Induced oligomerization targets Golgi proteins for degradation in lysosomes

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ABSTRACT Manganese protects cells against forms of Shiga toxin by down-regulating the cycling Golgi protein GPP130. Down-regulation occurs when Mn binding causes GPP130 to oligomerize and traffic to lysosomes. To determine how GPP130 is redirected to lysosomes, we tested the role of GGA1 and clathrin, which mediate sorting in the canonical Golgi-to-lysosome pathway. GPP130 oligomerization was induced using either Mn or a self-interacting version of the FKBP domain. Inhibition of GGA1 or clathrin specifically blocked GPP130 redistribution, suggesting recognition of the aggregated GPP130 by the GGA1/clathrin-sorting complex. Unexpectedly, however, GPP130’s cytoplasmic domain was not required, and redistribution also occurred after removal of GPP130 sequences needed for its normal cycling. Therefore, to test whether aggregate recognition might be a general phenomenon rather than one involving a specific GPP130 determinant, we induced homo-oligomerization of two unrelated Golgi-targeted constructs using the FKBP strategy. These were targeted to the cis- and trans-Golgi, respectively, using domains from mannosidase-1 and galactosyltransferase. Significantly, upon oligomerization, each redistributed to peripheral punctae and was degraded. This occurred in the absence of detectable UPR activation. These findings suggest the unexpected presence of quality control in the Golgi that recognizes aggregated Golgi proteins and targets them for degradation in lysosomes.

INTRODUCTION The Golgi membrane protein GPP130 normally cycles between its steady-state location in the cis-Golgi and endosomes (Puri et al., 2002). An increase in Golgi manganese causes it to leave this itinerary and instead traffic to lysosomes, where it is degraded (Mukhopadhyay et al., 2010). Although the role of GPP130 is unknown, this trafficking switch is fortuitous because the bacterially produced Shiga toxin and Shiga toxin 1 (STx/STx1), which cause severe diarrhea and fatal kidney failure (Beddoe et al., 2010), depend on GPP130 for their invasion of host cells (Natarajan and Linstedt, 2004; Mukhopadhyay and Linstedt, 2012). The toxins bind GPP130 in endosomes and piggyback a ride to the Golgi, which allows them to escape degradation in the lysosome (Mukhopadhyay and Linstedt, 2012; Mukhopadhyay et al., 2013). Indeed, Mn-induced down-regulation of GPP130 prevents STx/STx1 trafficking, and Mn protects cells and mice against Shiga toxicosis (Mukhopadhyay and Linstedt, 2012).

The switch in sorting fate of GPP130 caused by Mn is an instance of an important general regulatory mechanism in which sorting during membrane trafficking is used as a control point. The best-known examples of this type of sorting switch are those induced by ligand binding to cell surface receptors. Ligand binding induces conformational changes and/or posttranslational modifications of cytoplasmically exposed sorting signals. This alters the interaction of these signals with vesicle coat adaptors driving endocytosis (Traub and Bonifacino, 2013; Piper et al., 2014). A related scenario takes place when cholesterol regulates the endoplasmic reticulum (ER) exit of the sterol regulatory element–binding protein (SREBP) cleavage–activating protein (SCAP)/SREBP complex. Cholesterol binding changes SCAP’s conformation, leading to binding of another factor that masks SCAP’s sorting signal for ER exit (Brown and Goldstein, 1997; Sun et al., 2007). There are also disease-related examples.
Human immunodeficiency virus type 1 protein Nef alters the sorting of the major histocompatibility complex-1 by mediating the indirect interaction of MHCI with the AP-1 adaptor protein at the trans-Golgi network (TGN; Le Gall et al., 1998). Mutation of the amloid precursor protein in Alzheimer’s disease blocks its interaction with the adaptor AP-4 at the TGN, resulting in greater cleavage to the pathogenic amyloid-β peptide (Roeth et al., 2004). Control of sorting is also a critical aspect of metal ion homeostasis, and this may be the proper context in which to place Mn control of GPP130 trafficking. Similar to Mn-induced GPP130 trafficking, elevated copper causes redistribution of the Golgi proteins ATP7A and ATP7B, which are copper-translocating pumps. The pumps move from the TGN to the cell surface, where they expel excess copper (Polishchuk and Lutsko, 2013).

For each case of regulated sorting, understanding the underlying mechanism responsible for enrichment of the sorted membrane protein is critical to elucidating the switch. As in several of the foregoing examples, local enrichment is typically achieved by direct or adaptor-mediated interactions of the sorted cargo with an assembl ing vesicle coat complex, resulting in selective export in a budding vesicle or membrane tubule (Bonifacino and Glick, 2004; Traub and Bonifacino, 2013). Alternatively, local enrichment may be driven by accumulation in particular lipid domains (Surma et al., 2012) or interaction with localized domains of assembled cytoskeleton (Irmnejad and von Zastrow, 2014).

Significantly, recent work indicates that the switch in GPP130 sorting may involve a novel mechanism for its local enrichment in the plane of the membrane. When Mn is elevated, the GPP130 luminal stem domain binds Mn, and this binding causes the GPP130 oligomerization/aggregation that is required and sufficient for its altered sorting (Tewari et al., 2014). Here we sought to extend our understanding of this pathway by answering the following questions. Is a TGN-localized vesicle coat complex adaptor involved? If so, are specific sequence elements in GPP130 required, or is this sorting switch something more general such that it acts on any oligomerized/aggregated Golgi protein? Our work implicates γ-adaptin homologous ARF-interacting protein 1 (GGA1) and clathrin in a sorting switch that appears general to Golgi proteins.

