A selective ER-phagy exerts procollagen quality control via a Calnexin-FAM134B complex

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Abstract

Autophagy is a cytosolic quality control process that recognizes substrates through receptor-mediated mechanisms. Procollagens, the most abundant gene products in Metazoa, are synthesized in the endoplasmic reticulum (ER), and a fraction that fails to attain the native structure is cleared by autophagy. However, how autophagy selectively recognizes misfolded procollagens in the ER lumen is still unknown. We performed siRNA interference, CRISPR-Cas9 or knockout-mediated gene deletion of candidate autophagy and ER proteins in collagen producing cells. We found that the ER-resident lectin chaperone Calnexin (CANX) and the ER-phagy receptor FAM134B are required for autophagy-mediated quality control of endogenous procollagens. Mechanistically, CANX acts as co-receptor that recognizes ER luminal misfolded procollagens and interacts with the ER-phagy receptor FAM134B. In turn, FAM134B binds the autophagosome membrane-associated protein LC3 and delivers a portion of ER containing both CANX and procollagen to the lysosome for degradation. Thus, a crosstalk between the ER quality control machinery and the autophagy pathway selectively disposes of proteasome-resistant misfolded clients from the ER.

Keywords autophagy; Calnexin; collagen; endoplasmic reticulum; FAM134B

Subject Categories Autophagy & Cell Death; Membrane & Intracellular Transport

Introduction

Macroautophagy (hereafter referred to as autophagy) is a homeostatic catabolic process devoted to the sequestration of cytoplasmic material in double-membrane vesicles (autophagic vesicles, AVs) that eventually fuse with lysosomes where cargo is degraded (Mizushima, 2011). Autophagy is essential to maintain tissue homeostasis and counteracts both the onset and progression of many disease conditions, such as ageing, neurodegeneration and cancer (Levine et al., 2015).

Substrates can be selectively delivered to AVs through receptor-mediated processes. Autophagy receptors harbour a LC3 or GABARAP interaction motif (LIR or GIM, respectively) that facilitate binding of the cargo to LC3 or GABARAP proteins, which decorate autophagosomal membranes (Stolz et al., 2014; Rogov et al., 2017). Proteins and entire organelles or their portions can be targeted to autophagy via receptor-mediated processes. A notable example is represented by ER-phagy, a selective form of autophagy in which portions of the ER are sequestered within AVs and transported to the lysosomes for degradation (Fregno & Molinari, 2018; Grumati et al., 2018). To date, the yeast Atg39, Atg40 and the mammalian FAM134B, SEC62, RTN3 and CCGP1 proteins have been identified as ER-phagy receptors (i.e. as LC3-binding proteins that decorate specific ER subdomains for capture by AVs) (Khaminets et al., 2015; Mochida et al., 2015; Fumagalli et al., 2016; Grumati et al., 2017; Smith et al., 2018). ER-phagy mediates the...
turnover of ER membranes and promotes recovery after ER stress, bacterial and viral infections (Khaminets et al., 2015; Chiramel et al., 2016; Fumagalli et al., 2016; Grumati et al., 2017; Lennemann & Coyne, 2017; Moretti et al., 2017; Smith et al., 2018).

ER homeostasis relies on ER quality control mechanisms to prevent the accumulation of unevenly folded cargoes within its lumen. Misfolded proteins are dislocated from the ER to the cytosol to be degraded by the 26S proteasome, a process known as ER-associated degradation (ERAD) (Preston & Brodsky, 2017). However, not all misfolded ER proteins are eligible for ERAD and thus must be cleared from the ER through other processes. Autophagy-dependent and autophagy-independent lysosomal degradation of proteins from the ER has also been reported (Ishida et al., 2009; Hidvegi et al., 2010; Houck et al., 2014; Fregno et al., 2018). However, the mechanism by which misfolded ER luminal proteins are recognized by the cytosolic autophagic machinery and delivered to the lysosomes remains to be understood.

Collagens are the most abundant proteins in animals, and type I and type II collagen (COL1 and COL2) are the major protein components of bone and cartilage, respectively (Bateman et al., 2009). They are synthesized as alpha I and alpha II chains and folded into triple helices of procollagen (PC) in the ER. Properly folded PCs associate with the heat shock protein 47 (HSP47) chaperone and then leave the ER through sub-regions called ER exit sites (ERES), within COPII-coated carriers, and move along the secretory pathway (Malhotra & Erllmann, 2015). Previous studies estimated that approximately 20% of newly synthesized type I PC (PC1) is degraded by lysosomes as a consequence of inefficient PC1 folding or secretion (Bienkowski et al., 1986; Ishida et al., 2009). In case of mutations in PC or HSP47, the fraction of PC degraded increases significantly (Ishida et al., 2009). Similarly, a fraction of type II PC (PC2) produced by chondrocytes of the growth plates is degraded by autophagy, and inactivation of this catabolic pathway results in PC2 accumulation in the ER and defective formation of the extracellular matrix (Cinque et al., 2015; Bartolomeo et al., 2017; Settembre et al., 2018). Overall, these data clearly indicate that aberrant PC molecules represent ERAD-resistant substrates where autophagic clearance emerges as a crucial and physiologically relevant event in the maintenance of cellular and organ homeostasis. However, to date, the mechanism by which ER-localized PCs are selectively disposed of by autophagy is still unknown.

In this study, we sought to uncover the mechanisms that select non-native PC in the ER lumen for lysosomal delivery and clearance. We found that the misfolded PC molecules (e.g. HSP47 negative) are cleared from the ER through FAM134B-mediated ER-phagy. Notably, FAM134B binds PC molecules in the ER through the interaction with the transmembrane ER chaperone Calnexin (CANX) that acts as a specific FAM134B ER-phagy co-receptor for misfolded PCs. The formation of this complex allows the selective delivery of PC molecules to the lysosomes.

