Sorting of oligomerized proteins: Implications in toxin trafficking and quality control

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ABSTRACT

Shiga toxin (STx)–producing bacteria including *Shigella dysenteria* and the shigatoxigenic group of *Escherichia coli* (STEC) cause severe diarrhea that can develop into life-threatening hemolytic uremic syndrome. STx invades cells by binding to a cell surface receptor and undergoing endocytosis into early endosomes. Instead of subsequent lysosomal degradation, it actively sorts into early endosomal tubules by binding GPP130 (Golgi Phosphoprotein of 130KDa) a cis- Golgi resident integral membrane protein that constitutively cycles between early endosomes and Golgi. This directs the toxin to the Golgi where it continues its retrograde trafficking to exert its cytotoxic effects. Interestingly, elevated, non-toxic levels of manganese (Mn) protect cells from STx induced death via downregulation of GPP130. Mn binds to GPP130 in the Golgi, causes GPP130 to oligomerize/aggregate and the oligomerized protein gets diverted to lysosomes. Surprisingly, based on experiments using the self-interacting FM domain, it appears generally true that oligomerization of a Golgi protein can switch its trafficking itinerary leading to its lysosomal degradation via a GGA1-clathrin dependent pathway. However, how this sorting switch at the Golgi is mediated remains unknown. This has important implications for our understanding of regulation of protein trafficking as well as quality control pertaining to aggregated proteins in the Golgi. Here we provide evidence that Mn-induced exit of GPP130 from the Golgi towards lysosomes is mediated by the sorting receptor, sortilin. Sortilin was found to interact with GPP130 in cells within a ~50 residue stretch of the GPP130 lumenal stem domain.

Interestingly, Mn induced turnover of another Golgi membrane protein, TMEM165 was also found to be sortilin-dependent. In contrast, FM-induced lysosomal trafficking of GPP130 as well as the Golgi protein galactosyltransferase (GT) was sortilin independent and occurred even in the absence of the native lumenal domain of GT. Thus, sortilin-dependent as well as sortilin-independent sorting mechanisms target aggregated Golgi membrane proteins for lysosomal degradation.

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CHAPTER 1

INTRODUCTION

This thesis addresses the question of how oligomeric proteins in the Golgi are recognized and targeted for lysosomal degradation. Scenarios in which selective protein degradation occurs include: steady state protein turnover, protein regulation in cellular function and homeostasis, and protein quality control (Vogel and Marcotte 2012; Goldberg 2003; Rothman 2010). Although conceptually distinct, these scenarios can overlap in their degradation machinery. Protein quality control can be defined as accelerated turnover of proteins with the end goal of maintaining only proteins that are properly folded and assembled. Persistently misfolded, transiently unfolded or inefficiently folding proteins are recognized primarily via their exposed hydrophobic patches by the quality control machinery and targeted for degradation (Bukau, Weissman, and Horwich 2006; Shao and Hegde 2016). This cellular process is vital to minimize accumulation of aberrant, non-functional proteins that can eventually disrupt protein homeostasis and cause cell death. Inefficient quality control machinery is therefore associated with aging and various neurodegenerative disorders (Koga, Kaushik, and Cuervo 2011; Hol and Scheper 2008). Protein quality control occurs in various cellular compartments. One of the most well-studied quality control pathways is the endoplasmic reticulum (ER) quality control (Vembar and Brodsky 2008; Brodsky and Skach 2011). Although, it is a stringent process, some aberrant proteins are known to escape recognition and become substrates of post-ER quality control. Additionally, post-ER quality control

substrates may arise if they pass the ER checkpoint and subsequently acquire aberrant features. The evidence for post ER quality control is compelling but the examples are few and a clear mechanistic understanding is lacking (Arvan et al. 2002; Hammond and Helenius 1995). In particular, quality control at the Golgi is an important, yet understudied, process. Here we define Golgi quality control as a process where the initial recognition event takes place in the Golgi presumably marking the protein substrates somehow to cause their degradation. These substrates must escape ER quality control but possess aberrant features that are recognized at the Golgi. This can include misfolded proteins with exposed hydrophobic patches, transmembrane proteins containing charged residues in their TMDs or aggregated proteins. A lot remains to be discovered regarding the molecular players and regulatory processes operating in Golgi quality control, especially in mammalian cells. The degradation pathways known to operate on substrates at the Golgi that escape ER quality control include the canonical Golgi to lysosome pathway, autophagy, and proteasomal degradation. Additionally, ER retrieval and subsequent ERAD (ER-associated degradation) of substrates that escape to the Golgi has been observed. This chapter summarizes our current knowledge on substrates that escape ER quality control and undergo degradation initiated at the Golgi in an attempt to extract insights as to how this process operates. We start with the predominant pathway, lysosomal degradation as a means of Golgi quality control (1.1). We then briefly touch on autophagy (1.2) and proteasomal degradation (1.3) that operate in a minor capacity in Golgi quality

control before concluding with the outstanding questions in the field and the specific questions that we aimed to address (1.4).

1.1 LYSOSOMAL DEGRADATION AS A MEANS OF GOLGI QUALITY CONTROL

There are a number of examples of proteins that escape ER quality control and reach the Golgi and are then targeted for lysosomal degradation. How these defective proteins escape ERAD and what key molecular players at the Golgi recognize and traffic these substrates to the lysosome remains unclear. It is possible that the nature of the defects in these proteins plays a role in their escape from ERAD, but this has not been established. This pathway is best studied in yeast. The sections below, divided by the type of quality control substrate, briefly describe examples of this pathway in both yeast and mammalian systems.

1.1.1 Integral membrane proteins

Initial hints of the existence of a Golgi quality control pathway in yeast stemmed from studies characterizing a class of mutations in the plasma membrane ATPase encoding gene, Pma1 (Pizzirusso and Chang 2004). This multi-pass transmembrane protein having a half-life of ~11h, functions to regulate cytoplasmic pH and drives nutrient uptake (Chang and Fink 1995). When two residues on its cytoplasmic segment are mutated (P434A and G789S), this temperature sensitive protein (termed Pma1-7) undergoes ubiquitination and consequently vacuolar degradation with a half-life of 20min. The Rsp5-Bul1-Bul2 ligase complex mediates its ubiquitination, and in Rsp5 deficient cells, Pma1-7 is stable and cell surface

localized. It is interesting that Pma1-7 escapes ER quality control in contrast to some other Pma1 mutants (Wang and Ng 2010). What features in Pma1-7 allow its detection as a potentially misfolded protein in the Golgi and how this triggers its ubiquitination remains unclear.

Wsc1p is another membrane protein that, when mutated, escapes ER quality control and undergoes vacuolar degradation (Wang and Ng 2010). A non-essential single-pass type I transmembrane protein, it functions in plasma membrane integrity signaling. Wsc1p lacks N-linked glycans so it is not considered a conventional ERAD substrate. The tested mutated versions included a single non-conservative point mutation. Both fail to bind the ERAD chaperone Kar2p. Interestingly, their escape of ERAD may be due to a dominant ER exit signal. Deletion of this signal causes a Wsc1p variant to become an ERAD substrate. Support for this view comes from the misfolded ERAD substrate CPY*. CPY* with an appended ER exit signal escapes ER quality control and undergoes vacuolar degradation. Additionally, over expression of CPY* causes excess CPY* to traffic to the vacuole for degradation indicating that although Golgi quality control operates for substrates not recognized by ERAD, it can also kick in when ERAD is saturated (Spear and Ng 2003).

Another feature that may predispose membrane proteins to Golgi quality control is the presence of mutations that introduce polar or charged residues in a transmembrane domain (TMD). An example is the yeast syntaxin-like SNARE, Pep12 that normally functions in late endosomes. An L->D mutation within its

transmembrane domain causes it to be targeted from the Golgi to the MVB for vacuolar degradation but its ER exit is unperturbed. Polar residues do not present a problem for membrane insertion as long as the remainder of the TMD is sufficiently hydrophobic. Tul1p, a Golgi-localized integral membrane ubiquitin ligase, was shown to recognize the Pep12 mutant protein (Fulvio Reggiori and Pelham 2002). The RING domain of Tul1p binds the ubiquitin donor Ubc4 and facilitates ubiquitin transfer onto the cytoplasmic portion of Pep12. Interestingly, Tul1p also mediates ubiquitination of CPS1 (carboxy peptidase S) and Phm5 (polyphosphate phosphatase), two proteins with moderately polar transmembrane domains, which naturally undergo MVB sorting.

How does Tul1p recognize polar TMDs? More than 50% of single pass proteins in the yeast proteome contain ~ 3-5 polar residues in their TMD, ~30% contain 1-2 strongly polar residues while ~ 25% contain one charged residue. The human single pass transmembrane proteome shows a much lower fraction polar/charged residues in their TMDs (Worch et al. 2010). This raises the question of how does Tul1p selectively recognize mutated Pep12, CPS1 or Phm5? Tul1p is comprised of seven TMDs that contain multiple polar residues. Perhaps these residues form a polar patch that recognizes exposed polar residues in the substrate (Fulvio Reggiori and Pelham 2002). An additional possibility might have been that Tul1p recognizes an aggregated state of its targets. This is ruled out, at least for non-mutated substrates such as Cps1 and Phm5 because they do not appear to form oligomers (Fulvio Reggiori and Pelham 2002; F Reggiori, Black, and Pelham 2000). Clearly,

more work is needed. It is not even known whether Tul1p substrate recognition is direct or indirect.

There is also another pathway involving recognition of polar transmembrane domains. The integral membrane protein Bsd2 binds the ubiquitin ligase Rsp5 and ensures efficient ubiquitination and sorting of CPS1 and Phm5 (Hettema, Valdez-Taubas, and Pelham 2004). The recognition of CPS1 and Phm5 depends on polar residues in their transmembrane domains. Bsd2 is also implicated in ubiquitination and targeting of the yeast Mn^{+2} transporter Smf1p for vacuolar degradation under physiological levels of Mn^{+2}. This appears Mn^{+2}-dependent because in Mn^{+2} deficient conditions, Smf1p is localized at the cell surface (Liu and Culotta 1999). Thus, studies in yeast have identified at least two transmembrane proteins, Tul1p and Bsd2, that recognize and play a role in the ubiquitination of transmembrane proteins containing polar TMDs for lysosomal degradation (Zaliauskiene et al. 2000).

Golgi to lysosome targeting of misfolded membrane proteins has also been observed in mammalian cells. The first studies on the cell surface localized T cell receptor complex showed that excess free subunits or partially assembled subunits are targeted for lysosomal degradation before they reach the surface (Minami et al. 1987). Also, insertion of two positively charged residues into the alpha helical TMD of influenza hemagglutinin (HA^{++}) causes a fraction of the mutated population to undergo lysosomal degradation (Fayadat and Kopito 2003). Whether the pathway is

direct from the Golgi is not established. Whether there is an ubiquitin ligase similar to Tul1p in this pathway is also not known. Another example is the plasma membrane localized Aquaporin 2 water channel that, when mutated in its cytoplasmic domain (E258K), is rerouted from the Golgi to the MVBs for degradation (E. J. Kamsteeg et al. 1999; E.-J. Kamsteeg et al. 2008). The E258K mutation is dominant and causes nephrogenic diabetes insipidus because it trimerizes with the functional wildtype protein causing degradation of the whole complex leading to a deficit in surface levels of functional monomeric wild type protein. Another example is the corona virus E1 membrane protein for which certain mutations cause its trafficking from the Golgi to lysosomes (Armstrong, Patel, and Riddle 1990). A final example is the R142W mutant version of the gap junction plasma membrane protein, Connexin32. This mutation lies within the third transmembrane domain of Cx32 that is occupied by a basic residue in members of the connexin family and is predicted to contribute to the gap junction channel pore. In PC12 cells, it exits the ER and undergoes lysosomal degradation within $\sim$ 3h. However, other transmembrane residue mutations cause ER retention (VanSlyke, Deschenes, and Musil 2000; Musil and Goodenough 1993). It is unknown why some mutations cause ER retention and others lead to ER exit and lysosomal degradation. In fact, the machinery involved in recognition of integral membrane quality control substrates in the mammalian Golgi is entirely unknown.

1.1.2 Soluble proteins

Golgi quality control has been observed for a variety of soluble proteins in the yeast secretory pathway that are either poorly secreted or unstable/misfolded. A number of these proteins are dependent on Vps10p (Vacuolar Protein Sorting) for their vacuolar sorting. Vps10p is a type I, Golgi localized integral membrane protein that transports soluble lysosomal hydrolases to the lysosomes by binding them via its large, ten-bladed beta-propeller lumenal domain (Hermey 2009; Quistgaard et al. 2009). The human homologue of Vps10p, sortilin performs a similar function (Canuel, Libin, and Morales 2009). One of the first insights into the Golgi quality control of misfolded soluble proteins was obtained by studying fusions of lambda repressor variants to the secreted protein invertase. Chimeras containing the wildtype repressor are secreted to the cell surface. However, mutations that decrease the thermodynamic stability of the repressor, cause vacuole targeting of the chimera that is Vps10p-dependent and saturable (Hong, Davidson, and Kaiser 1996). Similarly, beta lactamase is poorly secreted in yeast because it is misfolded as it exits the ER causing it to be directed from the Golgi to the vacuole via a Vps10p-dependent trafficking pathway (Holkeri and Makarow 1998).

Another relevant example is quality control of the soluble protein, alpha 1 antitrypsin (AT). The misfolded variant Z (ATZ) is a classic ERAD substrate. ATZ retrotranslocates from the ER and subsequently undergoes proteasomal degradation. However, when overexpressed, excess ATZ exits the ER and transits the Golgi to be sorted for vacuolar degradation via a Vps10p-dependent pathway

(Kruse, Brodsky, and McCracken 2005; Gelling et al. 2012). Interestingly, if ATZ is overexpressed, ATZ is now secreted indicating the pathway is saturable. Also, Vps10p deletion increases AT wildtype protein secretion and, in mammalian cells, overexpression of sortilin, the Vps10p mammalian homolog, decreases secretion. It is possible that Vps10p acts on the unfolded variants of AT that can potentially exist in dynamic equilibrium with folded AT and escape ER quality control. Given these findings, there seems to be an important role of Vps10p/sortilin as a generic mediator of Golgi quality control.

Bovine pancreatic trypsin inhibitor is a 58 amino acid soluble protein that is secreted when expressed in yeast. However, when mutated (Y35L), it is unstable yet escapes ERAD and is degraded in the vacuole (Coughlan et al. 2004). Interestingly, this degradation is independent of Vps10p suggesting that there are other recognition mechanisms for exogenously expressed unstable proteins that escape ER quality control.

At least one study also hints at the existence of Golgi quality control for misfolded soluble proteins in mammalian cells. The lipoprotein lipase is usually secreted. However, the mutation G142E dramatically reduces its secretion. This mutant version exits the ER and is sorted from the Golgi to lysosomes for degradation (Buscà et al. 1996). The role of sortilin in this process remains to be explored. In conclusion, although recognition and targeting of misfolded proteins is well

documented, the mechanistic details governing specific substrate recognition and the molecular players orchestrating this quality control remain to be unraveled

1.1.3 Induced Oligomers

High-order oligomeric protein assemblies are another class of substrates subject to Golgi quality control via degradation in lysosomes. One of the first links between oligomerization and lysosomal targeting came from the study of the transmembrane mammalian endopeptidase, furin. Furin was found to naturally assemble into large oligomers in the trans Golgi network (TGN) which are then targeted to lysosomes for degradation (Wolins et al. 1997). The purpose of these large aggregates is not known. This lysosomal targeting was mediated via the furin lumenal domain implying that cytoplasmic ubiquitination of furin is not involved.

Another example is the clearance of α-synuclein oligomeric intermediates. Proposed to function in synaptic transmission and neuronal plasticity, α-Synuclein is highly expressed in the brain in two forms. One is membrane-associated and the other is cytosolic (Perrin et al. 2000; McLean et al. 2000)., The formation of fibrillar α-synuclein aggregates is linked to pathogenicity in neurodegenerative disorders such as Parkinson's disease (Kelly A. Conway, Harper, and Lansbury 1998). The membrane-associated fraction moving through the Golgi has been shown to have a greater propensity to aggregate and in fact, seeds aggregation of the cytosolic form (Lee, Choi, and Lee 2002). During assembly of the pathogenic fibrillar aggregates there are non-toxic intermediates that are targeted for lysosomal degradation (Lee

et al. 2004). Blocking the lysosomal pathway causes a buildup of these aggregates, which eventually became pathogenic causing cytotoxicity in neuronal cells (Kelly A. Conway, Harper, and Lansbury 1998; K A Conway et al. 2000). Surprisingly, pathogenic aggregates are not cleared via the lysosomal pathway. Since non-toxic aggregates serve as intermediates to toxic aggregates one can speculate that investigating the recognition machinery in this quality control pathway could yield an important therapeutic avenue for neurodegenerative disorders.

