Lysosomal proteome analysis reveals that CLN3-defective cells have multiple enzyme deficiencies associated with changes in intracellular trafficking

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Numerous lysosomal enzymes and membrane proteins are essential for the degradation of proteins, lipids, oligosaccharides, and nucleic acids. The CLN3 gene encodes a lysosomal membrane protein of unknown function, and CLN3 mutations cause the fatal neurodegenerative lysosomal storage disorder CLN3 (Batten disease) by mechanisms that are poorly understood. To define components critical for lysosomal homeostasis that are affected by this disease, we first quantified the lysosomal proteome in cerebellar cells derived from a CLN3 knock-in mouse model of human Batten disease and control cells. We purified lysosomes from SILAC-labeled, and magnetite-loaded cerebellar cells by magnetic separation and analyzed them by MS. This analysis identified 70 proteins assigned to the lysosomal compartment and 3 lysosomal cargo receptors, of which most exhibited a significant differential abundance between control and CLN3-defective cells. Among these, 28 soluble lysosomal proteins catalyzing the degradation of various macromolecules had reduced levels in CLN3-defective cells. We confirmed these results by immunoblotting and selected protease and glycosidase activities. The reduction of 11 lipid-degrading lysosomal enzymes correlated with reduced capacity for lipid droplet degradation and several alterations in the distribution and composition of membrane lipids. In particular, levels of lactosylceramides and glycosphingolipids were decreased in CLN3-defective cells, which were also impaired in the recycling pathway of the exocytic transferrin receptor. Our findings suggest that CLN3 has a crucial role in regulating lysosome composition and their function, particularly in degrading sphingolipids, and, as a consequence, in membrane transport along the recycling endosome pathway.

More than 60 soluble acid hydrolases and numerous lysosomal membrane proteins are essential for the degradation of proteins, lipids, oligosaccharides, and nucleic acids reaching lysosomes via endocytic and autophagic pathways, and for the transport of metabolites and ions across the membrane driven by a proton gradient that is maintained by the vacuolar H+ ATPase (vATPase). In addition to these functions, lysosomes are involved in fusion processes with the plasma membrane and other organelles, nutrient sensing, and gene regulation [1–3]. The physiological importance of lysosomal homeostasis is demonstrated by the existence of numerous diseases caused by mutations in genes encoding lysosomal proteins, which result in general lysosomal dysfunction associated with accumulation of nondegraded materials in lysosomes [4].

Mutations in the CLN3 gene coding for a glycosylated multiscanning lysosomal membrane protein of 438 amino acids, result in the fatal neurodegenerative lysosomal storage disorder CLN3 (also called juvenile neuronal ceroid lipofuscinosis (JNCL) or Batten disease). The world-wide most common mutation causes a 1-kb deletion in CLN3 gene that leads to the loss of exons 7 and 8, and is predicted to produce a truncated

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The MS proteomics datasets have been deposited to the Proteome X-change consortium with the dataset identifier PXD004548 (lysosomal proteome analysis).

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3 The abbreviations used are: vATPase, vacuolar H+ ATPase; ACN, acetonitrile; ANOVA, analysis of variance; ARSB, arylsulfatase B; buffer A, 10 mM HEPES, 250 mM sucrose, 1.5 mM KCl, 1.5 mM MgAc, 1 mM CaCl2, and 1 mM EDTA; CMV, cytomegalovirus; ER, endoplasmic reticulum; LacCer, lactosylceramides; M6P, mannose 6-phosphate; MES, 2-(N-morpholino)ethanesulfonic acid; Mpr300, 300-kDa mannose 6-phosphate receptor; SILAC, stable isotope labeling by amino acids in cell culture; TGN, trans-Golgi network; Trf, transferrin; TrfR, Trf receptor.
protein (5) that is retained in the endoplasmic reticulum (ER). The accumulation of heterogeneous autofluorescent ceroid lipopigment aggregates in lysosomes is not clearly disease-specific (6). The mechanisms underlying neuronal degeneration in CLN3 disease and the function of CLN3 are still unknown. It has been proposed that CLN3 contributes to the regulation of lysosomal size, pH, arginine, lipid, and Ca^{2+} homeostasis (7–11). Furthermore, CLN3 has been implicated in various intracellular membrane transport processes such as anterograde and retrograde transport between trans-Golgi network (TGN) and endosomes, formation of autophagolysosomes, late steps of the endocytic pathway, and vesicular trafficking along axons (5, 12–15).

To gain insight into mechanisms underlying neuronal degeneration, and to define components critical for lysosomal homeostasis in CLN3 disease, we performed a SILAC-based comparative mass spectrometric analysis of isolated lysosomes from cerebellar cell lines of WT and Cln3 knock-in mice (Cln3^{Δex7/8}) exhibiting the 1-kb deletion in the Cln3 gene (12). We found that the protein concentration of 28 soluble lysosomal enzymes was significantly reduced in Cln3^{Δex7/8} lysosomes associated with various changes in the lipid composition of Cln3^{Δex7/8} cerebellar cells. These were biochemically evaluated and found to contribute to differentially affected transport routes of endocytic cargo receptors.

