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The Batten disease gene *CLN3* confers resistance to endoplasmic reticulum stress induced by tunicamycin

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ABSTRACT

Mutations in *CLN3* gene cause juvenile neuronal ceroid lipofuscinosis (JNCL or Batten disease), an early-onset neurodegenerative disorder that is characterized by the accumulation of ceroid lipofuscin within lysosomes. The function of the CLN3 protein remains unclear and is presumed to be related to Endoplasmic reticulum (ER) stress. To investigate the function of *CLN3* in the ER stress signaling pathway, we measured proliferation and apoptosis in cells transfected with normal and mutant *CLN3* after treatment with the ER stress inducer tunicamycin (TM). We found that overexpression of *CLN3* was sufficient in conferring increased resistance to ER stress. Wild-type CLN3 protected cells from TM-induced apoptosis and increased cell proliferation. Overexpression of wild-type CLN3 enhanced expression of the ER chaperone protein, glucose-regulated protein 78 (GRP78), and reduced expression of the proapoptotic protein CCAAT/-enhancer-binding protein homologous protein (CHOP). In contrast, overexpression of mutant CLN3 or siRNA knockdown of CLN3 produced the opposite effect. Together, our data suggest that the lack of CLN3 function in cells leads to a failure of management in the response to ER stress and this may be the key deficit in JNCL that causes neuronal degeneration.

1. Introduction

Neuronal ceroid lipofuscinoses (NCLs) are a class of inherited progressive neurodegenerative diseases. NCLs are autosomal recessive and are classified by the age of onset and its unique gene mutations [1]. The diseases are clinically characterized by epileptic seizures, progressive motor and cognitive decline, and loss of vision. All NCLs share lysosomal accumulation of the autofluorescent storage material: ceroid. However, the causes or consequences of this accumulation and its relationship to neuronal degeneration are unclear. The most common form of NCLs is the juvenile neuronal ceroid lipofuscinosis (JNCL) and its onset usually ranges between 5 and 7 years of age. Mutations in the *CLN3* gene are responsible for the disease. CLN3 is a 438 amino acid transmembrane protein and its functions are unclear. Approximately 85% of all JNCL patients harbor a 1.02-kb deletion in *CLN3*, resulting in a frame-shift and a premature termination. The truncated CLN3 polypeptide contains 181 amino acids, of which the last 28 amino acids are not contained in the original wild-type protein [2]. Although the function of CLN3 remains unknown, its sequence is highly conserved among eukaryotes; therefore, it is safe to predict that the CLN3 may play the same functions among all eukaryotes [3]. A variety of approaches have implicated that CLN3 plays important roles in many cellular biological processes, including intracellular protein trafficking [4], lysosomal homeostasis [5], and mitochondrial functions [6]. JNCL's neuropathology mostly results from excessive neuronal and photoreceptor cell apoptosis [7]. The CLN3 protein is anti-apoptotic and it has previously been demonstrated that its integrity is necessary for neuronal and photoreceptor cell survival [8,9].

The endoplasmic reticulum (ER) fulfills multiple cellular functions. The lumen of the ER is an oxidative environment and is critical for the formation of disulfide bonds and the proper folding of proteins destined for secretion or display on the cell surface. The disruption of cellular redox regulation causes the accumulation of unfolded proteins in the ER [10]. Disturbances in the normal functions of the ER lead to an evolutionarily conserved ER stress (ERS) response. ERS is alleviated by proteins that increase the protein folding capacity of the ER. ERS-mediated apoptosis has been reported in NCLs, including JNCL [11–13], as well as other lysosomal storage disorders and many neurodegenerative diseases [14–16]. Similarly, ER and mitochondrial functions are impaired

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by oxidative stress, generating further oxidative loads in the cells. Recently, it was reported that *CLN3* genetically interacts with the oxidative stress signaling pathways in *Drosophila* [17]. In this study, we investigated the function of *CLN3* in the ER stress signaling pathway. Our data suggest that the lack of CLN3 function in cells leads to a failure of management in the response to ER stress and this may be the key deficit in JNCL that causes neuronal degeneration.

