Alterations in EGF-Endocytosis, Lysosomal Enzyme Transport and Maturation of Cathepsins in Juvenile Neuronal Ceroid Lipofuscinosis Fibroblasts

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Abstract

Background: Juvenile neuronal ceroid lipofuscinosis (JNCL), one of the most frequent forms of the NCL storage diseases, is known to be caused by loss-of-function mutations in ceroid-lipofuscinosis neuronal protein 3 (CLN3), but its cell function has not been fully elucidated. We previously reported increased lysosomal pH in CLN3 deficient cells. In the present study, we analysed the consequences of this effect in the endo-lysosomal pathways in CLN3 cells.

Methods: The present study investigated different endo-lysosomal pathways in control, CLN2, CLN3 human skin fibroblasts under high and low proteolysis conditions. Cell surface biotinylation after EGF (2 ng/mL) stimulation, EGF phosphorylation (Tyr-845), retromer and cation-independent mannose-6-phosphate receptor (CI-MPR) levels and stability, EGF degradation pathways and cathepsin L and D levels were analysed by western blots. Caveolae mediated endocytosis was analysed by flow cytometry. CIMPR subcellular localization was ascertained by immunocytochemistry, confocal microscopy and further image analysis.

Results: Whereas caveolae-mediated endocytosis was not affected in CLN3 cells, clathrin-mediated epidermal growth factor (EGF) internalization was reduced, along with EGF receptor (EGFR) phosphorylation. In addition, cell surface EGFR levels and recycling to the cell membrane were increased. EGFR lysosomal degradation was impaired and our results suggest that the receptor was diverted to proteasomal degradation. We also analysed the machinery responsible for lysosomal hydrolase transport to the lysosome and found increased stability of CIMPR, a major receptor implicated in the transport of hydrolases. The subcellular distribution of the CI-MPR was also altered in CLN3 cells, since it accumulated within the Trans-Golgi network (TGN) and did not progress into the lysosomes. In addition, we found a reduced turnover of retromer subunits, a complex that retrieves the CI-MPR from endosomes to the TGN. Finally and as a possible consequence of these alterations in lysosomal enzyme transport, cathepsin L and D maturation were found suppressed in CLN3 cells.

Conclusion: Altogether, these results point to increased lisosomal pH as a pivotal event causing various alterations in intracellular traffic associated to the development of JNCL disease.

Keywords: Juvenile neuronal ceroid lipofuscinosis; Lysosomal pH; Clathrin mediated endocytosis; Caveolae-mediated endocytosis; Cathepsin L; Cathepsin D; CI-MPR (CI-MPR); Retromer; Epidermal Growth Factor Receptor.

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Introduction

Neuronal Ceroid Lipofuscinoses (NCLs) are the most common group of rare autosomal recessive neurodegenerative disorders of childhood. NCL are histologically characterized by the building up of autofluorescent pigments within lysosomes. Among the NCLs, the juvenile CLN3 disease,[1,2] also known as juvenile NCL (JNCL)[3] or Batten disease,[4] is the most frequent. Increasing visual failure, seizures, motor and cognitive skill reduction, and early death are characteristic of JNCL.[2] In more than 80% of patients with CLN3 deficient cells there is a 1.02 kb genomic DNA deletion in the CLN3 gene (*CLN3*) that predicts a non-functional protein.[2] The *CLN3*-encoded protein (CLN3) is a highly conserved, multi-pass transmembrane glycoprotein that is confined to endosomes and lysosomes,[5,6] but its precise cell role is still unknown. Studies in yeast have reported that *BTN1*, which encodes the ortholog of *CLN3*, is involved in the regulation of the vacuolar pH[7-9] and the phospholipid contents of mitochondrial and vacuolar membranes.[10] It was reported that in yeast, BTN2/CLN3 expression is up-regulated upon deletion of BTN1 the yeast ortholog of Hook1, a retromer accessory protein involved in retrieval of specific cargo from late endosomes to Golgi.[11,12] Further

studies in yeast concluded that BTN1 regulates retrograde sorting by mediating vesicle fusion through SNARE protein phosphorylation and assembly, which could be altered in JNCL.[13]

In mammalian cells, a role for CLN3 in endocytic trafficking has been proposed.[14-17] Luiro et al showed that CLN3 overexpression in HeLa cells induces aggregation of Hook1, causing its dissociation from the microtubules in endosomal fusion events.[14] They linked in CLN3 fibroblasts the observed abnormalities to a delay in the lysosomal delivery of low-density lipoprotein and to increased recycling rates of transferrin.[14]

Other studies using mammalian cells have also indicated that CLN3 is directly implicated in the regulation of lysosomal pH[18] or in the delivery of lysosomal enzymes.[19,20]

Soluble acid hydrolases, once modified with mannose 6-phosphate residues in the Golgi, are commonly recognized by mannose 6-phosphate receptors and then transported to the endo/lysosomal system.[21] For example, two of the main lysosomal cathepsins, D and L, are transported by the cation-independent mannose 6-phosphate receptor (CI-MPR).[22] Cathepsin D is known to degrade ATP synthase subunit c, which typically accumulates in CLN3 deficient cells.[23] In lysosomes, cathepsin L has been found to cleave CLN7p, a protein deficient in a variant of the late-infantile NCL.[24] Cathepsin L has also been localized in secretory vesicles containing neuropeptides and found to play an essential role in neurotransmitter production.[25]

