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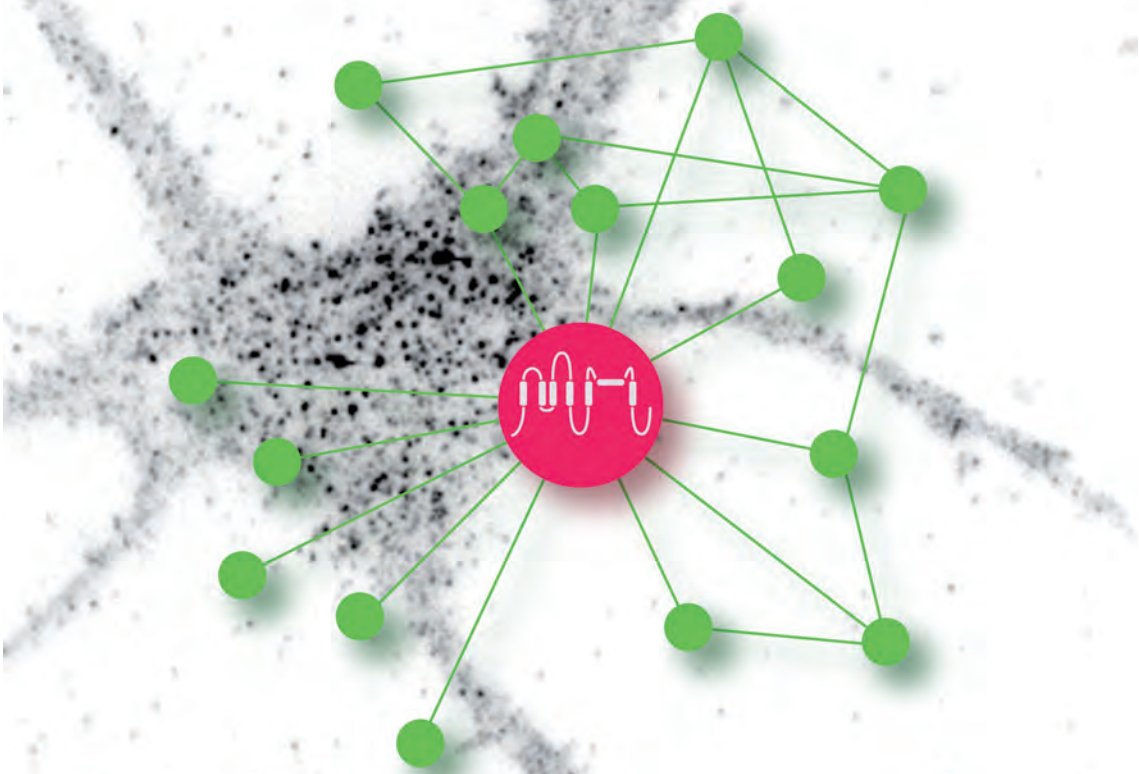
Kristiina Uusi-Rauva

# Molecular Interactions of Neuronal Ceroid Lipofuscinosis Protein CLN3



RESEARCH 82

Kristiina Uusi-Rauva

# Molecular Interactions of Neuronal Ceroid Lipofuscinosis Protein CLN3

## ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Medicine, University of Helsinki, for public examination in Lecture Hall 2, Biomedicum Helsinki, on Thursday May 24th, 2012, at 12 noon.

National Institute for Health and Welfare (THL), Helsinki, Finland
and
Department of Medical Genetics, University of Helsinki, Finland

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To my family

## Abstract

Kristiina Uusi-Rauva. Molecular Interactions of Neuronal Ceroid Lipofuscinosis Protein CLN3. National Institute for Health and Welfare (THL). Research 82/2012. 169 pages. Helsinki, Finland 2012.
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Neuronal ceroid lipofuscinoses (NCLs) are a collective group of recessively inherited severe neurodegenerative diseases of childhood. NCLs are characterised by progressive, selective neuronal death and lysosomal accumulation of autofluorescent storage material, ceroid lipofuscin, which lead to variable but progressive symptoms, including epileptic seizures, blindness, motor and mental deterioration, and premature death. Genetics of NCLs is well described, and several causative genes have been identified. However, due to the poor knowledge on the functions of the proteins primarily defective in NCLs, the intracellular changes critical to the pathogenesis of NCLs are not known. In this thesis study, NCL was studied in terms of its classic juvenile onset form, juvenile CLN3 disease, caused by mutations in a *CLN3* gene. *CLN3* encodes primarily late endosome/lysosome-localised transmembrane protein linked to a variety of intracellular processes. In order to determine the primary function(s) of CLN3, the protein interactions of CLN3 were carefully dissected utilising several different interaction analyses. Identified CLN3 interactions were further studied by analysing the characteristics of CLN3-interacting proteins and associated processes in *Cln3*$^{-/-}$ mouse model, patient fibroblasts, and mammalian cell lines.

CLN3 was determined to interact with the cell surface-localised $Na^+$, $K^+$ ATPase and its interacting partners, fodrin cytoskeleton and 78 kDa glucose-regulated protein/immunoglobulin heavy chain binding protein (GRP78/BiP). The ion-pumping function of $Na^+$, $K^+$ ATPase was found to be unaffected in Cln3-deficient primary cortical neuron cultures, but clear changes were observed in its basal plasma membrane association and ouabain-induced endocytosis. Ouabain is a cardiotonic steroid that not only inhibits the ion pumping activity of the $Na^+$, $K^+$ ATPase, but also regulates its recently discovered activity in intracellular signalling, apoptosis and calcium oscillations. Therefore, it was concluded that CLN3 may play an important role in the non-pumping functions of $Na^+$, $K^+$ ATPase. Furthermore, analyses of fodrin in Cln3-deficient brain sections and patient fibroblasts revealed putative structural changes in the fodrin cytoskeleton suggesting that fodrin-associated events in axonal and synaptic intracellular trafficking, synaptic transmission, and neuritogenesis may also be compromised in early stage of the pathogenesis of juvenile CLN3 disease.

The finding that CLN3 also interacts with the microtubule-binding, endocytic Hook1 was the first indication that CLN3 may act directly in membrane trafficking

processes. Subsequently, CLN3 was found to interact with motor proteins, dynein-dynactin and kinesin-2-dynactin, most likely through its direct interactions with Rab7 GTPase and its effector Rab7-interacting lysosomal protein (RILP). Previously, it has been reported that together with oxysterol-binding protein-related protein 1L (ORP1L), Rab7 and RILP facilitate the recruitment of dynein-dynactin motor protein to late endosomal/lysosomal membranes and thus, enhance the minus end-directed movement of late endosomes and lysosomes. Rab7-containing effector complex has also been suggested to regulate kinesin-dependent movement of organelles, and thus, may link CLN3 to these functions as well. Interestingly, several observations indicated that the late endosomal/lysosomal membrane trafficking is affected in CLN3 deficiency. First, disease-associated CLN3 mutants were found to interact differently with Rab7 and RILP proteins. Second, late endosomes and lysosomes in CLN3 mutant-expressing cells were observed to be abnormally clustered to the perinuclear area. Third, functional GTP/GDP cycle of Rab7 was found to be affected in CLN3 deficiency. Fourth, CLN3-deficient cells exhibited delays in the late endosomal transport of endocytosed cargo, and kinesin-dependent movement of late endosomes/lysosomes. These results suggested that Rab7-guided microtubular motor protein functions in neurons, such as axonal retrograde trafficking of neurotrophins, neurite outgrowth and maturation, and transportation of neuronal autophagic vesicles could also be dysregulated in juvenile CLN3 disease.

This thesis work has provided important novel data on the functions of CLN3 and the primary intracellular defects possibly resulting in CLN3 disease. This study also contributes to the determination of the pathogenesis of other NCLs and general processes of neurodegeneration.

Keywords: CLN3, lysosomal storage disease, intracellular membrane trafficking, lysosome, neurodegeneration, neuronal ceroid lipofuscinosis, protein function, protein interaction

## Tiivistelmä

Kristiina Uusi-Rauva. Molecular Interactions of Neuronal Ceroid Lipofuscinosis Protein CLN3 [Neuronaalisessa seroidilipofuskinoositaudissa vioittuneen CLN3-proteiinin molekyylitason vuorovaikutukset]. Terveyden ja Hyvinvoinnin laitos (THL). Tutkimus 82/2012. 169 sivua. Helsinki, 2012.
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Neuronaaliset seroidilipofuskinoosit eli NCL-taudit ovat peittyvästi periytyviä, lapsuusiässä ilmeneviä keskushermostoa rappeuttavia sairauksia. NCL-taudeille on tunnusomaista etenevä selektiivinen hermosolujen kuolema ja autofluoresoivan materiaalin, seroidilipofuskinoosin, kertyminen lysosomeihin. Tämä johtaa epileptisiin kohtauksiin, sokeuteen, psyko-motoriikan heikentymiseen ja ennenaikaiseen kuolemaan. NCL-tauteja aiheuttavia geenimuutoksia on löydetty useista eri geeneistä mutta on epäselvää, miten kyseisten geenien mutaatiot johtavat taudin puhkeamiseen proteiinitasolla. Tämä johtuu ennen kaikkea siitä, että NCL-tautien alttiusgeenit koodaavat proteiineja, jotka ovat toiminnallisilta ominaisuuksiltaan huonosti tunnettuja. Tämä väitöskirjatutkimus käsittelee NCL-tautien nuoruusiässä ilmenevää, CLN3-geenin mutaatioista johtuvaa alatyyppiä, klassista nuoruusiän CLN3-tautia. CLN3-proteiini on kalvoproteiini, joka sijaitsee myöhäisissä endosomeissa ja lysosomeissa ja jonka on ehdotettu liittyvän useisiin eri solunsisäisiin toimintoihin. Tässä väitöskirjatyössä on tutkittu CLN3:n ensisijaista toimintaa selvittämällä sen vuorovaikutuksia muiden proteiinien kanssa. Tunnistettuja CLN3:n kanssa vuorovaikuttavia proteiineja analysoitiin edelleen Cln3-poistogeenisessä hiirimallissa, potilaiden fibroblastisoluissa sekä nisäkässolulinjoissa.

Tutkimusten perusteella voitiin todeta, että CLN3 vuorovaikuttaa solukalvolla sijaitsevan $Na^+$, $K^+$ ATPaasin ja siihen liittyvän GRP78/BiP:n (engl. 78 kDa glucose-regulated protein/immunoglobulin heavy chain binding protein) ja solukalvotukirankaproteiini fodriinin kanssa. Cln3-poistogeenisten hiirten aivoista valmistetuissa hermosoluviljelmissä tehdyt kokeet osoittivat, että vaikka $Na^+$, $K^+$ ATPaasin ionipumppausaktiivisuudessa ei ole havaittavissa merkittäviä muutoksia Cln3:n puuttumisen seurauksena, kyseisen proteiinin suhteelliset määrät solukalvolla sekä sen ouabaiinilla indusoituva endosytoosi ovat kuitenkin häiriintyneet. Ouabaiini on kardiotoninen steroidi, joka säätelee $Na^+$, $K^+$ ATPaasin ionipumppausaktiivisuuden lisäksi sen toimintaa signaalinvälityksessä, apoptoosissa ja kalsiumvasteessa. Näin ollen on mahdollista, että CLN3 on toiminnallisesti kytköksissä $Na^+$, $K^+$ ATPaasin ionipumppausaktiivisuudesta riippumattomiin toimintoihin. Cln3-poistogeenisen hiiren aivoleikkeillä sekä potilassoluilla tehdyt analyysit osoittivat lisäksi, että fodriinitukiranka on mahdollisesti vioittunut CLN3-puutteellisissa soluissa. Koska fodriinin on raportoitu liittyvän aksonien ja synapsien solukalvoliikenteeseen, hermoimpulssin välittymiseen solusta toiseen sekä

hermosolujen ulokkeiden kasvuun, on mahdollista, että myös nämä toiminnot ovat vioittuneet CLN3-taudissa.

CLN3:n vuorovaikutus mikrotubuluksia sitovan solukalvoliikenteessä toimivan Hook1-proteiinin kanssa oli puolestaan ensimmäinen osoitus siitä, että CLN3 liittyy toiminnallisesti suoraan solukalvoliikenteeseen. Jatkotutkimukset paljastivat, että CLN3 vuorovaikuttaa lisäksi mikrotubuluksia pitkin organelleja ja vesikkeleitä liikuttavien moottoriproteiinien kanssa ja että nämä vuorovaikutukset tapahtuvat todennäköisesti Rab7 GTPaasin ja sen ns. efektoriproteiini RILP:n (engl. Rab7-interacting lysosomal protein) välityksellä. Rab7:n ja RILP:n on raportoitu muodostavan ORP1L-proteiinin (engl. oxysterol-binding protein-related protein 1L) kanssa kompleksin, joka aktivoi dyneiini-dynaktiini moottoriproteiinin kytkeytymisen myöhäisten endosomien ja lysosomien pinnalle edistäen kyseisten organellien kuljetusta mikrotubuluksia pitkin kohti solun keskiosaa. Lisäksi Rab7-efektorikompleksien on raportoitu osallistuvan kinesiinimoottoriproteiinin alaiseen, solun ulkoreunoja kohti tapahtuvaan organellien kuljetukseen. Tässä tutkimuksessa tehdyt havainnot viittasivatkin siihen, että edellämainitut myöhäisten endosomien ja lysosomien solukalvoliikenteeseen liittyvät tapahtumat ovat häiriintyneet CLN3-taudissa. CLN3-mutanttien osoitettiin vuorovaikuttavan eri affiniteetilla Rab7:n ja RILP:n kanssa, myöhäisten endosomien ja lysosomien solunsisäisen sijainnin havaittiin muuttuneen CLN3-mutantteja ilmentävissä soluissa ja Rab7:n toiminnallisen GTP/GDP-kierron todettiin olevan häiriintyneen potilassoluissa. Lisäksi CLN3-proteiinia poikkeavan vähän ilmentävillä nisäkässoluilla ja CLN3-puutteellisilla potilassoluilla tehdyt kokeet osoittivat, että lysosomien kinesiini-välitteinen liikkuminen sekä endosytoidun materiaalin kulkeutuminen myöhäisiin endosomeihin ja lysosomeihin ovat hidastuneet CLN3-proteiinin alentuneen ilmentymisen seurauksena. Kaiken kaikkiaan em. havainnot viittaavat vahvasti siihen, että myös Rab7-välitteiset hermosolujen sisäiset tapahtumat kuten autofagosomien solukalvoliikenne sekä kasvutekijöiden aksonaalinen kuljetus ja siihen liittyvä hermosolujen ulokkeiden kasvun säätely ovat häiriintyneet CLN3-taudissa.

Tämä väitöskirjatutkimus on antanut tärkeää uutta tietoa CLN3:n toiminnoista ja niistä mahdollisista solunsisäisistä tapahtumista, jotka ovat ensisijaisesti vioittuneet CLN3-taudissa. Tämä tutkimus edistää myös muiden NCL-tautien syntymekanismien sekä yleisten hermosolujen rappeutumiseen johtavien prosessien määrittämistä.

Avainsanat: CLN3, lysosomaalinen kertymätauti, lysosomi, neurodegeneraatio, neuronaalinen seroidilipofuskinoositauti, proteiinitoiminto, proteiinivuorovaikutus, solukalvoliikenne

## Contents

Original publications

## List of original publications

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals. In addition, some unpublished data is presented.

I **Uusi-Rauva Kristiina**, Luiro Kaisu, Tanhuanpää Kimmo, Kopra Outi, Martín-Vasallo Pablo, Kyttälä Aija., Jalanko Anu. Novel interactions of CLN3 protein link Batten disease to dysregulation of fodrin- $Na^+$, $K^+$ ATPase complex. Experimental Cell Research 2008; 314(15): 2895-2905.

II Luiro Kaisu, **Yliannala Kristiina**, Ahtiainen Laura, Maunu Heidi, Järvelä Irma, Kyttälä Aija, Jalanko Anu. Interconnections between CLN3, Hook1 and Rab proteins link Batten disease to defects in the endocytic pathway. Human Molecular Genetics 2004; 13(23): 3017-3027.

III **Uusi-Rauva Kristiina**, Kyttälä Aija, van der Kant Rik, Vesa Jouni, Tanhuanpää Kimmo, Neefjes Jacques, Olkkonen Vesa M., Jalanko Anu. Neuronal ceroid lipofuscinosis protein CLN3 interacts with motor proteins and modifies location of late endosomal compartments. Cellular and Molecular Life Sciences 2012; doi 10.1007/s00018-011-0913-1.

Publication II has appeared previously in the doctoral thesis of MD, PhD Kaisu Luiro (2006).

## Abbreviations

| | |
|---|---|
| AMPA | 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid |
| AP | adaptor protein complex |
| AP-1, AP-2, etc. | adaptor protein complex 1, adaptor protein complex 2, etc. |
| Arf | ADP-ribosylation factor |
| ATP | adenosine triphosphate |
| ATPase | adenosine triphosphatase |
| BiP | immunoglobulin heavy chain binding protein |
| Btn1p/*BTN1* | yeast protein/gene orthologue of human CLN3 |
| *btn1*Δ | yeast strain deleted for *BTN1* |
| cDNA | complementary deoxyribonucleic acid |
| CLN1, CLN2 etc. | human ceroid lipofuscinosis, neuronal 1 protein; ceroid lipofuscinosis, neuronal 2 protein etc. |
| *CLN1, CLN2* etc | human CLN1, CLN2 etc. genes |
| *CLN3/Cln3* | human/mouse CLN3 gene |
| CLN3/Cln3 | human/mouse CLN3 protein |
| CLN3E295K | mutated CLN3 protein (amino acid substitution from glutamic acid (E) to lysine (K) at residue 295) |
| COPI | coat protein complex I |
| COPII | coat protein complex II |
| COS-1 cells | African green monkey kidney cells |
| CSPα | cysteine string protein alpha |
| DNA | deoxyribonucleic acid |
| EE | early endosome |
| EEA1 | early endosomal antigen 1 protein |
| EGFP | enhanced green fluorescence protein |
| EPMR | progressive epilepsy with mental retardation (Northern epilepsy) |
| ER | endoplasmic reticulum |
| FRAP | fluorescence recovery after photobleaching |
| FYCO1 | FYVE and coiled-coil domain containing 1 |
| GABA | γ-aminobutyric acid |
| GAD65 | glutamic acid decarboxylase |
| GAP | guanosine triphosphatase activating protein |
| GEF | guanine nucleotide exchange factor |
| GGA | γ-ear containing, ADP ribosylation factor-binding protein |
| GRP78 | 78 kDa glucose-regulated protein |
| GST | glutathione S-transferase |
| GDF | GDI displacement factor |
| GDI | GDP dissociation inhibitor |
| GDP | guanosine diphosphate |

| | |
|---|---|
| GTP | guanosine triphosphate |
| GTPase | guanosine triphosphatase |
| HeLa cells | human cervical tumour cells |
| Hook1/hook/Btn2p | human/*Drosophila melanogaster*/*Saccharomyces cerevisiae* orthologue |
| HOPS | homotypic fusion and vacuole protein sorting |
| HRP | horseradish peroxidase |
| IPTG | isopropyl β-D-1-thiogalactopyranoside |
| kb | kilobase(s) |
| kDa | kilodaltons |
| LAMP-1 | lysosome-associated membrane protein 1 |
| LAMP-2 | lysosome-associated membrane protein 2 |
| LBPA | lysobisphosphatidic acid |
| LDL | low-density lipoprotein |
| LE | late endosome |
| LIMP-1 | lysosomal integral membrane protein type 1 |
| LIMP-2 | lysosomal integral membrane protein type 2 |
| LSD | lysosomal storage disorder |
| mRNA | messenger RNA |
| MFSD8 | major facilitator superfamily domain containing 8 |
| MPR | mannose 6-phosphate receptor |
| NCL | neuronal ceroid lipofuscinosis |
| NM | non-muscle myosin |
| NMHC | non-muscle myosin heavy chain |
| NPC1 | Niemann-Pick C1 |
| NSF | N-ethylmaleimide-sensitive factor |
| OD600 | optical density at 600 nm |
| ORP1L | oxysterol-binding protein-related protein 1L |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| PPT1 | human palmitoyl protein thioesterase 1 protein (CLN1) |
| qPCR | quantitative real-time polymerase chain reaction |
| Rabring7 | Rab7-interacting RING finger protein 7 |
| RILP | Rab7-interacting lysosomal protein |
| RNA | ribonucleic acid |
| RT | room temperature |
| Sar1 | secretion-associated and Ras-related protein 1 |
| saposin (SAP) | sphingolipid activator protein |
| SBDS | Shwachman-Bodian-Diamon syndrome protein |
| SD | synthetic defined |
| Sdo1/*SDO1* | Yeast protein/gene orthologue of human Shwachman-Bodian-Diamon syndrome protein |

| | |
|---|---|
| SDS | sodium dodecyl sulfate |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| siRNA | small interfering RNA |
| SNAP-25 | 25-kDa synaptosome-associated protein |
| SNARE | soluble N-ethylmaleimide-sensitive factor attachment protein receptor |
| TBST | Tris-buffered saline supplemented with Tween-20 |
| TGN | trans-Golgi network |
| TIRF | total internal reflection fluorescence |
| TPP1 | human tripeptidyl peptidase 1 protein (CLN2) |
| vATPase | vacuolar adenosine triphosphatase |
| YTH | yeast two-hybrid |

# 1 Introduction

The past two decades, the era of molecular genetics, have provided a tremendous amount of data on genetics of human hereditary traits. However, interpretation of the data on genetic variations has become a challenge that is far from being overcome, especially in terms of functional genomics and disease pathomechanism. The outcome of a genetic defect is ultimately dependent on the changes that the defect may cause on the functions of the proteins. Therefore, the detailed analysis of the associated proteins is the prerequisite for understanding the meaning of disease-associated genetic defects. In the best case, the normal function of the affected protein has already been dissected paving the way for further functional analysis downstream on the disturbed intracellular pathway(s), hopefully leading to drug discovery and treatment of the disease. In many cases, the defective protein is, however, uncharacterised, and may not even show homology to known proteins or functional protein domains. In these cases, analysis of the protein has to start from the scratch, from basic protein characterisation, followed by studies utilising animal models, patient samples, and microarray, metabolomics, and modern gene-silencing technologies to map defective intracellular processes. As exemplified by the studies on CLN3, the topic of this thesis work, defects of a particular protein may lead to a spectrum of disturbances at a molecular and cellular level, and consequently, cell death. However, only some of the affected cellular processes may be closely connected to a given protein and thus, critical for the disease pathogenesis. Therefore, functional dissection of disease pathogenesis remains incomplete until the interactome of the defective protein is determined and analysed in terms of disease-causing mutations. Besides providing clues to molecular background of a given hereditary disorder, detailed analysis of the disease-associated proteins and functional pathways provides important information usable in research of other related diseases. Furthermore, novel findings on intracellular functions, and the roles that associated proteins play in them, will further improve our understanding on a complexity of a cell and a human body.

Neuronal ceroid lipofuscinosis (NCLs) are severe and yet incurable recessively inherited progressive neurodegenerative disorders of childhood. NCLs are characterised by unifying accumulation of lysosomal storage material, neuronal death in the central nervous system, epilepsy, and mental and motor decline. Although NCLs are mostly diagnosed at birth up to middle school ages, they may also have an onset in adulthood (Haltia, 2003; Haltia, 2006). NCLs result from mutations in different genes (Kousi et al., 2012). Although first NCL-associated genes were identified more than 15 years ago (Consortium, 1995; Vesa et al., 1995), interactions of NCL proteins are poorly known (Kyttala et al., 2006; Getty and

Pearce, 2011). Consistently, the link between disease-causing mutations and neuropathogenesis in NCLs is inadequate (Kyttala et al., 2006).

Mutations in CLN3 are most common contributors to juvenile onset NCL (juvenile CLN3 disease or CLN3 disease, classic juvenile). Juvenile CLN3 disease has unique properties among NCL diseases, including vacuolated lymphocytes and production of autoantibodies (Chattopadhyay et al., 2002; Haltia, 2003). CLN3 encodes a lysosomal polytopic membrane protein with unclear functions. More than 300 reports linked to CLN3 have been published to date, with much of the data being achieved using mouse and yeast models (Cooper et al., 2006; Phillips et al., 2006). However, prior to the initiation of the current study, interactions of CLN3 were poorly known. Therefore, the aim of this study was to find interaction partners of CLN3 and to study them in terms of CLN3-deficiency and juvenile CLN3 disease. Overall, results of this study showed that CLN3 is functionally and molecularly linked to intracellular membrane trafficking.

# 2 Review of the literature

## 2.1 Intracellular membrane trafficking

### 2.1.1 General features of membrane trafficking

Intracellular membrane trafficking is a highly orchestrated system indispensable to eukaryotic cells due to their compartmentalised nature (reviewed in Aridor and Hannan, 2002; Olkkonen and Ikonen, 2006). Eukaryotic cell functions are performed within specialised compartments, organelles, enclosed within membranes. Communication between organelles, and with the environment, is based on the transportation of organelles and small membrane-enclosed structures, transport vesicles and tubules, along cytoskeletal tracks. Biogenesis of transport vesicles and association of two membrane-enclosed structures are dependent on dynamic membrane fission and fusion reactions. This basic model of intracellular membrane trafficking is based on the pioneering findings and methodological achievements of Claude, de Duve, Palade, and colleagues (Claude, 1975; Duve, 1975; Palade, 1975). Over the past half century research on membrane trafficking has greatly enhanced our understanding on the details of membrane trafficking networks and associated components (reviewed in Mellman and Warren, 2000; Bonifacino and Glick, 2004).

Two main intracellular membrane trafficking pathways exist, the secretory/biosynthetic pathway and the endocytic pathway (**Figure 1**). Following synthesis and post-translational modifications at endoplasmic reticulum (ER) and Golgi complex, newly synthesized membrane-bound or lumenal proteins and lipids destined to the compartments beyond the Golgi complex are either secreted to the extracellular space within specialised secretory vesicles or transported through a vesicular transport system to the plasma membrane or to endosomal compartments to support a particular cellular function (reviewed in Mellman and Warren, 2000; Bonifacino and Glick, 2004; Saftig and Klumperman, 2009) (**Figure 1**). The secretory/biosynthetic pathway is accompanied by the flow of the in-coming material (reviewed in Mellman and Warren, 2000; Bonifacino and Glick, 2004; Saftig and Klumperman, 2009) derived from the cell surface or extracellular milieu by the cellular uptake, endocytosis (reviewed in Doherty and McMahon, 2009) (endocytic pathway). The endocytosed material may be targeted to late endosomes (LEs) and lysosomes for degradation and further utilisation in cellular metabolism, but may also represent late endosomal/lysosomal housekeeping membrane proteins that, following the plasmalemmal transport, utilise endocytic pathway as an alternative trafficking route to reach their resident compartment (reviewed in Pryor and Luzio, 2009; Saftig and Klumperman, 2009) (**Figure 1**).

The secretory/biosynthetic pathway and the endocytic pathway are balanced by recycling pathways that are responsible for organelle homeostasis and the re-use of the components of transporting machineries. Recycling occurs at the level of endosomes with the main organelle being the early endosome (EE) which receives cargo from both main trafficking pathways. In association with recycling endosomes, EEs act as a central station for sorting of proteins for the reutilisation at the plasma membrane or trans-Golgi network (TGN) (for example, receptors and membrane fusion complexes) from proteins destined for degradation (for example, receptor ligands) (**Figure 1**). Another recycling organelle, LE, further delivers proteins back to the TGN and thus, together with other endosomal compartments connect the endocytic and secretory/biosynthetic routes (reviewed in Bonifacino and Rojas, 2006; Saftig and Klumperman, 2009; Johannes and Wunder, 2011).

