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Early mouse caudal development relies on crosstalk between retinoic acid, Shh and Fgf signalling pathways

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The progressive generation of embryonic trunk structures relies on the proper patterning of the caudal epiblast, which involves the integration of several signalling pathways. We have investigated the function of retinoic acid (RA) signalling during this process. We show that, in addition to posterior mesendoderm, primitive streak and node cells transiently express the RA-synthesizing enzyme *Raldh2* prior to the headfold stage. RA-responsive cells (detected by the RA-activated RARE-*lacZ* transgene) are additionally found in the epiblast layer. Analysis of RA-deficient *Raldh2*$^{-/-}$ mutants reveals early caudal patterning defects, with an expansion of primitive streak and mesodermal markers at the expense of markers of the prospective neuroepithelium. As a result, many genes involved in neurogenesis and/or patterning of the embryonic spinal cord are affected in their expression. We demonstrate that RA signalling is required at late gastrulation stages for mesodermal and neural progenitors to respond to the Shh signal. Whole-embryo culture experiments indicate that the proper response of cells to Shh requires two RA-dependent mechanisms: (1) a balanced antagonism between Fgf and RA signals, and (2) a RA-mediated repression of *Gli2* expression. Thus, an interplay between RA, Fgf and Shh signalling is likely to be an important mechanism underpinning the tight regulation of caudal embryonic development.

KEY WORDS: Retinoids, Gastrulation, Neurogenesis, Spinal cord, Somites, Sonic hedgehog, Gli, Fibroblast growth factor, Mouse

INTRODUCTION

In the vertebrate embryo, the trunk structures (spinal cord, notochord, somites and lateral plate mesoderm) are progressively generated from the caudal embryonic region, in which gastrulation proceeds during extension of the body axis. The epiblast adjacent to the anterior part of the primitive streak contributes mainly to paraxial mesoderm and neuroectoderm (Tam and Beddington, 1987). Fate maps in chick showed that spinal cord precursors are located in the epiblast lateral to Hensen's node (Brown and Storey, 2000; Mathis et al., 2001). Studies in mouse and chicken have shown that neural and somitic tissues are also derived from the node and node-streak border (Beddington, 1994; Cambray and Wilson, 2002; Cambray and Wilson, 2007; Mathis and Nicolas, 2000; Nicolas et al., 1996). Selleck and Stern (Selleck and Stern, 1991) demonstrated that cells from the chick node contribute iteratively to the notochord. Thus, from very early in development, the formation of the posterior central nervous system and the axial and paraxial mesoderm are tightly linked.

According to Nieuwkoop's 'activation-transformation' model (Nieuwkoop et al., 1952; Slack and Tannahill, 1992), further elaborated by Stern and colleagues (Foley et al., 2000; Stern, 2001), neural induction produces cells with a rostral forebrain character that, upon the action of 'transforming' factors identified as Wnts, fibroblast growth factor (Fgf) and retinoic acid (RA), acquire caudal neural character. These three signalling pathways are used repeatedly during caudal patterning in the neuroepithelium and the mesoderm (Diez del Corral and Storey, 2004; Maden, 2002; Olivera-Martinez and Storey, 2007; Stern, 2005), suggesting that they interact differently and use distinct co-factors according to the time and tissue where they are active.

The development of caudal structures relies on the spatio-temporal distribution of RA, resulting from regulated expression of both synthesizing (Raldh1, Raldh2 and Raldh3) and metabolizing enzymes (Cyp26a1, Cyp26b1 and Cyp26c1). Early RA signalling in mouse embryos relies on Raldh2 function (Niederreither et al., 1999). Complementary expression patterns of *Raldh2* and *Cyp26a1* are observed; during somitogenesis, *Raldh2* is expressed in somites and rostral presomitic mesoderm (Blentic et al., 2003; Niederreither et al., 1997; Vermot et al., 2005), whereas *Cyp26a1* expression is restricted to caudalmost tissues (Fujii et al., 1997; Reijntjes et al., 2004). Analysis of mouse mutants deficient in RA signalling (*Raldh2*$^{-/-}$ and *Rara*$^{-/-}$;*Rarg*$^{-/-}$ mutants) or vitamin A-deficient (VAD) quail embryos revealed that RA acts as a caudalizing signal in the developing rhombencephalon. Additionally, the entire posterior region of these embryos is severely shortened. Neural induction occurs in *Raldh2*$^{-/-}$ embryos (Molotkova et al., 2005; Niederreither et al., 1999); however, the neuroepithelium remains abnormally thin. In addition, these mutants exhibit smaller somites, probably as a consequence of increased *Fgf8* expression in the tail bud (Molotkova et al., 2005; Vermot et al., 2005). Although *Raldh2* is already expressed during gastrulation in the newly formed mesenchyme adjacent to the node and primitive streak (Niederreither et al., 1997; Zhao et al., 1996), little is known about its function at this stage.

As development proceeds, the processes of neurogenesis and mesodermal segmentation rely, at least in part, on a balance between Fgf and RA signalling (Diez del Corral et al., 2003; Dubrulle et al., 2001). *Fgf8* is expressed as a posterior (high) to anterior (low) gradient in caudal tissues, and a crucial threshold (the 'determination front') determines the location of intersomitic boundaries and the

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commitment of cells to a given axial identity (Dubrulle et al., 2001). Additionally, Fgf8 maintains an undifferentiated 'stem' zone adjacent to the regressing primitive streak (Akai et al., 2005; Mathis et al., 2001) [see Rozsko et al. (Rozsko et al., 2007) for a description of stem cell-like spinal cord progenitors]. Simultaneously, RA emanating from the anterior presomitic mesoderm (PSM) and the somites attenuates Fgf signalling and allows neural progenitors to initiate differentiation. A recent study implicated canonical Wnt signalling in mediating the transition from Fgf to retinoid activity (Olivera-Martinez and Storey, 2007).

The acquisition of a neural cell type is concomitant to the onset of differentiation, and exposure to both RA and sonic hedgehog (Shh) simultaneously is an obligatory step in the emergence of correct ventral patterning (Diez del Corral et al., 2003; Novitch et al., 2003). The mechanism by which RA and Shh signals interact is unknown. RA also acts to regulate Hox genes, many of which contain functional RA-response elements (RAREs) (e.g. Bel-Vialar et al., 2002; Gould et al., 1998; Huang et al., 1998; Mainguy et al., 2003; Oosterveen et al., 2003; Packer et al., 1998). Positional identity is encoded by the combination of Hox genes expressed at a given axial level. Expression of *Hoxa1* is downregulated in the caudal region of *Raldh2*$^{-/-}$ embryos (Niederreither et al., 1999), raising the issue of the anteroposterior identity of neural tube cells in the absence of RA signalling.

In this study, we investigate the function of RA during caudal patterning of the mouse embryo, using *Raldh2*$^{-/-}$ mutants as a model system. We show that RA is required as early as E7.5 for correct patterning of the caudal neural plate, and later for the onset of spinal cord neurogenesis. We demonstrate that lack of RA signalling interferes with the response of cells to Shh, a defect which may partly result from abnormal Fgf activity. We also report that an Fgf-independent upregulation of *Gli2* may be a key mediator of the early embryonic RA-*deficiency* phenotypes.

MATERIALS AND METHODS

Mouse lines and embryo culture

Raldh2 mutants and RARE-*lacZ* transgenic mice have been described (Niederreither et al., 1999; Rossant et al., 1991). Culture experiments were performed as described (Ribes et al., 2008), with embryos collected at E7.5. Recombinant mouse Shh-N (R&D systems) was rehydrated in culture medium at 2.5 μM, and SU5402 (Calbiochem) in DMSO at a stock concentration of 85 mM. All-trans-RA (Biomol) was rehydrated in 95% ethanol at 20 mM. Control embryos were always cultured in presence of the drug's vehicle. Drug efficacy was tested by analyzing target genes for each signalling pathway (see Fig. S1 in the supplementary material; Fig. 6). At least two independent experiments were performed for each drug condition. After culture, the embryos were fixed overnight in 4 % paraformaldehyde and processed for in situ hybridization.

