

Genetic Modifiers of *Drosophila* Palmitoyl-Protein Thioesterase 1-Induced Degeneration

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ABSTRACT

Infantile neuronal ceroid lipofuscinosis (INCL) is a pediatric neurodegenerative disease caused by mutations in the human *CLN1* gene. *CLN1* encodes palmitoyl-protein thioesterase 1 (PPT1), suggesting an important role for the regulation of palmitoylation in normal neuronal function. To further elucidate Ppt1 function, we performed a gain-of-function modifier screen in *Drosophila* using a collection of enhancer-promoter transgenic lines to suppress or enhance the degeneration produced by overexpression of Ppt1 in the adult visual system. Modifier genes identified in our screen connect Ppt1 function to synaptic vesicle cycling, endo-lysosomal trafficking, synaptic development, and activity-dependent remodeling of the synapse. Furthermore, several homologs of the modifying genes are known to be regulated by palmitoylation in other systems and may be *in vivo* substrates for Ppt1. Our results complement recent work on mouse *Ppt1*^{-/-} cells that shows a reduction in synaptic vesicle pools in primary neuronal cultures and defects in endosomal trafficking in human fibroblasts. The pathways and processes implicated by our modifier loci shed light on the normal cellular function of Ppt1. A greater understanding of Ppt1 function in these cellular processes will provide valuable insight into the molecular etiology of the neuronal dysfunction underlying the disease.

THE neuronal ceroid lipofuscinoses (NCLs) are a set of predominantly recessive pediatric neurological diseases that cause neurodegeneration in the retina, cortex, and cerebellum, leading to symptoms that include loss of vision, motor dysfunction, intellectual decline, and seizures (WISNIEWSKI *et al.* 2001). The NCLs have a worldwide occurrence of 0.1–7/100,000 births (WISNIEWSKI 2005). A subset of a wider group of disorders, each NCL subtype is classified by its characteristic lysosomal inclusion pathology and age of onset. Genetic analysis of the NCLs has identified eight loci, *CLN1–6*, *CLN8*, and *CTSD*, each with a different age of onset (WISNIEWSKI 2005; SIINTOLA *et al.* 2006). Most patient cells display the signature inclusions and an accumulation of autofluorescent lipopigment although only neurons undergo degeneration (KIDA *et al.* 2001; WISNIEWSKI 2005). Infantile onset NCL (INCL), produced by loss of palmitoyl-protein thioesterase 1 (PPT1) function, is the earliest and most severe form of NCL with symptoms appearing as early as 6 months of age and culminating with death between 24 months and 53 years of age (WISNIEWSKI 2005). Although primarily associated with INCL, mutations in *Ppt1* can also give rise to late-infantile, juvenile, and adult-onset NCL (MITCHISON *et al.* 1998; VAN DIGGELEN *et al.* 2001). This

variation in age of onset is likely related to residual enzyme activity produced by the specific mutation(s) a patient carries (WISNIEWSKI 2005). INCL is characterized at the cellular level by the presence of granular osmiophilic deposits (GRODs), the lysosomal accumulation of saposins A and D, and an almost complete loss of cortical neurons at the time of patient death (WISNIEWSKI *et al.* 2001).

An open question in the understanding of NCLs and other lysosomal storage disorders is the identification of the underlying molecular cause of the disease phenotype. In particular, is the inclusion pathology the primary insult to the cell or do specific disease pathways, whether or not they lead to inclusions, underlie the physical symptoms and eventual neuronal cell death (reviewed in FUTERMAN and VAN MEER 2004)? Recent work on several lysosomal storage disorders has shown that the storage phenotype may be secondary to more significant changes in cell physiology produced by the loss of a particular protein. This loss of protein function may have a primary effect on specific cellular processes or signal transduction mechanisms, leading directly or through secondary effects to the observed disease pathology (reviewed in FUTERMAN and VAN MEER 2004). Work on NCLs, including INCL, in mice, sheep, and humans has described the amount and regional location of cellular storage material in affected brains and compared it to the progressive cellular pathology seen in each subtype (BIBLE *et al.* 2004; MITCHISON *et al.* 2004). These analyses suggest no clear connection

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between the amount of storage material and the observed neurodegeneration in these systems, leading to the hypothesis that it is the disruption of certain cellular processes or signaling pathways in the patient's neuronal cells that produces dysfunction and degeneration (OSWALD *et al.* 2005). Identifying the earliest cellular insults in INCL patient neurons requires the characterization of the normal cellular function of PPT1 so that a more targeted approach to understanding the disease process can be undertaken.

The identification of human *PPT1* as the gene mutated in INCL indicates that there is a significant role for the regulation of palmitoylation in normal neuronal function (VESA *et al.* 1995). Palmitoylation, a dynamic post-translational modification, is critical for the cellular localization and modulation of many signaling proteins (MILLIGAN *et al.* 1995; SMOTRYS and LINDER 2004). Consistent with an important role in neurons, several proteins integral to the development and regulation of the synapse are known to be palmitoylated (EL-HUSSEINI and BREDT 2002). As expected from a lysosomal enzyme, histochemical and biochemical analysis of PPT1 has demonstrated localization within the endo-lysosomal compartment in neuronal and non-neuronal cell lines (CAMP and HOFFMAN 1993; HELLENSTEN *et al.* 1996; VERKRUUSE and HOFFMANN 1996; CHO and DAWSON 2000; VIRMANI *et al.* 2005). In cultured neuronal cells, PPT1 has also been found associated with lipid raft membrane microdomains and synaptosomes, suggesting a neuron-specific function for the protein that may be important in the development of the disease (LEHTOVIRTA *et al.* 2001; AHTIAINEN *et al.* 2003; GOSWAMI *et al.* 2005). PPT1's importance for neuronal function is underscored by the expression of *Ppt1* mRNA and protein in the retina and brain in a regulated pattern that coincides with major developmental changes in the nervous system (ISOSOMPPPI *et al.* 1999; SUOPANKI *et al.* 1999a,b; ZHANG *et al.* 1999). Finally, a model of excitotoxicity in the rat brain confirmed a presynaptic localization for the protein and suggests that PPT1 may be neuro-protective during an excitotoxic event (SUOPANKI *et al.* 2002).

Recently, progress has been made toward understanding the primary cellular defects underlying INCL disease pathology. Expression of PPT1 has been shown to modulate apoptotic cell death mediated by the activation of the Ras-AKT pathway and neutral sphingomyelinase 2 in several different neural-derived cell lines (CHO and DAWSON 2000; GOSWAMI *et al.* 2005). Work in cultured *Ppt1*^{-/-} mouse neuronal cells demonstrated a reduction in synaptic vesicle pool size in the absence of large amounts of storage material, suggesting that synaptic abnormalities may contribute to the early progression of INCL (VIRMANI *et al.* 2005). Changes in endo-lysosomal function have also been associated with the accumulation of saposin A and D, the main protein component of INCL storage material,

in cultured *Ppt1*^{-/-} fibroblasts (AHTIAINEN *et al.* 2006). Finally, Ppt1 was identified in a cell-based screen as a modifier of amyloid precursor protein shedding, a process that is regulated in part by endocytosis (SCHOBEL *et al.* 2006). A picture is now emerging on the basis of these results whereby Ppt1 may impact the efficient synaptic function of neurons by regulating specific signaling pathways and modulating endocytosis.

