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Lysosomal integral membrane protein type-2 (LIMP-2/SCARB2) is a substrate of cathepsin-F, a cysteine protease mutated in type-B-Kufs-disease

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ABSTRACT

The lysosomal integral membrane protein type-2 (LIMP-2/SCARB2) has been identified as a receptor for enterovirus 71 uptake and mannose-6-phosphate-independent lysosomal trafficking of the acid hydrolase β-glucocerebrosidase. Here we show that LIMP-2 undergoes proteolytic cleavage mediated by lysosomal cysteine proteases. Heterologous expression and *in vitro* studies suggest that cathepsin-F is mainly responsible for the lysosomal processing of wild-type LIMP-2. Furthermore, examination of purified lysosomes revealed that LIMP-2 undergoes proteolysis *in vivo*. Mutations in the gene encoding cathepsin-F (CTSF) have recently been associated with type-B-Kufs-disease, an adult form of neuronal ceroid-lipofuscinosis. In this study we show that disease-causing cathepsin-F mutants fail to cleave LIMP-2. Our findings provide evidence that LIMP-2 represents an *in vivo* substrate of cathepsin-F with relevance for understanding the pathophysiology of type-B-Kufs-disease.

1. Introduction

The lysosomal integral membrane protein type-2 (LIMP-2) is an abundant protein of the lysosomal membrane and has been identified as a mannose-6-phosphate-independent lysosomal transport receptor for β-glucocerebrosidase (GC) [1]. Recently, the crystal structure of the ectodomain of LIMP-2 was determined [2] supporting the idea that GC binds to a region comprised of the apical helices 4, 5 and 7 [1–3]. Binding of LIMP-2 and GC occurs in the endoplasmic reticulum (ER), leading to the transport of the enzyme/receptor-complex to lysosomes, where both proteins dissociate in a pH-dependent manner [4]. LIMP-2 also serves as a plasma membrane receptor for uptake of enterovirus 71 and coxsackievirus 16 associated with hand, foot and mouth disease [5,6]. Additionally, a role for LIMP-2 in the control of endosomal transport, lysosomal biogenesis and lipid transport has been suggested [2,7]. Mutations in SCARB2, the gene encoding LIMP-2, have been described as causative for a rare form of progressive myoclonus epilepsy, which can be associated with renal failure, called action myoclonus and renal failure syndrome [8,9].

Recently, it has been shown that many lysosomal membrane proteins undergo proteolysis which is mainly mediated by cathepsin proteases like cathepsin-L [10–12]. One member of the cathepsin-family is the ubiquitously expressed cysteine protease cathepsin-F [13], which localizes to acidic endosomal/lysosomal-like vesicles upon heterologous expression [14]. *In vitro* assays with recombinant cathepsin-F demonstrated its proteolytic activity in the degradation of the histocompatibility complex class II associated invariant chain and the apolipoprotein B-100 [15,16]. However, to date no physiological substrate of cathepsin-F has been described.

So far, four missense mutations, one splice site mutation and one single nucleotide deletion that results in a frameshift and

Abbreviations: LIMP-2, lysosomal integral membrane protein type-2; GC, β-glucocerebrosidase; ER, endoplasmic reticulum; Baf, bafilomycin A1; Leu, Leupeptin; EST, E-64-d; CathF, cathepsin-F; CathW, cathepsin-W; CathL, cathepsin-L; NCL, neuronal ceroid lipofuscinosis; F (dg), deglycosylated LIMP-2 fragments; fl (dg), deglycosylated full-length LIMP-2.

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subsequent nonsense mutation of cathepsin-F have been identified as causative for type-B-Kufs-disease, an autosomal recessive adult form of neuronal ceroid-lipofuscinosis (NCL) [17,18]. NCLs belong to the lysosomal storage disorders and are characterized by progressive neurodegeneration and accumulation of autofluorescent lipopigments in neuronal and peripheral tissues; onset varies from the neonatal period to adult life [19]. Due to the lack of natural cathepsin-F substrates, no functional characterization of the type-B-Kufs-disease-causing mutations could be performed so far.

In this report we provide evidence that lysosomal localized LIMP-2 represents a substrate of cathepsin-F. Alterations in a helical bundle motif of the ectodomain of LIMP-2, which is needed for ligand binding, increased the fragmentation of the protein. We also provide evidence that disease-causing mutations of cathepsin-F result in their inability to cleave LIMP-2, suggesting that they represent loss-of-function mutations.

