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The Receptor protein tyrosine phosphatase PTP69D antagonizes Abl tyrosine kinase to guide axons in *Drosophila*

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Abstract

During *Drosophila* embryogenesis, both the cytoplasmic Abelson tyrosine kinase (Abl) and the membrane bound tyrosine phosphatase PTP69D are required for proper guidance of CNS and motor axons. We provide evidence that PTP69D modulates signaling by Abl and its antagonist, Ena. An *Abl* loss-of function mutation dominantly suppresses most *Ptp69D* mutant phenotypes including larval/pupal lethality and CNS and motor axon defects, while increased Abl and decreased Ena expression dramatically increase the expressivity of *Ptp69D* axonal defects. In contrast, *Ptp69D* mutations do not affect *Abl* mutant phenotypes. These results support the hypothesis that PTP69D antagonizes the Abl/Ena genetic pathway, perhaps as an upstream regulator. We also find that mutation of the gene encoding the cytoplasmic *Src64B* tyrosine kinase exacerbates *Ptp69D* phenotypes, suggesting that two different cytoplasmic tyrosine kinases, Abl and Src64B, modify PTP69D-mediated axon patterning in quite different ways.

Keywords

Axon guidance; RPTP; Src; Signal transduction; Tyrosine kinase; Tyrosine phosphatase

INTRODUCTION

Axon guidance establishes the nervous system by connecting neurons with their synaptic targets during development (Goodman, 1996). Many of the proteins involved in axon guidance regulate the state of tyrosine phosphorylation (Flanagan and Vanderhaeghen, 1998; Gallo and Letourneau, 1999; Stoker and Dutta, 1998). In mammals, for example, the cytoplasmic tyrosine kinases Src and Fyn mediate axonal outgrowth in the presence of cell adhesion molecules L1 and N-CAM respectively (Beggs et al., 1994; Ignelzi et al., 1994). In *Drosophila*, mutations in Abl tyrosine kinase cause certain axons to terminate prior to reaching peripheral muscle targets (Wills et al., 1999a). Abl interacts with two key regulators of actin polymerization, Enabled (Ena) and Chickadee, the *Drosophila* homologue of Profilin (Krause et al., 2002; Wills et al., 1999b). Misregulation of this pathway causes specific axonal defects. For example, both *ena* loss of function and *Abl* gain of function mutations cause CNS axons to cross the

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midline inappropriately and peripheral motor axons to grow past their targets (Bashaw et al., 2000; Wills et al., 1999a). These and other results strongly implicate tyrosine phosphorylation as a key regulator of actin dynamics in axon guidance.

Many tyrosine phosphatases, which reverse reactions catalyzed by tyrosine kinases, are also required by growing axons during axon guidance in both vertebrates (Stoker et al., 1995) and *Drosophila* (Chien, 1996; Desai et al., 1996). For example, mammalian receptor protein tyrosine phosphatases RPTPδ, RPTPκ and RPTPμ (Burden-Gulley et al., 2002; Drosopoulos et al., 1999; Wang and Bixby, 1999) and chick PTPσ (Rashid-Doubell et al., 2002) have been implicated in the growth and guidance of several populations of developing vertebrate neurons. In *Drosophila*, at least 5 membrane bound tyrosine phosphatases, DLAR, PTP10D, PTP52F, PTP69D and PTP99A, function in axon guidance during embryogenesis (Desai et al., 1997; Krueger et al., 1996). For example, *Ptp69D* is an essential gene involved in motor, central and retinal axon guidance, suggesting a pivotal role in nervous system development (Desai et al., 1996; Garrity et al., 1999).

The logic and mechanism behind the interplay of tyrosine kinases and phosphatases during axon guidance is a current area of interest. *dlar* mutations suppress the CNS midline crossing defect of *Abl* mutants (Wills et al., 2002), while *Abl* alleles suppress the intersegmental nerve b (ISNb) guidance defects of *Dlar* mutants. DLAR binds to Abl and these proteins phosphorylate/dephosphorylate one another *in vitro* (Wills et al., 1999a). Like DLAR, cytoplasmic domains of PTP69D can also bind to Abl *in vitro* (Wills et al., 1999a) and can substitute for DLAR in retinal and motor axon guidance (Desai et al., 1997; Maurel-Zaffran et al., 2001), suggesting potential involvement of PTP69D in Abl mediated signaling (Wills et al., 1999a). However, the physiological relevance of the *in vitro* interaction of Abl with PTP69D has not been determined. Recently identified and characterized *Ptp69D* alleles (Desai and Purdy, 2003; Marlo and Desai, 2006) show a broad range of developmental defects and reveal a requirement for enzymatic activity in the proximal tyrosine phosphatase domain (D1) for proper axonal guidance.

In this article, we show that the phenotypes of *Ptp69D* mutants are suppressed by *Abl* mutations and enhanced by overexpression of Abl. Furthermore, Abl gain-of-function phenotypes are supressed by PTP69D overexpression. On the other hand, *Abl* mutant phenotypes are not affected by loss of function or null mutation of *Ptp69D*. These results show that PTP69D antagonizes the effects of Abl and suggest that Abl may function downstream of PTP69D. Consistent with these conclusions, and with previous data showing that Abl acts by inhibiting Ena (Fox and Peifer, 2007; Gertler et al., 1995; Gertler et al., 1990; Grevengoed et al., 2003), *ena* mutations enhance *Ptp69D* defects. Mutations in *Ptp69D* also interact with mutations in another non-receptor tyrosine kinase, Src64B, a *Drosophila* member of the Src-family kinases (SFK). Strikingly however, *Src64B* mutations enhance rather than suppress most *Ptp69D* axonal defects. This finding raises the possibility that the relationship of tyrosine kinases and phosphatases is more complex than the evident antagonism of their biochemical activities.

RESULTS

Abl mutations suppress lethality of *Ptp69D*

Heteroallelic combinations of *Ptp69D* mutants confer partial lethality ranging from 56%- 91% (Desai and Purdy, 2003). We therefore examined the effect of *Abl* mutations on the lethality of *Ptp69D* mutants as a test for genetic interactions. *Abl* mutations dominantly suppressed lethality of various *Ptp69D* genotypes. For example, lethality of the hypomorphic genotype ($Ptp69D^{10}/Df(3L)^{8ex34}$) was decreased by reduction of Abl from 91% to 49%. Moreover, removal of one copy of Abl suppressed the embryonic/larval lethality of the null genotype ($Ptp69D^{1}/Df(3L)^{8ex34}$), allowing survival of a significant fraction of *Ptp69D* null animals to

pupal stages (Table 1). Molecular information and severity of *Ptp69D* alleles are described in Materials and Methods. In brief, $Ptp69D^{1}$ is null and $Ptp69D^{10}$ is temperature sensitive and can be weaker or stronger than null depending on temperature and assay (Desai and Purdy, 2003).

