Noncanonical autophagy at ER exit sites regulates procollagen turnover

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Type I collagen is the main component of bone matrix and other connective tissues. Rerouting of its procollagen precursor to a degradative pathway is crucial for osteoblast survival in pathologies involving excessive intracellular buildup of procollagen that is improperly folded and/or trafficked. What cellular mechanisms underlie this rerouting remains unclear. To study these mechanisms, we employed live-cell imaging and fluorescent light and electron microscopy (CLEM) to examine procollagen trafficking in both wild-type mouse osteoblasts and osteoblasts expressing a bone pathology-causing mutant procollagen. We found that although most procollagen molecules successfully trafficked through the secretory pathway in these cells, a subpopulation did not. The latter molecules appeared in numerous dispersed puncta colocalizing with COPII subunits, autophagy markers and ubiquitin machinery, with more puncta seen in mutant procollagen-expressing cells. Blocking endoplasmic reticulum exit site (ERES) formation suppressed the number of these puncta, suggesting they formed after procollagen entry into ERESs. The punctate structures containing procollagen, COPII, and autophagic markers did not move toward the Golgi but instead were relatively immobile. They appeared to be quickly engulfed by nearby lysosomes through a bafilomycin-insensitive pathway. CLEM and fluorescence recovery after photobleaching experiments suggested engulfment occurred through a noncanonical form of autophagy resembling microautophagy of ERESs. Overall, our findings reveal that a subset of procollagen molecules is directed toward lysosomal degradation through an autophagic pathway originating at ERESs, providing a mechanism to remove excess procollagen from cells.

procollagen | autophagy | ERES | microautophagy | lysosome

Significance

Type I collagen, a major component of bone, skin, and other connective tissues, is synthesized in the endoplasmic reticulum (ER) and passes through the secretory pathway. Rerouting of its procollagen precursor to a degradative pathway is crucial for reducing intracellular buildup in pathologies caused by defects in procollagen folding and trafficking. Here, we identify an autophagy pathway initiated at ER exit sites (ERESs). Procollagen proteins following this pathway accumulate at ERESs modified with ubiquitin, LC3, and other autophagy machinery. Modified ERESs carrying procollagen are then engulfed by lysosomes through a microautophagy-like mechanism, not involving conventional, double-membrane autophagosomes. Procollagen homostasis thus involves a noncanonical mode of autophagy initiated at ERESs, which might also be important in degradation of other secretory proteins.


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confocal microscopy and correlative light and electron microscopy (CLEM) to study procollagen trafficking in normal osteoblasts and osteoblasts expressing bone pathology-causing mutant molecules. We discovered that a subset of folded/misfolded procollagen molecules were diverted to lysosomes from ERESs. These ERESs were decorated with ubiquitin, LC3, and other autophagy effectors before being engulfed by nearby lysosomes through a noncanonical, autophagic process resembling microautophagy. Our findings identify a pathway for regulating procollagen homeostasis, which is initiated at ERESs and involves a noncanonical mode of autophagic degradation.

**Results**

**Following Procollagen Trafficking in Osteoblasts.** To study the intracellular pathways followed by procollagen, we made fluorescent procollagen probes in which the proα2(I) chain of mouse procollagen type I was labeled with a fluorescent protein (FP) at its N-terminus [i.e., FP-proα2(I)], similar to the probes described in ref. 21. In addition to this wild-type construct, we created a construct to mimic a bone pathology-causing Gly610-to-Cys (G610C) substitution in the triple helical region of proα2(I), that is, FP-proα2(G610C)(I). The constructs were then individually expressed in an MC3T3 osteoblast cell line (22).

The G610C substitution was previously used to engineer a mouse model of osteogenesis imperfecta (23). A study of osteoblasts from these mice in vivo and in culture revealed that the G610C substitution increased procollagen misfolding, intracellular accumulation, and autophagy without perturbing cell function until several weeks of culturing (4). Most mutant molecules were folded, secreted, and incorporated into extracellular collagen fibers; only a small fraction of them was misfolded. The increased misfolding caused accumulation of procollagen aggregates in the ER over time, but the resulting severe ER dilation and osteoblast malfunction were only observed after several weeks (4). In the present study, we examined the cells within 18–24 h after transfection with FP-proα2(G610C)(I) vs. FP-proα2(I), before a significant detrimental effect of the transfection on osteoblast function.

Imaging of cells expressing either FP-proα2(I) or FP-proα2(G610C)(I) revealed similar distributions of these markers, both in intracellular pools and in extracellular collagen fibers (Fig. 1A). We confirmed coassembly of these chains with proα1(I) chains into procollagen molecules by observing colocalization of FP-proα2(I) or FP-proα2(G610C)(I) with a similarly fluorescently labeled TagBFP2 proα1(I) chain as well as with an antibody that recognizes properly folded procollagen (SI Appendix, Fig. S14).

