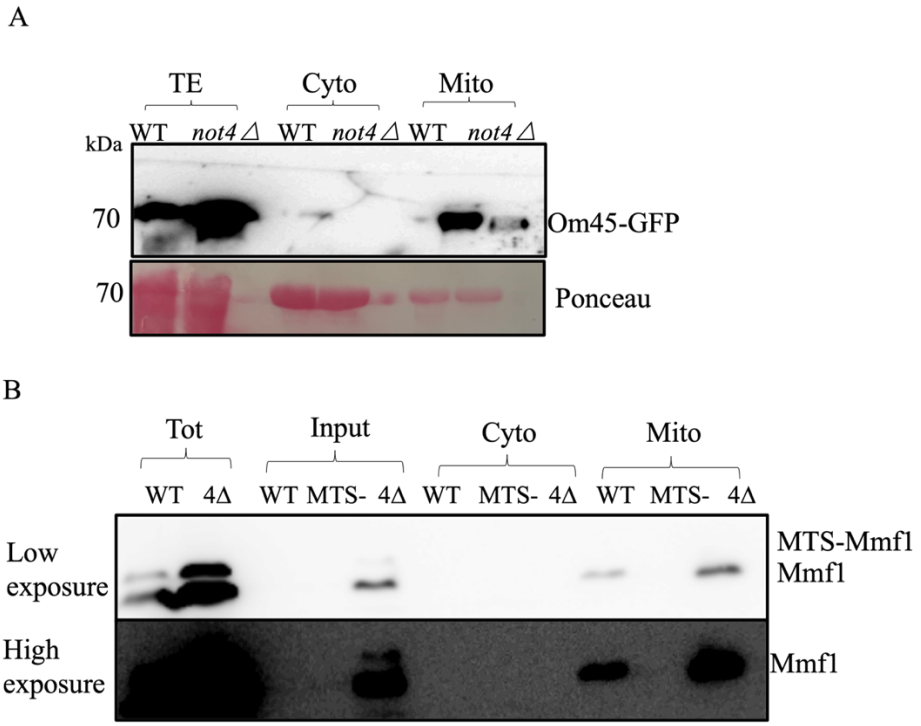

Figure 28. Overexpressed Mmf1 precursor accumulates in the cytoplasm in *not4Δ*.

Panel A: Extracts from wild type and *not4Δ* cells expressing Om45-GFP from its own locus were prepared and fractionated using a commercial kit. The total extract (TE), then cytosolic (Cyto) and mitochondrial (Mito) fractions were analyzed by western blotting with a GFP antibody, to verify the fractionation approach. B: The same experiment as in panel A was performed with WT or Not4 deleted cells overexpressing the *MMF1* reporter with or without an MTS sequence (MTS-) as indicated, after a 10 min copper induction. The western blot was revealed with antibodies to Flag and a low (upper panel) and high (lower panel) exposure is shown.

Materials and methods in appendix

All, plasmids, primers, strains and antibodies used are listed in **Table 4**. The plasmid pMAC1377 expressing MCP fused to mScarlet was constructed from plasmid pMAC1105 digested by XhoI and co-transformed in yeast with a PCR product amplified from pE697 with oligos 1115 and 1116, followed by plasmid rescue. MS2 loops were added in the pMAC1211 and pMAC1327 plasmids by co-transforming into yeast the pMAC1211 and pMAC1327 plasmids digested with SacI and a PCR fragment obtained with oligos 1087 and 1088 and pE659, leading to pMAC1365 and pMAC1367. For both plasmids the URA3 marker was swapped to the LEU2 marker by transforming pUL9 (pE24) digested with StuI and selection of Leu+ Ura- colonies, followed by plasmid rescue leading to plasmids pMAC1390 and pMAC1391. The strain expressing Not4-GFP from its endogenous locus (MY14341) was made with F2 and R1 oligos and pE85 by homologous recombination according to standard procedures, Not4 5' and Not4-V4 were used to verify the corrected Not4-GFP strian.

Table 4. List of plasmids, primers, strains and antibodies for appendix.