2. Material and methods

2.1. Materials

DNA oligonucleotides, Bafilomycin A1, Leupeptin, Triton WR1339 (Tyloxapol) were purchased from Sigma Aldrich (Steinheim, Germany). Recombinant human cathepsin-F enzyme (BML-SE568-0005) and E-64-d (EST) were obtained from Enzo Life Science (Farmingdale, USA). Restriction enzymes, Pierce Silver Stain Kit, protein ladders, ligases, TurboFect™ and other reagents for molecular biology were obtained from Thermo Scientific (Waltham, USA). Western immunoblotting reagents and BCA protein assay kit were purchased from Pierce (Rockford, IL) and GE Healthcare Bio-Science (Pittsburgh, USA), respectively. Complete™ protease inhibitor and PNGaseF were obtained from Roche (Mannheim, Germany). Standard chemicals and blotting membranes were acquired from Roth (Karlsruhe, Germany). Cell culture media was purchased from PAA (Pasching, Austria). Recombinant human luminal domain of LIMP-2 was purchased from R&D System (1966-LM-050, Minneapolis, USA).

2.2. Plasmids and cell lines

Murine(m) LIMP-2 [1] and *human(h)* cathepsin-F wild-type (WT) [20] and mutants [2,4,17] as well as *h*cathepsin-W cDNA were expressed in pFrog-Vector (derivative of pcDNA3.1) whereas *m*cathepsin-L cDNA was expressed in pcDNA3.1. All constructs were verified by sequencing. Human embryonic kidney 293T (HEK) and murine neuroblastoma (N2a) cells were used for overexpression experiments. Cells were transiently transfected using TurboFect™ following the manufacturer's instructions. LIMP-2 mice were previously described in Gamp et al. [21].

2.3. Antibodies

Tagged proteins were detected with mouse anti-myc (9B11, Cell Signaling), goat-anti-myc (GTX30518, Genetex) or with a peroxidase-conjugated anti-HA (HA-POD, 3F10, Roche) antibody. Endogenous LIMP-2 was detected using an antibody against the LIMP-2-C-terminus [4]. For endogenous detection of cathepsin-L an antibody from R&D Systems (AF1515) was used.

2.4. Tritosome preparation

Tritosome preparation was performed as described previously [11,22]. Livers were prepared according to european guidelines for the care and use of experimental animals.

2.5. SDS-PAGE and Western blotting

Cells were harvested and Western blotting was performed as described previously [23].

2.6. PNGaseF-digestion from cell lysates

An appropriate amount of protein was incubated with peptide-N-glycosidase F (PNGaseF) following the manufacturer's instructions of New England Biolabs (Ipswich, USA) for PNGaseF digest.

2.7. Inhibition tests

24 h after transfection cells were incubated for 12 h with the appropriate inhibitor in culture medium at the following concentrations: Bafilomycin A1: 100 nM; NH_4Cl: 25 mM; Leupeptin: 100 μM, E-64-d (EST): 50 μM. Cells were then harvested and subjected to SDS-PAGE and immunoblotting.

2.8. In vitro assay and silver gel staining

Three micrograms of LIMP-2 and 10 nM cathepsin-F were incubated at 37 °C in 20 mM MES, 150 mM NaCl, 2.5 mM EDTA, 1 mM DTT, pH 6.0 for the indicated time points. For enzyme inhibition with EST, cathepsin-F was preincubated for 5 min in MES buffer containing 50 μM EST. Silver gel staining was performed according to the manufacturer's instructions (Pierce Silver Stain Kit, Thermo Scientific).

2.9. Statistical analysis

Intensities of resulting immunoblot bands were measured using Image J software (http://imagej.nih.gov/ij/). Analyses of statistical significances were performed using one way ANOVA with a Tukey's post hoc test (GraphPad Prism software). All data are presented as the mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns = not significant.

