

TGF β 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 (TGF β) signals regulate axon growth. In most contexts, TGF β signals are tightly linked to Smad transcriptional activity. Although known to exist, how Smad-independent pathways mediate TGF β responses *in vivo* is unclear. In *Drosophila* mushroom body (MB) neurons, loss of the TGF β 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 TGF β 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 Netrin (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, *twinstar* (*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 (TGF β) pathway are involved. The TGF β 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 TGF β pathway consists of a core complex of type 1 and type 2 transmembrane receptor serine/threonine kinases, which are activated by secreted TGF β ligands [bone morphogenetic proteins (BMPs) or TGF β /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 Smad2 (also known as dSmad2 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; Foletta 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 TGF β signals are Smad-independent events. In *C. elegans*, mutations in a TGF β signal (*unc-129*) result in dorsal-ventral axon guidance defects (Colavita et al., 1998). Mutation analyses of other TGF β

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components, such as receptors or Smads, do not reveal this phenotype, suggesting that axon guidance in worms involves atypical TGF β signalling mechanisms. TGF β 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 TGF β 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 TGF β 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*, *RhoGAPp190*, *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*³², *babo*⁵², *UAS-activated babo*_a Q302D (*CA babo*) (Brummel et al., 1999); *tkv*⁴, *tkv*⁷ (Penton et al., 1994); *tkv*^{4a21} (Gibson and Perrimon, 2005); *UAS-putΔI*, *UAS-tkvΔGSK* (*DN tkv*), *UAS-saxΔI* (*DN sax*), *UAS-tkv1A* (HA) Q199D (*CA tkv*), *UAS-saxA* (HA) Q263D (*CA sax*) (Haerry et al., 1998); *sax*⁴ (Singer et al., 1997); *sax*^P, *UAS-put* (Nellen et al., 1994); *UAS-babo-a*, *UAS-babo-b::Flag*, *UAS-baboΔI* (*DN babo*) (Zheng et al., 2006) (a gift from M. O'Connor, HHMI/University of Minnesota, Minneapolis and Theo Haerry, Florida Atlantic University, Boca Raton); *wit*^{A12}, *wit*^{B11}, *wit*^{G15}, *P{wit genomic}* (*P{wit⁺}*), *P{wit tailless}* (*P{witΔC}*), *UAS-wit*, *UAS-witΔC* (Marques et al., 2002); *UAS-witΔI* (McCabe et al.,

2003); *put*¹³⁵, *UAS-put* (Ruberte et al., 1995); *put*⁶² (Simin et al., 1998); *Mad*¹² (Sekelsky et al., 1995); *Med*¹³ (Hudson et al., 1998); *Smad2*¹ (Zheng et al., 2003); *UAS-Dad* (Tsuneizumi et al., 1997); *UAS-MYC::tum* (*RacGAP50C*) (Goldstein et al., 2005); *UAS-EcR-B1* (Lee et al., 2000); *Df(1)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

