CLN5 DEFICIENCY RESULTS IN ALTERATIONS IN THE ACTIVATION OF AUTOPHAGY

by

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B.S., Kansas State University, 2011

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Biology
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2014

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Abstract

CLN5 is one of several proteins that when mutated result in the lysosomal storage disorder (LSD) Neuronal Ceroid Lipofuscinosis (NCL). CLN5 is a soluble lysosomal protein that has no known function at this time. Previously we showed that eight asparagine residues in CLN5 are N-glycosylated, and that this modification is important for the protein's transport and function. Now, we have identified a link between the activation of autophagy and CLN5 deficiency. The autophagy-lysosomal protein degradation system is one of the major pathways the cell uses to degrade intracellular material and recycle cellular building blocks. It was recently shown that other CLN proteins affect the relative level of autophagy, indicating a potential link between the autophagy pathway and the NCLs.

By knocking down endogenous CLN5 in HeLa we showed that, upon stress induction, cells responded with higher levels of autophagy activation. Consistent with these knockdown experiments, there is a higher level of the autophagy marker protein, LC3-II, in CLN5 patient cells that are naturally deficient for the CLN5 protein. Pharmaceutical induction of autophagy through different means also showed higher LC3-II levels compared to control, though patterns differed in the type of autophagy induced. In summary, we discovered that the autophagy pathway is altered in CLN5 deficient cells, indicating a potential role for CLN5 in autophagy. Further analyses of the autophagy pathway will shed light on where CLN5 is acting and the mechanism by which defective CLN5 causes NCL.

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Acknowledgements

I would first like to thank my major advisor, Dr. Stella Y. Lee, for her endless support and teaching during my tenure here and for giving me that opportunity to begin with. I would also like to thank the other members of my committee, Dr. Sherry Fleming and Dr. Anna Zolkiewska for their feedback and flexibility in helping me during this process. Thanks also go out to the lab of Dr. Jeroen Roelofs for their constructive criticism during lab meetings and their support in all my endeavors in both labs. This would also have not been possible without the aid of my fellow graduate students and the support of the several undergraduate researchers that have come and gone in my time here.

I am eternally grateful for the support of the staff and faculty here at Kansas State University that I received over my six years here. I owe the bulk of what I know today to the many professors and teaching assistants here at the university, and the knowledge I take with me from this education is something I will always treasure. Kansas State has been a second home for me nearly my entire life, and I will miss it tremendously.

Dedication

There are numerous people that deserve the credit for making all of this possible. First, to the friends I brought with me to K-State as well as the countless I made during my tenure here, I thank you for the innumerable memories and experiences we shared together that made this the most memorable time of my life as well as for keeping me grounded and connected to the world outside the classroom. I also dedicate this to my family and my canine companion Tinuviel, whose unconditional support carried me through this sometimes arduous road. Finally and most importantly, this goes out to Emily Williams who provided endless support in scholastic and personal matters, and who carried me through the most trying parts of this process.

For Frodo.

Chapter 1

Introduction

Lysosomal Storage Disorders

Lysosomes

Lysosomes are organelles first described in the late 1950's that are found in almost all eukaryotic organisms[1,2]. These organelles help maintain homeostasis in the cell by breaking down a variety of materials including wastes, cellular debris, and even invasive bacteria[3]. Intracellular proteins targeted for degradation reach the lysosome through a network of different pathways while extracellular substrates are endocytosed into the cell forming an early endosome[4-6]. This early endosome, as it is called, matures into a late endosome and eventually a lysosome itself, in which the substrates will be metabolized (Fig. 1.1)[5,7]. Lysosomes are distinguished from early and late endosomes by the increased acidity of the lumen. They are also differentiated by a lack of mannose-6 phosphate receptors (M6PRs) which are present in the early and late endosomes.

Endosomes and lysosomes distinguish themselves with the presence of Rab proteins. Rab monomeric G-proteins 5, 7, and 9 are markers used to further differentiate these structures. Early endosomes recruit Rab5 protein which acts in complex with a phosphatidylinositol (3) kinase and later recruits Rab7 to late endosomes when GTP bound[5]. The association of Rab7 converts Rab5-GTP to GDP thus dissociating Rab5 from the endosome making Rab7, which is involved in membrane trafficking, a marker for late endosomes[5]. Rab9 is located mainly in lysosomes and also plays a role in trafficking to the Trans-Golgi Network[5].

Mature lysosomes contain over fifty different acid hydrolases of varying natures including peptidases, proteases, and nucleases among other enzyme types[3]. The induction of

the acidic environment (lumen pH of 4.5-5) is brought about by proton pumps embedded in the lysosomal membrane, and aids in the structural denaturation of waste molecules[8]. These H+ ion pumps are ATPases that require a constant dose of ATP to work against equilibrium and maintain the acidity of the lumen[9]. The low pH surroundings are also necessary for the optimal catalytic performance of the hydrolases in the mature lysosome[10]. Without normally functioning lysosomes, the vital tasks they maintain fall into disarray and lead to disease.

Lysosomal Storage Disorders

The lysosomal storage disorders (LSDs) are a major disease group characterized by the mutation of one of many possible lysosomal proteins leading to the erroneous storage of particular substrates in the lysosome lumen[11]. Besides a few X-linked diseases, most of the LSDs are autosomal recessive disorders, meaning a mutated gene is required from both parents. Incidence of LSDs is relatively low, even when all disease cases are combined: approximately one LSD per 5,000 children born[12]. There are dozens of different LSDs all characterized by their individual accumulation of lipopigments (fats/proteins that appear yellow or green under UV light) as well as the age of onset and types of symptoms caused by the disease. LSDs are neurodegenerative diseases in nature, brought on by the buildup of specific substrates in the lysosome. As patients age, it is common to see the nervous system deleteriously affected, though symptoms of LSDs and the age of onset vary widely across different diseases. In fact, age of onset can range anywhere from birth to full adulthood in

Figure 1.1: Endosome Maturation and General Protein Trafficking

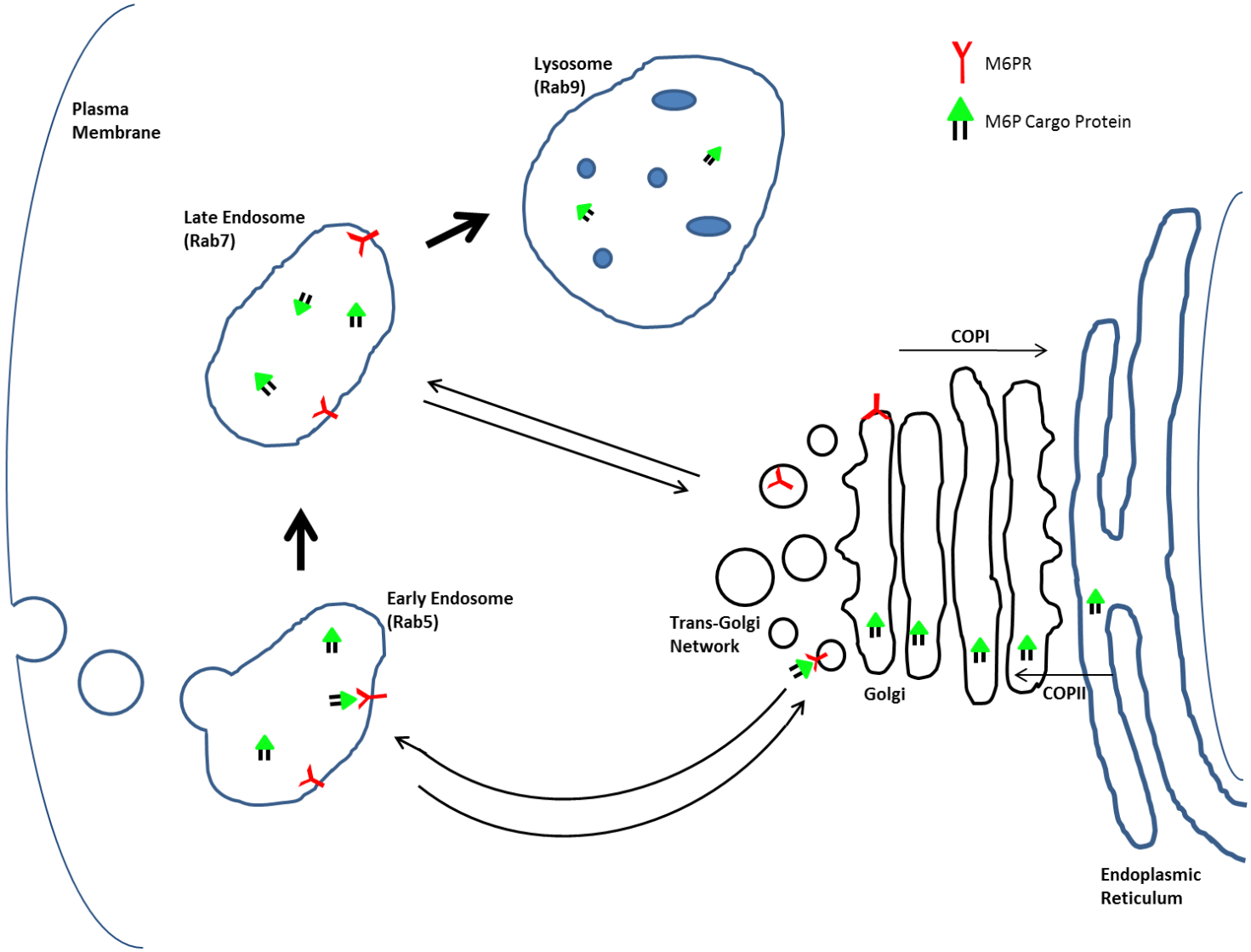

Figure 1.1-Endosome Maturation and General Protein Trafficking. Simplified graphic of the maturation of endosomes and protein trafficking. New cargo proteins, many of which are acid hydrolases destined for the lysosome, are trafficked from the ER to the Golgi where they receive an M6P tag in the cis Golgi which is then recognized by M6PRs. Cargo is then transported to early endosomes by M6PR which dissociates from the cargo protein at the late endosome and is trafficked back to the Trans-Golgi Network. The late endosome later matures into the lysosome wherein the cargo protein reaches its final destination in the organelle's lumen.

one's 30s or later[13]. The most prevalent and obvious symptoms caused by the diseases are those affecting the brain, specifically mental retardations and seizures; pulmonary, cardiac, skeletal muscle systems and vision irregularities are also common across the range of LSDs[14]. Prognoses for these diseases are not good, as many patients will not live to see adulthood (depending on onset) due to the highly degenerative nature of their particular conditions[15]. The deleterious nature of these disorders is understandable as many LSDs are shown to affect a large variety of critical cellular pathways including the apoptotic pathway, signaling involving inflammation and cellular calcium equilibrium, and autophagy[15]. The effects of LSDs are wide ranging and as a whole, they constitute a group of diseases that require further study to understand and develop treatments.

Neuronal Ceroid Lipofuscinoses

Neuronal Ceroid Lipofuscinoses (NCLs) are another important group of diseases brought on by the dysfunction of the lysosome. The disease family is also called Batten's disease, named for the individual who first characterized it by studying a family that produced multiple disease-bearing offspring[16]. There is some debate as to how to differentiate LSDs and NCLs, especially over the last 20 years with the characterizations of the NCLs. Classical LSDs, as they are now distinguished by some, retain a set of characteristics not seen in NCLs and vice versa. Named for the buildup of a particular lysosomal substrate, classical LSDs are defective for one of many acid hydrolases and disease arises from the specific undigested substrate targeted by these hydrolases; in other words, incorrect storage is a direct result of a defective lysosomal

protein[17]. NCLs that develop due to dysfunctional soluble hydrolases show no direct relation between the mutated protein of interest and the incorrectly stored material[17]. However, NCLs and LSDs do share strikingly similar symptom patterns across the spectrum of individual diseases as well as the obvious improper lysosomal storage issues and enzyme malfunction patterns. NCLs are a particularly unique group of LSDs, and what makes them unique is what has driven increased interest and research over recent years.

It wasn't until the mid-1990s that the genes coding for the mutated proteins causing NCLs were identified[15]. They have slowly been characterized over the last two decades, and are called ceroid-lipofuscin (CLN) proteins, now numbering 1-10[13]. Each CLN corresponds to a particular protein, and within each NCL protein of interest there are numerous patient mutations that have been documented (NCL Mutation Database -- http://www.ucl.ac.uk/ncl/mutation.shtml) and show varying pathologies and symptoms[18-20]. Diseases in the NCL family are characterized in different ways (Table 1.1). As with LSDs, there is a wide range in the age of onset of disease symptoms and the deterioration of the patient[21]. Early onset cases include infantile (from birth) and juvenile while late onset cases can occur during the second decade of life or even full adulthood[22-25]. There are also variants based on the region in which a certain mutation is abundant due to the defective gene persisting in a family (or several families) line[26,27]. For example, NCLs in general are more prevalent in regions like Scandinavia and other eastern parts of the continent leading to the classification of Finnish and Turkish NCL variants. Each variant carries with it a specific subset of symptoms unique to that protein and mutation. NCL is a complex disease with many intricacies that help us

recognize them as separate and distinct conditions, and this complexity only gets deeper at the molecular level.