Significant pools of FP-proα2(I) or FP-proα2(G610C)(I) were found in the ER and Golgi, based on their colocalization with the ER marker (Ii33-RFP) and Golgi marker (Cherry-GM130) (Fig. 1B). The extent of colocalization was similar to that found for endogenous procollagen detected with antibodies in primary osteoblasts, with no changes in ER or Golgi morphologies seen upon expression of the procollagen probes (SI Appendix, Fig. S1B). In cells expressing FP-proα2(I) or FP-proα2(G610C)(I), a continuous flux of procollagen moving from ER to Golgi was evident from experiments in which we selectively photobleached the Golgi pool of these markers (Movie S1).

Data from the above indicated that the majority of FP-proα2(I) and FP-proα2(G610C)(I) molecules in our MC3T3 osteoblasts were successfully assembled into heterotrimers, exported to the Golgi, and later released as mature collagen fibers. It remained possible, however, that subpopulations of procollagen molecules in these cells, in particular misfolded forms, were being routed differently.

**Rerouting of Some Procollagen Molecules to a Degradative Pathway.** To investigate whether any intracellular pools of procollagen were diverted from the above secretory trafficking route toward degradation, we coexpressed markers for autophagosomes (i.e., Cherry-LC3) or lysosomes (LAMP1-Cherry) in MC3T3 cells expressing FP-proα2(I) or FP-proα2(G610C)(I). Notably, we observed a small fraction of either FP-proα2(I) or FP-proα2(G610C)(I) present in dispersed puncta containing LC3/LAMP1 (Fig. 1C and D). At steady state, the percentage of FP-proα2(I) and FP-proα2(G610C)(I) proteins localized to LC3/LAMP1 puncta was ∼3–5% of total FP-labeled procollagen in these cells.

Further analyses of the above images revealed that the percentage of all Cherry-LC3 puncta that contained FP-proα2(I) in cells coexpressing these constructs was ∼30%, whereas Cherry-LC3 puncta that contained FP-proα2(G610C)(I) in cells coexpressing these constructs was ∼55% (Fig. 1E). A similar increase was also observed in transfected primary osteoblasts (SI Appendix, Fig. S2). We also found that in cells cotransfected with FP-proα2(I) and LAMP1-Cherry, ∼28% of LAMP1-positive lysosomes contained the procollagen probe, with the percentage increasing to 33% in FP-proα2(G610C)(I)-expressing cells (Fig. 1F). All these observations suggested that the mutation increased the diversion of procollagen toward autophagic degradation in lysosomes.

![Image](https://www.pnas.org/cgi/doi/10.1073/pnas.1814552115)
Characteristics of the Degradative Pathway Followed by Procollagen.

To clarify the pathway followed by procollagen toward autophagosomes and lysosomes, we employed FP-procoα2G610C(I) as our procollagen marker, since more of it colocalized with autophagosomes/lysosomes compared with FP-procoα2(I). We began by asking whether LC3-labeled puncta positive for FP-procoα2G610C(I) colocalized with other autophagy markers. The markers tested included Atg14, a member of the PI3 kinase complex I required for recruitment of LC3 lipidation machinery (24); the transmembrane protein Atg9, thought to provide a membrane source for autophagosomes (25, 26); ubiquitin, which marks substrates for autophagy (27); and an adaptor protein p62, which contains a ubiquitin binding domain and an LC3-interacting region (28). Notably, we observed each of these autophagy markers colocalizing with the procollagen-containing LC3-puncta (Fig. 2D), albeit to differing extents (discussed below). This confirmed that the puncta containing FP-procoα2G610C(I) and LC3 were authentic autophagic structures.

We next investigated the intracellular site from which procollagen was targeted toward these autophagic structures. An obvious possibility was the ER, since prior work has shown that whole ER cisternae or their fragments can be engulfed by phagophore membranes and delivered to lysosomes in a process called ER-phagy (29, 30). ER-phagy has also been linked to activation of the unfolded protein response (UPR), an ER stress pathway for unfolded/misfolded proteins (29, 31). To test whether ER-phagy was responsible for the autophagic fate of procollagen seen in our cells, we coexpressed FP-procoα2G610C(I) with an ER lumen marker, ssFP-KDEL, or with ER membrane markers, Ii33-FP or FP-Sec61 (Fig. 2B). ER-phagy would result in colocalization of misfolded procollagen with ER lumen and membrane markers in autophagosomes. We observed no ER proteins in LC3-positive puncta containing FP-procoα2G610C(I). This argued against the ER-phagy hypothesis.