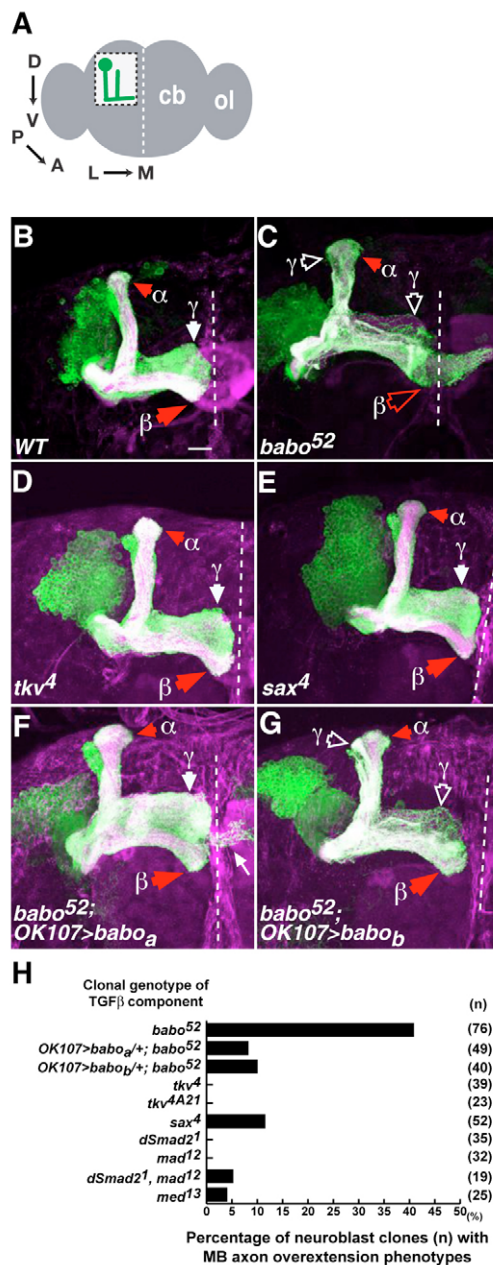
Loss-of-function clones were generated using the MARCM method (Lee and Luo, 1999). Neuroblast and single-cell $\alpha\beta$ clones were generated as previously described (Ng et al., 2002). Neurons were visualised using the *Gal4-OK107* driver expressing *UAS-mCD8::GFP*. Misexpression studies were performed 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, 7 and 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 (Caltag, 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 WitΔC-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 , $\alpha'\beta'$ and $\alpha\beta$), 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 $\alpha'\beta'$ or $\alpha\beta$ 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 TGF β 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*⁵²) neuroblast clones had axon overextension phenotypes in $\alpha\beta$ neurons, with 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



projections in adult brains (open white arrowheads in Fig. 1C). In wild-type adults, each γ neuron re-extends a single medial branch after axon pruning and the γ lobe appears more defasciculated along the dorsal-ventral axis (Fig. 1B). By mutant clonal analysis or by dominant-negative (DN) misexpression, loss of *tkv* or *sax* did not result in these defects (Fig. 1D,E,H; data not shown). These results suggest that Babo regulates axon growth, particularly of the β lobe.

Babo α and Babo β isoforms regulate axon growth cell-autonomously

Recent data suggest that different Babo isoforms have distinct neural functions (Zheng et al., 2006). Expression of the Babo α , but not Babo β , 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

Fig. 1. Babo inactivation results in axon overextension. Babo regulates axon growth through Babo α and Babo β 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 anteroventrally 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 *babo*⁵² (C), *tkv*⁴ (D) and *sax*⁴ (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 *babo*⁵² neuroblast clones expressing either *UAS-babo α* (F), or *UAS-babo β* (G). Many of the axons in the *UAS-babo α* 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 α* 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 γ (weakly stained) and $\alpha\beta$ (strongly stained) axons (appearing white when overlapping with mCD8::GFP). Dashed white line, midline. Scale bar: 20 μ m. (H) Quantification of axon overextension defects in the indicated genotypes. n, number of neuroblast clones examined.

were performed. In a wild-type background, ectopically expressed Babo α or Babo β was detected in all MB lobes and did not disrupt axonal projections (see Fig. S2 in the supplementary material). Babo α or Babo β expression in *babo*⁵² neuroblast clones rescued the axon overextension defect, as most β lobes terminated correctly (Fig. 1F-H). Thus, either Babo isoform can regulate axon growth.

Consistent with a cell-autonomous role, Babo inactivation in single $\alpha\beta$ 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).

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-babo β* expression rescued *babo*⁵² axon overextension but not the axon pruning defects (Fig. 1G). Similarly, RhoGEF2 coexpression also suppressed DN

