FUNCTION OF HUMAN SURF4 IN THE EARLY SECRETORY PATHWAY

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Dekan

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Summary

Transport along the early secretory pathway is mediated by vesicles that shuttle proteins and lipids between organelles. Highly organized machineries assure correct trafficking in anterograde and retrograde directions as well as homeostasis of the organelles. A unique position in this system hold transmembrane cargo receptors. They are specialized in recognition of soluble luminal proteins and are able to link them to transport machineries on the cytoplasmic side such as vesicular coats. Cargo receptors are abundant proteins, but their inactivation leads to rather limited secretion phenotypes, illustrating the strict selectivity of receptors for a subset of soluble secretory cargo. Increasing evidence links cargo receptors to human diseases. In humans inactivation of the cargo receptor ERGIC-53 leads to inefficient secretion of the blood coagulation factors V and VIII which is already enough to provoke bleeding disorders. Recently the p24 family member p23 was linked to Alzheimer's disease by regulating amyloid precursor protein trafficking. These studies show the importance and need to characterize the function of cargo receptors in more detail.

The identification of Erv29p in the yeast *Saccharomyces cerevisiae* as a cargo receptor for pro-α-factor (gpαf) opened new insights into the mechanism of cargo selection by recognizing the Ile-Leu-Val (ILV) sequence motif located in the pro-region of gpαf. Furthermore deletion of *ERV29* leads to a delay in transport of carboxypeptidase Y (CPY) and proteinase A (PrA) as well as to stabilization of the soluble ER associated degradation (ERAD) substrates CPY* and PrA*. So far Erv29p is the only known cargo receptor required for efficient transport of soluble secretory proteins and efficient degradation of misfolded ERAD substrates, suggesting a much wider function than only packaging correctly folded soluble proteins. The mammalian ortholog Surf4 is poorly characterized and gpαf as the cargo for Erv29p does not allow any speculation about a potential secretory cargo for Surf4 in humans. Therefore characterization of Surf4 would give new enlightenment into the mechansims of protein transport within the early secretory pathway in human cells.

In order to characterize Surf4 we localized endogenous Surf4 within the early secretory pathway. Mutational analysis of the conserved di-lysine retrieval motif identified Surf4 to cycle between the ER and Golgi in a lysine signal-dependent manner, similarly to the cargo receptor ERGIC-53. The hallmark of cycling

transmembrane proteins is their ability to form homo- and heterooligomers. Well known examples are the hexamerization of ERGIC-53 and heterooligmerization of p24 family members. In search of the function of Surf4 we attempted to identify interacting proteins by Blue Native-PAGE. The ability of Surf4 to form hetrooligomeric complexes with other cycling transmembrane proteins such as members of the p24 family and ERGIC-53, well known to mediate interactions with the machinery required for vesicle formation, opens new insights into the multifunctional behaviour of cargo receptors. Depletion of Surf4 together with ERGIC-53 disrupted the early secretory pathway, as depletion of the p24 family member p25, by redistributing COPI from Golgi and ERGIC membranes. Consequently COPI-mediated retrograde transport is reduced leading to disruption of the Golgi apparatus and reduction in ERGIC structures.

To test the cargo receptor function of Surf4 for secretory proteins, pulse-chase analysis was performed with cells depleted of Surf4 by short interference RNA (siRNA). Surf4 depletion resulted in reduced secretion of a subset of secretory proteins, implying cargo-receptor function. Is Surf4 also required for efficient degradation of soluble ERAD substrates as Erv29p? Given that alpha-1-antitrypsin Z variant (A1PiZ) is an ERAD substrate in both yeast and human and is stabilized in Erv29p depleted cells, it is a valid model substrate to study the role of Surf4 dependent stabilization of ERAD substrates in humans. Pulse-chase analysis in combination with Surf4 siRNA-mediated protein knockdown revealed normal degradation of A1PiZ, suggesting no requirement of Surf4 to clear the ER of accumulated soluble ERAD substrates. This work could confirm a potential cargo receptor function for Surf4, while it was not required for efficient degradation of the soluble ERAD substrate A1PiZ.

In conclusion the studies on Surf4 revealed that cargo receptors have at least two functions. They assure efficient anterograde transport of secreted proteins by their luminal domain and mediate efficient retrograde transport by controlling COPI recruitment via their cytosolic domain. Thereby cargo receptors facilitate exocytic transport and contribute to organelle maintenace.

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1. Introduction

1.1 The secretory pathway of eukaryotic cells

The secretory pathway of eukaryotic cells is composed of an elaborate endomembrane system that regulates delivery of newly synthesized secretory proteins, carbohydrates and lipids to the cell surface (Figure 1), a necessity for growth and homeostasis. Membrane traffic along these stations follows highly organized directional routes. Secretory cargo is synthesized in the endoplasmic reticulum (ER), transported to the ER-Golgi intermediate compartment (ERGIC) and the Golgi. After passage through the Golgi and arrival at the *trans* Golgi network (TGN), it is sorted into post-Golgi carriers that move to and fuse with the plasma membrane (PM).

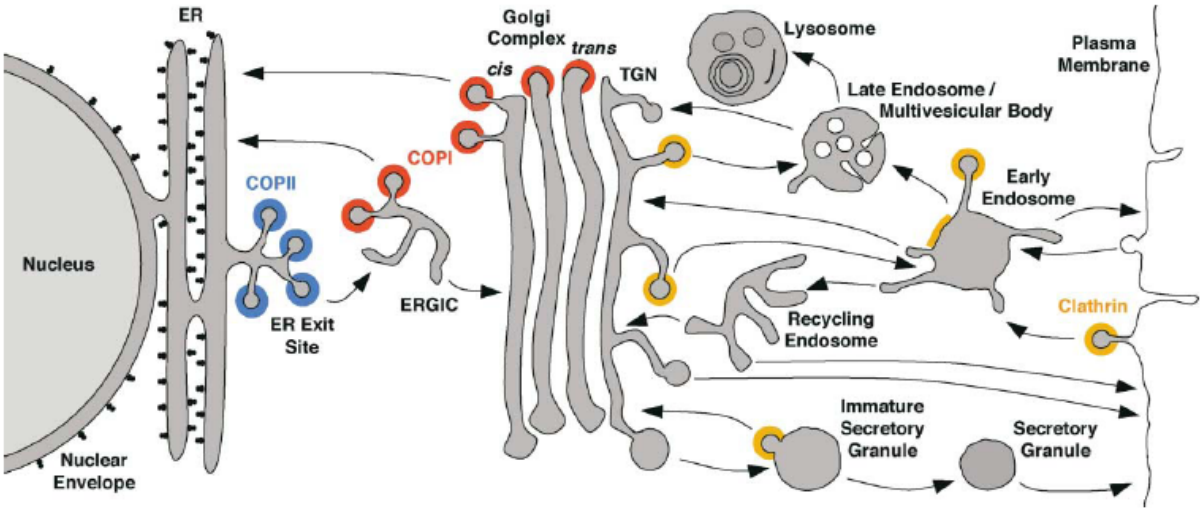

Figure 1: The secretory pathway of eukaryotic cells

The scheme depicts the endomembrane system describing the secretory, endocytic and lysosomal/vacuolar trafficking pathways. The distinct compartments are interconnected through vesicular transport steps (indicated by arrows). The membrane association of coat protein II (COPII) is depicted in blue, coat protein I (COPI) in red and clathrin in orange. The early secretory pathway (including ER, ERGIC and Golgi) mediates anterograde transport via COPII coated vesicles and retrograde transport via COPI coated vesicles. Transport through the Golgi is believed to involve a combination of COPI mediated vesicular transport and cisternal maturation.

(Reproduced from [1])

This forward movement of membranes is balanced by retrieval pathways that bring membrane and selected proteins back to the compartment of origin. The secretory pathway can be subdivided into an early and a late secretory pathway. The early secretory pathway includes the ER, ERGIC and Golgi, while the late secretory pathway defines transport steps happening between the Golgi apparatus and the PM (Figure 1).

The early secretory pathway, composed of the ER, ERGIC and Golgi, is a highly organized network that regulates protein synthesis, sorting and transport. Each organelle of the early secretory pathway is a specialized unit that harbours tightly regulated processes to assure proper transport of secretory cargo from one organelle to the other.

1.2 ER: the first station of secretory transport

1.2.1 Entry of newly synthesized proteins into the ER

The ER is the largest organelle of the cell and appears as a reticular structure, segregating the nuclear contents from the cytoplasm. It can be subdivided into at least two morphologically distinct subdomains: the ribosome-free smooth ER and the ribosome-covered rough ER. The smooth ER defines the site of lipid, cholesterol and steroid biosynthesis as well as detoxification. The rough ER is the entry point into the secretory pathway for newly synthesized secretory and membrane proteins. Newly synthesized proteins enter the ER lumen via co-translational translocation at ribosomes that dock onto a protein pore in the ER membrane [2]. The N-terminal signal sequence of secretory and membrane proteins is recognized by the signal recognition particle that directs the ribosome-nascent polypeptide chain complex to the membrane receptor (SRP receptor) [3]. Binding to SRP receptor targets the ribosome-nascent polypeptide chain complex to the Sec61 pore complex of the ER membrane. Once the ribosome is targeted to the Sec61 pore complex the nascent polypeptide chain is moved from the ribosome to the pore complex into the lumen of the ER.

1.2.2 Protein folding and quality control in the ER

The primary role of the ER is to provide a milieu that facilitates protein folding and modification. Many secretory and membrane proteins acquire during and after

translocation into the ER co- and posttranslational modifications, including N-glycosylation, disulfide bond formation and chaperone-assisted folding. An elaborate quality control system in the ER assures sorting of incorrectly from correctly folded proteins in the ER.

Two main quality control systems can be distinguished: The Hsp70 dependent folding and the calnexin/calreticulin (CNX/CRT) dependent folding of proteins. The Hsp70 system depends only on the recognition of hydrophobic residues within an unfolded protein, which is performed by the ER chaperone glucose regulated protein 78 (Bip) of the Hsp70 family [4]. Bip most likely aids folding by preventing off-pathway intermediates and thereby keeping the protein in a folding competent state [5]. The CNX/CRT cycle in contrast selects only proteins that contain monoglucosylated N-linked glycans [6-8].

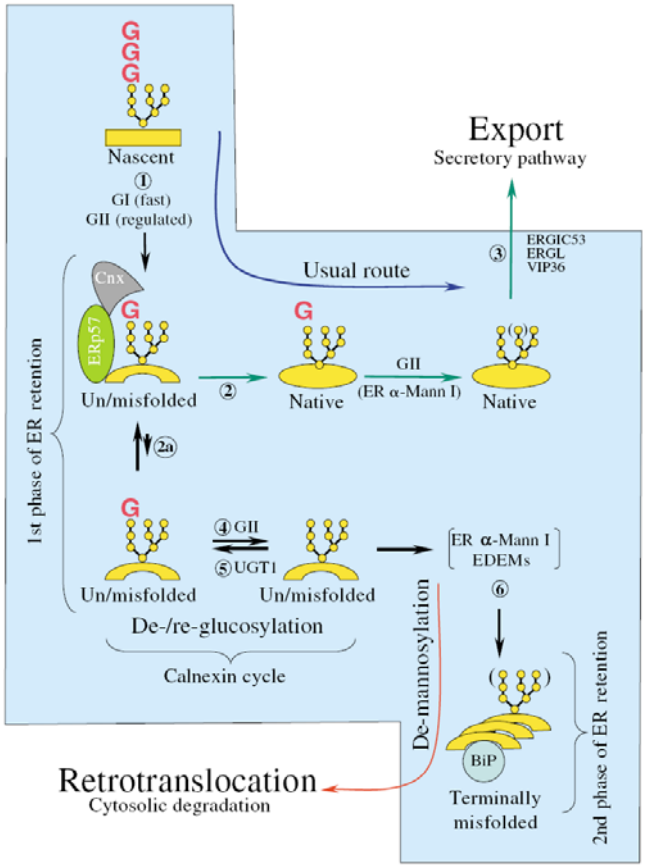

Figure 2: the quality control machinery. After the addition of the pre-assembled oligosaccharide the two outermost glucoses are removed (step1) and the nascent polypetide associates with calnexin/calreticulin (Cnx) and ERp57. correctly folded glycopeptides are released as native proteins (step 2) and exit the ER via cargo receptors (step3). Un/misfolded polypeptides enter cycles of dissociation/reassociation (steps 2a, 4 and 5) with Cnx until they reach the correct conformation. Terminally misfolded polypetides are retrotranslocated into the cytosol and degraded (step 6). (Reproduced from [8]).

Prior to the binding to the homologous lectins CNX and CRT newly synthesized proteins are co-translationally N-glycosylated and trimmed to the monoglucosylated form. The core glycan composed of three glucoses, nine mannoses and two N-

acetyl-glucosamines ($Glc_3Man_9GlcNAc_2$) is transferred en bloc by the oligosaccharyltransferase to the glycosylation sequon (Asn-X-Ser/Thr) of the protein. Glucosidase I and glucosidase II trim the core glycan by removing the two outermost glucose residues, generating the substrate ($Glc_1Man_9GlcNAc_2$) for CNX and CRT (Figure 2). Binding of incompletely folded glycoproteins to CNX and CRT prevents their aggregation and export from the ER. At the same time CNX and CRT expose the unfolded protein to the thiol-disulfide oxidoreductase Erp57. Erp57 acts as cofactor that catalyzes proper disulfide bond formation during the ongoing folding process [9]. The monoglucosylated glycoprotein is liberated from the CNX/CRT cycle by glucosidaseII, which removes the last glucose from the glycan (Figure 2). Misfolded glycoproteins are reglucosylated by the UDP-glucose:glycoprotein glucosyltransferase and rebound by CNX or CRT until the glycoprotein is correctly folded. Proteins that fail to fold correctly are targeted to ER-associated degradation (ERAD), which prevents accumulation of unsalvageable, misfolded proteins in the ER (Figure 2). Entry of a misfolded glycoprotein into ERAD requires trimming of the glycan by ER mannosidase I. ER mannosidase I trims the glycan to the $Man_{5-6}GlcNAc_2$ form [10]. The trimmed glycoprotein is recognized by ER-degradation enhancing α-mannosidase-like protein (EDEM) [11, 12] and targeted for retrotranslocation and ubiquitin-proteasome degradation into the cytosol.

Correctly folded proteins in contrast escape the CNX/CRT cycle and are competent to leave the ER and enter the secretory pathway (Figure 2). Some exceptions show that even misfolded proteins are capable of leaving the ER [13]. Yeast studies suggest that misfolded proteins require transport between the ER and Golgi for degradation [14, 15].

1.2.3 Exit from the ER

Immunofluorescence studies with the temperature sensitive vesicular stomatitis virus glycoprotein (ts045 VSVG) indicate that correctly folded proteins are segregated from the chaperone containing environment into ER domains designated ERES. Under restrictive conditions misfolded ts045 VSVG fails to co-localize with ERES, which are also devoid of ER resident chaperones. In contrast under permissive conditions correctly folded ts045 VSVG segregates from ER resident chaperones and becomes associated with ERES, the site of COPII vesicle formation [16]. Transport competent proteins that have accessed ERES may enter transport vesicles at their prevailing

concentration in the ER or are enriched up to 50 fold compared to their prevailing concentration. These two models of ER exit are termed “bulk flow-mediated ER exit” for proteins exiting the ER at the prevailing concentration and “receptor-mediated ER exit” describing the enrichment of cargo in vesicles. The interaction of the cytoplasmic coat with distinct sorting signals on the cytoplasmic tail of certain transmembrane cargos assures enrichment of transport competent proteins.

1.2.4 Cargo Receptors

In contrast to transmembrane cargos that expose a cytoplasmic tail to the coat, soluble cargo and GPI-anchored proteins have to interact with specific transmembrane receptors that link the luminal cargo to the cytoplasmic coat. In yeast Erv29p represents the cargo receptor for the precursor of the soluble pheromone α-factor and packages pro-α-factor into COPII coated vesicles by recognizing a hydrophobic ER export signal [17, 18]. Additionally to pro-α-factor Erv29p is believed to package other soluble cargos like carboxypeptidase Y and proteinase A, since these proteins are delayed in transport when Erv29p is deleted [14]. In mammalian cells the best characterized cargo receptor of the early secretory pathway is the mannose-specific lectin ERGIC-53 [19, 20]. ERGIC-53 is required for the secretion of a number of glycoproteins, including the lysosomal proteins cathepsin Z and cathepsin C as well as the secreted clotting proteins factor V and factor VIII [21-24]. Additionally to loss of function mutations in ERGIC-53, the multiple coagulation factor deficiency 2 gene (MCFD2) was identified as a second locus responsible for blood coagulation factor V and VIII deficiency [24]. Chemical cross-linking of factor VIII to MCFD2 and ERGIC-53, suggests that MCFD2 and ERGIC-53 operate together as a cargo receptor complex [25].

GPI-anchored proteins are luminal anchored proteins that have no cytoplasmic exposed signal for the coat, suggesting a requirement for a transmembrane cargo receptor. In yeast the GPI-anchored protein Gas1 can be cross-linked to the p24 family member Emp24 and packaging of Gas1 into transport vesicles is reduced in Emp24-depleted cells [26]. Interestingly Emp24 seems to specify a subpopulation of vesicles, since GPI-anchored proteins enter transport vesicles that are distinct from those that carry other cargo proteins such as pro-α-factor [27].

1.3 Vesicular transport between organelles

How is transport competent cargo transported between organelles? The isolation and analysis of temperature-sensitive “sec” mutants in yeast that were defective in protein secretion [28] identified inter alia small vesicles of 60-100nm diameter that as we know today correspond to transport carriers. Transport between organelles was further assessed by a cell free assay in which transport of VSVG from a “donor” Golgi fraction lacking the enzyme N-acetyl-glucosamine (GlcNAc) transferase I to an “acceptor” Golgi fraction was measured [29]. These studies support the vesicular transport hypothesis, which states that the transport of cargo between organelles is mediated by shuttling transport carriers. Transport competent cargo is selectively incorporated into vesicles that bud from a “donor” compartment, while resident proteins of the “donor” compartment are excluded.

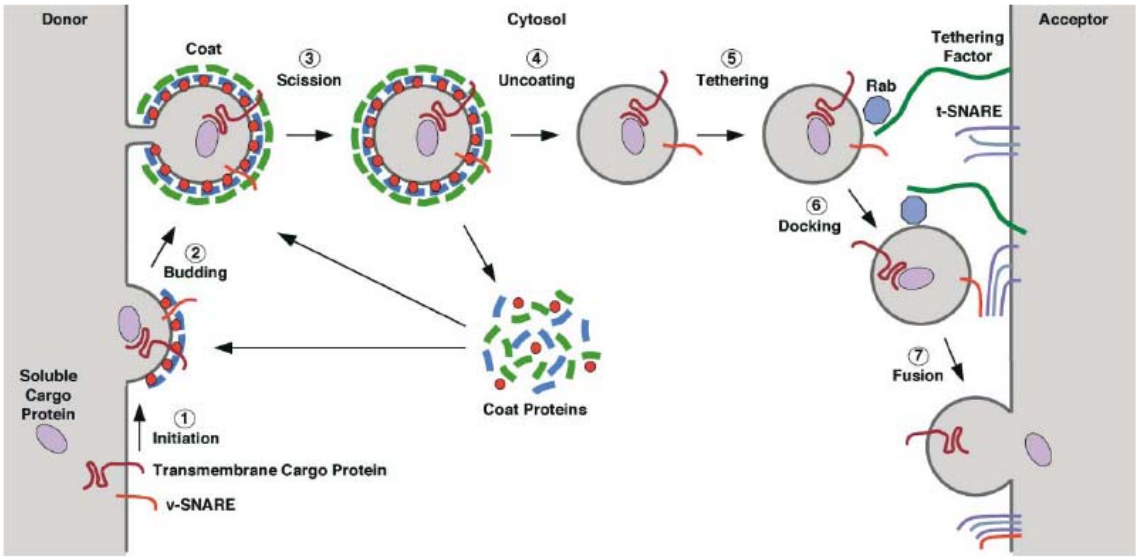

Figure 3: The mechanism of vesicle budding and fusion

(1) Initiation of coat assembly. The membrane-proximal coat components (blue) are recruited to the donor membrane by binding to a membrane-associated GTPase (red) and/or to a specific phosphoinositide. (2) Budding. The membrane-distal coat components (green) are assembled and polymerize into a mesh-like structure. Cargo becomes concentrated and membrane curvature increases. (3) Scission. The vesicle is released from the donor compartment. (4) Uncoating. The coat is released from the vesicle by inactivation of the small GTPase, phosphoinositide hydrolysis and uncoating enzymes. The cytosolic coat recycles for another cycle of vesicle budding. (5) Tethering. After transport the vesicle is tethered to the acceptor compartment by GTP bound Rab and tethering factors. (6) Docking. The v- and t-SNAREs assemble into a four-helix bundle. (7) Fusion. This *trans*-SNARE complex promotes fusion of the vesicle and acceptor membrane.

(Reproduced from [1])

The vesicles are targeted to a specific “acceptor” compartment into which they release their cargo after fusion. Figure 3 describes in detail the mechanism of vesicle budding and fusion.

The generation of a vesicle requires the recruitment of cytosolic coat components to the membrane. The coats deform the membrane into round buds and also participate in cargo recruitment by recognizing sorting signals present in the cytosolic tails of transmembrane cargo. Vesicular transport within the early secretory pathway is driven by two types of vesicles: COPII and COPI coated vesicles. COPII coated vesicles mediate export from the ER, while COPI coated vesicles are involved in intra-Golgi transport and retrograde transport from the Golgi to the ER (Figure 1).

1.3.1 COPII coated vesicles

COPII coated vesicles are generated at ribosome-free subdomains of the ER, the ERES. The minimal machinery to drive COPII coat formation requires the GTPase Sar1p, the Sec23p-Sec24p complex and the Sec13p-Sec31p complex [30]. This minimal machinery is sufficient to drive cargo capture, deformation of the budding membrane, scission of the forming vesicle and uncoating of the vesicle (Figure 4) [31]. COPII coat assembly is initiated through the activation of the small GTPase Sar1p. Activation of Sar1p requires the membrane bound guanine nucleotide exchange factor (GEF) Sec12p, which catalyzes the exchange of GDP for GTP on Sar1p. The membrane bound Sar1p-GTP sequentially recruits two cytosolic complexes, the Sec23p-Sec24p heterodimer and the Sec13p-Sec31p heterotetramer (Figure 4). The recruitment of Sec23p-Sec24p by Sar1p initiates selection of transmembrane cargo and soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins into the forming vesicle. Several lines of evidence suggest that the Sec23p-Sec24p complex is the component for cargo recognition [32-34] and recognizes signals at the cytoplasmic side of transmembrane cargo (see below). The Sec24p subunit has been ascribed a role in cargo recognition since it has various isoforms that exhibit distinct capacities to export different cargo from the ER. X-ray crystallography and mutagenesis studies suggest at least three cargo recognition sites for the yeast Sec24p [35, 36]. In yeast the Sec24p subunit has two isoforms termed Lst1 and Iss1 that exhibit distinct capacities to export different cargo from the ER [37, 38]. Mammalian Sec24p has four isoforms, termed Sec24A, Sec24B, Sec24C and Sec24D. Double knockdowns of these isoforms indicate

isoform-selective transport by binding preferentially to specific cytoplasmic signals of transmembrane cargo [39].

The Sec23p subunit is not only part of the coat but is also proposed to be the GTPase activating protein (GAP) for Sar1p. The Sec23p-mediated GAP activity is accelerated by the binding of the Sec13p-Sec31p complex, leading to release of Sar1p-GDP and uncoating of the vesicle (Figure 4). Thus the internal timer for Sar1p release by GTP hydrolysis is controlled by the stepwise assembly of the coat.

1.3.2 Cargo capture by COPII

The mechanism underlying receptor-mediated ER export requires signals that mediate concentrative sorting of cargo into COPII coated vesicles. The model protein VSVG for studying protein export from the ER contains a di-acidic (DXE) motif which is required for efficient ER export [40]. In yeast the DXE motif is also found in the transmembrane proteins Sys1p and Gap1p. Furthermore the binding of Sys1p to Sec23p-Sec24p and formation of a pre-budding complex of Gap1p with the Sar1-Sec23-Sec24 complex depends on the DXE motif [41, 42]. The di-acidic motif is not the only ticket for ER export. ER export of mammalian ERGIC-53 is directed via aromatic (FF, YY, FY) and di-hydrophobic (LL, II) residues or a single C-terminal valine [43, 44]. Additionally to ERGIC-53 the di-aromatic motifs are also found in the transmembrane proteins of the p24 family [45, 46] and the Erv41p-Erv46p complex [47]. Many of these proteins form oligomeric complexes, such that a given exported protein would presumably display multiple signals to the COPII coat. Indeed, detailed studies on the ER export of ERGIC-53 suggest that beside the ER export motif, proper oligomerization is a prerequisite for efficient ER exit [48]. Similarly, transient expression of the p24 family member p27 fails to leave the ER unless other p24 family proteins are coexpressed [49]. Erv41p and Erv46p although both contain ER export motifs, have to form a complex to exit the ER [47].