Our previous studies indicated that lysosomal pH was increased in isolated cells from JNCL patients, resulting in reduced fusion of autophagosomes with lysosomes.[26] In the present study, we further examined the consequences of increased pH in CLN3 cells in other lysosomal related processes. Throughout the text, CLN3-deficient cells will be referred to as CLN3 cells. Cells carrying the tpp1 mutation (CLN2 cells) have been used as control cells along with non-mutated cells (control cells). We report in these cells alterations in receptor-mediated endocytosis and in delivery of lysosomal cathepsins to lysosomes. Our data are consistent with the idea that increased lysosomal pH plays a central role in determining the phenotype of cells from JNCL patients.

Results

CLN3 cells have increased EGFR recycling

To examine receptor-mediated endocytosis, we analysed the phosphorylation state of the EGFR by Western blot and the rate of ligand endocytosis using EGF bound to Alexa Fluor 488 (AF488-EGF) by flow cytometry. To avoid possible aging effects, all experiments were done comparing cells at a similar passage number (10–14) in the cell culture. Upon ligand binding, EGFR phosphorylation increases at 5, 10 and 15 min in CLN3 cells as compared to control cells (Figure 1A). On the contrary, internalization of EGF was similar in CLN3 and control cells (Figure 1B, left panel). Flow cytometry analysis of internalized BODIPY FL C5-Ceramide, a specific marker of caveolae-mediated endocytosis,[27] shows that this type of internalization was not altered either in CLN3 fibroblasts as compared to controls (Figure 1B, right panel). However, plasma membrane composition
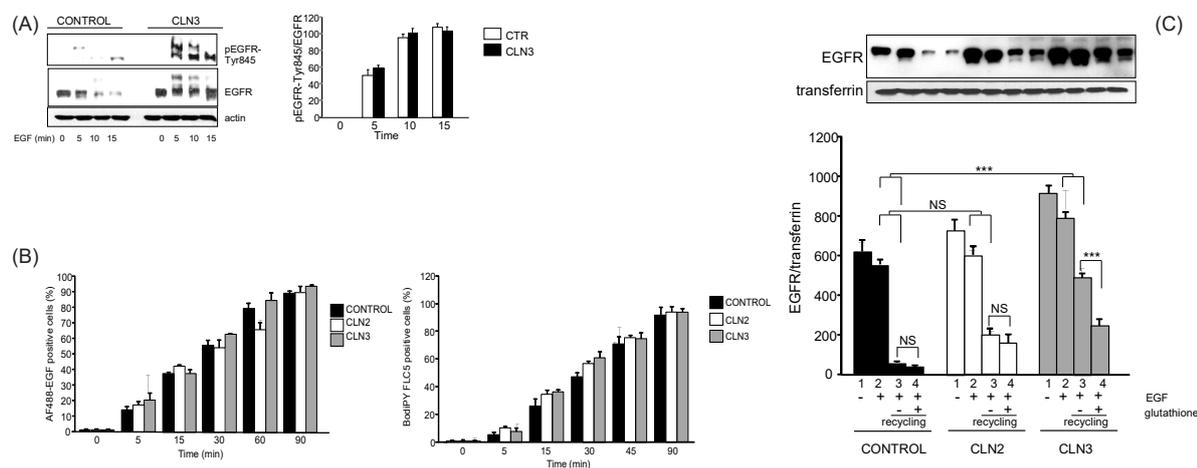

Figure 1. Alterations of EGF endocytosis in CLN3 cells. (A) Control and CLN3 fibroblasts were incubated with EGF (100 ng/mL) in high proteolysis medium for the indicated time periods. A representative blot of total cell extracts analysed with antibodies that recognize phosphorylated (Tyr845) EGFR and actin is shown. Bands were quantified and the ratios of EGFR to actin are shown below. (B) Comparison of EGF (left panel) or BODIPY FL C5-Ceramide (right panel) internalization in control, CLN2 and CLN3 fibroblasts incubated at 37 °C with AF488-EGF (200 ng/mL) or BODIPY FL C5-Ceramide (0.5 μM), respectively, for the indicated times. After washing, measurements were carried out by flow cytometry as described in Materials and Methods and are shown as percentage of fluorescently labelled cells. (C) Exponentially growing control, CLN2 and CLN3 fibroblasts incubated in high proteolysis medium were surface biotinylated, stimulated with EGF (2 ng/mL) and treated with reduced glutathione as explained in Materials and Methods. Samples were pulled-down with NeutrAvidin agarose-beads and subsequently analyzed by immunoblotting using anti-EGFR and, as an internal control, anti-TfR antibodies. Representative blots are shown and EGFR bands from three independent experiments similar to those shown were densitometred and normalized to the corresponding transferrin bands. Data from three independent experiments are expressed in % relative to cells at time 0 min. All results are the mean ± SD from three separate experiments, with triplicated measurements, and stars indicate statistically significant differences from control values at each time point.

is likely altered, as flow cytometry analysis revealed that binding to cholera toxin B (ChTxB), which binds to cell surface GM1 gangliosides,[28] is severely impaired in CLN3 fibroblasts (Figure S1).