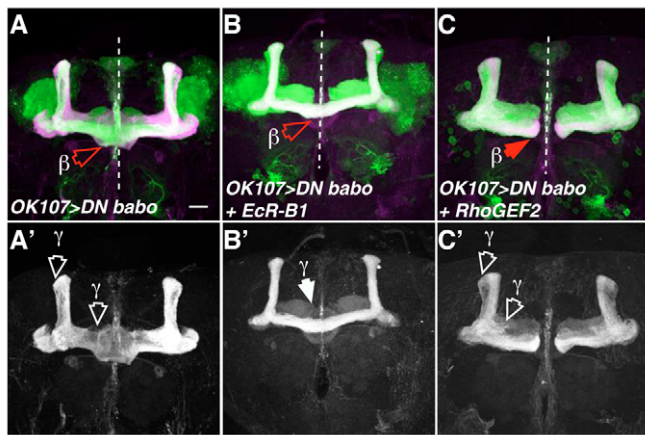


Fig. 2. Babo regulates axon pruning and axon growth independently. *Drosophila* MB neurons misexpressing *DN babo* (A), *DN babo* plus *Ecr-B1* (B), or *DN babo* plus *RhoGEF2* (C). Additional images (A', B', C') indicate the corresponding Fas2-positive (magenta in A, B, C) axon projections. Note the β lobe overextensions (open red arrowheads) present in A and B, but absent in C, and the axon pruning phenotypes (open white arrowheads highlight the aberrant γ -dorsal and medial branches), which are visible in A' and C', but absent in B'. Cell body sections were removed from C to clearly show MB axons. Scale bar: 20 μ m.

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* *Mad* 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 [classified 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). Nevertheless, these axon projections resembled those of the wild type (*CA tkv*, 100% as wild-type, $n=26$ hemispheres; *CA sax*, 92.1% as wild-type, $n=38$ hemispheres; Fig. 4B, C). These results again suggest that Babo, but not Tkv or Sax, regulates axon growth in vivo.

To determine whether the truncation phenotypes reflect an initial failure of axon extension, as opposed to axons failing to stabilise and subsequently retracting, *CA babo*-misexpressing animals were developmentally staged and analysed from wandering L3 larvae (data not shown) through to puparium formation. The results suggest that CA Babo resulted in early extension defects in developing axons (see Fig. S3 in the supplementary material).

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 $\alpha\beta$ 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 TGF β 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, *Rok*, also suppressed CA Babo.

When other Rho family members, *Cdc42* and *Rac* (*Rac1*, *Rac2* or *Mtl*), were tested, loss of *Rac1* (using the hypomorphic allele *J10*), or a combined loss of one copy of *Rac2* and *Mtl* (using null Δ

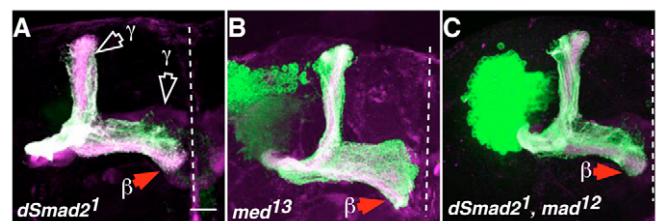


Fig. 3. Babo-regulated axon growth is Smad-independent. *Drosophila* *Smad2*¹ (A), *Med*¹³ (B) and double *Smad2*¹ *Mad*¹² (C) neuroblast clones do not show β lobe overextensions. Scale bar: 20 μ m.

