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Wnt signals provide a timing mechanism for the FGF-retinoid differentiation switch during vertebrate body axis extension

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Differentiation onset in the vertebrate body axis is controlled by a conserved switch from fibroblast growth factor (FGF) to retinoid signalling, which is also apparent in the extending limb and aberrant in many cancer cell lines. FGF protects tail-end stem zone cells from precocious differentiation by inhibiting retinoid synthesis, whereas later-produced retinoic acid (RA) attenuates FGF signalling and drives differentiation. The timing of RA production is therefore crucial for the preservation of stem zone cells and the continued extension of the body axis. Here we show that canonical Wnt signalling mediates the transition from FGF to retinoid signalling in the newly generated chick body axis. FGF promotes Wnt8c expression, which persists in the neuroepithelium as FGF signalling declines. Wnt signals then act here to repress neuronal differentiation. Furthermore, although FGF inhibition of neuronal differentiation involves repression of the RA-responsive gene, retinoic acid receptor β (*RARβ*), Wnt signals are weaker repressors of neuron production and do not interfere with RA signal transduction. Strikingly, as FGF signals decline in the extending axis, Wnt signals now elicit RA synthesis in neighbouring presomitic mesoderm. This study identifies a directional signalling relay that leads from FGF to retinoid signalling and demonstrates that Wnt signals serve, as cells leave the stem zone, to permit and promote RA activity, providing a mechanism to control the timing of the FGF-RA differentiation switch.

KEY WORDS: Neurogenesis, FGF, Retinoic acid, Wnt, Differentiation, Stem cells, Chick

INTRODUCTION

Regionalisation of the vertebrate nervous system is a fundamental process that involves global patterning mechanisms. It is well established in both higher and lower vertebrates that three factors, fibroblast growth factor (FGF), Wnt and retinoic acid (RA) play primary roles in imposing caudal/posterior character on rostral/anterior neural tissue (reviewed by Gamse and Sive, 2000; Schier, 2001; Stern et al., 2006). In particular, in the chick embryo, FGF and Wnt have been shown to act together in a dose-dependent manner to specify midbrain and hindbrain regions of the CNS (Nordstrom et al., 2002) whereas specification of spinal cord identity additionally involves retinoid signalling (Delfino-Machin et al., 2005; Muhr et al., 1999; Nordstrom et al., 2006). FGF, Wnt and RA signalling have also been shown in various contexts to regulate caudal (Cdx) genes, which are part of the mechanism that defines rostro-caudal identity in the body axis via regulation of Hox gene expression (Nordstrom et al., 2006) (reviewed in Lohnes, 2003). However, how all three pathways interact to assign caudal identity is still not clear. Several studies have addressed the regulatory relationships between FGF, Wnts and RA pathways (Blumberg et al., 1997; Domingos et al., 2001; Kudoh et al., 2002; McGrew et al., 1997; Moreno and Kintner, 2004; Shiotsugu et al., 2004), but these experiments involve misexpression of signalling factors or their antagonists throughout the early frog or fish embryo, making it difficult to determine a precise sequence of tissue interactions and signalling events (see Diez del Corral and Storey, 2004; Stern et al., 2006). Importantly, in higher vertebrates patterning of caudal neural tissue does not simply involve subdivision of the neural plate but is intrinsically linked to the progressive generation of new neural tissue as the body axis extends. This at once makes the problem more complex, but has the advantage that the temporal sequence of events underlying differentiation and pattern progression becomes spatially separated in the extending axis and the regulation of these steps can therefore be more easily investigated.

As caudal hindbrain and spinal cord regions are generated sequentially, the finding that increasing levels of FGF and Wnt signalling lead to more caudal character (Nordstrom et al., 2002; Nordstrom et al., 2006) can also be viewed in terms of the progressive assignment of rostro-caudal character; cells that reside close to the caudally regressing source of FGF and Wnt signals (the primitive streak) remain undifferentiated and acquire progressively more caudal fates (see Vasiliauskas and Stern, 2001). Importantly, FGF, Wnt and RA stimulate distinct cell behaviours as well as inducing expression of caudal marker genes. FGF and Wnt can both stimulate proliferation (Chenn and Walsh, 2002; Dickinson et al., 1994; Lee et al., 1997; Megason and McMahon, 2002; Qian et al., 1997; Zechner et al., 2003), while in contrast, RA signalling drives differentiation and can promote cell cycle exit (reviewed by Diez del Corral and Storey, 2004), and these behaviours are important when we consider the roles of these signals in the extending body axis. We have shown recently that FGF-dependent Notch signalling maintains an undifferentiated cell state in stem zone (caudal neural plate) cells that lie adjacent to the regressing primitive streak at the tail end of the embryo (Akai et al., 2005; Diez del Corral et al., 2002; Mathis et al., 2001). This cell population progressively gives rise to new neural progenitors (Brown and Storey, 2000) and has been shown to harbour a resident stem cell-like population in the mouse (Cambray and Wilson, 2002; Mathis and Nicolas, 2000) and most likely in the chick (Mathis et al., 2001; Delfino-Machin et al., 2005). As cells leave this stem zone, FGF-dependent Notch signalling declines and cells enter the transition zone (preneural tube) where they encounter RA, which is synthesised by *Raldh2* in the adjacent rostral presomitic mesoderm. We have found that retinoid signalling downregulates *Fgf8*, in both the presomitic mesoderm and the neuroepithelium and in this way drives and coordinates the differentiation of these tissues. Conversely, FGF signalling represses

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Raldh2 expression in caudal regions protecting stem zone and caudal presomitic mesoderm cells from precocious differentiation (Diez del Corral et al., 2003). FGF and RA pathways therefore act antagonistically in this context and we have shown that they have opposing effects on neuronal differentiation and ventral patterning onset in the newly formed neural tube (Diez del Corral et al., 2003; Novitch et al., 2003). In addition, the opposition of these two pathways in the presomitic mesoderm defines the position of the future somite boundary during the process of segmentation [(Diez del Corral et al., 2003); although this appears to be restricted to early stages in the mouse embryo (see Sirbu and Duester, 2006)]. The transition from FGF to retinoid signalling thus serves as a differentiation switch in the extending body axis. Importantly, a similar relationship between these pathways has been observed in the extending limb and in some cancer cell lines, indicating that this is a fundamental and conserved molecular mechanism that regulates differentiation progression (reviewed by Diez del Corral and Storey, 2004).

There is growing evidence that caudal Hox gene expression depends on FGF and not retinoid signalling in the stem zone or caudal neural plate (Bel-Vialar et al., 2002; Delfino-Machin et al., 2005; Liu et al., 2001). However, later, more rostral domains of caudal Hox gene expression then switch to dependence on RA (Muhr et al., 1999; Oosterveen et al., 2003). As onset of more caudal Hox genes commences in the stem zone we have proposed a model in which caudal Hox genes are progressively induced by FGF signalling and the expression of these genes then becomes 'fixed' as cells leave the stem zone and encounter retinoid signals (Diez del Corral and Storey, 2004). This also implies that the switch from FGF to RA regulates both differentiation status and progressive assignment of rostro-caudal character.

Here we focus on the regulatory relationships between FGF, Wnt and RA pathways during this critical transition from FGF to retinoid signalling. We demonstrate that FGF promotes expression of a caudal Wnt and that Wnt signalling acts, following decline of FGF activity in the body axis, to permit RA activity in the neuroepithelium and to promote RA synthesis in the neighbouring presomitic mesoderm. Wnt signals thus function as an intermediary between FGF and RA signalling and facilitate the spatial and temporal separation of signalling events in the extending body axis.

MATERIALS AND METHODS

Embryo culture

Fertilised hens' eggs (Henry Stewart farm, Lincolnshire), incubated at 38°C to yield embryos of stages HH6-9 (Hamburger and Hamilton, 1951) were set up in EC culture (Chapman et al., 2001) for operations or bead grafts. Presomitic mesoderm was removed unilaterally from HH7-9 embryos after brief exposure to 0.1% trypsin.

