A STUDY OF NEURONAL CEROID LIPOFUSCINOSIS PROTEINS CLN5 AND CLN8

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ABSTRACT

Neuronal ceroid lipofuscinoses (NCLs) are a group of neurodegenerative lysosomal storage disorders which is the most frequent group of inherited neurodegenerative disorders that affect children leading to severe pathological conditions such as progressive loss of motor neuron functions, loss of vision, mental retardation, epilepsy, ataxia and atrophy in cerebral, cerebella cortex and retina and eventually premature death. Among the many genes that cause NCL, mutations in CLN5 leads to different forms of NCL (infantile, late infantile, juvenile and adult) and mutations in CLN8 leads to progressive epilepsy with mental retardation (EPMR) and a variant late infantile form of NCL. The function(s) of both CLN5 and CLN8 proteins remain elusive.

CLN5 is a glycosylated soluble protein that resides in the lysosome. We observed that endogenous CLN5 protein exist in two forms and identified a previously unknown C-terminal proteolytic processing event of CLN5. Using a cycloheximide chase experiment we demonstrated that the proteolytic processing of CLN5 is a post-translational modification. Furthermore treatment with chloroquine showed the processing occurs in low pH cellular compartments. After treatment with different protease inhibitors our results suggested the protease involved in the processing of CLN5 could be a cysteine protease. Using two glycosylation mutants of CLN5, retained in the endoplasmic reticulum (ER) or the Golgi we showed the proteolytic processing occurs in an organelle beyond the ER. This study contributes to understanding the characteristics of the CLN5 protein.

CLN8 is an ER resident transmembrane protein that shuttles between the ER and the ER-Golgi intermediate compartment (ERGIC). In our study we identified a potential interaction between CLN8 and a PP2A holoenzyme complex consisting regulatory subunit A α isoform and regulatory subunit B α isoform. Using two CLN8 patient derived fibroblast cell lines we were able to show that the phosphorylated levels of PP2A target kinase Akt was reduced at both of its regulatory sites Ser473 and Thr308 and the activity of PP2A was increased. A delay of ceramide transport from ER to Golgi in CLN8 deficient patient cell lines was observed using BODIPY FL C5-Ceramide staining. Our results provide evidence for CLN8 protein being involved in the regulation of PP2A activity and trafficking of ceramide from ER to Golgi.

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CHAPTER 1

INTRODUCTION

Lysosomal Storage Disorders (LSD)

Lysosomal storage disorders (LSD) are a group of heterogeneous hereditary disorders, named so because of the characteristic excessive accumulation of undigested or partially undigested compounds including lipids, proteins, carbohydrates and trace amounts of metal ions in the lysosomes (1). Lysosomes are an important membrane bound organelle which acts as the waste disposal system of cells and has an acidic pH at which the many digestive enzymes in it are activated. About 50 – 60 soluble hydrolases and at least 7 integral membrane proteins have been identified to be involved in the enzymatic digestion of various substrates (2). More than 50 disorders have been recognized at present as LSDs since the introduction of this category by Hers in 1963 while studying the Pompe disease (glycogen storage disease type II) caused by a deficiency in α-glucosidase, which leads to the accumulation of glycogen in lysosomes (1, 3).

Classical LSDs occur because of the absence or defects in lysosomal hydrolases, leading to the accumulation of one or more of the undigested substrates in the lysosomal lumen. Examples such as Fabry disease caused due to deficiency or mutations in α-Galactosidase A or Gaucher disease caused by defects in β-Glucosidase are LSDs resulting from defects in lysosomal hydrolases (1, 4). It has also been discovered that apart from deficiencies in lysosomal hydrolases, the absence of or mutations in non-enzyme proteins such as integral membrane proteins involved as transporters of particular substrates or activator proteins can also lead to the accumulation of undigested material in the lysosomes (1, 5, 6). For instance, sphingolipid activator proteins (GM2 activator protein and Saposins A, B, C, D) are non-enzymatic proteins present in the lysosome. But they are essential in activating the step wise degradation of glycosylceramides during recycling of sphingolipids, by facilitating the interaction between membrane embedded glycosylceramides and the soluble hydrolases in the lysosomal lumen (7, 8). Thus the absence of

or mutations in sphingolipid activator proteins will lead to the accumulation of glycolipids in the lysosome (2). Mutations in proteins required for intracellular trafficking of lysosomal enzymes can also lead to LSDs. Mucolipidosis is an example of a group of LSDs caused due to a deficiency in a phosphotransferase which is necessary for mannose 6-phosphate (M6P) modifications of the lysosomal enzymes for trafficking them to the lysosomes. Therefore altered intracellular trafficking can lead to lysosomal enzymes not reaching the lysosome and by that causing the accumulation of its substrate material. The majority of the lysosomal soluble and membrane proteins are synthesized in the endoplasmic reticulum (ER) and at this site they undergo proper folding, quality control, N-glycosylation and sulfatase modifications. Afterwards they are transported from the ER along the secretory pathway through the Golgi apparatus, trans-Golgi network (TGN), endosomes and late endosomes before finally reaching the lysosomes. Modifications on the attached oligosaccharides, lipid modifications and proteolytical cleavages that occur along the pathway are required for the proper trafficking and maturation of lysosomal proteins (9). Therefore defects in proteins, either enzymatic or non-enzymatic, which are required in these processes can lead to defective lysosomal proteins or a deficiency of the lysosomal proteins, in turn causing LSDs. Multiple sulphatase deficiency is one such LSD caused because of mutations in sulphatase modifying factor 1 (10).

Although with time, improved understanding has been gained in how various types of defects in lysosomal proteins, problems in protein trafficking and posttranslational modifications lead to lysosomal accumulations, there are certain groups of lysosomal disorders like the neuronal ceroid lipofuscinosis (NCL) where it has been difficult in explaining the relationship between the accumulated material and the deficiencies in the NCL associated proteins (1, 4).

Many of the LSDs are known to cause neuropathology (1, 2, 5). Neuronal cells are post mitotic cells and are not able to regenerate themselves as readily as other tissue cells, making them highly susceptible to lysosomal accumulations and resulting alterations in cellular homeostasis. The direct link between lysosomal accumulations and neurodegeneration has not yet been understood but several mechanisms have been proposed such as altered cell signaling, altered Ca^{2+} homeostasis, decreased or increased autophagy and apoptosis (2, 4, 6). It has been observed in mucopolysaccharidose disease, the lysosomal storage of undigested or partly digested glycosaminoglycan causes altered cell signaling by functioning as ligands and non-specifically leading to the activation of certain receptors like TLR4 (Toll like receptor 4) (11, 12). Modification of the response of receptors, as observed in Hurler syndrome where deficiency of α-L-iduronidase ultimately results in reduced affinity of FGF-2 to the FGF receptor (13) is another mechanism leading to altered cell signaling. An example of altered receptor localization is the disturbed endosomal and lysosomal trafficking observed in Niemann-Pick Type C disorder that causes less degradation of TLR4 receptor in the lysosomes and accumulation on endosomes. This leads to intensified TLR4 signaling causing increased cytokine levels and inflammation (14). Inflammation causes subsequent neuronal dysfunction. In Gaucher disease accumulation of glycosylceramides have been observed to increase the release of Ca^{2+} from the ER by sensitizing one of the two ligand gated Ca^{2+} channels in the ER (15, 16). The ER acts as a Ca^{2+} reservoir in cells and Ca^{2+} is necessary for the activity of molecular chaperones such as calnexin and calreticulin which are involved in proper folding of proteins synthesized in the ER. Aggregation of misfolded proteins in the ER leads to the activation of the unfolded protein response (UPR) pathway. Although UPR is a protective mechanism, prolonged activation of the pathway can lead to apoptosis and loss of neuronal cells causing neuropathology. Another important pathway

affected in LSDs is the autophagy pathway. The survival of neuronal cells depends on proper regulation of autophagy. This is because of their post-mitotic nature and the need to mediate fast turnover of cellular proteins to maintain their integrity as required for proper neuronal function. The lysosome plays an important role in the autophagy pathway since the autophagosomes carrying damaged organelles, proteins and other cargo have to fuse with the lysosome where the cargo is exposed to lysosomal hydrolases and degraded. However in LSDs it has been observed that accumulation of material in the lysosomes can cause defects in autophagosome and lysosome fusion. Increased autophagy has been observed in a number of LSDs and in many of the subtypes of NCL disorders including CLN1, CLN2, CLN3 and CLN5 disease (1, 2, 4, 6).

Neuronal Ceroid Lipofuscinosis (NCL)

Neuronal ceroid lipofuscinoses (NCLs) are a collective group of neurodegenerative lysosomal storage disorders which make up the most frequently observed group of inherited progressive neurodegenerative disorders affecting children, with selective degeneration and loss of neurons in the cerebral, cerebellar cortex and retina leading to epileptic seizures, progressive loss of motor neuronal functions and loss of vision, mental retardation and premature death. Estimates of the incidence of NCL disorders report a range from 1 in 12, 500 to 1 in 100, 000 live births around the world (1, 17). Although NCLs are considered as a comparatively rare group of disorders they nevertheless have a devastating impact on affected children and their families and subsequent health care systems for long periods of time (18-20). At present NCL disorders are associated with mutations in 13 different genes, whose protein products are named as ceroid neuronal lipofuscinosis (CLN) proteins CLN1 – CLN14 (Table 1.1). Although genetically and

clinically heterogeneous, mutations in CLN proteins all have in common an accumulation of autofluorescent lipopigments, lipofuscin like ceroid material in the lysosomal compartments along with proteinaceous deposits of mainly subunit C of mitochondrial ATP synthase or saposins A and D depending on the subtype of the disease (1). NCLs are categorized as lysosomal storage disorders because of these lysosomal accumulations. Lysosomal storage disorders can be caused by the dysfunction in lysosomal components; hydrolases and hydrolase activators or due to impaired vesicular trafficking, as discussed in previous section (1). The NCL associated CLN proteins display a lot of heterogeneity in cellular localization where CLN1, CLN2, CLN5, CLN10, CLN13 are soluble proteins present in the lysosomal lumen, CLN3, CLN7 and CLN12 are lysosomal transmembrane proteins, CLN6 and CLN8 are transmembrane proteins localized in the endoplasmic reticulum (ER) and CLN4 and CLN14 localized in the cytosol associated with vesicular membranes (18, 21). The function of most of the CLN proteins and information about what substrates they act upon remain unclear. Genetic studies, analysis of protein and lipid profiles, biochemical studies using cultured NCL patient cells or animal models representing various subtypes of NCL disorders have helped in understanding or predicting the potential cellular functions of many CLN proteins as well as an understanding about the type of cellular pathways involved (17-19, 21). NCL disorders have also been classified based on their clinical onset as infantile (INCL), late infantile (LINCL), juvenile (JNCL) and adult (ANCL). Furthermore several variant forms having later onset or less severity has been recognized (19, 20).

Based on biochemical and analytical studies followed on cultured NCL patient cells, animal models of NCL or cerebella samples obtained from NCL disease patients, it has been observed that most CLN mutations and deficiencies lead to altered lipid profiles and altered lipid

metabolism and more specifically alterations in sphingolipid metabolism (22). Sphingolipids play an important role in the regulation of pro-death and pro-survival signaling in cells apart from being structural constituents of the cellular membranes. Relative to other body tissues, nervous tissues contain much higher levels of sphingolipids (23). Altered lipid and sphingolipid metabolism has been observed in other neurodegenerative disorders such as Alzheimer's, Huntington's, Gaucher disease and prion disease (22). Abnormal sphingolipid profiles have been observed in CLN1, CLN2, CLN3, CLN5, CLN6 and CLN8 deficient cells leading to the speculation that these proteins might be involved in a common pathway. Table 1.1 lists the known functions and predicted functions of CLN1 – CLN14. Although the functions of some CLN proteins have been identified, the link between those functions and the pathological observation of NCLs; the accumulation of ceroid lipopigments and the proteins subunit C of ATP synthase or saposins A and D have not been clearly understood (1).

This thesis work focuses mainly on two of the NCL associated proteins CLN5 and CLN8. The function(s) of both proteins remain elusive. In the study on CLN5, which is a lysosomal soluble protein, we aim to understand the characteristics of CLN5 by analyzing a potential processing occurring at the C-terminus of the protein. Many of the lysosomal proteins undergo trimming on attached oligosaccharides or proteolytical processing during vesicular transport from ER to lysosome or after reaching the lysosome, and these steps are considered important for the protein to mature. The objective of the study on CLN8 which is a transmembrane protein residing mainly in the ER, was to identify other proteins that would potentially interact with it in order to gain some understanding of the probable function(s) of CLN8. Some possible functional interactions between CLN5 and CLN8 have been discussed in literature (18, 21, 24-26). However the work followed in this thesis does not address this information.

Table 1-1: NCL associated proteins, their subcellular localization and proposed functions (1-4)

NCL Gene	Protein	Subcellular localization	Function
CLN1	CLN1/ Palmitoyl thioesterase 1 (PPT 1)	Lysosomal lumen, extra lysosomal vesicles associated with presynaptic areas of neurons	Palmitoyl thioesterase activity. Associated with recycling of synaptic vesicles, lipid metabolism and apoptosis
CLN2	CLN2/ Tripeptidyl-peptidase 1 (TPP 1)	Lysosomal lumen	A serine protease. Associated with autophagy and endocytosis
CLN3	CLN3	Late endosomal and lysosomal membrane	Unknown function. Proposed roles in endocytosis and autophagy, maintenance of lysosomal pH
CLN4	CLN4/ CPSα/DNAJC5	Cytosol associated with vesicular membranes/ associated with synaptic vesicles in neuronal cells	Hsc70 co-chaperone: predicted function in pre-synaptic endo/exocytosis
CLN5	CLN5	Lysosomal lumen	Unknown function. Predicted to be involved in synthesis and transport of sphingolipids, role in autophagy, Endosome sorting
CLN6	CLN6	ER membrane	Unknown function. Associated with endocytosis and autophagy
CLN7	CLN7/ MFSD8	Lysosomal membrane	Unknown function. Proposed to be involved in transport of small molecules across cellular membranes

CLN8	CLN8	ER and ER-Golgi intermediate compartment membrane	Unknown function. Predicted to have a role in the synthesis and transport of sphingolipids. Also has been related to oxidative and ER stress response and calcium homeostasis
CLN10	CLN10/ Cathepsin D (CTSD)	Lysosomal lumen and extracellular	Aspartyl endopeptidase. Plays an important role in autophagy and apoptosis
CLN11	CLN11/ Progranulin	Extracellular	Unknown function. Associated with autophagy, embryogenesis, inflammation
CLN12	CLN12/ ATPase 13A2/ PARK9	Lysosomal membrane	Unknown function. Proposed a role in shuttling cations, heavy metals and lipids across cellular membranes, protection against α-synuclein toxicity
CLN13	CLN13/ Cathepsin F	Lysosomal lumen	Cysteine protease. Involved in autophagy, lipoprotein degradation, associated with proteasomal degradation
CLN14	CLN14/ Potassium channel tetramerization domain-containing protein 7 (KCTD7)	Cytosol, partially associated with the plasma membrane	Unknown function. Predicted to regulate K^+ conductance in neurons, maintaining the polarization of neuronal membrane

CLN5

CLN5 is one of the 13 genes that have been associated with NCL disorders. Mutations in the CLN5 gene were in the beginning mainly related to a Finnish variant of late infantile NCL (LINCL) (27), although recently this variant has been discovered among NCL patients of broader ethnicity (28). At present about 36 CLN5 disease causing mutations have been discovered (http://www.ucl.ac.uk/ncl/CLN5mutationtable.htm) and novel CLN5 mutations have been identified and associated with early juvenile as well as adult forms of NCLs (18, 28-30). At the mRNA level it has been observed that CLN5 expression in mouse as well as humans is ubiquitous in all tissues (17, 18, 21). In the mouse embryonic brain, higher expression of CLN5 has been noted in cerebral cortex, cerebellum and in the ganglionic eminence. In the adult brain most expression of CLN5 has been observed in Purkinje cell layer of the cerebellum, in the cerebral cortex, cortical neuronal cells, hippocampal cell layers and hypothalamic neurons (17, 18, 21). The major storage component accumulated in the lysosomes of CLN5 disease cells is the subunit C of mitochondrial ATP synthase (31). In most cases the clinical onset of CLN5 disease occurs around ages 2 - 7 with motor clumsiness observed. Progressive visual impairment, mental and motor degeneration is followed by ataxia, myoclonus and seizures in the later stages of the disease. The age of death varies from ages around 10 – 30 (17, 18, 21, 32). Based on studies involving imaging of the brain or autopsy of brain specimens, it has been shown that cerebella atrophy is prominent in CLN5 disease (32).

The CLN5 gene has been mapped to chromosome 13q22. The gene consists of four exons which lead to the transcription of three CLN5 transcripts (17). Human CLN5 protein consists of 407 amino acids and contains an N-terminal signal peptide sequence which is cleaved after entering the ER during protein synthesis (33). The predicted amino acid sequence of CLN5 shows no

homology compared with other proteins. An earlier study has predicted CLN5 protein to contain a transmembrane region (34) while recent studies have shown CLN5 as a soluble protein (32, 35) Nonetheless, it is without doubt agreed that CLN5 is localized to the lysosome as it had been shown to co-localize with the lysosome marker protein LAMP-1 (32, 35). CLN5 protein contains eight predicted N-glycosylation sites and it has been shown all of these sites are utilized and have a role in proper folding, lysosome trafficking or function (35). It has been demonstrated that transfection of CLN5 cDNA in mammalian cells results in a ~ 60 kDa protein which is reduced to ~ 35 kDa when treated with Endoglycosidase H (Endo H) (35). CLN5 is synthesized and undergoes folding in the ER and is trafficked through the secretory pathway destined to reach the lysosomes and it is believed based on immunofluorescence studies and proteomic analysis studies that CLN5 is transported in a mannose 6-phosphate (M-6-P) dependent manner by which most of the lysosomal soluble proteins are targeted to their destination. The M-6-P receptor in the TGN can recognize these moieties and facilitate the proper sorting of these proteins to the endosome and lysosome (35, 36).