Abl strongly suppresses *Ptp69D* axon guidance defects

Ptp69D mutants display significant CNS and motor axon defects (Desai and Purdy, 2003; Marlo and Desai, 2006). For example, embryos hemizygous for $Ptp69D^{7}$ ($Ptp69D^{7}/Df(3L)^{8ex34}$), which has a mutation in the proximal phosphatase domain(D1) (Desai and Purdy, 2003), displayed CNS midline crossing defects in 54% of segments (Fig.1C, Table 2), and 64% of ISNb motor nerves bypassed the ventrolateral muscle (VLM) layer (Fig. 1D, Table 2), similar to the phenotypes observed for another D1 domain mutant allele, $Ptp69D^{21}$(Desai and Purdy, 2003). To test the ability of *Abl* to suppress *Ptp69D* axon defects, we introduced a heterozygous *Abl* mutation into embryos bearing various *Ptp69D* alleles. *Abl* mutations strongly suppressed CNS defects characteristic of several *Ptp69D* mutations. For example, the CNS defects of $Ptp69D^{7}/Df(3L)^{8ex34}$ dropped from 54% to 24% of segments when one copy of an *Abl* mutation was introduced (Fig. 1E, Table 2). Similarly, we found that *Abl* suppresses ISNb defects associated with *Ptp69D* mutations. In $Ptp69D^{1}/Ptp69D^{7}$, the ISNb bypassed its target VLMs and followed the ISN pathway towards the area of dorsal muscles in 58% of hemisegments (Fig. 1D, Table 2). In the presence of $Abl^{1}/+$, the bypass rate dropped to 23% (Fig. 1F, Table 2). These results support the hypothesis that *Abl* antagonizes *Ptp69D* axon guidance function in both CNS and motor neurons.

Ptp69D mutant phenotypes are enhanced by *Abl* gain-of-function

Since *Abl* mutations suppressed *Ptp69D* phenotypes, we expected that increasing Abl levels would exacerbate *Ptp69D* mutant phenotypes. Previous experiments demonstrated that *UAS-GAL4* mediated Abl overexpression can cause mild axon defects in CNS and ISNb (Bashaw et al., 2000; Fogerty et al., 1999; Wills et al., 1999a). To test the prediction that increased *Abl* expression should enhance *Ptp69D* defects, we examined the nervous system of embryos with reduced *Ptp69D* expression and increased Abl expression. Under our conditions, neither loss of *Ptp69D* nor simple overexpression of Abl in the nervous system caused CNS defects (Fig. 2A, B). When combined, however, reduced *Ptp69D* together with increased *Abl* caused severe CNS midline defects (Fig.2C, D). We tested additional *Ptp69D* genotypes; in all cases, neuronal overexpression of Abl dramatically increased both the CNS and motor axon defects of *Ptp69D* mutants (Table 2). Conversely, Abl over-expression conferred the ISNb bypass phenotypes in about 24% of hemisegments (Fig. 2F, Table 2), and for all *Ptp69D* alleles tested, this phenotype was dramatically increased in expressivity by reduction of *Ptp69D*. For example, when a single copy of $Ptp69D^{7}$ was combined with Abl overexpression, the rate at which ISNb nerves completely bypassed the VLM muscle field rose from 24% to 50%. The heterozygous $Ptp69D^{7}$ by itself did not cause ISNb bypass (Fig. 2E). Moreover, heterozygosity for *Ptp69D* is specific in its interaction with *Abl* pathway and does not promiscuously interact with all mutations that affect ISNb. Thus, for example, reduction of Notch activity produces a bypass phenotype similar to that from Abl overexpression ((Crowner et al., 2003), but the *Notch* phenotype is not enhanced by *Ptp69D* heterozygosity (Supplementary Table 1).

These data indicate that excess Abl exacerbates defects caused by reduced PTP69D activity and *vice versa*, consistent with a model in which PTP69D acts in opposition to Abl function. This model is further supported by the suppression of Abl overexpression phenotypes by excess PTP69D. Specifically, the rate of ISNb bypass defects in embryos overexpressing Abl was reduced from 25% to 0% by the simultaneous overexpression of PTP69D (Fig. 2H). This result supports the hypothesis that PTP69D acts to antagonize Abl.

Is *Abl* downstream of *Ptp69D*?

Since *Abl* appears to modify *Ptp69D* mutant phenotypes, we also examined the ability of *Ptp69D* mutations to modify the viability and axon phenotypes of *Abl* mutants. Abl^{3}/Abl^{4} shows 60% lethality prior to adult stage, and this rate is unchanged when *Ptp69D* alleles are introduced (Table 1). We obtained a similar negative result when we examined the effects of *Ptp69D* on *Abl* axon phenotypes. Stalled ISNb axons are an obvious neuronal phenotype of *Abl* mutants. Specifically, in both Abl^{1}/Abl^{3} and Abl^{3}/Abl^{3} embryos, ISNb terminates at the proximal edge of muscle 13 in 52% and 54% of hemisegments, respectively (Table 3). We introduced heterozygous or homozygous *Ptp69D* mutations in these *Abl* backgrounds, but observed no significant change in the frequency or severity of this ISNb stall phenotype, with defects ranging from 49% to 63%. Formally, these results suggest that *Abl* may be epistatic to *Ptp69D*, i.e. genetically downstream.

