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Identifying Cellular Pathways Modulated by *Drosophila* Palmitoyl-protein Thioesterase 1 Function

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Abstract

Infantile-onset Neuronal Ceroid Lipofuscinosis (INCL) is a severe pediatric neurodegenerative disorder produced by mutations in the gene encoding palmitoyl-protein thioesterase 1 (Ppt1). This enzyme is responsible for the removal of a palmitate post-translational modification from an unknown set of substrate proteins. To better understand the function of Ppt1 in neurons, we performed an unbiased dominant loss-of-function genetic modifier screen in *Drosophila* using a previously characterized Ppt1 gain-of-function system. The enhancers and suppressors identified in our screen make novel connections between Ppt1 and genes involved in cellular trafficking and the modulation of synaptic growth. We further support the relevance of our screen by demonstrating that Garland cells from *Ppt1* loss-of-function mutants have defects in endocytic trafficking. Endocytic tracer uptake and ultrastructural analysis of these non-neuronal cells points to Ppt1 playing a role in modulating the early stages of vesicle formation. This work lays the groundwork for further experimental exploration of these processes to better understand their contributions to the INCL disease process.

Keywords

Neuronal Ceroid Lipofuscinosis; Batten Disease; Palmitoylation; Palmitoyl-protein thioesterase 1; Trafficking; Endocytosis; *Drosophila* Models

Introduction

The Neuronal Ceroid Lipofuscinoses (NCL) are a group of primarily recessive neurodegenerative disorders characterized by regionally specific neurodegeneration in the retina, cortex, and cerebellum (Haltia, 2006). Commonly known as Batten Disease, the loss of major neuronal populations leads to clinical symptoms that include epilepsy, sleep disorders, ataxia, blindness and progressive mental and physical decline (Williams et al., 2006). There are 10 identified forms, each of which is characterized by the presence of lipofuscin-like pathology, distinct lysosomal inclusion pathology and differing age of onset (Haltia, 2006; Williams et al., 2006). Patients with the infantile onset form begin to show

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atrophy of the cortex and cerebellum by 13 months of age, culminating in a substantial loss of neurons at the time of death (Mitchison *et al.*, 1998; van Diggelen *et al.*, 2001, Wisniewski, 2005, Williams *et al.*, 2006).

Infantile NCL is caused by mutations in the *CLN1* gene that encodes the soluble lysosomal enzyme, palmitoyl-protein thioesterase 1 (PPT1) (Vesa *et al.*, 1995). Palmitoylation is a dynamic post-translational modification of proteins in which a 16-carbon palmitate moiety is covalently attached to cysteine residues found in cytoplasmic and integral membrane proteins. (Nadolski and Linder, 2007). This lipidation event plays an important regulatory role for modulating protein trafficking and localization, vesicle fusion, and signal transduction mechanisms (el-Husseini and Brecht, 2002; Nadolski and Linder, 2007). Ppt1 is one of the enzymes that can remove this lipid modification from specific substrates during protein degradation within the lysosomes. The enzyme is present in all cell types, but only neuronal cells undergo cell death suggesting that there is an important role for palmitoylation in normal neuronal function (Isosomppi *et al.*, 1999; Suopanki *et al.*, 1999b, 1999a; Zhang *et al.*, 1999).

Work on several different NCL models has shown that the location and density of intracellular inclusions within the brain does not necessarily correlate with the ultimate loss of neuronal cells (Bible *et al.*, 2004, Oswald *et al.*, 2005). Thus, a more in depth understanding of the cellular location and function of the Ppt1 protein will likely inform our understanding of the particular pathways and processes which, when perturbed, lead to the neuronal dysfunction typical of the disease. The fruit fly has been a powerful model system for studying neurological disorders including lysosomal storage disorders like the NCLs (reviewed in Muquit and Feany, 2002; Myllykangas *et al.*, 2005 (*Ctsd*); Hickey *et al.*, 2006 (*Ppt1*); Kuronen *et al.*, 2009 (*Ctsd*); Tuxworth *et al.* 2009 (*Cln3*)). Loss-of-function *Ppt1* mutations have been generated in *Drosophila* that produce cytoplasmic inclusions and autofluorescent storage material (Hickey *et al.*, 2006). In addition to loss of function approaches, we have also produced an over expression model of Ppt1 function in the adult visual system that was the basis for a previously reported dominant gain-of-function genetic modifier screen that implicated specific pathways and proteins as points of potential *in vivo* regulation by Ppt1's depalmitoylating activity (Korey and MacDonald, 2003; Buff *et al.*, 2007).

In this current study, we present the results of an F1 dominant loss-of-function modifier screen that identified several enhancers and one suppressor of Ppt1-induced degeneration. Our approach permitted an unbiased identification of loci that potentially interact with Ppt1 in a cellular context. These modifiers support a role for Ppt1 in regulating trafficking and suggest several novel interactions with proteins known to play a role in neuronal function. We have extended our gain-of-function screen to show that loss-of-function *Ppt1* mutants exhibit a decrease in fluid-phase endocytosis in Garland cells without major defects in the endo-lysosomal compartments. Fluorescent tracer experiments and ultrastructural analysis of these cells demonstrate a reduction in uptake and suggest that loss of Ppt1 impacts an early step in vesicle formation during endocytosis.

Materials and Methods

Drosophila Stocks and Fly Husbandry

Flies were raised on standard media and all crosses were carried out at 25°C. The chromosomal deficiency screen was performed with the full classic deficiency kit (X, 2nd, 3rd, and 4th chromosomes) and followed up with Exelixis and DrosDel Deficiencies. All of the deficiency stocks and alleles of candidate genes (except as noted below) were obtained from Bloomington Stock Center. We also obtained the EDGP lethal 1, BDGP lethal 2, and

BDGP lethal 3 sets from the Bloomington Stock Center. The *UAS:Ppt1* transgenic lines were previously described in Korey and MacDonald, 2003. The *Ppt1^{A179T}* and *Ppt1^{S77F}* point mutations are characterized loss-of-function alleles that were induced with ethyl methanesulfonate on the *w¹¹¹⁸* background (Hickey *et al.*, 2006). The neuronal specific inclusions observed in these point mutations are also completely rescued by *elav-Gal4* driven expression of a *UAS:Ppt1* transgene (Hickey *et al.*, 2006). We obtained the following published alleles from specific labs: *foi^{16.33}/foi^{20.713}* (Van Doren *et al.*, 2003), *Csk^{S01709}* (Read *et al.*, 2004), *Imp⁸* (Munro *et al.*, 2006), *UAS:Dyn* (Entchev *et al.*, 2000), *UAS:Sax^{Act}* (Yu *et al.*, 2004), *UAS:Sax^{DN}* (Nguyen *et al.*, 1998), *UAS:gbp^{9.1}* (Khalsa *et al.*, 1998), *RapGap1²²* and *RapGap1⁴⁷*, (Chen *et al.*, 1997).

