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An over-expression system for characterizing *Ppt1* function in *Drosophila*

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Abstract

Background: The infantile onset form of Neuronal Ceroid Lipofuscinoses (INCL) is the earliest and most severe form of NCL, with neurological symptoms that reflect massive neurodegeneration in the CNS and retina. INCL is due to recessively inherited mutations at the *CLN1* locus. This locus encodes the evolutionarily conserved enzyme palmitoyl-protein thioesterase 1 (PPT1), indicating an essential role for protein palmitoylation in normal neuronal function.

Results: To begin to elucidate the specific role that *Ppt1* plays in neuronal cells, we have developed a *Ppt1* over-expression system in *Drosophila*. We report that over-expression of *DmPpt1* in the developing *Drosophila* visual system leads to the loss of cells through apoptotic cell death. This *DmPpt1* over-expression phenotype is suppressed by *DmPpt1* genomic deficiencies. Moreover, over-expression of *DmPpt1S123A*, which bears a catalytic site serine 123 to alanine mutation, does not lead to the severe eye phenotype observed with over-expression of wild-type *DmPpt1*. Thus, cell loss in *DmPpt1* flies is directly related to the dosage of wildtype *DmPpt1*.

Conclusions: Although INCL is due to the loss of PPT1; increased levels of DmPpt1 also lead to neurodegeneration possibly via a detrimental effect on some aspect of PPT1's normal function. This suggests that the precise levels of PPT1 activity are important for neuronal cell survival. The *Drosophila DmPpt1* over-expression system provides a resource for genetic experiments that aim to identify the processes by which PPT1 regulates the palmitoylation-state of its essential protein substrates.

Background

The most common of the pediatric neurodegenerative diseases (1 in 12,500 births) are a set of primarily recessive disorders termed Neuronal Ceroid Lipofuscinoses (NCLs) due to the loss of central nervous system neurons and the accumulation of auto-fluorescent lipopigment [1]. While most cells contain inclusions, neurons are primarily affected leading to symptoms that include loss of vision, motor dysfunction, intellectual decline, and seizures [2]. Each NCL subtype is classified by its characteristic membrane/protein lysosomal inclusion pathology and age of onset [2]. Genetic analysis of the NCLs has identified 8 loci, *CLN1-8*, that are associated with the differing ages of onset of the disorders. Six of the eight loci have been mapped showing that *CLN1* and *CLN2* encode soluble enzymes with known functions while *CLN 3*, *5*, *6*, and *8* are putative transmembrane proteins of unknown function [2].

Infantile onset NCL (INCL) is the earliest and most severe form of NCL with symptoms that include loss of vision, motor dysfunction, intellectual decline, and seizures due to massive neurodegeneration in the CNS and retina [2]. INCL is caused by mutations in the *CLN1* gene which encodes palmitoyl-protein thioesterase 1 (PPT1), suggesting that there is an important role for the regulation of palmitoylation in normal neuronal function [3]. This post-translation modification is the addition of a palmitate fatty acid chain to proteins. PPT1 is one of the enzymes that catalyze the removal of palmitoyl groups from specific protein targets. Unlike permanent fatty-acid chain additions, such as myristolation, palmitoylation is dynamic and very little is known about its role in the regulation of protein function [4].

Histochemical and biochemical analysis of PPT1 has shown that it appears to be present within the endo-lysosomal compartment and possibly in the cytoplasm [5–8]. Consistent with an important role for PPT1 in neurons, the protein is found with synaptic vesicles and synaptosomes in neuronal cell culture [9,10]. A model of excitotoxicity in the rat brain confirms a presynaptic localization for the protein and suggests that PPT1 may be neuroprotective during an excito-toxic event [11]. Finally, overexpression of PPT1 in a neuronal cell line protected cells from the induction of apoptosis suggesting that de-palmitoylation of $p21^{Ras}$ and other substrate proteins may play a role in the regulation of neural death [8]. While this recent work is beginning to shed light on the protein's function, there is still little understanding of the role PPT1 plays in different cellular compartments, including tissue specific substrates and signaling pathways that it may modulate.

Drosophila has been an important model system for the study of human disease and has made important contributions to the understanding of several kinds of neuronal degeneration including Huntington's, Parkinson's, Alzheimer's, and Machado-Joseph Diseases [reviewed in [12,13] and references therein]. The powerful genetic tools available in *Drosophila* as well as the high degree of conservation of gene function between *Drosophila* and higher vertebrates makes it an ideal system to study the cellular function of PPT1. The adult visual system in particular has been especially useful for characterizing human disease genes in *Drosophila* and for identifying modifiers of their function. In this study we present the development of an over-expression system for the study of the *Drosophila Ppt1* gene.