1.3.3 COPI coated vesicles

Whereas COPII dependent traffic is unidirectional the COPI mediated traffic can be anterograde between stacks of the Golgi cisternae or retrograde involving trafficking from the Golgi and the ERGIC to the ER. The COPI coat is a complex of seven subunits, termed α, β, β', γ, δ, ε and ζ subunits. In contrast to COPII the COPI coat is

recruited by activated Arf1 from the cytosol as a preassembled complex composed of all seven subunits. Although the coat is recruited en block it can be divided into two functionally distinct subcomplexes, the F-COPI subcomplex (β, γ, δ, ζ) and the B-COPI subcomplex (α, β', ε) [45]. The first step of COPI formation involves the activation of Arf1, which stimulates the exchange of GDP for GTP on Arf1, a process that can be inhibited by the fungal metabolite Brefeldin A (BFA) [50]. This nucleotide exchange causes a conformational change that leads to exposure of an N-terminal myristoyl-anchor allowing stable membrane association [51]. Further work revealed that Arf1-GDP is first recruited by p23 and probably p24 before being activated by GEF [52, 53]. The Arf1-p23 complex dissociates upon exchange of GDP for GTP. The COPI coat binds Arf1-GTP through the β and γ subunit and p23 through the γ subunit [54]. The coat complex can also bind to cytoplasmic tails of membrane proteins bearing a KKXX signal [55]**.** Indeed recruitment of the COPI coat via Arf1-GTP in the absence of such cytoplasmic tails is poor (Bremser et al., 1999). The binding of the coat to cargo like members of the p24 family leads to bending of the membrane and vesicle formation (Figure 4). Prior to fuse with the target membrane the GTPase activity of Arf1 is enhanced by Arf1GAP leading to GTP hydrolysis and uncoating of the vesicle [31].

1.3.4 Cargo capture by COPI

Compared to signal recognition of COPII, interaction of the COPI coat with its cargo seems more simple. As mentioned above the KKXX and KXKXX motifs interact directly with the COPI coat [55, 56]. Numerous ER resident and cycling transmembrane proteins have cytoplasmic KKXX motifs that are recognized by the COPI coat. Escaped ER residents as well as cycling transmembrane proteins therefore use the COPI system for efficient retrieval and recycling to the ER. Contradictory results suggest that the α- and β'-COP subunits are required for the binding to the KKXX motif [57], while others found the γ subunit interacting with the KKXX sequence [54, 58]. This discrepancy reflects most likely the presence of several binding sites for KKXX motifs on coatomer.

A special case are the p24 family members, since several members were described to interact with the COPI coat, although only one member (p25 of the p24 family) contains a KKXX motif in its cytoplasmic tail. Indeed binding to the coat relies more on the conserved FF motif than on the two basic residues that do not have a

true KKXX sequence [45, 59, 60]. Another ER retrieval motif is the RXR sequence found in subunits of the ATP-sensitive K^+ channel [61]. COPI coat can bind to the RXR motif and assures retrieval of individual subunits to the ER. However, after assembly of the subunits to a functional receptor multiple RXR motifs are able to bind to 14-3-3 proteins that compete for the retrieval via COPI [62, 63].

Luminal ER resident proteins that escape the ER have to be retrieved back. The KDEL sequence present at the C-terminal end of these proteins was identified as their retention signal [64]. Proteins with such a sequence bind to their transmembrane receptor (KDEL-R) in a pH dependent manner. Binding of a KDEL protein to the KDEL-R induces redistribution of the KDEL-R from the Golgi to the ER via the COPI pathway [65, 66].

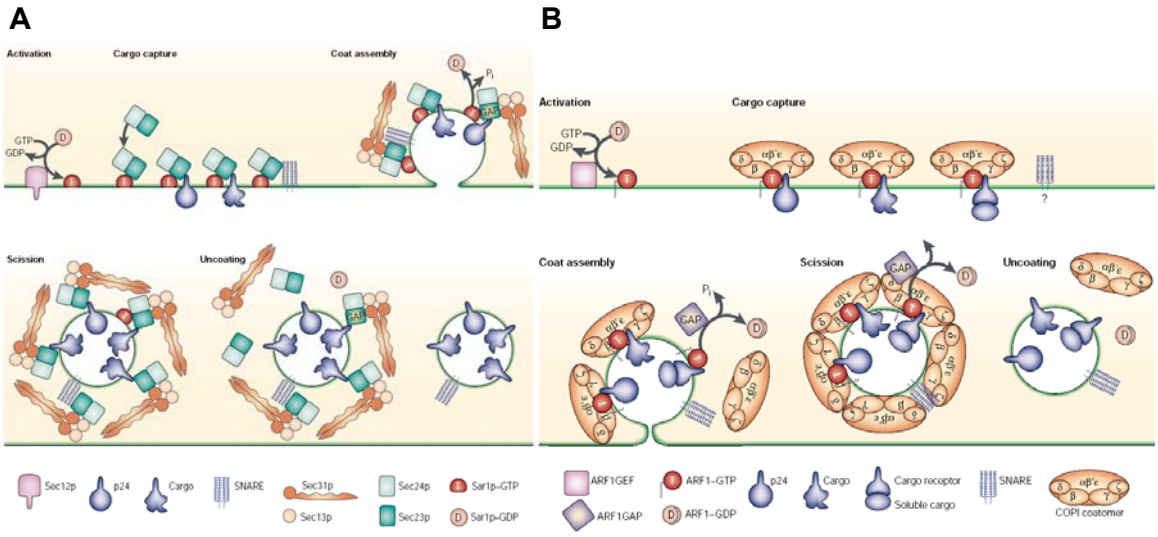

Figure 4: COPII and COPI coated vesicle formation

A) COPII coated vesicle formation. Coat assembly is activated by Sar1p-GTP recruitment to the membrane. The Sec23p-Sec24p complex binds to Sar1p-GTP, which initiates recruitment of cargo. The outer layer Sec13p-Sec31p assembles on top of the inner layer Sec23p-Sec24p, leading to membrane deformation. When the coat is complete, the vesicle buds. The GTPase activity of Sar1p is enhanced by Sec23p, which acts as a timer, leading to inactivation of Sar1p and uncoating. B) COPI coated vesicle formation. Coat assembly is activated by the recruitment of ARF1-GTP to the membrane. This allows the binding of the COPI coat and cargo recruitment. Membrane deformation occurs at the same time as coat recruitment. When the coat is complete, the vesicle buds. The GTPase activity of Arf1 is enhanced by ARF1GAP, which acts as a timer, leading to inactivation of ARF1 and uncoating.

(Reproduced from [30])

1.3.5 Targeting and fusion

After a vesicle looses its coat, it must be correctly delivered to and fuse with the appropriate acceptor compartment (Figure 3). Targeting of the vesicle requires a combination of GTP bound Rab and tethering factors that bring the vesicle into close proximity of the acceptor membrane. V-SNAREs (vesicular SNAREs) present on the vesicle then assemble with t-SNAREs (target SNAERs) on the acceptor membrane into a four-helix bundle leading to docking of the vesicle. This assembled SNARE complex (called *trans*-SNARE complex) promotes fusion of the vesicle with the acceptor membrane.

Tethering of vesicles is mediated by proteins containing extensive coiled-coil domains and large multisubunit complexes. Tethering of COPII vesicles is initiated by the coiled-coil protein p115. P115 is a cycling protein of the early secretory pathway, localizing to ERES, the ERGIC and the Golgi [67, 68]. Inactivation of p115 by microinjecting anti-p115 antibodies or depletion of p115 from cells prevents ER-to-Golgi transport of VSVG in vesicular structures [69]. Furthermore p115 is present on COPII vesicles generated in vitro [70]. In vitro studies with the yeast homologue Uso1p showed that Uso1p is required for tethering COPII vesicles to Golgi membranes [71]. These findings suggest that p115 might be necessary for the fusion of ER-derived COPII vesicles with later compartments of the secretory pathway. In addition to Uso1p and p115 a second large oligomeric complex is likely to be inolved in anterograde vesicle tethering. The transport protein particle I (TRAPP I) complex targets COPII vesicles to the Golgi in yeast and to the ERGIC in mammals [69].

Tethering of COPI vesicles requires a tripartite tether composed of p115, GM130 and Giantin that form a molecular bridge between the vesicle and the target membrane [72]. The model postulates that Giantin in COPI vesicles binds p115, which then binds GM130 on the acceptor *cis*-Golgi membrane [73]. Additionally to coiled-coil proteins, multisubunit complexes were described to be involved in tethering of COPI vesicles. The conserved oilgomeric Golgi (COG) complex consists of eight subunits (COG1-COG8) [69]. COG3 depletion in HeLa cells leads to the accumulation of vesicles containing the Golgi SNAREs GS15 and GS28 and the *cis*-Golgi glycoprotein GPP130 [74]. Anterograde transport of VSVG is not affected in COG3 depleted cells, while retrograde traffic of Shiga toxin is inhibited. According to these results the COG complex is believed to function in intra-Golgi recycling of COPI vesicles.

The final step of the lifetime of a vesicle is fusion of the vesicle with the acceptor

membrane. Initiation of fusion requires SNARE proteins that interact in specific combinations to bring the vesicles and acceptor membranes into close proximity and drive fusion. The model for SNARE-mediated membrane fusion postulates that the t-SNARE is composed of three subunits, a syntaxin-like heavy chain and two light chains composed of either one or two additional SNAREs. The v-SNARE is a monomeric protein that has to be on the membrane opposite of the t-SNARE assembly. The three t-SNAREs are assembled into an acceptor complex. The acceptor complex interacts with the monomeric v-SNARE which leads to the formation of a four-helical *trans*-complex leading to fusion of the vesicle with the membrane. During fusion, the strained *trans*-complex relaxes into a *cis*-configuration. The *cis*-complexes are then disassembled by the AAA+ (ATPases associated with various cellular activities) protein NSF (N-ethylmaleimide sensitive factor) together with SNAPs (soluble NSF attachment protein) that function as cofactors [75].

1.4 ERGIC: the first sorting station for anterograde and retrograde cargo

The ERGIC, also termed vesicular tubular clusters (VTCs), was originally described as tubulovesicular membrane clusters distributed between ERES and the Golgi [76-79]. Morphologically the ERGIC is best described by the cycling membrane protein ERGIC-53 [19] and the COPI coat subunit β-COP [77]. ERGIC-53 is a type I transmembrane protein that continuously cycles between the ER, ERGIC and the early Golgi. Other proteins enriched in the ERGIC are p24 family members [80-82], Kdel-R [83] and proteins of the targeting/fusion machinery directing ER to Golgi transport such as the small GTPases Rab1 and Rab2 [84-86] and the SNARE proteins syntaxin 5 [87, 88], rBet1 [89], Sec22 [90], and syntaxin 18 [91].

A characteristic feature of the ERGIC is its resistance to the fungal metabolite Brefeldin A (BFA). BFA binds Arf1-GDP, preventing its activation and thereby inhibits binding of COPI coats to ERGIC and Golgi membranes [92]. Upon BFA treatment the Golgi tubulates and fuses with the ER, while the ERGIC clusters keep their identity and become larger and more uniformly distributed in the cell. Several cycling proteins such as ERGIC-53, Kdel-R and proteins of the p24 family were shown to accumulate in the ERGIC after BFA treatment [49, 81, 93-95].

Studying transport of ts045 VSVG and the E1 glycoprotein of Semliki forest virus identified ERGIC-53 positive membranes as intermediates in ER to Golgi

transport [96, 97]. Currently there are two models describing transport through the ERGIC as transport intermediate station. Direct visualization of the GFP-tagged secretory marker protein tsO45 VSVG in living cells suggested that the ERGIC is a mobile membrane structure that itself carries secretory material along microtubules from the ER to the Golgi [98, 99]. These mobile structures were termed transport complexes (TC) which gave rise to the TC model [100, 101]. In this model ER-derived COPII vesicles form de novo the ERGIC by homotypic fusion. The ERGIC then migrates to and fuses with or gives rise to the *cis*-Golgi, delivering secretory cargo to the Golgi. Retrograde cargo is sorted in to COPI vesicles generated from the ERGIC and retrieved back to the ER.

The stable compartment model describes the ERGIC not as TC that fuses with the Golgi but rather considers the ERGIC as a true compartment receiving cargo from the ER and generating carriers destined for the Golgi [102]. This model is based on the findings that GFP-tagged ERGIC-53 localizes to long-lived stationary membrane structures that show no net movement towards the Golgi while TC carrying GFP-tagged tsO45 VSV-G do [103]. In this view ER-derived COPII vesicles carrying secretory cargo fuse with stationary ERGIC clusters which operate as sorting station for anterograde and retrograde traffic. Anterograde cargo is sorted into anterograde carriers that move towards and fuse with the *cis*-Golgi, while retrograde cargo is sorted into COPI vesicles and retrieved back to the ER. In both models the ERGIC is considered to be the first post-ER sorting station for anterograde and retrograde cargo.

1.5 The Golgi apparatus

The Golgi is the central station along the secretory pathway. It receives newly synthesized proteins and lipids from the ERGIC and distributes them to the plasma membrane and to the endosomal/lysosomal system. It operates as a carbohydrate factory for the processing and modification of proteins and lipids moving through the secretory pathway [104]. In mammalian cells the Golgi is typically located around the centrosome, where it remains due to interactions with microtubules and serves as a membrane scaffold onto which diverse signaling, sorting and cytoskeleton proteins adhere [105, 106].

Structurally the Golgi is composed of flat cisternae grouped into several stacks that are interconnected by tubular networks, which together form a continuous

membranous ribbon (Figure 5) [107, 108]. This organelle may be subdivided in three main morphologically distinct compartments: the *cis*-, *medial* and *trans*-Golgi, with three basic structural elements: stacks of flat cisternae, tubular-reticular networks and vesicles (Figure 5). The *cis* side of the Golgi harbours small tubules and vesicles forming the so-called vesicular tubular clusters (VTCs) that make up the ERGIC and the *cis*-Golgi network (CGN), a tubular network composed of branching tubules connected with the *cis*-most cisterna of the Golgi. The CGN is followed by the stack of flat cisternae. The stack of cisternae located between the CGN and the *trans*-Golgi network (TGN) makes up the *medial* Golgi. It contains enzymes that are involved in post-translational modification of newly synthesized proteins and lipids (for example, phosphorylation, acylation, glycosylation, methylation and sulphation) and form distribution gradients in the *cis*-to-*trans* direction of Golgi stacks [109]. Additionally to the cisternae the *medial* Golgi possesses significant tubular and vesicular elements.

The TGN forms the exit pole of the Golgi complex, where proteins are directed to their final destination. It is involved in the terminal glycosylation of proteins as well as in cargo packaging into membrane carriers destined for the PM or the endosomal/lysosomal system [110].

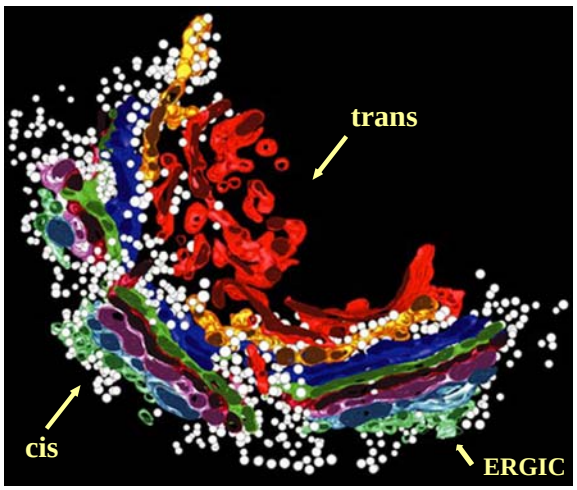

Figure 5: Model of part of the Golgi ribbon in mammalian cells

The Golgi ribbon in this model is composed of seven cisternae. The trans most cisterna (red) and the penultimate trans-element, C6 (gold) are dissociated from the stack and are fragmented by tubulation. C1 (light blue) is highly fenestrated and constitutes the CGN. C2 (pink) to C5 (blue) is composed of aligned and stacked sheets. A layer of docked and fused vesicular tubular clusters (light green, ERGIC) precedes the CGN. Numerous, small, clathrin-negative vesicles (white) occupy much of the space between C5, C6 and C7.
(Reproduced from [109]).

1.5.1 Architecture of the Golgi apparatus

The localization and tight organization of the Golgi apparatus within the cell requires a network of machineries and proteins that contribute. In mammalian cells the position of the Golgi correlates with that of the microtubule (MT)-organizing centre. Disruption of microtubules with agents such as colchicine and nocodazole that cause MT depolymerization results in dispersal of the Golgi into mini-stacks spread throughout the cell. Therefore MTs are important for the formation of the Golgi ribbon and positioning it adjacent to the centrosome and nucleus. The highly organized and polarized structure of the Golgi apparatus is not only dependent on MTs. Electron microscopy studies identified filamentous material linking Golgi cisternae and after detergent extraction a proteinaceous exoskeleton retaining the characteristic organization of the Golgi remained. This proteinaceous exoskeleton is referred to as the Golgi matrix and is known to contain members of the golgin family of Golgi-localized coiled-coil proteins and the GRASP (Golgi reassembly stacking proteins) family of Golgi stacking proteins [111, 112].

The golgins share a common predicted structural feature, the presence of long regions of coiled-coil motifs known to from an extended rod-like structure. One of the best characterized functions of golgins is their role in membrane tethering events, as described above for the golgins, p115, GM130 and giantin. Additionally to their tethering function of vesicles these golgins have also been implicated in the stacking of Golgi cisternae when the Golgi reforms following mitosis, a case of membrane tethering without subsequent fusion [113], suggesting that hey might form the Golgi matrix.

A second important component of the Golgi matrix is the GRASP family of proteins identified using a functional assay for the post-mitotic reassembly of Golgi stacks. Inhibition of this assay by NEM allowed the identification of GRASP55 and GRASP65. These proteins are cytosolic peripheral membrane proteins associated with the membrane of the *cis*-Golgi in the case of GRASP65 and the *medial*-Golgi for GRASP55 by N-terminal myristoylation. The role of GRASPs in stacking of Golgi membranes was elucidated in an in vitro assay in which reassembly of the Golgi into stacked cisternae following mitosis was blocked with antibodies specific to GRASP55 or GRASP65 [114, 115]. Additionally one important way in which GRASPs regulate Golgi structure is their interaction with members of the golgin family. GM130 is targeted to *cis*-Golgi membranes by its tight binding to GRASP65. Depletion of GM130 leads to destabilization of GRASP65 and converts the Golgi ribbon into a

perinuclear collection of short ministacks [116]. This supports the view that GM130 together with GRASP65 might be involved in Golgi structure maintenance by allowing lateral membrane fusion to obtain an intact Golgi ribbon. Similarly GRASP55 is the binding partner of golgin-45. Depletion of golgin-45 results in the dispersal of the Golgi apparatus and inhibition of protein transport [117].

1.5.2 Cargo movement through the Golgi

Conceptually there are three possibilities how cargo might be transported through the Golgi cisternae. First, a constant formation of cisternae can move cargo forward. Second, vesicles can move cargo from one cisterna to the other. Third, membrane-tubules can allow proteins and lipids to diffuse between cisternae.

How the cell can maintain an asymmetric distribution of enzymes, while at the same time ensuring vectorial transport of newly synthesized proteins to the PM is explained basically by two models: The vesicular transport model and the cisternal maturation model (Figure 6) [118]. In the vesicular transport model post-ER compartments are viewed as being biochemically distinct and stable. They receive newly synthesized proteins from an upstream compartment, subject them to processing and then pass them to a downstream compartment by vesicular transport. In contrast the cisternal maturation model views post-ER compartments such as Golgi cisternae as bulk carriers of cargo on the way to the cell surface. The vesicular transport model relies on stationary and stable compartments, each with its own composition of processing enzymes, whereas the cisternal maturation model relies on continuous remodeling of maturing dynamic compartments. In the vesicular transport model the input of anterograde proteins and membranes from upstream compartments is balanced by an equal output to downstream compartments. Escaped enzymes are returned via COPI vesicles to the proper compartment. In the cisternal maturation model the anterograde bulk movement of enzymes, secretory cargo and lipids is balanced by retrograde transport via COPI vesicles which maintains the asymmetric distribution of processing enzymes in the Golgi.

Beside the bi-directional vesicular transport and compartment maturation models a third principle of traffic via continuities was proposed (for review see [119]). This model predicts that different compartments of the early secretory pathway are interconnected via tubules with each other and that these tubules might serve as pipelines for cargo flow. Still the vesicular transport and compartment maturation

models are favoured to the traffic model via continuities.

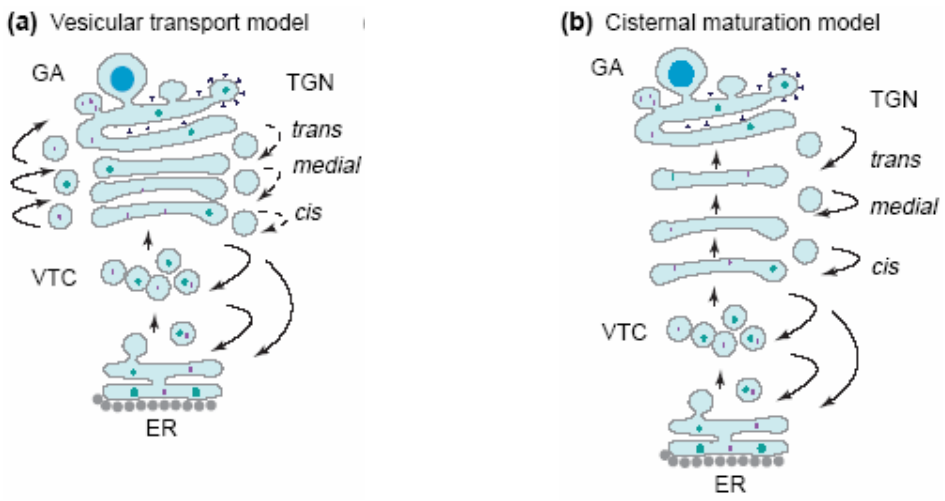

Figure 6: Vesicular transport and cisternal maturation models of secretory transport through the Golgi complex. In (a), the vesicular transport model, newly synthesized cargo proteins are transported in the forward or anterograde direction by vesicles coated with COPI. At the same time, a low level of intra-Golgi retrograde transport by COPIcoated vesicles is expected to offset leakage of resident proteins from one compartment to another. In (b), the cisternal maturation model, the cisternae themselves are the carriers for cargo, and COPI-coated vesicles function to transport resident Golgi components in the retrograde direction to produce cisternal maturation. Arrows between the cis-Golgi and the vesicular tubular carrier (VTC) and the endoplasmic reticulum (ER) mark the retrieval of components that shuttle between the ER and the Golgi complex (GA).
Reproduced from [118].

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2. Aim of the thesis

2.1 Characterization of Surf4 and Surf4 interacting proteins

Transmembrane cargo receptors are major constituents of anterograde and retrograde transport vesicles that mediate protein sorting by linking soluble luminal cargo to cytoplasmic coat assembly. Their ability to cycle within the early secretory pathway and to sort cargo depends on their oligomeric state. Erv29p is the best described cargo receptor in yeast but its human ortholog known as Surf4 is poorly characterized. Characterization of Surf4 may give new insights into the function of cargo receptors within the early secretory pathway.

In this thesis I characterized human Surf4 and it's cycling properties by site-directed mutagenesis and immunofluorescence-based protein localization studies. Since oligomerization is a hallmark of cycling transmembrane proteins, it was also attempted to find Surf4 interacting proteins. Blue Native-PAGE and mass spectrometry analysis together with co-immunoprecipitation studies indeed allowed the identification of new interacting partners for Surf4. The characterization of Surf4 interacting proteins in combination with short interference RNA (siRNA)-mediated protein knockdowns uncovered a novel role of cargo receptors in maintaining the architecture of the early secretory pathway.

2.2 Cargo receptor function of Surf4

The cargo receptor function of Erv29p in yeast points to a similar function of Surf4 in mammalian cells. Erv29p specifically recognizes an export signal on glycosylated pro-α-factor (gpαf) and packages it into COPII coated vesicles. Deletion of *ERV29* leads to a delay in transport of the soluble proteins gpαf, carboxypeptidase Y (CPY) and proteinase A (PrA) as well as stabilization of the soluble ER associated degradation (ERAD) substrates CPY* and PrA*. So far Erv29p is the only cargo receptor known to be involved in transport of soluble correctly folded cargo as well as stabilization of soluble ERAD substrates. What is the cargo for human Surf4? And does Surf4 contribute to clear the ER from accumulated ERAD substrates?

In this thesis pulse-chase analysis in combination with siRNA-mediated Surf4 knockdown gave insights into the cargo receptor function of Surf4 in human cells. A potential role of Surf4 in ERAD was also tested using misfolded α1-antitrypsin as a

substrate.

