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CLN5 is cleaved by members of the SPP/SPPL family to produce a mature soluble protein

Felix Jules [*,1], Etienne Sauvageau [*,‡,1], Karine Dumaresq-Doiron [¶], Javier Mazzaferri [¶], Martina Haug-Kröper [†], Regina Fluhrer [†,^], Santiago Costantino [¶,§] and Stephane Lefrancois [*,‡,2]

[*] Centre INRS-Institut Armand-Frappier, INRS, Laval, Canada H7V 1B7
[‡] Department of Anatomy and Cell Biology, McGill University, Montreal, Canada H3A 2B2
[§] Département d'Ophtalmologie et Institut de Génie Biomédical, Université de Montréal, Montréal, Canada H3T 1J4
[¶] Centre de Recherche de l'Hôpital Maisonneuve-Rosemont, Montréal, Canada H1T 2M4
[†] Biomedical Center (BMC), Institute for Metabolic Biochemistry, Ludwig-Maximilians University Munich, Munich, Germany
[^] DZNE – German Center for Neurodegenerative Diseases, Munich, Germany

Running Title: *CLN5 is cleaved by SPPL3 to produce a soluble protein*

[1] These authors contributed equally to this work

[2] To whom correspondence should be addressed: Stephane Lefrancois, Centre INRS-Institut Armand-Frappier, INRS, Laval, Canada H7V 1B7 Tel.: 450-687-5010 ext. 8860; Fax: 450 686-5501; E-mail: stephane.lefrancois@iaf.inrs.ca

Abstract

The Neuronal ceroid lipofuscinoses (NCLs) are a group of recessive disorders of childhood with overlapping symptoms including vision loss, ataxia, cognitive regression and premature death. 14 different genes have been linked to NCLs (*CLN1-CLN14*), but the functions of the proteins encoded by the majority of these genes have not been fully elucidated. Mutations in the *CLN5* gene are responsible for the Finnish variant late-infantile form of NCL (Finnish vLINCL). CLN5 is translated as a 407 amino acid transmembrane domain containing protein that is heavily glycosylated, and subsequently cleaved into a mature soluble protein. Functionally, CLN5 is implicated in the recruitment of the retromer complex to endosomes, which is required to sort the lysosomal sorting receptors from endosomes to the trans-Golgi network. The mechanism that processes CLN5 into a mature soluble protein is currently not known. Herein, we demonstrate that CLN5 is initially translated as a type II transmembrane protein and subsequently cleaved by SPPL3, a member of the SPP/SPPL intramembrane protease family, into a mature soluble protein consisting of residues 93-407. The remaining N-terminal fragment is then cleaved by SPPL3 and SPPL2b and degraded in the proteasome. This work further characterizes the biology of CLN5 in the hopes of identifying a novel therapeutic strategy for affected children.

Highlights

- CLN5 is initially translated as a type II integral membrane protein
- CLN5 is cleaved after residue 92
- CLN5 cleavage is mediated by SPPL3

Key Words

CLN5, Signal Peptide Peptidase-like proteases, Neuronal Ceroid Lipofuscinosis, endosomes, neurodegeneration, intracellular trafficking

Introduction

Neuronal ceroid lipofuscinoses (NCLs) are recessive disorders that are the most common neurodegenerative diseases of childhood [1]. Common symptoms of NCLs include gradual dementia, progressive vision loss, ataxia and seizures and they almost always result in premature death [2]. At the cellular level, NCL patients present an accumulation of autofluorescent lipopigments in cellular subcompartments [3]. Germline mutations in 14 different genes (*CLN1-CLN14*) have been shown to lead to human disease [4], but the exact function of many of the proteins encoded by these genes remains unknown or poorly understood hampering the development of therapies.

CLN5 is encoded on chromosome 13q21–q32 [5, 6] and produces a 407 amino acid protein of a predicted molecular mass of 46 kDa that is heavily glycosylated [7-9]. Mutations in the CLN5 gene were first identified in 18 families in Finland thus naming this variant of NCL: late-infantile Finnish variant (Finnish vLINCL) [10, 11]. Since the initial identification of the disease and mapping of the gene responsible, patients with NCL derived from a mutation in the CLN5 gene have been found in various regions around the globe [9, 12].

Several studies have shown that CLN5 localizes to the lysosomal compartment [8, 13] but little data is available as to the function of CLN5. We have shown a role for CLN5 in controlling the itinerary of the lysosomal sorting receptors by regulating retromer recruitment at late endosomes [14]. Indeed, we found that CLN5 was required for the activation and localization of Rab7 and subsequently, the recruitment of retromer to endosomal membranes. Furthermore, we found that in CLN5-depleted cells, both sortilin and the cationic independent mannose-6-phosphate receptor (CIMPR) were degraded in lysosomes, a similar phenotype as observed in retromer-depleted cells [15, 16]. Although CLN5 plays a role in the efficient endosome-to-trans Golgi Network (TGN) trafficking of these receptors, there is still uncertainty regarding the characterization of the protein.

Most prediction tools suggest that CLN5 could have at least one transmembrane domain and experimental evidence supports this [17-19], while other studies have shown that it is a soluble protein [19, 20]. A more recent study showed that CLN5 has one transmembrane domain and that the soluble form of the protein was potentially membrane anchored [21]. It is clear that in lysosomes, CLN5 is a soluble protein within the lumen of that organelle [8, 13]. *In vitro* experiments have suggested that CLN5 could have 4 different initiator sites [13]. However, it appears that regardless of the site used, CLN5 is cleaved around residue 96 to produce the mature soluble protein [8]. The mechanism that cleaves CLN5 into a soluble protein has not been identified. Signal Peptide Peptidase (SPP) and its homologues the Signal Peptide Peptidase-like proteases (SPPL) are intramembrane aspartyl proteases known to cleave a variety of type II transmembrane proteins including TNFα [22, 23], invariant chain [24], British dementia protein-2 [25] and foamy virus envelope protein [26]. So far 5 members of the SSPP/SPPL family have been identified in the mammalian genome (SPP, SPPL2a, SPPL2b, SPPL2c and SPPL3) that all share a common conserved catalytic motif of GxGD [27]. In this work, we found that CLN5 is produced as a type II transmembrane domain protein and cleaved by a member of the SPP/SPPL after residue 92 to produce a soluble lysosomal protein (residues 93 - 407).

EXPERIMENTAL

Antibodies, cDNA constructs and other reagents

All reagents, unless specified, were purchased from Fisher Scientific (Ottawa, ON). The HA-CLN5 and CLN5-HA constructs were purchased from Genecopoeia (Germantown, MD) and sequenced to ensure accuracy. The $CLN5^{Y392*}$ and $CLN5^{W75*}$ mutants were generated using the QuikChange II XL Mutagenesis kit (Stratagene, Mississauga, ON) to introduce a stop codon (*). The prosaposin-CLN5 chimera was engineered by cutting PSAP-myc [28] with NheI and inserting residues 94 - 407 of CLN5. The following mouse monoclonal antibodies were used: anti-hemagglutinin (anti-HA) antibody (MMS-101P, Cedarlane Laboratories, Burlington, ON); anti-Lamp-2 antibody (ab25631, Abcam, Cambridge, MA); anti-EEA1 antibody (610417, BD Bioscience, Mississauga, ON). The following rabbit polyclonal antibodies were used: anti-Lamp2 (L0668, Sigma-Aldrich, Oakville, ON); anti-HA antibody (ab20084, Abcam), anti-calnexin (ADI-SPA-865, Cedarlane Laboratories). Monoclonal anti-SPPL3 antibody was previously described [26]. Goat polyclonal anti-CLN5 antibody (sc-49928, Santa Cruz Biotechnology, Santa Cruz, CA). Rabbit monoclonal anti-CLN5 antibody (ab170899, Abcam). Anti-mouse monoclonal and anti-goat polyclonal antibodies conjugated to AlexaFluor 488 and AlexaFluor 594 used for immunofluorescence were obtained from Life Technologies (Burlington, ON). The protease inhibitor cocktail was purchased from Sigma-Aldrich (Oakville, ON). ENDO H and PNGase F were obtained from New England Biolabs (Whitby, ON). Leupeptin, pepstatin, E64 were purchased from Sigma-Aldrich (Oakville, ON) and $(Z\text{-}LL)_2$-ketone from EMD Millipore (Etobicoke, ON).

