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Proteolytic processing of the neuronal ceroid lipofuscinosis related lysosomal protein CLN5

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Abstract

CLN5 is a soluble lysosomal glycoprotein. Deficiency in CLN5 protein causes neuronal ceroid lipofuscinosis, an inherited neurodegenerative lysosomal storage disorder. The function of CLN5 and how it affects lysosome activity are unclear. We identified two forms of the CLN5 protein present in most of the cell lines studied. The molecular mass difference between these two forms is about 4 kDa. The fibroblast cells derived from two CLN5 patients lack both forms. Using transient transfection, we showed one of these two forms is a proprotein and the other is a C-terminal cleaved mature form. Using cycloheximide chase analysis, we were able to demonstrate that the C-terminal processing occurs post-translationally. By treating cells with several pharmaceutical drugs to inhibit proteases, we showed that the C-terminal processing takes place in an acidic compartment and the protease involved is most likely a cysteine protease. This is further supported by overexpression of a CLN5 patient mutant D279N and a glycosylation mutant N401Q, showing that the C-terminal processing takes place beyond the endoplasmic reticulum, and can occur as early as from the trans Golgi network. Furthermore, we demonstrated that CLN5 is expressed in a variety of murine tissues.

Keywords

Neuronal ceroid lipofuscinosis; CLN5; Proteolytic processing; Lysosomal storage disorder

1. Introduction

The neuronal ceroid lipofuscinoses (NCLs) are a group of genetically inherited lysosomal storage and neurodegenerative disorders. The age of onset varies with a range from early childhood to adulthood. The clinical symptoms of NCLs include loss of vision and motor

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function, seizures, mental retardation and premature death [1,2]. Similar to other lysosomal storage disorders, NCL is considered a rare disease. It has an estimated occurrence rate ranging from 0.5 to 8 per 1:100,000 live births, depending on the regions [3]. The incidence is higher in certain regions, such as Northern Europe and Northern America. Recently, through the exome sequencing technique, more NCL associated genes are identified, including CLN4, CLN11, CLN12, CLN13, and CLN14 [4–8]. Altogether there are thirteen genetically distinct subtypes of the disease named based on the defective gene [9–11]. For instance, CLN5 deficient NCL is now named CLN5 disease.

The major proteinaceous buildups in the lysosomes of NCL patients are either the subunit C of mitochondrial ATP synthase [12] or saposins A and D [13] depending on the subtype of the disease [14]. Intriguingly, despite all of them affecting lysosomal degradation process, the NCL associated proteins display high heterogeneity in cellular localization. For example, CLN1 (palmitoyl protein thioesterase 1, PPT1), CLN2 (tripeptidyl peptidase 1, TPP1), CLN5, CLN10 (cathepsin D, CTSD), CLN13 (cathepsin F, CTSF) are soluble proteins present in the lysosomal lumen, CLN3, CLN7 and CLN12 (ATP13A2) are lysosomal transmembrane proteins, CLN11 (progranulin) is an extracellular protein, CLN6 and CLN8 are transmembrane proteins localized in the endoplasmic reticulum (ER), and CLN4 (cysteine string protein α, CSPα) and CLN14 localized in the cytosol associated with vesicular membranes [15]. Among the soluble lysosomal CLN proteins, PPT1, TPP1, CTSD, and CTSF are acid hydrolases with different enzymatic activity and substrate specificities. CLN5, however, does not possess any homology to any known proteins and its function remains elusive [16,15].

CLN5 disease was originally identified as a rare variant form of NCL restricted to Finnish and other Northern European populations [16,17]. However, a more recent study has identified CLN5 disease in a variety of ethnic backgrounds and suggests that CLN5 mutations are more common in patients with NCL than previously thought [18]. Human CLN5 consists of 407 amino acids with an N-terminal signal sequence that is cleaved in the ER co-translationally [19]. In human CLN5 there are eight N-glycosylation consensus sites (N-X-T/S) present (Asparagine residues on position 179, 192, 227, 252, 304, 320, 330, and 401) and all are utilized, with roles involving proper folding, lysosomal targeting and function [20]. A previous study showed CLN5 contains a C-terminal amphipathic helix region that is tightly associated with the membrane [21]. Several studies indicate interactions among other NCL proteins and CLN5 [22,23], proposing molecular networking between NCL proteins. However, the significance of these interactions is not understood. CLN5 has also been suggested to play a role in endosomal sorting as an interaction between CLN5 and the lysosomal sorting receptor sortilin has been identified [24].

CLN5 is ubiquitously expressed in human and mouse tissues based on northern blot analysis [16,25]. Specific regions in brain tissues have also been examined for CLN5 distribution using *in situ* hybridization and immunohistochemistry techniques [26,25]. Enhanced CLN5 expression has been found in cerebellar Purkinje cells, cortical neurons, and hippocampal pyramidal cells [25]. However, the protein level of CLN5 in tissues has never been directly examined by Immunoblotting. In this report, we examine CLN5 protein expression in a panel of murine tissues and in various mammalian cell lines. We also discover previously

unknown C-terminal proteolytic processing of CLN5 during maturation process in the lysosome.

2. Materials and methods

2.1. Cell culture and transfections

Cell culture media and reagents were purchased from Gibco and Hyclone. Cell lines used in this study are A431 (ATCC CRL-1555), HEK293 (ATCC CRL-1573), HeLa (ATCC CCL-2), HepG2 (ATCC HB-8065), HT1080 (ATCC CCL-121), SH-SY5Y (ATCC CRL-2266), NIH-3T3 (ATCC CRL-1658), control fibroblasts GM00037 and GM00498 (Coriell). CLN5 patient fibroblasts #1 (homozygous c.694C > T, p.Gln232X) and #2 (c. 671G > A, p.Trp224X and exon 4 deletion) were received from Massachusetts General Hospital CHGR NCL Disorders Clinical Database and Biorepository. All cells were grown and maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 20 mM HEPES and gentamicin at 37 °C in a humidified incubator with 5% CO_2. For transfection, HEK293 cells were seeded in culture dishes for 24 h before transfection. The TransIT-LT1 transfection reagent (Mirus Bio) was used for overexpression of CLN5-Myc–His, and Lipofectamine RNA iMAX reagent (Life Technologies) was used for siRNA gene silencing. Transfections were done according to manufacturer's protocol. Opti-MEM reduced serum medium (Gibco) was used for reagent/ nucleotides complexes formation.

2.2. Plasmids

The wild type, D279N, and N401Q CLN5 in pcDNA3.1/Myc–His (-) A constructs for C-terminal Myc–6 × His tagged CLN5 overexpression were described previously [20]. The siRNA resistant CLN5 construct was generated using site-directed mutagenesis to create silent point mutations in the CLN5 siRNA target site (GAACCT AC<u>C</u>TA<u>C</u>CTGGGAA, underlined nucleotides are mismatched with the original sequence). DNA sequences corresponding to CLN5 amino acids 200–300, 200–220, and 240–300 were inserted to pGEX6pk-1 to generate constructs for Glutathione S Transferase (GST) fusion protein expression.

2.3. siRNAs

The siGENOME Control siRNA (D-001210-02-05) and CLN5 siRNA (target sequence GAACCTACTTATCTGGGAA) were purchased from Dharmacon. All siRNA were used at 20 nM working concentrations.

2.4. GST fusion protein expression and peptide blocking experiments

Bacteria strain Rosetta (DE3)pLysS was used for GST and GST fusion protein expression. Overnight bacterial culture was expanded and induced with 0.1 mM IPTG for 2 h at 37 °C. Cell pellets were lysed with 1 × sample buffer and run on SDS-PAGE for Coomassie Blue staining and immunoblotting analysis. For peptide blocking experiments, the rabbit monoclonal antibody against CLN5 was diluted with TBST and pre-incubated with a blot containing either GST or GST-CLN5 200–300 at room temperature for 1 h before applying to immunoblotting of HEK293 lysates.

