Published in final edited form as:

Mol Cell Neurosci. 2015 January ; 64: 24–31. doi:10.1016/j.mcn.2014.11.002.

Structure-function analyses of tyrosine phosphatase PTP69D in giant fiber synapse formation of *Drosophila*

LaTasha H. Lee and

Department of Biological Sciences, Florida Atlantic University, John D MacArthur Campus, Jupiter, Florida, United States of America

Tanja A. Godenschwege

Department of Biological Sciences, Florida Atlantic University, John D MacArthur Campus, Jupiter, Florida, United States of America

Abstract

PTP69D is a receptor protein tyrosine phosphatase (RPTP) with two intracellular catalytic domains (Cat1 and Cat2) and has been shown to play a role in axon guidance of embryonic motoneurons as well as targeting of photoreceptor neurons in the visual system of *Drosophila melanogaster*. Here, we characterized the developmental role of PTP69D in the giant fiber (GF) neurons, two interneurons in the central nervous system (CNS) that control the escape response of the fly. Our studies revealed that PTP69D has a function in synaptic terminal growth in the CNS. We found that missense mutations in the first immunoglobulin (Ig) domain and in the Cat1 domain, present in *Ptp69D*¹⁰ and *Ptp69D*²⁰ mutants, respectively, did not affect axon guidance or targeting but resulted in stunted terminal growth of the GFs. Cell autonomous rescue experiments demonstrated a function for the Cat1 and the first Ig domain of PTP69D in the GFs but not in its postsynaptic target neurons. In addition, complementation studies and structure-function analyses revealed that for GF terminal growth Cat1 function of PTP69D requires the immunoglobulin and the Cat2 domains, but not the fibronectin III or the membrane proximal region domains. In contrast, the fibronectin III but not the immunoglobulin domains were previously shown to be essential for axon targeting of photoreceptor neurons. Thus, our studies uncover a novel role for PTP69D in synaptic terminal growth in the CNS that is mechanistically distinct from its function in photoreceptor targeting.

Keywords

receptor protein tyrosine phosphatase; synapse formation; *Drosophila*; PTP69D; giant fiber

Introduction

Receptor protein tyrosine phosphatases (RPTPs) are a family of transmembrane proteins that are strongly expressed in the nervous system and are crucial for the formation of functional neuronal circuits (Stoker, 2014; Van Vactor, 1998). RPTPs have modular ectodomains,

Corresponding author: Tanja A. Godenschwege, Florida Atlantic University, John D MacArthur Campus, 5353 Parkside Drive, Jupiter, FL 33458, U.S.A., Phone: + 01 561 799 8055, godensch@fau.edu.

which resemble cell adhesion molecules, and intracellular tyrosine phosphatase domains, which antagonize tyrosine kinase signaling. PTP69D is a *Drosophila* RPTP that has been shown to play an important role during neurodevelopment (Desai and Purdy, 2003; Desai et al., 1996; Garrity et al., 1999). The extracellular domain of PTP69D is comprised of two immunoglobulin (Ig) domains, three fibronectin type III (FNIII) domains and a membrane-proximal region (MPR), while the intracellular region has two protein tyrosine phosphatase domains (Cat1 and Cat2, Figure 1A). PTP69D has a role during periods of axon outgrowth (Desai and Purdy, 2003) and along with other RPTPs, such as DLAR and PTP99A, is required for proper motor axon guidance (Desai et al., 1996; Desai et al., 1997; Song et al., 2008). PTP69D is also known to play a role in axon fasciculation and branching in the mushroom bodies (Kurusu and Zinn, 2008). In the *Drosophila* visual system, PTP69D is required for lamina specific targeting of the photoreceptor neurons R1-R6 (Garrity et al., 1999). These structure-function studies revealed that the FNIII domains, but not the Ig domains, are required for axon targeting. It was shown that mutations disrupting the function of both Cat1 and Cat2 domains prevented proper targeting of R1-R6 to the lamina, but catalytic activity of one of the two domains was sufficient for normal neuronal development. Furthermore, phosphatase activity of PTP69D was also shown to be involved in R7 targeting to the medulla (Hofmeyer and Treisman, 2009; Newsome et al., 2000).

In the present work, we characterize the function of PTP69D in the adult giant fiber circuit (GFC), which mediates the escape response of the fly (Allen et al., 2006). The two giant fiber (GF) neurons extend their axons in the brain during the L3 larval stage, and the axons project into the second thoracic neuromere during early pupal development. During mid pupal development, they establish electrical and chemical synapses with the peripheral synapsing interneurons (PSIs) and the tergotrochanteral motoneurons (TTMns, Fig. 1B). Here, we show that PTP69D, in addition to its previously characterized roles in axon guidance and targeting, also has a function in terminal growth in the central nervous system (CNS). We found that the disruption of the catalytic function of the membrane proximal Cat1 domain in *Ptp69D*²⁰ mutants had no effect on axon guidance or targeting, but resulted in severely stunted GF terminals that are dysfunctional, yet still connected to the TTMn. Structure-function analyses revealed that Cat1 function in GF terminal growth required the Ig domains, but not the FNIII domains. In summary, our studies suggest that during GF terminal growth outside-in signaling of PTP69D involves the Cat1 and the Ig domains and this is mechanistically distinct from how it functions in axon targeting.

