Golgi-situated endoplasmic reticulum α-1, 2-mannosidase contributes to the retrieval of ERAD substrates through a direct interaction with γ-COP

Shujuan Pan[a], Xiaoyun Cheng[a,b], and Richard N. Sifers[a,c,d]
Departments of [a]Pathology and Immunology, [c]Molecular and Cellular Biology, and [d]Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX 77030; [b]Department of Lymphoma and Myeloma, University of Texas, MD Anderson Cancer Center, Houston, TX 77054

ABSTRACT **Endoplasmic reticulum (ER) α-1, 2-mannosidase (ERManI) contributes to ER-associated protein degradation (ERAD) by initiating the formation of degradation signals on misfolded N-linked glycoproteins. Despite its inferred intracellular location, we recently discovered that the mammalian homologue is actually localized to the Golgi complex. In the present study, the functional role of Golgi-situated ERManI was investigated. Mass spectrometry analysis and coimmunoprecipitation (co-IP) identified a direct interaction between ERManI and γ-COP, the gamma subunit of coat protein complex I (COPI) that is responsible for Golgi-to-ER retrograde cargo transport. The functional relationship was validated by the requirement of both ERManI and γ-COP to support efficient intracellular clearance of the classical ERAD substrate, null Hong Kong (NHK). In addition, site-directed mutagenesis of suspected γ-COP–binding motifs in the cytoplasmic tail of ERManI was sufficient to disrupt the physical interaction and ablate NHK degradation. Moreover, a physical interaction between NHK, ERManI, and γ-COP was identified by co-IP and Western blotting. RNA interference–mediated knockdown of γ-COP enhanced the association between ERManI and NHK, while diminishing the efficiency of ERAD. Based on these findings, a model is proposed in which ERManI and γ-COP contribute to a Golgi-based quality control module that facilitates the retrieval of captured ERAD substrates back to the ER.**

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INTRODUCTION

The selective clearance of conformationally aberrant proteins is a fundamental process required for maintaining protein homeostasis in living cells (Balch *et al.*, 2008). Endoplasmic reticulum (ER)-associated degradation (ERAD) is part of a proteostasis network that functions in the eukaryotic secretory pathway. It consists of functional modules that regulate distinct steps of the disposal process, including substrate tagging for dislocation into the cytoplasm and elimination by 26S proteasomes (Plemper and Wolf, 1999; Kincaid and Cooper, 2007a; Bernasconi and Molinari, 2011).

ERAD machinery has been speculated to function primarily in the ER because 1) the vast majority of associated components are localized to this organelle (Christianson *et al.*, 2012) and 2) many misfolded proteins are statically retained in this compartment prior to their retrograde transport into the cytoplasm for proteasomal degradation (Nehls *et al.*, 2000; Spiliotis *et al.*, 2002). However, in yeast, numerous studies have demonstrated that proteins carrying subtle conformational defects can escape the ER and travel through more distal compartments of the secretory pathway (Chang and Fink,

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Address correspondence to: Richard N. Sifers (rsifers@bcm.edu).

Abbreviations used: AAT, α1-antitrypsin; ATZ, AAT variant Z; BFA, brefeldin A; co-IP, coimmunoprecipitation; COPI, coat protein complex I; ER, endoplasmic reticulum; ERAD, ER-associated protein degradation; ERManI, ER α-1, 2-mannosidase; FBS, fetal bovine serum; GRP78/BiP, glucose-regulated protein, 78 kDa/immunoglobin-binding protein; GST, glutathione *S*-transferase; IgG, immunoglobulin G; NHK, null Hong Kong; RNAi, RNA interference.

1995; Jenness *et al.*, 1997). Also, in both fission yeast and mammalian cells, proteins that exhibit a severe misfolded conformation can travel to post-ER compartments (Spear and Ng, 2003; Kincaid and Cooper, 2007b). These observations imply that the capacity of the ER to retain misfolded proteins is limited, requiring the involvement of post-ER surveillance systems as a means to prevent premature secretion and to ensure the elimination of escaped misfolded proteins.

Evidence for involvement of the Golgi complex as a component of ERAD has emerged from observations in both yeast and mammalian cells, in which a number of ERAD substrates undergo vesicular cycling through early Golgi compartments (Hammond and Helenius, 1994; Caldwell *et al.*, 2001; Vashist *et al.*, 2001; Kincaid and Cooper, 2007b). Direct involvement of this organelle in ERAD has been further supported by a recent finding in which the three traditional mammalian Golgi-localized α-1, 2-mannosidases (IA, IB, and IC) contribute to the intracellular degradation of misfolded glycoproteins (Hosokawa *et al.*, 2007). Irrespective of these observations, little is known regarding the mechanism by which the Golgi-localized machinery might contribute to the operation of a potential, possibly secondary, quality control module within an expanded ERAD network.

ER α-1, 2-mannosidase I (ERManI) is a type II transmembrane protein capable of initiating the ERAD of numerous misfolded N-glycosylated protein substrates (Jakob *et al.*, 1998; Gonzalez *et al.*, 1999; Tremblay and Herscovics, 1999; Cabral *et al.*, 2000; Hosokawa *et al.*, 2003; Wu *et al.*, 2003). This role was first identified in *Saccharomyces cerevisiae* as a mannosidase that catalyzes the cleavage of a terminal α-1, 2-mannose unit from the middle B branch of the N-linked glycan Man9GlcNAc to generate Man8GlcNAc (Jelinek-Kelly *et al.*, 1985). The corresponding gene cloned from *Saccharomyces cerevisiae*, designated *MNS1* (Camirand *et al.*, 1991), was implicated in ERAD, because its deletion was sufficient to severely impair the intracellular degradation of misfolded N-glycosylated protein substrates (Jakob *et al.*, 1998). Following this lead, the mammalian orthologue was cloned and designated "ERManI" (Gonzalez *et al.*, 1999; Tremblay and Herscovics, 1999). The inhibition of ERManI activity, either with the α-1, 2-mannosidase–specific inhibitor kifunensine or small interfering RNA (siRNA)-mediated knockdown, significantly hindered the intracellular degradation of luminal ERAD substrates such as α1-antitrypsin (AAT) variant null Hong Kong (NHK) (Cabral *et al.*, 2000, 2002; Hosokawa *et al.*, 2003, 2007; Wu *et al.*, 2003; Karaveg *et al.*, 2005; Avezov *et al.*, 2008).

Based on the aforementioned observations, ERManI was designated as a critical component in the mammalian glycoprotein ERAD network that contributes to the generation of a glycan-based component of a bipartite degradation signal, in which nonnative protein structure functions as the additional signal component (Sifers, 2010b). The function of ERManI in cleaving the terminal mannose unit from the B branch appears to be synergistic with the catalytic activity of EDEM1 in unmasking the penultimate α-1, 6–linked mannose on the C branch of the N-glycan, which generates the glycan-based component of the degradation signal in both yeast and mammalian cells (Hirao *et al.*, 2006; Olivari *et al.*, 2006; Clerc *et al.*, 2009; Hosokawa *et al.*, 2009, 2010). Subsequent recognition of the modified glycans by the ER-situated lectins OS9 and XTP3-B is suspected to initiate a series of events that culminates in the retrograde translocation of N-glycosylated ERAD substrates into the cytoplasm for ubiquitinylation and elimination by 26S proteasomes (Satoh *et al.*, 2010). The catalytic activity of ERManI is relatively weak under basal conditions due to an extremely low level of the enzyme (Wu *et al.*, 2003), implying that mannose units are preferentially removed from glycoproteins that have lingered for long periods in the early secretory pathway. Based on this series of experimental observations, ERManI was designated as a "timer" that initiates the ERAD of newly synthesized N-glycosylated proteins unable to attain native structure (Cabral *et al.*, 2001).