Next, we examined if EGFR recycling was altered in CLN3 fibroblasts in comparison to control and CLN2 fibroblasts by cell surface protein biotinylation, in non-treated cells (lane 1) and in cells treated with EGF at low (2 ng/mL) concentration and reduced glutathione (50 mM); at such low EGF concentration, most of the receptor is recycled to the cell membrane.[29] Internalized levels of biotinylated EGFR did not change among CLN3, CLN2, and control fibroblasts (lanes 1 and 2 in Figure 1C), in agreement with the observed similar EGF endocytosis. After subsequent treatment with EGF (2 ng/mL) and with reduced glutathione (50 mM), EGFR levels decreased in CLN3 fibroblasts as compared to cells not treated with activated glutathione, but the corresponding levels did not change in control or CLN2 fibroblasts (lanes 3 and 4 in Figure 1C). This indicates that EGFR recycling was increased in CLN3 fibroblasts. Also, the comparison of biotinylated EGFR relative to transferrin levels in control versus CLN3 fibroblasts (lane 1) indicates, as expected, that cell surface levels of EGFR are increased in fibroblasts from JNCL patients.

Lysosomal degradation of EGFR is delayed and the receptor is diverted towards the proteasomal pathway in CLN3 fibroblasts

To further examine the EGFR endocytic pathway upon internalization, receptor levels were analysed by Western blot after incubation with EGF (100 ng/mL). At this high concentration of ligand, 90 % of the receptor is headed towards degradation.[29] Ligand-induced degradation of EGFR was significantly delayed in the CLN3 fibroblasts as compared to their respective controls at all-time points (Figure 2A). As an increased lysosomal pH could prevent the receptor from being degraded in lysosomes, we examined the possibility that EGFR could undergo proteasomal degradation. Indeed, proteasome inhibition has also been shown to attenuate ligand-induced degradation of EGFR in HeLa cells.[30] We thus analysed the relative contribution of lysosomal and proteasomal degradation pathways to degradation of EGFR in CLN3 cells. Inhibitors were added 4 h prior to EGF (100 ng/mL) treatment, and EGFR degradation was analysed by Western blot for 1 additional hour in the presence of the inhibitors and in high proteolysis medium to maximize degradation. In the presence of the proteasome inhibitor lactacystin, the difference in EGFR levels between CLN3 cells and control cells was increased by 45% as compared to the absence of inhibitors, but not in CLN2 cells (Figure 2B and not shown). On the contrary, in the presence of the lysosomal inhibitors NH_4Cl plus leupeptin the difference in EGFR levels decreased by 55 % when compared with absence of inhibitors (Figure 2B). These results suggest that proteasome degradation of EGFR is increased in CLN3 fibroblasts, possibly as a compensatory mechanism for the impaired lysosomal degradation in these cells.

The observed variable steady-state EGFR levels in control and in CLN3 cells should not be affecting the described tendencies as similar results were obtained in cells with different EGFR levels (data not shown).

The stability of the CI-MPR and of the retromer subunits is increased in CLN3 fibroblasts under high proteolysis conditions

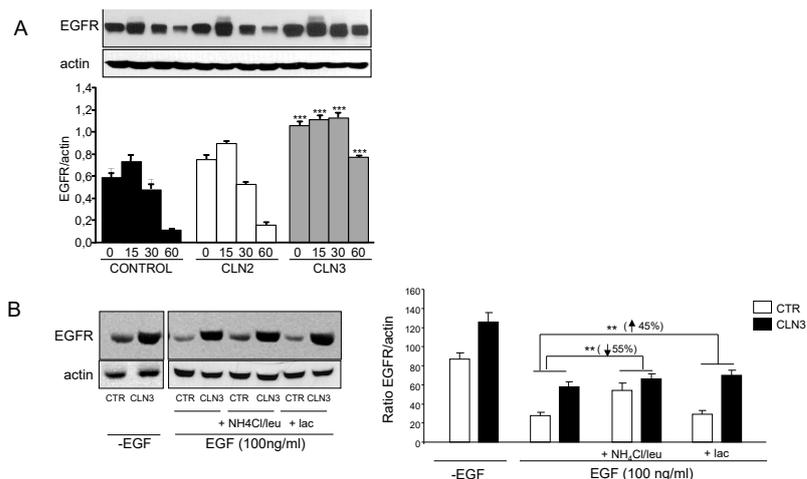Next, we examined levels and turnover in high and