**Results**

**Lysosomal proteomics identifies differential abundance of acid hydrolases in Cln3^{Δex7/8} cerebellar cells**

We performed SILAC-based comparative proteomics and quantified the relative amounts of lysosomal proteins at steady state in lysosomal fractions isolated from WT and Cln3^{Δex7/8} cerebellar cells (PXD004548; lysosomal proteome analysis) exhibiting storage material (Fig. S1) by means of internalized dextran-stabilized magnetite transported to LysoTracker-positive organelles (Fig. S2). Gene ontology (GO) enrichment analysis revealed that 197 and 170 of the identified proteins were annotated to vacuole and lysosome annotation in the database, containing 502 and 411 mouse proteins, respectively. We found in the isolated lysosomal fractions 104 of 185 experimentally confirmed lysosomal proteins comprising 47 soluble and 23 core membrane proteins (Table S1), and 34 associated proteins on the cytoplasmic lysosomal membrane. The majority exhibited a statistically significant differential abundance. In addition, the concentrations of three cargo receptors, 300-kDa mannos 6-phosphate receptor (Mpr300), LDL receptor-related protein 1 (Lrp1), and Lrp2 (also known as megalin), involved in trafficking of M6P-containing and nonphosphorylated lysosomal enzymes (16–19) were significantly changed in Cln3^{Δex7/8} cells (Table S1). Furthermore, numerous peripheral membrane proteins on the cytoplasmic side of lysosomes involved in vesicular targeting, positioning, and signaling have been covered by our proteomic analysis. Six lysosomal enzymes were increased 1.5- to 2.3-fold in lysosomal fractions of Cln3^{Δex7/8} cerebellar cells compared with WT controls (Fig. 1A): dipeptidylpeptidase 2 (Dpp7), tripeptidyl-peptidase 1 (Tpp1), RNase T2 (Rnaset2), lysosomal α-glucosidase (Gaa), α-galactosidase A (Gla), and palmitoyl-protein thioesterase 1 (Ppt1). In contrast, the amounts of 28 proteins were clearly decreased. Among these are enzymes involved in the degradation of glycans (aryl sulfatase B (Arsb), β-glal (Gb1), β-hexosaminidase subunit α (Hexa) and β (Hexb), GaINAc-6-sulfatase (Galns), β-mannosidase (Manba), β-glucuronidase (Gusb), and α-1-fucosidase (Fucα1)), or lipids (Glb1, N-acetylenolamine-hydrolyzing acid amidase (Naaα), Hexa, Hhexb, prosaposin (Psap), putative phospholipase B-like 2 (Pld2b), galactosylceramide-biosidase (Galc), aryl sulfatase A (Arsα), acid ceramidase (Asah1), group XV phospholipase A2 (Pla2g15), and lysosomal acid lipase (Lipa)). These proteins, as well as the following were decreased by 16 to 80% in Cln3^{Δex7/8} lysosomal fractions compared with WT: neuronal ceroid-lipofuscin protein 5 (Cln5), Niemann Pick type C2 protein (Npc2), cathepsin D (Ctsd), DNase-2-α (Dnase2), cathepsin Z (Ctsz), lysosomal Pro-X carboxypeptidase (Prpc), Creg1, legumain (Lgmn), carboxypeptidase Q (Cpq), sialate O-acetyleresterase (Sae), and mammalian ependymin-related protein 1 (Epdr1).

To verify the proteomic data, the expression levels of nine soluble lysosomal proteins were determined by Western blotting and enzymatic activity in whole cell lysates (Fig. 1, B and C). The amounts of immunoreactive Ctsd, Ctsz, and Creg1 were strongly reduced or almost absent in Cln3^{Δex7/8} cells (Fig. 1B). In agreement with the comparative proteomic data, the activities of Ppt1 and Gla, representing the most enriched proteins in lysosomal fractions of Cln3^{Δex7/8} cells, were significantly increased, whereas the activities of Hexb, Arsa, Manba, and Gusb were reduced in Cln3^{Δex7/8} cells (Fig. 1C). Next, we examined whether changes in the amounts of lysosomal membrane proteins in Cln3^{Δex7/8} cells affect other lysosomal functions. Although three subunits of the vATPase (Atp6p1, Atp6g1, and Atp6v1h) and the accessory protein 1 (Atp6ap1) were significantly changed in their abundance in lysosomal fractions of Cln3^{Δex7/8} cerebellar cells (Fig. 2A and Table S1), ratiometric pH measurements showed unchanged lysosomal H^{+} concentrations in mutant cells (pH 4.53 ± 0.08 (WT) versus 4.49 ± 0.09 (Cln3^{Δex7/8}); Fig. 2B). Therefore, our data do not support a role of CLN3 in lysosomal pH maintenance of neuronal cells, although previous studies reported on changes in vacuolar pH in yeast cells lacking the CLN3 homologue btnt1 (8, 20).

Clonal effects responsible for the observed phenotype in Cln3^{Δex7/8} cerebellar cells are unlikely because we found similar reductions in Ctsd and Ctsz protease or Lrp1 level in primary embryonic fibroblasts isolated from Cln3^{Δex7/8} mice (Fig. S3).

**Increased Mpr300-mediated uptake of 125I-aryl sulfatase B in Cln3^{Δex7/8} cells**

The proteomic analysis of lysosomal fractions revealed a 2.2-fold increased concentration of Mpr300 in Cln3^{Δex7/8} cerebellar cells compared with WT controls (Fig. 3A) which was confirmed by Western blotting (Fig. 3B). Furthermore, the relative number of Mpr300 was also determined by ligand-binding experiments on saponin-treated cerebellar cells (representing the total number of Mpr300) or on untreated cells (representing Mpr300 localized at the cell surface) using recombinant...
human ¹²⁵I-labeled arylsulfatase B (¹²⁵I-ARSB) as lysosomal indicator ligand. Binding studies with ¹²⁵I-ARSB at 4 °C revealed about doubled Mpr300 sites at the surface of Cln3<sup>−/−</sup>H9004<sup>ex7/8</sup> cells compared with WT cells, which corresponded to the 2.2-fold higher expression in Cln3<sup>−/−</sup>H9004<sup>ex7/8</sup> cerebellar cells determined in the presence of saponin (Fig. 3C).