We then examined whether procoα2G610C(I) was diverted into the autophagy pathway after entering the Golgi apparatus. To address this, we utilized the drug brefeldin A (BFA), known to disassemble the Golgi apparatus and block further protein transport through the secretory pathway (32–34). As expected, BFA treatment blocked procollagen secretion (SI Appendix, Fig. S3D). However, it reduced neither the total number of autophagic structures nor the number of autophagic structures containing procollagen (Fig. 2C and SI Appendix, Fig. S3B). Moreover, there was no effect of BFA treatment on procollagen content in biochemical analyses of primary osteoblasts (SI Appendix, Fig. S3C). Therefore, procoα2G610C(I) did not enter the autophagic pathway at the level of the Golgi or further downstream in the secretory pathway.

Recent work has revealed that COPII-coated structures such as ERESs can act as players in the formation of autophagic membranes (35–39). We therefore examined whether ERESs have any role in the formation of autophagic puncta positive for FP-procoα2G610C(I). As a simple way to explore this, we treated cells with the small-molecule PKA inhibitor H89, which prevents ERES formation by disrupting COPII coat assembly (40, 41). Within 1 h of H89 treatment, procollagen secretion was blocked (SI Appendix, Fig. S3D). Significantly, the overall number of both LC3-positive autophagic structures and that of FP-LC3–positive autophagic structures that contained FP-procoα2G610C(I) decreased (Fig. 2D and SI Appendix, Fig. S3A). Also, biochemical analyses in primary osteoblasts showed decreased autophagic flux after H89 treatment (SI Appendix, Fig. S3C). This suggested that diversion of procollagen to an autophagic pathway possibly occurs at or downstream of ERESs.

To further test this idea, we cotransfected cells with FP-procoα2G610C(I), FP-LC3, and the COPII coat proteins FP-sec23 and FP-sec31 to mark the COPII coat. As a simple way to explore this, we treated cells with the small-molecule PKA inhibitor H89, which prevents ERES formation by disrupting COPII coat assembly (40, 41). Within 1 h of H89 treatment, procollagen secretion was blocked (SI Appendix, Fig. S3D). Significantly, the overall number of both LC3-positive autophagic structures and that of FP-LC3–positive autophagic structures that contained FP-procoα2G610C(I) decreased (Fig. 2D and SI Appendix, Fig. S3A). Also, biochemical analyses in primary osteoblasts showed decreased autophagic flux after H89 treatment (SI Appendix, Fig. S3C). This suggested that diversion of procollagen to an autophagic pathway possibly occurs at or downstream of ERESs.
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Fig. 3. Misfolded procollagen enters autophagic structures at ERES. (A) Procollagen autophagic structures marked with GFP-prox2\(^{G610C}\)(I) and TagBFP2-LC3 were imaged in MC3T3 cells also transfected with FP-tagged components of COPII coat Cherry-Sec31 and Halo-Sec23. Outline of an LC3-positive punctum is projected onto the other channels in zoomed confocal single-slice images to visualize colocalization. Colocalization was confirmed by analysis of the full 3D z-stack (SI Appendix, Fig. S4). (B) Similar imaging of TagBFP2-Sec23 and Cherry-Sec31 colocalization with procollagen autophagic structures positive for GFP-prox2\(^{G610C}\)(I) and Halo-LC3. LC3 puncta outlines show only the structures in which the colocalization was confirmed by 3D z-stack analysis (SI Appendix, Fig. S4). The number of FP-Sec23/FP-prox2\(^{G610C}\)/FP-LC3 colocalized puncta represented 9.8 \(\pm\) 1.4% of total LC3 puncta (n = 21 cells) and 4.7 \(\pm\) 0.7% of total Sec23 puncta (n = 20 cells). (C) None of the puncta marked with GFP-prox2(\(^{G610C}\), TagBFP2-LC3, and FP-Sec23 (arrows) contained either Halo-Sec61 (ER membrane) or ssHalo-KDEL (ER lumen) markers. (D) Puncta marked with FP-Sec23, GFP-prox2(\(^{G610C}\), I), and TagBFP2-LC3 contained autophagic markers Apple-ATG14, Apple-ATG9, Halo-LC3, p62, or Apple-Ub. Images of C. Bottom are Airyscan single slices; all other images are confocal single slices. In all images, individual blue channels are displayed in cyan for better visualization. [Scale bars: 10 \(\mu\)m (whole cell) and 2 \(\mu\)m (zoom).]
FP-\(\alpha_2^{G610C}(I)\), FP-LC3, and LAMP1-FP (Fig. 6E, untreated), but this fraction increased to \(\sim 50\%\) when cells were treated for 6 h with leupeptin to block lysosomal hydrolases (Fig. 6B and E, leupeptin). This suggested that puncta containing Sec23, procollagen, and LC3 were being delivered to lysosomes for degradation, but upon arrival there Sec23 was degraded by lysosomal hydrolases faster than procollagen and LC3.

