CHAPTER 1

Introduction
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1 Juvenile Neuronal Ceroid Lipofuscinosis

1.1 Clinical features

Juvenile Neuronal Ceroid Lipofuscinosis (JNCL, MIM 204200) is a severe autosomal recessive hereditary neurodegenerative disorder (reviewed in Rapola, 1993). It is the juvenile form of the most common lysosomal storage disorders of childhood, the neuronal ceroid lipofuscinoses (NCLs). This lysosomal storage disease (LSD) is also called Batten, Spielmeyer and Vogt disease, or Batten disease. Patients suffer from gradual decline of the nervous system, starting at an early age. The first symptom, loss of vision, becomes apparent between four and nine years of age. Ocular fundi show macular degeneration, optic atrophy, and retinal degeneration. Symptoms progress to generalized or complex partial type epileptic seizures, psychomotor deterioration, followed by dementia and a vegetative state, and patients eventually die usually between 20 and 40 years (Goebel et al., 1999). This disease is distributed worldwide and has an incidence of 1.45 in 100 000 births. In patient cells accumulation of lipopigments can be found. These accumulations resemble the pathogenic pigment ceroid and the age pigment lipofuscin in their autofluorescent and staining characteristics, although chemically and ultrastructurally different. Stored materials can be found in lysosomes of a variety of cells and tissues, although only neurons appear to be pathologically affected. Most of the lipopigment accumulations have typical fingerprint profiles when observed using electron microscopy (Figure 1), and were found to mainly consist of Subunit c of the mitochondrial ATP synthase (Wisniewski et al., 1988, Palmer et al., 1992).

1.2 Positional cloning of the CLN3 gene

Linkage analysis was used to map the JNCL locus to chromosome 16, by demonstration of linkage to the haptoglobin locus. A collaboration of researchers performed linkage analysis with microsatellite markers to define flanking markers. This was followed by haplotype analysis and cosmid walking, which resulted in identification of the cosmid that contained the microsatellite locus D16S298 for which most JNCL disease chromosomes carry allele 6. Trapped exons of this cosmid were used to screen a fetal brain cDNA library for candidate transcripts, eventually yielding a clone of which the exons were found to flank the microsatellite locus. The cDNA was found to be a transcript of the CLN3 gene, mutations in which are causative of JNCL (IBDC, 1995, MIM 607042). The most common mutation detected in the CLN3 gene of Batten disease patients is a 1.02 kb deletion on genomic sequence level, which corresponds to 217 bp of cDNA sequence. Due to this deletion exons seven
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and eight are deleted, which probably leads to a truncated protein of 181 amino acids, consisting of the first 153 residues of the CLN3 protein and 28 novel amino acids (IBDC, 1995). 85% of JNCL disease chromosomes carry this mutation, which is in linkage disequilibrium with D16S298 allele 6. Other mutations in the CLN3 gene cause similar pathology, although a protracted disease course was observed for one mutation (Mole et al., 2001). Delayed progression has been described for some genotypes, but on the other hand this might be caused by the common phenotypic variability that also is present in JNCL patients, and probably modulated by modifier genes and environmental influences (Mole et al., 1999).

Figure 1 Pure fingerprint body, typical of juvenile NCL (CLN3), X 75000. Fingerprint bodies consist of alternating electron lucent, and dense paired parallel lines. The origin of this material is not certain, however, the frequent merging with the lysosomal matrix may indicate lysosomal origin, and this matrix may play a role in the typical fingerprint organization of the stored material. The intervening line may vary considerably in width, sometimes appearing as a mesh of unorganized single membranes that somewhat resemble fingerprint lamellae. Pure fingerprint bodies can be found in neurons of the peripheral nervous system in classic JNCL, Finnish variant NCL (CLN5), and in the variant late infantile/early juvenile NCL (CLN6). (from The Neuronal Ceroid Lipofuscinosis (Batten disease); Biomedical and Health Research, Volume 33, H.H. Goebel, S.E. Mole, and B.D. Lake, IOS press)
1.3 The CLN3 gene and protein

The CLN3 gene was mapped to locus 16p12.1, and contains 15 exons, spanning 15 kb of genomic sequence (IBDC, 1995). The cDNA sequence consists of 1689 bp and contains an open reading frame of 1314 bp, which is predicted to encode a 438 amino acid protein with a molecular mass of 48 kDa, with many post-translational modification sites, such as N-glycosylation sites, phosphorylation sites, and myristoylation sites, and has at least five transmembrane spanning domains (Phillips et al., 2005).

The CLN3 protein is conserved throughout most eukaryotic organisms, suggesting that its function is of fundamental importance for the eukaryotic cell (Taschner et al., 1997). Comparing protein sequences shows several nearly completely conserved regions throughout the eukaryote phylogeny (De Voer et al., 2001). The conservation of these regions suggests they are essential for protein function. In the CLN3 protein sequence no other domains could be recognized. All nine missense mutations found in CLN3 affect residues conserved across species as dog, mouse, rabbit, the nematode C. elegans, the fruit fly Drosophila melanogaster, and yeast (NCL mutation database, http://www.ucl.ac.uk/ncl).
2 Lysosomes, operation/activity

2.1 Lysosomes and disease

Lysosomes, the cell’s degradation compartments, were first identified by De Duve in 1949 and in his review paper of 1963 lysosomes were already hypothesized to be involved in pathogenic mechanisms in a number of ways. Indeed, lysosomes were found to be involved in many disorders and in the future may be discovered to play a role, one way or another, in other diseases (Futerman and Van Meer, 2004, Vellodi et al., 2005). Research into the etiology of lysosomal disease has elucidated mechanisms concerning formation, degradation and secretion and recycling of lysosomal compartments and components. This better understanding of common lysosomal processes may eventually lead to the development of treatments of diseases caused by lysosomal defects.

2.2 Lysosomes function in degradation

Lysosomes are dynamic membrane bound organelles essentially containing hydrolytic enzymes in an acidic internal environment in which digestion takes place (Bainton, 1981). After completion of the degradation process vesicles may bud off the lysosomes to secrete the left-over debris, recycle the lysosomal enzymes, or possibly take up the building blocks that result from the break-down process and use them in anabolism. It is the degradative activity of one of the lysosomal enzymes, acid phosphatase, that led to the discovery of lysosomes. Gentle homogenization of rat liver cells allowed the lysosomes to remain intact, thereby retaining all enzymes inside of the organelle. This caused a rather unexpected decrease of enzymatic acid phosphatase activity, compared to drastic homogenization that disrupted the lysosomal membrane thereby releasing the enzymes and permitting measurement of all acid phosphatase activity (De Duve, 1963). At that time the presence of many other enzymes had been established, altogether allowing degradation of proteins, nucleic acids, and polysaccharides in a slightly acidic environment. Due to the common lytic, or digestive, activities of the enzymes contained by the organelles, they were named “lysosomes”. Currently more than fifty lysosomal acid hydrolases are known, including phosphatases, nucleases, glycosidases, proteases, peptidases, sulfatases, and lipases, functioning in hydrolysis of biological compounds (Bainton, 1981).
2.3 Lysosomal enzymes are transported by multiple routes

The acid hydrolases are lysosomal enzymes that originate from the endoplasmic reticulum and move through the Golgi apparatus where they are glycosylated and prepared for the sorting process (see figure 2 for an overview of transport routes between compartments in the cell). In the cis-Golgi network, the mannose residues present on the lysosomal hydrolase precursors are provided with a phosphate completing the mannose 6-phosphate (M6P) marker (Brown et al., 1986). The lysosomal hydrolase precursors with M6P groups progress through the Golgi apparatus and bind M6P receptors at pH 7, which permits M6P-receptor-ligand complex formation, in the trans-Golgi network. Multiple M6P receptors bound to their hydrolytic cargo gather in clathrin coated vesicles (CCV) that bud off from the Golgi apparatus and travel through the cytoplasm to engage other vesicles that contain material destined to be degraded. Upon fusion of these vesicles filled with hydrolases with an endosome, the internal pH of the resulting vesicle is lowered thereby releasing the hydrolytic enzymes from their receptors. The empty M6P receptors gather once

![Figure 2 Trafficking pathways between different compartments in the cell](image)

In this scheme the three known transport routes that lysosomal proteins use to reach the lysosome are depicted: A the ‘direct’ mannose-6-phosphate receptor mediated transport route, by which lysosomal proteins travel to the early or late endosome, B the ‘indirect’ pathway, by which lysosomal proteins first travel to the plasma membrane followed by their endocytosis, through which they reach the early endosome, C the ‘direct’ pathway independent of the mannose-6-phosphate receptor. (ER endoplasmic reticulum, TGN trans-Golgi network) Adapted from Sachse et al., 2002.
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more into a vesicle to bud off from the late endosome and recycle back to the Golgi apparatus where they can again bind the hydrolytic precursors. The late endosome, containing the hydrolytic precursors but not the M6P receptors, acidifies to about pH 5 allowing the hydrolytic enzymes to become active and start degradation, although some hydrolytic enzymes are expected to be at least partially active in late-endosomes. It should be noted however, that the M6P-receptor transport route is not the sole pathway by which hydrolytic enzymes are transported to lysosomes. The membrane-bound precursor of lysosomal acid phosphatase (LAP), for example, is transported to the plasma membrane. After endocytosis, the precursor is translocated to the lysosome, in which proteolysis cleaves the precursor from the membrane-bound part releasing the soluble LAP (Suter et al., 2001). In addition, transport of the lysosomal aspartyl protease cathepsin D to lysosomes can be independent of M6P residues as was shown in hepatocytes (Rijnboutt et al., 1991). In cultured lymphoblastoid cells from I-cell disease patients phosphotransferase activity is absent, but cathepsin D can be identified in dense lysosomes (Glickman and Kornfeld, 1993). Mannose-6-phosphate deficient mice also have cathepsin D targeted to their lysosomes, although this was cell-type specific (Dittmer et al., 1999). Thus, hydrolytic enzymes use multiple routes to travel to lysosomes in cell-type specific manner, and we can not exclude the existence of other yet undiscovered lysosomal transport routes.

2.4 The lysosome has a characteristic bounding membrane

Another striking characteristic of the lysosome is its degradation-resistant bounding membrane, separating and thereby controlling degradation and protecting the cytoplasm from the potentially deleterious lytic mixture of enzymes present inside. This unique membrane has a characteristic phospholipid composition, contains tremendous amounts of carbohydrates and is rich in lysosome-specific membrane proteins with which the lysosome maintains its internal environment (Eskelinen et al., 2003). Other functions of the resident proteins of the lysosomal membrane include the acidification of the lysosomal internal milieu, translocation of breakdown products for reuse, and vesicular fusion and fission. Lysosomal membrane proteins are transported from the trans-Golgi network to late-endosomes / lysosomes through either direct intracellular trafficking or indirectly via the plasma membrane. Adapter protein-3 (AP-3) is involved in direct trafficking of some lysosomal membrane proteins, whereas other heterotetrameric and monomeric adapter proteins are likely to play similar roles in the indirect pathway (Luzio et al., 2003).

