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Non-canonical Notch function in motor axon guidance is mediated by Rac GTPase and the GEF1 domain of Trio

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Abstract

The receptor Notch interacts with the Abl tyrosine kinase signaling pathway to control axon growth and guidance in *Drosophila* motor neurons. In part, this is mediated by binding to Trio, a guanine nucleotide exchange factor (GEF) for Rho GTPases. We show here that one of the two GEF domains of Trio, the Rac-specific GEF1, is essential for Trio-dependent motor axon guidance and for the genetic suppression of Notch function in motor axon patterning, but the Rho-specific GEF2 domain is not. Consistent with this, we show that Rac, and not Rho1 or Cdc42, interacts genetically with Notch in a manner indistinguishable from that of bona fide Abl signaling components. We therefore infer that Rac is a key component of Abl signaling in *Drosophila* motor axons, and specifically that it is the crucial Rho GTPase in “non-canonical” Notch/Abl signaling.

Keywords

Notch; Trio; Rac; Abl; axon guidance; *Drosophila*

Introduction

As an axon navigates through its environment during nervous system development, the growth cone at its tip responds to signals from many guidance cues by executing dynamic rearrangements of the actin cytoskeleton. The Rho subfamily of small GTPases - Rho, Rac and Cdc42 – is critical for this modulation of the actin cytoskeleton (Hall, 1992; Tapon and Hall, 1997; Hall, 1998). The different Rho GTPases are thought to act upon different kinds of actin structures. For example, Rho stimulates formation of focal adhesions and stress fibers (Ridley and Hall, 1992), Rac promotes lamellipodial structures (Ridley et al., 1992) and Cdc42 activates filopodia (Nobes and Hall, 1995).

Among the Rho GTPases, Rac has been the most enigmatic. Activation of Rac displays many effects on cell morphology, cell polarity and cell migration (Etienne-Manneville and Hall, 2002; Jaffe and Hall, 2005). Specifically in the growth cone, Rac plays pivotal roles in outgrowth, branching and guidance of axons (Luo, 2000b; Guan and Rao, 2003). In *Drosophila*, Rac mutant embryos display severe axon growth defects both in CNS and PNS (Hakeda-Suzuki et al., 2002). A very large number of molecules have been identified as Rac binding partners, and it is not clear which among them are the key effectors for actin rearrangement in particular contexts (Bishop and Hall, 2000; Luo, 2000a).

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A variety of axon guidance signals seem to act through Rac GTPases (Tapon and Hall, 1997; Luo, 2000b). For example, in *C. elegans*, Rac acts downstream of UNC-40, a netrin receptor (Gitai et al., 2003). Axonal repulsion by Slit-Robo signaling is mediated by Rac and restricted Rac function also limits Slit-Robo signaling (Hakeda-Suzuki et al., 2002; Fan et al., 2003; Yang and Bashaw, 2006). The intracellular domain of Plexin B, a semaphorin receptor, binds to Rac-GTP and reduces active Rac by sequestering it from its target Pak, resulting in repulsion of axons (Vikis et al., 2000; Hu et al., 2001). Rac1 deficient cerebellar granule neurons in primary culture display reduced PAK1 phosphorylation and mislocalization of WAVE complex from the growth cone membrane (Tahirovic et al., 2010)

Like other GTPases, Rac GTPases cycle between an active GTP-bound state and an inactive GDP-bound state (Fig 1H). Rac activity is controlled by three main classes of regulatory proteins, Guanine nucleotide Exchange Factors (GEFs), GTPase –Activating Proteins (GAPs), and Guanine nucleotide Dissociation Inhibitors (GDIs). GEFs initiate the release of GDP resulting in accumulation of GTP-bound active GTPases (Rossman et al., 2005). GAPs convert the active state to the inactive GDP-bound form (Moon et al., 2003). GDIs bind to the GDP-bound form, preventing the release of GDP and keeping Rac in the inactive state (Dovas and Couchman, 2005). The overwhelming number of GEFs (~60) and GAPs (~70) in mammals (Etienne-Manneville and Hall, 2002) suggests that each of these proteins likely has its own specific roles in different contexts.