Prion protein, which is normally a cell surface GPI anchored protein provides another example. Its aberrant version forms aggregates at the plasma membrane that cause fatal and transmissible neurodegenerative disorders (Ashok and Hegde 2009). It was found that the chemical compound Suramin induces the wildtype protein to form non-infectious aggregates in the Golgi that are then rerouted to the lysosome for degradation. Significantly, this process interfered with prion propagation in cell culture and infected mice (Gilch et al. 2001).

Perhaps the most well-studied example is manganese (Mn) induced oligomerization and lysosomal degradation of the membrane protein, GPP130. Rather than quality control, this is more likely to be an example of protein downregulation as a part of cellular Mn response. Nevertheless, work on this reaction led to an important example of Golgi quality control for aggregated proteins. GPP130 is a type II, cis-Golgi localized membrane protein with a half-life of ~18h that constitutively cycles between the Golgi and early endosomes (Linstedt et al. 1997). Exposure to low

doses of extracellular Mn cause rapid redistribution of GPP130 to MVBs within ~1h, and complete degradation in ~8h (Mukhopadhyay et al. 2010). The Mn response of GPP130 requires the activity of the Golgi localized Ca2+/Mn2+ pump SPCA1, indicating that increased intra-Golgi Mn levels cause its lysosomal rerouting (Mukhopadhyay and Linstedt 2011). Consistent with the role of intra-Golgi Mn, the coiled-coil domain of GPP130 within the Golgi lumen is required for Mn responsiveness. Subsequent studies found that the main trigger for lysosomal targeting is Mn-induced oligomerization of GPP130. Upon an increase in intra-Golgi Mn levels, Mn binds GPP130, causes it to oligomerize and this oligomerization targets the protein to lysosomes for degradation (Figure 1.1) (Ritika Tewari, Jarvela, and Linstedt 2014).

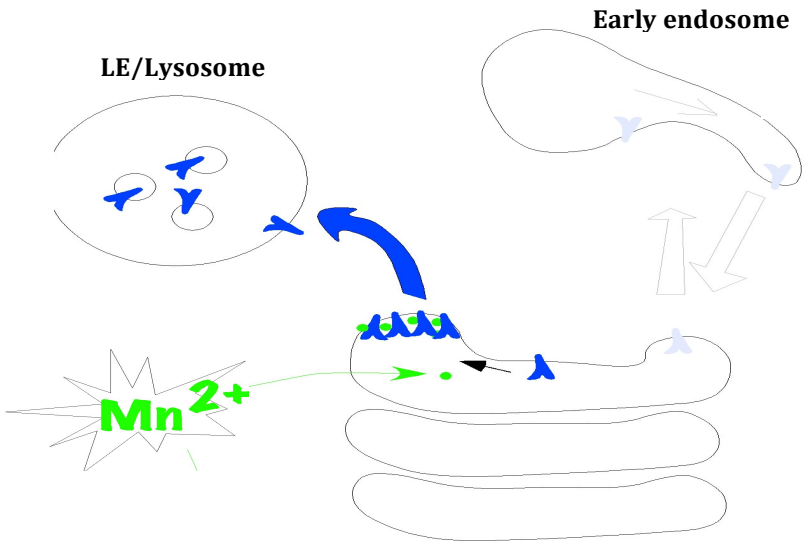

Figure 1.1 Mn-induced trafficking of GPP130: In its native state, GPP130 is a steady-state Golgi resident protein that constitutively cycles between the Golgi and early endosomes. Upon increase in intra-Golgi Mn (green) levels, Mn binds to the coiled-coil domain of GPP130 (blue), causes it to oligomerize and this oligomerization targets the protein to lysosomes for degradation

Mn-induced trafficking from the Golgi to lysosomes is direct because it is Rab-7-dependent and does not involve either early endosomes or the plasma membrane. Mapping and mutational analyses identified four critical residues in the coiled-coil domain ($_{88}$DFLV$_{91}$) that are required for Mn binding and oligomerization (Ritika Tewari, Jarvela, and Linstedt 2014).

Significantly, oligomerization was found to be sufficient to target several unrelated Golgi proteins for lysosomal degradation, suggesting the presence of a quality control pathway in the Golgi for aggregated proteins (R. Tewari, Bachert, and Linstedt 2015). These results were obtained by appending an array of three copies of a modified version (phenylalanine was mutated to methionine i.e. $F_{36}M$) of the FKBP12 domain, termed an FM domain (Rizzo et al. 2013; Rivera et al. 2000), to the lumenal domain of type-II Golgi membrane proteins. The FM domain self-interacts but this can be prevented by incubating cells with AP12998 (AP), a modified version of FK506 that is a competitive inhibitor of the interaction (Rivera et al. 2000). Three different type-II Golgi resident proteins, GPP130, mannosidase-I and beta 1,4-galactosyltransferase appended to FM domains, redistributed to lysosomes within 30mins of aggregation and complete protein degradation occurred within ~ 4h.

Studies to unravel the molecular players involved in this pathway revealed that lysosomal targeting of both Mn-induced GPP130 oligomers and FM-induced oligomers is GGA1 and clathrin dependent (Figure 1.2). Further, the redistribution is independent of an intact microtubule network showing that it does not require an

intact Golgi ribbon because the Golgi ribbon unlinks in the absence of microtubules (Mukhopadhyay et al. 2010). Neither Mn-induced nor FM-induced oligomers are likely to be misfolded and, consistent with this, their aggregation did not activate the UPR pathway (R. Tewari, Bachert, and Linstedt 2015). Also, targeting in both these processes occurs independently of the cytoplasmic domains making ubiquitination, as a mode of targeting, unlikely. However, it should be noted that an indirect role for ubiquitination is still possible, wherein some other protein with an ubiquinated cytoplasmic domain associates with the aggregates. Interestingly, Bsd2 serves a somewhat related function in yeast where its self-ubiquitination and association with the Mn^{+2} transporter Smf1p regulates the transporter's levels at the Golgi (Liu and Culotta 1999).

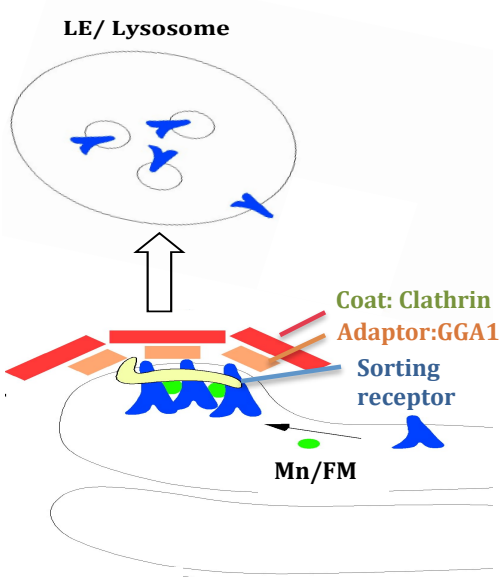

Figure 1.2 Lysosomal trafficking of GPP130 and FM-induced oligomers. Mn-induced trafficking of GPP130 as well as lysosomal trafficking of FM-induced oligomers is GGA1-clathrin dependent. A transmembrane sorting receptor could be mediating this process selectively for oligomeric proteins.

What is the mechanism underlying preferential redistribution of higher order

oligomers to lysosomes as opposed to their non-oligomeric counterparts? There are multiple possibilities, one of them being avidity. Avidity refers to the increased overall affinity of an interaction due to multiple parallel contacts such as an oligomer binding simultaneously to multiple binding sites on a receptor. Another possibility is that oligomers might simply be more efficiently transported to the lysosome compared to native molecules due to each receptor/cargo interaction capturing more cargo when the cargo is oligomerized. Another mechanism that might partially explain lysosomal targeting of integral membrane proteins is a hydrophobic mismatch. A hydrophobic mismatch refers to a disparity between membrane thickness and transmembrane domain length. Tilting of a long transmembrane domain is a strategy to maximize hydrophobic contact in a thinner membrane but this strategy may fail for a higher-order oligomer because it requires significant displacement of the transmembrane domains relative to one another. Thus, a protein that has a relatively long transmembrane domain might move to thicker membrane domains upon oligomerization (Lin and London 2013). However, it is unknown how this mechanism would mediate specific targeting to lysosomes or, more specifically, how GGA1 and clathrin would recognize the thicker membrane domains. Also, hydrophobic mismatch would not explain sorting of membrane associated induced oligomers, such as GPI-anchored proteins.

In summary, protein substrates escape the ER quality control pathway and are targeted from the Golgi for degradation if A) the substrate has a strong ER export signal, B) the ER quality control pathway is saturated, C) the substrate oligomerizes

in the Golgi environment to form large aggregates, or D) the substrate exhibits rapid reversibility between its folded and unfolded states. Additionally, another category might be subtle folding defects, such as those that may arise from a non-debilitating point mutation. The predominant mechanism of lysosomal targeting appears to be ubiquitin mediated. The ubiquitin ligases Rsp5, Bsd2 and Tul1p play an important role in targeting many misfolded and polar TMD containing substrates for lysosomal degradation in yeast. This machinery may or may not work in conjunction with Vps10p/sortilin, which is an important receptor for misfolded proteins that are soluble in the lumen. Despite these insights, how the quality control machinery selectively recognizes its mutated, misfolded and/or unnaturally oligomerized substrates is a significant avenue for future research.

1.2 AUTOPHAGY AS A MEANS OF GOLGI QUALITY CONTROL

Autophagy is a lysosome-dependent protein and membrane degradation system operational in eukaryotic cells (Boya, Reggiori, and Codogno 2013). Usually considered as a non-specific bulk degradative process, it is now being recognized as a highly selective quality control mechanism to turnover substrates that include aggregated/misfolded proteins, damaged/excess membranes and pathogens (Kraft, Peter, and Hofmann 2010; Alexandra Stolz, Andreas Ernst, and Ivan Dikic 2014). This helps mount an appropriate response under stress conditions in order to maintain cellular homeostasis. However selective autophagic clearance implies specific recognition of the cargo to be degraded and examples of this are few and far between, especially for Golgi localized cargo.

One example of selective autophagy of aggregated cargo at the Golgi is the degradation of modified apolipoprotein B (apoB). ApoB is synthesized and secreted primarily by hepatocytes and intestinal cells. Regulation of its secretion is critical since apoB is an essential component of atherogenic lipoproteins and dysregulation of its secretion can trigger atherosclerosis, a major health concern worldwide. As one means of regulation, a significant amount of apoB is degraded via the ER-associated degradation (ERAD) pathway (Fisher et al. 2001). Interestingly, in hepatocytes, autophagy also regulates the amount of apoB that is available for secretion. The pathway is induced by reactive oxygen species (ROS) generated by Ω-3 fatty acids. In the presence of ROS, when apoB exits the ER, it undergoes oxidation dependent aggregation. Upon leaving the Golgi, apoB converts into larger aggregates that fail to be secreted. These aggregates undergo autophagy, which involves PI3K signaling (Fisher et al. 2001; Pan et al. 2008). This pathway, therefore, is a late Golgi quality control process that plays a role in secretory regulation, independent of ERAD. Although this is an instance of selective cargo destruction, how large apoB aggregates are recognized and targeted for degradation remains to be investigated.

Non-specific protein degradation at the Golgi also has also been shown to occur via an alternative autophagic pathway, known as GOMED (Golgi membrane-associated degradation), that is conserved from yeast to mammals (Nishida et al. 2009; Yamaguchi et al. 2016). This pathway operates independently of Atg5/Atg7 but is otherwise similar to conventional autophagy. It predominates in cells lacking the

canonical autophagy process but can also operate in wildtype cells to play a subsidiary function. The membranes of autophagic structures formed via this process are primarily Golgi derived. In mammals, molecular players that orchestrate alternative autophagy include Unc-51 like kinase (Ulk1), Beclin1 and Rab9, while Atg9, Atg5, Atg7, and LC3 are not required (Shimizu, Arakawa, and Nishida 2010). In yeast, factors that disrupt anterograde trafficking such as reduced Golgi PI4P levels, arfaptin 1 silencing or GGA1/GGA2 knockout are sufficient to trigger this process (Yamaguchi et al. 2016). Overall, alternative autophagy is a non-specific but highly regulated quality control mechanism that can function to degrade unused or undelivered proteins that accumulate at the Golgi apparatus.

In general, recognition for engulfment into autophagosomes can be primarily attributed to ubiquitination, regardless of whether the cargo is secretory cargo in vesicles or cytoplasmic proteins or organelles (Fulvio Reggiori et al. 2012). Autophagic adaptors such as p62, NBR1, OPTN and NDP53 link the cargo of interest to the autophagosome by simultaneously binding the autophagosomal membrane protein LC3 on one end and the ubiquitinated cargo on the other end (Kraft, Peter, and Hofmann 2010; Alexandra Stolz, Andreas Ernst, and Ivan Dikic 2014). In the case of ubiquitinated Golgi proteins, however, they can also be recognized and degraded via the ubiquitin-proteasome system (UPS) or the MVB dependent lysosomal pathway. The control of this interplay between multiple quality control pathways at the Golgi remains to be discerned.

1.3 PROTEASOMAL DEGRADATION AS A MEANS OF GOLGI QUALITY CONTROL

Although not common, proteasomal degradation is another route of Golgi protein quality control. An example is apoB-100, which is an isoform of apoB synthesized exclusively in liver cells. The amount of apoB-100 secretion is tightly regulated in liver cells primarily via control over the amount degraded before secretion. ApoB-100 exists in a transmembrane form as well as a soluble form in HepG2 cells (Shelness, Morris-Rogers, and Ingram 1994; Shelness et al. 1999). The transmembrane form exits the ER and is transported to the Golgi where it undergoes ubiquitin-independent proteasomal degradation (Liao et al. 2003; Shelness et al. 1999). The soluble form, however, is subject to ER quality control and undergoes ubiquitin-dependent proteasomal degradation. Why different quality control mechanisms are at work to regulate these two versions of apoB-100 is unclear. Proteasomal degradation at the Golgi also operates in yeast. Yeast strains deficient in key ERAD components are still competent to efficiently degrade the ERAD substrate CPY*, a misfolded form of CPY. The degradation is mediated via an ubiquitin-dependent proteasome pathway but it requires CPY* trafficking to the Golgi (Haynes, Caldwell, and Cooper 2002). This pathway requires a functional Unfolded Protein Response (UPR) possibly to handle increased levels of misfolded ERAD substrates, while the ERAD pathway itself is sufficient under lower substrate loads. Interestingly, the pathway involves the cytoplasmic ubiquitin ligase Rsp5p but how CPY*, which is lumenal, gains access to Rsp5p remains to be determined. In summary, proteasomal degradation of proteins that have reached the Golgi takes

place via both ubiquitin-dependent and independent mechanisms, but how they are specifically recognized remains unknown.

1.4 CONCLUSION

In summary, we have little understanding of how Golgi quality control operates on a mechanistic level. This leaves many important questions to be addressed. How are the substrates selectively recognized? How are they sorted into appropriate TGN-exit domains? What regulatory controls exist governing the process? The work present in this thesis focuses on addressing these questions in the context of higher-order oligomeric protein substrates. Mn-induced oligomerization of the Golgi protein GPP130 provides us with a unique, controlled and well studied system to understand how higher-order oligomers can be recognized and sorted at the TGN. GPP130 is the endosome-to-Golgi host cell trafficking receptor for Shiga toxin. Therefore, this study is relevant not only to Golgi quality control but also towards understanding how a GPP130-directed therapy might protect cells against Shiga toxin induced death. GPP130 binds Mn at the Golgi and undergoes oligomerization-dependent lysosomal sorting. As mentioned, this sorting is GGA1 and clathrin dependent. But, how are GPP130 oligomers selectively recognized and partitioned into membrane domains coated with GGA1 and clathrin? Is there a membrane sorting receptor that binds GPP130 to alter its sorting? If there is a GPP130 receptor, does it also sort other Mn-sensitive proteins at the Golgi? Indeed, might it be even more general and also mediate sorting of the aforementioned FM-induced Golgi oligomers? These questions are experimentally addressed in Chapter 2.

Briefly, the results reveal that the sorting receptor sortilin binds GPP130 and is required for its Mn-induced trafficking as well as that of another Mn-sensitive Golgi protein. However, sortilin is not required for FM-induced lysosomal targeting. Thus, Golgi quality control can be mediated by both sortilin-dependent and sortilin-independent mechanisms to target aggregated Golgi proteins for lysosomal degradation.