3. Results

3.1 The cargo receptors Surf4, ERGIC-53 and p25 are required to maintain the architecture of ERGIC and Golgi

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The cargo receptors Surf4, ERGIC-53 and p25 are required to maintain the architecture of ERGIC and Golgi

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Abstract

Rapidly cycling proteins of the early secretory pathway can operate as cargo receptors. Known cargo receptors are abundant proteins, but it remains mysterious why their inactivation leads to rather limited secretion phenotypes. Studies of Surf4, the human homolog of the yeast cargo receptor Erv29p, now reveal a novel function of cargo receptors. Surf4 was found to interact with ERGIC-53 and p24 proteins. Silencing Surf4 together with ERGIC-53 or silencing the p24 family member p25 induced an identical phenotype characterized by a reduced number of ERGIC clusters and fragmentation of the Golgi apparatus without effect on anterograde transport. Live imaging showed decreased stability of ERGIC clusters after knockdown of p25. Silencing of Surf4/ERGIC-53 or p25 resulted in partial redistribution of COP I but not Golgi matrix proteins to the cytosol and partial resistance of the *cis*-Golgi to brefeldin A. These findings imply that cargo receptors are essential for maintaining the architecture of ERGIC and Golgi by controlling COP I recruitment.

Introduction

The secretory pathway of higher eukaryotic cells is composed of the three membrane organelles ER, ERGIC and Golgi (Appenzeller-Herzog and Hauri, 2006; Bonifacino and Glick, 2004). Maintenance of these organelles requires a balance of anterograde (secretory) and retrograde vesicular traffic. Anterograde traffic from ER to ERGIC is mediated by COP II (coat protein II) vesicles that form at ER exit sites (Aridor et al., 1995; Zeuschner et al., 2006) and fuse with the ERGIC that consists of a few hundred tubulovesicular membrane clusters in close vicinity of ER exit sites (Appenzeller-Herzog and Hauri, 2006). Transport from ERGIC to Golgi is mediated by pleomorphic vesicles (Ben-Tekaya et al., 2005) that carry COP I (coat protein I) (Presley et al., 1997; Scales et al., 1997) although the mechanism of their formation remains unknown. Retrograde traffic mediated by COP I vesicles can occur from ERGIC or Golgi and recycles membrane proteins that possess either di-lysine signals, including ERGIC-53 and KDEL-receptor, or di-phenylalanine signals, such as members of the 24 protein family. This rapid COP I-dependent recycling is distinct from the slow Golgi to ER recycling of Golgi resident proteins that is COP I-independent and can be either constitutive or induced (Storrie, 2005).

Major constituents of anterograde and retrograde transport vesicles are transmembrane cargo receptors that mediate protein sorting by linking soluble cargo on the luminal side and coat assembly on the cytoplasmic side. To date only few cargo receptors have been studied in detail. The polytopic transmembrane protein Erv29p is known to cycle between ER and Golgi in yeast and to operate as a cargo receptor (Belden and Barlowe, 2001). Erv29p is required for efficient packaging of the glycosylated α-factor pheromone precursor into COP II vesicles departing from the ER. Maturation of carboxypeptidase Y and proteinase A, but not other secretory proteins such as invertase, also depend on Erv29p (Caldwell et al., 2001). In support of the cargo receptor concept, a hydrophobic sorting signal was identified in α-factor which is required for its interaction with Erv29p and efficient transport (Belden and Barlowe, 2001; Otte and Barlowe, 2004). Erv29p is conserved among eukaryotes and the mammalian ortholog has been designated Surf4 (Reeves and Fried, 1995). Although its function is unknown, it is possible that Surf4 has a similar role ER-to-Golgi transport in mammalian cells given the extent of homology with Erv29p that includes a di-lysine retrieval motif.

The best characterized cargo receptor in mammalian cells is the mannose-

specific leguminous type lectin ERGIC-53 (Appenzeller-Herzog and Hauri, 2006; Hauri et al., 2000). ERGIC-53 is a hexameric type I membrane protein in complex with the luminal EF-hand protein MCFD2 (Nyfeler et al., 2006; Zhang et al., 2003). This cargo receptor complex cycles between ER and ERGIC (Klumperman et al., 1998; Nyfeler et al., 2006) and facilitates ER to ERGIC transport of the lysosomal enzymes glycoproteins cathepsin C (Nyfeler et al., 2005; Vollenweider et al., 1998), cathepsin Z (Appenzeller et al., 1999) and the blood coagulation factors V and VIII (Nichols et al., 1998; Zhang et al., 2003). MCFD2 is dispensable for the transport of the lysosomal enzymes but required for the transport of factors V and VIII (Nyfeler et al., 2006). In the ER, high-mannose cathepsin Z binds to ERGIC-53 by a combined glycan/β-hairpin signal and is subsequently released from ERGIC-53 in the ERGIC (Appenzeller-Herzog et al., 2005).

Yet another major cargo receptor is Emp24p in yeast. Emp24p is the founding member of the p24 protein family (Kaiser, 2000) and is required for efficient ER to Golgi transport of GPI-(glycosylphosphatidylinositol) anchored proteins (Muniz et al., 2000; Schimmoller et al., 1995). It is conceivable that mammalian p24 proteins also operate as cargo receptors although no cargo protein has been identified. Mammalian p24 proteins are localized in the early secretory pathway and rapidly cycle between the ER and Golgi. In order to achieve their correct targeting within the early secretory pathway they are in a dynamic equilibrium to form homo- and heterodimers with each other (Jenne et al., 2002). All p24 family members are type I membrane proteins and share a common structure, with a short cytoplasmic tail containing binding signals for COPI and COPII coat complexes and a luminal domain with potential secretory cargo binding capabilities (Dominguez et al., 1998; Fiedler et al., 1996; Muniz et al., 2000; Sohn et al., 1996). Proteomics analysis revealed that p24 family members are major constituents of COPI coated vesicles (Stamnes et al., 1995). Their involvement in COPI vesicle formation was identified in vitro by using liposomes with Golgi-like lipid composition. Liposomes incubated with the cytoplasmic components Arf1, coatomer and GTP alone are unable to induce vesicle formation unless cytoplasmic domains of p24 family proteins are present (Bremser et al., 1999). P24 proteins appear to have some morphogenetic potential. p23 of the p24 family is an essential gene in mammals, and a heterozygous deletion reduces the levels of this protein and other family members resulting in dilation of Golgi cisternae (Denzel et al., 2000). In cell cultures overexpression of p23 leads to its mislocalization to the ER which causes expansion and clustering of smooth ER

membranes. Mislocalization of p23 to the ER also leads to depletion of endogenous p23 from the Golgi, resulting in dispersion of this organelle (Rojo et al., 2000).

In the present study we have characterized human Surf4 and found it to localize to and cycle in the early secretory pathway similar to ERGIC-53. Surf4 forms multi-protein complexes with ERGIC-53 and p24 family members. Unexpectedly, silencing of Surf4 together with ERGIC-53 or silencing p25 of the p24 protein family disrupted the Golgi apparatus and led to instability of the ERGIC in conjunction with partial dissociation of COP I.

Results

Human Surf4 localizes to the ERGIC and cycles in the early secretory pathway

Although discovered quite some time ago, mammalian Surf4 remains largely uncharacterized. Even its subcellular localization is uncertain. N-terminally tagged Surf4 localizes to the ER, whereas C-terminally tagged Surf4 localizes to the Golgi in transfected cells (Reeves and Fried, 1995). In contrast, endogenous Surf4 was identified by mass spectrometry in an ERGIC fraction isolated from brefeldin A (BFA)-treated HepG2 cells (Breuza et al., 2004). To characterize endogenous Surf4, we prepared polyclonal antibodies to the N-terminal 60 amino acids of Surf4 fused to GST and purified them by affinity chromatography. Affinity-purified anti-Surf4 recognized a protein of about 22 kD on Western blots in reasonable agreement with the Mr of Surf4 deduced from conceptual translation (Fig. S1A). The specificity of the antibody was confirmed by silencing Surf4 in HeLa cells using siRNA. In control cells anti-Surf4 gave an immunofluorescence pattern similar to that of the ERGIC marker ERGIC-53, whereas the staining disappeared after siRNA-mediated knockdown of Surf4 without affecting the distribution of ERGIC-53 (Fig. S1B). These results indicate specificity of our antibodies against Surf4.

To more precisely establish the localization of Surf4, co-localization studies with various organelle markers were performed by immunofluorescence microscopy. Surf4 prominently stained peripheral ERGIC clusters positive for ERGIC-53 (Fig. 1A, also see Fig. S1B) and partially co-localized with the ER marker BAP31 and the Golgi marker giantin (Fig. 1A). The predominant localization of endogenous Surf4 in the ERGIC suggests that Surf4 might be a cycling protein of the early secretory pathway. To test this, the distribution of endogenous Surf4 was studied in HeLa cells treated with BFA that is known to accumulate rapidly cycling proteins in the ERGIC (Lippincott-Schwartz et al., 1990). Indeed, this treatment concentrated Surf4 in ERGIC-53-positive structures (Fig. 1A) supporting the notion that Surf4 is a cycling protein.

Surf4 is a multispanning membrane protein with its C-terminus predicted to face the cytosol. The cytosolic tail carries lysine residues in positions -2, -3 and -4 from the C-terminus. Two lysines in positions -3 and -4 are known to function as ER targeting signal mediating retrieval (Teasdale and Jackson, 1996) or retention (Andersson et al., 1999), depending on amino acids in position -1 and -2. We tested the functionality of the lysine motif by mutating the three lysines to serines in

hemagglutinin-tagged Surf4 (HA-Surf4SSS). HA-Surf4 localized to ER and ERGIC very much in contrast to HA-Surf4SSS that localized to the Golgi region (Fig. 1B). This result is consistent with and explains the Golgi localization of C-terminally tagged Surf4 (Reeves and Fried, 1995). In that study, the C-terminal tagging obviously inactivated the di-lysine signal, which is known to be position dependent (Teasdale and Jackson, 1996). Collectively, our results indicate that Surf4 cycles early in the secretory pathway in a lysine signal-dependent way, similarly to ERGIC-53.

Surf4 interacts with members of the p24 protein family and ERGIC-53

In search of the function of Surf4 we attempted to identify interacting proteins. To maximize such interactions, Surf4 was accumulated in the ERGIC by treating HepG2 cells with BFA. Both non-transfected and HA-Surf4-transfected cells were analyzed. ERGIC membranes were isolated by Nycodenz gradient centrifugation (Breuza et al., 2004) and the gradient fractions were analyzed for organelle markers by Western blotting. Surf4 largely co-distributed with the ERGIC marker ERGIC-53 (Fig S2). The ERGIC fractions were collected, and the membranes subjected to Blue Native PAGE. Western blotting revealed that both Surf4 and HA-tagged Surf4 formed protein complexes of about 60 kD and 232 kD (Fig. 2A). Since HA-Surf4 behaved like endogenous Surf4 on Blue Native gels (Fig. 2A), and was more abundant, some of the further experiments were performed with HA-Surf4. Separation of the protein complexes by SDS-PAGE in a second dimension showed distinct protein spots of 15 to 37 kD which were identified by mass spectrometry as Surf4 and members of the p24 protein family (Fig. 2B). This approach also identified the previously described protein complex of p23, p24, p25 and p27 (Fullekrug et al., 1999), demonstrating the accuracy of the method (Fig. 2B). The 60 kD complex appears to contain Surf4 and KDEL-receptor, but the possibility of an interaction of the two proteins has not been investigated in the current study.

To confirm the interaction between Surf4 and p24 family members, co-immunoprecipitation experiments were performed. Since the antibody against Surf4 did not immunoprecipitate endogenous Surf4, the HA-tagged protein was studied in transfected HepG2 cells. Fig 2C shows that both anti-p23 and anti-p24 pulled down HA-Surf4. Inversely, anti-HA pulled down both p23 and p24. Surprisingly, a highly specific monoclonal antibody against ERGIC-53, used as (presumed) negative control, also pulled down HA-Surf4 (Fig. 2C). This unexpected result was confirmed

for endogenous Surf4 in HeLa cells. Anti-ERGIC-53 pulled down Surf4 but not p23 (Fig. 2D). We conclude that Surf4 forms heterooligomeric complexes with members of the p24 family and in addition interacts with ERGIC-53.

Silencing of Surf4 and ERGIC-53 or p25 disrupts the Golgi

To obtain more insight into the function of Surf4 we took a silencing approach using siRNA (Fig. S3A, B). A knockdown of Surf4 to 10% in HeLa cells, had no effect on the distribution of organelle markers for ER (unpublished data), ERGIC and Golgi (Fig. 3B, S1B, S4A), nor was total secretion of ^{35}S-methonine-labeled proteins impaired 3 days after siRNA transfection (Fig. S4B). The serendipitous finding of co-immunoprecipation of ERGIC-53 and Surf4 mentioned above led us to test the combined requirement of Surf4 and ERGIC-53. Strikingly, a double knockdown of Surf4 and ERGIC-53 by siRNA disrupted the Golgi apparatus as visualized by staining for giantin (Fig. 3A). Quantification showed that 70% of the cells had a dispersed Golgi (Fig. 3B). In contrast, a single knockdown of ERGIC-53 to 10% (Fig. S3A, S3B) had no effect on Golgi morphology (Fig. 3B, S4A), consistent with previous knockdown data (Nyfeler et al., 2006) and the observation that mislocalization of ERGIC-53 to the ER did not induce changes of the early secretory pathway (Vollenweider et al., 1998). Since ERGIC-53 is known to form a complex with the soluble protein MCFD2 and a knockdown of ERGIC-53 leads to secretion of MCFD2 (Nyfeler et al., 2006), we wondered if the Golgi change was due to the lack of MCFD2 rather than ERGIC-53. However, a double knockdown of Surf4 and MCFD2 had no effect on Golgi morphology (not shown), strongly suggesting the specific involvement of ERGIC-53 in maintaining normal Golgi structure together with Surf4.

Next we asked whether the silencing of p24 proteins would also affect Golgi structure. Besides their proposed role as cargo receptors, p24 family members are believed to function as morphogens in the early secretory pathway. Such a function has mainly been derived from overexpression studies (Blum et al., 1999; Rojo et al., 2000). In addition, the inactivation of one allele of p23 in mice induces structural changes in the Golgi apparatus and reduces the levels of p23, p24 and p25 (Denzel et al., 2000). It is noteworthy that siRNA-based knockdown experiments have not been reported for p24 proteins. The known hetero-oligomerization and interdependence of p24 family members complicates such an analysis. Accordingly, we found that a knockdown of p24 reduced p23 levels and vice versa (not shown).

Depletion of p25 to 25% (Fig. S3A, B), however, did not affect the protein levels of p24 or p23 which led us to focus on p25 (not shown). Strikingly, the knockdown of p25 in HeLa cells induced a change in Golgi morphology that was indistinguishable from that obtained by the Surf4/ERGIC-53 double knockdown (Fig. 3A). 90% of the transfected cells showed fragmentation of the Golgi as visualized by immunfluorescene microscopy (Fig. 3B). Importantly, the knockdown of p25 did not change the protein levels of Surf4 or ERGIC-53 and vice versa (Fig. S3C).

Are the changed Golgi structures identical in Surf4/ERGIC-53 and p25 knockdowns? As a test we analyzed the silenced cells by transmission electron microscopy. This analysis indicated that under both knockdown conditions the Golgi ribbon was converted to mini-stacks that otherwise looked unchanged. In particular, the cisternae were not swollen and cisternal stacking was intact suggesting normal *cis*-*trans* topology (Fig. 3C). Thus, the changes in Golgi morphology induced by a knockdown of Surf4 and ERGIC-53 or p25 are indistinguishable by both light and electron microscopy.

Cargo receptor silencing destabilizes the ERGIC without affecting ER exit sites or protein secretion

The finding that a double knockdown of Surf4 and ERGIC-53 and a single knockdown of p25 induced a Golgi phenotype was unexpected since all three proteins are mainly associated with the ERGIC although they also cycle through the Golgi to some extent (Dominguez et al., 1998; Klumperman et al., 1998; Schweizer et al., 1988). Further, a study on the reconstitution of the secretory pathway in a cell-free assay suggests that p25 plays a role in the *de novo* formation of the ERGIC (Lavoie et al., 1999). Based on these findings we considered the possibility that the knockdowns might also induce changes at the level of the ERGIC. To detect such changes, HeLa cells depleted of Surf4/ERGIC-53 or p25 were double labeled for the ERGIC/*cis*-Golgi marker KDEL-receptor and the Golgi marker giantin. Peripheral ERGIC structures were quantified in the knockdown cells which could readily be identified by a dispersed giantin pattern (Fig. 4A). Quantification showed that control cells exhibited 490 KDEL-receptor-positive ERGIC structures on average, whereas cells depleted of Surf4/ERGIC-53 had only 230 and cells depleted of p25 only 300 ERGIC structures per cell (Fig. 4A, B). The reduction of KDEL-receptor-positive ERGIC structures was not due to reduced levels of KDEL-receptor (S3C). Clearly, both the Surf4/ERGIC-53 knockdown and the p25 knockdown reduced the number of

peripheral ERGIC clusters.

ER export activity is known to be modulated by the cargo load (Aridor et al., 1999; Guo and Linstedt, 2006). Accordingly, the depletion of cargo receptors may impair ER export that would explain the reduction in ERGIC cluster numbers. If true, one would expect that the number of ER exit sites is reduced in parallel. Therefore, we determined the number of ER exit sites labeled by antibodies against the COP II coat protein Sec31 (Fig. 5A). P25 knockdown cells showed 400 ER exit sites on average, which was comparable to the 420 ER exit sites counted in control cells (Fig. 5B). Surf4/ERGIC-53 knockdowns exhibited a slightly reduced number of 320 ER exit sites (Fig. 5B). These numbers show that the reduction of ERGIC clusters is not paralleled by a similar reduction of ER exit sites.

Do the structural changes of ERGIC Golgi impair total protein secretion? We used a pulse-chase approach to address this question. HeLa cells in which p25 or Surf4 together with ERGIC-53 had been silenced, were pulse-labeled with ^{35}S-methionine and the radioactive proteins secreted into the medium during chase were quantified by scintillation counting after TCA precipitation. Fig 5C shows that neither silencing Surf4/ERGIC-53 nor p25 significantly affected total protein secretion after 3 days, although after 4 days the secretion was less efficient (not shown). Since maximal protein silencing was reached already after 3 days of transfection, we conclude that secretion is initially unaffected by the two knockdown conditions.

To study the dynamics of the ERGIC we used live cell imaging of HeLa cells stably expressing GFP-ERGIC-53. In this cell line GFP-ERGIC-53 behaves like endogenous ERGIC-53 (Ben-Tekaya et al., 2005). Strikingly after p25 knockdown, stationary ERGIC structures hovered about in place more actively and disappeared faster than in control cells (Fig.6; supplementary movie 1). Tracking peripheral ERGIC structures revealed that their relative life span was reduced by 35% (Fig. S5). Thus, the reduction of ERGIC clusters in p25 knockdown cells can, at least in part, be attributed to a shorter half-life. The ERGIC structures in Surf4/ERGIC-53 knockdown cells could not be analyzed in living cells since no acceptable GFP-tagged marker was available to identify the ERGIC-53 in the absence of ERGIC-53. We speculate, however, that the ERGIC structures in Surf4/ERGIC-53 depleted cells would behave similarly.

Collectively, the morphological, biochemical and live cell imaging results indicate that cargo receptor silencing destabilizes the ERGIC without initial impairment of overall protein secretion.

Golgi matrix proteins remain associated with the dispersed Golgi

P24 family members are known to form complexes with the Golgi matrix proteins GM130, GRASP65 and GRASP55 (Barr et al., 2001). These matrix proteins are required for normal Golgi morophology. GM130 is a *cis*-Golgi localized coiled-coil protein targeted to membranes via the peripheral membrane protein GRASP65 (Barr et al., 1998; Barr et al., 1997). It also binds the vesicle tethering factor p115 (Nakamura et al., 1997; Nelson et al., 1998). GM130 and GRASP65 are key determinants for maintaining Golgi morphology as their knockdown transforms the Golgi ribbon to ministacks (Puthenveedu et al., 2006; Sohda et al., 2005). The knockdowns of p25 and Surf4/ERGIC-53 produced a Golgi phenotype reminiscent of that observed after a knockdown of GM130 and GRASP65 (Puthenveedu et al., 2006; Sohda et al., 2005). This led us to study the distribution of Golgi matrix proteins in p25- and Surf4/ERGIC-53-depleted cells. Fig. 7 clearly shows that GM130, GRASP65 and p115 remained associated with the dispersed Golgi in both p25 and Surf4/ERGIC-53 knockdown cells. We conclude that the morphological changes of the Golgi are unlikely to be due to impaired binding of matrix proteins to Golgi membranes.

Silencing of Surf4 and ERGIC-53 or p25 dissociates COPI

Apart from cycling, a common feature of Surf4, ERGIC-53, and p25 is a di-lysine ER retention/recycling signal. Since di-lysine signals mediate COP I binding we wondered if the depletion of the three cycling proteins would affect COP I binding. To this end, Surf4 and ERGIC-53 or p25 were silenced in HeLa cells and the COP I coat subunit β-COP was localized by immunofluorescence microscopy. Strikingly, the overall signal for β-COP was reduced in both knockdown conditions (Fig. 8A, 8B). The reduction of β-COP signal was not due to lower protein levels (Fig. S3C). The Golgi region identified by GM130 showed less prominent staining for β-COP when compared to cells treated with control siRNA. β-COP staining of the ERGIC clusters was also reduced. The results indicate that Surf4/ERGIC-53 or p25 are required for COP I binding to membranes of the early secretory pathway.

A loss of COP I from Golgi membranes is known to change the structure of this organelle to the extent that it rapidly tubulates and fuses with the ER. Such an outcome is well known for cells treated with the fungal metabolite BFA. The Golgi changes induced by silencing Surf4 and ERGIC-53 or p25 are clearly different from

those induced by BFA. We wondered if knockdown cells would respond normally to BFA. Fig. 9 shows that a 30 min BFA treatment of control cells induced an almost complete disappearance of the Golgi. As expected, giantin showed an ER-like pattern and GM130 redistributed to the ERGIC (Fig. 9B). In contrast, after Surf4/ERGIC-53 or p25 silencing, GM130 was largely resistant to BFA and remained in the juxtanuclear area (Fig. 9A), very much in contrast to KDEL-receptor that redistributed to the ERGIC (Fig. 9A) and the two Golgi markers giantin and GPP130 which redistributed to the ER (unpublished data).

In summary, these data indicate that silencing Surf4 together with ERGIC-53 or silencing of p25 leads to partial dissociation of COP I and partial resistance of the *cis*-Golgi to BFA.

DISCUSSION

In this study we characterized human Surf4 and found it to be associated with the ERGIC and to cycle in the early secretory pathway in a di-lysine signal-dependent manner. Erv29p, the yeast ortholog of Surf4, acts as a cargo receptor for glycosylated alpha-factor in yeast (Belden and Barlowe, 2001; Otte and Barlowe, 2004). Although a knockdown of Surf4 had no effect on total protein secretion, it remains possible that human Surf4 also operates as a cargo receptor for a limited set of proteins that would not be apparent in a global secretion assay. Previous studies have also implicated Erv29p in ER quality control. In yeast cells lacking Erv29p, misfolded soluble proteins are stabilized, and it was proposed that efficient degradation of these misfolded proteins requires transport between ER and Golgi mediated by Erv29p (Caldwell et al., 2001). We found no equivalent function for human Surf4. An efficient knockdown of Surf4 had no effect on the degradation of the Z mutant of alpha1-antitrypsin a prototype ERAD substrate (not shown). This observation argues against a general role of Surf4 in ER degradation of misfolded soluble proteins as suggested for Erv29p.

The characterization of Surf4-interacting proteins uncovered a novel role of cargo receptors in maintaining the architecture of ERGIC and Golgi. Surf4 was found to form at least two protein complexes, one of which has a Mr of 232 kD and comprises p23, p24 and p25, and another one of about 60 kD which was not further characterized but may contain KDEL-receptor. The serendipitous finding of a co-immunoprecipitation of Surf 4 and ERGIC-53 suggests the existence of a third complex. Since ERGIC-53 forms homo-hexamers (Schweizer et al., 1988) this complex can be expected to be very large so that it may not have entered the Blue Native gel. It is widely recognized that p24 family proteins form heterooligomeric complexes with one another which complicates the functional analysis of these proteins (Dominguez et al., 1998). The current study suggests that the situation is even more complex. The major known cargo receptors can form various protein complexes with one another with functional implications for organelle maintenance. While this was unexpected, an even greater surprise was the observation that a double knockdown of Surf4/ERGIC-53 and a single knockdown of p25 resulted in an identical Golgi and ERGIC phenotype, particularly since the Surf4/ERGIC-53 knockdown did not affect p25 levels and vice versa. There are no indications, however, for a major difference of the phenotypes resulting from the two different

knockdowns, neither at the light nor at the ultrastructural level. The phenotype is characterized by a reduced number of ERGIC clusters and fragmentation of the Golgi apparatus whereby the Golgi elements were not randomly distributed in the cytoplasm but largely remained in the original area of the initially compact Golgi.