Though many distinctions exist between types of NCL, they all share several common symptoms including types of dementia, seizures, deterioration of sight and physical wellbeing, and most notably premature death[15]. The average life expectancy across all NCL types is around 15 years of age[28]. These symptoms are all, of course, subject to varying levels of severity between disease types and cases. Neurobehavioral aspects also vary widely depending on the form of NCL in question. One study showed that there was an increased incidence of aggression, restlessness, difficulty sleeping, depression, irritability, and general fears in many NCLs; a lower but still significant and alarming number of patients also displayed varying levels of psychosis in the forms of hallucinations and delusions[29-31]. Symptoms of NCLs vary widely in scope and severity, due to the many biochemical pathways that are affected in patients.

In NCLs, the main storage bodies seen are subunit C of ATP synthase and saposins A and D, the latter of which are activators of lysosomal lipases. Subunit C of mitochondrial ATP synthase is part of the F_O/V_O transmembrane portion of the ATPase; ten of these subunits form an oligomer that make up the F_O rotor that is rotated by the flux of protons through the ATPase channel[32]. Numerous biochemical systems are disrupted by the lysosome's inability to degrade these products. It begins with the mutated gene product, the CLN proteins, which are also not all completely understood in terms of function. A few have well characterized functions like being soluble hydrolases in the lysosomes, while others like CLN5 have no concrete function to date[19,29,33,34]. Autophagy, a pathway involving the systematic recycling of cellular components (which will be discussed in length later in this review), is one such system affected in several

forms of NCL. Defects in some CLN proteins also result in disruption of endocytosis as well as protein trafficking within the cell. Mutations or ablations of CLN3 and CLN5, specifically, have recently shown perturbations in the cell's ability to maintain proper protein movement within the cell causing changes in organelle morphology, number, and distribution within the cell[35,36]. Meanwhile, recent CLN1 studies have shown that defects in the protein decrease the cell's ability to undergo endocytosis as seen in Garland cells and CLN1 deficient patient fibroblasts[37,38]. Trafficking in synapses is also affected by NCL related proteins, as seen in the case of CLN1. When deficient, a number of synaptic vesicle proteins remain membrane bound there causing an imbalance in the amount of vesicles that can be released freely into the synapse[39,40]. NCLs have also been implicated in defects seen in the apoptotic pathway, oxidative pathway, and in endoplasmic reticulum (ER) stress response[41-44]. The symptoms and deleterious effects of NCLs vary greatly in how they manifest on a macro and micro scale, and while many of the causative proteins have a well-defined function others have yet to be elucidated.

CLN5

One such NCL related protein is CLN5. CLN5 is a 407 amino acid, soluble lysosomal protein with no known function to date (though it is predicted to have a role in vesicular trafficking and has been implicated in recent studies to have a hand in several cellular pathways)[45]. It is ubiquitously expressed in all body tissues, but as with all NCLs, the characteristic occlusion bodies that are the dysfunctional lysosomes are most prevalent in

Table 1.1: Summary of NCL Genes

Gene	Chromosome	Age of Onset	Localization	Function	Storage Material
CLN1	1p32	Infantile; late- infantile; juvenile; adult	Lysosome lumen	Enzyme for lipid modified proteins (PPT1)	Saposin A and D
CLN2	11p15	Late-infantile; juvenile	Lysosome lumen	Enzyme for N-terminal tripeptides (TPP1)	SCMAS
CLN3	16p12	Juvenile	Lysosome membrane (integral)	UNKNOWN	SCMAS
CLN4	20q13.33	Adult	Plasma membrane; synaptic vesicle	UNKNOWN	SCMAS
CLN5	13q22	Late-infantile	Lysosome lumen	UNKNOWN	SCMAS
CLN6	15q21	Late-infantile; adult	ER membrane (integral)	UNKNOWN	SCMAS
CLN7	4q28	Late-infantile	Lysosome membrane (integral)	Membrane transport (MFSD8)	SCMAS
CLN8	8q23	Late-infantile	ER membrane (integral)	UNKNOWN	SCMAS
CLN9	UNKNOWN	Juvenile	UNKNOWN	UNKNOWN	SCMAS
CLN10	11p15	Congenital	Lysosome lumen	Aspartic protease (CTSD)	Saposin A and D

Table 1.1: NCL Related Proteins. PPT1-Palmitoyl protein thioesterase 1; TPP1-Tripeptidyl peptidase 1; MFSD8-Major facilitator superfamily containing 8; CTSD-Cathepsin D; SCMAS-Subunit c of mitochondrial ATP synthase

neuronal tissue[46,47]. The predicted size of the protein, based on its amino acid sequence, is about 46 kDa while in vitro translation studies display slightly smaller versions of the protein[45,48,49]. A sizable portion of the protein at the N-terminal end acts as a signal peptide, and this peptide is cleaved in the endoplasmic reticulum before CLN5 makes its path to the lysosome through the trans-Golgi network[48]. The protein itself is also heavily glycosylated as it maintains 8 N-linked glycosylation sites now confirmed by our previous study in which removal of glycosylation sites by mutation or chemical treatment results in an appropriate size decrease on SDS-PAGE[50,51]. With both modifications of signal peptide cleavage and glycosylation, the molecular weight of mature CLN5 is around 55 kDa[51].

Though its function is unknown, studies have linked CLN5 to a number of possibly related proteins and cellular pathways. Recent CLN5 co-immunoprecipitation analyses have shown that CLN5 interacts with a number of the other NCL proteins including CLN1, CLN2, CLN3, CLN6, and CLN8[48,52]. Most notably amongst these, CLN1 has been shown to restore proper localization of CLN5 mutant Y392X when they are overexpressed together[53].

Autophagy

Autophagy is a catabolic cellular process in which the cell purposefully degrades its own unwanted, unnecessary, non-functional and damaged components in order to perpetuate the important functions and life of the cell under stressful conditions like starvation[54]. Through a series of mechanisms, organelles and proteins are broken down to their most basic components so as to be recycled by the cell for its most pressing needs when the situation

demands it, though it has been shown in several cell types that there is a baseline level of autophagy occurring at all times[55,56]. This low level of consistent cellular degradation helps maintain the homeostatic nature of the cell as the nutrients provided from lysosomal breakdown are shuttled into the cytosol for future use[57]. The pathway is regulated by several signaling cascades, many of those intertwined with autophagy's "sister" pathway, apoptosis[58]. When deregulated, however, the alterations in the pathway have been shown to have a role in a multitude of different disease types ranging from neurodegenerative and cardiovascular to different cancer types.[59]. The regulation of autophagy is paramount in maintaining cellular health and in the prevention of several human diseases relying on homeostasis. Several different forms of autophagy exist inside the cell based on how and what kind of components are degraded. Among the major types are macroautophagy, microautophagy, and chaperone-mediated autophagy with several subtypes amongst these bearing specificity for a certain protein or organelle[60].

Macroautophagy

Macroautophagy, the form of autophagy we are most interested in, involves the nonspecific digestion of whole organelles and proteins through sequestration in a vesicle-like structure followed by lysosome-mediated degradation[57]. Through a mechanism that is currently not fully understood (see Figure 1.2), a membrane known as an isolation membrane or phagophore is synthesized around the cytosolic components that are to be degraded[61].

Recent studies and electron microscopy analyses now indicate that the phagophore originates from lipids of the endoplasmic reticulum[62-64]. The sequestered contents can range from a random conglomeration in a portion of the cytoplasm to a specific set of proteins or organelles. The phagophore continues to expand around the selected cytosolic components until the membrane completely fuses around them into a vesicle-like structure now identified as an autophagosome. From there, the autophagosome is targeted to the lysosome and can potentially join with other endosomes to consolidate all their components before its membrane fuses with that of the lysosome[65-67]. This new structure containing the components of both vesicles is known as the autolysosome. Acid hydrolases in the lysosome spill into the autophagosome and degrade the cellular components it holds after the fusion of the membranes is complete. Building blocks from the proteins or organelles can now be released from the autolysosome through permeases on the lysosomal membrane to be recycled by the cell for a range of tasks including protein synthesis[68-70].

The main biochemical sensor used to measure the level of autophagy (macroautophagy specifically) occurring in cells is the autophagy hallmark protein LC3, the mammalian homologue of Autophagy-related protein 8 (ATG8) from yeast[71]. Microtubule-associated protein 1A/1B- light chain 3 (LC3) is mainly found in two forms in eukaryotic cells: LC3-I and LC3-II along with a pro-LC3 that is quickly modified after translation[71-73]. As mentioned, autophagy is always occurring at low, baseline levels. When at low levels, most of the LC3 present is in the LC3-I form throughout the cell. LC3-I is a soluble cytosolic protein with a predicted molecular weight of 14.13 kDa (14.69 kDa before modification of pro-LC3). When autophagy is activated however, LC3-I is recruited to the autophagosome where it is lipidated

by the addition of phosphatidylethanolamine (PE)[71]. Cleavage at Met 121 of pro-LC3 results in the exposure of carboxy-terminal Gly 120 where lipidation can occur[74]. This process converts LC3-I to LC3-II, which due to mobility changes caused by the addition of the lipid make it appear slightly smaller on SDS-PAGE, around 12 kDa (though predicted molecular weight is 14.87 kDa). The newly formed LC3-II is embedded into the autophagosome membrane where it remains until the autophagosome fuses with the lysosome resulting in the degradation of the inner-autophagosomal components by lysosomal acid hydrolases[75]. The role of LC3, along with other proteins recruited to the autophagosomal membrane, is currently believed to be the nucleation and eventual expansion of the autophagosome through enabling curvature of the membrane[76]. Mutations in LC3 result in problems in the formation of autophagosomes, supporting this hypothesis[77]. Several studies also show a relationship between the amount of LC3-II and the size of autophagosomes that are eventually formed[71,77-79]. Because of its abundance in conjunction with times of increased autophagy activity, LC3-II and its turnover in particular are the preeminent indicators used to measure macroautophagy.

Microautophagy and Chaperone-Mediated Autophagy

Microautophagy and chaperone-mediated autophagy perform very similar functions in a more straightforward manner. In the case of microautophagy, cytoplasmic components (again ranging from organelles to simple proteins) are taken up directly by the lysosome through a mechanism similar to endocytosis as opposed to transport by an autophagosome[80]. This creates a vesicle in the lysosome whose membrane is slowly degraded thus exposing the

targeted cellular components to the acid hydrolases, leading to their own degradation[81-83]. Chaperone-mediated autophagy occurs in much the same manner as microautophagy in that the substrates of interest are directly targeted and degraded by the lysosome and no vesicle to vesicle transfer occurs. However, the mechanism by which the cellular components actually reach the lysosome in this particular pathway is much more complex. Unfolded proteins are the sole target in chaperone-mediated autophagy; these unfolded proteins are shuttled to the lysosome by any number of chaperones specific to this pathway much like what is seen in the ubiquitin-proteasome pathway in which proteins are directed by chaperones to the proteasome for unfolding and degradation[84]. Once unfolded proteins reach the lysosome by means of chaperones, they enter the organelle by means of lysosome-associated membrane protein 2A (LAMP-2A)[85]. This channel protein allows the substrate to enter the lysosome and there be degraded by the lysosomal hydrolases[86]. As with macroautophagy, both of the other forms of autophagy result in the formation of amino acids and other basic cellular components that are exported from the lysosome by membrane permeases. This provides a foundation for other important cellular processes while at the same time maintaining a healthy level of homeostasis thus preventing the cell from entering apoptosis.