In situ hybridization, X-gal assays and immunohistochemistry

Whole-mount in situ hybridization was performed using an Intavis InSituPro robot (for details, http://empress.har.mrc.ac.uk/, gene expression section). Template plasmids were generated in our institute, or kindly provided by Drs B. De Crombrugghe (*Sox10*), S. Ang (follistatin), J. Deschamps (*Cdx1*, *Hoxb8*), D. Echevarria (*Mkp3*), R. Kageyama (*Hes5*), R. Krumlauf (*Hoxb9*), P. Gruss (*Pax3*), F. Guillemot (*Ngn2*), B. Herrmann (brachyury), C. C. Hui (*Gli1-3*), M. Kmita/D. Duboule (*lacZ*), J. Lewis (*Delta1*), R. Lovell-Badge (*Sox2*), G. Martin (*Spry2*), A. McMahon (*Shh*, *Wnt3a*), A. Nieto (*Snail*), M. Petkovitch (*Cyp26a1*), B. Robert (*Msx1*) and C. Tabin (*Ptch1*). Following in situ hybridization, some embryos were embedded in 1% agarose and vibratome sectioned.

Whole-mount X-gal assays and immunolabelling were performed as described previously (Ribes et al., 2006; Rossant et al., 1991). For immunohistochemistry on sections, embryos were fixed for 20 minutes, impregnated with 10% sucrose and cryosectioned at 10 μm. All antibodies were incubated in a solution of PBS, 10% foetal calf serum, 3% BSA and 0.1% triton. Primary antibodies (anti-Foxa2, anti-neurofilament, anti-Shh-N, Developmental Studies Hybridoma Bank) and secondary peroxidase-coupled goat anti-mouse antibody (Jackson Immunoresearch) were incubated overnight at 4°C, whereas goat anti-mouse AlexA4880 or AlexA594-conjugated (Molecular probes) were incubated for 45 minutes at room temperature.

RESULTS

RA activity in the caudal region of gastrulating mouse embryos

To characterize the role of RA signalling during gastrulation and early neurulation, we performed a detailed analysis of *Raldh2* and *Cyp26a1* expression in comparison with cells actively responding to RA in RARE-*lacZ* transgenic embryos (Rossant et al., 1991). *Raldh2* expression starts at the mid-primitive streak stage within the posterior embryonic region (E6.75; data not shown). At the neural plate stage (E7.5), it is expressed in posterior mesoderm (Fig. 1A,B), in primitive streak (Fig. 1A,B′) and in most of the node cells (Fig. 1A′). RARE-*lacZ* activity was similarly detected in the node (Fig. 1C′) and primitive streak (Fig. 1C). Although *Raldh2* is not expressed in the epiblast (Fig. 1B), the RARE-*lacZ* transgene is strongly active in this layer (Fig. 1D,D′). At headfold stage (E7.75), RA-responsive cells match the distribution of RA-producing cells (Fig. 1E,F). However, close examination showed that *Raldh2* expression has been downregulated in node and primitive streak cells (Fig. 1E′), whereas some primitive streak and node cells are still positive for the reporter transgene (Fig. 1F′). RARE-*lacZ* activity extinguishes progressively from these cells, especially within the node where it is detected at least until the four-somite stage (data not shown) (Sirbu and Duester, 2006). At later stages, RA signalling is restricted to somites, rostral presomitic mesoderm and neural tube (Vermot et al., 2005).

Expression of *Cyp26a1* appears by E6.5 in the anterior epiblast and mesendoderm (Fujii et al., 1997; MacLean et al., 2001). At headfold stage, *Cyp26a1*-expressing cells are adjacent to the rostralmost RARE-*lacZ*-positive cells (Fig. 1F,G) (Sirbu et al., 2005). Interestingly, *Cyp26a1* expression appears in scattered cells of the primitive streak whereas the RA-responsive transgene becomes downregulated (Fig. 1F′,G′). In addition, at this stage, *Cyp26a1* expression appears in posterior endodermal cells (Fig. 1G′, inset). Eventually, its expression expands anteriorly and laterally within the caudal neural plate (CNP) and primitive streak to reach the node cells (data not shown) (Fujii et al., 1997; MacLean et al., 2001).

Thus, RA activity is highly dynamic during gastrulation. Interestingly, primitive streak cells are transiently RA responsive, RA activity being gradually excluded from the streak and the CNP at the late headfold stage. Node cells transiently express *Raldh2*, leading to RA activity both in the node ectoderm and mesendoderm until late headfold stage, after which activity is selectively maintained in the node ectoderm (see also Sirbu and Duester, 2006).

Raldh2$^{-/-}$ embryos display caudal patterning defects

We addressed whether RA activity plays a role in early development of caudal tissues by analyzing *Raldh2*$^{-/-}$ mutants, using marker genes of the posterior epiblast and mesoderm. Follistatin is expressed from E6.5 in the ectodermal and mesodermal layers near the primitive streak (Fig. 2A-A″). Its expression in mesoderm becomes restricted to the primitive streak cells at the four-somite-stage (Fig. 2C,C′). At E7.75 (Fig. 2B-B″, *n*=5/5) and in three-somite

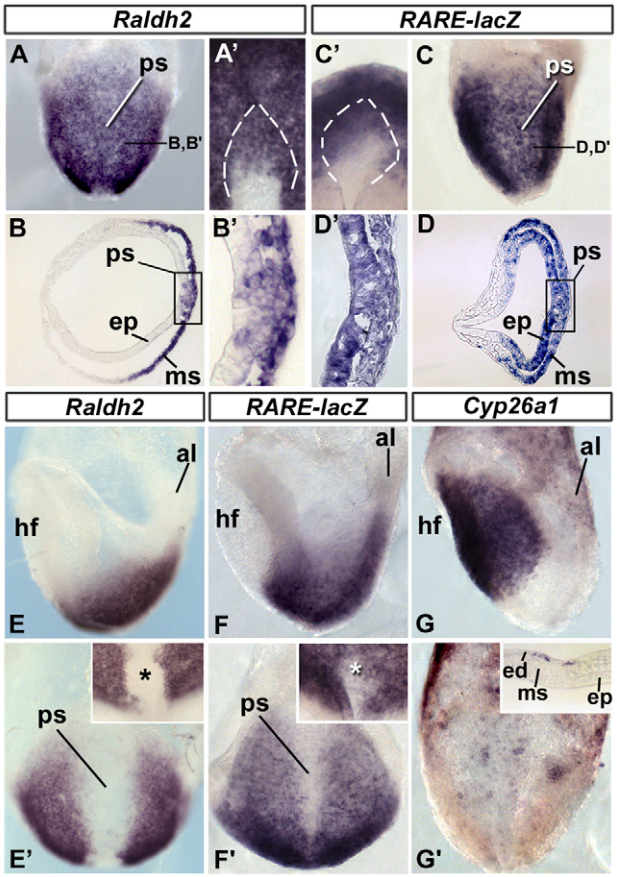

Fig. 1. Dynamic RA signalling in primitive streak, node and caudal neural plate. Whole-mount detection of *Raldh2* (**A-B'**,**E**,**E'**) and *Cyp26a1* (**G**,**G'**) transcripts in wild-type embryos, and *lacZ* mRNA (**C-D'**,**F**,**F'**) in RARE-*lacZ* embryos, at E7.5 (A-D') and E7.75 (E-G'). A,C,E',F',G', caudal views; E,F,G, lateral views; A',C', insets in E',F', details of the node region (broken lines or asterisks); B,D, transverse sections of the embryos shown in A,C, at the level of the medial region of the primitive streak (ps); B',D', high-power magnifications of the primitive streak (boxed in B,D); inset in G', transverse section of the embryo through the mid-primitive streak. al, allantois; ed, endoderm; ep, epiblast; hf, headfold; ms, mesoderm; ps, primitive streak.

stage (Fig. 2D-D″, *n*=3/3) *Raldh2*$^{-/-}$ embryos, follistatin expression within the epiblast and posterior mesoderm expands more laterally and anteriorly compared to wild-type embryos (brackets in Fig. 2C,D). Brachyury expression in the node and notochord is not significantly altered in mutants (Fig. 2G,H). However, similarly to follistatin, brachyury expression within the mutant epiblast and mesoderm expands more laterally and anteriorly than in wild-type embryos (Fig. 2E-F″, E7.75; Fig. 2G-H″, early somite-stage; *n*=4/5 and 8/9). The general level of brachyury expression also appears increased in mutants. We confirmed this patterning defect by analyzing *Wnt3a,* a gene required for brachyury expression (Yamaguchi et al., 1999), which exhibited a similar expansion in pre- and early somite stage *Raldh2*$^{-/-}$ embryos (Fig. 2I-J″; *n*=5/6 and 4/4, respectively).

