N-glycan processing selects ERAD-resistant misfolded proteins for ER-to-lysosome-associated degradation

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Abstract

Efficient degradation of by-products of protein biogenesis maintains cellular fitness. Strikingly, the major biosynthetic compartment in eukaryotic cells, the endoplasmic reticulum (ER), lacks degradative machineries. Misfolded proteins in the ER are translocated to the cytosol for proteasomal degradation via ER-associated degradation (ERAD). Alternatively, they are segregated in ER subdomains that are shed from the biosynthetic compartment and are delivered to endolysosomes under control of ER-phagy receptors for ER-to-lysosome-associated degradation (ERLAD). Demannosylation of N-linked oligosaccharides targets terminally misfolded proteins for ERAD. How misfolded proteins are eventually marked for ERLAD is not known. Here, we show for ATZ and mutant Pro-collagen that cycles of de-/re-glucosylation of selected N-glycans and persistent association with Calnexin (CNX) are required and sufficient to mark ERAD-resistant misfolded proteins for FAM134B-driven lysosomal delivery. In summary, we show that mannose and glucose processing of N-glycans are triggering events that target misfolded proteins in the ER to proteasomal (ERAD) and lysosomal (ERLAD) clearance, respectively, regulating protein quality control in eukaryotic cells.

Keywords ERAD; ERLAD; ER-phagy; N-glycan processing; Protein quality control

Subject Categories Autophagy & Cell Death; Membranes & Trafficking; Post-translational Modifications & Proteolysis

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Introduction

Proteins synthesized in the ER are covalently modified with pre-assembled oligosaccharides on asparagine residues in -N-X-S/T- sequons (Fig 1A, single letter code for asparagine (N), any amino acid but proline (X), serine (S), or threonine (T)) (Aebi et al, 2010). Rapid removal of glucose residues 1 and 2 by sequential action of the castanospermine (CST)-sensitive ER α-glucosidases I and II (GI and GII, Fig 1A) (Elbein, 1991) generates a mono-glucosylated trimming intermediate that recruits Calnexin (CNX) (Hammond et al, 1994). CNX is an ER lectin chaperone that assists folding of newly synthesized polypeptides and protects them from unwanted selection for ERAD via the ubiquitin proteasome system (UPS) (Moore & Spiro, 1993; Hammond et al, 1994; Helenius, 1994; Kears et al, 1994; Hebert et al, 1996; Liu et al, 1997, 1999; Ayalon-Soffer et al, 1999; Molinari et al, 2002; Molinari et al, 2003; Oda et al, 2003; Helenius & Aebl, 2004; Molinari, 2007; Olivari & Molinari, 2007; Lederkremer, 2009; Aebi et al, 2010; Adams et al, 2019; Sun & Brodsky, 2019). Folding-defective polypeptides are subject of extensive de-mannosylation of their N-linked oligosaccharides (i.e., removal of the light green mannose residues by members of the glycosyl hydrolase 47 family including the kifunensine (KIF)-sensitive ER-resident α-mannosidase I (αMI) and EDEM proteins, Fig 1A) (Moremen & Molinari, 2006). This has two consequences: first, removal of mannose A (Fig 1A) eliminates the acceptor for the re-glucosylation of the oligosaccharide’s branch A by the ER quality control factor UDP-glucose: glycoprotein glucosyltransferase (UGGT1, Fig 1A). This prevents the generation of mono-glucosylated N-glycans and irreversibly extracts non-native polypeptides from the CNX chaperone system (Olivari et al, 2006). Second, it engages ER lectins that bind N-linked oligosaccharides displaying dark green terminal mannoses (Fig 1A). These mannose-binding lectins (OS-9 and XTP3-B) shuttle misfolded polypeptides to supramolecular complexes built around E3 ubiquitin ligases. This promotes dislocation of terminally misfolded polypeptides across the ER membrane for degradation by cytosolic proteasomes (ERAD) (Bernasconi et al, 2008; Christianson et al, 2008; Hosokawa et al, 2009; Bernasconi et al, 2010; Sun & Brodsky, 2019).

Misfolded proteins that cannot be dislocated across the ER membrane for ERAD are segregated in specialized ER subdomains displaying ER-phagy receptors at the limiting membrane (Fregno et al, 2018; Schultz et al, 2018; Cui et al, 2019; De Leonibus et al, 2019; Forrester et al, 2019; Fregno & Molinari, 2019; Hubner & Dikic, 2020; Wilkinson, 2020; Molinari, 2021). ER-phagy receptors including FAM134B drive, with contribution of yet elusive additional factors, the scission of these ER subdomains from the bulk ER (Bhaskara et al, 2019; Jiang et al, 2020) and recruit select cytosolic autophagy gene products. These control delivery to lysosomes and...
Figure 1.
cleavage of the ER-derived vesicles containing misfolded proteins via client-specific avenues including LC3-dependent vesicular transport (Fregno et al., 2018), macro- (Cui et al., 2019; Forrester et al., 2019), or micro-ER-phagy (Omari et al., 2018). In this manuscript, we use the term ER-to-Lysosome-Associated Degradation (ERLAD) to define the catalytic pathways that remove ERAD-resistant misfolded proteins from the ER (Fregno et al., 2018; De Leonibus et al., 2019; Forrester et al., 2019; Fregno & Molinari, 2019; Sun & Brodsky, 2019; Wilkinson, 2019, 2020; Hubner & Dikic, 2020; Molinari, 2021).

The Z mutation of the SERPINA1 gene characterizes about 95% of the patients affected by alpha-1 antitrypsin (AAT) deficiency, a lung and liver disease. The Z mutation results in a Glu342Lys substitution that encodes for alpha-1 antitrypsin-Z (ATZ) (Strnad et al., 2020). Misfolded ATZ is translocated across the ER membrane for proteasomal degradation. However, a significant fraction of the polypeptide undergoes polymerization and cannot be translocated across the ER membrane. Instead, ATZ polymers are delivered to the endolysosomal district in an ERLAD pathway that relies on the endolysosomal SNAREs Syntaxin17 and VAMP8. Misfolded Pro-collagen is also segregated in FAM134B-positive ER-derived vesicles, but these are different from autophagosomes that fuse with endolysosomes (Forrester et al., 2019).

Puzzlingly, CNX, which we recall is an ER-resident lectin that protects misfolded N-glycosylated polypeptides from proteasomal degradation, is a major interactor of the ER-phagy receptor FAM134B (Grumati et al., 2017; Fregno et al., 2018). CNX deletion significantly inhibits FAM134B-driven ERLAD of ATZ polymers via LC3-dependent vesicular transport (Fregno et al., 2018) and FAM134B-driven ERLAD of misfolded Pro-collagen via macro-ER-phagy (Forrester et al., 2019). This led us to hypothesize that the role of N-glycans as quality control appendices, whose extensive de-mannosylation determines selection of misfolded proteins for ERAD, could also extend to define, upon appropriate processing, a signal that marks aberrant polypeptides for ERLAD.

### Results

#### Formation and delivery of ATZ polymers to endolysosomes for clearance

The wild-type AAT protein and the disease-causing ATZ variant display 3 N-glycans on asparagine residues at position N46, N83, and N247 (Fig 1B, AATNNN and ATZNNN, respectively). The Z mutation (blue in Fig 1B) enhances formation of ERAD-resistant polymers, which are delivered to lysosomal compartments for clearance both in mammalian and in yeast cells (Teckman & Perlmutter, 2000; Kamimoto et al., 2006; Kroeger et al., 2009; Hidvegi et al., 2010; Perlmutter, 2011; Pastore et al., 2013; Yamamura et al., 2014; Fregno et al., 2018; Cui et al., 2019; Strnad et al., 2020). ATG5 and ATG7 regulate the covalent association of the ubiquitin-like protein LC3 with the membrane lipid phosphatidyl ethanolamine. Lipidated LC3 displayed at the limiting membrane of autophagosomes or endolysosomes is engaged by autophagy receptors in catabolic processes, where cells clear their own components (Bento et al., 2016).

