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Effect of retinoic acid signaling on Wnt/β-catenin and FGF signaling during body axis extension

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Abstract

Cell-cell signaling regulated by retinoic acid (RA), Wnt/β-catenin, and fibroblast growth factor (FGF) is important during body axis extension, and interactions between these pathways have been suggested. At early somite stages, Wnt/β-catenin and FGF signaling domains exist both anterior and posterior to the developing trunk, whereas RA signaling occurs in between in the trunk under the control of the RA-synthesizing enzyme retinaldehyde dehydrogenase-2 (Raldh2). Previous studies demonstrated that vitamin A deficient quail embryos and *Raldh2*$^{-/-}$ mouse embryos lacking RA synthesis exhibit ectopic expression of *Fgf8* and *Wnt8a* in the developing trunk. Here, we demonstrate that *Raldh2*$^{-/-}$ mouse embryos display an expansion of FGF signaling into the trunk monitored by *Sprouty2* and *Pea3* expression, and an expansion of Wnt/β-catenin signaling detected by expression of *Axin2*, *Tbx6*, *Cdx2*, and *Cdx4*. Following loss of RA signaling, the caudal expression domains of *Fgf8*, *Wnt8a*, and *Wnt3a* expand anteriorly into the trunk, but no change is observed in caudal expression of *Fgf4* or *Fgf17* plus caudal expression of *Fgf18* and *Cdx1* is reduced. These findings suggest that RA repression of *Fgf8*, *Wnt8a*, and *Wnt3a* in the developing trunk functions to down-regulate FGF signaling and Wnt/β-catenin signaling as the body axis extends.

Keywords

Retinoic acid signaling; Wnt/β-catenin signaling; FGF signaling; Spry2; Pea3; Axin2; Fgf8; Wnt8a; Wnt3a; Tbx6; Cdx; axis extension

1. Results and discussion

During vertebrate embryogenesis, the process of body axis extension begins when somitogenesis commences. As somites form, the body extends along the anteroposterior axis forming a new domain (the developing trunk) located between the headfolds and the epiblast/primitive streak. Several secreted cell-cell signaling molecules control body axis extension including Wnt (Grigoryan et al., 2008), fibroblast growth factor (FGF) (Del Corral and Storey, 2004), and retinoic acid (RA) (Duester, 2008). Some of the actions of Wnt ligands are transduced through stabilization of β-catenin which can then enter the nucleus and bind to the LEF/TCF family of transcription factors (Logan and Nusse, 2004). During the early phase of body axis extension, Wnt/β-catenin signaling domains are limited to regions on either end of

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the developing trunk in the headfold and epiblast/primitive streak (Nakaya et al., 2005). Likewise, FGF signaling domains during the early phase of body axis extension are limited to regions on either end of the developing trunk in cardiac mesoderm (Sirbu et al., 2008) and epiblast/primitive streak (Sirbu and Duester, 2006). Although FGF signaling and Wnt/β-catenin signaling pathways play important roles in body axis extension, the mechanisms by which individual FGFs or Wnts control the processes of body patterning are largely unknown.

Interactions between the Wnt/β-catenin, FGF, and RA signaling pathways have been reported in RA-deficient embryos generated either through vitamin A deficiency which removes the precursor of RA, or through genetic loss of retinaldehyde dehydrogenase-2 (*Raldh2*) which controls RA synthesis (Duester, 2008). Loss of RA signaling in vitamin A deficient quail embryos and *Raldh2*$^{-/-}$ mouse embryos up-regulates caudal *Fgf8* expression and results in segmentation defects during body axis extension including a shortening of the body along the anteroposterior axis and somite left-right asymmetry (Del Corral et al., 2003; Vermot et al., 2005). Further studies with *Raldh2*$^{-/-}$ embryos demonstrated that loss of RA signaling results in an anterior expansion of *Fgf8* expression from the epiblast into the posterior neuroectoderm (Sirbu and Duester, 2006), and a posterior expansion of *Fgf8* mRNA and FGF signaling from cardiac mesoderm into trunk lateral plate mesoderm (Ryckebusch et al., 2008; Sirbu et al., 2008); together, these two events reduce the size of the Fgf8-free zone where the trunk initially develops. Loss of RA signaling in *Raldh2*$^{-/-}$ embryos and vitamin A deficient quail embryos also results in ectopic trunk expression of *Wnt8a* (avian ortholog is *Wnt8c*), suggesting that Wnt/β-catenin signaling may also be down-regulated by RA signaling in the developing trunk (Niederreither et al., 2000; Olivera-Martinez and Storey, 2007).

