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RESEARCH ARTICLE

STEM CELLS AND REGENERATION

A bi-modal function of Wnt signalling directs an FGF activity gradient to spatially regulate neuronal differentiation in the midbrain

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ABSTRACT

FGFs and Wnts are important morphogens during midbrain development, but their importance and potential interactions during neurogenesis are poorly understood. We have employed a combination of genetic and pharmacological manipulations in zebrafish to show that during neurogenesis FGF activity occurs as a gradient along the anterior-posterior axis of the dorsal midbrain and directs spatially dynamic expression of the Hairy gene *her5*. As FGF activity diminishes during development, Her5 is lost and differentiation of neuronal progenitors occurs in an anterior-posterior manner. We generated mathematical models to explain how Wnt and FGFs direct the spatial differentiation of neurons in the midbrain through Wnt regulation of FGF signalling. These models suggested that a negative-feedback loop controlled by Wnt is crucial for regulating FGF activity. We tested Sprouty genes as mediators of this regulatory loop using conditional mouse knockouts and pharmacological manipulations in zebrafish. These reveal that Sprouty genes direct the positioning of early midbrain neurons and are Wnt responsive in the midbrain. We propose a model in which Wnt regulates FGF activity at the isthmus by driving both FGF and Sprouty gene expression. This controls a dynamic, posteriorly retracting expression of *her5* that directs neuronal differentiation in a precise spatiotemporal manner in the midbrain.

KEY WORDS: Sprouty, Hairy, Neurogenesis, Chemical genetics, Zebrafish, Mathematical modelling

INTRODUCTION

Organ formation requires the coordinated movement, proliferation and differentiation of many cells over time. Signalling pathways are crucial for regulating these decisions and dictate cell behaviour through activity gradients from organiser centres. In the developing brain, the mid-hindbrain boundary (isthmus) is one such organiser that secretes Wnts and FGFs (Liu and Joyner, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). At early stages, Wnt and FGF specify identity of the midbrain and anterior hindbrain, but their later expression at the isthmus appears to be important for controlling cell fate decisions (Joyner et al., 2000; Liu and Joyner, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). Mutant mice with attenuated Wnt or FGF signalling show specific perturbations of posterior, but not anterior, midbrain structures (McMahon et al., 1992; Meyers et al., 1998; Brault et al., 2001; Chi et al., 2003; Basson et al., 2008). This might reflect different requirements for FGF activity along the anterior-posterior (A-P) axis of the midbrain via an activity gradient. The existence of a gradient originating from the isthmus and extending along the A-P axis of the midbrain has been proposed based on evidence from FGF receptor binding assays (Chen et al., 2009). Whether FGF signalling actually regulates cell fate decisions along the A-P axis of the midbrain at later stages in development is unclear, but posterior shifts in neurogenesis in Fgf receptor 1 (*Fgfr1*) mutant mice might reflect such a role (Jukkola et al., 2006).

During establishment of the isthmus and patterning of the midbrain, FGFs and Wnts maintain and control each other's expression at the isthmus in a regulatory network involving FGF, Wnt, Pax, Engrailed and Lmx genes (Liu and Joyner, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001; Canning et al., 2007; Wittmann et al., 2009). When neurogenesis commences, Wnt-FGF interactions are thought to control patterning events important for the specification of distinct neuronal subtypes (Jaeger et al., 2011; Lahti et al., 2011; Yang et al., 2013). Tissue-specific knockouts of FGF and Wnt signalling in the midbrain show similar midbrain phenotypes, consistent with a model involving FGF-Wnt interactions promoting each other's activity at the isthmus (Chi et al., 2003; Yang et al., 2013). However, overexpression analyses reveal that Wnt drives cell proliferation whereas FGF signalling drives patterning at neurogenesis stages, indicating potential differences in how each pathway functions during midbrain development (Lee et al., 1997; Brault et al., 2001; Chi et al., 2003; Panhuysen et al., 2004). This might reflect different temporal requirements for Wnt and FGF signalling that cannot be easily determined using genetic mutants due to the complex nature of the Wnt-FGF interactions.

To dissect Wnt-FGF interactions we have employed a combination of pharmacological and genetic approaches to simultaneously manipulate FGF and Wnt activity. By applying small-molecule compounds and correlating spatial changes of gene expression with alterations to neurogenesis, we have generated mathematical models for how Wnt and FGF regulate midbrain development. Our models lead us to propose that Wnt has a bi-modal role in regulating FGF activity during midbrain development and that this controls where and when neurons form. We show that positive- and negative-feedback loops drive this process and that Sprouty genes are Wnt targets during midbrain development and act to repress FGF activity. This has implications for other systems in which FGF and Wnt signalling are active and can potentially cross-

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regulate each other, as Sprouty genes are also FGF regulated and so represent a buffer to limit FGF activity.

RESULTS

Dorsal midbrain neurons form through an anterior-posterior process

To dissect signalling pathway function during midbrain neurogenesis, we characterised the early development of dorsal brain neurons, as they are easily identifiable. The first-born dorsal midbrain neurons in amniotes are the mesencephalic trigeminal nucleus (MTN) neurons; they are the only sensory neuron population in the central nervous system and they innervate jaw muscles (Stainier and Gilbert, 1990; Chédotal et al., 1995). Therefore, we tested if application of DiI to jaw muscles can label MTN neurons in zebrafish. Application of DiI to the adductor mandibulae (a.m.) muscle of 5 day post-fertilisation (dpf) larvae labelled trigeminal motoneurons and large centrally located neurons in the anterior dorsal midbrain (Fig. 1A,B). Labelled midbrain cells are large unipolar neurons with a single process that branches into two axons projecting laterally and posteriorly to the isthmus; one axon projects to trigeminal motoneurons in the hindbrain and the other projects to the trigeminal nerve (Fig. 1C). These features are hallmarks of MTN neurons and indicate that zebrafish possess MTN neurons in the midbrain.

We characterised the spatial and temporal origin of MTN neurons in *Tg[dlx5a6a:gfp]* transgenic fish, as back-labelling with DiI revealed that MTN express GFP at 5 dpf in this line (supplementary material Fig. S1A-F). At 24 hours post-fertilisation (hpf), presumptive MTN GFP$^+$ neurons were present at the anterior midbrain in addition to GFP$^+$ neurons of the nucleus of the tract of the posterior commissure (nTPC) in the posterior diencephalon (Fig. 1D,E). MTN neurons grew axons posterior-laterally in the midbrain and pioneered an axon tract parallel to the medial longitudinal fasicle (mlf). Our observations of this axon tract pioneered by the MTN indicate that it is very similar to the dorsal tract of the mesencephalic trigeminal (dtmesV), described in medaka fish and in amniotes, and hence we describe this tract as the dtmesV (Fig. 1F,G; supplementary material Movie 1).