RESULTS
Role of the canonical Golgi-lysosome pathway in oligomerization-induced trafficking
Manganese-induced GPP130 trafficking involves redistribution to multivesicular bodies (MVBs), where GPP130 is internalized to intraluminal vesicles and then appearance in LAMP2 positive lysosomes, where its degradation is blocked by leupeptin inhibition of lysosomal hydrolases (Mukhopadhyay et al., 2010). The redistribution is insensitive to a dominant-negative (DN) version of dynamin, microtubule disruption or DN-Rab5 indicating that neither endocytosis nor early endosome trafficking is involved (Mukhopadhyay et al., 2010). In contrast, it is blocked by DN-Rab7-T22N (Mukhopadhyay et al., 2010), which inhibits trafficking from MVBs to lysosomes (Bucci et al., 2000; Vanlindingham and Ceresa, 2009). The canonical route from the Golgi toward lysosomes is mediated by Golgi-associated GGA1 and clathrin (Black and Pelham, 2000; Puertollano et al., 2001b; Zhu et al., 2001). Thus we used a DN version of GGA1 and a small-molecule inhibitor of clathrin to test whether the oligomerization-induced trafficking of GPP130 occurs via this canonical route.

For GGA1, HeLa cells were transfected with either a wild-type version or a version lacking the clathrin-binding domains (GGA1-DN), which is known to block export of lysosome-directed cargo from the TGN (Puertollano et al., 2001b). The constructs were green fluorescent protein (GFP) tagged, and each was Golgi localized upon expression (Figure 1A, rows 1 and 2). Cells were then treated with Mn for 5 h, and GPP130 redistribution was determined. In cells expressing the wild-type GGA1, GPP130 moved from its predominant Golgi localization to peripheral punctae (Figure 1A, row 3), indicating movement to lysosomes, as previously described (Mukhopadhyay et al., 2010). Note that there may have been some interference by the wild-type GGA1 construct. Although the number of punctae arising in Mn-treated untransfected cells (Mukhopadhyay et al., 2010; Tewari et al., 2014) was essentially identical to that in GGA1-transfected cells, loss of GPP130 from the Golgi was slower in GGA1-expressing cells, suggesting that GGA1 overexpression may

| A | GFP | GPP130 | Merge | Threshold (GPP130) |
|---|-----|--------|-------|-------------------|
| + Mn (5h) | GGA1 | * |
| GGA1-DN | |

(B) Cells expressing GGA1 or GGA1-DN were treated with monensin for 30 min to induce GPP130 trafficking to endosomes. (C) Quantified appearance of GPP130 in peripheral punctae of cells expressing GGA1 or GGA1-DN after Mn or monensin (Mon) treatment (mean ± SEM, n = 3, >10 cells/experiment, *p < 0.05). (D) Total cell fluorescence of GPP130 in cells expressing GGA1 or GGA1-DN before or after Mn (mean ± SEM, n = 3, >10 cells/experiment).

FIGURE 1: Dominant-negative GGA1 blocks Mn-induced GPP130 trafficking. (A) Cells were transfected with GFP-tagged GGA1 or GGA1-DN and then either left untreated (Ø) or treated with Mn for 5 h. Images show GFP fluorescence from the transfected proteins and anti-GPP130 staining, with the final panel using uniform thresholding of the GPP130 image to aid visualization of small punctae. Note that GGA1-DN blocked GPP130 appearance in punctae. Bar, 5 μm. (B) Cells expressing GGA1 or GGA1-DN were treated with monensin for 30 min to induce GPP130 trafficking to endosomes. (C) Quantified appearance of GPP130 in peripheral punctae of cells expressing GGA1 or GGA1-DN after Mn or monensin (Mon) treatment (mean ± SEM, n = 3, >10 cells/experiment, *p < 0.05). (D) Total cell fluorescence of GPP130 in cells expressing GGA1 or GGA1-DN before or after Mn (mean ± SEM, n = 3, >10 cells/experiment).
FIGURE 2: Lysosomal redistribution of GPP130-FM. (A) Cells were cotransfected with HA-tagged GPP130-FM and GFP-tagged Rab7 and then treated with a 1-h AP washout. Images compare GFP fluorescence from the transfected proteins and anti-HA staining, with the indicated regions expanded and presented as insets. At the 1-h time point, cells vary in their extent of residual Golgi-localized GPP130-FM. To better illustrate our analysis, the cell shown here has mostly punctae-localized GPP130-FM but equivalent results were obtained in all cells. Arrowheads mark examples of costained punctae. Bars, 10 μm. (B) The percentage of total GPP130-FM punctae counted that were positive for Rab7 is quantified for the indicated time points (mean ± SEM, n = 3, >10 cells/experiment, p < 0.05 for 0 h vs 1 or 5 h). (C, D) Blot and quantification showing level of GPP130-FM in cell extracts prepared at the indicated times after AP washout with tubulin as a loading control (mean ± SEM, n = 3). (E, F) Blot and quantification showing level of GPP130-FM in cell extracts prepared after either 0 or 5 h of AP washout or 24 h of pretreatment with pepstatin and leupeptin (+inh) followed by 5 h of AP washout (mean ± SEM, n = 3).

Given these results for Mn-induced GPP130 redistribution, we also tested the role of GGA1 for GPP130-FM, a previously characterized construct (Tewari et al., 2014) in which GPP130 resides 1–247 are followed by three copies of a modified version of the FKBP12 domain termed FM (after its F36M substitution). The FM domain self-interacts in a manner that is inhibited by the drug AP21998 (AP), an analogue of rapamycin. Washout of AP forms large complexes due to multiplexing of the three FM domains, and, upon washout, GPP130-FM leaves the Golgi, redistributes to peripheral puncta, and is degraded over time (Tewari et al., 2014). As shown, many of the puncta colocalize with rab7, a marker of late endosomes (Figure 2, A and B), and degradation, which occurs over ~5 h (Figure 2, C and D), is blocked by lysosomal inhibitors (Figure 2, E and F). Thus GPP130-FM redistributes to endosomes and lysosomes, where it is degraded. As expected, neither GGA1 nor GGA1-DN altered GPP130-FM localization before AP washout (Figure 3, rows 1 and 2), and redistribution of GPP130-FM was readily apparent in cells expressing wild-type GGA1 (Figure 3, row 3). Of interest, GGA1 partially redistributed along with GPP130-FM, consistent with the possible persistence of a GGA1-based sorting complex associated with GPP130-FM beyond the TGN. In contrast, in cells expressing GGA1-DN the appearance of GPP130-FM in peripheral punctae was significantly reduced (Figure 3, row 4). The inhibitory effect of GGA1-DN was significant (Figure 3B).

