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Retinoic Acid Promotes Limb Induction through Effects on Body Axis Extension but is Unnecessary for Limb Patterning

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Summary

Retinoic acid (RA) is thought to be a key signaling molecule involved in limb bud patterning along the proximodistal or anteroposterior axes functioning through induction of *Meis2* and *Shh*, respectively [1]. Here, we utilize *Raldh2*$^{-/-}$ and *Raldh3*$^{-/-}$ mouse embryos lacking RA synthesis [2] to demonstrate that RA signaling is not required for limb expression of *Shh* and *Meis2*. We demonstrate that RA action is required outside the limb field in the body axis during forelimb induction, but that RA is unnecessary at later stages when hindlimb budding and patterning occurs. We provide evidence for a model of trunk mesodermal RA action in which forelimb induction requires RA repression of *Fgf8* in the developing trunk similar to how RA controls somitogenesis [3,4] and heart development [5]. We demonstrate that pectoral fin development in RA-deficient zebrafish embryos can be rescued by an FGF receptor antagonist SU5402. In addition, embryo ChIP assays demonstrate that RA receptors bind the *Fgf8* promoter in vivo. Our findings suggest that RA signaling is not required for limb proximodistal or anteroposterior patterning but that RA inhibition of FGF8 signaling during the early stages of body axis extension provides an environment permissive for induction of forelimb buds.

Results and Discussion

Retinoic acid (RA) is an important cell-cell signaling molecule that directly regulates genes through a nuclear RA receptor (RAR) bound to a RA response element (RARE) [2]. RA has been proposed to control chick limb anteroposterior patterning by inducing *Shh* posteriorly [6,7]. However, studies in mice carrying a RA-reporter transgene demonstrated that limb RA activity is distributed equally along the anteroposterior axis, although RA is located differentially along the proximodistal axis with highest activity proximally [8]. Genetic studies in mice have demonstrated that RA synthesis is controlled by retinaldehyde dehydrogenase-2 (*Raldh2*) expressed in trunk mesoderm lying proximal to the limb bud, but not in the limb bud itself [9,10]. Further studies in chick embryos suggested that RA may control the limb proximodistal axis through a mechanism in which RA generated by *Raldh2* proximally in the

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flank induces *Meis1* and *Meis2* (proximal limb markers), and that fibroblast growth factor (FGF) generated distally in the apical ectodermal ridge (AER) represses these *Meis* genes [11]. However, gene inactivation studies have shown that *Meis* genes are not essential for normal limb development, at least individually [1,12]. Although genetic studies have demonstrated the requirement for a distal FGF signaling center during proximodistal patterning [13] and the requirement for a distal region of RA degradation controlled by *Cyp26b1* to prevent RA-induced limb teratogenesis [14], there is no clear evidence that a proximal RA signaling center is required to establish the limb proximodistal axis [1]. Furthermore, *Raldh2*$^{-/-}$ mouse embryos lacking RA synthesis fail to undergo forelimb induction suggesting that RA plays a role in limb development prior to limb patterning [9,10]; also, zebrafish *raldh2* mutants lack pectoral fins [15]. *Raldh2*$^{-/-}$ embryos rescued by maternal dietary RA supplementation undergo limb induction resulting in forelimbs that are undersized but hindlimbs appear relatively normal [8,16]; as mutants rescued with a low RA dose express *Meis2* in forelimb buds despite a lack of RA-reporter activity in limb mesoderm, a role for RA induction of *Meis* during proximodistal patterning is questionable [16]. Although rescued *Raldh2*$^{-/-}$ embryos display normal hindlimb buds, another potential source of RA exists near the hindlimb provided by *Raldh3* expressed in the mesonephros [10]. Thus, the requirement of RA signaling for limb induction or patterning remains unclear, particularly as forelimb and hindlimb buds may differ in this regard. Here, we explore the role of RA during limb development by examining mouse embryos lacking either *Raldh2* or both *Raldh2* and *Raldh3* to eliminate all endogenous RA synthesis during induction and patterning of forelimbs and hindlimbs.