At 24 hpf, MTN and nTPC neurons expressed *isl1*, *drg11* (*drgx* – Zebrafish Information Network), *brn3a* (*pou4f1* – Zebrafish Information Network) and *tlx3a*, as is typical of sensory neurons, and were immunoreactive for Elavl3 (HuC) protein (Fig. 1H; supplementary material Fig. S1H,I). Intriguingly, despite the anterior restriction of MTN neurons to the anterior midbrain, we note that *elavl3*-expressing cells are present along the A-P extent of the dorsal midbrain (Fig. 1I; supplementary material Fig. S1G). Using *Tg[elavl3:egfp]* transgenic embryos, we characterised the temporal and spatial progression of neuronal differentiation in the dorsal midbrain. We find that GFP expression in this line correlates with markers of MTN identity (supplementary material Fig. S1J-L, Movie 2) (Park et al., 2000; Lyons et al., 2003; Coolen et al., 2012). Time-lapse analysis from 16 hpf reveals that GFP$^+$ neurons are first present at the anterior midbrain from 18 hpf: they divide across the midline, similar to spinal cord and hindbrain neurons (Tawk et al.,

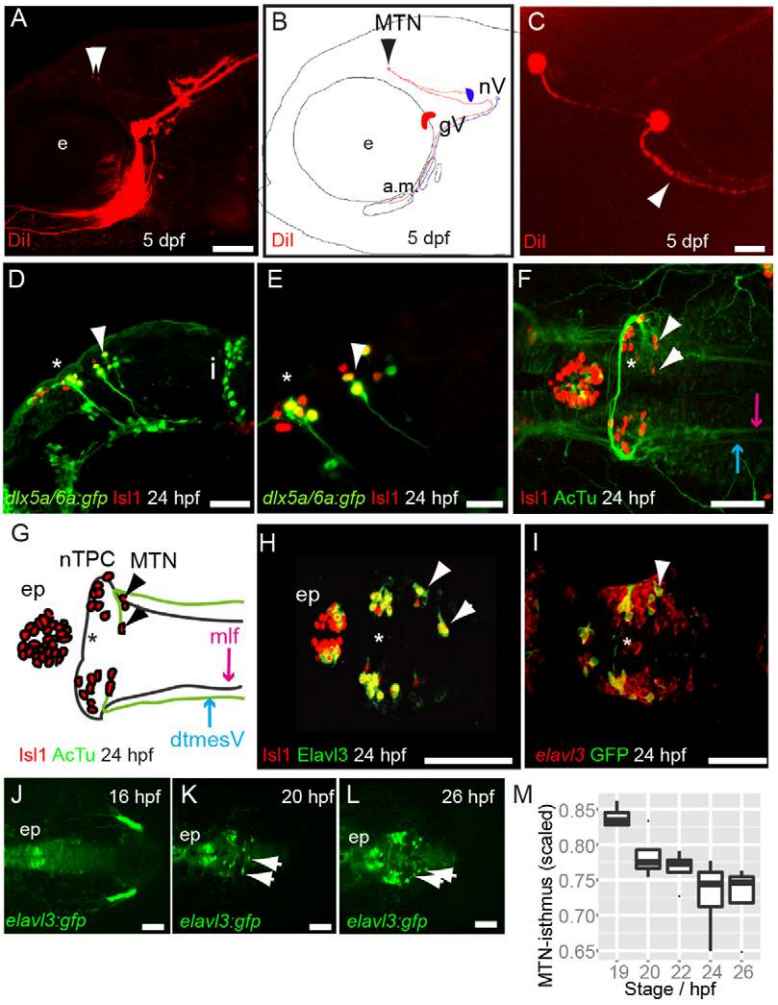

Fig. 1. MTN neurons are the first differentiating neurons in the dorsal midbrain and innervate jaw muscles. Lateral view (A) and schematic (B) of 5 dpf zebrafish larvae with DiI (red) applied to the a.m. muscle showing labelled axons and MTN neurons (arrowheads) in the midbrain. Dorsal view (C) of DiI-labelled MTN neurons reveals a single axonal process that splits into two branches (arrowhead). Lateral views of 24 hpf *Tg[dlx5a/6a:egfp]* embryos with nTPC (asterisk) and MTN (arrowheads) labelled by GFP and Isl1 (D,E). Dorsal view and schematic of 24 hpf embryos with anti-Isl1 and acetylated tubulin (AcTu) labelling reveal that MTN neurons (arrowheads) pioneer the dtmesV (blue) and that nTPC neurons (asterisk) contribute to the mlf (purple) axon tracts (F,G). Dorsal views of 24 hpf embryos reveal that MTN and nTPC neurons are positive for Isl1 and Elavl3 (HuC) and express GFP in a *Tg[elavl3:gfp]* transgenic line (H,I); by contrast, more posteriorly located *elavl3*-expressing progenitor cells do not express GFP at similar stages (I). Dorsal views of *Tg[elavl3:gfp]* embryos at various stages reveal that MTN neurons (arrowheads) arise at the dorsal midline in the anterior midbrain and pioneer axon tracts ventrolaterally (J-L). A plot of the distance between MTN neurons and the isthmus, corrected for midbrain size, reveals that MTN neurons are formed at progressively posterior positions over time (M). gV, trigeminal ganglia; e, eye; a.m., adductor mandibulae; nV, trigeminal motoneurons; i, isthmus; mlf, medial longitudinal fasicle; ep, epiphysis; MTN, mesencephalic trigeminal nucleus; nTPC, nucleus of the tract of the posterior commissure; dtmesV, dorsal tract of the mesencephalic trigeminal. Scale bars: 100 µm in A,D,F-L; 20 µm in C,E.

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2007), and rapidly move laterally while growing axons that pioneer the dtmesV (Fig. 1J-L). By 24 hpf, anterior GFP$^+$ neurons were Elavl3$^+$ Isl1$^+$ and later born MTN neurons formed at progressively posterior levels. We compared MTN position with developmental stage and found strong support for a model that links MTN neuron position with time (Fig. 1M; supplementary material Table S1). Our finding that MTN neuron formation occurs in a spatiotemporal manner along the A-P axis of the midbrain suggested that there is a mechanism spatially controlling the differentiation of neurons across the midbrain.

MTN formation is regulated by Wnt and FGF signalling

Wnts and FGFs are key regulators of midbrain development and their expression persists in the isthmus at stages when MTN neurons form, suggesting that they might regulate the A-P onset of MTN formation in the midbrain. We tested whether Wnt and FGF signalling regulate MTN development using zebrafish mutants, transgenics and small-molecule regulators. Abrogation of FGF signalling in *fgf8a* hypomorphic mutants or after treatment with the FGF receptor inhibitor SU5402 from 14 hpf, when midbrain specification has occurred (Scholpp et al., 2003), resulted in an increased number of MTN neurons (Fig. 2A,B,K); by contrast, upregulation of FGF activity by overexpression of a constitutively active Fgf receptor 1 (CA-fgfr1) at 16.5 hpf resulted in fewer MTN neurons than in control animals (Fig. 2H,I,K). Inhibition of Wnt signalling, by overexpression of the Wnt-binding protein Dickkopf 1 (Dkk1) or application of the Tankyrase inhibitor IWR-1, resulted in fewer MTN neurons (Fig. 2D-G,J). By contrast, adding the Gsk3 inhibitor BIO from 14 hpf resulted in an increased number of MTN neurons (Fig. 2A,C,J).

As both BIO and SU5402 application resulted in more MTN neurons, we tested whether proliferation was affected prior to MTN formation by measuring the number of GFP$^+$ cells in the midbrain of *Tg[her5:egfp]* embryos that expressed phospho-Histone H3 or the neuronal specifying gene *elavl3*, but found no difference (P>0.05; supplementary material Fig. S2F-K). Furthermore, MTN, but not nTPC, neuron number was specifically affected by these treatments and there was no change to the midbrain or diencephalic identity as assessed by *pax7* or *pax6* expression relative to differentiated neurons (supplementary material Fig. S2B-E; data not shown). Therefore, manipulation of Wnt or FGF from 14 hpf affected the rate of neuronal formation specifically in the midbrain, but did not affect midbrain identity or cell proliferation. If FGF activity regulates the number of neurons that form in the midbrain, there should be a dose-dependent effect of FGF activity on MTN number. We observed a statistically significant difference between the change in the number of MTN neurons when exposed to 10 µM versus 20 µM SU5402, revealing an FGF activity-dependent regulation of MTN development (supplementary material Fig. S2A). Intriguingly, our results showed that Wnt and FGF signalling regulate MTN development in an opposite manner: Wnt promotes MTN formation, whereas FGF inhibits it.