Figure 2. In CLN3 fibroblasts, EGFR degradation is diverted from the lysosomal to a proteasomal pathway. (A) Control and CLN3 fibroblasts were incubated for 60 min with EGF (100 ng/mL), and total extracts were analyzed by Western blot. Representative blots and quantification of EGFR levels by densitometry are shown. Data in the histogram are shown in percentage relative to time 0. Stars indicate statistically significant differences from control values at each time point. (B) Control (CTR) and CLN3 fibroblasts were treated with EGF and with the inhibitors where indicated as described in the materials and methods section. Total extracts were immunoblotted with anti-EGFR and, as a loading control, anti-actin. A representative blot is shown. Bands corresponding to EGFR were quantified by densitometry and the ratios of EGFR to actin were plotted on the right. Stars indicate statistically significant differences in control and CLN3 fibroblasts treated with inhibitors from the corresponding values in the absence of inhibitors.

low proteolysis media of the CI-MPR, a major receptor of soluble hydrolases that shuttles from the TGN to endosomes. Different medium conditions were used in these experiments for each cell type, as we wanted to observe differences under stressed (high proteolysis, H) and non-stressed lysosomal enzyme transport (low proteolysis, L). In the presence of cycloheximide (CHX), CI-MPR stability was found increased in CLN3 fibroblasts compared to controls under both conditions (Figure 3A). To analyze if this was due to CI-MPR missorting, we also analyzed the levels of retromer subunits because retromer mediates endosome-to-TGN retrieval of the CI-MPR.[31,32] Retromer is a pentameric complex which includes two sorting nexin family members (typically, SNX1 and SNX2), that curve the endosomal membrane, and three additional subunits, Vps26, Vps 29 and Vps 35, which constitute the complex core and determine cargo sorting.[33] We found that the stability of retromer subunits Vps26 and Vps35 (Figure 3B) and SNX1 and SNX2 (Figure 3C) are increased in high and low proteolysis media in CLN3 fibroblasts. The increased stability of retromer under the studied conditions suggests impairment of endosome-to-TGN retrieval and possible alterations in the delivery of lysosomal enzymes.

CI-MPR accumulates in TGN in CLN3 fibroblasts

To explain the reduced turnover of the CI-MPR (along with retromer), we hypothesized that cycling of CI-MPR

between TGN and early endosomes might be affected in the NCL cells. Thus, we compared the localization of CI-MPR with that of the TGN-resident protein TGN46[34] by confocal microscopy. Both are transmembrane proteins that cycle back and forth from the TGN to the early endosomes,[35] although most of TGN46 is maintained at the TGN at steady state, similarly to the CI-MPR.[36,37] In CLN3 fibroblasts, CI-MPR colocalized with TGN46 in tubular TGN-like structures (Figure 4). According to our results CI-MPR would not function as a proper enzyme shuttle as it would not leave the TGN.

Processing of procathepsin L and D into cathepsin L and D is abrogated in CLN3 fibroblasts

Accumulation of CI-MPR in TGN could affect the transport of acid hydrolases to the lysosome. Cathepsins L and D have been shown to be shuttled to the lysosomes mainly by CI-MPR.[22] Therefore, we examined the processing of procathepsin L and D to their mature forms. Fibroblasts were cultured in high or low proteolysis medium as we wanted to observe differences under stressed (High proteolysis) and non-stressed lysosomal enzyme transport (Low proteolysis). Levels of procathepsin and cathepsin L and D were analysed by Western blot. We found that procathepsin L was not converted into cathepsin L in CLN3 fibroblasts (Figure 5). Processing of procathepsin D into cathepsin D was also affected in CLN 3 fibroblasts under the same conditions, but to a lesser extent (Figure
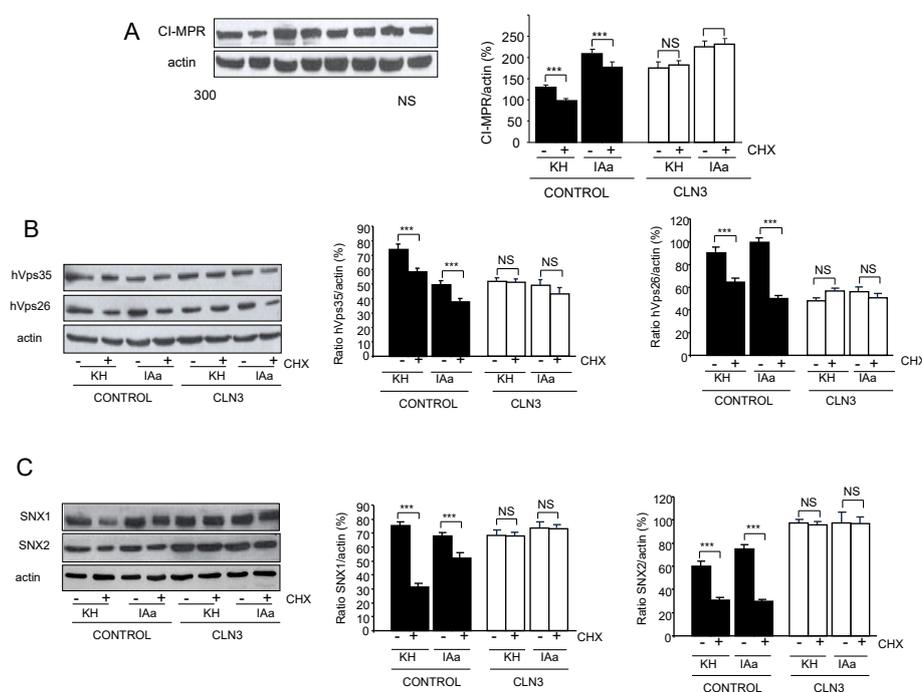