Results

**Autophagy promotes degradation of intracellular procollagens preventing their accumulation in the ER**

Using three different collagen producing cell lines, mouse embryonic fibroblasts (MEFs) and human osteoblasts (Saos2) stably expressing the autophagosome membrane marker LC3 fused with GFP (GFP-LC3) (Kabeya et al., 2000), and rat chondrosarcoma cells (RCS) immunolabelled for LC3, we observed co-localization of LC3-positive vesicles (hereafter referred as autophagic vesicles, AVs) with PC1 (MEFs and Saos2) and PC2 (RCS) (Fig 1A–D). Similarly, we observed the co-localization of PC1 spots with the GFP-tagged double-FYVE domain-containing protein 1 (DFCP1), which labels sites for autophagosome biogenesis (omegasomes) (Fig EV1A and B). In vivo, osteoblasts of the mandible in Medaka fish embryos (stage 40), showed the presence of AVs containing PC2 molecules (Fig EV1C–E).

When MEFs, Saos2 and RCS cells were treated with the lysosomal inhibitor bafilomycin A1 (BafA1), PC molecules accumulated in the lumen of swollen endo/lysosomes (LAMP1-positive organelles, hereafter referred as lysosomes) (Fig 1E–G). These data were validated by PC1 immuno-electron microscopy (IEM) (Fig 1H). Western blot analysis confirmed the accumulation of intracellular PCs, as well as of the autophagy markers LC3-II and SQSTM1/p62, in cells treated with BafA1 compared to untreated cells (Fig EV2A). BafA1 washout induced a rapid clearance of PC1 and PC2 from lysosomes of MEFs and RCS, respectively, in line with the notion that PCs are degraded in this compartment (Fig EV2B and C).

Lysosomal storage disorders (LSDs) are genetic diseases characterized by a defective lysosomal degradative capacity due to mutations in genes encoding for lysosomal proteins. As a result, lysosomal substrates progressively accumulate within the lumen of lysosomes causing lysosomal swelling and cell dysfunction. We sought to determine whether PC molecules accumulate in the lysosomes of LSD osteoblasts. Saos2 osteoblasts in which the alpha-L-iduronidase gene was deleted using CRISPR-Cas9 technology (CRISPR-IDUA) represent a disease model of mucopolysaccharidosis type I (MPS I), a lysosomal storage disorder with severe skeletal manifestations (Oestreich et al., 2015). Similar to cells treated with BafA1, CRISPR-IDUA showed swollen lysosomes, suggesting an accumulation of undigested substrates in the lysosomal lumen (Fig 1I). Most importantly, the level of PC1 in lysosomes, and in the whole cell lystate, was higher in CRISPR-IDUA Saos2 compared to control cells (Fig 1I and J).

To verify at which trafficking stage PC became an autophagy substrate, we performed a temperature shift assay where PC accumulates in the ER during incubation at 40°C, and is released from the ER upon shift of the temperature to 32°C. U2OS cells expressing GFP-LC3, mCherry-PC2 and ER marker RDEL-HALO, were imaged upon shift to 32°C (time 0 s). We observed that PC2 spots formed at the ER and progressively accumulated GFP-LC3 (Fig 2A and Movie EV1). Similarly in U2OS cells expressing phosphatidylinositol 3-phosphate (PtdIns(3)P) -recognition domain construct GFP-2FYVE, mCherry-PC2 and ER marker RDEL-HALO, the PC2 was visible at an area of GFP-2FYVE-positive ER, and dissociated from the main tubular ER structure releasing a vesicle positive for ER, GFP-2FYVE and PC2 (Fig 2B and Movie EV2). Co-localization between GFP-LC3, the ER chaperone CANX and PC1 was also observed by Airyscan super-resolution confocal microscopy (Fig 2C). Similarly, we observed co-localization of PC1 spots with GFP-DFCP1 and CANX in MEFs and Saos2 cells (Fig EV3A). We also performed correlative light electron microscopy (CLEM) and electron tomography of GFP-LC3 expressing Saos2 cells, showing that PC1 and CANX are found together in a small vesicle contained within a larger LC3-positive vesicle (Fig 2D and E). Taken together, these data suggest that PC
molecules are sequestered within LC3-positive vesicles when they are still within the ER.

The collagen-specific chaperone HSP47 was excluded from the AVs containing PC1 in MEFs, strongly suggesting that autophagy sequesters non-native PC1 molecules in the ER (Fig EV3B), in line with previous results (Ishida et al., 2009; Cinque et al., 2015). To further corroborate this notion, we studied two missense mutations in the COL2A1 protein (R789C and G1152D) that induce misfolding.
Figure 2. Autophagy sequesters PC molecules in the ER.

A, B U2OS expressing (A) GFP-LC3 or (B) GFP-2-FYVE (green), mCherry-PC2 (red) and RDEL-HALO (blue) were imaged live by spinning disc microscopy. Single and merge channels time-lapse stills at higher magnification (A = x3.93; B = x3.42) from the boxed region are shown on the right. Scale bar = 10 μm.

C Airyscan analysis of Saos2 cells expressing GFP-LC3 (green) and immunolabelled for PC1 (405, blue) and CANX (647, red). The insets show higher magnification (x5.26) and single colour channels of the boxed area. Scale bar = 10 μm.

D Correlative light electron microscopy (CLEM) and electron tomography of Saos2 cells transfected with GFP-LC3 (green) and labelled for PC1 (568, red and nanogold particles) and CANX (647, blue). Cells were first imaged by confocal microscopy (top left panel), and then, the same region was retraced in EM (upper middle panel) and overlay is shown (upper right panel). Arrow indicates a LC3-positive vesicle containing CANX and PC1 molecules.