2.5. Western blotting

Cells grown on 10 cm, 6 well or 12 well culture dishes were scraped and washed once with 1 × phosphate buffered saline, pH 7.4 and centrifuged for 3 min at 1500 × *g*. Cell pellets were lysed using RIPA lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (G-Biosciences). After incubation for 30 min on ice, extracts were centrifuged at 20,000 × *g* for 10 min at 4 °C. The supernatant was collected as the whole cell lysates. Protein concentrations were determined with Bradford assay when necessary. Aliquots of total extracts were incubated with sample buffer containing 100 mM DTT at 37 °C for 10 min. Samples were separated by SDS-PAGE. Proteins were transferred to PVDF membranes (Millipore) followed by immunoblotting. ECL detection was performed according to manufacturer's instructions (Millipore) and blots were imaged with G-Box (Syngene) or C-DiGit (Li-COR). C-DiGit software was used for quantification. Human tissue lysates megablot for western was purchased from BioChain. For medium samples, during transfection/mock transfection the cells were grown on OPTI-MEM. Medium samples were collected 24 h post transfection and concentrated with spin column (Pierce concentrator, 10 K MWCO) before proceeding to protein gels.

2.6. Murine tissues

The murine tissues were kindly provided by Sherry Fleming (Kansas State University). To prepare protein extracts, the frozen tissues were lysed in cold lysis buffer (20 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100) supplemented with protease inhibitors and homogenized using a handheld tissue homogenizer. The samples were further incubated for 30 min on ice. The homogenized lysates were centrifuged at 20,000 × *g* for 10 min at 4 °C. The supernatant was collected as the tissue protein extracts.

2.7. Antibodies

Mouse monoclonal antibodies used in this study were against the Myc epitope (9E10, hybridoma cell line from ATCC (CRL 1729)), beta-Actin (GenScript, A00702), and GAPDH (BioChain). Rabbit monoclonal CLN5 antibody used in this study was raised against human CLN5 (Abcam, ab170899). HRP-conjugated secondary antibodies for Western blotting were purchased from Jackson Laboratory.

2.8. Drug treatment experiments

2.8.1. Deglycosylation—After 24 h transfection, cells were collected and lysed in RIPA lysis buffer. Deglycosylation of samples with PNGase F (New England Biolabs) was performed according to the manufacturer's recommendations. Digestion with the enzyme was carried out for 2 h at 37 °C. Medium samples were first concentrated with spin column before proceeding to PNGase F digestion.

2.8.2. Cycloheximide chase—For cycloheximide treatment, 24 h post transfection the medium was replaced with DMEM containing 50 μg/ml cycloheximide (Fisher Scientific). Cells were collected at every 1 h from 0 to 5 h post treatment.

2.8.3. Protease inhibition—For treatment with different protease inhibitors HEK293 cells were seeded in culture dishes for 24 h before transfection. DMSO

(Cellgro), 50 μM Chloroquine (MP Biomedicals), 5 μM Pepstatin A (Sigma), 100 μM Leupeptin (Cayman Chemical), 10 μg/ml E-64 (Cayman Chemical) or 150 μM AEBSF (Cayman Chemical) were added to medium 4 h post transfection. Cells were collected 24 h after the start of drug treatment.

3. Results

3.1. The endogenous CLN5 glycoprotein exists in two forms

To study the expression of CLN5 in different cell lines at the protein level, total cell lysates of human cell lines A431, HEK293, HeLa, HepG2, HT1080, SH-SY5Y and GM00498 (a healthy control skin fibroblasts) were analyzed by CLN5 immunoblotting. These cell lines were chosen to represent a variety of tissue origins. For instance, A431 is from skin epidermis, HEK293 is from embryonic kidney, HeLa is from cervis, HepG2 is from liver, HT1080 is from connective tissue, and SH-SY5Y is derived from bone marrow of a neuroblastoma patient. The results of CLN5 immunoblotting (Fig. 1A) showed two major bands, an upper band around ~56 kDa and a lower band around ~52 kDa. Among the cell lines analyzed HEK293, HepG2, HT1080, SH-SY5Y and GM00498 predominantly had the lower band. The intensity of both bands was similar in A431 and HeLa cells. To verify the specificity of the antibody against CLN5 used, we performed a peptide blocking experiment. The antigen of this rabbit monoclonal antibody is within the region of amino acids 200–300 of CLN5 protein (based on the sequence of NP_006484, and it is equivalent to amino acids 150– 250 of UnitProKB O75503 referenced by Abcam). By pre-incubating the antibody with amino acids 200–300 of CLN5 fused with GST (GST-CLN5 200–300), the immunoreactive signal of CLN5 in HEK293 cells can be completely blocked (Fig. 1B). This indicates that both upper and lower bands were from CLN5. We further examined the CLN5 signals from skin fibroblasts of two CLN5 de-ficient patients (CLN5#1 and CLN5#2). As shown in Fig. 1C, both patients do not have either form of CLN5 proteins compared to two healthy control skin fibroblasts (GM00037 and GM00498). This further supports that the CLN5 antibody is specific for endogenous human CLN5 as shown by the absence of immunoreactive full length CLN5 bands in patient fibroblasts. The patient CLN5#1 has a homozygous 694C > T point mutation in DNA, resulting in a premature stop codon and CLN5 protein truncation (Gln232X), whereas patient #2 has exon 4 deletion in one allele and 671G > A point mutation in the other allele, resulting in a premature stop codon and CLN5 protein truncation (Trp224X). Since we did not observe any signals from patient cells using the CLN5 antibody, there is a possibility that this CLN5 antibody cannot recognize the truncated CLN5 in these two patients. We tested this by generating two constructs covering either 200– 220 amino acids or 240–300 amino acids of CLN5 to narrow down the possible immunogen region (Fig. 1D). Fig. 1E shows the positions of the immunogen and patient truncations. It is clear that the CLN5 antibody recognizes an epitope within the region of 240– 300 amino acids of CLN5. Therefore we cannot exclude the possibility that there are truncated CLN5 present in these two CLN5 patients.

The N-terminal portion of CLN5 contains the signal peptide, which is cleaved co-translationally during biosynthesis in the ER [19], and therefore is not suitable for tagging. In our previous work when we used an overexpression system to follow C-terminal Myc–6 × His tagged versions of CLN5, we did not observe two distinct bands [20]. We decided to use this CLN5 antibody to revisit the overexpression system. To minimize the signals from endogenous CLN5, we treated cells with CLN5 specific small interfering RNA (siRNA) for up to 72 h to knockdown CLN5 expression. Treatment with CLN5 siRNA resulted in a substantial reduction of CLN5 protein level after 48 h and continued to reduce the amount of endogenous CLN5 at 72 h (Fig. 2, left panel). This further confirms that the protein recognized by the CLN5 antibody used in this study is indeed CLN5. The less prominent upper endogenous CLN5 band was not readily visible in the HEK293 lysates in this experiment. In a separate set of experiment, after siRNA treatment for 48 h, cells were transfected with a siRNA resistant CLN5 construct for another 24 h. Interestingly, while after 72 h the level of endogenous CLN5 was dramatically reduced, over-expression of siRNA resistant CLN5 showed two major bands of ~60 kDa and 52 kDa when blotted with CLN5 antibody. The overexpressed CLN5 used in this study contains a C-terminal Myc and 6 × His tag, resulting in ~4 kDa increase in the upper band (~60 kDa) compared to the endogenous CLN5 upper band of ~56 kDa. For simplicity, hereafter only Myc tag will be mentioned in the text since we were not following the His tag. The lower band of overexpressed CLN5 has the same mobility as the lower band of endogenous CLN5. However while using Myc antibody only the higher band of ~60 kDa showed up (Fig. 2, right panel). This suggests that the Myc tag at the C-terminal end of CLN5 has been cleaved and is no longer present in the lower band. This explains why we did not observe two bands in our previous study using the Myc antibody. This data indicates that CLN5 undergoes post-translational proteolytic cleavage. From this point forward we will use the terms proprotein and mature protein to describe the upper and lower band of CLN5, respectively.