Her5 is FGF regulated and directs MTN number and positioning

In zebrafish, Her5 acts to prevent the differentiation of *elavl3*$^+$ neuronal progenitors at the isthmus and so ensures that cells required for later growth of midbrain structures are maintained in a progenitor state (Geling et al., 2003; Geling et al., 2004). We noted that *her5* is also expressed in the dorsal midbrain at stages prior to MTN differentiation. There are many *elavl3*$^+$ cells along the entire A-P extent of the dorsal midbrain, but differentiating Elavl3$^+$ MTN

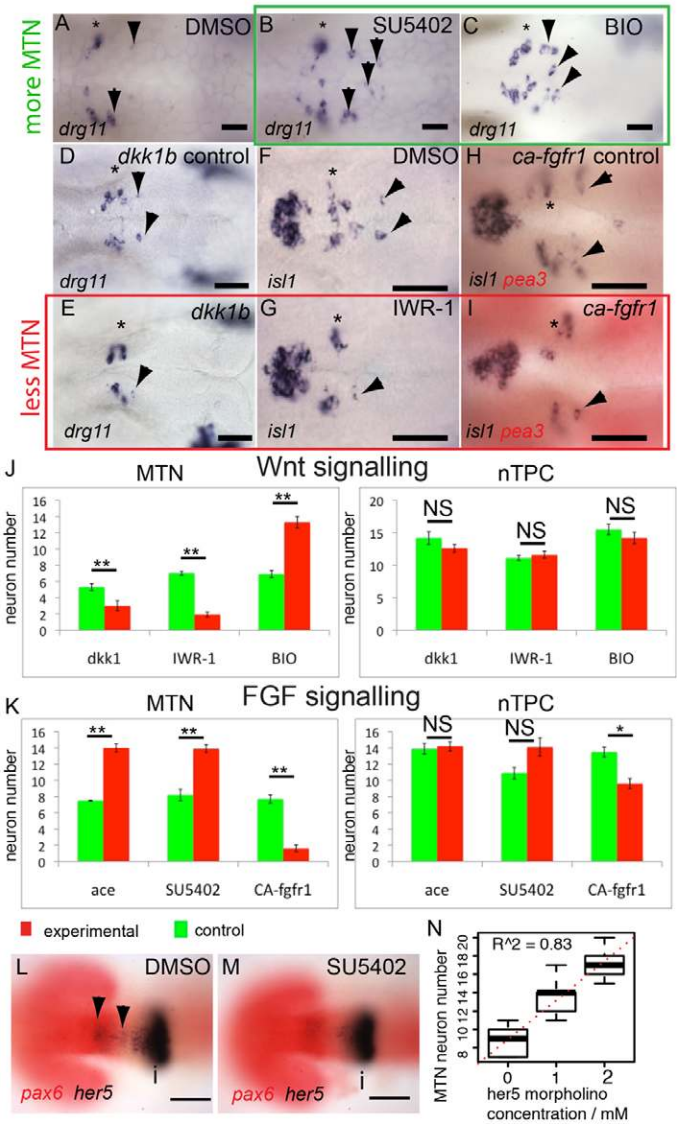

Fig. 2. FGF, Wnt and Her5 dictate the number of MTN neurons that form in the midbrain. *In situ* hybridisation with probes for *drg11* (A-E) and *isl1* (F-I) reveals increased numbers of MTN neurons in zebrafish embryos exposed to 40 µM SU5402 (B) or 4 µM BIO (C) from 14 hpf (green box), relative to DMSO treatment (A). Overexpression of Dkk1b in *Tg[hsp70l:dkk1b-egfp]* embryos (D,E), treatment with 40 µM IWR-1 (F,G) or overexpression of a constitutively active Fgfr1 in *Tg[hsp70:ca-fgfr1]* embryos (H,I) also results in fewer MTN neurons (red box). Arrowheads indicate MTN neurons; asterisks indicate nTPC neurons. Quantification of neurons in animals with altered FGF (K) or Wnt (J) activity in the midbrain reveals a highly significant increase in MTN (P<0.01) but not nTPC (P>0.01) neuron number at 24 hpf for all conditions. ace, acerebellar. *her5* expression in the dorsal midbrain (arrowheads) is lost by 20 hpf after application of 20 µM SU5402 at 14 hpf (M), but isthmus expression if unaffected relative to DMSO-treated controls (L). Plots of MTN and nTPC numbers in Her5 morphants reveals that loss of Her5 function causes a dose-dependent increase in MTN (R^2=0.83) by 24 hpf (N). Error bars indicate s.e.m. **P<0.01; *P<0.05; NS, not significant; unpaired t-tests were used to compare between conditions; n=10 for each condition. i, isthmus. Scale bars: 100 µm in L,M; 20 µm in A-I.

neurons arise initially in the anterior midbrain and not simultaneously at all positions. We therefore investigated whether Her5 acts to regulate the sequential differentiation of the MTN along the dorsal midbrain. Morpholino knockdown of Her5 affected MTN, but not nTPC, formation and the number of MTN neurons increased

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in a dose-responsive manner relative to Her5 morpholino concentration (Fig. 2N). Both ablation of Her5 function and inhibition of FGF activity from 14 hpf resulted in an increase in MTN neuron number. This implies that FGF signalling may control Her5 function, as suggested by analyses of *fgf8a* hypomorphic mutants (Tallafuss and Bally-Cuif, 2003).

We tested the temporal requirement for FGF signalling by *her5* during midbrain development by applying SU5402 from 14-24 hpf and observed a loss of *her5* expression specifically in the dorsal midbrain, but not in the isthmus (Fig. 2L,M). Our results revealed that FGF activity regulates *her5* expression; this suggests that a graded FGF activity across the midbrain could dictate the spatial limit of *her5* expression along the A-P extent of the midbrain and hence control the spatial onset of neuronal differentiation.

FGF activity shows a dynamic posterior movement prior to MTN formation

We found that the level of FGF signalling activity regulates the number of MTN neurons that form. As MTN neurons form at progressively posterior positions over time, this might reflect a posterior shift of FGF activity. In support of this, we found that *her5* and *pea3* (*etv4* – Zebrafish Information Network) showed a posterior retraction towards the isthmus between 14 and 24 hpf, suggesting that FGF activity across the midbrain becomes reduced at stages prior to MTN formation (supplementary material Fig. S3A-F).

If FGF activity acts in a gradient to drive the differentiation of MTN neurons along the A-P axis of the midbrain, an increase in MTN neuron number after reducing FGF activity should correlate with neurons lying closer to the isthmus. We found a strong correlation between MTN neuron numbers and their proximity to the isthmus after manipulating either Wnt or FGF signalling (Fig. 3A). This was not due to alterations of midbrain size, as midbrain size did not correlate with MTN or nTPC neuron number or the concentration of BIO or SU5402 (supplementary material Fig. S3G,H,K,L). Rather, increased MTN neuron number correlated with reduced FGF activity (Fig. 3B).

As FGF signalling is required for *her5* expression in the dorsal midbrain prior to MTN differentiation and we have found that FGF activity directs where MTN neurons form along the A-P axis, we hypothesised that FGF-directed Her5 function dictates both MTN positioning and number. Indeed, we noted a strong correlation between MTN number and position along the A-P axis of the midbrain at various levels of Her5 activity (Fig. 3C). This indicates that FGF activity dictates the spatial expression of Her5 along the midbrain and that Her5 in turn acts in a dose-responsive manner to regulate the number and positioning of MTN neurons.