Among the many Rac-specific GEFs and GAPs, the GEF Trio is perhaps one of the best characterized Rac regulators in axon patterning. Trio has two tandem Dbl-homology (DH-PH) GEF domains. GEF1 is a specific activator of Rac GTPase. This was shown by genetic epistasis in *Drosophila*, as a *Rac* mutation blocks the dominant effects of expressing the GEF1 domain in isolation. It was also verified by direct biochemical experiments, as Trio GEF1 activates Rac *in vitro*, but not Rho or Cdc 42, both for *Drosophila* Trio (Newsome et al., 2000) and mammalian Trio (Bellanger et al., 1998). GEF1 was shown genetically to be critically required for Trio function in photoreceptor axon guidance in *Drosophila*, in part to regulate the activity of PAK kinase (Newsome et al., 2000). The GEF2 domain of Trio, in contrast, is a specific activator of Rho (Bellanger et al., 1998; Spencer et al., 2001) and is not required for photoreceptor axon guidance in the fly eye (Newsome et al., 2000; Vanderzalm et al., 2009). A specific axonal function of GEF1 activity was also found in *C. elegans trio (UNC73)* (Vanderzalm et al., 2009), while GEF2 regulates pharynx and vulva musculature, synaptic neurotransmission (Steven et al., 2005) and P cell migration (Spencer et al., 2001).

The role of Trio in axon guidance has been linked to Abl tyrosine kinase (Liebl et al., 2000). Abl and Trio mutants have similar axon guidance phenotypes by themselves, and in combination they interact synergistically. Abl is one of the key molecules required in axon pathfinding (Wills et al., 2002; Forsthoefel et al., 2005; Song et al., 2008). The notion that Rac activity is required for Abl function has also been suggested in other contexts (reviewed in Hern·ndez 2004). Rac promotes the activities of oncogenic constitutively-activated forms of Abl such as p210Bcr-Abl and v-Abl in mammalian cultured cells (Renshaw et al., 1996; Bassermann et al., 2002). Abl activates Rac in conjunction with receptor tyrosine kinase signaling in part by phosphorylation of the Ras GEF, Sos-1 (Sini et al., 2004) and is also required for Rac activation following stimulation of cadherin-mediated cell-cell adhesion (Zandy et al., 2007).

We have shown previously that Abl and Trio participate in a non-canonical function of the receptor Notch in axon patterning in *Drosophila* (Giniger, 1998; Crowner et al., 2003). In contrast to the usual Notch signaling mechanism, the function of Notch during axon guidance does not require the canonical molecular events of nuclear translocation of the

intracellular domain to control target gene expression mediated by the transcription factor Su(H). Instead, Notch is present *in vivo* in a multiprotein complex together with Trio, and also with Disabled, another core component of Abl signaling, as shown by co-immunoprecipitation of Notch with Trio and Disabled proteins from wild type *Drosophila* extracts (Le Gall et al., 2008; Song et al., 2010). This physical association of Notch with Trio and Disabled is essential for Notch-dependent control of axon growth and guidance (Le Gall et al., 2008).

Motivated by these observations, we investigated the potential involvement of small Rho GTPases in non-canonical Notch signaling during axon guidance in *Drosophila* embryos. Here, we first show that the Rac-specific GEF1 activity of Trio is selectively required for Trio-dependent axon patterning in embryonic motor nerves, and specifically for the interaction with Notch. Furthermore, we show a selective genetic interaction of Rac, and not Rho1 or Cdc42, with Notch, modifying its axonal function. These data support the hypothesis that Rac is a critical player in the Abl- and Trio-dependent mechanism by which Notch controls axon growth and guidance.

Results

Trio GEF1 activity is essential for motor axon guidance

Motor nerve guidance in the fly embryonic nervous system provides a powerful system for quantitatively assaying the contribution of signaling proteins to axon growth and guidance *in vivo*. In late stage 16 embryos, subsets of motor neurons display simple and distinguishable axonal projections. Inter-Segmental Nerve b (ISNb) has 7 motor axons that exit from the ISN root at a specific choice point to innervate ventrolateral muscles (VLM) (Fig 1A). Abl and its accessory signaling components such as Neurotactin (Nrt), Disabled (Dab), Failed Axon Connections (Fax) and Trio, are essential for proper growth and guidance of this motor nerve (Gertler et al., 1989;Hill et al., 1995;Song et al., 2010)

Like other mutations in Abl signaling, *trio* mutants display a specific axonal defect in ISNb, ‘stalling’ in the middle of the target field (Fig.1B and Table 1)(Awasaki et al., 2000;Bateman et al., 2000). We found that expression of a *trio* transgene with a mutation inactivating the GEF1 domain (*UAS-trio*^{GEF1mu})(Newsome et al., 2000) was unable to rescue the ISNb axonal phenotypes of a *trio* mutant. In contrast, expression of a transgene bearing the equivalent lesion in GEF2 (*UAS-trio*^{GEF2mu}) rescued the axonal defects of *trio* as effectively as a wild type transgene (*UAS-trio*^{WT})(Fig 1C, D and E and Table 1). We verified by immunostaining that all three Trio derivatives accumulated to similar levels and trafficked properly to axons (data not shown). Therefore, the activity of GEF1 is essential for the function of Trio in motor axon guidance whereas GEF2 is not. This is consistent with previous data showing that the GEF1 domain of Trio is preferentially required for sensory axon guidance in photoreceptor cells, while GEF2 is dispensable in this context (Newsome et al., 2000).

