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Sustained interactive Wnt and FGF signaling is required to maintain isthmic identity

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Abstract

Fibroblast growth factor 8 (FGF8) is expressed at the mid–hindbrain boundary and is an important signal emanating from the isthmic organizer. *Wnt1* is expressed in the caudal midbrain juxtaposed to *Fgf8* expression and has been implicated in its regulation. In this study, we examine the requirement for continuous Wnt signaling in the maintenance of *Fgf8* expression at the isthmus. We demonstrate that prior to HH10, ongoing Wnt signaling is required to maintain the normal pattern of isthmic *Fgf8* expression *in ovo*. Similarly, in explant assays, sustained Wnt signaling is essential to maintain *Fgf8* expression in rhombomere 1. The mechanism by which Wnt signaling regulates isthmic *Fgf8* expression is likely to be a maintenance response rather than an inductive effect. Finally, we show that Wnt maintenance of *Fgf8* expression is dependent upon positive feedback by FGF signaling itself, and that rhombomere 1 does not receive instructive cues from the posterior hindbrain. In summary, these findings establish that a sustained reciprocal interaction between Wnt and FGF signaling is essential to maintain isthmic identity.

Keywords: Isthmus; *Fgf8*; *Wnt*; Maintenance; Rhombomere 1; Chick

Introduction

The isthmic organizer (IsO) situated at the mid–hindbrain boundary (MHB) is a key signaling centre that controls regional identity in both the midbrain and anterior hindbrain (Alvarado-Mallart, 2005; Nakamura and Watanabe, 2005; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001) and positions both the rhombomere 1 (r1) – rhombomere 2 (r2) and diencephalic–mesencephalic boundaries (Irving and Mason, 2000; Scholpp and Brand, 2003). Fibroblast growth factor 8 (FGF 8) is expressed across the morphological isthmic constriction in amniotes in a territory known to be the most anterior part of r1 (Crossley et al., 1996; Shamim et al., 1999; Wingate and Hatten, 1999). Studies in mouse, fish and chick show that FGF8 is an important signaling factor emanating from the IsO and is sufficient to mimic organizer activity first detected in heterotopic grafts of the IsO itself (Chi et al., 2003; Crossley et al., 1996; Irving and Mason, 1999; Lee et al., 1997; Liu et al., 1999; Liu and Joyner, 2001; Martinez et al., 1999; Meyers et al., 1998; Reifers et al., 1998; Sato and Nakamura, 2004; Shamim et al., 1999). Another key signaling molecule, *Wnt1*, is expressed rostral and immediately adjacent to *Fgf8* at the MHB and is required for proliferation and survival in the mid–hindbrain region and for maintenance of the IsO in the mouse embryo (Chi et al., 2003; Danielian and McMahon, 1996; Lee et al., 1997; Mastick et al., 1996; McMahon et al., 1992; McMahon and Bradley, 1990; Panhuysen et al., 2004; Sato and Nakamura, 2004; Serbedzija et al., 1996; Thomas and Capecchi, 1990; Trokovic et al., 2003).

We have previously shown that juxtaposing midbrain and r1 tissue both *in vitro* and *in vivo* induces *Fgf8* expression in r1 and generates tissue with the molecular characteristics of the IsO (Irving and Mason, 1999). The identity of the inducing factor(s) was not determined in those studies but trans-filter experiments implicated a diffusible molecule(s). Hence, Wnt1, a secreted factor, and by virtue of its expression domain relative

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to that of *Fgf8* expression (see Fig. 1), was an attractive candidate. However, studies of Wnt1 function in the regulation of *Fgf8*, undertaken in a range of vertebrate embryos, have produced apparently conflicting results.

Mice homozygous for Wnt1 null alleles display a loss of midbrain and anterior hindbrain by E9.5. *Fgf8* expression is initially induced but is subsequently lost (McMahon et al., 1992; McMahon and Bradley, 1990), and a conditional approach to inactivate β-catenin in the neural tube yielded a similar phenotype (Brault et al., 2001). Collectively, these studies demonstrated that Wnt/β-catenin signaling may regulate isthmic *Fgf8* expression in mouse. However, when Wnt1 was expressed under the control of the EN1 locus, *Fgf8* expression did not expand (Panhuysen et al., 2004), implying that in mouse Wnt signaling seems insufficient to induce ectopic *Fgf8* expression. In addition, the overexpression of *En2* in mouse *Wnt1*$^{-/-}$ mutants led to a rescue of the mid–hindbrain phenotype and *Fgf8* expression was now detected (Danielian and McMahon, 1996). This result suggests the existence of two distinct events in the regulation of isthmic genes. First, *En2* may act directly downstream of Wnt signaling to regulate *Fgf8* expression at the MHB. However, the same does not hold true in the chick, where ectopic *En1* expression is insufficient to induce *Fgf8* in the anterior hindbrain (Shamim et al., 1999). Alternatively, the absence of *Wnt1* in mouse leads to a loss of mesencephalic–metencephalic (mes–met) tissue, and the subsequent loss of *Fgf8* may be a secondary effect. This implies that Wnt1 in mouse may not directly regulate *Fgf8* expression. However, as will be discussed in this report, in the chick, Wnt1 appears to play a direct role in the maintenance of *Fgf8* expression, and when Wnt signaling is downregulated at the MHB, a loss of mesencephalic–metencephalic tissue is not observed. These discrepancies further highlight the subtle differences in how isthmic genes and the organizer itself are regulated differentially between the mouse and chick, reinforcing the need to understand these precise regulatory events in detail in different developmental organisms.

Additionally, in chick, studies have suggested that ectopic expression of Wnt1 can alter *Fgf8* expression through an interaction involving Lmx1b, a LIM homeodomain transcription factor (Lee et al., 1997; Matsunaga et al., 2002). Similarly, in zebrafish Lmx1b.1/2 regulates *Wnt1* and *Fgf8* expression, suggesting that these molecules are involved in a regulatory loop (Adams et al., 2000; O'Hara et al., 2005).

A recent study in the chick reports that Wnt and FGF activity is involved in the early establishment of a functional isthmus marked by *Fgf8* expression (Olander et al., 2006). Moreover, it is suggested that these activities are not required post-gastrulation. The differences highlighted in the above studies cloud the issue as to how and when isthmic *Fgf8* is influenced by Wnt signaling. Indeed, the precise relationship between *Wnt1* and *Fgf8* expression in the emergent IsO has not been well characterized.

