# *Frizzled-3a* and *Wnt-8b* genetically interact during forebrain commissural formation in embryonic zebrafish

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**Keyfindings:**

- Knockdown of Wnt8b results in an absence of the anterior commissure and a reduction of the post-optic commissure
- Knockdown of Wnt8b results in a expansion of *slit2* at the rostral midline and a reduction of the glial bridge
- The phenotype observed following knockdown of Wnt8b is similar to that seen following knockdown of Fzd3a
- Combined loss of both Wnt8b and Fzd3a caused a synergistic increase in commissural defects and abnormal *slit2* expression
- Fzd3a and Wnt8b act in the same genetic pathway to control normal patterning of the commissural plate

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**Abstract**

The commissural plate forms the rostral surface of the embryonic vertebrate forebrain and provides a cellular substrate for forebrain commissural axons. We have previously reported that the Wnt receptor *frizzled-3a* (*fzd3a*) restricts the expression of the chemorepulsive guidance ligand *slit2* to a discrete domain of neuroepithelial cells in the commissural plate of embryonic zebrafish. Loss-of-*Fzd3a* function perturbed *slit2* expression and disrupted the formation of glial bridges which guide the formation of forebrain commissures. We now show that Wnt8b is also necessary for anterior commissural formation as well as for patterning of *slit2* expression at the midline. Knock down of Wnt8b produced the same phenotype as loss of Fzd3a which suggested that these genes were acting together to regulate axon guidance. Simultaneous sub-threshold knock down of both Fzd3a and Wnt8b led to a greater than additive increase in the penetrance of the mutant phenotype which indicated that these two genes were indeed interacting. We have shown here that Fzd3a/Wnt8b signaling is essential for normal patterning of the commissural plate and that loss-of-function in either receptor or ligand causes Slit2-dependent defects in glial bridge morphology which indirectly attenuated axon midline crossing in the embryonic vertebrate forebrain.

## 1. Introduction

Axon tracts in the vertebrate central nervous system are typically organised along three principal axes during early embryogenesis. Pioneer axons initially establish anterioposterior oriented tracts which are then connected across the mediolateral axis by commissures. Axons also course between dorsal and ventral populations of neurons to create tracts perpendicular to the longitudinal tracts. The early scaffold of axon tracts that forms in the embryonic vertebrate brain appears topographically similar in zebrafish (Hjorth and Key, 2002), mice (Mastick and Easter, 1996) and humans (Muller and O'Rahilly, 1990). This basic three-axis organisation is maintained into adulthood, and is also evident in the human brain which undergoes complex folding and asymmetrical proliferation during morphogenesis (Wedeen et al., 2012). While the mouse has been the preferred model for studying late brain development we have championed the use of zebrafish for understanding axon guidance at the earliest stages of forebrain development (Hjorth and Key, 2001; Devine and Key, 2003; Gaudin et al., 2012; Hofmeister et al., 2012).

The rostral end of the neural tube in zebrafish gives rise to the forebrain, with the telencephalon emerging dorsally and the diencephalon arising ventrally. A small set of neurons referred to as the ventrorostral cluster initially differentiates in the ventral diencephalon. Axons from these neurons project caudally to form the longitudinally directed tract of the post-optic commissure. Some axons from this cluster also grow rostrally and cross the midline in the post-optic commissure. Another small set of neurons called the dorsorostral cluster differentiates dorsally in the telencephalon. This cluster gives rise to both the anterior commissure, which bilaterally interconnects these neurons, and to the supraoptic tract, which contains axons that project ventrally to join the tract of the post-optic commissure. This simple scaffold of neuronal

clusters, commissures and axon tracts forms a circuit which acts as a template for the rapidly expanding forebrain. The rostral surface of the embryonic vertebrate forebrain is formed by the closure of the lateral lips of the anterior neuropore by a thin neuroepithelial membrane which is historically referred to as the lamina terminalis (Smith, 1903; Johnston, 1909). Part of this structure thickens and forms the commissural plate (Muller and O'Rahilly, 1986), which contains the anterior and post-optic commissures (Johnston, 1909). These commissures are both phylogenetically and functionally preserved pathways. They develop early and are present in many species from primitive fish to humans (Herrick, 1910; Northcutt and Puzdrowski, 1988). Behavioural studies in goldfish, pigeons and primates have consistently revealed that these commissures are involved in contralateral transfer of visually learned information (Black and Myers, 1964; Sullivan and Hamilton, 1973; Risse et al., 1978; Francesconi et al., 1982; Hemsley and Savage, 1989).

We are interested in deciphering the mechanisms underlying the formation of early forebrain commissures. Following on from reports that the Frizzled-3 receptor in mice is necessary for the formation of the anterior commissure (Wang et al., 2002; Wang et al., 2006a; Wang et al., 2006b), we recently confirmed that the role of this receptor was conserved in zebrafish (Hofmeister et al., 2012). Loss of Fzd3a function in zebrafish embryos perturbed the formation of the anterior and post-optic commissures. The absence of Fzd3a increased the expression of the chemorepulsive ligand *slit2* within the commissural plate, which in turn affected the formation of a substrate of glial cells. Glial cells normally form bridges across the midline, facilitating the crossing of commissural axons (Barresi et al., 2005; Barresi et al., 2010). The loss of these bridges leads to aberrant commissure formation. However, the Wnt ligand responsible for initiating these downstream events remains unknown. Interestingly, a number of Wnt genes including, Wnt8b, Wnt9, Wnt5 and Wnt7 are all expressed in the zebrafish rostral

forebrain (Thisse and Thisse, 2005; Danesin et al., 2009; Cox et al., 2010; Beretta et al., 2011). Given the previously reported roles for Wnt8b in zebrafish forebrain development and patterning (Lekven et al., 2001; Danesin et al., 2009) we reasoned that this Wnt was a prime candidate for controlling commissural formation.

In the present study we found that zebrafish *wnt8b* and *frizzled-3a* (*fzd3a*) genetically interact to regulate the patterning of guidance cues in the rostral forebrain. Knock down of Wnt8b results in an expansion of *slit2,* a repulsive guidance cue at the rostral midline, reduction or absence of forebrain commissures and a loss of midline glia. Loss of both Wnt8b and Fzd3a caused a synergistic increase in commissural defects and abnormal *slit2* expression. Together, these results revealed that *wnt8b* and *fzd3a* act in the same genetic pathway to pattern the commissural plate, thereby indirectly regulating the crossing of axons across the rostral midline in the embryonic forebrain of zebrafish.

## 2. Results

### *2.1 Wnt8b is necessary for formation of the anterior and post-optic commissures*

The anterior surface of the early embryonic forebrain of vertebrates is defined by the commissural plate. This plate of neuroepithelial cells contains the two earliest brain commissures: the anterior commissure, which connects neurons in the dorsorostral cluster of the presumptive telencephalon; and the post-optic commissure, which connects neurons in the ventrorostral cluster of the ventral diencephalon (Fig. 1). We have recently shown that *fzd3a* indirectly regulates the formation of these commissures by modulating the expression of the chemorepulsive ligand *slit2* (Hofmeister et al., 2012). Given that *wnt8b* is expressed adjacent to the commissural plate and that it plays a role in forebrain patterning (Kim et al., 2000; Houart et al., 2002; Kim et al., 2002; Lee et al., 2006; Danesin et al., 2009; Paridaen et al., 2009) we reasoned that this ligand was a prime candidate for mediating Fzd3a-dependent commissural formation.

