Published in final edited form as:
Nat Cell Biol. 2006 March ; 8(3): 271–277. doi:10.1038/ncb1374.

Retinoic-acid signalling in node ectoderm and posterior neural plate directs left–right patterning of somitic mesoderm

Ioan Ovidiu Sirbu[1] and **Gregg Duester**[1,2]

[1] OncoDevelopmental Biology Program, Burnham Institute for Medical Research, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA

Abstract

Somitogenesis requires bilateral rhythmic segmentation of paraxial mesoderm along the antero-posterior axis[1]. The location of somite segmentation depends on opposing signalling gradients of retinoic acid (generated by *retinaldehyde dehydrogenase-2; Raldh2)* anteriorly and fibroblast growth factor (FGF; generated by *Fgf8)* posteriorly[2,3]. Retinoic-acid-deficient embryos exhibit somite left–right asymmetry[4–6], but it remains unclear how retinoic acid mediates left–right patterning. Here, we demonstrate that retinoic-acid signalling is uniform across the left–right axis and occurs in node ectoderm but not node mesoderm. In *Raldh2*$^{-/-}$ mouse embryos, ectodermal *Fgf8* expression encroaches anteriorly into node ectoderm and neural plate, but its expression in presomitic mesoderm is initially unchanged. The late stages of somitogenesis were rescued in *Raldh2*$^{-/-}$ mouse embryos when the maternal diet was supplemented with retinoic acid until only the 6-somite stage, demonstrating that retinoic acid is only needed during node stages. A retinoic-acid-reporter transgene marking the action of maternal retinoic acid in rescued *Raldh2*$^{-/-}$ embryos revealed that the targets of retinoic-acid signalling during somitogenesis are the node ectoderm and the posterior neural plate, not the presomitic mesoderm. Our findings suggest that antagonism of *Fgf8* expression by retinoic acid occurs in the ectoderm and that failure of this mechanism generates excessive FGF8 signalling to adjacent mesoderm, resulting initially in smaller somites and then left–right asymmetry.

In the ‘clock and wave front’ model of somitogenesis, a moving wavefront of *Fgf8* gene expression in the tailbud regresses posteriorly as the body axis extends, and mesodermal segmentation occurs just anterior to the *Fgf8*-expression domain[7,8]. The anterior extent of *Fgf8* expression is limited by retinoic acid that is generated in the presomitic mesoderm and antagonizes *Fgf8* expression in the tailbud[2,9]. Retinoic acid synthesized in the presomitic mesoderm by RALDH2 is required to maintain bilateral symmetry between the left and right somite columns[4–6]. Presomitic mesoderm in mouse *Raldh2*$^{-/-}$ embryos displays left–right asymmetric expression of *Hes7* and *Lfng*, genes involved in oscillator function[4], suggesting that a loss of retinoic acid allows left–right asymmetry to occur in presomitic mesoderm where it normally does not occur. However, lateral-plate mesoderm in *Raldh2*$^{-/-}$ embryos still maintains asymmetric expression of *Nodal* and *Pitx2*, genes required for left-right asymmetry[10]. Retinoic acid acts as a buffer to prevent left–right asymmetry from occurring in presomitic mesoderm, but its mechanism of action is unclear.

2Correspondence should be addressed to G.D. (duester@burnham.org).

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

Published online at http://www.nature.com/naturecellbiology

Genetic studies in mice have demonstrated that oxidation of retinol to retinaldehyde is ubiquitously catalysed by several alcohol dehydrogenases[11], whereas oxidation of retinaldehyde to retinoic acid occurs in a tissue specific manner, under the control of *Raldh2* (*Aldh1a2*)[12,13]. All-*trans*-retinoic acid synthesized by RALDH2 is the endogenous retinoid ligand needed for embryogenesis and functions through nuclear retinoic-acid receptors (RARs), whereas 9-*cis*-retinoic acid (which binds the retinoid X receptor, RXR), is undetectable in mouse embryos and unnecessary for embryogenesis[14]. In early-somite-stage mouse embryos, *Raldh2* expression was limited to somitic mesoderm and anterior presomitic mesoderm (Fig. 1a). Retinoic-acid activity detected in mouse embryos carrying the *retinoic-acid response element* (*RARE)–lacZ*-reporter transgene[15] demonstrated that retinoic-acid signalling occurs in somitic and presomitic mesoderm, as well as in adjacent neural plate (Fig. 1b; $n = 5$ of 5). Transverse sections through the node revealed no left–right difference in retinoic-acid signalling activity in presomitic or lateral plate mesoderm at early somite stages (Fig. 1d; $n = 3$ of 3). *Raldh2*$^{-/-}$ embryos carrying *RARE–lacZ* lose all *RARE–lacZ* expression from presomite to 10-somite stages, demonstrating that *RARE–lacZ* is specific for detection of retinoic-acid activity and that RALDH2 is responsible for all retinoic-acid synthesis (Fig. 1c; $n = 6$ of 6). A sagittal section of a 4-somite wild-type embryo double-stained for *RARE–lacZ* and *Fgf8* expression indicated retinoic-acid activity extending from the posterior neural plate into the node ectoderm where it meets the *Fgf8* expression domain in the epiblast (Fig. 1e). Although retinoic-acid activity exists in presomitic mesoderm (Fig. 1b, d), it was absent in the node mesoderm (Fig. 1e). As our data shows that retinoic-acid signalling is absent in the node mesoderm and equivalent in left and right presomitic and lateral plate mesoderm, they do not support the hypothesis that cilia-driven node-vesicular parcels carry retinoic acid from right to left mesoderm as previously proposed[16]. Instead, our findings suggest that retinoic acid functions in the node ectoderm during left–right patterning of somites.

