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REVIEW

Interactions of the proteins of neuronal ceroid lipofuscinosis: clues to function

Amanda L. Getty · David A. Pearce

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Abstract Neuronal ceroid lipofuscinoses (NCL) are caused by mutations in eight different genes, are characterized by lysosomal accumulation of autofluorescent storage material, and result in a disease that causes degeneration of the central nervous system (CNS). Although functions are defined for some of the soluble proteins that are defective in NCL (cathepsin D, PPT1, and TPP1), the primary function of the other proteins defective in NCLs (CLN3, CLN5, CLN6, CLN7, and CLN8) remain poorly defined. Understanding the localization and network of interactions for these proteins can offer clues as to the function of the NCL proteins and also the pathways that will be disrupted in their absence. Here, we present a review of the current understanding of the localization, interactions, and function of the proteins associated with NCL.

Keywords Batten disease · Lysosomal storage disorder

A. L. Getty · D. A. Pearce (✉)
Sanford Children's Health Research Center,
Sanford Research USD, Sanford School of Medicine
of the University of South Dakota, 2301 East 60th Street North,
Sioux Falls, SD 57104-0589, USA
e-mail: David.Pearce@sanfordhealth.org

A. L. Getty
Department of Biochemistry and Biophysics,
University of Rochester School of Medicine and Dentistry,
Rochester, NY 14642, USA

D. A. Pearce
Department of Pediatrics,
Sanford School of Medicine of the University
of South Dakota, Sioux Falls, SD 57117, USA

Introduction

Lysosomal storage disorders (LSD) are characterized by accumulation of storage material in lysosomes, and all LSD are caused by a protein deficiency due to genetic mutation in the corresponding gene (reviewed in [1]). While causative genetic mutations may be identified in many lysosomal storage disorders, the mechanism underlying why storage material accumulates often remains unclear. The neuronal ceroid lipofuscinoses (NCLs) are characterized by accumulation of autofluorescent storage material and progressive neurodegeneration. NCLs are grouped by the gene that bears an autosomal recessively inherited genetic mutation and the onset of disease symptoms [2]. Mutations in eight genes have been identified that cause an NCL: *CLN1* (*PPT1*) in infantile (INCL), *CLN2* (*TPP1*) in late infantile (LINCL), *CLN3* in juvenile (JNCL), *CLN5* in Finnish variant LINCL, *CLN6* in variant LINCL, *CLN7* in variant LINCL or Turkish variant LINCL, *CLN8* in epilepsy with mental retardation (EPMR) or LINCL variants, and cathepsin D or *CTSD* (*CLN10*) in congenital-NCL. The incidence of NCL worldwide is 1 in 12,500 live births [2], and the most common NCL results from mutations in *CLN3,* causing JNCL (also known as Batten disease). The pathological progression of these diseases has been reviewed in [3, 4]. There is no cure, and treatments are limited to palliative care. Development of NCL treatments is limited by our narrow understanding of the disease.

Three forms of NCL are associated to mutations in soluble lysosomal proteins with known enzymatic function, namely: palmitoyl protein thioesterase-1 (PPT1), tripeptidyl peptidase-1 (TPP1), and cathepsin D (CTSD). Therefore, preclinical research is ongoing for enzyme replacement therapy [5, 6], gene therapy [7, 8], and stem cell therapy [9], which aims to functionally restore these

missing enzymes. Conversely, the functions of the remaining proteins associated with NCL diseases are not fully characterized. CLN5 is soluble, but CLN3 and CLN7 are integral membrane proteins, and each is trafficked to the lysosome. CLN6 and CLN8 are integral membrane proteins associated with the endoplasmic reticulum (ER). Despite these different localizations, dysfunction of any of these proteins results in characteristic autofluorescent storage material and a broadly similar, untreatable disease.

The accumulated storage material in NCL varies in composition, but is generally a combination of proteins, proteolipids, and metals [10]. A main component of the storage material that accumulates in the late-infantile variants and juvenile-NCL is subunit C of the mitochondrial ATP-synthase [11]; however, sphingolipid activating proteins (saposins A and D) are enriched in the infantile NCL (reviewed in [12]). Additionally, saposin D accumulates in congenital-NCL [13]. The heterogeneity of the storage material indicates that the accumulation of storage material may involve disruption of several pathways.

In the past decade, much work in the NCL field focused on small animal models of several of these proteins (reviewed in [4, 14]). However, the primary function of the many of the NCL proteins remains unknown. In this review, we explore the biochemistry of the NCL protein interaction network and its implication for NCL protein function.

NCL-associated soluble proteins in the lysosome

The most rapidly progressing NCL variants are associated with mutations in soluble lysosomal enzymes (Fig. 1a, Table 1). Deficiencies in these enzymes probably cause specific lack of digestion of metabolic substrates, which may contribute to lipofuscin accumulation directly. However, these complex diseases cause accumulation of heterogeneous mixtures of lipofuscin, which indicates that these proteins may be involved in elaborate pathways. While congenital-NCL, INCL, and LINCL are caused by loss of the specific enzymatic activities of CTSD, PPT1, and TPP1, endogenous substrates that are critical for the development of NCL remain to be identified. CLN5, another soluble lysosomal protein with mutations that cause NCL, has unknown function. For each of these proteins, determining the critical function that is lost in NCL will be dramatically important for understanding not only NCL pathology, but also the fundamental role these proteins pay in the lysosome and the cell.

Cathepsin D

Mutations in cathepsin D (*CTSD* or *CLN10*), a lysosomal asparatyl endopeptidase [15, 16], cause accumulation of autofluorescent storage material and neurodegeneration typical of NCL pathology, with very rapid progression causing death at or before birth [13, 17]. One patient with a LINCL-like progression of symptoms had two missense mutations in *CTSD* (W383C, F229I) causing defects in posttranslational processing and targeting to the lysosome of CTSD, as well as diminished enzyme activity [18]. Evidence for NCL pathology due to mutations or deletions of *CTSD* homologs has also been found in *Drosophila*, mice, sheep, and American bulldogs [19–21]. Severity of the phenotype seems to correlate with residual enzyme activity as point mutations in some patients [18], and American bulldogs [19] have a milder phenotype than mutations that result in inactivation or truncation of the protein, which result in congenital NCL pathology [13, 21]. *Ctsd*$^{-/-}$ mice exhibit normal embryonic development, but postnatal apoptosis is seen in several tissues, including the retina and thymus, and mice die by postnatal day 26 with neuronal loss, autofluorescent storage material accumulation, astrogliosis and microglia activation, and intestinal necrosis and atrophy [22, 23]. Recently, the peripheral pathology of these mice was examined further by viral (AAV) vector-mediated gene transfer of *CTSD* either administered to the viscera or brain of the mice [24]. Only native AAV-*CTSD* injected into forebrains of *Ctsd*$^{-/-}$ mice prolonged lifespan, prevented CNS pathology, and restored visceral organ integrity [24]. Enzyme activity was required to rescue these certain pathologies in *Ctsd*$^{-/-}$ mice [24]. CTSD resides in the lysosome, but during certain conditions CTSD has been found to leak out of the lysosome or be secreted out of cells, such as neurons, which could explain the rescue of visceral pathology with forebrain injection of AAV-*CTSD* [24], and in certain cancers [25] reviewed in [16].

Though the optimum pH for CTSD activity is acidic, as in the lysosome, CTSD can cleave Tau at pH 7.0 indicating a potential involvement in Alzheimer's disease [26] or pro-apoptotic Bcl-2 protein Bid at pH 6.2, perhaps mediating apoptosis pathways directly [27]. CTSD has many proposed functions other than as an aspartyl endopeptidase, which may be pathologically relevant. The numerous substrates demonstrated for CTSD and accompanying proposed functions for CTSD have been reviewed in [16], but those potentially relevant for NCL disease will be discussed here.