2. Materials and methods

2.1. Cell culture and vector construction

SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (PAA Laboratories, Linz, Austria) at 37 °C with 5% CO_2. The full-length *CLN3* cDNA and mutant cDNA *CLN3*$^{\Delta ex7/8}$ (loss of 1.02-kb genomic sequence from base 598 to 814 of the cDNA [2]) were subcloned in the PAAV-IRES-GFP vector (Stratagene). These clonings resulted in two expression vectors named PAAV-IRES-GFP-CLN3 and PAAV-IRES-GFP-CLN3$^{\Delta ex7/8}$.

2.2. Transfection and RNAi

SH-SY5Y cells were plated at a density of 2×10^5/well in a 6-well plate one day before transfection. Cells were transfected by Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Short hairpin RNAs were synthesized (siCLN3: GGGCCUUUGCAACAACUUCUC UUAU at position 455; siCLN3-scramble: GCCUCUAUUUCGUUACGCUAGAC AU) (JiMa, Shanghai, China) and used for transfection into cells to knock down CLN3.

2.3. qRT-PCR

Total RNA was isolated from transfected cells using the TRIzol reagent (Invitrogen). First-strand cDNA was synthesized from mRNA by reverse transcription using a TransScript II First-Strand cDNA Synthesis Super Mix kit (Transgen Biotech Inc, China) following the manufacturer's protocol. The qRT-PCR reaction was set up with first-strand cDNA using a SYBR Premix Ex Taq kit (Takara, Japan). Briefly, the 25-μL reaction volume included 1 μL of first-strand cDNA (from 20-μL total volume from the reverse transcription kit), 200 nM of each primer, and 7.5 μL of 12.5 × SYBR Mix. Primers used for qRT-PCR were: human *CLN3* forward primer, 5′-GATCCGCTGTGCTCCTGGCGGACATC-3′; *CLN3* reverse primer, 5′-CCGCTCGAGTCACACACCACACAGGCTGGTC-3′; *Chop* forward primer, 5′-TGAACGGCTCAAGCAGGAA-3′; *Chop* reverse primer, 5′-CGGCGAGTCGCCTCTACTT-3′; *Grp78* forward primer, 5′-GAGATCATCGCCAACGATCAG-3′;*Grp78* reverse primer, 5′-ACTTGATGTCCTGCTGCACAG-3′; *GAPDH* forward primer, 5′-GAAGGTGAAGGTCGGAGTC-3′; *GAPDH* reverse primer, 5′-GAAGATGGTGATGGGATTTC-3′.*GAPDH* was used as an internal control. The PCR reaction conditions were as follows: denaturation at 95 °C for 30 s; 40 cycles of 95 °C for 5 s, and 60 °C for 30 s. Levels of mRNA expression were quantitatively analyzed using the ABI 7000 Sequence Detection System (Applied Biosystems, USA).

2.4. Tunicamycin treatment

Transfected SH-SY5Y cells were plated in 96-well plates at a density of 2×10^3 cells/well. After 24 h of incubation, cells were supplemented with a medium that included TM (0, 2.5, 5.0 μg/mL) for the indicated times and assessed for cell viability. For apoptosis analysis, cells were treated with 5 μg/mL TM for 36 h. Expression analysis of the molecular chaperone GRP78 and the transcription factor CHOP was carried out after treatment with 5 μg/mL TM for 12 h (for RNA) or 24 h (for protein).

2.5. Cell viability

Cell viability and growth curves were obtained using the Cell Counting Kit-8 (CCK-8) following the manufacturer's protocol (Dojindo, Kumamoto, Japan). Briefly, after TM treatment, 10 μl of CCK-8 solution was added to each well. Following incubation at 37 °C for 2 h in a humidified CO_2 incubator, absorbance at 540 nm was measured with the SpectraMax Plus 384 microplate reader (Molecular Devices, USA). The absorbance rates (absorbance at different treatment time points with respect to starting time point) were used to calculate cell viability. The viability rate of the treatment controls (TM Teated group) at the start time was set to 100%.