Wnt signalling controls MTN neuron number and positioning by regulating FGF activity

Reduced FGF activity or elevated Wnt activity causes an increased number of MTN neurons to form (Fig. 2J,K). As Wnt and FGF have been shown to co-regulate each other's expression at the isthmus, it is unclear why opposite manipulations of their activity affect MTN development in the same manner. We therefore tested how simultaneous manipulation of both pathways affected MTN neuron number and positioning. BIO and SU5402 were simultaneously applied at a variety of concentrations and unified models were tested for their fit to the data and for how well they explained the relative importance of BIO, SU5402 and any BIO-SU5402 interactions. Simple linear models explain the number of MTN neurons (n) and their position relative to the isthmus (d), dependent on the BIO and

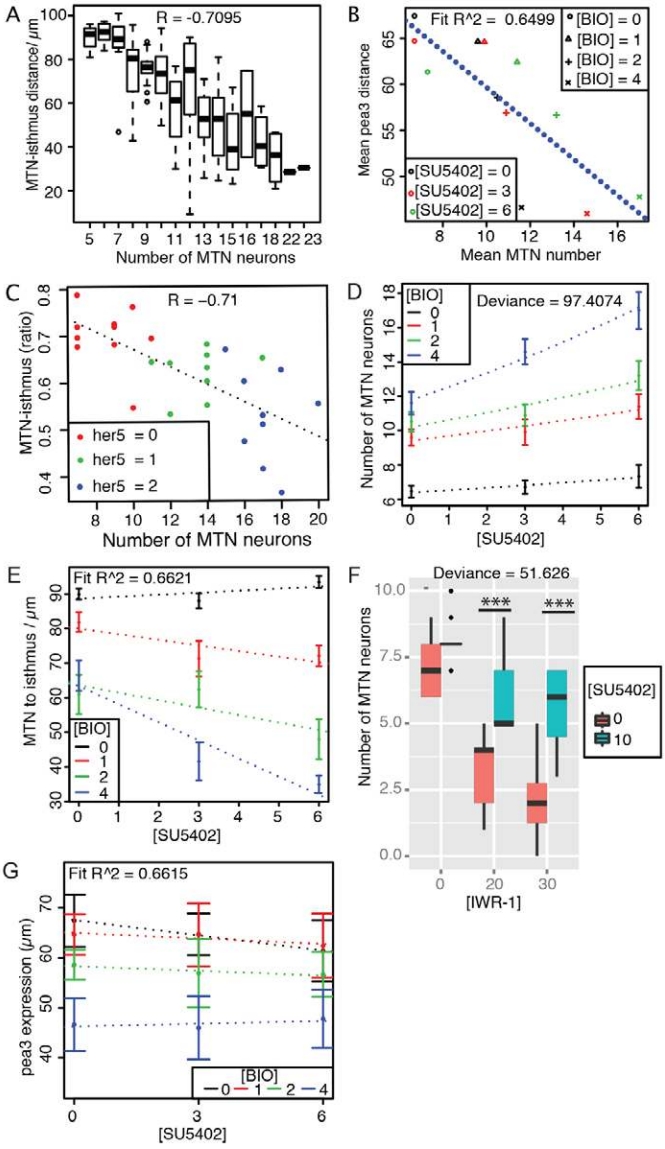

Fig. 3. An FGF gradient regulates the positional onset of neuronal differentiation in the midbrain and is modified by Wnt activity. A plot of distances between the most posterior MTN neurons and the isthmus reveals a strong correlation with the number of MTN neurons (R=–0.709, n=120) at 24 hpf (A). A plot of *pea3* expression relative to MTN neuron number under varying BIO and SU5402 concentrations reveals that MTN neuron number increases when *pea3* expression is reduced (B, R^2=0.649, n=240). Increasing doses of Her5 morpholino (1 nl of 0, 1, 2 mM) correlates with an increase in MTN neuron number and closer proximity of MTN neurons to the isthmus (C, R=–0.71, n=30). In plots of MTN neuron numbers (D) and position relative to the isthmus (E) at 24 hpf after simultaneous application of SU5402 (0, 3, 6 µM) and BIO (0, 1, 2, 4 µM) from 14 hpf, lines were fitted to the data and the slope represents how MTN neuron number or position changes relative to different SU5402 or BIO doses. Increasing doses of BIO enhances the concentration-dependent responses of MTN neuron number (deviance=97.4074) and position (R^2=0.66) to SU5402. Plots of MTN number at 24 hpf after addition of 0 or 10 µM SU5402 and IWR-1 at varying concentrations reveals a significant increase in MTN neuron number (***P<0.001) when SU5402 is added (F). Plots of *pea3* expression after BIO or SU5402 is added at 14 hpf reveals that BIO results in lower *pea3* expression and reduces the responsiveness of *pea3* to SU5402 (R^2=0.66), as reflected in the changing slopes of the fitted lines (G). Data show average and s.e.m. (D,E,G); the model agreement is reported by the R^2 value for linear models (E,G) and the deviance for generalised linear models with a Poisson link function (D,F).

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SU5402 concentration ([BIO], [SU5402]), with rates l and k describing how the concentration of BIO and SU5402 and the interaction between BIO and SU5402 dictate d and n, respectively:

$$d_0 = d_0 + k_{BIO}[\text{BIO}] + k_{SU5402}[\text{SU5402}] + k_{Interaction}[\text{BIO}][\text{SU542}],$$

$$n_0 = n_0 + l_{BIO}[\text{BIO}] + l_{SU5402}[\text{SU5402}] + l_{Interaction}[\text{BIO}][\text{SU5402}].$$

The probability for each rate reflects their importance in regulating MTN neuron number and positioning (supplementary material Tables S2 and S3). We found that elevated Wnt activity enhances the effects of decreased FGF activity on MTN formation. This revealed that Wnt can regulate the spatial response of neuronal progenitors to FGF activity across the midbrain (Fig. 3D,E). Wnt-FGF interactions were also important for dictating neuronal number and positioning, but the nature of this interaction is unclear. As FGF activity determines the spatial distribution of MTN neurons and the models predict that Wnt-FGF interactions are important for regulating MTN number and positioning, it was important to clarify how Wnt and FGF signalling interact. Our hypothesis was that Wnt signalling acts to prevent the inhibitory action of FGF activity on MTN formation. We tested this by simultaneous inhibition of Wnt and FGF signalling using SU5402 and IWR-1 and asked whether MTN development could be rescued in the absence of Wnt activity if FGF is also inhibited. We observed a dose-dependent rescue of MTN neuron number by IWR-1 in the presence of SU5402, confirming our hypothesis (Fig. 3F; supplementary material Table S4).

Wnt-FGF interactions regulate FGF activity across the midbrain

Our modelling suggested that Wnt-FGF interactions are important for regulating MTN positioning. We therefore aimed to understand how these interactions direct FGF activity across the midbrain and used a similar approach to that above to show how simultaneous application of SU5402 and BIO affected FGF activity by measuring *pea3* expression at the isthmus. As Wnt activity is increased, *pea3* expression is reduced and the response of *pea3* to SU5402 is attenuated (Fig. 3G; supplementary material Fig. S3I,J). Non-linear models describe the responses of *pea3* to SU5402 at different BIO concentrations and revealed that elevated Wnt activity acts to reduce FGF activity across the midbrain (supplementary material Table S5). This implies that increased Wnt signalling causes the formation of MTN neurons at more posterior locations of the midbrain by reducing FGF activity across the midbrain.