Dependence on GGA1 implies a role for clathrin. As a test, we used treatment with Pitstop2, a selective clathrin inhibitor. Pitstop2 occupies the clathrin terminal domain groove, preventing this domain’s interaction with clathrin box ligands, which stalls clathrin-coated-pit dynamics (von Kleist et al., 2011). Only short-term treatments were used to minimize indirect and toxic effects of the drug. HeLa cells expressing GPP130-FM were transferred to serum-free medium (serum interferes with Pitstop2) in the continued presence of AP. Then the washout of AP was carried out for 10 min with medium containing either dimethyl sulfoxide (DMSO) alone or DMSO containing Pitstop2. As expected, the 10-min washout yielded only a partial redistribution in control cells, but the peripheral punctae were clearly evident. In contrast, no such redistribution occurred in cells treated with the clathrin inhibitor, and this was confirmed by quantification (Figure 4). Taken together, the data in this section indicate that trafficking of oligomerized GPP130

have affected exit. In contrast, cells expressing GGA1-DN showed little or no GPP130 redistribution (Figure 1A, row 4). (Also note that thresholding of the GPP130 channel [last column] is presented to illustrate the peripheral punctae, which are dim due to GPP130 degradation. Identical parameters were used for thresholding and all other analyses within an experiment to allow direct comparison.)

Monensin treatment provided a convenient control to test the specificity of the GGA1 inhibition. Monensin is a proton ionophore that neutralizes acidic compartments (Tartakoff, 1983), causing GPP130 redistribution to endosomes presumably because GPP130 endosome-to-Golgi retrieval is pH dependent (Linstedt et al., 1997; Starr et al., 2007). Thus TGN export of GPP130 upon monensin treatment is expected to occur via a distinct pathway from lysosome-directed cargo and occurs independently of GGA1. Indeed, the marked appearance of GPP130 in peripheral endosomes after monensin addition was unabated by expression of either wild-type or GGA1-DN (Figure 1B).

The specific dependence of Mn-induced GPP130 redistribution on GGA1 was confirmed by image analysis for independent experiments quantifying the number of distinct GPP130 punctae per cell. A significant block was evident for GGA1-DN after Mn treatment but not after monensin treatment (Figure 1C). Further, the loss of total GPP130 fluorescence per cell after Mn treatment, which is a measure of its degradation in lysosomes (Mukhopadhyay et al., 2010), was abrogated in cells expressing GGA1-DN (Figure 1D).

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2–11 of the 12-residue cytoplasmic domain in our FM-domain GPP130 construct to create Δcyto-GPP130-FM. As expected, the construct was localized to the Golgi in the continued presence of AP, whereas after AP washout, Δcyto-GPP130-FM redistributed to peripheral punctae in a manner indistinguishable from wild type (Figure 5A). Image analysis confirmed the significant increase in punctae (Figure 5B), as well as the loss of total staining over time, reflecting degradation (Figure 5C). Thus the cytoplasmic domain of GPP130 is not needed for its oligomerization-induced trafficking.

Next we turned our attention to the GPP130 luminal coiled-coil domain, which contains sequence elements that independently mediate Shiga toxin binding and Mn binding, as well as Golgi targeting and endosome cycling (Bachert et al., 2001; Mukhopadhyay et al., 2010; Tewari et al., 2014). The entire coiled-coil stretches from residue 36 to 245. Previous work indicated that a deletion of 88–245 shifts the steady-state location of the protein to the trans-Golgi, blocks its cycling to endosomes, and blocks its Mn responsiveness (Bachert et al., 2001; Mukhopadhyay et al., 2010). Therefore we tested a GPP130-1-87-FM construct to see whether it retained the oligomerization-induced trafficking response. The construct was Golgi localized before AP washout, and, upon washout, it moved into peripheral punctae, ultimately undergoing degradation (Figure 6A). Also shown are the quantified results for both its appearance in peripheral punctae (Figure 6B) and its loss of staining over time (Figure 6C). Thus, even without most of its coiled-coil stem domain, GPP130 underwent oligomerization-induced trafficking and degradation. We reasoned that further deletions might be problematic due to the requirement for Golgi targeting in any construct to be tested. Also, we began to consider the possibility that the response might not be specific to GPP130 but instead part of a general pathway in the Golgi. Therefore we next tested the behavior of cis- and trans-Golgi markers after their forced oligomerization.

Induced oligomerization causes lysosomal targeting of other Golgi proteins

We first studied the cis-Golgi enzyme mannosidase-1 because a suitable construct (mannosidase-1 [Man1]–FM) had already been characterized in which tandem copies of the FM domain and a hemagglutinin (HA) tag were appended to the C-terminus of a truncated mouse α-1,2 mannosidase-1B composed of its cytoplasmic, transmembrane, and stem domains (Rizzo et al., 2013). The construct shows normal cis-Golgi localization in the presence of AP and migration to the trans-Golgi after a short AP washout (Rizzo et al., 2013).

