# Mechanistic insights into the protein targeting mediated by signal recognition particle

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# Mechanistic insights into the protein targeting mediated by signal recognition particle

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Par

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# RÉSUMÉ
## *(In French)*

Chez les eucaryotes supérieurs, la translocation des protéines sécrétées et des protéines membranaires à travers le réticulum endoplasmique (RE) est un processus co-traductionnel. Dans la cellule eucaryote, beaucoup des protéines destinées à entrer dans le RE sont synthétisées sur les ribosomes liés au RE et sont transloquées co-traductionnellement dans le RE. Une fois dans la lumière du RE, elles sont amenées vers leur destination cellulaire finale via la voie de sécrétion. La particule de reconnaissance du signal (SRP) et son récepteur membranaire (SR) sont des machines moléculaires conservées dans tous les règnes du vivant et impliquées dans l'adressage co-traductionnel des protéines à l'intérieur du RE. Le SRP des mammifères est composé d'un petit ARN de 300 nucléotides et de 6 polypeptides nommés selon leurs masses moléculaires : SRP9, SRP14, SRP68, SRP72, SRP19, SRP54 . SRP peut être divisé en deux domaines fonctionnellement distincts après digestion par la nucléase micrococcale (FIG. 3). Le domaine S comprend la partie centrale de l'ARN, ainsi que les protéines SRP19, SRP54, SRP68 et SRP72 . Le domaine *Alu*, quant à lui, est constitué des parties 5' et 3' terminales de l'ARN et des protéines SRP9 et SRP14.

SRP se lie à la séquence signale de la chaîne naissante émergeant du ribosome. Cette liaison entraîne une pause dans l'élongation du polypeptide naissant correspondant à la fonction d'arrêt d'élongation de SRP. Le complexe formé de SRP, du ribosome et de la chaîne naissante interagit ensuite avec le récepteur membranaire via SRP. Cette interaction est contrôlée par la liaison du GTP et son hydrolyse. Elle induit une série de changements de conformation permettant une liaison séquentielle qui aboutit au relâchement de la chaîne naissante à l'intérieur du translocon. Il y a alors dissociation du complexe SRP-SR et SRP est relâché dans le cytoplasme, libre pour un nouveau cycle d'adressage.

Au début de ma thèse, la majorité des études concernant les fonctions de SRP étaient effectuées *in vitro* grâce à des systèmes de traduction « cell free » et des membranes de RE de mammifères purifiées (microsomes). Ces systèmes permettaient de comprendre les mécanismes du fonctionnement de SRP, mais les conséquences cellulaires de ces mécanismes n'étaient alors pas élucidées. Des études avaient aussi été menées chez les bactéries, les levures et chez trypanosome pour comprendre la voie cellulaire de SRP. Cependant ces organismes sont relativement différents des systèmes mammifères dans leur organisation cellulaire et dans leur fonction. Aucune étude précédente n'avait été effectuée sur des cellules mammifères, c'est pourquoi nous nous sommes intéressés à la caractérisation du rôle de SRP dans les cellules de mammifères.

Pendant la première partie de ma thèse, j'ai étudié *in vivo* le rôle de SRP dans les cellules mammifères. Nous avons réduit la quantité cellulaire de trois protéines SRP différentes : SRP14, SRP54 et SRP72, en utilisant la méthode « RNA interference ». L'inhibition de chacune de ces protéines a entraîné une baisse significative de la quantité cellulaire de l'ARN SRP, démontrant qu'il ne restait que très peu de SRP fonctionnel. Nous avons aussi montré que la baisse de SRP endogène a un effet sur la croissance cellulaire. Dans les cellules les plus touchées, celle-ci a en effet été réduite de 50%. Ces cellules montraient aussi une diminution significative dans l'accumulation de protéines reporter possédant cinq différentes localisations cellulaires, due à une déficience dans l'adressage au RE. Nos résultats ont ensuite montré que la déplétion en SRP affecte sévèrement mais sélectivement le trafic membranaire post-RE. Le transport antérograde de VSV G et le transport rétrograde de la sous-unité de la Shiga toxine B se trouvent bloqués au niveau du Golgi. Ces résultats indiquent que dans ces cellules, la fonction du Golgi est altérée. De plus, dans

ces cellules, le recyclage du récepteur de la transferrine est déficient, ce qui provoque son accumulation dans les endosomes (Golgi-recyclage). Au contraire, la baisse du taux de SRP endogène ne perturbe pas le trafic de la membrane plasmique au lysosome.

En résumé, ces résultats ont montré que l'efficacité de l'adressage au RE et de la translocation était nécessaire pour assurer le bon fonctionnement du Golgi lors du trafic antérograde et rétrograde. Ceci suggère un lien entre les endosomes de recyclage et le Golgi. Il est intéressant de noter que ces phénotypes sont moins prononcés lorsque les taux cellulaires de SRP sont diminués de 80%. Une étude précédente a aussi montré qu'il n'y avait pas d'effet sur la croissance cellulaire si les taux cellulaires de SRP étaient diminués de 80%, indiquant que les cellules possèdent un excès de SRP. Nos études ont ensuite montré que quelques protéines sont transloquées normalement en présence de faible quantité de SRP, indiquant que les études de déplétion de SRP peuvent être utilisées comme outil pour caractériser la voie post-traductionnelle dans les cellules de mammifères.

Pendant la seconde partie de ma thèse, je me suis intéressé à la signification de la fonction d'arrêt d'élongation dans les cellules de mammifères. Des études précédentes avaient caractérisé la fonction d'arrêt d'élongation le plus souvent dans des systèmes *in vitro*. Dans notre laboratoire, l'étude *in vitro* a montré que l'activité d'arrêt d'élongation était dépendante d'un court motif présent dans la partie C-terminal de SRP14. Ce motif est composé principalement d'acides aminés basiques. Afin de comprendre le rôle physiologique de l'activité d'arrêt d'élongation dans les cellules de mammifères, j'ai développé un système de complémentation. Nous avons inhibé l'expression de la protéine endogène SRP14 dans les cellules de mammifères grâce à un shRNA ciblant la partie 3'UTR du gène SRP14 et nous avons complémenté les cellules avec des versions mutantes de SRP14 ne possédant pas d'activité d'arrêt d'élongation. La déplétion de la protéine SRP14 endogène et la complémentation avec des SRP14 mutés déficients pour l'activité d'arrêt d'élongation restaure des taux cellulaires fonctionnels de SRP. Des expériences de marquage par « pulse » ont montré que la perte de la fonction d'arrêt d'élongation dans les cellules affectait négativement et sévèrement la translocation des protéines dans le RE. Ceci résulte en une diminution de l'accumulation de protéines endogènes ou de protéines reporter à leur localisation respective conduisant à une baisse de la croissance cellulaire, la plupart des cellules s'accumulant à la phase G0/G1 du cycle cellulaire. Les phénotypes observés pouvaient être contrebalancés en réduisant le taux de synthèse des protéines cellulaires de quatre fois et en augmentant de deux fois l'expression des deux sous-unités du récepteur de SRP. Ces résultats montrent que l'inhibition de la traduction après la reconnaissance de la séquence signale par SRP est nécessaire pour s'ajuster au nombre limitant de sites de réception présents sur la membrane. En absence de la fonction d'arrêt d'élongation, les chaînes naissantes deviennent probablement trop longues avant d'entrer en contact avec un site de réception et donc empêchent un adressage et une translocation efficaces. Ces résultats montrent aussi que la vitesse de traduction doit corréler avec la vitesse d'adressage des chaînes naissantes vers le translocon. Ce besoin de ralentir la vitesse de traduction afin de l'ajuster à la vitesse d'adressage pourrait aussi représenter un mécanisme de régulation. Il favoriserait l'adressage des protéines dont la séquence signale possède une forte affinité pour SRP, puisque celles-ci se dissocieront moins rapidement de SRP.

# *ABSTRACT*

The signal recognition particle (SRP) and its membrane bound receptor constitute universally conserved molecular machines, which ensure the efficient targeting of proteins into the endoplasmic reticulum. SRP binds to the signal sequence of the nascent chain emerging out of the ribosome. This binding elicits a pause in the elongation of the nascent polypeptide defined as the elongation arrest function of SRP. The SRP-bound ribosome-nascent chain complex interacts with the membrane bound SRP receptor (SR). This interaction is controlled by GTP binding and hydrolysis. The interaction further induces a series of conformational changes enabling ordered binding and finally the release of the nascent chain into the translocon. The release of the nascent chain into the translocon is simultaneously associated with the GTP hydrolysis leading to the release of SRP and SR, which are now ready for the next cycle of targeting.

At the beginning of my thesis, most of the work characterizing the functions of SRP was done in the *in vitro* systems composed of cell free translation system and purified mammalian ER membranes (microsomes). These systems elucidated the mechanistic aspects of SRP function, but it was unclear as to what are the cellular consequences of such a mechanism induced by SRP. Studies were also done in bacteria, yeast and trypanosomes to understand the role of SRP pathway in the process of protein translocation across the ER. However these organisms are sufficiently different from their mammalian counterparts in their cellular organization. Previously, no studies were done on SRP functions in mammalian cells, which got us interested in characterizing the role of SRP in mammalian cells.

During the first part of my thesis, I was interested in understanding the role of SRP in protein translocation in mammalian cells. To examine the *in vivo* roles of SRP in mammalian cells, we reduced the cellular levels of three different SRP proteins, SRP14, SRP54 and SRP72 by more than 90% using RNA interference. All the three knockdowns resulted in a significant down- regulation of SRP RNA revealing that very little functional SRP was left. The cells with less than 10% of SRP showed prominent growth defects and the growth rate was decreased by 50%. These cells were significantly diminished in the accumulation of reporter proteins from five different cellular locales due to inefficient ER-targeting. Our studies further showed that depletion of SRP results in severe but selective defects in post ER membrane trafficking. The anterograde transport of the VSV-G and the retrograde transport of the Shiga toxin B subunit were stalled at the level of Golgi. These results indicate that the SRP knock-down cells have a functionally impaired Golgi. Furthermore, these cells showed a defect in the recycling of the transferrin receptor resulting in its accumulation in Golgi/recycling endosomes. In contrast, reduced SRP levels did not disturb plasma membrane to lysosome traffic. Overall, these results reveal a requirement for efficient ER-targeting and translocation to ensure proper Golgi function in antero and retrograde protein trafficking and suggest a link between early/recycling endosomes and the Golgi. Interestingly, these phenotypes were less pronounced when the cellular SRP levels were decreased by 80%. A previous study also showed that there were no growth defects when the cellular SRP levels were down by 80% indicating that these cells require much less SRP, than what they possess to survive. Our studies further showed that some proteins are translocated normally at the lower levels of SRP, probably by utilizing SRP independent targeting machinery. Hence, the SRP depletion studies could be used as tool to characterize the post-translational pathway in mammalian cells.

During the second part of my thesis, I was interested in understanding the significance of elongation arrest function in mammalian cells. Earlier studies

characterizing the elongation arrest function were mostly done in the *in vitro* systems. *In vitro* studies in our lab revealed that elongation arrest function is dependent on a short motif in the C-terminal region of SRP14 comprising mostly of basic amino acid residues. To understand the physiological importance of elongation arrest function in mammalian cells I developed a complementation assay system. I silenced the expression of endogenous SRP14 in mammalian cells using an shRNA against the 3' UTR of the gene and complemented the cells with mutant versions of SRP14 lacking the elongation arrest function. Depletion of endogenous SRP14 and complementation with mutant SRP14 lacking elongation arrest function successfully restored the functional SRP levels. Pulse labeling experiments showed that the cells lacking elongation arrest function have a pronounced defect in protein translocation into the ER. This resulted in diminished accumulation of endogenous and reporter proteins in their respective locales. Furthermore, we observed a slow down in the cell growth, with most of the cells accumulating in the G0/G1 phase of the cell cycle. The observed phenotypes were reversed by reducing the cellular protein synthesis rate by four fold using elongation inhibitors. A similar reversion of the phenotypes was also observed when the expression levels of both the subunits of SRP receptor were increased by two fold. These results indicate that the slow down in the translation after the recognition of the signal sequence by SRP is necessary to adjust to the limiting number of receptor sites present on the membrane. In the absence of the elongation arrest function, the nascent chains presumably become too long before they encounter a receptor site and this prevents successful targeting and translocation. These results show that the rate of translation has to be matched with the rate of targeting of nascent chains to the translocon. Furthermore, the elongation arrest activity could also function as a regulatory mechanism by utilizing the time window during the arrest to dissociate the signal sequences with weak affinity for SRP and allow the specific targeting of only those nascent chains, which have a signal sequence with higher affinity for SRP.

# *1. INTRODUCTION*

## *1.1 Proteins and Membranes*

Cell membranes are crucial to the life of the cell. The plasma membrane encloses the cells, marks the boundaries and maintains the difference between inside and outside the cell (Bruce Alberts). Inside the eukaryotic cell, the membranes enclosing different organelles such as endoplasmic reticulum (ER), Golgi, mitochondria, lysosomes etc maintains the characteristic differences between the contents of each organelle and the cytosol. Although the lipids provide the basic structure of a biological membrane, it is the proteins in the membrane, which perform most of the functions. A typical plasma membrane is made up of 50% of proteins. In contrast membranes of some organelles such as inner membrane of mitochondria is made up of 75% of proteins. Proteins make up 50% of the dry weight of the cells. Proteins confer upon each organelle its characteristic structural and functional properties. They are key to maintaining the integrity of the organelles and performing various functions in an organelle specific manner. Most proteins are synthesized in the cytoplasm of the cell. This raises the question of how proteins are transported from the cytoplasm to other destinations within or outside the cell? Cells have evolved specialized targeting machinery, which takes the proteins synthesized in the cytosol to the membrane. Once at the membrane, the crossing of the lipid bilayer is facilitated by proteinaceous channel present on the membrane called the translocon.

The entry point for all the proteins in the secretory pathway (this includes proteins for Golgi, lysosomes, secretion and plasma membrane proteins) is the ER. The ER is the largest endomembrane system within the eukaryotic cells and performs a wider variety of functions. The ER is composed of two morphologically distinct sub-compartments: the rough endoplasmic reticulum (RER) and the smooth endoplasmic reticulum (SER). The RER is studded with the ribosomes and it is mainly acts as the site for the protein synthesis on the ER. The SER is involved in metabolic pathways such as lipid detoxification.

The journey of a typical secretory or a membrane protein begins by its synthesis in the cytoplasm and then it is targeted to the ER membrane. This targeting event is facilitated by specific targeting sequence or signal sequence present in the protein. The signal sequence of a secretory protein is a highly hydrophobic N-terminal extension in higher eukaryotes [1-3]. These sequences are 15-30 residues in length and contain a positively charged N-terminus, a central hydrophobic core and a C-terminal region predominating in polar residues that are often negatively charged. The polar region contains a recognition site for signal peptidases, which are enzymes known to cut the signal peptide, once its targeting function has been completed. The cleavage occurs either during translocation or soon after completion of translocation by crossing the ER. Signal peptides can direct the proteins into two different translocational pathways in eukaryotes: the cotranslational translocation pathway and the posttranslational translocation pathway.

In cotranslational translocation pathway, the substrate is translocated across the membrane concurrent with its synthesis by the membrane bound ribsosome. The emergence of the signal sequence from the ribosome in the cytosol is recognized by a universally conserved ribonucleoprotein called as the signal recognition particle (SRP) [1, 3-5]. The complex of SRP-ribosome nascent chain is then targeted onto the ER membrane by an interaction with the SRP receptor (SR) [6]. At the membrane the SRP releases the signal sequence and the ribosome nascent chain (RNC) is released

into the translocon. The targeting cycle is culminated with the release of RNC into the translocon and recycling of SRP and SR for the next substrate.

In the posttranslational translocation pathway, the substrate is fully synthesized in the cytosol and then is translocated in a ribosome independent fashion. In eukaryotes this pathway has been extensively studied in yeast. Not much is known about this pathway in mammalian cells. The essential translocation apparatus in yeast have been identified as seven protein Sec complex and the luminal chaperone of the ER, BiP [7]. The Sec complex selectively binds to the substrate and mediates the targeting to the translocon in a single mechanistic step unlike in cotranslational targeting.

As we are mostly interested in understanding the mechanism of SRP mediated cotranslational protein targeting, I will initially describe the structure and functions of the signal recognition particle (SRP) components and then I will describe the mechanism involved in protein targeting mediated by SRP.

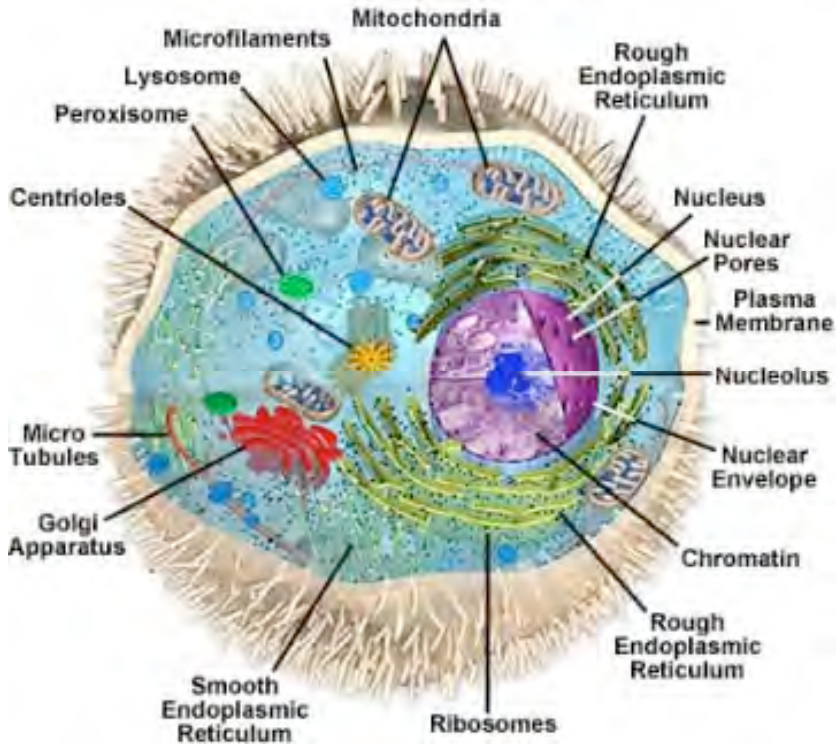



**Figure 1**: Compartmentalization of the cell. The cartoon depicts the different sub cellular compartments of a mammalian cell (adapted from Molecular expressions $^{TM}$).

## *1.2 SRP mediated protein targeting*

In higher eukaryotes translocation across the ER is a cotranslational process occurring concomitantly with the biosynthesis of the secretory and the membrane proteins. In eukaryotic cell many of the proteins destined to enter the ER are synthesized on the ribosomes bound to the ER and cotranslationally translocated into the ER. Once they are in the lumen of the ER, they are routed to the correct destination in the cell via the secretory pathway. The signal recognition particle (SRP) is a highly conserved ribonucleoprotein involved in cotranslational protein translocation into the ER [4, 8]. The main function of the SRP is to deliver the signal sequence bearing nascent polypeptides to the ER membrane

SRP-dependent protein targeting consists of two steps (a) signal sequence recognition and (b) association with the target membrane via its interaction with SRP receptor (SR). In the first step SRP binds to a N-terminal hydrophobic signal sequence of the nascent chain as soon as it emerges form the ribosomal polypeptide exit tunnel. This binding of the SRP to the ribosome nascent chain (SRP-RNC) leads to a pause in the elongation of the nascent chain. This function of SRP is termed the elongation arrest function (step1, Fig. 2). The resulting complex comprising of the ribosome, the nascent chain and the SRP is called as the targeting complex. The SRP-RNC, which is in a GTP bound state is then delivered to the ER membrane via an interaction with the SR, which is also in its GTP bound state. Notably, the GTP binding state of both SRP and SR is a prerequisite for the complex formation. (step 2, Fig. 2). The interaction between the SRP and SR results in series of conformational changes resulting in the release of the nascent chain into the translocon (step 3, Fig. 2). The ribosome nascent chain complex is now docked onto the translocation channel, which is formed by the Sec61 complex [9-11]. Once the nascent chain is released into the translocon the translation arrest induced by the SRP is released. The nascent chain translation is resumed at normal speed with the nascent chain passing into the lumen of the ER through the translocation channel. The release of the nascent chain by the SRP leads to rearrangement within the SRP-SR complex leading to the GTP hydrolysis. The GTP hydrolysis results in the dissociation of both SRP and the SR from each other. The free SRP and the SR are now available for a new targeting cycle.

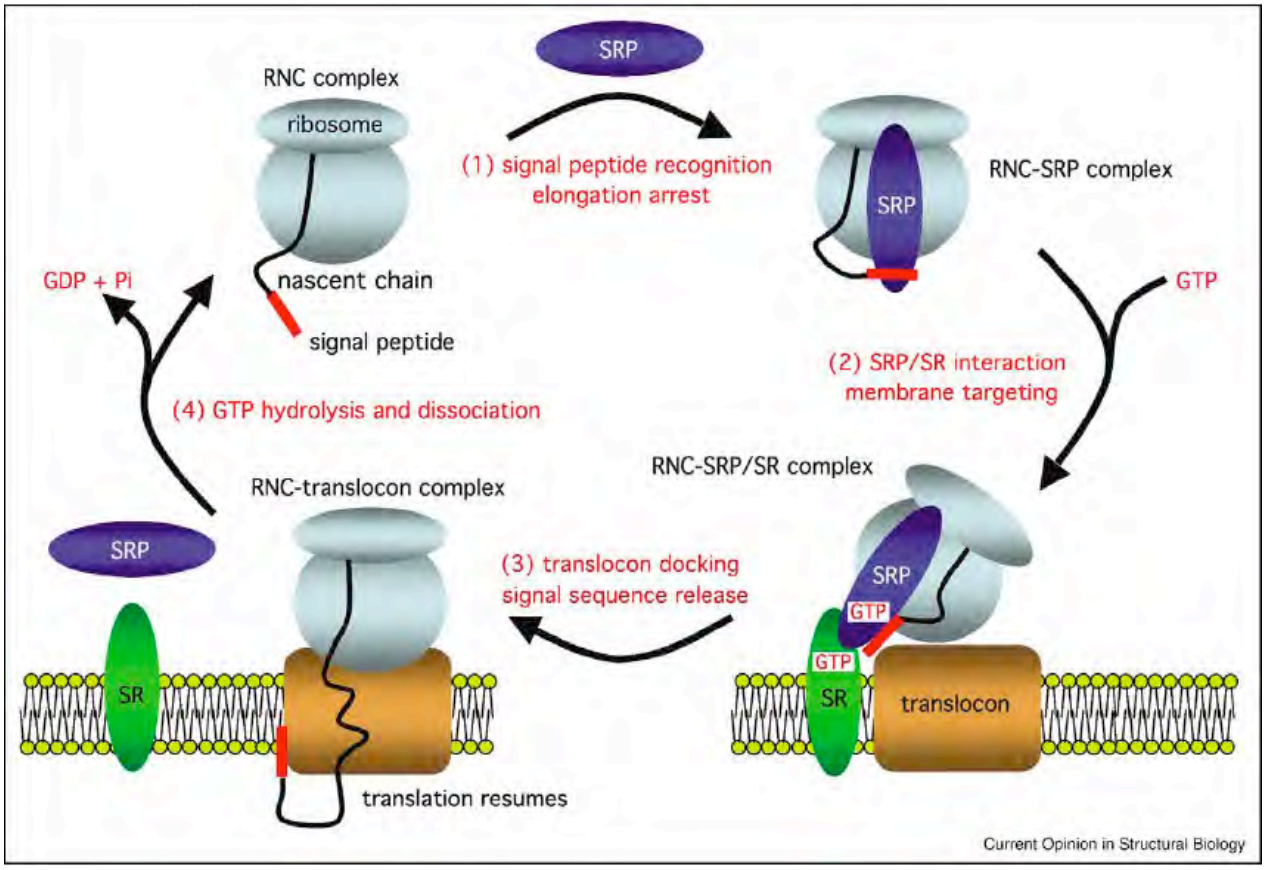



**Figure 2: The SRP cycle**. The cartoon depicts various steps involved in the SRP mediated protein targeting. For the details of various steps see the text (adapted from [6]).

## *1.3 Components of SRP*

The mammalian signal recognition particle is composed of a small RNA of 300 nucleotides and six polypeptides named according to their apparent molecular masses: SRP9, SRP14, SRP68, SRP72, SRP19, SRP54 [4, 8]. SRP can be divided into two functional domains upon treatment with micrococcal nuclease (Fig. 3). The S domain is comprised of the central part of the RNA and SRP19, SRP54, SRP68, SRP72 proteins. The *Alu* domain is made up of the 5' and 3' ends of the RNA along with the SRP9 and SRP14 proteins.

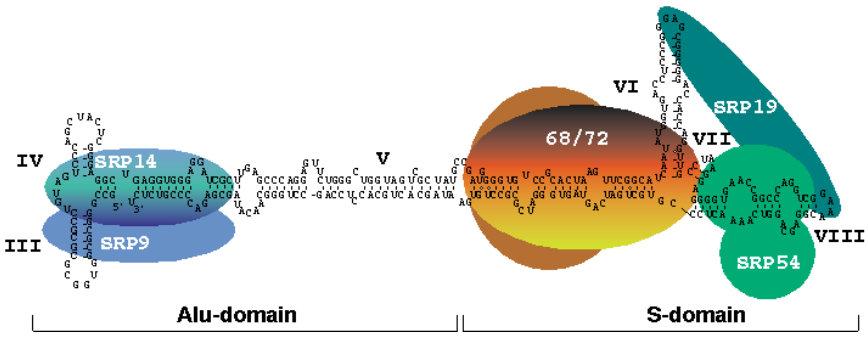



**Figure 3:** Schematic representation of mammalian SRP which is functionally divided into two domains. The *Alu* domain consists of SRP9 and SRP14 proteins. The S domain is composed of SRP19, SRP54, SRP68 and SRP72 (adapted from KS lab).

### *1.3.1 SRP RNA*

SRP RNA forms the structural lattice onto which all the SRP proteins bind [12]. The SRP RNA is a RNA polymerase III transcript encoded by the 7SL RNA gene. One of the main functions of SRP RNA is to ensure proper assembly of all the SRP subunits. The secondary structure of SRP RNA has been determined both by phylogenetic analysis and experimental approaches [13-16].

Structurally, the 300 nucleotide long human SRP RNA can be divided into 12 helices [17]. The *Alu* domain portion of the SRP RNA is made up of helices 2-4 and a portion of helix 5. Similarly, the S domain part of the SRP RNA constitutes helices 6-8 and a conserved part of helix 5. Due to the spatial separation of the two domains by

the variable portion of SRP RNA helix 5, the mammalian SRP RNA has an overall elongated dumbbell shape (Fig. 4).

Evolutionarily the 7SL RNA gene is considered to be the progenitor of the *Alu* family of DNA repeats in primates and of the B1 family in rodents [18]. The SRP RNA of eubacteria is apparently far simpler than its eukaryotic counterpart. In E.*coli*, SRP is composed of 4.5S RNA [19, 20]. The entire *Alu* domain is absent in eubacteria, except in some bacillus species and some species of methanoccocus. The most conserved motif with respect to size and primary sequence is found in the S domain. It constitutes the tertranucleotide loop and two bulges found in the helix 8. Recent studies on bacterial 4.5S RNA show that SRP RNA can accelerate the rate of SRP-SR complex formation by over two orders of magnitude [21, 22]. It thus represents a unique example of an RNA that catalytically modulates the behavior of proteins and is likely to play an important mechanistic role in promoting essential conformational changes. In contrast to the eubacteria, the archaeabacterial SRP RNA resembles very closely to the mammalian SRP RNA except for an additional helix, which is formed by the pairing of 3' and the 5' ends of the RNA.

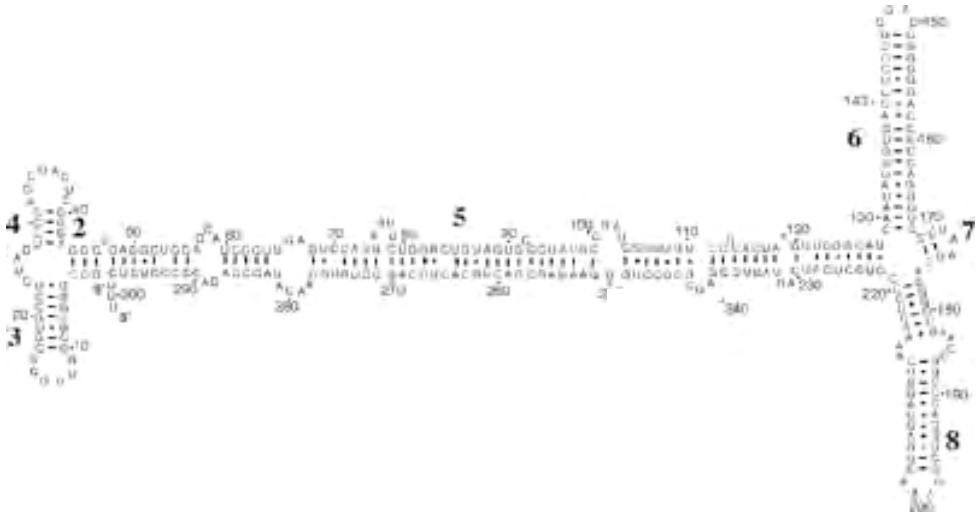



**Figure 4:** Secondary structure of human SRP RNA. The different helices are named according to the recently developed nomenclature. Helix 8 is most conserved among all the different species (adapted from [17]).

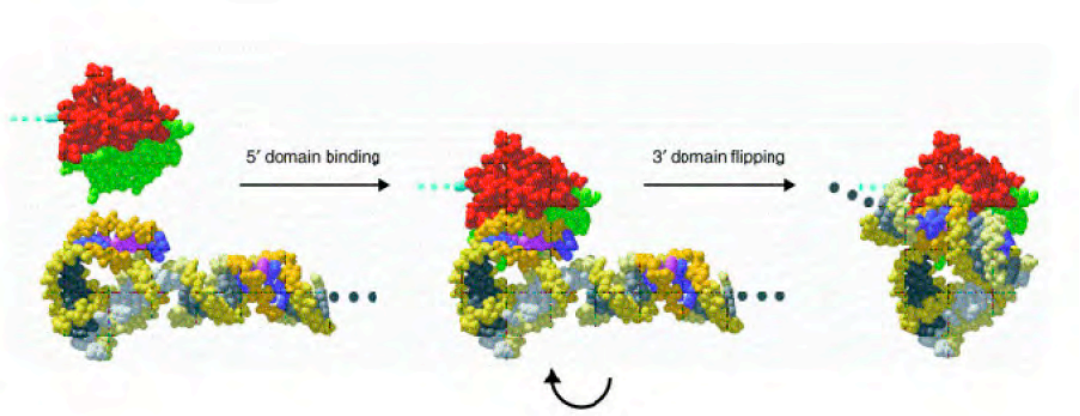
### *1.3.2 Alu domain binding proteins*

SRP9 and SRP14 along with 5' and 3' ends of the 7SL RNA constitute the *Alu* domain of SRP. *In vitro* studies show that mammalian SRP9 and SRP14 form a heterodimer in the absence of SRP RNA.[23]. The *Alu* domain is specifically required for the elongation arrest function of SRP (see Introduction 1.6.3).

SRP9 and SRP14 are structurally homologous and contain αββββα fold (Fig. 3). The heterodimer consists of six stranded antiparallel beta sheet stacked against four alpha helices with a pseudo two-fold symmetry [24]. SRP9/14 binds specifically to the *Alu* sequences of SRP RNA via its concave β-sheet surface [25]. *In vitro* studies have deduced a sequential assembly pathway for the SRP *Alu* domain. In the first step SRP9 and SRP14 form heterodimers. In the second step, the SRP9/14 heterodimer binds 7SL RNA. This binding occurs on a highly conserved position at the 5' region of 7SL RNA. This region is composed of two helical hairpins which are connected to a helical stem by a conserved U-turn [26] (Fig. 5B). In the final step of the assembly the heterodimer induces the RNA to fold back on itself in such a way that the 3' and the 5' domains become adjacent to each other [26]. This flipping of the 7SL RNA upon itself is a reversible step. In mammalian cells the Alu domain is essential for the final processing events of the 7SL RNA and it is also further implicated in the export of the whole particle to the cytoplasm [27]. Furthermore, the highly organized 5' region of 7SL RNA along with the SRP9/14 is found to be essential for efficient transcription of 7SL RNA [28].

SRP14 proteins have been identified in several mammalian species: human, primate, canine, murine. No homolog of SRP14 has been identified in prokaryotes although their binding sites are conserved in the archaeal SRP RNA. In *Bacillus subtilis*, an additional protein called as HBsu has been identified which binds to the *Alu* domain. This histone like binding protein shares a substantial structural homology with SRP9/14 heterodimer. It is not yet know whether this protein is a functional homolog of SRP9/14. Homologues of mammalian SRP9 have so far been identified mostly in eumetazoans and plants. More recently homologues of SRP9 and SRP14 have been identified in *Plasmodium falciparum* and *Chalamydomonas reinhardtii*. In *S.cerevisiae*, no homolog of SRP9 is identified. Two copies of SRP14 are thought to form a homodimer in *S.cerevisiae* [29]. It is not yet clearly understood whether the homodimer can functionally replace SRP9/14 heterodimer.

**A**




**B**

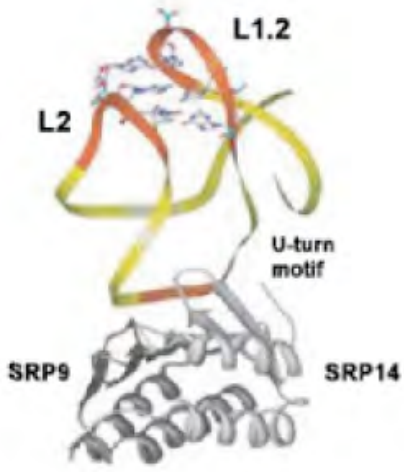



**Figure 5: (A)** Assembly of the SRP *Alu* domain. The heterodimerization of SRP9 (red) and SRP14 (green) is essential for the binding at the 5' region of the RNA. This interaction induces the RNA 3' domain to flip around and bind across the interface of 5' domain. **(B)** The structure of the SRP *Alu* 5' domain is presented on the left. SRP9 and SRP14 are coloured in grey. The nucleotides from loops L2 and L1.2 that are involved in tertiary base pairing between the loops are shown as wire frame. The U turn motif is indicated in the figure (adapted from [25]).

### *1.3.3 S domain binding proteins*

The S domain of mammalian SRP harbors four proteins and is essential for proteins targeting.

**SRP54**

One of the key proteins of S domain is SRP54. It performs two equally important functions. (i) Recognition of the signal sequence emerging from the exit site of the ribosome (see Introduction 1.6.2) (ii) docking of the RNC on to the ER membrane by interacting with SRP receptor present on the membrane (see Introduction 1.6.4).

SRP54 is a multidomain protein consisting of three distinct functional domains; an N-terminal four-helix bundle (N-domain), a GTPase domain (G-domain) and a C-terminal methionine-rich domain (M-domain) [30, 31] (Fig. 6). The N and G-domains are responsible for the GTP regulation during the protein. The M-domain is responsible for binding to the signal sequence. In the recent years many crystal structures of M domain have been solved [32-34]. The C-terminal part of M-domain (Mc) is very similar and well ordered in all structures. The N-terminal part of the M-domain shows differences, especially in the finger loop, which is closing the hydrophobic groove. These differences are probably because of the flexibility of the finger loop, which might be the basis to bind to a variety of signal sequences. This also means that signal sequence binding induces structural changes in the N terminal part of the M domain [35].

SRP54 and helix 8 of the SRP RNA are universally conserved and are sufficient to build a minimal SRP as seen in E.*coli*. The prokaryotic homolog of SRP54 is Ffh and it shows similar properties to that of its mammalian counterpart.

**SRP19**

SRP19 is considered to play a major role in the assembly of SRP. The binding of SRP19 is thought to be a pre-requisite for binding of SRP54 [36].

SRP19 is a $\alpha\beta$-type protein with a central three-stranded antiparallel $\beta$ sheet packed against two helices and has a $\beta\alpha\beta\beta\alpha$ topology [31]. Crystal structure studies reveal a complex protein-RNA binding interface; with long flexible loops of SRP19 recognizing the particular shape of stem loop RNA [31]. These studies further reveal a binding site for SRP19 in the distal loop of helix 8 apart from helix 6 (Fig. 7). This binding to both the helices, brings them in close proximity leading to a conformational change in helix 8 and results in the exposure of normally cryptic SRP54 binding site [30, 37].

Several homologues of mammalian SRP19 have been identified in organisms that have helix 6 of the SRP RNA. No homologue of SRP19 has been identified in eubacteria. In archaeabacteria, the SRP19 is thought be involved in the assembly of SRP54 similar to that of eukaryotes. A recent study has shown that SRP19 is dispensable for archaeabacteria. The common feature to all the SRP19 homologues is the presence of high density of positive charges near their C-terminus.

**SRP68/72**

The least understood proteins in terms of structure and function in mammalian SRP are SRP68 and SRP72. The SRP68/72 heterodimer binds the SRP RNA in the S domain independent of SRP19 and of SRP54 (Siegel and Walter, 1988b). The function of the heterodimer in the particle is still unknown, but it might be involved in translocation since inactivation of SRP68/72 by alkylation's results in SRP, that has lost the ability to promote translocation but which can still arrest elongation (Siegel and Walter, 1988c; Siegel and Walter, 1988d). Early studies on the assembly of SRP68 and SRP72 show that SRP68 binds first to the RNA and induces a conformational change allowing the binding of SRP72 [38]. Recent biochemical studies have shown that SRP72 binds to SRP RNA at the helical sections of 5e and 5f and the binding was independent of the presence of SRP68 [39]. Using bioinformatics approach nine tertratricopeptide repeats (TPR) were assigned within the first 500 amino acid residues of SRP72. Similar analysis in SRP68 has mapped the RNA binding site to be present between amino acid residues 52-252 in SRP68. Mapping of SRP68-SRP72 interaction domains show that C-terminus of SRP68 binds to first four predicted TPR motifs in SRP72 [40].

Homologues of mammalian SRP68 and SRP72 have been identified only in eukaryotes. Recent studies have identified the mammalian homologues of SRP68 and SRP72 in trypanosomes [41].

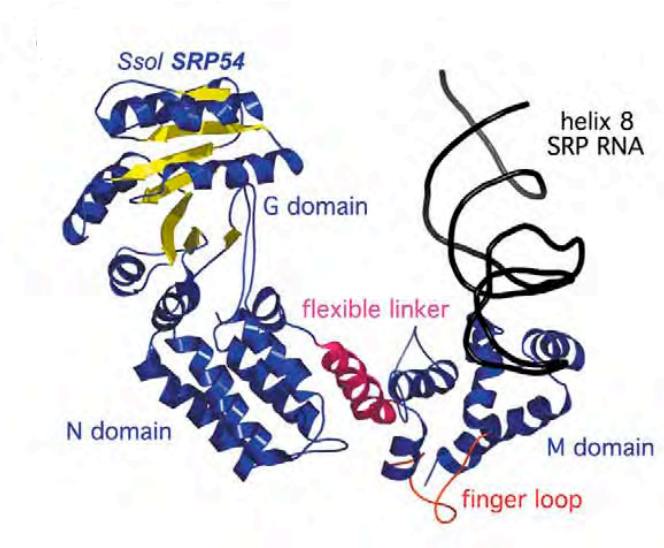



**Figure 6:** Crystal structure of Sulfolobus solfataricus Ffh subunits (SRP54). Bound to the helix 8 of the SRP RNA. The N, G and M domains can be visualized. Also seen is the linker (designated in pink) between the G and M domains (adapted from [6]).

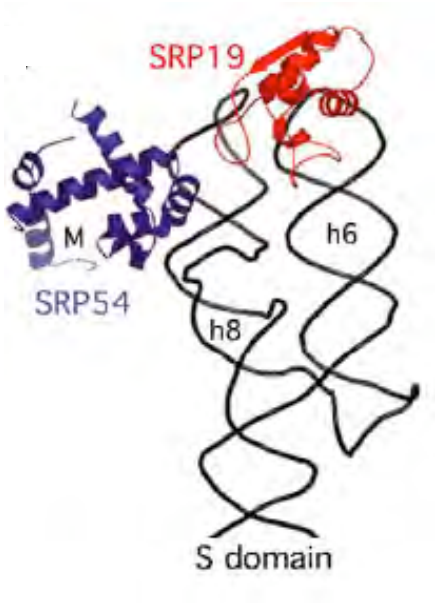



**Figure 7:** Structure of the SRP19 bound to the helix 6 and helix 8 of SRP RNA. Also seen is he M-domain of SRP54 bound to the helix 8 (adapted from [6]).

