Exploring new routes for secretory protein export from the *trans*-Golgi network

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ABSTRACT **Sorting of soluble proteins for transport to intracellular compartments and for secretion from cells is essential for cell and tissue homeostasis. The *trans*-Golgi network (TGN) is a major sorting station that sorts secretory proteins into specific carriers to transport them to their final destinations. The sorting of lysosomal hydrolases at the TGN by the mannose 6-phosphate receptor is well understood. The recent discovery of a Ca^{2+}-based sorting of secretory cargo at the TGN is beginning to uncover the mechanism by which cells sort secretory cargoes from Golgi residents and cargoes destined to the other cellular compartments. This Ca^{2+}-based sorting involves the cytoplasmic actin cytoskeleton, which through membrane anchored Ca^{2+} ATPase SPCA1 and the luminal Ca^{2+} binding protein Cab45 sorts of a subset of secretory proteins at the TGN. We present this discovery and highlight important challenges that remain unaddressed in the overall pathway of cargo sorting at the TGN.**

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INTRODUCTION

Biosynthetic transport of soluble proteins

Soluble proteins delivered to the secretory pathway include resident proteins such as endoplasmic reticulum (ER) chaperones, lysosomal hydrolases, and secretory proteins. Most soluble proteins contain a signal sequence that targets them to the ER (Blobel, 1980). In the ER these proteins are folded, glycosylated, and if properly folded, packaged into coat protein II (COP II)-coated vesicles for transport to the Golgi apparatus (GA) (Barlowe *et al.*, 1994; Schekman *et al.*, 1995; Malkus *et al.*, 2002). Subsequently, these proteins passage through the GA and they are sorted at the *trans*-Golgi Network (TGN) for transport to their final destinations (Anitei and Hoflack, 2011). These destinations include endosomes, lysosomes, secretory storage granules and the plasma membrane (De Matteis and Luini, 2008; Guo *et al.*, 2014; Kienzle and Blume, 2014). Furthermore, an additional level of complexity is the transport of proteins to different directions for delivery to various cell domains (Mellman and Nelson, 2008) for instance in mature epithelial cells and neurons that have functional and morphological polarization. Moreover, migrating cells, which develop a leading edge for forward movement, require polarized vesicular transport (Miller *et al.*, 2009; Veale *et al.*, 2010). To achieve high accuracy of protein transport into distinct exit routes, cells employ elaborate cargo sorting machineries to package cargo into the right transport carriers for targeting to the right destinations.

Sorting of transmembrane proteins at the TGN. The mechanism of TGN sorting of many transmembrane proteins has been well studied in the past three decades. Most of these proteins contain cytosolic domains that are recognized by adapter proteins that recruit clathrin triskelia, thereby forming a coating structure that concentrates the cargo molecules into a clathrin-coated vesicle. These sorting motifs have been identified for proteins directed to the endosomal system and for some basolateral-directed cargoes (Fölsch *et al.*, 2003; Ang and Fölsch, 2012; Bonifacino, 2014). It has also been postulated that glycosylphosphatidylinositol (GPI)-anchored proteins have a particular affinity for sphingolipid and cholesterol-rich membrane domains. This feature allows them to coalesce with these lipids and accumulate in TGN microdomains

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Abbreviations used: APEX, engineered ascorbate peroxidase; BioID, proximity dependent biotin identification; Cab45, 45 kDa calcium-binding protein; Cdc42, cell division control protein 42 homologue; CgA, chromograninA; COMP, Cartilage Oligomerizing Matrix Protein; COP II, coat protein II; CPA4, Carboxpeptidase A4; ER, endoplasmic reticulum; ERGIC, ER Golgi intermediate compartment; Fam20C, family with sequence similarity 20, member C; GC, β-glucocerebrosidase; GPI, glycosylphosphatidylinositol; GSIS, glucose-stimulated insulin secretion; LIMP-2, lysosomal integral membrane protein type 2; LyzC, LysozymeC; M6P, mannose 6-phosphate; MMP9, Matrix Metalloproteinase9; MPR, mannose 6-phosphate-receptor; P-domain, phosphorylation domain; Pak1, p21-activated kinase; PAUF, pancreatic adenocarcinoma up-regulated factor; PC, procollagen; SGCII, secretograninII; SPCA1, Secretory Pathway Calcium ATPase1; ss-HRP, signal sequence horseradish peroxidase; Tango1, Transport and Golgi organization protein 1; TGN, *trans*-Golgi network; TIMP1, tissue inhibitor of matrix proteinases; TSP, Thrombospondin; VPS10p, vacuolar sorting protein 10p.