Cell culture

HeLa, HEK293T and COS-7 cells were cultured in DMEM containing L-glutamine (Life Technologies, Burlington ON) supplemented with 10% Fetal Bovine Serum (FBS), 5% penicillin/streptomycin and maintained in 5% CO_2 at 37 °C. Cells were transfected using 1 µg of DNA per 10-cm^2 plate with Lipofectamine LTX Transfection Reagent (Life Technologies, Burlington ON) following the manufacturer's protocol or using linear 25kDa polyethylenimine (Polysciences, Inc., Warrington, PA) as previously described [29]. RNAi of SPPL3 was previously described [26]. Briefly, siGENOME SMARTpool siRNAs targeting human SPPL3 (M-006042-02-0005) and controls were purchased from Dharmacon. siRNAs were transfected using Lipofectamine RNAiMAX per manufactureres instructions (Invitrogen). Cells were analyzed on day 4 or 5 post-transfection.

Membrane separation Assay

24 hours post-transfection, cells were collected, snap frozen with liquid nitrogen and allowed to thaw at room temperature. Samples were resuspended in buffer 1 (0.1 M Mes-NaOH pH 6.5, 1 mM MgAc, 0.5 mM EGTA, 200 µM sodium orthovanadate, 0.2 M sucrose) and centrifuged at 10 000 g for 5 min at 4°C. The supernatant containing cytosolic proteins was collected (C, cytosolic fraction) and the pellet was resuspended in buffer 2 (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100) and spun at 10 000 g for 5 min at 4 °C.

The supernatant containing membrane proteins was collected (M, membrane fraction) for further analysis [30].

Sodium carbonate extraction

24 hours post-transfection, cells were collected and resuspended in ice-cold 0.1 M sodium carbonate at pH 11.5. Cells were passed 10 times through a 23G syringe and the samples incubated on ice for 30 min. Samples were then centrifuged at 100 000 g for 1 hr at 4 °C. The supernatant containing soluble proteins was collected and the pellet containing transmembrane proteins was resuspended in ice-cold 0.1 M sodium carbonate at pH 11.5. Samples were then analyzed by Western blot.

Membrane Protein Topology

24 hours post-transfection, cells were split into 3 groups. In group 1, the cells were lysed in ice-cold TNE (150 mM NaCl, 10 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.5% Triton X-100) containing protease inhibitors. In group 2, cells were treated with 20 mM digitonin in KHM buffer (110 mM potassium acetate, 20 mM HEPES and 2mM $MgCl_2$) on ice for 10 min prior to lysis in TNE. In group 3, cells were treated with digitonin followed by a 30 min treatment with trypsin (1/30 dilution of 0.25% Trypsin-EDTA (Life Technologies, Oakville ON) solution diluted in KHM) on ice prior to lysis in TNE.

Purification of CLN5 for mass spectrometry analysis

HEK 293T cells were seeded in 5 x 15 cm dishes and transfected with a plasmid coding for CLN5-HA. 24 hours post- transfection, cells were treated with 5 mM sodium butyrate for 24h to increase protein expression. Cells were then washed with PBS and detached with PBS/EDTA (5 mM) and lysed in lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 5 mM EDTA, 1% Triton X-100, 0.1% SDS) containing protease inhibitors for ~2 hours at 4 °C under gentle agitation. The lysates were clarified by centrifugation at 14 000 rpm for 30 minutes at 4 ºC and the supernatant was precleared with protein-G sepharose beads for 1h. A monoclonal antibody against the HA epitope and protein-G sepharose were added to the supernatant followed by an overnight incubation at 4 ºC. The precipitate was then washed five times in lysis buffer and the proteins eluted 1 h at room temperature in 50 µl of 2X SDS-PAGE loading buffer (125 mM Tris-HCl, pH 6.5, 5% SDS, 20% glycerol, 0.2% bromophenol blue, 10% β-mercaptoethanol). Proteins were then resolved on SDS-PAGE and the gel colored with coomassie blue. The band with a molecular weight corresponding to mature CLN5 was cut and sent for mass spectrometry analysis to the Proteomics facility at the Institute for Research in Immunology and Cancer (IRIC) where LC-MS/MS was performed. The results were analysed using Scaffold 4 software.

Western blotting

HeLa cells seeded in 6-well plates were transfected with the indicated plasmids. Approximately 24 hours after transfection, cells were washed with PBS then detached with PBS/EDTA (5 mM) and lysed in TNE buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.5% Triton X-100, 0.5%) containing protease inhibitors for 1 hour at 4 °C under gentle agitation. Lysates were clarified by centrifugation at 14 000 rpm for 15 minutes at 4 ºC

and the supernatants were mixed with sample buffer 4X to obtain a final concentration of 1X SDS-PAGE loading buffer (62.5 mM Tris-HCl pH 6.5, 2.5% SDS, 10% glycerol, 0.01% bromophenol blue). Proteins were then resolved on SDS-PAGE, transferred to nitrocellulose membranes and detected by immunoblotting using the indicated antibody.

Immunofluorescence, image analysis and quantification

COS-7 or HeLa cells were grown on glass coverslips for 24 hours and co-transfected with CLN5-HA and PSAP-CLN5-myc. 24 hours post-transfection, cells were fixed with 4 % paraformaldehyde in PBS for 15 min. Cells were subsequently incubated with polyclonal anti-HA and monoclonal anti-myc antibodies at 1:1000 dilutions in 0.1% BSA, 0.5 % saponin in PBS for 2 hours. Following two 5 min washes with PBS, the cells were incubated with anti-mouse AlexaFluor 488 and anti-rabbit AlexaFluor 594 for 1h in the same buffer as above. After two 5 min washes in PBS, the coverslips were mounted on slides using Fluoromount-G (SouthernBiotech, Birmingham, AL,) and imaged using an inverted microscope Olympus IX71 equipped with a confocal module (Thorlabs, Newton, NJ). Co-localization was assessed using the method described in [31, 32] using scripts programmed in Matlab (Mathworks, Natick, MA). First, thresholds for both channels are determined automatically so as the pixel intensity above them has positive Pearson's linear correlation coefficient. Finally, the co-localization is defined as the fraction of intensity in the HA channel that co-localizes with the myc channel above the intensity thresholds.

RESULTS

HA tagged CLN5 is glycosylated and cleaved into mature CLN5.

Prediction tools such as TOPCONs (http://topcons.cbr.su.se/) routinely find at least one transmembrane (TM) domain in CLN5 from residues 73 - 93 based on the 407 amino acid protein (Fig. 1A). CLN5 is first translated as a 407 amino acid protein with a predicted transmembrane domain (Fig. 1A, yellow box), while in lysosomes, CLN5 is found as a soluble luminal protein [8, 13]. In order to understand the translation and processing of CLN5, we expressed HA-CLN5 and CLN5-HA in HeLa cells and performed a Western blot (Fig. 1B). For HA-CLN5, we found bands slightly below 50 kDa (Fig. 1B, open arrowhead), slightly above 75 kDa (Fig. 1B, solid arrow) and slightly below 20 kDa (Fig. 1B, closed arrowhead). These bands correspond to uncleaved, unglycosylated CLN5 (preproCLN5, predicted molecular mass of 46.3 kDa, open arrowhead), uncleaved, glycosylated CLN5 (proCLN5, solid arrow) and the cleaved N-terminal fragment (ntfCLN5, solid arrowhead). When we expressed CLN5-HA, we found almost exclusively one band, slightly above 50 kDa (Fig. 1B, open arrow), corresponding to cleaved and glycosylated mature CLN5 (mCLN5, open arrow) with a small fraction of proCLN5 (Fig. 1B, solid arrow). To confirm that that preproCLN5 (open arrowhead) is the unglycosylated form of CLN5, we treated samples with either EndoH or PNGase F (Fig. 1C). As expected, EndoH or PNGase F treatment did not affect the molecular weight of preproCLN5, whereas proCLN5 was entirely converted in preproCLN5 after deglycosylation (Fig. 1C). We also observed a shift in molecular weight corresponding to the deglycosylated form of the mature protein in samples containing CLN5-HA. As predicted the band corresponding to preproCLN5 remained unchanged in all samples (Fig. 1C). These data suggest that HA tagged CLN5 can be glycosylated and cleaved in a similar fashion to endogenous CLN5.

CLN5 is initially translated as a type II transmembrane protein.

Recently, using a topology assay on isolated microsomal fractions, CLN5 was found to contain at least 1 transmembrane domain [21]. To confirm these results, we performed a topology assay in perforated intact cells rather than on isolated microsomal membranes. We expressed HA-CLN5, CLN5-HA, a soluble lysosomal protein, prosaposin-myc (PSAP-myc,) and a type I transmembrane protein (sortilin-myc) in HeLa cells. Following permeabilisation with digitonin, which induces pore formation in the plasma membrane but leaves internal membranes intact preventing the trypsin from digesting proteins within the lumen of organelles, the cells were incubated in cold diluted tryspin solution. As predicted, we found that PSAP-myc was not digested, while the cytosolic C-terminal tail of sortilin-myc was (Fig. 2A). We found that the N-terminal tail of CLN5 was digested (Fig. 2A) suggesting that it was in the cytosol and therefore accessible to tryspin. Conversely, mature CLN5 appears to be a soluble protein within the lumen of organelles as it was protected from tryspin digestion (Fig. 2A). This data supports a model that preproCLN5 (unglycosylated) and proCLN5 (glycosylated) are type II transmembrane proteins cleaved into a mature soluble protein.