Screen Genetics

Loss-of-function 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 lethal insertion name nor the molecular location of the insertion known to the screeners. The insertion lines were unblinded after the secondary screens. Non-specific modification due to the effects on the Gal4/UAS system were eliminated by their ability to also modify *GMR:Gal4/+* adults

Electron Microscopy

Transmission Electron Microscopy (TEM) of HRP uptake in Garland cells was done according to Chang *et al.*, 2002. Briefly, freshly dissected Garland cells were incubated with PBS containing 0.7% HRP type VI (Sigma) for 5 minutes at room temperature. The cells were then washed, fixed, and stained using a DAB substrate kit (Vector). Following the development of the stain, the cells were post fixed, embedded, and sectioned for TEM. All quantification of HRP positive vesicle uptake was done blind. For 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 2x 100% ethanol. They remained 100% ethanol until critical point drying and sputter coating for scanning electron microscopy at the Northeastern Electron Microscopy facility.

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 20ul of ddH₂O and 10ul of substrate (0.375 mg/ml 4MU-6S-palm-b-Glc substrate (Moscerdam Substrates); 0.2 M Na phosphate/0.1 M citrate, pH 4.0; 15 mM DTT; 0.09% BSA, 5 U/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 two hours at 30°C. The reaction was stopped by adding 100ul of stop buffer (0.5M NaHCO₃/Na₂CO₃ pH 10.7, 0.025% triton X-100) and then the amount of fluorescence at 460nm was read on a Perkin Elmer HTS7000 BioAssay Reader. All samples were done in triplicate.

Garland Cell Uptake Assay and Immunohistochemistry

Individual larvae of the appropriate genotypes were pinned down on Sylgard™ dishes and fresh garland cells were removed. The cells were placed in Schneider's Cell Media (Invitrogen, Carlsbad, CA) containing 1 μ M Lyotracker-Green (Molecular Probes, Carlsbad, CA) and Texas Red-conjugated avidin. This was defined as time zero. The cells were placed under a cover slip and a complete Z-stack was acquired every 5 minutes for 40

minutes using an Olympus IX71 Spinning Disc Confocal Microscope. The Z-stacks were then deconvolved using Slidebook. For garland cell immunohistochemistry, fresh garland cells were dissected, fixed, and stained with rabbit anti-Hook (1:500) (Kramer and Phistry, 1996). Staining was visualized with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (Molecular Probes, Carlsbad, CA). To examine Ppt1 localization, *UAS:GFP-Ppt1* was expressed using *Act5C-Gal4*. Garland cells were isolated and placed in 1 μ M LysoTracker-Red (Molecular Probes, Carlsbad, CA)/PBS for 3 minutes at room temperature. The tissue was then placed on a slide and immediately imaged with an Olympus IX71 Spinning Disc Confocal Microscope.

Transgenic Line

The construction of the *pUAST:GFP-Ppt1* vector using the Gateway system has been previously described (Bannan *et al.*, 2008). 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, and the insertions were mapped using standard techniques. Six independent transgenic lines were isolated. We have previously confirmed that the fusion protein is localized to lysosomes when transiently transfected into S2 cells (Bannan *et al.*, 2008).

Results

Over-expression models of gene function in the fly have been shown to be valuable tools for dissecting the cellular role of many proteins including those involved in human neurological disease (Fortini and Bonini, 2000). The expression of *Ppt1* at higher than wildtype levels in the *Drosophila* eye, using the Gal4/UAS system, produces a degeneration of the developing adult visual system that has proven to be a useful assay to identify other genetic loci that may modify this phenotype in order to shed light on Ppt1 function (Korey and MacDonald, 2003). We previously published a dominant gain-of-function modifier screen that examined the effect of co-overexpressing 1948 different genes on this degenerative phenotype (Buff *et al.*, 2007). This screen identified 20 genetic modifiers of Ppt1 function that made novel connections between Ppt1 and genes involved in synaptic vesicle endocytosis and synaptic function/homeostasis. This approach was limited in two ways. First, it only examined 14% of the approximately 14,000 annotated genes in the *Drosophila* genome. In addition, this approach identified loci that showed genetic interactions with Ppt1 when over-expressed and would miss loci that may show modifying effects only when the modifier gene was present in the cell at lower than normal levels.

To complement our first screen and scan a larger fraction of the *Drosophila* genome, we performed a second, non-redundant and unbiased F1 dominant loss-of-function screen using the complete chromosomal deficiency kit from the Bloomington *Drosophila* Stock Center and a collection of 1263 lethal P-element insertions that were generated by the *Drosophila* Gene Disruption Project (Torok *et al.*, 1993; Bellen *et al.*, 2004). This set of deficiency chromosomes provides a mechanism by which approximately 92% of the genome can be broadly screened (flystocks.bio.indiana.edu). The P-element insertion lines directly target a specific set of 1263 genes that span the X, 2nd and 3rd chromosomes of the fly. Together these two resources provide significant coverage of a large proportion of the *Drosophila* genome. The screens described below combined the *GMR:Gal4; UAS:Ppt1* phenotype with either a defined chromosomal deficiency or a lethal P-element insertion to identify a collection of genes that when reduced in copy number could modify the *Ppt1*-induced degeneration. In both approaches, 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 and/or produced a further breakdown of crystalline ommatidial structure on the eye surface.