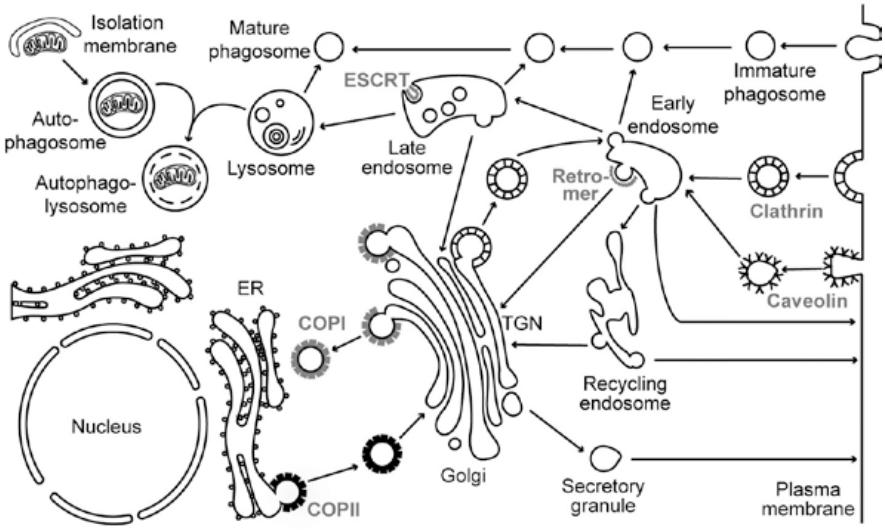


**Figure 1.** Main pathways of intracellular membrane trafficking. Transport steps (arrows) are indicated. Transport vesicles/compartments bud from the donor membrane by mechanisms usually involving vesicle coat or coat-like protein complexes assembled on the cytosolic face of a vesicle/compartment. Position of COPI, COPII, clathrin, caveolin, retromer and ESCRT coats/coat-like structures in the vesicular trafficking network is presented (see Chapter 2.1.2.1). In addition, two other processes of membrane trafficking are presented, phagocytosis and autophagy, which mediate the delivery of extracellular and intracellular material, respectively, to be degraded in endolysosomal compartments. Abbreviations: COPI, coat protein complex I; COPII, coat protein complex II; ER, endoplasmic reticulum; ESCRT, endosomal sorting complex required for transport; TGN, trans-Golgi network. Modified from Bonifacino and Glick, 2004 and Jahn and Scheller, 2006. Some of the figure components were produced using Servier Medical Art (www.servier.com).

### 2.1.2 The life cycle of a transport vesicle – basic mechanisms of membrane budding, movement and fusion

Transport vesicles mediate cargo delivery between distinct intracellular compartments. A conventional transport vesicle is spherical and 60–100 nm in diameter but larger and more tubulated carrier compartments may also exist (Bonifacino and Lippincott-Schwartz, 2003). The life cycle of a transport vesicle involves following steps: 1) assembly of the vesicular components in association with cargo selection and clustering, 2) fission from the donor compartment, 3) vesicle movement along cytoskeletal trails, 4) tethering and docking at the target compartment, and 5) fusion with the target membrane (**Figure 2**) (Bonifacino and Glick, 2004). In endolysosomal system, cargo delivery may additionally involve compartment conversion/maturation and direct fusions between "permanent" organelles (Rink et al., 2005; Vonderheit and Helenius, 2005; and Luzio et al., 2010) but nevertheless these alternative delivery mechanisms are dependent on membrane fission/fusion processes as well as cytoskeletal motor proteins.

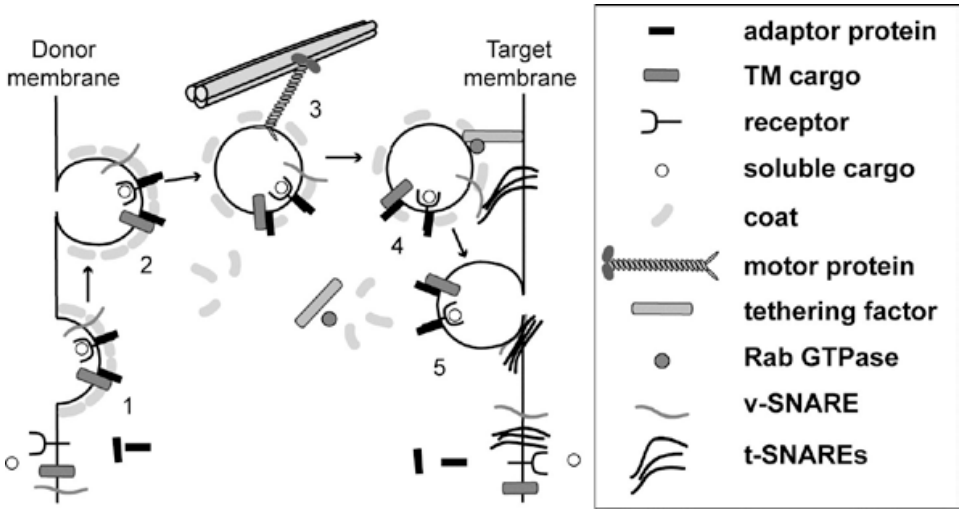


**Figure 2.** The life cycle of a transport vesicle. Soluble and transmembrane cargos (TM) are selectively recruited to the budding site of a donor membrane by specific receptor and/or adaptor proteins. Budding also involves coat formation on the cytosolic face of the budding vesicle (1). Vesicle is released (2) and transported along cytoskeletal trails by motor proteins (3). At a correct destination, the transport vesicle is tethered at a target membrane by a specific Rab GTPase, tethering factors, and possibly, vesicle coats (4). Membrane fusion (5) between two compartments occurs by the assembly of a SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins) complex composed of components from both the donor and the target membrane (v-SNARE and t-SNARE). For details, see next chapters. For the sake of clarity, formation of only one SNARE complex is shown. Modified from Bonifacino and Glick, 2004 and Cai et al., 2007. Some of the figure components were produced using Servier Medical Art (www.servier.com).

### *2.1.2.1 Vesicle assembly and fission*

The best known mechanism leading to carrier vesicle or tubule formation involves vesicle coat assembly. Vesicle coats are curved multimeric cage-like structures, scaffolds, on the cytosolic face of the vesicular membrane (**Figure 2**). Vesicle coats are composed of multiple protein species that have an important role in the life cycle of a carrier vesicle/compartment. As will be mentioned in this and following chapters, coat complexes play a role in membrane deformation, cargo selection and vesicle fission as well as vesicle tethering on the target membrane. The three best studied vesicle coats **clathrin**, **COPI** (coat protein complex I) and **COPII** (coat protein complex II) represent the major coats involved in membrane trafficking (reviewed in Kirchhausen, 2000; McMahon and Mills, 2004). COPII coat is involved in the transport from the ER to the Golgi compartment whereas COPI coat mediates trafficking in the opposite direction, from the Golgi back to the ER, but also between individual Golgi stacks (Orci et al., 1986; Barlowe et al., 1994; Letourneur et al., 1994). Clathrin, the first coat to be discovered (Roth and Porter, 1964; Pearse, 1975), has a wider range of action. Clathrin assembly may occur on TGN, the plasma membrane and endosomes (McNiven and Thompson, 2006; Johannes and Wunder, 2011) (**Figure 1**). These classical coats form an electron dense layer on membranes and thus are detectable under the electron microscope. COPI, COPII and clathrin are composed of two distinct protein layers that, upon activation, are formed in situ mostly from cytosolic oligomers primarily by protein-driven mechanisms. The two layers have distinct functions. The inner layer recognises and recruits the cargo in vesicle budding and interacts with proteins involved in vesicle tethering at the target membrane. The outer segment of vesicle coat is responsible for scaffolding and shaping the membrane in vesicle invagination (reviewed in Kirchhausen, 2000; McMahon and Mills, 2004). Initiation of COP and clathrin-coated vesicle budding is dependent on the small guanosine triphosphatases (GTPases) namely secretion-associated and Ras-related protein 1 (Sar1) and ADP-ribosylation factors (Arfs). Activation, i.e. exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP), of Sar1 and Arfs leads to exposure of their aminoterminal amphipathic α-helix which tends to dive into the membrane inducing an asymmetric expansion of one leaflet of the lipid bilayer and subsequently promoting membrane curvature. Most importantly, activated Sar1 and Arfs facilitate recruitment of cargo recognition and membrane scaffolding components. Whereas Sar1 is involved in the sequential recruitment of COPII components, Sec23/Sec24 heterodimer and Sec13/Sec31 heterotetramer, in the ER (Kirchhausen, 2000; Jensen and Schekman, 2011), Arfs operate at the TGN and the cell surface where they recruit COPI and clathrin coats (D'Souza-Schorey and Chavrier, 2006). COPI is composed of heptameric complexes, coatomers, which contain F-subcomplex (β/γ/δ/ζ protein) and B-subcomplex (α/β′/ε) (reviewed in Kirchhausen, 2000; and Beck et al., 2009). The major constituent of clathrin coats is clathrin which is recruited as triskelion structures of three clathrin light chains and three heavy chains

(Kirchhausen and Harrison, 1981; Ungewickell and Branton, 1981). Clathrin coats are additionally characterised by heterotetrameric adaptor protein complexes (APs), and Golgi-localised, γ-ear containing, ADP ribosylation factor-binding proteins (GGAs) that recognise well-characterised sorting signals located in the cytoplasmic domains of transmembrane cargo proteins (Bonifacino and Traub, 2003; Braulke and Bonifacino, 2009). Adaptor protein 2 complex (AP-2)/clathrin functions at the cell surface while GGA and other APs, AP-1, AP-3, and AP-4, mediate sorting from trans-Golgi network to lysosomes (Bonifacino and Traub, 2003).

In addition to above-mentioned conventional coats, several other types of coats are involved in vesicle budding. **Caveolae** invaginations and subsequent vesicles, caveosomes, occur at the plasma membrane and are mainly composed of caveolins and cavins specifically enriched in cholesterol and sphingolipid-containing membrane microdomains (reviewed in Bastiani and Parton, 2010). Caveolae biogenesis is different from that of conventional vesicle coats in many respects. Membrane-embedded components of caveolae, caveolins are preassembled in the secretory pathway and transported to the plasma membrane where cytosolic cavins are recruited to the caveolar domains (reviewed in Bastiani and Parton, 2010; Hayer et al., 2010 and references therein). Therefore, activation of caveolar coats at the plasma membrane does not involve major coat assembly. Furthermore, unlike conventional vesicle scaffolds (reviewed in Bonifacino and Lippincott-Schwartz, 2003) caveolar coats are stable structures which do not undergo cycles of assembly and disassembly (Tagawa et al., 2005). Other notable coat-like structures, **retromer** and **ESCRT** (endosomal sorting complex required for transport) complexes, contribute to retrograde trafficking from EEs to TGN and sorting of proteins from LEs to lysosomes, respectively. ESCRT is exceptional among intracellular coats as it mediates the inward budding of cargo clustered on the flat bilayered clathrin-coated areas of late endosomal limiting membrane (reviewed in Saftig and Klumperman, 2009).

Polymerising coats close themselves as spheres that are subsequently scissored out of the donor membrane by accessory proteins, such as the large GTPase dynamin, or by components of the coat itself (Hinshaw and Schmid, 1995; Bielli et al., 2005; Lee et al., 2005).

#### *2.1.2.2 Membrane compartments on the move – microtubular molecular motors*

Motor protein-mediated transport along the cytoskeletal tracks provides an efficient system to transport vesicles and organelles. Two cytoskeletal networks and three types of motor proteins are involved in the motor protein-mediated transport; the actin and the microtubule cytoskeletons, and myosin, dynein and kinesin motor proteins. In most cultured cell types, microtubules are organized in a radial manner, with one end anchored near the nucleus (minus end) and the other end facing the cytoplasm (plus end). The more randomly oriented actin meshwork is considered to

facilitate transport in areas where there are few microtubules, for example in the cell periphery, synaptic regions and growth cones, and in the interspaces of microtubular patches in neuronal projections. In these areas, myosins contribute to synaptic exocytosis, recycling of cell surface components from endosomes back to the plasma membrane, and transporting nascent endocytic vesicles to the EEs. Whereas actin cytoskeleton and myosins mediate slower and short-range local transport, the microtubular network in association with dynein and kinesin motors supports the long-range, high-speed vesicle movement (reviewed in Mallik and Gross, 2004; Soldati and Schliwa, 2006; Hirokawa et al., 2010). Microtubular motor protein functions are especially important in cells with elongated structures, for example in neuronal axons that may be up to a metre or more in length (Bloom and Goldstein, 1998).

There are a total of 45 kinesin genes (also known as KIFs) in a mammalian genome, with 38 genes expressed in the brain. Due to alternative mRNA splicing, the total number of kinesin proteins has been suggested to be at least twice the number of the genes (Miki et al., 2001). Phylogenetically, kinesins are classified into 14 families termed kinesin 1 to kinesin 14, with kinesin 14 family including two subfamilies, kinesin 14A and 14B (Lawrence et al., 2004). Based on the localisation of the motor domain within the molecule, kinesins can be grossly grouped into three functional classes. N-type kinesins posses the motor domain at or near the N-terminus, M-type kinesins bear the domain in the middle region of the protein, and C-type kinesins are characterised by a C-terminally located motor domain. Generally, N-type kinesins drive plus-end-directed and C-type kinesins minus-end-directed motilities, while M-type kinesins have been implicated in microtubule depolymerisation. Since most of the kinesin protein families comprise N-type motors, kinesins are often referred to as anterograde motor proteins (reviewed in Hirokawa et al., 2009). For dynein, two major functional groups exist, axonemal and cytoplasmic dyneins. Axonemal dyneins are involved in ciliary/flagellar beating while two cytoplasmic dynein complexes represent the major microtubule minus end-directed motor proteins of the cell. Cytoplasmic dynein 2 mediates bi-directional transport of particles along the flagellar/ciliary microtubules while cytoplasmic dynein 1 (termed from now on dynein) serves the intracellular transport (Pfister et al., 2005). It is suprising that cells use this single dynein for intracellular minus end-directed microtubular transport whereas a wide range of kinesins are utilised to transport cargo in the plus end direction.

Kinesin and dynein motors are composed of several different proteins, and the detailed composition and macromolecular structure vary between different motors. However, heavy chain is the basic component in each motor complex (reviewed in Hirokawa et al., 2010). For dynein and most of the kinesins, dimerisation, mediated by the kinesin/dynein heavy chains, is the basis for movement along the microtubule. In the folded conformation, the head of each heavy chain contains motor domains which bind and hydrolase ATP and associate with the microtubule

(**Figure 3**). Two motor domains provide an efficient way to avoid detachment from the cytoskeletal track and thus, allow kinesins to move in a highly processive manner. According to the "walking" or "hand-over-hand" model, kinesins use motor domains one at a time, one motor domain staying attached to the microtubule while the other one is stepping forward. "Walking" is powered by ATP hydrolysis, with one ATP being consumed in each step (reviewed in Marx et al., 2009). The tail of the folded heavy chain dimer binds an array of kinesin light chains, or dynein intermediate chains, dynein light intermediate chains, and dynein light chains which then mediate the cargo binding via specific adaptor proteins (reviewed in Hirokawa et al., 2009; Kardon and Vale, 2009; Hirokawa et al., 2010) (**Figure 3**). Dynactin, a large multisubunit complex, is an essential motor protein adaptor as it has been shown to interact with both dynein and kinesin motors (Blangy et al., 1997; Deacon et al., 2003; Berezuk and Schroer, 2007) (**Figure 3**). Dynactin has been shown to target dynein to microtubule plus ends, link dynein to cargo, and enhance dynein/kinesin processivity, i.e. ability of the motor to take multiple steps along the microtubule without detaching (Berezuk and Schroer, 2007; reviewed in Kardon and Vale, 2009).

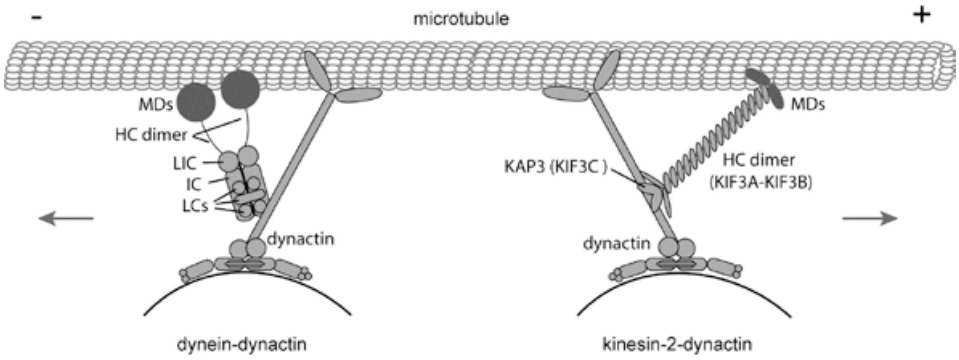


**Figure 3.** The structure of two motor protein complexes, dynein-dynactin and kinesin-2-dynactin. In dynein, a heavy chain (HC) dimer binds two intermediate chains (ICs) and two light intermediate chains (LICs). ICs interact with light chains (LCs) (Kardon and Vale, 2009). KIF3A and KIF3B subunits form a kinesin-2 heavy chain dimer which associates with KAP3 (KIF3C) and dynactin. Dynactin enhances the processivity of the motor proteins. The heavy chains contain motor domains (MDs). Modified from Hirokawa et al., 2010.

### *2.1.2.3 Membrane fusion*

Membrane fusion reactions are carried out by soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) proteins in collaboration with tethering factors, Rab proteins and vesicle coat complexes (reviewed in Jahn and Scheller, 2006; Cai et al., 2007; Brocker et al., 2010; Angers and Merz, 2011)

(**Figure 2**). Prior to the membrane fusion reactions opposing membranes need to be brought in close proximity. Vesicles and organelles moving along cytoskeletal tracks are captured on a correct target membrane by tethering factors (**Figure 2**). Tethering factors are generally considered to bridge two membrane compartments by interacting with specific Rab GTPases and SNARE proteins (reviewed in Cai et al., 2007; Brocker et al., 2010; Angers and Merz, 2011). In addition, vesicle coat components have been reported to interact with tethers (reviewed in Cai et al., 2007; Angers and Merz, 2011), which argues against the original hypothesis that vesicles must remove their coats early after vesicle budding in order to be recognised at the target membrane (reviewed in Kirchhausen, 2000; Bonifacino and Glick, 2004).

With almost 40 different SNARE proteins and a large repertoire of assistant proteins mammalian cells are able to direct membrane fusions with a high fidelity (McNew et al., 2000; Bock et al., 2001; Angers and Merz, 2011). SNARE proteins represent the core elements in the process (Sollner et al., 1993; Weber et al., 1998; reviewed in Jahn and Scheller, 2006). SNARE proteins have C-terminal transmembrane domain or are at least anchored to the membrane by a fatty acid modification. The transmembrane domain is linked to the cytoplasmic SNARE domain by a short linker region (**Figure 4**). Neuronal SNAP-25 (25-kDa synaptosome-associated protein) and SNAP-25-like proteins are exceptional among SNARE proteins since they contain two SNARE motifs (reviewed in Jahn and Scheller, 2006). Each transport vesicle is loaded by one type of SNARE protein, originally termed v-SNARE, while each target membrane usually contains three types of SNARE proteins, termed t-SNAREs (Sollner et al., 1993; Rothman, 1994) (**Figure 4**). A more adequate classification scheme is based on the amino acid identity of the critical residue in the SNARE domain; R-SNARE and Q-SNARE for arginine and glutamine, respectively, with Q-SNARE proteins being further divided into three subtypes, Qa, Qb and Qc (Bock et al., 2001). The latter classification is more adequate in organelle fusions, especially in its homotypic form. SNARE domain sequences of SNARE proteins are related to following neuronal proteins: vesicle-associated membrane protein (VAMP2, also known as synaptobrevin) (R-SNAREs), syntaxin 1A (Qa-SNAREs), N-terminal segment of SNAP-25 (Qb-SNAREs) and C-terminal segment of SNAP-25 (Qc-SNAREs) (Bock et al., 2001).

Interactions of appropriate SNARE proteins at membrane contact sites lead to SNARE complex formation (docking) (**Figure 4**). The core of the SNARE complex is a bundle of four parallel intertwined α-helical SNARE domains each representing one of four SNARE subtypes (R-, Qa-, Qb- or Qc-SNARE). In case of SNAP-25 (and related proteins), only two distinct t-SNARE proteins are required since SNAP-25 provides two SNARE domains on the t-SNARE complex. During the fusion process the configuration of the SNARE complex is transformed from a trans-SNARE, where opposing SNAREs are in separate membranes, to a cis-SNARE where both components reside in the same membrane (reviewed in Jahn and Scheller, 2006) (**Figure 4**). To maintain cellular homeostasis, vesicular components

of the fusion machinery are dissociated from the t-SNARE proteins and recycled back to the donor membrane. The disassembly of the cis-SNARE complex is mediated by NSF linked to the SNARE complex by soluble NSF attachment protein (α-SNAP) (Zhao et al., 2012).

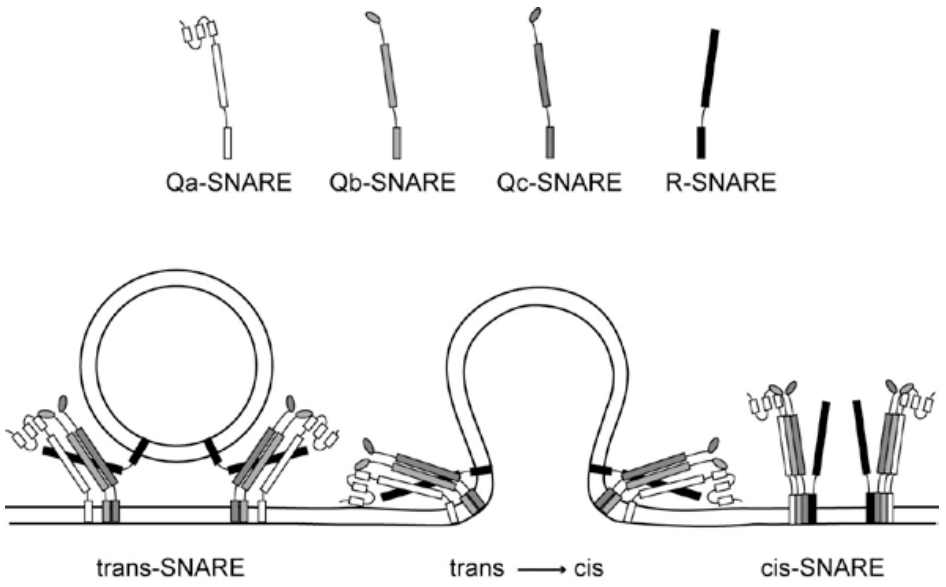


**Figure 4.** The structure and complex formation of soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) proteins. Three SNAREs, termed Qa-, Qb, and Qc-SNARE, on an acceptor membrane associate with one R-SNARE on a donor membrane. Resulting complex, a bundle of four parallel intertwined α-helical SNARE domains, proceeds from trans-configuration through trans-to-cis-transition state to a cis-configuration during membrane fusion reaction. Modified from Jahn and Scheller, 2006.

### 2.1.3 Rab GTPases

Rab (Ras-like proteins in brain) GTPases represent the largest subgroup of the Ras superfamily of small monomeric GTPases. Small GTPases are molecular switches that are activated by GTP-binding and inactivated upon GTP hydrolysis. At least 70 different Rab GTPases are encoded in mammals, and 11 orthologues (Ypt proteins and Sec4 protein) in yeast (Lazar et al., 1997; Jiang and Ramachandran, 2006).

#### *2.1.3.1 Basic characteristics of Rab GTPases*

Functions of Rab GTPases are tightly coupled to membrane association and nucleotide status (reviewed in Stenmark, 2009). Rabs are synthesised as GDP-bound soluble proteins that are presented to geranylgeranyltrasferase (RabGGTase) by Rab escort proteins (REPs). Geranylated Rab is then captured by GDP dissociation inhibitor (GDI) which masks the novel lipid modification at the C-terminus of the protein (reviewed in Goody et al., 2005). At the correct destination, Rab is released from the GDI by a membrane-bound GDI displacement factor (GDF) and associates with a membrane via the geranyl-geranyl moiety (Dirac-Svejstrup et al., 1997; Sivars et al., 2005). Due to their low intrinsic GDP-release and GTP-hydrolysis activities Rabs need guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) to catalyse the GDP to GTP conversion and GTP hydrolysis, respectively. The GDP-GTP transit results in conformational changes in the Rab protein and subsequently, interactions with downstream interaction partners (effectors) (reviewed in Barr and Lambright, 2010). These interactions are active until Rab undergoes GTP hydrolysis, and is extracted from the membrane by GDI (reviewed in Goody et al., 2005). Rabs remain in the cytosol until they are recruited again to the membrane to activate intracellular function specific for them. A schematic model of the functional cycle of Rab GTPases is represented in **Figure 5**.

Rab GTPases have characteristic, although partly overlapping membrane localisation within the cell (reviewed in Stenmark, 2009; Hutagalung and Novick, 2011). Mechanisms that determine the compartment-specific membrane positioning of Rabs are incompletely understood. It has been suggested that membrane-bound GDFs play an important role in the Rab targeting as they are able to expose the prenyl moiety and thus enhance membrane insertion of the Rab protein. However, to date only one GDF has been identified in mammals and it seems to interact with multiple Rab proteins (Dirac-Svejstrup et al., 1997; Sivars et al., 2005). Therefore, it is currently uncertain whether Rab GTPases are localised to specific membranes via interactions with compartment-specific GDFs. It has also been suggested that GDFs may only mediate the initial delivery of Rab proteins to the membrane, and other factors, including lipid microdomain constituents, GEFs and/or downstream Rab interacting proteins (effectors) may direct the steady-state localisation of each individual Rab protein (Pfeffer, 2005).

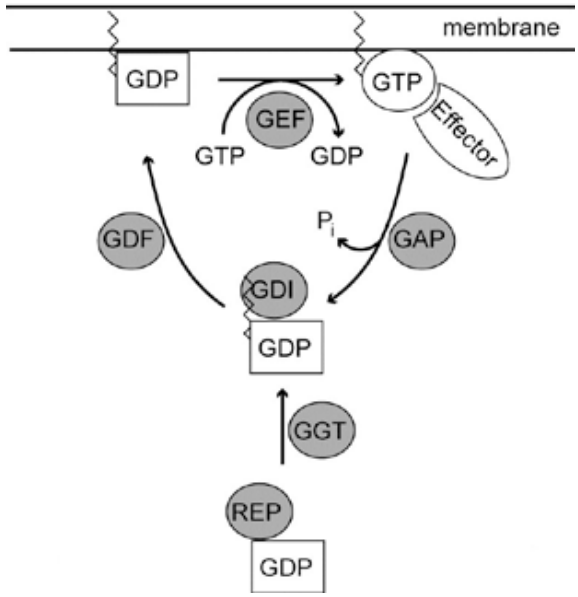


**Figure 5.** The nucleotide and the membrane association/dissociation cycle of Rab GTPases. A Rab escort protein (REP) presents a newly synthesised, inactive GDP-bound Rab GTPase to a geranyl-geranyltransferase (GGT). A geranyl-geranyl moiety is masked by a GDP dissociation inhibitor (GDI), which keeps Rab in the cytoplasm. The mask is removed by a membrane-bound GDI displacement factor (GDF), which allows Rab to associate with the membrane. Exchange of GDP to GTP by a guanine exchange factor (GEF) results in a conformational change of Rab GTPase. The active GTP-bound conformation is recognised by a specific Rab effector(s) and a resulting Rab GTPase effector complex is then able to execute its function(s) until the inactive conformation of Rab is restored by a GTPase-activating protein (GAP) and extracted from the membrane by the GDI.