To examine the functional significance of increased amounts of Mpr300 in Cln3<sup>−/−</sup>H9004<sup>ex7/8</sup> cerebellar cells, we performed Mpr300-dependent internalization assays using ¹²⁵I-ARSB as Mpr300 ligand. Because there is a continuous and constitutive exchange between Mpr300 localized at the cell surface and Mpr300 in all intracellular membranes with the same kinetics (21), the uptake is proportional to the total number of Mpr300. Cerebellar cells were incubated with ¹²⁵I-ARSB for 20 to 100 min. The specificity of Mpr300-mediated uptake of ¹²⁵I-ARSB was demonstrated by the complete inhibition by M6P (Fig. 3D, lanes 6 and 12). In both WT and Cln3<sup>−/−</sup>H9004<sup>ex7/8</sup> cerebellar cells, the amounts of ¹²⁵I-ARSB precursors increased with incubation time. Quantification of both cell-associated radioactivity and measurement of radioactivity in excised ¹²⁵I-ARSB bands from the gel demonstrated 2-fold higher amounts of ¹²⁵I-ARSB in Cln3<sup>−/−</sup>H9004<sup>ex7/8</sup> cerebellar cells compared with WT controls (Fig. 3E).

### Lipidome analysis reveals alterations of glycosphingolipids in Cln3<sup>−/−</sup>H9004<sup>ex7/8</sup> cerebellar cells

Because the amounts of 11 soluble lysosomal proteins involved in lipid degradation are significantly reduced in lysosomes of Cln3<sup>−/−</sup>H9004<sup>ex7/8</sup> cerebellar cells, we analyzed the lipidome...
of WT and Cln3<sup>3ax<sub>ex7/8</sub></sup/>cerebellar cells by electrospray ionization mass spectrometry (ESI-MS). To increase the sensitivity of MS detection, cerebellar cells of three independent preparations were combined, and anionic and neutral lipids were analyzed separately (Tables S2 and S3). The eight anionic lipid species which showed the most pronounced change in their relative amount included several GM3 and GD1 ganglioside species with a sphingosine (d18:1) backbone and variable acyl chains. The levels of GM3 species were increased 1.4- to 2.8-fold in Cln3<sup>3ax<sub>ex7/8</sub></sup/>cerebellar cells compared with the WT cells, whereas GD1 species were reduced by 50%. Particular phosphatidylinositol (PI) species (38:3) had 1.6-fold elevated values in Cln3<sup>3ax<sub>ex7/8</sub></sup/>cerebellar cells (Table S2). In addition, numerous alterations in the relative amounts of neutral lipid species were detected in Cln3<sup>3ax<sub>ex7/8</sub></sup/>cerebellar cells. Lactosylceramides (LacCer) 24:0 and 16:0 showed the strongest increase (5.0- and 4.7-fold, respectively) for these cells included lysophosphatidylethanolamines (LPE) 38:3 and 40:3. At the same time SM14:0 and 15:0, PE40:4, phosphatidylethanolamines (PE) 38:3 and 40:3, and phosphatidylylcholine (PC) 38:4, lysophosphatidylylcholine (LPC) 20:4, and hexose cerebrosides (HexCer) 18:0, 20:0, 22:0, and 23:0 were reduced to 30–60% of the WT levels (Table S3). GC with mass selective detection (GC-MS) revealed losses of essential arachidonic acid, 20:4n-6 (in PE40:4, PC38:4) compensated by increases of its precursor 20:3n-6 and nonessential 20:3n-9 (in LPE20:3, PE38:3, PE40:3) (Table S4).

In addition, immunofluorescence intensity of perilipin-2 (Plin2), an envelope protein of lipid droplets (22), was increased in Cln3<sup>3ax<sub>ex7/8</sub></sup/>cerebellar cells to WT cells (Fig. 5, A and B). Furthermore, loading of the cells with oleate revealed a stronger expansion in cytosolic Plin2-associated lipid droplets in Cln3<sup>3ax<sub>ex7/8</sub></sup/>cerebellar cells, suggesting impaired degradative capabilities.

Taken together, the comparative lipidome analyses of cerebellar cells revealed distinct alterations, in particular in the relative contents of glycosphingolipids in Cln3<sup>3ax<sub>ex7/8</sub></sup/>cerebellar cells, which can be partially explained by changes in the steady-state expression levels of lysosomal sphingolipid-degrading hydrolases.

To determine whether re-expression of CLN3 could reverse alterations in lysosomal homeostasis, we infected WT and Cln3<sup>3ax<sub>ex7/8</sub></sup/>cerebellar cells with lentiviruses expressing human CLN3 N-terminally tagged with GFP (GFP-CLN3) or GFP alone under a CMV promoter driving high transgene expression. After antibiotics selection for GFP- and GFP-CLN3-positive cells (Fig. S4), in both cell lines GFP-CLN3 was found primarily localized in the ER and to some extent in lysosomes (Fig. S5). However, re-expression of GFP-CLN3 neither affected the reduced cathepsin D and Z levels and decreased activities of lysosomal glycosidases, nor the oleate-induced formation/accumulation of Plin2-positive lipid droplets in Cln3<sup>3ax<sub>ex7/8</sub></sup/>cerebellar cells (Fig. S6).

