Drosophila melanogaster Hedgehog cooperates with Frazzled to guide axons through a non-canonical signalling pathway

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Runninghead: Hh and Fra in axonal guidance

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Highlights:

- We analysed the effects of Hh in midline axon guidance in *Drosophila melanogaster*
- We show that Hh is expressed in the ventral nerve cord of *Drosophila melanogaster* embryos
- Overexpression of *hh* in the midline causes ectopic midline crossing of FasII-positive axonal tracts
- A non-canonical Hh signaling pathway affects axonal guidance by cooperating with Netrin/Frazzled chemoattraction

Abstract

We report that the morphogen Hedgehog (Hh) is an axonal chemoattractant in the midline of *D. melanogaster* embryos. Hh is present in the ventral nerve cord during axonal guidance and overexpression of *hh* in the midline causes ectopic midline crossing of FasII-positive axonal tracts. In addition, we show that Hh influences axonal guidance via a non-canonical signalling pathway dependent on Ptc. Our results reveal that the Hh pathway cooperates with the Netrin/Frazzled pathway to guide axons through the midline in invertebrates.

1. Introduction

The ventral nerve cord (VNC) of *Drosophila melanogaster* is a well-known model system wherein to study axon guidance. The fly midline is similar to the vertebrate floor plate, in that it plays an essential part in cell fate determination in the central nervous system (CNS) and an important later role in axonal pathfinding. During development, commissural neurons send axons that project toward and, subsequently, across the midline, which acts as an intermediate target and influences axonal trajectories by expressing attractive and repulsive cues (Evans and Bashaw, 2010).

In the last few years, significant advances have been made in the identification of the ligands and receptors that dictate the trajectory taken by an individual axon, both in vertebrates and invertebrates. More recently, the repertoire of potential guidance factors has been expanded to include morphogens (reviewed in (Schnorrer and Dickson, 2004) (Charron and Tessier-Lavigne, 2005)). To date, the morphogens shown to have important and evolutionarily conserved roles in axon guidance in vertebrates include members of the Bone Morphogenetic Protein (BMP), Hedgehog (Hh), Wnt and Fibroblast Growth Factor (FGF) families (Charron and Tessier-Lavigne, 2005; Sánchez-Camacho and Bovolenta, 2009). So far, there is no evidence that Hh guides commissural axons in *D. melanogaster*.

In vertebrates, Sonic Hedgehog (Shh) secreted from the floor plate has multiple roles in nervous system development, from neuronal cell fate specification to axonal guidance. In addition to the Netrin1/DCC signalling system, the growth of spinal cord commissural axons

towards the midline requires floor plate-derived Shh (Charron et al., 2003). According to these authors, this morphogen acts through a Smo-dependent signalling mechanism, which regulates 14-3-3 protein levels and leads directly to axonal movement (Charron et al., 2003; Yam et al., 2012; Yam et al., 2009). However, the co-occurrence of transcription-dependent and -independent mechanisms involving canonical and alternative components has been reported to be involved in mediating Shh functions in axonal guidance (Sánchez-Camacho and Bovolenta, 2009{Parra, 2010 #1200; Yam et al., 2009). In addition, the intracellular signalling events that take place in Hh-mediated axonal guidance are mostly unknown. Thus, further work and distinct animal models are required to define the precise mechanism for each response. To this end, due its characteristics and well-characterised midline, the best model organism to molecularly and genetically dissect and clarify the involvement of Hh in axonal pathfinding is *D. melanogaster*.

Here, we show that *hh* is expressed at low levels and, most likely, secreted by cells in the VNC during commissural axon guidance, and that its receptors, Ptc and Smo, localise to axons in the VNC. Most importantly, we demonstrate that Hh helps to attract commissural axons *in vivo*. In the light of our results, we propose that Hh is a midline chemoattractant in *D. melanogaster* acting via its receptor Ptc in a non-canonical pathway. In addition, we provide evidence that Hh participates in midline axonal guidance by cooperating with the Netrin/Frazzled pathway and suggest it does so by activating Src42A.

2. Results and Discussion

2.1. *hh* mutants show axonal guidance phenotypes at the midline

Drosophila embryos mutant for members of the Hh pathway have been reported to have strong axonal guidance phenotypes that have mainly been attributed to mis-specification and neuronal loss (Merianda et al., 2005; Patel et al., 1989). To clarify the involvement of Hh in midline axon guidance in *D. melanogaster*, we examined late neurodevelopment and axonal guidance phenotypes of *hh* mutant embryos (Fig. 1). We observed that in addition to their strong patterning defects, *hh* mutants showed CNS axonal disruptions. Commissures and connectives were disorganised and in many segments commissures were fused and the longitudinal fibres were thinner. In addition, commissures were missing in many segments and FasII-positive axonal bundles rarely crossed the midline (Fig.1B and F). Given the pleiotropy of these mutants, and to overcome the effects of Hh during early neurodevelopment, we inactivated this morphogen in late developmental stages (see Materials and methods) using a temperature-sensitive (ts) allele (Hummel et al., 2002). We inactivated *hh* at approximately 10h (st13) and 11:30h (st15) after egg laying (AEL). As expected, inactivation at stage 15 generated only mild (31%, n=48) or no defects in the embryonic CNS (Fig.1D, H). We observed a slight thickening of the connectives accompanied by thinning of some commissures (Fig.1D) and guidance defects on the medial, FasII-m, fascicle (Fig.1H, arrowhead). Inactivation at stage 13 induced stronger phenotypes with guidance defects mainly at the commissural level (Fig. 1 C and G, arrows). To control for possible neuronal fate changes, we analysed neuronal differentiation in these embryos (Fig. 4 J and M) and concluded that there were no apparent changes in fate. These results suggest a role for Hh during guidance stages and are in accordance with previous results showing that when *ptc* mutants are rescued for their early neurogenesis defects, they continue to display axonal guidance phenotypes (Merianda et al., 2005).

2.2. Hh is expressed by a subset of VNC glial cells

Hh is a secreted segmentation protein. During embryogenesis, its expression in epidermal cells is restricted to the posterior compartment of each segment. In addition, it is also expressed in the midline of the CNS, where developmental compartments apparently do not form (Bossing and Brand, 2006; Tabata et al., 1992; Watson et al., 2011). In order to determine which CNS cells express *hh* at later stages of embryonic development, we started by analysing its expression in stage 15 embryos by means of a *hh-lacZ* reporter (Fig.2). *hh* expression was detected in the middle of the VNC and at the level of both commissural and connective fibers in the CNS, at early and at late stages (Fig. 2A-C). At embryonic stage 13, *hh* was detected in cells located at the posterior side of the posterior commissure and in those surrounding the longitudinal connectives. Most importantly, we detected *hh* expression in some cells adjacent to the midline (Fig. 2A-C, arrows and arrowheads).