The function of CLN5 is unknown. Some studies have demonstrated interactions between CLN5 and other NCL associated proteins CLN1, CLN2, CLN3, CLN6 and CLN8 suggesting the possibility of a molecular network. Although these interactions do not demonstrate a function for CLN5, it has been proposed to be involved in the trafficking of other NCL proteins as shown in a study involving the trafficking of CLN1 protein. The trafficking of lysosomal soluble protein CLN1 was impaired and it was retained in the ER in CLN5 depleted cells (26). It has been proposed to be involved in the recycling process of endosomal sorting proteins sortilin and LIMP-II (37). These proteins are involved in the sorting of proteins and anterograde trafficking (from Golgi to endosomes). When the vesicles carrying sortilin, LIMP-II and cargo proteins

reach the endosomes, another protein complex called the retromer is recruited from the cytosol and it mediates the recycling of most of the sortilin and LIMP-II through retrograde trafficking (endosomes to Golgi). Depletion of CLN5 interrupts with this recycling and leads to the degradation of sortilin and LIMP-II in the lysosomes thereby proposing CLN5 may have a role in the recruitment of retromer complex and endosomal sorting (37). However, as CLN5 does not have a cytoplasmic region, this recruitment role might be indirect. CLN5 protein has also been suggested to be involved in the synthesis or transport of sphingolipids (25) due to altered lipid profiles and lipid transport observed in CLN5 depleted cells. Myelination defects observed in CLN5 knockout mice are also supported by gene profiling data from a CLN5 deficient mouse brain displaying down regulation of myelin associated genes and brain atrophy (24, 25, 32).

CLN8

Mutations in the CLN8 gene causes two distinct NCL phenotypes; a juvenile onset NCL variant, progressive epilepsy with mental retardation (EPMR) and a more severe variant of late infantile NCL (vLINCL). EPMR (also known as Northern epilepsy) and the CLN8 vLINCL both display autofluorescent storage material with curvilinear like profiles and granular material mainly consisting of the accumulated subunit C of mitochondrial ATP synthase (17, 21, 38). Both disorders were first identified in small populations in Turkey and Finland, but are now reported in other ethnic origins as well (21, 39). The age of onset for EPMR is about 5 – 10 years old while CLN8 vLINCL may onset at a much younger age. The life expectancy of patients suffering from CLN8 disorders vary from teenage years to adulthood and more than 30 different mutations causing deletion or missense of nucleotides in the CLN8 gene have been linked to these

disorders (http://www.ucl.ac.uk/ncl/CLN8mutationtable.htm). Early clinical symptoms include delay or difficulty in speech, retarded development, visual impairment and ataxia. With the progression of the disorder epilepsy, progressive motor and cognitive decline, blindness, mental retardation occurs subsequently leading to premature death (38, 39).

The motor neuron degeneration (mnd) mouse model carries a homozygous mutation in its gene orthologous to human CLN8. The mutation leads to a truncated form of the protein and a deficiency in CLN8 protein (17, 18, 21, 38-40). The human gene for CLN8 is located on the short arm of chromosome 8. Human and mouse CLN8 share 82 % sequence similarity at nucleotide level and 85 % similarity at polypeptide level (41). The expression of CLN8 has been studied at mRNA level and in prenatal mouse embryos; CLN8 mRNA is majorly expressed in the gastrointestinal tract, dorsal root ganglia and brain. Analysis of CLN8 mRNA levels in the developing mouse brain has shown higher levels of expression in the cortex and hippocampus (42).

CLN8 gene product is a 286 amino acid transmembrane protein with five predicted α-helical transmembrane regions. Studies do not report any post-translational modifications of CLN8. The CLN8 peptide sequence contains one potential N-glycosylation site but it has been demonstrated by Endo H digestion studies that this N-glycosylation site is not utilized, therefore CLN8 remains a non-glycosylated protein (38). The CLN8 protein is ~ 33 kDa and pulse – chase analysis experiments have shown that it does not undergo any proteolytical processing (38). CLN8 protein is mainly localized to the ER and in the ER-Golgi intermediate compartment (ERGIC). CLN8 protein contains an ER retrieval signal at its C-terminus, a dilysine motif (KKXX) KKRP which suggests that CLN8 shuttles between the ER and the Golgi. CLN8 localization has been demonstrated through immunofluorescence studies. This has also been

confirmed by the mutation of the dilysine motif that leads to CLN8 being localized to the Golgi (38). None of the disease causing mutations identified at present affects the localization of CLN8 protein.

The function of the CLN8 is unknown. It belongs to a protein family with TLC (TRAM-Lag1p-CLN8) domains (43). Sixteen human proteins are known to contain the TLC domain including ceramide synthases 1- 6 involved in acyl-CoA dependent *de novo* synthesis of ceramide in the ER compartments, TRAMs; a protein family that facilitates the translocation of newly synthesized membrane proteins into the ER, CLN8 protein and six other novel proteins. Although the function(s) of most of the TLC domain family members remain unknown (apart from the ceramide synthases 1 - 6), functions involved in sensing and trafficking of lipids, regulation of lipid metabolism has been proposed (17, 25, 40).

A potential role for CLN8 in lipid homeostasis has been proposed since it contains a TLC domain spanning 200 amino acids (62 – 262 aa). A study involving mass spectrometry analysis of the levels of sphingolipids in fibroblast cells derived from the mnd mouse showed decreased generation of certain ceramide species and their downstream metabolites compared to normal fibroblast cells derived from healthy mouse (25). This study also demonstrated that the overexpression of CLN8 cDNA in normal human fibroblast cells, lead to an increase in certain ceramide species suggesting that CLN8 might have role in ceramide synthesis by itself or by activating ceramide synthases. Lipid profile analysis by liquid chromatography and mass spectrometry of cerebral brain samples obtained from EPMR patients showed reduced ceramide, galactosyceramide, lactosylceramide and sulfatide and also a decrease in long fatty acyl chain containing sphingolipids molecules also indicating a role for CLN8 in ceramide synthesis or

regulation of ceramide trafficking (44). Deficiency in galactolipids, delayed myelination and oligodendrocyte maturation has been observed in CLN8 mnd mouse neuronal tissue (24, 45). CLN8 has also been proposed to be involved in the regulation of ER stress response since several early ER stress indicators were discovered to be upregulated in the CLN8 mnd mouse (24, 46). Some studies indicate that CLN8 deficiency can lead to the dysfunction of mitochondrial Ca^{2+} clearance (47). It has been shown that spatial and functional interactions may exist between the mitochondria and ER. Furthermore abnormal morphology for mitochondria has been observed in NCL neuronal cells (48). Several studies have shown using CLN8 mnd mouse and cultured hippocampal neuronal cells derived from the CLN8 mnd mouse, that Ca^{2+} uptake from the mitochondria was significantly altered compared to healthy neuronal cells and this alteration seemed more prevalent with the age of the mice (48, 49). Prolonged intracellular elevated levels of Ca^{2+} can lead to excitotoxicity by the activation of a number of enzymes such as proteases like calpain, phospholipases and endonucleases which can cause damage to the cell membrane, cytoskeleton and DNA compromising the integrity of neuronal cells and eventually causing neuronal death (17, 48, 49).

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CHAPTER 2

PROTEOLYTIC PROCESSING OF NEURONAL CEROID LIPOFUSCINOSIS RELATED LYSOSOMAL PROTEIN CLN5

ABSTRACT

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

INTRODUCTION

The neuronal ceroid lipofuscinoses (NCLs) are a group of genetically inherited lysosomal storage and neurodegenerative disorders. NCLs have a variety of onsets and cause a range of clinical symptoms including loss of motor function and vision, seizures, mental retardation and premature death (1, 2). It is a severe disorder and considered the most frequent neurodegenerative disease in children. Similar to other lysosomal storage disorders, NCL is rare with an estimated occurrence of 1:100,000 live births. The incidence is higher in certain regions, such as Northern Europe and Northern America (3, 4). Eight genes are clearly identified to associate with childhood onset of NCLs (CLN1, CLN2, CLN3, CLN5, CLN6, CLN7, CLN8, and CLN10), whereas some other genes are linked to adult onset forms (e.g. CLN4 and CLN11). Recently more genes have been identified CLN11-14 (5).

The new nomenclature system names the subtype of NCLs based on the affected gene. For instance, CLN5 deficient NCL is now named CLN5 disease (6). The majority of the accumulated proteinaceous buildups in NCL patients are either the subunit C of mitochondrial ATP synthase (7) or saposins A and D (8) depending on the subtype of the disease. Intriguingly, in spite of affecting lysosomal degradation process, these NCL proteins reside in various locations within the cells such as the endoplasmic reticulum (ER) membrane (CLN6 and CLN8), the cytoplasm (CLN4), and the lysosomes (CLN1, CLN2, CLN3, CLN5, CLN7, CLN10, CLN12 and CLN13) (9). Among the lysosome-localized proteins, CLN1 (PPT1), CLN2 (TPP1), CLN5, CLN10 (Cathepsin D) and CLN13 (Cathepsin F) are luminal soluble proteins, whereas CLN3, CLN7 and CLN12 are multi-spanning transmembrane proteins. PPT1, TPP1, Cathepsin D and Cathepsin F are acid hydrolases with different enzyme activity and substrate specificities. CLN5, however, does not possess any homology to any known proteins and its function remains

unclear. CLN5 disease was originally identified as a rare variant form of NCL restricted to Finnish and other Northern European populations (10, 11). However, a more recent study has identified CLN5 disease in a variety of ethnic backgrounds and suggests that CLN5 mutations are more common in patients with NCL than previously thought (12).

The human CLN5 gene encodes a protein of 407 amino acids with an N-terminal signal sequence that is cleaved in the ER (13). Human CLN5 protein consist of eight putative N-glycosylation consensus sites (N-X-T/S) and it has been shown that all of these sites are utilized, with roles involving proper folding, lysosomal targeting and function (14). The glycosylation site at 401 aa in human CLN5 has been shown important in its sorting and trafficking from Golgi to the lysosome, by the Mannose-6-phosphate receptor (MPR) dependent pathway (14). However it has been shown CLN5 can reach the lysosomes in a MPR-independent pathway(s) as well during absence of MPR (13). CLN5 protein is about ~ 60 kDa since it is highly glycosylated. Treatment with EndoH reduces the size of CLN5 protein to ~ 35 kDa (14). A previous study showed CLN5 contains a C-terminal amphipathic helix region that is tightly associated with the membrane (15). CLN5 is ubiquitously expressed in human and mouse based on northern blot analyses (10). A study following the CLN5 mRNA expression in the mouse brain throughout embryo development report enhanced CLN5 expression in cerebellar Purkinje cells, cerebral neurons, hippocampal pyramidal cells and hippocampal interneurons. They also report the expression pattern can be correlated to the brain atrophy in CLN5 disease patients (16). Another study linked CLN5 expression with neurogenesis during embryo development. They observed that CLN5 was more prominently expressed in the regions of the mouse brain that give rise to new neuronal cells (17). It has also been observed CLN5 expression in mouse brain is different between neuronal cells and glia (18). Microglia functions as macrophages of the central nervous

system (CNS) that involves scavenging plaques, damaged neurons and infectious foreign material. This study demonstrated higher activation of microglia in CLN5 deficient mice during early development (18). High activity of microglia can lead to neurotoxicity. They also observed altered myelination in neuronal cells. Hypo myelination can be due to problems in oligodendrocyte maturation or function. Altered sphingolipid metabolism may also contribute to reduced myelination.

CLN5 protein has also been linked with sphingolipid metabolism. A study observed altered ceramide levels in CLN5 deficient fibroblast cells with decreased sphingomyelin and glycosphingolipids (19). Several studies indicate interactions among other NCL proteins and CLN5 (20, 21) proposing molecular networking between NCL proteins; however the significance of these interactions has not been understood. CLN5 has also been suggested to play a role in endosomal sorting as an interaction between CLN5 and the lysosomal sorting receptor sortilin has been identified (22).

Although the CLN5 expression has been analyzed at mRNA level, the protein level of CLN5 in tissues has never been directly examined. In this report, we examine CLN5 protein expression in a panel of various mammalian cell lines. We also discover a previously unknown C-terminal cleavage of CLN5 which might be involved in the maturation of the protein.

MATERIALS AND METHODS

Cell culture and transfections

Cell culture media and reagents were purchased from Gibco and Hyclone. Cell lines used in this study are A431 (ATCC CRL-1555), HEK293 (ATCC CRL-1573), HeLa (ATCC CCL-2), HepG2 (ATCC HB-8065), HT1080 (ATCC CCL-121), SH-SY5Y (ATCC CRL-2266), control fibroblasts GM00037 and GM00498 (Coriell). CLN5 patient fibroblasts #1 (homozygous c.694C>T, p.Q232X) and #2 (c.671G>A, p.W224X and exon 4 deletion) were received from Massachusetts General Hospital CHGR NCL Disorders Clinical Database and Biorepository. All cells were grown and maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 20 mM HEPES and gentamicin at 37 ºC in a humidified incubator with 5% CO_2. For transfection, HEK293 cells were seeded in culture dishes for 24 h before transfection. The TransIT-LT1 transfection reagent (Mirus Bio) was used for overexpression of CLN5-Myc-His, and Lipofectamine RNA iMAX reagent (Life Technologies) was used for siRNA gene silencing. Transfections were done according to manufacturer's protocol. Opti-MEM reduced serum medium (Gibco) was used for reagent/nucleotides complexes formation.

Plasmids

Wild type, D279N, and N401Q CLN5 in pcDNA3.1/Myc-His (-) A were described previously (14). The siRNA resistant CLN5 construct was generated using site-directed mutagenesis to create silent point mutations in the CLN5 siRNA target site (GAACCT AC<u>C</u>TA<u>C</u>CTGGGAA, underlined nucleotides are mismatched with the original sequence). DNA sequences

corresponding to CLN5 amino acids 200-300, 200-220, and 240-300 were inserted to pGEX6pk-1 to generate constructs for Glutathione S Transferase (GST) fusion protein expression.

siRNAs

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

GST fusion protein expression and peptide blocking experiments

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

Western blotting

Cells grown on 10 cm, 6 well or 12 well culture dishes were scraped and washed once with 1 X phosphate buffered saline, pH 7.4 and centrifuged for 3 min at 1,500xg. Cell pellets were lysed using RIPA lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (G-Biosciences). After incubation for 30 min on ice, extracts were centrifuged at 20,000xg for 10 min at 4 $^{\circ}$C. The

supernatant was collected as the whole cell lysates. Protein concentrations were determined with Bradford assay when necessary. Aliquots of total extracts were incubated with sample buffer containing 100 mM DTT at 37 °C for 10 min. Samples were separated by SDS-PAGE. Proteins were transferred to PVDF membranes (Millipore) followed by immunoblotting. ECL detection was performed according to manufacturer's instructions (Millipore) and blots were imaged with G-Box (Syngene) or C-DiGit (Li-COR). C-DiGit software was used for quantification. Medium samples were concentrated with spin column (Pierce concentrator, 10 K MWCO) before proceeding to protein gels.

Antibodies

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

Drug treatment experiments

Deglycosylation

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

Cycloheximide chase

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

Protease inhibition

For treatment with different protease inhibitors HEK293 cells were seeded in culture dishes for 24 h before transfection. DMSO (Cellgro), 50 µM Chloroquine (MP Biomedicals), 5 µM Pepstatin A (Sigma), 100 µM Leupeptin (Cayman Chemical), 10 µg/ml E-64 (Cayman Chemical) or 150 µM AEBSF (Cayman Chemical) were added to medium 4 h post transfection. Cells were collected 24 h after the start of drug treatment.

RESULTS

The endogenous CLN5 glycoprotein exists in two forms

To study the expression of CLN5 in different cell lines at the protein level, total cell lysates of human cell lines A431, HEK293, HeLa, HepG2, HT1080, SH-SY5Y and GM00498 (a healthy control fibroblast cell line) were analyzed by CLN5 immunoblotting. The results of CLN5 immunoblotting (Figure 2.1 A) showed two major bands, an upper band around ~56 kDa and a lower band around ~52 kDa. Among the cell lines analyzed HEK293, HepG2, HT1080, SH-SY5Y and GM00498 predominantly had the lower band. The intensity of both bands was similar in A431 and HeLa cells. To verify the specificity of the antibody against CLN5, we

performed a peptide blocking experiment. The antigen of this rabbit monoclonal antibody is within the region of amino acids 200-300 of CLN5 protein (based on the sequence of NP_006484). By pre-incubating the antibody with amino acids 200-300 of CLN5 fused with GST (GST-CLN5 200-300), the immunoblotting signal can be completely blocked (Figure 2.1 B). This indicates that both upper and lower bands were from CLN5. We further examined the CLN5 signals from skin fibroblasts of two CLN5 deficient patients (CLN5#1 and CLN5#2). As shown in Figure 2.1 C, both patients do not have either form of CLN5 proteins compared to two healthy control skin fibroblasts (GM00037 and GM00498). The patient CLN5#1 has a homozygous 694C>T point mutation in DNA, resulting in a premature stop codon and CLN5 protein truncation (Gln232X), whereas patient #2 has exon 4 deletion in one allele and 671G>A point mutation in the other allele, resulting in a premature stop codon and CLN5 protein truncation (Trp224X). Since we did not observe any signals from patient cells using the CLN5 antibody, there is a possibility that this CLN5 antibody cannot recognize the truncated CLN5 in these two patients. We tested this by generating two constructs covering either 200-220 amino acids or 240-300 amino acids of CLN5 to narrow down the possible immunogen region (Figure 2.1 D). It is clear that the CLN5 antibody recognizes an epitope within the region of 240-300 amino acids of CLN5. Therefore we cannot exclude the possibility that there are truncated CLN5 present in these two CLN5 patients. Figure 2.1 E shows the locations of the immunogen and patient truncations.