In this study, we analyze the interactions between Wnt and FGF signaling *in vivo* and in neural explant assays. We provide a precise description of the spatial and temporal relationship between *Wnt1* and *Fgf8* expression in the chick embryo from the time their transcripts are first detected until their abutting expression domains are established. We find that their expression patterns initially overlap significantly and are subsequently refined to adjacent domains at the MHB. Although Wnt signaling is not required post-gastrulation to initiate the IsO (Olander et al., 2006), we demonstrate *in vivo* that Wnt signaling is required from the time *Fgf8* transcripts begin to be expressed to maintain a normal pattern of *Fgf8*

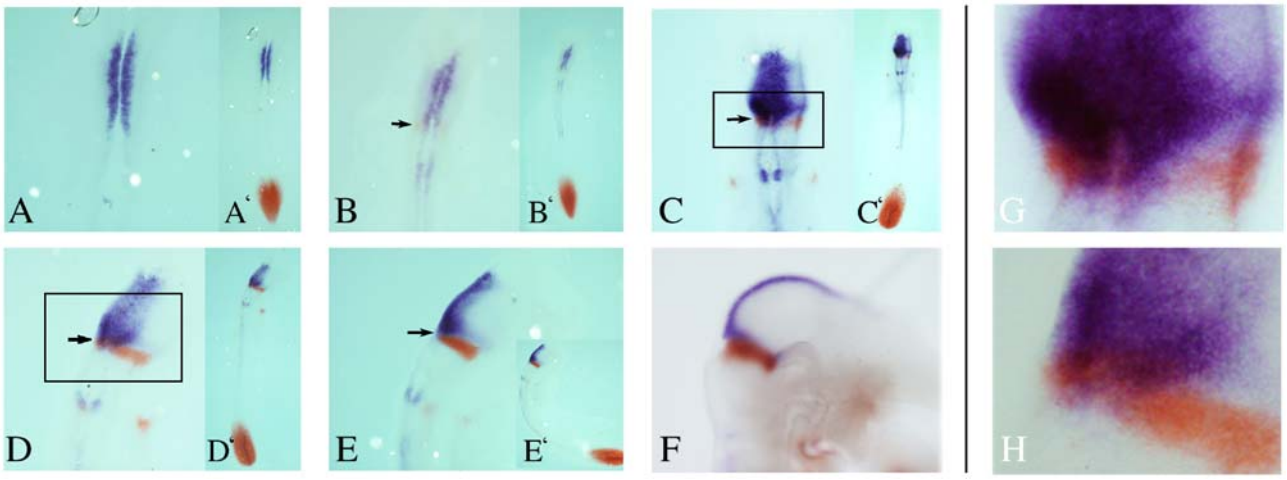

Fig. 1. *Wnt1* and *Fgf8* expression overlap considerably during the emergence of the isthmic organizer. (A–H) Temporal expression analysis of *Wnt1* (blue) and *Fgf8* (red) in the neural plate and neural tube. Endogenous *Wnt1* is first detected in the neural plate at HH6 and although *Fgf8* is expressed in other regions of the embryo it is not detected in the neural plate at HH8+ (A). At HH9−, (B) *Fgf8* transcripts are first detectable in the neural folds overlapping with the caudal-most domain of *Wnt1* (black arrow). At HH10+ the dorsal and caudal-most expression of *Wnt1* overlaps with isthmic *Fgf8* (C, black arrow) and continues to do so at HH11 (D, arrow) and HH12 (E, arrow). By HH16, the expression domains of *Wnt1* and *Fgf8* are completely separate and lie adjacent to each other at the MHB (F). (A–E) High magnification (6×) images of presumptive MHB regions with corresponding lower magnification (1.6×) images of whole embryos (A′–E′). Panels G and H are higher magnifications of the boxed regions in panels C and D, respectively. Embryos (A–H) are flat-mounted preparations under glass coverslips. Anterior is up (A–E) and to the right (F).

expression and IsO identity. Through modulation of Wnt activity at different stages in the embryo, we show that sustained Wnt signaling is required to maintain the correct expression of *Fgf8* at the isthmus. How this spatial expression of *Fgf8* is restricted to anterior rhombomere 1 is not understood. However, we demonstrate that posterior hindbrain signals are unlikely to be involved. Finally, we demonstrate that midbrain-derived Wnt signals are dependent on positive feedback mediated by FGF signaling itself to maintain isthmic identity. This forward regulatory mechanism seems to involve the transcription factors *Pax2* and *En2*. Taken together, these results reveal a previously unrecognized requirement for a continuous interaction between Wnt and FGF signaling to maintain isthmic identity.

Materials and methods

Electroporation of DNA constructs

Fertilized Bovans Goldline eggs were incubated at 38 °C in a humid atmosphere. pCAGGSmWnt1 (2 μg/μl) (Nishihara et al., 2003) or CS2dn-mWNT1 (2 μg/μl) (Hoppler et al., 1996) was electroporated together with pCAGGSeGFP (1 μg/μl) using an Intracel TSS20 at the following settings; 10 V, 4 pulses, 50 ms duration, space 950 ms. HH6 embryos were dissected, washed in PBS and placed in a custom-made Perspex electroporation chamber. Through a small hole in the vitelline membrane, DNA was injected over the neural plate region and current was delivered dorso-ventrally. The EC culture method was used (Chapman et al., 2001) and embryos were harvested between HH10 and HH12.

In situ hybridizations and immunohistochemistry

In situ hybridization used digoxigenin (DIG)-labeled probes as previously described (Irving and Mason, 2000; Shamim et al., 1999). For double *in situ* hybridisation, DIG- and fluorescein (FITC)-labeled probes were added simultaneously. Alkaline phosphatase (AP)-conjugated anti-DIG and anti-FITC (Roche) antibodies were added sequentially. NBT:BCIP (Roche) was used to detect the first reaction and INT:BCIP (Roche) or BCIP (Roche) alone were used to detect the second reaction. Inactivation of AP after the first colour reaction was carried out in TBST at 70 °C for 45 min. Embryos were flat-mounted as previously described (Irving and Mason, 2000).

For antibody staining, explants were fixed, dehydrated and rehydrated (Irving and Mason, 2000), blocked in PBSTx (PBS plus 0.1% Triton 100, 1% serum) and incubated with anti-dpERK (Sigma M8159, 1:250), anti-GFP (Calbiochem, 1:1000) anti-phospho-histone H3 (Upstate Biotech, 1:300). Explants were washed in PBSTx 5–6 times, blocked in PBSTx and incubated with a horseradish peroxidase (HRP) conjugated secondary antibody (Dako, 1:400). HRP detection was carried out using DAB tablets (Sigma). TUNEL staining was performed as per the manufacturer's recommendations (Roche).

Collagen explant assays and reagents

Explant tissues were prepared as previously described (Irving and Mason, 1999). All neural explants were isolated from HH12 embryos and cultured overnight until explants corresponded to HH16. In each experimental setup, all explants used for an individual figure were cultured and processed simultaneously such that experimental conditions were comparable. Collagen solution was made by adding 100 μl 10× MEM (Sigma) and 100 μl bicarbonate buffer (0.1 M NaOH, 240 mM $NaHCO_3$) to 0.8 ml collagen (Vitrogen). Explants were cultured alone in 75% (v/v) Optimem: 25% (v/v) F-12 (Invitrogen), or supplemented with WNT3a (0.1 μg/ml; R&D Systems), SFRP2 (1.0 μg/ml; R&D Systems), 40 mM lithium chloride (Sigma) or SU5402 (50 μM; Calbiochem). SU5402 or GSK-3β inhibitor XI (100 nM; Merck) were either injected directly into the lumen of the neural tube, or affigel blue beads (BioRad) were soaked in SU5402 and inserted into the neuroepithelium.