Numerous situations are known in which the Golgi assumes a fragmented phenotype. How do these phenotypes compare with that observed in the present study? The classical phenotype of dispersed Golgi is due to disruption of microtubules by microtubule-active drugs, such as nocodazole. By contrast, silencing of Surf4/ERGIC-53 or p25 had no effect on microtubules (unpublished data) and the Golgi mini-stacks were not randomly distributed in the cytoplasm as in nocodazole-treate cells. Some other knockdown conditions can lead to Golgi fragmentation similar to that described here although effects on the ERGIC have not been studied. For instance, silencing the SNARE protein syntaxin 5 results in Golgi fragmentation which barely affects anterograde transport of VSV-G, but the underlying mechanism is unknown (Suga et al., 2005). Silencing of KAP3, the non-motor subunit of kinesine 2, also results in fragmentation of the Golgi (Stauber et al., 2006). Again anterograde secretory traffic is unaffected but KDEL-receptor-dependent retrograde transport is abrogated presumably due to an unexplained redistribution of the KDEL-receptor to the ER. Thus, this phenotype is different. Yet another type of Golgi fragmentation results from silencing golgin-84 (Diao et al., 2003). However, this phenotype is accompanied by changes of the ER and has been attributed to a defect in anterograde trafficking. Comparing all the known Golgi fragmentation phenotypes, the Golgi phenotype induced by cargo receptor silencing is strikingly similar to that recently reported for knockdowns of the Golgi matrix proteins GM130 and GRASP65 (Puthenveedu et al., 2006). Either knockdown prevents lateral linking of Golgi stacks resulting in ministacks. GM130 mediates stabilization and targeting of GRASP65 and the two proteins are required for Golgi ribbon formation. As a further similarity to the current work, secretory transport is independent of GM130-mediated Golgi ribbon formation (Puthenveedu et al., 2006). Importantly, however, there was no indication of dissociation of GM130 or GRASP65 in cargo receptor knockdowns in the current study, indicating that these two Golgi matrix proteins are not sufficient for Golgi ribbon formation. Moreover, a knockdown of GM130 has no effect on the stability of the ERGIC (our unpublished observations).

Reduced COPI binding for both knockdowns of Surf4/ERIGC-53 and p25 provided a mechanistic explanation for at least some aspects of the phenotype.

There are at least two different functions of COP I: vesicle formation and stabilization of membranes (Bethune et al., 2006a; Klausner et al., 1992; Rothman, 1994; Storrie, 2005). COP I vesicles mediate membrane traffic within the Golgi, from *cis*-Golgi to ERGIC, and from ERGIC to ER. Some rapidly cycling transmembrane proteins are actively recruited to retrograde vesicles by a dilysine signal of their cytosolic tail that directly interacts with COPI subunits (Bethune et al., 2006a; Cosson and Letourneur, 1994; Jackson et al., 1990). Surf4, ERGIC-53 and p25 contain such a dilysine signal that is functional in all three proteins ((Emery et al., 2003; Itin et al., 1995) and this study). In vitro, the formation of COP I vesicles requires the presence of the cytoplasmic domains of p24 family proteins (Bremser et al., 1999). Thus COP I dissociation from *cis*-Golgi and ERGIC observed in the current study renders retrograde traffic less efficient. Because anterograde secretory traffic is unaffected this obviously leads to a shortage of ERGIC membranes which would explain the reduced number and perhaps also shortened life span of ERGIC clusters. For such an outcome with reduced ERGIC-53 cluster numbers one would have to also postulate that in the knockdown cells ERGIC-to-ER transport, although reduced, is slightly more efficient than *cis*-Golgi to ERGIC transport. This is plausible in view of the close proximity of ERGIC and ER but cannot be assessed experimentally with current technology.

A function of COPI in membrane stabilization is known from experiments with BFA. Upon BFA treatment, COP I dissociates from Golgi membranes, and these membranes rapidly tubulate and fuse with the ER. Obviously COPI protects membranes from tubulation and thereby guarantees organelle integrity and identity. Importantly, neither silencing Surf4/ERGIC-53 nor p25 induced Golgi tubulation despite considerable dissociation of COPI. Under these knockdown conditions COP I dissociation can be assumed to occur at the level of the ERGIC and *cis*-Golgi, that are the recycling sites of these cargo receptors. Clearly, COP I dissociation induced by cargo receptor silencing does not result in a BFA-like effect. Thus, COP I-depletion cannot explain the absence of tubulation of the *cis*-Golgi. Together with the partial resistance of the *cis*-Golgi to BFA after cargo receptor silencing, the lack of tubules implies that cargo receptors are required for efficient tubulation. A likely scenario is that cargo receptor tails mediate the interaction of *cis*-Golgi membranes with microtubules. Microtubules are required for BFA-induced tubulation of Golgi membranes after COPI dissociation and their subsequent consumption by the ER (Lippincott-Schwartz et al., 1990). Receptor tails may recruit kinesine-type motor

proteins, such as kinesine II (Stauber et al., 2006), in the absence of protective COP I coats. Consistent with such a mechanism, the tubulation of anterograde transport intermediates also depends on cargo receptor tails as microinjection of cytosolic tails of p23 and p24 efficiently inhibits tubule formation (Simpson et al., 2006). Obviously p24 and presumably other cargo receptor tails have an inherent tubulation potential which needs to be controlled by COP I coats to maintain Golgi integrity.

Is the Golgi fragmentation in Surf4/ERGIC-53 or p25 knockdown cells due to COP I dissociation? The close similarity of phenotypes resulting form matrix or cargo receptor knockdowns raises the question if an interaction of the two classes of proteins is required for maintaining the Golgi ribbon. If so, a knockdown of either protein class would cause an identical Golgi ministack phenotype. Such a notion is not entirely hypothetical since p23, p24 and p25 have been reported to be in a complex with GRASP65, GRASP55 and GM130 in vivo and purified GRASPs directly binds to cytoplasmic tails of p24 and p25 via two terminal valines (Barr et al., 2001). In contrast to these observations we have not seen an interaction of p25, Surf4 or ERGIC-53 with GM130 in immunoprecipitation experiments with antibodies to GM130 (not shown). Thus more detailed studies will be required to assess a putative dual interaction of cargo receptors with COP I and matrix proteins. It is worth noting, however, that the ERGIC phenotype induced by cargo receptor silencing is unlikely to be due impaired matrix/tail interactions, since GM130 is primarily associated with the first Golgi cisterna at steady state (Nakamura et al., 1995; Taguchi et al., 2003) and is not detectable in the ERGIC (Fig. 7). An alternative possibility to explain the Golgi phenotype induced by receptor silencing is a disturbed balance of the amount of Golgi membranes and matrix proteins. Reduced retrograde traffic from *cis*-Golgi to ERGIC may result in an increase in Golgi membranes without a corresponding increase in matrix proteins which may affect Golgi ribbon maintenance.

Why does a single knockdown of Surf4 or ERGIC-53 not change Golgi morphology while p25 does? Currently we can only speculate about the underlying mechansim. One possibility is that the individual levels of ERGIC-53 and Surf4 in the *cis*-Golgi are lower than those of p25 and therefore only a combined knockdown of Surf4 and ERGIC-53 leads to sufficient dissociation of COP I from the *cis*-Golgi. While no information for Surf4 is available, the levels of ERGIC-53 in the *cis*-Golgi are indeed low since the recycling of ERGIC-53 between ERGIC and ER largely bypasses the *cis*-Golgi (Klumperman et al., 1998, Tekaya et al., 2005). Alternatively p25 may not act in isolation as it forms complexes with other p24 proteins which are

known to interact with COP I coats via a di-phenylalanine rather than a di-lysine signal (Bethune et al., 2006a; Bethune et al., 2006b). By indirectly affecting other p24 family members, silencing of p25 may have a greater impact.

In conclusion we propose the following model for the changes of the early secretory pathway induced by the depletion of Surf4/ERGIC-53 or p25 (Fig. 10). The reduction of cargo receptor tails reduces COP I binding to *cis*-Golgi and ERGIC and impairs retrograde vesicular traffic. Since anterograde traffic is initially unchanged this defect results in fewer ERGIC clusters. The reduction of cargo receptors in the *cis*-Golgi also leads to Golgi ministacks either due to insufficient crosslinking of cargo receptor tails with Golgi matrix or due to an imbalance of Golgi membranes and Golgi matrix. According to the maturation model ministack formation would start at the *cis*-Golgi and gradually be completed as the first *cis*-Golgi cisterna moves and matures in *cis*-to-*trans* direction. Whatever the precise mechanism, the current study shows that networks of established and putative cargo receptors are required to maintain the architecture of ERGIC and Golgi. Thus cargo receptors of the early secretory pathway can have multiple functions by operating both individually and in concert with one another. This striking dual mode of operation will have to be taken into consideration in future attempts to understand the organization and function of the secretory pathway.

Materials and Methods

Antibodies: The following mouse monoclonal antibodies were used: G1/93 against ERGIC-53 (Schweizer et al., 1988) (ALX-804-602; Alexis, Lausen, Switzerland), A1/182/5 against BAP31 (Klumperman et al., 1998) (ALX-804-601; Alexis, Lausen, Switzerland), G1/133 against giantin (Linstedt and Hauri, 1993) (ALX-804-600-C100; Alexis, Lausen, Switzerland), anti-βCOP (kind gift of Thomas Kreis), GM130 (BD Transduction Laboratories), 12CA5 against the HA epitope. Rabbit polyclonal antibodies against the following proteins were used: KDEL-receptor (Majoul et al., 1998) (kind gift of H.-D. Söling, Max-Planck-Institut für Biophysikalische Chemie, Göttingen, Germany), Sec31 (Shugrue et al., 1999) (kind gift of F. Gorelick, Yale University), p23, p24, and p 25 (Jenne et al., 2002) (kind gifts of F. Wieland, University of Heidelberg, Germany), p115 and GM130 (Barroso et al., 1995) (Nelson et al., 1998) (kind gift of D.S. Nelson, University of Alabama Medical School, Birmingham), GRASP65 (Sutterlin et al., 2002) (kind gift of V. Malhotra, Division of Biology University of California, San Diego). Alexa 488-, Alexa 568- (Molecular Probes Europe BV, Leiden, NL) and horseradish proxidase-coupled antibodies (Jackson ImmunoResearch Inc.) were used as secondary antibodies. Polyclonal antibodies against human Surf4 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 1-60 of Surf4 fused to GST. The N-terminal sequence of Surf4 was amplified by PCR using the primers 5'-CAGGATCCCCGGCCAGAACGACCTGATG-3' and 5'-CGAATTCTTATTACATGTACTGTTTGGGGGAGCTCTC-3' and cloned into pGEX-5X2 vector via BamHI and EcoRI. The recombinant hybrid protein was expressed in E. coli BL21 and purified by glutathione-Sepharose 4B column chromatography according to the manufacturer's instruction (Amersham, Biosciences). The antiserum was affinity-purified by sequential adsorption to Affigel 15 (BioRad) immobilized GST and GST-Surf4 followed by acid elution.

Cell culture: HeLa cells and HeLa cells stably expressing GFP-ERGIC-53 (Ben-Tekaya et al., 2005) were grown in DMEM, supplemented with 10% fetal bovine serum and 1x nonessential amino acids. HepG2 cells and HepG2 cells stably expressing HA-Surf4 (Breuza et al., 2004) were grown in MEM medium, supplemented with 10% fetal bovine serum. For metabolic labeling and immunoblotting cells were grown in six-well plates. For immunofluorescence

microscopy cells were grown on coverslips in twelve-well plates.

Purification of ERGIC membranes and Blue Native-PAGE: 5 days post confluence HepG2 cells were treated with brefeldin A (BFA, 10μg/ml; Epicentre, Madison, WI, USA) for 90 min. and ERGIC membranes were isolated by subcellular fractionation using Nycodenz gradients (Breuza et al., 2004). Fractions of the Nycodenz gradient enriched in the ERGIC marker ERGIC-53 were pooled and diluted five times with PBS. The membranes were centrifuged at 100'000xg for 1 h, followed by solubilization in 25mM Bis-Tris-HCl pH 7.0, 2% digitonin, 500mM 6-amino-caproic acid. The lysates were cleared at 100'000 xg for 1h and separated by Blue Native PAGE (Hunte et al., 2003).

Mass Spectrometry: Protein complexes separated by Blue Native PAGE were separated in a second dimension by SDS-PAGE, and proteins were visualized by Colloidal Blue (Invitrogen, Switzerland). Gel pieces were excised and washed five times for 1 min with 50 μl 40% n-propanol followed by five washes (1 min each) with 30 μl 0.2 M NH_4HCO_3 containing 50% acetonitrile. The gel pieces were dried in a SpeedVac concentrator (Savant, Farmingdale, NY, USA) and re-swollen in 10 μl 100 mM NH_4HCO_3 containing 0.5 μg modified trypsin (Promega, Madison, WI, USA). Trypsin digestion was performed at 37°C for 18 hrs. The supernatants were collected and the gel pieces were extracted with 15 μl 0.1% formic acid for 5 min, followed by 15 μl acetonitrile for 1 min. Extraction was repeated twice and all supernatants were pooled and dried by SpeedVac. For desalting, the peptides were dissolved in 0.1% TFA and adsorbed on C18 ZipTips (P10 size, Millipore). The peptides adsorbed on the tips were washed with 0.1% TFA and eluted with 1.5 μl 80 % AcCN, 0.1%TFA, containing 1 μg/μl α-cyano-4-hydroxycinnamic acid (CHCA, Aldrich Chemical Co., Milwaukee, IL). 500 nl of the eluate was deposited onto anchor spots of a Scout 400 μm/36 sample support (Bruker Daltonik, Bremen, Germany) and the droplet was left to dry at room temperature. Mass spectra were recorded on a Bruker Scout 26 Reflex III instrument (Bruker Daltonik, Bremen, Germany). The instrument was calibrated with angiotensin II, substance P, bombesin, and ACTH. The peptides were analyzed in reflector mode using delayed ion extraction with a total acceleration voltage of 23 kV. 50 to 100 single-shot spectra were acquired to improve the signal-to-noise ratio. Spectrum calibration and peak assignment was carried out with the

XMASS 5.0 software package provided by the manufacturer. The Mascot search software (http://www.matrixscience.com) was used for protein identification.

Immunoprecipitation: HepG2 cells were solubilized in 50mM Tris-HCl/pH 7.4, 1% digitonin, 150mM NaCl, 2mM $CaCl_2$ and protease inhibitors for 1h at 4°C, followed by centrifugation at 100'000xg for 1h. Supernatants were incubated with anti-ERGIC-53 and anti-HA antibodies covalently coupled via dimethyl pimelimidate to protein A-Sepharose beads (Harlow and Lane, 1999). Beads were washed four times in 50mM Tris-HCl/pH 7.4, 0.1% digitonin, 150mM NaCl, 2mM $CaCl_2$. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting.

siRNA transfection: siRNA oligos were purchased from Eurogentec (Seraing, Belgium) and Qiagen (Venlo, The Netherlands). Three siRNA oligos were designed against Surf4 and two against p25. SiRNA oligos for ERGIC-53 knockdown were described previously (Nyfeler et al., 2006). The most efficient siRNA oligo was determined by immunoblotting and chosen for all further experiments. Surf4 was knocked down using 5'-CGUAUAUUUCAACGCCUUCdTdT-3' as sense and 5'-GAAGGCGUUGAAAUAUACGdTdT-3' as antisense oligo. P25 was knocked down using 5'-CCUCAGAAUCACAGUGUUAdTdT-3' as sense and 5'-UAACACUGUGAUUCUGAGGdTdG-3' as antisense oligo. Nonsilencing control siRNA was purchased from Qiagen, Switzerland. The siRNA was used at a final concentration of 5nM for transfection directly after cell plating using Hiperfect (Qiagen, Switzerland)) according to the manufacturer's instructions. All knockdown experiments were performed 72 h post-transfection.

Transmission electron microscopy: HeLa cells treated with control siRNA, p25 siRNA or Surf4/ERGIC-53 siRNA were fixed with 3% paraformaldehyde. 0.5% glutaraldehyde in 10mM PBS pH 7.4. After washing in PBS the cells were post-fixed in 1% osmium tetroxide. Fixed samples were dehydrated, embedded in Epon 812 resin (Fluka, Buchs, Switzerland). Sections were stained with 6% uranyl acetate and lead acetate and analyzed with an EM912 Omega EFTEM electron microscope (LEO Electron microscopy, Oberkochen, Germany).

Immunofluorescence microscopy and quantification: Cells were fixed in 3% para-fromaldehyde and permeabilized for 5 min in PBS containing 0.2% Triton X-

100, 3% BSA, and 20 mM glycine. For the staining with anti-Surf4 0.5% SDS was included in the permeabilization buffer. Primary antibodies were added for 30 min in PBS containing 3% BSA and 20 mM glycine. After washing, secondary antibodies were added for 30 min in PBS containing 3% BSA. Cells were embedded in Mowiol and analyzed by laser scanning confocal microscopy (TCS NT; Leica, Wetzlar, Germany). For the quantification of ERGIC clusters and ER exit sites, cells were stained for KDEL-receptor and Sec31, respectively, and co-stained for giantin. Knockdown cells were chosen based on a dispersed Golgi pattern indicated by giantin. The Golgi area was subtracted from the KDEL-receptor-stained image and the ERGIC clusters were counted using ImagePro5 software. For the quantification of ER exit sites spots positive for Sec31 were counted.

Live cell imaging: HeLa cells expressing GFP-ERGIC-53 were treated with sodium butyrate overnight and plated at a density of $4.5x10^4$ cells/ml on 18 mm round coverslips, followed by transfection with siRNA. 80 h post-transfection, living cells were imaged (Ben-Tekaya et al 2005). Images were acquired with a CCD camera (Orca-3CCD; Hamamtsu photonics) using a Lambda DG4 (Sutter instruments) for high speed filter switching. ImagePro®Plus software (Media Cybernetics®) was used for image recording and processing. Additionally an Edge filter was used to decrease the background signal. Life spans of individual ERGIC clusters were assessed in eight cells for each condition using the automatic tracking tool of ImagePro®Plus. Diameter and intensity filters were used to exclude the Golgi area and to monitor only prominent ERGIC structures. The life span corresponds to the average life span of all ERGIC structures counted per cell. It was measured for no longer than 5 min with image acquisition every 2 sec because with these conditions movement of spots could be tracked pixel by pixel. Increasing the analysis time (and imaging interval) resulted in tedious manual tracking and gave similar results. Statistical significance ($P \leq 0.05$) of the life span between control and knock-down conditions was probed by Student's t-test.

Immunoblotting: Cells were lysed for 1h at 4°C in PBS containing 1% digitonin, supplemented with protease inhibitors. Lysates were centrifuged at 20'000xg for 30 min at 4°C. 40μg protein per lane were separated by SDS-PAGE, transferred to nitrocellulose membranes, immunoblotted sequentially with primary and secondary antibodies and visualized by enhanced chemiluminescence (Amersham Pharmacia

Biosciences).

Metabolic labeling: HeLa cells were deprived of L-methionine for 20 min, pulsed for 10 min with 100 μCi ^{35}S-methionine (Perkin Elmer, Wellesley, MA, USA) and chased for the indicated times in HeLa culture medium containing 10mM L-methionine. At the end of the chase, the medium was collected and centrifuged for 10 min at 10'000xg to remove cell debris. For the 0 min chase time cells of parallel cultures were homogenized in PBS by passing them 10x through a 25G needle. 5 μl aliquots of homogenate and media were TCA-precipitated and radioactivity was measured by scintillation counting. Total protein secretion into the medium was normalized to the total counts in cell homogenates at 0 min chase.

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Abbreviations

BFA, brefeldin A

COP I, coat protein I

COP II, coat protein II

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Figure Legends

Figure 1

Surf4 localizes mainly to the ERGIC. (A) Localization of endogenous Surf4 in HeLa cells by confocal immunofluorescence microscopy using affinity purified antibodies against Surf4 in combination with antibodies against ERGIC-53, giantin and BAP31. HeLa cells were treated with 10µg/ml BFA for 90min (+BFA) and labeled with antibodies against Surf4 and ERGIC-53. (B) HeLa cells were transfected with HA-Surf4 or HA-Surf4SSS. The tagged versions of Surf4 were localized with anti-HA, anti-ERGIC-53, and anti-giantin. Arrowheads indicate co-localization of HA-Surf4 with ERGIC-53. Bars, 10µm.

Figure 2

Surf4 forms protein complexes with p24 family members and ERGIC-53. (A) ERGIC membranes were isolated from parent HepG2 celles (lane 1) and HepG2 cells stably expressing HA-Surf4 (lane 2)(see supplementary Fig. 1). Isolated membranes were separated by BN-PAGE followed by Western blotting with antibodies against Surf4 and the HA epitope. (B) ERGIC membranes of HepG2 cells stably expressing HA-Surf4 were separated by Blue Native-PAGE as in (A), followed SDS-PAGE in a second dimension. Proteins were stained with Coomassie blue and excised for mass spectrometry analysis. Black circles indicate the protein complex containing HA-Surf4 and the p24 family members p23, p24 and p25. Open circles indicate complexes of identified proteins that were not further analyzed. Astersiks, proteins not identified by mass spectrometry. (C) Co-immunoprecipitation experiments: ERGIC membranes of parent HepG2 cells (-) and HepG2 cells stably expressing HA-Surf4 (+) were isolated, lysed and subjected to immunoprecipitation with anti-HA, anti-ERGIC-53, anti-p23 and anti-p24 followed by Western blotting using antibodies against the HA-epitope, p23, p24 and p25. (D) HeLa cell lysates were immunoprecipitated with anti-ERGIC-53 antibodies coupled to beads or with beads alone. $^{1}/_{20}$ of the total lysate was loaded as indicator for protein amount in the cell. Proteins were visualized by Western blotting with antibodies to ERGIC-53 or p23.

Figure 3

Double knockdown of Surf4/ERGIC-53 or knockdown of p25 knockdown leads to Golgi dispersal. (A) HeLa cells were transfected with control siRNA, Surf4/ERGIC-53 siRNA ord p25 siRNA. The Golgi was visualized by immunofluorescence microscopy using anti-giantin. (B) Quantitative analysis of the Golgi phenotype in Surf4/ERGIC-53 and p25 knockdowns. More than 100 cells of three independent experiments were counted for each condition and the percentage of cells with fragmented Golgi plotted. Results are means ± s.d.. Bar, 10µm. (C) Cells treated with control, Surf4/ERGIC-53 or p25 siRNA were processed for electron microscopy and sections of 10'000x and 20'000x magnifications are shown. Arrows, Golgi ribbon; asterisks, dispersed Golgi stacks; bars, 0.5µm.

Figure 4

ERGIC structures are reduced in cells depleted of Surf4/ERGIC-53 or p25. (A) HeLa cells transfected with control, Surf4/ERGIC-53, or p25 siRNA were immunostained with antibodies against KDEL-receptor and giantin, and analyzed by confocal microscopy. The giantin staining was used as indication for efficient knockdown of Surf4/ERGIC-53 and p25. The cells borders are outlined in white. Bars, 10µm. (B) Quantitative analysis of the ERGIC structures. More than 18 cells per condition of three independent experiments were analyzed. KDEL-receptor-positive ERGIC structures were counted after removal of the Golgi area defined by giantin staining (see Materials and Methods). Results are means ± s.d.

Figure 5

ER exit site formation and anterograde transport are not affected in Surf4/ERGIC-53 or p25 knockdown cells. (A) HeLa cells transfected with control, Surf4/ERGIC-53 or p25 siRNAs were processed for immunofluorescence microscopy using antibodies against Sec31 and giantin. The giantin staining was used as indication for efficient knockdown of Surf4/ERGIC-53 and p25. The cells borders are outlined in white. Bars, 10µm. (B) Quantitative analysis of ER exit sites. More than 25 cells per condition of three independent experiments were analyzed. ER exit sites were counted according to the Sec31 staining (see Materials and Methods). Results are means ± s.d. Bars, 10µm. (C) HeLa cells were transfected with control, Surf4/ERGIC-53 and p25 siRNA and subjected to pulse-chase analysis using [^{35}S]-methionine. Media from cells were collected and assayed for incorporated

radioactivity. Results are means ± s.d. of at least three independent experiments.