Ena is involved in PTP69D signaling

Enabled (Ena) is the sole *Drosophila* member of the Ena/VASP (Vasodilator-Stimulated Phosphoprotein) family of proteins that influence actin cytoskeleton architecture (Krause et al., 2003). For example, Ena/VASP proteins are involved in actin filament elongation via the recruitment of actin-profilin complexes to sites of active actin rearrangement (Reinhard et al., 1995), and also influence the activity of the Arp2/3 complex and counteract the inhibition of actin polymerization by capping proteins (Skoble et al., 2001). Ena is an antagonistic component of the Abl signaling pathway. Reduction of Ena rescues the lethality and axonal phenotypes of *Abl* mutations and mutant interactions (Gertler et al., 1990). It binds to the SH3 domain of Abl via a proline rich region and is an Abl substrate (Ahern-Djamali et al., 1999; Gertler et al., 1995; Gertler et al., 1990; Grevengoed et al., 2003; Grevengoed et al., 2001). We reasoned that PTP69D might function in the same biochemical pathway as Ena because both *Ptp69D* and *ena* mutants interact genetically with *Abl* and display ISNb bypass defects. In addition, Ena associates with the PTP69D D2 domain *in vitro* (Desai et al., 1997; Wills et al., 1999a). Finally, Abl binds to and phosphorylates both Ena and the D2 domain of PTP69D *in vitro* (Wills et al., 1999a). To test potential interaction between *ena* and *Ptp69D*, we first examined adult lethality. *ena* mutations dominantly enhanced the adult lethal phenotype of *Ptp69D* mutants (Table 1). Next, we explored the effect of *ena* mutation on the neural phenotypes of *Ptp69D* mutants. *ena* mutations strongly increased the axon defects of *Ptp69D* mutants. Specifically, the expressivity of CNS crossover defects of *Ptp69D* mutant embryos increased from 5% to 69% when one copy of *ena* was eliminated (Table 2, Figs. 3A, C). Also, ena^{GC5} enhanced ISNb bypass phenotypes in *Ptp69D* animals from 25% to 77% (Table 2, Fig. 3B, D). These results suggest that PTP69D modulates axon guidance signaling by Abl and Ena.

Src64B acts positively in PTP69D signaling

Our results so far suggest that PTP69D negatively regulates Abl, an interpretation consistent with their opposite biochemical functions and reminiscent of the antagonism between DLAR and Abl. On the other hand, it has been reported that RPTPs are positively regulated by Src family kinases (SFK). For example, neurotrophin signaling through SFK in mammalian neurons is promoted by LAR (Yang et al., 2006). We wondered whether the opposite nature of the reported interactions of Abl and Src with RPTPs reflect differences in the two biological systems assayed or different specificities of SFK *vs* Abl. To address this question, we tested genetic interactions of *Ptp69D* with a *Drosophila Src* gene, *Src64B.* Interestingly, heterozygosity for the hypomorphic Src64B allele, $Src64B^{\Delta 17}$ enhanced the adult lethality of a *Ptp69D* mutant, increasing it from 74% to 100% , and a heterozygous deficiency of the Src64B region gave similar results (Table 1). Furthermore, this allele dramatically increased the axonal defects of *Ptp69D* embryos. Removing one copy of *Src64B* converted the mild, low

penetrance *Ptp69D* midline crossing phenotype to severe, highly penetrant *robo*-like defects (Table 2, Figs 4A, B). *Src64B*$^{\Delta 17}$ also both quantitatively increased the expressivity of the ISNb phenotype of *Ptp69D* mutants and qualitatively enhanced the phenotype from a partial to a complete bypass defect (Table 2, Figs. 4C, D). A heterozygous *Src* mutant alone does not produce axonal defects (not shown). These results reveal that Src64B acts cooperatively with PTP69D in embryonic axon guidance and are in striking contrast to the antagonistic interaction between PTP69D and Abl.

DISCUSSION

Two enzyme classes, tyrosine kinases and tyrosine phosphatases, dynamically maintain protein phosphotyrosine modifications that are critical for axon guidance. Studies that revealed physical interactions between members of these families (Wills et al., 1999a; Wills et al., 2002) led us to investigate the relationship between the membrane bound tyrosine phosphatase, PTP69D and cytoplasmic tyrosine kinases. Here we provide evidence that PTP69D modulates signaling by the tyrosine kinase, Abl, and its substrate Ena. First, *Ptp69D* mutant phenotypes, including adult lethality, embryonic CNS and ISNb motor axon defects, are significantly suppressed by loss of *Abl* function, and dramatically enhanced by gain of *Abl* function (Table 1, Fig. 1 and 2). Second, *Ptp69D* does not suppress *Abl*, suggesting that their interaction is asymmetric(Table 1 and 3). Third, Ena, a strong suppressor and a downstream substrate of Abl, (Gertler et al., 1995; Grevengoed et al., 2003) dominantly exacerbates the defects of *Ptp69D* (Fig. 3).

Abl mutants display evident phenotypes such as adult lethality and ISNb arrest defects (Gertler et al., 1990; Wills et al., 1999b). We expected to observe bi-directional suppression between *Ptp69D* and *Abl* by analogy to *Dlar* mutations, which interact symmetrically with *Abl* for such phenotypes as midline crossing defects in the CNS (Wills et al., 2002). However, although *Abl* mutants modify *Ptp69D* phenotypes, we failed to observe any evidence of reciprocal suppression by introducing *Ptp69D* mutations into *Abl* embryos despite trying various combinations of alleles (Table 1 and 3). While several models might explain this, one simple interpretation is that *Abl* is epistatic to *Ptp69D*, i.e. *Ptp69D* acts through *Abl*.

What could be downstream targets of PTP69D and Abl? As a substrate of Abl and antagonistic genetic component of the Abl pathway, Ena is an excellent candidate (Gertler et al., 1995; Gertler et al., 1990; Grevengoed et al., 2003; Grevengoed et al., 2001), and indeed, we found that *ena* mutations enhanced the lethality and axonal defects of *Ptp69D* mutants. Ena is known to play a role in cell motility (Goh et al., 2002; Krause et al., 2002), and likely supports F-actin assembly within cells by antagonizing capping protein at the barbed ends of actin and reducing filament branching (Bear et al., 2000). In *Drosophila*, Ena associates with the PTP69D D2 domain and is phosphorylated by Abl *in vitro* (Wills et al., 1999a), and its specific cellular localization is regulated by Abl (Grevengoed et al., 2003). The consistent pattern of interactions of *Ptp69D* with *Abl* and *ena* – suppressed by *Abl* mutations and enhanced by the *Abl* antagonist, *ena* – supports the idea that the Abl effector, Ena is also a key to signaling by PTP69D.