CTSD undergoes post-translational processing during its maturation to the lysosome. In the ER, prepro-CTSD receives N-linked glycosylation on two asparagine residues, and then is transported to the Golgi for further processing and phosphorylation at position six of mannose residues. These mannose-6-phosphates (M6P) are recognized by mannose-6-phosphate receptors (MPR) that segregate lysosomal enzymes in the *trans*-Golgi network for transport to the endosomes and lysosomes. Once in the acidic environment of

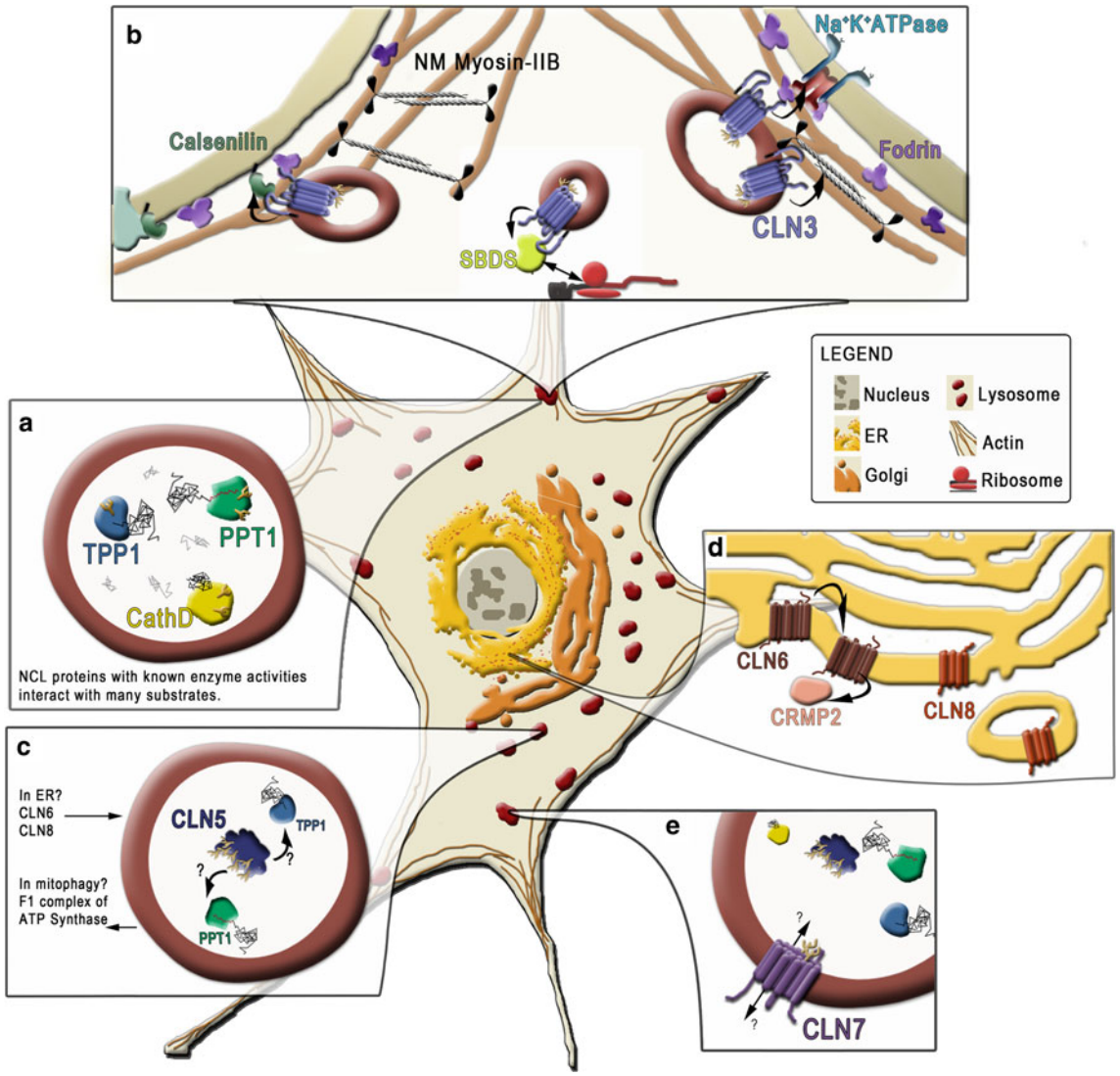

Fig. 1 The interactions and locations of NCL proteins. **a** Soluble NCL proteins with known enzymatic activity: *PPT1*, *TPP1*, and cathepsin D (*CathD*), are localized to the lysosome and interact with their substrates in the lysosome lumen. **b** *CLN3* has been reported to localize to several subcellular locations and, as described in the text for the purposes of this review, is depicted in the lysosomal membrane, and interacts with several proteins including proteins at the cell periphery. CLN3 interacts with *SBDS*, *calsenilin*, and *myosin-IIB* through the C-terminus, and the fodrin-Na^+–K^+–ATPase complex through a cytosolic loop. CLN3 most likely does not interact with all of these proteins at the same time, but rather these interactions are probably dynamic. **c** *CLN5* is a highly glycosylated protein is localized to the lysosome. It has potential interactions with NCL proteins and has been shown to interact with *CLN6* and *CLN8*, potentially in the ER. Interactions of CLN5 and PPT1 have been described with the F_1-complex of the ATP-Synthase, which may occur during mitochondrial degradation. **d** *CLN6* and *CLN8* are both transmembrane proteins that reside in the ER, and CLN6 interacts with *CRMP-2*. **e** *CLN7*, a putative transporter (MFSD8) resides in the lysosomal membrane, but the interactions or function of CLN7/MFSD8 has not been elucidated. A representative lysosome (*red*) is highlighted for each NCL protein, though these lysosomal proteins probably exist together in many lysosomes

the lysosome, the M6P-proenzyme-CTSD is dephosphorylated and activated by several cleavage events in the lysosome to form the mature aspartyl protease, CTSD (reviewed in [15]). CTSD has also been associated with the sortilin pathway and the sphingolipid activator precursor protein prosaposin, which indicate two MPR-independent sorting mechanisms for CTSD to the lysosome, where the acidic pH is optimum for CTSD enzymatic activity [15]. Because $Ctsd^{-/-}$ mice develop normally prenatally, it was suggested that the loss of the endopeptidase activity of CTSD is compensated by other proteins [28]. However, CTSD may be essential for activation of specific regulatory pathways, substrates, growth factors or receptors that have not yet been identified as players in the generation of NCL pathology.

The role of CTSD in apoptosis has been debated [25]. CTSD is protective in retinal, thymus, and neuroblastoma

Table 1 Biochemical properties of the NCL proteins

NCL onset	Gene	Protein	Amino acids and size	Solubility and topology	Posttranslational modifications	Localization	Interactions	Function
Congenital	*CLN10*	Cathepsin D	412 aa	Soluble	Cleaved pro-enzyme	Lysosome	Many substrates	Aspartic protease
	CTSD		45 kDa		Glycosylated	Secreted		Lipid homeostasis
Infantile	*CLN1*	PPT1	306 aa	Soluble	Glycosylated	Lysosome;	Substrates	Thioesterase of fatty acyl chains (14–18 carbons) such as palmitate residues
			34 kDa			Synaptic vesicles	F1 complex of ATP synthase	
						Lipid rafts		
Late-Infantile	*CLN2*	TPP1	563 aa	Soluble	Cleaved pro-enzyme	Lysosome	Substrates	Serine protease, cleaves N-terminal tripeptides
			46 kDa		Glycosylated		e.g. subunit C of ATP Synthase	
							neuromedin B	
							Bid	
Juvenile	*CLN3*	CLN3	438 aa	Membrane	Glycosylated	Lysosome	Na–K-ATPase, Fodrin	Unknown
			~47 kDa	6-TM domains	Phosphorylated	Endosome	Hook1	
				both termini in cytosol	Farnesylated	Golgi	Calsenilin/DREAM/KChIP3	
						Synaptosomes	SBDS	
						Lipid Rafts	Myosin-IIB	
Finnish	*CLN5*	CLN5	358 aa	Soluble	Glycosylated	Lysosome	Proposed:	Unknown
LINCL			55 kDa			ER/Golgi?	PPT1, TPP1, CLN3	
							CLN6, CLN8	
							F1 complex of ATP Synthase	
vLINCL	*CLN6*	CLN6	311 aa	Membrane	None Known	ER	CRMP-2	Unknown
			36 kDa	7-TM domains			Dimerizes	Neuronal Maturation and Polarity?
				N-terminus in cytoplasm				
				C-terminus in ER lumen				
vLINCL	*CLN7MFSD8*	CLN7	518 aa	Membrane	Glycosylated	Lysosome	None shown	Transporter
		MFSD8	100 and 70 kDa	12-TM domains		Plasma membrane		
EPMR	*CLN8*	CLN8	28 6aa	Membrane	None known	ER/ERGIC	None shown	Unknown
vLINCL			33 kDa	4–6 TM domains		Neuronal processes		Lipid Binding?

cells [22, 23, 28, 29], but has pro-apoptotic characteristics as well (reviewed in [25]). During induced apoptosis, lysosomal CTSD is found in the cytosol due to lysosomal membrane permeabilization [30], where it can cleave Bid and perpetuate apoptosis cascades [27]; however, microinjection of catalytically inactive CTSD exhibits the same effect on apoptosis, indicating that there may be undetermined protein interactions of CTSD in the cell [31]. Whether CTSD is a pro- or anti-apoptotic factor may be tightly regulated during certain specific cell contexts. In NCL pathology, CTSD function could be required for regulation of unidentified substrates in the neuron, or may function as a proapoptotic factor in its pro-enzyme or inactive form. Proenzyme CTSD levels were increased in sheep with a missense mutation in CTSD, which exhibited congenital NCL pathology [32]. Autophagic stress markers precede apoptosis in the brains of *Ctsd*$^{-/-}$ mice, and alterations in phosphatidylinositol-3-kinase signaling, such as decrease in phosphorylation of Akt and GSK3β at postnatal day 25–26 (P25–26), were shown [33]. It remains unclear what role CTSD directly plays in apoptosis pathways, but this role could contribute directly to the survival of neuronal populations in NCL.