Luciferase assays

In ovo luciferase assays were carried out on chick neural tube, electroporated at HH10 with reporter plasmids and isolated at HH16 at the level of rostral midbrain to rhombomere 3. For transcription assays, 1.0 μg of TOPFLASH reporter (gift from Hans Clevers) and 2.0 μg of CAGGSmWnt1 effector plasmid were co-injected with 1.0 μg of CMV Renilla Luciferase (Promega) as an internal control. For control embryos, the CAGGSmWnt1 effector was omitted and replaced with empty CAGGS vector. In LiCl-treated embryos, DNA was injected as per controls, and 40 μM LiCl was applied ectopically to the embryo. Embryos were lysed in passive lysis buffer, and luciferase assays were carried out according to the manufacturer's instructions (Promega). The average values were obtained from 8 independent experiments for each condition and data retrieved was shown to be statistically significant.

Results and discussion

WNT activity is required from HH6 onwards for the maintenance of correct Fgf8 expression at the isthmus

Previous studies examined the ability of Wnt1 to regulate isthmic *Fgf8* expression in chick at HH10, a stage when both *Wnt1* and *Fgf8* expression domains are already established (Matsunaga et al., 2002; Ye et al., 2001). We therefore undertook a precise temporal study of *Wnt1* and *Fgf8* expression in the presumptive MHB region to assess the onset of their expression and the precise spatial relationship between these two essential signaling molecules. We first detected *Wnt1* expression in the neural plate at HH6 (data not shown) and demonstrated that at HH8, although *Fgf8* is expressed in the posterior primitive streak and elsewhere in the embryo, transcripts were not yet detected in the neural plate. At this time, however, *Wnt1* was expressed in a broad domain in the neural plate (Fig. 1A). By HH9−, we detected weak transcripts of *Fgf8* at the most caudal domain of *Wnt1* expression, consistent with previous findings (Shamim et al., 1999) (Fig. 1B, arrow). A previously unreported overlap of *Wnt1* and *Fgf8* expression between HH9 and HH12 was observed in the dorsal most neural tube (Figs. 1C–E, see arrows, G and H). However, by HH16 the expression domains of both genes refine to abut each other at the MHB (Fig. 1F). These initially broad and overlapping domains of expression suggest that previous reports in which FGF8-coated beads induced ectopic *Wnt1* expression may have been due to the maintenance of an originally broad *Wnt1* domain that is refined by HH16 (Crossley et al., 1996; Shamim et al., 1999).

Given their early and overlapping expression domains, we investigated the interaction between Wnt1 and FGF8 before the initiation of *Fgf8* expression and later as the respective expression domains are being refined. Electroporation of a full length Wnt1 and a dominant inhibitory form of Wnt1 were carried out at HH6 whereby embryos were allowed to develop overnight until they reached HH10–12. Similarly, both constructs were electroporated at HH10 and embryos were incubated until they reached HH16.

By HH10–12, ectopic expression of Wnt1 induced a posterior expansion of isthmic *Fgf8* expression ($n=18/24$, Figs. 2B and C), compared to control unelectroporated embryos (Fig. 2A). *Fgf8* expression was expanded and detectable only in r1 (Figs. 2B, C). Notably, ectopic transcripts were never observed in the midbrain or more posterior in the hindbrain as observed morphologically and demarcated by double *in situ* for *Fgf8* and *Hoxa2* (data not shown). Co-electroporation of a GFP construct indicated ectopic Wnt1 expression throughout these regions (Fig. 2C, brown immunostain; right inset in Fig. 2B). By contrast, expression of a dominant inhibitory form of Wnt1 resulted in a dramatic reduction of endogenous *Fgf8* transcripts at the isthmus, ($n=8/12$; Fig. 2D). These results indicated that a precise level of Wnt activity is required around the time *Fgf8* expression is initiated and must be sustained to maintain *Fgf8* expression at the MHB.

We therefore sought to address whether sustained activation of the Wnt pathway was required between HH10 and HH16 *in vivo*. Forced expression of Wnt1 at HH10 and examination at HH16 revealed an expanded *Fgf8* domain in almost all embryos analyzed ($n=38/45$; Figs. 2F, H). GFP was co-electroporated with Wnt1 and observed throughout the posterior half of the midbrain and to the level of r3/r4 (Fig. 2G). However, ectopic *Fgf8* transcripts were never observed in the midbrain or caudal hindbrain (compare electroporated embryo Fig. 2F with control unelectroporated embryo Fig. 2E). Perturbation of the Wnt pathway using a dominant negative form of Wnt1 resulted in a reduction of isthmic *Fgf8* expression ($n=28/38$; Fig. 6J, arrow). *Fgf8* expression is never completely abolished, which may be the result of mosaic delivery of expression vectors, or that the dominant inhibitory protein is not completely effective in blocking Wnt signaling. Similarly, we cannot rule out the possibility that additional regulatory factor(s) may be involved.

A recent finding using explant culture reported that Wnt signaling is unnecessary after gastrulation for the establishment of the IsO (Olander et al., 2006). In the presence of their Wnt inhibitor, which differs from that used in this study, both *Wnt1* and *Fgf8* are expressed in the neural plate region albeit at lower levels than controls. The inhibitory Wnt1 construct used in our study rescued the duplicated axis induced by canonical Wnt signaling in *Xenopus* and is therefore functional (data not shown; Hoppler et al., 1996). Although Olander et al. (2006) observe the initial induction of *Fgf8* in the presence of a Wnt inhibitor, it may be that *Fgf8* is subsequently downregulated at later stages in their explant assays. However, in our loss of function assays, we never see a complete loss of *Fgf8* expression at the isthmus, implying that Wnt signaling acts to maintain rather than induce *Fgf8* expression. This is in agreement with mouse genetic data, where *Wnt1* is expressed in *Pax2*$^{-/-}$ mutants, but *Fgf8* transcription is not initiated at the mid–hindbrain boundary (Ye et al., 2001). Our findings, combined with results detailing the importance of mesendoderm-derived Wnt signaling in zebrafish (Rhinn et al., 2005), demonstrate that Wnt signaling *in vivo* is required after gastrulation to maintain *Fgf8* expression as the IsO is being established.