The N-terminal portion of CLN5 contains the signal peptide which is cleaved co-translationally during biosynthesis in the ER (13) and therefore not suitable for tagging. In our previous work when we used an overexpression system to follow C-terminal Myc-6xHis tagged versions of CLN5, we did not observe two distinct bands (14). We decide to use this CLN5 antibody to

revisit the overexpression system. To minimize the signals from endogenous CLN5, we treated cells with CLN5 specific small interfering RNA (siRNA) for up to 72 h to knockdown CLN5 expression. Treatment with CLN5 siRNA resulted in a substantial reduction of CLN5 protein level after 48 h and continued to reduce the amount of endogenous CLN5 at 72 h (Figure 2.2, left panel). This further confirms that the protein recognized by the CLN5 antibody used in this study is indeed CLN5. The less prominent upper endogenous CLN5 band was not readily visible in the HEK293 lysates in this experiment. In a separate set of experiment, after siRNA treatment for 48 h, cells were transfected with a siRNA resistant CLN5 construct for another 24 h. Interestingly, while after 72 h the level of endogenous CLN5 was dramatically reduced, overexpression of siRNA resistant CLN5 showed two major bands when blotted with CLN5 antibody. The overexpressed CLN5 used in this study contains a C-terminal Myc and 6xHis tag. For simplicity, hereafter only Myc tag will be mentioned in the text since we were not following the His tag. The lower band of overexpressed CLN5 has the same mobility as the lower band of endogenous CLN5. However by using Myc antibody only the higher band showed up (Figure 2.2, right panel). This suggests that the Myc tag at the C-terminal end of CLN5 has been cleaved and is no longer present in the lower band. This explains why we did not observe two bands in our previous study using the Myc antibody. This data indicates that CLN5 undergoes post-translational proteolytic cleavage.

Cycloheximide chase analysis demonstrates *in vivo* processing of CLN5

To verify the processing of CLN5 occurs *in vivo* during protein synthesis and maturation and not as an artifact of cell lysis conditions, a cycloheximide chase experiment was performed in cells overexpressing Myc tagged CLN5 (Figure 2.3). At 24 h post transfection, cells were treated with

cycloheximide to inhibit *de novo* protein biosynthesis. Immunoblotting with CLN5 showed the intensity of the ~60 kDa band corresponding to the pro-protein decreasing with increasing time in the presence of cycloheximide. Simultaneously the lower band of ~52 kDa increased with increasing time of cycloheximide chase. The intensity of upper band was 2.7-fold over the lower band at 0 h. After 5 h chase, the intensity of lower band was about 2.5-fold of the upper band. Immunoblotting with Myc also showed that the pro-protein was decreased with increasing time of cycloheximide chase. This data shows that the processing of CLN5 happens *in vivo* and is not an artifact of cell lysis conditions.

CLN5 processing takes place in low pH environment

To further investigate the proteolytic process of CLN5, we analyzed the conditions in which the processing occurs. CLN5 is a lysosomal lumen protein and the lysosome or TGN/endosome could be a potential location where the processing may occur. To address this, we treated cells with chloroquine, a weak base that can accumulate in acidic compartments such as lysosomes neutralizing the pH. As a consequence, the lysosome acid hydrolases become inactivated. Chloroquine was added to the cells 4 h post transfection and continued incubation for another 24 h. As seen in Figure 2.4, the intensity of the lower band was reduced dramatically, with an upper to lower band intensity ratio of 16.9:1. In fact, the weak lower band is probably the endogenous CLN5. This means that C-terminal processing of CLN5 requires an acidic environment. To understand what kind of protease would be involved in the processing, HEK293 cells were treated with different protease inhibitors 4 h post transfection. Incubation was continued for another 24 h before the samples were analyzed. Treatment with Pepstatin A, an aspartic protease inhibitor, did not result in inhibition of the lower band forming, with a similar upper to lower

band intensity ratio 1.9:1 as compared to the DMSO treatment of 2.4:1 ratio. However treatment with Leupeptin, a serine and cysteine protease inhibitor resulted in a reduced amount of the processed form, suggesting that a serine or cysteine protease could be involved in the processing. E-64 and AEBSF, inhibitor of cysteine protease and serine protease respectively, were also used to test the specificity of the protease involved in the processing. The ratio of the intensity of the upper band versus the lower band of each treatment is quantified and shown below the lanes. Treatment with E-64 demonstrated similar results as to treatment with Leupeptin with low amount of processed band. AEBSF seemed to affect transfection and/or protein expression. Nevertheless, the ratio between upper and lower processed band is comparable to the control DMSO treatment. This data suggests that the protease involved in the processing of CLN5 is probably a cysteine protease.

The different CLN5 protein forms are not due to glycosylation variants

To further examine the property of the two bands observed, we digested the lysates with PNGase F. PNGase F is a glyco-amidase which can cleave between the innermost N-acetyl glucosamine and the asparagine residue of the protein, thereby removing all traces of glycosylation from the protein. We analyzed endogenous CLN5, overexpressed wild type CLN5, a patient mutant D279N CLN5 and N401Q CLN5 (Figure 2.5 A). In non-transfected samples and overexpressed wild type CLN5 samples, two major bands can be seen before and after PNGase F treatment. Note that a weaker band in between two major bands in overexpressed wild type CLN5 samples appears to be the endogenous CLN5. The CLN5 D279N mutant protein has an extra glycosylation site and is retained in the ER (13, 14). Since the proteolytic processing takes place in an acidic environment, here D279N form is used as a negative control to show processing

does not occur in the ER of which the pH is neutral. Indeed, only one major CLN5 band was observed in D279N sample and it is slightly higher than the overexpressed wild type band, due to extra glycosylation. After PNGase F digestion removing all the glycans, there was no difference in size. The minor bands seen can be related to the endogenous CLN5 expressed in HEK293. This confirms that CLN5 D279N does not undergo proteolytic cleavage. When blotted with antibody against the Myc tag, only the upper band can be seen in each overexpressed sample (Figure 2.5 A, right panel), again confirming the C-terminal end of the lower CLN5 band has been cleaved. N401Q does not reach lysosomes but instead accumulates in Golgi/TGN and can be found in the media as well (14). In the N401Q sample, we observed the processed form when blotted with the CLN5 antibody. This suggests that the proteolytic processing starts in the TGN, which is mildly acidic. This data confirms that the two forms of CLN5 are not glycosylation variations from biosynthetic pathway but are due to proteolytic cleavage and this processing occurs in a cellular location beyond the ER. Since overexpressed CLN5 can be secreted and the processing starts in the TGN, we were interested to study if the processed form can be observed in the media as well. The media was collected and also digested with PNGase F after 24 hours post transfection of wild type CLN5. Interestingly, only the non-processed (Myc tagged) CLN5 form could be seen in the media samples (Figure 2.5 B). The slower migration in gel of CLN5 in media is probably due to continuing modification/glycosylation such as fucosylation and sialylation when proteins transported onward from TGN to outside of the cells (23). We also did not observe the processed form of endogenous CLN5 in the media (Figure 2.5 C). In fact, we did not observe any form of endogenous CLN5 in the media collected after 24 hours cell growth. This indicates that the processed form is been actively transported to the endosome/lysosomes, or the majority of the processing occurs once the protein has passed TGN in route to the

endosome/lysosome. The latter will suggest that the proteolytic processing of N401Q may be due to transient overexpression overwhelming the system.

DISCUSSION

In this report, we analyzed the protein levels of CLN5 in different mammalian cell lines. One of the obstacles in the NCL field is the availability of antibodies that can recognize endogenous NCL proteins. For some NCL proteins, another obstacle is the low expression in tissues. For CLN5, several groups have attempted to generate antibodies without much success in detecting endogenous protein expressions (13, 16, 24-26). Here we identified an antibody that is able to recognize endogenous CLN5 in various tissues and cell lines. We confirmed the antibody specificity by demonstrating: 1. the immunoblotting signal can be blocked by CLN5 fragment a.a. 200-300 (Figure 2.1 B), 2. Lack of signal in the CLN5 patient derived fibroblast cells which are deficient of the full length CLN5 protein (Figure 2.1 C), and 3. the ability to decrease the signals by knocking down CLN5 with small interference RNA (Figure 2.2).

Another important observation is that two forms of CLN5 are present in most cells and tissues examined. We and other groups have been using transient transfection overexpression systems and tagged versions to study CLN5, again in part due to the lack of verified antibodies for detecting endogenous CLN5. As shown in this report and our previous study (14), using Myc antibody only detects the upper, non-processed pro-protein. We demonstrated the lysosomal localization of unprocessed Myc-tagged CLN5 (14), which are consistent with our current study showing that the C-terminal proteolytic processing occurs in acidic compartments. Intriguingly,

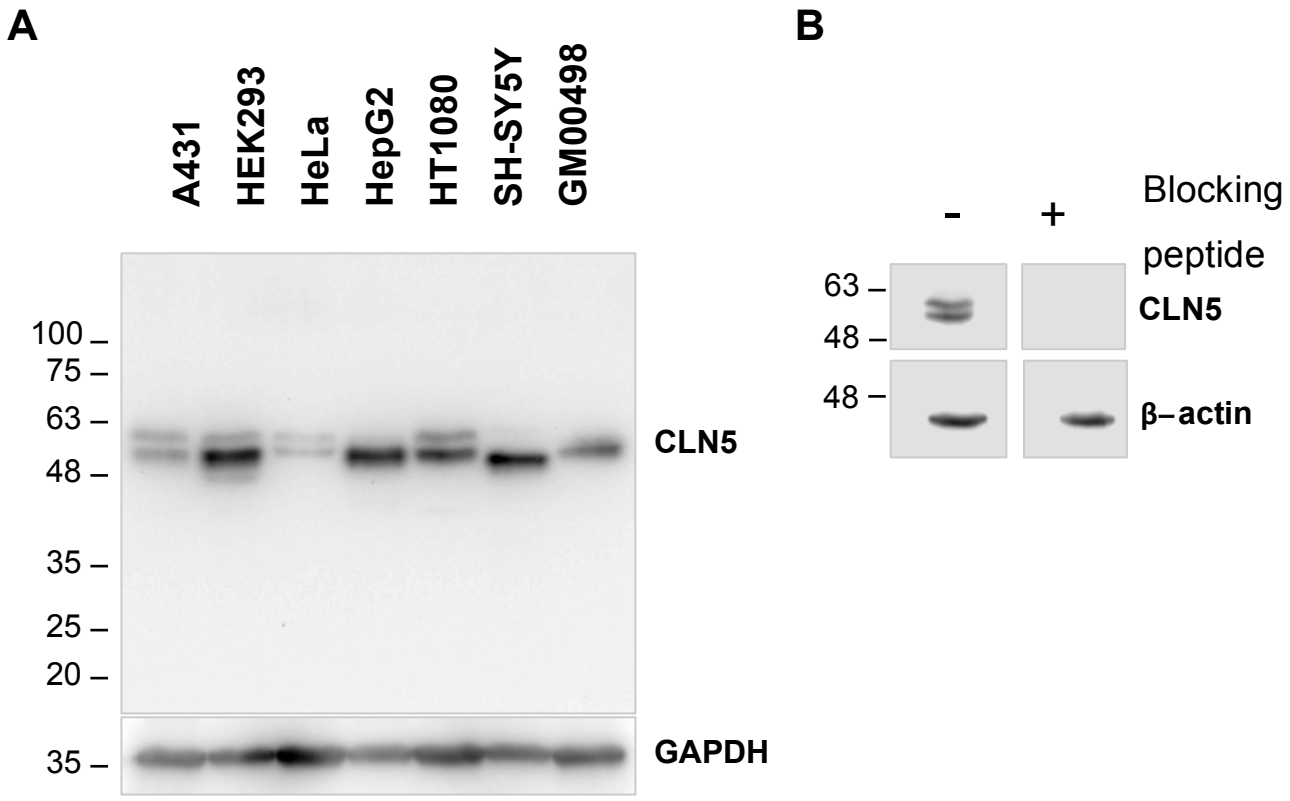
we found overexpressed CLN5 mutant N401Q can be cleaved. Previously we showed by immunofluorescence studies, N401Q does not reach the lysosomes which is the final destination. Instead, it is localized to the Golgi/TGN and can be secreted to the media. It was postulated that glycan moiety on N401 contains Man-6-P necessary for endosome/lysosome transport. Without this glycosylation site, CLN5 is not able to reach the lysosome (14). If the proteolytic cleavage occurs in the TGN the processed form, which most likely loses the N401 and therefore the Man-6-P, will not be able to use Man-6-P mediated pathway to transport to the endosome/lysosome but instead will be secreted. In this report when analyzing the media, we could not detect the processed form of wild type CLN5 (both endogenous and overexpressed) in the media while in the pellets it is the more prominent form and can be easily detected. This suggests that proteolytic processed form of CLN5 is normally transported to or generated in the lysosomes. It will be interesting to know whether this form is functional or is transported for degradation. With many of the cell lines surveyed having the processed form as most prominent band we favor the idea that the processed form is the functional CLN5.

During biosynthesis, CLN5 first undergoes co-translational cleavage to remove the N-terminal signal sequence. Based on the PNGase F digestion assay, it is clear that the upper form we observed does not contain the signal sequence. After removing the carbohydrate moieties the upper band of the endogenous CLN5 is about 33 kDa, which fits well with the molecular weight of CLN5 without the signal peptide. Furthermore, the formation of the lower band can be greatly reduced by using chloroquine to raise the pH of the lysosomes and therefore block the activity of most acid hydrolases. This confirms that CLN5 proteolytic processing occurs post-translationally. The electrophoretic mobility difference between the two forms of endogenous CLN5 is about 4 kDa (lower band ~52kDa and upper band ~56kDa). The Myc-His tagged

overexpressed version added another 4 kDa to the upper band to ~60kDa. Based on the amino acid sequence of CLN5, the proteolytic process will remove the glycan moiety on N401 and ~10 to 15 amino acids from the C-terminus. This would position the cleavage site behind the amphipathic helix region identified previously (15). This region was reported to associate with membrane tightly. However, we can readily detect the unprocessed form of overexpressed CLN5 from the media (in current study and (14)) suggesting CLN5 is fairly soluble in our system.

Our results demonstrate there is a proteolytic processed form of CLN5 present in the cells. Potentially this is a means to regulate the yet to be discovered activity/function of CLN5 In line with this idea, many soluble lysosomal enzymes including TPP1 (CLN2), CTSD (CLN10), CTSF (CLN13), and acid alpha-glucosidase, undergo proteolytic cleavage and maturation in the lysosomes (27-31). Another NCL polytopic membrane protein, CLN7, has also been observed to undergo cysteine protease-dependent proteolytic cleavage in the lysosome (32). Further investigation will be needed to understand the importance of CLN5 proteolytic processing and to reveal the function of CLN5.