Next, we performed a membrane separation assay to determine which form of CLN5 was membrane bound and which was soluble [30]. As preproCLN5 and proCLN5 presumably reside in the early secretory pathway, we focused our analysis using endoplasmic reticulum (ER) localized proteins. Our membrane separation was successful as the integral membrane protein calnexin was found almost exclusively in the membrane fraction (Fig. 2B) while the cytosolic protein α-tubulin was found in the cytosolic fraction (Fig. 2B). The soluble ER protein BiP was found in the membrane fraction, suggesting that our assay was isolating intact ER membranes (Fig. 2B). We found that the majority of HA-CLN5 was associated with the membrane fraction while a very small amount, was present in the soluble fractions (Fig. 2B). This suggested that preproCLN5 and proCLN5 are membrane associated and combined with data from our topology assay (Fig. 2A), supports the conclusion that preproCLN5 and proCLN5 are type II transmembrane domain proteins. Next we performed the assay using two truncated variants of CLN5 found in human disease, $CLN5^{Y392*}$ and $CLN5^{W75*}$, which correspond to the Finnish major mutation leading to a protein of 391 amino acids [18] and Finnish minor variant of the protein that leads to a truncated protein of 75 amino acids (truncated prior to its predicted transmembrane domain) [18]. As with wild-type protein, we found that HA-$CLN5^{Y392*}$ was significantly in the membrane fraction with very little found in the soluble fraction. This result suggests that the Finnish major mutant is initially translated as a transmembrane protein. In the case of the Finnish minor mutant, we found that HA-$CLN5^{W75*}$ was no longer associated with membranes and found most of the protein in the soluble fraction. The loss of membrane association infers that the first 75 amino acids comprised of the N-terminal region are not sufficient for CLN5 membrane interaction or for its internalization into the lumen of organelles suggesting that this region does not contain a signal peptide. Our results strongly suggest wild-type preproCLN5, proCLN5 and proCLN5^{Y392*} are integral membrane proteins. We next performed a membrane separation assay using CLN5-HA. As expected, when internal endosomal and lysosomal membranes are left intact, CLN5-HA is found in the membrane fraction suggesting that mCLN5 is found within a membrane compartment, presumably the lysosomal compartment (Fig. 2C). However, when membranes are linearized with Na_2CO_3, CLN5-HA, along with the soluble lysosomal proteins cathepsin D and prosaposin-myc, are

released into the soluble fraction (Fig. 2C). This result is in stark contrast to a previous publication showing that mCLN5 is tightly membrane bound [21]. However, in that study, internal soluble lysosomal proteins such as cathepsin D and prosaposin were not used as control, so it is possible that the NA_2CO_3 treatment used was not sufficient to rupture internal membranes or that their centrifugation protocol lead to the isolation of protein complexes. Our data strongly supports our hypothesis that preproCLN5 and proCLN5 are transmembrane forms of CLN5 and that mCLN5 is a soluble protein found inside the lumen of organelles.

CLN5 is cleaved after residue arginine 92 to produce a soluble mature protein.

It is clear that proCLN5 is cleaved to produce a mature soluble protein. The exact site of cleavage has never been determined although experimental evidence points to residue 96 [8]. To verify whether or not cleavage occurs at residue 96, we expressed CLN5-HA in HEK 293T cells and purified the protein using immunoprecipitation with anti-HA antibodies and performed mass spectrometry analysis on the purified band (Fig. 3A). A list of the peptides recovered with their corresponding abundance is shown (Fig. 3B). Mass spectrometry analysis identified CLN5 fragments beginning from residue 93 suggesting that CLN5 is cleaved prior to this residue. Mouse CLN5 is thought to consist of only 358 amino acids rather than the 407 found in humans [33]. In this shorter form, SignalIP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) predicts a cleavage site at precisely between arginine 92 and valine 93 that was not predicted in the 407 amino acid form of the protein. Furthermore, as these two amino acids are the predicted to be near the membrane interface with the organelle lumen, it appears that cleavage occurs nears this interface to release mCLN5. However, due to the nature of the mass spectrometry analysis, we are not able to completely exclude cleavage at residues upstream from this site, as the smallest fragment we can recover is 5 amino acids and trypsin digestion for mass spectrometry analysis cleaves at arginine residues. Therefore, our results support that mature soluble CLN5 appears to include residues 93 - 407 (Fig. 3C) producing a 314 amino acid mature protein but could possibly include residues 89 - 407. Other analysis would be required to resolve this discrepancy.

CLN5 cleavage is inhibited by $(Z\text{-}LL)_2$-ketone.

Since proCLN5 is a type II transmembrane protein and cleavage occurs within the membrane, we treated cells with $(Z\text{-}LL)_2$-ketone that is known to inhibit the function of most of the SPP/SPPL family members who are known to cleave type II transmembrane domain proteins [23]. We tested the effects of $(Z\text{-}LL)_2$-ketone and other inhibitors on the processing of CLN5 from proCLN5 with an apparent molecular weight above 75 kDa to mature CLN5 (mCLN5) with a molecular weight above 50 kDa. We tested leupeptin (a cysteine, serine and threonine peptidases inhibitor), pepstatin (an aspartyl protease inhibitor), E64 (a cysteine protease inhibitor) and 3 concentrations of $(Z\text{-}LL)_2$-ketone (an inhibitor of the SPP/SPPL family) in HeLa cells transfected with CLN5-HA. We found that only $(Z\text{-}LL)_2$-ketone prevented the cleavage of CLN5 from proCLN5 (Fig. 4A, solid arrow) to mCLN5 (Fig. 4A, open arrow) as the other inhibitors had no effect (Fig. 4A). To verify that the band appearing at 75kDa was in fact *de novo* synthesized proCLN5 that was not cleaved, we performed a cycloheximide chase experiment in the presence of the inhibitor $(Z\text{-}LL)_2$-ketone, this time using both HA-CLN5 and CLN5-HA (Fig. 4B). In cells treated with cycloheximide, we found no proCLN5 (Fig. 4B, solid arrow) in CLN5-HA transfected cells suggesting that the band appearing at 75 kDa was indeed newly synthesized

proCLN5 that was not processed to mCLN5. Since CLN5 was not processed in $(Z\text{-}LL)_2$-ketone treated cells, we hypothesized that HA-CLN5 would not be cleaved and we would therefore expect an increase in the proCLN5 at 75 kDa and a decrease in the N-terminal cleaved fragment below 20 kDa. We found an increase in proCLN5 in the cells expressing HA-CLN5 treated with the $(Z\text{-}LL)_2$-ketone (Fig. 4B, solid arrow), but to our surprise, we also found more of the N-terminal fragment (ntf) (Fig. 4B, solid arrowhead), suggesting that SPP/SPPL proteases could also be involved in the cleaving and degradation of the N-terminal fragment (ntf) by generating an intracellular domain (icd) that could be degraded by the proteasome. To test if the cleavage of ntfCLN5 into an icdCLN5 fragment leads to its degradation, HeLa cells were transfected with HA-CLN5 and treated with different concentrations or a fixed concentration for different amounts of time with the proteasome inhibitor MG132. ntfCLN5 was visible as a slight band in non-treated cells (Fig 4C). Treatment with MG132 dramatically increased the level of a band slightly smaller than ntfCLN5 suggesting the possibility of a further cleavage event (Fig. 4C, solid arrowhead) even though it had very little effect on the preproCLN5 (Fig. 4C, open arrowhead) and proCLN5 (Fig. 4C, solid arrow). Moreover, a significant increase in the level of the new band is already observable after only 30 min of MG132 treatment, showing that this new cleavage product is rapidly degraded (Fig. 4C, solid arrowhead, right panel). We hypothesized that the higher molecular weight fragment was ntfCLN5 within the membrane, while the smaller fragment, icdCLN5, was cleaved from ntfCLN5 and degraded in the proteasome. To test this experimentally, we performed a membrane assay on HeLa cells that had been transfected with HA-CLN5 and treated with MG132 (Fig. 4D, solid arrow). Indeed, we found the higher molecular weight ntfCLN5 in the membrane fraction (M), while in the cytosolic fraction (C), we found icdCLN5 that was not degraded due to the MG132. This suggests that ntfCLN5 is further cleaved resulting in the release of a soluble icdCLN5 that is subsequently rapidly degraded by the proteasome.