2.6. Analysis of apoptosis

Treated cells were fixed on glass coverslips for 10 min in phosphate buffered saline (PBS) containing 3% paraformaldehyde. The coverslips were washed twice for 3 min with PBS, air-dried, then stained for 10 min in 10 μg/mL Hoechst 33258 (BiYunTian, Beijing, China). Coverslips were then mounted in 50% glycerol containing 20 mM citric acid and 50 mM orthophosphate, and stored at −20 °C before analysis. Nuclear morphology was evaluated by fluorescent microscopy. Apoptotic cells were identified and a minimum of 200 cells were counted. Data were expressed as a percentage, relative to the total number of cells counted (mean ± SD; $n = 3$ determinations).

2.7. Western blotting

Whole cell lysates were prepared with lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1% Triton X-100) containing protease inhibitor cocktail (Roche). After incubation on ice with vortex for 1 h, cell lysates were centrifuged at 13,000*g* for 10 min. Protein concentrations were determined by the BCA Protein Assay Kit (Pierce, France) according to the manufacturer's instructions. Proteins were resolved on 12% SDS–PAGE gel and transferred onto nitrocellulose membranes. After blocking with 5% nonfat milk for 1 h at room temperature, the membranes were incubated overnight at 4 °C with primary antibodies. The antibodies used were: mouse monoclonal to CLN3 (1:500, Abcam, USA), CHOP (1:1500, Cell Signaling, USA), GRP78 (1:1000, Cell Signaling, USA), Flag (1:2000, Sigma, USA), and actin (1:5000, Zhongshan Goldbridge). After extensive washes with TBST, membranes were then incubated with rabbit anti-mouse IgG conjugated to HRP (1:2000, Abcam), followed by enhanced chemiluminescence detection (Pierce).

2.8. Statistical analysis

Data are expressed as mean ± SD. One-way analysis of variance was used to compare differences using SPSS 19.0 software (SPSS, Chicago, USA). A p-value of less than 0.05 was considered as statistically significant.

3. Results

3.1. Effect of CLN3 on cell proliferation after ERS induced by TM

Overexpression of CLN3 and mutant CLN3 (CLN3$^{\Delta ex7/8}$) in SH-SY5Y cells was confirmed by Western blot (Fig. 1A). Analysis of *CLN3* mRNA levels by quantitative real-time PCR (qRT-PCR)