The analysis of the *Drosophila* homologs of the human cathepsin D and PPT1 genes suggests that the fruit fly is a valuable model system for studying lysosomal storage disorders and the NCLs in particular. Mutations in the fly homolog of the lysosomal aspartyl protease cathepsin D produce the characteristic autofluorescent storage material and GRODs associated with human cases of congenital NCL as well as a low level of age-dependent neurodegeneration in the adult *Drosophila* brain (MYLLYKANGAS *et al.* 2005). *Ppt1*⁻ flies are viable, have a reduced life span, and show a central nervous system (CNS)-specific accumulation of autofluorescent storage material. The cytoplasmic inclusions, however, are different in morphology in contrast to the granular osmiophilic deposits typical of INCL patients (HICKEY *et al.* 2006). These two mutants suggest an evolutionary conservation of function in the fly that make it a powerful model for understanding the molecular etiology of human NCL phenotypes (MYLLYKANGAS *et al.* 2005; HICKEY *et al.* 2006). Previously, we showed that the targeted overexpression of *Ppt1* in the developing *Drosophila* visual system leads to the loss of cells, including neurons, through apoptotic cell death both early in eye development and after ommatidial differentiation has finished, yielding black ommatidial spots (KOREY and MACDONALD 2003). Taken together, the fly models of Ppt1 function indicate the importance of the proper regulation of enzyme activity for normal cellular function.

The development of both loss-of-function and gain-of-function models for *Ppt1* in *Drosophila* is critical for the future examination of the cellular basis of neuronal dysfunction and degeneration in INCL patients. An advantage of *Drosophila* and other small eukaryotic model systems lies in the ability to perform large-scale second-site genetic modifier screens (MUQUIT and FEANY 2002; PHILLIPS *et al.* 2006). This approach allows for the unbiased identification of specific genes and cellular pathways that are relevant to protein function and disease progression. To identify *in vivo* substrates and signaling pathways that are regulated by Ppt1 activity, we have here performed an F₁ dominant gain-of-function modifier screen using our Ppt1 overexpression model and a collection of enhancer-promoter lines (RORTH *et al.* 1998). The modifiers identified in this screen further support a role for Ppt1 in the regulation of synaptic vesicle endocytosis. Our results also suggest that Ppt1 modulates the activity of several pathways known to play a role in synaptic development either directly through its depalmitoylation activity

or indirectly through effects on general endocytic mechanisms.

MATERIALS AND METHODS

Drosophila stocks and fly husbandry: Flies were raised on standard media and all crosses were carried out at 25°. The enhancer–promoter (EP) stocks and other stocks were obtained from both the Szeged and Bloomington Stock Centers. The enhancer–yellow (EY) stocks were obtained from the Bloomington Stock Center. The *UAS:Ppt1* transgenic lines were previously described in KOREY and MACDONALD (2003). All other lines used in this study were obtained from Bloomington except for the following: *UAS:mys^{βPS}* (BEUMER *et al.* 1999), *fasII^{fb112}* (GRENNINGLOH *et al.* 1991), *UAS:fasII* (HOLMES and HEILIG 1999), *faf^{BX4}*, and *UAS:faf* (HUANG *et al.* 1995), *endoA^{Δ4}* and *UAS:endoA* (VERSTREKEN *et al.* 2003), *UAS:dFos* (ERESH *et al.* 1997), *UAS:sax* (YU *et al.* 2004), *UAS:syt1* (LITTLETON *et al.* 1999), and *UAS:stonedA* and *UAS:stonedB* (ESTES *et al.* 2003).

Screen genetics: EP and EY genetic modifiers of Ppt1-induced degeneration were identified by their ability to modify *GMR:Gal4*, *UAS:Ppt1^{8.1}/+*; *UAS:Ppt1^{2.1}/+* adult flies. All screen crosses were done blinded, with neither the EP or EY line number nor with the molecular location of the insertion known to the screeners. The EP/EY lines were unblinded after the secondary screen. Nonspecific modification due to the effects on the Gal4/upstream activating sequence (UAS) system were eliminated by their ability to also modify two unrelated phenotypes produced by *UAS:Tau^{V337M}/+*; *GMR:Gal4/+* adults (WHITMANN *et al.* 2001; SHULMAN and FEANY 2003) and *GMR:Gal4/+* adults at 29° (FERNANDEZ-FUNEZ *et al.* 2000). The *UAS:Tau^{V337M}* line expresses a mutant Val³³⁷ → Met (V337M) human Tau protein that is associated with early onset, familial dementia (WITTMANN *et al.* 2001).

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 subjected to a series of ethanol dehydration steps. Initially placed in 25% ethanol, they were moved to 50% ethanol after a 12-hr incubation time. This process continued through the following dilutions: 50, 75, 95, and 2× 100% ethanol. They remained 100% ethanol until critical point drying and sputter coating for scanning electron microscopy at the Northeastern Electron Microscopy facility.

In situ hybridization: EP/EY lines that were unable to be confirmed using *UAS* lines were further analyzed using *in situ* hybridization. Each line was crossed to *Gal4* driver lines and the resulting *trans*-heterozygote embryos or larvae were processed for *in situ* hybridization as previously described (VAN VACTOR and KOPCZYNSKI 1999). cDNA clones of modifier genes were used for production of sense and antisense probes. The clones were identified with the Berkeley Drosophila Genome Project database and obtained from the Drosophila Genomic Research Center.

Ppt1 enzyme assay: Ppt1 enzyme activity levels were assayed as described in HICKEY *et al.* (2006). The heads of single flies of the correct genotype were dissected and placed in individual wells of a 96-well plate on ice. Each well was then filled with 20 μl of ddH₂O and 10 μl of substrate (0.375 mg/ml 4MU-6S-palm-β-Glc substrate (Moscerdam Substrates); 0.2 M Na phosphate/0.1 M citrate, pH 4.0; 15 mM DTT; 0.09% BSA; and 5 units/ml β-glucosidase). The individual heads were homogenized in the substrate buffer using a 96-well plate homogenizer (Burkard Scientific, UK). The homogenate was incubated for 2 hr at 30°. The reaction was stopped by adding 100 μl of stop buffer (0.5 M NaHCO₃/Na₂CO₃, pH 10.7,

0.025% Triton X-100) and then the amount of fluorescence at 460 nm was read on a Perkin-Elmer (Norwalk, CT) HTS7000 BioAssay Reader. All samples were done in triplicate.