Ultrastructural Analysis of Procollagen-Laden ERESs Undergoing Autophagy. To explore the mechanism by which puncta containing procollagen, Sec23, and LC3 were directed toward lysosomes, we performed CLEM. Cells expressing FP-tagged versions of procollagen, Sec23, and LC3 were fixed and structures positive for these markers were imaged using enhanced resolution Airyscan microscopy. The images were then aligned with transmission electron microscopy (Fig. 4).

**Fig. 4.** Procollagen/Sec23/LC3 puncta colocalize with ubiquitination machinery. (A) Airyscan slice showing TagBFP2-LC3 and GFP-\(\alpha_2^{G610C}(I)\) puncta colocalization with Halo-Sec23 and Apple-CUL3, an E3 ubiquitin ligase. All large FP-CUL3 puncta were confirmed to be colocalized with FP-LC3 (Bottom Left image of the whole cell) and procollagen (zoomed panels, Right), and some also contained FP-Sec23 (zoomed panels, outline). (B) Airyscan slice showing colocalization of TagBFP2-LC3 puncta with Halo-KLHL12 adaptor for CUL3 ubiquitin ligase (Bottom Left) and with GFP-\(\alpha_2^{G610C}(I)\) and Apple-Sec23 (zoomed panels, outlines). All Halo-KLHL12 puncta colocalized with GFP-\(\alpha_2^{G610C}(I)\) were confirmed to be also colocalized with TagBFP2-LC3 (top images of the whole cell). (C) Airyscan slice showing colocalization of Halo-LC3 puncta with Apple-Keap1 adaptor for CUL3 ubiquitin ligase (Bottom Left) and with GFP-\(\alpha_2^{G610C}(I)\) and TagBFP2-Sec23 (zoomed panels, outlines). In all zoomed panels, yellow outlines of LC3-positive puncta are projected in white onto other channels. Individual blue channels are displayed in cyan. [Scale bars: 10 \(\mu m\) (whole cell) and 2 \(\mu m\) (zoom).]

**Fig. 5.** Procollagen/LC3 structures rapidly form at COPII puncta, retain COPII coat, and remain relatively stationary. MC3T3 cells transfected with Cherry-Sec23, GFP-\(\alpha_2^{G610C}(I)\), and TagBFP2-LC3 (Left) were imaged by Airyscan (5 s) time-lapse microscopy (Movie S4). Single-slice, time-lapse images (Right) show preexisting long-lived \(\alpha_2^{G610C}(I)\)/LC3/LC3-positive autophagic structures (white arrows) and formation of a new \(\alpha_2^{G610C}(I)\), LC3, and Sec23-positive autophagic structure (white circle). Blue channels are displayed in cyan for better visualization. [Scale bars: 10 \(\mu m\) (whole cell) and 1 \(\mu m\) (zoom).]
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lysosomes, which would prevent any exchange of bleached FP-Sec23 within puncta with unbleached molecules in the cytoplasm (Fig. 9 D–F, SI Appendix, Fig. S8, and Movie S5).

These results further argued against macroautophagy as a mechanism for delivery of puncta containing procollagen, Sec23, and LC3 to lysosomes. As shown in Fig. 10A, macroautophagy would predict that Sec23 should stop exchanging with cytoplasmic pools as soon as it is enveloped by a phagophore membrane. As this should occur before a Sec23/LC3-labeled autophagosome interacts with a lysosome, exchange of Sec23 with cytoplasmic pools should not occur at LAMP1-positive puncta and should not be impacted by leupeptin treatment. The data instead supported a microautophagy mechanism implied by the above CLEM data, in which lysosomes envelope puncta containing procollagen, Sec23, and LC3 (see model in Fig. 10B). In this model, Sec23 would continue to exchange on and off LC3-modified ERESs as nearby lysosomes start to envelope these sites. Once the ERES is fully enveloped by the lysosome, Sec23 exchange with the cytoplasm would stop. Leupeptin treatment would give rise to higher levels of Sec23 that show no recovery after photobleaching since this treatment prevents degradation of procollagen, Sec23, and LC3 inside fully enveloped lysosomes.