Experimental Methods

Flystocks and genetics

The wild type control (*w*¹¹¹⁸, #3605), UAS-mCD8-GFP (#5137) and *Df(3L)*^{8ex34} (#109087) stocks were obtained from the Bloomington *Drosophila* Stock Center (BDSC) and the UAS-*Ptp69D*-RNAi (RNA interference) line (ID104761) from the Vienna *Drosophila* Resource Center (Dietzl et al., 2007). The following PTP69D stocks have been previously described: *Ptp69D*¹⁰, *Ptp69D*²⁰, UAS-*Ptp69D*^{WT}, UAS-*Ptp69D*^{Ig}, UAS-*Ptp69D*^{Ig,FNIII}, UAS-*Ptp69D*^{FNIII}, UAS-*Ptp69D*^{FNIII,MPR}, UAS-*Ptp69D*^{Cat1-DA}, UAS-*Ptp69D*^{Cat1/2-DA}, and UAS-*Ptp69D*^{Cat2} (Desai and Purdy, 2003; Garrity et al., 1999). The c17 Gal4-driver,

which drives expression in the GF, but not in its target neurons, and the *ShakB* Gal4-driver, which only drives expression in the postsynaptic target neurons of the GF were described previously (Allen et al., 1999; Godenschwege et al., 2002; Jacobs et al., 2000). The R78G07 (#47022) and R91H05 (#40594) lines obtained from BDSC, were identified in a screen of the Janelia Farm Gal4 lines, as drivers that express selectively in the GF but not its postsynaptic target neurons (Jenett et al., 2012; Pfeiffer et al., 2008). Expression of these Gal4 lines in the GF is already present in the L3 larval stage during axon outgrowth and they maintain strong expression in the adult. For the characterization of *Ptp69D* mutant alleles, *Ptp69D*¹⁰ and *Ptp69D*²⁰ animals were reared at 25°C. For the rescue experiments the *c17/CyO*; *Ptp69D*^{20 or 10}/TM6β and *ShakB/CyO*; *Ptp69D*^{20 or 10}/TM6β stocks were generated and crossed to the various UAS-*Ptp69D* lines, that had been previously recombined onto the *Df(3L)^{8ex34}* deficiency chromosome (Garrity et al., 1999). All genetic crosses were performed on standard fly media at 25°C and 2-5 day old flies were used in all of the experiments.

Electrical stimulation of the neurons of the GFC and monitoring of muscle potentials

The method of obtaining electrophysiological recordings from the GFC has been described in detail previously (Allen and Godenschwege, 2010). In brief, the GFs were stimulated (Grass instruments S48 stimulator) with pulses (0.03 ms, 50 V) in the brain using the pair of tungsten electrodes. Glass electrodes (resistances of 40-60 MΩ) filled with saline were used for recordings from the tergotrochanteral muscles (TTMs) and dorsal longitudinal muscles (DLMs). The responses were amplified (Microelectrode amplifier Model 5a, Getting Instruments) and the traces were recorded, stored and analyzed using pClamp 10 software (Molecular Devices). To determine the response latency (RL) ten individual stimuli were given with a 5 second interval between the stimuli and the shortest RL of each fly was averaged for each genotype. The following frequency (FF) was determined as the number of responses in percent of ten trains of ten stimuli at 100 Hz with a two second interval between the trains. In mutant animals that had a reduced ability to follow stimuli at 100 HZ, we stimulated the motoneurons in the thorax directly in order to confirm that functional defects were not at the neuromuscular junction but at the GF to TTMn (GF-TTMn) connections. Data analysis using a two-way ANOVA on groups, followed by post-hoc t-tests between groups with a Bonferroni correction to determine statistical significance (SigmaPlot 12).

Dye injections and immunohistochemistry of the GF

Dye injection and immunohistochemistry methods have previously been described in detail (Boerner and Godenschwege, 2010, 2011). In brief, the dissected animal CNS was mounted dorsal side up on VECTABOND™ (Vector Labs) coated 0.9-1.1 mm etched slides. A glass electrode (80-100 MΩ) filled with a dye solution of 10% w/v neurobiotin (Vector Labs) and tetramethyl rhodamine-labeled dextran (Invitrogen) backfilled with 2 M potassium acetate was used to inject the dyes into the GF axons by passing depolarizing current. Samples fixed in 4% paraformaldehyde were prepared for confocal microscopy as described previously (Boerner and Godenschwege, 2010, 2011). The following antibodies were used: streptavidin-Cy2 conjugate (Jackson ImmunoResearch; 1:750), anti-GFP A11122 (Invitrogen, 1:500), goat anti-rabbit-Cy2 (Jackson ImmunoResearch, 1:500 dilution) to

visualize neurobiotin or GFP. Samples were scanned at a resolution of 1024×1024 pixels, 2.5× zoom, and 0.5 μm step size with a Nikon C1si Fast Spectral Confocal system using a 60× oil immersion objective lens. Images were processed using Nikon Elements Advance Research 4.0 and Adobe Photosuite CS4 software.

Results

Characterization of *Ptp69D* missense mutants in the GFC

Ptp69D null mutants exhibit lethality and typically do not survive past the larval stage (Desai et al., 1996). Therefore, we characterized the phenotypes of the viable *Ptp69D*^{I0} and *Ptp69D*^{D20} alleles, which carry missense mutations in the first Ig domain and Cat1 domain, respectively, in adult GF neurons (Fig. 1 A) (Desai and Purdy, 2003; Desai et al., 1996). The GF axons can be reliably identified in the cervical connective using differential interference contrast microscopy. We used dye injections of rhodamine-dextran and neurobiotin into the GF axons to reveal the morphology of the terminals and to determine if the GFs are coupled to their synaptic targets via the gap junctions of their electrical-chemical synapses. All GFs in both mutant alleles reached the synaptic target area in the second thoracic neuromere without any guidance defects and only disrupted the GF-TTMn connection anatomically and functionally, but not the GF to PSI (GF-PSI) connection (Fig. 1 B-E, Fig. 2, data not shown). In homozygous *Ptp69D*^{I0} (n=22) and *Ptp69D*^{D20} (n=20) mutants, the GF terminals were dramatically shorter when compared to control animals (Fig. 1 C-E). However, all GF terminals dye-coupled with the TTMns, although often weakly, suggesting that terminal growth and not synaptic targeting was disrupted in these mutants.