To test this idea we knocked down zebrafish Wnt8b using previously described antisense translational-blocking morpholinos (MOs) (Riley et al., 2004). While injection of 2ng of *wnt8b* MO resulted in embryos with abnormal gross morphology (data not shown), we found most embryos developed normally following injection of 0.5-1.0ng of *wnt8b* MO. Analysis of the rostral forebrain in these latter embryos revealed selective malformation of the commissures (Fig. 2). Embryos injected with 1ng of *wnt8b* MO showed an absence of the anterior commissure (unfilled arrowhead, cf. Fig. 2D with 2A) and a notable reduction of the post-optic commissure (filled arrowhead, Fig. 2D) (penetrance of 62%, n=26, $p<0.01$, Table I) in comparison to embryos injected with an equivalent amount of standard control (Std-cont) MO

(Fig. 2A). We then co-immunostained the same brains with anti-GFAP antibodies to visualise glial cells. This staining showed a concomitant reduction in the bridge of glial cells (cf. Fig. 2B-C with Fig. 2E-F) that acts as a pathway for the commissural axons (Barresi et al., 2005). Commissural axon defects following knock down of Wnt8b were accompanied by a loss of GFAP-positive glial structures at the anterior commissure (unfilled arrowhead, Fig. 2E) and a severe reduction at the post-optic commissure (filled arrowhead, Fig. 2E). This is consistent with an indirect role of Wnt8b in axon guidance.

*2.2 Knock down of Wnt8b results in expansion of slit2 at the midline*

Since GFAP-positive glia and commissural axons are known to be repelled by Slit2 expressed at the midline (Barresi et al., 2005), we next examined the effect of knock down of Wnt8b on *slit2* expression by *in situ* hybridisation. *Slit2* is expressed by a narrow wedge of midline neuroepithelial cells in the commissural plate (Barresi et al., 2005). This expression pattern was not affected by injection of 1ng Std-cont MO (Fig. 3A). Double staining embryos for *slit2* expression together with HNK-1 antibodies for axons revealed the spatial relationship of the axon tracts to this wedge of neuroepithelium (Fig. 3B-L). Interestingly, analysis of each of the single coronal scans through the depth of the *slit2*-expressing wedge revealed that the anterior commissure axons coursed superficially to *slit2*-expressing cells (Fig. 3D-F). That is, these axons always crossed the midline in regions devoid of *slit2* stained cells. As previously reported following knock down of Fzd3a (Hofmeister et al., 2012), injection of 1ng of *wnt8b* MO resulted in a large expansion of the *slit2* domain (dotted outline, cf. Fig. 3A with 3G and Fig. 4). This expansion of *slit2* also specifically correlated with an absence of the anterior commissure (unfilled arrowhead, Fig. 3H) and an absence or severe reduction of the POC (filled arrowhead, Fig. 3H) in 62% of embryos (Table I). Single slice confocal scans at the level of commissural

crossing (Fig. 3J-L) confirmed the presence of *slit2* neuroepithelial cells (Fig. 3J) in a region normally devoid of chemorepulsive ligand (bracket, Fig. 3D). In controls, the AC axons cross in this *slit2* negative region (Fig. 3E-F). In contrast, in the Wnt8b knock down embryos, the anterior commissure axons failed to cross through the expanded domain of *slit2* expressing cells (Fig. 3K-L). These results are consistent with those we previously observed following knock down of Fzd3a (Hofmeister et al., 2012).

*2.3 Fzd3a and Wnt8b genetically interact to control slit2 patterning in the rostral forebrain*

Based on the similarity of commissural plate phenotypes following knock down of either Fzd3a (Hofmeister et al., 2012) or Wnt8b we postulated that Wnt8b was the Fzd3 ligand acting to spatially restrict expression of *slit2* to a narrow wedge at the rostral midline. To test whether *fzd3a* and *wnt8b* were acting in the same genetic pathway, low doses of both MOs were co-injected into embryos. If the combined effect is significantly greater than the addition of each individual MO, then the two genes are genetically interacting. Injection of 0.5ng of either *zfzd3a*-AUG MO or *wnt8b* MO resulted in the majority of embryos with both normal *slit2* expression (dotted outline, Fig. 5A, D) as well as commissure formation (Fig. 5B-C, E-F). In contrast, co-injection of 0.5ng*wnt8b* MO and 0.5ng *fzd3a*-AUG MO resulted in a significantly greater than additive increase in the number of embryos (Table I) as well as an expansion of the *slit2* expression domain (dotted outline, Fig. 5G; 71%, n=17 and Fig. 4). This coincided with an absence of the anterior commissure (unfilled arrowhead, Fig. 5H) and a severe reduction and defasciculation of POC fibers (filled arrowhead, Fig. 5H). Analysis of midline glial cells in these co-knock down animals revealed that in addition to the commissural defects and *slit2* expansion there is also disruption of the glial growth substrate (Fig. 6). Sub-threshold knock down of either Wnt8b (Fig. 6A-C) or Fzd3a (Fig. 6D-F) did not affect axon crossing or the

formation of the GFAP-positive glial bridge that span the midline. In contrast, co-injection of sub-threshold amounts of both Wnt8b and Fzd3a MOs caused severe disruption to commissure formation as a result of the aberrant glial substrate (Fig. 6G-I). This phenotype was similar to the disruption of the glial bridges following injection of 1ng of *wnt8b* MO (Fig. 2) and is consistent with the repulsive effect that *slit2* expression domain has on these glial cells (Baressi et al., 2005). These results indicate that *wnt8b* and *fzd3* act together in the same genetic pathway to spatially restrict the *slit2* expression domain in the commissural plate.

## 3. Discussion

The Wnt family of ligands have been implicated in tissue patterning, cell proliferation and differentiation, cell migration and axon pathfinding during embryonic development. In many cases the identity of the Wnt ligand is not clear since only Wnt signaling (Houart et al., 2002; Kapsimali et al., 2004; Yu et al., 2008; Joksimovic et al., 2009;) or the Frizzled receptor (Wang et al., 2002; Wada et al., 2006; Wang et al., 2006a; Wang et al., 2006b) has been investigated. Matching Wnt ligand and Fzd receptor to specific developmental events remains a key goal in the field. We have revealed here for the first time that *wnt8b* modulates commissure formation in the anterior forebrain of zebrafish. Loss-of-*wnt8b* function zebrafish embryos phenocopy previously reported defects associated with knock down of Fzd3a (Hofmeister et al., 2012). There is expansion of *slit2* expression in the anterior midline of the commissural plate, absence of glia that form the pathway substrate for commissural axons and a concomitant loss of midline crossing of commissural axons. In *fzd3a* loss-of-function embryos the glia and axon defects are rescued by knock down of Slit2, indicating that Fzd3a was indirectly affecting axon guidance. These conclusions are supported by previous reports that *Slit2* is essential for glial bridge formation and subsequent axon growth across the midline (Barresi et al., 2005). By performing simultaneous partial knock downs of Wnt8b and Fzd3a we have demonstrated here that these two genes are genetically interacting to regulate commissure formation in the anterior forebrain. This is the first time that specific vertebrate Wnt and Fzd genes have been shown to be cooperating genetically to influence axon guidance either directly or indirectly *in vivo*. While zebrafish with insertional mutations in the *wnt5b* gene have recently been shown to exhibit aberrant post-optic commissure formation, the underlying cell and molecular mechanisms remain unresolved (Baressi et al., 2010).