SPPL3 cleaves proCLN5 into mCLN5 in the early secretory pathway.

Several mutations are thought to retain CLN5 in the early secretory pathway [34], including R112P [8]. In at least 4 mutations that result in the retention of the protein, CLN5 is still cleaved into mCLN5 suggesting cleavage occurs prior to exit from the Golgi apparatus [7]. First, we performed a Western blot to determine the cleavage pattern of HA-CLN5, HA-$CLN5^{R112P}$, CLN5-HA and $CLN5^{R112P}$-HA. We found that HA-$CLN5^{R112P}$ and $CLN5^{R112P}$-HA showed the same expression pattern, glycosylation (based on molecular mass) and processing as HA-CLN5 and CLN5-HA (Supp. Fig 1), suggesting that cleavage occurs in the early secretory pathway prior to Golgi exit of mCLN5.

The members of the SPP/SPPL family of proteases function at various intracellular locations. As such, SPP and SPPL2c are localized to the ER, SPPL2a is found in the lysosomal compartment [35], SPPL2b at the plasma membrane and SPPL3 is localized to the ER and Golgi apparatus [23, 36]. Most SPP/SPPL family members require cleavage by a sheddase prior to their function [27]. Since cleavage from proCLN5 to mCLN5 was inhibited by $(Z\text{-}LL)_2$-ketone, it is most likely performed by a member of the SPP/SPPL family or a protease/protease cascade that is activated by a member of the SPP/SPPL family. SPPL3 has been shown to function without the requirement for prior cleavage by a sheddase [26]. Therefore, in order to test whether SPPL3 is the protease implicated in cleaving proCLN5 to mCLN5, we co-transfected HeLa cells with CLN5-FLAG and either wild-type HA-SPPL2b, HA-SPPL3 or their catalytically inactive forms

(HA-SPPL2b^{D421A} and HA-SPPL3^{D272A}). 24-hours post-transfection, we performed a Western blot using antibodies to either FLAG or HA. CLN5-FLAG was cleaved to mCLN5 in the cells co-transfected with HA-SPPL2b, HA-SPPL2b^{D421A} and HA-SPPL3 (Fig. 5A, open arrow). However, in cells co-transfected with HA-SPPL3^{D272A}, we found an accumulation of proCLN5 not found in the other co-transfections (Fig. 5A, solid arrow). To confirm this data, we depleted SPPL3 from HEK 293T cells using siRNA. The depletion of SPPL3 was efficient when tested by Western blotting (Fig. 5B). In cells depleted of SPPL3, we found an accumulation of proCLN5 Fig. 5B, solid arrow) in cell lysates not found in the mock-depleted cells (Fig. 5B). In addition to a block in cleavage, mCLN5 also appeared at a slightly higher molecular weight most likely due to hyperglycosylation induced by reduced SPPL3 expression [37].

CLN5 does not contain a classic signal sequence

Classical signal sequences are usually 15 to 20 amino acids long [38] and are usually cleaved during translation. CLN5 does not appear to have a classical signal sequence as cleavage occurs after residue 92 and more than likely after insertion into the membrane since we observe both HA-CLN5 and CLN5-HA by Western blotting. We therefore generated a construct that switched the N-terminal portion of CLN5 with that of prosaposin (PSAP) fusing residues 1 - 28 of prosaposin with residues 94 - 407 of CLN5. PSAP is a soluble lysosomal protein whose trafficking requires sortilin [39]. The N-terminal portion of PSAP contains a classical signal peptide 16 residues long cleaved from the mature protein presumably by signal peptidase during translation [40] and not SPPL3 or a related SPPL family member. First, we determined whether the PSAP/CLN5 chimera would enter the lumen of organelles. We transfected HeLa cells with PSAP-myc, PSAP/CLN5-myc and CLN5-HA. We found that all 3 constructs entered the secretory pathway, as expected, as they were localized to the membrane fraction in the standard assay suggesting that they were luminal proteins (Fig. 5C). Next, we sought to determine whether or not switching the N-terminal portion of CLN5 for the signal peptide of prosaposin affected its intracellular distribution. We tested the ability of the PSAP/CLN5-myc (Supp. Fig. 2A, green) chimera to co-localize with CLN5-HA (Supp Fig. 2B, red) in COS-7 and HeLa cells. COS-7 cells were chosen for their large cytoplasm, allowing for a clearer distinction between different cytoplasmic organelles. We found the PSAP/CLN5-myc chimera is in the same structures as CLN5-HA (Supp Fig. 2C) suggesting that the N-terminal portion of CLN5 was not required for its localization. In fact, co-localization analysis showed that CLN5-HA co-localized with PSAP/CLN5-myc 96.2% (S.D. +/- 3.1) of the time in COS-7 cells (Supp Fig. 2D, black bar). Significant co-localization between CLN5-HA and PSAP/CLN5-myc was also observed in HeLa cells. Image analysis revealed that CLN5-HA co-localized with PSAP/CLN5-myc 96.1% (S.D. +/- 4.6) of the time (Supp Fig. 2D, white bar). This data suggests that CLN5 is sorted and transported to the lysosomal compartment post-cleavage by a mechanism independent of its N-terminal fragment.

Next, we tested whether PSAP/CLN5-myc chimera was cleaved by SPPL3. We co-transfected HeLa cells with PSAP/CLN5-myc and HA-SPPL3 or HA-SPPL3^{D272A} and found no difference in the cleavage of PSAP/CLN5-myc (Fig. 5D). This suggested that the classical signal peptide of the chimera was cleaved co-translationally by another mechanism, presumably signal peptidase within the ER and not by SPPL3. Furthermore, this supports a role for SPPL3 in cleaving CLN5 and not affecting it glycosylation. If the effects observed were due to glycosylation, expressing wild-type SPPL3 would result in a lower molecular weight band for

both wild-type CLN5 as well as the PSAP/CLN5 chimera due to decreased glycosylation as previously observed for other proteins [37]. This indicates that SPPL3 either directly cleaves CLN5 into proCLN5 and ntfCLN5 after translation and insertion into the membrane as a type II transmembrane domain or induces its cleavage by activating other proteases.

SPPL3 and SPPL2a/b sequentially cleave the foamy virus envelope protein [32]. Therefore we tested if SPPL2b and/or SPPL3 cleave ntfCLN5 by overexpressing WT or catalytically dead forms of these proteins with FLAG-CLN5 in HeLa cells. Overexpression of HA-SPPL3^{D272A} significantly increased the amount of ntfCLN5 whereas HA-SPPL2b^{D421A} only had a moderate effect (Fig. 5E, solid arrowhead) suggesting that both these enzyme may be implicated in the cleavage of ntfCLN5 (Fig. 5E). To verify the existence of the ntfCLN5 and icdCLN5, we expressed HA-CLN5 in HEK 293T cells and treated the cells with DMSO, 10μM MG132 for 30 minutes, 25 μM (Z-LL)$_2$-ketone for 4 hours or co-expressed HA-SPPL3^{D272A} and performed a Western blot with anti-HA antibody (Figure 5F). In the MG132 treated cells, we found a band that was slightly smaller (icdCLN5) than the band recovered in the (Z-LL)$_2$-ketone treated cells or cells expressing HA-SPPL3^{D272A} (ntfCLN5). This suggests that SPPL3 and possibly other SPPL family members are implicated in the cleavage of ntfCLN5 to icdCLN5, which is released into the cytosol and degraded in the proteasome.

Discussion

Several conclusions can be drawn from the data presented in this paper. First, we confirmed that CLN5 is initially translated as a type II transmembrane protein in intact cells and is subsequently cleaved into a mature soluble protein. Second, we demonstrate that a member of the SPP/SPPL family, most likely SPPL3 mediates this cleavage after residue 92. Finally, we show that the N-terminal fragment is not implicated in the intracellular localization of CLN5 and is further processed by members of the SPP/SPPL family, to allow its degradation in the proteasome.

CLN5 contains a hydrophobic region that is predicted to be a transmembrane domain between residues 73 and 93. A previous study using isolated microsomes found CLN5 is a type II transmembrane domain protein [21]. We confirmed this result using a topology assay in intact cells gently permeabilised with digitonin enabling us to test the topology of CLN5 without isolating microsomes using high-speed centrifugation, leaving CLN5 in a more physiological condition. We found that preproCLN5 and proCLN5 are type II transmembrane domain proteins while mature CLN5 (mCLN5) is within the lumen of organelles and localizes to the lysosomal compartment. A recent study also demonstrated a further processing step as mCLN5 is processed in the lysosomal compartment as leupeptin blocked this final processing step [41]. Next to determine whether mCLN5 was soluble or membrane bound, we performed a Na_2CO_3 extraction and found that a significant portion of mCLN5 is soluble. This result differs from a previous publication that found mCLN5 to be tightly membrane bound [21]. However, those previous experiments lacked a soluble lysosomal protein as a control. In the previous paper, the authors showed that two peripheral membrane proteins the endosomal protein EEA1 and the Golgi GM130 were released into the cytosolic fraction, but not mCLN5. They did not provide direct evidence that their assay resulted in the release of soluble lysosomal proteins such as cathepsin D or prosaposin.