Function of Trio in non-canonical Notch signaling during axonal guidance is mediated by GEF1 function

We next examined the interaction of Trio with the receptor Notch in axon growth and guidance. Inactivation of Notch at the time of axon growth selectively misroutes some motor axons, causing specific guidance defects. For example, in ISNb, *Notch* mutant (N^{ts1}) axons often grow past the “choice point” at which they should exit the main ISN and enter the ventrolateral muscle domain (VLM). This results in an abnormal “bypass” innervation pattern, with few or no axon projections into the VLM (36% of total hemisegments defective, dotted area in Fig. 2B and Supplementary Fig 1) under appropriate temperature

shifting conditions (details in Experimental Procedures). Guidance errors also occur in Segmental Nerve a (SNa) in N^{ts1} mutants (Supplementary Fig 2). The axonal action of Notch is mediated by a non-canonical signaling mechanism by which Notch locally inhibits the activity of Abl and associated cofactors(Crowner et al., 2003). Consistent with this, reduction of Abl signaling components, including Trio, suppresses the axon patterning phenotypes of N^{ts1}. For example, heterozygosity for a loss of function *trio* mutation significantly restores the ability of ISNb axons to enter the VLM field in a *Notch* mutant genetic background (N^{ts1}*; trio*$^{123.4}$/+, 28%, n=190, P<0.05 (Fig 2); N^{ts}*; trio*^{8}/+, 27%, n=236, P<0.001). The effect of *trio* heterozygosity on the *Notch* phenotype is quantitatively modest, but it is observed with unrelated *trio* alleles, and is similar to the degree of suppression produced by heterozygosity for other components of the Abl signaling pathway, such as Abl and Nrt (Crowner et al., 2003). In the Discussion (below) we expand on the motivation and significance of using hypomorphic manipulations, such as heterozygous mutations, in analyzing the *Notch/trio* interaction.

We used the Notch/Trio interaction to further dissect the mechanism of Trio action in Notch-dependent axon guidance. We found that the suppression of *Notch* phenotype by a *trio* mutation was reverted by pan-neuronal expression of *UAS-trio*^{WT} (*elav-Gal4* driven), once again causing ISNb to bypass the VLM as in N^{ts1} (Fig. 2D and G, 86% restoration of the VLM bypass phenotype [P<0.005], and Table 1). This suggests that neuronal *trio* is largely responsible for the genetic interaction of *Notch* with *trio*. Next, we examined which domains of Trio, in particular which GEF domains, contribute to the Notch-Trio interaction. Trio constructs bearing mutations that selectively inactivate either GEF1 or GEF2 domain, *UAS-trio*^{GEF1mu} and *UAS-trio*^{GEF2mu}, were pan-neuronally expressed in the N^{ts1}*; trio*$^{123.4}$/+ background. We did not detect any significant alteration of the Notch-Trio interaction with *elav-GAL4*-driven *trio*^{GEF1mu} expression (Fig. 2E and G, 23% suppression [Not significant, P=0.14] and Table 1), while *trio*^{GEF2mu} expression, in contrast, restored the bypass phenotype nearly as effectively as did *UAS-trio*^{WT} (Fig. 2F and G, restore 81% of N^{ts1} phenotype [P<0.005] and Table 1). Pan-neuronal overexpression of wild type *trio* or GEF-inactive *trio* transgenes did not produce any dominant axon patterning defects in a wild type background (data not shown). These results suggest that GEF1 activity of Trio is selectively required for the interaction with Notch in axon patterning. Consistent with this hypothesis, expression of the constitutively active Trio GEF1 domain (Newsome et al., 2000; Ferraro et al., 2007) alone mimics the *Notch* ISNb phenotype (48% bypass, n=456 driven by *elav-Gal4*), whereas expression of Trio (GEF2) alone does not perturb ISNb patterning (less than 2% bypass, n=228, driven by *elav-Gal4*).