We then focused our analysis on ectodermal markers. *Sox2*, an early marker of neural progenitor cells, is expressed by mid-late streak stages (E7.0-7.5) in the anterior presumptive neuroectoderm (Avilion et al., 2003). Eventually, it expands posteriorly and is detected in most of the CNP of three- to four-somite stage wild-type embryos (Fig. 3A) (Wood and Episkopou, 1999; Delfino-Machin et al., 2005). In stage-matched *Raldh2*$^{-/-}$ embryos, *Sox2* transcripts are undetectable in the CNP, but are present more rostrally in the neural tube (Fig. 3B; *n*=7/7). This defect is transient, as eight-somite stage wild-type and mutant embryos show comparable *Sox2* patterns in the CNP (data not shown; *n*=6/6) (Molotkova et al., 2005). *Wnt8a* is expressed in mitotic progenitors in the neural plate, and upon neural tube differentiation is maintained only in pre-rhombomere 4 (Fig. 3C). *Wnt8a* expression was shown to be maintained along the whole neural tube of *Raldh2*$^{-/-}$ embryos (Niederreither et al., 2000). However, *Wnt8a* expression within the CNP of mutants was consistently weaker, from E7.75 (data not shown; *n*=2/5) to at least the four-somite stage (Fig. 3D, bracket showing lack of expression in lateral regions; *n*=9/10).

We next analyzed *Cdx1*, which encodes a transcription factor required for caudal patterning and directly regulated by RA signalling (Houle et al., 2003; Subramanian et al., 1995; van den Akker et al., 2002). *Cdx1* is expressed in both the mesoderm and ectoderm of the CNP, and eventually in the neural tube (Meyer and Gruss, 1993). In four- to five-somite stage *Raldh2*$^{-/-}$ embryos, *Cdx1* expression is absent from the neural tube (Fig. 3F) and the lateral cells of the caudal region (Fig. 3E,F, brackets) (*n*=4/4). As Cdx proteins regulate Hox gene expression (Lohnes, 2003), we examined specification of the caudal neural tube in *Raldh2*$^{-/-}$ embryos by analyzing several Hox genes. *Hoxb9* expression is decreased in caudal ectoderm and mesoderm, and virtually undetectable in nascent neural tube, of five- to six-somite stage *Raldh2*$^{-/-}$ embryos (Fig. 3G,H, main panels; *n*=7/7), probably reflecting a delay in activation. However, expression of other Hox genes, including *Hoxb8* (Fig. 3G,H, insets; *n*=4/4), *Hoxb6*, *Hoxc6* and *Hoxc8* (data not shown) is comparable with control embryos. Thus, RA deficiency affects only some Hox genes in the caudal region, and, collectively, Hox expression patterns reveal that the posterior neural tube of *Raldh2* mutants acquires a spinal character.

In conclusion, lack of RA signalling leads to an enlargement of the domains of expression of primitive streak markers at E7.5. At early somite stages, the patterning of the caudal region is altered, ectopic expression of mesodermal markers is observed lateral to the node and in anterior/lateral regions of the CNP, whereas prospective neuroepithelial markers are undetectable or shifted anteriorly (see Fig. 9 for a summary).

Impaired neurogenesis in *Raldh2*$^{-/-}$ embryos

The delay in *Sox2* expression and persistence of *Wnt8a* expression in the neural tube (Fig. 3) suggest that neurogenesis may be altered in *Raldh2* mutants. Previous studies demonstrated a requirement for RA signalling during specification of neural progenitors along the spinal cord dorsoventral axis (Novitch et al., 2003; Diez del Corral et al., 2003; Wilson et al., 2004; Molotkova et al., 2005). Here, we show that RA is required for the proper molecular specification of spinal cord neural crest cells (NCCs), as observed by a delay in the appearance of *Wnt3a* (Fig. 4A,B, bracket, and data not shown; *n*=3/3 at E8.75; 4/4 at E9.5) and markedly decreased expression of *Msx1* (Fig. 4C,D; *n*=4/4) and *Pax3* (data not shown). Lack of *Sox9*, *Sox10* (Fig. 4E,F and data not shown; *n*=4/4 for both genes) and *Snail* (data not shown) transcripts further indicate an abnormal specification of NCCs in mutants.

Previous work in chick and quail has demonstrated that RA is required for the proper induction of genes that control spinal cord neurogenesis, including *Ngn1*, *Ngn2* and *Delta1* (Diez del Corral et

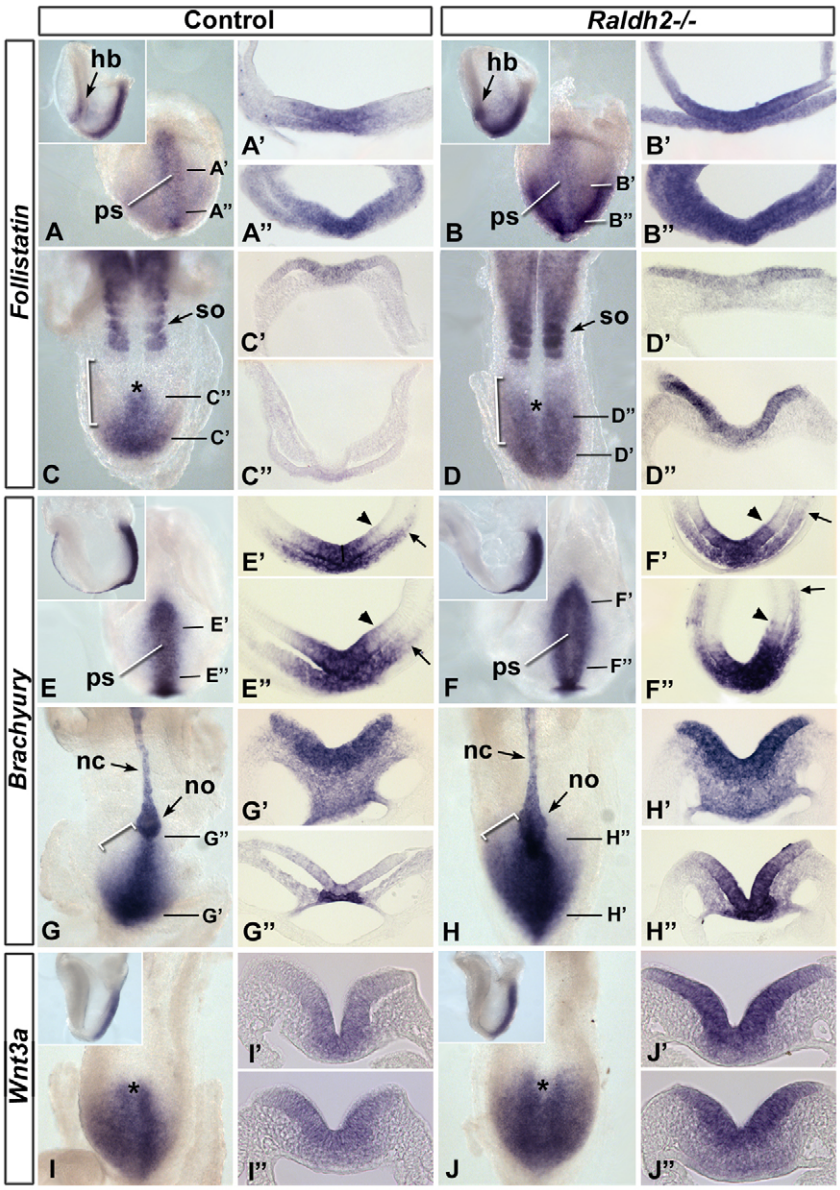

Fig. 2. RA is required to restrict the expression of primitive streak markers. Whole-mount in situ hybridization of follistatin (**A-D″**), brachyury (**E-H″**) and *Wnt3a* (**I-J″**) in control and *Raldh2*$^{-/-}$ embryos (genotypes indicated above) at E7.75 (A-B″,E-F″I,J, insets) and three- to four-somite stages (C-D″,G-J″). Main panels, posterior views; insets, lateral views. Transverse sections at the level of the node (″) and caudal primitive streak region (′) are also shown (approximate section levels indicated in main panels). Brackets show lateral expansion of follistatin (C,D) and brachyury (G,H) in mutants. Arrowheads and arrows (E′-F″) indicate the lateral border of brachyury expression in epiblast and mesoderm, respectively. hb, hindbrain; nc, notochord; no (or asterisks), node; ps, primitive streak; so, somites.