The data we report define a functional relationship among PTP69D, Abl and Ena, but what could be their physical relationship? Extrapolating from genetic interactions to molecular mechanism is not straightforward, however, an attractive speculation that could provide one framework for further thinking is the idea that PTP69D, Abl and Ena may coexist in a complex, where PTP69D inhibits Abl, which in turn inhibits Ena. Such a model would be consistent with the available biochemical evidence (Wills et al., 1999a) , as well as with the genetic interactions we observed. Many other models are equally possible, however, and a great deal of additional experimentation would be required to establish this hypothesis. For example, we have not investigated whether these three proteins co-immunoprecipitate, nor have we demonstrated

that they act simultaneously in the same cell. Moreover, although the kinase activity of Abl is required for its axonal function (Wills et al., 2002), it is thought that tyrosine phosphorylation of Ena is not the sole function of Abl (Comer et al., 1998). Axon guidance by Abl seems also, for example, to be associated with the action of small Rho family GTPases, particularly Rac (Hakeda-Suzuki et al., 2002; Luo, 2002; Maurel-Zaffran et al., 2001), and any model for the mechanism of axon guidance by Abl and its partners will have to take these data into account.

The patterns of genetic interactions of *Ptp69D* with *Abl* and *ena* described in our paper bear some similarities to those of *Dlar* (Wills et al., 1999a; Wills et al., 2002). Does either RPTP substitute for each other? Previous studies demonstrated that Ptp69D and DLAR cooperate at growth cone choice points along one nerve, ISNb, while along another nerve, ISN, they do not act together (Desai et al., 1997). In the adult eye, moreover, a *Dlar* transgene rescues *Ptp69D* R7 axon phenotype, but not *vice versa* (Maurel-Zaffran et al., 2001). Thus, the relationship between PTP69D and DLAR is complex and depends on cellular context.

We extended our analysis of PTP69D by investigating the functional interaction of PTP69D with a *Drosophila* Src gene, Src64B. Mammalian Src protein (c-src) has been shown to regulate the stability and remodeling of actin structures (Boschek et al., 1981; Boyce et al., 1992). In *Drosophila*, Src64B has been shown to function in nervous system development in the embryo (unpublished data described in Wills et al., 1999a), the mushroom body of the adult brain and in the adult eye (Kussick et al., 1993; Nicolai et al., 2003), making it a plausible candidate for interacting with PTP69D in axon guidance. Moreover, in mouse the RPTP CD45 functions to positively regulate SFK in T cells (Hermiston et al., 2003). Indeed, our data show that *Ptp69D* does interact with *Src64B*, but in a sense opposite to that with Abl: *Ptp69D* and *Src64B* interact synergistically rather than antagonistically (Fig. 4 and Table 2).

Hints as to a possible mechanism that could underlie the interaction of PTP69D with Src64B are suggested by experiments in mammals. The SFK Fyn binds to LAR and phosphorylates the LAR D2 domain. In turn, LAR dephosphorylates a c-terminal inhibitory motif of Fyn (Tsujikawa et al., 2002), increasing Fyn activity. Our data as well could potentially be explained by an analogous model whereby PTP69D derepresses Src activity by removing an inhibitory phosphate, though other models are clearly possible and more study is required to test this speculation. It is interesting that the biochemical association of LAR with Fyn in mammals is reminiscent of that observed for DLAR with Abl in *Drosophila* (Wills et al., 1999a), but the biological consequences in the two cases are quite different, and in fact opposite : activation of Fyn activity, but suppression of Abl.

Superficially, it seemed surprising that two cytoplasmic kinases had opposite interactions with PTP69D, antagonizing Abl but cooperating with Src64B. To test this further, we examined the genetic interaction between Src64B and Abl. Although $Src64B^{\Delta 17}$ did not show any significant effect on *Abl* lethality, it dramatically suppressed the ISNb stall defect of Abl mutants (Supplementary Table 2), further supporting the hypothesis that Src64B and Abl kinases may have opposing functions in axon guidance.

In summary, the receptor protein tyrosine phosphatase PTP69D interacts both with the Abl-Ena tyrosine kinase pathway and with Src64B to control axon patterning in *Drosophila*. PTP69D antagonizes Abl, perhaps as an upstream regulator, but functions synergistically with Src64B, thus revealing previously unrecognized specificity in the action of these tyrosine kinase pathways.

MATERIALS AND METHODS

Fly stocks

All experiments were done at 25°C except analysis of interaction with *Notch^{ts}* (Crowner et al., 2003). *Ptp69D* alleles were described previously (Marlo and Desai, 2006; Desai and Purdy, 2003; Desai et al., 1996). *Ptp69D^{1}* is a null allele, which lacks all but the first 115bp of the 5'UTR and sequences that encode most of the extracellular domain. *Ptp69D^{7}* has a 3 amino acid deletion in the D1 domain. *Ptp69D^{7}* has a phenotype stronger than that of a null allele, as is true of other D1 domain mutants such as *Ptp69D^{21}*. However, as *Ptp69D^{7}* is recessive, and a *Ptp69D^{7}* homozygote phenotype is suppressed by duplication of the wild type gene (Desai and Purdy, 2003), it is unlikely that *Ptp69D^{7}*and *Ptp69D^{21}* are neomorphic, but rather that they act negatively within the wild type PTP69D genetic pathway. *Ptp69D^{10}* is a temperature sensitive allele and has a V to D mutation at the junction between the first and second IgG domains. At the temperature used in this study (25°C), *Ptp69D^{10}* shows substantial axonal defects but is viable. Severity of alleles may be ordered as *Ptp69D^{7}=Ptp69D^{1} > Ptp69D^{10}* for animal lethality and *Ptp69D^{7} > Ptp69D^{10} ≥ Ptp69D^{1}* for axon patterning. *Abl*, *Src64B* and *ena* stocks were obtained from Van Vactor (Harvard University), Liebl (Danison University) and the Bloomington Drosophila Stock Center. Gal4 expression was driven by neuronal specific P[elav-GAL4] driver chromosomes (obtained from C.S. Goodman). Stocks were balanced over *TM6B, Tb* or *TM6B-T8-lacZ, Tb* (gift of Dr. Peter Kolodziej).

Viability test / Scoring

10 males and 25−30 virgin females were placed in a food vial at room temperature and parents were transferred to a fresh food vial every 12 hours for 7 days. For hypomorphic combinations (*Ptp69D^{1}/ Ptp69D^{10} and Ptp69D^{10}/Df(3L)^{8ex34}*), pre-adult lethality was assayed by daily counting of hatched F1 adults. The null combination,*Ptp69D^{1}/Df(3L)^{8ex34}* produced almost no viable pupae and was considered to be embryonic/larval lethal. For this genotype, lethality was determined by counting pupal cases, including both hatched pupal cases and dead pharate adults, after no new adults eclosed for at least 24hr. *Ptp69D* null animals did not survive to adulthood regardless of the *Abl* genotype.