In addition to alterations in phosphatidylinositol-3-kinase signaling, reduced CTSD levels cause disruptions in lipid homeostasis. Through positive modulation of ATP-binding cassette protein A1 (ABCA1), phospholipid and cholesterol efflux in macrophages and hepatocytes increases [34], showing that CTSD is involved in intracellular lipid trafficking and secretion. Further indicating that CTSD is involved in lipid homeostasis, CTSD protein levels are increased with Niemann-Pick type C1 (Npc1) inactivation in hepatocytes [35]. In the brain, it would be interesting to know if CTSD in glia has a similar effect, which could affect glial–neuron cell interactions. Loss of CTSD in the brain of *Ctsd*$^{-/-}$ mice causes an increase or accumulation of bis(monoacylglycero)phosphate (BMP) and GM2/GM3 gangliosides in both neurons and glial cells, accompanied with impaired processing of prosaposin, a precursor to saposins A to D [36]. Myelination is also disrupted in *Ctsd*$^{-/-}$ mice: significant decreases of three major myelin proteins (PLP, MBP, and CNP) have been demonstrated, significant thinning of axonal myelin sheets accompanied by axonal degeneration has been observed by EM, as well as hypermyelination in subsets of neurons, and finally, mice do not survive past P26, a time of active myelination, which could indicate a delay in myelin assembly [37]. Accumulation of cholesteryl esters in the brain of P24 *Ctsd*$^{-/-}$ mice also indicates that there is degradation of myelin in the brains of these mice [36]. Taken together, cholesterol and lipid homeostasis and trafficking are disrupted in *Ctsd*$^{-/-}$ mice.

It is clear that CTSD is an aspartyl endopeptidase; however, CTSD function affects multiple pathways in the cell beyond protein degradation in the lysosome. In NCL pathology, roles in apoptosis and lipid homeostasis are particularly relevant, and may be linked. Study of the disruptions in CTSD deficiency, which precipitate congenital-NCL, may reveal clues as to NCL pathways that are tangentially related by pathology.

PPT1

Palmitoylation is the addition of palmitate to cysteine through thioester linkage (S-acylation which tethers proteins to membranes, and this modification can be critical to certain recognition complexes for polarized protein trafficking (reviewed in [38]). Mutations in palmitoyl protein thioesterase (PPT1; *CLN1*) result in infantile-onset NCL (INCL) [39–43]. PPT1 was characterized as a thioesterase (Fig. 1a) based on its ability to depalmitoylate hRas in vitro [44]. The severity of INCL patient pathology correlates with residual PPT1 enzyme activity [45–47]; therefore, enzyme replacement gene therapy has been explored to treat INCL [48, 49]. The crystal structure of PPT1 revealed a soluble, globular monomer with an α/β hydrolase fold and a catalytic triad (S115, D233, H289) [50]. PPT1 is N-linked glycosylated at two asparagine residues (N197 and N232), which are required for PPT1 activity, transport to the lysosome through the MPR pathway, and prevention of degradation in the lysosome [50–53]; an additional glycosylation on N212 has also been described [54]. The most common mutations in PPT1 cause decreases in enzyme activity (V_{max}), altered palmitate binding (K_m), and reduced levels of protein, which could reflect degradation of misfolded PPT1 in the ER [45]. Though the structure [50] and thioesterase activity of PPT1 [55] have been identified, we still do not understand PPT1's critical role that is lost in INCL.

PPT1 is a lysosomal protein [43]. Inhibitors of lysosomal proteins or of acidification of the lysosome, cause an accumulation of palmitoylated proteins that could be potential PPT1 substrates. This indicates that lysosomal proteolysis involves depalmitoylation of proteins, perhaps dependent on PPT1 activity [54]. The localization of PPT1 extends beyond the lysosome down neuronal axons and in synaptic compartments [52, 56–59]; however in one study, colocalization with synaptic vesicle markers was not observed in cortical neurons in culture [60]. Interestingly, processing and trafficking of PPT1 differs between non-neuronal and neuronal cell types [52]. In neurons, PPT1 transport differs from lysosomal aspartylglucoaminidase (AGA), which goes directly to perinuclear vesicles, while PPT1 is endocytosed from the cell surface and maintains a diffuse localization, by antibody uptake assay [52]. Therefore, PPT1 may have distinct functions in neurons, reflected by unique roles for palmitoylation in neuronal function.

Palmitoylation is reversible and is particularly important in neuronal regulation and function. For example, palmitoylation is required in the targeting of synaptic proteins involved in neurotransmission: synaptosomal-associated protein-25 (SNAP25), glutamic acid decarboxylase-65 (GAD65), neural cell adhesion molecule (NCAM), and post-synaptic density-95 (PSD95, (reviewed in [38]). These proteins require depalmitoylation for vesicular trafficking, and thus PPT1 deficiency would cause these proteins to maintain anchorage in the membrane, preventing recycling of synaptic vesicle components. A progressive loss of the releasable synaptic vesicle pool was observed in cultured *Ppt1*$^{-/-}$ cortical neurons and aging *Ppt1*$^{-/-}$ mouse brains over time [59, 60]. This was observed as a reduction of depalmitolyated proteins (VAMP2, SNAP25, syntaxin 1) in the soluble fractions of synaptosomes, indicating that these proteins remained anchored in the membrane. A reduction in the number of synaptic vesicles at nerve terminals as observed by transmission electron microscopy also indicates that PPT1 deficiency prevents normal synaptic vesicle recycling following release of neurotransmitters at the presynaptic terminal [59, 60]. PPT1 clearly has important roles in synapse integrity, which may contribute to neurodegeneration in INCL.

PPT1 deficiency results in accumulation of autofluorescent storage material that is enriched with saposins A and D in INCL pathology [61]. Lipid oxidation induced apoptosis was initially suggested as a mechanism of cell death in PPT1 deficiency [54]. Cells with PPT1 deficiency exhibit elevated markers for oxidative and ER stress and activation of the unfolded protein response (UPR), with accompanied induction of apoptosis [62–65]. Specifically, caspase-4, caspase 9, and caspase-12 levels are increased in PPT1-deficient cells, which are accompanied by increases in detectable reactive oxygen species (ROS), superoxide dismutase (SOD1), and cleaved PARP [62, 63]. Increased levels of S-acylated proteins are also found in the ER of PPT1 deficient cells, which could activate UPR [65]. Sensitivity to ER stress conditions is not unique to INCL, but has been found associated to other LSD [66], indicating a connection between the lysosome and the ER, and between PPT1 and oxidative stress-mediated apoptosis. In INCL, ER stress may reflect accumulation of unfolded proteins that are trafficked back to the ER due to lysosomal dysfunction, rather than a direct role of PPT1 in mediating apoptosis [64]. Alternatively, PPT1-deficient cells were resistant to TNFα-induced apoptosis, showing a reduced amount of cleaved caspase-9, cleaved caspase-3, Bid, or a decrease in cytochrome C release when treated with TNFα [66]. This insensitivity was not found in cells deficient for other NCL proteins (CLN3 or CLN5), and may indicate that regulation of protein palmitoylation is important for propagation of apoptosis pathways. A requirement for palmitoylation on the TNFα-receptor in order for receptor internalization was proposed, which directly could explain the insensitivity of PPT1-deficient cells to TNFα, but this was not shown experimentally [66]. Thus, PPT1-deficient cells exhibit an increased level of oxidative and ER stress, but are insensitive to TNFα-mediated apoptosis.

Increased ER stress will cause release of ROS and Ca^{2+} from the ER, which could contribute to neurodegeneration. Specifically in the cerebellum of *Ppt1*$^{-/-}$ mice, there is Purkinje cell loss, reactive gliosis, granule cell apoptosis, microglia activation, and demyelination [67]. Transcript profiling of *Ppt1*$^{-/-}$ mouse brains indicated upregulation of genes involved in cholesterol metabolism, neuronal maturation, and calcium homeostasis, i.e. significant increases in α-synuclein [68]. These alterations are supported by a gene modifier screen completed in *Drosophila* linking PPT1 with genes involved in synaptic vesicle recycling, endosomal trafficking, and synaptic development [69, 70]. Cholesterol metabolism was confirmed to be altered in *Ppt1*$^{-/-}$ neurons both by upregulation of the rate of sterol synthesis [68] and by an increased uptake of apolipoprotein A-1 (apoA-I) [71]. Together, these studies indicate a potential dysfunction of neuronal–glia cell interactions during neuronal maturation and synaptogenesis in the developing PPT1-deficient brain [71].

Despite numerous studies describing PPT1 involvement in many linked pathways, it is still unclear what key substrates of PPT1 are not processed during PPT1 deficiency. The in vivo substrates and interactions of PPT1 must be known in non-neuronal and neuronal cells in order to fully understand PPT1 and therefore treat INCL effectively. Initially, PPT1 was not found to interact with any other proteins by yeast-2-hybrid [72]; however, numerous interactions of PPT1 have been described recently. Purified PPT1 secreted from CHO cells was found to interact with the F_1-complex of the ATP synthase by GST-pull-down and surface plasmon resonance [71]. PPT1 deficiency correlated with alterations in the amount of F_1-subunits in the neuronal plasma membrane by total internal reflection fluorescence microscopy (TIRF); however, colocalization of PPT1 and β-subunit of the F_1-complex in neurons or fibroblasts has not been reported, bringing into question whether interaction between these two proteins would occur in the cell [71].