Figure 2-1


A
A431
HEK293
HeLa
HepG2
HT1080
SH-SY5Y
GM00498
100
75
63
48
35
25
20
35
CLN5
GAPDH
B
-
+
Blocking peptide
63
48
48
CLN5
β–actin

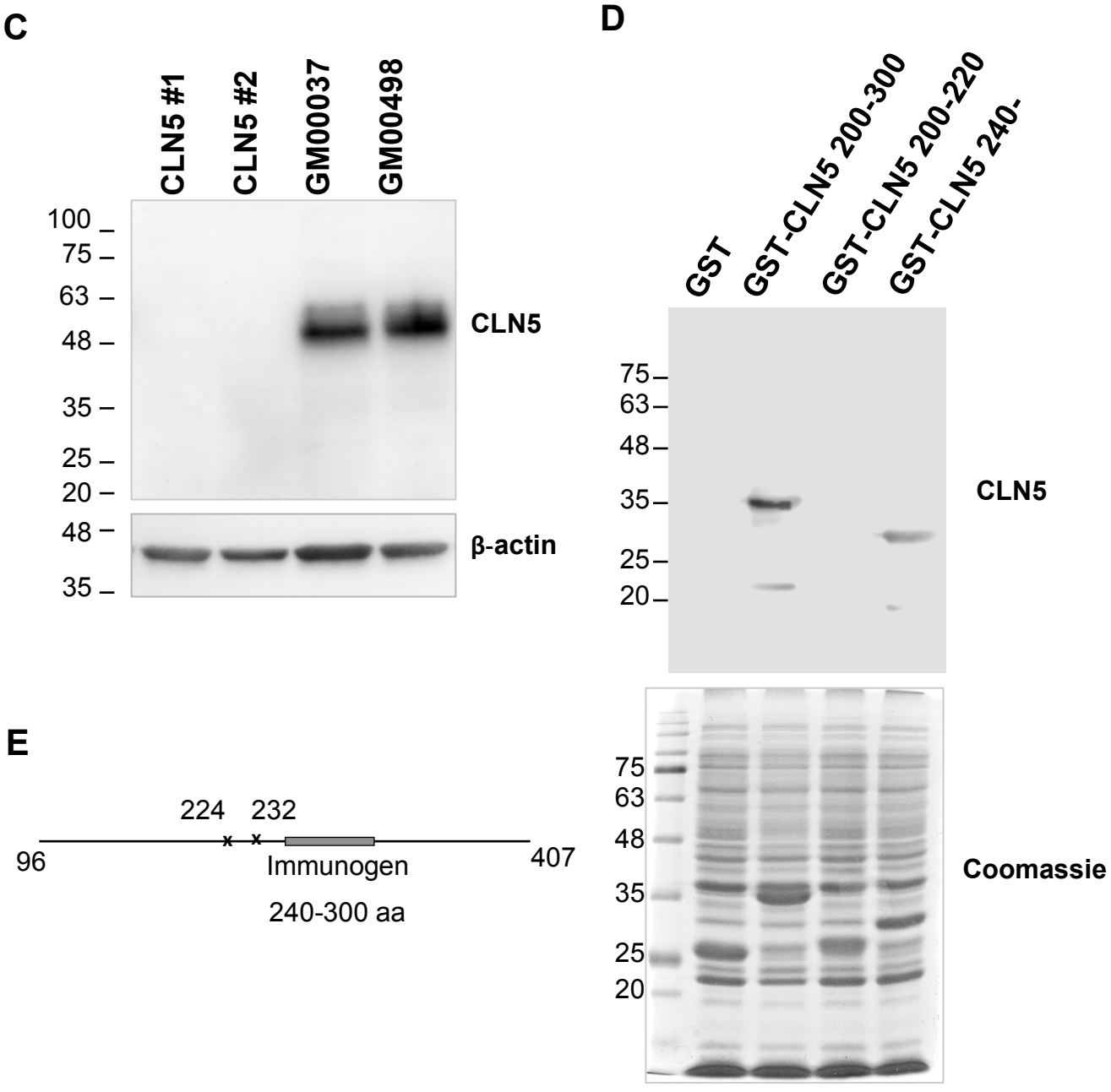

C
CLN5 #1
CLN5 #2
GM00037
GM00498
100
75
63
48
35
25
20
48
35
CLN5
β-actin
D
GST
GST-CLN5 200-300
GST-CLN5 200-220
GST-CLN5 240-
75
63
48
35
25
20
CLN5
75
63
48
35
25
20
Coomassie
E
224
232
96
407
Immunogen
240-300 aa

Figure 2.1: The endogenous expression of CLN5 (A) The endogenous CLN5 exist in two forms. The whole cell lysates of (50 μg) of human cell lines A431, HEK293, HeLa, HepG2, HT1080, SH-SY5Y and GM00498 control fibroblasts were analyzed for the expression of endogenous CLN5 protein. (B) The CLN5 antibody specifically recognizes endogenous CLN5 in HEK293 cells. The antigen for this rabbit monoclonal antibody is within the 200 – 300 aa region of human CLN5 protein. To show the specificity, GST-CLN5 200-300 was used as a blocking peptide. CLN5 antibody was pre-incubated with GST (-) or GST fused with the 200 – 300 aa of CLN5 (+) before applying to immunoblotting of HEK293 lysates. (C) Both bands observed are derived from CLN5. The whole cell lysates of two CLN5 patient fibroblast cell lines CLN5 #1 and CLN5 #2 and two control fibroblast cell lines GM00037 and GM00498 were collected and analyzed by immunoblotting for endogenous CLN5. CLN5 #1 and CLN5 #2 patient fibroblast cells carry mutations in the CLN5 gene resulting in truncation of the protein. (D) The locations of the immunogen was narrowed down to 240-300 aa of human CLN5. Whole cell lysates of bacteria expressing GST or GST fused with 200 – 300 aa, 200 – 220 aa, 240 – 300 aa were immunoblotted with CLN5 antibody. Coomassie blue stained gel shows the expression of GST and GST fusion proteins. (E) The locations of the immunogen and patient truncations are shown. β-actin and GAPDH were blotted as loading controls.

Figure 2-2

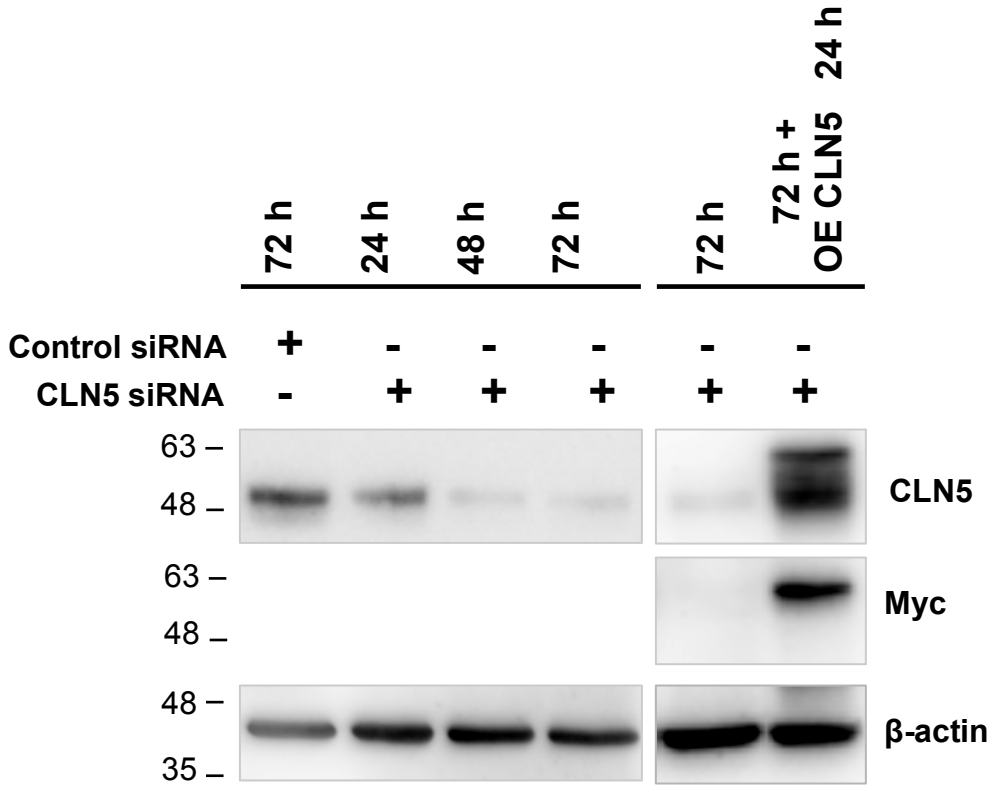


Figure 2.2: The two forms of CLN5 protein are derived from a processing event at the C-terminus. (A) HEK293 cells were transfected with either control siRNA or CLN5 siRNA. The cells were collected 24, 48, and 72 h post transfection and analyzed by immunoblotting for CLN5 (left panel). In a separate experiment, after 48 h CLN5 siRNA transfection, cells were transfected with wild type CLN5 for 24 h to overexpress (OE) Myc-tagged CLN5 (right panel). Immunoblotting was followed by CLN5 antibody and Myc antibody. β-actin was blotted as loading control.

Figure 2-3

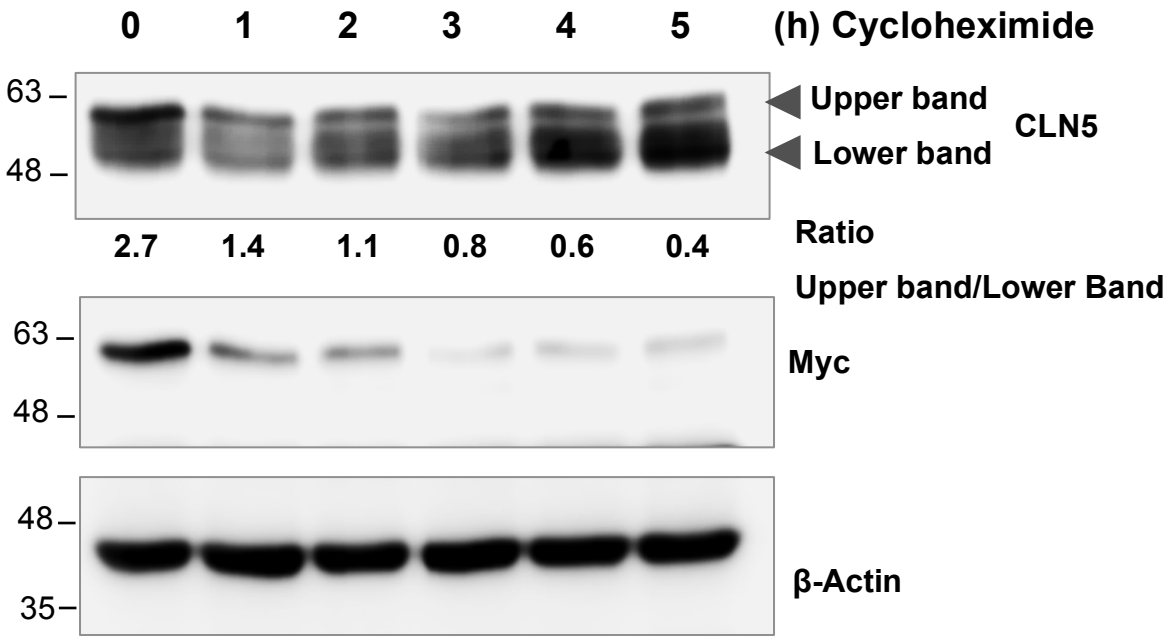


Figure 2.3: Post-translational proteolytic processing of CLN5 at the C-terminus. The CLN5 pro-protein is gradually cleaved and the processed form of CLN5 increases following cycloheximide treatment. HEK293 cells were overexpressed with Myc-tagged CLN5. 24 h after transfection cells were incubated with DMEM containing cycloheximide (50 μg/ml) for the indicated time intervals. The whole cell lysates were collected and analyzed by Western blotting. β-actin was blotted as a loading control.

Figure 2-4

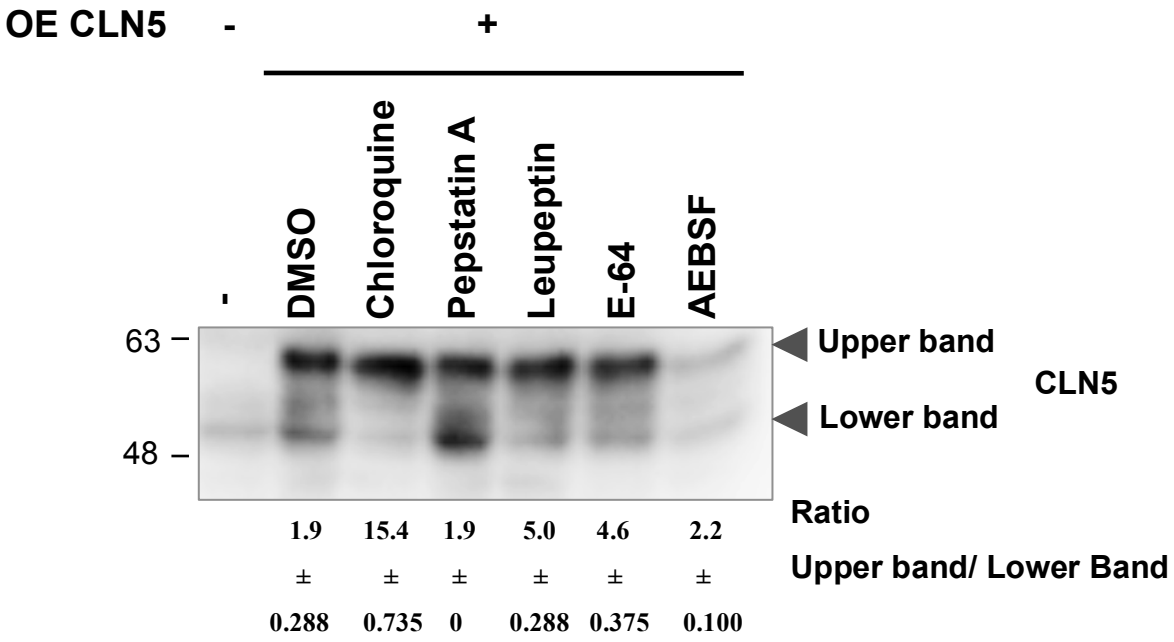


Figure 2.4: CLN5 processing takes place in low pH environment and is sensitive to cysteine protease inhibitors. HEK293 cells were transfected with wild type CLN5. DMSO, Chloroquine (50 μM), Pepstatin A (5 μM), Leupeptin (100 μM), E-64 (10 μg/ml) and AEBSF (150 μM) were added to the medium 4 h post transfection. Cells were incubated with drug and transfection reagent for another 24h. The whole cell lysates were collected and analyzed by Western blotting. The intensity of upper and lower bands was quantified and the ratio is indicated. The image and data is representative of three independent experiments.

Figure 2-5

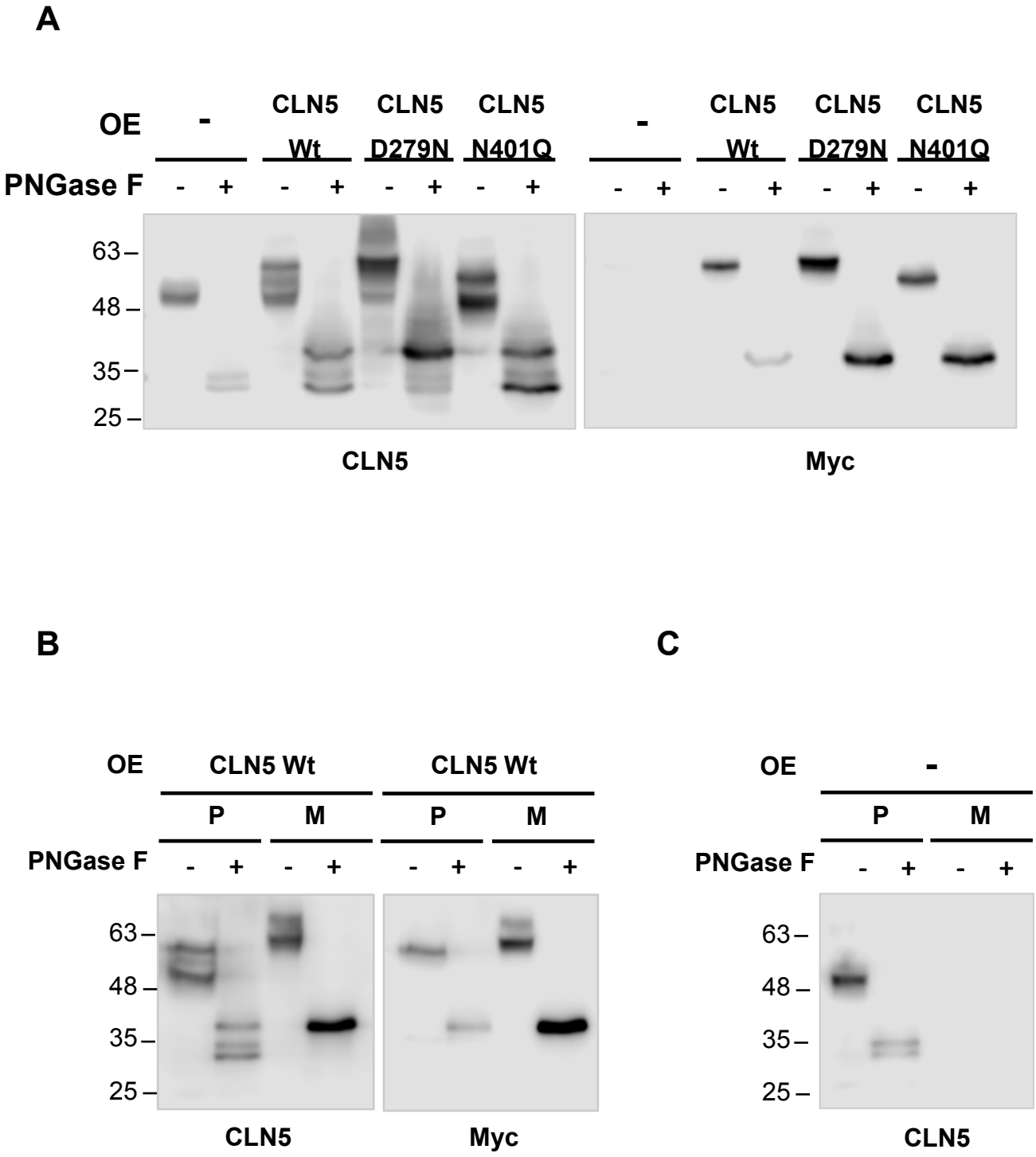

A
OE
-
CLN5 Wt
CLN5 D279N
CLN5 N401Q
-
CLN5 Wt
CLN5 D279N
CLN5 N401Q
PNGase F
- + - + - + - + - + - + - + - +
63
48
35
25
CLN5
Myc
B
OE
CLN5 Wt
CLN5 Wt
P
M
P
M
PNGase F
- + - + - + - +
63
48
35
25
CLN5
Myc
C
OE
-
P
M
PNGase F
- + - +
63
48
35
25
CLN5

Figure 2.5: The different CLN5 forms are not due to glycosylation variants. (A) Glycosylation variation does not contribute to the two forms of CLN5. HEK293 cells were either mock transfected or transfected with wild type CLN5 (Wt), CLN5 mutants D279N or N401Q for 24 h. Total lysates were treated with or without PNGase F and analyzed by immunoblotting with CLN5 and Myc antibodies. (B) The processed form of CLN5 protein is not secreted. Cell pellets and media were collected from HEK293 cells grown in Optimem media and transfected with wild type CLN5 (Wt). Total lysates were treated with or without PNGase F and analyzed by immunoblotting with CLN5 and Myc antibodies. (C) Cell pellets and media collected from non-transfected HEK293 cells grown in Optimem to analyze the endogenous CLN5. Total lysates were treated with or without PNGase F and analyzed by immunoblotting with CLN5 antibody.

