Distinct Early Molecular Responses to Mutations Causing vLINCL and JNCL Presage ATP Synthase Subunit C Accumulation in Cerebellar Cells

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Abstract

Variant late-infantile neuronal ceroid lipofuscinosis (vLINCL), caused by CLN6 mutation, and juvenile neuronal ceroid lipofuscinosis (JNCL), caused by CLN3 mutation, share clinical and pathological features, including lysosomal accumulation of mitochondrial ATP synthase subunit c, but the unrelated CLN6 and CLN3 genes may initiate disease via similar or distinct cellular processes. To gain insight into the NCL pathways, we established murine wild-type and CbCln6<sup>ncdf/ncdf</sup> cerebellar cells and compared them to wild-type and CbCln3<sup>ex7/8/ex7/8</sup> cerebellar cells. CbCln6<sup>ncdf/ncdf</sup> cells and CbCln3<sup>ex7/8/ex7/8</sup> cells both displayed abnormally elongated mitochondria and reduced cellular ATP levels and, as cells aged to confluence, exhibited accumulation of subunit c protein in Lamp 1-positive organelles. However, at sub-confluence, endoplasmic reticulum PDI immunostain was decreased only in CbCln6<sup>ncdf/ncdf</sup> cells, while fluid-phase endocytosis and LysoTracker<sup>®</sup> labeled vesicles were decreased in both CbCln6<sup>ncdf/ncdf</sup> and CbCln3<sup>ex7/8/ex7/8</sup> cells, though only the latter cells exhibited abnormal vesicle subcellular distribution. Furthermore, unbiased gene expression analyses revealed only partial overlap in the cerebellar cell genes and pathways that were altered by the <i>Cln3<sup>ex7/8</sup></i> and <i>Cln6<sup>ncdf</sup></i> mutations. Thus, these data support the hypothesis that CLN6 and CLN3 mutations trigger distinct processes that converge on a shared pathway, which is responsible for proper subunit c protein turnover and neuronal cell survival.


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Introduction

The neuronal ceroid lipofuscinoses (NCLs) collectively account for most cases of childhood-onset neurodegenerative disease worldwide, with clinical features of blindness, seizures, psychosis, motor and cognitive decline, and premature death (see recent reviews [1,2]). A defining feature of NCL is lysosomal storage of autofluorescent ceroid lipofuscin, which contains proteolipid and dolichol, and in most forms of NCL, the mitochondrial ATP synthase, subunit c protein [2,3,4,5].

Childhood-onset NCL is recessively inherited, and rare recessive and dominant adult-onset forms of NCL have also been described [2,6,7,8]. To date, 10 genetic loci are linked to NCL, and it is likely more are yet to be discovered [2,6]. The genes linked to NCL encode proteins primarily localized to either acidic organelles (late endosomes and lysosomes) or to the endoplasmic reticulum (ER). Several of the proteins are enzymes (PPT1, TPPI, cathepsin D), but the others are novel, mostly transmembrane proteins, with no known function (see recent reviews [2,6,9]).

Given the overlapping clinical symptoms and disease pathology in the different forms of NCL, it has been proposed that the NCL genes encode proteins that function together or at different points in a common pathway, which most likely involves lipid and protein trafficking pathways and/or ion homeostasis [2,9]. Consistent with this hypothesis, protein-protein interaction between several NCL-linked proteins has been implicated by studies in overexpression or pull-down assay systems [10,11], and cross-correction of growth defects in patient cells by other CLN-linked genes has been described [12]. However, clinical and pathological differences in the different NCL sub-types are also recognized, including distinctive ultrastructure of the storage material and differences in the age at onset and order of symptom onset [13,14,15].

The most common form of NCL, with juvenile onset (JNCL), is caused by CLN3 mutation [16]. The CLN3 gene encodes a novel multipass transmembrane protein (battenin or CLN3p) that primarily localizes to the late endosome and lysosome in most cell types. CLN3p is implicated in regulation of lysosomal pH [17,18], endocytosis [19,20,21], autophagy [22,23], cell growth and survival [24,25], palmitoyl desaturase activity [26], and lysosome-targeted protein trafficking [20,27]. However, the precise protein activity of CLN3p remains unknown.

CLN6 mutation causes a non-classical, hence ‘variant’, late-infantile NCL (vLINCL) [28,29]. The CLN6-encoded protein, linclin or CLN6p, encodes an ER-resident, multipass transmem-
brane protein. Though GLN6p function has not yet been well studied, it is also implicated in trafficking and regulating lysosomal function [30,31].

We previously established genetically precise murine and cell-based models for JNCL, Cln3Δex7/8 mice and CbCln6Δex7/8 cerebellar cells [20,32]. Here, we have created an analogous cellular model from Cb6ex8 spontaneous mutant mice, originally identified at The Jackson Laboratories [33], which harbor a mutation in the murine Cln6 gene that is also found in vLINCL patients [28,29]. With this set of genetic cell culture reagents, we have performed detailed, comparative phenotyping in order to determine the degree of overlap in abnormal cellular processes resulting from distinct NCL mutations.

Results

**CbCln3Δex7/8 and CbCln6Δnclf neuronal precursor cell lines**

We previously established CbCln3Δex7/8 neuronal precursor cell lines by conditionally immortalizing cerebellar granule neurons from postnatal wild-type, heterozygous or homozygous littersmate Cb3Δex7/8 mice [20]. These mice bear a ~1 kb genomic deletion in the endogenous murine Cln3 gene that is analogous to the most common ~1 kb genomic deletion in juvenile NCL patients. To permit a comparison of the effects of the vLINCL mutation, we have now created wild-type, heterozygous, and homozygous CbCln6Δnclf neuronal precursor cell lines from postnatal Cb6Δnclf mice [33], which bear a frameshift-producing, single base-pair insertion in the murine Cln6 gene, which is also found in human vLINCL patients [28,29]. The generation of the murine vLINCL cell panel is described in detail in Materials and Methods, along with the characterization demonstrating expression of the neural stem cell marker, nestin, confirming a neuronal lineage (Figure S1).

**Homozygous CbCln6Δnclf and CbCln3Δex7/8 cells accumulate ATP synthase, subunit c**

The pathological hallmark of NCL is an abnormal lysosomal accumulation of the pore-forming subunit c of the mitochondrial F0 ATP synthase complex. At sub-confluent density, a mitochondrial marker, anti-gpr75, which has revealed significant elongation of mitochondria in homozygous CbCln3Δex7/8 cells [20] (mean circularity index 0.81 ± 0.002), also revealed abnormally elongated mitochondria in homozygous CbCln6Δnclf cerebellar cells (mean circularity index 0.79 ± 0.003), compared to wild-type cells (CbCln3+/+ or CbCln6+/+; mean circularity index 0.85 ± 0.002) (Figure 1A). Similar to homozygous CbCln3Δex7/8 cells, the homozygous CbCln6Δnclf cerebellar cells also displayed a significant reduction (~30–40%, p ≤ 0.01) in total cellular ATP levels, relative to wild-type or heterozygous cells (Figure 1B), strongly suggesting altered mitochondrial morphology and function.