2.5 Lysosomes and endocytosis

Cells use endocytosis for uptake of extracellular macromolecules that subsequently will be transported to lysosomes for degradation. Phagocytosis, or cell eating, is mainly performed by specialized cells such as macrophages or neutrophils that aid in
neutralization of large pathogens and clear out cellular debris (Aderem and Underhill, 1999). In contrast, pinocytosis, or cell drinking, is a more general mechanism existing in different forms: macropinocytosis, caveolin- or clathrin-mediated endocytosis, or clathrin- and caveolin-independent endocytosis, in which lipid rafts presumably are involved and probably comprise more than one pathway (Conner and Schmid, 2003). The best understood form is clathrin-mediated endocytosis (CME), in which clathrin-coated vesicles with their receptor-bound macromolecular ligands form at the plasma membrane, and travel through cytoplasm to encounter and deliver their cargo to vesicles containing hydrolytic enzymes. Different hypotheses explain how material taken up by endocytosis eventually arrives in lysosomal compartments (Luzio et al., 2003). For example, vesicle maturation presumably takes place in endosomes, as the receptors release their ligands destined to be degraded and return to the plasma membrane, and hydrolytic enzymes are introduced (Murphy 1991, Mellman and Warren, 2000). All proposed mechanisms may be intertwined. Vesicular traffic may carry the receptors back to the plasma membrane or trans-Golgi network (Mellman and Warren, 2000), and kiss-and-run occurrences may deliver hydrolytic enzymes to endosomes (Storrie and Desjardins, 1996, Bright et al., 2005). Moreover, direct fusion between lysosome and endosome yielding a hybrid organelle also was shown to occur (Mullock 1998). The whole biological process of endocytosis may actually comprise a mixture of all proposed models with other complementary mechanisms to be discovered.

2.6 Secretion and recycling

Lysosomes are at the end of the endo-lysosomal pathway and a terminal degradative stage for most of the internalized materials, but these compartments should not be considered as dead-end organelles (Bainton, 1981). Most of the specific lysosomal components, e.g. hydrolytic enzymes, can be recycled and the breakdown products can be secreted or reused as building blocks (Luzio et al., 2003). The recycling of endosomal markers, such as M6P receptors, to the trans-Golgi network is thought to occur mostly from endosomes as late-endosomes contain relatively small quantities of them. Other lysosomal transport routes certainly exist: cholesterol transport from late endosomes to the trans-Golgi network is modulated by the integral membrane protein NPC1 (Liscum, 2000). Transport of lysosomal contents can be studied in specialized cells, e.g., osteoclasts, cytotoxic T cells, and natural killer cells, which contain secretory lysosomes that are characterized by their dual function in degradation and secretion (Blott and Griffiths, 2002). Secretory lysosomes resemble conventional lysosomes in their structural diversity, acid hydrolytic contents, and ability to fuse with the plasma membrane, e.g. lysosomes are thought to be involved in membrane repair processes (Andrews, 2000). Regulated secretion by secretory lysosomes involves several distinct steps stimulated by a mostly external signal, causing the mobilization of the granules and transport to the site of stimulation, followed by docking and release of its contents.
3 Lysosomal storage diseases

3.1 Introduction

Genetic lesions affecting any aspect of the lysosomal processes described above may result in metabolic diseases caused by abnormal lysosomal function (Scriver et al., 2001). Logically, when an acid hydrolase does not perform correctly the substrate will not be degraded, and will most likely accumulate and subsequently may disrupt the other processes that were proceeding in the organelle. Suboptimal performance of acid hydrolases can have various causes, e.g. mutations in the gene encoding the enzyme, incorrect trafficking of the enzyme or other requirements for degradation, such as co-enzymes or transporters, or suboptimal maintenance of the environment in which the enzyme is supposed to operate. Incorrect functioning of lysosomes may indirectly cause depletion of compounds used in anabolism, since lysosomes also play a role in recycling of cellular building blocks. Additional effects may arise when accumulated substances cause lysosomes to become enlarged and inflexible, possibly clogging the cell. Such a relatively small cellular defect may have devastating effects on the whole organism.

Review of the currently known LSDs and their causes makes clear that the majority is caused by lysosomal acid hydrolase defects, leading to the accumulation of their substrates. Therefore, enzyme replacement therapy (ERT) could be a possible approach to improve the quality of life of patients suffering from these diseases. Pompe and Gaucher disease patients, e.g., were shown to have significant improvements in quality of life during and after enzyme replacement therapy (Beck, 2007). The other group of LSDs consists of disorders that are caused by multiple different underlying defects: affected enzyme trafficking or enzyme regulation, abnormal transport of a substrate or reaction product, mutated cofactors leading to dysfunctional enzymes, aberrant vesicle trafficking or biogenesis, and still unknown mechanisms. Cellular metabolism involves many other genes and proteins, some of which may become associated with LSDs in the future. This group probably contains many essential genes and proteins, thus mutations in this group might never be found as they will cause lethality.

A comprehensive overview of detailed descriptions of all LSDs will not be provided in this dissertation, but can be found in Scriver et al. (2001). Here I will discuss a few examples of enzyme and alternative defects causative of lysosomal storage, and descriptive of basic lysosomal processes.
3.2 Enzyme defects leading to LSD

3.2.1 Glycogen storage disease
Many LSDs are caused by mutations in genes encoding lysosomal hydrolytic enzymes. The first LSD, in which defects in a degradative lysosomal enzyme were found, was glycogen storage disease type II, also known as acid α-glucosidase, acid maltase deficiency, or Pompe disease (Hers, 1963). This inherited disorder of glycogen metabolism is the result of reduced lysosomal hydrolase acid α-glucosidase activity, causing intralysosomal accumulation of normally structured glycogen in numerous tissues, most markedly in cardiac and skeletal muscle. Classical Pompe disease patients present with prominent cardiomegaly, hypotonia, hepatomegaly, and death due to cardiorespiratory failure, usually before the age of two (Pompe, 1932). After the genetic and metabolic defects were elucidated, varying clinical presentations could be diagnosed, revealing a highly variant disease progression, including degrees of myopathy, age of onset, and extent of organ involvement. Mild acid α-glucosidase deficiency presents as late as the sixth decade of life with slow progressive proximal myopathy, only in skeletal muscle. Enzyme activity is measured to confirm clinical diagnosis, and generally correlates to severity of the disease. Identification and characterization of the gene involved in this disease allowed DNA analysis for carrier detection and additional diagnostic means. The gene was designated acid alpha-1,4-glucosidase (GAA), and was found to encode an extensive posttranslationally modified 952 amino acid protein, in which multiple mutations have been identified (Hirschhorn and Reuser, 2001).

The effects of enzyme replacement therapy by intravenous infusion of recombinant human acid α-glucosidase were investigated. From an initial clinical trial could be concluded that the patients tolerated the infusions well, moreover, the patients heart size decreased, cardiac function could be maintained for more than one year, and the patients survived over the critical age of one year. In addition to the continued normal cardiac function, skeletal muscle function improved and muscle biopsies were used to show a dramatic decrease in glycogen accumulation in muscle cells (Amalfitano et al., 2001). In another study recombinant human acid α-glucosidase that was isolated from milk from transgenic rabbits was intravenously injected in four classic infantile Pompe disease patients (Winkel et al., 2003). After 72 weeks of treatment all patients had normal acid α-glucosidase activity in muscle cells, while the glycogen concentration decreased only in the least affected patient. Furthermore, after treatment all patients were in a better clinical condition, but only in the least affected patient substantial improvement of the muscle architecture was observed. All patients were alive after four years of treatment and the least affected patient achieved motor milestones comparable to a normal child (Van den Hout et al., 2004). The variability of the response to acid α-glucosidase injection was suggested to depend on the degree of glycogen storage at the start of the treatment, thus for the treatment to have optimal effect it has to be started as early as possible. A three year treatment of three patients suffering from juvenile Pompe’s disease with infusion of acid α-glucosidase isolated from rabbit milk resulted in improved muscle strength, most prominently in the youngest patient that abandoned
his wheelchair after two years of treatment. Quality of life had increased for all three patients after treatment (Winkel et al., 2004). In overview of the ERTs can be stated that treatments with recombinant acid α-glucosidase have demonstrated the safety and efficacy for ameliorating the condition of infantile and juvenile Pompe’s disease patients, indicating similar treatments might also be effective in other diseases caused by lysosomal hydrolytic enzyme defects.

3.2.2 Glycogen storage disease type IIb, Danon disease
Another form of glycogen storage disease is glycogen storage disease type IIb, a disorder characterized by prominent cardiac abnormalities, involvement of skeletal muscle, and variable mental retardation. This disease is also known as Danon disease, and was found to be caused by mutations in the LAMP-2 gene, encoding the lysosome associated membrane protein-2 (Hirschhorn and Reuser, 2001). Although a definitive protein function has to be established, the protein is thought to be involved in protecting the lysosomal membrane from intralysosomal proteolytic enzymes, and in protein import into lysosomes, using its receptor function (Sugie et al., 2002). Currently, no therapy is available but cardiac problems could be ameliorated by inserting pacemakers (Charron et al., 2004).

3.2.3 Gaucher’s disease
The lysosomal glycolipid storage disorder in which glucosylceramide or glucocerebroside is accumulated is called Gaucher’s disease. There are three subtypes of this disorder (Beutler and Grabowski, 2001, Grabowski, 2005). Type 1 is the most common form, and type 1 patients have no primary nervous system symptoms, whereas the acute type 2 and subacute type 3 forms are neuronopathic. In Gaucher disease patients lysosomal accumulation of glucosylceramide leads to hepatosplenomegaly, anemia, and bone disease. Neurological symptoms, for the type 2 and 3 forms, at the onset of the disease include strabismus and other eye abnormalities, for type 2 this starts early in life and progresses into severe neuronal disease and death usually before the patient is 2 years old. Whereas, type 3 patients have a later onset and neuronal symptoms may include progressive intellectual impairment, mental retardation, and myoclonic seizures. This disease is caused by mutations in the gene encoding acid β-glucosidase (Tsuji et al., 1988). A possible therapy for this defective lysosomal hydrolase may be introduction of correct acid β-glucosidase or inhibition of the substrate of the hydrolase.

Gaucher disease type 1 patients can be treated with ERT. A patient that was treated with human placental glucocerebrosidase had increased hemoglobin levels and platelet counts, decreased phagocytic activity in the spleen and improved skeletal structure (Barton et al., 1990). Recombinant forms of glucocerebrosidase, alglucerase or later imiglucerase, were produced by Genzyme corporation to enable production of sufficient enzyme to treat many more patients. Evaluation of long-term ERT indicates that imiglucerase infusions comprise a safe treatment of Gaucher disease (Starzyk et al., 2007). Other therapies, such as gene therapy, chaperone therapy, substrate reduction therapy and bone marrow transplantation, are also under investigation to find a cure for all forms of the disease.
3.3 Affected cofactors, and coactivators of hydrolytic enzymes

3.3.1 Variant metachromatic leukodystrophy
Hydrolytic enzymes use coactivators and cofactors to proceed with degradation. Mutations in genes encoding the coactivators and cofactors were also found to be causative of LSDs. This is exemplified by the LSDs variant metachromatic leukodystrophy (MLD) and G_{M2} gangliosidoses, which can be caused by affected coactivator proteins. Variant MLD is caused by mutations in the gene encoding prosaposin, a sphingolipid activator protein (SAP) that exist in multiple forms (Rafi et al., 1990). These non-enzymatic co-factors aid arylsulfatase A in the lysosomal degradation of sphingolipids. Patients with defective prosaposin may have disease symptoms similar to juvenile MLD that present between 4 years of age and puberty, with gait disturbances and mental regression, while lysosomal hydrolase arylsulfatase A activity, which is affected in MLD is normal (Wrobe et al., 2000, Von Figura et al., 2001). Additional clinical symptoms can be blindness, loss of speech and seizures. Retro-viral re-introduction of the correct prosaposin gene in cultured cells of a variant MLD patient restored SAP-1 protein levels and metabolism of endocytosed sulfatide (Rafi et al., 1992). In a case report of a two year old patient that was treated with bone marrow transplantation and clinically followed for three years the patient initially transiently deteriorates, followed by improvement of peripheral nervous system functions, however, eventually the patient condition worsened, thus at present treatment of variant MLD is not possible (Landrieu et al., 1998).