Results

Generation of DmPpt1 *Over-expression Lines*

The *Drosophila* PPT1 homolog (*DmPpt1*) is ~55% identical and ~74% similar to the human protein at the amino acid level [14]. The *DmPpt1* transcript appears to be expressed ubiquitously at low levels during embryonic and larval development (data not shown). Consistent with the levels of mRNA, DmPpt1 enzymatic activity is present at varying levels in all tissues that have been tested [14]. In order to produce a *Drosophila* model to study PPT1 function, we generated 10 independent UAS:*DmPpt1* insertion lines and used the GMR-Gal4 driver line to over-express DmPpt1 in the developing visual system. The GMR Gal4 driver expresses GAL4 in all cell types including neuronal photoreceptors as the eye differentiates during the larval and pupal stages [15]. We confirmed that the UAS:*DmPpt1* overexpression lines did indeed over-express *DmPpt1* message by performing *in situ* hybridizations on third instar eye imaginal discs (data not shown). Expression of the *DmPpt1* transgene was consistent with the previously described GMR GAL4 expression pattern [15]. We were not able to analyze the levels of protein produced or subcellular localization of the over-expressed protein due to the lack of a specific antibody reagent.

We analyzed the surface of the eyes in *DmPpt1* overexpression adults with scanning electron microscopy (Figure 1) and at the light microscope level and found a range of morphological defects, both weak (Figure 1C) and strong (Figure 1D) depending on the UAS-*DmPpt1* insertion line. The eyes show a change in size, a disruption of the individual ommatidia number and spacing as well as an absence of some sensory bristles (Figure 1A,1B,1C,1D), suggesting that there was cell loss occurring during the development of each ommatidium. Furthermore, when examined under the light microscope, the *DmPpt1* over-expressing eyes contain black ommatidia (Figure 1I). These black spots do not form progressively over the life of the fly. Quantitative counting experiments over a 15 day period indicates that the number present at eclosion, while variable from fly to fly, is constant throughout the life of the individual fly (data not shown). The black spots, therefore, appear to be indicative of a degeneration event later in development once the eye has fully differentiated.

DmPpt1 *Over-expression Induces Cell Death*

To determine whether the underlying cellular architecture of the eye was compromised, we preformed semi-thin sections on several over-expression lines, one showing a weak phenotype and one showing a strong phenotype, along with control lines (GMR:Gal4 and UAS insertion lines alone). These analyses revealed a striking loss of photoreceptors and other cell types that was correlated with the severity of the external eye morphology. Photoreceptor cell loss was quantified in the weak phenotypic line (*GMR:Gal4/UAS:DmPpt1*$^{8.1}$) by counting individual rhabdomeres per ommatidia in semi-thin retinal sections. This quantification showed that the eyes had an average of