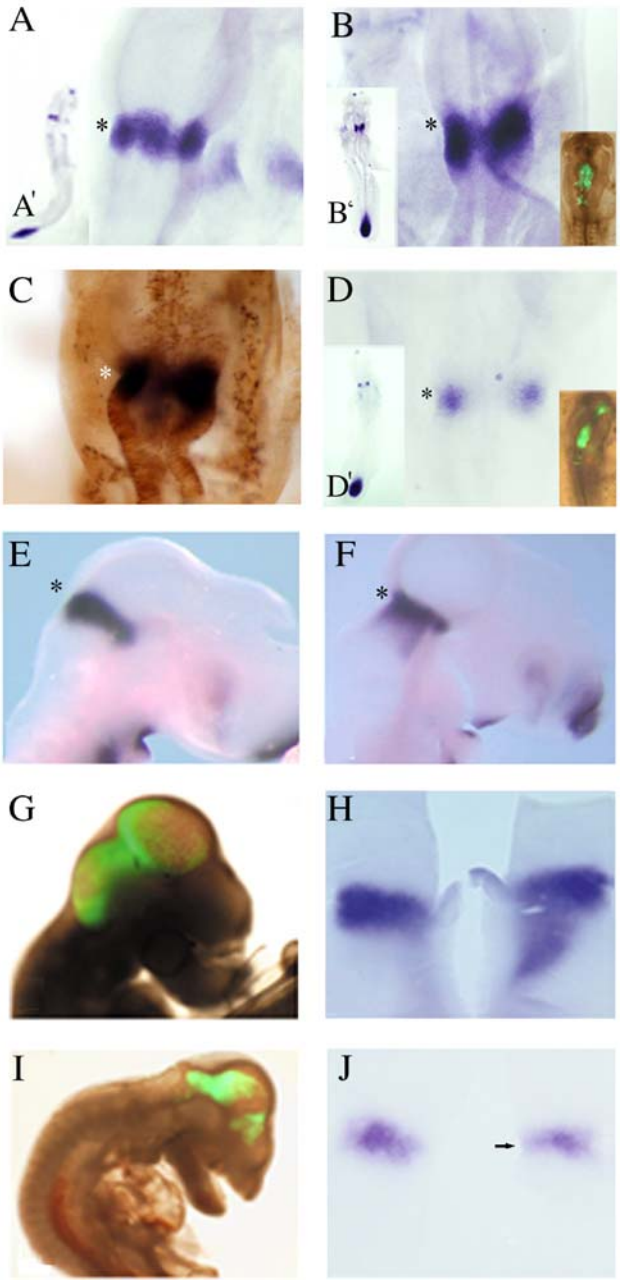

Fig. 2. Perturbation of the Wnt pathway alters *Fgf8* expression at the isthmus. Increased Wnt signaling from electroporation at HH6 results in an expanded *Fgf8* expression domain at the isthmus by HH10–12 (B, B′, C), while inhibition of the Wnt pathway results in decreased *Fgf8* transcript levels (D, D′) compared to untreated controls (A, A′). Panels A, B and D are higher magnifications focusing on the MHB region of flat-mounted whole embryos (A′, B′and D′). Lower right insets (B, D) show co-electroporated GFP expression extending throughout the midbrain and hindbrain. (C) GFP expression is detected throughout the midbrain and anterior hindbrain as shown by immunostaining (brown), however the expansion of *Fgf8* is restricted to the anterior hindbrain (dark blue). The asterisk (A–F) demarcates the isthmic constriction. (E–J) HH16 embryos showing *Fgf8* expression (blue; E, F, H and J) and corresponding GFP expression (green; G and I). Misexpression of Wnt1 at HH10 results in an expanded isthmic *Fgf8* domain (F, lateral view, right) compared to control uninjected embryo (E). (H) Flat-mount preparation of embryo in panel F showing an expansion of *Fgf8* only on the electroporated side (right). (J) Flat-mount preparation of an HH16 embryo electroporated with a dominant inhibitory form of Wnt1 resulting in a decrease in isthmic *Fgf8* expression on the electroporated side (right, arrow) compared to the control contralateral side (left).

WNT signaling is required to maintain Fgf8 expression in rhombomere 1 explants

We used explant assays to investigate how sustained Wnt signaling across the neuroepithelium contributes to the identity of r1. As a positive control, midbrain to r2 (mb–r2) explants were cultured overnight and shown to express Fgf8 similar to that observed in intact embryos (Fig. 3B, $n=15/15$). Mb–r2 tissue cultured in the presence of recombinant Wnt3a (a related Wnt1 family member) displayed an expansion of *Fgf8* expression within r1 (Fig. 3C, $n=8/10$). To determine whether our effects were mediated via canonical Wnt signaling, we cultured mb–r2 in the presence of LiCl (40 mM), which constitutively activates the pathway through inhibition of GSK-3β and leads to the activation of Wnt-dependant transcription in the brain (Hong et al., 1997; Klein and Melton, 1996; O'Brien et al., 2004; Stambolic et al., 1996). Expansion of *Fgf8* expression in r1 was also observed (Fig. 3D, $n=14/14$). Similarly, Wnt1 electroporation and treatment with LiCl *in vivo* resulted in a 4-fold activation of a TCF reporter construct (TOPFLASH) in neural tube lysates (Fig. 3E, $n=8$ for each experiment). In contrast, inhibition of Wnt activity using a secreted frizzled related protein (SFRP2) resulted in a severe reduction of endogenous *Fgf8* transcripts (Fig. 3Q, $n=8/8$). However, a substantial reduction of endogenous *Wnt1* transcripts was not detected (Fig. 3Q). Notably, LiCl activation of Wnt signaling rescued the effect of SFRP2 (Fig. 3R, $n=4/4$). The expanded *Fgf8* expression domain was confined to anterior r1, as shown by the gap between expanded *Fgf8* and that of *Hoxa2*, a r2-specific marker (Fig. 3R, arrow). We subsequently sought to determine whether LiCl was sufficient to modulate *Fgf8* expression in isolated r1 explants. In the absence of a visible r1–r2 boundary, it was estimated that rhombomere 1 occupied the anterior two thirds of this single neuromere.