3.3.2 G_{M2} gangliosidoses
The G_{M2} gangliosidoses are caused by defective degradation of ganglioside G_{M2}, leading to its accumulation (Gravel et al., 2001). This neurodegenerative disease is clinically variable: the most severe form, classical Tay-Sachs disease, presents with early developmental retardation, paralysis, dementia, and death usually in the second or third year of life. Other forms of this disease may display a protracted disease course of the neurological symptoms. Lysosomal G_{M2} ganglioside hydrolysis is modulated by β-hexosaminidase, which consists of two protein subunits encoded by the HEXA and HEXB genes (Neufeld, 1989). Another requirement for effective degradation is G_{M2} ganglioside binding to the G_{M2}/G_{M2} activator. Mutations in any of the genes encoding Hexosaminidase A (of HEXA), Hexosaminidase B, or the activator protein may lead to this disease. Treatment of this disease is not available, although the possibilities of enzyme replacement therapy, bone marrow transplantation, gene therapy and substrate deprivation therapy are currently being investigated.
3.4 Disorders of lysosomal enzyme localization, processing, and protection

3.4.1 I-cell disease and pseudo-Hurler polydystrophy
The mislocalization or misregulation of intact lysosomal enzymes may lead to impaired degradation of internalized compounds. I-cell disease (Mucolipidosis II, ML-II) and pseudo-Hurler polydystrophy (Mucolipidosis III, ML-III) are caused by abnormal transport of lysosomal enzymes due to defects in the GNPTAB gene encoding the N-acetylglucosamine-1-phosphotransferase alpha or beta subunit precursor, or GNPTG encoding the N-acetylglucosamine-1-phosphotransferase gamma subunit precursor, respectively (Kornfeld and Sly, 2001). After normal modification of lysosomal enzymes with M6P markers in the Golgi apparatus, these enzymes can become targeted to the lysosome by binding M6P-receptors. However, mutations in one of three genes encoding phosphotransferase complex subunits lead to a phosphotransferase that is catalytically active but unable to specifically recognize and bind its substrate, mannose residues of lysosomal enzymes. This results in mistargeting of lysosomal enzymes that can be found in the serum and body fluids of patients. Affected individuals present with progressive psychomotor retardation and premature death, usually in the first decade, although as in all LSDs clinical features may vary in age of onset and severity. Phase dense inclusions were detected in patient cells, which were called inclusion cells, hence the name I-cell disease. Although molecular diagnosis and carrier detection can be performed, definitive treatment is not available.

3.4.2 Multiple Sulfatase Deficiency
Affected sulfatase processing due to mutations in sulfatase-modifying factor-1, SUMF1 can result in a deficiency of all twelve known sulfatases (Dierks et al., 2003, Cosma et al., 2003). Sulfatases require post-translational generation of α-formylglycine residues, which presumably have a function in sulfate ester cleavage and this modification step is partially mediated by sulfatase-modifying factor-1. Incomplete formation of α-formylglycine residues on sulfatases was found to affect sulfatase processing. Multiple sulfatase deficiency clinically resembles Metachromatic Leukodystrophy, which is caused by affected enzymatic activity of arylsulfatase A or non-enzymatic saposin B. The clinical presentation and disease progression are variable for both disorders. Patients generally first present themselves with gait disturbance and mental regression. Subsequently, childhood variants usually display blindness, loss of speech, and seizures, whereas adult variants present themselves with behavioral disturbance and dementia. Diagnosis is performed biochemically and prenatal diagnosis and carrier detection is reliable. There is no treatment for patients suffering from this disease.

3.4.3 Galactosialidosis
Transport of lysosomal enzymes β-galactosidase and neuraminidase to the lysosome and protection against intralysosomal degradation is mediated by normal protective protein/cathepsin A (CTSA) that is associates with these enzymes. Additional
The ch-3 genes of Caenorhabditis elegans

CTSA functions comprise cathepsin A/deaminase/esterase activities on a subset of neuropeptides at both acidic and neutral pH. Mutations in the gene encoding CTSA were found to be involved with galactosialidosis, a lysosomal storage disorder in which sialyloligosaccharides accumulate in lysosomes and are excreted in body fluids (Zhou et al., 1996, d’Azzo et al., 2001). This disease is clinically heterogeneous, but most common features include dysmorphism, skeletal dysplasia, visceromegaly, cardiac and renal involvement, progressive neurologic manifestations, ocular abnormalities, angiokeratoma, and early death. Phenotypic variance and different age of onset distinguish three galactosialidosis subtypes, early infantile, late infantile, and juvenile/adult. Biochemical diagnosis, in patients and prenatal, is performed by demonstrating combined β-galactosidase/neuraminidase deficiency. Disease therapy is not present, but in Ctsa knockout mice systemic organ pathology can be fully corrected by bone marrow transplantation from mice overexpressing human CTSA in hematopoietic lineages (d’Azzo et al., 2001), possibly providing for treatment of patients in the future.

3.5 Disorders caused by aberrant substrate or product transport

3.5.1 Niemann-Pick disease type C
Niemann-Pick disease type C is caused by defects in cellular trafficking of cholesterol (Schuchman and Desnick, 2001). Mutations in the NPC1 gene, encoding a protein containing multiple transmembrane and a sterol-sensing domain, mostly cause progressive neurologic disease, occasionally accompanied by severely affected liver tissue and lethality. Age of onset and clinical presentation are highly variable. The diagnosis is based on the clinical presentations together with neurophysiological tests and tissue biopsies and can be confirmed by analyzing characteristic cholesterol staining patterns and measuring cholesterol esterification. Although symptomatic treatment of some of the accompanying clinical features is possible, disease progression can not be altered.

3.5.2 Nephropathic Cystinosis
Mutations in the CTNS gene, encoding cystinosin, which transports cystine out of lysosomes, leads to lysosomal accumulation of cystine in several tissues (Anikster et al., 1999). The most affected organs are the kidneys, which malfunction and continuously waste water and nutrients. As a result, a failure to thrive, polyuria, dehydration, hypophosphatemic rickets, hypokalemia, and acidosis are observed as the earliest symptoms, with other symptoms appearing later in life. Patients can be treated with cysteamine, which enters the lysosomes and converts cystine thereby permitting depletion of 95% of the cells cystine content. This treatment preferably is initiated as early as possible in life of the patient, in order to minimalize kidney damage and to achieve normal growth rates (Gahl, 2003).
3.5.3 Infantile sialic acid storage disorder (ISSD) and Salla disease
Impaired sialic acid transport was found to be the cause for infantile sialic acid storage disorder and Salla disease (Wreden et al., 2005). Both disorders share similar features, and differ in severity of clinical presentation. ISSD patients display intra-uterine hydrops, neonatal ascites, dysmorphic features, and death by 2 years of age. Salla disease is characterized by developmental delay with marked cognitive and motor impairment noticeable at 6–12 months of age, and patients usually reach adulthood. Mutations were identified in a gene, SLC17A5, encoding a transport protein that presumably acts as a symporter transporting protons and sialic acid (Verheijen et al., 1999). Therapy for this disease is not available.

3.6 Disorders caused by affected lysosome biogenesis

3.6.1 Hermansky-Pudlak syndrome
The Hermansky-Pudlak syndromes 1-7 (HPS 1-7) is a group of diseases with characteristic symptoms, including oculocutaneous albinism, loss of visual acuity, prolonged bleeding times due to platelet storage pool deficiency, storage of ceroid, and premature death caused by fibrotic lung disease (Huizing et al., 2001, Li et al., 2004). Mutations in different genes are associated with the various forms of the disease. The HPS1 and HPS4 genes, involved in Hermansky-Pudlak syndrome 1 and 4, respectively, encode subunits of a complex, termed BLOC-3 for biogenesis of lysosome-related organelles complex–3 (Dell’Angelica, 2004). HPS1 and HPS4 gene products were shown to interact, and together were suggested to regulate late-endosome and lysosome localization, with possibly an additional role in melanosome biogenesis (Nazarian et al., 2003). The HPS2 gene encodes a subunit of the adaptor protein 3 complex, AP3β1, which is involved with protein transport to lysosomes (Dell’Angelica et al., 1999). HPS3, HPS5, and HPS6 proteins were found to associate in the BLOC-2 complex (Di Pietro et al., 2004), and HPS7 protein was reported to be a member of the BLOC-1 complex (Li et al., 2004). It is obvious to assign a function in vesicular biogenesis to each of the BLOC complexes due to the cellular defects observed in tissues from patients and knock-out mice, harboring mutations in HPS gene orthologues. Among the affected organelles are melanosomes, lysosomes, and platelet dense granules, suggesting particular common aspects in their biogenesis or function (Spritz, 1999B). Patients suffering from this disease can not be treated at present.

3.6.2 Chediak-Higashi syndrome
Chediak-Higashi syndrome (CHS) resembles HPS in the oculocutaneous albinism and platelet storage pool deficiency (Huizing et al., 2001). However, CHS is distinct from HPS, and CHS patients also suffer from abnormally increased susceptibility to infections and a typical accelerated phase, in which patients develop a lymphoproliferative syndrome (Spritz, 1999A). Mutations in the LYST gene, lysosomal trafficking regulator, were found in CHS patients (Karim et al., 1997). The LYST protein may be involved in the sorting of endosomal resident proteins into
late multivesicular endosomes (Introne et al., 1999, Zarzour et al., 2005). Patients can be treated with stem cell therapy before the accelerated phase has been reached, decreasing their susceptibility to infections. Neurological complications, however, can not be reversed.

3.6.3 Mucolipidosis type IV
Mucolipidosis type IV (MLIV) clinically presents with psychomotor deterioration, ophthalmological abnormalities, and premature death. Lysosomal hydrolase activity and trafficking appeared intact for this lysosomal storage disorder (Chen et al., 1998). Markers for endocytosis were used to show that MLIV fibroblasts were affected in their rate of efflux from the lysosomes compared to wildtype cells, suggesting defects in late endosomal-lysosomal transport. In MLIV patients mutations in \textit{MCOLN1} were found as a cause for their disease (Bargal et al., 2000). The MCOLN1 protein, mucolipin-1, was shown to be a Ca$^{2+}$-permeable cation channel that is transiently modulated by changes in intracellular calcium (LaPlante et al., 2004). This suggests that calcium traffic and distribution in the cell is impaired, which could result in abnormal slow and inefficient late endosome-lysosome fusion in MLIV patients, leading to accumulation of lipids and other materials. A cure for this disease is not available at present.
The Neuronal Ceroid Lipofuscinoses

4.1 Historical description

The Neuronal Ceroid Lipofuscinoses originally belonged to a group of diseases collectively termed amaurotic familial idiocy (AFI) in 1896 (Rapola, 1993 and references therein). This cohort of diseases contained familial infantile neurological disorders that clinically presented with failure of psychomotor development, blindness, and early death. Although considerable clinical and pathological variance was present, this term has been used until far into the twentieth century and encompassed Tay-Sachs disease, a GM\textsubscript{2}-gangliosidosis, besides the NCLs. Despite the important contributions of Batten, Spielmeyer, and Vogt, who described several NCL families early in the twentieth century and at that time made distinctions between NCL and Tay-Sachs, the definitive separation occurred only after description of the ultrastructure of the storage material and identification of GM\textsubscript{2}-ganglioside. As the storage material in NCLs could be distinguished from stored materials found in other diseases and resembled ceroid and lipofuscin in staining characteristics, the term ‘Neuronal Ceroid Lipofuscinosis’ was introduced, obviously also bearing the neurological pathology in mind.