Immunohistochemistry

Embryos were fixed, collected, stained, analyzed and photographed as described (Desai et al., 1996). Mutant embryos from stocks balanced by *TM6B T8-lacZ, Tb* were identified by their failure to stain with anti-ß-galactosidase sera (Jackson Laboratories, Bar Harbor, ME).

Statistics

All data were analyzed with Microsoft excel 11.0 software. Statistically-significant differences were determined by CHITEST. Difference with a p value greater than 0.05 were considered to be not significant (NS).

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Supplementary Material

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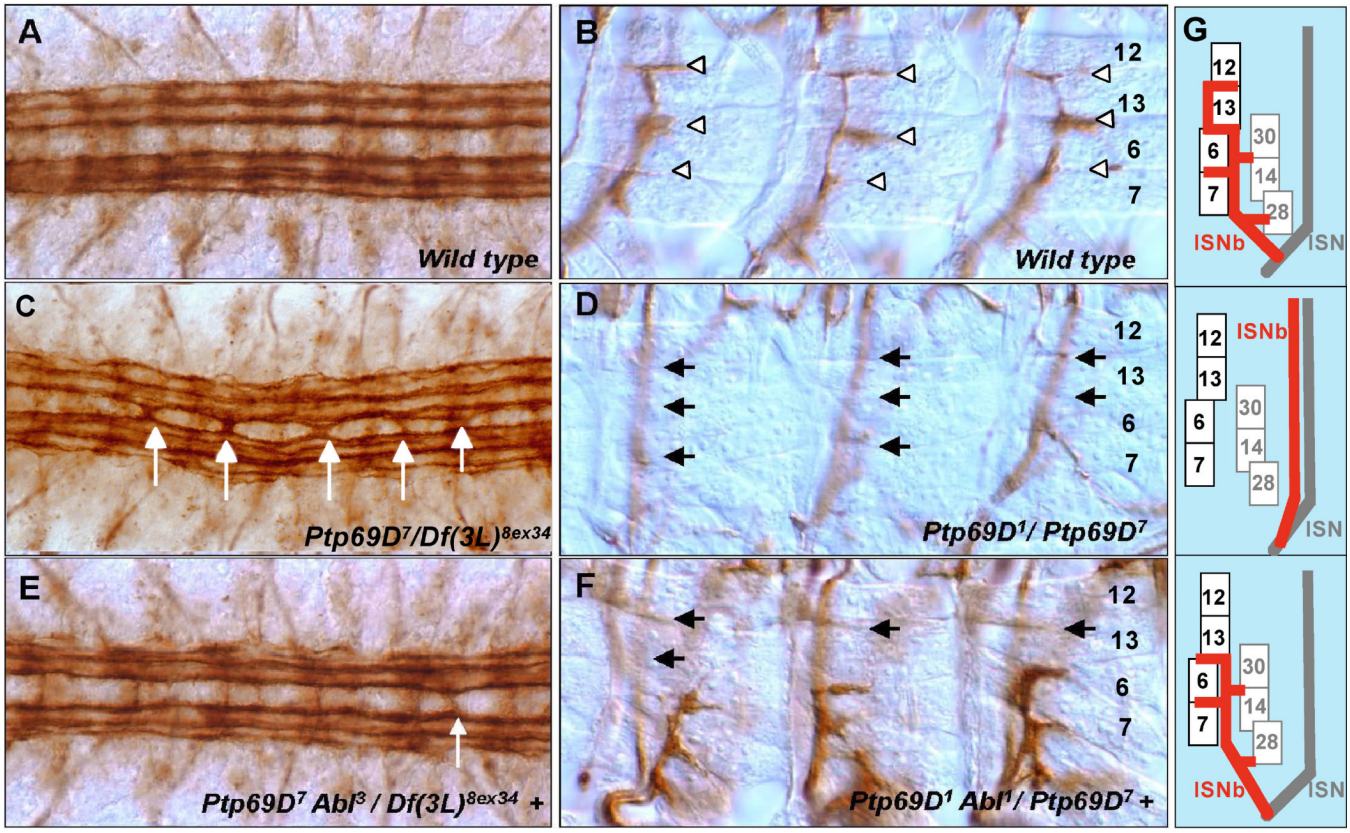

Fig.1. Abl suppresses axon defects of Ptp69D

Late stage 16 through early stage 17 embryos were stained with anti fasciclin II (mAb 1D4) and dissected to visualize FasII positive axons in the CNS (A, C, E) and VLM(Ventro-Lateral Muscle) innervation by the ISNb (B, D,F). (A) Wild-type CNS pattern showing three pairs of longitudinal bundles flanking the midline. (C, E) CNS patterns of *Ptp69D* mutants in embryos that are wild type (C) or heterozygous mutant (E) for an *Abl* mutation. (C) $Ptp69D^{7}/Df(3L)^{8ex34}$ embryo showing a severe CNS midline defect. In contrast to wild-type, axons cross the midline in most segments (white arrows). (E) $Ptp69D^{7}Abl^{3}/Df(3L)^{8ex34}$ + embryo showing highly rescued CNS phenotype. Occasional segments contain a thin bundle of axon staining across the midline (white arrow) (B) Wild-type ISNb pattern showing synaptic branches in the clefts between individual VLM fibers (white arrowheads). Numbers on the right are muscle identities. (D, F) ISNbs of *Ptp69D* mutants in embryos that are wild type (D) or heterozygous mutant (F) for an *Abl* mutation. (D) Bypass phenotype in an $Ptp69D^{1}/Ptp69D^{7}$ embryo; most ISNb axons extend past the VLM field (out of focus) and fail to innervate it (black arrows). (F) In this $Ptp69D^{1}Abl^{1}/Ptp69D^{7}$ + embryo, most ISNb axons enter and innervate VLM field, although they often fail to target distal muscle fibers (black arrows). (G) Schematic of a cross section through panels B,D and F. Top : In wild type, ISN is superficial to muscles. ISNb enters muscle field. Center : Ptp69D bypasses the muscle field ; ISNb associates with ISN. Bottom: Abl rescues ISNb ability to enter muscle field, though sometimes fails to reach muscle 12. Numbered rectangles represent muscles.

