The role of the lectin VIP36 in the early secretory pathway

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Summary

Lectins are of emerging importance for quality control and intracellular transport of glycoproteins in mammalian cells. One of the most prominent lectins involved in intracellular transport is ERGIC-53, which belongs to the family of L-type lectins. ERGIC-53 mediates the ER export of several glycoproteins like cathepsin Z, α1-antitrypsin (α1-AT) or blood coagulation factors. VIP36 belongs to the same family as ERGIC-53, but its cellular function remains poorly understood. VIP36 is a type I membrane protein. It cycles within the early secretory pathway and binds high mannose glycans. In order to gain insight into the function of VIP36 we decided to search for a luminal interaction partner for VIP36.

We used a YFP-protein fragmentation complementation (YFP-PCA) based FACS screen of a human adult liver library to unravel an interaction partner for VIP36. Complementation of YFP is irreversible. Therefore, the YFP-PCA is well suited to detect weak interactions, like those between mammalian lectins and glycoproteins. YFP2-VIP36 was used as the bait in our screen. The human liver library was tagged with YFP1. Our screen identified α1-AT as an interaction partner for VIP36. VIP36 recognized high mannose containing α1-AT, which is consistent with the previously obtained results about the glycan affinity of VIP36. This interaction was increased upon inhibition of complex glycosylation by kifunensine. The complex formed by α1-AT and VIP36 was localized to the Golgi and the ER. α1-AT was previously identified as a cargo for ERGIC-53. Knockdown of ERGIC-53 slowed down α1-AT transport, consistent with a role for ERGIC-53 in ER export of α1-AT. In contrast, knockdown of VIP36 accelerated transport of endogenous α1-AT in HepG2 cells. This effect was specific for α1-AT, as the non-glycosylated protein albumin showed no acceleration in transport. In addition, VIP36 knockdown did not affect general protein secretion. This finding makes it unlikely that VIP36 acts as an anterograde cargo receptor for α1-AT. Further studies on the dynamics of the complex formed by VIP36 and α1-AT revealed that VIP36 recycles α1-AT back to the ER, which argues for a role of VIP36 in post-ER quality control. This notion is further supported by the finding that the chaperone BiP co-immunoprecipitated with the complex of VIP36 and α1-AT. This chaperone was previously described as an interaction partner for VIP36. This argues for a complex consisting of VIP36 and BiP acting together in post-ER quality control to detect misfolded α1-antitrypsin in the Golgi and retrieve it back to the ER.

Apart from searching for an interaction partner, I also determined the effect of depletion of VIP36 on the morphology of the secretory pathway. The rationale behind this is the

observation that cargo receptors contribute to the structural integrity of organelles of the secretory pathway. Knockdown of VIP36 had no effect on ER exit sites or on the ERGIC. However, VIP36 knockdown resulted in fragmentation of the Golgi apparatus. The fragmented Golgi was not the consequence of disturbed bidirectional protein transport and not due to effects on microtubules. Knockdown of VIP36 reduced COPI staining on the Golgi. VIP36 is likely to provide COPI binding sites on the Golgi via its cytosolic tail and thereby contribute to Golgi structural integrity. Our results underscore the importance of cargo receptors, not only for intracellular transport within the secretory pathway, but also to maintain the integrity of the secretory pathway itself.

In conclusion, my thesis provides a deeper insight into the function of VIP36 in the early secretory pathway.

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Introduction

1.1 The secretory pathway

The journey of a secretory protein starts in the ER, where it is synthesized. It exits the ER in vesicles, reaches the ER-Golgi intermediate compartment (ERGIC) and continues to travel to the Golgi (Fig. 1). There it is further sorted to reach the plasma membrane or to enter the endosomal compartments.

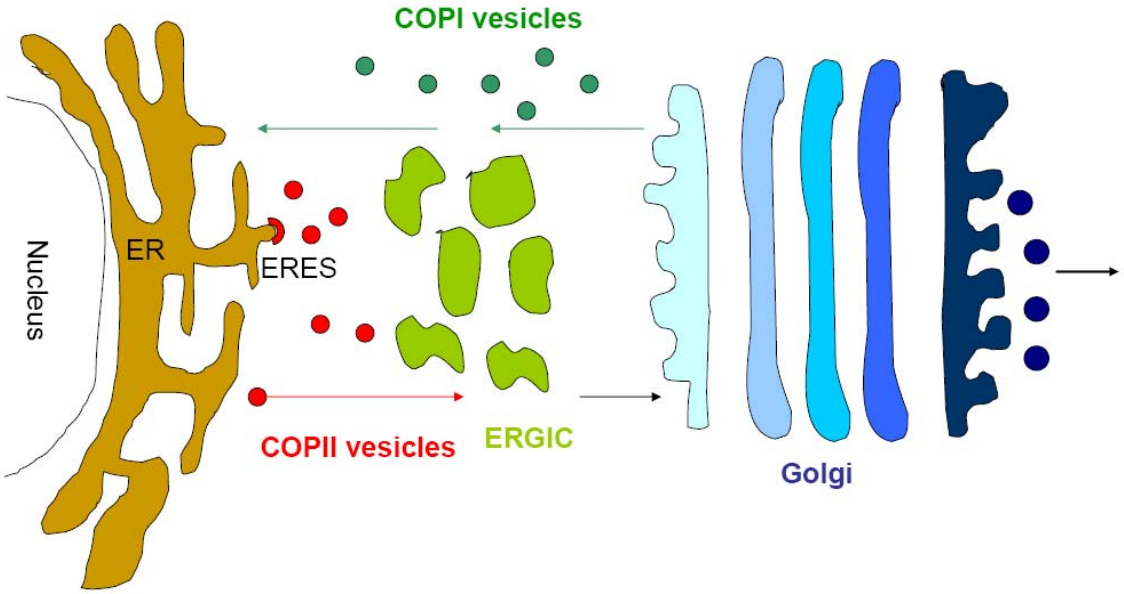


Figure 1: ***Early secretory pathway****: Proteins are synthesized in the ER. They are packed into COPII vesicles at ER exit sites (ERES). In the ER-Golgi intermediate compartment (ERGIC) anterograde secretory cargo is segregated from retrograde cargo. Subsequently anterograde cargo is transported to the Golgi. Secretory proteins traverse the Golgi and acquire enzymatic modifications. At the trans-Golgi network the protein is sorted into carriers that target them to their final destinations. COPI vesicles transport recycling cargo receptors and ER resident proteins back to the ER.*

1.1.1 Export from the endoplasmic reticulum

Correct protein synthesis in the rough endoplasmic reticulum (ER) is warranted by the quality control machinery. By this, proper protein folding and (in the case of oligomeric proteins) assembly into higher-order complexes is assured. Once this is achieved, secretory proteins are prepared for export from the ER. Initially it has been thought that this export process is non-selective. In this scenario, proteins carrying an ER retention motif would stay in the ER, all other proteins would be transported out of the ER by default. This turned out to be not true. There is meanwhile a plethora of evidence for an active concentrative ER export process (Balch et al., 1994; Ma et al., 2001; Malkus et al., 2002). In mammalian cells, ER export takes

place at ribosome-free regions of the rough ER, originally referred to as transitional ER elements (TEs) or ER exit sites (ERES) (Orci et al.,1991; Zeuschner et al.,2006). ERES are COPII coated and exhibit up to 350nm long membrane evaginations with omega shaped budding profiles (Bannykh et al.,1996). In mammalian cells, ERES are the major sites for COPII vesicle formation. COPII vesicles are 50nm-80nm large carriers and are responsible for export of most known cargo molecules out of the ER. COPII vesicle formation is initiated by the recruitment of the small 21kDa GTPase Sar1 to the ER membrane. This is mediated by the membrane bound GDP exchange factor (GEF) Sec12 (Barlowe and Schekman, 1993). Active Sar1 on the ER membrane inserts its NH2-terminus into the lipid bilayer, which is important for deformation of the ER membrane and for vesicle fission (Bielli et al., 2005). Active Sar1 recruits the heterodimeric complex consisting of Sec24 and Sec23. This complex consisting of Sar1, Sec23 and Sec24 is termed the pre-budding complex. Sec24 interacts with the cytosolic portions of transmembrane cargo proteins. Sec23 serves as a GTPase activating protein (GAP) for Sar1. Two mammalian isoforms exist (Sec23A and Sec23B) (Paccaud et al., 1996). Three distinct cargo binding sites have been indentified in yeast Sec24p. The A-site and B-site recognize two different motifs in Sed5p. The B-site also binds to the v-Snare Bet1p and the Golgi protein Sys1p. The C-site recognizes a motif in Sec22 (Mossesova et al., 2003). Sec24p has two homologs in yeast: Lst1p (Roberg et al., 1999) and Iss1 (Kurihara et al., 2000). These homologs are mostly redundant in their function. In humans four Sec24 isoforms were described (Sec24A-D) (Pagano et al., 1999). The first cargo binding site in a mammalian Sec24 isoform was found in Sec24D (Farhan et al., 2007). There, the ^{733}DD^{734} motif binds to an arginine residue in the C-terminus of the GABA transporter 1. Later, Mancias and Goldberg (2008) combined X-ray crystallographic and biochemical analysis and thereby identified further binding motifs in all Sec24 isoforms. The presence of four Sec24 isoforms was always thought to provide a broader range for differential cargo recognition. Wendeler et al (2007) studied the dependence of ERGIC-53 ER export on Sec24 isoforms. They found that ERGIC-53 export is dependent on Sec24A and Sec24B. This interaction is mediated by the FF-motif on its extreme C-terminus. Interestingly, substituting the FF-motif by two valines changed ERGIC-53 export dependence to Sec24C and D.

The next step in the COPII assembly cascade is recruitment of the heterotetramer Sec13-Sec31. This forms the outer layer of the COPII coat. Recruitment of Sec13-Sec31 has been shown to stimulate the GAP-activity of Sec23p towards Sar1p by about 10fold (Antonny et al., 2001). Recently, it was proposed that the presence of the Sec13-Sec31 complex is essential for COPII vesicle fission. This was based on a finding from patients with Cranio-

lenticulo-sutural dysplasia (CLSD). This developmental disease is caused by a point-mutation in Sec23A (Boyadjief et al., 2006). This mutant Sec23A fails to recruit Sec31 and thus there is no increase in the GAP activity of Sec23 anymore (Bi et al., 2007; Fromme et al., 2007).

COPII is a cytosolic complex. Thus, it can only interact with ER export motifs in the cytosolic portion of transmembrane proteins. Several types of ER export motifs were reported in the literature. Hydrophobic motifs (Fiedler et al., 1996; Kappeler et al., 1997; Dominguez et al., 1998), di-acidic motifs, and also di-basic motifs have been described. In addition the first di-acidic ER export motif was found in the cytoplasmic tail of the vesicular stomatitis virus G (VSVG) protein tail (Nishimura and Balch, 1997). Substitution of the two acidic amino acids of this DXE motif by alanines reduced ER export of VSVG in a pulse chase experiment. Other proteins with di-acidic export motifs are: the potassium channel protein Kir2.1 (Ma et al., 2001) and the Golgi proteins Sys1p (Votsmeier and Gallwitz, 2001) and Gap1p (Malkus et al., 2002). A di-hydrophobic/ di-aromatic export motif consisting of two phenylalanines was found in the extreme C-terminus of ERGIC-53 (Kappeler et al., 1997). This motif can be substituted by a single phenylalanine or tyrosine in position -2, two leucines or isoleucines at position -1 and -2 or a single valine in position -1 (Nufer et al., 2002). Other di-hydrophobic motifs (FF, FY) were discovered in p24 family members (Fiedler et al., 1996) and the Erv41-Erv46 complex (Otte and Barlowe, 2002). A dibasic motif has been described for a Golgi resident glycosyltransferase. This motif is quite distinct from the previous described ER export motifs. It is located proximal to the transmembrane domain and it interacts with Sar1 and not with Sec24 (Giraudo and Maccioni, 2003).

In contrast to transmembrane proteins, soluble secretory proteins within the lumen of the ER have no direct access to the ER export machinery. Two models have been proposed to explain ER export of soluble proteins: the bulk flow model and the receptor mediated export model. Initially it seemed that all soluble proteins exited the ER simply by bulk flow. This was based on experiments were glycosylated tripeptides lacking an ER retention motif were rapidly secreted (Wieland et al., 1987). This model seems to hold true for the export of amylase and chymotrypsinogen, as no concentration into COPII coated buds could be observed. The concentration of these proteins seems to occur at a later step. Nevertheless it appears that at least for a part of secretory proteins an active sorting process is required for efficient ER export. Members of the p24 family enhanced transport of invertase and the GPI-anchored protein Gasp1 in yeast (Schimmoler et al., 1995; Muniz et al., 2000) Glycopro-α-factor, carboxypetidase Y and proteinase A need ERv29p for their efficient sorting into COPII vesicles (Belden and Barlowe, 2001; Caldwell et al., 2001). The lectin ERGIC-53 serves as a

cargo receptor for several glycoproteins including cathepsin Z, cathepsin C, blood coagulation factors and alpha1-antitrypsin (Appenzeller et al., 1999; Nyfeler et al., 2008).

Beside proteins that are destined for secretion, there are also proteins that recycle between ER and Golgi. At some point all these proteins have to be transported back to the ER. This step is mediated by COPI vesicles. COPI vesicles are also implicated in the retrieval of escaped ER resident proteins back to the ER and are involved in intra-Golgi trafficking.

COPI vesicle formation is initiated by the activation of the small GTPase Arf1 and the subsequent binding of coatomer/COPI. This is a heteromeric complex consisting of seven subunits (α, β, β',γ, δ, ε, ζ) . Dilysine motifs in the cytoplasmic carboxylic tail of cargo proteins directly interact with coatomer (Cosson and Letourneur, 1994). These motifs are only functional if they are either located in position -3 and -4 (KKXX-COOH) or in position -5 and -3 (KXKXX-COOH) (Jackson et al., 1990). These dilysine motifs ensure the ER localization of type I membrane proteins by two different mechanisms: ER retention or ER retrieval (Andersson et al., 1999). ER retention does not depend on a functional COPI coat. Another type of dibasic motif, a di-arginine motif located close to the N-terminus mediates ER retention of type II membrane proteins (Teasdale and Jackson, 1996). This signal is found in multimeric membrane proteins usually forming channels or receptors. There is evidence that the arginine motif also mediates interaction with COPI (Michelsen et al., 2007). Diarginine and dilysine motifs bind to different sites in coatomere subunits. In contrast soluble recycling proteins cannot directly interact with COPI components. They interact via their KDEL amino acid sequence with the KDEL-receptor (Munro and Pelham, 1987; Lewis and Pelham, 1992)

1.1.2 Traffic through the ER-Golgi-intermediate compartment (ERGIC)

The ERGIC is a complex and highly dynamic compartment found only in higher eukaryotic cells. As its name already describes, this compartment is located at the interface of the ER-Golgi boundary. This compartment corresponds to the place where viral membrane proteins were shown to accumulate upon a temperature block of 15°C (Saraste and Kuismanen, 1984; Balch et al., 1986; Saraste and Svensson, 1991; Schweizer et al., 1990). This compartment is also known as intermediate compartment or VTCs (vesicular-tubular clusters) (Balch et al., 1994).

The main marker of the ERGIC is a type-I transmembrane protein of 53 kDa named ERGIC-53 (Schweizer et al., 1988). The discovery of ERGIC-53 as a marker protein for the intermediate compartment allowed characterization of this compartment more precisely. Experiments with VSVG showed that it continues its trafficking route from the ERGIC to the

Golgi upon temperature increase from 15°C to 32°C. In contrast ERGIC-53 localized still to the ERGIC (Schweizer et al., 1990). The nature of the ERGIC was for very long a matter of hot and controversial debate. Very early, the ERGIC was considered a distal subcompartment of the ER. It was described as a salvage subcompartment, where the KDEL receptor binds escaped ER resident proteins to retrieve them to the ER (Sitia and Meldolesi, 1992). This view was supported by electron microscopy that showed connections of the VTCs with the ER (Griffiths et al., 1994; Krijnse-Locker et al., 1994; Stinchcombe et al., 1995). Another group suggested that the KDEL receptor binds to its targets at the level of the Golgi. Therefore they proposed that the intermediate compartment corresponds to an early compartment of the Golgi (Mellman and Simons, 1992).

Subcellular fractionation experiments (Schweizer et al., 1991) and further morphological studies at an ultrastructural level finally showed that ERGIC membranes are non-continuous with either the ER or the cis-Golgi and also differ in their protein composition from these two compartments (Klumperman et al., 1998).

Today, the ERGIC is accepted to be distinct from the ER and the Golgi. This agreement is, however, only on structural aspects of the ERGIC, but not on the functional ones. There are two hypotheses that explain the function of the ERGIC, the transient compartment model and the stable compartment model. According to the transient-compartment/maturation hypothesis, the ERGIC represents transport intermediates on their way to the Golgi. These transport intermediates are formed by the homotypic fusion of COPII vesicles. This is supported by in vitro experiments that showed that COPII vesicles can homotypically fuse to generate larger carriers (Xu and Hay, 2004) and that this process is dependent on the tethering complex TRAPP-I (Cai et al., 2007). ERGIC clusters move along microtubules to the Golgi (Bannykh and Balch, 1997; Presley et al., 1997). Subsequently the ERGIC clusters either fuse with each other to generate the new cis-Golgi cisterna or they fuse with a pre-existing cis-Golgi cisterna. This is supported by the observation that VSVG containing transport containers (TC) where shown to move towards the Golgi and fused with it (Presley et al., 1997). A closer examination of VSVG–containing TCs revealed that they exhibit a polarized distribution of COPI and cargo. VSVG was preferentially localized to the side of the TC that faced the Golgi and COPI localized to the opposite direction (Shima et al., 1999). For procollagen-I, ER to Golgi transport was shown to occur inside the tubular portion of the VTCs (Bonfanti et al., 1998). A subsequent study suggested that procollagen-I as well as VSVG travel in these tubular carriers emerging from the ER to the Golgi (Mironov et al., 2003). The main problem of the maturation model is that it is mainly based on the

examination of the overexpressed viral protein VSVG. VSVG is exported in a cargo wave from the ER, therefore the secretory pathway might accommodate to these needs and the physiological transport conditions may not be correctly reflected (Appenzeller-Herzog and Hauri, 2006).

The maturation hypothesis is opposed by the stable compartment hypothesis. According to this hypothesis, the ERGIC forms an independent stable compartment. Cargo is exported from the ER in COPII vesicles that subsequently fuse with pre-existing ERGIC clusters. Further transport from the ERGIC to the Golgi is mediated via carriers that still need to be characterized. This is supported by live cell imaging experiments of cells expressing GFP-ERGIC-53 and a luminal version of dsRed (ss-dsRed) (Ben-Tekaya et al., 2005). In this study cells were incubated at 15°C to block secretory traffic in the ERGIC. After re-warming, ss-dsRed segregated from GFP-ERGIC-53. While ss-ds-Red moved to the Golgi, GFP-ERGIC-53 remained in peripheral ERGIC structures. There is evidence that anterograde transport from the ERGIC is COPI-dependent. VSVG transport was blocked at the level of the ERGIC, after microinjection of an antibody against a COPI subunit (Pepperkok et al., 1993). As depicted already in the maturation model the VSVG containing carriers were coated with COPI (Scales et al., 1997; Stephens et al., 2000; Presley et al., 2002). In case of the stable compartment model these VSVG containing carriers would correspond to the anterograde carriers segregating from the ERGIC. COPI would be therefore involved in trafficking of the transport carriers in the anterograde direction and in the recycling of proteins back to the ER. How are these opposite directed processes regulated? Goldberg (2000) showed with in vitro experiments that different cargo proteins differentially affected the GTPase activity of the small GTPAse Arf1, depending on the type of COPI interaction motif. Cargo containing a di-arginine interaction motif inhibited GTPase activity, while cargo with a dilysine interaction motif showed no effect. He concluded that the dilysine containing proteins excluded themselves from the anterograde vesicles. It is still possible that COPI only plays a role in retrograde transport and that the observed effects are indirect and further studies are required to resolve this mystery. Finally the question comes up if the ERGIC only serves as a sorting station or if also enzymatic processes occur. So far only UDP-glucose:glycoprotein glucosyltransferase and glucosidase-II, two enzymes also resident in the ER, were found to be enriched in the ERGIC by immunoelectron-microscopy: (Zuber et al., 2001). If ERGIC specific enzymes exist has to be further investigated.

1.1.3 Golgi apparatus

The next compartment along the secretory pathway is the Golgi apparatus. The Golgi apparatus consists of stacks of cisternae. These cisternae are composed of flattened disk shaped membranes, which form a ribbon-like organelle in mammalian cells. The stacks form the so-called compact zones (Thorne-Tjomsland et al., 1998; Jackson et al., 2009). Tubulovesicular regions, also known as non-compact zones connect these Golgi stacks laterally with each other (Fig.2). The cisternae are organized in a polarized fashion. Each cisterna is defined by structural and biochemical criteria. The cis-Golgi cisterna is followed by the medial-Golgi and then by the trans-Golgi. The cis-Golgi is preceded by the cis-Golgi network and the trans-Golgi is followed by the trans-Golgi network. Both networks show tubulovesicular morphology.

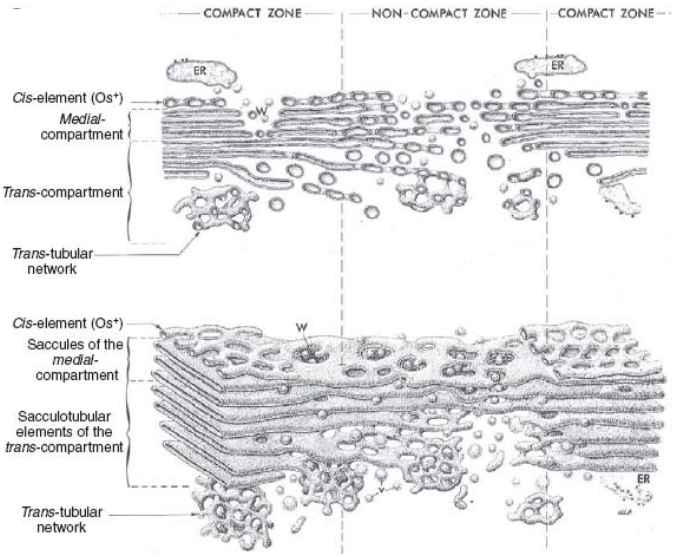


Figure 2: ***Golgi morphology*** *The Golgi apparatus is formed by compact zones and non-compact zones. The compact zones correspond to the Golgi stacks and consist of flattened cisternae. The Golgi stacks are connected via non compact zones composed of tubulovesicular regions. The cisternae are ordered in a polarized fashion with the cis-Golgi first, followed by the medial and the trans-Golgi. The cis-Golgi is preceded by the cis-Golgi network and the trans- Golgi is followed by the trans-Golgi network.(reproduced from Rambourg and Clermont, 1990)*

To maintain the Golgi structure the Golgi cisternae are linked via filamentous material (Franke et al., 1972), which is sensitive to protease treatment (Cluett and Brown, 1992). Extraction of the Golgi with a detergent revealed a proteinaceous skeleton (Slusarewicz et al., 1994) which functions as Golgi matrix. The Golgi matrix is formed by golgins and GRASPs (Short and Barr, 2003; Barinaga-Rementeria Ramirez and Lowe 2009). Golgins are Golgi localized proteins that harbour large coiled-coil domains. Golgins are either transmembrane proteins integrated into the Golgi membrane (giantin, golgin-84, CLASP) or they associate via adaptors with the Golgi (GM130, golgin 45). Golgins also interact with the small GTPases Rabs, ARLs or ARFs to mediate Golgi recruitment (Short et al., 2005). Knockdown or

overexpression of golgins often results in a loss of integrity of the Golgi. Microinjection of an antibody against p115 leads to fragmentation of the Golgi apparatus (Puthenveedu and Linstedt, 2001). Also the lack of GM130 in a temperature sensitive mutant cell line LdLG resulted in a fragmentation of the Golgi, but only if cells were incubated at higher temperature (Vasile et al., 2003). Moreover an siRNA induced knockdown of GM130 resulted in a fragmentation of the Golgi (Puthenveedu et al., 2006). Contradictory results were obtained from microinjection of an antibody against GM130 which showed no effect on Golgi integrity (Puthenveedu and Linstedt, 2001). Beside membrane tethering, golgins are also involved in tethering of arriving vesicles at the Golgi. The mechanism of vesicle docking to the Golgi was recently elucidated in detail for GMAP-210 (Drin et al., 2008). GMAP-210 is a golgin whose depletion causes fragmentation of the Golgi (Pernet-Gallay et al., 2002; Rios et al., 2004). The N-terminus of GMAP-210 contains an ALPS (amphipathic lipid-packing sensor) motif which senses highly curved membranes. Thereby, the N-terminus binds small vesicles. The C-terminal region of GMAP-210 binds to more flat membranes where Arf1 is present. This would correspond to the Golgi membrane. A model was suggested where GMAP-210 would be recruited to the Golgi by Arf1. Its N-terminus reaches into the cytoplasm like a tentacle waiting for a vesicle. Binding of a vesicle to the N-terminus of GMAP-210 induces a conformational change thereby bringing the vesicle closer to the Golgi. The Drosophila homologues of GMAP-210 and GM130 were shown to have several binding sites for different rabs. Based on these findings, a model of a tentacular Golgi was suggested where coiled-coil proteins that surround the Golgi capture Rab-containing membranes (like vesicles) but exclude other structures (Sinka et al., 2008).

Another type of Golgi matrix proteins are the GRASPs (Golgi reassembly and stacking proteins). So far two GRASPs have been discovered in mammalian cells GRASP55 and GRASP65 (Barr et al., 1997; Shorter et al., 1999). Both are peripheral membrane proteins and are anchored to the membrane via an N-terminal myristoyl group. Knockdown of GRASP65 (Puthenveedu et al., 2006) as well as the knockdown of GRASP55 (Feinstein and Linstedt, 2008) result in an unlinking of the Golgi ribbon. There are different hypothesis about the role of GRASPs in linking membranes. It was suggested that they anchor golgins to the Golgi membranes and enable them to function in tethering reactions (Barr et al., 1998). Another hypothesis suggests that GRASPs form oligomers and thereby link membranes together. GRASP65 was shown to form high-order oligomers. In addition GRASP65 which was coupled to magnetic beads, formed trans-oligomers and caused aggregation of the beads (Wang et al., 2003).

Beside GRASPs and golgins, other proteins are also involved in maintaining Golgi structure. Disruption of the microtubule network results in Golgi disassembly into several mini-stacks (Rogalski et al., 1984). The fungal product brefeldin A (BFA) causes disassembly of the Golgi and rapid redistribution of Golgi membranes to the ER (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989). Degradation of a COPI subunit in a mutant cell line led to a fragmented Golgi (Guo et al., 1994). A combined knockdown of two cargo receptors (ERGIC-53 and Surf4) also led to Golgi fragmentation (Mitrovic et al., 2008). Many other proteins from different classes are also important for integrity of the Golgi. These include cargo receptors from the p24 protein family, retromer components or the conserved oligomeric Golgi complex (Rojo et al., 2000; Seaman, 2004; Shestakova et al., 2006; Koegler et al., 2009), enzymes like protein kinase D (Anel and Malhotra, 2005) and proteins of the fusion machinery like the SNARE GS15 (Xu et al., 2002).

Proteins that pass the Golgi are transported from the *cis*-side to the *trans*-side of the Golgi and undergo enzymatic modifications. These include N-glycosylation steps, O-glycosylation (Helenius and Aebi, 2001; Wopereis et al., 2006) and sulfation of sugar- and tyrosine residues (Honke and Tanigichi, 2002). In addition the pro-protein convertases/endoproteases furin acts in the *trans*-Golgi, where it process protein precursors into their mature form (Nakayama, 1997; Shapiro et al., 1997). Glycosylation steps occur in a sequential order. In the *cis*-cisternae mannose trimming occurs. N-acetylglucosamine is added in the medial Golgi. Addition of fucose residues also occurs in the medial Golgi. Finally, addition of galactose and sialic acid takes place in the *trans*-Golgi and the TGN (Kornfeld and Kornfeld, 1985). This model of sequential action was supported by microscopic studies that showed compartmentalization of the enzymes to the different cisternae. Galactosyltranferase was located to the trans Golgi cisternae (Roth and Berger, 1982) and N-acetylglucosamine transferase I to the medial Golgi cisterna (Dunphy et al., 1985) by electron microscopy. However, this strict compartmentalization model does not seem to be entirely true. More recent results favor a model of a concentration gradient along the cisternae. Myc-tagged N-acetylglucosaminyltransferase I (GnT I) localized to the medial as well as to the *trans*-Golgi. In the trans Golgi it overlapped with endogenous beta 1,4 galactosyltransferase (GalT) (Nilsson et al., 1993). Also mannosidase II localized to the medial Golgi cisterna as well as to the *trans*-Golgi cisterna. In the trans Golgi cisterna overlapping staining of mannosidase II with the trans Golgi enzyme sialyltransferase was observed (Rabouille et al., 1995). Localization of the Golgi enzymes was also shown to vary with the cell type. Depending on the cell line mannosidase II was found in the medial, medial and trans or only in the trans-

Golgi (Velasco et al., 1993). Nilsson et al (2009) hypothesized that the different Golgi enzymes present in the same compartment form complexes. These complexes would then provide specific and efficient processing of the oligosaccharide structures. So far this is only a hypothesis and further studies are necessary to confirm it.

Intra-Golgi protein transport is still a matter of debate. Two different models for the organization and transport of cargo through the Golgi have been proposed (Fig. 3). These models are known as the cisternal maturation model and the vesicular transport model (Rothman and Wieland, 1996; Glick et al., 1997). The cisternal maturation model proposes that cargo enters the first cisterna (*cis*-cisterna) and this cisterna matures to become the medial cisterna and finally the trans-cisterna. The trans-cisterna finally disintegrates into post-Golgi vesicles that mediate further transport. Support for the cisternal maturation model came from a study on procollagen transport. Procollagen-I, is a very large protein, which was found to stay in the same cisterna during intra-Golgi transport (Bonfanti et al., 1998). The transmembrane protein VSVG was shown to move through the Golgi at the same rate as procollagen-I (Mironov et al., 2001). This study indicates that large as well as small cargo molecules are transported via cisternal maturation. The cisternal maturation model raises the question of how the *cis*-to-*trans* Golgi enzyme gradient is maintained. A possibility is that the gradient is generated by COPI vesicles that transport Golgi resident proteins to their home cisternae. This is supported by the observation that COPI vesicles contain several Golgi resident proteins but no or very little secretory cargo (Love et al., 1998). In contrast to this observation Orci et al. (1997) found COPI vesicles involved in anterograde transport of VSVG and Volchuk et al. (2000) discovered megavesicles involved in anterograde intra-Golgi transport of large protein aggregates. Another explanation is that inter-cisternal tubular connections are formed. Cargo matures in these tubules but enzymes stay in their cisterna. Alternatively, cargo matures within cisternae and enzymes slide back in tubules. The existence of tubules was observed by Trucco et al (2004) when a wave of VSVG arrived at the Golgi. In this model COPI vesicles would only serve in recycling of the fusion machinery, but do contain neither secretory cargo nor resident Golgi enzymes.

The vesicular transport model (Fig. 3) is based on the assumption of stable Golgi cisternae. Anterograde and retrograde transport between the cisternae is mediated by COPI vesicles. Anterograde COPI vesicles transport the secretory proteins from cisterna to cisterna where the posttranslational modifications occur. These anterograde COPI vesicles exclude resident Golgi enzymes from further transport. This model is supported by a number of biochemical and microscopical evidences. COPI vesicles containing anterograde cargo could be produced

in an in vitro budding reaction, using Golgi membranes (Malsam et al., 1999). COPI vesicles containing anterograde (VSVG) and retrograde (KDEL-receptor) cargo were also visualized by electron microscopy (Orci et al., 1997). These KDEL receptor-containing vesicles excluded the Golgi enzyme mannosidase-II from transport (Cosson et al., 2002). Another electron microscopy study suggested that anterograde COPI vesicles differ from retrograde ones in their SNARE composition. The Golgi restricted v-SNARE GOS28 could be localized to anterograde VSVG containing COPI vesicles, but not to retrograde KDEL receptor containing vesicles (Orci et al., 2000). The remaining question is how large cargo that does not fit into COPI vesicles is transported. As a kind of a salomonic solution Pelham and Rothman (2000) suggested, that both models have their justification. According to their suggestion vesicular transport mediates most of the transport of proteins through the Golgi in mammalian cells. Cisternal maturation serves to explain rapid protein secretion in yeast and transport of large macromolecular aggregates in mammalian cells.