Although ~50% homologous to the primary amino acid sequences of Golgi α-1, 2-mannosidases IA, IB, and IC (Gonzalez *et al.*, 1999; Tremblay and Herscovics, 1999), human ERManI was initially predicted to function in the ER based on the localization of its yeast orthologue MNS1 (Burke *et al.*, 1996). However, the subcellular localization of the mammalian orthologue has been controversial, mostly due to the poor specificity of available anti-ERManI polyclonal antibodies that were useful only for transient expression studies (Gonzalez *et al.*, 1999; Avezov *et al.*, 2008). Recently, after generating a panel of highly specific monoclonal antibodies against human ERManI, we made the discovery that the mammalian orthologue is actually localized to the Golgi complex, and several subsequent lines of evidence supported that conclusion (Pan *et al.*, 2011). The spatial separation of this central ERAD component from the remaining ER-situated machinery raised a question as to where it functions as a participant in the mammalian ERAD system. In a subsequent set of experiments, directed localization of recombinant ERManI to the ER led to an enhanced rate of mannose removal, without accelerating ERAD substrate degradation over that of the wild-type molecule (Pan *et al.*, 2011). These observations strongly argued against the previously accepted concept that ERManI functions as an enzyme in the ER and support a model in which ERManI's role in ERAD requires residence in the Golgi complex. Consistent with this notion, addition of the ER-to-Golgi recycling amino acid sequence KDEL at the carboxy terminus of NHK significantly hindered intracellular degradation of this molecule (Le *et al.*, 1990).

A central goal for the present study was to experimentally investigate how Golgi-localized ERManI contributes to the proposed temporospatial expansion of the mammalian ERAD network (Pan *et al.*, 2011). To this end, a direct interaction was identified between ERManI and γ-COP, a subunit of COPI coatomers that promote Golgi-to-ER retrograde vesicular transport. Additional supporting lines of evidence, including a detectable association between ERManI, γ-COP, and NHK, support a model in which the mannosidase contributes to the establishment of a multifunctional gatekeeper that recruits escaped ERAD substrates into COPI vesicles for retrograde transport to the ER.

RESULTS

Direct interaction between ERManI and γ-COP

For obtaining mechanistic insight about the functional significance of Golgi-localized ERManI in ERAD, endogenous immunocomplexes were isolated from MCF7 (a human breast epithelial cell line) cells with 1D6, a well-characterized anti-ERManI monoclonal antibody previously generated by our lab (Pan *et al.*, 2011). MCF7 cells were chosen because of the relatively high abundance of ERManI in this cell line (Supplemental Figure S1). The immunocomplexes were resolved by SDS–PAGE and detected by silver staining (Figure 1A). Immunoprecipitated proteins were subjected to in-gel digestion followed by mass spectrometry analysis. ~100 proteins were identified to be specifically associated with ERManI (Supplemental Table S1). Among these, several Golgi-localized proteins (Table 1) were chosen for further validation using reciprocal coimmunoprecipitation (co-IP). As shown in Figure 1, B and C, immunocomplexes containing ERManI and γ-COP were readily detected using this methodology. A similar phenomenon was also observed when the MCF7 cells were replaced with HeLa cells (Figure S2A). Two of the additional

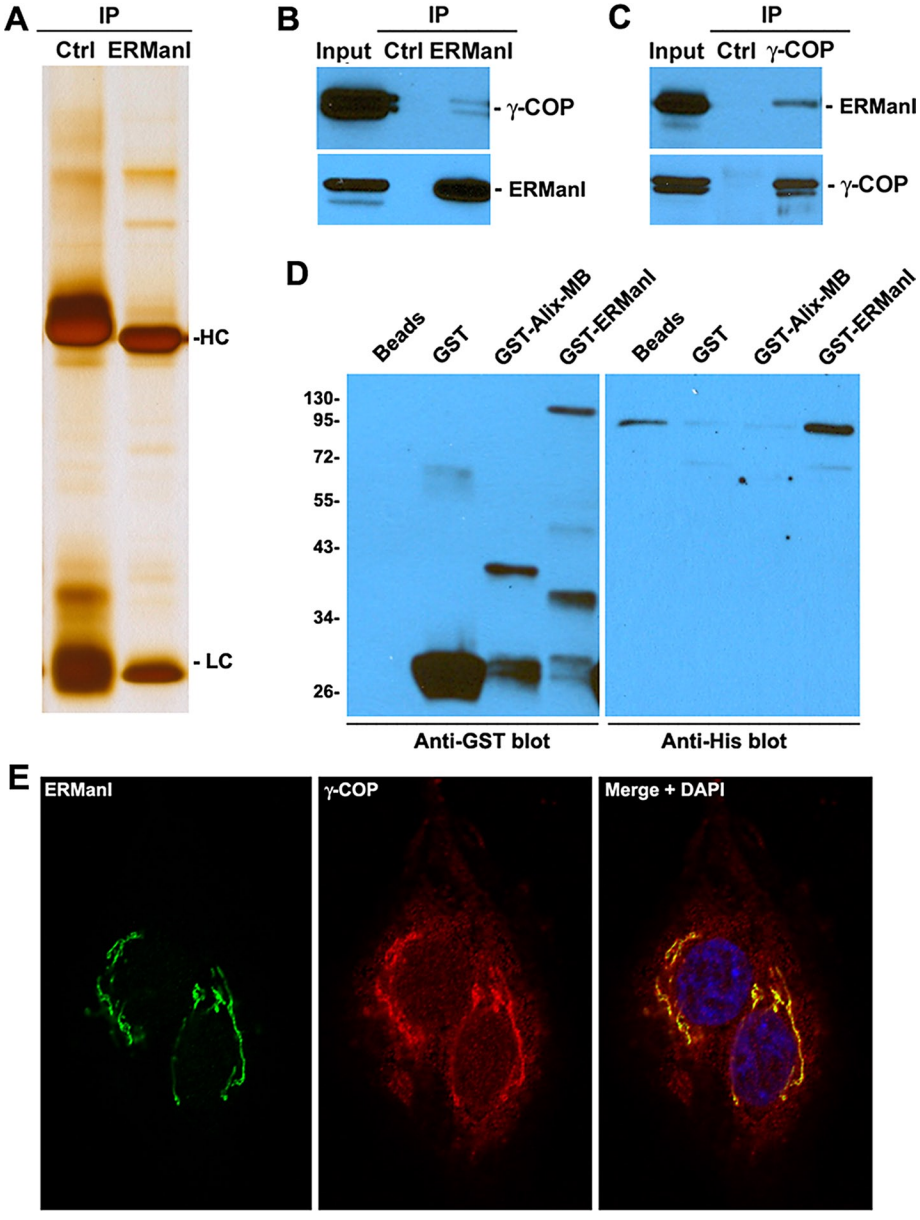

FIGURE 1: ERManI directly binds to γ-COP. (A) Image of a silver-stained SDS–PAGE gel showing proteins differentially immunoprecipitated by 10 μg either mouse IgG (Ctrl) or anti-ERManI antibody 1D6 (ERManI) from extracts derived from 1 ml MCF7 cells. HC, heavy chain; LC, light chain. (B) Extracts from 20 μl of MCF7 cells were subjected to IP with 1 μg of either mouse IgG (Ctrl) or antibodies against ERManI, and the proteins bound to the beads were eluted and separated on SDS–PAGE; this was followed by Western blotting with antibodies against γ-COP or ERManI. Input: 5% of the extracts were loaded in parallel. (C) Immunocomplexes isolated from 20 μl MCF7 cell extracts by either rabbit IgG (Ctrl) or anti–γ-COP antibodies were separated on SDS–PAGE; this was followed by Western blotting with antibodies against γ-COP or ERManI. Input was 5% of the extracts loaded in parallel. (D) Ten microliters of blank glutathione agarose beads or agarose beads conjugated with 0.5 μg purified GST, GST-Alix-MB, or GST-ERManI recombinant proteins was incubated with 0.5 μg purified His-tagged γ-COP. After stringent washing, the protein-bound beads were eluted and resolved by SDS–PAGE; this was followed by Western blotting with anti-His antibodies (right). The membrane was subsequently stripped; this was followed by Western blotting with anti-GST antibodies (left). (E) Confocal images of HeLa cells costained with antibodies against ERManI and γ-COP. The primary antibodies were detected with secondary antibodies conjugated with Alexa Fluor 488 (for ERManI) and Alexa Fluor 555 (for γ-COP), respectively. The cells were subsequently counterstained with 4′,6-diamidino-2-phenylindole.

candidate proteins, including GBF1 and β′-COP, could be detected in ERManI immunocomplexes, but no ERManI was detected in reciprocal IPs (Figure S2, B and C). Another candidate protein, COG1, could not be validated by IP in either direction (Figure S2D). These results implied that a stable interaction exists between ERManI and γ-COP, whereas weaker or indirect interactions exist between ERManI and β′-COP, GBF1, or COG1. To determine whether the ERManI/γ-COP interaction is direct, we performed an in vitro binding assay using glutathione *S*-transferase (GST)-ERManI and His-γ-COP purified from bacteria. As shown in Figure 1D, His-γ-COP was pulled down with GST-ERManI, but not by GST or an irrelevant protein Alix-MB (a truncated form of an irrelevant protein named Alix), indicating a direct interaction.