Autophagy Signaling

A variety of natural and synthetic methods to regulate autophagy are known to us today. During amino acid starvation, macroautophagy, hereafter referred to as autophagy, is stimulated in the cell resulting in various cellular components being shuttled to the lysosome to

Figure 1.2: Formation of Autolysosome in Macroautophagy

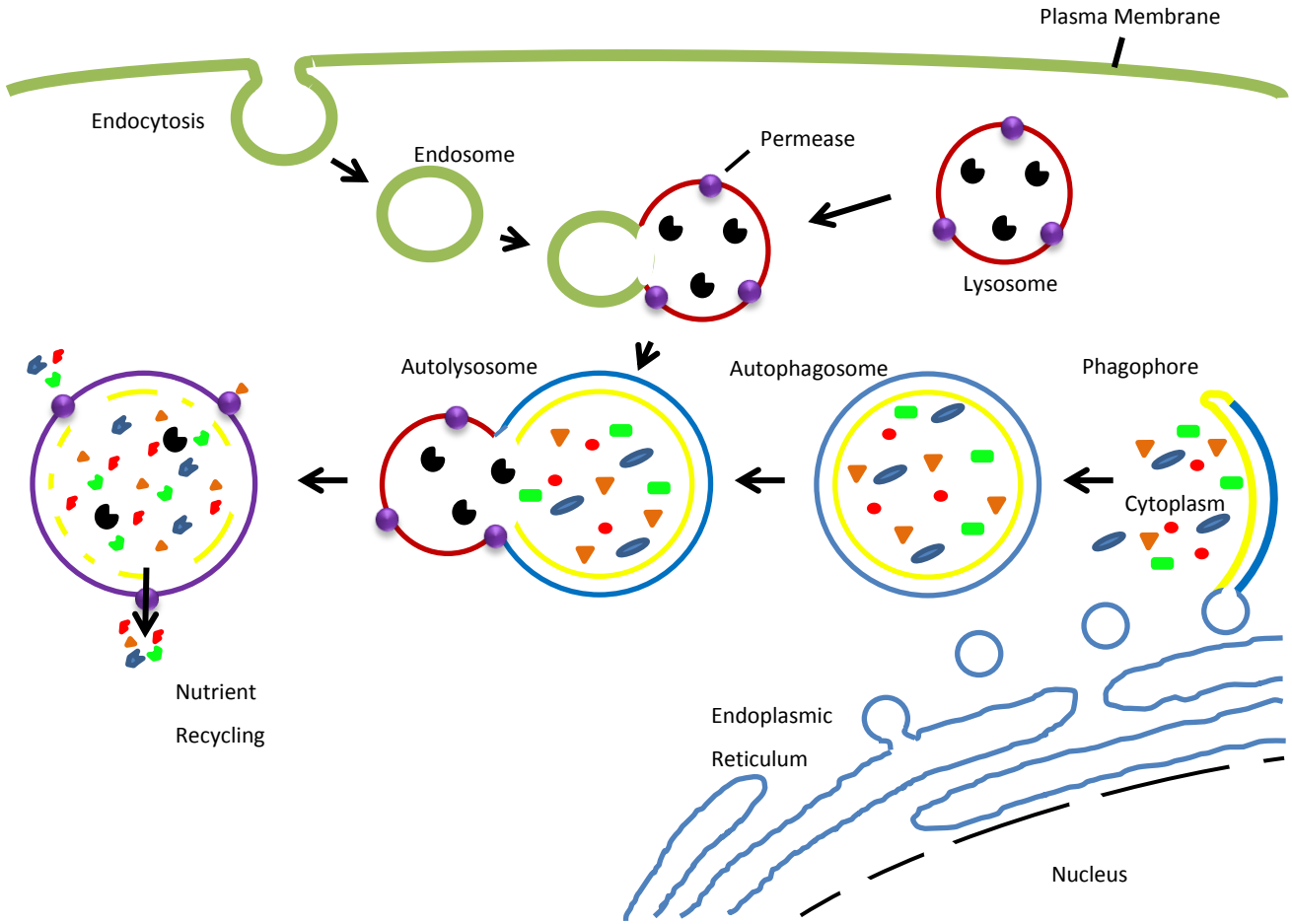

Figure 1.2-Formation of Autolysosome in Macroautophagy. Basic overview of the mechanism of general autophagy. A phagophore is synthesized from lipids provided by the ER until it encases cellular components and becomes a mature autophagosome. The autophagosome shuttles these materials to the lysosome and fuses with it, releasing enzymes into the autophagosome lumen (now called the autolysosome). These acid hydrolases degrade organelles and proteins into essential nutrients needed for survival which are then recycled back into the cytoplasm through permeases present on the membrane the lysosome.

be recycled and keep the cell alive by preventing the apoptosis cascade[87-89]. This activation of autophagy is in part due to the inhibition of the mTOR pathway (Fig. 1.3); normally, addition of amino acids to cell culture results in stimulation of mammalian target of rapamycin (mTOR) signaling. However, depletion of amino acids inhibits mTOR signaling and results in the activation of autophagy[90-92]. mTOR signaling is normally directly regulated by Rheb, a GTPase that binds and activates mTOR when in the GTP-bound form[93]. Other cellular activators of autophagy include Beclin1, a protein also involved in autophagosome formation, and phosphatidylinositol 3-kinase class III (PI3K class III)[94]. Beclin1 first dissociates from its complex with anti-apoptotic protein Bcl-2[95]. It is then able to form a new complex with PI3K class III and directly activate autophagy[96]. This complex is activated further by UV irradiation resistance-associated gene (UVRAG), increasing the cell's level of autophagy[97]. In some cells, the addition of amino acids induces cell swelling that is sensed by integrins; the swelling of the cell results in the activation of p38MAPK which inhibits autophagy[98,99]. Integrins are another substrate that can inhibit autophagy through p38MAPK activation by sensing cell swelling[100]. The presence of amino acids also inhibits autophagy through inactivating the MAPK kinase of Erk1/2, Raf-1, by stimulating Ser^{259} phosphorylation[101]. Without phosphorylation by Raf-1, Erk1/2 cannot stimulate GAIP, a GTPase-activating protein that inhibits trimeric Gi3 protein thus activating autophagy[102].

Mitophagy

The specific subset of macroautophagy we are most interested in is mitophagy,

distinguished by its targeted degradation of mitochondria. This interest is due to the presence of subunit C of mitochondrial ATP synthase found in the lysosomes of NCL patients with a CLN5 mutation[103-105]. Exploring a possible link between improper storage of this mitochondrial protein in CLN5 patients and mitophagy offers a potential mechanism for discovering the pathogenesis of the disease. Mitophagy itself is a term that was coined by Lemasters a decade ago although the phenomenon had been observed and described far earlier[106,107]. Originally viewed as a nonselective pathway, there are now numerous autophagy studies that indicate selective targeting of specific cellular bodies to phagosomes and eventually the lysosome[108-110]. These include not only mitochondria but also other organelles, invasive bacteria, and protein aggregates[111-113].

Mitophagy is induced in mammals by the loss of mitochondrial membrane potential[114,115]. This can be accomplished through a number of different ways, among them the use of carbonyl cyanide m-chlorophenylhydrazone (CCCP), an ionophore that disrupts the mitochondrial membrane potential[116]. By transferring protons from one side of the inner mitochondrial membrane to the other, this drug dissipates the proton gradient that is used to power ATP synthesis, making it highly toxic to the organism[117]. Mitochondria are seen in long chain formations under normal conditions, but when damaged or disrupted it has been shown that they become highly fragmented thus allowing autophagosomes to target and engulf the damaged organelles[118-120]. Under normal conditions, only superfluous or damaged mitochondria are targeted for degradation by autophagy[120].

CLN5 and Autophagy

Drawing links between autophagy and NCLs appears very feasible when their processes are considered; NCLs result from the buildup of proteins, and autophagy seeks to remove and recycle proteins along with other cellular bodies. Because of recent studies implicating other CLN proteins in the alteration of autophagy, we wanted to test whether CLN5 had a similar effect. We postulate that CLN5 deficiency increases the activity of autophagy based on these previous studies. As CLN5 localizes to the lysosome lumen, it is hard to imagine the protein being involved in the formation or transport of autophagosomes. It was discussed earlier that the autophagy and mTOR pathways are intertwined, and as such it would be interesting to look into CLN5 fitting in the cascade of either of these pathways. Being a luminal lysosomal protein, CLN5 could potentially be interacting with a membrane-bound protein which in complex is involved in the binding and/or fusion of autophagosomes to lysosomes thus allowing them to spill their contents into the lysosome. Linking CLN5 to autophagy will be critical in drawing further connections between NCL diseases and autophagy, and finding a link between NCLs and autophagy will be a large step toward elucidating a function for the many CLN proteins including CLN5.

Figure 1.3: Autophagy Signaling and Regulation by mTOR

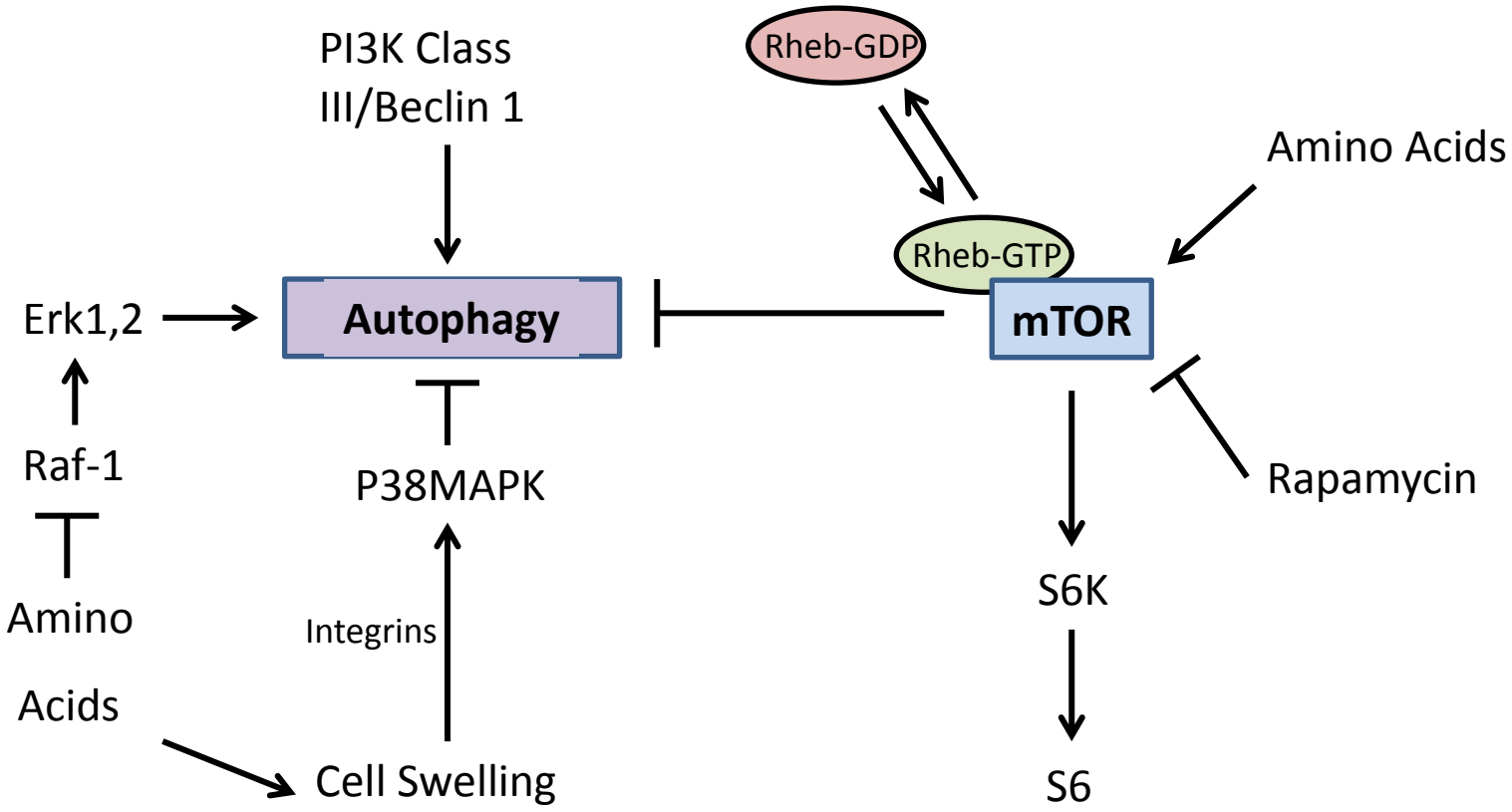

Figure 1.3: Autophagy Signaling and Regulation by mTOR. One of the main regulators of the autophagy activation is the mTOR complex, which itself is regulated by Rheb-GTPase, the abundance of amino acids, and rapamycin. S6K and S6 are downstream effectors of mTOR whose expression is being examined in regards to CLN5 deficiency in current and future experiments. Autophagy is also directly regulated by PI3K/Beclin I complex, P38MAPK, and Erk1,2.