Genome-wide Deficiency Modifier Screen

We screened 271 deletion lines using this *GMR:Gal4; UAS:Ppt1* over-expression system. Progeny from crosses between *GMR:Gal4; UAS:Ppt1* and each deficiency line were compared to control flies expressing *Ppt1* alone to determine if the deleted region enhanced or suppressed the degenerative effect. In each case we tested whether a reduction in gene dose by half for the genes in the deleted region was able to alter the degeneration produced by *Ppt1*. After the primary screen, all the modifying deficiencies went through a secondary screen to confirm the repeatability of the enhancer or suppressor effect. Those that did not yield the result obtained in the primary screen were eliminated. Flybase was used to determine the extent of the chromosomal deletion for those lines that consistently showed a modifying effect. The minimal deleted region that still produced the enhancing or suppressing effect was determined by further sub-dividing the region with smaller deficiency chromosomes (Supplemental Materials, Table S1). When possible, we used the isogenic Exelixis and DrosDel deletion collections for fine mapping (Parks *et al.*, 2004; Ryder *et al.*, 2007). In addition to providing smaller deletions that remove fewer genes, these deficiency lines were created on different genetic backgrounds than those found in the original deficiency kit. The observation of a consistent suppression or enhancement while we mapped the minimal region with these deficiencies suggested that the effect was likely due to the loss of a particular gene(s) within the deletion rather than a non-specific modification due to genetic background.

Only nine regions of the genome produced significant and repeatable modification, 8 enhancers and 1 suppressor, of the *Ppt1* degenerative phenotype (Supplementary Material, Table S1). The minimal chromosomal regions produced by fine mapping were used to query Flybase to identify the candidate modifier genes in these regions. Many annotated genes in these regions lack characterized loss-of-function mutations or there are uncharacterized transposable element insertions that have not been shown to inactivate gene function. We only obtained alleles for those genes for which loss-of-function mutations have been characterized and published. These were then crossed to the *GMR:Gal4; UAS:Ppt1* line to determine if a reduction in gene dose for these individual genes had a modifying effect that was consistent with what we observed with the deletions in the region. Using this approach we were able to identify one candidate *Ppt1* modifying locus. Alleles of *twisted gastrulation* (*tsg*; 11A1) phenocopied the enhancement effect we observed within the 10F7-11A7 minimal region that was originally identified with the *Df(1)N-105* deletion (Figure 2C; Table 1; Supplementary Material, Table S1).

Lethal P-element Modifier Screen

Similar to our approach with the chromosomal deficiencies, we screened 1263 lethal insertions using the *GMR:Gal4; UAS:Ppt1* over-expression system. Progeny from crosses between *GMR:Gal4; UAS:Ppt1* and each insertion line were compared to control flies expressing *Ppt1* alone to determine if a reduction in gene dose produced by the p-element insertion enhanced or suppressed the degenerative effect. All identified modifier insertion lines went through a secondary screen to confirm the repeatability of the enhancer or suppressor effect. Those that did not yield the same result obtained in the primary screen were eliminated. After the primary genetic screen, 27 putative enhancers and suppressors (including those from the previously described deficiency screen) were put through a series of elimination criteria in order to produce a final list of modifiers (Figure 1).

The putative modifiers could affect the Gal4/UAS system employed in the screen rather than *Ppt1*'s cellular activity. For example, suppression could be produced by a reduction in the expression of the Gal4 protein rather than effects on *Ppt1*'s cellular activity. To account for this possibility, we crossed alleles of the modifier genes to *GMR:Gal4* alone and raised the

progeny at 29°C. Due to high levels of Gal4 expression, *GMR:Gal4* flies develop a rough eye when grown at 29°C (Fernandez-Funez *et al.*, 2000). If any of the candidate Ppt1 modifiers produced genetic modification in the progeny of this cross that was similar to that which we observed in our original screen, we assumed the modifier mutation might have a generalized effect on the Gal4/UAS expression system and it would be eliminated. This control eliminated nine modifier loci. In addition, enhancers of Ppt1 could be producing a more disordered eye through a nonspecific additive effect if flies heterozygous for the insertions also had rough eyes on their own. All of the enhancers had no alterations in eye morphology in the heterozygous state suggesting that these lines had *Ppt1*-specific genetic interactions (*data not shown*).

We also used a flouragenic assay designed for the diagnosis of INCL in humans to confirm that the modification 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* is 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). We found that none of the modifiers when co-expressed with Ppt1 significantly modified enzyme activity levels as compared to the expression of Ppt1 alone (*data not shown*). All assays used loss-of-function *Ppt1* mutations as a negative control.

After passing these initial secondary screens, we controlled for the effects of genetic background by obtaining characterized loss-of-function alleles of the remaining modifying loci. We eliminated 11 modifier genes for which independent alleles of the gene did not phenocopy the effect of the original insertion line. We generated precise excisions for one insertion, *P[lacW]Vps28^{sk16503}*, for which we could not obtain alleles. The excision line was then crossed to the original *GMR:Gal4; UAS:Ppt1* line to determine if the modifying effect was retained. If the excision line retained the suppressor or enhancer effect in the absence of the P-element insertion it likely did so because of genetic alterations other than the presence of the insertion and it would be eliminated from consideration. We found that the excision of the *lacW* transgene did not revert the modifying effect of the *P[lacW]Vps28^{sk16503}* insertion and thus this modifier was eliminated.

Modifiers of Ppt1 Degeneration

Of the original 1263 lines and candidates from the genome wide deficiency screen, five enhancers and one suppressor of the *Ppt1* eye phenotype survived our stringent prioritization criteria. These are described in Table 1 with the accompanying functional information and verification procedures.

In particular, Drosophila C-terminal Src Kinase (*dcsk*) was identified as an enhancer of the Ppt1 phenotype. In Drosophila, *dcsk* is a regulator of Src-family kinases during cell proliferation and morphological changes (Stewart *et al.*, 2003; Pedraza *et al.*, 2004; O'Reilly *et al.*, 2006). Modification by *dcsk* was confirmed with the *dcsk^{SO1709}* allele (Figure 2G).

Mutations in the fear of intimacy (*foi*) gene were also identified as enhancers (Figure 2D). *Foi* is thought to be a zinc ion transmembrane transporter that regulates the influx of zinc into the cytoplasm, and plays an important role in regulating several developmental processes, including glial cell migration and gonad morphogenesis (Van Doren *et al.*, 2003; Pielage *et al.*, 2004; Matthews *et al.*, 2005). The enhancement was verified with the *foi* alleles, *foi^{16.33}* and *foi20*.