Proteins that interact with Rabs usually in their GTP-bound state and mediate their regulatory actions are termed Rab effectors. Rabs and their effectors are directly involved in membrane trafficking and in fact, they play a role at almost every stage of vesicular trafficking. Rabs are pivotal in vesicle tethering and fusion, like mentioned in Chapter 2.1.2.3. In addition, Rabs are involved in cargo selection, coat assembly and vesicle uncoating. Furthermore, Rabs also mediate vesicle and organelle motility along actin and microtubules and they seem to be important co-ordinators in attaching the right carrier vesicle/organelle to the right myosin, kinesin or dynein motor protein complex. (reviewed in Stenmark, 2009; Hutagalung and Novick, 2011).

#### *2.1.3.2 Rab7*

Rab7 has been localised to LEs, lysosomes, melanosomes and (auto)phagosomal membranes (Chavrier et al., 1990; Feng et al., 1995; Meresse et al., 1995; Vitelli et al., 1997; Bucci et al., 2000; Gomez et al., 2001; Gutierrez et al., 2004; Jager et al., 2004). Trafficking experiments using different ligands/cargos have indicated that Rab7 is required for the late steps of endocytosis, although it has remained controversial as to whether Rab7 regulates membrane trafficking between early and late endosomes, or between late endosomes and lysosomes, or both (Feng et al., 1995; Meresse et al., 1995; Mukhopadhyay et al., 1997; Vitelli et al., 1997; Press et al., 1998; Bucci et al., 2000; Vonderheit and Helenius, 2005; Ceresa and Bahr, 2006; Vanlandingham and Ceresa, 2009). Most of the reports support the view that at least the trafficking between LEs and lysosomes is dependent on Rab7. In fact, Rab7 seems to be the only Rab-family protein shown to regulate the membrane trafficking step between these two compartments. In addition, Rab7 is needed for maturation of late autophagic vesicles (Gutierrez et al., 2004; Jager et al., 2004), axonal retrograde trafficking of neurotrophins and neurite outgrowth (Saxena et al., 2005; Deinhardt et al., 2006; Cogli et al., 2010). The role that Rab7 plays in neurotrophin transport has been suggested (Mitra et al., 2011) to contribute to neurodegeneration of sensory and motor neurons in Charcot-Marie-Tooth type 2B (CMT2B) disease, a rare autosomal recessive axonal neuropathy caused by gain-of-function mutations in the GTP binding and hydrolysis domain of Rab7 (Verhoeven et al., 2003; Spinosa et al., 2008). Rab7 mutations may also cause autosomal recessive hereditary sensory and autonomic neuropathy (HSAN) (Klein et al., 2005).

Several Rab7 effectors have been identified, namely Rabring7 (Rab7-interacting RING finger protein) (Mizuno et al., 2003), RILP (Rab7 interacting lysosomal protein) (Cantalupo et al., 2001), ORP1L (oxysterol-binding protein-related protein 1L) (Johansson et al., 2005), FYCO1 (FYVE and coiled-coil domain containing 1) (Pankiv et al., 2010), Rubicon (Sun et al., 2010), retromer (Rojas et al., 2008; Seaman et al., 2009) and HOPS (homotypic fusion and vacuole protein sorting) tethering complex (Price et al., 2000; Wurmser et al., 2000). These have significantly contributed to the understanding of Rab7 functions in the last few years (reviewed in Wang et al., 2011). Several Rab7 effector complexes play a role in microtubular transport, and both dynein and kinesin motor proteins seem to be regulated by Rab7 effector complexes. Rab7-FYCO1-kinesin complex is involved in plus-end directed microtubular transport of autophagic vesicles (Pankiv et al., 2010). RILP and ORP1L are part of the same Rab7-RILP-ORP1L complex which recruits the dynein-dynactin motor complex to LEs/lysosomes and thus, facilitates minus end-directed movement of these organelles (Jordens et al., 2001; Johansson et al., 2005; Johansson et al., 2007). Furthermore, Rab7-RILP-ORP1L complex is able to regulate the intracellular positioning of LEs on the basis of the luminal cholesterol content and subsequent interactions of ORP1L with an ER membrane protein (Rocha et al., 2009). Rabring7 may also be involved in dynein-mediated transport of

LEs since it tends to induce their perinuclear aggregation (Mizuno et al., 2003). Rabring7 has also E3 ligase activity and it affects the degradation of epidermal growth factor (Mizuno et al., 2003; Sakane et al., 2007). However, it does not ubiquitinate EGF (Sakane et al., 2007). Due to the finding that RILP also interacts with the ESCRT complex, which is involved in the lysosomal sorting of ubiquitinated proteins (Progida et al., 2006; Wang and Hong, 2006; Progida et al., 2007), it was suggested that perhaps also Rabring7 is somehow involved in the process (Sakane et al., 2007). ESCRT is not the only coat complex that Rab7 may bind. Rab7 associates with retromer and regulates its recruitment to LEs (Rojas et al., 2008; Seaman et al., 2009). Finally, the yeast orthologue of Rab7, Ypt7, has been shown to enhance late endosomal membrane fusion by recruiting the HOPS tethering factor (Price et al., 2000; Wurmser et al., 2000; reviewed in Wang et al., 2011).

## 2.2 Lysosomes

### 2.2.1 Characteristics of lysosomes

Lysosomes, first discovered and named by de Duve over 50 years ago (Duve, 1975), are primarily characterised as acidic organelles which contain the primary hydrolysis machinery of the cell required for the degradation of proteins, lipids, and carbohydrates, but also for whole organelle degradation. Lysosomes are globular or tubular-shaped vacuoles with variable electron-dense constituents. Their lumen is acidic (pH 4.5 – 5) and contains membrane sheets and a few intraluminal vesicles. Lysosomes are limited by a single phospholipid bilayer rich in glycosylated proteins which form a so-called protective glycogalyx on the luminal side of the limiting membrane. In addition, lack of mannose 6-phosphate receptors is characteristic to lysosomes. Lysosomal compartments receive their resident proteins via different targeting mechanisms, and macromolecules destined for lysosomal degradation enter the lysosome directly via specific membrane transporters or indirectly by membrane fusion reactions with LEs and (auto)phagosomal organelles. Degradation products are transported across the lysosomal membrane and released into the cytosol to allow their reuse in cell metabolism. Lysosomes also release degradative enzymes into the cytosol in order to contribute to apoptosis, or extracellular space via fusing with the plasma membrane in order to degrade extracellular material and to target lysosomal constituents to the cell surface. (Reviewed in Eskelinen et al., 2003; Luzio et al., 2007; Braulke and Bonifacino, 2009; Lubke et al., 2009; Schroder et al., 2010).

### 2.2.2 Proteome and functions of lysosomes

Lysosomes contain more than 60 different soluble acid hydrolases and non-enzymatic cofactors (e.g., glycosidases, lipases, nucleases, peptidases, phosphatases, proteinases, sulfatases) (Schroder et al., 2010 and references therein). Soluble lysosomal proteins are synthesised as inactive large precursors. The precursors contain an N-terminal signal sequence which mediates protein translocation into the ER lumen and subsequently, is cleaved by a signal peptidase. Lysosomal proteins usually become heavily glycosylated during their transport along the biosynthetic pathway. This glycosylation process initiates in the ER and continues in the Golgi compartment (reviewed in Braulke and Bonifacino, 2009). Oligosaccharide modifications play a significant role in the endolysosomal targeting, a process that occurs in a receptor-mediated fashion (reviewed in Braulke and Bonifacino, 2009; Saftig and Klumperman, 2009). Most nascent soluble lysosomal proteins receive a mannose 6-phosphate moiety in the Golgi. The moiety is then captured by two different mannose 6-phosphate receptors (MPRs) in the TGN; 46 kDa cation-dependent MPR (CD-MPR or MPR46) and 300 kDa cation-independent MPR (CI-MPR or MPR300). MPRs contain dileucine and tyrosine-based sorting signals in their cytoplasmic domains which are recognised by clathrin-associated AP-1 and/or GGA adaptor proteins in the TGN. These adaptor proteins then recruit MPR-associated lysosomal cargo into clathrin-coated vesicles to be transported along the biosynthetic pathway, usually directly to the endosomal system, although, some may also escape to the cell surface. Upon entering the acidic environment of LEs, cargo become dephosphorylated [except in the brain (Sleat et al., 1996; Sleat et al., 2005)], and subsequently, are released from the receptor (reviewed in Braulke and Bonifacino, 2009; Saftig and Klumperman, 2009). This allows delivery of lysosomal soluble proteins to their final destination whereas MPRs are recycled back to the TGN (Braulke and Bonifacino, 2009; Saftig and Klumperman, 2009). In addition, soluble lysosomal proteins undergo a limited proteolysis in the acidic environment of Les, which results in their activation. This is an efficient system to avoid cellular damages caused by prematurely active lysosomal enzymes.

MPR-mediated transport is not the only way to reach the lysosomal lumen, as evidenced by the fact that some soluble lysosomal proteins are able to reach the lysosome in conditions defective for the mannose 6-phosphate targeting system (Owada and Neufeld, 1982; Waheed et al., 1982; Little et al., 1987; Dittmer et al., 1999; Gelfman et al., 2007). Concomitantly, alternative receptor proteins have been identified. For example, β-glucocerebrosidase is specifically transported to lysosomes in association with lysosomal integral membrane protein type 2 (LIMP-2), while the sphingolipid activator proteins (saposins), the soluble hydrolases cathepsin D and H, and acid sphingomyelinase may use sortilin in their lysosomal targeting (Petersen et al., 1997; Lefrancois et al., 2003; Ni and Morales, 2006; Reczek et al., 2007; Canuel et al., 2008).

First lysosomal membrane proteins, namely lysosome-associated membrane proteins (LAMPs) and lysosomal integral membrane proteins (LIMPs), were described in the 1980s (Chen et al., 1985; Lewis et al., 1985; Barriocanal et al., 1986). However, a more thorough understanding on lysosomal membrane constituents has awaited for development of appropriate mass spectrometric analyses, and many new lysosomal membrane proteins have just recently been identified (reviewed in Lubke et al., 2009; Schroder et al., 2010). Depending on the study, the number of identified lysosomal membrane proteins ranges from one hundred to several hundreds (Schroder et al., 2010 and references therein). Compared to soluble lysosomal proteins, less is known on the targeting of lysosomal membrane proteins. Many of them have been observed to utilise clathrin/AP-1/GGA-dependent mechanisms to exit from the TGN (reviewed in Bonifacino and Traub, 2003; Braulke and Bonifacino, 2009; Saftig and Klumperman, 2009). Lysosomal membrane proteins may travel to the endolysosomal system either directly or indirectly via the cell surface. Together with the observations that some lysosomal membrane proteins may contain several distinct sorting motifs, and that their targeting may not depend on conventional clathrin/GGA-adaptor system, this indicates that lysosomal membrane proteins may use distinct trafficking pathways in different cellular conditions (Saftig and Klumperman, 2009).

Functions of lysosomal membrane proteins involve the establishment of the pH gradient between cytoplasm and lysosomal lumen, compartmentalisation of hydrolases, membrane fusions with other organelles, and transport across the lysosomal membrane (Saftig et al., 2010). The most abundant protein constituents of lysosomal membranes are the highly glycosylated lysosome-associated membrane proteins 1 and 2 (LAMP-1 and LAMP-2, respectively), and lysosomal integral membrane protein type 1 and 2 (LIMP-1 and LIMP-2) that together account approximately 50% of the total membrane protein content of the lysosome (Winchester, 2001). These proteins form a protective glycocalyx coat on the inner leaflet of the lysosomal membrane but are implicated in other lysosomal functions as well (see below) (Eskelinen et al., 2003; Saftig and Klumperman, 2009). V-type $H^+$ ATPase is a functionally important lysosomal membrane protein as it establishes the internal acidic pH essential for enzymatic activities and maturation of hydrolases (reviewed in Hinton et al., 2009). Most importantly, lysosomes comprise several types of transporters that either export lysosomal catabolites from the lysosomal lumen to cytosol or import macromolecules for degradation (reviewed in Eskelinen et al., 2003; Schroder et al., 2010). Indeed, more than 20 different transport processes have been demonstrated for the lysosomal membrane, including transport of amino acids, carbohydrate derivatives, inorganic ions, nucleotides and small peptides (Sagne and Gasnier, 2008). For example, sialin and cystinosin transport the degradation products sialic/glucuronic acids and cystine, respectively, to the cytosol (reviewed in Ruivo et al., 2009). Another lysosomal integral membrane protein, NPC1 (Niemann-Pick C1), has been implicated in the removal of LDL-derived

cholesterol from lysosomes (reviewed in Peake and Vance, 2010). Fusion of lysosomes with LEs, (auto)phagosomes, and the plasma membrane are mediated by a specific R-SNARE protein, vesicle-associated membrane protein 7 (VAMP7) (reviewed in Luzio et al., 2007; Luzio et al., 2010). Furthermore, lysosomal membrane transiently associates with motor proteins, most likely dynein, kinesin-1 and kinesin-2 (Brown et al., 2005 and references therein; Loubery et al., 2008), which move lysosomes to membrane fusion sites. These two above mentioned processes are regulated by Rab7 GTPase which is specifically localised to LEs and lysosomes as discussed in Chapter 2.1.3.2.

Lysosomes are involved in a wide range of cellular activities. Lysosomes and their constituents are utilised in the turnover of long-lived proteins and organelles, downregulation of activated plasma membrane receptors, cholesterol homeostasis, plasma membrane repair, antigen processing and presentation, and inactivation of pathogens (reviewed in Eskelinen et al., 2003; Saftig and Klumperman, 2009; Schroder et al., 2010). Furthermore, lysosomal hydrolases are released into the extracellular space via constitutive secretion or lysosomal exocytosis, and utilised in tumor metastasis and propagation, angiogenesis, bone remodelling and cell death signalling (reviewed in Watts, 2011). LAMP-1 and especially LAMP-2 have an essential role in the lysosomal function as they participate in the (auto)phagosomal maturation which is an important process contributing to multiple cellular activities (reviewed in Eskelinen et al., 2003; Eskelinen and Saftig, 2009; Saftig et al., 2010). LAMPs have also been indicated to regulate the dynein/dynactin-dependent movement of (auto)phagosomes to perinuclear areas in order to facilitate their fusion with lysosomes and thus, final maturation (Huynh et al., 2007 and references therein). In addition, LAMP-2A isoform interacts with a subset of cytosolic proteins to enhance their direct transport across the lysosomal limiting membrane in a process termed chaperone-mediated autophagy (Cuervo and Dice, 1996). Distinct from its (auto)phagosomal functions, LAMP-2 is additionally involved in endosomal/lysosomal cholesterol export (Schneede et al., 2011). The LAMPs are an excellent example showing that lysosomal proteins may have effects on a wide range of cellular activities.

### 2.2.3 Lysosomal storage disorders

Lysosomal storage disorders (LSDs) (Hers, 1965) mirror the physiological importance of the lysosomal system. Indeed, defects in more than 40 different lysosomal or lysosomal system-associated proteins have so far been implicated as causing storage disorder with widespread tissue and organ involvement. Individually, LSDs are rare but more common as a group, with a combined prevalence being about 1:5000 (Futerman and van Meer, 2004).

LSDs are mostly recessively inherited, fatal diseases characterised by a progressive accumulation of undegraded metabolite(s) in the lysosome but also in

other intracellular and extracellular locations. Storage in a given LSD can be rather heterogeneous. Several types of macromolecules have been identified to be stored in LSDs, including sphingolipids, mucopolysaccharides, oligosaccharides, glycoproteins, lipids, sulfatides, and specific proteins and amino acids (Futerman and van Meer, 2004; Ballabio and Gieselmann, 2009). Identity of accumulated material has been used as a basis for classification of LSDs; for example, lipids accumulate in lipidosis and mucopolysaccharides in mucopolysaccharidosis. However, this classification scheme does not necessarily depict the type of primary cellular defect in a given LSD as defects in functionally different proteins may lead to accumulation of similar intracellular compound(s). Therefore, LSDs have alternatively been grouped according to the characteristics of the defective protein (Futerman and van Meer, 2004; Bellettato and Scarpa, 2010). Most of the LSDs are due to mutations in soluble lysosomal hydrolases. Mutations can directly reduce their catalytic activity but may also affect indirectly via disturbing their folding and glycosylation in the ER and the Golgi complex. Lysosomal storage can also result from mutations in non-enzymatic lysosomal proteins (including above-mentioned sialin, cystinosin, NPC1 and LAMP-2), or even in non-lysosomal proteins, and both soluble and integral membrane proteins can be involved. These cases are due to defects of proteins acting either in the activation/stabilisation, trafficking, or posttranslational modification of lysosomal enzymes, or transportation of degradation end products or ions across the lysosomal limiting membrane (Futerman and van Meer, 2004; Ruivo et al., 2009). Furthermore, LAMP-2 deficiency has shown that defective organelle fusion and motility may also underlie lysosomal storage accumulation (Ruivo et al., 2009). Examples of primary functional defects leading to lysosomal storage disorders are presented in **Table 1**. Although the genetic defect has been identified in the known LSDs, the primary functional insult has remained elusive in some cases. A representative example are neuronal ceroid lipofuscinoses, a group of diseases caused by unknown defects in lysosomal and non-lysosomal proteins (see Chapter 2.3.1).

**Table 1.** Examples of intracellular defects that may result in lysosomal storage disorder.

| Defective intracellular function/process | Primary defective protein | Protein function | Associated disease |
|---|---|---|---|
| **Enzymatic activity of hydrolases** | β-glucosidase (soluble lysosomal hydrolase) | degradation of glucocerebroside | Gaucher disease |
| **Enzyme cofactor** | sphingolipid activator protein C (soluble lysosomal protein) | activator of β-glucosidase | Gaucher disease |
| | cathepsin A (soluble lysosomal protein) | protection for processing, sorting and stability of β-galactosidase and neuraminidase | Galactosialidosis |
| **Trafficking of hydrolases** | N-acetylglucosamine-1-phosphate transferase (soluble Golgi enzyme) | mannose 6-phosphorylation of hydrolases | I-cell disease (mucolipidosis II) |
| **Posttranslational modification of hydrolases** | Cα-formylglycine-generating enzyme (soluble ER enzyme) | convertion of critical cysteine residue to a Cα-formylglycine residue | Multiple sulfatase deficiency |
| **Export of degradation products** | sialin (lysosomal membrane protein) | sialic acid transporter | Sialic acid storage disease (Salla disease) |
| | NPC1 (lysosomal membrane protein) | involved in export of cholesterol | Niemann-Pick type C |
| | NPC2 (soluble lysosomal protein) | involved in export of cholesterol | Niemann-Pick type C |

In addition to the accumulation of storage material, LSDs are characterised by their tendency to affect several tissues and organs. However, each individual LSD has a distinct clinical and pathological picture, a phenomenon most likely resulting from differences in the nature of stored material and subsequently, in the effects they have on cellular activities (Ballabio and Gieselmann, 2009). Moreover, most LSDs may manifest themselves as various clinical phenotypes from early severe disease to late-onset mild phenotypes. However, phenotype may not necessarily depend on a given genotype, as highlighted by the fact that disease course can be highly variable among patients carrying the same disease mutation. Together, these observations

suggest that the disease outcome may additionally depend on modifying genes and environmental factors (Futerman and van Meer, 2004).

Approximately two-thirds of LSDs affect the brain where the disease manifests as seizures, mental and physical retardation, sensory loss, sleep and behavioural problems and neurodegeneration (Walkley, 2009). The mechanisms that connect storage accumulation and cellular dysfunction are not fully understood but they seem to occupy several biochemical and cellular pathways. Indeed, many processes have been shown to be affected in LSDs including intracellular signalling, general lysosomal degradation, membrane trafficking, autophagosytosis, intracellular $Ca^{2+}$ homeostasis, mitochondrial functions and lipid metabolism (Ballabio and Gieselmann, 2009; Bellettato and Scarpa, 2010; Parkinson-Lawrence et al., 2010). It is considered that the pathogenesis of neurodegenerative LSDs is a multistep process where the accumulation of storage material induces a cascade of secondary events which then ultimately lead to neuronal dysfunction, microglia activation and subsequently, collapse of the neuronal homeostasis (Walkley, 2009; Bellettato and Scarpa, 2010). Moreover, it is noteworthy that some LSD-associated proteins have been suggested to localize to and exhibit functions in non-lysosomal compartments, including the plasma membrane, synaptic vesicles and LAMP-1-negative vesicles along the neuronal projections and synaptosomes (Mencarelli et al., 2005; Ruivo et al., 2009 and see Chapter 2.3.1.2). Therefore it is possible that defects in extralysosomal functions may be of high importance in the pathogenesis of some LSDs.

## 2.3 Juvenile CLN3 disease (earlier referred to as juvenile neuronal ceroid lipofuscinosis, JNCL, Batten disease)

### 2.3.1 Neuronal ceroid lipofuscinosis (NCL)

#### *2.3.1.1 Main characteristics of NCLs*

Neuronal ceroid lipofuscinoses (NCLs) are a group of hereditary progressive neurodegenerative disorders usually occurring in childhood. Except a few rare cases, NCLs follow autosomal recessive type of inheritance. NCLs are characterised by variable but progressive symptoms including epileptic seizures, blindness, decline in motor and cognitive skills, cerebellar and cerebral cortical atrophy, and premature death (reviewed in Haltia, 2003; and Haltia, 2006). The most unifying feature of the NCLs is the lysosomal accumulation of autofluorescent storage material, termed ceroid lipofuscin or lipofuscin-like, in a variety of tissues, including brain (reviewed in Haltia, 2003; Mole et al., 2005; and Seehafer and Pearce, 2006) and thus, NCLs are also considered as lysosomal storage disorders (LSDs). However, the stored material in NCLs is not a disease-specific substrate. The storage deposits in NCLs are mainly composed of mitochondrial ATP synthase subunit c (Palmer et al., 1989; Fearnley et al., 1990; reviewed in Haltia, 2003; and Mole et al., 2005) or saposin A

and D (Tyynela et al., 1993; reviewed in Haltia, 2003; and Mole et al., 2005) which may accumulate in other lysosomal storage disorders as well (Morimoto et al., 1990; Elleder et al., 1997b). Based on the ultrastructural appearance, NCL storage deposits are classified into four basic types: granular osmiophilic deposits (GROD), curvilinear profiles (CLP), fingerprint profiles (FPP), and rectilinear profiles (RLP). The storage material in a particular case may predominantly show only one of the four main morphologies or may have a mixed fine structure (reviewed in Haltia, 2003; and Mole et al., 2005). Examples of these storage materials are shown in **Figure 6**.

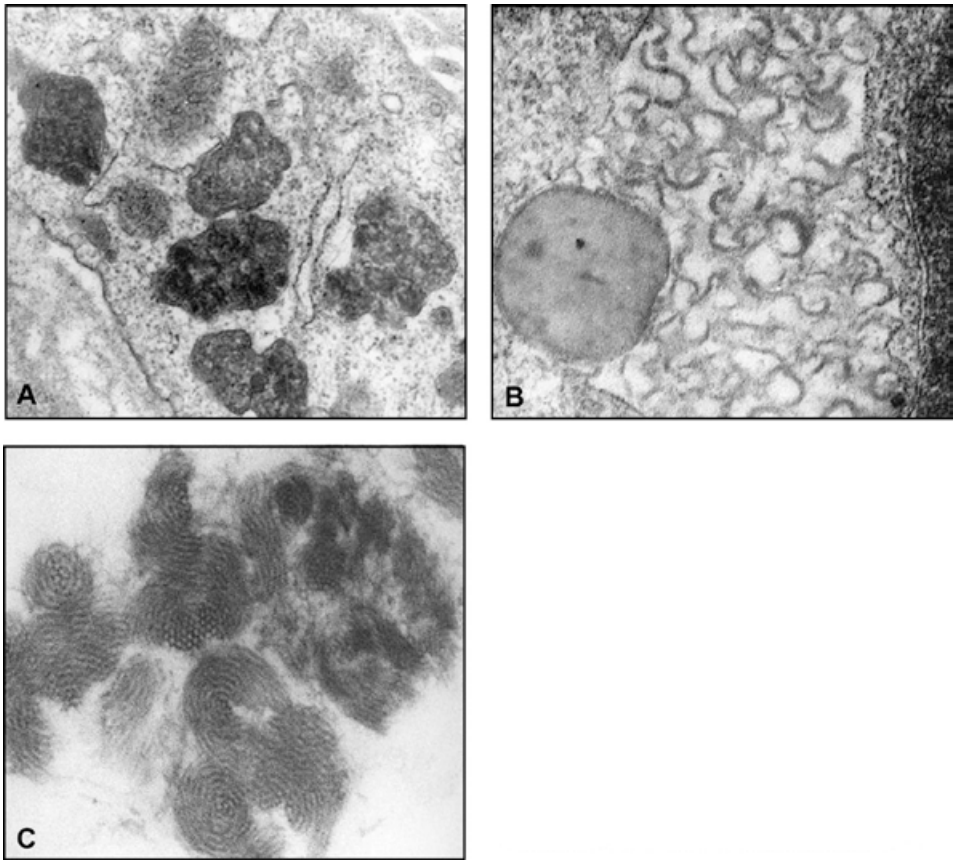


**Figure 6.** Ultrastructural appearance of storage deposits characteristic to neuronal ceroid lipofuscinoses. A) Granular osmiophilic deposits (GROD), B) curvilinear profiles (CLP), and C) fingerprint profiles (FPP). Figure courtesy of Professor Juhani Rapola, University of Helsinki.

The human NCLs were originally classified as infantile (INCL, Haltia-Santavuori), late infantile (LINCL, Jansky-Bielschowsky), juvenile (JNCL, Spielmeyer-Vogt or Batten disease), and adult NCL (ANCL), the latter of which can be separated in three clinical phenotypes, recessively inherited Kufs disease type A or type B and dominantly inherited Parry disease. With time, it has become evident that NCLs are clinically and genetically heterogeneous, and several causative genes and phenotypic variant forms are now recognised, especially among late infantile onset families (thus earlier referred to as variant LINCL, or vLINCL, to distinguish from classic LINCL patients) (reviewed in Haltia, 2003; Mole et al., 2005; and Kousi et al., 2012). Therefore, a new classification has been adopted. New nomenclature is primarily based on the affected gene, specified with the age of onset and other main clinical and pathological features. For example, juvenile onset NCLs associated with *CLN3* mutations are classified as CLN3 disease, classic juvenile (juvenile CLN3 disease) while infantile onset cases with CLN3 defects are referred to as CLN3 disease, infantile (or infantile CLN3 disease) (Kousi et al., 2012; NCL Resource, 2012). To date, nine causative genes have been identified for NCLs, including *PPT1* (*CLN1*), *TPP1* (*CLN2*), *CLN3*, *DNAJC5* (*CLN4*), *CLN5*, *CLN6*, *MFSD8* (*CLN7*), *CLN8*, and *CTSD* (*CLN10*) (Noskova et al., 2011; reviewed in Kousi et al., 2012). In addition, heterozygous mutations in two additional genes encoding chloride channel 6 (CLCN6) and N-sulfoglucosamine sulfohydrolase (sulfamidase, SGSH), have been reported in some patients (Poet et al., 2006; Sleat et al., 2009). Reported mutations in NCL-related genes are collected in NCL Mutation Database (NCL Resource). In addition, several additional NCL susceptibility genes are thought to exist. Juvenile onset NCL not due to mutations in *CLN3* (designated as juvenile CLN9 disease), and a variant form of late infantile NCL present in Turkish families, and negative for *CLN6*, *CLN8* and *MFSD8* (*CLN7*), are expected to result from mutations in so far unidentified genes (reviewed in Siintola et al., 2006a; Siintola et al., 2007; Kousi et al., 2009; reviewed in Kousi et al., 2012). Furthermore, despite the recent progress in genetics of adult onset NCL a significant fraction of associated autosomal dominant adult onset NCL families and families associated with recessive Kufs type B are still of unknown genetic etiology (Sleat et al., 2009; Arsov et al., 2011; Noskova et al., 2011). Interestingly, a possible genetic interplay between NCL genes has recently been demonstrated (Kousi, 2011). Several heterozygous mutations in NCL loci were observed to associate with disease-causing mutations in other NCL loci. This suggests that other NCL genes may act as modifiers of a given NCL phenotype (Kousi, 2011). A summary of NCL genes, respective proteins, clinical phenotypes, and main characteristics of the associated storage material are shown in **Table 2**.