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Chapter 2

CLN5 Deficiency Results in Alterations in Autophagy

Abstract

The neuronal ceroid lipofuscinoses (NCLs) are a set of genetically inherited lysosomal storage diseases that have a variety of onsets and cause a range of clinical symptoms including loss of motor function and vision, seizures, and retardation. These diseases result from the mutations of CLN proteins including CLN5, a soluble lysosomal protein with no known function at this time. CLN5 disease is phenotypically characterized by the accumulation of subunit c of mitochondrial ATP synthase. Other NCL related-proteins, including CLN2 and CLN3 have recently been shown to affect autophagy, a cellular mechanism that entails the basal and stress induced self-digestion of general or specified cellular components for recycling to maintain the survival of the cell. By siRNA knockdown in HeLa and Hek293 as well as the utilization of CLN5 human patient fibroblasts we examined the effect of the absence of CLN5 on the relative basal amounts of LC3-II, a hallmark protein of autophagy used to measure the level of autophagy activation. With these tests we showed that the deficiency of CLN5 results in an increase in the amount of LC3-II within all cell types. By inducing autophagy in the aforementioned cell types with several different drug treatments, we found that treated cells in the absence of CLN5 produced higher LC3-II levels than treated cells with CLN5 and that the pattern and amounts of LC3-II expressed in these experiments varied depending on the form of autophagy induced. These studies indicate the absence of CLN5 increases the activation of autophagy degradation pathway.

Introduction

The Neuronal ceroid lipofuscinoses (NCLs), more commonly known as Batten's disease, are a set of related debilitating neurodegenerative lysosomal storage disorders. These diseases are most often seen in young children, yet the onset of symptoms can range from birth to adulthood[1]. Main symptoms of these disorders include loss of vision and some motor function, epileptic seizures, and varying severities of mental retardation[2]. Due to early onset in the majority of patients, the average life expectancy for NCL patients is fifteen. Behavioral symptoms throughout the life of the patient can include aggression, restlessness, difficulty sleeping, depression, and general irritability[3-5]. NCL classification is further determined by the variant or region the causative mutation is most prominent[6,7]. As lysosmal storage disorders, all NCLs are unable to degrade and remove specific proteins from their lysosomal lumens; the main accumulated culprits seen in NCLs are Saposin A and D as well as subunit c of mitochondrial ATP synthase[8]. These phenotypes arise from mutations in one of any of the known ceroid lipofuscin (CLN) proteins, now numbering from 1 to 10[9]. The function of many of these proteins is still unknown, though several of them have been identified as lysosomal enzymes and integral membrane transport proteins[4,10-12]. To date, mutated NCL related proteins (CLN proteins) have been implicated in causing defects in endoplasmic reticulum stress response as well as the autophagy, oxidative, and apoptotic pathways[13-16].

CLN5 is a 407 amino acid protein found in the lysosomal lumen; it currently has no known function[17]. This protein is found in all body tissues, but as with other CLNs the characteristic occlusion bodies found in patient cells are far more prevalent in neuronal

tissue[18,19]. CLN5 is highly glycosylated with its eight putative N-glycosylation sites, all of which were confirmed by our earlier study utilizing mutation and chemical treatment to observe cleaved protein size[20,21]. Recently, co-immunoprecipitation studies showed CLN5 interacts with other NCL proteins including CLN1, CLN2, CLN3, CLN6, and CLN8[22,23]. Interestingly, some of these same CLN proteins were recently shown to have a distinct effect on autophagy which piqued our interest in the relationships between these CLN proteins and this degradation pathway[24].

Autophagy is one of the major degradation pathways in the cell along with the ubiquitin-proteasome pathway. Unlike apoptosis, this mechanism is used to maintain the survival of the cell for as long as possible by degrading old, damaged, or non-essential cellular components. Autophagy is activated when cells are introduced to stressful conditions like starvation and certain drug treatments, though it has been shown that low levels of autophagy are occurring at all times[25-27]. Depending on the method of activation or cellular component in need of degradation, different forms of autophagy are utilized to fit the situation. Known forms of autophagy include macroautophagy, microautophagy, and chaperone-mediated autophagy. Microautophagy and chaperone-mediated autophagy involve the direct transport of proteins into the lysosome through the lysosomal membrane[28,29]. Macroautophagy entails the engulfment of general or specified targets by an autophagosome that is then directed to the lysosome for degradation[30]. During starvation, general macroautophagy is induced to non-specifically degrade proteins and organelles for the purpose of nutrient acquisition. Other forms of macroautophagy like mitophagy (mitochondria-specific autophagy) can specifically target damaged proteins or organelles to the lysosome to maintain the homeostatic nature of

the cell[31]. To measure autophagy, the level of LC3-II protein is measured as its formation correlates with autophagosome formation. It is worth noting other methods of measuring autophagy exist, and these will be covered later in the discussion section. As all methods of autophagy detection have their own caveats, LC3-II detection was selected as it is a commonly used, tried and proven method. LC3 has two main forms, LC3-I and LC3-II (along with a pro-LC3 that is quickly modified after translation to the LC3-I form)[32]. Most of the LC3 pool is in the LC3-I form when autophagy is at its basal level. However, when the degradation pathway is induced LC3-I is lipidated with the addition of phosphatidylethanolamine; it then becomes LC3-II, which is directed to autophagosomes where it becomes anchored in the membrane[32]. After the autophagosome fuses to a lysosome, LC3-II is degraded by acid hydrolases. There is a direct relationship between the amount of autophagosome formation and the levels of LC3-II, thus the lipidated form of the protein is used as a marker for the level of autophagy activation.

Here we investigated the effect of CLN5 protein on different forms of autophagy. We utilized siRNA knockdown techniques to silence CLN5 in HeLa and Hek293 cell cultures and monitor the effect of its deficiency on LC3-II levels. Initial results indicated that knockdown of CLN5 resulted in an increase in the amount of LC3-II, a difference between experimental and control cells that was further heightened by the synthetic induction of autophagy. We also studied a number of fibroblasts from patients without functional CLN5 (CLN5 human fibroblasts), three of which we showed to be deficient for full length CLN5 (CLN5 patient #2 being the only exception). In comparison to normal human fibroblasts, all four CLN5 patient fibroblasts initially displayed a higher baseline level of LC3-II and thus a higher level autophagy activity. When these cell lines were treated to induce different forms of autophagy, different

patterns and levels of autophagy activation were recorded between patients and control. Patient cells consistently showed higher levels of LC3-II than control with and without synthetic induction of autophagy. Understanding CLN5 has been a slow process with the function of the protein still unknown. With these experiments, however, we take a step in a promising direction as we show a novel reaction in autophagy to the deficiency of CLN5 protein. Our studies indicate that CLN5 plays a role in the alteration of the autophagy degradation pathway.

Materials and Methods

Cell Culture and Transfection Methods

Media used for cell growth and maintenance for all cell lines was Dulbecco's Modified Eagle Medium (DMEM) that was supplemented with 10% fetal bovine serum (FBS), Glutamax, HEPES and gentamycin at 37°C in a humidified incubator with a consistent supply of 5% CO_2. Cells were split and seeded into either 6 or 12-well plates 18-24 hours before transfection at a confluence dependent on the cell type. Cell types used in these experiments include HeLa, Hek293, control fibroblast lines GM00498 and GM00037, and patient fibroblasts "CLN5#1, CLN5#2, CLN5#3, and CLN5#4." HeLa (Cat. No. ATCC CCL-2) and Hek293 (Cat. No. ATCC CRL-1573) cells were purchased from American Tissue Culture Collection (ATCC). Control skin fibroblasts (from non-diseased human) were purchased from the Corelli Institute for Medical Research while CLN5 patient cells were obtained from Massachusetts General Hospital, Boston. For experimentation, HeLa and Hek293 cells were seeded at ~20% confluence while patient fibroblasts were seeded at ~40% to ~50% depending on the growth rate of the cell line. Aging fibroblasts were consistently replaced by frozen stock after ~20 passages due to extremely diminished cell growth. Cells were normally transiently transfected for 24 hours using Trans IT-LT1 (Mirus Bio) in OPTI-MEM media. For double transfection of siRNA and plasmid DNA, Trans IT-TKO (Mirus Bio) was used. Initial transfection tests using Manufacturer's recommendations resulted in a significant amount of cell death, therefore the volume of reagent, media, and DNA was reduced while maintaining the same ratios of reagent and DNA. For troubleshooting

transfection in fibroblasts the transfection reagents used were Lipofectamine from Invitrogen, FuGENE HD from Promega, Trans IT-3T3 from Mirus, and Trans IT-2020 from Mirus Bio. The use of these reagents resulted in nearly complete cell death when used according manufacturer's specifications, so reagent volumes were reduced while maintaining identical ratios between media, nucleic acid and reagent resulting in minimal cell death. Cells were collected by scraping 24 hours after transfection. HeLa cell pellets were either used immediately for SDS-PAGE and western blotting experimentation or were stored at -80°C until use. Fibroblast pellets were always used immediately upon harvesting to address concerns of protein degradation at -80°C.

Drug Treatment

Reagents used in autophagy induction were carbonyl cyanide m-chlorophenylhydrazone (CCCP) (ENZO Life Sciences), Hank's Buffered Saline Solution (HBSS)/10 mM HEPES, and bortezomib (BZ) (LC Laboratories). CCCP was dissolved in dimethyl sulfoxide (DMSO) and was added to cells at a final concentration of 10 µM for a period of 2 hours before cells were harvested. DMSO alone was also used as control in CCCP experiments at a volume of 1µL which was similar to the amount of CCCP added. 20 nM BZ dissolved in DMSO was added to cells for a period of 24 hours and as with CCCP experiments 1µL of DMSO was used as control. Starvation was induced by HBSS use after media was removed and washed with PBS. 1mL or 2mL was added to 12-well or 6-well plates, respectively, for a period of 2 hours. Lysosomal degradation was blocked by chloroquine (CQ) (MP Biomedicals), which was dissolved in water, at a

concentration of 50μM for a period of 2 or 24 hours depending on what autophagy induction method was used.

SDS-PAGE and Western Blotting

Cell pellets were lysed in a RIPA lysis buffer consisting of 50 mM Tris pH 8, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate and protease inhibitor mix) and placed on ice for 30 minutes. Samples were then spun down at 20,000 rpm for 10 minutes and the soluble lysate was separated for further use. Sample buffer was added to the lysate which was then boiled for five minutes. Completed samples were loaded onto 15% SDS-PAGE and electrophoretically separated at 100 V before next being transferred onto a PVDF membrane at 4°C for ~1 hour at 100 V. Membranes were blocked with a 5% milk solution in TBST (20 mM Tris-HCl pH 7.4, 150 mM NaCl and 0.05% Tween20) and incubated at room temperature for 45 minutes to 1 hour. The membranes were then incubated with primary antibody for ~1 hour at room temperature or overnight at 4°C. After brief washings in TBST the membranes were incubated with the peroxidase-conjugated secondary antibody at a dilution of 1:50,000 in TBST for ~45 minutes at room temperature. Finally, membranes once again received brief TBST washes and were then developed using Chemiluminescent HRP substrate for ~3 minutes before images were taken using the G-Box imager from Syngene. Quantification of raw data was completed using Syngene software. Normalization and significance using the student t-test were completed using Microsoft Excel.

Antibodies

Mouse monoclonal antibody (α-Myc) producing hybridoma cell line 9E10 was purchased from ATCC (CRL 1729), grown in RPMI-1640 with 10% FBS, Glutamax, HEPES and gentamycin. Media supernatant was collected and used in 1:10 dilution (all antibodies diluted in TBST). Mouse α β-actin was received from Genscript (Cat. No. A00702) and used at a 1:1000 dilution. Rabbit α LC3-B was purchased from Abcam (Cat. No. ab51520 and used at a 1:2000 dilution. Rabbit α CLN5 antibody was also purchased from Abcam (Cat. No. ab170899) and was diluted to 1:1000. HRP-conjugated secondary antibodies (donkey α mouse/rabbit) used to complete western blotting were purchased from Jackson Laboratories.

siRNA Knockdown and Co-transfection

For endogenous CLN5 knockdown in HeLa and Hek293 cells, siRNA specific to CLN5 was overexpressed for a period of 24 hours (see sequence below). The siRNA was purchased from Dharmacon and used at a concentration of 20 nM. siGenome non-target siRNA also purchased from Dharmacon was used as control; oligomer targeted to firefly luciferase (Accession #U47296). For CLN5 rescue experiments in HeLa and Hek293 cells, siRNA was co-transfected with siRNA resistant CLN5 plasmid (in pcDNA3.1/Myc-His (-) A, siRNA resistant sequence below in Table 2.2) using Trans IT-TKO (technical knockout) transfection reagent. 2.5 µL TKO was used per well of a 12-well plate after first attempting manufacturer's recommendations followed by extensive troubleshooting.