Mutations in *zipper* (*zip*) produced an enhancement of the degenerative phenotype. Zip is the heavy chain homolog of fly non-muscle myosin II. It is involved in tissue dynamics and morphogenesis. For example, Zip plays a crucial role in epithelial movements that occur during embryonic dorsal closure particularly in the leading edge cells (Harden, 2002, Jacinto *et al.*, 2002). The *zipper* alleles, *zip*¹ and *zip*² were used to verify the enhancement produced by this locus (Figure 2H).

Mutations at the *Drosophila* IGF-II mRNA-binding protein (*Imp*) locus also enhanced the degenerative phenotype. *Imp* belongs to a family of RNA binding proteins that play a role in mRNA localization/translation and it has recently been shown to play a role in synapse function in the fly (Munro *et al.*, 2006; Boylan *et al.*, 2008). The *imp* allele *imp*⁸ also produced enhancement (Figure 2F).

dSmt3 mutations were the lone suppressor modifiers found in this screen. This locus encodes the *Drosophila* homolog of the small ubiquitin-related modifier (SUMO-1) (Huang *et al.*, 1998). The addition of this peptide to proteins has been shown to play a role in the regulation of a wide-range of cellular processes including protein stability and subcellular localization. The suppressive effect was confirmed with the *dSmt3* allele, *dSmt3*⁰⁴⁴⁹³ (Figure 2E).

Fine mapping of the *Df(1)N-105* deficiency and candidate gene testing identified *twisted gastrulation* (*tsg*) as the modifier locus within this region (Table 1, Supplementary Material Table S1, Figure 2C). The *tsg* alleles *tsg*² and *tsg*⁴ both enhanced the rough eye phenotype produced by Ppt1 expression. *Tsg*, a secreted glycoprotein, is a conserved regulator of Bone Morphogenetic Protein (BMP) signaling during early fly development (Ross *et al.*, 2001). Our previous gain-of-function modifier screen also identified a connection between BMP signaling and Ppt1 function (Buff *et al.*, 2007). That screen showed that over-expression of the wildtype BMP type I receptor Saxophone (*sax*) suppressed the degeneration produced by Ppt1 expression (Buff *et al.*, 2007). In light of the identification of *tsg* alleles in this screen, we further examined candidate Ppt1-BMP interactions by obtaining UAS lines that expressed activated and dominant negative versions of *sax* and a line that expressed the BMP ligand, *glass bottom boat* (*gbb*). We found that like over-expression of wildtype *sax*, the activated version of the receptor also suppressed the degeneration while a dominant negative *sax* receptor enhanced the Ppt1 degenerative phenotype (Figure 3B–C). GMR-driven expression of only the dominant negative *sax* receptor had no effect on eye development indicating that the modification we observed is specific to Ppt1-function (*data not shown*). Finally, the over-expression of the *gbb* ligand in parallel with Ppt1 also had a suppressing effect (Figure 3D). The identification of *tsg* in this unbiased screen combined with a further analysis of other candidates from this pathway strongly suggests a connection between Ppt1 and BMP signaling at the cellular level.

Ppt1 mutants reduce endocytosis in a non-neuronal cell type

Our current screen, as well as a previous screen (Buff *et al.*, 2007), suggests a role for Ppt1 in cellular trafficking. Since these connections to trafficking (protein localization, endo-lysosomal trafficking and autophagy) are based on over-expression systems and INCL is a recessive disorder, we wanted to extend and verify our genetic screen results by determining if there were trafficking defects in the more disease relevant *Ppt1* loss-of-function mutations. To do this we chose to use Garland cells, a nephrocytic-like cell type in larvae that undergoes rapid fluid phase endocytic uptake from the hemolymph (Narita *et al.*, 1989). We examined the localization of Ppt1 in these cells, the rate of tracer endocytosis, and performed an ultrastructural comparison between mutant and wildtype cells.

Before examining endocytosis in Garland cells, we determined the localization of Ppt1 in this particular cell type. Our lab and others have previously attempted to produce an antibody to Ppt1 to examine endogenous protein localization in *Drosophila* without success. This has prevented a careful analysis of the endogenous localization of the Ppt1 protein. To circumvent this technical issue, we chose to produce a *UAS:GFP-Ppt1* transgenic fly line to shed some light on this question. We have previously shown that this fusion protein localizes to lysosomes in transiently transfected S2 cells (Bannan et al., 2008). To demonstrate that the GFP-Ppt1 protein retains thioesterase activity *in vivo* we expressed the *UAS-GFP-Ppt1* transgene using *GMR-Gal4* and then ran fluorogenic Ppt1 enzyme assays on individual dissected adult heads. A comparison of *GMR-Gal4; UAS:GFP-Ppt1* and *GMR-Gal4* alone controls shows a significant increase in enzyme activity in GFP-Ppt1-expressing flies as compared to *GMR/CyO* (wildtype Ppt1 levels) and *Df(1)446-20* (Ppt1 deletion) ($p < 0.0002$), indicating that the fusion protein retains normal thioesterase activity (Supplemental Figure 2). To look at *in vivo* localization of the fusion protein in the fly, we used Act5C-*Gal4* to drive expression in garland cells. Live confocal imaging of GFP-Ppt1 expression in freshly dissected Garland cells shows that the fusion protein co-localizes with Lysotracker-red stained lysosomes in all these cells (Figure 4A). This is consistent with the *in vivo* localization of Ppt1 in all other model systems examined.

Garland cells are a well-characterized system for assaying fluid phase endocytosis in *Drosophila* (Narita et al., 1989). This assay is particularly useful since it can be used to compare endocytosis rates between freshly isolated mutant cells and wildtype controls. Many proteins involved in endocytosis show alterations in fluorescent tracer uptake when examined with this system (Chang et al., 2002; Satoh et al., 2005; Vijayakrishnan et al., 2009). In order to observe endocytosis, we dissected live garland cells from 3rd instar larvae and monitored endocytosis over a period of 40 minutes by incubating the cells in cell culture medium containing Texas Red-conjugated Avidin (TR-Avidin) and Lysotracker green. The Lysotracker green is membrane permeable and rapidly accumulates in acidic cellular compartments, primarily lysosomes. In contrast, the Avidin must be actively endocytosed into the cell. This combination of markers allowed us to observe the endocytic process in this cell type. Previously published work on proteins involved in endocytic trafficking, such as Hsc-70, Rab11, and Benchwarmer, has shown that significant uptake in wildtype cells occurs rapidly within the first few minutes (Chang et al., 2002; Satoh et al., 2005; Dermaut et al., 2005). A detailed kinetic analysis has not been done, but localization with lysotracker appears soon after Avidin addition with strong co-localization occurring by 20 minutes in one study (Dermaut et al., 2005).