CHAPTER 2

MANGANESE-INDUCED TRAFFICKING AND TURNOVER OF GPP130 IS MEDIATED BY SORTILIN

2.1 ABSTRACT

Elevated, non-toxic doses of manganese (Mn) protect against Shiga toxin-1 induced cell death via down regulation of GPP130, a cycling Golgi membrane protein that serves as an endosome-to-Golgi trafficking receptor for the toxin. Mn binds to GPP130 in the Golgi, causes GPP130 to oligomerize/aggregate and the complexes are diverted to lysosomes. In fact, based on experiments using the self-interacting FM domain, it appears generally true that aggregation of a Golgi protein leads to its lysosomal degradation. How such oligomers are selectively sorted out of the Golgi is unknown. Here we provide evidence that Mn-induced exit of GPP130 from the trans Golgi network (TGN) towards lysosomes is mediated by the sorting receptor sortilin interacting with the lumenal stem domain of GPP130. In contrast, FM-induced lysosomal trafficking of the Golgi protein galactosyltransferase was sortilin independent and occurred even in the absence of its native lumenal domain. Thus, sortilin-dependent as well as sortilin-independent sorting mechanisms target aggregated Golgi membrane proteins for lysosomal degradation.

2.2 INTRODUCTION

Over a million deaths occur annually due to Shiga toxin (STx)–producing bacteria, which include *Shigella dysenteriae* and the Shiga toxigenic group of *Escherichia coli* (Beddoe *et al.*, 2010). Shiga toxins invade cells by binding to a cell surface glycolipid followed by endocytosis to early endosomes (Mallard and Johannes, 2003). There, they evade lysosomal degradation by actively sorting into membrane tubules destined for the Golgi complex, a key step in their retrograde trafficking to exert cytotoxicity (Johannes and Wunder, 2011; Mukhopadhyay and Linstedt, 2013). Significantly, for two toxin types, STx and STx1, this sorting is blocked in cells that have had prior exposure to elevated extracellular manganese (Mn). Indeed, non-toxic doses of Mn protect cultured cells and mice from STx1-induced death (Mukhopadhyay and Linstedt, 2012).

Mn-sensitivity of STx/STx1 arises because Mn downregulates GPP130, which serves as a host-cell trafficking receptor for these toxins (Mukhopadhyay and Linstedt, 2012). GPP130 is a cis-Golgi localized integral membrane protein that constitutively cycles between early endosomes and the Golgi (Linstedt *et al.*, 1997). A surface loop present in the STx/STx1 pentameric B subunit binds at or near a seven-residue stretch in the coiled-coil lumenal stem domain of GPP130 allowing the toxin to "piggyback" its way to the Golgi (Mukhopadhyay *et al.*, 2013). In the absence of GPP130, the toxins are degraded because instead of gaining access to Golgi-directed membrane tubules, they remain in the central cavity of endosomes, which then

mature into multivesicular bodies (MVBs) and lysosomes (Mukhopadhyay and Linstedt, 2012).

GPP130 downregulation by Mn may relate to homeostatic control of cellular Mn. While Mn is essential, high cytoplasmic levels are toxic (Missiaen *et al.*, 2004; Olanow, 2004; Bouchard *et al.*, 2007). The Mn transporter SPCA1 is Golgi-localized and required for GPP130 downregulation (Mukhopadhyay and Linstedt, 2011). Loss of SPCA1 sensitizes cells to Mn exposure and a hyperactive mutation of SPCA1 protects against toxic Mn levels (Culotta *et al.*, 2005; Leitch *et al.*, 2010; Mukhopadhyay and Linstedt, 2011). It is thought that active transport of Mn by SPCA1 reduces cytoplasmic Mn concentrations allowing Mn to be expelled by exocytosis.

The mechanism of Mn-induced GPP130 downregulation is beginning to emerge. Upon Mn treatment, GPP130 leaves its Golgi-endosome recycling route and instead is diverted from the TGN to MVBs and then to lysosomes, where it is degraded (Mukhopadhyay *et al.*, 2010). Redistribution is via the canonical TGN-to-lysosome pathway as it requires the clathrin vesicle coat complex and its γ-adaptin homologous ARF-interacting protein 1 (GGA1) adaptor (Tewari *et al.*, 2015). The trafficking depends on the late endosome GTPase Rab7 but not its early endosome counterpart Rab5 (Mukhopadhyay *et al.*, 2010). The trigger appears to be Mn-induced clustering of GPP130 dimers into higher order oligomers. Mn directly binds the GPP130 lumenal stem domain and the binding causes reversible formation of

higher-order oligomers (Tewari *et al.*, 2014). Clustering occurs in the Golgi and, critically, Mn-induced oligomerization is both required and sufficient for GPP130 redistribution to lysosomes (Tewari *et al.*, 2014).

Rather than being unique to GPP130, oligomerization may be a general trigger of Golgi protein exit and lysosomal degradation, possibly used for Golgi quality control (Tewari *et al.*, 2015). That the region of GPP130 that binds Mn and oligomerizes contains a transferable activity suggests that other Golgi proteins contain the potential for this trafficking behavior if forced to form higher order oligomers (Tewari *et al.*, 2014). Indeed, the placement of an array of self-interacting FM domains on the lumenal domain of a number of Golgi proteins causes their oligomerization-induced degradation in lysosomes (Tewari *et al.*, 2015). Similar to Mn-induced GPP130 redistribution, FM-induced Golgi protein redistribution is clathrin and GGA1 dependent (Tewari *et al.*, 2015). Also, another Mn-sensitive Golgi protein was recently identified. Although the role of oligomerization was not tested, TMEM165 moves to lysosomes in Mn-treated cells in a fashion analogous to GPP130 (Potelle *et al.*, 2017). Additionally, the TGN-localized protease furin forms aggregates correlating with its exit from the Golgi and degradation in lysosomes (Wolins *et al.*, 1997).

This apparent generality raises a significant question. If formation of higher-order oligomers is sufficient to alter trafficking of unrelated Golgi proteins, how are they selectively recognized and partitioned into membrane domains coated with GGA1

and clathrin? GPP130 redistribution occurs in the absence of its cytoplasmic domain suggesting participation of a membrane spanning sorting receptor. Here, we tested the role of sortilin because it is a known TGN-to-lysosome sorting receptor with a lumenal domain capable of binding diverse cargo and a cytoplasmic domain that engages GGA1 and clathrin (Lefrancois *et al.*, 2003; Canuel *et al.*, 2008; Canuel *et al.*, 2009; Campbell *et al.*, 2016). We provide evidence that sortilin binds GPP130 and is required for its Mn-induced trafficking. Mn-induced redistribution of TMEM165 was also sortilin dependent. Intriguingly, however, sortilin was not required for FM-induced lysosomal targeting and this occurred even when lumenal sequences were deleted. Thus, there are both sortilin-dependent and sortilin-independent mechanisms to target aggregated Golgi proteins for lysosomal degradation.

2.3 RESULTS

Sortilin is required for Mn induced redistribution of GPP130 to lysosomes

We focused on sortilin because it mediates TGN sorting towards lysosomes for a diverse set of cargo using the clathrin coat complex and the GGA1 adaptor. HeLa cells were transfected with 20 nM sortilin siRNA and assessed for knockdown 48 h post transfection by immunoblotting (Figure 2.1A). Sortilin levels dropped to ~20% of the controls at 48 h post transfection. This time point was used for subsequent experiments.

By immunofluorescence microscopy, the steady-state localization of GPP130 and the Golgi marker giantin were unaffected by the knockdown treatment (Figure 2.1B,

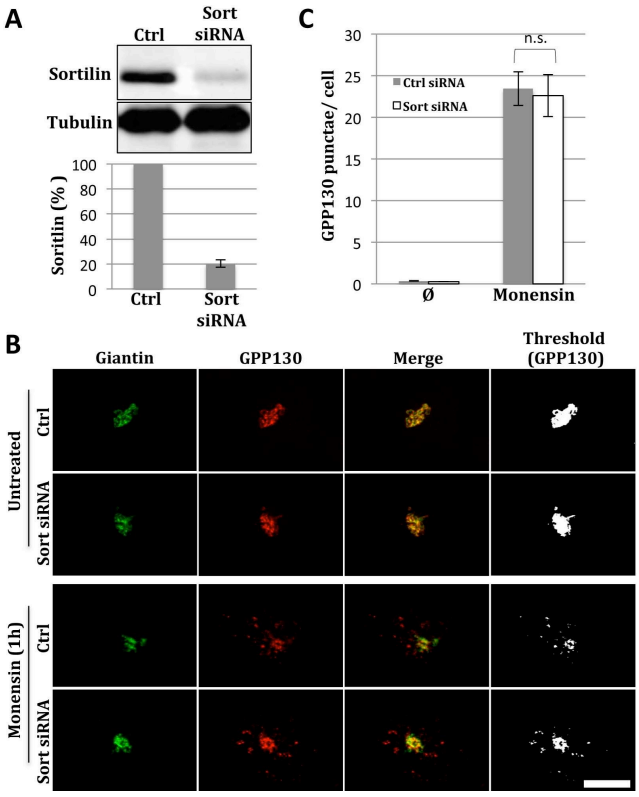

Figure 2.1 Normal GPP130 Golgi localization and redistribution to endosomes in sortilin knockdown cells. (A) To assess knockdown, HeLa cells were untreated or sortilin siRNA-transfected and lysed after 48 h followed by immunoblotting with anti-sortilin and anti-tubulin antibodies. Quantification shows average values normalized to control cells (±SEM, n=3). (B) Control and sortilin siRNA transfected cells were left untreated (upper rows) or treated with 10μM monensin for 1h (lower rows) and stained with anti-giantin (green) or anti-GPP130 (red) antibodies. Merged and thresholded (GPP130 only) images are shown to better visualize the extent of redistribution of GPP130 into endosomal punctae. Bar=5μm. (C) Quantified appearance of GPP130 in peripheral punctae of cells that were either control or sortilin siRNA-transfected before or after monensin (Mon) treatment (mean±SEM, n=3, >15 cells per experiment).

untreated). To ensure that sortilin knockdown did not disrupt the Golgi exit of GPP130 along its normal cycling path to endosomes, we treated the cells with monensin, which is a proton ionophore that neutralizes acidic compartments (Tartakoff, 1983). Monensin has no effect on TGN exit of GPP130 but it blocks its retrieval thereby trapping the protein in early endosomes (Bachert *et al.*, 2001; Puri *et al.*, 2002). In our experiment, a 1 h monensin treatment trapped a fraction of GPP130 in punctae and this was equally evident for the sortilin knockdown cells

(Figure 2.1B, monensin). As expected, giantin in both control and knockdown cells remained Golgi localized. Thresholded images of the GPP130 channel are also shown to better visualize the punctae. Quantification of the GPP130 appearance in punctae for independent trials confirmed the lack of effect of sortilin knockdown (Figure 2.1C). Thus, TGN exit of GPP130 under the conditions of the monensin treatment was unaffected by sortilin knockdown.

Next, control and sortilin knockdown cells were subjected to Mn treatment. As expected, after 1 or 2 h exposures to 500 μM $MnCl_2$, control cells showed a fraction of GPP130 redistributed to punctate structures (Figure 2.2A, Ctrl) previously identified as pre-lysosomes or lysosomes based on colocalization with Rab7-GFP among other measures (Mukhopadhyay *et al.*, 2010; Tewari *et al.*, 2015). Colocalization with Rab7-GFP was also confirmed for the present experiments (Figure 2.3). In striking contrast, sortilin knockdown cells showed little if any GPP130 redistribution (Figure 2.2A, siSort). Note that giantin staining was unperturbed under these conditions and that the GPP130 channel is again shown after thresholding to better detect faint punctae. Immunoblotting of independent trials confirmed knockdown while quantification of the peripheral punctae clearly supported a strong inhibitory effect of sortilin knockdown (Figure 2.2B).

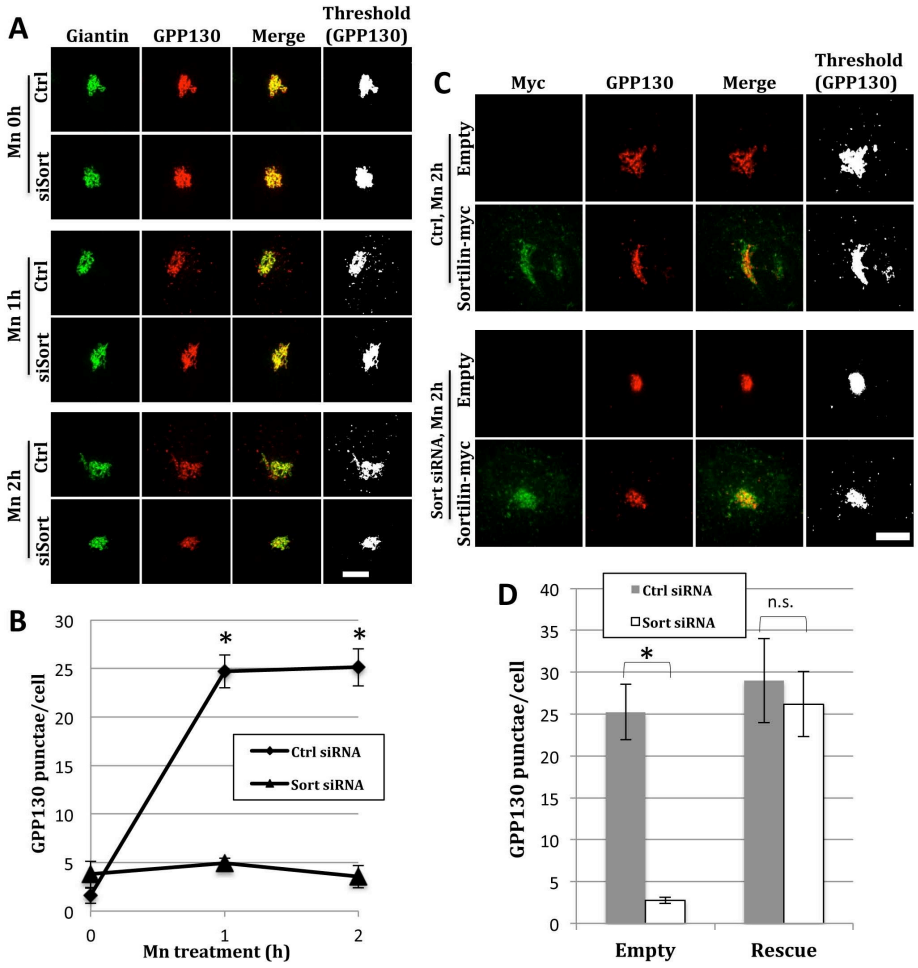

Figure 2.2 Knockdown and rescue of sortilin dependent, Mn induced GPP130 redistribution to lysosomes (A) Control and sortilin knockdown cells were subjected to a 0, 1 or 2 h Mn treatment (500μM) and fixed and stained using anti-GPP130 (red) and anti-giantin (green) antibodies. Merged and thresholded (GPP130 only) images are also shown for visualization of GPP130 punctae. Bar=5μm. (B) Quantification shows the number of GPP130 punctae at each time point of Mn treatment (n=3±SEM, >15 cells per experiment, *p<0.0006). (C) To assess rescue, control and sortilin knockdown cells were transfected with a siRNA immune version of the sortilin-myc plasmid or an empty vector. The cells were treated 24 h post plasmid transfection with 2 h of Mn treatment and then were stained using anti-GPP130 (red) and anti-myc (green) antibodies. Bar=5μm. (D) The average number of punctae after the 2 h Mn treatment is quantified for control and knockdown cells (±SEM, n=3, >10 cells per experiment, *p<0.002).

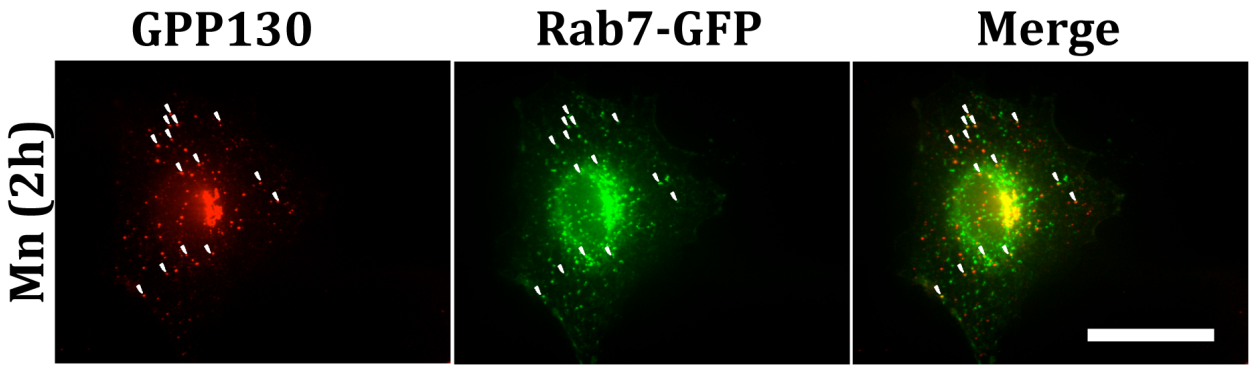

Figure 2.3 Colocalization of GPP130 punctae with Rab7-GFP. Cells were transiently transfected to express Rab7-GFP and after 48 h they were subjected to a 2h Mn exposure followed by staining with anti-GPP130 antibodies (red) and visualization of GFP fluorescence (green). Arrowheads indicate colocalization in punctae. Bar=5μm.