The nuclei of most glial cells in the CNS can be visualised using an antibody against the glial marker protein Reversed polarity (Repo) (Halter et al., 1995). Using this antibody we observed that some of the cells expressing *hh* in the VNC were glial cells (Fig.2D' arrowheads). However, *hh* was not expressed in midline glia as evaluated by analysis of co-localization of both Hh and Sim or Wrapper proteins (Fig.2C''), but in Repo-positive glial cells adjacent to the midline (Fig.2D' arrowheads). We confirmed these results expressing UAS-GFP using a HhGAL4 (Fig.2E). In addition, when we analysed the presence of *hh* mRNA at embryonic stages 11 and 13, we could detect the presence of this morphogen, albeit faintly, in the CNS of embryos at these stages (Fig.2 F, arrow and arrowheads). This indicates that Hh is expressed by some glial cells at the VNC as late as stage 13 of embryonic development.

Ptc is the canonical receptor for Hh, and its expression is an indication that a cell can respond to Hh signalling (Chen and Struhl, 1996). Therefore, if Hh is expressed in the CNS and has a role in axonal guidance, Ptc should be present in axons to relay the chemoattractant or chemorepellent signal intracellularly. Ptc expression has been detected in many cells of the CNS (Hooper and Scott, 1989; Watson et al., 2011); however, we specifically checked for the presence of Ptc in axons using a monoclonal antibody against

the first extracellular domain of this receptor, which in normal conditions detects Ptc mainly in early endocytic vesicles (Torroja et al., 2004). To mark the midline of the CNS we used a btlGAL4::NLSredstinger stock where all Btl positive cells are marked by means of a fluorescence nuclear marker. Ptc protein was detected in axons of the CNS both at early and late stages of CNS development (Fig.2 G-H). We also observed the presence of Smo in axons (Fig.2J). Taken together, these results confirm the presence of both Hh, Ptc and Smo in the CNS during embryonic stages of axonal guidance.

2.3. The morphogen Hh is an attractant for CNS axons

Due to the pleiotropy of *hh* mutants, which is unavoidable even when using temperature-sensitive mutations, misexpression experiments provide a more direct test of the capacity of *hh* to influence axonal guidance, separately from its patterning effects. Overexpression of *hh* in the CNS using general drivers like *scabrous* (*sca*) or *embryonic lethal abnormal vision* (*elav*) induces many strong neuronal defects ((Bossing and Brand, 2006) and data not shown). Therefore, we used *single-minded* GAL4 (*simGAL4*) to express *hh* only in CNS midline cells (Nambu et al., 1990) and checked for guidance defects using BP102 and anti-FasII antibodies (Fig.3A-D). These experiments showed that overexpression of *hh* in the midline causes midline crossing in FasII fibers (Fig.3 B,D). More specifically, the medial FasII (FasII-m) positive fascicle, which does not normally cross the midline, showed an average of 4-7 crosses per embryo (n=40) in comparison to none in the wt (n=28); the intermediate and lateral fascicles did not show significant crosses between them (Fig. 3 D,E). When axons were marked by means of BP102 antibody (Fig.3 A,B) or an antibody against HRP (Fig. 4B, D), midline crossing defects were either very mild (Fig.3B) or undetectable (50%, n=30), suggesting that the effect of having excess of Hh in the midline is mostly specific to the FasII-m axons.

In many mutants that affect the differentiation and/or number of midline cells, such as *tramtrack* (*ttk*), *pointed* (*pnt*) or *single-minded* (*sim*), or in situations when there is massive CNS cell death, (Hidalgo and Brand, 1997; Hidalgo et al., 1995) both FasII- and BP102-

positive axon bundles collapse at the midline (Booth et al., 2000; Crews et al., 1988; Giesen et al., 1997; Nambu et al., 1990). Therefore, our results suggest that overexpression of *hh* in the midline causes specific crossing in a subset of axons rather than a random collapse of the midline of the VNC as a result of major cell fate/number changes. In addition, the overexpression phenotype was clearly detected in FasII-m-positive axons and almost undetectable when using pan-axonal markers like BP102 and HRP. These findings suggest that the phenotypes observed are more specific of FasII-m-expressing fascicles. To confirm these results, we overexpressed *hh* using *enGAL4* which is segmentally expressed in cells of the VNC and in cells in the midline (Bossing and Brand, 2006; Nambu et al., 1990). We detected 1-4 midline crossings per embryo (n=10), confirming that excess Hh in the midline and adjacent cells induces ectopic crossing (Fig.3 I and J-J', arrow).

To better understand how excess Hh in the midline affected axonal pattern formation, we used a single-cell marker that reveals contralateral projections. In wt embryos, the SemaIIb:τmyc marker (Rajagopalan et al., 2000) is expressed in only one neuron per hemineuromere; this neuron projects its axon across the midline and the axon then follows a specific path within the longitudinal connectives, just medial to the FasII-i fascicle (Fig. 3F) (Spitzweck et al., 2010). When *hh* was overexpressed in the midline, the specification and positioning of the SemaIIb neurons was not affected and the overall axonal trajectories were unchanged. However, the arrangement of the SemaIIb:τmyc-expressing axons was shifted towards the CNS midline in all mutant embryos observed (n=8), despite the position of the SemaIIb neurons remaining unchanged (Fig.3F and G, arrows). Although the normally straight axonal projections across the midline appeared irregular in some segments (Fig. 3G, asterisk), ectopic crossings of the CNS midline were not observed for SemaIIb positive axons. These results suggest that Hh is responsible for ectopic midline crossings only in connective fascicles extending closer to the midline. In order to clarify if the FasII-m and FasII-l fascicles are closer to the midline when *hh* is overexpressed, we quantified these distances at embryonic stage 16 (approx. 14h AEL) and compared them to wt and *frazzled (fra)* mutants (Fig. 3H). Overall, *hh* overexpression in the midline induces a shift of Fas-

positive fibres towards the midline. The opposite occurs in *fra* mutant embryos (Fig. 3H and (Bhat, 2005)), suggesting that *hh* is acting through a chemoattractive pathway. In addition, in hhts embryos moved to the restrictive temperature after neuronal differentiation, the distance between FasII-m fascicles is slightly increased in relation to the wt (Fig. 3H), further suggesting and involvement of Hh in axonal attraction.