We and others recently identified FAM134B (and Atg40p, the yeast parologue of FAM134B) as the LC3-binding ER-phagy receptor that regulates removal of ER portions containing ERAD-resistant misfolded proteins from mammalian or yeast cells (Fregno et al., 2018; Schultz et al., 2018; Cui et al., 2019; Forrester et al., 2019). Relevant for this study, ERAD-resistant ATZ polymers (Fregno et al., 2018) are segregated in ER subdomains enriched in FAM134B. These vesiculate and ER-derived vesicles directly release their content within LAMP1/RAB7-positive endolysosomes under control of the SNAREs Syntaxin17 and VAMP8. Misfolded Pro-collagen is also segregated in FAM134B-positive ER-derived vesicles, but these are first captured by autophagosomes that then fuse with endolysosomes (Forrester et al., 2019).

Figure 1. N-glycan processing and delivery of ATZNNN to endolysosomes.

A N-glycan. Glucoses, blue; mannososes, green; N-acetylglucosamines, black. GI removes glucose 1. GII removes glucose 2 and 3. UGGT1 adds-back glucose 3. UGGT1 can only modify the terminal mannose on branch A (black circle). xMI and EDEM1, EDEM2, and EDEM3 remove light green, α1,2-bonded, mannosose residues. CST inhibits GI and GII. KIF inhibits α1,3-mannosidases.

B ATTNNN and ATZNNN. N-glycans in red. The E3A2K mutation of ATZNNN is in blue. CNX (black) and EDEMs (green) are recognized by the monoclonal antibody 2C1 (Miranda et al., 2010; Ronzoni et al., 2021). They are transported from the ER to the extracellular space or are segregated in FAM134B-positive ER-derived vesicles, but these are first captured by autophagosomes that then fuse with endolysosomes (Forrester et al., 2019).

C Control of polimerization propensity for the Halo-tagged versions of AAT (lanes 1 and 4) and of ATZ (lanes 2 and 5). Polymers and monomers corresponding to (Miranda et al., 2010; Ronzoni et al., 2021) are seen with anti-Halo (lanes 1–3) or with the polymer-specific 2C1 antibody (lanes 4–6). Synthesis control in SDS-polyacrylamide gel by visualization of tetramethylrhodamine (TMR) ligand-labeled Halo-AATNNN and Halo-ATZNNN (lanes 7–9).

D HaloTag pulse-chase analysis in WT MEF monitoring fluorescent Halo-AATNNN during up to 6 h of chase in the presence of BafA1. The fluorescent JF646-Halo ligand and the polymer-specific 2C1 antibody stains are shown.

E Same as (D) for fluorescent Halo-ATZNNN.

F Quantification of accumulation of fluorescent Halo-AATNNN and Halo-ATZNNN within LAMP1-positive endolysosomes. Mean ± SEM, n = 20, 11, 10, 13 for Halo-AATNNN, n = 18, 12, 12, 13 for Halo-ATZNNN. Two-way ANOVA and Sidak’s multiple comparison test, ns P > 0.05, ***P < 0.001.

Data information: Scale bars 10 μm.

Source data are available online for this figure.
start a chase of 0, 2, 4, or 6 h (Fig 1D–F). Bafilomycin A1 (BafA1) is added to the cell culture media during the chase to ensure accumulation within the LAMP1-positive compartments of the fluorescently labeled protein selected for lysosomal clearance (Klionsky et al., 2008; Fregno et al., 2018). The 15 min pulse generates a population of fluorescent Halo-AATNNN (Fig 1D, Halo, 0 h chase) or Halo-ATZNNN (Fig 1E, Halo, 0 h chase). Image analyses and unbiased quantification with LysoQuant, a deep learning approach for segmentation and classification of fluorescence images (Morone et al., 2020), show significant and progressive accumulation within LAMP1-positive endolysosomes only for Halo-ATZNNN (Fig 1E, 2C1/LAMP1 and Insets, quantification in Fig 1F, 0 to 6 h chase). This conforms to the fact that the secretory protein AATNNN is efficiently released from cells, whereas a fraction of ATZNNN enters polymers.
that are retained intracellularly and are eventually cleared by lysosomal enzymes (Fig 2B) (Teckman & Perlmutter, 1996, 2000; Kamimoto et al., 2006; Kroeger et al., 2009; Hideg et al., 2010; Perlmutter, 2011; Pastore et al., 2013; Yamamura et al., 2014; Fregno et al., 2018; Cui et al., 2019).

The ER-phagy receptor FAM134B, and not the functional homologs SEC62 and TEX264, is involved in lysosomal delivery of ERAD-resistant ATZ\textsubscript{N\textit{NN}} polymers

In wild-type MEF, confocal laser scanning microscopy with anti-HA (Fig 2A, HA/LAMP1, Fig 2H) and 2C1 antibodies (Fig 2A, 2C1/LAMP1, Fig 2I) reports on delivery of HA-tagged ATZ\textsubscript{N\textit{NN}} polymers within LAMP1-positive endolysosomes, where ATZ\textsubscript{N\textit{NN}} accumulates upon inactivation of hydrolytic enzymes with BafA1 (Fig 2A). Consistently, cell exposure to BafA1 substantially delays the clearance of 2C1-positive polymers (Fig 2B) (and thoroughly analyzed in (Fregno et al., 2018)). In MEF lacking the ER-phagy receptor FAM134B (Fig EV1A, lanes 1–2), delivery of ATZ\textsubscript{N\textit{NN}} polymers within the LAMP1-positive degradative compartments is defective (Fig EV1B and EV1C, cells expressing HA-tagged ATZ\textsubscript{N\textit{NN}} and mock-transfected with empty pcDNA3 plasmid). Delivery of ATZ\textsubscript{N\textit{NN}} polymers within endolysosomes is rescued upon back-transfection of V5-tagged FAM134B (Fig EV1C and E), but not upon back-transfection of FAM134BLIR (Fig EV1D and E). This latter is a mutant form of FAM134B, where the LC3-interacting region -453DDFELL458- has been mutated to -453AAAAAA458- to abolish LC3 binding and to render the ER-phagy receptor inactive in starvation-induced ER-phagy and in ERLAD (Khaminets et al., 2015; Fregno et al., 2018). A co-immunoprecipitation assay confirms that both FAM134B and FAM134BLIR do associate with CNX (Fig EV1F, Co-IP, lanes 4 and 5, upper panel) (Fregno et al., 2018) and with the ERLAD client ATZ\textsubscript{N\textit{NN}} (Co-IP, lanes 4 and 5, middle panel). However, only FAM134B engages LC3 (Co-IP, lanes 4 and 5, lower panel), which is required for formation of ER-derived vesicles containing polymers and their delivery to the degradative endolysosomal compartment (Fig EV1C–E) (Fregno et al., 2018). Deletion of SEC62 or of TEX264 (Fig EV1A, lanes 3–4 and 5–6, respectively), two ER-phagy receptors that regulate ER clearance during recovery from ER stress (Fumagalli et al., 2016; Loi et al., 2019; Loi & Molinari, 2020) or upon nutrient deprivation (An et al., 2019; Chino et al., 2019; Liang et al., 2020), does not affect delivery of ATZ\textsubscript{N\textit{NN}} polymers within the LAMP1-positive compartment (Fig EV1G–K). These results reveal that FAM134B is required and sufficient for lysosomal clearance of ATZ\textsubscript{N\textit{NN}} polymers generated in the ER (Fregno et al., 2018).