**Impaired recycling of transferrin receptor in Cln3<sup>3ax<sub>ex7/8</sub></sup/>cerebellar cells**

Because the intracellular distribution and transport of membrane proteins between different subcellular compartments, in particular along the endocytic and autophagic pathways, depend on the lipid composition of the membranes (23–25), we examined the endocytosis of fluorophore-labeled transferrin (Trf-Alexa Fluor<sup>546</sup>) mediated by transferrin receptor (TrfR). The TrfR protein level was not changed in Cln3<sup>3ax<sub>ex7/8</sub></sup/>cerebellar cells (Fig. S7). However, the amount of Trf-Alexa Fluor<sup>546</sup> detected intracellularly after 30-min incubation was significantly increased in Cln3<sup>3ax<sub>ex7/8</sub></sup/>cerebellar cells compared with WT cells (Fig. 6, A and D). The continuously internalized Trf-Alexa Fluor<sup>546</sup> is detectable beneath the plasma membrane, in small vesicles evenly distributed over the whole cell, and in larger, more dilated structures in perinuclear regions partially co-localizing with Rab11a and the Golgi marker protein Gm130 characteristic for the transferrin-recycling pathway (Fig. S8) (26, 27). The identity of organelles mainly retaining Trf in Cln3<sup>3ax<sub>ex7/8</sub></sup/>cerebellar cells needs to be studied.

To examine whether the increased accumulation of Trf-Alexa Fluor<sup>546</sup> in Cln3<sup>3ax<sub>ex7/8</sub></sup/>cerebellar cells is because of elevated uptake or reduced recycling and exocytosis, the cells were incubated for 30 min with Trf-Alexa Fluor<sup>546</sup>, washed, and further incubated for 15 min in the presence of Trf-Alexa Fluor<sup>546</sup> 488 (Fig. 6B). As expected, the Alexa Fluor<sup>546</sup>
546 fluorescence completely disappeared in WT cells because of the release of Apo-Trf into the medium accompanied by the accumulation of Trf-Alexa Fluor/1488 (Fig. 6, B and D, upper row). In contrast, in Cln3\textsuperscript{ex7/8} cells both Trf-coupled fluorophores partially co-localized in the same intracellular membrane structures (Fig. 6, B and D, lower row). During a further 15-min chase in medium lacking Trf-Alexa Fluor\textsuperscript{®} ligands, almost no ligand-related fluorescence was observed in WT cells (Fig. 6, C and D, upper row), whereas both Trf-Alexa Fluor\textsuperscript{®} 546 and Trf-Alexa Fluor\textsuperscript{®}
Figure 6. Alteration in transferrin receptor recycling in Cln3<sup>Δex7/8</sup> cells. A–C, confocal fluorescence images of WT and Cln3<sup>Δex7/8</sup> cells after incubation with Trf-Alexa Fluor<sup>®</sup> 546 (red) for 30 min at 33 °C (A), and subsequent incubation with Trf-Alexa Fluor<sup>®</sup> 488 (green) for 15 min at 33 °C (B). After removal of the medium, WT and Cln3<sup>Δex7/8</sup> cells were further incubated with fresh medium in the absence of Trf-Alexa Fluor<sup>®</sup> (chase) for 15 min at 33 °C (C). Confocal immunofluorescence images were captured with identical exposure settings. Nuclei were visualized using DAPI (blue). Scale bars = 10 μm. D, quantification of Trf-Alexa Fluor<sup>®</sup> 546 and Trf-Alexa Fluor<sup>®</sup> 488 fluorescence intensities at each time point. Statistical significance was determined using one-way ANOVA followed by Dunnett’s multiple comparison test. Data are presented as mean ± S.E. of three independent experiments (15 cells were analyzed per experiment), (ns, not significant; *, p ≤ 0.05, ***, p ≤ 0.001).
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488 were still detectable in Cln3<sup>ex7/8</sup> cells (Fig. 6, C and D, lower row). These data demonstrate that the exocytic pathway of Trf from the recycling endosome to the plasma membrane is strongly disturbed in Cln3<sup>ex7/8</sup> cells.

**Discussion**

The homeostasis of lysosomes relies on the balance between the biosynthetic replenishment of lysosomal components, degradation, and recycling of macromolecules obtained by endocytosis and autophagy. Our analysis of the lysosomal proteome of Cln3<sup>ex7/8</sup> cerebellar cells provides novel insight into complex regulatory mechanisms linking altered composition of lysosomes and dysregulated membrane transport. We found that the amounts of only few enzymes are increased, whereas the majority is decreased in Cln3<sup>ex7/8</sup> lysosomes (Fig. 1A and Table S1). Therefore, we predicted impaired capabilities for degradation of multiple classes of substrates in Cln3<sup>ex7/8</sup> lysosomes, which was supported by transmission EM revealing heterogeneous storage material in enlarged lysosomes composed by dense and multilamellar bodies, electron-lucent floccular bodies and curvilinear and fingerprint-like material in the cytoplasmic side of lysosomes are relatively similar. Furthermore, the high sensitivity of MS leads to the identification of a considerable number of nonlysosomal “contaminating” proteins. Because the role of lysosomes as purely degradative organelles changed in the past years toward central players in the cytoplasmic side of lysosomes are relatively similar. Furthermore, the high sensitivity of MS leads to the identification of a considerable number of nonlysosomal “contaminating” proteins. Because the role of lysosomes as purely degradative organelles changed in the past years toward central players in autophagy/mitophagy/ribophagy, energy metabolism, nucleus signaling, physical contacts with other organelles, or adaptive intracellular positioning (28–31), numerous co-purified contaminating proteins may be functionally linked to diverse functions of lysosomes.