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(Keller and Simons, 1997; Simons and Ikonen, 1997; Harder *et al*., 1998; Paladino *et al*., 2004; Lingwood and Simons, 2010; Simons and Gerl, 2010; Surma *et al*., 2012).

Since soluble cargoes do not contain bona fide membrane binding domains, their sorting is less well understood; only a few mechanisms have been proposed and studied.

Sorting of soluble lysosome hydrolases. Sorting lysosomal hydrolases as well as secretory storage granules targeted proteins have been well studied in past three decades, and we refer to excellent reviews for more detailed information (Kornfeld and Mellman, 1989; Borgonovo *et al*., 2006). Kornfeld and colleagues described the first sorting receptor-dependent route for acid hydrolases from the TGN to endolysosomes that is mediated by mannose 6-phosphate (M6P) recognition. Most newly synthesized lysosomal hydrolases acquire these M6P moieties on their N-linked oligosaccharide chains as a unique marker that is recognized by the M6P-receptor (MPR) at the TGN. MPRs then bind to luminal hydrolases and to cytoplasmic adaptors that recruit clathrin coats on the cytosolic face of the TGN membrane. These clathrin-coated vesicles deliver their contents to late endosomes via early endosomes (Reitman and Kornfeld, 1981; Hoflack and Kornfeld, 1985; Griffiths *et al*., 1988; Kornfeld and Mellman, 1989; Le Borgne and Hoflack, 1997; Traub and Kornfeld, 1997; Ghosh and Kornfeld, 2004; Niehage *et al*., 2014). There are also M6P-independent sorting pathways that include sortilin, a member of the vacuolar sorting protein 10p (VPS10p) family of sorting receptors. Sortilin mediates the sorting and lysosomal trafficking of sphingolipid activator proteins prosaposin and acid sphingomyelinase (Braulke and Bonifacino, 2009). Furthermore, sorting of the acid hydrolase β-glucocerebrosidase (GC) by lysosomal integral membrane protein type 2 (LIMP-2) to lysosomes has been reported to be M6P-independent (Reczek *et al*., 2007). In 2014, a study of Zhao *et al.* questioned the view on LIMP-2 as an M6P-independent trafficking receptor. LIMP-2 crystal structure revealed a M6P residue at N325 suggesting MPR binding to LIMP-2 (Zhao *et al*., 2014). By contrast, studies in living cells showed that LIMP-2 and GC localize to lysosomes independently of the M6P pathway (Blanz *et al*., 2015). Whether GC sorting is facilitated by LIMP-2 in an M6P-independent manner remains to be elucidated. These studies underline the complexity of cargo sorting at the TGN.

Sorting of large and rigid extracellular matrix proteins. Sorting, packaging, and export of fibrillar rigid procollagens (PC) that contain rod-like triple helical domains and can reach 450 nm in length is challenging since procollagens are too large to fit into canonical COPII-coated vesicles that reach a diameter of 60–90 nm. Transport and Golgi organization protein 1 (Tango1) has been described to be essential for collagen export at the ER (Bard *et al*., 2006; Saito *et al*., 2009). These results suggest a unique mechanism to modulate COPII vesicle size to support the exit of rigid and bulky cargo such as for PC VII. However, the mechanism how these bulky cargoes are sorted at the TGN remains unknown. Interestingly, it has been shown that collagen IV and laminin require a low pH, for their sorting to the basolateral membrane in cultured glomerular epithelial cells (Natori *et al*., 1992). Furthermore, it has been demonstrated that polarized secretion of laminin and heparan sulfate proteoglycans in Madin–Darby canine kidney cells requires an acidic pH (Caplan *et al*., 1987). To date, the mechanistic features of the sorting of these proteins have not been investigated.