Homozygous CbCln3Δex7/8 cells, when aged at confluent cell density, accumulated subunit c-positive puncta [20]. Assessment of the wild-type and CbCln6Δnclf cerebellar cells revealed subunit c-positive puncta in aged homozygous mutant CbCln6Δnclf cerebellar cells, similar to that observed in the aged homozygous CbCln3Δex7/8 cells (Figure 2). Consistent with the abnormal accumulation occurring in a lysosomal compartment, little to no overlap of subunit c signal was observed with mitochondrial gpr75 in confluent aged homozygous CbCln3Δex7/8 cells and only limited overlap of the subunit c and gpr75 immunostain was observed in the confluent aged homozygous CbCln6Δnclf cerebellar cells (Figure 2A), while the subunit c immunostain in confluent aged wild-type cells exhibited moderate overlap with gpr75 immunostain (Figure 2A). Moreover, in the aged wild-type cerebellar cells, the subunit c immunostain did not overlap with Lamp 1 immunostain, whereas the strongly immunopositive subunit c puncta in both the homozygous CbCln3Δex7/8 and CbCln6Δnclf cerebellar cells aged at confluent density were Lamp 1-positive (Figure 2B), though the overlap was imperfect, particularly in the homozygous CbCln6Δnclf cerebellar cells. In both homozygous CbCln3Δex7/8 and CbCln6Δnclf cells aged at confluent density, the Lamp 1 immunostain also revealed enlarged or aggregated lysosomes, which were not often observed in the aged wild-type cells, consistent with lysosomal defects.

Thus, cerebellar cells homozygous for either a vLINCL mutation or the major JNCL mutation exhibited aberrant mitochondrial morphology and, when aged at confluent density, accumulated mitochondrial ATP synthase subunit c in enlarged, Lamp 1-positive vesicles, albeit with slightly different staining characteristics.

**Homozygous CbCln6Δnclf and CbCln3Δex7/8 cells display similar but distinct membrane abnormalities**

To determine whether similar accumulation of subunit c in Lamp 1-positive vesicles, might reflect similar or distinct membrane organelle perturbations, CbCln6Δex7/8/nclf and CbCln3Δex7/8/nclf cerebellar cells were assessed using a panel of organelle markers [20]. We found no obvious morphological differences in the cis- and trans-Golgi in either homozygous CbCln6Δex7/8 or CbCln3Δex7/8 cerebellar cells (data not shown). However, the ER marker protein, PDI, consistently displayed reduced staining intensity in homozygous CbCln6Δnclf cerebellar cells, compared to wild-type (CbCln6+/+) cells (Figure 3, top panels). Homozygous CbCln3Δex7/8/nclf cells did not display altered PDI immunostain (Figure 3). Interestingly, immunostain for another ER-associated protein, BiP [34], was not decreased in homozygous CbCln6Δnclf cerebellar cells and highlighted a morphologically intact ER network (Figure 3). Notably, PDI immunostain was also decreased in variant late-infantile NCL patient lymphoblast cells, compared to normal lymphoblasts (Figure 3, bottom panels), suggesting that the vLINCL mutation may specifically alter PDI epitope availability or distribution within the ER of diverse cell types. Total PDI levels were not obviously different in the homozygous CbCln6Δnclf cerebellar cells versus wild-type cells by immunoblot analysis (data not shown).

To determine whether subconfluent, non-stressed CbCln6Δnclf cerebellar neuronal precursor cells displayed abnormalities in the endosomal-lysosomal system, which is significantly disrupted in subconfluent homozygous CbCln3Δex7/8 cells [20], we assayed fluid phase endocytosis using a fluorescently labeled dextran uptake assay (dextran, Alexa Fluor® 488) and acidic organelles were probed using LysoTracker® Red. As expected, homozygous CbCln3Δex7/8 cells showed consistently reduced numbers of dextran-Alexa 488 labeled vesicles and LysoTracker® stained vesicles, along with a reduced perinuclear distribution of the labeled vesicles, compared to wild-type (CbCln3+/+) or heterozygous (CbCln3+/Δex7/8) cells (Figure 4). Homozygous CbCln6Δnclf cells also showed consistently reduced dextran-Alexa 488 labeled vesicles and LysoTracker® stained vesicles, compared to wild-type (CbCln6+/+) or heterozygous (CbCln6+/Δex7/8) cells. However, in contrast to what was observed in homozygous CbCln3Δex7/8 cells, the distribution of the labeled vesicles in homozygous CbCln6Δnclf cells was not obviously altered (Figure 4A). Notably, the expanded and/or aggregated lysosomal compartment, which was evident by Lamp 1 immunostain in the confluent density aged homozygous CbCln3Δex7/8 and CbCln6Δnclf cerebellar cells, as shown in Figure 2B, was not observed in the subconfluent, LysoTracker-stained homozygous CbCln3Δex7/8/nclf and CbCln6Δnclf cells here. Automated image analysis demonstrated that the numbers of labeled vesicles were more dramatically decreased (p < 0.001) in the homozygous CbCln3Δex7/8 cells (~65–75% reduced from wild-type
Distinct Responses to Cln3 and Cln6 Mutations

Homozygous CbCln3\textsuperscript{Δex7/8} and CbCln6\textsuperscript{nclf} cerebellar cells exhibit mostly distinct gene expression changes

The membrane organelle survey revealed similarities but also differences in the impact of the vLINCL and JNCL mutations that implied distinct underlying processes might be involved. To assess this idea at the molecular level, we performed unbiased global gene expression analyses on total RNA isolated from wild-type (CbCln3\textsuperscript{+/-} and CbCln6\textsuperscript{+/-}) and homozygous CbCln3\textsuperscript{Δex7/8} and CbCln6\textsuperscript{nclf} cerebellar cells (see Materials and Methods).

In a probe-level analysis, we identified 981 significantly changed probes in the homozygous CbCln3\textsuperscript{Δex7/8} cerebellar cells (p < 0.01, absolute fold-change > 1.5; Figure 5, Table S2). Among these, only a small number, 36 probes, were significantly changed in both homozygous CbCln3\textsuperscript{Δex7/8} and CbCln6\textsuperscript{nclf} cells, with the majority (28) discordant in the direction of change (Figure 5, Table S2). The reliability of the datasets was assessed by principal components analysis (PCA) (Figure S2) and real-time qRT-PCR of selected genes (Table S3).

