Published in final edited form as:

Biosci Rep. 2009 June ; 29(3): 173–181. doi:10.1042/BSR20080143.

CLN6 MUTANTS ASSOCIATED WITH NEURONAL CEROID LIPOFUSCINOSIS ARE DEGRADED IN A PROTEASOME DEPENDENT MANNER

Kristina Oresic[¶], Britta Mueller[†], and Domenico Tortorella^{¶,‡}

[¶]Mount Sinai School of Medicine, Department of Microbiology, New York, NY 10029

[†]Whitehead Institute for Biomedical Research, Cambridge, MA 02142

Abstract

Neuronal ceroid lipofuscinoses (NCL), a group of inherited neurodegenerative lysosomal storage diseases predominantly affecting children, are due to autosomal recessive mutations within one of the nine *cln* genes. The wild type *cln* gene products are comprised of membrane and soluble proteins that localize to the lysosome or endoplasmic reticulum. However, the destiny of the Cln variants has not been fully characterized. To explore a possible link between ER quality control and processing of Cln mutants, we investigated the fate of two NCL-related Cln6 mutants found in patient samples (Cln6^{G123D} and Cln6^{M241T}) in neuronal-derived human cells. The point mutations are predicted to be in the putative transmembrane domains and most likely generate misfolded membrane proteins that are subjected to ER quality control. Consistent with this paradigm, both mutants underwent rapid proteasome-mediated degradation and complexed with components of the ER extraction apparatus, Derlin-1 and p97. In addition, knock down of SEL1L, a member of an E3 ubiquitin ligase complex involved in ER protein extraction rescued significant amounts of Cln6^{G123D} and Cln6^{M241T} polypeptides. The results implicate ER quality control in the instability of the Cln variants that likely contributes to the development of NCL.

INTRODUCTION

Neuronal ceroid lipofuscinoses (NCL), also referred to as Batten disease, are the most common pediatric neurodegenerative diseases having an incidence rate of 1 in 12,500 in the US population [1]. Their significant clinical feature is progressive encephalopathy confined to macular cerebral degeneration [2]. The symptoms of NCL encephalopathy include epileptic seizures, dementia, progressive psychomotor decline, visual retrocession, and blindness and ultimately leads to premature death [3]. The histological hallmark of NCL is the accumulation of proteolipid pigment deposits in lysosomes of many cell types [4,5]. The patients suffering from Batten disease show intracellular inclusions predominately in neurons caused by lysosomal accumulation of ceroid and lipofuscin with mitochondrial ATP synthase subunit C [6].

Most NCL are inherited as autosomal recessive mutations within 6 identified and 3 unidentified genes denominated *cln1-9*. Defective Cln proteins result in lysosomal dysfunction and accumulation of lysosomal storage material in the brain of affected children, leading to development of several pathogenic processes and neurodegeneration. Current genetic

[‡]Address correspondence to: Domenico Tortorella, Department of Microbiology, One Gustave L. Levy Place, Box 1124, New York, NY 10029, Office: 212-241-5447 Fax: 212-241-7336 Email: Domenico.Tortorella@mssm.edu

classification of NCLs vary in clinical phenotypes from more benign to extremely severe depending on the specific genetic defect of the encoded protein. They are divided into four main groups, infantile NCL, late infantile NCL, juvenile JNCL, and adult NCL and four other less frequent entities. The Cln polypeptides are both soluble and membrane proteins located in either endosomal-lysosomal compartments (Cln1:palmitoyl-protein thioesterase, Cln2:tripeptidyl peptidase I, Cln3, Cln5) or the ER (Cln6, Cln8) [7]. Cln4, Cln7, and Cln9 proteins remain to be identified. Interestingly, the processing and fate of aberrant Cln proteins is not clear. In fact, hereditary mutations within cellular genes can result in clinically significant pathologies such as cystic fibrosis, hereditary emphysema [8], nephrogenic diabetes insipidus [9], and oculocutaneous albinism [10]. Since ER quality control has been implicated in these diseases, it is likely that it plays a role in processing of Cln variants and development of NCL.

Mutations within the *cln6* gene cause both the classical late-infantile and juvenile forms of NCL [11]. Our study explores the role of ER quality control in the early processing events of ER localized Cln6 mutant proteins [12-14]. Experiments revealed that Cln6 mutant proteins were degraded in a proteasome dependent manner. In addition, knock down of SEL1L, a protein involved in disposal of aberrant ER proteins, rescued the degradation of the Cln6 mutants. The data supports a paradigm that ER quality control may contribute to the onset of NCL type 6 and offers insight towards potential therapy against NCL.

EXPERIMENTAL

Cell lines and antibodies

Human U373-MG astrocytoma cells expressing Cln6 polypeptides (U373_{Cln6wt}, U373_{Cln6G123D} and U373_{Cln6M241T} cells) were generated and maintained as described [15]. Anti-PDI [16], anti-SEL1L [17], and anti-calnexin antibody (AF8 [18], gift from M. Brenner (Harvard Medical School) were utilized as described. Anti-GAPDH, anti-p97, anti-Derlin-1, and anti-BiP antibodies were purchased from Chemicon International Inc., Sigma, and Stressgen, respectively.

cDNA constructs

The Cln6 chimeras were cloned from the Cln6 template (ATTC Image ID 3878776) with an amino-terminus hemagglutinin (HA) epitope tag (AYPYDVPDYA) into the retroviral vector pLpCX (Clontech). Cln6^{G123D} and Cln6^{M241T} site-directed mutant cDNAs were generated from PCR fragments [15].