Loss of retinoic-acid signalling results in left–right asymmetry of somites after the eight somite stage[4–6]. This was also observed in 8–14-somite *Raldh2*$^{-/-}$ embryos by analysis of *Uncx4.1*, a homeobox gene involved in somite patterning (Fig. 2a–b; $n = 7$ of 7) and *Papc*, endoding a paraxial protocadherin involved in convergent extension (Fig. 2c–d; $n = 4$ of 4). *Mesp2* expressed in the presomitic mesoderm establishes segmental borders through suppression of Notch activity[17]. The *Xenopus laevis* homologue of *Mesp2* was reported to require retinoic acid for expression[3]. *Mesp2* expression in *Raldh2*$^{-/-}$ embryos at somite stages 4–5 shifted bilaterally and anteriorly, but no significant reduction in expression was observed (Fig. 2e–f; $n = 3$ of 3). Double-staining for *RARE–lacZ* and *Mesp2* expression in a 5-somite wild-type embryo showed that retinoic-acid signalling activity is normally detected in the *Mesp2* expression domain at this stage (Fig. 2g). Our results indicate that mouse *Mesp2* does not require retinoic acid for expression, but that retinoic acid is required to set its position along the antero-posterior axis. *Tbx18* encodes a T-box transcription factor that marks the anterior domain of each somite[18]. *Tbx18* expression in *Raldh2*$^{-/-}$ embryos at somite stages 5–14 demonstrated that somites were smaller all along the antero-posterior axis, but it was also observed that *Tbx18* expression was always reduced or lost in somites 3–14 on both the left and right sides (Fig. 2h–k; $n = 7$ of 7). This differs from *Uncx4.1* expression which was differentially reduced on the right side after the 8-somite stage (Fig. 2b). These findings indicate that before the left–right somite patterning defect, a loss of retinoic acid results in reduced somite size that is associated with a bilateral anterior shift of *Mesp2* expression and bilateral reduction of *Tbx18* expression.

Fgf8 expression in the posterior region of retinoic-acid-deficient embryos is upregulated and in some cases left–right asymmetric expression is observed[2,4–6]. As *Fgf8* is normally expressed in both the presomitic mesoderm and the epiblast (primitive ectoderm), the deregulated *Fgf8* expression observed in retinoic-acid-deficient embryos was examined in more detail. *Fgf8* expression in *Raldh2*$^{-/-}$ embryos with 11–13 somites was found to be higher on the right side

than on the left in both neuroectoderm and presomitic mesoderm (Fig. 3a, b; $n = 3$ of 3). At somite stages 3–5, left–right asymmetric expression of *Fgf8* in *Raldh2*$^{-/-}$ embryos was not observed, nor was any effect on expression in presomitic mesoderm; however, a bilateral anterior shift of *Fgf8* expression into node ectoderm and neural plate, where it is not normally expressed, was evident (Fig. 3c–f; $n = 4$ of 4). *Raldh2*$^{-/-}$ embryos at the one-somite stage already displayed the anterior shift of *Fgf8* expression in the ectoderm (Fig. 3g,h); sagittal sections revealed that a loss of retinoic acid allows *Fgf8* expression to expand from epiblast into the node ectoderm and the neural plate ectoderm (Fig. 3i–l; $n = 2$ of 2). Furthermore, a loss of retinoic acid did not result in an anterior advance of *Fgf8* expression in the presomitic mesoderm (where expression remained unchanged in early somite embryos), and *Fgf8* expression was still absent in the node mesoderm (Fig. 3i–l). Thus, we concluded that retinoic acid antagonizes *Fgf8* expression in ectoderm (but not mesoderm) at early somite stages, and that left–right asymmetric expression of *Fgf8* in presomitic mesoderm of later stage retinoic acid-deficient embryos is a secondary event.

To determine whether retinoic acid is required for patterning all somites during embryogenesis, we took advantage of the ability to rescue *Raldh2*$^{-/-}$ embryos by supplementing the maternal diet with low doses of retinoic acid[10,14]. It is also possible to detect embryonic sites of retinoic-acid signalling by analysing these rescued *Raldh2*$^{-/-}$ embryos carrying the *RARE–lacZ* transgene. When maternal-dietary retinoic-acid supplementation was ended at embryonic-day (E)8.25 (approximately six-somite stage) and embryos were examined at E9.5 (22–24 somites) for *Uncx4.1* expression, all somites in rescued *Raldh2*$^{-/-}$ embryos were of normal size and exhibited normal left–right bilateral symmetry (Fig. 4a, b; $n = 3$ of 3). Double-staining of these embryos for *RARE–lacZ* and *Uncx4.1* expression indicated that retinoic-acid signalling activity was completely absent in the somitic and presomitic mesoderm of rescued *Raldh2*$^{-/-}$ embryos (Fig. 4a, b). Similar experiments demonstrated that *Mesp2* was expressed normally in the presomitic mesoderm of E9.5 *Raldh2*$^{-/-}$ embryos rescued to only E8.25 (Fig. 4c, d; $n = 5$ of 5). In 4–7-somite *Raldh2*$^{-/-}$ embryos rescued to E8.25, the antero-posterior border of *Fgf8* expression was returned to normal, and retinoic-acid signalling activity was undetectable in the mesoderm but present in the neural plate where it was adjacent to *Fgf8* expression in the epiblast (Fig. 3m, n; $n = 3$ of 3). These findings indicate that retinoic acid is not required for later-forming somites and that retinoic acid limits ectodermal *Fgf8* expression to the epiblast during early somite stages.

These observations prompted us to examine more closely the expression patterns of *Raldh2* (the retinoic acid source) and *Fgf8* (a target of retinoic acid action). *Raldh2* is initially expressed at E7.5, before somitogenesis in paraxial mesoderm, where it functions as a source of the retinoic acid required for hindbrain antero-posterior patterning[19]. At the two-somite stage, *Raldh2* mRNA is localized in somitic and presomitic paraxial mesoderm but not the node, whereas *Fgf8* mRNA is found in presomitic mesoderm and epiblast, with an anterior expression boundary at the posterior lip of the node (Fig. 4e, f). Thus, *Raldh2* and *Fgf8* expression overlaps in the presomitic mesoderm at this early stage. Double-staining demonstrated that, as development proceeds, a gap between the *Raldh2* and *Fgf8* expression domains occurs in the presomitic mesoderm by the 6-somite stage (Fig. 4g), but that the close relationship between the *Mesp2* and *Fgf8* expression domains remains constant (Fig. 4h). Thus, after six somites, *Raldh2* expression does not occur in presomitic mesoderm. Double-staining of wild-type embryos for *RARE–lacZ* and *Mesp2* expression shows that retinoic-acid activity retracts anteriorly from somite stages 7–10 such that by 10 somites there is no longer retinoic-acid activity around the *Mesp2* expression domain (Fig. 4i–k). These findings provide evidence that after the node regresses (10 somites) *Raldh2* may no longer be needed to limit *Fgf8* expression. The timing of this separation is consistent with our observation that maternal dietary retinoic-acid supplementation is not needed after the 6-somite stage to achieve correct somite patterning in rescued *Raldh2*$^{-/-}$ embryos.