Gene ontology analysis of the gene lists did not reveal obvious functional overlap among the genes with altered expression in both CbCln3\textsuperscript{Δex7/8} and CbCln6\textsuperscript{nclf} cells, suggesting the pathways most dramatically affected by the Cln3\textsuperscript{Δex7/8} and Cln6\textsuperscript{nclf} mutations were mostly different (Tables S3 and S4). PCA analysis of the CbCln3\textsuperscript{Δex7/8} significant probes in the CbCln6\textsuperscript{nclf} dataset, and vice versa, further supported this conclusion (Figure S3).

Notably, in our Affymetrix gene expression array analysis, Cbl3 and Cbl6 gene expression were not significantly altered by the vLINCL and JNCL mutations, respectively (Tables S1 and S2).

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**Figure 1. Mitochondrial abnormalities in homozygous CbCln6\textsuperscript{nclf} and CbCln3\textsuperscript{Δex7/8} cells.** A. Representative micrographs of wild-type, CbCln3\textsuperscript{Δex7/8} and CbCln6\textsuperscript{nclf} cells immunostained with antibody recognizing the mitochondrial matrix protein, grp75, are shown. Note the elongated appearance of mitochondria in homozygous CbCln3\textsuperscript{Δex7/8} and CbCln6\textsuperscript{nclf} cells, compared to wild-type cells in the zoomed insets. A lower magnification of a representative field of cells is also shown to demonstrate the subconfluent culture conditions. The altered mitochondrial morphology was also quantified by automated image analysis, showing a significantly reduced circularity index of the labeled mitochondria in the mutant cells, compared to wild-type cells (see Results). Wild-type panel is representative of both CbCln3\textsuperscript{+/-} and CbCln6\textsuperscript{+/-} cell lines. Heterozygous cell lines were indistinguishable from wild-type (not shown). All images were captured at 40× magnification and were taken on the same day with identical settings. B. The bar graph depicts relative total cellular ATP levels in wild-type, heterozygous, or homozygous CbCln3\textsuperscript{Δex7/8} and CbCln6\textsuperscript{nclf} cells, determined using the CellTiter-GLO\textsuperscript{®} Luminescent Cell assay. Relative luciferase units were normalized to the wild-type cell lines and were pooled from 2–3 independent assays per cell line, each tested in 3–10 wells per assay. For reference, absolute RLUs for CbCln3\textsuperscript{+/-} and CbCln6\textsuperscript{+/-} cell lines were 1995830±27506 and 626172±151671, respectively. * p<0.01 in a Student’s t-test; n.s. = not significant. doi:10.1371/journal.pone.0017118.g001
Distinct Responses to *Cln3* and *Cln6* Mutations

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However, subsequent qRT-PCR analysis revealed reduced expression of mutant Cln3 mRNA in CbCln3\textsubscript{ex7/8} cerebellar cells (5-fold downregulated), consistent with previous reports [20,32], and reduced expression of mutant Cln6 mRNA in CbCln6\textsubscript{ex7/8} cerebellar cells (6-fold downregulated). We also detected a 1.6-fold upregulation of Cln6 mRNA in homozygous CbCln3\textsubscript{ex7/8} cerebellar cells and an ~2-fold downregulation of Cln3 mRNA in homozygous CbCln6\textsubscript{ex7/8} cerebellar cells by qRT-PCR (Table S3). This apparent discrepancy between the Affymetrix array and qRT-PCR data at the level of the Cln3 and Cln6 genes could be due to the gene regions being probed in the two different formats, which differed (e.g. reflecting detection of different splice variants), or the different sensitivities of the two methodologies. Among the other NCL loci present on the Affymetrix array (Ppt1, Tpp1, Cln3, Mjfd8, Csd1, Cln6), only Cln3, which is mutated in another form of non-classical ‘variant’ LINCL [33], displayed a significant change (1.8-fold upregulated), and only in the homozygous CbCln6\textsubscript{ex7/8} cerebellar cells (Table S1).

Though our probe-level analysis suggested only limited overlap in the genes affected by the Cln3\textsubscript{ex7/8} and Cln6\textsubscript{ex7/8} mutation, we also sought to examine the data at a pathways level. Gene Set Enrichment Analysis (GSEA) has proven to be a sensitive method in the genes affected by the Cln3\textsubscript{ex7/8} and Cln6\textsubscript{ex7/8} mutation, we also sought to examine the data at a pathways level. Gene Set Enrichment Analysis (GSEA) has proven to be a sensitive method of identifying pathways relevant to human disease from gene expression datasets (e.g. [36,37,38,39]). GSEA is predicated on the idea that genes with related biological functions are often coordinately regulated, and that even small gene expression changes, which escape identification through traditional single gene analysis approaches, are biologically meaningful if in the context of related gene changes [36,40]. We further analyzed our unbiased gene expression data in the sigPathway program, which is an extension of the original GSEA platform [41], using our entire CbCln3\textsubscript{ex7/8} and CbCln6\textsubscript{ex7/8} datasets.

The significantly altered gene sets (false discovery rate<0.01) in homozygous CbCln3\textsubscript{ex7/8} and CbCln6\textsubscript{ex7/8} are shown in Tables S6 and S7, respectively. Overall, more changed gene sets (597 out of 2077 total screened) were identified in the homozygous CbCln6\textsubscript{ex7/8} cerebellar cells than in the homozygous CbCln3\textsubscript{ex7/8} cells (222 out of 2077 total screened). Consistent with our probe-level analysis results, inspection of the top 20 ranked (NTK Rank) gene sets, which reflected those gene sets determined by the sigPathway software to have the most changes among all gene sets screened (see Materials and Methods and [41]), revealed limited overlap in the most dramatically changed pathways in the CbCln3\textsubscript{ex7/8} and CbCln6\textsubscript{ex7/8} cells (Tables 2 and 3). Only the gene set for the oxidative phosphorylation KEGG pathway scored within the top 20 ranks for both the homozygous CbCln3\textsubscript{ex7/8} (NTK Rank = 1) and CbCln6\textsubscript{ex7/8} (NTK Rank = 17) gene set lists. However, supporting the notion that there is convergence in the biological processes affected by the NCL and vLINCL mutations, further inspection of the complete lists of significantly changed gene sets in the two different conditions revealed common pathways (Table S8).