Hindlimb Budding and Patterning does not Require RA

As *Raldh2*$^{-/-}$ embryos fail to grow beyond E8.5, mutants were rescued using maternal dietary RA supplementation [4]; the low doses of dietary RA used here (0.1-0.25 mg RA per g food) have been shown to provide embryos an amount of RA in the normal physiological range [17]. In order to detect RA activity in rescued *Raldh2*$^{-/-}$ embryos, we used embryos carrying the *RARE-lacZ* RA-reporter transgene [18]. We found that *Raldh2*$^{-/-}$ embryos provided brief RA treatment (0.1 mg RA per g food from E6.75-E8.25), then analyzed at E10.5, display RA activity in the neural tube and in the mesonephros adjoining the proximal hindlimb bud (Figure 1A-D; $n = 6/6$). We examined transverse sections of E10.5 wild-type embryos and found that *Raldh3* mRNA is expressed in the mesonephric duct adjacent to the hindlimb bud (Figure 1E-F). *Raldh3*$^{-/-}$ embryos have normal limb buds [19], so we tested the potential role of *Raldh3* in providing RA for hindlimb development by generating *Raldh2*$^{-/-}$;*Raldh3*$^{-/-}$ double mutants. As *Raldh2*$^{-/-}$;*Raldh3*$^{-/-}$ embryos exhibit early lethality similar to *Raldh2*$^{-/-}$ mutants [19], we examined rescued *Raldh2*$^{-/-}$;*Raldh3*$^{-/-}$ embryos at E10.5 following brief RA treatment from E6.75-E8.25. Hindlimb buds of a normal size were observed in rescued *Raldh2*$^{-/-}$;*Raldh3*$^{-/-}$ embryos ($n = 7/7$) despite a complete absence of RA activity (monitored by *RARE-lacZ* expression) in the mesonephros and hindlimb mesoderm (Figure 1G; $n = 3/3$). Similar to *Raldh2*$^{-/-}$ embryos, rescued *Raldh2*$^{-/-}$;*Raldh3*$^{-/-}$ embryos always exhibited forelimbs smaller than their hindlimbs (Figure 1G-H). These findings demonstrate that *Raldh3* is a source of RA for the mesonephros, but that RA synthesized by RALDH3 is not required in rescued *Raldh2*$^{-/-}$ embryos for hindlimb induction or early outgrowth.

RA activity was also absent at earlier stages in the hindlimb field of rescued *Raldh2*$^{-/-}$ embryos. In an E9.5 RA-rescued *Raldh2*$^{-/-}$ embryo (25-somite stage when the hindlimb field is forming), RA activity was not observed in hindlimb mesoderm (adjacent to somites 23-25) but a small region of *RARE-lacZ* expression was seen in the mesonephros likely due to *Raldh3* expressed in that tissue (Figure. 2D; see Figure S1 for transverse sections).

Genes required for hindlimb induction and patterning were examined in *Raldh2*$^{-/-}$;*Raldh3*$^{-/-}$ embryos including *Fgf8* [13], *Shh* [20], *Tbx4* [21], and *Pitx1* [21]. Following brief RA

treatment, *Raldh2*$^{-/-}$;*Raldh3*$^{-/-}$ hindlimbs at E10.5 displayed relatively normal expression of *Fgf8* in the AER needed for proximodistal patterning (Figure 1I-J;Figure S2) and normal expression of *Shh* in the zone of polarizing activity (ZPA) needed for anteroposterior patterning (Figure 1K-L;Figure S2). We also detected normal hindlimb expression of *Tbx4* and *Pitx1* which function in hindlimb induction (Figure 1M-P). We also analyzed early proximodistal patterning of the hindlimb which can be visualized with probes for expression of *Meis2* (stylopod) and *Hoxa11* (zeugopod) [1]. Analysis of these markers at E10.5 in RA-rescued *Raldh2*$^{-/-}$;*Raldh3*$^{-/-}$ hindlimbs showed that these segments of the limb are present indicating that early proximodistal patterning does not depend on RA signaling (Figure 1Q-T). Moreover, although *Meis2* expression is proposed to be dependent on RA from the flank [11], our data do not support this hypothesis. As rescued *Raldh2*$^{-/-}$;*Raldh3*$^{-/-}$ hindlimb buds lack RA activity but undergo normal induction and patterning, these results suggest that RA is not required to establish limb patterning along either the anteroposterior or proximodistal axes. This conclusion is further supported by mutation of retinol dehydrogenase *Rdh10* (acting upstream of *Raldh2* for RA synthesis) which results in a phenotype with small forelimbs and normally-patterned hindlimbs reminiscent of rescued *Raldh2* mutants [22]; the *Rdh10* mutant does not require a small dose of RA to survive until hindlimb budding occurs, but nevertheless displays the same *RARE-lacZ* pattern as a rescued *Raldh2*$^{-/-}$;*Raldh3*$^{-/-}$ embryo with RA activity detected in the neural tube but not the limb buds.