Sorting by formation of protein complexes or aggregates. Professional secretory cells store peptide hormones in secretory storage granules that localize in the cytosol closed to the plasma membrane. In contrast to constitutive secretion, these proteins are released upon an extracellular stimulus that induces the fusion of the granule with the cell membrane. The prohormone VGF is an important factor regulating animal metabolism including insulin secretion in pancreatic β cells. Pro-VGF is sorted into dense core secretory granules and is proteolytically processed into secreted peptides (Possenti *et al*., 1999; Trani *et al*., 2002; Stephens *et al*., 2012). It has been shown that the secretion of the C-terminal VGFP peptide leads to increase of glucose-stimulated insulin secretion (GSIS) and promotes β cell survival (Stephens *et al*., 2012). Loss of VGF in isolated islet β cells and conditional knockout mice leads to a decrease of GSIS and to the accumulation of granule cargo chromograninA (CgA) at the TGN, indicating that VGF also facilitates efficient exit of granule cargo thereby controlling granule biogenesis and insulin biosynthesis in islet β cells (Stephens *et al*., 2017). The formation of protein complexes or aggregates has been postulated to segregate these soluble cargo proteins, by clustering-induced sorting (Arvan and Castle, 1998; Arvan *et al*., 2002; Borgonovo *et al*., 2006; Bartolomucci *et al*., 2011; Fargali *et al*., 2014). VGF, CgA, and secretograninII (SGCII), are sorted by aggregation that depends on millimolar Ca^{2+} concentrations and on a mildly acidic pH (TGN pH is 6.2). Ca^{2+}/pH dependent aggregation of proteins is mediated by structural features in the cargoes that often contain numerous acidic amino acids distributed over vast areas of the folded polypeptide chains (Gerdes *et al*., 1989; Bartolomucci *et al*., 2011; Fargali *et al*., 2014). This mechanism has been proposed for cargoes destined for regulated secretion that need to be sorted away from the cargo of the conventional sorting pathway. Therefore, the exact mechanism how these cargoes are packaged into storage granules is still poorly understood. The following section will describe a novel sorting process sharing similar features.

Discovery of Ca^{2+}-based sorting at the TGN. A genome-wide screen demonstrated the requirement for the actin-severing protein twinstar, for secretion of signal sequence horseradish peroxidase (ss-HRP) from *Drosophila* S2 cells (Bard *et al*., 2006). Further examination of the process revealed that twinstar and its orthologues, in both yeast (cof1) and mammalian cells (ADF and cofilin), are required to sort a subset of cargo molecules at the TGN (Blume *et al*., 2009; Curwin *et al*., 2012). We showed that a pool of cofilin localizes transiently to TGN membranes and regulates the Ca^{2+} influx into the TGN by interacting with the Secretory Pathway Calcium ATPase1 (SPCA1) (Blume *et al*., 2011).

SPCA1 pumps Ca^{2+}, as well as Mn^{2+}, into the lumen of the TGN in an ATP-dependent manner (Van Baelen *et al*., 2004). The concentration of Ca^{2+} in the Golgi apparatus is heterogeneous, and it was suggested that there is a Ca^{2+} gradient across the secretory pathway from the ER to the TGN (Pizzo *et al*., 2011). At steady state, The ER has the highest concentration (400 μM), while the *cis* Golgi contains 250 μM and the TGN around 100 μM. However, the TGN Ca^{2+} level oscillates over time (unpublished data) (Dolman and Tepikin, 2006). Tulio Pozzan and colleagues have shown that TGN Ca^{2+} uptake relies solely on SPCA1 (Lissandron *et al*., 2010). Using purified proteins, we found that the SPCA1 phosphorylation domain (P-domain), crucial for pump activation, interacts with F-actin in a cofilin dependent manner (Kienzle *et al*., 2014). When expressed in HeLa cells, the P-domain inhibits Ca^{2+} entry into the TGN and causes missorting of secretory cargo. Furthermore, mutation of four amino acids in the SPCA1 cofilin binding site impairs Ca^{2+} import into the TGN and affects secretory cargo sorting (Kienzle *et al*., 2014).