As maternal dietary supplementation of *Raldh2*$^{-/-}$ embryos to E8.25 is sufficient for correct patterning of early and late somites, we examined these embryos carrying *RARE–lacZ* at E8.25 to detect embryonic sites of retinoic-acid signalling that were stimulated by maternally derived retinoic acid. *RARE–lacZ* detected in rescued embryos must be derived from maternal retinoic acid (the levels of which have been elevated by dietary retinoic-acid supplementation). Surprisingly, rescued *Raldh2*$^{-/-}$ embryos at somite stages 2–10 exhibited no *RARE–lacZ* expression in somitic or presomitic mesoderm (normally the source of retinoic acid), but *RARE–lacZ* was expressed in the posterior neural plate (Fig. 5a; $n = 5$ of 5). Transverse sections verified the absence of *RARE–lacZ* expression in *Raldh2*$^{-/-}$ mesoderm (Fig. 5f, g). Para-sagittal and mid-sagittal sections through a two-somite-stage rescued *Raldh2*$^{-/-}$ embryo indicated the presence of strong *RARE–lacZ* expression in the posterior neural plate and node ectoderm, but no expression in somitic mesoderm, presomitic mesoderm, or node mesoderm (Fig. 5h, i). However, *RARE–lacZ* expression was observed in the cranial mesoderm of rescued *Raldh2*$^{-/-}$ embryos, indicating that the absence of the *RARE–lacZ* signal in trunk-paraxial mesoderm is authentic and not caused by mutant mesodermal retinoic-acid activity being below the limit of detection (Fig. 5h). These observations indicate that retinoic-acid signalling does not need to function in the presomitic mesoderm where it is synthesized, but instead retinoic acid travels to the adjacent ectoderm to function in somitogenesis.

Our findings suggest that a low dose of maternal dietary retinoic acid functions preferentially in the posterior neuroectoderm, rather than the somitic or presomitic mesoderm. One possibility is that retinoic acid may be preferentially degraded in certain tissues — as demonstrated in the hindbrain, which induces the retinoic-acid-degrading enzyme encoded by *Cyp26c1* to create a retinoic-acid boundary[19]. However, expression of *Cyp26a1*, *Cyp26b1* and *Cyp26c1* in early somite-stage mouse embryos does not occur in the somitic mesoderm[19,20]. A second possibility is that incoming retinoic acid may be preferentially sequestered by cellular retinoic-acid-binding proteins (CRABPs) that facilitate retinoic-acid signalling[21]. Consistently, we found that *Crabp2* was preferentially expressed in the posterior neural plate at somite stages 6–8 (Fig. 5b, c; $n = 3$ of 3). A final possibility is that although retinoic-acid receptors are widely expressed throughout the embryo at early stages[22], they may not respond to retinoic acid in certain tissues because of the presence of factors that antagonize retinoic-acid-mediated transcription, such as the orphan nuclear receptor, COUP–TF, which binds the same DNA response element as retinoic-acid receptors and functions as a repressor[23]. At somite stages 3–5 we found that *COUP–TFII* was expressed in the somitic and anterior presomitic mesoderm but was absent in the neural plate (Fig. 5d, e; $n = 2$ of 2). Expression of *Crabp2* and *COUP–TFII* at early somite stages is consistent with low-dose maternal retinoic-acid functioning preferentially in the posterior neuroectoderm.

Here, we provide significant insight into the role of retinoic acid during left–right patterning and antagonism of *Fgf8*. Our *RARE–lacZ* experiments indicate that retinoic-acid signalling does not occur in the node mesoderm (that is, ventral node) during establishment of left–right symmetry, and that retinoic-acid signalling does not occur at higher levels in mesoderm on the left side of the embryo. This finding contrasts with a study that reports the use of antibodies to detect retinoic acid on the surface of nodal vesicular parcels released from the ventral node and transported by leftward nodal flow[16]. This model predicts higher retinoic-acid signalling in left mesoderm, but this was not observed in our studies. Previous studies have also indicated a role for retinoic-acid signalling in the positioning of somite boundaries[2,3] and somite left–right patterning[4–6], presuming that retinoic acid functions in the presomitic mesoderm during each cycle of somite formation. As *Raldh2* is expressed in the presomitic mesoderm it was reasonable to hypothesize that retinoic acid may function in somite patterning in this tissue. However, our studies demonstrate that retinoic acid is needed only during the early somite stages (when the node is present) and that retinoic acid synthesized in the presomitic mesoderm by RALDH2 travels to the adjacent neural plate and node ectoderm where it regulates early

somite patterning. Also, as *Fgf8* is expressed in both presomitic mesoderm and epiblast ectoderm it was reasonable to assume that retinoic-acid antagonism of *Fgf8* expression may occur in presomitic mesoderm. However, our data indicate that antagonism occurs only in ectoderm. Thus, our conclusion that retinoic acid acts on ectoderm rather than presomitic mesoderm to exert its effects on left–right patterning is based, not only on *RARE–lacZ* expression in the ectoderm of rescued *Raldh2*$^{-/-}$ embryos, but also on ectopic expression of *Fgf8* in the same ectodermal tissue of unrescued mutants. This conclusion is also supported by our observation of selective expression of *Crabp2* (a stimulator of retinoic-acid signalling) in ectoderm and expression of *COUP–TFII* (an antagonist of retinoic-acid signalling) in mesoderm. It has also been observed that conditional loss of *Fgf8* expression in presomitic mesoderm does not have an effect on somitogenesis[24].