In our experiments, HeLa cells were transfected with the Man1–FM construct in the presence of AP. After 24 h, the AP was removed,
GRASP65 (Figure 7A). Similar to our previous observations after induced oligomerization of GPP130 (by either Mn addition for endogenous GPP130 or AP washout for GPP130-FM), Man1-FM clearly redistributed to endosome-like structures. The peripheral punctae were easily detected by 15 min of AP washout and continued to be present, whereas the overall staining became more and more reduced. GRASP65 in the same cells showed no change.

The effect was quantified by determining both the number of punctae per cell (Figure 7B) and the total fluorescence per cell (Figure 7C). An immunoblotting assay was also used to confirm degradation. As expected, Man1-FM recovery in cell extracts was strongly reduced upon AP washout (Figure 7D), yielding a reduction of >90% by 5 h (Figure 7E). Endogenous GPP130 served as a control and showed no change. Similar to Mn-induced GPP130 and GPP130-FM, redistribution of Man1-FM to peripheral punctae occurred in cells expressing GGA1 but not GGA1-DN (Figure 8A), and this was confirmed by quantifying appearance in punctae after 1 h of AP washout (Figure 8B). Again, the wild-type GGA1 construct may have slowed exit somewhat, but the inhibition by GGA1-DN was significantly greater. Further, the peripheral puncta colocalized with Rab7 (Figure 8, C and D). Taken together, these findings suggest that Man1-FM redistribution occurs via the same pathway as GPP130.

Because both Man1 and GPP130 are predominately in the cis-Golgi at steady state, we next tested whether a trans-Golgi protein would also respond to oligomerization by leaving the Golgi and undergoing degradation. A similar strategy was used in which the cytoplasmic, transmembrane, and stem domains of the trans-Golgi protein galactosyltransferase (GT) were fused to the

and the construct’s localization was determined over time using anti-HA antibodies. As expected, in the presence of AP, Man1-FM was strongly localized to the Golgi, as indicated by comparing its staining pattern in the same cells with that of the cis-Golgi marker

FIGURE 4: Clathrin inhibitor blocks oligomer-induced GPP130 trafficking. (A) GPP130-FM–transfected cells in the presence of AP were washed into serum-free medium lacking AP and containing either vehicle alone (DMSO) or vehicle containing the clathrin inhibitor Pitstop2. The cells were fixed and analyzed after 10 min to minimize indirect and toxic effects of Pitstop2. Images show GFP fluorescence from GPP130-FM and anti-giantin staining to mark the Golgi, as well as thresholded GPP130 images to mark cytoplasmic punctae. Note that Pitstop2 blocked GPP13-FM appearance in punctae. Bar, 5 μm. (B) Quantified appearance of GPP130-FM in peripheral punctae of control and Pitstop2-treated cells after AP washout (mean ± SEM, n = 3, >10 cells/experiment, *p < 0.05).

FIGURE 5: Oligomer-induced GPP130 trafficking is independent of its cytoplasmic domain. (A) Cells were transiently transfected with ∆cyto-GPP130-FM in the presence of AP and then examined at the indicated times after AP washout. Images show GFP fluorescence from ∆cyto-GPP130-FM and anti-giantin staining to mark the Golgi. The final panels present the GPP130 images after uniform thresholding. Note redistribution of ∆cyto-GPP130-FM. Bar, 5 μm. (B) Quantified appearance of ∆cyto-GPP130-FM in peripheral punctae at 0 and 1 h after AP washout (mean ± SEM, n = 3, >10 cells/experiment, *p < 0.01). (C) Determination of total fluorescence per cell after AP washout normalized to average value before AP washout (mean ± SEM, n = 3, >10 cells/experiment, error bars too small to be seen).
transfection alone elevated the level of Bip somewhat. However, AP
GPP130-FM. Transfection frequency was

to test the effect of AP washout, we needed to transfect the cells with

UPR (Oslowski and Urano, 2011). As expected, Bip levels increased

treated with tunicamycin to block N-glycosylation and activate the

which is a standard UPR marker. As a positive control, HeLa cells were

purpose, we assayed up-regulation of the ATPase chaperone Bip,

proteins also activated the unfolded protein response (UPR). For this

quality control, it seemed important, even if unlikely, to test whether

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anterograde direction within the Golgi is most easily explained by

their size-based exclusion from retrieval vesicles (Rizzo et al., 2013).

Of interest, redistribution and degradation of GPP130 and GPP130-

FM are insensitive to nocodazole (our unpublished results; Mukhopadhyay et al., 2010), indicating that an intact Golgi ribbon is not

required, which differs from the intra-Golgi transport of large or ag-

gregated soluble cargo (Lavieu et al., 2014). The requirement for

GGA1 and clathrin suggests that upon reaching the TGN, the Golgi

protein complexes are engaged by the canonical Golgi-to-lysosome

pathway. However, based on GPP130, the sorting appears indepen-

dent of a cytoplasmic sorting signal, raising the possibility that it

also involves yet-to-be-described machinery. Elucidating the mech-

anism of recognition is an important next step, given that it appears
to extend to at least several Golgi membrane proteins, and for each,
it must distinguish between their normal and oligomerized states.

Taken together, these observations suggest the presence of a qual-

yty control pathway in the Golgi based on recognition of aggrega-

tions in the Golgi in which clustered Golgi proteins are routed for degradation.