Immunoprecipitation

Immunoprecipitations were performed as described [15]. In brief, cells (1×10^6) were lysed in 0.5% Nonident P (NP)-40 lysis mix followed by incubation with the respective antibody and protein A-agarose beads. The immunoprecipitates were resolved using SDS-PAGE (12.5%) and subjected to immunoblot analysis using the respective immunoglobulin.

Pulse-chase analysis

Pulse-chase experiment was performed as described [15]. Cells were labeled with ³⁵S-methionine and chased in cold methionine (25 mM). Cln6 proteins were recovered from NP-40 cell lysates using anti-HA antibodies and resolved using SDS-PAGE (12.5%). The polyacrylamide gel was dried, exposed to autoradiography film and polypeptides were quantified by densitometry analysis using an Alpha Imager 3400.

Immunofluorescence microscopy

Cells were fixed with methanol/acetone solution (v/v 1:1) and incubated with blocking solution (1% BSA, 0.5% cold water fish gelatin (Sigma), PBS pH 7.2) followed by the respective antibody. The cells were then incubated with the respective anti-mouse^{FTTC} and anti-rabbit^{Texas Red} (Molecular Probes) immunoglobulins. Fluorescence microscopy was carried out using an Olympus IX70/IX-FLA inverted fluorescence microscope and a Sony DKC-5000 digital camera. Images were created with Adobe Photoshop software.

RESULTS

Cln6 mutant molecules are stabilized by inclusion of proteasome inhibition

Cln6 is a 311 amino acid non-glycosylated ER membrane protein predicted to contain either five or six transmembrane domains [12,14]. To investigate the processing of Cln6 mutants implicated in the development of NCL, a hemagglutinin (HA) epitope tag was introduced at the amino terminus of Cln6^{wt} and Cln6 variants (Cln6^{G123D} and Cln6^{M241T}) with missense mutations in the predicted transmembrane domains [19]. These mutants were chosen for study because they were identified from NCL patients [12-14]. These polar residues within the membrane bilayer most likely cause the mutant to exist in a misfolded conformation and potential a target for the ER quality control apparatus. Hemagglutinin-epitope tagged versions of Cln6^{wt}, Cln6^{G123D}, and Cln6^{M241T} were transduced into U373 cells (U373^{Cln6wt}, U373^{Cln6G123D}, and U373^{Cln6M241T} cells) and the stability of the Cln6 proteins was examined by pulse-chase analysis (Figure 1A). U373^{Cln6wt}, U373^{Cln6G123D}, and U373^{Cln6M241T} cells were metabolically labeled for 15 min and chased for up to 45 min. Equivalent levels of wild type Cln6 polypeptides were recovered throughout the chase period (Figure 1A, lanes 1-4), a result consistent with protein stability. In contrast, levels of Cln6^{G123D} polypeptides rapidly decreased during the chase period (Figure 1A, lanes 5-8) with a half-life of ~20 min (Figure 1B). Interestingly, the migration difference of Cln6^{G123D} versus wild type Cln6 or Cln6^{M241T} is probably due to a conformational change in the overall structure that is specific to the mutation. A similar result was observed in mutants of the multi-spanning membrane protein Sec61p and the soluble protein p97 [20,21]. Even more striking was the instability of Cln6^{M241T} polypeptides (Figure 2A, lanes 9-12). Only a minute amount of Cln6^{M241T} polypeptides was recovered from the 0 min chase period and even less from the 15 min chase point suggesting a half-life of less than 15 min (Figure 2A, lane 10). These data demonstrate that Cln6 mutants are degraded with fast kinetics.

To examine whether the Cln6 mutants are degraded by the proteasome, Cln6 protein levels were examined from U373^{Cln6wt}, U373^{Cln6G123D}, and U373^{Cln6M241T} cells treated with or without the proteasome inhibitor carboxybenzyl-leucyl-leucyl-leucyl vinyl sulfone (ZL₃VS) (10μM, 5 hr) [22]. Cln6 proteins recovered from the respective cells were analyzed using an immunoprecipitation/immunoblot experiment (Figure 2A). U373 cells transduced with empty vector were used as a negative control (Figure 2A, lanes 9 and 11). Equivalent levels of Cln6^{wt} were recovered independently of inhibitor treatment (Figure 2A, lanes 1-2 and 5-6), whereas a minimal number of polypeptides was recovered from U373^{Cln6G123D} and U373^{Cln6M241T} cells (Figure 2A, lanes 3 and 7). However, significant amounts of Cln6^{G123D} and Cln6^{M241T} polypeptides were recovered from ZL₃VS-treated cells (Figure 2A, lanes 4 and 8). Because Cln6^{G123D} is degraded with slower kinetics than Cln6^{M241T}, we observed increased levels of Cln6^{G123D} than Cln6^{M241T} from proteasome treated cells (Figure 2A, lane 4 vs. 8). Consistent with this data, higher molecular weight, most likely ubiquitinated, Cln6^{G123D} and Cln6^{M241T} species (*) were recovered from proteasome inhibitor-treated cells (Figure S1). Collectively, these data verify that Cln6^{G123D} and Cln6^{M241T} are extracted from the ER to the cytosol and degraded by the proteasome.