Colocalization of ERManI and γ-COP

γ-COP (Stenbeck *et al.*, 1992; Wegmann *et al.*, 2004), β′-COP (Stenbeck *et al.*, 1993), and GBF1 (Garcia-Mata and Sztul, 2003) are known Golgi-associated proteins that all contribute to the formation of COPI vesicles, which are responsible for the retrieval of Golgi-localized proteins back to the ER (Lowe and Kreis, 1998; Nickel *et al.*, 2002). For example, γ-COP and β′-COP are the core subunits of COPI coatomers (Nickel *et al.*, 2002), and a direct interaction between γ-COP and GBF1 initiates the formation of COPI-coated vesicles on the Golgi membrane (Deng *et al.*, 2009). The detection of these proteins in the immunocomplexes implied that ERManI is localized to the Golgi membrane in proximity to where COPI-coated vesicles are formed. Coimmunostaining in HeLa cells was next performed to identify the intracellular location where ERManI and γ-COP interact. As shown in Figure 1E, ERManI staining decorated a typical Golgi structure, which overlapped with the staining for γ-COP. Besides the localization in the Golgi, γ-COP also exhibited punctate staining in the cytoplasm, the latter likely representing the ER–Golgi intermediate compartment in which COPI vesicles are often located (Bannykh *et al.*, 1998; Lowe and Kreis, 1998; Marie *et al.*, 2009). However, no ERManI was observed in these cytoplasmic punctate structures. Considering the presence of both GBF1 and γ-COP in ERManI immunocomplexes, the result strongly indicated that the interaction between ERManI and γ-COP occurs on the Golgi membrane, where γ-COP is recruited (Waters *et al.*, 1991) through a process assisted by GBF1 (Szul *et al.*, 2007). In addition, because COPI

Protein name	Accession number	Peptide identified
Coatomer subunit β′ [*Homo sapiens*]	gi4758032	VFNYNTLER
Coatomer subunit γ [*Homo sapiens*]	gi11559929	SVPLATAPmAEQR
Golgi-specific BFA-resistant guanine nucleotide exchange factor 1 isoform 1 (GBF1) [*Homo sapiens*]	gi4758416	LLENISPADVGGmEETR mQALTYLQR
Conserved oligomeric Golgi complex subunit 1 (COG1) [*Homo sapiens*]	gi21237783	aTAATSPALKR STAGDPTVPGSLFR

Name, accession number, and identified peptide of Golgi-localized proteins present in ERManI immunocomplexes.

TABLE 1: ERManI-associated Golgi-localized proteins identified by mass spectrometry.

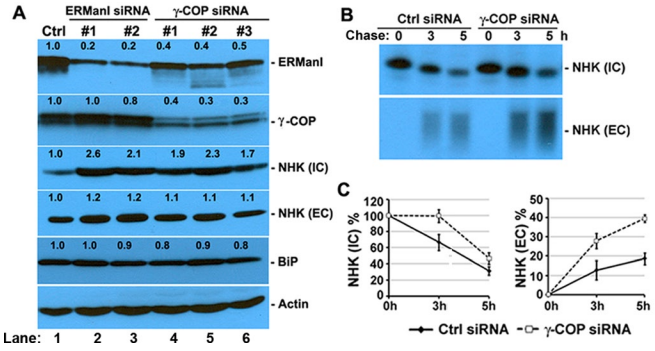

FIGURE 2: γ-COP is required for efficient ERAD. (A) HeLa cells were transfected with NHK together with scrambled siRNA (Ctrl), anti-ERManI siRNA 1 and 2, or anti-γ-COP siRNA 1, 2, and 3. At 48 h posttransfection, cells were switched to serum-free medium for 6 h before both the cells and the medium were separately collected. The cells were lysed, and ~20 μg of the total extracted proteins were separated by SDS–PAGE; this was followed by Western blotting using antibodies against ERManI, γ-COP, AAT, BiP, and actin. The NHK present in the medium was isolated by IP and then treated by PNGase prior to SDS–PAGE and Western blotting using antibodies against AAT. (B) HeLa cells were transfected with NHK together with either scrambled siRNA (Ctrl) or γ-COP–specific siRNA 1. At 24 h posttransfection, cells were evenly divided into four groups in six-well plates and cultured for an additional 24 h. Subsequently, cells were pulse-labeled with [^{35}S]methionine for 30 min and chased for 3 h and 5 h. NHK present both in the cells (IC) and the medium (EC) was collected at each time point by IP, resolved on SDS–PAGE, and detected by autoradiography. (C) Percentages of NHK present in both IC and EC at each indicated time point after quantification of images obtained from three replicate experiments shown (B). Error bars represent SDs.

coatomers are recruited to the membrane en bloc (Hara-Kuge *et al.*, 1994), ERManI is likely localized at, or near, sites on the Golgi membrane where COPI coats are formed.

γ-COP participates in the efficient clearance of NHK

Considering the involvement of ERManI in ERAD, the direct interaction identified above raised the possibility that γ-COP functionally participates in ERAD as well. For testing this hypothesis, HeLa cells were transfected separately with three siRNAs, each of which targets a distinct sequence on the γ-COP mRNA. Effects on ERAD were evaluated by monitoring changes in the concentration of the terminally misfolded genetic variant NHK of AAT, which is a representative N-glycosylated ERAD substrate. Transfections with two separate ERManI siRNAs served as positive controls. At 48 h posttransfection, both ERManI siRNAs resulted in ~80% knockdown of endogenous ERManI without altering γ-COP expression (Figure 2A, lanes 2 and 3). In contrast, the manipulation resulted in a twofold increase in the intracellular concentration of NHK and an ~20% increase in extracellular NHK, implying that impaired degradation led to enhanced secretion of the molecules (Figure 2A, compare lanes 2 and 3 with lane 1). Because NHK is missing amino acids required to achieve conformational maturation, these data imply that the intracellular retention system had been saturated to an even greater extent than under control conditions. Importantly, individual transfections with each of the three γ-COP–specific siRNAs led to a 60–70% of knockdown in γ-COP expression, resulting in a 1.7- to 2.3-fold increase in intracellular NHK and ~10% increase in extracellular NHK (Figure 2A, lanes 4–6). Such changes did not reflect induction of the unfolded protein response, as the level of GRP78/BiP (glucose-regulated protein, 78 kDa/immunoglobin-binding protein) was unaffected (Figure 2A, compare lanes 4–6 with lane 1). To further determine whether the increased intracellular and extracellular levels of NHK were due to the hindrance of its degradation, we cotransfected HeLa cells with NHK together with one of the γ-COP siRNAs and monitored the degradation of NHK by metabolic pulse–chase radiolabeling. As shown in Figure 2, B and C, 30% and ~20% of intracellular and extracellular NHK levels, respectively, remained at 5 h after synthesis in cells transfected with control siRNAs. In contrast, ~45% and ~40% of the intracellular and extracellular NHK, respectively, remained in response to transfection with γ-COP–specific siRNAs, indicating NHK degradation was significantly hindered as a result of γ-COP knockdown. These results, in addition to the direct interactions demonstrated above, indicate that ERManI and γ-COP functionally partner in orchestrating the ERAD of an N-glycosylated ERAD substrate.

