

Structural features within the nascent chain regulate alternative targeting of secretory proteins to mitochondria

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Protein targeting to specified cellular compartments is essential to maintain cell function and homeostasis. In eukaryotic cells, two major pathways rely on N-terminal signal peptides to target proteins to either the endoplasmic reticulum (ER) or mitochondria. In this study, we show that the ER signal peptides of the prion protein-like protein shadoo, the neuropeptide hormone somatostatin and the amyloid precursor protein have the property to mediate alternative targeting to mitochondria. Remarkably, the targeting direction of these signal peptides is determined by structural elements within the nascent chain. Each of the identified signal peptides promotes efficient ER import of nascent chains containing α -helical domains, but targets unstructured polypeptides to mitochondria. Moreover, we observed that mitochondrial targeting by the ER signal peptides correlates inversely with ER import efficiency. When ER import is compromised, targeting to mitochondria is enhanced, whereas improving ER import efficiency decreases mitochondrial targeting. In conclusion, our study reveals a novel mechanism of dual targeting to either the ER or mitochondria that is mediated by structural features within the nascent chain.

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Introduction

Protein sorting to specific compartments is a fundamental cellular process. Targeting to the endoplasmic reticulum (ER) and mitochondria is mainly mediated by N-terminal signal peptides that ensure transport to the respective locale and initiate translocation. Both the mode of ER/mitochondrial targeting and the features of signal peptides are different. ER targeting occurs predominantly cotranslationally while proteins destined for mitochondria are targeted in a post-translational process. Interestingly, every secretory protein has an individual signal sequence, although the targeting peptide is usually removed during transmembrane transport and therefore not part of the mature protein. The current understanding is that ER signal peptides have an intrinsic activity to mediate cotranslational ER import independently of the sequence of the rest of the protein. Thus, ER signal peptides can mediate ER import of heterologous polypeptides when fused to their N-terminus. However, there are differences in ER import efficiency due to the variability of ER signal sequences. As a consequence, the ratio between the ER/cytosolic fraction may vary for some secretory pathway proteins. Regulated ER import can serve different purposes. First, the cell can temporarily limit the ER protein load, for example, under stress conditions (Kang *et al*, 2006; Oyadomari *et al*, 2006). This pathway usually includes degradation of non-translocated polypeptides to prevent toxic effects in the cytosol. Second, localization of a protein to more than one cellular compartment can expand its physiological function. A prominent example for such a mechanism is calreticulin, which plays a major role in protein folding and calcium homeostasis in the ER lumen. In addition, cytosolic calreticulin, generated due to inefficient ER import, can influence the functional activity of the glucocorticoid receptor in gene activation (Shaffer *et al*, 2005). Dual localization of proteins can also be achieved by two distinct translation products that differ in the presence or absence of a targeting signal, for example, as a result of differential splicing (reviewed in Danpure, 1995 and Small *et al*, 1998). Furthermore, bimodal targeting of proteins to the ER and mitochondria is mediated by N-terminal peptides that carry essential elements of both ER and mitochondrial targeting signals. Such signal sequences require activation by either proteolytic cleavage or phosphorylation, as described for cytochrome P450 proteins and the amyloid precursor protein (APP) (reviewed in Anandatheerthavarada *et al*, 2003; Karniely and Pines, 2005 and Avadhani *et al*, 2011).

Apart from the sequence of the N-terminal signal peptide, structural features within the nascent chain can regulate ER import efficiency. We and others demonstrated that intrinsically disordered domains fail to be productively imported into the ER lumen (Zanusso *et al*, 1999; Heske *et al*, 2004; Miesbauer *et al*, 2009). In this context, it is important to note

that formation of secondary structure already occurs in the ribosomal exit tunnel (Whitley *et al*, 1996; Mingarro *et al*, 2000; Woolhead *et al*, 2004, 2006; Lu and Deutsch, 2005; Bhushan *et al*, 2010). Moreover, the polypeptide structure within the ribosomal exit tunnel can modulate the translocation of distal parts of the nascent chain (Daniel *et al*, 2008), and ribosome-induced folding of transmembrane segments can trigger structural rearrangements of multiple components in and on both sides of the ER membrane (Lin *et al*, 2011).

In a classical view of protein function, the physiological activity is linked to the ability of the polypeptide chain to adopt a stable secondary/tertiary structure. This concept has been extended when it became evident that intrinsically disordered domains and proteins have defined physiological activities and play a major role in several protein classes including transcription factors, scaffold proteins and signaling molecules (reviewed in Fuxreiter *et al*, 2008; Tompa *et al*, 2009; Uversky and Dunker, 2010; Fuxreiter and Tompa, 2012; and Longhi, 2012). By studying the biosynthesis of naturally occurring intrinsically disordered secretory proteins, we now made the unexpected observation that the ER signal peptides of the prion protein-like protein shadoo (Sho), the neuropeptide hormone somatostatin (Som) and the APP are able to mediate alternative targeting to mitochondria. Here, we show that the targeting direction of these ER signal peptides is determined by structural features within the nascent chain. While proteins containing structured domains are efficiently imported into the ER, polypeptides with extended intrinsically disordered domains are targeted to mitochondria.

Results

A C-terminal glycosylphosphatidylinositol signal sequence promotes translocation of intrinsically disordered proteins into the ER

We have previously shown that ER-targeted polypeptides dominated by unstructured domains fail to efficiently translocate into the ER lumen (Heske *et al*, 2004; Miesbauer *et al*, 2009). Interestingly, shadoo (Sho), a highly conserved member of the mammalian prion glycoprotein family, appears to lack a stable secondary structure, based on *in silico* predictions and CD analysis of recombinant mouse Sho (Watts *et al*, 2007; Daude *et al*, 2010). Yet, studies in cultured cells, primary neurons and mice conclusively demonstrated that Sho is efficiently imported into the ER, modified with N-linked glycans of complex structure and targeted to the outer leaflet of the plasma membrane via a C-terminal glycosylphosphatidylinositol (GPI) anchor (Miesbauer *et al*, 2006; Watts *et al*, 2007; Sakthivelu *et al*, 2011; Westaway *et al*, 2011) (Figure 1A and B). Since the GPI anchor signal sequence is predicted to adopt an α -helical conformation (PSIPRED; <http://bioinf.cs.ucl.ac.uk/psipred>) we speculated whether this domain can promote ER import of the intrinsically disordered protein. To investigate this possibility, we generated a deletion mutant lacking the C-terminal signal sequence (Δ 128–151, Sho Δ GPI). In theory, deletion of the GPI anchor signal sequence should only prevent the post-translational addition of a GPI anchor, resulting in secretion of the mutated protein. This is what we observed using the cellular prion protein (PrP^C) as an

example for another GPI-anchored protein. The corresponding mutant PrP Δ GPI is efficiently imported into the ER and secreted from cultured cells and neurons in the brain of transgenic animals (Winklhofer *et al*, 2003; Chesebro *et al*, 2005) (Figure 1A and C). In contrast, deletion of the GPI signal sequence of Sho did not result in its secretion. Whereas PrP Δ GPI was found in the conditioned medium of transfected cells, Sho Δ GPI could not be detected in the medium. Western blot analysis of total cell lysates revealed that the relative protein amounts of Sho Δ GPI and PrP Δ GPI were comparable, indicating that decreased secretion was not due to an impaired expression of Sho Δ GPI (Figure 1C). Productive ER import usually correlates with the cleavage of the N-terminal signal peptide. To analyse this modification, we compared the migration of Sho Δ GPI on SDS/PAGE with that of a double mutant lacking both the N-terminal ER signal peptide and the C-terminal GPI signal sequence (Δ SP \bullet Sho Δ GPI). As shown in Figure 1D, Sho Δ GPI migrated more slowly on SDS/PAGE than Δ SP \bullet Sho Δ GPI, suggesting that Sho Δ GPI contains an uncleaved N-terminal ER signal peptide. In sum, this analysis revealed that Sho, in contrast to PrP, is critically dependent on its GPI signal sequence to be translocated into the secretory pathway.

To address the possibility that the GPI signal sequence itself mediates ER import, we expressed Δ SP \bullet Sho, a mutant lacking the N-terminal ER signal peptide but bearing the C-terminal GPI signal sequence. Δ SP \bullet Sho was only found in the cytosol, revealing that the GPI anchor signal sequence is not sufficient to mediate ER import of Sho (Figure 1E). In line with these results, we did not observe ER import of Δ SP \bullet PrP, a PrP mutant devoid of the N-terminal ER signal peptide but containing the C-terminal GPI signal sequence (data not shown).

To analyse the role of the C-terminal GPI anchor signal sequence in the ER import of Sho in more detail, we mutated the cleavage/attachment site (ω site) for the GPI anchor. In Sho-mtGPI, the residues 125, 126 and 127 have been replaced by threonine which is expected to impair the efficiency of GPI anchoring (Nuoffer *et al*, 1993). In this way, the original length of the nascent Sho polypeptide chain was preserved without allowing the GPI anchor attachment to occur. The western blot analysis indicated that Sho-mtGPI was glycosylated, that is, was productively imported into the ER lumen (Figure 1G). In a next step, we replaced the GPI signal sequence by (i) a heterologous CD4 transmembrane domain and (ii) helix 2 of PrP or (iii) helices 2 and 3 of PrP. Indeed, Sho-CD4, Sho- α 2 and Sho- α 2 α 3 were imported into the ER evidenced by the appearance of a glycosylated fraction (Figure 1G). Please note that only wildtype Sho and Sho-CD4 were complex glycosylated, indicating that the processing of core glycans into complex type glycans requires membrane anchoring of Sho. Interestingly, a similar phenomenon has been described previously for PrP (Winklhofer *et al*, 2003).