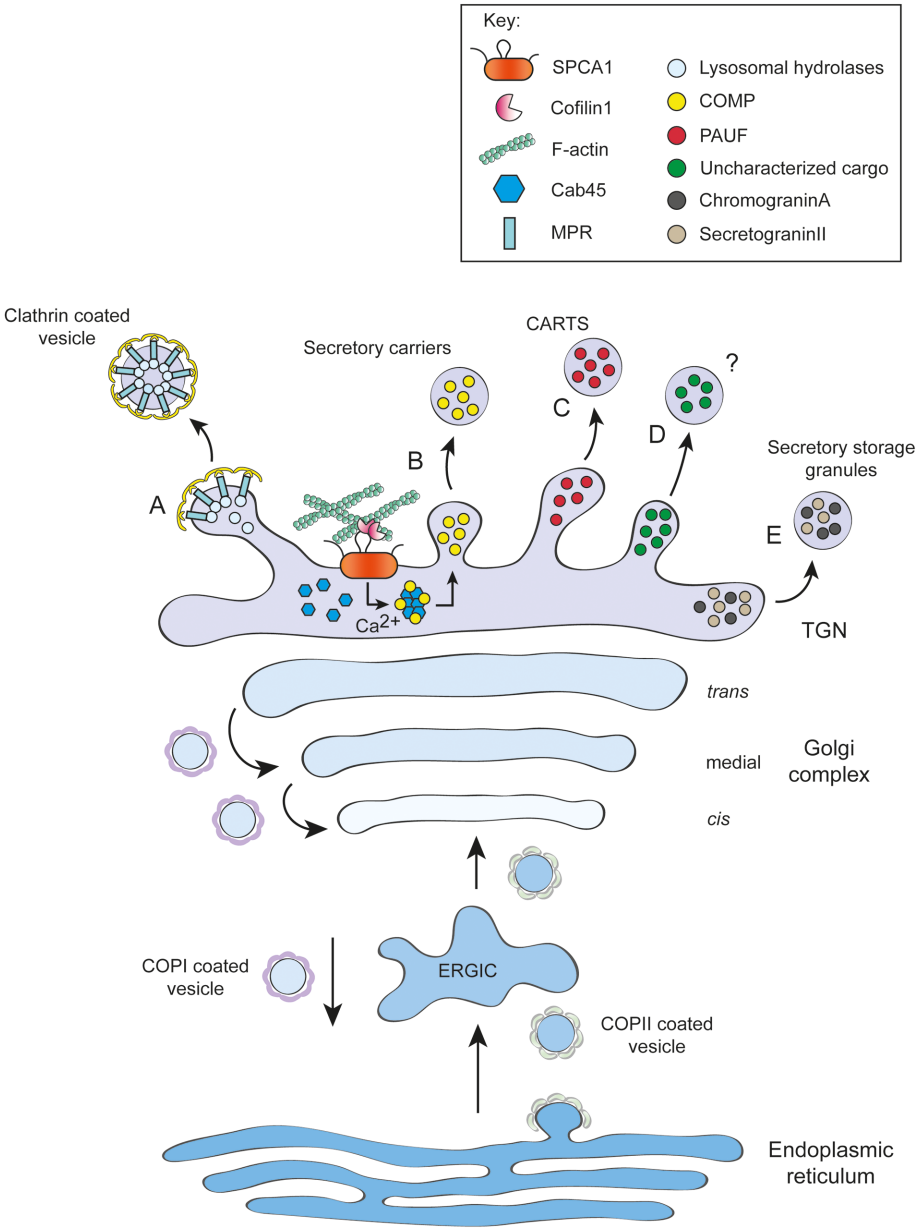

FIGURE 1: Protein transport and cargo sorting in the secretory pathway. Proteins containing a signal sequence are cotranslationally inserted into the endoplasmic reticulum (ER). Secretory proteins leave the ER in coat protein complex II (COPII)-coated vesicles and are transported via the ER Golgi intermediate compartment (ERGIC) to the Golgi apparatus (GA). After transport through the *cis*- and medial Golgi compartments, proteins enter the *trans*-Golgi network (TGN) and are sorted to their correct destination. (A) Mannose 6-phosphate (M6P) modified lysosomal hydrolases are captured by M6P-receptor (MPR) and packaged into clathrin-coated vesicles. (B) The Secretory Pathway Calcium ATPase 1 (SPCA1) pumps Ca^{2+} into the TGN in a cofilin and F-actin dependent manner. Ca^{2+} influx leads to calcium binding protein 45 (Cab45) oligomerization and sorting of soluble secretory cargo such as cartilage oligomerizing matrix protein (COMP) into secretory carriers. (C) Carriers from the TGN to the cell surface (CARTS) transport pancreatic adenocarcinoma up-regulated factor (PAUF). (D) Alternative cargo sorting mechanisms of yet uncharacterized cargoes remain only poorly understood. (E) In specialized cells, secretory storage granule proteins chromograninA (CgA) and secretograninII (SGCII) are sorted by aggregation into secretory storage granules under high Ca^{2+} concentrations and midly acidic pH. ER resident proteins are retrograde transported in COPI-coated vesicles.

The next major question was how luminal Ca^{2+} facilitates the sorting process. Interestingly, cells that are depleted of ADF/cofilin or SPCA1 mis-sort secretory proteins and also secrete the soluble Golgi-resident protein, 45 kDa calcium-binding protein (Cab45) (Blume *et al.