al., 2003). We investigated the timing of induction of these genes in the murine RA-deficient spinal cord. We found that, in wild-type embryos, *Delta1* is activated in scattered neural tube cells from the four- to five-somite stages (Fig. 4G). No *Delta1*-positive cells are present in *Raldh2*$^{-/-}$ neural tube until at least the six- to seven-somite stages (Fig. 4H; *n*=6/6). However, at the 14- to 15-somite stage, *Delta1* expression has become comparable with wild type (Fig. 4I,J; *n*=4/4). Similarly, the effector of Notch signalling *Hes5* is induced with a delay of about 10 hours in *Raldh2*$^{-/-}$ spinal cords (Fig. 4K-N; *n*=3/3 and 5/5, respectively). Neurogenin 2 (*Ngn2*) is normally activated at early somite stages (Scardigli et al., 2001; Ribes et al., 2008), whereas it only appears in *Raldh2*$^{-/-}$ spinal cord and hindbrain cells around E9.0 (Fig. 4O,P; *n*=6/6 and 7/7, insets and main panels, respectively). This is in agreement with the identification of an enhancer element recapitulating the early expression of *Ngn2*, the activity of which is dependent on RA signalling (Ribes et al., 2008). We also analyzed spinal neuron differentiation. In E9.5 wild-type embryos, neurofilaments are present along the dorsal root ganglia and the developing motoneuron axons (Fig. 4Q). No neurofilament-positive cells are observed along the *Raldh2*$^{-/-}$ spinal cord (Fig. 4R; *n*=5/5), although the hindbrain ganglia express this protein (data not shown) (Niederreither et al., 2000). These results corroborate data obtained in avian systems (Novitch et al., 2003; Diez del Corral et al., 2003) by showing that RA synthesized by Raldh2 is required, in the mouse embryo, for the onset of neurogenesis and neural differentiation at the trunk level. The delayed induction of various genes in the spinal cord of *Raldh2*$^{-/-}$ mutants may reflect the presence of other sources of RA in this tissue (Mic et al., 2002).

Decreased efficiency of Shh signalling in *Raldh2*$^{-/-}$ embryos

Previous studies have shown that in several tissues, Shh and RA signalling converge and influence target gene regulation (e.g. Bertrand and Dahmane, 2006; Helms et al., 1994; Helms et al., 1997). We investigated whether crosstalk between these two signalling pathways occurs during caudal development. We analyzed Shh transcripts and protein distribution in *Raldh2*$^{-/-}$

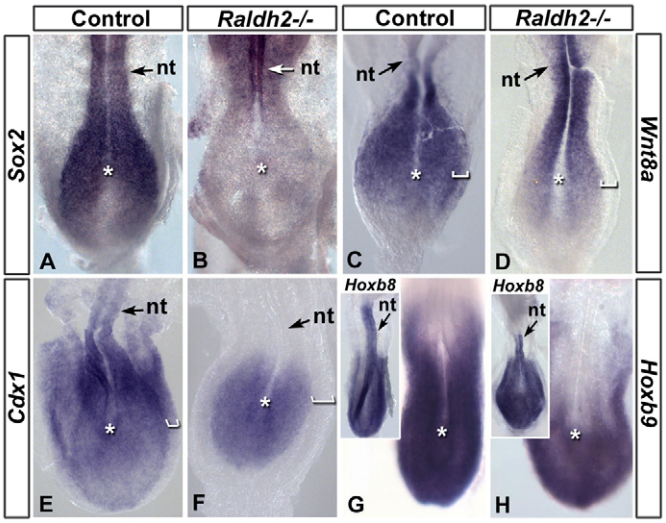

Fig. 3. RA deficiency affects expression of caudal neural plate markers. (**A-H**) Whole-mount in situ hybridization of *Sox2* (A,B), *Wnt8a* (C,D), *Cdx1* (E,F), *Hoxb9* (G,H) and *Hoxb8* (G,H, insets) in control and *Raldh2*$^{-/-}$ embryos (genotypes indicated above) at the three- to four- (A-F) and five- to six- (G,H) somite stages. Asterisks indicate the node. Brackets (C-F) indicate a lack of *Wnt8a* and *Cdx1* expression in lateral regions of mutants. Owing to overall shortening of the trunk, the *Hoxb8* domain appears shorter in the mutant neural tube (nt).

mutants, and found that *Shh* is correctly transcribed and translated in the prechordal plate, notochord and node until the 12- to 14-somite stages (Fig. 5A-B′; *n*=6/6; and data not shown). However, at the 14- to 15-somite stages (Fig. 5C) and at E9.5 (Fig. 5D), Shh protein is almost undetectable in *Raldh2*$^{-/-}$ floor-plate cells (Fig. 5E,F, and data not shown; *n*=5/5 at each stage). This is not due to a lack of floor plate, as both *Shh* transcripts and Foxa2 protein are similarly detected in ventral midline cells in controls and mutants (Fig. 5I,J and data not shown; *n*=9/9 for *Shh* in mutants). Shh transcript and protein expression in notochord is unaffected by RA deficiency, at least until E9.0 (Fig. 5C-F,G,I; notice the enlarged notochord with low *Shh* expression at E9.5 in 5J).

Among three transcription factors (Gli1, Gli2, Gli3) acting combinatorially to regulate Shh-dependent gene expression, *Gli1* is the only direct Shh target gene (Bai et al., 2004; Ingham and McMahon, 2001; Lee et al., 1997; Ruiz i Altaba et al., 2003; Sasaki et al., 1997; Stamataki et al., 2005). *Gli1* expression is markedly downregulated in *Raldh2*$^{-/-}$ embryos as early as E7.75 (Fig. 5K-L′; *n*=4/4). In wild-type embryos, *Gli1* is expressed in axial mesoderm, medial neural plate, newly formed mesoderm (Fig. 5K, bracket), lateral epiblast (Fig. 5K′; bracket) and node (asterisk). In *Raldh2*$^{-/-}$ embryos, *Gli1* expression is present in the anterior neural plate (Fig. 5L), but is downregulated in mesoderm (Fig. 5L, bracket), lateral epiblast (Fig. 5L′, bracket) and node (asterisk). At E8.5, *Gli1* is severely downregulated in most *Raldh2*$^{-/-}$ tissues (Fig. 5M-N″; *n*=9/9). Noteworthy, its distribution in the posterior hindbrain of mutants (Fig. 5N′) is not ventrally restricted as in controls (Fig. 5M′). Patched 1, another Shh target gene (Goodrich et al., 1996; Marigo et al., 1996), showed the same spatial and temporal downregulation in *Raldh2*$^{-/-}$ embryos (data not shown). These results suggest that in the absence of RA activity, Shh signalling cannot proceed efficiently in the prospective spinal cord and adjacent tissues.

RA activity is required for proper response to Shh signalling

The downregulation of Shh target genes in *Raldh2*$^{-/-}$ mutants suggests that cells require RA during late gastrulation and early neurulation to respond properly to the Shh signal. Should it be the case, administration of active Shh protein (Shh-N) may not rescue Shh signalling, and therefore *Gli1* expression, in *Raldh2*$^{-/-}$ embryos. We tested this hypothesis using whole-embryo culture. Headfold stage (E7.5) embryos were cultured for 14 hours in presence or absence of Shh-N. An increase in *Gli1* expression is observed in wild-type embryos (5/6 embryos) in both mesodermal and neural tissues after addition of Shh-N, already at a physiological concentration of 5 nM (Fig. 6A,A′,C,C′,E,E′,G,G′). By contrast, the addition of 5 nM, 50 nM or 150 nM Shh-N does not augment significantly *Gli1* expression in *Raldh2*$^{-/-}$ embryos (0/2; 0/9; 2/15 embryos showing elevated *Gli1* levels, respectively; Fig. 6B,B′,D,D′,F,F′). *Gli1* upregulation in mutants is only seen by increasing Shh-N to 200 nM (5/9; Fig. 6H,H′). Higher Shh-N doses (300, 400 nM) drastically affect both wild-type