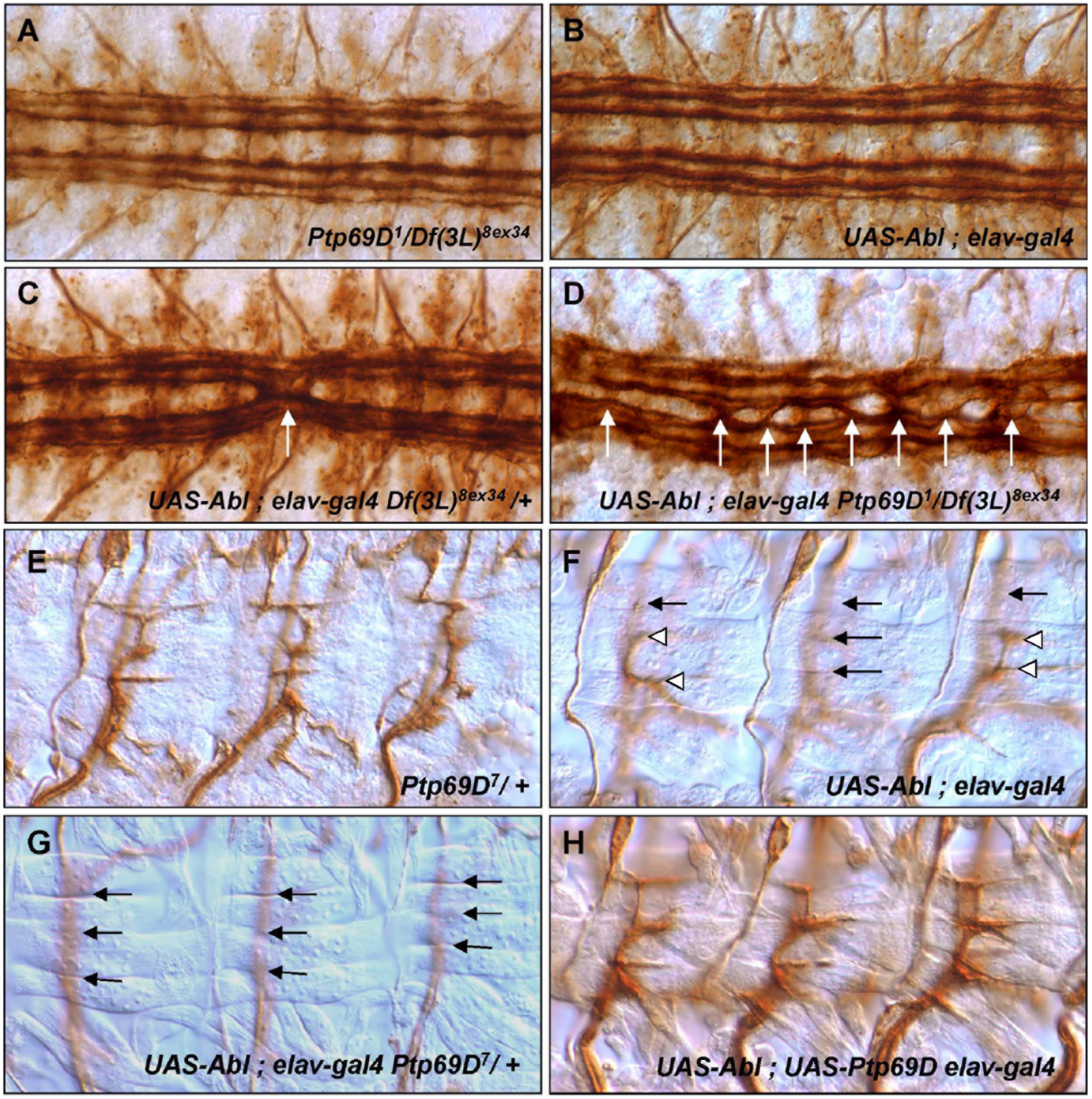

Fig. 2. Excess Abl function enhances *Ptp69D* axonal defects

Axon patterns of CNS (A-D) and ISNb (E-H). (A) *Ptp69D*^{1}*/Df(3L)*^{8ex34} null. FasII positive axons cross the midline very rarely (4% of total segment of T2−3 and A1−8). (B) P[UAS-*Abl*]/+; P[elav-GAL4]/+. Abl overexpressing embryos resemble the wild-type; FasII positive axons do not cross the midline. (C) In P[UAS-*Abl*]/+; *Df(3L)*^{8ex34} P[elav-GAL4]/++ embryos, FasII axons(white arrows) frequently cross the midline (32% of segments, Table 2). (D) P [UAS-*Abl*]/+; *Ptp69D*^{1} P[elav-GAL4] */Df(3L)*^{8ex34} + embryos display severe midline crossing defects in most segments (white arrows). (E) *Ptp69D*^{7}/+ embryos display normal ISNbs. (F) P[UAS-*Abl*$^{+}$]/+; P[elav-GAL4]/+ embryos display partial ISNb bypass phenotypes, in which some axons fail to enter the VLM field while others enter and successfully innervate their target

muscles (white arrowheads). (G) P[UAS-*Abl*+]/+; *Ptp69D*^{7} P[elav-GAL4]/+ + embryos display the complete ISNb bypass phenotype in most hemisegments (arrows). (H) Overexpression of PTP69D in P[UAS-*Abl*+]/+; P[UAS-*Ptp69D*] P[elav-GAL4]/+ + embryos fully rescue ISNb defects associated with Abl gain of function (compare to F).