Recently a third model called rapid-partitioning model was proposed (Fig. 3). In this model the Golgi stacks are interconnected and secretory cargo as well as resident Golgi enzymes move in both directions through the Golgi. Although the Golgi forms a continuous system, it is segregated into differential membrane domains. A two-phase membrane system allows differential partitioning of transmembrane cargo proteins and resident Golgi enzymes in subdomains. Export domains are enriched in sphingolipids/cholesterol and represent the place of cargo sorting. Processing domains are glycerolipid rich microdomains and form the processing platforms in the Golgi where the glycosylation enzymes act (Patterson et al., 2008). Recently a group of prominent Golgi researchers made the attempt to find a consensus on a model for intra-Golgi transport (Emr et al., 2009). They agreed on the cisternal maturation model as the most likely one and on the existence of tubules connecting the Golgi cisternae in a stack. Still there is no consensus about the contents and the transport directionality of COPI vesicles, but future work will help to clarify this issue.

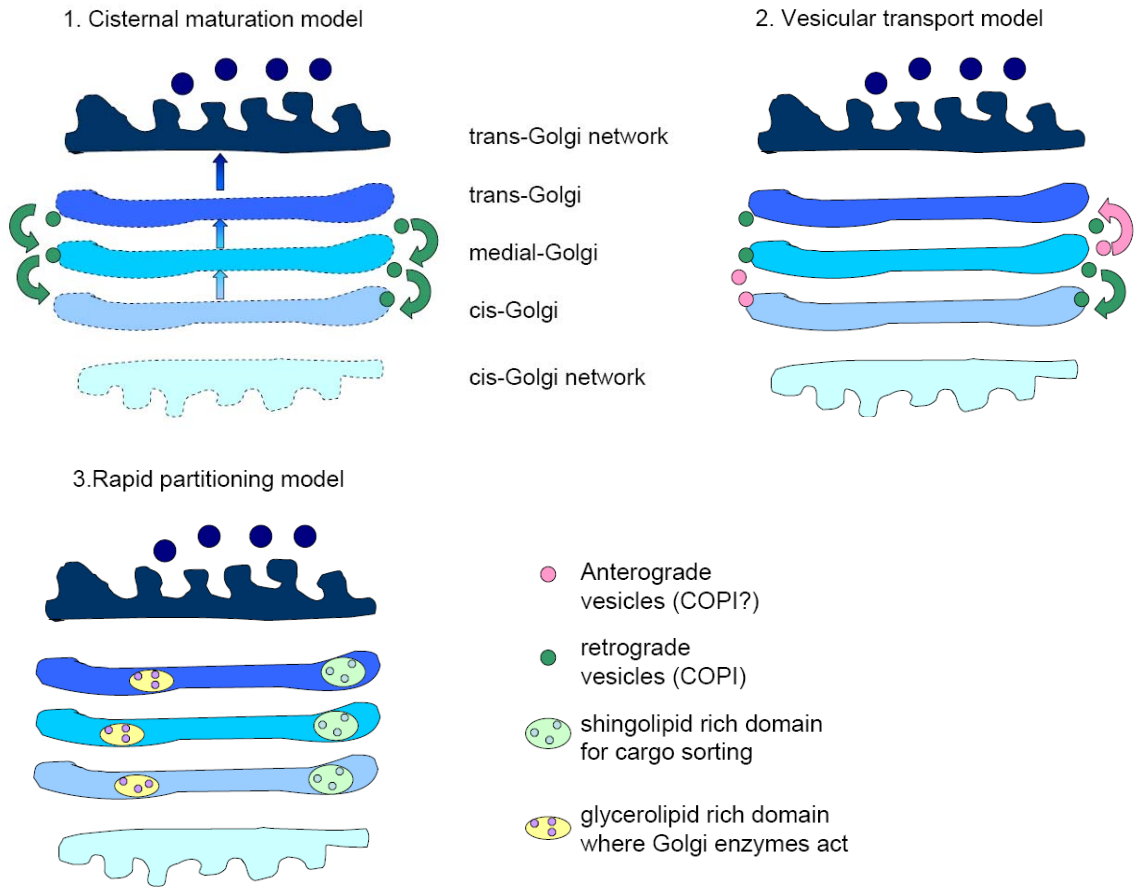


Figure 3: ***Intra-Golgi transport models:***

1.In the ***cisternal maturation model*** *anterograde cargo travels within the same cisterna through the Golgi. The cisterna receives the Golgi enzymes specific for the following cisterna via COPI vesicles (green).*

2.In the ***vesicular transport model*** *cargo transport is mediated by anterograde COPI vesicles (pink) that move between the cisternae. Retrograde COPI vesicles (green) recycle transport components of the fusion machinery back to their home cisterna.*

3 In the ***rapid partitioning model*** *segregation between anterograde cargo and resident Golgi proteins is mediated via lipid microdomains (light-green oval and yellow oval)*

1.2 N-glycosylation and oligosaccharide processing

N-glycosylation is one of the most common protein modifications (Apweiler et al., 1999). It plays an important role in glycoprotein folding, ER quality control (Spiro, 2004), protein secretion (Helenius and Aebi, 2004; Vagin et al., 2009), cell-cell communication (Zhao et al., 2008), development (Haltiwanger and Lowe, 2004), wound repair (Lackie and Adam, 2006) and innate immunity (Marth and Grewal, 2008). In addition N-glycosylation was shown to be important for enzyme activity and substrate specificity (Skropeta, 2009). N-glycosylation is crucial for the stability of a glycoprotein (Shental-Bechor and Levy, 2009) and also protects it from the action of proteases by steric hindrance and from non-specific interactions with other proteins (Rudd et al., 2001). Three main types of N-glycans can be distinguished: high

mannose, complex and hybrid-N-glycans. All types share a common pentasaccharide core structure of three mannose residues (Man) and two N-acetylglucosamine residues (GlcNAc): *Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc* (α and β indicate type of glycosidic linkage between the sugar residues). This $Man_3GlcNAc_2$ core is linked to an asparagine residue in the glycoprotein chain. The high mannose structure consists of the pentasaccharide core structure with two to six additional mannose residues attached. The complex-type structure is formed by the pentasaccharide core, to which oligosaccharide antennae are attached by the action of N-acetylglucosaminyltransferases (GnTs). The hybrid structure contains features of both the high mannose and the complex glycosylated structure. The hybrid structure is formed by mannose residues attached to the Manα1–6 arm and one or two antennae, which are attached to the Manα1–3 arm of the $Man_3GlcNAc_2$ core (Stanley et al., 2008)

1.2.1 N-glycosylation in the ER

The initial step in N-glycosylation takes place in the endoplasmic reticulum and is mediated by the oligosaccharide transferase (Roth, 2002). This integral membrane protein complex consists of eight subunits. It transfers a $Glc_3Man_9GlcNAc_2$ residue (where Glc is glucose) from the lipid carrier dolichol-P-P to an asparagine residue in the newly synthesized protein (Fig. 4) (Elbein, 1979). The asparagine is part of the consensus sequence N-X-S/T., where X is any amino acid except proline.

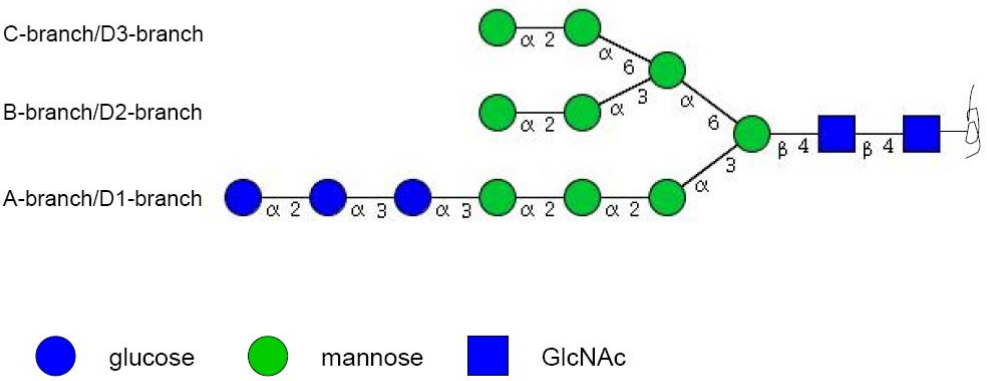


Figure 4 ***Basic N-glycan structure*** *attached to the N-glycosylation site of a glycoprotein. The basic structure consists of two N-acetylglucosamine residues, nine mannose residues and three glucose residues. The nomenclature of the different branches and the type of glycosidic linkage is indicated. The structure was created with the glycan builder (Ceroni et al., 2007)*

The attached oligosaccharide is then further processed by two ER localized α-glucosidases (Fig. 5). The integral type-II membrane protein α-glucosidase-I removes the terminal α1,2

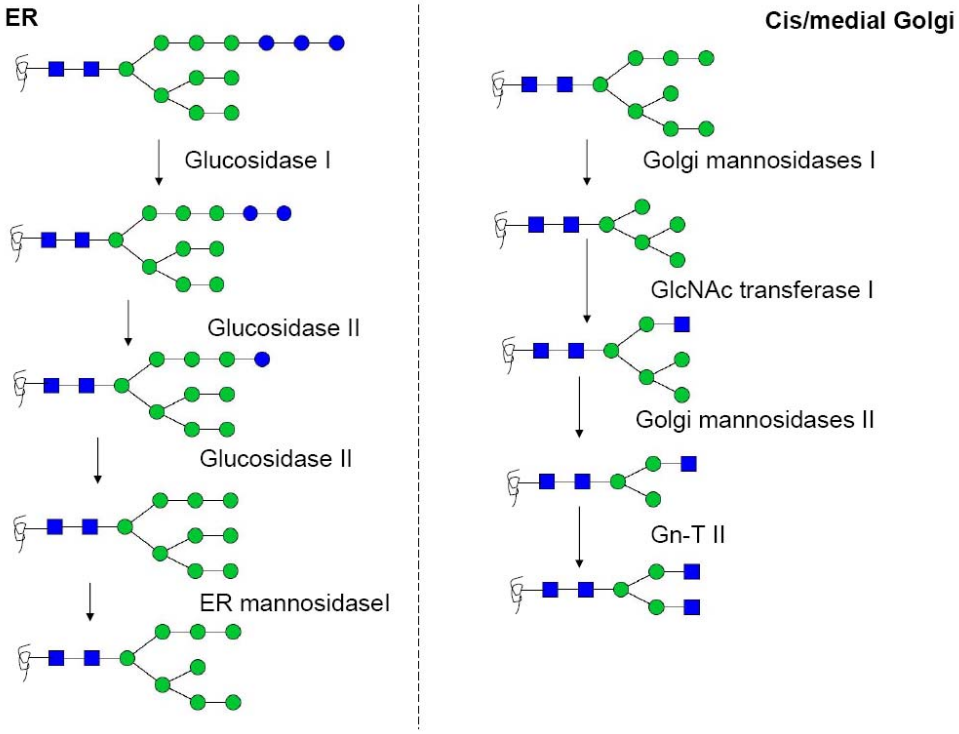
linked glucose (Shailubhai et al., 1991). Subsequently the soluble glucosidase-II removes the two α1,3 linked glucose residues. Glucosidase-II is composed of an α-subunit and β-subunit. The α-subunit contributes the active site and the β-site mediates ER retention (Trombetta et al., 1996; Helenius et al., 1997). Additionally the β-subunit shows sequence homology to the mannose-6-phosphate receptor (MRH domain). The residues involved in mannose binding are conserved in the β-subunit. The two glucose residues are cleaved with different kinetics. The first residue is cleaved very rapidly, but the second residue is cleaved more slowly. This allows the monoglucosylated glycoprotein to enter the calnexin/calreticulin cycle, before the second glucose residue is cleaved. Release from this cycle is catalyzed by a concerted action of ER mannosidase-I and glucosidase-II. ER mannosidase-I (Gonzalez et al., 1999) removes one mannose residue from the B-branch and the glucosidase-II removes the remaining glucose residue from the A-branch. Correctly folded proteins which exit the ER carry a $Man_8(GlcNAc)_2$ glycan chain. Further processing of the glycan chain occurs in the Golgi

1.2.2 N-glycosylation in the Golgi

A combination of trimming (by glycosidases) and elongation (by glycosyltransferases) reactions converts the high mannose to a complex type oligosaccharide (Fig. 5). So far three Golgi α1,2-mannosidase-I isoforms have been discovered: Golgi α1,2-mannosidase-I A, B and C (Herscovics et al., 1994; Lal et al., 1994; Tremblay and Herscovics, 2000). They process the $Man_8GlcNAc_2$ glycan to the $Man_5GlcNAc_2$ which constitutes the basis for complex glycosylation. GlcNAc transferase-I (GnT-I) adds a single GlcNAc residue to the $Man_5GlcNAc_2$ thereby generating $GlcNAc_1Man_5GlcNAc_2$ (Schachter et al., 1984). This is the prerequisite for further formation of N-acetyllactosamine branches. After modification by GnT-I, Golgi mannosidase-II removes two further mannose residues (Tulsiani et al., 1982; Moremen, 2002). The resulting $GlcNAc_1Man_3GlcNAc_2$ chain is further modified by the addition of another N-acetylglucosamine residue to the free mannose residue by GlcNAc transferase-II (GnT-II) (Oppenheimer et al., 1981; Brockhausen et al., 1988). The $GlcNAc_2Man_3GlcNAc_2$ chain is the precursor for complex oligosaccharides with three or four outer branches. At this stage several subsequent reactions are possible. Fucosyltransferase can transfer a fucose residue to the innermost GlcNAc residue of the glycan chain (Uozumi et al., 1996). GnT-IV adds a GlcNAc residue to the α1,3 mannose. GnT-V adds a GlcNAc residue to the α1,6 mannose (Kornfeld and Kornfeld, 1985). GnT-VI adds an additional GlcNAc residue to the α1,6 mannose and GnT-IX adds an additional GlcNAc residue to the α1,3 mannose (Takahashi et al., 2009). GnT-III introduces a GlcNAc residue at the base of the

trimannosyl core. This step prevents further branching, as the other GlcNAc transferases cannot process this oligosaccharide chain anymore (Narasimhan, 1982).

The final modifications of the glycan chains involve addition of galactose (Gal) residues by galactosyltransferases and sialylation by sialyltransferases. Two main families of galactosyltransferases can be distinguished. The β4-galactosyltransferases catalyze the transfer of a galactose residue via β1-4 linkage to a GlcNAc residue. The β3-galactosyltransferases catalyze the transfer of a galactose residue via β1-3 linkage to a GlcNAc based acceptor (Hennet, 2002). Chain elongation occurs by the alternate action of the GnTs adding a GlcNAc residue to a Gal at the end of the chain and the GalTs adding a Gal to the terminal GlcNAc of the chain (Ujita et al., 1999). Sialylation follows upon galactosylation and is usually the terminal modification of a glycan chain (Berger and Rohrer, 2008). Sialyltransferases mediate transfer of sialic acid/neuraminic acid (Neu) to galactose residues.



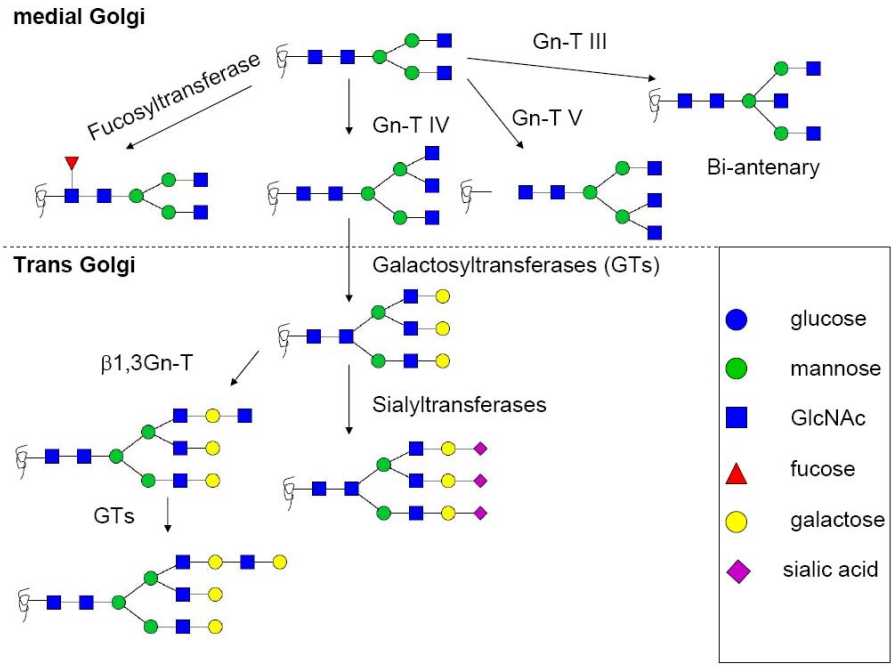


Figure 5 ***Processing and maturation of N-glycans*** *Oligosacharyltransferase transfers the $Glc_3Man_9GlcNAc_2$ residue to the glycoprotein (not indicated). The processing steps in the ER include removal of the three glucose residues by glucosidase-I and glucosidase-II and removal of one mannose residue by ER mannosidase-I. Further processing in the Golgi includes further removal of two mannose residues by mannosidase-I. After addition of GlcNAc by GnT-II, Golgi mannosidase-II removes two mannose residues. Fucosyltransferase may add a fucose residue to the innermost GlcNAc. Addition of GlcNAc to the terminal mannose residues by GnTs allows branching. Galactosyltransferases and sialyltransferases perform the final modification by adding galactose and sialic acid residues. Poly-N-acetyllactosamine (= Gal-GlcNAc-Gal-GlcNAc) structures are created by the sequential action of β1,3 Gn-T and galactosyltransferases. All structure were created using glycan builder (Ceroni et al., 2007)*

1.3 Leguminous type lectins (L-type lectins)

Lectins are sugar binding proteins, lacking any enzymatic activity. They exert different intra- and extracellular roles. Lectins are involved in quality control, protein sorting and cellular adhesion. The domain responsible for sugar binding is referred to as the carbohydrate recognition domain (CRD). This domain can be formed by different secondary structure elements. In P-type lectins it is a unique β-sheet-rich structure, in C-type lectins it is formed by a unique mix of α–helices and β-sheets, in galectins and in L-type lectins it is a β-sandwich. The immunoglobulin family is the basis for the structure in I-type lectins and a β-trefoil in R-type lectins (Dodd and Drickamer, 2001). The first L-type lectin discovered in vertebrates was ERGIC-53. Like in the other L-type lectins, its luminal portion containing the CRD corresponds to the single folded domain in leguminous plant lectins (Sharon and Lis, 1990). Because of this homology they were termed leguminous (L-type) lectins. The L-type lectin group in mammals comprises the four members ERGIC-53, VIP36, VIPL and ERGL (Fig. 6).

1.3.1 ERGIC-53 (ER-Golgi-intermediate compartment protein of 53kDa)

ERGIC-53 is a 53kDa, type I transmembrane protein. It was originally discovered in a monoclonal antibody screen for organelle marker proteins (Schweizer et al., 1988). The rat homolog p58 was identified as a marker for the cis-Golgi (Saraste et al., 1987). MR60 was discovered by Pimpaneau et al (1991) as a new mammalian monocytic D-mannose receptor. Later it turned out that the protein MR60 is nearly identical to ERGIC-53 (Arar et al., 1995). ERGIC-53 serves as a marker of the ER-Golgi intermediate compartment (ERGIC) where it is present at high concentrations. ERGIC-53 is also found at lower levels in the ER and in the first fenestrated cisterna in the cis-Golgi (Schweizer et al., 1988; Chavrier et al., 1990). ERGIC-53 is an unglycosylated protein and forms homodimers and homohexamers directly after synthesis. Oligomerization is mediated by two luminal cysteine residues Cys466 and Cys475 that form disulfide bridges (Appenzeller et al., 1999; Lahtinen et al., 1999). Mutation of one of the cysteines abolished hexamere formation. If both cysteines are mutated, ERGIC-53 is only present in its monomeric form (Nufer et al., 2003). Moreover, efficient transport of ERGIC-53 was impaired, when Cys 475 was mutated. Surprisingly, when the two corresponding cysteines (Cys473, Cys482) were mutated in the rat homologue of ERGIC-53, p58, the subcellular distribution visualized by immunofluorescence was the same as observed for the wildtype, suggesting that its trafficking was unimpaired (Lahtinen et al., 1999). Later the same group published that an ERGIC-53 mutant where both cysteines had been substituted by alanines, still cofractionated with endogenous ERGIC-53 in a sucrose gradient (Neve et al., 2005). They proposed that ERGIC-53 exists only in hexamers and not in dimers. These hexamers are either formed by covalent disulfide-linked or non-covalently linked disulfide dimers (Neve et al., 2005). The different observations might be explained by the different experimental settings. The immunofluorescence data and the subcellular fractionation experiments provide information about the steady state. The pulse chase-experiment focuses more on the kinetic behaviour of the ERGIC-53 mutants.

Efficient transport of ERGIC-53 also depends on an ER export motif localized in the cytoplasmic carboxyl-terminus, although additional determinants are required (Nufer et al., 2003). The diphenylalanine (FF) COPII interaction motif in position -1 and -2 mediates the anterograde transport (Kappeler et al., 1997). In addition disulfide bond-stabilized oligomerization, residues in the transmembrane domain and a glutamine residue in the cytoplasmic domain all support efficient ER export (Nufer et al., 2003). As ERGIC-53 cycles between the ER and the ERGIC, a motif mediating retrograde transport has also to be present. The dilysine COPI interaction motif in position -3 and -4 fulfils this requirements. A peptide

corresponding to the cytoplasmic tail of ERGIC-53 was shown to bind to COPI components (Kappeler et al., 1997). Similar results were obtained with the carboxy-terminus of ERGIC-53 coupled to GST. It was shown to bind to COPI and binding could be specifically inhibited by an antibody directed against the C-terminus (Tisdale et al., 1997).

ERGIC-53 binds to high mannose glycans in a Ca^{2+} dependent manner (Itin et al., 1996). The conserved Asp121 and Asn156 residues are involved in sugar ligand binding. Mutation of either of these residues abolished binding of ERGIC-53 to a mannose column (Itin et al., 1996). Binding of ERGIC-53 to mannose is pH sensitive. The binding affinity of ERGIC-53 for glycoproteins decreases below a pH of 7 (Appenzeller-Herzog et al., 2004). ERGIC-53 was shown to be involved in the transport of several glycoproteins. A Cathepsin Z related protein (CatZr) was the first glycoprotein shown to interact directly with ERGIC-53 (Appenzeller et al., 1999). As ERGIC-53 is a lectin it is likely that the interaction with its ligands is carbohydrate dependent. Accordingly, a carbohydrate binding-deficient ERGIC-53 mutant cannot interact with CatZr anymore (Appenzeller et al., 1999). Further investigation revealed that CatZr is identical to procathepsin Z. In addition to the N-glycan, a β-hairpin structure in procathepsin Z was also important for this interaction. This indicates that the interaction is not solely dependent on glycans (Appenzeller-Herzog et al., 2005). ERGIC-53 is also involved in the transport of cathepsin C (Vollenweider et al., 1998). Furthermore ERGIC-53 interacts with the chaperone ERp44 and together they are involved in the quality control of IgM polymerization and therefore their ER export (Anelli et al., 2007). Nyfeler et al (2008) added recently another important protein to the list of cargos. In a YFP-based protein fragment complementation based screen of a human liver library, alpha1-antitrypsin was identified as a cargo for ERGIC-53. ER to Golgi transport of α1-AT was shown to be impaired upon ERGIC-53 knockdown. Mutations in the ERGIC-53 gene locus are the cause of combined deficiency of blood coagulation factors V and VIII, leading to a rare bleeding disorder. Affected individuals exhibit a reduction of plasma level of factor V and factor VIII by about 70-95% (Nichols et al., 1998). Further work on this subject revealed that transport of blood coagulation factors is not mediated by ERGIC-53 alone. ERGIC-53 associates with the soluble glycoprotein MCFD2 (multiple coagulation factor deficiency 2) in a Ca^{2+}-dependent manner (Zhang et al., 2003). The sequence of events in ERGIC-53/MCFD2/FactorVIII complex formation is not entirely solved. The current model suggests that factor VIII binds to MCFD2 in a carbohydrate-independent manner and that this complex is further stabilized by ERGIC-53 which interacts with MCFD2 as well as with carbohydrate side chains of factor VIII (Zhang et al., 2005). MCFD2 is not involved in binding to the other glycoproteins like

cathepsin C or cathepsin Z to ERGIC-53 (Nyfeler et al., 2006). All these examples show that ERGIC-53 serves as a transport receptor for soluble glycoproteins. Therefore it is surprising that ERGIC-53 was also found to interact with the membrane protein FGF receptor 3 (Lievens et al., 2008). Again this interaction was shown to be carbohydrate dependent.

Overall the role of ERGIC-53 is to facilitate export of different glycoproteins from the ER. Upon change in the concentration of Ca^{2+} and the pH in the ERGIC, the affinity for glycoproteins drop and cargo is released. After ERGIC-53 has released its cargo it can recycle back to the ER for another round of transport.

1.3.2 VIP36 (vesicular integral membrane protein of 36kDa)

VIP36 was originally discovered in an attempt to identify proteins involved in apical and basolateral sorting at the *trans*-Golgi (Fiedler et al., 1994). VIP36 seems to have appeared quite late in evolution as orthologs are only present in higher organisms. VIP36 is expressed in many organs like liver, kidney, intestine or spleen. Only low expression levels of VIP36 have been detected in the brain and in the heart on mRNA levels (Fiedler and Simons, 1996). VIP36 has a 44 aa signal sequence, a lectin like domain, a transmembrane domain and a cytoplasmic tail. In contrast to ERGIC-53, VIP36 seems only to exist as a monomer. The luminal domain of VIP36 is not able to form disulphide linked oligomeres or stable-non-covalent oligomeres (Fiedler and Simons, 1996). VIP36 has an N-glycosylation site and becomes complex glycosylated in the Golgi apparatus (Fiedler and Simons, 1996; Fullekrug et al., 1999). Two hours after synthesis most of endogenous VIP36 is found to be complex glycosylated (Fullekrug et al., 1999). VIP36 is a relatively short-lived protein with a half-life of only 5 hours (Neve et al., 2003).

While there is general agreement that VIP36 is a sugar binding L-type lectin, there is no final consensus on its intracellular localization. Overexpressed myc-tagged VIP36 was found to localize to the Golgi apparatus, vesicular structures, endosomes and the plasma membrane (Fiedler et al., 1994). A follow-up study by the same group revealed that endogenous VIP36 localizes mainly to the cis/medial Golgi and pre-Golgi structures (Fullekrug et al., 1999). Depending on the cell type, VIP36 was found in pre-or post Golgi compartments. In Vero cells, VIP36 was detected in the intermediate compartment (Fullekrug et al., 1999). In GH3 cells (a rat pituitary cell line), VIP36 was localized by electron microscopy also to 70-100nm pre-Golgi transport vesicles (Shimada et al., 2003a). In rat parotid acinar cells, VIP36 was detected in post-Golgi secretory vesicles (Shimada et al., 2003b). In MDCK cells VIP36 was even found at the plasma membrane (Hara-Kuge et al., 2002).

Which motifs/signals regulate the trafficking of VIP36 was never investigated. Therefore, we can only speculate on possible functional motifs. The carboxyl-terminus of VIP36 contains a potential retrieval motif. It is formed by a lysine in position -4 and an arginine in position -3 (KRXX). This motif resembles a dilysine (KKXX), COPI-binding, retrieval motif. Therefore, it is likely that VIP36 cycles within the secretory pathway. This is supported by the observation that endogenous VIP36 localized to the ERGIC after BFA treatment (Füllekrug et al., 1999). Dahm et al (2001) used fluorescence recovery after photobleaching (FRAP) experiments to study VIP36 trafficking. They used a C-terminally YFP-tagged version of VIP36. According to their data, VIP36 is transported from the ER to the Golgi with a halftime of 105±39 minutes. The retrograde transport of VIP36 is much faster. The half-time for Golgi-to-ER transport was only 1.67 ± 0.45minutes. This extremely fast retrograde transport suggests that the KRXX-motif of VIP36 is very potent. However, it has to be mentioned here that the authors used a C-terminally tagged VIP36. This should actually have inactivated this position-dependent motif. Why this was not the case, remains elusive. Thus, these results should be taken cautiously.

As a lectin, VIP36 is supposed to bind glycoproteins. Several attempts were made to gain further insight in its affinity for glycans and specificity for different sugar groups. Originally the luminal domain of VIP36 bound to glycopeptides isolated from [^{3}H]galactose-labeled cells. These glycopeptides could be eluted with GalNac. Glycopeptides isolated from [^{3}H] mannose-labeled cells did not bind to the column. The authors concluded that VIP36 binds to N-acetyl-D-galactosamine found in O-linked glycans (Fiedler and Simons, 1996). These results were challenged by later studies. Yamashita's group performed binding experiments of post-nuclear supernatant proteins to the purified VIP36-CRD (Hara-Kuge et al., 1999). This binding could be competed by the addition of $Man_{7\text{-}9}GlcNAc_2$ high mannose type glycopeptides. The discrepancy in the results was explained by the fact that in the study of Fiedler and Simons (1996) [^{3}H]mannose-labeled glycopeptides were created by the digestion with pronase. Pronase can hydrolyze high mannose type glycans and could have led to reduced binding to VIP36 (Hara-Kuge et al., 1999). Frontal affinity chromatography (FAC) experiments confirmed the findings of Yamashita's group (Kamiya et al., 2005). FAC data showed a preference of VIP36 for high mannose type oligosaccharides containing eight, nine and seven mannose residues. VIP36 preferred isomers containing the Manα1-2- Manα1-2Man branch (D1) (Kamiya et al., 2005). According to these data, VIP36 would have the highest affinity for glycoproteins before the D1 arm is trimmed by the Golgi mannosidase-I in the cis-Golgi. Thus, VIP36 is assumed to bind glycoproteins before they are processed in the

Golgi. VIP36 binds these glycoproteins in a pH-dependent manner. As revealed by FAC data VIP36-CRD shows an optimal sugar binding at a pH value of 6.5. This mildly acidic pH is usually found in the early Golgi (Kim et al., 1996; Wu et al., 2001). The pH of the ER is typically around 7.4 (Wu et al., 2001) and the pH of the ERGIC (although never formally shown) is expected to be between that of the ER and the Golgi. Based on these data, VIP36 should bind glycoproteins in the ERGIC or the cis-Golgi.

The residues Asp-131, Asn-166, His-190 in the CRD of VIP36 seem to play a major role in sugar binding. Mutation of Asp-131 diminished binding of VIP36-CRD to proteins of a postnuclear supernatant (Hara-Kuge et al., 1999). The involvement of Asn-166 in sugar binding is concluded from the fact that this residue is conserved in ERGIC-53. His-190 is also a conserved residue, mutation of which abolished sugar binding of the purified VIP36 CRD to its sugar ligands (Kamiya et al., 2005). All these experimental findings were confirmed by co-crystallization of the luminal part of VIP36 together with its carbohydrate ligand. All three residues (Asp131, Asn-166 and His190) were found to form the binding site and interact directly with carbohydrate ligands (Satoh et al., 2007). In addition also Gly260, Asp261 and Leu262 bind via hydrogen bonds to the mannose of the carbohydrate ligand (Satoh et al., 2007). The binding of leguminous lectins to their carbohydrate ligands is in general Ca^{2+} and Mn^{2+}dependent. Mn^{2+} allows binding of Ca^{2+} (Sharon and Lis, 1990). There are two conserved residues in plant L-type lectins, an aspartate and a glutamate residue that are required for Mn^{2+} binding. These two residues are not conserved in mammalian L-type lectins. This suggests that mammalian L-type lectins do not need Mn^{2+} for sugar binding. In fact sugar binding of VIP36 seems to be only Ca^{2+}dependent (Fiedler and Simons, 1996) although there are also controversial data on this fact. Hara-Kuge et al (1999) found that the purified CRD of VIP36 bound glycoproteins independently of Ca^{2+} and Mg^{2+}. In contrast crystallization data supported the Ca2+ dependency. Crystals of VIP36 could only be obtained in a Ca2+ bound form. Additionally, calcium binding showed to fix the position of three residues involved in sugar binding, indicating that calcium is required for sugar binding (Satoh et al., 2007).