To explore the possibility that a GPI signal sequence has a general activity to promote ER import, we followed up different approaches. First, we tested two model substrates that are not translocated into the ER. PrP \bullet 115 and PrP \bullet 115–115 are composed of the N-terminal ER signal peptide of PrP (aa 1–22: PrP \bullet) followed by one or two copies of the intrinsically disordered N-terminal domain (aa 23–115: 115) of PrP (Miesbauer *et al*, 2009). To facilitate the analysis of

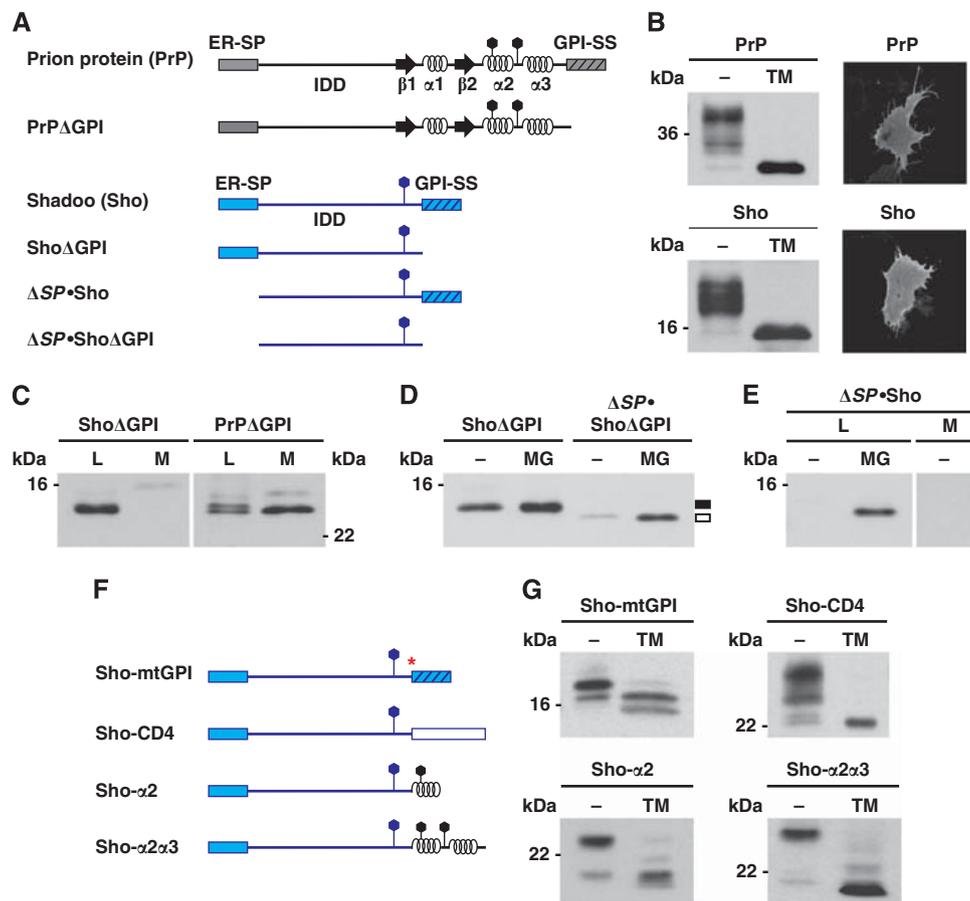


Figure 1 A GPI anchor signal sequence promotes translocation of intrinsically disordered proteins into the ER. (A) Schematic presentation of the constructs analysed. ER-SP, endoplasmic reticulum signal peptide; IDD, intrinsically disordered domain; β , beta-sheet; α , α -helical region; filled hexagon, N-linked glycans; GPI-SS, glycosylphosphatidylinositol anchor signal sequence. (B) Wildtype PrP and wildtype Sho are complex glycosylated and located at the outer leaflet of the plasma membrane. N2a cells expressing the constructs indicated were cultured in the absence or presence of the glycosylation inhibitor tunicamycin (TM, 0.5 μ g/ml for 16 h). Total cell lysates were prepared and PrP and Sho detected by western blotting (left panel). SH-SY5Y cells were transiently transfected with PrP or Sho and localization of the constructs was analysed by indirect immunofluorescence of non-permeabilized cells (right panel). (C) Deletion of the GPI signal sequence interferes with ER import of Sho. N2a cells were transiently transfected with the constructs indicated. Conditioned medium (M) and total cell lysates (L) were analysed by western blotting. (D) Sho Δ GPI contains an uncleaved ER signal peptide. Transiently transfected N2a cells expressing Sho mutants lacking the C-terminal GPI signal sequence (Sho Δ GPI) or lacking both the N- and C-terminal signal sequences (Δ SP•Sho Δ GPI) were treated with the proteasomal inhibitor MG132 dissolved in DMSO (MG, 30 μ M for 3 h) or DMSO only. Total cell lysates were analysed by western blotting. Filled rectangle: uncleaved signal peptide; unfilled rectangle: cleaved signal peptide. (E) The GPI signal sequence requires an N-terminal ER signal peptide in order to promote ER import. Conditioned medium (M) and total cell lysates (L) from N2a cells expressing a Sho mutant without the ER signal peptide (Δ SP•Sho) but with the GPI signal sequence were analysed by western blotting. Cell lysates of transiently transfected N2a cells treated with MG132 (MG, 30 μ M for 3 h) were analysed in parallel. (F, G) ER import of Sho can be promoted by C-terminal α -helical domains. (F) Schematic presentation of the constructs analysed. (G) N2a cells expressing the constructs indicated were cultured in the presence of tunicamycin (TM, 0.5 μ g/ml for 16 h) or left untreated. Total cell lysates were prepared and analysed by western blotting.

productive ER import, the constructs contain an acceptor site for N-linked glycosylation shortly behind the ER signal peptide. Thus, ER import can be analysed by the appearance of a glycosylated protein fraction. Corroborating earlier results (Miesbauer *et al*, 2009), in cells transiently expressing PrP•115 or PrP•115–115 only an unglycosylated species was detectable (Figure 2A and B). To test the activity of the GPI signal sequence to promote ER import of PrP•115 and PrP•115–115, we fused the GPI signal sequence of PrP (aa 231–254) to the C-terminus of the respective constructs. Indeed, addition of the GPI signal sequence promoted ER import, as reflected by the appearance of a glycosylated fraction of PrP•115GPI and PrP•115–115GPI (Figure 2B). Notably, the GPI anchor signal sequence of the glial cell line-derived neurotrophic factor receptor α (GFR α) had a similar activity to promote ER import of PrP•115 (Figure 2A

and B; PrP•115GPI-GFR α). Immunofluorescence analysis further demonstrated that PrP•115GPI was present at the outer leaflet of the plasma membrane, while PrP•115 was exclusively located in the cytosol (data not shown, Figure 4D). The observation that C-terminal α -helical domains can also restore ER import of PrP•115–115 (Figure 2A and B; PrP•115–115 α 2 α 3) supports the concept that the activity to promote ER import resides in the propensity to adopt an α -helical conformation.

In a second approach, we analysed PrP•Q159X (corresponding to human Q160X), a pathogenic mutant linked to inherited prion diseases in humans. PrP•Q159X lacks part of the C-terminal domain, including the highly structured α -helical core and the GPI signal sequence. As a consequence, ER import of PrP•Q159X was compromised, and moreover, the cytosolic fraction induced cell death (Heske *et al*, 2004;

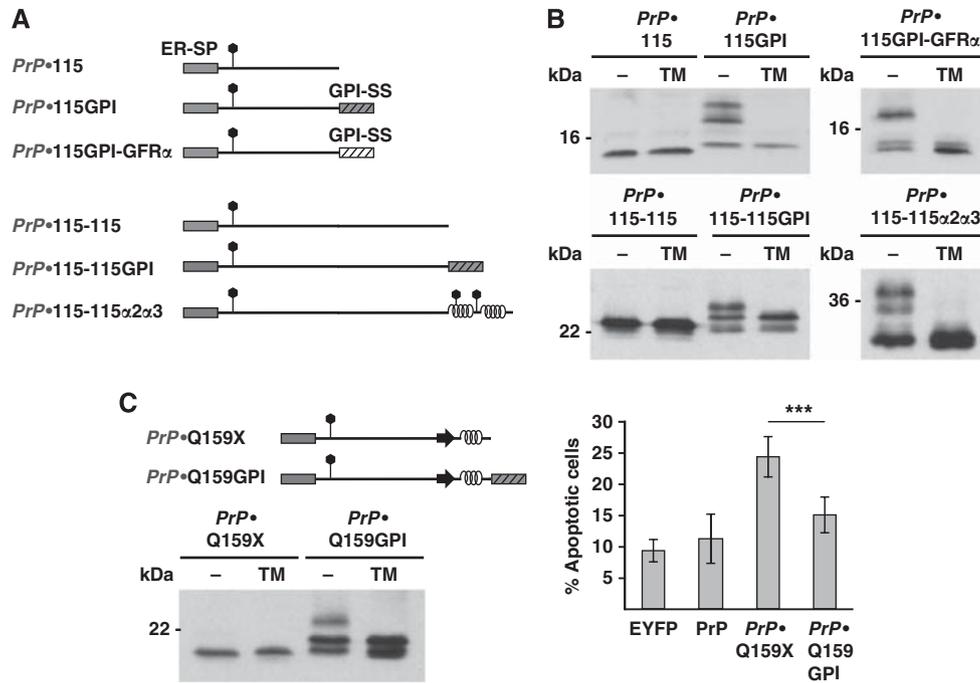


Figure 2 A GPI anchor signal sequence alleviates toxic effects of a pathogenic PrP mutant. (A, B) A GPI signal sequence and C-terminal α -helices promote ER import of polypeptides impaired in translocation. (A) Schematic presentation of the constructs analysed. (B) N2a cells expressing the constructs indicated were cultured in the presence of tunicamycin (TM, 0.5 μ g/ml for 16 h) or left untreated. Total cell lysates were prepared and analysed by western blotting. (C) A GPI signal sequence alleviates toxic effects of the pathogenic PrP mutant Q159X. SH-SY5Y cells were cotransfected with the constructs indicated and EYFP as transfection marker. For determination of apoptotic cell death, cells were fixed, permeabilized and stained for activated caspase-3. Apoptosis was quantified in transfected cells based on at least four independent experiments with triplicates each. For each construct indicated, at least 1000 cells per experiment were assessed. *** $P < 0.0005$.

Rambold *et al*, 2006) (Figure 2C). Supporting the activity of the GPI anchor to promote ER import, PrP•Q159GPI was partially glycosylated, indicative of productive ER import (Figure 2C, lower panel). Moreover, increased ER import correlated with decreased toxicity of this mutant: apoptotic cell death was significantly reduced in cells expressing PrP•Q159GPI in comparison to PrP•Q159X-expressing cells (Figure 2C, right panel).

Loss of the α -helical prodomain interferes with ER import of an intrinsically disordered peptide hormone

The data shown above further suggest that nascent chains dominated by unstructured domains are inefficiently imported into the ER. Another secretory protein predicted to be intrinsically disordered is the neuropeptide hormone somatostatin (Som) (using POODLE-W as an identifier of mostly disordered proteins <http://mbs.cbrc.jp/poodle/index.html>). Interestingly, somatostatin is synthesized as a larger precursor, containing a prodomain (pro) in addition to the N-terminal signal peptide. After ER import and cleavage of the ER signal peptide, the prodomain is proteolytically removed in the Golgi apparatus to generate the biologically active hormone (Patel and Galanopoulou, 1995) (Figure 3A). In contrast to the hormone domain, the prodomain of somatostatin is predicted to be α -helical. To assess whether the α -helical prodomain is required for ER import, we generated mammalian expression constructs of wildtype human somatostatin and two mutants, one lacking the α -helical prodomain (Som Δ pro) and another one lacking the unstructured hormone domain (Som Δ hor) (Figure 3A). After transient expression in N2a cells and pulse labelling with

[³⁵S]-methionine, the cell culture medium was analysed by immunoprecipitation. Since N2a cells do not contain convertases required for processing of the precursor, the unprocessed propeptide was secreted from N2a cells. The mutant lacking the hormone domain (Som Δ hor) was efficiently secreted like wildtype somatostatin. However, deletion of the prodomain completely abolished secretion of this somatostatin mutant: conditioned medium from cells transfected with Som Δ pro did not contain detectable levels of the mutant protein (Figure 3B, chase M). The analysis of total cell lysates revealed that the two somatostatin mutants were expressed at comparable levels, indicating that impaired secretion of Som Δ pro was not due to reduced synthesis (Figure 3B, pulse L).