DISCUSSION

Our interest in the targeting of Golgi pro-

teins to the lysosome started with Mn-

induced degradation of GPP130 (Mukhopadhyay et al., 2010). Subsequently, using mutagenesis, cross-linking, fluorescence re-

covery after photobleaching analysis, and

FM-mediated oligomerization, we found that Mn oligomerizes GPP130 and that oligomerization is required and sufficient to alter GPP130 trafficking such that it moves from the Golgi to lysosomes, where it is de-

graded (Tewari et al., 2014). Here we report the surprising finding that other, unrelated Golgi membrane proteins also left the Golgi and were degraded when their oligomeriza-

tion was induced. We tested membrane proteins with a type II topology, which is the characteristic topology of Golgi glycosyl-

ation enzymes, but proteins of other topolo-

gies present in the Golgi will need to be
tested. Movement of the complexes in an
tandem array of FM domains followed, in this case, by GFP. Before AP washout, the construct was strongly Golgi localized, but after washout, it selectively redistributed to peripheral punctae, and, over time, the total GT-FM levels per cell were reduced (Figure 9A). These changes were marked compared with giantin in the same
cells, and both appearance in punctae (Figure 9B) and selective loss
(Figure 9C) were confirmed by image analysis. As expected, degra-
dation was apparent by immunoblot analysis (Figure 9, D and E).

Cycloheximide treatment showed that all of our constructs (GPP130-

FM, Man1-FM, and GT-GM) were stable in the Golgi in their nono-

ligomerized forms, with redistribution and degradation occurring

only upon oligomerization induced by AP washout (Supplemental

Figure S1).

FIGURE 6: Redistribution of truncated GPP130-FM. (A) Cells expressing GPP130-1-87-FM (cycling deficient and Mn insensitive) were incubated in the absence of AP for the indicated times. Images show thresholded GFP fluorescence from GPP130-1-87-FM and anti-giantin staining to mark the Golgi. Note redistribution and then loss of GPP130-1-87-FM. Bar, 5 μm. (B) Quantified appearance of GPP130-1-87-FM in peripheral punctae after AP washout (mean ± SEM, n = 3, >10 cells/experiment). (C) Total GPP130-1-87-FM or giantin fluorescence as a percentage of starting value and at the indicated times of AP washout (mean ± SEM, n = 3, >10 cells/experiment, error bars too small to be seen).

FIGURE 6: Redistribution of truncated GPP130-FM. (A) Cells expressing GPP130-1-87-FM (cycling deficient and Mn insensitive) were incubated in the absence of AP for the indicated times. Images show thresholded GFP fluorescence from GPP130-1-87-FM and anti-giantin staining to mark the Golgi. Note redistribution and then loss of GPP130-1-87-FM. Bar, 5 μm. (B) Quantified appearance of GPP130-1-87-FM in peripheral punctae after AP washout (mean ± SEM, n = 3, >10 cells/experiment). (C) Total GPP130-1-87-FM or giantin fluorescence as a percentage of starting value and at the indicated times of AP washout (mean ± SEM, n = 3, >10 cells/experiment, error bars too small to be seen).

Involvement of the Golgi in quality control, although perhaps un-

derappreciated, is not new. Studies in both yeast and mammalian
cells have shown that misfolded proteins may leave the ER before

being trafficked back to the ER for dislocation and proteasome-me-

diated degradation (Hammond and Helenius, 1994; Jenness et al.,

1997; Dusseljee et al., 1998; Vashist et al., 2001; Pan et al., 2013;

Iannotti et al., 2014), and the UPR up-regulates ER-to-Golgi trafficking

factors (Travers et al., 2000; Caldwell et al., 2001). Trafficking of se-

lected substrates to the Golgi may assist in ER quality control, but

the mechanistic basis is poorly understood (Caldwell et al., 2001;
In contrast to all of these examples, we assume that the oligomerization/clustering taking place in our experiments is not causing folding defects. The Mn–GPP130 interaction is not yet structurally characterized. Because it is a direct interaction, however, it presumably involves simultaneous coordination of the metal by GPP130 residues on the outer surface of its coiled coil (Tewari et al., 2014). This would cluster the GPP130 dimers without perturbing folding. Similarly, the FM domains we used are positioned well away from the membrane within the Golgi lumen and undergo a well-characterized interaction with one another. Binding via this mechanism is unlikely to cause folding defects, and the UPR was not activated in our experiments. Nevertheless, the FM domain on membrane-anchored proteins creates polymers constrained from entering 40- to 80-nm vesicles and tubules (Rizzo et al., 2013), and it is capable of stapling single cisternae via luminal interactions in trans (Lavieu et al., 2014).

How might oligomerized/clustered Golgi proteins be differentially recognized by GGA1/clathrin in the TGN? At this point, we can only speculate. One possibility is that recognition is based on avidity. For the sake of argument, assume that many membrane proteins share a generic determinant that binds weakly to a hypothetical multiadhesive sorting receptor. Under normal conditions, when the cargo is in its native oligomeric state, the weak interaction with its fast off-rate would yield little sorting. If, on the other hand, the cargo becomes clustered, allowing its many copies to interact simultaneously with the multiadhesive sorting receptor, then sorting would occur. Given that sorting of GPP130 is independent of its cytoplasmic domain, we would predict a luminal cargo/receptor interaction and a cytoplasmic receptor/GGA1 interaction. Among known receptors, both mannose-6-phosphate receptors and sortilins use GGA1 and clathrin to mediate Golgi-to-lysosome trafficking of their cargo (Puertollano et al., 2001; Takatsu et al., 2001; Canuel et al., 2009). Although it is important to test the possible role of these receptors, they are not known to act on integral membrane cargo, and there is no indication of how they would discriminate between clustered and nonclustered cargo.

Another possibility is differential partitioning of oligomerized cargo in the plane of the membrane. By this view, clusters of transmembrane domains would favor lipid microenvironments that are also recognized by GGA1. Arguably, clustering, if it extends to the transmembrane domains, can impose a rigidity of protein orientation due to mismatch of transmembrane domain length with a given bilayer width. A monomer or dimer with a relatively long transmembrane domain would favor lipid microenvironments that are far removed from the sorting receptors and sortilins, which recognize GGA1 and clathrin in the TGN (Reggiori and Pelham, 2002). Among known receptors, both mannose-6-phosphate receptors and sortilins use GGA1 and clathrin to mediate Golgi-to-lysosome trafficking of their cargo (Puertollano et al., 2001; Takatsu et al., 2001; Canuel et al., 2009). Although it is important to test the possible role of these receptors, they are not known to act on integral membrane cargo, and there is no indication of how they would discriminate between clustered and nonclustered cargo.