To date, Wnts have been shown to affect axon guidance in the vertebrate central nervous system by directly acting on axons. For instance, both Wnt4 and wnt5a are chemoattractants for mouse spinal commissural axons (Lyuksyutova et al., 2003; Shafer et al., 2011). After crossing the ventral midline these axons have been proposed to respond directly to gradients of Wnt4 and Wnt5a which cause them to ascend the spinal cord. Interestingly, the response of an axon to a particular Wnt ligand is context dependent since Wnt5a can act either as a chemorepellent (Liu et al., 2005; Fenstermaker et al., 2010; Blakely et al., 2011) or as a chemoattractant (Lyuksyutova et al., 2003; Fenstermaker et al., 2010; Shafer et al., 2011) for different neuronal populations. However, in both cases, Wnt5a acts directly on axons to mediate pathfinding. In contrast, we have shown in the forebrain that *wnt8b* cooperates with *fzd3a* to regulate *slit2* expression which then affects the patterning of a glial substrate essential for commissural formation. This is similar to the function of *wnt5b* which has recently been shown to affect post-optic commissure formation in zebrafish due to aberrant formation of the glial bridge (Baressi et al., 2010).

Wnt8b has previously been shown to regulate patterning events in zebrafish. During gastrulation, inhibition of Wnt8b signaling by the anterior neural ridge cells establishes the anterior brain territory in the neural ectoderm (Houart et al., 2002). In the diencephalon, Wnt8b signaling promotes ventral fates (Kapsimali et al., 2004) and regulates neuronal number (Russek-Blum et al., 2008). *Wnt8b* is expressed in the dorsal telencephalon and ventral diencephalon (caudal to the commissural plate), where it is involved in patterning of the anterior-posterior neuroectoderm and telencephalon (Kelly et al., 1995; Lekven et al., 2001; Rhinn et al., 2005; Lee et al., 2006; Danesin et al., 2009). While we have shown that *wnt8b* regulates the expression of *slit2,* the downstream signaling events remain unknown. Wnt8a has been traditionally classified as an activator of canonical/β-catenin signaling (Katoh, 2005; Lee et al., 2006) and in zebrafish has been shown to also interact with other Frizzled family members (Kim et al., 2002). In

contrast, the downstream events following Fzd3 activation appear to vary depending on the developmental context which most likely reflects the Wnt ligand and co-receptors involved. Loss of function studies in mouse and fish have linked Fzd3 to the planar cell polarity pathway (Wang et al., 2002; Lyuksyutova et al., 2003; Wada et al., 2006; Wang et al., 2006a; Wang et al., 2006b). *In vitro* studies indicate that mFzd3 can cause an increase in intracellular $Ca^{2+}$ levels (Slusarski et al., 1997a; Slusarski et al., 1997b; Liu et al., 1999; Kuhl et al., 2000; Sheldahl et al., 2003;), thereby implicating the Wnt/$Ca^{2+}$ signaling pathway. In *Xenopus,* Fzd3 has been repeatedly linked to the activation of β-catenin signaling in neural crest formation and axis specification (Umbhauer et al., 2000; Deardorff et al., 2001; Carron et al., 2003). However, *Xenopus* Fzd3 may also act through recruitment of the metastasis associated kinase to inhibit canonical Wnt signaling in the neuroectoderm (Kibardin et al., 2006).

### *3.1 A model for commissural plate formation*

A number of studies have independently revealed the role of *fgf8 (ace)* (Shanmugalingam et al., 2000), *lhx2* (*belladonna*) (Seth et al., 2006), *shh* (Barresi et al., 2005) and *fzd3a* (Hofmeister et al., 2012) in the development of the early rostral forebrain commissures in zebrafish. *Shh*, *wnt8b* and *lhx2* each inhibit the expression of *slit2* in the commissural plate which allows the formation of glial bridges across the rostral midline and the subsequent crossing of commissural axons. While *fgf8* appears to act upstream of *shh* and the closely related *twhh* (Shanmugalingam et al., 2000), it is not known if *wnt8b* and *lhx2* lie in the same or an independent pathway. A recent report indicates that *shh* is upstream of both *foxg1* and *wnt8b*, however this pathway is active in neuronal specification in the ventral telencephalon rather than in the commissural plate (Danesin et al., 2009).

The role of slit in the correct positioning of midline glia formation and the subsequent formation of the corpus callosum has also recently been revealed in mice (Unni et al., 2012). These results indicate the fundamental importance of glia-axon interactions in brain commissure formation. Interestingly, the navigation of axons across the midline in the spinal cord appears to be very different to that in the brain. Spinal commissural axons instead rely primarily on chemorepulsive and attractive ligands acting directly on axons rather than on glia (Chedotal, 2011).

Using the power of zebrafish we have begun to unravel the importance of Wnt-Fzd interactions in development of the rostral forebrain commissures in the vertebrate brain. Given that the function of Fzd3 is essential for anterior commissure in both fish and mice, which are separated by approximately 450 million years (Nobrega and Pennacchio, 2004), it is clear that Fzd3-Wnt signaling is a biologically significant process for preserving neural circuitry common across species. It now remains to be determined whether the role of Wnt8 itself is also conserved between these species.

## 4. **Experimental Procedures**

### *4.1 Zebrafish maintenance*

Zebrafish were maintained on a 14-hour light and 10-hour dark cycle at the University of Queensland fish facility. Experimental embryos were produced using light induced spawning and raised at 28.5$^{o}$C. All work was carried out according to institutional and Australian government animal ethics and experimentation guidelines.