To explore the interactions between Wnt and FGF signalling at neurogenesis stages in the midbrain we examined the expression of *fgf8a*, *pea3*, *wnt1*, *lef1* and *dgfp* (which encodes destabilised GFP) in a Wnt reporter line (*Tg[top:dgfp]*) after upregulation or downregulation of Wnt or FGF signalling from 14-16.5 hpf (Fig. 4A-D; supplementary material Fig. S4A-E). Inhibition of FGF signalling led to reduced expression of Wnt-responsive (*lef1*, *axin2*, *dgfp*) and FGF-responsive (*pea3*) genes and of *wnt1* and *fgf8a*. By contrast, overactivation of FGF signalling led to increased expression of all of these genes except for *fgf8a*, suggesting a key requirement for FGF activity in regulating Wnt signalling during midbrain neurogenesis (Fig. 4B). Inhibition of Wnt signalling also resulted in a loss of Wnt- and FGF-responsive genes, but did not affect *wnt1* or *fgf8a* (Fig. 4D). Overactivation of Wnt signalling resulted in elevated expression of Wnt-responsive genes as predicted, but FGF-responsive genes were downregulated. In contrast to upregulation of FGF signalling, elevated Wnt signalling led to a loss of *wnt1* expression and to upregulation of *fgf8a* expression (Fig. 4D). This reveals that manipulations of Wnt and FGF signalling do not elicit the same responses: fluctuations of FGF activity affect both pathways in the same way, whereas changes to Wnt signalling will inhibit FGF activity across the midbrain.

To investigate the requirements for FGF activity by Wnt signalling, we tested whether overactivation of Wnt signalling (by β-catenin overexpression or BIO) affected the changes in gene expression caused by loss of FGF activity (by SU5402). *pea3* and *wnt1* were downregulated, arguing that FGF activity is needed for their expression regardless of Wnt activity (supplementary material Fig. S4M,Q). *fgf8a* was not highly upregulated when β-catenin was overexpressed, in contrast to BIO application, but in the presence of SU5402 β-catenin overexpression rescued *fgf8a* expression, revealing that *fgf8a* expression is regulated by Wnt signalling (supplementary material Fig. S4F-I). As inhibition of FGF activity rescued MTN neuron formation when Wnt signalling is reduced (Fig. 3F), we examined whether Wnt signalling regulates FGF target gene expression across the midbrain independently of FGF signalling by applying both IWR-1 and SU5402. FGF-responsive (*pea3*) and Wnt-responsive (*lef1*) genes were downregulated, whereas *fgf8a* and *wnt1* were unaffected (supplementary material Fig. S5). Our analyses revealed that fluctuations of Wnt activity inhibit FGF activity across the midbrain.

As we found that FGF signalling directs *her5* expression across the midbrain, we predicted that either elevated Wnt or reduced FGF signalling would reduce the spatial expression of *her5* and *pea3* at stages prior to MTN formation. We tested this by examining *her5* and *pea3* expression at several stages after exposure to varying BIO or SU5402 concentrations. Polynomial quadratic equations were fitted to plots of gene expression to reveal the temporal responses of *pea3* and *her5*. Both genes showed similar responses to high concentrations of BIO and SU5402 (Fig. 4F-H). This agrees with our predictions that Wnt signalling regulates FGF activity across the midbrain and that elevated Wnt activity will inhibit responses to FGF signalling. Similar to *pea3* (Fig. 4Dd), *her5* expression at 20 hpf is reduced when Wnt signalling is inhibited at 16.5 hpf (Fig. 4I-K), revealing a requirement for Wnt activity to maintain FGF-dependent gene expression; by contrast, shortly after Wnt activity is inhibited by applying IWR-1 at 14 hpf, *her5* expression appeared to be upregulated (Fig. 4L,M), although this was not significant ($P<0.05$; data not shown). If Wnt signalling controls *her5* expression along the dorsal midbrain by regulating FGF activity, overactivation of Wnt should exacerbate reductions in *her5* caused by reduced FGF activity. We tested this by overexpressing β-catenin in the presence or absence of SU5402 and noted that both upregulating Wnt and downregulating FGF caused a further reduction in *her5* expression than either manipulation alone (Fig. 4N-Q). Therefore, Wnt signalling can regulate the spatial expression of *her5* in the dorsal midbrain and this might explain why we observe more posteriorly located MTN neurons in embryos with elevated Wnt signalling. Intriguingly, lower BIO concentrations did not cause a simple downregulation of *pea3* and *her5* expression, but led to a fluctuating response that might reflect attempted compensation by Wnt-FGF feedback loops (Fig. 4H).

Wnt regulation of Sprouty genes modulates FGF activity in the midbrain

We found that changes to Wnt signalling result in decreased FGF activity in the midbrain. As we have shown that Wnt signalling is regulated by FGF activity, this implies that Wnt directs a negative-feedback loop to inhibit FGF signalling at the isthmus. Sprouty

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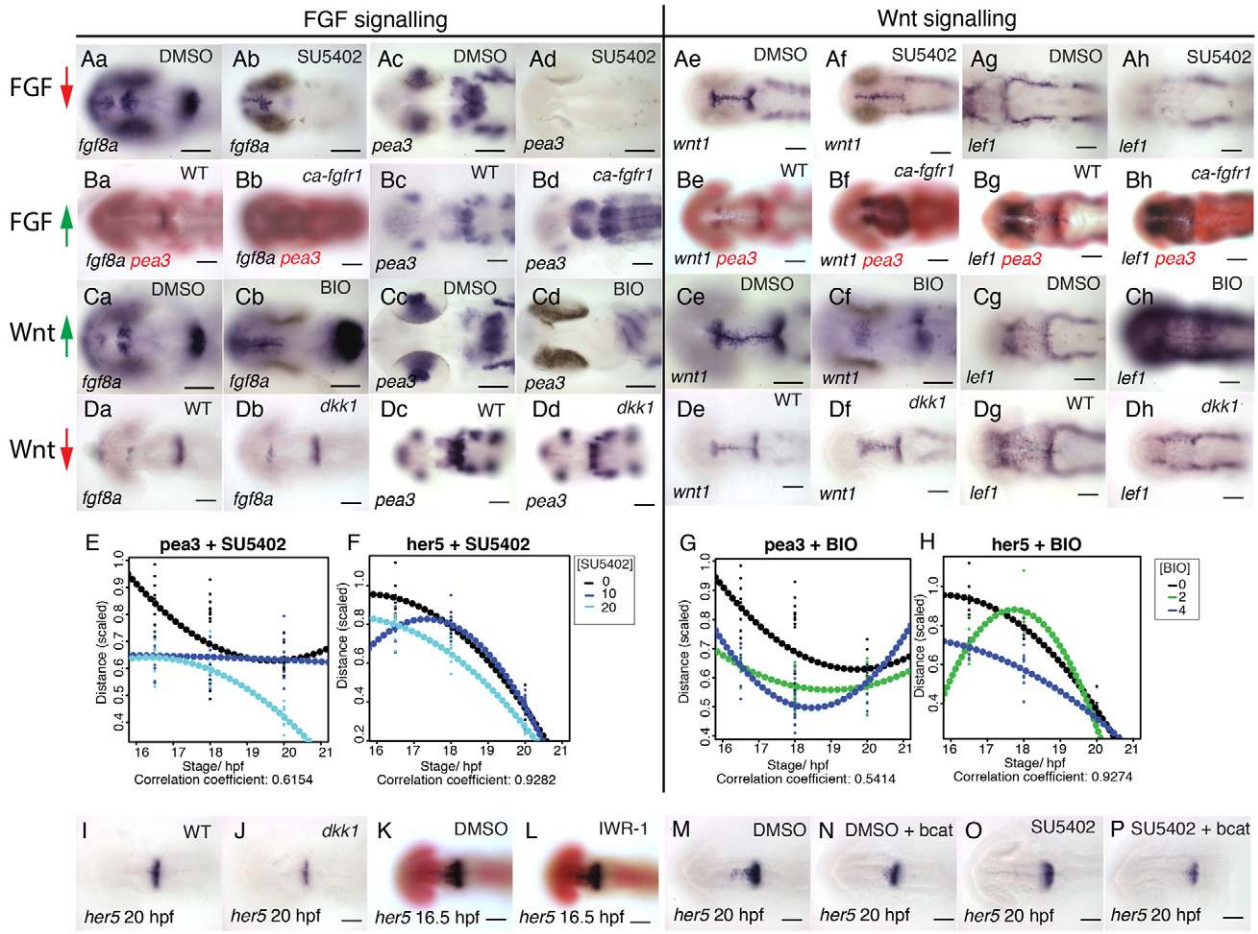