Here, we examine FGF and Wnt/β-catenin signaling in mouse *Raldh2*$^{-/-}$ embryos during the early phase of body axis extension. *Raldh2*$^{-/-}$ embryos are completely devoid of RA signaling from E7.5-E8.5 (0-10 somites) when body axis extension commences (Sirbu and Duester, 2006; Sirbu et al., 2005). As a marker of FGF signaling we examined expression of *Sprouty2* (*Spry2*) which is induced by FGF signaling (Minowada et al., 1999) and acts as a negative-feedback regulator of the pathway (Hanafusa et al., 2002). Whereas *Spry2* mRNA is normally expressed in two separate domains anterior and posterior to the developing trunk, *Spry2* was greatly up-regulated in *Raldh2*$^{-/-}$ embryos such that the two domains are nearly joined in the developing trunk (Fig. 1A-B). We also examined another marker of FGF signaling, *Pea3* encoding an Ets transcription factor induced by FGF (Raible and Brand, 2001), and observed an anterior extension of its caudal expression domain in *Raldh2*$^{-/-}$ embryos (Fig. 1C-D). These findings indicate that a loss of RA signaling in *Raldh2*$^{-/-}$ embryos results in an increase in FGF signaling.

Previous studies have demonstrated that caudal expression of *Fgf8* extends ectopically into posterior neuroectoderm following loss of RA at the 1-3 somite stages (Sirbu and Duester, 2006). Here, we show that a 5-somite *Raldh2*$^{-/-}$ embryo exhibits an anterior extension of the caudal *Fgf8* expression domain plus a posterior extension of the cardiac *Fgf8* expression domain (Fig. 1G-H). As *Fgf4*, *Fgf17*, and *Fgf18* are also expressed caudally and may have overlapping functions with *Fgf8* in body axis extension (Maruoka et al., 1998; Niswander and Martin, 1992), we examined these genes in *Raldh2* mutants. *Fgf4* and *Fgf17* expression was not significantly changed in *Raldh2*$^{-/-}$ embryos compared to wild-type (Fig. 1E-F, I-J). *Fgf18* mRNA was either lost or greatly down-regulated in *Raldh2*$^{-/-}$ embryos (Fig. 1K-M); a similar down-regulation of *Fgf18* was previously reported (Vermot et al., 2005). Taken together with the findings on *Spry2* and *Pea3*, these observations suggest that the large increase in FGF signaling observed after loss of RA signaling is due to a specific increase in *Fgf8* expression but not expression of *Fgf4*, *Fgf17* or *Fgf18*. As *Fgf18* is induced by RA signaling, this gene appears to play a much different role in caudal development than *Fgf8* which is repressed by RA.

Two caudally-expressed Wnt genes most often associated with vertebrate Wnt/β-catenin signaling during body axis extension are *Wnt3a* in mouse (Nakaya et al., 2005; Takada et al., 1994) and *Wnt8* in *Xenopus* and zebrafish (Lekven et al., 2001; Smith and Harland, 1991). Previous studies in vitamin A deficient quail embryos demonstrated a large up-regulation of *Wnt8c* expression in trunk neuroectoderm (Olivera-Martinez and Storey, 2007). We examined expression of *Wnt8a*, the mouse homolog of *Wnt8c*, in *Raldh2*$^{-/-}$ embryos. At the 1-somite stage, when *Wnt8a* expression is normally observed continuously along the anteroposterior axis from the posterior hindbrain to the epiblast, *Wnt8a* expression was down-regulated in the hindbrain of *Raldh2*$^{-/-}$ embryos compared to wild-type (Fig. 2A-B). At the 5-somite stage, when *Wnt8a* expression normally resolves into two domains, i.e. epiblast and hindbrain rhombomere 4 (Niederreither et al., 2000), we found that *Raldh2*$^{-/-}$ embryos had lost *Wnt8a* expression in rhombomere 4 but the caudal *Wnt8a* domain now extended much further anteriorly from the epiblast into the trunk neuroectoderm (Fig. 2C-D). We also examined expression of *Wnt3a* which plays an essential role during body axis extension in the mouse (Nakaya et al., 2005; Takada et al., 1994). At both the 1-somite and 4-somite stages, *Wnt3a* expression was unchanged in *Raldh2*$^{-/-}$ embryos compared to wild-type (Fig. 2E-H). However, at the 7-somite stage we observed an anterior extension of the caudal *Wnt3a* expression domain relative to the node (Fig. 2I-J). These findings indicate that RA-deficient mouse embryos exhibit an early increase in caudal *Wnt8a* expression, followed by a later increase in caudal *Wnt3a* expression.