3. Results

3.1. LIMP-2 ligand binding mutants are subject to proteolysis by cysteine proteases

In previous studies we demonstrated that LIMP-2 harbors a GC-binding domain that is comprised of a helical bundle, which is mainly formed by helices 5 to 7 [1,3,4]. During these analyses we examined several point mutants within this region for their GC-binding capacity (Fig. 1A) and observed that some of the LIMP-2 mutants investigated showed two specific C-terminal fragments at 40 kDa and 34 kDa (Fig. 1B). The fragments could be separated after deglycosylation through treatment of cellular lysates with PNGaseF. Interestingly, this cleavage pattern was only observed for those LIMP-2 constructs carrying mutations within helix 5, indicating that alterations in this region make LIMP-2 more accessible for proteolysis.

In order to characterize the responsible protease(s) HEK cells were treated with leupeptin and EST (E-64-d), two inhibitors of lysosomal proteases of the cysteine family (cathepsins) and pepstatin A, an inhibitor for the aspartyl protease cathepsin D [24,25] after heterologous expression of the LIMP-2 I156D mutant, which was most susceptible to proteolysis (Fig. 1B). For better separation of the cleavage products, the indicated cellular lysates were treated with PNGaseF. The levels of the cleaved fragments were prominently reduced only after EST and leupeptin treatment (Fig. 1C). Pepstatin A