Several attempts have been made to identify cargos for VIP36. Potential interaction partners are α-amylase, clusterin and BiP. Hara Kuge et al (2002) chose to investigate VIP36's interaction with clusterin, as clusterin is a main secretory glycoprotein in MDCK cells. VIP36 co-immunoprecipitated with clusterin and seems to be involved in its transport. Although it remains to be elucidated at which stage of transport VIP36 is actually involved. α-amylase was detected together with VIP36 in secretory vesicles of the rat parotid gland. VIP36 bound

to high mannose containing amylase in these secretory vesicles (Hara-Kuge et al., 2004). The antibody used in this study to detect VIP36 might also detect VIPL, therefore it is not entirely clear that it is really VIP36, that α-amylase interacts with. Another important point is that VIP36 is for sure not involved in the late steps of secretion of human amylase. Human amylase is secreted in a complex glycosylated and a non-glycosylated variant. Both are not able to interact with VIP36. Another protein that was found to interact with VIP36 was BiP (Nawa et al., 2007). Complex glycosylated VIP36 interacts with BiP in a carbohydrate independent manner. The authors proposed that VIP36 binds to misfolded glycoproteins at the level of the Golgi and cycles them back to the ER. There it delivers the misfolded glycoproteins to the ER chaperone BiP. All these results show that the function of VIP36 is still not clarified.

1.3.3 VIP36-like protein (VIPL)

VIPL was identified by a profile based data base scanning for L-type lectins (Nufer et al., 2003). In the same year the group of Petterson found VIPL during a database search using the conserved carbohydrate recognition domain as a search string (Neve et al., 2003). VIPL exhibits 68% similarity to VIP36 (Fig. 6). There are orthologs of VIPL in mouse, fly, worm and S. pombe, but not in S.cervisiae. VIPL is expressed in several organs. The highest level was found in skeletal muscles and in the kidney. Like VIP36, VIPL is a type I transmembrane protein and has also an N-glycosylation site. But in contrast to VIP36, VIPL does not become complex glycosylated (Neve et al., 2003; Nufer et al., 2003)

Until now it was not possible to define the subcellular localization of endogenous VIPL due to its low expression level. Overexpressed VIPL was found to localize to the ER by immunofluorescence and subcellular fractionation (Nufer et al., 2003). Neve et al (2003) detected overexpressed VIPL partly at the level of the Golgi. VIPL carries the same potential export motif (FY at position -1 and -2) as VIP36 in its cytoplasmic carboxyl-terminus. In contrast to VIP36 (KR at position -4 and -3), VIPL has an efficient ER retention motif in its C-terminus (RKR at position -5 to –3). This explains why upon BFA treatment, VIPL showed no alterations in its subcellular distribution and continued to show a pure ER pattern. This might indicate that VIPL does not cycle between the ER and the Golgi. Interestingly, substitution of the whole RKR retention motif by serines localized VIPL to the plasma-membrane (Nufer et al., 2003). Like its homologue VIP36, VIPL binds to high mannose residues (Yamaguchi et al., 2007). It shows a binding profile comparable to VIP36, with a preference for deglucosylated trimannose in the D1 branch (Kamiya et al., 2008). In contrast to VIP36, which binds to sugars at rather low pH, VIPL shows most efficient binding at pH

7.5, which is a value found in the ER. The physiologic function of VIPL is poorly understood. VIPL may be involved in glycoprotein secretion. Upon VIPL knockdown the secretion of two non-further characterized glycoproteins decreased (Neve et al., 2003). The VIPL orthologue in zebrafish embryos is involved in early development. Zebrafish embryos lacking VIPL are touch insensitive (Golling et al., 2002). The mechanism leading to this observation still needs to be clarified. Also its principal role in the secretory pathway is not clear. VIPL overexpression was shown to change the localization of ERGIC-53 to the ER, indicating that some sort of interplay exists between the two proteins (Nufer et al., 2003). It has been suggested that VIPL might bind to proteins released from the calnexin-calreticulin cycle and hand them over to ERGIC-53 (Yamaguchi et al., 2007).

1.3.4 ERGL (ERGIC-53 like)

ERGL was identified in a prostate specific EST-cluster.

ERGL was only detected in prostate, spleen, salivary gland and in parts of the brain on mRNA level. Until now no data on protein expression are available. ERGL shows high homology to ERGIC-53 (Fig. 6), but its carboxyl-terminus lacks an ER-export or retrieval motif (Yerushalmi et al., 2001). Like ERGIC-53, ERGL has a transmembrane domain. Interestingly there seems to be a splice variant lacking the transmembrane domain, indicating that there is a soluble form of this protein. Further work on its subcellular localization and its function needs to be conducted.

Figure 6: ***Sequence alignment of L-type lectins in mammals:*** *Alignment of the four human version of ERGIC-53 (LMAN1) ERGL (LMAN1L), VIP36 (LMAN2) and VIPL (LMAN2L). The sequence alignment was performed using Clustal W software (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Blue: acidic amino acids (D,E), red: small hydrophobic amino acids (A, V, F, P, M, I, L, W), magenta: basic residues(R, K) and green: amino acids containing hydroxyl and amine residues (S, T, Y, H, C, N, G, Q).*

1.3.5 Calnexin and Calreticulin

Calnexin and calreticulin are both involved in ER quality control. Calreticulin was originally identified as a calcium binding protein in the sarcoplasmic reticulum of the skeletal muscle (Ostwald and MacLennan, 1974). The authors termed it high-affinity calcium binding protein. Several years later, Calreticulin was rediscovered, but the authors named it CAB-63/calregulin (Waisman et al., 1985). There, it was described as a new calcium binding protein from bovine liver. In the same year, a protein called ERp60 was discovered, that later turned out to be also the same as calreticulin (Lewis et al., 1985). Later on, calreticulin was rediscovered a fourth time as CRP55, a major calcium binding protein in the lumen of the ER (Macer et al., 1988). The name calreticulin was proposed by Koch's group, describing that this protein binds calcium and that it localizes to the endoplasmic reticulum (Smith and Koch, 1989). Also calnexin was discovered under different names (pp90, p88, IP90) by several groups. Wada et al (1991) identified the phosphoprotein pp90 during their search for proteins that were phosphorylated by the kinase activity associated with ER membranes. They noticed a high sequence similarity with calreticulin and therefore they proposed the name calnexin (Wada et al., 1991). Degen and Williams (1991) found p88 in search of interaction partners for class I histocompatibility molecules. There it was already suggested that the dissociation of the class I molecules from p88 might be a rate limiting step in ER to Golgi transport. Hochstenbach et al (1992) identified IP90 as a 90kDa protein that immunoprecipitated with the T-cell receptor, during its assembly.

Calreticulin is a luminal protein and calnexin is a type I membrane protein. They share several features and have an overall sequence similarity of 39% (Williams, 2006). Both proteins are composed of a P-domain and a globular domain. The globular domain is responsible for sugar binding. This lectin-like domain interacts with the terminal glucose residue that is found in the core oligosaccharide ($Glu_1Man_9GlcNac_2$). This oligosaccharide is formed after cleavage of $Glu_3Man_9GlcNac_2$ by the ER glucosidases. Both, Calnexin and calreticulin bind ATP but do not possess ATPase activity (Ou et al., 1995; Corbett et al., 2000). Whether ATP binding is absolutely required for sugar binding in vivo remains unanswered.

The ER localization of calnexin is mediated by the RKPRRE sequence (Rajagopalan et al., 1994). As a soluble protein, Calreticulin has a KDEL retrieval sequence to ensure its ER localization (Sonnichsen et al. 1994). Additionally, its calcium binding domain seems also to contribute to the ER localization of calreticulin but the mechanism behind this is not clear.

Calreticulin and Calnexin bind transiently to membrane and soluble glycoproteins and the interaction is prolonged if these glycoproteins are misfolded or misassembled. Both proteins

have specificity for monoglucosylated polymannose proteins (Hammond and Helenius, 1994). This was initially shown for calnexin by the Helenius group who found that calnexin bound to an ER retained temperatures sensitive mutant of VSVG, in its monoglucosylated form (Hammond et al., 1994). Additionally, treatment with glucosidase inhibitors like castanospermine or deoxyonjirimicin inhibits association of calnexin with its substrate (Hammond et al., 1994; Helenius et al., 1997). Calnexin and calreticulin show a high degree of similarity and therefore it is not surprising that they can partially associate also with the same glycoproteins. The influenza hemagglutinin (HA), a transmembrane glycoprotein, interacted with calnexin and also calreticulin simultaneously (Hebert et al., 1997). They can also bind both to class I MHC molecules, but in this case consecutive binding was observed. First calnexin associates with the heavy chain of MHC-I, but as soon as the heavy chain has oligomerized with the β2-microglobulin subunit it is replaced by calreticulin (Sadasivan et al., 1996). Some other glycoproteins bind exclusively only to one of the two chaperones. Keller et al (1998) found that the nicotinic acetylcholine receptor α-chain only associated with calnexin, but not with calreticulin. By contrast, coagulation factor V was shown to interact only with calreticulin, but not with calnexin (Pipe et al., 1998).

As with all lectins, it remains a matter of debate whether calnexin/calreticulin only bind to the glycans on proteins or whether they also interact with the protein directly. A study of Ihara et al. (1999) suggested that calnexin detects unfolded regions in a protein and binds to them. Additional evidence for a lectin-independent activity came from studies with mutants lacking lectin function. Lectin-deficient mutants of calnexin and calreticulin were still able to work as a functional chaperone in MHC-I processing (Leach and Williams, 2004; Ireland et al., 2008).

1.3.6 ER degradation enhancing α-mannosidase-like proteins (EDEMs)

EDEMs are proteins that are involved in ER associated degradation (ERAD) (Olivari and Molinari, 2007). So far three isoforms have been discovered: EDEM1, EDEM2 and EDEM3 (Mast et al., 2005; Olivari et al., 2005; Hirao et al., 2006). Originally it seemed that EDEM1 is a type II integral membrane protein. This was suggested using HA-tagged EDEM1 overexpressed in COS cells (Hosokawa et al., 2001). Later it turned out that all EDEMs are soluble ER localized proteins (Olivari et al., 2005; Hirao et al., 2006; Olivari and Molinari, 2007). An inefficient cleavage of the signal sequence in different cell lines might explain the different results. All EDEMs are N-glycosylated proteins, containing high mannose residues indicating that they are not transported to the Golgi apparatus. They all show sequence similarity to α-1,2 mannosidases. Until recently, EDEM1 and EDEM2 were considered to lack enzymatic activity. Meanwhile, a recent paper demonstrated increased de-mannosylation

activity upon overexpression of EDEM1, although a direct proof for enzymatic activity is still missing (Olivari et al., 2006). EDEM3 was also shown to have α-1,2 mannosidase activity. Overexpressed EDEM3 stimulated mannose trimming of a misfolded α1-antitrypsin mutant and accelerated its degradation. A catalytic inactive mutant of EDEM3, had no effect on mannose processing and showed only slight acceleration of ERAD (Hirao et al., 2006). This again suggests that EDEM3 is catalytically active.

Htm1p the yeast homolog of EDEM, that is implicated in ERAD of glycoproteins, is catalytically inactive (Jakob et al., 2001). EDEM1 binds preferentially to glycoproteins containing a $Man_8GlcNac_2$ glycan, a modification which has been shown to serve as a tag for degradation in yeast (Jakob et al., 1998). EDEM1 and EDEM2 overexpression accelerates the degradation of misfolded α1-antitrypsin (Hosokawa et al., 2001; Olivari et al., 2005). EDEM1 was shown to interact directly with calnexin (Molinari et al., 2003). This indicates that EDEM accepts misfolded glycoproteins directly from calnexin. Overexpression of EDEM1 enhances this process leading to an accelerated degradation of the terminally misfolded substrate (Hosokawa et al., 2006). EDEM1 prevents aggregation of ERAD substrates, which would disturb retrotranslocation. EDEM was further shown to inhibit aggregation of a folding defective α1-antitrypsin mutant or BACE457 (Hosokawa et al., 2006; Olivari et al., 2006). All these data show that EDEMs are somehow involved in ERAD, but the precise mechanisms need to be further elucidated.

1.3.7 OS-9 and XTP-3/Erlectin

OS-9 and XTP-3 are two lectin-like proteins with a role in ER associated degradation. OS-9 was originally discovered using a chromosome microdissection-based hybrid-selection strategy to identify genes upregulated in osteosarcoma (Su et al., 1996). XTP-3/erlectin was discovered in a proteomic approach in search for interaction partners of the Dickkopf co-receptor Kremen 2 (Cruciat et al., 2006). The yeast homologue Yos9p exhibits 15% identity with OS-9 and XTP-3 (Bhamidipati et al., 2005). Yos9p is part of a complex involved in ERAD of glycoproteins (Buschhorn et al., 2004; Bhamidipati et al., 2005; Kim et al., 2005; Szathmari et al., 2005). A similar role has been demonstrated for OS-9 and XTP-3 (Christianson et al., 2008). OS-9 and XTP-3 are both ER resident glycoproteins, containing high mannose glycans. Both were shown to interact via the SEL1 adaptor with Hrd1 (Hosokawa et al., 2008). Hrd1 is an ubiquitin ligase involved in ERAD. Knockdown of OS-9, but not of XTP-3 slowed down the degradation of a misfolded α1-antitrypsin mutant (Christianson et al., 2008). A study by Hosokawa et al (2009) showed that OS-9 binds to *N*-glycans lacking the terminal mannose from the C-branch in vitro. This interaction is mediated

by the mannose 6-phosphate receptor homology (MRH) domain of OS-9. OS-9 interacts with the chaperone Grp94, which might improve substrate recognition (Christianson et al., 2008). XTP-3 has two MRH domains, which implicates that it is also involved in N-glycan recognition. XTP-3 bound to a misfolded antitrypsin mutant and this interaction was dependent on the MRH domain (Yamaguchi et al., 2009). Interestingly XTP-3 was also shown to interact with a misfolded non-glycosylated variant of transthyretin, that OS-9 could not bind (Christianson et al., 2008). Further work has to be conducted on the exact mode of actions of these two proteins,

1.3.8 Mannose-6 phosphate receptors

Mannose-6-phosphate receptors (MPRs) belong to the family of P-lectins (Dahms and Hancock, 2002). The P-lectins are characterized by the ability to recognize phosphorylated mannose residues. The P-lectins comprise only two members in mammals the cation dependent MPR (CD-MPR/MPR46) (Hoflack and Kornfeld, 1985) and the cation independent MPR/insulin-like growth factor II receptor (CI-MPR/MPR300) (Sahagian et al., 1981; Ghosh et al., 2003). They are both type-I transmembrane proteins that localize to the TGN, early sorting endosomes, late endosomes and also at the plasma-membrane (Geuze et al., 1985; Griffiths et al., 1988) The MPRs mediate the transport of lysosomal hydrolases to the lysosomes (Kyle et al., 1988; Jin et al., 1989). The interaction of the MPRs with the lysosomal enzymes occurs via binding to their mannose-6-phosphate (M6P) residues (Braulke and Bonifacino, 2009). Lysosomal hydrolases acquire this modification by phosphorylation of mannose residues in their high mannose glycan chains in the Golgi (Lazzarino and Gabel, 1989). Binding of MPRs to lysosomal enzymes occurs in the TGN (Geuze et al., 1985). CD-MPR has one M6P binding site and CI-MPR has two high affinity and one low affinity M6P binding site (Hancock et al., 2002; Reddy et al., 2004). The receptor-ligand complex is sorted into clathrin coated transport intermediates. The sorting signal for this process resides in the cytosolic tail of the MPRs. The minimal motif consists of DXXLL and is often surrounded by other acidic amino acids. These signals are known as acidic-cluster-dileucine signals (DDSDEDLL" in CI-MPR and EESEERDDHLL" in CD-MPR) (Johnson and Kornfeld, 1992a; Johnson and Kornfeld 1992b; Chen et al., 1997). This motif mediates interaction with clathrin adaptor proteins called GGAs (Puertollano et al., 2001; Zhu et al., 2001; Misra et al., 2002). In addition, the MPRs were shown to interact with AP-1, an adaptor protein found in clathrin-coated vesicles (Mauxion et al., 1996). After the receptor/ligand complex has reached the endosomes, dissociation of the MPR from the acidic hydrolases occurs and the MPR cycles back to the Golgi for another round of transport (Duncan and Kornfeld, 1988). As is

the problem with many lectins, the actual site where receptor and ligand dissociate is not clear. It is still a matter of debate if the MPR/ligand complexes enter early (Ludwig et al., 1991; Press et al., 1998; Waguri et al., 2003) or late endosomes (Bucci et al., 2000) and where the complex finally dissociates. Recycling of the MPR to the Golgi is achieved by TIP47 and the retromer complex. The retromer is a conserved complex of the five proteins Vps35p, 29p, 26p, 17p, and 5p, originally discovered in yeast. The complex is involved in transport from endosomes to the TGN (Seaman et al., 1997; Seaman et al., 1998; Haft et al., 2000). In cells derived from Vps26 knockout mice, the CI-MPR lost its Golgi localization and was found at the plasma membrane (Seaman, 2004). Several other proteins have been implicated in retrograde transport of MPRs like clathrin, AP-1 (Meyer et al., 2000; Meyer et al., 2001) and PACS-1 (Wan et al., 1998; Crump et al., 2001).

In addition to its role in lysosomal enzyme transport the CI-MPR has an important role in the regulation of IGF-II levels. About 10% of the CI-MPR escape the retrieval mechanism back to the TGN and reach the plasma-membrane where it is involved in the endocytosis of IGF-II. After internalization, IGF-II is targeted to lysosomes for degradation. The recognition of IGF-II occurs independently of mannose-phosphate residues (Ghosh et al., 2003). Although the MPRs comprise only two members, several proteins comprise a domain homologous to the MPRs (**m**annose 6-phosphate **r**eceptor **h**omology :MRH) like glucosidase-II, OS-9 and XTP-3.

1.4 Quality control

Quality control of proteins ensures that only correctly folded and correctly assembly newly synthesized proteins can exit the ER. This requires correct co-and posttranslational processing. Problems in this process, either caused by changes in the protein structure due to genetic mutations or by environmental influences like temperature or pH changes, lead to misfolded proteins (Herczenik and Gebbink, 2008).

It is important for cell survival to avoid the formation or at least the longer persistence of misfolded proteins within a cell. Otherwise misfolded proteins form intra-or extracellular deposits and induce cellular toxicity (Broadly and Hartl, 2009). This mechanism is observed in the pathophysiology of many diseases like Alzheimer's disease, Parkinson's disease or Huntington's disease (Herczenik and Gebbink, 2008). Misfolded proteins induce ER stress and this leads to a process called the unfolded protein response (UPR). UPR is an intracellular signalling pathway activated in response to accumulation of unfolded proteins in the ER. This includes transcriptional upregulation of proteins involved in quality control and protein transport (Ng et al., 2000; Nyfeler et al., 2003; Acosta-Alvear et al., 2007), attenuation of

global protein translation to reduce protein load and degradation of unfolded proteins. In case these mechanisms fail to restore the balance in the cell, apoptosis is induced by mitochondria-dependent and independent mechanisms (Malhotra and Kaufman, 2007). As a cell wants to avoid all these negative effects, there are a couple of mechanisms involved in quality control of proteins (Ellgaard et al., 1999). Primary quality control affects all newly synthesized proteins in the ER and is therefore usually referred to as ER quality control (Ellgaard and Helenius, 2003). Secondary quality control is only necessary for selected proteins and protein families.

1.4.1 ER quality control

ER quality control was first discovered studying the intracellular transport of viral proteins. Different misfolded viral membrane proteins were shown to be retained at the site of synthesis (Gething et al., 1986; Kreis and Lodish, 1986). ER quality control is controlled by chaperone and co-chaperone proteins.

1.4.1.1 Chaperones

A main role in ER quality control is accomplished by chaperones. Chaperones are necessary to avoid aggregation, promote correct folding and correct assembly of the newly synthesized proteins and lead to their correct membrane translocation. Chaperones were originally defined as a family of unrelated classes of proteins that mediate the correct assembly of other polypeptides, but are not themselves components of the final functional structures (Ellis and Hemmingsen, 1989). In general they bind to protein folding intermediates, but not to correctly folded and assembled proteins. They recognize these folding intermediates via exposed hydrophobic patches or via exposed reactive cysteines. The lectin chaperones like calnexin and calreticulin also recognize sugar groups.

The major part of ER chaperones is made up by three families of heat shock proteins (Hsp) Hsp70, Hsp40 and Hsp90. The Hsp70 family includes BiP (immunoglobulin binding protein) which is also called glucose-regulated protein of 78 kDa (GRP78) and GRP170 (Ellgaard et al., 1999). BiP/Grp78 is one of the most widely studied and most abundant chaperones. It is involved in protein translocation by sealing the Sec61 translocation channel and by binding to freshly synthesized proteins as they leave the translocation channel to prevent them from slipping back (Vogel et al., 1990). BiP also helps in protein folding (Knittler and Haas 1992). Whereas interaction with newly synthesized proteins during the translocation process is only transient, BiP stays covalently bound to unfolded or aggregated proteins (Gething et al., 1986; Marquardt and Helenius, 1992). Two domains are important for BiP's chaperone function: the

N-terminal ATPase domain and the C-terminal substrate binding domain. These domains are conserved among the Hsp70s family members (Saibil, 2008). BiP binds predominantly aliphatic amino acids (Flynn et al., 1991; Blond-Eguindi et al., 1993). Binding as well as the release of the proteins is dependent on binding to and hydrolysis of ATP (Flynn et al., 1989)

BiP also regulates the UPR as it binds to the three main proteins involved in ER stress response ATF6 (Shen et al., 2005), IRE1 (Okamura et al., 2000) and PERK (Bertolotti et al., 2000) and keeps them inactive. An increase in amount of folding intermediates will release BiP from ATF6, IRE1 or PERK, thereby increasing their activity and inducing the UPR.

Much less is known about GRP170 the other ER localized member of the Hsp70's family. Its yeast homolog Lhs1 was shown to bind Kar2p/BiP where it might serve as a nucleotide exchange factor for Kar2p. Kar2p stimulates ATP hydrolysis of Lhs1p in return (Steel et al., 2004). Whether GRP170 works the same way in mammalian cells has to be investigated.

GRP94/gp96 belongs to the Hsp90 family. It was found in a complex with BiP and newly synthesized immunoglobulin chains (Melnick et al., 1992). Later it was shown that the two chaperones work in a consecutive manner. BiP binds to a disulfide intermediate of the newly synthesized immunoglobulin chain. After BiP has released the protein, Grp94 binds to the fully oxidized form of the immunoglobulin chain (Melnick et al.; 1994). Substrates of Grp94 include mutant alpha-1-antitrypsin (Schmidt and Perlmutter, 2005) thyroglobulin (Kuznetsov et al., 1994) and apolipoprotein B (Linnik and Herscoitz, 1998). The ability to bind and hydrolyze ATP is essential for the function of GRP94 (Ostrovsky et al., 2009).

1.4.1.2 Co-chaperones

Chaperones are assisted by Co-chaperones like ERdjs or Sil1/BAP, but also by proline isomerases (PPIases, cyclophilins) or thiol-disuphide oxidoreductases (PDI, Ero1α and β).

ERdjs are members of the Hsp40s/DNAJ family (Qiu et al., 2006). So far seven isoforms have been discovered (Dudek et al., 2009) which all contain a J-domain. They interact with BiP via their J-domain and are able to stimulate ATP hydrolysis in vitro. ERdj3 binds to misfolded and unfolded proteins and recruits BiP (Shen et al., 2005). ERdj5/JPDI has in addition to its J-domain, a PDI-like domain and a thioredoxin domain (Cunnea et al., 2003; Hosoda et al., 2003). Erdj5 cleaves disulfide bonds of misfolded proteins and is involved in ER associated degradation (Ushioda et al., 2008).

Another BiP cofactor is Sil1/BAP. This protein works as a nucleotide exchange factor for BiP (Chung et al., 2002).

Thiol-disulfide-oxidoreductases and peptidyl-prolyl-isomerases are factors that assist chaperones. Thiol-disulfide oxidoreductases catalyse oxidation, isomerisation and reduction

of disulfide bonds. Inter- and intramolecular disulfide bond formation is a rate-limiting step in the process of protein folding (Hatahet and Ruddok, 2007; Creighton, 1979). The protein disulfide isomerase (PDI) family plays an important role in the correct disulfide bond formation. The PDI family comprises 19 members (Appenzeller-Herzog and Ellgaard, 2008). They all share a domain that is homologous to the cytosolic reductase thioredoxin and is therefore called thioredoxin-like domain which is characterized by a CXXC motif (Ellgaard and Ruddock, 2005). During disulfide bond formation the two cysteines in the CXXC motif become reduced and are re-oxidized by the flavoenzyme Ero1. There are two isoforms in humans, Ero 1-Lα and Ero 1-Lβ (Cabibo et al., 2000; Pagani et al., 2000). In addition, PDI is involved in disposal of misfolded proteins via ERAD (Molinari et al., 2002). Another important member of the PDI family is Erp57, an oxidoreductase specialized on glycoproteins (Molinari and Helenius, 1999; Oliver et al., 1999). Erp57 interacts with calnexin and calreticulin (Ellgaard et al., 2001; Frickel et al., 2002; Leach et al., 2002). It is involved in correct folding of several glycoproteins like clusterin and several integrins (Jessop et al., 2007). The activity of Erp57 seems to depend on the lectin activity of calnexin/calreticulin. Treatment with glucosidase inhibitors that inhibit association of glycoproteins with calnexin and calreticulin was found to inhibit also their association with Erp57 (Di Jeso et al., 2005). Thiol-oxidoreductases, especially Erp44 are involved in a process called thiol-mediated retention (Isidoro et al., 1996; Reddy and Corley, 1998; Anelli et al., 2003; Anelli et al., 2007). The thiol-oxidoreductase interacts with free cysteines of the substrate and keeps it thereby in the ER. One example for thiol-mediated retention was shown for IgMs. B-cells usually do not secrete IgM, because thiol-mediated retention inhibits IgM secretion. Mutation of Cys575 in IgM inhibits its thiol-medited retention by Erp44 and therefore IgM becomes secreted (Alberini et al., 1990; Anelli et al., 2003). Erp44 cooperates with ERGIC-53 to allow only export of correctly assembled IgM polymers (Anelli et al., 2007).

Finally, peptidyl-prolyl isomerases catalyze the *cis-trans* isomerisation of peptidyl-prolyl bonds (Gothel and Marahiel, 1999; Chantal et al., 2008). Three main families exist. The cyclophilins, the FK506-binding proteins (FKBP) and the parvulins. Cyclophilins and FKBP interact with chaperones in the ER. Mouse FKBP32 was shown to interact with BiP in a calcium dependent manner and to modulate its ATPase activity (Zhang et al., 2004; Wang et al., 2007). Cyclophilin has been found to be part of a whole ER chaperoning network (Meunier et al., 2002). This network consists of BiP, GRP94, CaBP1, PDI, ERdj3, cyclophilin B, ERp72, GRP170, UDP-glucosyltransferase and SDF2-L1.

1.4.1.3 N-glycan dependent quality control-the calnexin/calreticulin cycle

Glycoproteins undergo a different kind of quality control than non-glycoproteins. This N-glycan dependent quality control was initially postulated by Hammond and Helenius (1994). The initial step in N-glycan dependent quality control is the addition of the 14 saccharide core unit ($Glc_3Man_9GlcNAc_2$) from a dolichol P-P derivative to an Asn residues in an N-X-S/T motif in a nascent polypeptide chain (Helenius and Aebi, 2004). This glycan chain is then further modified through the action of two glucosidases (glucosidase I and glucosidase II). The resulting $Glc_1Man_9GlcNAc_2$ chain serves as a substrate for the ER resident lectin chaperones calnexin and calreticulin.

Two different modes have been proposed for CNX and CRT: the lectin-only-model (i.e. only sugar-dependent) and the dual binding model (i.e. sugar- and peptide-dependent). In the lectin-only-model the interaction is mediated primarily by monoglucosylated glycans. CNX and CRT showed the same affinity for monoglucosylated RNAseB irrespective of whether the protein was native or not (Rodan et al., 1996). In this model the lectin substrate is bound while other enzymes/proteins like Erp57 may act on it and influence its dissociation from CNX/CRT (Kornfeld and Kornfeld, 1985; Oliver et al., 1999). The lectin-only model is supported by experiments with the glucosidase inhibitor castanospermine. Castanospermine blocks glucose trimming of the core oligosaccharide preventing binding to CNX or CRT. Treatment with castanospermine inhibited association of tyrosinase with calnexin which resulted in a more rapid folding, but also in an inactive enzyme (Branza-Nichita et al., 1999).

The dual binding model proposes that a combined interaction with the oligosaccharide and the protein backbone occurs (Ihara et al., 1999). In this model CNX/CRT would bind for example hydrophobic patches in non-native glycoproteins and thereby suppress their aggregation (Williams, 2006). A combination of glycans and polypeptide determinants would increase the affinity for a substrate. Zhang et al (1995) proposed a model where the glycans are only important for initial substrate binding. As soon as the complex between calnexin and its substrate is formed the sugar would be less or not important anymore. In this model dissociation would occur after glucose trimming by glucosidase-II combined with a conformational change of the substrate.

If the protein is not correctly folded it is reglucosylated by the UDP-Glc: glycoprotein glucosyltransferase (GT) (Parodi et al., 1984). GT is primarily a soluble ER resident protein, but it also localizes to pre-Golgi intermediates (Zuber et al., 2001). So far two isoforms of GT have been discovered in mammalian cells (Arnold, 2000). They share about 55% sequence identity. But only the isoform GT1 seems to be functional, at least in an in vitro assay. GT utilizes UDP-glucose as a glucosyl donor. GT adds a single glucose to the terminal mannose

of the α (1-3)-α(1-2) branch/A-branch and forms again the $Glc_1Man_9GlcNAc_2$ chain (Helenius et al., 1997; Van Leeuwen and Kearse, 1997). The activity of GT depends on calcium (Trombetta and Parodi, 1992) and the amount of mannose molecules present in the oligosaccharide side chain of the substrate (usually between seven and nine). GT does not recognize high mannose oligosaccharides alone. The oligosaccharide needs to be attached to incompletely folded proteins where GT interacts with hydrophobic patches (Trombetta and Parodi, 1992; Caramelo et al., 2003). This suggests that GT is involved in a later step in glycoprotein folding. As soon as the glycoprotein is back in its monoglucosylated form, the glycoprotein re-enters the Calnexin/Calreticulin cycle to obtain correct folding (Fig.7). This process is repeated until the correct conformation is achieved (Van Leeuwe and Kearse, 1997; Wada et al., 1997). Some proteins need several turns of this cycle. This is for example true for the human β-secretase BACE 501. In contrast, the VSVG protein needs only one turn for complete folding (Soldá et al., 2007).