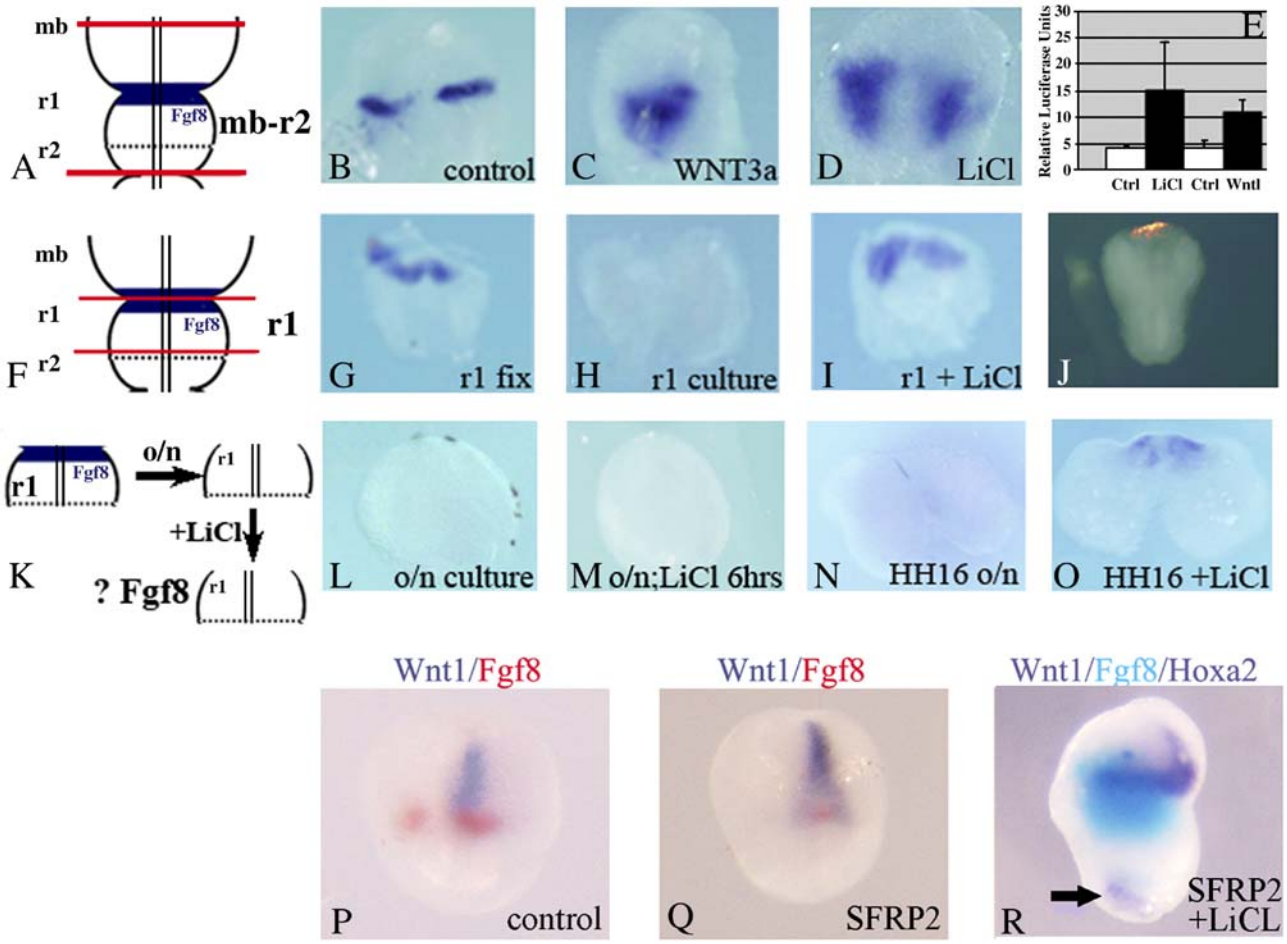

Fig. 3. Sustained Wnt signaling is required to maintain *Fgf8* expression in r1. Mb–r2 tissue (B–D) cultured overnight displays a normal isthmic-like stripe of *Fgf8* expression (B), and treatment with Wnt3a (C) or LiCl (D) induced an expansion of the *Fgf8* domain. r1 isolates (G–I, L–O) are positive for *Fgf8* expression at the time of explant (G). Isolated r1 loses *Fgf8* transcripts after overnight culture (H), but LiCl treatment (minimum of 3 h) maintains *Fgf8* expression (I). (L) r1 cultured overnight does not maintain *Fgf8* expression and subsequent treatment with LiCl for a further 6 h is insufficient to induce detectable transcripts (M). HH16 r1 loses *Fgf8* expression in culture (N) but at this stage is still competent to respond to activated Wnt signaling and maintain *Fgf8* expression (O). Wnt1 over-expression and treatment with LiCl in vivo activate a TOPFLASH reporter similarly (E). DiI labeling (red) at rostral end of explants ensures correct orientation (J). Inhibition of Wnt signaling using SFRP2 lead to a severe reduction of *Fgf8* expression (Q, red) compared to control mb–r2 explants (P). *Fgf8* expression is rescued by the application of LiCl (R). Explants are stained for the expression of *Fgf8* (blue, B–O; red, P and Q; light blue, R), *Wnt1* (purple, P–R) and *Hoxa2* (R, purple, arrow). The diagrams (A, F and K) illustrate the isolated tissues. Anterior is up in all explants.

When r1 tissue was dissected (Fig. 3G) and fixed immediately, all explants were positive for *Fgf8* expression ($n=7/7$), demonstrating that r1 tissue could be accurately dissected. By contrast, when r1 was cultured overnight in the absence of midbrain tissue, all explants lost *Fgf8* expression (Fig. 3H, $n=11/11$). This result supported our previous findings that continuous signaling by a midbrain-derived secreted factor is required for maintenance of isthmic *Fgf8* expression (Irving and Mason, 1999). To test directly that this signal is a member(s) of the canonical Wnt family, r1 was cultured in the presence of either Wnt3a or LiCl, and all explants maintained the expression of *Fgf8* (Fig. 3I; Wnt3a $n=6/6$, data not shown; LiCl $n=8/8$). Although we cannot exclude the fact that lithium chloride modulates alternative signaling pathways, in our assays we provide strong evidence that it activates a canonical Wnt-like response resulting in expansion of *Fgf8* expression.

To address whether Wnt signaling played an inductive or maintenance role in the regulation of isthmic *Fgf8* expression, r1 explants that lost *Fgf8* expression after overnight culture (Fig. 3L, $n=4/4$) were incubated with LiCl for a further 6 h to determine whether they were able to re-initiate *Fgf8* expression (see diagram in Fig. 3K). The addition of LiCl after *Fgf8* expression was lost did not induce detectable *Fgf8* transcripts (Fig. 3M, $n=4/4$), implying that Wnt signals act as maintenance factor(s). As a control, we demonstrated that at HH16 r1 explants still require midbrain-derived Wnt signals to maintain *Fgf8* expression (Figs. 3N and O). Taken together, these results substantiate the requirement for sustained Wnt signaling to maintain *Fgf8* expression from before HH10 until after HH16.

In mouse mutants, where *Fgf8* is conditionally inactivated, significant cell death is observed at the isthmus by E10. Similar results were also observed for both *Wnt1*$^{-/-}$ and *En1*$^{-/-}$ mutants (Chi et al., 2003). Therefore we wished to investigate changes in proliferation and cell death in our explant assays. As expected, the LiCl-mediated activation of the Wnt pathway resulted in an increase in proliferation in mb–r2 explants, compared to control explants (see Supplementary Fig. 1). Of interest, we did not observe any significant loss of proliferation when explants were incubated with SFRP2 (see Supplementary data). However, in agreement with mouse genetic data, we observed a moderate but detectable increase in apoptosis in r1–r2 explants that lose *Fgf8* expression due to the absence of midbrain-derived Wnt signals (see Supplementary data). It is difficult to assess from these experiments whether the loss of *Fgf8* expression is due primarily to the absence of Wnt signals or is in fact secondary to increased apoptosis. In support of this, Chi et al. (2003) demonstrate that while some mes–met markers are lost before a substantial level of apoptosis is observed, others are lost at the time when the highest levels of apoptotic cells are detected. Although r1–r2 explants when cultured alone lose *Fgf8* expression, they do maintain the expression of hindbrain markers, as shown by the expression of *Gbx2* (see Fig. 4). In summary, our results are similar to those observed in *En1*$^{-/-}$, *Wnt1*$^{-/-}$ and *Fgf8*$^{-/-}$ mouse mutants, in that Fgf8 is required for normal cell survival in the mid–hindbrain region.