Collectively, the analysis of Sho and Som mutants support the concept that ER import of intrinsically disordered domains is significantly impaired. Productive import can be promoted by α -helical domains or a C-terminal GPI signal sequence (Figure 3C).

ER signal peptides of shadoo and somatostatin can mediate targeting to mitochondria

To follow the fate of the Sho and Som mutants that are not imported into the ER, we performed an indirect immunofluorescence analysis. As expected for secretory proteins, wildtype Sho and Som were predominantly found in the Golgi (Figure 3E and F). In addition, Sho is found at the plasma membrane, where it is localized via a GPI anchor (Figure 1B). Unexpectedly, the immunofluorescence pattern of Sho Δ GPI and Som Δ pro was reminiscent of mitochondrial localization. Indeed, costaining with MitoTracker Red

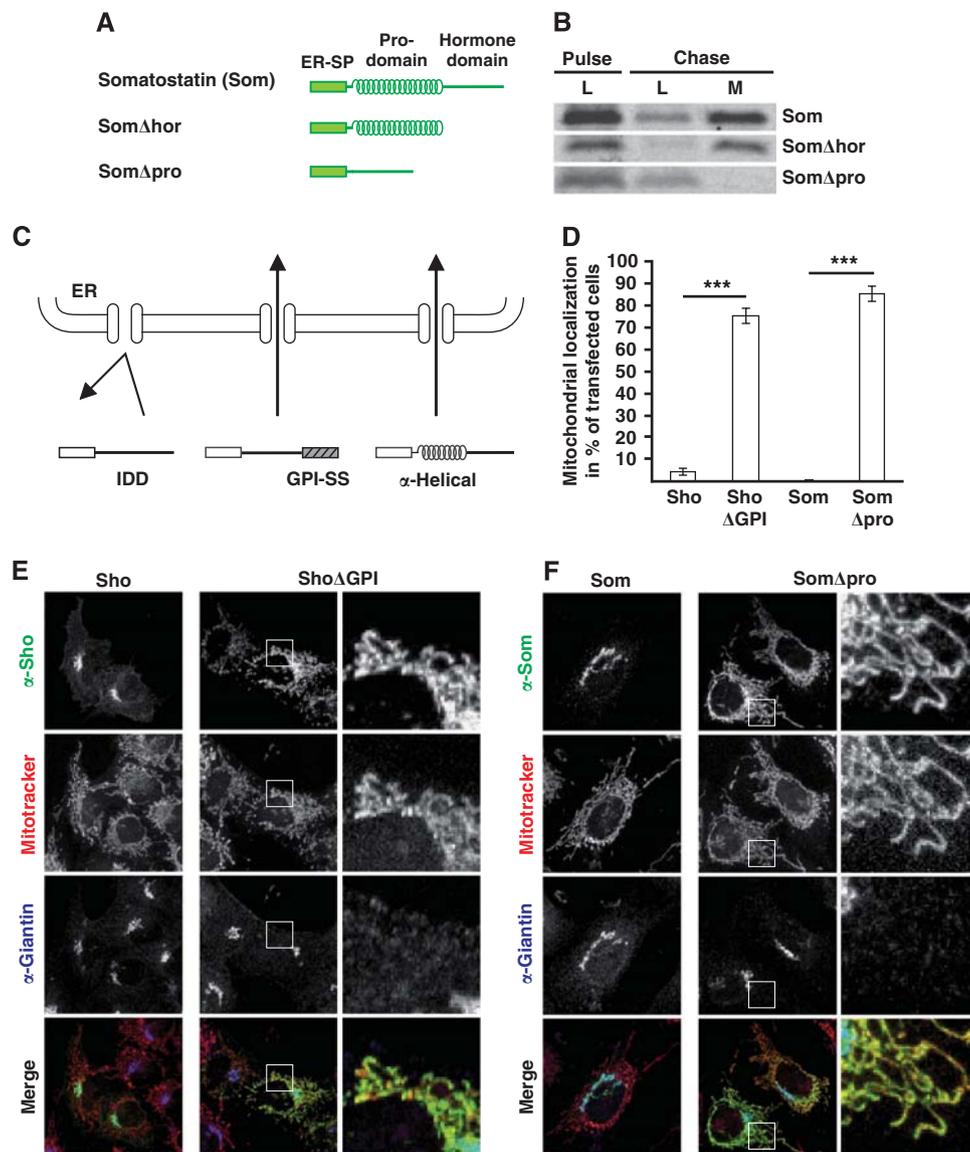


Figure 3 Non-translocated secretory proteins can be targeted to mitochondria. (A) Schematic presentation of the constructs used. (B) Deletion of the α -helical prodomain impairs ER import of somatostatin. Transiently transfected N2a cells were labelled with L-[³⁵S]-methionine for 15 min and either harvested directly (pulse) or after further incubation in fresh medium for 1 h (chase). Proteins present in cell lysates (L) or in conditioned medium (M) were analysed by immunoprecipitation and SDS-PAGE. (C) Schematic summary. ER import of intrinsically disordered domains can be promoted by GPI signal sequences or α -helical domains. (D–F) Non-translocated Sho and Som mutants are targeted to mitochondria. Transiently transfected SH-SY5Y cells expressing the constructs indicated were analysed by indirect immunofluorescence. Mitochondrial localization of proteins was determined by colocalization with MitoTracker Red CMXRos. Giantin was used as a marker for the Golgi compartment. (D) Quantification is based on three independent experiments. In total, at least 600 transfected cells per construct were counted. *** $P < 0.0005$. (E, F) Representative immunofluorescence images. Enlargement of the white frame is shown in the right panel.

CMXRos confirmed that Sho Δ GPI and Som Δ pro predominantly colocalize with mitochondria (Figure 3D–F). To evaluate the possibility that the reduced length of Som Δ pro might modulate targeting, we fused the unstructured domain of Sho to Som Δ pro to generate Som Δ pro-Sho. The immunofluorescence analysis revealed that Som Δ pro-Sho, which is even longer than wildtype Sho, is targeted to mitochondria similarly to Som Δ pro (Supplementary Figure 1A).

Since the ER signal peptide of cytosolic Sho Δ GPI is not cleaved off (Figure 1D), we reasoned that the uncleaved ER signal peptide of Som and Sho can mediate targeting to mitochondria. Therefore, we analysed Δ SP•Sho Δ GPI, Δ SP•Sho and Δ SP•Som, three mutants that are not imported into the ER because they lack the N-terminal signal peptide.

The immunofluorescence analysis clearly indicated that these mutants are present in the cytosol, but do not colocalize with mitochondria (Supplementary Figure 1B).

Structural elements within the nascent chain regulate direction of targeting

The results shown above revealed that the ER signal peptides of Som and Sho can mediate both ER and mitochondrial targeting. The targeting direction seems to be determined by structural elements within the nascent chain, such as α -helical domains and/or a GPI signal sequence. To study this mechanism in more detail, we fused the Sho or Som signal peptide to wildtype PrP, a protein with α -helical domains and a C-terminal GPI signal sequence. For the

analysis of unstructured polypeptides, we used the intrinsically disordered N-terminal domain of PrP (aa 23–115: 115) or a fragment of the Tau protein (aa 103–197: Tau). In addition, we included the ER signal peptide of the APP into our analysis based on its previously described activity to mediate mitochondrial targeting (Anandatheerthavarada *et al*, 2003). To predict the subcellular localization of the proteins studied, we used TargetP 1.1 (Emanuelsson *et al*, 2000). The location assignment is based on the predicted presence of any of the N-terminal presequences: chloroplast transit peptide, mitochondrial targeting peptide (mTP) or secretory pathway signal peptide (ER-SP). As expected, all four signal peptides got the highest scores for an ER location (Figure 4B). As a positive control for mitochondrial targeting, we used the N-terminal mitochondrial targeting peptide of subunit 1 of the ubiquinol cytochrome c reductase complex (UCR).

Western blot analysis of transiently transfected cells indicated that each of the three heterologous signal peptides promotes efficient import of full-length PrP into the secretory pathway, leading to the formation of complex glycosylated PrP (Figure 4C). Immunofluorescence analysis of transfected cells confirmed that all constructs are located at the plasma membrane (Supplementary Figure 1C). As shown before (Figure 1B; Miesbauer *et al*, 2009), the intrinsically unstructured proteins (PrP•115, PrP•Tau) were not efficiently imported into the ER. Similarly, the ER signal peptides of Sho, Som or APP did not promote ER import of the intrinsically disordered N-terminal domain of PrP (115) or the Tau fragment (Supplementary Figure 1D and E). However, the intracellular localization of these constructs was different from that of PrP•115 and PrP•Tau. Whereas PrP•115 and PrP•Tau were distributed throughout the cytosol and did not colocalize with mitochondria, the unstructured domains equipped with Som, Sho or APP signal peptide were targeted to mitochondria (Figure 4D and E).

Since the 115 (SP + 93 aa) and Tau (SP + 108 aa) constructs are rather short, it appeared possible that the reduced length impairs cotranslational targeting of the nascent chain to the ER and thereby the subsequent translocation event. Therefore, cotranslational targeting to the ER membrane was investigated *in vitro*. Stalled ribosome–nascent chain complexes (RNCs) were generated by translating truncated mRNAs lacking a stop codon in reticulocyte lysate supplemented with [³⁵S]-methionine. After synthesis in the presence of canine pancreatic microsomal membranes, RNCs bound to ER membranes were separated from untargeted nascent chains by sedimentation. The percentage of nascent chains recovered in this way was quantified by SDS-PAGE and autoradiography. To analyse whether structural features of the nascent chain modulate this step, we compared cotranslational targeting of PrP•115 with that of PrP• α 2 α 3, a construct composed of α -helical domains. As a negative control, we included a construct lacking the N-terminal signal peptide (Δ SP•115). This comparative analysis revealed that both constructs are targeted cotranslationally to ER membranes and that the structure of the nascent chain does not seem to modulate ER targeting, at least under the *in vitro* conditions tested (Figure 4F).

In sum, these experiments revealed that three unrelated ER signal peptides derived from Sho, Som and APP have the ability to mediate targeting to mitochondria. Targeting direction was determined by structural elements within the

nascent chain. While productive ER import was mediated for proteins containing α -helical domains, unstructured nascent chains were predominantly targeted to mitochondria.