3.2. Cycloheximide chase analysis demonstrates in vivo processing of CLN5

To verify the processing of CLN5 occurs *in vivo* during protein synthesis and maturation and not as an artifact of cell lysis conditions, a cycloheximide chase experiment was performed in cells overexpressing Myc tagged CLN5 (Fig. 3). At 24 h post transfection, cells were treated with cycloheximide to inhibit *de novo* protein biosynthesis. Immunoblotting with CLN5 showed the intensity of the ~60 kDa band corresponding to the proprotein (p) decreasing with increasing time in the presence of cycloheximide. Simultaneously the mature form (m) of ~52 kDa increased with increasing time of cycloheximide chase. The intensity of proprotein was 2.7-fold over the mature protein at 0 h. After 5 h chase, the intensity of the mature form was about 2.5-fold over the proprotein. Immunoblotting with Myc also showed that the proprotein was decreased with increasing time of cycloheximide chase. This data shows that the processing of CLN5 happens in real time and is not an artifact of cell lysis conditions.

3.3. CLN5 processing takes place in low pH environment

To further investigate the proteolytic process of CLN5, we analyzed the conditions in which the processing occurs. CLN5 is a lysosomal lumen protein and the lysosome or TGN/endosome could be a potential location where the processing may occur. To address this, we

treated cells with chloroquine, a weak base that can accumulate in acidic compartments such as lysosomes and neutralize the pH of the compartments. As a consequence, the lysosome acid hydrolases become inactivated. Chloroquine was added to the cells 4 h post CLN5 transfection and continued incubation for another 24 h. As seen in Fig. 4, the intensity of the mature form was reduced dramatically, with proprotein to mature protein intensity ratio of 16.9:1. In fact, the weak signal of the lower band is probably the endogenous mature CLN5 already present in the cells before chloroquine was added. The band between overexpressed proprotein and mature protein is most likely the endogenous proprotein (e. p). This means that C-terminal processing of CLN5 requires an acidic environment. To understand what type of protease would be involved in the processing, HEK293 cells were treated with different protease inhibitors 4 h post CLN5 transfection. Incubation was continued for another 24 h before the samples were analyzed. Treatment with Pepstatin A, an aspartic protease inhibitor, did not result in inhibition of the mature protein forming, with a similar p/m ratio of 1.9:1 as compared to the DMSO treatment of p/m ratio of 2.4:1. However treatment with Leupeptin, a serine and cysteine protease inhibitor resulted in a reduced amount of the mature form (p/m ratio 5:1), suggesting that a serine or cysteine protease is involved in the processing. To distinguish between these two proteases, E-64 and AEBSF, inhibitor of cysteine protease and serine protease respectively, were used to test the specific protease involved in the processing. Treatment with E-64 demonstrated similar results as to treatment with Leupeptin with low amount of the mature CLN5 formed. AEBSF seemed to affect transfection and/or protein expression. Nevertheless, the p/m ratio is comparable to the control DMSO treatment. The ratio of the intensity of proprotein to the mature protein of each treatment is quantified and shown below the lanes. In sum, this data suggests that the protease involved in the proteolytic processing of CLN5 is a cysteine protease.

3.4. The different CLN5 protein forms are not due to glycosylation variants

To further examine the property of the two bands observed, we digested the lysates with PNGase F. PNGase F is a glyco-amidase which can cleave between the innermost N-acetyl glucosamine and the asparagine residue of the protein, thereby removing all traces of glycosylation from the protein. We analyzed endogenous CLN5, overexpressed Myc-tagged wild type CLN5, a patient mutant D279N CLN5 and a glycosylation mutant N401Q CLN5 (Fig. 5A). Note that in all cases, since the mature form of endogenous and overexpressed CLN5 is the same size, the “m” band we observed in the overexpressed samples represents the total mature form from both origins. In non-transfected samples, the two endogenous CLN5 bands are still visible after PNGase F digestion. In fact, these two forms became more distinguishable after digestion. Similarly, in overexpressed wild type CLN5 samples, two major bands can be seen before and after PNGase F treatment, representing proprotein (p) and mature protein (m). Note that a weaker band in between appears to be the endogenous proprotein of CLN5 (e. p). The CLN5 D279N mutant protein has an extra glycosylation site and is retained in the ER [19,20]. Since the proteolytic processing takes place in an acidic environment, here D279N mutant is used as a negative control to show processing does not occur in the ER of which the pH is neutral. Indeed, only one major CLN5 band was observed in D279N sample and it is slightly higher than the overexpressed wild type proprotein band due to extra glycosylation. After PNGase F digestion removing all the glycans, there was no difference in size comparing proprotein of wild type and D279N

CLN5. There was no processed mature form derived from D279N proprotoein in both N-glycosylated and non-N glycosylated samples. The two minor bands seen in D279N samples can be related to the proprotein and the mature endogenous CLN5 expressed in HEK293. This confirms that CLN5 D279N does not undergo proteolytic cleavage. When blotted with antibody against the Myc tag, only the proprotein band can be seen in each over-expressed sample (Fig. 5A, right panel). N401Q, a CLN5 glycosylation mutant, does not reach lysosomes but instead accumulates in Golgi/TGN and can be found in the media as well [20]. Interestingly, in the N401Q sample, we observed the proprotein as well as the mature form when blotted with the CLN5 antibody. This suggests that the proteolytic processing can occur in the TGN, which is mildly acidic. This set of data further confirms that the two forms of CLN5 are not glycosylation variants from biosynthetic pathway but are derived from proteolytic cleavage, and this processing occurs in a cellular location beyond the ER.

Since overexpressed CLN5 can be secreted [27,20,8]. and we found its proteolytic processing can occur in the TGN where secretory vesicles are packaged, we examined if the mature form can be detected in the media as well. The media was collected and also digested with PNGase F after 24 h post transfection of wild type CLN5. Interestingly, only the proprotein of overexpressed CLN5 could be seen in the media samples (Fig. 5B). The slower migration in protein gel of non-processed CLN5 in the media sample (M) is probably due to continued modification/glycosylation, such as fucosylation and sialylation, when proteins are transported onward from TGN to outside of the cells [28]. We also did not observe the mature form of endogenous CLN5 in the media (Fig. 5C). In fact, we did not observe any form of endogenous CLN5 in the media collected after 24 h cell growth. This indicates that, for endogenous CLN5, the majority of the processing occurs once the protein has passed TGN en route to the endosome/lysosome. Therefore no endogenous CLN5 was detected in the media. This would suggest that the observation of proprotein of overexpressed CLN5 in the media and proteolytic processing of N401Q CLN5 at TGN maybe due to transient overexpression overwhelming the system.