4.2 The different forms of NCL

The NCLs can be distinguished by age of onset of the first symptom, although for each form of NCL starting age can be variable, by ultrastructure of the stored material, and by causative gene (Goebel et al., 1999, Wisniewski et al., 2001b). An overview of the different forms of NCL and some of their characteristics is shown in Table 1 on the next page.

4.2.1 Infantile NCL, CLN1

Children suffering from the infantile form of NCL (INCL, Haltia-Santavuori disease) usually lose their eye-sight between six months and two years of age. Ultrastructural analysis, using electron micrographs, of INCL storage bodies revealed the so-called granular osmiophilic deposits (GRODs). INCL is caused by mutations in the \textit{CLN1} gene, encoding a lysosomal enzyme, palmitoyl protein thioesterase (PPT). Dysfunctional PPT causes accumulations mainly consisting of the very hydrophobic saposins A and D. In the other NCLs different ultrastructural storage patterns are observed and the main component of the stored material is Subunit c of the mitochondrial ATP synthase. The variability of the characteristic starting age of infantile NCL is demonstrated by the adult age of onset, 31 and 38 years, in two INCL patients that had mutations in the \textit{CLN1} gene and had decreased palmitoyl-protein thioesterase activity (Van Diggelen et al., 2001).
Table 1 An overview of the different NCL forms and some characteristics

<table>
<thead>
<tr>
<th>Disease type</th>
<th>Gene symbol</th>
<th>Eponym</th>
<th>Genomic location</th>
<th>OMIM</th>
<th>Protein</th>
<th>Age of onset</th>
<th>EM storage profile</th>
<th>Inheritance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infantile NCL</td>
<td>CLN1</td>
<td>Haltia-Santavuori</td>
<td>1p32</td>
<td>256730</td>
<td>Palmitoyl protein thioesterase I, lysosomal</td>
<td>0.1 - 38</td>
<td>GROD</td>
<td>autosomal recessive</td>
</tr>
<tr>
<td>Late infantile NCL</td>
<td>CLN2</td>
<td>Jansky-Bieleschowsky</td>
<td>11p15</td>
<td>204500</td>
<td>Tripeptidyl peptidase I, lysosomal</td>
<td>2 - 8</td>
<td>CV, mixed</td>
<td>autosomal recessive</td>
</tr>
<tr>
<td>Juvenile NCL</td>
<td>CLN3</td>
<td>Batten-Spielmeyer-Vogt</td>
<td>16p12</td>
<td>204200</td>
<td>CLN3 lysosomal transmembrane protein</td>
<td>4 - 10</td>
<td>FP, mixed</td>
<td>autosomal recessive</td>
</tr>
<tr>
<td>Adult NCL</td>
<td>CLN4</td>
<td>Kufs</td>
<td>not known</td>
<td>204300</td>
<td>not known</td>
<td>11 - 55</td>
<td>FP, granular</td>
<td>autosomal recessive (1)</td>
</tr>
<tr>
<td>Finnish variant late infantile NCL</td>
<td>CLN5</td>
<td></td>
<td>13q31-32</td>
<td>256731</td>
<td>CLN5 lysosomal membrane protein</td>
<td>4 - 7</td>
<td>FP, CV, RL</td>
<td>autosomal recessive</td>
</tr>
<tr>
<td>Variant late infantile, early juvenile NCL</td>
<td>CLN6</td>
<td>Lake-Cavanagh</td>
<td>15q21-23</td>
<td>601780</td>
<td>CLN6 endoplasmic reticulum membrane protein</td>
<td>1.5 - 8</td>
<td>CV, FP, RL</td>
<td>autosomal recessive</td>
</tr>
<tr>
<td>Turkish variant late infantile NCL</td>
<td>CLN7</td>
<td></td>
<td>not known</td>
<td>600143</td>
<td>not known</td>
<td>1 - 6</td>
<td>RL, FP</td>
<td>autosomal recessive (2)</td>
</tr>
<tr>
<td>Turkish variant late infantile NCL, Northern epilepsy</td>
<td>CLN8</td>
<td></td>
<td>8p23</td>
<td>600143</td>
<td>CLN8 endoplasmic reticulum membrane protein</td>
<td>5 - 10</td>
<td>CV or GROD like</td>
<td>autosomal recessive</td>
</tr>
<tr>
<td>CLN9 deficiency</td>
<td>CLN9</td>
<td></td>
<td>not known</td>
<td>609055</td>
<td>unknown protein involved in dihydroceramide synthase pathway</td>
<td>4</td>
<td>FP, CV, GROD</td>
<td>autosomal recessive (2)</td>
</tr>
<tr>
<td>Congenital NCL</td>
<td>CLN10</td>
<td></td>
<td>11p15.5</td>
<td>610127</td>
<td>Cathepsin D, lysosomal</td>
<td>0</td>
<td>GROD</td>
<td>autosomal recessive (2)</td>
</tr>
</tbody>
</table>

Abbreviations: GROD: granular osmiophilic deposits, CV: curvilinear, FP: fingerprint, RL: rectilinear

(1) The majority of Adult NCL is autosomal recessively inherited, some cases of autosomal dominant inheritance have been described as Parry disease

(2) Most probable mode of inheritance as shown with pedigree, segregation or linkage analysis
4.2.2 Late infantile NCL, CLN2
The late infantile form of NCL (LINCL, Janský-Bielschowsky disease) has an average age of onset of seizures, ataxia and myoclonus between two and four years of age. Classical LINCL is caused by mutations in the CLN2 gene, encoding the lysosomal enzyme tripeptidyl peptidase, and LINCL storage patterns mainly have curvilinear profiles. Three additional genetically distinct variant forms of LINCL are discussed below.

4.2.3 Adult NCL, CLN4
The adult form of NCL (ANCL, Kufs disease) is a rare form of NCL, for which the CLN4 gene has not yet been identified. The diagnosis of ANCL is difficult due to the relatively low frequency of the disease combined with an overlap of clinical features with other diseases. In addition, autosomal dominant and recessive patterns of inheritance have been observed. This suggests that genetic heterogeneity underlies the phenotypical variation observed. The average age at which epilepsy or behavioral changes occur is 30 years, but cases with starting ages from 11 until 60 years have also been described. Consistent with genetic heterogeneity, the storage patterns in ANCL are also variable: all storage profiles observed in other types of NCL can be found in ANCL patient tissues.

4.2.4 Finnish variant late infantile NCL, CLN5
Three other variant forms of LINCL could be reclassified due to mapping to alternative loci or alternative disease characteristics. The first form, Finnish variant late infantile NCL (Finnish LINCL), is caused by mutations in the CLN5 gene, which encodes a transmembrane protein with unknown function (Savukoski et al., 1998). This variant form of NCL originally was described in patients from Finland and later also found in other countries (Santavuori et al., 1991, Pineda-Trujillo et al., 2005). The first symptoms, slight motor clumsiness and muscular hypotonia, become apparent between 4.5 and 6 years of age. Multiple storage pattern profiles are also found when performing ultrastructural analysis on tissues of Finnish LINCL patients.

4.2.5 Variant late infantile-early juvenile NCL, CLN6
Variant late infantile-early juvenile NCL patient tissues shared features of late infantile and juvenile NCLs, and could only be distinguished from other NCLs when the causative gene, CLN6, was mapped. Recently, the CLN6 gene has been cloned and was found to encode a transmembrane protein (Gao et al., 2002). Patients suffer from motor delay and seizures starting between 18 months and 8 years of age, and just less than half of the patients clinically appear similar to classic LINCL, although distinguishable using electron microscopy, since a mixture of fingerprint, curvilinear, and rectilinear profiles is present in patient lysosomes. This variant late infantile form differs subtly from classical late infantile NCL, in that the storage material also was present as fingerprint profiles, in addition to the curvilinear profiles predominantly found in classical LINCL (Williams, et al., 1999).
4.2.6 Turkish variant late infantile NCL, CLN7 - Northern Epilepsy, CLN8

Turkish variant late infantile NCL and Northern Epilepsy or progressive epilepsy with mental retardation (EPMR) initially were thought to be genetically distinct disorders, due to their predominance in different populations, Turkish and Finnish, respectively. However, mutations in CLN8, the gene involved in Northern Epilepsy, were also found in Turkish variant LINCL patients, although not all patients were homozygous for the used marker alleles (Ranta et al., 2004, Ranta et al., 1999). Clinically, both diseases can clearly be distinguished: Turkish variant LINCL patients display typical NCL symptoms, starting between one and six years of age with seizures, motor impairment, mental retardation and loss of vision. Storage patterns are either fingerprint or curvilinear-fingerprint mixture profiles in the Turkish variant. Northern Epilepsy, on the other hand, presents between five and ten years of age with seizures and mental retardation, while loss of vision is absent and clinical progression is slower. Mainly curvilinear storage patterns are detected by ultrastructural analysis. In the two distinct populations different mutations were found in the same gene (Ranta et al., 2004). A clear genotype-phenotype correlation, however, could not be made, and the Turkish patients with CLN8 mutations could clinically not be distinguished from Turkish LINCL patients without them. A subgroup of the latter patients is assumed to be linked to the unidentified CLN7 locus.

4.2.7 CLN9 deficiency, CLN9

The initial characterization of a small group of patients with typical NCL disease characteristics but without mutations in any of the earlier found NCL genes or in any of other lysosomal storage disease gene suggested the existence of another variant of juvenile NCL (Schulz et al., 2004). Cultured patient cells used to analyse the defect underlying CLN9 had a decreased dihydroceramide synthase activity, which could be partially rescued by introducing other genes that increase the activity of the dihydroceramide synthase pathway (Schulz et al., 2006). These results indicate that the CLN9 protein may be involved in the dihydroceramide synthase pathway, although the gene causing this disease has not been identified.

4.2.8 Congenital NCL, CTSD

The gene involved in congenital ovine NCL was identified more than five years ago as the sheep CTSD gene encoding Cathepsin D (Tyynela et al., 2000). Its human counterpart, congenital NCL, is the very rare most severe form of the NCL and has been characterized genetically with CTSD mutations just recently (Siintola et al., 2006). Congenital NCL patients clinically present with respiratory insufficiency, epileptic seizures and death occurs within hours to weeks after birth. The brain of patients is extremely small and show severe neuronal loss and accumulation of autofluorescent material that has granular osmiophilic nature at ultrastructural level. The severity of the pathology of congenital NCL depends on the mutation in the CTSD gene. In an individual in which residual cathepsin D activity could be measured the disease progressed slower than in patients without cathepsin D activity (Steinfeld et al., 2006).
How mutations in the cathepsin D gene result in this pathology on a molecular level is unknown but the involvement of cathepsin D in NCL emphasizes the study of the naturally occurring sheep NCL and the artificially generated cathepsin D mutants in *Drosophila melanogaster* and *Mus musculus*, which allow for detailed investigation of the molecular mechanisms that underlie this disease.