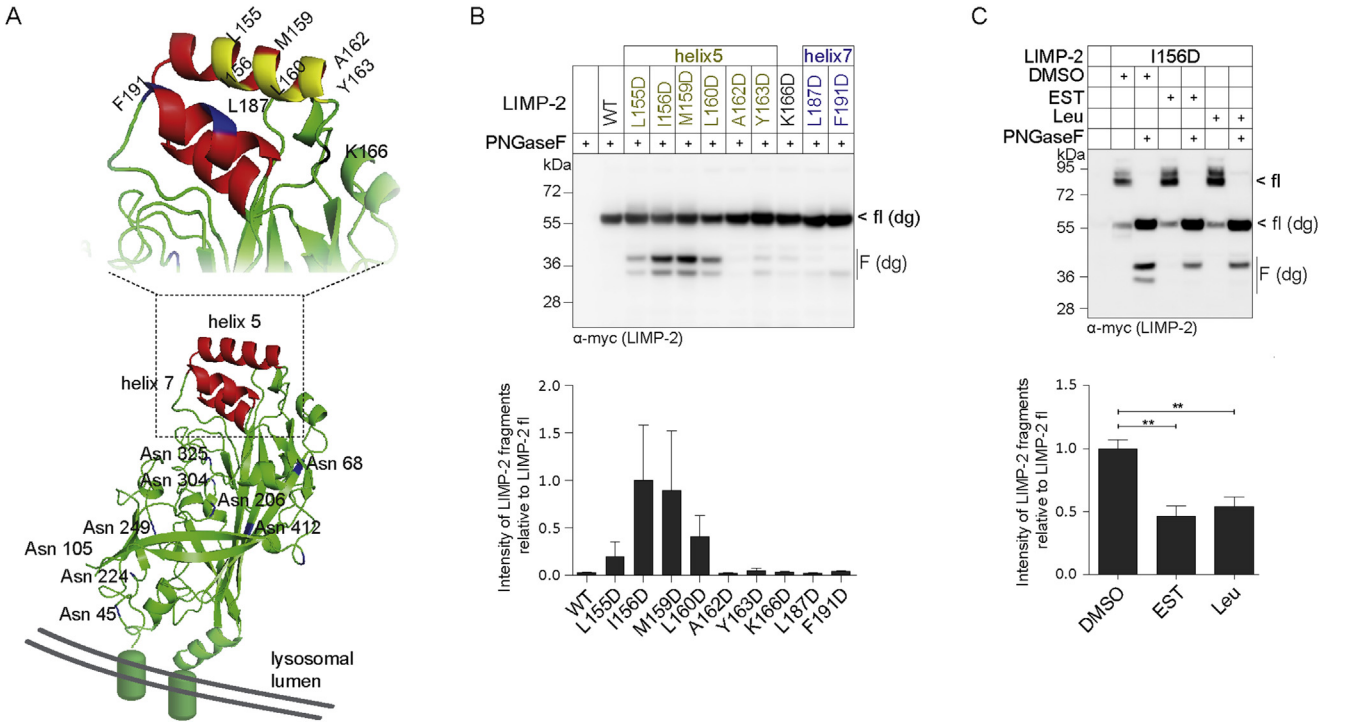

Fig. 1. LIMP-2 ligand binding mutants are subject to proteolysis by cysteine proteases. A) 3D-reconstruction of human LIMP-2 (PDB Code 4F7B) using PyMOL software. The helices of the binding region are highlighted in red. Murine analogs of the mutants are indicated in the magnification in yellow (helix 5), blue (helix 7) and black (interhelical). Glycosylated asparagines are additionally indicated. B) Immunoblot of HEK-cells transiently transfected with different myc-tagged LIMP-2 mutant constructs. Samples were treated with PNGaseF. LIMP-2 full-length and cleavage fragments were detected using an anti-myc antibody. C) Immunoblot of HEK cells overexpressing LIMP-2 I156D-myc (I156D) treated with EST or leupeptin for 12 h. Indicated protein lysates were treated with PNGaseF. Graphs of B/C: Densitometric analysis of two (B) or three (C) independent experiments was performed. Ratios of full-length LIMP-2 and the cleavage fragments were calculated.

did not significantly affect the formation of the cleavage products (data not shown). These data point to a critical role of thiol-dependent cathepsins in the proteolysis of the LIMP-2 helix 5 mutants.

3.2. Cathepsin-F induces LIMP-2 cleavage

To specifically address which cysteine protease is responsible for the observed LIMP-2 cleavage and to determine if wild-type LIMP-2 can be proteolytically processed, we co-expressed LIMP-2 with C-terminally HA-tagged versions of different cysteine proteases such as cathepsin-F and W as well as untagged cathepsin-L. In this assay LIMP-2 proteolysis appeared to be predominantly mediated by cathepsin-F and to a minor degree by cathepsin-L (Fig. 2A). Interestingly, expression of cathepsin-W, which belongs to the same subfamily as cathepsin-F [26], had no apparent effect on LIMP-2 cleavage.

Next, we explored the role of cathepsin-F in LIMP-2 cleavage and included a catalytically inactive form of cathepsin-F in our analysis by mutating the cysteine in the catalytic center of the protease to an alanine (C295A) [13]. This cathepsin-F mutant was unable to generate proteolytic fragments of LIMP-2 (Fig. 2B). In order to directly verify the cleavage of LIMP-2 by cathepsin-F, we performed an *in vitro* cleavage assay. Therefore, the recombinant luminal domain of LIMP-2 was incubated with recombinant cathepsin-F. Finally, processed and unprocessed LIMP-2 protein was visualized using silver staining. Due to an IgG-tag, the luminal domain LIMP-2 migrated at about 130 kDa. The experiment revealed that cathepsin-F mediates LIMP-2 proteolysis, resulting in the time dependent generation of several LIMP-2-specific cleavage fragments and a significant decrease in the amount of the full-length protein (Fig. 2C). Preincubation of cathepsin-F with EST prevented the fragmentation of LIMP-2.

In summary our study indicates that wild-type LIMP-2 can be cleaved by cathepsin-F and that proteolysis likely occurs in the helical bundle, which forms the head of the luminal ectodomain.

3.3. Endogenously expressed LIMP-2 is processed by cathepsin-F in lysosomes

Next, we addressed the question in which cellular compartment cathepsin-F cleavage of LIMP-2 occurs. Therefore, processing of LIMP-2 by cathepsin-F was analyzed in the presence of lysosomal proteolysis inhibitors. HEK cells overexpressing LIMP-2 and cathepsin-F were treated with Bafilomycin A1 or NH_4Cl. Both substances increase the lysosomal/endosomal pH leading to an inactivation of lysosomal hydrolases [27,28]. Cellular treatment with these drugs significantly inhibited cathepsin-F mediated fragmentation of LIMP-2 (Fig. 3A). In addition, an LIMP-2-ER-retention mutant [1,4] was not processed by cathepsin-F (Fig. 3B), suggesting that cathepsin-F-mediated proteolytic cleavage of LIMP-2 occurs in post-ER compartments most likely in late endosomes and lysosomes.