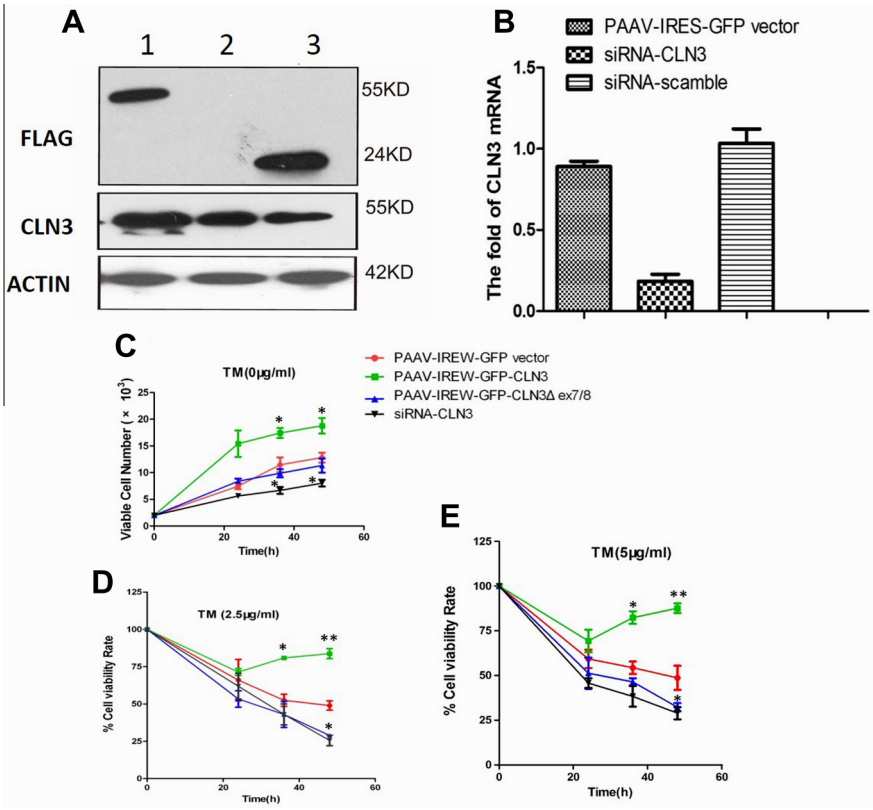

Fig. 1. Overexpression of CLN3 increases the rate of SY5Y cell growth. (A) Western blot analysis of transit transfected wild-type CLN3 and mutant CLN3 (upper panel); endogenous CLN3 (Middle panel); and β-Actin as loading control (lower panel). (B) Real-time PCR analysis of CLN3 mRNA level. The fold of CLN3 mRNA was calculated by CLN3 mRNA levels relative to the internal control GAPDH mRNA levels. (C–E) Effect of CLN3 overexpression on SY5Y cells proliferation at the indicated times without TM treatment (C); with 2.5 μg/ml TM treatment (D); with 5.0 μg/ml TM treatment (E). Each data point is an average of three experiments each of which was carried out in triplicate. $^{*}P < 0.05$; $^{**}P < 0.001$.

revealed a reduction in *CLN3* expression in CLN3 knockdown cells compared to control cells. There was no significant difference in *CLN3* mRNA levels between cells transfected with a scrambled siRNA and cells transfected with the empty vector (Fig. 1B). Under normal conditions, overexpression of CLN3 increased SH-SY5Y cell proliferation compared with cells transfected with mutant CLN3, or an empty vector. CLN3 down regulation further reduced cell proliferation rates (Fig. 1C). TM treatment dramatically reduced cell viability. However, overexpression of CLN3 exhibited marked resistance to cell death induced by TM (2.5 μg/ml or 5 μg/ml) and reversed the reduction in proliferation by 24 h while both overexpression of mutant CLN3 and down regulation of CLN3 showed hypersensitivity to TM treatment (Fig. 1D and E). These results demonstrate that CLN3 plays an important role in cell proliferation under ERS.

3.2. Effect of CLN3 on apoptosis after ERS induced by TM

We further investigated the effect of CLN3 on apoptosis induced by ERS. Apoptosis assay by Hoechst 33258 staining demonstrated that cells transfected with wild-type *CLN3* were protected from TM-induced apoptosis compared with cells transfected with empty vector (5.847% ± 1.464% versus 69.977% ± 5.957%, $p < 0.001$). Mutant $CLN3^{\Delta ex7/8}$ transfected or CLN3 down regulated cells increased apoptosis compared with control cells (84.939% ± 2.3% and 88.805% ± 4.074%, $p < 0.05$) (Fig. 2A and B).

We also noticed that in the absence of TM treatment, transfection with full-length or mutant *CLN3* has no effect on apoptosis ($p > 0.05$). This result indicated that the protective function of CLN3 from apoptosis was only apparent after treatment with TM.