Mitochondrial targeting by ER signal peptides can interfere with mitochondrial function

To extend our analysis, we studied a model substrate that is composed of the intrinsically disordered N-terminal domain of PrP followed by the second and third α -helical domain of PrP (115 α 2 α 3). In this context, it is important to note that *in vitro* experiments indicated that isolated PrP fragments comprising helices 2 and 3 or helix 3 only show complete structural autonomy, that is, independently adopt an α -helical conformation (Eberl and Glockshuber, 2002; Gallo *et al*, 2005). The addition of the α -helical domains to the unstructured part of PrP partially restored ER import, as evidenced by the appearance of a glycosylated fraction. This phenomenon is illustrated by the western blot analysis of lysates from N2a cells transiently expressing 115 α 2 α 3 equipped with the signal peptide from PrP, Sho, Som or APP. The slower migrating species (Figure 5B, filled arrowhead) are composed of glycosylated molecules, since they disappear in cell lysates that had been treated with PNGase F, an enzyme that cleaves N-linked oligosaccharides. The faster migrating bands represent unglycosylated molecules that have failed to be productively imported into the ER. A construct without any signal peptide (Δ SP•115 α 2 α 3) migrated faster on SDS/PAGE, suggesting that the unglycosylated fractions of 115 α 2 α 3 containing the signal peptide of PrP, Sho, Som or APP contain an uncleaved N-terminal ER signal peptide. The ratio between the ER and cytosolic fraction varied among the different constructs, emphasizing the ability of signal peptides to modulate translocation efficiency in a sequence-specific manner (reviewed in Hegde and Kang, 2008). In this setting, the APP signal peptide was the least efficient to promote productive translocation of 115 α 2 α 3 into the ER (Figure 5B).

For the immunofluorescence analysis, we included UCR•115 α 2 α 3, a construct containing the authentic mitochondrial targeting peptide of UCR. The cytosolic fraction of PrP•115 α 2 α 3 did not colocalize with mitochondria, similarly to the construct without a signal peptide (Δ SP•115 α 2 α 3). As expected, UCR•115 α 2 α 3 colocalized with mitochondria (Figure 5C). Interestingly, staining with MitoTracker RED CMXRos, a dye that labels only mitochondria that are maintaining a membrane potential, revealed a dissipation of the mitochondrial transmembrane potential in cells expressing Sho•115 α 2 α 3, Som•115 α 2 α 3 or APP•115 α 2 α 3. Costaining with an antibody against the mitochondrial protein Tom 20 indicated that these constructs were targeted to mitochondria (Figure 5C and D). Importantly, the expression of UCR•115 α 2 α 3 did not induce an obvious impairment of mitochondrial membrane potential, indicating that the mitochondrial targeting of 115 α 2 α 3 *per se* has no adverse effects (Figure 5C and D).

Support for the notion that mitochondria are functionally impaired in cells expressing APP•115 α 2 α 3 was obtained by analysing biogenesis of the nuclear encoded mitochondrial chaperone tumour necrosis factor receptor associated protein-1 (TRAP1) that is located in the mitochondrial matrix. Employing western blotting and immunofluorescence analysis we could show that in APP•115 α 2 α 3-expressing

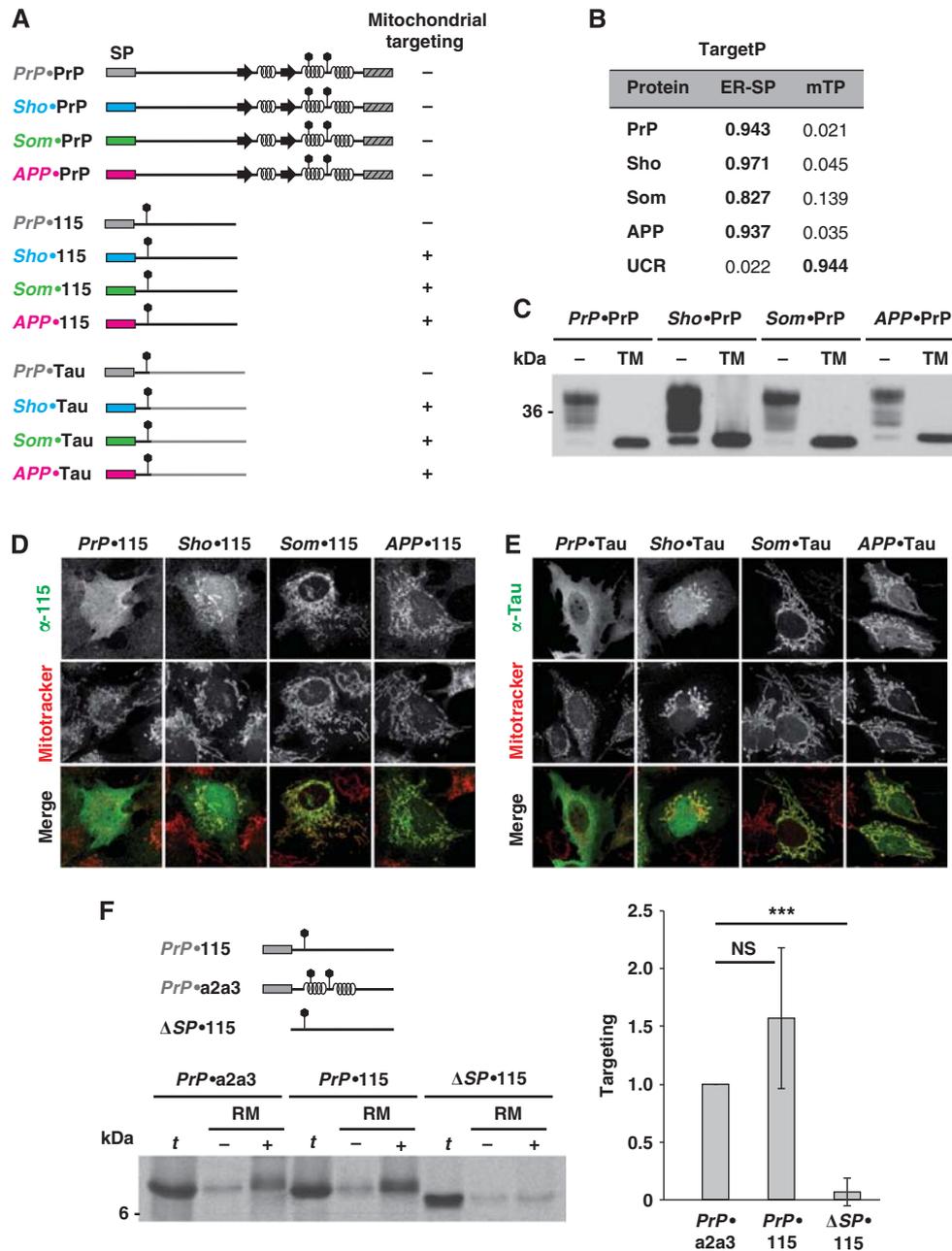


Figure 4 Structural features within the nascent chain regulate alternative targeting of secretory proteins to mitochondria. (A) Schematic presentation of the constructs analysed. Targeting of the respective constructs to mitochondria is indicated on the right. SP, signal peptide. (B) The N-terminal ER signal peptides of Sho, Som and APP are predicted to have no significant mitochondrial targeting activity. The subcellular locations of the respective wildtype proteins were predicted by using the TargetP 1.1 Server. Complete sequences had been analysed without selecting a cutoff. ER-SP, ER signal peptide; mTP, mitochondrial targeting peptide. (C) The signal peptides of Sho, Som and APP mediate productive ER import of full-length PrP. N2a cells were transiently transfected with the mutants indicated and cultured for 16 h in the presence or absence of tunicamycin (TM, 0.5 μ g/ml for 16 h). Total cell lysates were analysed by western blotting. (D, E) The signal peptides of Sho, Som and APP target intrinsically disordered proteins to mitochondria. SH-SY5Y cells were transiently transfected with the indicated constructs and localization of the proteins expressed was analysed by indirect immunofluorescence. To visualize mitochondria, cells were stained with MitoTracker Red CMXRos. (F) Intrinsically disordered nascent chains are cotranslationally targeted to ER membranes *in vitro*. Truncated mRNAs of the constructs depicted (upper left panel) lacking a stop codon were translated *in vitro* using rabbit reticulocyte lysate supplemented with L-[³⁵S]-methionine in the presence (+) or absence (-) of rough microsomal membranes (RM). Targeted nascent chains were pelleted by centrifugation over a sucrose cushion for 3 min at 50 000 g. In parallel, polysomes of translation reactions without microsomes were sedimented by centrifugation for 30 min at 50 000 g (*t*). Samples were analysed by SDS-PAGE and autoradiography (lower panel). Targeting efficiency was determined by quantifying the band intensities of the respective constructs in the + RM fraction relative to the amount present in the total fraction (right panel). Data represent mean \pm s.e.m. of at least three independent experiments. Targeting efficiency of PrP• α 2 α 3 was set as 1. ****P* < 0.0005; NS, not significant.

cells the precursor of TRAP1, containing an uncleaved N-terminal mitochondrial targeting peptide, accumulates in the cytosol, indicating an impairment of mitochondrial

import. Mitochondrial import of TRAP1 was not impaired in UCR•115 α 2 α 3-expressing cells (Supplementary Figure 2).