*, 2012). Cab45 is evolutionary conserved in higher eurkaryotic organisms with highest sequence homology in vertebrates. There are no Cab45 homologues reported in fungi, indicating a specialized enhancement in vertebrates due to expanded secretory cargo complexity such as the emerging of an increased variety of extracellular matrix proteins that require sorting to be secreted in order to support cell adhesion and migration (Tabach *et al.*, 2013a,b).

Lodish and colleagues identified Cab45 as a Golgi-resident protein with 6 Ca^{2+} binding EF hand domains (Scherer *et al.*, 1996). Consistent with this observation, we recently demonstrated that Cab45 localization in the Golgi is sensitive to Ca^{2+} levels, and disrupting Golgi Ca^{2+} gradients induces Cab45 secretion by cells (Blume *et al.*, 2009, 2011). The knockdown of Cab45 affects cargo sorting similar to ADF/cofilin or SPCA1 depletion. Also, Cab45 binds several secretory proteins in a Ca^{2+}-dependent manner, and this binding appears to be required for cargo sorting at the TGN (Blume *et al.*, 2012). Taken together these results indicated that Cab45 is a component of the cofilin/F-actin/SPCA1 sorting machinery. What is the role of Cab45 in this process?

Cab45 forms oligomers in the presence of Ca^{2+} in vitro and living cells (Crevenna *et al.*, 2016). Furthermore, Cab45 changes its secondary structure upon Ca^{2+} binding, possibly to enable it to interact with its target cargo proteins. Moreover, we observed that only the oligomeric form of Cab45 binds selectively to specific cargo molecules such as Cartilage Oligomerizing Matrix Protein (COMP) and LysozymeC (LyzC), but not to cathepsin D in vitro. Finally, three-dimensional structured illumination microscopy showed that Cab45, SPCA1, and cargo colocalize in specific clusters at the TGN. We conclude from this data that upon SPCA1-dependent Ca^{2+} influx into the lumen of the TGN, Cab45 binds Ca^{2+}, triggering a conformational change and allowing oligomerization. These oligomers then bind specific proteins, thereby sorting cargo from noncargo (Crevenna *et al.*, 2016).