RESULTS

Ppt1 dominant modifier screen: Previously we have shown that overexpressed Drosophila Ppt1 has a degenerative effect on the developing adult visual system (KOREY and MACDONALD 2003). This effect is dose dependent and requires a catalytically active Ppt1 enzyme. The overexpression phenotype was generated with the Gal4/UAS system that permits the expression of a gene in a specific tissue or during a particular developmental stage (BRAND and PERRIMON 1993). We found that *GMR:Gal4*-directed expression of a *UAS:Ppt1* transgene in the developing eye led to the loss of cells, including neurons, through apoptotic cell death both early in eye development and also after ommatidial differentiation had finished, yielding black ommatidial spots (KOREY and MACDONALD 2003). Finally, the *Ppt1* overexpression phenotype is suppressed by coexpression of a *UAS:Ppt1-RNAi* transgene, indicating that the phenotype is specific to *Ppt1* function (HICKEY *et al.* 2006; data not shown).

This degenerative phenotype provides a useful assay to identify other genetic loci that may modify this phenotype and thus elucidate the cellular role of Ppt1. We performed an F₁ dominant gain-of-function modifier screen using a subset of the EP and EY collections available from the Bloomington and Szeged Drosophila stock centers (RORTH *et al.* 1998). EP/EY lines have a *UAS*-containing transposable *P*-element randomly inserted within the genome. If the element is within the 5' untranslated region or close to the transcriptional start site of an open reading frame, it can direct the expression of this gene in a Gal4-dependent fashion. We combined our previous *GMR:Gal4; UAS:Ppt1* phenotype with the EP/EY lines to identify a collection of genes that could modify the *Ppt1*-induced degeneration when coexpressed.

We screened 1948 EP/EY lines using the *GMR:Gal4; UAS:Ppt1* overexpression system (Figure 1, A and B). Progeny from crosses between *GMR:Gal4; UAS:Ppt1* and each EP/EY line were compared to control flies expressing *Ppt1* alone to determine if the unknown gene being coexpressed enhanced or suppressed the degenerative effect. Suppressors showed a restoration of the outer ommatidial surface of the eye and an increased surface area, while enhancers further reduced the size of the eye with a concomitant loss of ommatidial structure on the eye surface.

After the primary genetic screen, putative enhancers and suppressors were put through a series of elimination criteria to produce a final list of modifiers (Figure 1C). First, all identified modifier lines went through a secondary screen to confirm the repeatability of the enhancer

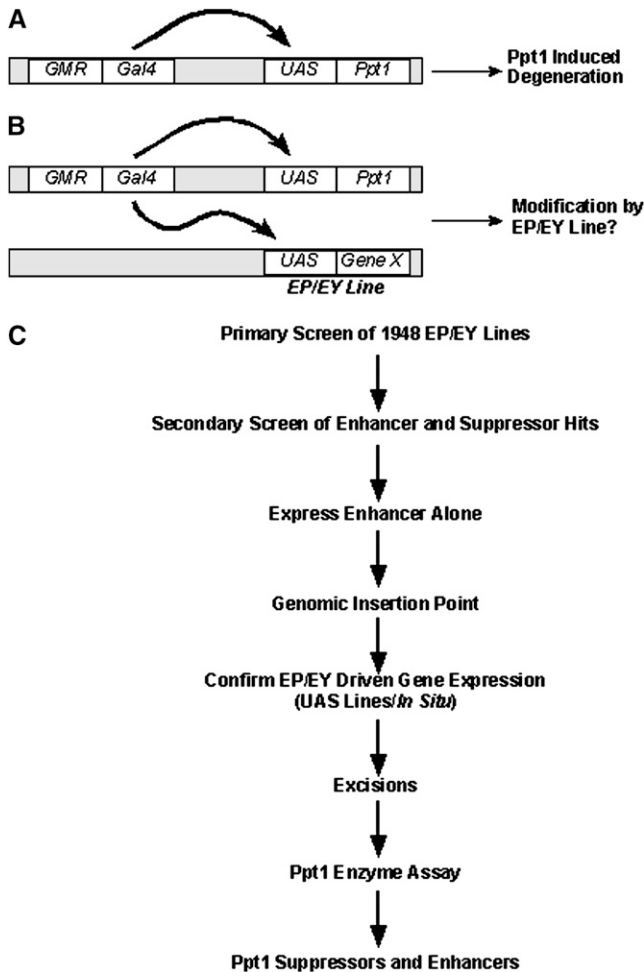


FIGURE 1.—Gain-of-function modifier screen. (A) *Ppt1* was overexpressed using the Gal4/UAS system. Transcription factors binding to the GMR promoter initiate transcription of Gal4 that in turn binds to the upstream activating sequence, allowing the synthesis of Ppt1. (B) When EP/EY lines were crossed to flies diagrammed in A, Gal4 turned on a random gene driven by the EP/EY transgene as well as *Ppt1*. Progeny in the F₁ were screened to determine if the unknown gene being coexpressed with *Ppt1* enhances or suppresses the degeneration produced by Ppt1 activity. (C) A schematic of the secondary screening procedures used to finalize the genetic modifiers of Ppt1 listed in Table 1.

or suppressor effect. Those that did not yield the result obtained in the primary screen were eliminated. Confirmed enhancers were then crossed to *GMR:Gal4* alone to determine if the lines produced eye defects on their own. A rough eye indicated that the EP/EY line enhancement might be due to a nonspecific additive effect of the expression of both proteins rather than an enhancement specific to Ppt1 function. Thus these lines were also eliminated.

The genomic insertion point of the transgene was then checked using the resources available at NCBI to determine if the EP/EY element was inserted in the correct position for producing expression of the asso-

ciated open reading frame. Those that were inserted within the gene but past the start codon, and therefore were likely to produce a truncated form of the protein, were eliminated. Transgene insertions >1000 bp away from an open reading frame and those in a region with no identifiable open reading frames were also eliminated. Modifier lines advanced to further screening if the EP/EY element was inserted within 500 bp of the gene, before the start codon, and in the correct orientation for gene expression.

Some of the modifiers could affect the Gal4/UAS system employed in the screen rather than Ppt1's cellular activity. To account for this possibility, we crossed all of the remaining EP/EY lines to two unrelated Gal4-induced rough-eye phenotypes: *GMR:Gal4* alone and *GMR:Gal4; UAS:Tau^{V337M}*. Due to high levels of Gal4 expression, *GMR:Gal4* flies develop a rough eye when grown at 29° (FERNANDEZ-FUNEZ *et al.* 2000). *GMR*-driven *UAS:Tau^{V337M}* is a previously published model for tauopathies in *Drosophila* (SHULMAN and FEANY 2003). If a candidate Ppt1 modifier produced genetic modification in the progeny of both of these crosses that was similar to that which we observed in our original screen, we assumed that the EP/EY line might have a generalized effect on the driver system, and these lines were eliminated as well.