We used two well-characterized (see materials and methods) point mutations, *Ppt1*^{A179T} and *Ppt1*^{S77F}, to perform the garland cell endocytic tracer uptake experiments. We observed significant uptake of the tracer in wildtype garland cells 5 minutes into the incubation as indicated by the ring of Texas-Red-positive vesicles/early endosomes surrounding the periphery of the cells (Figure 4B). At the 5-minute time-point in *Ppt1* garland cells we observed far fewer Texas-red-positive vesicles/early endosomes in the cell periphery suggesting a decrease in the rate of tracer uptake in mutant cells (Figure 4B'). After 30 minutes we observed almost complete co-localization between the Texas-red avidin and the Lysotracker green compartments in wildtype cells (Figure 4C). In contrast, *Ppt1* garland cells at 30 minutes showed some co-labeling of compartments, but many Texas-red positive vesicles are still distinct from the Lysotracker labeled regions of the cell (Figure 4C'). This suggests that, although endocytosis and endo-lysosomal trafficking does occur in *Ppt1* mutant garland cells, there is a reduced level of uptake and a decreased rate of trafficking to the lysosomes. These results are representative of three separate uptake experiments in cells obtained from *Ppt1*^{A179T} and *Ppt1*^{S77F} larvae.

The decreased rate of tracer uptake in *Ppt1* mutant garland cells prompted us to look at early endosomal organization in these cells. We stained wildtype and *Ppt1* mutant garland cells with an anti-Hook antibody (Supplemental Figure 2A). Hook (Hk) is a cytosolic protein associated with early endosomes. We observed no significant difference in the staining between *wildtype* garland cells and *Ppt1* mutant cells, indicating that the reduced levels of endocytosis in mutant cells are not due to gross disorganization of the early endocytic compartments as has been observed in mutants in endocytic genes such as *Hsc70-4* (Chang *et al.*, 2002). In addition, given *Ppt1*'s localization to lysosomes in these cells, we examined the overall lysosomal morphology of these cells as highlighted by Lysotracker-red in freshly dissected tissue (Supplemental Figure 2B). We observed no drastic changes in the lysosomal compartment using this visualization method.

In order to better quantify the decreased rate of tracer uptake in *Ppt1* mutant garland cells we observed with TR-Avidin, we used horseradish peroxidase (HRP) as a tracer for electron microscopy (EM). This approach allowed us to more precisely analyze vesicle uptake in Garland cells (Koenig and Ikeda, 1990; Chang *et al.*, 2002; Vijayakrishnan *et al.*, 2009). Freshly dissected Garland cells from *wildtype* and *Ppt1*^{A179T} mutant larvae were incubated with HRP for 5 minutes at room temperature, fixed, and processed for electron microscopy. HRP labeled vesicles were easily identifiable in sections from either wildtype or *Ppt1*^{A179T} mutant Garland cells (Figure 5A). We counted the number of HRP positive vesicles present in sections through the middle of distinct identified Garland cells from both genotypes. Similar to our TR-Avidin experiments, we found a reduction in the number of HRP positive vesicles per section in the *Ppt1* mutant cells as compared to the *wildtype* controls (*wildtype*: 52.3 ± 12.4 , n=3 cells; *Ppt1*^{A179T}: 12.42 ± 4.86 , n=6 cells). To control for differences in cell size, we divided the number of observed vesicles in each section by the area of the cell cross section. After controlling for cell size, we still observed significantly less labeled vesicles per cell area in the *Ppt1*^{A179T} cells as compared to wildtype cells (Figure 5B; *wildtype*: 0.24 ± 0.06 , n=3 cells; *Ppt1*^{A179T}: 0.08 ± 0.04 , n=6 cells; $p < 0.003$, t-test). This is an approximately 68% reduction in HRP tracer uptake. Together, our endocytic tracer experiments demonstrate that *Ppt1* is necessary for normal endocytic uptake in this non-neuronal cell type.

In addition to the HRP uptake assay, we examined the cellular ultrastructure of mutant and wildtype cells to determine if there were morphological changes in *Ppt1* cells that accompanied the functional changes. Previous work on Garland cells has shown that they have two distinct concentric regions, the central region and the cortical region (Narita *et al.*, 1989; Koenig and Ikeda, 1990). The cortical region of the cell is characterized by a distinctive network of labyrinthine channels that extends $\sim 3\mu\text{M}$ into the cell's cytoplasm (Koenig and Ikeda 1990). These channels are thought to be endocytic intermediates since they are observed to enlarge at the restrictive temperature in *shibire* (Dynamin) temperature sensitive mutants that block endocytosis (Koenig and Ikeda, 1990). Transmission electron microscope images of wildtype Garland cells clearly show these long narrow tubular structures that are contiguous with the extracellular space (Figure 5 C–D). Images of four distinct *Ppt1*^{A179T} Garland cells show distended and highly vacuolarized labyrinthine channels (Figure 5 E–H). These types of labyrinthine channel structures have also been observed in Garland cells with other mutations that alter endocytosis and are consistent with a partial block in endocytosis in *Ppt1* mutant cells (Koenig and Ikeda, 1990; Satoh *et al.*, 2005).