We examined Wnt/β-catenin signaling in wild-type and *Raldh2*$^{-/-}$ embryos. *Axin2* encodes a negative feedback inhibitor of Wnt/β-catenin signaling that is induced by this signaling pathway in a wide range of tissues (Jho et al., 2002). In 4-somite *Raldh2*$^{-/-}$ embryos, an ectopic domain of *Axin2* mRNA domain was observed in the developing trunk (Fig. 3A-D). The ectopic domain of *Axin2* mRNA observed in *Raldh2*$^{-/-}$ embryos was in a region which overlaps the ectopic domain of *Wnt8a* expression (compare Fig. 2D and Fig. 3B); *Axin2* up-regulation is most likely due to up-regulation of *Wnt8a* which occurs prior to up-regulation of *Wnt3a* in *Raldh2*$^{-/-}$ embryos. We also examined expression of *Tbx6* which is induced by Wnt/β-catenin signaling during mouse body axis extension (Dunty et al., 2008). In 5-somite *Raldh2*$^{-/-}$ embryos we observed an anterior extension of the caudal *Tbx6* expression domain relative to the node (Fig. 3E-F). From these findings it appears that a loss of RA does lead to a significant increase in trunk Wnt/β-catenin signaling as monitored by both *Axin2* and *Tbx6* expression.

Cdx1, *Cdx2*, and *Cdx4* play essential roles in vertebrate caudal development through their ability to regulate posterior *Hox* gene expression (Van den Akker et al., 2002; van Nes et al., 2006). In zebrafish, expression of *Cdx1a* and *Cdx4* is strongly reduced in Wnt3a/Wnt8 double knock-down embryos, and both Wnt3a/Wnt8 and Cdx1a/Cdx4 knockdowns result in complete loss of somitogenesis during mid-segmentation, indicating that *Cdx1a* and *Cdx4* mediate Wnt-dependent body axis extension (Shimizu et al., 2005). In mouse, the early caudal expression domains of *Cdx1* and *Cdx4* are directly induced by Wnt/β-catenin signaling (Pilon et al., 2006; Pilon et al., 2007), and the *Cdx2* promoter contains binding sites for LEF/TCF factors regulating Wnt/β-catenin signaling (Wang and Shashikant, 2007). As another means of determining whether a loss of RA increases Wnt/β-catenin signaling, we examined expression of the *Cdx* gene family in *Raldh2*$^{-/-}$ embryos. *Cdx1* expression was not increased following loss of RA signaling, but was instead significantly decreased in the posterior neuroectoderm and primitive streak mesoderm (Fig. 4A-D). Both *Cdx2* and *Cdx4* were upregulated in *Raldh2*$^{-/-}$ embryos, but in different tissues; *Cdx2* expression exhibited an anterior extension in neuroectoderm (Fig. 4E-F) whereas *Cdx4* exhibited an anterior extension in lateral plate mesoderm (Fig. 4G-H). Thus, our findings on *Cdx2* and *Cdx4* expression provide further evidence (along with our *Axin2* and *Tbx6* results), that caudal Wnt/β-catenin signaling increases following a loss of RA. Our observation that *Cdx1* expression is greatly reduced in *Raldh2*$^{-/-}$ embryos can be explained by previous observations showing that *Cdx1* is induced

by RA as well as *Wnt3a* (Prinos et al., 2001). As the *Cdx1* promoter contains a retinoic acid response element essential for high-level expression of *Cdx1* caudally (Houle et al., 2003), a loss of RA signaling may prevent *Cdx1* from being able to respond to an increase in Wnt/β-catenin signaling.