Discussion

In this paper, we provide evidence that a subset of folded/misfolded procollagen molecules are directed toward lysosomal degradation through a pathway initiated at ERESs. In this pathway, procollagen-laden ERESs decorated with both COPII coat proteins and autophagy-related proteins are directly engulfed by nearby lysosomes through a microautophagy-like process (Fig. 10B). Since expression of mutant procollagen led to increased internalization of ERESs, which increases procollagen misfolding relative to expression of wild-type procollagen, entry into this pathway, the pathway’s role is likely for routing misfolded molecules toward degradation.

Other pathways for removal of excess and/or misfolded procollagen, such as ER-phagy, are possible and could depend on the extent of procollagen misfolding, collagen type, cell type, or experimental conditions. However, no procollagen turnover by ER-phagy was ever observed. This suggested that ER-mediated degradation of procollagen was a major pathway for disposal of misfolded/abnormal procollagen in our cells. While the precise amount of procollagen routed into this pathway was unclear, ~3–5% of intracellular FP-α2(G610C) was found in LC3-positive structures. Given that ~50% of all LC3-positive structures contained procollagen, the pathway accounted for a major fraction of the autophagic flux in the cell (Fig. 1E).

Prior work has reported that a subpopulation of ERESs interact with autophagy modulators/receptors, serving as a membrane source for precursors of autophagic structures (20). Our analysis of procollagen trafficking suggests that ERESs might also contribute to entry points for unwanted secretory cargo into a noncanonical autophagy pathway. This pathway would begin by influx of misfolded molecules like procollagen into the elaborate membrane domains comprising an ERES. Due to the presence of the misfolded proteins, the ERES would become modified with ubiquitin, causing autophagy machinery to get recruited to it. Nearby lysosomes would then engulf the ERES through the process of microautophagy (Fig. 10B).

What is the most important evidence for this model? First, time-lapse imaging revealed puncta containing FP-α2(G610C) (I), FP-Sec23, and FP-LC3 appeared abruptly and then remained immobile before their delivery to lysosomes (Fig. 5 and Movie S4). This suggested they were modified ERESs rather than ER-to-Golgi transport intermediates, which are typically transient in nature and move quickly away from ER-Golgi membranes (21). Second, puncta containing FP-α2(G610C) (I), FP-Sec23, and FP-LC3 could be seen inside lysosomes by CLEM (Fig. 7). Third, these internal structures contained lysosomal membrane, implying a microautophagy rather than macrolamellar (22) delivery of these structures. Consistent with this possibility, delivery of the puncta to lysosomes was not blocked by the microautophagy inhibitor bafilomycin A1 (Fig. 8). Furthermore, Sec23 subunits continued to exchange on and off the puncta even after the puncta became localized with lysosomes. Envelopment by phagophore membranes in the process of macrolamellar autophagy precludes such exchange of Sec23 with surrounding cytoplasm. In a microautophagy delivery mode, however, such exchange would be possible if engulfment by the lysosome was slow.

Given this model for ERES-initiated autophagy, many questions arise. One issue is how cargo like misfolded procollagen is recognized for entry into the pathway. Misfolding of the procollagen triple helix, which can result in gelatin-like aggregates, is distinct from misfolding of globular proteins (4, 6) in that it does not expose large hydrophobic surfaces that bind BIP or other ER chaperones, which normally prevent unfolded/misfolded globular proteins from entering the secretory pathway (46, 47). This could help explain why in prior studies of procollagen autophagy...
in primary osteoblasts no UPR activation was seen (4). Therefore, misfolded procollagen molecules might escape chaperone-based ER quality control and enter ERESs on their own, or together with normally folded molecules. We envision that the presence of sufficient levels of misfolded procollagen in an ERES would then delay/disrupt formation of Golgi-bound carriers, perhaps by being too bulky, triggering ERES modification and recruitment of autophagic machinery.

Another question is how an ERES that contains misfolded procollagen becomes modified with autophagic machinery. Prior work has shown that the CUL3 ubiquitin ligase adaptor protein KLHL12 helps to ubiquitinate Sec31 (48, 49). One possibility, therefore, is that buildup of misfolded procollagen pools at an ERES leads to ubiquitination of ERES surface proteins, initiating downstream recruitment of autophagy machinery. Consistent with this scenario we found that KLHL12, CUL3, and an additional adaptor Keap1 were associated with puncta containing FP-proc20610C(1), FP-Sec23, and FP-LC3 (Fig. 4). Other autophagy proteins were also seen associated with these puncta, including FP-Atg14 and FP-Atg9, as well as the autophagy adaptor FP-p62 (Fig. 3).