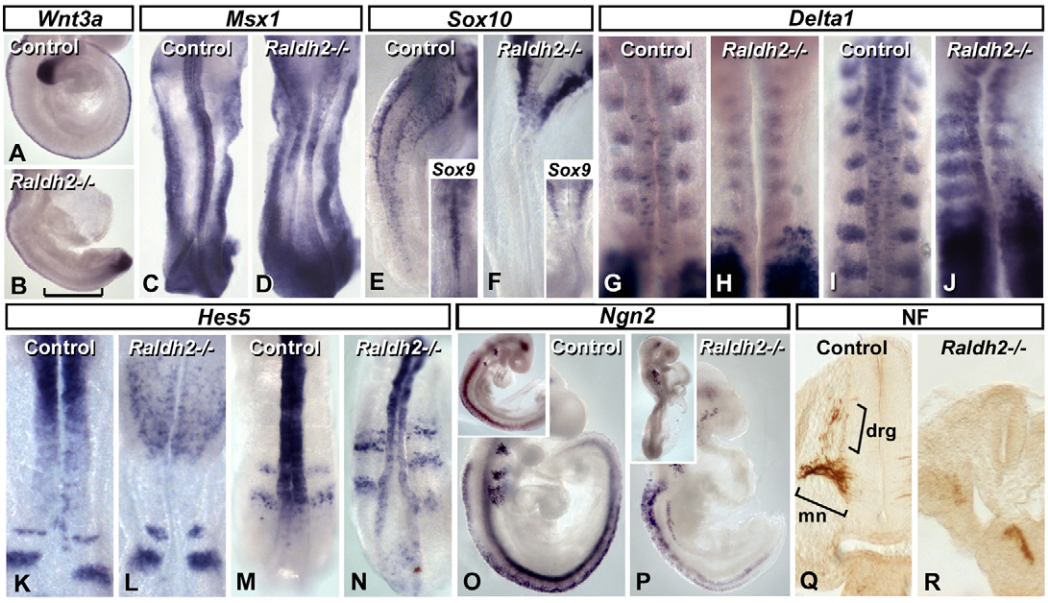

Fig. 4. Impaired neurogenesis in *Raldh2*$^{-/-}$ embryos. (**A-P**) Whole-mount in situ hybridization of *Wnt3a* (A,B), *Msx1* (C,D), *Sox10* (E,F, main panels), *Sox9* (E,F, insets), *Delta1* (G-J), *Hes5* (K-N) and *Ngn2* (O,P). (**Q**,**R**) Anti-neurofilament immunolabelling on transverse brachial spinal cord sections. Genotypes are as indicated. Developmental stages are four- to five-somite stage (G,H,K,L), 13-15 somites (C-F,I,J,M,N, insets in O,P) and E9.5 (A,B,O,P,Q,R). drg, dorsal root ganglion; mn, motoneurons.

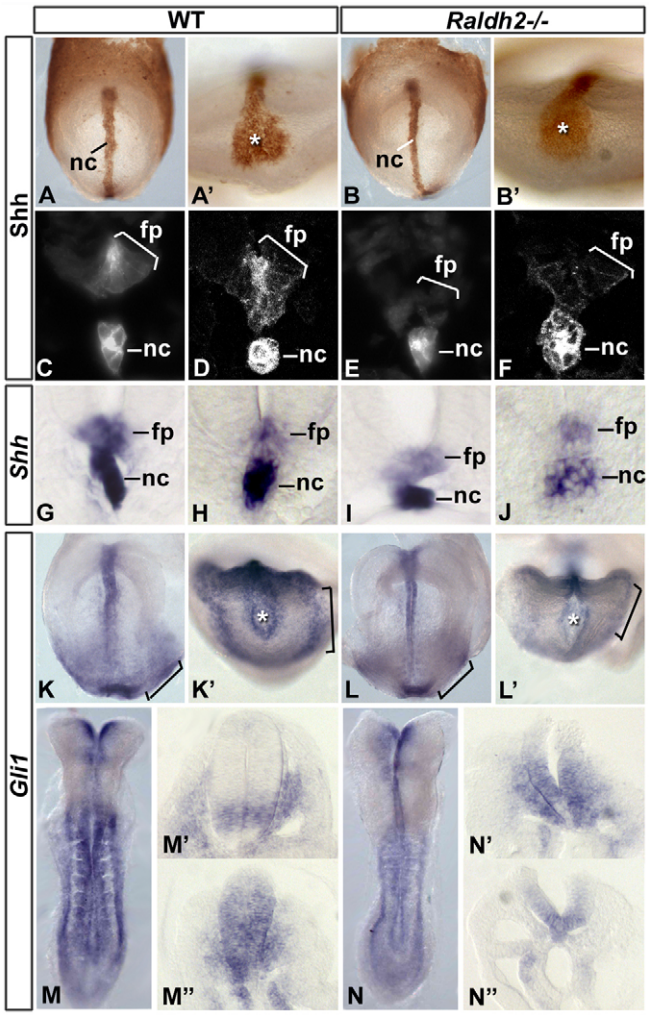

Fig. 5. Decreased Shh signalling in *Raldh2*$^{-/-}$ embryos. Immunodetection of Shh on whole-mount embryos (**A-B'**) and on transverse cryosections of the brachial spinal cord (**C-F**). Whole-mount in situ hybridization of *Shh* (**G-J**) and *Gli1* (**K-N"**). G-J,M',M",N',N" are vibratome transverse sections of the brachial spinal cord (G-J,M",N") and hindbrain (M',N'). Genotypes are indicated above. Developmental stages are E7.75 (A-B',K-L'), 6 somites (M,N), 11-12 somites (M'-N"), 14-15 somites (C,E,G,I) and E9.5 (D,F,H,J). Brackets indicate *Gli1* expression in newly formed mesoderm (K,L) and lateral epiblast (K',L'). Asterisks indicate the node. nc, notochord; fp, floor plate.

and *Raldh2*$^{-/-}$ embryos, preventing further analysis (data not shown). These experiments demonstrate that tissues of *Raldh2*$^{-/-}$ embryos are not able to respond to exogenous Shh at physiological or even supra-physiological concentrations, although they do respond to Shh-N if administered at a concentration about 40-fold over the physiological range.

We then investigated whether addition of all-*trans*-RA (AT-RA) could rescue the competence of cells to respond to Shh signalling in mutant embryos. After 14 hours' culture with 200 nM AT-RA, *Gli1* levels markedly increase both in controls (12/14) and in mutant (8/8) embryos (Fig. 6I-L). This suggests that the addition of RA is sufficient either to make the cells competent to respond to endogenous Shh, or to regulate *Gli1* expression directly. To distinguish between these possibilities, embryos were analyzed after shorter (6 hour) exposure to 200 nM AT-RA. *Gli1* levels did not significantly change, both in control and mutants (8/24, 0/8 embryos with elevated *Gli1* levels; data not shown) compared with untreated controls, making it unlikely that AT-RA directly regulates expression of *Gli1*. We further analyzed whether a low dose of AT-RA may facilitate the response of *Raldh2*$^{-/-}$ cells to exogenous Shh-N. Following a 14 hour culture with 10 nM AT-RA (and without Shh-N), *Gli1* expression is not increased in control embryos (Fig. 6M; 8/43 embryos with elevated *Gli1* levels) and remains downregulated in half of the mutants (7/15) (Fig. 6N). The other half (8/15) of the mutants show the same level of expression as the wild-type littermates (not shown), suggesting that the rescue is not fully penetrant. The combined addition of 10 nM AT-RA and 50 nM Shh-N led to an increase of *Gli1* expression, both in controls (72/76) and in almost all mutants (20/22) (Fig. 6O,P). In this situation, the *Raldh2*$^{-/-}$ embryos show the same level of expression as the wild-type embryos (Fig. 6O,P), a level highly increased when compared with untreated controls (Fig. 6I). These findings support the idea that addition of RA is sufficient to restore, within 14 hours, the competence of cells to respond to exogenous Shh. Altogether, these data show that RA signalling during late gastrulation and early neurulation regulates the cells response to Shh signal.