E Single tomography slice (left panel, taken from boxed are in D at a magnification of x2.84), overlay with immunofluorescence (IF) (central panel) and IF 3D rendering of AV (green) and the CANX positive vesicle containing gold particles of labelled collagen (blue and white, respectively) inside an AV (right panel).
of the PC2 triple helix and accumulation within the ER of chondrocytes. The mutations cause a type II collagenopathy in humans, named spondyloepiphyseal dysplasia congenita (SEDC) (Murray et al., 1989). When expressed in chondrocytes, the R789C and G1152D mutants were targeted to the lysosomes at higher rates compared with WT COL2. Notably, pharmacological enhancement of autophagy with the autophagy inducing peptide Tat-BECLIN-1 (Shoji-Kawata et al., 2013) increased targeting of WT and of mutant PC2 molecules to lysosomes. Opposite results were observed by treating cells with the autophagy inhibitor SAR405 (Fig EV3C). Taken together, these data suggest that autophagy preferentially degrades non-native PC molecules and prevents their accumulation in the ER.

**FAM134B is required for autophagy of procollagen**

Distinct autophagy-related (ATG) proteins and receptors play an essential role in autophagosome formation and cargo recognition, respectively (Suzuki et al., 2017). To characterize the machinery that enables the delivery of PC molecules to lysosomes, we silenced genes belonging to different functional autophagy clusters in Saos2 cells treated with BafA1 and quantified the levels of PC1 within lysosomes. As expected, we found that the silencing of all genes tested involved in AV biogenesis significantly inhibited the delivery of PC1 to the lysosomes. Notably, among autophagy and ER-phagy receptors, we found that FAM134B silencing most effectively inhibited PC1 delivery to lysosomes (Fig 3A). Our siRNA data were further validated using MEFs knocked out for genes involved in AV biogenesis, namely Fip200 (Fip200−/−), Atg7 (Atg7−/−) or Atg16l1−/− as well as in MEFs lacking Fam134b expression (CRISPR Fam134b) (Figs 3B and EV4A). The effect of Fam134b knockout was specific, since MEFs lacking Sec62 expression (CRISPR Sec62), a different ER-phagy receptor (Fumagalli et al., 2016), showed a normal rate of PC1 delivery to the lysosomes (Fig 3B, bottom panels). Western blot and immunofluorescence analyses confirmed the accumulation of intracellular PC1 in CRISPR Fam134b MEFs compared to control cells (Fig 4A and B). Notably, there was not a generalized accumulation of other ER proteins (VAPA, Sec23a and the soluble ER chaperone protein disulphide isomerase [PDI]) (Fig EV4B). The impaired delivery of PC1 to lysosomes in CRISPR Fam134b MEFs was rescued by reintroducing WT human FAM134B, but not a FAM134B protein lacking the (LIR) motif (FAM134Blir), in which interaction with LC3 is abolished (Khaminets et al., 2015) (Fig 4C). Taken together, these data strongly suggest a primary role of FAM134B in mediating the delivery of ER-resident PC molecules to lysosomes.

**Calnexin is required for autophagy of procollagen**

FAM134B is not predicted to have an ER luminal domain, so a direct interaction with PC molecules in the ER is unlikely. We also hypothesized that the PC molecules destined for degradation need to be selectively recognized by ER quality control machinery in order to be subjected to FAM134B-mediated ER-phagy. Thus, we investigated the involvement of ER chaperones in autophagy of PC. Taking advantage of a published list of putative PC1 and FAM134B ER interactors (DiChiara et al., 2016; Grumati et al., 2017), we silenced different ER genes by RNAi. The silencing of the transmembrane chaperone CANX most effectively inhibited the delivery of PC1 to lysosomes in Saos2 cells treated with BafA1 (Fig 5A).

Similar to what we observed in CRISPR Fam134b MEFs, Canx−/− MEFs showed an accumulation of intracellular PC1 but not of other ER-resident proteins (VAPA, Sec23a and PDI; Figs 5B and EV4C). When WT MEFs were treated with BafA1, the intracellular PC1 levels increased as consequence of defective lysosomal degradation (Fig 5B). Conversely, in Canx−/− MEFs the accumulation of PC1 was evident even in the absence of BafA1 treatment (Fig 5B). MEFs lacking Canx or Crt (Calreticulin) expression had an impaired PC1 delivery to lysosomes (Fig 5C). Similarly, MEFs lacking Erp57, a protein disulphide isomerase that cooperates with CANX and CRT to ensure a proper folding of proteins (Oliver et al., 1999), also showed a defective PC1 delivery to lysosomes (Fig 5C). The binding of CANX and CRT to target substrates occurs through the recognition of monoglucosylated oligosaccharide residues generated either by ER glucosidases I and II or by UDP-glucose:glycoprotein glycosyltransferase (UGT1) proteins (Hebert et al., 1995; Keller et al., 1998; Soldà et al., 2007). Pharmacological inhibition of glucosidase activities with castanospermine (CST) or deletion of Ugt1 in MEFs also inhibited PC1 delivery to lysosomes (Fig 5C). Taken together, these data indicate that all the components of the CANX/CRT cycle are required to operate the PC folding quality control and to select the misfolded PC destined to autophagy.