3.5. CLN5 expression of murine tissues and cell lines

Since no CLN5 protein expression data in tissues by immunoblotting has been reported, we decided to survey the CLN5 levels in different tissues (Fig. 6). Based on the information provided by the company, the CLN5 antibody should recognize the mouse CLN5 as well. The Western blotting data shows differential distributions in adult murine tissues. Two major bands close to 48 kDa can be identified in many different tissues, indicating proteolytic processing occurs in murine CLN5 as well (Fig. 6A). Interestingly, besides the two major bands, there is a higher molecular weight band around 63 kDa in the heart and kidney. It may be the preproprotein containing the signal peptide. However in HEK293 embryonic kidney cells we did not observe such a high molecular weight band (Fig. 1A). An extra lower band (~40 kDa) was detected in kidney, liver and muscle samples, suggesting there might be other forms of modification or CLN5 in those tissue samples were partially degraded. Similarly to the higher CLN5 expression levels in human cell lines HEK293 (kidney origin) and HepG2 (liver origin) (Fig. 1A), mouse kidney and liver have higher levels of CLN5 expression as well. Compared to other tissues, the level of CLN5 in the brain was low. The relatively low level of CLN5 in the brain was also observed in human

tissues (data not shown). To further validate the CLN5 signals observed in mouse tissues, we compared CLN5 in mouse spleen (with two distinct bands visible in Fig. 6A), mouse embryonic fibroblast cell line NIH3T3, and human HEK293 cells. As shown in Fig. 6B, the size of mouse CLN5 from both spleen and NIH3T3 cells was slightly smaller than the CLN5 in human HEK293 cells, consistent with a previous observation [25]. In NIH3T3, only the mature form of CLN5 was observed. The deglycosylated mature CLN5 in NIH3T3 is also smaller compared to the human form (Fig. 6C).

4. Discussion

One of the obstacles in the NCL field is the lack of antibodies that can recognize endogenous NCL proteins. For some NCL proteins it's mainly due to the low expression in tissues. For CLN5, several groups have attempted to generate antibodies without much success in detecting endogenous protein expression [27,25,29,30,19]. Here we identified an antibody that is able to recognize endogenous CLN5 in various tissues and cell lines from human and mice. We confirmed the antibody specificity by demonstrating: (1) the immunoreactive signal can be blocked by CLN5 fragment amino acids 200–300 corresponding to the immunogen region, (2) lacking the full length CLN5 signals in skin fibroblasts derived from CLN5 deficient patients, and (3) the ability to decrease the signals by knocking down CLN5 with siRNA.

We found higher level of CLN5 protein expression in kidney and liver in both mouse tissues and human cell line origins by western blotting. This is in agreement with a previous study showing high levels of gene expression in adult mouse kidney and liver using northern blotting [25]. In addition to kidney and liver, we also detect CLN5 signals in a variety of mouse tissues, consistent with earlier northern blotting studies [16,25]. This may indicate a more global role for CLN5. In comparison with other tissues, we observed relatively low levels of CLN5 protein level in both adult mouse and human brain tissue (not shown). Depending on the developmental stage, the levels of CLN5 might be differentially expressed. For example, in developing brain CLN5 mRNA was found abundant in the developing cerebral cortex, cerebellum, and ganglionic eminance, whereas in adult brain CLN5 mRNA was enriched in the cerebellar Purkinje cells and cerebral cortex [25].

Another important finding is that two forms of CLN5, derived from the C-terminal proteolytic processing, are present in most cells and tissues examined. We and other groups have been using transient transfection overexpression systems to study CLN5, again in part due to the lack of verified antibodies for detecting endogenous CLN5 [27,25,19–21]. As shown in this report and our previous study [20], Myc antibody only detects the upper, non-processed proprotein of C-terminal Myc-tagged CLN5. We demonstrated the lysosomal localization of unprocessed Myc-tagged CLN5 [20], which is consistent with our current study showing that the C-terminal proteolytic processing occurs in acidic compartments. Intriguingly, we found overexpressed CLN5 mutant N401Q can be cleaved. Previously we showed by immuno-fluorescence studies, N401Q does not reach the lysosomes. Instead, it is localized to the Golgi/TGN and can be secreted to the media. It was postulated that glycan moiety on N401 contains Man-6-P necessary for endosome/lysosome transport. Without this glycosylation site, CLN5 is not able to reach the lysosome [20]. If the C-terminal proteolytic

cleavage occurs in the TGN the processed form, which most likely loses the N401 and therefore the Man-6-P on N401, will not be able to use Man-6-P mediated pathway to transport to the endosome/lysosome but instead will be secreted. However, a proteomics study identified two other potential Man-6-P sites in CLN5 (N320 and N330) besides the N401 [31]. It is therefore possible that in certain conditions, these sites can be phosphorylated and contribute to the endosome/lysosome transport. Alternatively, CLN5 endosome/lysosome traf-ficking can also be mediated by non-Man-6-P pathway [19]. In the current report when analyzing the media, we could not detect the mature form of wild type CLN5 (in both non-transfected and CLN5 transfected samples) in the media while in the pellets it is the more prominent form and can be easily detected. This suggests that proteolytic processed mature form of CLN5 is normally transported to or generated in the lysosomes. Supporting the latter, we can detect the unprocessed proprotein CLN5 in the lysosomes [20]. It will be interesting to know whether this form is the mature functional form or is on its way for degradation. With many of tissues and cell lines surveyed having the processed form as the most prominent band, we favor the idea that the processed form is the functional CLN5. In the future it will be interesting to examine whether higher levels of mature CLN5 present correlates with a functional role of CLN5 in the tissues.

During biosynthesis, CLN5 first undergoes co-translational cleavage to remove the N-terminal signal sequence. Based on the PNGase F digestion assay, it is clear that the upper form we observed does not contain the signal sequence. After removing the carbohydrate moieties the proprotein of the endogenous CLN5 is about 33 kDa, which fits well with the molecular weight of CLN5 without the signal peptide. Furthermore, we found the formation of the mature CLN5 relies on acidic environment and can be greatly reduced by cysteine specific protease inhibitors, Leupeptin and E-64. Identifying the cysteine protease involved in the C-terminal processing of CLN5 will provide more information on the proteolytic processing of CLN5.

The electrophoretic mobility difference between the proprotein and mature forms of endogenous CLN5 is about 4 kDa. Based on the amino acid sequence of CLN5, the proteolytic process will remove the glycan moiety on N401 and ~10 to 15 amino acids from the C-terminus (including N401). Supporting this observation, our results in Fig. 5, after PNGase F digestion removing all glycans, showed the size difference between proprotein and mature form is ~2 kDa. This would position the cleavage site behind the amphipathic helix region identified previously [21]. This region was reported to associate with the membrane tightly. However, we can readily detect the unprocessed form of overexpressed CLN5 from the media (in current study and [20]), suggesting CLN5 is fairly soluble in our system. This is consistent with a previous study showing CLN5 was recovered in the aqueous phase after Triton X-114 phase separation [25]. It will be interesting to examine which residues in CLN5 are involved in the C-terminal processing and if the processing is compromised in any CLN5 patient mutants.

The processed form of CLN5 has been observed previously [25,19] using antibodies that recognize overexpressed CLN5. Holmberg et al. [25] detected two CLN5 bands of 48 and 50 kDa from CLN5 transfected samples. Schmiedt et al. [19] used pulse-chase experiments to demonstrate the existence of proprotein and mature CLN5 with molecular weight of ~60

kDa and 50 kDa, respectively. In both studies, non-tagged CLN5 were used in over-expression studies. In our study, we estimate the size of the overexpressed CLN5 with Myc–6 × His tag at the C-terminus is ~60 kDa. With the new CLN5 antibody we characterized in this study, we were able to detect the endogenous proprotein and mature CLN5 of ~56 kDa and 52 kDa. Since all the estimation of molecular weight is based on the electrophoretic mobility in protein gels, the size difference in these three studies can be due to different protein molecular weight markers and/or different percentage of protein gels used. Schmiedt et al. [19] used a rabbit antibody (C/32) raised against C-terminal end (amino acids 393–407) of human CLN5 and was able to immunoprecipitate both proprotein and mature form of CLN5, suggesting the proteolytic processing is within last few C-terminal amino acids, which is consistent with our estimation of 10–15 amino acids from the C-terminus. Further analysis of the C-terminal region of CLN5 will be useful in revealing the nature of the processing.

