TGFβ signals regulate axonal development through distinct Smad-independent mechanisms

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Proper nerve connections form when growing axons terminate at the correct postsynaptic target. Here I show that Transforming growth factor beta (TGFβ) signals regulate axon growth. In most contexts, TGFβ signals are tightly linked to Smad transcriptional activity. Although known to exist, how Smad-independent pathways mediate TGFβ responses in vivo is unclear. In Drosophila mushroom body (MB) neurons, loss of the TGFβ receptor Baboon (Babo) results in axon overextension. Conversely, misexpression of constitutively active Babo results in premature axon termination. Smad activity is not required for these phenotypes. This study shows that Babo signals require the Rho GTPases Rho1 and Rac, and LIM kinase1 (LIMK1), which regulate the actin cytoskeleton. Contrary to the well-established receptor activation model, in which type 1 receptors act downstream of type 2 receptors, this study shows that the type 2 receptors Wishful thinking (Wit) and Punt act downstream of the Babo type 1 receptor. Wit and Punt regulate axon growth independently, and interchangeably, through LIMK1-dependent and -independent mechanisms. Thus, novel TGFβ receptor interactions control non-Smad signals and regulate multiple aspects of axonal development in vivo.

KEY WORDS: Neural development, Signal transduction, Cytoskeleton, Drosophila

INTRODUCTION

In developing neurons, axon and dendrite extensions are directed by specialised motile structures termed growth cones. These extensions are often long and intricate, but once nerve growth cones have reached their targets, cell extensions stop and synaptogenesis begins. How this takes place in vivo is unclear. Extracellular cues often direct growth cone motility through cytoskeletal reorganisation. Many (if not all) axon guidance cues regulate the nerve cell cytoskeleton through Rho family GTPases (Luo, 2002). Multiple aspects of axonal development are regulated by Rho GTPases. Although Rac generally mediates axon extension and attractive responses, and Rho1 (also known as RhoA) generally mediates axon retraction and repulsion, these distinctions can be complex. For example, Drosophila genetic studies show axon outgrowth and attractive responses mediated by Nettin (Forsthoefel et al., 2005), and axon repulsive cues mediated by Robo (Fan et al., 2003; Matsuura et al., 2004), both of which depend on Rac subfamily GTPases. Similarly, Drosophila Rho1 signals can mediate axon retraction (Billuart et al., 2001) and attraction (Bashaw et al., 2001) in different neurons. These and many other studies highlight the key and complex roles that Rho GTPases play in growth cone responses.

Studies on mushroom body (MB) neurons in the Drosophila brain have shown that Rho proteins regulate axon growth through LIM kinase (LIMK)-dependent and -independent pathways, and that they can act antagonistically (Ng and Luo, 2004). LIMK regulates actin filament turnover by phosphorylating, and thereby inactivating, an actin depolymerisation and severing factor, ADF/cofilin (Bamburg, 1999). LIMK1 misexpression in neurons, in vitro or in vivo, leads to axon growth inhibition. Consistent with a role in ADF/cofilin regulation, this phenotype is suppressed by increasing cofilin activity, either by coexpressing wild-type cofilin or a form (S3A) that cannot be phosphorylated, or by expressing the cofilin phosphatase, Slingshot (Ssh) (Endo et al., 2003; Ng and Luo, 2004). In Drosophila, one homologue of ADF/cofilin exists, twinsclare (tsr), and its inactivation results in growth cone morphology and axon growth defects. These results suggest that cofilin phosphoregulation is essential for axon growth.

How extracellular cues pattern axons through Rho GTPase and cofilin regulation in vivo is unclear. Here I show that components of the Transforming growth factor beta (TGFβ) pathway are involved. The TGFβ pathway regulates many morphogenic events, including cell fate specification, cell migration, proliferation and apoptosis (Hogan, 1996; Massague et al., 2000; Raftery and Sutherland, 1999). The conserved TGFβ pathway consists of a core complex of type 1 and type 2 transmembrane receptor serine/threonine kinases, which are activated by secreted TGFβ ligands [bone morphogenetic proteins (BMPs) or TGFβ/Activins] (Feng and Derynck, 2005; Shi and Massague, 2003). The presence of ligand dimers triggers a signalling cascade involving the receptor complex. The following events are essential: phosphorylation of type 1 receptors by the type 2 receptor kinase; phosphorylation of receptor activated Smads (R-Smads) by the type 1 receptor kinase; R-Smad complex formation with a common Smad (co-Smad); translocation of Smad complexes into the nucleus to elicit gene transcription. In Drosophila, there are three type 1 receptors, Baboon (Babo), Thickveins (Tkv) and Saxophone (Sax), and two type 2 receptors, Wishful thinking (Wit) and Punt (Put). The activated receptors phosphorylate two R-Smads, Mad and Sma2 (also known as S2mad and Smox – FlyBase), which form a trimeric complex with the co-Smad Medea (Med). In most models, Smad activation is an obligate effector response upon ligand binding.