TPP1

Late-infantile NCL (LINCL) is caused by mutations in tripeptidyl peptidase-1 (TPP1; CLN2) [73–75] (reviewed in [76]). TPP1 is a lysosomal serine protease with N-terminal exopeptidase activity, weak endoproteolytic activity, [77, 78] and known structure [79, 80] (reviewed in [76]) (Fig. 1a). It is the only known eukaryotic member of the

sedolisin family of carboxy-peptidases, by homology [81]. TPP1 is glycosylated on several residues, but N-glycosylation of Asn-286 is required for activity and maturation through the MPR pathway to the lysosome [80, 82–84]. In the lysosome, it undergoes autocatalytic cleavage for activation at acidic pH [78]. Interestingly, recent evidence suggests the cleaved prosegment (176 aa) is a slow-binding inhibitor of TPP1, and prosegment binding also slows the rate of TPP1 inactivation at neutral pH [85]. Prosegment binding to TPP1 outside of the lysosome could permit activity at alkaline pH, in other regions of the cell; however, TPP1 activity has been examined mostly in vitro. In cultured hippocampal neurons, TPP1 is contained in lysosomes, shown by colocalization with lysosomal membrane protein, LAMP1, and is not found in synpatophysin-positive synaptic vesicles in neurons [86].

TPP1 activity is lost in the brain, liver, kidney, heart, and intestine of LINCL patients and the expression of TPP1 is developmentally controlled, reaching peak expression at the same age as the initial age of symptoms onset (2–4 years) [87]. Residual TPP1 protein activity can be detected by histology in certain patients, which may correlate with protracted phenotype [87, 88]. TPP1's endogenous preferred substrates are unclear, but TPP1 has activity on angiotensin-I and –II [89], cholecystokinin [90], neuropeptide neuromedin B [91], Bid during apoptosis [92], and the mitochondrial ATP-synthase subunit C, which is a major component of the storage material accumulated in LINCL [93]. TPP1 likely has numerous protein substrates, and highest efficiency cleavage for Arg-Ala-Gly peptides has been specifically shown [94] (reviewed in [76]). Activity of TPP1 was increased in *CLN5*$^{-/-}$ patient fibroblasts, which could indicate a common disruption or link between the two proteins [95]. Recently, TPP1 expressed with patient missense mutations in CHO cells exhibited low activity and multiple routes of clearance, with some following the ubiquitin/proteasome system and others being hyper-secreted, indicating that misfolding may contribute to the dysfunction of mutated TPP1 [96]. Loss of TPP1 activity contributes to the accumulation of products in the lysosome, and residual activity in mutated TPP1 can alter LINCL pathology [88]; however, the storage material that accumulates in LINCL patients is complex.

Like PPT1, TPP1-deficient cells are resistant to TNFα-induced apoptosis, which may indicate a link between these two proteins or lysosomal involvement in the perpetuation of apoptotic signaling pathways in general [92]. TPP1 has been reported to interact with NCL proteins, which will be discussed later. The role that TPP1 plays outside the lysosome or on specific substrates has not been fully characterized, but identification of in vivo substrates, interacting partners, or primary functions of TPP1 in neurons would aid in our understanding of LINCL pathology.

CLN5

The function of CLN5, the protein mutated in Finnish variant late infantile NCL (FinLINCL), is currently unknown (Fig. 1c). CLN5 is a 407 amino acid protein with a predicted molecular weight of 46 kDa [97]. Four in-frame alternative initiator codons for CLN5 have been examined at Met-1, Met-30, Met-50, and Met-62 [97], which can produce polypeptides of molecular weights of 46.3, 43.4, 41.5, and 40.3 kDa; and all have been seen in reticulate cell-free translation systems, in vitro [95, 98]. In a recent study, CLN5 was tagged on the N-terminus with GFP to determine if the predicted signal sequence on the N-terminus is cleaved [99]. Using a new CLN5 antibody directed at the C-terminus of CLN5, cleavage of the N-terminus of overexpressed CLN5 in COS-1 cells was confirmed for all four variants of CLN5 [99], which indicates that several forms of CLN5 may exist in perhaps different cell types or regulatory conditions.

CLN5 was originally described to have two transmembrane domains, as predicted by BCM Transmembrane Prediction Program and by Kyte and Doolittle hydrophobicity plot analysis [97]; however, the solubility of CLN5 has been debated [98, 100]. CLN5 is most likely a soluble protein based on the presence of CLN5 in culture medium due to secretion from transiently transfected BHK-21 cells [98], the ability to isolate CLN5 by mannose-6-phosphate affinity purification [101], and the likelihood that a N-terminal signal peptide is cleaved from CLN5 in its maturation to the lysosome [98, 102]. Further, both mouse and human CLN5 were reported to be soluble by Triton X-114 fractionation with the appropriate controls, using PDI (a soluble protein) and transferrin receptor (a membrane protein) for confirmation [100]. CLN5 is highly glycosylated at up to eight sites (Fig. 1c), resulting in an observed size of 60–80 kDa (Table 1) [95, 98], and glycosylations are both sensitive to EndoH (highly complex or mannose type sugars) and PNGaseF (N-type linked) treatments, indicating complex glycosylations [95, 98, 99]. Mouse CLN5 was confirmed to contain three mannose-6-phosphate (M6P) residues making it likely that CLN5 follows the MPR pathway to the lysosome [103], but in MPR deficient fibroblasts, CLN5 was found in LAMP1^{+} vesicles [99], indicating that CLN5 may use alternate routes to the lysosome.

CLN5 predominantly colocalizes with lysosomal associated membrane protein-1 (LAMP1) in several studies [95, 98–102, 104–106]. Mutations in *CLN5* result in Finnish variant late infantile NCL (Fin-vLINCL) [97, 107, 108]; recently, mutations in CLN5 have been found outside Finnish populations [97, 102, 106, 109, 110]. Pathogenic mutations seem to cause retention of CLN5 in the ER/Golgi, in immunocytochemistry of cells overexpressing mutated CLN5 [98, 99, 102]. The presence of complex

glycosylations on several mutated forms of the CLN5 proteins, such as the major Finnish mutation (Trp392Stop), indicates that at least partial populations of the mutated proteins leave the ER to the Golgi apparatus for glycosylation [95]. In summary, evidence suggests that CLN5 is synthesized at one or all of four initiator codons, depending on condition or cell type, as a preproprotein. In the ER, the N-terminal signal peptide is cleaved, and the protein receives mannose-type sugars, at which point it is trafficked to the Golgi apparatus for additional glycosylation and modification to the mature 50 kDa form. The mature form then completes its journey to the lysosomes through either the MPR or secretory pathway [99].

Several interactions of CLN5 with other NCL proteins have been described [95] and will be discussed in a later section. Perhaps due to the extensive post-translational modifications of CLN5, there are several caveats with studies of this protein. Antibodies generated to CLN5 do not recognize the endogenous CLN5, and therefore can only be used on overexpressed protein [95, 98, 102]. All these studies are done in overexpression systems in COS-1 or BHK cells, which provide valuable information, but the data must be examined with the caveat that, at endogenously regulated levels, subtle variations and treatments of this important CLN5 protein may be missed. It is clear that CLN5 has important functions due to the pathology that results from its loss, but the function remains elusive. Due to massive glycosylations, it is possible that it may be a sensor important in trafficking or integrity of lysosomes, but we have no evidence yet to suggest the role it plays in lysosomes or neurons.

NCL-associated membrane proteins

Mutations in four distinct transmembrane proteins are associated with an NCL disease (Fig. 1b, d, e), each encoded separately with no clear homology to other proteins. CLN3 and CLN7 are found in the lysosome, while CLN6 and CLN8 are localized to the ER, but dysfunction in each causes lysosomal storage accumulation. The primary function of each membrane protein associated with NCL disease is unknown. Much of what we have learned thus far about these proteins has come from loss of function models such as yeast and mice constructed to lack these proteins. Not surprisingly, studying the effect of protein loss has implicated each protein in several biological pathways. Studies focused on elucidating interaction partners should reveal more detail as to the precise function of these hard to study proteins. Due to similar disease pathology, direct interactions between the NCL proteins have been proposed by several studies; however, as we detail later in this review, participation in a common pathway is unlikely.

The interaction network of CLN3

Mutations in *CLN3* result in the most common NCL: juvenile-onset NCL (JNCL) [2, 111]. The most common mutation in patients is a frameshift mutation after amino acid Cys-153, which results in the addition of 28 novel amino acids and a termination stop codon [111]. While some debate continues concerning the possibility of residual function being retained by this predicted truncated protein product, it appears that the mRNA transcripts are degraded, resulting in an absence of CLN3 protein and a complete loss of CLN3 function [112]. Despite a decade of research into the primary cause of JNCL, the function of CLN3 remains unknown. Following the characterization of CLN3 topology, localization, and posttranslational modifications, protein interacting partners have been examined to determine the endogenous function of CLN3 (Table 1). CLN3 has been reported to be present in the nucleus [113], Golgi [114–116], mitochondria [117], plasma membrane [113], endosomes and lysosomes [114, 115, 118, 119], neuronal processes and synaptosomes [120, 121]. The localization experiments were conducted using several techniques and have been completed with different CLN3 antibodies, expression constructs, epitope tags, and cell types. The validity of these localization studies has been critically reviewed in [122].