To control for the effects of genetic background, excisions of the EP/EY element were produced for each modifier line. Excision lines were crossed to the original *GMR:Gal4; UAS:Ppt1* line to determine if the modifying effect was retained. Those lines that retained the suppressor or enhancer effect in the absence of the EP/EY insertion likely do so because of genetic alterations other than the presence of the transgene and were eliminated from consideration. Finally, the remaining EP/EY modifier genes were further characterized to determine whether the associated gene was being expressed. For a subset of lines (7/20), we were able to confirm expression with available published UAS lines that are known to express a specific gene. The expression of the remaining EP/EY lines (13/20) was confirmed by driving the associated locus with a tissue-specific Gal4 line and visualizing mRNA production by *in situ* hybridization.

Finally, we used a fluorogenic assay designed for the diagnosis of INCL in humans to confirm that the suppression or enhancement observed in these modifier lines was not due to significantly decreased or increased levels of Ppt1 (VAN DIGGELEN *et al.* 1999). Since the expression of *Ppt1* and the EP/EY modifier lines was under the control of *GMR:Gal4* in the developing adult visual system, we used a modified version of the assay in which single adult heads of the appropriate genotypes were dissected and homogenized in the presence of the 4-methylumbelliferyl-6-thiopalmityl-β-D-glucoside substrate (HICKEY *et al.* 2006). While the assay does not directly assay for total Ppt1 protein concentration, enzyme activity provides a reliable proxy for the determination

TABLE 1
Genetic modifiers of *Ppt1*-induced degeneration

Gene	Mammalian homolog	Line	Insertion	Modification ^a	Verification ^b	Loss-of-function alleles ^c
Endocytosis/trafficking						
<i>Endophilin A</i>	Endophilin A	<i>EP(3)464</i>	91D4	Enh	<i>UAS</i>	<i>endoA^{Δ4}/NE</i>
<i>CG14709</i>	Multidrug resistance-associated protein	<i>EP(3)430</i>	86E11	Enh	<i>In situ</i>	NA
<i>Blue cheese</i>	ALFY	<i>EY(3)2503</i>	26A6	Enh	<i>In situ</i>	NA
<i>CG5991</i>	Phosphatidylserine decarboxylase	<i>EY(3)3559</i>	95D5	Enh	<i>In situ</i>	NA
<i>CG32138</i>	Formin-like 2	<i>EY(3)3931</i>	70D1	Enh	<i>In situ</i>	NA
Ubiquitination						
<i>Fat facets</i>	Ubiquitin protease 9	<i>EY(3)2018</i>	100E1	Enh	<i>UAS</i>	<i>faj^{βX4}/NE</i>
<i>UbcE2H</i>	Ubc-E2H	<i>EP(X)1303</i>	7D6	Sup	<i>In situ</i>	NA
<i>CG7023</i>	Ubiquitin protease 12-like	<i>EY(3)249</i>	94C4-6	Sup	<i>In situ</i>	NA
Cell adhesion						
<i>Myospheroid</i>	β-integrin	<i>EP(X)1033</i>	7D3-5	Enh	<i>UAS</i>	<i>mys¹/NE</i>
<i>Fasciclin 2</i>	NCAM2	<i>EP(X)1462</i>	4B1-3	Sup	<i>UAS</i>	<i>fasII^{6b112}/NE</i>
<i>Miniature</i>	Novel	<i>EP(X)406</i>	10D8	Sup	<i>In situ</i>	<i>m¹/NE</i>
Signaling pathways						
<i>Misshappen</i>	Ste20-related kinase	<i>EP(3)549</i>	62E6-7	Enh	<i>UAS</i>	<i>msn¹⁰²/NE</i>
<i>Kayak</i>	FosB	<i>EY(3)283</i>	99C2	Sup	<i>UAS</i>	<i>kay^{stro-1}/NE</i>
<i>Saxophone</i>	TGF-β receptor type I	<i>EY(2)4377</i>	43E18	Sup	<i>UAS</i>	<i>sax⁵/NE</i>
<i>Mesr4</i>	Novel	<i>EP(2)386</i>	54C3-7	Sup	<i>In situ</i>	NA
<i>Fs(1)N</i>	Novel	<i>EP(X)1336</i>	1F01-02	Sup	<i>In situ</i>	<i>fs(1)N¹/E</i>
Miscellaneous						
<i>Hsc70-3</i>	BiP chaperone	<i>EP(X)1507</i>	10E3-4	Sup	<i>In situ</i>	NA
<i>CG18177</i>	N-acetyltransferase	<i>EP(3)3301</i>	67C5	Enh	<i>In situ</i>	NA
<i>CG3654</i>	Novel	<i>EY(3)142</i>	67B9	Enh	<i>In situ</i>	NA
<i>CG5859</i>	Integrator complex subunit 8	<i>EP(2)2090</i>	53D13	Sup	<i>In situ</i>	NA

^aEnh, enhancer; Sup, suppressor.

^bUAS, published line; *In situ*, *In situ* hybridization.

^cLoss-of-function alleles used for each gene; NE, no effect; E, enhance; NA, no specific alleles available.

of major changes in enzyme function. We found that none of the 20 modifier lines significantly changed the levels of Ppt1 activity compared to the control *GMR:Gal4; UAS:Ppt1* line used in the screen (data not shown). Of the original 1948 lines, 10 enhancers and 10 suppressors of the *Ppt1* eye phenotype survived our stringent prioritization criteria. These are described in Table 1 with the accompanying functional information and verification procedures.

Endocytosis and endosomal trafficking: A major class of modifiers of our *Ppt1*-induced degenerative phenotype is associated with endocytic mechanisms. *EP(3)464*-induced expression of *endophilin A* (*endoA*) was found to be an enhancer of the Ppt1 phenotype (Figure 2E). *EndoA*, the fly homolog of the SH3-domain-containing protein endophilin, plays a role in synaptic vesicle endocytosis and recycling. We confirmed the enhancement by overexpression of *endoA* using a *UAS-endoA* transgene (Table 1).

The enhancer *EY(3)2503* is predicted to drive the expression of *blue cheese* (*bchs*). *Bchs* is localized to CNS neurons/axons, and loss-of-function mutants in *Drosophila* show progressive neural degeneration (Finley *et al.* 2003). The presence of BEACH and FYVE domains

in *Bchs* suggests a role for *Bchs* in endo-lysosomal maturation and trafficking in neurons (FINLEY *et al.* 2003). We confirmed the overexpression of *bchs* by *EY(3)2503* by mRNA *in situ* hybridization (Table 1).

Hsc70-3, when driven by *EP(X)1507*, was found to be a suppressor. *Hsc70-3* is the fly homolog of BiP, a chaperone for secretory and membrane proteins in endoplasmic reticulum. This protein is upregulated in *Aplysia* models of long-term memory and may be required for the efficient trafficking of new proteins in support of activity-dependent synaptic changes (KUHL *et al.* 1992).