The size of mature CLN5 in murine samples appears to be smaller than in humans. This is not due to lacking one glycosylation site in mouse sequence (which is equivalent to human sequence on N401 position), because after PNGase F digestion the deglycosylated mouse CLN5 is still smaller than the human one (Fig. 6C). This is consistent with a study overexpressing untagged mouse CLN5 (Holmberg 2004). Based on the sequences (human, NP_006484; mouse, AAI41315), and signal peptide prediction (SignalP 4.1 Server, Technical University of Denmark), human CLN5 polypeptide after signal peptide removed (92–407) has a molecular weight of 37.02 kDa and mouse CLN5 (34–341) is 36.12 kDa after signal peptide removal. However, the size difference in SDS-PAGE seems to be greater than 0.9 kDa. Further investigation on CLN5 C-terminal processing and actual signal peptide cleavage site will be needed to address the size differences between these two species.

Our results here clearly demonstrate there is a proteolytic processed mature form of CLN5 present in the cells. Potentially this is a means to regulate the yet to be discovered activity/function of CLN5. In line with this idea, many soluble lysosomal enzymes including TPP1 (CLN2), CTSD (CLN10), CTSF (CLN13), and acid alpha-glucosidase undergo proteolytic cleavage and maturation in the lysosomes [32–36]. Another NCL polytopic membrane protein, CLN7, has also been observed to undergo cysteine pro-tease-dependent proteolytic cleavage in the lysosome [37]. Further investigation will be needed to understand the importance of CLN5 proteolytic processing and to reveal the function of CLN5.

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References

1. Bennett MJ, Hofmann SL. The neuronal ceroid-lipofuscinoses (Batten disease): a new class of lysosomal storage diseases. J. Inherit. Metab. Dis. 1999; 22:535–544. [PubMed: 10407785]
2. Haltia M. The neuronal ceroid-lipofuscinoses: from past to present. Biochim. Biophys. Acta. 2006; 1762:850–856. [PubMed: 16908122]

3. Augestad LB, Flanders WD. Occurrence of and mortality from childhood neuronal ceroid lipofuscinoses in norway. J. Child Neurol. 2006; 21:917–922. [PubMed: 17092455]
4. Noskova L, Stranecky V, Hartmannova H, Pristoupilova A, Baresova V, Ivanek R, Hulkova H, Jahnova H, van der Zee J, Staropoli JF, Sims KB, Tyynela J, Van Broeckhoven C, Nijssen PC, Mole SE, Elleder M, Kmoch S. Mutations in DNAJC5, encoding cysteine-string protein alpha, cause autosomal-dominant adult-onset neuronal ceroid lipofuscinosis. Am. J. Hum. Genet. 2011; 89:241–252. [PubMed: 21820099]
5. Bras J, Verloes A, Schneider SA, Mole SE, Guerreiro RJ. Mutation of the parkinsonism gene ATP13A2 causes neuronal ceroid-lipofuscinosis. Hum. Mol. Genet. 2012; 21:2646–2650. [PubMed: 22388936]
6. Smith KR, Damiano J, Franceschetti S, Carpenter S, Canafoglia L, Morbin M, Rossi G, Pareyson D, Mole SE, Staropoli JF, Sims KB, Lewis J, Lin WL, Dickson DW, Dahl HH, Bahlo M, Berkovic SF. Strikingly different clinicopathological phenotypes determined by progranulin-mutation dosage. Am. J. Hum. Genet. 2012; 90:1102–1107. [PubMed: 22608501]
7. Staropoli JF, Karaa A, Lim ET, Kirby A, Elbalalesy N, Romansky SG, Leydiker KB, Coppel SH, Barone R, Xin W, MacDonald ME, Abdenur JE, Daly MJ, Sims KB, Cotman SL. A homozygous mutation in KCTD7 links neuronal ceroid lipofuscinosis to the ubiquitin-proteasome system. Am. J. Hum. Genet. 2012; 91:202–208. [PubMed: 22748208]
8. Smith KR, Dahl HH, Canafoglia L, Andermann E, Damiano J, Morbin M, Bruni AC, Giaccone G, Cossette P, Saftig P, Grotzinger J, Schwake M, Andermann F, Staropoli JF, Sims KB, Mole SE, Franceschetti S, Alexander NA, Cooper JD, Chapman HA, Carpenter S, Berkovic SF, Bahlo M. Cathepsin F mutations cause Type B Kufs disease, an adult-onset neuronal ceroid lipofuscinosis. Hum. Mol. Genet. 2013; 22:1417–1423. [PubMed: 23297359]
9. Williams RE, Mole SE. New nomenclature and classification scheme for the neuronal ceroid lipofuscinoses. Neurology. 2012; 79:183–191. [PubMed: 22778232]
10. Kollmann K, Uusi-Rauva K, Scifo E, Tyynela J, Jalanko A, Braulke T. Cell biology and function of neuronal ceroid lipofuscinosis-related proteins. Biochim. Biophys. Acta. 2013; 1832:1866–1881. [PubMed: 23402926]
11. Mole, SE.; Cotman, SL. Genetics of the neuronal ceroid lipofuscinoses (Batten disease). Biochim. Biophys. Acta. 2015. http://dx.doi.org/10.1016/j.bbadis.2015.05.011. (Epub ahead of print)
12. Palmer DN, Fearnley IM, Walker JE, Hall NA, Lake BD, Wolfe LS, Haltia M, Martinus RD, Jolly RD. Mitochondrial ATP synthase subunit c storage in the ceroid-lipofuscinoses (Batten disease). Am. J. Med. Genet. 1992; 42:561–567. [PubMed: 1535179]
13. Tyynela J, Palmer DN, Baumann M, Haltia M. Storage of saposins A and D in infantile neuronal ceroid-lipofuscinosis. FEBS Lett. 1993; 330:8–12. [PubMed: 8370464]
14. Radke, J.; Stenzel, W.; Goebel, HH. Human NCL Neuropathology. Biochim. Biophys. Acta. 2015. http://dx.doi.org/10.1016/j.bbadis.2015.05.007. (Epub ahead of print)
15. Carcel-Trullols, J.; Kovacs, AD.; Pearce, DA. Cell biology of the NCL proteins: what they do and don't do. Biochim. Biophys. Acta. 2015. http://dx.doi.org/10.1016/j.bbadis.2015.04.027. (Epub ahead of print)
16. Savukoski M, Klockars T, Holmberg V, Santavuori P, Lander ES, Peltonen L. CLN5, a novel gene encoding a putative transmembrane protein mutated in Finnish variant late infantile neuronal ceroid lipofuscinosis. Nat. Genet. 1998; 19:286–288. [PubMed: 9662406]
17. Holmberg V, Lauronen L, Autti T, Santavuori P, Savukoski M, Uvebrant P, Hofman I, Peltonen L, Jarvela I. Phenotype-genotype correlation in eight patients with Finnish variant late infantile NCL (CLN5). Neurology. 2000; 55:579–581. [PubMed: 10953198]
18. Xin W, Mullen TE, Kiely R, Min J, Feng X, Cao Y, O'Malley L, Shen Y, Chu-Shore C, Mole SE, Goebel HH, Sims K. CLN5 mutations are frequent in juvenile and late-onset non-Finnish patients with NCL. Neurology. 2010; 74:565–571. [PubMed: 20157158]
19. Schmiedt ML, Bessa C, Heine C, Ribeiro MG, Jalanko A, Kyttala A. The neuronal ceroid lipofuscinosis protein CLN5: new insights into cellular maturation, transport, and consequences of mutations. Hum. Mutat. 2010; 31:356–365. [PubMed: 20052765]
20. Moharir A, Peck SH, Budden T, Lee SY. The role of N-glycosylation in folding, trafficking, and functionality of lysosomal protein CLN5. PloS One. 2013; 8:e74299. [PubMed: 24058541]