If the morphogen Hh is an attractive signal for axonal migration, its misexpression outside the CNS midline should attract axons towards the new expression sites. To address this question, we misexpressed *hh* in *apterous* (*ap*)-expressing neurons, using apGAL4. There are three *ap*-expressing interneurons per abdominal segment, located outside the lateral FasII-positive fascicle (Lundgren et al., 1995) (Fig. 3K). When *hh* was expressed in these neurons, we detected mild turning of the lateral fascicle axons towards the Hh source. In contrast to axons of the medial fascicle when *hh* is expressed in the midline, we did not detect any major misguidance events, only mild indentations of the FasII-l fascicle towards the chemoattractant source (85% of embryos, n=22, Fig.3L). In addition, we observed some defasciculation events within the other FasII fascicles (Fig.3L, arrowheads). In extreme cases (15% of embryos, n=22), we observed the occasional exit of some of these nerves towards the Hh source (Fig. 3M, arrow). Taken together, these observations suggest that axons may require an extra factor(s) in order to read the Hh chemoattractant cues and that these factor(s) are present in higher amounts in the midline than in surroundings of the CNS periphery. Taken together, these mis/overexpression results suggest that Hh acts as a chemoattractant for *D. melanogaster* CNS axons.

2.4. The effect of Hh in axons is not caused by midline cell losses or major fate changes

Although these results support the notion that excess Hh may induce midline crossing, they do not address how this morphogen exerts this effect. Instead of acting as a guidance molecule, eliciting the subsequent growth cone movement, Hh could be simply repatterning cells in the midline and inducing similar phenotypes. However, in the majority of patterning

mutants, phenotypes are strong when all axons are analysed using BP102 or HRP, which does not happen when we overexpress *hh* using *simGAL4* (see Fig.1). Nevertheless, in order to check the integrity of midline cells upon overexpression of *hh*, we labelled these embryos with antibodies directed against the Wrapper protein, which specifically labels midline glia (Noordermeer et al., 1998). In all cases observed, Wrapper-expressing cells were detected at the CNS midline (Fig. 4B). Thus, the extra midline crossing of axons in embryos with excess Hh in the midline cannot be ascribed to an absence of midline glial cells. Nonetheless, we cannot rule out the possibility that slight defects in glial positioning contribute to some aspects of this phenotype, for example, the mild defects of separation of the anterior and posterior commissures in some segments (Fig. 4B). Consistent with our results, it has been shown that *hh* expression in Sim positive cells reduces Wrapper protein levels (Watson et al., 2011). However, *wrapper* mutant embryos show very mild guidance phenotypes in the midline (Noordermeer et al., 1998). Hence, our strong axonal crossing phenotypes in simGAL4::UASHh embryos cannot be attributed to the alterations observed in Wrapper levels in some cells in the midline.

In order to obtain a generalized view of glial cell number and position at the midline, we analysed the numbers and positions of Repo positive cells in embryos expressing Hh in midline glia. Overall, no major differences in glial cell number nor in glia position were detected (Fig. 4 C,D). Interestingly, we cannot discard a certain displacement of some glial cells towards the midline upon overexpression of *hh* (Fig. 4D), which may be possible since Ptc is expressed by some migrating glial cells (reviewed in (Klämbt, 2009)).

Another explanation for the observed phenotypes would be an interference with midline cell differentiation. Hh is essential to induce and maintain late *en* expression in the midline. Major axonal defects are detected upon Hh expression using an early pan-neural driver, and these fate phenotypes caused by ectopic Hh are due to the subsequent induction of *en* in this tissue (Bossing and Brand, 2006). In order to discard whether the same fate determination defects are responsible for the axonal guidance phenotypes observed when Hh is expressed in Sim-positive cells, we overexpressed *en* in the midline using the same

driver. Upon the induction of higher levels of En in the midline, we detected guidance defects in 80% of all embryos examined (n=20). These ranged from mild guidance phenotypes in FasII-m axons (Fig.4G, arrowhead), to minor midline crossing (Fig.4G, arrow; from one to three per embryo in 75%) and some fascicle collapses at the midline (Fig.4G, asterisk; 15%). In addition, when all the axons were visualised with BP102 antibody, we observed the absence of some commissures (data not shown). Therefore, *simGAL4::UASen* phenotypes are stronger and more general than *simGAL4::UAShh* phenotypes suggesting that the defects observed when *hh* is expressed in the midline are not due to En-induced problems in differentiation. In addition, En protein levels and localization were not majorly changed in *sim>hh* embryos (Fig.4F).

We also analysed neuronal fate in embryos where Hh was overexpressed in the midline and we could not identify any early problems in neurogenesis (Fig. 4 I and L). Taken together, these results indicate that the axonal phenotypes observed are not due to loss or major changes in midline and neuronal cell identity upon expression of Hh in the midline.

2.5. *hh* is epistatic to *fra*

If indeed Hh is a chemoattractant involved in commissural guidance, we asked whether, as in vertebrates, Hh is acting together with the Netrin/Fra pathway. To answer this question, we analysed the effect of overexpressing Hh in a *fra* mutant background and quantified the numbers of axons crossing the midline when *hh* is driven in Sim-positive cells in the absence of *fra* (Fig.5 A-D). We observed that we could decrease the numbers of midline crosses to nearly wt levels when overexpressing *hh* in *fra* mutant embryos (Fig. 5D) meaning that in the absence of Fra, Hh cannot exert its attractive influence in FasII positive axons. When all axons are visualized by the BP102 antibody, we could not detect any major changes in commissural axon crossing, indicating that excess Hh is not sufficient to rescue the absence of the Fra receptor (Fig. 5 E-F). Interestingly, these results shows that Hh cooperates with the chemoattraction mediated by the Netrins, via their receptor Fra. In addition, it also

reinforces our argument that Hh overexpression is not changing the midline cell fates and inducing midline crossing due to repatterning events.

2.6. Patched is the receptor for the Hh signal coming from the midline

To test whether the observed higher levels of midline crossing in the presence of elevated Hh expression at the midline are dependent on Hh's receptor Ptc, we used an engineered form of Ptc, which is composed of the N-terminal signalling portion of Hh joined via HA tags to the Ptc receptor (Casali and Struhl, 2004). This form of Ptc, named Hh-Ptc, behaves as a cell-autonomous, constitutively-bound to Hh form of Ptc (Casali and Struhl, 2004). When overexpressed in the Ptc domain of expression, using a PtcGAL4 driver Hh-Ptc induces defects similar to the lack of Ptc receptor, i.e. resulting in the activation of the Hh pathway (Butí et al., 2014). In order to check when and where midline crossing was dependent upon Hh binding to Ptc, we expressed this "activated" form of Ptc at various stages and/or in different cells of nervous system development (Fig.6).