Fig. 4. FGF and Wnt signalling interact to regulate each other's activity. Dorsal views of zebrafish embryos processed for *in situ* hybridisation with probes to *fgf8a* (a,b in A-D), *pea3* (c,d in A-D), *wnt1* (e,f in A-D) and *lef1* (g,h in A-D) following treatment with 40 µM SU5402 (Aa-h) or 4 µM BIO (Ca-h) or DMSO from 14 hpf or in transgenic *Tg[hsp70:dkk1b-egfp]* (Da-h), *Tg[hsp70:ca-fgfr1]* (Ba-h) and non-transgenic siblings after heat shock induction at 16.5 hpf. Spatial expression of *pea3* (E,G) and *her5* (F,H) across the dorsal midbrain was measured at 16.5, 18 and 20 hpf (n=10 for each stage) following exposure to SU5402 (E,F; 0, 10, 20 µM) or BIO (G,H; 0, 3, 6 µM). Polynomial plots (dotted line) were generated from quadratic equations describing the expression of each gene over time under the various conditions. Dorsal views of embryos processed by *in situ* hybridisation with probes to *her5* (I-P) and *pax6* (red, K,L) following treatment with 10 µM SU5402 (O,P), 40 µM IWR-1 (L) or DMSO from 14 hpf (K,M,N) in wild type (K,L), transgenic *Tg[hsp70:dkk1b-egfp]* (J) and non-transgenic siblings (I) or *Tg[UAS:HA-bcat]; Tg[hsp70:gal4]* (N,P) and non-transgenic siblings (M,O) after heat-shock induction at 16.5 hpf. Scale bars: 100 µm.

proteins are well-known inhibitors of FGF receptors and a number of Sprouty genes have been shown to be regulated by FGF signalling (Hacohen et al., 1998; Minowada et al., 1999; Fürthauer et al., 2001; Hanafusa et al., 2002).

In zebrafish, *spry4* is the principal Sprouty family gene regulating midbrain development and is therefore a good candidate for regulating FGF activity at the isthmus (Fürthauer et al., 2001). *spry4* is restricted to the isthmus and expression becomes more restricted from 14 to 24 hpf (Fig. 5A). As *spry4* is described as an FGF-responsive gene, we measured how *spry4* expression is affected by treatment with variable doses of SU5402. Polynomial plots of *spry4* expression revealed that the overall expression profile was unaffected by SU5402 despite a reduction in expression at high doses of SU5402 (Fig. 5F). By contrast, treatment with BIO or IWR-1 dramatically altered the expression profile and caused a change to the level of *spry4* expression (Fig. 5B-E,G). These data suggested that *spry4* is regulated by Wnt signalling independently of FGF signalling. We therefore compared the response of *spry4* to a well-characterised FGF-regulated gene, *pea3*, to see whether they showed similar responses to a loss of Wnt signalling. We found that the *spry4* response to Dkk1 overexpression was significantly more rapid than that of *pea3*, with a significant change at 18 hpf, compared with *pea3* for which differences were not apparent until 20 hpf (Fig. 5H; supplementary material Table S6).

To determine whether this response of *spry4* to a loss of Wnt signalling reflects a different response to other FGF-regulated genes we analysed global gene expression changes after manipulation of Wnt signalling. Using a combined score indicating how responsive genes are to the upregulation and downregulation of Wnt activity, we note that *spry4* was highly responsive to Wnt signalling, similar to Wnt target genes such as *sp5* and *axin2*. By contrast, *pea3*, *her5* and *fgf8a* did not show such a Wnt-responsive change in expression (supplementary material Fig. S6A; data available from ArrayExpress with accession number E-MTAB-1887).

As our data imply that *spry4* is both FGF and Wnt responsive, it should therefore be able to respond to both pathways. If this is the case, increased Wnt signalling should drive *spry4* expression when FGF activity is reduced. We tested this by application of BIO and SU5402 and measured *spry4* expression. We found that there was increased expression of *spry4* when BIO was added with SU5402

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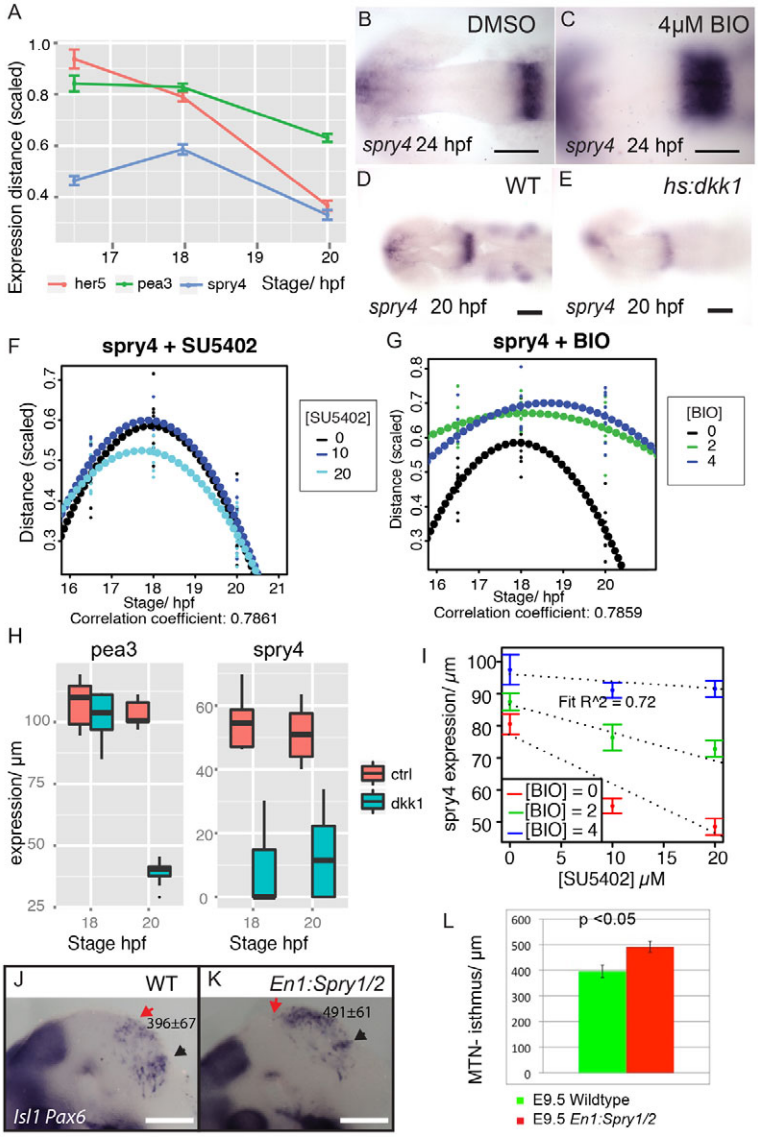