In vitro explant culture

Explants (indicated in each figure) were isolated from HH7-8 chick embryos and cultured in collagen as described previously (Diez del Corral et al., 2002; Placzek and Dale, 1999). In all experiments control and experimental explants were derived from the same embryo, processed individually and scored as pairs. Two quail somites (last formed somites HH7-8) were combined with single chick caudal neural plate explants.

Manipulating FGF signalling

Heparin beads soaked in human FGF4 (200 μg/ml) or mouse FGF8 (250 μg/ml) (R&D Systems) were grafted under the caudal neural plate and the embryos allowed to develop for 6 to 18 hours. Explants were treated with human FGF4 at 200 ng/ml or mouse FGF8 at 250 ng/ml in the presence of heparin (0.1 ng/ml) and BSA (0.0001%). Explants were exposed to SU5402 (10 μM) (Calbiochem) or to DMSO only in controls.

Retinoic acid treatments

Explants were treated with 9-cis RA (10 μM, Sigma) or the RA receptor (RAR) agonist TTNPB (1 μM, a kind gift from C. Tickle) or DMSO only for controls. Vitamin A-deficient quails (VAD) have been described previously (Dersch and Zile, 1993).

Manipulating Wnt signalling

COS7 cells (ECACC) were transiently transfected with either the empty vector (control cells) or Wnt8c-IRES-GFP/PCINeo (Wnt8c cells) (kindly provided by R. Lahder and C. Hume, NCBI #AB193181). Transfection efficiency was measured as the percentage of GFP-positive cells (typically 55-75%), immediately before making aggregates by the hanging drop culture technique; each experiment was carried out in parallel with a positive control for Wnt8c protein activity (either loss of *NeuroM* in preneural tube explants or induction of *Raldh2* in caudal presomitic mesoderm). Explants were cultured with either LiCl (5 mM) (Klein and Melton, 1996), DKK1 (1 μg/ml, R&D Systems) (Glinka et al., 1998) or the casein kinase I inhibitor CKI-7 (Chijiwa et al., 1989) (200 μM, United States Biological). Neither LiCl (5 mM) nor CKI-7 (200 μM) increased cell death in explants as assessed with the LIVE/DEAD Viability/Cytotoxicity kit (Molecular Bioprobes) (data not shown). Affiblue gel beads soaked in mouse sFRP2 (R&D Systems, 2 μg/μl) were grafted in contact with caudal presomitic mesoderm, and embryos incubated for 8 hours. Embryos HH8-10 were cultured on filters for 4 hours as described previously (Delfino-Machin et al., 2005) in culture media with either LiCl (10 mM), SU5402 (60 μM in DMSO), or CKI-7 (400 μM in ethanol 100%) or the corresponding control media.

In ovo electroporation

The Wnt8c-IRES-GFP/PCINeo construct or the empty vector were introduced in the caudal neural plate and preneural tube at 9-10 HH using standard in ovo electroporation. After 10-18 hours embryos were processed for double in situ hybridisation (ISH). *NeuroM*-positive cells and total nuclei (DAPI) were counted in the electroporated half of the neural tube in at least ten sections per embryo.

In situ hybridisation, immunocytochemistry

Standard methods for whole-mount ISH and double ISH were used. Automated in ISH was carried out for explants using a robotic InsituPro machine (protocol available on request). Quail cells were detected with QCPN antibody (DSHB).

RESULTS

Expression patterns of caudal Wnt genes in the extending body axis identify *Wnt8c* as a potential target of FGF and retinoid signalling

To understand better the signalling events that control differentiation onset in the forming body axis we examined the expression patterns of key Wnt genes (*Wnt3a*, *Wnt5a* and *Wnt8c*) expressed in the caudal end of the chick embryo in relation to components of the FGF, Wnt and retinoid pathways. This analysis is presented in Fig. S1 in the supplementary material and identifies *Wnt8c* (Hollyday et al., 1995; Hume and Dodd, 1993) as a likely regulator of differentiation onset in the extending neural axis. In particular, *Wnt8c* expression overlaps caudally with *Fgf8* (Fig. 1A-B′), but extends more rostrally into the preneural tube and is then sharply downregulated at the level of the somites where *Raldh2* is expressed (Fig. 1C,C′). We therefore next examined the regulation of *Wnt8c* by FGF and retinoid signals.

FGF signalling is required to maintain *Wnt8c* expression

To test whether *Wnt8c* expression depends on signals from the presomitic mesoderm (a source of FGF signals), this tissue was unilaterally removed in HH7-9 embryos (Fig. 1D). After 4-6 hours in culture, *Wnt8c* levels were reduced in half the cases (11/22) (Fig.