**Table 2.** The neuronal ceroid lipofuscinoses classified according to the affected gene. Clinical phenotypes (typical phenotypes are shown in bold), main storage component(s), and main ultrastructure(s) of the storage material are presented.

| Gene | Protein | Clinical phenotype | Main ultrastructure of the storage | Storage component |
|---|---|---|---|---|
| ***PPT1 (CLN1)*** | PPT1 | **CLN1 disease, classic infantile** | GROD | SAPs A and D |
| | | CLN1 disease, late infantile | GROD | |
| | | CLN1 disease, juvenile | GROD | |
| | | CLN1 disease, adult | GROD | |
| ***TPP1 (CLN2)*** | TPP1 | **CLN2 disease, classic late infantile** | CLP | subunit c |
| | | CLN2 disease, infantile | CLP | |
| | | CLN2 disease, juvenile | CLP | |
| ***CLN3*** | CLN3 | **CLN3 disease, classic juvenile** | FPP or FPP/CLP | subunit c |
| | | CLN3 disease, protracted | FPP or FPP/CLP | |
| | | CLN3 disease, infantile | No inclusions observed | |
| ***DNAJC5* (*CLN4*)** | CSPα | **adult NCL disease (autosomal dominant, Parry disease)** | GROD | SAPs A and D |
| ***CLN5*** | CLN5 | **CLN5 disease, late infantile variant** | FPP | subunit c |
| | | CLN5 disease, infantile | GROD | |
| | | CLN5 disease, juvenile | FPP | |
| | | CLN5 disease, adult | GROD | |
| ***CLN6*** | CLN6 | **CLN6 disease, late infantile variant** | FPP/CLP | subunit c |
| | | CLN6 disease, adult (autosomal recessive, Kufs type A) | FPP or GROD | |
| ***MFSD8 (CLN7)*** | MFSD8 | **CLN7 disease, late infantile variant** | FPP | n.d. |
| | | CLN7 disease, juvenile | n.d. | |
| ***CLN8*** | CLN8 | **CLN8 disease, late infantile variant** | FPP, FPP/CLP, FPP/CLP/GROD | subunit c |
| | | CLN8 disease, EPMR | CLP | |
| **n.d. (*CLN9*)** | n.d. | **CLN9 disease, juvenile variant** | GROD/ CLP | subunit c |
| ***CTSD (CLN10)*** | Cathepsin D | **CLN10 disease, congenital** | GROD | SAP D[1)] |
| | | CLN10 disease, juvenile | GROD | |

Abbreviations: CLN1 etc., ceroid lipofuscinosis, neuronal 1 etc.; n.d., not determined; CLP, curvilinear profiles; DNAJC5, DnaJ homologue subfamily C member 5; CSPα, cysteine string protein alpha; EPMR, progressive epilepsy with mental retardation; FPP, fingerprint profiles; GROD, granular osmiophilic deposits; MFSD8, major facilitator superfamily domain containing 8; PPT1, palmitoyl protein thioesterase 1; RLP, rectilinear profiles; SAP, saposin; TPP1, tripeptidyl peptidase 1.

[1)] Testing of saposin A was not reported.

References: Schultz et al., 2004; Siintola et al., 2006b; Noskova et al., 2011; Kousi et al., 2012.

NCLs are rare disorders with estimated collective incidence ranging from 0.6 in 100,000 to 13.6 in 100,000 live births, with juvenile NCL being the most common form of NCL worldwide (Rider and Rider, 1988; Santavuori, 1988; Claussen et al., 1992; Cardona and Rosati, 1995; Crow et al., 1997; Elleder et al., 1997a; Uvebrant and Hagberg, 1997; Taschner et al., 1999; Augestad and Flanders, 2006; Moore et al., 2008). Finland is enriched with NCLs, especially those associated with mutations in *CLN1*, *CLN3*, *CLN5*, and progressive epilepsy with mental retardation (EPMR)-causing mutations in *CLN8* (Mitchison et al., 1995; Santavuori et al., 2000; Anttonen et al., 2012).

#### *2.3.1.2 Proteins defective in NCLs*

Genes mutated in NCLs encode proteins that mostly represent two intracellular compartments, lysosomes and the ER. Four known NCL proteins, palmitoyl protein thioesterase 1 (PPT1), tripeptidyl peptidase 1 (TPP1), CLN5, and cathepsin D are soluble lysosomal proteins (reviewed in Kyttala et al., 2006; Schmiedt et al., 2010). Remaining known NCL proteins, with the exception of cysteine string protein alpha (CSPα), are polytopic integral membrane proteins. CLN3 and MFSD8 localise to lysosomal membranes (Siintola et al., 2007) (for CLN3 references, see Chapters 2.4.2 and 2.4.3) whereas CLN6 and CLN8 mainly reside in the ER (reviewed in Kyttala et al., 2006).

A common feature between PPT1, CLN3 and CLN8 in neurons is their tendency to localise to additional vesicular compartments in the cell periphery, which suggests that the proteins may have specific functions in excitatory cells (Isosomppi et al., 1999; Heinonen et al., 2000; Lehtovirta et al., 2001; Ahtiainen et al., 2003; Lonka et al., 2004) (for CLN3 references, see Chapter 2.4.3). In addition, CSPα is anchored to the surface of synaptic vesicles, among other exocytotic organelles (reviewed in Johnson et al., 2010), further emphasizing a potential role of NCL proteins in synaptic compartments.

CSPα, the best characterised NCL-related protein, exhibits chaperone activity and plays a pivotal role in the protein quality control machinery required for continued synaptic transmission (Johnson et al., 2010; Sharma et al., 2011). Instead, the biological roles of the more commonly defective NCL proteins are less well characterised. They either lack homology to known proteins or functional domains (CLN5 and CLN6), or their homology does not reveal specific action of the protein (CLN3, MFSD8 and CLN8). MFSD8 belongs to the major facilitator superfamily of transporter proteins, substrates of which include sugars, drugs, inorganic and organic cations, and various metabolites (Saier et al., 1999; Siintola et al., 2007). By sequence similarity with members of the TLC (TRAM-Lag1-CLN8) family of proteins, CLN8 has been suggested to operate in sensing, biosynthesis, and trafficking of lipids (Winter and Ponting, 2002). CLN3 will be discussed in more detail in Chapter 2.4. Three of the NCL proteins represent lysosomal hydrolases with several reported *in vitro* substrates (PPT1, TPP1 and cathepsin D) (reviewed in

Kyttala et al., 2006; Jalanko and Braulke, 2009; and Getty and Pearce, 2011). However, their native substrates have not been confirmed and thus, the specific role of these NCL proteins are not clear either. PPT1 removes palmitate residues from proteins (Camp and Hofmann, 1993), TPP1 catalyses the removal of N-terminal tripeptides from the proteins (Sleat et al., 1997; Vines and Warburton, 1998), and human cathepsin D is an aspartic protease (Zaidi et al., 2008). Loss of the NCL proteins have been shown to affect several intracellular processes (reviewed in Kyttala et al., 2006; Jalanko and Braulke, 2009; and Getty and Pearce, 2011). Some of these affected pathways are common to different NCL proteins, including apoptosis (Cho and Dawson, 2000; Korey and MacDonald, 2003; Guarneri et al., 2004; Zhang et al., 2006b; Benes et al., 2008; Autefage et al., 2009; Tardy et al., 2009; Vantaggiato et al., 2009), intracellular trafficking (Ahtiainen et al., 2006; Buff et al., 2007; Saja et al., 2010; Cao et al., 2011), lysosomal pH homeostasis (Holopainen et al., 2001; Virmani et al., 2005), synaptic functions (Battaglioli et al., 1993; Mennini et al., 1998; Mennini et al., 2002; Virmani et al., 2005; Ahtiainen et al., 2007; Buff et al., 2007; Kielar et al., 2009; Saja et al., 2010), lipid metabolism (Vance et al., 1997; Griffin et al., 2002; Kakela et al., 2003; Hermansson et al., 2005; Ahtiainen et al., 2007; Benes et al., 2008; Jabs et al., 2008; Lyly et al., 2008), and oxidative stress (Bertamini et al., 2002; Guarneri et al., 2004). The finding that NCL proteins may affect same intracellular pathways suggests that NCL proteins are possibly functionally linked. This hypothesis is supported by the analyses indicating that NCL genes may act as modifiers of phenotype associated with other NCL genes (Kousi, 2011), and that at least some of the NCL proteins interact with each other (Vesa et al., 2002; Persaud-Sawin et al., 2007; Lyly et al., 2009). CLN5 and PPT1 have also been reported to have a common interaction partner outside the NCL protein family. The interaction with the $F_1$ complex of ATP synthase may link both PPT1 and CLN5 to lipid metabolism (Lyly et al., 2009).

### 2.3.2 Clinical and pathological findings in juvenile CLN3 disease

The first clinical symptom of classic juvenile onset NCL caused by mutations in *CLN3* (juvenile CLN3 disease or CLN3 disease, classic juvenile; MIM#204200) is almost invariably a visual failure, noticed between 4-10 years of age, and leading to blindness within a few years (Jarvela et al., 1997; reviewed in Mole et al., 2011). Seizures usually appear around the age of 10 years (Jarvela et al., 1997; Aberg et al., 2000b). With age, the seizure frequency and severity tend to increase. Signs of cognitive impairment can be detected at an early stage of the disease (Lamminranta et al., 2001). The greatest decline in intelligence as well as motor functions occurs between 11 and 15 years of age (Jarvela et al., 1997). Motor disturbances include impaired imbalance, rigidity, hypokinesia, stooped posture, and shuffling gate. Speech becomes dysarthric and speech difficulties usually accompany motor deficits (Jarvela et al., 1997). Magnetic resonance imaging and computed tomography show

cerebral and cerebellar atrophy usually after the age of 10-14 years (Raininko et al., 1990; Autti et al., 1996). The severity of cerebellar atrophy has been shown to correlate positively with disturbances in motor functions (Raininko et al., 1990; Nardocci et al., 1995; Autti et al., 1996). Juvenile CLN3 disease patients suffer from a number of behavioural and psychiatric problems such as aggressiveness, depression, sleep problems, hallucinations (Santavuori et al., 1993; Jarvela et al., 1997; Backman et al., 2005; Adams et al., 2006). Patients may also exhibit cardiac dysfunction and hyperandrogenism (Aberg et al., 2002; Ostergaard et al., 2011). CLN3 disease is also characterised by vacuolated lymphocytes (reviewed in Haltia, 2003; and Mole et al., 2005). Juvenile CLN3 disease leads to premature death at second or third decade, with the mean age at death being 24 years (Jarvela et al., 1997).

At the postmortem examination, juvenile CLN3 disease brains are almost normal in their macroscopic appearance but are moderately or even severely reduced in weight due to cerebral and cerebellar atrophy (Autti et al., 1997 and references therein). There is a mild to moderate loss of neurons in all parts of the affected brains. However, the overall degree of neuronal loss varies in different subfields (Autti et al., 1997; Haltia et al., 2001; Tyynela et al., 2004). Detailed neuropathological analyses have revealed a selective loss of neurons in the cerebral cortical layers II and V, cerebellar cortex (especially granule cells), and hippocampal formation (especially within CA3 and CA2 subfields) (Braak and Goebel, 1978; Braak and Goebel, 1979; reviewed in Haltia, 2003; and Mole et al., 2011). Loss of cortical and hippocampal γ-aminobutyric acid (GABA)ergic interneurons is also evident (Braak and Goebel, 1978; Braak and Goebel, 1979; Tyynela et al., 2004). These changes are accompanied by microglial and astrocyte activation, and loss of myelin (Autti et al., 1997; Haltia et al., 2001; Tyynela et al., 2004). The visual system shows macular and retinal degeneration, optic atrophy, thinning of the vessels and storage accumulation in the peripheral retina (reviewed in Bozorg et al., 2009; and Mole et al., 2011).

The storage inclusions are mainly composed of the subunit c of mitochondrial ATPase and have mostly the appearance of fingerprint-like deposits in ultrastructural analysis using electron microscopy (EM) (Palmer et al., 1992; reviewed in Haltia, 2003; and Mole et al., 2005). Degree of neuronal storage also varies in different brain areas. However, neuronal loss is not closely related to the extent of the storage and the presence of storage material does not necessarily result in neurodegeneration. Thus, it is possible that the damage induced by storage may not be the main neurodegenerative component in juvenile CLN3 disease underlining the influence of the other mechanisms on determining neuronal survival (Autti et al., 1997; Tyynela et al., 2004; reviewed in Mole et al., 2011).

Unlike other NCLs, juvenile CLN3 disease patients also display patterns of an autoimmune response evidenced by the presence of circulating autoantibodies against glutamic acid decarboxylase (GAD65), α-fetoprotein (AFP) and other, so far

unidentified antigens present in brain tissue (Chattopadhyay et al., 2002; Lim et al., 2006; Castaneda and Pearce, 2008).

### 2.3.3 Phenotypes and pathological findings in juvenile CLN3 disease mouse models

No naturally occurring mouse (or any animal) models exist for juvenile CLN3 disease and therefore, artificial murine models have been generated (**Table 3**). To date, four different mouse models for CLN3 disease have been generated, including *Cln3*$^{\Delta ex1\text{-}6}$ (*Cln3*$^{-/-}$) knock-out mouse, *Cln3*$^{\Delta ex7\text{-}8\ Katz}$ mouse, *Cln3*$^{\Delta ex7\text{-}8\ Cotman}$ mouse, and *Cln3*$^{LacZ}$ β-galactosidase reporter model, each representing different gene targeting strategies. While the first CLN3 disease mouse model, *Cln3*$^{\Delta ex1\text{-}6}$ (*Cln3*$^{-/-}$) mouse, was created by replacing the start codon and first six exons of *Cln3* with a neo cassette, resulting in a null allele (*Cln3*$^{-/-}$) (Mitchison et al., 1999), two other CLN3 disease mouse models replicate the most common disease-causing *CLN3* mutation (Katz et al., 1999; Cotman et al., 2002). In the knock-out model of Katz et al., *Cln3*$^{\Delta ex7\text{-}8\ Katz}$ mouse, *Cln3* was replaced with a targeting vector in which most of exon 7, the entire intron 7 and exon 8, and part of intron 8 of *Cln3* were replaced with a neo cassette (Katz et al., 1999). Cotman et al. (2002) generated a knock-in mouse in which exons 7 and 8 of *Cln3* were entirely removed without leaving the neo cassette in a final mutant allele. In addition, Eliason et al. (2007) have created a mouse model that harbors a bacterial β-galactosidase reporter gene (*lacZ*) inserted in place of exons 1-8 of *Cln3* and transcribed by native *Cln3* promoter.

All CLN3 disease mouse models recapitulate the accumulation of autofluorescent storage material, neurodegeneration, and neurological defects, although the age of onset and the severity of the changes vary, with *Cln3*$^{\Delta ex7\text{-}8\ Cotman}$ mouse model exhibiting a more aggressive phenotype than the other models (**Table 3**). Visual defects are not that apparent. *Cln3*$^{\Delta ex7\text{-}8\ Katz}$ mouse is the only CLN3 disease mouse model that has been reported to exhibit altered retinal function (Seigel et al., 2002; Katz et al., 2008; Osorio et al., 2009). Interestingly, examination of the optic nerve of *Cln3*$^{-/-}$ mice, the most extensively studied mouse model of CLN3 disease, revealed other more prominent deficits, including reduction in optic nerve axonal density and myelination, hypertrophy of individual axons, impaired fast axonal transport of amino acids, and decreased conduction velocity of action potentials (Seigel et al., 2002; Sappington et al., 2003; Weimer et al., 2006). Furthermore, the stereological analysis revealed that the thalamic nuclei receiving input from the retina and relaying it to visual cortex displayed selective cell loss (Weimer et al., 2006). Based on these findings, it was suggested that visual deterioration in juvenile CLN3 disease may result from pathological events occurring outside of the retina (Weimer et al., 2006).

In addition, autoantibodies and loss of GABAergic interneurons, observed in CLN3 disease patients, have also been detected in Cln3-deficient mice (Mitchison et

al., 1999; Chattopadhyay et al., 2002; Pontikis et al., 2004; Pontikis et al., 2005; Castaneda and Pearce, 2008). However, just like the appearance of visual defects, the significant loss of GABAergic interneurons does not seem to be a unifying feature among the CLN3 disease mouse models (Mitchison et al., 1999; Pontikis et al., 2004; Pontikis et al., 2005). Instead, early glial activation has been observed in two analysed models (Pontikis et al., 2004; Pontikis et al., 2005; Weimer et al., 2009). Detailed studies on the early onset gliosis in the cerebellum of *Cln3*$^{-/-}$ mice revealed that activated cerebellar Bergmann glias were clustered adjacent to regions devoid of Purkinje cells. Surviving Purkinje cells exhibited defective neuritogenesis, seen as dendritic mis-orientation and altered dendritic spine density, and were defective in migration and/or maturation (Weimer et al., 2009). Interestingly, these changes were shown to precede deficits in motor coordination and balance (Kovacs et al., 2006). This suggests that an insult to cerebellar Purkinje cells is the primordial event resulting in decline in motor skills in juvenile CLN3 disease.

**Table 3.** Phenotypes of CLN3 disease mouse models.

| Mouse model | Onset of storage | Auto - antibodies | Visual defects | Retinal degeneration | Onset of neurological signs | Brain atrophy | Gliosis | Loss of GABAergic neurons | Mortality |
|---|---|---|---|---|---|---|---|---|---|
| ***Cln3*$^{\Delta ex1-6}$ (*Cln3*$^{-/-}$)** (1) | by 3 months | + | - | subtle (defects in optic nerve) | present at 2 weeks | - | + | + | no survival from 20 months |
| ***Cln3*$^{\Delta ex7-8\ Katz}$** (2) | present at 5 weeks | n. d. | + | loss of inner retinal neurons | present at 24 months | + | n.d. | n.d. | mean survival 103 weeks |
| ***Cln3*$^{\Delta ex7-8\ Cotman}$** (3) | < birth | n. d. | - | selective loss of cone cells | neonatal | only in thalamus | + | - | from 7 months |
| ***Cln3*$^{LacZ}$** (4) | present at 3 months | n .d. | n.d. | n. d. | by 2 months | n.d. | n.d. | n.d. | n.d. |

References: (1) Mitchison et al., 1999; Chattopadhyay et al., 2002; Seigel et al., 2002; Pontikis et al., 2004; Castaneda and Pearce, 2008; Weimer et al., 2009; (2) Katz et al., 1999; Wendt et al., 2005; Katz et al., 2008; (3) Cotman et al., 2002; Pontikis et al., 2005; Osorio et al., 2009; (4) Eliason et al., 2007.
Abbreviations: n.d., not determined.

## 2.4 CLN3

### 2.4.1 *CLN3* gene and disease mutations

Most cases of juvenile onset NCL are caused by mutations in *CLN3* which maps to the chromosomal region 16p11.2-12.1 spanning 15 kilobases (kb) with its 15 exons and 14 introns (Consortium, 1995; Mitchison et al., 1997a). The open reading frame of CLN3 is 1314 bp in length (Consortium, 1995). *Alu* elements are overrepresented in a *CLN3* locus, a phenomenon suggested to be the underlying cause for the existence of numerous deletion and insertion mutations in the gene (Mitchison et al., 1997a).

To date, 59 disease-causing mutations in *CLN3* have been identified, with the majority of the mutations affecting residues of the second and third lumenal loop of the polytopic CLN3 protein (Kousi et al., 2012) (**Figure 7**). This suggests that these domains represent functional hotspot in juvenile CLN3 disease. The most common mutation in *CLN3* is 1.02-kb deletion (c.462-677del, g.6060-7025del), also referred to as CLN3Δex7-8. It is present on 80 - 85% of disease chromosomes and affects approximately 300 families (Consortium, 1995; Munroe et al., 1997a and NCL Resource). The removal of exons 7 and 8 due to the 1.02-kb deletion results in a frameshift and a truncated protein consisting of the first 153 residues of the protein, followed by 28 novel amino acids before the stop codon (Consortium, 1995). Most of the remaining juvenile CLN3 disease cases are compound heterozygous for the 1.02-kb deletion and another mutation each accounting only a few families (Consortium, 1995; Munroe et al., 1997a, and NCL Resource). In Finland, most of the compound heterozygous patients bear a 2.8-kb deletion mutation found uniquely in Finland (Munroe et al., 1997b and Karhu, V. et al., unpublished data).

Controversial evidence exists for the presence of the 1.02-kb deletion protein product in mutated cells (Kitzmuller et al., 2007; Chan et al., 2008; Sarpong et al., 2009). It has even been suggested that, due to alternative splicing of messenger ribonucleic acid (mRNA), the 1.02-kb deletion mutant may also encode a CLN3 mutant protein that contains a portion of the C-terminus (Cotman et al., 2002; Fossale et al., 2004). In any case, the 1.02-kb deletion as well as other mutations resulting in severely truncated protein may lead to loss of CLN3 function in a correct intracellular compartment due to a lack of a full collection of intracellular targeting signals (see Chapter 2.4.3) and thus retention of the protein in the ER (Jarvela et al., 1999; Kitzmuller et al., 2008). A few mutations that affect the splicing of mRNA may result in mis-spliced transcripts encoding a protein with partial function (Munroe et al., 1997a; Munroe et al., 1997b). All identified missense mutations affect residues conserved between species. Most of the missense mutations, if not all, allow the protein to traffic to lysosomes but may interfere with the conformation and/or protein interactions and functions (Zhong et al., 1998; Jarvela et al., 1999; Golabek et al., 2000; Haskell et al., 2000; Golabek et al., 2001;

Persaud-Sawin et al., 2002; Gachet et al., 2005; Hobert and Dawson, 2007; Kitzmuller et al., 2008; Haines et al., 2009).

*CLN3* mutations predicted to give rise to severely truncated proteins or some missense mutations presumably affecting critical residues in the protein cause almost inclusively a classic juvenile NCL with highly concordant onset of visual failure (Jarvela et al., 1997; Munroe et al., 1997b; Kwon et al., 2005). However, inter- as well as intrafamilial variation in the severity of other symptoms has been observed indicating that environmental factors and/or modifying genes may influence the clinical phenotype (Jarvela et al., 1997). The clinical variation has been reported to be more prominent within compound heterozygote patients, and a proportion of these individuals display an atypical disease course associated with visual impairment but less severe mental and motor dysfunctions, with some cases having only visual failure (Jarvela et al., 1997; Munroe et al., 1997a; Wisniewski et al., 1997; Wisniewski et al., 1998; Lauronen et al., 1999). Furthermore, it has been suggested that undiscovered missense mutations may associate with a benign phenotype and thus, may not be diagnosed with juvenile CLN3 disease (Jarvela et al., 1997; Munroe et al., 1997a). A recent study, however, suggested that there are no differences in the behavioural phenotype between individuals homozygous or heterozygous for the major CLN3 mutation (Adams et al., 2010).

### 2.4.2 Structure and post-translational modifications of CLN3

*CLN3* encodes a 438-amino acid protein with a predicted molecular weigh of 43 kilodaltons (kDa) (Consortium, 1995). CLN3 has no homology with other proteins or functional domains, except a distant similarity with equilibrative nucleoside transporter family SLC29 and fatty acid desaturases (Baldwin et al., 2004; Narayan et al., 2006b). Nevertheless, CLN3 is highly conserved across species indicating that the protein has a fundamental role in cells (Taschner et al., 1997; Muzaffar and Pearce, 2008; Tuxworth et al., 2009).

The membrane topology of the human CLN3 has been studied extensively both by experimental (Ezaki et al., 2003; Mao et al., 2003a; Mao et al., 2003b; Kyttala et al., 2004) and computational methods (Janes et al., 1996; Mitchison et al., 1997b; Mao et al., 2003a; Nugent et al., 2008). Consensus is that CLN3 is a type III transmembrane protein, since it contains multiple membrane spanning domains, and its carboxy terminus faces the cytoplasm. However, predictions differ in the exact number of transmembrane segments (4-10) as well as in the positioning of the amino terminus. *In vivo* data favours the cytoplasmic positioning of the N-terminus (Ezaki et al., 2003; Kyttala et al., 2004). When this is combined with the experimental data on the other hydrophilic domains of CLN3 (Mao et al., 2003a; Kyttala et al., 2004) and with the most recent computational prediction analysis (Nugent et al., 2008), the emerging topology model of CLN3 supports a structure with six transmembrane segments, cytoplasmic N- and C-termini, one large cytoplasmic loop domain, and

three large lumenal loops, one of which putatively contains an amphipathic helix (Nugent et al., 2008). This model (**Figure 7**) is generally considered a valid topology model until a CLN3 crystal structure is available. The prediction that the yeast orthologues of CLN3 might produce different topologies (Nugent et al., 2008) remains to be confirmed experimentally. This is important considering that yeast models have widely been used to determine CLN3 functions.

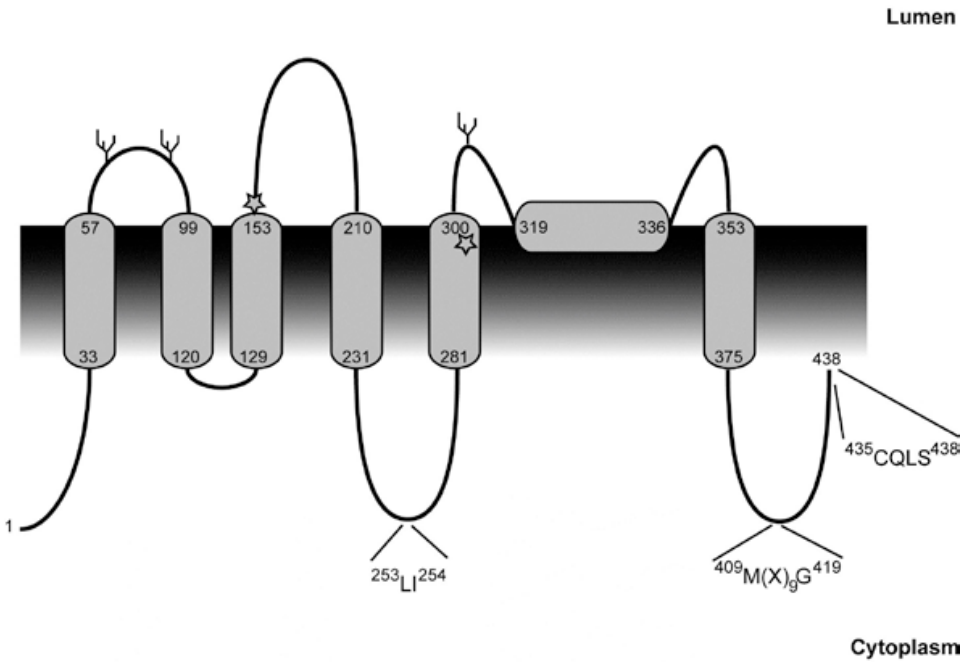


**Figure 7.** A schematic model for the topology of human CLN3 modified from Kyttälä et al. (2004) and Nugent et al. (2008). Experimentally determined positioning of lumenal and cytoplasmic loops, glycosylation sites (antennas), and lysosomal targeting signals and sorting motifs (LI, $M(X_9)G$, CQLS) are indicated. Proposed amphipathic helix (amino acids 319-336) and amino acid residues at the domain interfaces are also presented. Two disease-associated mutations referred to in the text, the classical CLN3Δex7-8 (c.462-677del), and the protracted disease causing CLN3E295K (c.883G>A), affect the second lumenal loop and the fifth transmembrane segment of the protein, respectively (stars). C-terminus may adopt a loop structure through prenylation on the CQLS motif, and subsequent attachment to the membrane.