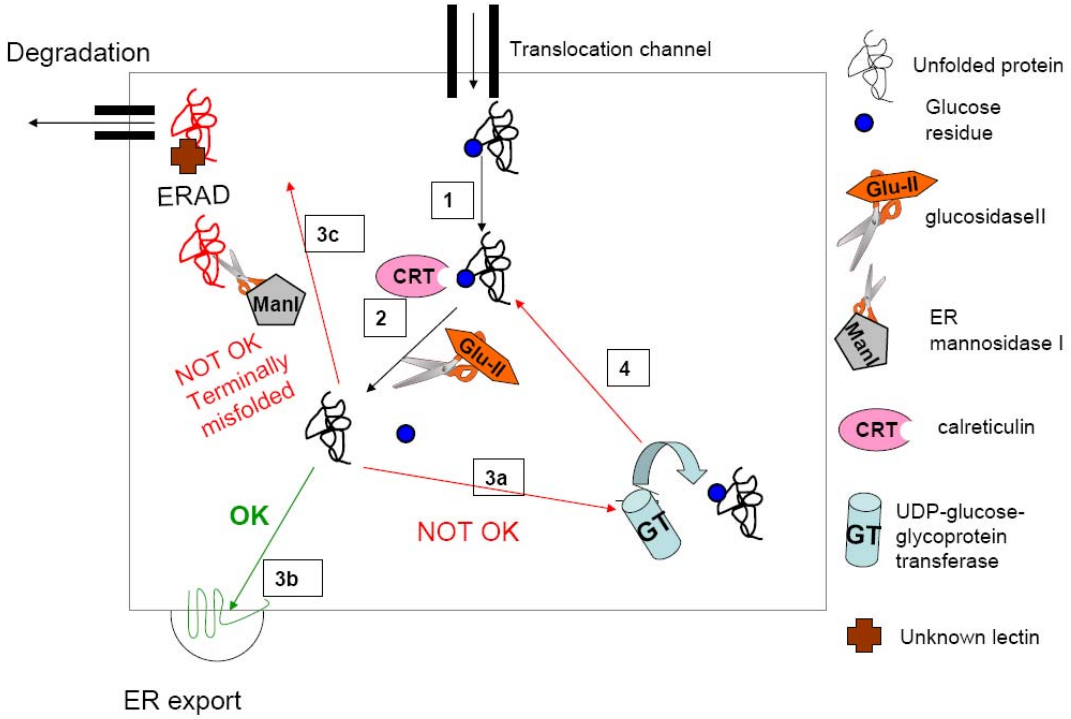


Figure 7 ***Calnexin/Calreticulin cycle****: Proteins are translocated into the lumen of the endoplasmic reticulum and the core oligosaccharide is added. Upon removal of two glucose residues by glucosidase I and glucosidase II, the glycoprotein enters the calnexin/calreticulin cycle in its monoglucosylated form [1 .Calreticulin assists in the folding of the glycoprotein. The glycoprotein is released from CNX/CRT upon the cleavage of the glucose residue by glucosidase II (GluII) [2]. In case the protein is folded correctly it will be targeted for ER export [3b]. Otherwise it becomes reglucosylated by the UDP-glucose-glycoprotein transferase (GT) [3a] and the cycle starts from new [4]. In case the protein is terminally misfolded it will be targeted to ER associated degradation. This process is initiated by ER mannosidase I [3c] that cleaves one mannose residue from the B-branch of the glycoprotein. The protein is then bound by an unknown lectin (perhaps EDEM) and then subjected to degradation.*

1.4.2 Secondary quality control

Secondary quality control includes different mechanisms that only apply for a specific set of proteins (Elgaard et al., 1999). Proteins involved in secondary quality control help in the assembly of the proteins or work as escort proteins. Typically these proteins are dedicated to assist only a particular protein. In yeast Vma12p-Vma22p assist the vacuolar H^+-ATPase in the correct assembly of its subunits (Graham et al., 1998). Escort proteins assist their binding partner in trafficking through the secretory pathway. The receptor associated protein (RAP) binds to the lipoprotein receptor-related protein (LRP). This leads to aggregation of LRP and reduced ligand binding. As soon as the complex reaches the Golgi it dissociates upon pH change. The KDEL receptor binds to the HNEL sequence in the C-terminus of RAP and transports it back to the ER for another round of escort (Bu et al., 1995).

Some proteins need specialized chaperones for their folding. The large protein procollagen needs Hsp47 and prolyl-4-hydroxylase for its correct folding. Prolyl-4-hydroxylase recognizes and retains unfolded procollagen in the ER (Walmsley et al., 1999). Hsp47 seems to bind unfolded as well as correctly folded procollagen. Hsp47 binds to procollagen in the ER and releases it in the Golgi (Satoh et al., 1996).

If all quality control mechanisms fail to provide a correctly folded and correctly assembled protein, this terminally misfolded protein will be targeted to degradation.

1.4.3 ER-associated degradation (ERAD)

Terminally misfolded proteins undergo a degradation process known as ER associated degradation (ERAD). Around 30% of all newly synthesized proteins were shown to undergo this degradation process. Initial evidence for an ER associated degradation pathway came from work on the T-cell receptor complex where the degradation of some subunits could not be inhibited by lysosomal inhibitors. Therefore the idea came up that degradation either occurs in the ER itself or in an unidentified compartment (Chen et al., 1988; Lippincott-Schwartz et al., 1988). It was speculated that an unidentified protease might be involved in this process (Finger et al., 1993). Subsequent work in yeast showed that degradation did not occur in the ER itself. A loss of function mutant of an ubiquitin conjugating enzyme (UBC6) rescued the protein translocation defect induced by a Sec61 mutant (Sommer and Jentsch, 1993). The same results were obtained for the loss of function mutant of another ubiquitin conjugating enzyme (Biederer et al., 1996). These findings implied a role of the proteasome in the ER associated degradation. The first evidence that mammalian cells use a similar mechanism for protein disposal came from work on CFTR. CFTR is a transmembrane protein that works as a chloride channel. The folding of the wild type protein is already very

inefficient. Around 40% of the newly synthesized proteins are degraded (Lukacs et al., 1994). Jensen et al. (1995) found that ER associated degradation could be inhibited with proteasomal inhibitors. Upon proteasomal treatment a polyubiquitinated form of CFTR accumulated, indicating that degradation was dependent on ubiquitination (Ward et al., 1995). Further work showed that this process also applied to soluble proteins. Misfolded carboxypeptidase Y (CPY*) was shown to be degraded in a proteasome-dependent manner by retrotranslocation from the ER (Hiller et al., 1996).

There is a tight connection between chaperones and ERAD. Chaperones maintain solubility of the ERAD substrates. If ERAD substrates aggregate, they are not degraded and accumulate. Lumenal soluble proteins are targeted to the ERAD with the help of BiP (Nishikawa et al., 2001). In mammals BiP substrates subsequently interact with the cytoplasmic protein Herp. Knockdown of Herp affected specifically non-glycosylated BiP substrates, but had no effect on the degradation of glycosylated CNX/CRT-substrates (Okuda-Shimizu and Hendershot, 2007). Herp was found in a complex with derlin1 and the 26S proteasome and could therefore directly link the substrate to the proteasome. Membrane proteins with a large cytosolically localized portion need the help of cytosolic chaperones. Misfolded CFTR associates with cytoplasmic Hsc70. Drug induced dissociation of Hsc70 from the CFTR either by downregulation of Hsc70 or binding competition lead to an increased export of the protein. (Jiang et al., 1998; Rubenstein and Zeitlin, 2000). Certain co-factors help the chaperones to target the substrates to degradation. In the case of CFTR degradation the E3-ubiquitin-protein ligase CHIP acts as a co-factor for Hsc70.

ERAD processes for glycoproteins depend on a different subset of proteins. One of the proteins that play a major role in degradation is ER mannosidase I (Herscovics, 2001). ER mannosidase I is a slow-acting α1,2-mannosidase. Inhibition of the ER mannosidase I by kifunensine and deoxymannojirimycin inhibits degradation of misfolded glycoproteins (Su et al., 1993). This has been shown for several glycoproteins like misfolded alpha1-antitrypsin (Liu et al., 1999) or ribophorin 1 (Kitzmüller et al., 2003). ER mannosidase I cleaves one mannose residue resulting in $Man_8GlcNac_2$ isomer B. This serves two purposes. First the affinity of GT for mannose trimmed substrates is much lower and reglucosylation is less likely to occur (Sousa et al., 1992). Second this glycan is the ideal target for the lectin-like proteins EDEMs **(**ER degradation enhancing α-mannosidase-like proteins) (Hosokawa et al., 2001). It has been proposed that EDEMs accept substrates directly from calnexin and target them to degradation (Molinari et al., 2003; Oda et al., 2003). The cleavage of the mannose residue is therefore a rate limiting step in the degradation process. These observations led to

the mannose timer hypothesis (Su et al., 1993). The slow action of the mannosidase provides a window in which correct folding should occur. If the proteins are terminally misfolded, the ER mannosidase I finally cleaves the mannose residue and the protein is targeted to ERAD. It seems to be important that the mannose residue on the A branch, which serves as an acceptor for the glucose transferred by GT is removed (Foulquier et al., 2002; Frenkel et al., 2003; Kitzmüller et al., 2003; Foulquier et al., 2004). ER mannosidase also cleaves additional mannose residues from the B-branch but very high concentrations are required for this (Herscovics et al., 2002). To achieve such high levels in the cell, it would be necessary to concentrate the enzyme at certain sites. It has been suggested that this occurs in a special compartment termed ER quality control compartment (Kamhi-Nesher et al., 2001; Avezov et al., 2008). This compartment is probably a subcompartment of the ER and referred to as the ER quality control (ERQC) compartment. The ERAD substrate asialoglycoprotein receptor (H2a) accumulated in this compartment together with calnexin and calreticulin after proteasomal inhibition (Kamhi-Nesher et al., 2001). Beside the ER mannosidase also other proteins have been implicated in the process of mannose removal. The lectin-like proteins EDEM1, and EDEM3 were both shown to accelerate mannose removal upon overexpression (Hirao et al., 2006). Whether this is due to a direct or indirect effect remains to be clarified. Based on this we may have to modify the initial timer hypothesis. In this revised model mannose trimming and reglucosylation take turns, until the GT is not able to reglucosylate the protein anymore. Then the protein is targeted to ERAD (Lederkremer and Glickman, 2005). The group of Lederkremer provided a model where part of this process takes place in the ERQC. The ERAD substrate would move into the ERQC for mannose removal and then it recycles back to the peripheral ER for reglucosylation (Kamhi-Nesher et al., 2001).

Beside the oligosaccharide composition, the context of the N-glycan in the protein also contributes to the selection for ERAD (Spear and Ng, 2005; Mbonye et al., 2006; Mbonye et al., 2008). The misfolded protein CPYΔ1 is recognized by the ER quality control machinery, but not targeted to ERAD. The deletion removed the N-glycosylation site nearest to the C-terminus. Experiments in htm1p deletion strains suggested that this glycan interacts specifically with htm1p and is thereby targeted to ERAD (Spear and Ng, 2005).

Protein retrotranslocation is assumed to occur via a channel. There is growing evidence that the translocation channel Sec61 could fulfill this role in yeast as well as in mammals (Wiertz et al., 1996; Plemper et al., 1997). Mutant Sec61, delayed for example, ERAD of CPY* in yeast (Plemper et al., 1997). However, there is growing evidence that retrotranslocation of misfolded proteins is not only achieved by Sec61, because retrotranslocation still occurs in

Sec61 mutants (Huyer et al., 2004). In addition not all soluble proteins seem to depend on Sec61 for retrotranslocation. In vitro experiments showed that retrotranslocation of nonglycosylated pro-alpha factor was dependent on Der1p (Degradation in the ER) rather than on Sec61 (Wahlman et al., 2007). Other studies support the view that Der1p may constitute part of the retrotranslocation channel in yeast (Knop et al., 1996; Carvalho et al., 2006). Der1p has three homologs in mammals: Derlin-1, Derlin-2 and Derlin-3 (Ye et al., 2004; Oda et al., 2006). There is increasing evidence suggesting that Derlin-1 might also constitute part of a retrotranslocation channel in mammals (Lilley and Ploegh, 2004; Ye et al., 2004; Younger et al., 2006). Beside derlins, the E3 ubiquitin ligases Doa10p and Hrd1 were also proposed to form a translocation channel. Both proteins are multispanning membrane proteins, which would allow intramembrane substrate recognition. They could play a role in degradation of misfolded integral membrane proteins (Nakatsukasa and Brodsky, 2008). Further work is needed to validate this hypothesis.

Retrotranslocated proteins destined for degradation are covalently modified by the attachment of ubiquitin molecules. This polyubiquitin chain may then function in a ratcheting mechanism, preventing the substrate of slipping back into the ER (Riezman, 1997; Tsai et al, 2002). Upon inhibition of ubiquitination proteins do not accumulate in the cytosol, but associate with the lumen of the ER (de Virgilio et al., 1998). This could mean that polyubiquitination is needed for the process of retrotranslocation itself. The polyubiquitin chain could also serve as a targeting signal recognized by ubiquitin binding proteins (Flierman et al., 2003).

Depending on the location of the defect, different ERAD pathways were suggested. ERAD-C is for proteins with a defect in their cytosolic domain, ERAD-M is for proteins with defects in their transmembrane region and ERAD-L is for proteins with defects in their luminal domain (Swanson et al., 2001; Vashist and Ng, 2004; Carvalho et al., 2006; Gauss et al., 2006). ERAD-C requires the ubiquitin ligase Doa10p (Swanson et al., 2001). ERAD-L needs a complex consisting of the transmembrane ubiquitin ligases Hrd1p/Hrd3p, Der1p and Usa1p. This complex associates with the lectin-like protein Yos9p. Hrd1p/Hrd3p is also necessary for ERAD-M, which differens from ERAD-L by being independent of Usa1p and Der1p. In addition to ubiquitination, glycoproteins are deglycosylated by peptide: N-glycanase (PNGase) before degradation (Hirsch et al., 2003; Blom et al., 2004). PNGase is able to distinguish between folded and unfolded proteins. However, PNGase is not essential for glycoprotein degradation but its function may rather be to increase the efficiency of glycoprotein degradation.

Proteins belonging to the AAA+ family (**A**TPases **a**ssociated with various cellular **a**ctivities) play and important role in the degradation of misfolded proteins (Dai et al., 1998; Ye et al., 2001; Jarosch et al., 2002). In yeast the cytosolic ATPase complex named Cdc48p fulfils this role. Its mammalian homolog is called valosin containing protein (VCP)/p97. Cdc48/VCP/p97 forms a hexameric ring, which associates with the ER membrane and is thought to pull the ERAD substrate through its central pore out of the ER. Several studies showed that Cdc48p/VCP/p97 recognizes the ubiquitin tag of proteins directly (Dai and Li, 2001; Rape et al., 2001; Rabinovich et al., 2002). A chain of at least four ubiquitins is needed to send the protein to the proteasome (Jarosch et al., 2002). This polyubiquitin-tag is presumably recognized by polyubiqitin binding proteins that escort the substrates to the proteasome (Kim et al., 2004; Medicherla et al., 2004; Richly et al., 2005; Kim et al., 2006; Raasi and Wolf, 2007).

1.4.4 Post ER quality control

There is evidence that quality control continues beyond the ER (Arvan et al., 2002). In case quality control acts at a later step, the misfolded protein is either targeted back to the ER or is sent to the vacuole/lysosome for degradation. Most of this evidence originates from data obtained in yeast. The CPY cargo receptor Erv29p (Belden and Barlowe, 2001) was shown to be involved in degradation of a misfolded mutant of CPY (CPY*). Strains lacking Erv29p showed a stabilization of the misfolded protein, indicating that ER to Golgi transport is somehow involved in the degradation process (Caldwell et al., 2001). This could be possibly due to the need for a modification of the protein in the Golgi prior to degradation. A subsequent study by a different group showed that ER to Golgi transport in general is a prerequisite for ER associated degradation of CPY* (Taxis et al., 2002). Vashist et al (2001) compared degradation of a membrane and a soluble protein. They found that the membrane protein was directly retained in the ER while the soluble protein was transported to the Golgi in a COPII-dependent manner. From the Golgi, the protein is retrieved in COPI vesicles and subjected to ERAD. Post ER quality control was also shown to be present in mammalian cells. A misfolded VSVG mutant was not retained in the ER completely, reached the ERGIC and the cis-Golgi, but was then cycled back to the ER (Hammond an Helenius, 1994). During this transport round BiP remained bound to VSVG the whole time. Misfolding of VSVG which was trapped in ERES (by incubation at 10°C) did not allow exit from the ER. Instead VSVG was re-routed to the reticular ER. In contrast, misfolding of VSVG which was trapped in the ERGIC (by incubation at 15°C), did not result in its retrieval. Instead the protein was transported onward to the Golgi (Mezzacasa and Helenius, 2002). Another example for post

ER quality control was shown for the overexpressed T-cell receptor alpha chain (TCRα). When overexpressed, unassembled TCRα becomes degraded. However, unassembled TCRα cycles between ER and the Golgi. TCRα that escapes the ER remains bound to BiP. BiP is recognized by the KDEL receptor which mediates retrieval of the TCRα-BiP complex back to the ER (Yamamoto et al., 2001). Additional evidence for a post-ER quality came from electron microscopy studies of Zuber et al (2001). They found some of the proteins involved in quality control enriched in the ERGIC namely calreticulin, GT and glucosidaseII.

Beside degradation in the ER, proteins may also be targeted to the vacuole/lysosome for degradation. Again far more is known in yeast than in mammalian cells about this mechanism. Bacterial β-lactamase is targeted from the Golgi apparatus to the vacuole for degradation (Holkeri and Makarow, 1998). A fusion protein between yeast invertase and a mutated form of bacterial lambda repressor was also shown to be targeted to the vacuole for degradation. This pathway is saturable, as overexpression of the fusion protein leads to its secretion (Hong et al., 1996; Holkeri and Makarow, 1998). A mutant of the plasma membrane ATPase Pma1p is not subjected to ERAD, but instead transported to the Golgi and finally delivered to the endosomal/vacuolar system for degradation (Chang and Fink, 1995). A mutant arginine permease, $Can1^{ts}$, and a mutant alpha mating factor receptor Ste2-3 (both membrane proteins) are also subjected to vacuolar degradation. Mutants that block Golgi to vacuole trafficking inhibited degradation of mutant Ste2-3 (Li et al., 1999). A similar disposal pathway has been described for the coronavirus membrane protein E1 in mammalian cells. Wild type E1 localizes to the Golgi, but deletions of membrane spanning sequences or a deletion of a large part of the carboxyl-terminus results in lysosomal degradation (Armstrong et al., 1990). The lysosomal degradation pathway is also used for a lipoprotein lipase mutant. This mutant carries a point mutation that sends the protein to lysosomal degradation. The authors speculated that a different oligosaccharide processing in this mutant caused increased affinity for the mannose-6-phosphate receptor (Buscá et al., 1996). So far supporting experimental evidence for this hypothesis is lacking.

1.5 Alpha-1-antitrypsin

Alpha-1-antitrypsin (α1-AT) is a member of the serine protease inhibitor superfamily. It is a 52kDa plasma protein with a half life of 4-5 days. The main function of α1-AT is the inactivation of serine proteases (Travis and Salvesen, 1983), especially of the human neutrophil elastase (Baugh and Travis, 2002). Because several serin proteases are important mediators of inflammation, α1-AT is critical for inflammation control. In fact, α1-AT is an

acute phase protein and undergoes a manifold increase after temperature elevation and inflammation.

α1-AT is N-glycosylated at the three residues N70, N107 and N271. This numbering includes the signal sequence. In some papers α1-AT is numbered excluding the signal sequence. The first and the third N-glycosylation sites are occupied in 100% of the cases, while the second was occupied at an average of 99±1,4% under physiological conditions (Hulsmeier et al., 2007). This second glycosylation site seems also to be most affected in several individuals with a congenital disorder of glycosylation type-I (Hulsmeier et al, 2007). Mutation of the second N-glycosylation decreased the secretion of α1-AT more than a single mutation of the other two N-glycosylation sites (Samandari and Brown, 1993). In accordance with this, Nyfeler et al. (2008) found that mutation of the second glycosylation site of α1-AT has the strongest impact on the affinity to its transport receptor ERGIC-53. Hepatocytes secrete the highest amount of α1-AT, but also other cells like bronchial epithelial cells (Cichy et al.,1997), type II pneumocytes (Venembre et al., 1994), neutrophile granulocytes (Paakko et al., 1996), and mononuclear phagocytes (Paakko et al., 1996) are able to produce it, although at lower amounts. Different mutations in the α1-AT gene have been identified that result in a partial or total α1-AT-deficiency due to secretion problems. This can cause emphysema in the third and fourth life decade. In addition, asthma is significantly more common in people suffering from α1-AT deficiency (Eden et al., 1997). α1-AT deficiency can also cause liver diseases like neonatal hepatitis or hepatic cirrhosis which ultimately leads to the development of hepatocellular carcinoma (Sveger, 1976; Eriksson et al., 1986). Especially the misfolded α1-AT Z variant (PIZ; Q347K) can lead to liver diseases, already early in childhood (Sveger, 1976). One out of 27 of North European populations is heterozygote for this mutation (Gooptu et al., 2009). In homozygotes only 15% of the α1-AT is secreted, whereas 85% is retained in the ER forming aggregates (Lomas et al., 1992). Several other mutants have been described. The truncation of carboxy terminal amino acids results, for example, in the null Hong-Kong mutant. There is also one mutant which is less efficiently secreted due to a mutation in its first N-glycosylation site (Bristol) (Lovegrove et al., 1997). Antitrypsin was also shown to be closely related to the HIV infectivity outcome. The amount of total and active antitrypsin in the plasma of HIV patients changes during disease progression. Asymptomatic HIV patients show normal α1-AT levels, but the amount of active α1-AT is decreased. Symptomatic pre-AIDS HIV patients have normal values of active α1-AT, but the total amount of antitrypsin is increased (Bristow et al., 2001). α1-AT was shown to have

antiviral effects against the HIV virus. α1-AT inhibits entry of HIV and its intracellular production (Congote, 2007). Due to the important role of α1-AT in human pathologies, it is a key to understand its sub-cellular trafficking. A first important contribution has been made by Nyfeler et al (2008) who used yellow fluorescence protein (YFP) protein complementation assay to identify ERGIC-53 as the cargo-receptor for α1-AT.

1.6 Protein complementation assays

Several methods have been developed to study protein-protein interactions (Tord et al., 2007) including the yeast two hybrid system (Fields and Song, 1989), co-immunoprecipitation, covalent crosslinking (Phizicky and Fields, 1995; Ren et al., 2003) or fluorescence resonance transfer (FRET) (Jares-Erijman and Jovin, 2003) and protein complementation assays (PCA) (Morell et al., 2009).

The pre-requisite for a protein complementation based assay is a protein reporter that can be cleaved into two non-active fragments, which reassemble upon close contact. Each of these fragments is then fused to one of two hypothetical interacting proteins (bait and prey). If the two proteins interact with each other, the attached reporter fragments are brought into close contact and assemble non-covalently to the full reporter. Usually a linker sequence of serines and glycines is introduced between reporter and bait/prey protein to provide flexibility. All protein complementation based methods were developed to trap transient protein-protein interactions. Several proteins were shown to be suited as reporter proteins like ubiqiquitin, dihydrofolate reductase (DHFR), β-lactamase, TEV protease, green fluorescent proteins and variants or luciferase.

Split ubiquitin was the first protein used for protein complementation assays (PCA) (Johnsson and Varshavsky, 1994) (Fig.8). Ubiquitin is usually cleaved very rapidly by intracellular proteases. The split ubiquitin fragments are not targeted by proteases. The N-terminal ubiquitin fragment (Ub_N) is fused to protein A (A- Ub_N). The C-terminal ubiquitin fragment (Ub_C) is fused to protein B (B- Ub_C). In case protein A and protein B interact, the ubiquitin fragments are brought in close contact and form a native-like ubiquitin. Subsequently the native-like ubiquitin is cleaved by ubiquitin proteases. The Ub_C is fused to a small monomeric non-host reporter protein like HA-tagged dihydrofolate reductase. This reporter protein will be released upon cleavage of the ubiquitin. Transcription factors are also suitable as reporter proteins. Upon ubiquitin cleavage the transcription factor is released, moves into the nucleus and activates transcription of reporter genes (Stagljar et al., 1998).

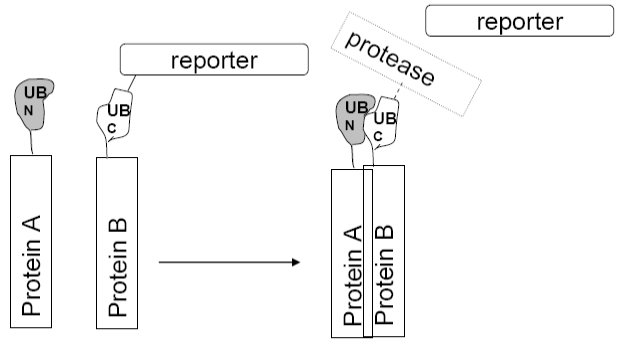
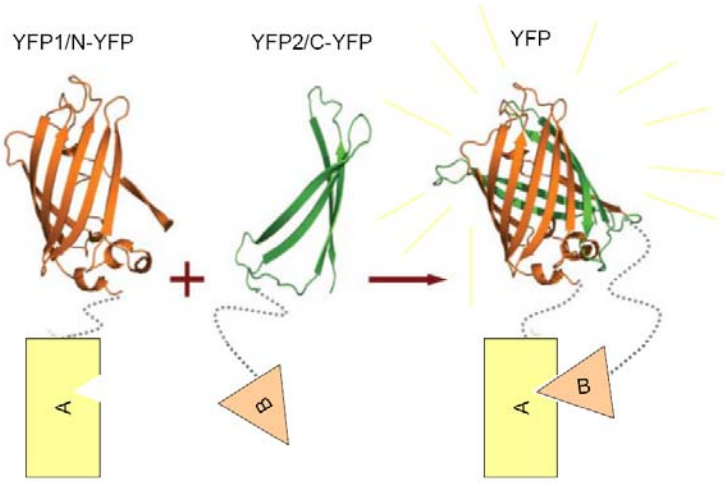


Figure 8 ***Split-ubiquitin system****: Protein A is tagged with the N-terminal part of the ubiquitin (UB_N) and protein B carries the C-terminal part of the ubiquitin (UB_C). A reporter molecule is attached to the UB_C. If the two proteins interact the fragments of the ubiquitin are brought in close contact and reform the full ubiquitin. Ubiquitin protease cleaves the ubiquitin and releases the reporter molecule from the ubiquitin. The kind of reporter determines the readout.*

Murine dihydrofolate reductase (DHFR)-based protein complementation was used to measure protein-protein interaction in bacteria in vivo (Pelletier et al., 1998). The reconstituted mammalian DHFR confers resistance towards the drug trimethoprim that targets bacterial DHFR with high affinity. Upon successful interaction of the two tested proteins, murine DHFR is reconstituted and the bacteria will survive on a trimethoprim containing agar plate. β-lactamase-based protein complementation is suitable to detect protein interactions in vitro, but also in vivo in mammalian cells. Upon complementation of the two β-lactamase fragments its enzymatic activity is reconstituted. A chromogenic substrate added to the in vitro reaction changes its color upon hydrolysis by β-lactamase (Galarneau et al., 2002).

Split tobacco etch virus protease (TEV) is another system that can be used to validate protein-protein interactions in mammalian cells (Wehr et al., 2006; Wehr et al., 2008). The TEV fragments are coupled to either a transcription factor or to luciferase. Upon reconstitution of the protease activity the transcription factor is released enters into the nucleus and activates a reporter gene. In case of using luciferase as reporter gene lucferin has to be added to visualize it.

The bimolecular fluorescent complementation assay is based on the reconstitution of green fluorescent protein (GFP) or one of its variants. So far ten fluorescent proteins are suitable to support fluorescence complementation (Shyu and Hu, 2008). Reconstitution of the two non-fluorescent fragments results in a fluorescent signal that can be measured by a plate reader or can be visualized by fluorescence microscopy. In case of using yellow fluorescent protein (YFP) as a reporter the N-terminal part N-YFP/YFP1 harbors amino acid 1-158 and the C-terminal part C-YFP/YFP2 harbors amino acid 159-239 (Fig.9).



YFP1
MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTFGYGL
QCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNIL
GHKLEYNYNSHNVYIMADKQ

YFP2
KNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSYQSALSK
DPNEKRDHMVLLEFVTAAGITLGMDELYK

Figure 9: Basic principle of fluorescence complementation. Protein A is fused to the N-terminal fragment of YFP (YFP1/N-YFP) and protein B is fused to the C-terminal fragment of the YFP protein (YFP2/C-YFP). In case protein A and protein B interact with each other the two fragments complement and YFP is reconstituted. Adapted from Morell et al., 2007
In the lower panel the protein sequences of YFP1 (orange) and YFP2 (green) are indicated.

The intensity of the fluorescent signal is highly dependent on the interaction of the bait and prey protein to which the fragments are fused (Morell et al., 2007). In most studies the reconstituted YFP complex was irreversible (Kerppola, 2008), although there are a few studies that claimed the interaction to be at least partially reversible (Guo et al., 2005; Cole et al., 2007). Bimolecular fluorescent complementation has been used successfully in bacteria, yeast, *C. elegans* and in mammalian cells (Hu et al., 2002; Cole et al., 2007; Min et al., 2007). The first study that showed the proof of principle for fluorescent complementation was performed in E.coli. GFP was split into two fragments and fused to two artificial interacting proteins, showing a high affinity (Ghosh et al., 2000). Shortly after YFP fragments were used in mammalian cells to visualize the Ca^{2+}dependent interactions between calmodulin and its target peptide M13 (Nagai et al., 2001). Several other applications to study protein interactions in mammalian cells followed, like oligomerization of receptors (Vidi et al., 2008), interaction between components of the splicosom (Ali et al., 2008), interaction between components of the nucleosome (Cherukuri et al., 2008) and interaction between the cargo receptor ERGIC-53 and its secretory cargo (Nyfeler et al., 2005; Nyfeler et al., 2008). YFP-PCA based approaches were further used to visualize post-translational modifications, protein folding and protein aggregation (Shyu and Hu, 2008).

The multicolor BiFC assay allows the visualization of multiple protein interactions (Fig. 10). The N-terminal fragments of two different fluorescent proteins are fused to two proteins (*e.g.* protein A fused to N-YFP, protein B fused to N-CFP). The C-terminal fragment (*e.g.* C-CFP) that is able to complement with both N-termini and reconstitute a fluorescent protein is fused to a third protein (X fused to C-CFP). Interaction of protein A with protein X results in complemented YFP and a yellow fluorescent signal is measured. Interaction of protein B with protein X results in complemented CFP and in a blue fluorescent signal. This approach allows to study efficiencies of complex formation between different interaction partners (Hu and Kerppola, 2003). The multicolor BiFc assay was used to study the competing interaction of seven different γ-subunits of G-proteins with the same β1-subunit (Mervine et al., 2006).

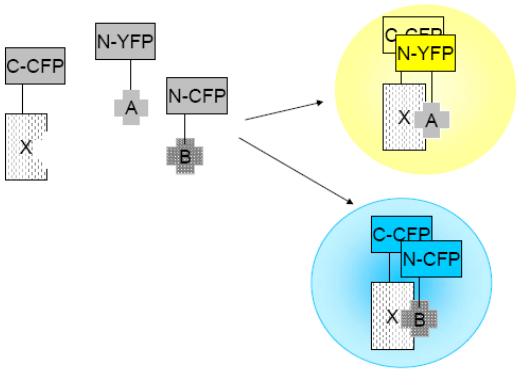


Figure 10 ***Multicolor BiFc:*** *Protein X is tagged with the C-terminal fragment of CFP (C-CFP). C-CFP interacts with the N-terminal YFP fragment (N-YFP) to reconstitute the YFP protein. Interaction with the N-terminal fragment of CFP (N-CFP) results in a reconstituted CFP. Therefore interaction of protein X with protein A results in a YFP signal and interaction of protein X with protein B results in a CFP signal.*

A BiFc assay combined with a FRET assay allows even to monitor formation of ternary complexes (Shyu et al., 2008) (Fig.11). Two interacting proteins (A and B) are fused to the two fragments of YFP and a third protein (X) is fused to CFP. The interaction of protein A and protein B reconstitutes YFP. This complemented YFP is a good FRET partner for CFP. If proteins A, B and X trimerize, YFP will be complemented and a FRET signal will be measurable. The association of a ternary transcription factor complex was monitored by this technique (Shyu et al., 2008).

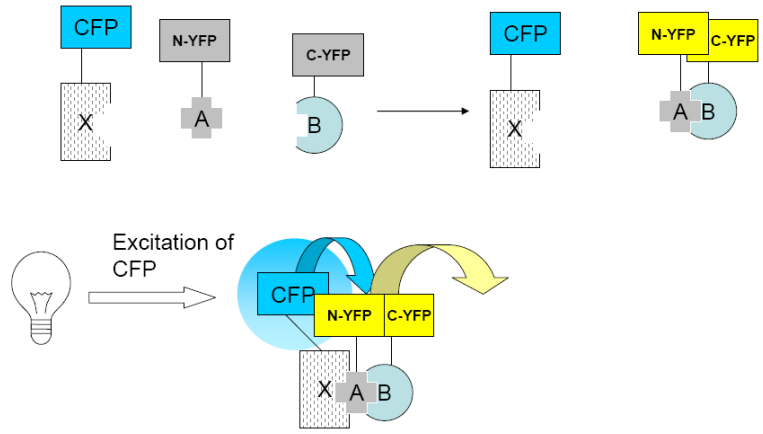


Figure 11 ***BiFc-FRET****: Protein A fused to N-YFP interacts with protein B fused to C-YFP. Upon interaction the YFP is reconstituted. Subsequently protein A bound to protein B interacts with protein X fused to full length CFP. Excitation of CFP leads to the emission of blue light (blue arrow) which excites the reconstituted YFP. Upon excitation YFP emits yellow light (yellow arrow) which indicates interaction of all three proteins.*

Overall bimolecular fluorescent complementation assays have several advantages. The readout is direct (no enzymatic assay is needed for it). The intracellular compartment where the interaction takes place can be visualized by fluorescent microscopy. The assay detects even weak and transient interactions (Morell et al., 2007). One disadvantage of the fluorescent complementation is that the irreversibility of the interaction precludes studies of dynamic interactions between proteins. A luciferase based complementation assay provides a solution for this problem, as this interaction is reversible (Luker et al., 2004; Remy and Michnick, 2006).