Taken together, cofilin binds to SPCA1 at the TGN and recruits F-actin (Figure 1), resulting in pump activation, thereby inducing Ca^{2+} influx into a specific domain of the TGN. This transient, local increase in Ca^{2+} recruits Cab45, which has a high affinity for Ca^{2+} and oligomerizes and binds cargo. Subsequent dissociation of the Cab45-cargo complex occurs either upon a decrease in Ca^{2+} or by a signal such as phosphorylation, resulting in the segregation of

cargo for sorting into a particular class of transport carrier. We have named this Cab45 sorting oligomer a cernosome, from the Latin *cernere*, which means to choose, sift, separate, decide, or distinguish. Thus we suggest that this is a unique way to export cargo molecules independent of a bona fide cargo receptor.

OPEN QUESTIONS

SPCA1 and Cab45-dependent sorting have evolved as a unique pathway to sort proteins such as LyzC, tissue inhibitor of matrix proteinases (TIMP1), Thrombospondin (TSP) 1, and 3, Matrix Metalloproteinase9 (MMP9) and COMP while, for instance, Carboxpeptidase A4 (CPA4), Fibulin1, Fibrillin1, and Fibronectin are sorted via a different pathway (Blume *et al.*, 2009; Kienzle and Blume, 2014). However many open questions remain to be elucidated to understand the mechanism of this process.

Does actin control SPCA1 activation?

SPCA1 Ca^{2+} uptake- and sorting activity requires its binding to F-actin via cofilin. However, the precise role of this interaction has not yet been elucidated. The TGN has a low Ca^{2+} concentration, and we hypothesize that SPCA1 pumps high Ca^{2+} only at specific subdomains of the TGN (Blume *et al.*, 2011; Aulestia *et al.*, 2015). Previous work has shown that specific lipids such as cholesterol regulate clustering of proteins at the plasma membrane (Goswami *et al.*, 2008). Also, the activity of SPCA1 is determined by the cholesterol and sphingolipid composition in living cells and in a reconstituted system (Baron *et al.*, 2010; Chen *et al.*, 2017). F-actin and cofilin might mediate the clustering of SPCA1 into a specific lipid environment rich in cholesterol and sphingomyelin. Furthermore, SPCA1 clustering could favor a model of high local Ca^{2+} influx, leading to spatially regulated Cab45 oligomer formation and cargo sorting at distinct lipid domains that could promote secretory vesicle formation. In this respect, Burd and colleagues have described a new class of unknown TGN derived sphingomyelin-rich vesicles (Deng *et al.*, 2016). Inhibition of sphingomyelin synthesis has been shown to affect the trafficking to the plasma membrane of several proteins including vesicular stomatitis virus G protein, influenza hemagglutinin, and pancreatic adenocarcinoma up-regulated factor (PAUF) (Subathra *et al.*, 2011; Tafesse *et al.*, 2013; Wakana *et al.*, 2015). Sphingomyelin has structural functions by decreasing membrane fluidity (Barenholz and Thompson, 1980; Van Blitterswijk *et al.*, 1981). Furthermore, it serves as a source of important signaling molecules (Hla and Dannenberg, 2012). How Sphingomyelin signaling and structural features are potentially involved during secretory cargo sorting, remains to be elucidated.

Another important question is whether the dynamics of F-actin polymerization versus depolymerization regulates SPCA1 Ca^{2+} pumping cycles. It has been shown that expressed LIM kinase (LIMK) localizes to the Golgi and regulates cofilin activity by phosphorylation at serine3 (Arber *et al.*, 1998; Rosso *et al.*, 2004). LIMK is activated by p21-activated kinase (Pak1) through a cell division control protein 42 homologue (Cdc42) signaling cascade (Edwards *et al.*, 1999). This process might also be directly linked to vesicle generation at the TGN since others (Almeida *et al.*, 2011; Pylypenko *et al.*, 2016) and we (unpublished results) have already identified the involvement of myosins in this pathway. The question remains whether cofilin activation at the Golgi by LIMK is temporally regulated by upstream stimuli or cofilin is activated in a stochastic manner leading to Ca^{2+} influx cycles.

How is cargo recognized by Cab45?