Although Smad-independent pathways are known (Derynck and Zhang, 2003; Moustakas and Heldin, 2005; Fioletta et al., 2003; Lee-Hoeflich et al., 2004; Ozdamar et al., 2005), how they affect development in vivo is unclear. In many instances, Smad-independent pathways exhibit cross-regulatory effects, which either regulate Smads or are under Smad regulation. However, some TGFβ signals are Smad-independent events. In C. elegans, mutations in a TGFβ signal (unc-129) result in dorsal-ventral axon guidance defects (Colavita et al., 1998). Mutation analyses of other TGFβ signals are Smad-independent events. In C. elegans, mutations in a TGFβ signal (unc-129) result in dorsal-ventral axon guidance defects (Colavita et al., 1998). Mutation analyses of other TGFβ signals are Smad-independent events. In C. elegans, mutations in a TGFβ signal (unc-129) result in dorsal-ventral axon guidance defects (Colavita et al., 1998). Mutation analyses of other TGFβ signals are Smad-independent events. In C. elegans, mutations in a TGFβ signal (unc-129) result in dorsal-ventral axon guidance defects (Colavita et al., 1998). Mutation analyses of other TGFβ signals are Smad-independent events. In C. elegans, mutations in a TGFβ signal (unc-129) result in dorsal-ventral axon guidance defects (Colavita et al., 1998). Mutation analyses of other TGFβ signals are Smad-independent events. In C. elegans, mutations in a TGFβ signal (unc-129) result in dorsal-ventral axon guidance defects (Colavita et al., 1998).
components, such as receptors or Smads, do not reveal this phenotype, suggesting that axon guidance in worms involves atypical TGFβ signallng mechanisms. TGFβ signals also regulate dorsal-ventral axon guidance in the developing mouse spinal cord. BMP7 expression in the dorsal roof plate acts to repel spinal cord neurons and guide their projections ventrally (Augsburger et al., 1999; Butler and Dodd, 2003). Whether Smads are involved is unclear; nonetheless, the rapid axonal responses would seem to preclude transcriptional events.

Recent studies have shown that BMP4 and BMP7 treatment in mammalian non-neuronal and neuronal cell cultures, respectively, leads to LIMK activation, resulting in a rapid increase in cofilin phosphorylation (Foletta et al., 2003; Lee-Hoeflich et al., 2004). This requires a direct interaction between the C-terminal tail of a BMP receptor (BMPR2), which is dispensable for Smad signalling, and LIMK. Lee-Hoeflich et al. (Hoeflich et al., 2004) have further shown that the BMPR2 C-terminus is required for dendritogenesis in cultured cortical neurons. Mammalian BMPs also regulate growth cone turning responses in cultured Xenopus spinal neurons (Wen et al., 2007). BMP7 exposure causes attractive or repulsive growth cone turning behaviours by regulating cofilin through LIMK1 or Ssh activities, respectively.

Drosophila LIMK1 is essential for synaptic stability controlled by BMPs. Genetic analysis of the Drosophila neuromuscular junction (NMJ) reveals that the stability of presynaptic terminals requires a retrograde BMP-type signal, Glass bottom boat (Gbb), that acts through Wit (the Drosophila homologue of BMPR2). Like BMPR2, Wit binds to LIMK1 via its C-terminal extension. Without this interaction, NMJ synapses can grow (through Wit signalling via the Drosophila Smads, Mad and Medea) but they have defects in synaptic stability (Eaton and Davis, 2005). How TGFβ receptor interactions regulate LIMK1 is unclear (Foletta et al., 2003; Lee-Hoeflich et al., 2004). Nor is it clear how LIMK1 regulates synapses, as cofilin phosphoregulation does not appear to be essential (Eaton and Davis, 2005).

Here, I show that TGFβ signals regulate distinct aspects of axonal development. Loss of Babo results in MB axon overextension, whereas in other neurons axon outgrowth and targeting defects are observed. The results show that Babo acts together with Wit and Put, but is independent of Smads. Babo signals depend on Rho1, Rac and LIMK1. Consistent with a role in LIMK1 regulation, babo and wit genetically interact with LIMK1. babo and LIMK1 gain-of-function phenotypes are similar, and both are suppressed by increasing cofilin activity. Contrary to the canonical receptor activation model, the type 2 receptors Wit and Put both act downstream of the Babo type 1 receptor, and distinct LIMK1-dependent and -independent pathways are required.

MATERIALS AND METHODS

**Drosophila strains**

LIMK1, tsr, ssh, RhogEF2, pbl, trio, sif, RhinoGAPp190, Rac, Rho, Cdc42, Pak and Rok mutant and transgenic strains have been described previously and are referenced therein (Ng and Luo, 2004). The following additional strains were used: babo[76], babo[77], UAS-activated babo[Q302D] (Ca babo) (Brummel et al., 1999); tkv[78], tkv[79] (Penton et al., 1994); tkv[80] (Gibson and Perrimon, 2005); UAS-put△, UAS-tkv△GSK (DN tkv), UAS-sax△ (DN sax), UAS-tkv.A (HA) Q199D (CA tkv), UAS-sax△.Q199D (CA sax) (Haery et al., 1998); sax[81] (Singer et al., 1997); sax[82], UAS-put (Nellen et al., 1994); UAS-babo-a, UAS-babo-b, Flag, UAS-babo-Δ1 (DN babo) (Zheng et al., 2006) (a gift from M. O’Connor, HHMI/University of Minnesota, Minneapolis and Theo Haery, Florida Atlantic University, Boca Raton); wit[83], wit[84], wit[85] (P(wit genomic); P(wit)), P(wit tailless); P(witAC), UAS-wit, UAS-wit△C (Marques et al., 2002); UAS-wit△ (McCabe et al., 2003); put[86], UAS-put (Ruberte et al., 1995); put[87] (Simin et al., 1998); Mab[88] (Sekelsky et al., 1995); Med[89] (Hudson et al., 1998); Ssh[90] (Zheng et al., 2003); UAS-Dad (Tsuneyama et al., 1997); UAS-MYC::tum (RacGAP50C) (Goldstein et al., 2005); UAS-Ecr-B1 (Lee et al., 2000); Df(l)HF368, UAS-RhoGEF2 (Bloomington Drosophila Stock Center). Constitutively active (CA) forms of type 1 receptors result from a conserved Gln (Q) to Glu (D) mutation leading to constitutively active kinase activity (Wieser et al., 1995). Dominant-negative (DN) forms of type 1 and type 2 receptors derive from cytoplasmic deletions, with the loss of intracellular domains (cited above). Genetic crossing schemes used in this study are available upon request.