### *4.2 Morpholino microinjections*

Morpholino (MO) phosphodiestermer oligonucleotides were purchased from Gene Tools LLC (Philomath, USA). Standard control (Std-cont MO) (5'- CCT CTT ACC TCA GTT ACA ATT TAT A-3) was designed and synthesized by Gene tools. Previously described MOs against *fzd3a* -MO (5'-AGA ACC ATG GTG CGT CCG TCT GTG C-3'; (Hofmeister et al., 2012)) and *wnt8b*-MO (5'-AGG GAG ACT TTC TTC ACC TTT CAC-3'; (Riley et al., 2004)) were used. The *wnt8b* morpholino has been widely used and shown not to produce off-target effects (Lee et al., 2006; Knowlton et al., 2008; Russek-Blum et al., 2008; Danesin et al., 2009). The specificity of the *wnt8b* morpholino was confirmed here by our genetic interaction analysis and is consistent with previous reports that that this morpholino is selective for *wnt8b* acting through *frizzled* (Russek-Blom et al., 2008), downstream of *foxg1* (Danesin et al., 2009) and upstream of *lef1* (Lee et al., 2006). Morpholinos were injected into the yolk of zygotes. For combinatorial knock down experiments of Fzd3a and Wnt8b, MOs were separately injected into single cell embryos. Std-cont MO was injected into control embryos at matched concentrations. The use of

subthreshold levels of morpholinos to identify genetic interactions have been described previously (Ekker and Larson, 2001; Rikin et al., 2010)

*4.3 In situ hybridization and immunofluorescence*

Wholemount zebrafish brains were immunolabeled for acetylated α-tubulin as previously described (Devine and Key, 2003) using an anti-mouse IgG conjugated to Alexa 594 secondary antibody (1:200). To label glial cells, a rabbit anti-goldfish GFAP antibody (Nona et al., 1989) was used as previously described (Barresi et al., 2005). Wholemount *in situ* hybridization using Fast Red (Hoffman-La Roche Ltd.) as a substrate combined with HNK-1 or acetylated α-tubulin immunocytochemical staining was performed as described previously (Hjorth and Key, 2001). Brains were mounted anteriorly between two coverslips and serial parasagittal optical sections were collected every 1μm using an Olympus BX61 confocal microscope and z-stacks were compiled using Image-J (Abramoff et al., 2004). Axon immunostaining was used to assess the AC and POC phenotype, which was characterized by absence of continuity of the large AC and POC fascicles across the midline or the presence of only several axons crossing. The significance of difference in penetrance of phenotypes was assessed using Fisher's exact test.

**References**


Abramoff, M.D., Magalhaes, P.J. and Ram, S.J. (2004) Image Processing with ImageJ. Biophotonics international 11, 36-42.

Barresi, M.J., Burton, S., Dipietrantonio, K., Amsterdam, A., Hopkins, N. and Karlstrom, R.O. (2010) Essential genes for astroglial development and axon pathfinding during zebrafish embryogenesis. Dev Dyn 239, 2603-18.

Barresi, M.J., Hutson, L.D., Chien, C.B. and Karlstrom, R.O. (2005) Hedgehog regulated Slit expression determines commissure and glial cell position in the zebrafish forebrain. Development 132, 3643-56.

Beretta, C.A., Brinkmann, I. and Carl, M. (2011) All four zebrafish Wnt7 genes are expressed during early brain development. Gene Expr Patterns 11, 277-84.

Black, P. and Myers, R.E. (1964) Visual Function of the Forebrain Commissures in the Chimpanzee. Science 146, 799-800.

Blakely, B.D., Bye, C.R., Fernando, C.V., Horne, M.K., Macheda, M.L., Stacker, S.A., Arenas, E. and Parish, C.L. (2011) Wnt5a regulates midbrain dopaminergic axon growth and guidance. PLoS One 6, e18373.

Carron, C., Pascal, A., Djiane, A., Boucaut, J.C., Shi, D.L. and Umbhauer, M. (2003) Frizzled receptor dimerization is sufficient to activate the Wnt/beta-catenin pathway. J Cell Sci 116, 2541-50.

Chedotal, A. (2011) Further tales of the midline. Curr Opin Neurobiol 21, 68-75.

Cox, A.A., Jezewski, P.A., Fang, P.K. and Payne-Ferreira, T.L. (2010) Zebrafish Wnt9a,9b paralog comparisons suggest ancestral roles for Wnt9 in neural, oral-pharyngeal ectoderm and mesendoderm. Gene Expr Patterns 10, 251-8.

Danesin, C., Peres, J.N., Johansson, M., Snowden, V., Cording, A., Papalopulu, N. and Houart, C. (2009) Integration of telencephalic Wnt and hedgehog signaling center activities by Foxg1. Dev Cell 16, 576-87.

Deardorff, M.A., Tan, C., Saint-Jeannet, J.P. and Klein, P.S. (2001) A role for frizzled 3 in neural crest development. Development 128, 3655-63.

Devine, C.A. and Key, B. (2003) Identifying axon guidance defects in the embryonic zebrafish brain. Methods Cell Sci 25, 33-7.

Ekker SC and Larson JD. (2001) Morphant technology in model developmental systems.

Genesis 30, 89-93.

Fenstermaker, A.G., Prasad, A.A., Bechara, A., Adolfs, Y., Tissir, F., Goffinet, A., Zou, Y. and Pasterkamp, R.J. (2010) Wnt/planar cell polarity signaling controls the anterior-posterior organization of monoaminergic axons in the brainstem. J Neurosci 30, 16053-64.

Francesconi, W., Fogassi, L. and Musumeci, D. (1982) Interocular transfer of visual discriminations in Wulst-ablated pigeons. Behav Brain Res 5, 399-406.

Gaudin, A., Hofmeister, W. and Key, B. (2012) Chemoattractant axon guidance cues regulate de novo axon trajectories in the embryonic forebrain of zebrafish. Dev Biol.

Hemsley, J.P. and Savage, G.E. (1989) Interocular transfer of preoperatively trained visual discriminations in goldfish (Carassius auratus) following selective commissure transections. Behav Brain Res 32, 297-304.

Herrick, C.J. (1910) The morphology of the forebrain in amphibia and reptilia. Journal of Comparative Neurology and Psychology 20, 413-547.

Hjorth, J.T. and Key, B. (2001) Are pioneer axons guided by regulatory gene expression domains in the zebrafish forebrain? High-resolution analysis of the patterning of the zebrafish brain during axon tract formation. Dev Biol 229, 271-86.

Hjorth, J.T. and Key, B. (2002) Development of axon pathways in the zebrafish central nervous system. Int J Dev Biol 46, 609-19.

Hofmeister, W., Devine, C.A., Rothnagel, J.A. and Key, B. (2012) Frizzled-3a and slit2 genetically interact to modulate midline axon crossing in the telencephalon. Mech Dev.

Houart, C., Caneparo, L., Heisenberg, C., Barth, K., Take-Uchi, M. and Wilson, S. (2002) Establishment of the telencephalon during gastrulation by local antagonism of Wnt signaling. Neuron 35, 255-65.

Johnston, J.B. (1909) The morphology of the forebrain vesicle in vertebrates. The Journal of Comparative Neurology and Psychology 19, 458-539.

Joksimovic, M., Yun, B.A., Kittappa, R., Anderegg, A.M., Chang, W.W., Taketo, M.M., McKay, R.D. and Awatramani, R.B. (2009) Wnt antagonism of Shh facilitates midbrain floor plate neurogenesis. Nat Neurosci 12, 125-31.

Kapsimali, M., Caneparo, L., Houart, C. and Wilson, S.W. (2004) Inhibition of Wnt/Axin/beta-catenin pathway activity promotes ventral CNS midline tissue to adopt hypothalamic rather than floorplate identity. Development 131, 5923-33.