Impaired Fgf signalling in *Raldh2*$^{-/-}$ embryos accounts in part for the defects in Shh signalling

Studies in chick and mouse have shown that RA activity controls the extent of *Fgf8* expression in caudal tissues (Diez del Corral et al., 2003; Vermot et al., 2005; Molotkova et al., 2005; Sirbu and Duester, 2006). Fgf and Shh signalling pathways interact in several developing systems (see Introduction), and exposure of chicken caudal neural tissue to Fgf inhibits onset of *Shh* and *Ptc2* expression (Diez del Corral et al., 2003). To investigate whether alterations in Fgf signalling could be responsible for the decrease in Shh response in *Raldh2*$^{-/-}$ embryos, we analyzed two targets of this pathway: sprouty 2 (Minowada et al., 1999) and *Mkp3* [(Dickinson et al., 2002); for studies in chick (Eblaghie et al., 2003; Kawakami et al., 2003)]. Sprouty 2 is expressed at E7.75 in the CNP nearby the primitive streak of wild-type embryos (Fig. 7A). Its domain of expression expands laterally in *Raldh2*$^{-/-}$ embryos (Fig. 7B; *n*=5/5), reminiscent of the expansion of brachyury and follistatin at the same stage (Fig. 2). Then, at early somite stages, sprouty 2 expression in the caudal region of *Raldh2*$^{-/-}$ embryos is comparable with that seen in controls (Fig. 7C,D; *n*=5/5). At E9.5, sprouty 2 is markedly downregulated in caudal tissues of mutants (Fig. 7E,F, *n*=4/4).

In early somite-stage wild-type embryos, *Mkp3* is expressed in caudal tissues, with higher levels around the node (Fig. 7G). *Mkp3* caudal expression is comparable in *Raldh2*$^{-/-}$ mutants, although higher expression levels are seen in a domain posterior to the node (Fig. 7H; *n*=9/9). Whereas expression is observed in the somites and caudal tissues of wild-type embryos from the 12- to 13-somite stages, *Mkp3* expression is abnormally low in the caudalmost region of the mutants (Fig. 7I,J, brackets; *n*=12/12). In summary, Fgf signalling is laterally expanded within the CNP of *Raldh2*$^{-/-}$ embryos at the late gastrulation stage. However, at early somite stages, no ectopic anterior expression of Fgf target genes is seen in the *Raldh2*$^{-/-}$ neural plate and presomitic mesoderm, and by the 15- to 16-somite stage Fgf signalling is downregulated both in caudal tissues and somites. These results indicate that Fgf signalling is only transiently expanded in caudal tissues of RA-deficient embryos prior to E8. Consistent with previous observations in the VAD quail model, we found no evidence of ectopic expression of target genes of Fgf signalling within the developing neural tube (Diez del Corral et al., 2003).

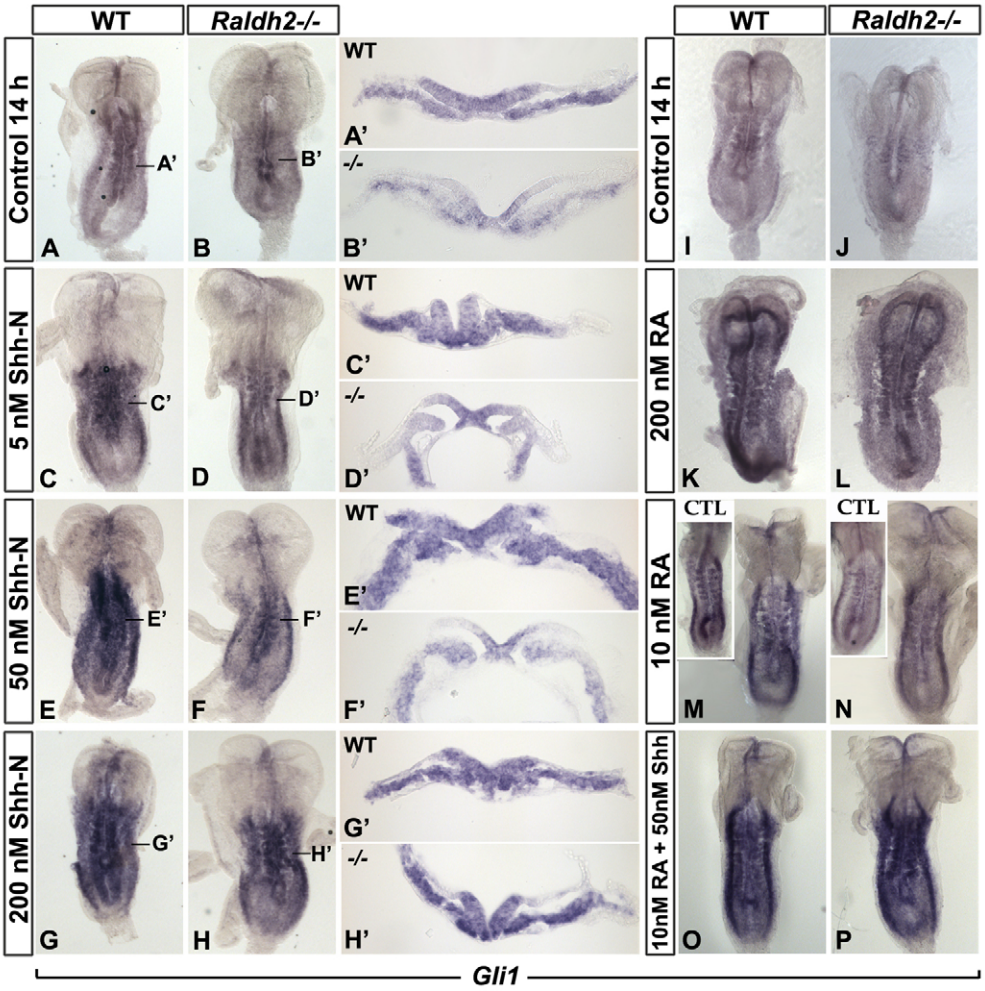

Fig. 6. Exogenous Shh-N does not rescue *Gli1* expression in *Raldh2*$^{-/-}$ embryos, even at supra-physiological concentrations. (**A-P**) Whole-mount in situ hybridization of *Gli1* on E7.5 embryos cultured for 14 hours in medium containing no drug (A-B′), and 5 nM (C-D′), 50 nM (E-F′) or 200 nM (G-H′) Shh-N, and embryos cultured for 14 hours in medium containing 0.001% ethanol (vehicle, I,J, insets in M,N), and 200 nM AT-RA (K,L), 10 nM AT-RA (M,N), or 10 nM AT-RA and 50 nM Shh-N (O,P). Genotypes are indicated above.

To test whether the ectopic Fgf activity in gastrulating *Raldh2*$^{-/-}$ embryos contributes to the decrease in Shh signalling efficiency, embryos were cultured for 6 or 14 hours in the presence of 50 μM SU5402, an inhibitor of Fgf receptor signalling (Mohammadi et al., 1997). Blockade of Fgf signalling for 14 hours dramatically increases *Gli1* expression in both controls (9/9) and mutants (3/3) (Fig. 7J-Q). An increase of *Gli1* expression is observed in few controls (3/13), but not in mutants (0/5), after 6 hours of SU5402 administration (Fig. 7N-Q), suggesting that its effect on *Gli1* is indirect, similar to the effect of AT-RA. These findings suggest that the abnormal Fgf signalling observed in *Raldh2*$^{-/-}$ mutants contributes to the decrease of Shh signalling efficiency. As SU5402 increases *Gli1* levels even in tissues lying at a distance from the ventral source of Shh, they might also suggest that *Gli1* can be stimulated independently of Shh.

RA negatively controls *Gli2* expression independently of Fgf signalling

To further investigate at which level RA interferes with Shh signalling, we analyzed *Gli2* and *Gli3*. Expression of *Gli3* is comparable in *Raldh2*$^{-/-}$ mutants and wild-type controls, at least until 13- to 14-somite stages (data not shown; *n*=7/7). By contrast, *Gli2* expression is markedly elevated in *Raldh2*$^{-/-}$ embryos as early as the late gastrulation stages (Fig. 8A-D) (*n*=12/12). Increased and/or ectopic expression is observed in the mesoderm and epiblast (Fig. 8A,B, insets), and the anterior neural plate (Fig. 8A,B, main panels). *Gli2* levels are also increased in *Raldh2*$^{+/-}$ embryos, albeit to a lesser extent than in null mutants (data not shown). This observation suggests that *Gli2* expression is highly sensitive to embryonic RA levels.