Modification of COPII subunits in response to procollagen accumulation could be a critical factor in mediating ERES autophagy. Monoubiquitination of Sec31 by the CUL3/KLHL12 complex has been proposed to be involved in the formation of giant COPII vesicles, enabling procollagen molecules to leave the ER (48, 49). We found COPII subunits, CUL3 and KLHL12 were associated with procollagen puncta marked with LC3. These puncta were relatively stationary (Figs. 3–5 and Movie S4), unlike the rapidly moving, Golgi-bound vesicles with procollagen. The Golgi-bound vesicles lacked a COPII coat (SI Appendix, Fig. S5 and Movies S2 and S3), as suggested in prior studies examining procollagen movement to the Golgi (50, 51). These results raise the possibility that modification of COPII subunits by ubiquitination machinery is involved in recognizing misfolded forms of procollagen at ERESs and in redirecting them into the ERES autophagy pathway. This role would be distinct from that previously described for ubiquitinated COPII in trafficking of procollagen into the secretory pathway (48, 49).

Further work is necessary for understanding how disease-causing COPII mutants induce pathologies in collagen-rich tissues, which include a severe form of osteogenesis imperfecta (14, 16, 17), considered to be primarily a type I procollagen disorder.

Fig. 9. Sec23 dynamics in procollagen autophagic ERESs engulfed by lysosomal membranes. (A and C) Single-slice confocal images of GFP-proc20610C(1), TagBFP2-LC3, Apple-Sec23, and LAMP1-Halo–positive puncta in untreated MC3T3 cells (A) and after 6-h 100 μM leupeptin treatment (C). Individual blue channels are displayed in cyan. (Scale bars: 2 μm.) (B and D) High-magnification, single-slice Airyscan microscopy images of the boxed regions before and 0, 1, 3, 5, 7, and 9 min after Apple-Sec23 photobleaching within the circled area (selected from full 30-s-per-image time-lapse sets shown in Movie S5). Individual blue channels are displayed in cyan. (Scale bars: 1 μm.) (E) Average kinetics of Apple-Sec23 fluorescence recovery in untreated cells and in cells pretreated for 6 h with 100 μM leupeptin. In the treated cells, the kinetics are shown only for the puncta that exhibited no fluorescence recovery (~65% of all puncta); average kinetics of fluorescence recovery in the other 35% of the puncta is shown in SI Appendix, Fig. S8. Graph displays mean intensity relative to the prebleach value ± SEM.

Fig. 10. Noncanonical ERES microautophagy model of procollagen degradation. Schematics of macroautophagy (A) and microautophagy (B) pathways of ERES degradation. In macroautophagy, the cargo is first internalized inside a double-membrane autophagosome followed by autophagosome-lysosome fusion. In microautophagy, the cargo is directly engulfed by a lysosome. Misfolded procollagen aggregates appear to enter ERESs on their own or together with normally folded molecules, preventing formation of Golgi-bound carrier vesicles and activating autophagy machinery (perhaps because of their size/structure). These ERESs are then degraded by microautophagy, as suggested by the following observations. (i) Airyscan and CLEM microscopy show LC3 membranes intermixing with (expected in microautophagy) rather than encapsulating expected in macroautophagy) Sec23 and procollagen. (ii) CLEM shows LC3 inside but not on the LAMP1-positive lysosome surface, as expected after lysosomal engulfment but not autophagosome-lysosome fusion. (iii) CLEM also shows encapsulation of LAMP1-positive membranes together with autophagic ERES inside the lysosome, as expected after lysosomal engulfment but not autophagosome-lysosome fusion. (iv) No effect of bafilomycin A1 on lysosomal internalization of autophagic ERESs containing procollagen is consistent with micro- but not macroautophagy. (v) Sec23 photobleaching experiments show rapid exchange of Sec23 between cytoplasm and LAMP1-positive autophagic ERESs, which is possible at ERESs partially engulfed by lysosomes in microautophagy but not after autophagosome-lysosome fusion.
Procollagen folding, secretion, and degradation were occurring at ERESs described here.

A final set of questions relates to how lysosomes target LC3-decorated ERESs containing procollagen and whether ERES microautophagy is activated by stresses other than procollagen misfolding. Currently, we do not know the mechanism by which lysosomes move to ERESs to engulf them. Likewise, it is unclear whether this pathway is triggered by other conditions. For instance, under amino acid starvation, COPII coat proteins (52, 53) have been shown to be involved in a role in nonlysosomal microautophagy. Perhaps direct rerouting of secretory cargo from ERESs to lysosomes through ERES microautophagy under starvation conditions could help resupply the cell with amino acids from a now-disposable source (i.e., secretion). Regardless of whether it is activated by other conditions, ERES microautophagy seems to be a natural pathway for diverting proteins that escape the ER lumen quality control from the secretory route to lysosomal degradation.