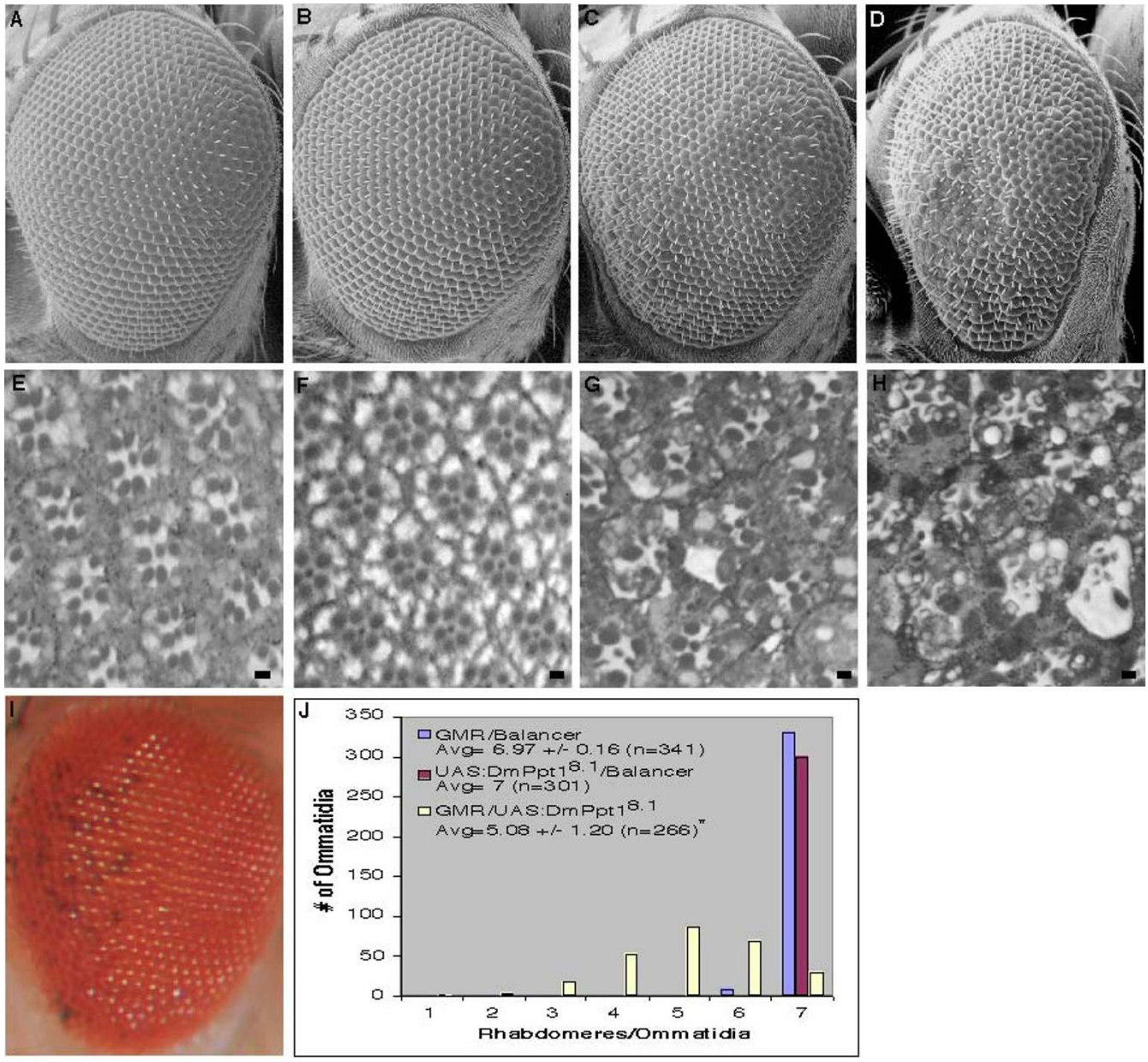

Figure 1

Analysis of *DmPpt1* over-expression phenotypes. A-D, Scanning Electron Micrographs showing the external surface of control and *DmPpt1* over-expression eyes (200×). E-H, Semi-thin sections through fixed retinal tissue showing the internal structure of control and *DmPpt1* over-expression retinas. Scale bar = 2 μm. A and E, *GMR:Gal4/CyO*. B and F, *UAS:DmPpt1*$^{8.1}$*/CyO*. C and G, *GMR:Gal4/UAS:DmPpt1*$^{8.1}$. D and H, *GMR:Gal4/UAS:DmPpt1*$^{2.1}$. I, A light microscope image of a *GMR:Gal4/UAS:DmPpt1*$^{8.1}$ eye showing the black ommatidia present in the fully differentiated eye. J. Quantification of the loss of rhabdomeres in *GMR:Gal4/UAS:DmPpt1*$^{8.1}$ eyes in relation to the *GMR:Gal4/CyO* and *UAS:DmPpt1*$^{8.1}$*/CyO* controls. *p < .00001 using Ttest.

5.08 +/- 1.20 rhabdomeres/ommatidia (n= 266). This was compared to a *GMR:Gal4/CyO* control line with an average of 6.97 +/- 0.16 (n = 341) and to a *UAS:DmPpt1/CyO* control line with 7 rhabdomeres/ommatidia (n = 301) (Figure 1J). As indicated by the quantification, the weak phenotypic line, *UAS:DmPpt1*$^{8.1}$, showed less than the full complement of 7 rhabdomeres visible in a section of control eyes (Figure 1J). Furthermore, those photoreceptor