**Procollagens are the main substrates that accumulate in Fam134b−/− and Canx−/− cells**

We performed quantitative proteome analysis using mass spectrometry (MS) label-free protein quantification approach in Canx−/− and Fam134b−/− MEFs versus wild-type MEFs. Canx−/− and Fam134b−/− samples were prepared and run in parallel in order to minimize the variability due to the MS calibration and sample preparation. We identified 95 upregulated and 142 downregulated proteins in Fam134b−/− MEFs. Specifically, both Col1a1 and Col1a2 peptide chains were among the most significantly increased (−Log Student’s t-test P-value: 8.55 and 8.2, respectively, for Col1a1 and Col1a2; Fig 6A and Dataset EV1). Gene ontology analysis confirmed the accumulation of collagens in MEFs lacking Fam134b (Fig EV4D). In Canx−/− cells, we identified 384 upregulated and 278 downregulated proteins. Col1a1 and Col1a2 peptides were identified as significantly increased also in Canx−/− MEFs (−log Student’s t-test P-value: 3.06 and 2.37, respectively, for Col1a1 and Col1a2; Fig 6B, Dataset EV1). Interestingly, only 17 identified peptides were commonly upregulated in both Fam134b−/− and Canx−/− MEFs. Among these, collagens (Col1a1, Col1a2, Col6a1, Col6a2, Col5a1) and collagen interacting proteins (procollagen C-endopeptidase enhancer 1, SPARC/osteonectin) were the most represented categories (Fig 6C). These data clearly show that FAM134B and CANX are important regulators of PC proteostasis and that they might cooperate for the selective removal of misfolded procollagens in the ER.

A CANX-FAM134B ER-phagy complex acts as PC autophagy receptor

Mass spectrometry analysis identified CANX as a putative FAM134B interactor (Grumati et al., 2017). We confirmed this interaction by co-immunoprecipitation experiments (Fig 7A and B). CANX has a N-terminal ER luminal domain, a single transmembrane helix and a short acidic cytoplasmic tail. FAM134B instead is composed of a
N-terminal cytosolic domain, a reticulon homology domain (containing alpha helices and a cytosolic loop) and a C-terminal cytosolic domain (Fig 7A). Thus, CANX and FAM134B could potentially interact either in the cytosol or in the ER membrane. We found that the interaction between CANX and FAM134B is lost when co-immunoprecipitation experiments were performed using a mutant version of FAM134B that lacked the intramembrane part of the reticulon homology domain, suggesting that FAM134B interacts with CANX in the ER membrane (Fig 7A and B). Notably, the FAM134Blir mutant still interacts with CANX in co-immunoprecipitation experiments (Fig 7A and B).

FAM134B-CANX interaction was not modulated by PCs, since it occurs also in HeLa (Kyoto) cells that do not express significant amounts of collagens (Hein et al., 2015; Fig EV5A). Functionally, CANX is not required for FAM134B-mediated ER-phagy, as FAM134B is recruited to LC3-positive vesicles with the same efficiency in both Canx<sup>−/−</sup> and WT MEFs (Fig EV5B and C). We postulated that FAM134B interacts with misfolded PC molecules via CANX. To test this hypothesis, we generated a human osteosarcoma cell line (U2OS) expressing PC2 molecules tagged with HALO at the N terminus. HALO-PC2 was normally secreted and, similarly to endogenous PC2, accumulated in lysosomes upon BafA1 treatment (Fig EV5D and E) indicating that the presence of the HaloTag did not alter the intracellular processing of PC2. HA-resin-mediated pull-down experiments using HA-tagged FAM134B or FAM134Blir showed that both HALO-PC2 and CANX interact with FAM134B, irrespective of whether it contained the LIR domain or not (Fig 7C). Conversely co-precipitation of LC3II was dependent on a functional

Figure 3. FAM134B is required for autophagy recognition of PC1.
A Bar graph shows quantification of lysosomes (LAMP1<sup>+</sup>) containing PC1 expressed as % of total number of lysosomes (mean ± SEM) in Saos2 cells mock transfected or transfected with siRNA against the indicated genes and treated with 100 nM BafA1 for 9 h. n = 20 cells per condition; three independent experiments. One-way ANOVA with Dunnett’s multiple comparisons test performed, ***p < 0.0001.
B MEF cell lines lacking the expression of indicated genes were treated for 12 h with 50 nM BafA1, fixed and immunolabelled for PC1 (568, red) and LAMP1 (488, green). Scale bar = 10 μm. Insets show magnification of the boxed area. Bar graph (on the left) shows quantification of LAMP1 vesicles positive for PC1, expressed as % of total lysosomes (mean ± SEM), n = 12, 10, 12, 10, 7, 10 cells per genotype, respectively; three independent experiments. One-way ANOVA with Dunnett’s multiple comparisons test performed and P-value adjusted for multiple comparisons. ns ≥ 0.05, ***p < 0.0001.
LIR motif in FAM134B (Fig 7C), consistent with previous results (Khaminets et al., 2015). CST treatment diminished the level of HALO-PC2 co-precipitated by FAM134B-HA (Fig 7C) without perturbing the co-precipitation of CANX and LC3II. Taken together, these data suggest a model by which the interaction of PC with FAM134B is mediated by CANX and that the selective degradation of PC mediated by FAM134B is dependent on PC binding to CANX.
Figure 5. **CANX is required for autophagic targeting of PC1.**

A Bar graph shows quantification of lysosomes (LAMP1+) containing PC1 expressed as % of lysosomes (mean ± SEM) in Saos2 cells mock transfected or transfected with siRNA against the indicated genes, treated with 100 nM BafA1 for 9 h. n = 18 cells/treatment; three independent experiments. One-way ANOVA with Dunnett’s multiple comparisons test performed, **P < 0.005, ***P < 0.0001.

B WT and Canx–/– MEFs were untreated or treated with BafA1 (10 μM) for 6 h, lysed and analysed by Western blot with indicated antibodies. Filamin and β-actin were used as loading control. Dashed line indicates that unnecessary lanes were removed. Western blot is representative of three independent experiments.

C MEF cell lines lacking the indicated genes were treated for 12 h with 50 nM BafA1 fixed and immunolabelled for PC1 (568, red) and LAMP1 (488, green). CST was added where indicated. Scale bar = 10 μm. Inset panels show magnification of the boxed area. Bar graph on the right shows quantification of LAMP1 vesicles positive for PC1, expressed as % of total lysosomes (mean ± SEM), n = 8, 8, 12, 8, 8 cells, respectively; three independent experiments. One-way ANOVA with Dunnett’s multiple comparisons test performed and P-value adjusted for multiple comparisons. ***P < 0.0001.