Table 2.1: List of CLN5 Patient Mutations

CLN5 Patient	Mutation
CLN5#1	c.671G>A, p.Trp224X and c. 1103_1106del, pLys368SerfsX15
CLN5#2	c.61C>T, p.Pro21Ser and Exon 3 Deletion
CLN5#3	Homozygous c.694C>T, p.Gln232X
CLN5#4	c.671G>A, p224X and Exon 4 Deletion

Table 2.2: Sequence Data for HeLa RNAi and Rescue Experiments

Description	Sequence
CLN5 siRNA Oligomer	GAACCUACUUAUCUGGGAAUU
CLN5 WT Target	GAACCTACTTATCTGGGAA
CLN5 Mutation for siRNA Resistance	GAACCTACCTACCTGGGAA

Results

Autophagy Activation in HeLa

To determine our ability to induce and measure autophagy in HeLa cells, cells were treated to induce autophagy through starvation (HBSS) and mitochondrial uncoupling using CCCP (Fig. 2.1A). CQ was also added to observe the effect on LC3-II levels when lysosomal degradation was blocked. The LC3 antibody we used specifically recognized an isoform of the protein, LC3B. LC3-I and LC3-II ran on SDS-PAGE at ~14 kDa and 12 kDa, respectively. Cells treated for the induction of autophagy displayed a higher level of LC3-II than non-treated cells, as expected (Fig. 2.1A and 2.1C). Levels of autophagy induction for specific treatments were consistent in terms of elevation compared to non-treatment, though there was little consistency in the level of that elevation. Over several experiments, however, a pattern was noticed wherein starvation induced a lower elevation of autophagy activity than did mitochondrial uncoupling CCCP treatment. Additionally, CQ treatment in tandem with autophagy induction resulted in even more substantial LC3-II accumulation than with autophagy induction alone. This elevated LC3-II in drug-treated HeLa cells confirmed our ability to experimentally induce autophagy and block lysosomal degradation.

CLN5 Knockdown Effect on LC3-II

We next examined the effects of CLN5 deficiency on autophagy activation. To achieve CLN5 knockdown, we employed RNAi experiments using siRNA to silence CLN5 in HeLa cells.

Figure 2.1: **Autophagy Induction and CLN5 RNAi Silencing in HeLa**

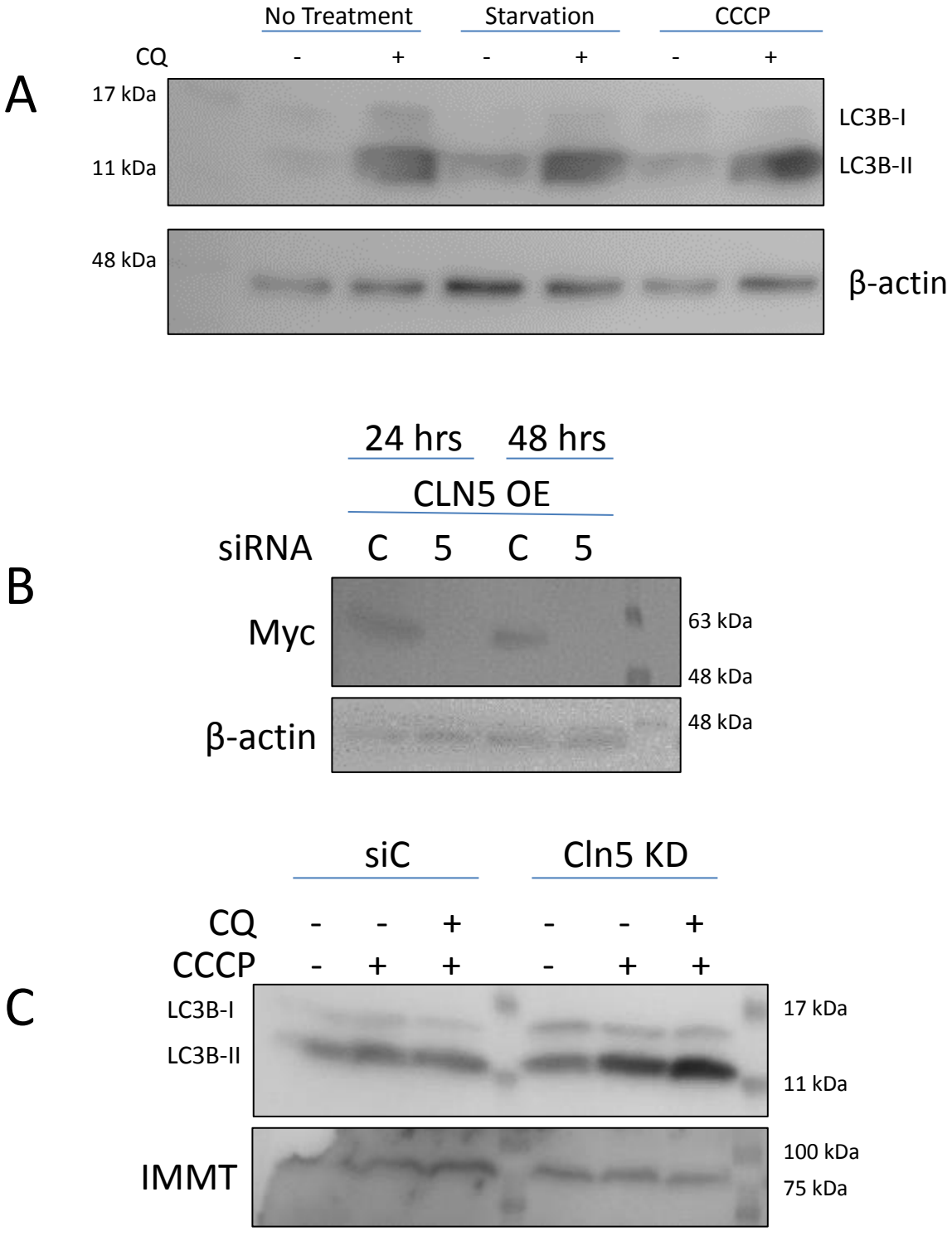


Figure 2.1-Autophagy Induction and CLN5 RNAi Silencing in HeLa. A. Hela cells were treated with 1 mL HBSS for starvation or 10 μM CCCP for mitophagy for 2 hrs. CQ was added at a concentration of 50 μM, also for 2 hrs. B. siRNA oligomers for non-specific control (C) use and for CLN5 (5) were transfected at a 20 nM concentration for 24 or 48 hrs along with siRNA resistant CLN5. C. siRNA and resistant CLN5 plasmid were transfected together for 24 hrs; CCCP and CQ were added during the final two hours at the concentration described above. Whole cell lysates were run on 15% SDS-PAGE for western; CLN5 was detected by use of mouse α-Myc antibody and LC3-II was detected with rabbit α-LC3-B antibody. β-actin (A and B) and IMMT (C) (inner membrane protein, mitochondrial) were used as loading control.

RNAi experiments were first optimized with the amount of RNAi oligomers and TKO transfection reagent (data not shown). CLN5 specific siRNA was administered for 24 and 48 hours to determine the most appropriate knockdown conditions. During the time this set of experiments was being conducted there was no antibody that could recognize endogenous CLN5 available to us, so we overexpressed Myc-tagged CLN5 to assess the knockdown effects (Fig. 2.1B). siRNA oligomers and Myc-tagged siRNA resistant CLN5 were simultaneously transfected using the TKO transfection reagent. Compared to control knockdown, CLN5 knockdown was greatly reduced for both 24 and 48 hour siRNA transfection conditions.

We then treated the HeLa cells with the CLN5 siRNA oligomers for 24 hours in tandem with CCCP to induce mitophagy. This experiment was designed to show the effect of the lack of CLN5 on LC3-II levels in HeLa cells both with and without the induction of autophagy. Knockdown of CLN5 did not seem to change basal levels of LC3-II (Fig. 2.1C). However, with the induction of mitophagy cells that were CLN5 silenced displayed an elevated LC3-II level compared to control cells that were CCCP treated. This observation creates two possibilities: either LC3-II is being produced at a higher level due to the activation of autophagy, or the lack of CLN5 causes a defect in lysosomal degradation that prevents LC3-II from being turned over. The addition of CQ along with CCCP treatment causes further accumulation of LC3-II in CLN5 knockdown cells, indicating the lysosome is functional in CLN5 knockdown cells and suggesting a higher level of LC3-II has been produced under mitophagy. These knockdown experiments suggested a link between the lack of CLN5 and the level of autophagy activation in cells experiencing artificially induced autophagy. It can also be said that the elevation of LC3-II may be due to the increased formation and recruitment of LC3-II and not its diminished degradation.

CLN5 Knockdown and Overexpression

To examine the functionality of CLN5 and to further assess if the observed effects on autophagy were due to the absence of CLN5, we tested whether overexpression of siRNA resistant CLN5 could rescue the effects of CCCP treatment (Fig. 2.2). siRNA specific for endogenous CLN5 was transfected in tandem with Myc-tagged, siRNA resistant CLN5 WT or N192S[21]. CLN5 patient mutant N192S is a glycosylation mutant that is known to properly localize to the lumen of the lysosome and yet must be functionally defective due to its disease causing nature[21]. Numerous rescue experiments were undertaken over an extended period of time, and unfortunately no conclusion could be drawn from the results rendered. A portion of the time, cells overexpressing resistant CLN5 WT plasmid reduced LC3-II back to levels comparable to or even lower than CLN5 silenced. This result held with and without the induction of autophagy using CCCP. Notably, the reduction of LC3-II in autophagy induced/CLN5 silenced cells was even greater than cells that had not been treated for autophagy. However, the opposite of this effect was seen at a nearly equal incidence. CLN5 overexpression at times showed no ability to diminish LC3-II back to control levels with or without autophagy induction. Throughout the time these experiments were undertaken, CLN5 N192S overexpression results on autophagy activation were equally inconsistent. No discernable pattern of LC3-II expression could be obtained through our analyses of the CLN5 glycosylation mutant between the many experiments. There are several possible explanations: since we couldn't examine the level of endogenous CLN5, we cannot be certain that the knockdown was equally efficient in every set of experiments. Overexpression efficiency may not be high enough to see a consistent rescuing effect. During extensive troubleshooting, we