Arvan et al., 2002). The proteasome is also involved in turnover of cytoplasmically exposed Golgi proteins, at least under certain conditions (Puthenveedu and Linstedt, 2001). Lysosomal-mediated degradation of misfolded Golgi proteins has also been observed (Wang et al., 2011). In yeast, there is a Golgi-localized ubiquitin ligase, Tul1p, that promotes sorting of mutated proteins with polar transmembrane domains to MVBs (Reggiori and Pelham, 2002). Further, at a nonpermissive temperature, the mutated yeast plasma membrane H+ATPase Pma1p1-7 is targeted directly from the Golgi to the endosomal system for vacuolar degradation (Chang and Fink, 1995). In mammalian cells, certain mutated forms of human lipoprotein lipase fail to be secreted and are targeted from the Golgi to lysosomes for degradation (Busca et al., 1996), and the TGN glycoprotein furin is somehow aggregated by lysosomal protease inhibitors, causing its transport to lysosomes (Wolins et al., 1997). Finally, under acute ER stress, glycosylphosphatidylinositol-linked, ER-associated degradation substrates traffic through the Golgi to the cell surface before being degraded in lysosomes (Satpute-Krishnan et al., 2014).
MATERIALS AND METHODS

Antibodies and other reagents
Polyclonal antibodies against GPP130 and giantin and monoclonal antibodies against GPP130 and GFP have been described (Puri et al., 2002; Mukhopadhyay et al., 2010). For immunoblots, monoclonal anti-HA (Sigma-Aldrich, St. Louis, MO) was used at 1:1000, polyclonal anti-Bip (Abcam, Cambridge, MA) was used at 1:500, and anti-\(\gamma\)-tubulin (Sigma-Aldrich) was used at 1:1000. Horseradish peroxidase–conjugated goat anti-rabbit or anti-mouse secondary antibodies were used at 1:3000 (Bio-Rad, Hercules, CA). For immunofluorescence, Alexa Fluor 488 and Alexa Fluor 568 (Invitrogen, Carlsbad, CA) were used at 1:400. AP (now called D/D solubilizer) was purchased from Clontech (Mountain View, CA). Pitstop2 was from Abcam, and tunicamycin was from Sigma-Aldrich. Leupeptin and pepstatin were purchased from Sigma-Aldrich.

Constructs
GPP130-FM, which contains a GFP tag, was previously described (Tewari et al., 2014). A version substituting an HA tag in place of the GFP tag was used for cotransfection with GGA1 constructs. \(\Delta\)cyto-GPP130-FM was engineered by looping out residues 2–11 from the 12-residue cytoplasmic domain in GPP130-FM using a PCR-based loop-out modification of the QuikChange protocol (Stratagene, La Jolla, CA), as described (Tewari et al., 2014). Similarly, looping out residues 88–247 from GPP130-FM created GPP130-1-87-FM. Subcloning residues 1–75 of galactosyltransferase in-frame before the FM repeats and GFP using Nhe1 and EcoRI restriction sites generated GT-FM. All constructs were confirmed by restriction analysis and sequencing. GGA1-GFP and containing relatively long transmembrane domains into thicker sections of bilayer (Lin and London, 2013).

A final possibility is that the clustered proteins are traveling to the lysosome by default. That is, the clustered complexes are left behind after all of the active sorting events take place at the TGN (including retrieval of their nonclustered counterparts), and these residual membranes head to MVB/lysosomes. This contradicts the view that the cell surface is the default pathway in mammalian cells. Of greater importance, it fails to say anything about the role of GGA1 and clathrin or, more generally, how “default” vesicles acquire targeting and fusion factors necessary for delivery to MVB/lysosomes.

We have shown that several Golgi proteins leave the Golgi upon oligomerization and are degraded, suggesting the presence of an ER-independent, Golgi-based quality control system sensitive to oligomeric state. This process seems to provide another level of quality control in the secretory pathway, in addition to the widely known quality control process in the ER. The primary purpose may be to rid cells of clustered or aggregated proteins that accumulate in the Golgi due to defective Golgi-based processing. The pathway may also prove amenable to manipulations, making it therapeutically useful. Oligomerization and the ensuing lysosomal degradation of GPP130 protect against Shiga toxinosis in cultured cells and mice (Mukhopadhyay and Linstedt, 2012). The drug suramin can aggregate the prion protein PrP in a post-ER/Golgi compartment, redirecting it to lysosomes and delaying onset of prion disease (Gilch et al., 2001). Future studies will likely reveal additional components in the oligomer-induced degradation of Golgi proteins and provide new potential targets for therapeutic intervention.
noted otherwise, used for experiments 24 h after transfection. For GGA1 coexpression experiments, only cells expressing moderately high levels of GGA1 and GGA1-DN were analyzed to ensure adequate levels for inhibition by GGA1-DN.

**Controlled polymerization**

Cells were transfected with the FM constructs, and after 16–18 h, they were placed in fresh medium containing 1 μM AP for 24 h. This was followed by another change into medium containing 1 μM AP and 100 μg/ml cyclohexamide for 30 min. Next, the cells were incubated in medium containing only 100 μg/ml cycloheximide for various times of AP washout before fixation and imaging. Only cells exhibiting moderate expression levels were analyzed. Note that the control cells contained both cycloheximide and AP throughout the course of the experiment. For the nocodazole experiments, HeLa cells were treated with 1 μg/ml nocodazole and 100 μg/ml cycloheximide for 3 h, followed by either Mn addition for 1 or 5 h or AP removal for 1 h in GPP130-FM–transfected cells. Nocodazole and cycloheximide were present during the entire course of the experiment. For Pitstop2 experiments, 24 h posttransfection, the GPP130-FM transfected cells were serum deprived for 45 min in the presence of AP, followed by AP washout for 10 min in the presence of Pitstop2 (30 μM) in DMSO or DMSO alone. We also confirmed that this period of serum deprivation did not affect GPP130-FM redistribution to endosomal punctae.