**MARCM and Gal4-UAS expression studies**

Loss-of-function clones were generated using the MARCM method (Lee and Luo, 1999). Neuroblast and single-cell ββ clones were generated as previously described (Ng et al., 2002). Neurons were visualised using the Gal4-OK107 driver expressing UAS-mCD8::GFP. Misexpression studies were performed using the same driver. For CA and DN misexpression studies, unless indicated otherwise, multiple copies (2–4) of the UAS transgene were used to derive the strongest possible phenotypes. The strength of CA Babo phenotypes was correlated with Babo expression levels, using one, two or four copies of UAS-CA babo (data not shown; Figs 4, 5 and 7 see Fig. S2 and Fig. S6D in the supplementary material). The data shown in Figs 4, 5 and 7 were obtained using two copies (UAS lines 1B and 9B). MARCM clones were visualised by immunostaining using anti-CD8 (Cattag, clone CT-CD8a, 1:100) and anti-Fas2 (a gift from G. Tear, King’s College London; clone 1D4; 1:5) antibodies. In misexpression studies, neurons were visualised using epifluorescent CD8::GFP together with anti-Fas2 staining. Additional antibodies used were HA (Santa Cruz, Y11, 1:500), Babo (Abcam, ab14681, 1:50), Wit (a gift from H. Aberle, MPI Developmental Biology, Tübingen; clone 23C7; 1:10) and FLAG (Sigma, clone M5, 1:200). These were used to estimate the level and localisation of ectopic Sax-HA, Tkv-HA, Babo, Wit and WitAC-FLAG proteins, respectively, in neurons. Although endogenous Babo and Wit were detected throughout brain tissue, ectopic levels were distinguished using these antibodies. Drosophila brains were dissected, fixed and stained as previously described (Ng et al., 2002). Confocal images were generated with a Zeiss LSM510 confocal microscope, using Zeiss LSM510, Image J and Adobe Photoshop software.

**RESULTS**

**MB intrinsic neurons (‘Kenyon’ cells) in the Drosophila brain are well characterised with respect to their cell division, differentiation and projection patterns (Ito et al., 1997; Kurusu et al., 2002; Lee et al., 1999). There are three different sets of adult MB neurons (γ, αβ and ββ), which are born at different periods from common neuroblast progenitors and have distinct axonal projections (Lee et al., 1999) (Fig. 1A,B). Each neuron extends a primary neurite that gives rise to dendrites near the cell body, and a single axon that projects anteroventrally through the peduncle. Axons of αβ or ββ neurons bifurcate to form a dorsal and a medial branch, whereas γ neurons extend only a medial branch (branches are also referred to collectively as ‘lobes’). All axons terminate either medially, close to the midline, or close to the anterior dorsal cortex (Fig. 1A,B).**

**Babo inactivation results in MB axon overextension**

To study the role of TGFβ signals in MB neurons, mutant clones were generated using strong loss-of-function or null alleles of the type 1 receptors babo, tkv and sax. babo-null (babo[76]) neuroblast clones had axon overextension phenotypes in ββ neurons, where β lobes overextending across the midline (Fig. 1, compare C with B, quantified in H). Consistent with previous studies (Zheng et al., 2003), babo clones also exhibited axon pruning defects, characterised by the presence of larval-stage dorsal and medial
Baboa and Babob isoforms regulate axon growth
suggest that Babo regulates axon growth, particularly of the MB axon pruning defects. Conversely, nor were axon pruning defects observed, with β lobes fusing at the midline (Fig. 1F-H). Thus, either Babo isoform can regulate axon growth.

Consistent with a cell-autonomous role, Babo inactivation in single αβ neurons resulted in similar axon overextensions. Interestingly, non-cell-autonomy was also observed, as single babo neurons caused heterozygote axons to similarly overextend across the midline (see Fig. S1 in the supplementary material).

Baboa and Babob isoforms regulate axon growth cell-autonomously
Recent data suggest that different Babo isoforms have distinct neural functions (Zheng et al., 2006). Expression of the Baboa, but not Babob, isoform rescues the babo MB axon pruning phenotypes. By contrast, either isoform rescues the babo axon extension defects of dorsal cluster (DC) neurons in the optic lobe. To test whether different Babo isoforms regulate MB axon growth, similar assays were performed. In a wild-type background, ectopically expressed Baboa or Babob, was detected in all MB lobes and did not disrupt axonal projections (see Fig. S2 in the supplementary material). Baboa or Babob expression in babo52 neuroblast clones rescued the axon overextension defect, as most β lobes terminated correctly (Fig. 1F-H). Thus, either Babo isoform can regulate axon growth.