Source data are available online for this figure.
Discussion

In this work, we have investigated the mechanism by which autophagy selectively recognizes PC molecules destined for degradation in the ER. We have shown that the ER transmembrane chaperone CANX, by interacting with FAM134B and LC3, forms a novel ER-phagy complex with specific protein targeting capabilities. This complex is responsible for a specific ER clearance mechanism of PCs and links a non-native large protein within the ER lumen to the cytosolic autophagy machinery.

Firstly, we have found that silencing genes belonging to functionally different complexes involved in autophagosome biogenesis inhibited lysosomal delivery of PCs, indicating that autophagy is mediating the delivery of PC to lysosomes.

Secondly, we have identified the ER-resident autophagy receptor FAM134B as a key mediator of PC delivery to the lysosomes. FAM134B was recently identified as an ER-phagy receptor that mediates turnover of portions of the ER via autophagy (Khaminets et al., 2015). Our data suggest that FAM134B-dependent ER-phagy also functions as an ER quality control pathway for PCs. Our quantitative proteomic analysis in Fam134b−/− MEFS suggests that PCs are the main clients of FAM134B-mediated ER-phagy. The identification of multiple ER-phagy receptors also suggests that different cargoes might be subjected to different types of ER-phagy. Notably, a recent study showed that the disruption of CCPG1-mediated ER-phagy leads to the accumulation of ER insoluble proteins in acinar cells (Smith et al., 2018).

Thirdly, we have demonstrated that the chaperone CANX is a key player in PC disposal via ER-phagy. CANX is a molecular
Figure 7. CANX and FAM134B interact and deliver PC to autophagosomes.

A Schematic representation of FAM134B WT, lir mutant and delta reticulon constructs.
B U2OS cells were transfected with empty vector control (pcDNA3), FAM134B-HA WT or mutant constructs as indicated.
C U2OS cells were transfected with HALO-PC2, FAM134B-HA or FAM134Blir-HA constructs, treated with 100 nM BafA1 for 6 h and with CST where indicated, (B, C) Complexes were immune-isolated with HA-magnetic beads, separated by Western blot and visualized with antibodies against HALO, CANX, FAM134B, LC3 and β-actin. 5% of the input is shown. Western blots are representative of three independent experiments. Dashed line indicates that unnecessary lanes were removed.
D Proposed model of collagen recognition by autophagy. After synthesis, PC chains are subjected to quality control operated by the CANX/CRT chaperone system. Properly folded PC associates with HSP47 and is then secreted, whereas the misfolded fraction is sequestered by the CANX-FAM134B complex and delivered to lysosomes through ER-phagy.

Source data are available online for this figure.
chaperone that assists the folding of monoglucosylated glycoprotein in the ER. CANX forms transient but relatively stable complexes with unfolded ER proteins until they either become folded or are degraded (Williams, 2006). The genetic or pharmacological inhibition of ER enzymes that mediate the binding of substrates to CANX impairs the delivery of PC to lysosomes, suggesting that the N-glycans-mediated recognition of PC by CANX (and CRT) represents a prerequisite for PC targeting to autophagosomes. Consistently co-immunoprecipitation experiments demonstrate that PC2 binding to FAM134B complex depends on CANX substrate affinity, since it can be reduced by CST treatment.

It is currently unknown whether additional ER partners also aid CANX. For example, ERp29, a CANX binding protein, has recently been shown to mediate the retention of immature PC1 in the ER (DiChiara et al., 2016).

Finally, we have also provided biochemical evidence indicating that FAM134B interacts with CANX. This interaction seems to occur within the ER membrane since it is mediated by the transmembrane regions of the reticulon homology domain of FAM134B. The reticulon homology domain generates membrane curvature by increasing the area of the cytoplasmic leaflet (Zurek et al., 2011). The observation that CANX:FAM134B binding is rather stable and not modulated by PCs suggests that the binding of PC to CANX might induce a conformational change of the FAM134B reticulon homology domain that increases ER membrane curvature, favouring vesicle formation. Indeed, CLEM analysis confirmed the presence of both PC molecules and CANX within a small vesicle contained within a large autophagosome, supporting the model by which portions of the ER containing both CANX and PC1 are sequestered into AVs (Fig 7D).

Mass spectrometry analyses clearly show that the CANX:FAM134B interplay is devoted to the degradation of different types of collagens, suggesting that cells may have evolved a specific mechanism to cope with the difficulties associated with the production and secretion of procollagens in the ER. This is not surprising if we consider that collagens are the most abundant proteins of our body (about 25% of our dry weight), and that its production represents a major task for cells.

We have recently reported that CANX delivers proteasome-resistant polymers of alpha 1-antitrypsin Z (ATZ) to ER subdomains en route for FAM134B-mediated vesicular transport to the lysosomes for degradation (Fregno et al., 2018). ATZ clearance, however, shows substantial differences compared to the quality control autophagy of endogenous PC that we studied in collagen-producing cells. In particular, delivery of PC molecules to lysosomes fully relies on components of the autophagosome biogenesis machinery. Conversely, many of them (e.g. ULK1/2, ATG9 and ATG13) are dispensable for ATZ clearance, suggesting that the CANX:FAM134B complex can mediate ER cargo clearance though different vesicular pathways.

Both in quality control autophagy of PC1 and ATZ clearance, the lectin chaperone CANX delivers the misfolded cargo in ER subdomains to be cleared from cells on stable interactions with FAM134B. However, other components of the CANX chaperone system (i.e. CRT, UCT1 and ERp57) cycle are required in quality control autophagy of PC1, but are dispensable for ATZ clearance.