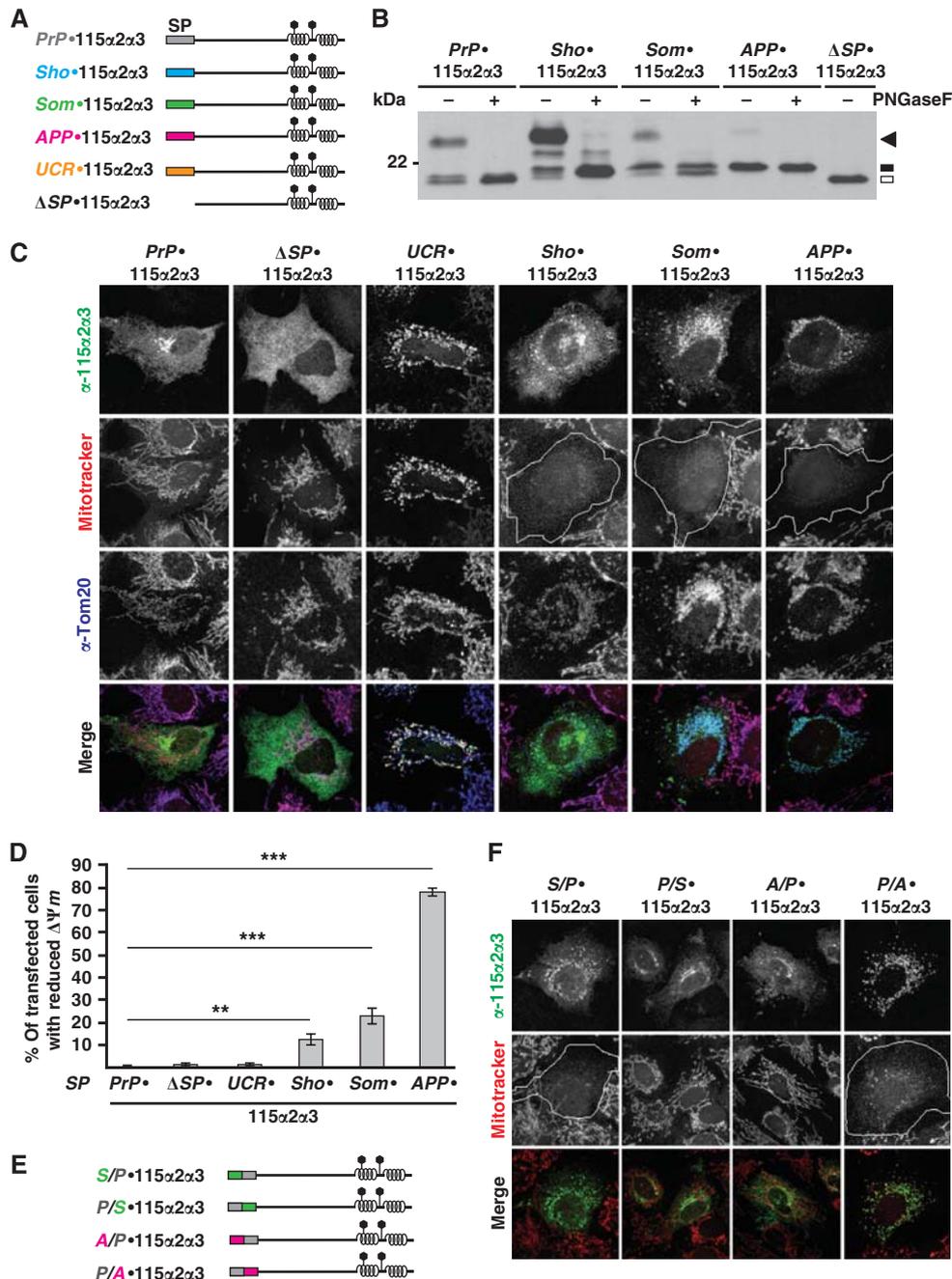


Figure 5 Mitochondrial targeting by ER signal peptides can impair mitochondrial function. (A, B) α-Helical domains can partially restore ER import of intrinsically disordered domains. (A) Schematic presentation of the constructs analysed. SP, signal peptide. (B) N2a cells were transiently transfected with the constructs indicated. Total cell lysates were either treated with PNGase F or left untreated and analysed by western blotting. Filled arrowhead: glycosylated fraction; filled rectangle: uncleaved signal peptide; unfilled rectangle: cleaved signal peptide. (C, D) Mitochondrial targeting by ER signal peptides can induce dissipation of the mitochondrial membrane potential. (C) SH-SY5Y cells transiently transfected with the constructs indicated were analysed by indirect immunofluorescence. Mitochondria were visualized by immunofluorescence analysis using an anti-Tom20 antibody and by staining with Mitotracker Red CMXRos, a dye whose mitochondrial localization is dependent on an intact mitochondrial membrane potential. Transfected cells with a dissipated membrane potential are indicated by a white frame. (D) Dissipation of the mitochondrial membrane potential was quantified by counting transfected cells without or with a strongly decreased MitoTracker Red CMXRos staining. Quantification is based on at least three independent experiments. In total, at least 600 transfected cells per construct were counted. ***P* < 0.005, ****P* < 0.0005. (E, F) Chimeric ER signal sequences can mediate mitochondrial targeting. (E) Schematic presentation of the constructs analysed. (F) SH-SY5Y cells transiently transfected with the constructs indicated were analysed by indirect immunofluorescence as described under (C).

In order to identify the region/amino acids within the signal peptide responsible for the mitochondrial targeting activity, we generated chimeric PrP/Som (P/S and S/P) and PrP/APP (P/A and A/P) signal peptides and evaluated their targeting activity in transiently transfected cells. This

approach revealed that the ability to mediate mitochondrial targeting was located in the N-terminal half of the Som signal peptide, while the C-terminal half of the APP signal peptide determined mitochondrial localization (Figure 5E and F). However, examining the biochemical and biophysical

properties of the ER signal peptides Sho, Som or APP did not reveal any features that could be specifically linked to their activity to mediate mitochondrial targeting (data not shown).

In an attempt to unravel the mechanism underlying the toxic activity of APP•115 α 2 α 3, we mutated the two cysteine residues located in helices 2 and 3 to alanines to generate APP•115 α 2 α 3AA (Figure 6A). These cysteines form a disulphide bridge in the ER under physiological conditions. Thus, it is possible that oxidation of the cysteine residues by the MIA (mitochondrial intermembrane space assembly) pathway (reviewed in Stojanovski *et al*, 2012) contributes to the toxic effects of APP•115 α 2 α 3. APP•115 α 2 α 3AA was targeted to mitochondria and expressed at levels comparable to that of APP•115 α 2 α 3. However, substitution of the two cysteine residues significantly reduced the toxic activity of APP•115 α 2 α 3 to induce dissipation of the mitochondrial membrane potential (Figure 6B–D).

ER signal peptides can mediate targeting into the mitochondrial matrix

To investigate the localization of the mitochondrially targeted constructs in more detail, we analysed mitochondria isolated from transfected cells. Cell homogenates prepared from transiently transfected SH-SY5Y cells expressing APP•115 α 2 α 3 or Som Δ pro were fractionated to separate the cytosol from mitochondria. APP•115 α 2 α 3 and Som Δ pro cofractionated with mitochondria and were barely present in the cytosolic fraction. Moreover, after a limited proteolytic digestion of intact mitochondria, APP•115 α 2 α 3 and Som Δ pro were

protected similarly to Tim 23, a protein located in the inner mitochondrial membrane (IMM). In contrast, the import receptor Tom 20, located at the outer mitochondrial membrane (OMM), was degraded under these conditions (Figure 7A and B). To evaluate the submitochondrial localization, we took three different approaches. First, we prepared mitochondria without their outer membranes (mitoplasts). After a limited proteolytic digestion of intact mitoplasts the IMM protein Tim 23 was degraded, whereas the mitochondrial matrix protein Tim 44 was protected. Similarly, APP•115 α 2 α 3, APP•115 α 2 α 3AA, UCR•115 α 2 α 3 and Sho Δ GPI were protected under these conditions, indicating that at least a fraction of these proteins was imported into the mitochondrial matrix (Figure 7C). Second, we treated isolated mitochondria with increasing concentrations of digitonin. Under conditions in which proteins located in the mitochondrial intermembrane space or the IMM (Omi and Tim 23) were completely degraded by proteinase K, APP•115 α 2 α 3, APP•115 α 2 α 3AA and UCR•115 α 2 α 3 were still partially protected (Supplementary Figure 3). Finally, we performed an immunofluorescence analysis of semi-permeabilized cells. This approach exploits digitonin concentrations under which the plasma membrane and the OMM are disrupted, while the IMM stays intact. Thus, antibodies have no access to proteins located in the mitochondrial matrix. As a control, the immunofluorescence analysis was performed with cells that had been treated with Triton X-100 to disrupt all cellular membranes. Indeed, APP•115 α 2 α 3, APP•115 α 2 α 3AA and UCR•115 α 2 α 3 could only be detected in cells at conditions under which antibodies had access to the mitochondrial matrix (Figure 7D). In sum, these different approaches indicated that the ER signal peptides of Sho, Som and APP have the ability to mediate targeting to the mitochondrial matrix.

Mitochondrial targeting correlates inversely with ER import efficiency

The findings presented so far indicated that among the three mitochondrially targeted constructs APP•115 α 2 α 3 has the highest activity to disrupt the mitochondrial membrane potential and the lowest ER import efficiency (Figure 5B and D). In a next step, we quantified the relative amount of the glycosylated fraction of Sho•115 α 2 α 3, Som•115 α 2 α 3 and APP•115 α 2 α 3 and compared it with the activity of the same construct to induce dissipation of the mitochondrial membrane potential. This revealed an inverse correlation between ER import efficiency and mitochondrial targeting (Figure 8A). Based on this observation, we tested the hypothesis that enhancing ER import reduces mitochondrial targeting and *vice versa*. Since a C-terminal GPI signal sequence can significantly improve ER import efficiency (Figure 2), we investigated whether mitochondrial targeting and toxicity of APP•115 α 2 α 3 can be reduced by a C-terminal GPI anchor signal sequence. Indeed, this approach significantly enhanced ER import efficiency as reflected by the appearance of glycosylated species of APP•115 α 2 α 3GPI (Figure 8B, upper panel). Moreover, in contrast to APP•115 α 2 α 3 expression of APP•115 α 2 α 3GPI did not induce dissipation of the mitochondrial membrane potential (Figure 8B, lower panel).

To assess mitochondrial targeting under conditions of impaired ER translocation, we used different experimental approaches. First, we tested a possible effect of p58^{IPK},

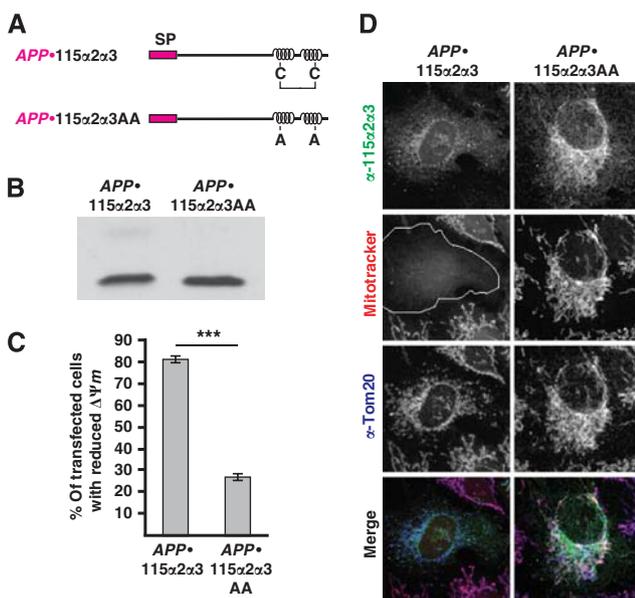


Figure 6 Mutation of the cysteine residues decreases the toxic potential of APP•115 α 2 α 3. (A) Schematic presentation of the constructs analysed. SP, signal peptide. (B) Transiently transfected N2a cells expressing the constructs indicated (upper panel) were analysed by western blotting (lower panel). (C, D) Transiently transfected SH-SY5Y cells expressing APP•115 α 2 α 3 or APP•115 α 2 α 3AA were analysed by immunofluorescence. Mitochondria were visualized by immunofluorescence analysis using an anti-Tom20 antibody and by staining with Mitotracker Red CMXRos (D). The percentage of transfected cells with impaired mitochondrial membrane potential was quantified from at least four independent experiments. In total, at least 700 transfected cells per construct were counted. *** $P < 0.0005$.