Katoh, M. (2005) Comparative genomics on Wnt8a and Wnt8b genes. Int J Oncol 26, 1129-33.

Kelly, G.M., Greenstein, P., Erezyilmaz, D.F. and Moon, R.T. (1995) Zebrafish wnt8 and wnt8b share a common activity but are involved in distinct developmental pathways. Development 121, 1787-99.

Kibardin, A., Ossipova, O. and Sokol, S.Y. (2006) Metastasis-associated kinase modulates Wnt signaling to regulate brain patterning and morphogenesis. Development 133, 2845-54.

Kim, C.H., Oda, T., Itoh, M., Jiang, D., Artinger, K.B., Chandrasekharappa, S.C., Driever, W. and Chitnis, A.B. (2000) Repressor activity of Headless/Tcf3 is essential for vertebrate head formation. Nature 407, 913-6.

Kim, S.H., Shin, J., Park, H.C., Yeo, S.Y., Hong, S.K., Han, S., Rhee, M., Kim, C.H., Chitnis, A.B. and Huh, T.L. (2002) Specification of an anterior neuroectoderm patterning by Frizzled8a-mediated Wnt8b signalling during late gastrulation in zebrafish. Development 129, 4443-55.

Knowlton, M.N., Li, T., Ren, Y., Bill, B.R., Ellis, L.B., and Ekker, S.C. (2008) A PATO compliant zebrafish screening database (MODB): management of morpholino knockdown screen information. BMC Bioinformatics 9, 7.

Kuhl, M., Sheldahl, L.C., Park, M., Miller, J.R. and Moon, R.T. (2000) The Wnt/Ca2+ pathway: a new vertebrate Wnt signaling pathway takes shape. Trends Genet 16, 279-83.

Lee, J.E., Wu, S.F., Goering, L.M. and Dorsky, R.I. (2006) Canonical Wnt signaling through Lef1 is required for hypothalamic neurogenesis. Development 133, 4451-61.

Lekven, A.C., Thorpe, C.J., Waxman, J.S. and Moon, R.T. (2001) Zebrafish wnt8 encodes two wnt8 proteins on a bicistronic transcript and is required for mesoderm and neurectoderm patterning. Dev Cell 1, 103-14.

Liu, T., Liu, X., Wang, H., Moon, R.T. and Malbon, C.C. (1999) Activation of rat frizzled-1 promotes Wnt signaling and differentiation of mouse F9 teratocarcinoma cells via pathways that require Galpha(q) and Galpha(o) function. J Biol Chem 274, 33539-44.

Liu, Y., Shi, J., Lu, C.C., Wang, Z.B., Lyuksyutova, A.I., Song, X.J. and Zou, Y. (2005) Ryk-mediated Wnt repulsion regulates posterior-directed growth of corticospinal tract. Nat Neurosci 8, 1151-9.

Lyuksyutova, A.I., Lu, C.C., Milanesio, N., King, L.A., Guo, N., Wang, Y., Nathans, J., Tessier-Lavigne, M. and Zou, Y. (2003) Anterior-posterior guidance of commissural axons by Wnt-frizzled signaling. Science 302, 1984-8.

Mastick, G.S. and Easter, S.S., Jr. (1996) Initial organization of neurons and tracts in the embryonic mouse fore- and midbrain. Dev Biol 173, 79-94.

Muller, F. and O'Rahilly, R. (1986) The development of the human brain and the closure of the rostral neuropore at stage 11. Anat Embryol (Berl) 175, 205-22.

Muller, F. and O'Rahilly, R. (1990) The human brain at stages 18-20, including the choroid plexuses and the amygdaloid and septal nuclei. Anat Embryol (Berl) 182, 285-306.

Nobrega, M.A. and Pennacchio, L.A. (2004) Comparative genomic analysis as a tool for biological discovery. J Physiol 554, 31-9.

Nona, S.N., Shehab, S.A., Stafford, C.A. and Cronly-Dillon, J.R. (1989) Glial fibrillary acidic protein (GFAP) from goldfish: its localisation in visual pathway. Glia 2, 189-200.

Northcutt, R.G. and Puzdrowski, R.L. (1988) Projections of the olfactory bulb and nervus terminalis in the silver lamprey. Brain Behav Evol 32, 96-107.

Paridaen, J.T., Danesin, C., Elas, A.T., van de Water, S., Houart, C. and Zivkovic, D. (2009) Apc1-mediated antagonism of Wnt/beta-catenin signaling is required for retino-tectal pathfinding in the zebrafish. Zebrafish 6, 41-7.

Rhinn, M., Lun, K., Luz, M., Werner, M. and Brand, M. (2005) Positioning of the midbrain-hindbrain boundary organizer through global posteriorization of the neuroectoderm mediated by Wnt8 signaling. Development 132, 1261-72.

Rikin, A., Rosenfeld, G.E., McCartin, K. and Evans, T. (2010) A reverse genetic approach to test functional redundancy during embryogenesis. J Vis Exp.

Riley, B.B., Chiang, M.Y., Storch, E.M., Heck, R., Buckles, G.R. and Lekven, A.C. (2004) Rhombomere boundaries are Wnt signaling centers that regulate metameric patterning in the zebrafish hindbrain. Dev Dyn 231, 278-91.

Risse, G.L., LeDoux, J., Springer, S.P., Wilson, D.H. and Gazzaniga, M.S. (1978) The anterior commissure in man: functional variation in a multisensory system. Neuropsychologia 16, 23-31.

Russek-Blum, N., Gutnick, A., Nabel-Rosen, H., Blechman, J., Staudt, N., Dorsky, R.I., Houart, C. and Levkowitz, G. (2008) Dopaminergic neuronal cluster size is determined during early forebrain patterning. Development 135, 3401-13.

Seth, A., Culverwell, J., Walkowicz, M., Toro, S., Rick, J.M., Neuhauss, S.C., Varga, Z.M. and Karlstrom, R.O. (2006) belladonna/(Ihx2) is required for neural patterning and midline axon guidance in the zebrafish forebrain. Development 133, 725-35.

Shafer, B., Onishi, K., Lo, C., Colakoglu, G. and Zou, Y. (2011) Vangl2 promotes Wnt/planar cell polarity-like signaling by antagonizing Dvl1-mediated feedback inhibition in growth cone guidance. Dev Cell 20, 177-91.

Shanmugalingam, S., Houart, C., Picker, A., Reifers, F., Macdonald, R., Barth, A., Griffin, K., Brand, M. and Wilson, S.W. (2000) Ace/Fgf8 is required for forebrain commissure formation and patterning of the telencephalon. Development 127, 2549-61.

Sheldahl, L.C., Slusarski, D.C., Pandur, P., Miller, J.R., Kuhl, M. and Moon, R.T. (2003) Dishevelled activates Ca2+ flux, PKC, and CamKII in vertebrate embryos. J Cell Biol 161, 769-77.

Slusarski, D.C., Corces, V.G. and Moon, R.T. (1997a) Interaction of Wnt and a Frizzled homologue triggers G-protein-linked phosphatidylinositol signalling. Nature 390, 410-3.