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Fig. 1. Babo inactivation results in axon overextension. Babo regulates axon growth through Baboa and Babob isoforms. (A) Schematic of the adult Drosophila brain. The boxed region shows mushroom body (MB) neurons in the left hemisphere of the central brain (cb). Arrows show the MB axon trajectory extending from posterior dorsal cell bodies, projecting anterogravely and then turning towards the midline. The MB images shown are either from the left hemisphere in this orientation, or of the central brain, showing both hemispheres. Dashed white lines indicate the midline. ol, optic lobe; D, dorsal; V, ventral; P, posterior; A, anterior; L, lateral; M, medial. (B) A wild-type MB neuroblast clone. Typical adult wild-type clones generated from newly hatched larvae have axonal projections that terminate either in the dorsal anterior cortex or just prior to the midline. Only γ, α and β projections are indicated. (C-E) Representative images of babo52 (C), tkv4 (D) and sax4 (E) neuroblast clones. Note the β lobe overextensions (open red arrowhead) across the midline in babo clones. In these and subsequent figures, open white arrowheads indicate γ axon pruning defects. (F-G) Representative images of babo52 neuroblast clones expressing either UAS-baboa (F), or UAS-babob (G). Many of the axons in the UAS-baboa rescue exhibited small protrusions that were not characteristic of any lobe (thin white arrow in F). These represent ectopic projections of a subclass of MB axons induced in the OK107-babo, genetic background. In these and subsequent figures, solid red or white arrowheads indicate normal α and β or γ lobe termination points, as indicated. All images in this and subsequent figures are z-projections of confocal sections. Green, expression of the marker mCD8::GFP on all MB, neuroblast or single-cell MARCM clones (sometimes multiple single-cell clones); magenta, Fas2 staining of all MB (weakly stained) and αβ (strongly stained) axons (appearing white when overlapping with mCD8::GFP). Dashed white line, midline. Scale bar: 20 μm. (H) Quantification of axon overextension defects in the indicated genotypes. n, number of neuroblast clones examined.

Babo regulates MB axon growth independently of axon pruning
Using a different approach, a DN form of Babo was misexpressed in MB neurons. Like the null phenotype, axon pruning and overextension phenotypes were observed, with β lobes fusing at the midline (Fig. 2A,A'; 65.2% fusion defects, n=23 brains). To determine whether axon overextension was secondary to axon pruning defects, DN babo was misexpressed together with the Ecdysone receptor B1 isoform (EcR-B1). Similar to previous results (Zheng et al., 2003), these axon pruning defects were suppressed by ectopic EcR-B1 (Fig. 2B'). However, β lobe fusions remained visible (64.5%, n=31; Fig. 2B). Therefore, DN Babo axon overextension was not secondary to the axon pruning defects. Conversely, nor were axon pruning defects a consequence of axon overextension, as UAS-baboa expression rescued babo52 axon overextension but not the axon pruning defects (Fig. 1G). Similarly, RhoGEF2 coexpression also suppressed DN
Babo axon overextension but not the axon pruning defects (Fig. 2C,C′; see below). Thus, Babo regulates axon pruning and axon growth independently.

Babo regulates axon growth independently of Smads

Babo functions through Smad2 (Brummel et al., 1999; Das et al., 1999; Zheng et al., 2003). When Smad2 strong loss-of-function clones were analysed, axon overextension defects were not detected, although, consistent with previous data (Zheng et al., 2003), axon pruning defects were (Fig. 3A, quantified in Fig. 1H). Null clones of Medea (Med, the Drosophila homologue of the co-Smad Smad4) also did not exhibit overextension defects (Fig. 3B and Fig. 1H). Recent data suggest that, under certain in vitro conditions, Babo can signal through Mad (Gesualdi and Haerry, 2007). When Mad-null, or Smad2 Med double mutant clones were analysed, axon overextensions were not observed either (data not shown; Fig. 3C and Fig. 1H). Similarly, in a different strategy, misexpression of an inhibitory form of Smad, Dad, also did not perturb these axons (data not shown; 100% as wild-type, n=26 hemispheres). As Smads could play a redundant role, their role was tested in a sensitised background. Using a Babo gain-of-function phenotype, one mutant copy of either Smad2, Mad or UAS-Dad was introduced with constitutively active (CA) babo (Fig. 5A,B; see below). Reducing Smad levels did not suppress CA Babo. In fact, loss of Mad, or Dad misexpression, enhanced CA Babo phenotypes. Together, these results suggest that Babo regulates axon growth independently of Smads.

Expression of constitutively active Babo inhibits axon growth

To determine how Babo functions independently of Smads, a gain-of-function approach was taken. CA forms of type 1 receptors were misexpressed in MB neurons. CA Babo expression resulted in axon truncation phenotypes, with the loss of dorsal and/or medial branches (Fig. 4A,A′; for quantification see Fig. 5). Axon guidance defects were also observed; however, this phenotype represented a small fraction of animals [classed as misguidance (MG) in Figs 5, 7; see Fig. S2A,B in the supplementary material]. To test whether CA Babo phenotypes were simply due to increased levels of Babo protein, ectopic wild-type Babo levels were compared with CA Babo levels (see Fig. S2 in the supplementary material). The results showed that the dominant CA Babo phenotype is due to the Q302D mutation, which results in higher kinase activity. High levels of CA Tkv and CA Sax protein were detected in MB axons (data not shown).