Drosophila Dynamin is a genetic modifier of *Ppt1* function

To further substantiate a connection to early stages of endocytosis, we examined *Drosophila* Dynamin as a possible candidate genetic modifier of *Ppt1*-induced degeneration in our over-expression system. Dynamin is a small GTPase intimately associated with the invagination

and release of clathrin-coated pits during general endocytosis and synaptic vesicle recycling. Other genes shown to play a role in endocytosis in *Drosophila*, such as Hsc70–4, have also been shown to genetically interact with Dynamin in this type of assay (Chang *et al.*, 2002). To examine a potential genetic interaction we co-expressed a wildtype version of Dynamin (Dyn) with Ppt1 using the Gal4/UAS system. As has been previously stated, expression of wildtype Ppt1 in the adult visual system using *GMR-Gal4* alters the external and internal organization of the adult retina (Figure 6A). When we co-expressed UAS-Dyn with Ppt1 we found a significant enhancement of the rough eye phenotype (Figure 6C). In contrast, when UAS-Dyn is expressed alone in the adult visual system, we observed no defects in the outer morphology and organization of the eye (Figure 6B). This control provides strong evidence that the genetic interaction is specific to Ppt1 function and not a non-specific additive effect. The interaction we observe is consistent with the early uptake defects we characterized in Garland cells.

Discussion

We have completed a genome-wide dominant loss-of function screen that identified several novel *Ppt1*-modifying loci. The combination of defined deletions and P-element insertions allowed us to cover greater than 90 percent of the genome in an unbiased fashion. Each of the loci we characterized modified a *Ppt1*-specific degenerative phenotype in the adult suggesting a cellular connection between these new proteins and Ppt1 function. In particular our modifiers suggest novel links to glial cell migration (*foi*), Src kinase signaling (*dcsk*), and mRNA localization/translation (*Imp*). While the visual system degeneration phenotype we employed is not an INCL disease-specific phenotype, a clearer understanding of a disease gene's cellular function can be achieved using this over-expression approach in the fly (Fortini and Bonini, 2000). In addition to our work on INCL, a recent CLN3 gain-of-function system highlighted the benefit of this approach by identifying Notch and Jun N-terminal Kinase signaling pathways as playing potential roles in the Juvenile-onset form of NCL (Tuxworth *et al.* 2009). Since NCL diseases are primarily recessive, the final step in this gene-discovery process is to confirm the possible role of these newly identified pathways in disease pathogenesis by examining them in relevant loss-of-function disease models.

Multiple modifying loci have pointed to a role for Ppt1 in regulating endocytosis, thus we followed-up on our gain-of-function results by examining endocytic function in an easily accessible non-neuronal cell type in *Drosophila* *Ppt1* loss-of-function mutants. Our results demonstrated a decreased rate of TR-Avidin and HRP tracer uptake in *Ppt1* mutant cells that is consistent with a role for Ppt1 in the modulation of early endocytic processes. *Drosophila* genes that have been shown to play a major role in endocytosis, such as Rab11, Dynamin, Rolling Blackout (RBO), and Hsc70–4, all demonstrate a complete block in the uptake of tracer (fluorescent avidin or HRP) in mutant cells (Koenig and Ikeda, 1990; Satoh *et al.*, 2005; Chang *et al.*, 2002; Vijaykrishnan *et al.*, 2009). A reduction, but not complete cessation of uptake in *Ppt1* mutant cells suggests that Ppt1 is not required for endocytosis but rather that its function is necessary for robust endocytosis to occur. This phenotype is consistent with what has been observed in other INCL models; a reduction in fluid-phase and receptor-mediated endocytosis in INCL patient fibroblasts as well as impaired synaptic vesicle recycling in mouse knock-out models (Ahtiaienen *et al.*, 2006; Virmani *et al.*, 2005; Kim *et al.* 2008). Our ultrastructural analysis of *Ppt1* garland cells demonstrates altered labyrinthine channel morphology with channels that are often distended and highly vacuolated. A phenotype similar to this is seen in some *Drosophila* endocytic mutants. While labyrinthine channels are absent in Rab11 and Hsc70–4 cells, cells that lack Dynamin or Rolling Blackout (EFRS integral membrane lipase) function show ultrastructural phenotypes similar to Ppt1 (Koenig and Ikeda, 1990; Vijaykrishnan *et al.*, 2009). Work on

both Dynamin and RBO indicate that the enlarged and vacuolated channels are due to a block in vesicle formation during endocytosis (Koenig and Ikeda, 1990; Vijayakrishnan *et al.*, 2009). Our garland cell experiments and the genetic interaction with Dynamin in the adult eye are consistent with the loss of Ppt1 function impacting endocytosis at the point of vesicle formation.

The role of palmitoylation in controlling protein trafficking and synaptic vesicle endocytosis/recycling has been well established (Reviewed in El-Husseini and Bredt, 2002; Nadolski and Linder, 2007). Genetic modifiers identified using fly screens, our results on endocytosis in a non-neuronal cell type, and the work of others in many different INCL model systems all point to the mis-regulation of these cellular processes in INCL patients as an important contributor to disease pathogenesis (Buff *et al.*, 2007; Ahtiaenen *et al.*, 2006; Virmani *et al.*, 2005; Kim *et al.* 2008). One important outstanding question is which protein(s) involved in vesicle endocytosis/recycling are substrates for Ppt1 and thus may be mis-regulated in the absence of its normal enzymatic function in patients. Our modifier screens have identified several interacting loci, such as *dynamin*, *endophilin A*, *stoned A*, and *synaptotagmin 1*, that are intimately involved in vesicle exo/endocytosis (Buff *et al.* 2007). While none of these proteins have been biochemically shown to be palmitoylated in *Drosophila*, bioinformatic analysis using CSS-Palm 2.0 suggest Dynamin (at Cysteine (Cys) 438), Endophilin A (at Cys 309 and 310), and Synaptotagmin I (at Cys 133) are all potentially palmitoylated (Ren *et al.*, 2008). In addition, recent work on the palmitoyl transferase Hip14 in *Drosophila* has shown that the exocytic proteins Cysteine String Protein (CSP) and SNAP25 are palmitoylated in the fly (Ohyama *et al.*, 2007; Stowers and Isacoff, 2007). It has been shown that the correct palmitoylation of CSP is crucial for proper synaptic function in *Drosophila* (Ohyama *et al.*, 2007). Recent work on Ppt1-knockout mouse synaptosomes demonstrated a persistent membrane-association of palmitoylated synaptic vesicle components including Syt1, SNAP-25, and Syntaxin 1 in the absence of Ppt1 function (Kim *et al.* 2008). Taken together, this work supports the hypothesis that several important synaptic vesicle proteins are potential substrates for Ppt1 and that the dysregulation of these proteins' palmitoylation cycling hinders a neurons ability to maintain a functional pool of synaptic vesicles. In fact, an alteration in synaptic vesicle pools is observed in primary neuronal cultures that showed no other signs of dysfunction suggesting that changes to neural transmission may be a very early manifestation of the disease (Virmani *et al.* 2005). A recent genetic screen in a *Drosophila Cathepsin D* model (congenital NCL) also found modifiers associated with endocytosis, including Dynamin, suggesting that alterations in this process may be a common mechanism in several different NCL subtypes.