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CHAPTER 3

IDENTIFYING INTERACTING PROTEINS AND THE FUNCTIONAL ROLE OF CLN8

ABSTRACT

CLN8 is a 286 aa transmembrane protein with 5 predicted α-helical transmembrane regions and a C-terminal endoplasmic reticulum (ER) retrieval signal (KKXX). CLN8 is mainly localized to the ER and ER-Golgi intermediate compartment and is believed to shuttle between the two compartments. Mutations in CLN8 gene lead to progressive epilepsy with mental retardation (EPMR) and a variant late infantile-onset form of neuronal ceroid lipofuscinosis (NCL), a neurodegenerative lysosomal storage disorder that is the most common inherited neurodegenerative disorder observed to affect children. Affected patients suffer from epilepsy, progressive degeneration of cognitive and motor functions, mental retardation, blindness, ataxia and premature death. The CLN8 protein contains a TLC (TRAM-Lag1-CLN8) domain found in a family of membrane associated proteins with functions involving in lipid synthesis, lipid sensing and trafficking. Studies followed using a CLN8 deficient mouse model, patient derived cell lines and brain samples of CLN8 patients have shown altered sphingolipid profiles and myelination defects. Our study was directed towards identifying proteins that interact with CLN8 to help explore the function of it. We identified a potential interaction between CLN8 and a PP2A holoenzyme complex consisting regulatory subunit A α isoform and regulatory subunit B α isoform. Using two CLN8 patient derived fibroblast cell lines we were able to show that the phosphorylated levels of PP2A target kinase Akt was reduced at both of its regulatory sites Ser473 and Thr308 and the activity of PP2A was increased. A delay of ceramide transport from ER to Golgi in CLN8 deficient patient cell lines was also observed using BODIPY FL C5-Ceramide staining. Our results provide evidence for CLN8 protein being involved the trafficking of ceramide from ER to Golgi.

INTRODUCTION

NCLs are a group of neurodegenerative lysosomal storage disorders which is the most frequent inherited neurodegenerative disorders that affect children leading to severe pathological conditions such as progressive loss of motor neuron functions, loss of vision, mental retardation, epilepsy, ataxia and atrophy in cerebral, cerebella cortex and retina and eventually premature death (1, 2). Most NCL disorders display an autosomal recessive inheritance and they are classified according to their gene mutations or else the age of onset such as congenital, infantile, late infantile, juvenile and adult onset. So far mutations in 13 different genes have been associated with NCL which are named ceroid neuronal lipofuscinosis (CLN) proteins CLN1 – CLN14 (1, 2). The NCL associated CLN proteins display a lot of heterogeneity in cellular localization where CLN1 (PPT1), CLN2 (TPP1), CLN5, CLN10 (Cathepsin D), CLN13 (Cathepsin F) are soluble proteins present in the lysosomal lumen, CLN3, CLN7 and CLN12 are lysosomal transmembrane proteins, CLN6 and CLN8 are transmembrane proteins localized in the endoplasmic reticulum (ER) and CLN4 and CLN14 localized in the cytosol associated with vesicular membranes (2, 3). Despite the heterogeneity in localization and clinical onset, mutations in all CLN proteins lead to the accumulation of autofluorescent lipopigments and the subunit C of mitochondrial ATP synthase or saposins A or D in the lysosomes (1-5). The function(s) of most of the NCL proteins remain elusive because of the lack of understanding of the substrates or pathways involved. Analysis of NCL disorder model organisms; prominently mouse models, studies on cultured patient cells and case studies have provided valuable insight about the pathological mechanisms and the role(s) of mutated NCL proteins (2, 6).

Progressive epilepsy with mental retardation (EPMR) and CLN8 variant late infantile-onset forms of NCL are both caused due to mutations in the CLN8 gene (7-9). Subunit C of

mitochondrial ATP synthase is the prominent storage material accumulated in the lysosomes of affected patient cells. Epilepsy, blindness, and progressive degradation of motor neuronal functions leading to ataxia, progressive cognitive decline are observed as main pathological effects of CLN8 disorders (1-6).

The CLN8 gene encodes a 286 amino acid transmembrane protein of ~ 33 kDa with five predicted α-helical transmembrane regions and a C-terminal endoplasmic reticulum (ER) retrieval signal (KKRP) (10). CLN8 is a non-glycosylated protein with no subsequent proteolytical processing and is localized mainly to the ER and ER-Golgi intermediate compartment (ERGIC) (10). It is believed that the CLN8 protein is shuttled between the ER and the ERGIC due to the presence of the ER retrieval signal via retrograde transport (Golgi to ER) (10). Most known patient mutations do not interfere with the localization of CLN8 protein (10, 11). A spontaneous mutation mouse model known as the CLN8 mnd (motor neuron degeneration) mouse model, that mimic the CLN8 v-LINCL phenotype due to a homozygous mutation in the orthologous mouse CLN8 gene has been used in various studies in order to understand the cellular homeostasis and probable function(s) of CLN8 protein (2, 8, 10). Several studies involving analysis of CLN8 disease mouse model and analysis of brain specimens of affected patients have provided evidence that has linked CLN8 protein with altered lipid metabolism (12, 13), problems in calcium buffering, calcium homeostasis (14), dysfunction of mitochondria and ER stress response (15).

CLN8 protein contains a TLC (TRAM-Lag1p-CLN8) domain of 200 aa (62 – 262 aa) that is found in a family of membrane associated proteins which also include all the mammalian ceramide synthase family members and a group of proteins known as TRAM that facilitates the translocation of nascent polypeptides from cytoplasm to ER during protein synthesis (16, 17).

Lag1 is the name of the yeast orthologue to mammalian ceramide synthases (16). It has been shown that ceramide synthases are involved in acyl-CoA dependent ceramide synthesis from sphingosine bases and fatty acyl-CoA (18). Deficiency of these ceramide synthases has been shown to result in altered lipid profiles and accumulation of lipofuscin like material in mouse cerebella Purkinje cells (19).

Lipid profile analysis of CLN8 mnd mouse brain and fibroblast cells, CLN8 patient cerebella samples and CLN8 deficient human fibroblast cells have demonstrated reduction in certain ceramide species (particularly C18, C24 and C24:1 species) (12, 13). Furthermore overexpression of CLN8 demonstrated an increase in these ceramide species (12). A different study demonstrated by the analysis of mRNA expression the activity of UDP galactose: ceramide galactosyltransferase the key enzyme involved in the synthesis of galactolipids was reduced in CLN8 mnd mouse brain (20). This experimental evidence suggests a role for CLN8 in activating or facilitating ceramide synthesis. Reduced levels of galactosylceramides and their derivative sulfatides have been reported in analysis of brain samples of EPMR patients (13). The evidence supports a role for CLN8 in either facilitating synthesis or transport of these sphingolipids from ER to Golgi for modifications.

Proper regulation of lipid metabolism is essential for nervous tissue, since they contain the highest lipid concentration among all body tissues after adipose tissue. Regulation of sphingolipid metabolism is particularly important because sphingomyelin and glycosphingolipids are important constituents of the neuron and glial cell membranes, especially in the lipid rafts and in the myelin sheath of the neuronal axons. They also serve as signaling molecules important for neuronal cell maturation, particularly oligodendrocyte maturation. Ceramide species are also

important second messengers involved in the regulation of cellular apoptosis, growth and differentiation (21).

Sphingolipid synthesis is initiated at the cytosolic leaflet of the ER where various ceramide species are synthesized through the *de novo* pathway by the addition of fatty acids (with varying fatty acyl-CoA chains) to a sphingosine backbone derived by non-sphingolipid precursors. This is achieved through the combined activity of Serine Palmitoyl transferase, 3 – Keto-dihydrosphingosine reductase, (dihydro) Ceramide synthases and (dihydro) Ceramide desaturase (22). The ceramides which are membrane bound and display low solubility in the cytoplasm are transported from the ER to the Golgi compartments where further modifications are applied to ceramide molecules. The transport of ceramide from ER to Golgi occurs via two pathways; vesicular transport or through the ceramide transfer protein (CERT). Once ceramide molecules reach the Golgi they are modified into sphingomyelins by sphingomyelin synthases through the addition of phosphatidyl choline or phosphatidyl ethanolamine. They can also be modified to glucosylceramides by glucosyltransferase through the addition of UDP-glucose (22). Galactosylceramides are synthesized in the ER but they are transported to the Golgi for further modifications and synthesis of sulfatides (22). Sulfatides are highly enriched in the myelin sheath (22). Further sugar modifications of the glycosphingolipids can produce cerebrosides and gangliosides. The *de novo* pathway is the prominent pathway utilized for ceramide synthesis, however two other pathways; salvage pathway in which sphingolipids are recycled by acid hydrolase activity in the lysosomes and sphingomyelinase mediated release of ceramide from sphingomyelin at the plasma membrane also exist (22).

Several studies indicate interactions between CLN8 and other NCL proteins. One study proposed NCL proteins share a common pathway (23, 24). They suggested this because CLN3, CLN6 and

CLN8 proteins (all membrane bound) were able to complement each other and correct growth defects and apoptosis (23). Using co-immunoprecipitation and immunofluorescence studies they showed CLN2, CLN3, CLN6 and CLN8 might interact (23). A relationship between CLN8 and CLN5 has also been proposed (12, 24). One study showed CLN8 protein corrected growth defects and apoptosis observed in CLN5 deficient fibroblast cells (12). They also provide evidence of altered sphingolipid levels in both CLN5 deficient and CLN8 deficient fibroblast cells. Therefore they suggest CLN8 and CLN5 might have related function(s) (12). The significance of these interactions has not been clearly understood although it has been linked with sphingolipid metabolism. One study attempted in identifying proteins interacting with CLN8 in a membrane environment using split-ubiquitin membrane-based yeast two-hybrid (MYTH) system (25). This study identified some target proteins such as VAPA (Vesicle-associated membrane protein-associated protein A) and GATE16 (Golgi-associated ATPase enhancer of 16 kDa) with functions implicated in lipid metabolism or Golgi vesicular transport (25).

In our study we aim to obtain an understanding of the possible function(s) of CLN8 protein by attempting to identify interacting protein partners by a GST pulldown approach and analyze a possible role of CLN8 in ceramide transport.

MATERIALS AND METHODS

Reagents

Chemicals and reagents used in the preparation of cell lysis buffers, protease inhibitors and phosphatase inhibitors, and reagents used in SDS-PAGE and western blotting were purchased from Amresco, Sigma Aldrich, Fisher, Macron and Calbiochem. Lysozyme, Ampicillin, Chloramphenicol and Isopropyl thio beta D-Galactoside (IPTG) were purchased from Gold Bio technology.

Optimization of GST fusion protein expression in bacteria

E.coli strains (BL21 (DE3) competent *E.coli*) carrying plasmids pGEX6PK-1 (for GST expression), and with insertion coding for GST-CLN8 (151-225) and GST-CLN8 (246-286) were grown overnight in 2 mL of Luria-Bertani (LB) broth supplemented with Ampicillin (100 mg/mL) and Chloramphenicol (35 mg/mL) at 37 ^{0}C shaker. From each culture 400 µL was added to fresh LB broth supplemented with antibiotics as earlier and allowed to grow at 37 ^{0}C shaker for 3 h (~ 0.6 O.D.). IPTG was added to the cultures at a 0.1 mM final concentration to induce protein expression and 100 µL from each culture was removed and the cell pellets collected at every 1 h from 0-4 h to analyze total protein and soluble protein fractions for the expression of GST and GST fusion proteins. For total protein fraction the cell pellets were lysed in 1x Sample buffer containing 100 mM DTT and boiled at 95 ^{0}C. For Soluble protein fraction each pellet was lysed on ice for 20 min with lysis buffer (20 mM Tris HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mg/mL Lysozyme, protease inhibitors and 1% Triton-X-100) and centrifuged at 20, 000xg for 10 min at 4 ^{0}C. The collected lysates were boiled at 95 ^{0}C with 1x

Sample buffer. All samples were separated on a 12% SDS-PAGE and stained with Coomassie blue.

GST and GST fusion protein purification

From each overnight culture 2 mL was transferred to 48 mL (total 50 mL) LB broth and continue to grow and induce recombinant protein expression as described earlier. After 3 h induction the cultures were centrifuged at 4500 rpm for 10 min at room temperature. Each pellet was suspended in 2.5 mL of lysis buffer and lysed for 30 min on ice and briefly sonicated on ice. After sonication the lysates were centrifuged at 20, 000xg for 10 min at 4 ^{0}C. The lysates were mixed with 50 µL Glutathione HiCap Matrix beads (Qiagen) equilibrated with lysis buffer. The bead and lysate slurry was allowed to tumble gently for 1 h at 4 ^{0}C. The slurry was centrifuged at 1000 rpm for 10 min at 4 ^{0}C to sediment the beads now bound with GST or GST-CLN8 (246-286) proteins. The beads were washed 3 times with 1x Phosphate buffered saline (PBS) pH 7.4, resuspended in fresh 1x PBS with 0.1% Triton-X-100 and stored at 4 ^{0}C until future use.

GST-CLN8 (246-286) Pulldown experiment

Cell pellets were collected from HEK293 cells grown to 100% confluency in 10 cm/60 cm^2 culture dishes. A total of 6 pellets were lysed in 200 µL of lysis buffer (20 mM Tris HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 05% NP-40, 0.5 mM $MgCl_2$, 1% Protease inhibitors) for 30 min on ice. Lysed cells were centrifuged at 20, 000xg for 10 min at 4 ^{0}C to remove cell debris and collect cell lysate. The cell lysate was pre-cleared using fresh glutathione beads equilibrated with lysis buffer by tumbling at 4 ^{0}C for 1 h and centrifugation at 1000 rpm, 4 ^{0}C for 5 min. The pre-cleared HEK293 lysate was divided into two equal portions and mixed with 10 µL of GST bound

or GST-CLN8 (246-286) fusion protein bound beads equilibrated with lysis buffer and incubated at 4 ^{0}C with gentle tumbling for 2 h. The slurries were centrifuged at 1000 rpm, 4 ^{0}C for 5 min to sediment the beads and washed 2x with cold lysis buffer and 1x with cold lysis buffer not containing NP-40. The beads were resuspended in 1x sample buffer and separated on a 12% SDS-PAGE followed by Coomassie blue staining.

Mass spectrometry analysis of the above resulting SDS-PAGE gel was followed at the biochemistry and molecular biology recombinant DNA and protein core facility at Oklahoma State University.

Cell culture and transfections

Cell culture media and reagents were purchased from Gibco and Hyclone. Cell lines used in this study are HEK293 (ATCC CRL-1573), control fibroblast cell lines GM00498/C1 and GM05757/C2 (Coriell Institute for Medical Research, NJ), CLN8 deficient patient fibroblast #1 (homozygous c. 63 G>A, p. Trp21x) was received from Massachusetts General Hospital CHGR NCL Disorders Clinical Database and Biorepository, CLN8 deficient patient fibroblast #2 (heterozygous c. 581 A>G, p. Q194R and c. 66 del G, p. Gly22fs> x78) was received from Institute of Basic Research, Staten Island, NY. All cells were grown and maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 20 mM HEPES and gentamycin at 37 ^{0}C in a humidified incubator with 5% CO_2.

For the analysis of the total lysates of control fibroblast cell lines C1 and C2 and the CLN8 deficient patient fibroblast cell lines CLN8 #1 and #2, cell pellets were collected from cells reached 100% confluency in 10 cm culture dishes. For the drug treatment experiment the cell lines C1, C2, CLN8 #1 and #2 were seeded in 6 well culture dishes and incubated at 37 ^{0}C in a

humidified incubator with 5% CO_2 for 72 h and treated with DMSO (Cellgro) or Cantharidin (Cayman) 25 μM for 1 h before cells were collected and the cell pellets were either used immediately for western blotting or else stored at - 80 ^{0}C until future use.

SDS-PAGE and western blotting

Cells grown on 10 cm or 6 well culture dishes were scraped and washed once with 1xPBS and centrifuged for 3 min at 1, 500xg to pellet cells. Cell pellets used for analysis by SDS-PAGE were lysed using RIPA lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (GBiosciences) and additionally with (50 mM Sodium fluoride, 2 mM Sodium orthovanadate, 10 mM Sodium pyrophosphate tetra basic) when the purpose was to analyze phosphorylation levels of target proteins. Cells were lysed for 30 min on ice and centrifuged at 20, 000xg for 10 min at 4 ^{0}C. The supernatant was collected as the whole cell lysates. Protein concentrations were determined by the Bradford assay. Aliquots of the whole cell lysates were incubated with 1x sample buffer in a 37 ^{0}C water bath for 10 min and separated by 10% or 12% SDS-PAGE.

Protein samples separated electrophoretically by SDS-PAGE were then transferred onto PVDF membranes (Millipore). Following western transfer the membranes were blocked with 5% milk in TBST (20 mM Tris-HCl pH 7.4, 150 mM NaCl and 0.05% Tween20) and incubated with the respective primary antibody for 1-2 h at room temperature or overnight at 4 ^{0}C. The membranes were then washed with TBST and incubated with horse-radish-peroxidase (HRP) conjugated secondary antibody diluted to 1:50, 000 in TBST for 45 min at room temperature and developed using Chemiluminescent HRP substrate (Millipore). The membranes were imaged using G-Box

imager from Syngene or C-DiGit imager from Li-COR. The C-DiGit software was used for quantification.