In conclusion, inhibition of FGF signaling by RA signaling has been observed during body axis extension, and this appears to operate through the ability of RA to repress *Fgf8* (Del Corral et al., 2003; Sirbu and Duester, 2006; Sirbu et al., 2008; Vermot et al., 2005). Other studies suggest that RA may directly repress *Fgf8* since it was discovered that the *Fgf8* promoter is controlled differentially by a retinoic acid response element, with expression of the major isoform Fgf8b being repressed when RA is present (Brondani et al., 2002). From the studies reported here on *Raldh2*$^{-/-}$ embryos, we show that loss of RA signaling results in the up-regulation of *Spry2* and *Pea3*, markers for sites of FGF signaling. RA has also been suggested to inhibit Wnt/β-catenin signaling based on studies in vitamin A deficient quail embryos that display a large increase in *Wnt8c* expression (Olivera-Martinez and Storey, 2007); in those studies a feedback loop was proposed in which FGF signaling stimulates *Wnt8c* expression in posterior neuroectoderm leading to induction of *Raldh2* in mesoderm which then results in release of RA that represses both *Fgf8* and *Wnt8c* as the body axis extends. The FGF-Wnt portion of this feedback loop is supported by studies in *Xenopus* demonstrating that Fgf8a induces Wnt8 (Hong et al., 2008). In the developing trunk of mouse *Raldh2*$^{-/-}$ embryos, we observe increases in expression of *Wnt8a* (early) and *Wnt3a* (late), and a corresponding increase in Wnt/β-catenin signaling in the trunk monitored by *Axin2*, *Tbx6*, *Cdx2*, and *Cdx4* expression. *Wnt3a* appears to be more important than *Wnt8a* for normal mouse development as *Wnt3a* knockout embryos exhibit arrested body axis extension at the 7-9 somite stage (Takada et al., 1994), whereas *Wnt8a* knockout mice have no obvious developmental phenotype (van Amerongen and Berns, 2006). Thus, *Wnt3a* expression appears to be much more efficient than *Wnt8a* in stimulating caudal Wnt/β-catenin signaling in mouse, but our studies demonstrate that *Wnt8a* over-expression in *Raldh2*$^{-/-}$ embryos can induce ectopic Wnt/β-catenin signaling. In addition to regulating *Raldh2* as previously suggested (Olivera-Martinez and Storey, 2007), it will be interesting to determine whether *Wnt8a* regulates other genes involved in body axis extension.

2. Experimental procedures

2.1. Generation of Raldh2$^{-/-}$ Embryos

Generation of *Raldh2*$^{+/-}$ adult mice were previously described (Mic et al., 2002). Embryos from *Raldh2*$^{+/-}$ crosses were genotyped by PCR analysis of yolk sac DNA to identify *Raldh2*$^{-/-}$ embryos. Embryos were staged according to somite number. All mouse studies conformed to the regulatory standards adopted by the Animal Research Committee at the Burnham Institute for Medical Research.

2.2. In situ Hybridization

Detection of mRNA was performed by whole-mount in situ hybridization as previously described (Mic et al., 2002). Wild-type and *Raldh2*$^{-/-}$ embryos were treated under identical hybridization conditions and stained for the same length of time. For each gene analyzed, we collected data from at least three *Raldh2*$^{-/-}$ embryos and three wild-type embryos at a similar stage to draw conclusions.