Figure 6

Live imaging of GFP-ERGIC-53 reveals a shorter life span of ERGIC structures in p25 knockdown cells. (A) Time series from supplementary material movies 1 and 2. Cells were transfected with control or p25 siRNA and imaged with an interval of ~2 sec. Representative frames from a control cell show stationary ERGIC structures that hardly move throughout the imaging period (top panel, arrowheads). In p25 knockdown cells the stationary ERGIC structures do not move either but disappear with time (lower panel, arrowheads). (B) Life span of ERGIC structures in p25 depleted cells. Quantification of the relative life span of GFP-ERGIC-53 structures presented in Figure6. The average life span is plotted in percentage. Note that in p25 knockdown cells the life span of ERGIC structures is reduced by ~35%. This difference is statistically significant (Student's t-test, P≤0.05). Results are mean ± s.d. (control siRNA, *n= 8*; p25 siRNA, *n=8*).

Figure 7

Golgi matrix proteins remain associated with the dispersed Golgi. HeLa cells in which Surf4/ERGIC-53 or p25 was silenced by siRNA were processed for immunofluorescence microscopy using anti-GM130, anti-GRASP65 and anti-p115 in combination with anti-giantin. The giantin staining was used as indication for efficient knockdown of Surf4/ERGIC-53 and p25. Bars, 10µm.

Figure 8

β-COP is dispersed in Surf4/ERGIC-53 and p25 knockdown cells. (A) HeLa cell treated with Surf4/ERGIC-53 or p25 siRNA were immunostained for β-COP and the *cis*-Golgi marker GM130. Shown is a representative image of three independent experiments. (B) enlargement of panel (A) with β-COP stained in green and GM130 in red. Bars, 10µm.

Figure 9

***Cis*-Golgi remains partially resistant to BFA in Surf4/ERGIC-53 and p25 depleted cells.** HeLa cells transfected with Surf4/ERGIC-53 or p25 siRNA were treated with 10µg/ml BFA for 30 min. Cells were processed for immunofluorescence microscopy using anti-GM130 and anti-KDEL-receptor. Bars, 10µm.

Figure 10

Model depicting the effect of silencing Surf4/ERGIC-53 or p25 on the early secretory pathway. In the presence of cargo receptors (+cargo receptors), the architecture of the organelles is guaranteed by balanced anterograde and retrograde trafficking indicated by arrows. Depletion of cargo receptors such as Surf4/ERGIC-53 or p25 (- cargo receptors) dissociated COPI coats from *cis*-Golgi and ERGIC membranes leading impairing retrograde transport from *cis*-Golgi to ERGIC and ERGIC to ER. The sum of this reaction results in dispersal of the Golgi apparatus and reduction of ERGIC structures.

Supplementary Figures

Figure S1

Specificity of antibodies against Surf4. (A) HeLa cell lysates were analyzed by Western blotting with crude antibodies (lane 1) and affinity-purified antibodies (lane 2) raised against human Surf4 (2). (B) HeLa cells were transfected with control siRNA or Surf4 siRNA and processed for indirect immunofluorescence with the affinity-purified anti-Surf4 antibody and anti-ERGIC-53 antibody. Bars, 10µm.

Figure S2

Purification of ERGIC membranes containing Surf4. HepG2 cells were treated with 10µg/ml BFA for 90 min. The cells were homongenized and the post-nuclear supernatant was loaded on a 13-29% Nycodenz gradient and centrifuged for 3h at 80'000xg. Gradient fractions were collected and analyzed by Western blotting with antibodies against Surf4, BAP31, and ERGIC-53. The ERGIC fractions 9-12 were pooled and used for further analysis by Blue Native-PAGE.

Figure S3

Efficient knockdown of Surf4/ERGIC-53 or p25 does not affect protein levels of other proteins analyzed in this study. (A) HeLa cell transfected with control, Surf4/ERGIC-53 or p25 siRNA were lysed and equal protein amounts were processed for SDS-PAGE and analyzed by Western blotting using anti-p25, anti-

Surf4 and anti-ERGIC-53. (B) Quantification of knockdown efficiency of p25, Surf4 and ERGIC-53. Three independent experiments were quantified. Results are means ± s.d. (C) Control, p25 and Surf4/ERGIC-53 siRNA treated HeLa cells were analyzed as in (A) using anti-Sec31, anti-β-COP, anti-tubulin and anti-KDEL-receptor. Shown are representative images of three independent experiments.

Figure S4

Single knockdowns of Surf4 and ERGIC-53 do not affect Golgi morphology or total protein secretion. HeLa cells were transfected with control, Surf4 or ERGIC-53 siRNA. The Golgi was visualized by immunofluorescence using anti-giantin antibodies. Bar, 10µm. (B) HeLa cells were transfected with control, Surf4 or ERGIC-53 siRNA and subjected to pulse-chase analysis using [^{35}S]-methionine. Conditioned media were collected and assayed for incorporated radioactivity. Results are means ± s.d. of at least three independent experiments.

Figure1

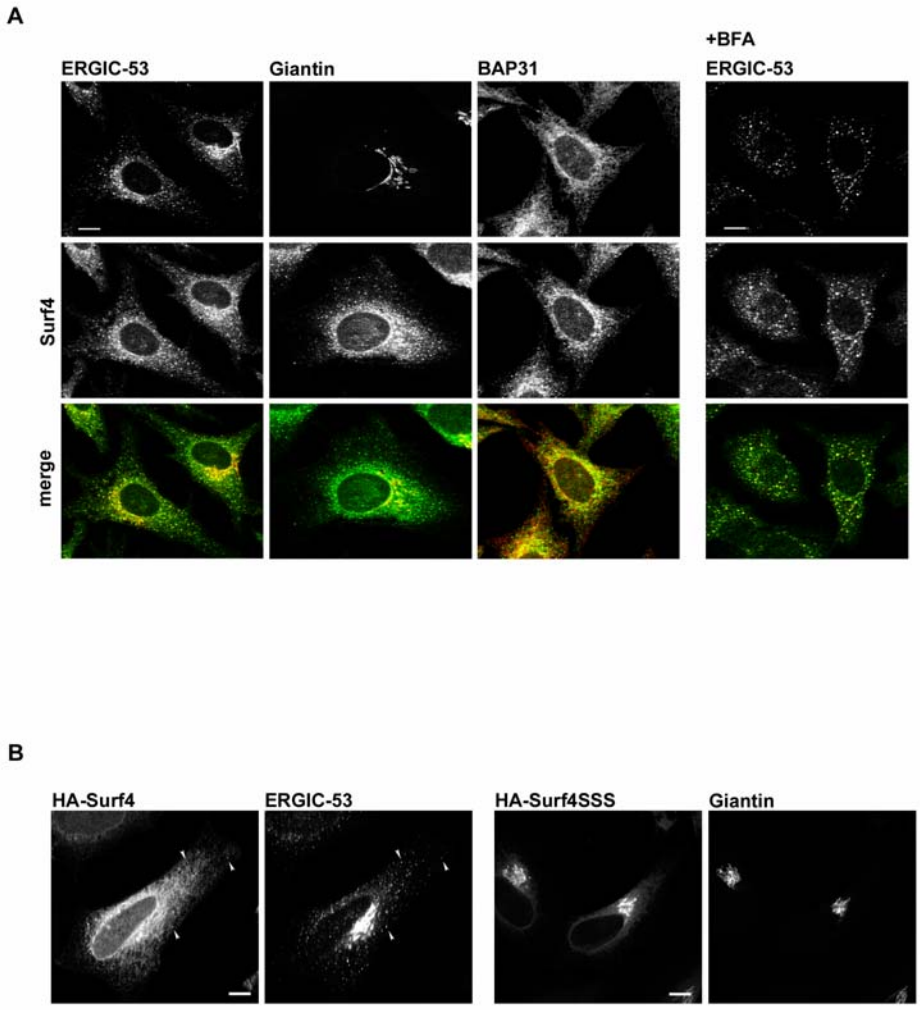

A
ERGIC-53
Giantin
BAP31
+BFA
ERGIC-53
Surf4
merge
B
HA-Surf4
ERGIC-53
HA-Surf4SSS
Giantin

Figure2

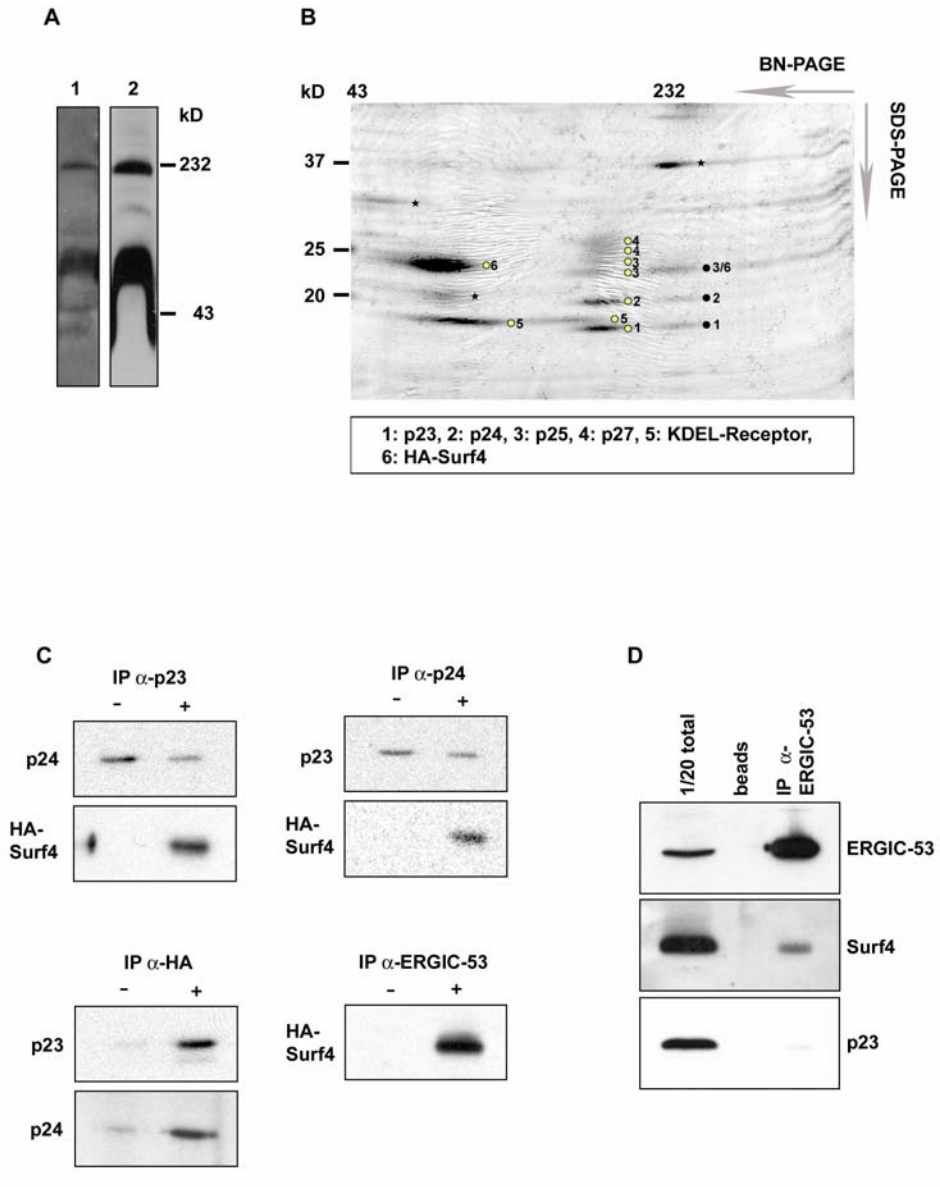

A
1
2
kD
232
43
B
BN-PAGE
kD
43
232
37
25
20
SDS-PAGE
1: p23, 2: p24, 3: p25, 4: p27, 5: KDEL-Receptor,
6: HA-Surf4
C
IP α-p23
p24
HA-Surf4
IP α-p24
p23
HA-Surf4
IP α-HA
p23
p24
IP α-ERGIC-53
HA-Surf4
D
1/20 total
beads
IP α-ERGIC-53
ERGIC-53
Surf4
p23

Figure3

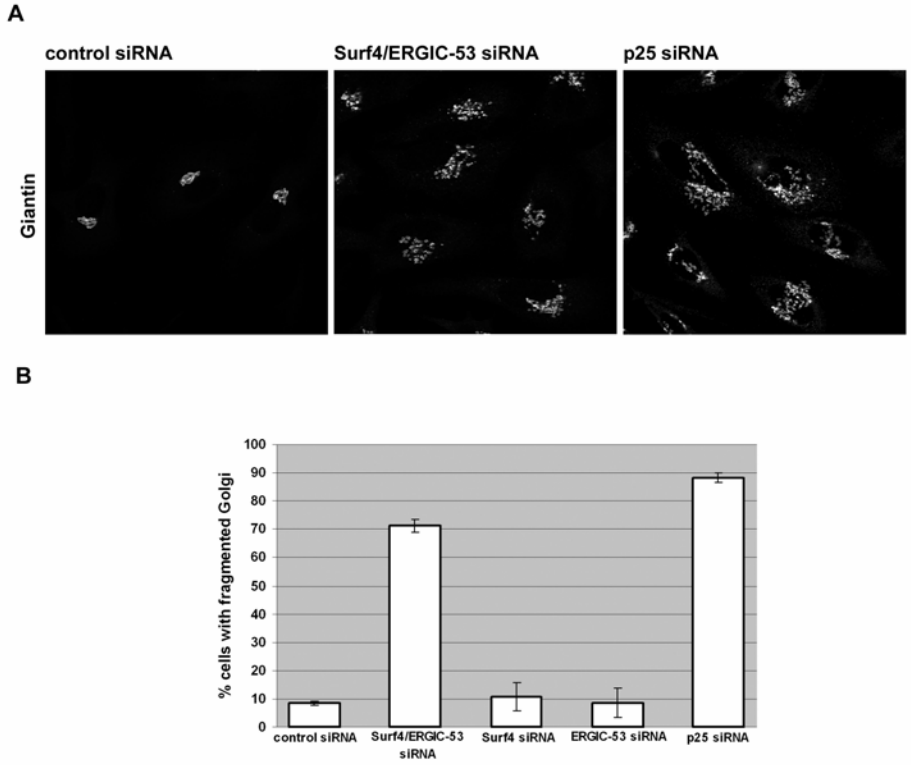
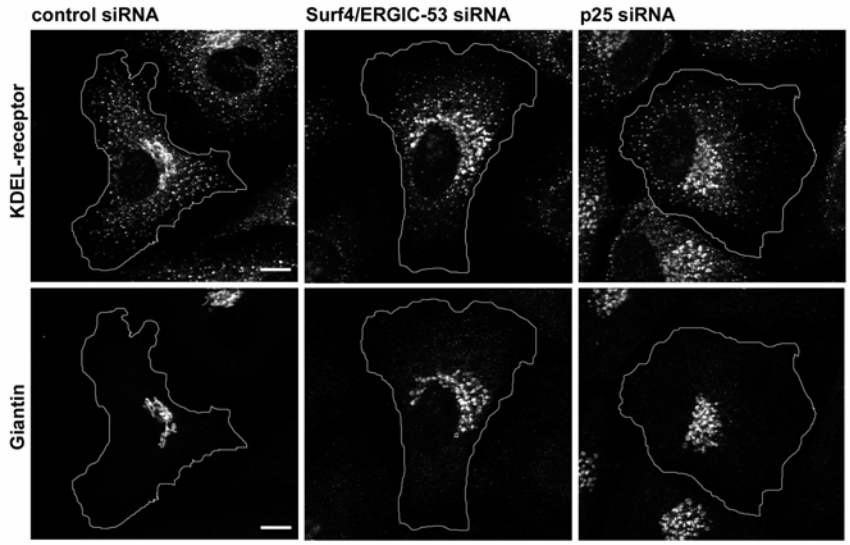

A
control siRNA
Surf4/ERGIC-53 siRNA
p25 siRNA
Giantin
B
% cells with fragmented Golgi
100
90
80
70
60
50
40
30
20
10
0
control siRNA
Surf4/ERGIC-53 siRNA
Surf4 siRNA
ERGIC-53 siRNA
p25 siRNA
C
control siRNA
Surf4/ERGIC-53 siRNA
p25 siRNA
10'000x
20'000x

Figure4

A


control siRNA
Surf4/ERGIC-53 siRNA
p25 siRNA
KDEL-receptor
Giantin

B

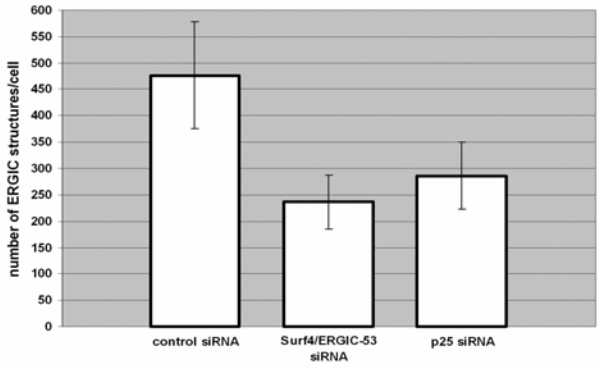
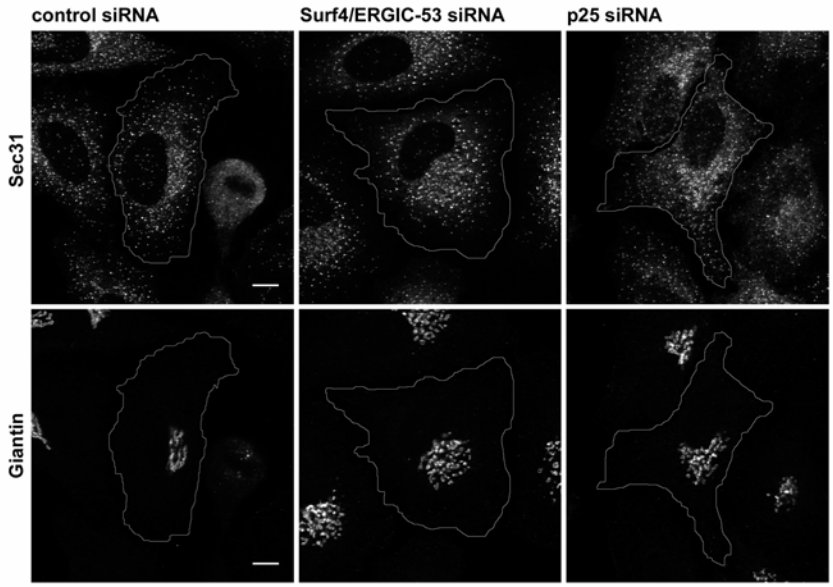
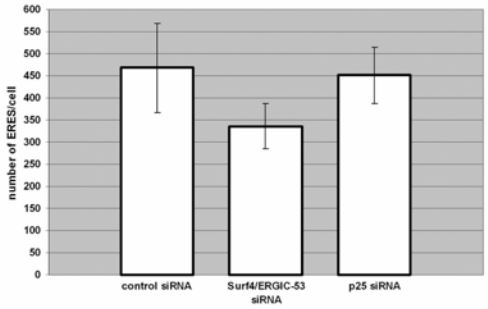

number of ERGIC structures/cell
600
550
500
450
400
350
300
250
200
150
100
50
0
control siRNA
Surf4/ERGIC-53 siRNA
p25 siRNA

Figure5

A


control siRNA
Surf4/ERGIC-53 siRNA
p25 siRNA
Sec31
Giantin

B


number of ERES/cell
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550
500
450
400
350
300
250
200
150
100
50
0
control siRNA
Surf4/ERGIC-53 siRNA
p25 siRNA

C

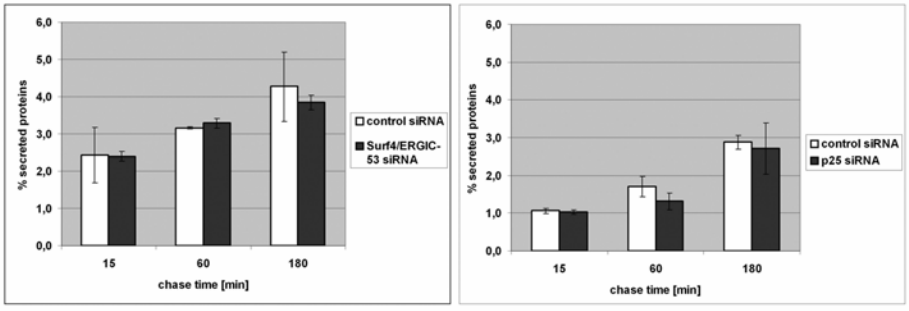
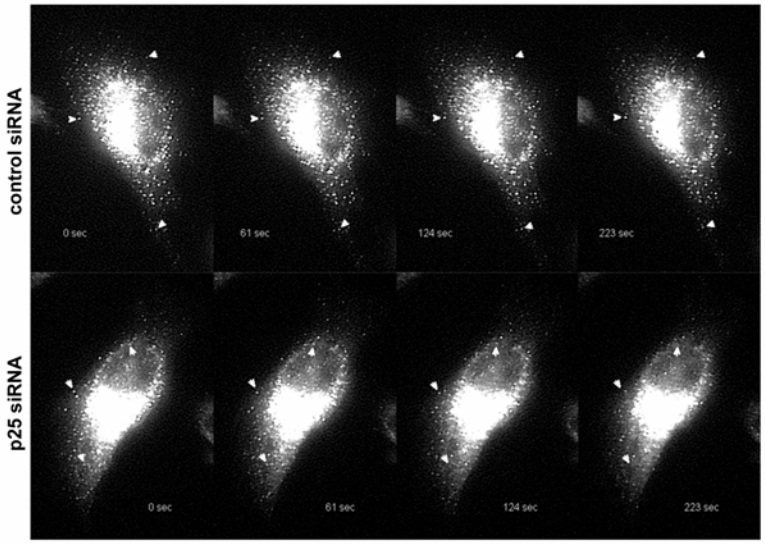

% secreted proteins
6,0
5,0
4,0
3,0
2,0
1,0
0,0
15
60
180
chase time [min]
control siRNA
Surf4/ERGIC-53 siRNA
control siRNA
p25 siRNA

Figure6

A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p25 siRNA
0 sec
61 sec
124 sec
223 sec
0 sec
61 sec
124 sec
223 sec

B

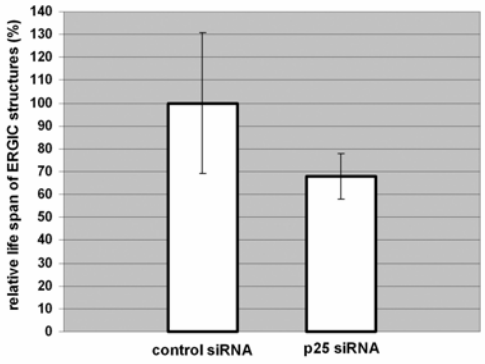
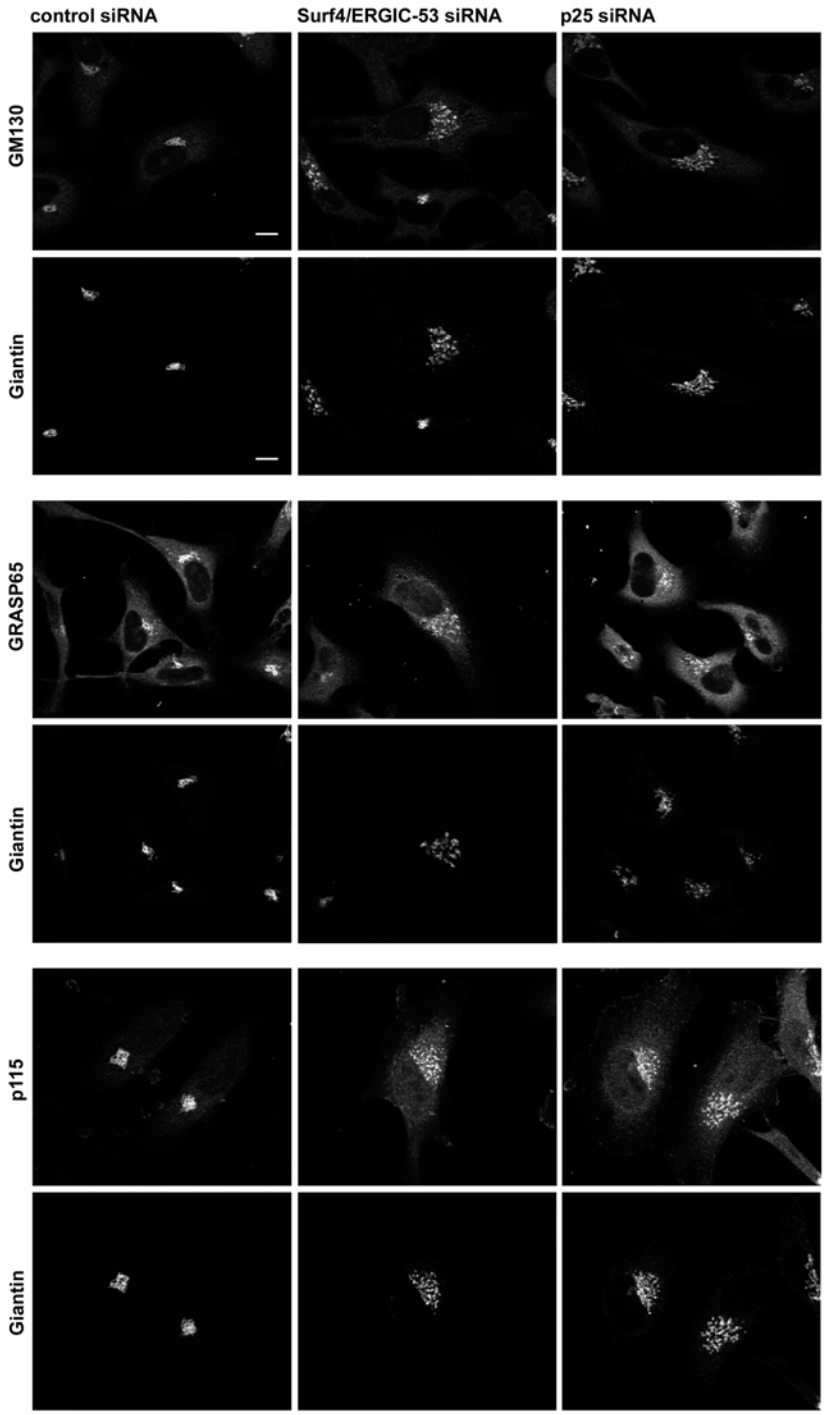

relative life span of ERGIC structures (%)
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
control siRNA
p25 siRNA