When Hh-Ptc was expressed in all neuroblasts and their progeny from embryonic stage 10/11 using a *scabrous* (*sca*) Gal4 driver (Klaes et al., 1994), a strong neuronal phenotype was observed in about 60% of the embryos (n=34). Neurons in these embryos extended very few projections, and the CNS was highly disorganised like in *ptc* mutants (data not shown). We also identified milder phenotypes (40%, as shown in Fig. 6B). These observations are possibly attributable to an impairment of neuronal differentiation and axonal extension upon activation of the Hh pathway in the entire neuroectoderm from early stages. Using an *elav*GAL4 driver, we proceeded to express Hh-Ptc pan-neuronally, but only in postmitotic neurons from stage 12 to the end of embryogenesis, (Robinow and White, 1988). Upon this misexpression, we detected midline crossing and general guidance problems in all three FasII positive fascicles (Fig. 6C and 5G). Given that these phenotypes were induced by misexpression of Hh-Ptc in postmitotic neurons they are likely to be the result of the direct effect on axonal guidance and not caused by indirect effects in cell fate changes. In view of these results, we propose that the binding of Hh to Ptc at the axonal membrane makes

these axons more prone to crossing the midline upon *fra* activation. However, we cannot rule out the possibility that these phenotypes were due to excess activation of the Hh pathway in the surrounding neurons. In order to clarify this, we activated the downstream response to the Hh pathway by expressing Ci^{Act} (Hepker et al., 1997) using the same *elavGAL4* driver under the same conditions. Activation of the canonical Hh pathway, via Ci, in postmitotic neurons did not produce any axonal phenotypes (Fig. 6D) in contrast to its effects in tracheal development when expressed in the *ptc* domain (Butí et al., 2014). This finding confirms that the observed phenotypes are not due to overactivation of the canonical Hh pathway in post-mitotic neurons.

Having observed a cooperation between Hh and Fra, we asked whether the effect of Hh-Ptc in axons could be altered in a *fra* mutant background. Indeed, when Hh-Ptc was expressed in all post-mitotic *fra* mutant neurons, we detected no midline crossing in the majority of embryos (73%, n=11, Fig. 6E). These results indicate that Hh binding to the Ptc receptor in axons is responsible for the guidance phenotypes observed and suggest that this is dependent on Fra and accomplished via a non-canonical Hh signalling pathway.

In vertebrates, Shh guides axons through a non-canonical Src-dependent pathway (Yam et al., 2009). Therefore, we asked if this could also be involved in the *D. melanogaster* response to Hh. We observed that overexpression of Src42A in postmitotic neurons induces midline crossing (Fig.6F and 5G). To monitor Src activity in the CNS, we used antibodies directed to the phosphotyrosine residue of Src that is essential for its activation (Y400 of Src42A, called pSrc (Shindo et al., 2008)) and we could detect higher activation of pSrc in commissural axons upon *hh* overexpression in the midline (Fig. 6G and Fig.5E). In addition, we were able to decrease midline crossing in the absence of Src42A in conditions where Hh was overexpressed in the midline (average of 2 midline crossing events per embryo, n=8, Fig. 6 G). In view of these results we would like to propose that Hh guides axons in *D. melanogaster* through a non-canonical Src-dependent pathway.

2.7. Conclusions

The ventral midline of *D. melanogaster* provides an ideal system for comprehensive studies of axonal growth and guidance. Here we show that Hh is involved in the attractive response mediated by Netrin/Fra, thereby establishing this organism as a new model system for the study of this morphogen, as well as of its downstream effectors, in axonal guidance. Our *D. melanogaster* phenotypes reveal that, like floor plate cells expressing Shh, *D. melanogaster* midline cells secreting Hh can reorient and attract the growth of FasII-positive axons towards their source. In agreement with our results, we propose that other mediators are also required for this attraction. As in vertebrates, in *D. melanogaster*, many axons still orient correctly and grow towards the midline in the absence of netrins (Brankatschk and Dickson, 2006). Therefore, we propose that Hh acts in synergy with netrins, via Fra, to attract commissural axons to the midline.

Here, we provide evidence that, in axons of the VNC, the receptor for the Hh guidance signal is Ptc. Not only Ptc and Smo are expressed in axons at the VNC, but guidance defects are also induced in axons by the expression of a chimeric Hh-Ptc molecule that mimics constitutive binding of Hh to its receptor. Nevertheless, many questions remain open on how Hh is sensed by axons in order for these to change their trajectories accordingly. We are now in the position to be able to, by using *D. melanogaster*, to dissect this response genetically and molecularly.

To conclude, our data reveal a previously unknown role of Hh in midline axonal guidance in *D. melanogaster* and identify Ptc as the axonal receptor of this morphogen. We also demonstrate that Hh/Ptc cooperate with Netrin/Fra *via* a non-canonical Hh signalling mechanism and propose that this may be accomplished through direct axonal activation of Src42A. Of course, we cannot rule out the possibility of this being accomplished via other molecular effectors or through interactions with distinct axonal guidance pathways {O'Donnell, 2013 #1694}(Wouda et al., 2008). Future work is required to further dissect the molecular mechanisms by which Hh binding to Ptc is translated into cellular movement and to determine whether this pathfinding is conserved in other neuronal cell types in *D. melanogaster*.

4. Experimental Procedures

4.1. *D. melanogaster* stocks and genetics.

The following stocks are described in FlyBase (http://flybase.bio.indiana.edu): *sim*Gal4, fra^{3}, *Src42A*^{E1}, *hh*^{ts}, *hh*GAL4, *hh*^{AC}. We also used ptc^{Ilw}, UAS-Hh and UAS-Hh-Ptc (from A. Casali), sema2BτmyC (from G. Tear) and UASci^{Act} (from T. Orenic). For wild-type we used *yw*. *elav*Gal4 (from G.Tear) was used as a late pan-neural driver, *sca*Gal4 (from M. Krasnow) as an early neuronal driver, *sim*GAL4 (from G. Tear) as a midline driver, *en*GAL4UASGFP (from J. Casanova) as a midline segmental driver and *ap*GAL4UASGFP (from M. Milán) for expressions in *apterous* positive cells. *D. melanogaster* stocks and crosses were kept under standard conditions at 25°C and times AEL are defined at this temperature. All overexpression experiments were conducted at 29°C unless otherwise stated. To bypass the early requirement of *hh* in the nervous system, we used the *hh*^{ts} mutant. For this purpose, we grew flies at a permissive temperature (18ºC) for either 21h or 24 h and then shifted them to a restrictive temperature (29ºC) for 4 h.