These findings prompted us to examine if endogenous LIMP-2 is a substrate for cathepsin-F-mediated lysosomal cleavage. Therefore, we used highly enriched lysosomes of murine liver (tritosomes) that were stained for LIMP-2. Immunoblot analysis revealed several C-terminal fragments, suggesting that endogenous LIMP-2 undergoes lysosomal proteolysis *in vivo* (Fig. 3C). We also demonstrated cleavage of endogenous LIMP-2 in murine neuroblastoma cells (N2a) after overexpression of active but not catalytically inactive cathepsin-F (Fig. 3D). The main fragment runs at 53 kDa as lysates were not treated with PNGaseF. These results strongly suggest that LIMP-2 is cleaved in lysosomes and that the proteolysis also occurs at the endogenous level.

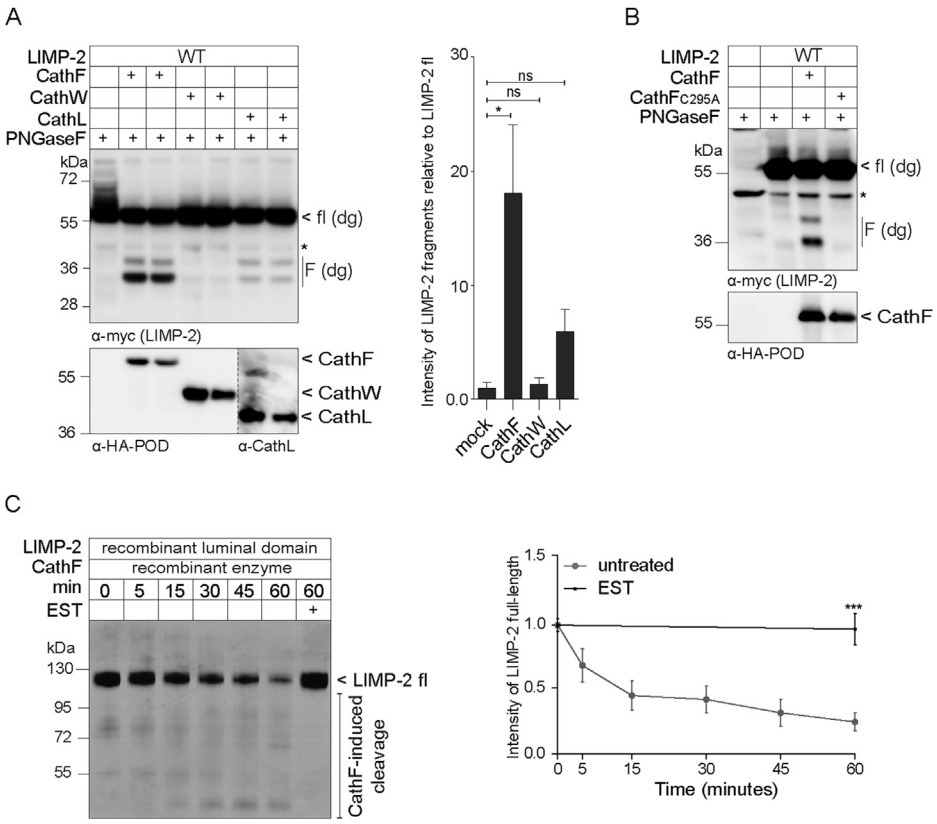

Fig. 2. Cathepsin-F induces LIMP-2 cleavage. A) Immunoblot of wild-type LIMP-2-myc co-expressed with HA-tagged cathepsin-F, cathepsin-W and untagged cathepsin-L (CathF/W/L) in HEK cells. Obtained protein lysates were treated with PNGaseF. LIMP-2 was detected using the anti-myc antibody. Tagged cathepsin-F and cathepsin-W were detected using an HA-POD antibody and anti-cathepsin-L antibody was used to detect the untagged protein. * indicates an unspecific band. Graph: Densitometric analyses of three independent experiments were performed. Ratios of full-length LIMP-2 and the cleavage fragments were calculated. B) Immunoblot of HA-tagged wild-type LIMP-2 co-expressed with cathepsin-F-myc and an inactive mutant of cathepsin-F-myc (C295A) in HEK cells. Protein lysates were PNGaseF treated and separated by SDS-PAGE. Expression was detected using antibodies against myc- or HA. C) Recombinant luminal domain of LIMP-2 fused to a human IgG-tag was incubated with recombinant cathepsin-F and as a control treated with EST for the indicated time at 37 °C. Proteins were separated by SDS-PAGE and silver staining was performed. Graph: Densitometric analysis of three independent experiments was performed.