ERManI requires γ-COP to function in ERAD

Previous studies have demonstrated that the overexpression of transfected recombinant ERManI accelerates the intracellular degradation of NHK (Hosokawa *et al.*, 2003; Pan *et al.*, 2011). For determination of whether this capacity requires the involvement of γ-COP, HeLa cells transfected with NHK cDNA, were cotransfected with either control siRNA or γ-COP–specific siRNA. The fate of newly synthesized NHK was monitored by ^{35}S metabolic pulse–chase radiolabeling 48 h posttransfection. In the presence of γ-COP–specific siRNA, the increased intracellular degradation rate of NHK due to overexpressed ERManI was completely inhibited, and the amount of NHK secreted into the medium was significantly increased as compared with control (Figure 3, B and C). Although the total ERManI level was moderately diminished in the presence of γ-COP–specific siRNAs (Figure 3A, compare lane 3 with lane 2), this could not account for the complete inhibition of intracellular degradation of NHK, because the overall ERManI level was still greater than the endogenous protein detected in untransfected cells (Figure 3A, compare lane 3 with lane 1). These results indicated that the capacity of ERManI to accelerate ERAD of NHK is dependent on γ-COP.

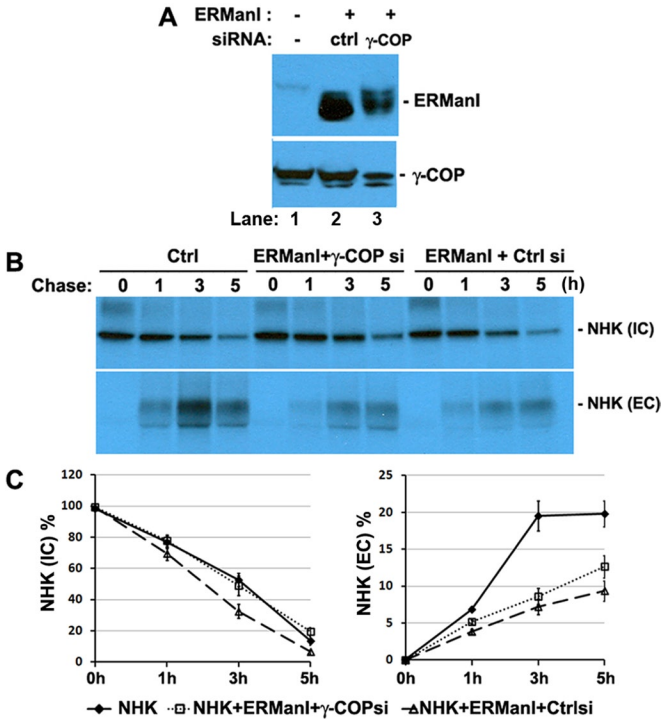

FIGURE 3: γ-COP is required for overexpressed ERManI to accelerate degradation of NHK. HeLa cells were transfected with NHK alone or NHK together with either scrambled siRNA (ctrl) or γ-COP–specific siRNA 1 in combination with ERManI cDNA constructs. At 24 h posttransfection, cells were evenly divided into five groups in six-well plates and cultured for an additional 24 h. (A) One group of cells was lysed directly, and ~20 μg of the extracted proteins was separated on SDS–PAGE; this was followed by Western blotting with antibodies against ERManI or γ-COP. (B) Four groups of cells were pulse-labeled with [^{35}S]methionine for 30 min and chased for 1 h, 3 h, and 5 h. NHK present in both IC and EC portions was collected at each time point by IP, resolved on SDS–PAGE, and detected by autoradiography. (C) Quantified mean values from three parallel experiments were plotted. Error bars represent SDs.

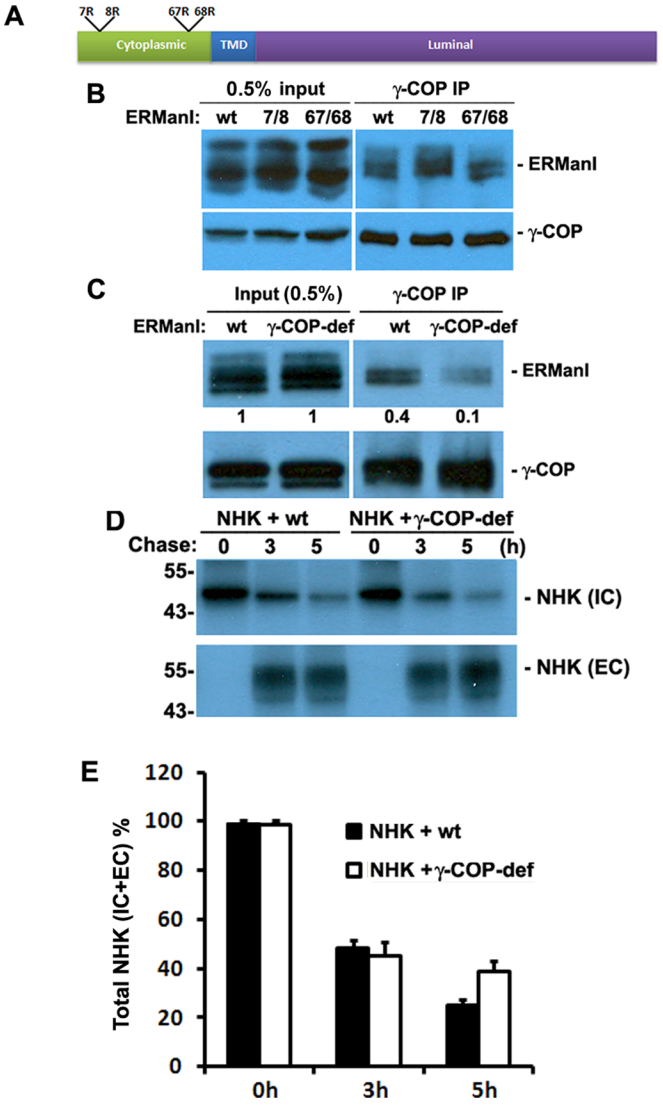

FIGURE 4: γ-COP–binding motifs are required for ERManI to facilitate ERAD. (A) Illustration of di-arginine residues localized at the cytoplasmic tail of ERManI. TMD, transmembrane domain. (B) HeLa cells transfected with either wild-type ERManI (wt) cDNA or ERManI cDNAs containing arginine-to-alanine mutations at residues 7 and 8 or 67 and 68. At 48 h posttransfection, cells were lysed; this was followed by IP of γ-COP. The amounts of ERManI present in the immunocomplexes were detected by Western blotting. Loading control: 5% of total extracts. (C) Either wild-type (wt) ERManI or ERManI cDNA containing arginine-to-alanine mutations at positions 7, 8, 67, and 68 (γ-COP-def) were transfected into HeLa cells. At 48 h posttransfection, cells were lysed, and the cell extracts were subjected to IP with anti–γ-COP antibodies; this was followed by Western blotting with either anti-ERManI antibodies or γ-COP antibodies. Numbers show the relative quantities of ERManI in the γ-COP immunocomplex as compared with that in the 5% of the lysates. (D) HeLa cells were transfected with NHK together with either wild-type ERManI cDNA (wt) or γ-COP-def. At 48 h posttransfection, cells were subjected to pulse–chase assay, and the NHK present both in the cells (IC) and in the media was isolated and detected by autoradiography. (E) Total amounts of NHK (combined IC and EC portions) quantified from of images obtained from three replicate experiments, as shown in (D). Error bars represent SDs.