To further confirm that the instability of Cln6^{G123D} and Cln6^{M241T} polypeptides was due to their transport out of the ER, we examined the effect of eeyarestatin, an inhibitor of the dislocation reaction [23]. U373, U373_{Cln6wt}, U373_{Cln6G123D}, and U373_{Cln6M241T} cells were treated with or without eeyarestatin (5 µg/mL) or the proteasome inhibitors lactacystin [24] (10 µM) or ZL₃VS (5 µM) for 12 hr. Total cell lysates were subjected to immunoblot analysis using anti-HA antibodies (Figure 2B). An anti-GAPDH immunoblot verified equal protein loading (Figure 2B, lanes 20-38). As expected, treatment with the proteasome inhibitors lactacystin and ZL₃VS caused a significant increase in both Cln6^{G123D} and Cln6^{M241T} polypeptides (Figure 2B, lanes 10, 12, 16, and 18, respectively). Moreover, eeyarestatin treatment caused increased levels of Cln6^{G123D} and Cln6^{M241T} polypeptides (Figure 2B, lanes 4 and 6), albeit to a lower level than the proteasome inhibitors. This is probably due to the inability of eeyarestatin to completely attenuate dislocation of ER substrates [23]. Eeyarestatin treatment caused a greater accumulation of Cln6^{G123D} than Cln6^{M241T} probably due to differences in their degradation kinetics (Figure 1B). In summary, Cln6^{G123D} and Cln6^{M241T} polypeptides were stabilized upon inclusion of proteasome or dislocation inhibitors.

Stabilized Cln6 mutant proteins are localized to the ER

The localization of the stabilized Cln mutant Cln6^{G123D} was examined using fluorescence microscopy (Figure 3). U373_{Cln6wt} and U373_{Cln6G123D} cells treated with or without ZL₃VS (2.5 µM, 12 hr) were probed for Cln6 or PDI polypeptides using anti-HA (Figure 3, panels 1-2 and 7-8) and anti-PDI (Figure 3, panels 3-4 and 9-10) antibodies, respectively. The merged images represent the co-localized polypeptides (Figure 3, panels 5-6 and 11-12). The intensity of the Cln6^{wt} fluorescence signal was independent of proteasome-inhibitor treatment (Figure 3, panels 1-2) and co-localized with the ER resident protein PDI (Figure 3, panels 5-6). In contrast, a significant increase in the fluorescence signal was observed in U373_{Cln6G123D} cells treated with proteasome inhibitor (Figure 3, compare panels 7 and 8). The ER localization of Cln6^{G123D} polypeptides upon proteasome inhibition was confirmed by their co-localization with PDI (Figure 3, panel 12). In addition, a subcellular fractionation experiment [25] revealed that the majority of Cln6^{G123D} and Cln6^{M241T} mutant polypeptides was observed from the membrane fraction upon proteasome inhibitor treatment (Figure S2). These results demonstrate that stabilized Cln6 mutants are localized to the ER.

Cln6^{G123D} and Cln6^{M241T} polypeptides interact with the dislocation complex

The dislocation of ER proteasomal substrates is mediated through the interaction with both ER and cytosolic proteins. Derlin-1, an ER membrane protein essential for dislocation of certain ER degradation substrates, together with the cytosolic complex of AAA ATPase p97/Ufd1/Npl4 facilitates the extraction of ER proteins out of the ER [26]. To further define the processing of the Cln6^{G123D} and Cln6^{M241T} mutants, we examined whether Cln6^{G123D} and Cln6^{M241T} complex with the specific components of the dislocation apparatus, Derlin-1 and p97. Derlin-1 was recovered from U373_{Cln6wt}, U373_{Cln6G123D}, and U373_{Cln6M241T} cells treated with or without ZL₃VS (10 µM, 5 hr) using anti-Derlin-1 antibodies. The immunoprecipitates and total cell lysates were subjected to an anti-HA (Figure 4A, lanes 1-14) and anti-Derlin-1 (Figure 4A, lanes 15-28) immunoblot. As expected, Derlin-1 polypeptides were recovered from all samples (Figure 4A, lanes 15-28). Consistent with previous data (Figure 2), Cln6^{G123D} and Cln6^{M241T} were stabilized in the presence of proteasome inhibitor (Figure 4A, lanes 11 and 13). Both Cln6^{G123D} and Cln6^{M241T} polypeptides co-precipitated with Derlin-1 molecules from proteasome inhibitor-treated U373_{Cln6G123D} and U373_{Cln6M241T} cells (Figure 4A, lanes 4 and 6). The lack of a polypeptide signal from U373 and U373_{Cln6wt} cells (Figure 4A, lanes 1-2 and 7) confirmed the specificity of the anti-Derlin-1 immunoprecipitation. These results suggest that Derlin-1 is probably involved in the dislocation of Cln6^{G123D} and Cln6^{M241T} mutants.