3.4. Cathepsin-F mutants identified in type-B-Kufs-disease patients are unable to cleave LIMP-2

Mutations in cathepsin-F lead to type-B-Kufs-disease, an adult-onset NCL [17]. Since physiological substrates for cathepsin-F have not yet been revealed and type-B-Kufs-disease mutations are only thought to be inactive due to structural prediction experiments [17] we assessed their ability to cleave LIMP-2 in comparison to wild-type cathepsin-F. Immunoblot analysis of lysates derived from HEK cells co-transfected with LIMP-2 and cathepsin-F demonstrated that all five of the originally described type-B-Kufs-disease-causing mutations were unable to process LIMP-2 and therefore failed to generate any of the LIMP-2 fragments identified so far (Fig. 4). These data strongly suggest that disease-associated mutations in cathepsin-F lead to a loss of their proteolytic function.

4. Discussion

In this study we demonstrated that LIMP-2 undergoes proteolysis in late endosomal/lysosomal compartments and that cleavage is mediated mainly by cathepsin-F.

Recent studies also revealed that other lysosomal membrane proteins such as heparan sulfate acetyl-CoA alpha-glucosaminide N-acetyltransferase (HGSNAT), neuronal ceroid lipofuscinosis protein 7 (CLN7) and disrupted in renal carcinoma 2 (DIRC2) are proteolytically cleaved by cathepsins. This process can, at least in the case of HGSNAT, modulate their activity [10–12]. However, it is conceivable that the proteolysis of lysosomal membrane proteins including LIMP-2 reflects their conventional degradation pathway and thereby regulates the half-life of these proteins. In general, lysosomal membrane proteins have a long lifetime [29,30] and their complex and dense glycosylation protects them and the underlying membrane from hydrolytic damage mediated by acid hydrolases [31]. While the tight glycocalyx-like inner luminal part of lysosomal membrane proteins is physiologically important to maintain the integrity of lysosomes [31], more distally exposed non-glycosylated domains of lysosomal membrane proteins are likely to be more susceptible to degradation by cathepsins. Such initial cleavage events may further trigger subsequent hydrolysis and degradation of the membrane proteins. Our data on LIMP-2 proteolysis are in agreement with such a model and highlight the role of lysosomal cysteine proteases, particularly cathepsin-F, in LIMP-2 processing.

A well-characterized function of LIMP-2 is the transport of GC from the ER to lysosomes [1] where both proteins dissociate [4]. In this study we show that alterations in helix 5 but not in helix 7 induce the fragmentation of LIMP-2. These helices are part of a helical bundle mainly formed by the three α-helices 4, 5 and 7 [2] composing the ligand binding site of LIMP-2 for interaction with GC [2] or enteroviruses [32]. It has been proposed that the helical bundle adopts two different pH-dependent conformations [33]. Helices 5 and 7 form a tight bundle at neutral pH but a more lose structure at acidic pH. Mutations in helix 5 as shown in this study may inhibit the formation of a tight bundle, making the helices more accessible for proteases such as cathepsin-F. The exposed ligand binding region that is not covered by glycosylation sites