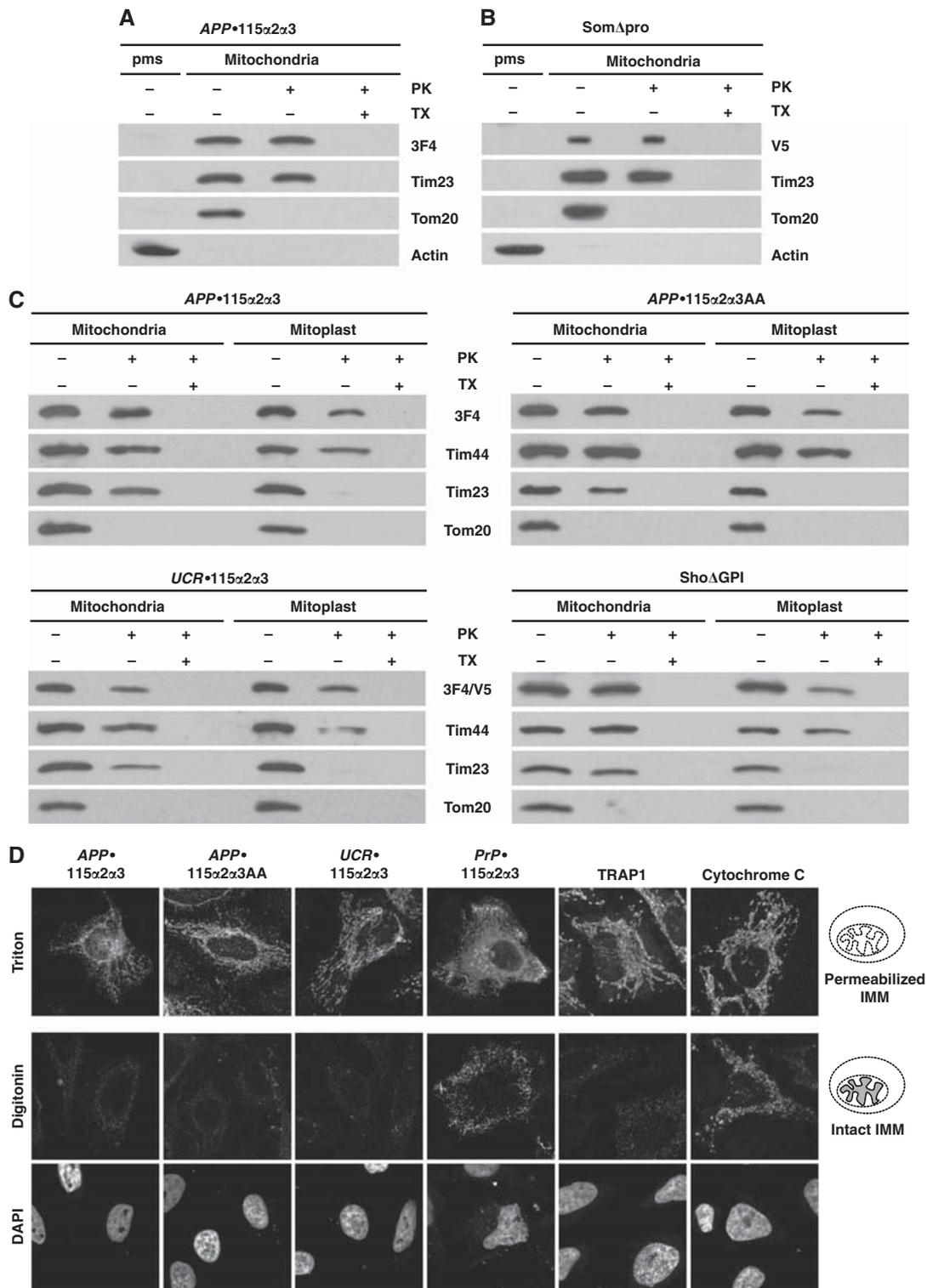


Figure 7 The ER signal peptides mediate targeting to the mitochondrial matrix. (A, B) *APP•115 α 2 α 3* (A) and *SomΔpro* (B) are translocated into mitochondria. Isolated mitochondria from transiently transfected SH-SY5Y cells expressing *APP•115 α 2 α 3* were digested with proteinase K (PK) (50 μ g/ml, 30 min on ice) in the absence or presence of Triton X-100 (TX) (1%) or left untreated. The samples were analysed by western blotting to detect the proteins indicated. The post-mitochondrial supernatant (pms) was analysed in parallel. (C, D) The ER signal peptides can mediate targeting to the mitochondrial matrix. (C) Mitochondria or mitoplasts from transiently transfected SH-SY5Y cells expressing the indicated constructs were digested with PK in the absence or presence of TX or left untreated. The samples were analysed by western blotting to detect the proteins indicated. The mAb 3F4 detects all *115 α 2 α 3* constructs. *SomΔpro* and *ShoΔGPI* were detected using the mouse monoclonal anti-V5 antibody. (D) HeLa cells transfected with the indicated constructs were subjected to either complete permeabilization (0.15% Triton X-100) or selective permeabilization of the plasma membrane and the outer mitochondrial membrane only (0.5 μ g/ml digitonin for 3 min). Submitochondrial localization of the constructs was analysed by indirect immunofluorescence. In parallel, antibody access to cytochrome c, located in the mitochondrial intermembrane space, and the matrix protein TRAP-1 was determined. IMM, inner mitochondrial membrane. (A–D) Marker proteins: actin (cytosol), Tom 20 (outer mitochondrial membrane), Omi (intermembrane space), Tim 23 (inner mitochondrial membrane), and Tim 44 (mitochondrial matrix).

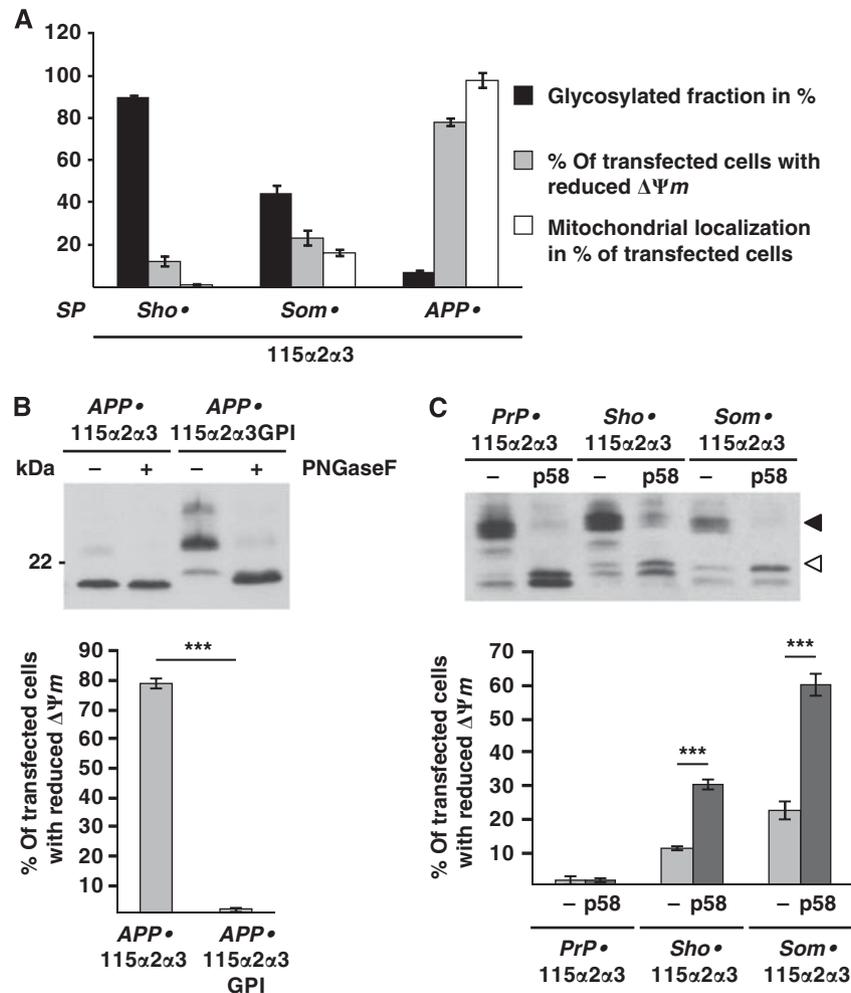


Figure 8 Mitochondrial targeting correlates inversely with ER import efficiency. (A) Inverse correlation of mitochondrial impairment and ER translocation. Transiently transfected N2a cells expressing the constructs indicated were analysed by western blotting and the relative amounts of the glycosylated fraction were quantified. For statistical analysis, the samples of three independent experiments were analysed by western blotting and measured by a luminescence reader. The percentage of the ER-translocated fraction was determined as ratio between the signal of the glycosylated fractions and the total protein signal (black bars). In parallel, mitochondrial targeting of the same constructs (white bars) and their activity to disrupt the mitochondrial membrane potential (grey bars) are shown. (B) Enhancing ER translocation decreases mitochondrial targeting. Transiently transfected N2a cells expressing APP•115α2α3 with or without a GPI signal sequence were analysed by western blotting. To monitor N-linked glycosylation, parallel samples were treated with PNGase F (PNGaseF +) (upper panel). To determine mitochondrial impairment, cells were stained with Mitotracker Red CMXRos. Loss of mitochondrial membrane potential was quantified by counting transfected cells with strongly decreased or lost Mitotracker Red CMXRos staining. Quantification is based on at least four independent experiments. In total, at least 800 transfected cells per construct were counted. *** $P < 0.0005$ (lower panel). (C) Decreasing ER translocation enhances mitochondrial targeting. N2a cells transiently cotransfected with the constructs indicated and either p58^{IPK} (p58) or pcDNA were analysed by western blotting. SP: signal peptide, filled arrowhead: glycosylated fraction, unfilled arrowhead: unglycosylated fraction. For quantification of mitochondrial impairment, an immunofluorescence analysis of transiently transfected cells was carried out as described in Figure 6C and D. Quantification is based on at least three independent experiments. In total, at least 600 transfected cells per construct were counted. *** $P < 0.0005$.

a Hsp40 cochaperone that can interfere with the translocation of secretory proteins into the ER (Oyadomari *et al*, 2006; Rutkowski *et al*, 2007; Petrova *et al*, 2008; Miesbauer *et al*, 2009). This effect is illustrated in the western blot analysis of cells co-expressing PrP•115α2α3 and p58^{IPK} (Figure 8C, upper panel). The relative amount of the glycosylated fraction of PrP•115α2α3 was reduced in these cells, concomitant with an increase in the unglycosylated fraction localized in the cytosol. Similarly, p58^{IPK} co-expression decreased the glycosylated fractions of Som•115α2α3 and Sho•115α2α3 and increased the respective non-translocated fractions (Figure 8C, upper panel). The reduced ER import of

these constructs obviously increased mitochondrial targeting. Co-expression of p58^{IPK} and Som•115α2α3 or Sho•115α2α3 significantly increased the percentage of cells with a dissipated membrane potential (Figure 8C, lower panel).