As *Fgf8* is essential for regulation of left–right patterning[25] and given our data indicate that retinoic acid functions in the ectoderm during somite left–right patterning, we propose that retinoic acid controls left–right patterning through its ability to control the anterior limit of *Fgf8* mRNA in the posterior ectoderm. The effects seen on the mesodermal expression of *Mesp2*, *Papc*, *Uncx4.1*, and *Tbx18* in *Raldh2*$^{-/-}$ embryos may be attributed to the anterior encroachment of ectodermal *Fgf8* mRNA; this may lead to production and release of FGF8, which increases FGF signalling in adjacent presomitic mesoderm and thus pushes somite formation further anterior. By correctly establishing the antero-posterior limit of ectodermal *Fgf8* expression during the node stages, retinoic acid signalling may ensure that *Fgf8* expression in presomitic mesoderm does not become subject to later signals that would push it to asymmetry along the left–right axis and thus result in asymmetric somite formation. As the requirement for retinoic acid in somitogenesis ends after the node has regressed, this suggests that retinoic-acid signalling coordinates the early simultaneous functions of FGF8 in controlling somite formation (through action in the presomitic mesoderm) and left–right asymmetry (through action in the node). These findings also provide more evidence that retinoic acid synthesized in the paraxial mesoderm does not function in this tissue, but instead operates in a non-cell autonomous manner, as a signal to adjacent tissues (including hindbrain neuroectoderm19, spinal cord neuroectoderm9, foregut endoderm26) and to posterior neural plate and node ectoderm as detailed here.

METHODS

Generation of *Raldh2*$^{-/-}$ embryos and rescue with a physiological dose of retinoic acid

Raldh2$^{-/-}$ mice have been previously described[13]. Embryos derived from timed matings of *Raldh2*$^{+/-}$ mice were genotyped by PCR analysis of yolk-sac DNA. After mating, we designated noon on the day of vaginal plug detection as E0.5. Embryos were staged according to somite number. Rescue of *Raldh2*$^{-/-}$ embryos by maternal dietary retinoic-acid supplementation was performed as previously described with a retinoic-acid dose that has been demonstrated to be within the normal physiological range14. This dose stimulates development of the heart, somites and other structures up to E13.5 (ref. 10). Briefly, all-*trans*-retinoic acid (Sigma, St Louis, MO) was dissolved in dimethylsulphoxide (50 mg ml), diluted 1:10 with corn oil, and mixed with powdered mouse chow to provide a final retinoic-acid concentration of 0.1 mg g^{-1} for treatment from E6.75–E8.25. Food was prepared fresh twice each day (morning and evening) and provided *ad libitum*. In some cases embryos were analysed at E8.25 when the mother was still receiving the retinoic-acid-supplemented diet. For embryos analysed after E8.25, mothers received standard mouse chow from E8.25 until the point of analysis at E9.5.

Retinoic-acid detection and *in situ* hybridization

Detection of retinoic-acid activity was performed in embryos carrying the *RARE–lacZ*–retinoic-acid-reporter transgene, which places *lacZ* (encoding β-galactosidase) under the transcriptional control of a retinoic-acid response element (RARE)[15]. β-galactosidase activity was detected using either X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) to produce a blue reaction product or salmon-gal (6-chloro-3-indolyl-β-D-galactopyranoside; Labscientific, Inc., Livingstone, NJ) to produce a red reaction product19. Whole-mount *in situ* hybridization was performed as previously described[19]. Double-staining to examine both retinoic-acid activity (*RARE–lacZ*) and mRNA localization was performed by first staining for 1 h for β-galactosidase activity, followed by processing for whole-mount *in situ* hybridization as previously reported[19]. Stained embryos were embedded in 3% agarose and sectioned at 20–50 μm with a vibratome.

Acknowledgments

We thank the following for mouse cDNAs used to prepare *in situ* hybridization probes: G. Martin (*Fgf8*), Y. Saga (*Mesp2*), E. DeRobertis (*Papc*), A. Kispert (*Tbx18*), V. Giguere (*Crabp2*), M. Tsai (*COUP-TFII*) and A. Mansouri (*Uncx4.1*). We also thank J. Rossant for providing *RARE–lacZ* mice, and A. Molotkov, N. Molotkova and X. Zhao for helpful discussions. This work was funded by National Institutes of Health grant GM62848.

References

1. Pourquié O. The segmentation clock: converting embryonic time into spatial pattern. Science 2003;301:328–330. [PubMed: 12869750]
2. Del Corral RD, et al. Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension. Neuron 2003;40:65–79. [PubMed: 14527434]
3. Moreno TA, Kintner C. Regulation of segmental patterning by retinoic acid signaling during *Xenopus* somitogenesis. Dev Cell 2004;6:205–218. [PubMed: 14960275]
4. Vermot J, et al. Retinoic acid controls the bilateral symmetry of somite formation in the mouse embryo. Science 2005;308:563–566. [PubMed: 15731404]
5. Vermot J, Pourquié O. Retinoic acid coordinates somitogenesis and left-right patterning in vertebrate embryos. Nature 2005;435:215–220. [PubMed: 15889094]
6. Kawakami Y, et al. Retinoic acid signalling links left-right asymmetric patterning and bilaterally symmetric somitogenesis in the zebrafish embryo. Nature 2005;435:165–171. [PubMed: 15889082]
7. Dubrulle J, McGrew MJ, Pourquié O. FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal *Hox* gene activation. Cell 2001;106:219–232. [PubMed: 11511349]
8. Aulehla A, Herrmann BG. Segmentation in vertebrates: clock and gradient finally joined. Genes Dev 2004;18:2060–2067. [PubMed: 15342488]
9. Molotkova N, Molotkov A, Sirbu IO, Duester G. Requirement of mesodermal retinoic acid generated by *Raldh2* for posterior neural transformation. Mech Dev 2005;122:145–155. [PubMed: 15652703]
10. Niederreither K, et al. Embryonic retinoic acid synthesis is essential for heart morphogenesis in the mouse. Development 2001;128:1019–1031. [PubMed: 11245568]
11. Molotkov A, et al. Stimulation of retinoic acid production and growth by ubiquitously-expressed alcohol dehydrogenase. Adh3 Proc Natl Acad Sci USA 2002;99:5337–5342.
12. Niederreither K, Subbarayan V, Dollé P, Chambon P. Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. Nature Genet 1999;21:444–448. [PubMed: 10192400]
13. Mic FA, Haselbeck RJ, Cuenca AE, Duester G. Novel retinoic acid generating activities in the neural tube and heart identified by conditional rescue of *Raldh2* null mutant mice. Development 2002;129:2271–2282. [PubMed: 11959834]