### *1.3.4 SRP receptor*

The mammalian SRP receptor (SR) is a heterodimer composed of two distinct subunits SRα, a peripheral membrane protein that is tightly associated with SRβ, an integral membrane protein [6]. Both SRα and SRβ contain GTPase domains [42-44]. Contact between SRP54 and SRα leads to the transfer of nascent chain from SRP to the translocon complex [9, 10]. SRP54 and SRα are similar in their GTPase domains and form a distinct subfamily of GTPases. They have low affinity for the nucleotide and are relatively stable in their empty states [45, 46]. In contrast SRβ is more closely related to Sar1, a member of Arf subfamily of GTPases. Mutations in the GTPase domains of SRβ disrupt the function of SR *in vivo* [47]. Recent studies also point out that SRβ also functions in recruiting the SRP-nascent polypeptide to the protein-conducting channel [48].

SRα consists of three domains, the N-terminal X-domain, which interacts with SRβ, the N-domain, which builds a four-helix bundle, and the G-domain, which binds GTP. The NG domain of the receptor is structurally and functionally homologous to the SRP54 NG domain. Recent studies have revealed the crystal structure of SRβ in complex with SRα [49]. The SRβ is composed of six β sheets surrounded by five α helices similar to that of Arf GTPase superfamily. Furthermore the studies also showed that SRβ requires being in a GTP bound form to efficiently bind to SRα (Fig. 8). Hence concluding that SRβ and SRα are conditional heterodimers and not obligate heterodimers.

The SRα is universally conserved. In bacteria and archaea, SRα are single subunits proteins called as FtsY. FtsY is either soluble or loosely associated with bacterial inner membrane [50, 51]. The SRβ subunit is expressed only in eukaryotes. So far no homologues of SRβ have been discovered in bacteria. Recently homologues of mammalian SRβ have been found in *Arabidopsis thaliana* and *Caenorhabditis elegans*.

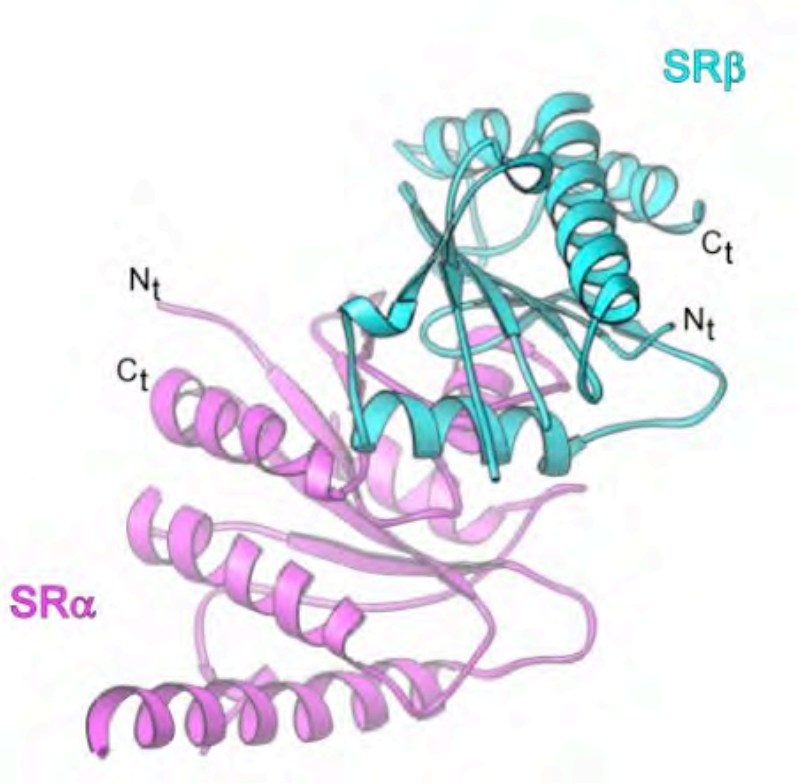



**Figure 8:** Structure of the SRb-GTP:SRα complex from yeast, with the β subunit in cyan and the SRX domain of the α subunit in magenta (adapted from [49]).

## *1.4 Biogenesis of SRP*

Several studies support the notion that SRP is assembled in the nucleolus, which is the main site for the assembly of ribosomes. Nuclear microinjected fluorescent SRP RNA initially localized into the nucleolus before appearing in the cytosol. Further microinjection studies of mutant SRP RNA's revealed that the nuclear localization elements were located in the *Alu* domain of SRP RNA as well as helix 8 in the S domain [52]. These studies were confirmed by biochemical fractionation studies, which showed that the endogenous SRP RNA was localized in nucleolus [53]. Like SRP RNA, SRP19, SRP68 and SRP72 localized into nucleolus and cytoplasm when over expressed as GFP fusion proteins suggesting that the nucleolar localization of these SRP components represents a step in SRP assembly [54]. In contrast GFP fused SRP54 did not display any nuclear localization. *In vitro* SRP assembly studies had revealed that SRP54 does not bind SRP RNA until SRP19 has first bound. Binding of SRP19 induces a stable conformational change facilitating the binding of SRP54 [37, 55, 56]. Similar experiments in yeast indicated that the GFP fused Srp14p, Srp21p, Srp68p, Srp72p accumulate in the nucleus as shown by the co-localization experiment using Nop1p, which is a nucleolar marker [57]. The nucleolar localization of the four yeast core SRP proteins, together with the fact that disruption of any of these protein's causes destabilization as well as nuclear accumulation of scR1, strongly suggest that an SRP sub particle (pre-SRP) containing these proteins and scR1 is assembled in the nucleolus. In Trypanosomes, down regulation of SRP68 and SRP72 using RNAi resulted in the accumulation of the 7SL RNA in the nucleoli and this coordinated with a decreased levels of 7SL RNA in the cytoplasm. This indicated that the presence of SRP68 and SRP72 is essential for the export of the 7SL RNA from the nucleoli to the cytoplasm [41]. Furthermore, these studies suggest that Trypanosome SRP assembly takes place in the nucleoli similar to that of mammalian and yeast SRP assembly. The specific sites of localization of the SRP RNA in nucleolus was examined in mammalian cells and it was found that it differed from the classical ribosome synthesis sites. SRP RNA is associated with rRNA deficient regions and a small portion was found to be associated with granular component of nucleolus thus confirming that SRP RNA is physically not associated with ribosome assembly [58].

SRP proteins are imported into the nucleolus by a pathway mediated by Pse1p and Kap123p. Studies on the export pathway of SRP RNA in yeast revealed that SRP RNA utilizes an Xpo1p mediated pathway. A defect in Xpo1p/CRM1 pathway led to the accumulation of SRP RNA in the nucleolus along with 28S rRNA [59, 60]. The nuclear export of this particle requires the presence of all four SRP core proteins and an intact scR1 3' end, and is mediated by the exportin Xpo1p and the nucleoporins Nsp1p (Srx1p) and Nup159p (Rat7p) [57].

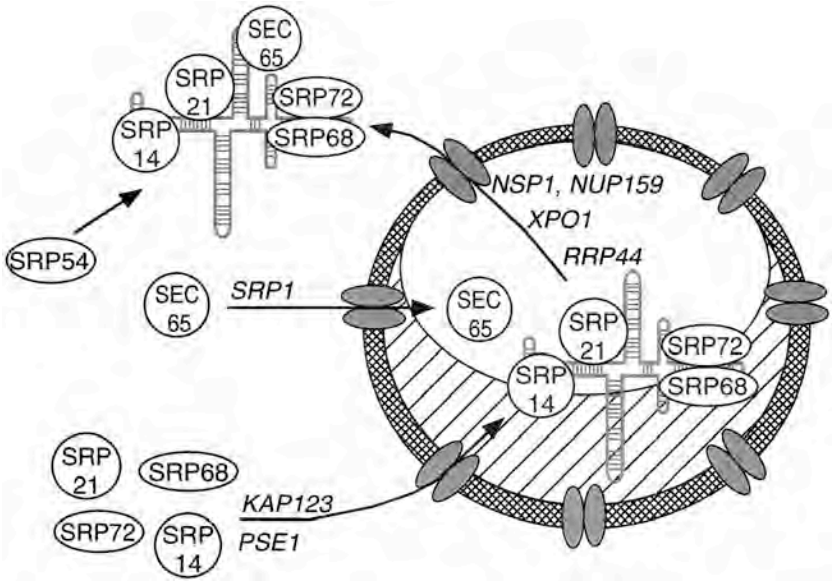



**Figure 9:** Cartoon depicting the assembly of functional SRP particle in yeast. Except SRP54 all other components of SRP are imported into the nucleoli where they assemble into a SRP sub particle. This partially assembled complex is now exported out of the nucleus in cytoplasm where it binds to SRP54 and forms a fully functional SRP (adapted from [57]).

## *1.5 Effects of SRP depletion in various organisms*

Components of SRP pathway are conserved in all the three kingdoms of life. Several studies in the yeast *S.cerevisiae,* showed that SRP is not essential for growth of yeast cells but it is required for efficient targeting of proteins to ER whereas SRP depletion was found to be lethal in *S.pombe* and *Y.lipolytica* [61, 62]. *S.cerevisiae* lacking SRP grows poorly and exhibits a multifaceted physiological response to the absence of SRP [63]. There was induction of heat shock genes and decrease in the biosynthesis of ribosomes and RNA suggesting an adaptation of the cell to the loss of the SRP pathway. However, a sudden drop in cellular SRP levels, such as observed at higher temperature with the sec65-1 mutant, is lethal for *S.cerevisiae* [64, 65]. In *E.coli,* Ffh (SRP54) and FtsY (SR) are essential genes. Ffh was found to be important for proper insertion and assembly of membrane proteins [66] whereas depletion of FtsY led to decreased expression of membrane proteins [67]. Further analysis showed that ribosomes accumulate on the membranes in Ffh depleted cells in contrast to the FtsY depleted cells where the amount of membrane bound ribosomes decreased substantially [68].

RNAi studies on SRP were first done in trypanosomes. The trypanosome SRP is unique when compared to other SRP complexes, because it contains two RNA molecules, the 7SL RNA and a tRNA like molecule [69]. Silencing of SRP54 using RNAi was lethal for trypanosomes [70]. The three parameters considered in the knock

out analysis are nuclear content, number of kinetoplasts and the shape of the cells. After 4 days of silencing, cells were multinucleated, changed their normal shape and the flagella was lost. In general, the cells became disorganized. To elucidate specifically the translocation defects arising due to the silencing, four specific proteins were examined. All four proteins were translocated quite efficiently however microscopic analysis revealed mislocalisation of these proteins. This indicated the presence of an alternate protein translocation pathway in trypanosomes. Mislocalization may be caused as a secondary effect resulting from improper sorting of other polytopic membrane proteins that are essential to maintain cell organization. More recent studies in trypanosomes show that SRP in trypanosomes is mainly essential for the biogenesis of polytopic membrane proteins [71].

In mammalian cells, the knock down of SRP54 and SRP72 to 20% of wild type levels did not appear to interfere with normal cell growth and did not reveal a significant phenotype. There was a decrease in the number of death receptor 4 (DR4) molecules on the plasma membrane. In contrast there was no significant change in the levels of death receptor 5 (DR5). The decrease in the levels of DR4 is possibly because of a defect in localization as observed by the slight accumulation of DR4 in Golgi[72]. Recent studies showed that macrophages infected with a protozoan parasite Leishmania showed a prominent decrease in the levels of SRP RNA upon successful infection [73]. This decrease coordinated with decreased secretion of a reporter protein indicating defective protein secretion in these cells. The decrease in the SRP RNA was proposed to compromise the secretion of immunocompetent proteins. This would result in the cells becoming less resistant to the parasitic infections.

## *1.6 Sequential events in SRP mediated protein targeting*

### *1.6.1 Interaction of SRP with the ribosome*

SRP has a salt sensitive low affinity for all the ribosomes engaged in the protein synthesis [74-76]. This binding of SRP is in the proximity of the nascent chain [77]. The SRP and the ribosome can therefore form a transient complex called sampling complex. The role of this complex is to sample the nascent chains for the presence of the signal sequences[64]. Once the signal sequence emerges out of the ribosome, SRP immediately recognizes it and the affinity of the SRP-RNC increases remarkably [74-76]. This stable complex is now termed as targeting complex and is targeted to the ER membrane. Cross linking studies have shown that S domain of SRP interacts with the ribosome near the tunnel exit site of the large ribosomal subunit involving ribosomal proteins L23 and L35 [78]. Similar cross-linking studies have shown that the interactions between the *Alu* domain and the ribosome are dynamic and change upon the binding to the signal peptide [79]. More recent studies using cryo-electron microscopy have depicted the binding the SRP to an elongation-arrested ribosome [80]. The images show SRP spanning from the peptide exit site to the elongation factor-binding site in a kinked conformation (Fig. 8). The SRP core is positioned with the SRP54N interacting with L23p and the SRP54M with the hydrophobic groove

bound with the signal sequence, sits right on the top of the exit site. The *Alu* domain is positioned in the elongation factor-binding site (see Introduction 1.6.3).

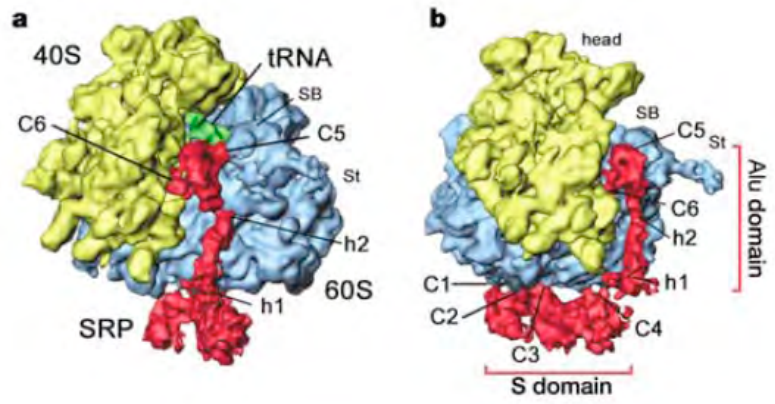



**Figure 10:** Cryo electron microscopy images of SRP bound to an elongation-arrested ribosome. The *Alu* domain is positioned at the elongation factor-binding site and the s domain is positioned at the peptide exit site. C5 and C6 are the connections of the *Alu* domain with the ribosome. C1-C4 are the connections of the S domain of SRP with the ribosome. In yellow is the 40s ribosome subunit and in blue is the 60s ribosome subunit h1 and h2 are hinges of the 7S RNA backbone of SRP; St, stalk; SB, stalk base (adapted from [80]).

## *1.6.2 Interaction of the SRP with signal sequence*

Signal sequences are generally present at the N-terminus of the protein and are between 20-30 amino acids and α helical in structure. They encode a short positively charged N-terminal region, a central hydrophobic core of 10 to 15 residues with a marked preference to leucine or alanine [81] and a more polar C-terminal region, which includes the site for the cleavage by signal peptidase (Fig. 11). The signal sequences are remarkably tolerant to the amino acid substitutions, as long as their central hydrophobic character is retained [82, 83]. However a single mutation in the hydrophobic core of the signal sequence can disrupt its function. Mammalian SRP appears to interact with the signal peptides that vary widely in their hydrophobicity whereas yeast and bacterial SRPs bind only to the signal sequences having high hydrophobicity index [84-86].

Signal sequence binding to the SRP is via the M domain of SRP54 as demonstrated by the crosslinking studies [87]. The M domain contains typically high percentage of methionines. Methionine has a highly flexible hydrophobic side chain because it is unbranched and displays unique conformational properties of the thioether linkage [87, 88]. These features led to the hypothesis that methionines and other hydrophobic residues in the M domain of SRP54 are arranged such that their flexible side chains form the hydrophobic binding site for the signal sequence and also

provide sufficient plasticity to recognize the wide variety of signal sequences [89, 90]. X-ray crystallography studies and *in vivo* studies on protein secretion in yeast have indicated the presence of electrostatic interaction between the basic amino acids in the N-terminus of the signal sequence and phosphate backbone of SRP RNA [32, 91].


**Figure 11.** Tripartite structure of signal sequences. The signal sequence can be divided into three domains. A hydrophilic **n** region composed of 15-20 aa. A hydrophobic core **h** composed of 6-12 aa. A polar C-terminal region **c** composed of 5-6aa (adapted from [113]).

### *1.6.3 Elongation arrest function of SRP*

The interaction of the signal sequence with SRP is followed by a transient pause in the elongation of the nascent chain termed the elongation arrest function of SRP. Elongation arrest function was first discovered as a pause in the translation of signal peptide encoding proteins upon binding of SRP [92]. The *Alu* domain of SRP, which is comprised of SRP9, SRP14 and the 5' and 3' ends of the 7SL RNA performs the elongation arrest function [93]. In the initial studies, it was observed that the SRP lacking either the *Alu* domain or SRP9/14 heterodimer showed a reduction in the translocation efficiency [77]. Later experiments have shown that these are defective in signal sequence independent binding to the ribosomes [77, 94]. A C-terminal truncation of murine SRP14 resulting in the loss of 20 amino acids disrupted the elongation arrest function [95]. The ribosome binding capacity of this mutant was intact. A similar truncation of SRP14 in *S.cerevisiae* (Srp14pΔ29) resulted in the loss of elongation arrest function [96]. These studies suggested that the C-terminus of SRP14 plays an important role in the elongation arrest function.

Most of the studies elucidating the details of this function were done in cell free translation systems using reconstituted SRP. In heterologous cell free translation/translocation systems using canine microsomes, wheat germ lysate and SRP, the recognition of signal sequence by SRP was followed by an arrest in the translation at one or more sites [44, 92, 97, 98]. In homologous system using rabbit reticulocyte lysate, SRP causes a delay in the accumulation of full-length proteins rather than an arrest as observed with the wheat germ system [99]. However, specific pause sites of the ribosomes at the level of mRNA were revealed in these studies.

The loss of elongation arrest function results in defective translocation in heterologous cell free systems [95, 100]. This led to the hypothesis that elongation

arrest increases the time window during which the nascent chain remains in a translocation competent state. In *S.cerevisiae*, loss of elongation arrest function did not induce any apparent growth defects or any defects in translocation into the ER under normal conditions. The strain is temperature sensitive for growth. A defect in tight coupling between translation and translocation was observed as demonstrated by the ubiquitin translocation assay [101]. A mathematical model proposed to explain the effects of SRP on translation and translocation proposed that translocation into the ER requires only the catalytic action of SRP and is determined by the accumulation of protein synthesizing ribosomes at the sites of SRP receptor. It also predicts that the translation inhibition is required only when the SRP receptor concentration is limiting [102].

More recent studies depicting the cryo-electron microscopy images of SRP bound to an elongation-arrested ribosome positioned the *Alu* domain in the elongation factor-binding site. Eventhough it is not possible to assign a contact site for the SRP14 on ribosome, the electron densities of the *Alu* domain and the elongation factor-2 at the ribosome had a high resemblance. This suggests that *Alu* domain might interfere with the binding of elongation factors and execute the elongation arrest function.

### *1.6.4 Targeting to the membrane*

In addition to the signal sequence recognition the other major function of SRP is to interact with the SR present on the ER membrane to ensure translocation of the nascent chain into the lumen of ER [42, 43]. Numerous biochemical experiments have shown that formation of a stable SRP-SR complex requires both the GTPases to bound with GTP. Assembly of the SRP-RNC complex slows down the elongation of the nascent chain and it induces stable GTP binding to SRP54. This results in the primed state of SRP54 with a conformation that is ready to interact productively with the SRα. In the ER membrane, the contact of SRβ with the free translocon induces GTP binding by SRβ, which results in formation of the SRα-SRβ complex [103]. The SRP-RNC complex is then targeted to the ER membrane where it interacts with SRα. SRP54 and SRα NG domains interact in a GTP dependent manner, which brings GTP into the catalytic centre. Recent studies depicting the crystal structures of the GTPase domains of bacterial Ffh and FtsY show that these GTPases undergo large-scale conformational changes upon binding with each other [104]. Both the GTPases interact as head to head, quasi-two fold symmetrical heterodimer. The interaction is extensive and involves residues from both the N and G domain. A readjustment of the relative position of N and G domains takes place allowing the N domain of both proteins to bend towards its binding partner and form an additional face of interaction (Fig. 8). Following the delivery of the ribosome nascent chain to the empty translocon, SRP and SR reciprocally stimulate the hydrolysis of GTP of each other [105, 106]. This results in the dissociation of the targeting complex allowing SRP and SR to participate in subsequent targeting reactions. Recent studies performed by isolating distinct classes of mutant GTPases defective in different steps of the interaction between SRP and SR suggests the presence of extensive conformational changes during the activation of SRP-SR complex [107]. All these conformational changes ensure the binding and release of the cargo at the appropriate place and time.


**Figure 12:** The heterodimer of Ffh and FtsY from Thermophilus *acquaticus* shown in two orientations. In Ffh the alpha helices and beta strands are indicated by blue and yellow color. Green and pink colors indicate the same in FtsY. The quasi-two-fold axis is indicated in black and the IBD loops are shown in red (adapted from [6]).

### *1.6.5 Translocation into the ER*

The Sec61 complex is responsible for directing the translocation and integration of membrane and secretory proteins in the endoplasmic reticulum. The heterotrimeric Sec61 complex has been identified as a central component of the translocation machinery. In mammals, the Sec61 complex contains an α subunit with 10 membrane-spanning domains, and β and γ subunits, each of which spans the membrane once [108]. Initially, cross-linking experiments provided evidence that the Sec61 complex forms the actual channel [109]. This complex functions in association with a variety of proteins including signal peptidase, TRAM and TRAP. Signal sequences and transmembrane domains are recognized by the translocon at the membrane. This event is essential in distinguishing the translocon substrates from other proteins, which are present in the surrounding environment of the translocon [35]. Cross-linking studies performed in yeast and mammalian system suggests that signal sequence binds to a site that is at the interface of the Sec61 channel and the surrounding lipid bilayer. Biochemical analysis of Sec61 has identified helices 2 and 7 as the binding sites. TRAM is implicated in binding the hydrophilic region preceding the hydrophophobic core of the signal sequence [10, 110]. The role of TRAP is less clearly understood. It is highly unlikely that all these components would come together to form a single translocon instead they could form translocons, which have different components for different substrates. The physical transfer of the ribosome nascent chain complex from the targeting complex to the translocon is mediated by SR. Previous studies have shown that SR interacts with the ribosome and also with the SRP. More recent cryo electron microscopy structures have predicted that SR interaction with SRP results in a conformational change resulting in loss of interaction with the ribosome [111]. This exposes the two ribosomal proteins L23e

and L35, which now become available to bind to the translocon. Once the nascent chain is inserted into the translocon pore the translation is restored. As the nascent chain is further elongated, the signal sequence is cleaved off by the signal peptidase complex at a recognition site following the hydrophobic core [112]. After cleavage, the signal peptide remains in the lipid bilayer where it is subsequently processed by a signal peptide peptidase. Fragments of the signal sequence are then released into the cytosol [113].

During the translocation of the proteins into the lumen of the ER, the pore must prevent the free movement of small molecules such as ions and other metabolites. Recent studies have pointed out towards two possible models, but the exact mode of maintenance of permeability barrier is still controversial and unresolved (for review see [114]).

Fluorescent quenching studies show that in order to maintain the permeability barrier during the insertion of proteins into the ER, the ribosome and BiP gate the cytosolic side and the luminal side of the translocon alternatively. BiP mediated gate seals the translocon until the nascent chain reaches at least 70 amino acids. When the nascent chain reaches 70 amino acids, BiP assumes the ATP bound open pocket confirmation [115]. Further after completion of the translation, BiP reseals the translocon pore on the luminal side. Another model, which has been deduced by the crystal structure, suggests that the membrane barrier is formed by the channel itself with both the plug and the pore ring contributing to the seal [116]. The function of the plug is to lock the channel in the inactive state (Fig. 13). In the acive state when the plug is open, the pore ring would fit like a gasket around the polypetide chain to prevent small molecules from entering the lumen [117]. The translocated protein undergoes further modification and is transported to its final destination from the ER.

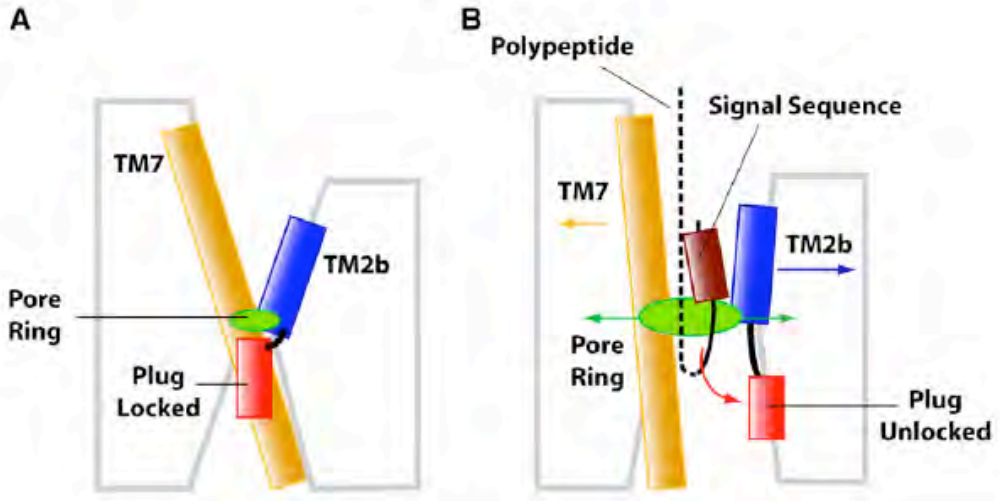



**Figure 13**: Maintenance of permeability barrier by the plug domain. (A) Resting channel with the locked state of the plug domain. (B) In the presence of the substrate the plug is unlocked and the pore ring forms a gasket like structure around the substrate which is entering the translocon to prevent small ions from diffusing from the ends (adapted from [116]).

## *1.7 Aim of the thesis*

Several studies have been done in the past 25 years to understand the cotranslational protein-targeting pathway mediated by the SRP. Most of the work characterizing the SRP functions was done in a cell free translation system and in the presence of canine microsomes. These studies helped us to understand in great detail as to how the different components of SRP work as a complex. As of today the structural details of most of the SRP components is known, which helped us in understanding the interactions between the various SRP components and other components present in the targeting pathway. Furthermore, the *in vitro* studies have clearly elucidated the mechanistic details of the different steps involved in the SRP mediated protein targeting. Eventhough we now know the structure and function of SRP in great detail, it is always important to understand these functions in the context of a cell or an organism. Studies have been done in bacteria, yeast and other organisms to understand the functions of SRP in a cellular context. However, these organisms were sufficiently different from mammalian cells both in their cellular organization and functions. During the beginning of my thesis, no studies have been performed on the role of SRP in mammalian cells. In this context, it was important for us to understand the cellular and mechanistic functions of SRP in mammalian cells.

**Aim 1**: The main aim of this study was to elucidate the cellular roles of SRP in mammalian cells. We utilized the RNAi technology to successfully knockdown the different subunits of SRP. Using these SRP depleted cells; we further tried to understand the importance of SRP for post ER membrane trafficking in mammalian cells.

**Aim 2:** Elongation arrest function of SRP has been studied in the *in vitro* systems and the existence of this function in mammalian cells has been a point of major debate. The main aim of this study was to characterize the elongation arrest function of SRP in mammalian cells and also to understand the reason for the existence of this function.

# *2. RESULTS*

## *2.1 Inefficient targeting to the endoplasmic reticulum by the signal recognition particle elicits selective defects in post-ER membrane trafficking.*

### *2.1.1 Summary*

Although many studies were done in the cell free assay systems to understand the mechanistic aspects of SRP functioning, there were no significant studies elucidating the cellular roles of SRP in mammalian cells. We decided to investigate the effect of low levels of functional SRP on the localization and accumulation of ER bound proteins and also its effect on post-ER membrane trafficking in mammalian cells. To achieve this, we depleted the levels of endogenous SRP proteins using RNAi.

To lower the levels of functional SRP subunits using RNA interference (RNAi), we initially generated *in vitro* synthesized double stranded siRNA. Using these siRNA we were able to successfully down regulate exogenously expressed SRP proteins. However, when we tried to down regulate the endogenous SRP proteins, the down regulation was not very significant at 72 h post transfection. This might be because of the high stability of the SRP proteins. Also, the siRNA have relatively short half-lives and previous studies have shown that beyond 72 h siRNA do not function efficiently. To efficiently knock down the endogenous SRP proteins, we generated vectors encoding short hairpin RNAs (shRNA) against three different subunits of SRP: SRP14, SRP54, SRP72. The shRNAs are expressed constitutively over longer period of time enabling the down regulation of stable proteins. The three different SRP proteins chosen for the knockdown presumably effect different functions of SRP (see introduction). We were interested in knowing, if the three knockdowns would produce similar or different phenotypes. The shRNA constructs were transfected individually into either HEK 293T or HeLa cells. Time course experiments revealed that the three SRP proteins were down regulated with maximum efficiency between 144-168 h post transfection. SRP RNA was also significantly down regulated in all the three knockdown cells, albeit with highest efficiency in SRP14 knockdown, indicating that very little functional SRP was present in these cells. The low levels of SRP RNA also indicated that all these three proteins are important for its accumulation in the cell.

To analyze the cellular roles of SRP, we examined the effect of SRP knockdown on the accumulation and localization of ER targeted proteins. We chose four different reporter proteins, which are targeted to different locations in the cell. We observed that several endogenous and the four reporter proteins failed to accumulate at the desired levels in their respective locales. This was accompanied by mislocalization of certain proteins such as VSV-G and transferrin receptor (TfnR) to the Golgi.

In order to determine whether the defects we observed above were a result of defective translocation of proteins into the ER, we monitored for the increased accumulation of precursor protein. We detected an increased accumulation of protein in two of the reporters in the presence of proteasomal inhibitor confirming defective translocation into the ER.

A previous study in yeast established that low levels of SRP become sufficient to ensure its normal function in ER-targeting by lengthening the time window during which SRP can functionally interact with the ribosome. Slowing down the rate of translation elongation using sub-lethal doses of cycloheximide rescued the translocation efficiency at lower levels of SRP. Cycloheximide acts on the translation elongation by preventing the transfer of peptidyl tRNA from A site to the P site. Anisomycin, which inhibits the translation elongation by interfering with the transpeptidylation reaction, cannot rescue the defective translocation. This suggests that SRP interacts with the nascent at a specific step in elongation. Addition of cycloheximide is thought to increase the time window for low levels SRP to interact

with nascent chain and ensure efficient translocation. We observed that reporter proteins accumulated in higher amounts at the respective locales in the presence of sub-lethal concentrations of cycloheximide and not in the presence of anisomycin in mammalian cells indicating that defects we observe are specific to down regulation of functional SRP. These studies also suggested that similar to yeast the mammalian SRP also binds to the ribosome at a specific step in the elongation.

The mislocalization of proteins in SRP depleted cells indicated defects in post ER membrane trafficking. These cells can no longer sustain efficient anterograde protein traffic. In addition, these cells also failed to perform retrograde traffic of shiga toxin B subunit (STxB) with most of the protein once again accumulating in the Golgi. Recombinant STxB undergoes retrograde transport and accumulates in the ER under normal conditions. Furthermore, endocytosed transferrin receptor failed to recycle back to the plasma membrane and accumulated in Golgi. This indicated that the cells are now limiting in crucial components of Golgi complex, which are essential to maintain anterograde and retrograde traffic in the cells. In contrast, no defects were observed in the endocytic pathway to lysosomes in the cells depleted of SRP.

To gain further insights into the cellular roles of SRP, we analyzed the cells depleted of SRP for growth defects. We observed a significant slow down in the cell growth in the cells depleted of SRP indicating an important role for SRP in maintaining cell survival (Fig. S1). Furthermore there was an activation of stress response in these cells as indicated by the two fold increased accumulation of BiP in the SRP14 depleted cells.

We investigated the effect of depletion of one SRP subunit on the other subunits. We see a differential effect on the steady state protein levels of different SRP subunits (Fig. S2). Together, all these results show that SRP is crucial for maintaining normal cellular functions in mammalian cells.

## *2.1.2 Publication*

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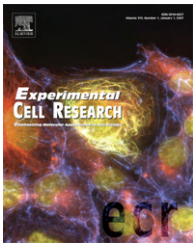

Research Article

# Inefficient targeting to the endoplasmic reticulum by the signal recognition particle elicits selective defects in post-ER membrane trafficking

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ABSTRACT

The signal recognition particle (SRP) is required for protein translocation into the endoplasmic reticulum (ER). With RNA interference we reduced its level about ten-fold in mammalian cells to study its cellular functions. Such low levels proved insufficient for efficient ER-targeting, since the accumulation of several proteins in the secretory pathway was specifically diminished. Although the cells looked unaffected, they displayed noticeable and selective defects in post-ER membrane trafficking. Specifically, the anterograde transport of VSV-G and the retrograde transport of the Shiga toxin B-subunit were stalled at the level of the Golgi whereas the endocytosed transferrin receptor failed to recycle to the plasma membrane. Endocytic membrane trafficking from the plasma membrane to lysosomes or Golgi was undisturbed and major morphological changes in the ER and the Golgi were undetectable at low resolution. Selective membrane trafficking defects were specifically suppressed under conditions when low levels of SRP became sufficient for efficient ER-targeting and are therefore a direct consequence of the lower targeting capacity of cells with reduced SRP levels. Selective post-ER membrane trafficking defects occur at SRP levels sufficient for survival suggesting that changes in SRP levels and their effects on post-ER membrane trafficking might serve as a mechanism to alter temporarily the localization of selected proteins.



## Introduction

Intracellular sorting of newly synthesized proteins is essential for generating and maintaining cell structures and functions. Translocation into the endoplasmic reticulum (ER) is the first step in the sorting pathway of luminal and membrane proteins of cellular compartments as well as of plasma membrane, nuclear envelope and secretory proteins. The common hallmark of these proteins is the N-terminally located hydrophobic signal sequence [1]. SRP and its receptor in the ER membrane are a molecular machine associated with the specific targeting of proteins into the ER (for review, see [2]). From the ER, cargo proteins move through Golgi and beyond by vesicular transport in a vectorial fashion (for reviews, see [3,4]). The anterograde transport is counterbalanced by retrograde transport of lipids, ER and Golgi components as well as of external components taken up at the plasma membrane including toxins (for review, see [5]).

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The basic mechanism of SRP-mediated ER-targeting is highly conserved in evolution and consists of the recognition of signal sequence-bearing nascent chains by SRP followed by their specific delivery to the site of translocation in the ER (for reviews, see [6–9]). SRP is composed of a 300 nts-long RNA (SRP RNA) and six protein subunits (Fig. 1A). The signal recognition and targeting functions were assigned to SRP54 and the RNA stem that constitutes its binding site. SRP9/14 bound to the Alu portion of the RNA is required to delay nascent chain elongation during targeting. Elongation arrest activity has been found to be important for efficient translocation. Consistent with their functions, SRP54 and the Alu domain bind closely to the nascent chain exit site and in the elongation factor-binding site of ribosomes, respectively [10–12]. Although essential for co-translational targeting, still little is known about the exact functions of SRP68 and SRP72.

Bacterial SRP is essential for growth [13] because of its important role in biosynthesis of polytopic membrane proteins ([14,15]; for review, see [16]). Deletion of SRP is also lethal in *Y. lipolytica* and in *S. pombe* [17,18] whereas *S. cerevisiae* continues to grow, albeit poorly, in the absence of SRP [19]. It survives by first increasing the expression of chaperones followed by decreasing biosynthesis of ribosomes [20]. However, a sudden drop in cellular SRP levels, such as observed at higher temperature with the sec65-1 mutant, is lethal for *S. cerevisiae* [21,22]. In bacteria and yeast, only a subset of proteins uses the SRP-dependent pathway whereas the other proteins reach the plasma/ER membrane via a SRP-indepen-

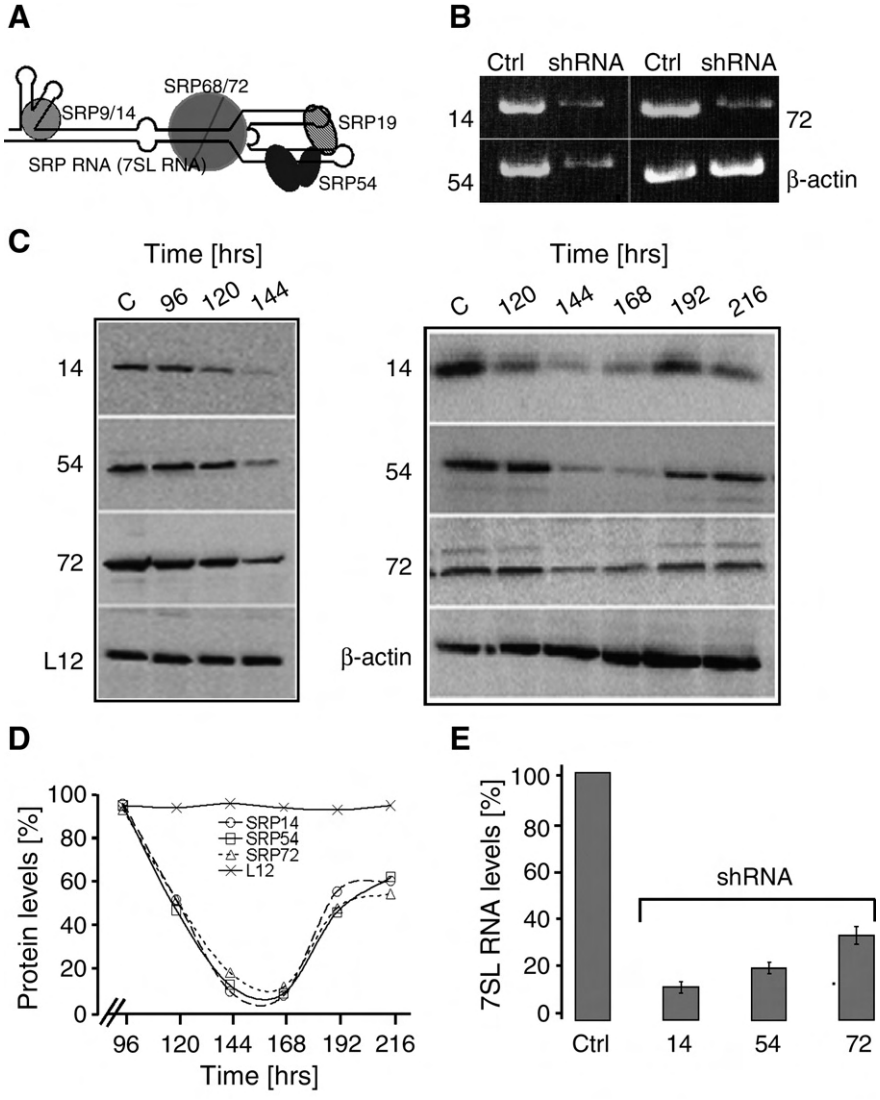



**Fig. 1 – Reducing cellular levels of SRP with RNAi against individual subunits. (A) Schematic representation of SRP. (B) 2% agarose gel of the mRNA-specific PCR products (25 cycles) obtained from total cellular RNA of HEK 293T cells harvested at 72 h post transfection with shRNA-expressing plasmids against SRP14, SRP54 and SRP72. (C) Time course of the expression levels of SRP proteins revealed with subunit-specific antibodies after RNAi against SRP14, SRP54 and SRP72. Equal amounts of cell extracts were loaded in each lane. Control proteins: The ribosomal protein L12 and β-actin. (D) Quantification of the Western blots. Expression levels were normalized to control cells and represent the average of two independent experiments. (E) The relative expression levels of SRP RNA in SRP14, SRP54 and SRP72-depleted cells 144 h post transfection: 14: 11.8±0.3%; 54: 20.8± 0.3%; 72: 34.5±0.3% ($n$=3).**

dent posttranslational route. In trypanosomes, cellular roles of SRP were studied using RNA interference (RNAi). Upon depletion of SRP54 or SRP19, the cells displayed aberrant morphologies and proteins became mislocalized before the cells eventually died [23]. Silencing of SRP68 or SRP72 resulted in the sudden death of the parasite. The acute phenotype of SRP68 and SRP72 depletions may be explained by a severe defect in SRP RNA assembly leading to a rapid drop in SRP levels [24].