To test this hypothesis, wild-type and mutant embryos were cultured for 6 hours in the presence of 200 nM AT-RA. *Gli2* levels are comparable and relatively low in vehicle and RA-treated control embryos (Fig. 8E,F). However, in the presence of AT-RA, *Gli2* is downregulated in most heterozygous and null mutants, especially at the trunk level, when compared with vehicle-treated controls (*n*=12/15 and 7/16) (Fig. 8G,H; data not shown). Similar results were obtained upon longer (14 hour) cultures with 200 nM AT-RA (data not shown). *Gli2* was also found to be downregulated following a 6-hour culture of wild-type embryos in the presence of BMS493 (5 μM), a pan-RAR antagonist that stabilizes the association of RAR/RXR heterodimers with transcriptional co-

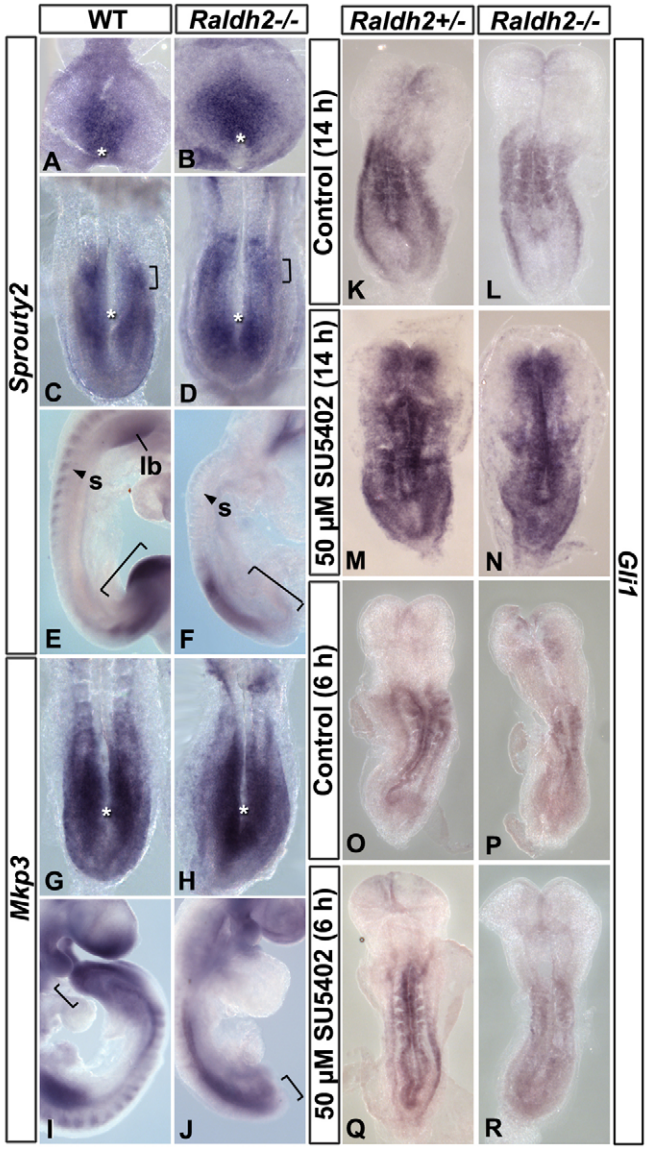

Fig. 7. Altered Fgf signalling in *Raldh2*$^{-/-}$ embryos. (**A-J**) Whole-mount in situ hybridization of sprouty 2 (A-F) and *Mkp3* (G-J) in wild-type and *Raldh2*$^{-/-}$ embryos (genotypes indicated above) at E7.75 (A,B), 4-5 somites (C,D,G,H) and E9.5 (E,F,I,J). Asterisks indicate the node. Brackets in C,D indicate expression in condensing somites as a landmark, and in E,F,I,J indicate regions with abnormally low expression. lb, limb bud; s, somites. (**K-R**) Whole-mount *Gli1* in situ hybridization on embryos (genotypes indicated above) collected at E7.5 and cultured for 14 h (K-N) or 6 h (O-R) in medium containing 0.1% DMSO (vehicle, K,L,O,P) and 50 µM SU5402 (M,N,Q,R).

repressors (Germain et al., 2002) (n=31/40; data not shown), suggesting that it may be directly regulated by RARs. This effect of BMS493 seems paradoxical, but probably reflects a forced conformation of RAR/RXRs in a repressive state, also called 'inverse agonist' activity. Paradoxical effects observed upon treatment of mouse embryos with BMS493 have already been discussed (Niederreither et al., 2001). We checked whether the regulation of *Gli2* is dependent on Fgf signalling by analyzing embryos cultured in the presence of SU5402. After 6 or 14 hours of culture, *Gli2* expression is comparable in vehicle-treated (n=7 and 10) and SU5402-treated embryos (n=8 and 11) (Fig. 8I-L). These experiments indicate that RA signalling represses *Gli2* expression independently of Fgf signalling (Fig. 9B).

DISCUSSION

We have addressed the functions of RA signalling in the generation of posterior structures in the mouse embryo. We show that during late gastrulation, RA activity regulates spatial and temporal patterning of the epiblast, thereby limiting cell ingression through the primitive streak and promoting neural induction. Importantly, the control of these processes is mediated by the modulation of Fgf and Shh signalling at multiple levels (see Fig. 9). We show in particular that the response of cells to endogenous Shh in late gastrulating embryos is potentiated by RA activity and by inhibition of Fgf signalling, and more unexpectedly that the expression of Gli2 is repressed by RA signalling.

RA signalling controls the partitioning of the epiblast into neural and mesodermal domains by inhibiting Fgf signalling

The generation of caudal structures relies on the orchestration within the epiblast of two processes: cell ingression movements through the primitive streak occurring during gastrulation and neural induction. The segregation of these processes probably relies on the early subdivision of the epiblast into mesodermal and neural domains (Delaune et al., 2005; Sheng et al., 2003). Our data indicate that the transition between gastrulation and neural induction occurs in mouse around the late-headfold stage, when epiblast cells along the anterior primitive streak start expressing early neural markers such as *Sox2* (Fig. 3 and data not shown). This raises the question of how is the commitment of anterior epiblast cells towards a neural fate temporally and spatially regulated? Studies in chick led to a model stating that this commitment relies on opposing actions of RA emanating from the somites and anterior presomitic mesoderm and Fgf signalling within caudalmost tissues (Diez del Corral and Storey, 2004; Delphino-Machin et al., 2005). The present results lead us to refine this model.

First, we found that RA signalling is dynamically regulated prior to the onset of somitogenesis and that, from mid-gastrulation to headfold-stage, all caudal progenitors, including primitive streak and node cells respond to the RA signal. Thus, during gastrulation and early neural induction, RA and Fgfs act in conjunction within the epiblast. Second, we show that this early RA activity is required to restrict the expression of mesodermal markers, including *Wnt3a*, brachyury and follistatin (Fig. 2; Fig. 9A). Although Wnt3a directly regulates brachyury (Yamaguchi et al., 1999), RA signalling is another candidate to regulate its expression. Upon RA treatment, wild-type mouse embryos display within 4 hours a downregulation of brachyury expression (Iulianella et al., 1999). In RA-deficient embryos, the expansion of these mesodermal markers probably accounts for the abnormal accumulation of mesodermal cells within the tail bud. Indeed, brachyury is necessary for caudal elongation, and analysis of mouse chimeras showed that it controls cell movements in the posterior mesoderm cell autonomously (Wilson et al., 1995). Therefore, the expansion of brachyury expression in *Raldh2*$^{-/-}$ embryos could alter mesodermal cell motility. Moreover, by limiting the expansion of mesodermal markers and preventing the ingression of the epiblast, RA signalling may control the timing of anterior epiblast cells commitment towards a neural fate. This