Slusarski, D.C., Yang-Snyder, J., Busa, W.B. and Moon, R.T. (1997b) Modulation of embryonic intracellular Ca2+ signaling by Wnt-5A. Dev Biol 182, 114-20.

Smith, G.E. (1903) XII. On the Morphology of the Cerebral Commissures in the Vertebrata, with Special Reference to an Aberrant Commissures found in the Forebrain of certain Reptiles. Transactions of the linnean Society of London. 2nd Series: Zoology 8, 455-500.

Sullivan, M.V. and Hamilton, C.R. (1973) Memory establishment via the anterior commissure of monkeys. Physiol Behav 11, 873-9.

Thisse, C. and Thisse, B. (2005) High Throughput Expression Analysis of ZF-Models Consortium Clones. ZFIN Direct Submission (http://zfin.org).

Umbhauer, M., Djiane, A., Goisset, C., Penzo-Mendez, A., Riou, J.F., Boucaut, J.C. and Shi, D.L. (2000) The C-terminal cytoplasmic Lys-thr-X-X-X-Trp motif in frizzled receptors mediates Wnt/beta-catenin signalling. EMBO J 19, 4944-54.

Unni, D.K., Piper, M., Moldrich, R.X., Gobius, I., Liu, S., Fothergill, T., Donahoo, A.L., Baisden, J.M., Cooper, H.M. and Richards, L.J. (2012) Multiple Slits regulate the development of midline glial populations and the corpus callosum. Dev Biol 365, 36-49.

Wada, H., Tanaka, H., Nakayama, S., Iwasaki, M. and Okamoto, H. (2006) Frizzled3a and Celsr2 function in the neuroepithelium to regulate migration of facial motor neurons in the developing zebrafish hindbrain. Development 133, 4749-59.

Wang, Y., Guo, N. and Nathans, J. (2006a) The role of Frizzled3 and Frizzled6 in neural tube closure and in the planar polarity of inner-ear sensory hair cells. J Neurosci 26, 2147-56.

Wang, Y., Thekdi, N., Smallwood, P.M., Macke, J.P. and Nathans, J. (2002) Frizzled-3 is required for the development of major fiber tracts in the rostral CNS. J Neurosci 22, 8563-73.

Wang, Y., Zhang, J., Mori, S. and Nathans, J. (2006b) Axonal growth and guidance defects in Frizzled3 knock-out mice: a comparison of diffusion tensor magnetic resonance imaging, neurofilament staining, and genetically directed cell labeling. J Neurosci 26, 355-64.

Wedeen, V.J., Rosene, D.L., Wang, R., Dai, G., Mortazavi, F., Hagmann, P., Kaas, J.H. and Tseng, W.Y. (2012) The geometric structure of the brain fiber pathways. Science 335, 1628-34.

Yu, W., McDonnell, K., Taketo, M.M. and Bai, C.B. (2008) Wnt signaling determines ventral spinal cord cell fates in a time-dependent manner. Development 135, 3687-96.

**Fig. 1. Zebrafish forebrain commissures.**

Commissures in the 28-30hpf zebrafish commissural plate. (A). Diagrammatic representation of an oblique rostrolateral view of the rostral end of the brain. In the telencephalon neurons of the dorso-rostral cluster (drc) have extended axons across the midline to form the anterior commissure (AC) and ventrally towards the ventro-rostral cluster (vrc) forming the supra-optic tract (SOT). Similarly in the diencephalon, neurons of the vrc have projected contralaterally to form the post-optic commissure (POC). (B) represents compiled optical sections of the wholemount zebrafish brain showing the two commissures at the rostral end of the brain. Axons were labelled with an antibody against acetylated α–tubulin (green) revealing that they grow in close association with the GFAP stained glia (red).

**Fig. 2. Wnt8b is required for commissure formation**

Compiled optical sections of wholemount zebrafish brains at 28-30hpf stained for GFAP (B, E and in red C, F) and acetylated tubulin (A, D and in green C, F). Rostral is facing and dorsal to the top in all panels. In embryos injected with Std-cont MO, GFAP-positive glia are present at the midline where the anterior commissure (AC) and post-optic commissure (POC) form (A-C). In contrast, loss of Wnt8b results in a severe reduction of GFAP-positive glia (arrowheads, E), correlating with a loss or severe reduction of the AC (unfilled arrowhead, D) and POC (filled arrowhead, D). Scale bar in F is 20μm.

**Fig. 3. Knock down of Wnt8b results in expansion of *slit2* expression domain**

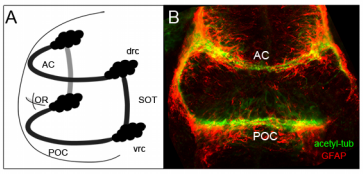
Z-stack (A-C, G-I) or single 1μm optical slices (D-F, J-L) of wholemount zebrafish brains at 28-30hpf. Embryos were injected with Std-cont MO (A-C) or Wnt8b MO (G-I). *slit2* RNA expression (A, D, G, J; and in red in C, F, I, L) is shown in relation to commissures labeled with

HNK-1 (B, E, H, K; and in green in C, F, I, L). Injection of *wnt8b MO* resulted in an expansion of the *slit2* domain in the commissural plate (compare outline in A to G), which correlated with a failure of the both commissures to cross the midline (H, K unfilled arrowheads; H filled arrowhead respectively). Single optical slices show *slit2* expression in the region where the AC (unfilled arrowheads K) would normally cross the rostral midline (compare J to D). Scale bar in C, 20μm.

**Fig 4. Embryos with abnormal comissure show an expansion of the *slit2* expression domain.** Embryos were sorted according to their commissural phenotype and the area of the *slit2* expression domain measured using image J as indicated in Figure 1 (outline A-G). Area measurements were compared in Graph Pad Prism 5.1 using a one-way ANOVA and a Tukey post-test ($p<0.05$). Embryos with abnormal commissure formation showed a significant expansion in *slit2* expression compared to control embryos. Abbreviations; morpholino (MO), significant at $p<0.05$ (*), highly significant at $p<0.001$ (***).

**Fig. 5. Fzd3a genetically interacts with Wnt8b to restrict the *slit2* expression domain in the rostral neuroepithelium.** Wholemount zebrafish brains at 28-30hpf. Embryos were injected with 0.5ng Fzd3a-AUG MO + 0.5ng Std-cont MO (A-C), 0.5ng Wnt8b MO + 0.5ng Std-cont MO (D-F), or 0.5ng Fzd3a MO +0.5ng Wnt8b MO (G-I). *slit2* expression is shown individually in panels A, D, and G. Commissures stained with anti-acetylated α-tubulin are shown in panels B, E andH. Panels C, F and I show co-staining for slit2 (red) and acetylated α-tubulin (green). Co-injection of 0.5ng Fzd3a-AUG MO and 0.5ng Wnt8b MO resulted in a synergistic increase in embryos with an expanded *slit2* expression domain compared to embryos injected with a low dose of either 0.5ng Fzd3a-AUG MO or 0.5ng Wnt8b MO (compare outline in G to A and D). Expansion of *slit2* at the region of commissure formation correlated with a failure of the AC and POC to form normally (unfilled and filled arrowheads in H, respectively). Scale bar in C, 20μm.