Antibodies

Mouse monoclonal antibodies used in this study were against Myc (produced from 9E10 hybridoma cell line purchased from ATCC (CRL 1729)), beta-Actin (GenScript, A00702) and alpha-Tubulin (Developmental Studies Hybridoma Bank-University of Iowa, 12G10). Rabbit polyclonal antibodies used were CPVL-Carboxypeptidase, vitellogenic-like (GeneTex, GTX106020) and Protein phosphatase 2A (PP2A) regulatory subunit A (GeneTex, GTX102206). Rabbit monoclonal antibodies used in this study were all purchased from Cell signaling technology and against total-Akt (C67E7), phosphorylated Akt-Ser^{473} (D9E), phosphorylated Akt-Thr^{308} (C31E5E), phosphorylated GSK3β-Ser^{9}, total-p70 S6 Kinase (49D7), phosphorylated p70 S6 Kinase-Thr^{389} (108D2), PP2A regulatory subunit B alpha isoform (100C1) and PP2A catalytic subunit C (52F8). HRP conjugated secondary antibodies for western blotting was purchased from Jackson laboratories.

Ceramide staining and immunofluorescence microscopy

Control fibroblast cell lines C1, C2 and CLN8 patient fibroblast cell lines CLN8 #1 and #2 were grown on circular coverslips placed in 6 well culture dishes up to 70-80% confluency for 24 h at 37 ^{0}C in a humidified incubator with 5% CO_2. Bovine serum albumin (BSA) complexed BODIPY FL C_5-Ceramide lyophilized form was purchased from Molecular Probes (B-22650). To reconstitute, 5 mg of the BSA complexed BODIPY FL C_5-Ceramide was dissolved in 150 µL of sterile deionized water. The resulting stock solution contained 0.5 mM sphingolipid and 0.5 mM BSA (1:1 mol:mol). Cells grown on coverslips were incubated with ice cold DMEM and

placed in ice for 10 min. The coverslips were rinsed 2x with ice cold Hanks' balanced salt solution (HBSS, Cellgro) supplemented with 10 mM HEPES (Hyclone). In the meantime a 0.05 μM staining solution was prepared by diluting the BODIPY FL C_5-Ceramide stock solution in ice cold HBSS/HEPES. 1 mL of the ice cold staining solution was added on to the coverslips and incubated on ice in dark for 10 min. The coverslips were then rinsed 2x with ice cold HBSS/HEPES and 1 mL of DMEM warmed to 37 ^{0}C was added and the coverslips were incubated at 37 ^{0}C. Coverslips for each cell line C1, C2, CLN8 #1 and #2 were removed at every 5 min time point from 0-20 min and fixed in 4% Formaldehyde for 20 min. The coverslips were washed with PBS and mounted onto glass slides with mounting media and stored in dark at 4 ^{0}C until imaging. The cells were imaged using a Zeiss LSM-5 PASCAL laser scanning confocal microscope.

RESULTS AND DISCUSSION

The functional role of CLN8 protein is not clear. The link between mutations in CLN8 gene and the observed neuropathological events are also largely unknown. CLN8 protein has been proposed to be involved in sphingolipid synthesis and trafficking, facilitating nascent polypeptide translocation across the ER membrane, regulation of calcium homeostasis and ER stress response. The implication of CLN8 protein in sphingolipid biosynthesis and lipid trafficking has been proposed largely due to it being a member of a family of transmembrane proteins containing a TLC (TRAM-Lag1p-CLN8) domain. Proteins in this family are supposed

to function in the synthesis, sensing or trafficking of lipids (16) Furthermore CLN8 deficiency has been associated with alterations in sphingolipid profiles in CLN8 mnd mouse, cultured CLN8 deficient cells and analyzed patient brain specimens, leading to hypo myelination and defects in oligodendrocyte maturation (12, 13, 20).

Optimization of GST fusion protein expression in bacteria

To identify proteins that may potentially interact with or complex with CLN8 protein, two constructs were prepared to express aa 151-225 and aa 246-286 portions of CLN8 fused with the GST protein in order to employ them in a GST pulldown experiment. The aa 151-225 is a large luminal region of the protein and aa 246-286 is the C-terminal cytoplasmic region (Figure 3.1 A). The 151 – 225 aa region was chosen since a number of patient mutations A155V, T170M, Y158C, H157R, R204C, Q194R and R204L have been discovered in this region (http://www.ucl.ac.uk/ncl/CLN8mutationtable.htm). Two patient mutations have been discovered in CLN8 246 – 286 aa region W263C and Q269V. Furthermore CLN8 protein is known to shuttle between the ER and the Golgi. The cytoplasmic tail of the protein contains an ER retrieval signal (KKRP) which facilitates its retrograde transport (Golgi to ER) and it is most likely to interact with other proteins throughout the process.

The expression and solubility of the GST fusion proteins GST-CLN8 (151-225) and GST-CLN8 (246-286) were analyzed by separating via SDS-PAGE, the total and soluble cell lysates of bacteria grown at 37 ^{0}C and induced by IPTG for 0-4 h. The results after Coomassie staining the SDS gel showed that at 3 h after induction of protein expression GST-CLN8 (246-286) was expressed well and observed at ~ 32 kDa and also present in the soluble fraction (Figure 3.1 B). GST-CLN8 (151-225) was expressed very weakly and a faint band was observed at ~36 kDa which was not present in the soluble fraction (Figure 3.1 B). The experiment was repeated with

varying temperatures to optimize the expression as well as solubility of GST-CLN8 (151-225) but was not successful (data not shown). GST-CLN8 (246-286) protein which could be successfully expressed and solubilized was produced in a larger quantity and purified to be used in a GST pulldown experiment.

GST-CLN8 (246-286) pulldown

To identify proteins that may potentially interact with or complex with CLN8 protein, a GST pulldown experiment was followed using GST-CLN8 (246-286) fusion protein loaded on to glutathione beads and incubated with HEK293 cell lysates. GST protein was used as a control. After incubation the beads were pelleted and washed to clear off unbound proteins and then analyzed by separating via SDS-PAGE. After Coomassie blue staining, bands specific for GST-CLN8 (246-286) pulldown of HEK293 cell lysates could be observed on the SDS gel ranging from 35 – 135 kDa (Figure 3.2 A).

Mass spectrometry analysis of the digested samples extracted from the SDS gel produced some unique hits or target proteins for GST-CLN8 (246-286) compared with the GST control. Two subunits from the serine/threonine-protein phosphatase 2A (PP2A) holoenzyme; PP2A regulatory subunit B alpha isoform (~ 55 kDa) and PP2A regulatory subunit A alpha isoform (~ 65 kDa) were identified as unique hits for CLN8. Protein SET isoform 2 (32 kDa) also known as I_2PP2A which is known as an endogenous non-competitive inhibitor of PP2A was also identified. Another serine/threonine phosphatase family member PP1G (59 kDa) was also observed as a unique hit for GST-CLN8 (246-286) pulldown. CLN8 protein has been studied to transport between the ER and Golgi and it carries an ER retrieval signal on the C-terminus (10). This retrograde transport of proteins is mediated by COPI coated vesicles. COPI or Coatomer is a protein complex made of seven subunits. The GST-CLN8 (246-286) pulldown experiment

identified two subunits from this complex; Coatomer subunit beta (102 kDa) and Coatomer subunit alpha isoform 2 (138 kDa). Based on protein sequence homology, proteins of the TLC domain family are proposed to aid transfer of nascent polypeptides in to the ER (16). Signal recognition particle (SRP) complex is involved in the docking of proteins with ongoing synthesis that contain a signal peptide to the ER, via a translocon. The SRP complex is composed of six subunits and the GST-CLN8 (246-286) pulldown experiment identified two subunits from this complex; SRP 68 isoform 1 (71 kDa) and SRP 72 isoform 1 (75 kDa). A serine carboxypeptidase also known as CPVL (54 kDa) and predicted to be involved in trimming of peptides for antigen presentation (26) was identified as a unique hit for the GST-CLN8 (246-286) pulldown.

To confirm some of the hits, the GST pulldown experiment was repeated and western blot analysis was followed by immunoblotting for some of the target proteins identified by mass spectrometry. The interactions between GST-CLN8 (246-286) and two of the target proteins; CPVL and PP2A subunit A alpha isoform could be observed by western blot (Figure 3.2 B). The PVDF membrane was stained with Coomassie blue to show that approximately same amounts of GST or GST-CLN8 (246-286) bound beads were used in the experiment.

Since the intensity observed for CPVL western signal in the GST-pulldown was prominent, we attempted to study whether CPVL would co-localize with overexpressed CLN8-Myc protein using confocal microscopy (data not shown). However this experiment showed CPVL to be localized in punctate vesicle like structures and did not co-localize with CLN8 protein in the ER. Further analysis is required to determine whether CLN8 interacts with CPVL physiologically, because the pulldown employed the cytoplasmic region of CLN8 and CPVL is predicted to be a luminal protein and was found to be localized in vesicle like structures.

Studies on the phosphorylation of PP2A target kinases

Mass spectrometry analysis and western blotting data from the GST-CLN8 (246-286) pulldown experiment suggested a possible interaction between CLN8 protein and the PP2A holoenzyme. PP2A is a highly conserved serine/threonine protein phosphatase that reverses the actions of protein kinases involved in most major signaling pathways (27). PP2A is a trimetric holoenzyme complex made of subunits A, B and C. Subunit A is ~ 65 kDa and acts as a scaffolding unit that can bind to both C and B subunits (28). Subunit C ~ 36-38 kDa is the catalytic subunit and is always bound with subunit A. Both A and C subunits of PP2A exist as alpha or beta isoforms encoded by separate genes but sharing a sequence similarity of 87% and 97% respectively (29). Biochemical studies have shown the existence of several different regulatory B subunits mainly divided into four classes; B, B', B'' and B''', each class containing multiple isoforms. The binding of different B subunits with the AC complex leads to the diverse substrate specificity displayed by PP2A (27, 29, 30).

The mass spectrometry analysis of the GST-CLN8 (246-286) pulldown experiment identified PP2A subunit A alpha isoform (PR65α) and PP2A subunit B alpha (PR55α or B55α) isoform. It has been discovered that PP2A subunit A alpha isoform would interact and bind B55α subunit but PP2A subunit A beta isoform would not (29). This evidence and our results suggest that we were able to identify a particular combination of the PP2A holoenzyme complex.

PP2A has been shown to be involved in the regulation of a number of cellular signaling pathways in mammalian cells affecting protein synthesis, cell cycle progression, apoptosis, metabolism and stress response (27, 29, 30). PP2A has been shown to regulate the activities of more than 30 protein kinases by *in vitro* experiments and some protein kinases have been found to form stable complexes with PP2A (27). Several of these protein kinases are involved in major signaling pathways important for cell growth and survival (27). For example, Akt (Protein kinase

B) is a serine/threonine protein kinase involved in the PI3Kinase/Akt/mTOR pathway and other important signaling pathways regulating cell proliferation, transcription, apoptosis, glucose metabolism and cell migration. Phosphorylation at two regulatory sites Thr308 and Ser473 is necessary for the full activation of Akt (31) and PP2A is known to negatively regulate Akt by dephosphorylation at these sites (27, 29, 32). Protein interaction studies have demonstrated that PP2A A/B55α/C holoenzyme is associated with Akt protein kinase endogenously and *in vitro* pulldown assays have shown direct interaction between PP2A subunit B alpha isoform and Akt (33). Biochemical studies either overexpressing or knockdown of PP2A subunit B alpha isoform have shown respectively impaired or enhanced phosphorylation at both regulatory sites Thr308 and Ser473 of Akt (33). It is debated in some studies that the effect of PP2A on Thr308 site is more profound than the effect on Ser473 while other studies demonstrate both sites to be affected in a similar manner (30, 33-35).

P70 S6 kinase is a serine/threonine protein kinase that acts downstream of PI3K/Akt/mTOR pathway and is involved in stimulating cell growth by activating S6 ribosomal protein which promotes transcription and protein synthesis and inhibits autophagy. P70 S6 kinase activity is regulated at Thr389 and it is deactivated by dephosphorylation. It has been shown that deactivation of its upstream kinases or direct dephosphorylation by PP2A can lead to inactivation of p70 S6 kinase (27, 36). Glycogen synthase kinase-3β (GSK-3β) is another kinase known to act downstream of Akt and also targeted by PP2A (32, 37). GSK-3β inactivates glycogen synthase by phosphorylation and negatively regulates glucose homeostasis. Akt inactivates GSK-3β by phosphorylation at Ser9 regulatory site and PP2A has been shown to activate GSK-3β directly by dephosphorylation at Ser9 or indirectly by regulating the activity of Akt (35, 37).

To examine how CLN8 might affect PP2A activity, we compared the phosphorylation levels of Akt at Thr308 and Ser473 and its downstream targets GSK-3β at Ser9 and p70 S6 kinase at Thr389 in control fibroblast cells and CLN8 deficient patient fibroblast cell lines. The CLN8 deficient #1 patient fibroblast cell line carries a homozygous mutation c. 63 G>A, p. Trp21x and therefore full length endogenous CLN8 protein is absent in these cells and it is likely that the truncated protein is degraded. The CLN8 deficient #2 patient fibroblast cell line carries a heterozygous mutation c. 581 A>G, p. Q194R and c. 66 del G, p. Gly22fs>78x Therefore full length endogenous CLN8 protein, if not degraded, exists with a point mutation or the level of endogenous CLN8 protein may be very low due to the frame shift and truncation at the other allele.

Phosphorylation levels of Akt at Thr308 and Ser473, GSK-3β at Ser9 and p70 S6 kinase at Thr389 were compared in the total cell lysates of control fibroblast cell line C1 and CLN8 deficient patient fibroblast cell lines #1 and #2 (Figure 3.3 A). All cell pellets were lysed in the presence of phosphatase inhibitors. Phosphorylation of Akt at Thr308 and Ser473 was reduced in the patient cell lysates compared to control cell lysate while the total level of Akt was similar in all cell lysates. Phosphorylation of GSK-3β at Ser9 was also reduced in the patient cell lysates although not as a large reduction as observed for Akt, therefore dephosphorylation of GSK-3β observed in this experiment can be indirect dephosphorylation resulting from the inactivation of Akt (37). The dephosphorylation observed for p70 S6 kinase at Thr389 was similar as observed for Akt showing low or almost no phosphorylated protein present while the total p70 S6 kinase levels remained similar among all three cell lysates. To ensure this observation was consistent we compared the CLN8 deficient patient fibroblast cell lysates with another control fibroblast cell line C2 (Figure 3.3 B). Analyzing the phosphorylation of Akt at Thr308 and Ser473

demonstrated that patient cell lysates have reduced phosphorylation compared to both control fibroblast cell lines. The cell lysates from control fibroblast cell lines C1, C2 and the CLN8 deficient patient fibroblast cell lines #1 and #2 were immunoblotted to observe the protein level expression of PP2A subunit A alpha isomer, PP2A subunit B alpha isomer and PP2A subunit C (Supplement Figure 3.1). The results indicate that the protein level expression of the PP2A subunits immunoblotted for were not elevated in the CLN8 deficient patient fibroblast cells compared to control fibroblast cells and the effect observed on Akt phosphorylation could be due to higher activity of PP2A holoenzyme.

To study whether the reduction in phosphorylation of the kinases was due to an increase in PP2A activity we treated the control fibroblast cells C1 and C2 and the CLN8 deficient patient fibroblast cell lines #1 and #2 with cantharidin a potent inhibitor of PP2A (38, 39). Cantharidin is dissolved in DMSO; therefore DMSO was used as a control during this experiment. Control and CLN8 deficient patient fibroblast cell lines were treated with DMSO or 25 μM cantharidin for 1 h at 37 ^{0}C and the cell pellets were collected and lysed in the presence of phosphatase inhibitors and analyzed on western blot to observe the phosphorylation levels of Akt at Thr308 and Ser473. Western blot analysis showed that in cells treated with only DMSO, the phosphorylation levels of Akt at Thr308 and Ser473 was reduced in the CLN8 deficient patient fibroblast cell lines compared to control fibroblast cell lines (same as in Figure 3.3B). By contrast, in the cells treated with 25 μM cantharidin the phosphorylation levels of Akt at Thr308 and Ser473 was now increased in the CLN8 deficient patient fibroblast cells compared to the patient cells treated with only DMSO (Figure 3.3 C). This observation confirms that the alterations in the phosphorylation levels of Akt, GSK-3β and p70 S6 kinase seen in CLN8

deficient patient cells are a consequence of increased PP2A activity. These data suggests that CLN8 protein may have a role in regulating the activation of PP2A.

Ceramide staining and immunofluorescence microscopy

The CLN8 protein belongs to a protein superfamily containing a TLC (TRAM-Lag1p-CLN8) domain. It has been proposed that proteins which contain this domain is likely to be involved in the sensing and trafficking of lipids or else function as acyl-CoA dependent ceramide synthases involved in the biosynthesis of ceramide (16). As mentioned previously the levels of ceramide derived complex sphingolipid species such as sphingomyelin, glucosylceramide, galactosylceramides and other complex glycosphingolipid species have been observed to be reduced in CLN8 mnd mouse brain, CLN8 mnd mouse derived cell lines and EPMR patients brain samples. This can result from either defect(s) in the synthesis of ceramides or else a defect in the transport of ceramides from ER to the Golgi where they are modified into complex sphingolipid forms.