Figure 3. Increased stability of the CI-MPR and retromer subunits in CLN3 fibroblasts. Fibroblasts were incubated under high (H) or low (L) proteolysis conditions in the presence of cycloheximide (CHX, 100 µg/mL for 6 h). Total extracts were immunoblotted with the following antibodies: (A) anti-CI-MPR, (B) anti-hVps35 and anti-hVps26 and (C) anti-SNX1 and anti-SNX2. Anti-actin was used as a loading control. The bands that correspond to the analyzed proteins were quantified by densitometry and normalized to actin. Representative gels are shown and the histograms depict the corresponding protein/actin ratios. Stars indicate statistically significant differences between cycloheximide-treated and untreated fibroblasts.
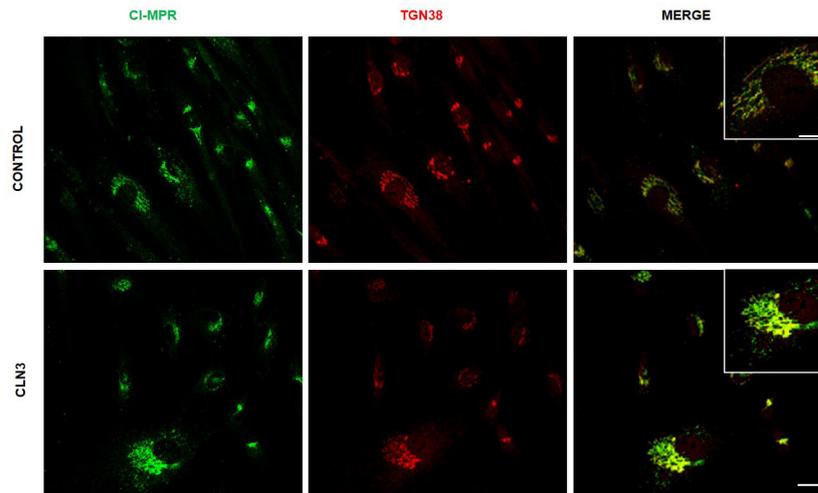
Figure 4. In CLN3 fibroblasts, CI-MPR accumulates in the TGN. Representative fluorescence microscopy images of control and CLN3 fibroblasts incubated at 37°C under high proteolysis conditions in the presence of lysosomal inhibitors, fixed, and probed with anti-CI-MPR and anti-TGN46. Cells were examined by confocal microscope.

5). These results reflect the consequences of the shown alterations in the cell machinery responsible for TGN to endosome communication.

Discussion

In a previous report, we found that CLN3 cells, as opposed to CLN2 cells, had an increase of 0.5 units in lysosomal pH over normal (wild type) cells.[26] The current study, using fibroblasts derived from Juvenile CLN3 patients (called CLN3 fibroblasts), analyzes the potential effects of this increased pH in EGFR traffic and lysosomal enzyme transport. EGFR/ligand dissociation and dissociation of the transported lysosomal enzymes from CI-MPR are both dependent on pH.[29,38] In this study, we conclude that EGFR degradation is impaired as a consequence of steady

Figure 5. Processing of procathepsin L and D into the corresponding mature forms is impaired in CLN3 fibroblasts. Fibroblasts were incubated under high (H) or low (L) proteolysis conditions in the presence of cycloheximide (CHX, 100 µg/ml for 6 h). Total extracts were immunoblotted with antibodies recognizing the precursor and mature forms of cathepsin D and cathepsin L, using actin as a loading control.

recycling of the receptor to the cell surface. Furthermore, our results suggest that CI-MPR, the major lysosomal enzyme receptor, is also constantly recycled back from endosomes to the TGN, and, therefore, enzyme transport to lysosomes is altered.

EGFR auto phosphorylation, that occurs immediately after ligand binding, and is required for endocytic processing of the EGFR/ligand complex,[39] was increased in CLN3 fibroblasts. Nevertheless, receptor-mediated endocytosis, that occurs immediately thereafter, was not affected. Once the EGFR/ligand complex arrives to the early endosomes, it can be recycled to the cell membrane or delivered to late endosomes and lysosomes for degradation. In HeLa cells, only 30% of the internalized EGFR is degraded at low (1.5 ng/mL) EGF concentrations, while most of it is recycled. However, at high (100 ng/mL) EGF concentrations, approximately 90% of the internalized receptor is degraded.[31] In CLN3 cells, we found both increased recycling and impaired degradation of the EGFR at low and high concentrations of EGF, respectively. The increase in recycling could explain the increased amounts of EGFR at the cell membrane as well as the increase in receptor phosphorylation in the CLN3 cells. The decreased EGFR degradation could account for its higher steady state levels. Our results indicate that the internalized EGFR is not transported to lysosomes but is recycled back to the cell membrane in CLN3 cells.