Next, we addressed the question whether wildtype Sho, Som or APP is targeted to mitochondria under conditions of impaired ER import. First, we employed eeyarestatin I (Eey1), a drug that blocks Sec61-mediated protein translocation at the ER (Cross *et al*, 2009). The immunofluorescence analysis clearly demonstrated that in Eey1-treated cells wildtype Sho is targeted to mitochondria (Figure 9A). In a different approach, we used siRNA-mediated silencing of the *SEC61A1*

gene in SH-SY5Y cells. A previous study employing this strategy demonstrated that cells functionally depleted of the Sec61 complex can be analysed in a defined time window before toxic effects occur (Lang *et al*, 2012). Similarly to the findings in Eey1-treated cells, wildtype Sho was targeted to mitochondria in cells depleted of the Sec61 complex (Figure 9B; Supplementary Figure 4A). Finally, we corroborated and extended these findings in a different cell line. After depleting HeLa cells of Sec61 by siRNA-mediated gene silencing, wildtype Sho, Som or APP was targeted to mitochondria (Figure 9C–E; Supplementary Figure 4B). Notably, non-translocated wildtype PrP was not redirected to mitochondria under these conditions (Supplementary Figure 4C).

These findings further support the concept that the signal peptides of Som, Sho and APP initially target nascent chains to the Sec61 translocon. However, under conditions of impaired ER import, caused by a lack of structural domains in the nascent chain or by an impairment of the translocon, these ER signal peptides have the potential to mediate mitochondrial targeting (Figure 9F).

Discussion

It is generally assumed that an N-terminal ER signal peptide has an autonomic activity to dictate cotranslational targeting to specific cellular compartments, independently of the primary/secondary structure of the rest of the protein. Our study provides experimental evidence that several ER signal peptides have the potential to mediate both ER and mitochondrial targeting and that structural features within the nascent chain can influence the targeting direction.

Intrinsically disordered secretory proteins contain α -helical prodomains or GPI anchor signal sequences to promote import into the ER

From previous studies with pathogenic mutants of the prion protein and artificial model proteins it emerged that intrinsically disordered proteins are inefficiently imported into the ER (Heske *et al*, 2004; Miesbauer *et al*, 2009). Based on these findings, we had a closer look at intrinsically disordered secretory proteins that are obviously efficiently imported into the ER since they are transported to the plasma membrane or secreted. As examples for this class of secretory pathway proteins we analysed the GPI-anchored protein shadoo (Sho) and the secretory hormone somatostatin (Som). We noticed that the primary translation products of Sho and Som contain domains that are not part of the mature protein. Employing different deletion mutants we could demonstrate that ER import of Sho or Som is critically dependent on the presence of the GPI signal sequence or the prodomain, respectively. How can this effect be explained? We suggest that the activity of the prodomain to promote ER import of Som is based on its propensity to adopt an α -helical conformation. Interestingly, such a propensity was described previously for the prodomains of various neuropeptide hormones (Kizer and Tropsha, 1991). Most likely, the effect of the GPI anchor signal sequence on ER import is similar. A unifying feature of the different GPI signal sequences used in our study (PrP, Sho, GFR α) is that they are predicted to adopt an α -helical conformation (PSIPRED; <http://bioinf.cs.ucl.ac.uk/psipred>). We assume that GPI anchor signal sequences in general can promote ER import. This

concept is based on previous findings (Holscher *et al*, 2001; Gu *et al*, 2008) and our approach to use the GPI anchor signal sequences of Sho, PrP and GFR α to increase ER import of intrinsically disordered proteins.

Our data provide evidence for the concept that ER import of intrinsically disordered proteins is an inefficient process, which can be significantly enhanced by including α -helical domains in the polypeptide. Obviously, this strategy has successfully been followed by evolution. Intrinsically disordered secretory pathway proteins are synthesized as larger precursors containing domains that facilitate translocation into the ER. After ER import, these domains are removed and thereby not part of the mature protein.

Mitochondrial targeting by ER signal peptides is determined by structural domains within the nascent chain

Another major finding of our study is the observation that different ER signal peptides have the propensity to mediate targeting to mitochondria. This unexpected result emerged from the analysis of Sho and Som mutants that failed to be productively imported into the ER. The transport of these mutants to mitochondria was dependent on an uncleaved ER signal peptide, indicating that the cytosolic fraction of these secretory proteins was not generated via a classical ERAD pathway. Whereas dual or alternative targeting of secretory proteins to mitochondria has been described before, our findings revealed a novel mechanism. It has been established that a protein can be equipped with different N-terminal signal peptides due to differential splicing or based on two distinct translation products (reviewed in Danpure, 1995 and Small *et al*, 1998). Alternatively, composite signal sequences, described for cytochrome P450 proteins and the APP, require activation either by proteolytic cleavage or by phosphorylation (reviewed in Anandatheerthavarada *et al*, 2003; Karniely and Pines, 2005 and Avadhani *et al*, 2011). Here, we show that the ER signal peptides of Sho, Som and APP have the propensity to mediate mitochondrial targeting that seems to be independent of post-translational modifications. Instead, the targeting direction is determined by structural features within the protein. This concept is experimentally supported by the finding that targeting of chimeric proteins composed of the signal peptide of Sho, Som or APP fused to heterologous proteins was exclusively determined by structural features of the heterologous protein. All three signal peptides mediated efficient ER import of full-length PrP, a protein containing α -helical domains and a GPI anchor. However, in the context of intrinsically disordered proteins, these signal peptides induced mitochondrial targeting. So far, it remains elusive why the ER signal peptides of Sho, Som or APP have the ability to mediate mitochondrial targeting, since we could not identify a unifying motif linked to this activity.

Mitochondrial targeting by ER signal peptides can interfere with mitochondrial function

It was previously shown that mitochondrial targeting of APP interferes with mitochondrial function. Toxicity was attributed to an impaired mitochondrial translocation of APP due to its acidic C-terminal sequence (aa 220–290) (Anandatheerthavarada *et al*, 2003; Devi *et al*, 2006; Walls *et al*, 2012). In our study, a mitochondrially targeted fragment

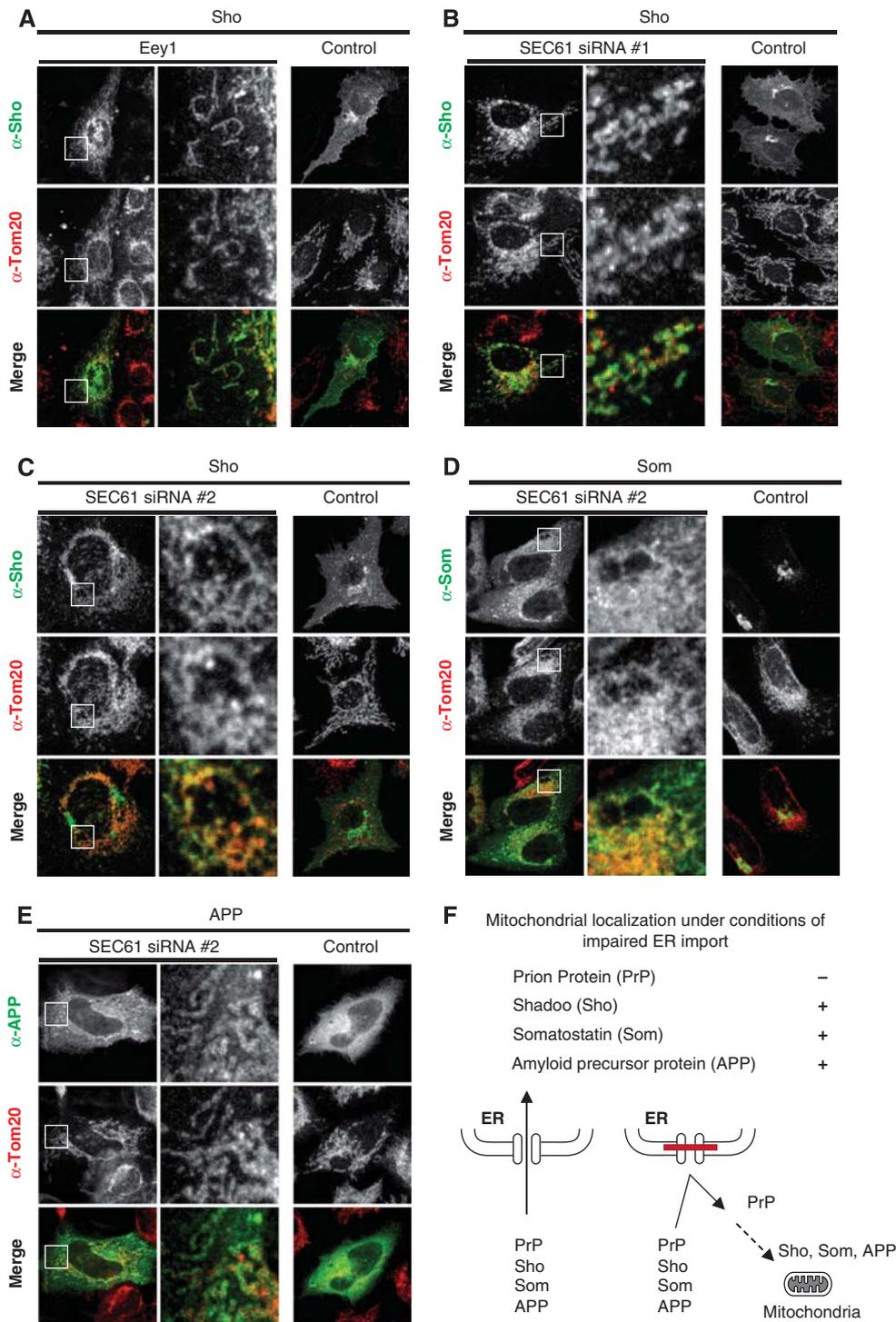


Figure 9 Wildtype Sho, Som and APP are targeted to mitochondria under conditions of impaired ER import. **(A)** Inhibition of Sec61-mediated protein translocation leads to mitochondrial targeting of wildtype Sho. SH-SY5Y cells transiently expressing Sho were treated with Eeyarestatin 1 (Eey1, 8 μ M) or DMSO (control) for 4 h prior to immunofluorescence analysis. Mitochondrial localization of Sho was determined by costaining of endogenous Tom20. **(B, C)** SEC61A1 gene silencing leads to mitochondrial targeting of wildtype Sho, Som and APP. **(B)** SH-SY5Y cells were reversely transfected with siRNA against Sec61 subunit alpha isoform 1 (SEC61 siRNA#1) or control siRNA (control). Forty-eight hours later, cells were transfected with Sho and analysed after additional 24 h by indirect immunofluorescence. Mitochondrial localization of Sho was determined by costaining of endogenous Tom20. Silencing efficiency was evaluated by western blot analysis (Supplementary Figure 4A). **(C-E)** HeLa cells were reversely transfected with siRNA against Sec61 subunit alpha isoform 1 (SEC61 siRNA #2) or control siRNA (control). Twenty-four hours later, the transfection with the siRNA was repeated. After another 24 h, cells were transfected with wildtype Sho **(C)**, Som **(D)** or APP **(E)**. Ninety-six hours after plating, the cells were analysed by indirect immunofluorescence. Mitochondrial localization of the transfected proteins was evaluated by costaining of endogenous Tom20. Silencing efficiency was evaluated by western blot analysis (Supplementary Figure 4B). **(F)** Schematic summary. Under normal conditions, the wildtype proteins indicated are efficiently translocated into the ER. Under conditions of impaired ER import, non-translocated PrP remains in the cytosol, whereas Sho, Som and APP are targeted to mitochondria.