Rac small GTPase is selectively required in Notch mediated motor axon guidance

The observation that activity of Trio GEF1 is necessary and sufficient for the functional interaction of Notch with Trio in axon patterning led us to investigate whether Rac, the specific target for activation by GEF1, acts in this context. First, we investigated whether genetic reduction of Rac levels modifies motor axon phenotypes of N^{ts1}, and found that heterozygosity for a Rac triple mutant, *Rac1*^{J10}*Rac2*$^{\Delta}$*Mtl*$^{\Delta}$/+ suppressed the axonal defects of N^{ts1} (Table 2, 23% of hemisegments defective, N=132, *vs* 36% for N^{ts1}, P<0.05). In contrast, heterozygosity for mutations of other small Rho GTPases (i.e. *Cdc42*^{4} *and Rho1*^{rev220}) did not alter the expressivity of ISNb bypass in N^{ts1} (Table 2). This result suggests that among Rho GTPases, Rac is preferentially required in the genetic pathway of Notch signaling for patterning ISNb motor axons.

To test this hypothesis further, we employed dominant negative or constitutively active forms of Rac. Expression of a constitutively active form of Rac, Rac^{V12} significantly increased the occurrence of ISNb bypass in N^{ts1} embryos (Fig. 3C and Table 2), while expression of the dominant negative Rac^{N17} suppressed the ISNb bypass phenotype (Fig. 3B

and Table 2). In contrast, the ISNb phenotype of N^{ts1} was not significantly modulated by expression of dominant negative or constitutively active forms of Cdc42 or Rho1 (Fig. 3D and Table 2). None of the GTPase transgenes produces dominant bypass phenotypes on their own under the conditions that we used, though Rac^{N17} produces a low frequency of trio-like stall phenotypes (11%). Moreover, the effect of *UAS-Rac1*^{WT} expression in N^{ts1} is not statistically significant (Table 2), suggesting that Rac activity, rather than amount, is critical for the genetic interaction with Notch. Taken together, our results suggest that Rac selectively modulates Notch function in motor axon guidance, while Rho1 and Cdc42 do not have strong effects in this context.

Discussion

The guanine exchange factor Trio physically associates with Notch *in vivo* (Le Gall et al., 2008) and is a genetic component of Notch signaling in motor axon guidance (Crowner et al., 2003; Le Gall et al., 2008). In this paper, we found that one of the tandem GEF domains of Trio, the Rac-specific GEF1, is essential for this genetic interaction. Consistent with this observation, the axonal phenotypes of a *Notch* mutant are suppressed by reducing the level of the three Rac paralogs, to a degree similar to that caused by reduction of *trio*. This interaction appears to be specific, because mutations of other closely related small GTPases, Rho1 and Cdc42, did not modify the axonal phenotypes of N^{ts1}. This was further confirmed by testing the effect of dominant Rac transgenes. Expression of a dominant negative Rac^{N17} suppressed the ISNb phenotype of a *Notch* mutant, while a constitutively active Rac^{v12} enhanced it. In contrast, introduction of other dominant transgenes such as $Cdc42^{N17}$ did not alter the *Notch* mutant phenotypes. Thus, Rac appears to be the key Rho GTPase for control of motor axon guidance by Notch.

Our data support the hypothesis that Rac is a key component of Abl signaling in *Drosophila* motor axons. Notch-dependent axon patterning is executed by an alternate, “non-canonical” Notch signaling pathway defined by the Abl tyrosine kinase (Giniger, 1998). Notch protein associates in vivo with the Abl cofactors, Disabled and Trio, and genetic experiments suggest that Notch antagonizes the activity of the Abl signaling pathway (Crowner et al., 2003; Le Gall et al., 2008). We find here that Rac interacts functionally with Notch in the same way as do the core Abl signaling proteins. Reduction of Rac activity suppresses *Notch* axonal phenotypes, just as do reduction of Abl pathway components or expression of the Abl antagonist, Enabled. Enhancement of Rac activity exacerbates *Notch* axonal phenotypes, just as does activation of Abl signaling or mutation of Enabled. These data are therefore consistent with the hypothesis we proposed previously that the key role of Notch in ISNb guidance is to limit Abl-dependent adhesion of ISNb growth cones to the ISN nerve pathway (Crowner et al., 2003). By this model, excessive Abl, Rac-dependent substratum adhesion in a *Notch* mutant prevents ISNb growth cones from defasiculating from the ISN to enter the target muscle field. Modulation of Rac activity directly modifies this adhesion, aiding or hindering the *Notch*-dependent release of the growth cone from the ISN pathway at the choice point. These observations are also consistent with results from vertebrate cell culture models that suggested a crucial role for Rac in Abl-dependent signaling and cell adhesion (Zandy et al., 2007; Zandy and Pendergast, 2008).