**Fig 6. Loss of glial structures following double knockdown of Fzd3a and Wnt8b**. Compiled confocal sections of zebrafish brains at 28-30hpf stained for acetylated α-tubulin (A, D, G and in green C, F, I) and GFAP (B, E, H and in red C, F, I). Rostral is facing and dorsal to the top in all panels. Embryos were injected with 0.5ng of *wnt8b* MO (A-C) or 0.5ng of *fzd3a*-AUG MO showed normal formation of commissures as previously observed (Fig. 3, Table I). Analysis of these embryos revealed normal formation of glial structures. In contrast following co-injection with 0.5ng of *wnt8b* MO + 0.5ng Std-cont MO (A-C) or 0.5ng of *fzd3a*-AUG MO +0.5ng Std cont MO embryos that displayed a commissural defect also showed an absence of glial cells at the anterior commissure (unfilled arrowhead, H) and a reduction at the post-optic commissure (filled arrowhead, H). Scale bar in I, 20μm.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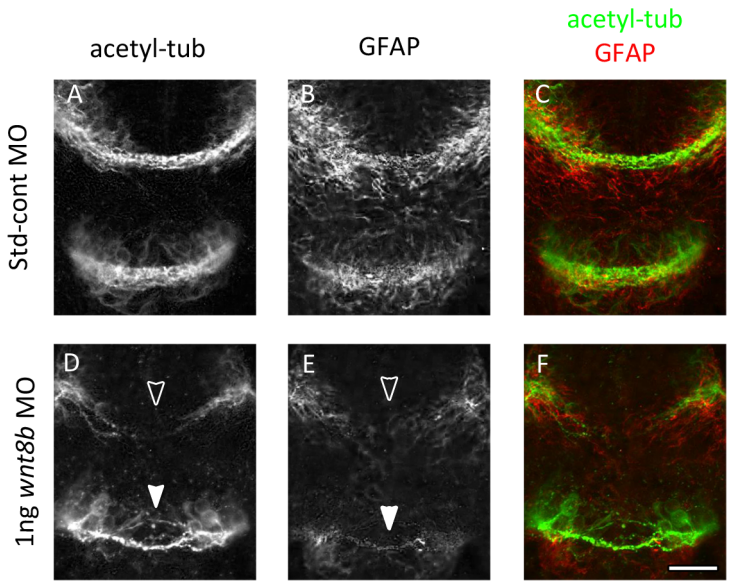
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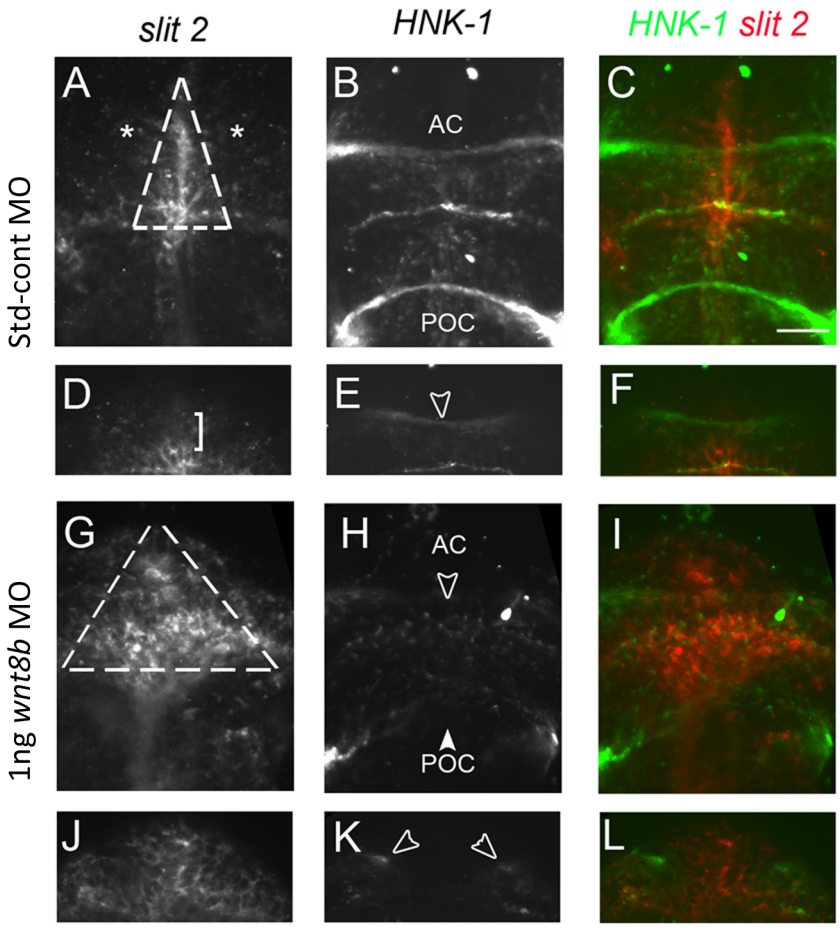
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1ng wnt8b MO
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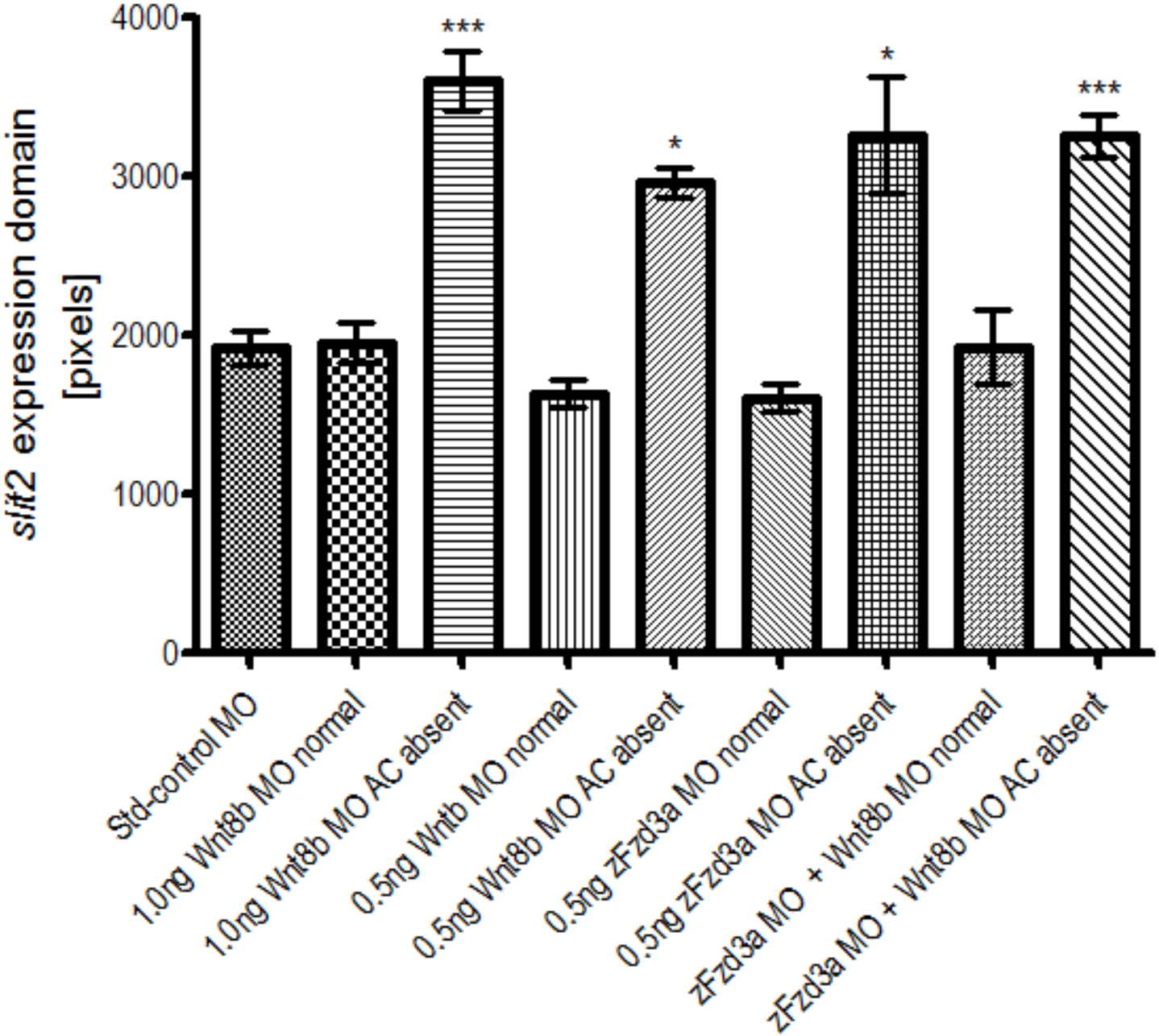
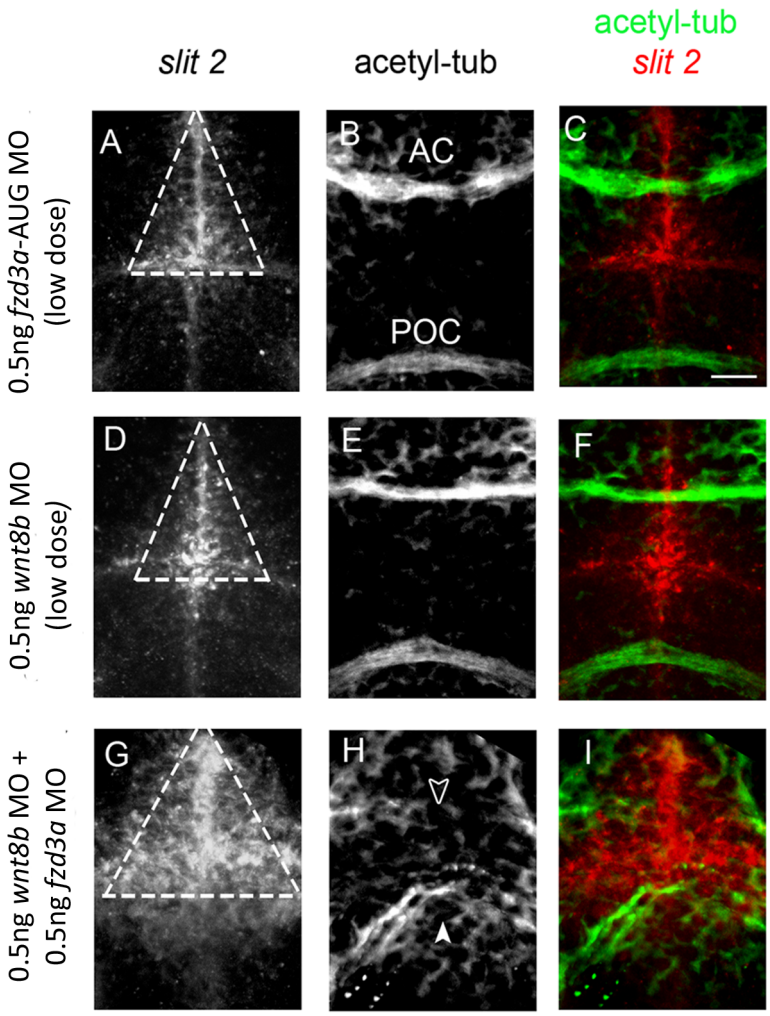
# Area measurement of *slit2* expression domain