In mammalian cells, CLN3 has most often been localized to the endosome and lysosome, and for the purpose of this review, this localization will be considered the primary location of CLN3 [118, 119, 123–125]. CLN3 is expressed at very low endogenous levels and is a highly hydrophobic protein, which has made generating an antibody for studying CLN3 localization and function difficult. Peptide-derived polyclonal antibodies are used most frequently to study CLN3, reviewed in [122], however, in only one study has the specificity of the CLN3 antibody been truly verified [118]. Experimentally, CLN3 has six transmembrane domains with both termini facing into the cytoplasm (Fig. 1b); though a five membrane domain topology has also been suggested [118, 122, 125–128]. Using site-directed mutagenesis, loss of CLN3 glycosylation had no effect on the localization of CLN3, but loss of C-terminal farneslyation caused an enrichment of CLN3 at the cell surface [119], indicating the importance of this modification for localization of CLN3. One interpretation for multiple localizations of CLN3 may be that this protein has functions in multiple regions of the cell, or perhaps even that CLN3 has a specific or conditional function in certain cell types. However, CLN3 most likely functions at the lysosome, but has transient roles in dynamic vesicles that interact with lysosomes at specific regions in the cell during specific conditions. CLN3 has been reported to interact with several proteins (Fig. 1b), which further indicates that

it is a multifunctional protein and suggests that at certain times some of the cellular pool of CLN3 might have alternate localizations.

A common technique used to determine protein interaction partners is yeast-2-hybrid (Y2H), which has been employed to identify interacting partners of CLN3 in several studies [129]. The classic technique requires fusion proteins to enter the nucleus to initiate reporter gene expression, which is not ideal for assaying highly hydrophobic proteins, such as the NCL membrane proteins. An alternate Y2H, the CytoTrap (Stratagene), interactions occur in the cytosol and associated with the plasma membrane, rather than in the nucleus. The disadvantage of both methods is identification of false positive interactions; therefore, all candidates must be validated by co-immunoprecipitation and colocalization in mammalian cells, which are limited by poor antibody reagents for NCL proteins. Additionally, screening transmembrane proteins in Y2H systems is challenging, so many studies screened the hydrophilic regions of the proteins that would exposed to the cytosol or lumen of an organelle, rather than full-length proteins. All interactions of CLN3 were initially identified using Y2H, and these interactions have revealed a complex interaction network for CLN3 throughout the cell, each of which will be discussed.

CLN3 was shown to interact with the cytoskeletal protein β-fodrin and the Na^+-K^+-ATPase complex (Fig. 1b) [130]. Based on the six-transmembrane domain topology, the N-terminus and the second cytoplasmic loop of CLN3 were screened for interactions using the *LacZ*/β-galactosidase Y2H [130]. The interactions were validated by immunoprecipitation of overexpressed CLN3 and endogenous candidate interactors from COS-1 cell extracts, using CLN3 antibodies [130]. It was shown that CLN3 co-immunoprecipitated with subunits of the Na^+-K^+-ATPase and fodrin (β-II-spectrin chain), but it is not clear which protein CLN3 interacts with directly, or if the three proteins interact in a complex. Fodrin is a heterotetrameric complex of two α and two β chains that binds the plasma membrane through pleckstrin homology (PH) domains and tethers membrane protein complexes to the actin cytoskeleton [131, 132]. The spectrin-ankryin-actin skeleton at the plasma membrane maintains and stabilizes the polarization of proteins (reviewed in [132]). CLN3 may be trafficked to the lysosome through the adaptor protein-1 and -3 (AP-1 and AP-3) complexes binding CLN3 dileucine lysosomal sorting motifs, and loss of these motifs resulted in CLN3 enriched in the plasma membrane [123–125, 127]. CLN3 would be capable of interacting with plasma membrane proteins during a trafficking pathway that reached the plasma membrane on the way to the lysosome, or it could interact within lysosomes close to the plasma membrane. Distribution of fodrin architecture in JNCL fibroblasts and *Cln3*$^{-/-}$ mouse brain sections was altered [130], and these changes indicate a possible role for CLN3 in this structure either in the plasma membrane, or perhaps in a special function in neurons. Synapsin-1 interacts with fodrin (β-spectrin) at the synapse and synaptic transmission of hippocampal neurons was blocked in vitro by treatment with antibodies to β-spectrin, thus fodrin complexes may have unique roles in neurons [133]. Fodrin controls cell surface distribution of the Na^+-K^+-ATPase directly in polarized cells such as Madin-Darby canine kidney (MDCK) cells [134] and in the inner segment of rod photoreceptors [135]; therefore, at the plasma membrane these two interacting partners of CLN3 probably interact as a multi-protein complex.

The Na^+-K^+-ATPase maintains chemical gradients at the cell surface by pumping Na^+ out and K^+ into the cell [136], and its function is integral to neuronal function. The Na^+-K^+-ATPase exists in several isoforms, based on heterogenic combinations of α, β subunits, and FXYD regulatory proteins, with tissue and region-specificity (reviewed in [137–139]). CLN3 interacts with the ubiquitously expressed α_1-β_1 isoform by co-immunoprecipitation from COS-1 cells, using CLN3 antibodies and antibodies to endogenous α_1-β_1 subunits [130]. There is no defect in the ion pumping activity of Na^+-K^+-ATPase in *Cln3*$^{-/-}$ neurons; however, the dynamic trafficking of the Na^+-K^+-ATPase at the plasma membrane is altered in *Cln3*$^{-/-}$ [130]. By total internal reflection fluorescence (TIRF) microscopy, the neuron-specific α_3-β_1 isoform was increased at the plasma membrane in *Cln3*$^{-/-}$ neurons [130]. Ouabain treatment inhibits the Na^+-K^+-ATPase and induces its internalization from the plasma membrane, and following oubain treatment, internalization of Na^+-K^+-ATPase was decreased in *Cln3*$^{-/-}$ neurons [130]. Defects in endocytosis due to CLN3 loss were examined in another study because of a weak interaction between CLN3 and Hook1 [140], a microtubule binding protein involved in endocytosis [141–143]. The rate of transferrin receptor uptake from the cell surface by endocytosis was not altered by CLN3 deficiency; however, the recycling of the receptor back to the plasma membrane was increased in JNCL fibroblasts [140]. Importantly, further investigation of Hook1's interaction with Ankyrin-G at the spectrin-Ankyrin-actin cytoskeleton in *Cln3*$^{-/-}$ mice revealed no defects [144]. Therefore, it does not appear that CLN3 is directly involved in general endocytosis pathways for internalization of the transferrin receptor, or perhaps the Na^+-K^+-ATPase subunits. Therefore, the increased Na^+-K^+-ATPase subunits at the plasma membrane of *Cln3*$^{-/-}$ neurons may reflect an indirect consequence of CLN3 loss. The association of fodrin and the Na^+-K^+-ATPase in *Cln3*$^{-/-}$ was not examined directly, but alterations in fodrin and Na^+-K^+-ATPase subunit distribution in *Cln3*$^{-/-}$

neurons and JNCL fibroblasts indicate that CLN3 could be required for Na^+-K^+-ATPase turnover at the plasma membrane in a tethering, structural, or trafficking role.

Beyond functions in ion homeostasis, the Na^+-K^+-ATPase has functions in cell polarity, adhesion, endocytosis, and in mediating calcium oscillations (reviewed in [145]). Importantly, glutamate-mediated neurotransmission requires coupling of glutamate transporters and the Na^+-K^+-ATPase to drive both transport and maintenance of the chemical gradient, either directly through Na^+-K^+-ATPase and glutamate transporter protein complexes [146], and also through Na^+-K^+-ATPase activity regulated AMPA receptor turnover in neurons [147]. Cerebellar granule cell neurons from *Cln3*$^{-/-}$ mice exhibit increased sensitivity to AMPA-mediated excitotoxicity [148] accompanied by an increase in glutamate in JNCL patients [149]. CLN3 has been found in the synaptosomal compartment [120, 150], and it would be interesting to know if endogenous CLN3 interacts with fodrin and the Na^+-K^+-ATPase down axonal projections of neurons. If the absence of CLN3 causes dysregulation of fodrin-Na^+-K^+-ATPase complex dynamics and turnover, it could contribute to previously recognized pathology in *Cln3*$^{-/-}$ or JNCL through modulation of ion homeostasis in neurons during synaptic transmission and in neuronal excitotoxicity.