We next examined a possible interaction between Cln6^{G123D} and Cln6^{M241T} and p97 (Figure 4B and C). Cln6 polypeptides recovered from U373^{Cln6wt}, U373^{Cln6G123D}, and U373^{Cln6M241T} cells treated with or without ZL₃VS (5 μ M, 12 hr) were subjected to immunoblot analysis. As expected, significant amounts of Cln6^{G123D} and Cln6^{M241T} proteins were recovered from the proteasome inhibitor treated cells (Figure 4B and C, lane 9). p97 exclusively co-precipitated with Cln6^{G123D} and Cln6^{M241T} from ZL₃VS-treated cells (Figure 4B and C, lane 4) and not with Cln6^{wt} (Figure 4A and B, lanes 1-2). Collectively, these results suggest that Derlin-1 and p97 probably mediate the extraction of Cln6^{G123D} and Cln6^{M241T} polypeptides from the ER and into the cytosol.

Cln6^{G123D} and Cln6^{M241T} are dislocated in a SEL1L dependent manner

Ubiquitination of ER degradation substrates through a specific E3 ligase is a prerequisite for the dislocation of most degradation substrates [27]. In fact, ER localized membrane proteins that complex with E3 ligases play an important role in the dislocation process. Sel-1 suppressor of lin-12-like (SEL1L), an ER membrane protein that complexes with the E3 ligase HMG Co-A reductase degradation protein 1 (Hrd1), Derlin-1, Derlin-2, and p97 [28,29] has been implicated in the dislocation of both membrane and soluble degradation substrates [17,30]. Is SEL1L involved in the degradation of Cln6^{G123D} and Cln6^{M241T}? To address this question, we examined the steady state levels of Cln6 polypeptides in cells that stably express shRNA against SEL1L (shRNA-SEL1L) or GFP (shRNA-GFP) [17]. The shRNA-GFP cells were utilized as a negative control. U373^{Cln6wt}, U373^{Cln6G123D}, and U373^{Cln6M241T} cells expressing shRNA-SEL1L or shRNA-GFP were treated with proteasome inhibitor ZL₃VS (10 μ M, 5 hr). The total cell lysates were subjected to immunoblot analysis to determine the levels of Cln6 polypeptides (Figure 5). Cln6^{wt} levels were not altered upon inclusion of proteasome inhibitor or expression of shRNAs (Figure 5, lanes 1-3 and 10-12). As expected, in shRNA-GFP expressing cells, the levels of Cln6^{G123D} and Cln6^{M241T} increased upon the treatment with proteasome inhibitor (Figure 5, lanes 4-6 and 7-9, respectively). Strikingly, significant levels of both Cln6^{G123D} and Cln6^{M241T} polypeptides were observed from SEL1L knock down cells (Figure 5, lanes 13-18). Since SEL1L was knocked down only ~50% (Figure S3), a greater attenuation of Cln6^{G123D} and Cln6^{M241T} degradation would be probably observed upon a complete knock down of SEL1L. The GAPDH immunoblot confirmed equal loading of cell lysates (Figure 5, lanes 20-38). These results strongly support the hypothesis that Cln6^{G123D} and Cln6^{M241T} degradation is dependent on SEL1L and is regulated by ER quality control.

DISCUSSION

NCL are neurodegenerative childhood diseases caused by mutations within 9 different *cln* genes [31]. Cln mutants are either truncated versions of the protein or contain point mutations yielding enzymatically inactive polypeptides or molecules that never reach their final destination. The early processing events within the ER can dictate the fate of these mutant proteins. In fact, specific genetic diseases including cystic fibrosis, emphysema, Fabri disease, Gaucher disease, GM1 Gangliosidosis, and Tay-Sachs disease are caused by mutations within the proteins that are subjected to ER quality control [32,33]. The present study shows for the first time that the Cln6 mutants Cln6^{G123D} and Cln6^{M241T} implicated in the onset of NCL type 6 are unstable and degraded in a proteasome dependent manner. Hence, the development of NCL may be attributed to the instability of the mutant polypeptide implying that ER quality control contributes to the onset of disease.

ER quality control has evolved to prevent the egress of defective membrane or secretory polypeptides from the ER. In most cases, the misfolded proteins are recognized by ER chaperones such as BiP, calnexin, and PDI [34,35] and extracted through the ER membrane via a proteinaceous channel to be degraded by the proteasome. The recruitment of the

degradation substrate to the dislocon occurs through an interaction with membrane-bound ubiquitin-conjugating enzymes (i.e. Ubc7, Ubc1, Ubc6), E3 ubiquitin ligases (i.e. gp78, Hrd1/Der3, Doa10, HsHrd1, trc8, and TEB4) [27], and factors such as the Derlin family of proteins and SEL1L. Once at the site of dislocation, p97 complexed with Npl4 and Ufd1 extracts the ubiquitin-conjugated dislocated substrate through the membrane [29,36]. The N-linked glycan attached to the polypeptide would then be efficiently removed followed by deubiquitination and degradation by the proteasome. The mutant Cln6 proteins are degraded as classical misfolded proteins as observed by their association with ER chaperones calnexin and BiP (data not shown) as well as with Derlin-1 and p97 (Figure 4). Since the knock down of SEL1L caused an increase in levels of Cln6 mutants, it is possible that SEL1L contributes to the instability of Cln6^{G123D} and Cln6^{M241T} proteins. Collectively, the data is consistent with the paradigm that Cln6^{G123D} and Cln6^{M241T} mutant proteins are recognized and disposed of by the ER dislocation/degradation apparatus.