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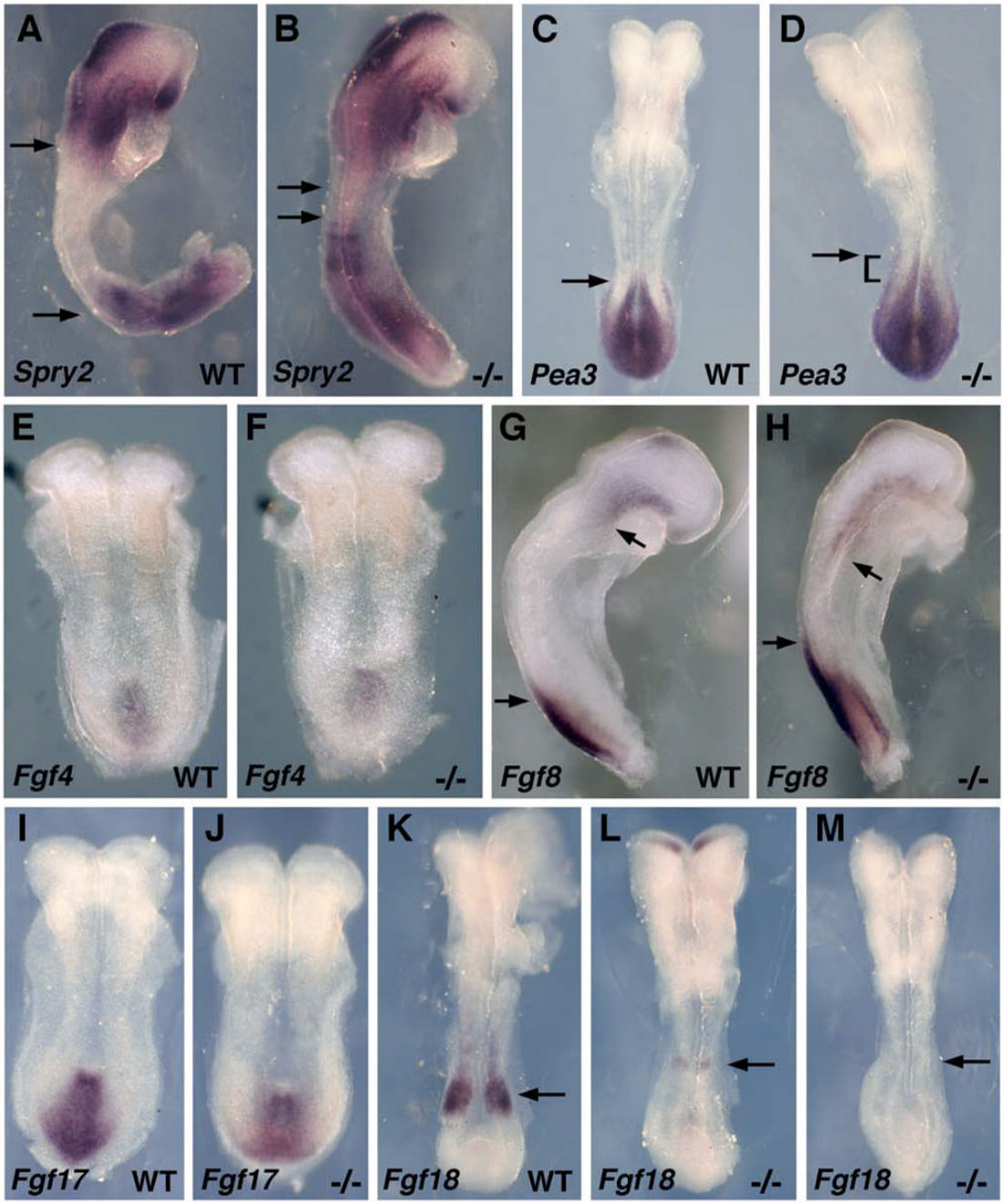

Fig. 1.

FGF signaling following loss of RA synthesis. (A-B) *Spry2* mRNA and (C-D) *Pea3* mRNA at the 6-somite stage; arrows indicate that the *Raldh2* mutant exhibits a large increase in *Spry2* expression in the developing trunk, and an anterior extension of the caudal *Pea3* expression domain. (E-F) *Fgf4* mRNA at the 4-somite stage; no difference is observed between wild-type and *Raldh2* mutant embryos. (G-H) *Fgf8* mRNA at the 5-somite stage; arrows indicate that the anterior and posterior domains of *Fgf8* expression extend further into the trunk in the *Raldh2* mutant. (I-J) *Fgf17* mRNA at the 4-somite stage showing no difference between wild-type and mutant. (K-M) *Fgf18* mRNA at the 7-somite stage; arrows indicate that caudal *Fgf18* expression is either lost or greatly reduced in *Raldh2* mutants.