The spatial restriction of Fgf8 expression in r1 is independent of signals from rhombomere 2

The mechanism by which *Fgf8* transcripts are restricted to anterior r1 remains to be understood, but signals from the posterior hindbrain may serve to limit the posterior domain of *Fgf8* expression in r1. To address whether r2-derived signals are involved in the regulation of isthmic *Fgf8* expression, a number of explants comprising different regions of the neural tube were cultured in the presence or absence of activated canonical Wnt signaling. As observed previously, mb–r2 explants continued to express endogenous *Fgf8* ($n=8/8$), and expanded *Fgf8* in the presence of LiCl ($n=8/8$) (Figs. 4B, C). Explants comprising mb–r1 ($n=6/6$), but lacking r2 appeared identical to mb–r2 samples (Figs. 4E, F). By contrast, when r1–r2 ($n=10/10$) or r1 ($n=12/12$) alone were cultured, neither tissue maintained the expression of endogenous *Fgf8* (Figs. 4H and K). No differences were observed between r1–r2 explants and r1 tissue alone, as activation of the Wnt pathway was sufficient to maintain *Fgf8* expression in both explants (Figs. 4I and L). Notably, even in the absence of r2 tissue, ectopic activation of the canonical Wnt pathway could not induce expression of *Fgf8* in posterior r1 (Fig. 4L). *In situ* hybridization for *Fgf8* (light blue), *Wnt1* (midbrain, dark blue), *Hoxa2* (rhombomere 2, dark blue) and *Gbx2* (hindbrain, dark blue) confirmed that the explants comprised the intended tissues (Figs. 4M–P). As described above, LiCl-induced expansion of *Fgf8* expression was only detected in anterior r1 (Figs. 4N and O), as a gap is observed between the expanded *Fgf8* domain and that of *Hoxa2* in mb–r2 explants (Fig. 4N). Similarly, mb–r1 explants display an *Fgf8* negative domain in posterior r1, which is also negative for the r2 marker, *Hoxa2* (Fig. 4O). As a control for the viability of r1–r2 tissue, explants were tested for expression of *Gbx2,* which was detected, and *Wnt1*, which was absent (Fig. 4P). Together, these results reinforce that a Wnt-mediated maintenance signal is derived from the midbrain, and that the presence of r2 or posterior hindbrain does not influence the spatial refinement of isthmic *Fgf8* expression.

Sustained interplay between FGF and Wnt signaling is necessary to maintain isthmic identity

It has previously been shown that FGF signaling itself is involved in the maintenance of MHB identity in an autocrine manner through FGFR1 (Scholpp et al., 2004; Trokovic et al., 2003; Trokovic et al., 2005; Ye et al., 1998). Hence, we addressed whether active FGF signaling is required in concert with Wnt signaling to maintain *Fgf8* expression at the isthmus. We used a chemical inhibitor of FGFR signaling, SU5402 (Mohammadi et al., 1997), to block signaling downstream of FGF8 at the isthmus (Sato and Nakamura, 2004). We tested a number of concentrations of SU5402 in our explant assays. When used below 10 μM, no significant reduction in *Fgf8* expression was detected (data not shown). A recent study of MAPK/ERK signaling in presomitic mesoderm revealed that 10 μM SU5402 did not inhibit ERK activity, but that 100 μM

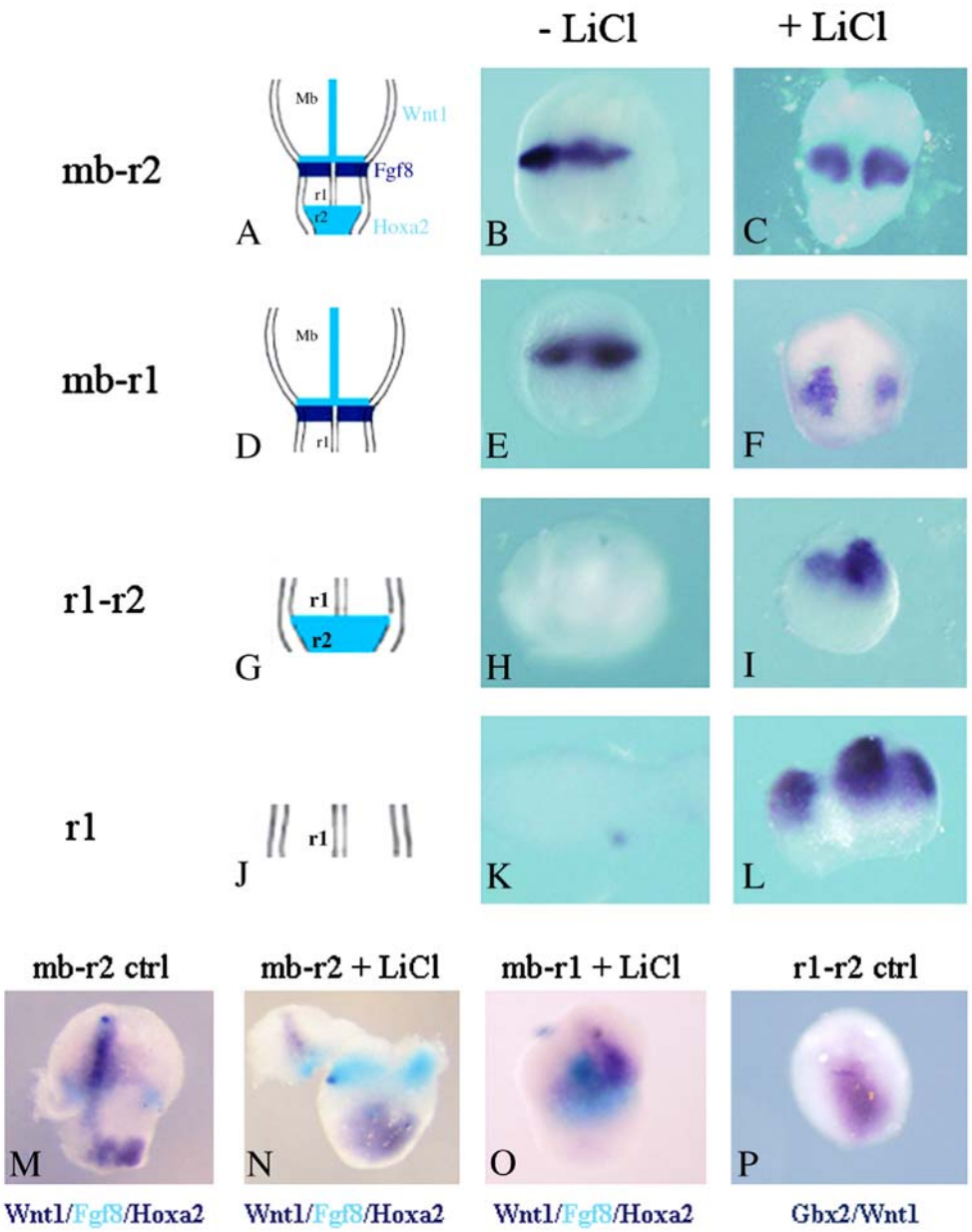

Fig. 4. Rhombomere 2 is not required for the correct spatial regulation of *Fgf8* expression. An isthmic-like *Fgf8* domain is observed in mb–r2 and mb-r1 explants cultured overnight (B and E), but *Fgf8* expression is lost when midbrain tissue is removed (H and K). Activation of canonical Wnt signaling (+LiCl) maintains *Fgf8* expression in r1–r2 and r1 explants (I and L) and expands the domain when midbrain tissue is included (C and F). (M and N) mb–r2 explants express *Wnt1*, *Hoxa2* (purple) and *Fgf8* (light blue), whereas only *Wnt1* (purple) and *Fgf8* (light blue) are observed in mb–r1 (O). r1–r2 explants are devoid of *Fgf8* and *Wnt1* expression but are positive for *Gbx2* (P). The explant in panel N was damaged during processing; the anterior end is displaced to the left. (A, D, G, J) Schematic of regional dissections showing the normal expression domains of *Wnt1/Fgf8/Hoxa2*.