21. Larkin H, Ribeiro MG, Lavoie C. Topology and membrane anchoring of the lysosomal storage disease-related protein CLN5. Hum. Mutat. 2013; 34:1688–1697. [PubMed: 24038957]
22. Vesa J, Chin MH, Oelgeschlager K, Isosomppi J, DellAngelica EC, Jalanko A, Peltonen L. Neuronal ceroid lipofuscinoses are connected at molecular level: interaction of CLN5 protein with CLN2 and CLN3. Mol. Biol. Cell. 2002; 13:2410–2420. [PubMed: 12134079]
23. Lyly A, von Schantz C, Heine C, Schmiedt ML, Sipila T, Jalanko A, Kyttala A. Novel interactions of CLN5 support molecular networking between Neuronal Ceroid Lipofuscinosis proteins. BMC Cell Biol. 2009; 10:83. [PubMed: 19941651]
24. Mamo A, Jules F, Dumaresq-Doiron K, Costantino S, Lefrancois S. The role of ceroid lipofuscinosis neuronal protein 5 (CLN5) in endosomal sorting. Mol. Cell. Biol. 2012; 32:1855–1866. [PubMed: 22431521]
25. Holmberg V, Jalanko A, Isosomppi J, Fabritius AL, Peltonen L, Kopra O. The mouse ortholog of the neuronal ceroid lipofuscinosis CLN5 gene encodes a soluble lysosomal glycoprotein expressed in the developing brain. Neurobiol. Dis. 2004; 16:29–40. [PubMed: 15207259]
26. Heinonen O, Salonen T, Jalanko A, Peltonen L, Copp A. CLN-1 and CLN-5, genes for infantile and variant late infantile neuronal ceroid lipofuscinoses, are expressed in the embryonic human brain. J. Comp. Neurol. 2000; 426:406–412. [PubMed: 10992246]
27. Isosomppi J, Vesa J, Jalanko A, Peltonen L. Lysosomal localization of the neuronal ceroid lipofuscinosis CLN5 protein. Hum. Mol. Genet. 2002; 11:885–891. [PubMed: 11971870]
28. Klein A. Human total serum N-glycome. Adv. Clin. Chem. 2008; 46:51–85. [PubMed: 19004187]
29. Bessa C, Teixeira CA, Mangas M, Dias A, Sa Miranda MC, Guimaraes A, Ferreira JC, Canas N, Cabral P, Ribeiro MG. Two novel CLN5 mutations in a Portuguese patient with vLINCL: insights into molecular mechanisms of CLN5 deficiency. Mol. Genet. Metab. 2006; 89:245–253. [PubMed: 16814585]
30. Lebrun AH, Storch S, Ruschendorf F, Schmiedt ML, Kyttala A, Mole SE, Kitzmuller C, Saar K, Mewasingh LD, Boda V, Kohlschutter A, Ullrich K, Braulke T, Schulz A. Retention of lysosomal protein CLN5 in the endoplasmic reticulum causes neuronal ceroid lipofuscinosis in Asian sibship. Hum. Mutat. 2009; 30:E651–E661. [PubMed: 19309691]
31. Sleat DE, Zheng H, Qian M, Lobel P. Identification of sites of mannose 6-phosphorylation on lysosomal proteins. Mol. Cell. Proteom. 2006; 5:686–701.
32. Richo G, Conner GE. Proteolytic activation of human procathepsin D. Adv. Exp. Med. Biol. 1991; 306:289–296. [PubMed: 1812719]
33. Santamaria I, Velasco G, Pendas AM, Paz A, Lopez-Otin C. Molecular cloning and structural and functional characterization of human cathepsin F, a new cysteine proteinase of the papain family with a long propeptide domain. J. Biol. Chem. 1999; 274:13800–13809. [PubMed: 10318784]
34. Ishidoh K, Kominami E. Processing and activation of lysosomal proteinases. Biol. Chem. 2002; 383:1827–1831. [PubMed: 12553719]
35. Golabek AA, Kida E, Walus M, Wujek P, Mehta P, Wisniewski KE. Bio-synthesis, glycosylation, and enzymatic processing in vivo of human tripeptidyl-peptidase I. J. Biol. Chem. 2003; 278:7135–7145. [PubMed: 12488460]
36. Moreland RJ, Jin X, Zhang XK, Decker RW, Albee KL, Lee KL, Cauthron RD, Brewer K, Edmunds T, Canfield WM. Lysosomal acid alpha-glucosidase consists of four different peptides processed from a single chain precursor. J. Biol. Chem. 2005; 280:6780–6791. [PubMed: 15520017]
37. Steenhuis P, Froemming J, Reinheckel T, Storch S. Proteolytic cleavage of the disease-related lysosomal membrane glycoprotein CLN7. Biochim. Biophys. Acta. 2012; 1822:1617–1628. [PubMed: 22668694]

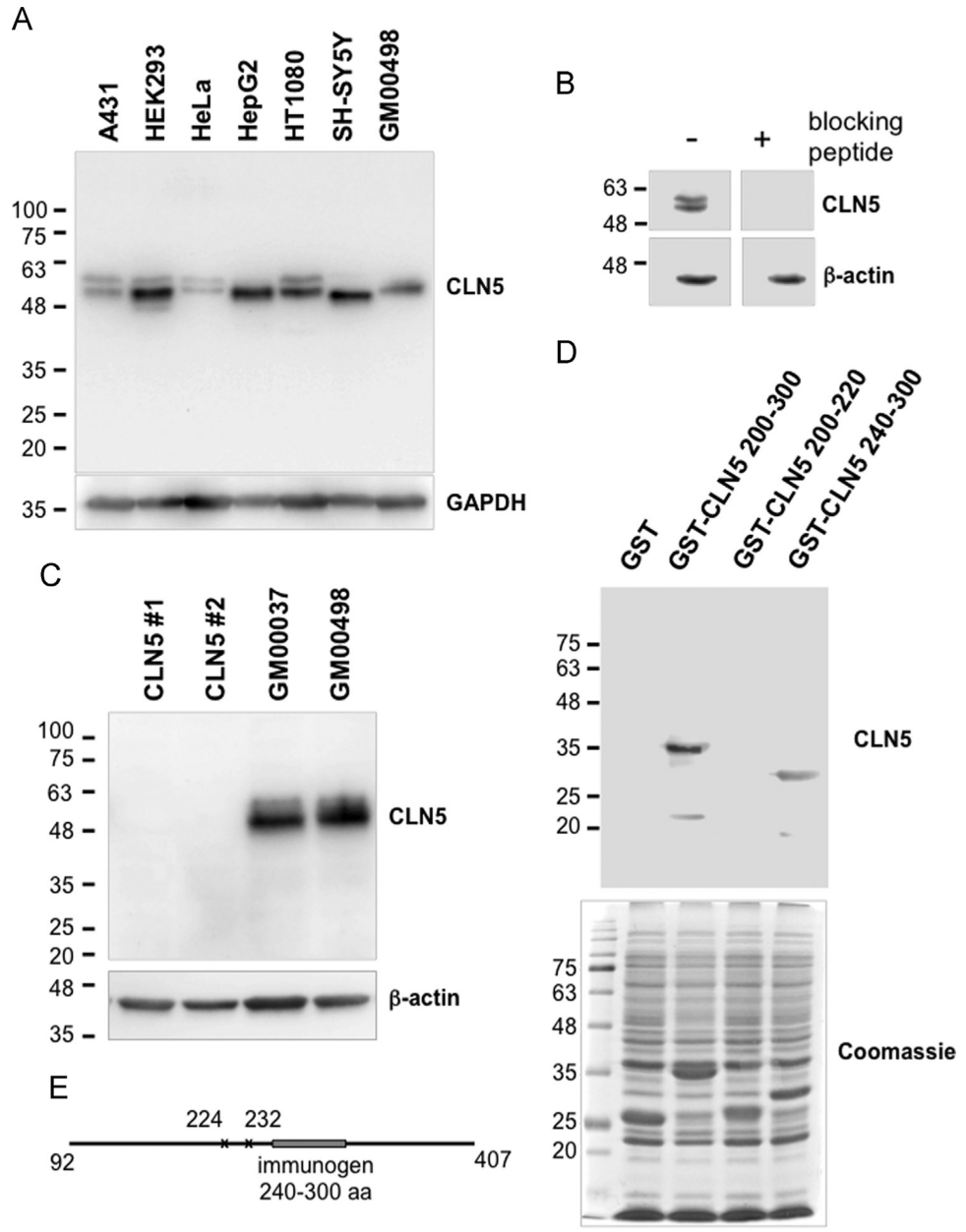

Fig. 1.