γ-COP–binding motifs endow ERManI with the capacity to retrieve ERAD substrates

γ-COP is known to directly interact with the cytoplasmic tails of type I transmembrane proteins via a C-terminal KKxx (x represents any amino acids) motif (Harter *et al.*, 1996). Whether γ-COP directly interacts with a similar motif in the N-termini of type II transmembrane proteins is still unclear. Several studies have, however, demonstrated that dibasic motifs (RRxx or RKxx) within the N-terminal cytoplasmic tails of type II transmembrane proteins directs their COPI-mediated vesicular retrieval from the Golgi complex back to the ER (Schutze *et al.*, 1994; Lowe and Kreis, 1998). In support of this notion, two suspected sets of dibasic residues at positions 7–8 and 67–68 were detected in the N-terminal cytoplasmic tail of ERManI (Figure 4A). For determining whether these might mediate direct binding to γ-COP, each set was mutated to di-alanine (AA) residues, and the effect on the ERManI/γ-COP interaction was determined by co-IP. As shown in Figure 4B, as compared with the amount of wild-type ERManI pulled down by γ-COP, neither of the individual mutations (ERManI^{7A8A}, ERManI^{67A68A}) disrupted the association. To determine whether disruption of the ERManI/γ-COP interaction requires mutations at both dibasic motifs, we mutated all four arginine residues into alanines, and the consequences were evaluated by co-IP. As shown in Figure 4C, the combination of mutations significantly inhibited the ERManI interaction with γ-COP (~80%), confirming their dual involvement. For subsequent experiments, the

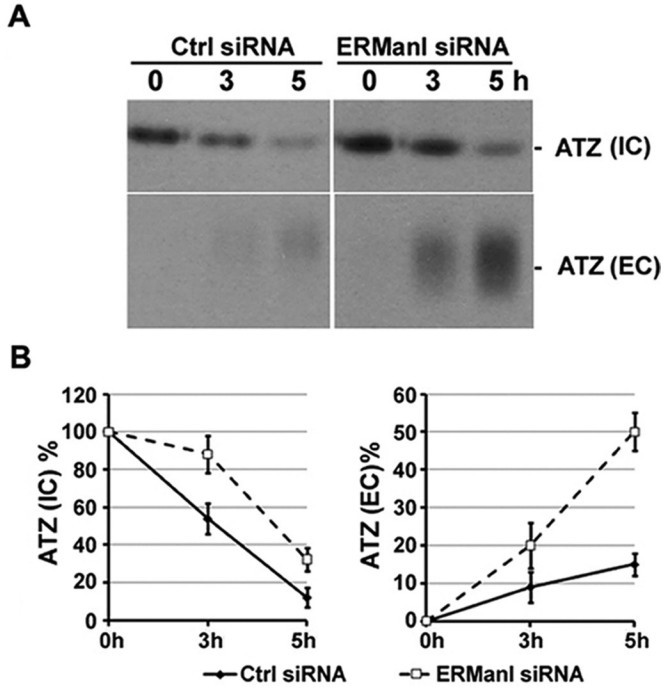

FIGURE 5: ERManI is required for ERAD substrate retention. (A) ATZ cDNA was cotransfected with either control siRNA or ERManI-specific siRNA. At 48 h posttransfection, cells were labeled with [^{35}S]methionine for 30 min and chased for 3 h and 5 h. ATZ immunoprecipitated from cells (IC) and medium (EC) was resolved by SDS–PAGE and detected by autoradiography. (B) Quantified ATZ present in IC and EC portions from images obtained from three replicate experiments as shown in (E). Error bars represent SDs.

recombinant protein containing both mutations was designated ERManI$^{\gamma\text{-COP-def}}$.

For determining whether the functional dependence between ERManI and γ-COP reflects their direct interaction, either wild-type ERManI or ERManI$^{\gamma\text{-COP-def}}$ cDNA was cotransfected, along with NHK, into HeLa cells. At 48 h posttransfection, the fate of NHK was monitored by [^{35}S]methionine pulse-labeling. As compared with the effects generated by the wild-type ERManI, expression of the mutated protein led to a significant increase in NHK secretion (Figure 4D), resulting in a 50% increase of total NHK (intracellular plus extracellular) levels at 5 h of chase (Figure 4E), possibly reflecting impaired degradation and enhanced secretion as sequential events. Importantly, the results obtained through the expression of ERManI$^{\gamma\text{-COP-def}}$ were not observed when replaced with either single di-arginine motif mutant (Figure S3). These findings demonstrated that ERManI lost its capacity to accelerate NHK degradation in response to the mutation of both di-arginine groups in the cytoplasmic tail, indicating that direct binding of ERManI to γ-COP is required for the efficient disposal of this ERAD substrate.

ERManI contributes to ERAD substrate retention

It is noteworthy that the failure of recombinant ERManI$^{\gamma\text{-COP-def}}$ to accelerate NHK degradation is mainly reflected by enhanced secretion as compared with cells expressing the wild-type recombinant protein (Figure 4, D and E). The latter observation indicated that the uncoupling of ERManI from ERAD, via loss of γ-COP–binding motifs, was sufficient to exert a dominant-negative effect on ERAD substrate retention, thereby demonstrating the interconnected nature of substrate retention and retrieval. To confirm that our observation of ERAD substrate retention was not restricted to NHK, we used metabolic pulse–chase radiolabeling and IP in HeLa cells to examine the consequences of endogenous ERManI knockdown on the fate of another AAT variant, designated ATZ. An ~90% knockdown of endogenous ERManI led to a breach in the intracellular retention of radiolabeled ATZ as judged by a threefold-enhanced secretion into the medium as compared with untreated cells (Figure 5, A and B). We concluded that loss of the actual ERManI molecules, rather than the inhibition of ERAD, had led to enhanced ATZ secretion, because this phenomenon has never been observed (in any cell line) in response to the inhibition of the essential α-1, 2-mannosidase activity (Cabral *et al.*, 2000, 2002; Hosokawa *et al.*, 2003; Wu *et al.*, 2003; Avezov *et al.*, 2008).

Physical interaction between ERManI, γ-COP, and ERAD substrates

Results generated from the preceding set of experiments are consistent with the hypothesis that ERManI functions as part of the Golgi-based retention system that precedes the incorporation of captured ERAD substrates into COPI vesicles en route to the ER. γ-COP is a major component of COPI coatomers that mediate the retrograde transport of protein cargo. The functional interdependence of ERManI and γ-COP during ERAD therefore raised the possibility that the ERAD substrates recognized by ERManI in the Golgi complex can be retrieved back to the ER via COPI vesicles. By localizing at COPI vesicle assembly sites and being endowed with γ-COP–binding motifs, ERManI could contribute to the retention system in a manner that loads ERAD substrates into COPI vesicles. As an initial step in the testing of this critical hypothesis, it was important to determine whether a physical association between ERManI, γ-COP, and ERAD substrates exists. To this end, NHK was transfected into HeLa cells, and its association with the endogenous candidate proteins was examined by a series of co-IP experiments. Consistent with the hypothesis, NHK was identified in the ERManI immunocomplexes (Figure 6A), and both ERManI and NHK were detected in γ-COP immunocomplexes (Figure 6B), indicating their physical associations.