was effective (Delfini et al., 2005). Here, 50 μM SU5402 efficiently blocked FGF signaling which led to a loss of *Fgf8* expression in mb–r2 explants (Fig. 5C). Similarly, we detected a loss of *Wnt1* transcripts in mb–r2 explants cultured in the presence of SU5402, suggestive of a feed-forward interaction between Wnt and FGF signaling (see Supplementary Fig. 2). Significantly, SU5402 also prevented LiCl-mediated maintenance of *Fgf8* in mb–r2 and r1 explants (Figs. 5D and H). The efficacy of SU5402 treatment was demonstrated by inhibition of ERK1/2 activation (Fig. 5J) compared to mb–r2 explants cultured under normal conditions (Fig. 5I). Hence, the maintenance of *Fgf8* expression by the canonical Wnt pathway is dependent upon FGF signaling itself. These results extend those of Olander et al. (2006) who report that neither Wnt nor FGF signaling are required post-gastrulation to induce an isthmic organizer, as determined by the expression of isthmic markers in the presence of Wnt and FGF inhibitors. We demonstrate that both signaling pathways are required to maintain isthmic identity. The importance of FGF signaling is also supported by another study carried out in mouse. Blocking FGFR3 signaling resulted in the loss of midbrain dopaminergic and rostral hindbrain 5-hydroxytryptamine neurons. Although *Fgf8*

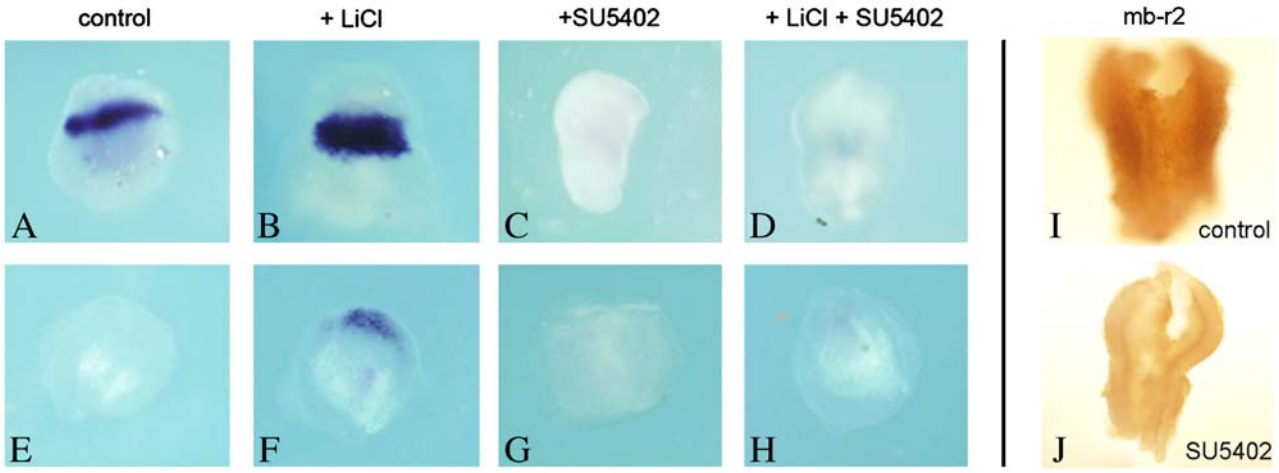

Fig. 5. Combined Wnt and FGF signaling are required for the maintenance of isthmic *Fgf8* expression. Mb–r2 explants (A–D) express *Fgf8* (blue), but r1–r2 tissues (E–H) lose *Fgf8* transcripts after overnight culture (E). LiCl treatment expands the *Fgf8* domain (B) and rescues its expression in r1–r2 explants (F). When FGF signaling is blocked in explants treated with SU5402 overnight, *Fgf8* expression is lost in mb–r2 and r1–r2 (C and G). This is not rescued by treatment with LiCl (D and H). SU5402-treated explants have reduced phospho-ERK1/2 levels (J, brow stain) compared to controls (I), demonstrating the effectiveness of the treatment.

isoforms were initially detected, transcription of all but one was completely lost by 4 days, coincident with a loss of neuronal markers (Ye et al., 1998). Similarly, positive feedback at the level of FGFR1 has been implicated in regulating *Fgf8* and other genes expressed at the MHB in mouse and zebrafish (Scholpp et al., 2004; Trokovic et al., 2003, 2005). These observations, combined with our findings, point to a combinatorial mechanism in which both Wnt and FGF signaling act in concert to maintain isthmic identity.

Since our neural explant data suggested a role for Wnt and FGF signaling in the maintenance of isthmic *Fgf8* expression, we also examined this in the context of the whole embryo. At HH10, a glycogen synthase kinase-3 β inhibitor (GSK-3βI) or SU5402 was injected directly into the lumen of the neural tube at the level of the mes–met region and embryos were incubated until they reached approximately HH14. In the presence of the GSK3-βI, which directly stimulates the canonical Wnt pathway (similar to the actions of LiCl incubation demonstrated in neural explants), *Fgf8* expression at the MHB expanded, but only within rhombomere 1 (Fig. 6B) compared to control embryos injected with DMSO (Fig. 6A). Conversely, injection of SU5402 resulted in a complete loss of isthmic *Fgf8* expression (Fig. 6C), compared to controls (Fig. 6A). Of particular note, the *Fgf8* expression domain in the posterior primitive streak of control, GSK3-βI, and SU5402-injected embryos is comparable (Figs. 6A, B and C, respectively). In addition, *Wnt1* transcripts were visibly reduced (Fig. 6C), consistent with results observed in explants (Supplementary Fig. 2). For comparison, beads were soaked in SU5402 and implanted directly into the neuroepithelium and similar results were observed (see Supplementary Fig. 2). Collectively, these results further support the existence of a regulatory loop between FGF signaling and Wnt1 in the maintenance of isthmic identity. Our experiments demonstrate that WNT signaling is necessary but not sufficient to regulate *Fgf8* expression at the isthmus, and suggest that the maintenance of *Fgf8* expression by the canonical WNT pathway is dependent upon FGF signaling itself.

Pax2 and En1/2 are implicated in the Wnt1/Fgf8 maintenance of isthmic identity

Above we demonstrated the importance of a sustained interaction between Wnt and FGF signaling in the maintenance of isthmic *Fgf8* expression both in neural explant and *in vivo* assays. We next sought to investigate whether other mes–met markers were also influenced by the misexpression of *Wnt1* and concomitant expansion of *Fgf8* expression. Although we detect a considerable expansion of *Fgf8* expression in r1 after *Wnt1* misexpression, no ectopic expression of endogenous *Wnt1* transcripts were observed (Fig. 6E). However, we do not rule out the possibility that endogenous *Wnt1* transcript levels may be elevated. Under these electroporation conditions, mouse *Wnt1* transcripts were only detected on the right side of the neural tube, coincident with an expansion of *Fgf8* expression (Fig. 6F). As observed throughout this study, the expanded *Fgf8* domain was restricted to anterior r1, despite the fact that ectopic mouse *Wnt1* transcripts were present in the midbrain and the hindbrain.