14. Mic FA, Molotkov A, Benbrook DM, Duester G. Retinoid activation of retinoic acid receptor but not retinoid X receptor is sufficient to rescue lethal defect in retinoic acid synthesis. Proc Natl Acad Sci USA 2003;100:7135–7140. [PubMed: 12782789]

15. Rossant J, et al. Expression of a retinoic acid response element-*hsplacZ* transgene defines specific domains of transcriptional activity during mouse embryogenesis. Genes Dev 1991;5:1333–1344. [PubMed: 1907940]

16. Tanaka Y, Okada Y, Hirokawa N. FGF-induced vesicular release of Sonic hedgehog and retinoic acid in leftward nodal flow is critical for left–right determination. Nature 2005;435:172–177. [PubMed: 15889083]

17. Morimoto M, Takahashi Y, Endo M, Saga Y. The Mesp2 transcription factor establishes segmental borders by suppressing Notch activity. Nature 2005;435:354–359. [PubMed: 15902259]

18. Bussen M, et al. The T-box transcription factor Tbx18 maintains the separation of anterior and posterior somite compartments. Genes Dev 2004;18:1209–1221. [PubMed: 15155583]

19. Sirbu IO, Gresh L, Barra J, Duester G. Shifting boundaries of retinoic acid activity control hindbrain segmental gene expression. Development 2005;132:2611–2622. [PubMed: 15872003]

20. MacLean G, et al. Cloning of a novel retinoic-acid metabolizing cytochrome P450, *Cyp26B1*, and comparative expression analysis with *Cyp26A1* during early murine development. Mech Dev 2001;107:195–201. [PubMed: 11520679]

21. Noy N. Retinoid-binding proteins: mediators of retinoid action. Biochem J 2000;348:481–495. [PubMed: 10839978]

22. Ruberte E, Dolle P, Chambon P, Morriss–Kay G. Retinoic acid receptors and cellular retinoid binding proteins. II. Their differential pattern of transcription during early morphogenesis in mouse embryos. Development 1991;111:45–60. [PubMed: 1849812]

23. Qiu Y, Tsai SY, Tsai M-JCOUP-TF. An orphan member of the steroid–thyroid hormone receptor superfamily. Trends Endocrinol Metab 1994;5:234–239. [PubMed: 18407213]

24. Perantoni AO, et al. Inactivation of FGF8 in early mesoderm reveals an essential role in kidney development. Development 2005;132:3859–3871. [PubMed: 16049111]

25. Meyers EN, Martin GR. Differences in left–right axis pathways in mouse and chick: functions of FGF8 and SHH. Science 1999;285:403–406. [PubMed: 10411502]

26. Molotkov A, Molotkova N, Duester G. Retinoic acid generated by *Raldh2* in mesoderm is required for mouse dorsal endodermal pancreas development. Dev Dyn 2005;232:950–957. [PubMed: 15739227]

27. Mansouri A, et al. Paired-related murine homeobox gene expressed in the developing sclerotome, kidney, and nervous system. Dev Dyn 1997;210:53–65. [PubMed: 9286595]

28. Kim SH, et al. The role of paraxial protocadherin in selective adhesion and cell movements of the mesoderm during *Xenopus* gastrulation. Development 1998;125:4681–4691. [PubMed: 9806917]

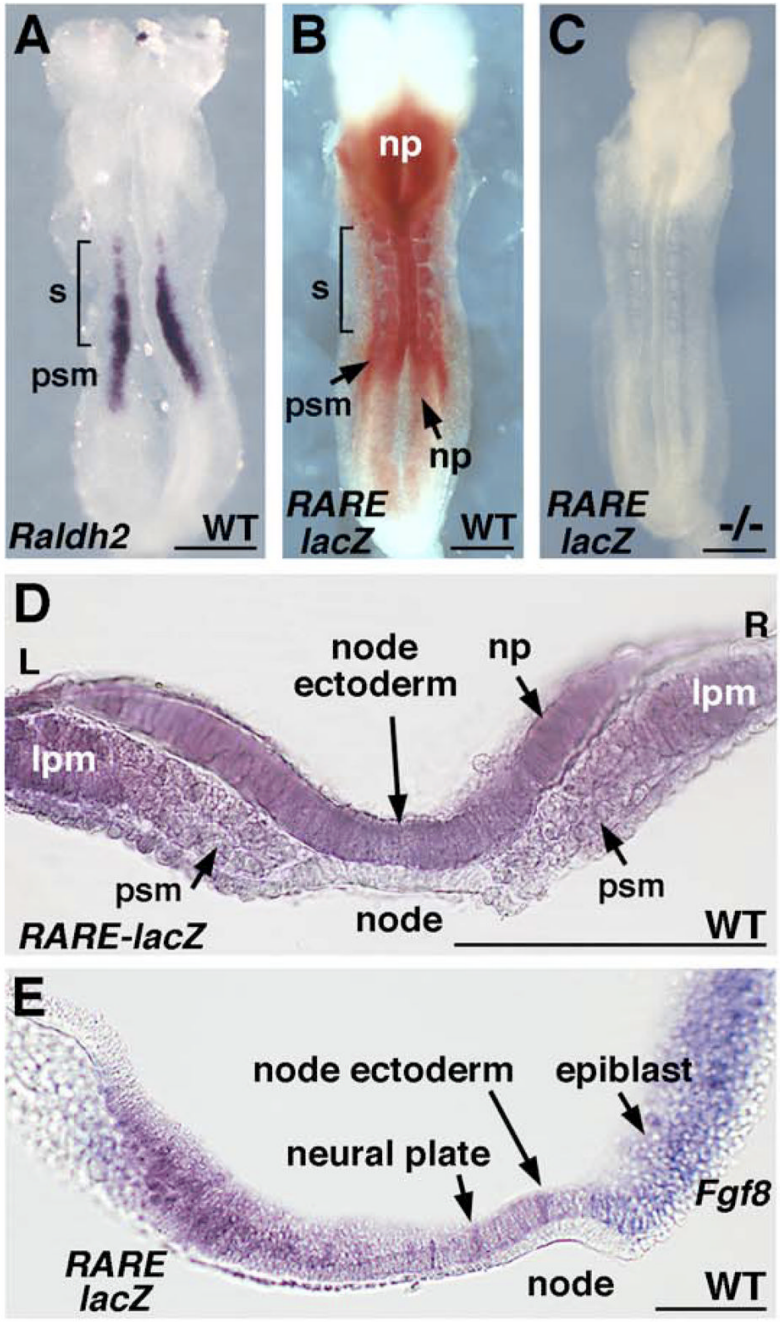

Figure 1.