The endogenous expression of CLN5 (A) two immunoreactive bands were detected by a rabbit monoclonal antibody against human CLN5. The whole cell lysates 50 μg of human cell lines A431, HEK293, HeLa, HepG2, HT1080, SH-SY5Y and GM00498 (a healthy control fibroblasts) were analyzed by Western blotting for the expression of endogenous CLN5 protein. (B) The CLN5 antibody specifically recognizes endogenous CLN5 in HEK293 cells. The antigen for this rabbit monoclonal antibody is within the 200–300 aa region of human CLN5 protein. To show the specificity, GST-CLN5 200–300 was used as a blocking peptide. CLN5 antibody was pre-incubated with GST (−) or GST-CLN5 200–300 (+) before applying to immunoblotting of HEK293 lysates. (C) No immunoreactive bands were detected in CLN5 #1 and CLN5 #2 patient fibroblast cells. The whole cell lysates of

two CLN5 deficient patient skin fibroblasts CLN5 #1 (Gln232X) and CLN5 #2 (Trp224X) and two control skin fibroblasts GM00037 and GM00498 were analyzed by Western blotting. No immunoreactive bands were detected in CLN5 #1 and CLN5 #2 patient fibroblast cells carry mutations in the CLN5 gene resulting in truncation of the protein. (D) The epitope that the CLN5 antibody recognizes is within 240–300 amino acids of human CLN5. The whole cell lysates of bacteria expressing GST or GST fused with 200–300 aa, 200–220 aa, 240–300 aa were immunoblotted with CLN5 antibody. After immunoblotting, the blot was stained with Coomassie blue to visualize the protein expression. Coomassie blue stained gel shows the expression of GST and GST-CLN5 fusion proteins corresponding to the expected size in each sample. (E) The positions of the immunogen and patient mutations are shown. The N-terminal region 1–91 amino acid not shown here is predicted to be the signal peptide (SignalP 4.1 Server Technical University of Denmark). β-actin and GAPDH were blotted as loading controls.

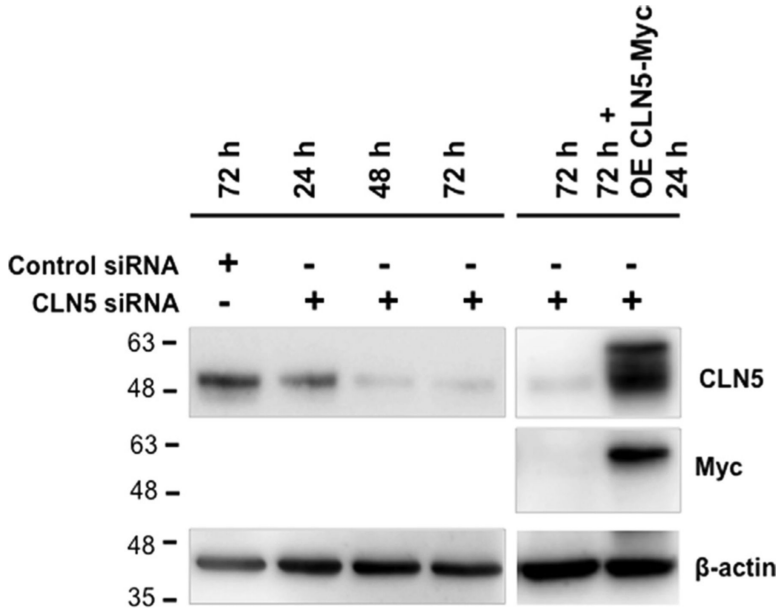

Fig. 2.

The two forms of CLN5 protein are derived from a processing event at the C-terminus. (A) HEK293 cells were transfected with either control siRNA or CLN5 siRNA. The cells were collected 24, 48, and 72 h post transfection and analyzed by immunoblotting for CLN5 (left panel). In a separate experiment, after 48 h CLN5 siRNA transfection, cells were transfected with wild type CLN5 for 24 h to over-express (OE) Myc-tagged siRNA resistant CLN5 (right panel). Immunoblotting was followed by CLN5 and Myc antibodies. β-actin was blotted as a loading control.

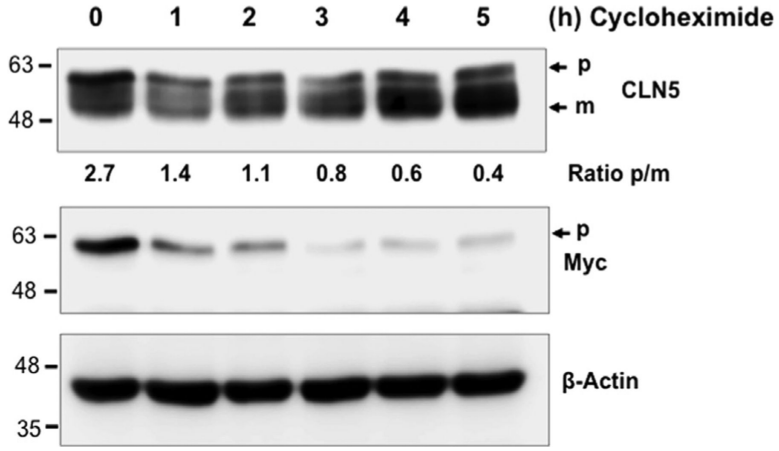

Fig. 3.

Post-translational proteolytic processing of CLN5 at the C-terminus. The CLN5 proprotein (as indicated “p”) is gradually cleaved and the processed mature form (as indicated “m”) of CLN5 increases following cycloheximide chase treatment. HEK293 cells were overexpressed with Myc-tagged CLN5. 24 h after transfection cells were incubated with DMEM containing cycloheximide (50 μg/ml) for the indicated time intervals. The whole cell lysates were analyzed by Western blotting with CLN5 and Myc antibodies. β-actin was blotted as a loading control. The intensity of proprotein (p) and mature form (m) of CLN5 was quantified using C-DiGit software (Li-COR) and the ratio is as indicated.

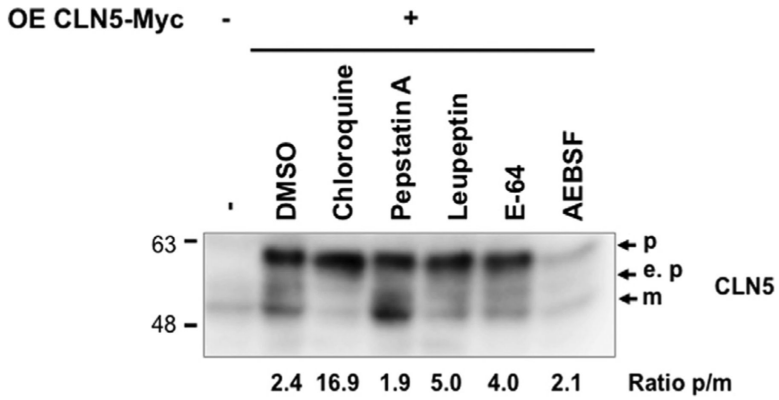

Fig. 4.