Electrochemical gradients drive neuronal excitation and synaptic transmission, therefore ion homeostasis in neurons is particularly well regulated. Calcium cations (Ca^{2+}) are involved in many signaling pathways in all cells, and are required for presynaptic transmission in neurons. Calsenilin is a multi-functional Ca^{2+}-dependent protein that interacts with presenilin proteins and modulates amyloid β-peptide (Aβ), acts as a transcriptional repressor (DREAM), and binds the N-terminus of Kv4 α-subunits of A-type voltage-gated K^+ channels (KChIP3) [151–156]. This protein is encoded by a single gene, but clearly calsenilin/KChIP3/DREAM (hereafter referred to as calsenilin) is involved in different functions which may be mediated through localization and protein binding partners [157]. Using a Y2H screen to identify calsenilin interaction partners, calsenilin was found to interact with the C-terminus of CLN3 (amino acids 385-438) [158], and this interaction was further confirmed by immunoprecipitation. Calsenilin binds Ca^{2+} through its EF-hand domains, [159] and although CLN3 has not been shown to directly bind Ca^{2+} or EF-hand domains, increasing Ca^{2+} concentration in vitro and in cells decreases the CLN3-calsenilin affiliation in a concentration-dependent manner [158]. Calsenilin modulates neuronal sensitivity to Ca^{2+} transients in a pro-apoptotic manner, and therefore mediates Ca^{2+} toxicity [153]. Overexpression of CLN3 in SH-SY5Y cells led to protection from Ca^{2+}-mediated apoptosis and calsenilin expression was reduced in this same overexpression system, indicating a strong interplay between these two proteins [158]. Calsenilin binding to Kv4 subunits of A-type voltage-gated K^+ channels dramatically affects localization and posttranslational modifications of channel subunits, and members of the KChIP family bind to Kv4 subunits in a tightly controlled and nonredundant manner [160]. An interaction between calsenilin and CLN3 could be caused by co-trafficking of CLN3 with channels to specific cell regions, such as at the cell periphery where binding could be responsive to Ca^{2+} transients.

A-type voltage-gated potassium channels and their interacting proteins modulate the excitation and repolarization of both neurons and myocytes [161, 162]. In *Cln3*$^{-/-}$ mice, neurons are selectively sensitive to AMPA-mediated excitotoxicity, and the loss of the interaction between calsenilin and CLN3 may contribute to this neuron response [148]. Recently, researchers proposed a new pathway for astrocyte differentiation involving the pituitary adenylate cyclase-activating polypeptide (PACAP)-cAMP-Ca^{+2}–calsenilin cascade because of the increased numbers of astrocytes and neurons in *Calsenilin*$^{-/-}$mice [163]. Increased astrocytosis and gliosis are characteristic of *Cln3*$^{-/-}$ mice [164] as well, and it is tempting to consider if the loss of the CLN3-calsenilin interaction could contribute to that pathology. Calsenilin was initially identified as an interactor of presenilins, which combine with nicastrin, APH-1, and presenilin enhancer-2 (PEN-2) to compose the γ-secretase complex [152, 165]. The γ-secretase complex cleaves single-pass transmembrane proteins at the plasma membrane, such as amyloid precursor protein (APP) and Notch, and though calsenilin is not required for catalysis, Aβ peptides were decreased in *Calsenilin*$^{-/-}$ mice, linking calsenilin with γ-secretase function [166]. Notch mediates cell–cell communication in numerous processes, such as development and function of neurons [167]. Examination of the cerebellum of *Cln3*$^{-/-}$ mice revealed an increase in Notch2 expression and thinning of the internal granule cell layer (IGL), accompanied by increased number of proliferating cells (BrdU+) in *Cln3*$^{-/-}$ cerebellum [168]. In the developing cerebellum, granule neuron precursors (GNP) proliferate in the external granule cell layer (EGL), then differentiate and migrate to the IGL; and Notch signaling contributes to the fate decisions of proliferating or differentiating GNP cells [169]. If CLN3 and calsenilin interact close to the cell periphery in order to regulate Ca^{2+} transients in response to either A-type voltage-gated potassium channel ion flux, or to regulate γ-secretase localization or activity, then these pathways would be disrupted with CLN3 dysfunction.

In *Drosophila*, genetic interactions of *CLN3* with the Notch and Jun N-terminal kinase (JNK) signaling pathways were observed; specifically, overexpression of CLN3 caused decreased Notch signaling and activated JNK signaling [170]. Interestingly, CLN3 overexpression had no

effect on Notch protein expression levels or downstream signaling capability, which was shown by co-expression of cleaved Notch with CLN3; therefore, the cause of Notch-like phenotypes were most likely due to alterations in the Notch protein function itself [170]. The authors suggested that overexpression of CLN3 altered the cleavage or processing of Notch in certain cell types or developmental periods [170]; however, the cleavage of Notch was not directly assayed. If CLN3 overexpression caused an increased association with calsenilin, alterations in the γ-secretase complex presenilin levels may result, causing altered cleavage of Notch at the plasma membrane in these specific cell types. At this time, calsenilin's numerous functions in the cell are still being elucidated, and CLN3 may interact with calsenilin under one of these specific conditions. It is interesting that, in the developing mouse cerebellum, Notch expression was increased in the absence of CLN3 [168], and in *Drosophila*, overexpression of CLN3 caused the opposite effect: decreased Notch signaling and Notch-like phenotypes [170]. This is suggestive of a genetic interaction between CLN3 and the Notch signaling pathway.

Overexpression of CLN3 in the fly eye caused degeneration, and this phenotype was used to complete a genetic screen for genetic interactions [170]. This screen identified *mago nashi*, a constituent of the exon-exon-junction complex and a gene with roles in RNA transport and cell polarity in the developing oocyte of *Drosophila* [170–172] as an enhancer of the eye degeneration phenotype. Interestingly, it was recently reported that CLN3 interacts with the Shwachman-Bodian-Diamond Syndrome protein, SBDS [173]. *SBDS* is ubiquitously expressed and highly conserved [174], and due to the high conservation of this protein, studies to examine the function of Sdo1p, the yeast homolog of SBDS, have revealed further important information as to the function of this protein in RNA processing and ribosomal biogenesis [175, 176]. Specifically, Sdo1p is required for the release and recycling of ribosome maturation factor Tif6 from the pre-60S ribosomes in the cytoplasm [175]. SBDS is a multifunctional protein, involved in a complex interactome with proteins of the large ribosomal subunit (RPL4) and DNA metabolism (DNA-PK and RPA70), and cells depleted of SBDS exhibit increased sensitivity to DNA damage and ER stress [177]. The C-terminus of CLN3 was found by Cytotrap Y2H to interact with SBDS and this interaction was confirmed by co-immunoprecipitation of CLN3 and SBDS expressed in NIH-3T3 cells [173]. The CLN3–SBDS interaction is evolutionarily conserved since the *Saccharoymyces cerevesiae* homologs to CLN3 (Btn1p) and SBDS (Sdo1p) interact [173]. Examination of this interaction indicated that during pH stress, Sdo1p may regulate Btn1p's functional modulation of the V-ATPase [173]. Shwachman-Diamond Syndrome is a disease characterized by exocrine pancreatic insufficiency, bone marrow deficiency, and skeletal dysplasia [178], which differs from JNCL pathology. However, some brain structural irregularities have been seen in SBDS patients [179]. CLN3 expression has been found in pancreatic islet cells [180], and both CLN3 and SBDS are expressed in adult mouse brain (Table 2). The interaction between Sdo1p and Btn1p functionally links lysosomal and ion homeostasis with the ribosome, possibly through a novel regulating mechanism.

The reported CLN3 genetic interaction with *mago nashi*, and the CLN3 physical interaction with SBDS, may potentially signify a conditionally regulated link in specific conditions. For example, SBDS was enriched in the pseudopod of *Dictostelium* ameobae migrating towards a chemoattractant [181], and likewise, the loss of SBDS in patient polymorphonuclear leukocytes prevented orientation and motility towards a chemoattractant [182]. CLN3 and SBDS may couple ribosome and localized translation to the pseudopod, where the chemoattractant signal is sensed and degraded in the lysosome. *Cln3*$^{-/-}$ mouse embryonic fibroblasts exhibit cell motility defects, which could be due to the loss of an interaction of the C-terminus of CLN3 and nonmuscle myosin-IIB [220]. This interaction was found by Y2H and confirmed by co-immunoprecipitation of expressed CLN3 with endogenous myosin-IIB. An interaction of myosin-IIB with CLN3 has interesting implications for CLN3 function as it is an actin-binding motor protein with increased expression in the brain during development, and is involved in cell motility, division, and polarity [183]. Calsenilin, SBDS, and myosin-IIB interact with the C-terminus of CLN3, indicating that this region of CLN3 may contain a novel binding motif or more likely exhibits labile or conditional interaction with each protein.

Table 2 NCL gene expression in mouse central nervous system

Gene	E11	E15	P7	P42
CLN10/CTSD	+++	+++	+++	++
CLN1	n/a	n/a	n/a	n/a
CLN2	+	+++	++	n/a
CLN3	+	+++	++	++
CLN5	–	+	–	n/a
CLN6	+	++	+++	++
CLN7/MFSD8	n/a	n/a	n/a	n/a
CLN8	+	+	+	+[a]

Relative expression levels indicated as: *n/a* not available in database, – no expression, + low expression, ++ moderate expression, +++ high expression, as determined qualitatively by in situ on the Brain Gene Expression Map (stjudebgem.org).