Cumulating evidence delineates a scenario where multiple catabolic pathways ensure efficient removal of misfolded proteins from the ER lumen, which is crucial to maintain the function of this biosynthetic organelle. ER-associated-degradation (ERAD) collectively defines the many client-specific pathways engaged by misfolded proteins generated in the ER for delivery at, and dislocation across the ER membrane preceding clearance by cytosolic proteasomes (Preston & Brodsky, 2017). An increasing number of faulty gene products are shown to be excluded from the ERAD pathways (Noda & Farquhar, 1992; Fujita et al., 2007; Ishida et al., 2009; Hidvegi et al., 2010; Houck et al., 2014; Fregno et al., 2018). The vast heterogeneity of gene products synthesized in the ER lead us to predict that, like the multiple pathways operating for ERAD, client-specific pathways also ensure delivery of proteasome-resistant misfolded proteins to specialized ER subdomains that are eventually transported to lysosomal compartments for ER-to-lysosome-associated degradation (ER-LAAD).

Our results highlight the complexity of quality control pathways operating in mammalian cells to surveil the ER lumen and prevent accumulation of toxic by-products of protein biogenesis.

Lack of ER homeostasis and protein accumulation has been shown to be an underlying cause for various diseases, opening up this pathway for development as a potential therapeutic target.

## Materials and Methods

### Cell culture, transfections, siRNA and plasmids

#### Cell culture

MEFs and RCS cell lines were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin at 37°C in 5% CO₂. Saos2 and U2OS cells were purchased from ATCC and cultured in McCoy’s medium with 15% (Saos2) or 10% (U2OS) FBS and 1% penicillin/streptomycin at 37°C in 5% CO₂. For collagen experiments, medium was supplemented with 50 μg/ml ascorbic acid. Wild-type, Atg5⁻/⁻ and Atg7⁻⁻ MEFs were gifts from M. Komatsu and N. Mizushima. Atg16⁻⁻ MEFs were from T. Saitoh. Fip200⁻⁻ MEFs were from J.L. Guan. The generation of the Sec62 CRISPR-Cas9 knockout MEF cell line was previously described (Fumagalli et al., 2016). Canx⁻⁻ MEF cell lines were previously described (Kraus et al., 2010). Fam134b CRISPR-Cas9 MEF cell line was described in Fregno et al (2018). Fam134b⁻⁻ MEF cell line was described in Khaminets et al (2015). Crt⁻⁻, Erp57⁻⁻ and Ugt1⁻⁻ were previously described (Moliniari et al., 2004; Soldà et al., 2006, 2007).

Saos2 IDUA CRISPR-Cas9 MEF cell line was generated as follows: For construction of the guideRNA-Cas9 plasmid, pSpCas9(BB)-2A-Puro plasmid (PX459) was obtained from Addgene (Plasmid #62988). To clone the guide sequence into the sgRNA scaffold, two annealed oligonucleotides (5’-CACCAGGCACTCAACCTCGCCTATG-3’, 5’-AAACCATAGCCAGGTAGGCTCAGTGC-3’) were inserted into the pSpCas9 (BB)-2A-Puro plasmid using BbsI restriction site. Saos2 cells were transfected with the plasmid using Lipofectamine LT and Plus Reagent (Invitrogen, Thermo Fisher Scientific) following a reverse transfection protocol. Two days after transfection, the medium was supplemented with 1 μg/ml puromycin. Puromycin-resistant clones were isolated, and gene KO was verified by sequencing. CRISPR-IDUA and WT Saos2 cells were kept in medium containing 1 mg/ml dexamethasone (Sigma-Aldrich) for 48 h before any experiment was performed.
Transfection
Cells were reverse-transfected using Lipofectamine LTX and PLUS reagent (Invitrogen) according to manufacturer’s instructions. In Fig 4C, cells were transfected with JetPrime transfection reagent (PolyPlus) following the manufacturer’s protocol. For siRNA experiments, siGENOME SMARTpool siRNAs (Dharmacon Thermo Scientific) were transfected to a final concentration of 100 nM and cells harvested 72 h after transfection.

Plasmids
GFP–LC3 was from Dr. Yoshimori. GFP-2-FYVE was a gift from Dr. S. Tooze. FAM134Blir-HA and FAM134B-GFP was previously described (Khaminets et al, 2015). Site-directed mutagenesis plasmids: R789C and G1152D were created using the Agilent QuikChange XL Site-Directed mutagenesis kit using the mCherry-C2-Col2A1 plasmid (Venditti et al, 2015). Site-directed mutagenesis plasmids were described in Khaminets et al (2015). Site-directed mutagenesis plasmids: R789C and G1152D mutations were created using the Agilent QuikChange XL Site-Directed mutagenesis kit using the mCherry-C2 backbone.

Immunofluorescence
Cells were seeded on coverslips at least 24 h before treatment and fixed for 10 min in 4% PFA, or for the detection of endogenous LC3, fixed for 10 min in ice-cold methanol. Cells were blocked and permeabilized for 30 min in blocking buffer (0.05% (w/v) saponin, 0.5% (w/v) BSA, 50 mM NH₄Cl and 0.02% NaN₃ in PBS, pH 7.4). For LAMP1 immunolabelling, 15 mM glycine was added to blocking buffer. Cells were incubated for 1 h with the primary antibodies: collagen I (SP1.D8, Hybridoma Bank), collagen II (II-II6B3, Hybridoma Bank), LAMP1 (Abcam, ab24170), or LAMP1 (Hybridoma Bank, ID48 was deposited to the DSHB by August, J.T.), CANX (Enzo Life Sciences ADI-SPA-860-D), LC3 (NB100-2220; Novus Biologicals), HSP47 (Abcam, ab77609); HA (Sigma, H6908), washed 3 times in PBS; incubated for 45 min with secondary antibody (Alexa Fluor-labelled goat anti-rat A11077, goat anti-guinea pig A11073, goat anti-rabbit A11011/A11008, and goat anti-mouse A11001, A11004; Life Technologies, Thermo Fisher Scientific); washed three times in PBS; incubated for 20 min with 1 µg/ml Hoechst 33342, and finally mounted in Mowiol (Sigma-Aldrich) or Vectashield (Vector Laboratories) supplemented with 4',6-diamidino-2-phenylindole (DAPI).