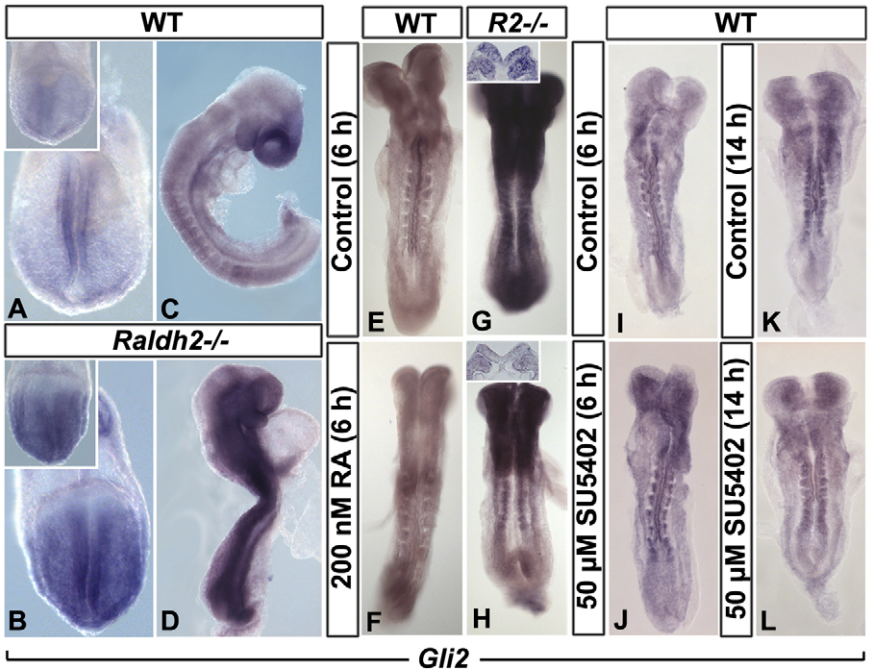

Fig. 8. Retinoic acid negatively regulates *Gli2* expression, independently of Fgf signalling. (**A-D**) Whole-mount in situ hybridization of *Gli2* in control and *Raldh2*$^{-/-}$ embryos at E7.75 (A,B, main panels, anterior views; insets, posterior views) and 13- to 14-somite stages (C,D). (**E-L**) *Gli2* in situ hybridization on embryos cultured for 6 hours (E-J) or 14 hours (K,L) in medium containing 0.001% ethanol (E,G), 0.1% DMSO (I,K), 200 nM AT-RA (F,H) or 50 µM SU5402 (J,L). Genotypes indicated above.

is supported by our findings in *Raldh2*$^{-/-}$ embryos, including a delay in *Sox2* induction in the CNP and an absence of *Wnt8a* expression postero-laterally to the node (Fig. 9A).

Despite observations of an anteriorization of the *Fgf8* caudal gradient in *Raldh2*$^{-/-}$ embryos (Vermot et al., 2005; Sirbu and Duester, 2006), our analysis of target genes of Fgf signalling did not confirm an anterior expansion of this signal within the newly formed spinal cord or somites. However, during gastrulation and early neurulation, a transient ectopic Fgf activity mimics the lateral expansion of primitive streak markers in the caudal region of *Raldh2*$^{-/-}$ embryos, arguing that RA modulates Fgf signalling within epiblast cells. Because, in chick and *Xenopus*, high levels of Fgf signalling enhance mesoderm formation and cell ingression through the primitive streak, whereas lower levels drive the epiblast towards a neural fate (Delphino-Machin et al., 2005; Delaune et al., 2005), we conclude that this abnormal Fgf activity could be one of the cues responsible for the expansion of mesodermal markers in Raldh2 mutants. Altogether, our results indicate that within the primitive streak, RA activity is required to attenuate Fgf signalling, thereby limiting the progression of mesodermal markers and the cell ingression movements through the primitive streak.

RA signalling sustains the cells response to Shh activity during specification and differentiation of neural and mesodermal derivatives

Previous studies have shown that in several developmental processes, Shh and RA signalling converge and influence target gene regulation (e.g. Bertrand and Dahmane, 2006; Helms et al., 1994; Helms et al., 1997). Some key target genes may actually be regulated by direct combinatorial inputs from both signalling pathways, as recently demonstrated in the case of the enhancer responsible for *Ngn2* onset of expression, which contains functional Gli and RAR-RXR-binding sites (Ribes et al., 2008). By overexpressing a dominant-negative RARα, Novitch et al. (Novitch et al., 2003) reported a lack of response of cells to Shh signalling within the chicken neural tube. Our present work strongly suggests that a decrease in Shh signalling efficiency demonstrated in *Raldh2*$^{-/-}$ embryos contributes to defects in both neural and mesodermal patterning and differentiation. Many known Shh target genes are downregulated in mutant tissues, such as the markers of somite differentiation *Pax1* and *Myf5*, or genes involved in specification of the ventral neural tube *Olig2*, *Pax6*, *Nkx6.2* and *Nkx2.2* (Briscoe and Ericson, 2001; Diez del Corral et al., 2003; Lee and Pfaff, 2001; Molotkova et al., 2005) (V.R., I.L.R. and P.D., unpublished). We provide further evidence that RA activity acts at least at two different levels of Shh signalling (Fig. 9B), by controlling the cells response to Shh and the levels of *Gli2*.

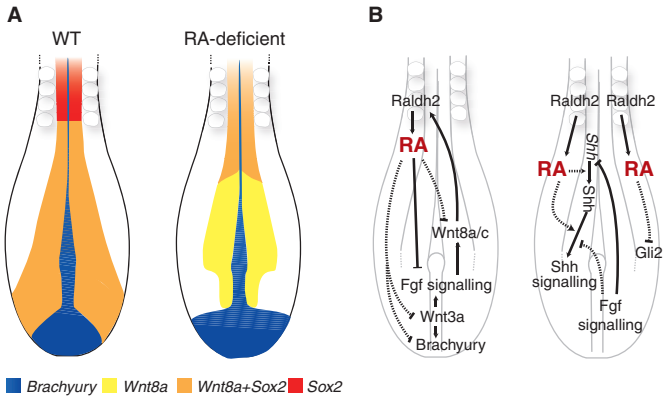

Fig. 9. Summary of retinoic acid-dependent events during body axis extension and caudal development. Schematic overview of the abnormal caudal phenotype of early somite-stage *Raldh2*$^{-/-}$ embryos (**A**) and of the main regulatory interactions underlying these defects (**B**). This summarizes regulatory loops occurring at pre- and early somite stages, characterized by previous work on avian models (see Diez del Corral and Storey, 2004; Olivera-Martinez and Storey, 2007) and supported (or uncovered) in our mouse model (broken lines).

As early as E7.5, *Raldh2*$^{-/-}$ neural and mesodermal tissues exhibit abnormally low levels of expression of Shh target genes (*Gli1*, *Ptch1*), despite unaltered levels of Shh mRNA and protein. Using embryo culture experiments, we show that: (1) *Raldh2*$^{-/-}$ embryos are less efficient that wild-type embryos to respond to exogenous Shh protein; (2) *Gli1* and *Ptch1* expression can be rescued following exposure for 14 hours, but not 6 hours, to exogenous RA, arguing for an indirect regulation; (3) exogenous RA enhances the response of cells to Shh in both wild-type and RA-deficient conditions. These results support the idea that RA is a necessary signal for cells to efficiently respond to Shh.

Importantly, we found that RA negatively regulates expression of *Gli2* (see Fig. 9B), a key player of the Shh signalling pathway. An upregulation of *Gli2* has been reported in mice deficient for Shh (Bai and Joyner, 2001), although at later stages than in the RA-deficient mutants. This may suggest that *Gli2* upregulation in *Raldh2* mutants is a consequence of decreased Shh signalling. This is, however, unlikely, as we have not observed any changes in *Gli2* levels upon administration of Shh-N for 14 hours to cultured embryos, either wild-type or *Raldh2*$^{-/-}$ (data not shown).

We propose that the upregulation of *Gli2* in RA-deficient embryos could be one of the molecular cues underlying the specification defects both in the caudal region and the forming somites (Fig. 9B). Experiments in *Xenopus* and mice have demonstrated that Gli2 is involved in maintenance and AP patterning of the mesoderm, and establishment of ventral identities within the spinal cord (Ruiz i Altaba, 1999; Borycki et al., 1998; Brewster et al., 1998; Mullor et al., 2001; Bai et al., 2002; Bai et al., 2004). Interestingly, Gli2 ability to induce ectopic expression of mesodermal markers relies on an activating form of the protein (Ruiz i Altaba, 1999; Brewster et al., 1998; Mullor et al., 2001), suggesting that, in RA-deficient embryos, this transcription factor is acting as an activator. Thus, unlike Gli3, which requires Shh signalling in order not to be processed into a negative form (Litingtung et al., 2002; Persson et al., 2002; Wang et al., 2000), Gli2-activating function can be maintained independently of Shh signalling. This indicates that, in mice, as described in chick and *Xenopus* (Borycki et al., 1998; Brewster et al., 1998; Ruiz i Altaba, 1999), Gli2 may not only be the major mediator of Shh signalling in neural and mesodermal tissues, but could also have independent functions. From our results, Gli2 could play a pivotal function by integrating the RA and Shh signals. As these two signalling pathways crosstalk in various cells types, including cancer cells (Goyette et al., 2000; So et al., 2004), our data might suggest new routes to combat diseases due to alteration in the transduction of these pathways, such as basal cell carcinoma (So et al., 2006; So et al., 2004).

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/4/665/DC1

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