Consistent with the morphological defects of the GF terminal, we found that the function of the GF-TTM pathways was severely impaired. In control animals, the GF-TTM pathway is able to respond in a 1:1 ratio when the GFs are stimulated with 10 pulses at 100 Hz (Fig. 1 A, Fig. 2 A). In contrast, the average FF of the GF-TTM pathway was severely reduced in *Ptp69D*^{I0} (FF=27%) and *Ptp69D*^{D20} (FF=32%) animals (Fig. 2 B, C). Correlating with the decrease to follow stimulations at 100 Hz, the average RL of the GF-TTM pathway to an individual stimulus significantly increased in *Ptp69D* mutants (1 ms), when compared to the control animals (0.8 ms) (Fig. 2 D). In control animals the RL remained constant in individual flies, when 10 stimuli were given at 5 Hz (Fig. 2B). In contrast, in the *Ptp69D* mutants the RL varied and the duration usually increased further (Fig. 2B) or the GF-TTM pathways failed to respond, similar to repetitive stimulation seen at 100 Hz (Fig. 2 A) revealing a severe weakening of the synaptic connection. When we used thoracic stimulation to bypass the GFs and activate the TTMns directly, 100% FF of the neuromuscular junctions and a RL below 0.8 ms were observed (data not shown). This confirmed that the site of the synaptic defect was at the GF-TTMn synapse. The functional defects were slightly enhanced when the *Ptp69D*^{I0} and *Ptp69D*^{D20} alleles were tested over a chromosome deficient (*Df(3L)^{8ex34}*) for the *PTP69D* gene demonstrating that the observed defects are PTP69D-specific (Fig. 2 C, D). Finally, we generated *Ptp69D*^{I0}/*Ptp69D*^{D20} transheterozygous animals in order to test whether *Ptp69D*^{I0} can complement for the lack of catalytic function in *Ptp69D*^{D20} animals. The function of the GF-TTMn connection remained severely disrupted

(Fig. 2 C, D), and the anatomical defects (n=8) were indistinguishable from homozygous *Ptp69D*¹⁰ and *Ptp69D*²⁰ mutants.

Structure-function analyses of *Ptp69D* signaling via the Cat1 domain

The glycine to serine missense mutation in the Cat1 domain in *Ptp69D*²⁰ mutants was shown to inhibit catalytic activity (Marlo and Desai, 2006), and our characterization of these mutants revealed that the Cat1 domain has a function in GF terminal growth, but not in guidance or targeting. In order to further analyze the spatial and functional requirements for the signaling of the Cat1 domain we expressed wild type and mutant *Ptp69D* constructs in a *Ptp69D*²⁰/*Df*(3L)^{8ex34} background. Presynaptic expression of UAS-*Ptp69D*^{WT} with the c17 Gal4 driver in this mutant background restored the synaptic function in virtually all animals (Fig. 3 A) and resulted in normal sized terminals (Fig. 3 B). In contrast, postsynaptic expression of wild type PTP69D protein using the *ShakB*-Gal4 driver did not rescue the function of the GF-TTM pathway, and the GF terminal remained severely stunted in these animals (Fig. 3 A, C). An analysis of variance showed that the expression of UAS-*Ptp69D*^{WT} was significant ($F(2,58) = 213.14$, $p < 0.001$). Post hoc analyses using the Bonferroni correction for significance indicated that the presynaptic, but not postsynaptic, expression of wild type PTP69D was significantly higher than *Ptp69D*²⁰/*Df*(3L)^{8ex34}, rescuing the phenotype ($M = 47.8$, $SD = 10$, $p \text{ value} < 0.001$). This suggests that Cat1 activity of *Ptp69D* is required in the GF for terminal growth.

In order to determine what domains are required for outside-in signaling of *Ptp69D*, we expressed mutant constructs of *Ptp69D* presynaptically in a *Ptp69D*²⁰/*Df*(3L)^{8ex34} background whereby either the Ig domains (IG), FNIII domains (FN), both domains (IG+FN), or FNIII and MPR domains (FN+MPR) were lacking (Fig. 1 A). In addition, we tested if constructs with an intracellular deletion of the Cat2 domain (Cat2) or aspartic acid to alanine missense mutation in both catalytic domains (Cat1/2-DA) could rescue the defects of *Ptp69D*²⁰/*Df*(3L)^{8ex34} animals. We found that the expression of constructs with deleted Ig domains (IG and IG+FN) did not rescue the function of the GF synaptic terminal (Fig. 3 A). This suggests that *Ptp69D* requires the Cat1 and the Ig domains for GF terminal growth. Similarly, neither the expression of UAS-*Ptp69D*^{Cat2} nor UAS-*Ptp69D*^{Cat1/2-DA} with the c17 driver was able to rescue the synaptic or morphological defects of *Ptp69D*²⁰/*Df*(3L)^{8ex34} animals. Thus, GF terminal function and growth involves both catalytic domains (Fig. 3 A, E). In contrast, the expression of PTP69D^{FN} or PTP69^{FN+MPR} in *Ptp69D*²⁰/*Df*(3L)^{8ex34} mutants was able to rescue the synaptic function, as well as the GF terminal anatomy (Fig. 3 A, D). This demonstrates that Cat1 function does not require the FNIII or the MPR domains in GF terminal growth.