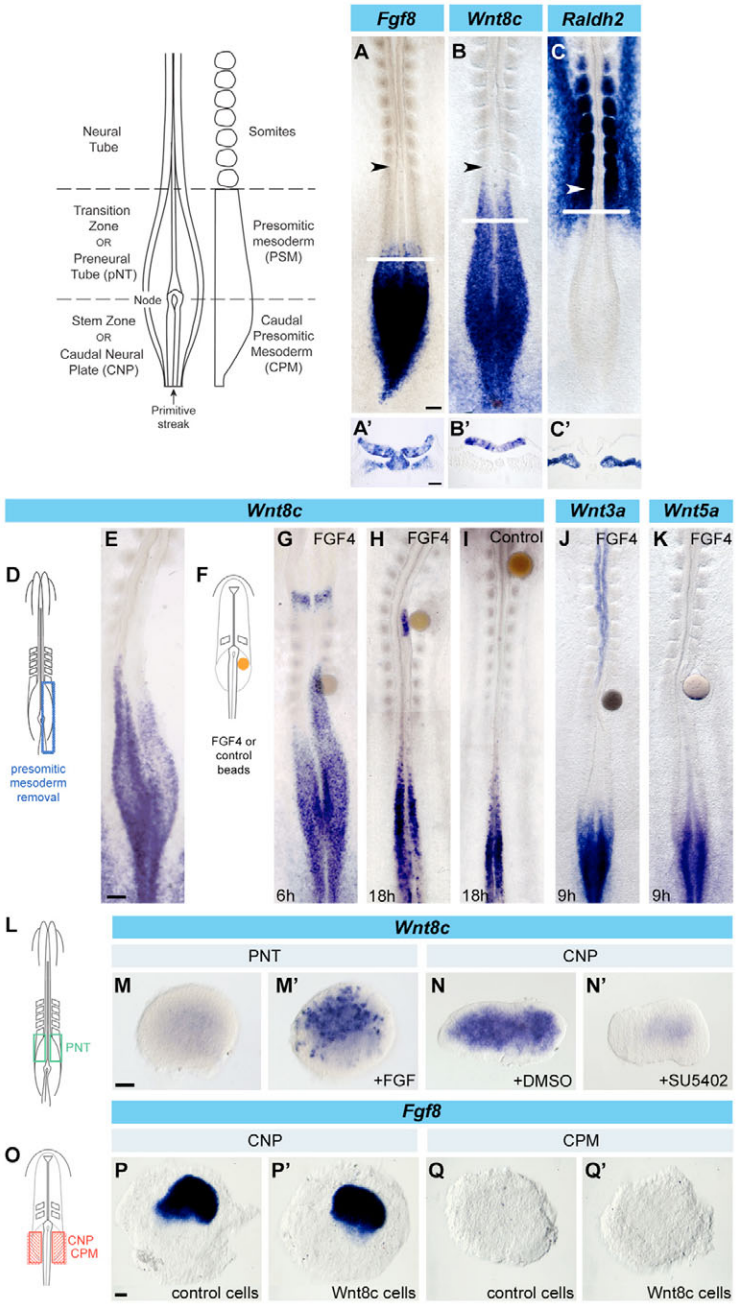

Fig. 1. FGF signalling is required to maintain *Wnt8c* expression. (**A-C′**) Expression of *Fgf8* (A,A′), *Wnt8c* (B,B′) and *Raldh2* (C,C′) in HH10 chicken embryos and corresponding transverse sections (at position of white lines): arrowheads indicate the last somite formed. Diagram on the left defines the different regions of the caudal end of the embryos at stage HH10. (**D**,**E**) *Wnt8c* is downregulated 6 hours after presomitic mesoderm removal. (**F-I**) FGF4 beads beneath caudal neural plate (F) ectopically maintain *Wnt8c* 6 hours (G) and 18 hours (H) later, whereas control PBS beads do not (I). (**J**,**K**) Caudal *Wnt3a* (J) or *Wnt5a* (K) expression is not maintained rostrally by FGF4 beads (9 hours later). (**L**) Origin of explants used in M,M′. (**M**,**M′**) *Wnt8c* loss in preneural tube explants (pNT) cultured 24 hours (M) is prevented by FGF4 (M′). (**O**) Origin of caudal neural plate (CNP) explants used in **N**,**N′**,**P**,**P′** and caudal presomitic mesoderm (CPM) explants used in **Q**,**Q′**. (N,N′) *Wnt8c* is still expressed in CNP explants after 8 hours in DMSO control media (N) but is downregulated by SU5402 (N′). *Fgf8* transcripts are still detected in CNP with control cells after 24 hours (P) but not in CPM explants (Q). Explants (P-Q′) are derived from the same embryo.Wnt8c cells do not alter *Fgf8* expression (P′,Q′). Scale bars: 100 μm in A for A-C and in E for E,G-K; 50 μm in M for M-N′ and in P for P-Q′.

1E), suggesting that signals from the presomitic mesoderm may promote *Wnt8c*. Many *Fgfs* are expressed by the caudal presomitic mesoderm and primitive streak including *Fgf4* and *Fgf8* (reviewed by Diez del Corral and Storey, 2004), and FGF4- or FGF8-soaked beads grafted under the caudal neural plate (Fig. 1F) maintain *Wnt8c* expression rostrally into the neural tube (FGF4, 9/11; FGF8, 8/10) (Fig. 1G,H), whereas control PBS-soaked beads have no effect (six control embryos) (Fig. 1I). In contrast, the other caudal Wnt ligands, *Wnt3a* and *Wnt5a*, were not maintained more rostrally by FGF4 beads in this same assay (Wnt3a, 0/5 and 3 control PBS beads; Wnt5a, 0/5 and 3 control PBS beads) (Fig. 1J,K). These experiments therefore indicate that *Wnt8c*, but not other caudally expressed Wnt genes, is promoted by FGF signalling.

In preneural tube explants (Fig. 1L) cultured for 24 hours, *Wnt8c* is similarly maintained by both FGF4 (7/8) and FGF8 (5/6, data not shown), whereas in untreated controls *Wnt8c* is now barely detected (14 cases) (Fig. 1M,M′). This indicates that FGF signalling can sustain *Wnt8c* expression directly in the neuroepithelium. Furthermore, whereas caudal neural plate explants (Fig. 1O) express *Wnt8c* strongly after 8 hours (5/5) (Fig. 1N) and weakly after 24 hours (5/5) (data not shown), *Wnt8c* is dramatically reduced in the presence of the FGF receptor inhibitor SU5402 after 8 hours (5/5) (Fig. 1N′) and is lost completely after 24 hours (5/5) (although the pan neural marker *Sox2* is still detected, 2/2, 24 hours, data not shown). *Wnt8c* expression in caudal neural tissue thus requires FGF signalling.

Finally, Wnt8c might reciprocally maintain expression of *Fgf8*; however, this seems unlikely because *Wnt8c* transcripts persist after *Fgf8* has declined in the neural axis (compare Fig. 1A and 1B). *Fgf8* is detected in caudal neural plate explants after 24 hours in culture but is not present in caudal presomitic mesoderm explants derived from the same embryo (compare Fig. 1P and 1Q and see details Fig. S2 in the supplementary material). Exposure to Wnt8c (provided by Wnt8c secreting COS7 cells) does not sustain or lead to more extensive *Fgf8* in either caudal neural plate (4/4) (Fig. 1P′) or caudal presomitic mesoderm (4/4) (Fig. 1Q′) (while the same Wnt8c cells grafted on the same day promoted expression of Wnt regulated genes, see below Fig. 5A-B′). Together these findings define the regulatory relationship between FGF signalling and *Wnt8c* expression in the extending axis. Although Wnt8c does not promote *Fgf8*, caudal FGF signals, provided in part by the presomitic mesoderm, are required specifically within the neuroepithelium to maintain *Wnt8c* expression.

Somite-derived retinoic acid downregulates *Wnt8c* via the FGF pathway

Wnt8c has a sharp rostral boundary in the neural tube at the level of somitogenesis onset (Hume and Dodd, 1993) (Fig. 1B) and somitic RA signalling accelerates the loss of *Fgf8* transcripts (Diez del Corral et al., 2003) and so we next assessed whether somites and RA signalling are also responsible for *Wnt8c* downregulation. *Wnt8c* expression was examined in pairs of chick-derived caudal neural plate explants cultured either alone or in contact with quail somites (Fig. 2A). In most cases, *Wnt8c* is reduced by somite signals (after 4 hours, 4/9; 12 hours, 3/6 and 24 hours, 6/8) (Fig. 2B,B′). Consistent with this

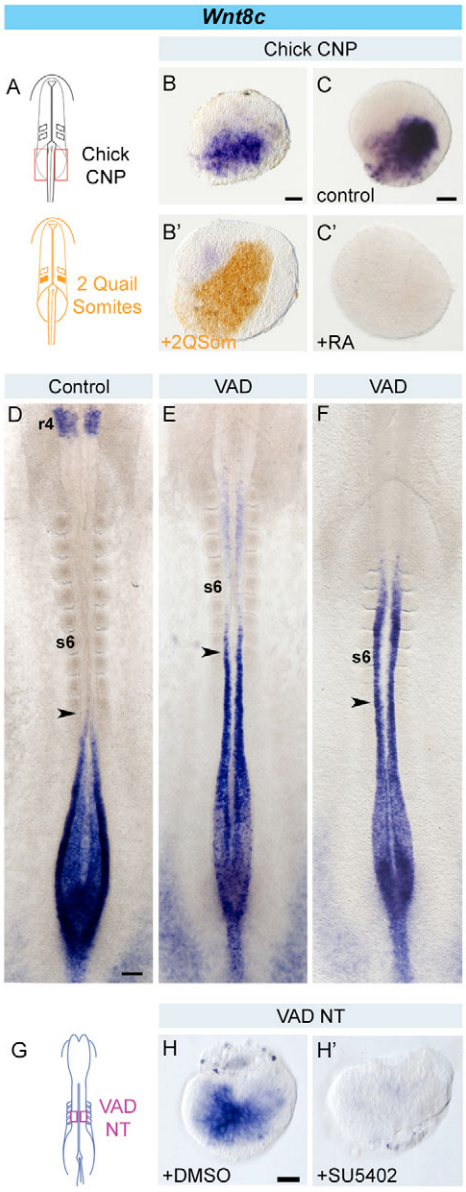

Fig. 2. Somites and retinoic acid inhibit *Wnt8c* expression. (**A**) Origin of explants used in **B-C′**. (B) Caudal neural plate (CNP) expresses *Wnt8c* (purple) after 24 hours culture whereas its counterpart explant pair (B′) loses *Wnt8c* next to the quail somites (QCPN, orange). CNP explants in DMSO (control) (C) express *Wnt8c* after 24 hours, whereas 9cisRA represses *Wnt8c* (C′). *Wnt8c* expression in control (**D**) and VAD quails (**E**,**F**). *Wnt8c* is ectopically expressed in VAD neural tube. Arrowheads indicate the last somite formed; r4, prospective rhombomere 4; s6, somite 6 which indicates the level of the prospective hindbrain/spinal cord boundary. (**G**) Origin of explants derived from VAD quails used in (**H**,**H′**). VAD neural tube (VAD NT) explants still express *Wnt8c* after 6 hours in DMSO (H), but this is lost with SU5402 (H′). r4, prospective rhombomere 4; s6, somite 6. Scale bars: 50 μm in B for B-C′; 100 μm in D for D-F; 25 μm in G for H,H′.

and compared with the DMSO-treated controls, 9-*cis* RA (8/8) or the RA agonist TTNPB (5/5) inhibited dramatically *Wnt8c* in caudal neural plate explants cultured for 24 hours (Fig. 2C,C′). These findings suggest that somite-derived RA suppresses *Wnt8c* expression in the neuroepithelium.