It has been suggested that ceramide activates PP2A, whether all forms of PP2A are ceramide activated has not been understood (40). As our study implicates an increase in PP2A activity it suggests that perhaps the synthesis of certain ceramide species are not altered at the cytosolic leaflet of ER but it is the transport of ceramide from ER to Golgi that is affected in CLN8 deficient cells.

Ceramide is an important and bioactive sphingolipid intermediate mainly synthesized at the surface of the ER or in ER associated membranes by the *de novo* pathway where ceramide is synthesized from less complex molecules or by the salvage pathway which utilizes long chain sphingoid bases to form ceramide through the action of ceramide synthases. The ceramide synthesized at the ER is transported to the Golgi apparatus where they can be further

metabolized into other sphingolipids such as sphingomyelin and glucosylceramides. Studies have identified that ceramide transport from ER to the Golgi apparatus occurs in two methods; non-vesicular transport by the involvement of a soluble ceramide transfer protein known as CERT and CERT independent vesicular transport (41). It has also been discovered that the PI3Kinase/Akt pathway is involved in the regulation of vesicle mediated transport of ceramide from ER to Golgi (42). It has been demonstrated that decreased phosphorylated Akt levels inhibit vesicular transport of ceramide from ER to Golgi by either chemically inhibiting PI3Kinase which is required to activate Akt or by overexpressing a dominant negative Akt and showing that ceramide transport was specifically impaired in the vesicular mediated pathway and biosynthesis of sphingomyelin and glucosylceramide was significantly reduced (42, 43).

Our results suggest that in the CLN8 deficient patient fibroblast cell lines studied, the PP2A activity is relatively higher and it leads to comparatively decreased phosphorylated Akt levels. Literature indicates that decreased phosphorylated Akt levels can impair vesicular mediated transport of ceramide from ER to Golgi (43). Furthermore CLN8 protein is known to shuttle between the ER and Golgi as suggested by its localization studies and the presence of an ER retrieval signal in its C-terminus. Therefore a possibility exist that CLN8 protein could be involved in the trafficking of ceramide cargo from ER to Golgi in a vesicular mediated pathway.

We attempted to observe whether there is a difference in ceramide transport from ER to Golgi between control fibroblast cell lines C1, C2 and CLN8 deficient patient fibroblast cell lines #1 and #2 by utilizing a BODIPY FL fluorophore incorporated C5 ceramide. This BODIPY dye labeled ceramide has been a useful tool to qualitatively observe the process of ceramide transport from ER to Golgi by following their redistribution in cells (44).

All four fibroblast cell lines grown on coverslips up to 70-80% confluency incubated at 37 ^{0}C were metabolically inactivated by replacing the 37 ^{0}C DMEM with ice cold DMEM and incubating on ice for 10 min. At this cold temperature the ceramide transport is attenuated. The staining of cells with 0.05 μM BODIPY FL C5-Ceramide was also followed at ice cold conditions. The BODIPY FL C5-Ceramide would be absorbed by the plasma membrane easily due to the short sphingoid base. Once the cells are reintroduced to 37 ^{0}C with fresh media the BODIPY FL C5-Ceramide molecules will be rapidly incorporated to the ER. The transport of BODIPY FL C5-Ceramide from ER to Golgi was observed in the control and CLN8 deficient patient fibroblast cells at different time points (Figure 3.4). The transport of ceramide from ER to Golgi in the control and patient fibroblast cells were qualitatively compared by observing the redistribution of BODIPY FL C5-Ceramide in the cells. After labelling with BODIPY FL C5-Ceramide at 0 min all cells displayed staining of the ER. By 5 min, in control cells most of the fluorescence was accumulated in the perinuclear region, representative of the Golgi apparatus. But in the CLN8 deficient patient cells much less of fluorescence was accumulated in the Golgi region. In the control cells most of the fluorescence which was accumulated in the Golgi had dissipated by 15 min indicating that those ceramide molecules have already been utilized for the synthesis of complex sphingolipids and trafficked out of the Golgi towards their varied destinations. At 15 min there is some fluorescence accumulation in the perinuclear region, representative of the Golgi in CLN8 deficient patient #1 cells. However in CLN8 deficient patient #2 cells most of the fluorescence is still in the ER even at 15 min. Altogether the experiment suggests that ceramide transport from ER to Golgi seems lagged in the CLN8 deficient patient fibroblast cells used in this study compared to the control fibroblast cells. This experiment however does not distinguish between CERT mediated and vesicular mediated

ceramide transport from ER to Golgi and further studies would be necessary to more clearly observe the involvement of CLN8 in ceramide transport.

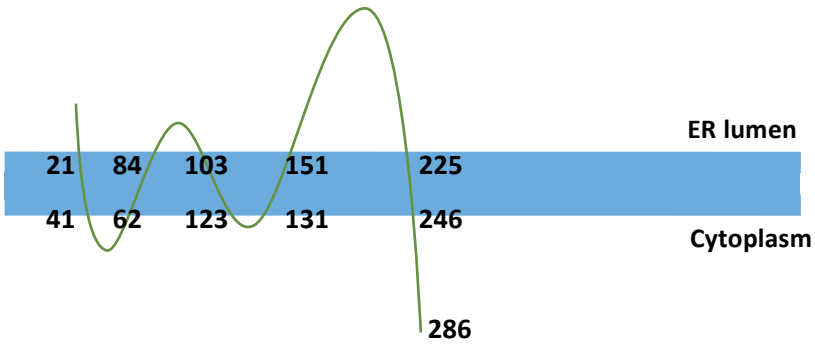
Studies have indicated CERT mediated transport is mainly involved in delivering ceramide from ER to the Golgi site for sphingomyelin synthesis and has little impact on ceramide transport for glycosphingolipid biosynthesis (22). It has been shown that knockdown of CERT lead to a significant but not complete reduction of ceramide transport for the utilization for sphingomyelin synthesis, but did not display a similar effect on the utilization of ceramide for glycosphingolipid synthesis (41). This indicates that the CERT independent vesicular mediated pathway is the main contributor in transporting ceramide from ER to Golgi for glycosphingolipid synthesis. The CLN8 protein can shuttle between the ER and Golgi, and since it is a transmembrane protein it is probably shuttled in vesicles. Our GST-pulldown and MS analysis showed two COPI complex proteins involved in the retrograde transport of proteins from Golgi to ER was found as CLN8 interacting proteins. During this cycling between ER and Golgi, the CLN8 protein may have a role in facilitating the transport of ceramide species along with it.

Our attempt in this study was to identify proteins interacting with CLN8, and analyze their role in cells in order to gain some insight to the function of the CLN8 protein. We were able to identify an interaction between CLN8 and PP2A holoenzyme. Although there are many different PP2A complexes in the cells our analysis showed CLN8 might be interacting with a particular complex of PP2A containing subunit A alpha isoform and subunit B alpha isoform. Using two CLN8 patient derived fibroblast cell lines we were able to show that the phosphorylated levels of Akt at both of its regulatory sites Ser473 and Thr308 were reduced and the activity of PP2A was increased. We also provide some evidence for CLN8 protein being involved in the trafficking of ceramide from ER to Golgi.

Altogether the data in this study, although it is not sufficient to draw major conclusions on the function(s) of the CLN8 protein it provides some evidence into its involvement in ceramide trafficking and possible regulation of PP2A activity. The information derived in this study will be important in further research where the impact of CLN8 on vesicular medicated ceramide transport or regulation of PP2A activity can be explored in order to discover the function of this protein.

Figure 3-1

A



- The large luminal region from aa 151 – 225
- C-terminal cytosolic region from aa 246 – 286

The selected regions were fused with GST (Glutathione S-transferase)

B

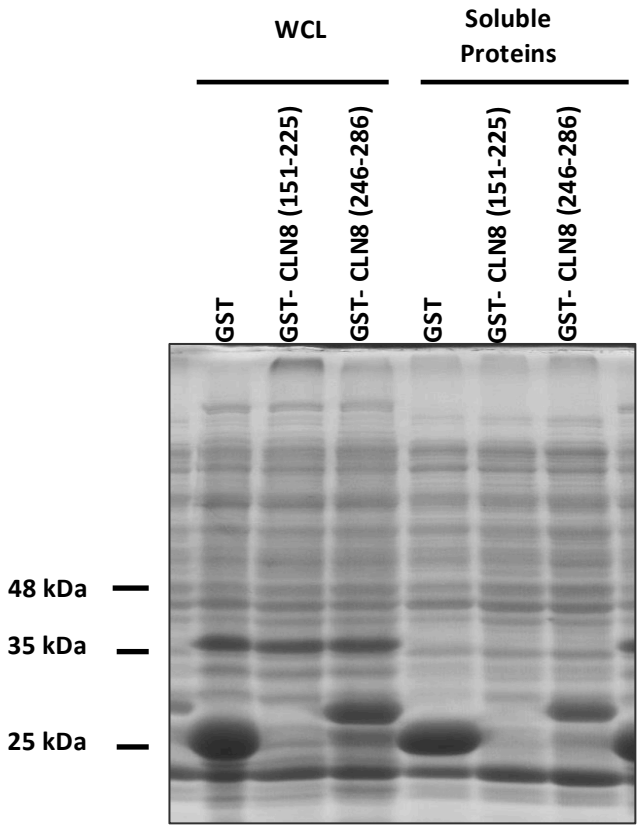


Figure 3.1: Optimization of GST fusion protein expression in bacteria. (A) Schematic diagram of human CLN8 protein. CLN8 protein consists of 286 amino acids and 5 predicted transmembrane domains that span through the ER membrane. The N-terminus of the protein exists in the ER lumen while the C-terminus faces the cytoplasm. The large luminal region spanning from aa 151-225 and the cytoplasmic tail region from aa 246-286 were recombined with GST in pGEX6PK-1 GST expression vector to express GST fusion proteins. These proteins can be used in a pulldown experiment in order to identify proteins that may potentially interact with the CLN8 protein. (B) Expression and solubility analysis of GST and GST-CLN8 fusion proteins. Bacteria constructs carrying sequences for GST, GST-CLN8 (151-225) and GST-CLN8 (246-286) were grown at 37 ^{0}C and induced for recombinant protein expression. Lysates were separated by SDS-PAGE and stained with Coomassie blue.

Figure 3-2

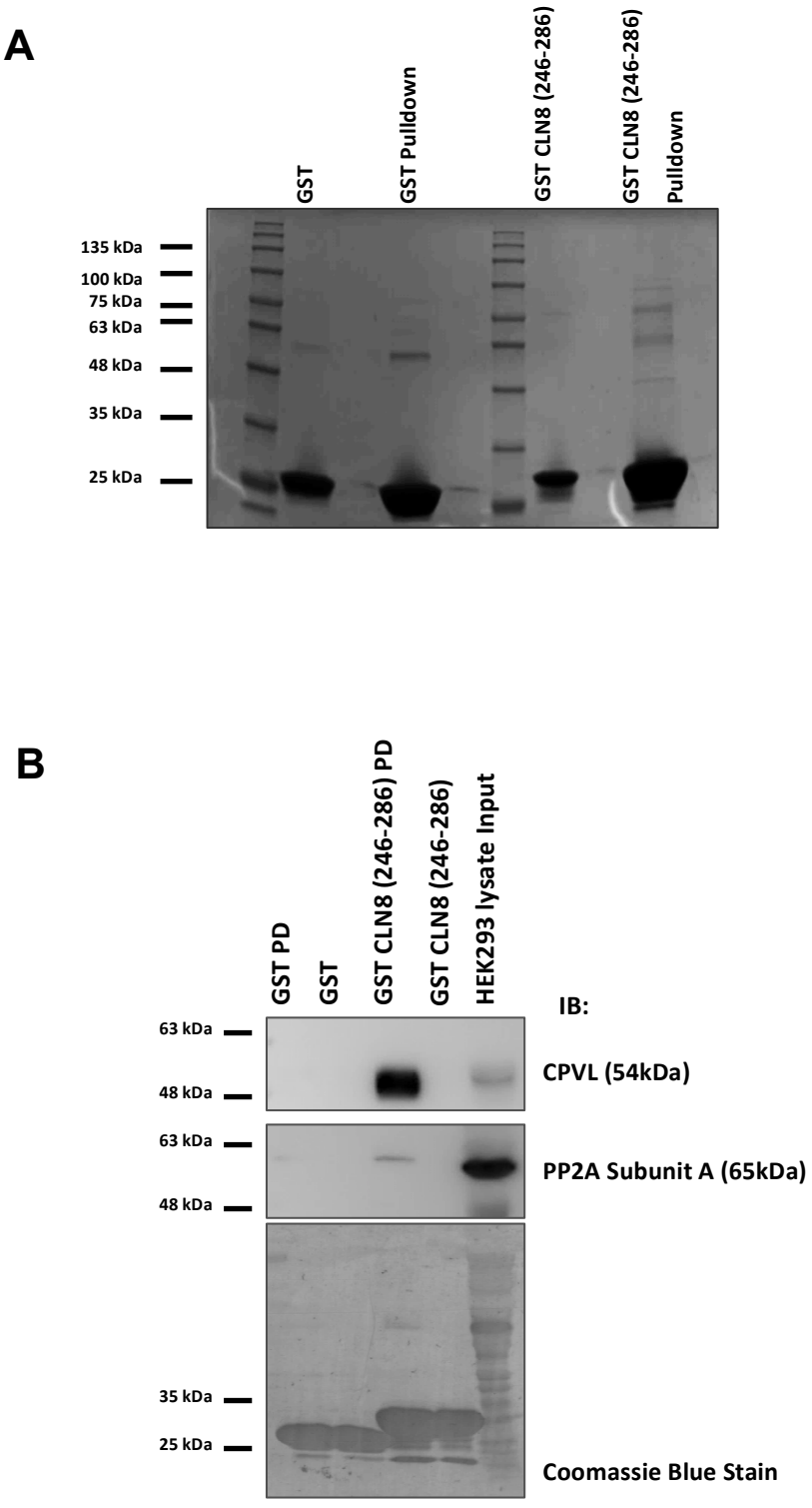

A
GST
GST Pulldown
GST CLN8 (246-286)
GST CLN8 (246-286) Pulldown
135 kDa
100 kDa
75 kDa
63 kDa
48 kDa
35 kDa
25 kDa
B
GST PD
GST
GST CLN8 (246-286) PD
GST CLN8 (246-286)
HEK293 lysate Input
IB:
63 kDa
48 kDa
CPVL (54kDa)
63 kDa
48 kDa
PP2A Subunit A (65kDa)
35 kDa
25 kDa
Coomassie Blue Stain

Figure 3.2: GST-CLN8 (246-286) pulldown of HEK293 cell lysates. HEK293 cell lysates were pre-cleared with GST beads and then incubated with GST or GST-CLN8 (246-286) bound beads at 4 ^{0}C with gentle tumbling for 2h. GST or GST-CLN8 (246-286) bound beads not combined with cell lysate was used as controls. All samples were separated on 12% SDS-PAGE. (A) Coomassie blue stained SDS-PAGE of GST-CLN8 (246-286) pulldown. Bands specific for the GST-CLN8 (246-286) pulldown were observed between 35 – 135 kDa. (B) Western blot analysis of GST-CLN8 (246-286) pulldown. Immunoblotting was followed for some target proteins discovered by the mass spectrometry analysis of the GST-CLN8 (246-286) pulldown SDS-PAGE. Serine carboxypeptidase (CPVL) and protein phosphatase 2A (PP2A) subunit A could be confirmed by western blot. The western blot was stained with Coomassie blue to show that approximately same amounts of GST or GST-CLN8 (246-286) bound beads were used in the experiment.
PD: Pulldown