γ-COP is required for the dissociation of NHK from ERManI-containing complexes

On the positioning of ERAD substrates in close proximity to COPI vesicles, ERManI itself could either be recruited into the vesicles or remain in the Golgi complex. To distinguish between these two possibilities, we transfected NHK into HeLa cells and performed coimmunostaining of ERManI, NHK, and γ-COP. A distinct Golgi localization of ERManI was observed, irrespective of the presence or absence of NHK (Figure 6C), supporting the latter possibility. Furthermore, much of the NHK was dispersed throughout the cytoplasm in a reticular pattern, with only a small portion colocalizing with ERManI and γ-COP in the Golgi complex. This observation implied that only a small portion of NHK escapes beyond the ER at any one time, consistent with previous observations that misfolded proteins are primarily retained in the ER (Nehls *et al.*, 2000; Spiliotis *et al.*, 2002). Finally, if ERManI detaches from NHK after delivering it to COPI vesicles, then eliminating the latter event should result in more ERManI associated with NHK. For testing this possibility, HeLa cells were transiently transfected with NHK together with an siRNA targeting γ-COP or a scrambled siRNA as control. At 48 h posttransfection, NHK was pulled down, and the amounts of ERManI and γ-COP in the immunocomplexes were examined by Western blotting and quantified by densitometry. As shown in Figure 6D, an ~50% knockdown of γ-COP resulted in an approximately fivefold increase

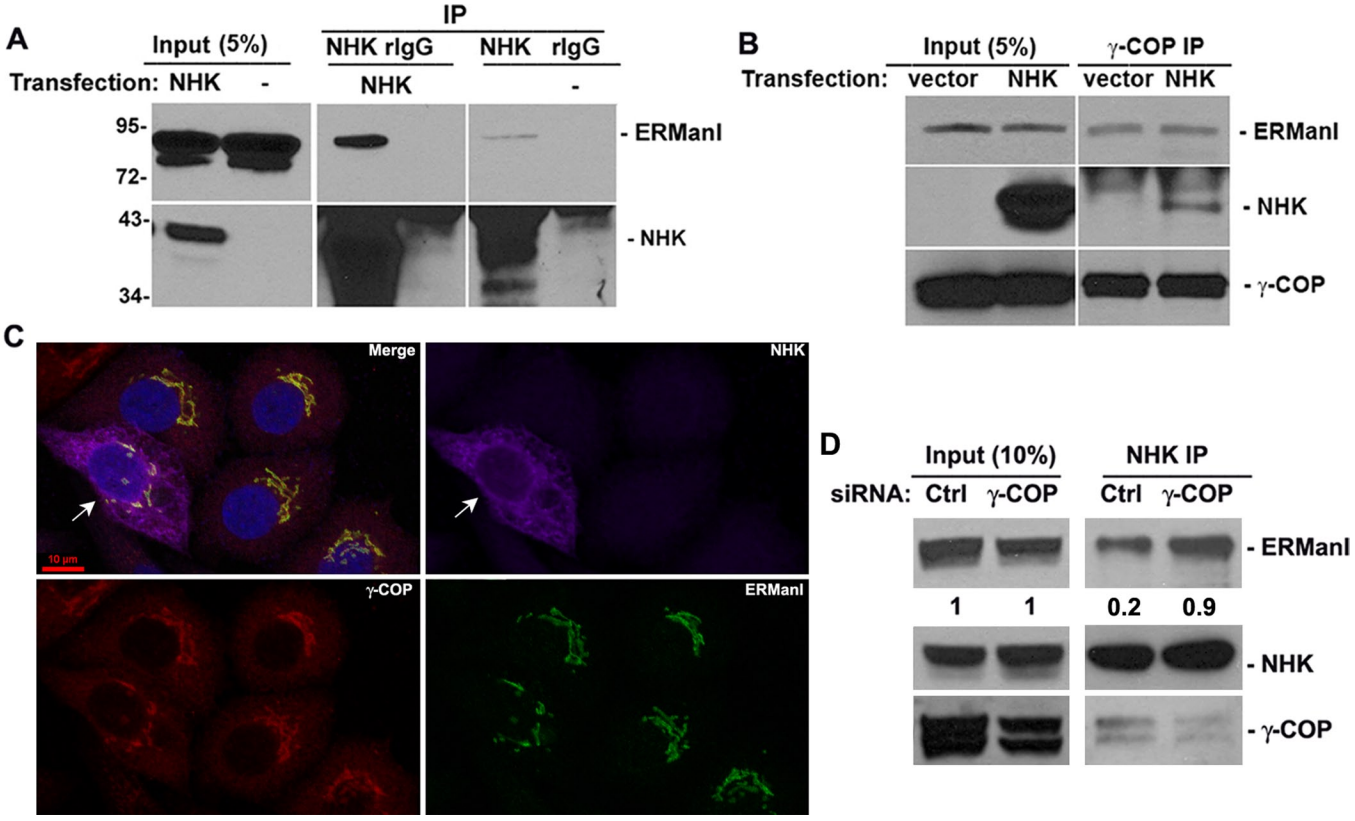

FIGURE 6: ERManI contributes to ERAD substrate loading into COPI vesicles. (A) HeLa cells were lysed 48 h following transfection with NHK or empty vector (–), and extracts were used for IP using anti-AAT antibodies for NHK or control rabbit IgG (rIgG). Immunoprecipitated proteins were resolved by SDS–PAGE. ERManI and NHK were detected by Western blotting. Input: 5% of the cell extracts. (B) Extracts from HeLa cells transfected with empty vector or NHK for 48 h were subjected to IP with anti–γ-COP antibodies. The presence of ERManI, NHK, and γ-COP in the immunocomplexes was detected by Western blotting. Input control: 5% of the total cell extracts. (C) Confocal images of HeLa cells transfected with NHK followed by coimmunostaining with antibodies against ERManI (green), γ-COP (red), and AAT (purple). Arrows show cellular expression of NHK. (D) NHK cDNA was cotransfected with either control siRNA or γ-COP–specific siRNA. At 48 h posttransfection, cells were lysed, and the extracts were used for IP of NHK. The amounts of ERManI, NHK, and γ-COP in the complex were determined by Western blotting. Numbers indicate the relative amounts of ERManI associated with NHK, as compared with that in 10% of the input extracts.

in the amount of ERManI associated with NHK. These results imply that γ-COP is required for the release of ERAD substrates from ERManI-containing complexes during the loading of COPI vesicles en route to the ER.

Finally, to validate that our findings were not restricted to NHK and/or HeLa cells, the human hepatoma cell line Huh7 was transiently transfected with the secretion-impaired AAT variant designated ATZ (Graham *et al.*, 1990; Le *et al.*, 1990). Its subcellular localization was determined by costaining with antibodies against AAT and ERManI. Unlike NHK, which mainly displayed a reticular pattern throughout the cytoplasm, the localization of ATZ varied in response to its expression level. In cells expressing high levels of ATZ, the majority of the molecules were localized in the cytoplasm as large puncta, possibly reflecting ATZ's polymerized form, as previously reported (Le *et al.*, 1992; An *et al.*, 2005; Sifers, 2010a). In these cells, only a small portion of ATZ colocalized with the endogenous ERManI in the Golgi complex. As compared with the cytoplasm-localized ATZ, the staining of Golgi-localized molecules was less intense and more homogenous, indicating a subpopulation of ATZ can escape the ER and travel to the Golgi complex, where ERManI is localized. In cells expressing low levels of ATZ, the majority of the ATZ molecules were localized to the Golgi complex, with the staining pattern overlapping that of ERManI. On the other hand, ERManI exhibited a distinct Golgi-localization pattern, irrespective of the ATZ expression level (Figure 7A). These observations support the notion that a portion of the ATZ population can escape the ER and travel to the Golgi complex, where it can be recycled.

DISCUSSION

The traditionally accepted role for ERManI in ERAD is that it functions as a mannosidase in the ER, contributing to the generation of degradation signals in response to the persistent retention of misfolded glycoproteins in the early secretory pathway of mammalian cells (Wu *et al.*, 2003; Lederkremer, 2009). However, our recent discovery of Golgi-localized ERManI, and the fact that a portion of misfolded glycoproteins can escape the ER (this study; Sifers *et al.*, 1989; Hosokawa *et al.*, 2007) and are recycled through the Golgi complex prior to proteasome-mediated intracellular disposal in both yeast and mammalian cells (Hammond and Helenius, 1994; Caldwell *et al.*, 2001; Vashist *et al.*, 2001; Kincaid and Cooper, 2007b), demanded a revision of the functional mechanism in which ERManI operates in the Golgi complex as a component of ERAD.