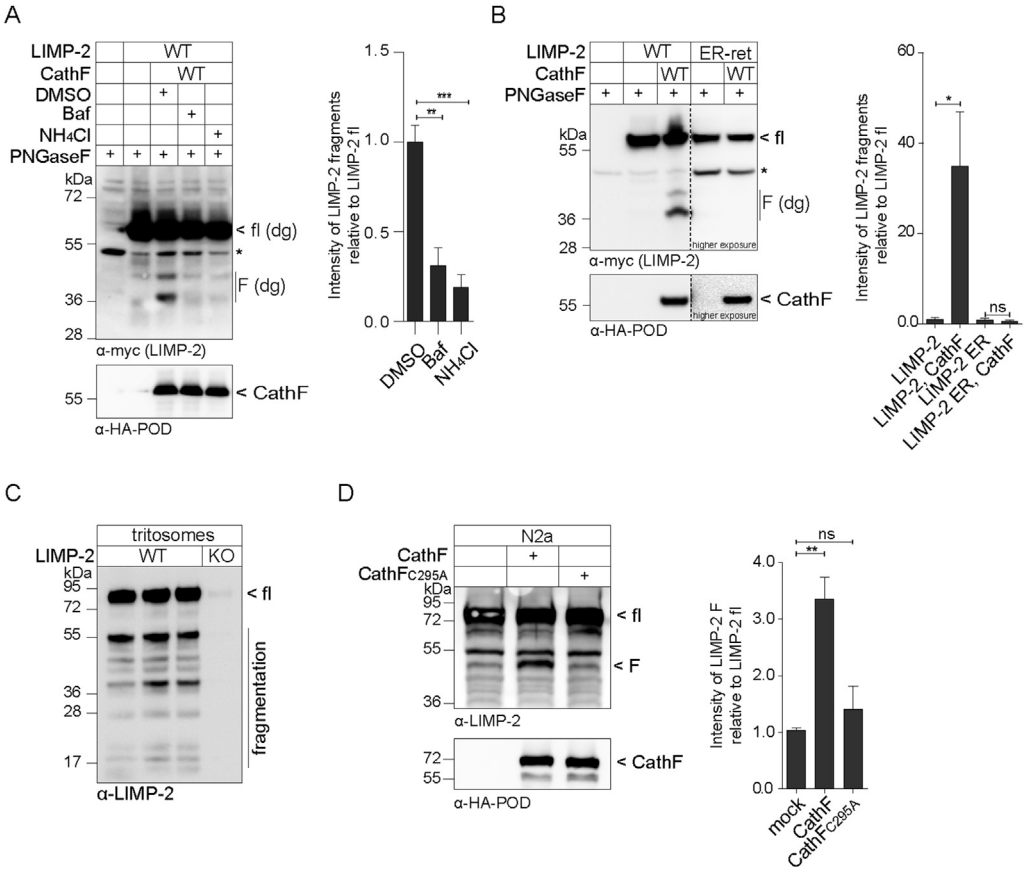

Fig. 3. Endogenously expressed LIMP-2 is processed by cathepsin-F in lysosomes. A) LIMP-2-myc and cathepsin-F-HA were co-expressed in HEK cells. Cells were incubated with Bafilomycin A1 or NH_4Cl for 12 h. Protein lysates were PNGaseF treated and separated by SDS-PAGE and analyzed by Western blotting. Resulting membranes were stained against myc or HA. * indicates an unspecific band. B) Immunoblot analysis of the cleavage of myc-tagged LIMP-2-wild-type (WT) and an LIMP-2 ER-retention (ER-ret) mutant co-expressed with cathepsin-F-HA in HEK cells. Protein lysates were treated with PNGaseF. Proteins were detected using anti-myc or anti-HA-POD antibody. C) Immunoblot of tritosome preparation from murine livers. Endogenous LIMP-2 full-length (fl) and additional smaller LIMP-2 fragments are detectable in wild-type (WT) tritosomes but are absent in LIMP-2 knock out (KO) livers. D) HA-tagged wild-type cathepsin-F and the inactive cathepsin-F (C295A) mutant were overexpressed in N2a cells. Obtained protein lysates were tested for endogenous LIMP-2 cleavage via immunoblot. Cathepsin-F expression was controlled using the HA-POD antibody. Graph of A, B, D: Densitometric analysis of four (A, B) or three (D) independent experiments was performed. Ratios of full-length LIMP-2 and the cleavage fragments were calculated.