Medaka stocks
Samples of the Cab strain of wild-type medaka fish were kept and staged as described previously (Iwamatsu, 2004; Carrella et al, 2015). All studies on fish were conducted in strict accordance with the institutional guidelines for animal research and approved by the Institutional Animal Care and Use Committee of the Department of Fish Science, National Institute of Agrobiological Resources, National Institute of Agrobiological Science, Japan.

Confocal microscopy
Scanning laser confocal experiments were acquired using a Zeiss LSM 880 or Leica TCS SP5 confocal microscope equipped with a 63x/1.4 numerical aperture oil objective. Airyscan microscopy was performed using a Zeiss LSM 880 confocal microscope, equipped with Plan-Apochromat 63x/1.4 numerical aperture oil objective and pixel size of 8.7 nm. Images were subjected to post-acquisition Airyscan processing. Image acquisition and processing were
performed with Zen Blue software and co-localization analysis and image presentation was performed using ImageJ FIJI software or Photoshop (Adobe).

Live cell imaging

U2OS cells were transiently transfected with mCherry-PC2 and RDEL-HALO plus GFP-LC3 or GFP-2-FYVE. Cells were incubated on a Tokai Hit stage top incubator heated stage in 5% CO₂ at 40°C in the presence of far red HALO ligand for 3 h. Immediately prior to imaging, medium was supplemented with ascorbic acid and rutin (routinely used to decrease photobleaching). Imaging was initiated at temperature switch to 32°C. Frames were acquired at 1-s intervals. Imaging was performed on a Nikon Inverted Spinning Disk confocal with sCMOS Prime95B camera (Photometrics) with pixel size of 11 μm, using a 100× CFPlan Apo objective with 1.4 NA. Image acquisition was performed with Metamorph 7.7.6 software (Molecular Devices, France) and processing in ImageJ FIJI software.

Correlative electron light microscopy (CLEM) and Tomography

Saos2 cells were grown on gridded MatTek glass-bottomed dishes (MatTek Corporation) transfected with GFP-LC3 and fixed with 0.05% glutaraldehyde in 4% paraformaldehyde (PFA) and 0.1 M HEPES buffer for 10 min, washed once in 4% PFA, then incubated in fresh 4% paraformaldehyde in 0.1 M HEPES buffer for 30 min. Subsequently, cells were incubated for 30 min in blocking buffer and immunolabelled for collagen I (SP1.D8 Hybridoma Bank) and CANX (ADI-SPA-860-D Enzo Life Sciences), visualized with AlexaFluor546 fluoro-nanogold Fab’ conjugate (Nanoprobes) and AlexaFluor647 Rabbit Ab, respectively. Nanogold was enlarged using gold enhancement kit (Nanoprobes) according to manufacturer’s instructions. Samples were then post-fixed with 1.5% potassium ferricyanide, 1% OsO₄ in 0.1 M cacodylate buffer for 1 h on ice and en bloc stained overnight with 1% uranyl acetate. Samples were dehydrated in ethanol and embedded in epoxy resin (Sigma). After baking for 48 h at 60°C, the resin was released from the glass coverslip by temperature shock in liquid nitrogen. Serial sections (70–90 nm) were collected on carbon-coated formvar slot grids and imaged with a Zeiss LEO 512 electron microscope. Images were acquired with a 2k × 2k bottom-mounted slow-scan Precoscan camera controlled by EsiVisionPro 3.2 software.

For electron tomography, tilted series were acquired with a 200 kV Tecnai G2 20 electron microscope (FEI, Eindhoven) at a magnification of 11.5 k, resulting in pixel size of 1.95 nm. Single, tilted image series (± 60°) according to a Saxton scheme with the initial tilt step of 2° were acquired using Xpolor3D (FEI) with an Eagle 2,048 × 2,048 CCD camera (FEI). Tilted series alignment and tomographic reconstructions were done with the IMOD software package. Image segmentation was done by MIB software (BW thresholding) and visualized using IMOD.

Transmission electron microscopy

Cells were fixed in 1% glutaraldehyde in 0.2 M HEPES buffer and then post-fixed in uranyl acetate and in OsO₄. After dehydration through a graded series of ethanol, samples were cleared in propylene oxide, embedded in epoxy resin (Epon 812) and polymerized at 60°C for 72 h. From each sample, thin sections were cut with a Leica EM UC6 ultramicrotome and images were acquired using a FEI Tecnai –12 (FEI) electron microscope equipped with Veleta CCD camera for digital image acquisition.

Immunoprecipitation experiments

HA-tag precipitation: U2OS cells were transiently transfected with plasmids encoding HALO-PC2 and FAM134B-HA. On the day of experiment, plates were treated with 1 mM CST where indicated for 2 h, then all plates treated with 100 nM BafA1 and 50 μg/ml ascorbic acid for 4 h. Cells were detached with trypsin–EDTA and centrifuged. The cell pellets were washed three times with ice-cold PBS and then resuspended in 1 ml MCLB lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris/HCl pH 8). The cell suspension was lysed by passing it through a 24-G needle for 10-15 times. The lysates were incubated on ice for 20 min with gentle swirling and centrifuged at 18,000 x g to pellet nuclei and cell debris. The supernatants were collected and subjected to protein quantification using BCA protein assay kit (Thermo Fisher Scientific) and rotated at 4°C overnight. The precipitated proteins were washed three times with MCLB lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris/HCl pH 8) and two times with the same lysis buffer, detergent free. The protein complexes were resuspended in 1 v/v 2× Laemmli sample buffer and analysed by SDS–PAGE in a 7–14% gradient gel.