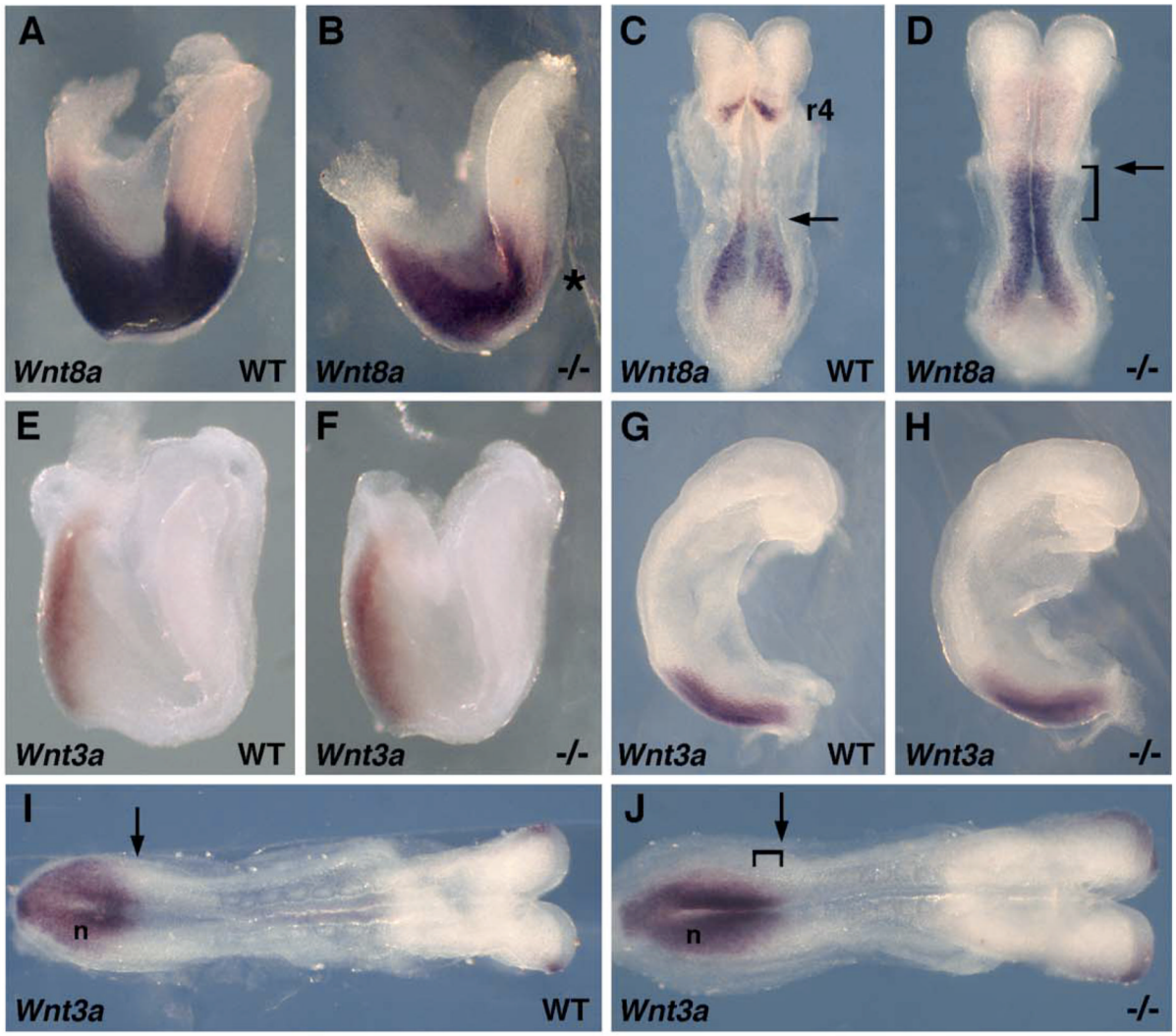

Fig. 2.