CNX and glucose processing enzymes regulate delivery of ERAD-resistant ATZ\textsubscript{N\textit{NN}} polymers (and misfolded Pro-Col2A1) to LAMP1-positive endolysosomes

N-glycosylation and processing of N-linked oligosaccharides (i.e., deglucosylation by Gl and GlI, re-glucosylation by the UGGT1, demannosylation by αM1 and EDEM proteins, Fig 1A) are crucial events in ER protein quality control. They enhance solubility of unfolded nascent chains in the crowded ER environment and determine their access and retention in the CNX chaperone system. CNX association promotes structural maturation and protects not-yet-folded polypeptides from unwanted selection for ERAD (Moore & Spiro, 1993; Hammond et al., 1994; Helenius, 1994; Kears et al., 1994; Hebert et al., 1996; Ayalaon-Soffier et al., 1999; Liu et al., 1999; Molinari et al., 2002; Molinari et al., 2003; Oda et al., 2003; Helenius & Aebl, 2004; Molinari, 2007; Olivari & Molinari, 2007; Lederkremer, 2009; Aebi et al., 2010; Adams et al., 2019; Sun & Brodsky, 2019).

Formation of 2C1-immunoreactive ATZ\textsubscript{N\textit{NN}} polymers does not require the lectin chaperone CNX (Fig 2C, panel 2C1, Fig EV2A, CNX-KO, Fig EV2B, lane 4) (Fregno et al., 2018). However, delivery of ATZ\textsubscript{N\textit{NN}} polymers within the degradative LAMP1-positive compartment for clearance is substantially impaired in CNX-KO MEF (Fig 2C, 2C1/LAMP1, Fig 2H and I). Characterization of another folding-defective polypeptide, the disease-causing Arg989Cys (R989C) variant of Pro-Col2A1 (Fig EV3A) (Hintze et al., 2008; Zhang et al., 2011), another emblematic ERLAD client (Fig EV3B–F) (Forrester et al., 2019), confirms defective polypeptides delivery from the ER to degradative endolysosomes in cells lacking CNX (Figs EV3G and 3H and M). These data confirm that delivery of misfolded polypeptides from the ER to the endolysosomal compartment (i.e., ERLAD) requires CNX association (Fregno et al., 2018; Forrester et al., 2019). Interestingly, dislocation across the ER membrane and proteasomal degradation of misfolded polypeptides (i.e., ERAD) is in contrast prevented by CNX association (Moore & Spiro, 1993; Hammond et al., 2006; Kroeger et al., 2019).
et al., 1994; Helenius, 1994; Karse et al., 1994; Hebert et al., 1996; Liu et al., 1997, 1999; Ayalon-Soffer et al., 1999; Molinari et al., 2002; Molinari et al., 2003; Oda et al., 2003; Helenius & Aebi, 2004; Molinari, 2007; Olivari & Molinari, 2007; Lederkremer, 2009; Aebi et al., 2010; Adams et al., 2019; Sun & Brodsky, 2019).

Further experiments reveal that pharmacologic modulation of N-glycan processing affects ERLAD and ERAD pathways differently. For instance, lysosomal delivery of ATZ\textsubscript{NNN} polymers is unaffected in cells exposed to KIF (Figs 2D and H and I and EV3I and M for Pro-Col2A1\textsubscript{R989C}). Thus, mannose removal from N-glycans, which is

![Image](image-url)

**Figure 3.**
a potent trigger of ERAD (Aebi et al., 2010; Adams et al., 2019; Sun & Brodsky, 2019), is dispensable for ERLAD. On the same line, CST, which abolishes glycoproteins association with CNX and substantially accelerates ERAD (Aebi et al., 2010; Adams et al., 2019; Sun & Brodsky, 2019), drastically reduces the selection of ERAD-resistant ATZNNN polymers for lysosomal delivery (Figs 2E and H and I and EV3J and M for Pro-Col2A1R989C).

FAM134B is inserted in the ER membrane but it does not protrude in the ER lumen (Khaminets et al., 2015). Thus, client association is mediated by its interacting partner CNX (Fregno et al., 2018; Forrester et al., 2019). Notably, lysosomal delivery of ATZNNN is defective in cells that express a short (s)Cnx variant (Figs 2F and H, EV3K and M for Pro-Col2A1R989C). sCnx conserves the transmembrane and cytosolic domains that ensure the formation of the functional complex with FAM134B. However, it is characterized by splicing by between bases 307-725, corresponding to a deletion from residues 104 to 242 in the polypeptide chain. The deletion removes D118, C161, Y165, K167, Y186, M189, C195, E217, which are essential for the lectin function of CNX (Denzel et al., 2002; Pieren et al., 2005). Consistently, like CNX (Fig EV1F, Co-IP, upper panel, lanes 3-5, Fig EV4A, Co-IP, lower panel, lane 1), sCnx binds FAM134B (Fig EV4A, Co-IP, lower panel, lane 2). However, lacking the lectin site, sCnx does not bind clients (Denzel et al., 2002; Pieren et al., 2005) and, consistently, it does not bind ATZNNN (Fig EV4A, Co-IP, upper panel, lane 2). This prevents the formation of a complex that contains functional FAM134B:lipidated LC3-II:CNX:ATZ, which is required for productive ERLAD (Fig EV1F, Co-IP, lanes 3-5 and Fig EV4A, Co-IP, lanes 1-4) (Fregno et al., 2018).

Thus, the role of the CNX:FAM134B complex in client delivery to endolysosomes for clearance requires the LIR function of FAM134B (Fig EV1B-E) and the glucose-binding function of CNX (for ATZNNN, Figs 2A and C and E and F and H and I, and EV4A; for Pro-Col2A1R989C, Fig EV3G and H, and J, and K, and M).

The quality control factor UGGT1 selects ERAD-resistant ATZNNN polymers (and misfolded Pro-Col2A1) for ERLAD

Newly synthesized proteins engage CNX via mono-glucosylated oligosaccharides generated by the sequential removal of the two outermost glucose residues by GI and GII (Fig 1A, thin arrows). Execution of the folding program is followed by removal of glucose 3 by GII (Fig 1A, thick arrow), release from CNX, and transport of the native polypeptide at the intra- or extra-cellular site of activity (Adams et al., 2019). Release of non-native proteins such as ATZNNN from CNX is delayed by repeated cycles of GI-mediated deglucosylation (Fig 1A, thick arrow) followed by immediate re-glucosylation by the ER quality control factor UGGT1 (Fig 1A, curved arrow) (Adams et al., 2019). This can be disabled upon deletion of UGGT1, with an overall reduction of CNX engagement (Molinari et al., 2005). For ATZNNN, hampered CNX engagement is shown by the reduction in CNX that co-precipitates with ATZNNN in cells lacking UGCT1 compared to WT cells (Fig EV4B, Co-IP, top panel, lanes 2 vs. 5). In Uggt1-KO MEF, ATZNNN abundantly associates with the Hsp70 family member BiP (Fig EV4B, Co-IP, lower panel, lanes 2 vs. 5) that prevents secretion of misfolded proteins eventually released by CNX (Molinari et al., 2005). In Uggt1-KO MEF, ATZNNN polymers are not delivered to the endolysosomal compartment (Fig 2G-J and M). Lysosomal delivery is rescued on back-transfection of catalytically active UGTT1 (Fig 2K and M), but not when cells are complemented with a mutant form of UGTT1 (Fig 2L and M) that cannot re-glycosylate misfolded proteins because it lacks the 1452DQDLPN1457 residues of the active site (Arnold et al., 2000). These experiments show that the catalytic activity of UGTT1 is required for lysosomal delivery of ATZNNN polymers and imply that multiple de-glucosylation/re-glucosylation cycles and persistent retention by CNX are required for ERLAD selection.