Next we examined if retinoid signalling is required to repress *Wnt8c* by assessing its expression in vitamin A-deficient (VAD) quails. *Wnt8c* is ectopically expressed in the VAD neural tube flanked by somites (4/4) in comparison with control quails (four control embryos) (Fig. 2D-F). In the most severe case *Wnt8c* transcripts were detected along the entire length of the VAD hindbrain and spinal cord (Fig. 2F) due to the combined caudal expansion of the prospective rhombomere 4 (r4) (Dupe and Lumsden, 2001) and perdurance of stem zone *Wnt8c*. These findings thus strongly suggest that RA is required for downregulation of *Wnt8c* as the neural tube forms.

It is possible that retinoid signals inhibit *Wnt8c* indirectly by attenuating FGF signalling. We therefore tested whether the persistent *Wnt8c* domain in VAD neural tube is still dependent on FGF signalling [even though *Fgf8* transcripts are not detected in VAD neural tube (see Diez del Corral et al., 2003)], or if it resulted from the direct loss of retinoid signalling. In the majority of cases, VAD neural tube explants still expressed *Wnt8c* after 6 hours in culture (3/5) (Fig. 2G,H) and in these, the contralateral explant pair treated with SU5402 showed a dramatic reduction in *Wnt8c* (3/3) (Fig. 2H′). This indicates that RA is unlikely to repress directly *Wnt8c* expression, which is FGF-dependent even in the VAD neural tube. This suggests that during normal development RA indirectly represses *Wnt8c* via its ability to attenuate FGF signalling.

Wnt8c inhibits neuronal differentiation

As *Wnt8c* is induced by FGF signalling, this molecule likely mediates some FGF activities. We therefore tested whether maintaining *Wnt8c* expression could delay onset of neuronal differentiation, as indicated by expression of *NeuroM*. Electroporation of a Wnt8c-IRES-GFP/PCINeo expression vector into the caudal neural plate and preneural tube HH8-10 lead to a significant reduction in the number of *NeuroM*-positive cells in the neural tube (11/12, embryos) compared with the control empty vector (nine controls) (Fig. 3A-C). A similar reduction was also observed for the proneural gene homologue *Neurogenin1* (3/4 embryos and 3 controls, data not shown). These in vivo data are further supported by the effects of Wnt8c on explanted neural tissue; comparison of explants of the preneural tube (Fig. 3D) combined with COS7 cells transiently transfected either with the Wnt8c-IRES-GFP/PCINeo vector (Wnt8c cells) or the empty control vector (control cells) shows that after 24 hours explants combined with control cells consistently express *NeuroM* (9/9) (Fig. 3E) (Diez del Corral et al., 2002), but contralateral explants cultured with Wnt8c cells contain less *NeuroM*-positive cells than the controls (6/9) (Fig. 3E′). Wnt8c is generally thought to signal through the β-catenin pathway, so we next cultured preneural tube explants in the presence of LiCl, an inhibitor of GSK3β that can mimic canonical Wnt signalling (Klein and Melton, 1996). After 24 hours, *NeuroM* expression was reduced in LiCl-treated explants compared with untreated controls (11/15) (Fig. 3F,F′). This result is comparable to the effects of Wnt8c cells and is consistent with previous work identifying canonical/β-catenin signalling as an inhibitor of neuronal differentiation (Megason and McMahon, 2002). Wnt signalling thus appears to be a relay of FGF activity in the newly formed neuroepithelium, where it acts to prevent precocious neuronal differentiation.

FGF signalling can inhibit neurogenesis independently of Wnt signalling

It is, however, unclear whether Wnt and FGF signalling act via the same mechanism to inhibit neuronal differentiation. Indeed, whereas FGF dramatically inhibits *NeuroM* expression in neural

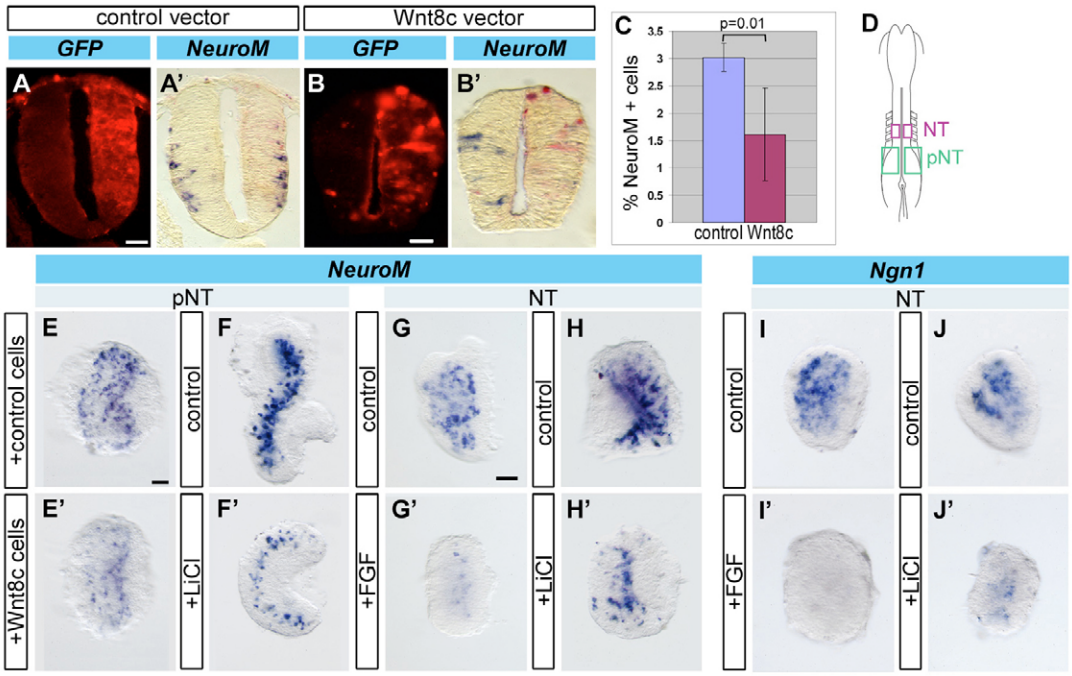

Fig. 3. Canonical Wnt signalling inhibits neuronal differentiation. (**A**,**A′**) Electroporation of control IRES-GFP/PCINeo vector (A, Fast Red, GFP transcripts) has no effect on *NeuroM* (A′). (**B**,**B′**) Electroporation of Wnt8c-IRES-GFP/PCINeo (B), reduces the amount of *NeuroM*-positive cells (B′). (**C**) Quantification of results shown in (A-B′): the percentage of *NeuroM*-positive cells on the electroporated side is significantly lower in presence of Wnt8c (four sectioned embryos), compared with the control (five sectioned embryos) (*t*-test, *P*=0.01). (**D**) Origin of neural tube (NT), preneural tube (pNT) and underlying presomitic mesoderm (PSM) explants used in (**E-J′**). (E-F′) pNT explants either combined with control cells (E) or Wnt8c cells (E′), or cultured in control media (F) or media supplemented with LiCl (F′). Fewer *NeuroM*+ cells arise in pNT exposed for 24 hours to Wnt8c or to LiCl. (G-H′) NT in control media (G-J), with FGF4 (G′,I′) or with LiCl (H′,J′). Fewer *Ngn1*+ cells are present in NT exposed to FGF4 (I′) or to LiCl (J′). Scale bars: 25 μm in A for A,A′; 20 μm in B for B,B′; 50 μm in E for E-F′ and in G for G-H′.