Babo and wit genetically interact with LIMK1

LIMK1 misexpression results in similar MB axon phenotypes to those described above (Fig. 4, compare D with A) (Ng and Luo, 2004). However, in contrast to LIMK1, which also led to γ lobe truncations, only αβ lobes were truncated in CA babo-misexpressing animals. Additionally, in CA babo, β lobes were predominantly disrupted (Fig. 4A′; see quantification in Fig. 5A,B).

To study the link between TGFβ and LIMK1, receptor mutants were introduced to determine whether they could modify the LIMK1 misexpression phenotype (Fig. 4E). Loss of one copy of babo or wit suppressed the LIMK1 phenotype. LIMK1 misexpression was not suppressed by other type 1 receptors, such as tkv or sax, or by the other type 2 receptor, put. These genetic assays suggest that Babo and Wit positively interact with LIMK1.

Babo-regulated axon growth requires components of the Rho1 and Rac pathway

Drosophila LIMK1 is regulated by Rho GTPases (Rho1, Rac and Cdc42) through the effector kinases, Rok and Pak (Ng and Luo, 2004). To determine whether Babo-regulated axon growth requires the Rho GTPase pathway, genetic interaction assays were performed using CA babo (Fig. 5A). Lowering the level of Rho1 signals, by loss of one copy of Rho1 or of the Rho1 activator RhoGEF2, resulted in suppression of the CA Babo phenotype. Loss of the Rho1 effector kinase, Sok, also suppressed CA Babo.

When other Rho family members, Cdc42 and Rac (Rac1, Rac2 or Ml), were tested, loss of Rac1 (using the hypomorphic allele J10), or a combined loss of one copy of Rac2 and Ml (using null Δ
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Alleles, also suppressed CA Babo (Fig. 5A). Stronger allelic combinations of Rac enhanced the CA Babo phenotypes (unpublished observations). This is expected, based on previous observations that Rac GTPases can play opposite roles in promoting and inhibiting MB axon growth (Ng and Luo, 2004; Ng et al., 2002).

Loss of Cdc42 did not suppress CA Babo. Loss of the Cdc42/Rac effector kinase Pak also did not suppress CA Babo, but instead resulted in stronger CA Babo phenotypes. These results suggest that in addition to Rho1, CA Babo-mediated axon growth inhibition also requires Rac, but not Cdc42 or Pak.

In contrast to RhoGAP2, loss of pebble (pbl, another Rho1 activator) did not suppress CA Babo. Loss of the Rac activators trio and still life (sif) also did not suppress CA Babo. This suggests that Babo regulates Rho1 through RhoGAP2. Whether Babo regulates axon growth via RacGTPases is unclear, although Sif and Trio are unlikely mediators.

Whether inhibiting Rho pathways through RhoGAPs affects CA Babo was then tested (Fig. 5B). In a wild-type background, single-copy expression of UAS-RhoGAPp190 or UAS-tumbleweed (tum, also known as RacGAP50C) did not disrupt normal axonal projections, although, as previously described, RhoGAPp190 caused a mild dorsal lobe overgrowth defect (Billuart et al., 2001; Goldstein et al., 2005). RhoGAPp190, which acts as a Rho1 inhibitor, strongly suppressed CA Babo (Fig. 5B; data not shown). This is consistent with previous findings that ectopic RhoGAPp190 also suppresses LIMK1 misexpression phenotypes (Ng and Luo, 2004). Tum expression also suppressed CA Babo (Fig. 5B; data not shown). Drosophila tum genetically interacts with Rac1 in the wing and eye (Sotillos and Campuzano, 2000) and tum mutant clones exhibit MB axon extension defects (Goldstein et al., 2005).

Together, this suggests that Babo-regulated axon growth requires the Rho1 and Rac GTPases and involves RhoGAPs (RhoGAP2) and RhoGAPs (RhoGAPp190 and Tum) (Fig. 7E; see below).

DN Babo-induced axon overextension is suppressed by increased Rho1 activity

Based on these results, one would predict that DN Babo-induced axon overextension (Fig. 2A; 65.2% fusion defects, n=23 brains) would be suppressed by increased Rho1 signals. Thus, when RhoGAP2 was coexpressed with DN Babo, axon overextension was suppressed (Fig. 2C; 8.7% fusion defects, n=46 brains). RhoGEF2 did not affect the DN Babo axon pruning defect (Fig. 2C'). Similarly, Rok coexpression also suppressed DN Babo axon overextension, but not the axon pruning phenotype (11.8%, n=34; data not shown).

Other RhoGEFs were tested, but none of these suppressed the DN babo-induced axon overextensions (UAS-pbl, 51.9%, n=77; UAS-trio, 63.3%, n=60; UAS-sif, 43.9%, n=41; data not shown). Taken together, these results suggest that Babo-regulated axon growth requires Rho1 through the activator RhoGEF2 and the effector kinase Rok (Fig. 7E).