3.3. CLN3 confers resistance to ERS induced by TM

To determine whether CLN3 affects the progression of ERS, we analyzed mRNA and protein levels of the ERS marker genes *Grp78* and *Chop* after TM treatment. TM significantly increased the expression of *Grp78* and *Chop* at the mRNA level, indicating activation of the ER unfolded protein response (UPR). The degree of enhancement of Grp78 expression shows differences in various cell groups after treatment. Overexpression of CLN3 increased *Grp78* level compared with control cells (5.433 ± 0.192 versus 2.386 ± 0.11 fold, $p < 0.001$). Down regulation of CLN3 reduced the *Grp78* level compared with control cells (0.797 ± 0.06 versus 2.386 ± 0.11 fold, $p < 0.001$). Mutant CLN3 showed a similar pattern compared to cells with down regulated CLN3 (0.829 ± 0.141 versus 0.797 ± 0.06 fold, $p > 0.05$) (Fig. 3A). *Chop* mRNA expression levels were increased in all cells after treatment with TM. However, the effect of CLN3 on the degree of enhanced expression of *Chop* was different to that seen with *Grp78*. Overexpression of CLN3 decreased *Chop* expression compared with control cells (3.429 ± 0.633 versus 6.415 ± 0.204 fold, $p < 0.001$). Down regulation of CLN3 showed higher levels of *Chop* expression compared with control cells (9.556 ± 0.559 versus 6.415 ± 0.204 fold, $p < 0.001$). Overexpression of mutant *CLN3* showed a higher degree of enhanced expression of *Chop* compared with control cells (7.675 ± 0.247 versus 6.415 ± 0.204 fold, $p < 0.05$) (Fig. 3B). The

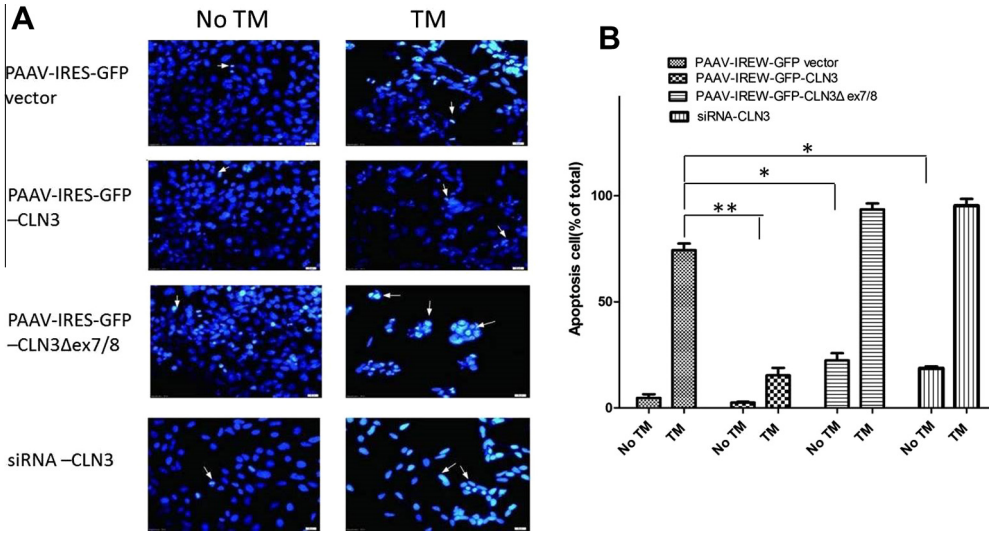

Fig. 2. Effect of CLN3 on apoptosis after ERS induced by TM. (A) Apoptotic cells were identified by UV-microscopy. Nuclei of normal cells were revealed by Hoechst 33342 staining (blue). Nuclei features of apoptotic cells shows white (arrowhead). Scale bar = 20 μm. (B) Counting a minimum of 200 total cells in each group from A. (mean ± SD; $n = 3$). $^{*}P < 0.05$, $^{**}P < 0.001$ by one-way ANOVA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