Materials and Methods

Cell Lines and Primary Cell Culture. MC3T3–E1 Subclone 4 osteoblast cell lines were acquired from ATCC (ATCC CRL-2593). Cells were cultured in αMEM + Glutamax (32571-036; Gibco) supplemented with 10% FBS (Sigma-Aldrich) and 1% Pen/Strep (Corning). To stimulate procollagen synthesis and secretion, ascorbic acid 2-phosphate (Sigma-Aldrich) was supplemented 18–24 h before imaging experiments. Primary osteoblasts were extracted from mice harboring the G610C mutation and their wild-type littermates (66.12F/V8–Col1α2m1Brcrl; Jackson Laboratories), which were maintained on the C57BL/6J background (4, 23). Osteoblasts were extracted from parietal bones of 3- to 8-old-day mice as previously described (4). All care and procedures were performed in accordance with a U niece Kennedy Shriver National Institute of Child Health and Human Development Animal Care and Use Committee-approved protocol.

Constructs. Two FP-prox2() constructs were generated. In one construct, FP CDNA was placed between the signal sequence and exon 6 Col1α2 CDNA (Origene), replacing exons 1–5 that encode the N-propeptide and its cleavage site, following the cloning protocol generously provided by Sarah Dallas (21, S4). In another construct, FP CDNA replaced exons 2–3, retaining the cleavage site and the minor triple helix of the N-propeptide. Both constructs demonstrated identical trafficking and secretion patterns. FP-prox2() construct was similarly shown to be a useful model for FPs and was used in the following constructs: GM130-Cherry modified from GM130-CFP (34), I33-Cerulean (55), and I33-mRFP (55). FP-LC3 constructs were modified from CFP-LC3 (56); LAMP1-FP from LAMP1-Cherry (57); Sec23 from YFP-Sec23A, a gift from David Stephens, University of Bristol, Bristol, United Kingdom (Addgene 66611) (10); FP-Sec31 modified from pECP-Sec31A, a gift from CFP-LC3 (56); LAMP1-FP from LAMP1-Cherry (57); Sec23 from YFP-Sec23A, a gift from David Stephens, University of Bristol, Bristol, United Kingdom (Addgene 66611) (10); FP-UB modified from GFP-UB, a gift from Nico Dantuma, Karolinska Institutet, Stockholm (Addgene 11928) (S8); FP-p62 modified from pMXs-puro GFP-p62, a gift from Noboru Mizushima, University of Tokyo, Tokyo (Addgene 38277) (S9); sHalo-KDEL modified from mEmerald-ER-3, a gift from Michael Davidison, Florida State University, Tallahassee, FL (Addgene 54002); Halo-Sec61 modified from mApple-Sec61α-C-18, a gift from Michael Davidison (Addgene 54946); Halo-KLH12 modified from XE50-pCDNA3.1 +neo–VSV-KLH12-OX5, a gift from Randall Moon, University of Washington, Seattle (Addgene 16759); and FP-Halo modified from pENTR4-HaloTag (w876-1), a gift from Eric Campeau, University of Massachusetts, Worcester, MA (Addgene 29644). The following FPs were utilized: eGFP-N1 (GFP), mCherry-N1 (Cherry), HaloTag-N1 (Halo), mApple-N1 (Apple), mVenus-N1 (Venus), mCerulean-N1 (Cerulean), and mTagBFP2-N1 (TagBFP2). Janelia Fluor D646 (60, 61) was utilized for marking Halo-tagged molecules in live cells.

FP-prox2() and FP-prox1() with GFP and Venus FP had low transfection efficiencies but were otherwise well-expressed and appeared to have minimal or no effect on procollagen synthesis and trafficking as well as cell function, unless dramatically overexpressed. Only the cells with low or moderate expression levels of FP-procollagen were utilized for imaging experiments. Cells that overexpressed GFP or Venus FP accumulated large aggregates within the ER lumen, although physiologically relevant and recapitulating in vivo observations of ER stress (4), were not utilized for studies of trafficking or degradation. TagBFP2, Cerulean, or Apple FP-procollagen constructs had even lower transfection efficiencies than GFP and Venus constructs but displayed no abnormal distributions. FP-procollagen with Cherry had an abnormal localization pattern inside the cell, which appeared to be caused by Cherry dimerization.

Transfection and Treatments. MC3T3 cells were transfected with Fugene 6 (Promega) and primary cells were transfected with TransIT-LT1 (Mirus Bio), using the manufacturer’s protocols. Cells were imaged 18–24 h after transfection and subsequent incubation in αMEM + Glutamax media supplemented with 100 μM ascorbic acid 2-phosphate (Sigma-Aldrich) and 10% FBS from Valley Biomedical (lot no. NC0550 tested for supporting osteoblast differentiation). Fifty micromolar BFA (Sigma-Aldrich), 5 μg/mL BFA (Cell Signaling), 10 μM bafilomycin A1 (Sigma-Aldrich), and 100 μM leupeptin (Sigma-Aldrich) were added to the cell culture media as needed at the time points indicated in the text.