Figure 2. Subunit c deposits co-localize with Lamp 1 in homozygous Cb Cln3\textsubscript{ex7/8} and Cb Cln6\textsubscript{ex7/8} cells. A. Representative micrographs of confluency aged wild-type, CbCln3\textsubscript{ex7/8} and CbCln6\textsubscript{ex7/8} cells, co-immunostained with antibodies recognizing subunit c (green) and the mitochondrial marker, grp75 (red). Note the large accumulations of subunit c immunostain (white arrows) in both CbCln3\textsubscript{ex7/8} and CbCln6\textsubscript{ex7/8} cells, but which are not common in the wild-type cells. Moderate overlap of the subunit c and grp75 immunostains is observed in wild-type cells (yellow in overlay), but little to no overlap is seen in CbCln3\textsubscript{ex7/8} and CbCln6\textsubscript{ex7/8} cells. Also, again note the elongated mitochondrial morphology revealed by the grp75 immunostain in CbCln3\textsubscript{ex7/8} and CbCln6\textsubscript{ex7/8} cells, compared to wild-type cells. B. Representative micrographs of confluency aged wild-type, CbCln3\textsubscript{ex7/8} and CbCln6\textsubscript{ex7/8} cells, co-immunostained with antibodies recognizing subunit c (green) and Lamp 1 (red). Limited overlap of subunit c and Lamp 1 immunostains is observed in wild-type cells (yellow in overlay), but Lamp 1 strongly, though not perfectly, overlaps with the accumulated subunit c in CbCln3\textsubscript{ex7/8} and CbCln6\textsubscript{ex7/8} cells. Note that in confluency aged cultures, the Lamp 1-labeled compartment appears expanded and/or aggregated in the mutant cells, which was not observed under sub-confluent culture conditions (not shown). A,B. Insets provide a zoomed view of the degree of immunostain overlap (yellow). Blue = DAPI stain. Images were captured with a 40X objective and, for like stains, were taken on the same day with identical settings.

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Figure 3. Altered PDI immunostain in homozygous Cb Cln6\textsubscript{ex7/8} cells. Representative micrographs of CbCln6 (CbCln6\textsubscript{+/+} and CbCln6\textsubscript{ex7/8}) and CbCln3 cells (CbCln3\textsubscript{ex7/8/Lex7/8}) immuno-stained for the ER marker protein PDI (red) are shown. Note the decreased PDI signal in CbCln6\textsubscript{ex7/8} compared to wild-type (CbCln6\textsubscript{+/+}) and CbCln3\textsubscript{ex7/8/Lex7/8} cells. PDI immunostain of normal and variant late-infantile (vLINCL) lymphoblast cells is also shown. PDI immunostain was comparable across all of the cerebellar cell lines, but is only shown for the CbCln6\textsubscript{ex7/8} and CbCln6\textsubscript{ex7/8} cells. PDI was not assessed in lymphoblast cells. All images were taken at 40× magnification, and for like stains, on the same day, with identical instrument settings. DAPI nuclear counter stain is shown in blue.

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genetic cell models revealed a significant negative correlation between them, reflecting common pathways that were changed in the opposite direction in Cb\textsuperscript{Cln3\textsuperscript{Dex7/8}} and Cb\textsuperscript{Cln6\textsuperscript{nclf}} cells (Figure S4).

Intriguingly, consistent with biological data supporting a lysosomal localization and function for the Cln3-encoded protein, lysosomal function-related (GO:0005773, GO:0000323, GO:0005764) gene sets were among the top 20 in the homozygous Cb\textsuperscript{Cln3\textsuperscript{Dex7/8}} list, but not in the homozygous Cb\textsuperscript{Cln6\textsuperscript{nclf}} gene set list (Table 2). Conversely, consistent with the ER localization of the Cln6-encoded protein, ER-function (GO:0005783), protein biosynthesis (GO:0009059, GO:0006412) and protein transport-related (GO:0015031, GO:0045184, GO:0008104) gene sets were among the top 20 in the homozygous Cb\textsuperscript{Cln6\textsuperscript{nclf}} list (Table 3), but were ranked substantially lower in the homozygous Cb\textsuperscript{Cln3\textsuperscript{Dex7/8}} gene set list.

Thus, our unbiased global gene expression analysis of Cb\textsuperscript{Cln3\textsuperscript{Dex7/8}} and Cb\textsuperscript{Cln6\textsuperscript{nclf}} cerebellar cells lends further support for the hypothesis that Cln3 and Cln6 mutations initiate disease via distinct molecular and cell biological processes that converge on a common pathway. Moreover, a number of potentially relevant pathways have been identified, including oxidative phosphorylation, that merit further investigation into their role in the NCL disease process.

**Discussion**

The NCLs, while genetically heterogeneous, share a common pathological feature, the accumulation and storage of ceroid lipofuscin, which appears to mostly be comprised of dolichol lipids and the hydrophobic protein, subunit c of mitochondrial ATP synthase
was specifically decreased in homozygous Cb
8 (22%) were concordant in their direction of change in the
analyses in sub-confluent cultured homozygous Cb
synthase subunit c in Lamp-1 positive compartments by aging in
cellular bodies for subsequent lysosomal delivery and degradation
which was consistent with biological data in this study and others
reviewed in [9]). Moreover, relatively specific changes in gene sets
related to metabolic processes, such as fatty acid and amino acid
metabolism, and in ion transport in CbCln5\textsuperscript{ex7/8} cerebellar cells,
which were not evidently dramatically changed in homozygous
CbCln6\textsuperscript{nclf} cells, suggests focusing studies aimed at Cln3p
function on these processes.

It is noteworthy that homozygous mutation of Cln3 or Cln6 in
our cerebellar cell models of JNCL and vLINCL did not
dramatically alter expression at the other NCL loci, at least by
Affymetrix array analysis, supporting distinct primary functions for
the differing NCL related proteins, consistent with the observation
that NCL patients have varied storage material ultrastructure, age-at-onset,
and order of symptom onset that typically correlates with the
genetic etiology [13,14]. However, subtler gene expression
changes in the other NCL loci were detected, in particular, in
follow-up analysis by the more sensitive method of qRT-PCR,
supporting the hypothesis that the NCL gene functions converge
on a common pathway.

Biological areas that merit further investigation, because they
were commonly altered in cerebellar cells in response to CLN6p
and Cln3p dysfunction, are aspects of vesicle/membrane
trafficking, protein transport, and altered metabolism. For
example, future study of alterations in Sna\textsubscript{p95} and Fas1, which were
two of the validated gene changes in homozygous CbCln6\textsuperscript{nclf} and
Cln3\textsuperscript{mclf} cells (Table 1, Table S3), could lead to an improved
understanding of the altered trafficking in these two forms of NCL.
Sna\textsubscript{p95} (a.k.a. ESCRT-II complex subunit VPS22), which is involved
in sorting endocytosed and ubiquitinated proteins into multivesicular
bodies for subsequent lysosomal delivery and degradation
[43], was significantly upregulated in homozygous CbCln3\textsuperscript{ex7/8} cells,
and significantly downregulated in homozygous CbCln6\textsuperscript{nclf} cells
(Tables 1 and S3). Fas1 (fascin-1), an actin-bundling protein
highly expressed in brain (reviewed in [44]), was downregulated in
both homozygous CbCln3\textsuperscript{ex7/8} and homozygous CbCln6\textsuperscript{nclf} cells
(Tables 1 and S3). The importance of the actin cytoskeleton in
membrane trafficking is well documented (reviewed in [45]), and a
role for CLN3p in membrane-cytoskeletal interactions has already
been proposed [46,47].