Finally, the subtle but not statistically significant decrease of the average FF of the GF-TTM pathway by the expression of UAS-*Ptp69D*^{Cat1/2-DA} with the c17 driver in a wild type background (Fig. 3 F), indicates a putative dominant negative effect. However, the expression of all other diverse mutant constructs had no effect on the function of the GF-TTM pathway when expressed with the c17 Gal4 driver in a wild type background (Fig. 3F). This suggests that these mutant PTP69D proteins have no poisonous or dominant negative effects at this expression level. The fact that the expression of PTP69D^{FN} and

PTP69D^{FN+MPR} rescued the GF defects and the expression of PTP69D^{Ig} and PTP69D^{Cat2} rescued the loss of function defects in the visual system (Garrity et al., 1999), demonstrates that these transgenes produce proteins with residual functions that are expressed at the plasma membrane of neurons and are not subject to general retention in the endoplasmic reticulum. Because the expression of the same transgenic lines had opposite effects with the respect to their rescue ability, it suggests that the differences are due to distinct domain-specific requirements during photoreceptor targeting and GF terminal growth.

Temporal and spatial requirements for PTP69D in the GFC

The rescue experiments of the *Ptp69D*²⁰ mutants demonstrate a presynaptic function for the Cat1 domain of PTP69D in GF terminal growth. Expression of PTP69D^{Cat2} or PTP69D^{Cat1-DA} was able to rescue the photoreceptor targeting defects of PTP69D null mutants, revealing that the activity of one catalytic domain is sufficient for photoreceptor targeting (Garrity et al., 1999). The aspartic acid to alanine (D-A) conversion in the first catalytic domain of the latter construct is predicted to prevent dephosphorylation; consequently, the RPTPs with this mutation function as “substrate traps” because they remain tightly bound to the target protein (Flint et al., 1997; Tiganis and Bennett, 2007). We used three distinct Gal4-lines which drive expression in the GF during development and in the adult, but do not drive expression in the postsynaptic target neurons. The c17 lines only turn on expression in the GF after it has exited the brain and entered the thoracic ganglion during early pupal stages (Godenschwege et al., 2002; Jenett et al., 2012; Pfeiffer et al., 2008). In addition, we used two Gal4 lines, R78G07 and R91H05 which we recently identified in a screen of the Janelia FlyLight Gal4 Collection (Jenett et al., 2012; Pfeiffer et al., 2008). Both lines drive expression in the GF more robustly and selectively and are already turned on during GF neurite outgrowth in L3 larvae. For postsynaptic expression in the TTMn, throughout development and in the adult, we used the *ShakB*-Gal4 line (Jacobs et al., 2000).

Pre- but not postsynaptic expression of UAS-*Ptp69D*^{Cat1-DA} in a wild type background disrupted the anatomy and function of the GF (Fig. 4 A-D). The majority of the GF terminals, were severely stunted (Fig. 4 A, C, D) and the ability of the GF-TTM pathway to follow high frequency stimulation was significantly impaired (Fig. 4 B), when UAS-*Ptp69D*^{Cat1-DA} was expressed strongly with the R91H05 and R78G07 drivers. PTP69D^{Cat1} was sufficient for photoreceptor targeting (Garrity et al., 1999), but its expression in the GF caused morphological and functional GF phenotypes similar to *Ptp69D*^{I0} and *Ptp69D*²⁰ mutants. This suggests that PTP69D^{Cat1-DA} has a dominant negative effect on terminal growth by possibly reducing signaling of Cat1-specific targets in the GF. Taken together, these results also further support a cell-autonomous role in the GF for PTP69D signaling involving the Cat1 domain during terminal growth.

In addition, we expressed wild type PTP69D in a *Ptp69D*^{I0} mutant background to test for the spatial requirements of the Ig-domains. We find that the expression of wild type PTP69D in the GF but not in the TTMn was able to rescue the synaptic defects of *Ptp69D*^{I0} mutants

(Fig. 4E). This demonstrates that the first Ig domain of PTP69D is required in the GF for proper signaling during terminal growth as well.

Finally, we expressed a RNA interference (RNAi)-inducing line directed against a coding region of PTP69D to down regulate signaling of all PTP69D domains (Dietzl et al., 2007). In some animals expressing UAS-*Ptp69D*^{RNAi} with the R78G07 (4 out of 14 recordings) and the R91H05 (7 out of 25 recordings) Gal4 drivers, but not with the c17 Gal4 driver, we were not able to obtain any electrophysiological recordings from the GF-TTM pathway or from the GF to DLM (GF-DLM) pathway; this suggests that these GF did not reach the target area. In this genotype we could not visualize all GFs with differential interference contrast microscopy in the cervical connective for dye injections, therefore we co-expressed transmembrane-bound mCD8-GFP presynaptically with UAS-*Ptp69D*^{RNAi} to label the GFs. Correlating with the electrophysiological phenotype, we found that some GFs failed to exit the brain and often projected towards the retina instead (Fig. 5 A and B, arrow). However, all GFs that exited the brain reached the synaptic target area and dye-coupled with the PSIs. With all three presynaptic drivers we found that the expression of UAS-*Ptp69D*^{RNAi} disrupted the growth and function of the GF terminal in the target area similar to *Ptp69D*¹⁰ and *Ptp69D*²⁰ mutants (Fig. 5 C-G). However, while most GFs dye-coupled with their postsynaptic target neurons, some GFs did not dye-couple with the TTMns and the GF-TTMn terminal was completely lacking (Fig. 5 D). Consistent with the anatomical phenotypes, the function of the GF-TTMn pathway was severely impaired in this genotype (Fig. 5 F). In contrast, the expression of UAS-*Ptp69D*-RNAi postsynaptically did not affect the GF anatomy or the function of the GF to TTM pathway (Fig. 5 E-G). The majority of defects observed were similar to *Ptp69D*¹⁰ and *Ptp69D*²⁰ mutants suggesting that the RNAi construct down regulates PTP69D and the guidance and target defects seen are consistent with previously described functions for PTP69D. While we can not exclude PTP69D nonspecific effects of the RNAi or the UAS-*Ptp69D*^{Cat1-DA} line, the finding that these lines did not disrupt the TTMn function when expressed with the *ShakB*-Gal4 line suggests that they do not generally affect the development of all neurons.