**Immunoblot analysis**

HeLa cells from 60-mm plates were harvested in Tris-SDS lysis buffer (25 mM Tris-Cl, pH 6.7, 1.5% SDS, 0.1% β-mercaptoethanol, and 1 mM phenylmethylsulfonylfluoride), passed through a 25-gauge needle 10 times, and clarified by centrifugation at 15,000 rpm in a microfuge.

**GGA1-DN-GFP** (Puertollano and Alonso, 1999) were a kind gift from Juan Bonifacino (National Institutes of Health, Bethesda, MD). GPP130-GFP (residues 1–247; Mukhopadhyay et al., 2010), GFP-Rab7 (Mukhopadhyay et al., 2010), and GRASP65-GFP (Sengupta et al., 2009) have been described previously.

**Cell culture and transfection**

HeLa cells were grown in MEM with 100 IU/ml penicillin-G and 100 μg/ml streptomycin (Fisher Scientific, Hanover Park, IL) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and maintained at 37°C in a 5% CO₂ incubator. Manganese treatment was performed by adding freshly prepared MnCl₂ to the culture medium at 500 μM for the indicated times. DNA transfections were performed using the JetPEI transfection reagent (PolyPlus, Illkirch, France) according to the manufacturer’s protocol. Cultures were transfected 24 h after plating and, unless otherwise noted, used for experiments 24 h after transfection. For GGA1 coexpression experiments, only cells expressing moderately high levels of GGA1 and GGA1-DN were analyzed to ensure adequate levels for inhibition by GGA1-DN.

**Microscopy and image analysis**

Immunofluorescence on 3% paraformaldehyde–fixed cells was performed exactly as described (Tewari et al., 2014) using a

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**Figure 9:** Induced oligomerization causes degradation of galactosyltransferase. (A) Images show thresholded GFP fluorescence of GT-FM and anti-giantin antibody staining after the indicated times of AP washout. Bar, 5 μm. (B) Quantified number of cytoplasmic punctae per cell is shown for GT-FM and the giantin control at the indicated time points after AP washout (mean ± SEM, n = 3, >10 cells/experiment). (C) Total cell fluorescence levels of GT-FM and giantin were determined using ImageJ and normalized to the level of AP treated cells before washout (mean ± SEM, n = 3, >10 cells/experiment). (D, E) Blot and quantification showing level of GT-FM in cell extracts prepared at the indicated times after AP washout with GPP130 as a loading control (mean ± SEM, n = 3).
spinning-disk confocal microscope and sectioning at 0.3-μm steps. Display images are maximum value projections, except for those that are thresholded to highlight cytoplasmic punctae. For these, a uniform threshold was applied after background subtraction. The images were analyzed using ImageJ (National Institutes of Health, Bethesda, MD) as described (Tewari et al., 2014). To measure fluorescence per cell, background was subtracted from each Z-section, and average value projections were created. Cells were outlined using the Gaussian blur filter, and mean fluorescence was measured using the Measure plug-in of ImageJ. The number of cytoplasmic punctae per cell was determined using the Analyze Particle plug-in of ImageJ. The number of cytoplasmic punctae was determined using the Measure plug-in of ImageJ. The number of cytoplasmic punctae was determined using the Measure plug-in of ImageJ. The number of cytoplasmic punctae was determined using the Measure plug-in of ImageJ. The number of cytoplasmic punctae was determined using the Measure plug-in of ImageJ. The number of cytoplasmic punctae was determined using the Measure plug-in of ImageJ.

FIGURE 10: Oligomerization-induced trafficking is not associated with UPR activation. (A) Immunoblots using anti-Bip or anti-tubulin antibodies of extracts from nontransfected (lanes 1–3) or cells transfected with GPP130-GFP or GPP130-FM (lanes 4–6). The cells were untreated (Ø), tunicamycin treated (Tun) to induce the UPR, Mn treated (Mn) to cause endogenous GPP130 redistribution, left in AP (AP), or subjected to AP washout (w/o) to cause oligomerization of GPP130-FM. (B) Quantification of Bip recovery as a percentage of Bip levels in untreated cells (mean ± SEM, n = 3).

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REFERENCES

Arvan P, Zhao X, Ramos-Castaneda J, Chang A (2002). Secretory pathway quality control operating in Golgi, plasmalemmal, and endosomal systems. Traffic 3, 771–780.

Bachert C, Lee TH, Linstedt AD (2001). Lumenal endosomal and Golgi-retrieval determinants involved in pH-sensitive targeting of an early Golgi protein. Mol Biol Cell 12, 3152–3160.

Beddoe T, Paton AW, Le Nours J, Rossjohn J, Paton JC (2010). Structure, biological functions and applications of the ABX toxins. Trends Biochem Sci 35, 411–418.

Black MW, Pelham HR (2000). A selective transport route from Golgi to late endosomes that requires the yeast GGA proteins. J Cell Biol 151, 587–600.

Bonifacino JS, Glick BS (2004). The mechanisms of vesicle budding and fusion. Cell 116, 153–166.

Brown MS, Goldstein JL (1997). The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell 89, 331–340.

Bucci C, Thomsen P, Nicoziani P, McCarthy J, van Deurs B (2000). Rab7: a key to lysosome biogenesis. Mol Biol Cell 11, 467–480.

