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in vivo localization of the neuronal ceroid lipofuscinosis proteins, *CLN3* and *CLN7*, at endogenous expression levels

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ABSTRACT

The neuronal ceroid lipofuscinoses are a group of recessively inherited, childhood-onset neurodegenerative conditions. Several forms are caused by mutations in genes encoding putative lysosomal membrane proteins. Studies of the cell biology underpinning these disorders are hampered by the poor antigenicity of the membrane proteins, which makes visualization of the endogenous proteins difficult. We have used *Drosophila* to generate knock-in YFP-fusions for two of the NCL membrane proteins: *CLN7* and *CLN3*. The YFP-fusions are expressed at endogenous levels and the proteins can be visualized live without the need for overexpression. Unexpectedly, both *CLN7* and *CLN3* have restricted expression in the CNS of *Drosophila* larva and are predominantly expressed in the glia that form the insect blood-brain-barrier. *CLN7* is also expressed in neurons in the developing visual system. Analogous with murine *CLN3*, *Drosophila CLN3* is strongly expressed in the excretory and osmoregulatory Malpighian tubules, but the knock-in also reveals unexpected localization of the protein to the apical domain adjacent to the lumen. In addition, some *CLN3* protein in the tubules is localized within mitochondria. Our *in vivo* imaging of *CLN7* and *CLN3* suggests new possibilities for function and promotes new ideas about the cell biology of the NCLs.

1. Introduction

The neuronal ceroid lipofuscinoses (NCLs) are a collection of inherited neurodegenerative lysosomal storage disorders predominantly affecting children (Jalanko and Braulke, 2009). They share symptoms that include visual failure, seizures, psychiatric and behavioral changes and a progressive decline in mental and motor functions followed by premature death. The NCLs have a common histopathological hallmark: accumulation of autofluorescent lysosomal storage material in most cells, including neurons, indicative of lysosomal dysfunction or failure. Another common histopathological feature of the disorders is an early glial activation, which precedes selective neuronal loss (reviewed in Cooper et al., 2015).

The NCLs are recessively inherited monogenic disorders (with the exception of one rare adult-onset autosomal dominant form). To date mutations have been identified in 14 genes responsible for NCL with varying ages of onset. These encode soluble intra-lysosomal proteins and enzymes (*CLN1*, *CLN2*, *CLN5*, *CLN10* and *CLN13*), late endosomal/lysosomal transmembrane proteins (*CLN3*, *CLN7* and *CLN12*), ER/ERGIC membrane proteins (*CLN6* and *CLN8*), cytosolic proteins (*CLN4* and *CLN14*) and an extracellular protein (*CLN11*) (reviewed in Carcel-Trullols et al., 2015). The cell biology underpinning the NCLs is not well understood despite many years of study, nor is it clear why pathology is almost entirely restricted to the CNS despite many of the *CLN* genes being widely expressed. We have turned to the fruit fly, *Drosophila*, to study two of the *CLN* genes encoding putative lysosomal membrane transporters, *CLN7* and *CLN3*. *Drosophila* expresses only a subset of the *CLN* genes and we hypothesized that these are likely to have core functions conserved in vertebrates.

Mutations in *CLN7/MFS-domain containing 8* (*CLN7/MFSD8*) are responsible for late-infantile onset NCL (or CLN7 disease), with disease onset at 1.5–5 years of age (Kousi et al., 2009). The CLN7 protein is predicted to be a member of the multi-facilitator superfamily of transporters, each of which has twelve membrane spanning domains (Siintola et al., 2007). However, its function and any possible substrate it may transport remains unknown. In cell culture experiments with tagged forms, CLN7 protein is localized primarily in lysosomes (Sharifi et al., 2010; Siintola et al., 2007; Steenhuis et al., 2010) and has been identified by lysosomal proteomics (Chapel et al., 2013). Consistent with this, phenotypes in mutant mice suggest a role for CLN7 in autophagy (Brandenstein et al., 2016). In a related project that will be reported

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elsewhere, we have identified neurodevelopmental defects in *CLN7* mutant *Drosophila* (O'Hare, Mohammed, Tuxworth and Tear, in prep).