Although many mechanistic details of SRP-mediated ER-targeting have been elucidated using mammalian SRP and microsomes, still very little is known about the cellular roles of SRP in mammalian cells. It was recently found that the successful infection of macrophages with *Leishmania* was coupled to the down regulation of SRP RNA levels. Low levels of SRP were proposed to neutralize the cellular defense mechanisms by preventing the cells from secreting the proteins necessary to counteract the parasitic infection [25]. In addition, low levels of SRP54 and SRP72 were found to interfere with death receptor 4 (DR4)-induced apoptosis due to reduced DR4 levels in the plasma membrane [26]. In contrast, another death receptor, DR5, was still expressed normally in the plasma membrane. Interestingly, HeLa cells with stable depletions of SRP54 or SRP72 displayed no growth defects or aberrant morphologies. The selective defects in DR4 localization, which accumulated in Golgi in SRP-depleted cells, together with the observed mislocalization of proteins in trypanosomes suggested that low levels of SRP may interfere with post ER membrane trafficking.

To get more insight into the question of how low levels of SRP affect accumulation and localization of ER-targeted proteins as well as protein trafficking, we depleted HeLa and HEK 293T cells of three different SRP protein subunits using RNAi. We observed that at ten-fold reduced levels SRP, accumulation of endogenous and reporter proteins in different cellular locales was diminished to variable degrees and two proteins were mislocalized. In addition, our results confirmed that inefficient ER-targeting at low levels of SRP leads to selective defects in post-ER membrane trafficking. Efficient targeting by SRP is therefore critically required to maintain proper post-ER membrane trafficking in mammalian cells. In addition, since SRP-depleted cells display no deleterious phenotype, changes in SRP levels and their effects on post-ER membrane trafficking might serve as a mechanism to alter temporarily the localization of selected proteins.

## Materials and methods

### *Cell culture and RNAi*

Human HeLa and HEK293T cells were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (both from Sigma). shRNAs were produced from the expression vectors pSUPER.retro.puro (Oligoengine) containing the appropriate inserts (pSR54, pSR72, pSR14 and firefly luciferase) complementary to the following target sequences: 14 sense: 5′-AGGGCATACATTTCCTGCT-3′, 54 sense: 5′-GAAATGAACAGGAGTCAAT-3′, 72 sense: 5′-GAAGGAGCTTTATGGACAA-3′ and firefly luciferase sense: 5′-CGTACGCGGAATACTTCGA-3′. The cells were transfected by the calcium phosphate protocol and 24 h post transfection, they were grown for 24 h in the presence of 3 μg/ml of puromycin dihydrochloride (Sigma) to select transfected cells. Where applicable, cells were grown in the presence of cycloheximide or anisomycin (Sigma) between 120 and 144 h post transfection at the concentrations indicated.

### *Real Time-PCR*

Total RNA was extracted at 144 h post transfection from cells using RNeasy Mini Kit (Qiagen GmbH). 10 ng total RNA was used for the quantification of SRP RNA in cell extracts. A one step quantitative real time PCR was performed using the Quantitect custom assay kit (Qiagen GmbH) and monitored with the iCycler (BIO-RAD). The relative amounts of SRP RNA were obtained by comparison to standard curves produced from different amounts of total RNA from wild type cells. The experiment was repeated three times. Primers for PCR: SRP RNA sense: 5′-TAAGTTCGGCATCAATATGGT-3′, SRP RNA antisense: 5′-GATCAGCACGGGAGTTTT-3′, Quanti Probe: 5′-GTCGGAAACGGAGCAGG-3′. For the measurement of mRNA levels of SRP14, SRP54, SRP72, total RNA was extracted at 72 h post transfection using RNeasy Mini Kit. 200 ng of total RNA was used for the reverse transcription reaction using Quantitect custom primers against the three different mRNAs and SYBr green (BIO-RAD) as an indicator. PCR products had the expected sizes. For quantification, the values obtained were normalized to β actin mRNA levels. Primers for mRNA amplification: SRP14 sense: 5′-ACATGGATGGGCTGAAGAAGAGAG-3′, antisense: 5′-TTGCTGCTGCTGTTGTTGCTG-3′ SRP54 sense: 5′-GCCGCCACAAACAAGAAGACTC-3′, antisense: 5′-TCACAAGCCTGCCCAATGGAG-3′, SRP72 sense: 5′-AGGAGTTAAAGCATAAACCAGGCATG-3′, antisense: 5′-ACCTCAATGGCACTATCAATATCTTCTTC-3′, β Actin sense: 5′-AGATGTGGATCAGCAAGCAGGAG-3′, antisense: 5′-CGCAAGTTAGGTTTTGTCAAGAAAGG-3′.

### *Enzymatic assays for SEAP and LDH*

HEK 293T cells grown in 10 cm plates were co-transfected with pSEAP2 control plasmid and one of the plasmids pSR54, pSR72 and pSR14. The growth medium was changed every 24 h starting from 72 h post transfection. 15 μl of the growth medium was used to perform the assay in a 96 well plate. Cell extracts (10 μg) were used to determine the activity of intracellular SEAP with the Great EscAPe SEAP kit (BD Biosciences) following the manufacturer's instruction. The chemiluminescence signals were collected by the Chameleon multiplate reader (Hidex) and the data were analyzed by Microwin software. The LDH assay was performed from the same extracts. 10 μg of the cell extract was added to the reagent solution (10 mg/ml NADH, 10 mg/ml sodium pyruvate in TRIS buffer pH 7.5) and the absorbance of NADH at 340 nm was monitored for 6 min. The data were plotted to observe the kinetics of the enzymatic reaction.

### *CD63-GFP, NAGT1, ECFP-ER and GFP-SRα quantification*

Cells were co-transfected with the reporter plasmid and either pSR14, pSR54, pSR72 or empty vector plasmid and the fluorescence was monitored. The reporter constructs were

CD63-GFP [27], pNAGT1 [27] and pECFP-ER (Invitrogen). pTfnR-GFP and pGFP-SRα were obtained by cloning the human cDNAs of TfnR (kind gift from J. Gruenberg, University of Geneva) and of SRα (Invitrogen) into pEGFP-N1 and pEGFP-C1, respectively. CD63-GFP expression was analyzed using FACS (FACS Calibur). The data were quantified from the scatter plots. The fluorescent intensities of NAGT1-GFP, ECFP-ER and TfnR-GFP were quantified by capturing 100 images of cells present in different microscopic fields with same intensity of light for both the control cells and the RNAi induced cells. The average surface intensity (voxel/cell) of the images was determined with the IMARIS software.

### *VSV infection, immunofluorescence and microscopy*

Cells expressing shRNA were infected with VSV as described [28]. The surface staining of VSV-G was quantified using IMARIS software. Immunofluorescence was done as described previously [28]. For internal staining cells were permeabilized with saponin (0.05%) during the incubation with the first antibody. Cover slips were mounted on slides containing Mowiol (Sigma) solution mixed with DAPI (0.25 mg /ml, Invitrogen). VSV-G, TfnR and the LAMP1 proteins were labeled with the monoclonal antibodies 17.2.21.4 ([29], 1:100 dilution), H68.4 (Zymed Lab Inc. 1:200) and CD107a (S. Carlson, Umea University Sweden, 1:100), respectively. For Golgi stain, live cells were incubated with GS-II lectin (Molecular Probes). GS-II specifically stains the medium and trans Golgi stacks at a concentration of 30 μg/ml. To monitor transport from plasma membrane to lysosomes, rhodamine dextran (Rhod-DEX) was co-localized with LAMP1 as described previously [30]. The retrograde transport assay using Cy3-labeled STxB was done as described previously [31], and 200 cells were examined visually for the quantification of the phenotypes.. The cover slips were analyzed by the fluorescent microscope Zeiss Axiovert 135T using a 100× (Plan Neofluar, NA-1.40) magnification. Images were captured using a charge-couple device camera (photometric CE200A) with Open Lab software.

### *Western blotting*

Cells grown in 6 cm plates were washed with ice cold PBS and lysed in 10 mM Hepes pH 7.4, 300 mM potassium chloride, 5 mM magnesium chloride, 1 mM EGTA, 1% Triton X-100 supplemented with a cocktail of protease inhibitors. Lysates were cleared by centrifugation at 10,000×*g* for 15 min at 4 °C. and, after dilution, the protein content determined with Bradford reagent (BIO-RAD). Equal amounts of protein (25–40 μg) are displayed by PAGE. Anti-SRP14 and anti-SRP19 antibodies were used as described previously [32]. SRP54, SRP72, SRP68 and L12 antibodies were raised in rabbits using the peptide CADDFRAGAFDQLKQ (Sigma-Genosys) and the urea-soluble recombinant proteins (Gramsch Laboratories), respectively. Immunopurification and immunoblotting was done as described previously [12]. Antibodies were used at the following dilutions: SRP68 and SRP72: 1:100, SRP54, SRP19 and L12 and VSV-G: 1:500, LDH (Fitzgerald): 1:1000, GFP (Molecular Probes) 1:1000, VSV-G [33] 1:500. Sec61α, β-actin and calnexin antibodies were from Abcam and used as suggested by the manufacturer.

## Results

### *SRP levels are reduced ten-fold with RNAi against individual SRP proteins*

To reduce SRP levels in mammalian cells, we expressed short hairpin RNAs (shRNAs) with complementarities to regions of the mRNAs encoding SRP14, SRP54 and SRP72 (Fig. 1A). In cells, shRNAs are further cleaved into siRNAs, which suppress the expression of the desired proteins [34]. The vectors targeting any of the three proteins were transfected individually into HEK 293T and into HeLa cells and the transfected cells selected 24 h post transfection with puromycin for 24 h. As a negative control, we used either the empty vector or the vector expressing shRNA against firefly luciferase. Similar results were obtained with both vectors. The levels of SRP14, SRP54 and SRP72 mRNAs were decreased to 32, 34 and 36%, respectively, at 72 h post transfection as determined by real time PCR (Fig. 1B). The negative control, actin mRNA, was unchanged. We did not quantify the mRNA levels at later time points. However, based on the analysis of the protein levels (see below), they were likely to decrease further.

The protein levels were analyzed by quantitative immunoblotting (Figs. 1C, D). The cellular levels of any of the three proteins followed a similar time course over 216 h. It started to decrease at 120 h and was diminished about ten-fold between 144 and 168 h post transfection. Beyond 168 h, it increased again, because RNAi became ineffective. At 216 h post transfection, the cellular protein levels had increased again to 60% as compared to control cells (Fig. 1D). In previous studies, siRNAs were used to reduce the expression levels of SRP72, SRP54 and 7SL RNA [25,26]. In those experiments a significant reduction was observed already 72 h post transfection. In our hands, the reduction of SRP components was more efficient with shRNAs than with siRNAs. However, it became effective later, most likely because of the time needed to express and process shRNAs. The levels of two cytosolic control proteins, the ribosomal protein L12 and β-actin, remained unchanged in all experiments confirming the specificity of the RNAi (Fig. 1C). In agreement with previous studies [26], we noticed that even during the time period with lowest SRP levels, cells continued to divide normally and had a normal morphology. In HeLa cells we obtained the same results (not shown), and HEK 293T and HeLa cells were used simultaneously or alternatively in the subsequent experiments and they always gave the same results (see below).

In yeast, SRP RNA levels decrease rapidly in the absence of any of the SRP proteins [35]. We examined whether this is also the case in mammalian cells. Mammalian SRP RNA (7SL RNA) was quantified with real time PCR at 144 h post transfection and its level was reduced in cells depleted of any of the three SRP subunits (Fig. 1E), albeit the level was lowest in cells depleted of SRP14. 7SL RNA levels were reduced nine-fold in SRP14-depleted cells as well as five-fold and three-fold in cells with reduced levels of SRP54 and SRP72, respectively. Hence, a full set of SRP proteins is required for 7SL RNA to accumulate at normal levels.

In mammalian cells, practically all SRP54 and SRP72 are assembled into SRP [32] and unpublished results) and both

**Table 1 – Relative expression levels of different ER-targeted and control proteins in SRP-depleted cells**

| | Protein | Cellular locale | Method [a] | Relative expression levels [%] [b] | | | |
|---|---|---|---|---|---|---|---|
| | | | | shRNA | | | |
| | | | | 14 | 54 | 72 | Average |
| Reporter proteins | VSV-G, viral | Plasma membrane | IF, cell surface | 35±1.6 | 37±1.8 | 38±1.8 | 37±2 |
| | | | W | 71±2.2 | | | 71±2 |
| | VSV-G, plasmid | | W | 26±3.0 | | | 26±3 |
| | SEAP | Secretory protein | E | 16±0.3 | 26±1.3 | 34±1.1 | 25±9 |
| | CD63-GFP | Endosomes | FACS | 47±4.2 | 56±1.8 | 43±2.9 | 49±7 |
| | | | W | 45 | | | 45 |
| | NAGT1-GFP | Golgi | FI | 29±2.4 | 36±0.5 | 20±1.7 | 28±8 |
| | ECFP-ER | ER | FI | 26±1.8 | 25±2 | 35±1.4 | 29±6 |
| | GFP-SRα | ER | W | 99±0.8 | – | – | 99±1 |
| | TfnR-GFP | Plasma membrane | FI | 26±3.1 | | | 26±3 |
| Endogenous proteins | | | W | 28±3.6 | | | 28±4 |
| | TfnR | Plasma membrane | IF, cell surface | 49±2.6 | | | 51±3 |
| | | | W | 69±2.7 | | | 69±3 |
| | Calnexin | ER | W | 31±2.3 | – | – | 31±2 |
| | Sec61α | ER | W | 69±2.2 | – | – | 69±2 |
| | L12 | Cytosol | W | 96±3.3 | 94±2.2 | 98±4.2 | 99±3 |
| | β-actin | Cytosol | W | 95±4.2 | 92±6 | 97±1.2 | 99±4 |
| | LDH | Cytosol | E, W | 98±3.3 | 94±3.3 | 97±1.9 | 99±3 |

[a] IF: Immunofluorescence; W: Western; E: Enzymatic assay; FI: Fluorescence intensity.
[b] Normalized to control cells which were arbitrarily set to 100%, $n=2$ or 3.

proteins are essential for SRP functions [36,37]. Their depletion therefore results in a corresponding reduction in the levels of functional SRP (ten-fold). SRP14 exists in a free form in primate cells in excess over SRP [32,38] and it was conceivable that the ten-fold depletion of SRP14 preferentially affected the free pool. However, since its depletion lead to a dramatic decrease in SRP RNA (Fig. 1E), we concluded that the cellular levels of functional SRP were also reduced by at least nine-fold in SRP14-depleted cells.

### *Effects of low SRP levels on the accumulation and localization of ER-targeted proteins*

Next, we examined the expression levels of five reporter and three endogenous proteins from different cellular locales. In addition, we also monitored several control proteins. The expression studies were done at 144 h post transfection, the earliest time point at which the level of functional SRP is reduced ten-fold (Fig. 1). The quantified results from these studies are summarized in Table 1.

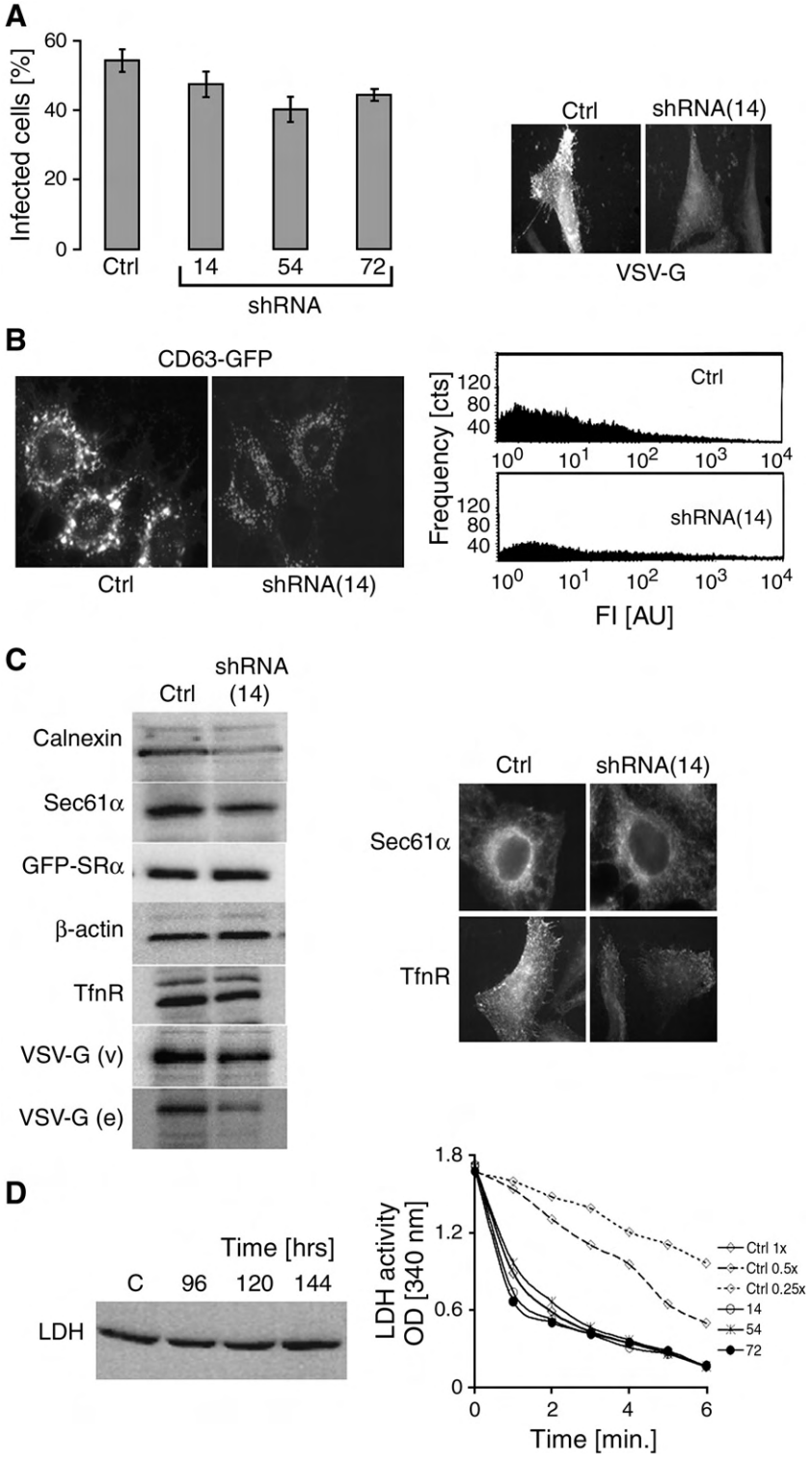
The G protein of vesicular stomatitis virus (VSV-G) was expressed by viral infection of HeLa cells. Its expression level in the plasma membrane was analyzed by immunofluorescent-staining of VSV-G in the absence of detergent. The percentage of cells positive for VSV-G staining was comparable, albeit slightly diminished in shRNAs expressing cells as compared to control cells (Fig. 2A, left panel). In about 70% of the infected cells, the surface expression level of VSV-G was strongly reduced as compared to control cells (Fig. 2A, right panel). The other 30% of cells were stained like control cells. Those cells were probably not significantly depleted of SRP. On average, the surface staining intensity was decreased to 37% as compared to the control cells and there was little difference between cells depleted of different SRP subunits (Table 1, IF). Taking into account that about 30% of cells have wild type staining, plasma membrane expression of VSV-G was most likely diminished to about 10% in the cells that were efficiently depleted of SRP. We also quantified the VSV-G protein by immunoblotting cell extracts with VSV-G antibodies (Fig. 2C, Table 1). Surprisingly, the total content of cellular VSV-G was

**Fig. 2 – Effects of SRP-depletion on the expression of ER-targeted proteins. (A) VSV-G expression at the plasma membrane in HeLa cells. Left panel: Percentage of infected cells after infection with 1 MOI VSV; right panel: Cell surface staining of HeLa cells with anti-VSV-G antibodies in the absence of saponin (B) Left panel: Fluorescent images of HeLa cells expressing CD63-GFP; right panel: CD63-GFP expression profiles of control and of SRP14-depleted cells obtained by FACS FI: fluorescence intensity; cts: counts. (C) Left panel: Western blots of cell extracts from SRP14-depleted and control HEK 293T cells using antibodies against the proteins indicated. VSV-G (v): Expression after viral infection, VSV-G(e): Episomally-expressed protein; right panel: Immunofluorescent images from HeLa cells labeled with anti-Sec61α or anti-TfnR antibodies (D) Protein levels and activity of lactate dehydrogenase. Left panel: Western blot with anti-LDH antibodies against the M subunit. Right panel: The activity of LDH was monitored by following the changes in OD of the co-factor NADH at 340 nm over a period of 6 min in cell extracts produced from cells that were used to monitor SEAP secretion. Ctrl: Control cells. 1×: undiluted, 0.5×: diluted 2-fold, 0.25×: diluted 4-fold. The error rate for all data points was lower than ± 0.07 OD, $n=2$.**

only reduced to 71% indicating that a fraction of the protein was not at the plasma membrane but mislocalized to another cellular locale (see below).

The expression vector for the alkaline phosphatase SEAP, which is a secreted version of this enzyme [39], was co-transfected with the shRNA-expressing plasmids into 293T cells. The secretion efficiency was determined by measuring the accumulation of SEAP in the medium 120–144 h after transfection with an enzymatic assay. During this time period, SRP levels decrease from 50 to ≤10% (Fig. 1D, Table 1). In average, the amount of secreted protein was only 25% compared to control cells, all values standardized to cell mass (Table 1). In the case of SEAP, we noticed slight differences between cells depleted of any of the three subunits (Table 1 and Fig. 1).

CD63 is a tetraspanning protein of late endosomal and lysosomal membranes [40], although in certain cells it is also found in the plasma membrane. We used a fusion protein of



CD63 and green fluorescent protein (CD63-GFP), which has previously been demonstrated to be functional [27]. Plasmids expressing CD63-GFP and shRNA were co-transfected into 293T and HeLa cells. Fluorescent images taken from HeLa cells showed a punctuate staining pattern consistent with an endosomal/lysosomal localization of the CD63-GFP protein (Fig. 2B). The results were quantified with 293T cells, which are more efficiently transfected, using the fluorescence activated cell sorter (FACS, Fig. 2B, right panel). The comparison between the areas of the two histograms revealed that overall fluorescence in shRNA-expressing cells was in average 49% of the one observed in control cells (Table 1). CD63-GFP quantified by immunoblotting gave a similar result as the level was reduced to 45% as compared to control cells.

ECFP-ER contains the ER-targeting sequence of calreticulin, a soluble ER protein, fused to the enhanced cyan fluorescent protein. NAGT1-GFP is a fusion protein of GFP and N-Acetyl glucosamine transferase 1, a component of the Golgi complex. Both proteins were found in the expected subcellular compartments (results not shown) and fluorescence intensities were strongly diminished in cells with reduced levels of SRP. The quantitative analysis of the images revealed that depletion of any of the three subunits reduced the expression levels of ECFP-ER and NAGT1-GFP to 29 and 28%, respectively (Table 1).

We also examined the expression levels of three endogenous ER-targeted proteins (Fig. 2C). Like most of the reporter proteins, the cellular level of calnexin, a membrane-bound ER-chaperone, was strongly reduced (Table 1, 31%) whereas the level of Sec61α, a subunit of the Sec61 protein complex, which forms the core of the translocation apparatus, was only reduced to 69%. To confirm that Sec61α detected by immunoblotting was properly localized, we made fluorescent images of HeLa cells decorated with anti-Sec61α antibodies (Fig. 2C, right panel). The images looked similar for shRNA-expressing and control cells with a perceptible decrease in the staining intensity of cells with reduced levels of SRP14. The expression level of transferrin receptor (TfnR) in the plasma membrane was determined by immunofluorescence (Fig. 2C) and it was reduced to 51% (Table 1). In contrast, the total protein content analyzed by Western was only reduced to 69% (Fig. 2C and Table 1). This result suggested that some of the protein was mislocalized in SRP-depleted cells (see below).

As a negative control, we analyzed the activity of the endogenous cytosolic enzyme lactate dehydrogenase (LDH) in the same cells that were used to monitor SEAP secretion. LDH catalyzes the conversion of pyruvate to lactate in the presence of NADH as a cofactor. The kinetic assay is very sensitive as illustrated by the kinetic curves obtained with diluted and undiluted extracts from control cells (Fig. 2D, Ctrl1×, 0.5×, 0.25×). Compared to these standard curves, the LDH activities of SRP-depleted and control cells, as well as the protein levels (Fig. 2D left and right panels, Table 1), were essentially the same. Two other control proteins, endogenous β-actin and GFP-SRα, were also unaffected by reduced levels of SRP14 (Fig. 2C and Table 1). GFP-SRα represents the SRP receptor α subunit fused to GFP. SRα is a membrane-associated protein and its targeting to the ER is SRP-independent [41].

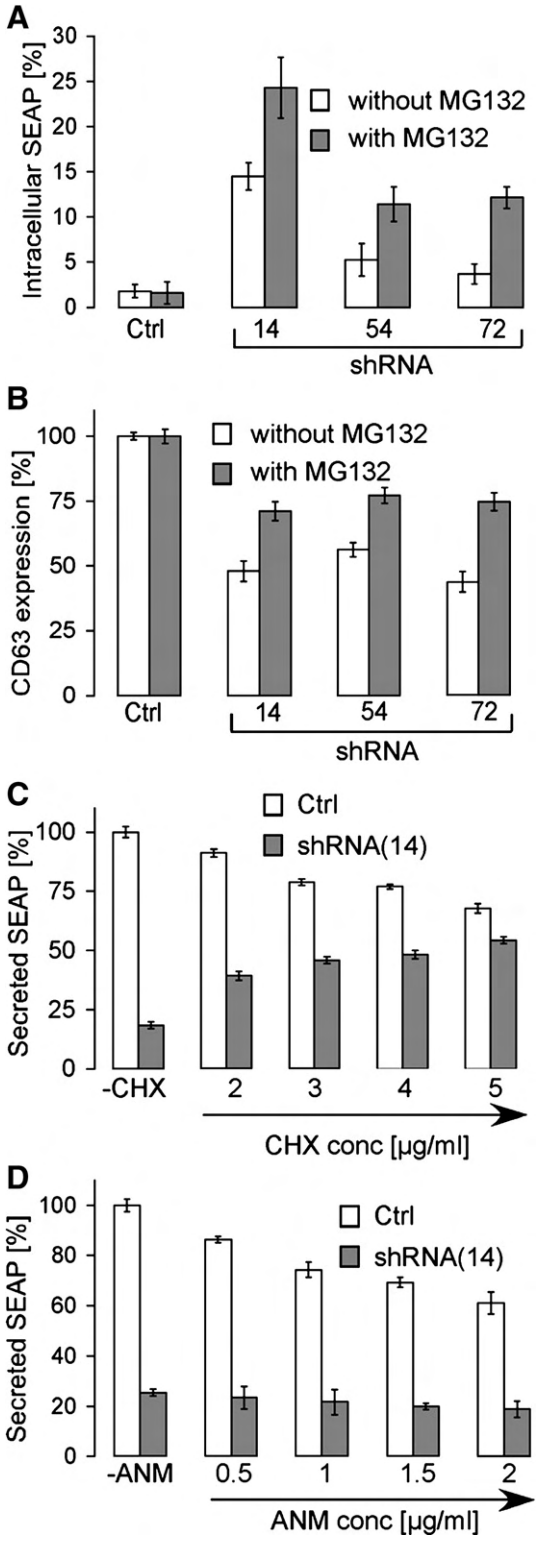
When the cellular level of SRP is reduced ten-fold, the expression levels of ER-targeted proteins are decreased to various degrees. In the cases studied, depletion of any of the three SRP proteins had comparable negative effects on the expression levels confirming that the effect was specifically due to depletion of SRP and not explained by "off target" effects of shRNAs. The effects were also selective for ER-targeted proteins because the activity as well as the levels of several cytosolic control proteins remained unchanged. For two plasma membrane proteins (VSV-G and TfnR), we found a difference in the reduction of plasma membrane expression and of total protein levels suggesting that these proteins became partially mislocalized in SRP-depleted cells.

### *SRP levels reduced by ten-fold are insufficient to ensure efficient targeting*

To reveal targeting defects at low SRP levels, we tried to detect precursor proteins in SRP-depleted cells with pulse-labeling experiments using SEAP and CD63-GFP as reporter proteins. Unlike in yeast and in bacteria, where an increase in precursor proteins can easily be detected upon depletion of SRP, we could only detect a single band for SEAP (presumably the protein without the signal sequence) and the already glycosylated CD63-GFP protein (not shown). We reasoned that this failure might be due to very rapid degradation of precursor proteins in mammalian cells by the proteasome. We therefore used two other approaches to provide evidence for inefficient ER-targeting at reduced SRP levels. Firstly, we examined whether we could monitor the accumulation of the secretory protein SEAP in the cytosol in the presence of the proteasome inhibitor MG132 [42] as previously observed [25]. MG132 was added at 120 h after transfection and SEAP activity in cell extracts was determined at 24 h later. Very little intracellular SEAP activity is detected in control cells and it remained unchanged in the presence of the proteasome inhibitor (Fig. 3A). It represents less than 2% of the SEAP activity that accumulates in the cell medium over 24 h with both values standardized to cell mass. SEAP activity already increased in the absence of MG132 in cells with reduced levels of SRP. The strongest effect was observed in SRP14-depleted cells. The intra- and extracellular SEAP activities were almost identical (compare Fig. 3A and Table 1). In all three cases, the presence of the proteasome inhibitor significantly increased the intracellular SEAP activity. Similarly, we also observed an increase of CD63-GFP in the presence of MG132 (Fig. 3B). These results supported the interpretation that at low levels of SRP, proteins become targeted inefficiently and that non-targeted precursors are rapidly degraded.

Secondly, we took advantage of the previous finding that growth and protein translocation defects caused by low levels of functional SRP in yeast, could be overcome by slowing down nascent chain elongation with sublethal doses of the protein synthesis inhibitor cycloheximide ([21], CHX). The rationale behind these findings, which was developed in the yeast study, is that reduced elongation rates lengthen the time span during which the signal sequence of a nascent chain can be recognized by SRP and the nascent chain be targeted cotranslationally to the ER. At reduced elongation rates, low

amounts of SRP become therefore enough for efficient ER-targeting. Furthermore, the delay in elongation had to occur at a specific step in elongation, since the inhibitor anisomycin, which inhibits the transpeptidylation reaction [43], failed to rescue cell growth [21]. To establish a link between the observed defects and inefficient targeting of newly synthesized proteins by SRP, we monitored the secretion efficiencies of SEAP at different sublethal concentrations of cycloheximide and anisomycin (Figs. 3C, D) in cells with low levels of SRP and in control cells. As expected, adding increasing amounts of cycloheximide and anisomycin to control cells decreased the total amount of secreted SEAP. Anisomycin is lethal for cells at lower concentrations than cycloheximide [44]. At the highest cycloheximide concentration, SEAP synthesis was diminished to about 70%. Concomitantly, the relative amounts of secreted protein from SRP14-depleted cells increased. In the presence of 5 μg/ml cycloheximide, 80% of the protein was secreted whereas in the absence of cycloheximide only 20% of the protein was secreted as compared to control cells. In cells treated with anisomycin, SEAP levels remained low (Fig. 3D). Hence like in yeast, the lower amounts of functional SRP became sufficient for efficient targeting of SEAP to the ER in the presence of cycloheximide but not in the presence of anisomycin. Ten-fold reduced cellular levels of SRP are therefore insufficient to assume fully its function in ER-targeting at normal elongation rates, although they appear sufficient to sustain survival ([26] and as shown here).




### *Low levels of SRP result in selective defects in post-ER membrane trafficking*

Our experiments so far indicated that the two plasma membrane proteins TfnR and VSV-G, were mislocalized to other cellular locales. We therefore examined their intracellular distribution. After viral infection, intracellular VSV-G was visualized by immunofluorescent staining in the presence of saponin. In control HeLa cells, VSV-G had a punctuate staining pattern consistent with its location in the different compartments of the secretory pathway (Fig. 4A, upper panel). In contrast, in the cells depleted of any of the three SRP subunits, we observed specific accumulation of VSV-G in a Golgi-like structure (Fig. 4A, lower panel). Quantification of the results showed that 60 to 70% of the cells displayed an accumulation of VSV-G in Golgi-like structures (Fig. 4B). As observed before, 30–40% of the cells gave the same staining pattern as control cells most likely because shRNA levels were too low for effective RNAi. To confirm that VSV-G accumulated in Golgi, we performed a co-localization assay in which Golgi was stained with a lectin dye. It was chosen as a marker because the expression levels of Golgi proteins such as NAGT1 were strongly

**Fig. 3 – Low levels of SRP lead to inefficient targeting of proteins to the ER. (A) Intracellular SEAP activity was determined in HEK 293T cells 144 h after transfection with shRNA-expressing plasmids using an enzymatic assay. Activities were standardized to equal amounts of protein and normalized to control cells. (B) Intracellular fluorescence of CD63-GFP determined from expression profiles obtained with FACS. The expression levels represent the area of the histograms obtained by FACS normalized to control cells ($n$=3). Cells were treated with MG132 120–144 h post transfection and analyzed thereafter. (C) HEK 293T expressing SEAP were incubated in fresh medium containing cycloheximide (CHX) or anisomycin (ANM) at the concentrations indicated at 120 h posttransfection. At 144 h, the medium was collected and SEAP activity quantified with an enzymatic assay ($n$=2). Ctrl: control cells.**

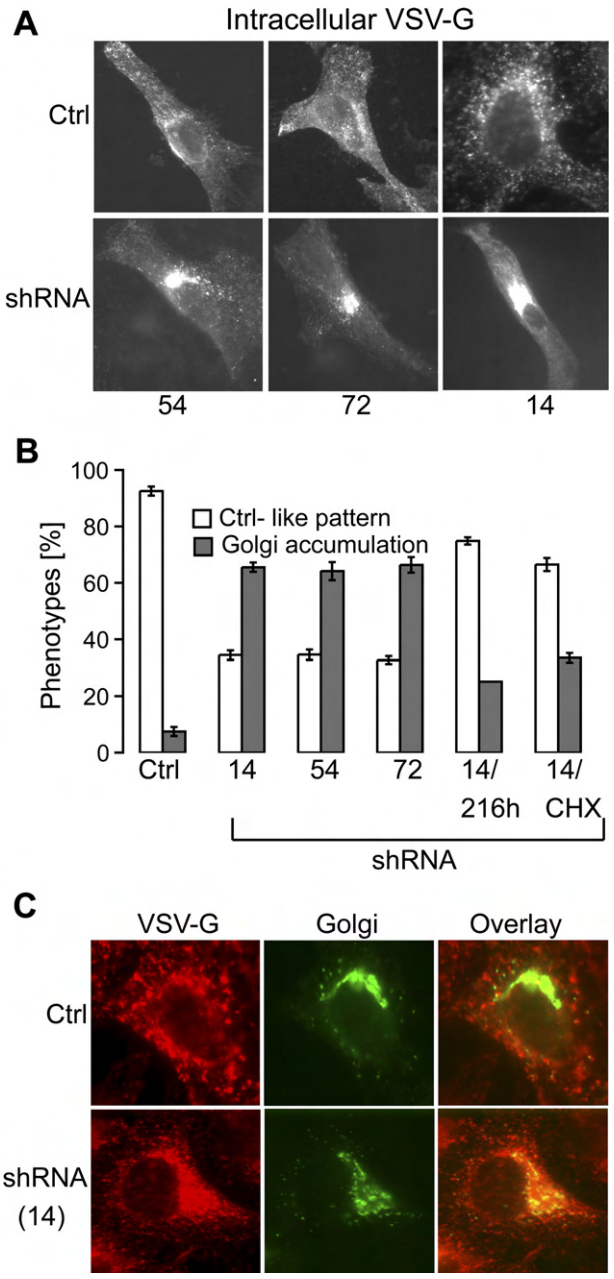



**Fig. 4 – Accumulation of VSV-G in Golgi upon SRP-depletion. (A) SRP-depleted and control HeLa cells were infected with VSV 144 h after inducing RNAi against SRP14. After treatment with saponin, intracellular VSV-G was visualized with immunofluorescence. (B) Phenotypes were quantified by counting 200 cells of each sample manually ($n$=3). 14/CHX: in the presence of cycloheximide at 5 μg/ml; 14/216: phenotypes were counted at 216 h posttransfection. (C) HeLa cells depleted of SRP14 (lower panel) and control cells (upper panel) infected with VSV. Golgi was labeled with the fluorescent lectin GS-II (green). There is a significant overlay between VSV-G and the Golgi marker in SRP14-depleted cells.**

diminished in SRP-depleted cells and they were therefore not suitable for colocalization experiments. The staining pattern obtained with the Golgi-specific lectin was quite similar in SRP-depleted and control cells (Fig. 4C). It remained concentrated around the nucleus but was less dense in about 50% of the cells exposed to RNAi consistent with lower levels of glycosylated proteins and/or less efficient glycosylation of proteins. The data revealed a specific overlap of VSV-G with Golgi in SRP depleted cells but not in the control cells (Fig. 4C).

SEAP and CD63-GFP are proteins that travel beyond the Golgi compartment and might therefore display a similar phenotype. However, we failed to detect their accumulation in Golgi (see Fig. 2B for CD63-GFP). When VSV-G was expressed from a plasmid, we also failed to observe an accumulation of the protein in the Golgi apparatus (not shown), and we noticed that its expression was more importantly diminished by RNAi than the virally expressed protein (Fig. 2C, Table 1). Upon viral infection, the host translation machinery is entirely dedicated to the synthesis of viral proteins whereas the translation of host proteins is inhibited ([45]). This suggests that the remaining SRP was also exclusively dedicated to the targeting of viral proteins, which may result in higher amounts of VSV-G entering the ER. The capacity of the Golgi to ensure the efficient export of higher amounts of VSV-G to the plasma membrane is now surpassed in SRP-depleted cells. The low amounts of episomally-expressed VSV-G that might reach the Golgi, however, were undetectable.

To examine whether the observed defect was linked to inefficient targeting of newly synthesized proteins by SRP, we repeated the experiments in the presence of cycloheximide when, due to slower elongation rates, reduced levels of SRP become sufficient for its normal function. In the presence of cycloheximide fewer cells still displayed an accumulation of VSV-G in the Golgi (Fig. 4B) indicating that the observed block in the secretory pathway is a direct consequence of inefficient targeting by SRP. The defect was also reversed at a later time point, when SRP levels were again increased to 60% of control cells (Figs. 4B, 1D).