CLN5 processing takes place in low pH environment and is sensitive to cysteine protease inhibitors. HEK293 cells were transfected with Myc-tagged wild type CLN5. DMSO, Chloroquine (50 μM), Pepstatin A (5 μM), Leupeptin (100 μM), E-64 (10 μg/ml) and AEBSF (150 μM) were added to the medium 4 h post transfection. Cells were incubated with drug and transfection reagent for another 24 h. The whole cell lysates were analyzed by Western blotting with CLN5 antibody. The intensity of proprotein (p) and mature form (m) of CLN5 was quantified using C-DiGit software (Li-COR) and the ratio is as indicated. “e. p” indicates endogenous proprotein. OE: overexpression.

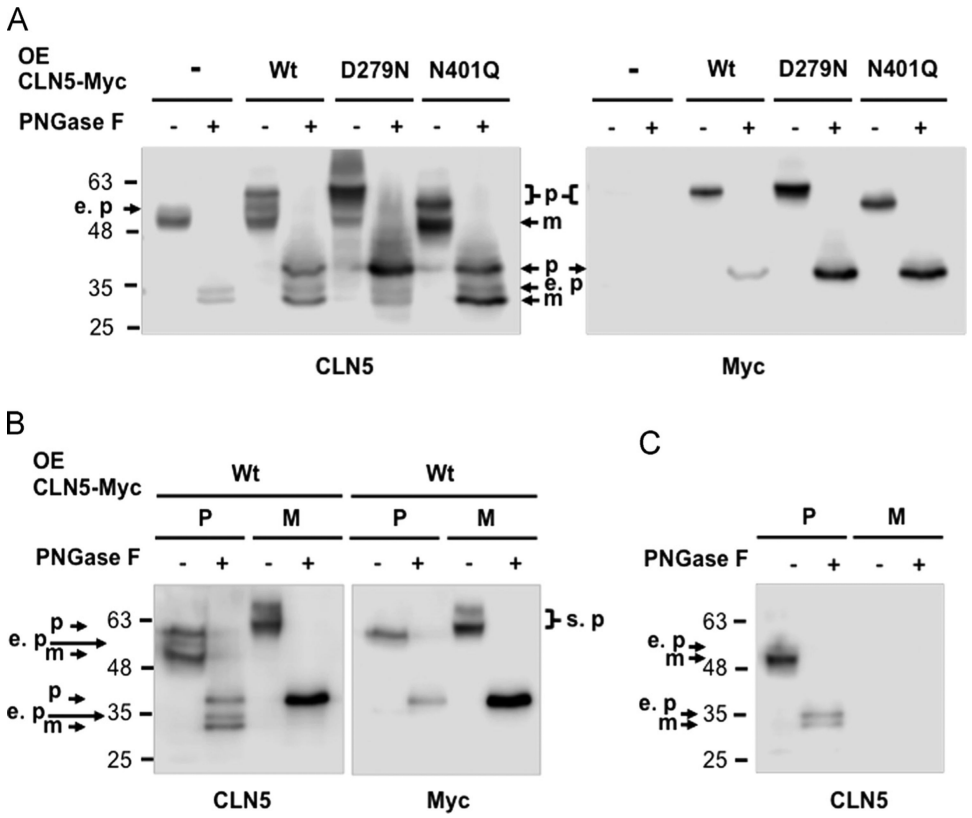

Fig. 5.

The different CLN5 forms are not due to glycosylation variants. (A) The two forms (p and m) of CLN5 are not derived from heterogeneous glycosylation. HEK293 cells were either mock transfected or transfected with Myc-tagged wild type CLN5 (Wt), CLN5 mutants D279N or N401Q for 24 h. Total lysates were treated with or without PNGase F and analyzed by immunoblotting with CLN5 and Myc antibodies. (B) The mature form of CLN5 protein is not secreted. Cell were transfected with Myc-tagged wild type CLN5 (Wt) in Opti-MEM for 24 h. Cell pellets and media were collected, treated with or without PNGase F, and analyzed by Western blotting with CLN5 and Myc antibodies. (C) Cell pellets and media were collected from non-transfected HEK293 grown 24 h in Opti-MEM. Samples were treated as described in (B). OE, overexpression; p, proprotein; m, mature form; e. p, endogenous proprotein; s. p, secreted proprotein; P, pellet; M, medium.

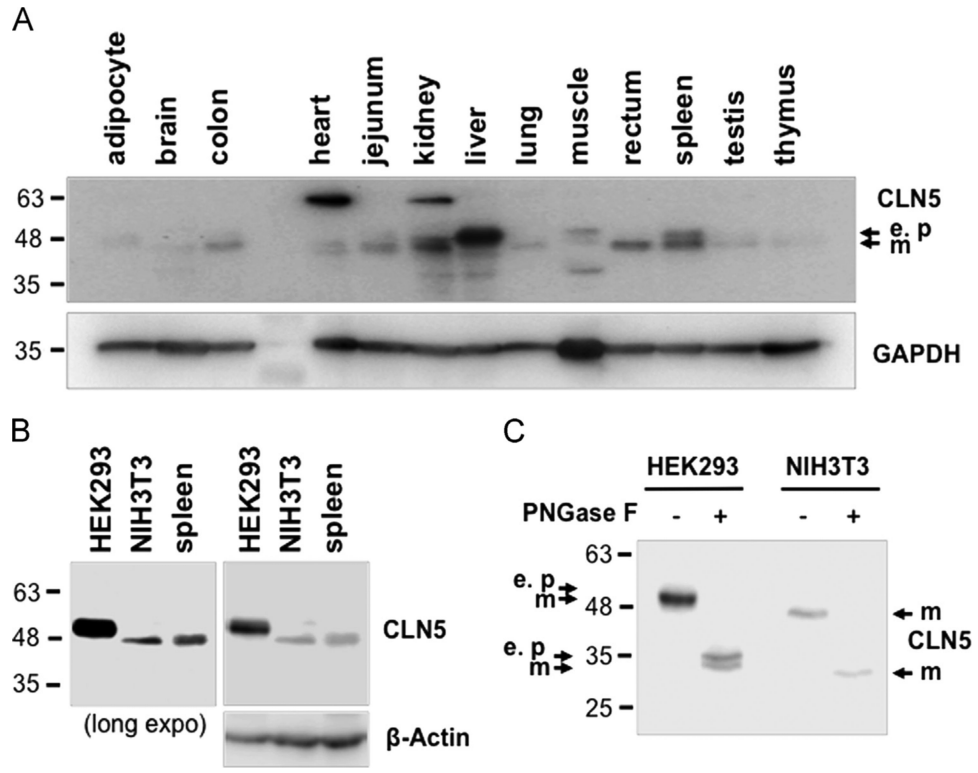

Fig. 6.

CLN5 expression in murine tissues and cell lines. (A) Surveying CLN5 levels in different mouse tissues. A variety of mouse tissues as indicated were collected and the protein extracts (50 μg) were analyzed by Western blotting with CLN5 antibody. (B) Mouse CLN5 is smaller in size compared to human CLN5 in HEK293 cell line. Total cell lysates from mouse spleen (120 μg), mouse cell line NIH3T3 (120 μg), and human cell line HEK293 (60 μg) were analyzed by Western blotting with CLN5 antibody. Long exposure of CLN5 immunoblotting is shown on the left. (C) Deglycosylation of CLN5 from HEK293 and NIH3T3 by PNGaseF treatment. β-actin was blotted as a loading control. e. p, endogenous proprotein; m, mature form.