4.2. Immunohistochemistry, image acquisition and data analysis

We used antibodies that recognise FasII (mAb1D4, DSHB), GFP (Molecular Probes and Roche), βGal (Cappel and Promega), Repo (DSHB), Smo (DSHB), Ptc (A. Casali), pSrc (Invitrogen) and mAbBP102 (DSHB); and HRP fluorescently conjugated (Roche) or Alexa488 and Alexa-555 conjugated secondary antibodies (Jackson ImmunoResearch). For HRP histochemistry, the signal was amplified using the Vectastain-ABC kit (Vector Laboratories) when required. In addition, the signal for the DAB reaction was intensified with $NiCl_2$, except for double stainings where it was omitted from one of the reactions. *In situ* hybridisation was performed following standard protocols. ribo-*hh* was generated using the whole cDNA as a template and using the Megascript kit (Ambion). Photographs were taken using a Nikon Eclipse 80i microscope. Fluorescent images were obtained with confocal microscopy (Leica TCS-SPE-AOBS system) and processed using Fiji (Schindelin J, 2012)

and Adobe Photoshop. Images are maximum projections of confocal Z-sections.
To quantify midline crossing, those between the two medial fibers (midline), and between the lateral and intermedial and intermedial and medial fibers (other) were counted in 40 simGAL4;UASHhh embryos and in 28 wild-type embryos at stage 16-17. Midline FasII fascicle measurements were taken using Fiji. Phenotypes were scored using Nomarski optics on a Nikon Eclipse 80i microscope with 20x and 40x objective. Box plot was done using R version 2.11.1 (2010-05-31) Copyright (C) 2010. The R Foundation for Statistical Computing ISBN 3-900051-07-0. Other graphs and statistics were done using Microsoft Excel.

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Figure legends

Figure 1 – Axonal guidance defects in the ventral nerve cord of *hh* mutant embryos.
Embryos are stained with BP102 to visualise the axon scaffold (A-D) and anti-FasII to mark all FasII-positive axons (E-H). A, wt embryo showing the anterior and posterior commissures and the longitudinal connectives. B and F, *hh* mutant embryos, both commissures and longitudinal connectives are collapsed. C,D and G,H, temperature-sensitive *hh* mutant embryos shifted to the restrictive temperature (RT) at stage 13 (approx. 10h AEL at 25°C) of embryogenesis (C and G) or stage 15 (approx. 11:30 h) of embryogenesis (D and H) and allowed to develop until stage 16 (approx. 15h). Embryos shifted to RT at stage 13 show defects in commissures and longitudinal connectives (arrows in C and G). Embryos shifted to RT at stage 15 display very mild phenotypes (arrowhead in H). Anterior is up.

Figure 2 – Hh is expressed in subset of glial cells and Ptc and Smo are detected in axons
Hh-lacZ embryos (A-D) and wild-type (E-H and J) of embryonic stage 11 (F), stage 13 (E, F', G, I and J) and stage 15 (A-D and H). A, embryos stained with BP102 to visualise the axon scaffold and anti-β-galactosidase (βgal) as a reporter for Hh, showing Hh-positive cells in the midline of the CNS (arrow) and Hh-positive cells surrounding the axons. B and E, embryos stained with anti-FasII and anti-βgal, showing Hh positive cells in the midline and surrounding the FasII positive axons. C, D, embryo stained with anti-Repo and anti-βgal, showing that some of the Hh-positive cells are repo-positive glia (arrow and arrowheads); D is a detail of C, a 2x digital zoom view of C with different number of Z-sections used for the projection (20 in C and 7 in D, all Z sections are 0.5 µm apart). C' is an orthogonal projection of C showing the position of the Hh positive cells in relation to the repo-positive glia. C'' is an orthogonal projection of and embryos stained for anti-Wrapper in green and anti-βgal in red showing the position of the Hh positive cells in relation to the midline wrapper-positive glia.

F, *in situ* hybridization of wt embryos with a RNA probe for detection of *hh* mRNA; arrow in F points a hh expression in the CNS at stage 11; arrowheads in F' point at very faint hh detection in the CNS of stage 13 embryos. G and H, embryos expressing a nuclear reporter for all *breathless*-expressing cells, to better visualise the position of the midline, stained with anti-Patched antibody and showing the presence of this receptor in all axons of the CNS; compare with BP102 stainings in A; dashed line marks the midline. Expecting lower signals and to avoid immunohistochemistry artifacts, we did not use other antibodies against proteins in the nervous system. G'' and H'' are B&W inversions of G' and H' for better visualization. I is a negative control of G and H, a anti-Ptc staining of *ptc* null embryos. J, staining of wt embryos with anti-Smo antibody, showing the presence of this receptor in all axons of the CNS. Anterior is left in all panels.

Figure 3 – Hh overexpression induces more axonal midline crossing

Embryos were stained with BP102 to visualise all axons in the CNS (A and B) and anti-FasII to mark FasII-positive axons (C and D). B and D show the midline of stage 16 embryos where *hh* is being expressed in all Sim-positive cells in the midline. Arrow in B shows extra midline crossing and the arrowhead marks the thinning of the longitudinal connectives as a result of midline guidance mistakes. The arrowhead in D depicts a characteristic midline cross in embryos with excess Hh in the midline. E, a box plot was used to represent the data from the quantification of extra crossing in FasII-fibers. The boxes show the interquartile range. The upper line of the box represents the maximum value, and the bottom line represents the minimum. Black lines show medians and small bars at the ends of dotted lines display the upper and the lower values. Small circles show the normal outliers. "midline" refers to midline crossing and "other" to crossing between fascicles (arrow in D). F and G are embryos carrying a SemaIIb:τmyc reporter and stained with anti-Myc to visualise the SemaIIb positive axons; black arrows mark the distance between fascicles and white arrows the distance between the neuronal cell body and the longitudinal connective formed by its axon; the asterisk marks mild guidance problems in the commissures upon expression

of Hh in the midline. H, displays the quantification of the distance in μm between fascicles (FasII-m and FasII-i) and compares wt with *sim>hh*, *frazzled* mutant and hh^{ts} embryos at stage 16 (approx. 15h AEL) n=23 in all lanes and $p<0.001$ between controls and experiments by Student's T-test. I and J, J' are confocal projections of the VNC of embryos expressing GFP in all *en*-expressing cells and stained for GFP and FasII (only FasII in J'). I, shows overexpression of Hh in the same *en* cells; the arrow shows a characteristic midline cross in these embryos. K, L and M are confocal projections of the VNC of embryos expressing GFP in all *ap*-expressing interneurons and their projections, stained for GFP and FasII. K and M show overexpression of Hh in the same *ap*-expressing cells; the arrows and arrowhead depict the guidance phenotypes produced in FasII fibers in these embryos. Anterior is up in all panels except in F and G where it is left.