We also examined the intracellular staining of TfnR. Control cells revealed the punctuated staining pattern of early/recycling endosomes typical for internalized TfnR (Fig. 5A). In contrast, in SRP14-depleted cells, TfnR accumulated in or in the proximity of the Golgi. Quantification revealed that more than 50% of cells displayed an accumulation of receptor in or close to the Golgi (Fig. 5B). Again, this phenotype was reversed, when cells were treated with cycloheximide to ensure efficient ER-targeting at low levels of SRP and at later time points when SRP levels were increased (Fig. 5B). The synthesis rate of endogenous TfnR is very low [46] and it was therefore unlikely that the TfnR signal observed in or in proximity of the Golgi represented newly synthesized protein. To confirm this interpretation, we examined the effects of SRP-depletion on the accumulation and on the intracellular location of ectopically expressed human TfnR fused to GFP (TfnR-GFP). As previously observed for SEAP and CD63-GFP, the accumulation of hTfnR-GFP was strongly diminished in SRP-depleted cells and no accumulation of newly synthesized hTfnR could be detected in or close to Golgi (Table 1 and Fig. 5C, left panel). We therefore concluded that the endogenous TfnR observed

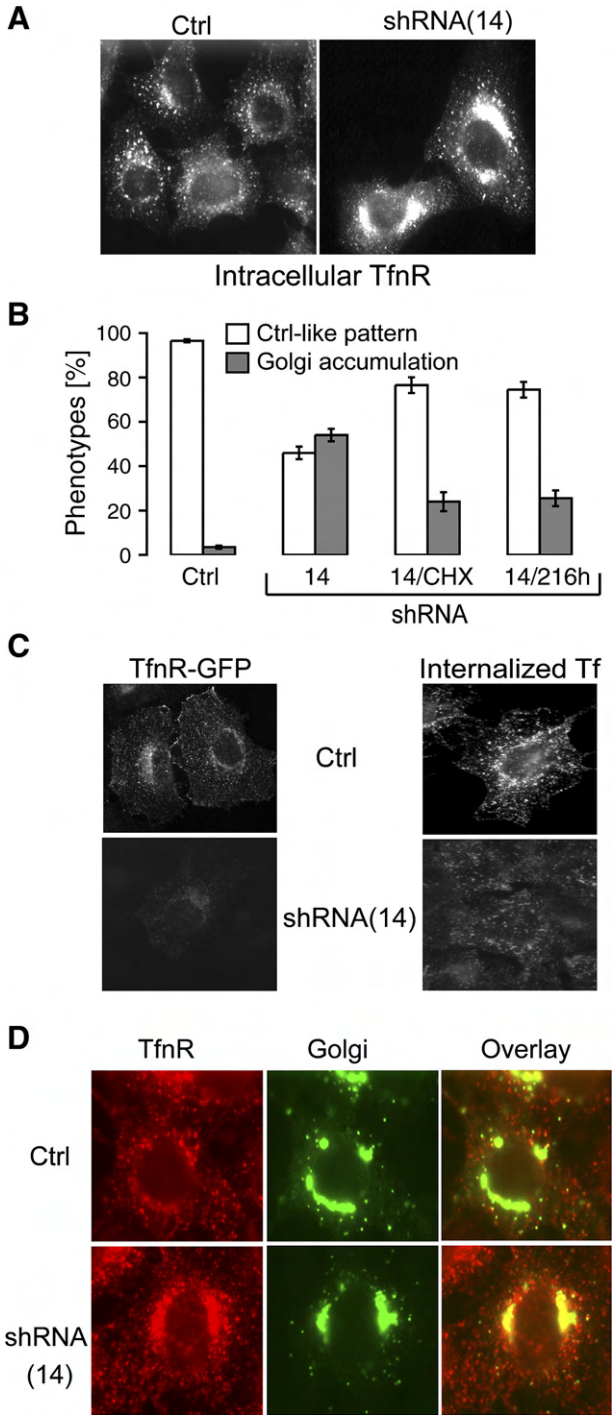



**Fig. 5 – Effects of SRP14 depletion on TfnR recycling. (A) HeLa cells labeled with fluorescent antibodies against TfnR in SRP14-depleted and control cells. (B) Quantification of the cells that show an accumulation of TfnR in Golgi. 200 cells in different fields were counted for each sample ($n$=2). Ctrl, 14: 144 h post transfection; 14/CHX: in the presence of cycloheximide at 5 μg/ml; 14/216: phenotypes were counted at 216 h posttransfection. (C) Left panel: Fluorescent images of HeLa cells expressing TfnR-GFP. Right panel: internalization of fluorescently-labeled Tf. (D) Co-localization of TfnR and Golgi components. Golgi was labeled with the fluorescent lectin GS-II.**

in Golgi represents receptor that was endocytosed but failed to be recycled to the plasma membrane in SRP-depleted cells.

If TfnR was no longer recycled to the surface, the uptake of transferrin (Tf) should be severely diminished. To test this hypothesis, we examined the uptake of rhodamine-labeled transferrin in shRNA-expressing and control cells. The results supported our interpretation. The control cells revealed the expected punctuate staining pattern of early/recycling endosomes whereas the uptake of rhodamine-labeled transferrin was decreased by 67% in SRP-depleted cells (Fig. 5C, right panel). Co-localization experiments with a Golgi-specific lectin confirmed that TfnR was localized in the Golgi compartment (Fig. 5D) or possibly in early/recycling endosomes close to the Golgi. The resolution of our images did not allow us to exclude the latter possibility. In control cells, there is no detectable localization of TfnR in Golgi.

Our results were consistent with the notion that rapidly after SRP-depletion membrane trafficking between Golgi and the plasma membrane becomes defective. We therefore wondered whether there was also a defect in the retrograde transport from the plasma membrane to the ER. To address this question, we decided to use the Shiga toxin B-subunit (STxB). Toxins have become a widely used tool to study trafficking pathways in cells [47]. For our experiments, we used recombinant Cy3 STxB [48]. The non-toxic STxB can undergo retrograde transport in the absence of the toxic A subunit and will accumulate in the ER [31]. Like before, we studied the retrograde transport of recombinant STxB in shRNA-expressing and control cells at the earliest time point at which SRP levels were reduced by ten-fold (144 h after transfection). The retrograde transport was monitored by fluorescent imaging at different time points after internalization of STxB and incubation of cells at 37 °C [48]. In agreement with the previously published time course of events, we observed the protein in endosomes at 15 min and in the Golgi at 45 min after internalization in SRP depleted and control cells (Fig. 6A). After 5 h at 37 °C, STxB in the control cells displayed a diffuse pattern as expected if STxB were localized to the ER. In contrast, in cells with low SRP levels the STxB was still localized to Golgi in 64% of the cells (Figs. 6A, B) whereas only 20% of the cells showed Golgi localization in control cells. The defects in retrograde traffic were reversed in the presence of cycloheximide and at 216 h post transfection (Fig. 6B). Hence, the observed defects are a direct consequence of inefficient targeting of newly synthesized proteins by SRP.

Because of the results with TfnR, we decided to study the endocytic pathway. We monitored the uptake and localization of rhodamine-labeled dextran to lysosomes at 144 h post transfection in SRP14-depleted cells. The lysosomes were labeled with LAMP1 antibodies. Analysis of the images from both control cells and the SRP depleted cells, revealed no significant differences between them (Fig. 6C). This demonstrated that there is no detectable defect in the endocytic pathway to the lysosomes. This result is consistent with the fact that the rate of infection with VSV, which is dependent on endocytosis [28], is not changed significantly upon SRP depletion (Fig. 2A).

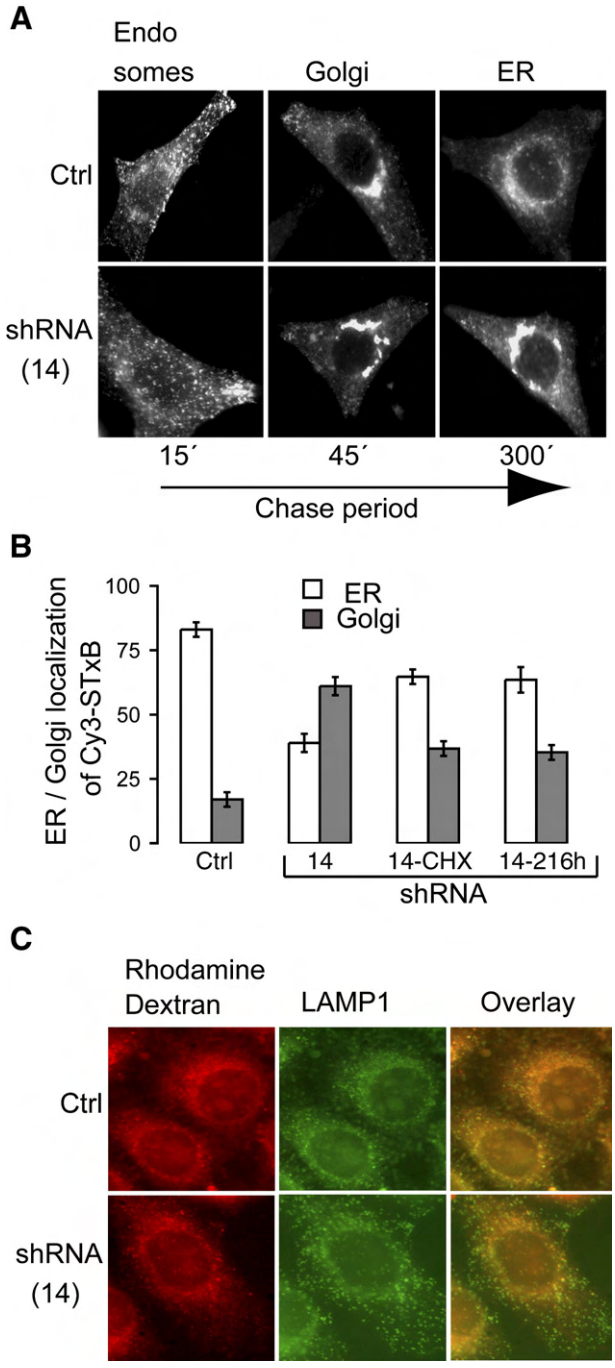



**Fig. 6 – Effect of SRP depletion on retrograde transport and on endocytosis. (A) Intracellular trafficking of Cy3-labeled STxB followed over time. (B) Quantification of cells showing accumulation of STxB in a Golgi-like structure at 5 h post internalization in SRP14-depleted and control cells. 200 cells from four different fields were counted for both samples in two independent experiments. (C) Co-localization of rhodamine-labeled dextran (Rhod-DEX) and the lysosomal marker LAMP1 in SRP14-depleted and control cells 30 min after internalization of Rhod-DEX.**

## Discussion

In this study we demonstrate that if SRP levels are reduced ten-fold or more they become insufficient to ensure efficient targeting of all proteins to the ER in mammalian cells. The deficit in SRP reduced the accumulation of several endogenous and reporter proteins in their respective locales to various extents, and interfered with Golgi functions in antero- and retrograde transport as well as with TfnR recycling. Targeting defects were revealed by interfering with the rapid degradation of newly synthesized preproteins in the cytoplasm and by slowing down nascent chain elongation with cycloheximide. As previously shown for yeast [21], in mammalian cells low levels of SRP also become sufficient for efficient targeting of the secretory protein SEAP at reduced elongation rates. Notably, all the observed defects in post-ER membrane trafficking such as mislocalization of VSV-G and TfnR as well as the retention of the Shiga toxin B-subunit in Golgi are reversed by the treatment with cycloheximide. Hence, the defects in post-ER membrane trafficking are a direct consequence of inefficient ER-targeting at low levels of SRP and are not explained by a direct role of SRP in membrane trafficking. In addition, to suppress the selective defects in membrane trafficking, almost normal levels of SRP are required, since slight defects were still observed at SRP levels reduced by 40%.

Interestingly, even during the time period with the lowest SRP levels, the cells displayed no noticeable phenotype and low levels of SRP failed to induce an apoptotic response, since the cells continued to grow normally at later time points. In previous studies, stable cell lines depleted of any of the two SRP proteins, SRP54 and SRP72, showed no growth defects [26]. Similarly, we observed no growth defects in cells which stably expressed shRNAs after viral infection and in which the SRP14 levels were reduced by 80% (A.K.K.L. and K.S., unpublished results). This suggests that far less SRP is required for survival than normally present in cells. The minimal SRP level required for cell survival could not be determined, since we failed to obtain stable cells lines with SRP levels below 10–20%. It is likely that at levels below this threshold, cell death might finally come about after massive internal cell damage through mislocalization of proteins as was described for trypanosomes [23]. One adaptation of yeast to low levels of SRP is to reduce the number of ribosomes [20]. Over the limited time period we studied, we failed to observe a significant decrease in ribosomes as deduced from the levels of the ribosomal protein L12 (Fig. 1C).

The observation that selective post-ER membrane trafficking defects occur at SRP levels sufficient for survival suggests that changes in SRP levels might be used as a cellular mechanism to interfere with post-ER membrane trafficking such that certain proteins alter their localization. This notion is supported by the differential effects on the cell surface expression of the death receptors DR4 and DR5 [26]. DR4 accumulated in the Golgi upon depletion of SRP72 whereas DR5 remained in the plasma membrane. The mechanism by which DR4 becomes selectively mislocalized in SRP72-depleted cells is as yet unclear. In agreement with our results, it is conceivable that selective defects in membrane trafficking may account for the observed difference. In addition and as

mentioned before, it has been observed that the successful infection of macrophages with *Leishmania* was coupled to the down regulation of SRP RNA levels. Low levels of SRP were proposed to interfere with the secretion of proteins necessary to counteract the parasitic infection [25]. Hence, viruses and parasites may take advantage of the fact that SRP levels can be down regulated to prevent plasma membrane expression of proteins required to trigger the immune response without killing the cells. Cell surface receptors involved in immune response often have high turnover rates and their accumulation in the plasma membrane might therefore be especially sensitive to a block in secretion at the level of Golgi as we observed for the viral protein VSV-G. The observation that the surface receptor TfnR can be trapped in Golgi/recycling endosomes at low SRP levels also supports the notion that cells might use such a mechanism to transiently remove proteins (receptors) from the cell surface.

For most proteins, the expression levels in their specific locales are reduced by 50–80% (Table 1). The observed variations could be determined by factors such as turnover rate and the capacity of proteins to effectively recruit SRP even at low abundance. The expression level of Sec61α, which appears to be properly localized based on the immunofluorescent images, is least affected by SRP-depletion, since its level is only reduced by 31%. It might therefore represent a protein with a very low turnover rate and/or with a high capacity to recruit SRP. Alternatively, it might be able to use a posttranslational chaperone-mediated targeting pathway. Although this pathway has so far only been characterized in yeast and in bacteria [49,50], it is likely to exist in mammalian cells as well. Like in yeast [51], signal sequences might influence the choice between the two pathways in vitro and in vivo [52,53,54].

TfnR constitutively recycles between the plasma membrane and early/recycling endosomes [55]. Its accumulation in or near the Golgi in SRP-depleted cells cannot be explained primarily by a defect in the biosynthetic pathway for several reasons: (i) its *de novo* synthesis is very low in normal cells and is expected to be even more reduced in SRP-depleted cells, since targeting of TfnR to the ER is SRP-dependent [56] (ii) in agreement with the previous statement no accumulation of episomally-expressed hTfnR-GFP could be detected in SRP14-depleted cells, and (iii) the total amount of TfnR was much less decreased than its cell surface expression in SRP14-depleted cells (Table 1). Its accumulation in the Golgi or in early/recycling endosomes close to Golgi is therefore due to a defect in the recycling of the internalized receptor.

There have been multiple observations that components of the exocytic pathway transiently localize to endocytic compartments [57–59]. Specifically, recycling endosomes have been identified recently as intermediates in the exocytic pathway of MDCK cells [60]. The fact that TfnR is unable to recycle back to the plasma membrane in SRP-depleted cells indicates that recycling endosomes may not only serve as an intermediate in the exocytic pathway but that their capacity to recycle back to the plasma membrane is actually dependent on active Golgi to plasma membrane protein transport. Alternatively, TfnR may normally transit through Golgi in the recycling pathway and now becomes trapped because of the defect in the exocytic pathway. To our knowledge TfnR has so far not been observed in Golgi.

The most plausible explanation for the observed defects is to assume that one or several components might become limiting to the extent that their absence selectively impairs post-ER membrane trafficking. More functional and structural experiments will be required to identify the critical component(s) and the exact process(es) that are defective. However, since the morphology of the Golgi is not dramatically changed at least based on low resolution images, (e.g. it is not significantly fragmented), it appears more likely that the limiting factors are essential for vesicular transport rather than structural integrity.

SRP-depletion studies have further substantiated the fundamental role of the SRP-mediated targeting pathway in mammalian cells for maintaining cellular structures and functions. Furthermore, our studies suggest that SRP-depletion experiments might provide a useful tool for the analysis and identification of critical components in membrane trafficking.

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## REFERENCES

[1] G. Blobel, B. Dobberstein, Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma, J. Cell Biol. 67 (1975) 835–851.
[2] R.J. Keenan, D.M. Freymann, R.M. Stroud, P. Walter, The Signal Recognition Particle, Annu. Rev. Biochem. 70 (2001) 755–775.
[3] B.B. Allan, W.E. Balch, Protein sorting by directed maturation of Golgi compartments, Science 285 (1999) 63–66.
[4] M.C. Lee, E.A. Miller, J. Goldberg, L. Orci, R. Schekman, Bi-directional protein transport between the ER and Golgi, Annu. Rev. Cell Dev. Biol. 20 (2004) 87–123.
[5] K. Sandvig, B. van Deurs, Transport of protein toxins into cells: pathways used by ricin, cholera toxin and Shiga toxin, FEBS Lett. 529 (2002) 49–53.
[6] M.R. Pool, Signal recognition particles in chloroplasts, bacteria, yeast and mammals (review), Mol. Membr. Biol. 22 (2005) 3–15.
[7] K. Wild, K.R. Rosendal, I. Sinning, A structural step into the SRP cycle, Mol. Microbiol. 53 (2004) 357–363.
[8] A.E. Johnson, M.A. van Waes, The translocon: a dynamic gateway at the ER membrane, Annu. Rev. Cell Dev. Biol. 15 (1999) 799–842.
[9] A.R. Osborne, T.A. Rapoport, B. van den Berg, Protein Translocation by the Sec61/Secy Channel, Annu. Rev. Cell Dev. Biol. 21 (2005) 529–550.

[10] M. Halic, T. Becker, M.R. Pool, C.M. Spahn, R.A. Grassucci, J. Frank, R. Beckmann, Structure of the signal recognition particle interacting with the elongation-arrested ribosome, Nature 427 (2004) 808–814.
[11] M.R. Pool, J. Stumm, T.A. Fulga, I. Sinning, B. Dobberstein, Distinct modes of signal recognition particle interaction with the ribosome, Science 297 (2002) 1345–1348.
[12] L. Terzi, M.R. Pool, B. Dobberstein, K. Strub, Signal recognition particle Alu domain occupies a defined site at the ribosomal subunit interface upon signal sequence recognition, Biochem. 43 (2004) 107–117.
[13] G.J. Phillips, T.J. Silhavy, The *E. coli* ffh gene is necessary for viability and efficient protein transport, Nature 359 (1992) 744–746.
[14] J. MacFarlane, M. Müller, Functional integration of a polytopic membrane protein of *E. coli* requires the bacterial signal recognition particle, Biochem. Soc. Trans. 23 (1995) 560.
[15] N.D. Ulbrandt, J.A. Newitt, H.D. Bernstein, The *E. coli* signal recognition particle is required for the insertion of a subset of inner membrane proteins, Cell 88 (1997) 187–196.
[16] J. Luirink, G. von Heijne, E. Houben, J.-W. de Gier, Biogenesis of inner membrane proteins in *Escherichia coli*, Annu. Rev. Microbiol. 59 (2005) 329–355.
[17] S.M. Althoff, S.W. Stevens, J.A. Wise, The Srp54 GTPase is essential for protein export in the fission yeast *Schizosaccharomyces pombe*, Mol. Biol. Cell 14 (1994) 7839–7854.
[18] F. He, D. Yaver, J.M. Beckerich, D. Ogrydziak, C. Gaillardin, The yeast *Yarrowia lipolytica* has two, functional, signal recognition particle 7S RNA genes, Curr. Genet. 17 (1990) 289–292.
[19] B.C. Hann, P. Walter, The signal recognition particle in *S. cerevisiae*, Cell 67 (1991) 131–144.
[20] S.C. Mutka, P. Walter, Multifaceted physiological response allows yeast to adapt to the loss of the signal recognition particle-dependent protein-targeting pathway, Mol. Biol. Cell 12 (2001) 577–588.
[21] S.C. Ogg, P. Walter, SRP samples nascent chains for the presence of signal sequences by interacting with ribosomes at a discrete step during translation elongation, Cell 81 (1995) 1075–1084.
[22] C.J. Stirling, J. Rothblatt, M. Hosobuchi, R. Deshaies, R. Schekman, Protein translocation mutants defective in the insertion of integral membrane proteins into the endoplasmic reticulum, Mol. Biol. Cell 3 (1992) 129–142.
[23] L. Liu, X.H. Liang, S. Uliel, R. Unger, E. Ullu, S. Michaeli, RNA interference of signal peptide-binding protein SRP54 elicits deleterious effects and protein sorting defects in trypanosomes, J. Biol. Chem. 277 (2002) 47348–47357.
[24] Y. Lustig, H. Goldshmidt, S. Uliel, S. Michaeli, The *Trypanosoma brucei* signal recognition particle lacks the Alu-domain-binding proteins: purification and functional analysis of its binding proteins by RNAi, J. Cell Sci. 118 (2005) 4551–4562.
[25] S. Misra, M.K. Tripathi, G. Chaudhuri, Down-regulation of 7SL RNA expression and impairment of vesicular protein transport pathways by Leishmania infection of macrophages, J. Biol. Chem. 280 (2005) 29364–29373.
[26] Y.G. Ren, K.W. Wagner, D.A. Knee, P. Aza-Blanc, M. Nasoff, Q.L. Deveraux, Differential regulation of the TRAIL death receptors DR4 and DR5 by the signal recognition particle, Mol. Biol. Cell 15 (2004) 5064–5074.
[27] T. Kobayashi, M.H. Beuchat, J. Chevallier, A. Makino, N. Mayran, J.M. Escola, C. Lebrand, P. Cosson, T. Kobayashi, J. Gruenberg, Separation and characterization of late endosomal membrane domains, J. Biol. Chem. 277 (2002) 32157–32164.
[28] I. Le Blanc, P.P. Luyet, V. Pons, C. Ferguson, N. Emans, A. Petiot, N. Mayran, N. Demaurex, J. Faure, R. Sadoul, R.G. Parton, J. Gruenberg, Endosome-to-cytosol transport of viral nucleocapsids, Nat. Cell Biol. 7 (2005) 653–664.
[29] J. Gruenberg, K.E. Howell, Immuno-isolation of vesicles using antigenic sites either located on the cytoplasmic or the exoplasmic domain of an implanted viral protein. A quantitative analysis, Eur. J. Cell Biol. 38 (1985) 312–321.
[30] M. Kauppi, A. Simonsen, B. Bremnes, A. Vieira, J. Callaghan, H. Stenmark, V.M. Olkkonen, The small GTPase Rab22 interacts with EEA1 and controls endosomal membrane trafficking, J. Cell Sci. 115 (2002) 899–911.
[31] T. Falguieres, F. Mallard, C. Baron, D. Hanau, C. Lingwood, B. Goud, J. Salamero, L. Johannes, Targeting of Shiga toxin B-subunit to retrograde transport route in association with detergent-resistant membranes, Mol. Biol. Cell 12 (2001) 2453–2468.
[32] F. Bovia, M. Fornallaz, H. Leffers, K. Strub, The SRP9/14 subunit of the signal recognition particle (SRP) is present in more than 20-fold excess over SRP in primate cells and exists primarily free but also in complex with small cytoplasmic Alu RNAs, Mol. Biol. Cell 6 (1995) 471–484.
[33] T.E. Kreis, Microinjected antibodies against the cytoplasmic domain of vesicular stomatitis virus glycoprotein block its transport to the cell surface, EMBO J. 5 (1986) 931–941.
[34] W. Filipowicz, RNAi: the nuts and bolts of the RISC machine, Cell 122 (2005) 17–20.
[35] J.D. Brown, B.C. Hann, K.F. Medzihradszky, M. Niwa, A.L. Burlingame, P. Walter, Subunits of the *Saccharomyces cerevisiae* signal recognition particle required for its functional expression, EMBO J. 13 (1994) 4390–4400.
[36] S. Hauser, G. Bacher, B. Dobberstein, H. Lütcke, A complex of the signal sequence binding protein and the SRP RNA promotes translocation of nascent proteins, EMBO J. 14 (1995) 5485–5493.
[37] V. Siegel, P. Walter, Each of the Activities of Signal Recognition Particle (SRP) Is Contained within a Distinct Domain: Analysis of Biochemical Mutants of SRP, Cell 52 (1988) 39–49.
[38] D.Y. Chang, N. Sasaki-Tozawa, L.K. Green, R.J. Maraia, A trinucleotide repeat-associated increase in the level of Alu RNA-binding protein occurred during the same period as the major Alu amplification that accompanied anthropoid evolution, Mol. Cell. Biol. 15 (1995) 2109–2116.
[39] M.E. Lowe, Site-specific mutations in the COOH-terminus of placental alkaline phosphatase: a single amino acid change converts a phosphatidylinositol-glycan-anchored protein to a secreted protein, J. Cell Biol. 116 (1992) 799–807.
[40] M. Ageberg, A. Lindmark, Characterisation of the biosynthesis and processing of the neutrophil granule membrane protein CD63 in myeloid cells, Clin. Lab. Haematol. 25 (2003) 297–306.
[41] D.W. Andrews, L. Lauffer, P. Walter, V.R. Lingappa, Evidence for a two-step mechanism involved in assembly of functional signal recognition particle receptor, J. Cell Biol. 108 (1989) 797–810.
[42] J.L. Cowan, S.J. Morley, The proteasome inhibitor, MG132, promotes the reprogramming of translation in C2C12 myoblasts and facilitates the association of hsp25 with the eIF4F complex, Eur. J. Biochem. 271 (2004) 3596–3611.
[43] E.F. Gale, E. Cundliffe, P. Reynolds, M.H. Richmond, M.J. Waring, The molecular basis of antibiotic action, 2nd ed., J. Wiley and Sons, London, 1981.
[44] A. Joiakim, P.A. Mathieu, A.A. Elliott, J.J. Reiners Jr., Superinduction of CYP1A1 in MCF10A cultures by cycloheximide, anisomycin, and puromycin: a process independent of effects on protein translation and unrelated to suppression of aryl hydrocarbon receptor proteolysis by the proteasome, Mol. Pharmacol. 66 (2004) 936–947.
[45] J.H. Connor, D.S. Lyles, Inhibition of host and viral translation during vesicular stomatitis virus infection. eIF2 is responsible

for the inhibition of viral but not host translation, J. Biol. Chem. 280 (2005) 13512–13519.
[46] E.A. Rutledge, C.A. Mikoryak, R.K. Draper, Turnover of the transferrin receptor is not influenced by removing most of the extracellular domain, J. Biol. Chem. 266 (1991) 21125–21130.
[47] F. Mallard, L. Johannes, Shiga toxin B-subunit as a tool to study retrograde transport, Methods Mol. Med. 73 (2003) 209–220.
[48] L. Johannes, D. Tenza, C. Antony, B. Goud, Retrograde transport of KDEL-bearing B-fragment of Shiga toxin, J. Biol. Chem. 272 (1997) 19554–19561.
[49] T.A. Rapoport, K.E. Matlack, K. Plath, B. Misselwitz, O. Staeck, Posttranslational protein translocation across the membrane of the endoplasmic reticulum, Biol. Chem. 380 (1999) 1143–1150.
[50] A.J. Driessen, E.H. Manting, C. van der Does, The structural basis of protein targeting and translocation in bacteria, Nat. Struct. Biol. 8 (2001) 492–498.
[51] D.T. Ng, J.D. Brown, P. Walter, Signal sequences specify the targeting route to the endoplasmic reticulum membrane, J. Cell Biol. 134 (1996) 269–278.
[52] B. Jungnickel, T.A. Rapoport, A posttargeting signal sequence recognition event in the endoplasmic reticulum membrane, Cell 82 (1995) 261–270.
[53] D. Belin, S. Bost, J.D. Vassalli, K. Strub, A two-step recognition of signal sequences determines the translocation efficiency of proteins, EMBO J. 15 (1996) 468–478.
[54] C.G. Levine, D. Mitra, A. Sharma, C.L. Smith, R.S. Hegde, The efficiency of protein compartmentalization into the secretory pathway, Mol. Biol. Cell 16 (2005) 279–291.
[55] J.L. Goldstein, M.S. Brown, R.G. Anderson, D.W. Russell, W.J. Schneider, Receptor-mediated endocytosis: concepts emerging from the LDL receptor system, Annu. Rev. Cell Biol. 1 (1985) 1–39.
[56] M. Zerial, P. Melancon, C. Schneider, H. Garoff, The transmembrane segment of the human transferrin receptor functions as a signal peptide, EMBO J. 5 (1986) 1543–1550.
[57] W. Stoorvogel, H.J. Geuze, J.M. Griffith, G.J. Strous, The pathways of endocytosed transferrin and secretory protein are connected in the trans-Golgi reticulum, J. Cell Biol. 106 (1988) 1821–1829.
[58] E. Harsay, R. Schekman, A subset of yeast vacuolar protein sorting mutants is blocked in one branch of the exocytic pathway, J. Cell Biol. 156 (2002) 271–285.
[59] B. Leitinger, A. Hille-Rehfeld, M. Spiess, Biosynthetic transport of the asialoglycoprotein receptor H1 to the cell surface occurs via endosomes, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 10109–10113.
[60] A.L. Ang, T. Taguchi, S. Francis, H. Folsch, L.J. Murrells, M. Pypaert, G. Warren, I. Mellman, Recycling endosomes can serve as intermediates during transport from the Golgi to the plasma membrane of MDCK cells, J. Cell Biol. 167 (2004) 531–543.

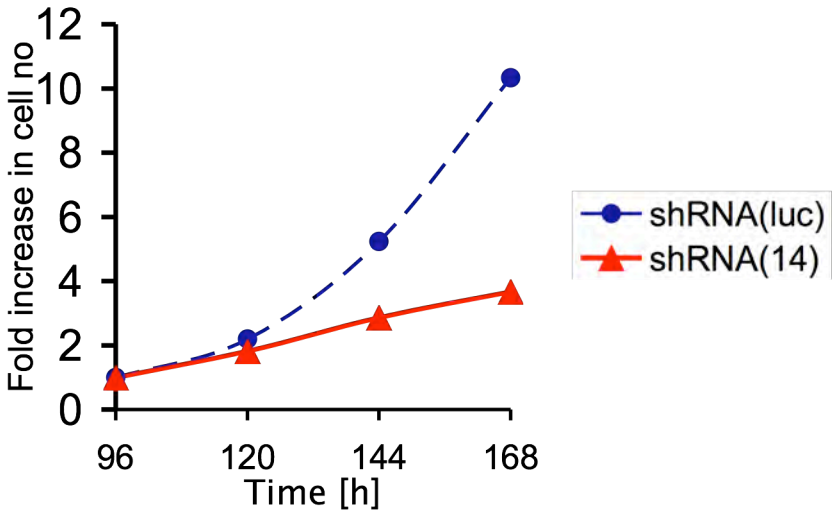
## *2.2 Unpublished Supplementary data*

### *2.2.1 Effect of low levels of SRP on cell growth*

We examined the effect of SRP14 depletion on mammalian cell growth. Equal number of cells were transfected either with shRNA encoding luciferase or shRNA against SRP14. Cell growth was quantified by counting the cells manually every 24 h starting from 96 h. The cell count is expressed as a fold increases in the number of cells present at the beginning of the count. There is no growth defect observed between 96 and 120 h when the SRP14 level decreases to about 50%. However, after 120 h there is a significant decrease in the cell growth in the cells depleted of SRP14. Calculation of doubling times revealed that cells transfected with shRNA (luc) have a doubling time of 21 h where as cells transfected with shRNA(14) have a doubling time of 47 h. After 168 h the levels of SRP14 increase again and thereby limiting this study to 168 h. If the cells are grown under the persistent loss of functional SRP for a longer periods of time, they would become limiting in many crucial proteins essential for cell survival and might ultimately die under such conditions, These results show that depletion of SRP14 strongly interferes with cellular physiology by altering the growth rates of the cells.

We examined the expression level of BiP, which is a luminal protein in the endoplasmic reticulum. We observed that cells depleted of SRP14 showed an increase of 2.2 fold in the accumulation of BiP (Fig. S1C), when compared to the control cells that were treated with shRNA (luc). BiP is an ER stress sensor and its level is upregulated upon ER stress. The increase in the BiP levels in SRP14 depleted cells clearly suggested an activation of stress response in these cells.

**A**




**B**

| 293T cells | Doubling Time (120-168 h) |
|---|---|
| shRNA (luc) | 21 ± 4 |
| shRNA (14) | 47 ± 3 |

**C**

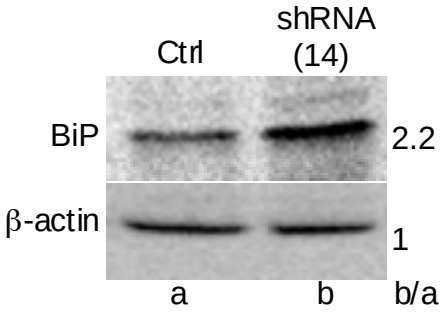



Figure S1: **(A)** Cells were plated at equal densities and transfected with either shRNA (luc) or shRNA (14). Live cells were counted manually starting from 96 h post transfection (n=3). **(B)** The doubling times were calculated between 120-168 h. **(C)** Western blot with anti BiP and anti beta actin antibodies of the cell extracts from from SRP14 depleted and control cells

### *2.2.2 Effects of SRP subunits depletion on SRP*

We investigated the effect on all of the SRP proteins when one of them was depleted using RNAi. The levels of SRP proteins that were not the target of siRNAs were differentially affected. In SRP14-depleted cells, SRP54 and SRP19 levels were reduced to 55 and 70%, respectively, whereas no significant changes were observed for SRP72 and SRP68 proteins. (Fig. S2A and D). In SRP54-depleted cells, SRP19, SRP68 and SRP72 were down regulated to 70-80% of their wild type levels (Fig. S2B and E). SRP14 protein levels remained unchanged. In SRP72-depleted cells, only SRP68 was significantly down regulated when compared to control cells (Fig. S2C and F). In all cases, the protein L12 of the large ribosomal subunit that was used as a control remained unchanged.

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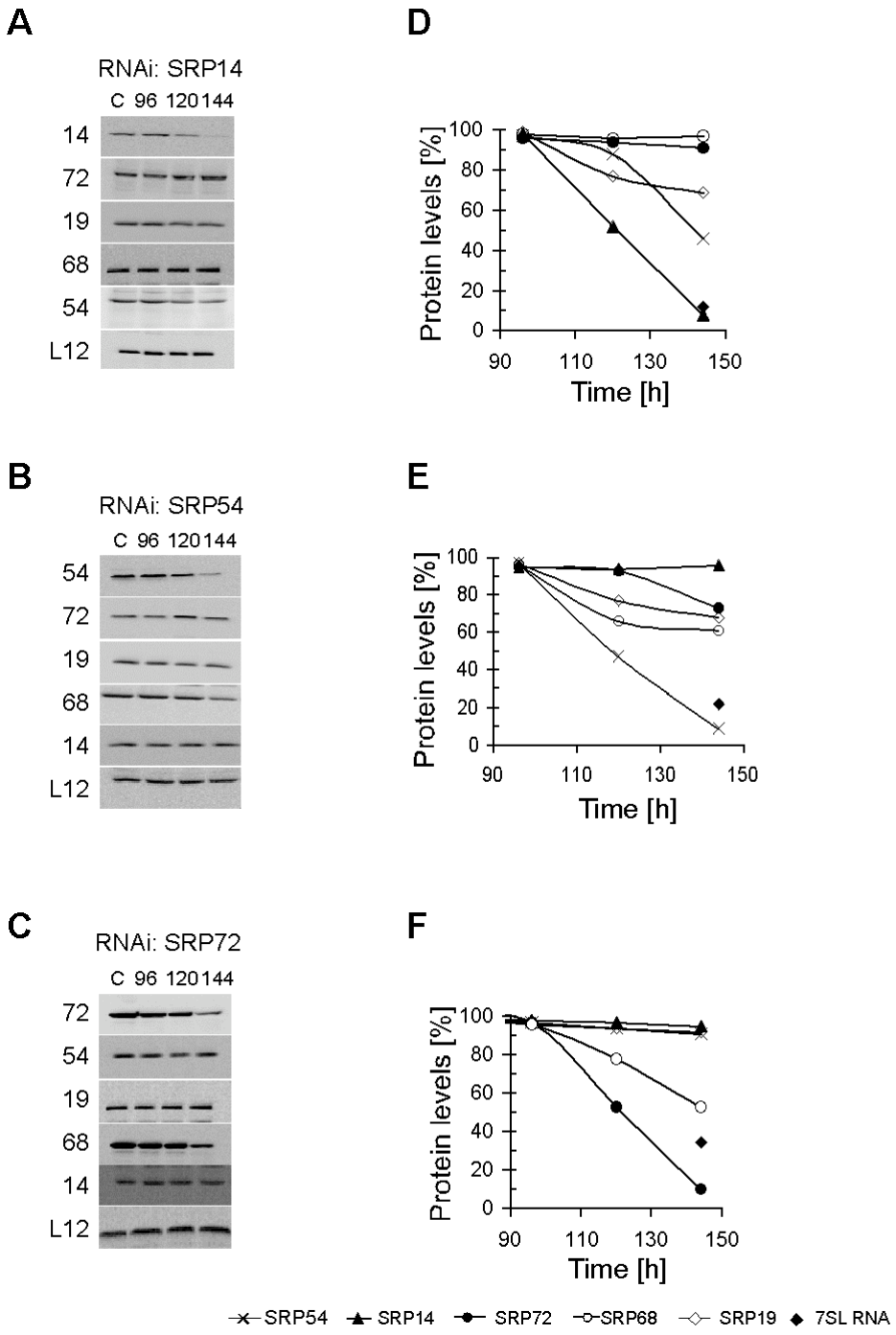


Fig S2: Expression levels of SRP proteins after inducing RNAi against individual subunits. Time course of the expression levels of SRP proteins revealed with subunit-specific antibodies. RNAi against SRP14 (**A**), SRP54 (**B**) and SRP72 (**C**). Equal amounts of cell extract were loaded in each lane. L12: Protein of the large ribosomal subunit. **(D**, **E**, **F**) Quantification of the Western blots. Expression levels were normalized to control cells. The expression levels of SRP RNA (closed diamonds) were 11.8±0.3%, 20.8±0.3% and 34.5±0.3% for SRP14, SRP54 and SRP72-depleted cells, respectively.

## *2.3 SRP maintains nascent chains translocation-competent by slowing translation rates to match limiting numbers of targeting sites*

### *2.3.1 Summary*

Cotranslational protein targeting is facilitated by signal recognition particle (SRP) and its membrane bound receptor, by recognizing the signal sequence in the nascent polypeptide chain. The identification of signal sequence by SRP is followed by a transient pause in the elongation of the nascent chain termed as elongation arrest function of SRP. The *Alu* domain of SRP, which is comprised of SRP9, SRP14 and the 5' and 3' ends of the 7SL RNA, performs the elongation arrest function. Several *in vitro* studies performed in cell free translation systems have characterized the elongation arrest function. Previous studies in our lab have shown that elongation arrest function is dependent on the presence of a five amino acid motif in the C-terminal domain of SRP14. The absence of this motif resulted in defects in translocation in the *in vitro* system.

Until today the relevance of elongation arrest function in mammalian cells has not been established and the very existence of elongation arrest function in mammalian cells is a question of major debate. To solve this problem, we decided to address two major questions: 1) Is elongation arrest function a physiologically relevant event in mammalian cells? 2) If it is indeed relevant then what is the reason for the existence of the delay in nascent chain elongation?

To answer the above-mentioned questions we utilized a complementation system in mammalian cells. We down regulated the endogenous SRP14 using an shRNA targeting the 3' UTR of the gene. Cells depleted of endogenous SRP14 were complemented with either wild type or elongation arrest defective GFP-SRP14 chimeras. The GFP-SRP14 chimeras were properly assembled into functional SRP as analyzed by glycerol gradients and restoration of functional levels of SRP was analyzed by measuring the levels of 7SL RNA, which were restored upon complementation with GFP-SRP14 chimeras.

The cells expressing elongation arrest defective GFP-SRP14 chimeras showed defects in accumulation of secretory and membrane proteins. Further, a significant decrease in the cell surface glycoprotein content was observed in these cells indicating the importance of elongation arrest function in mammalian cells. Pulse labeling experiments have confirmed that the above-mentioned defects are due to decreased translocation efficiency in these cells, as observed by the accumulation of precursor proteins in these cells.

Further we examined the effect of loss of elongation arrest activity on the cells growth. We observed that cells show a decreased growth rate and specifically accumulated in G0/G1 phase of cell cycle. This appears plausible, since protein synthesis is shut down in the G2/M phase, which makes SRP-mediated targeting unnecessary. Multiple factors such as decreased levels of growth receptors in the plasma membrane and diminished secretion of endocrine growth factors are likely to interfere with normal cell growth. The absence of elongation arrest activity will ultimately result in cell death, since essential components will eventually become limiting. These results indicate that elongation arrest activity is an essential function in mammalian cells

To gain mechanistic insights into the elongation arrest function, we examined whether slowing down the nascent chain elongation using inhibitors can rescue the defects observed due to lack on elongation arrest function. We observed that slowing down the nascent chain speed by four fold using elongation specific inhibitors rescued

the cells from the defects caused by the lack of elongation arrest activity. This slowing down of nascent chain is essential for efficient targeting indicating that elongation arrest increases the time window during which the nascent chain can be retained translocation competent.

Furthermore it can be hypothesized that slowing down of the nascent chain is essential because, one or more components downstream of SRP binding to nascent chain are limiting in their cellular amounts. The two events that happen downstream of SRP binding to the nascent chain are binding with the SRP receptor on the membrane and release of the nascent chain into the free translocon. Having translocon as a rate-limiting step was highly improbable because translocon components are also involved in posttranslational proteins targeting and having one of them in limiting amounts could interfere with SRP independent targeting pathways. Overexpression of both the SRP receptor subunits resulted in the cells defective in elongation arrest function resulted in the rescue of secretory defects indicating that SRP receptor is the rate-limiting factor.