We also identified two genes involved with lipid metabolism that may impact endocytic mechanisms. *EY(3)3559*, an enhancer of the Ppt1 eye phenotype, is predicted to direct the expression of *CG5991*, a gene that encodes the enzyme phosphatidylserine decarboxylase, which converts phosphatidylserine to phosphatidylethanolamine in the mitochondrial inner membrane. Overexpression of *CG14709*, a protein with homology to ATP-binding cassette/multi-drug-resistance-associated proteins, by *EP(3)430* was identified as an enhancer. This family of integral membrane proteins is involved in the transport of various substrates across the lipid bilayer.

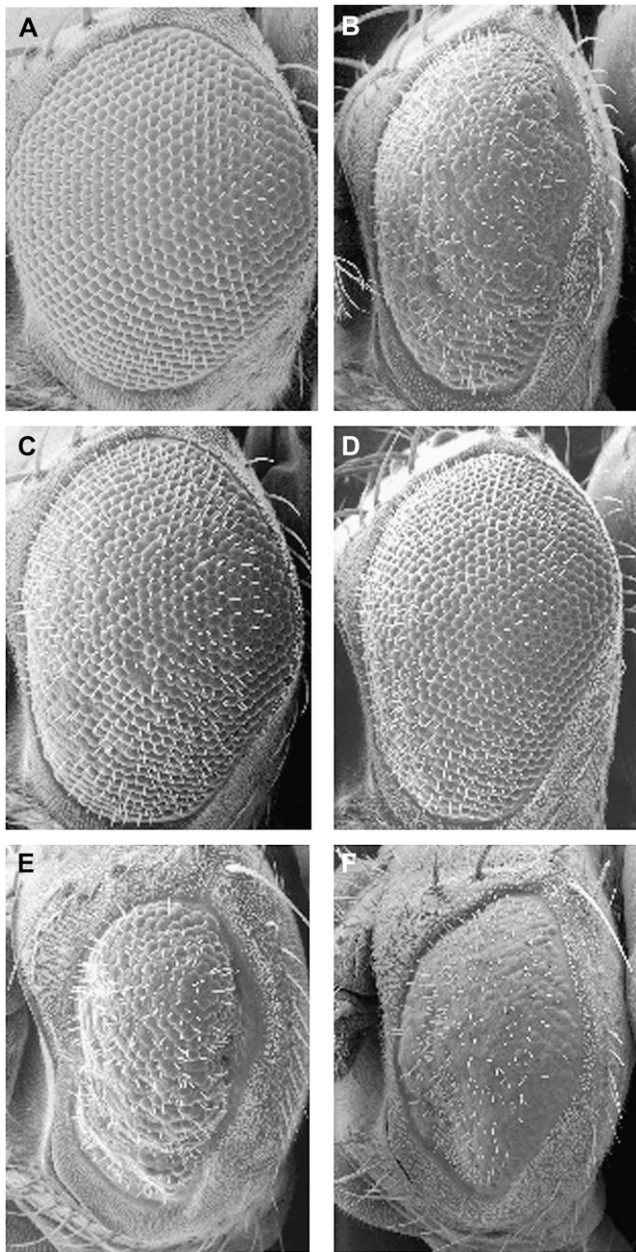


FIGURE 2.—Enhancers and suppressors of Ppt1-induced degeneration scanning electron images of adult eyes ($\times 200$). (A–F) SEM images showing the external surface of control and identified *Ppt1* modifiers. (A) *UAS:Ppt1^{8.1}/CyO*. (B) *GMR:Gal4*, *UAS:Ppt1^{8.1}/+*; *UAS:Ppt1^{2.1}/+*. (C) *EP(X)1462/+*; *GMR:Gal4*, *UAS:Ppt1^{8.1}/+*; *UAS:Ppt1^{2.1}/+*. (D) *GMR:Gal4*, *UAS:Ppt1^{8.1}/+*; *UAS:Ppt1^{2.1}/EP(3)283*. (E) *GMR:Gal4*, *UAS:Ppt1^{8.1}/+*; *UAS:Ppt1^{2.1}/EP(3)464*. (F) *GMR:Gal4*, *UAS:Ppt1^{8.1}/+*; *UAS:Ppt1^{2.1}/EP(3)549*.

Since several modifiers from our screen, particularly those associated with neuronal function, implicated Ppt1 in endocytic processes, we obtained published *UAS* lines that expressed proteins known to play a role in synaptic vesicle endocytosis. For example, synaptotagmin is a calcium sensor for synaptic vesicle fusion and facilitates endocytosis (YOSHIHARA and MONTANA 2004) while stoned A and B are atypical adaptor proteins

that bind to and facilitate synaptotagmin recycling (FERGESTAD *et al.* 1999; PHILLIPS *et al.* 2000). We examined *UAS* lines for *synaptotagmin* (*syt1*), *stoned A* (*stnA*), and *stoned B* (*stnB*) for their ability to modify the *Ppt1* phenotype when coexpressed using *GMR-Gal4*. We found that both *syt1* and *stnA* suppressed eye degeneration (Figure 3, B and C) while *stnB* had no effect as compared to expression of *Ppt1* alone (data not shown). In contrast to genes involved in endocytosis, we found that a *UAS* line that expresses the ATPase *Hsc70-4*, a protein involved in synaptic vesicle exocytosis (BRONK *et al.* 2001), had no modifying effect (data not shown).

Ubiquitination: In addition, we identified two proteases and one conjugating enzyme associated with the process of protein ubiquitination. The enhancer *EY(3)2018* drives the expression of the gene *fat facets* (*faf*), the fly homolog of ubiquitin-specific protease 9. In *Drosophila*, *faf* is important for eye differentiation and synaptic development at the larval neuromuscular junction (FISCHER and OVERSTREET 2002). We confirmed the enhancement produced by *EY(3)2018* using a *UAS:faf* transgene (Table 1). The other predicted protease found to be a suppressor when driven by *EY(3)249* is *CG7023*, the fly homolog of human ubiquitin-specific protease 12-like and mouse Ubh1. Finally, we identified *EP(X)1303* as a suppressor. This line is predicted to drive the expression of the fly homolog of the ubiquitin-conjugating enzyme, E2H.

Cell adhesion: Our modifier collection also revealed several genes that control cell adhesion. The β -integrin homolog *mysospheroid* (*mys*), driven by *EP(X)1033*, was identified as an enhancer of Ppt1-induced degeneration. In *Drosophila*, *mys* is involved in several cell adhesion-dependent processes during development, including axon guidance and activity-dependent synaptic development (HOANG and CHIBA 1998; BEUMER *et al.* 1999; ROHRBOUGH *et al.* 2000). We confirmed *mys*-specific modification of *Ppt1* degeneration using a *UAS:mys* transgene (Table 1; data not shown).