Figure 2.2: CLN5 Rescue in HeLa Cells

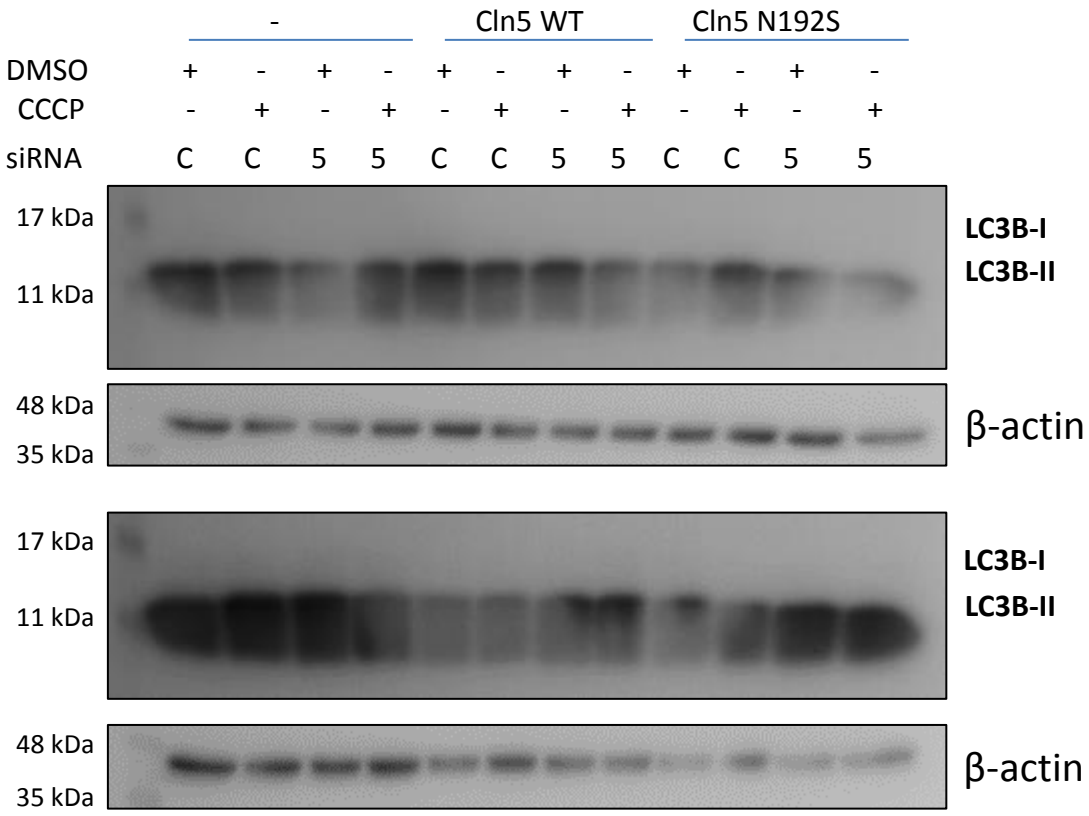


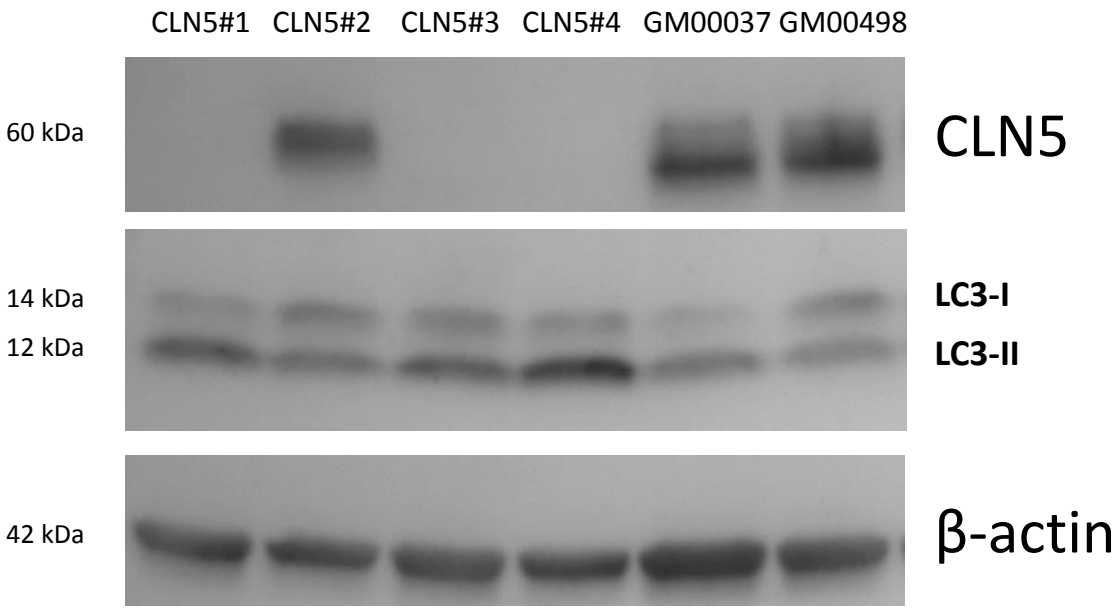
Figure 2.2-CLN5 Rescue in HeLa Cells. Cells were tandem transfected with CLN5-Myc, CLN5 N192S mutant and control/CLN5 oligomers for 24 hours. Treatment with DMSO (1 µL) and CCCP (10 µM) were added to cells 22 hours into the transfection period for 2 hrs. Cells were harvested and whole cell lysates were run on 15% SDS-PAGE for western with rabbit α-LC3-B to observe LC3-II. B-actin was used as loading control. Data is representative of at least five experiments (two shown).

purchased the CLN5 specific antibody previously mentioned. Although the myc antibody validated that we were knocking down most if not all overexpressed CLN5, the antibody specific for endogenous CLN5 showed the samples contained varying levels of endogenous CLN5 present. Despite our understanding of the problem, CLN5 patient fibroblast became available to us at this time. Thus, after confirming the absence of CLN5 in these cells, we decided it was more prudent to use CLN5 human fibroblasts for our project. Because of this, the RNAi approach was abandoned in favor of analyses that involved the use of CLN5 patient fibroblasts that were naturally deficient for CLN5 protein.

Basal LC3-II in CLN5 Patients

Skin fibroblasts of CLN5 deficient patients (excluding CLN5 patient #2, which will be addressed in the discussion section) were obtained from the Massachusetts General Hospital repository. Four patient samples were acquired, though originally two were thawed for initial experimentation. We first wanted to observe the baseline levels of LC3-II in patient fibroblasts. For comparison, control human skin fibroblasts purchased from the Coriell Institute for Medical Research (GM00037 and GM00498) were used. Total cell lysates of the control cells and the patient skin fibroblasts (dubbed CLN5#3 and CLN5#4) were run on SDS-PAGE and western blotted the CLN5 specific antibody. This antibody revealed that the patient cells were completely deficient for CLN5 (Fig. 2.3). Control fibroblasts, on the other hand, displayed a relatively high level of the CLN5 protein. Even more notably, the control fibroblasts expressed a markedly lower level of LC3-II protein than did the CLN5 mutant cells. With this revelation, the

Figure 2.3: CLN5 Patient Fibroblasts Display Elevated Basal LC3-II



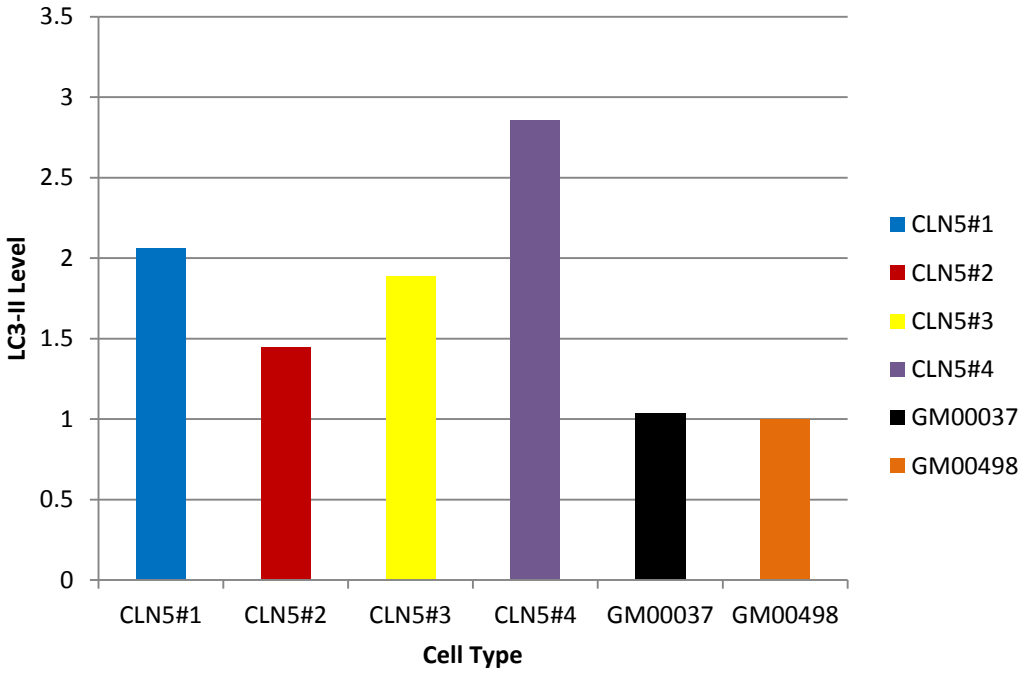


Figure 2.3-CLN5 Patient Fibroblasts Display Elevated Basal LC3-II. Whole cell lysates of control fibroblasts (GM00037 and GM00498) and CLN5 patient fibroblasts (CLN5#1-4) were run on 15% SDS-PAGE for western. LC3-II and CLN5 were detected by rabbit α-LC3-B and rabbit α-CLN5 antibodies, respectively. β-actin was used as loading control. LC3-II levels were normalized to β-actin, then normalized again to GM00498 when set at a value of 1.

decision was made to abandon the RNAi protocol used in HeLa cells as the patient fibroblast system showed more promise and removed the potential for incomplete siRNA knockdown affecting experimental results.

CLN5 Patients and Autophagy: Mitochondrial Uncoupling

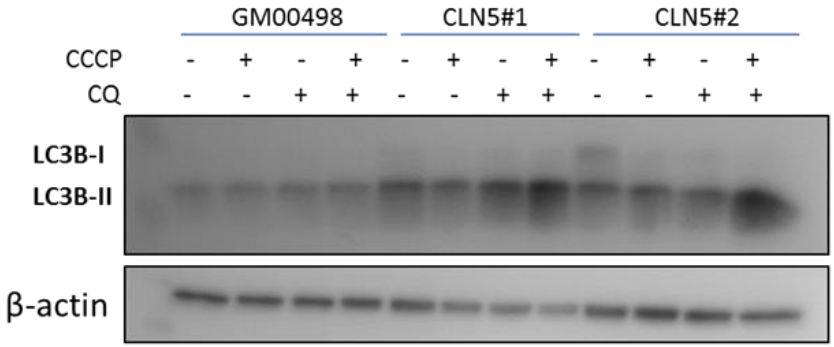
After determining CLN5 deficient patient cells had elevated LC3-II levels, we proceeded to look into the cause of this elevation as well as the effect of different forms of autophagy induction in this system. Experiments were run with two patient cell lines at a time along with a single control line, GM00498. This division in patient samples between experiments was necessary to accommodate the number of lanes available in our SDS-PAGE system. Additionally, control fibroblast line GM00037 growth proved too slow and became a detriment in an experimental and time management sense, so it was discarded in favor of GM00498. Patient fibroblasts were subjected to mitophagy induction experiments with the use of CCCP (Fig. 2.4). Cells were treated for a period of 2 hours with the drug at a concentration of 10µM as well as with and without 50µM CQ for the same period. Cell death from the use of CCCP at this concentration and incubation period was minimal. Cells were lysed and run on SDS-PAGE, then β-actin and LC3-II levels were measured with western blotting. Raw data was quantified by normalizing all LC3-II bands to their respective β-actin loading control. These data were then used to determine the fold change to control cells with no treatment which was set to a value of one. With this method we were able to properly analyze the effects of the drugs on the cells and draw definitive conclusions.

Patient cell lines CLN5#1, CLN5#2, and CLN5#4 all showed LC3-II elevation across all four treatment types (including no treatment) compared to GM00498 levels (Fig. 2.4). This was especially true for CLN5#2 and CLN5#4, which both showed a five-fold increase in the amount of LC3-II seen in control cells with a combination of CCCP and CQ treatment. These two patients also showed statistically relevant increases in LC3 compared to control under different treatment conditions (all treatments for CLN5#2, combination of CCCP and CQ treatment in CLN5#4). Notably, patient line CLN5#3 again showed similar autophagy activation in comparison to GM00498 for all treatment types. This pattern was not expected in this analysis as several times during CCCP treatment experiments CLN5#3 patient cells showed elevated LC3-II levels like those in the other mutant lines. The previous result may be due to the age and health of the cells used, and will be discussed further in the discussion. With these results we were able to conclude that specific autophagy for mitochondria is increased in patient cells CLN5#1, CLN5#2, and CLN5#4 that are deficient for functional CLN5 protein.

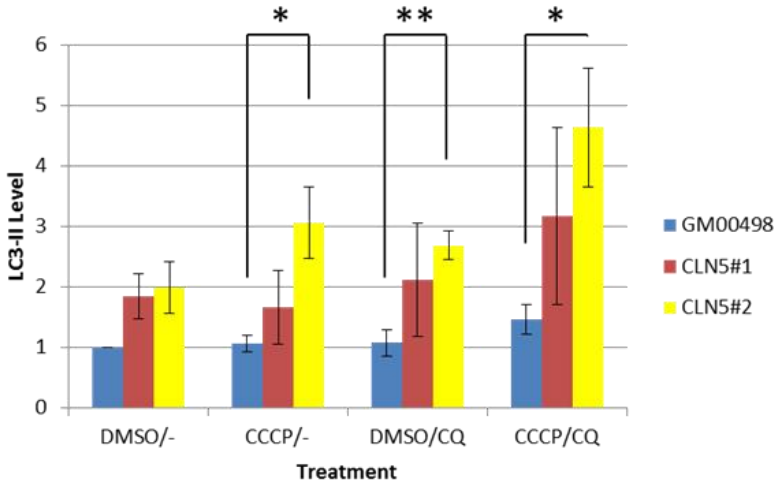
CLN5 Patients and Autophagy: Protein Aggregation

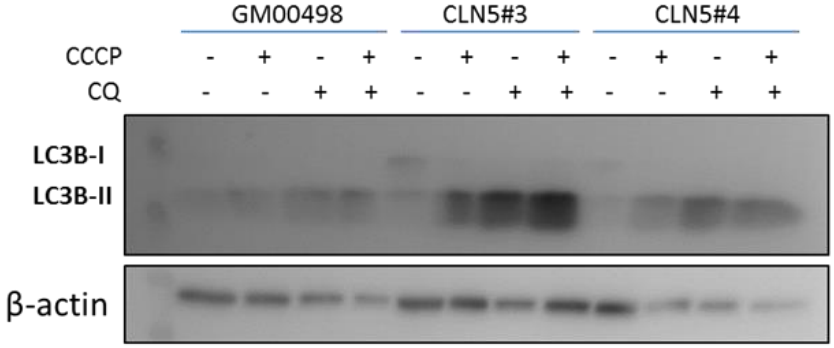
One of the three autophagy induction methods used for these assays was the use of the proteasome inhibitor bortezomib (BZ). This drug blocks proteasome function resulting in the accumulation of protein aggregates, thus rendering it incapable of being catalytically cleaved by the proteasome. Autophagy is activated to replace lost function of the ubiquitin-proteasome mechanism in this scenario. Cells were treated with 20nM BZ for ~24 hours, with and without the addition of 50μM CQ to block lysosomal degradation. Numerical data was quantified and

Figure 2.4: LC3-II Elevation and Mitophagy Studies in CLN5 Patients



*Cells treated with 10 μM CCCP; 50 μM Chloroquine for 2 hours at 37°C.