In addition to synaptic vesicle cycling proteins, CSSPalm 2.0 analysis suggests that several of the modifier proteins we identified in our screen have the potential to be palmitoylated and thus may be substrates for Ppt1. Specifically, the enhancers Fear of Intimacy (at Cys 9, 12, and 13) and C-terminal Src Kinase (at Cys 9) both may be regulated by palmitate addition. The identification of the modifier *tsg* in our screen and *sax* and *gbb* in a follow-up candidate gene approach makes a strong case for Ppt1 regulation of BMP signaling in *Drosophila*. CSS-Palm analysis of BMP signaling components indicates that the type I receptors Saxophone (Cys 569) and Thickveins (Cys 201) as well as the type II receptors Wishful Thinking (Cys 322) and Punt (Cys 515) are all potentially palmitoylated. While speculative, these bioinformatic results combined with the genetic modifiers suggest that further work in characterizing the intersection of Ppt1 and BMP signaling in the fly is warranted. Given the large body of work on the role of this pathway in synaptic development and homeostasis in the fly (Keshishian and Kim, 2004), a confirmation of a regulatory role for Ppt1 in BMP signaling would be a significant step in our understanding of INCL pathogenesis.

A key question in the understanding of the function of Ppt1 and thus INCL is its localization and interaction with putative substrate proteins, many of which are cytoplasmic, in the context of neuronal function. While several groups have provided some evidence for an extra-lysosomal pre-synaptic role for Ppt1 (Lehtovirta *et al.* 2001; Kim *et al.* 2008), it has been well established that the protein is localized to the lysosomal lumen in neuronal and non-neuronal cells in a variety of systems (Camp and Hoffman, 1993; Verkruyse and Hoffman, 1996; Hellensten *et al.*, 1996; Cho and Dawson, 2000; Virmani *et al.*, 2005). This study used a GFP-Ppt1 fusion protein to demonstrate that the fly Ppt1 protein is also localized to lysosomes *in vivo*. With out direct access to the cytoplasmic compartment, one mechanism that may be underlying the Garland cell phenotypes in the fly and INCL disease phenotypes could be a gradual build-up of toxic palmitoylated peptides, possibly still membrane bound, that interfere with the regulation of a subset of important neuronal proteins in patient cells (Virmani *et al.*, 2005).

Finally, there is a growing body of evidence from many model systems that suggests infantile onset NCL could be categorized as a synaptopathy where early synaptic deficits precede the large-scale neuronal cell death that occurs in patients. This hypothesis is supported by work in mice and on human patient samples that show a disruption of synaptic vesicle cycling (Virmani *et al.*, 2005; Kim *et al.*, 2008). In addition, recent analysis of mouse models of several NCL subtypes, including INCL, has revealed early changes in the expression of important synaptic proteins at presymptomatic stages before large-scale brain pathology occurs (Kielar *et al.* 2009). This suggests that early insults to synaptic homeostasis are a major component of the molecular etiology of INCL. The work presented here continues to support a role for Ppt1 in endocytosis and therefore synaptic vesicle cycling. It also points to alterations in synaptic growth pathways as a potentially important contributor to the neural dysfunction associated with INCL. These two points of impact on synaptic processes provide a valuable focus for future work. The success of the fly as a model for INCL, as well as JNCL and congenital NCL, suggests that the exploration of these processes in *Drosophila* will continue to make important contributions to the understanding of these diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Loss-of-Function Modifier Screen

A schematic diagramming the secondary screening procedures used to finalize the genetic modifiers of Ppt1-induced degeneration listed in Table 1.

Figure 2. Enhancers and Suppressors of Ppt1-induced degeneration

Scanning Electron Microscopy images of adult Eyes (200x) from representative Ppt1 modifiers. **A–F**, SEM images showing the external surface of control and identified Ppt1 modifiers. **A**. An image of the normal eye morphology that is observed without the expression of Ppt1 (*UAS:Ppt1*^{12.1/+}). **B**. An image of the Ppt1 alone over-expression control (*GMR:Gal4,UAS:Ppt1*^{8.1/+}; *UAS:Ppt1*^{2.1/+}) **C**. The enhancement produced by a reduction in *tsg* expression (*tsg*^{4/+}; *GMR:Gal4,UAS:Ppt1*^{8.1/+}; *UAS:Ppt1*^{2.1/+}) **D**. The enhancement produced by a reduction in *foi* expression (*GMR:Gal4,UAS:Ppt1*^{8.1/+}; *UAS:Ppt1*^{2.1/foi}^{16.33}) **E**. The suppression produced by a reduction in *smt3* expression (*GMR:Gal4,UAS:Ppt1*^{8.1/smt3}⁰⁴⁹⁹³; *UAS:Ppt1*^{2.1/+}) **F**. The enhancement produced by a reduction in *imp* expression (*imp*^{8/+}; *GMR:Gal4,UAS:Ppt1*^{8.1/+}; *UAS:Ppt1*^{2.1/+}) **G**. The enhancement produced by a reduction in *csk* expression (*GMR:Gal4,UAS:Ppt1*^{8.1/+}; *UAS:Ppt1*^{2.1/csk}^{S01709}) **H**. The enhancement produced by a reduction in *zip* expression *GMR:Gal4,UAS:Ppt1*^{8.1/zip}²; *UAS:Ppt1*^{2.1/+}.