Figure 3-3

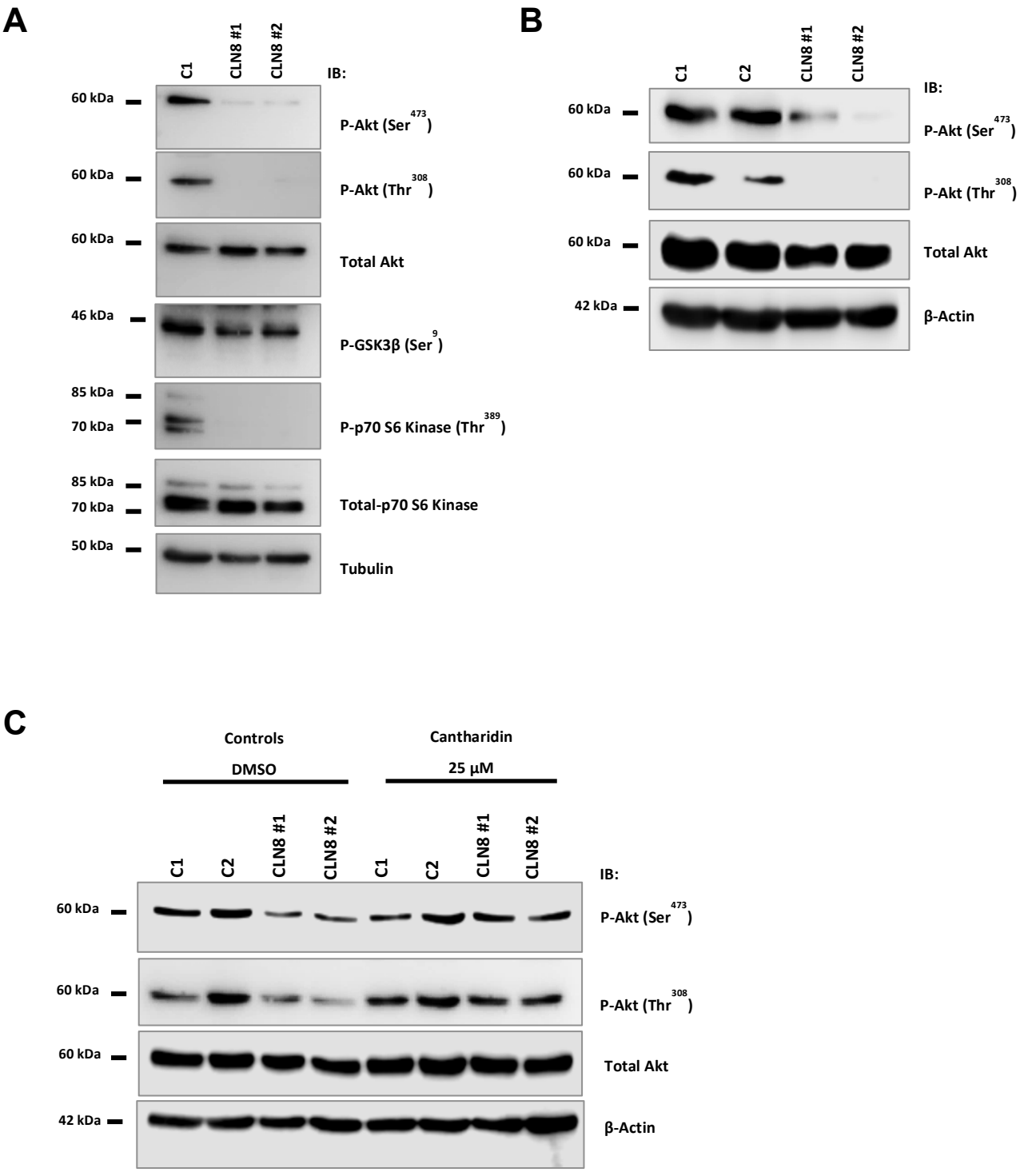

A
C1
CLN8 #1
CLN8 #2
IB:
60 kDa
P-Akt (Ser473)
60 kDa
P-Akt (Thr308)
60 kDa
Total Akt
46 kDa
P-GSK3β (Ser9)
85 kDa
70 kDa
P-p70 S6 Kinase (Thr389)
85 kDa
70 kDa
Total-p70 S6 Kinase
50 kDa
Tubulin
B
C1
C2
CLN8 #1
CLN8 #2
IB:
60 kDa
P-Akt (Ser473)
60 kDa
P-Akt (Thr308)
60 kDa
Total Akt
42 kDa
β-Actin
C
Controls
DMSO
Cantharidin
25 µM
C1
C2
CLN8 #1
CLN8 #2
C1
C2
CLN8 #1
CLN8 #2
IB:
60 kDa
P-Akt (Ser473)
60 kDa
P-Akt (Thr308)
60 kDa
Total Akt
42 kDa
β-Actin

Figure 3.3: Phosphorylation of PP2A target kinases. Phosphorylated protein levels of PP2A target kinases were analyzed in control fibroblast cell lysates and CLN8 deficient patient fibroblast cell lysates. All cells were lysed in the presence of phosphatase inhibitors. It was observed CLN8 deficient patient cell lysates have reduced phosphorylated protein levels of PP2A target kinases. (A) Total cell lysates of control fibroblast cell line C1 and CLN8 deficient patient fibroblast cell lines CLN8 #1 and #2 analyzed for the phosphorylation at both Ser^{473} and Thr^{308} of Akt. Phosphorylation of GSK3β at Ser^{9} and p70 S6 kinase was also analyzed. Tubulin was immunoblotted as a loading control. (B) Phosphorylation level of Akt at Ser^{473} and Thr^{308} were studied in the CLN8 deficient patient fibroblast cell lysates against two control fibroblast cell lines C1 and C2. (C) Cell lines C1, C2, CLN8 #1 and CLN8 #2 grown in 6 well culture dishes for ~ 72 h were treated with 25 μM Cantharidin a potent PP2A inhibitor for 1 h at 37 ^{0}C. Cells treated with DMSO were used as controls. Cell lysates were analyzed by western blot and immunoblotted for phosphorylated Akt at Ser^{473} and Thr^{308}.

Figure 3-4

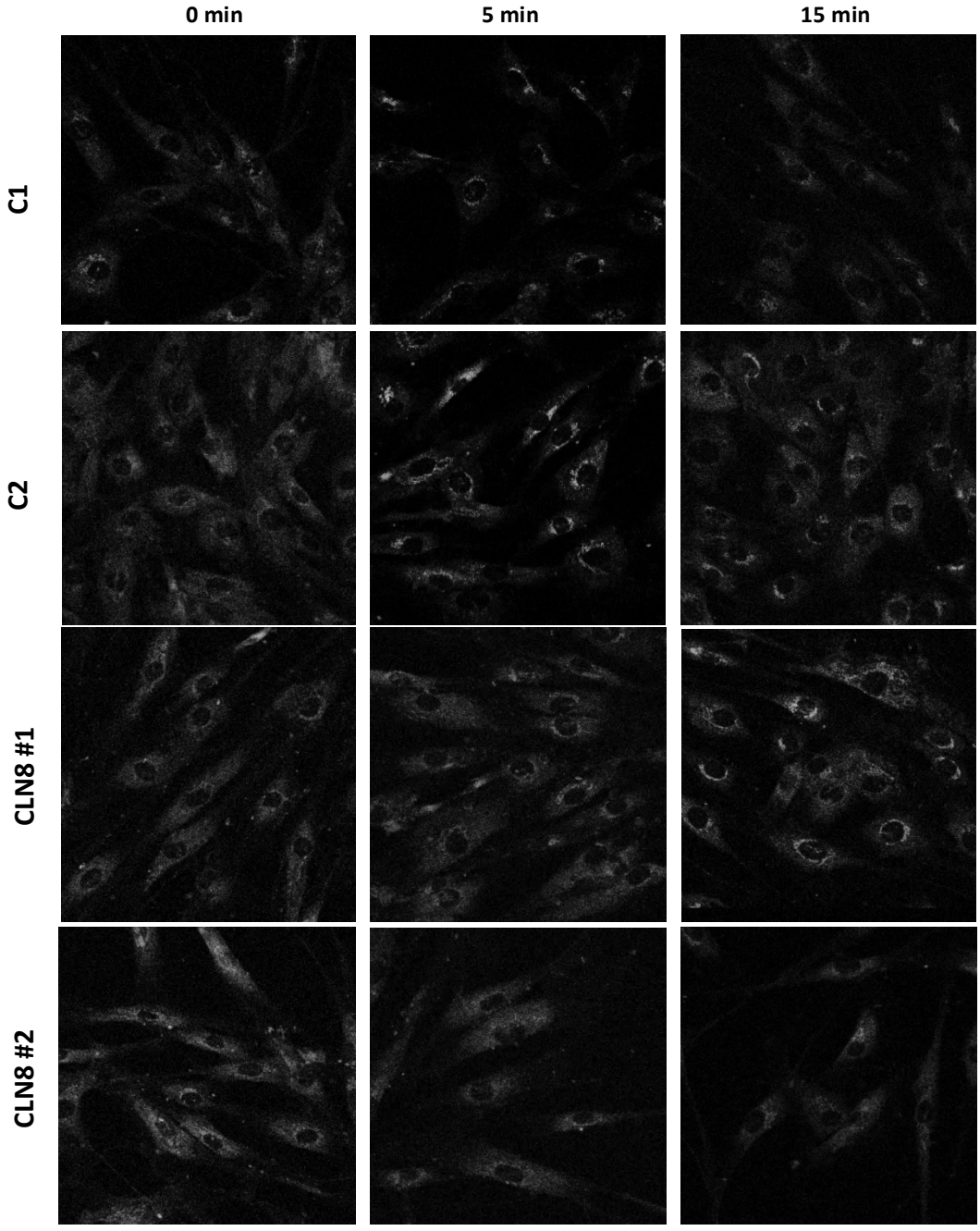
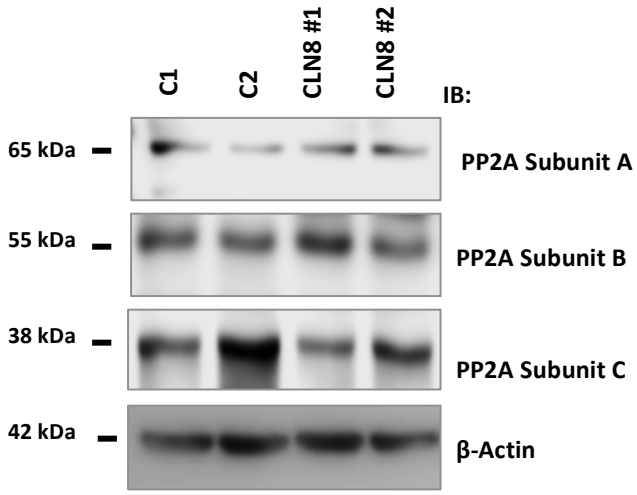


Figure 3.4: Ceramide staining and confocal microscopy. The transport of ceramide from ER to Golgi in the control and patient fibroblast cells were qualitatively compared by observing the redistribution of BODIPY FL C5-Ceramide in the cells. After labelling with BODIPY FL C5-Ceramide at 0 min all cells displayed staining of the ER. In control cells most of the fluorescence was accumulated in the perinuclear region, representative of the Golgi apparatus by 5 min, but in the CLN8 deficient patient cells the accumulation of fluorescence in the Golgi region was clearly reduced. In the control cells most of the fluorescence which was accumulated in the Golgi had dissipated by 15 min indicating that those ceramide molecules have already been utilized for the synthesis of complex sphingolipids and trafficked out of the Golgi towards their varied destinations. At 15 min there is some fluorescence accumulation in the perinuclear region, representative of the Golgi in CLN8 deficient patient #1 cells. However in CLN8 deficient patient #2 the accumulation of fluorescence in the Golgi is more strongly reduced even at 15 min. Altogether the ceramide transport from ER to Golgi seems lagged in the CLN8 deficient patient fibroblast cells compared to control fibroblast cells.

Supplementary Figure S1



Supplement Figure 3.1: Protein expression levels of PP2A subunits. Total cell lysates of control fibroblasts cell lines C1 and C2 and CLN8 fibroblast patient cell lines CLN8 #1 and #2 were analyzed on western blot to observe the protein expression levels of PP2A holoenzyme subunits A, B and C. β-Actin was immunoblotted as a loading control.

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CHAPTER 4

DISCUSSION AND FUTURE PROSPECTIVE

DISCUSSION AND FUTURE PROSPECTIVES

NCL has been identified as the most common group of inherited neurodegenerative disorders affecting children. Currently 13 genes associated with NCL disorders have been discovered. However the function(s) of most of the NCL associated proteins still remain elusive. Among these proteins CLN1 (PPT1), CLN2 (TPP1), CLN10 (Cathepsin D) and CLN13 (Cathepsin F) which are all lysosomal soluble proteins, has been linked with a specific function, although how the aberration of these functions would lead to the neuropathological events observed is not clear yet (1-3). Therapies for NCL disorders are still evolving with the use of animal models and NCL patient derived cell lines (1). Understanding the function(s) of NCL proteins, what kind of cellular signaling pathways they affect and identifying particular characteristics of these proteins such as specific folding and post translational modifications they undergo for proper trafficking and maturation are all important in aiding the development of therapeutics.

In this study we were able to identify a post-translational C-terminal processing of the CLN5 protein which was previously unknown. The scarcity of specific antibodies against endogenous CLN proteins has been a major challenge in the studies of these proteins. In this study we were able to identify an antibody that can recognize endogenous CLN5 protein. The specificity of this antibody against CLN5 was demonstrated using a GST fused blocking peptide targeting the antigen region 200 – 300 aa of the CLN5 protein and small interference RNA mediated knockdown of CLN5. By analyzing the expression of CLN5 at protein level in several mammalian cell lines we observed two forms of CLN5 which were both absent in two CLN5 deficient patient derived skin fibroblast cell lines. Furthermore analysis of transiently transfected wild type CLN5 with a C-terminus Myc tag showed only the upper band when immunoblotted

with Myc antibody and both upper and lower bands when immunoblotted with CLN5 antibody. This suggested the potential C-terminal processing of the CLN5 protein. By treatment with cycloheximide over a period of time we were able to demonstrate that the processing occurs as a post-translational event and with treatment of chloroquine it was shown the processing was inhibited suggesting that the processing occurs in an acidic compartment of the cell. The CLN5 protein is synthesized in the ER and trafficked through the Golgi and endosomes to the lysosomes. The trans Golgi network (TGN), endosomes and lysosomes are all acidic compartments of the cell with varying acidity. After analyzing transiently transfected CLN5 patient mutant D279N which is retained in the ER due to an extra glycosylation site, we were able to observe that it lacked the processed form of CLN5, confirming that the processing occurs in an organelle beyond the ER and the processing requires an acidic environment. A previous study showed the trafficking of the CLN5 glycosylation mutant N401Q is impaired and it is retained in the Golgi/TGN (4) and do not reach the lysosomes. Western blot analysis of transiently transfected N401Q mutant CLN5 with CLN5 antibody showed both unprocessed and processed forms of CLN5. This observation suggested that the processing of CLN5 can occur from the TGN onwards.

Many of the lysosomal proteins undergo trimming on attached oligosaccharides or proteolytical processing during transport from ER to lysosome or after reaching the lysosome, and these steps are important for the protein to mature (5). Proteolytic processing has been observed for several NCL lysosomal proteins such as CLN1 (PPT1) (6, 7), CLN2 (TPP1) (8, 9), CLN7 (10) and CLN10 (Cathepsin D) (11) proteins at acidic pH environment. Based on the electrophoretic mobility ~ 4 kDa shift was observed between unprocessed and processed forms of CLN5 and this would correspond to a glycan moiety (~2.5 kDa) on N401 and ~15 amino acids from the C-

terminus of the protein. The potential cleavage site would not interfere with an amphipathic helix region (aa 353-373) previously identified (12).

Several patient mutations have also been identified in this region; Leu358Ala, Phe361Leu, Lys368Ser (http://www.ucl.ac.uk/ncl/CLN5mutationtable.htm). It would be interesting to study whether any of these patient mutants would lack the processed form and such information would provide evidence for the importance of the proteolytic processing event of CLN5. Although the present study does not contribute to understanding the function(s) of CLN5, it provides new evidence with regard to the characterization of the protein and the report of a CLN5 antibody able in detecting endogenous CLN5, would be beneficial to further research followed in this field.

The objective of the study followed on the CLN8 protein was to identify interacting protein partners of CLN8. The proteins identified by the GST-pulldown method and mass spectrometry analysis were two subunits from the serine/threonine-protein phosphatase 2A (PP2A) holoenzyme; PP2A regulatory subunit B alpha isoform 2 and PP2A regulatory subunit A alpha isoform, I_2PP2A an endogenous inhibitor of PP2A, serine/threonine phosphatase family member PP1G, Coatomer subunit beta and Coatomer subunit alpha isoform 2, two subunits from the signal recognition particle complex; SRP 68 isoform 1 and SRP 72 isoform 1 and a serine carboxypeptidase also known as CPVL.

In the present study we were able to identify an interaction between CLN8 and PP2A holoenzyme containing subunit A alpha isoform and subunit B alpha isoform. By analyzing the phosphorylated levels of Akt at both of its regulatory sites Ser473 and Thr308 we demonstrated that the activity of PP2A was increased in two CLN8 deficient patient derived fibroblast cell lines studied compared to two control fibroblast cell lines. We studied the phosphorylation of

two downstream targets of Akt; p70 S6 kinase and GSK3β, which showed both kinases with reduced phosphorylation at their respective regulatory sites. Akt, p70 S6 kinase and GSK3β are all important kinases in mammalian cells, involved in the regulation of cell proliferation, survival and growth, gene expression, glucose metabolism, autophagy and apoptosis (13, 14). Analysis of downstream targets of these particular kinases would provide more insight that might help link CLN8 to a particular signaling pathway or function.

It has been proposed that the reduction of Akt phosphorylation has a negative effect on vesicular mediated transport of ceramide from the ER to the Golgi (15). This study provides evidence that CLN8 deficient fibroblast cells used in the study have a delay in ceramide transport from ER to Golgi. However further studies are required to understand whether CLN8 protein itself is involved in the trafficking or whether it is a secondary event resulting from dephosphorylation of Akt. Also we did not distinguish between vesicular mediated ceramide transport and CERT mediated transport. CERT deficient cell lines can be utilized to observe the impact of CLN8 protein on the vesicular mediated pathway. Furthermore treatment with cantharidin can be used to understand whether CLN8 deficiency, PP2A activity and Akt dephosphorylation are all linked with ceramide transport.

The absence of a proper antibody against endogenous CLN8 protein has been a drawback in studies on this protein. Although studies have been continued using animal models and CLN8 patient derived cell lines, more direct observations can be obtained for CLN8 protein and its predicted functions with an antibody to follow knockdown of the protein. Therefore developing an antibody against endogenous CLN8 is a major future prospective of this study.

As the activity of PP2A was increased in the two CLN8 patient derived fibroblast cell lines studied, and we were able to identify two subunits of PP2A and also I_2PP2A the endogenous

inhibitor of PP2A through the GST-pulldown experiment, it would be interesting to study whether CLN8 protein is involved in the regulation of PP2A activity by interacting with I_2PP2A. It would be important to confirm these interactions by co-immunoprecipitation reactions.

As time permitted we were not able to explore the interactions between CLN8 and the other proteins identified by the GST-pulldown and subsequent mass spectrometry analysis, further analysis of those target proteins may also provide evidence to help understand the function(s) of CLN8 protein.

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