Rescue with a siRNA immune version of sortilin was used to control against off-target effects. Expression of the myc epitope tagged rescue construct, sortilin-myc, in control cells had no effect on Mn-induced GPP130 redistribution because the redistribution occurred equally in cells transfected with either an empty vector or the same vector containing sortilin-myc (Figure 2.2C, Ctrl). In contrast, the sortilin-myc construct rescued in that sortilin knockdown blocked redistribution in cells transfected with the empty vector, but the redistribution was restored in cells expressing sortilin-myc (Figure 2.2C, Sort siRNA). Rescue (≥10 punctae/cell) was observed in 93±7% of cells expressing sortilin-myc (identified by anti-myc staining of the Golgi) compared to a background of 6±3% for all of the control transfected cells. Quantification of GPP130 punctae for independent trials also confirmed the rescue (Figure 2.2D) showing that the block of the Mn-induced GPP130 redistribution was a specific effect of sortilin absence.

Previous studies have shown that it takes 4 to 8 h of Mn treatment to cause complete redistribution of GPP130 from the Golgi and its loss of staining due to degradation in lysosomes (Mukhopadhyay *et al.*, 2010). Therefore, we extended the Mn treatment in control and sortilin knockdown cells. As expected, control cells showed a significant loss at 4 h and a nearly complete loss at 8 h (Figure 2.4A, Ctrl). In contrast, the knockdown cells showed a markedly attenuated response (Figure 2.4A, Sort siRNA). Quantification revealed that the 25% remaining at 4 h was increased to 75% and that the 5% remaining at 8 h was increased to 45% (Figure 2.4B). Giantin levels remained stable. That this was a reduction and not a block is typical for membrane trafficking in which cargo gain access to alternate routes or may simply be due to incomplete knockdown. Thus, overall, Mn-induced redistribution and degradation of GPP130 in lysosomes is strongly sortilin dependent. Interestingly, whereas Mn treatment resulted in progressive loss of GPP130 over time, sortilin remained stable (Figure 2.5A-B). Thus, if sortilin binds and sorts GPP130 during TGN exit, it is likely that the complex subsequently dissociates so that sortilin can recycle.

Sortilin binds GPP130

To test whether sortilin interacts with GPP130 we used co-immunoprecipitation from cell lysates generated using RIPA lysis buffer, which is non-denaturing but stringent (Seddon *et al.*, 2004). HeLa cells were transfected with GPP130-GFP and the sortilin-myc plasmid. Both transfected constructs were primarily Golgi localized. Immunoprecipitation with anti-myc antibodies yielded a clear recovery of GPP130-

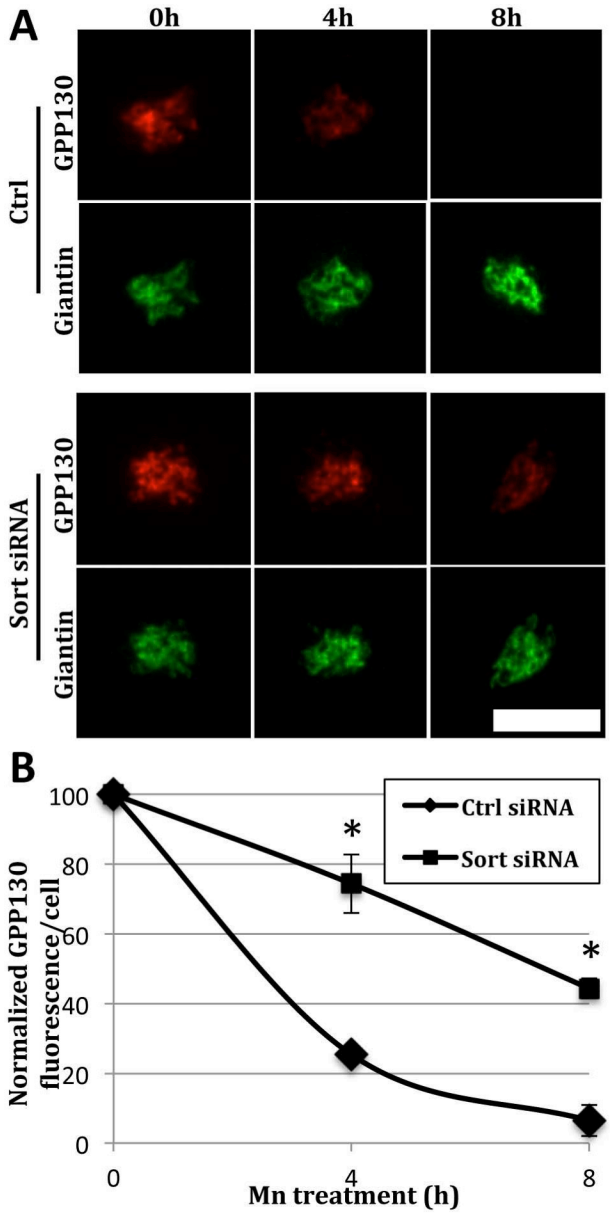

Figure 2.4 Sortilin knockdown attenuates Mn-induced GPP130 degradation. (A) Control and sortilin knockdown cells were subjected to Mn treatment (500μM) for 0, 4 and 8 h and then stained for GPP130 (red) and giantin (green). Note attenuated decrease of Golgi GPP130 after sortilin knockdown. Bar=5μm. (B) Average fluorescence of Golgi localized GPP130 was quantified at each time point of Mn treatment (±SEM, n=3, >15 cells per experiment, *p<0.004).

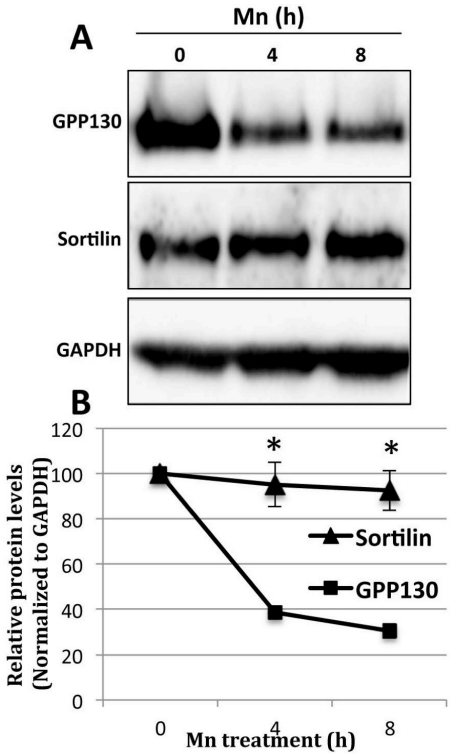

Figure 2.5 Sortilin levels remain stable in Mn treated cells. (A) Cells were subjected to Mn treatment (500μM) for 0, 4 and 8 h and then immunoblotted to detect sortilin, GPP130 and the loading control GAPDH. (B) Average levels of sortilin and GPP130 were determined after normalization to GAPDH (mean±SEM, n=3, *p<0.004).

GFP from both untreated and Mn treated cells (Figure 2.6A). In contrast, there was no recovery of GPP130-GFP from cells transfected with the control empty myc vector or in cells not expressing GPP130-GFP. As a further evidence of specificity, co-transfection of sortilin with the GPP130-related Golgi protein GP73 (Kladney *et al.*, 2000) yielded little GP73-GFP recovery with sortilin-myc under the identical conditions (Figure 2.6C, lane 1). There was also little or no interaction detected for endogenous GPP130 presumably because of its low level relative to transfected GPP130-GFP. The interaction is likely direct because, for it to be indirect, the endogenous level of the hypothetical linking component would have to be

unexpectedly high relative to endogenous GPP130. It was interesting that sortilin bound GPP130 in untreated cells and that there was no enhancement for Mn treated cells. This was also the case when 1mM Mn was added to the lysis buffer in an attempt to preserve oligomerized GPP130 complexes. We interpreted this to mean that GPP130 can form complexes with sortilin even when not oligomerized by Mn but how Mn enhances sortilin-dependent trafficking remains unclear (see Discussion).

To validate the interaction we attempted the reciprocal co-immunoprecipitation. Lysates from cells that were not Mn treated were subjected to immunoprecipitation using anti-GPP130 antibodies and anti-myc immunoblotting was used to test for association of sortilin-myc. As shown, there was clear recovery of sortilin-myc only if both GFP-GPP130 and sortilin-myc were present (Figure 2.6B). Together, the anti-myc and anti-GFP co-precipitation experiments provide strong evidence that the sorting receptor sortilin and its putative cargo GPP130 form reasonably stable complexes when co-expressed, even in the absence of Mn treatment.

Next, we attempted to identify a sequence stretch responsible for the interaction. Our focus was on the GPP130 lumenal stem domain because it mediates Mn-induced trafficking of GPP130 (Mukhopadhyay *et al.*, 2010). To investigate whether the lumenal stem domain of GPP130 (residues 36-247) contains a sortilin binding site, we used a previously generated construct in which the lumenal domain is appended to GP73 (Bachert *et al.*, 2007; Mukhopadhyay *et al.*, 2010). GP73 itself does not

undergo Mn-induced redistribution to lysosomes but this behavior is conferred by the addition of the GPP130 stem domain (Mukhopadhyay *et al.*, 2010). Significantly, while GP73 itself yielded only weak recovery, co-expression of GP73-GPP130$_{36\text{-}247}$-GFP with sortilin-myc yielded a strong interaction as indicated by immunoprecipitation with anti-myc antibodies (Figure 2.6C). To refine the

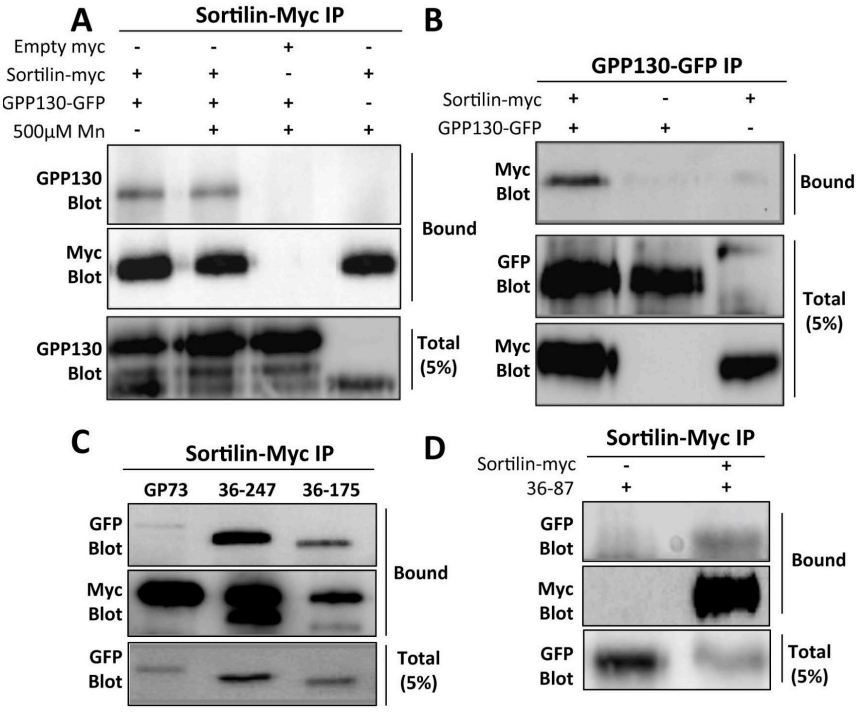

Figure 2.6 GPP130 co-precipitates with sortilin. (A) HeLa cells were transfected with GPP130-GFP, sortilin-myc, or an empty myc plasmid as indicated and then left untreated or Mn-treated (2h) before lysis and immunoprecipitation using anti-myc antibodies. The immunoblot shown was obtained using anti-GPP130 and anti-myc antibodies and is representative of 3 trials. Where shown, the 5% total is directly comparable to its corresponding bound fraction, which analyzed in its entirety. The exposures were identical. (B) The reciprocal experiment was identical except that anti-GPP130 was used for the co-immunoprecipitation (n=3). (C) Cells were transfected with sortilin-myc and GP73, GP73-GPP130$_{36\text{-}247}$, or GP73-GPP130$_{36\text{-}175}$ and immunoprecipitation was carried out using anti-myc followed by immunoblotting with anti-GFP or anti-myc (n≥2). (D) Cells were transfected with sortilin-myc or GP73-GPP130$_{36\text{-}87}$ as indicated immunoprecipitation was with anti-myc

interacting domain, we carried out the same experiment for two additional chimeric constructs, GP73-GPP130$_{36\text{-}175}$-GFP and GP73-GPP130$_{36\text{-}87}$. Each construct was

recovered in the presence of sortilin-myc indicating that the sortilin-binding site in GPP130 likely resides in residues 36-87 (Figure 2.6C-D). Interestingly, while GP73-GPP130$_{36-175}$-GFP retains sensitivity to Mn, GP73-GPP130$_{36-87}$ is not responsive (Mukhopadhyay *et al.*, 2010) presumably because this construct lacks the residues 88-91 implicated in Mn binding (Tewari *et al.*, 2014). That the sortilin-binding site in GPP130 remains binding competent when separated from the putative Mn-binding site supports the conclusion that sortilin binds GPP130 even in the absence of Mn-induced oligomerization of GPP130.

Mn-induced redistribution of TMEM165 is sortilin dependent

TMEM165 is a conserved, multi-pass H^+/Ca^{2+} exchanger in the Golgi (Demaegd *et al.*, 2013; Colinet *et al.*, 2016; Potelle *et al.*, 2016). Similar to GPP130, elevated Mn causes TMEM165 to redistribute to lysosomes where it is degraded (Potelle *et al.*, 2017). Therefore, we asked whether TMEM165 redistribution also depends on sortilin. TMEM165 was Golgi localized in untreated cells and then partially redistributed to peripheral punctae after a 2h Mn exposure (Figure 2.7A, Ctrl). The kinetics and pattern of the redistribution were similar to GPP130. Whereas sortilin knockdown itself had no effect on TMEM165 targeting to the Golgi, it profoundly blocked TMEM165 appearance in punctae after Mn treatment (Figure 2.7A, siSort). The dependence of TMEM165 redistribution on sortilin was confirmed by quantification of both TMEM165 appearance in punctae (Figure 2.7B) and TMEM165 loss from the Golgi (Figure 2.7C).