All in all, ATZNNN polymers are delivered within LAMP1-positive endolysosomes for clearance (Fig EV5C, 2C1/LAMP1) (Teckman & Perlmutter, 2000; Kamimoto et al., 2006; Kroeger et al., 2009; Hidvegi et al., 2010; Perlmutter, 2011; Pastore et al., 2013; Yamamura et al., 2014; Fregno et al., 2018; Strnad et al., 2020). The fraction of polymeric ATZNNN that accumulates within endolysosomes upon cell exposure to BaF1 does not co-localize with the ER marker CLIMP63 (Fig EV5C, 2C1/CLIMP63). In cells lacking CNX or UGTT1, ATZNNN polymers are not delivered within LAMP1-positive endolysosomes (Fig EV5D and E, 2C1/LAMP1). Rather, they remain in the ER (or in a ER-derived compartment) as shown by their co-localization with the ER marker CLIMP63 (Fig EV5D and E, 2C1/CLIMP63).

Time course, confocal light scanning microscopy analyses of the defective delivery of ATZNNN polymers to the endolysosomal district in Cnx- and in Uggt1-KO MEF

HaloTag pulse-chase reveals that in WT MEF exposed to BaF1, fluorescently labeled, polymeric, Halo-ATZNNN progressively accumulates within LAMP1-positive, inactive endolysosomes (Fig 1E, Insets, 6 h chase, Fig 1F). If the same experiment is performed in the absence of BaF1, fluorescent Halo-tagged polymers of ATZNNN are degraded as soon as they are delivered within the LAMP1-positive degradative compartment rather than accumulating in their lumen (Fig EV4F). In these cells, monomeric Halo-ATZNNN is degraded by ERAD and polymeric Halo-ATZNNN by ERLAD (Perlmutter, 2011), with overall disappearance of the labeled protein from cells (Fig EV4F, 0 vs. 6 h chase).

In cells lacking CNX (Fig EV4G) or UGTT1 (Fig EV4H), the fraction of Halo-tagged ATZNNN labeled during the 45 min fluorescent pulse (red in Halo/LAMP1) remains in the ER (or in a ER-derived
Chinese Hamster Ovary (CHO) cells, where ATZNNN is not delivered to mouse 3T3 cells (see Fig 4G and EV5A). Embryonic kidney cells (HEK293, Fig EV5A) transiently or stably expressing the model proteins, including human BiP. ATZNNN expressed in compartment), where it extensively co-localizes with BiP (Halo/Bip). ATZNNN expressed in Crc- or in Uggt1-KO cells with inactive ERLAD is more prone to enter in a detergent-insoluble fraction, from which it is extracted upon dissolution in 8 M urea (Fig EV4I, lanes 2 and 3). This is a phenotype that we will again observe and discuss below, where analysis of ERLAD-incompetent glycosylation variants of ATZ reveals their proneness to enter in a detergent-insoluble fraction.

**Context-specific N-oligosaccharides select ERLAD-resistant ATZNNN polymers (and misfolded Pro-Col2A1) for lysosomal delivery**

To definitely assess the role of N-linked oligosaccharides in client selection for ERLAD, we monitored the intracellular fate of the full series of ATZNNN glycosylation mutants generated by individual and combined mutations of the N-glycans acceptor asparagine residues (N) at positions 46, 83, and 247 to glutamine (Q) (Fig 3A). All 8 glycosylation variants do form 2C1-immunoreactive polymers as shown in confocal laser scanning microscopy analyses (Fig 3B–J, 2C1), in flow cytometry and native gels (Fig EV2C–D) and by immunoprecipitation (Fig 4A and B). However, only ATZ polymers displaying the N-glycan at position 83 are efficiently delivered within LAMP1-positive, degradative compartments (i.e., ATZQ (Fig 3B and J, and K), ATZQ (Fig 3C and J, and K), ATZQ (Fig 3E and J, and K), ATZQ (Fig 3G and J, and K)). The variants lacking this oligosaccharide (i.e., ATZ (Fig 3D and J, and K), ATZ (Fig 3F and J, and K), ATZ (Fig 3H and J, and K), and ATZ (Fig 3I–J)) are very inefficiently, if at all, delivered within the lysosomal compartments. Thus, the oligosaccharide at position 83 is the N-glycan whose modification marks ATZ for lysosomal delivery. These data were confirmed in a variety of cell lines transiently or stably expressing the model proteins, including human embryonic kidney cells (HEK293, Fig EV5A–E), mouse hepatocytes (HEPA 1-6, Fig EV5F–J), human HeLa cells (Fig EV5K–O), and mouse 3T3 cells (see Fig 4G–J). The only notable exception is Chinese Hamster Ovary (CHO) cells, where ATZNNN is not delivered within LAMP1-positive endolysosomes (Fig EV5P). Consistently, a recent work shows that BafA1 has no effect on clearance of soluble ATZ polymers in CHO cells and reports on the substantial deposition of this species in detergent insoluble pellet (Ronzoni et al., 2021). We have no explanation as for the reasons of defective transport of ATZ polymers from the ER to endolysosomes in CHO cells, but we consider this finding worth-reporting because CHO cells are widely used in cell biology labs, including those investigating ATZ biology.

The relevance of N-glycans as a tag for lysosomal delivery of ERAD-resistant polypeptides is reinforced by data showing that for both the WT and the RaspC form of Pro-Col2A1, the elimination of the protein-bound oligosaccharide at position 1388 (Fig EV3A) substantially impairs delivery within LAMP1 degradative compartments for clearance (Fig EV3B–F).

**Chaperone engagement and formation of insoluble aggregates by ERLAD-competent versus ERLAD-incompetent ATZ glycosylation variants**

Next, we selected 2 ERLAD-competent (ATZNNN and ATZQ) and 2 ERLAD-resistant variants of ATZ (ATZQ and ATZQ) to monitor polymer’s fate and chaperone association (Fig 4, note that all experiments shown in this figure are performed in the absence of BafA1). Cells expressing the individual model proteins were metabolically labeled with 35S methionine and cysteine and chased for 10, 120, and 240 min. At the end of each chase time, the residual population of radiolabeled ATZ and ATZ polymers was immunosolubilized from detergent-lysates with anti-HA (Fig 4A, IP:HA) or with the polymer-specific 2C1 antibody (IP:2C1), respectively. For the ERLAD-competent variants, radiolabeled polymers peak 120 min after synthesis (ATZNNN and ATZQ), Fig 4A, IP:2C1, lanes 1–3 and 4–6, respectively, Fig 4B). Their disappearance at longer chase times (Fig 4A, lanes 3 and 6, Fig 4B) is ascribed to their lysosomal delivery and clearance (Fig 3B and G, and J, and K) (Teckman & Perlmutter, 2000; Kamimoto et al., 2006; Kroeger et al., 2009; Hidvegi et al., 2010; Perlmutter, 2011; Pastore et al., 2013; Yamamura et al., 2014; Fregno et al., 2018). In contrast, the ERLAD-incompetent ATZQ and ATZQ polymers, which are not delivered to the

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**Figure 4. Intracellular fate of ERLAD-competent vs. ERLAD-incompetent ATZ glucosylation variants.**

A Radiolabeled ATZ enter in 2C1-positive polymers that are immunoprecipitated at the end of the given chase times. Total ATZNNN is immunoprecipitated with HA. Protein load and 35S-methionine/cysteine incorporated in nascent polypeptides are shown.