Plasmid number	Description	Reference
pMAC1377	FUR4p-Myc6-Flag-MS2-mScarlett-CYC1t	This work
pMAC1105	FUR4p-6Myc-Flag-MS2-CYC1t	Kassem et al., 2017
pMAC1367	CUP1p-MTS-less-MMF1-FLAG-MS2bps-URA3	This work
pMAC1391	CUP1p-MTS-less-MMF1-FLAG-MS2bps-ura3::LEU2	This work
pMAC1365	CUP1p-MMF1-FLAG-MS2bps-URA3	This work
pMAC1390	CUP1p-MMF1-FLAG-MS2bps-ura3::LEU2	This work
pE85	tag GFP 3'	Euroscarf
pE24	ura3::LEU2	pUH7 Daignan Fournier lab
pE116	pRS315	Euroscarf
pE697	yOM-mScarletI-CaURA	Addgene 118457

primer number	Sequence
1115	CTCGGCAATCGCAGCAAACTCCGGCATCTACGATATCGTCGACCTCGAGATGGTTAGTAAAGGTGAAGCTG
1116	GGGGGGAGGGCGTGAATGTAAGCGTGACATAACTAATTACATGACCCGGGTTATTTATACAATTCATCCATTCCTCCG
1087	TATCGCTGTTGAAAAGAATGCGGCCGCACTAGTATCGATGGATTACAAGGATGACGACGATAAGATCTGACCGCTCTAGAACTAGTGGAT
1088	GTAGACAAGCCGACAACCTTGATTGGAGACTTGACCAAACCTCTGGCGAAGAATTGTTAATTAAGAGCTCATTAAGGGTTGTCGAGATCG
Not4 R1	ATTATGGTTAATGCAAACAAGAAAAATATTTAGAGTCGGAGAATTCGAGCTCGTTTAAAC
Not4 F2	AAATCAACTAATCAACGGAAGGAAAATTATCGCCGGTAATCGGATCCCCGGGTTAATTAA
Not4 5’	CCGAAGACGTGAAACGCTATAG
Not4_V4	CAACAACACGCGCGCAAGCT

strain number	Genotype	Reference
11884	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 atp5::ATP5-GFP-HIS3MX4*	Lars Steinmetz lab
14341	*MAT*α *his3 leu2 ura3 lys2 not4::NOT4-GFP-KANMX4*	3415 + Not4-GFP by PCR clone 3 with pE85
12328	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 atp5::ATP5-GFP-HIS3MX4 not4::NATMX4*	11884 + PCR to disrupt NOT4 using DNA from MY 6993

12094	*MAT*a *leu2Δ20 ura3Δ met15Δ his3Δ1 om45::OM45-GFP-HIS3MX4*	Steinmetz lab
12225	*MAT*a *leu2Δ20 ura3Δ met15Δ his3Δ1 om45::OM45-GFP-HIS3MX4 not4::NATMX4*	PCR to disrupt *NOT4* in strain 12094
13472	*MATa ade2-1 his3-11 trp1-1 leu2-3 leu2-112 can1-100 ura3-1::ADH1p-OsTIR1*	Shore Lab
13771	*MATa ade2-1 his3-11 trp1-1 leu2-3 leu2-112 can1-100 ura3-1::ADH1p-OsTIR1 not4::NOT4-AID-Myc9-NATMX4*	Collart lab
13523	*MATa ade2-1 his3-11 trp1-1 leu2-3 leu2-112 can1-100 ura3-1::ADH1p-OsTIR1 not1::NOT1-AID- Myc9-NATMX4*	Collart lab

Antibodies	**Reference**
PAP（Peroxidase Anti Peroxidase）	Sigma
Rps3	Martin POOL
GFP	Roche

Protein extracts, SDS- PAGE and Western blotting

Total protein extracts were prepared by incubating pelleted yeast cells in 0.1 M NaOH for 10 min at RT. After a quick spin in a microfuge, the cell pellet was resuspended in 2 X sample buffer (post-alkaline lysis). Samples were subjected to SDS-PAGE and Western blotting according to standard procedures. Following the electrophoresis and transfer to NC membranes, the blots were incubated with the indicated antibodies.

Confocal Microscopy

For imaging cells were grown in selective synthetic media for plasmid selection as indicated to an OD_{600} between 0.6 and 1.2. 2 OD_{600} of cells was collected by the centrifugation at 3000 g for 5 min at RT. The cell pellets were washed twice with 1 ml PBS. The final cell pellets from 0.5 OD_{600} of cells were resuspended in 200 ml PBS, 20 ml of which was loaded on 1 % agarose

gel coated coverslips, with an even distribution of cells. Then the coverslips were mounted by nail polish. The prepared slides were immediately imaged with a standard confocal microscope (LSM800 Airyscan) with a 63 X oil objective (NA=1.4) that was used for image acquisition. Each image was acquired by z-stacking. The image J software was used to process the images for co-localization analysis and for this co-localization analysis, more than 20 cells of each sample were evaluated. The acquired person's R value of co-localization was statistically analyzed by Prism9.