The Cln6^{G123D} and Cln6^{M241T} mutants are degraded with fast kinetics (Figure 1). Given the fact that the Cln6 protein spans the membrane seven times, we would expect that the degradation kinetics would be similar to the degradation kinetics of CFTR mutants [37]. Why are these aberrant proteins degraded in such a fast manner? Since both Cln6 mutations lie within the transmembrane regions, the amino acid changes may disrupt the stability of the transmembrane domains and induce a drastically misfolded protein that is quickly discarded for degradation by the proteasome. The severity of the misfolded state of the protein can directly dictate the half-life of the polypeptide. Therefore, Cln6^{G123D} would be in a more native state than Cln6^{M241T}. Nevertheless, the dramatic instability of the Cln6 mutants can prove to be a useful model system to study how multi-membrane spanning proteins are dislocated across the ER membrane.

The degradation of aberrant Cln6 polypeptides implies that those proteins are thwarted in their cellular function that eventually leads to the accumulation of insoluble proteolipid material in the lysosomes. It remains to be clarified whether increasing the stability of these mutants would allow for the Cln mutant to regain at least partial wild type activity. Therefore, proteasome inhibitors may contribute to the considered as potential therapeutics for NCL type 6 if the stabilized Cln6 mutant would retain at least partial activity. In fact, understanding the process of ER quality control in the development of specific genetic diseases has already given promising results in the treatment of Fabry disease caused by mutations in alpha-galactosidase polypeptides. Namely, 1-deoxygalactonojirimycin, a competitive inhibitor of alpha-galactosidase could stabilize alpha-galactosidase mutant and correct its trafficking defect [38]. In addition, chemical chaperones could rescue some lysosomal storage diseases caused by inherited mutations such as in Gaucher disease, GM1 Gangliosidosis and Tay-Sachs disease [39-41]. The current study serves as a preliminary guide for identifying potential new treatments against NCL that could be examined in the available animal models. A number of spontaneous animal forms of NCL have been described including sheep, dogs, and mice [42-44]. The mouse models of NCLs, *Ppt1*^{-/-} [45], *Ppt1*^{Δex4} knockout [46], *Tpp1*^{neo^{ins}Arg446His} knockout [47], *Cln5*^{-/-} knockout [48], Cln6^{nclf} spontaneous mutant [49], Cln8^{mnd} spontaneous mutant [50], *Cln3*^{-/-} knockout [51], *Cln3*^{Δex7/8} knock-in [52], *Cln3* knockout [53] demonstrate phenotypes that closely resemble the major features of human NCLs. They all show clinically relevant behavioral and pathological changes that exhibit a progressive neurodegenerative disorder. The value of animal models for neurodegenerative disorders lies largely in the utility of these models for evaluating potential therapeutic interventions in the future [54].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

We thank Caroline Ng for critical analysis of the manuscript.

Funding: This study was supported by the National Institutes of Health Grants [# AI060905 and #U19 AI062623]. DT is partially supported by the Irma T. Hirsch Trust.

References

1. Rider JA, Rider DL. Batten disease, a twenty-eight-year struggle: past, present and future. *Neuropediatrics* 1997;28:4–5. [PubMed: 9151308]
2. Goebel HH, Wisniewski KE. Current state of clinical and morphological features in human NCL. *Brain Pathol* 2004;14:61–69. [PubMed: 14997938]
3. Kohlschutter A, Gardiner RM, Goebel HH. Human forms of neuronal ceroid-lipofuscinosis (Batten disease): consensus on diagnostic criteria, Hamburg 1992. *J Inherit Metab Dis* 1993;16:241–244. [PubMed: 8411970]
4. Wisniewski KE, Rapin I, Heaney-Kieras J. Clinico-pathological variability in the childhood neuronal ceroid-lipofuscinoses and new observations on glycoprotein abnormalities. *Am J Med Genet Suppl* 1988;5:27–46. [PubMed: 3146325]
5. Prasad VV, Pullarkat RK. Brain lysosomal hydrolases in neuronal ceroid-lipofuscinoses. *Mol Chem Neuropathol* 1996;29:169–179. [PubMed: 8971694]
6. Palmer DN, Tyynela J, van Mil HC, Westlake VJ, Jolly RD. Accumulation of sphingolipid activator proteins (SAPs) A and D in granular osmiophilic deposits in miniature Schnauzer dogs with ceroid-lipofuscinosis. *J Inherit Metab Dis* 1997;20:74–84. [PubMed: 9061571]
7. Kyttila A, Lahtinen U, Bräulke T, Hofmann SL. Functional biology of the neuronal ceroid lipofuscinoses (NCL) proteins. *Biochim Biophys Acta* 2006;1762:920–933. [PubMed: 16839750]
8. Qu D, Teckman JH, Omura S, Perlmutter DH. Degradation of a mutant secretory protein, alpha1-antitrypsin Z, in the endoplasmic reticulum requires proteasome activity. *J Biol Chem* 1996;271:22791–22795. [PubMed: 8798455]
9. Hirano K, Zuber C, Roth J, Ziak M. The proteasome is involved in the degradation of different aquaporin-2 mutants causing nephrogenic diabetes insipidus. *Am J Pathol* 2003;163:111–120. [PubMed: 12819016]
10. Halaban R, Svedine S, Cheng E, Smicun Y, Aron R, Hebert DN. Endoplasmic reticulum retention is a common defect associated with tyrosinase-negative albinism. *Proc Natl Acad Sci U S A* 2000;97:5889–5894. [PubMed: 10823941]
11. Kida E, Golabek AA, Wisniewski KE. Cellular pathology and pathogenic aspects of neuronal ceroid lipofuscinoses. *Adv Genet* 2001;45:35–68. [PubMed: 11332776]
12. Wheeler RB, Sharp JD, Schultz RA, Joslin JM, Williams RE, Mole SE. The gene mutated in variant late-infantile neuronal ceroid lipofuscinosis (CLN6) and in nclf mutant mice encodes a novel predicted transmembrane protein. *Am J Hum Genet* 2002;70:537–542. [PubMed: 11727201]
13. Gao H, Boustany RM, Espinola JA, Cotman SL, Srinidhi L, Antonellis KA, Gillis T, Qin X, Liu S, Donahue LR, Bronson RT, Faust JR, Stout D, Haines JL, Lerner TJ, MacDonald ME. Mutations in a novel CLN6-encoded transmembrane protein cause variant neuronal ceroid lipofuscinosis in man and mouse. *Am J Hum Genet* 2002;70:324–335. [PubMed: 11791207]
14. Heine C, Quitsch A, Storch S, Martin Y, Lonka L, Lehesjoki AE, Mole SE, Bräulke T. Topology and endoplasmic reticulum retention signals of the lysosomal storage disease-related membrane protein CLN6. *Mol Membr Biol* 2007;24:74–87. [PubMed: 17453415]
15. Oresic K, Noriega V, Andrews L, Tortorella D. A structural determinant of human cytomegalovirus US2 dictates the down-regulation of class I major histocompatibility molecules. *J Biol Chem* 2006;281:19395–19406. [PubMed: 16687410]
16. Tortorella D, Story CM, Huppa JB, Wiertz EJ, Jones TR, Bacik I, Bennink JR, Yewdell JW, Ploegh HL. Dislocation of type I membrane proteins from the ER to the cytosol is sensitive to changes in redox potential. *J Cell Biol* 1998;142:365–376. [PubMed: 9679137]
17. Mueller B, Lilley BN, Ploegh HL. SEL1L, the homologue of yeast Hrd3p, is involved in protein dislocation from the mammalian ER. *J Cell Biol* 2006;175:261–270. [PubMed: 17043138]