tube explants [17/17; after Diez del Corral et al. (Diez del Corral et al., 2002)] (Fig. 3G,G′), stimulating Wnt signalling with LiCl inhibits *NeuroM* in just half the cases (6/12) and leads to only a modest reduction in *NeuroM*-positive cells (Fig. 3H,H′). Similarly, *Neurogenin1* expression was dramatically reduced in neural tube explants treated with FGF4 (12/13) (Fig. 3I,I′) but only partially inhibited by LiCl in half the cases (5/9) (Fig. 3J,J′). This suggests that Wnt signalling is a less efficient repressor of neurogenesis. Furthermore, in neural tube explants (which no longer express *Fgf8* or *Wnt8c* at the time of excision) exposure to FGF4 does not re-activate *Wnt8c* (8/8) (Fig. 4A,A′), indicating that Wnt8c is not necessary for FGF to inhibit neurogenesis. It is possible that FGF suppresses neuronal differentiation in this context via other canonical Wnt ligands *Wnt1* and *Wnt3a*, which are normally present in the dorsal neural tube (Hollyday et al., 1995). Indeed, neural tube explants express both *Wnt1* (4/4) and *Wnt3a* (3/4) after 24 hours in culture (Fig. 4B,C) but treatment with FGF4 strongly inhibits *Wnt1* (4/4) (Fig. 4B′) and can downregulate *Wnt3a* expression in some cases (2/4) (Fig. 4C′). To test directly whether FGF relies on Wnt signalling to inhibit neuronal differentiation we next simultaneously exposed neural tube explants to FGF and blocked Wnt signalling, using the casein kinase I inhibitor CKI-7 (Chijiwa et al., 1989; Price, 2006) (see below). CKI-7 by itself has no consistent effect on *NeuroM* (8/8) (Fig. 4D,D′) and neural tube explants in the presence of FGF4 and CKI-7 still lose *NeuroM* expression (3/4) (Fig. 4E,E′). These findings indicate that inhibition of neuronal differentiation by FGF is unlikely to rely on canonical Wnt signalling in the neural tube and further, strongly suggest that FGF and Wnt act via different mechanisms to interfere with neurogenesis. To address this possibility we next assessed the regulatory relationship between these two signalling pathways and the retinoid pathway, which is necessary for neuron production in this context (Diez del Corral et al., 2003).

FGF but not canonical Wnt signalling represses *RARβ* expression

The promoter of *RARβ* contains a retinoic acid response element (RARE), making *RARβ* transcript levels a useful reporter of retinoid pathway activity (de The et al., 1990). To dissect the mechanism(s) by which FGF and Wnt exert their effects on neuronal differentiation we therefore tested whether these signals act by attenuating *RARβ* levels. *RARβ* transcripts are normally detected in the neural tube (Fig. 4F,F′) and as expected are present in explants of this tissue cultured for 24 hours (8/8) (Fig. 4G). Strikingly, FGF treatment strongly inhibits *RARβ* expression in neural tube explants (8/8) (Fig. 4G′) indicating that FGF can interfere with RA signal transduction within the neuroepithelium. As *Wnt8c* is expressed in the preneural tube, we used this tissue to test whether Wnt signalling also acts by repressing *RARβ*. In contrast to FGF treatment, Wnt8c/control cells or LiCl produce no consistent change in levels of *RARβ* in preneural tube explants, after 8 or 24 hours culture (Wnt8c cells, 8h, 6/6; LiCl, 24 hours, 4/4, data not shown; Wnt8c cells, 24 hours, 6/6 Fig. 4H,H′). These observations suggest that FGF and canonical Wnt signalling repress neuronal differentiation via different mechanisms: FGF, but not Wnt signalling, interferes with the retinoid pathway, either at the level of *RARβ* transcription and/or RA signal transduction.

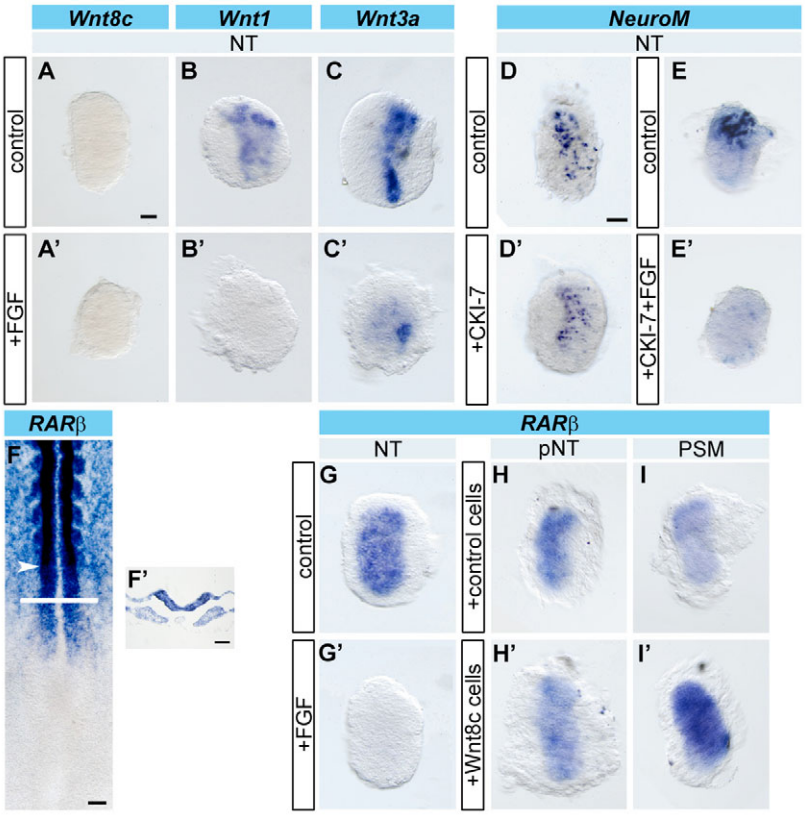

Fig. 4. Wnt and FGF signalling inhibit neurogenesis via different mechanisms. (**A-C′**) Neural tube (NT) explants in control media do not express *Wnt8c* at the time of excision or 24 hours later (A) but do express *Wnt1* (B) and *Wnt3a* (C). FGF4 does not reactivate *Wnt8c* (A′), reduces strongly *Wnt1* (B′) and mildly *Wnt3a* (C′). (**D-E′**) NT explants in control media with ethanol (D,E), CKI-7 (D′) or FGF4 and CKI-7 (E′). Inhibiting Wnt signalling with CKI-7 does not alter *NeuroM* expression in NT (within 24 hours) and FGF4 in the presence of CKI-7 is still able to inhibit *NeuroM* (24 hours, compare E with E′ and see Fig. 3G,G′). (**F**,**F′**) *RARβ* is expressed in the transition zone and the underlying rostral presomitic mesoderm at HH10 (F′: section of F indicated by a white line). (**G**,**G′**) NT explants in control media (G), with FGF4 (G′). pNT explants combined with control cells (**H**) or Wnt8c cells (**H′**). PSM explants combined with control cells (**I**) or Wnt8c cells (**I′**). *RARβ* is dramatically inhibited by FGF4 in neural tube explants, but is not consistently affected by Wnt8c in pNT. *RARβ* appears enhanced in underlying PSM by Wnt8c in 24 hours of culture. Scale bars: 50 μm in A for A-C′,H-I′, in D for D-E′,G-G′ and in F′; 100 μm in F.