The altered lysosomal protein levels in Cln3<sup>ex7/8</sup> cells might be caused by changes in their mRNA level, differences in lysosomal stability, and/or intracellular transport efficiency. Although it is rather unlikely that the low expression of cathepsin D and Z in Cln3<sup>ex7/8</sup> lysosomes results in stabilization of few selected proteins, it was shown that CLN3 is necessary for normal protein trafficking along the secretory, TGN-endosome, and endocytic pathway (12, 32, 33). Consistent with these observations, we show here that in Cln3<sup>ex7/8</sup> cells the transport efficiency of two endocytic cargo receptors, the Mpr300 and TrfR, is affected by two different mechanisms: alteration of the steady-state receptor protein concentration (Mpr300) (Fig. 3, B and C), and changes in the recycling efficiency (TrfR) (Fig. 6). The altered levels of Mpr300 correspond to alterations in ligand uptake and lysosome delivery (Fig. 3, D and E). Our ratio-metric pH measurements (Fig. 2B) exclude that changes in H<sup>+</sup> concentration are responsible for impaired dissociation processes of receptor-ligand complexes or lysosomal dysfunction in Cln3<sup>ex7/8</sup> cells and provide no evidence for a role of CLN3 in lysosomal pH maintenance of neuronal cells as reported previously (28) for the vacuolar pH in yeast cells lacking the CLN3 homolog bt1 (8, 20).

The concentration of transferrin receptors was not changed in Cln3<sup>ex7/8</sup> cells, whereas the amount of internalized transferrin was increased. We show here that the accumulation of transferrin in Cln3<sup>ex7/8</sup> cells is caused by slowdown of the exocytic route and subsequent release of apotransferrin (Fig. 6). Although the molecular mechanism, the identity, and protein-lipid composition of vesicular structures accumulating internalized transferrin in Cln3<sup>ex7/8</sup> cells are unknown, it is likely that e.g. alterations in lipid composition of membranes affect the recycling route of transferrin. Indeed, our lipidomic studies based on total cell extracts show abnormal lipid compositions in Cln3<sup>ex7/8</sup> cerebellar membranes, in particular of gangliosides, sphingomyelin, ceramide, and cerebroside species (Table 1 and Tables S2 and S3), known to impair protein sorting, trafficking, and the autophagic pathway (25, 33–36). Therefore, it is possible that the reduction of distinct sphingomyelin species (14:0 or 15:0) in Cln3<sup>ex7/8</sup> cells (Table S3) which are highly enriched in microdomains of purified recycling endosomes (37) might impair the transport of transferrin to the plasma membrane. Our findings are consistent with data on the coordinate mislocalization of microdomain-associated proteins to the plasma membrane in immortalized brain endothelial cells from Cln3-null mice (33).

Table 1

| Lipid species       | WT     | Cln3<sup>ex7/8</sup> | Ratio (Cln3<sup>ex7/8</sup>/WT) |
|---------------------|--------|----------------------|----------------------------------|
| **Anionic**         |        |                      |                                  |
| GM5                 | d18:1/24:1 | 0.029 | 0.080 | 2.76 |
| GM3                 | d18:1/16:0 | 0.077 | 0.153 | 1.99 |
| PI                  | 38:3    | 3.363 | 5.361 | 1.59 |
| PS                  | 38:3    | 2.577 | 4.093 | 1.57 |
| GD1                 | d18:1/16:0 | 2.927 | 1.440 | 0.49 |
| GD1                 | d18:1/22:0 | 0.622 | 0.265 | 0.43 |
| GD1                 | d18:1/25:0 | 0.339 | 0.145 | 0.43 |
| **Neutral**         |        |                      |                                  |
| LacCer              | 24:0    | 0.001 | 0.005 | 5.00 |
| LacCer              | 16:0    | 0.0003 | 0.014 | 4.67 |
| PE                  | 38:3    | 0.354 | 0.738 | 2.08 |
| Cer                 | 24:0    | 0.015 | 0.029 | 1.93 |
| Cer                 | 24:1    | 0.034 | 0.056 | 1.65 |
| PE                  | 40:3    | 0.030 | 0.097 | 1.57 |
| SM                  | 24:1    | 0.373 | 0.579 | 1.55 |
| LPE                 | 20:3    | 0.111 | 0.170 | 1.53 |
| LPE                 | 20:4    | 0.870 | 0.527 | 0.61 |
| HexCer              | 18:0    | 0.054 | 0.030 | 0.56 |
| HexCer              | 22:0    | 0.102 | 0.056 | 0.55 |
| HexCer              | 23:0    | 0.030 | 0.013 | 0.43 |
| HexCer              | 20:0    | 0.034 | 0.010 | 0.29 |
tor protein precursor prosaposin, which is proteolytically processed to saposins A–D in lysosomes making lipid substrates accessible to lysosomal galactosylceramidase, sulfatide arylsulfatase A, glucocerebrosidase, and acid ceramidase, respectively, might partially explain the accumulation or reduction of sphingolipids in Cln3<sup>Δex7/8</sup> cells (38). The intracellular compartments affected by the changes in sphingolipids in Cln3<sup>Δex7/8</sup> cerebellar cells remain to be further studied.

In agreement with our data, elevated levels of GM3 gangliosides have been reported in Cln3<sup>Δex7/8</sup> cerebellar cells, which are caused by transcriptional down-regulation of GM2 synthetase and hexosaminidase A responsible for conversion of GM3 into GM2 and the lysosomal degradation of GM2 to GM3 gangliosides, respectively (40). The mechanisms, molecules, and sensors initiating the differential expression and abundance of acid hydrolases in lysosomes in the absence of Cln3, in particular lipid-degrading enzymes, are poorly defined. Different transcription factors have been described that control transcriptional expression of acid lipase (FoxO1), ceramidase (KLF6), and sphingomyelinase (Sp1, AP-2), suggesting that Cln3 deficiency and subsequent lysosomal dysfunctions impair various signaling pathways (41–43). Our own data show that the mTORC1 pathway is activated in Cln3<sup>Δex7/8</sup> cerebellar cells (Fig. S9). The subsequent downstream targets, however, affecting the protein and lipid composition of lysosomes and membranes in Cln3<sup>Δex7/8</sup> cerebellar cells remain to be examined.