The biological function of Rac has been difficult to investigate in *vivo*. First, Rac performs a wide range of functions in many cells, so experimental modulation of Rac often produces complex combinations of effects. Second, the phenotypes caused by overexpression of constitutively active Rac are often similar to those produced by overexpression of a dominant negative form, rather than being opposite, making interpretation of experimental manipulations extremely challenging (Luo et al., 1994; Luo, 2000a). For example, both increase and decrease of Rac activity can cause growth cone stalling in *Drosophila* neurons.

At the molecular level, however, this occurs for opposite reasons: extreme activation of Rac causes excessive stabilization of actin filaments, while inactivation of Rac excessively destabilizes them (Luo et al., 1994). In either event, however, the result is to block growth cone advance. These properties motivated us to introduce two technical modifications to our experiments that have been essential to obtaining clear conclusions. First, we used a sensitized genetic background in which a single molecular process was limiting for a specific axon guidance decision. This minimized the confusions normally introduced by the pleiotropic functions of Rac. We achieved this by employing a precise temperature shift of a temperature-sensitive allele of *Notch* in a synchronized population of embryos, and assaying the turning of a single nerve comprising seven closely related axons at a precise point in their trajectory. Second, rather than using severe manipulation of Rac level or activity we used the mildest manipulations we could achieve and averaged over a large number of trials to detect quantitative modulation of an intermediate (hypomorphic) *Notch* phenotype by Rac. Axon growth and guidance rely on a cycle of actin dynamics. Extreme manipulations run the risk of halting that cycle altogether. We reasoned that more modest manipulations would allow us to interrogate sensitively the effect of a particular signaling molecule on the dynamics of the actin cycle. We therefore employed heterozygous *Rac* mutations rather than homozygotes for investigating genetic interactions with *Notch*. Moreover, when expressing dominant *Rac* transgenes, we searched for GAL4 drivers that expressed at low, rather than high level, and that did not produce phenotypes on their own in a wild type genetic background. These perturbations nonetheless sufficed to produce significant quantitative effects on axon phenotype in the sensitized N^{ts1} background. While the subtle manipulations used here necessarily produced effects that were relatively modest quantitatively, they were consistent across genotypes, such as different mutant alleles, or reduction of different genes within the Abl pathway, and they were internally consistent when comparing different kinds of perturbations, such as gain- *vs* loss-of-function experiments. In contrast, use of more extreme manipulations, such as interaction with homozygous mutations in the Abl pathway, were often uninterpretable due to defects in other, unrelated developmental processes.

The data reported here suggest that Rac, acting downstream of Trio, is a major player in the non-canonical signaling pathway by which Notch controls axon growth and guidance. The key challenges now are to uncover the molecular mechanism by which Notch antagonizes Abl signaling, and to understand how and why suppression of Abl signaling by Notch promotes proper growth and guidance of Notch-dependent axons.

Experimental Procedures

Fly stocks

Fly stocks were raised on standard Drosophila media at room temperature (23–25°C) except for Notch mutant, N^{ts1} in 18°C. We obtained fly stocks as follows: $trio^{123.4}$-E. Leibl (Dennison University, OH, USA); *Gal4-60* -G. Technau (University of Mainz, Mainz, Germany); $Rho1^{rev220}$, S. Parker (Fred Hutchinson Cancer Research Center, WA, USA); *elav-Gal4*, Y.N. Jan (UCSF, CA, USA); $Rac1^{J10}Rac2^{\Delta}Mtl$, *UAS-*$Rac^{N17}$, *UAS-*$Rac^{V12}$, *UAS- Rac1*, *UAS-*$Cdc42^{V12}$, *UAS-*$Cdc42^{N17}$ (L. Luo, Stanford University, CA, USA); $Cdc42^{4}$ (R. Fehon, Duke University, NC, USA); $trio^{1}$,*UAS-*$trio^{WT}$, *UAS-*$Rho1^{N19}$, *UAS-*$Rho1^{v14}$ -the Bloomington Drosophila Stock Center (Bloomington, IN, USA). To examine the specificity of GEF activity of Trio, we generate transgenic lines of GEF1-inactive (UAS-$trio^{GEF1mu}$) and GEF2-inactive (*UAS-*$trio^{GEF2mu}$)(mutant *trio* constructs were kindly provided by B.J. Dickson). Chromosome balancer containing β-galactosidase (*TM6B-T8-LacZ* and *CyOact-LacZ*) were used in all genetic experiments.