It is reasonable to postulate that the commonly altered
molecular genes and pathways in homozygous CbCln6\textsuperscript{nclf} and
Cln3\textsuperscript{mclf} cells may culminate in the abnormal accumulation
of mitochondrial ATP synthase, subunit c protein that becomes manifest when homozygous CbCln6\textsuperscript{nclf} and CbCln3\textsuperscript{ex7/8} cells are
stressed by aging at confluent cell density. Our co-staining analyses
of the formed deposits was suggestive that in both homozygous
CbCln6\textsuperscript{nclf} and CbCln3\textsuperscript{ex7/8} cells, the accumulation of the subunit
c protein occurs within acidic organelles rather than in the
mitochondrion itself, consistent with a defect in the autophago-
The somal-lysosomal pathway in these forms of NCL [22,48,49]. The subunit c-positive foci that formed in the homozygous Cb
Cln6
nclf and Cb
Cln3
D
ex7/8 cells were morphologically heterogeneous, so, while dramatic differences were not apparent, it would also be
worthwhile to determine whether the vLINCL and JNCL mutations may give rise to subtle differences in the relative
kinetics and features of subunit c turnover.

In summary, our data are consistent with distinct functions for the CLN3p and CLN6p proteins, that likely primarily work in
distinct processes regulating shared downstream biological path-
ways that are connected to the lysosome, the mitochondrion and
subunit c turnover, in a manner that is essential for proper
neuronal cell survival. Our findings, therefore, support the
interconnected goals of developing therapeutics based on a full
understanding of CLN3p and CLN6p functions, which may prove
to be disease-specific, as well as therapeutics aimed at circum-
venting or preventing the changes proximal to the overall
dysfunction of cellular energetics and membrane function that
result in storage of ceroid lipofuscin and neuronal cell death.

Materials and Methods
Ethics Statement
All mouse protocols were in accordance with the National
Institutes of Health Guide for the Care and Use of Laboratory
Animals and were reviewed and approved by the Massachusetts

| Probe       | Gene Symbol | Cb
Cln6
nclf Cells Fold Change | Cb
Cln3
D
ex7/8
Cells Fold Change |
<table>
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<td>Hars</td>
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<td>−1.6</td>
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<td>2.2</td>
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<td>Bmp2k</td>
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<td>−7.0</td>
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<td>Usp14</td>
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<td>−1.6</td>
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<tr>
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<td>Usp14</td>
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<td>−1.5</td>
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<td>1451818_at</td>
<td>Mib1</td>
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<td>−1.6</td>
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<td>Wdr36</td>
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<td>1457632_s_at</td>
<td>Meis2</td>
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*Validated by qRT-PCR.
doi:10.1371/journal.pone.0017118.t001
Table 2. 20 highest-ranked gene sets significantly altered in CbCln3\textsuperscript{aex7/B\textae7/B} cerebellar cells.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>CbCln3\textsuperscript{aex7/B\textae7/B} Cells Dataset</th>
<th>CbCln3\textsuperscript{aex7/B\textae7/B} Cells Dataset</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NTK Rank\textsuperscript{a}</td>
<td>NTK Stat</td>
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<tr>
<td>KEGG:Oxidative phosphorylation</td>
<td>1.0</td>
<td>12.3</td>
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<tr>
<td>GO:0015078 hydrogen ion transporter activity</td>
<td>2.0</td>
<td>11.4</td>
</tr>
<tr>
<td>GO:0015077 monovalent inorganic cation transporter activity</td>
<td>3.3</td>
<td>11.0</td>
</tr>
<tr>
<td>KEGG:Fatty acid metabolism</td>
<td>4.0</td>
<td>10.9</td>
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<tr>
<td>GO:0003954; GO:0050136; GO:0008137; GO:0015081 NADH dehydrogenase activity</td>
<td>5.3</td>
<td>10.9</td>
</tr>
<tr>
<td>KEGG:Porphyrin and chlorophyll metabolism</td>
<td>5.3</td>
<td>10.8</td>
</tr>
<tr>
<td>GO:0016655 oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor</td>
<td>7.0</td>
<td>10.6</td>
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<tr>
<td>KEGG:Valine, leucine and isoleucine degradation</td>
<td>8.0</td>
<td>10.1</td>
</tr>
<tr>
<td>GO:0015399 primary active transporter activity</td>
<td>9.0</td>
<td>10.1</td>
</tr>
<tr>
<td>GO:0016667 oxidoreductase activity, acting on sulfur group of donors</td>
<td>10.3</td>
<td>–9.7</td>
</tr>
<tr>
<td>GO:0015036 disulfide oxidoreductase activity</td>
<td>10.7</td>
<td>–9.6</td>
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<tr>
<td>KEGG:Ribosome</td>
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<td>8.9</td>
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<tr>
<td>GO:0006119 oxidative phosphorylation</td>
<td>13.0</td>
<td>8.8</td>
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<tr>
<td>GO:0046873 metal ion transporter activity</td>
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<td>8.7</td>
</tr>
<tr>
<td>GO:0042773 ATP synthesis coupled electron transport</td>
<td>15.0</td>
<td>8.4</td>
</tr>
<tr>
<td>GO:0005773 vacuole</td>
<td>16.0</td>
<td>8.0</td>
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<tr>
<td>GO:0003323 lytic vacuole GO:0005764; lysosome</td>
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<td>7.9</td>
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<tr>
<td>GO:0016491 oxidoreductase activity</td>
<td>18.0</td>
<td>7.8</td>
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<tr>
<td>GO:0019370 leucotriene biosynthesis</td>
<td>18.3</td>
<td>–7.8</td>
</tr>
<tr>
<td>GO:0006691 leucotriene metabolism</td>
<td>21.3</td>
<td>–7.5</td>
</tr>
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</table>

\textsuperscript{a}The top 20 NTK ranked gene set pathways in the CbCln3\textsuperscript{aex7/B\textae7/B} dataset are shown (NTK Rank), ranked by the sigPathway program according to the average NTK statistics (NTK Stat). NTK statistics reflect significant changes within a given gene set (the higher the absolute value of the statistic, the greater its significance), and the direction of change (indicated by positive or negative statistics). For comparison, the CbCln3\textsuperscript{aex7/B\textae7/B} dataset NTK Rank and NTK Stat values are also shown. Replicated gene sets were grouped together. For example, four different gene sets, categorized ‘NADH dehydrogenase activity’, gave identical statistics, so they are grouped together into one row of the table, with the individual gene set identifiers listed in the ‘Pathway’ column.

doi:10.1371/journal.pone.0017118.t002

General Hospital (MGH) Subcommittee of Research Animal Care (SRAC), which serves as the Institutional Animal Care and Use Committee (IACUC) for MGH (Protocol #2005N000289). The use of anonymous, de-identified human patient lymphoblast cell lines for our NCL research was reviewed by the Partners Institutional Review Board and deemed exempt (2010P-001489).