Our work has shown that oligomeric Cab45 binds to secretory proteins and we propose that these clusters sort cargo. It is not yet clear how Cab45 recognizes its target proteins. One possibility would be that there is a sorting sequence present in Cab45 dependent cargoes, such KDEL that has been shown for escaped ER resident chaperones (Munro and Pelham, 1987). So far a defined consensus sequence for Cab45 cargo could not be identified. It might be also the case that Cab45 recognizes different classes of cargoes through multiple interaction surfaces as it has been shown for Calmondulin (Tidow and Nissen, 2013). Similarly, Cab45 oligomers could bind to intrinsically disordered cargo binding sites that fold up upon Cab45 binding. In contrast, since lysosomal hydrolases such as cathepsin D do not interact with Cab45, they are captured with high affinity by MPR and targeted to clathrin-coated vesicles.

To solve this problem we need to increase our repertoire about Cab45 target proteins. In addition, the binding surfaces in Cab45 mono- and oligomers have to be identified biochemically and by structural biology to finally characterize the mechanism of binding.

How and where does Cab45 dissociate from the cargo?

Since Cab45 is a Golgi resident, it must somehow separate from cargo before being packaged into a transport carrier. This process might occur upon a drop of Ca^{2+} after complex formation of the oligomer with cargo. Furthermore, we imagine that Cab45 cargo dissociation occurs by a posttranslational modification such as phosphorylation. The serine/threonine kinase family with sequence similarity 20, member C (Fam20C) (Tagliabracci *et al.*, 2012, 2015) and the extracellular tyrosine-protein kinase PKDCC (Bordoli *et al.*, 2014) were reported to phosphorylate several resident as well as secreted proteins throughout the secretory pathway.

CONCLUSIONS AND FUTURE DIRECTIONS

Secretory proteins are essential for many crucial cellular events. Cells secrete signaling molecules such as hormones or neurotransmitters, digestive enzymes, antibodies, mucus, and extracellular matrix proteins such as collagens that provide mechanical strength and tissue integrity. For instance, matrix metalloproteinases (MMPs) in monocytes are specifically secreted to invadosomes that are cell matrix contacts with an actin-rich core. This local MMP secretion facilitates the lysis of extracellular matrix components at invadosomes being key features in both physiological and pathological cell invasion (Linder *et al.*, 2011). The general view of secretory protein sorting into the constitutive pathway in the TGN was that proteins traverse and exit the Golgi independent of sorting signals (Pfeffer and Rothman, 1987). In contrast, research in recent years has shown that at least a subset of secretory proteins such as LyzC, COMP, TSP1, TSP5, TIMP1 and MMP9 are actively sorted at the TGN. Malhotra and colleagues have identified carriers from the TGN to the cell surface (CARTS) that transport PAUF but not collagen I (Wakana *et al.*, 2012). We have proposed a mechanism that involves F-actin/cofilin/SPCA1/Ca^{2+} and Cab45 that form a functional sorting module in a particular TGN subdomain to direct LyzC, TIMP1, TSP1 and 5, Matrix MMP9 and COMP to the cell surface (Kienzle and Blume, 2014; Blank and Blume, 2017). Importantly, we found that there are other cargoes such as interleukins (unpublished data) and other proteins (Blume *et al.*, 2009) that are sorted in a Cab45 independent manner for instance Carboxpeptidase A, Fibulin1, Fibrillin1, Fibronectin (Blume *et al.*, 2009). This highlights the fact that there are additional alternative sorting events that remain to be elucidated. These may also be cell type specific and differ during development of an organism.

The TGN, as a highly dynamic organelle, is challenging to study. Nevertheless, tremendous progress in technology will help

to elucidate the mechanism of SPCA1 dependent sorting as well as the identification these new exit routes. Genome editing now allows monitoring sorting and transport of tagged endogenous proteins by live-cell microscopy with high temporal resolution as well as super-resolution microscopy. Furthermore, biochemical approaches such as proximity dependent biotin identification (BioID) and engineered ascorbate peroxidase (APEX) show promise for the identification of new protein-protein interactions in the highly dynamic TGN environment. These interactions can be reconstituted in vitro to give a comprehensive understanding of the mechanism of protein sorting at the TGN. Future studies should therefore be aimed at answering remaining questions about secretory cargo sorting at the TGN.

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