Figure7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Surf4/ERGIC-53 siRNA
p25 siRNA
GM130
Giantin
GRASP65
Giantin
p115
Giantin

Figure8

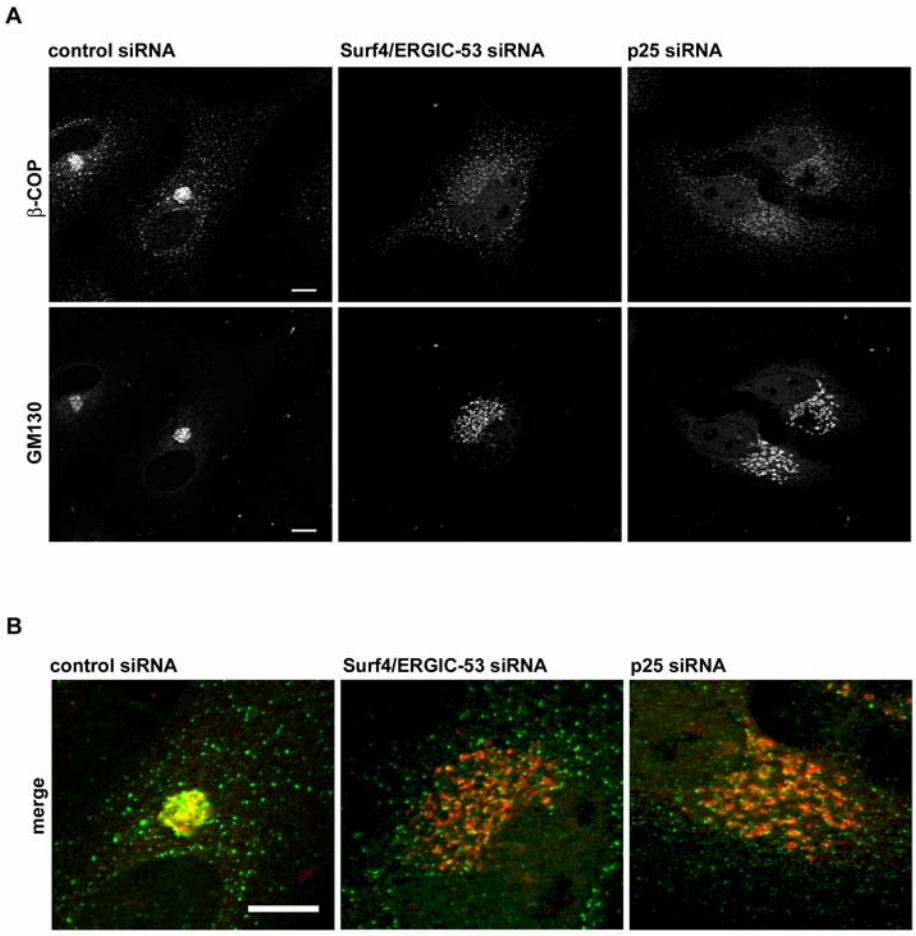
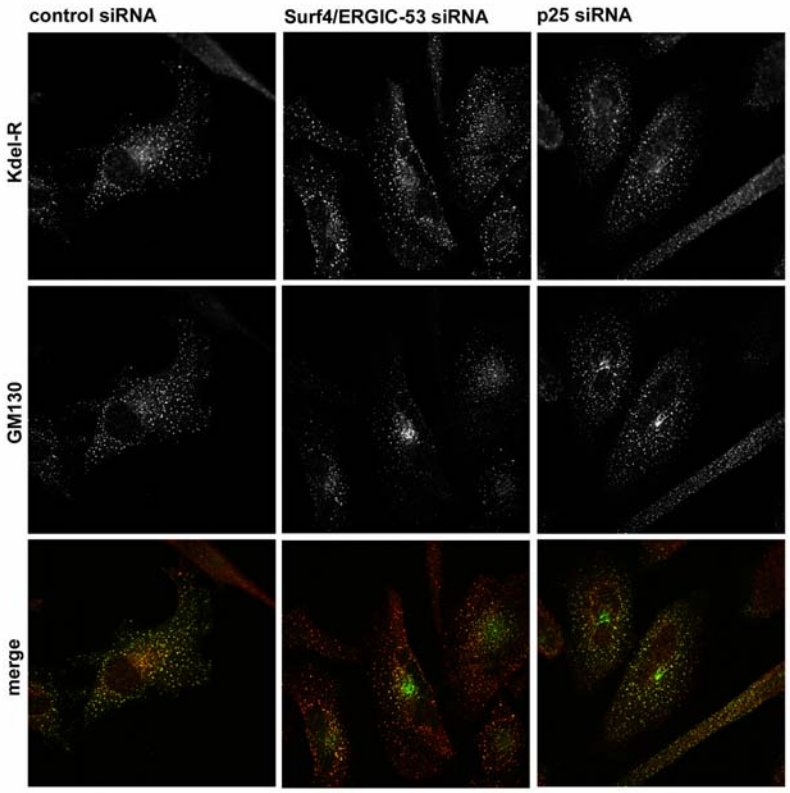

A
control siRNA
Surf4/ERGIC-53 siRNA
p25 siRNA
β-COP
GM130
B
control siRNA
Surf4/ERGIC-53 siRNA
p25 siRNA
merge

Figure9

A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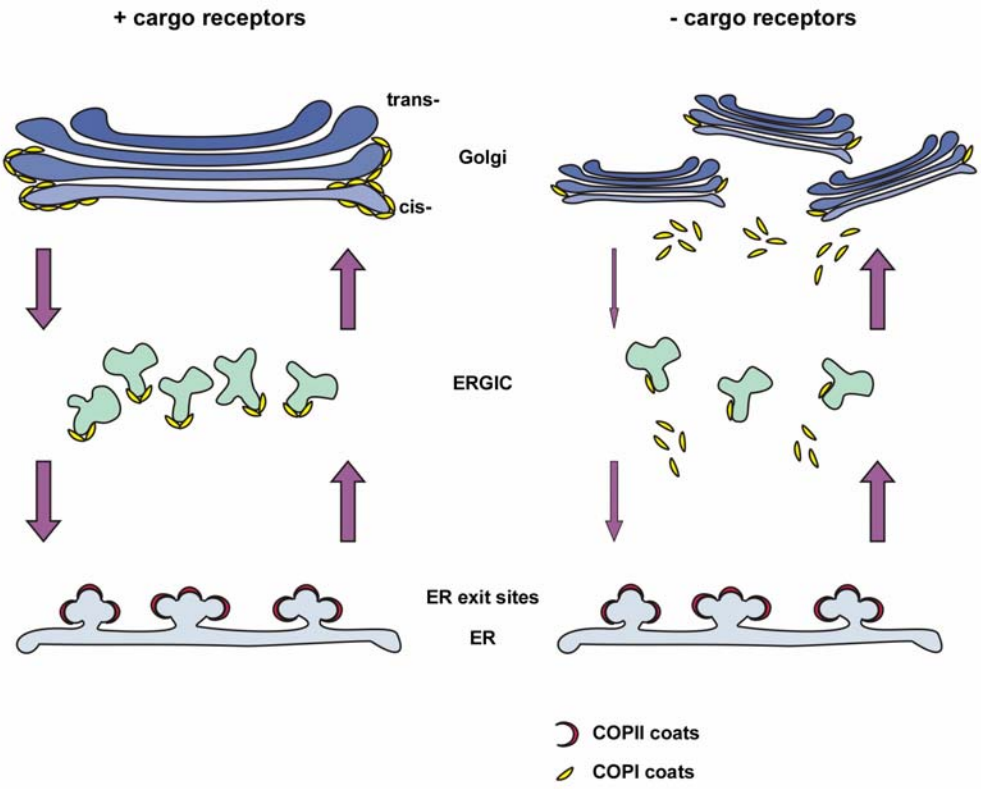
Surf4/ERGIC-53 siRNA
p25 siRNA
KdeI-R
GM130
merge

Figure10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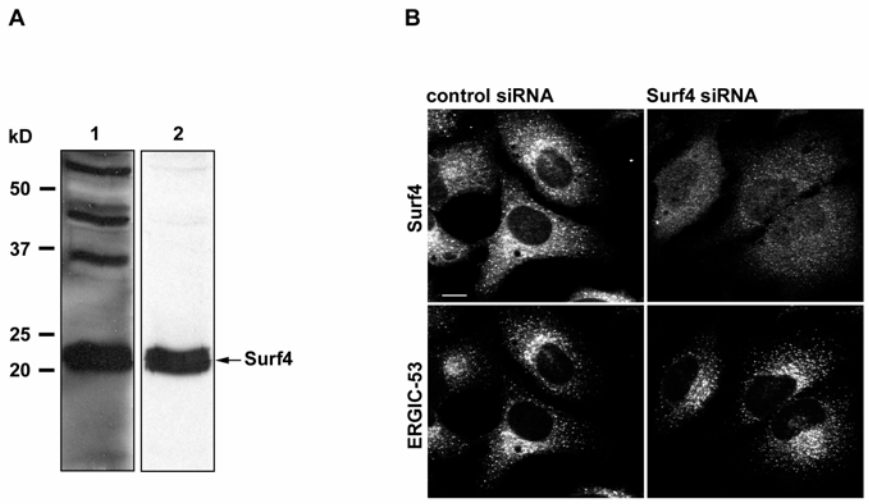
- cargo receptors
trans-
Golgi
cis-
ERGIC
ER exit sites
ER
COPII coats
COPI coats

Supplementary1



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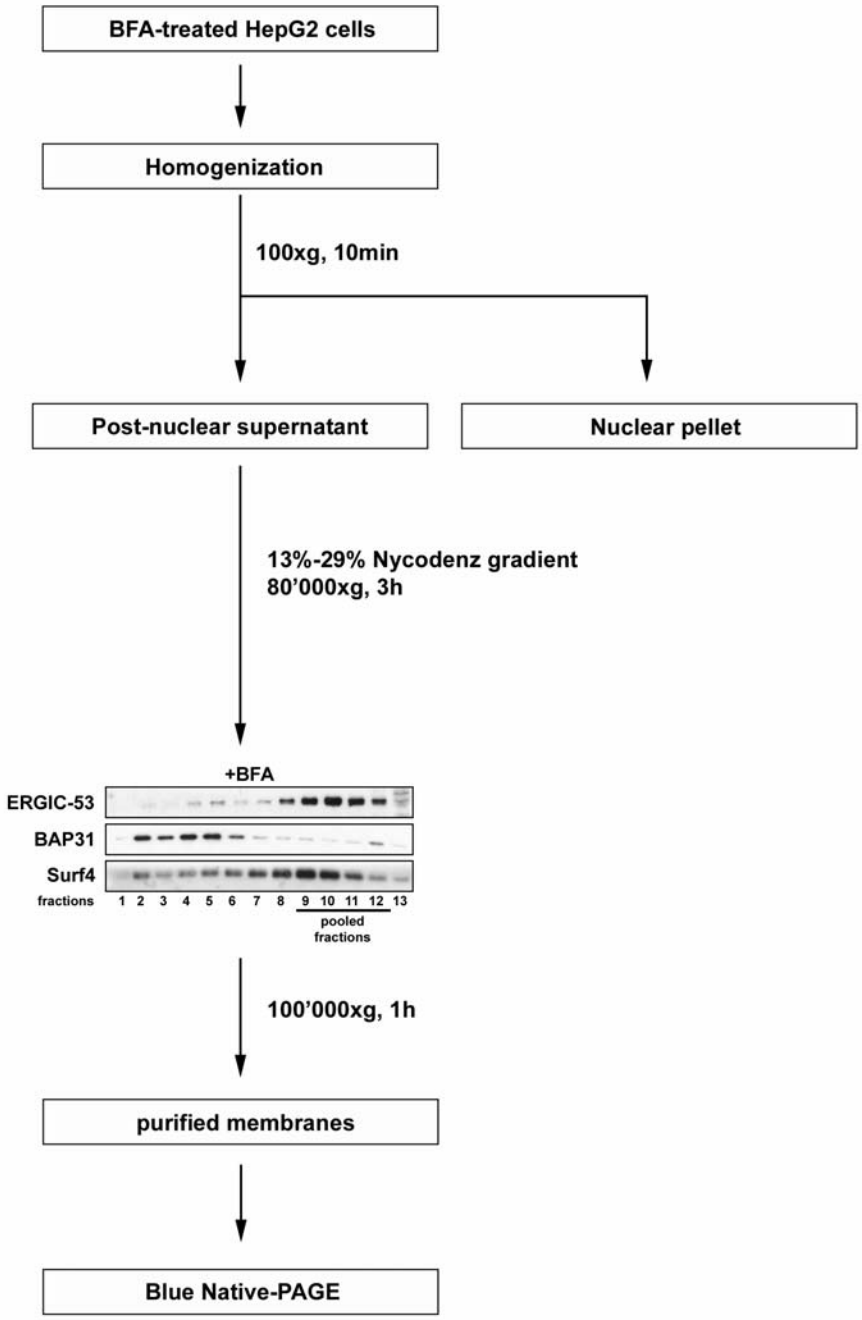
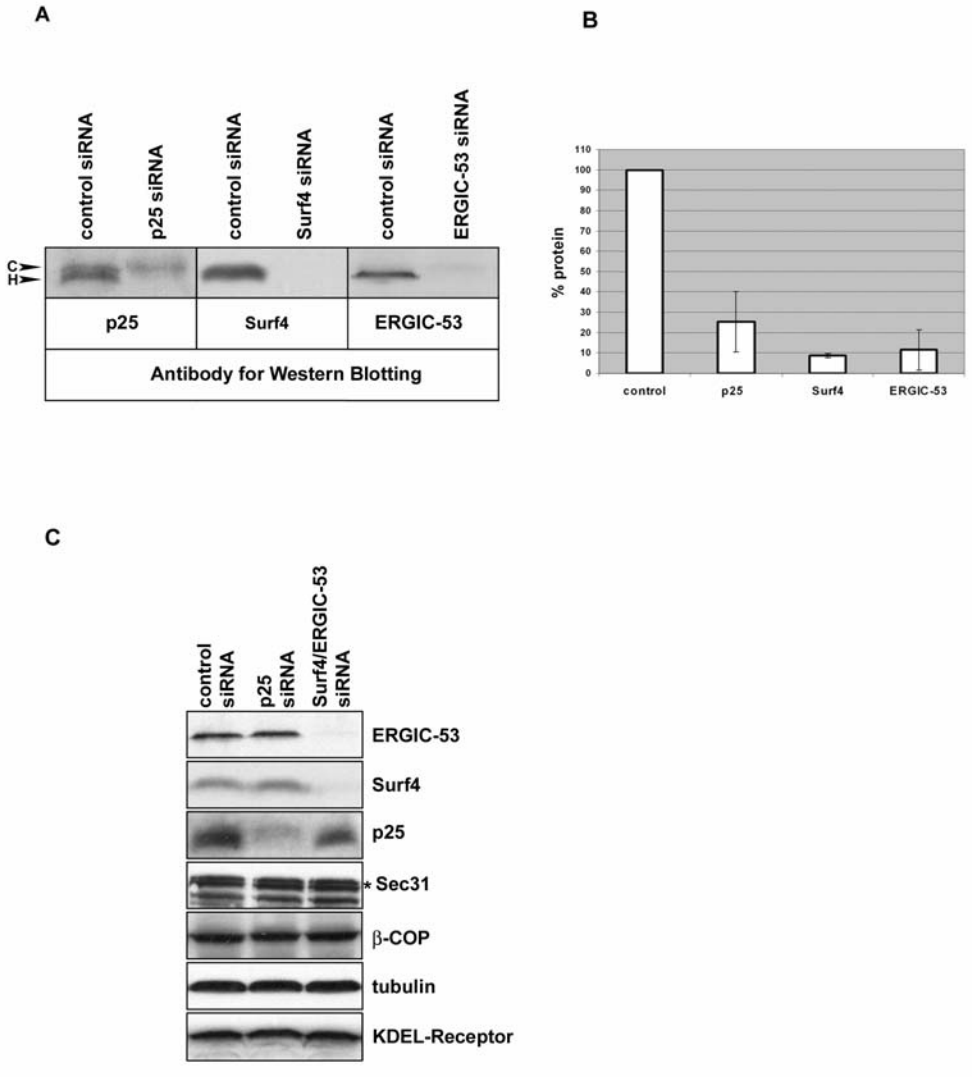
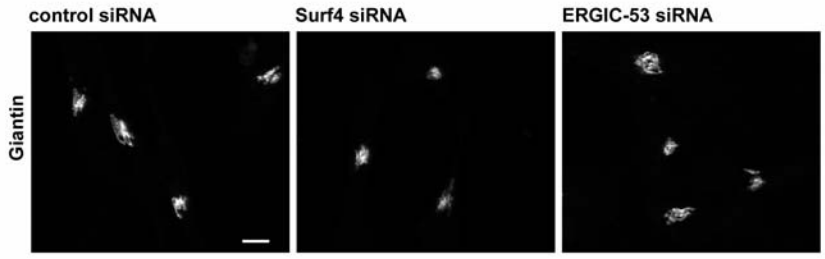


Supplementary3

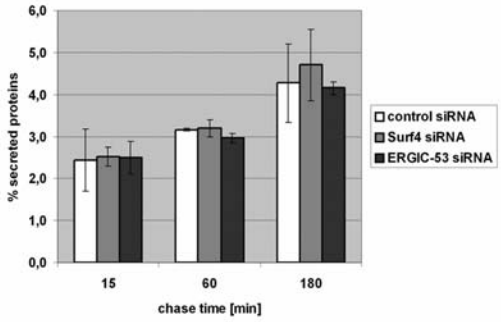


Supplementary4

A



B



3.2 Additional data: Surf4 exhibits cargo receptor properties required for efficient transport of a subset of secretory proteins

Abstract

In yeast ER exit is not only required for efficient ER export of secretory proteins but is also required for efficient degradation of some misfolded proteins. Efficient export of correctly folded soluble proteins and misfolded soluble substrates for ER associated degradation (ERAD) require cargo receptors that link the luminal protein to the cytoplasmic coat. The cargo receptor Erv29p maintains a special position within the early secretory pathway accomplishing both tasks. Erv29p is required for efficient transport of the soluble cargos CPY and PrA as well as for efficient degradation of the misfolded soluble ERAD substrates CPY* and PrA*. The mammalian ortholog Surf4 is far less characterized. We found by analyzing total protein secretion in Surf4 depleted cells, that Surf4 is required for efficient transport of a subset of secretory proteins. In contrast to Erv29p, depletion of Surf4 did not affect degradation of alpha-1-antitrypsin Z variant, a model substrate for ERAD, or transport of the secreted wild type alpha-1-antitrypsin.

Introduction

Secretory proteins entering the secretory pathway are first translocated into the endoplasmic reticulum (ER) where they undergo co- and posttranslational modification, including N-linked glycosylation, oligomerization, di-sulfide bond formation and chaperone assisted folding. An elaborate quality control system in the ER assures sorting of incorrectly from correctly folded proteins in the ER. Misfolded proteins are selectively retained in the ER by the quality control system and if unable to refold correctly, the misfolded proteins are retrotranslocated to the cytosol where they are ubiquitinated and degraded by the proteasome [1-4].

Correctly folded secretory proteins are packaged into COPII-coated vesicles for transport through the secretory pathway. Packaging of at least some soluble secretory proteins requires transmembrane receptors that link the soluble proteins with the coat components. In yeast Erv29p was described to function as a cargo receptor in ER exit for a subset of soluble proteins. It directly binds to the soluble secretory protein glycosylated α-factor pheromone precursor (gpαf) and packages it into COPII vesicles for transport to the Golgi [5, 6]. Beside gpαf, CPY and PrA were shown to be delayed in transport to the Golgi in Erv29p deleted cells [7]. It is believed that after delivery to the Golgi, Erv29p-cargo complexes dissociate and empty receptors are recruited to COPI vesicles by a di-lysine motif present on the cytoplasmic tail of Erv29p and retrieved back to the ER.

Although an active mechanism for retaining misfolded proteins in the ER has been proposed [8], a number of misfolded proteins exit the ER and traffic to the Golgi. Interestingly *ERV29* and other genes involved in transport between the ER and Golgi are up-regulated by the unfolded protein response (UPR) and are required for efficient degradation of soluble misfolded substrates [7, 9]. The up-regulation of genes involved in vesicle budding from the ER (SEC12, SEC13, SEC16 and SEC24) and retrograde transport from the Golgi to the ER (ERD2, RER2, RET2 and SEC26) suggests that ER to Golgi transport is required for efficient degradation [9]. Especially Erv29p was reported to be required to clear the ER of accumulated misfolded proteins like CPY* and PrA* when ERAD becomes saturated [7]**.** These ERAD substrates require Erv29p dependent cycling between the ER and Golgi for efficient degradation. Altogether Erv29p is required for packaging of correctly folded secretory proteins as well as for ER exit of misfolded soluble proteins that are subsequently degraded.

Mammalian Surf4 is far less characterized compared to its yeast homolog

Erv29p. Studies on the localization and trafficking of Surf4 report that Surf4 localizes to and cycles within the early secretory pathway [10, 11]. Similarly to Erv29p Surf4 contains a di-lysine motif that when inactivated leads to mistargeting of Surf4 to the Golgi. Given the extent of homology between Erv29p and the mammalian ortholog Surf4 it is likely that Surf4 performs a similar function to Erv29p in ER to Golgi transport of correctly folded secretory proteins or misfolded ERAD substrates.

In this study the potential role of Surf4 in human cells to act as a cargo receptor for a soluble misfolded ERAD substrate and correctly folded secretory proteins was assessed. The soluble model substrate for ERAD in mammalian cells alpha-1-antitrypsin Z variant (A1PiZ) showed no stabilization upon depletion of Surf4 by siRNA. Also transport of correctly folded alpha-1-antitrypsin (A1Pi) to the Golgi was not affected in cells depleted of Surf4. However, analysis of the proteins secreted from cells depleted of Surf4 showed a delay in transport of a subset of secretory proteins. The selective delay of a subset of proteins suggests that Surf4 similarly to its yeast homologue Erv29p might serve as a cargo receptor for a subset of soluble secretory proteins that remain to be identified. Unlike Erv29p, however, Surf4 is not involved in ERAD of A1PiZ.

Results and Discussion

A1PiZ does not require Surf4 for efficient degradation by ERAD

In humans, the classical form of A1Pi deficiency results from the Z variant, which is secretion incompetent and accumulates in the hepatic ER, giving rise to liver disease and juvenile emphysemia [12, 13]. A1PiZ is an ERAD substrate in both yeast and human. Interestingly deletion of the *ERV29* gene in yeast leads to stabilization of the soluble ERAD substrate A1PiZ [14], suggesting a similar degradation pathway as for CPY* and PrA*. The degradation dependence of a non-yeast protein like A1PiZ on Erv29p in yeast, indicates that the mammalian ortholog Surf4 might serve a similar function for A1PiZ in humans. Therefore A1PiZ can be considered a model substrate to study the role of Surf4 dependent ER to Golgi transport in efficient degradation of ERAD substrates in human cells.