slit 2
acetyl-tub
acetyl-tub
slit 2
0.5ng fzd3a-AUG MO
(low dose)
0.5ng wnt8b MO
(low dose)
0.5ng wnt8b MO +
0.5ng fzd3a MO
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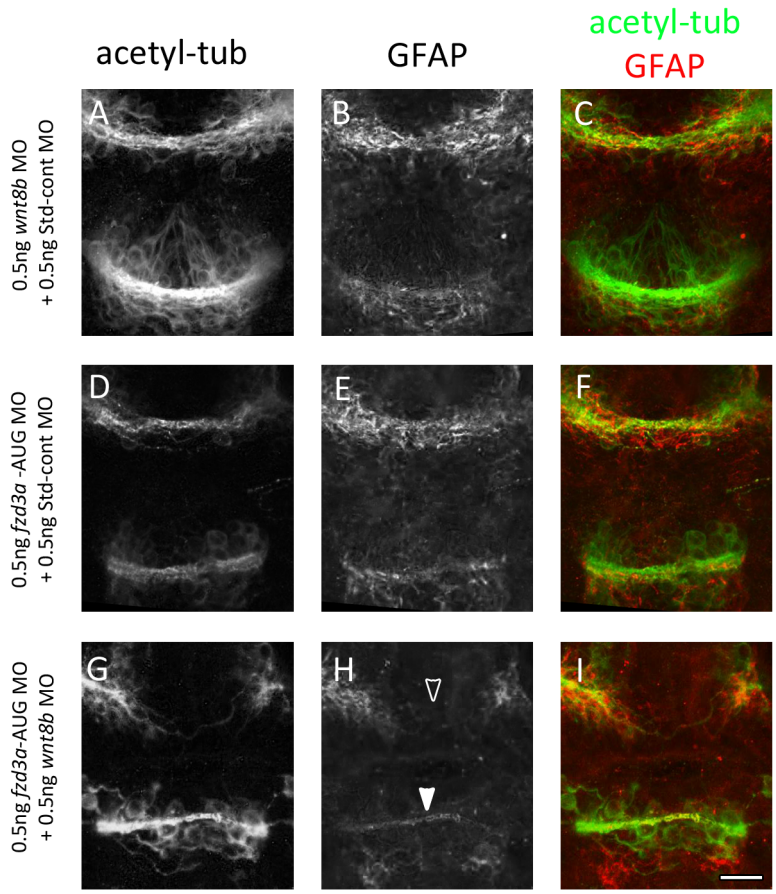
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0.5ng wnt8b MO
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0.5ng fzd3a-AUG MO
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