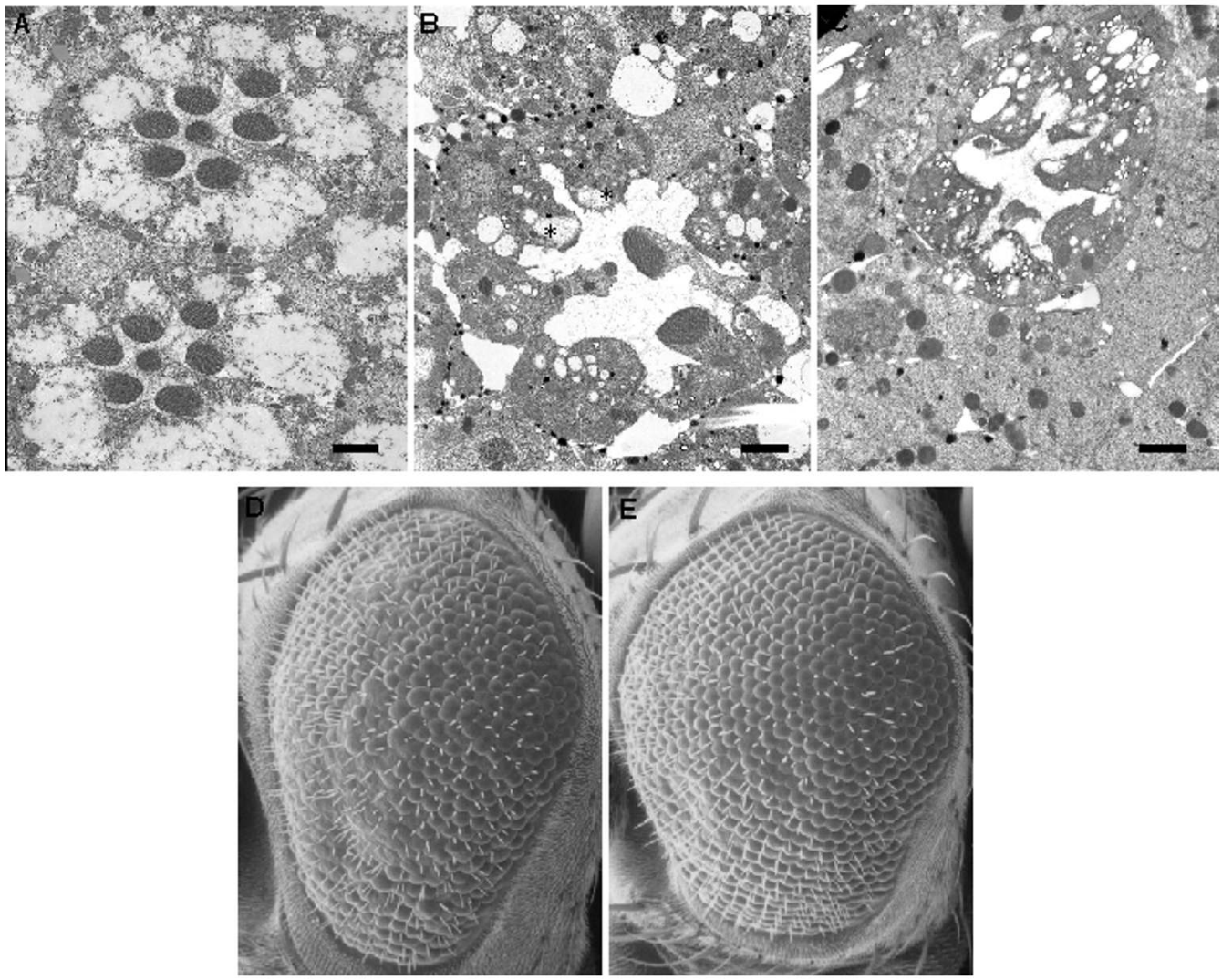

Figure 2
DmPpt1 over-expressing eyes undergo apoptotic cell death. A-C, Transmission electron micrographs. A, *UAS:DmPpt1*$^{8.1}$/*CyO* control showing normal ommatidial structure. Scale bar = 2 μm. B, An image of *GMR:Gal4*/*UAS:DmPpt1*$^{8.1}$ over-expression ommatidium showing individual degenerating photoreceptors (labeled with an asterisk). Scale bar = 1 μm. C, An image of *GMR:Gal4*/*UAS:DmPpt1*$^{2.1}$over-expression ommatidium showing all photoreceptor undergoing cell death with no rhabdomeres present. Scale bar = 1 μm. D, Scanning electron micrograph (200×) of a *GMR:Gal4, UAS:DmPpt1*$^{8.1}$/+; *UAS:DmPpt1*$^{2.1}$/+ eye. E, Scanning electron micrograph (200×) of a *GMR:Gal4, UAS:DmPpt1*$^{8.1}$/*pGMR:p35 ; UAS:DmPpt1*$^{2.1}$/+ eye.

cells that were missing rhabdomeres appeared highly pigmented suggesting they were undergoing cell death [16]. The strong phenotypic line, *UAS:DmPpt1*$^{2.1}$, was not analyzed in this manner due to the severity of the defects and the fact that almost all rhabdomeres were missing, precluding reliable quantification.

We further analyzed the ultrastructure of the degenerating photoreceptor neurons with transmission electron microscopy (TEM) focusing on sections of the weak *UAS:DmPpt1*$^{8.1}$ line, the strong *UAS:DmPpt1*$^{2.1}$ line, and a control insertion alone line. TEM sections of UAS:*DmPpt1* over-expression lines showed photoreceptor neurons that had become highly vacuolized with rhabdomeres in various stages of degeneration (Figure 2A,2B,2C). In a strong phenotypic line, this abnormal neuronal cell phenotype was more pronounced, with almost all ommatidia appearing highly pigmented, full of vacuoles and missing