Taken together this study shows for the first time that elongation arrest function is an essential event in mammalian cells. These results also show that the normal cellular concentration of SRP receptor is too low to allow targeting to proceed at the same rate as translation in the absence of SRP elongation arrest function.

## *2.3.2 Publication (submitted)*

# SRP maintains nascent chains translocation-competent by slowing translation rates to match limiting numbers of targeting sites

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## Abstract

SRP is essential for targeting nascent chains to the endoplasmic reticulum, and it delays nascent chain elongation in cell-free translation systems. However, the significance of this function has remained elusive. We show that efficient protein translocation into the ER is incompatible with normal cellular translation rates due to rate-limiting concentrations of SRP receptor (SR). We complemented mammalian cells depleted of SRP14 by expressing mutant versions of the protein lacking the elongation arrest function. The absence of a delay caused inefficient targeting of preproteins leading to defects in secretion, depletion of proteins in the endogenous membranes and reduced cell growth. The detrimental effects were reversed by either reducing the cellular protein synthesis rate or by increasing SR expression. SRP therefore ensures that nascent chains remain translocation-competent during the targeting time window dictated by SR. Since SRP-signal sequence affinities vary, the delay also regulates which proteins are preferentially targeted.

## Introduction

Efficient delivery of proteins to their subcellular locations and to the outside of the cell is important to maintain cell function and organization. Thus, cells have developed competent mechanisms to deliver proteins to their target sites. The universally conserved signal recognition particle (SRP) and its membrane-associated receptor (SRP receptor (SR) or docking protein) are responsible for cotranslational targeting of secretory and membrane proteins to the endoplasmic reticulum (ER).

SRP-mediated targeting is achieved via a series of ordered steps that are closely coordinated (for review, see Keenan et al., 2001; Pool, 2005). The hydrophobic signal sequence, a common hallmark of ER-targeted proteins, is first recognized by the SRP54 subunit of SRP, and their association causes a delay in the elongation of the nascent chain that is termed the elongation arrest (Walter and Blobel, 1981). The ribosome-nascent chain-SRP complex (RNC-SRP) is then docked to the ER membrane through the interaction of SRP with SR (Gilmore et al., 1982; Meyer et al., 1982), both in their GTP-bound form (Connolly and Gilmore, 1986). After docking of the ribosome onto the protein-conducting channel (translocon), SRP and SR dissociate from the ribosome and from each other by hydrolyzing their bound GTPs (Connolly et al., 1991) and translation resumes at its normal speed. Such a coordinated mechanism requires SRP and SR to switch dynamically between multiple functional states in response to cargo binding (Shan et al., 2004).

Mammalian SRP is composed of a single RNA and six protein subunits and can be divided into two domains. The heterodimer SRP9/14 and the 5' and 3' ends of the SRP RNA form the *Alu* domain, which holds the elongation arrest function (Siegel and Walter, 1985). SRP lacking the *Alu* domain or SRP9/14 can still promote translocation in cell-free assays, albeit at reduced efficiency. However, it lacks the capacity to bind RNCs lacking signal sequences (Hauser et al., 1995; Powers and Walter, 1996). A C-terminal truncation of murine SRP14 abrogates the elongation arrest function, but does not interfere with its ribosome binding capacity (Thomas et al., 1997). A similar truncation in S. cerevisiae SRP14 (Srp14pΔ29) also leads to the loss of the elongation arrest function (Mason et al., 2000). These results suggest an elongation arrest-specific role for the C-terminal region in SRP14.

Cryo-electron microscopy images of SRP bound to artificially arrested ribosomes showed that the *Alu* domain of SRP is located in the elongation factor-

binding site (Halic et al., 2004). Furthermore, SRP was found to interact with the ribosome at the step of the EF2-catalyzed translocation of the tRNA from the A to the P site in yeast and in mammalian cells (Ogg and Walter, 1995; Lakkaraju et al., 2007). The *Alu* domain might then delay the elongation cycle by preventing the binding of EF2. Cross-linking studies in ongoing translation revealed that the interactions between the *Alu* domain and the ribosome are dynamic and change upon signal sequence recognition (Terzi et al., 2004).

The elongation arrest function has been studied mostly in cell-free translation/translocation systems. In the heterologous translation/translocation assay using wheat germ lysate and canine microsomes and SRP, signal sequence recognition by SRP induces an arrest in the elongation of several ER-targeted proteins at one or multiple sites in the nascent chain (Walter and Blobel, 1981; Meyer et al., 1982; Lipp et al., 1987; Okun and Shields, 1992; Wolin and Walter, 1993). In the homologous mammalian and yeast translation/translocation systems, SRP causes a delay in the accumulation of full-length protein rather than an accumulation of arrested fragments (Wolin and Walter, 1989; Mason et al., 2000), although specific pause sites of the ribosome could be revealed at the level of the mRNA. The delay in elongation of the nascent chain by SRP leads to the stacking of ribosomes at the pause sites (Wolin and Walter, 1988; Wolin and Walter, 1989; Wolin and Walter, 1993).

Abrogating the elongation arrest function of SRP reduces the translocation efficiency in the heterologous as well as in the yeast homologous translation/translocation systems (Siegel and Walter, 1986; Thomas et al., 1997; Mason et al., 2000) leading to the hypothesis that the elongation arrest activity may increase the time window of opportunity for the SRP-RNC complex to interact with SR (Siegel and Walter, 1985). A time window for SRP-mediated targeting also served as a parameter to develop a mathematical model of the translation/translocation process, which predicted that translation inhibition is not required for efficient translocation *in vivo* unless SR concentrations would be strongly limiting (Rapoport et al., 1987). The mutant *S. cerevisiae* strain expressing an elongation arrest-defective version of the SRP14 subunit failed to reveal growth and translocation defects under normal conditions (Mason et al., 2000). The strain is temperature-sensitive for growth and, at non-permissive temperature, small amounts of the untargeted precursor protein of Pho8p could be detected whereas DPAPB was still translocated efficiently even though both proteins are SRP-dependent for ER targeting (Ng et al., 1996). The

ubiquitin-assisted translocation assay (Johnsson and Varshavsky, 1994) also revealed a defect in the tight coupling of translation and translocation.

Although absent in many bacteria, the *Alu* domain of SRP is otherwise highly conserved in evolution, consistent with its having an important function. It therefore remained a key task to understand the significance of the function for protein translocation into the ER. Based on previous SRP protein depletion experiments (Lakkaraju et al., 2007), we developed a complementation assay in mammalian cells. It was used to investigate the phenotypes caused by mutations in human SRP14 that abrogate exclusively the elongation arrest function of SRP in cell-free assays without interfering with its signal recognition and targeting activities. The mutant versions of the h14 subunit assembled well and restored normal SRP levels, but caused significant growth and translocation defects. The defects could be rescued i) by specifically slowing down the elongation step in protein synthesis or ii) by increasing the cellular levels of the receptor subunits SRα and SRß. Our results demonstrate that the elongation arrest activity has an essential function in mammalian cells. SRP reduces the elongation rate of nascent chains to maximize the in vivo efficiency of protein translocation into the ER through a limited number of SR targeting sites. In doing so, SRP also functions in a regulatory role by favoring the targeting of RNCs whose signal sequences bind to SRP with high affinity.

## Results

### A short basic region in SRP14 is essential for the elongation arrest function of SRP

The C-terminal region of human SRP14 (h14) is composed mainly of highly conserved basic amino acid residues in positions 96-107, followed by an alanine tail which is unique to primates (Figure 1A) and dispensable for elongation arrest activity (Bovia et al., 1997). In murine SRP14, truncation of the C-terminal residues 91 to 110 abrogated elongation arrest activity of SRP (Thomas et al., 1997). Crystal structures of the protein-RNA complex revealed that amino acid residues 93 to 95 make contacts with SRP9 (Weichenrieder et al., 2000) whereas residues after amino acid 95 could not be traced. This suggested that residues past amino acid 95 might be important for elongation arrest activity. We therefore decided to change the basic amino acid residues 96-107 in h14 (Figure 1A) either completely (h14A12) or partially (h14A5, h14A6-12). Since h14 functions in complex with h9, we purified the recombinant h14 proteins as heterodimeric complex with recombinant h9 as described (Terzi et al., 2004).

To analyze the effects of the mutations, it was necessary to reconstitute particles from wild type and mutated h9/14 proteins together with all other SRP proteins and synthetic SRP RNA. The activities of the particles were assayed by adding the reconstitution reactions directly to wheat germ lysate programmed for translation with synthetic mRNAs encoding preprolactin (a secreted protein) and a truncated form of cyclin D (a cytosolic protein). The relative inhibition of preprolactin synthesis as compared to cyclin D synthesis was monitored to determine elongation arrest activity.

As expected, particles reconstituted with h9/14 (RCwt) showed maximal elongation arrest activity confirming the assembly of active SRP *in vitro* (Figure 1B) whereas the negative controls (-RC and RC(-14)) displayed strongly reduced elongation arrest activities. The results of the negative controls confirmed that our assay system was dependent on exogenous SRP and that h9/14 is essential for the activity. Of the three mutated proteins we analyzed, h9/14A12 and h9/14A5 lacked the capacity to reconstitute elongation arrest-competent particles whereas h9/14A6-12 was able to do so, albeit slightly less efficiecently than the wild-type protein. With insulin as a secretory protein in the elongation arrest assay, we obtained the same results (not shown).

Although unlikely based on structure information, h14A12 and h14A5 might fail to assemble into SRP. To examine this possibility, we fractionated the reconstitution reactions on glycerol gradients. The fractionation profiles of h14 and the synthetic SRP RNA were comparable in the three reconstitution reactions (Figure S1). Hence, h9/14A12 and h9/14A5 are assembly competent.

Next, we examined processing of preprolactin into prolactin in the presence of microsomes (Figure 1C). All the reconstituted particles were able to promote translocation of preprolactin confirming that they possessed intact signal recognition and targeting activities. Importantly, the lack of elongation arrest activity reduced the translocation efficiency. RCA6-12 promoted efficient translocation.

To examine the targeting capacities of elongation-arrest-defective particles, we monitored the capacity of RCwt and RCA5 to target artificially arrested RNCs, which carry a 86 amino acid-long nascent chain of preprolactin, to microsomes (Flanagan et al., 2003). RCA5 and RCwt had comparable targeting efficiencies (Figure 1D).

Hence, the conserved amino acid residues 96-100 in h14 are essential for the elongation arrest function of SRP and for efficient translocation into microsomes. In contrast, the signal recognition and the targeting functions were not affected by the mutations.

**GFP-h14 proteins are properly assembled and restore normal SRP levels**

To analyze the physiological importance of elongation arrest activity in mammalian cells, we developed a complementation assay. Endogenous h14 was depleted in HEK 293T cells by expressing shRNAs (Lakkaraju et al., 2007). Endogenous h14 levels are reduced to about 50% at 120 hr and to less than 5% at 144-168 hr post transfection. As a negative control, we expressed shRNA against firefly luciferase. The h14 proteins characterized in the cell-free assay system were expressed as C-terminal fusions with GFP (G14, G14A12, G14A5 and G14A6-12) to complement cells for the absence of h14. To monitor GFP-h14 assembly into functional SRP, we fractionated cell extracts on glycerol gradients (Figure S1B). The migration of h19, another SRP subunit, in fractions 5 and 6 designated the presence of SRP. In mock-depleted cells, h14 was seen in the first six fractions consistent with the presence of an excess of h14 in human cells (Bovia et al., 1995) whereas in h14-depleted cells, the protein was hardly detectable (middle and lower panels). G14 and G14A5 proteins also migrated in fractions 5 and 6 demonstrating that both proteins

were assembled into SRP. In addition, all h19 migrated with SRP in cells expressing G14A5 (lower panel) confirming that SRP assembly was normal and efficient.

7SL RNA (human SRP RNA) levels decrease rapidly upon depletion of individual SRP proteins (Lakkaraju et al., 2007). The 7SL RNA level is therefore a sensitive tool to monitor the assembly and the cellular levels of SRP. In cells depleted of h14, the levels of 7SL RNA are decreased nine-fold (Figure S1C). Significantly, the levels of 7SL RNA in cells expressing any of the four different GFP-h14 protein chimeras were restored to approximately 90% as compared to wild-type levels. These results confirmed that the GFP-h14 protein chimeras assemble into SRP and thereby restore almost normal levels of cellular SRP.

**Elongation arrest-defective SRP impairs the accumulation of membrane and secreted proteins**

To examine protein secretion and membrane protein accumulation in cells expressing elongation arrest-defective GFP-h14 proteins, we monitored two specific reporter and one endogenous protein: SEAP, a secreted version of alkaline phosphatase, the plasma membrane proteins VSV-G and transferrin receptor (TfnR). The secretion efficiency of SEAP was determined by measuring the enzymatic activity accumulating in the medium between 144-168 hr after transfection. VSV-G and TfnR cell surface accumulation was determined at 168 hr after the initial transfection by labeling the protein with fluorescent antibodies in the absence of permeabilizing detergents. As expected from previous results (Lakkaraju et al., 2007), depletion of h14 without complementation (shRNA(14)/GFP) resulted in a strongly reduced amount of SEAP secreted into the medium whereas the complementation with wild-type G14 restored normal levels of SEAP secretion (Figure 2A). The secretion efficiencies of cells in which h14 was replaced with G14A5 and G14A12 was reduced to about 50% of the wild-type levels. The G14A6-12 protein restored normal secretion levels.

VSV-G and TfnR cell surface accumulation was also reduced in G14A12 and G14A5 expressing cells (Figure 2B). The quantification (C and D) revealed similar results as observed with SEAP. Notably, cells depleted of h14 that failed to express GFP-h14 chimeras were disregarded in the quantification. The decrease in VSV-G and TfnR expression is therefore specifically due to the presence of the elongation arrest-defective SRP and not due to low levels of SRP resulting from h14 depletion.

Moreover, the decrease in the cell surface expression of TfnR was not due to a recycling defect, as we could not detect an internal accumulation of TfnR in the cells expressing G14A5 and G14A12 (data not shown). Since we measured steady state levels of proteins in these experiments, and since TfnR has a low turnover rate (19±6 h, Rutledge et al., 1991), it might not be surprising that the observed effects were diminished as compared to SEAP and VSV-G.

Next, we compared the cell surface glycoprotein expression of G14 and G14A5 expressing cells. The cell surface proteins were labeled with fluorescent GS-II lectin under non permeabilizing conditions (Figure 2E). There was a difference of 30% in the surface staining intensities of glycoproteins between cells complemented with either G14A5 or G14 (Figure 2F) taking into account cells that expressed the GFP-h14 proteins. Considering that cell surface proteins have turnover rates of 30-100 h (Hare, 1990), the observed decrease in glycoprotein expression is highly significant and indicates that many proteins are affected by the absence of the elongation arrest function.

We further examined the levels of several proteins associated with ER translocation: Sec61α, ß, TRAM and TRAPα as well as the chaperone BiP (Alder and Johnson, 2004; Rapoport, 2007). Of the five proteins only the level of BiP was notably decreased. The levels of three other proteins were even increased. This observation may in part be explained by experimental variations (Figure S2). Moreover, the Sec61α protein was not mislocalized. The results are consistent with a very low turnover rate of these components. If the proteins are as stable as SRP subunits, a significant decrease in their levels would only be expected to occur at 96 hr after the lack of elongation arrest activity has become effective (120 hr + 96 hr). These findings suggested that decreased levels of translocon components were not the major cause for the defects observed in the absence of the elongation arrest function. In addition, overall protein synthesis was not affected in these experiments as shown later.

Hence, replacing endogenous h14 with either of two elongation arrest-defective proteins G14A12 and G14A5 caused specific and significant defects in protein secretion and membrane protein accumulation.

**The elongation arrest function is required for efficient translocation *in vivo***

Inefficient translocation of proteins can be revealed by monitoring the accumulation of preproteins (proteins with an uncleaved signal sequence). Bovine preprolactin, a well-studied SRP substrate in cell-free translocation systems, was chosen as a reporter and was modified with a triple flag-tag at its C-terminus (pPrl3f). The translocation efficiency was studied in pulse-labeling experiments with 293T cells grown in the presence of proteasome inhibitor (MG132). In its absence, no preprotein was detectable, presumably due to the its rapid degradation (not shown). Immunoprecipitation of prolactin and preprolactin indicated that the relative translocation efficiency was reduced to 44% in G14A5 cells (Figure 3A). Precursor accumulation was also detected by immunoblotting extracts that were prepared from cells grown in the presence of MG132 for 8 hr (Figure 3B) and the relative amount of prolactin secreted into the medium during this time period was reduced to 45% in G14A5-expressing cells. The perceptible accumulation of modified and unmodified prolactin in cells expressing G14A12 and G14A5 suggested that steps later in the secretory pathway might have slowed down as a consequence of inefficient translocation of membrane proteins and modifying enzymes.

We also analyzed the secretory protein SEAP which, in order to recognize the preprotein, was modified with a triple flag-tag at its very N-terminus (p3fSEAP). The relative size difference between the precursor and the mature SEAP proteins is very small and the pulse-labeled proteins immunoprecipated with SEAP antibodies therefore migrated together in SDS-PAGE. However, the specific analysis of the [$^{35}$S]methionine-labeled preprotein immunoprecipitated with the flag antibodies showed its accumulation in G14A5-expressing cells during the pulse of 3 min. (Figure 3C). A similar precursor accumulation was also revealed in extracts from cells grown in the presence of MG132 for 8 h (not shown).

To examine the accumulation of preTfnR, we used a plasmid expressing the TfnR-GFP fusion protein. (Figure 3D). We observed an accumulation of unmodified TfnR-GFP, which most likely represents preTfnR-GFP, in cells with elongation arrest-defective SRP. Moreover, accumulation of mature TfnR-GFP was decreased by 40% in the cells expressing G14A12 and G14A5 consistent with the results shown previously for the endogenous TfnR (Figure 2D).

These results demonstrated that the elongation arrest function is essential for efficient translocation in vivo and its absence leads to defects in protein secretion and membrane protein accumulation.

**Expression of the mutant versions of h14 significantly reduces cell growth**

Next, we examined whether replacement of h14 with G14A12 and G14A5 impaired cell growth. Experiments were started with an equal number of cells and cell growth was quantified by counting manually the live cells collected every 24 hours starting at 96 hr. The cell count is expressed as fold increase in the number of cells present at the start (Figure 4A). Between 96 and 120 hr, when the endogenous h14 levels decrease from 100-50% in shRNA(14)-expressing cells (Lakkaraju et al., 2007), there is no detectable growth difference. However, after 120 hr, when cells became dependent on the GFP-h14 proteins for proper SRP assembly, G14A12 and G14A5-expressing cells displayed a significant growth defect. The doubling time of mock-depleted cells and of cells complemented with wild-type G14 was in the range of 21-23 hr. In contrast, it was 30-34 hr for cells expressing the elongation arrest-defective chimeras G14A12 and G14A5 (Figure 4B).

To analyze whether cell growth was delayed at a particular stage in cell cycle, we quantified the cells present in different phases of the cell cycle using propidium iodide staining followed by flow cytometric analysis (Figure 4C). The analysis revealed a specific and statistically significant increase in accumulation of the cells in G0/G1 phase in G14A5 cells as compared to the G14 cells (Figure 4D). Hence, reduced cell growth is, at least partially, explained by a delay in the G0/G1 phase of the cell cycle. After 168 hr, endogenous h14 levels start to increase again (Lakkaraju et al., 2007) and the negative effects of G14A12 and G14A5 expression disappeared rather rapidly (not shown). Our studies on cell growth were therefore limited in time. Cells expressing shRNAs stably after viral infection could not be used in the complementation assay, because they displayed higher levels of endogenous h14 than the cells in the transient transfection experiments (A.K.K.L. and K.S., unpublished results).

**Slowing down nascent chain elongation with antibiotics reverts the translocation defect caused by mutant proteins.**

Next, we examined whether slowing down elongation of the nascent chains with low doses of protein synthesis inhibitors could rescue the mutant phenotype. We used two reversible inhibitors anisomycin and cycloheximide. Anisomycin prevents peptide bond formation, whereas cycloheximide interferes with translocation of the

tRNA from the A to the P site (Gale et al., 1981) in the elongation cycle. SEAP secretion was monitored to follow the effects of the antibiotics on translocation. At 136 hr, 293T cells were treated for 2 hr with varying low doses of antibiotics. Subsequently, the medium was changed to remove already secreted SEAP and the cells were incubated for another 6 hr with the antibiotics before collecting the medium.

In the absence of antibiotics, we observed the same reduction in SEAP secretion for G14A12 and G14A5-expressing cells as already reported (Figure 2A and 5A). Adding increasing amounts of anisomycin increased the relative amounts of SEAP secreted by cells with an elongation arrest-defective SRP (G14A12 and G14A5) when compared to cells with fully functional SRP (G14). At the lowest dose of antibiotics, the relative secretion efficiency of SEAP was already improved and it became equal at a concentration of 0.03 μg/ml of anisomycin, (Figure 5A, see Figure S3 for the results with HeLa cells). The antibiotic specifically rescued the phenotype caused by G14A12 and G14A5, since it failed to restore secretion levels of cells depleted of h14 and complemented with GFP. With cycloheximide, we saw the same dose-dependent increase in secretion efficiency (Figure 5B). In contrast to anisomycin, cycloheximide was capable to compensate for low levels of SRP at higher concentrations. This is because SRP is thought to interact with the ribosome at the step in the elongation cycle, which is slowed down by cycloheximide (Ogg and Walter, 1995). By slowing down this step, the low amounts of SRP present in h14-depleted cells become sufficient for efficient ER-targeting (Lakkaraju et al., 2007).

Next, we monitored the relative cellular protein synthesis rate by quantifying the [$^{35}$S]-uptake in the presence of the antibiotics. There was no difference in the incorporation between the mock-depleted cells and the cells complemented with h14-GFP proteins (Figure 5, legend). The cellular protein synthesis rate was reduced by 4-4.5-fold at the concentration of anisomycin or cycloheximide required to rescue the secretion defect caused by the absence of the elongation arrest function (anisomycin 0.03 μg/ml, cycloheximide at 3 μg/ml, Figure 5E and F).

To confirm that the antibiotics restored secretion by increasing the translocation efficiency, we monitored preprolactin accumulation in anisomycin-treated cells. In the presence of anisomycin, the preprotein was barely detectable in G14A5-expressing cells (Figure 5D).

The reversion of the phenotype was specific for inhibitors of translation elongation, since hippuristanol, an inhibitor of initiation (Bordeleau et al., 2006) failed to restore SEAP secretion (Figure 5C and G). At five-fold decreased protein synthesis rate, SEAP secretion was only increased to 65%. Notably, hippuristanol increased the secretion efficiency partially in a dose-dependent fashion consistent with a subsequent rate-limiting step.

These results demonstrated that a delay in elongation is essential for efficient translocation of preproteins and indicated the presence of a rate limiting step subsequent to the formation of the SRP-RNC complex.

**Cellular SR levels are rate-limiting in targeting.**

To determine whether SR was rate-limiting in targeting, we expressed exogenous human Srα and SRß from two separate plasmids transfected into HeLa cells at the same time as the expression plasmids for the GFP-h14 chimeras. When expressed together, the levels of SRα and SRβ were increased by 2.4 and 2.1 fold, respectively, as compared to the endogenous proteins and both proteins were found associated with the ER (Figure 6A and B). The subunits expressed individually also accumulated stably and were properly localized (Figure 6 and data not shown). As before, without the elongation arrest function, SEAP secretion was reduced to 44% as compared to control cells (Figure 6C). Expression of individual receptor subunits increased SEAP secretion only slightly. Expression of both subunits rescued secretion to 72% as compared to cells not depleted of h14. These results showed that the availability of SR is rate-limiting in targeting.

In the presence of the elongation arrest function, when all nascent chains become successfully targeted to the translocon, and in the absence of exogenous h14 (at low levels of SRP) the expression of Srα and SRß did not significantly change the secretion efficiency (Fig. 6C, G14 and GFP). Similar results were obtained in a set of experiments which were done in 293T cells with the receptors subunits expressed as fusion proteins tagged with GFP (GFP-Srα) and three flag epitopes (SRß-3f, Figure S4).

## Discussion

The results of these studies demonstrate for the first time that elongation arrest activity has a fundamental role in maintaining the structure and function of mammalian cells. Its absence leads to the depletion of proteins in the endogenous membrane system and to the reduction of protein secretion with profound consequences on cell growth. SRP lacking elongation arrest activity causes defects in protein translocation that are reversed when the overall rate of nascent chain elongation is decreased by four-fold. This result demonstrates that when the cellular translation elongation rate of signal sequence-containing proteins exceeds the rate of targeting those proteins to the ER membrane, translocation into the ER does not proceed efficiently. The rate-limiting factor in targeting in vivo is the SRP receptor: if its concentration is insufficient, delays in the targeting of RNCs to the ER occur because of a shortage of operational targeting sites. As a consequence, not all nascent chains will reach the translocon in time for successful engagement into translocation. But importantly, this effect may also serve a regulatory purpose. Since SRP will dissociate preferentially from SRP-RNC complexes with low affinity while awaiting a free SR, nascent chains will be targeted and processed more frequently at the ER if their SRP-signal sequence affinity is high rather than low.

In pancreatic microsomes, SR is about two-fold less abundant than active translocons (Guth et al., 2004), consistent with a rate-limiting function in secretion. Moreover, the translocon might become rate-limiting at more than two-fold increased SR levels providing an explanation for the incomplete rescue of translocation in these experiments. Exogenous SR expression in wild-type cells did not improve secretion efficiency suggesting that subsequent steps in protein translocation and secretion become limiting. SR expression also failed to improve secretion at low SRP levels consistent with the interpretation that SRP is rate-limiting under these conditions. Very little is known about the relative abundance of SRP and the membrane components in different cell types or tissues, and this issue needs to be explored more thoroughly to understand whether elongation arrest activity has the same fundamental importance for all cell types as suggested by our experiments and whether it might also be used for regulatory purposes. *S. cerevisiae* failed to reveal growth or translocation defects under normal growth conditions in a strain expressing an elongation arrest-defective version of the Srp14p subunit. It has a temperature-

sensitive growth defect similar to the one observed for strains lacking Srp14p altogether (Mason et al., 2000). The difference may be explained by increased cellular levels of SR and by a higher capacity of *S. cerevisiae* to translocate proteins post-translationally.

A kinetic study in COS-1 cells revealed that the average targeting time for a reporter protein was very short (about 5 s) (Goder et al., 2000). During this time period, nascent chains only elongate by 40 amino acid residues (with an average elongation rate of 8 aa/s), making the elongation arrest function presumably unnecessary. As already discussed in (Goder et al., 2000), this time period represents the average time for all ribosomes and it is conceivable that the targeting time for the first ribosome is considerably longer than the one of the subsequent ribosomes. One factor which may shorten the average targeting time is SRP-mediated ribosome stacking, which was shown in cell-free translation systems (Wolin and Walter, 1988 Wolin and Walter, 1989).

Based on these observations, our results may be explained with the following model (Figure 7). At limiting SR concentrations, the first targeting event is critical because in the absence of a delay mediated by SRP or antibiotics, nascent chains extend beyond a critical length which renders them translocation-incompetent. With SRP and antibiotics, the average length of nascent chains is decreased during the time window required for targeting, and ribosomes stack along the mRNA by switching the mode of translation from initiation-limited (Sonenberg et al., 2000) to elongation-limited. The first ribosome will be arrested by signal sequence binding of SRP, whereas the following ribosome stacks behind the first one without the need for SRP. This effect may reduce the number of SRP bound to polysomes, consistent with the 20-50-fold lower abundance of SRP as compared to ribosomes (Bovia et al., 1995; Raue et al., 2007). With antibiotics, the ribosomes will stack close to the translation start site. SRP will bind to the leading ribosome as soon as the signal sequence emerges. In both cases, targeting of the leading ribosome will increase the concentration of short nascent chains in close proximity of the membrane-bound SR. Although each nascent chain may still need to be targeted individually (Rapoport et al., 1987), the process may be accelerated and thereby decreases the average targeting time. Both antibiotics have a compensatory effect indicating that the stage at which the ribosome is arrested in the elongation cycle is apparently not critical for restoring efficient translocation.

At higher levels of membrane-bound SR, the targeting frequency of SRP-RNCs is increased resulting in the successful targeting of more nascent chains, even in the absence of a delay in elongation and subsequent ribosome stacking. The average occupancy of mRNAs is in the range of one ribosome every 80-100 nucleotides (Sonenberg et al., 2000).

Nascent chains may loose competence for translocation because translation has already terminated or nascent chains have elongated beyond a critical length after which they cannot be targeted (Siegel and Walter, 1985; Wiedmann et al., 1987; Flanagan et al., 2003). Why nascent chain length interferes with targeting still remains to be elucidated. It was first suggested that SRP might no longer recognize the signal sequence in longer nascent chains because it becomes sequestered (Siegel and Walter, 1985). However, more recent experiments demonstrated that SRP binds with the same affinity to RNCs with preprolactin nascent chains of different length (Flanagan et al., 2003). It is conceivable that longer nascent chains may interfere sterically with SR and/or translocon recognition.

The cellular generation time is increased by 50% in the absence of the elongation arrest function. Plausibly, the delay in growth is most prominent in the G1 phase of the cell cycle when cells depend on rapid synthesis of membrane and secretory proteins. We expect the absence of elongation arrest activity to result in cell death, since essential membrane components will eventually become limiting causing severe defects in cell structure and function.

The essential motif of only five amino acid residues in the C-terminal region of SRP14 is highly conserved. The dramatic effect of the mutation indicates that very defined and limited interactions, most likely with the ribosome, effect the arrest in elongation. Based on available atomic structure data (Weichenrieder et al., 2000) combined with cryo-electron microscopy densities (Halic et al., 2004), it is not yet possible to assign a contact site in the ribosome for the SRP14 motif. A similar small basic peptide in the ER membrane protein Erj1p inhibits translation initiation (Dudek et al., 2005). In contrast to the *Alu* domain of SRP, the protein was found to bind at the nascent chain exit site (Blau et al., 2005).

An *Alu* domain-like RNA structure is present in most eukaryal and archaeal SRPs, and also in a few eubacterial SRPs. However, SRP14 proteins have only been identified in eukaryal species. Organisms lacking SRP14 may have developed other mechanisms to interfere with elongation. For example, SRP in trypanosomes

comprises a small tRNA-like RNA (sRNA-85) instead of SRP9/14 (Lustig et al., 2005) and sRNA-85 may well replace the function of SRP14 (Liu et al., 2003).

The described complementation assay may serve as a tool to study the physiological relevance of mutations in other SRP subunits and may prove useful in exploring the functions of other ribonucleoprotein particles.

**EXPERIMENTAL PROCEDURES**

**Plasmid construction and SRP assembly in vivo.** Please refer to the supplementary Experimental Procedures for a detailed description.

**Western blotting and antibodies.** 7SL RNA analysis and Western blotting with most antibodies are described in Lakkaraju et al., 2007. Additional antibodies: Anti-flag M2 (1:10'000, Sigma), anti-SRα (1:2'000, Abcam), anti-SRβ (1:500, kind gift of Dr. P. Walter (UCSF, California).

**Cell culture.** Human HeLa (CCL-2) and HEK 293T cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Sigma). Equal numbers of cells were initially transfected with shRNA(14) and, for mock depletion, with shRNA(Luc). After 24 hr, cells were selected for 24 hr in puromycin (3 μg/ml). At 72 hr, cells were plated at equal numbers into 6 cm dishes and transfected with GFP-h14-expressing plasmids (or GFP as a control) and, if applicable, with a plasmid expressing a reporter protein. Antibiotics were added initially for 2 hr at 136 hr, then the medium was changed and the cells were incubated for another 6 hr with antibiotics before collecting the medium and lysing the cells (Lakkaraju et al., 2007). The cell number was quantified manually by counting the number of live cells. Growth rates were calculated for every 24 hr. Cell cycle assays are described in supplemental Experimental Procedures.

**SEAP assay, glycoprotein, VSV-G, and TfnR-GFP cell surface quantification and microscopy.** Methods are described in Lakkaraju et al., 2007. Cell surface glycoprotein staining is described in supplemental Experimental Procedures.

**Pulse labeling.** At 168 h after initial transfection, MG132 (25 μM) was added and after 45 min. 293T cells were labeled with 200 μCi/ml of [$^{35}$S]methionine/cysteine mix (Hartmann Analytic) for 3 min. Cells were lysed with 200 μl of 1% SDS in 0.1M Tris, pH 8 directly in the plate. The lysate was transferred into a microtube and heated to 70°C with occasional vortexing until the lysate became less viscous. The sample was diluted (3-fold) in the IP buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS 50mM Tris-Hcl pH 8, 1mM EDTA and 1x protease inhibitor cocktail) and the labeled proteins incubated overnight with the antibodies. The antibodies were immobilized on protein G-Sepharose (40 μl) for 4 hr. The precipitates were washed 3x with IP buffer for 15 min. and the bound proteins displayed by 12% SDS-PAGE. Visualization and quantification was done with the Bio-Rad phosphorimager.

**Protein and RNA expression, purification, reconstitution of SRP and activity assays.** Proteins and synthetic SRP RNA were expressed and purified as described in supplemental Experimental procedures and in (Huck et al., 2004) and (Terzi et al., 2004). The targeting assay was done as described in (Flanagan et al., 2003). Cycloheximide, SRP and EKRM were used at concentrations of 500 μM, 200 nM and 1 eq/reaction, respectively. EKRM membranes were pre-saturated as described in (Schaletzky and Rapoport, 2006). The protein contents of the supernatants and pellets were analyzed on a 15% Tricine gel and the radioactivity was detected and quantified with a Bio-Rad phosphorimager.

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## References


Alder, N. N., and Johnson, A. E. (2004). Cotranslational membrane protein biogenesis at the endoplasmic reticulum. J Biol Chem *279*, 22787-22790.

Blau, M., Mullapudi, S., Becker, T., Dudek, J., Zimmermann, R., Penczek, P. A., and Beckmann, R. (2005). ERj1p uses a universal ribosomal adaptor site to coordinate the 80S ribosome at the membrane. Nat Struct Mol Biol *12*, 1015-1016.

Bordeleau, M. E., Mori, A., Oberer, M., Lindqvist, L., Chard, L. S., Higa, T., Belsham, G. J., Wagner, G., Tanaka, J., and Pelletier, J. (2006). Functional characterization of IRESes by an inhibitor of the RNA helicase eIF4A. Nat Chem Biol *2*, 213-220.

Bovia, F., Fornallaz, M., Leffers, H., and Strub, K. (1995). The SRP9/14 subunit of the signal recognition particle (SRP) is present in more than 20-fold excess over SRP in primate cells and exists primarily free but also in complex with small cytoplasmic Alu RNAs. Mol Biol Cell *6*, 471-484.

Bovia, F., Wolff, N., Ryser, S., and Strub, K. (1997). The SRP9/14 subunit of the human signal recognition particle binds to a variety of Alu-like RNAs and with higher affinity than its mouse homolog. Nucleic Acids Res *25*, 318-326.

Connolly, T., and Gilmore, R. (1986). Formation of a functional ribosome-membrane junction during translocation requires the participation of a GTP-binding protein. J Cell Biol *103*, 2253-2261.

Connolly, T., Rapiejko, P. J., and Gilmore, R. (1991). Requirement of GTP hydrolysis for dissociation of the signal recognition particle from its receptor. Science *252*, 1171-1173.

Dudek, J., Greiner, M., Muller, A., Hendershot, L. M., Kopsch, K., Nastainczyk, W., and Zimmermann, R. (2005). ERj1p has a basic role in protein biogenesis at the endoplasmic reticulum. Nat Struct Mol Biol *12*, 1008-1014.

Flanagan, J. J., Chen, J. C., Miao, Y., Shao, Y., Lin, J., Bock, P. E., and Johnson, A. E. (2003). Signal recognition particle binds to ribosome-bound signal sequences with fluorescence-detected subnanomolar affinity that does not diminish as the nascent chain lengthens. J Biol Chem *278*, 18628-18637.

Gilmore, R., Blobel, G., and Walter, P. (1982). Protein translocation across the endoplasmic reticulum. I. Detection in the microsomal membrane of a receptor for the signal recognition particle. J Cell Biol *95*, 463-469.

Goder, V., Crottet, P., and Spiess, M. (2000). In vivo kinetics of protein targeting to the endoplasmic reticulum determined by site-specific phosphorylation. Embo J *19*, 6704-6712.

Guth, S., Volzing, C., Muller, A., Jung, M., and Zimmermann, R. (2004). Protein transport into canine pancreatic microsomes: a quantitative approach. Eur J Biochem *271*, 3200-3207.

Halic, M., Becker, T., Pool, M. R., Spahn, C. M., Grassucci, R. A., Frank, J., and Beckmann, R. (2004). Structure of the signal recognition particle interacting with the elongation-arrested ribosome. Nature *427*, 808-814.

Hare, J. F. (1990). Mechanisms of membrane protein turnover. Biochim. Biophys. Acta *1031*, 71-90.

Hauser, S., Bacher, G., Dobberstein, B., and Lutcke, H. (1995). A complex of the signal sequence binding protein and the SRP RNA promotes translocation of nascent proteins. Embo J *14*, 5485-5493.

Huck, L., Scherrer, A., Terzi, L., Johnson, A. E., Bernstein, H. D., Cusack, S., Weichenrieder, O., and Strub, K. (2004). Conserved tertiary base pairing ensures proper RNA folding and efficient assembly of the signal recognition particle Alu domain. Nucleic Acids Res *32*, 4915-4924.

Johnsson, N., and Varshavsky, A. (1994). Split ubiquitin as a sensor of protein interactions in vivo. Proc Natl Acad Sci U S A *91*, 10340-10344.

Keenan, R. J., Freymann, D. M., Stroud, R. M., and Walter, P. (2001). The signal recognition particle. Annu Rev Biochem *70*, 755-775.

Lakkaraju, A. K., Luyet, P. P., Parone, P., Falguieres, T., and Strub, K. (2007). Inefficient targeting to the endoplasmic reticulum by the signal recognition particle elicits selective defects in post-ER membrane trafficking. Exp Cell Res *313*, 834-847.

Lipp, J., Dobberstein, B., and Haeuptle, M. T. (1987). Signal recognition particle arrests elongation of nascent secretory and membrane proteins at multiple sites in a transient manner. J Biol Chem *262*, 1680-1684.

Liu, L., Ben-Shlomo, H., Xu, Y. X., Stern, M. Z., Goncharov, I., Zhang, Y., and Michaeli, S. (2003). The trypanosomatid signal recognition particle consists of two RNA molecules, a 7SL RNA homologue and a novel tRNA-like molecule. J Biol Chem *278*, 18271-18280.

Lustig, Y., Goldshmidt, H., Uliel, S., and Michaeli, S. (2005). The Trypanosoma brucei signal recognition particle lacks the Alu-domain-binding proteins: purification and functional analysis of its binding proteins by RNAi. J Cell Sci *118*, 4551-4562.

Mason, N., Ciufo, L. F., and Brown, J. D. (2000). Elongation arrest is a physiologically important function of signal recognition particle. Embo J *19*, 4164-4174.

Meyer, D. I., Krause, E., and Dobberstein, B. (1982). Secretory protein translocation across membranes-the role of the "docking protein'. Nature *297*, 647-650.

Ng, D. T., Brown, J. D., and Walter, P. (1996). Signal sequences specify the targeting route to the endoplasmic reticulum membrane. J Cell Biol *134*, 269-278.

Ogg, S. C., and Walter, P. (1995). SRP samples nascent chains for the presence of signal sequences by interacting with ribosomes at a discrete step during translation elongation. Cell *81*, 1075-1084.

Okun, M. M., and Shields, D. (1992). Translocation of preproinsulin across the endoplasmic reticulum membrane. The relationship between nascent polypeptide size and extent of signal recognition particle-mediated inhibition of protein synthesis. J Biol Chem *267*, 11476-11482.

Pool, M. R. (2005). Signal recognition particles in chloroplasts, bacteria, yeast and mammals (review). Mol Membr Biol *22*, 3-15.

Powers, T., and Walter, P. (1996). The nascent polypeptide-associated complex modulates interactions between the signal recognition particle and the ribosome. Curr Biol *6*, 331-338.

Rapoport, T. A. (2007). Protein translocation across the eukaryotic endoplasmic reticulum and bacterial plasma membranes. Nature *450*, 663-669.