The lentiviral-mediated re-expression of GFP-CLN3 in Cln3<sup>Δex7/8</sup> cerebellar cells did not reverse any of the altered lysosomal components or functions tested here (Fig. S6). This might be explained by the high CMV promotor driven expression of the transgene in the functionally unrelated ER compartment in relation to the small population of CLN3 in lysosomes (Fig. S5). These data are in agreement with studies on high-dosage systemic scAAV9 delivery of CLN3 which failed to restore motor activity and glial activation in Cln3<sup>Δex7/8</sup> mice (44). Moreover, a marginal 2.9-fold increase of CLN3 over basal level of CLN3 led to a degenerative eye phenotype in Drosophila (45). This suggests that tightly regulated levels of CLN3 are critical to maintain lysosomal functions and communication with other organelles. Therefore, antibodies suitable to determine the endogenous level of CLN3 in intracellular membranes, are a prerequisite to define conditions for re-expression experiments in CLN3-defective cells and organisms. Additionally, it cannot be excluded that the GFP-fusion interferes with the so far unknown function of CLN3.

In summary, our work indicates that Cln3 has a more generalized role in cellular and in particular lysosomal homeostasis than previously recognized. The low abundance of several lysosomal enzymes in the absence of Cln3 affecting the lysosomal proteome, the associated degradation of substrates, and secondary cellular protein trafficking processes makes Cln3 a central player that is functionally connected to many other proteins and signaling pathways in neuronal cells.

**Experimental procedures**

**Antibodies and materials**

The following antibodies were used: mouse anti-α-tubulin (Sigma-Aldrich); polyclonal goat anti-Ctsd (Santa Cruz Biotechnology), goat anti-Ctsz and goat anti-Creg1 (R&D Systems), mouse anti-Gm130 (BD Biosciences), mouse anti-TrfR (Thermo Fisher Scientific), rabbit anti-Plin2 (Abcam), mouse anti-PDI (Enzo Life Sciences), rat anti-Lamp1 (clone 1D4B, Developmental Studies Hybridoma Bank), anti-Lrp1 (Abcam), rabbit anti-Rab11a (Cell Signaling Technology), and rat anti-Mpr30 (kindly provided by Dr. K. von Figura, University of Göttingen, Germany); horseradish peroxidase (HRP)–coupled secondary antibodies (Dianova); Alexa Fluor® 546–coupled anti-mouse IgG, Alexa Fluor® 488–coupled anti rabbit IgG, and Alexa Fluor® 488–coupled anti rat IgG (Thermo Fisher Scientific). Recombinant human ARSB was kindly provided by Dr. M. Vellard (BioMarin). Sodium 125I/13C<sub>6</sub>/15N<sub>2</sub>/15O<sub>2</sub>/H<sub>2</sub>O (75 terabecquerels/mmol) was purchased from Hartmann Analytic. M6P sodium salt, penicillin/streptomycin, BSA, protease inhibitor mixture, and BSA were obtained from Sigma-Aldrich. FBS, L-lysine 13C<sub>6</sub> sulfate, 4-methylumbelliferyl (4MU)-6-thio-palmitate-nitrocatechol, 4-nitrophenyl-N-acetyl-β-D-glucosaminide, p-nitroacetohexyl fluoride, 4-methylumbelliferyl (4MU)-6-thio-palmitate-β-D-glucopyranoside, 4MU-α-β-D-galactopyranoside, 4MU-β-β-D-mannopyranoside, 4MU-β-D-glucuronide, paraformaldehyde, nigericin, and monensin, and other common laboratory reagents were obtained from Sigma-Aldrich. FBS, 1-lysine 13C<sub>6</sub> isotopes, IODO-GEN® , DMEM, geneticin, GlutaMAX®M, Oregon Green® 488–dextran, Alexa Fluor® 546– or Alexa Fluor® 488–coupled transferin were from Thermo Fisher Scientific. LiquidMAG® QD (dextran-stabilized magnetite nanoparticles) and FluoreMAG® A (fluorescent dextran magnetite nanoparticles) and MACS LS separation columns were purchased from Liquid Research Ltd and Miltenyi Biotech, respectively. μ-Slides (8 well) were purchased from ibidi.

**Cell culture**

Cultured cerebellar cells derived from Cln3<sup>Δex7/8</sup> and WT mice, respectively, were described previously (12) and maintained in DMEM containing 10% heat-inactivated FBS, 1× GlutaMAX®TM, 24 mm KCl, 200 μg/ml geneticin, and penicillin/streptomycin at 33°C and 5% CO<sub>2</sub>. All experiments have been performed with 70 to 80% confluent cerebellar cells exhibiting heterogeneous storage material ranging from dense and multilamellar bodies, electron-lucent floccular bodies and fingerprint-like structures in Cln3<sup>Δex7/8</sup> cells (Fig. S1). Isolation and cultivation of primary embryonic mouse fibroblasts (MEF) from WT and Cln3<sup>Δex7/8</sup> mice were performed as described (18).

**Endocytosis assay of 125I-ARSB**

ARSB was iodinated with sodium 125I and IODO-GEN® as described (21) to specific activities of 15–20 μCi/μg protein. Cerebellar cells grown in 3.5-cm plates were pre-incubated with DMEM containing 0.1% BSA for 1 h, followed by incubation with 125I-labeled ARSB (~250,000 cpm/ml) in DMEM/
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0.1% BSA in the absence or presence of 10 mM M6P for the indicated time points. Cells were washed with PBS and either harvested or chased as indicated. Cell lysates prepared in PBS containing 0.2% Triton X-100 and protease inhibitors were separated by SDS-PAGE and visualized by autoradiography.