Busca R, Martinez M, Vieilla E, Pognonec P, Deeb S, Auwerx J, Reina M, Vilario S (1996). The mutation Gly142→Glu in human lipoprotein lipase produces a missorted protein that is diverted to lysosomes. J Biol Chem 271, 2139–2146.

Caldwell SR, Hill KJ, Cooper AA (2001). Degradation of endoplasmic reticulum (ER) quality control substrates requires transport between the ER and Golgi. J Biol Chem 276, 23296–23303.

Canuel M, Libin Y, Morales CR (2009). The interactomics of sortilin: an ancient lysosomal receptor evolving new functions. Histol Histopathol 24, 481–492.

Chang A, Fink GR (1995). Targeting of the yeast plasma membrane [H+] ATPase: a novel gene AST1 prevents mislocalization of mutant ATPase to the vacuole. J Cell Biol 128, 39–49.

Dusseljee S, Wubbolts R, Verwoerd D, Tulp A, Janssen H, Calafat J, Neefjes J (1998). Removal and degradation of the free MHC class II beta chain in the endoplasmic reticulum requires proteasomes and is accelerated by BFA. J Cell Sci 111, 2217–2226.

Gilch S, Winklhofer KF, Groschup MH, Nunziante M, Lucassen R, Spielhaupter C, Muranyi W, Riesner D, Tatzelt J, Schatzl HM (2001). Intracellular recycling of the MHC class II α and β chains involves multiple recycling steps. Cell 105, 395–396.

Hammond C, Helenius A (1994). Quality control in the secretory pathway: retention of a misfolded viral membrane glycoprotein involves cycling between the ER, intermediate compartment, and Golgi apparatus. J Cell Biol 126, 41–52.

He X, Chang WP, Koelsch G, Tang J (2002). Memapsin 2 (beta-secretase) cytosolic domain binds to the VHS domains of GGA1 and GGA2: implications on the endocytosis mechanism of memapsin 2. FEBS Lett 524, 183–187.

Iannotti MJ, Figard L, Sokac AM, Sifers RN (2014). A Golgi-localized mannosidase (MAN1B1) plays a non-enzymatic gatekeeper role in protein biosynthetic quality control. J Biol Chem 289, 11844–11858.

Irannejad R, von Zastrow M (2014). GPCR signaling along the endocytic pathways. Traffic 3, 771–780.
clathrin adaptor complexes and reveals a cryptic sorting signal in MHC I molecules. Immunity 8, 483–495.

Lin Q, London E (2013). Altering hydrophobic sequence lengths shows that hydrophobic mismatch controls affinity for ordered lipid domains (rafts) in the multitransmembrane strand protein perfringolisyn O. J Biol Chem 288, 1340–1352.

Linstedt AD, Mehta A, Suhan J, Reggio H, Hauri HP (1997). Sequence and overexpression of GPP130/GMPCP: evidence for saturable pH-sensitive targeting of a type II early Golgi membrane protein. Mol Biol Cell 8, 1073–1087.

Mukhopadhyay S, Bachert C, Smith DR, Linstedt AD (2010). Manganese-induced trafficking and turnover of the cis-Golgi glycoprotein GPP130. Mol Biol Cell 21, 1282–1292.

Mukhopadhyay S, Linstedt AD (2012). Manganese blocks intracellular trafficking of Shiga toxin and protects against Shiga toxicity. Science 335, 332–335.

Mukhopadhyay S, Redler B, Linstedt AD (2013). Shiga toxin-binding site for host cell receptor GPP130 reveals unexpected divergence in toxin-trafficking mechanisms. Mol Biol Cell 24, 2311–2318.

Natarajan R, Linstedt AD (2004). A cycling cis-Golgi protein mediates endosome-to-Golgi traffic. Mol Biol Cell 15, 4798–4806.

Olsowksi CM, Urano F (2011). Measuring ER stress and the unfolded protein response using mammalian tissue culture system. Methods Enzymol 490, 71–92.

Pan S, Cheng X, Sifers RN (2013). Golgi-situated endoplasmic reticulum alpha1, 2-mannosidase contributes to the retrieval of ERAD substrates through a direct interaction with gamma-COP. Mol Biol Cell 24, 1111–1121.

Piper RC, Dicki I, Lukacs S (2014). Sorting of mannose 6-phosphate receptors mediated by the GGAs. Mol Biol Cell 24, 2311–2318.

Traub LM, Bonifacino JS (2013). Cargo recognition in clathrin-mediated endocytosis. Cold Spring Harb Perspect Biol 5, a016790.

Travers KJ, Patil CK, Wodicka L, Lockhart DJ, Weissman JS, Walter P (2000). Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. Cell 101, 249–258.

Vanlandingham PA, Ceresa BP (2009). Rab7 regulates late endocytic trafficking downstream of multivesicular body biogenesis and cargo sequestration. J Biol Chem 284, 12110–12124.

Vashist S, Kim W, Belden WJ, Spear ED, Barlowe C, Ng DT (2001). Distinct retrieval and retention mechanisms are required for the quality control of endoplasmic reticulum protein folding. J Cell Biol 155, 3049–3058.

Wang S, Thibault G, Ng DT (2011). Distinct retrieval and retention mechanisms are required for the quality control of endoplasmic reticulum protein folding. J Cell Biol 155, 3049–3058.

Wolins N, Bosshart H, Kuster H, Bonifacino JS (1997). Aggregation as a determinant of protein fate in post-Golgi compartments: role of the luminal domain of furin in lysosomal targeting. J Cell Biol 139, 1735–1745.

Zhu Y, Doray B, Poussa A, Lehto VP, Kornfeld S (2001). Binding of GGA2 to the lysosomal enzyme sorting motif of the mannose 6-phosphate receptor. Science 292, 1716–1718.