Wnt signalling promotes *Raldh2* onset in the presomitic mesoderm

We reasoned that although Wnt signalling does not inhibit RA transduction in the preneural tube it might interfere with this process or retinoid synthesis in the mesoderm, as *Lef1*, a key component of the Wnt signal transduction machinery, is expressed at high levels in the rostral presomitic mesoderm (Schmidt et al., 2004) (see Fig. S1E,E′ in the supplementary material). We therefore tested whether Wnt8c could inhibit *RARβ* expression in the presomitic mesoderm. However, unexpectedly half of the presomitic mesoderm explants cultured in contact with Wnt8c cells showed enhanced *RARβ* expression compared with the control cells (4/8, 8 hours) (Fig. 4I,I′). This suggests that in this tissue Wnt signalling actually promotes RA signalling. One way in which the Wnt pathway might promote *RARβ* is by increasing RA synthesis. We therefore next manipulated Wnt signalling in the presomitic mesoderm and examined the onset of *Raldh2* in this tissue. Caudal presomitic mesoderm explants (Fig. 5A) weakly express *Raldh2* after 8 hours in culture with control cells (3/11 are slightly positive) (Fig. 5B) whereas in the presence of Wnt8c cells, *Raldh2* is clearly upregulated in most explant pairs (9/11) (Fig. 5B′), indicating that Wnt signalling can promote *Raldh2* onset.

To test whether canonical Wnt signalling upregulates *Raldh2* in vivo, we next briefly exposed whole embryos to LiCl and control and treated embryos were then processed strictly in parallel to allow comparison of the intensity of ISH signals. This revealed that although the Wnt target *Lef1* is clearly upregulated (LiCl, 6/6 and 5 control embryos) (Fig. 5C,D), LiCl enhances *Raldh2* expression in only a minority of cases (LiCl, 2/7 and 6 control embryos) (Fig. 5E,F). As we have shown previously that FGF signalling inhibits the onset of *Raldh2* (Diez del Corral et al., 2003) it may be that in vivo *Raldh2* expression requires Wnt activity in the context of low or no FGF signalling (a condition achieved in explanted caudal paraxial mesoderm, which rapidly loses *Fgf8* expression, see Fig. S2 in the supplementary material). Treatment of embryos with the FGF receptor inhibitor SU5402 results in the loss of the FGF responsive gene *Sprouty2* (SU5402, 5/5 and 5 control embryos) (Fig. 5G,H) whereas *Raldh2* expression is largely unaffected (SU5402, 5/6 and 6 control embryos) (Fig. 5I,J). However, exposure to both SU5402 and LiCl consistently enhances *Raldh2* expression (SU5402 + LiCl, 11/12 and 9 control embryos) (Fig. 5K,L) and a subset of these embryos (4/11) exhibits a caudal expansion of the *Raldh2* domain (Fig. 5L). These data therefore suggest that Wnt signals promote *Raldh2*, once FGF signalling has declined.

We next carried out a series of experiments to test whether *Raldh2* onset requires Wnt signalling. To inhibit this pathway in whole embryos we first grafted beads soaked in SFRP2 protein between the neuroepithelium and presomitic mesoderm and embryos were allowed to develop for 8 hours. In most cases *Raldh2* onset is shifted rostrally with respect to the unoperated side of the embryo (Fig. 6A,B) (SFPR2 beads 7/9, control PBS beads *n*=7), indicating that local inhibition of Wnt signalling delays *Raldh2* expression. Consistent with this finding, *Lef1* expression in the presomitic mesoderm is also attenuated by sFRP2 beads (3/3, 3 control PBS beads, data not shown). These data suggest that there is a specific requirement for Wnt signalling for mesoderm maturation. SFRP2 can inhibit both canonical and non-canonical Wnt signals (for a review, see Kawano and Kypta, 2003) and we therefore next tested the effects of CKI inhibition, as this molecule is a key mediator of the canonical Wnt pathway (Chijiwa et al., 1989; Price, 2006). For treatment with this drug embryos were prepared in filter culture, exposed to media containing CKI-7 and processed in parallel with control embryos, as above. This revealed that in most cases CKI-7 attenuates both *Lef1* (Fig. 6C,D) (CKI-7, 4/5 and 5 control embryos)

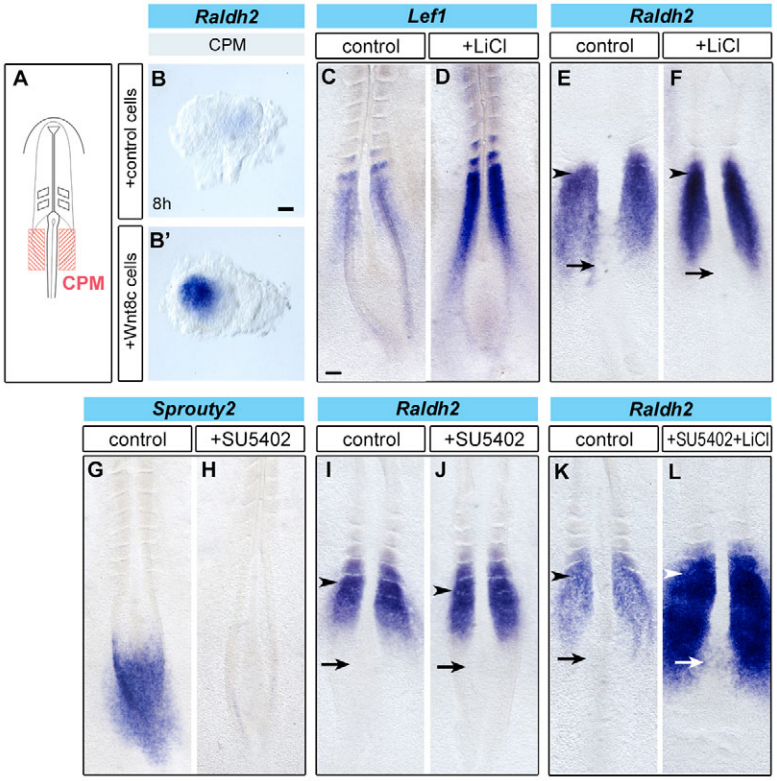

Fig. 5. Onset of RA synthesis is promoted by canonical Wnt signalling once FGF signalling has declined. (**A-B'**) *Raldh2* is weakly expressed in caudal presomitic mesoderm (CPM) explants (drawn in A) cultured for 8 hours with control cells (B), whereas it is activated in presence of Wnt8c cells (B′). (**C-F**) Embryos cultured in control media (C,E) or media with LiCl (D,F). The Wnt responsive gene *Lef1* is upregulated by LiCl, whereas *Raldh2* expression is not consistently upregulated. (**G-J**) Comparison of embryos treated with DMSO (control media G,I) or SU5402 (H,J). SU5402 inhibits the expression of the FGF responsive gene *Sprouty2* but *Raldh2* is not affected by this treatment. (**K**,**L**) Compared with embryos in DMSO media (K), embryos cultured in presence of SU5402 and LiCl combined (L) show enhanced *Raldh2* expression, and in some cases (L) this expands caudally. Arrow, node; arrowhead, last somite formed. Scale bars: 50 μm in B for B,B′; 100 μm in C for C-L.

and *Raldh2* expression (Fig. 6E,F) (CKI-7, 5/9 and 11 control embryos), supporting a requirement for canonical Wnt signalling for *Raldh2* onset.