18. Hochstenbach F, David V, Watkins S, Brenner MB. Endoplasmic reticulum resident protein of 90 kilodaltons associates with the T- and B-cell antigen receptors and major histocompatibility complex antigens during their assembly. *Proc Natl Acad Sci U S A* 1992;89:4734–4738. [PubMed: 1584811]
19. Teixeira CA, Espinola J, Huo L, Kohlschutter J, Persaud Sawin DA, Minassian B, Bessa CJ, Guimaraes A, Stephan DA, Sa Miranda MC, MacDonald ME, Ribeiro MG, Boustany RM. Novel mutations in the CLN6 gene causing a variant late infantile neuronal ceroid lipofuscinosis. *Hum Mutat* 2003;21:502–508. [PubMed: 12673792]
20. Scott DC, Schekman R. Role of Sec61p in the ER-associated degradation of short-lived transmembrane proteins. *J Cell Biol* 2008;181:1095–1105. [PubMed: 18573918]
21. Wang Q, Li L, Ye Y. Regulation of retrotranslocation by p97-associated deubiquitinating enzyme ataxin-3. *J Cell Biol* 2006;174:963–971. [PubMed: 17000876]
22. Wiertz EJ, Tortorella D, Bogyo M, Yu J, Mothes W, Jones TR, Rapoport TA, Ploegh HL. Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. *Nature* 1996;384:432–438. [PubMed: 8945469]
23. Fiebigler E, Hirsch C, Vyas JM, Gordon E, Ploegh HL, Tortorella D. Dissection of the dislocation pathway for type I membrane proteins with a new small molecule inhibitor, eeyarestatin. *Mol Biol Cell* 2004;15:1635–1646. [PubMed: 14767067]
24. Omura S, Fujimoto T, Otoguro K, Matsuzaki K, Moriguchi R, Tanaka H, Sasaki Y. Lactacystin, a novel microbial metabolite, induces neuritogenesis of neuroblastoma cells. *J Antibiot (Tokyo)* 1991;44:113–116. [PubMed: 1848215]
25. Baker BM, Tortorella D. Dislocation of an endoplasmic reticulum membrane glycoprotein involves the formation of partially dislocated ubiquitinated polypeptides. *J Biol Chem* 2007;282:26845–26856. [PubMed: 17650499]
26. Lilley BN, Ploegh HL. A membrane protein required for dislocation of misfolded proteins from the ER. *Nature* 2004;429:834–840. [PubMed: 15215855]
27. Kostova Z, Tsai YC, Weissman AM. Ubiquitin ligases, critical mediators of endoplasmic reticulum-associated degradation. *Semin Cell Dev Biol* 2007;18:770–779. [PubMed: 17950636]
28. Lilley BN, Ploegh HL. Multiprotein complexes that link dislocation, ubiquitination, and extraction of misfolded proteins from the endoplasmic reticulum membrane. *Proceedings of the National Academy of Sciences* 2005;102:14296–14301.
29. Ye Y, Shibata Y, Kikkert M, van Voorden S, Wiertz E, Rapoport TA. Inaugural Article: Recruitment of the p97 ATPase and ubiquitin ligases to the site of retrotranslocation at the endoplasmic reticulum membrane. *Proceedings of the National Academy of Sciences* 2005;102:14132–14138.
30. Kopito RR. Aggresomes, inclusion bodies and protein aggregation. *Trends Cell Biol* 2000;10:524–530. [PubMed: 11121744]
31. Haltia M. The neuronal ceroid-lipofuscinoses: From past to present. *Biochim Biophys Acta*. 2006
32. Hebert DN, Molinari M. In and out of the ER: protein folding, quality control, degradation, and related human diseases. *Physiol Rev* 2007;87:1377–1408. [PubMed: 17928587]
33. Aridor M. Visiting the ER: the endoplasmic reticulum as a target for therapeutics in traffic related diseases. *Adv Drug Deliv Rev* 2007;59:759–781. [PubMed: 17681635]
34. Brodsky JL. The protective and destructive roles played by molecular chaperones during ERAD (endoplasmic-reticulum-associated degradation). *Biochem J* 2007;404:353–363. [PubMed: 17521290]
35. Nakatsukasa K, Brodsky JL. The recognition and retrotranslocation of misfolded proteins from the endoplasmic reticulum. *Traffic* 2008;9:861–870. [PubMed: 18315532]
36. Ye Y, Meyer HH, Rapoport TA. The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. *Nature* 2001;414:652–656. [PubMed: 11740563]
37. Cheng SH, Gregory RJ, Marshall J, Paul S, Souza DW, White GA, O'Riordan CR, Smith AE. Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell* 1990;63:827–834. [PubMed: 1699669]
38. Yam GH, Zuber C, Roth J. A synthetic chaperone corrects the trafficking defect and disease phenotype in a protein misfolding disorder. *FASEB J* 2005;19:12–18. [PubMed: 15629890]
39. Beck M. New therapeutic options for lysosomal storage disorders: enzyme replacement, small molecules and gene therapy. *Hum Genet* 2007;121:1–22. [PubMed: 17089160]