### 4.3 Investigation of CLN protein function in cultured cells and model organisms

#### 4.3.1 Infantile NCL, palmitoyl protein thioesterase

To understand how a reduced palmitoyl protein thioesterase (PPT) activity results in the clinical manifestation of INCL we need to know the normal function of the PPT protein. The presumed function of the PPT hydrolytic enzyme is to catalyze the cleavage of thioester bonds between target protein cysteine residues and their palmitoyl fatty acid side groups (Lu and Hofmann, 2006). The enzyme was found to facilitate the removal of palmitate from H-Ras *in vitro*, and may perform this activity on its natural substrates in the lysosome (Camp *et al.*, 1994, Kim *et al.*, 2006). The PPT protein localizes to lysosomes in non-neuronal cells, and enzyme activity could be measured at acidic pH, with the optimal activity at approximately pH 4 (Voznyi *et al.*, 1999), although activity at neutral pH was also reported (Verkruyse and Hofmann, 1996). PPT may also have an extracellular function, as the protein was found to be secreted (Camp *et al.*, 1994, Verkruyse and Hofmann, 1996). Since the disease manifests itself primarily in the nervous system, PPT may have a neuron-specific function. In neuronal cells, the enzyme is found in axons and presynaptic region localized to synaptic vesicles and synaptosomes (Ahtiainen *et al.*, 2003). In the brain, the expression is developmentally regulated, suggesting that PPT is involved in maturation and growth of neural networks (Isosomppi *et al.*, 1999). In addition, PPT may be involved in apoptosis, since PPT was found to exert an anti-apoptotic effect (Cho and Dawson, 2000). A second *PPT* gene, *PPT2*, was also detected in humans, but it was shown to encode a lysosomal thioesterase with a substrate specificity that was different from PPT1 (Soyombo and Hofmann, 1997). Furthermore, PPT2 could not complement the metabolic defect of PPT1 deficient cells. Despite these advances, our knowledge of the affected molecular mechanisms underlying INCL is limited. Therefore additional investigations in model organisms are required to elucidate the INCL etiology at the molecular level.

#### 4.3.2 INCL mouse models

Several INCL mouse models are available to investigate PPT protein function and INCL pathogenesis. Knock-out strains of the murine *Ppt1* gene encoding the lysosomal palmitoyl protein thioesterase were generated (Gupta *et al.*, 2001, Jalanko *et al.*, 2005). The *Ppt1*<sup>−/−</sup> knock-out was shown to result in viable and fertile mice that developed spasticity. The *Ppt1*<sup>−/−</sup> mice showed progressive pathology, presenting as motor abnormalities and premature death at 10 months of age. Furthermore, in 6 months old *Ppt1*<sup>−/−</sup> mice autofluorescence levels were increased compared to 10 months old
wildtype mice, and granular osmiophilic deposits (GRODs) were easily identified in Ppt1\(^{/-}\) neurons while absent from wildtype mice. Neuronal degeneration was obvious in sections from 6 months old Ppt1\(^{/-}\) mice, of which apoptosis was suggested to be the underlying mechanism. Thus, the Ppt1\(^{/-}\) mouse represents an INCL model that displays several hallmarks of the disease and will provide a substrate for testing therapy (Gupta et al., 2001). In addition, a mouse knock-out for Ppt2 was generated. Mice, in which this lysosomal palmitoyl protein thioesterase activity was disrupted, were viable and fertile and spastic. Compared to the Ppt1 knock-out, the Ppt2 \((-/-)\) mices displayed no other motor abnormalities and only slightly decreased survival, while autofluorescence levels were normal and GRODs were absent (Gupta et al., 2001).

In another murine INCL model, Ppt1\(^{\text{lox/lox}}\), exon 4 of Ppt1 was eliminated, resulting in a frame-shift and premature stop codon in exon 3. This mutation deletes the active site of the Ppt1 enzyme and resembles the most common Ppt1 mutation (Jalanko et al., 2005). These mice were viable, fertile and gross morphology was normal. In the Ppt1\(^{\text{lox/lox}}\) mice loss of vision was demonstrated to be significant at 14 weeks of age, the disease gradually progressed into seizures at four months and myoclonic jerks at six months of age, and the average age of death was 6.5 months, at which the brain was shown to be severely decreased in weight. In homozygous Ppt1\(^{\text{lox/lox}}\) mutants no Ppt1 enzyme activity could be detected, and increased autofluorescence and GRODs were observed similar to the other INCL mouse model and human patients. Furthermore, in Ppt1\(^{\text{lox/lox}}\) mutant mice prominent neuronal loss could be demonstrated. Expression profiling experiments performed on cerebra of 6 months old Ppt1\(^{\text{lox/lox}}\) mutants indicate involvement of the immune system in neuronal degeneration. Inflammation was also reported in the Cln3 knockout mouse and in other neurodegenerative diseases, such as Alzheimer’s, and Parkinson’s disease. Although these INCL mouse models display most of the disease characteristics, they may also be too complicated to unravel the molecular mechanisms that underlie the pathogenesis. Thus simpler model organisms, such as flies and worms, may be required to achieve this.

4.3.3 PPT1 genes of Drosophila melanogaster and Caenorhabditis elegans

PPT1 protein homologues were identified in the invertebrate fruitfly and nematode, to establish relatively simple models, in which molecular mechanisms underlying INCL could be elucidated. The homologous proteins were shown to exhibit an enzymatic activity that could be measured with the same assay as human PPT1 activity, indicating protein function conservation (Glaser et al., 2003, Porter et al., 2005, Van Diggelen et al., 1999). Although Drosophila Ppt1 knockouts are being generated, gene and protein function were mainly investigated in flies that overexpress Ppt1 in the developing visual system (Korey and MacDonald, 2003). Overexpression of Ppt1 leads to neuronal degeneration, manifested as black omatidia in the Drosophila eye presumably due to apoptosis. Identification of genetic modifiers of the observed phenotypes are expected to facilitate the unraveling of the pathways in which Ppt1 is involved.

In C. elegans, ppt-1 knockouts were isolated and characterized (Porter et al., 2005). Nematode ppt-1 mutants displayed developmental and reproductive phenotypes:
Making C. elegans models for Juvenile Neuronal Ceroid Lipofuscinosis

development was delayed for four hours compared to wildtype, the number of embryos contained within the gonad consistently was higher compared to wildtype. The actual life span was similar to wildtype, but the animals were less motile and appeared aged earlier than wildtype. At the ultrastructural level, ppt-1 nematodes were shown to contain abnormal mitochondria, already at day one of the adult life stage, indicating PPT1 has an influence on mitochondrial integrity. Further investigation of these models for INCL will be valuable to reveal the affected molecular mechanisms that cause this disease.

4.3.4 Late infantile NCL, tripeptidyl peptidase 1

The lysosomal serine protease, tripeptidyl peptidase 1 (TPP1), which when affected leads to late infantile NCL (LINCL) cleaves tripeptides of the amino terminus of small proteins (Lin et al., 2001,Sleat et al., 1997, Bernardini and Warburton, 2001). Although the natural substrates of TPP1 have not been identified, its enzymatic activity can be assayed in vitro (Wisniewski et al., 2001). The enzyme appeared to have an acidic pH optimum and was found to be localized to lysosomes. To investigate the function of TPP1 and how changed function may lead to LINCL, a Cln2−/− mouse model was generated (Sleat et al., 2004). This mouse model recapitulates the phenotype of the disease in humans, starting with body tremors that progress in severity throughout the life of the animal. Furthermore, Cln2−/− mice present with locomotor difficulties, including what appear to be epileptic seizures at end stage of the disease, and lifespan was considerably shorter than wildtype animals. In addition, fluorescent storage material filled the cytoplasm of cells of most brain regions in mutant mice of 100 days or older. In the moderately atrophic brain of Cln2−/− mutant mice, disruption of myelin was observed and neurons contained curvilinear storage bodies, a hallmark of LINCL in humans. Autofluorescent storage material was also observed outside of the brain. Interestingly, the CLN2−/− mouse has been used to test the therapeutic efficacy of injection of adeno-associated virus (AAV) vectors carrying Cln2, as a cure for this disease in mice (Passini et al., 2006). With this gene transfer a significant decrease in storage material was achieved, but the effects on neurodegeneration and behavior are unclear.

4.3.5 Finnish variant late infantile NCL, CLN5

The function of the CLN5 protein is still unknown. In cultured human cells the CLN5 protein was found to be glycosylated and localizes to lysosomes, although a cytoplasmic soluble form of this protein was found as well. The CLN5 protein with the most common mutation identified in Finnish variant late infantile NCL patients did not localize correctly to lysosomes (Isosomppi et al., 2002). Using co-immunoprecipitation studies, the CLN5 protein was found to interact with the CLN2 and CLN3 proteins, while disease causing mutations in CLN5 disrupted the interaction with CLN2 but not with CLN3 (Vesa et al., 2002). These interactions were the first indications that CLN proteins interconnect at the molecular level, suggestive of a common pathogenic pathway underlying these diseases. Strengthening this hypothesis is the observation that in fibroblasts from human patients with mutations in CLN5,
and TPP1, the CLN3 mRNA levels were decreased to 65% and 73% of wildtype mRNA levels, respectively (Bessa et al., 2006). A Cln5 mouse model was generated with a targeted deletion of exon 3 (Kopra et al., 2004). The progressive pathology of these mice resembles the pathology observed in human patients, with severe loss of vision occurring on average at week 21, prominent accumulation of autofluorescent material in the brain of six months old mutant mice. The accumulated material contained curvilinear and fingerprint profiles. Moreover, microarray expression analysis of cerebra of Cln5−/− mice indicated differential expression of genes involved in inflammatory processes, neuronal degeneration, and encoding myelin components, which is in agreement with the hypomyelination and the other neuronal problems observed in this model. The expression profile of Cln5 mutant cerebra appears to display parallels to expression profiles of aging brain in mice, which suggests that premature aging may play a role in the pathogenesis of Finnish variant late infantile NCL.