Expression of an *En1* transgene under control of a WNT1 enhancer is sufficient to rescue the expression of *Fgf8* and mid–hindbrain tissue in the *WNT1*$^{-/-}$ mutant background (Danielian and McMahon, 1996). This study demonstrates a *Wnt1/En1* genetic interaction in mouse that may be involved in the regulation of *Fgf8* expression. When we examined the expression of *En2* in the chick at HH16, after misexpression of *Wnt1*, we detected both a caudal expansion of *En2* expression in the hindbrain and a rostral expansion in the midbrain (Fig. 6H). This was accompanied by a caudal expansion of *Fgf8* transcripts. Although previous findings demonstrated that misexpression of *En2* under the control of a viral promoter failed to induce ectopic *Fgf8* expression in

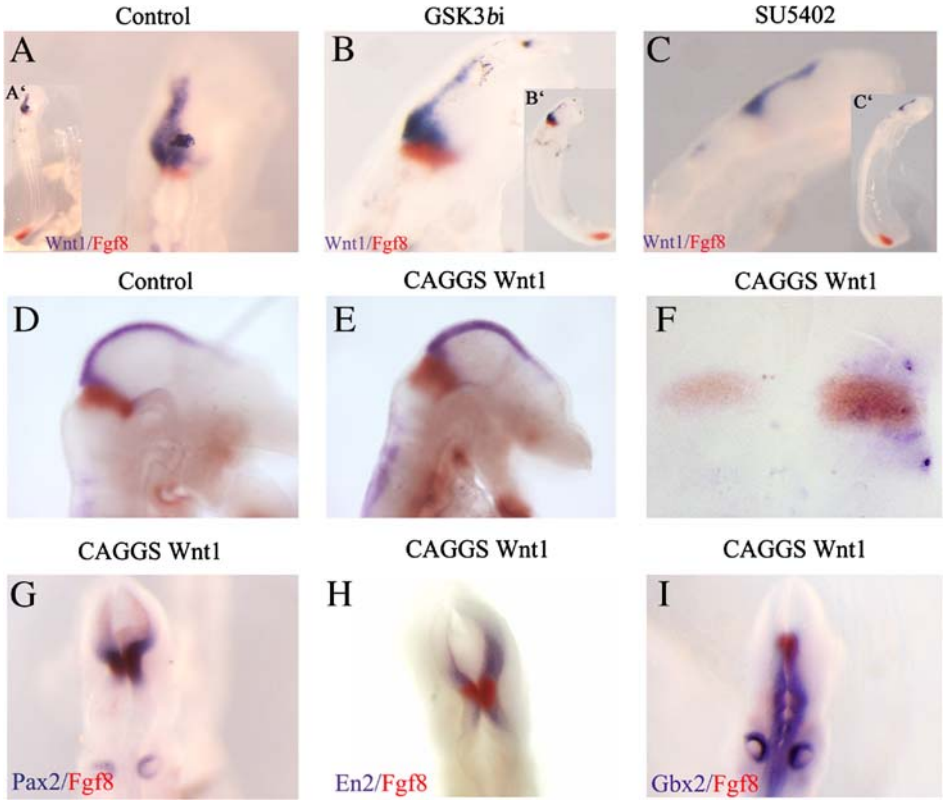

Fig. 6. Combined Wnt and Fgf signaling act to regulate isthmic *Fgf8* expression and *Pax2* and *En2* are implicated in this forward regulatory mechanism. Application of a GSK-3β inhibitor results in an expansion of *Fgf8* expression at the isthmus (B) compared to controls (A) injected with DMSO. Injection of 50 μM SU5402 into the lumen of the neural tube resulted in a loss of isthmic *Fgf8* expression (red) and a reduction of *Wnt1* expression (blue) (C). (D and E) Lateral views of HH16 embryos stained for *Wnt1* (blue) and *Fgf8* (red). Misexpression of Wnt1 results in an expansion of isthmic *Fgf8* caudally, whereas no spatial change in the endogenous *Wnt1* expression domain is observed (E, compared to control unelectroporated embryo D). Flat-mount preparation of a HH16 embryo (F) electroporated with Wnt1 demonstrates ectopic mouse Wnt1 transcripts (blue) on the experimental side (right) concomitant with an expansion of *Fgf8* expression in r1 (red). Compare with control contralateral side (left). Panels G, H and I are dorsal views of HH16 embryos stained with *Fgf8* (red) and *Pax2*, *En2*, or *Gbx2* (blue). Upon misexpression of Wnt1, *Pax2* (G) and *En2* (H) expression expanded both rostrally in the midbrain and caudally in the hindbrain on the experimental side (right). (I) Conversely, no change in the spatial limit of *Gbx2* expression is observed (I, blue).

anterior hindbrain (Shamim et al., 1999), our data reinforce the observation in the mouse that En/Wnt/FGF signaling are genetically linked. However, when Wnt1 was expressed under the control of *En1* regulatory sequences in mouse, *Fgf8* expression did not expand in the anterior hindbrain (Panhuysen et al., 2004). This again underlies subtle differences that may exist between vertebrate species. *Pax2*, a member of the paired box family of transcription factors, has been shown to be necessary and sufficient for the induction of *Fgf8* at the isthmus (Ye et al., 2001). Similar to the observations made for *En2*, in the presence of activated Wnt signaling, we observed an expansion of *Pax2*, again extending rostrally in the midbrain and caudally in the anterior hindbrain (Fig. 6G). These observations are consistent with previous reports demonstrating that the isthmus and *Fgf8* expression lie within a *Pax2/En1/2* domain (Hidalgo-Sanchez et al., 2005; Liu and Joyner, 2001; Ye et al., 2001). The spatial domains of either *Otx2* in the midbrain (data not shown) or *Gbx2* in the hindbrain (Fig. 6I) did not change under the influence of altered Wnt or FGF signaling. Again, we cannot exclude that changes in the levels of these two transcripts may have occurred. These findings highlight once again the complex combinatorial interactions of transcription factors and signaling molecules in the regulation of isthmic identity.

Concluding remarks

The multifaceted regulatory mechanism through which *Fgf8* expression is continuously refined underlies the importance of the maintenance of the IsO during different developmental stages. We demonstrate that Wnt signaling post-gastrulation is involved in maintaining the early pattern of *Fgf8* expression at the mid–hindbrain boundary. We also reveal that sustained Wnt signaling is vital for the maintenance of *Fgf8* expression in anterior r1, and that the spatial restriction of *Fgf8* expression is independent of posterior hindbrain signals. Furthermore, Wnt-mediated maintenance of *Fgf8* expression is dependent on FGF signaling itself. Finally, we demonstrate that *Pax2* and *En1/2* are likely to be involved in the Wnt1/FGF8 forward regulatory mechanism that maintains isthmic identity. Our findings indicate that Wnt and FGF signaling are required at multiple developmental stages to regulate isthmic identity, and demonstrate that sustained interplay between the two pathways is essential to that process.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.02.009.

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