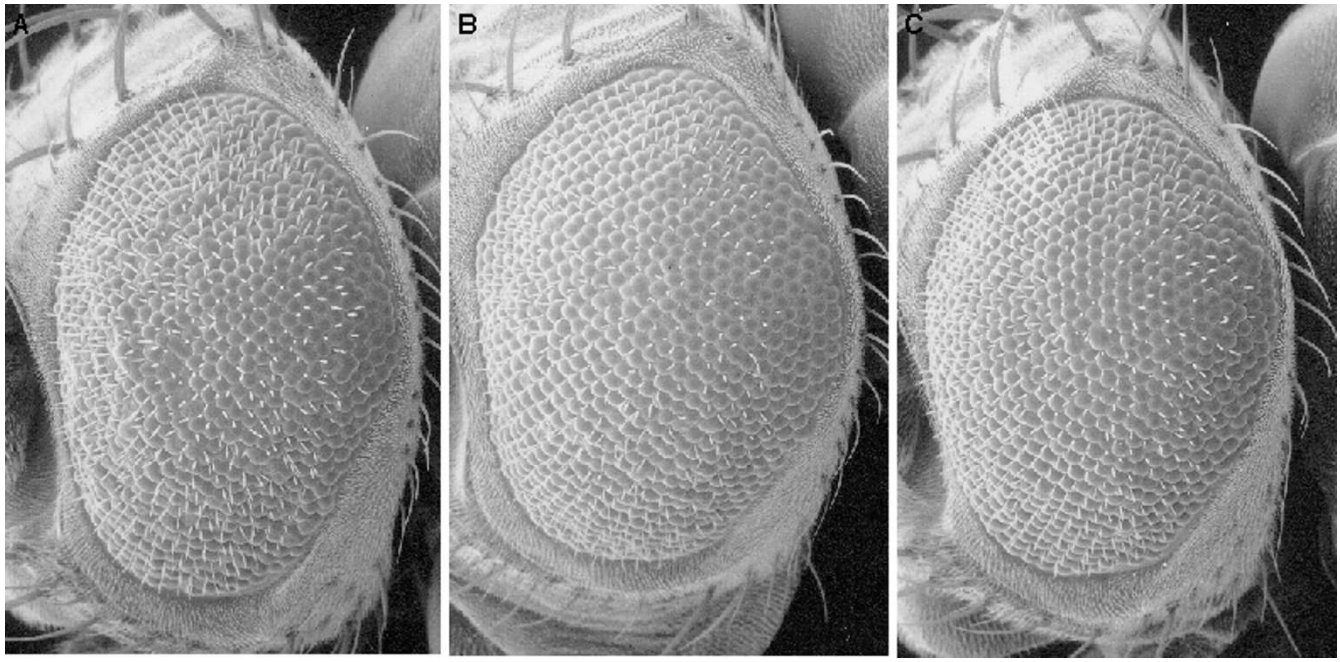

Figure 3
Deficiency chromosome suppression of the *DmPpt1* over-expression phenotype. A-C, Scanning electron micrographs (200×). A, +/+; *GMR:Gal4, UAS:DmPpt1*$^{8.1}$/+; *UAS:DmPpt1*$^{2.1}$/+ control. B, *Df(1) RA2/+; GMR:Gal4, UAS:DmPpt1*$^{8.1}$/+; *UAS:DmPpt1*$^{2.1}$/+. C, *Df(1) KA12/+; GMR:Gal4, UAS:DmPpt1*$^{8.1}$/+; *UAS:DmPpt1*$^{2.1}$/+.

all of their rhabdomeres. This type of ultrastructural appearance is indicative of photoreceptors undergoing programmed cell death [16].

To further test the idea that the defects we observed in eye development were the result of apoptotic cell death, we examined whether the eye morphology phenotype is altered by the presence of the baculoviral anti-apoptotic protein p35 [17]. Co-expression of *p35* and *DmPpt1* using the GMR promoter significantly reduced the rough eye defects associated with *DmPpt1* over-expression (Figure 2D,2E). This suggests that, consistent with our TEM analysis, the defects we see are due in large part to cells dying through apoptosis.

DmPpt1 *Over-expression Mechanism*

We took two approaches to address whether the abnormal eye phenotypes are a result of increased levels of wildtype *DmPpt1* or are due to an ectopic, non-wildtype function of the protein. First, two independent deficiency chromosomes that remove the genomic region containing the *DmPpt1* locus were crossed into the *DmPpt1* over-expression background. The rough eye defects were suppressed when the dose ofwildtype *DmPpt1* was decreased (Figure 3). This suggests that the defects are produced by increased levels of wildtype *DmPpt1*.