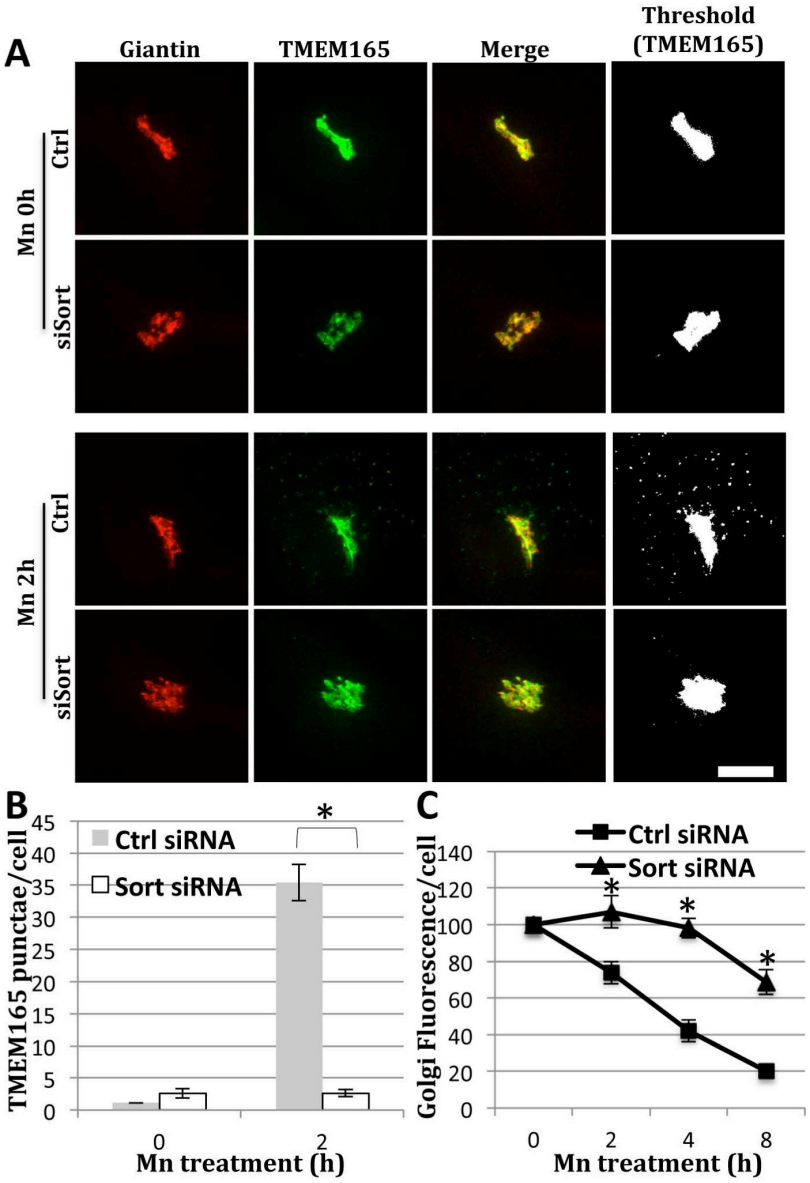

Figure 2.7 TMEM165 redistribution depends on sortilin. (A) Control and sortilin knockdown cells were subjected to a 0 or 2 h Mn treatment (500μM) and fixed and stained using anti-TMEM165 (green) and anti-giantin (red) antibodies. Merged and thresholded (TMEM165 only) images are also shown. Bar=5μm. (B) Quantification shows the average number of TMEM165 punctae at each time point (±SEM, n=3, >15 cells per experiment, *p<0.0003). (C) Control and sortilin knockdown cells were subjected to Mn treatment (500μM) for 0, 2, 4 and 8 h and then stained for TMEM165 and giantin. Average fluorescence of Golgi localized TMEM165 was quantified (±SEM, n=3, >15 cells per experiment, *p<0.003).

Sortilin is not required for redistribution of an FM-induced oligomer

Previous work showed that induced oligomerization of several different Golgi resident proteins causes their lysosomal degradation in a manner similar to Mn-

induced GPP130 degradation (Tewari *et al.*, 2015). These results were obtained by appending an array of three copies of a modified version of the FKBP12 domain, termed an FM domain, to the lumenal domain of the type 2 Golgi membrane proteins (Rizzo *et al.*, 2013). The FM domain self-interacts but this can be prevented by incubating cells with AP12998 (AP), a modified version of FK506 that is a competitive inhibitor of the interaction (Rivera *et al.*, 2000). Thus AP washout leads to oligomerization and then redistribution from the Golgi to lysosomes. We used an FM-tagged construct based on the Golgi enzyme beta 1, 4 galactosyltransferase (GT), to test if its redistribution depended on sortilin (Tewari *et al.*, 2015). Control and sortilin knockdown cells were transfected with GT-FM-GFP and cultured in the presence of AP. The construct was Golgi localized and the sortilin knockdown was confirmed by immunoblot. After 24 h, the AP was removed and redistribution of the construct was assayed over time. Redistribution was clearly evident at the 1 and 2 h time points in the control cells and equally evident in the sortilin knockdown cells (Figure 2.8A). As expected the Golgi marker giantin remained Golgi localized. Thresholded images of the GT-FM-GFP channel substantiated its presence in peripheral punctae. Quantification of such images for independent trials further validated the lack of effect of sortilin knockdown on redistribution of GT-FM-GFP (Figure 2.8B). We also tested a version of GPP130 containing FM domains that was previously shown to redistribute upon AP washout (Tewari *et al.*, 2014). In both

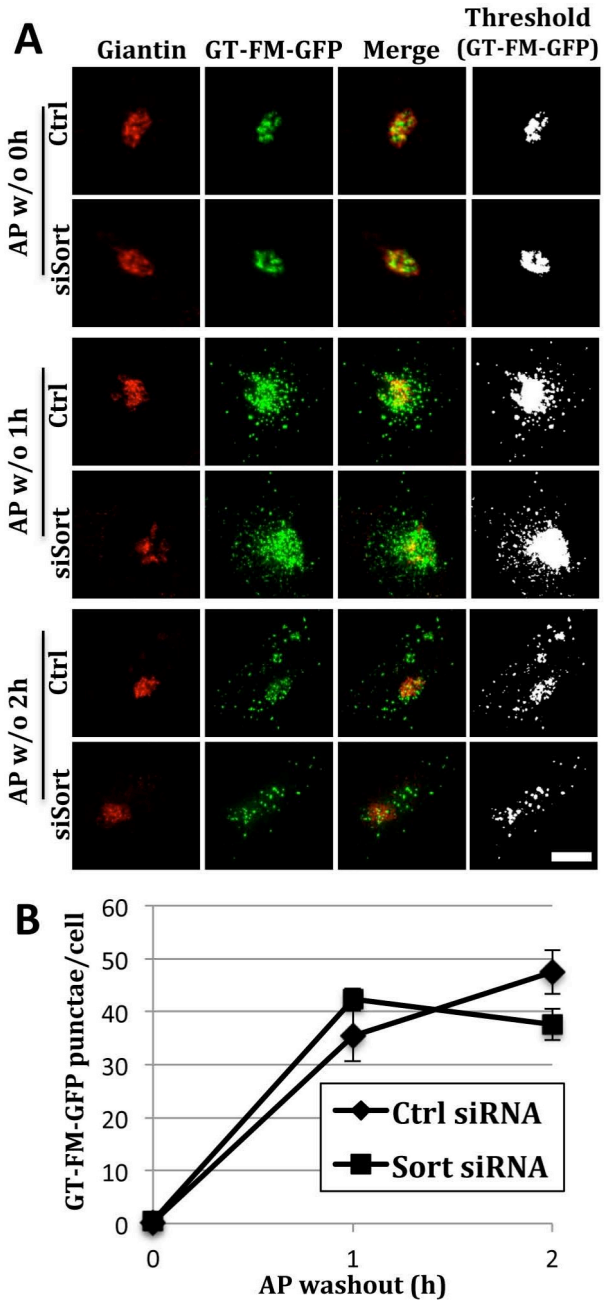

Figure 2.8 FM-induced oligomers redistribute to lysosomes independent of sortilin. (A) Control and sortilin knockdown cells were transfected with GT-FM-GFP and, after 24 h, were subjected to AP washout for 0, 1 or 2 h before staining with anti-giantin antibodies (red) and visualization of GFP fluorescence (green). Merged and thresholded (GFP only) images are also shown. Bar=5μm. (B) Quantification shows the number of GT-FM-GFP punctae at each time point (mean±SEM, n=3, >12 cells per experiment, p≥0.1).

control and sortilin-knockdown cells, the construct robustly redistributed to punctae after AP washout (Figure 2.9) showing that a GPP130 construct can

redistribute independent of sortilin if oligomerized by FM domains. Unfortunately, insolubility of the FM constructs in lysis buffers that preserve the GPP130/sortilin interaction prevented us from testing their ability to bind sortilin. Nevertheless, these findings suggest the presence of a predominant, sortilin-independent mechanism for degradation of Golgi proteins after forced oligomerization by appended FM domains.

Given the sortilin independence and the lack of any obvious sequence similarity among the Golgi proteins that undergo FM-induced degradation (Tewari *et al.*, 2015) we wondered whether the mechanism might be independent of any sequence requirement, at least for the lumenal domains of the Golgi proteins. As a test, we removed all remaining GT lumenal sequence from GT-FM-GFP to yield $GT_{\Delta lum}$-FM-GFP. With its cytoplasmic and transmembrane domain left intact to mediate its Golgi targeting, and in the continued presence of AP to prevent its oligomerization, the construct was Golgi localized (Figure 2.10A, 0h). However, upon AP washout, it redistributed to lysosomes in a manner indistinguishable from GT-FM-GFP (Figure 2.10A, 1-2h). The result was confirmed by quantifying the punctae present for independent trials (Figure 2.10B). Thus, while Mn-induced GPP130 oligomerization and FM-induced Golgi protein oligomerization are similar in certain respects, the former requires a lumenal sequence stretch and binding to sortilin while the latter requires neither.

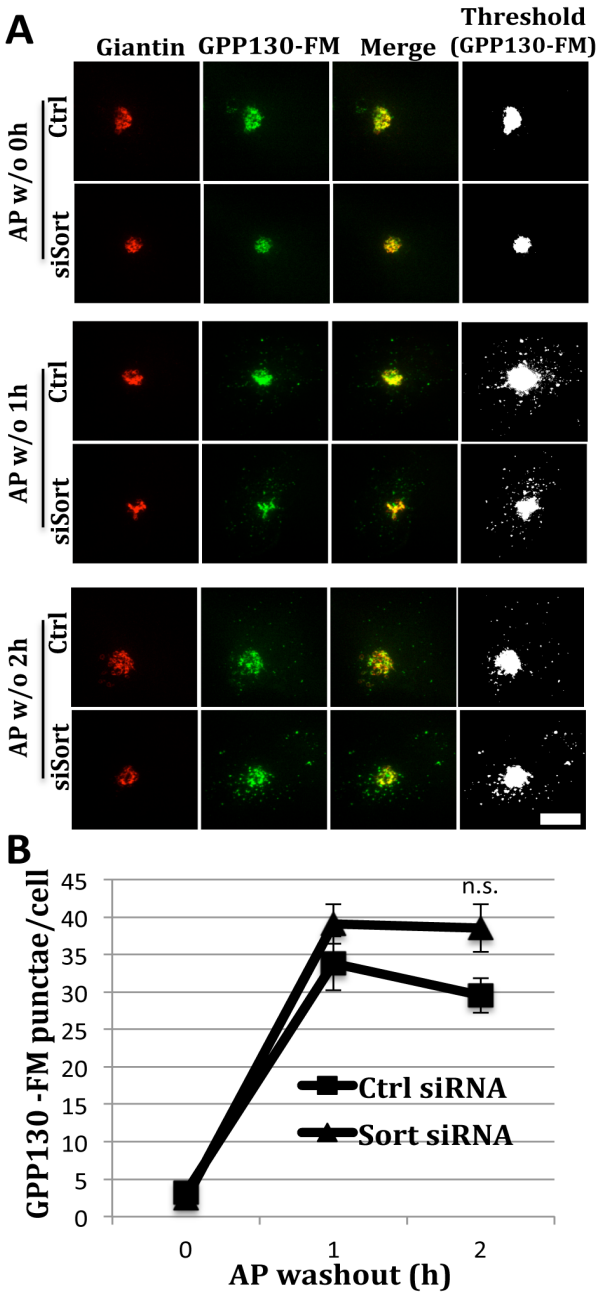

Figure 2.9 GPP130-FM redistributes to lysosomes independent of sortilin. (A) Control and sortilin knockdown cells were transfected with GPP130-FM-GFP and, after 24 h, were subjected to AP washout for 0, 1 or 2 h before staining with anti-giantin antibodies (red) and visualization of GFP fluorescence (green). Merged and thresholded (GFP only) images are also shown. Bar=5μm. (B) Quantification shows the number of GPP130-FM-GFP punctae at each time point (mean±SEM, n=3, >10 cells per experiment, n.s.=p>0.8).

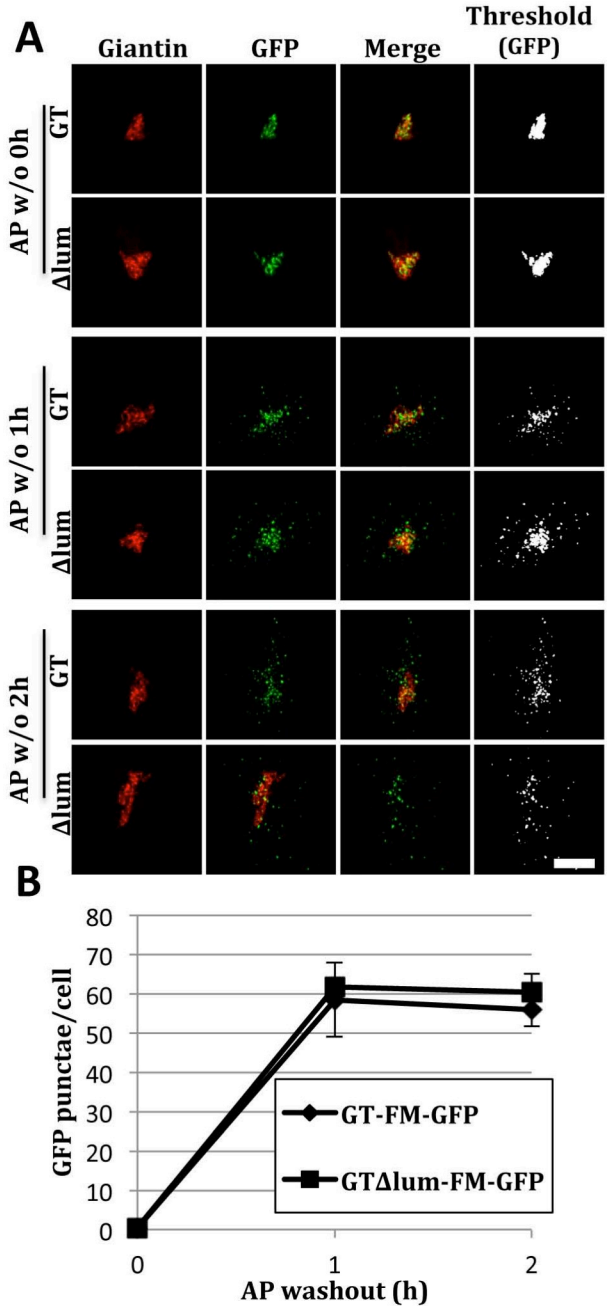

Figure 2.10 Lysosomal redistribution of $GT_{\Delta lum}$-FM-GFP. (A) Cells were transfected with either GT-FM-GFP or GTΔlum-FM-GFP (all GT lumenal sequence removed) and subjected to an AP washout for 0, 1 or 2 h prior to staining for giantin and visualization of GFP. Merged and thresholded (GFP only) images are also shown. (B) Quantification shows the number of GFP punctae for the constructs at each time point (mean±SEM, n=3, >12 cells per experiment, p≥0.3).

2.4 DISCUSSION

This study identifies the trafficking receptor sortilin as a key mediator of Mn-induced lysosomal transport and turnover of GPP130. Sortilin knockdown selectively blocked Mn-induced GPP130 trafficking and this was confirmed by rescue. Sortilin bound GPP130 and the interaction involved a segment of the GPP130 lumenal domain that is required for GPP130 sensitivity to Mn. Because sortilin bound GPP130 even in untreated cells we speculate that the interaction is normally occurring to mediate turnover of GPP130 and that Mn enhances sorting into this pathway. Interestingly, whereas lysosomal redistribution of TMEM165 was also sortilin dependent, the redistribution FM-induced oligomers of either GPP130 or GT did not depend on sortilin and even occurred without any of the GT lumenal sequence.

At the outset we entertained several ideas for how Mn-induced oligomerization might switch GPP130 sorting away from its normal retrieval and early endosome pathways and into the lysosomal pathway. One was hydrophobic mismatch, referring to a disparity between membrane thickness and transmembrane domain length (Destainville *et al.*, 2016). Tilting of a long transmembrane is a strategy to maximize hydrophobic contact in a thinner membrane but this strategy may fail for a higher-order oligomer because it requires significant displacement of the transmembrane domains relative to one another. Thus, to minimize hydrophobic mismatch of its transmembrane domain, GPP130 might segregate into thicker membrane domains upon oligomerization. The GPP130 transmembrane domain

comprises 22 residues, which is relatively long for a Golgi protein. Another idea was avidity in which the overall affinity of an interaction is increased due to multiple parallel contacts. To achieve avidity-based sorting, a GPP130 oligomer could conceivably engage multiple sites on a putative multivalent receptor. The involvement of sortilin favors the avidity model, especially because sortilin has a ten-bladed beta-propeller lumenal domain suggestive of multivalency (Quistgaard *et al.*, 2009). However, this model predicts increased recovery of GPP130 with sortilin in Mn treated cells, which was not observed (Figure 2.6A).