Sedimentation through a sucrose cushion

For polysome sedimentation 100 OD_{600} of cells were harvested at exponential growth. Cells were disrupted by 0.2 ml glass beads in the presence of 0.4 ml lysis buffer (20 mM Hepes pH 7.5, 20 mM KCl, 10 mM $MgCl_2$, 1 % Triton X-100 ,1 mM DTT,1 mM PMSF, supplemented with a cocktail of protease inhibitors). 20 ml of lysate was taken as input control; 200 ml of the remaining lysate was loaded onto a 60 % sucrose cushion in 0.5 ml mini-ultracentrifuge tubes. Following ultracentrifugation (85000 rpm, 90 minutes, 4°C) in a Sorvall S120-AT2 Fixed-Angle Micro-Ultraspeed Rotor, the pellet at the bottom of the mini-ultracentrifuge tube was resuspended with 200 ml of lysis buffer. The resuspended pellet was analyzed by SDS-PAGE.

Mitochondria isolation

The Mitochondrial Yeast Isolation kit and protocol (ab178779; Abcam) were used to fractionate yeast cells by differential centrifugation. Briefly, cells with the different reporters were grown in synthetic drop-out media at 30 °C until the logarithmic growth phase. 25 OD_{600} of cells were collected by centrifugation (3000 rpm, 5 min, RT). Cell pellets were resuspended with the buffer A containing 10 mM DTT, then buffer B containing Lysis Enzyme Mix provided by the supplier, to form spheroplasts. From this step onwards, the procedure was on ice. The spheroplasted cells were resuspended by homogenization in Buffer C provided by the supplier + a protease inhibitor cocktail provided, transferred to a glass douncer, and broken by 10 –15 strokes. 20 ml of lysate was put aside as input. Following centrifugation at 600 g for 5 min at 4°C, the supernatant, which contained the intact mitochondria, was collected. Further centrifugation of the supernatant (12000 g, 10 min, 4 °C), led to a sedimented fraction containing mitochondria. 600 ml of the supernatant at this step (cytoplasm) was taken and

mixed with 120 ml 100% trichloroacetic acid solution and incubated for 10 min at 4°C to precipitate the cytoplasmic proteins. The input, mitochondrial and cytoplasmic pellet fractions were mixed with 2 X SB and analyzed by western blotting.

Flow cytometry

Flow cytometry analysis was performed on isolated crude mitochondria fractions using a commercial mitochondria isolation kit. The analysis was conducted on the CYTOFLIX flow cytometry, which located at flow cytometry platform. The fluorescence channels were set to detect GFP, PE-A, and PE610. This choice was made because the excited wavelength of mScarlet, a fluorescent protein of interest, closely aligns with the excited wavelengths detected by PE-A and PE 610. To establish gating parameters, a negative control sample was utilized, in which cells did not contain fluorescence. The fluorescence intensity of both GFP and red fluorescence (mScarlet) was taken into account when defining the gates.

The experiments mentioned above were conducted as the initial version to visualize the localization of *MMF1* mRNA. Upon submission of the manuscript, we received feedback from the reviewer which made us realize that the image quality of mScarlet was not sufficiently robust. In a previous study, two copies of tandem GFP were used to visualize mRNA localization, whereas we utilized only one copy of mScarlet, making it difficult to distinguish the true signal from the noise. In response to this challenge, I tried two approaches. Firstly, I came up the idea of demonstrating the localization of *MMF1* mRNA to mitochondria through flow cytometry. The data obtained indeed provided evidence supporting the localization of *MMF1* mRNA to mitochondria. However, due to the weak signal of mScarlet, it is challenged to accurately compare the proportion of colocalized *MMF1* mRNA with mitochondria between WT and mutants. On the other hand, I used new MS2 system by employing MCP-4GFP as a reporter to track *MMF1* mRNA localization and Su9-mCherry to indicate the location of mitochondria. This approach yielded new data, which we have presented in the results section. By using the new MS2 system, we were able to overcome the limitations associated with mScarlet and obtain clearer and more reliable data regarding *MMF1* mRNA localization. Additionally, I optimized the protocol for isolating relatively pure mitochondria, better than the use of commercial kits. I have described and presented the new data results from this optimization in the results section. It is worth noting that we did not include the above results

in the revised publication. This decision was made because the newly developed MS2 system, which provided improved and more reliable data on *MMF1* mRNA localization. The new generated results have been acknowledged by the reviewing experts.