Animals

\textit{Cln6}\textsuperscript{-/-} spontaneous mutant mice were originally purchased from The Jackson Laboratory, and were subsequently maintained as a breeding colony on the C57Bl6/J background, at Massachusetts General Hospital. Genotypes were determined by the mouse nclf exon 4 insertion assay, from tail biopsy DNA, as previously described [20]. \textit{Cln3}Dex7/8\textsuperscript{-/-} mutant mice were previously described [20].

Antibodies and Cell Staining Reagents

Commercial antibodies used were anti-nestin (Rat 401, Developmental Studies Hybridoma Bank, maintained by The University of Iowa, Department of Biological Sciences), anti-GFAP (cat#Z0334, Dako Corporation), anti-PDI (H-160, cat#sc-20132, Santa Cruz Biotechnology), anti-GM130 (cat#G65120, BD Transduction Laboratories), anti-tubulin (Sigma), anti-BiP (Abcam), anti-EEA1 (C-15, cat#sc-64114, Santa Cruz Biotechnology), anti-Rab7 (C-19, cat#sc-6563, Santa Cruz Biotechnology), anti-Lamp1 (D4B, cat#sc-19992, Santa Cruz Biotechnology), anti-Grp75 (cat#S8-P325, Stressgen). All fluorescent secondary antibodies used were obtained from Molecular Probes/Invitrogen and were used at a 1:200 dilution. Cell staining reagents used were LysoTracker\textsuperscript{®} Red DND-99 (500 nM; cat#D-7528, Invitrogen) and 10,000 MW dextran-Alexa Fluor 488 (1 mg/ml; cat#D-22910, Invitrogen).

A new subunit c antibody was generated to replace the diminishing stocks of the previously described subunit c antibody generated by Dr. Kominami [20,32,50]. A peptide corresponding to amino acids 62–73 of the subunit c protein (the N-terminus of the mature protein) was synthesized with an additional C-terminal cysteine residue for carrier protein conjugation by the MGH Peptide Core Facility. Keyhole limpet hemocyanin (KLH)-conjugated peptide was used for rabbit immunization, and antisera were collected and affinity purified according to standard procedures (Quality Controlled Biochemicals). The new anti-subunit c antibody was tested alongside the previously described subunit c antibody [20,32,50] and was found to perform similarly in all immunostaining and immunoblot assays (data not shown). All subunit c data contained herein were obtained with the new subunit c antibody, used at a 1:200–1:500 dilution.

Generation and maintenance of CbCln6\textsuperscript{nef} and CbCln3\textsuperscript{aex7/B\textae7/B} cerebellar neuronal precursor cell lines

CbCln6\textsuperscript{nef} cerebellar neuronal precursor cell lines were established in the same manner as CbCln3\textsuperscript{aex7/B\textae7/B} cerebellar neuronal precursor cells, which were previously described [20].
Briefly, postnatal day 4 (P4) cerebella were dissected from wild-type, heterozygous, and homozygous Cln6<sup>ex7/8</sup> (or Cln6<sup>Δex3</sup>/Δex3) littermate mice, and primary cultures were established that were enriched for cerebellar granule neurons, according to previously established procedures [51]. Cultures were then transduced with a retroviral vector containing the tsA58/U19 temperature-sensitive p53, which allowed for selection of clones for growth in 400 μg/ml G418, and multiple clonal cell lines were isolated for each genotype that expressed the targeted protein (GFAP) expression by immunofluorescence and, in some cases, also confirmed by Western blot, to verify a neuronal lineage. 

For maintenance, CbCln6<sup>ex7/8ex7/8</sup> cells, consistent with previous reports [31,53] (data not shown). 

For immunostaining, CbCln6<sup>ex7/8ex7/8</sup> and CbCln6<sup>Δex3/Δex3</sup> cerebellar neuronal precursor cells were grown between 30 and 90% confluency on plastic tissue culture dishes in ‘Cbc’ media (Dulbecco’s Modified Eagle Medium [DMEM; Gibco BRL], 10% heat-inactivated fetal bovine serum [FBS; Sigma #F1206C], 24 mM KCl, penicillin/streptomycin/glutamine [1X, Gibco BRL], and G418 [200 μg/ml] to maintain selection), in a water-jacketed, humidified incubator maintained at 33°C, 5% CO<sub>2</sub> atmosphere. Passage number was recorded and cells were used for experiments up to ~passage 15, without apparent impact on phenotypes. All phenotypes were tested in 2–3 independent cell lines per genotype to ensure they represented a genotype-phenotype relationship, rather than just inter-subclone variability. Except where noted, data shown were from representative cell lines. 

**Lymphoblast cell culture**

Patient lymphoblast cell lines were previously collected [28] and were grown as previously described [54]. The Cln6 patient lymphoblast line was from a male with Costa Rican ancestry harboring a homozygous mutation in exon 3 (c.214G>T, p.Glu72X) that predicts a prematurely truncated protein product [28]. 

**Immunostaining**

For immunostaining of cultured cells and subsequent confocal microscopy, cells were seeded onto 18 mm diameter glass No. 1 coverslips (Fisher Scientific), inside a 12-well tissue culture petri dish, at a density of 104 cells/well, and cells were grown overnight in Cbc media, at 33°C, 5% CO<sub>2</sub>. The following day, coverslips were fixed inside the well with either ice-cold 4% formaldehyde/PBS, pH 7.4, incubated for 20’ at room temperature, or with ice-cold methanol:acetone (1:1), for 10’ at −20°C, following by air drying, depending on the antibody. Following

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**Table 3. 20 highest-ranked gene sets significantly altered in CbCln6<sup>Δex3/Δex3</sup> cerebellar cells.**