Figure 4– Hh overexpression in the midline does not produce major differentiation changes

Stage 16 embryos stained with HRP to visualise the axon scaffold and anti-Wrapper to stain midline glia (A and B). B shows the midline of stage 16 embryos where *hh* is being expressed in all Sim-positive cells in the midline. C and D stage 16 embryos stained with HRP to visualise the axon scaffold and anti-Repo to stain all glia, except midline glia.
E, F and G show embryos stained with FasII (brown) and *en* (black) and respective characteristic phenotypes. E is the wt, F the *hh* overexpression in Sim-positive cells and G the *en* overexpression in Sim-positive cells; in F, arrow shows midline cross; in G arrow shows midline cross, arrowhead mild indentation of the FasII-m fascicle towards the midline, and the asterisk the collapse of the axonal fascicles at the midline. H, I and J, *even-skipped(eve)* expression in stage 13 embryos. K,L and M, FasII expression in stage 13 embryos. Anterior is up in all panels.

Figure 5 – *hh* is epistatic to *fra*

Stage 16 embryos stained with FasII (A-D) and BP102 (E-F). A, wt; B, *fra* mutant embryo; C and D overexpression of Hh in Sim–positive cells in *fra* heterozygotes and *fra* homozygotes, respectively; E, *fra* mutant embryos, arrow points at commissural defect; F, *fra* mutant embryos where *hh* has been overexpressed in the midline, arrowhead points at misguided commissural axons. G, quantification of midline crossing in wt, sim>hh, fra/+;sim>hh, fra/fra;sim>hh, elav>Hh-Ptc and elav>Src42A.

Figure 6 – Hh chemoattraction is mediated by Ptc via non-canonical Hh signalling

Stage 16 embryos stained with anti-FasII (A-F). A, wt; B, activation of Ptc signaling by Hh-Ptc in all neuroblasts from stage 10/11; C, activation of Ptc signaling by Hh-Ptc in all post-mitotic neurons; D, activation of Hh signalling via Ci in all post-mitotic neurons; E, activation of Ptc signaling by Hh-Ptc in all post-mitotic neurons in a *fra* mutant background; F, activation of Src42A in all post-mitotic neurons; G, overexpression of Hh in Sim-positive cells in a *Src42A* mutant background; H and I, stage 14 embryos stained with Fas-II to visualise neurons and axons in the VNC and anti-pSrc to detect phosphorylated (activated Src kinase) (F' and G'). Anterior is up in all panels.

Figure 1 - Butí et al

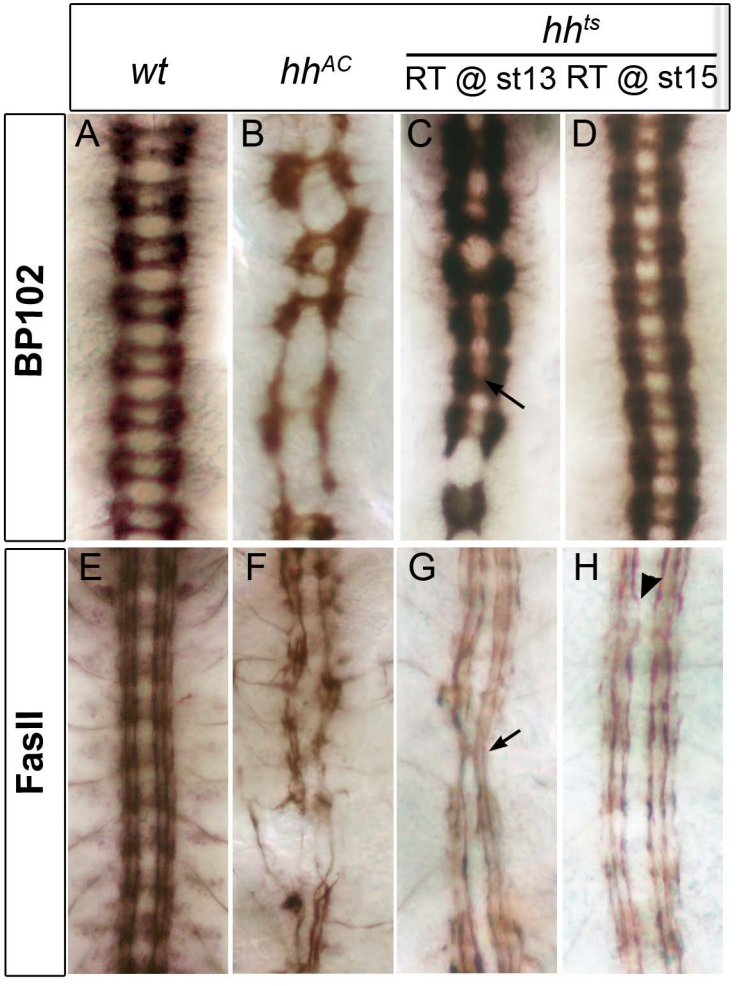


Figure 2 - Butí et al.