Rapoport, T. A., Heinrich, R., Walter, P., and Schulmeister, T. (1987). Mathematical modeling of the effects of the signal recognition particle on translation and translocation of proteins across the endoplasmic reticulum membrane. J Mol Biol *195*, 621-636.

Raue, U., Oellerer, S., and Rospert, S. (2007). Association of protein biogenesis factors at the yeast ribosomal tunnel exit is affected by the translational status and nascent polypeptide sequence. J Biol Chem *282*, 7809-7816.

Rutledge, E. A., Mikoryak, C. A., and Draper, R. K. (1991). Turnover of the transferrin receptor is not influenced by removing most of the extracellular domain. J Biol Chem *266*, 21125-21130.

Schaletzky, J., and Rapoport, T. A. (2006). Ribosome binding to and dissociation from translocation sites of the endoplasmic reticulum membrane. Mol Biol Cell *17*, 3860-3869.

Shan, S. O., Stroud, R. M., and Walter, P. (2004). Mechanism of association and reciprocal activation of two GTPases. PLoS Biol *2*, e320.

Siegel, V., and Walter, P. (1985). Elongation arrest is not a prerequisite for secretory protein translocation across the microsomal membrane. J Cell Biol *100*, 1913-1921.

Siegel, V., and Walter, P. (1986). Removal of the Alu structural domain from signal recognition particle leaves its protein translocation activity intact. Nature *320*, 81-84.

Terzi, L., Pool, M. R., Dobberstein, B., and Strub, K. (2004). Signal recognition particle Alu domain occupies a defined site at the ribosomal subunit interface upon signal sequence recognition. Biochemistry *43*, 107-117.

Thomas, Y., Bui, N., and Strub, K. (1997). A truncation in the 14 kDa protein of the signal recognition particle leads to tertiary structure changes in the RNA and abolishes the elongation arrest activity of the particle. Nucleic Acids Res *25*, 1920-1929.

Walter, P., and Blobel, G. (1981). Translocation of proteins across the endoplasmic reticulum III. Signal recognition protein (SRP) causes signal sequence-dependent and site-specific arrest of chain elongation that is released by microsomal membranes. J Cell Biol *91*, 557-561.

Weichenrieder, O., Wild, K., Strub, K., and Cusack, S. (2000). Structure and assembly of the Alu domain of the mammalian signal recognition particle. Nature *408*, 167-173.

Wiedmann, M., Kurzchalia, T. V., Bielka, H., and Rapoport, T. A. (1987). Direct probing of the interaction between the signal sequence of nascent preprolactin and the signal recognition particle by specific cross-linking. J Cell Biol *104*, 201-208.

Wolin, S. L., and Walter, P. (1988). Ribosome pausing and stacking during translation of a eukaryotic mRNA. Embo J *7*, 3559-3569.

Wolin, S. L., and Walter, P. (1989). Signal recognition particle mediates a transient elongation arrest of preprolactin in reticulocyte lysate. J Cell Biol *109*, 2617-2622.

Wolin, S. L., and Walter, P. (1993). Discrete nascent chain lengths are required for the insertion of presecretory proteins into microsomal membranes. J Cell Biol *121*, 1211-1219.

## Figure legend

**Figure 1. Amino acid residues 96-100 in SRP14 are critical for elongation arrest activity of SRP.**

**(**A) Sequence alignment of the C-terminal portions of wild type and mutated human SRP14 proteins (h14).

(B) and (C) Elongation arrest (black) and translocation (gray) activities of SRPs (RCs) reconstituted with different h14 proteins. The right panels: quantification of the results (n≥3). EKRM: 0.2 eq./reaction. Activities were normalized to RCwt (100%.) The elongation arrest and the translocation activities of RCwt were 72±7% and 79±10%, respectively.

(D) Targeting of pPL86-RNCs to microsomes. Targeting efficiencies (T [%]) were monitored by sedimenting microsomes through a sucrose cushion. EKRM: 1 eq./reaction; S: Supernatant, P: Pellet. Right panel: Quantification of the assays (n=3). T [%] was normalized to RCwt (100%).

–RC: Buffer. RC(-14): SRP without h9/14. EKRM: SRP-depleted microsomes.

**Figure 2. G14A12 and G14A5 fail to complement h14-depleted cells for efficient protein secretion and membrane protein accumulation.**

(A) SEAP activity in medium of 293T cells collected between 144-168 hr and standardized to the amount of protein present in extracts prepared from the secreting cells. shRNA(Luc)/G14: Activities were normalized to the one of mock-depleted cells expressing G14 (n=3).

(B) HeLa cells stained with antibodies for cell surface expression of VSV-G and TfnR in the absence of detergent.

(C) and (D) Surface staining intensities of HeLa cells expressing the GFP-h14 protein as indicated (n=2) were quantified for 200 cells each and normalized to the one of G14/shRNA(Luc).

(E) Cell surface expression of glycoproteins revealed in HeLa cells stained with GS-II lectin.

(F) Quantification of cell surface glycoprotein staining in G14A5 cells normalized to cells complemented with G14 (n=2).

**Figure 3. Elongation arrest-defective SRP causes a defect in preprotein translocation.**

(A) Preprolactin (pPL3f) and prolactin (PL3f) pulse-labeled with [$^{35}$S]methionine and immunoprecipitated with anti-flag antibodies. T: translocation efficiency.
(B) Preprolactin (pPL3f) and prolactin (PL-3f and *PL3f) accumulation revealed with anti-flag antibodies. *PL: Phosphorylated prolactin in transit for secretion. *PL3f/SUP: relative levels of *PL in the medium.
(C) Same as (A) with pSEAP3f. 3fSEAP and p3fSEAP were precipitated with antibodies against SEAP (upper panel). p3fSEAP was precipitated with flag-tag antibodies (lower panel).
(D) TfnR-GFP was revealed with anti-GFP antibodies. preTfnR-GFP most likely represents the preprotein. *TfnR-GFP: modified mature protein. The preTfnR-GFP values were standardized to actin and normalized to shRNA(Luc)/G14 (n=2).
All experiments were done in 293T cells and equal amounts of cell extracts were loaded in each lane for Western blot analysis.

**Figure 4. The absence of the elongation arrest function impairs cell growth.**
(A) 293T cells were plated at equal densities and live cells counted at the times indicated (n=3). h14 depletion starts at 120 hr (Lakkaraju et al., 2007). No increased cell death was observed with GA12 and GA5 cells (see 0-300 AU in C).
(B) The doubling time was calculated for cell counts between 120-168 hr. Doubling times of 30-34 hr represent a 50-60% increase as compared to 21 hr of the mock treated cells.
(C) Propidium iodide-stained 293T cells were analyzed for cell cycle progression using FACSort. FI: Fluorescence intensity in arbitrary units [AU].
(D) Quantification of cells in different cell cycle phases (n=5). , The sum of cells in the three phases was set to 100%. $p<0.05$ for cells in G0/G1phase indicates statistical significance of the data at 95% confidence levels.

**Figure 5. The mutant phenotypes of G14A12 and G14A5 are specifically rescued by slowing down nascent chain elongation.**
(A) (B) and (C) SEAP secretion from 293T cells in the presence of anisomycin (ANM), cycloheximide (CHX) or hippuristanol. For each concentration, the secretion activities were normalized to the one of shRNA(Luc)/G14 cells (n=3). The absolute activities of mock-depleted cells were decreased to 11, 16 and 15 % at 0.03 µg/ml of

anisomycin, at 3 µg/ml cycloheximide and at 0.75 µM hippuristanol, respectively, as compared to control cells.

(D) Preprotein accumulation in the presence of anisomycin at 168 hr. Equal amounts of cell extracts were displayed by SDS-PAGE and pPL3f revealed with anti-flag antibodies (n=2).

(E), (F) and (G) Effects of antibiotics on the cellular protein synthesis rate in mock-depleted 293T cells. Cells were labeled for 15 min. with [$^{35}$S]methionine/cysteine, labeled proteins were acid-precipitated and quantified (n=3). In parallel experiments we found that the [$^{35}$S]-uptake in shRNA(Luc)-G14, shRNA(14)-G14 and shRNA(14)-G14A5 cells without antibiotics was 31'354, 33'047 and 29'149 total counts/$10^6$ cells, respectively, indicating that protein synthesis was unaffected by GFP-h14A5 expression.

**Figure 6. Increasing receptor levels compensate the effects of protein mutants**

(A) Western blot analysis from HeLa cells co-expressing either G14 or G14A5 with one or both SRα and SRβ. b/a indicates the increase in SR subunit expression standardized to ß-actin.

(B) Cellular localization of SRα and SRβ. Immunofluorescent images from HeLa cells expressing G14A5 and both SR subunits labeled with anti-SRα and anti-SRß antibodies.

(C) SEAP secretion of cells co-expressing either G14A5 or, as controls, G14 or GFP and one or both SR subunits.

**Figure 7. A model for SRP-mediated targeting**.

A. The elongation arrest function of SRP reduces nascent chain length and induces ribosome stacking during the targeting process to ensure highest efficacy in protein translocation at rate-limiting SR levels. Limiting targeting sites lengthen the time window required for targeting and thereby may increase the selectivity of substrate recognition (see also text). SRP: red; SRP with black cross: SRP without the elongation arrest function; SR: green.

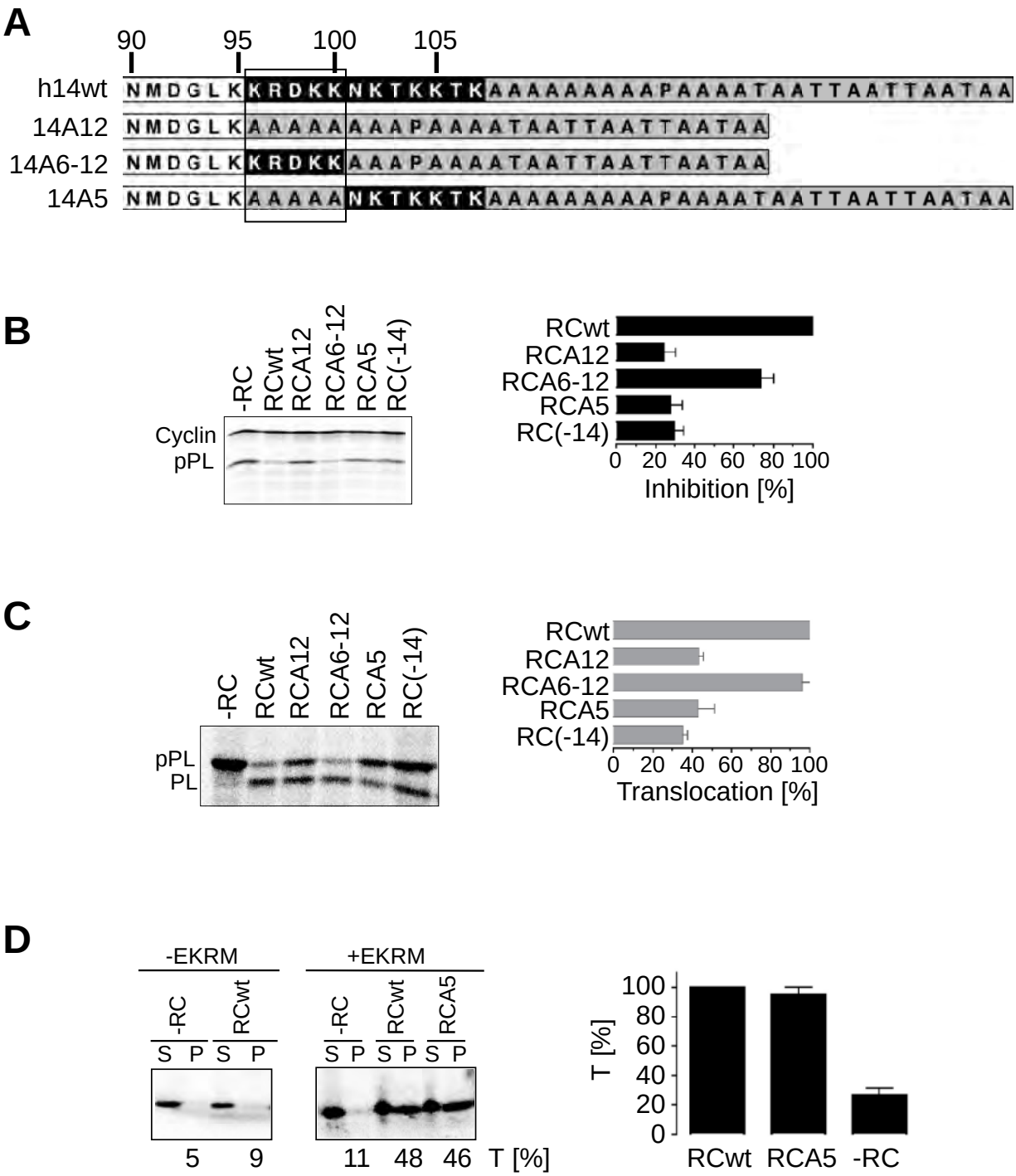


Figure 1
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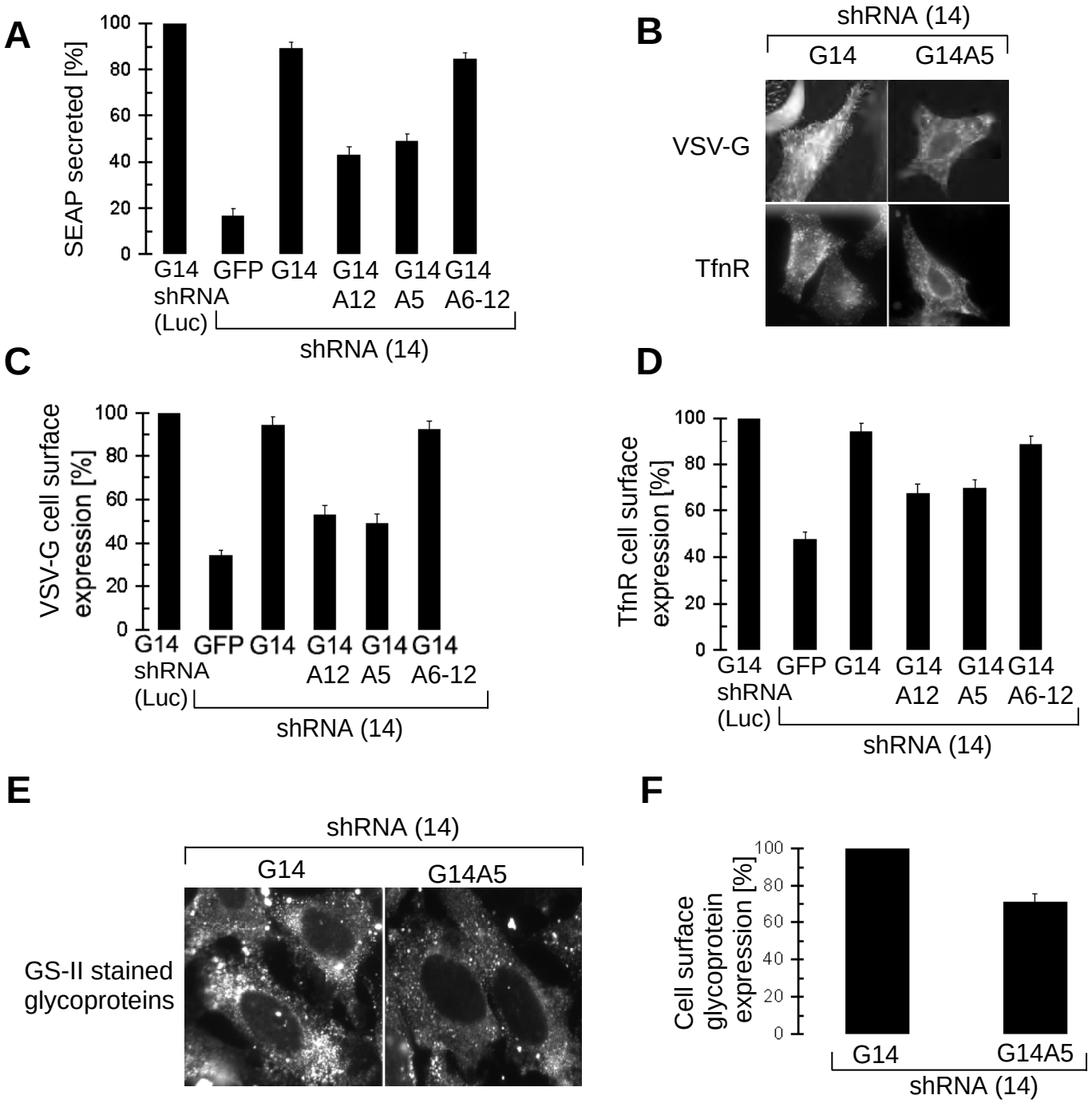


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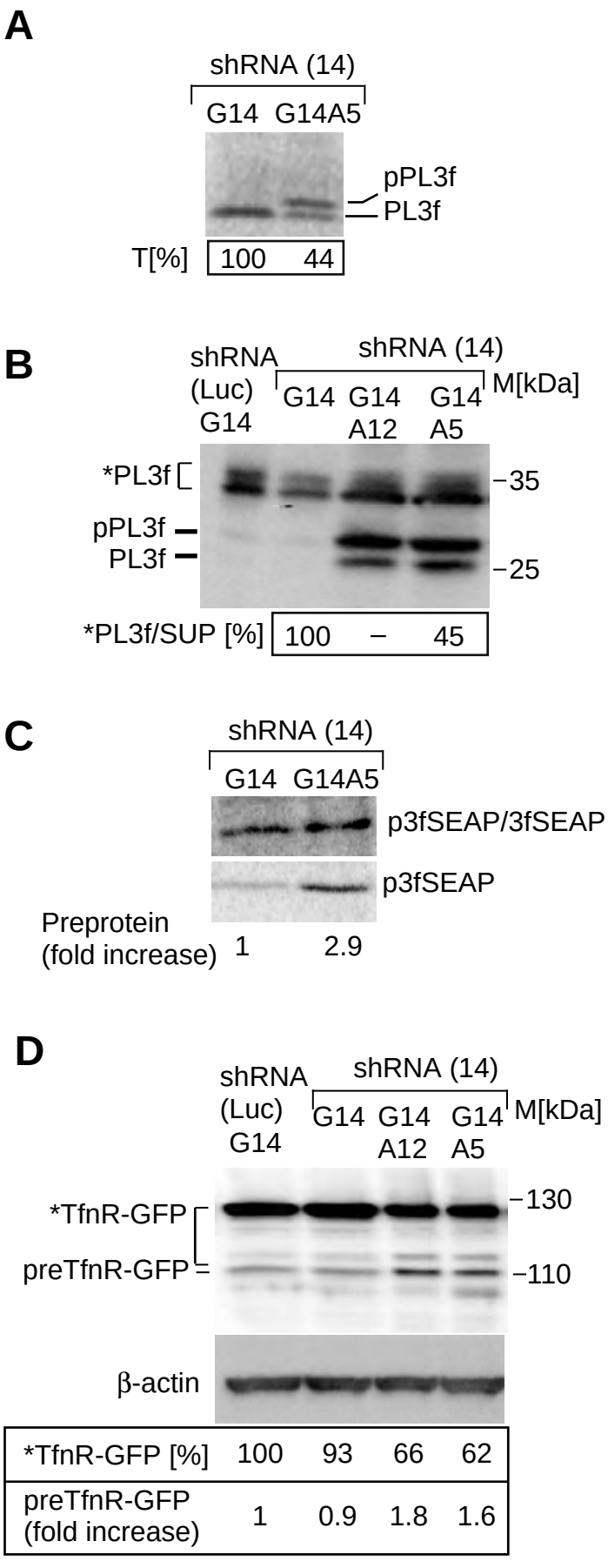
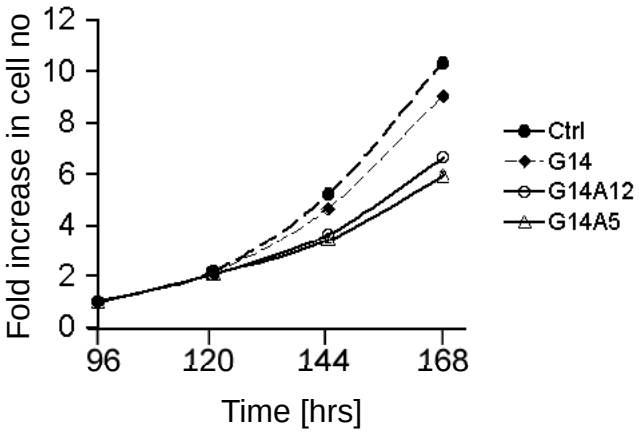



| *TfnR-GFP [%] | 100 | 93 | 66 | 62 |
|---|---|---|---|---|
| preTfnR-GFP (fold increase) | 1 | 0.9 | 1.8 | 1.6 |

Figure 3
Lakkaraju et al.

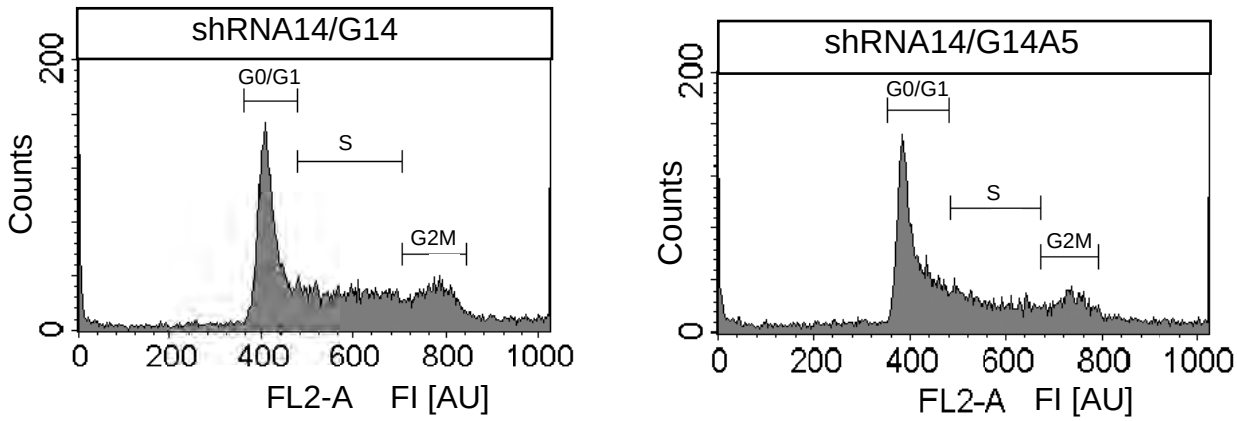
**A**




**B**

| h14 protein | doubling time (120-168) [hrs] |
|---|---|
| mock | 21 |
| G14 | 23 |
| G14A12 | 30 |
| G14A5 | 34 |

**C**




**D**

| | G0/G1 [%] | S [%] | G2/M [%] |
|---|---|---|---|
| G14 | 44±2 | 40±3 | 14±4 |
| G14A5 | 50±4 | 35±4 | 15±2 |
| | p<0.05 | p>0.05 | p>0.05 |

Figure 4
Lakkaraju et al.

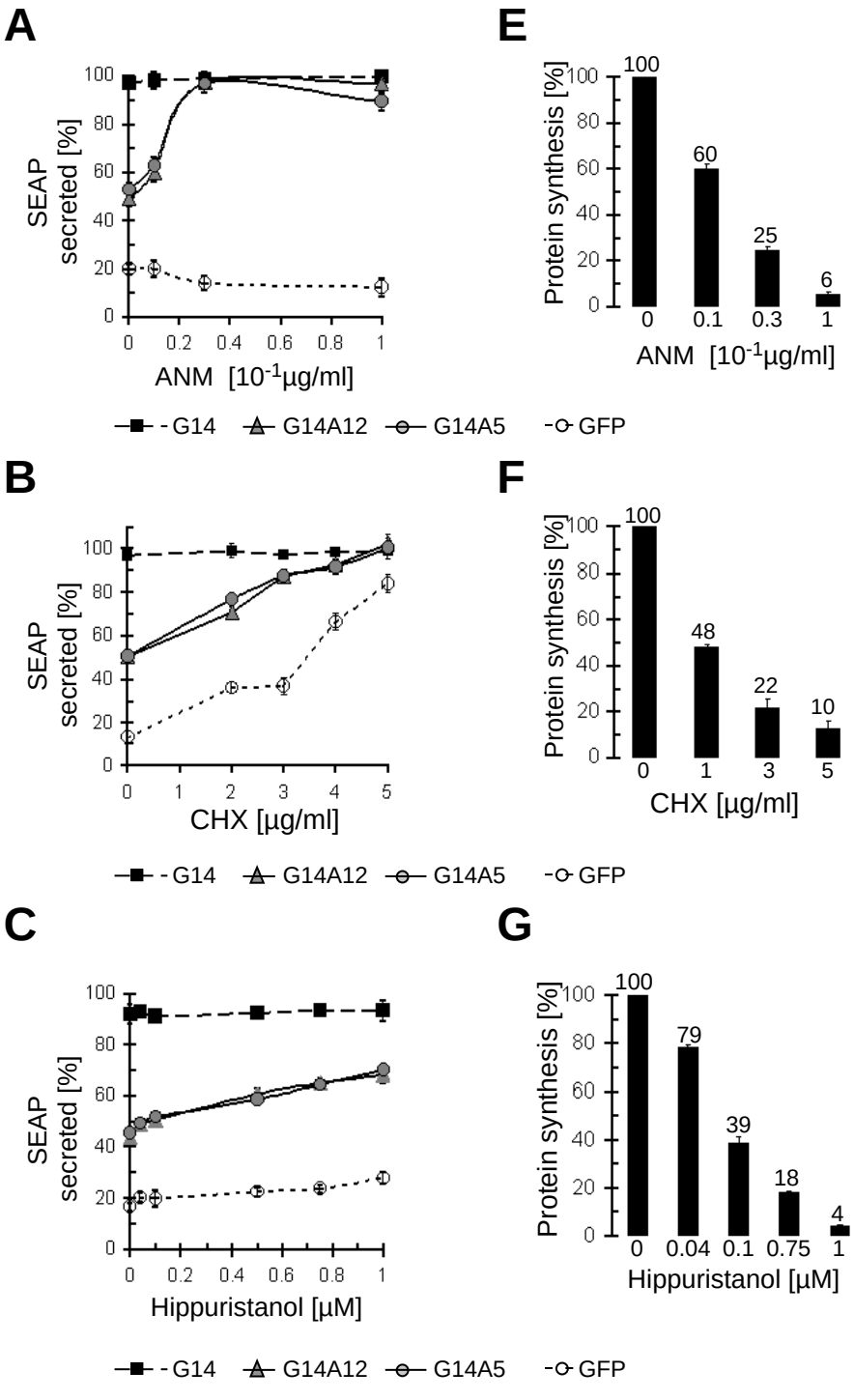



Figure 5
Lakkaraju et al.

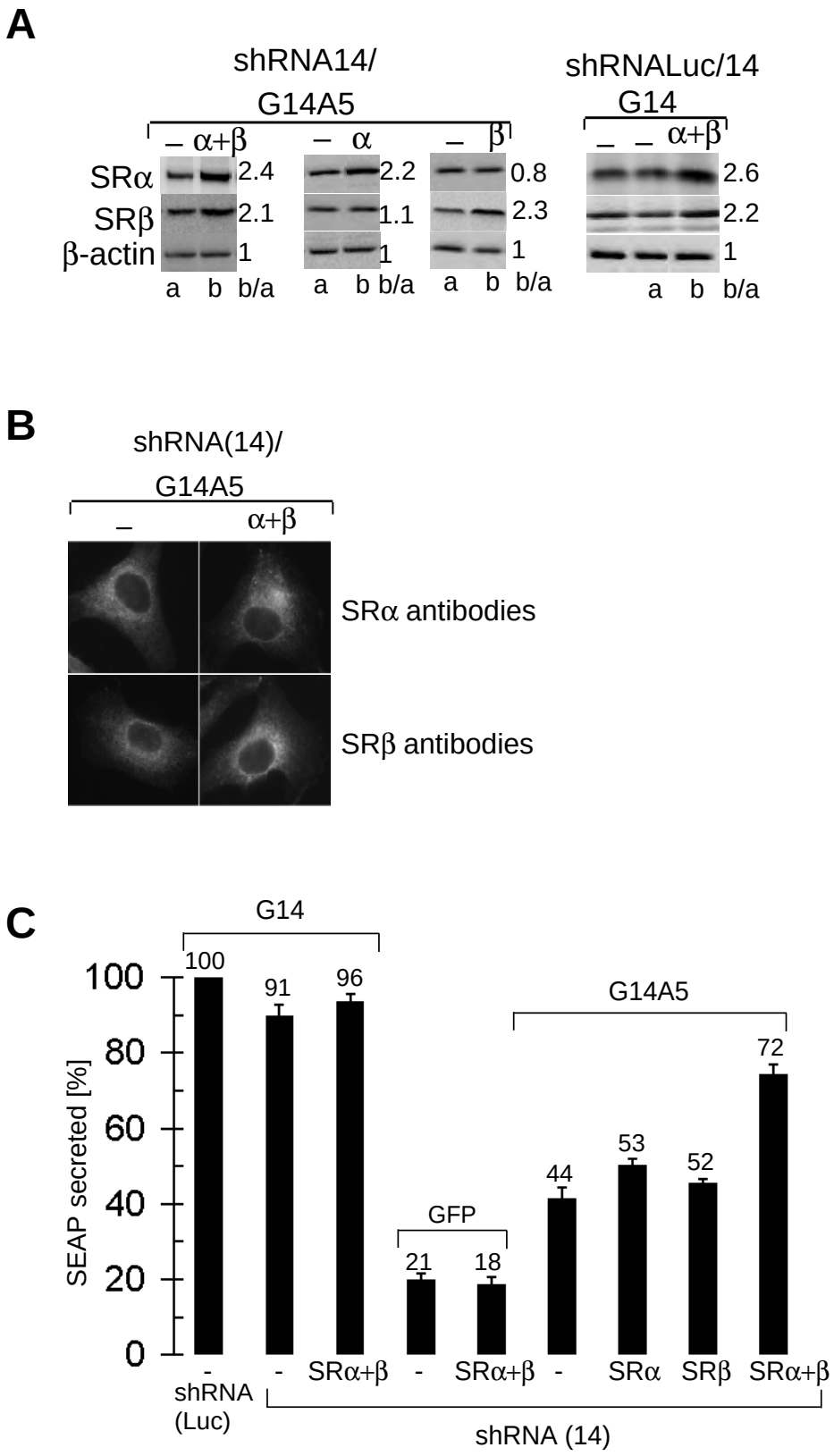



Figure 6
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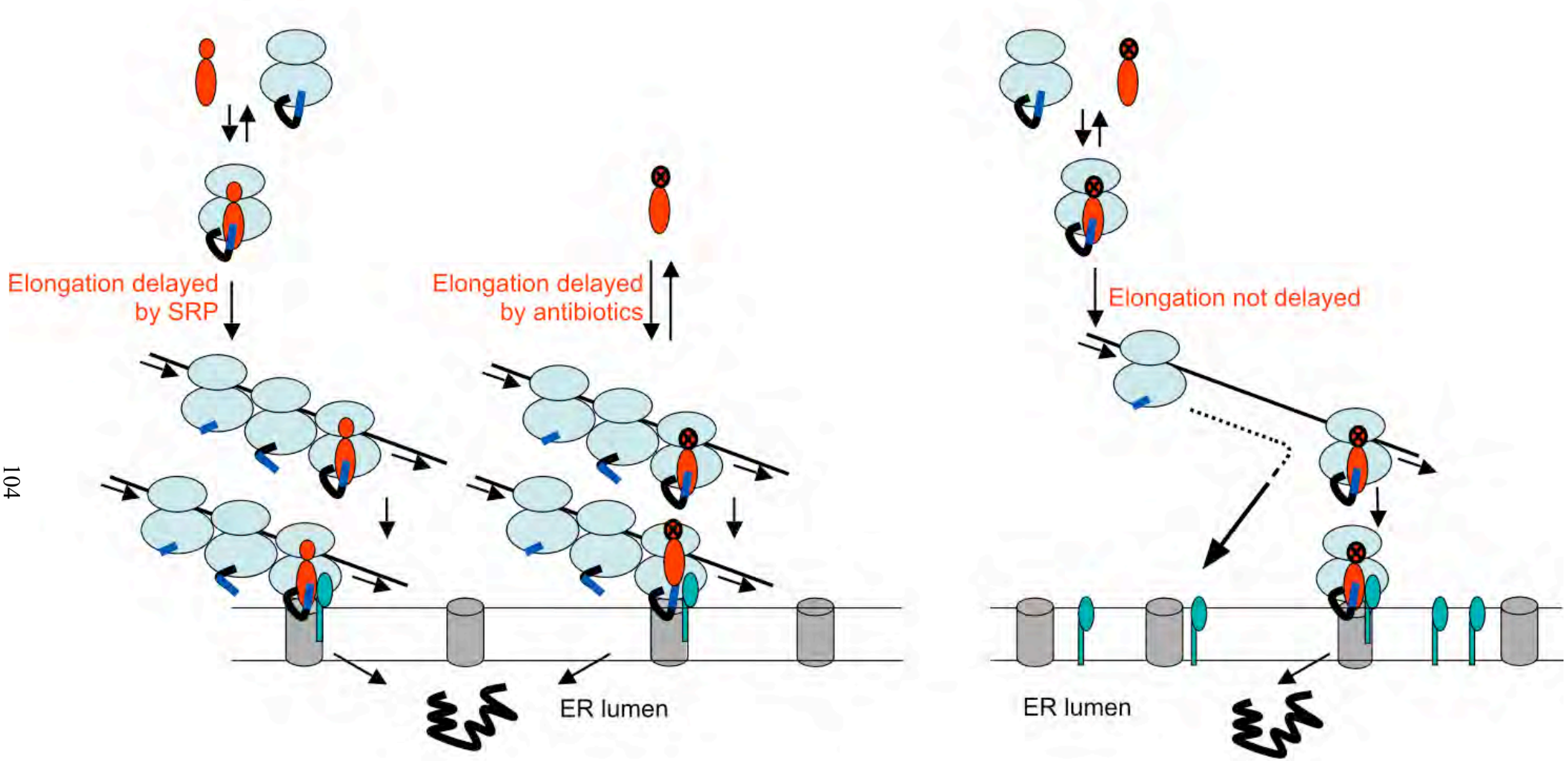


Figure 7
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# SUPPLEMENTAL DATA

## Supplemental Experimental Procedures

**Plasmid construction.** Plasmids coding for the mutated versions of the h14 protein were obtained using the Quickchange method (Stratagene). Mutated nucleotides in primers were flanked by 15 nucleotides on both sides (Microsynth). pG14 was generated by cloning the cDNA of h14 into pEGFP-C1. Clones pEth14A12 and pG14A12 were generated by removing the nucleotides encoding amino acid residues 96-108 in pEth14 (Bovia et al., 1997) and pG14 respectively. For pEth14A5 and pG14A5, the sequences of the pEth14 and pG14 plasmids were changed to encode alanine residues from positions 96 to 100. For pEth14A6-12 and pG14A6-12, the sequences were changed to encode KRDKK at position 96-100. p3F-SEAP was made by cloning the cDNA into pCMV-3xFlagN. pSRß, pPL3F and pSRβ-3F were obtained by cloning the respective cDNAs and, where applicable, a triple flag-tag into pCK. pCMV 3xflagN and pCK were kind gifts of Dr. Didier Picard, University of Geneva. pTfnR-GFP, pGFP-SRα and shRNA-expressing plasmids were described previously (Lakkaraju et al., 2007). The VSV-G expression plasmid pMD2G was a generous gift from Dr. Patrick Salmon (CMU, Geneva). All the plasmids were sequenced.

**Cell cycle analysis.** To analyze different stages of cell cycle, 293T cells grown in 6 cm plates were pelleted down and fixed with 70% ethanol for 1 h on ice, followed by the treatment with 50 μg/ml propidium iodide (Sigma) and 0.1 mg/ml RNase A (Invitrogen) for 15 min at room temperature. 20000 cells were cytometrically analyzed using FACSort (Becton Dickinson). The data was analyzed using Cell Quest software. The data was quantified using a two-tailed t-test and $p<0.05$ was considered statistically significant.

**Glycoprotein analysis.** To analyze cell surface glycoprotein content, HeLa cells were fixed in 4% paraformaldehyde and incubated with 25 μg of GS II Alexa 594 lectin (Invitrogen). GS-II binds with high specificity to terminal non-reducing α– and β-N-acetyl-D-glucosaminyl (GlcNAc) residues of glycoproteins. The fluorescence intensities were quantified by capturing 200 images of cells present in different

**SRP assembly in vivo and in vitro.** The assembly of SRP was analyzed using glycerol gradients. Either reconstituted SRP particles (4 pmoles) diluted to a final volume of 120 µl containing 50 mM Hepes pH 7.5, 120 mM KOAc, 2.5 mM MgOAc, 1 mM DTT, 0.01% (v/v) Nikkol, 25 mM sucrose or 200 µg of 293T cell extract was layered on the top of a 4 ml 12-30% (v/v) glycerol gradient for fractionation as described (Bovia et al., 1995). Fractions collected were used for Western and Northern blotting. For Northern analysis, each fraction was digested with 0.06 µg/µl proteinase K (Roche Diagnostic) for 30 min at 55°C and then ethanol precipitated in the presence of 5 µg of glycogen. RNA samples were denatured with three volumes of denaturing buffer [70%(v/v) de-ionized formamide, 2.6 M formaldehyde, 20 mM MOPS (pH 7.0), 65 mM NaOAc, 1 mM EDTA] at 55°C for 15 min and chilled on ice. Denatured RNA samples were diluted with 2 volumes of 20X SCC and transferred to an optimized nylon membrane using MilliBlot-S system (Millipore). The membrane was UV cross-linked, hybridized with a [$^{32}$P]-labeled specific SRP RNA probe (5'-GTGCGGACACCCGATCGGC-3'), washed, and quantified by autoradiography using a phosphorimager (BioRad).

**Western blotting.** The following antibodies were used: Anti Sec61α (1:2'000) and anti-Sec61β (1:10'000) both from Abcam; anti-BiP (1:100), a kind gift of Dr. V.R. Lingappa (UCSF); anti-TRAM (1:5'000) and anti-TRAPα (1:5'000), kind gifts of Dr. R.S. Hegde (NIH).

## References

Bovia, F., Fornallaz, M., Leffers, H., and Strub, K. (1995). The SRP9/14 subunit of the signal recognition particle (SRP) is present in more than 20-fold excess over SRP in primate cells and exists primarily free but also in complex with small cytoplasmic Alu RNAs. Mol. Biol. Cell *6*, 471-484.

Bovia, F., Wolff, N., Ryser, S., and Strub, K. (1997). The SRP9/14 subunit of the human signal recognition particle binds to a variety of Alu-like RNAs and with higher affinity than its mouse homolog. Nucleic Acids Res. *25*, 318-326.

Huck, L., Scherrer, A., Terzi, L., Johnson, A. E., Bernstein, H. D., Cusack, S., Weichenrieder, O., and Strub, K. (2004). Conserved tertiary base pairing

microscopic fields with the same intensity of light. The images were further analyzed by IMARIS software by calculating the average surface intensity (voxel/cell). The cover slips were analyzed by the fluorescence microscope Zeiss Axiovert 135T using a 100x (Plan Neofluar, NA-1.40) magnification. Images were captured using a charge-couple device camera (photometric CE200A) with Open Lab software.

**[$^{35}$S]methionine labeling.** Cells were grown in presence of varying concentrations of antibiotics and labeled with 50 μCi/ml of [$^{35}$S]methionine for 15 min and harvested in the lysis buffer followed by acid precipitation. The precipitated proteins were transferred onto a nitrocellulose membrane using a MilliBlot-S system (Millipore). [$^{35}$S]methionine uptake was quantified using a phosphorimager (Bio-Rad).

**Analysis of preproteins in cultured cells.** At 160 hr after the initial transfection, the cells were treated with MG132 (10μM) (Sigma) for 8 hr. Cells were harvested in lysis buffer and the amount of total protein was quantified using Bradford reagent. Equal amounts of protein were loaded on a gel to perform SDS-PAGE followed by Western blotting.

**Protein synthesis and purification.** The recombinant human proteins h14, h14A12, h14A5, h14A6-12, h9 and h19 were expressed from the plasmids pEth14, pEth14A12, pEth14A5 and pEth14A6-12 pEth9, pE19, in bacteria and purified as described (Huck et al., 2004.) The concentrations of the proteins were calculated from the absorbance at 280 nm using their specific molar extinction coefficients. Canine SRP54 and SRP68/72 were purified as described (Siegel and Walter, 1985).