*Cells treated with 10 μM CCCP; 50 μM Chloroquine for 2 hours at 37°C.

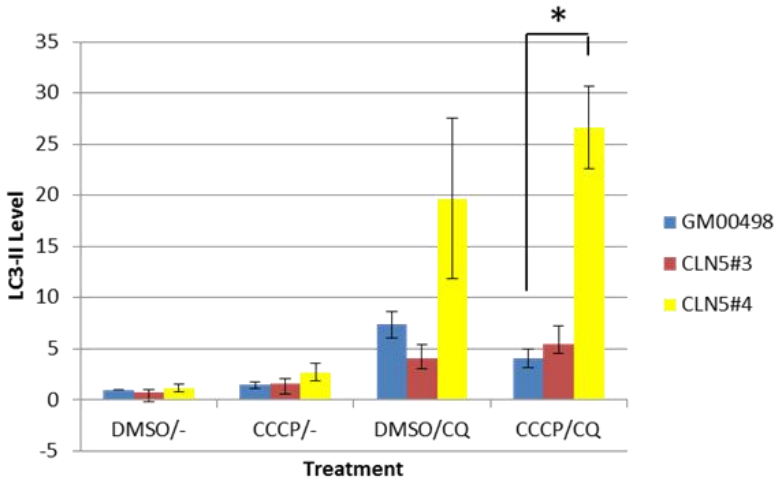
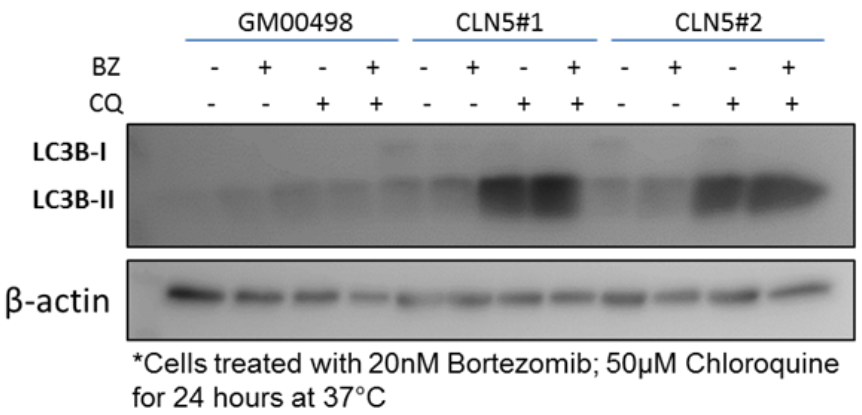


Figure 2.4-LC3-II Elevation and Mitophagy Studies in CLN5 Patients. Cells were treated with 10 μM CCCP and 50 μM CQ for 24 hrs. -/- denotes no autophagy induction drug and no CQ use. Whole cell lysates were run on 15% SDS-PAGE for western; LC3-II was detected by using rabbit α-LC3-B antibody. β-actin was used as loading control. Western blots are representative of 3-5 experiments. Graphs denote arbitrary levels of LC3-II normalized to β-actin with overall control -/- set to a value of 1. Student t-test was used to determine significance. * =P < .05, ** =P < .01

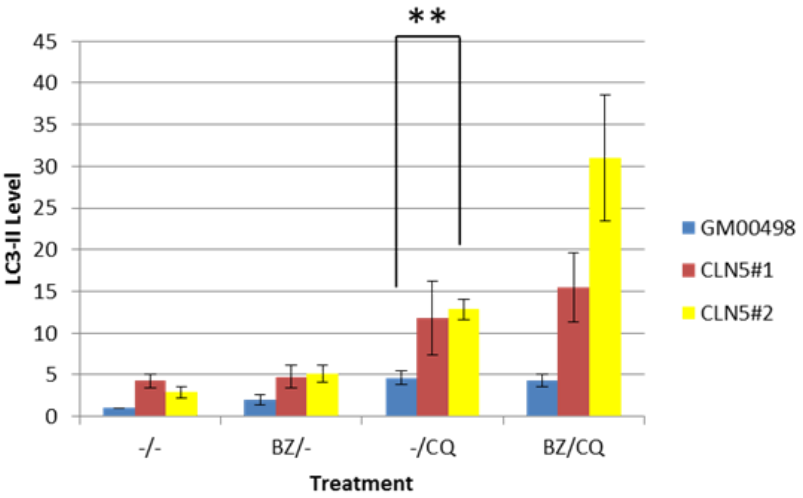
normalized as described in the mitochondrial uncoupling section.

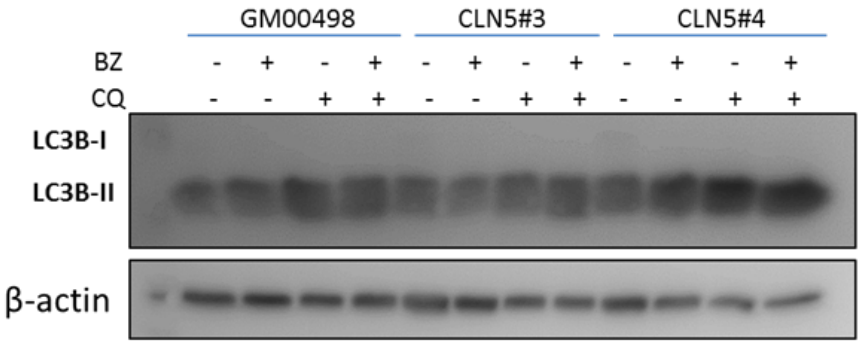
The LC3-II changes in these experiments showed similar patterns to those seen in mitochondrial uncoupling experiments which was expected as both are forms of autophagy specific for targeting and degrading materials that may damage the cell. Again, lines CLN5#2 and CLN5#4 showed significant LC3 increase in CQ and BZ/CQ combination experiments respectively, though CLN5#1, #2, and #4 all displayed a noticeable pattern of increase in all experiments. Both patient lines CLN5#1 and CLN5#2 showed a higher level of LC3-II compared to GM00498 without any drug treatment, which is similar to what was in Fig. 2.3 in the initial patient fibroblast experiment. The addition of proteasome inhibitor increased LC3-II for all cell lines, though the level continued to be moderately more elevated in CLN5#1 and CLN5#2 Fig. 2.5). CQ treatment to prevent LC3-II degradation showed the same pattern, as did the same treatment with the addition of BZ: both CLN5 patient lines showed a markedly larger increase in LC3-II levels than their control counterpart. The same experiments on patient CLN#4 showed an identical pattern of elevated LC3-II in regards to GM00498 with all treatment combinations. Once again, CLN5#3 did not follow this trend as was expected. CLN5#3 showed lower LC3-II levels than control in BZ, CQ, and the combination treatment. From these experiments we can conclude that the deficiency of CLN5 for three of the four mutants in conjunction with proteasome inhibition mediated autophagy results in a high level of autophagy activation.

Figure 2.5: LC3-II Elevation and Protein Aggregation in CLN5 Patients



*Cells treated with 20nM Bortezomib; 50μM Chloroquine for 24 hours at 37°C





*Cells treated with 20nM Bortezomib; 50μM Chloroquine for 24 hours at 37°C

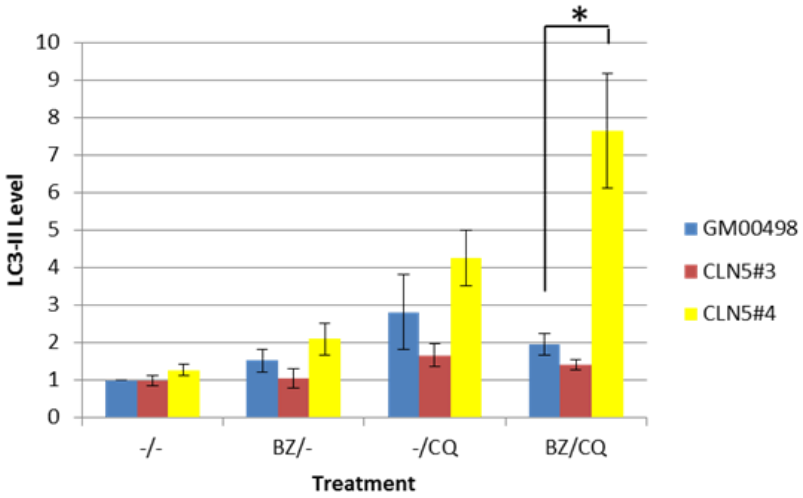


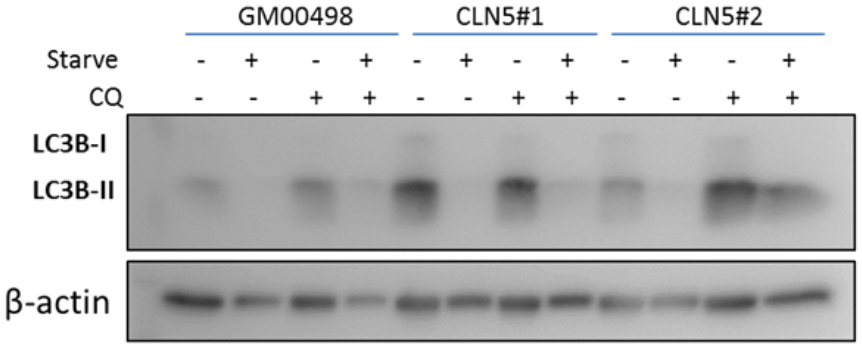
Figure 2.5-LC3-II Elevation and Protein Aggregation in CLN5 Patients. Cells were treated with 20 nM BZ and 50 μM CQ for 24 hrs. -/- denotes no autophagy induction drug and no CQ use. Whole cell lysates were run on 15% SDS-PAGE for western; LC3-II was detected by using rabbit α-LC3-B antibody. β-actin was used as loading control. Western blots are representative of 3 experiments. Graphs denote arbitrary levels of LC3-II normalized to β-actin with overall control -/- set to a value of 1. Student t-test was used to determine significance. * =P < .05, ** =P < .01

CLN5 Patients and Autophagy: Starvation

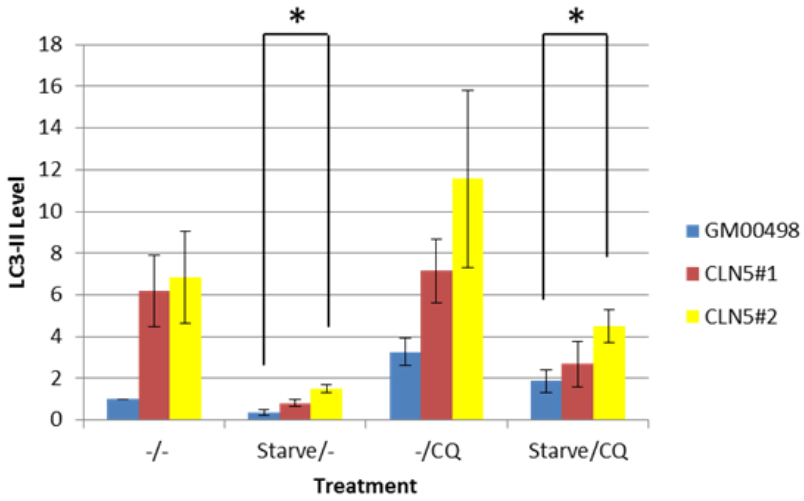
The final autophagy induction method used to assay LC3-II amounts in patient fibroblasts was starvation. Cell cultures were washed with PBS for removal of any residual growth media and HBSS was added in its place; as with previous treatments, CQ was also used to observe the effect starvation had on autophagy activation when degradation was blocked. Incubation occurred over a two hour period before cells were harvested for lysis and loading on SDS-PAGE.