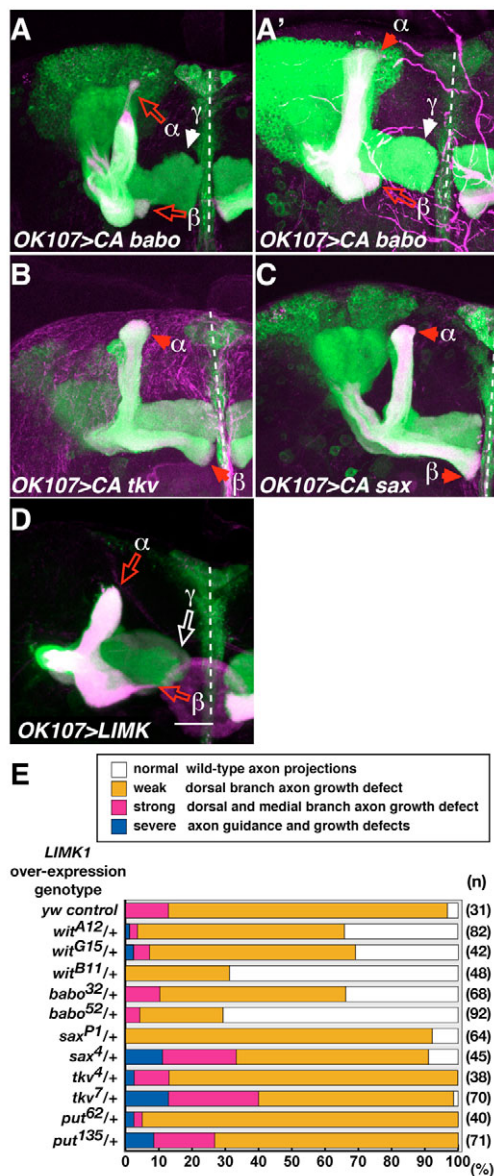


Fig. 4. CA *babo* misexpression results in MB axon truncation. *babo* and *wit* genetically interact with *LIMK1*. (A–D) *Drosophila* MB neurons misexpressing CA *babo* (A,A'), CA *tkv* (B), CA *sax* (C) or *LIMK1* (D). Solid red or white arrowheads indicate normal α and β or γ lobe termination points, as indicated. Open red or white arrows indicate axon truncations in α and β lobes, or in γ lobes, respectively. In D, the cell body section was removed to clearly reveal axon phenotypes. Scale bar: 20 μ m. (E) Quantification of axon growth defects in *LIMK1*-overexpressing neurons (using intermediate expression line F4) in the presence of control (y, w), or one copy of each TGF β receptor mutant. Phenotypic quantifications were carried out as described previously (Ng and Luo, 2004), and are briefly summarised in the key. n, number of hemispheres examined.

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 *RhoGEF2*, 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 RhoGEF2. Whether Babo regulates axon growth via RacGEFs 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 RhoGEFs (RhoGEF2) 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 RhoGEF2 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, β lobe overextensions were 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 TGF β 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{wit Δ C}*), 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-put Δ I*) 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-wit Δ C* (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 TGF β 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 TGF β 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-wit Δ I*), 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-put Δ I* 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

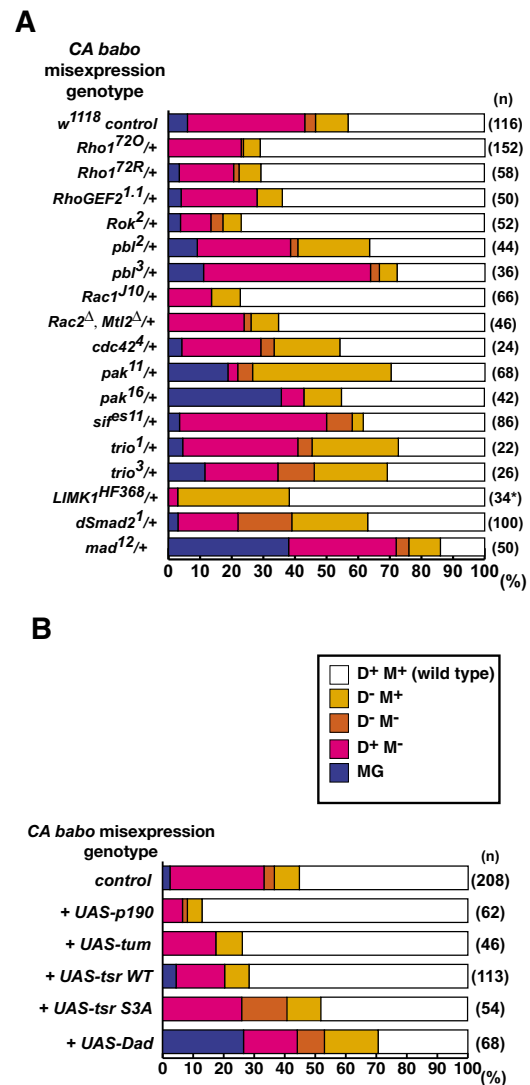


Fig. 5 CA babo genetically interacts with the components of the Rho GTPase pathway. (A) Quantification of CA Babo defects in the presence of control (*w¹¹¹⁸*) or one mutant copy of Rho or Smad, as indicated. CA Babo phenotypes were classed according to the loss or truncation of dorsal (D⁻M⁺), medial (D⁺M⁻) or both (D⁻M⁻) lobes. Axon fasciculation defects were also observed (classed as misguidance, MG; see Fig. S1 in the supplementary material). Based on the level of Babo expression (see Materials and methods), misguidance represents the strongest phenotype and loss of dorsal lobes the weakest phenotype (MG > D⁺M⁻ > D⁻M⁺ > D⁺M⁺). The asterisk denotes CA Babo-induced β lobe overextension upon the loss of one copy of *LIMK1*. (B) Quantification of CA Babo defects in control (*UAS-mCD8::GFP*), or with one copy of the indicated transgene. n, number of hemispheres examined.