Our results show that the CI-MPR – as well as subunits of the retromer complex, which mediates CI-MPR retrieval from endosomes to TGN – is more stable in CLN3 fibroblasts. The increased stability of CI-MPR and retromer levels might be a possible cell compensatory mechanism to try to preserve their altered functions. In accordance with our results, a role of the CLN3p in CI-MPR traffic from the TGN has been already shown in HeLa cells with a transfected chimaeric CI-MPR reporter
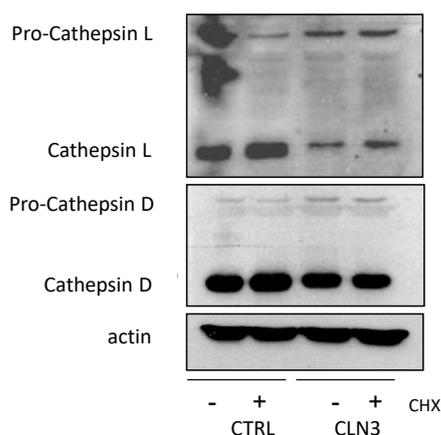
(CD8-CI-MPR) by Metcalf et al.[20] These authors found that ablation of CLN3p caused a 50% reduction in exit of the CI-MPR reporter from the TGN. In accordance to our findings concerning CI-MPR stability, Qureshi et al[40] showed in HeLa cells transfected with a plasmid expressing the a c.A959G mutant CLN5p, which has been physically and functionally related with the CLN3p,[41,42] that mutant CLN5p was deficiently glycosylated and was abnormally detained in the endoplasmic reticulum resulting in reduced delivery to the endolysosomal compartment. Most of ours and the previously published work agree with the notion that impaired CLN3 function is related to altered trafficking and localization of CI-MPR.

A failure in CI-MPR localization, as well as the inability to deliver its cargo to the endo-lysosomal compartment due to changes in pH, could have a deep impact in delivery of lysosomal enzymes, such as cathepsins, whose precursors are transported by the CI-MPR.[22] Thus, Arighi et al observed that depletion of hVps26 by siRNA causes CI-MPR lysosomal degradation and reduced cathepsin D levels in HeLa cells.[31] Similarly, Seaman showed a defect in cathepsin D maturation in HeLa cells after mVPS26 knock down in spite of seeing no effect in CI-MPR turnover.[32] The study from Qureshi et al also showed a significant decrease in procathepsin D to cathepsin D conversion.[40] A past study in *Drosophila* cells shows that retromer is needed for the right delivery of cathepsin L to lysosomes, but independently of the *Drosophila* homolog of the CI-MPR[43]. Our results are consistent with these reports as we found mislocalized CI-MPR and deficient cathepsin D maturation in CLN3 cells. A recent study by Carcel-Trullols et al further showed that in Baby Hamster Kidney cells, stably expressing myc-tagged human CLN3, activity of cathepsin D decreased as CLN3 expression increased under hyperosmolar conditions.[44] Several studies in animal models have indicated that there are close ties between maturation and activity of cathepsin D and NCL disease.[45-47] In addition, a reduction in the maturation of cathepsin D, which is known to degrade the subunit c of the ATP synthase, could explain its accumulation, a distinctive marker of the NCL phenotype.[23] According to our results, the observed alterations in lysosomal enzyme transport in CLN3 cells are also reflected in a completely abrogated processing of procathepsin L into cathepsin L in CLN3 cells. Procathepsin L is mainly transported by the CI-MPR, although in neurons, SORLA/SORL1, an APP receptor which dysfunction is implicated in Alzheimer's disease, has also been involved in this process.[48] Cathepsin L has been postulated as a central nervous system neuroprotease that participates in peptide neurotransmission and in neurodegenerative diseases, and expression of human cathepsin L has been found to prevent neuronal cell death and consequent neurodegeneration in cells of the cerebral cortex and in Purkinje cells of the cerebellum.[49,50] An animal model with a combined deficiency in cathepsins B and L resulted in early-onset neurodegeneration in mice reminiscent of NCL in humans.[50] Other reports suggest that cathepsin L may be linked to Parkinsonian neurodegeneration by means of 6-hydroxydopamine induced apoptosis.[51] Thus, the lack of procathepsin L maturation could be the linkage between lysosomal alterations and neurodegeneration.

In conclusion, the results presented in this study show that in juvenile CLN3 disease, increased lysosomal pH might be responsible for the reported alterations in EGFR recycling and degradation. Along with these alterations the showed CI-MPR mislocalization likely plays a very important role in the alterations of cathepsin L and D maturation that may critically affect JNCL and other types of related NCL diseases.