CLN3 has been suggested to undergo several posttranslational modifications, including phosphorylation, glycosylation, myristoylation and prenylation. Based on the latest sequence analysis by Nugent et al. (2008), CLN3 is predicted to contain nine potential phosphorylation sites. However, to date, the only experimental evidence for the phosphorylation of CLN3 has been obtained in a study showing that a radiolabeled phosphate could be incorporated into the green fluorescence protein (GFP)-tagged CLN3 and that the labelling could be removed by alkaline phosphatase (Michalewski et al., 1999). Same study suggested that several kinases and phosphatases affect the level of CLN3 phosphorylation. Further studies are needed to map the specific phosphorylation sites in CLN3 and their functional importance for the protein. A study where the effects of CLN3 mutants on cell growth rates were analysed showed that the phosphorylation of CLN3 is at least not associated with the apoptotic functions of the protein (Persaud-Sawin et al., 2002).

Several studies have indicated that CLN3 is a glycosylated protein (**Figure 7**) and that the level of CLN3 glycosylation varies in different cell types or tissues (Jarvela et al., 1998; Golabek et al., 1999; Ezaki et al., 2003; Mao et al., 2003a; Storch et al., 2007). There are four potential *N*-glycosylation sites (residues 49, 71, 85, 310), two putative *O*-glycosylation sites (residues 80 and 256), and two potential glycosaminoglycan sites (residues 162 and 186) within CLN3 (Consortium, 1995). The *N*-glycosylation sites at asparagine residues 71 and 85 have been validated experimentally (Storch et al., 2007). In addition, *in vitro* studies by Mao et al. (2003a) suggested that asparagine residue 310 is also *N*-glycosylated but this was not supported by *in vivo* experiments of Storch et al. (2007). *N*-glycosylation is not required for the delivery of CLN3 to lysosomal compartments (Kida et al., 1999; Storch et al., 2007) but might be important for certain functional activities (Persaud-Sawin et al., 2002). Interestingly, the *Saccharomyces cerevisiae* homologue of CLN3 possibly uses glycosylation for its relocalisation under variable pH conditions (Wolfe et al., 2011) (see Chapter 2.4.4.2).

CLN3 sequence was predicted to contain lipid modification sites for N-terminal myristoylation ($^{2}$GGCAGS$^{7}$) and, at its C-terminal tail, a CAAX motif for prenylation ($^{435}$CQLS$^{438}$) (Consortium, 1995; Taschner et al., 1997; Jarvela et al., 1998). No direct experimental evidence exists for the myristoylation. However, the N-terminus is resistant to the Edman degradation, which indicates the presence of a post-translational modification at the amino terminus (Ezaki et al., 2003). Furthermore, CLN3 has been shown to undergo prenylation *in vitro* (Pullarkat and Morris, 1997; Kaczmarski et al., 1999) and *in vivo* (Storch et al., 2007), which creates an additional, C-terminal loop into the protein (**Figure 7**). The amino acid composition of the CAAX motif in CLN3 suggests that the prenylation of CLN3 involves rather farnesylation than geranylgeranylation (Pullarkat and Morris, 1997). Prenylation affects the trafficking of CLN3 along the endosomal/lysosomal compartments (Storch et al., 2007) (see Chapter 2.4.3).

### 2.4.3 Tissue expression and intracellular localisation of CLN3

*CLN3* is transcribed throughout the body, with placenta being the most prominent site of *CLN3* mRNA expression (Consortium, 1995; Chattopadhyay and Pearce, 2000; Su et al., 2004). Unfortunately, low endogenous protein levels and lack of reliable antibodies have challenged the determination of the localisation of CLN3 protein. Margraf et al. (1999) and Ezaki et al. (2003) have analysed the expression of the protein in brain and in a few extraneural tissues using antibodies against the N-terminus of CLN3. Although it seems that also at protein level both mouse and human *CLN3* are expressed in several tissues, antibodies used in the studies by Margraf and colleagues and Ezaki and colleagues gave conflicting results on the tissue specific expression levels of CLN3. A more comprehensive analysis on CLN3 tissue localisation has been obtained utilising CLN3-deficient reporter mice that express β-galactosidase under the native *Cln3* promoter (Eliason et al., 2007; Stein et al., 2010; Ding et al., 2011). The β-galactosidase reporter gene was translated in a wide variety of tissues confirming that CLN3 is ubiquitously expressed (Stein et al., 2010; Ding et al., 2011).

Several studies indicate that compared to extraneural tissues, *CLN3* expression in brain is relatively low (Consortium, 1995; Chattopadhyay and Pearce, 2000; Cotman et al., 2002; Ezaki et al., 2003; Stein et al., 2010). This suggests that the neural expression of CLN3 is tightly regulated. However, detailed information on CLN3 expression in brain is partly inconsistent. Nevertheless, hippocampus (especially the granular cells of the dentate gyrus), cortex, and cerebellum (especially the granular cell layer and Purkinje cells) have frequently been reported to express CLN3 (Pane et al., 1999; Chattopadhyay and Pearce, 2000; Luiro et al., 2001; Cotman et al., 2002; Eliason et al., 2007; Ding et al., 2011). Furthermore, the inner and the outer nuclear layers of retina have been reported to express notable amounts of CLN3 (Ding et al., 2011). In the CLN3-positive areas of the brain, the protein is predominantly expressed in neurons, although not all neurons are expressing the protein.

Most of the studies on intracellular localisation of CLN3 suggest that the protein primarily resides in LEs/lysosomes (Fossale et al., 2004; Storch et al., 2004; reviewed in Phillips et al., 2005; Storch et al., 2007; Tuxworth et al., 2009). The transport of CLN3 to lysosomes occurs slowly (Storch et al., 2007), possibly via the plasma membrane (Mao et al., 2003b; Storch et al., 2007), and requires at least three different sorting signals; a dileucine signal (LI) preceded by an acidic patch in the large cytoplasmic loop (Kyttala et al., 2004; Storch et al., 2004; Kyttala et al., 2005), a stretch of methionine and glycine separated by nine amino acid residues [$M(X)_9G$ motif] in the C-terminal domain (Kyttala et al., 2004; Kyttala et al., 2005), and the prenylation signal, a CAAX box motif ($^{435}$CQLS$^{438}$), also located in the carboxy terminus of CLN3 (Storch et al., 2007) (**Figure 7**). Although Storch et al. (2004) failed to demonstrate interaction between CLN3 and the adaptor proteins AP-1 and

AP-3, an independent study by Kyttälä et al. (2005) revealed that these adaptor proteins do recognise the dileucine signal in CLN3 and mediate the transport of the protein to lysosomal compartments. Molecules responsible for the recognition of the $M(X)_9G$ motif and the prenyl moiety are currently unknown. Although either the LI signal or the $M(X)_9G$ motif on their own can mediate the transport of CLN3 to lysosomes, both are required for optimal lysosomal delivery (Kyttala et al., 2004; Storch et al., 2004). However, the prenyl moiety executes its function differently. It is not essential for the lysosomal transport of CLN3 (Haskell et al., 2000; Storch et al., 2007) but rather enhances the lysosomal sorting of the protein in early endosomal compartments (Storch et al., 2007).

In addition to LEs and lysosomes, CLN3 has been suggested to localise to several other intracellular compartments including the nucleus, ER, Golgi, cytoplasm, mitochondria, EEs, recycling endosomes, lipid rafts, and plasma membrane (reviewed in Phillips et al., 2005). Since lysosomal transport of CLN3 extend to the EEs and possibly the cell surface, it is possible that these compartments may indeed contain some of the functional CLN3. Whether CLN3 is associated with and executes its function in the rest of the above-mentioned compartments remains to be confirmed. It is possible that some of the compartments suggested to contain CLN3, may represent false positives due to poor antibody specificity and protein over-expression. In addition, utilisation of terminal epitope tags has been reported to interfere with the normal targeting of CLN3 (Haskell et al., 1999; Kyttala et al., 2004).

In neuronal cells, CLN3 has also been localised to LEs/lysosomes, which in neurons are mostly retained in the cell soma (Jarvela et al., 1999; Luiro et al., 2001; Fossale et al., 2004; Kyttala et al., 2004; Storch et al., 2007). However, a substantial fraction of CLN3 is targeted to neuronal extensions and synaptosomes where the protein has been found to reside in EEs, presynaptic vesicles, and in so far unidentified vesicles (Jarvela et al., 1999; Haskell et al., 2000; Luiro et al., 2001; Kyttala et al., 2004; Storch et al., 2007).

### 2.4.4 Intracellular processes affected by CLN3 and its orthologues

Evolutionary conservation of CLN3 has enabled its functional analysis in several model organisms. In addition to numerous studies in mammals, the consequences of mutations or altered expression levels of CLN3 have been dissected in unicellular species and invertebrate animal models. Human CLN3 is able to complement functions of its orthologues, which further indicates the functional conservation among CLN3 orthologues (Pearce and Sherman, 1998; Kim et al., 2003; Gachet et al., 2005). During the past 16 years, CLN3 has been reported to associate with numerous intracellular processes, although some of them are likely secondary and may not be related to CLN3 directly. In addition to this chapter, CLN3-associated

processes will be further discussed in the context of the protein interactions of CLN3 (Chapter 2.4.5).

#### *2.4.4.1 Studies on CLN3 in mammals*

The observation that the mitochondrial ATP synthase subunit c accumulates in the NCL disease has prompted investigators to analyse the possible role of CLN3 in **autophagy**, a pathway that contributes to mitochondrial turnover. Indeed, CLN3 has been found to associate with autophagosomal membranes and the accumulation of subunit c protein has been detected in both autophagic vacuoles and lysosomes (Fossale et al., 2004; Cao et al., 2006). Analyses performed on Cln3-deficient mice, CLN3-silenced neuroblastoma cells, and juvenile CLN3 disease patient samples have further showed that loss of functional CLN3 impairs the maturation of autophagosomes but also activates autophagy, which may be due to prosurvival feedback responses of affected cells. Due to smaller size and trafficking defects of autophagic vacuoles in CLN3-deficient cells, it has been suggested that CLN3 may function in the trafficking of autophagic vacuoles to the perinuclear region for the fusion with LEs/lysosomes (Cao et al., 2006; Chang et al., 2011). In addition, abnormalities in **intracellular trafficking** of several other intracellular compartments have been associated with CLN3-deficiency. These include impaired exit of mannose 6-phosphate receptor from TGN, demonstrated in CLN3-silenced human cervical tumor cells (HeLa cells) (Metcalf et al., 2008), reduced fast axonal transport in optic nerves of *Cln3*$^{-/-}$ mice (Weimer et al., 2006), altered localisation of LEs/lysosomes, and defects in fluid-phase endocytosis in cerebellar neuronal precursor cells of *Cln3*$^{\Delta ex7\text{-}8\ Cotman}$ mice (Fossale et al., 2004; Cao et al., 2011). The role of CLN3 in intracellular trafficking was also studied in this thesis work.

CLN3 also affects other properties of lysosomal compartments. Studies in patient fibroblasts and human cell lines have shown that alterations in CLN3 expression levels result in changes in **lysosomal pH and size** (Golabek et al., 2000; Holopainen et al., 2001; Kitzmuller et al., 2008). In addition, several reports indicate that the **amount or activity/processing of lysosomal proteins**, including cathepsins and TPP1, deficient in congenital and late infantile NCL, respectively (Sleat et al., 1997; Siintola et al., 2006b), is altered in the brains of patients and juvenile CLN3 disease mouse models, and in CLN3-silenced human cell lines (Prasad and Pullarkat, 1996; Sleat et al., 1998; Junaid and Pullarkat, 1999; Mitchison et al., 1999; Golabek et al., 2000; Fossale et al., 2004; Eliason et al., 2007; Metcalf et al., 2008). Lysosomes from patient lymphoblasts also exhibit decreased lysosomal import of arginine, which together with subtle changes in mitochondrial arginine metabolism and plasma membrane arginine uptake in *Cln3*$^{-/-}$ mice suggest that loss of CLN3 may result in multiple disturbances in cellular **arginine metabolism** (Ramirez-Montealegre and Pearce, 2005; Chan et al., 2009).

Several studies have indicated that loss of CLN3 affects communication between neurons. GAD65 is an enzyme that converts the excitatory neurotransmitter

glutamate to the inhibitory neurotransmitter GABA. Brains of *Cln3*$^{-/-}$ mice have decreased activity of GAD65, due to the inhibition of this enzyme by the autoantibody. This results in early-onset elevated presynaptic levels of glutamate and decreased production of GABA in the brains of affected mice (Chattopadhyay et al., 2002; Pears et al., 2005). These alterations have been suggested (Chattopadhyay et al., 2002) to contribute to loss of GABAergic neurons observed in the patients (Braak and Goebel, 1978; Braak and Goebel, 1979; Tyynela et al., 2004) and *Cln3*$^{-/-}$ mice (Mitchison et al., 1999; Pontikis et al., 2004). Changes have also been observed in the functions of glutamaergic receptors. Cerebellar granule cells of *Cln3*$^{\Delta ex7\text{-}8\ Cotman}$ and *Cln3*$^{-/-}$ mice show increased sensitivity to 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid (AMPA) and/or *N*-Methyl-D-aspartic acid (NMDA)-type glutamate receptor-mediated excitotoxicity (Kovacs et al., 2006; Kovacs and Pearce, 2008; Finn et al., 2011; Kovacs et al., 2011). In addition, an independent analysis performed in *Cln3*$^{\Delta ex7\text{-}8\ Cotman}$ mice revealed changes in the levels of accessible receptor binding sites of glutamergic and cholinergic receptors (Herrmann et al., 2008). Juvenile CLN3 disease patients also show downregulation of dopamine transporters (Ruottinen et al., 1997; Aberg et al., 2000a) and dopamine receptors (Rinne et al., 2002). Furthermore, *Cln3*$^{-/-}$ mice exhibit alterations in dopamine catabolism (Weimer et al., 2007). Therefore, these findings suggest that CLN3-deficiency affects the **regulation of components involved in neurotransmission**, although the underlying mechanisms are as yet elusive.

In addition to the accumulation of mitochondrial protein as a component of the storage material in juvenile CLN3 disease, numerous other findings have evidenced the connection between the lack of CLN3 and **mitochondrial** dys**function**. These include changes in the size and morphology of mitochondria in the neurons of Cln3 mouse models (Fossale et al., 2004; Luiro et al., 2006), dysfunction of mitochondrial enzymes demonstrated by functional assays in patient fibroblasts, mitochondrial fractions isolated from patients and *Cln3* knock-out mice (Majander et al., 1995; Das and Kohlschutter, 1996; Dawson et al., 1996; Luiro et al., 2006), altered levels of high-energy phosphate compounds in patient fibroblasts and cerebellar precursor cells of *Cln3*$^{\Delta ex7\text{-}8}$ mice (Das et al., 2001; Fossale et al., 2004).

Integrity of the **apoptotic pathway, lipid metabolism, and oxidative homeostasis** in CLN3 deficiency has also been questioned. CLN3 has been suggested to exhibit anti-apoptotic activities due to the presence of apoptotic neuronal cells in juvenile CLN3 disease brain (Lane et al., 1996) and the ability of CLN3 to suppress apoptosis in cultured cells (Puranam et al., 1999; Narayan et al., 2006a). The view that CLN3 is also involved in lipid metabolism emerged from the studies showing the CLN3 expression correlates with palmitoyl-protein Δ9-desaturase activity (Narayan et al., 2006b) and synthesis of bis(monoacylglyceryl)phosphate (BMP; also termed lysobisphosphatidic acid, LBPA) (Hobert and Dawson, 2007; Narayan et al., 2008). Finally, altered levels of pro- and antioxidant molecules and increased protein oxidation in the brains of *Cln3*$^{-/-}$

and *Cln3*$^{\Delta ex7\text{-}8\ Cotman}$ mice, together with reduced survival of cerebellar precursor cells prepared from *Cln3*$^{\Delta ex7\text{-}8}$ mice from oxidative stress indicate that the oxidative stress response pathway may be defective due to loss of CLN3 (Benedict et al., 2007; Weimer et al., 2007; Herrmann et al., 2008).

#### *2.4.4.2 Studies on CLN3 in yeasts*

Both the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* have been utilised to analyse the function of CLN3 in simple unicellular organisms (Pearce and Sherman, 1998; Gachet et al., 2005). The yeast homologue of CLN3, termed Btn1p, is 39% and 30% identical, and 59% and 48% similar in *S. cerevisiae* and *S. pombe*, respectively (Mitchison et al., 1997b; Gachet et al., 2005). Like the human counterpart, the yeast homologues of CLN3 have been suggested to localise to more than one intracellular compartment. Btn1p in both yeast organisms was originally reported to localise to the vacuole, the analogous organelle to the lysosome (Croopnick et al., 1998; Gachet et al., 2005). In *S. pombe*, the protein was additionally localised to smaller, prevacuolar compartments that were later suggested to represent the Golgi compartment (Gachet et al., 2005; Codlin and Mole, 2009). In fact, it has subsequently been argued that Btn1p in both yeasts is predominantly located within the Golgi, but in overexpression, may escape to vacuolar compartments (Codlin and Mole, 2009; Kama et al., 2011). Nevertheless, both *S. pombe* and *S. cerevisiae* Btn1p show conditional intracellular localisation. In *S. pombe*, the protein is enriched in the endomembrane structures near the cell poles or septum at high temperature growth conditions (Codlin et al., 2008b). The *S. cerevisiae* homologue is re-localised to undefined punctuate spots in response to changes in extracellular pH (Wolfe et al., 2011).

The budding yeast deleted for the *CLN3* yeast orthologue *BTN1* (*btn1*Δ strain) manifests **changes in vacuolar pH, amino acid homeostasis, nitric oxide production, and phospholipid distribution**. The *btn1*Δ strain has a decreased vacuolar pH at early growth that continues to rise above normal at later growth points (Pearce et al., 1999a; Padilla-Lopez and Pearce, 2006). Concurrently, coupling of proton transport and ATPase activities of the vacuolar ATPase (vATPase), a major contributor to the acidic pH of the vacuole, is altered (Padilla-Lopez and Pearce, 2006) and the activity of the plasma membrane $H^+$ ATPase is increased in *btn1*Δ (Pearce et al., 1999a; Pearce et al., 1999b). Both processes have been suggested to act in order to compensate the imbalance in vacuolar pH homeostasis. Altered vacuolar pH has also been reported to underlie the transport defect of arginine into *btn1*Δ vacuoles (Kim et al., 2003). However, there is a decrease in both vacuolar and cytosolic arginine (and lysine) levels in *btn1*Δ cells (Kim et al., 2003). The findings that *btn1*Δ can grow in the absence of arginine and that high intracellular levels of arginine are, due to unknown reasons, toxic to *btn1*Δ strain, led to the suggestion that arginine is deliberately kept low in cells lacking Btn1 (Vitiello et al., 2007). Since arginine serves as the substrate for nitric oxide synthesis, this likely explains why

*btn1*Δ cells exhibit limited synthesis of nitric oxide and suppression of nitric oxide-dependent signalling pathways (Osorio et al., 2007). In addition, phospholipid levels and their subcellular distribution have been reported to be dysregulated in *S. cerevisiae* due to loss of Btn1p (Padilla-Lopez et al., 2012).

Finally, the *btn1*Δ strain upregulates *BTN2* (Pearce et al., 1999a). The protein product of *BTN2*, *S. cerevisiae* Btn2p, has been implicated in **intracellular trafficking**. Btn2p has been reported to affect the cell surface localisation of regulators of arginine uptake and salt tolerance (Chattopadhyay and Pearce, 2002; Kim et al., 2005), and to interact with components specifically involved in the retrieval of Golgi-associated vesicular targeting protein, Yif1p (Chattopadhyay et al., 2003; Kama et al., 2007). In fact, it was recently reported that also Btn1p works on retrograde late endosome-to-Golgi transport of Yif1p, but probably by different mechanism than Btn2p. While Btn2p localises to vacuoles and associates there with retrieval components, Golgi-localised Btn1p has been shown to execute its function by regulating SNARE phosphorylation and assembly, most likely in Golgi complex (Kama et al., 2007; Kama et al., 2011).

Deletion of Btn1p-coding gene in *S. pombe* has also pleiotropic effects. Btn1p-deficient fission yeast has larger and less acidic vacuoles than the wild type yeast (Gachet et al., 2005). The **vacuole size** strongly correlates with changes in Btn1p expression but is affected indirectly by Btn1p via modulation of the vacuolar pH (Gachet et al., 2005; Kitzmuller et al., 2008). *S. pombe btn1*Δ show also aberrant **cell-wall structure** and delayed **cytokinesis** under normal growth conditions (Gachet et al., 2005; Codlin et al., 2008a). Changes in **vacuole pH** may also explain some of the observed defects in cell-wall (Codlin et al., 2008a) but not in the cytokinesis (Gachet et al., 2005). This suggests that Btn1p impacts cytokinesis apart from its role in vacuolar pH homeostasis. The defect in cytokinesis is more severe at 37°C. After prolonged growth at high temperature, *S. pombe btn1*Δ cells lose their ability for polarised growth and eventually become lysed (Codlin et al., 2008b). This has been shown to be due to defects in the processes required for the **distribution of sterol-rich domains (lipid rafts)** to cell poles (Codlin et al., 2008b). In addition, Btn1p-deficient cells exhibit altered **Golgi morphology** and defective **sorting of the vacuolar hydrolase carboxypeptidase Y (Cpy1p)** in part due to delayed trafficking of its sorting receptor Vps10p through ER and the Golgi compartment (Codlin and Mole, 2009).

#### *2.4.4.3 Studies on CLN3 in Drosophila melanogaster and Caenorhabditis elegans*

Two small invertebrate animal models, *Drosophila melanogaster* and *Caenorhabditis elegans*, have been utilised in CLN3 research (de Voer et al., 2005; Tuxworth et al., 2009; Tuxworth et al., 2011). *Drosophila* homologue of CLN3, expressed in human embryonic kidney cells, has been reported to localise to late endosomal compartments, with a fraction of the protein also appearing on the plasma membrane and Rab11-positive recycling endosomes (Tuxworth et al., 2009).

A transgenic fly harbouring a null mutation in *CLN3* exhibits no obvious external phenotype, or accumulation of autofluorescent material (Tuxworth et al., 2011). Instead, over-expression of CLN3 has strong phenotypic consequences (Tuxworth et al., 2009; Tuxworth et al., 2011). Expression of CLN3 in the eye causes degeneration while ubiquitous expression leads to semi-lethality. Escaping adults exhibit several morphological changes that resemble those of Notch loss-of-function or JNK gain-of-function phenotypes. Not surprisingly, genetic interactions between CLN3 and the Notch or JNK signalling pathways were subsequently confirmed (Tuxworth et al., 2009). Furthermore, CLN3 over-expression has been demonstrated to affect the expression of Notch and JNK target genes (Tuxworth et al., 2009). The two signalling pathways are functionally connected and therefore, Tuxworth et al. suggested that increased expression of *CLN3* causes inhibition of **Notch signalling**, possibly via activation of the JNK signalling pathway (Tuxworth et al., 2009). Based on the finding that Notch signalling appears unaffected downstream of Notch cleavage, it was also suggested that increased levels of CLN3 most likely impair the processing or cleavage of the Notch receptor itself (Tuxworth et al., 2009). CLN3-expressing fly has also been used to systematically screen for genes that modify CLN3-dependent degenerative phenotypes in the eye and wing, to further recognise intracellular pathways and processes possibly involving CLN3. The analysis initially involved genes that changed the phenotype when their gene dosage was reduced (Tuxworth et al., 2009) and was later expanded to genes that modified phenotypes when co-expressed with *CLN3* (Tuxworth et al., 2011). Several intracellular processes or pathways emerged from the screens, including **stress response signalling** (Tuxworth et al., 2011) and **regulation of mRNA translation and localisation** (Tuxworth et al., 2009; Tuxworth et al., 2011). Detailed investigations revealed that among different environmental stress pathways, especially the oxidative stress response is compromised due to changes in CLN3 expression (Tuxworth et al., 2011). Consistently, CLN3 null flies were found to be hypersensitive to oxidative stress and to accumulate reactive oxygen species. Even so, CLN3 was not found to be a direct antioxidant effector, as its expression levels kept constant during exposure to stress conditions (Tuxworth et al., 2011). Further analysis downstream the oxidative stress response pathway should clarify the potential role of CLN3 in oxidative stress.

*C. elegans* has three CLN3 homologues, designated *cln-3.1*, *cln-3.2*, and *cln-3.3*. They encode proteins that show considerable homology across their complete amino acid sequences (De Voer et al., 2001; Mitchell et al., 2001). Thorough examination of each single *cln-3* deletion mutant or double and triple *cln-3* mutants revealed decreased life span or brood size but no significant alterations in behaviour, morphology, neuronal integrity, or lysosomal staining have been noticed. In addition, *cln-3* mutant worms do not exhibit the accumulation of autofluorescent storage material (de Voer et al., 2005; Phillips et al., 2006).

### 2.4.5 Protein interactions of CLN3

The first interaction of CLN3 was reported in 2002, 7 years after gene identification (Vesa et al., 2002). With time, several other binding partners of CLN3 have been identified including those discovered in this thesis work. A summary of CLN3 interactions, discussed below, is presented in **Table 4**.

**Table 4.** A summary of CLN3-interacting proteins reported by others. Interacting domains, research methods, and functions are presented.

| Interactor | Domain | CLN3 domain | Method | Function |
|---|---|---|---|---|
| **CLN5** [1)] | ? | ? | GST pull-down, co-IP | ? |
| **AP-1, 2, 3** [2)] | ? | 232-280 | GST pull-down | lysosomal targeting of CLN3 |
| **Calsenilin** [3)] | ? | 385-438 | YTH, GST pull-down, co-IP | regulation of $Ca^{2+}$-induced cell death |
| **SBDS** [4)] | N-terminus | 388-438 | YTH, co-IP | regulation of yeast vacuolar pH |
| **myosin IIB** [5)] | 585-1010 | 388-438 | YTH, co-IP | regulation of myosin distribution, cell migration |

References: [1)] Vesa et al., 2002 and Lyly et al., 2009; [2)] Kyttälä et al., 2005; [3)] Chang et al., 2007; [4)] Vitiello et al., 2010; [5)] Getty et al., 2010.
Abbreviations : AP-1, adaptor protein complex 1; SBDS, Shwachman-Bodian-Diamon syndrome protein; co-IP, co-immunoprecipitation; GST, glutathione S-transferase; YTH, yeast two-hybrid.

#### 2.4.5.1 NCL proteins

CLN3 has been demonstrated to interact with CLN5 (Vesa et al., 2002; Lyly et al., 2009). However, CLN3-CLN5 complex has not been studied further, except the finding that the interaction is at least not contributing to intracellular localisation of CLN3 (Lyly et al., 2009). Whether CLN3 interacts with rest of the NCL proteins is unclear due to conflicting results from different studies. Based on yeast two-hybrid (YTH) analysis, it has been suggested that no interactions exist among PPT1, TPP1 and CLN3 (Zhong et al., 2000). Later, an independent study using co-immunoprecipitation argued for the interactions among TPP1, CLN3, CLN6 and CLN8 (and PPT1 and TPP1) (Persaud-Sawin et al., 2007). However, both of these studies have potential pitfalls. In the yeast-two-hybrid analysis, full-length CLN3 was exploited, an approach which has been shown to be unfavourable in the YTH analyses of hydrophobic integral membrane proteins (Auerbach et al., 2002; Stagljar and Fields, 2002). In the co-immunoprecipitation analysis, samples were denatured by boiling for several minutes, a procedure which usually results in the aggregation of integral membrane proteins (including CLN3, CLN6, and CLN8) and subsequent loss of their signal in Western blotting. Further concerns have been discussed extensively elsewhere (Getty and Pearce, 2011).