Wnt signalling has been implicated in mesoderm formation (see Yamaguchi, 2001), which, despite our assessment of effects in embryos on filters after only 4 hours, might contribute to the observed reduction of *Raldh2* in the whole embryo when Wnt signalling is blocked. To assess directly the effects of Wnt signalling on the maturation of the presomitic mesoderm we therefore next manipulated this pathway in explants of this tissue. Incubation of caudal presomitic mesoderm explants for a long period leads eventually to *Raldh2* expression (Diez del Corral et al., 2003). We therefore cultured these explants for 18 hours either alone or in the presence of a Wnt inhibitor. As expected, explants cultured in control medium eventually came to express *Raldh2* by themselves (16/16) (Fig. 6G) whereas significantly, treatment with either CKI-7 (7/8) or the secreted canonical Wnt signalling inhibitor DKK1 (14/16) (Glinka et al., 1998) inhibited endogenous *Raldh2* onset (Fig. 6G′). Altogether these findings indicate that in the embryo, once FGF signalling has declined (which happens more rapidly in the mesoderm than in the neuroepithelium, see Fig. S2 in the supplementary material) canonical Wnt signalling acts specifically in the presomitic mesoderm to promote *Raldh2* expression.

DISCUSSION

We have determined the regulatory relationships between FGF, Wnt and RA signalling in the extending body axis and show how they work together to control and coordinate differentiation onset in newly generated neural and mesodermal tissue. FGF signalling interferes with RA signal transduction and is sufficient and necessary for maintenance of *Wnt8c* in the neuroepithelium, which is the only FGF promoted caudal Wnt signal (Fig. 7, step 1). As FGF signalling declines *Wnt8c* expression persists in the neuroepithelium and, in contrast to FGF, Wnt signals permit RA signal transduction. Decline of FGF signalling in the presomitic mesoderm now also

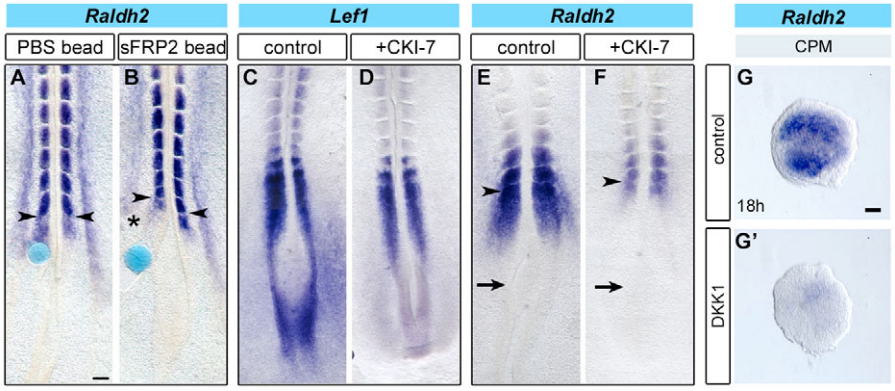

Fig. 6. Loss of Wnt signalling delays *Raldh2* onset. (**A**,**B**) Effects of control PBS beads (A) and beads soaked in sFRP2 (B) on *Raldh2* onset. Asterisk in B indicates region of *Raldh2* inhibition. (**C-F**) Embryos cultured in ethanol control media (C,E) or media supplemented with CKI-7 (D,F). Both *Lef1* and *Raldh2* are downregulated by CKI-7. (**G**,**G'**) After 18 hours in culture, CPM explants come to express *Raldh2* in control media (G), but this is prevented by DKK1 (G′). Arrow, node; arrowhead, last somite formed. Scale bars: 100 μm in A for A-F; 50 μm in G for G,G′.

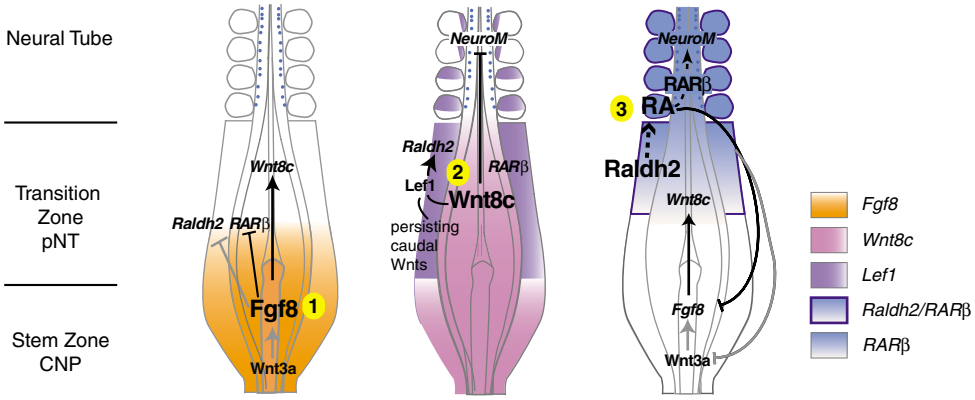

Fig. 7. Sequence of signalling events controlling maturation of the extending body axis. Model for the sequential action of Fgf8 (1), Wnt (2) and retinoid (3) signals in the extending body axis (see text for details). Grey lines indicate interactions shown elsewhere, and black lines those established in this work.

allows persisting caudal Wnt signals to promote expression of *Raldh2* and hence RA synthesis (Fig. 7, step 2). Once RA reaches sufficient levels, it acts back to inhibit *Fgf8* and hence *Wnt8c* (Fig. 7, step 3). These findings identify canonical Wnt signalling as a pivotal pathway that acts as FGF signals decline to promote and permit RA signalling in the extending body axis and hence mediate the transition from the proliferative undifferentiated caudal cell state to one in which differentiation and cell cycle exit are possible.

Wnt8c prevents neuronal differentiation onset but permits retinoid signalling

Our finding that exposure to Wnt8c or LiCl leads to a reduction in *NeuroM*- and *Ngn1*-positive cells is consistent with previous work showing that Wnt signalling maintains neuroepithelial cells in the cell cycle (Chenn and Walsh, 2002; Dickinson et al., 1994; Ikeya et al., 1997; Megason and McMahon, 2002; Zechner et al., 2003). FGF signalling also promotes proliferation in many contexts, including neural progenitors in which it can additionally accelerate the cell cycle (Lukaszewicz et al., 2002; Wilcock et al., 2007). Our data further support this distinction between Wnt and FGF signalling and suggest that Wnt signalling is a milder inhibitor of neurogenesis than FGF as indicated by the expression of *NeuroM* and *Ngn1*. These findings are consistent with data showing that although activation of β-catenin in the neuroepithelium maintains proliferation, some cells can still differentiate into neurons in this context (Zechner et al., 2003).

Although FGF does not depend on Wnt signalling to inhibit neuronal differentiation in our neural tube explant assay, it is still possible that FGF regulates Wnt activity in the neuroepithelium. FGF can influence the outcome of β-catenin activity in cortical progenitor cells maintained in vitro; when β-catenin is overexpressed with FGF it promotes proliferation whereas in the absence of FGF it can enhance neuronal differentiation (Israsena et al., 2004). There is also some evidence that Wnt signals promote neuron production by regulating *Ngn1* expression in cortical progenitors, however, this is stage dependent (Hirabayashi et al., 2004; Israsena et al., 2004) and appears not to correspond to neurogenesis in our neural tube assay, where Wnt signalling mildly reduces neuron production.