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2 Aim of the thesis

Screening for a VIP36 interaction partner and elucidating the function of VIP36 in the secretory pathway

Several studies demonstrated the importance of the L-type lectin ERGIC-53 in intracellular transport of glycoproteins. Much less is known about the function of the homologous L-type lectin, VIP36. We have information about the localization, 3D-structure and sugar binding affinity of VIP36. However, we have no information about the biological/cellular role of VIP36. Some studies suggested a role as a cargo receptor in intracellular transport, but it is not clear at which level of the secretory pathway VIP36 operates. VIP36 has also been proposed to act in quality control, but no experimental evidence supports this hypothesis so far. The aim of my project was to identify an interaction partner for VIP36, which should then enable me to draw further conclusion about its cellular function. Therefore, I used a YFP-fragmentation-complementation assay (YFP-PCA) based approach to screen a human liver library for luminal VIP36 interaction partners. The YFP-PCA shows a lot of advantages compared to other methods. One important aspect is that it allows trapping very weak interactions because of the irreversibility of the YFP complementation. This is important, as the carbohydrate based interaction between mammalian lectins and their glycoprotein interaction partners is usually of weak affinity. After completing the screen, I validated the interaction biochemically. YFP-PCA further allows visualizing the subcellular localization of complex formation. Again, knowing the localization of VIP36 and its potential client protein could give a hint about its function. I determined the effect of depletion of VIP36 (by siRNA mediated knockdown) on the identified interaction partner for VIP36. In addition, the effect of VIP36 knockdown on the morphology and function of the secretory pathway was determined. Taken together the results of this study should provide a deeper insight into the function of VIP36.

3 Results

3.1 Role of the lectin VIP36 in post-ER quality control of human α1-antitrypsin

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Role of the lectin VIP36 in post-ER quality control of human α1-antitrypsin

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Running title: VIP36 binds α-1-antitrypsin

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ABSTRACT

The L-type lectin VIP36 localizes to the Golgi apparatus and cycles early in the secretory pathway. In vitro, VIP36 binds high mannose glycans with a pH optimum of 6.5, a value similar to the luminal pH of the Golgi apparatus. Although the sugar binding properties of VIP36 in vitro have been characterized in detail, the function of VIP36 in the intact cell remains unclear since no convincing glycoprotein cargo has been identified. Here we used yellow fluorescent protein (YFP) fragment complementation to identify luminal interaction partners of VIP36. By screening a human liver cDNA library, we identified the glycoprotein alpha-1-antitrypsin (α1-AT) as a cargo of VIP36. The VIP36/ α1-AT complex localized to Golgi and ER. In the living cell, VIP36 bound exclusively to the high-mannose form of α1-AT. The binding was increased when complex glycosylation was prevented by kifunensine and abolished when the glycosylation sites of α1-AT were inactivated by mutagenesis. Silencing VIP36 accelerated α1-AT transport, arguing against a role of VIP36 in anterograde traffic. The complex formed by VIP36 and α1-AT in the Golgi recycled back to the ER. The combined data are most consistent with a function of VIP36 in post-ER quality control of α1-AT.

INTRODUCTION

N-glycosylation is one of the most common protein modifications in the cell (1). Two third of all proteins present in the SWISS-PROT protein sequence data bank have at least one potential N-glycosylation consensus site. N-glycosylation plays an important role in numerous cellular functions including development (2), wound repair (3), and innate immunity (4). In addition, N-glycosylation is important for quality control and transport of glycoproteins in the secretory pathway (5-8). These processes require interaction of the glycans with different lectins. The major lectins involved in ER quality control are the chaperone proteins calnexin and calreticulin. Other lectins, such as ERGIC-53, act as cargo receptors involved in intracellular protein transport. ERGIC-53 is the best characterized anterograde cargo receptor in higher eukaryotic cells (9, 10). ERGIC-53 is a member of the leguminous type (L-type) lectin family which also comprises ERGL, VIP36 and VIPL (11-13). L-type lectins are type 1 membrane proteins with a large luminal domain possessing the carbohydrate recognition domain (CRD) and a short cytosolic domain that mediates sorting within the early secretory pathway. ERGIG-53 localizes mainly to the ER-Golgi intermediate compartment (ERGIC) and to the ER (14, 15) and cycles between these two compartments. This lectin is involved in ER export of several glycoproteins, including cathepsin C, cathepsin Z, blood coagulation factors V and VIII, alpha 1-antitrypsin (α1-AT) and immunoglobulin M (16-20) by binding to high mannose glycans in a Ca^{2+}- and pH-dependent manner (21, 22).

VIP36 was discovered in a screen for proteins involved in apical sorting from the trans-Golgi network (23). VIP36 cycles between Golgi and ER (24). Its CRD exhibits 46% similarity to the CRD of ERGIC-53. Although sharing some similarities with ERGIC-53, VIP36 has several features that differ. In contrast to the unglycosylated ERGIC-53, VIP36 has one N-glycosylation site and acquires complex glycosylation (25). While ERGIC-53 forms homodimers and homohexameres (16), VIP36 does not oligomerize (25, 26). Moreover, VIP36 localizes mainly to the Golgi and only to some extent to the ERGIC (27). Other studies found VIP36 to also localize to post-Golgi secretory vesicles and to the plasma-membrane (28, 29). VIP36 interacts with high mannose glycans most efficiently at pH of 6.5 (30). Such a pH is characteristic for the Golgi apparatus, and it has been postulated that VIP36 may bind glycoproteins in the Golgi rather than the ER (31). Although the sugar binding properties of VIP36 have been elucidated in quite some detail, the biological function of VIP36 has remained an enigma. VIP36 was suggested to interact with rat α-amylase (32) and clusterin (28), and hence a role in post-Golgi anterograde transport was postulated. By contrast, a role

for VIP36 in quality control was proposed based on the finding that BiP can bind to complex-glycosylated VIP36 in the ER, but no corresponding glycoprotein cargo was identified (33). We reasoned that the identification of a convincing cargo glycoprotein may provide insight into the function of VIP36 in the secretory pathway. A major problem in identifying cargo glycoproteins of animal lectins is their low affinity and transient binding, but yellow fluorescent (YFP) protein fragment complementation (PCA) is a promising emerging technique to overcome these limitations (34, 35). Here we used a previously established YFP PCA-based screening approach (19) to identify new VIP36 interaction partners. By screening a human liver DNA library we identified α1-AT as a cargo glycoprotein of VIP36 and provide evidence for a role of VIP36 in post-ER quality control of α1-AT.

RESULTS

YFP PCA-based screening approach

In our search for VIP36 interaction partners we adopted a previously developed screening strategy that is based on a YFP protein complementation assay (YFP-PCA; (19)). YFP-PCA is well suited to detect weak interactions in the secretory pathway, as the complementation of YFP is irreversible (35, 36). This feature is important, because carbohydrate-based interactions of mammalian L-type lectins are of low affinity. To be suitable for YFP-PCA-based screening, the VIP36 construct should meet two principal requirements: correct localization in the secretory pathway and lectin activity. We cloned VIP36 without its signal sequence into a vector containing the signal sequence of calreticulin and the C-terminal fragment of YFP (YFP2)-fragment (YFP2-VIP36). The signal sequence of calreticulin ensures efficient translocation of the construct into the ER. The YFP2-VIP36 construct was expressed in HeLa cells to determine its localization (Figure 1 A-C). In cells with low to medium expression, YFP2-VIP36 mainly localized to the Golgi apparatus, identified by staining for the cis-Golgi marker GM130 and the medial Golgi marker giantin, and to a lesser extent to peripheral punctate structures identified as ERGIC (Figure 1A-C). This localization is identical to that described for endogenous VIP36 (27). To test if the construct has mannose lectin activity like wild-type VIP36 (37, 38), YFP2-VIP36 was expressed in HeLa cells and the cells were labeled with FITC-tagged mannose-BSA (22). As shown in Figure 1D, mannose-BSA bound to the Golgi region of YFP2-VIP36-expressing cells. Non-transfected cells gave no specific signal, most likely due to the only low amount of endogenous VIP36

and other endogenous mannose-binding lectins. The combined data indicate that our YFP-VIP36 probe fulfills the criteria required for use as functional bait in a DNA library screen.

Identification of α1-antitrypsin as interaction partner of VIP36

For the YFP PCA-based screening we co-expressed YFP2-VIP36 and a C-terminally-tagged human liver cDNA library in COS cells. COS cells were chosen because they express the large T antigen and can thus replicate transfected plasmids carrying the SV40 eukaryotic origin of replication. This ensures that the transfected plasmids are amplified and enough prey is provided for an interaction with the bait and allows for recovery of prey plasmids. Upon YFP complementation, YFP positive cells were isolated by fluorescence activated cell sorting (FACS). Cells co-transfected with YFP2-VIP36 and the YFP1-tagged human liver cDNA library exhibited more YFP-positive cells than cells transfected with YFP2-VIP36 alone (Figure 2A). 166 positive cells were sorted (Figure 2A, right panel), and library plasmids were isolated and transformed into bacteria. We recovered more colonies than sorted cells, as each cell contained several library plasmids. The colonies were then screened for the presence of an insert by PCR (Figure 2B). By this we wanted to make sure that we evaluated colonies that contained an insert and not only empty vectors. Nearly all tested colonies contained inserts, their size ranging from 750bp to 4000bp. Although COS cells are preferable for the initial screening due to high expression levels, false positive clones can arise under these conditions. We therefore retested the primary hits in HeLa cells. cDNAs encoding the potential interaction partners were co-transfected with YFP2-VIP36 and the signal obtained by fluorescence complementation was measured (Figure 2C) and compared to the negative control (YFP2-VIP36 alone). Interestingly, the plasmids leading to the highest fluorescence complementation with VIP36 encoded α1-antitrypsin (α1-AT). The YFP signal resulting from the interaction of YFP2-VIP36 and α1-AT-YFP1 was 30%-40% higher than the negative control. α1-AT-YFP1 appeared three times among 200 plasmids tested.

α1-AT is a 52 kDa soluble secretory glycoprotein. Its main role is inactivation of serine proteases in blood plasma (39). α1-AT deficiency, one of the most common genetic human diseases, results from the lack or misfolding of this protein. Misfolded α1-AT leads to liver cirrhosis and lung emphysema (40). We focused our further analysis on a characterization of the VIP36/α1-AT interaction.

Characterization of the interaction between VIP36 and α1-AT in the cell

We first visualized the VIP36/α1-AT interaction in HeLa cells by YFP PCA. YFP1-VIP36 and YFP2-α1-AT were co-expressed in HeLa cells, and the YFP signal resulting from the

complementation was visualized by fluorescence microscopy (Figure 3A). The YFP signal exhibited a Golgi- and ER-pattern. We confirmed the Golgi localization of the YFP complex by co-staining with the cis-Golgi marker GM130. Although this analysis does not indicate where the initial interaction takes place, it clearly shows that the YFP1-VIP36/YFP2-α1-AT complex is restricted to the early secretory pathway where the initial interaction of VIP36 and α1-AT can be expected to occur. As a negative control we used albumin-YFP2, a secretory protein that is not glycosylated. VIP36 showed no fluorescence complementation with albumin (Figure 3B). Of note, the expression levels of albumin-YFP2 and YFP2-α1-AT were similar. The reason for the low albumin signal in the cells is that it does not interact with VIP36 and is therefore rapidly secreted into the culture medium (Figure 3B).

To test if the interaction with α1-AT is sugar dependent, we used an N-glycosylation deficient α1-AṬmutant in which all three N-glycosylation sites were mutated to alanine termed triple mutant" (19). YFP PCA experiments showed a markedly reduced interaction of VIP36 with the triple mutant compared to wild type α1-AT (Figure 3B). We also tested a potential interaction of VIP36 with the two misfolded and ER-retained α1-AT variants Z-mutant and Null Hong-Kong mutant (NHK). The Z-mutant has a glutamic acid to leucine point mutation at position 342. The NHK mutant is a truncated variant. A dinucleotide deletion in the leucine at position 318 leads to a premature stop-codon. VIP36 interacted poorly with both the Z-mutant and the NHK mutant in the intact cell. These results are very similar to those previously reported for ERGIC-53 (19) but require different interpretations (see Discussion).

VIP36 interacts with the high mannose form of α1-AT in vivo

To test if in the intact cell the VIP36/α1-AT interaction occurs before or after the medial-Golgi, we co-expressed YFP1-α1-AT and YFP2-VIP36 in HeLa or COS cells for complementation and immunoprecipitated the complex from cell lysates with anti-YFP2 (Figure 4A,B). Digestion of the immunoprecipitated complex with endo-H showed that co-immunoprecipitated α1-AT was endoH-sensitive in both cell lines. This result indicates that VIP36 binds high-mannose and not complex-glycosylated α1-AT in the cell, hence the interaction occurs before the medial-Golgi. As a consequence, increasing the amount of high mannose α1-AT should result in an increase in fluorescence complementation of VIP36 with α1-AT. We tested this prediction by pre-treating the cells with kifunensine. Kifunensine inhibits Golgi mannosidase-I by 100% and ER mannosidase by 57% and thereby prevents

complex glycosylation (41). Treatment with kifunensine overnight increased the interaction with α1-AT by 41% (Figure 4C). α1-AT was still secreted from kifunensine-treated cells (Figure S1), and the intracellular localization of the VIP36/α1-AT complex was unchanged (Figure 4D). Since VIP36 is complex-glycosylated, we had to exclude the possibility that the increased α1-AT binding in response to kifunensine was due to a change in VIP36 glycosylation. To this end, we mutated the asparagine of the N-glycosylation consensus site to alanine (N183A). The resulting non-glycosylated VIP36-N183A still localized to Golgi and ERGIC (Figure 4E) and exhibited unchanged binding properties in the YFP PCA (Figure 4F). Clearly, the change in the glycosylation status of VIP36 is not responsible for the kifunensine effect. The preference for the high-mannose form excludes initial VIP36/α1-AT binding in medial- and trans-Golgi.

VIP36 silencing accelerates α1-AT transport

The interaction between VIP36 and α1-AT suggests an involvement of VIP36 in the intracellular transport of α1-AT. Provided VIP36 operates in anterograde transport one would expect that its knockdown inhibits the intracellular transport of α1-AT in a similar way as has been reported for ERGIC-53 (19). We tested this possibility in HepG2 cells that express α1-AT and VIP36 endogenously. In HepG2 cells VIP36 localizes to Golgi and ERGIC like in other cells (Figure 5A). Knockdown of VIP36 by siRNA was very efficient and reduced endogenous VIP36 levels to less then 10% (Figure 5B). We focused on early events of α1-AT secretion, because the complex of α1-AT and VIP36 localized to the ER-Golgi system. The cells were pulse-labeled with ^{35}S-methionine for 10 min and chased for 0, 15, and 30 min in the presence of unlabeled methionine in excess. After a 30 min chase, more than 50% of the labeled α1-AT was complex glycosylated, thus had passed the medial Golgi (Figure 5C). We calculated the percentage of complex glycosylated to total α1-AT as an indicator for transport efficiency. Remarkably, VIP36 knockdown accelerated α1-AT transport compared to control cells. After a 30 min chase, the amount of complex glycosylated α1-AT was increased by 12% and this increase was statistically significant. To rule out that a general acceleration of intracellular transport occurred, we followed the secretion of endogenously-expressed albumin over the same time period (Figure 5D). VIP36 silencing did not affect albumin secretion. The data indicate that VIP36 retards the intracellular transport of α1-AT.

Evidence for recycling of the VIP36/α1-AT complex from Golgi to ER

One possibility consistent with the result in this study would be a function of VIP36 in retrograde traffic, whereby a fraction of high-mannose α1-AT would be recycled from Golgi-to-ER. We addressed this notion by YFP-PCA. Visualizing the α1-AT-VIP36 complex should allow us to follow the route of transport when de novo protein synthesis is blocked. To study the fate of the α1-AT/VIP36 complex we expressed YFP2-α1-AT and YFP1-VIP36 in HeLa cells and treated them with cycloheximide to block protein synthesis. If VIP36 binds α1-AT in the Golgi and recycles it to the ER, the Golgi signal due to complex formation should decrease in favor of an increasing ER signal. This was indeed observed. 5 h after the addition of cycloheximide, the YFP fluorescence exhibited an ER rather than a Golgi pattern in most cells (Figure 6A). The percentage of cells with Golgi localization of the YFP signal decreased from 52% to 17% in response to cycloheximide (Figure 6B). The structure of the Golgi was unchanged, however, as indicated by the unchanged pattern of GM130 (Figure 6A). Similar results were obtained by a shorter 3 h cycloheximide treatment (not shown). It is noteworthy that cycloheximide has no effect on the bidirectional ER to Golgi traffic itself (42). This result indicates that VIP36 is involved in retrograde transport.

Why would VIP36 recycle a protein destined for secretion from the Golgi back to the ER? The most likely explanation is that VIP36 recognizes a fraction of α1-AT that escaped the ER quality control. It has been reported that VIP36 interacts with BiP (33). We wondered if the α1-AT/VIP36 complex formed in the Golgi interacts with BiP. To this end we expressed YFP2-VIP36, YFP2-α1-AT or YFP2.-VIP36 together with YFP1-α1-AT in Hela cells and immunoprecipitated YFP2. Figure 6C shows co-immunoprecipitation of BiP with the VIP36 construct. The amount of co-immunoprecipitated BiP was identical for singly expressed VIP36 and VIP36 co-expressed with the α1-AT. Less BiP co-immunoprecipitated with singly expressed α1-AT than with the α1-AT/VIP36 complex. Collectively, our results indicate that the α1-AT/VIP36 complex formed in the Golgi contains BiP and recycles to the ER where it is obviously trapped.

DISCUSSION

In the current work we have identified by YFP PCA α1-AT as cargo glycoprotein for the mammalian L-type lectin VIP36. The YFP signals after complementation were lower than those previously obtained for ERGIC-53 when identical expression levels were compared.

Together with the fact that all follow-up experiments confirmed that the VIP36/α1-AT interaction was specific, this difference indicates that a smaller fraction of α1-AT interacts with VIP36 than with ERGIC-53.

Our YFP PCA analysis of the VIP36/α1-AT interaction gave results seemingly similar to those previously obtained for the ERGIC-53/α1-AT interaction (19). In both cases no complementation occurred when the lectin was co-expressed with misfolded mutants or a glycosylation deficient mutant of α1-AT. However, an interpretation of these results has to take into account the pH optimum for glycan binding and the compartmentalization of the secretory pathway. VIP36 has a pH optimum of 6.5 (37) corresponding to the luminal pH of the cis/medial Golgi cisternae (43-45). By contrast, ERGIC-53 has a pH optimum of 7.4, the pH of the ER (21). Predictably, VIP36 cannot bind its cargo in the ER. Since the misfolded Z-mutant and NHK mutant of α1-AT are trapped in the ER and fail to be transported to the Golgi (46, 47) they do not meet VIP36 in an acidic compartment that allows glycan binding. Therefore, the lack of YFP complementation does not necessarily indicate that VIP36 cannot bind misfolded α1-AT. The case is different for the triple glycan mutant of α1-AT. A sizable fraction of this variant is secreted (48) and therefore meets VIP36 in the Golgi. The considerably reduced YFP PCA signal for the triple mutant therefore indicates that VIP36 is unable to recognize the non-glycosylated α1-AT.

Another key finding of our study is that silencing of VIP36 accelerates intracellular transport of α1-AT as opposed to silencing of ERGIC-53 which slows α1-AT transport. Importantly, albumin transport was unaffected, indicating that a VIP36 knockdown does not lead to a general acceleration of protein transport. These results argue against a cargo receptor function of VIP36 in the anterograde direction. Clearly, the presence of VIP36 slows intracellular transport of α1-AT1. We believe that VIP36 transiently retains only a small fraction of α1-AT1 because the YFP signal produced in the YFP PCA assay is considerably lower for the VIP36/α1-AT1 interaction than for the ERGIC-53/α1-AT1 interaction. By contrast, as expected for a cargo receptor mediating ER export, ERGIC-53 interacts with a large fraction (if not all) newly synthesized α1-AT1 (19).

What then is the function of VIP36 in α1-AT1 traffic? Since VIP36 shows highest binding to largely untrimmed high-mannose glycans in vitro (30, 37), and since the trimming inhibitor kifunensine increases the VIP36/α1-AT1 interaction, VIP36 may bind α1-AT1 molecules that fail to be trimmed in the Golgi and may recycle them to the ER. Although high-mannose glycoproteins are efficiently secreted, they have a considerably reduced half

life in the blood stream because they are rapidly endocytosed by the mannose receptor (49, 50). Thus, complex glycosylation is definitively required for protein stability in the organism. One of our results, however, is inconsistent with a role of VIP36 in mannose trimming. If VIP36 was involved in quality control at the level of mannose trimming in the Golgi, its knockdown should increase the fraction of high-mannose α1-AT1, but such an effect was not observed.

We propose that VIP36 acts in post-ER quality control in the Golgi by binding incompletely folded α1-AT1 that inadvertently escaped ER quality control and by recycling it back to the ER for an additional round of quality control. Consistent with a retrieval action, we observed a redistribution of the VIP36/α1-AT1 complex from Golgi to ER when de novo synthesis of VIP36 was blocked by cycloheximide. But why would the complex remain trapped in ER after recycling? A likely possibility is that due to the irreversible YFP complementation the VIP36/α1-AT1 complex forms larger assemblies with chaperones in the ER that cannot exit the ER anymore. Kifunensine not only prevents high mannose trimming, but also inhibits ER associated degradation (ERAD). Inhibition of ERAD allows misfolded α1-AT to escape the ER quality control and to be secreted (51). Therefore, we believe that the increased YFP PCA signal in response to kifunensine reflects complex formation of VIP36 with an increased amount of incompletely folded α1-AT that escaped ER quality control. How would VIP36 discriminate between folded and incompletely-folded α1-AT? One possibility is that the VIP36/α1-AT interaction requires both glycan and protein-protein interaction whereby the protein-protein interaction would only occur with incompletely-folded α1-AT. Glycan interaction alone would be insufficient to retain α1-AT in the Golgi. There is evidence that L-type lectins may not recognize solely glycans. Efficient binding of ERGIC-53 to its cargo cathepsin Z requires a combined carbohydrate/peptide motif (52). With ERGIC-53, however, the protein-protein interaction occurs only with the folded cargo. As an alternative possibility, the post-ER quality control of α1-AT by VIP36 may involve the chaperone BiP. BiP has indeed been shown to interact with complex-glycosylated VIP36 (33). We now show that BiP associates with the VIP36/α1-AT complex. In this scenario one could imagine the following events in quality control and transport of α1-AT. Newly synthesized α1-AT undergoes ER quality control in the ER involving the calnexin/calreticulin cycle (53, 54). Folded α1-AT can bind to ERGIC-53 and is exported actively from the ER (19). Some incompletely-folded α1-AT escapes ER quality control and reaches ERGIC and Golgi by default where it is bound to both VIP-36 and BiP under slightly acid conditions. BiP is known to have in part access to

post-ER compartments (55). The α1-AT/VIP36/BiP complex then recycles to the ER where α1-AT dissociates due to the increased pH and undergoes another round of folding.

In conclusion, the successful identification of α1-AT as a cargo glycoprotein of VIP36 has provided new insight into the function of VIP36. Our data, in combination with known features of VIP36, are most consistent with a glycan dependent quality control function in the Golgi. Molecular details of this mechanism, and whether this mechanism applies primarily to α1-AT or to additional glycoproteins, remain to be determined.

METHODS

Antibodies and inhibitors

The following antibodies were used: monoclonal mouse antibody (mAb) against BiP (BD Bioscience), monoclonal rabbit GFP antibody against the N-terminal YFP fragment (YFP1) for immunoblotting (Abcam), mAb against the C-terminal YFP fragment (YFP2) for immunoblotting, immunofluorescence and immunoprecipitation (Roche), and a rabbit polyclonal antibody (pAb) against GFP for immunofluorescence (Clonetech). A custom-made rabbit pAb was used to detect VIP36 by immunoblotting (Eurogentech). The pAb was raised against a synthetic peptide encompassing residues 298-312 (FLKSPKDNVDDPTGNC), using keyhole limpet hemocyanine as a carrier. Mouse mAb 1A2 against tubulin was kindly provided by Karl Matter (University College London, UK). Goat pAb against human α1-AT (MP Biomedicals) and sheep pAb against human albumin (The Binding Site) were used for immunoprecipitation. Antibodies used for immunofluorescence: Rabbit pAb against ERGIC-53 (56), mAb G1/133 against giantin (57), mouse mAb anti-GM130 (BD Transduction), anti-TGN46 (kind gift from Sreenivasan Ponnambalam, University of Leeds, UK) and rabbit pAb anti-VIP36 (27) (kind gift of Kai Simons, Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany). Alexa 488-, Alexa 568- (Molecular Probes Europe, Leiden, The Netherlands) and horseradish peroxidase-coupled antibodies (The Jackson ImmunoResearch Laboratories, West Grove, PA) were used as secondary antibodies. FITC-mannosidase BSA was a kind gift of Annie-Claude Roche (CNRS Orleans, France). The following inhibitors were used: Kifunensine (Calbiochem) and cycloheximide (Sigma).

siRNAs

SiRNA oligonucleotides against VIP36 were obtained from Qiagen (5'-AGAUAACUUCCACGGCUUA)dTdT-3'was used as sense and 5'-UAAGCCGUGGAAGUUAUCUdTdT-3' as antisense oligonucleotide) and from Ambion (5'-UGACUGCGGAUAUAACUGAtt-3' as sense and UCAGUUAUAUCCGCAGUCAca as an antisense oligonucleotide). Nonsilencing negative control siRNAs were obtained from Qiagen and Ambion.

Plasmids

VIP36 was amplified by PCR from the cDNA-NubG human liver library (DualSystem) without its signal sequence, using 5'-GGCCCTCGAGTTGATAT AACTGACGGCAACAG-3' as forward and 5'- GGCCCTCGAGTCAGTAGAAGCGCTTGTTCCG-3' as reverse primer. The PCR product was subcloned into the SScal-YFP2-pCMV vector (58) via XhoI restriction sites. The SScal-YFP1/YFP2-VIP36-pcDNA3 constructs (YFP1-VIP36, Y2-VIP36) were obtained by amplifying VIP36 with the following primers 5'-GGCCCTCGAGTTGATATAACTGACGGCAACAG-3'and 5'-GGCCTCTAGATCAGT AGAAGCGCTTGTTCCG-3'. The PCR product was digested with XhoI and XbaI and ligated into the SScal-YFP1-pcDNA3 or SScal-YFP2-pcDNA3 via XhoI and XbaI restriction sites (35). The construction of the SS-YFP2-alpha-1 antitrypsin mutants (YFP2-α1-AT) and albumin-YFP2 has already been described ((35, 58). The SS-YFP1-antitrypsin construct was obtained by subcloning α1-AT from the SS-YFP2-α1-AT plasmid into the SS-YFP1-vector. All constructs were verified by sequencing.

Generation of cDNA-YFP1 library

For the screening, a human adult liver cDNA-NubG library (DualSystems) was subcloned into vectors containing linker-YFP1 fragments covering all three reading frames. The previously described pcDNA3-YFP1 constructs (58) were digested with Xho and EcoR1 and the linker-YFP1 fragments were ligated into the pcDNA^{TM4}/TO vector (Invitrogen). The human adult liver cDNA-NubG library (Dualsystems) was digested with SfiI (NEB) at 50°C overnight. The inserts were separated by agarose gel electrophoresis. Inserts ranging from 0.75 kb to 4kb were excised. The inserts were ligated via two SfiI sites into the vector mix containing all three reading frames of pcDNA^{TM4}/TO-[YFP1]. Ligation products were transformed into XL-gold bacteria (Stratagene). The transformed bacteria were plated on agar plates containing 25ug/ml Zeocin for selection allowing only growth of colonies transformed with library plasmids. The plates were incubated overnight at 37°C. The bacterial colonies

were collected in liquid broth medium. Plasmid isolation was perfomed using HiSpeed Plamid Maxi Kit (Qiagen).

Cell culture and transfection

HeLa and COS-1 cells were cultured in DMEM, supplemented with 10% FCS, antibiotics and fungizone. HepG2 cells were grown in MEM supplemented with 10% FCS and antibiotics. Transfection of plasmids was performed using Fugene6^{TM} according to the manufacturer's instruction. SiRNA transfection was carried out using HiPerfect (Qiagen) according to the manufacturer's instruction.

Immunofluorescence microscopy

Cells were washed with PBS and fixed in 4% paraformaldehyde. Cells were permeabilized using 0,2% Triton X-100 in PBG buffer (20mM glycine and 3%BSA in PBS pH7,4). Cells were incubated with the appropriate primary antibody in PBG followed by incubation with Alexa Fluor secondary antibodies, and the coverslips were mounted in Mowiol. For co-staining with mannose-FITC-BSA, samples were co-incubated with the indicated antibody and 100ug/ml FITC-mannose-BSA together in the presence of 1mM $CaCl_2$ and 1mM $MgCl_2$.
For the visualization of the YFP complementation signal, cells were washed twice in PBS, fixed in 4% PFA, washed several times with PBS, and mounted in Mowiol. Images were acquired on a Leica SPE 5 confocal microscope.

YFP-PCA- based FACS screening and plasmid isolation

The screen was performed as described in Nyfeler et al (19) with the following modifications. The YFP2-VIP36 construct and the cDNA-YFP1 library were co-transfected at a ratio of 1:1. The DNA isolated from the sorted cells was transformed into electro-competent bacteria (MC1061). Transformed bacteria were plated on 15cm agar plates containing Zeocin (25µg/ml). The first 100 tested colonies were analyzed for the presence of inserts by PCR. As nearly all colonies contained an insert, this step was omitted later on.

PCR screen of colonies

Individual colonies were picked and transferred into 5ul H2O. Then the PCR mix including Taq polymerase (Roche) was added. The forward primer:
5'-TCCACGCTGTTTTGACCTCC-3' and the reverse primer
5'-CGACCAGGATGGGCACCACC-3' were used to screen for the presence of an insert. The PCR products were subjected to agarose gel electrophoresis and visualized by ethidium bromide staining.

Fluorometric analysis of the YFP signal

HeLa cells were grown in 6 well plates. 0.5µg DNA of each plasmid was transfected. 24 h after transfection cells were washed with PBS, collected in PBS and spun down at 500xg. The cell pellet was resuspended in 200µl PBS and transferred into black 96 microwell plates (Nunc). Fluorescence was measured with a Victor2 fluorometer. 485nm was used as excitation and 535nm as emission wavelength. To assess the expression levels of the different constructs, cells were subjected to SDS-PAGE followed by Western blotting using nitrocellulose membranes and enhanced chemiluminescence (GE Healthcare).

Metabolic labeling and pulse- chase experiments

HepG2 cells were used for studying α1-AT transport. 500,000 HepG2 cells were seeded into 3,5cm plates and reverse transfected with either 10nM siRNA (Qiagen, Ambion) or 10nM control siRNA using Hiperfect (Qiagen). 72 h after siRNA transfection the pulse chase experiment was performed. Cells were starved in methionine-free MEM medium supplemented with 10% dialyzed FCS and 10mM glutamine (starving medium) for 20 min. The cells were pulsed in starving medium containing 100µCi S35 (Easy tag) per sample. Following the pulse period the cells were washed once with MEM medium supplemented with 10% FCS and 10mM methionine, and then chased for different time periods. Finally the cells were lysed, α1-AT1 was immunopreciptated and subjected to SDS-PAGE followed by autoradiography. Radiolabeled bands were imaged and quantified using a phosphorimager (Molecular Dynamics).

Immunoprecipitation

Cells were washed with PBS and lysed in IP-buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 2mM CaCl2, 1% TritonX-100 and protease inhibitors). The lysate was incubated for 30 min on ice and centrifuged at 20,000xg for 30 min. The supernatant was incubated with Protein G-Sepharose preloaded with the indicated antibody. The reaction was incubated overnight. The Sepharose beads were washed three times and eluted proteins were analyzed by SDS-PAGE.