40. Brady RO. Emerging strategies for the treatment of hereditary metabolic storage disorders. *Rejuvenation Res* 2006;9:237–244. [PubMed: 16706651]
41. Sakuraba H, Sawada M, Matsuzawa F, Aikawa S, Chiba Y, Jigami Y, Itoh K. Molecular pathologies of and enzyme replacement therapies for lysosomal diseases. *CNS Neurol Disord Drug Targets* 2006;5:401–413. [PubMed: 16918392]
42. Ahmad-Annuar A, Tabrizi SJ, Fisher EM. Mouse models as a tool for understanding neurodegenerative diseases. *Curr Opin Neurol* 2003;16:451–458. [PubMed: 12869802]
43. Cooper JD, Russell C, Mitchison HM. Progress towards understanding disease mechanisms in small vertebrate models of neuronal ceroid lipofuscinosis. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* 2006;1762:873.
44. Hafezparast M, Ahmad-Annuar A, Wood NW, Tabrizi SJ, Fisher EM. Mouse models for neurological disease. *Lancet Neurol* 2002;1:215–224. [PubMed: 12849454]
45. Gupta P, Soyombo AA, Atashband A, Wisniewski KE, Shelton JM, Richardson JA, Hammer RE, Hofmann SL. Disruption of PPT1 or PPT2 causes neuronal ceroid lipofuscinosis in knockout mice. *Proc Natl Acad Sci U S A* 2001;98:13566–13571. [PubMed: 11717424]
46. Jalanko A, Vesa J, Manninen T, von Schantz C, Minye H, Fabritius AL, Salonen T, Rapola J, Gentile M, Kopra O, Peltonen L. Mice with Ppt1Deltaex4 mutation replicate the INCL phenotype and show an inflammation-associated loss of interneurons. *Neurobiol Dis* 2005;18:226–241. [PubMed: 15649713]
47. Sleat DE, Wiseman JA, El-Banna M, Kim KH, Mao Q, Price S, Macauley SL, Sidman RL, Shen MM, Zhao Q, Passini MA, Davidson BL, Stewart GR, Lobel P. A mouse model of classical late-infantile neuronal ceroid lipofuscinosis based on targeted disruption of the CLN2 gene results in a loss of tripeptidyl-peptidase I activity and progressive neurodegeneration. *J Neurosci* 2004;24:9117–9126. [PubMed: 15483130]
48. Kopra O, Vesa J, von Schantz C, Manninen T, Minye H, Fabritius AL, Rapola J, van Diggelen OP, Saarela J, Jalanko A, Peltonen L. A mouse model for Finnish variant late infantile neuronal ceroid lipofuscinosis, CLN5, reveals neuropathology associated with early aging. *Hum Mol Genet* 2004;13:2893–2906. [PubMed: 15459177]
49. Bronson RT, Donahue LR, Johnson KR, Tanner A, Lane PW, Faust JR. Neuronal ceroid lipofuscinosis (nclf), a new disorder of the mouse linked to chromosome 9. *Am J Med Genet* 1998;77:289–297. [PubMed: 9600738]
50. Cooper JD, Messer A, Feng AK, Chua-Couzens J, Mobley WC. Apparent loss and hypertrophy of interneurons in a mouse model of neuronal ceroid lipofuscinosis: evidence for partial response to insulin-like growth factor-1 treatment. *J Neurosci* 1999;19:2556–2567. [PubMed: 10087069]
51. Mitchison HM, Bernard DJ, Greene ND, Cooper JD, Junaid MA, Pullarkat RK, de Vos N, Breuning MH, Owens JW, Mobley WC, Gardiner RM, Lake BD, Taschner PE, Nussbaum RL. Targeted disruption of the Cln3 gene provides a mouse model for Batten disease. The Batten Mouse Model Consortium [corrected]. *Neurobiol Dis* 1999;6:321–334. [PubMed: 10527801]
52. Cotman SL, Vrbanc V, Lebel LA, Lee RL, Johnson KA, Donahue LR, Teed AM, Antonellis K, Bronson RT, Lerner TJ, MacDonald ME. Cln3(Deltaex7/8) knock-in mice with the common JNCL mutation exhibit progressive neurologic disease that begins before birth. *Hum Mol Genet* 2002;11:2709–2721. [PubMed: 12374761]
53. Katz ML, Shibuya H, Liu PC, Kaur S, Gao CL, Johnson GS. A mouse gene knockout model for juvenile ceroid-lipofuscinosis (Batten disease). *J Neurosci Res* 1999;57:551–556. [PubMed: 10440905]
54. Wendt KD, Lei B, Schachtman TR, Tullis GE, Ibe ME, Katz ML. Behavioral assessment in mouse models of neuronal ceroid lipofuscinosis using a light-cued T-maze. *Behavioural Brain Research* 2005;161:175. [PubMed: 15885820]