In a second approach, we changed the catalytic serine at amino acid 123 to alanine (S123A) through site directed mutagenesis of the *DmPpt1* cDNA. A similar mutation in the homologous amino acid in Bovine PPT1 produces an enzyme with severely reduced catalytic activity *in vitro* [18]. We isolated several independent UAS:*DmPpt1-S123A* insertion lines to test whether enzyme activity is required for the rough eye and black spot phenotypes observed with *DmPpt1* over-expression. We confirmed that the transgenic lines overexpressed *DmPpt1-S123A* message by *in situ* hybridization of eye imaginal discs (data not shown). Over-expression of three UAS:*DmPpt1-S123A* lines with GMR-Gal4 yielded no observable abnormal phenotypes when analyzed with SEM (Figure 4A,4B). We further compared two of these lines to wild-type *DmPpt1* over-expression by analyzing semi-thin sections of UAS:*DmPpt1-S123A* over-expressing eyes (Figure 4C,4D). For quantification, the number of rhabdomeres in each ommatidia of UAS:*DmPpt1-S123A* lines and the weak UAS:*DmPpt1*$^{8.1}$ phenotypic line were compared (Figure 4E). Our analysis of the two catalytic mutant lines, S123A^{1} and S123A^{4}, showed that they possessed 6.54 +/- 0.86 (n = 369) and 6.09 +/- 1.07 (n = 292) rhabdomeres per ommatidia, respectively. These data demonstrate that over-expression of *DmPpt1* catalytic domain mutants slightly decreases the number of rhabdomeres per omma-

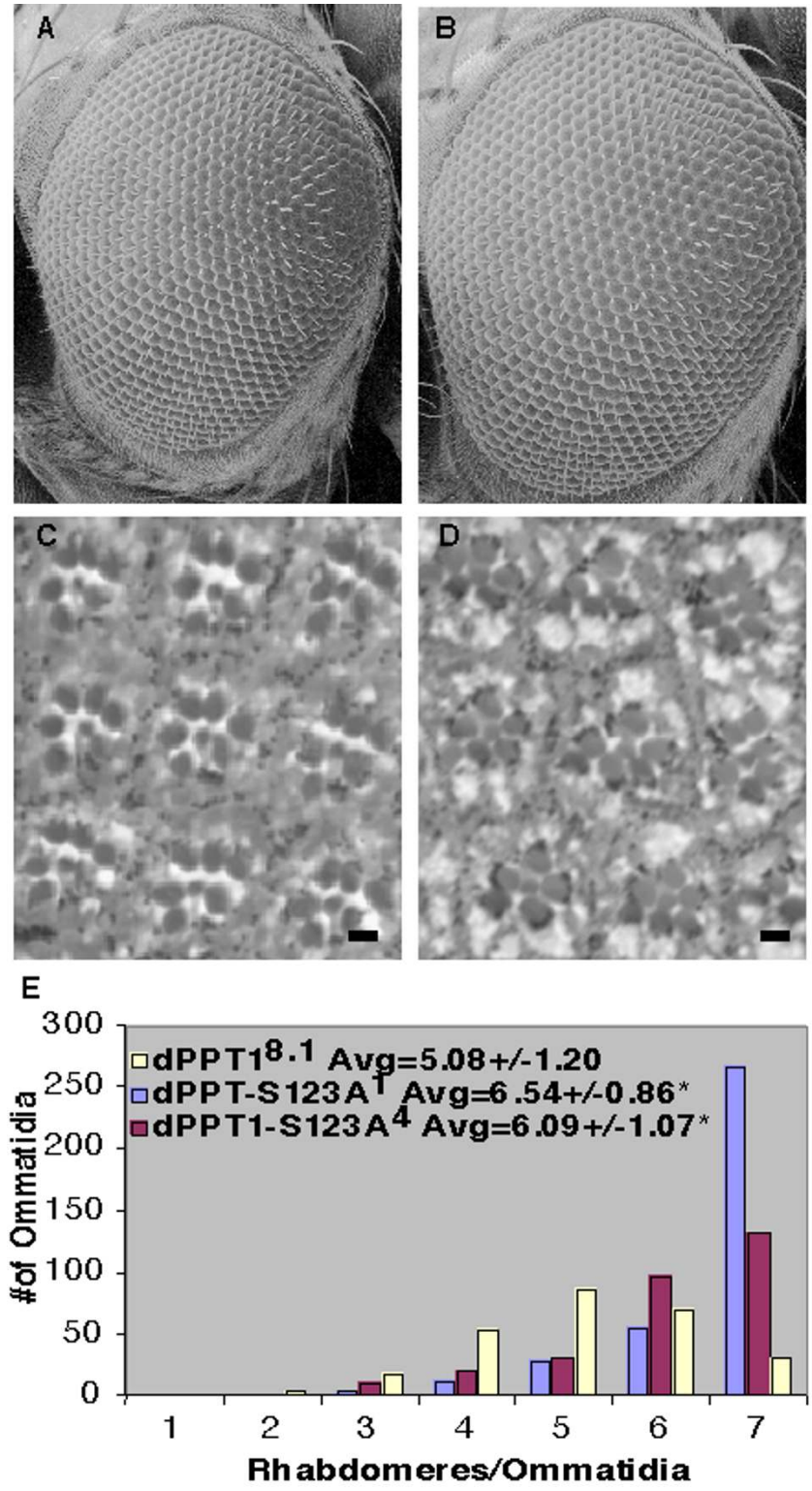

Figure 4
Analysis of *DmPpt1S123A* catalytic mutant over-expression. A-B, Scanning electron micrographs (200×). C-D, Light microscope images of semi-thin retinal sections. Scale bar= 2 μm. A and C, *GMR:Gal4/UAS:DmPpt1S123A*^{1}. B and D, *GMR:Gal4/UAS:DmPpt1S123A*^{4}. E. Quantification of the number of rhabdomeres per ommatidia in *DmPpt1S123A* over-expression eyes compared to *DmPpt1*$^{8.1}$ over-expression eyes. *p < .00001 by Ttest.