The hydrophobic region (amino acids 73 - 93) is not an actual transmembrane domain. It appears to serve as a signal anchor allowing the insertion of the C-terminal portion of CLN5

(amino acids 93 - 407) into the lumen of the ER [40]. Once inserted into the membrane, CLN5 is subsequently cleaved by a member of the SPP/SPPL protease family or a protease that is activated by one of these intramembrane aspartyl proteases. Our observation of the 2 forms of CLN5 depending on the placement of the HA tag (HA-CLN5 versus CLN5-HA) would suggest that cleavage of the overexpressed protein occurs inefficiently or after translocation and membrane insertion. If cleavage, under physiological conditions were also inefficient, it would be possible that CLN5 as a type II transmembrane protein could have biological activity in the ER or early Golgi, or that ntfCLN5 could have a biological function.

To identify the mechanism of cleavage of CLN5, we treated cells with a variety of inhibitors including (Z-LL_2)-ketone, which was previously shown to inhibit the SPP/SPPL, which specifically cleave type II transmembrane proteins. Not only did the inhibitor prevent cleavage from proCLN5 to mCLN5, it also blocked the degradation of ntfCLN5. Using catalytically dead mutants of SPPL2b and SPPL3, we found that SPPL3 affects the cleavage from proCLN5 to mCLN5, but SPPL2b does not. This is consistent with previous observations showing that SPPL3 is the only member of the SPPL family that does not require prior shedding for proteolysis [32]. However, SPPL3 is also capable of regulating cellular glycosylation processes, by cleavage of various glycosyltransferases [37]. This could also potentially account for the altered molecular weight of mCLN5 upon SPPL3 knock down.

SPPL3 is also capable of cleaving ntfCLN5 but our results suggest that SPPL2b and maybe another member(s) of the SPP/SPPL family could also be involved in the cleavage of ntfCLN5. Presumably, the cleavage of ntfCLN5 leads to its degradation as inhibition of the proteasome with MG132 rapidly increases the level of ntfCLN5. It is unclear at this point if ntfCLN5 has a cellular function but it does not appear to be implicated in the localization of CLN5 as replacing the N-terminal portion of CLN5 with the signal peptide of prosaposin, did not appear to affect it intracellular distribution. In fact, we found that CLN5-HA and PSAP/CLN5-myc co-localized more than 95% of the time within cells. However, based on our data, it appears that the ntf of CLN5 is a signal anchor and not a classical signal peptide. This is supported by previous work highlighting the cleavage site of GFP-CLN5 expressed in cells [8].

CLN5 most likely represents an additional novel SPPL3 substrate. However, we cannot fully exclude that SPP/SPPL proteases activate other proteases that cleave CLN5 in the ER/Golgi to generate its soluble variant mCLN5. Using mass spectrometry analysis of purified mature CLN5, we were able to identify the site of cleavage after residue 92, which is the predicted cleavage site of mouse CLN5. In mice and some other species, CLN5 is though to be 358 amino acids long, while in humans the longest form is 407 amino acids. ntfCLN5 in mice is predicted to be 42 amino acids with a predicted cleavage site between residues 42 and 43. This would be a highly usual signal peptide in terms of length, and may well be a target of SPPL3, but at this time we are not able to eliminate this possibility. Contrary to other aspartic proteases, no consensus cleavage sequence has been described for the SPP family of proteases. It has been suggested that the α-helical conformation of transmembrane domains (TMDs) hides peptide bonds from proteases and that intramembrane proteolysis requires the presence of helix destabilizing amino acids in the TMD. Indeed, reducing the α-helical content of the Bri2 TMD increases its cleavage by SPPL2b [25]. We have confirmed that in contrast to other SPP proteases, SPPL3 cleaves full-length proteins without prior shedding. Consequently, its mechanism of substrate recognition is likely to differ from other SPPs and further mutational analysis is required to identify the structural determinants responsible for the cleavage specificity.

Conclusions

In conclusion, we have shown that SPPL3 directly or indirectly mediates the cleavage of proCLN5 into mCLN5 extending our knowledge of the biology of this protein implicated in human disease.

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Author contributions

FJ, ES, JM, SC, MHK, RF and SL designed and planned experiments. FJ, ES, JM, MHK and KDD performed experiments. FJ, ES, SC, RF and SL interpreted results, analyzed data and wrote the manuscript.

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FIGURE LEGENDS

Figure 1: HA tagged CLN5 is cleaved and glycosylated.
(A) Schematic representation of CLN5 tagged at the N-terminal end (HA-CLN5) or the C-terminal end (CLN5-HA). Highlighted in yellow is the hydrophobic region predicted to be a possible transmembrane domain (residues 73-93). proCLN5 is represented as the full-length protein composed of 407 amino acids (solid arrow), the cleaved N-terminal fragment (ntfCLN5, solid arrow head) and mature CLN5 (mCLN5, open arrow). (B) HeLa cells were transfected with HA-CLN5 or CLN5-HA. 24 hours post-transfection, the cells were lysed in TNE and the

samples run on a 12% SDS-PAGE and analyzed by Western blot (Wb) with an antibody against HA. The bottom of the gel was overexposed to increase the visibility of the N-terminal fragment (ntfCLN5). The solid arrow indicates proCLN5 (fully glycosylated), solid arrowhead indicates ntfCLN5open arrow indicates mCLN5 and the open arrowhead represents preproCLN5 (unglycosylated). (C) HeLa cells were transfected with HA-CLN5 or CLN5-HA. 24 hours post-transfection, the cells were lysed in TNE and the cell lysates were treated with either EndoH or PNGase F and subsequently run on SDS-PAGE and Western blotted (Wb) with anti HA antibodies.

Figure 2: CLN5 is a type II transmembrane protein cleaved to a soluble protein.
(A) HeLa cells were transfected with HA-CLN5, CLN5-HA, prosaposin-myc (PSAP-myc) or sortilin-myc. 24 hours post-transfection, cells were split into 3 groups. In group 1, the cells were lysed in TNE. In group 2, cells were treated with 20 mM digitonin in KHM buffer on ice for 10 min prior to lysis. In group 3, cells were treated with digitonin followed by a 30 min treatment with trypsin diluted in KHM on ice prior to lysis. (B) HeLa cells were transfected with HA-CLN5, HA-CLN5^{Y392*} or HA-CLN5$^{W75*.}$ 24 hours post- transfection, a membrane separation assay (see materials and methods) was performed. Equal volumes of cytosolic and membrane fractions were run on a 12% SDS-PAGE and then analyzed by Western blot (Wb). Nitrocellulose membranes were sequentially stained with the following antibodies: mono-HA, poly-calnexin (an ER transmembrane protein), poly-BiP (a soluble ER protein) and poly-α-tubulin (a cytosolic protein) C: soluble cytosolic fraction, M: membrane fraction. (C) Cells were transfected with either HA-CLN5 or CLN5-HA and membranes were isolated by performing a membrane separation assay or sodium carbonate assay (see material and methods). Nitrocellulose membranes were sequentially stained with the following antibodies: mono-HA, and poly-Lamp2 (a lysosomal transmembrane protein), mono-myc and poly-cathepsin D (proCatD = proCathepsin D, mCatD = mature Cathepsin D). C: cytosolic fraction, M: membrane fraction.

Figure 3: CLN5 is cleaved after residue 92
(A) HEK 293T cells were transfected with CLN5-HA. 48 hours post-transfection, the cells were lysed in TNE and an immunoprecipitation with anti-HA antibody was performed. The post-IP protein eluted from the sepharose beads was run on SDS-PAGE and coomassie stained. (B) The band from A was cut out of the gel and sent for mass spectroscopy analysis at the proteomics facility of the Institute for Research in Immunology and Cancer (IRIC), University of Montreal. The CLN5 fragments recovered are listed with their abundance. (C) CLN5 amino acids sequence with the residues detected by mass spectrometry highlighted in green. This suggests that CLN5 is cleaved after residue 92 (red box) to produce a mature soluble protein. (D) HeLa cells were transfected with various arginine mutants of CLN5 to test if these residues were required for cleavage. 24 hours post-transfection, the cells were lysed in TNE and an SDS-PAGE was performed. Western blotting (Wb) with anti-HA was done.