CA Babo is suppressed by loss of LIMK1 and by increased cofilin activity

Given their similar phenotypes, the link between CA Babo and LIMK1 was analysed further. Loss of one copy of LIMK1 [using the deficiency Df(1)HF368] strongly suppressed the CA babo axon truncation phenotype (Fig. 5A). Intriguingly, LIMK1 loss of function was observed in many CA babo brains (15 out of 17 brains; see Fig. S4 in the supplementary material), suggesting that CA Babo promotes axon extension under low LIMK1 levels. As the LIMK1 misexpression phenotype is inhibited by Drosophila cofilin (Tsr) (Ng and Luo, 2004), tsr was coexpressed with CA babo. Consistent with its predicted role in regulating LIMK1, Tsr (tsr WT) expression suppressed CA Babo (data not shown; Fig. 5B). However, the results suggest that Babo does not regulate cofilin phosphorylation alone (see Discussion).
Type 2 receptors Wit and Put regulate axon growth independently and interchangeably

Whether TGFβ type 2 receptors regulate axon growth was tested. wit-null neuroblast clones exhibited β lobe overextensions similar to those of babo mutants (Fig. 6A,G, compare with Fig. 1C). Since the Wit C-terminal tail binds to LIMK1 (Eaton and Davis, 2005), the relevance of this region was analysed. Consistent with previous results, wit mutants are viable in the presence of the ‘tailless’ genomic rescue transgene (P[witΔC]), which lacks the Wit C-terminal region but includes the kinase region (Marques et al., 2002) (data not shown). However, compared with the wild-type full-length wit genomic construct (P[wit+]), the tailless wit transgene failed to suppress the wit-null overextensions (data not shown; Fig. 6G). This suggests that the C-terminal region is essential for Wit-regulated axon growth.

put strong loss-of-function clones also exhibited (albeit to a lesser extent) axon overextensions (Fig. 6B,G). This was also observed when a DN form of Put (UAS-putΔ) was misexpressed (Fig. 6C; 45.5% fusion defects; n=44 brains).

To test whether type 2 receptors can function interchangeably, UAS-put was expressed in wit clones. wit axon overextensions were suppressed by Put expression (Fig. 6D,G). Conversely, put phenotypes were rescued by UAS-put or UAS-wit (data not shown; Fig. 6E,G). However, put phenotypes were not rescued by the tailless UAS-witΔC (Fig. 6F,G). These results suggest that although Wit and Put regulate axon growth independently, they can function interchangeably. However, distinct mechanisms are employed, involving LIMK1-dependent and -independent pathways (Fig. 7E) (see Discussion).

The type 2 receptors Wit and Put act downstream of the type 1 receptor Babo

The results suggest that Babo, Wit and Put work together. In the canonical model of TGFβ signalling, type 1 receptors act downstream of type 2 receptors. Furthermore, activated type 1 receptors propagate Smad signals independently of ligands or type 2 receptors (Brummel et al., 1999; Wieser et al., 1995) and, in vivo, result in ectopic TGFβ responses independently of ligands (Haerry et al., 1998; Lecuit et al., 1996; Nellen et al., 1996). Using CA Babo, the relevance of this model was tested (Fig. 7A). Loss of one copy of wit suppressed CA Babo. Expression of a DN form of wit (UAS-witΔ), which alone did not disrupt MB axon projection (data not shown), also suppressed CA Babo. In similar assays, one mutant copy of put, or UAS-putΔI coexpression, also suppressed CA Babo. These results suggest that Babo regulates axon growth together with Wit and Put. However, contrary to the canonical model, CA Babo requires the presence of type 2 receptors.

To explore this further, genetic epistasis experiments were performed. Wit and Put were expressed in babo-null neurons (Fig. 7B,C, quantified in D). Ectopic Wit or Put suppressed the babo axon overextension but not the axon pruning phenotype (a Smad-dependent process). Collectively, these results suggest that in Babo-regulated axon growth, type 2 receptors act downstream of type 1 signals (Fig. 7E).

Babo regulates axon extension and targeting of AL and OL axons independently of Smads

To determine whether Babo regulates the axon patterning of other neurons, antennal lobe (AL) and optic lobe (OL) contralateral projection neurons were analysed (Ng and Luo, 2004) (Fig. 8A,B,F). As previously described, these neurons extend axons contralaterally into the opposite AL (Fig. 8A,B) or OL (Fig. 8A,F), respectively. babo AL and OL clones showed axonal defects (Fig. 8C,G, quantified in J). babo AL axons were disrupted in the target area and fewer axons extended across the midline. babo OL axons displayed a subtler phenotype: although the number of babo OL axons projecting into the initial target area appeared normal, terminal branches were less elaborated and ‘gaps’ were observed in terminal zones (open blue arrowheads in Fig. 8G; see Fig. S5 in the supplementary material). No gross
mispredictions were observed. These results suggest that Babo regulates axon extension and targeting in AL neurons, but only on axons targeting in OL neurons.

The relevance of Smads in AL and OL axonal development was also determined. Smad2 (Fig. 6D,H) and Smad1 (Fig. 5G) in AL and OL axons did not reveal any gross defects, although gaps similar to those observed for Smad2 were occasionally observed in Smad2 OL axons. Thus, as with MB neurons, Babo regulates AL and OL axonal development independently of Smads.

**DISCUSSION**

This study shows that non-canonical TGFβ signals play multiple roles in axonal development. Babo-regulated axon growth is Smad-independent, but requires the type 2 receptors Wit and Put. Contrary to the canonical receptor activation model, type 2 receptors act downstream of type 1 receptors in axon growth signalling. Type 2 receptors work independently and interchangeably, requiring LIMK1-dependent (Wit) and -independent (Put) signals. The experiments show that TGFβ signals act through Rhò1, Rac and LIMK1, in part by regulating cofilin. Finally, analysis of different neurons demonstrated that Babo signals do not simply restrict axon extension, but also promote axon extension and axon targeting.