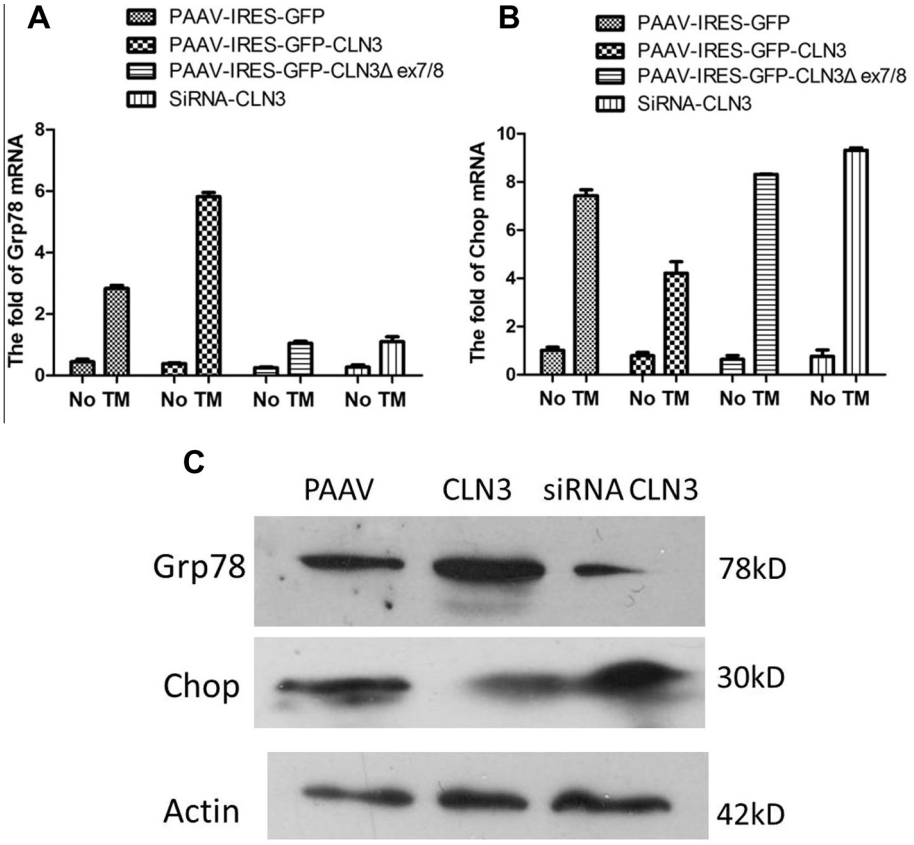

Fig. 3. CLN3 effect on expression of ERS markers. (A) Analysis of mRNA level of Grp78. The level of Grp78 mRNA was normalized to GAPDH mRNA level. (B) Analysis of mRNA level of Chop. The level of Chop mRNA was normalized to GAPDH mRNA level. (C) Western Blot analysis of GRP78 (upper panel) and CHOP (middle panel) protein level. The cells were treated with 5 μg/ml TM for 36 h. Forty micrograms of total cell lysis were loaded. β-Actin was detected as loading control (lower panel).

changes in protein expression levels of GRP78 and CHOP also showed a similar pattern with mRNA expression (Fig. 3C).

Transfection with different *CLN3* constructs did not reveal any quantitative differences in the expression levels of *Grp78* and *Chop* prior to treatment with TM. This indicated that transfection of these constructs did not induce ERS (Fig. 4A and B).

4. Discussion

Mutations in the human *CLN3* gene are responsible for JNCL, the most common form of the NCLs. Currently, the function of CLN3 and how the loss of this gene leads to the pathogenesis of JNCL are not clear [18]. Previous studies have reported that ERS

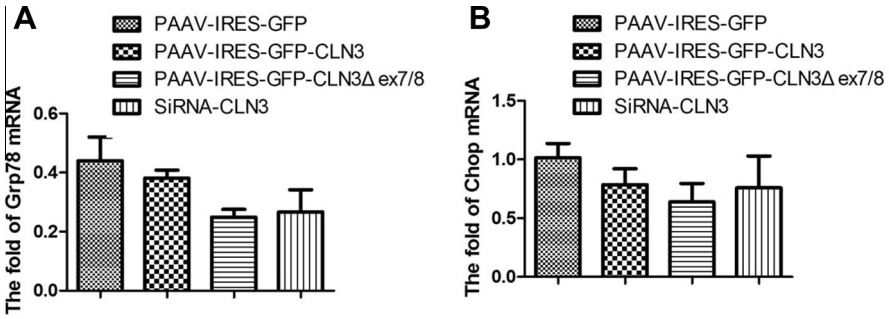

Fig. 4. The CLN3 expression alone is unable to induce ER response. (A) The expression mRNA level of Grp78 after transfection at 24 h without TM treatment. (B) The expression mRNA level of Chop after transfection at 24 h without TM treatment.