Currently, we favor the idea that the switch from normal cycling to degradation is the result of accelerated access to a normal, sortilin-mediated, turnover pathway. This might be attributed to two effects of oligomerization: steric exclusion from the other pathways and greater efficiency of the sortilin pathway due to the increased GPP130 in each sorting complex. Exclusion of large proteins or protein complexes from small vesicles, such as Golgi retrieval vesicles, has been observed (Rivera *et al.*, 2000; Rizzo *et al.*, 2013). In a similar manner, oligomerized GPP130 may be excluded from the Golgi retrieval and endosomal recycling pathways. Clearly, it is not excluded from the lysosomal pathway implying that this clathrin/GGA1-mediated pathway has the flexibility to accommodate large complexes. Anterograde transport of large cargo, including collagen, is well documented and takes place using alternative mechanisms and/or unexpected geometric flexibility in vesicle coat complexes (Malhotra and Erlmann, 2015; Saito and Katada, 2015; Gorur *et al.*, 2017). At the same time, if access of GPP130 to the canonical lysosomal pathway is

governed by binding to sortilin and sortilin is limiting (as might well be expected) then oligomers will be more efficiently sorted than dimers. That is, each sortilin-GPP130 interaction would capture many more GPP130 molecules when GPP130 is oligomerized. The size range of the GPP130 oligomers is not known so the stoichiometry cannot be estimated. Also, these stoichiometrically unbalanced sortilin-GPP130 complexes likely disassemble upon cell lysis leaving each sortilin bound only to the GPP130 dimer it directly contacted because the Mn-dependent contacts between GPP130 dimers are rapidly reversible upon chelation or dilution of Mn (Tewari *et al.*, 2014). This would explain the equal recovery of GPP130 by sortilin immunoprecipitation from untreated and Mn-treated cells (Figure 2.6A), although it is confounding that such dissociation, if present, was not counteracted by supplementing the lysis buffer with Mn. In any case, the binding of GPP130 to sortilin in untreated cells can be explained as mediating normal turnover of GPP130, which exhibits a half-life of roughly 18 h (Linstedt *et al.*, 1997). While constitutive, it would normally account for only a small fraction of GPP130 due to competition from the retrieval and recycling pathways. Then, upon oligomerization by Golgi Mn, the GPP130 clusters would bind sortilin with higher stoichiometry while simultaneously failing to enter the other routes because of steric exclusion.

Because FM-induced oligomers are sortilin independent and require neither cytosolic (Tewari *et al.*, 2015) nor lumenal sequence, their sorting mechanism might be quite novel. If it requires some shared aspect of the transmembrane domains it could be receptor-mediated or perhaps involve hydrophobic mismatch. While

hydrophobic mismatch could explain segregation of the oligomerized clusters in the membrane it would still require an explanation for how clathrin and GGA1 recognize this patch. Candidate receptors might include sortilin-related receptor proteins SorLA and SorCS or either of the mannose-6-phosphate receptors but each of these is known for binding lumenal cargo not transmembrane domains (Ghosh *et al.*, 2003; Willnow *et al.*, 2008; Braulke and Bonifacino, 2009; Hermey, 2009). Also, hydrophobic mismatch should depend on transmembrane domain length and shortening the transmembrane domain of an FM-containing construct by deleting five residues did not prevent lysosomal redistribution (our unpublished observations). It is notable that while Mn induced GPP130 degradation occurs over a period of ~8 h, rapid and almost complete loss of FM induced oligomers from the Golgi is achieved in ~4 h (Mukhopadhyay *et al.*, 2010; Tewari *et al.*, 2015). We expect the FM-induced oligomeric units to be more stable and perhaps much larger than Mn-induced GPP130 oligomers. They may also impart strong effects on membrane curvature because FM domains interact in an anti-parallel orientation that can zipper together opposed membranes (Rivera *et al.*, 2000; Rizzo *et al.*, 2013). Perhaps their sorting is simply the consequence of strong exclusion from any other pathway except towards lysosomes. This possibility highlights the significance of understanding the mechanism of size flexibility in the clathrin/GGA1 pathway.

In conclusion, this work shows that sortilin binds to the coiled coil lumenal domain of GPP130 and mediates its lysosomal trafficking and turnover under elevated Mn conditions. Lysosomal targeting of FM induced oligomers is sortilin independent

and indicates that there exists an alternate, possibly sequence-independent, quality control pathway operating at the Golgi that mediates their clearance. Future studies will aim to test for direct interaction between sortilin and GPP130, and map GPP130 residues required for this binding in order to elucidate the underlying interaction mechanism.

2.5 MATERIALS AND METHODS

Antibodies and general reagents. Monoclonal antibodies were against GPP130 (Linstedt *et al.*, 1997), myc (Evan *et al.*, 1985; Jesch *et al.*, 2001), HA (H3663, Clone HA-7, Sigma), giantin (Linstedt and Hauri, 1993), GFP (SAB2702197, clone GT859, Sigma), sortilin (Cat# 612100, clone 48/Neurotensin, BD Biosciences), and tubulin (T6557, clone GTU-88, Sigma). Polyclonal antibodies were against GPP130 (Puri *et al.*, 2002), TMEM165 (NBP1-90651, Novus Biologicals), GAPDH (14C10, Cell Signaling Technology) and sortilin (a kind gift from Dr. Claus M. Petersen, Aarhus University (Petersen *et al.*, 1997)). Secondary antibodies were: Alexa 488 anti-mouse (Cat#A28175, Thermo Fisher Scientific), Alexa 488 anti-rabbit (Cat#A27034, Thermo Fisher Scientific), Alexa 555 anti-rabbit (Cat#A27039, Thermo Fisher Scientific), Alexa 555 anti-mouse (Cat#A28180, Thermo Fisher Scientific), and horse radish peroxidase-conjugated goat anti-mouse (Cat#170-6516, Sigma) and goat anti-rabbit antibodies (Cat#170-6515, Sigma). AP12998 was from Clontech (called D/D solubilizer, Cat#635054). Cycloheximide (Cat#C7698) and monensin (Cat#M5273) were from Sigma. ECL blotting substrate (Cat#32209) and $MnCl_2$ (Cat#M87-500) were from Thermo Fisher Scientific.

Constructs and transfection reagents. Primers were purchased from Integrated DNA Technologies. The sortilin-myc construct was a kind gift from Dr. Stephane Lefrancois, McGill University (Lefrancois *et al.*, 2003). Silent mutations to confer siRNA resistance were introduced using the Phusion DNA polymerase (F530S, Thermo Fisher Scientific) as per manufacturer's protocols (forward primer 5'-CCTCCGTGGGACAGGAACA**A**TT**T**TA**C**TCTATTCTGGCAGCAAAT-3' with mutations highlighted). The construct was verified by sequencing. GT-FM-GFP and GPP130-FM-GFP are previously described (Rizzo *et al.*, 2013; Tewari *et al.*, 2015). To create $GT_{\Delta lum}$-FM-GFP, the remaining 20 amino acid lumenal domain of GT from the GT-FM-GFP plasmid was looped out (forward primer 5'-GGATCCGAGCTCGGTGAAGC-3') using Pfu Turbo DNA polymerase (Cat#600250, Agilent Technologies). The construct was verified by sequencing. The constructs Rab7-GFP, GP73-$GPP130_{36-247}$, GP73-$GPP130_{36-87}$, and GP73-$GPP130_{88-247}$ are previously described (Mukhopadhyay *et al.*, 2010). Control siRNA (5'-GACCAGCCAUCGUAGUACUtt-3') and sortilin siRNA (SORT1HSS109429, Catalog#:1299001) were purchased from Thermo Fisher Scientific. DNA and siRNA transfections were carried out using JetPEI (Cat#101-40N, Polypus-transfection) and JetPRIME (Cat#114-07, Polypus-transfection), respectively, as per the manufacturer's protocols.

Cell culture and experimental treatments. HeLa cells (Cat#ATCC-CCL-2, CVCL_0030) were grown in Minimum Essential Medium (Cat#10-010-CV, Corning) containing 10% Fetal Bovine Serum (FBS, Cat#S111150, Atlanta Biologicals) at 37°C, 5% CO_2. All cell lines were verified mycoplasma free every two months using

Hoechst staining. For immunofluorescence experiments, cells were plated on 12mm coverslips in 35mm or 24-well plates and, after 16 h, each plate (~50% confluent) was transfected with control or sortilin siRNA at a final concentration of 20nM. After 48 h, the media was replaced with fresh media alone or with 500 μM $MnCl_2$ or 10μM monensin for the indicated times. The coverslips were then collected and analyzed by immunofluorescence while the cells remaining behind in the 35mm plates were analyzed by immunoblot to determine knockdown efficiency. For rescue experiments, the cells were siRNA-transfected (20nM) at 80% confluence and then grown 24 h before being transfected with plasmid. Fresh media was substituted after 8h and then, after 24 h, 500μM $MnCl_2$ was added for the indicated times. Analysis was restricted to cells with moderate expression. Controlled polymerization experiments were essentially as described (Tewari *et al.*, 2015). Cells at 80% confluence were siRNA-transfected (20nM), then after 24 h they were plasmid-transfected and allowed to recover another 8 h. They were then cultured 16 h in fresh media containing 1 μM AP with the last 30 min containing both AP and cycloheximide. At this point the AP washout began for the indicated times and in the continued presence of cycloheximide. To determine sortilin stability, Hela cells grown in 35 mm plates to 80% confluency were treated as indicated then washed twice with PBS and lysed in reducing sample buffer (156mM Tris pH 6.8, 10% SDS, 50% glycerol, 5% β- mercaptoethanol, 0.1% bromophenol blue, 6M urea) prior to immunoblotting. For other immunoblotting experiments, cells at 50% confluence were siRNA-transfected (20nM) and allowed to recover for 2 days before treatment with 500μM $MnCl_2$ for the indicated times. For immunoprecipitation, HeLa cells

were grown in 10cm plates to ~70% confluency and transfected with the indicated pairs of plasmids. Fresh media was substituted after 8h and then, after 16h the media was left alone or adjusted to 500 μ M $MnCl_2$ for the final 2 h of incubation.

Immunofluorescence. Generally, cells were fixed with 3% paraformaldehyde for 15 min, washed 5x with PBS (137mM NaCl, 2.7mM KCl, 6.5mM Na_2HPO_4, 1.5mM KH_2PO_4) permeabilized for 30 min with block solution (PBS containing 0.1% Triton X-100, 5% FBS and 0.2 M glycine) and then incubated in ~150 μ l primary antibody solution (diluted in block) on a shaker at room temperature (RT) for 1 h. GPP130 and/or giantin were detected using a polyclonal anti-GPP130 and monoclonal anti-giantin respectively at 1:1000 dilution. After 5 washes with PBS, the coverslips were incubated with secondary antibodies at a 1:1000 dilution in block solution for 30 min at RT on a shaker. Following 5 final washes with PBS, the coverslips were attached to glass slides using Gelvatol mounting medium (10.5% polyvinyl alcohol and 21% glycerol in 100 mM Tris, pH 8.5) and observed under a spinning disc confocal microscope as previously described (Tewari *et al.*, 2014). Staining for TMEM165 was exactly the same except that fixation was with 0.5 ml methanol at -20°C for 20 min and the antibody was used at a 1:500 dilution. Images were analyzed using ImageJ (Version 1.50i, http://imagej.nih.gov/ij). To measure mean Golgi fluorescence, background subtraction was carried out on average value projections generated from individual z-sections of each image. Mean GPP130 or TMEM165 fluorescence in the Golgi was measured via the measure plugin of ImageJ, using giantin as a mask. Analysis of cytoplasmic punctae was performed as

previously described (Tewari *et al.*, 2014). Briefly, maximum value projections were generated from individual z-stacks of an image. These projections were background subtracted and uniformly thresholded to visualize punctae (Mukhopadhyay *et al.*, 2010).

Immunoprecipitation. Cells (10 cm plate) were washed 2X with PBS then lysed with 0.5 ml RIPA buffer (25mM Tris pH 6.8, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1%SDS, 1mM PMSF) and ~15 passes through a 25-gauge needle followed by centrifugation at 14000xg to remove insoluble material. Aliquots (5% input) of the supernatants were set aside and the remainders were pre-cleared by rotating with 10µl Protein G beads (17-0618-01, GE Healthcare) at 4°C for 30 min followed by brief centrifugation for 1 min at 14,000xg. The pre-cleared lysates were then incubated for 1 h at 4°C with antibody (3µl anti-myc monoclonal or 5ul anti-GPP130 monoclonal) followed by a 1h rotation with 10µl of Protein G beads (50% slurry). The beads were washed 3 x 2 min in 1 ml RIPA buffer at 4°C with rotation. The washed bead and input fractions were then boiled 10 min in reducing sample buffer prior to immunoblotting.

Immunoblotting. Samples were run alongside a molecular weight ladder (Cat#26619, Thermo Fisher Scientific) on 7% SDS PAGE gels and then transferred to nitrocellulose membranes (Cat#10600002, GE Healthcare) at 0.3A for 2 h. The membrane was blocked with 5% non-fat dry milk powder in PBST (PBS+ 0.1% Tween-20) for 15 min and then incubated in the same buffer containing the primary

antibody 1-18 h on a shaker. Polyclonal anti-GPP130 (1:1000), monoclonal anti-myc (1:500) and monoclonal anti-GFP (1:2000) was used to detect GPP130, sortilin-myc and GFP tagged GP73-GPP130 chimeric constructs respectively. Also used were monoclonal (1:500), and polyclonal anti-sortilin (1:2000), polyclonal anti-GAPDH and monoclonal anti-tubulin (1:3000) antibodies. The membrane was washed 10 x 6 min in PBS-T, followed by incubation with HRP-conjugated secondary antibody (1:2000) for 1 h and then another 10 x 6 min washes. The blots were soaked with the ECL substrate and imaged using ChemiDoc Touch Imaging System followed by analysis with Image Lab software (Version 5.2.1, Bio-Rad).

Statistical analyses. All findings presented were replicated in three or more independent experiments. Comparisons between two groups were performed using two-tailed Student's t test assuming equal variances. In general, $p < 0.05$ was considered significant, and the determined p values are provided in the figure legends. Asterisks in graphs, wherever present, denote statistically significant differences.

CHAPTER 3

CONCLUSIONS AND FUTURE DIRECTIONS

3.1 CONCLUSIONS

This study aims to investigate how the switch in trafficking itinerary between oligomerized GPP130 and its non-oligomerized counterpart is mediated at the Golgi and whether this mechanism can be generalized to any oligomerized Golgi protein. We found sortilin, a type 1 Golgi localized membrane protein to be a key mediator of this process. Sortilin is a GGA1 binding receptor that functions similar to the mannose-6-phosphate receptor to bind lysosomal hydrolases and other soluble cargo at the TGN and transport it to lysosomes (Canuel, Libin, and Morales 2009; Braulke and Bonifacino 2009). Sortilin knockdown in HeLa cells significantly blocked TGN exit/lysosomal redistribution of Mn bound oligomerized GPP130. Transfecting a siRNA immune version of sortilin in knockdown cells restored TGN exit. Additionally, control cells showed a significant loss of GPP130 Golgi fluorescence upon prolonged Mn treatment as compared to sortilin knockdown cells. Sortilin levels (unlike GPP130) remain relatively stable in cells subject to prolonged Mn treatment. This suggests that sortilin recycles back efficiently after concentrating and transporting GPP130 in GGA1-clathrin vesicles to late endosomes/lysosomes.

This led to the question of whether sortilin interacts with GPP130 at the TGN in the presence of Mn to traffic GPP130 to the lysosomes. Intriguingly, co-immunoprecipitation assays of HeLa cells transfected with GPP130 and sortilin showed a robust interaction between the two proteins even in the absence of Mn. The interaction is likely direct but this remains to be shown. In any case, these observations are the first evidence of a transmembrane cargo being trafficked by sortilin. GPP130 has a half-life of 18h (Linstedt et al. 1997). It is not known how its gradual turnover at the Golgi is mediated. That sortilin binds GPP130 in the absence of added Mn suggests that sortilin mediates the normal turnover of GPP130. How then is degradation enhanced by Mn-induced oligomerization? One straightforward consequence of oligomerization is that when sortilin binds GPP130 it will be engaging a complex of several GPP130 molecules. Thus, each sortilin could traffic a larger fraction of GPP130 molecules to lysosomes per unit time. Based on this, we expected enhanced GPP130 recovery upon sortilin immunoprecipitation but it is possible that we were unable to preserve the oligomeric GPP130 complexes. Further evidence will be required to validate the model that Mn-induced GPP130 oligomerization allows each sortilin to engage more GPP10 (see below in future directions)

Attempts to define the GPP130 region that binds sortilin, yielded a stretch of ~50 residues in the GPP130 lumenal coiled-coil domain. This stretch, residues 36-87, does not contain the residues $_{88}$DFLV$_{91}$ required for Mn binding to GPP130, supporting the finding that the GPP130-sortilin interaction is Mn independent.