**Reconstitution of SRP, cell-free activity and targeting assays.** SRP particles were reconstituted from recombinant SRP proteins, synthetic SRP RNA and canine SRP68/72 as described previously (Huck et al., 2004). SRP proteins and SRP RNA concentrations were 0.5 μM and 0.6 μM respectively. Activity assays and quantifications were done as described before (Thomas et al., 1997). Final concentration of reconstituted particles and EDTA and salt-washed rough microsomes (EKRM) (Walter and Blobel, 1983) were 100 nM and 0.02 eq/μl, respectively. The truncated form of the preprolactin mRNA encoding the N-terminal 86 amino acids (pPL86) was synthesized and purified as described (Terzi et al., 2004).

ensures proper RNA folding and efficient assembly of the signal recognition particle Alu domain. Nucleic Acids Res. *32*, 4915-4924.

Lakkaraju, A. K., Luyet, P. P., Parone, P., Falguieres, T., and Strub, K. (2007). Inefficient targeting to the endoplasmic reticulum by the signal recognition particle elicits selective defects in post-ER membrane trafficking. Exp. Cell Res. *313*, 834-847.

Siegel, V., and Walter, P. (1985). Elongation arrest is not a prerequisite for secretory protein translocation across the microsomal membrane. J. Cell Biol. *100*, 1913-1921.

Terzi, L., Pool, M. R., Dobberstein, B., and Strub, K. (2004). Signal recognition particle Alu domain occupies a defined site at the ribosomal subunit interface upon signal sequence recognition. Biochem. *43*, 107-117.

Thomas, Y., Bui, N., and Strub, K. (1997). A truncation in the 14 kDa protein of the signal recognition particle leads to tertiary structure changes in the RNA and abolishes the elongation arrest activity of the particle. Nucleic Acids Res. *25*, 1920-1929.

Walter, P., and Blobel, G. (1983). Disassembly and reconstitution of signal recognition particle. Cell *34*, 525-533.

## Figure legend

**Figure S1. Assembly of SRP in vitro and in vivo.**

(A) Fractionation of in vitro assembled SRP on glycerol gradients. Quantification of the relative amounts of synthetic SRP RNA (gray) and h14 protein (black) present in the fractions of the glycerol gradients. The sum of all fractions was set to 100%. The gradient fractions were analyzed for RNA and h14 with Northern and Western blotting, respectively.

(B) G14 and G14A5 proteins were expressed in 293T cells, which were mock-depleted (upper panel, shRNA(Luc)) or depleted of h14 (middle and lower panel, shRNA(14)). Cells were harvested at 168 hr post-transfection and post-nuclear supernatants fractionated through glycerol gradients. The fractions were analyzed by Western blotting using affinity-purified antibodies against h14 and h19. Upper panel: Mock depletion of h14 and expression of the chimera G14. In primate cells, all h19 is assembled into SRP whereas h14 exists in a free and 7SL RNA-bound form Bovia et al., 1995. Middle panel: Depletion of h14 and complementation with G14. Lower

panel: Depletion of h14 and complementation with the elongation arrest-defective G14A5. The excess of G14 and G14A5 proteins over the endogenous SRP proteins were present in lower molecular weight fractions, like the excess of endogenous h14, and, to a lesser extent, in higher molecular weight fractions. Possibly, the excess protein may form aggregates or bind to other cellular components like ribosomes or RNAs.

(C) Relative expression levels of 7SL RNA. Expression levels of 7SL RNA were normalized to the one of mock-depleted cells, which was set to 100% (n=3).

**Figure S2. Complementation with G14A5 does not significantly reduce the cellular levels of Sec61α and ß, TRAM and TRAP.**

(A) Equal amounts of cell extracts from 293T cells expressing G14 and G14A5 after depletion of h14 were displayed by SDS-PAGE and the endogenous proteins revealed by immunoblotting with specific antibodies. The relative levels of expression in G14A5 as compared to G14 and standardized to actin levels are indicated.

(B) Images from G14- and G14A5-expressing HeLa cells labeled with antibodies against the α subunit of Sec61.

**Figure S3. Effects of antibiotics on SEAP secretion in HeLa cells expressing different GFP-h14 proteins.**

(A) (B) and (C) SEAP secretion from HeLa cells in the presence of anisomycin (ANM), cycloheximide (CHX) or hippuristanol. For each concentration, the secretion activities were normalized to the one of shRNA(Luc)/G14 cells (n=2). The absolute activities of mock-depleted cells were decreased to 15, 18 and 21 % at 0.03 µg/ml of anisomycin, at 3 µg/ml cycloheximide and at 0.75 µM hippuristanol, respectively, as compared to control cells.

(D), (E) and (F) Effects of antibiotics on the cellular protein synthesis rate in mock-depleted HeLa cells. Cells were labeled for 15 min. with [$^{35}$S]methionine/cysteine, labeled proteins were acid-precipitated and quantified (n=2).

**Figure S4. Increasing receptor levels compensate the effects of protein mutants in 293T cells**

(A) Western blot analysis from 293T cells co-expressing either G14 or G14A5 and one or both GFP-SRα and flag-tagged SRβ (SRβ-3f). The antibodies detect the

endogenous and the over expressed proteins. b/a: fold increase in SR subunit expression standardized to ß-actin.

(B) Cellular localization of GFP-SRα and SRß-3f. Immunofluorescent images from HeLa cells expressing G14A5 and both tagged SR subunits labeled with anti-SRα and anti-SRß antibodies.

(C) SEAP secretion of 293T cells co-expressing either G14 or G14A5 together with one or both SR subunits. Expression of both SR subunits partially rescues the secretion defect caused by the absence of elongation arrest activity.

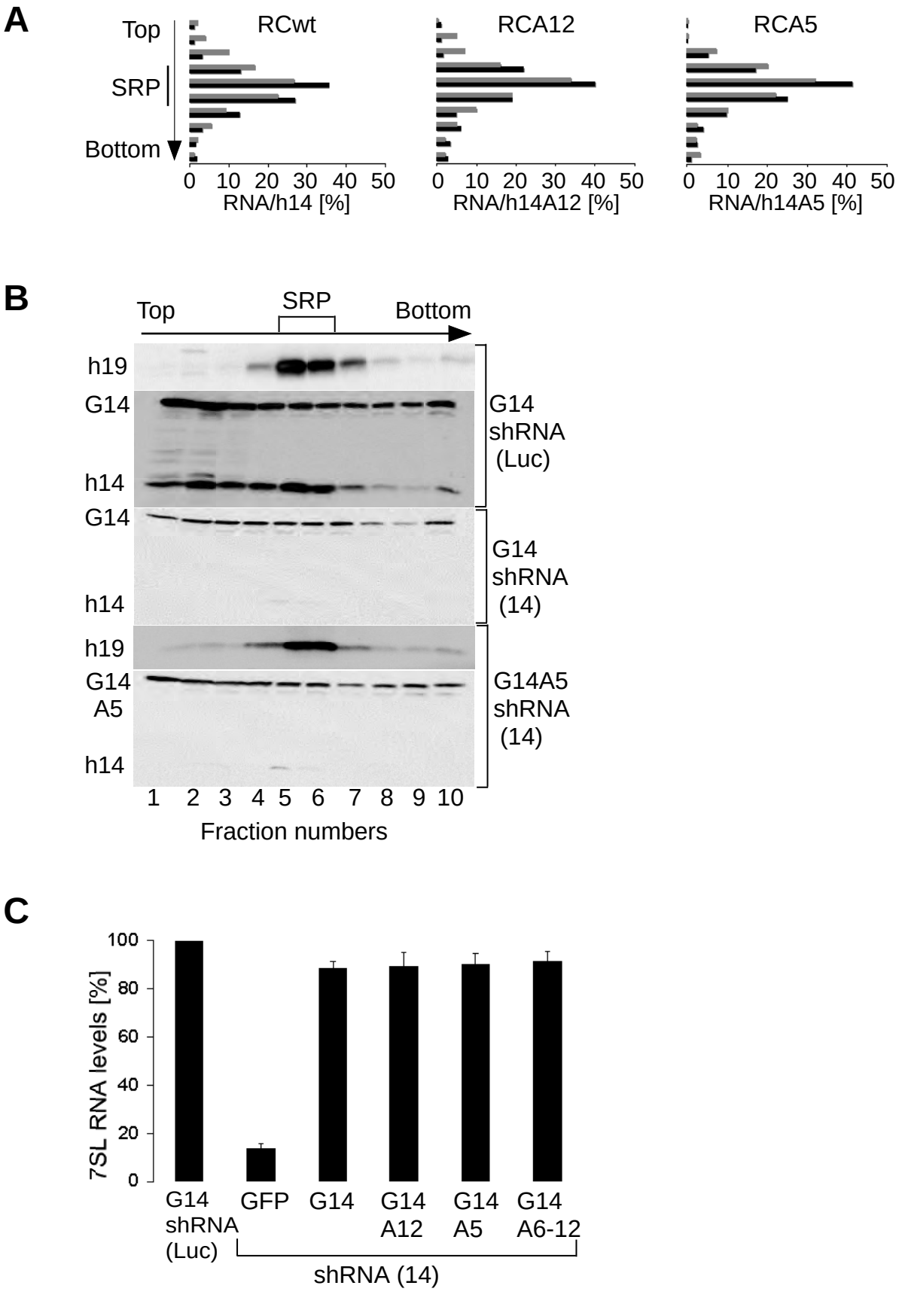


Figure S1
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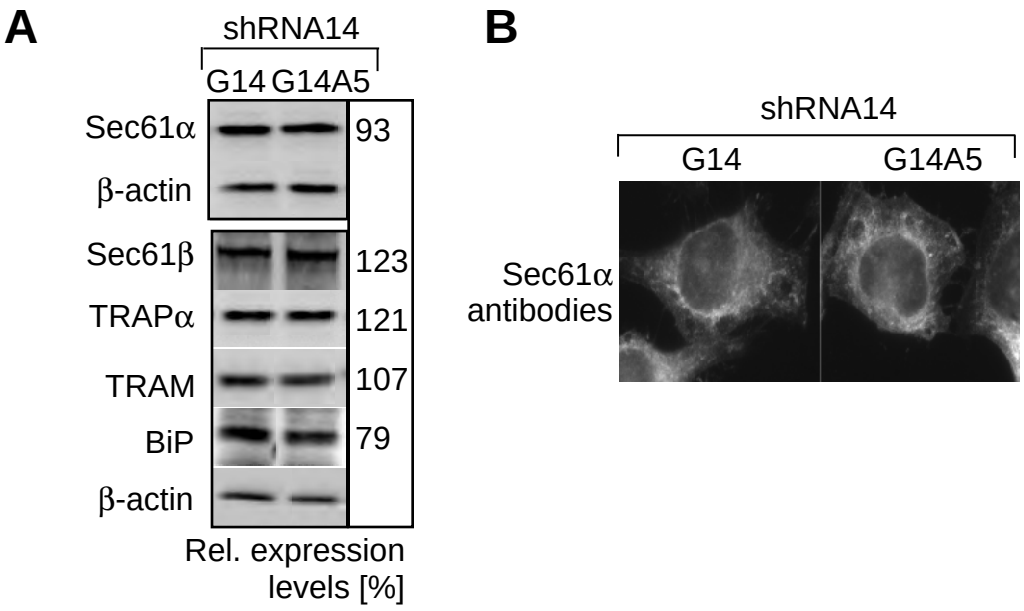


Figure S2
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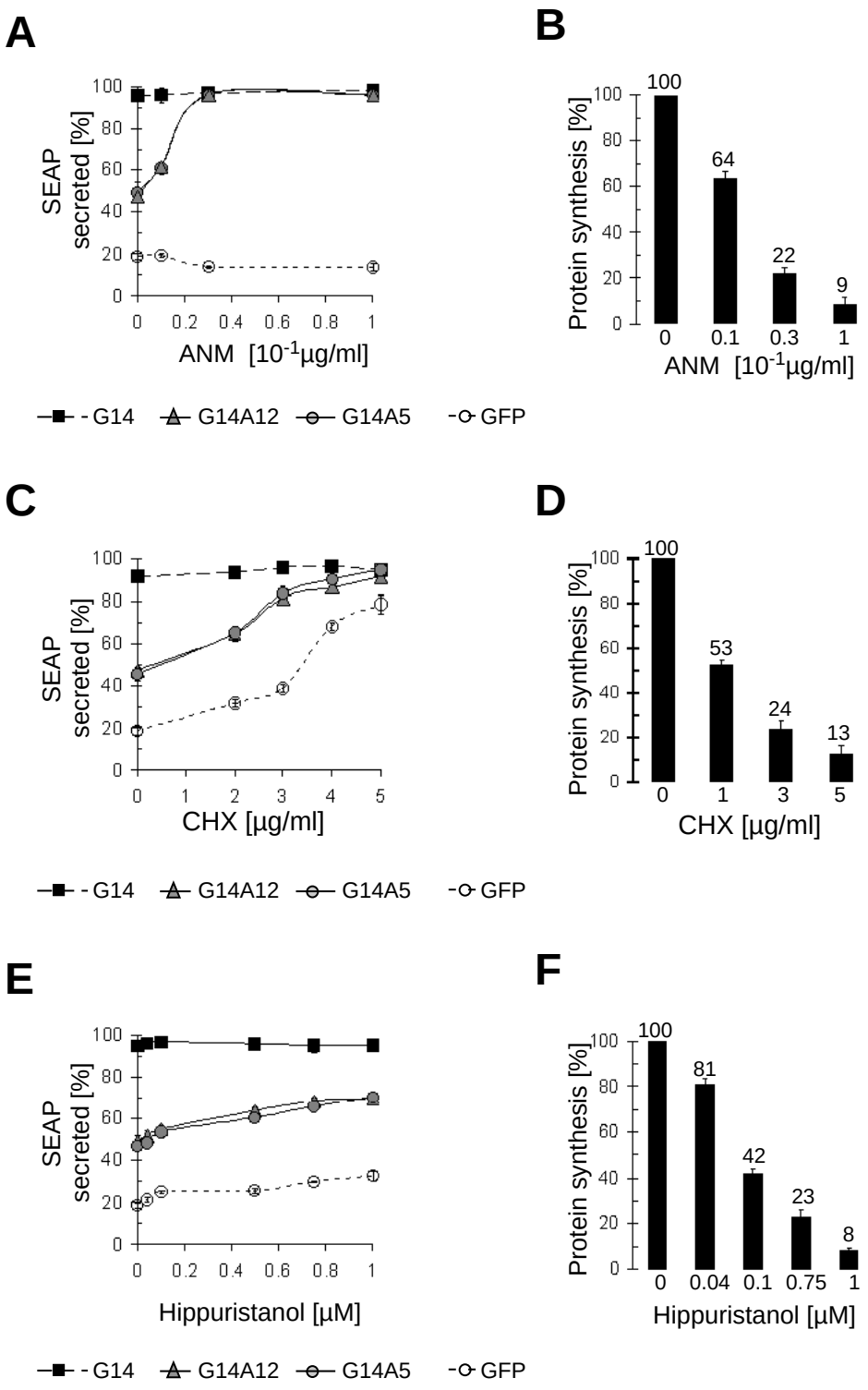


Figure S3
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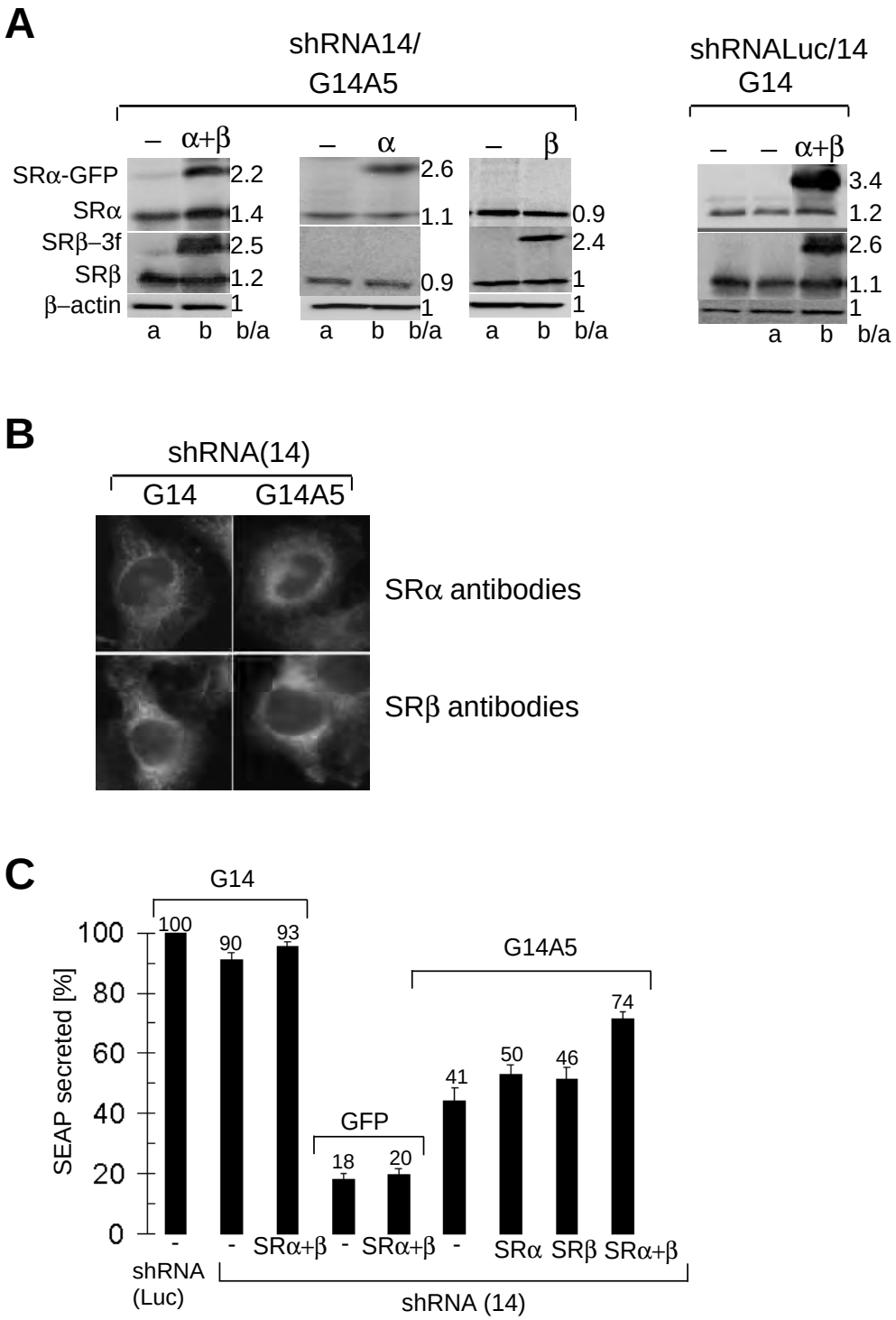


Figure S4
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# *3. DISCUSSION*

## *3.1 Elongation arrest function: secrets unveiled*

During the process of co translational protein targeting, SRP immediately binds to the nascent chain with a signal sequence, when it emerges out of the ribosome [4, 8]. Binding of SRP induces a pause in the further elongation of the nascent chain and this function of SRP is termed as the elongation arrest function [92]. Elongation arrest function has been characterized by in vitro studies (see Introduction 1.6.3). Interfering with the elongation arrest function resulted in decreased translocation efficiency into the ER [93, 95, 96]. So far, no study has been done elucidating the importance of elongation arrest function in mammalian cells. Moreover, the very existence of elongation arrest function in mammalian cells has been a major point of debate.

In our studies, we show that elongation arrest function is an essential event in mammalian cells. In the absence of the elongation arrest function, we observe that the cellular generation time is increased by 50%. The delay in the cell growth is caused because of the decreased translocation of proteins into the ER. We expect the absence of elongation arrest activity to eventually result in cell death, since essential components involved in cell structure and function will become limiting. In contrast to mammalian cells, a *S.cerevisiae* strain expressing an elongation arrest-defective version of the Srp14p subunit failed to reveal growth or translocation defects under normal growth conditions. It has a temperature-sensitive growth defect similar to the one observed for strains lacking Srp14p altogether [96]. The difference may be explained by a higher capacity of *S.cerevisiae* to translocate proteins post-translationally and/or by differences in the parameters determining the kinetics of the targeting process.

Our studies showed that elongation arrest is an essential event during co-translational protein translocation in mammalian cells. However the important question was to understand why is the elongation arrest function essential? All the studies on elongation arrest function of SRP have indicated that a slow down of nascent chain elongation by SRP would increase the time window during which the nascent chains could be efficiently targeted. We hypothesized that nascent chains have to be slowed down because one or more components downstream of SRP binding are limiting. The components that are downstream of SRP binding are the SRP receptor (SR) and the translocon machinery. A previous study has reported the concentrations of all the components involved in protein transport present in the pancreatic microsomes [118]. Interestingly, they found that both SR subunits were least abundant among all the components. They were two fold less than the Sec61 complex and about 1.5 fold less than other translocon components indicating that they could be the limiting components of the targeting pathway. Furthermore the translocon components are also involved in posttranslational pathway [119] and having one of them limiting appears to be highly unlikely. Exogenous expression of both the SR subunits in the cells lacking elongation arrest function rescued the defects in protein translocation. Thus we have identified the rate-limiting factor for the targeting of nascent chains in mammalian cells to be the SR. In the absence of elongation arrest function not all the nascent chains can reach the translocon in a translocation-competent state because of limiting number of targeting sites (SRP receptor sites) on the membrane and this leads to a defect in translocation.

Our studies also show that both the subunits of the SR are essential to reconstitute a functional SR in mammalian cells. Expression of either SR$\alpha$ or SR$\beta$

could not rescue the defects caused by the lack of elongation arrest function. Previous studies have shown that SRα is essential for the interaction with the SRP54 subunit in a GTP-dependent manner whereas SRβ is essential to anchor SRα to the membrane and also in the release of the nascent chain into the translocon [49]. In wild type cells we do not see an increased secretion in the presence of excess of receptor because under these conditions, when the receptor is over expressed by two-fold, the downstream components of the secretory pathway could now become limiting. This experiment shows a tight regulation in the secretory pathway. Just by increasing the expression of one of the components in the secretory pathway of the wild type cells does not increase in the overall secretory output.

## *3.2 Nascent chain length influences the targeting efficiency*

In the absence of elongation arrest, the nascent chains no longer remain in a translocation-competent state because the nascent chains become too long to be efficiently translocated, before they reach the membrane [120-122]. Our studies also confirmed the idea that nascent chains become too long to be targeted in the absence of the elongation arrest function. When the cells lacking the elongation arrest function were treated with inhibitors of elongation, we see the reversion of the phenotypes caused by the lack of elongation arrest. Earlier studies have suggested that SRP can no longer recognize the longer nascent chain because it becomes sequestered [93]. However, a recent study used fluorescent techniques to monitor the association of SRP with various RNC and has revealed that binding of SRP to the RNC is independent of the nascent chain length [120]. These experiments were done in equilibrium conditions where there was no time constraint on the targeting, hence raising a question about their validity in the *in vivo* conditions. However, our *in vivo* data also suggests that SRP could bind to the nascent chain independent of the length supporting this model. It is not yet clear as to which step in the targeting is compromised when the nascent chain length becomes too long. It might sterically interfere with the complex formation between SRP and SR or could interfere with binding of the RNC to the translocon. The latter seems more reasonable because the cytosol contains many ribosomes that are continuously synthesizing various cytoplasmic proteins. These ribosomes most probably diffuse around and collide with empty translocons. The presence of long nascent chains would interfere with the binding of such ribosomes to the empty translocon and thereby prevent the targeting of a wrong substrate to the ER. This bias against the ribosomes bearing longer nascent chains could serve as a selective mechanism to ensure proper targeting of the correct substrates into the ER.

Recent cryo-electron microscopy studies have suggested that release of nascent chain from RNC-SRP-SR complex requires rearrangement of the S domain of the SRP. The structures show that the NG domain of SRP54 is delocalized after the NG twin formation resulting in the exposure of L23/L35 ribosomal adaptor site for the binding to the translocon [111]. The presence of long and disordered nascent chain in such a complex could interfere with the exposure of the ribosomal adaptor sites. It is also important to remember that long nascent chain is not only a disordered structure but could also be a charged moiety depending on the type of protein. The presence of charge could further interfere with the normal interactions in the complexes. To clearly understand the effect of nascent chain length on protein translocation one has to perform experiments such as FRET between the nascent chain and the SRP

receptor or nascent chain and translocon. This should help us in understanding the targeting event, which is compromised by the presence of long nascent chains.

## *3.3 Stacking of ribosomes is the key to efficient targeting*

A study on the *in vivo* kinetics of protein targeting in mammalian cells has revealed that the average targeting time for a reporter protein in mammalian cell was very short (about 5 s). Such short targeting times could render the elongation arrest function dispensable [123]. However these targeting times represent an average of all the ribosomes. Initial targeting of the first ribosome may take significantly longer than targeting of the subsequent ribosomes in a polysome that is already tethered to the ER membrane. Furthermore the lower targeting times in the cells can be explained by the SRP induced piling up of the ribosomes at the pause sites as shown in the *in vitro* systems [99, 124]. A similar stacking of the ribosomes was observed in a prokaryotic system, when translation was artificially perturbed by using elongation inhibitors [125].

The elongation arrest function of the SRP and antibiotics have two effects: i) they decrease the average length of nascent chains during the time window required for targeting, and ii) they induce stacking of ribosomes along the mRNA by switching the mode of translation from initiation-limited [126] to elongation-limited. SRP has been shown to induce enhanced stacking of ribosomes at natural pause sites in two different cell-free translation systems [99, 124]. The first ribosome will be arrested by SRP, whereas the following ribosomes pile up behind the first one without the need for SRP. This effect may reduce the average number of SRP bound to polysomes, consistent with the 20-50-fold lower abundance of SRP as compared to ribosomes [127, 128]. In the presence of elongation inhibitors, ribosomes will stack close to the translation start site because of the slow elongation rate and SRP will bind to the leading ribosome as soon as the signal sequence emerges. In both cases, targeting of the leading ribosome will increase the concentration of short nascent chains in the vicinity of the membrane. Although each nascent chain may still need to be targeted individually [129], the process may be accelerated, thereby leading to a much shorter average targeting time. In the absence of the elongation arrest function, nascent chains become much longer during the same targeting event and, since ribosomes will be more distantly spaced over the mRNA, the average targeting time will increase. As a result, nascent chains will be translocated less efficiently.

As discussed above, the delay in the nascent chain elongation by inhibitors results in reversing the defects caused by the absence of elongation arrest function. To analyze if the reversion of the defects was due to a general decrease in the synthesis of proteins or specifically because of the slow down of elongation, we used a translation initiation inhibitor. When cells lacking elongation arrest function were treated with an initiation inhibitor only partial rescue was observed. Unlike the elongation inhibitors the initiation inhibitor will not induce the stacking of the ribosomes. The slight improvement in the secretion efficiency is attributed to the reduced rate of protein synthesis and the chance of now finding an empty receptor on the membrane before the nascent chain becomes too long is higher. However, a significant number of nascent chains are still not targeted in time.

## *3.4 Translocation of small proteins is independent of elongation arrest function*

During this study we have identified that not all proteins are dependent on elongation arrest function for targeting (unpublished results). We have identified that insulin, which is small protein of 110 aa, is targeted in an elongation arrest independent manner. We observed no preproinsulin (precursor) accumulation in the elongation arrest defective cells at the steady state levels. To understand if this phenomenon is specific to all small proteins or whether insulin was a special case, we generated different preprolactin constructs of smaller size. Interestingly, we observed no precursor accumulation for the preprolactin chains, which are shorter than 140 aa. These results indicated that this phenomenon we observe, is specific to small proteins. It is not yet clear whether these proteins are sensitive to elongation arrest function or not. If these proteins are sensitive to elongation arrest, then in the absence of elongation arrest much less protein should be secreted outside the cell. One of the possibilities is that these proteins are inherently defective in secretion. In the in vitro system insulin is translocated poorly across the microsomal membrane. It is possible that insulin is poorly translocated in the in vivo system too. This can be verified by performing pulse-labeling experiments to detect the presence of precursor in the wild type conditions. The other possibility is that short proteins could utilize both cotranslational and posttranslational pathways in mammalian cells. The third possibility is associated with a possible new function of SRP. It can be hypothesized that SRP binds to the nascent chain and prevents the termination of the nascent chain until it is targeted. In the absence of elongation arrest there are limiting number of targeting sites. Under such conditions, by the time SRP can access the receptor, the short proteins must be fully synthesized but cannot be terminated. We can assume that short proteins do not sterically hinder the access of the nascent chain to the translocon. Hence the short proteins could be translocated.

## *3.5 Elongation arrest function: A new level of regulation?*

We have observed that the translation rate is faster than the rate at which nascent chains get targeted in mammalian cells. The normal cellular amount of receptor is too low for the targeting to proceed at the same rate as translation. Nature has developed elongation arrest mechanism to slow down the translation of a specific subset of proteins that are to be targeted into the ER without interfering with the translation rates of other cytosolic proteins like the ribosomal proteins, which are needed in higher amounts by the cells. The question that arises here is why does a cell need to have limiting amounts of receptor? If it has higher amounts of receptor then it would not need elongation arrest function and could target more proteins quickly. From our studies it is clear that cells have chosen lower synthesis and efficient targeting over higher synthesis and lower targeting. The presence of elongation arrest function probably gives a selective advantage to the cell. In the presence of elongation arrest function the nascent chains having signal sequences with weaker affinity for SRP could be preferentially dissociated during the time window of the targeting allowing the translocation of the nascent chains whose signal sequences have a higher affinity for SRP. To experimentally validate this hypothesis we could perform experiments

where in we mutate the signal sequence of preprolactin in such a way that it has a decreased affinity for the SRP now. We could test the translocation efficiency of this mutant preprolactin in the presence and absence of elongation arrest function and in the presence of excess of receptor. In the presence of elongation arrest function we should see a decreased translocation of this mutant preprolactin, whereas in the absence of elongation arrest function and in the presence of excess receptor this mutant could now be targeted as efficiently as the wild type preprolactin. Another experimental way to confirm the same hypothesis would be to use substrates such as plasminogen activators inhibitor-2 (PAI-2), which are naturally translocated poorly into the ER [154]. The translocation of these substrates should now be tested in the presence of excess of the receptor in the cell. If our hypothesis is right, then it highly probable that PAI-2 will now be efficiently translocated into the ER.

It is also unclear whether the receptor is limiting in all the cell types other than the ones we have tested. A further detailed analysis of the amount of SRP and SRP receptor present in different cell types and different tissues should be performed to understand the importance of having receptor as rate limiting step in the targeting event.

## *3.6 Molecular basis for elongation arrest function*

Earlier studies have shown that truncation in the C-terminus of SRP14 abolishes the elongation arrest function [95, 96]. A similar truncation in *S.cerevisiae* also led to the loss of elongation arrest function. More recently, mutational analysis of the C-terminal domain of SRP has resulted in the identification a highly conserved five-amino acid motif, which is essential for elongation arrest function (data from Camille Mary). It is still unknown as to how these five amino acids execute the elongation arrest function. It is plausible that these five amino acids could be important in interacting with the ribosome and positioning the *Alu* domain in the correct position to block the elongation. Biochemical studies in yeast and our studies in mammalian cells show that SRP interacts with the ribosome when it has just completed the transpeptidylation reaction, but before it has undergone translocation of the peptidyl-tRNA from the A site to the P site [64]. Cross linking studies have shown that *Alu* domain and the ribosome interactions change dynamically upon the binding of signal sequence by SRP and further positions the *Alu* domain at the ribosomal subunit interface [79]. Recent cryo-electron microscopy studies have positioned the *Alu* domain in the elongation factor-binding site of the ribosomes [80]. Interestingly the contact sites used by the *Alu* domain are also used by the elongation factor-2 (EF-2) [130]. It is highly probable that the interactions between the *Alu* domain and ribosome interfere with function of EF-2 and thereby inducing a pause in the elongation. In the mutant that lacks the elongation arrest function, the interactions between the *Alu* domain and the ribosome must have been disrupted leading to the loss of an ordered structure.

## *3.7 Evolutionary conservation of Elongation arrest function*

Elongation arrest function is unique to the *Alu* domain of SRP [93]. An *Alu* domain like RNA structure is present in most of the eukaryal and archaeal SRPs. Certain eubacteria have SRP RNA with domains, that seem to be analogous to the *Alu* domain of mammalian SRP RNA. Such eubacterial RNAs with *Alu* domain have been identified in *Bacillus, Listeria Clostridium* and *Thermotoga*. The archaeabacterial SRP RNA is very similar to the human SRP RNA having an *Alu* domain [131]. SRP RNA of euglenazoa and alveolates show a large variation in their *Alu* domains. An example of such variation is observed in organisms such as *Trypanosoma, Tetrahymena, Theileria,* which possess a truncated *Alu* domain [41]. The *Saccaromyces* SRP RNAs have an insert in helix 5 adjacent to the conserved *Alu* hairpin.

The SRP9 and SRP14 proteins, which constitute the *Alu* domain binding proteins, have been identified only in eukaryal species. The *Alu* domain binding proteins have not been identified in eubacteria and archeabacteria. The only exception is a eubacterial organism *B.subtillus* where a histone like proteins HBsu binds to the *Alu* domain. This histone-like HBsu protein does not share significant sequence similarity with SRP9 or SRP14 but shares a substantial structural homology with SRP9/14 heterodimer [132]. It is not yet clear whether the HBsu functions in a similar way to the SRP9/14 heterodimer, as the elongation arrest function has not been demonstrated in *B.subtilis*. More studies are required to understand if HBsu is a functional homolog of mammalian SRP9/14. In archaeabacteria, which possess the *Alu* domain, no functional homologues of *Alu* binding proteins have yet been identified. A recent study in Trypanosomes has shown that it completely lacks the *Alu* domain binding proteins [41]. However, they possess a tRNA-like RNA molecule, which co-purifies with the SRP RNA in these organisms. It has been speculated that the tRNA like RNA molecule may functionally replace the *Alu* domain and mediate the elongation arrest function. However, more studies have to be performed to show that the tRNA like molecule actually performs the elongation arrest function. Both the *Alu* domain and the *Alu* binding proteins SRP9 and SRP14 seemed to have undergone a rapid evolution. From the available data, which show that the *Alu* binding proteins have been identified only in eukaryotes, it is highly tempting to speculate that the ancestral *Alu* domain was composed of only RNA. SRP9/14 proteins were later added to adjust to the subsequent evolution. The presence of SRP9/14 in eukaryotes suggests that these proteins must be important in increasing the specificity of a certain event in the cells, which is why they have undergone a positive selection. We can assume that this event might be the elongation arrest function. This raises the question whether the organisms, which lack *Alu* domain binding proteins, have no elongation arrest? In the organisms which lack the *Alu* domain altogether, it is yet unknown whether they can perform the elongation arrest function by some other means. It is also possible that these organisms might not need elongation arrest function. For example in organisms like E.*coli* where there is an active posttranslational pathway [85], the load of targeting on cotranslational pathway might be less. The number of proteins that are targeted by SRP pathway would be relatively less when compared to the mammalian cells. This ensures that there is enough SRP receptor present to efficiently target all the protein that is being synthesized. In mammalian cells most the protein translocation occurs in a cotranslational manner so they might require elongation

arrest function to ensure that all nascent chains find an empty receptor site for targeting.

## *3.8 How much SRP does a cell need?*

Several studies have been performed in various organisms to understand the cellular roles of SRP. Genetic studies have shown that depletion of SRP is lethal in E.*coli* and two yeast species *S.pombe* and *Y.lipolytica* [61, 66, 133-135]. In the absence of SRP, *S.cerevisiae* continues to grow but poorly [136]. In Trypanosomes the down regulation of SRP is lethal [70]. The depletion of SRP in mammalian cells by more than 90% results in severe growth defects (Fig.S1A). The cells grow at much slower rate with a doubling time of 47 h when compared to the wild type cells, which have a generation time of 21 h. One adaptation of yeast to low levels of SRP is to reduce the number of ribosomes and increase the expression of chaperones [136]. Over the limited time period we studied, we failed to observe a significant decrease in ribosomes as deduced from the levels of the ribosomal protein L12. However, we see an increased expression of BiP in these cells indicating an activation of stress response in these cells. More experiments have to be done to characterize the type of stress response generated in these cells and the implications of this stress response. Surprisingly, we failed to observe increased cell death at such low SRP concentrations and, when RNAi became ineffective (>168 h), the cells recovered again. This suggested that the major problems in protein trafficking observed at 144 hrs failed to trigger an intracellular apoptotic response such as the one observed upon tunicamycin treatment [137]. Why is cell death not immediate in mammalian cells? In the absence of receptors in the plasma membrane due to reduced accumulation of newly synthesized receptors and due to inefficient recycling of certain plasma membrane receptors, and in particular also due to the absence of death receptors [72], the cells might enter a quiescent state which makes them transiently resistant to outside signals and cell death. However, based on the severe defects in intracellular protein trafficking that we observed with SRP levels as low as 5-10% it is likely that significant damage will appear quite rapidly leading to cell death.

In order to further analyze the fate of the cells at such low levels of SRP we tried to generate stable cell lines using viral transfections. When the protein levels were analyzed it was observed that the SRP14 levels were reduced only by 80% (unpublished data). There could be a basic technical problem to generate stable cell lines with less than 10% of SRP. During the culturing of the cells, the cells having very little SRP levels probably do not survive after a few rounds of selection and we end up with cells having slightly higher amounts of SRP. These cells, which now 20-25% SRP have a less pronounced defect in secretion and membrane trafficking. Possibly, the cells we used in the experiments need only relatively low amounts of SRP to survive as it has been suggested by previous studies where stable cell lines depleted of about 80% of SRP showed no growth defects [72]. This suggested that the cells could survive with much lesser amounts of SRP than expected. We can hypothesize that most of the essential components that are necessary for cell survival and dependent on SRP for their translocation have a higher affinity to bind to SRP. At lower levels of SRP they might out compete other proteins with lesser affinity in binding to the SRP. This in turn could lead to the selective translocation of these essential components. Our studies clearly show that far less SRP is required for

survival in mammalian cells. Survival and death might therefore depend on a small difference in SRP levels.

It has been recently observed that the successful infection of the macrophages with *Leishmania* was associated with the down regulation of SRP RNA [73]. It is hypothesized that low levels of SRP would interfere the expression of major hisocompatibility complex proteins on the cell surface and thereby preventing the immune system of the host from secretion of proteins necessary to counteract the parasitic infections. When taken in context with our results, we could assume that parasites and other microorganisms, which invade the human cells, could take advantage of the fact that cells can survive with low levels of SRP. Hence parasites and other microorganisms could infect the host cells and down regulate the expression of SRP. This would result in the lack of immune response from the host cells facilitating successful invasion by the microbes.

## *3.9 Efficient functioning of Golgi is impeded upon SRP depletion*

The Golgi complex functions at the crossroads of the secretory pathway, receiving newly synthesized proteins and lipids from the ER [138, 139]. Cargo proteins undergo sequential modifications and are delivered to their final destinations from the trans Golgi network (TGN), which constitutes the main sorting station of cargo proteins. Depletion of SRP in mammalian cells results in the accumulation of many reporter proteins and a recycling receptor in the Golgi. During my thesis, a study of SRP depletion in mammalian cells was published where in the death receptor DR4, a plasma membrane protein, also accumulated in Golgi upon depletion of SRP72 [72].

In our studies we observed a partial block in the anterograde transport of VSV-G at the level of the Golgi in SRP-depleted cells. This phenomenon was observed when VSV-G was expressed upon viral infection and the episomally expressed VSV-G protein did not accumulate in the Golgi. Upon viral infection, the host translation machinery is entirely dedicated to the synthesis of viral proteins whereas the translation of host proteins is inhibited [140]. This suggests that the remaining SRP is also exclusively dedicated to the targeting of viral proteins, which may result in higher amounts of VSV-G entering the ER. Alternatively, VSV-G may also use an SRP-independent targeting pathway. The capacity of the Golgi to ensure the efficient export of higher amounts of VSV-G to the plasma membrane is now surpassed in SRP-depleted cells.