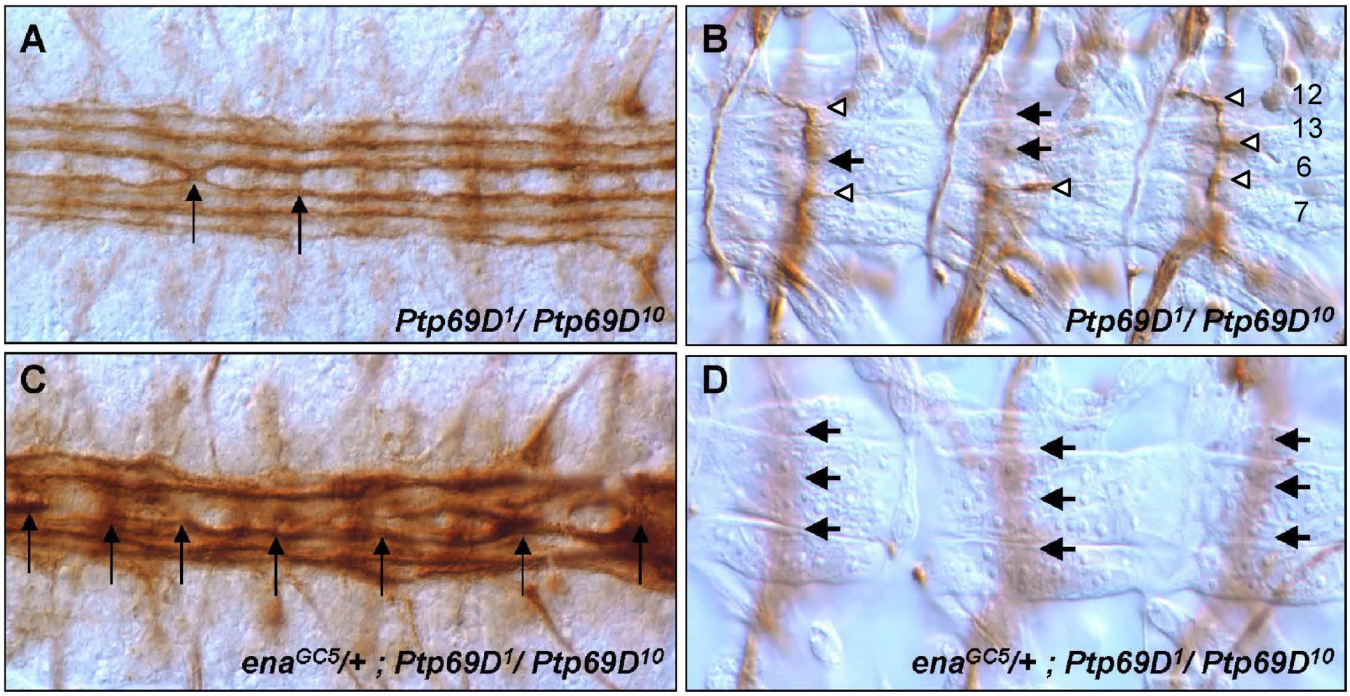

Fig. 3. *ena* enhances *Ptp69D* axon phenotypes

CNS (A, C) and ISNb (B, D) *Ptp69D* mutant phenotypes in the absence (A,B) and presence (C,D) of a heterozygous *ena* mutation. (A) CNS in $Ptp69D^{1}/Ptp69D^{10}$ embryo shows occasional midline crossovers (arrow). (C) CNS in $ena^{GC5}/+$; $Ptp69D^{1}/Ptp69D^{10}$ embryos display severe defects, including roundabouts, fusions and crossovers (arrows). (B) ISNbs in $Ptp69D^{1}/Ptp69D^{10}$ embryos often display partial bypass phenotypes (arrows), while still innervating some muscles(white arrowheads) (D) ISNbs in $ena^{GC5}/+$;$Ptp69D^{1}/Ptp69D^{10}$ embryos show complete bypass phenotypes (arrows).

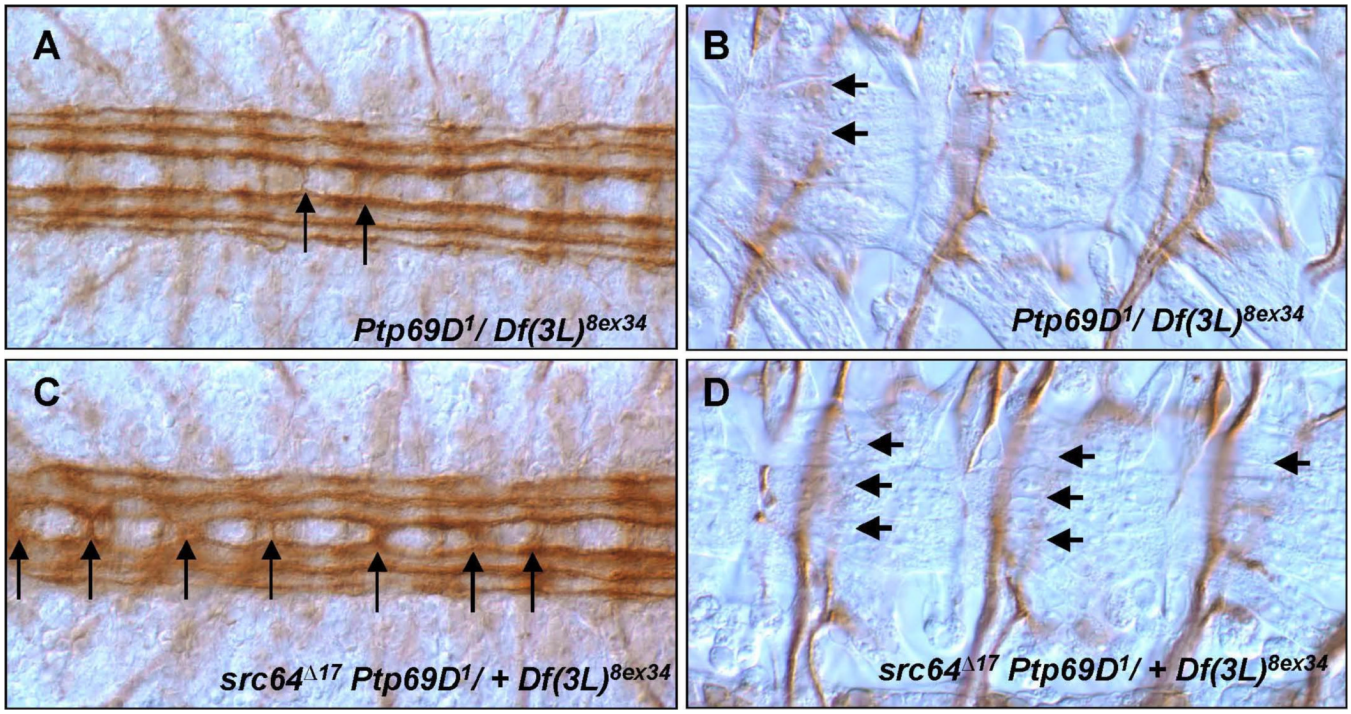

Fig. 4. *Src64B* enhances axonal defects of *Ptp69D* mutants

CNS (A, C) and ISNb (B, D) axon patterns of *Ptp69D* mutants in the absence (A, B) and presence(C, D) of a heterozygous *Src64B* mutation. (A) A few FasII positive axons occasionally cross the midline in $Ptp69D^{1}/Df(3L)^{8ex34}$ embryos (arrows). (C) Severe midline crossing phenotypes (arrows) characterize $Src64B^{\Delta 17}Ptp69D^{1}/+\ Df(3L)^{8ex34}$ embryos. (B) ISNbs in $Ptp69D^{1}/Df(3L)^{8ex34}$ embryos occasionally display partial bypass and early termination phenotypes (arrows). (D) ISNbs in $Src64B^{\Delta 17}Ptp69D^{1}/+\ Df(3L)^{8ex34}$ embryo frequently show the complete bypass phenotype. Most axons fail to enter VLM field.