[a] Cln8 expression is seen in postnatal brains in the cortex and hippocampus [219]

While the primary function of CLN3 remains elusive, it is apparent that CLN3 has multiple interaction partners with the potential to have an influence on many biological processes. However, the identification of interacting proteins is only a starting point for understanding the role of this protein in the CNS. The need for robust and verified antibodies that specifically bind CLN3 remains. Currently, immunoprecipitation of endogenous CLN3 is difficult, and experiments on the trafficking, localization, and function of CLN3 and CLN3 interactors present many challenges. Whether CLN3 interacts with these same proteins in all cell types, and in particular in all cell types of the CNS, at all points in development, remains unclear. It is possible that the regulation of these interactions occurs in both a temporal or spatial manner. Indeed, these interactions are not necessarily mutually exclusive. Moreover, many of the CLN3 interactors function at the cell periphery, and while CLN3 is likely primarily localized to the lysosome, CLN3 could have specific roles in vesicles at the cell periphery during certain conditions in integrating the response to alterations in localized ion homeostasis. The numerous interactions of CLN3 provide clues as to its function, though the primary functions are still unknown. Interactions have been described for CLN3 (Fig. 1b), implicating a functional role in cytoskeleton dynamics, ion channel trafficking, cell adhesion and migration, lysosomal/vacuolar homeostasis and modulation of ionic flux.

CLN6

Variant late infantile NCL (vLINCL) results from mutations in *CLN6* [184, 185]. CLN6 is found in the ER, and not in the Golgi or lysosomes [186–188]. CLN6 is a seven transmembrane domain protein with the N-terminus directed towards the cytoplasm and the C-terminus within the ER lumen (Fig. 1d) [188]. Through cross-linking experiments, CLN6 was shown to form homo-dimers which require both the N- and C-termini for dimerization [186, 188]. ER retention for CLN6 requires the N-terminus and transmembrane domains 6 and 7 [188]. Interestingly, similar cross-linking experiments did not reveal interactions between CLN6 and CLN8, both located in the ER membrane [188], indicating that these proteins do not form heterodimers and probably do not function together (Fig. 1d, Table 1).

CLN6$^{-/-}$ cells from patients (vLINCL), mutant mice (*nclf*), or mutant sheep (*OCL6*$^{-/-}$) do not show defects in synthesis, sorting or processing of cathepsin D, which was used in this study as a prototypical lysosomal protein to reveal defects in lysosomal protein maturation in the loss of CLN6 in the ER [186]. However, in both *OCL6*$^{-/-}$ and *CLN6*$^{-/-}$ patient cells, endocytosis of arylsulfatase A (ASA) from the plasma membrane by the M6PR pathway was increased, and this effect was specific to the M6PR pathway since transferrin-mediated endocytosis was normal in these cells [186]. Interestingly, this defect was not in kinetics of endocytosis, but rather in degradation of ASA, as examined by pulse-chase audioradiography [186]. This experiment clearly shows that the ER-resident protein, CLN6, is required for degradation pathways involving lysosomes, though the exact mechanism remains unclear.

Recently, peptide fragments of CLN6 were screened against a human fetal brain library using the CytoTrap Y2H, and CLN6 was shown to interact with collapsin response mediator protein-2 (CRMP-2), also known as dihydropyrimidinase-like-2 (DRP-2) [189]. This interaction was validated by co-immunoprecipitation of overexpressed proteins in NIH-3T3 cells [189]. CRMP-2 is involved in neuronal polarity, growth cone guidance, and interacts with Numb at the growth cone of neurons to mediate endocytosis of L1 [190–193]. CRMP-2 binds tubulin heterodimers and the Sra-1/WAVE1-actin complex and so mediates axon dynamics and specification [194, 195]. Therefore, an interaction of CLN6 with CRMP-2 may have implications in the CNS of vLINCL patients. CRMP-2 is involved in semaphorin signaling pathways to guide axonal growth cones, but no alterations in semaphorin-3A signaling were observed in *nclf* dorsal root ganglion explants [189]. However, there was a defect in the maturation of hippocampal neurons in a co-culture system of glia and hippocampal neurons from *nclf* mice [189]. Alterations in CRMP-2 protein expression levels were also seen in specific brain regions of the *nclf* mouse [189]. CRMP-2 and CLN6 are both well expressed in the developing mouse brain (Table 2). Thus, the interaction of CLN6 with CRMP-2 may have implications in the maturation and integrity of axonal outgrowth in vLINCL patients, though the exact function of this interaction or of CLN6 remains unknown.

CLN7/MFSD8

Variant late infantile NCL, resulting from mutations in *CLN7*, was originally termed the Turkish variant [196, 197], but was later found in broader populations [198, 199]. The CLN7 protein is predicted to be a 518 amino acid protein, approximately 58 kDa, with 12 predicted transmembrane domains and both the N- and C-terminus facing the cytosol (Fig. 1e, Table 1) [200, 201]. Recently, the protein was identified as a member of the major facilitator superfamily (MFS), by amino acid sequence homology; therefore, the gene locus was identified to be MFS-domain containing protein-8 (MFSD8) [200]. MFS domain proteins are ubiquitously expressed, conserved, and function as transporters of small solutes by chemiosmotic gradients [202], though the substrate specificity of CLN7/MFSD8 has not been determined [200]. Overexpressed GFP-tagged

CLN7/MFSD8 was localized to the lysosomes in COS-1 cells and HeLa cells (Fig. 1e), and CLN7/MFSD8 is thought to act as a novel lysosomal transporter [200, 201]. CLN7/MFSD8 is glycosylated at two positions (N371, N376), which are not required for lysosomal targeting of CLN7/MFSD8 [201]. Two dileucine-based sorting motifs, N-terminal [D/E]XXXL[L/I]-type ($E^{9}QEPL^{13}L^{14}$) and two YXXØ-type tyrosine-based motifs ($Y^{503}KRL$ and $Y^{513}GRI$) in the C-terminus both contribute to targeting the protein to the plasma membrane and clathrin-mediated endocytosis; however, the dileucine motif is the dominant signal for lysosomal targeting from the cell surface [201]. The interactions and primary functions of CLN7/MFSD8 have not been elucidated.

CLN8

CLN8 mutations result in progressive epilepsy with mental retardation (EPMR, also known as Northern Epilepsy), or variant late-infantile NCL progression [203–207]. *CLN8* mutations spontaneously occurring in mice resulted in the motor neuron degeneration phenotype (*mnd*) [204, 208]. The brains of *mnd* mice and NCL patients show accumulation of autofluorescent storage material and neurodegeneration. The CLN8 protein is a membrane protein of 286 amino acids and approximately 33 kDa that localizes to the endoplasmic reticulum (ER) and the ER to Golgi intermediate compartment (ERGIC), in BHK cells (Fig. 1d, Table 1) [209]. CLN8 contains an ER-retrieval signal (KKRP) that directs retention of the protein; patient mutations did not affect this ER localization [209]. CLN8 is not proteolytically processed or glycosylated [209]. In neuronal cells, transfected primary hippocampal neurons, CLN8 was found to colocalize with ER markers, but CLN8 staining extended beyond the ER markers towards the periphery of the neurons [210]. Similarly in polarized epithelial CaCo-2 cells, CLN8 staining was found beyond the ER and ERGIC compartment and extended to the basolateral surface of these cells [210]. Further, CLN8 from mouse brain extracts fractionated separately from ER and ERGIC, as well as synaptic markers, indicating that CLN8 may have a unique, unidentified vesicular localization in neurons [210]. It is possible that artifacts of CLN8 staining exist due to overexpression in these studies.

PSI-BLAST analysis identified CLN8 sequence similarity with yeast Lag1p, a protein involved in longevity and aging [211] and TRAM, a regulator of translocation into the ER [212], and through these similarities, functions for CLN8 were suggested in lipid synthesis, proteolipid trafficking, or lipid sensing [213], though these suggestions have not been tested directly. Interestingly, examination of brain samples from two EPMR patients by liquid chromatography/mass spectrometry revealed reduced levels of ceramide, galactosylceramide, lactosylceramide, and sulfatide accompanied with increases in polyunsaturated acyl chain species such as phosphatidylserines and phosphatidylethanolamines [214]. There were no changes in total phospholipid content, but these alterations in the composition of phospholipid and sphingolipid species may increase lipid oxidation or affect specific membrane dynamics and function of membrane receptors, contributing to the pathology of EPMR [214]. Similarly, *mnd* mice also have disturbances in lipid metabolism proteins in the liver [215], and taken together these studies support a role for CLN8 in lipid homeostasis, though the direct function of CLN8 protein in these pathways remains unclear. Interacting partners of CLN8 in the ER, ERGIC, and beyond these compartments in neurons have not been reported, but identification of interactions may offer valuable insight into the function of CLN8 in lipid homeostasis, or how the loss of CLN8 precipitates these changes.