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

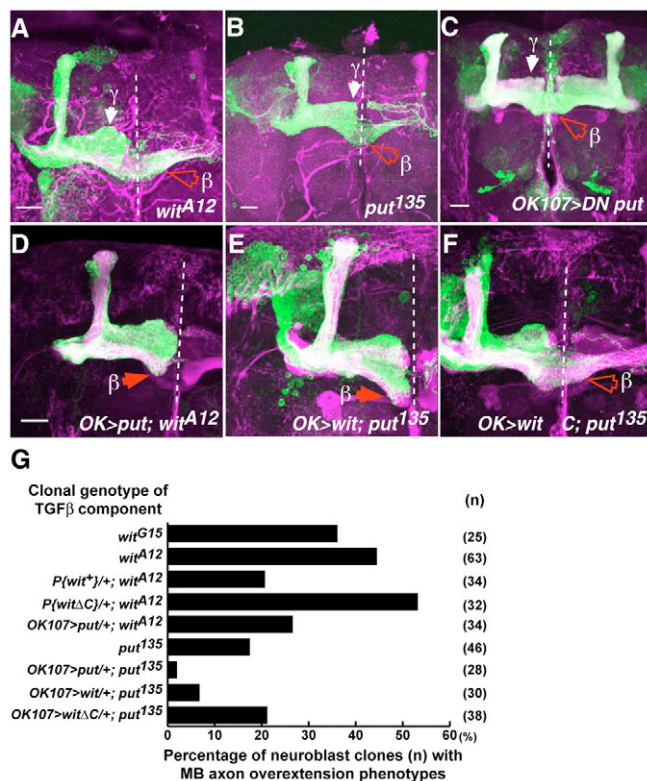


Fig. 6. The type 2 receptors Wit and Put regulate axon growth and can function interchangeably. (A,B) *wit^{A12}* (A) and *put¹³⁵* (B) *Drosophila* neuroblast clones show β lobe overextensions (open red arrowheads). (C) DN *put*-expressing neurons show similar overextensions. (D–F) *wit* clones expressing *UAS-put* (D), or *put* clones expressing either *UAS-wit* (E) or *UAS-wit ΔC* (F). (G) Quantification of these defects. n, number of neuroblast clones examined. Scale bars: 20 μ m.

misprojections were observed. These results suggest that Babo regulates axon extension and targeting in AL neurons, but only axon targeting in OL neurons.

The relevance of Smads in AL and OL axonal development was also determined. *Smad2* (Fig. 8D,H,J and see Fig. S5 in the supplementary material), *Med* (Fig. 8E,I,J) or *Mad* (data not shown; Fig. 8J) mutant clones did not reveal any gross AL or OL axon defects, although gaps similar to those observed for *babo* 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 TGF β 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 TGF β signals act through Rho1, 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 TGF β 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 TGF β ligands could signal to Babo (on MB axon growth cones) and stop axons growing further. In an alternative scenario, TGF β 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 $\alpha 3$ (Karyopherin $\alpha 3$ – 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 TGF β 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 Rho1, 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/Rho1/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 Rho1 and Rac components phenocopy *babo* phenotypes? β lobe overextensions are observed in *Rok* (Billuart et al., 2001), *Rho1* and *Rac* mutant neurons (unpublished observations). In MB neurons, Rac GTPases also control axon outgrowth, guidance and branching (Ng et al., 2002). Rho1 also has additional roles in MB neurons (Billuart et al., 2001). Although *Rho1* mutant neuroblasts have cell proliferation defects, single-cell $\alpha\beta$ clones do 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 Rho1

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 TGF β is a subset in MB morphogenesis.

Role of LIMK1 and cofilin 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 cofilin (Tsr), S3A Tsr, or the cofilin 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 cofilin to undergo cycles of phosphorylation and dephosphorylation (Meberg and Bamberg, 2000; Ng and Luo, 2004). As S3A forms of cofilin cannot be inactivated and recycled from actin-bound complexes, wild-type cofilin is more potent in actin cytoskeletal regulation.