of the prion protein (115 α 2 α 3) was toxic indicating that mistargeting of different proteins can interfere with mitochondrial function. Notably, mitochondrial targeting of 115 α 2 α 3 by any of the three signal peptides analysed (Sho, Som, APP) induced dissipation of the mitochondrial membrane potential. Proteinase K protection assays with isolated mitochondria and indirect immunofluorescence studies with semi-permeabilized cells indicated that the three ER signal peptides can mediate targeting to the mitochondrial matrix. Toxic effects of 115 α 2 α 3 were linked to two cysteines, predicted form a disulphide bridge in the ER under physiological conditions. Thus, one could speculate that toxicity is linked to the oxidation of the cysteine residues by the MIA pathway (reviewed in Stojanovski *et al*, 2012). However, our study also indicated that mitochondrial targeting of 115 α 2 α 3 was not toxic *per se*. In cells expressing UCR●115 α 2 α 3, a construct containing an authentic mitochondrial targeting peptide, the mitochondrial membrane potential was not dissipated, although UCR●115 α 2 α 3 was efficiently targeted to the mitochondrial matrix. Based on these findings, it appears that the toxic effects are due to a combination of aberrant mitochondrial targeting by an ER signal peptide and certain structural features of the targeted protein.

Mitochondrial targeting occurs after abortive ER translocation

The question arose how structured domains can determine the targeting direction of nascent chains. We suggest that the major function of α -helical domains or GPI anchor signal sequences is to promote productive ER import of secretory pathway proteins. The first evidence for such a model emerged from the analysis of wildtype Sho and Som. Both proteins were efficiently imported into the ER and did not colocalize with mitochondria. However, in the absence of domains promoting ER import, Sho Δ GPI and Som Δ pro were targeted to mitochondria. Second, mitochondrial toxicity of the 115 α 2 α 3 constructs containing the Sho, Som or APP signal peptide inversely correlated with ER translocation efficiency. Third, we could enhance mitochondrial targeting by impairing ER translocation efficiency. A GPI anchor signal sequence increased ER import of APP●115 α 2 α 3 and decreased its mitochondrial targeting. *Vice versa*, decreasing ER import of Sho●115 α 2 α 3 and Som●115 α 2 α 3 by coexpressing p58^{IPK} increased their mitochondrial targeting. Finally, we could show that wildtype Sho, Som and APP, which are efficiently imported into the ER under physiological conditions, were targeted to mitochondria in cells with impaired translocon activity.

Based on these findings, the following model appears plausible (Figure 9F). The ER signal peptides of PrP, Sho, Som and APP mediate targeting of nascent chains to the ER membrane, irrespective of whether the polypeptides are unstructured or composed of α -helical domains. If the protein cannot be productively imported, because either it lacks structured domains or the translocation pathway is impaired, then the non-translocated polypeptide is released into the cytosol. In a subsequent step, the protein either remains in the cytosol, where it can be subjected to proteasomal degradation (Hessa *et al*, 2011), or the uncleaved signal peptide can mediate targeting to mitochondria. We cannot rule out that structural features within the nascent chain modulate

targeting of the ribosome-nascent chain complex to the ER. However, we showed that a short unstructured protein, which is not productively imported into the ER, can be cotranslationally targeted to ER membranes at least under *in vitro* conditions employing stalled RNCs. In addition, the finding that a C-terminal GPI-anchor signal sequence is sufficient to promote efficient ER import of Sho indicates that the nascent chain remains translocation competent until the complete protein is synthesized.

Our study revealed that structural features within the nascent chain can act in concert with signal peptides to regulate protein sorting. The physiological function of dual targeting is documented for different proteins (reviewed in Shaffer *et al*, 2005; Carrie *et al*, 2009; Avadhani *et al*, 2011 and Yogev *et al*, 2011). The finding that one third of the yeast mitochondrial proteome seems to be dually localized (Ben-Menachem *et al*, 2011) might indicate that dual targeting serves important physiological functions and is more abundant than previously appreciated. It will now be important to address the question of whether mitochondrial targeting by ER signal peptides is a more general phenomenon and under which physiological or pathophysiological conditions this alternative targeting can occur. Probably, there are some more secretory proteins than the ones currently known with functional activities in the cytosol or mitochondria. However, it is also conceivable that the ER targeting process is leaky, resulting in mislocalization of secretory proteins in cytosolic or mitochondrial compartments. Moreover, such a mislocalization may challenge protein homeostasis and cause toxicity, contributing to pathomechanisms. Indeed, mistargeting of APP to mitochondria or cytosolic localization of the prion protein has been linked to neurodegeneration (Ma *et al*, 2002; Anandatheerthavarada *et al*, 2003; Devi *et al*, 2006; Rambold *et al*, 2006; Chakrabarti and Hegde, 2009; Wang *et al*, 2009; Walls *et al*, 2012), indicating that aberrant protein sorting under stress conditions and/or aging can contribute to cellular dysfunction.

Materials and methods

Immunofluorescence analysis

Transiently transfected cells were grown on glass coverslips. To analyse mitochondrial morphology and function, cells were incubated with MitoTracker Red CMXRos (50 nM) 30 min prior to fixation. Twenty-four hours after transfection, cells were fixed with 3.7% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 in PBS for 10 min, washed with PBS, blocked with PBS containing 0.1% Tween-20 and 5% horse serum for 1 h at room temperature, incubated with primary antibody in PBS containing 1% BSA for 1 h at 37°C, washed again with PBS and incubated with a fluorescently labelled secondary Alexa Fluor antibody in PBS containing 1% BSA for 1 h at room temperature. Cells were mounted onto glass slides and examined by fluorescence microscopy using either a Zeiss Axiovert 200 M microscope for imaging or a Zeiss Axioskop 2 plus microscope for quantification of mitochondrial targeting or impairment.

Apoptosis assay

SH-SY5Y cells were transiently cotransfected with the constructs indicated and EYFP as transfection marker. Three hours prior to fixation, cells were treated with the proteasomal inhibitor MG132 (30 μ M). Indirect immunofluorescence was performed as described above with the exception that the primary antibody against activated caspase-3 was incubated overnight at 4°C. Cells were mounted onto glass slides and examined by fluorescence microscopy using a Zeiss Axioskop 2 plus microscope. The numbers of

cells positive for activated caspase-3 of at least 1000 transfected cells per experiment were determined in a blinded manner. All quantifications were based on at least four independent experiments.

Sec61A1 silencing in SH-SY5Y cells

SH-SY5Y cells were reversely transfected with siRNA against Sec61 subunit alpha isoform 1 (UACCAGUACUUUGAGAUCUUCGUUA, Stealth RNAi siRNA, Invitrogen) or control siRNA (Stealth RNAi siRNA-negative control med GC, Invitrogen) at a final concentration of 30 nM using Lipofectamine RNAiMAX transfection reagent (Invitrogen). Forty-eight hours later, cells were transiently transfected with DNA using Lipofectamine with Plus Reagent (Invitrogen). Additional 24 h later cells were analysed by indirect immunofluorescence as described above. Silencing efficiency was evaluated by western blot analysis using anti-Sec61 alpha antibody and anti- β -actin as loading control.

Sec61A1 silencing in HeLa cells

HeLa cells were transfected with siRNA against Sec61 subunit alpha isoform 1 (5'-CACUGAAAUGUCUACGUUUtt-3', Applied Biosystems) or control siRNA (control; AllStars Negative Control siRNA, Qiagen) at a final concentration of 20 nM using HiPerFect Reagent (Qiagen) as described previously (Lang *et al*, 2012). Twenty-four hours later, the transfection with siRNA was repeated. Additional 12 h later cells were transfected with the constructs indicated or GFP as control using FuGene transfection reagent (Promega) according to manufacturer's recommendations. Finally 60 h after plating, cells were analysed by indirect immunofluorescence using the conditions and antibodies described above. Silencing was evaluated by western blot analysis using anti-Sec61 alpha antibody and anti- β -actin as loading control. The primary antibodies were visualized using ECL Plex goat anti-rabbit IgG-Cy5 antibody or ECL Plex goat anti-mouse IgG-Cy3 antibody (GE Healthcare, Freiburg, Germany) and the Typhoon-Trio imaging system (GE Healthcare) in combination with Image Quant TL software 7.0 (GE Healthcare).

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Statistical analysis

Quantifications were based on at least three independent experiments. Data were shown as means \pm s.e. Statistical analysis was performed using Student's *t*-test. *P*-values are as follows: **P* < 0.05; ***P* < 0.005; ****P* < 0.0005.

Detailed information on plasmids, antibodies and reagents, cell culture, transfection and secretion analysis, inhibitor treatment and protein deglycosylation, cell lysis and western blotting, immunofluorescence analysis of semi-permeabilized cells, *in vitro* targeting, isolation and treatment of mitochondria, metabolic labelling and immunoprecipitation is available under Supplementary Data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: NVP performed the experiments, did the image processing and generated the figures. DD performed the pulse-chase analysis and the immunofluorescence of Δ SS•Som. UKR, DD, CH and LMR analysed the mitochondrial sublocalization. SL carried out the SEC61-silencing experiments in HeLa cells. Cloning was done by NVP, DD and MM. SF, AN, RZ and KFW contributed to the project by co-ordination of experimental work and providing ideas. JT planned and coordinated the entire project. KFW and JT wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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