CLN3 has also been suggested to form non-glycosylated homodimeric complexes that are resistant to sodium dodecyl sulfate (SDS) in gel electrophoresis analysis (Storch et al., 2007).

#### 2.4.5.2 Adaptor proteins

Three adaptor protein complexes, namely AP-1, AP-2 and AP-3 bind CLN3 (Kyttala et al., 2005). AP-1 and AP-3 have been shown to facilitate endosomal/lysosomal targeting of CLN3 (Kyttala et al., 2005) (see Chapter 2.4.3). The meaning of the binding with AP-2, the adaptor of clathrin-mediated endocytosis at the plasma membrane, has remained unclear. Although CLN3 is capable of binding AP-2, it is not targeted to lysosomes via clathrin-mediated endocytosis at the plasma membrane demonstrated by the observation that CLN3 does not accumulate at the cell surface in the AP-2-deficient cells (Kyttala et al., 2005).

#### 2.4.5.3 Flotillin-1

In the study by Rakheja et al., the lipid raft marker protein flotillin-1 was found to be one of the proteins that immunoprecipitated with the CLN3 antibody from bovine brain homogenate (Rakheja et al., 2004). However, no controls were shown to confirm the specificity of the immunoprecipitation. Therefore, it remains elusive whether flotillin and other indicated proteins represent true interaction partners of CLN3.

#### 2.4.5.4 Calsenilin

Calsenilin, also named downstream regulatory element antagonist modulator (DREAM) or $K^+$ channel interacting protein 3 (KChIP3), is a $Ca^{2+}$-binding protein mainly expressed in the brain (Zaidi et al., 2002). It has been linked to several functions affecting different intracellular compartments. Calsenilin enhances apoptosis (Jo et al., 2001; Lilliehook et al., 2002) and through binding with ER-resident presenilin 1 and 2, increases cleavage of the amyloid precursor protein (APP) in a manner that elevates the formation of Alzheimer disease-associated β-amyloid peptide (Buxbaum et al., 1998; Jo et al., 2001; Jo et al., 2003; Lilliehook et al., 2003). Calsenilin also binds to cell surface A-type voltage-gated potassium channels (An et al., 2000), acts as a transcription repressor (Carrion et al., 1999), and is involved in the trafficking of Golgi glycosyltransferases (Quintero et al., 2008).

Interaction between calsenilin and the C-terminus of CLN3 was revealed in the search of calsenilin-interacting proteins (Chang et al., 2007). CLN3-calsenilin interaction was found to be impaired in increasing $Ca^{2+}$ concentrations. Furthermore, CLN3 negatively regulates the expression of calsenilin, induces its intracellular relocalisation, and subsequently, the co-localisation between the two proteins (Chang et al., 2007). There is a strong correlation between the ability of CLN3 to bind calsenilin and the susceptibility of cells to $Ca^{2+}$-induced cell death. Reduction in the amount of CLN3, or over-expression of CLN3 containing only the N-terminus, exposed cells to high ATP-induced intracellular $Ca^{2+}$ concentrations and $Ca^{2+}$-induced cell death. In contrast, cells expressing full-length CLN3, or the C-terminal part of CLN3 containing the calsenilin-binding region, displayed normal ATP-induced $Ca^{2+}$ transients and were protected from $Ca^{2+}$-induced cell death (Chang et al., 2007).

#### 2.4.5.5 Shwachman-Bodian-Diamon syndrome protein (SBDS)

SBDS protein is mutated in Shwachman-Bodian-Diamon syndrome (Boocock et al., 2003), an autosomal recessive disorder characterised by skeletal and hematologic abnormalities, exocrine pancreatic dysfunction, susceptibility to recurrent infections, defects in immunity and increased risk of leukaemia (reviewed in Burroughs et al., 2009). SBDS is a vital protein highly conserved across species and expressed throughout the body, especially in rapidly proliferating tissues (Boocock et al., 2003; Zhang et al., 2006a). The protein localises to the nucleus and cytoplasm (Austin et al., 2005). SBDS protein function has been associated with apoptosis (Watanabe et al., 2009), stabilisation of the mitotic spindle to prevent genomic instability (Austin et al., 2008), deoxyribonucleic acid (DNA) damage and ER stress response (Ball et al., 2009) and most of all, ribosomal functions. The yeast homologue of SBDS, Sdo1, is required for the late 60S ribosomal subunit maturation (Menne et al., 2007). The human homologue has been shown to associate with components of 60S ribosome (Ganapathi et al., 2007) and with the proteins nucleophosmin and NIP7 (Ganapathi et al., 2007; Hesling et al., 2007) known to direct nuclear export of

ribosomal subunits and pre-ribosomal RNA (pre-rRNA) processing, respectively (Maggi et al., 2008; Morello et al., 2011).

CLN3 binding to SBDS is also conserved among respective *S. cerevisiae* homologues, Btn1 and Sdo1 (Vitiello et al., 2010). Moreover, the two proteins were found to partially co-localise to unidentified punctate cytoplasmic structures putatively containing unglycosylated form of Btn1 (Vitiello et al., 2010; Wolfe et al., 2011). Using *SDO1* deletion yeast strain it was shown that similar to Btn1 deficiency, Sdo1 deficiency results in alterations in vacuolar pH and vATPase activity. *SDO1* deletion strain displayed decreased vacuolar pH, $H^+$ transport (possibly to compensate imbalance in vacuolar pH), vATPase dependent ATP hydrolysis, and decreased expression of vATPase at the vacuole (Vitiello et al., 2010). Based on the analyses of the effects of BTN1 over-expression on the phenotype and the vacuolar properties of *SDO1* deletion strain, it was suggested that Sdo1 and Btn1 operate in the same intracellular pathway and that Sdo1 regulates the function of Btn1 (Vitiello et al., 2010). Although it was shown that defects in the ribosomal maturation pathway, in general, affect the yeast vacuole (Vitiello et al., 2010), it remained elusive whether ribosomal functions of Sdo1 and the regulation of vacuolar Btn1 are associated and most importantly, whether the two proteins operate similarly in mammalian cells. Nonetheless, Vitiello et al. (2010) suggested that the action of SBDS/Sdo1 as a general stress-response protein extends to the lysosomal/vacuolar compartment via interaction with CLN3/Btn1.

#### *2.4.5.6 Non-muscle myosin IIB (NM IIB)*

Three non-muscle myosin heavy chain isoforms exist (NMHC IIA, NMHC IIB and NMHC IIC) and they determine the isoform of whole non-muscle myosin II molecule (NM IIA, NM IIB and NM IIC). NM IIB plays a role in the polarisation of the migration machinery and positioning of the intracellular compartments during migration (Lo et al., 2004; Vicente-Manzanares et al., 2007; Vicente-Manzanares et al., 2008). NM IIB is predominantly expressed in brain and neurons, and mice deleted for *MYH10*, the gene encoding NMHC IIB, show brain defects (Simons et al., 1991; Rochlin et al., 1995; Tullio et al., 2001; Ryu et al., 2006). In neurons, NM IIB has been implicated in axonal outgrowth, dendritic spine morphology, synaptic transmission, and growth cone motility (Bridgman et al., 2001; Tullio et al., 2001; Takagishi et al., 2005; Ryu et al., 2006).

C-terminal segment of CLN3 associates with non-muscle myosin heavy chain IIB (Getty et al., 2010). Using a scratch assay Getty et al. (2010) showed that primary mouse embryonic fibroblasts of *Cln3*$^{-/-}$ mice displayed a migration defect and unlike the wild type cells, the migration of Cln3-deficient cells was not affected by blebbistatin, an inhibitor of myosin II ATPase activity (Getty et al., 2010). This was suggested to indicate that the migration was already impaired in *Cln3*$^{-/-}$ cells, possibly due to dysregulation of myosin IIB. Furthermore, Cln3-deficient fibroblasts were shown to display changes in myosin IIB intracellular distribution as well as in

the general cell morphology. *Cln3*$^{-/-}$ cells were found to be more elongated and narrow, which likely explained the observed enhanced migration of the cells through the filter in Boyden chamber assay (Getty et al., 2010).

### 2.4.6 Recurrent themes in studies regarding CLN3

Based on the protein interaction analyses and biochemical studies in mammalian cell lines, CLN3 disease mouse models, and yeast and *Drosophila* models, it seems that CLN3 affects several intracellular processes. However, only some of them have been observed in more than one system, or are supported by a specific protein interaction partner. A summary of functions linked to CLN3 is presented in **Table 5**.

**Table 5.** A summary of intracellular functions linked to CLN3.

| Intracellular function | Studies in mammals | Studies in yeasts | Studies in *Drosophila* | Protein interaction studies |
|---|---|---|---|---|
| Apoptosis | x | | | Calsenilin, SBDS |
| Autophagy | x | | | |
| Arginine metabolism | x | x | | |
| Cell migration | x | | | Myosin IIB |
| Lipid metabolism | x | x | | |
| Lysosomal homeostasis | x | x | | SBDS, CLN5 (?) |
| Membrane trafficking | x | x | | Myosin IIB |
| Mitochondrial functions | x | | | |
| Neuritogenesis | x | | | Myosin IIB |
| Stress response | x | | x | SBDS |
| Synaptic functions | x | | | Myosin IIB |

# 3 Aims of the study

Prior to this study the localisation of CLN3 had been investigated and functional analyses were initiated. However, functional studies were mostly based on the phenotypes of CLN3-deficient cells and animal models, an approach which can not reliably confirm the direct role of the protein in respective intracellular events. Therefore, studies analysing the function of CLN3 via protein interactions were needed. The aim of this study was to identify novel CLN3 interactions and to study their relation to CLN3 deficiency. The specific aims of the study were the following:

- to screen for unknown protein interaction partners of CLN3 by yeast two-hybrid and glutathione S-transferase (GST) interaction pull-down methods
- to analyse the characteristics of two previously unidentified CLN3 interaction partners found in the yeast two-hybrid study, fodrin and $Na^+$, $K^+$ ATPase, in the *Cln3*$^{-/-}$ mouse and in the juvenile CLN3 disease patient cells
- to verify and analyse the potential interaction between CLN3 and the endocytic microtubule-binding protein Hook1
- to verify and analyse the potential interactions between CLN3 and proteins involved in late endosomal/lysosomal membrane trafficking
- to investigate intracellular membrane trafficking in the context of CLN3 deficiency

# 4 Materials and methods

## 4.1 Materials and methods used in the current study

Materials and methods used in this study are summarised in **Table 6**. Details of each material or method are described in the original publications or below in Chapter 4.2 (Additional information on materials and methods used in the original publications) and Chapter 4.3 (Unpublished materials and methods).

**Table 6.** A summary of materials and methods.

| Material or method | Detailed information |
|---|---|
| Cell cultures | I, II, III |
| Cloning of cDNA constructs | I, II |
| (Co-)immunoprecipitation | I, II, III, Chapter 4.2.2 |
| Confocal immunofluorescence microscopy | I, II, III |
| Cytoplasmic acidification | III |
| Dissection and culturing of mouse primary cortical neurons | I |
| Fluorescence recovery after photobleaching (FRAP) | III |
| GST pull-down of purified $His_6$-Rab7Q67L and $His_6$-RILP | III |
| GST interaction pull-down with *in vitro*-translated proteins | II |
| GST interaction pull-down with tissue extracts | Chapter 4.3.1 |
| LDL receptor-mediated endocytosis assay | II |
| Mammalian two-hybrid | III |
| Metabolic labeling | II |
| Polyclonal antibody production against synthetic peptide | II |
| Preparation of mouse brain tissue extracts | I |
| Protein detection by immunofluorescence | I, II, III |
| Protein detection by immunohistochemistry | I |
| Protein detection by Coomassie and silver staining | Chapter 4.3.1 |
| Protein detection by Western blotting | I, II, III, Chapter 4.2.1 |
| Protein production by *in vitro* translation | II |
| Protein production in *Escherichia coli* | II, Chapter 4.2.4 |
| Quantitative immunofluorescence image analysis | I, III |
| Quantitative real-time polymerase chain reaction (qPCR) | III |
| Quantitative Western blot analysis | I |
| $^{86}Rb^+$ uptake assay for $Na^+$, $K^+$ ATPase | I |
| RNA interference | III |
| Sequencing | I, Chapter 4.2.3 |
| Statistical analyses | I, II, III |
| Total internal reflection fluorescence (TIRF) microscopy | I |
| Transferrin uptake and recycling assay | II |
| Transient transfections | I, II, III |
| Yeast two-hybrid | I, Chapter 4.2.3 |

## 4.2 Additional information on materials and methods used in the original publications

### 4.2.1 Protein detection by Western blotting (I, II, III)

Western blotting was performed according to standard protocol. Briefly, protein samples were denatured in Laemmli buffer and if appropriate, boiled briefly. Samples intended for detection of CLN3 were not boiled to avoid protein aggregation. Proteins were separated on appropriate sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels under standard conditions and then transferred to a nitrocellulose membrane (GE Healthcare) by standard wet blotting method. Blotted membranes were blocked with Tris-buffered saline supplemented with 0.05% Tween-20 (TBST) and 5% skimmed milk powder for a minimum of 30 minutes at room temperature (RT) in a gentle rotation. After brief washing with TBST, primary antibodies diluted in TBST were added and membranes were incubated for a minimum of 1 hour at RT followed by washing with TBST. Membranes were then incubated with appropriate horseradish peroxidase (HRP)-conjugated polyclonal secondary antibodies (Dako Denmark A/S) for a minimum of 30 min at RT. Longer blocking or antibody incubations were performed at +4°C. Protein-bound antibody complexes were detected by enhanced chemiluminescence (ECL) reaction using a commercial ECL-kit (GE Healthcare).

### 4.2.2 Hook1 immunoprecipitation (II)

African green monkey kidney cells (COS-1 cells) were plated on a 6-well culture dish and transfected by the calcium-phosphate method followed by metabolic labelling with [$^{35}$S]cysteine. Cells were then collected by trypsinisation and resuspended in 100 µl of ice-cold lysis buffer (phosphate-buffered saline (PBS) supplemented with 1% Triton X-100 and Complete Protease Inhibitor Cocktail, Roche) followed by lysing by two freeze-thaw cycles. 10 µl of each lysate was analysed by Western blotting to check transfection efficiency and the remaining lysate was used for the immunoprecipitation analysis. Lysates diluted to 1 ml with immunoprecipitation buffer (Tris-buffered saline supplemented with 0.05% Tween-20 and 10 mg/ml bovine serum albumin, BSA) were pre-cleared three times with 50 µl of 10% heat-killed and formalin-fixed *Staphylococcus aureus* cells (standardised PANSORBIN® Cells, Calbiochem). Pre-cleared samples were then incubated either with 5 µl of Hook1 antibody 9005 or 9019 or 1 µl of Hook1 antibody hHK1 (antibodies are described in the original publication II) at +4°C overnight followed by incubation with 20 µl of 10% *S. aureus* for 3 hours on ice. Immobilised protein complexes were then carefully washed with washing buffer (0.6 M NaCl, 0.1% SDS, 0.05% Tween-20, 0.01 M Tris-HCl pH 7.4), resuspended in Laemmli buffer, boiled and separated by SDS-PAGE and finally analysed by autoradiography.

### 4.2.3 Yeast transformation, plasmid isolation and sequencing in yeast two-hybrid assay (I)

CLN3 bait and a library complementary DNAs (cDNAs) were transformed in tandem into MaV203 yeast strain using lithium acetate (LiAc) transformation protocol. A colony of MaV203 yeast strain was cultured overnight at +30°C in Yeast Extract Peptone Dextrose (YPD) medium. Next day, 50 ml of the medium was inoculated with the overnight culture to optical density at 600 nm (OD600) of 0.2 – 0.3, followed by incubation at +30°C until OD600 was between 0.9 and 1. Then, cells were spinned down and washed twice with 50 ml of sterile water and once with 1 ml of 100 mM LiAc. Cells were resuspended in 400 µl of 100 mM LiAc and divided into 50 µl aliquots. Cells were pelleted and following reagents were added on the cell pellet; 240 µl of 50% polyethylene glycol, 36 µl of 1 M LiAc, 25 µl of denatured 2 mg/ml single-stranded DNA, and 1 – 1.5 µg of appropriate cDNA diluted in 50 µl of sterile water. Cells were carefully resuspended and incubated for 30 minutes at +30°C followed by heat shock at +42°C for 30 minutes. After brief centrifugation, cells were resuspended in 200 µl of sterile water and plated on appropriate synthetic defined (SD) selective plate.

To isolate positive library cDNAs, a corresponding colony was picked and cultured in 3 ml of SD/-Leucine selective media overnight at +30°C. After centrifugation a yeast pellet was resuspended in 200 µl of 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris, pH 8.0 buffer. 200 µl of acid-washed glass beads (425 – 600 µm, Sigma) were added and suspensions were vortexed vigorously 5 times for 1 minute each time and kept on ice for 1 minute between the rounds. Proteins were denatured and precipitated by phenol/chloroform/isoamyl alcohol extraction, after which DNA was isolated by ethanol precipitation.

For nucleotide sequence determination, each positive library cDNA was polymerase chain reaction (PCR)-amplified with pACT2 vector primers (Proligo). PCR products were purified, analysed on agarose gels and sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

### 4.2.4 Production of GST-CLN3 fusion proteins in *Escherichia coli*

*Escherichia coli* DH5α strain was transformed with pGEX4T-3 cDNA constructs encoding either the plain GST, or the N-terminally GST-tagged CLN3 fusion proteins containing either the N-terminus (amino acids 1-33; GST-CLN3 1-33), the first luminal loop (amino acids 56-97; GST-CLN3 56-97) or the major cytoplasmic loop (amino acids 232-280; GST-CLN3 232-280) of CLN3. Transformed DH5α cells were then cultured overnight in Luria medium supplemented with selective antibiotic. Next day, appropriate amounts of selective Luria medium were inoculated with overnight cultures and incubated at +30°C until OD600 was approximately 0.6. Then, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and the cultures were further incubated for 4 hours. The

cultures were divided into smaller aliquots to enhance proper cell lysis and to avoid aggregation of GST-CLN3 fusion proteins. Cells were pelleted and resuspended in PBS supplemented with 0.2 mg/ml lysozyme, 0.4% Triton X-100 and Complete Protease Inhibitor Cocktail (Roche). Suspensions were incubated on ice for 4 hours, after which sarcosyl was added to a final concentration of 0.1% and suspensions were sonicated on ice in short periods. Sonicated suspensions were centrifuged and supernatants were collected and if not used immediately, frozen and stored at -70°C.

## 4.3 Unpublished materials and methods

### 4.3.1 GST interaction pull-down with tissue extracts

Equal amounts (10 - 80 µg) of Glutathione Sepharose 4B-immobilised GST (control), GST-CLN3 1-33, or GST-CLN3 232-280 fusion proteins were mixed with 1 – 2 mg of mouse whole brain extract (prepared as described in the original publication I) and diluted to the total volume of 1 ml with tissue extraction buffer. Mixtures were incubated at +4°C for 2 – 3 hours or overnight, depending on the case. Then, beads and immobilised protein complexes were washed 3-5 times with cold extraction buffer and resuspended in Laemmli buffer. Samples were analyzed on SDS-PAGE and protein bands were visualised either by Coomassie staining, silver staining or Western blotting.

For protein identification, SDS-PAGE gels were stained using mass spectrometric compatible Coomassie or silver staining protocols. In Coomassie staining, gels were incubated in 0.1% Coomassie blue R-350, 30% methanol, 0.48% acetic acid staining solution for 20 minutes at RT followed by destaining with 30% methanol, 0.48% acetic acid solution over two nights. In silver staining, proteins were visualised according to O´Connell and Stults (1997). Stained CLN3 specific protein bands were cut from the gel and sequenced by matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometric peptide mass fingerprint analysis at The Protein Chemistry Core Facility of the Institute of Biotechnology, University of Helsinki.

## 4.4 Ethical aspects

This study has been evaluated and approved by the Laboratory Animal Care and Use Committee of the National Insitute for Health and Welfare, Helsinki. This study has been carried out following good practice in laboratory animal handling and the regulations for handling genetically modified organism.

# 5 Results and discussion

## 5.1 CLN3 protein interaction screening (the original publication I, and unpublished data)

One aim of the current study was to screen for unknown CLN3 interactions employing two methods, yeast two-hybrid and GST interaction pull-down. Due to the hydrophobic nature of CLN3, utilisation of full-length protein was not applicable. Therefore, defined regions of the protein were employed separately. Two of the cytoplasmic domains of CLN3, the N-terminal domain and the major cytoplasmic loop domain, were used as baits to fish unknown CLN3 interaction partners expressed in brain tissues. The third major cytoplasmic segment of CLN3, the C-terminus, was excluded due to its toxicity to *E. coli* and autonomous transcription activation property observed in previous (Kaczmarski et al., 1997; Leung et al., 2001b) and in the current studies (data not shown).

### 5.1.1 GST-CLN3 interaction pull-down with mouse brain extracts

Amino acids 1-33 and 232-280 of the N-terminus and the major cytoplasmic loop domain of CLN3, respectively, were produced as N-terminally tagged GST fusion proteins and used to pull-down CLN3 interacting proteins from mouse whole brain extract. Plain GST was used to control the binding specificity.

The analyses revealed several putative CLN3 binding partners. However, the mass spectrometric identification of the CLN3-specific proteins turned out to be challenging due to binding of several other proteins with similar electrophoretic mobility. However, one of the GST-CLN3 232-280-bound proteins repeatedly found to associate with GST-CLN3 232-280 but not with GST control (**Figure 8A**), was recognised in mass spectrometry as immunoglobulin heavy chain binding protein, BiP, also known as 78 kDa glucose-regulated protein, GRP78. The binding of GRP78/BiP with CLN3 was verified by the subsequent Western blotting analysis of the GST-CLN3 interaction pull-down samples (**Figure 8B**) as well as by CLN3 co-immunoprecipitation (the original publication I, Figure 2C). Furthermore, GRP78/BiP was found to specifically associate with the loop domain but not with the N-terminal domain of CLN3 (**Figure 8B**). At the time of the interaction pull-down analysis, GRP78/BiP was well-known for its chaperone activity in protein folding (Beggah et al., 1996), and therefore, observed interaction between CLN3 and GRP78/BiP was initially considered to be associated with the synthesis and folding of CLN3 in the ER. However, during the course of the CLN3 yeast two-hybrid analysis and especially in the light of the novel findings on the GRP78/BiP, the interaction turned out to be putatively important also for the CLN3 protein

functions. This will be discussed in more detail in the context of CLN3/ $Na^+$, $K^+$ ATPase interaction (Chapter 5.2.3).

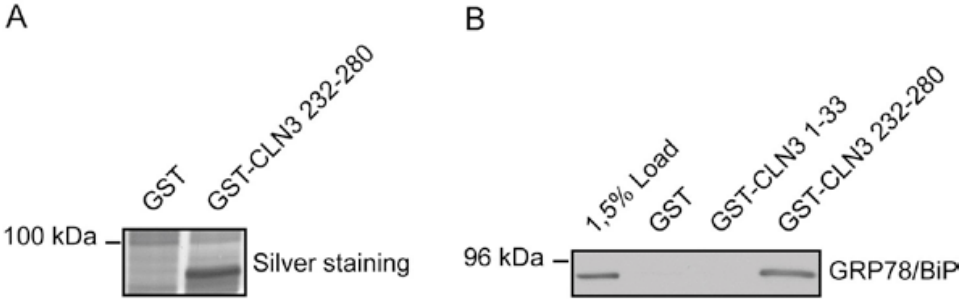


**Figure 8.** GRP78/BiP associates with CLN3 in GST interaction pull-down assay. (A) GST-CLN3 232-280 pull-down from mouse brain extract. Proteins in pull-down samples were visualised by silver-staining. Intensively stained protein band specifically found in GST-CLN3 232-280 sample was identified in mass spectrometry as mouse GRP78/BiP protein. (B) GST pull-down analysis with GST-CLN3 1-33 and GST-CLN3 232-280. Western blotting with anti-GRP78/BiP confirmed the interaction with CLN3 amino acid portion 232-280 but excluded the association with the N-terminus (amino acids 1-33) of CLN3.

### 5.1.2 CLN3 yeast two-hybrid assay

Prior to the current study, attempts to use yeast two-hybrid approach were unsuccessful in the identification of the interaction partners for CLN3, or its yeast orthologue Btn1p (Cottone et al., 2001; Leung et al., 2001a). This was partly due to attempts to use full-length protein or membrane-bound segments containing domains as baits in a classical YTH assay, an approach unsuitable for insoluble integral membrane proteins (Auerbach et al., 2002; Stagljar and Fields, 2002). At the time of initiating the current study, well-established YTH applications for full-length transmembrane proteins were not available and therefore, defined cytoplasmic domains of CLN3, amino acids 1-40 and 232-280, were utilised as baits in the traditional Fields´ GAL4 YTH assay (Fields and Song, 1989).

Similar to the GST-CLN3 interaction pull-down analysis, the N-terminal and the major cytoplasmic loop domains of CLN3 were found to associate with a number of putative interacting proteins. Altogether, analysis of 30 000 co-transformants in each CLN3 YTH assay resulted in 396 putative positive library clones for the CLN3 1-40 bait and 159 for the CLN3 232-280 bait, most of which were identified. Sequence homology analysis revealed that only a fraction of the putative positive clones represented previously identified proteins or uncharacterised proteins with partial homology to known proteins. Rest of the clones represented non-coding regions or yet unidentified proteins (expressed sequence tags (ESTs), untranslated regions

(UTR), clones, and sequences with no homology to known proteins or functional domains).

To evaluate their specificity for CLN3, each positive library clone classified to encode protein was reassayed with appropriate CLN3 bait and vector control. Those which were found to be CLN3-specific were taken as true positives. Altogether, approximately 40 proteins were identified as true CLN3 1-40 positives and six as true CLN3 232-280 positives, with three of them associating with both CLN3 baits (**Table 7**).

The biological relevance of each CLN3 interaction candidate was scored on the basis of its function, intracellular localisation and molecular interactions. As a result, several interesting proteins were estimated to be worth further analyses. However, due to the substantial number of the proteins, further selection was performed for initial functional analysis. Focus was set to proteins involved in intracellular membrane trafficking and/or cytoskeletal functions. These selection criteria were based on the ongoing analysis of putative CLN3/Hook1 interaction (the original publication II) and were further supported by later reports linking CLN3 to intracellular membrane trafficking (Fossale et al., 2004; Luiro et al., 2006; Weimer et al., 2006). In addition, proteins which were known to be part of the same protein interaction network or encoded by several independent clones were considered highly important. Among these, plasma membrane-associated cytoskeletal and endocytic protein fodrin and its multifunctional plasmalemmal/endosomal interaction partner $Na^+$, $K^+$ ATPase, both positive with the CLN3 1-40 bait, were the first candidates selected for further analysis (the original publication I).

Fodrin interacts indirectly with and regulates the plasma membrane-association of Na+, K+ ATPase (Morrow et al., 1989; Nelson and Hammerton, 1989; Kizhatil et al., 2009). Furthermore, four CLN3 1-40 positive clones were found to encode the β subunit of $Na^+$, $K^+$ ATPase heterodimer. Three clones encoded amino acids 88-303, 158-303 or 250-303 of the β1 isoform and one clone encoded amino acids 187-290 of the β2 isoform. Fodrin, a heterotetrameric α-β complex, was represented by one clone encoding the amino acids 2265-2364 of β-fodrin, also known as β-II-spectrin or spectrin beta chain, brain 1. The finding that $Na^+$, $K^+$ ATPase and associated proteins may represent true CLN3 interacting proteins was emphasised by the observation that also two other proteins, FXYD domain-containing ion transport regulator 6 (FXYD6, phosphohippolin) and beta-arrestin 1 (arrestin 2), both positive with the CLN3 1-40 bait, were later revealed to be functionally connected to $Na^+$, $K^+$ ATPase (Delprat et al., 2007; Kimura et al., 2007; Shiina et al., 2010). The analyses of the additional $Na^+$, $K^+$ ATPase -associated putative CLN3 interacting proteins among other interesting interaction candidates found in the CLN3 YTH assay are currently in progress (Scifo, E., Uusi-Rauva, K. et al., unpublished).