Loss of RA signaling up-regulates caudal *Wnt8a* and *Wnt3a* expression. (A-B) Detection of *Wnt8a* mRNA in wild-type (WT) and *Raldh2*$^{-/-}$ embryos at the 1-somite stage; overall, *Wnt8a* expression is lower in the mutant, and an asterisk points out down-regulation specifically in the hindbrain. (C-D) At the 5-somite stage it can be seen that *Wnt8a* expression is up-regulated in the trunk (arrows indicate that the anterior border of *Wnt8a* expression in the trunk is shifted anteriorly in the mutant); also, expression in rhombomere 4 (r4) of the hindbrain is lost in the mutant at 5-somites. Detection of *Wnt3a* mRNA at the 1-somite stage (E-F), 4-somite stage (G-H), and 7-somite stage (I-J) shows that initially no difference exists between wild-type and *Raldh2* mutant, but at 7-somites the mutant exhibits an anterior extension of the caudal *Wnt3a* expression domain relative to the node (n).

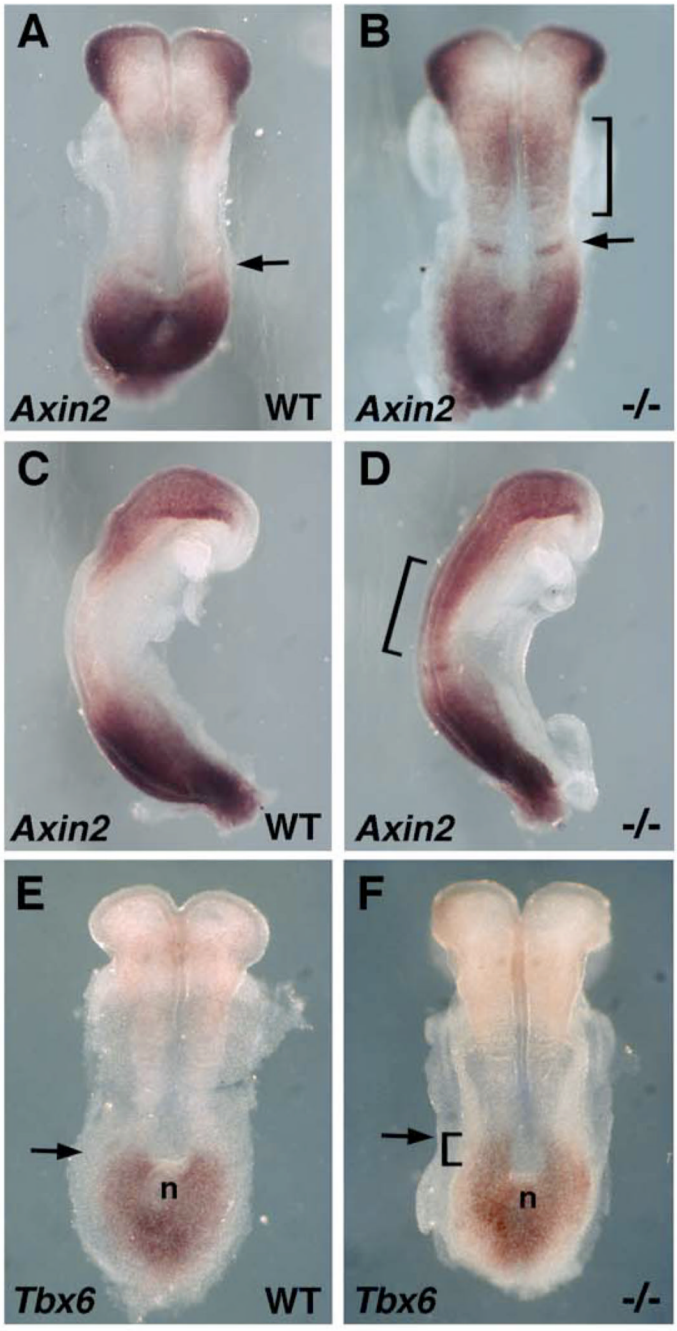

Fig. 3.

Analysis of Wnt/β-catenin signaling in embryos lacking RA synthesis. (A-D) Detection of *Axin2* mRNA at the 4-somite stage; brackets indicate a region in the developing trunk where the *Raldh2* mutant exhibits a significant increase in *Axin2* expression relative to wild-type; an arrow indicates that the mutant exhibits an increase in *Axin2* expression at the somite determination front. (E-F) *Tbx6* mRNA at the 5-somite stage shows that the *Raldh2* mutant exhibits an anterior extension of the caudal *Tbx6* expression domain relative to the node (n).