Retinoic-acid signalling is uniform across the left–right axis and present only in node ectoderm. (**a**) *Raldh2* mRNA expression in wild-type 5-somite mouse embryo (dorsal view). (**b**, **c**) *RARE–lacZ* expression (detected with salmon–gal) in 6-somite embryos indicates no left–right difference in retinoic-acid signalling activity in wild-type embryos and complete loss of retinoic-acid activity in *Raldh2*$^{-/-}$ embryos (dorsal view). (**d**) Transverse section through the node of 2-somite wild-type embryo stained for *RARE–lacZ* expression. No left–right difference in retinoic-acid activity is observed. (**e**) Double-staining for *RARE–lacZ* and *Fgf8* expression in a mid-sagittal section of a 4-somite wild-type embryo shows retinoic-acid activity in node ectoderm, but not in node mesoderm; also, abutment of retinoic-acid activity with *Fgf8* mRNA,

forming a border at the junction of the node ectoderm and epiblast, is noted. L, left; lpm, lateral-plate mesoderm; np, neural plate; psm, presomitic mesoderm; R, right; s, somites. Scale bars represent 100 μm.

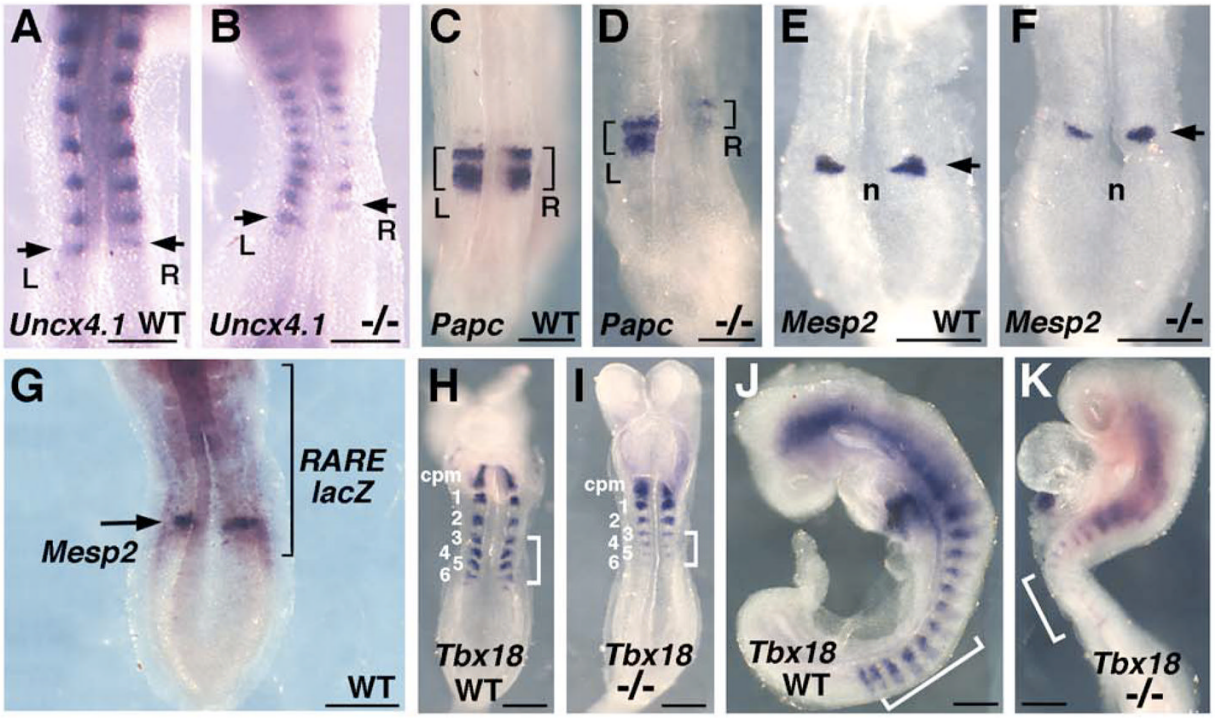

Figure 2.

Bilateral somite-size defects precede left–right defects in the absence of retinoic-acid signalling. (**a**, **d**) *Uncx4.1* mRNA[27] expression (**a**, **b)** at 11 somites marks the posterior domain of each somite and *Papc* mRNA[28] expression (**c**, **d)** at 14 somites marks the somite determination region, showing antero-posterior compression of somites (smaller somites) and left–right asymmetry in *Raldh2*$^{-/-}$ embryos (dorsal view). (**e**, **f**) *Mesp2* mRNA expression at 5 somites shows an anterior shift in its expression domain in *Raldh2*$^{-/-}$ embryos relative to wild-type, pushing it further anterior to the node (dorsal view). (**g**) Double-staining for *RARE–lacZ* and *Mesp2* expression in 5-somite wild-type embryo shows that retinoic-acid activity extends posterior to the *Mesp2* expression domain (dorsal view). (**h**, **k**) *Tbx18* mRNA expression at 6 somites (**h**, **i**; ventral view) and at 14 somites (**j**, **k**; lateral view) demonstrating reduced (or absent) expression in somite 3 and all subsequent somites of *Raldh2*$^{-/-}$ embryos (square brackets). cpm, cranial-paraxial mesoderm; L, left; N, node; R, right. Scale bars represent 100 μm.