**Binding of 125I-ARSB**

Cerebellar cells were cultured on 3.5-cm plates and chilled to 4 °C. To determine the relative number of Mpr300 at the cell surface, cerebellar cells were incubated with 125I-ARSB (~600,000 cpm/ml) in DMEM/0.1% BSA adjusted to 20 mM Hepes (pH 7.4) in the absence or presence of 10 mM M6P for 2 h at 4 °C. To measure the total number of M6P-binding sites, cells were pre-incubated at 4 °C in DMEM/0.1% BSA adjusted to 20 mM Hepes (pH 7.4) containing 0.1% saponin and M6P. Saponin was included in all cell buffers further used. After two washes with PBS, cells were incubated with 125I-ARSB for 2 h at 4 °C in the presence or absence of 10 mM M6P followed by four washes with PBS. The M6P-specific cell-associated radioactivity was determined and related to the cellular protein amount.

**Endocytosis/recycling of transferrin**

Cells were cultured on glass coverslips in 24-well plates and subsequently incubated in serum-free medium containing 25 μg/ml Alexa Fluor® 564– or Alexa Fluor® 488–coupled transferrin for 30 or 15 min at 33 °C, respectively, followed by a chase in serum-free medium for 15 min. Cells were then washed three times with PBS, fixed in 4% paraformaldehyde on ice for 30 min, and embedded in Aqua-Poly/Mount. Fluorescence was detected and images were obtained using a Leica DMIRE2 digital scanning confocal microscope (Leica Microsystems). Fluorescence intensities were quantified using the ImageJ 1.49v software (National Institutes of Health). Data represent the mean ± S.E. (n = 15) and significance was evaluated using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test. Statistics were performed using GraphPad PRISM (GraphPad Software).

**EM**

Ultrastructural analysis of cells was performed as described previously (46).

**Measurement of lysosomal pH**

Cerebellar cells were plated on glass-bottom live-cell dishes (MatTek) and ratiometric pH measurements were performed 24 h after plating (47). Lysosomes were loaded with 0.5 mg/ml Oregon Green® 488-dextran in growth medium overnight, followed by a 2-h chase. Images were acquired using a Zeiss Axiovert 200 equipped with a 100 × 1.30 NA oil-immersion lens with excitation at 440 and 488 nm delivered by a Polychrom V monochromator system (TILL Photonics). The emitted light was selected with a 535 ± 20 nm filter and captured with a Sensicam CCD camera (PCO AG). For each dish, at least 10 different cells were measured in 10 ml Hepes (pH 7.4), 150 mM NaCl, 1 mM MgCl2, 2 mM CaCl2, and 10 mM glucose and analyzed using a Fiji plug-in, in which regions of interest were defined as areas above a defined fluorescence threshold in the acquired images at 488 nm excitation. The mean intensity ratio between 488 and 440 nm excitation was calculated for each region of interest. At the end of each experiment, in situ pH calibration curves were obtained after treatment in isotonic K-based solutions (5 mM NaCl; 115 mM KCl; 1.2 mM MgSO4; 10 mM glucose; 25 mM Hepes, MES, or acetate, ranging in pH from 3.9 through 6.45) supplemented with 10 μM of both nigericin and monensin. Cells were equilibrated for at least 2 min for each pH. The resulting fluorescence intensity ratio (488/440) as a function of pH was fit to a sigmoid and used to interpolate pH values from the experimental ratio data.

**Western blotting**

Preparation of whole cell extracts, SDS-PAGE and Western blot analysis were performed as described previously (18). For Mpr300 Western blotting, cells were solubilized in 10 mM Tris/ HCl, pH 7.4, containing 300 mM NaCl, 1 mM EDTA, 1.5% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitor mixture (48). Anti-α-tubulin Western blotting was used as loading control. After incubation with secondary HRP-conjugated IgG antibody, the immunoreactive bands were visualized by enhanced chemiluminescence.

**Enzyme activity measurements**

The enzymatic activities of Ppt1, Hexb, and ArsA in whole cell extracts were determined as described previously (49, 50). To measure Gusb, Gla, or Manba activities, cell extracts were incubated with 1 mM 4MU-β-D-glucuronide, 0.75 mM 4MU-α-D-galactopyranoside, or 0.75 mM 4MU-β-mannopyranoside, respectively, in 0.2 M sodium-citrate (pH 4.6) containing 150 mM NaCl and incubated for 2 h (Gusb) or for 16 h (Gla, Manba) at 37 °C. The reactions were stopped by the addition of 0.4 M glycine/NaOH buffer (pH 10.4). The liberated 4MU was measured fluorimetrically with excitation at 360 nm and emission at 440 nm. The specific activities (nmol/h/mg cell protein) of Cln3<sup>Dex7/8</sup> cells were normalized to the WT cells.