Sample collection and preparation

Egg collection and fixation was carried out as previously described (Crowner et al., 2003) with a slight modification of temperature shift condition; 0–3 hour N^{ts1} embryos were placed at 18°C for 13 hours and then 32°C for 6 hours. In this late-shifting condition, a significant portion of motor axons display abnormal patterning including ISNb phenotype (Supplementary 1) without affecting relevant cell fates, including number and location of neurons and glia (confirmed by marker staining, data not shown) and morphogenesis of muscles. For scoring SNa phenotypes, embryos were placed at 18°C for 14 hours and then 32°C for 5.5 hours.

Immunohistochemistry

Collected embryos were stained with antibodies by standard methods (Bodmer 1987, Bier 1989). Anti-Sxl (M114, Developmental Studies Hybridoma Bank, Iowa, USA) was used at 1:50 for segregating hemizygote for X chromosome, negative for Sxl expression. Anti-Fasciclin II (1D4, Developmental Studies Hybridoma Bank) antibody was used at 1:150 for labeling motor nerves. Rabbit anti β-gal (1:1000, Cappel, pre-absorbed prior to use) was used for sorting negative embryos out for further analysis.

Statistical analysis

ISNb was scored in hemisegments A2–A7 by staining with anti-Fasciclin II, as described previously (Song et al., 2010). Data for a given genotype was pooled and significance vs control was assessed by χ^2 test. Average variation between duplicate trials was less than 15% of the mean value for a given condition (Table 2).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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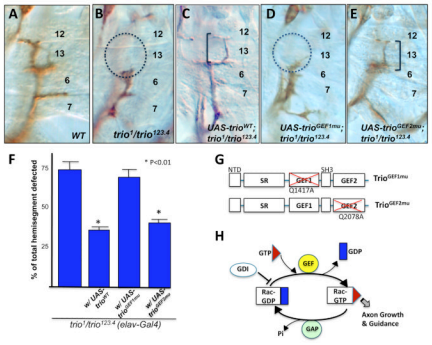

Figure 1. Trio-GEF1 activity is required for motor axon guidance

Embryos of the indicated genotypes were stained with anti fasciclin II (Fas II; 1D4) at late stage 16 and dissected. (A) *Wild type*. (B) Embryos of loss of function *trio* mutant ($trio^{1}/trio^{123.4}$) exhibit highly penetrant ISNb stall phenotype where axons cannot reach their distal target, the muscle 12/13 cleft (dotted circle). (C), (E) ISNb growth is partially rescued (brackets) by transgenes of $UAS\text{-}trio^{WT}$ (C) and $UAS\text{-}trio^{GEF2mu}$ (E). (D) $UAS\text{-}trio^{GEF1mu}$ fails to rescue ISNb growth (dotted circle). (F) Expressivities of *trio* phenotypes. For control (left bar), we scored embryos of *elav-Gal4;* $trio^{1}/trio^{123.4}$, which displayed similar expressivity to $trio^{1}/trio^{123.4}$ alone (74%, n=248). Asterisk (*) indicates that difference compared to the control is statistically significant (P<0.01). More than 200 hemisegments per each genotype were analyzed. (G) Schematic diagram of mutant *trio* transgenes. $trio^{GEF1mu}$ construct has Q1417A mutation abolishing GEF activities in GEF1 and $trio^{GEF2mu}$ has Q2078A mutation in GEF2 (Newsome et al., 2000). NTD; N-terminal Domain. SR; Spectrin Repeats. (H) Cycle of Rac GTPase in axonal growth and guidance. GEF: Guanine nucleotide Exchange Factor; GAP: GTPase Activating Protein; GDI: Guanine nucleotide Dissociation Inhibitor.