Figure 3. Ppt1-Induced Degeneration is Modified by Alterations in BMP Signaling

Scanning Electron Images of Adult Eyes (200x). **A–D**, SEM images showing the external surface of control and Ppt1 modifiers. **A**. An image of the Ppt1 alone overexpression control (*GMR:Gal4,UAS:Ppt1^{8.1/+}; UAS:Ppt1^{2.1/+}*) **B**. The suppression produced by activated Sax receptor co-expression (*GMR:Gal4,UAS:Ppt1^{8.1/+}; UAS:Ppt1^{2.1}/UAS:Sax^{act}*) **C**. The enhancement produced by dominant negative Sax receptor co-expression (*GMR:Gal4,UAS:Ppt1^{8.1/+}; UAS:Ppt1^{2.1}/UAS:Sax^{DN}*) **D**. The suppression produced by a gbb ligand co-expression (*GMR:Gal4,UAS:Ppt1^{8.1/+}; UAS:Ppt1^{2.1}/UAS:gbb^{9.1}*)

Figure 4. *Ppt1* mutant garland cells have reduced endocytosis but retain endolysosomal trafficking

A. To determine the subcellular localization of Ppt1 in Garland cells, *UAS-GFP:Ppt1* was driven with *Actin5C-Gal4*. Live dissected Garland cells were placed in LysoTracker and imaged live using an Olympus Spinning Disc Confocal Microscope. The panel shows the GFP channel, the LysoTracker-red channel and a merged image showing significant co-localization of GFP with the LysoTracker. The garland cells are also co-stained with DAPI (blue) to highlight the nucleus. The images shown are one section of a deconvolved Z-section. **B-B'** Live cell Texas Red Conjugated Avidin (TR-Avidin) uptake assays with freshly dissected garland cells from *wildtype* and *Ppt1*^{S77F} wandering 3rd instar larvae at the

5-minute time point. Each of the five *wildtype* cells (**B**) shows a significant uptake of the fluorescent tracer as indicated by the red vesicles in the cell periphery. The *Ppt1*^{S77F} cells show a significantly reduced uptake of the tracer at the same time point (**B'**). Dotted lines were drawn around the boundary of three of the mutant cells to clarify the edges of the cells in the absence of tracer uptake. Images are single sections of a deconvolved Z-stack taken at 40x magnification. **C**. Wildtype cells show significant co-localization of the endocytosed avidin tracer and the lysosomal marker Lysotracker-green at 30 minutes. The panel shows the Lysotracker channel, the TR-Avidin channel, and the merged image. An N labels the nuclei in several of the cells in the merged image. **C'** Although the *Ppt1*^{S77F} mutants show a reduced endocytosis of the tracer, TR-Avidin does reach the Lysosomes in these cells by the 30-minute time point. The panel shows the Lysotracker channel, the TR-Avidin channel, and the merged image from a single cell. The merged image is a three-view representation showing the co-localization of a TR-Avidin signal and the Lysotracker-Green in this cell (Arrowheads). Dotted line boxes highlight the same region in the red and green channel panels. These images are representative of three independent experiments with two separate alleles of *Ppt1* (*Ppt1*^{S77F} and *Ppt1*^{A179T}). Scale Bars = 10μm.

Figure 5. *Ppt1* Garland cells have a significant reduction in HRP-Tracer uptake and show altered labyrinthine channel ultrastructure

Horse radish peroxidase (HRP) was used as an endocytic tracer for EM analysis of *wildtype* and *Ppt1^{A179T}* Garland cells. Cells were incubated with HRP for 5 minutes, fixed, the HRP stain was developed and then processed for EM. **A.** An image showing HRP labeled vesicles (h), labyrinthine channels (la), the channel entrances (arrowheads) and an α -vacuole (a). **B.** Quantification of the reduced tracer uptake phenotype observed in *Ppt1^{A179T}* Garland cells. *Ppt1^{A179T}* cells show a significant reduction (~68%) in the number of HRP positive vesicles per cell area as compared to wildtype cells (wildtype: 0.24 ± 0.06 , n=3 cells; *Ppt1^{A179T}* 0.08 ± 0.04 , n=6 cells; * = p < 0.003) **C–H.** Transmission electron microscopy images of *wildtype*

and *Ppt1* cells. **C–D.** The *wildtype* images show numerous channels (two are highlighted in a purple box in each image) that can extend up to 3μm into the cortical region of the cells. The channel openings are labeled with arrowheads. **E–H.** Sections through *Ppt1*^{A179T} cells show distended channels and many that are highly vacuolated (asterisks). Scale Bars = 1μm.

Figure 6. *Ppt1* and *Dynamin* genetically interact

A–C, SEM images of the external surface of controls and the *Ppt1*-*Dynamin* interaction showing that co-expression of *Dynamin* enhances the *Ppt1*-induced cellular degenerations.

A. Expression of *Ppt1* in the adult visual system produces degeneration

(*GMR:Gal4,UAS:ppt1^{8.1/+}; UAS:ppt1^{2.1/+}*) **B**. Expression of *Dynamin* alone in the adult visual system has no effect (*GMR:Gal4/UAS:Dyn*) **C**. Co-expression of *Ppt1* and *Dynamin* produces an enhancement of the degeneration (*GMR:Gal4,UAS:ppt1^{8.1}/UAS:Dyn; UAS:ppt1^{2.1/+}*).

Table 1

Genetic Modifiers of *Ppt1* induced Degeneration

Gene	Cellular Function	Original Insertion	Locus	Modification ^a	Verification ^b
Lethal Screen					
<i>Fear of Intimacy</i>	Zinc Transporter	<i>P[lacW]/fop^{i8E8}</i>	66D5–66D6	Enh	<i>fop^{i6.33}, fop^{20.71}</i>
<i>C-terminal Src kinase</i>	Tyrosine Kinase	<i>P[lacW]/Csk^{i1D8}</i>	86 E13	Enh	<i>Csk^{S01709}</i>
<i>Smt3</i>	Sumoylation	<i>P[lacW]/smt3^{k06307}</i>	27C7	Sup	<i>Smt3⁰⁴⁴⁹³</i>
<i>Zipper</i>	Non-muscle Myosin II	<i>P[PZ]/zip⁰²⁹⁵⁷</i>	60 E12	Enh	<i>zip¹, zip²</i>
<i>Imp</i>	<i>IGF-II mRNA-binding protein</i>	<i>P[lacW]/Imp^{G0072}</i>	9F2	Enh	<i>Imp⁸</i>
Deficiency Screen					
<i>Twisted Gastrulation</i>	BMP Signaling	-	11A1	Enh	<i>tsg², tsg^d</i>

^aEnh, enhancer; Sup, suppressor.

^bCharacterized alleles listed