<table>
<thead>
<tr>
<th>Pathway</th>
<th>CbCln6&lt;sup&gt;Δex3/Δex3&lt;/sup&gt; Cells Dataset</th>
<th>CbCln6&lt;sup&gt;Δex7/8ex7/8&lt;/sup&gt; Cells Dataset</th>
</tr>
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<tbody>
<tr>
<td>GO:0001584 rhodopsin-like receptor activity</td>
<td>1.0</td>
<td>12.5</td>
</tr>
<tr>
<td>GO:0004930 G-protein coupled receptor activity</td>
<td>2.0</td>
<td>12.3</td>
</tr>
<tr>
<td>GO:0005783 endoplasmic reticulum</td>
<td>3.0</td>
<td>−12.1</td>
</tr>
<tr>
<td>GO:0000959 macromolecule biosynthesis</td>
<td>4.0</td>
<td>−11.8</td>
</tr>
<tr>
<td>GO:0006412 protein biosynthesis</td>
<td>5.0</td>
<td>−11.6</td>
</tr>
<tr>
<td>GO:0004888 transmembrane receptor activity</td>
<td>6.0</td>
<td>11.5</td>
</tr>
<tr>
<td>GO:0015031 protein transport</td>
<td>7.3</td>
<td>−11.1</td>
</tr>
<tr>
<td>KEGG: Neuroactive_ligand-receptor_interaction</td>
<td>8.0</td>
<td>11.1</td>
</tr>
<tr>
<td>GO:0045186 establishment of protein localization</td>
<td>9.3</td>
<td>−11.0</td>
</tr>
<tr>
<td>GO:0015268 alpha-type channel activity</td>
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<td>10.9</td>
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<tr>
<td>GO:0008104 protein localization</td>
<td>11.0</td>
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<tr>
<td>GO:0031090 organelle membrane</td>
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<tr>
<td>GO:0015267 channel or pore class transporter activity</td>
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<td>10.6</td>
</tr>
<tr>
<td>GO:0007186 G-protein coupled receptor protein signaling pathway</td>
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<tr>
<td>GO:0005216 ion channel activity</td>
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<tr>
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<td>−10.3</td>
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<tr>
<td>KEGG: Oxidative phosphorylation</td>
<td>17.3</td>
<td>−10.2</td>
</tr>
<tr>
<td>GO:0003735 structural constituent of ribosome</td>
<td>17.7</td>
<td>−10.2</td>
</tr>
<tr>
<td>GO:0030529 ribonucleoprotein complex</td>
<td>19.0</td>
<td>−9.8</td>
</tr>
<tr>
<td>GO:0042165 neurotransmitter binding</td>
<td>20.0</td>
<td>9.6</td>
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</table>

*The top 20 NTk ranked gene set pathways in the CbCln6<sup>Δex3/Δex3</sup> dataset are shown (NTK Rank), ranked by the sigPathway program according to the average NTK statistics (NTK Stat). NTK statistics reflect how significant changes were within a given gene set (the higher the absolute value of the statistic, the greater its significance), and the direction of change (indicated by positive or negative statistics). For comparison, the CbCln6<sup>Δex7/8/Δex7/8</sup> NTK Rank and NTK Stat values are also shown. doi:10.1371/journal.pone.0017118.t003
fixation, coverslips were rinsed with PBS, removed from the tissue culture dish, and processed for immunostaining, as previously described [20]. For buffer incubations, coverslips, set atop parafilm inside a large Petri dish, were overlaid with 100–300 µl of the appropriate solution, and aspiration from the coverslip edge was used to remove previous buffers. Following immunostaining, coverslips were mounted onto slides with ProLong® Gold antifade reagent with or without DAPI (Invitrogen), according to the manufacturer’s recommendations. Nail polish-sealed coverslips were imaged on a Leica SP5 AOBS scanning laser confocal microscope (Leica Microsystems). Like-stained wild-type and homozygous mutant samples were mounted on the same microscope slide and were imaged in the same session, with identical settings.

**LysoTracker® and Endocytosis Assay**

LysoTracker® staining and 10,000 molecular weight dextran-Alexa Fluor® 488 endocytic uptake was as previously described [32], but was adapted to a 96-well, high-content imaging format. Cerebellar cells were seeded into clear-bottomed, 96-well Costar® tissue culture plates (Corning Inc.) at a density of 5000 cells/well (100 µl volume). Following overnight incubation at 33 °C, in a 5% CO2 humidified tissue culture incubator, the media was aspirated and exchanged for pre-warmed, fresh media containing 500 nM LysoTracker® DND-99 and 1 mg/ml dextran-Alexa Fluor® 488, using a multispipette. Plates were immediately placed back in the tissue culture incubator. Following 30’ incubation, plates were fixed with ice-cold 4% formaldehyde in PBS, pH 7.4 on ice, for 20’. Wells were then rinsed with PBS, pH 7.4 five times, 10’ each. Following aspiration of the final PBS rinse, nuclei were counterstained with Hoechst dye for 5 minutes and rinsed twice. Following aspiration of the final PBS rinse, nuclei were

**Quantification of mitochondrial shape**

Images of grp75 immunostained cells were collected on the same day with the same settings, and were then analyzed to quantify mitochondrial shape using ImageJ software (v1.44k for Macintosh; http://rsweb.nih.gov/ij/). Images were first thresholded in ImageJ, then the ‘analyze particles’ function was used to segment and measure the mitochondrial circularity. A circularity index of ‘1’ indicates a perfect circle. At least 3 independent, random fields per coverslip, representing a total of ~100–150 cells per line, were analyzed.

**Gene expression analysis**

Gene expression experiments were carried out through the NIH Neuroscience Microarray Consortium, within the UCLA Center. Total RNA from three different cell lines per genotype was isolated at MGH using TRIzol reagent (Invitrogen), according to the manufacturer’s recommendations. Prepared RNA samples were submitted to the UCLA DNA Microarray Facility (microarray.genetics.ucla.edu) for cDNA preparation and hybridization. All RNA samples passed quality checks on the Nanodrop (Thermo Scientific) and Agilent Bioanalyzer (Agilent Technologies). cDNA was prepared and hybridized to MOE 430 2.0 Affymetrix GeneChip® oligonucleotide microarrays, according to standard protocols at the UCLA Facility. Microarray data were corrected for backgrounds and normalized using gcRMA (R, 2.6.2; Biobase, 1.16.3; gcrma, 2.10.0).

The microarray data are MIAME compliant, and the raw data have been deposited in NCBI’s Gene Expression Omnibus [55] and are accessible through GEO Series accession number GSE24368 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE24368).

Real-time qRT-PCR was performed to validate selected genes in the homozygous CbCln3+/− and CbCln3+/− cell lines, according to standard procedures, using Gapdh and/or β-actin (A43) as unchanged housekeeping gene controls (see Table S8 for specific primer information). From the same original total RNAs used for the array experiments, cDNA was generated from 1 µg RNA using SuperScript II reverse transcriptase (Invitrogen). Real-time PCR was performed using SYBR Green (Roche) according to the manufacturer’s instructions and analyzed on a LightCycler 400 instrument (Roche) using the following thermocycling conditions: an initial denaturation step of 95 °C for 5 minutes followed by 45 cycles of 95 °C for 10 seconds, 56 °C for 10 seconds, and 72 °C for 10 seconds.

The DAVID Bioinformatics Resource 6.7 [56,57] was used for further gene ontology analysis of significant probes.