Fig. 5. Wnt regulation of Sprouty genes controls FGF activity at the isthmus and directs neuronal positioning. Zebrafish *pea3*, *her5* and *spry4* midbrain expression between 16.5 and 20 hpf (A); distance is scaled by midbrain size; error bars indicate s.e.m., *n*=30. Expression of *spry4* at 24 hpf in embryos treated from 14 hpf with DMSO (B) or 4 μM BIO (C) or *spry4* at 20 hpf in *Tg[hsp70:dkk1b-egfp]* transgenic embryos (E) and siblings (D) after heat-shock at 16.5 hpf. Expression (scaled to midbrain size) of *spry4* in the dorsal midbrain between 16.5 and 20 hpf after exposure to SU5402 (F) or BIO (G) from 14 hpf. Quadratic polynomials (dotted line) were fitted to plots of *spry4* expression (μm) over time after DMSO, SU5402 or BIO exposure from 14 hpf at varying concentrations (*n*=10 for each condition). Plots of *pea3* and *spry4* expression at 18 and 20 hpf, following heat-shock induction of *dkk1* at 16.5 hpf in *Tg[hsp70:dkk1b-egfp]* embryos and non-transgenic siblings (H). Plots of *spry4* expression at 24 hpf after exposure to varying SU5402 and BIO concentrations from 14 hpf were fitted with lines; the decreasing slopes of expression as BIO concentration is increased reveals that BIO upregulates *spry4* and attenuates an SU5402-induced reduction of *spry4* expression (I); data are average with s.e.m. and the model agreement is reported by the R^2 value for linear models (*n*=90). Expression of *Isl1* and *Pax6* in E9.5 wild-type (J) and *En1:Spry1/2*$^{-/-}$ (K) mice was quantified (L) to reveal that MTN neurons are anteriorly displaced after loss of Sprouty function in the midbrain; values represent distance (μm) between the red arrow (MTN) and black arrow (isthmus); error bars indicate s.e.m. (*n*=10). Scale bars: 200 μm in J,K; 100 μm in D,E; 50 μm in B,C.

(Fig. 5I; supplementary material Fig. S6B-H). This Wnt-FGF regulation of *spry4* can be represented by the following linear model relative to BIO and SU5402 concentrations ([BIO], [SU5402]) and the interaction between them ([BIO] × [SU5402]):

$$d(spry4) = (77.20 + 4.75 \times [\mathrm{BIO}]) - (1.53 \times [\mathrm{SU5402}]) + (0.33 \times [\mathrm{BIO}] \times [\mathrm{SU5402}]).$$

This model predicts that *spry4* is upregulated by increased Wnt activity and decreases with reduced FGF activity. Interestingly, it also predicts an interaction effect between the two pathways, such that *spry4* is less sensitive to reductions in FGF activity when Wnt activity is upregulated (Fig. 5I). We therefore tested whether *spry4* expression can be regulated by Wnt when FGF activity is reduced, and found that overexpression of β-catenin rescued the reduced expression of *spry4* caused by SU5402 in the isthmus and telencephalon (supplementary material Fig. S6I-L). To test if Sprouty genes regulate FGF activity across the midbrain and hence MTN positioning, we then used transgenic mice with a tissue-specific loss of Spry1 and Spry2 in the midbrain. We found that ablation of Sprouty gene function results in an anterior displacement of MTN neurons in the midbrain; this contrasts with the posterior positioning of MTN neurons that occurs following a loss of FGF activity or upregulation of Wnt signalling (Fig. 5J-L). This would be predicted if Sprouty genes inhibit FGF activity across the midbrain and hence regulate the positioning of MTN neurons.

DISCUSSION

A major question in biology is how cells respond to multiple signalling pathways to generate diverse cell and phenotypic outputs. Neural patterning is an excellent paradigm for addressing this issue as neurons form at discrete locations and times during development under the influence of multiple signals. We have used the developing midbrain to dissect how interactions between two key signalling pathways direct where and when a discrete class of neurons is formed. A key finding from this work is that Wnt regulates the control of Sprouty genes and FGF signalling. This finding has potential implications for our understanding of how Wnt and FGF signalling may regulate cell decisions in a wide variety of biological situations in which both pathways are active.

Our results reveal that differential FGF activity across the midbrain regulates where and when neuronal progenitors differentiate through FGF regulation of Her5. As FGF activity

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diminishes across the midbrain, *her5* expression retracts posteriorly in the anterior dorsal midbrain and the first neurons differentiate. This function of Her5 in regulating neuronal differentiation in the midbrain of zebrafish is similar to that in the mouse, in which Hes1 expression in the midbrain requires FGF activity, and loss of FGF activity or Hes1/Hes3 function results in premature differentiation of ventricular zone progenitor cells (Hirata et al., 2001; Lahti et al., 2011). Strikingly, we observe that the level of Her5 function dictates not only the number, but also the positioning of neurons along the A-P axis, revealing a mechanism by which FGF activity can direct the spatial onset of neuronal differentiation. We find that this FGF-regulated Her5 expression domain is shaped by Wnt signalling, which has a bi-modal action, both activating FGF gene expression at the isthmus and attenuating FGF receptor activity. A number of models have been proposed that explain how Wnt and FGF co-regulate each other to pattern the midbrain and then control neurogenesis (Liu and Joyner, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). Our findings show that although FGF and Wnt co-regulate the expression of each other's ligands at the isthmus (and hence activity), Wnt signalling also acts to repress FGF receptor activity. We propose that this is principally through Wnt regulation of Sprouty genes in the isthmus that act to repress FGF activity. Sprouty genes have been considered to be principally FGF target genes and interpretation of Sprouty function *in vivo* has relied on this assumption (Jászai et al., 2003; Trokovic et al., 2003; Paridaen et al., 2009). However, human *SPRY4* has conserved LEF binding sites in its promoter (Katoh and Katoh, 2006) and Spry4 is upregulated by Wnt7a (Tennis et al., 2010), suggesting that Sprouty genes might also be Wnt regulated. Our results show that *spry4* does not respond similarly to *pea3* and *her5* when FGF or Wnt signalling are manipulated and, unlike these FGF-responsive genes, *spry4* responds rapidly to changes in Wnt signalling. Our identification of *spry4* as a Wnt target in the midbrain reveals a mechanism whereby Wnt signalling at the isthmus can regulate FGF activity across the midbrain and potentially direct where neurons form. By conditional ablation of Spry1/2 function in the isthmus of mice we have shown that Sprouty genes direct neuronal positioning. This is likely to occur through modulation of FGF activity, as we observe elevated *Pea3* expression in conjunction with anteriorly displaced neurons in these mice, comparable to zebrafish with overexpression of Ca-fgfr1 (data not shown). How Sprouty genes regulate FGF activity across the midbrain is not clear. We find that upregulation of *spry4* by Wnt in zebrafish correlates with elevated *fgf8a* expression in conjunction with repression of FGF activity, whereas loss of Spry1/2 in mice results in a loss of *Fgf8* expression at the isthmus (data not shown). This suggests that Sprouty proteins act to promote *fgf8a* expression, but how this results in an inhibition of FGF activity is unclear.

The regulation of neuronal differentiation in the brain is important to ensure that an appropriate number of neurons form at the correct time and place. In the dorsal midbrain, we show that Her5 directs neuronal formation and is spatially controlled by the level of FGF activity, similar to previously described roles of Hairy genes in ensuring the retention of progenitor cells at the mid-hindbrain boundary in zebrafish and mouse (Hirata et al., 2001; Geling et al., 2003; Tallafuss and Bally-Cuif, 2003). As there are many neuronal progenitor cells expressing *elavl3* present along the A-P extent of the midbrain, we propose that they are primed to differentiate following the posterior retraction of *her5* towards the isthmus. This is corroborated by the lack of changes we find in progenitor proliferation or neurogenic determination when Wnt, FGF or Her5 are perturbed at these stages. This and other studies reveal that FGF activity is a crucial regulator of where and when neurons form in the midbrain. Small fluctuations in FGF activity across the midbrain can therefore alter where early born neurons form and potentially lead to the premature differentiation of progenitor cells that contribute to later forming neuronal populations. During midbrain development, such fluctuations in FGF activity are buffered by compensatory feedback loops between FGF and Wnt signalling in order to ensure that neurons are formed in a controlled spatiotemporal manner (see model in Fig. 6).