4.3.6 Late infantile variant NCL, CLN6
The CLN6 protein was predicted to have seven transmembrane domains and localized to the endoplasmic reticulum (Wheeler et al., 2002, Gao et al., 2002, Mole et al., 2004). The same localization was observed with proteins harboring vLINCL disease causing mutations. Investigation of gene expression profiles of CLN6-deficient cultured fibroblasts has lead to the observation that cholesterol homeostasis is disrupted in patient cell lines. Additional problems with extracellular matrix remodelling, signalling cascades and vesicular traffic are likely to underlie vLINCL disease pathogenesis (Teixeira et al., 2006). Furthermore, overexpression of genes involved in apoptosis and inflammatory response was also observed, presumably reflecting cellular protection against stress and degeneration, and secondary inflammatory responses in cellular degeneration, respectively. Naturally occurring NCL disease models that are caused by mutations in genes homologous to CLN6 are present in mice and sheep (Tyynela et al., 2000, Wheeler et al., 2002, Gao et al., 2002). The nclf mice that were shown to have mutations in the murine homologue of CLN6, developed rear limb paresis after 8 months, which progressed into paralysis over several months and subsequent death (Bronson et al., 1998, Wheeler et al., 2002, Gao et al., 2002). These mice also suffered from retinal degeneration present in four months old nclf mice and neuronal degeneration in six months old animals. In nclf mice as young as 11 days accumulated material could already be observed in many different kinds of cells. The naturally occurring ovine NCL, is caused by mutations in a gene that was mapped to a region on sheep chromosome 7 that is syntenic to the human 15q21-23 region that contains CLN6 (Broom and Zhou, 2001, Tammen et al., 2001, Oswald et al., 2005). In these sheep brain mass started to decrease at 4 months of age compared to healthy age matched controls and by 12 months atrophy of cortical brain regions was macroscopically visible. Autofluorescent storage materials were present as early as 12 days in the cortical regions of the brain, and immunoreactivity of the Subunit c of the mitochondrial ATP synthase antibody was increased in the parieto-occipital cortex of brains of affected
sheep (Oswald et al., 2005). Both the murine and ovine animal models closely resemble the vLINCL in humans and are being used to investigate the molecular mechanisms underlying the disorder.

4.3.7 Turkish variant late infantile NCL and Northern Epilepsy, CLN8

The function of the CLN8 protein is still unknown. The CLN8 protein was predicted to be a transmembrane protein of 286 amino acids. This protein localized to ER membrane and ER Golgi intermediate compartment (ERGIC) when CLN8 cDNA was overexpressed in cultured cells and to ER in neurons, and this localization was not aborted when a disease causing mutations was introduced in the coding region (Lonka et al., 2004, Lonka et al., 2000). The mouse model for motor neuron degeneration (mnd) was the first described naturally occurring NCL disease model and it was caused by a mutation in the Cln8 gene (Bronson et al., 1993, Ranta et al., 1999). The murine Cln8 gene was shown to be ubiquitously expressed and its transcript could be identified throughout development of the mouse. The expression of the Cln8 gene was shown to be increased in mice that were electrically stimulated to induce epileptic seizures (Lonka et al., 2005). How a defect in the CLN8 protein may lead to this disease still has to be elucidated.

4.3.8 Congenital NCL, CTSD

The Cathepsin D protein is a lysosomal aspartic protease that belongs to the family of pepsin proteases and is involved in congenital NCL. The protein plays a role in several processes including: proliferation of cancer cells, apoptosis, inflammatory responses and regenerative processes. This peptidase is highly active and prominently present in the lysosomal compartment. The functions of the cathepsin D protein are conserved between species as diverse as humans, sheep, dogs, mice and fruitflies, since disruption of the cathepsin D gene leads to neurodegeneration and storage of autofluorescent materials in all of these organisms (Steinfeld et al., 2006, Myllykangas et al., 2005). Investigation of the Cathepsin D protein in these models may provide further insight into the etiology of Congenital NCL.
5 JNCL Disease Models

5.1 Mouse models for JNCL

For many lysosomal storage diseases that are caused by enzyme defects, a therapeutic strategy may be provided by gene transfer or enzyme replacement therapy. JNCL, however, is caused by mutations in a gene encoding a transmembrane protein of unknown function. Disease models in small vertebrates are imperative in the research of the molecular mechanisms underlying JNCL to minutely monitor the successive stages of disease progression and to develop and test therapeutic strategies (reviewed in Cooper et al., 2006). Furthermore, the molecular basis of the affected processes can be studied on a cellular level in cultured cells derived from these animals.

To investigate JNCL etiology in mice, three murine models were generated. Two Cln3 knock-out mouse models, exon 1-6 deletion mice and exon 7-8 deletion mice, were made by inserting a resistance cassette in the murine Cln3 gene, replacing exons 1 to 6 and 7 to 8, respectively (Mitchison et al., 1999, Katz et al., 1999). The third model was a knock-in model generated by deleting exons 7 and 8 using the Cre-loxP technology. This deletion mimics the most common mutation, which is the 1.02 kb deletion, found in 85% of the human JNCL patients (Cotman et al., 2002). These three models all display storage of autofluorescent material and neurodegeneration, thereby recapitulating important JNCL characteristics. Which mouse mutants eventually will be the most reliable model for JNCL is not yet clear. The models were generated in different genetic backgrounds, making the models difficult to compare. Each model needs to be backcrossed into the same genetic background before the mutants can be compared (Cooper et al., 2006). However, clinical symptoms in these mutant mice appear milder than the effects of human CLN3 mutations, e.g. the exon 1-6 deletion mice did not develop obvious neurological defects at 12 months of age (Mitchison et al., 1999). The pathologic process may require a longer time interval to produce detectable symptoms or it may affect neurons of which the functionality is not readily and reliably testable in mice. JNCL mouse models have been used to investigate differential gene expression and cell lines derived from these mutants can be cultured and studied to examine effects of Cln3 mutations at the cellular level.

Gene expression profiling of cerebella of exon 1-6 deletion mice showed altered expression levels of genes involved in neurotransmission, neuronal cell structure, development, immune response, lipid metabolism, and inflammation (Brooks et al., 2003). From this study, in which 10 week old Cln3 knock-out mice were compared with age-matched wild-types, it becomes clear that cerebellar expression levels are already changed before evidence of neurodegeneration can be observed. In another study, gene expression in whole brains of the same mouse model was compared with gene expression of whole brains of Cln1 knockout mice and wild-type controls.
Making C. elegans models for Juvenile Neuronal Ceroid Lipofuscinosis (Elshatory et al., 2003). The 10 week old Cln3 exon 1-6 deletion mice used in this study showed differential expression of genes involved in neurotransmitter metabolism compared to wild type gene expression levels. When the expression levels of the Cln3 mutants are compared with the Cln1 mutants, the absence of overlap in differential expression is striking. The most downregulated gene in Cln1 knock-out mice is the most upregulated gene in Cln3 knock-out mice. This suggests that the effects of the Cln3 and Cln1 mutations are fundamentally different, and while caused by distinct molecular mechanisms the mutations lead to similar pathologies. Since loss of vision is the first symptom in human JNCL patients, gene expression levels present in cells of the eye of Cln3 exon 1-6 deletion mice were determined and compared to wild type mice and with the cerebellar data set (Chattopadhyay et al., 2004). In the eye, 18 genes were shown to be differentially regulated compared to cerebellar expression levels. Three of the downregulated genes, Cytochrome oxidase I, Cytochrome B, ATP synthase subunit B, are involved in energy production in mitochondria. This is an interesting observation, since Subunit c of the mitochondrial ATP synthase is the major component of the storage material and mitochondrial dysfunction has been suggested to underlie cell death in the eye. Moreover, decreased activity of mitochondrial ATP synthase was reported previously (Palmer et al., 1992, Jolly et al., 2002, Das and Kohlschutter, 1996). In addition to the expression profiling of cerebellum, brain and retina, cultured neuronal precursor cells obtained from Cln3 knock-in mice were investigated. These cells resemble JNCL patient neurons in that Subunit c of the mitochondrial ATP synthase is accumulated (Fossale et al., 2004). Lysosomal enzyme processing was also affected and lysosomes appeared smaller and labeled less intensely with Lysotracker Red. Additional phenotypes of these Cln3 mutant cells in comparison to wild type cells encompassed decreased uptake of fluid phase markers, abnormally elongated mitochondria, and decreased ATP levels. Interestingly, autophagy and lysosomal trafficking were shown to be affected, possibly due to defective mitochondrial turnover, indicating that altered organelar maintenance may play a role in JNCL pathology (Cao et al., 2006). Expression analysis of these Cln3 mutant cells indicated differential expression of genes involved in three pathways or structural elements: glucose metabolism, cytoskeleton, and synaptosomes (Luiro et al., 2006). Mitochondrial function was slightly affected in these cells, consistent with their changed ultrastructural morphology. Synaptosomes were suggested to be abnormal as demonstrated by a delay in neuronal depolarization. These mitochondrial and synaptosomal defects may underly primary cellular events that together with defective cytoskeletal components could lead to JNCL. Although the investigations of these mouse models has provided additional insight in disease progression and affected molecular mechanisms, much is still unresolved and may require other experimental models to elucidate the full functional spectrum of the Cln3 protein.
5.2 Lower eukaryotic model organisms

Since studying molecular processes underlying JNCL pathology in complicated vertebrate disease models is a difficult task and the results may be difficult to interpret, detailed molecular analysis of the affected protein function may be more straightforward in less complex model organisms. Although JNCL is a human neurodegenerative disorder, CLN3 protein sequence comparison has lead to the identification of CLN3 homologues with high amino acid sequence similarity in

![Figure 3 Human CLN3 protein sequence aligned with corresponding model organism homologous](image)

Sequences were aligned using the multalin program. In the top sequence alignment the human sequence is compared with the three C. elegans (Ce) homologues sequences and in the bottom alignment with the Saccharomyces cerevisiae (Sc) and Schizosaccharomyces pombe (Sp) homologous sequences. Conserved amino acid residues are shown in black boxes, neutral substitutions in grey boxes.
Saccharomyces cerevisiae, Schizosaccharomyces pombe, and Caenorhabditis elegans. This conservation suggests that CLN3 protein function is partially conserved (Figure 3) (Taschner et al., 1997, Corpet, 1988). Thus functional analysis might be performed in small model organisms with their powerful genetic and biochemical toolset. Even model organisms without a nervous system, e.g. yeast, have proven to be invaluable to characterize many human disorders. The data obtained with the smaller model organisms may then be extrapolated to vertebrate disease models to unravel details about possible additional functional aspects in a more complex environment.

Part of the following section, of which de Voer and Taschner were co-authors, was derived from: Phillips SN, Muzaffar N, Codlin S, Korey CA, Taschner PEM, de Voer G, Mole SE, Pearce DA. 2006. Characterizing pathogenic processes in Batten disease: Use of small eukaryotic model systems. Biochim Biophys Acta. 1762:906-19.