A **B**

Figure 1. Cln6^{G123D} and Cln6^{M241T} are degraded with rapid kinetics

(A) U373^{Cln6wt}, U373^{Cln6G123D}, and U373^{Cln6M241T} cells were pulsed with ³⁵S-methionine for 15 min and chased for up to 45 min. Cln6 polypeptides were immunoprecipitated using anti-HA antibodies and resolved on an SDS-polyacrylamide gel. The gel was exposed to autoradiography film for 3 days. The Cln6 proteins and molecular weight markers are indicated. (B) Levels of Cln6 proteins were quantified by densitometry and plotted as a percentage to the 0 min chase point (100%).

Figure 2. Cln6^{G123D} and Cln6^{M241T} proteins are stabilized in the presence of proteasome and dislocation inhibitors

(A) Cln6 polypeptides were recovered from U373^{Cln6wt} (lanes 1-2, 5-6, and 10), U373^{Cln6G123D} (lanes 3-4), and U373^{Cln6M241T} (lanes 7-8) cells treated with or without proteasome inhibitor ZL₃VS. The anti-HA precipitates and total cell lysates from U373^{Cln6wt} and U373 cells were subjected to an anti-HA immunoblot. (B) Total cell lysates from U373^{Cln6wt} (lanes 1-2, 7-8, and 13-14), U373^{Cln6G123D} (lanes 3-4, 9-10, and 15-16), and U373^{Cln6M241T} (lanes 5-6, 11-12, and 17-18) cells treated with or without eeyarestatin (lanes 2, 4, and 6), lactacystin (lanes 8, 10, and 12), and ZL₃VS (lanes 14, 16, and 18) were subjected to anti-HA (lanes 1-19) and anti-GAPDH (lanes 20-38) immunoblots. U373 cell lysates were used as a negative control. The Cln6 proteins, immunoglobulin light chain (IgG LC), GAPDH, and molecular weight markers are indicated.

Figure 3. Stabilized Cln6 mutants localize to the ER

U373_{Cln6wt} (panels 1-6) and U373_{Cln6G123D} (panels 7-12) cells treated with or without proteasome inhibitor ZL₃VS were probed with anti-HA (panels 1-2 and 7-8) and anti-PDI (panels 3-4 and 9-10) antibodies and subjected to indirect immunofluorescence microscopy. The yellow regions in the merged images (panels 5-6 and 11-12) represent the co-localized material.

Figure 4. Cln6 mutants complex with the dislocation apparatus

(A) Derlin-1 was recovered from U373, U373^{Cln6wt}, U373^{Cln6G123D}, and U373^{Cln6M241T} cells treated with or without proteasome inhibitor ZL₃VS using anti-Derlin-1 antibodies. The precipitates (lanes 1-7 and 15-21) and total cell lysates (lanes 8-14 and 22-28) were subjected to immunoblot analysis using anti-HA (lanes 1-14) and anti-Derlin-1 (lanes 15-28) antibodies. Cln6 proteins were recovered from U373^{Cln6wt} (B and C), U373^{Cln6G123D} (B), and U373^{Cln6M241T} (C) cells treated with or without proteasome inhibitor ZL₃VS. The precipitates and U373^{Cln6wt} total cell lysates (B and C) were subjected anti-p97 (B and C, lanes 1-5) and anti-HA (B and C, lanes 6-10) immunoblots. Derlin-1, p97, Cln6 proteins, and molecular weight markers are indicated.

Figure 5. Knockout of SEL1L in cells attenuates Cln6^{G123D} and Cln6^{M241T} degradation

Total cell lysates from U373^{Cln6wt}, U373^{Cln6G123D}, and U373^{Cln6M241T} cells that express shRNA against either GFP (lanes 1-9 and 20-28) or SeL1L (lanes 10-19 and 29-38) were treated with proteasome inhibitor ZL₃VS. The samples were subjected to immunoblot analysis using anti-HA (lanes 1-19) and anti-GAPDH (lanes 20-38) antibodies. The Cln6 proteins, GAPDH, and molecular weight markers are indicated.