Discussion

In this study, we uncover and characterize a novel role for PTP69D in synaptic terminal growth in the CNS, which is distinct from its previously described functions in guidance and target recognition. A potential function for PTP69D in terminal growth in the peripheral nervous system has been previously implied by the finding that bouton numbers at the larval neuromuscular junction are reduced in *Ptp69D* null mutants, but functional requirements have not been determined (Hofmeyer and Treisman, 2009). Here, we used structure-function analyses to reveal the domains required in synaptic terminal growth of the GF.

In the visual system PTP69D is required in the axon for proper target selection (Garritty et al., 1999; Hofmeyer and Treisman, 2009). Without PTP69D the R1-6 and the R7 photoreceptor neurons pass by the target area in the lamina and medulla, respectively. In the *Ptp69D* mutant embryonic nervous systems, motoneurons did not innervate their respective muscles due to guidance defects (Desai and Purdy, 2003; Desai et al., 1996; Desai et al., 1997; Song et al., 2008). In addition, a role for PTP69D in axonal branching of mushroom

body neurons has been described (Kurusu and Zinn, 2008). We found that the missense mutations in the first Ig domain and the Cat1 domain of *Ptp69D*¹⁰ and *Ptp69D*²⁰ mutants did not affect GF guidance, branching or targeting. Though all GFs dye-coupled with their target TTMn motoneurons, they failed to grow a full-size terminal, resulting in a severely weakened synaptic connection (Fig. 1, 2). We demonstrated that PTP69D is required cell-autonomously in the GF for terminal growth as the synaptic phenotypes of *Ptp69D*¹⁰ and *Ptp69D*²⁰ mutants can be rescued with pre- but not postsynaptic expression of the wild type PTP69D. Further evidence for a presynaptic role is provided by the finding that pre- but not postsynaptic expression of PTP69D^{Cat1-DA} or a putative *Ptp69D*^{RNAi} line in a wild type background disrupted GF terminal growth and function.

Both catalytic domains in RPTPs are capable of dephosphorylating substrates; the catalytic domain that is more proximal to the membrane is thought to catalyze the majority of dephosphorylation reactions (Streuli et al., 1989). For R1-6 photoreceptors it was shown that targeting is disrupted when both catalytic domains are mutated, but not when either catalytic domain alone is mutated. This suggests the activity of one catalytic domain is sufficient for R1-6 photoreceptor targeting. In contrast, the activity of one catalytic domain is not sufficient for GF terminal growth as demonstrated by synaptic phenotypes in *Ptp69D*²⁰ mutants. Nevertheless, a function for both catalytic domains in terminal growth is implied by our findings that PTP69D^{Cat2} expression did not rescue the synaptic defects of *Ptp69D*²⁰ mutants.

These results suggest that the PTP69D signaling required for photoreceptor targeting is mechanistically distinct from that required for GF terminal growth. This hypothesis is further supported by rescue experiments demonstrating that R1-6 photoreceptors targeting requires the FNIII domain but not the Ig domain (Garrity et al., 1999), while we find the opposite to be true for GF terminal growth. We show that the expression of PTP69D lacking the FNIII domains (FN and FN+MPR), but not PTP69D lacking the Ig domains, was able to rescue the synaptic function of the GFs in *Ptp69D*²⁰ mutants. Interestingly, R7 targeting to the medulla requires the FNIII domains of tyrosine phosphatase DLAR, while the catalytic activity is provided by PTP69D and not DLAR (Hofmeyer and Treisman, 2009). This suggests a general function for the FNIII domains of receptor tyrosine phosphatases in axon targeting.

Ample evidence suggests that extracellular interactions of the Ig-domains are critical for proper regulation of Cat1 activity during GF terminal growth. First, anatomical and functional synaptic defects of *Ptp69D*¹⁰ animals are indistinguishable from *Ptp69D*²⁰ mutants demonstrating a requirement for the Ig and the Cat1 domains in this developmental process. Furthermore, *Ptp69D*¹⁰ and *Ptp69D*²⁰ mutants do not complement each other as transheterozygote *Ptp69D*¹⁰/*Ptp69D*²⁰ animals still exhibited a mutant phenotype (Fig. 2 C, D). This is consistent with the finding that the expression of PTP69D^{Ig} did not restore GF terminal growth in *Ptp69D*²⁰ mutants, although the same transgenic line was able to rescue axon targeting defects of photoreceptor neurons (Garrity et al., 1999). Finally, we find that the expression of PTP69D^{FN+MPR} lacking all extracellular domains but the Ig domains, was able to rescue the synaptic function of the GFs.