tidia compared to control eyes, but is not nearly as severe as even a weak *DmPpt1* line. This, along with the *DmPpt1* deficiencies, indicates that a majority of the abnormal *DmPpt1*-associated phenotypes that we observe are due to increased levels of *DmPpt1* message that likely leads to increased wildtype protein activity.

Discussion

In this study, we have presented and characterized the first system to study the function of the Infantile Neuronal Ceroid Lipofuscinosis (INCL) gene, *CLN1/Ppt1*, in *Drosophila*. This is also the first *Drosophila* model system designed to characterize the cellular function of a lysosomal enzyme. Specifically, we have shown that targeted over-expression of *DmPpt1* in the developing *Drosophila* visual system leads to the loss of cells through apoptotic cell death both early in eye development (rough eye) and also after ommatidial differentiation has finished, yielding black ommatidial spots. This phenotype is directly related to the increased dosage of wildtype *DmPpt1* mRNA. First, the *DmPpt1* over-expression phenotypes were suppressed by two genomic deficiencies that remove one copy of the endogenous *DmPpt1* locus. Moreover, the over-expression of a *DmPpt1* catalytic mutant, shown to have reduced catalytic activity [18], produces only a mild eye phenotype rather than the dramatic degeneration seen in wildtype *DmPpt1* over-expression. The mild eye phenotype associated with the expression of the S123A catalytic mutant suggests that this mutation is not a null but instead severely reduces DmPpt1 function. Thus, while over-expression may lead to non-specific consequences, our data suggests that the eye phenotypes in *DmPpt1* over-expression flies are primarily a result of over-expression of wild-type *DmPpt1*, implying a detrimental effect of increased catalytic activity on processes that are normally regulated by DmPpt1.

PPT1 is involved in the de-palmitoylation of substrate proteins. This function is clearly important to neuronal cells since loss of PPT1 function leads to massive degeneration of neurons in the central nervous system of INCL patients [2] and *Ppt1* knock-out mice [19]. The presence of lipofuscin and the build-up of endo-lysosomal inclusions suggest that PPT1's function may lie in the regulated subcellular trafficking and degradation of palmitoylated proteins. While these pathological defects associated with loss of PPT1 in humans and mice have been thoroughly characterized, there is very little functional data about the specific cell biological role that this protein plays.

The protein was initially purified through its ability to de-palmitoylate $p21^{Ras}$ [5]. *In vitro*, PPT1 has been shown to de-palmitoylate specific peptides suggesting that GAP43, the G_{α} subunit of heterotrimeric G-proteins, and rhodopsin are possible *in vivo* targets [20]. Further evidence that $p21^{Ras}$ may be an endogenous target was shown by the ability of over-expressed PPT1 to block apoptosis through a $p21^{Ras}$-Akt-Caspase pathway in neuroblastoma cells [8]. This inhibition was coincident with a decreased presence of $p21^{Ras}$ at the membrane suggesting that PPT1 may regulate $p21^{Ras}$ signaling by modulating its palmitoylation state [8]. This suggests that Drosophila Ras may be a candidate modifier of the *DmPpt1* over-expression phenotype.

Conclusions

Our findings indicate that, while recessive mutations that severely decrease PPT1 cause neuronal cell death in INCL patients, increased levels of PPT1 can also lead to neurodegeneration, revealing that the precise level of PPT1 is important for neuronal cell survival. A deeper understanding of PPT1's normal cellular function may therefore be necessary to the success of treatment strategies that aim to replace PPT1 in INCL patients. The over-expression system that we have developed in *Drosophila* will provide an opportunity to elucidate the role of DmPpt1 in neuronal and non-neuronal cells. The identification of genetic modifiers of this visual system phenotype will facilitate the identification of *in vivo* substrates and signaling pathways that *DmPpt1* may modulate. The insights gained from these results will further the understanding of PPT1 function and the molecular etiology of INCL.

Methods

Fly Husbandry

All crosses were performed at 25°C on standard Drosophila media.