B Quantification of radiolabeled polymers. Green columns are for the ERLAD-competent ATZNNN and ATZQ variants (see Fig 3B and G and, J and K), red columns are for the ERLAD-incompetent ATZQ and ATZQ (see Fig 3H–J) (mean, n = 5 for ATZNNN, n = 3 for ATZQ, n = 4 ATZQ, and n = 4 for ATZQ). Two-way ANOVA and Sidak’s multiple comparison test, ns P > 0.05, *P < 0.005, **P < 0.05, ***P < 0.005, ****P < 0.0001.

C Co-immunoprecipitation of ATZ (Bait, lower panel) with CNX (upper panel) and BIP (middle panel). Protein content in the total cell extracts (TCE) is shown.

D Quantification of ATZ association (n = 3). Unpaired two-tailed t-test, **P < 0.01***P < 0.001, ****P < 0.0001.

E Quantification of ATZ association (n = 3). Unpaired two-tailed t-test, **P < 0.01***P < 0.001, ****P < 0.0001.

F Detergent-insoluble fraction of ATZ association (n = 3).

G Confocal laser scanning microscopy in Flp-In 3T3 cells stably expressing ATZNNN in the absence of BafA1 (to allow clearance of misfolded model proteins delivered within endolysosomes). Co-localization with Caiexin (CNX).

H Same as G for BIP co-localization.

I Same as G for ATZQ.

J Same as H for ATZQ.

K HaloTag pulse-chase analysis in MEF monitoring fluorescent Halo-ATZNNN during up to 24 h.

L Same as K for fluorescent Halo-ATZQ. Arrowheads show select clusters of JF646-labeled Halo-ATZQ. Arrowheads show co-localization Halo-ATZQ:Bip.

Data information: Scale bars 10 μm. Source data are available online for this figure.
Figure 4.
endolysosomal district for clearance (Fig 3H–K), accumulate during the chase time (Fig 4A, lanes 7–9 and 10–12, Fig 4B).

Further analyses performed by immunoisolation of ATZ with the associated molecular chaperones (see methods section) reveal that the two ERLAD substrates ATZNNN and ATZQNQ abundantly associate with CNX as expected by the involvement of the lectin chaperone in selection for their lysosomal delivery. Misfolded proteins are de-glycosylated, poly-ubiquitylated, and eventually degraded by 26S-proteasomes (ERAD, upper part) (Sun & Brodsky, 2019). De/re-glycosylation cycles and persistent association with Calnexin (CNX) select ERAD-resistant misfolded proteins for segregation in ER subdomains. ER vesiculation is directly or indirectly promoted by ER-phagy receptors (e.g., FAM134B, SEC62, RTN3L, CCPG1). ER-derived vesicles containing misfolded polypeptides are delivered to the endolysosomal district by LC3-dependent transport, macro- or micro- ER-phagy (ERLAD, lower part) (Molinari, 2021).

To sum up, in cells with active ERLAD (WT MEF, Fig 4A, lanes 1–3), the ERLAD client ATZNNN abundantly associates with CNX (Fig EV4B, Co-IP, upper panel, lane 2). The ERLAD-resistant ATZQQQ abundantly associates with BiP (Co-IP, upper panel, lane 3). In cells with inactive ERLAD (Uggt1-KO MEF, Fig EV4B, lanes 4–6), the association of ATZNNN with CNX is substantially reduced (compare Co-IP, upper panel, lane 5 vs. lane 2) and the association with BiP is enhanced (Co-IP, lower panel, lane 5 vs. lane 2). These data further support the notion that association with CNX plays a crucial role to channel ERAD-resistant polypeptides in the ERLAD pathway and may reveal a role of BiP for ER retention of those misfolded polypeptides that fail to access the ERLAD pathway.

Analyses of the detergent-insoluble fractions show the propensity of the ERLAD-incompetent ATZQON and ATZQQO to enter in detergent-insoluble fractions, from which they are extracted upon dissolution in 8 M urea (Fig 4F, lanes 4, 5).

**Figure 5. Selection of misfolded polypeptides for proteasomal (ERAD) or endolysosomal degradation (ERLAD).**

De-mannosylation (i.e., removal of light green mannoses by ER α-mannosidase I (αM) and EDEM proteins) generates ligand for mannose-binding lectins (OS-9 and XTP-3B). These shuttle misfolded proteins to translocation sites. Translocons are built around ER membrane-embedded E3 ligases (e.g., HRDL, TEB4, GP78) and include co-factors that confer client-specificity and promote cytosolic delivery. Misfolded proteins are de-glycosylated, poly-ubiquitylated, and eventually degraded by 26S-proteasomes (ERAD, upper part) (Sun & Brodsky, 2019). De/re-glycosylation cycles and persistent association with Calnexin (CNX) select ERAD-resistant misfolded proteins for segregation in ER subdomains. ER vesiculation is directly or indirectly promoted by ER-phagy receptors (e.g., FAM134B, SEC62, RTN3L, CCPG1). ER-derived vesicles containing misfolded polypeptides are delivered to the endolysosomal district by LC3-dependent transport, macro- or micro- ER-phagy (ERLAD, lower part) (Molinari, 2021).
Stationary and time course, confocal light scanning microscopy analyses of lysosomal delivery of ATZ glycosylation variants

The different intracellular fate of ERLAD-competent versus ERLAD-incompetent ATZ glycosylation variants was monitored by confocal laser scanning microscopy in the absence of BafA1. Under these experimental conditions, ATZNNN polymers are degraded as soon as they are delivered within the LAMP1-positive endolysosomes (see above and Fig EV4F). Consequently, at steady state, ATZNNN is mostly in the ER, where it is synthesized and co-localizes with the ER chaperones CNX (Fig 4G) and BiP (Fig 4H). In contrast, ATZQQQ is seen in the biosynthetic compartment, where it accumulates in large clusters that exclude CNX (Fig 4I) and co-localize with BiP (Fig 4J). Altogether, the results are consistent with a model, where ERLAD clients associate with CNX and are efficiently delivered within endolysosomes for clearance, whereas terminally misfolded proteins that inefficiently associate with CNX (or that are in cells characterized by defective function of the CNX chaperone system) are inefficiently delivered into endolysosomes, are captured by BiP and eventually enter insoluble aggregates.