Although constructs lacking the FNIII and MPR domains fully rescued the *Ptp69D*²⁰ phenotypes, it does not imply that these domains have no function in target selection of the GF. The absence of any GF guidance or targeting defects in the *Ptp69D*¹⁰ and *Ptp69D*²⁰ mutants suggests that the affected domains are either not required during earlier developmental processes or that, similar to R1-6 photoreceptor targeting, the activity of one catalytic domain is sufficient and involves the regulation via the FNIII and MPR domains.

In summary, we conclude that PTP69D is essential for proper GF development and that the regulation of the Cat1 activity during GF terminal growth involves the Ig domains, but not the FNIII and MPR domains. In view of previously published data, we find that PTP69D has different functions in different neurons and developmental processes with distinct requirements for the Ig, FNIII and MPR domains.

Acknowledgements

This work was supported by a grant from the National Institutes of Health (R01HD050725) to TAG. LHL was supported by a Research Supplement to Promote Diversity in Health-Related Research from the National Institute of Child Health and Human Development (R01HD050725-S). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NICHD or the NIH. The authors declare no competing financial interests. We would also like to thank Kerstin Hofmeyer, Paul A. Garrity, Chan J. Desai and the Bloomington Stock Center as well as the Vienna Drosophila Resource Center for providing fly stocks. We are grateful to Drs D Baronas-Lowell and SB Uthaman for editorial support.

Abbreviations

CNS	central nervous system
RPTP	protein tyrosine phosphatase
GF	giant fiber
Ig	immunoglobulin
FNIII	fibronectin type III
MPR	membrane-proximal region
Cat	catalytic domain
GFC	giant fiber circuit
TTM	tergotrochanteral muscle
TTMn	tergotrochanteral motorneuron
PSI	peripheral synapsing interneuron
DLM	dorsal longitudinal muscle
RL	response latency
FF	following frequency

References

- Allen MJ, Godenschwege TA. Electrophysiological recordings from the *Drosophila* giant fiber system (GFS). Cold Spring Harbor protocols 2010. 2010 pdb prot5453.

- Allen MJ, Godenschwege TA, Tanouye MA, Phelan P. Making an escape: development and function of the *Drosophila* giant fibre system. *Seminars in cell & developmental biology*. 2006; 17:31–41. [PubMed: 16378740]
- Allen MJ, Shan X, Caruccio P, Froggett SJ, Moffat KG, Murphey RK. Targeted expression of truncated glued disrupts giant fiber synapse formation in *Drosophila*. *J Neurosci*. 1999; 19:9374–9384. [PubMed: 10531442]
- Boerner J, Godenschwege TA. Application for the *Drosophila* ventral nerve cord standard in neuronal circuit reconstruction and in-depth analysis of mutant morphology. *Journal of neurogenetics*. 2010; 24:158–167. [PubMed: 20615087]
- Boerner J, Godenschwege TA. Whole mount preparation of the adult *Drosophila* ventral nerve cord for giant fiber dye injection. *Journal of visualized experiments : JoVE*. 2011
- Desai C, Purdy J. The neural receptor protein tyrosine phosphatase DPTP69D is required during periods of axon outgrowth in *Drosophila*. *Genetics*. 2003; 164:575–588. [PubMed: 12807778]
- Desai CJ, Gindhart JG Jr. Goldstein LS, Zinn K. Receptor tyrosine phosphatases are required for motor axon guidance in the *Drosophila* embryo. *Cell*. 1996; 84:599–609. [PubMed: 8598046]
- Desai CJ, Krueger NX, Saito H, Zinn K. Competition and cooperation among receptor tyrosine phosphatases control motoneuron growth cone guidance in *Drosophila*. *Development*. 1997; 124:1941–1952. [PubMed: 9169841]
- Dietzl G, Chen D, Schnorrrer F, Su KC, Barinova Y, Fellner M, Gasser B, Kinsey K, Oppel S, Scheiblaue S, Couto A, Marra V, Keleman K, Dickson BJ. A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. *Nature*. 2007; 448:151–156. [PubMed: 17625558]
- Flint AJ, Tiganis T, Barford D, Tonks NK. Development of "substrate-trapping" mutants to identify physiological substrates of protein tyrosine phosphatases. *Proceedings of the National Academy of Sciences of the United States of America*. 1997; 94:1680–1685. [PubMed: 9050838]
- Garrity PA, Lee CH, Salecker I, Robertson HC, Desai CJ, Zinn K, Zipursky SL. Retinal axon target selection in *Drosophila* is regulated by a receptor protein tyrosine phosphatase. *Neuron*. 1999; 22:707–717. [PubMed: 10230791]
- Godenschwege TA, Simpson JH, Shan X, Bashaw GJ, Goodman CS, Murphey RK. Ectopic expression in the giant fiber system of *Drosophila* reveals distinct roles for roundabout (Robo), Robo2, and Robo3 in dendritic guidance and synaptic connectivity. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2002; 22:3117–3129. [PubMed: 11943815]
- Hofmeyer K, Treisman JE. The receptor protein tyrosine phosphatase LAR promotes R7 photoreceptor axon targeting by a phosphatase-independent signaling mechanism. *Proceedings of the National Academy of Sciences of the United States of America*. 2009; 106:19399–19404. [PubMed: 19889974]
- Jacobs K, Todman MG, Allen MJ, Davies JA, Bacon JP. Synaptogenesis in the giant-fibre system of *Drosophila*: interaction of the giant fibre and its major motoneuronal target. *Development*. 2000; 127:5203–5212. [PubMed: 11060245]
- Jenett A, Rubin GM, Ngo TT, Shepherd D, Murphy C, Dionne H, Pfeiffer BD, Cavallaro A, Hall D, Jeter J, Iyer N, Fetter D, Hausenfluck JH, Peng H, Trautman ET, Svirskas RR, Myers EW, Iwinski ZR, Aso Y, DePasquale GM, Enos A, Hulamm P, Lam SC, Li HH, Lavery TR, Long F, Qu L, Murphy SD, Rokicki K, Safford T, Shaw K, Simpson JH, Sowell A, Tae S, Yu Y, Zugates CT. A GAL4-driver line resource for *Drosophila* neurobiology. *Cell reports*. 2012; 2:991–1001. [PubMed: 23063364]
- Kurusu M, Zinn K. Receptor tyrosine phosphatases regulate birth order-dependent axonal fasciculation and midline repulsion during development of the *Drosophila* mushroom body. *Molecular and cellular neurosciences*. 2008; 38:53–65. [PubMed: 18356078]
- Marlo JE, Desai CJ. Loss of phosphatase activity in Ptp69D alleles supporting axon guidance defects. *Journal of cellular biochemistry*. 2006; 98:1296–1307. [PubMed: 16514605]
- Newsome TP, Asling B, Dickson BJ. Analysis of *Drosophila* photoreceptor axon guidance in eye-specific mosaics. *Development*. 2000; 127:851–860. [PubMed: 10648243]
- Pfeiffer BD, Jenett A, Hammonds AS, Ngo TT, Misra S, Murphy C, Scully A, Carlson JW, Wan KH, Lavery TR, Mungall C, Svirskas R, Kadonaga JT, Doe CQ, Eisen MB, Celniker SE, Rubin GM.