**Table 7.** Proteins confirmed to interact with CLN3 domain(s) in YTH assay.

| **CLN3 1-40 yeast two-hybrid** | **No of clones** |
|---|---|
| Activator of 90 kDa heat shock protein ATPase homologue 1 (AHA1) (O95433) | 1 |
| ADAMTS-9 (A disintegrin and metalloproteinase with thrombospondin motifs 9) (Q9P2N4) | 2 |
| Adenylate cyclase, type VI (O43306) | 1 |
| Alpha-2-macroglobulin (P01023) | 1 |
| ATP-dependent metalloprotease FtsH1 homologue (Q96TA2) | 1 |
| Atrial natriuretic peptide receptor B (guanylate cyclase B) (P20594) | 1 |
| Beta-arrestin 1, isoform 1A (P49407) | 1 |
| CB1 cannabinoid receptor-interacting protein 1 (Q96F85) | 2 |
| Clusterin (Apolipoprotein J) (P10909) | 1 |
| Collagen alpha 1(I) chain (P02452) | 2 |
| Collagen alpha 2(I) chain (P08123) | 1 |
| β-fodrin (Spectrin beta chain, brain 1, long isoform) (Q01082) | 1 |
| FSCN1 protein (Fascin 1) (Q16658) | 1 |
| FXYD domain-containing ion transport regulator 6 (FXYD6) (Q9H0Q3) | 1 |
| Glomulin (FKBP-associated protein) (Q92990) | 1 |
| Heat shock cognate 71 kDa protein (isoform 1) (P11142) | 1 |
| Heat shock protein HSP60 (Q9P0X2) | 1 |
| Heterogeneous nuclear ribonucleoprotein H (P31943) | 1 |
| *H. sapiens* heterogeneous nuclear ribonucleoprotein A2/B1 (HNRPA2B1) (Q9BWA9) | 1 |
| N-myc proto-oncogene protein (P04198) | 1 |
| Oxidation resistance 1 (Q9NWC7) | 1 |
| Peroxiredoxin 6 (P30041) | 1 |
| Protein arginine N-methyltransferase 6 (Q96LA8) | 1 |
| Putative uncharacterized protein DKFZp547J036 (ELAVL3 protein) (Q9H024) | 2 |
| Sodium/potassium-transporting ATPase beta-1 chain (P05026) | 3 |
| Sodium/potassium-transporting ATPase beta-2 chain (P14415) | 1 |
| Ubiquitin-activating enzyme E1C (Nedd8-activating enzyme E1C) (Q8TBC4) | 1 |
| Uncharacterized hypothalamus protein HT008 (Q8IWB9) | 1 |
| Voltage-dependent anion-selective channel protein 3 (VDAC-3) (Q9Y277) | 1 |
| Zinc finger protein clone 647 (P15622) | 2 |
| **CLN3 232–280 yeast two-hybrid** | |
| Adapter-related protein complex 1 gamma 1 subunit (O43747) | 1 |
| Synaptotagmin IV (Q9H2B2) | 2 |
| **CLN3 1-40 yeast two-hybrid / CLN3 232–280 yeast two-hybrid** | |
| Elongation factor 1-alpha 1 (P04720) | 1/4 |
| Microtubule-associated protein 1A (MAP 1A) (P78559) | 1/1 |
| THAP domain protein 11 (Q96EK4) | 1/2 |

The large number of positive library clones in the CLN3 YTH assay most likely results from the utilisation of short protein domains. It would be of great interest to test CLN3 in a novel YTH application designed for the full-length transmembrane proteins. This so-called split-ubiquitin yeast two-hybrid system (Stagljar et al., 1998; Stagljar and te Heesen, 2000) would perhaps provide additional CLN3 interacting proteins but also help in defining true interactions obtained in the classical YTH analysis.

## 5.2 CLN3 and $Na^+$, $K^+$ ATPase-fodrin complex (the original publication I)

Following experiments were performed to verify putative CLN3/fodrin and CLN3/ $Na^+$, $K^+$ ATPase interactions and to analyse their relation to CLN3-deficiency in mammalian cells.

### 5.2.1 CLN3 interacts with $Na^+$, $K^+$ ATPase and fodrin

Putative interactions of CLN3 with fodrin and $Na^+$, $K^+$ ATPase were verified by co-immunoprecipitation. Full-length CLN3 over-expressed in COS-1 cells was immunoprecipitated with the rabbit polyclonal antibody produced against amino acids 242-258 of the major cytoplasmic loop of CLN3. The presence of fodrin and $Na^+$, $K^+$ ATPase in the CLN3 co-immunoprecipitate was tested by Western blotting with antibodies against the endogenous proteins (the original publication I, Figure 2). Due to lack of Western blot compatible β-fodrin antibody, fodrin was assayed with an α-fodrin antibody. $Na^+$, $K^+$ ATPase was assayed with an antibody produced against the most widely expressed $Na^+$, $K^+$ ATPase α subunit, α1, as the immunoblot detection of the β1 subunit with the available β1 antibody was not possible due to similar electrophoretic size of $Na^+$, $K^+$ ATPase β1 (approximately 55 kDa) and the heavy chain of the precipitating antibody. As a result, both tested proteins were confirmed to co-immunoprecipitate with CLN3 (the original publication I, Figure 2A).

The interaction between CLN3 and $Na^+$, $K^+$ ATPase was also studied by testing whether CLN3 immunoprecipitates with $Na^+$, $K^+$ ATPase β1 antibody. Fodrin and GRP78/BiP known to interact with $Na^+$, $K^+$ ATPase were utilised as positive controls. This approach also showed that $Na^+$, $K^+$ ATPase and CLN3 co-immunoprecipitate as CLN3 and the positive controls were confirmed to immunoprecipitate with the $Na^+$, $K^+$ ATPase -precipitating antibody (the original publication I, Figure 2B).

These results support the molecular connection between full-length CLN3, fodrin and $Na^+$, $K^+$ ATPase.

### 5.2.2 Abnormal fodrin cytoskeleton in CLN3 deficiency

To study whether plasma membrane-associated fodrin cytoskeleton is affected in CLN3 deficiency, juvenile CLN3 disease fibroblasts and *Cln3*$^{-/-}$ mouse brain sections were analysed by immunofluorescence microscopy and immunohistochemistry, respectively. Due to the properties of available fodrin antibodies, antibodies recognising different subunits of fodrin tetramer were utilised. CLN3/Cln3 deficiency was found to be marked by abnormalities in the fodrin staining. This was demonstrated by the confocal immunofluorescence microscopy analyses of α-fodrin in the patient fibroblasts and control cells grown to equivalent confluence as well as immunohistochemical analyses of β-fodrin in brain sections of 1- and 3-month-old wild type and *Cln3*$^{-/-}$ mice. While most of the fodrin signal was diffuse in control cells, the structure of fodrin cytoskeleton appeared more punctuated in the patient fibroblasts (the original publication I, Figure 4). Furthermore, compared to the wild type brain sections (the original publication I, Figure 5A, C), *Cln3*$^{-/-}$ brain tissue showed more faint cell membrane immunostaining of fodrin in the hippocampal pyramidal neurons and abnormally faint filamentous staining in the cortex already in 1-month-old animals (the original publication I, Figure 5B, D). The abnormal immunostaining of fodrin in *Cln3*$^{-/-}$ mouse brain suggests structural abnormalities in the neuronal fodrin cytoskeleton as cell morphology (the original publication I, Figure 5E, F) and the expression level of fodrin (the original publication I, Figure 6) were found indistinguishable between wt and *Cln3*$^{-/-}$ samples. These findings suggest that CLN3/Cln3 deficiency results in changes in the plasma membrane-associated fodrin cytoskeleton.

### 5.2.3 Impaired ouabain-induced endocytosis of $Na^+$, $K^+$ ATPase in *Cln3*$^{-/-}$ neurons suggests defects in non-pumping functions of the protein

$Na^+$, $K^+$ ATPase represents the major pump for $Na^+/K^+$ exchange in neurons (reviewed in Benarroch, 2011). Therefore, it was first studied whether loss of Cln3 affects the plasmalemmal net ion pumping activity of $Na^+$, $K^+$ ATPase. The $Na^+$, $K^+$ ATPase activity was determined in wt and Cln3-deficient cortical primary neurons grown for 8 or 12 days after plating, using the classical $^{86}Rb^+$ uptake assay utilising the cardiotonic steroid ouabain as an $Na^+$, $K^+$ ATPase -specific inhibitor. No statistically significant differences in the total or in the ouabain-sensitive $^{86}Rb^+$ uptake were found between the control and Cln3-deficient neurons (the original publication I, Figure 3). The potential impact of $Na^+$, $K^+$, $2Cl^-$ cotransporter was excluded by repeating the assay in the presence of the specific inhibitor, furosemide (data not shown). Therefore, combined with the finding that also the intracellular $K^+$ concentrations were unaltered in Cln3-deficiency (data not shown), these results suggest that loss of Cln3 has no major effect on the basal plasmalemmal net ion pumping activity of $Na^+$, $K^+$ ATPase in the primary neuronal cultures.

$Na^+$, $K^+$ ATPase exists in two functionally distinct pools. While one pool is known for its role as a traditional $Na^+/K^+$ transporter (reviewed in Kaplan, 2002; and Benarroch, 2011), the other is engaged in cellular processes other than ion pumping (reviewed in Aperia, 2007; Liang et al., 2007; Schoner and Scheiner-Bobis, 2007; Tian and Xie, 2008; Bagrov et al., 2009; Lingrel, 2010). Interestingly, also the latter pool of the protein is regulated by ouabain. Following ouabain-binding, the non-pumping pool of the $Na^+$, $K^+$ ATPase accumulates in clathrin-coated pits and caveolaes induces cytosolic cascades of various intracellular events, including activation of signalling proteins, apoptosis and calcium oscillations (see references above). These events are eventually followed by endocytosis of the protein (Liu et al., 2002; Liu et al., 2004; Kesiry and Liu, 2005; Liu et al., 2005). This aspect of the $Na^+$, $K^+$ ATPase, relative to Cln3 deficiency, was explored utilising total internal reflection fluorescence (TIRF) microscopy and image analysis. First, the amount of different $Na^+$, $K^+$ ATPase subunits at the plasma membrane relative to that in the total cellular pool in Cln3-deficient and wt neurons was measured by determining the correlation value of co-localisation between TIRF and total fluorescence signals. Compared to that in control cells, the correlation values of α3 and β1 subunit signals were increased in *Cln3*$^{-/-}$ neurons indicating that Cln3-deficient cells had relatively more α3 and β1 in the plasma membrane-associated fraction. Correspondingly, all other analysed $Na^+$, $K^+$ ATPase subunits were found to be normally distributed between the cell surface and intracellular compartments (the original publication I, Figure 7). To study the observed defect in more detail, the absolute amounts of plasma membrane-associated α3 and β1 were measured both at basal condition and after ouabain-induced endocytosis utilising fluorescence intensity analysis. The analyses of TIRF images showed that in the basal condition, the averaged absolute plasmalemmal amounts of both subunits were abnormal in *Cln3*$^{-/-}$ neurons. The basal plasmalemmal amount of β1 was slightly increased (the original publication, I, Figure 8A, black bars) and the amount of α3 was remarkably decreased in the Cln3-deficient neurons (the original publication, I, Figure 8B, black bars). Furthermore, the analyses of the total fluorescence images revealed that the averaged total cellular expression of both subunits was decreased in Cln3-deficient cells (data not shown), α3 being more affected (the original publication I, Figure 9, compare A and E). These results suggest that although the total cellular expression of $Na^+$, $K^+$ ATPase α3 and β1 is decreased in *Cln3*$^{-/-}$ neurons, their targeting to the plasma membrane is favoured at the expense of the intracellular pool in Cln3 deficiency. Furthermore, Cln3-deficient neurons remained with higher levels of the protein at the cell surface after ouabain-treatment (the original publication I, Figure 8, grey bars and Figure 9, compare H and D, P and L). This indicates that the loss of Cln3 results in defects in the ouabain-induced endocytosis of the $Na^+$, $K^+$ ATPase α3 and β1. The fact that the absolute amount of $Na^+$, $K^+$ ATPase α3 at the cell surface of Cln3-deficient neurons was even increased during ouabain-treatment may be due to increased expression of the protein and subsequently, repleatment of the cell surface-associated pool (the

original publication I, Figure 9, compare E and G). This has been reported to occur also in other conditions (Tian et al., 2009).

In summary, the basal cell surface association of a neuron-specific $Na^+$, $K^+$ ATPase (α3/β1) is increased in *Cln3*$^{-/-}$ neurons most likely due to defective endocytosis of the protein. However, CLN3 may additionally contribute to the basal cell surface targeting of $Na^+$, $K^+$ ATPase already in the ER via the interaction with GRP78/BiP, the protein essential for maturation of $Na^+$, $K^+$ ATPase (Beggah et al., 1996). This study showed for the first time that ouabain also induces the endocytosis of $Na^+$, $K^+$ ATPase in neurons, which suggests that the ouabain-regulated non-pumping functions of $Na^+$, $K^+$ ATPase may also have an important role in neuronal cells. Most importantly, the abnormal response of *Cln3*$^{-/-}$ neurons to ouabain, together with the reported finding that GRP78/BiP is involved in ouabain-induced endocytosis of $Na^+$, $K^+$ ATPase (Kesiry and Liu, 2005), suggests that CLN3 may be involved in the above-mentioned ouabain-regulated non-pumping functions of $Na^+$, $K^+$ ATPase.

## 5.3 CLN3 and endosomal/lysosomal membrane trafficking (the original publications II and III)

In parallel with the screening of novel CLN3 interactions, an approach focusing on the confirmation of the potential interacting partners was also utilised. This study was originally initiated in response to findings obtained with a *CLN3* knock-out yeast model, *btn1*Δ. The *btn1*Δ strain has been reported to have increased expression of *BTN2* (Pearce et al., 1999a). Btn2p shows 38% similarity to human Hook1 protein (Pearce et al., 1999a). At the time of initiating the current study no reports on the function of Hook1 were published and its role in intracellular events could only be estimated based on the available information on its *Drosophila* and *S. cerevisiae* homologues. At that time, *S. cerevisiae* Btn2p had been suggested to have a role in maintenance of cellular pH homeostasis (Chattopadhyay et al., 2000) while *Drosophila* hook had been shown to be required for the trafficking in late endosomal/lysosomal compartments (Kramer and Phistry, 1996; Kramer and Phistry, 1999; Sunio et al., 1999). In the course of the study, it was reported that also Btn2p and possibly human Hook1 are involved in the intracellular trafficking processes as it was shown that Btn2p interacts with the yeast Yif1 protein (Chattopadhyay et al., 2003), a component of a Ypt1 (Rab1) complex required for the vesicular trafficking between the ER and Golgi (Barrowman et al., 2003) and that Hook1 belongs to a novel microtubule-binding protein family of Hook proteins (Walenta et al., 2001). Therefore, it was of interest to analyse the putative role of CLN3 in intracellular membrane trafficking. The study consisted of 1) the analyses of putative interactions between CLN3 and proteins involved in the intracellular membrane trafficking, 2) characterisation of the effects of CLN3 and its mutants on confirmed interacting

proteins, and 3) analyses on the possible defects in the intracellular membrane trafficking in CLN3-deficiency.

### 5.3.1 CLN3 interacts with several proteins involved in late endosomal/lysosomal membrane trafficking

#### *5.3.1.1 CLN3 interacts with Hook1*

The molecular association between CLN3 and Hook1 was tested by GST pull-down method utilising *in vitro*-translated $^{35}$S-labeled-Hook1 and different domains of CLN3 expressed as N-terminally tagged GST-fusion proteins. Densitometric autoradiographic analysis of three separate GST interaction pull-down experiments showed that compared to the GST control, both cytosolic CLN3 domains employed, the N-terminal domain (amino acids 1-40) and the major cytoplasmic loop domain (amino acids 232-280), but not the lumenal domain (amino acids 56-97), of CLN3 showed a weak but statistically significant interaction with cytosolic Hook1 protein (the original publication II, Figure 5). Inspired by the report on the physical interaction between Btn2p and Ypt1 (Rab1) yeast proteins (Chattopadhyay et al., 2003), the interaction of Hook1 with several different mammalian Rab proteins was also examined. As a result, Hook1 was found to interact with N-terminally enhanced green fluorescence protein (EGFP)-tagged Rab7, Rab9 and Rab11 while no association with negative controls, non-endocytic EGFP-Rab24 and bare EGFP, was detected (the original publication II, Figure 6). Furthermore, confocal immunofluorescence microscopy analysis of HeLa cells showed that Hook1 and EGFP-tagged Rab7 co-localise in the same intracellular structures. Furthermore, compared to the proteins expressed alone, Hook1 and EGFP-Rab7 in double-transfected cells showed altered intracellular distribution (the original publication II, Figure 7). This was shown to be specific to Rab7 as no similar effect on other tested Hook1-associated Rab proteins was observed (the original publication II, Figure 7). The finding that Hook1 interacts with and affects the intracellular localisation of late endocytic Rab7 was later supported by the report showing that Hook1 interacts with the Rab7-binding HOPS complex in mammalian cells (Richardson et al., 2004; Wang et al., 2011). This is possibly mediated by a Hook proteins-containing FTS/Hook/FHIP multiprotein complex (Xu et al., 2008). In addition, the interaction between Hook1 and Rab9, a mediator of vesicle transport from LE to the Golgi compartments, was later supported by yeast studies showing that Btn2p interacts with SNAREs, cargo proteins, and coat components involved in endosome-Golgi protein sorting and that Btn2p is specifically required for the retrieval of Yif1 back to the Golgi apparatus (Kama et al., 2007; Kanneganti et al., 2011). Therefore, it is likely that Hook1 represents a general player in a trafficking network and mediates vesicular trafficking between various compartments. Furthermore, it seems that in yeast, Hook1 (Btn2p) has a more diverse role than in *Drosophila* and mammals. In addition to its role in intracellular membrane trafficking and maintenance of cellular

pH homeostasis, Btn2p has been suggested to regulate the plasma membrane arginine uptake and ion homeostasis (Chattopadhyay and Pearce, 2002; Kim et al., 2005).

#### *5.3.1.2 CLN3 interacts with Rab7-RILP effector complex and associated motor proteins*

The results above suggested for the first time that due to the interaction with Hook1, mammalian CLN3 is connected to cytoskeletal and intracellular membrane trafficking processes at the molecular level. Most importantly, CLN3 may also have a physical interaction and a functional role in the processes guided by endocytic Rab GTPases, especially those implicated in the trafficking of CLN3-associated late endosomal/lysosomal compartments. Therefore, next step in the study was to test putative interaction between CLN3 and Hook1-interacting endosome/lysosome localised Rab7. This was done by co-immunoprecipitation. N-terminally Xpress-tagged Rab7 was expressed with or without (untransfected cell control) CLN3 in COS-1 cells and the lysates were processed for immunoprecipitation with or without (matrix control) CLN3 amino acids 242-258 antibody followed by Western blotting with CLN3 and Xpress antibodies. Xpress-Rab7 was found to co-immunoprecipitate with CLN3 while no signal was detected in control samples (the original publication III, Figure 7A). To test whether CLN3 also interacts with the Rab7 effector RILP, the co-immunoprecipitation experiment described above was also carried out with Xpress-tagged RILP protein, and results confirmed that CLN3 indeed interacts with RILP recombinant protein (the original publication III, Figure 7B). These results suggested that CLN3 interacts with the Rab7 effector complex.

The above-mentioned interactions of CLN3 were analysed in more detail. First, the Rab7 interaction domain(s) of CLN3, and the preferred form of Rab7 participating in the interaction were determined by mammalian two-hybrid assay (MTH). Two major cytoplasmic domains of CLN3, amino acids 1-40 and amino acids 232-273, were separately cloned into the GAL4 DNA-binding domain vector pM. The C-terminal segment of CLN3 was excluded from the analysis due to its autonomous transcriptional activation properties (Leung et al., 2001b) (Uusi-Rauva, K. et al., unpublished data). A wild type Rab7, a GTPase-deficient constitutively active Rab7Q67L mutant and a dominant-negative Rab7T22N mutant, unable to release GDP, were cloned into the activation domain vector pVP16. COS-1 cells were transfected with the hybrid vectors and subsequently, analysed for the expression of the GAL4-dependent CAT reporter gene by CAT ELISA assay. The results of three separate experiments clearly demonstrated that only the expression of CLN3 amino acids 1-40 with Rab7wt and Rab7Q67L resulted in higher expression of reporter gene than the controls (the original publication III, Figure 8A). This indicated that CLN3 preferably interacts with active GTP-bound form of Rab7 via its N-terminal domain.

Next, GST pull-down experiments and purified proteins were utilised to test whether CLN3 interacts directly with GTP-bound Rab7 or possibly via interaction with RILP. GST, GST-CLN3 1-33 or GST-CLN3 232-280 fusion proteins bound to the glutathione Sepharose were incubated with purified $His_6$-Rab7Q67L and/or $His_6$-RILP recombinant proteins. Immunoblot analysis with antibodies produced against Rab7 and RILP revealed relatively weak but experimentally consistent direct binding between the N-terminal domain of CLN3 and $His_6$-Rab7Q67L (the original publication III, Figure 8B). Instead, RILP recombinant protein was found to interact directly with the cytoplasmic loop of CLN3 (the original publication III, Figure 8B). The difference in the CLN3-domain specificities of Rab7 and RILP proteins was supported by the co-immunoprecipitation experiments utilising a CLN3 antibody produced against the Rab7 interacting domain of CLN3. Compared to that with CLN3 242-258 antibody, CLN3 1-33 antibody immunoprecipitated significantly less Xpress-Rab7 but did not interfere the immunoprecipitation of RILP (the original publication III, Online Resource 1). Since RILP immunoprecipitates with the antibody produced against amino acids 242-258 of the same cytoplasmic loop domain of CLN3 that binds RILP (the original publication III, Figure 8 and Online Resource 1), it most likely interacts with CLN3 via amino acids outside of this epitopic region.

Together, these results indicate that active, GTP-bound Rab7 and RILP proteins interact directly with CLN3 but through different cytoplasmic domains of CLN3. GST pull-down and mammalian two-hybrid experiments suggested that the interaction between the N-terminal segment of CLN3 and Rab7 is relatively weak. However, the interaction may be stronger between full-length proteins due to stabilisation of the interaction by other CLN3 interacting proteins such as RILP. In addition, several other proteins involved in the intracellular membrane trafficking of late endosomal/lysosomal compartments were determined to co-immunoprecipitate with CLN3 (the original publication III, Figure 6). Tubulin and endogenous components of both plus and minus end-directed late endosomal/lysosomal microtubular motor complexes, namely dynactin, dynein, and kinesin-2, were found to interact with CLN3 (the original publication III, Figure 6).

#### *5.3.1.3 Mutations in CLN3 disturb interactions with Rab7 and RILP proteins*

To analyse whether juvenile CLN3 disease-associated mutations in CLN3 result in disturbances in the interactions between CLN3 and late endosomal/lysosomal motor protein complexes, quantitative co-immunoprecipitation analyses were performed with wild type CLN3 and two disease-associated CLN3 mutants, lysosome-localised CLN3E295K and ER-retained CLN3Δex7-8 (the original publication III, Online Resource 3). Experiments involving CLN3Δex7-8 were performed with anti-CLN3 1-33 antibody, and experiments involving CLN3E295K were performed either with

anti-CLN3 1-33 (Online Resource 3c, d) or anti-CLN3 242-258 (Online Resource 3a) antibodies. Quantities of indicated interacting proteins "pulled-down" with CLN3 mutants were compared to that with wild type CLN3. Due to limited amount of available CLN3 antibodies only few replicates per condition were included. Based on these analyses, CLN3Δex7-8 shows a reduced binding affinity to Rab7 (the original publication III, Online Resource 3a, b). This observation is consistent with the intracellular localisation of the mutant. CLN3Δex7-8 is retained in the ER and thus, is incapable to interact with late endosomal/lysosomal proteins. Interaction between CLN3Δex7-8 and RILP was not tested due to the fact that CLN3Δex7-8 mutant lacks the RILP-interacting domain and naturally, cannot interact with the protein. There were also slight changes in the binding affinities between CLN3E295K and dynactin, although this was not significant at the chosen confidence level (0.05), and with only few replicates (the original publication III, Online Resource 3a). CLN3E295K mutant was found to bind significantly less RILP than the wild type CLN3, especially when Rab7 was co-expressed with RILP and CLN3 (the original publication III, Online Resource 3c,d). In addition, compared to wild type protein, CLN3E295K interacts less efficiently with Rab7 in cells simultaneously expressing Rab7 and RILP (Online Resource 3c, d). These results indicate that interactions between CLN3 and Rab7-RILP effector complex are disturbed in juvenile CLN3 disease.

### 5.3.2 Effects of CLN3 and its mutants on Hook1 protein

When co-localisation between CLN3 and Hook1 was studied by confocal immunofluorescence microscopy it was observed that high expression levels of native or EGFP-tagged CLN3 in HeLa or COS-1 cells resulted in drastic changes in the intracellular localisation of Hook1 (the original publication II, Figure 1; COS-1 cells and native CLN3 not shown). In basal conditions (data not shown) and in co-expression with EGFP-tagged control protein, sialin, Hook1 had a diffuse cytoplasmic staining pattern while co-expression with CLN3 induced dramatic relocalisation of the protein to large unidentified dot-like structures (the original publication II, Figure 1). Interestingly, when Hook1-expressing HeLa cells were treated with the microtubule-depolymerising agent, nocodazole, an experiment inspired by a novel report on microtubule-binding properties of Hook1 (Walenta et al., 2001), the similar Hook1 aggregates were observed (the original publication II, Figure 2C). Therefore, it is possible that the formation of CLN3-induced Hook1-aggregates also involves detachment of Hook1 from microtubules.

Although the exact content of the CLN3-induced Hook1 aggregates remained elusive, they were found to contain Hook1 in a form which had lost or hidden antigenic epitope sites. This was shown by an experiment where metabolically labelled Hook1, over-expressed in COS-1 cells either with vector control, control protein sialin, or with CLN3, was immunoprecipitated from cell lysates with three

separate Hook1 antibodies. While Hook1 was clearly immunoprecipitable when expressed with the vector control or the control protein, no protein was precipitated from Hook1/CLN3 cell lysates (the original publication II, Figure 2A and B, shown for one precipitating Hook1 antibody). Similar results were obtained with both tested disease-associated CLN3 mutants, CLN3Δex7-8 and CLN3E295K (the original publication II, Figure 2B).

Substantial changes in the properties of Hook1 due to high expression levels of CLN3 imply that Hook1 may be regulated by CLN3 via mechanisms that affect its intracellular localisation and association with microtubules.

### 5.3.3 Effects of CLN3 and its mutants on late endosomal/lysosomal compartments

The finding that CLN3 interacts with several important proteins involved in late endosomal/lysosomal trafficking is in line with the reported observation that immortalised neuronal precursor cells expressing the ER-retained CLN3 mutant, CLN3Δex7-8, displayed changes in the distribution of endosomal/lysosomal compartments (Fossale et al., 2004). In the current study, it was tested whether another disease-associated CLN3 mutant, lysosome-localised CLN3E295K, exhibits similar properties. Although CLN3E295K mutation is associated with protracted disease progression, it eventually causes rapid deterioration and premature death (Aberg et al., 2009). This suggests that this mutant possesses endosomal/lysosomal function(s) which *in vivo* do not result in acute, severe insult but rather in changes which become lethal over time. However, high levels of the mutant protein may accelerate these processes and thus provide a valuable tool to study the effect of the lysosomal CLN3 mutant. Therefore, wild type CLN3 and CLN3E295K were over-expressed in HeLa cells followed by analyses of late endosomal/lysosomal compartments by immunofluorescence microscopy.