Figure 4: (Z-LL)$_2$-Ketone prevents the cleavage of proCLN5 to mCLN5
(A) HeLa cells were transfected with CLN5-HA and subsequently treated with leupeptin, pepstatin, E64 or (Z-LL)$_2$-ketone at the concentration indicated for either 0, 3 or 6 hours. Following cell lysis, the lysates were run on a 12% polyacrylamide gel, transferred to nitrocellulose and blotted (Wb) with anti-HA antibody. (B) HeLa cells were transfected with either HA-CLN5 or CLN5-HA and treated with (Z-LL)$_2$-ketone with or without cycloheximide

for 0, 3 or 6 hours. Total cell lysate was subsequently run a 12% SDS-PAGE and Western blotted (Wb) with anti-HA or anti-actin antibody. (C) HeLa cells were transfected with HA-CLN5 and treated with the indicated concentrations of MG132 for 4hrs (left panel) or with 5 μM MG132 for the indicated time periods (right panel). Total cell lysate was subsequently run a 12% SDS-PAGE and Western blotted (Wb) with anti-HA. (D) HeLa cells were transfected with HA-CLN5. 24Hrs post-transfection, cells were treated (+MG132) with 5μm MG132 for 1 hour and a membrane assay was performed. The cytosolic fraction and membrane fraction were run on a 12% polyacrylamide gel and Western blotting (Wb) performed with anti-HA antibody. C: cytosolic fraction, M: membrane fraction.

Figure 5: SPPL3 cleaves proCLN5 to mCLN5

(A) HeLa cells were co-transfected with CLN5-Flag and HA-SPPL2b, HA-SPPL2b^{D421A}, HA-SPPL3 or HA-SPPL3^{D272A}. 24 hours post-transfection, cells were lysed in TNE and samples run on a SDS-PAGE. Western blotting (Wb) was performed with anti-FLAG and anti-HA antibodies. (B) siRNA was used to deplete SPPL3 in HEK 293T. Cells were transfected with HA-CLN5 and a Western blot (Wb) was performed with anti-CLN5 and anti-SPPL3 antibodies. Wb of calnexin was used as a loading control. (C) HeLa cells were transfected with PSAP-myc, PSAP/CLN5-myc or CLN5-HA. A membrane separation assay was performed to determine their distribution. C: cytosolic fraction, M: membrane fraction. (D) HeLa cells were co-transfected with PSAP/CLN5-myc and HA-SPPL3 or HA-SPPL3^{D272A}. 24 hours post-transfection, cells were lysed and run on SDS-PAGE followed by Western blotting (Wb) with anti-myc and anti-HA antibodies. (E) HeLa cells were co-transfected with Flag-CLN5 and HA-SPPL2b, HA-SPPL2b^{D421A}, HA-SPPL3 or HA-SPPL3^{D272A}. 24 hours post-transfection, cells were lysed in TNE and samples run on a SDS-PAGE. Western blotting (Wb) was performed with anti-FLAG and anti-HA antibodies. (F) HEK 293T cells were transfected with HA-CLN5 and treated with DMSO, 10μM MG132 for 30 minutes, 25 μM (Z-LL)$_2$-ketone for 4 hours or co-expressed HA-SPPL3^{D272A}. Total cell lysates were run on a 12% polyacrylamide gel and a Western blot (Wb) with anti-HA antibody was performed. (G) Schematic representation of the cleavage of proCLN5 after membrane insertion by SPPL3 into mCLN5 and ntfCLN5. icdCLN5 is cleaved from ntfCLN5 and degraded by the proteasome.

Supplemental Figure 1: CLN5^{R112P} is processed like wild-type CLN5.

HeLa cells were transfected with HA-CLN5, HA-CLN5^{R112P}, CLN5-HA or CLN5^{R112P}-HA. 24 hours post-transfection, cells were lysed and run on a SDS-PAGE. Western blotting (Wb) was performed with anti-HA antibody.

Supplemental Figure 2: The N-terminal tail of CLN5 is not required for its localization.

(A - C) COS-7 cells grown overnight on glass coverslips were transfected with CLN5-HA and PSAP/CLN5-myc, fixed in 4% paraformaldehyde and immunostained with monoclonal anti-myc (A, green) and polyclonal anti-HA (B, red) antibodies. The merge of the image is shown in C. Scale bar = 10μm. (D) Protein co-localization was quantitatively analyzed in COS-7 and HeLa cells. 44 and 41 cells were analyzed respectively. Error bar represents standard deviation.

Figure 1

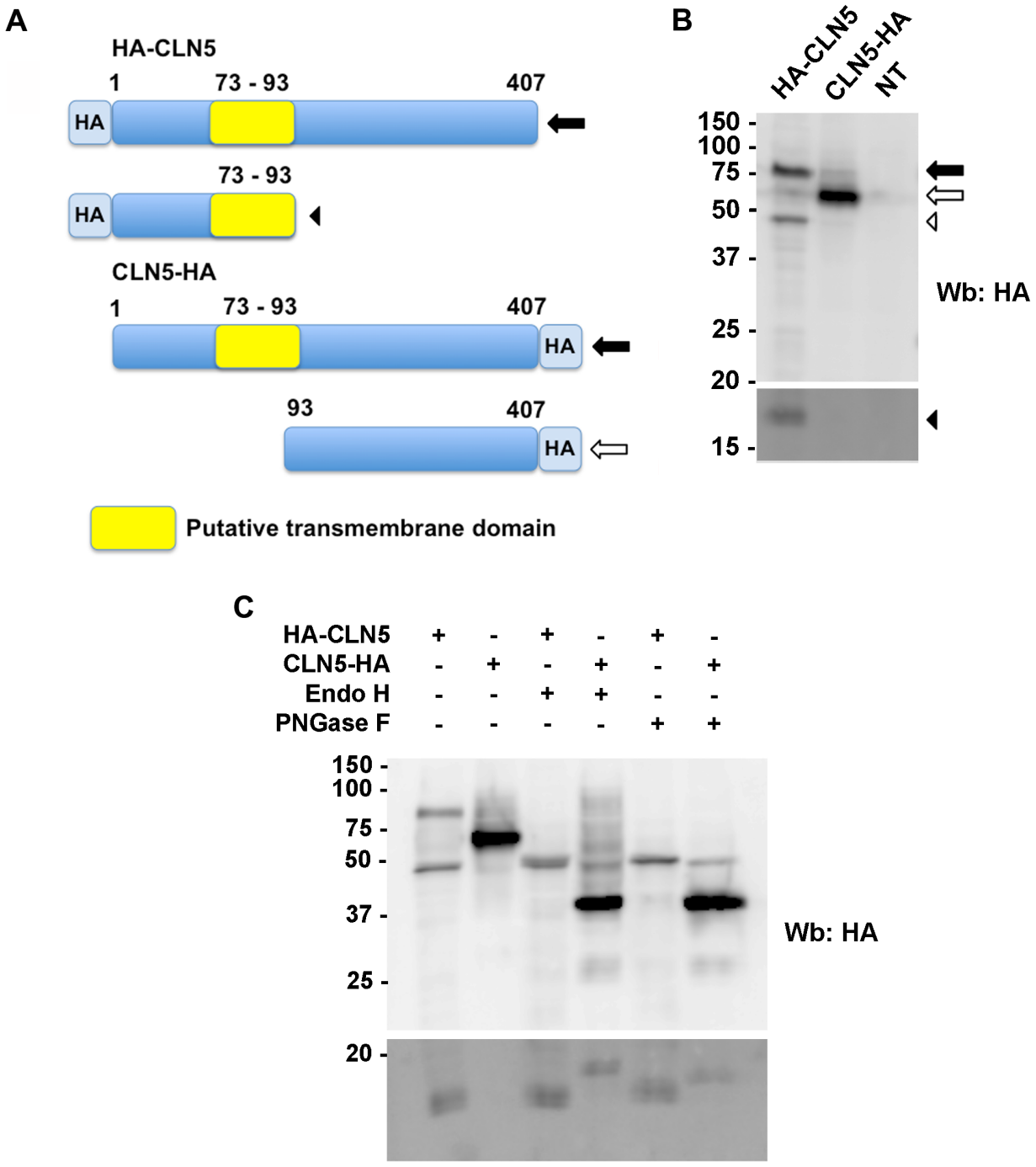

A
HA-CLN5
1
73 - 93
407
HA
73 - 93
HA
CLN5-HA
1
73 - 93
407
HA
93
407
HA
Putative transmembrane domain
B
HA-CLN5
CLN5-HA
NT
150
100
75
50
37
25
20
15
Wb: HA
C
HA-CLN5 + - + - + -
CLN5-HA - + - + - +
Endo H - - + + - -
PNGase F - - - - + +
150
100
75
50
37
25
20
Wb: HA