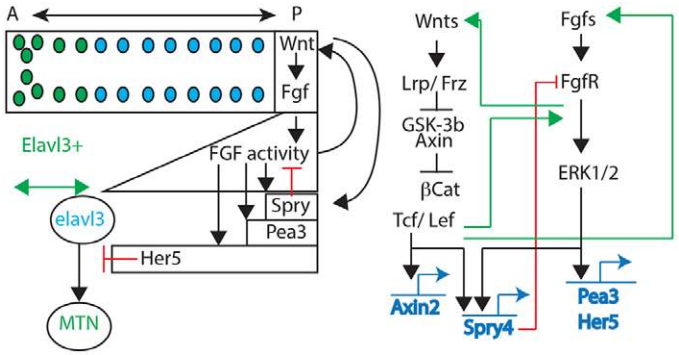

Fig. 6. Model describing how Wnt-FGF cross-regulatory interactions drive the spatiotemporal development of neurons in the dorsal midbrain. At early stages of development, an FGF activity gradient occurs throughout the midbrain from the mid-hindbrain boundary. Nested expression of Sprouty genes (Spry), *pea3* and *her5* reflects their response to differing levels of FGF activity along the anterior-posterior (A-P) axis of the midbrain. Expression of *her5* along the dorsal midbrain is dictated by the FGF activity level and Her5 acts to positionally restrict neuronal differentiation of *elavl3*-expressing progenitor cells. As development proceeds, FGF activity diminishes across the midbrain, resulting in a retraction of the *her5* domain. This leads to a loss of Her5-mediated repression of neuronal differentiation, resulting in the formation of Elavl3^{+} MTN neurons. FGF signalling drives Wnt signalling by regulating the expression of *wnt1*; in turn, Wnt signalling is able to regulate FGF signalling by modulating *fgf8* expression, although Wnt activity is not necessary for *fgf8* expression. Wnt also controls FGF activity through regulation of *spry4* expression; FGF activity also regulates *spry4*, providing a dual regulatory mechanism for controlling FGF activity at the isthmus. Feedback loops between Wnt and FGF mean that fluctuations in FGF activity lead to responses by Wnt signalling that in turn act to attenuate the FGF response and so confer robustness to the system and maintain a steady level of FGF activity. As this FGF activity decreases over time, this mechanism acts to promote a stable change in FGF activity over the midbrain and permits a controlled spatiotemporal onset of neuronal differentiation to occur.

Our model has implications for understanding the basis of mid-hindbrain malformations seen in a number of human patients (Barkovich et al., 2009), as we show that Wnt, FGF, Sprouty and Her genes interact to regulate the spatiotemporal differentiation of neurons across the A-P axis. Given the role that we have identified for Wnt in regulating FGF activity at the isthmus, perturbations of Wnt, Sprouty, Her or FGF function can potentially affect the differentiation of neurons in the tectum and cerebellum. Intriguingly, the positioning of MTN neurons along the A-P axis of the brain varies across the vertebrates (Weinberg, 1928). Our results reveal how MTN neurons in zebrafish and mouse are regulated by FGF activity; small changes in Wnt-directed FGF activity during evolution might therefore provide a potential mechanism for the differing position of MTN neurons among vertebrates. Changes to MTN positioning within the midbrain might have led to the potential to form new connections to adjacent neurons and facilitated the acquisition of new sensory targets of the MTN in jawed vertebrates, including the whisker pad, periodontal ligaments and cranial muscles.

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MATERIALS AND METHODS

Animal care and lines

Zebrafish were kept under standard conditions (Westerfield, 2007) in compliance with UK Home Office regulations. Lines used were: *fgf8a* (*acerebellar*) (Brand et al., 1996), *pax2.1a* (*no isthmus*) (Brand et al., 1996), *Tg[dlx5a/6a:gfp]* (Zerucha et al., 2000), *Tg[elavl3:gfp]* (Park et al., 2000), *Tg[her5:gfp]* (Tallafuss and Bally-Cuif, 2003), *Tg[hsp70l:dkk1b-gfp]* and *Tg[hsp70l:wnt8b-gfp]* (Weidinger et al., 2005; Stoick-Cooper et al., 2007), *Tg[top:dgfp]* (Dorsky et al., 2002), *Tg[hsp70:ca-fgfr1]* (Gonzalez-Quevedo et al., 2010) and *Tg[hsp70:gal4]* (Scheer and Campos-Ortega, 1999). A transgenic line *Tg[UAS:HA-beta-catenin]* was generated by injecting a plasmid containing a stabilised *Xenopus* β-catenin coding sequence cloned in frame with an N-terminal haemagglutinin (HA) tag under the control of the UAS response element into the T2KXIGΔ vector as previously described (Kawakami et al., 2004). All husbandry and procedures performed on mice were approved by the UK Home Office. Conditional *Spry1/Spry2* knockout mice were generated by crossing $En1^{Cre+/-}$ mice with mice carrying both loxP-flanked *Spry1* and *Spry2* alleles as previously described (Yu et al., 2011).

Drug treatments and morpholino injections

SU5402 (Sigma), BIO (Invitrogen) and IWR-1 (Merck) were dissolved in DMSO and diluted in E3. To knock down Her5 function, 1 nl *her5* morpholino diluted to 1 mM or 2 mM (Geling et al., 2003) was injected into one-cell stage zebrafish embryos.

Immunohistochemistry and *in situ* hybridisation

In situ hybridisation (Thisse and Thisse, 2004) and immunohistochemistry (Westerfield, 2007; Nusslein-Volhard and Dahm, 2002) were performed as described previously. Antibodies used were: mouse anti-acetylated tubulin (1:200; Sigma), rabbit anti-GFP (1:500; AMS Biotechnology), mouse anti-HuC/D (1:500; Invitrogen), rabbit anti-phospho-histone H3 (1:200; Sigma), mouse anti-Isl1 (1:200; DSHB) and rat anti-HA (1:300; Roche). Retrograde labelling of axons was performed by applying DiI to zebrafish larvae using a sharpened tungsten needle.

Confocal microscopy

Fluorescent image data were acquired on a Nikon C-1 Eclipse or on an Olympus FV500 microscope and processed using ImageJ (NIH) and Photoshop (Adobe).

Microarray analysis

RNA was extracted from GFP^+ (transgenic) and GFP^- (siblings) *Tg[hsp70l:dkk1-gfp]* and *Tg[hsp70l:wnt8b-gfp]* embryos 4 hours after heat-shock induction at 37°C. RNA was reverse transcribed and expression levels of genes determined using Zebrafish Microarray V2 (Agilent). Microarray hybridization data were analysed using scripts written in the statistical programming language R (R Development Core Team, 2010). From the two transgenic lines, two biological replicates of transgenics and of wild-type siblings each were analysed. Data were processed using the Bioconductor limma package. Background correction was performed using the normexp function. Differentially expressed genes in transgenic embryos relative to siblings were identified using a linear model and multiple testing correction. Microarray data are available from ArrayExpress with accession number E-MTAB-1887.

Quantification and mathematical modelling

Statistical analyses were performed using Excel (Microsoft) or R (R Development Core Team, 2010). *t*-tests were performed using a two-tailed distribution with normal variance after confirming that data showed a normal distribution. For linear models, the coefficient of determination R^2 is shown to quantify the agreement between model values and observations. In the case of generalised linear models, the deviance is used for the same purpose.

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Competing interests

The authors declare no competing financial interests.

Author contributions

R.K. designed the project with E.B., C.D. and M.A.B., and wrote the paper; E.B. and G.O. performed analyses; R.K., C.D., A.H., G.O., H.R. and T.Y. performed experiments.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.099507/-/DC1

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