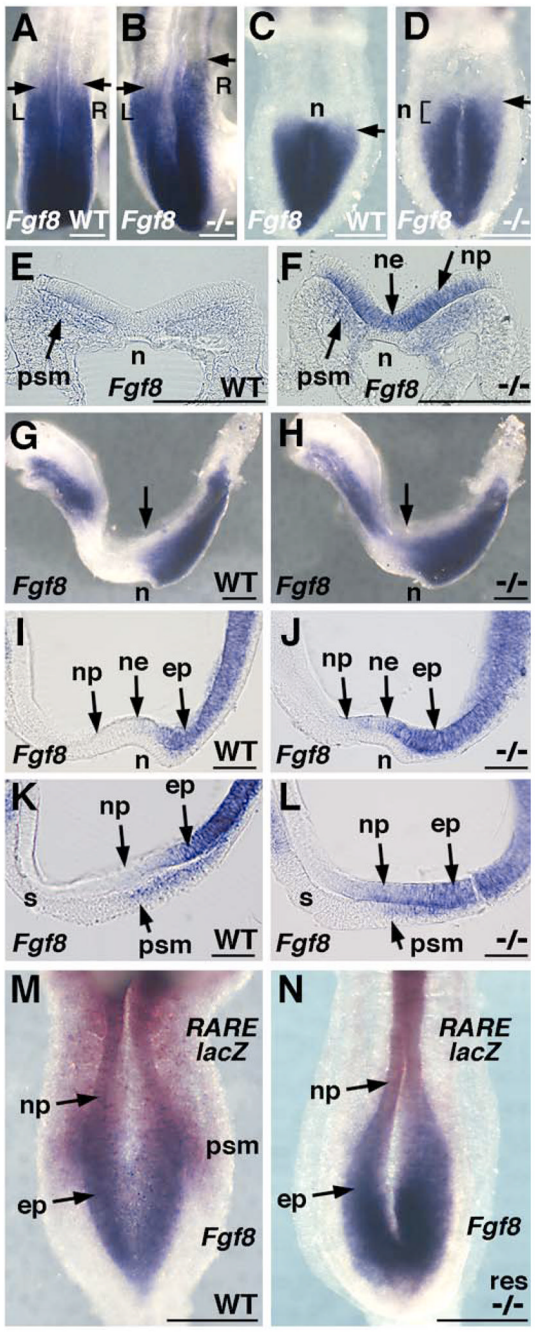

Figure 3.

Retinoic-acid signalling activity antagonizes *Fgf8* expression only in ectoderm. (**a**, **b**) *Fgf8* mRNA expression at 13 somites shows left–right asymmetry in an *Raldh2*$^{-/-}$ embryo (dorsal view). (**c**, **d**) *Fgf8* mRNA expression at 3 somites does not indicate left–right asymmetry in an *Raldh2*$^{-/-}$ embryo, but exhibits a bilateral anterior shift into node and neural plate ectoderm where it is not normally expressed (dorsal view). (**e**, **f**) Transverse sections through the node of embryos shown in **c** and **d** demonstrate that *Raldh2*$^{-/-}$ results in an anterior shift of *Fgf8* expression into node and neural plate ectoderm but not presomitic mesoderm. (**g–l**) Lateral views showing the tissue distribution of ectopic *Fgf8* mRNA expression in a 1-somite *Raldh2*$^{-/-}$ embryo; mid-sagittal sections indicate an anterior advance of *Fgf8* mRNA

expression from the epiblast into node ectoderm where it is not normally detected (**i**, **j**) and para-sagittal sections reveal an anterior advance of *Fgf8* mRNA expression from the epiblast into the neural plate where it is not normally detected. *Fgf8* expression in presomitic mesoderm was unchanged (**k**, **l**). (**m**, **n**) Double-staining for *RARE–lacZ* and *Fgf8* expression (dorsal view) in a wild-type embryo and a *Raldh2*$^{-/-}$ embryo rescued with maternal dietary retinoic-acid supplementation to E8.25. An absence of retinoic-acid activity in the mesoderm of the rescued mutant and abutment of retinoic-acid activity with *Fgf8* mRNA expression, forming a border in the posterior neural plate, is noted. ep, epiblast; L, left; N, node;ne, node ectoderm; np, neural plate; psm, presomitic mesoderm; R, right; S, somite; res, rescued.

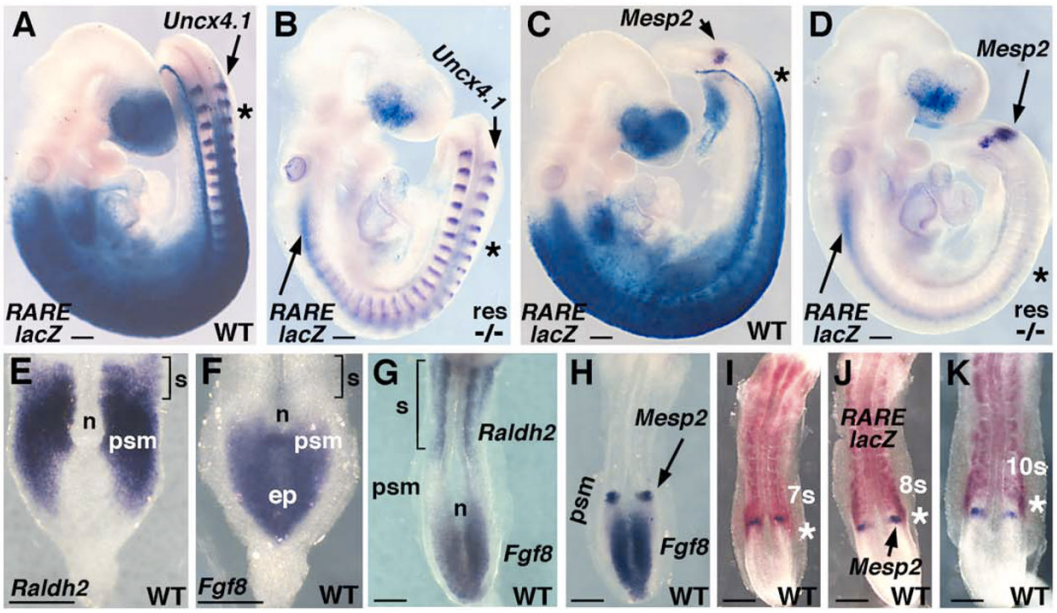

Figure 4.