Figure 2

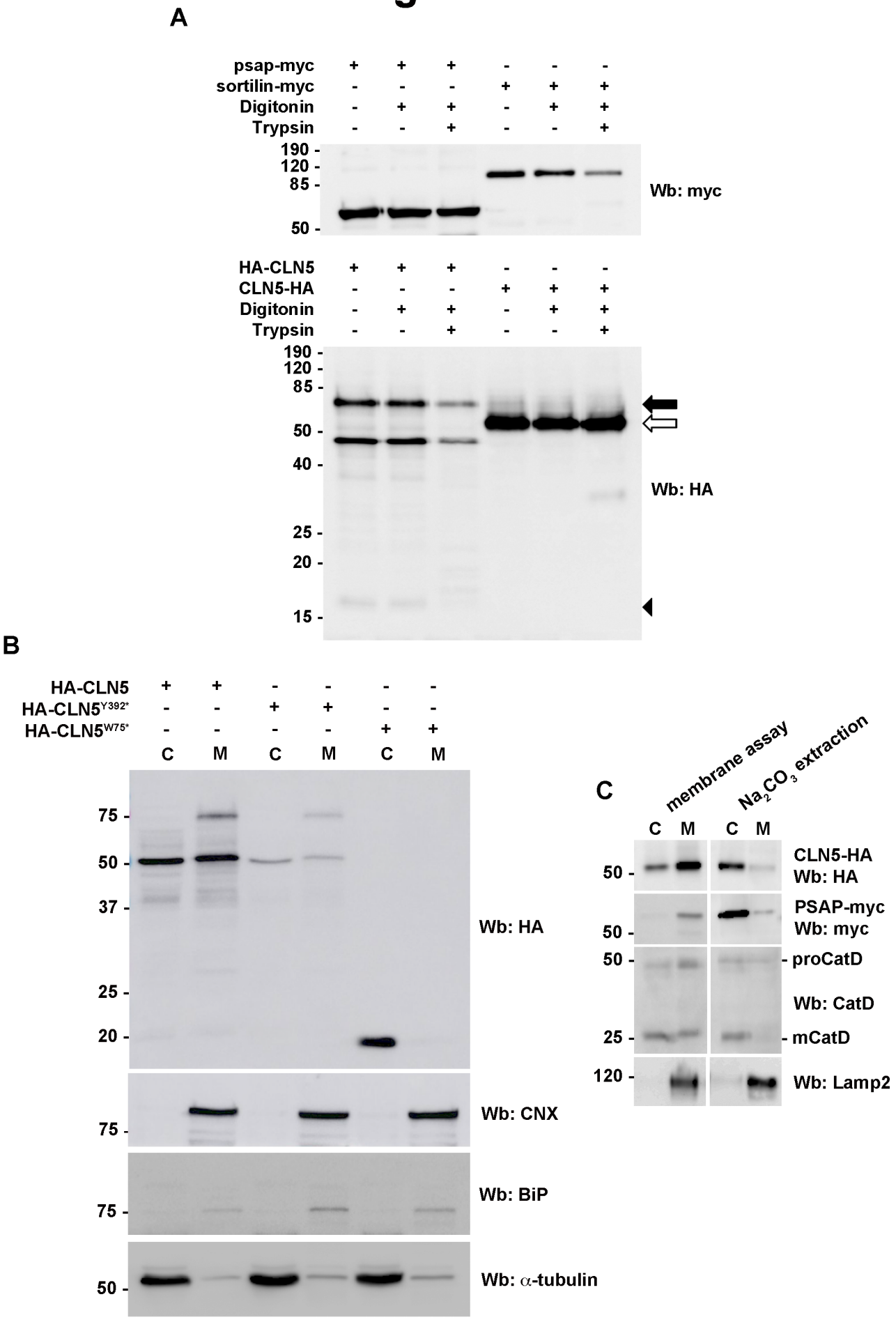
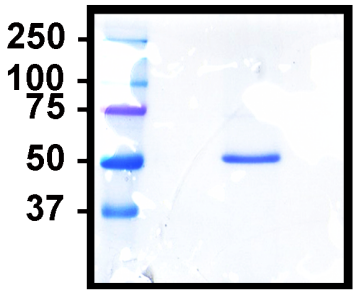

A
psap-myc + + + - - -
sortilin-myc - - - + + +
Digitonin - + + - + +
Trypsin - - + - - +
190
120
85
50
Wb: myc
HA-CLN5 + + + - - -
CLN5-HA - - - + + +
Digitonin - + + - + +
Trypsin - - + - - +
190
120
85
50
40
25
20
15
Wb: HA
B
HA-CLN5 + + - - - -
HA-CLN5Y392* - - + + - -
HA-CLN5W75* - - - - + +
C M C M C M
75
50
37
25
20
Wb: HA
75
Wb: CNX
75
Wb: BiP
50
Wb: α-tubulin
C
membrane assay
Na2CO3 extraction
C M C M
50
CLN5-HA
Wb: HA
50
PSAP-myc
Wb: myc
50
proCatD
Wb: CatD
25
mCatD
120
Wb: Lamp2

Figure 3

A



CLN5-HA

B

Peptides Recovered	#	Peptides Recovered	#
VSGIPSR	1	NIETNYTR	4
VSGIPSRR	2	TLGLAIK	16
HWPVPYK	11	TLGLAIKR	3
FDFRPKPDPYCQAK	5	FYYPFKPHLPTK	3
YTFCPTGSPIPVMEGDDDIEVFR	8	FYYPFKPHLPTKEFLLSLLQIFDAVIVHK	13
LQAPVWEFK	23	EFLLSLLQIFDAVIVHK	7
YGDLLGHLK	25	EFLLSLLQIFDAVIVHKQFYLFYNFEYWFLPMK	4
IMHDAIGFR	50	QFYLFYNFEYWFLPMK	8
GAETWFDSYDCSK	12	QFYLFYNFEYWFLPMKFPFIK	1
LAEFGAEFK	45	ITYEEIPLPIR	61
		ITYEEIPLPIRNK	9

C

MRRNLRLGPS SGADAQGQGA PRPGLAAPRM
LLPPASQASR GSGSTCSLM AQEVDTAQGA
EMRRGAGAAR GRASWCWALA LLWLAVVPGW
SRVSGIPSRR HWPVPYKRFD FRPQPDPYCQ
AKYTFCPTGS PIPVMEGDDD IEVFRLQAPV
WEFKYGDLLG HLKIMHDAIG FRSTLTGKNY
TMEWYELFQL GNCTFPHLRP EMDAPFWCNQ
GAACFFEGID DVHWKENGTL VQVATISGNM
FNQMAKWVKQ DNETGIYYET WNVKASPEKG
AETWFDSYDC SKFVLRTFNK LAEFGAEFKN
IETNYTRIFL YSGEPTYLGN ETSVFGPTGN
KTLGLAIKRF YYPFKPHLPT KEFLLSLLQI
FDAVIVHKQF YLFYNFEYWF LPMKFPFIKI
TYEEIPLPIR NKTLSGL

