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Identifying protein partners of CLN8, an ER-resident protein involved in neuronal ceroid lipofuscinosis

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ABSTRACT

Neuronal ceroid lipofuscinoses (NCLs) are a genetically heterogeneous group of neurodegenerative diseases characterized by cognitive and motor decline, epilepsy, visual loss and by lysosomal autofluorescent inclusions. Two distinct clinical phenotypes, the progressive epilepsy with mental retardation (EPMR) and a late-infantile variant of NCLs (CLN8-vLINCL) are associated with mutations in the *CLN8* gene that encodes a transmembrane protein predominantly located to the endoplasmic reticulum (ER). To gain insight into the function of CLN8 protein, we employed the split-ubiquitin membrane-based yeast two-hybrid (MYTH) system, which detects protein-protein interactions in a membrane environment, using the full-length human CLN8 as bait and a human brain cDNA library as prey. We identified several potential protein partners of CLN8 and especially referred to VAPA, c14orf1/hERG28, STX8, GATE16, BNIP3 and BNIP3L proteins that are associated with biologically relevant processes such as synthesis and transport of lipids, vesicular/membrane trafficking, autophagy/mitophagy and apoptosis. Interactions of CLN8 with VAPA and GATE16 were further validated by co-immunoprecipitation and co-localization assays in mammalian cells. Using a new C-terminal-oriented CLN8 antibody, CLN8-VAPA interaction was also confirmed by co-staining in close spatial proximity within different CNS tissues. The results of this study shed light on potential interactome networks of CLN8 and provide a powerful starting point for understanding protein function(s) and molecular aspects of diseases associated with CLN8 deficiency.

1. Introduction

The neuronal ceroid lipofuscinoses (NCLs) are a group of autosomal recessively inherited neurodegenerative disorders manifest mainly in childhood [1,2]. More rare adult forms are also described and can present with either dominant or recessive patterns of inheritance [1,3,4]. NCLs belong to the family of lysosomal storage diseases (LSDs), and are characterized by an intralysosomal accumulation of autofluorescent lipopigment and clinical symptoms such as progressive mental and motor deterioration, epilepsy, ataxia and visual loss. They vary in age of onset and severity of disease progression, and are genetically linked to distinct genes – *CLN1/PPT1* (MIM 256730), *CLN2/TPP1* (MIM 204500), *CLN3* (MIM 204200), *CLN4/DNAJC5* (MIM 611203), *CLN5* (MIM 256731), *CLN6* (MIM 601780), *CLN7/MFSD8* (MIM 610951), *CLN8* (MIM 600143), *CLN10/CTSD* (MIM 610127) – and different mutations. Recently, more genes have been identified: *CLN11/GRN* (MIM 138945), *CLN12/ATP13A2* (MIM 610513), *CLN13/CTSF* (MIM 603539; http://www.ucl.ac.uk/ncl), and *CLN14/KCTD7* (MIM 611725) [5–7]. *CLN* genes encode primarily soluble lysosomal proteins or membrane proteins localized to endosomes/lysosomes or endoplasmic reticulum, although their distribution in other compartments is also documented [2,8]. The exact functions of many CLN proteins remain to be defined and the pathogenic mechanisms underlying NCLs are generally unknown. Recent evidence suggests CLN proteins' involvement in cytoskeletal dynamics, sorting or recycling endosomes, including lysosomes and autophagosomes and synaptic membrane trafficking, and apoptosis [9–16].

CLN8 belongs to the group of CLN proteins and is mutated in two distinct clinical phenotypes: the progressive epilepsy with mental retardation (EPMR), a juvenile-onset phenotypic variant [17], and a more severe form that shares clinical and pathological similarities with the late-infantile NCL variants (vLINCL), including CLN5-, CLN6- and CLN7-associated diseases [1,18–20]. At least 24 different mutations, either deletion or missense, are linked to CLN8 diseases and mostly to the vLINCL form [1]. Using the naturally-occurring *mnd* (motor neuron degeneration) mouse model (*Cln8*^{mnd} mouse) exhibiting a disease phenotype similar to that of CLN8-vLINCL [17,21,22], numerous studies have shown altered lipid metabolism, oxidative and ER stresses, mitochondrial dysfunction, defects in calcium homeostasis, inflammation and apoptosis [23–29]. Changes in brain lipids and ER-stress responses in fibroblast cells have been also reported in EPMR patients [30,31]. However, due to the still undefined CLN8 function, the primary defect underlying CLN8-associated diseases remains an open question.