Materials and Methods
Materials
Minimum essential medium (MEM), human insulin, 3-methyladenine, NH_4Cl, PMSF, cycloheximide, EDTA, streptavidin, L-glutathione (reduced), anti-tubulin antibody and horseradish-peroxidase labelled secondary antibodies were purchased from Sigma Chemical Co. EZ-Link Sulfo-NHS-SS-Biotin and Biotinylation Kits (NHS-SS-Biotin) were purchased from Thermo Fisher Scientific. MEM amino acids 50x, MEM non-essential amino acids 100x, penicillin, MEM vitamins, streptomycin, foetal bovine serum and recombinant human EGF (PHG0311) were supplied by Life Technologies. Leupeptin and lactacystin were acquired from Peptide Institute, Inc. Phosphospecific antibodies that recognize phosphorylated (Tyr-845)-EGFR (#6963) were purchased from Cell Signalling. Specific antibodies that recognize total EGFR (ab2430), used in western blot experiments, and CI-MPR (ab2733) and TGN46 (ab16052), used in the immunofluorescence experiments, were purchased from Abcam. Rabbit polyclonal antibodies to human retromer subunits[52] have been described previously. A polyclonal anti-CI-MPR antibody was obtained as a gift from Dr. B. Hoflack. AF488-EGF (E-13345), AF488-ChtxB (C-34775), Alexa Fluor® 488 goat anti-mouse IgG, IgM (H+L) (A-10667) and Alexa Fluor® 546 goat anti-rabbit IgG (H+L) (A-11010) were purchased from Molecular Probes-Life Technologies. Other reagents, such as H_2O_2, Bovine Serum Albumin (BSA), trichloroacetic acid (TCA), and Purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole-3-thiol), were of the best analytical quality available.

Cell Culture
Control cells, CLN2, CLN3 human skin fibroblasts were obtained from the Coriell Institute for Medical Research (Camden, NJ, USA). Conditions for cell growth, cell viability and growth curves as well as high and low proteolysis conditions were described previously.[26]

Flow Cytometry

Determination by flow cytometry of clathrin-mediated endocytosis was performed essentially as described earlier[53] and caveolae-mediated endocytosis was performed as a modification of an existing protocol.[54] Subconfluent fibroblasts were incubated at 37°C under high proteolysis conditions to maximize internalization for the indicated times in the presence of AF488-EGF (200 ng/mL) or BODIPY FL C5-Ceramide (0.5 μM). Cells were then washed with cold PBS at 4°C and about 5×10^5 cells were immediately analyzed by flow cytometry (FACSCalibur flow cytometer from BD Biosciences). Cells were analyzed at excitation and emission wavelengths of 495 and 519 nm, respectively, for the AF488-EGF experiment, and at 505 nm and 620, respectively, for the BODIPY FL C5-Ceramide determination. Mean fluorescence values were obtained, and the % of positive cells was calculated for every experimental condition.

To determine the binding of ChTxB and GM1 cell membrane contents, cells were incubated in a buffer containing ChTxB bound to Alexa Fluor-488 (AF488-ChtxB) (1 μg/mL) in cold PBS containing sodium azide (0.1 %) at 4°C for one minute to prevent internalization, and then cells were washed three times in cold PBS containing sodium azide (0.1%) at 4°C. About 5×10^5 cells were immediately analyzed by flow cytometry (FACSCalibur flow cytometer, from BD Biosciences) at excitation and emission wavelengths of 495 and 519 nm, respectively, and mean fluorescence values were plotted.

Protein Determination and Western Blot

These procedures have been already described.[55] To determine protein degradation, cells were incubated for 6 hours with 100 μg/mL cycloheximide. All blots were developed using Lumi-Light Western Blotting Substrate and protein bands were quantified by densitometric analysis (Image Quant ECL, GE Healthcare).

For analysis of EGFR phosphorylation by immunoblot, cells were collected and lysed in lysis buffer (1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, 15% glycerol, 150 mM NaCl, 2 mM Na_3VO_4, 100 mM NaF, 20 mM $Na_4P_2O_7$, pH 7.4, containing 1 mM PMSF and 100 μM leupeptin,) at 4°C. Immunoblots were probed against phospho-specific EGFR and actin antibodies.

For semiquantitative determination of EGFR degradation, confluent fibroblasts were incubated at 37°C under high proteolysis conditions with or without EGF (100 ng/mL) for 15, 30 and 60 minutes. To inhibit protein degradation, cells were incubated 4 h before the addition of EGF with the lysosomal inhibitors NH_4Cl (20 mM) and leupeptin (100 μM), or with the proteasomal inhibitor lactacystin (30 μM). Cells were then washed with cold PBS and homogenized in RIPA buffer (150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing 0.1 mM leupeptin and 1 mM PMSF. Extracts were centrifuged at 12,000 g for 10 minutes at 4°C and the supernatants were collected for Western blot. Lysates (50 μg protein) were analyzed on 15% SDS-PAGE gels and proteins were transferred to PVDF membranes and probed with antibodies that recognize EGFR.