Expression of the neural cell adhesion molecule *fasciclin II* (*fasII*) by *EP(X)1462* was found to be suppressor (Figure 2C). FasII function is critical for axon path finding, synaptogenesis, and synaptic plasticity in the fly. We confirmed suppression of the eye phenotype using a *UAS:fasII* transgene (Table 1; data not shown). In addition, *EP(X)406*-driven expression of the novel gene *miniature* was a suppressor of the Ppt1 phenotype. We confirmed overexpression of *miniature* by *in situ* hybridization (Table 1).

Signaling: Components of known intracellular signaling pathways were also recovered in our modifier screen. *EP(3)549*, an enhancer of Ppt1, expresses the Ste-20-related serine/threonine kinase *misshapen* (*msn*) (Figure 2F). We confirmed the enhancement by overexpression of *misshapen* using a *UAS:msn* transgene (Table 1; data not shown). Recent work has identified *msn* as a participant in the regulation of dorsal closure through

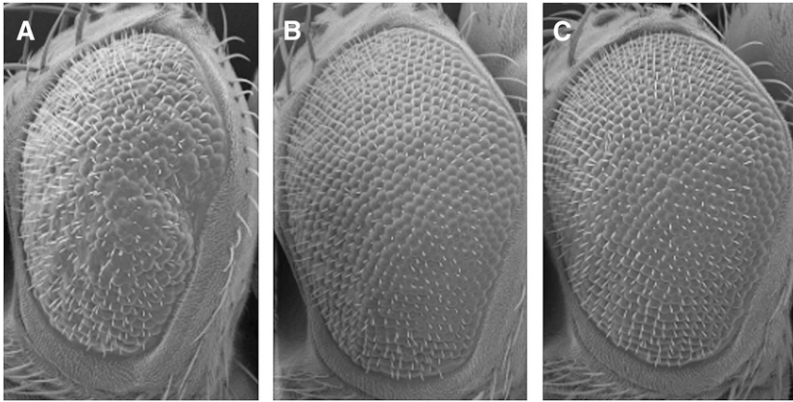


FIGURE 3.—Ppt1-induced degeneration is modified by genes involved in endocytosis scanning electron images of adult eyes ($\times 200$). (A–C) SEM images showing the external surface of control and identified Ppt1 modifiers. (A) *GMR:Gal4, UAS:Ppt1^{8.1}/+; UAS:Ppt1^{2.1}/+*. (B) *UAS:syt1/+; GMR:Gal4, UAS:Ppt1^{8.1}/+; UAS:Ppt1^{2.1}/+*. (C) *GMR:Gal4, UAS:Ppt1^{8.1}/UAS:stnA; UAS:dPpt1^{2.1}/+*.

the activation of Jun kinase (JNK) signaling and retinal axon guidance (DAN *et al.* 2001).

Kayak, driven by *EY(3)283*, was identified as a suppressor of Ppt1-induced degeneration (Figure 2D). Also known as *dFos*, *kayak* encodes a transcription factor that is part of the AP1 transcriptional activation complex with dJun (GOBERDHAN and WILSON 1998). AP1-stimulated transcription is activated by JNK and is involved in both embryonic dorsal closure and synaptic plasticity (GOBERDHAN and WILSON 1998; SANYAL *et al.* 2002). The suppression of the eye phenotype was confirmed using a *UAS:dfos* transgene (Table 1; data not shown).

Saxophone (sax), the fly homolog of a TGF- β type I receptor, was also identified as a suppressor. *EY(2)4377*-driven expression of *sax* was confirmed using a *UAS:sax* transgene (Table 1; data not shown). TGF- β signaling is known to play a role in multiple processes during *Drosophila* development, including the formation of the neuromuscular junction (MARQUES 2005).

EY(3)3931, an enhancer of the Ppt1 eye phenotype, expresses *CG32138*. *CG32138*, the fly homolog of the formin-like 2 protein, contains a formin homology-2, formin homology-3, and GTPase-binding domains. Formin-domain-containing proteins function in actin fiber formation and play a role in a diverse array of cellular processes, including stress fiber formation, cytokinesis, cell motility, and endocytosis (FAIX and GROSSE 2006).

EP(X)1336-driven expression of the novel protein *fs(1)Nasrat [fs(1)N]* suppressed the Ppt1 eye phenotype and was confirmed by *in situ* hybridization. We also found that the loss-of-function allele, *fs(1)N¹*, produces a dominant enhancement of the Ppt1 degeneration (Table 1; data not shown). Finally, we identified *EP(2)386* as an enhancer of the Ppt1 eye phenotype. *EP(2)386* drives the expression of *mesr4*, a transcription factor identified in a RAS1 genetic modifier screen, and expression was confirmed by *in situ* hybridization (HUANG and RUBIN, 2000).

Miscellaneous modifiers: We identified *CG18177*, driven by *EP(3)3301*, as an enhancer of Ppt1-induced degeneration. *CG18177* encodes a novel protein with predicted *n*-acetyltransferase activity. The enhancer *EY(3)142*

drives the expression of *CG3654*, a novel putative transcription factor. *CG5859*, the fly homolog of integrator complex subunit 8, suppresses Ppt1-induced degeneration when expressed under the control of *EP(2)2090*.

DISCUSSION

We have presented an F₁ gain-of-function genetic modifier screen that has identified 10 enhancers and 10 suppressors of degeneration induced by the expression of Ppt1 in the developing adult visual system. The use of a random collection of EP and EY lines spanning the X, second, and third chromosomes allowed for an unbiased identification of a characterized set of *Drosophila* Ppt1 modifier loci. These begin to place Ppt1 function in its cellular context and provide candidate *in vivo* substrates for the enzyme. Recent work on several different systems has demonstrated an involvement of Ppt1 in cellular processes associated with endocytosis and endo-lysosomal trafficking. The polarity and shape of neurons require efficient endosomal processes to maintain cellular structure, transport cellular metabolites, and allow rapid communication between the axon/dendrites and the cell soma (NIXON and CATALDO 1995; NIXON 2005). Changes in these pathways might lead to the symptoms associated with INCL due to the importance of the endosomal system for neuronal function (NIXON and CATALDO 1995; NIXON 2005). Furthermore, neurons also make use of rapid exo- and endocytic mechanisms to precisely control synaptic transmission between pre- and postsynaptic regions.