Accumulation of these proteins in Golgi reveals a severe defect in its function in anterograde transport. Recombinant shiga toxin B subunit (STxB) which undergoes retrograde transport from plasma membrane to the ER, also accumulated in Golgi exposing a defect in retrograde transport of proteins. The most plausible explanation for the observed defect is that depletion of SRP reduces the levels of one or several membrane proteins of the Golgi apparatus to the extent that it becomes non-functional. We have observed in our experiments, the level of the Golgi enzyme NATG1-GFP expressed from a plasmid was reduced to 25% in SRP-depleted cells. NAGT1 is a glycosylating enzyme present in the Golgi. It appears unlikely that the critical component(s) include a modifying enzyme because the STxB does not require a modification for retrograde transport. Depletion of SRP did not alter the Golgi

morphology (we used low resolution microscopy) suggesting that the reduced levels of matrix proteins might not be responsible for the functional defects in Golgi. Very little is known about the membrane components of Golgi that are involved in post Golgi transport. The question that arises here is what could be the components in Golgi, which become limiting when SRP is depleted? It could be a component(s) essential for vesicular transport between compartments such as SNAREs, tethering proteins and GTPases involved in coat recruitment [141-143]. SNAREs are predominantly tail-anchored proteins and their translocation is generally assumed to be SRP-independent [144]. However, recent data provides evidence that the translocation of the SNARE synaptobrevin-2 into the ER is SRP-dependent *in vitro* [145]. Furthermore it is possible that SRP-depletion, might indirectly affect integration of tail-anchored proteins and vesicle tethering proteins such a giantin. Rab GTPases, which ensure the specificity of the cargo flow in cells, also depend on membranous activators and effectors for their function [146]. More functional and structural experiments will be required to identify the critical components and the exact process that is defective.

## *4. A tool to characterize SRP assembly*

The classical view of the nucleolus as solely committed to ribosome biosynthesis has been modified by studies in the last decade pointing to additional roles for this nuclear structure. Earlier studies have shown that SRP is partially assembled in the nucleoli of the mammalian cells. Along with the SRP RNA three of the four S domain proteins, SRP19, SRP68, and SRP72, displayed nucleolar localization, as well as cytoplasmic localization [54]. In contrast, the fourth S-domain specific protein, SRP54, did not display nucleolar localization. The *Alu* domain proteins SRP9 and SRP14 also displayed nucleolar localization (unpublished data). In mammalian cells, depletion of SRP14 has the most dramatic effect on SRP RNA levels suggesting that it might be a key player in SRP assembly and possibly also in nuclear export as previously suggested by a model based on structural studies [25, 26]. This interpretation is also consistent with the result that nucleolar localization and nuclear export is dependent on the *Alu* portion of SRP RNA [147]. SRP proteins can be classified as three groups of functionally associated proteins. SRP9 and SRP14 bind as a heterodimer to SRP RNA, SRP54 requires SRP19 for its assembly into SRP and SRP68 and SRP72 can bind individually to the RNA, but in this process form a very stable heterodimer. We downregulated one protein from each of this group using shRNA. The time course of down regulation of each protein was similar and consistent with very long half-lives of SRP proteins. Like in yeast [148], depletion of SRP proteins had an immediate and strong effect on the levels of SRP RNA indicating that the RNA is degraded when only partially assembled into SRP. In addition, its transcription might be down regulated in the absence of SRP proteins. In contrast, certain SRP proteins are very stable in the absence of other subunits. The changes in the cellular levels of non-target proteins are different in mammalian cells and in yeast. For example depletion of SRP14 reduced the levels of SRP19 and SRP54 but not the ones of SRP68 and SRP72 in mammalian cells, whereas in *S. cerevisiae* just the opposite was observed [148]. Hence, the results reveal differences in the assembly pathways and their regulation between the two organisms. The SRP proteins that were not down regulated may exist in a free form or in partially assembled particles. Partially assembled particles are most likely present in SRP54- and SRP72-depleted cells, where the SRP RNA levels

are higher than the levels of SRP54 and SRP72, respectively. In all cases, the ribosomal protein L12 (L11 in *E. coli*) that was used as a control remained unchanged. The constant level of L12 confirmed that shRNAs against SRP proteins and reduced levels of SRP did not affect cellular levels of ribosomes. It is unclear yet whether the changes in the protein levels of non target proteins is because of the loss of their stability or because their transcription is affected, ultimately resulting in less of amount of protein being produced. From the preliminary data we have it is too hard to conclude the steps in the assembly of SRP.

Studies characterizing the assembly pathway in mammalian cells were done with GFP-tagged over expressed SRP proteins. These studies show the localization of these proteins without revealing any details of the sequential events in the assembly of fully functional SRP. To understand the sequential events, we could down regulate all the SRP subunits individually and then look the expression of the non-target SRP proteins by western blot. Further we should also analyze the transcription of all the non-target proteins to understand the regulation, if any, at the level of transcription. Another experiment that needs to be done is looking at the localization of all the non-target proteins when one of the SRP proteins is depleted. A combination of an elaborate microscopic and gene expression analysis of each of the subunits when one of them is depleted would probably help us in understanding the sequence of events taking place in the assembly of SRP. Overall the SRP depletion studies could be used as a tool to understand the events that take place during the assembly of SRP.

## *4.1 A tool to characterize posttranslational pathway in mammalian cells*

SRP functions in co-translational protein targeting and the observed effects in membrane trafficking when SRP is depleted are therefore plausibly explained by inefficient targeting into the ER. Unlike in *E. coli* and in *S. cerevisiae* [66, 149], the depletion of SRP affected the accumulation and the localization of all reporter proteins independently of the hydrophobicity of their signal sequences and of the number of transmembrane segments. These results strengthen the notion that SRP might be a more generally used targeting factor in mammalian cells than in yeast and bacteria. However it does not disprove the theory that certain proteins could use the posttranslational targeting pathway.

It is widely believed that signal sequences influence the selection of protein targeting pathways [35]. In bacteria and yeast the posttranslational pathway has been well characterized. It has been suggested that bacteria and yeast use SRP pathway to transport only those proteins, which rapidly loose translocation competence in the cytosol [150, 151]. Recent studies in trypanosomes have also suggested that SRP is mainly used in the biogenesis membrane proteins as they are highly hydrophobic and prone to aggregate formation if left in the in the cytosol [71]. In our analysis we observed that some proteins are targeted across the ER membrane when the levels of functional SRP were reduced. A previous study also reported that two death receptors DR4 and DR5 were differentially affected in their plasma membrane expression at lower levels of SRP [72]. Our studies on SRP receptor depletion also showed that certain proteins are targeted efficiently when the functional SRP pathway is abrogated. SRP receptor depletion leads to severe defects in cell growth. It further revealed a decreased accumulation of several endogenous and reporter proteins

including some of the translocon components such as TRAP and TRAM (unpublished results). One of the proteins that was targeted independent of SRP and SRP receptor depletion was immunoglobulin binding protein (BiP). BiP is an ER resident protein, whose levels go up under the conditions of stress in the cells [115, 152, 153]. So does BiP use a posttranslational pathway to get across the membrane? The signal sequence of BiP has a high affinity for SRP as measured by fluorescent binding experiments [120]. It is possible that BiP out competes all other proteins and gets targeted efficiently even at lower levels of SRP. One way to rule out the possibility that BiP out competes other proteins for SRP is to make point mutations in the signal sequence of BiP such that it decreases the affinity of the signal sequence to SRP but does not completely diminish it. Now under these conditions the mutant BiP must not be targeted since it has a weaker signal sequence. However if it is targeted, then it probably indicates that BiP uses a posttranslational pathway. The higher affinity of the signal sequence of BiP for SRP still does not explain the two-fold accumulation of BiP in the SRP depleted cells (unpublished results), which is relatively high compared to the amount of SRP left (<10%). It is highly probable that BiP uses a posttranslational pathway when the levels of SRP are down. BiP could be used as a reporter protein under these conditions to characterize the components involved in the posttranslational pathway. Cross-linking assays should be performed to characterize the interactions of BiP. Our studies have also identified few other proteins which are targeted efficiently even at low levels of SRP. It would very interesting to identify, if all these proteins follow the same pathway to enter the ER or there are different pathways of posttranslational targeting in mammalian cells. Hence our studies on SRP depletion and SRP receptor depletion could be used to characterize the posttranslational pathway in mammalian cells.

# 4. *REFERENCES*

[1] G. Blobel, P. Walter, C.N. Chang, B.M. Goldman, A.H. Erickson, V.R. Lingappa, Translocation of proteins across membranes: the signal hypothesis and beyond, Symp Soc Exp Biol 33 (1979) 9-36.
[2] B. Martoglio, M.W. Hofmann, J. Brunner, B. Dobberstein, The protein-conducting channel in the membrane of the endoplasmic reticulum is open laterally toward the lipid bilayer, Cell 81 (1995) 207-214.
[3] P. Walter, G. Blobel, Mechanism of protein translocation across the endoplasmic reticulum, Biochem. Soc. Symp. 47 (1982) 183-191.
[4] R.J. Keenan, D.M. Freymann, R.M. Stroud, P. Walter, The signal recognition particle, Annu. Rev. Biochem. 70 (2001) 755-775.
[5] P. Walter, G. Blobel, Signal recognition particle: a ribonucleoprotein required for cotranslational translocation of proteins, isolation and properties, Methods Enzymol. 96 (1983) 682-691.
[6] P.F. Egea, R.M. Stroud, P. Walter, Targeting proteins to membranes: structure of the signal recognition particle, Curr. Opin. Struct. Biol. 15 (2005) 213-220.
[7] S. Panzner, L. Dreier, E. Hartmann, S. Kostka, T.A. Rapoport, Posttranslational protein transport into the endoplasmic reticulum, Cold Spring Harb Symp Quant Biol 60 (1995) 31-40.
[8] M.R. Pool, Signal recognition particles in chloroplasts, bacteria, yeast and mammals (review), Mol. Membr. Biol. 22 (2005) 3-15.
[9] K.S. Crowley, S. Liao, V.E. Worrell, G.D. Reinhart, A.E. Johnson, Secretory proteins move through the endoplasmic reticulum membrane via an aqueous, gated pore, Cell 78 (1994) 461-471.
[10] D. Gorlich, T.A. Rapoport, Protein translocation into proteoliposomes reconstituted from purified components of the endoplasmic reticulum membrane, Cell 75 (1993) 615-630.
[11] D. Hanein, K.E. Matlack, B. Jungnickel, K. Plath, K.U. Kalies, K.R. Miller, T.A. Rapoport, C.W. Akey, Oligomeric rings of the Sec61p complex induced by ligands required for protein translocation, Cell 87 (1996) 721-732.
[12] V. Siegel, P. Walter, Binding sites of the 19-kDa and 68/72-kDa signal recognition particle (SRP) proteins on SRP RNA as determined in protein-RNA "footprinting", Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 1801-1805.
[13] C. Zwieb, Structure and function of signal recognition particle RNA, Prog. Nucleic Acid Res. Mol. Biol. 37 (1989) 207-234.
[14] C. Zwieb, Interaction of protein SRP19 with signal recognition particle RNA lacking individual RNA-helices, Nucleic Acids Res. 19 (1991) 2955-2960.
[15] C. Zwieb, N. Larsen, The signal recognition particle (SRP) database, Nucleic Acids Res. 20 Suppl (1992) 2207.
[16] C. Zwieb, N. Larsen, The signal recognition particle database (SRPDB), Nucleic Acids Res. 22 (1994) 3483-3484.
[17] C. Zwieb, R.W. van Nues, M.A. Rosenblad, J.D. Brown, T. Samuelsson, A nomenclature for all signal recognition particle RNAs, Rna 11 (2005) 7-13.
[18] D. Labuda, E. Zietkiewicz, Evolution of secondary structure in the family of 7SL-like RNAs, J. Mol. Evol. 39 (1994) 506-518.
[19] M.A. Poritz, H.D. Bernstein, K. Strub, D. Zopf, H. Wilhelm, P. Walter, An E. coli ribonucleoprotein containing 4.5S RNA resembles mammalian signal recognition particle, Science 250 (1990) 1111-1117.

[20] M.A. Poritz, K. Strub, P. Walter, Human SRP RNA and E. coli 4.5S RNA contain a highly homologous structural domain, Cell 55 (1988) 4-6.
[21] N. Bradshaw, P. Walter, The signal recognition particle (SRP) RNA links conformational changes in the SRP to protein targeting, Mol. Biol. Cell 18 (2007) 2728-2734.
[22] F.Y. Siu, R.J. Spanggord, J.A. Doudna, SRP RNA provides the physiologically essential GTPase activation function in cotranslational protein targeting, Rna (2006).
[23] K. Strub, P. Walter, Assembly of the Alu domain of the signal recognition particle (SRP): dimerization of the two protein components is required for efficient binding to SRP RNA, Mol. Cell. Biol. 10 (1990) 777-784.
[24] D.E. Birse, U. Kapp, K. Strub, S. Cusack, A. Aberg, The crystal structure of the signal recognition particle Alu RNA binding heterodimer, SRP9/14, EMBO J. 16 (1997) 3757-3766.
[25] O. Weichenrieder, K. Wild, K. Strub, S. Cusack, Structure and assembly of the Alu domain of the mammalian signal recognition particle, Nature 408 (2000) 167-173.
[26] O. Weichenrieder, C. Stehlin, U. Kapp, D.E. Birse, P.A. Timmins, K. Strub, S. Cusack, Hierarchical assembly of the Alu domain of the mammalian signal recognition particle, Rna 7 (2001) 731-740.
[27] Y. Chen, K. Sinha, K. Perumal, J. Gu, R. Reddy, Accurate 3' end processing and adenylation of human signal recognition particle RNA and alu RNA in vitro, J. Biol. Chem. 273 (1998) 35023-35031.
[28] G. Emde, A. Frontzek, B.J. Benecke, Secondary structure of the nascent 7S L RNA mediates efficient transcription by RNA polymerase III, Rna 3 (1997) 538-549.
[29] K. Strub, M. Fornallaz, N. Bui, The Alu domain homolog of the yeast signal recognition particle consists of an Srp14p homodimer and a yeast-specific RNA structure, Rna 5 (1999) 1333-1347.
[30] A. Kuglstatter, C. Oubridge, K. Nagai, Induced structural changes of 7SL RNA during the assembly of human signal recognition particle, Nat. Struct. Biol. 9 (2002) 740-744.
[31] K. Wild, I. Sinning, S. Cusack, Crystal structure of an early protein-RNA assembly complex of the signal recognition particle, Science 294 (2001) 598-601.
[32] R.T. Batey, R.P. Rambo, L. Lucast, B. Rha, J.A. Doudna, Crystal structure of the ribonucleoprotein core of the signal recognition particle, Science 287 (2000) 1232-1239.
[33] R.J. Keenan, D.M. Freymann, P. Walter, R.M. Stroud, Crystal structure of the signal sequence binding subunit of the signal recognition particle, Cell 94 (1998) 181-191.
[34] K.R. Rosendal, K. Wild, G. Montoya, I. Sinning, Crystal structure of the complete core of archaeal signal recognition particle and implications for interdomain communication, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 14701-14706.
[35] R.S. Hegde, H.D. Bernstein, The surprising complexity of signal sequences, Trends In the Biochemical Sciences 31 (2006) 563-571.
[36] P. Walter, G. Blobel, Disassembly and reconstitution of signal recognition particle, Cell 34 (1983) 525-533.

[37] M.A. Rose, K.M. Weeks, Visualizing induced fit in early assembly of the human signal recognition particle, Nat. Struct. Biol. 8 (2001) 515-520.
[38] H. Lutcke, S. Prehn, A.J. Ashford, M. Remus, R. Frank, B. Dobberstein, Assembly of the 68- and 72-kD proteins of signal recognition particle with 7S RNA, J. Cell Biol. 121 (1993) 977-985.
[39] E. Iakhiaeva, J. Yin, C. Zwieb, Identification of an RNA-binding domain in human SRP72, J. Mol. Biol. 345 (2005) 659-666.
[40] E. Iakhiaeva, S.H. Bhuiyan, J. Yin, C. Zwieb, Protein SRP68 of human signal recognition particle: identification of the RNA and SRP72 binding domains, Protein Sci. 15 (2006) 1290-1302.
[41] Y. Lustig, H. Goldshmidt, S. Uliel, S. Michaeli, The Trypanosoma brucei signal recognition particle lacks the Alu-domain-binding proteins: purification and functional analysis of its binding proteins by RNAi, J. Cell Sci. 118 (2005) 4551-4562.
[42] R. Gilmore, G. Blobel, P. Walter, Protein translocation across the endoplasmic reticulum. I. Detection in the microsomal membrane of a receptor for the signal recognition particle, J. Cell Biol. 95 (1982) 463-469.
[43] R. Gilmore, P. Walter, G. Blobel, Protein translocation across the endoplasmic reticulum. II. Isolation and characterization of the signal recognition particle receptor, J. Cell Biol. 95 (1982) 470-477.
[44] D.I. Meyer, E. Krause, B. Dobberstein, Secretory protein translocation across membranes-the role of the "docking protein', Nature 297 (1982) 647-650.
[45] J.D. Miller, H. Wilhelm, L. Gierasch, R. Gilmore, P. Walter, GTP binding and hydrolysis by the signal recognition particle during initiation of protein translocation, Nature 366 (1993) 351-354.
[46] P.J. Rapiejko, R. Gilmore, Protein translocation across the ER requires a functional GTP binding site in the alpha subunit of the signal recognition particle receptor, J. Cell Biol. 117 (1992) 493-503.
[47] S.C. Ogg, W.P. Barz, P. Walter, A functional GTPase domain, but not its transmembrane domain, is required for function of the SRP receptor beta-subunit, J. Cell Biol. 142 (1998) 341-354.
[48] T.A. Fulga, I. Sinning, B. Dobberstein, M.R. Pool, SRbeta coordinates signal sequence release from SRP with ribosome binding to the translocon, EMBO J. 20 (2001) 2338-2347.
[49] T. Schwartz, G. Blobel, Structural basis for the function of the beta subunit of the eukaryotic signal recognition particle receptor, Cell 112 (2003) 793-803.
[50] S.A. Ladefoged, G. Christiansen, A GTP-binding protein of Mycoplasma hominis: a small sized homolog to the signal recognition particle receptor FtsY, Gene 201 (1997) 37-44.
[51] J. Luirink, C.M. ten Hagen-Jongman, C.C. van der Weijden, B. Oudega, S. High, B. Dobberstein, R. Kusters, An alternative protein targeting pathway in Escherichia coli: studies on the role of FtsY, EMBO J. 13 (1994) 2289-2296.
[52] M.R. Jacobson, T. Pederson, Localization of signal recognition particle RNA in the nucleolus of mammalian cells, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 7981-7986.
[53] J.R. Mitchell, J. Cheng, K. Collins, A box H/ACA small nucleolar RNA-like domain at the human telomerase RNA 3' end, Mol. Cell. Biol. 19 (1999) 567-576.

[54] J.C. Politz, S. Yarovoi, S.M. Kilroy, K. Gowda, C. Zwieb, T. Pederson, Signal recognition particle components in the nucleolus, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 55-60.
[55] J.L. Diener, C. Wilson, Role of SRP19 in assembly of the Archaeoglobus fulgidus signal recognition particle, Biochemistry 39 (2000) 12862-12874.
[56] C. Oubridge, A. Kuglstatter, L. Jovine, K. Nagai, Crystal structure of SRP19 in complex with the S domain of SRP RNA and its implication for the assembly of the signal recognition particle, Mol. Cell 9 (2002) 1251-1261.
[57] H. Grosshans, K. Deinert, E. Hurt, G. Simos, Biogenesis of the signal recognition particle (SRP) involves import of SRP proteins into the nucleolus, assembly with the SRP-RNA, and Xpo1p-mediated export, J. Cell Biol. 153 (2001) 745-762.
[58] J.C. Politz, L.B. Lewandowski, T. Pederson, Signal recognition particle RNA localization within the nucleolus differs from the classical sites of ribosome synthesis, J. Cell Biol. 159 (2002) 411-418.
[59] C.N. Alavian, J.C. Politz, L.B. Lewandowski, C.M. Powers, T. Pederson, Nuclear export of signal recognition particle RNA in mammalian cells, Biochem. Biophys. Res. Commun. 313 (2004) 351-355.
[60] L.F. Ciufo, J.D. Brown, Nuclear export of yeast signal recognition particle lacking Srp54p by the Xpo1p/Crm1p NES-dependent pathway, Curr. Biol. 10 (2000) 1256-1264.
[61] S.M. Althoff, S.W. Stevens, J.A. Wise, The Srp54 GTPase is essential for protein export in the fission yeast Schizosaccharomyces pombe, Mol. Cell. Biol. 14 (1994) 7839-7854.
[62] F. He, D. Yaver, J.M. Beckerich, D. Ogrydziak, C. Gaillardin, The yeast Yarrowia lipolytica has two, functional, signal recognition particle 7S RNA genes, Curr. Genet. 17 (1990) 289-292.
[63] S.C. Mutka, P. Walter, Multifaceted physiological response allows yeast to adapt to the loss of the signal recognition particle-dependent protein-targeting pathway, Mol. Biol. Cell 12 (2001) 577-588.
[64] S.C. Ogg, P. Walter, SRP samples nascent chains for the presence of signal sequences by interacting with ribosomes at a discrete step during translation elongation, Cell 81 (1995) 1075-1084.
[65] C.J. Stirling, E.W. Hewitt, The S. cerevisiae SEC65 gene encodes a component of yeast signal recognition particle with homology to human SRP19, Nature 356 (1992) 534-537.
[66] N.D. Ulbrandt, J.A. Newitt, H.D. Bernstein, The E. coli signal recognition particle is required for the insertion of a subset of inner membrane proteins, Cell 88 (1997) 187-196.
[67] A. Seluanov, E. Bibi, FtsY, the prokaryotic signal recognition particle receptor homologue, is essential for biogenesis of membrane proteins, J. Biol. Chem. 272 (1997) 2053-2055.
[68] A.A. Herskovits, E. Shimoni, A. Minsky, E. Bibi, Accumulation of endoplasmic membranes and novel membrane-bound ribosome-signal recognition particle receptor complexes in Escherichia coli, J. Cell Biol. 159 (2002) 403-410.
[69] L. Liu, H. Ben-Shlomo, Y.X. Xu, M.Z. Stern, I. Goncharov, Y. Zhang, S. Michaeli, The trypanosomatid signal recognition particle consists of two RNA molecules, a 7SL RNA homologue and a novel tRNA-like molecule, J. Biol. Chem. 278 (2003) 18271-18280.

[70] L. Liu, X.H. Liang, S. Uliel, R. Unger, E. Ullu, S. Michaeli, RNA interference of signal peptide-binding protein SRP54 elicits deleterious effects and protein sorting defects in trypanosomes, J. Biol. Chem. 277 (2002) 47348-47357.
[71] Y. Lustig, Y. Vagima, H. Goldshmidt, A. Erlanger, V. Ozeri, J. Vince, M.J. McConville, D.M. Dwyer, S.M. Landfear, S. Michaeli, Down-regulation of the trypanosomatid signal recognition particle affects the biogenesis of polytopic membrane proteins but not of signal peptide-containing proteins, Eukaryot. Cell 6 (2007) 1865-1875.
[72] Y.G. Ren, K.W. Wagner, D.A. Knee, P. Aza-Blanc, M. Nasoff, Q.L. Deveraux, Differential regulation of the TRAIL death receptors DR4 and DR5 by the signal recognition particle, Mol. Biol. Cell 15 (2004) 5064-5074.
[73] S. Misra, M.K. Tripathi, G. Chaudhuri, Down-regulation of 7SL RNA expression and impairment of vesicular protein transport pathways by Leishmania infection of macrophages, J. Biol. Chem. 280 (2005) 29364-29373.
[74] G. Bacher, H. Lutcke, B. Jungnickel, T.A. Rapoport, B. Dobberstein, Regulation by the ribosome of the GTPase of the signal-recognition particle during protein targeting, Nature 381 (1996) 248-251.
[75] T. Powers, P. Walter, Protein targeting. The ribosome talks back, Nature 381 (1996) 191-192.
[76] P. Walter, G. Blobel, Signal recognition particle contains a 7S RNA essential for protein translocation across the endoplasmic reticulum, Nature 299 (1982) 691-698.
[77] S. Hauser, G. Bacher, B. Dobberstein, H. Lutcke, A complex of the signal sequence binding protein and the SRP RNA promotes translocation of nascent proteins, EMBO J. 14 (1995) 5485-5493.
[78] M.R. Pool, J. Stumm, T.A. Fulga, I. Sinning, B. Dobberstein, Distinct modes of signal recognition particle interaction with the ribosome, Science 297 (2002) 1345-1348.
[79] L. Terzi, M.R. Pool, B. Dobberstein, K. Strub, Signal recognition particle Alu domain occupies a defined site at the ribosomal subunit interface upon signal sequence recognition, Biochemistry 43 (2004) 107-117.
[80] M. Halic, T. Becker, M.R. Pool, C.M. Spahn, R.A. Grassucci, J. Frank, R. Beckmann, Structure of the signal recognition particle interacting with the elongation-arrested ribosome, Nature 427 (2004) 808-814.
[81] G. von Heijne, Ribosome-SRP-signal sequence interactions. The relay helix hypothesis, FEBS Lett. 190 (1985) 1-5.
[82] Q.A. Valent, D.A. Kendall, S. High, R. Kusters, B. Oudega, J. Luirink, Early events in preprotein recognition in E. coli: interaction of SRP and trigger factor with nascent polypeptides, EMBO J. 14 (1995) 5494-5505.
[83] G. von Heijne, Signal sequences. The limits of variation, J. Mol. Biol. 184 (1985) 99-105.
[84] J.W. de Gier, P.A. Scotti, A. Saaf, Q.A. Valent, A. Kuhn, J. Luirink, G. von Heijne, Differential use of the signal recognition particle translocase targeting pathway for inner membrane protein assembly in Escherichia coli, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 14646-14651.
[85] H.C. Lee, H.D. Bernstein, The targeting pathway of Escherichia coli presecretory and integral membrane proteins is specified by the hydrophobicity of the targeting signal, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 3471-3476.

[86] D.T. Ng, J.D. Brown, P. Walter, Signal sequences specify the targeting route to the endoplasmic reticulum membrane, J. Cell Biol. 134 (1996) 269-278.
[87] D. Zopf, H.D. Bernstein, A.E. Johnson, P. Walter, The methionine-rich domain of the 54 kd protein subunit of the signal recognition particle contains an RNA binding site and can be crosslinked to a signal sequence, EMBO J. 9 (1990) 4511-4517.
[88] K. Romisch, J. Webb, K. Lingelbach, H. Gausepohl, B. Dobberstein, The 54-kD protein of signal recognition particle contains a methionine-rich RNA binding domain, J. Cell Biol. 111 (1990) 1793-1802.
[89] S. High, B. Dobberstein, The signal sequence interacts with the methionine-rich domain of the 54-kD protein of signal recognition particle, J. Cell Biol. 113 (1991) 229-233.
[90] H. Lutcke, S. High, K. Romisch, A.J. Ashford, B. Dobberstein, The methionine-rich domain of the 54 kDa subunit of signal recognition particle is sufficient for the interaction with signal sequences, EMBO J. 11 (1992) 1543-1551.
[91] J.H. Peterson, C.A. Woolhead, H.D. Bernstein, Basic amino acids in a distinct subset of signal peptides promote interaction with the signal recognition particle, J. Biol. Chem. 278 (2003) 46155-46162.
[92] P. Walter, G. Blobel, Translocation of proteins across the endoplasmic reticulum III. Signal recognition protein (SRP) causes signal sequence-dependent and site-specific arrest of chain elongation that is released by microsomal membranes, J. Cell Biol. 91 (1981) 557-561.
[93] V. Siegel, P. Walter, Elongation arrest is not a prerequisite for secretory protein translocation across the microsomal membrane, J. Cell Biol. 100 (1985) 1913-1921.
[94] T. Powers, P. Walter, The nascent polypeptide-associated complex modulates interactions between the signal recognition particle and the ribosome, Curr. Biol. 6 (1996) 331-338.
[95] Y. Thomas, N. Bui, K. Strub, A truncation in the 14 kDa protein of the signal recognition particle leads to tertiary structure changes in the RNA and abolishes the elongation arrest activity of the particle, Nucleic Acids Res. 25 (1997) 1920-1929.
[96] N. Mason, L.F. Ciufo, J.D. Brown, Elongation arrest is a physiologically important function of signal recognition particle, EMBO J. 19 (2000) 4164-4174.
[97] J. Lipp, B. Dobberstein, M.T. Haeuptle, Signal recognition particle arrests elongation of nascent secretory and membrane proteins at multiple sites in a transient manner, J. Biol. Chem. 262 (1987) 1680-1684.
[98] M.M. Okun, D. Shields, Translocation of preproinsulin across the endoplasmic reticulum membrane. The relationship between nascent polypeptide size and extent of signal recognition particle-mediated inhibition of protein synthesis, J. Biol. Chem. 267 (1992) 11476-11482.
[99] S.L. Wolin, P. Walter, Signal recognition particle mediates a transient elongation arrest of preprolactin in reticulocyte lysate, J. Cell Biol. 109 (1989) 2617-2622.
[100] V. Siegel, P. Walter, Removal of the Alu structural domain from signal recognition particle leaves its protein translocation activity intact, Nature 320 (1986) 81-84.

[101] N. Johnsson, A. Varshavsky, Split ubiquitin as a sensor of protein interactions in vivo, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 10340-10344.
[102] T.A. Rapoport, R. Heinrich, P. Walter, T. Schulmeister, Mathematical modeling of the effects of the signal recognition particle on translation and translocation of proteins across the endoplasmic reticulum membrane, J. Mol. Biol. 195 (1987) 621-636.
[103] M.R. Pool, Getting to the membrane: how is co-translational protein targeting to the endoplasmic reticulum regulated?, Biochem. Soc. Trans. 31 (2003) 1232-1237.
[104] P.F. Egea, S.O. Shan, J. Napetschnig, D.F. Savage, P. Walter, R.M. Stroud, Substrate twinning activates the signal recognition particle and its receptor, Nature 427 (2004) 215-221.
[105] T. Connolly, R. Gilmore, GTP hydrolysis by complexes of the signal recognition particle and the signal recognition particle receptor, J. Cell Biol. 123 (1993) 799-807.
[106] P.J. Rapiejko, R. Gilmore, Empty site forms of the SRP54 and SR alpha GTPases mediate targeting of ribosome-nascent chain complexes to the endoplasmic reticulum, Cell 89 (1997) 703-713.
[107] S.O. Shan, R.M. Stroud, P. Walter, Mechanism of association and reciprocal activation of two GTPases, PLoS Biology 2 (2004) e320.
[108] E. Hartmann, M. Wiedmann, T.A. Rapoport, A membrane component of the endoplasmic reticulum that may be essential for protein translocation, EMBO J. 8 (1989) 2225-2229.
[109] W. Mothes, B. Jungnickel, J. Brunner, T.A. Rapoport, Signal sequence recognition in cotranslational translocation by protein components of the endoplasmic reticulum membrane, J. Cell Biol. 142 (1998) 355-364.
[110] S. High, N. Flint, B. Dobberstein, Requirements for the membrane insertion of signal-anchor type proteins, J. Cell Biol. 113 (1991) 25-34.
[111] M. Halic, M. Gartmann, O. Schlenker, T. Mielke, M.R. Pool, I. Sinning, R. Beckmann, Signal recognition particle receptor exposes the ribosomal translocon binding site, Science 312 (2006) 745-747.
[112] E.A. Evans, R. Gilmore, G. Blobel, Purification of microsomal signal peptidase as a complex, Proc. Natl. Acad. Sci. U. S. A. 83 (1986) 581-585.
[113] F. Lyko, B. Martoglio, B. Jungnickel, T.A. Rapoport, B. Dobberstein, Signal sequence processing in rough microsomes, J. Biol. Chem. 270 (1995) 19873-19878.
[114] T.A. Rapoport, Protein translocation across the eukaryotic endoplasmic reticulum and bacterial plasma membranes, Nature 450 (2007) 663-669.
[115] N.N. Alder, Y. Shen, J.L. Brodsky, L.M. Hendershot, A.E. Johnson, The molecular mechanisms underlying BiP-mediated gating of the Sec61 translocon of the endoplasmic reticulum, J. Cell Biol. 168 (2005) 389-399.
[116] W. Li, S. Schulman, D. Boyd, K. Erlandson, J. Beckwith, T.A. Rapoport, The plug domain of the SecY protein stabilizes the closed state of the translocation channel and maintains a membrane seal, Mol. Cell 26 (2007) 511-521.
[117] S.M. Saparov, K. Erlandson, K. Cannon, J. Schaletzky, S. Schulman, T.A. Rapoport, P. Pohl, Determining the conductance of the SecY protein translocation channel for small molecules, Mol. Cell 26 (2007) 501-509.
[118] S. Guth, C. Volzing, A. Muller, M. Jung, R. Zimmermann, Protein transport into canine pancreatic microsomes: a quantitative approach, Eur. J. Biochem. 271 (2004) 3200-3207.

[119] A.E. Johnson, M.A. van Waes, The translocon: a dynamic gateway at the ER membrane, Annu. Rev. Cell Dev. Biol. 15 (1999) 799-842.
[120] J.J. Flanagan, J.C. Chen, Y. Miao, Y. Shao, J. Lin, P.E. Bock, A.E. Johnson, Signal recognition particle binds to ribosome-bound signal sequences with fluorescence-detected subnanomolar affinity that does not diminish as the nascent chain lengthens, J. Biol. Chem. 278 (2003) 18628-18637.
[121] V. Siegel, P. Walter, The affinity of signal recognition particle for presecretory proteins is dependent on nascent chain length, EMBO J. 7 (1988) 1769-1775.
[122] M. Wiedmann, T.V. Kurzchalia, H. Bielka, T.A. Rapoport, Direct probing of the interaction between the signal sequence of nascent preprolactin and the signal recognition particle by specific cross-linking, J. Cell Biol. 104 (1987) 201-208.
[123] V. Goder, P. Crottet, M. Spiess, In vivo kinetics of protein targeting to the endoplasmic reticulum determined by site-specific phosphorylation, EMBO J. 19 (2000) 6704-6712.
[124] S.L. Wolin, P. Walter, Ribosome pausing and stacking during translation of a eukaryotic mRNA, EMBO J. 7 (1988) 3559-3569.
[125] A.E. Dahlberg, E. Lund, N.O. Kjeldgaard, Some effects of antibiotics on bacterial polyribosomes as studied by gel electrophoresis, J. Mol. Biol. 78 (1973) 627-636.
[126] N. Sonenberg, H. JWB, M. MB, Translational control of Gene Expression, Cold Spring Harbor Laboratory Press, New York, 2000.
[127] F. Bovia, M. Fornallaz, H. Leffers, K. Strub, The SRP9/14 subunit of the signal recognition particle (SRP) is present in more than 20-fold excess over SRP in primate cells and exists primarily free but also in complex with small cytoplasmic Alu RNAs, Mol. Biol. Cell 6 (1995) 471-484.
[128] U. Raue, S. Oellerer, S. Rospert, Association of protein biogenesis factors at the yeast ribosomal tunnel exit is affected by the translational status and nascent polypeptide sequence, J. Biol. Chem. 282 (2007) 7809-7816.
[129] S. High, D. Gorlich, M. Wiedmann, T.A. Rapoport, B. Dobberstein, The identification of proteins in the proximity of signal-anchor sequences during their targeting to and insertion into the membrane of the ER, J. Cell Biol. 113 (1991) 35-44.
[130] M.G. Gomez-Lorenzo, C.M. Spahn, R.K. Agrawal, R.A. Grassucci, P. Penczek, K. Chakraburtty, J.P. Ballesta, J.L. Lavandera, J.F. Garcia-Bustos, J. Frank, Three-dimensional cryo-electron microscopy localization of EF2 in the Saccharomyces cerevisiae 80S ribosome at 17.5 A resolution, Embo J 19 (2000) 2710-2718.
[131] M.A. Rosenblad, C. Zwieb, T. Samuelsson, Identification and comparative analysis of components from the signal recognition particle in protozoa and fungi, BMC Genomics 5 (2004) 5.
[132] K. Nakamura, S. Yahagi, T. Yamazaki, K. Yamane, Bacillus subtilis histone-like protein, HBsu, is an integral component of a SRP-like particle that can bind the Alu domain of small cytoplasmic RNA, J. Biol. Chem. 274 (1999) 13569-13576.
[133] J. Luirink, G. von Heijne, E. Houben, J.W. de Gier, Biogenesis of inner membrane proteins in Escherichia coli, Annu. Rev. Microbiol. 59 (2005) 329-355.

[134] J. Macfarlane, M. Muller, The functional integration of a polytopic membrane protein of Escherichia coli is dependent on the bacterial signal-recognition particle, Eur. J. Biochem. 233 (1995) 766-771.
[135] G.J. Phillips, T.J. Silhavy, The E. coli ffh gene is necessary for viability and efficient protein export, Nature 359 (1992) 744-746.
[136] B.C. Hann, C.J. Stirling, P. Walter, SEC65 gene product is a subunit of the yeast signal recognition particle required for its integrity, Nature 356 (1992) 532-533.
[137] T. Nakagawa, H. Zhu, N. Morishima, E. Li, J. Xu, B.A. Yankner, J. Yuan, Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta, Nature 403 (2000) 98-103.
[138] B.B. Allan, W.E. Balch, Protein sorting by directed maturation of Golgi compartments, Science 285 (1999) 63-66.
[139] M.C. Lee, E.A. Miller, J. Goldberg, L. Orci, R. Schekman, Bi-directional protein transport between the ER and Golgi, Annu. Rev. Cell Dev. Biol. 20 (2004) 87-123.
[140] J.H. Connor, D.S. Lyles, Inhibition of host and viral translation during vesicular stomatitis virus infection. eIF2 is responsible for the inhibition of viral but not host translation, J. Biol. Chem. 280 (2005) 13512-13519.
[141] M. Kauppi, A. Simonsen, B. Bremnes, A. Vieira, J. Callaghan, H. Stenmark, V.M. Olkkonen, The small GTPase Rab22 interacts with EEA1 and controls endosomal membrane trafficking, J. Cell Sci. 115 (2002) 899-911.
[142] C.L. Jackson, J.E. Casanova, Turning on ARF: the Sec7 family of guanine-nucleotide-exchange factors, Trends Cell Biol. 10 (2000) 60-67.
[143] D. Ungar, F.M. Hughson, SNARE protein structure and function, Annu. Rev. Cell Dev. Biol. 19 (2003) 493-517.
[144] N. Borgese, S. Colombo, E. Pedrazzini, The tale of tail-anchored proteins: coming from the cytosol and looking for a membrane, J. Cell Biol. 161 (2003) 1013-1019.
[145] B.M. Abell, M. Jung, J.D. Oliver, B.C. Knight, J. Tyedmers, R. Zimmermann, S. High, Tail-anchored and signal-anchored proteins utilize overlapping pathways during membrane insertion, J. Biol. Chem. 278 (2003) 5669-5678.
[146] M. Zerial, H. McBride, Rab proteins as membrane organizers, Nat. Rev. Mol. Cell. Biol. 2 (2001) 107-117.
[147] X.P. He, N. Bataille, H.M. Fried, Nuclear export of signal recognition particle RNA is a facilitated process that involves the Alu sequence domain, J. Cell Sci. 107 ( Pt 4) (1994) 903-912.
[148] J. Brown, D., B.C. Hann, K.F. Medzihradszky, M. Niwa, A.L. Burlingame, P. Walter, Subunits of the Saccharomyces cerevisiae signal recognition particle required for its functional expression, EMBO J. 13 (1994) 4390-4400.
[149] D.W. Andrews, A.E. Johnson, The translocon: more than a hole in the ER membrane?, Trends In the Biochemical Sciences 21 (1996) 365-369.
[150] D. Huber, D. Boyd, Y. Xia, M.H. Olma, M. Gerstein, J. Beckwith, Use of thioredoxin as a reporter to identify a subset of Escherichia coli signal sequences that promote signal recognition particle-dependent translocation, J. Bacteriol. 187 (2005) 2983-2991.
[151] B.M. Wilkinson, J.R. Tyson, C.J. Stirling, Ssh1p determines the translocation and dislocation capacities of the yeast endoplasmic reticulum, Dev Cell 1 (2001) 401-409.

[152] C.E. Arnold, K.D. Wittrup, The stress response to loss of signal recognition particle function in Saccharomyces cerevisiae, J. Biol. Chem. 269 (1994) 30412-30418.
[153] T. Dierks, P. Klappa, H. Wiech, R. Zimmermann, The role of molecular chaperones in protein transport into the endoplasmic reticulum, Philos. Trans. R. Soc. Lond. B Biol. Sci. 339 (1993) 335-341.
[154] D. Belin, S. Bost, J.D. Vassalli, K. Strub, A two-step recognition of signal sequences determines the translocation efficiency of proteins, EMBO J.15 (1996) 468-478.