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CLN8 is mainly an ER-resident transmembrane protein of predicted 286-amino acid (aa) residues and contains five-hydrophobic regions corresponding to a TLC (TRAM-LAG1-CLN8) domain of 200 aa (62–262), a luminal-oriented N-terminus and a functional C-terminal ER-retrieval KKRP signal, likely facing the cytosolic side, for recycling between ER and ER-Golgi intermediate compartment (ERGIC) [1,17,22,32]. Most *CLN8* mutations do not affect the protein localization at the ER [20,22,33], suggesting that CLN8-deficiency in the ER may be responsible for the onset of the disease. However, CLN8 is seen outside the ER in a specialized subcompartment of the polarized epithelial CaCo2 cells [33], in the Golgi, endosomes and lipid rafts of mouse fibroblasts [34], as well as in the proximity of plasma membrane of hippocampal neurons [20,33]. In addition, CLN8 may interact with other CLN proteins such as CLN2, CLN3, CLN5 and CLN6 in different subcompartments [34,36], even if an *in vivo* confirmation is still lacking and no interaction and heterodimerization between CLN8 and CLN6, another ER-resident CLN protein, are detected using different experimental approaches [37]. Although the precise function of CLN8 remains elusive, a potential role in lipid homeostasis is supposed on its homology to the TLC family and some evidence referring to its implication in sphingolipids' regulation [24,25,32]. Moreover, CLN8 overexpression has been found to induce cell proliferation and survival after a damaging insult [20].

To gain insight into CLN8 function and identify molecular partners, we here thought to use a split-ubiquitin membrane yeast two-hybrid (MYTH) screen which allows sorting out interactions in a membrane environment. The conventional MYTH system relies on the fact that severed C-terminal (Cub) and N-terminal (Nub) halves of ubiquitin spontaneously reassemble to form a functional ubiquitin molecule [38]. We then used a C-terminally Cub-tagged full-length CLN8 as a bait and an N-terminally NubG-tagged human brain cDNA library as a prey to search for CLN8 interactors through the MYTH system. These experimental conditions permit the identification of prey proteins, either membrane or cytosolic proteins, with the N-terminal portion exposed to the cytosol, thus excluding prey partners with a different topology like many CLN proteins [1]. Our study reports the identification of different binding partners of CLN8 and especially refers to a group of six interacting proteins, two of which supported herein by confirmation assays, that are involved in biologically relevant processes with potential links to the NCL pathophysiology.

2. Materials and methods

2.1. Split-ubiquitin membrane based yeast two-hybrid assay

The split-ubiquitin membrane based yeast two-hybrid assay (MYTH) was carried out in the version of DUALmembrane system Kit 2 (Dualsystems Biotech, Switzerland). For building CLN8-bait, the coding region of CLN8 was PCR amplified from SVPoly-CLN8, kindly provided by Drs. L. Lonka and A.E. Lehesjoki (Neuroscience Center and Folkhälsan Institute of Genetics, Biomedicum Helsinki, University of Helsinki, Finland), with primers containing SfiI restriction sites (Fw: 5′-GAATTCCCGGCCATTACGGCCATGAATCCTGCGAGCGATGGGGG-3′, Rv: 5′-GGGAATTCGGCCGAGGCGGCCGGCCTCTTCTTCCGCAGCAGCTG-3′). Polymerase chain reaction (PCR) fragments were cloned into the pCCW and pCCW-STE vectors to yield, respectively, pCCW-CLN8 and pCCW-STE-CLN8 plasmids encoding CLN8 baits fused to the Cub-LexA-VP16 cassette (Dualsystems Biotech, Switzerland). To verify the correct expression of baits and their eventual self-activation on the reporter genes, each bait plasmid was transfected with the positive pAI-Alg5 or negative pDL2-Alg5 control preys into the NMY32 yeast strain [MATa his3delta200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)4-HIS3 URA3::(lexAop)8-lacZ (lexAop)8-ADE2 GAL4] and the growth on the triple and quadruple dropout media (SD-LTA, SD-LTH and SD-LTHA) was analyzed. The human adult brain cDNA library was constructed in the pNubGx plasmid (Dualsystems Biotech) to express cDNAs fused to the C-terminus of NubG. Twenty-eight μg of the library was transformed into the NMY32 yeast expressing CLN8-bait and the screening was carried out by means of the growth on the quadruple dropout selection medium SD-LTHA. Colonies that were bigger and with white color, likely expressing interactions, were re-streaked on SD-LTHA medium with or without 5 mM 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of His3-protein. The selected colonies were screened further by the output of the third reporter gene, *LacZ*, throughout the semiquantitative β-galactosidase assay. From colonies developing the highest color, prey plasmid DNAs were isolated, transformed into *E. coli XL1blue* for the amplification and, finally, were re-transfected into CLN8-bait yeast for a second round of screening on SD-LTHA and SD-LTHA + 5 mM 3-AT media. Inserts of positive plasmids were sequenced using primers for the pNubG-X vector chosen from both ends of the inserts. Nucleotide sequences were compared to the GenBank database using BLAST program available at the National Center for Biotechnology Information.