Interestingly, although both wild type CLN3 and the CLN3E295K mutant located to LAMP-1-positive LEs/lysosomes (the original publication III, Figures 1C and F, respectively), the staining pattern of the CLN3 mutant and subsequently, LAMP-1-positive compartments, was different from that in wild type CLN3-transfected cells. The CLN3E295K mutant displayed a tight perinuclear distribution while the signal of wild type CLN3 was more dispersed (the original publication III, Figures 1D or G and A, respectively). As expected, compared to untransfected and wild type CLN3-transfected cells, high expression levels of the CLN3E295K mutant also resulted in notable changes in the position of LAMP-1-positive LEs/lysosomes. In CLN3E295K-transfected HeLa cells, LEs/lysosomes were aggregated on one side of the nucleus while in the control cells, these compartments appeared more dispersed around the nucleus and additionally, the cytoplasm (the original publication III, Figure 1E and B, respectively). Instead, analysis of early endosomal antigen 1 protein (EEA1)-positive compartments showed that CLN3E295K had no

detectable effect on the steady-state location of EEs (the original publication III, Figure 1H), thus suggesting that CLN3E295K specifically disturbs the steady-state distribution of late endosomal/lysosomal compartments. Interestingly, confocal immunofluorescence microscopy and image analysis of fibroblasts from healthy control and from two juvenile CLN3 disease patients carrying either CLN3Δex7-8/CLN3Δex7-8 (homozygous) or CLN3E295K/CLN3Δex7-8 (compound heterozygous) mutation demonstrated that late endosomal/lysosomal compartments in patient fibroblasts were also located abnormally close to a perinuclear region (the original publication III, Online Resource 2).

Supporting the finding that CLN3 interacts with proteins of late endosomal/lysosomal microtubular trafficking, the CLN3E295K-induced aggregation of LAMP-1-positive compartments was confirmed, by immunofluorescence microscopy analyses, to be dependent on the integrity of the motor protein complexes and microtubular network. Indeed, when CLN3E295K-transfected HeLa cells were disrupted either for the dynactin complex by over-expression of EGFP-tagged $p50^{dynamitin}$ (Echeverri et al., 1996), or for the microtubular network by nocodazole treatment, CLN3E295K and LAMP-1-positive compartments were observed to be dispersed into the cytoplasm (the original publication III, Figure 4).

### 5.3.4 Effects of CLN3 and its mutants on Rab7 GTPase

To further analyse late endosomal compartments in the context of juvenile CLN3 disease, the effects of disease-associated CLN3 mutants on Rab7 GTPase were studied. Using confocal immunofluorescence microscopy, it was first determined whether CLN3E295K results in changes in the intracellular localisation of EGFP-tagged Rab7 in HeLa cells. Compared to EGFP-Rab7- and CLN3/EGFP-Rab7-transfected control cells (the original publication III, Figure 2C,C´ and 2G,G´,H,H´, respectively), EGFP-Rab7 in CLN3E295K/EGFP-Rab7-transfected cells (Figure 2L,L´,M,M´) co-localised more efficiently on LEs/lysosomes, marked by CLN3 and LAMP-1-stainings. Quantitative analysis of the co-localisation between EGFP-Rab7 and endosomal/lysosomal markers under each condition showed that in CLN3E295K-expressing cells, the co-localisation of EGFP-Rab7 with CLN3 and LAMP-1 was increased by approximately 20% compared to control cells (the original publication III, Figure 3). These findings imply that in addition to the steady-state position of LEs/lysosomes, CLN3 contributes to the compartmentalisation of Rab7.

To test whether CLN3 deficiency affects functional GTP/GDP cycle of Rab7, control fibroblasts and CLN3 disease fibroblasts were transfected with EGFP-Rab7 and analysed by FRAP analysis. Perinuclear clusters of EGFP-Rab7-positive compartments were photobleached and the recovery of EGFP fluorescence was monitored for 500 s. In the control fibroblasts, the recovery curve reached nearly

60% of initial fluorescence after 500 s. Juvenile CLN3 disease fibroblasts carrying compound heterozygous mutation (CLN3E295K/CLN3Δex7-8), did not show substantial differences in the EGFP recovery compared to the control cells (the original publication III, Figure 9A). Instead, the recovery was significantly faster in fibroblasts carrying the homozygous mutation. Homozygous patient cells showed ≥ 50 % recovery already after 100 s and reached ≥ 80% recovery after 500 s (the original publication III, Figure 9A). Since the vesicular pattern of the recovering cells resembled the pattern before the photobleaching (shown for control cells and homozygous patient cells, the original publication III, Figure 9B), it was concluded that the recovery of the vesicular EGFP-Rab7 signal reflects recruitment of unbleached cytoplasmic Rab7 on endosomal/lysosomal membranes rather than the movement of unbleached Rab7-positive organelles. Therefore, these results suggest that the loss of CLN3 function in the lysosomal compartments (CLN3Δex7-8 homozygous) lead to unbalanced GTP/GDP cycle of Rab7 and thus, could have a major effect on the function of Rab7. The observation that compound heterozygous (CLN3E295K/CLN3Δex7-8) cells did not show notably changes in the GTP/GDP cycle of Rab7 suggests that endogenously expressed lysosome-localised CLN3E295K mutant protein result in late endosomal/lysosomal abnormalities not captured by the FRAP experiment.

Taken together, the findings that disease-associated CLN3 mutations affect the intracellular position of late endosomal/lysosomal compartments and Rab7 but also the functional cycle of Rab7, suggest that disturbed Rab7-associated functions may play a role in juvenile CLN3 disease. While the current study showed that LEs/lysosomes but not EEs are perinuclearly aggregated in both the CLN3E295K-transfected HeLa cells and patient fibroblasts carrying two different mutation types (CLN3E295K/CLN3Δex7-8 or CLN3Δex7-8/CLN3Δex7-8), both early and late endosomal compartments were reported to be dispersed in *Cln3*$^{\Delta ex7\text{-}8\ Cotman}$ cerebellar neurons (Fossale et al., 2004). This implies that the changes in the vesicular distribution due to CLN3 deficiency may be dependent on the cell type.

### 5.3.5 CLN3 is required for bi-directional late endosomal/lysosomal trafficking

The following experiments were performed to analyse whether CLN3-deficiency results in defects in the endocytic pathways or in the outward movement of endosomal/lysosomal compartments. First, the uptake and recycling of biotinylated transferrin was examined in wild type and juvenile CLN3 disease patient fibroblasts (the original publication II, Figure 3). Second, later steps of the endocytic pathway in the patient fibroblasts were examined by analysing microscopically the trafficking of fluorescent low-density lipoprotein (BODIPY FL-LDL) to the EEA1 and LBPA-positive early endosomal and late endosomal/lysosomal compartments, respectively

(the original publication II, Figure 4). Third, regarding the CLN3/kinesin-2 interaction, it was also tested, using cytoplasmic acidification assay, whether CLN3-silenced HeLa cells have defects in induced anterograde movement of LAMP-1-positive LEs and lysosomes (the original publication III, Figure 5).

In the uptake assay, biotinylated transferrin was bound to the cell surface of the fibroblasts on ice followed by internalisation at 37°C. The amount of intracellular biotin-transferrin at indicated time points at 37°C was then examined using enzyme-linked immunosorbent assay (ELISA). No difference in the internalisation was observed between the patient and control fibroblasts (the original publication II, Figure 3A). To analyse the recycling rate of endocytosed transferrin in the fibroblasts, cells were first loaded with the biotinylated transferrin at 17°C for 2 hours, which allowed transferring to reach the endosomes, and then incubated at 37°C. The amount of remaining intracellular transferrin at indicated time points was then determined. Results from three independent experiments revealed that compared to the control cells, the recycling of biotin-transferrin was slightly increased in the juvenile CLN3 disease fibroblasts (the original publication II, Figure 3B).

To examine the trafficking of endocytosed material in the degradative endocytic pathway, the patient and wild type fibroblasts, starved in lipoprotein-free medium overnight, were subjected to BODIPY FL-LDL internalisation for 30 min at 37°C and then, either fixed immediately or chased for 40 min or 2 h before processing for confocal immunofluorescence microscopy. Immediately after the uptake period, LDL in the control cells was found to localise both to EEA1-positive EEs (the original publication II, Figure 4A) and LBPA-positive LEs/lysosomes (the original publication II, Figure 4B) indicating that LDL had already reached the late endosomal compartments. In contrast, LDL in patient fibroblasts was mostly found in the EEA1-positive EEs and no co-localisation with the late endosomal marker was detected at this time point (the original publication II, Figure 4C and D, respectively). Instead, minor co-localisation between LDL and LBPA was detected after 2 h of chase indicating that LDL in the juvenile CLN3 disease cells reached the late endosomal compartments at the later time points (the original publication II, Figure 4E and F). These results indicate that the trafficking of endocytosed material to LEs/lysosomes is delayed in CLN3-deficient fibroblasts.

Cytoplasmic acidification has been reported to induce the relocalisation of LEs and lysosomes from the cell center to the periphery (Heuser, 1989), and has been used to analyse kinesin-mediated organelle transport (Nakata and Hirokawa, 1995; Tanaka et al., 1998; Brown et al., 2005). In the current study, the cytoplasmic acidification assay was used to analyse anterograde movement of LAMP-1-positive LEs and lysosomes in HeLa cells transfected with control siRNAs (small interfering RNAs) or siRNAs against CLN3 sequence. siRNA-transfected cells were treated with Ringer´s solution pH 7.2 or with Ringer´s solution pH 6.9 to induce the relocalisation, and processed for immunofluorescence microscopy. Cells were

classified in three phenotypic categories based on the pattern of LAMP-1 immunofluorescence staining (the original publication III, Figure 5A). Cells showing only perinuclear LAMP-1-positive compartments, with some dispersed vesicles, were classified as normal. Cells displaying loose peripheral clusters or tight peripheral clusters were classified as dispersed or extremely dispersed, respectively. In most of the control cells treated with Ringer´s, pH 7.2, LEs/lysosomes were already dispersed (the original publication III, Figure 5B). The percentage of cells with tight peripheral lysosomal clusters increased during incubation in a more acidic solution (pH 6.9), as expected (the original publication III, Figure 5B). Interestingly, relocalisation of LEs/lysosomes was less efficient in CLN3-silenced cells. 6% of CLN3siRNA-transfected cells treated with Ringer´s, pH 7.2, were classified with extremely dispersed LAMP-1-positive compartments, which was clearly less than in the respective control culture (18%). Moreover, CLN3-silenced cells incubated in the pH 6.9 solution showed significantly fewer cells with extremely dispersed LEs/lysosomes than the controls (41 vs. 69%) (the original publication III, Figure 5B). These results suggest that CLN3 is required for the outward movement of LEs and lysosomes.

The current study shows that CLN3-deficiency or reduced expression levels of CLN3 causes defects in the intracellular membrane trafficking, a finding later confirmed by several other studies. Indeed, trafficking abnormalities in several intracellular compartments have been detected in deficiency of CLN3 or its *S. pombe* orthologue, Btn1p. These include impaired Golgi protein sorting (Codlin and Mole, 2009) and exit of mannose 6-phosphate receptor from the trans-Golgi network (Metcalf et al., 2008), and defects in endocytosis (Fossale et al., 2004), fast axonal transport (Weimer et al., 2006) and LE – Golgi retrograde sorting (Kama et al., 2011). However, no supportive protein interactions related to the trafficking of corresponding compartments were reported prior to this thesis study. This emphasises the significance of the current findings showing that CLN3 physically interacts with the proteins of late endosomal/lysosomal membrane trafficking. It is likely that the loss or disturbances in the interactions between CLN3 mutants and Rab7/RILP explain the observed defects in late endosomal/lysosomal functions. It is noteworthy that CLN3, as determined in this study, and associated proteins (dynactin, Rab7, RILP) have all been reported to affect dynein as well as kinesin-dependent membrane trafficking and therefore, disturbed CLN3 interactions may equally, and apparently cell-type specifically, affect antero- and retrograde trafficking (Blangy et al., 1997; Deacon et al., 2003; Berezuk and Schroer, 2007; Pankiv et al., 2010).

## 5.4 A model for CLN3 interaction network

Based on this thesis study and reports by others, the current functional interaction network of CLN3 consists of the following proteins: CLN5, calsenilin, SBDS, non-muscle myosin IIB, GRP78/BiP, $Na^+$, $K^+$ ATPase, fodrin, Hook1, Rab7, RILP, dynactin, dynein, kinesin-2, and tubulin. A summary of CLN3 interactions identified in this study is presented in **Table 8**.

**Table 8.** A summary of CLN3 interactions determined in the current study. Interacting domains, methods and putative functions are presented.

| Interactor | | Domain | CLN3 domain | Method | Function of the interaction |
|---|---|---|---|---|---|
| **GRP78/BiP** | | ? | 232-280 | GST pull-down, co-IP | ? |
| **$Na^+$,$K^+$ ATPase** | **α1** | ? | ? | co-IP | regulation of plasma membrane-association of $Na^+$, $K^+$ ATPase |
| | **β1** | 250-303[1)] | 1-40 | YTH, co-IP | |
| | **β2** | 187-290 | 1-40 | YTH | |
| **β-fodrin** | | 2265-2364 | 1-40 | YTH, co-IP | regulation/maintenance of fodrin cytoskeleton |
| **Hook1** | | ? | | GST pull-down | movement of late endosomes/lysosomes |
| **β-tubulin** | | ? | ? | co-IP | |
| **kinesin-2** | | ? | ? | co-IP | |
| **dynein** | | ? | ? | co-IP | |
| **dynactin** | | ? | ? | co-IP | |
| **Rab7 (direct)** | | ? | 1-33, 1-40 | co-IP, MTH, GST pull-down | |
| **RILP (direct)** | | ? | 232-280 | co-IP, GST pull-down | |

[1)] In YTH, amino acid segments 88-303 and 158-303 were also positive.
Abbreviations: GRP78/BiP, 78 kDa glucose-regulated protein/immunoglobulin heavy chain binding protein; RILP, Rab7-interacting lysosomal protein; co-IP, co-immunoprecipitation; GST, glutathione S-transferase; MTH, mammalian two-hybrid; YTH, yeast two-hybrid.

The CLN3-interacting proteins represent proteins of different intracellular compartments as well as partly unrelated functions. The complexity of CLN3 interactome implies that CLN3 has several functions in multiple regions of the cell. Alternatively, the localisation and function of CLN3 may be conditional or even specific to certain cell types. Based on its protein interactions, the functions of CLN3 affect at least endosomal and lysosomal compartments, the plasma membrane and associated cytoskeleton, and putatively ER (**Figure 9**). CLN3 may interact with other proteins either during its trafficking to lysosomes or after its intracellular relocalisation in response to a certain stimulus. Fodrin, $Na^+$, $K^+$ ATPase, non-muscle

myosin IIB and putatively GRP78/BiP represent plasmalemmal or plasma membrane-associated cytoskeletal interactions of CLN3. Therefore, in order to interact with them CLN3 has to localise in the dynamic endosomal/lysosomal compartments close to the plasma membrane or alternatively at the plasma membrane (**Figure 9**). Most of the other interactions of CLN3 most likely occur at the late endosomal/lysosomal compartments (**Figure 9**).

Although fodrin and $Na^+$, $K^+$ ATPase are known to interact with each other, the hierarchy of the associated CLN3 interactions can not be defined by the methods employed in the current study. CLN3 may affect fodrin and $Na^+$, $K^+$ ATPase either separately or alternatively, via a physical CLN3-fodrin- $Na^+$, $K^+$ ATPase complex. Although the plasma membrane-associated spectrin skeleton has been suggested to function in endocytosis (Williams et al., 2004; Phillips and Thomas, 2006), it is likely, based on the transferrin uptake studies (the original publication II), that the CLN3/fodrin interaction is not involved in the general internalisation of endocytic material but rather contributes to other functions of the plasma membrane-associated spectrin cytoskeleton. Fodrin is rather poorly characterised in mammalian central nervous system. However, it has been associated with synaptic transmission, fast axonal transport and neurite extension (Sobue and Kanda, 1989; Sikorski et al., 1991; Sihag et al., 1996; Riederer and Routtenberg, 1999; Takeda et al., 2000; Zimmer et al., 2000). Fodrin has no exact orthologue in *Drosophila* and *C. elegans*, which contain a far more simple battery of spectrin proteins (McKeown et al., 1998; Thomas et al., 1998; Adams et al., 2000; Hammarlund et al., 2000). Nevertheless, studies in these lower organisms suggest that plasma membrane associated spectrin is required for the prevention of spontaneous breaks in mature neuronal processes (Hammarlund et al., 2007), axon guidance/pathfinding (Garbe et al., 2007; Hulsmeier et al., 2007), synapse stabilisation (Pielage et al., 2005; Pielage et al., 2006) and organisation of synaptic components required for neurotransmitter release (Featherstone et al., 2001). Some of these intracellular events have also been linked to CLN3 (see Chapters 2.3.3, 2.4.4, 2.4.5) suggesting a possible role for CLN3-fodrin interaction in these processes and thus, in the pathogenesis of juvenile CLN3 disease.

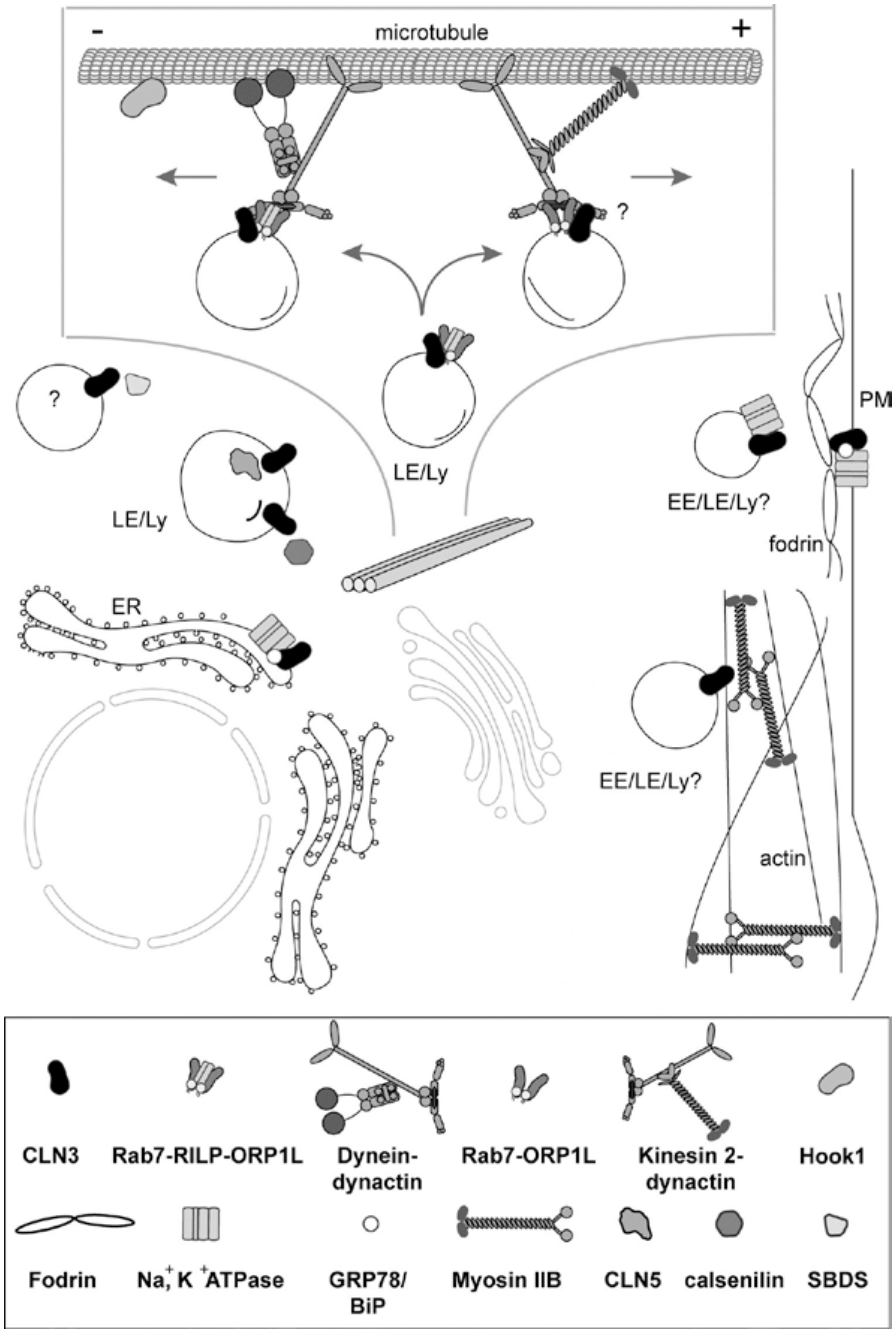


**Figure 9.** A schematic model of CLN3 interactions. CLN3 interacts with lysosomal lumenal proteins (CLN5), cytoplasmic proteins (calsenilin, SBDS), and plasma membrane (PM) proteins ($Na^+$, $K^+$ ATPase). Furthermore, CLN3 is associated with three cytoskeletal compartments: actin (via myosin IIB), fodrin, and microtubules (via motor protein complexes). The direct interaction between CLN3 and Rab7 effector complexes further highlights the role of CLN3 in microtubule-based movement of vesicles/organelles. Some of the figure components were produced using Servier Medical Art (www.servier.com).

Loss of CLN3 was shown to affect ouabain-induced endocytosis of $Na^+$, $K^+$ ATPase (the original publication I), which may have consequences on the activities of the protein in intracellular signalling, apoptosis and calcium oscillations (reviewed in Aperia, 2007; Tian and Xie, 2008; Lingrel, 2010). Interestingly, $Na^+$, $K^+$ ATPase also regulates neurotransmission through AMPA receptor turnover (Rose et al., 2009; Zhang et al., 2009) and it is possible that disruption of CLN3- $Na^+$, $K^+$ ATPase interaction is the basis for the AMPA-mediated excitotoxicity observed in juvenile CLN3 disease mouse models (Kovacs et al., 2006; Finn et al., 2011). Furthermore, the interaction may also be important for $Na^+$, $K^+$ ATPase activities in the heart and kidney as these organs are also affected in juvenile CLN3 disease (Stein et al., 2010; Ostergaard et al., 2011).

Most importantly, this study suggests that CLN3 and possibly Hook1 represent previously unidentified Rab7 effectors and thus may have pivotal roles regarding lysosomal functions. Further studies are needed to determine exactly how many separate protein complexes CLN3 forms with Hook1, Rab7, RILP, dynactin, dynein, kinesin-2 and tubulin and whether Hook1 associates with Rab7 and CLN3 in the same molecular complex. However, since over-expression of CLN3 induces aggregation of Hook1 and inability of the protein to co-immunoprecipitate with CLN3 (the original publication II), it is likely that Hook1 is not required for the interaction between CLN3 and Rab7. Instead, Hook1 may compete with CLN3 for binding to Rab7. The finding that CLN3 interacts with Rab7 and RILP, as well as with dynein-dynactin suggests that CLN3 associates with the Rab7-RILP-ORP1L dynein-dynactin activator complex. Rab7 and ORP1L also participate in the plus-end directed microtubular transport of autophagic vesicles in a manner uncoupled by RILP expression (Pankiv et al., 2010). Therefore, it is possible that Rab7 (and ORP1L) is also involved in the CLN3-kinesin-2-containing complex. Therefore, interactions of CLN3 with the components of late endosomal/lysosomal trafficking may involve at least the following protein complexes; CLN3-Hook1, CLN3-Rab7-RILP-ORP1L-dynein-dynactin and CLN3-Rab7-ORP1L-kinesin-2-dynactin (**Figure 9**).

Further studies are also needed to elucidate the consequences of impaired CLN3-Rab7 interaction in neurons. It would be interesting to study whether CLN3 deficiency affects Rab7-mediated axonal retrograde trafficking of neurotrophins and associated neurite outgrowth as well as maturation and transportation of neuronal autophagic vesicles. Interestingly, kinesin-2 has been shown to transport fodrin-associated vesicles (Takeda et al., 2000), thus, providing an unexpected connection between the two CLN3 interacting proteins, fodrin and kinesin-2. The transport of fodrin-containing vesicles by kinesin-2 has also been suggested to be important for neurite growth and the role of fodrin in this process is either to act as a linker protein between kinesin and cargo vesicle or to provide membraneous/cytoskeletal components essential for neurite extension (Takeda et al., 2000).

As a summary, neurite growth and neurite morphology are a common theme between CLN3 and its interaction partners (fodrin, kinesin-2, Rab7 and non-muscle myosin IIB) (Takeda et al., 2000; Tullio et al., 2001; Saxena et al., 2005; Ryu et al., 2006; Weimer et al., 2009). Therefore, it is plausible to argue that at least these processes are disturbed in juvenile CLN3 disease due to loss of functional CLN3 interactions.

Taken together, several important intracellular processes may be dysfunctional due to mutated CLN3. However, only fraction of the defects may be causative to disease. It is also possible that some of the defects may affect organs not equally sensitive to CLN3 deficiency as the central nervous system.

# 6 Conclusions and future prospects

In this thesis study, CLN3 functions and neurodegenerative juvenile CLN3 disease were investigated at a molecular level by studying CLN3-interacting proteins. The major findings of this study include 1) the determination of several previously unidentified CLN3 interactions with $Na^+$, $K^+$ ATPase, fodrin, GRP78/BiP, Hook1, Rab7, RILP, dynactin, dynein, kinesin-2, and tubulin, 2) putative structural abnormalities of neuronal fodrin cytoskeleton caused by CLN3 deficiency, 3) the connection of CLN3 to regulation of ouabain-dependent non-pumping functions of $Na^+$, $K^+$ ATPase in neurons, and 4) observations that CLN3 is required for the correct intracellular positioning of late endosomes/lysosomes, the trafficking of endocytosed material to late endosomal/lysosomal compartments, microtubule plus end-directed movement of late endosomes and lysosomes, and balanced functional cycle of Rab7. Together with the observed protein interactions, the latter findings strongly indicate that CLN3 regulates the movement and possibly, the membrane fusion of late endosomes and lysosomes.

In addition to juvenile CLN3 disease, this study provides clues to the pathogenesis of other NCLs and neurodegenerative disorders, as similar diseases may share defects in the same and/or functionally related intracellular pathways. Furthermore, interaction analyses of CLN3 provide candidates for potential modifiers of phenotypes associated with CLN3 or any other NCL protein and therefore, may contribute to better understanding of clinical variation in disease manifestation.

In terms of CLN3 and results of this thesis work, it would be essential to study the observed CLN3 interactions and associated intracellular events in neuronal cells. Followed by validation of the interactions in neurons, preferably done by *in vivo* methods, functional connections could be confirmed and further dissected utilising, for example, single and double mutant *Drosophila* models, as well as siRNA and high-content screening applications performed with mammalian neurons.

Urged by "the era of a genome", the current biological research is moving forward with one of the main focus being interactome mapping. Due to huge amount of protein interactions, and especially, due to the fact that interactions may form very complex networks in which binary interactions are linked to each other through indirect interactions forming "co-complex" interactome network (De Las Rivas and Fontanillo, 2010), design and development of more efficient large-scale interaction analysis tools are essential. Peptide microarray is one of the state-of-the-art technologies, and will revolutionise our vision on protein interaction analysis. Together with the current generally available high-throughput technologies, including tandem affinity purification-mass spectrometry (TAP-SM), traditional yeast-two hybrid analysis, and more sensitive *in vivo* methods such as fluorescence

resonance energy transfer (FRET), proximity ligation assay, and other fluorescence-based interaction detection methods, and in silico analysis tools, the peptide microarray analyses will speed up the mapping of the protein interactomes and pathways critical for human diseases.

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