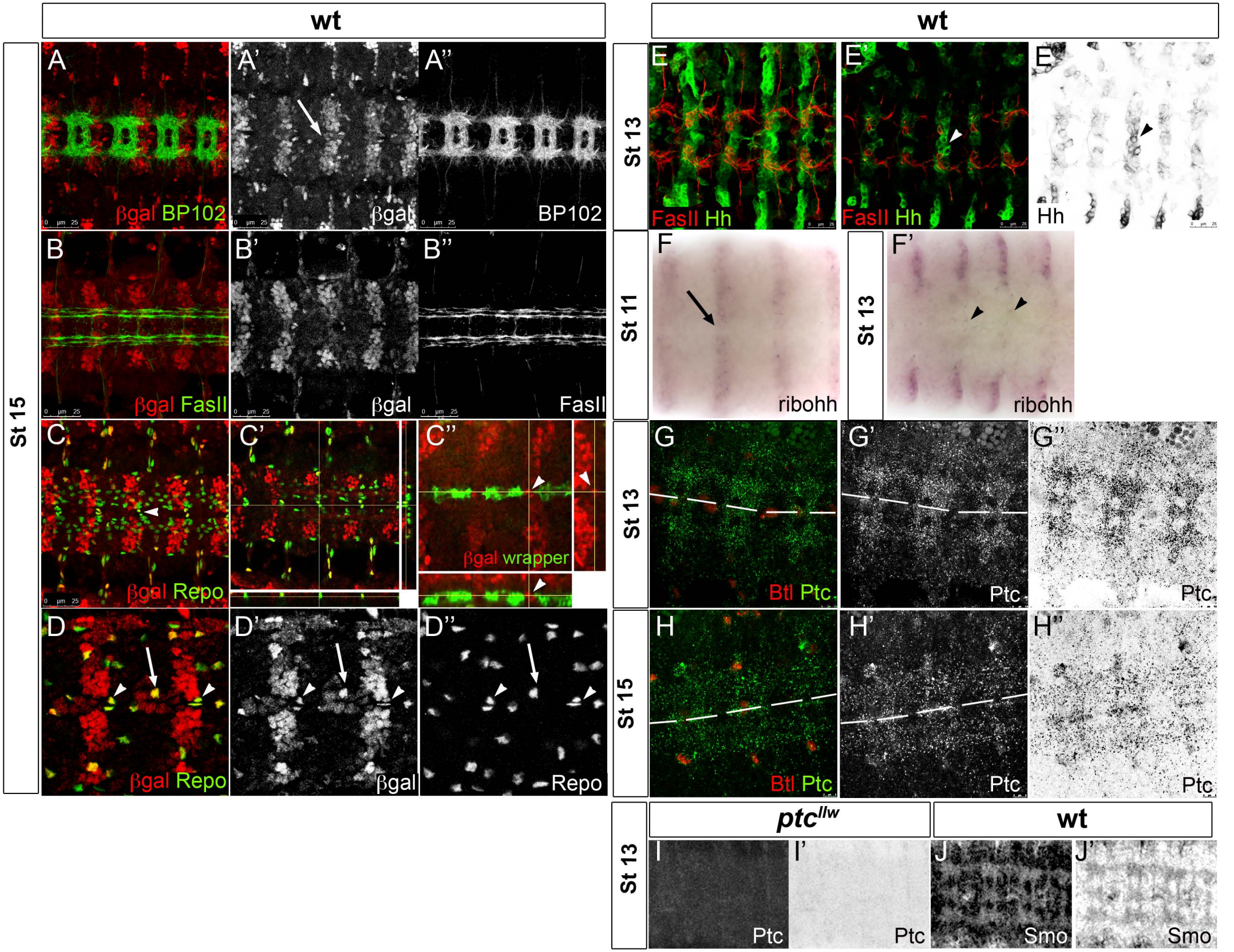


Figure 3 - Butí et al

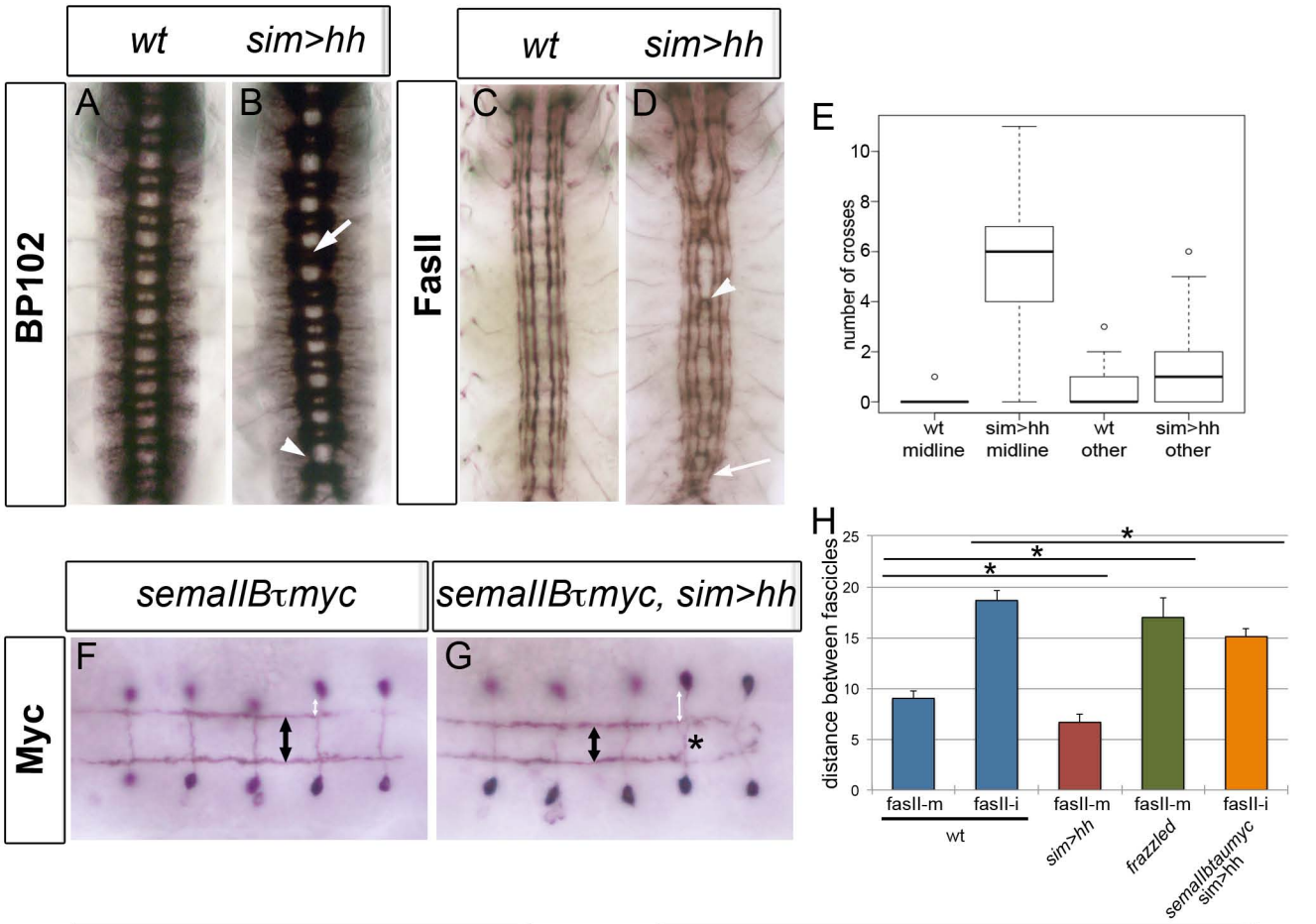
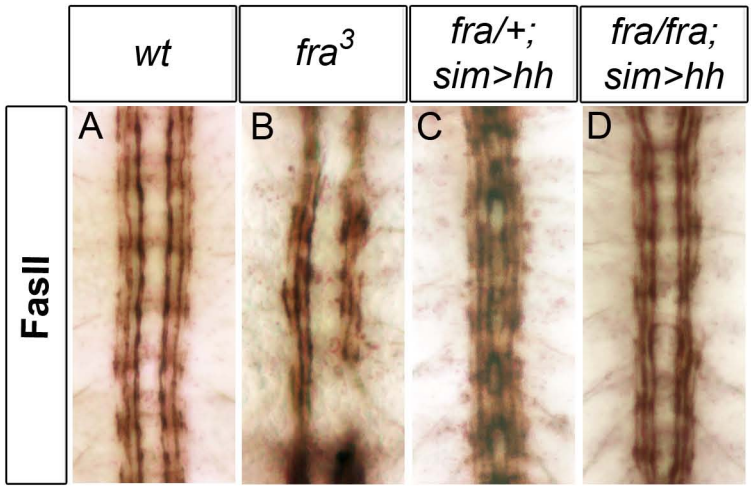