Endoglycosidase H digestion

Immunoprecipitates were released from protein G-Sepharose beads by boiling in endoH buffer (0.25M sodium-citrate, 50mM Tris pH 6.0, 0.6% SDS). Samples were incubated with 5mU endoglycosidase H (Roche) for 3 h at 37°C.

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FIGURE LEGENDS

Figure 1:

YFP2-VIP36 localizes to the early secretory pathway and binds mannose-BSA. HeLa cells expressing YFP2-VIP36 were stained with anti-ERGIC-53 for the ERGIC (A), GM130 for the cis-Golgi (B), and giantin for the medial-Golgi (C). (D) Hela cells expressing YFP2-VIP36 were fixed, permeabilized and stained with a YFP2-specific antibody and with FITC-mannose-BSA. Note co-staining of FITC-mannose-BSA in the Golgi region with YFP2-VIP36 (arrows). FITC-mannose-BSA does not localize to the Golgi region in untransfected cells (arrowheads).

Figure 2:

YFP-PCA-based FACS screening for VIP36 interacting proteins. (A) COS cells were transfected with YFP2-VIP36 alone or together with the YFP1-tagged library plasmids. After 48 h, FACS sorting was performed. YFP was restored upon complementation and YFP-positive cells were sorted (encircled white dots, right panel). Cells expressing YFP2-VIP36

only were used as a negative control and showed nearly no false positive cells (white circle, left panel). DNA was extracted from YFP-positive cells and transformed in bacteria. (B, C): Examples for the validation procedure. (B) Colonies were tested for the presence of an insert by PCR. PCR products were electrophoresed on a 1% agarose gel containing ethidiumbromide. Note that all colonies contained an insert. (C) Plasmids isolated from transformed bacteria were co-expressed with YFP2-VIP36 in HeLa cells and the signal resulting from YFP complementation was measured. Cells transfected with YFP2-VIP36 alone were used as a negative control. The fluorescence signal of the single tested library plasmids was expressed as percentage increase compared to the negative control. Library-plasmids that resulted in an increase of the fluorescence by more than 30% were sequenced. Red bars are the plasmids that turned out to encode α1-AT.

Figure 3

Localization of the VIP36/α1-AT complex and interaction of VIP36 with α1-AT mutants. (A) HeLa cells co-transfected with YFP1-VIP36 and YFP2-α1-AT were fixed, permeabilized and stained with anti-GM130 anti. (B) HeLa cells were transfected with YFP1-VIP36 and different YFP2-tagged alpha1-anitrypsin mutants or YFP2-tagged albumin. After 24 h fluorometric analysis was performed. Upper panel: Background-corrected values obtained by fluorometric measurement. For background corrections the values from cells expressing YFP1-VIP36 only were subtracted. Bars represent means ± SD from 3 independent experiments (paired t-test, * $P < 0.05$). Lower left panel: Immunoblot showing expression levels. Lower right panel: HeLa cells were tranfected with plasmids encoding YFP2-albumin and YFP1-VIP36. Immunoblot shows the expression level of intracellular (IC) and secreted (M) albumin-Y2.

Figure 4

VIP36 interacts with the high mannose form of α1-AT and this interaction increases upon inhibition of complex glycosylation

HeLa (A) or COS (B) cells were co-transfected with YFP1-α1-AT and YFP2-VIP36. After 24 h, the cells were lysed and YFP2-VIP36 was immunoprecipitated with anti-YFP2. The immunoprecipitate was subjected to endoH digestion followed by SDS-PAGE. Left panels: Co-immunoprecipitated YFP1-antitrypsin detected with anti-YFP1. Right panels: Immunoprecipitated YFP2-VIP36 detected with anti-VIP36. (C) HeLa cells expressing YFP2-α1-AT and YFP1-VIP36 were treated with kifunensine (25uM) overnight (kif) or with solvent

(Co). After 24 h, fluorometric analysis was performed. Results are expressed as percent of control. Bars: means ± SD (3 independent experiments; paired t-test, * =P <0.05). Expression levels were determined by Western blotting: Upper panel shows YFP2-α1-AT expression; lower panel shows YFP1-VIP36 expression. (D) The VIP36-α1-AT complex shows comparable localization under control conditions (upper panel) and after kifunensine treatment (lower panel). (E) Localization of the glycosylation-deficient VIP36 mutant (YFP2-VIP36-N183A) in HeLa cells. (F) HeLa cells were transfected with YFP2-α1-AT and YFP1-VIP36-WT or the glycosylation deficient mutant (YFP1-VIP36-N183A). The fluorescent signal was not significantly different between cells expressing wild type and mutant VIP36.

Figure 5

Expression of VIP36 in HepG2 cells and effect of VIP36 silencing on the transport of α1-AT and albumin. (A) HepG2 cells were fixed, permeabilized, and co-stained with anti-VIP36 and anti-GM130. (B) Knockdown efficiency of two siRNAs targeting VIP36 in HepG2 cells. The knockdown was validated by immunoblotting with anti-VIP36. Tubulin was used as a loading control. (C) Pulse-chase experiments. HepG2 cells were transfected with VIP36 siRNA (VIP) or control siRNA (Co). After 72 h, the cells were pulsed with 35S-methionine for 10 min and chased for the indicated time. α1-AT was immunoprecipitated from cell lysates and from the medium. Complex glycosylated form (arrow) and high mannose form (arrow head) are indicated. Bars: means ± SD (mean of 4 experiments; two experiments per siRNA). Radiolabeled bands were quantified by phosphorimaging. The percentage of complex glycosylated α1-AT (intracellular and medium) compared to total α1-AT (intracellular and medium, both high mannose and complex glycosylated) was calculated for the different time points. The results were analyzed using paired t-test (* =P <0.05). (D) HepG2 cells were transfected with VIP36 siRNA or control siRNA. After 72 h, cells were pulsed with 35S methionine for 10 min and chased for the indicated times. Albumin was immunoprecipitated from cell lysates and media. Means ± SD (3 independent experiments).

Figure 6

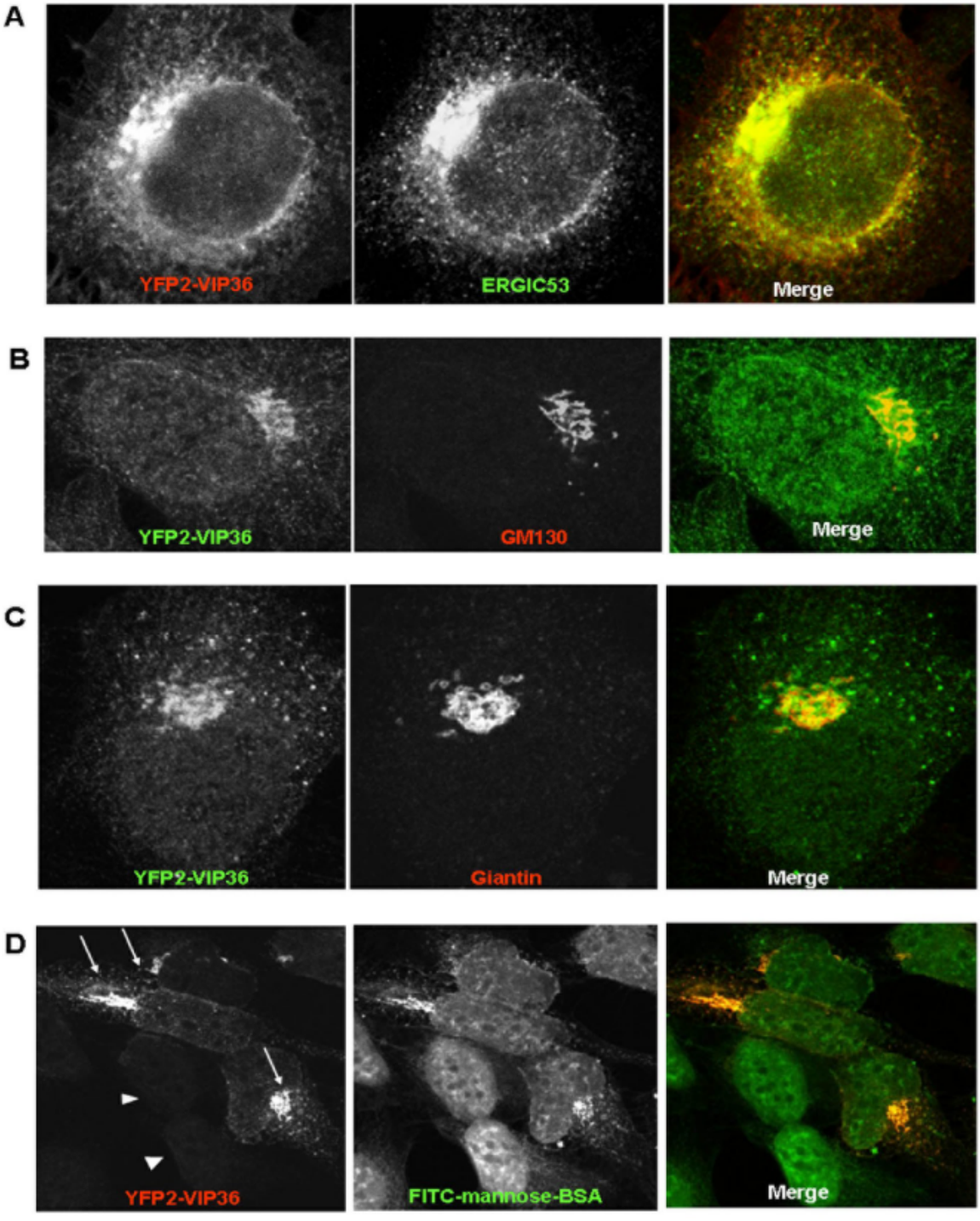
Recycling of the VIP36/α1-AT complex to the ER. (A) HeLa cells expressing YFP1-VIP36 and YFP2-α1-AT were treated with 100 ug/ml cycloheximide (+Cx) for 5 h or with solvent (Co). Cells were fixed, and the complex localization was determined by fluorescence microscopy. Note that the Golgi is not affected by cycloheximide treatment, as indicated by co-staining with the Golgi marker GM130. (B) Percentage of cells exhibiting Golgi

localization of the VIP36-α1-AT complex. Means + SD (3 independent experiments; paired t-test, * =P <0.05). (C) HeLa cells expressing YFP2-VIP36, YFP2-α1-AT, or YFP2-VIP36 together with YFP1-VIP36 were lysed and the YFP2-tagged proteins were immunoprecipitated. The immunoblot shows the amount of BiP that co-immunoprecipitated in the different samples.

Figure S1

Secretion of α1-AT from HepG2 cells after kifunensine treatment. HepG2 cells were pretreated with kifunensine, pulsed for 10 min with 35S-methionine, and chased for 60 min. Kifunensine was present during the entire experiment. α1-AT was immunoprecipitated from the medium. Half of each fraction was digested with endoH and the other half was left untreated. Samples were subjected to SDS-PAGE and radioactive bands were visualized by phosphorimaging.

Figure 1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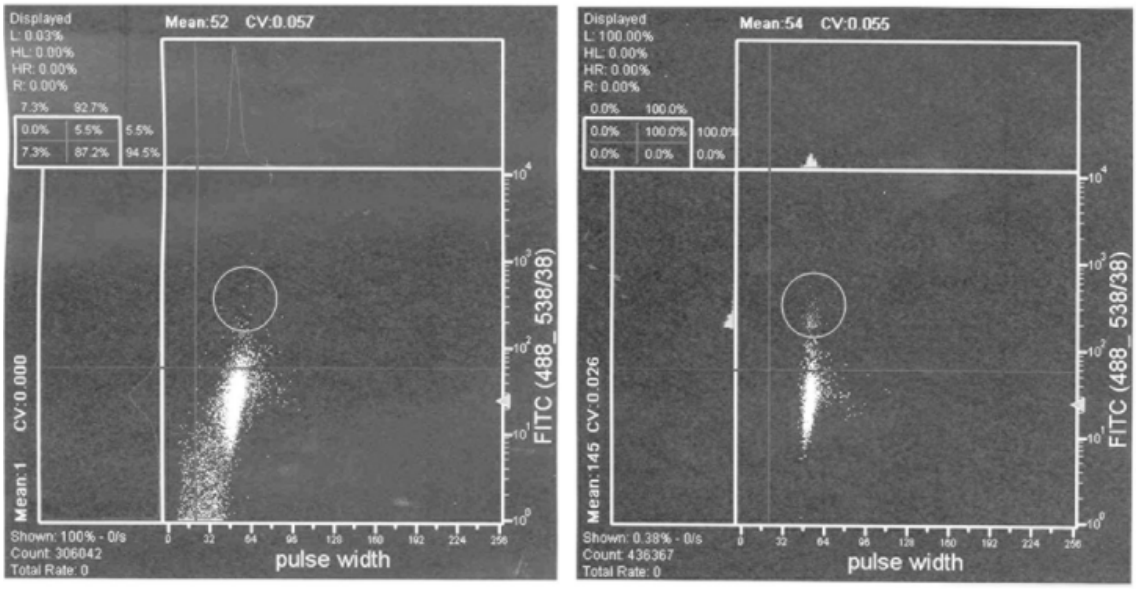
YFP2-VIP36
ERGIC53
Merge
B
YFP2-VIP36
GM130
Merge
C
YFP2-VIP36
Giantin
Merge
D
YFP2-VIP36
FITC-mannose-BSA
Merge

Figure 2

A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pulse width
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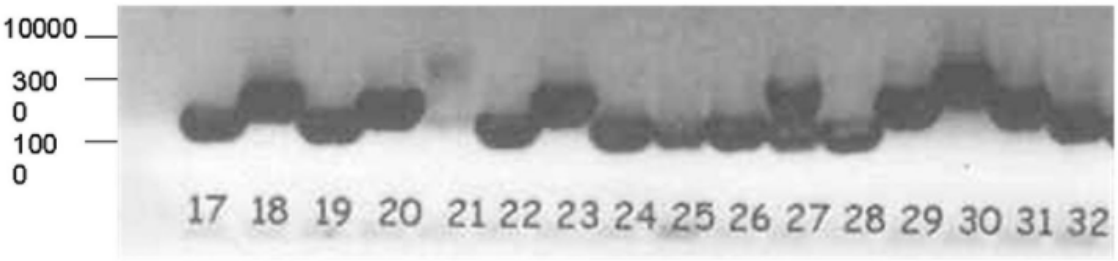
B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3000
1000
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C

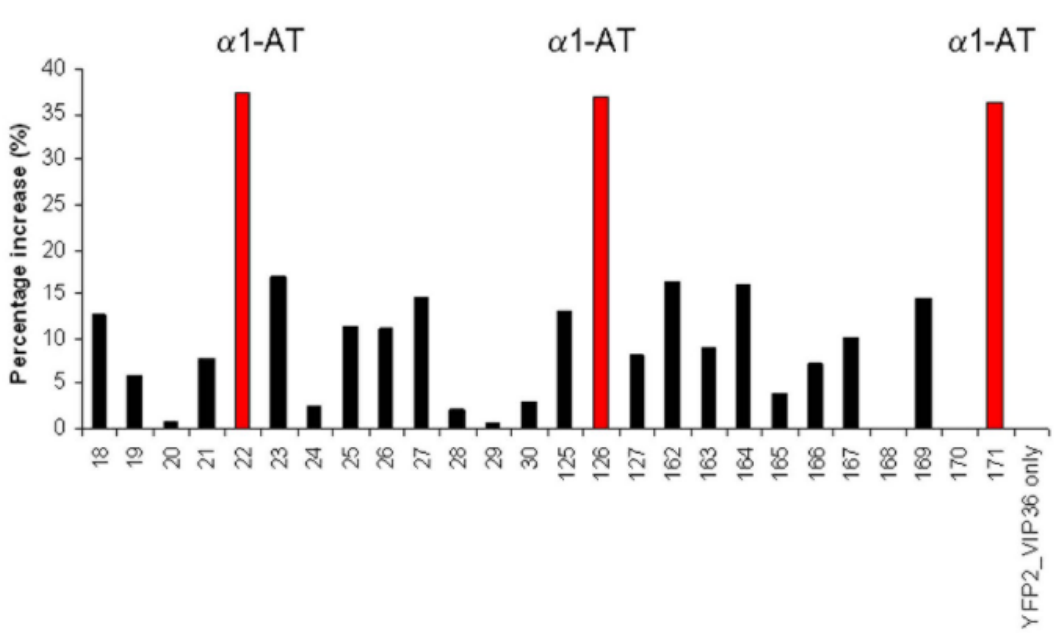

α1-AT
α1-AT
α1-AT
Percentage increase (%)
40
35
30
25
20
15
10
5
0
18 19 20 21 22 23 24 25 26 27 28 29 30 125 126 127 162 163 164 165 166 167 168 169 170 171
YFP2_VIP36 only

Figure 3

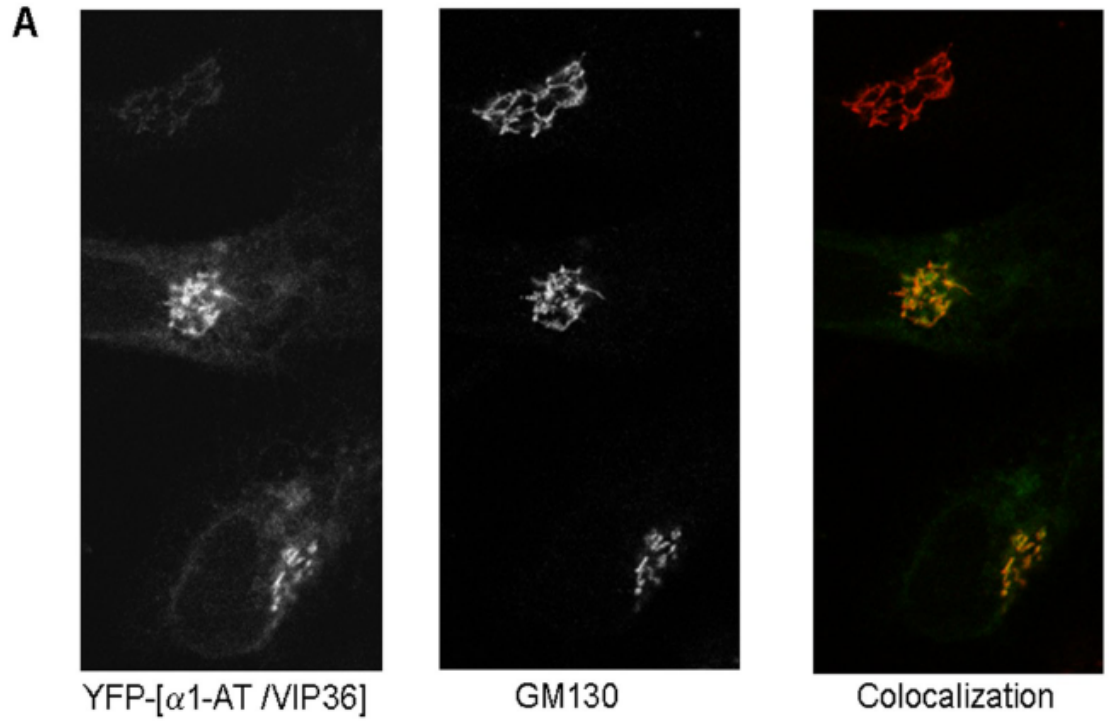
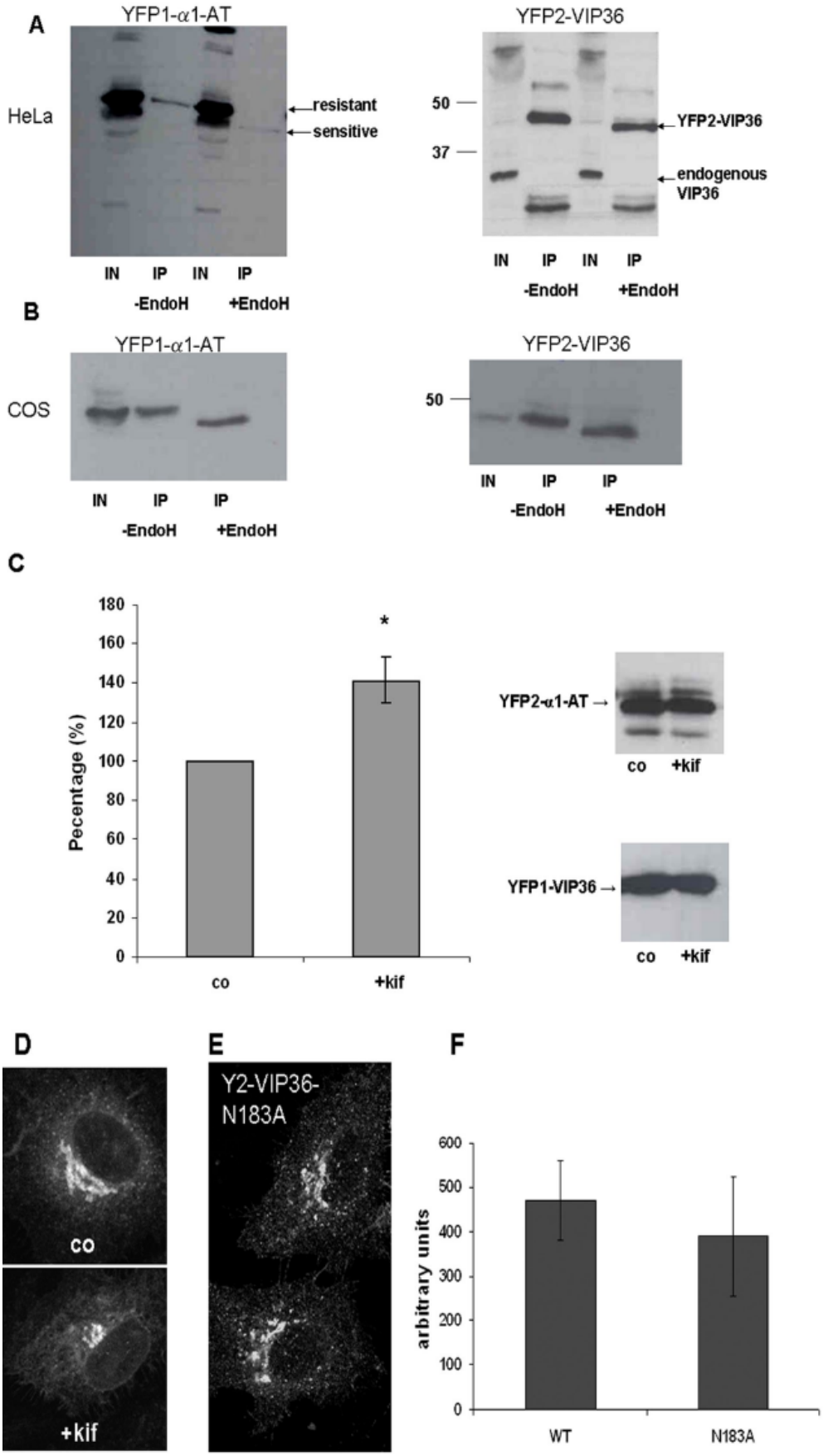

A
YFP-[α1-AT /VIP36]
GM130
Colocalization
B
arbitrary units
500
450
400
350
300
250
200
150
100
50
0
Y2-AT+ Y1-VIP36
Y2-NHK + Y1-VIP36
Y2-Z + Y1-VIP36
Y2-triple + Y1-VIP36
Y2-albumin + Y1-VIP36
YFP1-VIP36
a-GFP1
a-GFP2
YFP2- α1-AT -
WT
NHK
Z
Trip
alb
albumin-YFP2
75
IC
M

Figure 4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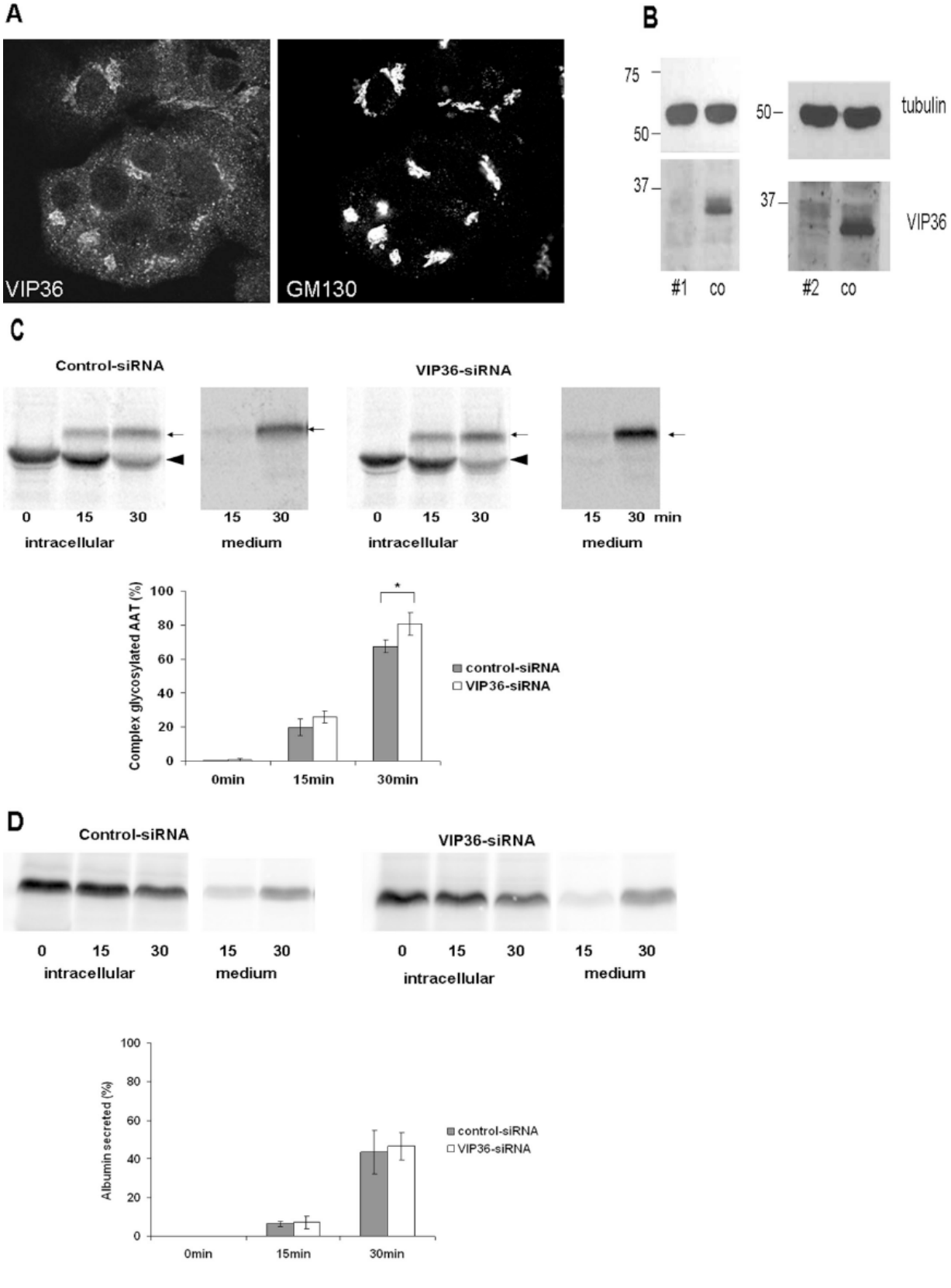
HeLa
resistant
sensitive
IN IP IN IP
-EndoH +EndoH
YFP2-VIP36
50
37
YFP2-VIP36
endogenous VIP36
IN IP IN IP
-EndoH +EndoH
B
YFP1-α1-AT
COS
IN IP IP
-EndoH +EndoH
YFP2-VIP36
50
IN IP IP
-EndoH +EndoH
C
Pecentage (%)
180
160
140
120
100
80
60
40
20
0
*
co
+kif
YFP2-α1-AT →
co +kif
YFP1-VIP36 →
co +kif
D
co
+kif
E
Y2-VIP36-N183A
F
arbitrary units
600
500
400
300
200
100
0
WT
N183A

Figure 5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VIP36
GM130
B
75
50
37
50
37
tubulin
VIP36
#1
co
#2
co
C
Control-siRNA
VIP36-siRNA
0
15
30
intracellular
15
30
medium
0
15
30
intracellular
15
30
min
medium
Complex glycosylated AAT (%)
100
80
60
40
20
0
*
control-siRNA
VIP36-siRNA
0min
15min
30min
D
Control-siRNA
VIP36-siRNA
0
15
30
intracellular
15
30
medium
0
15
30
intracellular
15
30
medium
Albumin secreted (%)
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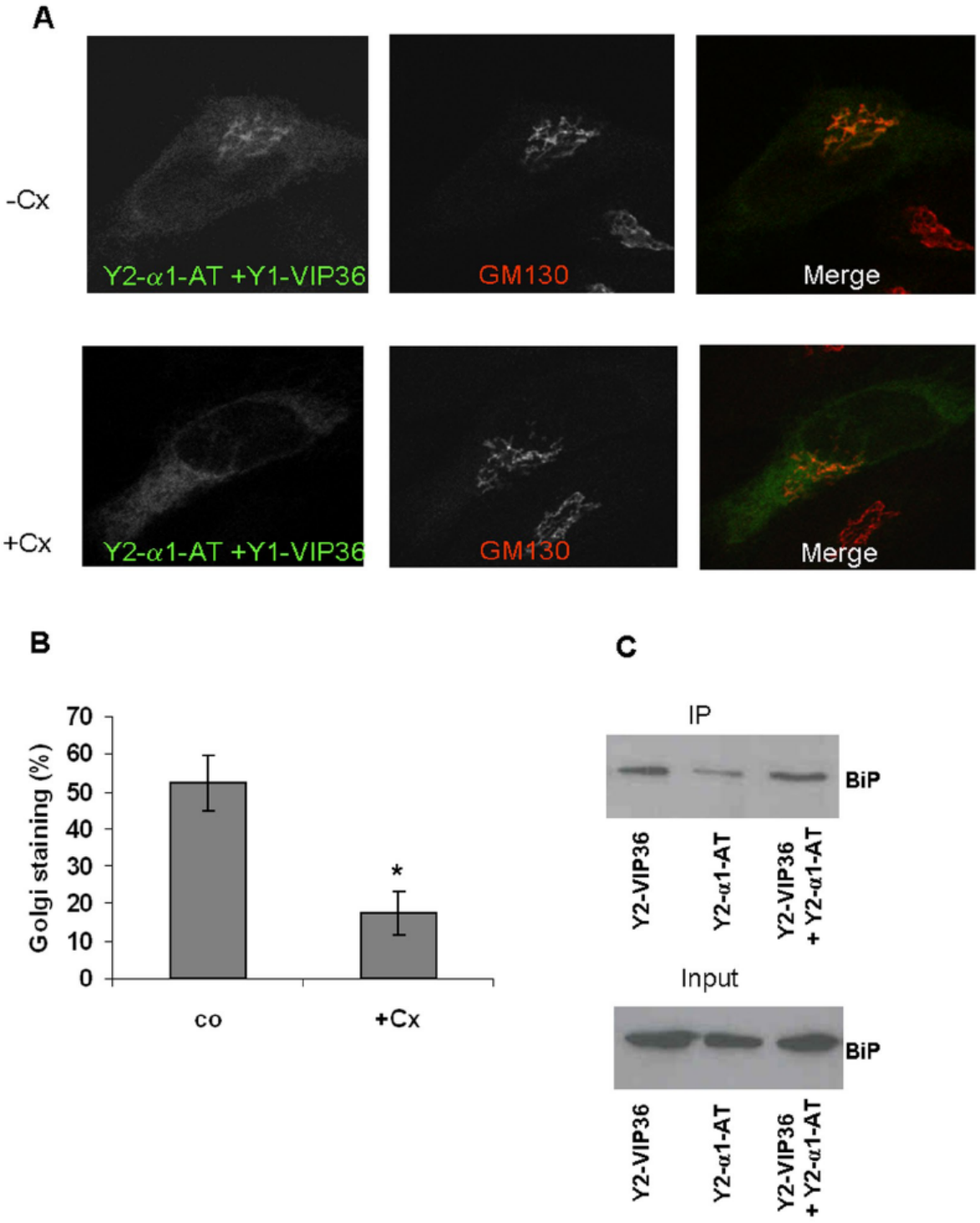
80
60
40
20
0
control-siRNA
VIP36-siRNA
0min
15min
30min

Figure 6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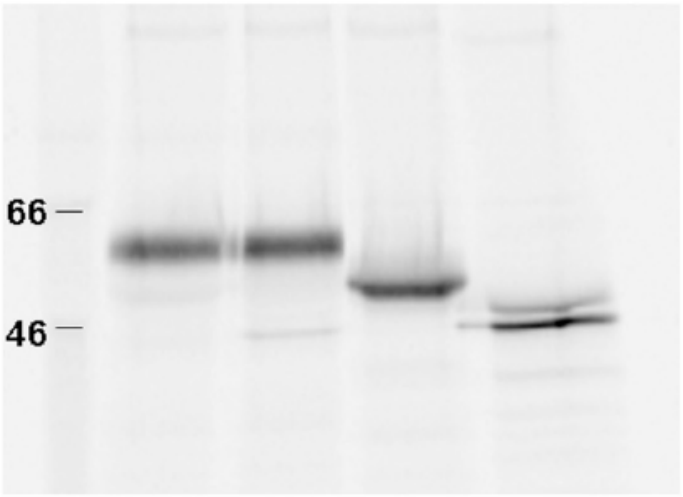
-Cx
Y2-α1-AT +Y1-VIP36
GM130
Merge
+Cx
Y2-α1-AT +Y1-VIP36
GM130
Merge
B
Golgi staining (%)
70
60
50
40
30
20
10
0
*
co
+Cx
C
IP
BiP
Y2-VIP36
Y2-α1-AT
Y2-VIP36 + Y2-α1-AT
Input
BiP
Y2-VIP36
Y2-α1-AT
Y2-VIP36 + Y2-α1-AT

Figure S1



Kifunensin	-	-	+	+
EndoH	-	+	-	+

3.2 Additional data: The role of VIP36 in Golgi integrity

The primary goal of my work was to investigate the function of VIP36 in the early secretory pathway focusing on the identification of a potential ligand. In addition, I was also interested whether VIP36 affected the organization of the secretory pathway itself. The results of this part of the study are presented and discussed in the following section.