For identification of significantly enriched pathways, we used sigPathway (1.6.0) (http://biociconductor.org/packages/release/bio/html/sigPathway.html) [41]. Following analysis using sigPathway software, ‘NEk statistics’ and ‘NTk statistics’ were output as measures of how significant changes were for a given gene set (the higher the absolute value of the statistic, the greater its significance), and the direction of change (indicated by positive or negative statistics). ‘NEk statistics’ represented enrichment statistics based on phenotype permutations, and ‘NTk statistics’ represented gene set permutation-based test results (60,000 permutations). A false discovery rate cut-off of less than 0.01 was applied for both NTk and NEk statistics, given a limited number of unique permutations to construct a null distribution of test statistics. Therefore, in the summary tables presented in Tables 2 and 3, only the NTk values are shown. Both NTk and NEk values are shown in the supporting information tables (Tables S6 and S7).

**Subunit c accumulation assay**

The subunit c accumulation assay was modified from that which has been previously described [20]. Cells were seeded onto 100 mm petri dishes at a density of 2×105 cells/plate and subsequently incubated at 33 °C, 5% CO2 for between seven and ten days. Separate plates for each cell line were maintained at sub-confluent density, as described above, for use as un-aged controls, which do not show significant subunit c accumulations in this assay.

For immunostaining, confluency aged or un-aged control cells were trypsinized and replated onto 18 mm No. 1 glass coverslips inside a 12-well tissue culture plate at a density of 8×104 cells/well. Replated cells were then incubated overnight at 33 °C, 5% CO2, 5%
CO₂, prior to fixation (methanol:acetone, 1:1) and immunostaining, which were carried out as described above.

**ATP assay**

Un- treated control and mutant cells were trypsinized and replated into clear-bottomed, 96-well Costar® tissue culture plates (Corning Inc.) at a density of 10, 000 cells/well (100 μl volume). Total cellular ATP levels were assayed using the CellTiter-GLO® Luminescent Cell Viability kit (Promega), according to the manufacturer’s recommendations and as previously described [20]. Experiments conducted on the set of CbCln3 cell lines (i.e. wild-type, heterozygous, and homozygous lines) were performed at the same time, and those on CbCln6 cell lines were performed at the same time, but independently from the CbCln3 cell lines. To enable comparison across experiments, absolute RLUs were normalized to the respective wild-type numbers.

**Supporting Information**

Figure S1 Marker immunostaining of CbCln6 wild-type cerebellar neuronal precursor cells. Representative micrographs of nestin- (green) and GFAP-(red) immunostained wild-type (CbCln6+/+), heterozygous (CbCln6+/−), and homozygous (CbCln6−/−) neuronal precursor cell lines are shown. Selected clones were further confirmed as positive or negative for the markers by immunoblot analysis (not shown). 20× magnification. (TIF)

Figure S2 Quality control of the CbCln3 ex7/+ and CbCln6 ex7/+ cell gene expression datasets using principal components analysis (PCA). PCA plots for CbCln6 wild-type cells (top row, red circles) and CbCln3 ex7/+ cells (bottom row, blue circles) are shown. In all plots, closed circles represent data from mutant cells and open circles represent data from wild-type cells. As expected, PCA plots for the entire genome-normalized datasets (‘Total Probes’) show good separation by genotype, but also some variation among biological replicates, which most likely arose from the original derivation of the cell lines, which were from different mouse pups of the same genotype. The use of biological replicates from independent animals for our gene expression study was desirable in order to achieve our goal of capturing the gene expression variation that was a consequence of the genetic mutation. To further explore the variation in our datasets, we performed additional PCA analyses on the most variable probes (‘Top 1000 Variable Probes’), the most variable significant probes (‘Significant Probes’), and the most variable non-significant probes (‘Non-significant Probes’). The PCA plots for the ‘Top 1000 Variable Probes’ were highly similar to the ‘Total Probes’ PCA plots, demonstrating that restricting our analysis to a smaller set of probes did not dramatically alter the variation structure among the plots, demonstrating that restricting our analysis to a smaller set of probes did not dramatically alter the variation structure among the plots, demonstrating that restricting our analysis to a smaller set of probes did not dramatically alter the variation structure among the plots, demonstrating that restricting our analysis to a smaller set of probes did not dramatically alter the variation structure among the plots, demonstrating that restricting our analysis to a smaller set of probes did not dramatically alter the variation structure among the plots, demonstrating that restricting our analysis to a smaller set of probes did not dramatically alter the variation structure among the plots, demonstrating that restricting our analysis to a smaller set of 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gene expression was not determined for the CbCln3 set of cells because this gene change was only identified in the CbCln6 dataset. (XLS)

Table S4 DAVID gene ontology analysis of significant CbCln3^ex7/8^ probes. The significantly enriched (p<0.01) gene ontology terms (GOTERM/Term) from the Biological Process and Cellular Component categories are shown, determined through analysis of the significant CbCln3^ex7/8^ probes in DAVID Bioinformatics Resources 6.7. The number of probes (and the % of the total) that were represented by the GO term (Probe Count) is indicated, and the Probe-IDs are listed (Probes). The relative enrichment value (Fold Enrichment) is also shown. (XLS)

Table S5 DAVID gene ontology analysis of significant CbCln6^mutif^ probes. The significantly enriched (p<0.01) gene ontology terms (GOTERM/Term) from the Biological Process and Cellular Component categories are shown, determined through analysis of the significant CbCln6^mutif^ probes in DAVID Bioinformatics Resources 6.7. The number of probes (and the % of the total) that were represented by the GO term (Probe Count) is indicated, and the Probe-IDs are listed (Probes). The relative enrichment value (Fold Enrichment) is also shown. (XLS)

Table S6 Significantly altered gene sets identified by sigPathway analysis of the CbCln3^ex7/8^ dataset. The significantly altered (q-value<0.01) gene sets, and associated statistics details, are shown for the CbCln3^ex7/8^ cells. For comparison, the statistics for these same gene sets are shown for the CbCln6^mutif^ cells. For a complete description of the NTk and NEk value determinations, see Materials and Methods. (XLS)

Table S7 Significantly altered gene sets identified by sigPathway analysis of the CbCln6^mutif^ dataset. The significantly altered (q-value<0.01) gene sets, and associated statistics details, are shown for the CbCln6^mutif^ cells. For comparison, the statistics for these same gene sets are shown for the CbCln3^ex7/8^ cells. For a complete description of the NTk and NEk value determinations, see Materials and Methods. (XLS)

Table S8 Primers used for qRT-PCR validation of expression array hits. For each selected gene target, primers used for qRT-PCR experiments are shown, with the sequence in the 5’ to 3’ orientation. (DOC)

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Author Contributions

Conceived and designed the experiments: SLC MEM. Performed the experiments: YC JAE SB JFS. Analyzed the data: SB JML SLC JFS. Contributed reagents/materials/analysis tools: SLC JML. Wrote the paper: SLC.

References