Figure 4 - Butí et al



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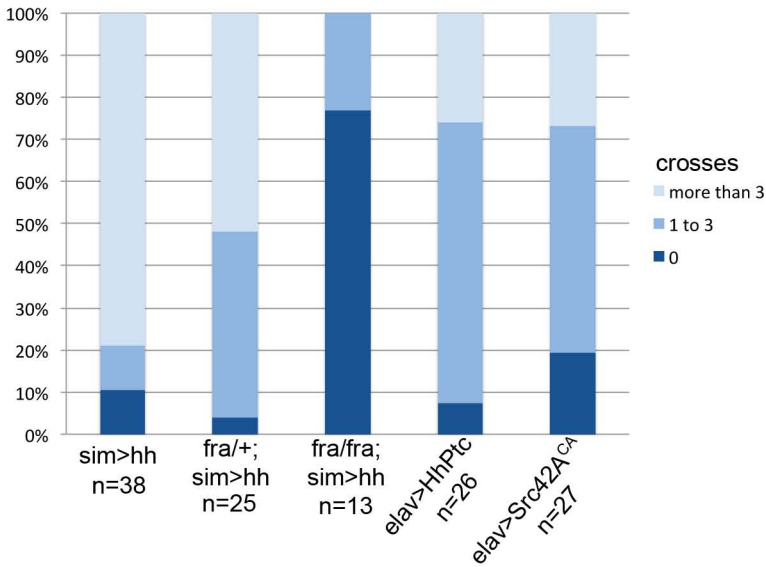
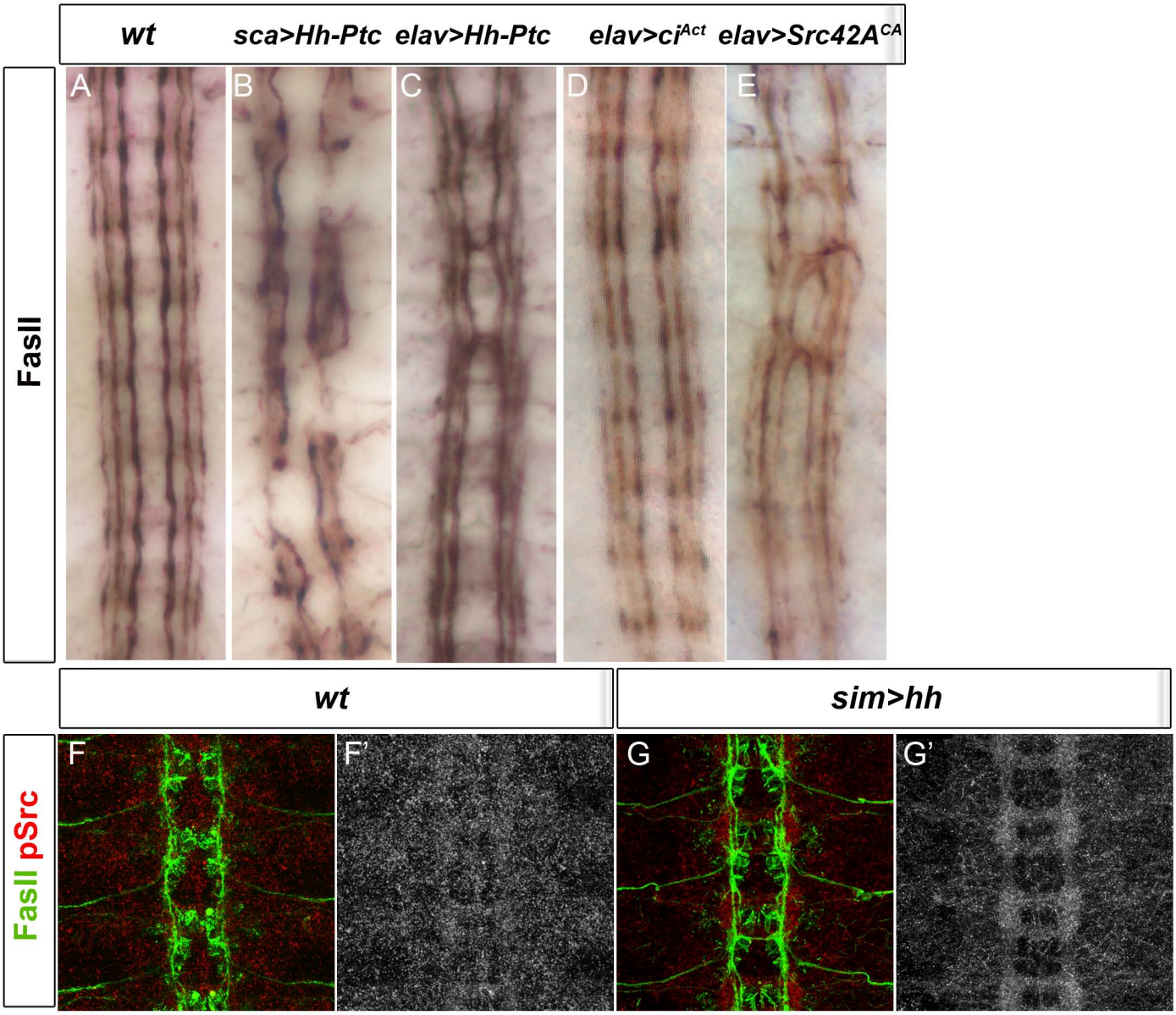


Figure 5 - Butí et al



Supplementary Figure 1 - Butí et al