Additionally, deletion of residues 36-87 significantly blocks Mn-induced redistribution of GPP130 supporting the proposed role of the sortilin-GPP130 interaction. It should be noted, however, that it is not yet known whether GPP130 lacking residues 36-87 remains oligomerization competent.

In order to test the generality of this finding, redistribution of another Mn sensitive protein TMEM165, was tested for sortilin dependence. TMEM165 is a multipass transmembrane H^+/Ca^{2+} exchanger localized at the Golgi that undergoes lysosomal trafficking upon Mn treatment (Potelle et al. 2017). Sortilin knockdown significantly blocked Mn induced TGN exit and loss of TMEM165 Golgi fluorescence. Unlike GPP130, which has a large lumenal coiled-coil domain, TMEM165 has very short lumenal stretches. Hence a direct interaction between TMEM165 and sortilin via their lumenal domains seems unlikely. Additionally, whether TMEM165 oligomerizes upon Mn treatment is not known. It is interesting that Mn-induced trafficking of TMEM165 is independent of SPCA1, the major transporter of Mn into the Golgi, while Mn-induced trafficking of GPP130 is SPCA1 dependent (Mukhopadhyay and Linstedt 2011; Potelle et al. 2017). It is possible that cytoplasmic, not lumenal Mn acts on TMEM165. While we considered the possibility that sortilin knockdown might indirectly affect GPP130 through an unexpected effect on SPCA1, this cannot be the case for TMEM165. Thus it is more likely that sortilin acts directly in sorting both of these Golgi proteins rather than via an indirect effect on SPCA1.

Surprisingly, sortilin did not mediate the TGN exit/redistribution of FM induced oligomers. Perhaps the FM oligomers are much larger than the Mn-induced GPP130 oligomers and this somehow engages distinct trafficking machinery. The sortilin-independent redistribution of GPP130-FM-GFP indicates that even a construct that should be able to bind sortilin uses an alternate pathway. Thus, the alternate path appears predominant. GT-FM-GFP redistributed independently of its native lumenal domain arguing against the possibility of a receptor recognizing the GT lumenal domain. We cannot rule out the possibility of a receptor interacting with the transmembrane domains of the FM-tagged Golgi proteins. Mannose-6- phosphate receptor is an unlikely candidate since it is known to interact with cargo that is soluble in the lumen containing the mannose-6-phosphate signal (Braulke and Bonifacino 2009). Similarly, two sortilin isoforms, SorCS and SorLA are primarily known for interacting with soluble cargo (Hermey 2009; Willnow, Petersen, and Nykjaer 2010). However, it remains formally possible that any of these receptors could act in an unexpected way. In that light it is interesting that both mannose-6 phosphate receptor and SorLA engage GGA1 and clathrin because it is this adaptor/coat complex that appears to mediate TGN exit of FM-tagged cargo

3.2 FUTURE DIRECTIONS

Map and mutate the GPP130-sortilin binding interface. Currently we have identified a ~50 residue region in the coiled-coil, lumenal, stem domain of GPP130 ($GPP130_{36-87}$) that binds sortilin. This stretch is also required to elicit efficient Mn-induced GPP130 redistribution (Mukhopadhyay et al. 2010). Further mapping showed that while the construct $GPP130_{50-100}$ exhibited reduced redistribution,

GPP130$_{60\text{-}100}$ showed no redistribution (Ritika Tewari, Jarvela, and Linstedt 2014). Since 36-87 is sufficient for sortilin binding, the above results hint at the importance of testing residues 36-60 (~ 24 residues) and, in particular, the stretch 50-60. Also, residues 50-60 contain a histidine that could confer pH dependent sortilin binding such that binding occurs in the TGN and release occurs in the lower pH of late endosomes/lysosomes. Interestingly, residues 50-60 in GPP130 contain the Shiga toxin binding site (Mukhopadhyay and Linstedt 2012; Mukhopadhyay, Redler, and Linstedt 2013), which is pH-dependent (unpublished). It will also be important to test each construct for Mn-induced oligomerization to show that even oligomerized GPP130 will fail to exit the TGN if it lacks a functional binding site for sortilin.

A similar approach can be employed to narrow down the binding interface in sortilin. Sortilin has two main domains that interact with soluble ligands in the Golgi lumen, a ~120 residue 10CC segment containing 10 conserved cysteines right next to the membrane, and a 10 bladed β propeller domain, Vps10 situated farther from the membrane interface (Westergaard et al. 2004). The binding segment on GPP130 (36-87) is right next to the membrane making 10CC a good candidate domain. So far, no consensus sequence has been defined for recognition by sortilin and no single site on sortilin is responsible for cargo binding (Quistgaard et al. 2009). Therefore, deletions that do not disrupt protein structure rather than point mutations would be an appropriate starting point. The constructs would be tested in rescue assays in sortilin knockdown cells. Constructs that fail to rescue Mn-induced GPP130 redistribution would identify functionally important residues. Although the

binding between the two proteins need not be direct, these experiments would help provide a clearer understanding of how the sortilin-GPP130 interaction mediates GPP130 sorting.

Test for direct binding between GPP130-sortilin. Several important issues can be addressed using an in vitro binding assay with purified versions of GPP130 and sortilin: A) pH dependence of the interaction, B) Mn sensitivity of the interaction under well-controlled Mn concentrations, C) precise binding affinity, and D) identity of residues that mediate direct binding. Importantly, soluble forms of a GST-tagged GPP130 stem domain and a His-tagged sortilin lumenal domain have previously been purified (Skeldal et al. 2012; Tewari, Jarvela, and Linstedt 2014).

Steric exclusion of oligomerized GPP130 from early endosomal pathway. In our work, we proposed that increased GPP130 turnover upon Mn is mediated by sortilin transporting a larger fraction of (oligomerized) GPP130 into the lysosomal pathway. This may or may not involve exclusion of oligomerized GPP130 from its normal trafficking to early endosomes. While the lysosomal pathway is GGA1-clathrin dependent and involves vesicles that can evidently accommodate oligomeric GPP130 complexes, molecular players governing TGN-exit of GPP130 towards early endosomes have not been identified. It will be important to test whether steric exclusion of large oligomeric GPP130 complexes from this early endosomal pathway contributes to its trafficking into the late endosomal/lysosomal pathway. Monensin disrupts the pH dependent retrieval of GPP130 from early

endosomes thereby trapping GPP130 in early endosomes (Linstedt et al. 1997; Bachert, Lee, and Linstedt 2001). Thus, monensin treatment provides a simple assay to test TGN-exit of GPP130 towards early endosomes and if it were carried out on Mn-treated cells, it would test whether oligomerized GPP130 can still access this pathway. To enhance the discriminating power of the experiment it could be carried out on sortilin knockdown cells, because this blocks TGN-exit of GPP130 towards lysosomes. We know that GPP130 traffics normally to early endosomes upon monensin treatment of sortilin knockdown cells. If Mn blocks this for wildtype but not oligomerization-deficient GPP130, it would strongly argue that oligomerization of GPP130 creates a steric block for access to the TGN-to-early endosome pathway.

Identification of molecular players involved in trafficking of FM induced oligomers. The mechanism underlying trafficking of FM induced oligomers and therefore an important aspect of Golgi quality control i.e. how a variety of proteins are trafficked out of the Golgi solely due to their oligomerization state, still remains unclear. A number of molecular players that might be mediating trafficking could be engaged in transient interactions with oligomerized proteins. An approach that is being widely utilized to detect these transient interactions is BIOID tagging. Fusion of the oligomerizing construct with a promiscuous biotin ligase would cause proximal or transiently interacting proteins to be biotinylated upon addition of biotin in the culture medium, which can be detected with a streptavidin pulldown and analyzed by mass spectrometry (Roux et al. 2012; Varnaitė and MacNeill 2016; Kim et al. 2016). This could be carried out with the tag on either side of the

membrane. The assays we have developed including knockdown/rescue and co-immunoprecipitation would then be used to test candidates for functional roles and direct interactions. This would be an important first step in unraveling the mechanism underlying quality control in the Golgi as it pertains to oligomeric state.

In summary this thesis work extends our knowledge of how Mn-induced lysosomal trafficking of GPP130 is mediated at the TGN and it opens several avenues that can now be explored to attain a deeper, mechanistic understanding of how trafficking is regulated and how quality control occurs in the Golgi complex.

REFERENCES

Alexandra Stolz, Andreas Ernst, and Ivan Dikic. 2014. “Cargo Recognition and Trafficking in Selective Autophagy.” *Nature Cell Biology* 16 (6): 495–501.

Armstrong, J., S. Patel, and P. Riddle. 1990. “Lysosomal Sorting Mutants of Coronavirus E1 Protein, a Golgi Membrane Protein.” *Journal of Cell Science* 95 (2).

Arvan, Peter, Xiang Zhao, Jose Ramos-Castaneda, and Amy Chang. 2002. “Secretory Pathway Quality Control Operating in Golgi, Plasmalemmal, and Endosomal Systems.” *Traffic (Copenhagen, Denmark)* 3 (11): 771–80.

Ashok, Aarthi, and Ramanujan S Hegde. 2009. “Selective Processing and Metabolism of Disease-Causing Mutant Prion Proteins.” *PLoS Pathogens* 5 (6). Public Library of Science: e1000479. doi:10.1371/journal.ppat.1000479.

Bachert, C, T H Lee, and A D Linstedt. 2001. “Lumenal Endosomal and Golgi-Retrieval Determinants Involved in pH-Sensitive Targeting of an Early Golgi Protein.” *Molecular Biology of the Cell* 12 (10): 3152–60.

Boya, Patricia, Fulvio Reggiori, and Patrice Codogno. 2013. “Emerging Regulation and Functions of Autophagy.” *Nature Cell Biology* 15 (7): 713–20.

Braulke, Thomas, and Juan S Bonifacino. 2009. “Sorting of Lysosomal Proteins.” *Biochimica et Biophysica Acta* 1793 (4): 605–14. doi:10.1016/j.bbamcr.2008.10.016.

Brodsky, Jeffrey L, and William R Skach. 2011. “Protein Folding and Quality Control in the Endoplasmic Reticulum: Recent Lessons from Yeast and Mammalian Cell Systems.” *Current Opinion in Cell Biology* 23 (4): 464–75. doi:10.1016/j.ceb.2011.05.004.

Bukau, Bernd, Jonathan Weissman, and Arthur Horwich. 2006. “Molecular Chaperones and Protein Quality Control.” *Cell* 125 (3): 443–51. doi:10.1016/j.cell.2006.04.014.

Buscà, R, M Martínez, E Vilella, P Pognonec, S Deeb, J Auwerx, M Reina, and S Vilaró. 1996. “The Mutation Gly142-->Glu in Human Lipoprotein Lipase Produces a Missorted Protein That Is Diverted to Lysosomes.” *The Journal of Biological Chemistry* 271 (4): 2139–46.

Canuel, Maryssa, Yuan Libin, and Carlos R Morales. 2009. “The Interactomics of Sortilin: An Ancient Lysosomal Receptor Evolving New Functions.” *Histology and Histopathology* 24 (4): 481–92.

Chang, A, and G R Fink. 1995. “Targeting of the Yeast Plasma Membrane [H+]ATPase: A Novel Gene AST1 Prevents Mislocalization of Mutant ATPase to the Vacuole.” *The Journal of Cell Biology* 128 (1–2): 39–49.

Conway, K A, S J Lee, J C Rochet, T T Ding, R E Williamson, and P T Lansbury. 2000. “Acceleration of Oligomerization, Not Fibrillization, Is a Shared Property of Both Alpha-Synuclein Mutations Linked to Early-Onset Parkinson’s Disease: Implications for Pathogenesis and Therapy.” *Proceedings of the National Academy of Sciences of the United States of America* 97 (2): 571–76.

Conway, Kelly A., James D. Harper, and Peter T. Lansbury. 1998. “Accelerated in

Vitro Fibril Formation by a Mutant α-Synuclein Linked to Early-Onset Parkinson Disease." *Nature Medicine* 4 (11): 1318–20. doi:10.1038/3311.
Coughlan, Christina M, Jennifer L Walker, Jared C Cochran, K Dane Wittrup, and Jeffrey L Brodsky. 2004. "Degradation of Mutated Bovine Pancreatic Trypsin Inhibitor in the Yeast Vacuole Suggests Post-Endoplasmic Reticulum Protein Quality Control." *The Journal of Biological Chemistry* 279 (15). American Society for Biochemistry and Molecular Biology: 15289–97. doi:10.1074/jbc.M309673200.
Fayadat, L., and Ron R Kopito. 2003. "Recognition of a Single Transmembrane Degron by Sequential Quality Control Checkpoints." *Molecular Biology of the Cell* 14 (3): 1268–78. doi:10.1091/mbc.E02-06-0363.
Fisher, E A, M Pan, X Chen, X Wu, H Wang, H Jamil, J D Sparks, and K J Williams. 2001. "The Triple Threat to Nascent Apolipoprotein B. Evidence for Multiple, Distinct Degradative Pathways." *The Journal of Biological Chemistry* 276 (30). American Society for Biochemistry and Molecular Biology: 27855–63. doi:10.1074/jbc.M008885200.
Gelling, Cristy L., Ian W. Dawes, David H. Perlmutter, Edward A. Fisher, and Jeffrey L. Brodsky. 2012. "The Endosomal Protein-Sorting Receptor Sortilin Has a Role in Trafficking α-1 Antitrypsin." *Genetics* 192 (3).
Gilch, S, K F Winklhofer, M H Groschup, M Nunziante, R Lucassen, C Spielhaupter, W Muranyi, D Riesner, J Tatzelt, and H M Schätzl. 2001. "Intracellular Re-Routing of Prion Protein Prevents Propagation of PrP(Sc) and Delays Onset of Prion Disease." *The EMBO Journal* 20 (15). European Molecular Biology Organization: 3957–66. doi:10.1093/emboj/20.15.3957.
Goldberg, Alfred L. 2003. "Protein Degradation and Protection against Misfolded or Damaged Proteins." *Nature* 426 (6968). Nature Publishing Group: 895–99. doi:10.1038/nature02263.
Hammond, Craig, and Ari Helenius. 1995. "Quality Control in the Secretory Pathway." *Current Opinion in Cell Biology* 7 (4): 523–29. doi:10.1016/0955-0674(95)80009-3.
Haynes, Cole M., Sabrina Caldwell, and Antony A. Cooper. 2002. "An HRD/DER-Independent ER Quality Control Mechanism Involves Rsp5p-Dependent Ubiquitination and ER-Golgi Transport." *The Journal of Cell Biology* 158 (1).
Hermey, Guido. 2009. "The Vps10p-Domain Receptor Family." *Cellular and Molecular Life Sciences* 66 (16). SP Birkhäuser Verlag Basel: 2677–89. doi:10.1007/s00018-009-0043-1.
Hettema, Ewald H, Javier Valdez-Taubas, and Hugh R B Pelham. 2004. "Bsd2 Binds the Ubiquitin Ligase Rsp5 and Mediates the Ubiquitination of Transmembrane Proteins." *The EMBO Journal* 23 (6): 1279–88. doi:10.1038/sj.emboj.7600137.
Hol, E. M., and W. Scheper. 2008. "Protein Quality Control in Neurodegeneration: Walking the Tight Rope Between Health and Disease." *Journal of Molecular Neuroscience* 34 (1). Humana Press Inc: 23–33. doi:10.1007/s12031-007-0013-8.
Holkeri, Heidi, and Marja Makarow. 1998. "Different Degradation Pathways for Heterologous Glycoproteins in Yeast." *FEBS Letters* 429 (2): 162–66. doi:10.1016/S0014-5793(98)00586-9.