**Role of Smad-independent signals in neural connectivity**

Once growing axons reach the correct postsynaptic target, axon outgrowth terminates and synaptogenesis begins. These studies suggest that TGFβ signals play a role. When Babo is inactivated, MB axon growth does not terminate properly and overextends across the midline. Consistent with this, CA Babo expression results in precocious termination, forming axon truncations. How Babo is spatially and temporally regulated remains to be determined. Analogous to the Drosophila NMJ, MB axon growth might be terminated through retrograde signalling. Target-derived TGFβ ligands could signal to Babo (on MB axon growth cones) and stop axons growing further. In an alternative scenario, TGFβ ligands might act as a positional cue that prevents MB axons from crossing the midline. Recent data have shown that Babo acting through Smad2 restricts individual R7 photoreceptor axons to single termini (Ting et al., 2007). Loss of Babo, Smad2, or the nuclear import regulator Importin α3 (Karyopherin α3 – FlyBase), results in R7 mutant axons invading neighbouring R7 terminal zones. With the phenotype described here, Babo could similarly be restricting MB axons to appropriate termination zones, its loss resulting in inappropriate terminations on the contralateral side.

In contrast to MB neurons, Babo inactivation in AL and OL neurons resulted in axon extension and targeting defects. This might reflect cell-intrinsic differences in the response in different neurons to a common Babo signalling programme. This may be the case for MB axon pruning and DC axon extension, which require Babo/Smad2 signals (Zheng et al., 2006). Whether these differences derive from cell-intrinsic properties, or from Babo signal transduction, they underline the importance of Smad-independent signals in many aspects of axonal development.

**Role of Rho GTPases in TGFβ signalling**

The results suggest that Smad-independent signals involve Rho GTPases. One caveat in genetic interaction experiments is that the loss of any given gene might not be dosage-sensitive with a particular assay. Nevertheless, all the manipulations together suggest that Babo-regulated axon growth requires Rhò1, Rac and LIMK1. How Babo signals involve Rho GTPases remains to be fully determined. In addition to LIMK1, which binds to Wit, one possibility, as demonstrated for many axon guidance receptors (Luo, 2002), is that the RhoGEFs, RhoGAPs and Rho proteins might be linked to the Babo receptor complex. Thus, ligand-mediated changes in receptor properties would lead to spatiotemporal changes in Rho GTPase and LIMK1 activities.

The data suggest that a RhoGEF2/Rhò1/Rok/LIMK1 pathway mediates Babo responses (Fig. 7E). Whether Rac activators are required is unclear, as tested RacGEFs do not genetically interact with Babo. In this respect, rather than through GEFs, Babo might regulate Rac through GAPs, by inhibiting Tum activity (Fig. 7E).

Do mutations in Rhò1 and Rac components phenocopy babo phenotypes? β lobe overextensions are observed in Rok (Billuart et al., 2001), Rhò1 and Rac mutant neurons (unpublished observations). In MB neurons, Rac GTPases also control axon outgrowth, guidance and branching (Ng et al., 2002). Rhò1 also has additional roles in MB neurons (Billuart et al., 2001). Although Rhò1 mutant neuroblasts have cell proliferation defects, single-cell αβ clones do not show β lobe extensions (unpublished observations). RhoGEF2 strong loss-of-function clones do not exhibit axon overextension (unpublished observations). As there are 23 RhoGEFs in the Drosophila genome (Adams et al., 2000; Hu et al., 2005), there might well be redundancy in the way Rhò1...
is activated. LIMK1 inactivation in MB neurons was reported previously (Ng and Luo, 2004). Axon overextensions were not observed as LIMK1 loss results in axon outgrowth and misguidance phenotypes. This suggests that LIMK1 mediates multiple axon guidance signals, of which TGFβ is a subset in MB morphogenesis.

**Role of LIMK1 and coflin phosphoregulation in Babo signalling**

Although their phenotypes are similar, several lines of evidence indicate that CA Babo does not simply reflect LIMK1 misregulation in MB neurons. First, whereas LIMK1 genetically interacts with most Rho family members and many Rho regulators (Ng and Luo, 2004), CA babo is dosage-sensitive only to Rho1 and Rac and specific Rho regulators (this study), suggesting that Babo regulates LIMK1 only through a subset of Rho signals.

Second, the LIMK1 misexpression phenotype is suppressed by expression of wild-type coflin (Tsr), S3A Tsr, or the coflin phosphatase Ssh (Ng and Luo, 2004). By contrast, only wild-type Tsr, but not S3A Tsr or Ssh (Fig. 5B; unpublished observations), suppresses CA Babo. The suppression by wild-type Tsr might reflect a restoration of the endogenous balance or spatial distribution of cofilin-on (unphosphorylated) and -off (phosphorylated) states within neurons. Indeed, optimal axon outgrowth requires coflin to undergo cycles of phosphorylation and dephosphorylation (Meberg and Bamburg, 2000; Ng and Luo, 2004). As S3A forms of coflin cannot be inactivated and recycled from actin-bound complexes, wild-type coflin is more potent in actin cytoskeletal regulation.

CA Babo might not simply misregulate LIMK1 but also additional coflin regulators. Recent data suggest that extracellular cues (including mammalian BMPs) can regulate coflin through Ssh phosphatase (Endo et al., 2007; Nishita et al., 2005; Wen et al., 2007) and phospholipase Cγ activities (Mouneimne et al., 2006; van Rheenen et al., 2007). In different cell types, coflin phosphorylation and phospholipid binding (which also inhibits coflin activity) states vary and potently affect cell motility and cytoskeletal regulation. Whether a combination of LIMK1, Ssh and phospholipid regulation affects coflin-dependent axon growth remains to be determined.