Immunofluorescence. Cells were fixed in freshly prepared methanol-free 2% formaldehyde (Thermo Fisher Scientific) solution in PBS, pH 7.4, for 10–15 min, washed in PBS, permeabilized in 0.4% Triton X in PBS for 10 min, and returned to PBS. After 30-min blocking in 3% BSA in PBS for 30 min, cells were incubated overnight at 4 °C with primary antibody diluted in the same blocking buffer then washed and incubated for 30–60 min with secondary Alexa Fluor-labeled antibodies (Thermo Fisher Scientific) diluted with 1.5% BSA in PBS. After the final PBS wash, cells were either imaged immediately or mounted with Prolong Diamond Antifade with DAPI (Thermo Fisher Scientific) for subsequent imaging. The following primary antibodies were utilized: anti-procollagen (AB765P, Millipore), anti-LC3 (3688; Cell Signaling), anti-SEC31 (13483; Cell Signaling), anti-GM130 (610822; BD Biosciences), and anti-PDI (103; Enzo Life Sciences).

Imaging. Live–fixed-cell imaging was performed on an LSM 880 microscope (Zeiss) with a 63× oil objective at standard confocal resolution or enhanced Airyscan resolution. Live-cell imaging was performed with line scanning whereas fixed-cell imaging was performed with frame scanning. For CLEM imaging, MC3T3 cells were grown on fibronectin-coated gridded cover glass (72265-50; EMS) and transfected as described above. After imaging for 18 h, cells were fixed with 2% formaldehyde and 0.1% glutaraldehyde (Sigma-Aldrich) in PBS for 10 min and imaged in PBS with Airyscan resolution. After imaging, the cells were additionally fixed in 2.5% glutaraldehyde, 2% formaldehyde, and 2 mM CaCl2 in 0.1 M sodium cacodylate, pH 7.4, for 15 min at room temperature followed by 45 min on ice. The coverslips were washed for 5 min four times, postfixed with 2% OsO4 for 2 h in the same buffer at 4 °C, after which, rinsed in water, stained with 2% uranyl acetate in water, dehydrated through series of increasing ethanol concentrations (30, 50, 70, and 90%, three changes of 100%) and embedded in EMBed 812 epoxy resin (EMS). After resin polymerization, the coverslip was removed with hydrofluoric acid. Cells previously imaged by light microscopy were identified by their position on the grid. A 1 × 1-mm area containing the cell(s) of interest was cut out using a jeweler’s saw, mounted on an aluminum holder, and trimmed to 300 μm × 300 μm. For technical validation of the approach, gridded 70- to 80-mm diameter 1-mL polystyrene cups were cut parallel to the plane of the coverslip and mounted on formvar-carbon-coated slot (0.5 × 2 mm) EM grids. Sections were stained with 2% uranyl acetate in 50% ethanol and imaged in an FEI Tecnai 20 transmission electron microscope operated at 120 kV. Images were recorded on ARTM XR81 widefield CCD camera. Light and electron microscopy images were manually aligned based on well-defined organelles.

Photobleach Corrections for Time-Lapse Videos of Fluorescence Recovery Experiments. Time-lapse sequences and videos were corrected for photo-bleaching associated with acquisition of multiple images from the same area by using a bleach correction plugin based on histogram matching within the Fiji image processing package (62). In FP-Sec23 fluorescence recovery after photo-bleaching experiments (Fig. 9 and SI Appendix, Fig. S8), intensity of FP- Sec23 puncta within the bleached fluorescence recovery area was normalized to the intensity of adjacent FP- Sec23 puncta in the same image outside this area.

Biochemical Assays. Procollagen folding, secretion, and degradation were measured by Western blotting and pulse-chase experiments with azidohomoalanine as described in SI Appendix, Fig. S3 C–E (4, 63–65).

Quantitation and Statistical Analysis. Images were quantitatively analyzed using custom generated macros for Fiji image processing package as illustrated in SI Appendix, Fig. S6. Two-way ANOVA with a Holm–Sidak post hoc test (SigmaPlot 13.0; SYSSTAT) was performed for analysis of transfected and treated primary osteoblasts (SI Appendix, Figs. 52 and S3E). One-way repeated-measures ANOVA with a Holm–Sidak post hoc test was performed on time-series data with BFA or H89 treatment (Fig. 2 C and D). Heteroscedastic, two-tailed t tests were performed for all other data.
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