The initial identification of *endoA* as an enhancer of Ppt1-induced degeneration led us to consider a role for Ppt1 in endocytosis. *EndoA*, a cytoplasmic protein essential for synaptic vesicle endocytosis, contains an SH3 domain that binds to other endocytic proteins such as synaptojanin and dynamin and a lysophosphatidic acid acyl transferase domain that plays a role in altering membrane curvature (REUTENS and BEGLEY 2002). The identification of both *EndoA* homologs and Ppt1 in a cell-based cDNA screen for genes that altered the

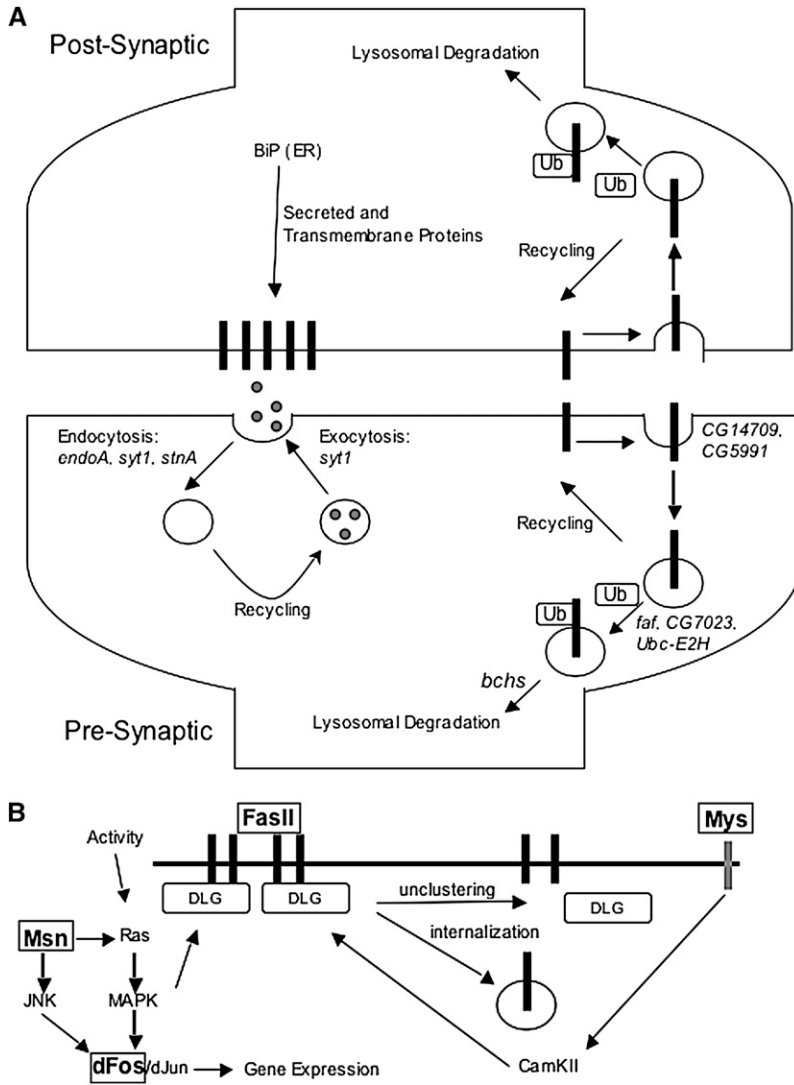


FIGURE 4.—A model of Ppt1-related cellular processes. (A) An illustration of pre- and postsynaptic compartments showing synaptic vesicle cycling and endocytic pathways. The modifiers identified in the screen are placed in the model on the basis of the hypothesis that Ppt1 is connected to these activities. (B) A representation of pathways known to play a role in neuromuscular junction development and activity-dependent remodeling of the synapse. The genes identified as modifiers in our screen are boxed and in bold-face type.

surface shedding of amyloid precursor protein (APP) suggests a connection between their functions (SCHOBEL *et al.* 2006). The expression of the mammalian homologs endophilin-A1 and endophilin-A3 was shown to activate APP shedding in this assay through the inhibition of endocytosis (SCHOBEL *et al.* 2006). These findings indicate that the overexpression of Ppt1 in the Drosophila retina may lead to degeneration by negatively impacting synaptic vesicle and general endocytosis. Similar to *endoA*, our other modifiers implicate Ppt1 function both in the regulation of synaptic vesicle endocytosis and in the modulation of signaling pathways important for neuronal function through the regulation of endocytosis (Figure 4A).

Synaptic vesicle cycling: In addition to the identification of endophilin in the primary screen, the demonstration that the expression of both *syt1* and *stnA* suppressed the *Ppt1* eye phenotype indicates a connection between Ppt1 function and synaptic vesicle endocytosis. As is observed in cultured *Ppt1*^{-/-} neurons from

mice, both *syt1* and *stn* mutants in Drosophila showed decreased synaptic vesicle density at the larval neuromuscular junction (FERGESTAD *et al.* 1999). Work on the fly has demonstrated that Stn proteins not only bind to Syt1, but also are necessary for its maintenance at the synapse (FERGESTAD *et al.* 1999; PHILLIPS *et al.* 2000). Furthermore, overexpression of *syt1* in a *stn* mutant background rescues the lethality and endocytosis defects associated with loss of *stn* (PHILLIPS *et al.* 2000). The suppression of the Ppt1-induced degeneration by expression of *syt1* or *stnA* links Ppt1 expression with the inhibition of synaptic vesicle endocytosis. Palmitoylation of human Syt1 has been shown to be critical for the sorting of the protein to presynaptic vesicle pools, and mutant Syt1 proteins that are not palmitoylated show an increase in general cell surface expression and a reduction in endocytosis (KANG *et al.* 2004). Although Syt1 in Drosophila has not been shown to be palmitoylated, the genetic interaction that we observe with Ppt1 indicates that Syt1 may be an *in vivo* substrate of Ppt1.

Signaling and endocytosis: Our screen identified several genes that have been shown to play a role in endo-lysosomal trafficking or whose cellular functions suggest that they are likely to play a role in this process. These modifiers further support a role for Ppt1 in endocytic mechanisms that was originally indicated by defects observed in Ppt1^{-/-} fibroblasts (AHTIAINEN *et al.* 2006). We identified two deubiquitinating enzymes and an ubiquitin-conjugating enzyme suggesting a connection to ubiquitination, a signal that serves as a molecular tag for internalization of surface proteins and maturation of late endosomes into multi-vesicular bodies for the proteolysis of cellular proteins (SETO *et al.* 2002). Another modifier, the conserved BEACH- and FYVE-domain-containing protein Blue Cheese, is thought to play a role in trafficking through the endosomal pathway (FINLEY *et al.* 2003). Our identification of the enhancer CG32138, a formin-like protein, may also indicate a potential connection to endocytosis in light of recent work that has linked several formin-domain-containing proteins to endosome transport along actin fibers (FAIX and GROSSE 2006). Finally, both phosphatidylserine decarboxylase and CG14709 modulate lipids that are known to play an important role in regulating membrane curvature, vesicle trafficking, and cell signaling (VAN MEER and SPRONG 2004; VANCE and STEENBERGEN 2005).

A large body of work has demonstrated how cellular signaling is intimately connected to endocytic mechanisms (SETO *et al.* 2002). Endocytosis not only is a downregulator of cell surface receptors, but also is important for the activation of signaling, shaping morphogen gradients, and the proper spatial and temporal localization of signaling molecules (SETO *et al.* 2002). These functions of endocytic trafficking are on display in all cell types, including neurons where it controls the development and activity-dependent modulation of synapses. The identification of the TGF- β type I receptor *sax* as a suppressor in our screen suggests a connection between Ppt1 function and a pathway whose modulation by endocytosis is critical for the development and maintenance of the neuromuscular junction in *Drosophila* (SWEENEY and DAVIS 2002; DERMAUT *et al.* 2005; MARQUES 2005).