If Surf4 operates as a cargo receptor to clear the ER from accumulated A1PiZ, depletion of Surf4 would lead to inefficient degradation that is stabilization of A1PiZ. We took advantage of the silencing approach in HeLa cells using siRNA against Surf4 [11]. The Surf4 knockdown efficiency in HeLa cells was described previously [11]. To identify a potential involvment of Surf4 in degradation of A1PiZ, a pulse-chase approach was used to study the degradation and transport of A1PiZ in control and Surf4 siRNA treated cells. A1PiZ transfected control cells and Surf4 depleted cells were pulse-labeled with ^{35}S-methionine. Cell lysates and medium were probed for ^{35}S-methionine-labeled A1PiZ after the indicated chase periods (Fig. 1A). Quantification of A1PiZ revealed that 30% of A1PiZ was secreted (Fig. 1B) which is consistent with previous studies which showed that about 15% of the misfolded protein is secreted [15]. The difference in secretion may be due to transient (this study) versus stably transfected cells [15]. Surprisingly, the rate of disappearance of the high mannose form of A1PiZ was unchanged in Surf4 siRNA compared to control siRNA treated cells (Fig. 1B), suggesting that degradation was not affected in Surf4 depleted cells. Likewise the secretion of A1PiZ remained comparable for Surf4 depleted cells and control cells. These results suggest that transport and degradation of A1PiZ proceeded independent of Surf4 in mammalian cells.

Silencing of Surf4 does not delay transport of A1Pi

Although degradation of A1PiZ is not affected in cells dpleted of Surf4, correctly folded transport competent A1Pi might still require a cargo receptor for efficient ER exit. A1Pi is a protease inhibitor that suppresses neutrophil-derived proteases in the

serum and elastase activity in lung tissue. Inefficient transport of A1Pi might have a strong effect on human health as seen by the accumulation of A1PiZ where inefficient secretion provokes liver disease and juvenile emphysemia. To answer the question if Surf4 might function as a cargo receptor for A1Pi, liver derived HepG2 cells were chosen to analyze the secretion of endogenous A1Pi. If Surf4 is a cargo receptor for A1Pi knockdown of Surf4 should result in inefficient secretion of A1Pi. HepG2 cells were co-transfected with either control or Surf4 siRNA and cell lysates were analyzed by Western blotting using antibodies against Surf4 and,as a loading control, against the ER protein CLIMP63 (Fig. 2A). Quantification revealed a Surf4 knochdown efficiency of 65% (Fig. 2A). To investigate the transport of A1Pi after knockdown of Surf4, pulse-chase experiments with ^{35}S-methionine were performed. Cell lysates and media of control and Surf4 siRNA transfected HepG2 cells were immunoprecipitated with antibodies against A1Pi after various chase times (Fig. 2B). Quantification of high mannose and complex glycosylated A1Pi revealed that after 30min of chase 50% of A1Pi was converted to the complex glycosylated form, in both control and Surf4 siRNA treated cells, suggesting that depletion of Surf4 did not delay the transport of A1Pi. Therefore we exclude that Surf4 acts as a cargo receptor for packaging A1Pi into COPII coated vesicles.

Interestingly recent findings in our lab identified A1Pi as specific interacting partner for ERGIC-53 by a YFP-protein fragment complementation assay (PCA) screen (Nyfeler et al., 2007 in preparation), suggesting that ERGIC-53 might be the cargo receptor for A1Pi. ERGIC-53 is already known to act as a cargo receptor for two lysosomal glycoproteins cathepsin Z and cathepsin C [16, 17]. Furthermore mutations in ERGIC-53 can lead to combined factor V and factor VIII deficiency in humans. Patients with loss of function mutations in ERGIC-53 show reduced levels of blood coagulation factors V and VIII in their plasma [18, 19]. As discussed above inefficient transport of A1Pi can lead to liver disease and juvenile emphysemia. Although, there is no evidence so far for a correlation between patients suffering from hemophelia and patients that show liver disease and juvenile emphysemia it would be of great interest if loss of function mutations in ERGIC-53 could provoke such a disease. If ERGIC-53 is indeed involved in efficient ER exit of A1Pi remains to be shown.

Testing an interaction between ERGIC-53 and A1PiZ by YFP-PCA revealed no interaction for these two proteins (Nyfeler et al., 2007 in preparation), suggesting that even ERGIC-53 most likely cannot act as a cargo receptor in mammalian cells for

A1PiZ as does Erv29p in yeast. So far there is no evidence in mammalian cells supporting the view that soluble misfolded ERAD substrates require cargo receptors for ER exit and efficient degradation.

Effect of siRNA-mediated Surf4 knock down on protein secretion

Erv29p is likely to have a different specificity for cargos than its mammalian ortholog Surf4, since the cargo gpαf of Erv29p is not expressed in mammalian cells. Nevertheless, Surf4 might operate as transport receptor for other cargo proteins. To test this, HepG2 cells depleted of Surf4 were subjected to a pulse-chase analysis. The medium was divided into con A bound and unbound fractions, to avoid protein overload on SDS-PAGE, followed by SDS-PAGE analysis and fluorometry (Fig. 3A, B). Interestingly a subset of secreted con A-bound (Fig. 3A) and unbound proteins (Fig. 3B) were less efficiently secreted in Surf4 depleted cells compared to control siRNA treated cells. This result suggests that Surf4 is required for efficient transport of a subset of soluble secretory proteins. Future mass spectrometry analysis is required to identifiy the potential cargos for Surf4, which would help to understand better the role of Surf4 within the early secretory pathway.

Membrane topology of Surf4

A recent study on the membrane topology of Erv29p opens new insights towards the understanding of the mechanisms of Erv29p-dependent protein transport. Prediction algorithms generate various models containing up to seven possible transmembrane domains for Erv29p. Experimental data in contrast show that Erv29p spans the membrane four times [20], with the N- and C-terminus exposed to the cytoplasm. The TMHMM prediction algorithm predicts five possible membrane spanning segments for Surf4 (Fig. 5A). At the C-terminus Surf4 contains a conserved di-lysine motif (Fig. 5B), a well characterized ER retrieval signal, which interacts with COPI subunits mediating Golgi to ER transport. Inactivation of the di-lysine motif identified Surf4 to cycle within the early secretory pathway in a lysine signal-dependent way, suggesting that the C-terminus faces the cytoplasm [11]. In contrast to Erv29p the N-terminus of Surf4 has been predicted to be translocated into the lumen (Fig. 5A, B and [21]). To determine the orientation of the N-terminus of Surf4, antibody accessibility of N-terminally hemagglutinin tagged Surf4 (HA-Surf4) was analyzed. At low concentrations digitonin permeabilizes the plasma membrane but leaves internal membranes intact, permitting the selective detection of cytoplasmic epitopes on

transmembrane proteins [22]. Fig. 5 shows that digitonin permeabilization allowed the detection of giantin with an antibody against its cytoplasmic domain [23], while an antibody against the luminal domain of GPP130 [24] gave no reaction. In contrast permeabilization of internal membranes with saponin allowed the detection of both cytoplasmic and luminal epitopes (Fig. 4). The N-terminally located HA epitope of Surf4 was detected under both permeabilization conditions, indicating that the N-terminus is exposed to the cytoplasm. Therefore the N-terminal part of Surf4 can be excluded to interact with luminal cargo.

Multiple sequence alignment of Surf4 among species shows that three predicted transmembrane domains of Surf4 overlap with the position of the experimentally confirmed transmembrane domains of Erv29p, implying that these domains might well be present in Surf4 (Fig. 5B). Since the N- and C-terminus both face the cytoplasm, Surf4 can contain maximally four transmembrane domains. Detailed analysis of the membrane topology would indicate which regions of Surf4 might interact with cargo on the luminal side. In light of the findings linking cargo receptors like ERGIC-53 to secretion of blood coagulation factors and to hemophilia [18] the identification of cargo for Surf4 is of highest interest. In combination with the elucidation of the membrane topology of Surf4, new insights into the mechanisms of cargo sorting would be achieved.

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Materials and Methods

Antibodies: The following mouse monoclonal antibodies were used: G1/133 against giantin [25] (ALX-804-603; Alexis, Lausen, Switzerland), A1/118 against GPP130 [24] (ALX-804-603; Alexis, Lausen, Switzerland), G1/296 against CLIMP63 [26] (ALX-804-604; Alexis, Lausen, Switzerland), 12CA5 against the HA epitope. Rabbit polyclonal antibody against Surf4 [11] was used and goat polyclonal antibody against A1Pi recognizing as well A1PiZ (ICN, Biomedicals) were used. Alexa 488-, Alexa 568- [Molecular Probes Europe, BV, Leiden, NL] and horseradish peroxidase-coupled antibodies [Jackson ImmunoResearch Inc.] were used as secondary antibodies.

Cell culture: HeLa cells were grown in DMEM, supplemented with 10% fetal bovine serum and 1x nonessential amino acids. HepG2 cells were grown in MEM medium, supplemented with 10% fetal bovine serum. For metabolic labeling and immunobotting cells were grown in 35 mm dishes. For immunofluorescence analysis cells were grown on coverslips in twelve-well plates.

siRNA and DNA transfections: The construction and purchase of nonsilencing control siRNA oligos and siRNA oligos against Surf4 were described previously [11]. The siRNA was transfected using Hiperfect (Qiagen, Switzerland) according to the manufacturer's instructions at a final concentration of 5nM directly after cell plating. All knockdown experiments were performed 72 h post-transfection. Molecular cloning of HA-Surf4 was described previously [10]. A1PiZ cDNA was a kind gift from M. Spiess [27, 28]. HA-Surf4 was transfected using FuGENE6 (Roche Diagnostics), according to the manufacturer's instructions. 24h after the transfection of control and Surf4 siRNA, A1PiZ was transfected as HA-Surf4.

Immunofluorescence microscopy: Cells were fixed in 3% para-formaldehyde and permeabilized for 2 min in PBS containing 20 μg/ml digitonin, 3% BSA and 20mM glycine. Alternatively cells were additionally permeabilized with 1 mg/ml saponin for 20 min. Primary and secondary antibodies were incubated sequentially in PBS containing 3% BSA. Saponin was used in all incubation steps for saponin-permeabilized cells. Cells were embedded in mowiol and analyzed by lase scanning confocal microscopy (TCS SPE; Leica, Wetzlar, Germany).

Immunoblotting: Cells were lysed for 1h at 4°C in PBS containing 1% Digitonin, supplemented with protease inhibitors. Lysates were centrifuged at 20'000xg for 30 min at 4°C. 50µg protein per lane were separated by SDS-PAGE, transferred to nitrocellulose membranes, immunoblotted sequentially with primary and secondary following visualization by enhanced chemiluminescence (Amersham Pharmacia Biosciences). Estern blots were quantified with a ChemImager™ device and AlphaEase™ software (Alpha Inotech Corporation, USA).

Metabolic labeling: HepG2 and A1PiZ transfected HeLa cells were deprived of L-methionine and pulsed for 30 min or 15 min with 100µCi ^{35}S-methionine (Perkin Elmer, Wellesley, MA, USA) and chased for the indictade times in MEM or DMEM medium containing 10mM L-methionine. The chase medium was cleared from cell debris by centrifugation at 10'000 x g for 10 min. Cells and chased medium were processed for immunoprecipitation analysis or total protein secretion.

Immunoprecipitation and quantification: For immunoprecipitation the cells were washed twice with ice-cold PBS and resuspended in lysis buffer (50mM Tris-HCl, 1% NP-40, 150mM NaCl, pH 7.4) supplemented with protease inhibitors. Cells were lysed by passing them three times through a 25-gauge needle. After 1 h on ice, the lysate was cleared by centrifugation at 100'000 × g for 1 h. The supernatant was added to protein A-Sepharose beads (Amersham Biosciences) to which antibodies had been prebound. After incubation for at least 1 h at 4°C, the beads were washed four times with lysis buffer, once with 50mM Tris-HCl, 150mM NaCl (pH 7.4) and once with 50mM Tris-HCl (pH 7.4). Immunoprecipitates were separated by SDS-PAGE and radiolabeled bands were imaged and quantified using a phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA).

Analysis of total protein secretion: For the analysis of total protein secretion cells were lysed as described for the immunoprecipitation and centrifuged for 20min at 20'000xg. The medium was incubated under constant agitation for 1h at room temperature with 30µl Con A beads. After 1h of incubation the unbound proteins were collected and precipitated by methanol. The beads were washed four times with Con A buffer (20mM Tris-HCl, 500mM NaCl, 1mM $CaCl_2$ pH 7.5). Bound proteins were eluted with 0.5M α-methyl-mannoside in Con A buffer. Unbound and Con A

bound proteins were precipitated by methanol and analyzed by SDS-PAGE. $^{1}/_{100}$ of the cell lysates collected at 0 min of chase was analyzed by SDS-PAGE as a control for ^{35}S-methionine incorporation.

Figure legends

Figure1

Degradation of A1PiZ in Surf4 depleted cells

(A) HeLa cells were transiently transfected with control or Surf4 siRNA duplexes. 24h post transfection HeLa cells were transiently transfected with A1PiZ and subjected to pulse-chase analysis using ^{35}S-methionine. 48 h after the last transfection A1PiZ was immunoprecipitated from cell lysates (intracellular) and from medium (secreted). (B) Quantification of the transport of A1PiZ analyzed in (A). Shown is one experiment. hA1PiZ represents the high mannose form and cA1PiZ the complex glycosylated form of A1PiZ.

Figure2

Secretion of A1Pi is unaffected by a knockdown of Surf4

(A) HepG2 cells were transiently transfected with control or Surf4 siRNA duplexes. 72h after transfection Surf4 and CLIMP63 were visualized by immunoblotting. The fluorograms of three independent experiments were quantified. Results are mean ± s.d. (B) HepG2 cells transiently transfected with control or Surf4 siRNA were subjected to pulse-chase analysis using ^{35}S-methionine. 72h after transfection A1Pi was immuniprecipitated from cell lysates (intracellular) and from medium (secreted). (C) Transport of A1Pi was quantified. Shown is one experiment. hA1Pi represents the high mannose form and cA1Pi the complex glycosylated form of A1Pi.

Figure3

Total protein secretion in Surf4 depleted cells

HepG2 cells transiently transfected with control or Surf4 siRNA were subjected to pulse-chase analysis. (A) At the indicated chase times secreted proteins were collected and isolated by Con A beads. (B) After incubation unbound proteins were collected. Con A bound proteins were eluted. Con A bound and unbound proteins were methanol precipitated and analyzed by 4-15% gradient SDS-PAGE. T represents $^{1}/_{100}$ of the cell lysates collected at 0 min of chase. Stars indicate proteins that are less efficiently secreted in Surf4 depleted cells compared to control cells.

Figure4

N-terminal orientation of Surf4

HeLa cells expressing HA-Surf4 were fixed and permeabilized with digitonin alone (upper panels) or digitonin and saponin (lower panles). The cells were then processed for immunofluorescence microscopy using anti-Giantin and anti-GPP130 in combination with anti-HA antibodies. Bars, 10μm.

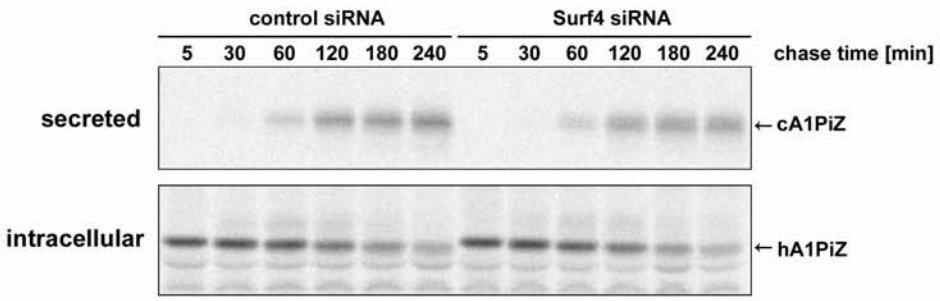
Figure5

Membrane topology of Surf4

(A) Transmembrane segment probability determined using TMHMM. The plot shows the probability that a residue sits in a transmembrane helix (transmembrane red), is exposed to the cytoplasm (inside blue) or the lumen (outside pink). (B) Protein sequence alignment of Surf4 orthologs. All sequences were aligned using ClustalW. Identical conserved residues are marked by an asterix. Sp indicates the swiss prot number of the proteins. Red bars represent the transmembrane domains recieved from the TMHMM prediction of the human Surf4 sequence and green bars represent the experimentally confirmed transmembrane domains for Erv29p [19].

Figure1

A


control siRNA
Surf4 siRNA
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chase time [min]
secreted
← cA1PiZ
intracellular
← hA1PiZ

B

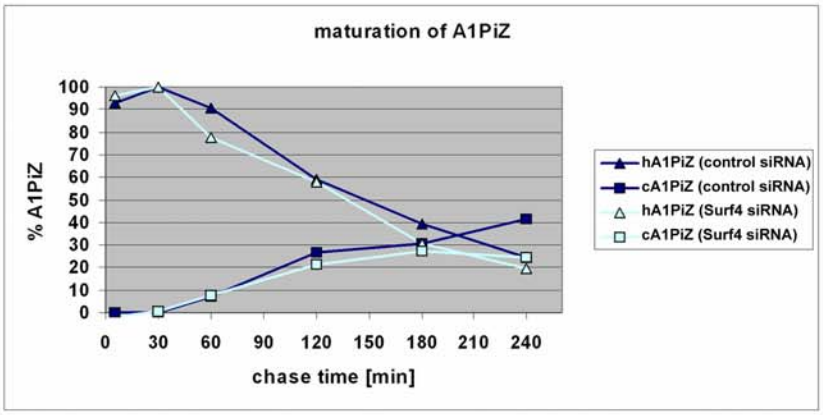
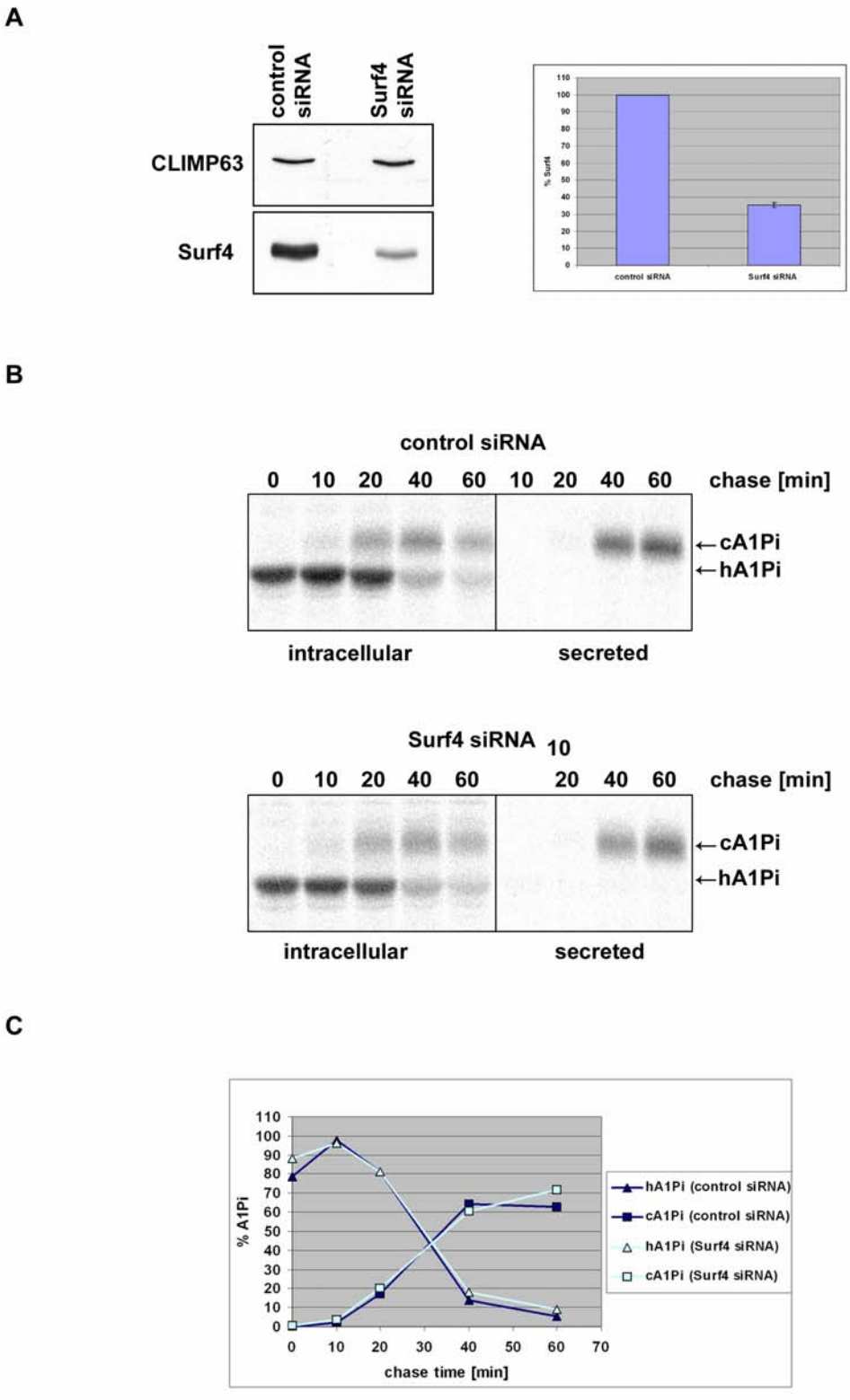

maturation of A1PiZ
% A1PiZ
100
90
80
70
60
50
40
30
20
10
0
0 30 60 90 120 150 180 210 240
chase time [min]
hA1PiZ (control siRNA)
cA1PiZ (control siRNA)
hA1PiZ (Surf4 siRNA)
cA1PiZ (Surf4 siRNA)

Figure2


A
control siRNA
Surf4 siRNA
CLIMP63
Surf4
% Surf4
control siRNA
Surf4 siRNA
B
control siRNA
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←hA1Pi
intracellular
secreted
Surf4 siRNA
0 10 20 40 60 10 20 40 60 chase [min]
←cA1Pi
←hA1Pi
intracellular
secreted
C
% A1Pi
chase time [min]
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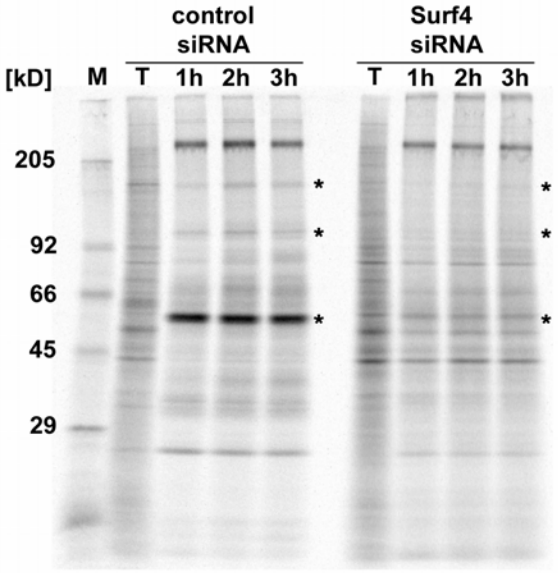
cA1Pi (Surf4 siRNA)

Figure3

A


control
siRNA
Surf4
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29
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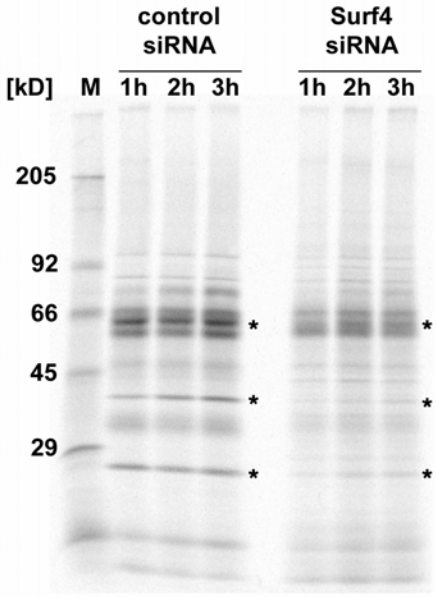
B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Surf4
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Figure 4