Table 1

Modification of *Ptp69D* and *Abl* lethality

Genotype of Ptp69D	Modifier	Lethality(%)	N	[1]Su	p value
*$Ptp69D^{10}/Ptp69D^{10}$	+/+	56	599	Control	
	Abl^{3}/+	1	662	++++	<0.0001
*$Ptp69D^{1}/Ptp69D^{10}$	+/+	74	505	Control	
	Abl^{1}/+	42	270	+++	<0.0001
	Abl^{3}/+	11	632	++++	<0.0001
*$Ptp69D^{10}/Df(3L)^{8ex34}$	+/+	91	643	Control	
	Abl^{3}/+	49	595	+++	<0.0001
@$Ptp69D^{1}/Df(3L)^{8ex34}$	+/+	94	614	Control	
	Abl^{3}/+	73	777	++	<0.0001

Genotype of Ptp69D	Modifier	Lethality(%)	N	[2]En	
$Ptp69D^{1}/Ptp69D^{10}$	+/+	74	505	Control	
	ena^{GC5}/+	99	222	++	<0.0001
	ena^{GC1}/+	100	180	++	<0.0001
	$src64^{\Delta 17}$/+	100	289	++	<0.0001
	Df(3L)10[H]/+	100	274	++	<0.0001

Genotype of Abl	Modifier	Lethality(%)	N	[2]En	
Abl^{3}/Abl^{4}	+/+	60	569	Control	
	$Df(3L)^{8ex34}$/+	64	533	−	NS
	$Ptp69D^{10}$/+	69	545	−	NS

NS; Difference from control not significant. p > 0.05

[1]Su; Degree of Suppression.

[2]En; Degree of Enhancement.

*Pre-adult lethality, determined by counting adult eclosers.

@Embryonic/larval lethality, determined by counting pupae. *Ptp69D* null animals did not survive to adulthood regardless of the *Abl* genotype.

Table 2

Modification of *Ptp69D* axonal phenotypes

Genotype	Modifier	[1]CNS midline cross				[2]ISNb bypass			
		%	N	[3]Su	p value	%	N	[3]Su	p value
Ptp69D^{1}/*Ptp69D*^{10}	+/+	5	152	Control		25	223	Control	
	Abl^{3}/+	2	88	−	NS	7	132	+++	< 0.0001
	Abl^{3}/*Abl*^{3}	4	96	−	NS	0	144	+++	< 0.0001
Ptp69D^{1}/*Ptp69D*^{7}	+/+	56	144	Control		58	287	Control	
	Abl^{1}/+	37	132	++	0.0003	23	244	++	< 0.0001
Ptp69D^{7}/*Df(3L)*^{8ex34}	+/+	54	112	Control		64	168	Control	
	Abl^{3}/+	24	128	++	< 0.0001	19	192	++++	< 0.0001
Ptp69D^{10}/*Df(3L)*^{8ex34}	+/+	15	104	Control		25	116	Control	
	Abl^{3}/+	3	160	++	0.0002	17	239	−	NS
UAS-Abl	+/+	0	112	Control		24	168	Control	
	UAS-Ptp69D	0	80	−	NS	0	120	++++	<< 0.0001

Genotype	Modifier	[1]CNS midline cross				[2]ISNb bypass			
		%	N	[4]En	p value	%	N	[4]En	p value
Ptp69D^{1}/*Ptp69D*^{10}	+/+	5	152	Control		25	223	Control	
	UAS-Abl	62	128	+++	< 0.0001	78	192	+++	< 0.0001
	ena^{GC5}/+	69	111	+++	< 0.0001	77	194	+++	< 0.0001
	src64^{417}/+	48	104	+++	< 0.0001	74	156	+++	< 0.0001
Ptp69D^{1}/*Ptp69D*^{7}	+/+	56	144	Control		58	287	Control	
	UAS-Abl	98	115	+++	< 0.0001	99	166	+++	< 0.0001
Ptp69D^{1}/*Df(3L)*^{8ex34}	+/+	4	152	Control		6	227	Control	
	UAS-Abl	74	120	++++	< 0.0001	59	170	+++	< 0.0001
	src64^{417}/+	55	110	++++	< 0.0001	59	132	+++	< 0.0001
Ptp69D^{7}/+	+/+	1	120	Control		0	120	Control	
	UAS-Abl	25	84	+++	< 0.0001	50	118	+++	< 0.0001
Df(3L)^{8ex34}/+	+/+	0	120	Control		0	120	Control	
	UAS-Abl	32	88	+++	< 0.0001	38	132	+++	< 0.0001

NS; Difference from control not significant. $p > 0.05$

[1]Segments (T2−3 and A1−8) harboring midline crosses were counted.

[2]Hemisegments (A2-A7) showing that ISNb bypass the layer of target muscles were counted. Distinguishable partial bypasses were also counted.

[3]Su; Degree of Suppression.

^{4}En; Degree of Enhancement.

Table 3

Modification of *Abl* axonal phenotypes

Genotype	Modifier	[1]ISNb stall		
		%	N	p-value
Abl^{1}/Abl^{3}	+/+	52	188	
	$Ptp69D^{1}/Ptp69D^{10}$	49	144	NS
Abl^{3}/Abl^{3}	+/+	54	168	
	$Ptp69D^{10}/+$	48	96	NS
	$Df(3L)^{8ex34}/+$	51	144	NS
	$Df(3L)^{8ex34}/Df(3L)^{8ex34}$	63	142	NS

NS; Difference from control is not significant. $p > 0.05$

[1]Most ISNb axons reached VLM properly but arrested in the ventral region (muscle 12,13) were considered as 'Stall' phenotype and counted per hemisegments of A2-A7.