This was further confirmed by the same HaloTag pulse-chase approach shown in Fig EV4F–H (i.e., performed in the absence of BafA1). Briefly, cells expressing Halo-ATZNNN or Halo-ATZQQQ (Fig 4K and L, respectively) are incubated with the cell permeable HaloTag fluorescent ligand JF646. After 45 min, JF646 is replaced by the “black ligand” 7-bromoheptanol to stop fluorescence incorporation. Fluorescent Halo-ATZNNN gradually disappears from cells within 24 h, consistent with its efficient lysosomal clearance (Fig 4K, gray scale, and see also Fig EV4F). Fluorescent Halo-ATZQQQ clusters in intracellular spots during the 24 h of chase (Fig 4L, gray scale). Characterization of the ATZQQQ clusters (red, Fig 4L) shows that they form outside the LAMP1-positive endolysosomes (Fig 4L, panel Merge, Halo/LAMP) and that they co-localize with BiP (Fig 4L, arrowheads). Thus, ERLAD clients expressed in ERLAD-incompetent cells that lack functional CNX or functional glucosidases and glucosyltransferase (Figs 2C and E–G, and J, and EV1B and Figs EV3H and J–L, and EV4D and E, and G, and H) or ERLAD clients depleted of the N-glycan required for ERLAD selection (Figs 3D and F, and H, and I, and 4I and J, and L, and EV3C and E, and EV5C and D, and H, and I, and M, and N) fail to be delivered to the lysosomal compartments for clearance and are more prone to form insoluble intracellular deposits.

Discussion

The ER is site of protein synthesis. Folding-incompetent and terminally misfolded polypeptides with highly divergent structure, size, modifications, physicochemical properties engage a variety of client-, cell-, tissue-specific and general pathways to be eventually removed from the biosynthetic compartment and degraded. A large fraction of them is delivered, via specialized translocation machineries built around membrane-embedded E3 ubiquitin ligases, across the ER membrane, for degradation by the cytosolic ubiquitin/proteasome system in catabolic processes collectively defined as ER-associated degradation (ERAD, upper part in Fig 5) (Sun & Brodsky, 2019).

The ER also generates misfolded polypeptides that fail to engage the ERAD machinery. Large size or aggregation/polymerization propensity generally prevent translocation across the ER membrane and activate alternative catabolic pathways that engage components of the ER-phagy machinery. ER-phagy programs are activated by pleiotropic or ER-centric signals that lead cells to eat their own ER to gain nutrients, to control ER size and function or, as in the case of misfolded proteins, to remove ER portions that contain them (Molinari, 2021). Under the control of a family of ER-phagy receptors, portions of the ER vesiculate and are delivered to the endolysosomal compartments for clearance. In the case of ER-to-lysosomes-associated degradation (ERAD), the ER portions detaching from the biosynthetic compartment contain ERAD-resistant misfolded polypeptides, which are delivered for lysosomal degradation via LC3-dependent vesicular transport, macro- and micro-ER-phagy (ERAD, lower part in Fig 5) (Fregno & Molinari, 2019).

How proteins are selected for ERAD is well understood. Strong experimental evidences support a mannose timer model, first proposed by Ari Helenius in the mid ’90s (Helenius, 1994). In this model, aberrant gene products generated in the ER are marked for proteasomal, ER-associated degradation (ERAD) upon removal of α1,2-bonded mannose residues (light green in Fig 1A). De-mannosylation is operated by members of the glycosyl hydrolase 47 family including the ER-resident α-mannosidase I and EDEM proteins. It interrupts CNX binding and generates N-glycan species engaging the mannose-binding ERAD lectins OS-9 and XTP3-B, which deliver terminally misfolded proteins to retro-translocation sites built around membrane-embedded E3 ubiquitin ligases (upper part in Fig 5) (Aebi et al, 2010; Adams et al, 2019; Sun & Brodsky, 2019). Mannose processing to select terminally misfolded proteins for ERAD and intervention of mannose-binding lectins to deliver terminally misfolded proteins to retro-translocation sites is conserved in yeast (Buschhorn et al, 2004; Bhamipati et al, 2005; Kim et al, 2005; Szathmary et al, 2005; Gaus et al, 2006; Kanehara et al, 2007; Clerc et al, 2009; Gaus et al, 2011).

The study of how ERAD-resistant proteins are selected for ERLAD is still in its infancy. Our analyses of the fate of ERAD-resistant ATZ polymers and Pro-Col2A1 variants in 5 mouse and human cell lines reveal, for ERLAD as well, the crucial role of N-linked glycans, their position within the polypeptide chain, and their processing. Our data reveal that selection of a misfolded polypeptide for ERLAD relies on Gl/GI/UGGT1-driven cycles of CNX association/release.

An interesting result emerging from our study is that one of the three ATZNNN oligosaccharides is required and sufficient for selection of ATZNNN polymers for ERLAD. The functional prevalence of one oligosaccharide in the context of multi-glycosylated proteins has previously been reported for N-glycan-regulated protein folding and for N-glycan selection for ERAD. For protein folding, Dan Hebert et al, showed that of the 7 N-linked oligosaccharides of influenza virus hemagglutinin, only the oligosaccharide at position 81 is absolutely required for maturation (Hebert et al, 1997). For ERAD, by studying the mechanistic details of clearance of the tetra-glycosylated CPY* and of the di-glycosylated PrA* proteins from the yeast ER, Spear and Ng revealed that only the oligosaccharide at position 479 of CPY* and the one at position 107 of PrA* are required to channel these two folding-defective polypeptides into the ERAD pathway (Spear & Ng, 2005). Here, by determining the fate of the 2C1-immunoreactive polymers formed by the 8 glycosylation variants of ATZ, we show that only the 4 variants displaying
the oligosaccharide at position 83 enter the FAM134B:CNX-driven ERLAD pathway. Thus, for protein folding (Hebert et al, 1997), for selection of misfolded proteins for proteasomal (ERAD, (Spear & Ng, 2005)), and for lysosomal degradation (ERLAD, this work) one of the oligosaccharides displayed on multi-glycosylated polypeptides plays a pivotal role as signaling appendix.

Surprisingly, our tests in a variety of cell lines revealed the lack of ATZ_{NNN} polymers delivery to endolysosomes in CHO cells. This confirms indirect evidences of defective ERLAD in CHO cells published by others (e.g., (Ronzoni et al, 2021)) and is in contrast to the efficient lysosomal delivery and clearance of ER portions containing ATZ_{NNN} polymers in human and mouse cell lines, primary hepatocytes, in a mouse model of the disease and even in yeast cells (Teckman & Perlmutter, 2000; Kamimoto et al, 2006; Kroeger et al, 2009; Hidvegi et al, 2010; Perlmutter, 2011; Pastore et al, 2013; Yamamura et al, 2014; Fregno et al, 2018; Cui et al, 2019; Strnad et al, 2020 and this work). We did not further investigate this issue, but we think that this merits a note in our discussion.