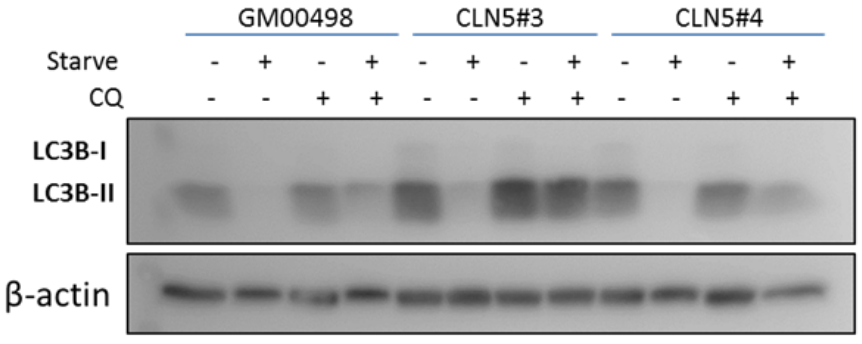
We expected the level of LC3-II to rise with the induction of autophagy through starvation as we saw with the use of CCCP and BZ. However, in this instance the exact opposite effect was observed. All cell types, patient and control, showed a large decrease in the amount of LC3-II under solely starvation (starve/-) conditions than samples that were not treated for autophagy induction via starvation (Fig. 2.6). Upon addition of CQ (starve/CQ), LC3-II levels were elevated compared to starvation alone, but were much lower than cells treated with CQ without starvation (-/CQ). This phenomenon has been documented in other studies, and may be due to the type of cell and/or the length of time cells were starved[32,33]. The pattern of baseline and autophagy induced LC3-II levels between control and CLN5 patient cells remained similar to that seen with the other induction experiments with the exception that patient CLN5#3 also showed increased levels. In these experiments, patients #2 and #4 again showed noticeable LC3 increases compared to control with all treatments. These experiments show that, as with previous fibroblast experiments, CLN5 deficiency results in an increase in LC3-II levels compared to control in many of the patients. However, overall LC3-II is decreased in all

Figure 2.6: LC3-II Elevation and Starvation Studies in CLN5 Patients



*Cells treated with 1 mL HBSS; 50 μM Chloroquine for 2 hours at 37°C.





*Cells treated with 1 mL HBSS; 50 μM Chloroquine for 2 hours at 37°C.

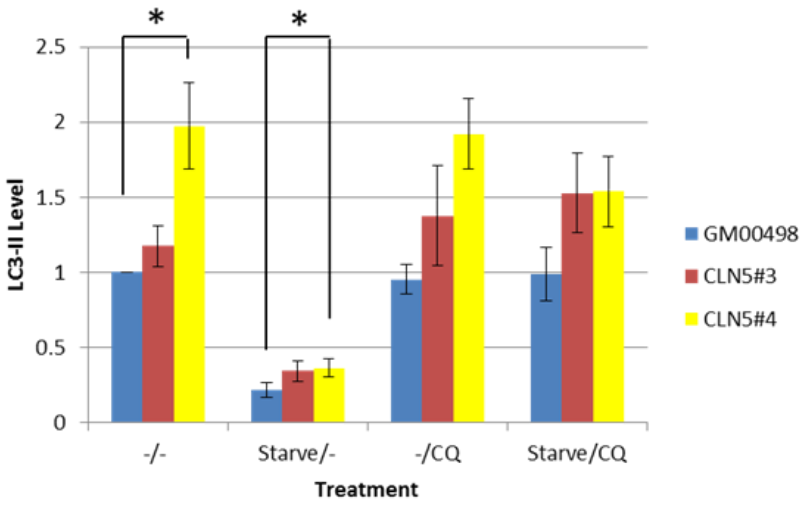


Figure 2.6-LC3-II Elevation and Starvation Studies in CLN5 Patients. Cells were treated with 1 mL HBSS and 50 μM CQ for 2 hrs. -/- denotes no autophagy induction drug and no CQ use. Whole cell lysates were run on 15% SDS-PAGE for western; LC3-II was detected by using rabbit α-LC3-B antibody. β-actin was used as loading control. Western blots are representative of 3 experiments. Graphs denote arbitrary levels of LC3-II normalized to β-actin with overall control -/- set to a value of 1. Student t-test was used to determine significance. * =P < .05

cells with the induction of starvation in comparison to non-induced samples.

Discussion

Although the function of the CLN5 protein is still unknown at this point, it is clear from our research here that lack of or defect in the protein plays a role in how the cell regulates autophagy-based degradation. Though this study does not directly elucidate the function of the protein, it points to a potential place for CLN5 in the autophagy cascade to be examined and elaborated upon in future studies. Of four CLN5 patient fibroblasts, only patient #2 has detectable level of CLN5. Mutation data of CLN5 patient #2 suggests a point mutation in the signal peptide of the protein results in the expression of dysfunctional CLN5 since the other allele is an exon deletion. With this knowledge, as well as that of the specific mutations for each patient, the most likely scenario is that the patient CLN5 proteins are unstable and thus degraded. CLN5 patient #2 cells, however, do produce mature full length CLN5 (Fig 2.3). We are currently investigating whether this full length CLN5 has wild-type CLN5 function. CLN5 patient #2 is the only patient whose CLN5 mutations include something other than an exon deletion or truncation. The CLN5 antibody we used for these is rabbit monoclonal antibody against the middle portion of CLN5, based on the information provided by Abcam. Thus, the conclusions herein are made with the knowledge that even with the use of our antibody specific for endogenous CLN5, some truncated non-functional forms of the protein may be present.

Our initial experiments with autophagy induction in CLN5 silenced HeLa cells showed our ability to induce autophagy and satisfactorily knockdown CLN5. They also showed that the level of LC3-II increase is much more marked in CLN5 silenced cells that have been treated for

autophagy induction than in those only silenced for the protein (Figure 2.1 C). Thus, although the lack of the protein may itself increase the level of autophagy activation, the effect is far more prominent when autophagy is already occurring at high levels. Whatever its function within autophagy may be, it seems as though its presence is most important during periods of high autophagy activity due to the difference in LC3-II between silenced cells and silenced, autophagy treated cells.

As discussed earlier, HeLa rescue experiments were determined to be inconclusive due to inconsistency between experiments. Though promising at times with decreased amounts of LC3-II with CLN5 overexpression, some unknown form of experimental variation caused many experiments to show opposing results. This may also be due to HeLa culture conditions or the passage number, as it was later seen in human skin fibroblasts that the level of LC3-II expressed and the health of the cells declined after many passages. After troubleshooting in many areas including cell culture treatment, storage, antibody use, and image development it was determined that the source of inconsistency was in siRNA knockdown of CLN5. Much more time, effort, and research will need to go into these rescue methods to make them viable and capable of producing solid conclusions.

In the CLN5 patient fibroblasts, we saw an elevation of LC3-II compared to the control skin fibroblasts which confirmed the RNAi in HeLa cell studies, supporting our hypothesis that the lack of CLN5 causes this increase. The experiments that followed determined how different forms of autophagy, whether specific or general, were affected by the absence of CLN5. The conclusion we were able to make from these experiments was that the elevation in LC3-II found

in CLN5 patients was due to an increase in its formation and not a decrease in its degradation. If decreased degradation had been the cause, treatment with CQ would have resulted in similar levels of undigested LC3-II as compared to no CQ treatment in CLN5 deficient cells.

For both of the specific forms of artificially induced autophagy (protein aggregation and mitophagy) similar patterns for most patient cell lines compared to control were observed. All patients except CLN5#3 showed increased amounts of LC3-II across all treatment types though much more noticeably when lysosome activity was blocked during induced autophagy activation. This reinforces our previous belief that whatever the function of CLN5 with regards to autophagy may be, it is more crucial in its regulation during times of increased autophagy activity than when the cell is under normal conditions. As for starvation, the general form of induced autophagy, all cell types including control showed reduced LC3-II levels when depleted of nutrients. This decrease may be due to enhanced lysosomal activity during starvation, the amount of time cells were starved, how these specific cells react to starvation, or possibly the method of starvation induction[34]. Some studies have shown that different cell types show LC3-II buildup at different rates when autophagy is induced, and also that LC3-II decreases exponentially after different periods of time depending on cell type[33]. In this case, more troubleshooting would need to be undertaken to determine whether more or less time is needed in starvation experiments to accurately capture the elevated LC3-II levels indicating autophagy activation. The method of starvation might also need to be changed to see the expected increase of LC3-II during starvation that is seen with other autophagy induction experiments. Though HBSS is used in several studies to induce starvation, this is not the true amino acid starvation that is known to activate autophagy. In these studies using balanced salt

solution, such as HBSS or EBSS, lower levels of LC3-II have been observed[35]. Without CQ blocking lysosome function, the reduced level of LC3-II suggests enhanced lysosome activity during induction of autophagy[35]. Our experiments deprive cells of amino acids and all other cellular nutrients excluding the basic salts found in HBSS, so another media concoction would be needed containing all normal cell culture media nutrients excluding amino acids and substrates that can be degraded to produce them. However, the decrease in LC3-II expression may not necessarily indicate a decrease in autophagy activity. Using these fibroblasts along with HBSS as a starvation method, another explanation might be a unique way in which these cells metabolize LC3-II under these conditions. With mitophagy and protein aggregation induced autophagy the cell is targeting a specific set of dysfunctional organelles or proteins to prevent them from causing further damage to the cell, processes that requires a high level of autophagy to remove the deleterious components as quickly as possible. Starvation, however, requires that the cell induce enough autophagy-based degradation for the purpose of replenishing necessary nutrients to allow the cell to survive; in essence, degrade enough cellular components to survive, but not thrive, until conditions approve. At the same time, the cell would want to turn over these components into usable cellular building blocks as quickly as possible to keep the cell alive. These factors may account for the diminished LC3-II seen with starvation treatment, including those treatments with the addition of CQ which shows reduced levels of the protein.

Across the many autophagy induction experiments using patient fibroblasts, only patients CLN5#2 and CLN5#4 showed significant change of autophagy activation in a few experiment types. That said, during these experiments and previous ones all patient cells

showed noticeable patterns of LC3-II elevation compared to control fibroblasts with and without CQ use. Some of the experiments seen in these figures were also very close (within 5%) of statistical relevance, so an increase in the number of repeats could potentially prove these to be relevant as several of these figures were produced with as few as three repeats (up to five). Again, the pattern of LC3-II increase in CLN5 deficient cells was very strong and noticeable from experiment to experiment. Even in the case of CLN5#3, whose low levels of LC3-II during the last several weeks of autophagy induction experiments could be explained by cells that had been passaged too far. Future experiments would entail using fibroblasts at identical passages once thawed, and discarding them all together before they reached an unhealthy state.

The phenomenon of lowered LC3-II in starvation experiments and in CLN5#3 (as opposed to earlier experiments) could also be circumvented all together with the use of additional autophagy activation markers including Beclin1 and p62. The use of these proteins in the assays used here would be beneficial in confirming the levels of autophagy in each of these experiments. Beclin1 is a protein that, as seen in the literature review, interacts with class III PI3K and is important in the localization of autophagosome-related proteins[36,37]. Up-regulation of Beclin1 in this complex results in the activation of autophagy, so its levels directly correlate with the level of autophagy. p62/SQSTM (sequestosome 1) is another protein complex that is often used to monitor autophagy, and may also improve our future studies. This complex interacts with protein aggregates that are eventually targeted to the autolysosome, so it too has a direct relationship with autophagy activation[38,39]. This method

would be especially important and relevant to further BZ induced autophagy experiments due to the specificity of the complex to protein aggregates.

Though most of the CLN5 patients show similarities in terms of displaying increased levels of LC3-II, the levels of that increase vary between mutant types. This may be accounted for by unknown metabolic discrepancies specific to certain mutants besides the absence of CLN5. Additionally, each patient possesses different CLN5 mutations, and as discussed before the complete ablation of CLN5 protein in fibroblasts has only been confirmed with antibody specific for endogenous CLN5. This may not account for possible truncated CLN5 protein in certain mutants than can result in semi-functional CLN5 that would restore some function to the protein's unknown role in autophagy regulation.

As of this point, much of the data we have is preliminary and further work is required to support some of the conclusions made and hypotheses postulated herein. This includes analyses into specific autophagy mechanism types and the status of potential truncated CLN5 protein in patient cells. Additionally, more work is required in the area of CLN5 rescue in patient fibroblasts to confirm replacement of lost CLN5 function as to date extensive troubleshooting in fibroblast transfection has been unsuccessful. Discussion has been held in regards to potentially using a retroviral transfection system. The future of this project is ultimately geared toward elucidating the function of CLN5 in the autophagy cascade. This study, however, has shown that the CLN5 protein has some role in the regulation of autophagy, and that its role is not involved in the degradation of lysosomal targets as our CQ experiments indicated the excess LC3-II was due to increased expression of the protein. We also showed

that the expression of LC3-II was dependent on the type of autophagy induced and differed between patients most likely due to difference in mutation. Although the function of CLN5 is still unknown, this study provides a mechanism to further explore to elucidate the protein's function.

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