5.2.1 The yeast Saccharomyces cerevisiae JNCL model

*S. cerevisiae* is used to study the juvenile form of NCL in which *CLN3*, encoding a yet uncharacterized protein, is mutated (IBDC, 1995). The homolog to CLN3 is called Btn1p in the budding yeast *S. cerevisiae*. Btn1p, also designated Yhc3p, is 39% identical and 59% similar in amino acid sequence to human CLN3 (Figure 3) (Mitchison et al., 1997). Similar to CLN3, Btn1p is a 46 kD integral membrane protein with several predicted phosphorylation, myristoylation, glycosylation, and farnesylation sites (Taschner et al., 1997, Golabek et al., 1999, Jarvela et al., 1998, Ezaki et al., 2003, Michalewski et al., 1999, Pullarkat and Morris, 1997, Kaczmarski et al., 1999). Importantly, the residues that are mutated in Batten disease patients are conserved in Btn1p, suggesting that the primary activity of the protein is conserved. Furthermore, Btn1p and CLN3 are functional homologs, because plasmid-derived CLN3 can complement for the absence of Btn1p in the *BTN1* deletion strain, *btn1-l*, confirming that the primordial function of CLN3 is conserved in yeast (Kim et al., 2003, Pearce and Sherman, 1998). This property of the yeast model potentially offers a powerful tool to test the functionality of CLN3 and Btn1p constructs. For example, it is unclear if GFP fusions of CLN3 are functional (Haskell et al., 1999), and a simple complementation assay in the yeast model could test this. Moreover, the yeast model has been used to test the ability of *CLN3* point mutants to complement *BTN1*, leading to the conclusion that disease severity correlated with the degree of complementation (Pearce and Sherman, 1998, Haskell et al., 2000). Human CLN3 has been localized to the lysosome in various studies and cell types (Golabek et al., 1999, Kytälä et al., 2004, and reviewed in Phillips et al., 2005, Pearce 2000). Similarly, Btn1p has been localized to the vacuole, the structure analogous to the higher eukaryotic lysosome, further emphasizing the relevance of the yeast model (Croopnick et al., 1998, Pearce et al., 1999b). To date, Btn1p has been implicated in three main cellular processes, two of which have been validated in mammalian models. Although these processes partially overlap, Btn1p has been linked to regulation of cellular pH, basic amino acid homeostasis, and nitric oxide production (Pearce et al., 1999b, Chattopadhyay et al., 2003, Pearce et al., 1999c, Pearce et al., 1999a).
5.2.2 Btn1p and the regulation of cellular pH

Using pH sensitive dyes, Pearce and colleagues first reported that the vacuolar pH in btn1-Δ cells was decreased compared to BTN1+ vacuoles at early growth and continued to increase throughout log and stationary phases (Pearce et al., 1999b). More recently, it has been shown that as btn1-Δ cells grow, vacuolar pH will rise above normal (Padilla-Lopez and Pearce, 2006). Moreover, vacuolar pH in both normal and btn1-Δ strains was shown to be altered by extracellular pH. In btn1-Δ cells, the activity of the plasma membrane H+-ATPase is increased, likely acting to buffer altered vacuolar pH. This led to the discovery of a plate phenotype for the btn1-Δ strain, which due to the increased plasma membrane H+-ATPase activity has an elevated rate of media acidification. This elevated media acidification allows btn1-Δ cells to grow in the presence of D(-)-threo-2-amino-1-[p-nitrophenyl]-1,3-propanediol (ANP), since the increased acidity of the medium renders ANP non-toxic to btn1-Δ cells, whereas ANP is toxic to BTN1+ cells (Pearce and Sherman, 1998, Pearce et al., 1999a). In addition, when the vacuolar pH of btn1-Δ cells was artificially increased using chloroquine, the activity of the plasma membrane ATPase decreased strengthening the link between external and vacuolar pH (Pearce et al., 1999b, Pearce et al., 1999c). The pH and ANP results are in contrast to the Schizosaccharomyces pombe model, where an increased vacuolar pH and sensitivity to ANP are observed in the deletion (Gachet et al., 2005)(see below). Subsequent follow up studies on the pH of lysosomes from human fibroblasts indicated that pH was slightly elevated in JNCL, suggesting that defects in human CLN3 also result in a disruption in the regulation of this organelle’s pH (Pearce and Sherman, 1998, Golabek et al., 2000). However, limitations in what can be explored in cell culture means that it is not feasible to explore the possibility of a correlation between the dynamic change from lower to higher pH of the lysosome and the disease. Nevertheless, it is clear that the processes that govern vacuolar/lysosomal pH are disrupted in the absence of a functional Btn1p/CLN3 ultimately leading to an elevated pH of this organelle.

5.2.3 A possible role of Btn1p and CLN3 in amino acid homeostasis

Besides altered vacuolar pH, alterations in arginine levels in btn1-Δ cells have been observed (Kim et al., 2003). Both cytoplasmic and vacuolar levels of arginine and lysine and ATP-dependent vacuolar arginine uptake are significantly decreased in btn1-Δ (Kim et al., 2003). This is interesting, since the vacuole acts as a storage organelle for sequestration of basic amino acids (Kitamoto et al., 1988, Wiemken and Durr, 1974). Like the pH defects, vacuolar arginine transport returns to normal when human CLN3 is expressed in btn1-Δ. Moreover, lysosomes isolated from JNCL patient human lymphoblast cell lines demonstrate decreased arginine transport (Kim et al., 2003, Ramirez-Montealegre and Pearce, 2005). The role of Btn1p in both regulating vacuolar pH and arginine transport has recently been clarified: the coupling of proton pumping and the activity of the vATPase was found to depend on extracellular pH (Padilla-Lopez and Pearce, 2006). Importantly, the btn1-Δ mutation results in an alteration in the coupling of proton pumping and the activity of the vATPase. Thus, defective arginine transport in btn1-Δ could result from an alteration
in the regulation of the electrochemical gradient driving this transport (Padilla-Lopez and Pearce, 2006). Interestingly, subsequent studies aimed at dissecting out whether alterations in intracellular arginine impact cells lacking Btn1p (btn1-Δ) have revealed that overexpression of Can1p, the plasma membrane basic amino acid transporter, is lethal (Regenberg et al., 1999, Phillips et al., 2006). It is tempting to speculate that the decrease in intracellular arginine and lysine levels may result from a buffering mechanism against arginine and lysine being toxic to cells lacking Btn1p (Regenberg et al., 1999, Phillips et al., 2006). Alternatively, it should be noted that BTN1+ cells with either endogenous or overexpressed levels of Can1p have similar rates of plasma membrane arginine uptake, suggesting that btn1-Δ cells may lack the ability to regulate the amount or activity of Can1p at the plasma membrane, resulting in arginine and lysine toxicity (Phillips et al., 2006). It is pertinent to point out that as amino acid levels, and possibly metabolism, are clearly affected by {\textit{EWQ}} cells have identical amino acid growth requirements to that of wild type to avoid studying artifacts of altered amino acid metabolism.

5.2.4 Btn1p involved in nitric oxide synthesis
A recent study has highlighted a third pathway affected by the absence of Btn1p, namely nitric oxide (NO) synthesis (Osorio et al., 2006). As arginine serves as the substrate for NO synthesis, this is also likely linked to the aforementioned alterations in arginine levels. Specifically, Osorio and colleagues demonstrated that {\textit{EWQ}} cells are more resistant to menadione due to defective synthesis of NO and consequent decreased levels of reactive oxygen and nitrogen species. If {\textit{EWQ}} cells are preincubated with arginine before menadione exposure, the phenotype is lost, suggesting that the decrease in nitric oxide results from the decrease in cellular arginine (Osorio et al., 2006). Although this phenotype may be the consequence of the primary defect associated to lack of Btn1p, it could also underlie the pathophysiological aspects of the disease. It will be important to recapitulate these observations in human cell lines.

5.2.5 Btn1p has a genetic interaction with Btn2p
Microarray analysis of {\textit{btn1-Δ}} strains revealed that BTN2 mRNA levels were increased in a {\textit{btn1-Δ}} background (Pearce et al., 1999b). Btn2p interacts with Yif1p, Rhb1p, and Ist2p, with deletion of BTN2 resulting in an altered localization of these proteins (Chattopadhyay et al., 2003, Kim et al., 2005, Chattopadhyay and Pearce, 2002). Yif1p is involved in ER to Golgi transport, Rhb1p is a small GTPase that has been implicated in plasma membrane arginine transport regulation, and Ist2p is a putative ion channel at the plasma membrane (Matern et al., 2000, Urano et al., 2000, Entian et al., 1999, Mannhaupt et al., 1994). Taken together, these observations would suggest that Btn2p is involved in trafficking. Btn2p does not have a true mammalian homolog, but as a cytosolic coiled-coil protein it shows very specific domain similarity to the higher eukaryotic protein Hook1. Hook1 is a microtubule binding protein involved in trafficking to the late endosome, multivesicular body formation, and endosomal
fusion (Kramer and Phistry, 1996, Kramer and Phistry, 1999, Richardson et al., 2004, Sunio et al., 1999, Walenta et al., 2001). Upregulation of Btn2p may be explained by the pH alterations, since decreases in vATPase activity can have an effect on protein trafficking and degradation (Mellman, 1992, Mellman et al., 1986, Trombetta et al., 2003, Nishi and Forgac, 2002, Hurtado-Lorenzo et al., 2006, Sun-Wada et al., 2003, Sun-Wada et al., 2004). Therefore, upregulation of Btn2p may result in a compensatory response to minimize disruptions in protein trafficking in btn1-Δ cells due to altered intracellular pH.

In summary, it appears that the primary defect in btn1-Δ involves a disrupted regulation of vacuolar pH, suggesting that Btn1p functions in a pH regulatory pathway. However, at this point a direct role for Btn1p in arginine homeostasis, nitric oxide production or protein trafficking cannot be ruled out. It is important to note that studies thus far have focused on determining the role for Btn1p through the use of btn1-Δ cells that lack Btn1p and are therefore based on a loss-of-function model, in which cells might be altering these pathways to compensate for the loss of Btn1p. It is possible that pH alterations are a secondary consequence of the loss of Btn1p, and the primary defect is yet to be elucidated. Future studies are focused on looking on the direct function of Btn1p, especially its role in coupling transport mechanisms with vATPase activity. S. cerevisiae has played and will continue to play a strong part in the efforts to understand JNCL.

5.2.6 JNCL in Schizosaccharomyces pombe, changes in pH and vacuole size

The fission yeast Schizosaccharomyces pombe homolog of human CLN3, termed Btn1p, is a predicted transmembrane protein of 396 amino acids that is 30% identical and 48% similar to its human counterpart (Figure 3). The S. pombe Δbtn1 strain, deleted for the btn1 gene is viable, but shows subtle and reproducible defects in cell cycle progression, which could be rescued by human CLN3 (Gachet et al., 2005). Average cell size is increased and the number of mitotic and dividing cells as well. In addition, Δbtn1 mutant cell vacuoles are larger than wild type cell vacuoles (mean size of 1.3 μm and 0.9 μm, respectively), Δbtn1 mutants have a broader vacuole size distribution, and Δbtn1 cells have an elevation in vacuole pH of 1 pH unit (pH 5.1 compared to pH 4.1 for wild type cells). A correlation is known to exist between increased vacuole size and increased pH in fission yeast (Iwaki et al., 2004), suggesting that Δbtn1 mutant pH regulation is affected, which in turn influences vacuole size (Gachet et al., 2005). The vacuolar size defect was restored to wild type levels when the Δbtn1 strain was grown in acidic media (pH 4) confirming that vacuole size is a reflection of the increased pH of the vacuole in Δbtn1 cells. In contrast to S. cerevisiae Δbtn1 mutants, S. pombe btn1Δ cells were found to be sensitive for growth in media containing 1 mM ANP. Although the mechanism for this sensitivity in fission yeast remains to be determined, growth of S. pombe Δbtn1 on plates containing 1 mM ANP was restored when this media was at pH 4, suggesting a relation between pH and ANP sensitivity (S. Codlin, unpublished). The basis for the difference in ANP sensitivity between S. cerevisiae and S. pombe may be in vacuolar pH (pH 6.2 and pH 4 respectively) (Gachet et al., 2005).
5.2.7 *S. pombe* btn1+ has a genetic interaction with vacuolar ATPase subunit vma1+

The vacuolar pH of *S. pombe* is similar to that of mammalian cells which are also acidic (Holopainen et al., 2001). Expression of Bn1 and human CLN3 in fission yeast deleted for *btn1*+ causes a decrease in pH of the vacuoles (Gachet et al., 2005). These results are in contrast to overexpression studies of CLN3 in HEK293 cells (Golabek et al., 2000), where lysosomal pH is increased but in agreement with studies on JNCL fibroblasts that had a slightly elevated lysosomal pH (Holopainen et al., 2001).