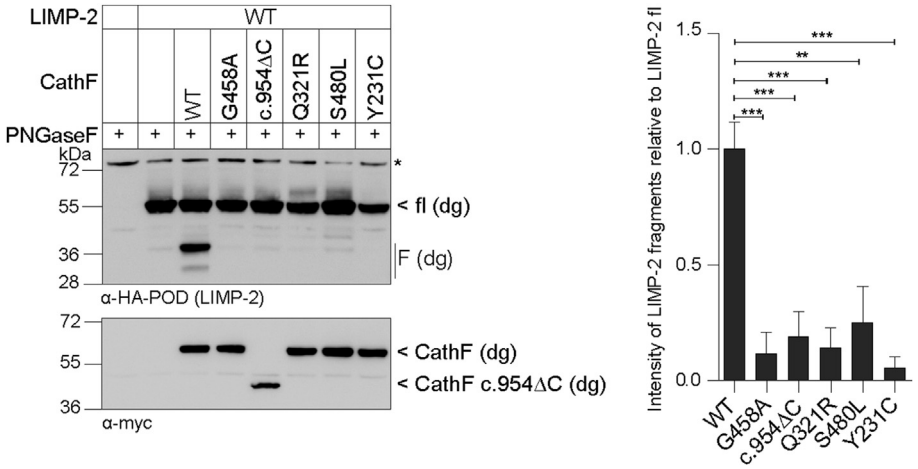

Fig. 4. Cathepsin-F mutants identified in type-B-Kufs-disease patients are unable to cleave LIMP-2. Immunoblot of LIMP-2-HA cleavage by type-B-Kufs disease-associated cathepsin-F mutants. cDNA was expressed in HEK cells and the samples were treated with PNGaseF. Deglycosylated LIMP-2 full length (fl dg) and indicated cleavage fragments (F dg) were detected using an HA-POD antibody, whereas expression of WT cathepsin-F and disease-causing mutants were detected using an anti-myc antibody. * indicates an unspecific band. Graph: Densitometric analysis of four independent experiments. Ratios of full-length LIMP-2 and the cleavage fragments were calculated.

seems to be a likely area for proteolysis. Therefore cleavage of LIMP-2 in the ligand binding site may be a regulatory mechanism to influence binding and lysosomal release of the ligands. Additionally, the luminal domain of LIMP-2 contains a tunnel structure, which at least in the case of the LIMP-2 homologs SR-B1 and CD36 acts as a lipid transport channel [2]. Disruption of the luminal LIMP-2 structure by proteolysis would certainly also affect similar transport activity. However, the molecules transported in this channel have not yet been identified.

In this report we firstly described the proteolysis of LIMP-2, which we identified as a novel substrate for cathepsin-F. This protease was described as a cysteine protease of the papain family, although it exhibits relatively low sequence homology with other family members. The protease is ubiquitously expressed in a similar pattern as described for cathepsin-L, B and H, with higher expression levels in brain, heart, skeletal muscle, testis and ovary [14]. Using recombinant cathepsin-F, functional redundancy between cathepsin-F and S, K and L in invariant chain processing or apolipoprotein B-100 degradation has been shown [15,16]. Even though cathepsin-F shows the highest similarity with cathepsin-W [34], our data suggest that LIMP-2 is apparently not cleaved by this protease. Cathepsin-L on the other hand is also able to produce LIMP-2 cleavage products, which may hint to a redundant function of cathepsin-F and L. As a cysteine protease cathepsin-F exhibits a catalytic triad of C295, H431 and N451 [17]. Mutation of C295 to alanine leads to an inactive form of cathepsin-F that was not able to cleave LIMP-2.

Mutations in the cathepsin-F gene cause type-B-Kufs-disease [17,18], an adult form of NCL. Patients show progressive neurodegeneration and accumulation of abnormal lipopigments predominantly in the brain [35], which in type-B-Kufs-disease patients manifests mainly as dementia and motor disturbances [36]. So far there are six known mutations within the cathepsin-F gene described that are associated with type-B-Kufs-disease [17,18]. The present study revealed that the intraluminal domain of LIMP-2 is a natural substrate of cathepsin-F. Importantly, the five originally described clinical cathepsin-F mutants failed to cleave LIMP-2 when expressed in our cell based assay. This confirms previous inferences based on structural modeling that at least some mutations lead to a loss of enzymatic activity [17]. It is not well understood how cathepsin-F mutations lead to the observed neurodegeneration and intralysosomal storage in type-B-Kufs-disease [17,37]. Similar to cathepsin-D [38], L and B [39], cathepsin-F may also be involved in the degradation of other lysosomal substrates. Our data suggest that lack of LIMP-2 cleavage may contribute to the pathogenesis of this disease by impaired lysosomal degradation. Leading to a reduced autophagic flow and accumulation of engulfed structures, a hallmark of many NCLs [40].

In conclusion, our findings provide additional evidence for the hypothesis that cysteine proteases, including cathepsin-F, are major regulators of lysosomal membrane proteins such as LIMP-2. Their proteolytic activity may be necessary to control both the half-life but also the function of lysosomal membrane proteins.

Conflict of interest

The authors declare no conflicts of interests.

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