- Tools for neuroanatomy and neurogenetics in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105:9715–9720. [PubMed: 18621688]
- Song JK, Giniger E, Desai CJ. The receptor protein tyrosine phosphatase PTP69D antagonizes Abl tyrosine kinase to guide axons in *Drosophila*. *Mechanisms of development*. 2008; 125:247–256. [PubMed: 18160268]
- Stoker AW. RPTPs in axons, synapses and neurology. *Seminars in cell & developmental biology*. 2014
- Streuli M, Krueger NX, Tsai AY, Saito H. A family of receptor-linked protein tyrosine phosphatases in humans and *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*. 1989; 86:8698–8702. [PubMed: 2554325]
- Tiganis T, Bennett AM. Protein tyrosine phosphatase function: the substrate perspective. *The Biochemical journal*. 2007; 402:1–15. [PubMed: 17238862]
- Van Vactor D. Protein tyrosine phosphatases in the developing nervous system. *Current opinion in cell biology*. 1998; 10:174–181. [PubMed: 9561841]

Figure 1. Anatomical phenotypes of *Ptp69D* missense mutants in the GFC

A) Schematic of PTP69D protein structure, indicating the mutation sites (*) representative of the *Ptp69D*¹⁰ and *Ptp69D*²⁰ alleles (Desai and Purdy, 2003). Deletions () in transgenic constructs used for structure-function analysis are depicted by lines adjacent to the molecule (Garrity et al., 1999). A construct with D-A conversions in both Cat1 and Cat2 domains is not depicted. **B)** Schematic of the CNS of *Drosophila* depicting the GFC within the brain and the ventral nerve cord (modified from Allen and Godenschwege, 2010). The two GFs (white), somas and dendrites are located in the brain. In the GF-TTM pathway, the GF axon forms a large synaptic terminal onto the TTMn (green) in the second thoracic neuromere, which innervates the TTM. In the GF-DLM pathway, the GF synapses with PSI (green). The PSI synapses with the DLMs (gray), which innervates the DLM. Placement of stimulation and recording electrodes as well as site of dye injections are indicated. **C-E)** GF synaptic terminals and the dye-coupling to the postsynaptic target neurons were visualized by co-injection of rhodamine-dextran (white) and neurobiotin (green) into the GF in the cervical connective. Projection views of confocal stacks of the GF terminals are shown. Scale bars are 20 μ m. In *w*¹¹¹⁸ wild type control animals (**C**), the GFs exhibited large GF terminals and dye-coupled with the TTMns and the PSIs. In homozygous *Ptp69D*¹⁰ (**D**) and *Ptp69D*²⁰ (**E**) mutants the GFs were severely stunted (arrowheads) but dye-coupled with the TTMns and the PSIs in all cases.

Figure 2. Electrophysiological phenotypes of *Ptp69D* missense mutants in the GFC

A) Electrophysiological traces of the GF-TTM pathway from wild type control animals (*w*¹¹¹⁸) and *Ptp69D* mutants. Control animals, but not *Ptp69D*¹⁰ and *Ptp69D*²⁰ mutants, were able to respond in a 1:1 ratio when the GFs were stimulated at 100 Hz. The failures to respond to a stimulus are indicated by asterisks. **B)** Ten overlaid TTM responses of an individual fly when the GF was stimulated at 5Hz. RL of control animals was 0.87 ms (dashed gray line) and remained the same for all 10 stimuli. In *Ptp69D* mutants the RL was increased and often shifted to longer durations when stimulated multiple times. **C)** The quantification of responses to 10 stimuli given at 100 Hz is depicted by the average FF in percent. The average FF is significantly reduced in *Ptp69D* mutants, when compared to control animals. ANOVA showed significance and a Bonferroni corrected post hoc t test indicates statistical significance (* = p value <0.05, ** = p value <0.001). **D)** The average RL of the GF-TTM pathway in control animals and *Ptp69D* mutants. Asterisks indicate significance (* = p value <0.05, ** = p value <0.001, two-way ANOVA and Bonferroni corrected post hoc t test).