Multiple client-specific ERAD pathways ensure rapid removal by the ubiquitin proteasome system of the large array of misfolded polypeptides generated within the eukaryotic cells ER (Leto et al, 2019; Sun & Brodsky, 2019; Molinari, 2021). It is therefore fair to predict that multiple client-specific pathways also exist to ensure intraluminal segregation and lysosomal removal of the vast array of misfolded polypeptides that do not enter ERAD pathways. These client-specific ERLAD pathways may rely on selective engagement of one of the many ER-phagy receptors expressed by nucleated cells (Molinari, 2021). ER-phagy receptors play major role in ERLAD. This prediction is supported by evidences including (i) the defective lysosomal clearance of ATZ polymers ((Fregno et al, 2018) and this work), misfolded Pro-collagen (Forrester et al, 2019), or NPC1 variants (Schultz et al, 2018) in cells lacking the ER-phagy receptor FAM134B (or of its yeast ortholog Atg40p (Cui et al, 2019)); (ii) the ER expansion upon accumulation of insoluble acinar secretory enzymes in mice expressing a non-functional form of the ER-phagy receptor CCPG1 (Smith et al, 2018); (iii) the accumulation of aberrant secretory proteins in the hepatocyte’s ER of mice lacking the liver-specific FAM134B-2 splice variant (Kohno et al, 2019) and reviewed in (Molinari, 2021). As it happened for the identification of the large variety of ERAD pathways, further studies involving analysis of other model, ERAD-resistant polypeptides will certainly reveal the involvement of other ER-phagy receptors in ERLAD. On this line, ER-phagy receptors TEX264, SEC62, ATL3, and FAM134B are expressed ubiquitously, with the latter highly expressed in peripheral neurons and in the brain; its splice variant FAM134B-2 in kidney, liver, white adipose tissue, and spleen; CCPG1 in pancreas, liver, kidney, and stomach (Smith et al, 2018; Chino et al, 2019; Kohno et al, 2019; Keles et al, 2020). Moreover, select ER-phagy receptors have specific distribution within the ER, with TEX264 in three-way ER junctions (An et al, 2019; Chino et al, 2019), FAM134 variants in ER sheets (Khaminet et al, 2015), ATL3 and RTN3L in ER tubuli (Grunati et al, 2017; Chen et al, 2019). We predict that misfolded polypeptides expressed in tissue-specific or in sub-organelar-restricted manner will engage specific ERLAD pathways driven by the relevant ER-phagy receptor.

Why it is important to investigate ERLAD in molecular details? In about 10% of patients carrying the Z mutation of the SERPINA1 gene, clearance of ATZ polymers from the ER is defective. This results in clinically significant hepatotoxicity, which is the major inherited cause of pediatric liver disease and transplantation (Sharp et al, 1969; Eriksson et al, 1986; Wu et al, 1994; Hidvegi et al, 2010; Perlmutter, 2011; Roussel et al, 2011; Marciniak et al, 2016). Notably, cell lines derived from patients with liver disease (susceptible cells) and without liver disease (protected cells) degrade a model ERAD substrate with equal efficiency. However, susceptible cells degrade ATZ and other polymerogenic serpins less efficiently (Teckman & Perlmutter, 1996). Altogether, we speculate that cells of susceptible hosts carrying the PiZZ mutation leading to expression of ATZ have additional mutations leading to a loss-of-function in the ERLAD pathway. This would enhance accumulation of mutant ATZ within the ER of liver cells and make the patients more susceptible to liver injury.

Finally, our report underscores the role of N-glycans as tag for protein quality control. Their context-specific localization determines their processing by ER-resident glycanases and the signal they will encode to eventually release the protein into the secretory, the ERAD or the ERLAD pathways. Specialized lectins must decode the signal to ensure the appropriate assistance to folded (mature, ready-for-secretion), folding (immature, to be retained in the folding environment), or terminally misfolded polypeptide (to be cleared by proteasomes or lysosomes). ER-targeted therapeutic approaches are sought to intervene in diseases caused by protein misfolding, to interfere with pathogens that hijack these machineries to invade host cells and generate their progeny, and to enhance production of proteins-of-interest. Understanding how N-glycosylation and processing of N-linked glycans determine polypeptide’s fate allows pharmacologic/genetic intervention to enhance production of functional proteins or to optimize proteome (via ERAD) or lysosomal (via ERLAD) removal of defective ones.

Materials and Methods

Expression plasmids and antibodies

HA-tagged ATZ, FAM134B-V5, and FAM134BLIR-V5 are subcloned in pcDNA3.1. mCherry-Pro-collagen 2A1 and the R_{sync}C disease-linked variant and UGGT1 expression plasmid were described in (Arnold et al, 2000; Fregno et al, 2018; Forrester et al, 2019). Halo-ATZ_{NNN}-HA, Halo-AATNNN-HA Halo-ATZ_{QQQ}-HA are subcloned in pcDNA5 vector. The ATZ glycosylation variants were prepared by replacing asparagine 46, 83 and/or 247 of the glycosylation sequons with glutamine residues (fwd N46Q 5’-GAAGAAGATATTTGTTGCTATT GGACTTGTGTCAGCTG-3’, rev N46Q 5’-CAGCTGACACAGCAT CCAATAGCCAATATCTTCTTC-3’; fwd N83Q 5’-CCGGAATCTCC TGGAGGGCC-3’, rev N247Q 5’-GTGAGATTGAAATTCAGGCCCTCCA-3’). The ATZ glycosylation variants were prepared by replacing asparagine 46, 83 and/or 247 of the glyclosylation sequons with glutamine residues (fwd N46Q 5’-GAAGAAGATATTTGTTGCTATT GGACTTGTGTCAGCTG-3’, rev N46Q 5’-CAGCTGACACAGCAT CCAATAGCCAATATCTTCTTC-3’; fwd N83Q 5’-CCGGAATCTCC TGGAGGGCC-3’, rev N247Q 5’-GTGAGATTGAAATTCAGGCCCTCCA-3’). The mCherry-Pro-collagen glycosylation variant was prepared by replacing asparagine 1388 of the glyclosylation sequon with a glutamine residue (fwd 5’-CGACCTGCTGATGTCGTGCTGGGAGCTCTTGCTG-3’, rev 5’-ACGGAAGGTCCTCCAGACATCACTACCCTACCTGCG-3’). Antibodies used in this study are the monoclonal 2C1 anti-polymeric ATZ (Hycult Biotech) (Miranda et al, 2010), polyclonal anti-HA
(Sigma), monoclonal anti-HA probe F7 (Santa Cruz Biotech.), anti-LAMP1 (Hybridoma Bank, 1D4B deposited by J.T. August), anti-GAPDH (Merck), anti-v5 (Invitrogen), anti-CLIMP63 (Proteintech), anti-Blp (Stressgen), anti-UUGT1 (Sigma), anti-CNx (kind gift A. Helenius), anti-FAM134B (kind gift I. Kurth), anti-SEC62 (kind gift R. Zimmermann), anti-HaloTag® monoclonal (Promega), anti-HA probe (F7, Santa Cruz), anti-LC3B (Sigma), and anti-TEX264 (Novus). Alexa-conjugated secondary antibodies were purchased from Thermo Fisher Scientific and Jackson Immunoresearch; HRP-conjugated from SouthernBiotech, Invitrogen and Bio-Rad; Protein A and Protein G beads were from Sigma and Thermo Fisher, respectively.

**Cell culture, transient transfection, and inhibitors**

MEF, HEK293, HeLa, CHO, and HEPA 1-6 cells were grown in DMEM supplemented with 10% FBS at 37°C and 5% CO₂. Ctx-KO and stCnx (Denzel et al., 2002; Pieren et al., 2005; Kraus et al., 2010) and Ugg1-KO MEF (Molinar et al., 2005; Soldà et al., 2007) have been previously described. Flp-In™-T3 cells (Thermo Fisher) stably expressing Halo-ATZQNN-HA or Halo-ATZQQQ-HA were generated following manufacturer instructions and cultured in DMEM supplemented with 10% FCS and 150 mg/ml Hygromycin. Transient transfections were performed using JetPrime transfection reagent (PolyPlus) following manufacturer’s protocol. BafA1 (Calbiochem) was used at 50 nM for 12 h or 100 nM for 6 h. CST (Sigma) and KIF (Toronto Research Chemicals) were used at 1 mM, and 200 μM, respectively.