Hong, E, A R Davidson, and C A Kaiser. 1996. “A Pathway for Targeting Soluble Misfolded Proteins to the Yeast Vacuole.” *The Journal of Cell Biology* 135 (3).
Kamsteeg, E J, T A Wormhoudt, J P Rijss, C H van Os, and P M Deen. 1999. “An Impaired Routing of Wild-Type Aquaporin-2 after Tetramerization with an Aquaporin-2 Mutant Explains Dominant Nephrogenic Diabetes Insipidus.” *The EMBO Journal* 18 (9). European Molecular Biology Organization: 2394–2400. doi:10.1093/emboj/18.9.2394.
Kamsteeg, Erik-Jan, Paul J. M. Savelkoul, Giel Hendriks, Irene B. M. Konings, Nicole M. I. Nivillac, Anne Karine Lagendijk, Peter van der Sluijs, and Peter M. T. Deen. 2008. “Missorting of the Aquaporin-2 Mutant E258K to Multivesicular Bodies/lysosomes in Dominant NDI Is Associated with Its Monoubiquitination and Increased Phosphorylation by PKC but Is due to the Loss of E258.” *Pfl?gers Archiv - European Journal of Physiology* 455 (6). Springer-Verlag: 1041–54. doi:10.1007/s00424-007-0364-6.
Kim, D. I., S. C. Jensen, K. A. Noble, B. KC, K. H. Roux, K. Motamedchaboki, and K. J. Roux. 2016. “An Improved Smaller Biotin Ligase for BioID Proximity Labeling.” *Molecular Biology of the Cell* 27 (8): 1188–96. doi:10.1091/mbc.E15-12-0844.
Koga, Hiroshi, Susmita Kaushik, and Ana Maria Cuervo. 2011. “Protein Homeostasis and Aging: The Importance of Exquisite Quality Control.” *Ageing Research Reviews* 10 (2): 205–15. doi:10.1016/j.arr.2010.02.001.
Kraft, Claudine, Matthias Peter, and Kay Hofmann. 2010. “Selective Autophagy: Ubiquitin-Mediated Recognition and beyond.” *Nature Cell Biology* 12 (9): 836–41. doi:10.1038/ncb0910-836.
Kruse, K. B., Jeffrey L Brodsky, and Ardythe A McCracken. 2005. “Characterization of an ERAD Gene as VPS30/ATG6 Reveals Two Alternative and Functionally Distinct Protein Quality Control Pathways: One for Soluble Z Variant of Human ?-1 Proteinase Inhibitor (A1PiZ) and Another for Aggregates of A1PiZ.” *Molecular Biology of the Cell* 17 (1). American Society for Cell Biology: 203–12. doi:10.1091/mbc.E04-09-0779.
Lee, He-Jin, Chan Choi, and Seung-Jae Lee. 2002. “Membrane-Bound Alpha-Synuclein Has a High Aggregation Propensity and the Ability to Seed the Aggregation of the Cytosolic Form.” *The Journal of Biological Chemistry* 277 (1). American Society for Biochemistry and Molecular Biology: 671–78. doi:10.1074/jbc.M107045200.
Lee, He-Jin, Farnaz Khoshaghideh, Smita Patel, and Seung-Jae Lee. 2004. “Clearance of α-Synuclein Oligomeric Intermediates via the Lysosomal Degradation Pathway.” *Journal of Neuroscience* 24 (8).
Liao, Wei, Benny Hung-Junn Chang, Michael Mancini, and Lawrence Chan. 2003. “Ubiquitin-Dependent and -Independent Proteasomal Degradation of apoB Associated with Endoplasmic Reticulum and Golgi Apparatus, Respectively, in HepG2 Cells.” *Journal of Cellular Biochemistry* 89 (5). Wiley Subscription Services, Inc., A Wiley Company: 1019–29. doi:10.1002/jcb.10538.
Lin, Qingqing, and Erwin London. 2013. “Altering Hydrophobic Sequence Lengths Shows That Hydrophobic Mismatch Controls Affinity for Ordered Lipid Domains (Rafts) in the Multitransmembrane Strand Protein Perfringolysin O.” *The Journal of Biological Chemistry* 288 (2): 1340–52.

doi:10.1074/jbc.M112.415596.
Linstedt, A D, A Mehta, J Suhan, H Reggio, and H P Hauri. 1997. “Sequence and Overexpression of GPP130/GIMPc: Evidence for Saturable pH-Sensitive Targeting of a Type II Early Golgi Membrane Protein.” *Molecular Biology of the Cell* 8 (6): 1073–87.
Liu, Xiu Fen, and Valeria Cizewski Culotta. 1999. “Mutational Analysis of Saccharomyces Cerevisiae Smf1p, a Member of the Nramp Family of Metal Transporters.” *Journal of Molecular Biology* 289 (4): 885–91. doi:10.1006/jmbi.1999.2815.
McLean, P J, H Kawamata, S Ribich, and B T Hyman. 2000. “Membrane Association and Protein Conformation of Alpha-Synuclein in Intact Neurons. Effect of Parkinson’s Disease-Linked Mutations.” *The Journal of Biological Chemistry* 275 (12): 8812–16.
Minami, Y, A M Weissman, L E Samelson, and R D Klausner. 1987. “Building a Multichain Receptor: Synthesis, Degradation, and Assembly of the T-Cell Antigen Receptor.” *Proceedings of the National Academy of Sciences of the United States of America* 84 (9). National Academy of Sciences: 2688–92.
Mukhopadhyay, Somshuvra, Collin Bachert, Donald R Smith, and Adam D Linstedt. 2010. “Manganese-Induced Trafficking and Turnover of the Cis -Golgi Glycoprotein GPP130.” *Molecular Biology of the Cell* 21: 1282–92. doi:10.1091/mbc.E09.
Mukhopadhyay, Somshuvra, and Adam D Linstedt. 2011. “Identification of a Gain-of-Function Mutation in a Golgi P-Type ATPase That Enhances Mn2+ Efflux and Protects against Toxicity.” *Proceedings of the National Academy of Sciences of the United States of America* 108 (2). National Academy of Sciences: 858–63. doi:10.1073/pnas.1013642108.
———. 2012. “Manganese Blocks Intracellular Trafficking of Shiga Toxin and Protects Against Shiga Toxicosis.” *Science* 335 (January): 332–35.
Mukhopadhyay, Somshuvra, Brendan Redler, and Adam D Linstedt. 2013. “Shiga Toxin-Binding Site for Host Cell Receptor GPP130 Reveals Unexpected Divergence in Toxin-Trafficking Mechanisms.” *Molecular Biology of the Cell* 24 (15): 2311–18. doi:10.1091/mbc.E13-01-0057.
Musil, Linda S., and Daniel A. Goodenough. 1993. “Multisubunit Assembly of an Integral Plasma Membrane Channel Protein, Gap Junction connexin43, Occurs after Exit from the ER.” *Cell* 74 (6): 1065–77. doi:10.1016/0092-8674(93)90728-9.
Nishida, Yuya, Satoko Arakawa, Kenji Fujitani, Hirofumi Yamaguchi, Takeshi Mizuta, Toku Kanaseki, Masaaki Komatsu, Kinya Otsu, Yoshihide Tsujimoto, and Shigeomi Shimizu. 2009. “Discovery of Atg5/Atg7-Independent Alternative Macroautophagy.” *Nature* 461 (7264). Nature Publishing Group: 654–58. doi:10.1038/nature08455.
Pan, Meihui, Vatsala Maitin, Sajesh Parathath, Ursula Andreo, Sharron X Lin, Carly St Germain, Zemin Yao, Frederick R Maxfield, Kevin Jon Williams, and Edward A Fisher. 2008. “Presecretory Oxidation, Aggregation, and Autophagic Destruction of Apoprotein-B: A Pathway for Late-Stage Quality Control.” *Proceedings of the National Academy of Sciences of the United States of America*

105 (15). National Academy of Sciences: 5862–67. doi:10.1073/pnas.0707460104.
Perrin, R J, W S Woods, D F Clayton, and J M George. 2000. "Interaction of Human Alpha-Synuclein and Parkinson's Disease Variants with Phospholipids. Structural Analysis Using Site-Directed Mutagenesis." *The Journal of Biological Chemistry* 275 (44). American Society for Biochemistry and Molecular Biology: 34393–98. doi:10.1074/jbc.M004851200.
Pizzirusso, M., and Amy Chang. 2004. "Ubiquitin-Mediated Targeting of a Mutant Plasma Membrane ATPase, Pma1-7, to the Endosomal/Vacuolar System in Yeast." *Molecular Biology of the Cell* 15 (5). American Society for Cell Biology: 2401–9. doi:10.1091/mbc.E03-10-0727.
Potelle, Sven, Eudoxie Dulary, Leslie Climer, Sandrine Duvet, Willy Morelle, Dorothée Vicogne, Elodie Lebredonchel, et al. 2017. "Manganese-Induced Turnover of TMEM165." *Biochemical Journal* 474 (9): 1481–93. doi:10.1042/BCJ20160910.
Quistgaard, Esben M, Peder Madsen, Morten K Grøftehauge, Poul Nissen, Claus M Petersen, and Søren S Thirup. 2009. "Ligands Bind to Sortilin in the Tunnel of a Ten-Bladed β-Propeller Domain." *Nature Structural & Molecular Biology* 16 (1). Nature Publishing Group: 96–98. doi:10.1038/nsmb.1543.
Reggiori, F, M W Black, and H R Pelham. 2000. "Polar Transmembrane Domains Target Proteins to the Interior of the Yeast Vacuole." *Molecular Biology of the Cell* 11 (11). American Society for Cell Biology: 3737–49.
Reggiori, Fulvio, Masaaki Komatsu, Kim Finley, and Anne Simonsen. 2012. "Autophagy: More than a Nonselective Pathway." *International Journal of Cell Biology* 2012. Hindawi: 219625. doi:10.1155/2012/219625.
Reggiori, Fulvio, and Hugh R. B. Pelham. 2002. "A Transmembrane Ubiquitin Ligase Required to Sort Membrane Proteins into Multivesicular Bodies." *Nature Cell Biology* 4 (2). Nature Publishing Group: 117–23. doi:10.1038/ncb743.
Rivera, V M, X Wang, S Wardwell, N L Courage, A Volchuk, T Keenan, D A Holt, et al. 2000. "Regulation of Protein Secretion through Controlled Aggregation in the Endoplasmic Reticulum." *Science (New York, N.Y.)* 287 (5454). American Association for the Advancement of Science: 826–30. doi:10.1126/science.287.5454.826.
Rizzo, Riccardo, Seetharaman Parashuraman, Peppino Mirabelli, Claudia Puri, John Lucocq, and Alberto Luini. 2013. "The Dynamics of Engineered Resident Proteins in the Mammalian Golgi Complex Relies on Cisternal Maturation." *The Journal of Cell Biology* 201 (7): 1027–36. doi:10.1083/jcb.201211147.
Rothman, Stephen. 2010. "How Is the Balance between Protein Synthesis and Degradation Achieved?" *Theoretical Biology & Medical Modelling* 7 (June). BioMed Central: 25. doi:10.1186/1742-4682-7-25.
Roux, Kyle J, Dae In Kim, Manfred Raida, and Brian Burke. 2012. "A Promiscuous Biotin Ligase Fusion Protein Identifies Proximal and Interacting Proteins in Mammalian Cells." *The Journal of Cell Biology* 196 (6): 801–10. doi:10.1083/jcb.201112098.
Shao, Sichen, and Ramanujan S. Hegde. 2016. "Target Selection during Protein Quality Control." *Trends in Biochemical Sciences* 41 (2): 124–37.

doi:10.1016/j.tibs.2015.10.007.
Shelness, G S, M F Ingram, X F Huang, and J A DeLozier. 1999. “Apolipoprotein B in the Rough Endoplasmic Reticulum: Translation, Translocation and the Initiation of Lipoprotein Assembly.” *The Journal of Nutrition* 129 (2S Suppl). American Society for Nutrition: 456S–462S.
Shelness, G S, K C Morris-Rogers, and M F Ingram. 1994. “Apolipoprotein B48-Membrane Interactions. Absence of Transmembrane Localization in Nonhepatic Cells.” *The Journal of Biological Chemistry* 269 (12): 9310–18.
Shimizu, Shigeomi, Satoko Arakawa, and Yuya Nishida. 2010. “Autophagy Takes an Alternative Pathway.” *Autophagy* 6 (2): 290–91.
Skeldal, Sune, Alex M Sykes, Simon Glerup, Dusan Matusica, Nickless Palstra, Henri Autio, Zoran Boskovic, et al. 2012. “Mapping of the Interaction Site between Sortilin and the p75 Neurotrophin Receptor Reveals a Regulatory Role for the Sortilin Intracellular Domain in p75 Neurotrophin Receptor Shedding and Apoptosis.” *The Journal of Biological Chemistry* 287 (52). American Society for Biochemistry and Molecular Biology: 43798–809. doi:10.1074/jbc.M112.374710.
Spear, E. D., and Davis T W Ng. 2003. “Stress Tolerance of Misfolded Carboxypeptidase Y Requires Maintenance of Protein Trafficking and Degradative Pathways.” *Molecular Biology of the Cell* 14 (7). American Society for Cell Biology: 2756–67. doi:10.1091/mbc.E02-11-0717.
Tewari, R., C. Bachert, and A. D. Linstedt. 2015. “Induced-Oligomerization Targets Golgi Proteins for Degradation in Lysosomes.” *Molecular Biology of the Cell*, October. doi:10.1091/mbc.E15-04-0207.
Tewari, Ritika, Timothy Jarvela, and Adam D Linstedt. 2014. “Manganese Induces Oligomerization to Promote down-Regulation of the Intracellular Trafficking Receptor Used by Shiga Toxin.” *Molecular Biology of the Cell* 25 (19): 3049–58. doi:10.1091/mbc.E14-05-1003.
VanSlyke, J K, S M Deschenes, and L S Musil. 2000. “Intracellular Transport, Assembly, and Degradation of Wild-Type and Disease-Linked Mutant Gap Junction Proteins.” *Molecular Biology of the Cell* 11 (6). American Society for Cell Biology: 1933–46. doi:10.1091/MBC.11.6.1933.
Varnaitė, Renata, and Stuart A. MacNeill. 2016. “Meet the Neighbors: Mapping Local Protein Interactomes by Proximity-Dependent Labeling with BioID.” *PROTEOMICS*, July. doi:10.1002/pmic.201600123.
Vembar, Shruthi S., and Jeffrey L. Brodsky. 2008. “One Step at a Time: Endoplasmic Reticulum-Associated Degradation.” *Nature Reviews Molecular Cell Biology* 9 (12). Nature Publishing Group: 944–57. doi:10.1038/nrm2546.
Vogel, Christine, and Edward M Marcotte. 2012. “Insights into the Regulation of Protein Abundance from Proteomic and Transcriptomic Analyses.” *Nature Reviews. Genetics* 13 (4). NIH Public Access: 227–32. doi:10.1038/nrg3185.
Wang, Songyu, and Davis T W Ng. 2010. “Evasion of Endoplasmic Reticulum Surveillance Makes Wsc1p an Obligate Substrate of Golgi Quality Control.” *Molecular Biology of the Cell* 21 (7). American Society for Cell Biology: 1153–65. doi:10.1091/mbc.E09-10-0910.
Westergaard, Uffe B, Esben S Sørensen, Guido Hermey, Morten S Nielsen, Anders

Nykjaer, Kirstine Kirkegaard, Christian Jacobsen, Jørgen Gliemann, Peder Madsen, and Claus Munck Petersen. 2004. "Functional Organization of the Sortilin Vps10p Domain." *The Journal of Biological Chemistry* 279 (48). American Society for Biochemistry and Molecular Biology: 50221–29. doi:10.1074/jbc.M408873200.

Willnow, Thomas E., Claus M. Petersen, and Anders Nykjaer. 2010. "VPS10P-Domain Receptors — Regulators of Neuronal Viability and Function." *Nature Reviews Neuroscience* 9 (12): 899–909. doi:10.1038/nrn2516.

Wolins, N, H Bosshart, H Küster, and J S Bonifacino. 1997. "Aggregation as a Determinant of Protein Fate in Post-Golgi Compartments: Role of the Luminal Domain of Furin in Lysosomal Targeting." *The Journal of Cell Biology* 139 (7): 1735–45.

Worch, Remigiusz, Christian Bökel, Sigfried Höfinger, Petra Schwille, and Thomas Weidemann. 2010. "Focus on Composition and Interaction Potential of Single-Pass Transmembrane Domains." *PROTEOMICS* 10 (23). WILEY-VCH Verlag: 4196–4208. doi:10.1002/pmic.201000208.

Yamaguchi, Hirofumi, Satoko Arakawa, Toku Kanaseki, Takeshi Miyatsuka, Yoshio Fujitani, Hirotaka Watada, Yoshihide Tsujimoto, and Shigeomi Shimizu. 2016. "Golgi Membrane-associated Degradation Pathway in Yeast and Mammals." *The EMBO Journal* 35 (18): 1991–2007. doi:10.15252/embj.201593191.

Zaliauskiene, L, S Kang, C G Brouillette, J Lebowitz, R B Arani, and J F Collawn. 2000. "Down-Regulation of Cell Surface Receptors Is Modulated by Polar Residues within the Transmembrane Domain." *Molecular Biology of the Cell* 11 (8). EMBO Press: 2643–55. doi:10.1091/MBC.11.8.2643.