Effect of VIP36 knockdown on the morphology of the early secretory pathway

VIP36 cycles between the ER and the Golgi (Dahm et al. 2001). Therefore an effect of VIP36 knockdown is most likely to manifest itself in the early secretory pathway. VIP36 knockdown was performed in HeLa cells and the cells were stained with markers for different compartments of the early secretory pathway. Knockdown of VIP36 had no appreciable effect on ER exit sites (ERES) as determined by staining for the COPII component Sec31 (Fig. 1A). There was also no effect on the peripheral ERGIC structures (Figure 1B). However, ERGIC-53 showed less accumulation in the Golgi region in knockdown compared to control cells. This observation points to a possible alteration of Golgi morphology caused by the VIP36 knockdown. Staining for the *cis*-Golgi marker GM130 and the medial-Golgi marker giantin revealed that the Golgi ribbon was fragmented (Fig. 1C). The effect on Golgi morphology was confirmed by two different siRNAs against VIP36. To further ensure that the effect on Golgi morphology was specific for cells in which VIP36 is depleted, we co-labeled VIP36 and GM130. Only those cells without a residual VIP36 staining exhibited a fragmented Golgi (Fig. 1D). Overexpression of VIP36 had no effect on Golgi morphology (Fig. 1E), further supporting the conclusion that the observed fragmentation of the Golgi is due to depletion of VIP36.

The Golgi fragments observed under VIP36 knockdown conditions are not connected anymore

Next we wanted to determine whether the Golgi fragments in VIP36 knockdown cells are still connected. We therefore performed a FRAP experiment (fluorescence recovery after photobleaching). HeLa cells were either transfected with control siRNA or VIP36 siRNA. After 48 h, a plasmid encoding GFP-tagged-galactosyltransferase was transfected to label the Golgi. FRAP experiments were performed 24 h afterwards. A small region of the Golgi was bleached and subsequently the recovery of the fluorescent signal was measured. As the Golgi is a connected ribbon in control cells, bleaching of a circular area was followed by rapid

recovery (Fig. 2). In VIP36 knockdown cells, the bleached Golgi region did not recover within the time-frame of the experiment (~55 sec) (Fig. 2). This indicates that the bleached region is not connected to the other Golgi fragments and thus cannot be filled by diffusion.

VIP36 knockdown does not affect the microtubule network but decreases the amount of COPI on the Golgi

Several conditions can account for a fragmentation of the Golgi. One of these conditions is depolymerization of the microtubules. However, knockdown of VIP36 did not change the gross organization of the microtubule network as judged by staining for α-tubulin (Fig. 3A). Disruption of the coatomer complex either by knockdown of β-COP (Styers et al. 2008) or by conditional depletion of ε-COP (Guo et al. 1994) also results in fragmentation of the Golgi. Importantly, co-knockdown of ERGIC-53 and Surf 4 fragmented the Golgi, partly by redistribution of COPI from the Golgi to the cytosol (Mitrovic et al. 2008). VIP36 is related to ERGIC-53, and it contains a putative (dibasic) COPI binding motif. Therefore it seemed worthwhile to analyze the effect of VIP36 knockdown on COPI distribution. HeLa cells were transfected with control or VIP36 siRNA and a co-staining for β-COP and giantin was performed 72 h after transfection. Knockdown of VIP36 fragmented the Golgi and the fluorescence of β-COP on these Golgi fragments was significantly reduced when normalized to the giantin signal (Fig. 3B). We therefore concluded that Golgi fragmentation could be due to changes in COPI distribution analogous to the observation made by co-knockdown of ERGIC-53 and SURF4.

VIP36 knockdown does not affect general glycoprotein secretion

A major function of the Golgi is the posttranslational modification of proteins and their secretion. As shown above (see manuscript) knockdown of VIP36 did not affect secretion of albumin which is a non-glycosylated protein. However, secretion of the glycoprotein α1-AT was accelerated. We therefore asked whether the fragmentation of the Golgi induced by VIP36 knockdown has any impact on general secretion of glycoproteins. To test this, HepG2 cells were transfected with siRNA against VIP36 or control siRNA. After 72 h, cells were metabolically labeled with [^{35}S]-methionine for 10 min. Media and cells were harvested after a chase period of 20 and 60 min. Intracellular and secreted glycoproteins were precipitated using Concanavalin A-Sepharose. Concanavalin A is a lectin that binds preferentially to high-mannose and hybrid type N-glycans. We compared the amount of intracellular glycoproteins to the amount of secreted glycoproteins. To ensure that the glycoproteins bound to

Concanavalin A were really of high-mannose type, we performed endoglycosidase H (endoH) digestion of intracellular glycoproteins. The most prominent bands appeared to be sensitive to endoH, indicating that they indeed contained high-mannose glycans. We could not observe an obvious difference in glycoprotein secretion between knockdown and control cells (Fig. 4A). In addition, there were also no differences in proteins not bound by Concanavalin A (Flow through) between knockdown and control conditions (Fig. 4B). This assay provides only a rough estimate of the functionality of the anterograde transport, but it is sufficient to show that the fragmented Golgi is still able to support anterograde transport.

Retrograde Golgi to ER transport is not affected by VIP36 knockdown.

Knockdown of the non-motor subunit of kinesin also leads to Golgi fragmentation (Stauber et al. 2006). No effect was observed on anterograde transport but retrograde (Golgi-to ER) transport was affected. Therefore, we wanted to test if the retrograde transport was affected by Golgi fragmentation in VIP36 knockdown cells. We used the ts045-VSVG-KDEL-receptor (VSVG-KDELr) based assay to test the functionality of the COPI dependent retrograde transport route. This reporter is composed of the KDELr which is retrieved in a COPI-dependent manner. The luminal part of the temperature sensitive VSVG is attached to the N-terminus of the KDELr (Cole et al. 1998). At the permissive temperature (32°C), this reporter cycles in the early secretory pathway and is at steady state in the Golgi. Upon shifting to the non-permissive temperature (40°C) the reporter is transported back to the ER, where it is trapped due to misfolding of the VSVG part. Conversion of the Golgi localization to an ER localization is indicative of an intact retrograde transport. If there is a disruption of retrograde trafficking, the VSVG-KDELr reporter will not be transported to the ER, and will remain in the Golgi. VSVG-KDELr distributed evenly to the ER and the Golgi in control as well as in VIP36 knockdown cells at the 32°C (Fig. 5 A,B). After incubation at 40°C for 2h, VSVG-KDELr localized to the ER in both control and VIP36 knockdown cells (Fig. 5 A,B). This indicates that the COPI-dependent retrograde transport is still supported by the fragmented Golgi.

Discussion

Beside a role of VIP36 in post-Golgi quality control our study revealed that VIP36 also plays a role in maintaining/establishing Golgi integrity. There are several possibilities how VIP36 could maintain Golgi integrity. Our study showed that neither the microtubule network, nor general anterograde or retrograde protein trafficking was affected by VIP36 knockdown.

Therefore we can exclude that a disruption of the microtubule network or a consequence of impaired protein trafficking are the reasons for the fragmented Golgi. According to our data VIP36 is involved in post-Golgi quality control of at least one glycoprotein: α1-AT. VIP36 is supposed to bind misfolded α1-AT and to transport it back to the ER. It is therefore tempting to speculate that VIP36 knockdown leads to an accumulation of misfolded glycoproteins in the Golgi, as they are not retrieved anymore. This accumulation of misfolded proteins could have a toxic effect on the Golgi leading to Golgi fragmentation. So far there are a few examples in the literature that showed a relation between misfolded proteins and the fragmentation of the Golgi (Gonatas et al. 2006). The most interesting one is a misfolded mutant of the growth hormone (Graves et al. 2001). Overexpression of this mutant causes massive Golgi fragmentation. The mutant itself was retained in the ER, but in addition located partially to Golgi fragments, indicating that part of the protein reached the Golgi. Overexpression of mutant growth hormone induced an anterograde trafficking defect. Golgi fragmentation could be either due to the disturbed ER export of proteins, but also due to accumulation of protein aggregates in the Golgi. This latter possibility was not tested. Organelle fractionation is needed here to determine whether protein aggregates are also found in Golgi fractions. However for α1-AT we did not find evidence for accumulation of misfolded proteins in HepG2 cells. On the contrary, knockdown of VIP36 led to an accelerated secretion of α1-AT. Also general secretion of glycoproteins did not differ between VIP36 knockdown and control cells. Therefore, our results do not support the accumulation of misfolded protein in the Golgi as a cause for Golgi fragmentation. Our results favor a different observation to underlie Golgi fragmentation. We found that VIP36 knockdown reduced COPI binding to the Golgi membrane. The importance of COPI for Golgi integrity is well supported. Treatment with BFA inhibits assembly of COPI coats on membranes due to the lack of activation of Arf1 by its exchange factor GBF1. This leads to dissociation of COPI from Golgi membranes and redistribution of the Golgi to the ER (Lippincott-Schwartz et al. 1989; Lippincott-Schwartz et al. 1998). Knockdown of β-COP (Styers et al. 2008) or conditional depletion of ε-COP (Guo et al. 1994) both resulted in fragmentation of the Golgi. We found that knockdown of VIP36 reduced the amount of Golgi-associated COPI. A recent study from our laboratory showed that recruitment of COPI to the Golgi depends on the presence of cargo receptors (Mitrovic et al. 2008). Double knockdown of ERGIC-53 and the putative cargo receptor SURF4 disrupt the Golgi and COPI dissociates partly from the Golgi. The same result was found for single knockdown of p25, a cargo receptor of the p24 family (Mitrovic et al. 2008). All of these proteins provide di-lysine COPI-binding motifs that are

critical for COPI recruitment. VIP36 has a dibasic signal in its C-terminus (KR on position –3 and –4), which are likely to serve as COPI binding sites. This is a possible explanation for the reduction of COPI association to the Golgi. The question arises now, why a single knockdown of VIP36 is sufficient to induce Golgi fragmentation, while a single knockdown of the related protein ERGIC-53 does not? ERGIC-53 is mainly localized to the ERGIC and the ER and a very small amount of it is found in the Golgi. VIP36 mainly localizes to the Golgi and may therefore contribute more COPI binding sites than ERGIC-53. Taken together our data demonstrate that VIP36 supports Golgi integrity mainly by providing binding sites for COPI.

Materials and methods:

Antibodies: The following antibodies were used for immunofluorescence: G1/133 against giantin (Linstedt et al. 1993), GM130 (BD, Bioscience), tubulin , mAb G1/93 against human ERGIC-53 (Schweizer et al. 1991), mouse mAb 1A2 against α-tubulin (kindly provided by Karl Matter, University College London, UK), rabbit pAb VIP36 (kindly provided by Kai Simons, Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany), mouse mAb 9E10 against the myc epitope, mouse mAb maD anti-βCOP (Pepperkok et al. 1993), pAb against giantin (kind gift of Adam Linstedt, Department of Biological Sciences, Carnegie Mellon University, Pittsburgh). Alexa 488-, Alexa 568 (Molecular Probes, BV, Leiden, NL) were used as secondary antibodies.

Cell culture:

HeLa cells were grown in DMEM, supplemented with 10% fetal bovine serum and antibiotics. HepG2 cell were grown in MEM supplemented with 10% fetal bovine serum and antibiotics.

siRNA and DNA transfection

Si RNA oligonucleotides against VIP36 were obtained from Qiagen (5'-AGAUAACUUCCACGGCUUA)dTdT-3'was used as sense and 5'-UAAGCCGUGGAAGUUAUCUdTdT-3' as antisense oligonucleotide) and from Ambion (5'-UGACUGCGGAUAUAACUGAtt-3' as sense and UCAGUUAUAUCCGCAGUCAca as an antisense oligonucleotide). Nonsilencing negative control siRNAs were obtained from Qiagen and Ambion. The siRNAs were transfected using Hiperfect (Qiagen, Switzerland) according to the manufacturer's instructions at a final concentration of 10nM. Plasmid transfection was performed using Fugene6 (Roche Diagnostics) according to the manufacturer's instruction.

Plasmids

Full length VIP36 was cloned from cDNA obtained by reverse transcription from HepG2 mRNA using oligodT. VIP36 was amplified using 5'-AATTCTCGAGATGGCGGCGGAAGGCTGGATT-3' as forward primer and 5'-GGCCTCTAGATCAGTAGAAGCGCTTGTTCCG-3'as a reverse primer. The PCR product was subcloned into a pcDNA3 vector via XhoI and XbaI restriction sites. The construct was verified by sequencing

Immunofluorecence microscopy:

Cells were fixed in 3% para-formaldehyde, permeablizied in 0,2% Triton in PBS containing 3% BSA and 20mM glycine (PBG) or 0,1% saponin (for anti-myc staining). Primary and secondary antibodies were incubated sequentially in PBS containing 3% BSA at room temperature. In case of saponin permeabilisation procedure saponin was present during all steps. For COPI staining cell were fixed in ice cold methanol/acetone (1:1) for 2 min. Following a one hour blocking step with PBG, cells were subsequently incubated with the primary antibodies in PBG and the secondary antibody in PBS containing 3% BSA. Cells were embedded in Mowiol and analyzed by laser scanning confocal microscopy (SPE5, Leica). For quantification of the β-COP staining, the fluorescence intensity of the giantin and the β-COP staining was determined within the same region of the Golgi. All regions quantitated had the same size. The intensity of β-COP staining was normalized to the intensity of the giantin staining. Images in knockdown and control conditions were acquired using the same settings (magnification and laser power).

Total glycoprotein secretion

HepG2 cells were seeded in 10cm plates and reverse transfected with either VIP36 or control siRNA. 72 h after knockdown cells were starved in methionine-cysteine free medium and pulsed afterwards with ^{35}S-labeled methionine/cysteine for 15 min. The medium was collected after a 60 min chase period and the samples were incubated with Concanavalin A-Sepharose for 1 h. The samples were washed four times with ConA buffer (20mM Tris-HCl, 500mM NaCl, 1mM $CaCl_2$, 1mM $MgCl_2$, pH 7.5) and glycoproteins were eluted in endoH buffer (0.25M sodium-citrate, 50mM Tris pH 6.0, 0.6% SDS). 50% of each sample was left untreated, 50% of it was incubated with 5mU endoglycosidase H (Roche) for 3 h at 37°C. Loading buffer was added and the samples were loaded on an SDS-gradient gel (4%-15%). The gel was fixed by incubation in 50%methanol and 10% acetic acid for 30 min. The radiolabeled bands were analyzed by phosphorimaging.

Fluorescence recovery after photobleaching:

HeLa cells were transfected with 10 nM siRNAs, 48 h later they were transfected with GalT-EGFP plasmid DNA. 72 h after siRNA transfection the cells were used for FRAP experiments on a LeicaSP5 microscopy at 37°C (× 40 objective, 1.4 NA). Before bleaching, a scan was obtained; the area of interest was then bleached with maximum laser power for one frame. Subsequently fluorescence recovery was monitored for 40 frames (each frame was 1.4 seconds). The recorded fluorescence intensities were digitized and averaged over the bleach region using ImageJ.

VSV-G-KDEL-receptor-based retrograde transport assay

HeLa cells were transfected with control or VIP36 siRNA. Two days later, they were transfected with myc-tagged ts045 VSV-G-KDEL receptor construct (kind gift from Victor Hsu, Harvard University). Subsequently cells were incubated at 32°C to allow VSVG to fold. Three days after siRNA transfection, the cells were either shifted for 2 h to 40°C or left at 32°C before fixation with 3%PFA. Immunofluorescence staining was performed using anti-myc antibody. A secondary anti-mouse-antibody coupled with Alexa568 was used. The amount of cells showing the VSVG-KDELR in the ER, respectively the Golgi were counted and expressed as percentage of total cells counted.

Figure legends:

Figure 1: VIP36 knockdown shows no effect on ER exit sites or the ERGIC, but fragments the Golgi. ***(A-C)*** Hela cells were transfected with VIP36 siRNA or control siRNA. After 72 h, cells were fixed and stained for Sec31 (A), ERGIC-53 (B), GM130 (C, upper row) or the medial Golgi marker giantin (C, lower row). Note that two different siRNAs fragmented the Golgi. ***(D)*** Hela cells were transfected with siRNA against VIP36. After 72 h, cells were fixed and co-stained for GM130 and VIP36. Cells without residual VIP36 staining showed fragmentation of the Golgi (arrow heads). ***(E)*** HeLa cells were transfected with cDNA encoding VIP36. After 24 h, cells were fixed and stained for VIP36 and GM130. Transfected cells were identified as those exhibiting a twofold higher fluorescence value over the Golgi region.

Figure 2: The fragments of the Golgi are not interconnected.

HeLa cells were transfected with siRNA against VIP36 or with control siRNA. Two days later cells were transfected with a plasmid encoding GalT-EGFP and the FRAP experiment was performed 24 h later as described in material and methods. Upper panel shows an example for

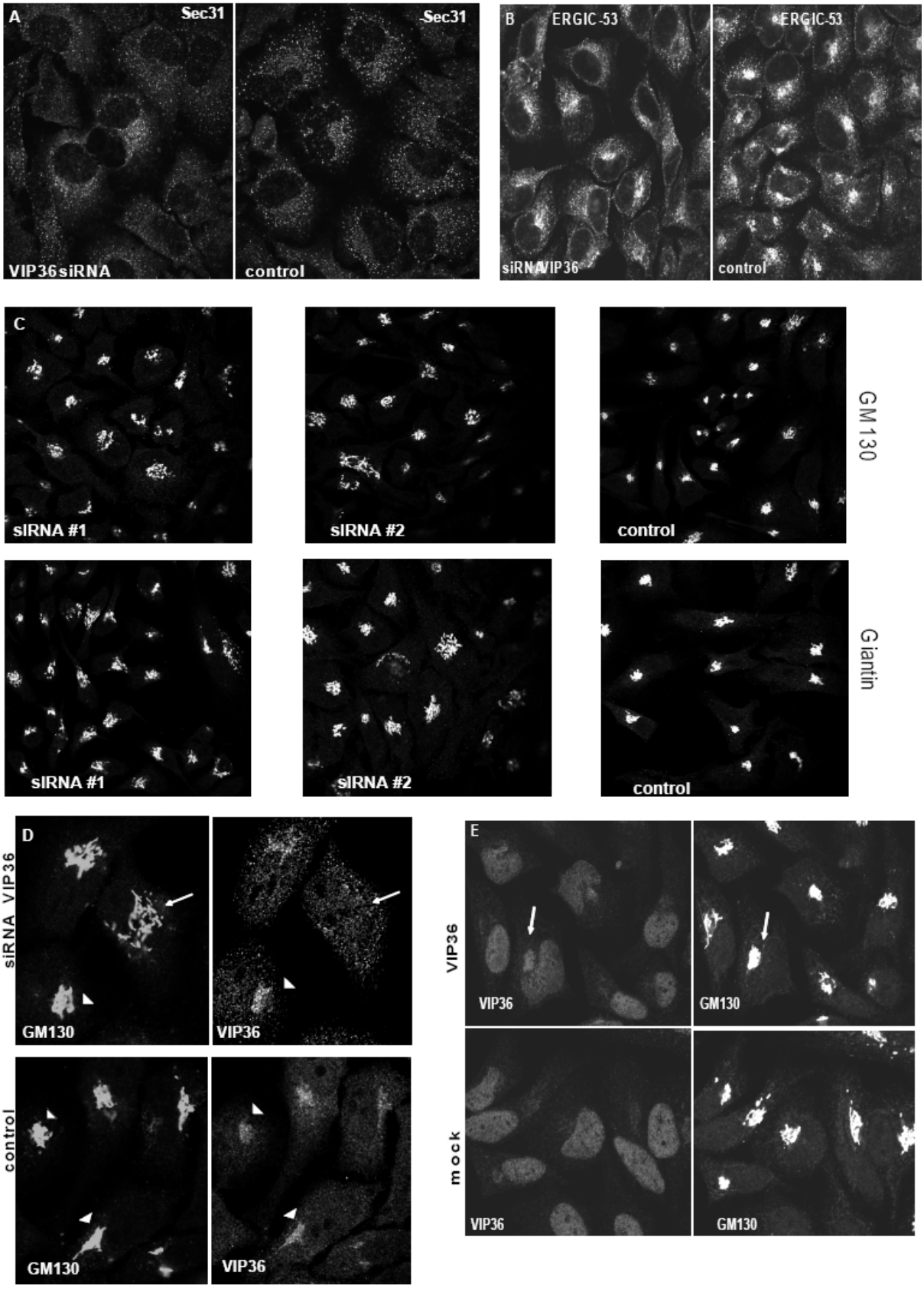
FRAP in VIP36 knockdown *vs.* control cells after the same period of recovery. Lower panel shows the evaluation of 5 cells for each condition (expressed as mean ± SD)

Figure 3: Effect of VIP36 knockdown on microtubule and COPI distribution.
(A,B) HeLa cells were treated with siRNA against VIP36 or with control siRNA. After 72 h, cells were stained with anti-tubulin antibody (A) or co-stained with β-COP and giantin (B). Fluorescence intensity of the β-COP staining was normalized to the intensity of the giantin staining.

Figure 4: VIP36 knockdown has no effect on general glycoprotein secretion.
(A,B) HepG2 cells were transfected with VIP36 siRNA (VIP) or control siRNA (co). 72 h after transfection the cells were labeled with [^{35}S]methionine for 10 min and chased for 0, 20 and 60 min. Cell lysate (c) and medium (M) was incubated with Concanavalin A. Panel A shows the fraction of (glyco-) proteins that bound to Concanavlin A. Samples were eluted and one half subjected to endo-H treatment (+) and the other half left untreated (-). Samples were subjected to SDS-PAGE and bands were visualized using phosphorimaging. Panel B shows a fraction of the flow through (2%) was also subjected to SDS-PAGE and visualized by phosphorimaging.

Figure 5: VIP36 knockdown does not affect COPI dependent retrograde transport. ***(A)*** HeLa cells were transfected with siRNA against VIP36 or control siRNA. After 48 h, cells were transfected with the myc-VSVG-KDEL-receptor construct. Cell were either left at 32°C (left panel) or incubated at 40°C for 2 h (right panel). Subsequently cells were fixed and stained for anti-myc. ***(B)*** Scoring of the percentage of cells showing a Golgi or ER pattern of the VSVG-KDELR under the different conditions. Bars show mean of two experiments. For each condition 44 to 58 cells were evaluated.

Figure 1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Sec31
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control
B
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ERGIC-53
siRNAVIP36
control
C
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control
GM130
siRNA #1
siRNA #2
control
Giantin
D
siRNA VIP36
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GM130
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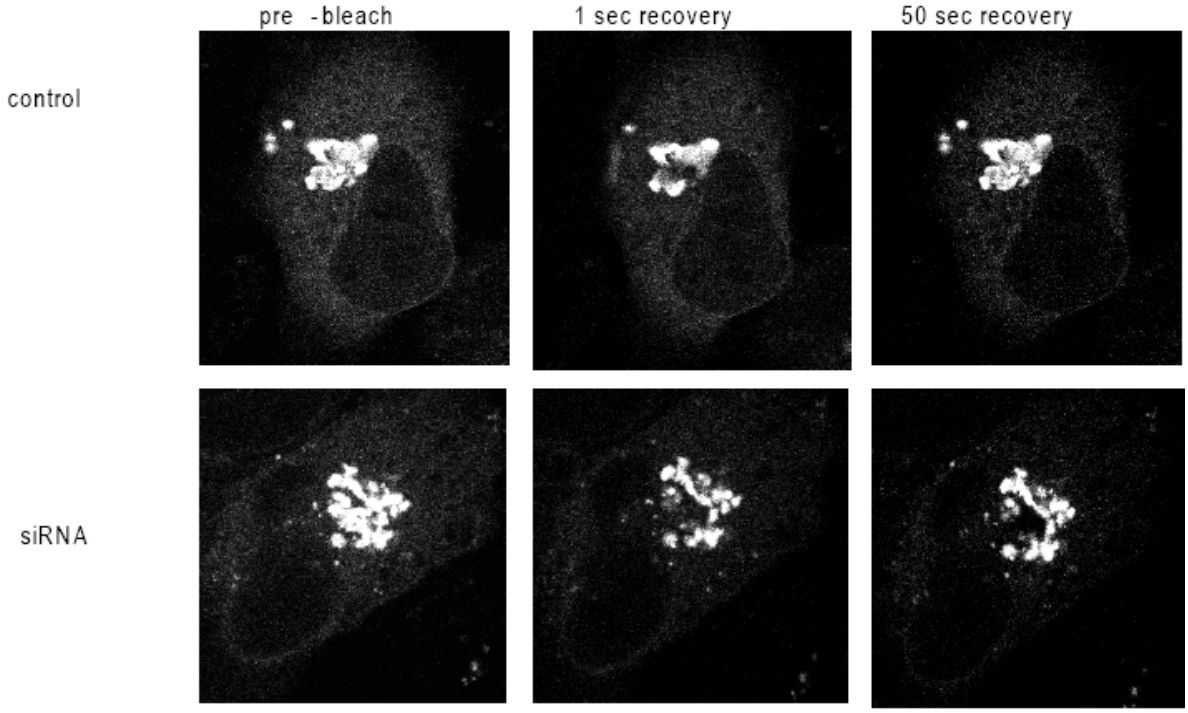
VIP36
VIP36
GM130
mock
VIP36
GM130

Figure 2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1 sec recovery
50 sec recovery
control
siRNA

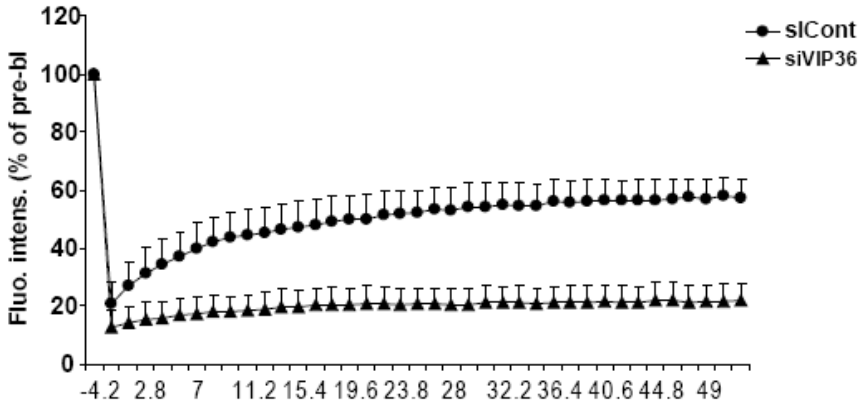
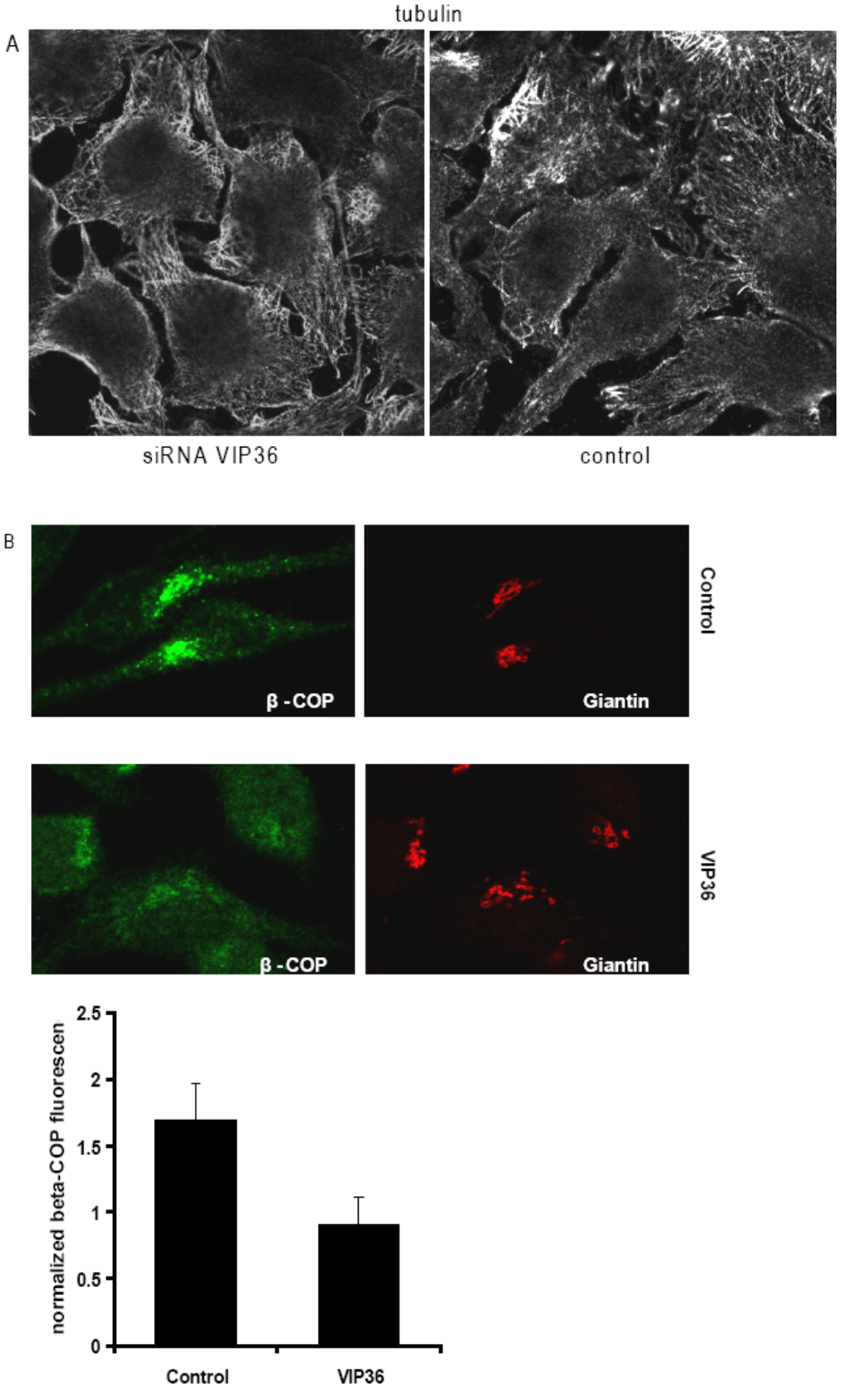

siCont
siVIP36
Fluo. intens. (% of pre-bl
120
100
80
60
40
20
0
-4.2 2.8 7 11.2 15.4 19.6 23.8 28 32.2 36.4 40.6 44.8 49