Transgenic Line

The full length *Ppt1* cDNA was obtained from Research Genetics. It was initially identified as the EST GM14257 using the BDGP EST database. The cDNA was cloned into the EcoRI and XhoI sites of the pUAST expression vector [21]. Transgenic flies were produced by standard methods using the pP{Wc **Δ**2,3} helper plasmid. Transformants were identified using the *white* marker gene contained in the pUAST vector. We further confirmed that the transgenics lines were over-expressing *DmPpt1* message through *in situ* hybridization on third instar eye imaginal discs. We used the GM14257 cDNA to *in vitro* transcribe sense and anti-sense RNAs that were then used as probes on fixed eye imaginal disc tissue.

Ppt1 mutagenesis

To produce the Serine 123 to Alanine mutation, we used PCR to change the Serine codon CTC to the Alanine codon CGC. We designed overlapping primers that contained the point mutation and used them in conjunction with two flanking primers to incorporate the mutation at Serine 123. The primers used were (the point mutation is

indicated in bold): S123A1:CGCCTTGCG**C**GAATC-CGATG; S123A2:CATCGGATTC**G**CGCAAGGCG.

Scanning Electron Microscopy

Newly eclosed adults of the specified genotypes were collected and aged for several days in a yeast-free food vial. These flies were then taken through a series of ethanol dehydration steps. They were first placed in 25% ethanol and after a 12-hr incubation time they were moved to 50% ethanol. This process continued, through the following dilutions: 50, 75, 95, and 2 incubations in 100% ethanol. They were left in 100% ethanol and taken to the Northeastern Electron Microscopy facility to be critical point dried and sputter coated for scanning electron microscopy.

Sectioning and Transmission Electron Microscopy

Newly eclosed adults of the specified genotypes were collected, their heads were removed and then placed in a fixative solution (1% gluturaldehyde, 2% Paraformaldehyde 0.1 M Na Phosphate pH 7.4) for 1 hour at room temperature. The heads were then washed three times in 0.1 M Na Phosphate and post fixed in osmium tetroxide (2% OsO_4, 0.1 M Na Phosphate pH 7.4) for 1 hour. Following the post fixation step, the heads were washed three times for 10 minutes each in 0.1 M Na Phosphate. They were then dehydrated with an ethanol series consisting of 5 minute incubations in 30%, 50%, 70%, 85%, 95% ethanol and 2 incubations in 100% ethanol for 10 minutes each. The heads were embedded in plastic. Semi-thin plastic sections were mounted and stained with Toludine blue. Thin EM sections were stained, mounted on grids and analyzed using a Transmission Electron Microscope.

Photoreceptor Quantification

For each genotype, the number of rhabdomeres present in each ommatidia were counted for semi-thin sections of 2 independent eyes per genotype. The following genotypes were used in the analyses: *GMR:Gal4/CyO*, *UAS:DmPpt1*$^{8.1}$*/CyO*, *GMR:Gal4/UAS:DmPpt1*$^{8.1}$, *GMR:Gal4/UAS:DmPpt1S123A*^{1}, and *GMR:Gal4/UAS:DmPpt1S123A*^{4}. All slides were blinded before scoring.

p35 and Deficiency Suppression

To determine the effect of p35 expression on the *DmPpt1* over-expression phenotype we compared *GMR:Gal4, UAS:DmPpt1*$^{8.1}$*/+; UAS:DmPpt1*$^{2.1}$*/+* (n = 12) to *GMR:Gal4, UAS:DmPpt1*$^{8.1}$*/pGMR:p35 ; UAS:DmPpt1*$^{2.1}$*/+* (n = 11). To determine the effect of removing one copy of the *DmPpt1* genomic locus on the over-expression phenotype, we obtained two deficiencies, Df(1)KA12 and Df(1)RA2, from the Bloomington Stock Center. We confirmed that they both removed the *Ppt1* locus by performing quantitative southern blots using the *DmPpt1* cDNA as a probe (data not shown). For the analysis, we collected and compare the following genotypes *+/+; GMR:Gal4, UAS:DmPpt1*$^{8.1}$*/+; UAS:DmPpt1*$^{2.1}$*/+* (n = 6), *Df(1)KA12/+; GMR:Gal4, UAS:DmPpt1*$^{8.1}$*/+; UAS:DmPpt1*$^{2.1}$*/+* (n = 5), and *Df(1)RA2/+; GMR:Gal4, UAS:DmPpt1*$^{8.1}$*/+; UAS:DmPpt1*$^{2.1}$*/+* (n = 5). Newly eclosed adults of the specific genotypes for both experiments were collected and analyzed by SEM as described above.

Authors' Contributions

CK and MM conceived of the study and designed the experiments described. CK carried out all of the experiments and drafted the manuscript. MM played an advisory role as the project developed and edited the draft manuscript. All authors read and approved the final manuscript.

Abbreviations

NCL, Neuronal Ceroid Lipofuscinosis; INCL, Infantile Neuronal Ceroid Lipofuscinosis; PPT1, Palmitoyl Protein-thioesterase 1

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