Mutations in the *CLN3* gene lead to Juvenile NCL, the most common form of NCL (also known as Batten disease or CLN3 disease) with onset usually at 5–7 years of age (Lerner et al., 1995). The *CLN3* gene is predicted to encode a hydrophobic six transmembrane domain protein (Ratajczak et al., 2014) and is conserved in many species including yeast and *Drosophila* but its function is unclear despite more than 20 years of study. Various studies of *CLN3* in different cell lines and models have suggested roles in regulation of lysosomal pH, anterograde and retrograde post-Golgi trafficking, autophagy, endocytosis, apoptosis, oxidative stress responses or Notch and JNK signalling (Tuxworth et al., 2011; Tuxworth et al., 2009 and reviewed in Carcel-Trullols et al., 2015). The expression pattern of the *CLN3* gene in mice is known from a combination of *in situ* hybridization studies and a knock-in reporter of gene expression (Ding et al., 2011; Eliason et al., 2007). The knock-in mouse, in particular, demonstrated an expression pattern in the CNS predominantly in the later stages of embryonic development and persisting in post-natal development. Interestingly, strong expression from early stages of development in the endothelia of the brain was maintained into adult life (Eliason et al., 2007). *CLN3* expression was also detected in endothelia in other organs, in epithelia and strongly in the renal tubules, where its expression is regulated by osmolality (Stein et al., 2010). Taken together, these data suggest an important role for *CLN3* in epithelia but since the reporter used was a nuclear-localized β-galactosidase, the sub-cellular localization of CLN3 protein in polarized epithelial cells could not be determined. CLN3 is considered primarily a lysosomal protein, based on numerous studies in cell culture with epitope-tagged or fluorescent fusion proteins (reviewed in Phillips et al., 2005) and it has been identified in lysosomal membranes by proteomics (Chapel et al., 2013). Studies of mammalian CLN3 localization have generally relied on overexpression; one of the few studies to detect endogenous CLN3 indicated a mitochondrial localization in Müller glia of the mouse retina and in the inner segments of photoreceptors (Katz et al., 1997). Knock-in approaches have been used in yeast to avoid overexpression artefacts and reveal CLN3 can be found in the Golgi (Kama et al., 2011) or at the vacuole (the yeast lysosomal equivalent) with sub-cellular localization regulated by intracellular pH (Wolfe et al., 2011). In *Dictyostelium*, GFP-CLN3 expressed at low levels localizes to the osmoregulatory contractile vacuole and other endocytic vesicles (Huber et al., 2014) but knock-in approaches have not been used to date to study CLN3 in a species with a complex nervous system.

The lack of reagents for reliable detection of endogenous protein localization *in vivo* for either CLN7 or CLN3 has hampered the search for their functions. To overcome these limitations, we used recombineering and CRISPR/Cas9 genome editing to generate seamless knock-in YFP fusions of *CLN7* and *CLN3* to report gene expression and protein localization in *Drosophila*. We show that *CLN7* is strongly expressed in glial cells in the CNS but largely absent from neurons other than in the developing visual system. *CLN3* is also expressed in glia and very strongly in Malpighian tubules, the insect organ orthologous to the kidney. Unexpectedly, CLN3 protein in tubules is localized to the apical domain and also to mitochondria. These findings alter our ideas of *CLN* gene function and suggest new possibilities for the causes of NCL disease.

2. Materials and methods

2.1. Drosophila stocks and husbandry

Flies were maintained in vials on standard agar/yeast-based media at 25 °C and 12-h light/dark cycle except during the genome editing and recombineering procedures where semi-defined medium was used to boost egg laying (recipes available from Bloomington Stock Center website). The control line used for all experiments was an isogenic w^{1118} strain (BL 6326).