**CRISPR**

Cas9 engineered MEF cell lines SEC62-deficient MEF cells (CRISPR SEC62) and FAM134B-deficient MEF cells (CRISPR FAM134B) were engineered using CRISPR/Cas9 system as previously described (Fumagalli et al., 2016; Fregno et al., 2018). For generation of TEX264 KO cells: the guideRNA-Cas9 plasmid, lenti-CRISPRv2-puro system (Addgene52961) was purchased from Addgene (http://www.addgene.org). Guide sequences were obtained from the Cas9 target design tools (crispr.mit.edu:8079 and/or www.addgene.org/pooled-library) and synthesized by Microsynth. Two annealed oligonucleotides (5’-CACCG GCTGTTGTGCTACCCCTGCC-3’, 5’-AA ACGGCAGGCTGAGCACACAGCC-3’ for murine TEX264) were inserted into the lenti-CRISPRv2-puro vector using the BsmBI restriction site. After transfection of MEF cells with JetPrime (Polyplus), cells were cultured in DMEM supplemented with 10% FBS. Two days after transfection, the medium was supplemented with 2 μg/ml puromycin. Puromycin-resistant clones were picked after 10 days, and gene KO was verified by WB analysis.

**Protein cross-linking with DSP**

Cells were washed with PBS followed by addition of 1 mM DSP in PBS. After 30 min incubation at room temperature, the reaction was stopped with 1 M Tris, pH 7.8 to a final concentration of 20 mM and incubated for 15 min at room temperature. Cells were washed with PBS containing 20 mM NEM and lysed with RIPA buffer (1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate in HBS, pH 7.4) for 20 min on ice.

**Cell lysis, immunoprecipitation, and Western blot**

After treatments, cells were washed with ice-cold PBS containing 20 mM NEM and lysed with 2% CHAPS (Anatrace) in HBS pH 6.8 supplemented with protease inhibitors and Apyrase (Sigma) to prevent client proteins release from BiP. Post-nuclear supernatants (PNS) were collected after centrifugation at 10,600 g for 10 min. Detergent-insoluble material was solubilized in 8 M Urea and dissolved in 0.1 M ammonium bicarbonate for further analyses. For immunoprecipitations, PNSs were diluted with lysis buffer and incubated with Protein A or Protein G beads (1:10 w/v, swollen in PBS) and select antibodies at 4°C. After three washes of the immunoprecipitates with 0.5% CHAPS in HBS pH 7.4, beads were denatured for 5 min at 95°C and subjected to SDS-PAGE. Proteins were transferred to PVDF membranes using the Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked with 10% (w/v) non-fat dry milk (Bio-Rad) in TBS-T and stained with primary antibodies diluted in TBS-T followed by HRP-conjugated secondary antibodies or Protein A diluted in TBS-T. Membranes were developed using Western Bright ECL or Quantum (Advansa) and signals captured on Fusion FX (Vilber). Images were quantified with the Evolution Capture Edge (Vilber). Membrane stripping for probing additional antigens was done using Re-Blot Plus Strong Solution (Millipore) following manufacturer’s instructions.

**Native gel electrophoresis**

After CHAPS lysis, PNSs were incubated for 15 min at RT in Native Sample Buffer (Bio-Rad) and run on 7.5% native acrylamide gel in Tris/Glycine Buffer (Bio-Rad). Proteins were then transferred onto PVDF membrane in Tris/Glycine Buffer (Bio-Rad). Immunoblot analysis was performed as described above.

**Metabolic labeling**

Seventeen hours after transient transfections, cells were pulsed with 0.05 mCi [35S]methionine/cysteine mix and chased for the indicated time points with DMEM supplemented with 5mM cold methionine and cysteine. Cells were detergent-solubilized and radiolabeled proteins were revealed with Typhoon FLA 9500, version 1.0 (GE Healthcare). Radioactive signals were quantified using ImageQuant software (Molecular Dynamics, GE Healthcare).

**Confocal laser scanning microscopy**

Cells were plated on Alcian Blue-treated glass coverslips (for HEK293 cells, glass coverslips were pre-treated with Poly-Lys). After two washes with PBS cells were fixed at room temperature for 20 min in 3.7% formaldehyde diluted in PBS. Cells were permeabilized for 15 min with permeabilization solution (PS) (0.05% saponin, 10% goat serum, 10mM HEPES, 15mM glycine). Primary antibodies were diluted 1:100 in PS for 90 min, washed for 15 min in PS, and then incubated with Alexa Fluor-conjugated secondary antibodies diluted 1:300 in PS for 45 min. Cells were rinsed with PS and water and mounted with Vectashield (Vector Laboratories) supplemented with or without 4′,6-diamidino-2-phenylindole (DAPI). Confocal pictures were acquired on a Leica TCS SP5 microscope with a Leica HCS PL APO lambda blue 63.0X1.40 OIL UV objective. Image analyses and quantification were performed with...
LysoQuant as described in (Morone et al., 2020). Image processing was also performed with Photoshop (Adobe).

**HaloTag pulse-chase**

HaloTag covalently and irreversibly binds synthetic ligands, including cell permeable ligands that are coupled to fluorescent probes (England et al., 2015). When placed at the N-terminus of ATZ, the HaloTag does not affect polymerization and lysosomal clearance of ATZ (Dickens et al., 2016; Fregno et al., 2018). The HaloTag pulse-chase protocol is described in (Fregno et al., 2018). Briefly, cells expressing Halo-ATZ are incubated for 30 min with a cell permeable “black ligand” (i.e., 6-chlorohexanol, Sigma) to quench fluorescent detection of cellular Halo-ATZ. The “black ligand” is replaced by the cell permeable fluorescent, far red, HaloTag ligands JF646 or tetramethylrhodamine (TMR) (Promega) to label newly synthesized Halo-ATZ for 15 or 45 min (fluorescent pulse). The fluorescent ligand is replaced with the “black ligand” 7-Bromohexanol (Sigma) to rapidly interrupt fluorescent labeling of Halo-ATZ (chase). Cells were then fixed at the indicated time points for 20 min in 3.7% formaldehyde diluted in PBS or detergent-solubilized. The intracellular fate of the ATZ population synthesized during the fluorescent pulse is monitored by confocal laser scanning microscopy.

**Flow cytometry**

Cells were transfected as described above and, after 24 h, detached from Petri dishes, washed with PBS, fixed with 3.7% PFA in PBS for 20 min at RT, washed 2 times with PBS, and permeabilized with saponin solution (10% goat serum, 15 mM glycine, 0.05% saponin, 10 mM HEPES in PBS). Permeabilized cells were exposed for 90 min at RT to anti-HA and 2C1 antibodies diluted in saponin solution. Fluorophore-conjugated secondary antibodies diluted in saponin solution were added after 2 washes in PBS for 60 min at RT. Finally, cells were washed 2 times in saponin solution, resuspended in MACS buffer (2% FCS, 2 mM EDTA), and run on a FACSCanto I flow cytometer (BD Biosciences). Data were analyzed and annotated using FlowJo software (FlowJo LLC).

**Statistical analysis**

Plots and statistical analyses were performed using GraphPad Prism 8 (GraphPad Software Inc.). In this study, one-way ANOVA with Dunnett’s multiple comparisons test, two-way ANOVA and Sidak’s multiple comparison test, and unpaired two-tailed t-test were used to assess statistical significance. An adjusted P-value < 0.05 was considered as statistically significant.

**Data availability**

No primary datasets produced in the study.

**Expanded View** for this article is available online.

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**Author contributions**

Conceptualization: IF, EF, TS, CG, and MM; Methodology: IF, EF, TS, CG, and MM; Investigation: MM, IF, EF, TS, CG, and MM; Writing original draft: MM; Writing, review, editing: IF, EF, TS, CG, and MM; Supervision: MM.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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