CA Babo might not simply misregulate LIMK1 but also additional cofilin regulators. Recent data suggest that extracellular cues (including mammalian BMPs) can regulate cofilin through Ssh phosphatase (Endo et al., 2007; Nishita et al., 2005; Wen et al., 2007) and phospholipase C γ activities (Mouneimne et al., 2006; van Rheen et al., 2007). In different cell types, cofilin phosphorylation and phospholipid binding (which also inhibits cofilin activity) states vary and potentially affect cell motility and cytoskeletal regulation. Whether a combination of LIMK1, Ssh and phospholipid regulation affects cofilin-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 in 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 the *babo* axon overextension phenotype, 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),

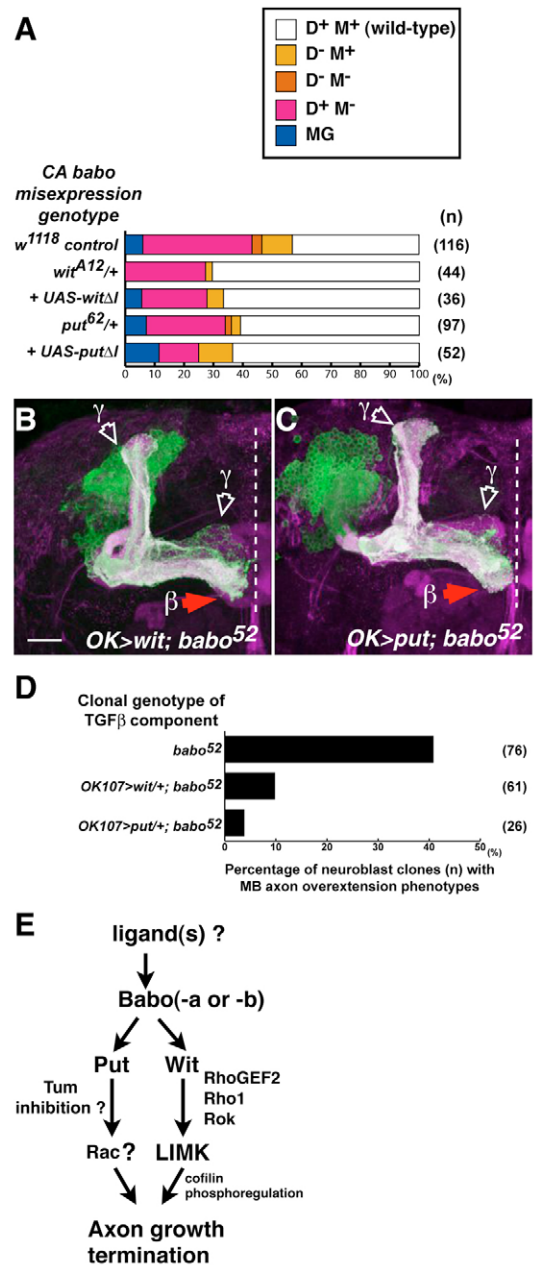


Fig. 7. Wit and Put act downstream of Babo. (A) Quantification of CA Babo defects in control (*w¹¹¹⁸*), or with one mutant copy of *wit*, *put*, *UAS-wit Δ I* or *UAS-put Δ I*, as indicated. n, number of hemispheres examined. (B, C) *babo*-null clones expressing either *UAS-wit* (B) or *UAS-put* (C). Wit or Put expression suppresses the *babo* axon overextension but not the axon pruning phenotype. Scale bar: 20 μ m. (D) Quantification of *babo* axon growth phenotypes in the presence of one copy of *UAS-wit*, or *UAS-put*, as indicated. (E) A model of Babo-regulated axon growth derived from data in this study.

it is likely that Babo requires the Wit C-terminus-LIMK1 interaction to relay cofilin 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