The difference in the impact of Wnt and FGF signalling on neuronal differentiation may well be explained by our finding that FGF, but not canonical Wnt signalling, can inhibit the retinoid pathway, which is required for neuronal differentiation. Indeed, there is evidence in cell lines that association of RARβ and β-catenin proteins can elicit activity at RAREs in the promoters of RA-responsive genes and additionally, that there can be competition for β-catenin association with either RARβ or TCF/Lef1, which could reduce either RA or Wnt activity (Easwaran et al., 1999). This suggests that direct interactions between RA and Wnt pathways could help to regulate neuronal differentiation within the neural tube. Together these observations underscore a key conclusion of this work: although Wnt signalling can restrain neuronal differentiation it permits RA activity, whereas FGF, as indicated by its dramatic inhibition of *RARβ*, abolishes RA signalling. The regulatory relationships between FGF, Wnt and RA pathways defined in this study may also help to explain why a combination of FGF and Wnt signalling leads to the acquisition of more caudal spinal cord character, as these signals are characteristic of the stem zone where progressively more caudal genes are expressed, and why exposure to retinoid signalling, which represses FGF/Wnt activity, gives more rostral spinal cord character (see Nordstrom et al., 2006).

Wnt signalling controls the timing of presomitic mesoderm maturation

Canonical Wnt signals are critical for multiple steps in the mesodermal lineage. These include: mesoderm induction (Szeto and Kimelman, 2004; Takada et al., 1994; Yamaguchi et al., 1999; Yoshikawa et al., 1997); regulation of cyclic gene expression and maintenance of *Fgf8* and hence the maturation wavefront underpinning segmentation (Aulehla et al., 2003; Dubrulle and Pourquie, 2004a; Ishikawa et al., 2004); as well as the promotion of myogenesis (reviewed by Tajbakhsh and Buckingham, 2000). Here we identify a new role for canonical Wnt signalling in controlling the timing of retinoid production in the extending body axis. We demonstrate in whole embryos that canonical Wnt signalling is required for *Raldh2* expression and that blocking this pathway with either a small molecule inhibitor or an endogenous secreted LRP5/6 co-receptor antagonist (DKK1) specifically in explants of the caudal paraxial mesoderm inhibits *Raldh2* onset. Furthermore, we show that Wnt signals are sufficient to accelerate onset of this gene in explanted caudal paraxial mesoderm. Importantly, such explants cultured for a long period without exposure to additional Wnt ligand do eventually express *Raldh2* and this is most likely due to prior exposure to Wnts, as blocking canonical Wnt signalling in this tissue inhibits *Raldh2* onset. This indicates that Wnt signalling acts normally in the presomitic mesoderm to control the timing of *Raldh2* expression.

However, in our short-term in vivo assay, acceleration of *Raldh2* expression requires both stimulation of canonical Wnt signalling and loss of FGF signalling. This reflects the ability of FGF to repress *Raldh2* (Diez del Corral et al., 2003), while the difference between in vivo and in vitro assays may be explained by the very rapid loss

of *Fgf8* expression observed in caudal paraxial mesoderm explants (see Fig. S2 in the supplementary material). This requirement for Wnt signalling in addition to attenuation of FGF for *Raldh2* onset in vivo is also consistent with previous work showing that inhibiting FGF signalling alone is insufficient for onset of *paraxis*, a later marker of somitic tissue (Delfini et al., 2005). Finally, by placing Wnt inhibitor-presenting beads between the neuroepithelium and the paraxial mesoderm we localize this requirement for Wnt signalling for *Raldh2* expression in vivo. This experiment also supports the possibility that it is Wnt signals provided by the neuroepithelium that regulate *Raldh2* onset. Importantly, *Wnt8c* is a good candidate to mediate this step as it is expressed by the neuroepithelium and is the only known canonical Wnt expressed in the vicinity of the *Raldh2* domain. We also demonstrate that Wnt8c can induce *Raldh2* in caudal presomitic mesoderm explants. So, although there may be a contribution from persisting Wnts transcribed more caudally in the mesoderm (Wnt3a, Wnt5a and Wnt8c) (see Nakaya et al., 2005), our experiments strongly suggest that local stimulation of Wnt signalling, as indicated by raised *Lef1* expression in the rostral paraxial mesoderm, is most likely provided by Wnt8c during normal development.

This conclusion further suggests that timely *Raldh2* onset depends on the differential loss of FGF signalling in presomitic mesoderm and caudal neuroepithelium, which would allow *Wnt8c* expression maintained by low-level FGF signalling to persist and act on the rostral presomitic mesoderm. We demonstrate the differential loss of FGF signalling by comparing *Fgf8* transcript levels in the caudal presomitic mesoderm and caudal neural plate explants taken from the same embryo (see Fig. S2 in the supplementary material). Our finding that *Fgf8* is lost more rapidly from the mesodermal layer together with a previous study which shows that this tissue contains only degrading *Fgf8* transcripts (Dubrulle and Pourquie, 2004b), suggests that transcription is only ongoing in the upper layer, the caudal neural plate. The sensitivity of *Wnt8c* to FGF signalling is demonstrated by its continued expression once *Fgf8* transcripts have declined in the neuroepithelium; *Wnt8c* is only downregulated at the level of the somites concomitant with *Sprouty2* (Chambers and Mason, 2000), a reporter of FGF signalling via MAPK (Minowada et al., 1999). Furthermore, we show that *Wnt8c* remains sensitive to loss of FGF signalling in the VAD neural tube, where even *Sprouty2* and activated MAPK are beneath detection levels (Diez del Corral et al., 2003), suggesting that *Wnt8c* is able to respond to very low levels of FGF signalling.

It is likely that Wnt signals are transduced via *Lef1* in the presomitic mesoderm, as this appears to be the main TCF expressed in this tissue (Schmidt et al., 2004). Previous studies indicate that *Lef1* expression in the rostral presomitic mesoderm is elicited by a combination of Shh and Wnt/β-catenin signalling and that this leads to induction of *MyoD* and subsequent myogenesis (Schmidt et al., 2000). We have shown previously that *Shh* expression in the neural plate is attenuated by FGF signalling and documented the onset of *Shh* in the floor plate at the level of somites (Diez del Corral et al., 2003), both of which suggest that Shh activity rises as FGF signalling declines. So, as FGF signalling diminishes, Shh levels increase and act together with Wnt8c to promote *Lef1* expression, which may then lead to the discrete onset of *Raldh2* (see Fig. 7). Interestingly, as well as Wnts, retinoid signalling promotes myogenesis in the embryo (Hamade et al., 2005; Maden et al., 2000) and can drive cell cycle exit and differentiation of myoblasts in vitro (Puri and Sartorelli, 2000). So, an early step in Wnt-directed myogenesis may be the promotion of RA synthesis in the presomitic mesoderm.

Crucially, once RA begins to be produced by the presomitic mesoderm, it acts back to inhibit *Wnt8c*. Our results support the idea that caudal *Wnt8c* is indirectly repressed by RA via its attenuation of FGF signalling; although *Wnt8c/8a* are dramatically expanded into the neural tube in the absence of retinoid signalling (this work) (Dupe and Lumsden, 2001; Niederreither et al., 2000), blocking FGF signalling in retinoid-deficient neural tube explants still leads to loss of *Wnt8c*. However, this does not rule out the possibility that RA also directly inhibits *Wnt8c*. Interestingly, caudal Wnt3a is also inhibited by exposure to retinoid signalling (Iulianella et al., 1999; Shum et al., 1999), suggesting that this regulatory loop may commence even earlier in the primitive streak where Wnt3a is required for maintenance of *Fgf8* expression (Aulehla et al., 2003). Significantly, although *Fgf8* then promotes *Wnt8c*, Fgf8 cannot induce *Wnt3a* (Kengaku et al., 1998) and we show here that Wnt8c does not induce *Fgf8*. These regulatory relationships are therefore directional and indeed a similar directional relay of Wnt and FGF signalling has been described during the initiation and outgrowth of the vertebrate limb bud (Kawakami et al., 2001). Wnt-FGF signalling relays therefore appear to be conserved mechanisms which underpin the spatial and temporal separation of signalling events during axis extension. In the case of the body axis described here, this directional signalling determines the precise spatial regulation of retinoid production and in this way controls the timing of the FGF/RA differentiation switch.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/11/2125/DC1

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