2.2. CRISPR/Cas9-mediated HDR of CLN7

A gRNA was selected using fly CRISPR Optimal Target Finder (tools.flycrispr.molbio.wisc.edu/targetFinder). Oligos corresponding to the gRNA sequence were cloned into BbsI digested pCFD3 (Port et al., 2014) and confirmed by Sanger sequencing. Templates for homology-directed repair was designed to incorporate the *Drosophila* codon-optimised Venus-YFP sequence immediately 3′ of the *CLN7* start codon followed by 15 bp encoding GGAGG as a linker. 500 bp of *CLN7* sequence either side of the site of the Cas9 digestion site were included as homology arms. In one version, an artificial intron from the *white* gene was incorporated into the YFP sequence to reduce the size of the expanded YFP-containing first exon (Fig. 1). qPCR suggested both intron-containing and intron-less genes were expressed at similar levels. Constructs were synthesized by GenScript. The gRNA and HDR template were co-injected as supercoiled plasmids into *vasa::Cas9* embryos (BL51323) at a mixed ratio of 250:750 μg/μl respectively at the Department of Genetics, University of Cambridge. Injected flies were crossed to a third chromosome balancer and successful incorporation of the YFP sequence into CG8596 detected by PCR from gDNA after mating. Germline transmission was followed after mating by single fly gDNA PCR.

2.3. Recombineering of CLN3

The Venus variant of YFP was incorporated immediately downstream of the ATG start codon of the *CLN3* locus by recombineering of

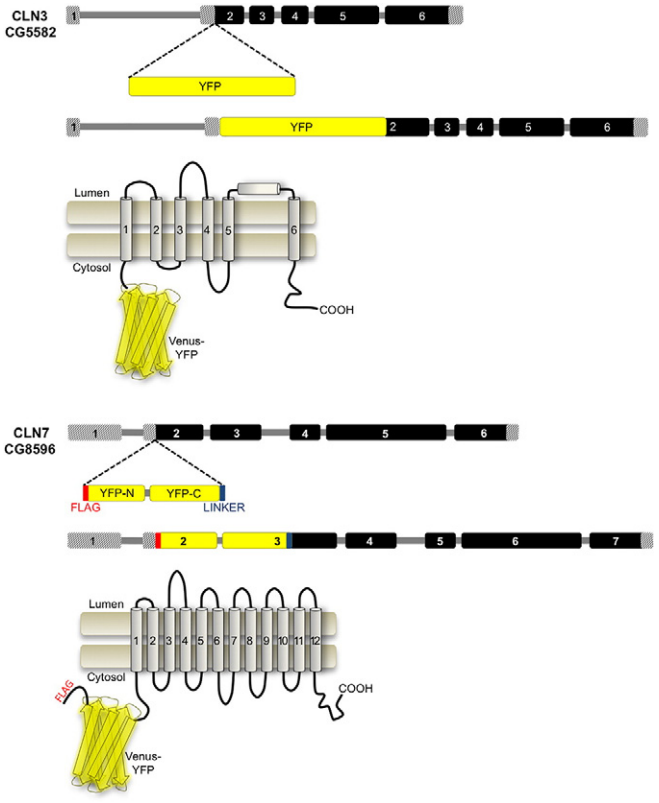

Fig. 1. Strategy to generate YFP-*CLN3* and YFP-*CLN7* knock-ins. The Venus variant of YFP was inserted after the ATG start codon of *CLN7* or *CLN3* to generate N-terminal fusion proteins. For *CLN3*, a BAC containing the *CLN3* locus and surrounding sequences was modified by seamless recombineering to include YFP after the ATG start codon then the BAC inserted into a landing site on chromosome II by ϕC31-mediated recombination. For *CLN7*, CRISPR/Cas9-mediated homologous recombination was used to insert YFP into the *CLN7* locus. A single FLAG-tag was included at the N-terminus of YFP and a short flexible linker between YFP and *CLN7*. An artificial intron from the *white* gene was included in the YFP sequence. Based on predicted topologies, YFP should be on the cytosolic side for both proteins. CLN3 is likely to exist as a dimer.