Cells deleted for *btn1*+ and vATPase subunit *vma1*+, which is involved in vacuolar acidification, exhibited slow growth and synthetic lethality at 30°C, indicating a genetic interaction between *Bn1* and the vATPase subunit (Gachet et al., 2005). While the molecular basis of this interaction has not been determined, previously described studies in *S. cerevisiae* implicating Btn1p in the coupling of proton pumping and the activity of the vATPase may provide a clue (Padilla-Lopez and Pearce, 2006).

5.2.8 Possible pre-vacuolar function of Btn1p

Overexpression of GFP-Btn1p and GFP-CLN3 constructs, which both have the GFP tags at the N-terminus, in Δ*btn1* cells complemented the vacuolar size and pH defects as well as the subtle cell growth defects, proving that Btn1p and CLN3 are functional homologs. In fission yeast, both Bn1p and CLN3 traffic to the vacuolar membrane via FM4-64 stained pre-vacuolar compartments, suggesting an endocytic trafficking route for Btn1p. Localization of Btn1p using a functional GFP-tagged Btn1p to the vacuole membrane was Ras GTPase Ypt7p dependent, with Btn1p being excluded from the vacuole and held in prevacuolar compartments in Δ*ypt7* cells (Gachet et al., 2005). Cells deleted for both *ypt7* and *btn1* show synthetic lethality at 36°C and vacuoles in these cells were larger than those of cells deleted for *ypt7* alone and again showed reduced pH. Bn1p must therefore have a functional role prior to reaching the vacuole, and this function impacts on vacuolar function.

5.2.9 Btn1p involved in osmoregulation in *S. pombe*

In addition to the subtle growth defects in cell cycle progression at 25°C, Δ*btn1* cells are also temperature sensitive for growth at 37°C (S. Codlin, unpublished). At this temperature, *btn1A* cells passed through no more than three cell cycles and subsequently lost their normal rod-shaped morphology, resulting in swollen and rounded cells. Cell lysis was found to be the subsequent cause of cell death. Electron microscopy reveals grossly thickened cell walls and septum regions, suggesting defects in the development of the main cell wall components, the α- and β-glucans. Indeed, Δ*btn1* cells were found to be highly sensitive to zymolase, a β-glucanase, but not to novozyme, an α-glucanase. Also, the swelling phenotype and cell lysis were completely rescued by the addition of 1 M sorbitol, an osmolyte, to the media, suggesting that the swelling and lysis may be caused by aberrant osmoregulation in Δ*btn1* cells (Gachet et al., 2005).

This interesting link between JNCL and pH alteration phenotypes in *S. pombe* supports the use of fission yeast to study NCLs.
6 Caenorhabditis elegans

6.1 Introduction to *C. elegans* biology

In 1972 Sidney Brenner and colleagues started to study the genetics of development and behavior in the relatively simple non-parasitic soil-borne nematode *Caenorhabditis elegans* (Brenner, 1974). The first report described methods to generate and study mutants in this small easily manageable model organism, and the first library of mutants that was generated. *Caenorhabditis elegans* has a body length of approximately 1 mm, and can be cultured in the lab on Petri dishes that contain an agar lawn spread with bacteria, on which this nematode feeds. The nematode exists as a hermaphrodite that can self-fertilize, which is a major advantage, e.g. after mutagenesis or when performing crosses, as homozygotes can be obtained without a subsequent cross. *C. elegans* males can spontaneously arise through non-disjunction of the X chromosome, which occurs at an approximate rate of 1/1000 during gametogenesis (Hodgkin and Doniach, 1997). The hermaphrodite itself can produce 300 progenitors by self-fertilization and many more progeny will result from a cross with a male nematode. The *C. elegans* life cycle starts when the hermaphrodite lays eggs, containing embryos of which the development had started in the hermaphrodite uterus. In the eggs the nematodes develop and hatch to become L1 larvae and proceed their life cycle through four larval stages until adult life begins. During development at the L2 larval stage, *C. elegans* can develop into an alternative third larval stage, called the dauer stage, an inactive form in which the nematode can survive adverse environmental conditions. When the environment becomes favourable again, this organism can leave dauer stage and develop into an L4 larva after which normal nematode life can proceed. The normal life cycle from egg to egg-laying adult without dauer stage lasts three days at 20°C, and *C. elegans* has a life span of approximately three weeks.

6.2 *C. elegans* anatomy and development

The extensive anatomical studies have shown that the hermaphrodite nematode body consists of 959 somatic cells of which 302 are neurons. The rest of the cells constitute musculature, intestinal tract, reproductive system, and hypodermal cells (Figure 4). Studies of the developing embryo and larva have lead to a complete understanding of the cell lineage that was found to be identical from one nematode to the next, a remarkable trait and of importance when investigating the development of an organism (Sulston *et al.*, 1988). The cells of the nervous system were mapped and were also shown to be invariably wired and connected, enabling analysis of how neuronal connectivity influences *C. elegans* behavior (White *et al.*, 1988). The study of this
Figure 4  An adult hermaphrodite *Caenorhabditis elegans* nematode

In the top light micrograph Nomarksi image the worm body and its major constituents are depicted. In the bottom epifluorescence micrograph the autofluorescence of the same nematode can be seen visualized with a standard FITC filter set. The autofluorescent gut granules are particularly bright.

(Bar=0.1 mm)
animal’s development was partly facilitated by its transparency, and virtually every cell can be observed using differential interference contrast microscopy. Due to this and the efforts made on ultrastructural level, the nematode body now is a well known entity, in which every cell has its place and function, generating the possibility to investigate the effects of changes to these cells.

6.3 C. elegans genetics and manipulability

The diploid genome of *C. elegans* is completely sequenced and comprises 100 million base pairs divided over six chromosomes, five autosomes and one sex chromosome (*C. elegans* sequencing consortium, 1998). The genetic map is freely available to all researchers and it is obtainable from the *C. elegans* Genetics Center (CGC). Furthermore, the CGC collects, maintains and distributes mutants that were generated and studied over the years. The *C. elegans* bibliography is also maintained at the CGC and they coordinate genetic nomenclature, as well as provide and update the *C. elegans* web server (*C. elegans* web server: http://elegans.swmed.edu). Nematode mutants can be obtained through forward and reverse genetics approaches. If not present at the CGC, additional mutants can be ordered from the *C. elegans* gene knock out consortium (http://celeganskoconsortium.omrf.org) or a mutant library can be made by the researcher (Zwaal *et al.*, 1993, Jansen *et al.*, 1997). Alternatively, knock-down of gene expression is easily performed by RNA interference (RNAi) in a number of ways. Firstly, dsRNAi targeted against the gene of interest can be injected in the animal after which it will spread through the animal and by the action of the dicer complex the mRNA of the gene of interest will be degraded. Secondly, the nematodes can be soaked in a solution containing the dsRNA that will be taken up in the intestine and spread through the worm body. Thirdly, the nematodes can be grown on bacteria that express a transgene producing dsRNA: the worms will digest the bacteria and internalize the dsRNA to exert its interfering effect (Ahringer, 2006). Fourthly, transgenic nematodes can be generated that harbor an extrachromosomal array consisting of plasmid DNA on which an inducible promoter regulates the expression of an inverted repeat derived from the gene that is targeted by the RNAi. Upon induction of this promoter the inverted repeat will be expressed, generating a double stranded RNA molecule that will knock-down the endogenous transcript. With the completion of the genome sequence, whole genome approaches became feasible and RNAi for all predicted coding sequences was started (Ahringer, 2006). An additional feature of the extensive *C. elegans* genetic toolset is transformation by microinjection of the hermaphrodite germline that allows generation of transgenic animals in less than a week (Mello *et al.*, 1991). A large set of expression vectors is available for promoter analysis, ectopic expression analysis, inducible expression, tagging of any sequence with different fluorescent proteins or LacZ, and for use in RNAi experiments (Mello and Fire, 1995). In addition, a comprehensive collection of methods to manipulate and investigate *C. elegans* is available (Epstein and Shakes, 1995, WormMethods: http://www.wormbook.org/toc_wormmethods.html).
6.4 C. elegans as a model organism

After comparing the genomes of man and nematode, it became clear that many protein sequences are conserved. Thus many human genes, in which mutations have been found causing genetic diseases, have homologues in the nematode, of which the function could be studied (Culetto and Sattelle, 2000). Although extrapolating the results obtained with the relatively simple nematode to a complex mammalian system may be a complicated task, the nematode could shed light on protein function and the molecular mechanisms they play a role in and how changes in these proteins affect their normal function. With its great number of homologues to human genes and its excellent genetic, biochemical, and cell biological experimental possibilities, C. elegans appears to be an ideal model organism to investigate the processes underlying human genetic disorders.
7 Aim and outline of this thesis

Juvenile Neuronal Ceroid Lipofuscinosis is a devastating lysosomal storage disorder disease of childhood for which there is no cure. While it is known to be caused by mutations in the CLN3 gene, this gene encodes a protein of unknown function. Although several mouse models and yeast models exist to study the processes underlying this disease, the molecular mechanisms leading to JNCL are unknown. The aim of the work described in this thesis has been to elucidate CLN3 function by the establishment and study of a nematode Caenorhabditis elegans model for JNCL. The information that would be acquired with the C. elegans model for JNCL was expected to be extrapolatable to other model organisms, e.g. the murine model, to provide better understanding of the pathology underlying JNCL, which might then lead to the development of new treatments.

In chapter 2, we explored the potential of C. elegans to be used as a model organism for lysosomal storage diseases and for studying different aspects of lysosomal function. Therefore, we identified in silico putative homologues of proteins involved in lysosomal storage diseases and other lysosomal proteins, by comparing the human protein sequences to all predicted protein sequences of C. elegans. In addition, we surveyed the available phenotypic information of nematodes with mutations in any of the homologues, in order to get an overview of possible lysosomal phenotypes in the nematode. The feasibility of using these phenotypes in genetic screens for modifier alleles was discussed.

To generate a nematode model for JNCL the C. elegans genes homologous to CLN3 were identified. This nematode turned out to have three homologues and characterization of the three worm cln-3 genes and their protein sequences was performed. The first cln-3 deletion mutant was reported and the initial analysis of the cln-3.1 mutant C. elegans strain was done (chapter 3). The function of the three cln-3 proteins may have diverged during evolution, but they also may have similar functions in different cells. Therefore, spatial and temporal expression patterns of the nematode cln-3 genes were determined. C. elegans cln-3 deletion mutants were generated and characterized with particular emphasis on the cell types in which the genes are expressed (chapter 4). Furthermore, the extensive genetic toolset that has been developed for investigations in the nematode has been exploited to examine worm cln-3 gene function.

We could not detect storage material in C. elegans cln-3 mutants, possibly due to the short life span of the nematode. Three weeks may not be sufficient for accumulation to occur. Therefore, we set out to induce accumulation of the main component of the storage material Subunit c of the mitochondrial ATP synthase. C. elegans that inducibly
overexpressed Subunit c were generated and studied. The results of this work are
described in chapter 5.

The work described in this thesis is concluded by a general discussion in chapter 6, in
which also future directions are provided.
References


Making *C. elegans* models for Juvenile Neuronal Ceroid Lipofuscinosis


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