Figure 4

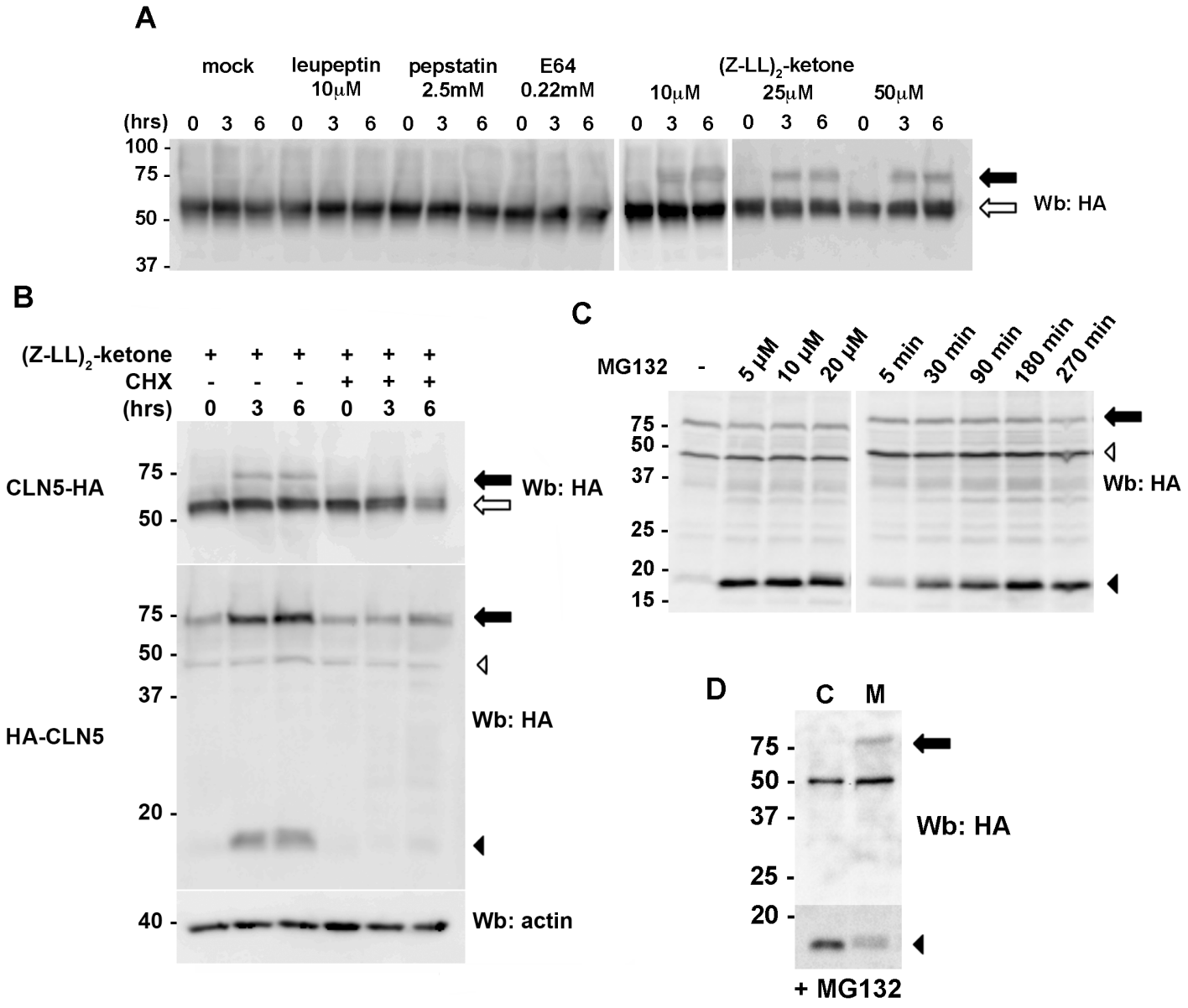
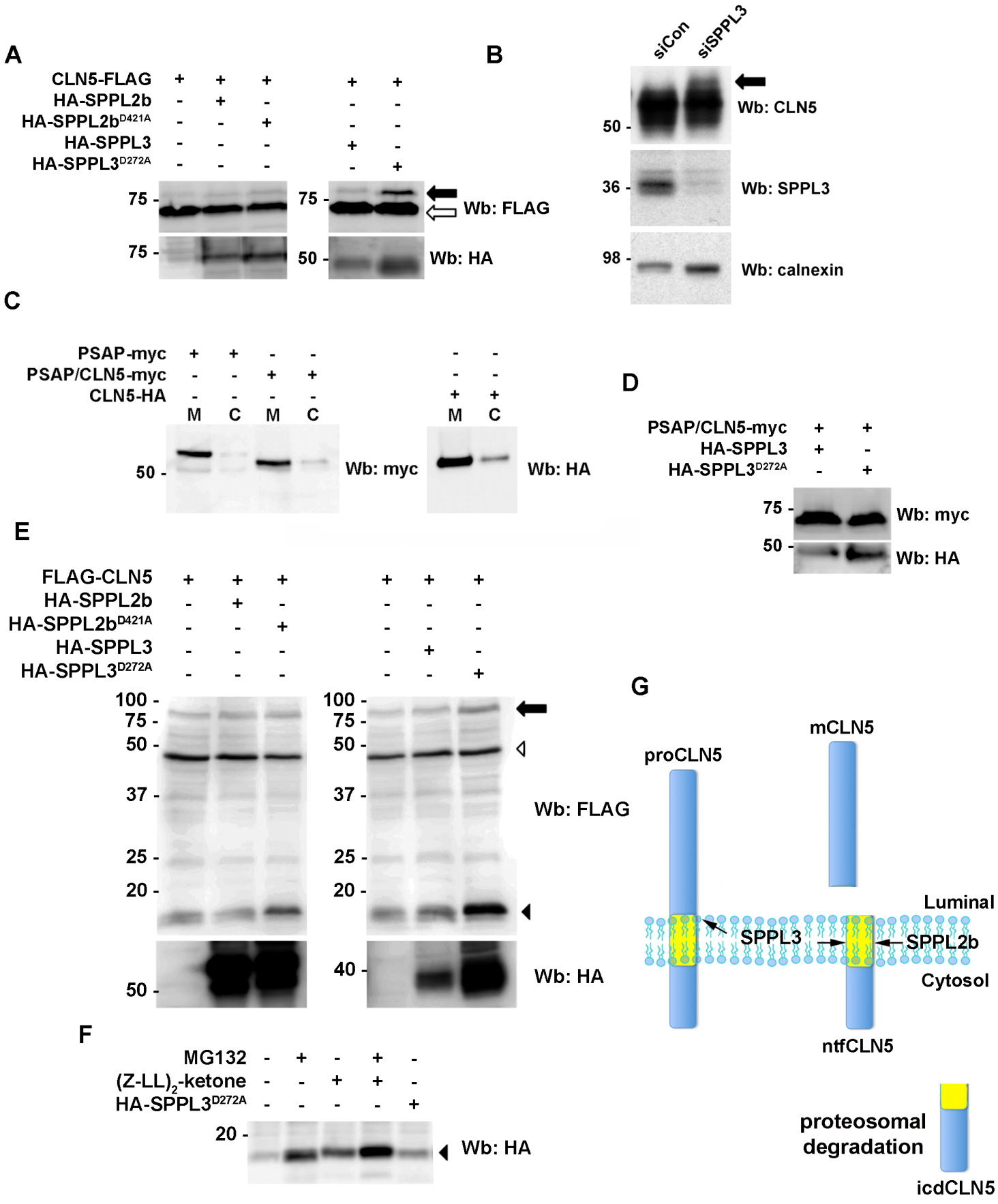
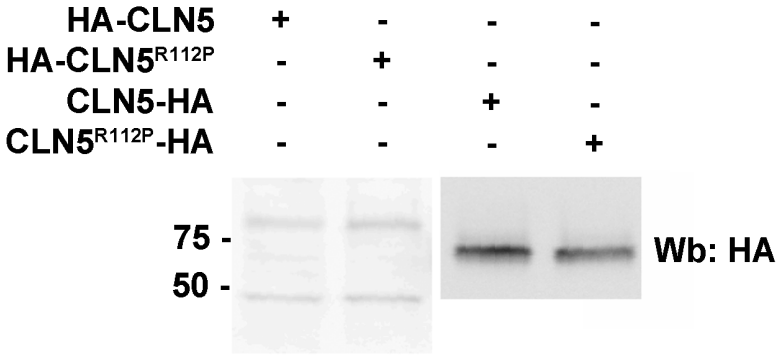

A
mock
leupeptin 10μM
pepstatin 2.5mM
E64 0.22mM
(Z-LL)2-ketone
10μM
25μM
50μM
(hrs) 0 3 6 0 3 6 0 3 6 0 3 6 0 3 6 0 3 6 0 3 6
100
75
50
37
Wb: HA
B
(Z-LL)2-ketone + + + + + +
CHX - - - + + +
(hrs) 0 3 6 0 3 6
CLN5-HA
75
50
Wb: HA
75
50
37
HA-CLN5
20
Wb: HA
40
Wb: actin
C
MG132 - 5 μM 10 μM 20 μM 5 min 30 min 90 min 180 min 270 min
75
50
37
25
20
15
Wb: HA
D
C M
75
50
37
25
20
Wb: HA
+ MG132

Figure 5


A
CLN5-FLAG
HA-SPPL2b
HA-SPPL2b^{D421A}
HA-SPPL3
HA-SPPL3^{D272A}
75
75
75
50
Wb: FLAG
Wb: HA
B
siCon
siSPPL3
50
36
98
Wb: CLN5
Wb: SPPL3
Wb: calnexin
C
PSAP-myc
PSAP/CLN5-myc
CLN5-HA
M C M C
M C
50
Wb: myc
Wb: HA
D
PSAP/CLN5-myc
HA-SPPL3
HA-SPPL3^{D272A}
75
50
Wb: myc
Wb: HA
E
FLAG-CLN5
HA-SPPL2b
HA-SPPL2b^{D421A}
HA-SPPL3
HA-SPPL3^{D272A}
100
75
50
37
25
20
50
40
Wb: FLAG
Wb: HA
F
MG132
(Z-LL)$_2$-ketone
HA-SPPL3^{D272A}
20
Wb: HA
G
proCLN5
mCLN5
Luminal
SPPL3
SPPL2b
Cytosol
ntfCLN5
proteosomal degradation
icdCLN5

Supplemental Figure 1



Supplemental Figure 2