Figure 3. Rescue of *Ptp69D²⁰* mutants with target expression of wild type and mutant *Ptp69D* constructs

A) Quantification of FF defects of the GF-TTM pathway when wild type and mutant *Ptp69D* constructs were expressed pre- or postsynaptically in a *Ptp69D²⁰/Df(3L)^{8ex34}* background. Only presynaptic expression of *UAS-Ptp69D^{WT}*, *UAS-Ptp69D^{FN}* and *UAS-Ptp69D^{FN+MPR}* with the c17 Gal4-driver improved the ability of the GF-TTM pathway to follow stimuli at 100 Hz to wild type levels (** = p value < 0.001, two-way ANOVA and Bonferroni corrected post hoc t test). **B)** The GF morphology and dye-coupling to the postsynaptic target neurons is normal on c17/+; *Ptp69D²⁰/UAS-PTP69D^{WT}*, *Df(3L)^{8ex34}* animals. **C)** The GF terminals were stunted in *ShakB-Gal4/+; Ptp69D²⁰/UAS-Ptp69D^{WT}*, *Df(3L)^{8ex34}* animals. **D)** Expression of *UAS-Ptp69D^{FN}* with the c17 Gal4 driver line resulted in normal sized GF terminals in a *Ptp69D²⁰/Df(3L)^{8ex34}* background. **E)** Presynaptic expression of PTP69D with a missense D to A mutation in both catalytic domains did not rescue the *Ptp69D²⁰* phenotypes. The GF terminals were severely stunted in c17/+; *Ptp69D²⁰/UAS-Ptp69D^{Cat1/2-DA}*, *Df(3L)^{8ex34}* animals. **F)** Quantification of FF defects of the GF-TTM pathway when wild type and mutant *Ptp69D* constructs were expressed with the c17 Gal4 driver in GFs of wild type flies.

Figure 4. Targeted expression of catalytically inactive mutant in the GFC

A) Quantification of morphological GF defects when *UAS-Ptp69D^{Cat1-DA}* was overexpressed pre- or postsynaptically. **B)** Quantification of FF defects of the GF-TTM pathway when *PTP69D^{Cat1-DA}* was expressed pre- or postsynaptically in the wild type background. Strong presynaptic expression with the R78G07 and R91H05 Gal4 drivers significantly disrupted the GF function (* = p value 0.05, ** = p value < 0.001, two-way ANOVA and Bonferroni corrected post hoc t test). **C)** Postsynaptic expression of *UAS-Ptp69D^{Cat1-DA}* with the *ShakB*-Gal4 line did not affect GF morphology. Rhodamine-dextran injections into the GF axons were used to reveal the GF morphology. Scale bar, 20 μ m. **D)** Presynaptic expression of *PTP69D^{Cat1-DA}* protein in the GF of the R78G07 line disrupted the growth of a GF terminal. Rhodamine-dextran injections into the GF axons were used to reveal the GF morphology. Scale bar, 20 μ m. **E)** Quantification of the GF-TTM pathway in wild type and mutant *Ptp69D¹⁰* animals with pre- or postsynaptic expression of *UAS-Ptp69D^{WT}* in a *Ptp69D¹⁰/Df(3L)8ex34* deficient background. Expression of wild type PTP69D in the GF (c17 Gal4) but not in the TTMn (*ShakB*-Gal4) rescued the ability of the GF-TTM pathway to follow stimuli at 100Hz (** = p value < 0.001, two-way ANOVA and Bonferroni corrected post hoc t test).

Figure 5. Phenotypes of PTP69D knockdown in the GFC

Knockdown of PTP69D in the GFC was achieved by pre- or postsynaptic expression of UAS-*Ptp69D*^{RNAi}. GF morphology in the brain and cervical connective was revealed by the co-expression of mCD8-GFP (A, B, scale bars are 50 μ m) or with dye injection (C-E, scale bars are 20 μ m), respectively. Projection views of confocal stacks are shown. A) In control animals all GFs exited the brain in the subesophageal ganglion (arrowheads), when mCD8-GFP was expressed with the R91H05 driver. B) In animals co-expressing mCD8-GFP and *Ptp69D*^{RNAi}, some GFs did not exit the brain and grew towards the retina instead (arrow). C) Presynaptic expression of UAS-*Ptp69D*^{RNAi} with the R91H05 driver resulted in some GFs that dye-coupled with the PSIs but not the TTMns. D) Example of presynaptic expression of UAS-*Ptp69D*^{RNAi} with the R78G07 driver. One GF is of normal size and the other GF is stunted (arrowhead) but both dye-coupled with the TTMns and the PSIs. E) Postsynaptic expression of UAS-*Ptp69D*^{RNAi} with *ShakB*-Gal4 did not affect the GF morphology or its dye-coupling with the PSI and the TTMn. F) Quantification of the GF anatomical phenotypes with knockdown of PTP69D by RNAi pre- or postsynaptically. G) Quantification of FF from the GF-TTM pathway for animals with defects in the synaptic target area. Animals without responses from the DLM and the TTM (indicating a guidance defect) were not included in the calculations. FFs were significantly decreased when UAS-*Ptp69D*^{RNAi} was expressed with presynaptic drivers, but not with the postsynaptic-expressing *ShakB*-Gal4 line (* = p value 0.05, ** = p value < 0.001, two-way ANOVA and Bonferroni corrected post hoc t test).