**Bodipy lactosylceramide labeling**

Cln3<sup>Dex7/8</sup> and WT cerebellar cells were plated on μ-slides (8 well) in a density of 10,000 cells/well. After the cells reached 70% confluency, they were cultured for 16 h in Golgi-RFP (CellLight<sup>TM</sup> BacMam 2.0 Reagents) supplemented culture medium according to manufacturer’s instructions, followed by 30-min incubation with 5 μM BODIPY® FL C<sub>3</sub>-Lactosylceramide complexed to BSA in Hanks’ Balanced Salt solution at 4 °C. For intravitral co-staining of BODIPY® FL C<sub>3</sub>-Lactosylceramide and lysosomes, 50 nM LysoTracker<sup>TM</sup> Deep Red probe was added to fresh medium after the BODIPY® FL C<sub>3</sub>-Lactosylceramide uptake and incubated for 30 min at 33 °C. Cells were analyzed by confocal fluorescence life cell microscopy. Fluorescence intensities were quantified in defined reference regions (Golgi- RFP– or LysoTracker-positive cell compartments) using the ImageJ 1.49v software (National Institutes of Health). Data represent the mean ± S.D. of three independent experiments (25 cells were analyzed per condition) and significance was evaluated with two-tailed Student’s t test. Statistics were performed using GraphPad PRISM (GraphPad Software).
The lysosomal eluate was concentrated in Amicon Centrifugal Filters at 14,000 × g and 4-fold SDS-sample buffer (250 mM Tris/HCl, pH 6.8, 8% (w/v) SDS, 40% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 0.004% (w/v) bromphenol blue) was added. After solubilization at 95 °C for 5 min, samples were cooled to room temperature and alkylation was performed by adding 1% acrylamide for 30 min at room temperature. The sample was separated by 10% SDS-PAGE, and the gel was then washed twice in HPLC water and stained with Coomassie Blue for 1 h. After destaining the gel with distilled water, the eluate band profile was divided into 10 fragments.

The gel slices were washed consecutively with water, 50% acetonitrile (ACN), and 100% ACN. Proteins were reduced with 20 mM DTT in 50 mM ammonium bicarbonate and alkylated with 40 mM iodoacetamide (in 50 mM bicarbonate). The slices were washed again and dehydrated with ACN. Dried slices were incubated with 400 ng sequencing grade trypsin at 37 °C overnight. The peptide extract was separated and remaining peptides extracted with 50% ACN. Peptides were dried in a vacuum concentrator and stored at −20 °C.

Peptides were dissolved in 8 μl 0.1% TFA and 1.5 μl were injected onto a C18 trap column (20 mm length, 100 μm inner diameter) coupled to a C18 analytical column (200 mm length, 75 μm inner diameter), made in house with 1.9 μm ReproSil-Pur 120 C18-AQ particles (Dr. Maisch, Ammerbuch, Germany). Solvent A was 0.1% formic acid. Peptides were separated during a linear gradient from 4 to 40% solvent B (80% ACN, 0.1% formic acid) within 80 min at a flow rate of 320 nl/min. The nanoHPLC was coupled online to an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). Ions between 330 and 1600 m/z were scanned in the Orbitrap detector with a resolution of 30,000 (maximum fill time 400 ms, automatic gain control target 106). The 25 most intense precursor ions (threshold intensity 5000) were subjected to collision-induced dissociation and fragments analyzed in the linear ion trap. Fragmented peptide ions were excluded from repeat analysis for 15 s. Raw data processing and analysis of database searches were performed with Proteome Discoverer software 1.4.1.14 (Thermo Fisher Scientific). Peptide identification was done with an in-house Mascot server version 2.4 (Matrix Science Ltd.). MS2 data were searched against mouse sequences from SwissProt (release 2014_01). Precursor ion m/z tolerance was 8 ppm, fragment ion tolerance 0.6 Da. Tryptic peptides were searched with up to two missed cleavages. Carbamidomethylation was set as a static modification (Cys), Oxidation (Met), acetylation (protein N terminus), Gln→pyro-Glu (N-term Q), Glu→pyro-Glu (N-term E), and 13C6 (Lys) were set as dynamic modifications. Mascot results from searches against SwissProt were sent to the percolator algorithm (53) version 2.04 as implemented in Proteome Discoverer. Spectra without high confident peptide match were sent to a second round of search with semi-tryptic specificity and phosphorylation as dynamic modification (Ser, Thr, Tyr). Only proteins with two peptides (maximum posterior error probability 1%) were considered identified. The false discovery rate (FDR) calculated by Percolator and peptide identifications were accepted with a cut-off of 0.01. Data were filtered for peptide–spectrum matches assigned to only one accession number and the median intensity computed across all
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peptide-spectrum matches identified for the respective protein. Protein intensities of all replicates were then combined into a feature table and such identified in only one replicate were excluded. Statistical analysis of the heavy to light ratios on the protein level was performed with the R package limma (54) whose applicability on MS data is described in Ref. 55. Corrected p values were computed with the R package q value (56) for false discovery rate estimation, using the corrected p values obtained from the Benjamini-Hochberg output. All data processing steps were implemented in Perl and R scripts. The MS proteomics datasets have been deposited to the Proteome Xchange consortium with the dataset identifiers PXD004548 (lysosomal proteome analysis).

Lipidomics

Total lipids were extracted in three independent experiments from 10 10-cm plates (~900 mg cells) and separated into a neutral and an anionic fraction by reversed-phase C18 columns (Agilent Technologies, Santa Clara, CA), as described previously (57). Aliquots of the neutral and anionic lipid fractions were analyzed by electrospray ionization-MS using Agilent 6490 Triple Quad LC/MS (Agilent Technologies) and Micromass Quattro Micro triple quadrupole mass spectrometers (Waters, Milford, MA), and lipid class-specific detection modes (57, 58).

Acyl chain alterations in the lipid fractions were studied by GC (Shimadzu GC-2010 Plus with FID, and Shimadzu GCMS-QP2010 Ultra with MSD, Kyoto, Japan) and employing standard protocols (**59**). Data represent the mean ± standard deviation (S.D.) or standard error of the mean (S.E.), and significance was evaluated with one-way ANOVA followed by Dunnett’s or Bonferroni’s multiple comparison test. A result was considered statistically significant if the p value was ≤ 0.05 (*), ≤ 0.01 (**), or ≤ 0.001 (***)

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