mediates apoptosis in many neurodegenerative disorders including NCLs. In this study, we found that overexpression of CLN3 protected cells from TM-induced apoptosis and increased cell proliferation. Both overexpression of mutant $CLN3^{\Delta ex7/8}$ that lacks CLN3 function and down regulation of CLN3 lead cells to become hypersensitive to ERS. Moreover, we demonstrated that CLN3 increased the expression of the ER chaperone GRP78 and decreased the expression of the pro-apoptotic marker CHOP. Our data suggest that CLN3 function in anti-apoptosis and pro-cell proliferation, at least partly through regulation of GRP78 and CHOP, in response to ERS.

Under normal conditions, cells containing mutant CLN3 showed similar proliferation rates as control cells (Fig. 1C); however, the rate was radically altered after TM treatment (Fig. 1D and E). This result indicated that mutant CLN3 was unable to regulate ERS. Two possible reasons could lead to this effect. First, mutant CLN3 protein misses the conserved C-terminus, which includes domains important for proliferation. The topological model for CLN3 reveals that the majority of mutations in CLN3 localize to the luminal side of the transmembrane structure within C-terminus. This strongly implies that ligand-substrate binding mediates CLN3 function [19]. Second, we used SH-SY5Y cells that contain the wild-type *CLN3* gene in our study, which may inhibit the effect of the deletion mutant.

Overexpression of CLN3 significantly protects cells from apoptosis induced by TM (Fig. 2). The CLN3 protein is important for the survival of both neurons and photoreceptors in JNCL patients [7]. One possible explanation for this is that the eye and the brain are both immune-privileged sites that cannot tolerate destructive inflammatory responses [20]. However, ERS-induced activation of the UPR serves as a signal amplification cascade favoring inflammatory cytokine production. UPR alone was shown to promote inflammation in different cellular and pathological models. [20]. Excessive ERS responses can induce destructive inflammatory responses to cellular apoptosis. Our findings on CLN3 protecting cells from apoptosis after TM treatment suggest that CLN3 directly interacts with ERS signaling pathway.

Our results showed that CLN3 increased expression levels of the ER chaperone protein GRP78 (Fig. 3). Chaperone proteins can stabilize protein structures and assist in the correct folding and unfolding of proteins. This function is important for the maintenance of cellular protein homeostasis [21]. Chaperones also respond to stress by initiating the destruction of stress-denatured proteins or by promoting apoptosis of damaged cells [22]. Chaperone deficiency in either amount or function is implicated in the etiology or pathogenesis of the central nervous system [23]. The chaperone GRP78 is a heat shock protein (HSP) 70 homolog residing in the ER. HSP70s have been implicated in the development of pathology of neurodegenerative diseases [24,25]. Overexpression of GRP78 can protect cells from AD (or PD)-specific damage [26,27]. Overexpression of CLN3 enhances expression of GRP78 while mutant CLN3 reduces levels of GRP78 after TM treatment. This indicates a specific role for CLN3 in the ERS response towards TM-induced apoptosis. Furthermore, it may suggest that a compromise of the ERS response may be a fundamental defect leading to neurodegeneration in JNCL caused by mutations of CLN3.

Our results also indicated that neither wild-type *CLN3* nor mutant *CLN3* directly cause an ER response (Fig. 4). It seems that CLN3 is unlikely to be a direct inducer of the ER response. The presence of mutant CLN3 might impair the ability of JNCL patients to manage ERS; however, it is not a direct cause of ceroid storage material accumulation.

Together, our data suggest that CLN3 protects cells from TM-induced apoptosis and reverses a decline in proliferation after TM treatment. This function is achieved, at least partly, through regulation of GRP78 and CHOP in response to ERS. These results will help centralize the search for CLN3 molecular functions and the pathogenic mechanism underlying JNCL. A number of questions remain unanswered. What is the mechanism underlying CLN3 involvement in ERS signaling pathways? Is there a role for the overexpression of GRP78 in suppression of JNCL-related phenotypes? Further research will be needed to address these questions.

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