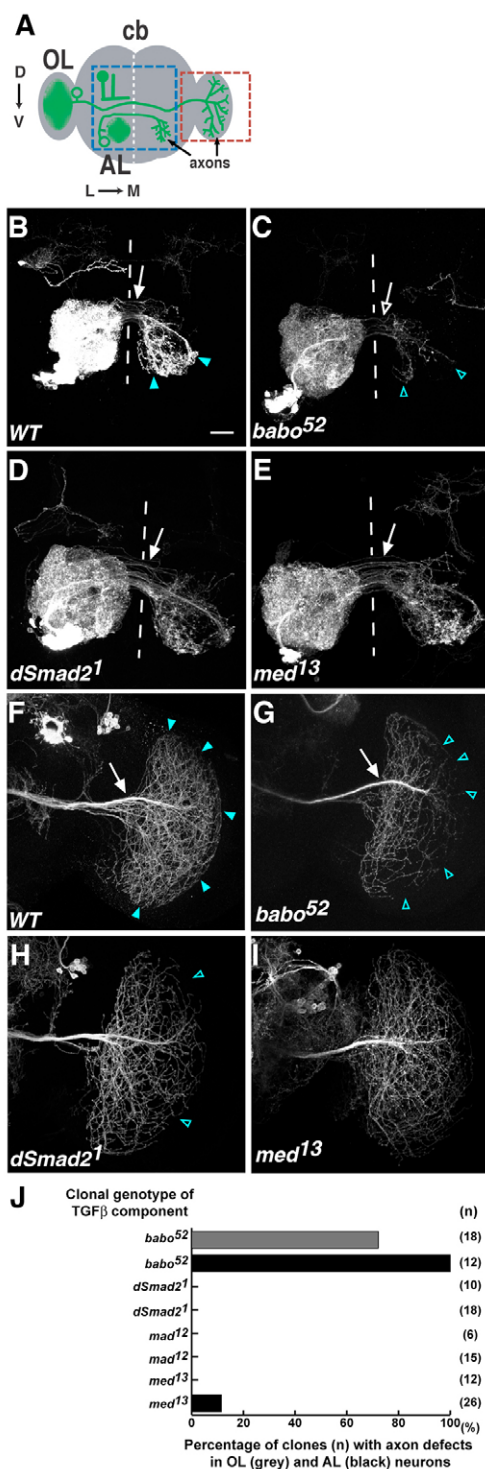


Fig. 8. Babo regulates extension and targeting of AL and OL axons independently of Smads. (A) Schematic of the adult

Drosophila brain. Shown from the left hemisphere, antennal lobe (AL) contralateral projection neurons elaborate dendrites (green) ipsilaterally to one AL, but project axons contralaterally to the opposite AL. The blue boxed region indicates the location of all represented AL images. Also, from the left hemisphere, optic lobe (OL) contralateral projection neurons elaborate dendrites (green) ipsilaterally to one OL, but project axons contralaterally to the opposite OL. The red boxed region indicates the orientation of all represented OL axons. Green circles indicate cell bodies. (B-I) Wild-type (B,F), *babo52* (C,G), *Smad21* (D,H) and *Med13* (E,I) AL (B-E) and OL (F-I) contralateral projecting neurons. White arrows indicate a wild-type number of axons that reach the target zone. Open white arrows indicate axon extension defects. Blue arrowheads indicate wild-type axon termination zones. Open blue arrowheads indicate targeting defects ('gaps'). Scale bar: 20 μ m. See also Fig. S5 in the supplementary material. (J) Quantification of these OL (grey bars) and AL (black bars) phenotypes. n, number of clones analysed.

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 *Babo_a* or *Babo_b* elicits Smad-independent responses, only *Babo_a* 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, *CA babo* misexpression (which confers ligand-independent signals) perturbs only $\alpha\beta$ axons (Fig. 4A,A' and see Fig. S2 in the supplementary material). Thus, cell-intrinsic properties might also be essential in determining Babo responses.

Many TGF β 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

TGF β 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. TGF β 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 (Grunert et al., 2003; Shook and Keller, 2003). TGF β -mediated changes in the actin cytoskeleton and adherens junctions are necessary for EMT. Although Smads are crucial, TGF β signals also involve the Cdc42-Par6 complex, resulting in cell de-adhesion and F-actin breakdown through Rho1 degradation

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.

(Ozdamar et al., 2005). In other studies, however, TGF β -mediated EMT has been shown to require Rho1 (Bhowmick et al., 2001), which can be regulated by Smad activity (Levy and Hill, 2005).

Many TGF β -driven events in *Drosophila* are Smad-dependent (Rafferty 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.

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Supplementary material

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

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