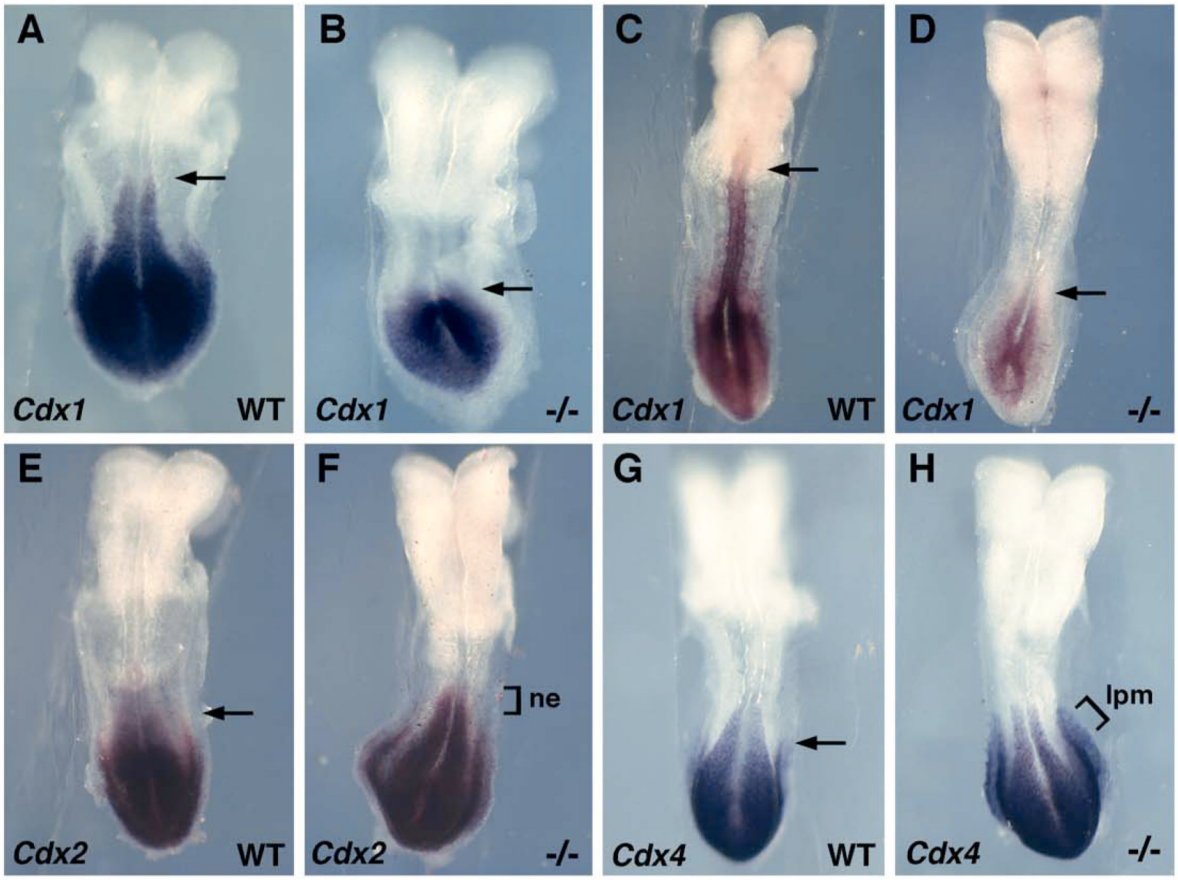

Fig. 4.

Regulation of *Cdx* genes by RA signaling. (A-B) *Cdx1* mRNA at 4-somite stage and (C-D) 7-somite stage; *Raldh2*$^{-/-}$ embryos exhibit a loss of *Cdx1* expression in the neuroectoderm (marked by arrows) and reduced expression in the primitive streak. (E-F) *Cdx2* mRNA at 6-somite stage showing an *Raldh2*$^{-/-}$ embryo exhibiting an anterior extension of the caudal *Cdx2* expression domain in neuroectoderm (ne). (G-H) *Cdx4* mRNA at the 6-somite stage showing an *Raldh2* mutant exhibiting an anterior extension of the caudal *Cdx4* expression domain in lateral plate mesoderm (lpm) but not neuroectoderm or paraxial mesoderm.