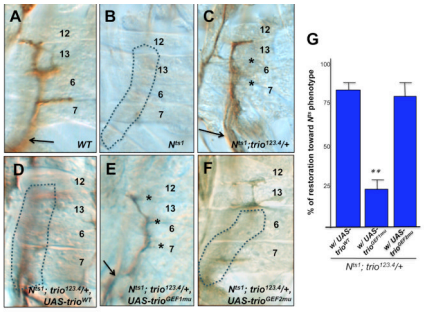

Figure 2. Modulation of ISNb axon patterning by expression of Trio derivatives in *Notch^{ts1}*

(A) *Wild type*. Anti-FasII stained ISNb axons normally exit from the ISN at a specific choice point (arrow). (B) *Notch^{ts1} (N^{ts1})*. ISNb axons fail to diverge from the ISN pathway and do not enter the ventrolateral muscle layer (VLM) and therefore are not visible in this plane of focus (dotted line). (C) *N^{ts1}; trio$^{123.4}$/+*. Reduction of *trio* gene dosage rescues the ISNb bypass phenotype of the *Notch* mutant. Note re- entrance of ISNb axons to VLM field at choice point (arrow), though in a fraction of the rescued hemisegments some of the normal muscle innervations fail to form properly (asterisks). (D – F) *N^{ts1};elav-GAL4; trio$^{123.4}$/+* embryos bearing the indicated *trio* transgenes. Pan-neuronal expression of wildtype *trio* (D) or *trio^{GEF2mu}* (F) reverts the suppression produced by a heterozygous trio mutation, restoring the expressivity of the original *N^{ts1}* phenotype (quantified in (G)). In contrast, expression of *trio^{GEF1mu}* (E) has no effect on the *N^{ts}; trio$^{-/+}$* genetic interaction (though in this genotype ISNb axons seem not always to fully invade the clefts between adjacent muscles (asterisks)). (G) Quantification of the effect of trio transgenes on the *Notch-trio* interaction. Effect of expressing the indicated *trio* transgene is graphed as percent restoration of the *N^{ts}* phenotype, relative to the phenoytpe of *N^{ts1}; trio$^{123.4/+}$*. Double asterisk (**) indicates that the difference between *N^{ts1}; UAS-trio^{WT}; trio$^{123.4}$/+* and *N^{ts1}; UAS-trio^{GEF1mu}; trio$^{123.4}$/+* is statistically significant (P<0.005).

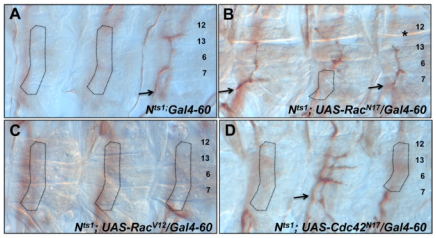

Figure 3. Modification of *Notch* ISNb axon patterning by dominant mutations of small GTPases

(A) N^{ts1};*Gal4-60*. Like N^{ts1}, ISNb axons often fail to diverge from the ISN pathway and do not enter the ventrolateral muscle layer (VLM) and therefore are not visible in this plane of focus (dotted line). (B) N^{ts1}; *UAS-*Rac^{N17}*/Gal4-60*. *Gal4-60* driven Rac^{N17}, a dominant negative form of Rac, significantly restores the *Notch* ISNb bypass phenotype, causing ISNb axons to enter the VLM field at the choice point (arrows), though in a fraction of the rescued hemisegments some of the normal muscle innervations are incomplete (asterisks). (C) N^{ts1}; *UAS-*Rac^{V12}*/Gal4-60. Gal4-60* driven Rac^{V12}, a constitutively active form of Rac, strongly enhanced *Notch* ISNb phenotype. However, dominant mutations of other small GTPases such as Cdc42 (D) or RhoA (not shown) did not change significantly the expressivity of *Notch* ISNb bypass phenotype.

Table 1

Genetic rescue of *trio* mutant and modification of *Notch-trio* interaction by *trio* transgenes in ISNb motor axon guidance.

Genotype	n	ISNb defects
Rescue of *trio* mutant by *trio* transgenes		
$trio^{1}/trio^{123.4}$	190	74%
$UAS\text{-}trio^{WT}; trio^{1}/trio^{123.4}$	240	35%*
$UAS\text{-}trio^{GEF1mu}; trio^{1}/trio^{123.4}$	242	70%^{ns}
$UAS\text{-}trio^{GEF2mu}; trio^{1}/trio^{123.4}$	132	40%*
Modification of *N-trio* interaction by *trio* transgenes		
N^{ts1}	562	36%
$N^{ts1}; trio^{123.4}/+$	300	27%
$N^{ts1}; UAS\text{-}trio^{WT}; trio^{123.4}/+$	286	35%**
$N^{ts1}; UAS\text{-}trio^{GEF1mu}; trio^{123.4}/+$	259	29%^{ns}
$N^{ts1}; UAS\text{-}trio^{GEF2mu}; trio^{123.4}/+$	284	35%**
$trio^{123.4}/+$	120	<2%

Abdominal segments A2 – A7 were examined in late stage 16 embryos for quantification of ISNb phenotypes. n, total hemisegments counted. All trio transgenes were expressed by pan-neuronal *elav-Gal4* driver.