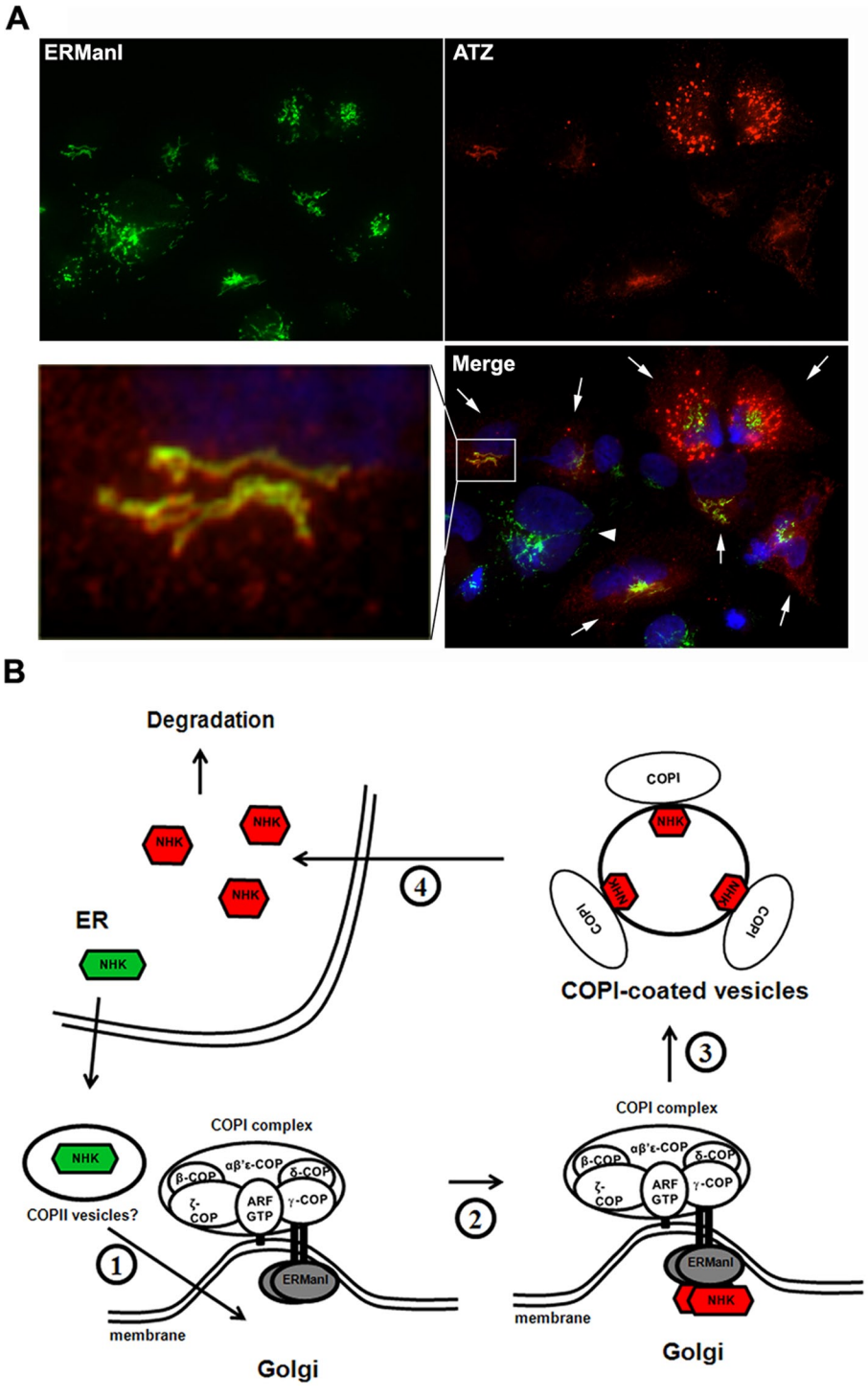

FIGURE 7: Substrate specificity and proposed functional model of ERAD expansion. (A) Confocal images of Huh7 cells transfected with myc-tagged ATZ followed by coimmunostaining with antibodies against ERManI (green) and Myc (red). Arrows point to cells expressing myc-ATZ, and an arrowhead identifies a cell that was not transfected. The bottom, left panel shows an enlarged image of the rectangle in the bottom, right panel. (B) Proposed functional model of mammalian ERAD expansion includes the following steps: 1) Misfolded N-linked glycoproteins, such as NHK, can escape quality control checkpoints in the ER and travel to the Golgi complex. The process is likely mediated by COPII vesicles, and the oligosaccharide structure attached to NHK is intact (green). 2) Once in the Golgi complex, NHK is recognized and captured by a protein complex that contains ERManI. Eventually, mannose trimming signals NHK as a terminally misfolded glycoprotein (red). ERManI, via a direct physical interaction with γ-COP, brings NHK into close proximity to coatomers where COPI vesicles are formed. 3) ERManI dissociates from NHK, allowing its loading into the COPI vesicles. 4) NHK undergoes retrograde transport back to the ER, where it is subsequently recognized for dislocation into the cytosol and degradation by 26S proteasomes.

Currently, COPI-mediated vesicle trafficking is the only known route for the recycling of Golgi-situated proteins back to the ER. Theoretically, this routing system would also serve as the prominent mechanism by which ERAD substrates are retrieved from post-ER compartments. The identification of a direct interaction between ERManI and γ-COP supports this idea, and served to validate the notion that γ-COP plays a functional role in the intracellular disposal of the classical ERAD substrate, NHK. The identification of γ-COP–binding sites in the ERManI cytoplasmic tail, plus the consequences of mutating them, provided additional validation for the functional partnership. Our study provides, for the first time, experimental evidence to support this hypothesis.

The incorporation of protein cargo into COPI vesicles is a selective process. The selection of luminal cargo is mediated by groups of transmembrane proteins designated as cargo receptors (Nickel *et al.*, 1998, 2002). In this study, we discovered that the ERManI/γ-COP complex associates with the luminal ERAD substrate NHK. Such an association likely brings ERAD substrates in close proximity to the COPI coatomers, thus allowing their selective recruitment into COPI vesicles. In this regard, ERManI functions similar to a cargo receptor that retains escaped proteins in the Golgi complex prior to facilitating their loading into COPI vesicles. In support of this notion, in the absence of ERManI, the retention of NHK is impaired, allowing its enhanced secretion. The interconnected and transient natures of the aforementioned events were further validated by the enhanced secretion of NHK in response to the mutation of γ-COP-binding sites in the ERManI cytoplasmic tail. Also, RNAi-mediated knockdown of endogenous γ-COP enhanced the association between NHK and ERManI, implying that their dissociation either precedes vesicle loading or is coupled to delivery of the associated ERAD substrate to the ER. Enhanced secretion, possibly caused by saturation of the retention process in the absence of dissociated ERManI, implies that the freed mannosidase is recycled to the Golgi complex where it can functionally support the retention process. Unlike classic cargo receptors recruited with their cargo into COPI vesicles, the movement of ERManI from the Golgi complex has not been detected in our studies.

On the basis of these findings, we propose a model in which ERManI contributes to a Golgi-based quality control module that captures ERAD substrates that have escaped from the ER and facilitates their loading into COPI vesicles via a dynamic process driven by an association with γ-COP (Figure 7B). However, considering the detection limits of our methodology, especially after proteins are diluted upon recycling back to the ER, our findings do not exclude the possibility that a trace amount of ERManI actually recycles in COPI vesicles. In fact, a previous proteomics study has indicated that ERManI is present in COPI vesicles (Gilchrist *et al.*, 2006), supporting this notion.

The absence of obvious interaction domains in the crystallized three-dimensional structure of the mannosidase (Dole *et al.*, 1997) suggests that the association between ERManI and luminal ERAD substrates likely takes place within the context of a much larger complex. In this regard, Cormier *et al.* (2009) reported the existence of a glycan-independent interaction between EDEM1, an evolutionary relative of ERManI, and NHK. Because the human orthologues of ERManI and EDEM1 share 33% sequence identity and 49% similarity (Kanehara *et al.*, 2007), it is possible that a similar interaction might exist between ERManI and NHK. Currently, intensive immunoaffinity purification/proteomic investigations are underway to test the validity of this hypothesis and/or to both identify and characterize the components of a much larger complex. An additional prediction, which must be revisited as the subject of future investigations, is that degradation signals initiated by the enzymatic removal of terminal α-1, 2–linked mannose units diverts the recycled molecules away from additional rounds of folding events in response to their recognition by ER-situated lectins (Kanehara *et al.*, 2007; Yoshida and Tanaka, 2010), all of which precedes their dislocation into the cytoplasm for proteasomal degradation.