Retinoic-acid-signalling requirement for somitogenesis occurs only during the early somite stages. (**a–d**) E9.5 wild-type embryos (WT) and *Raldh2*$^{-/-}$ embryos rescued with maternal dietary retinoic-acid supplementation to E8.25 (Res −/−). Double-staining for *Uncx4.1* mRNA (purple) and *RARE–lacZ* retinoic-acid signalling activity (X-gal blue staining) at 24-somites (**a**, **b**) and double-staining for *Mesp2* mRNA and *RARE–lacZ* at 24-somites (**c**, **d**), indicating normal late somitogenesis in *Raldh2*$^{-/-}$ embryos rescued with maternal retinoic acid only to early somite stages. (**e**, **f**) *Raldh2* mRNA expression (**e**) at 2 somites and *Fgf8* mRNA expression (**f**) at 2 somites (both dorsal views), showing overlap of expression in the psm. (**g**, **h**) Double-staining for *Raldh2* and *Fgf8* mRNAs (**g**) and *Mesp2* and *Fgf8* mRNAs (**h**) at 6 somites (dorsal view) showing that *Raldh2* expression has now retracted anteriorly, away from the *Fgf8* and *Mesp2* expression domains. (**i–k**) Double-staining for *RARE–lacZ* and *Mesp2* expression (dorsal view) showing that between somite stages 7–10 retinoic-acid activity retracts anterior to the *Mesp2* expression domain. Asterisks mark the posterior boundary of retinoic-acid signalling activity. ep, epiblast; N, node; psm, presomitic mesoderm; S, somites. Scale bars represent 100 μm.

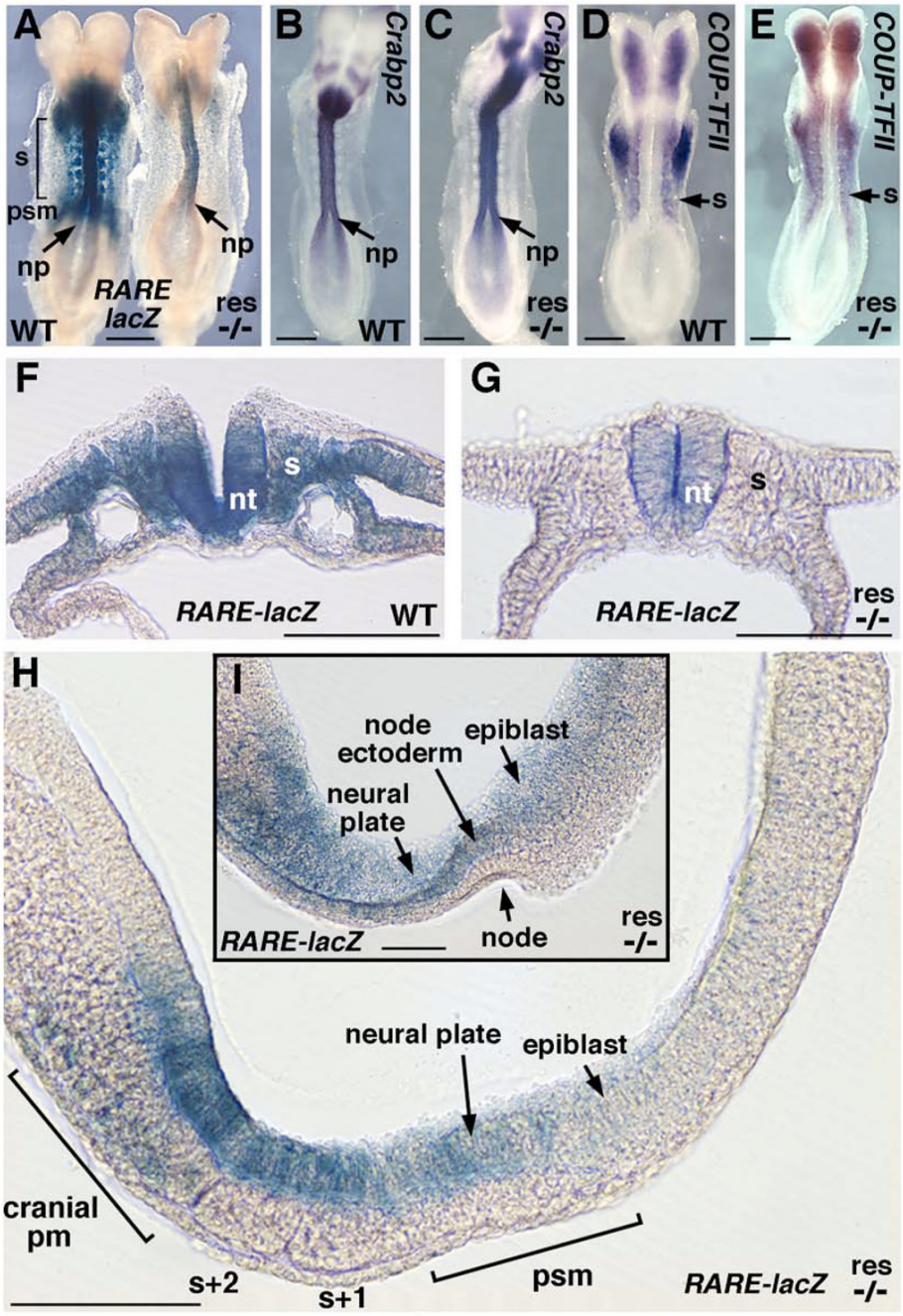

Figure 5.

The targets of retinoic-acid signalling for somitogenesis are the node ectoderm and the posterior neural plate. (**a–i**) E8.25 wild-type embryos (WT) and *Raldh2*$^{-/-}$ embryos rescued with maternal dietary retinoic-acid supplementation to E8.25 (Res −/−). *RARE–lacZ* and retinoic-acid signalling activity (**a**) in wild-type (left) and rescued mutant (right) both from a dorsal view. *Crabp2* mRNA expression (**b, c**) at 7 somites (dorsal view). *COUP–TFII* mRNA expression (**d, e**) at 5 somites (dorsal view). Transverse sections showing *RARE–lacZ* expression in 6-somite wild-type embryo (**f**) and rescued *Raldh2*$^{-/-}$ embryo (**g**). Para-sagittal (**h**) and mid-sagittal (**i**) sections of 2-somite rescued *Raldh2*$^{-/-}$ embryo showing *RARE–lacZ* expression in posterior neural plate and node ectoderm but not somitic, presomitic, or node

mesoderm. Cranial pm, cranial paraxial mesoderm; Np, neural plate; Nt, neural tube; Psm, presomitic mesoderm; S, somite. Scale bars represent 100 μm.