Third, by phalloidin staining, LIMK1, but not CA Babo, misexpression results in a dramatic increase in F-actin in MB neurons (see Fig. S6 in the supplementary material). Thus, CA Babo does not itself lead to actin misregulation. Fourth, Babo also regulates axon growth independently of LIMK1 (see below).

**Role of Babo, Wit and Put in neuronal morphogenesis**

This study differs significantly from the canonical model of Smad signalling (Feng and Derynck, 2005; Shi and Massague, 2003), in which type 1 receptors function downstream of the ligand-type 2 receptor complex (Wieser et al., 1995). In this study, the gain- and loss-of-function results suggest that type 2 receptors act downstream of type 1 signals. As ectopic Wit and Put only suppress babo axon overextension phenotypes, this implies that Smad-dependent and -independent signals have distinct type 1/type 2 receptor interactions. How these interactions propagate Smad-independent signals remains to be fully determined. Babo could act as a ligand-binding co-receptor with Wit and Put. In addition, Babo kinase activity could regulate type 2 receptor or Rho functions. The results suggest, however, that provided that Wit or Put signals are sufficiently high, Babo is not required. Whatever the mechanism(s), it is likely that Babo requires the Wit C-terminus-LIMK1 interaction to relay coflin phosphoregulatory signals (Fig. 7E). How Put functions is unclear. As the put135 allele (used in this study) carries a missense mutation within the kinase domain, this suggests that kinase activity is essential. put does not genetically interact with LIMK1. As Put lacks the C-terminal extension of Wit that is necessary for LIMK1 binding, this suggests that Put acts
independently of LIMK1. One potential effector is Rac, which, in the context of Babo signalling, also appears to be Pak1- and thus LIMK1-independent (Fig. 7E).

In MB neurons, Wit and Put can function interchangeably. In other in vivo paradigms, type 2 receptors are not interchangeable (Marques et al., 2002). However, as the Wit C-terminal tail is required to substitute for Put, this suggests that Wit axon growth signals are independent of its kinase activity. Together, this suggests that Smad-independent signals involve LIMK1-dependent and -independent mechanisms.

Distinct roles of Babo in neuronal morphogenesis

This study, together with Zheng et al. (Zheng et al., 2003), shows that Babo mediates two distinct responses in related MB populations. How do MB neurons choose between axon pruning and axon growth? The babo rescue studies suggest that whereas Baboa or Babob elicits Smad-independent responses, only Baboa mediates Smad-dependent responses. As Babo isoforms differ only in the extracellular domain, differences in ligand binding could determine Smad2 or Rho GTPase activation. However, it is worth noting that in DC neurons, either isoform mediates axon extension through Smad2 and Medea (Zheng et al., 2006). In addition, although expressed in all MB neurons, CAbabo misexpression (which confers ligand-independent signals) perturbs only αβ axons (Fig. 4A, A/H11032 and see Fig. S2 in the supplementary material). Thus, cell-intrinsic properties might also be essential in determining Babo responses.

Many TGFβ ligands signal through Babo (Gesualdi and Haerry, 2007; Lee-Hoeflich et al., 2005; Parker et al., 2006; Serpe and O’Connor, 2006; Zheng et al., 2003; Zhu et al., 2008). For example, Dawdle, an Activin-related ligand, patterns Drosophila motor axons (Parker et al., 2006; Serpe and O’Connor, 2006), whereas Activin (Activin-β, FlyBase) is required for MB axon pruning (Zheng et al., 2003). Whether these ligands regulate Babo MB, AL and OL axonal morphogenesis is unclear. Taken together, the evidence suggests that Babo signalling is varied in vivo and is involved in many aspects of neuronal development.

Smad-independent signals in cytoskeletal regulation and cell morphogenesis

TGFβ signals are responsible for many aspects of development and disease and, throughout different models, Smad pathways are closely involved. Although Smad-independent pathways are known, their mechanisms and roles in vivo are unclear. TGFβ signals often drive cell shape changes in vivo. During epithelial-to-mesenchymal transition (EMT), cells lose their epithelial structure and adopt a fibroblast-like structure that is essential for cell migration during development and tumour invasion (Grumet et al., 2003; Shook and Keller, 2003). TGFβ-mediated changes in the actin cytoskeleton and adherens junctions are necessary for EMT. Although Smads are crucial, TGFβ signals also involve the Cdc42-Par6 complex, resulting in cell de-adhesion and F-actin breakdown through Rho1 degradation.
(Ozdamar et al., 2005). In other studies, however, TGFβ-mediated EMT has been shown to require Rho1 (Bhowmick et al., 2001), which can be regulated by Smad activity (Levy and Hill, 2005).

Many TGFβ-driven events in Drosophila are Smad-dependent (Raftery and Sutherland, 1999). Whether Smad-independent roles exist beyond those identified in this study remains to be tested. Here, I provide a framework to understand how non-Smad signals regulate cell morphogenesis during development.

I thank the Bloomington Drosophila Stock Center and many colleagues who provided reagents for this study, especially Theo Haerry and Michael O’Connor. Uwe Drescher, Theo Haerry, Greg Jeffers, Michael O’Connor and Rosa Gonzalez-Quevedo provided comments on the manuscript. Michael Fletcher and Coralie Moore provided technical assistance, and Guy Tear helped with laboratory equipment. The Wellcome Trust (078045) supported this study.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/24/4025/DC1

References


Non-canonical TGFβ signals control axon growth