Currently, we are not aware of the precise mechanism responsible for regulating the concentration of Golgi-situated ERManI, although previously published experiments have indicated that a significant fraction of the newly synthesized molecules are eliminated by lysosomal proteases (Wu *et al.*, 2007). It is apparent, however, that the intracellular residence of the mannosidase does not rely on its direct binding to γ-COP, because ERManI$^{\gamma\text{-COP-def}}$ continued to localize to the Golgi complex (Figure S4A). Also, unlike the capacity of γ-COP knockdown to diminish the steady-state level of the wild-type mannosidase, the ERManI$^{\gamma\text{-COP-def}}$ did not exhibit a diminished intracellular concentration (Figure 4C), implying that the capacity of γ-COP to regulate the concentration of the mannosidase does not rely on a direct interaction. It should be noted, however, that the RNAi-mediated knockdown of γ-COP did result in the dispersal of endogenous ERManI throughout the cytoplasm (Figure S4B). In support of the notion that this phenomenon reflected fragmentation of the Golgi complex (Lippincott-Schwartz *et al.*, 1990; Beller *et al.*, 2008), we observed that incubating cells in media that contained brefeldin A (BFA), an antibiotic that fragments the Golgi complex by interfering with COPI complex assembly (Peyroche *et al.*, 1999; Mossessova *et al.*, 2003; Renault *et al.*, 2003), led to a significantly diminished steady-state level of ERManI (Figure S4C). However, in contrast to that notion, incubation with nocodazole, a potent drug that is able to induce Golgi disassembly via the depolymerization of microtubules (Lippincott-Schwartz *et al.*, 1990; Dinter and Berger, 1998), did not alter the steady-state level of ERManI (Figure S4C). Altogether, our observations support the idea that the steady-state level of ERManI is likely regulated by protein trafficking in the secretory pathway, rather than by the structural integrity of the Golgi apparatus.

In summary, our study indicates that Golgi-localized ERManI apparently serves as a linchpin in an expanded mammalian ERAD network in which functional modules are linked by the vesicular transport of cargo. This arrangement provides the temporospatial distancing of essential proteostasis events that would otherwise directly compete with one another if performed in the same compartment. In this regard, ERManI contributes to the establishment of a multifunctional gatekeeper.

MATERIALS AND METHODS

cDNA constructs and siRNAs

Generation of ERManI cDNA was described previously (Pan *et al.*, 2011). The di-arginine motif mutations were generated using the QuikChange site-directed mutagenesis kit purchased from Stratagene (La Jolla, CA), following the manufacturer's instructions. The mutagenic primers were designed using the QuikChange primer design program from Stratagene, and the sequences are available upon request. All cDNA constructs were verified by nucleotide sequencing. cDNA constructs of AAT NHK and ATZ were described previously (Wu *et al.*, 2003). MGC (mammalian gene collection) human clones containing γ-COP cDNA (IMAGE:6046736) were purchased from the American Type Culture Collection (Manassas, VA). For generation of C-terminal His-tagged γ-COP cDNA plasmid, γ-COP cDNA was amplified by PCR using a forward primer (5′-CCGGATCCGTTGAAGAAATTCGACAAGAAG-3′ and a reverse primer 5′-TTCTCGAGTCCCACAGATGCCAAGATGAT-3′. The inserts were then cloned into pET23b vector using *Bam*HI and *Xho*I restriction sites. GST-tagged ERManI plasmid was generated previously using pEGX4T1 vector (Pan *et al.*, 2011). GST-tagged AlixMB plasmid was generated previously using pEGX4T3 vector (Pan *et al.*, 2008). ERManI siRNAs used in this study were purchased from Ambion (Austin, TX), and their sequences were described previously (Pan *et al.*, 2011). The three γ-COP siRNAs were purchased from Sigma-Aldrich (St. Louis, MO), and the target sequences are 1) GAGATGTTACCCAGTATCT, 2) CTTGTGAGAGGTCAGACAA, and 3) CTTGTAATCTGGATCTGGA.

Antibodies

Anti-ERManI monoclonal antibodies were generated previously in our group (Pan *et al.*, 2011). Anti–γ-COP and anti-His polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santz Cruz, CA). Anti-actin polyclonal antibodies were purchased from Sigma-Aldrich. Anti-BiP monoclonal antibody was purchased from BD Biosciences (San Jose, CA). Anti–human AAT antibodies were purchased from MP Biomedicals (Solon, OH).

Cell lines

HeLa cells were cultured in DMEM (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA) and 1% ampicillin/streptomycin (Invitrogen, Carlsbad, CA). MCF7 cells were cultured in RPMI 1640 (Mediatech) supplemented with 10% FBS and 1% ampicillin/streptomycin.

Transient transfection, IP, Western blotting, and immunofluorescence staining

The day prior to transfection, cells were plated onto 60-mm dishes and allowed to reach 80% confluence by the time of transfection. The cDNA plasmids (10 μg) or 20 nM siRNA (20 μl) were transfected into each well with Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. At 24 h posttransfection, cells were replated into six-well plates, and their culture was continued for an additional 24 h prior to protein extraction or pulse–chase assay. For co-IP, cells were lysed in buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM $Na3VO_4$ (Sigma-Aldrich), 10 mM NaF (Sigma Aldrich), 0.5% CHAPS (Sigma-Aldrich), 2 mM phenylmethylsulfonyl fluoride

(Sigma-Aldrich), and additional protease inhibitors (GenDepot, Houston, TX). After centrifugation, the cell extracts were incubated overnight with primary antibodies conjugated to protein G agarose beads. After stringent washing, proteins associated with the beads were eluted and resolved on SDS–PAGE, which was followed by Western blotting, as described previously (Pan *et al.*, 2011). For immunofluorescence staining, cells cultured on 18-mm glass coverslips were fixed and stained, and the images were captured following the same procedure described previously (Pan *et al.*, 2011).

Purification of recombinant proteins from bacteria

pET23b-γ-COP, pGEX4T1-ERManI (Pan *et al.*, 2011), pGEX4T1, and pGEX4T3-AlixMB (Pan *et al.*, 2008) plasmids were each transfected into BL-21 *Escherichia coli* cells and cultured to ~0.6 of A600 before induction with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside at 37°C for 4 h. The cells were then harvested by centrifugation at 5000 × *g* for 10 min. The His-γ-COP recombinant proteins were purified under native conditions using QIAexpress Ni-NTA columns (Qiagen, Valencia, CA), following the manufacturer's instructions. GST, GST-ERManI, and GST-AlixMB were purified following procedures described previously (Pan *et al.*, 2006).

In vitro binding assay

Fifteen micrograms of purified GST, GST-ERManI, or GST-AlixMB recombinant proteins was conjugated to 15 μl glutathione agarose beads (Sigma-Aldrich) by rotating at 4°C for 4 h before being mixed with 5 μg γ-COP-His and rotated overnight at 4°C. The beads were washed with native lysis buffer (Qiagen) five times, and the bound proteins were eluted using Laemmli sample buffer. After being separated by SDS–PAGE, the bound proteins were subjected to Western blotting using anti-His antibodies.

Protein identification by mass spectrometry

MCF7 cell pellets (~1 ml) were used for large-scale IP with 10 μg of either mouse immunoglobulin G (IgG) or anti-ERManI monoclonal antibodies, and the isolated proteins were identified using mass spectrometry, following the same procedure as described previously (Pan *et al.*, 2011).

Metabolic radiolabeling and chase experiments

Cells cultured for at least 24 h were starved in methionine- and cysteine-free medium for 1 h, subjected to metabolic pulse-radiolabeling with [^{35}S]methionine for 20 min, and chased for different time points following methods described previously (Pan *et al.*, 2011). Cells and media were collected from each time point and were used for IP followed by autoradiography, as described previously (Pan *et al.*, 2011).

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