HeLa (Kyoto) cells and U2OS cells were transiently transfected with empty vector control, FAM134B-HA WT or mutant constructs. On the day of the experiment, cells were detached with trypsin–EDTA and centrifuged. Immunoprecipitation experiments were performed in the same conditions and analysed by SDS–PAGE in a 4–15% Mini-PROTEAN™ TGX™ Precast Protein gel.

Western blot analysis

Cells were washed twice with PBS and then scraped in RIPA lysis buffer (20 mM Tris [pH 8.0], 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate) in the presence of PhosSTOP and EDTA-free protease inhibitor tablets (Roche). Cell lysates were incubated on ice for 30 min, and then, the soluble fraction was isolated by centrifugation at 16,000 g for 20 min at 4°C. The total protein concentration in cellular extracts was measured by BCA protein assay kit (Pierce Chemical). Protein extracts, separated by SDS–PAGE and transferred onto membranes, were probed with antibodies against COLLAGEN I (Abcam, ab138492 for human cells; Abcam, ab21286 for mouse cells), LAMP1 (Abcam, ab24170), LC3B (NB100-2220; Novus Biologicals), CANX (Enzo Life Sciences ADI-SPA-860-D), HALO (Promega G928A) FAM134B (Sigma, HPA012077), SQSTM1/p62 (Abnova, H00008878-M01), SEC23A (PAI-069A; Thermo Fisher Scientific), VAP-A (15275-1-AP, Proteintech), PDI (Enzo Life Sciences ADI-SPA-891-F), Filamin (Abcam, ab76289), TUBULIN (T5168, Sigma-Aldrich) and β-actin (Novus Biologicals NB600-501), probed with horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit IgG antibody (1:2,000, Vector Laboratories; 8125, 8114; Cell Signaling Technology) and visualized with the Super Signal West Dura substrate (Thermo Fisher Scientific), according to the manufacturer’s protocol.
Mass spectrometry

Wild-type, *Fam134b* and *Canx* knockout MEFs were grown in DMEM media supplemented with 10% FBS. Cells were lysate in SDS-lysis buffer (4% SDS in 0.1 M Tris/HCl pH 7.6). Protein concentration was measured using BCA Kit (Pearce), and 50 µg of cells lysate was precipitated with ice-cold acetone and resuspended in 30 µl of GnHCl buffer (6 M guanidine hydrochloride, 50 mM Tris pH 8.5, 5 mM TCEP, 20 mM chloro-iodoacetamide). For label-free quantification-based proteome analysis of whole cell lysates, proteins were in-solution digested with the endopeptidase sequenc- ing-grade Lys-C (1:100 ratio) for 3 h at 37°C and subsequently with trypsin (1:100 ratio) overnight 37°C. Digestion was blocked with TFA 1% final concentration. Collected peptide mixtures were concentrated and desalted using the Stop and Go Extraction (STAGE) technique (Rappsilber et al, 2003).

Instruments for LC-MS/MS analysis consisted of a NanoLC 1200 coupled via a nano-electrospray ionization source to the quadrupole-based Q Exactive HF benchtop mass spectrometer (Thermo Scientific). Peptide separation was carried out according to their hydrophobicity on an in-house packed 20 cm column with 1.9 mm C18 beads (Dr Maisch GmbH) using a binary buffer system consisting of solution A: 0.1% formic acid (0.5% formic acid) and B: 80% acetonitrile, 0.1% formic acid (80% acetonitrile, 0.5% formic acid). 2 h gradients were used for each sample. Linear gradients from 5–38% B were applied with a following increase to 95% B at 400 nl/ min and a re-equilibration to 5% B. Q Exactive HF settings: MS fragmentation MS/MS spectra was set to 30,000 at 200 m/z. Resolution for MS/MS spectra of the 15 most intense peaks. Resolution for MS/MS spectra was set to 30,000 at 200 m/z, AGC target to 1E5, max injection time to 25 ms and the isolation window to 1.6 Th.

Statistics

Statistics were performed in GraphPad PRISM software. A two-tailed, paired and unpaired Student’s *t*-test was performed when comparing the same cell population with two different treatments or cells with different genotypes, respectively. One-way ANOVA and Dunnett’s post hoc test were performed when comparing more than two groups relative to a single factor (treatment). A *P*-value of 0.05 or less was considered statistically significant.

For mass spectrometry analysis, the raw files were processed using MaxQuant software (Cox et al, 2011). Parameters were set to default values. Statistical analysis, *t*-test and GO annotation enrichment were performed using Perseus software (Tyanova et al, 2016). Data are representative of three independent mass spectrometry analyses for each genotype.

Expanded View for this article is available online.

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Author contributions

AF, CDL and EF performed most of the experiments. PG and MS performed mass spectrometry experiments. LS and GB performed HA-pull-down studies. AM and IF performed IF experiments in KO MEF. IC and DI performed experiments using Medaka fish. AR performed CLEM and tomography. AM, IF, MP and MI helped with the quantification of the data. EVA generated and charac- terized HALO-PC2 plasmids. MADM provided critical suggestions, protocols and reagents. CS designed the project. CS, MM and ID supervised the study. CS and AF wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Chiramel AI, Dougherty JD, Nair V, Robertson SJ, Best SM (2015) FAM134B and MI helped with the quantification of the data. EVA generated and characterized HALO-PC2 plasmids. MADM provided critical suggestions, protocols and reagents. CS designed the project. CS, MM and ID supervised the study. CS and AF wrote the manuscript.

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References

Procollagen ER quality control by CNX


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