For rescue of trio mutant, single asterisks (*) denote statistically significant rescue by transgenes relative to $trio^{1}/trio^{123.4}$ ($P<0.005$).

For rescue of Notch-trio interaction, double asterisks (**) denote statistically significant modification by transgenes relative to $N^{ts1}; trio^{123.4}/+$ ($P<0.005$). P-values were determined by χ^2 test.

^{ns}Not statistically significant. P-value relative to czontrol was more than 0.1 in each comparison.

Table 2

Quantification of genetic interactions between *Notch* and *Rho* small GTPases in ISNb motor axon guidance.

Genotype	n[a]	%[b]	Control	n[a]	%[b]
Modification by mutation					
N^{ts1}	562	36			
N^{ts1}; $Rac1^{J10}Rac2^{\Delta}Mtl^{\Delta}$/+	132	23*	$Rac1^{J10}Rac2^{\Delta}Mtl^{\Delta}$/+	120	<2
N^{ts1};$Rho1^{rev220}$/+	192	36 *ns*	$Rho1^{rev220}$/+	120	<2
N^{ts1},$Cdc42^{4}$/N^{ts1},+	144	35 *ns*	$Cdc42^{4}$/+	120	<2
Modification by transgenes					
N^{ts1}; *Gal4-60*/+	230	35			
N^{ts1}; *elav-Gal4*/+	192	37			
N^{ts1}; *UAS-*Rac^{V12}*/Gal4-60*	180	55*	*UAS-*Rac^{V12}*/Gal4-60*	168	6
N^{ts1}; *UAS-*Rac^{N17}*/ Gal4-60*	156	27*[c]	*UAS-*Rac^{N17}*/ Gal4-60*	180	3
N^{ts1}; *UAS-*$Rac1^{WT}$*/ Gal4-60*	168	41 *ns*	*UAS-Rac1* ^{WT} */ Gal4-60*	132	5
N^{ts1}; *UAS-*$Rho1^{V14}$*/Gal4-60*	192	39 *ns*	*UAS-*$Rho1^{V14}$*/Gal4-60*	120	<2
N^{ts1}; *UAS-*$Rho1^{N19}$*/ elav-Gal4*	120	38*ns*	*UAS-*$Rho1^{N19}$*/elav-Gal4*	202	<2
N^{ts1}; *UAS-*$Cdc42^{V12}$*/Gal4-60*	300	39 *ns*	*UAS-*$Cdc42^{V12}$*/Gal4-60*	120	<2
N^{ts1}; *UAS-*$Cdc42^{N17}$*/elav-Gal4*	168	36 *ns*	*UAS-*$Cdc42^{N17}$*/elav-Gal4*	240	<2

Abdominal segments A2 – A7 were examined in late stage 16 embryos for quantification of ISNb bypass phenotypes. Asterisks denote statistically significant modification by mutants and transgenes (P<0.05). Comparison is to N^{ts1} for effect of mutations; comparison is to N^{ts1} with the appropriate GAL4 driver for transgenes. P-values were determined by χ^2 test. *UAS-*$Cdc42^{N17}$ and *UAS-*$Rho1^{N19}$ were pan-neuronally expressed by *elav-Gal4* driver. Since *elav*-driven expression of some dominant forms of GTPases (Rac^{N17}, Rac^{V12}, $Rho1^{V14}$ and $Cdc42^{V12}$) and *UAS-Rac1* ^{WT} show massive axonal defects in most hemisegments (also described in(Kaufmann et al., 1998; Fritz and VanBerkum, 2002), for these transgenes instead we used *Gal4-60*, *a* less active *GAL4* driver (Luo et al., 1994). In a completely independent set of experiments, the critical genotypes were assayed in a somewhat different temperature shift protocol and gave the same result, supporting the robustness of the genetic interaction (D.Crowner and E.G unpublished observation)

[a] Number of hemisegments of the designated genotype that were scored.

[b] The percentage of the hemisegments examined that exhibited ISNb bypass phenotype (described in Supplementary 1).

[c] Besides the bypass phenotype, we observed a different guidance defect, 11% of *trio*-like stall phenotypes.

ns Not statistically significant. P-values relative to control were > 0.4 in these comparisons.