Do NCL proteins interact and participate in a common pathway?

Deficiency in eight separate genes results in the accumulation of autofluorescent storage material. Thus, it is tempting to hypothesize that there is a functional link between the NCL proteins or that they interact directly in one pathway. This hypothesis was examined by classic Y2H of the NCL proteins, which showed that none interacted [216]; however, subsequent studies reexamined these potential interactions.

Initially, PPT1 was not found to interact with any other NCL proteins by co-immunoprecipitation (co-IP) of overexpressed proteins in COS-1 cells; however TPP1 and CLN3 were found to interact with CLN5 [95]. In this study, antibodies to PPT1, TPP1, CLN3, and CLN5 were all generated in rabbit and used for immunoprecipitation (IP) of overexpressed proteins from COS-1 cell extracts. To identify an interaction, an IP was performed with a rabbit-generated antibody and to detect the co-IP protein, the second protein was immunoblotted, using a second rabbit antibody [95]. Thus, unless unreported actions were taken to prevent antibody cross-reactivity, for instance cross-linking the IP antibody to protein-A agarose beads, the bands in these co-IP immunoblots may simply be IgG bands. This does not explain why PPT1 did not appear to IP, but it may explain why LAMP1 did not co-IP in the negative control, because the LAMP1 antibody (H4A3) is a mouse monoclonal [95], which would not result in the same IgG cross-reactivity. No sizes were reported on the immunoblot to indicate if the bands were in fact IgG or specifc to the NCL protein, so the validity of this experiment is unclear [95]. Antibodies to most of the NCL proteins are of questionable specificity and avidity; for

example, several reports indicate that antibodies to CLN5 do not recognize endogenously expressed CLN5, and study of CLN5 requires overexpression for both immunoblotting and immunocytochemistry [95, 98, 102]. Therefore, overexpressed and epitope-tagged proteins are used to identify localization and interactions, which is acceptable when the appropriate controls are used. Unfortunately, vector-only transfection controls are not presented in several localization studies of CLN5 [95, 98, 102], and not used in all immunoblotting experiments. Moreover, the specificity of the NCL antibodies used in most studies are usually not validated, verified, or reported.

Recently, CLN5 was again reported to interact with PPT1, TPP1, CLN3, CLN6 and CLN8 in vitro [95, 105]. GST-CLN5 expressed in *E. coli* and was used for in vitro binding assays to show interactions of CLN5 with the other NCL proteins [105]. GST-CLN5 was reported to interact with overexpressed PPT1, CLN3, CLN6-myc, and CLN8-HA in COS-1 cell extracts, but not with endogenously expressed LAMP1 [105]. It was also reported that GST-CLN5 interacted with the α and β subunits of the F_1-complex of the ATP synthase [105]. GST-CLN5 was also shown to interact with endogenous TPP1 [105]. Overall, the fact that the CLN5 used in these studies was fused with GST raises some questions about these interactions. While it was appropriately demonstrated that the GST alone did not interact with other NCL-proteins, CLN5 was ultimately isolated from *E. coli.* Expressing a highly modified protein like CLN5 in *E. coli* probably does not necessarily produce a native CLN5 protein; and native structure is required for a binding assay to identify relevant protein interactions. CLN5 would not be glycosylated properly in *E. coli,* the N-terminal signal sequence would probably not be cleaved, and the protein may even be incapable of folding correctly without ER-processing [95, 98, 99, 102, 105]. Further, the purity of the GST-CLN5 yielded from the glutathione-Sepharose purification, and used for affinity-precipitation experiments, was not shown; therefore, the interactions that were reported in [105] could be the result of many nonspecific interactions occurring with other contaminating proteins, or could be due to aggregation of the hydrophobic NCL proteins themselves.

In terms of the specific interactions shown in this study [105], PPT1 and CLN3, tagged CLN6 and tagged CLN8, were all overexpressed in COS-1 cells. Overexpression of these proteins may affect their structure or interactions, as certain NCL proteins can dimerize (for example, CLN6 [188]), and produce interactions in cell extracts that would not occur in the cell. Interactions with CLN6 and CLN8 would have to occur while CLN5 is present in the ER, and CLN3, PPT1, and TPP1 would interact with CLN5 at the lysosome; however, each of these interactions are possible in a cell extract where spatial constraints of the cell are lost. The effect of overexpression of a trafficking-deficient CLN5 (CLN5-TD) on the localization of the NCL proteins was examined, and showed that co-overexpressed PPT1 was also retained in the ER with CLN5-TD [105]. This is not surprising, as overexpression of proteins could result in ER retention, therefore any link between these proteins due to the common retention in the ER under overexpression conditions probably is not relevant to their function.

TPP1, CLN3, CLN6, and CLN8 were also reported to interact by co-immunoprecipitation and co-localization from lymphoblast and fibroblast cell lines derived from NCL patients or NCL mutant mice [217]. Unfortunately, this study is wrought with technical concerns such as a co-localization by immunocytochemistry that used improper pairing of secondary antibody species and resulted in false-positive co-localization [217]. A sheep anti-CLN6 antibody and a sheep anti-CLN8 antibody were used to co-localize CLN6 and CLN8 in human fibroblasts, which will result with anti-Sheep IgG cross-reactivity. Immunoprecipitation of CLN3 and CLN2 from lymphoblasts using rabbit anti-CLN3 and rabbit anti-CLN2 antibodies was followed by immunoblotting with the same rabbit antibodies for detecting co-immunoprecipitation, again causing anti-rabbit-IgG-HRP cross-reactivity [217]. Curiously, the immunoprecipitation immunoblots are in fact showing bands that are most likely the IgG from the antibodies used for the immunoprecipitation, rather than the NCL proteins. Additionally, the primary antibodies used were of dubious specificity to NCL proteins, as no validation of the antibodies was published with the study. CLN5 was not addressed in this work, and therefore any comparison between it and the work of Lyly et al. [105] is not appropriate.

When considering the interactions of the NCL proteins, it is important to consider the tissue and developmental expression of these genes and therefore the likelihood of an in vitro interaction having biological significance. The genes encoding NCL proteins are differentially expressed in the mouse brain in both a spatial and temporal manner, according to in situ images of the St. Jude Brain Gene Expression Map (Table 2) (http://www.stjudebgem.org/, 2010). For example, at embryonic day 15, TPP1 is well expressed in the ventricles of the developing mouse brain, a location and time point where there is little expression of CLN3, CLN5, CLN6, or CLN8, but high expression of cathepsin D. Indeed, CLN5 and CLN8 expression throughout mouse brain development are both quite low compared to other NCL proteins, and they do not correlate with expression of any of the other NCL proteins in this database. This database provides information only on the transcript expression of these proteins, and it is also possible that expression exists at sub-detectable levels, or at later stages of development since the oldest age examined was postnatal day 42.

Because mutations in many different NCL proteins result in common disease, it is tempting to simplify the

mechanism of pathology to a common pathway; however, it is likely that NCL is much more complicated. Not surprisingly, comparison of mouse models for different NCLs show similar disruptions, for example in *Cln1*$^{-/-}$ and *Cln5*$^{-/-}$ mice have similar alterations in brain gene expression profiles [218]. It is probable that NCL proteins are multi-functional cell players that interact with different proteins in various locations in the cell, and perhaps even in diverse types of cells in a temporally regulated manner. Inevitably, the context of a neuron must be considered for NCL protein function, as these are the cells that are most sensitive to the loss of these proteins. It is not clear if neurons are more sensitive to storage material accumulation, or to the processes that are disrupted to cause the accumulation of storage material. Experiments to transfer storage material to wild type cells could answer this question. It is likely the answer is a combination of the two. The localizations of NCL proteins indicate that NCL is a cellular disorder of different pathways converging on the same disease. Neurons are highly specialized, highly polarized, and post-mitotic cells that exist in a state of constant ionic flux and rely heavily on the secretory system for neurotransmission. PPT1 has been found down axons and in synaptic vesicles [56] and CLN3 has been found in synaptosomes [120], providing evidence that NCL proteins can be found in the processes of neurons beyond just the lysosome in the cell soma. These localizations could indicate specific roles for the NCL proteins in neurons that are not yet well understood.

Concluding remarks

Neuronal Ceroid Lipofuscinosis is a lysosomal storage disorder that is caused by mutations in eight different proteins that reside in the lysosome, ER, and other regions of the cell. The dysfunction caused by the loss of functional NCL proteins results in neurodegeneration and central nervous system pathology. Though we know the NCLs to be neurodegenerative diseases, the mechanism of the pathology has not been clearly defined; whether loss of neurons is precipitated by accumulation of toxic storage material in neurons, or by the loss of the functional proteins directly, is still a topic of debate. Additionally, the importance of NCL proteins in the support cells of the brain and other tissues has not been assessed. It is unlikely that interactions between the NCL proteins have biological significance; however, understanding the interactome of the each of the NCL proteins with their substrates or binding partners will offer insight into the pathways affected in NCL, and how these pathways converge in lysosomal dysfunction.

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