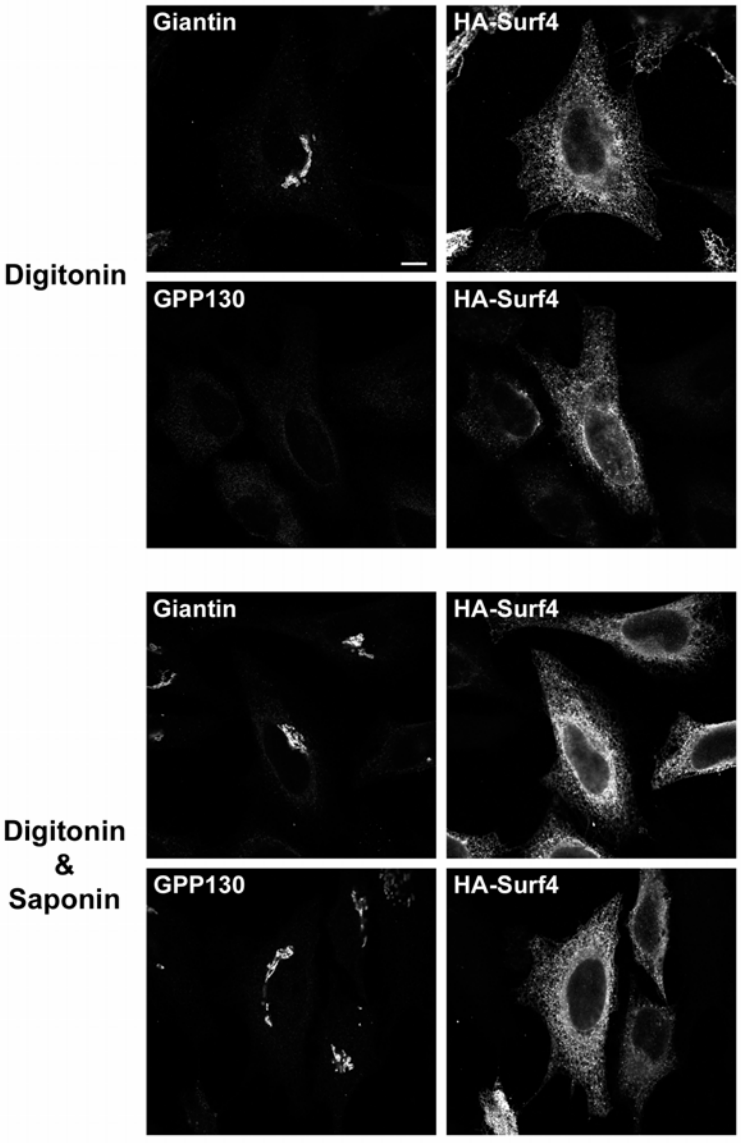
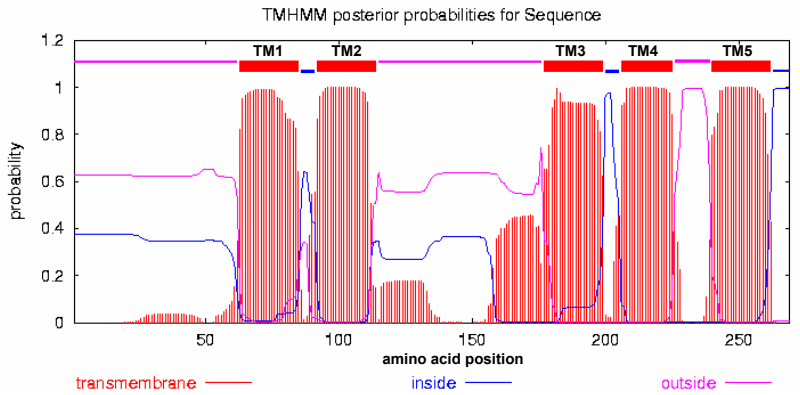

Giantin
HA-Surf4
Digitonin
GPP130
HA-Surf4
Giantin
HA-Surf4
Digitonin
&
Saponin
GPP130
HA-Surf4

Figure 5

A



B

```
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sp:Q64310:SURF4_Mouse.              M---------G-------QNDLMGTAE-----------------DFADQFLR--------
sp:O57590:SURF4_F.rubripes.         M---------G-------QEDLMNRAE-----------------DVADQFLR--------
sp:O18405:SURF4_D.melanogaster.     M---------SI------PNEYIAKTE-----------------DVAEQVIK--------
sp:Q18864:SURF4_C.elegans.          MNQFRA---PGG------QNEMLAKAE-----------------DAAEDFFR--------
sp:O74559:SURF4_S.pombe.            MTSRSP---FSTIPLSMNQDSYQTRTTV-GIRKKTFSERACQFMEQAETFMA--------
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                                    *

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sp:O57590:SURF4_F.rubripes.         ----VTKQYLPHLARLCLISTFLEDGIRMWFQWNEQRDYIEATWSCGYFLATCFVLLNLI
sp:O18405:SURF4_D.melanogaster.     ----RGKNVLPTVARLCLIATFFEDGLRMYIQWNEQREYMDMSWGCGKFLATVFVLVNLL
sp:Q18864:SURF4_C.elegans.          ----KTRTYLPHIARLCLVSTFLEDGIRMYFQWDDQKQFMQESWSCGWFIATLFVIYNFF
sp:O74559:SURF4_S.pombe.            ----PFTPYMPLLGRFLIVATYFEDAIRIVTQWPEQVSYMRDYRRFRFGTAPLLLFVCVV
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                                          *   *     *  **  *   **  *

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sp:O57590:SURF4_F.rubripes.         GQLGGCVLILSRNFVQYACFGLFGIIALQTVAYSILWDLKFLMRNLALGGGLLLLLAESR
sp:O18405:SURF4_D.melanogaster.     GQLGGCGMVMARFKVDIAVGLLFFIVVLQTVAYSILWDFQFLLRNFALIGALLLVLAEAR
sp:Q18864:SURF4_C.elegans.          GQFIPVLMIMLRKKVLVACGILASIVILQTIAYHILWDLKFLARNIAVGGGLLLLLAETQ
sp:O74559:SURF4_S.pombe.            LMLVGSTLVVFKKRQAYAIGSLLFVTLLQAFAYGLITSGEMFFRNMSVIGGLCLVASDTF
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                                          *   *     *   *          **    * *

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sp:Q64310:SURF4_Mouse.              SEGK-SMFAGVPTMRE-S-SPKQYMQLGGRVLLVLMFMTLLHFD---ASFFSIIQNIVGT
sp:O57590:SURF4_F.rubripes.         SEGK-SMFAGVPSMGE-S-SPKQYMQLGGRVLLVLMFMTLLHFD---FNFFSILQNLVGT
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sp:O18405:SURF4_D.melanogaster.     ILMVLVVLGYKTKLSALILVALLTILNLYHNAWWTIPSYKPLRDFLKYDFFQTLSVIGGL
sp:Q18864:SURF4_C.elegans.          ALITLVSIGYKTKLSAIVLVIWLFGLNLWLNAWWTIPSDRFYRDFMKYDFFQTMSVIGGL
sp:O74559:SURF4_S.pombe.            TACAMVVIGFKAKFFAAVLVLILSVANFIINSFWSVPRESPYRDFYRYDFFQTLSIVGGL
sp:P53337:ERV29_S.cerevisiae.       ---ICFAIGYKTKFASIMLGLILTFYNITLNNYWFYNNTK--RDFLKYEFYQNLSIIGGL
                                          * * *     *   *   *   *  *          **  * * *  *  ***

sp:O15260:SURF4_Human.              LLVVALGPGGVSMDEKKKEW
sp:Q64310:SURF4_Mouse.              LLVVALGPGGVSMDEKKKEW
sp:O57590:SURF4_F.rubripes.         LLVVALGPGGVSMDEKKKEW
sp:O18405:SURF4_D.melanogaster.     LMIVSLGPGGVSMDEHKKKW
sp:Q18864:SURF4_C.elegans.          LLVIAYGPGGVSVDDYKKRW
sp:O74559:SURF4_S.pombe.            LYLVNTGPGKFSVDEKKKIY
sp:P53337:ERV29_S.cerevisiae.       LLVTNTGAGELSVDEKKKIY
                                    *     * *  * *  **
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XXXXXXX predicted TM of Surf4
XXXXXXX experimentally confirmed TM of Erv29p

4. Discussion

Cargo receptors are abundant proteins and are essential for efficient transport of a subset of soluble secretory proteins. Inefficient transport of soluble proteins like factors V and VIII due to loss of function mutations in ERGIC-53 are manifested in diseases such as bleeding disorders [1, 2]. Although secretion deficiency of factors V and VIII result in disease, inactivation of cargo receptors leads to rather limited secretion phenotypes. In yeast deletion of p24 family members delays transport of GPI anchored proteins and deletion of Erv29p results in inefficient transport of gpαf, CPY and PrA with no effect on the majority of secreted proteins. The previously described involvement of Erv29p in ERAD implicates that cargo receptors might not only be involved in packaging of correctly folded secretory proteins. In yeast cells lacking Erv29p, misfolded soluble proteins are stabilized, and it was proposed that efficient degradation of these misfolded proteins requires transport between ER and Golgi mediated by Erv29p [3]. In order to obtain new insights into the function of cargo receptors I characterized Surf4, the mammalian ortholog of the cargo receptor Erv29p. Silencing Surf4 resulted in reduced secretion of a subset of soluble secretory proteins implicating that Surf4 possesses cargo receptor potential that remains to be confirmed by the identification of the putative proteins. In contrast to Erv29p, depletion of Surf4 did not affect the degradation efficiency of A1PiZ, the soluble model substrate for ERAD in mammalian cells.

Studies with p24 proteins implicate that the function of cargo receptors is not restricted to packaging cargo into vesicles. Budding assays with Golgi-like liposomes require tails of p24 proteins to induce COPI vesicle formation, suggesting an involvement of p24 proteins in vesicle formation [4]. The other prominent feature of these proteins is their morphogenic impact on organelles of the early secretory pathway. Heterozygous deletion in mice or overexpression in cell culture of p23 results in alterations of Golgi morphology [5].

The morphogenic function of cargo receptors is not limited to p24 proteins as shown in this thesis. Depletion of the Surf4/ERGIC-53 complex resulted in the same morphogenic phenotype as the depletion of the p24 family member p25. Detailed analysis revealed that silencing Surf4/ERGIC-53 or p25 disrupted the Golgi apparatus and led to instability of the ERGIC in conjunction with partial dissociation of COPI. These findings suggest a much wider function of cargo receptors than packaging soluble cargo into vesicles. How can we explain the morphogenic function

of cargo receptors on a molecular level? To answer this question it helps to analyze the conditions that maintain the organization of the early secretory pathway and its organelles.

4.1 Dynamics of organelles within the early secretory pathway

To understand how the individual proteins of the early secretory pathway are involved in organelle maintenance, it is informative to consider conditions that cause disruption of these organelles. Proteins that lead to depletion or fragmentation of the Golgi apparatus can be categorized into at least three classes: 1) proteins that are involved in anterograde trafficking [6, 7] 2) proteins that are involved in retrograde trafficking [[8, 9], this study] and 3) structural proteins that contribute to the organization and architecture of the Golgi [10-12] (table I). The examples listed in table I clearly indicate that the maintenance of the Golgi apparatus depends on dynamic processes such as anterograde and retrograde trafficking, implicating that the organelles itself are highly dynamic structures. This view of dynamic rather than stable compartments is best described for proteins localizing to the Golgi apparatus. GFP-based imaging of Golgi localized proteins and exploring their trafficking routes and residency times suggest that no class of Golgi proteins is stably associated with the Golgi. Integral membrane proteins such as processing enzymes (mannosidase II, galactosyltransferase, etc.) SNAREs and cargo receptors are continuously exiting and re-entering the Golgi apparatus [13-15], while peripheral membrane proteins such as Arf1, COPI coat, p115 and GRASPs exchange constantly between membrane and cytosolic pools [13, 15]. Although all these proteins localize to the Golgi their individual residency time within the Golgi varies considerably. Golgi processing enzymes are stably associated with the Golgi for about 60 min, followed by cargo receptors which remain for 10 min and peripheral proteins which remain for only 1 min in the Golgi [13, 15]. The dynamic feature of the Golgi apparatus is particularly apparent when routes between the ER and Golgi are blocked or accelerated. Overexpressing the inactive GDP-restricted mutant form of Sar1 [T39N], which blocks ER exit, results in complete loss of Golgi membranes with retargeting Golgi enzymes to the ER [13, 16]. Overexpression of the Golgi enzyme N-acetylgalactosaminyl transferase-2 induces increased COPII assembly on the ER and an overall increase in the size of the Golgi apparatus [17]. Mutant Arf1 with preferential affinity for GDP inhibits export from the ER and leads to redistribution of

β-COP from Golgi membranes to the cytosol resulting in the collapse of the Golgi into the ER [18]. These results show that maintenance of organelles in the early secretory pathway is highly sensitive to vesicular traffic and components that regulate vesicle formation.

Table I. Fragmentation of the Golgi apparatus

	depletion of	phenotype	function
anterograde transport	p115	ministacked fragmented Golgi delayed anterograde transport	tethering of COPII vesicles to the ERGIC or for homotypic fusion
	golgin-84	ministacked fragmented Golgi reduction of overall size of fragmented Golgi exaggerated ER delayed anterograde transport	tethering of retrograde vesicles to CGN tethering of CGN elements and promoting their lateral fusion
retrograde transport	cog3p	ministacked fragmented Golgi accumulation of nontethered transport vesicles inhibition of retrograde transport	tethering of retrograde directed intra Golgi vesicles
	syntaxin 5	fragmentation of the Golgi apparatus no effect on anterograde transport	tethering of retrograde directed intra Golgi vesicles (remains to be shown)
	Surf4/ERGIC-53 or p25	ministacked fragmented Golgi reduction of ERGIC strucrures redistribution of COPI from membranes	initiation of COPI vesicle formation from the *cis*-Golgi and ERGIC
structural proteins	GM130/GRASP65	ministacked fragmented Golgi intact anterograde transport	lateral fusion of Golgi cisternae
	golgin-45	fragmentation of Golgi apparatus redistribution of the *medial* Golgi enzyme N-acetylglucosaminyltransferase-I to the ER block of anterograde transport	structural maintenance of the Golgi apparatus by maintaining matrix intact (involvment in anterograde or retrograde transport remains to be established)
	GMAP-210	Golgi fragmentation with loss of pericentrosomal localization	assembly of the Golgi ribbon around the centrosome

4.2 Cargo can modulate vesicle formation

Anterograde transport from the ER is mediated by COPII coated vesicles, while COPI coated vesicles mediate retrograde traffic within the Golgi and from the Golgi to the ER. The COPII coat is formed by sequential recruitment of Sar1-GTP, the Sec23/Sec24 complex and the Sec13/31 complex. Several studies imply that the capacity of cargo to bind coats influences coat assembly by increasing avidity of the coat for the membrane [19-21]. Synchronized export of VSV-G stimulates COPII vesicle budding, suggesting that cargo contributes to the formation of vesicles budding from the ER [20]. Recently a study identified that overexpression of N-acetyl

galactosaminyl transferase-2 increased COPII assembly on the ER via a direct interaction with Sar1p [17], showing that cargo exiting the ER can modulate ER exit by directly interacting with COPII components.

The involvement of cargo in vesicle formation is more completely understood for the retrograde directed COPI vesicles. In vitro budding assays with liposomes of Golgi-like lipid composition indicate that the cytoplasmic components Arf1, coatomer and GTP alone are not sufficient for COPI vesicle budding. Addition of the cytoplasmic domains of p24 family proteins to the system resulted in efficient vesicle formation [4]. The initiation of COPI vesicle formation is the activation of cytosolic Arf1 complexed with GDP. Further studies revealed that p23 and probably p24 recruit Arf1-GDP to the Golgi membrane, and thereby act as primary Arf1-GDP receptors [22-24]. The exchange of GDP for GTP by GEFs dissociates the Arf1/p23 complex, exposing the remaining Arf1-GTP and p24 proteins for subsequent coatomer binding. Coatomer binds Arf1-GTP through the β- and γ- COP subunits [25, 26] and p23 through the γ-COP subunit [27].

The current study extends the knowledge on cargo dependent COPI recruitment. Blue Native-PAGE and co-immunoprecipitation studies identified Surf4 to be present in protein complexes with p24 family members and ERGIC-53. Depletion of p25 or Surf4 together with ERGIC-53 resulted in inefficient retrograde traffic by regulating COPI recruitment to Golgi and ERGIC membranes. P25 requires heterooligomerization with other p24 family members in order to achieve correct trafficking. Therefore depletion of p25 would affect heterooligomerization of p24 family members and their targeting, resulting in ineffcient recruitment of COPI coats to membranes. These results suggest that COPI vesicle formation requires a certain amount of cytoplasmic exposed tails. Therefore a COPI vesicle would only be formed if enough cargo is present, supporting the view that cargo receptors regulate coat assembly and not vice versa.

4.3 Coats give identity to cis-Golgi and ERGIC

Recent findings suggest the existence of distinct COPI vesicles. In algae and higher plants two populations of COPI vesicles were described. COPIa vesicles are restricted to the *cis*-Golgi presumably mediating retrograde transport to the ER, while COPIb vesicles are limited to *medial* and trans cisternae probably for retrograde

transport of Golgi resident proteins [28]. In mammalian cells subpopulations of COPI vesicles were described not according to different coats, but containing either p24 proteins or the Golgi-resident enzymes mannosidase I and II [29]. Immunoelectron microscopy studies identified two distinct populations of COPI vesicles containing either KDEL-receptor or proinsulin [30]. Similarly, the Golgi v-SNARE GOS28 is present in COPI vesicles devoid of KDEL-receptor and KDEL-receptor positive vesicles lack GOS28 [31]. Do different types of COPI vesicles in mammalian cells imply different composition of COPI coats? For the COPI coat two isotypic coatomer, γ2 and ζ2, were identified [32, 33]. Interestingly different combinations of coatomer isoforms with distinct combinations of γ- and ζ-COP subunits were reported [34]. The coatomer isoforms are differentially distributed within the Golgi, with γ1-COP and ζ2 found preferentially in the early Golgi and the γ2 isoform preferentially in the late Golgi. The distinct distribution of individual coatomer isoforms and the identification of distinct COPI vesicles suggests the presence of various types of COPI vesicles. Figure 1 shows a model of how different COPI vesicles composed of distinct COPI subunits might bud from different membranes. Depending on their site of formation they will contain distinct cargo. At present it is unknown how the specific coatomer isoforms are recruited to a certain Golgi cisterna and how subpopulations of COPI vesicles are formed. From the current study and the model depicted in figure 1 one might speculate that cargo receptors such as Surf4/ERGIC-53 and p25 might selectively recruit COPI coats composed of γ1ξ2 subunits and therefore give rise to a specific subset of COPI coated vesicles. By generating subpopulations of COPI vesicles cargo receptors might influence or regulate directly or indirectly the recruitment of a subset of cargo restricted to these vesicles. Additionally, the recruitment of γ1ξ2 subunits by cargo receptors might specify the identity of the cis-Golgi and ERGIC on the cytoplasmic side. By giving identity to the cis-Golgi and ERGIC this recruited coat would perform a platform for other cytosolic factors to be recruited specifically to the cis-Golgi or ERGIC. This might include factors that mediate interactions with the cytoskeleton, the Golgi matrix, factors involved in vesicle tethering or the differentiation of membrane subdomains.

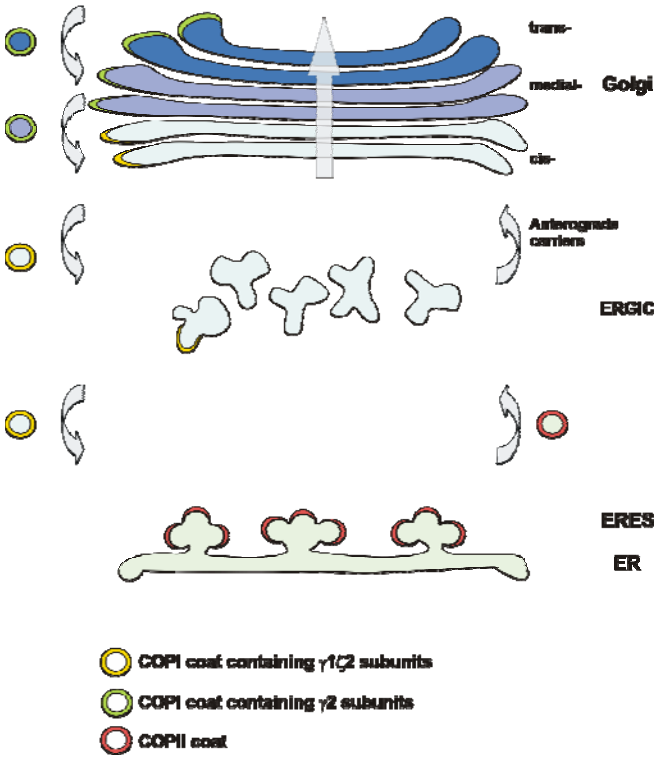

Figure1 Model of how different coats can give rise to distinct vesicles.

Anterograde transport is mediated by COPII vesicles from the ER to the ERGIC and by anterograde carriers from ERGIC to Golgi. Anterograde transport through the Golgi is mediated by cisternal maturation (transparent arrow) and/or percollating COPI vesicles, differing in content from retrograde COPI vesicles (not depicted). COPI vesicles are involved in inter-cisternal retrograde and *cis*-Golgi to ER transport. Different COPI coat subunits are recruited to distinct membranes, depending on the transmembrane protein content of the individual membrane.

4.4 Future Perspectives

Future work will require a more precise characterization of the cargo receptor potential of Surf4 as well as extension of the identification of new cytosolic interaction partners for Surf4. Since co-immunoprecipitation and cross-linking approaches failed to identify cargo proteins for Surf4 (unpublished data) it is of highest interest to identify the population of inefficiently secreted proteins by mass spectrometry analysis. Membrane topology predictions and studies on the membrane orientation of Surf4 indicate that Surf4 similarly to Erv29p passes the membrane four times with the N- and C-terminus exposed to the cytoplasm. Therefore Surf4 contains two luminal loops that might be involved in cargo selection. From topological studies on Erv29p and Surf4 one can only speculate about the position of the luminal exposed loops. Therefore, a more detailed analysis of the membrane orientation of Surf4 is required. Knowing the parts involved in potential cargo selection will allow to screen for interaction partners by a yeast two-hybrid screen using the luminal parts of Surf4 as bait.

Work in this thesis on the membrane topology of Surf4 suggests that the N-terminus, a large loop and the C-terminus face the cytoplasm, which determines more than half of the protein. One can speculate about the C-terminal part which contains a conserved di-lysine motif, most likely binding to the COPI coat. The function of the N-terminal part and the large cytoplasmic loop remain a mystery. Precise topological information will provide the basis for the analysis of cytoplasmic interaction partners of Surf4. Different approaches might be used to identify such partners. Pull down assay might be developed for cytoplasmic tails and loops expressed and purified from bacteria. Of special interest would be the identification of the cytoplasmic part which interacts with the COPII coat, since Surf4 does not contain a classical ER export signal. In order to identify new interacting partners other strategies would be pursued. The split-ubiquitin yeast two hybrid screen using full length Surf4 as a bait allows the identification of cytoplasmic interacting proteins [35]. An alternative would be the YFP-protein fragment complementation assay (PCA) in a mammalian system [36].

The identification of p24 family members as interaction partners for Surf4 may open new insights into the function of Surf4. Recently p23 was suggested to regulate amyloid precursor protein (APP) biosynthesis and trafficking [37]. Additionally, p23 was proposed to regulate intramembrane proteolysis controlling γ-secretase activity and thus preventing over-production of beta amyloid (Aβ) a key component associated with Alzheimer's disease [38, 39]. Since p23 knockdown leads to disruption of the Golgi apparatus [5] similarly to silencing p25 or Surf4/ERGIC-53, the question arises if Surf4 complexed with p23 or Surf4 complexed with ERGIC-53 are involved in APP trafficking or regulation of Aβ production. Simple pulse-chase analysis in cells depleted of Surf4 or Surf4 and ERGIC-53 may reveal a requirement of Surf4 in APP trafficking or regulation of Aβ production.

This thesis suggests that the depletion of Surf4/ERGIC-53 or p25 leads to inefficient recruitment of β-COP to membranes associated with inefficient retrograde transport leading to disruption of the Golgi apparatus and reduction of ERGIC structures. Disruption of the Golgi apparatus might not only be due to inefficient retrograde transport. A structural involvement of cargo receptors in organelle maintenance comes from studies with p24 family members which are supposed to interact with Golgi matrix proteins such as GM130 and GRASPs [40]. Depletion of GM130 leads to fragmentation of the Golgi apparatus due to inhibition of lateral fusion of cisternae. Especially since depletion of GM130 leads to a Golgi phenotype

comparable to the knockdowns analyzed in the current study [10] more effort is required to elucidate a potential involvement of Golgi matrix proteins and cargo receptors in maintaining architecture of the Golgi apparatus. This would include co-immunoprecipitation analysis of cargo receptors with the mentioned Golgi matrix proteins as well as comparison of the phenotypes obtained by depletion of cargo receptors and GM130.

In conclusion the present studies on Surf4 have opened avenues toward understanding the architecture and membrane trafficking of the early secretory pathway that may lead us into novel insights of diseases associated with the secretory pathway such as Alzheimer's disease and Cranio-lenticulo-sutural dysplasia [37, 38, 41, 42].

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Curriculum Vitae

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EDUCATION

PhD thesis at the Biozentrum, University of Basel, Switzerland. **2001-2007**
PhD thesis performed under the guidance of Professor Hans-Peter Hauri.
Title of the PhD thesis: "Function of human Surf4 in the early secretory pathway".

Study of Biology II at the University of Basel, Switzerland **1996-2001**
Master degree in Biochemistry, Microbiology, Cell Biology, Biophysics and Structural Biology.
Master thesis performed under the guidance of Professor Hans-Peter Hauri.
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Highschool at the Realgymnasium, Basel, Switzerland **1988-1996**

PUBLICATIONS

The cargo receptors Surf, ERGIC-53 and p25 are required to maintain the architecture of ERGIC and Golgi.
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