Figure 3


tubulin
A
siRNA VIP36
control
B
β -COP
Giantin
Control
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VIP36
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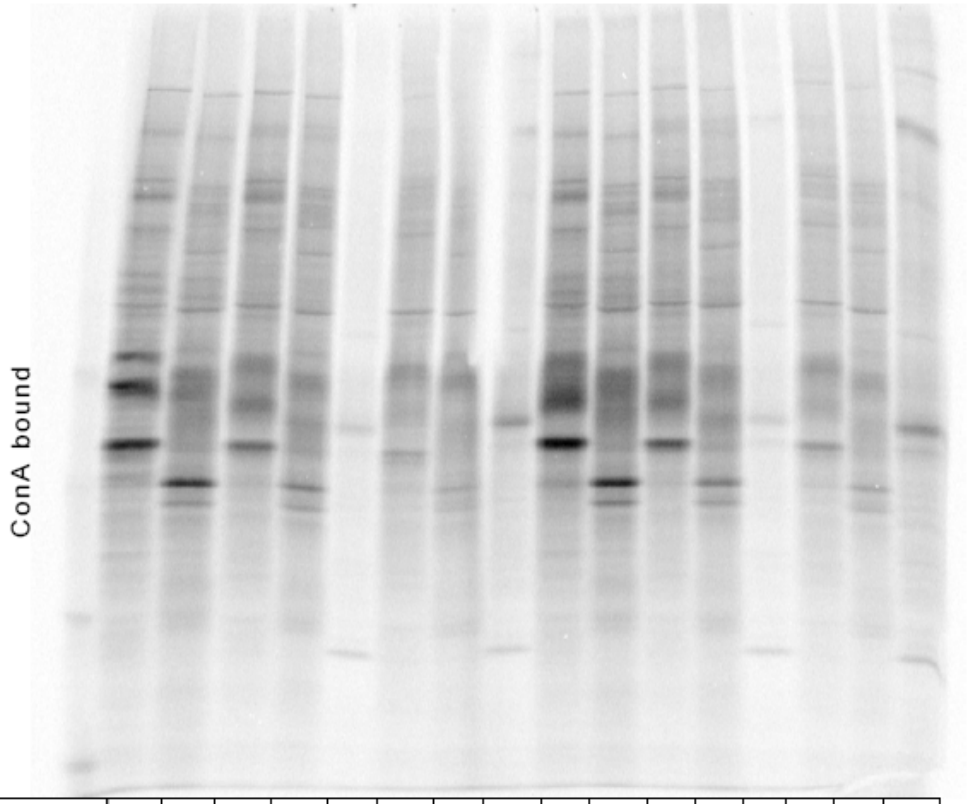
2.5
2
1.5
1
0.5
0
Control
VIP36

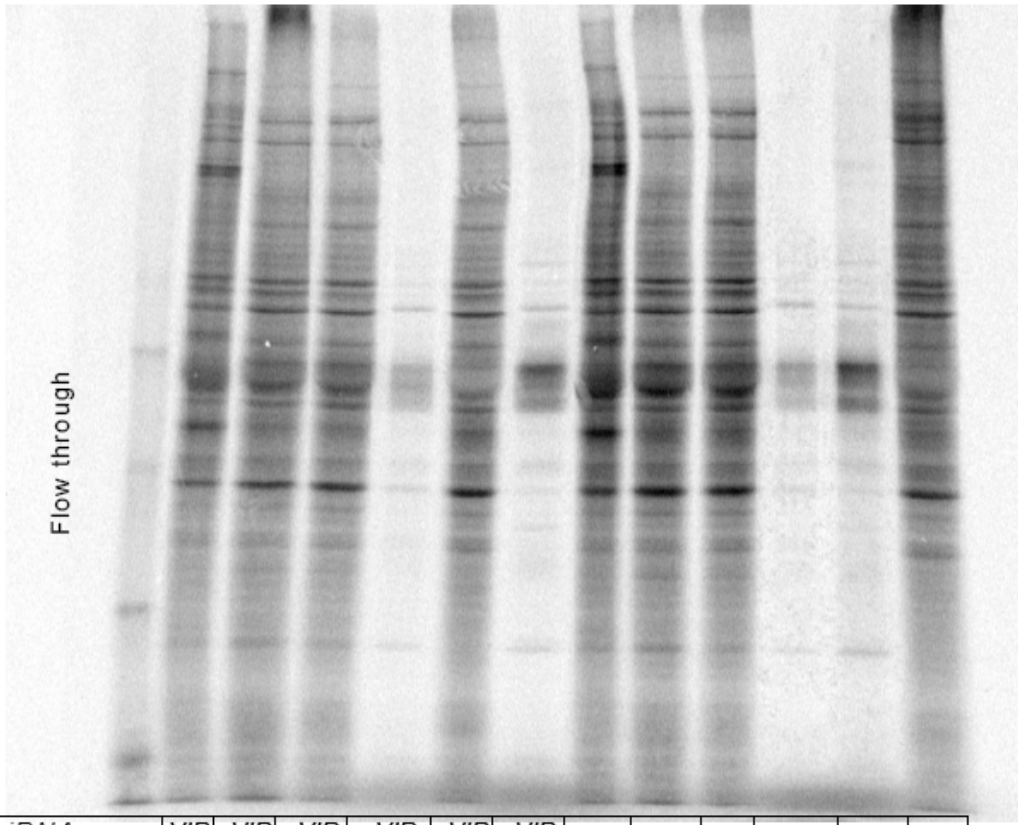
Figure 4

A



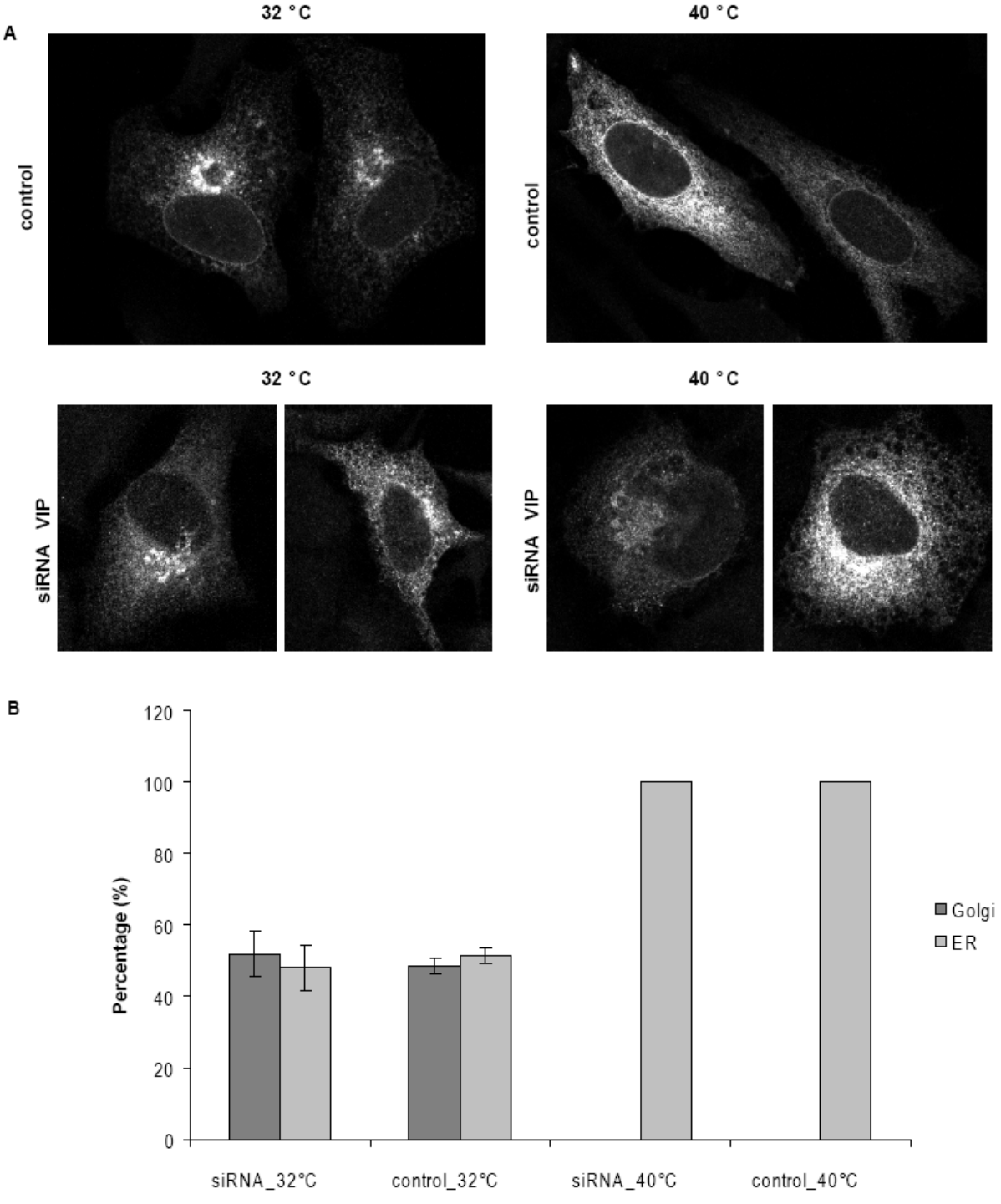
siRNA	VIP	VIP	VIP	VIP	VIP	VIP	VIP	VIP	co	co	co	co	co	co	co	co
endoH	-	+	-	+	-	-	+	-	+	-	+	-	-	-	+	-
chase time	0	0	20	20	20	60	60	60	0	0	20	20	20	60	60	60
cells/medium	c	c	c	c	M	c	c	M	c	c	c	c	M	c	c	M

B



siRNA	VIP	VIP	VIP	VIP	VIP	VIP	co	co	co	co	co	co
chase time	0	0	20	20	60	60	0	0	20	20	60	60
cells/medium	c	c	c	M	c	M	c	c	c	M	c	M

Figure 5


A
32 °C
40 °C
control
control
32 °C
40 °C
siRNA VIP
siRNA VIP
B
120
100
80
60
40
20
0
Percentage (%)
Golgi
ER
siRNA_32°C
control_32°C
siRNA_40°C
control_40°C

4 Discussion

Among the four L-type lectins in mammalian cells, only the role of ERGIC-53 in the early secretory pathway has been investigated in detail. The main function of ERGIC-53 is the ER export of a series of glycoproteins (Hauri et al. 2000). In addition, it acts together with Erp44 to ensure efficient polymerization and secretion of IgM polymers (Cortini et al. 2010). The function of the other three members of mammalian L-type lectins remains elusive. So far no study was conducted on the role of ERGL in the early secretory pathway. VIPL was implicated as a regulator for ERGIC-53, as overexpression of VIPL redistributed ERGIC-53 back to the ER (Nufer et al. 2003). In addition, knockdown of VIPL affected the secretion of two non-identified glycoproteins (Neve et al. 2003) but it remains unknown if VIPL directly affects the transport of these glycoproteins.

Several studies were conducted on VIP36, but no convincing data supporting a specific function of VIP36 was provided. Our approach to uncover the cellular function of VIP36 was to identify a cargo and study the function of VIP36 based on this cargo. We identified α1-AT by our YFP-PCA-based FACS screening as an interaction partner for VIP36. At first sight we were surprised to retrieve α1-AT as a possible interaction partner for VIP36 because we have previously uncovered α1-AT as a cargo for ERGIC-53. We first speculated that VIP36 might work as a backup receptor, a notion supported by the fact that VIP36 has a preference for high-mannose (ER-type) sugars and cycles in the early secretory pathway (Dahm et al. 2001; Kamiya et al. 2005). Both features are reminiscent of ERGIC-53. In addition, we found that the complex formed by α1-AT and VIP36 localized to ER and Golgi. This would be consistent with binding of α1-AT to VIP36 in the ER and subsequent transport to the Golgi. A backup receptor function would be analogous to COPII where four different versions of the cargo adaptor Sec24 (Sec24A,B,C,D) exist, which function redundantly (Wendeler et al. 2007). However, our data do not support a backup function of VIP36. The main point arguing against a backup-receptor hypothesis is the fact that a knockdown of VIP36 accelerates α1-AT transport. In addition, findings by K. Kato and colleagues argue against a role for VIP36 at the level of the ER. They found that VIP36 has its highest affinity for sugars at pH of 6.5 (Kamiya et al. 2005). This is a value typically attributed to the Golgi (Paroutis et al. 2004). Similarly, it is also unlikely that VIP36 acts in intra-Golgi transport. Depletion of an anterograde intra-Golgi transport receptor would result in slowed complex-glycosylation of its cargo glycoprotein. In contrast we observed accelerated complex-glycosylation of α1-AT in VIP36. Apart from that, this is not likely for another reason. The Golgi mannosidase-I cleaves $GlcNAc_2Man_8$ to $GlcNAc_2Man_5$ already in

the *cis*-Golgi. VIP36 has a very low affinity for Man_5 glycans (Kamiya et al. 2005). Therefore, VIP36 would not be able to support transport of glycoproteins after the *cis*-Golgi, making it an unlikely candidate for an intra-Golgi transport receptor.

One of the potential functions postulated for VIP36 is post-Golgi transport of glycoproteins (Hara-Kuge et al. 2002). Therefore, we considered the possibility that VIP36 may mediate post-Golgi transport of α1-AT. The idea that VIP36 acts in post Golgi transport is mainly supported by two studies (Hara-Kuge et al. 2002; Hara-Kuge et al. 2004). These authors described that VIP36 interacts with α-amylase in post-Golgi vesicles of rat parotid cells (Hara-Kuge et al. 2004). However, this is not relevant for human α-amylase. Rat α-amylase is secreted in a high-mannose form which can potentially be recognized by VIP36. By contrast, human α-amylase is secreted in its complex glycosylated form, which is unlikely to interact with VIP36 (Hara-Kuge et al. 2004). The second study focused on the secretory protein clusterin in MDCK cells. Clusterin has seven N-glycosylation sites, one of which remains in its high-mannose form even when it is secreted (Hara-Kuge et al., 2002). This high-mannose glycan could therefore serve as a binding site for VIP36 to mediate post-Golgi trafficking (Hara-Kuge et al. 2002). Yet only indirect evidence for a role of VIP36 in post-Golgi trafficking of clusterin was provided. Hara Kuge et al. (2002) localized VIP36 to the plasma-membrane in MDCK cells. The observation of VIP36 in the late secretory pathway is incompatible with our findings as well as with the findings of the Simons group (Fullekrug et al. 1999). The discrepancy may be due to the usage of different antibodies. The antibody used by Füllekrug et al. (1999) is directed against the C-terminus of VIP36. We used the same antibody in our studies and also localized VIP36 in the early secretory pathway. The polyclonal antibody used in the study of Hara Kuge et al (2002) is directed against the luminal domain of VIP36, which would be accessible to the extracellular milieu in case VIP36 would reach the plasma membrane. Hara Kuge et al (2002) detected VIP36 at the plasma-membrane in non-permeabilized cells, but the same antibody showed mainly an ER pattern for VIP36 in permeabilized cells. In addition, this antibody was raised against the complete CRD of VIP36 and is therefore likely to detect also VIPL, as the luminal domains are homologous. Thus the results obtained with this antibody have to be taken cautiously. Further studies are needed to clarify the role of VIP36 in clusterin transport in more detail. Nevertheless, the possibility remains that VIP36 is able to fulfill different functions dependent on the cell type. Regarding α1-AT, we are convinced that VIP36 is not involved in its post-Golgi transport for several reasons. The role assigned to VIP36 in post-Golgi trafficking was to regulate apical secretion. HepG2 cells (where we have studied α1-AT transport) are polarized cells and exhibit an

apical and a basolateral side. The canalicular membrane domain of HepG2 cells correspond to the apical domain and their sinusoidal and lateral domains correspond to the basolateral membrane (Zegers et al. 1998). α1-AT is a plasma-protein and as such it would be secreted via the sinusoidal and lateral membrane into the bloodstream. However, it is unlikely that VIP36 mediates basolateral-directed post-Golgi transport of α1-AT. Secretion of α1-AT into the medium was not slowed down under VIP36 knockdown conditions. Finally, there is no evidence that any of the three glycosylation sites of secreted α1-AT remains in its high-mannose state, which would be necessary to serve as a binding site for VIP36 (Gross et al. 1982; Mills et al. 2001). Overall, our data do not support a role for VIP36 as an anterograde cargo receptor at any stage for α1-AT.

So far all listed hypotheses fail to provide an explanation for the accelerated transport we have observed under VIP36 knockdown conditions. Which intracellular function of VIP36 can explain such an observation? Interestingly, acceleration of α1-AT transport was already described in the literature (Yeo et al 1985). Treatment with the mannosidase-II inhibitor swainsonine accelerates the transport of α1-AT and other glycoproteins (Yeo et al. 1985). Inhibition of mannosidase-II interferes with the conversion of Man_5 to Man_3, resulting in an accumulation of Man_5. The inhibition of trimming prevents the formation of complex-type glycans, and hybrid-type glycans are synthesized instead. The authors speculated that a Man_3- and Man_5-binding lectin may exist. Transport by the Man_3-binding lectin would be slower than transport by the Man_5-binding lectin. Therefore, accumulation of Man_5 will lead to a faster transport. It is very unlikely that VIP36 represents this elusive Man_3-binding lectin because VIP36 has a very low affinity for Man_3 glycans (Kamiya et al 2005).

Based on our data the most likely explanation for the accelerated transport under VIP36 knockdown would be a role in quality control at the level of the Golgi. According to this notion, VIP36 binds high-mannose α1-AT in the Golgi and transports it back to the ER and thereby retards its complex glycosylation. In case VIP36 is depleted, α1-AT is not retrieved, but subjected to complex glycosylation and finally secreted. This hypothesis explains all our observations. If VIP36 acts in post-ER quality control it is likely to interact only with a small fraction of proteins, which is reflected by the low YFP-PCA signal. Likewise, the small increase in transport velocity could be explained. We also demonstrated the recycling of the VIP36/α1-AT complex from the Golgi back to the ER, which further strengthens our hypothesis.

The principle of post-ER quality control *per se* is not without precedence. A well known example is the retrieval activity of the KDEL receptor. The KDEL receptor cycles

between ER and Golgi and ensures that escaped ER resident proteins are transported back to the ER. The KDEL receptor binds to the KDEL peptide sequence (lysine-aspartate-glutamic acid-leucine) present in a variety of soluble proteins, like BiP or calreticulin (Pelham 1989). Calreticulin retrieves sub-optimally loaded MHC-I molecules from the Golgi back to the ER (Howe et al. 2009). This shows that ER chaperones may also act at the level of the Golgi. The retained MHCI would be brought back to the ER and may only be released from calreticulin there. However, there is evidence that retention by and release from calreticulin may also take place directly in the Golgi. Endomannosidase was shown by electron-microscopy to localize to the Golgi (Zuber et al. 2000) and it was also shown to co-purify with calreticulin from Golgi extracts (Spiro et al. 1996). This suggests that endomannosidase removes glucose residues from calreticulin-glycoprotein complexes. The removal of glucose would release the glycoprotein from calreticulin at the level of the Golgi. Finally, misfolded mutants of the vasopressin receptor were shown to reach the ERGIC and were subsequently rerouted to the ER for degradation (Hermosilla et al. 2004). The mechanism behind retention and retrieval remains to be resolved.

Two different defects would theoretically render a glycoprotein prone to recognition by post-Golgi quality control: a problem in glycosylation or a folding defect. Glycosylation is important for protein stability and different cellular functions. Therefore, it would make sense to have a mechanism that assures correct glycosylation beyond the level of the ER. High-mannose-containing proteins in the plasma are recognized by the mannose receptor, endocytosed and degraded (Schlesinger et al. 1978). It was shown that high-mannose glycans shortens the half life of IgG1 in blood plasma (Wright et al. 1994). Therefore, cells have to prevent high-mannose glycoproteins from being secreted. This function may be exerted by VIP36. VIP36 could interact with α1-AT molecules that were not trimmed by Golgi mannosidase-I within a certain time frame and transport them back to the ER. This would prevent secretion of high-mannose containing α1-AT. Although this idea is appealing, our data do not support it. If VIP36 prevents the secretion of high-mannose α1-AT, then we would expect to detect these high-mannose forms of α1-AT in the medium of VIP36 knockdown cells. This was, however, not the case. We cannot completely rule out that single N-glycan chains of α1-AT may show subtle glycosylation differences upon VIP36 knockdown. Whether this is true cannot be clarified by SDS-PAGE. Analysis of secreted α1-AT from VIP36 knockdown cells by HPLC is needed to tackle this question.

Another possibility for VIP36 to act in post-ER quality control would be the removal of misfolded α1-AT from the Golgi. According to this hypothesis, VIP36 would recognize

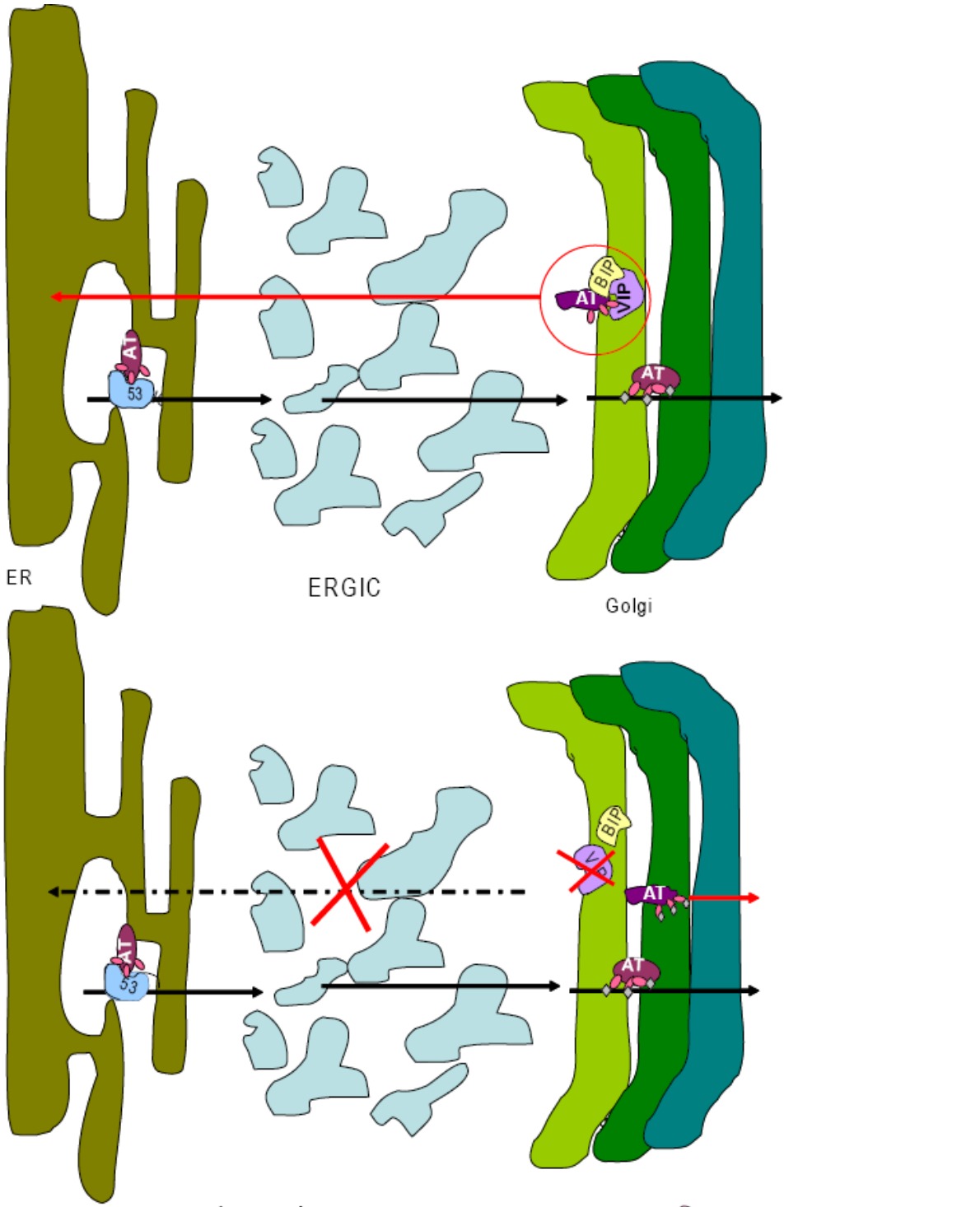
misfolded α1-AT that escaped the ER quality control. In this case, our observation that kifunensine treatment increases the interaction between VIP36 and α1-AT may be interpreted differently. Kifunensine does not only inhibit mannosidases, it also inhibits ERAD. Therefore, the increase in fluorescent complementation between VIP36 and α1-AT, upon kifunensine treatment, might reflect the interaction with α1-AT variants that escaped the ERAD. Results obtained by Nawa et al (2007) also support a role for VIP36 in post-ER quality control of misfolded proteins. They found that complex glycosylated VIP36 immunoprecipitated with BiP. The complex glycosylation of VIP36 indicates that VIP36 either binds BiP directly in the Golgi or recycles from the Golgi back to the ER and interacts subsequently with BiP. In both cases the interaction between BiP and VIP36 argues strongly for a role in post-ER quality control of misfolded proteins. We confirmed the interaction between BiP and VIP36 and in addition found that BiP associates with the complex formed by VIP36 and α1-AT. As it is currently not clear where the complex between VIP36 and BiP forms, we can only speculate about the mode how VIP36 and BiP cooperate in quality control. In case the interaction of VIP36 and BiP takes place in the ER, VIP36 would have to recognize the defective glycoprotein in the Golgi, recycle it back to the to the ER and hand it over to BiP. In this scenario, VIP36 would need to recognize its client protein by binding to non-native regions of the protein. There is already some evidence that lectins do not only interact with the glycan structure of a protein alone. ERGIC-53 does not only interact with high-mannose glycans of a glycoprotein, but also with peptide determinants (Appenzeller-Herzog et al. 2005). There is also evidence that calnexin and calreticulin bind to unfolded parts of the protein (Ihara et al. 1999; Saito et al. 1999; Jørgensen et al. 2000). Thus, it is possible that also VIP36 recognizes peptide determinant. VIP36 has an unpaired cysteine in its structure. It is tempting to speculate that this residue is involved in recognition of peptide determinants. According to the crystal structure this cysteine residue is localized on the opposite side of the glycan-recognition site. The glycan chains might suffice to span the distance. Co-crystallization of α1-AT and VIP36 will be required to test this notion.

In the alternative scenario VIP36 and BiP interact already in the Golgi. VIP36 recognizes the sugar determinants and BiP recognizes the misfolded (non-native) part of the protein. The complex made of BiP, VIP36 and misfolded α1-AT would then recycle back to the ER. Because of the pH change, VIP36 would release the high-mannose chains in the ER and BiP would stay bound to the misfolded protein and retain it in the ER. Our observation that BiP binds not only to VIP36 but also to the complex formed by α1-AT and VIP36 argues in favor of this interpretation. Such a mechanism would explain why the complex of VIP36

and α1-AT was retained in the ER after cycloheximide treatment. α1-AT bound to VIP36 is recycled back to the ER in a complex with BiP. As the YFP-PCA is irreversible VIP36 was not able to dissociate from α1-AT anymore and was retained together with α1-AT and BiP in the ER. The question arises now, why should BiP alone not be sufficient to bind the misfolded protein in the Golgi? One explanation is the difference in the environments of the Golgi and the ER. BiP may have decreased affinity towards its binding partners in the Golgi, presumably because of different pH. Another possibility is that BiP has a lower affinity for misfolded glycoprotein, than for non-glycoproteins. It has been shown that a glycoprotein that is N-glycosylated within the first 50 aa of its sequence does not interact with BiP (Molinari et al. 2000). Instead it interacts with calnexin or calreticulin. This criterion applies to α1-AT, as its first glycosylation is within the first 50 aa. It is possible that BiP needs the help of VIP36 to recognize α1-AT in the Golgi. VIP36 would interact with every high-mannose containing α1-AT arriving at the Golgi. In case the protein is correctly folded the affinity between VIP36 and α1-AT is not high enough to promote retention. In case non-native α1-AT arrives at the Golgi, VIP36 would be aided by BiP and the complex would be re-routed to the ER. As retrieval of BiP is mediated by the KDEL receptor, this could enhance the efficiency of the retrieval of the whole complex. BiP was already implicated in post-ER quality control of the T-cell receptor alpha (TCRα). Together with the KDEL receptor, BiP was shown to be involved in retrieval of incorrectly assembled, overexpressed TCRα (Yamamoto et al. 2001). Partially assembled TCRα is recognized by BiP in the Golgi followed by binding of BiP to the KDEL receptor. The KDEL receptor retrieves the whole complex back to the ER where TCRα is subjected to degradation. As the TCRα is a glycosylated protein, it would be interesting to test, whether VIP36 is also part of this complex or acts in addition to it to enhance the efficiency of the retrieval system.

If VIP36 binds misfolded proteins, why did the misfolded mutants of α1-AT not interact with VIP36? The two mutants used in our study are retained in ER. The Hong Kong mutant is not exported from the ER at all and the misfolded Z mutant is secreted at 10-fold lower rates than wild type α1-AT. Therefore, both mutants do not reach the Golgi at sufficient amount to be detectable by YFP-PCA. As the Z-mutant has a strong tendency to aggregate, it might be that the accessibility of the glycan chains is impaired, again lowering the probability to interact with VIP36.

In summary, our study suggests a cellular role for VIP36 as part of the post-ER quality control system in the Golgi. Our proposed working model is schematically illustrated below.



Model for VIP36 in α1-AT post-ER quality control

Upper panel:α1-AT is synthesized in the ER and binds to its cargo receptor ERGIC-53. ERGIC-53 mediates anterograde transport to the ERGIC/Golgi. In case α1-AT is correctly folded it is subjected to complex glycosylation and finally secreted. Misfolded α1-AT that escaped the ER quality control is recognised in the Golgi by VIP36. BiP interacts with VIP36 and improves binding to the misfolded protein. The complex consisting of VIP36,BiP and α1-AT is transported back to the ER. Upon the pH change VIP36 dissociates from α1-AT. Subsequently α1-AT is either subjected to degradation or to repair.

Lower panel shows the effect of VIP36 knockdown on α1-AT transport. α1-AT is exported from the ER and arrives at the Golgi. The transport of correctly folded α1-AT is not influenced. But misfolded α1-AT that arrives at the Golgi is not retrieved by VIP36 anymore. Glycosylation enzymes do not discriminate between the correct and incorrect folding status and α1-AT becomes complex glycosylated and secreted.

5 Future perspectives

There are still many open questions related to VIP36. I suggest the following questions to be tested experimentally in the future:

- Formally, the trafficking motifs in VIP36 have not been investigated. Is the retrieval motif functional? The classical retrieval motif is KK*XX*. The putative retrieval motif of VIP36 is KR*XX*. It would be interesting to test the ability of the VIP36 C-terminus to recruit COPI. This is important in light of the fact that we propose that depletion of VIP36 leads to less COPI at the Golgi. Equally important is the question of whether the putative ER export motif of VIP36 is functional and which Sec24 isoform it binds.

- What form of α1-AT is secreted by VIP36 knockdown cells compared to control conditions? This requires analysis of VIP36 secreted into the medium by HPLC or by isoelectric focusing.

- How does VIP36 bind to BiP? Mapping the domain in VIP36 binding to BiP will allow the construction of VIP36 mutants that fail to bind BiP. These mutants can be then tested in terms of interaction with α1-AT.

- If the VIP36-BiP complex retrieves α1-AT to the ER, how does the complex dissociate? This will involve studying the interaction of VIP36 and BiP at different pH levels (6.5 for a Golgi-relevant pH and 7.4 for an ER-relevant pH value). Also interesting would be to study the sequence of events in the assembly of the complex. Does the VIP36-BiP complex pre-exist in the absence of α1-AT? Is it induced upon engagement with α1-AT? Does VIP36 bind α1-AT first followed by recruitment of BiP? Does non-native α1-AT arrive at the Golgi already with BiP bound to it?

- What are the molecular details of the VIP36 α1-AT interaction? Co-crystallization of VIP36 and α1-AT will be required to tackle this question.

- What is the biological function of VIP36? The complete knockout of genes has often retrieved limited information about the function of the protein being studied. Tissue-specific (conditional) knockout models are more useful. A VIP36 knockout in the liver would be a potentially useful model to uncover the biological function of this lectin. VIP36 is highly expressed in the liver and α1-AT which we have identified as a binding partner for it, is synthesized and secreted in the liver.

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