2.2. β-Galactosidase assay

The semi-quantitative determination of the β-galactosidase activity was performed as described by Stagljar et al. [38] with slight modifications. Cells in 3 ml SD medium lacking tryptophan and leucine were grown at 30 °C up to late exponential phase. Cells (0.5 OD_{600}) were pelleted, washed once in buffer Z [113 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, and 1 mM $MgCl_2$, (pH 7.0)] and suspended in 200 μl of buffer Z. After three freeze/thaw cycles, cells were incubated with buffer Z (100 μl) containing 0.81% (v/v) 2-mercaptoethanol and 2-nitrophenyl-β-D-galactopyranoside (1 mg), for 5–15 min at 30 °C. Blue color development was monitored by eye.

2.3. Plasmids for co-immunoprecipitation and co-localization experiments

For confirmation assays, plasmids were generated by amplification of the full-length cDNAs followed by cloning into the appropriate epitope-tagging vectors.

1) pFLAG-CMV4-CLN8: amplimers Fw 5′-CCCAAGCTTATGAATCCTGCGAGCGATGGGG-3′ and Rv 5′-CCCAAGCTTCTATGGCCTCTTCTTCCGCAGCAG-3′, HindIII restriction enzyme, pFLAG-CMV4 vector (Sigma)
2) pMyc-CMV3-CLN8: amplimers, Fw 5′-GGAATTCACCATGAATCCTGCGAGCGATGG-3′ and Rv 5′-CCCAAGCTTCTATGGCCTCTTCTTCCGCAGCAG-3′, BamHI-EcoRI restriction enzyme, pCMV3-Tag2A vector (Stratagen)
3) pMyc-CMV3-VAPA or pFLAG-CMV4-VAPA: amplimers Fw 5′-CGGGATCCACCATGGCGA AGCACGAGCAGA-3′ and Rv 5′-GGAATTCCTACAAGATGAATTTCCCTAGAA-3′, BamHI-EcoRI or BglII site (Klenow-blunted) restriction enzymes, pCMV3-Tag2A or pFLAG-CMV4 vectors
4) pMyc-CMV3-GATE16 or pcDNA4-V5-GATE16: amplimers Fw 5′-CGGGATCCACCATGAA GTGGTGTTCAAGG-3′ and Rv 5′-GGAATTCTCAGAAGCCAAAAGTGTTCTCTC-3′, BamHI-EcoRI restriction enzyme, pCMV3-Tag2A or pcDNA4-V5-HisA vectors (Invitrogen)
5) pCMV4-FLAG-GATE16: GATE16 cDNA was excised from pcDNA4-V5-GATE16 plasmid with HindIII and EcoRI and cloned into pFLAG-CMV4 digested with the same restriction enzymes
6) pcDNA3-CLN8: CLN8 cDNA was excised from SVPoly-CLN8 plasmid with EcoRI and HindIII and cloned into pcDNA3 vector (Invitrogen) restricted with the same enzymes.

All constructs were checked by DNA sequence analysis.

2.4. Production of a polyclonal antibody anti-CLN8

To produce a polyclonal antibody against a His-tagged peptide of the C-terminal domain of human CLN8, the DNA region coding for aa's 238–286 of CLN8 was amplified from SVPoly-CLN8 plasmid, cleaved with restriction enzymes (BamHI and HindIII), and ligated into the pQE30 vector (Qiagen) to yield the pQE30-721-stop-CLN8 construct.