Cell Surface Biotinylation

The analysis of EGFR internalization and recycling was performed as described earlier.[56] Briefly, confluent fibroblasts were washed three times with PBS-CM (containing 0.2 mM $CaCl_2$ and 2 mM $MgCl_2$) and incubated for 30 min. in PBS-CM at 4°C containing the biotin reagent (NHS-SS-Biotin) at 0.5 mg/mL. A sample for each cell line was taken and frozen in RIPA buffer containing leupeptin (0, 1 mM) and PMSF (1 mM). After a first wash with PBS-CM to remove the excess of unbound reagent, and a second wash with MEM-BSA (MEM, 0.6 % BSA, 20 mM Hepes-NaOH pH 7.4 and 0.35 g/L $NaHCO_3$), supplemented with 100 mM glycine to quench the free succinimide groups, the cells were incubated at 37°C with 2 ng/mL EGF in MEM-BSA for 30 minutes. The ligand concentration used here was most suitable for semiquantitative determination of EGFR recycling. Afterwards, cells were incubated twice for two 20-minute periods in reduced glutathione at 50 mM in TAPs buffer (50 mM TAPS-NaOH pH 8.60, 100 mM NaCl and 0.2 % BSA) and washed three times with ice cold PBS-CM. A sample for each cell line was taken and processed as above. The rest of the plates were incubated in PBS-CM containing iodoacetamide (5 mg/mL) for 5 minutes at 4°C to inactivate glutathione, and cells were washed with PBS-CM. EGF 2 ng/mL was added to the cells for 30 min., Cells were washed three times with PBS-CM and a sample for each cell line was frozen as before. Finally, cells were incubated again twice for two 20-minute periods in reduced glutathione at 50 mM in TAPs buffer, washed three times with ice cold PBS-CM, and samples were frozen as detailed. Extracts from the different samples were centrifuged at 12 000 g for 10 minutes, supernatants were collected, biotinylated proteins were pulled-down with NeutrAvidin beads and analyzed by immunoblotting using antibodies to EGFR and the transferrin receptor.

Immunocytochemistry, Confocal Microscopy and Image Analysis

Subconfluent fibroblasts were cultured for 4 hours on glass coverslips under high proteolysis conditions with protease inhibitors (1 μM pepstatin and 100 μM leupeptin). Cells were then fixed for 10 minutes with 4% paraformaldehyde in PBS-CM. After quenching of the free aldehyde groups, cells were permeabilized, blocked, and incubated at 4°C overnight with anti-CI-MPR (1:100) and anti-TGN46 (1:200) to label the TGN. Secondary antibodies conjugated to Alexa 488 or to Alexa 595 were applied (1:500) for 1 hour at r.t. Slides were rinsed with

PBS, mounted and observed in a confocal fluorescence microscope. For analysis of co-localization, cells were observed using a Leica TCS-SP2-AOBS confocal microscope using a 63X oil immersion objective of 1.4 NA at 1024×1024 pixel resolution.

The presented images were processed using Adobe Photoshop software (Adobe Systems) by adjusting brightness and contrast levels to the same extension for all the experimental conditions showed in each experiment.

Statistics

The presented data for the different conditions were calculated as means ± S.D. of at least three independent experiments. GraphPad Prism version 5.0 software was used to perform statistical analysis. Two-tailed Student's *t* tests was used to ascertain differences between paired samples and for multiple sample comparison one-way ANOVA was used followed by Student-Newman-Keuls post hoc test. The significance has been determined at * $P<0.05$, ** $P<0.01$ and *** $P<0.005$, as showed in each case.

Conclusion

In the past two decades, considerable progress has been made in understanding the function of CLN3 protein in the development of the JNCL neurodegenerative disease. Despite knowing that CLN3p is an integral transmembrane glycoprotein that localizes to endosomes and lysosomes[5,6] little is known about its precise function and its role in the development of the disease, so further investigation could set new molecular targets that could prevent the onset of this devastating neurodegenerative disease. Our group published in 2013[26] that CLN3 cell lysosomes distinctively presented a statistically significantly increased pH of 0.5 units, possibly related to the reported defects in fluid phase endocytosis and impairment in autophagosome maturation in these cells. The presented study deepens in the study of the consequences of increased lysosomal pH in CLN3 cells as opposed to CLN2 and control cells. In this study we analyze the regulation of the endo-lysosomal pathways in CLN3 cells which might be directly affected by the increased lysosomal pH. Clathrin-mediated epidermal growth factor (EGF) internalization showed to be reduced, possibly as consequence of impaired delivery of the receptor to the lysosomes, along with EGF receptor (EGFR) phosphorylation. Most importantly, EGFR lysosomal degradation was impaired and EGFR was rerouted to the cell membrane. We analysed the machinery responsible for lysosomal hydrolase transport to the lysosome and found increased stability of CI-MPR and the retromer subunits which are in charge of its retrieval from the endo-lysosomal system to the TGN, CI-MPR accumulation within the TGN and thus not progression into the lysosomal membranes as expected. Finally, as a possible consequence of these alterations in lysosomal enzyme transport, cathepsin L and D maturation were found dramatically reduced in CLN3 cells.

Authors' contributions
The entire manuscript was written by the author. The only author collected literature, designed and wrote the manuscript, edited and prepared manuscript for submission.

Conflict of Interest Disclosures
The authors declare that they have no conflict of interests.

Ethical Statement
Not applicable.

Availability of Supporting Data
Supporting data is available from the author.

Supplementary Materials
Supplementary file 1 contains Figure S1.

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