The control of cell adhesion at the neuromuscular junction is important for synapse growth during development and activity-dependent synapse remodeling. Many pathways that control these processes in *Drosophila* converge on the neural cell adhesion molecule (NCAM) homolog *fasciclin II* (*fasII*), the main integrator of these signals at the synapse (Figure 4B). The levels and localization of Fas II at the synapse help to define the degree of synapse stability such that a reduction in FasII adhesion permits the expansion of the synapse (SCHUSTER *et al.* 1996a,b). Remodeling of the synapse is also controlled by an activity-dependent downregulation of FasII, possibly through an endocytic mechanism

(HOEFFER *et al.* 2003). Studies in *Aplysia* revealed that during long-term facilitation, synapse elaboration is achieved through a MAPK-dependent endocytosis of the *Aplysia* NCAM (BAILEY *et al.* 1992, 1997). In *Drosophila*, work from several labs has shown that activation of the Ras–MAPK pathway produces a similar downregulation of FasII, suggesting an evolutionary conservation of this synaptic plasticity mechanism (KOH *et al.* 2002; HOEFFER *et al.* 2003). Furthermore, MAPK activation causes the transcriptional upregulation of dFos which, with dJun, is part of the AP1 transcriptional activator complex that is involved in the activity-dependent activation of genes involved in long-term plasticity (SANYAL *et al.* 2002; HOEFFER *et al.* 2003). Finally, the endoplasmic reticulum chaperone protein BiP is upregulated during long-term facilitation in *Aplysia* (KUHL *et al.* 1992). It has been proposed that increased expression of this protein is important for the rapid trafficking of the secreted and membrane proteins required for synaptic remodeling (KUHL *et al.* 1992; RUBIO and WENTHOLD 1999).

We have demonstrated that *fasII*, *mysospheroid* (β -*integrin*), *kayak* (*dFos*), and *Hsc70-3* (*BiP*) all modify the degeneration produced by Ppt1 expression. Furthermore, both *misshapen* and *mesr4* were identified in a screen for modifiers of Ras1 function (HUANG and RUBIN 2000). In addition to the regulation of Ras signaling, the Ste20-related kinase Msn also activates JNK signaling during dorsal closure (DAN *et al.* 2001). Similar to the genetic interaction that we observe with *sax*, the identities of these modifiers strongly indicate that the overexpression of Ppt1 appears to have a targeted effect on a subset of pathways critical for the formation and remodeling of the synapse. In future work, it will be important to extend this work and examine the connection among Ppt1, trafficking, and signaling by directly assessing loss- and gain-of-function *Ppt1* lines for defects in endocytosis using several well-characterized tissues such as the larval neuromuscular junction (NMJ) and garland cells. Since published work has shown that neural degeneration does not directly correlate with the amount of storage material, modification of the *Ppt1* loss-of-function inclusion phenotype in the fly may not be relevant to the disease pathology (OSWALD *et al.* 2005). Thus, the results of future NMJ and endocytosis analyses will provide crucial phenotypic resources for the further characterization of the relationships between our modifier loci and Ppt1 function.

Ppt1 substrates: It is possible that some of the effects that we see are due to the misregulation of palmitoylation on specific substrate proteins. For example, the vertebrate NCAM homologs of *fasII* are known to be palmitoylated, providing a second membrane anchor for the protein that is important for NCAM lipid-raft localization (LITTLE *et al.* 1998). Mutant NCAM proteins that cannot be palmitoylated fail to localize correctly, and consequently their signaling activity is reduced

(NIETHAMMER *et al.* 2002). In light of the localization of Ppt1 to lipid rafts, it is possible FasII may also be a Ppt1 substrate.

Ppt1 was originally identified by its ability to depalmitoylate H-Ras (CAMP and HOFMANN 1993). While the palmitoylation state of the *Drosophila* homolog is unknown, the fly homolog does have a C-terminal cysteine residue that could serve as a site of palmitoylation. Since our modifier data suggest an interaction with the Ras-MAPK pathway, some of the genetic interactions that we observe may be due to misregulation of Ras palmitoylation. Work on palmitoylation in several systems has shown that there is no tight consensus sequence that indicates that a particular cysteine will be palmitoylated (SMOTRYS and LINDER 2004). Until recently, this has meant that the palmitoylation state of proteins has been determined on an individual basis. The development of a new biochemical modification technique for palmitoylated proteins has, for the first time, permitted the identification of modified proteins on a global scale in yeast (ROTH *et al.* 2006). In *Drosophila*, very little is known either about specific palmitoylated proteins or about the role of palmitoylation during development. To identify *in vivo* substrates of Ppt1, it will be important to characterize specific candidates, such as Syt1, FasII, and Ras, and to undertake proteome-wide studies of protein palmitoylation in the fly.

Is INCL a synaptopathy?: Many neurodegenerative disorders display cellular dysfunction before the death of specific populations of neuronal cells, particularly in processes related to endocytosis (BOSSY-WETZEL *et al.* 2004; NIXON 2005). For example, in Huntington's disease, changes in dendritic spine morphology and number precede cell death (LI *et al.* 2003) and symptoms can precede overt cell loss (MIZUNO *et al.* 2000). These observations, as well as studies in Huntington's disease mouse models, suggest that changes in synaptic function, including synaptic vesicle cycling and endocytosis, may explain the early progression of the disease (LI *et al.* 2003; SMITH *et al.* 2005). Changes in endosomal function are also one of the earliest pathological markers of Alzheimer's disease (NIXON 2005). Finally, these observations extend to lysosomal storage disorders such as Niemann-Pick Type C and juvenile-onset NCL (JNCL), where recent work has documented changes in endolysosomal trafficking and function (FOSSALE *et al.* 2004; NIXON 2005; CAO *et al.* 2006).

Consistent with the importance of these changes for the pathogenesis of JNCL, membrane-trafficking defects are observed in a cell culture model before the accumulation of the characteristic storage material (FOSSALE *et al.* 2004). Our results, combined with those of others working on Ppt1 models, support the extension of this observation to INCL. Our modifiers suggest the hypothesis that early trafficking changes in patients—both synaptic vesicle and signaling proteins involved in synaptic structure—may lead to a progres-

sive dysfunction of neurons, producing disease symptoms and ultimately widespread cell death. As with all large-scale screens, the modifiers that we have identified must be further validated in *Drosophila Ppt1* mutants and higher eukaryotic systems such as the INCL mouse, as well as in INCL patient cells. The pathways and processes implicated by our results will be valuable points of entry in the future development of therapeutics that aim to ameliorate the consequences of PPT1 deficiency, avoiding the neurodegeneration typical of later stages of INCL.

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