Forelimb Induction Requires RA Signaling in the Body Axis but not Limb Mesoderm

Although we find no requirement for RA during hindlimb budding or patterning, forelimb buds do appear to require RA for normal development. We further investigated RA signaling during budding of rescued forelimbs using *RARE-lacZ* and found that RA activity was not present in the small forelimb buds of E10.5 rescued *Raldh2*$^{-/-}$ embryos but RA was detected in the neural tube (Figure 2A-B; $n = 6/6$); RA activity was also not present in the small forelimb bud at E9.25 when it is first morphologically detectable (Figure 2C-D; Figure S3; $n = 5/5$). To complement this analysis of RA activity, we also examined expression of *Cdx1* in rescued forelimbs. Although *Cdx1* is not required for forelimb development [23], the *Cdx1* promoter contains a highly sensitive RARE that functions *in vivo* and therefore serves as an endogenous reporter of RA activity [24]. *Cdx1* was highly expressed in wild-type forelimbs, but was not expressed in small forelimb buds of rescued *Raldh2*$^{-/-}$ embryos suggesting they lack RA activity (Figure 2E-F; Figure S3; $n = 3/3$). At E8.5 (8-10 somites) the forelimb field has already been determined as evidenced by expression of Tbx5, the earliest known marker of the mammalian forelimb [25]. Examination of E8.5 rescued *Raldh2*$^{-/-}$ embryos revealed that RA activity was undetectable in the lateral plate mesoderm that gives rise to the forelimb field although RA was detected in neuroectoderm and endoderm (Figure 2G-J; $n = 7/7$). To further test whether RA signaling is absent in lateral plate mesoderm of rescued *Raldh2*$^{-/-}$ embryos, we examined expression of RARβ which possesses a potent RARE and is expressed in lateral plate mesoderm and neuroectoderm [26]; in rescued *Raldh2*$^{-/-}$ embryos, RARβ expression was detected in neuroectoderm but not lateral plate mesoderm (Figure 2K-L; $n = 4/4$). These findings demonstrate that our low-dose dietary method of RA administration to *Raldh2*$^{-/-}$ embryos provides less RA than *Raldh2* normally generates. Even though we presume that RA is entering the embryo uniformly during rescue (by diffusion from the uterus since there is no placenta at this early stage), when RA is provided at such a low level it does not stimulate gene expression in all cells where it normally would perhaps due to tissue-specific differences in expression of RA-binding proteins or RARs [4]. The normal source of RA during forelimb induction is *Raldh2* expressed in the somites and lateral plate mesoderm (Figure 2M-N). Thus, even though RA generated by *Raldh2* in wild-type embryos is normally present in somites and lateral plate mesoderm fated to become limb, and can induce *Cdx1* in limb mesoderm, RA is not acting

there to initiate forelimb development but instead RA is functioning in a paracrine fashion elsewhere in the body axis to permit forelimb induction.

RA is Required for Induction of Forelimb Buds but not for Anteroposterior or Proximodistal Patterning

We explored whether RA acts early during forelimb induction to establish the forelimb field by examining the effect of RA rescue on expression of *Tbx5* encoding a T-box transcription factor that is the earliest known marker of the mouse forelimb field [25]. Unrescued *Raldh2*$^{-/-}$ embryos failed to initiate *Tbx5* expression in the lateral plate mesoderm posterior to the heart indicating a failure in forelimb induction at the 13-somite (13s) stage (Figure 3A-B; $n = 2/2$). Rescued *Raldh2*$^{-/-}$ embryos exhibited *Tbx5* expression at 18s, although the size of the forelimb field was much smaller than normal (Figure 3C-D; $n = 6/6$). Double-staining for expression of *Tbx5* and the somite marker *Uncx4.1* [4] revealed that rescued *Raldh2*$^{-/-}$ embryos have no *Tbx5* expression at 10s (Figure 3E-F; $n = 4/4$), but that a small domain of *Tbx5* expression was observed at 13s (Figure 3G-H; $n = 3/3$). These findings indicate that brief RA treatment allows a domain of *Tbx5* expression to arise after a delay of a few hours, potentially leading to the small forelimbs observed later. However, the effect of RA on *Tbx5* must be indirect as we do not detect RA in the cells where *Tbx5* is induced (Figure 2H,J,L).

We find that forelimbs lacking RA activity still express *Shh* even though expression appears distally rather than posteriorly as normal (Figure 1L; Figure S2). However, exogenous RA treatment (high-dose beads) has been reported to induce expression of *Shh*, *Hoxb8*, and *Hand2* leading to the conclusion that RA is needed to establish anteroposterior patterning of chick limb buds; thus, we examined rescued E9.5 *Raldh2*$^{-/-}$ embryos for expression of *Hoxb8* and *Hand2* which are expressed prior to *Shh* [20]. In E9.5 rescued *Raldh2*$^{-/-}$ embryos we found that *Hoxb8* was still expressed in the posterior portion of forelimb buds (Figure 3I-J; Figure S4; $n = 3/3$), and that a small domain of *Hand2* was still expressed in the small forelimb bud that develops even though expression is localized distally rather than posteriorly (Figure 3K-L; Figure S4; $n = 2/2$). We examined proximodistal markers in E10.5 rescued *Raldh2*$^{-/-}$;*Raldh3*$^{-/-}$ embryos and found that *Meis2* (previously suggested to require RA for induction) was expressed in the proximal portion of the small forelimb that develops, and *Hoxa11* was expressed more distally as expected (Fig. 3M-P). Thus, taken together with our findings above demonstrating that rescued *Raldh2*$^{-/-}$ embryos lack forelimb RA activity, RA signaling is not required in the forelimb bud for induction of *Hoxb8*, *Hand2*, *Shh*, and *Meis2*. However, RA signaling is required outside the forelimb field during induction to obtain the correct size bud and the correct posterior expression domains of *Hand2* and *Shh*; we suggest that posterior domains may not be able to form properly when forelimb growth is retarded, thus resulting in distal expression.

Previous studies using pharmacological treatment of chick embryos with combined RAR/RXR antagonists to block RA receptor activity [6] or the RA synthesis inhibitor disulfiram [7] have shown down-regulation of *Shh* and *Hoxb8*. However, these chemicals may have non-specific effects as RXRs are heterodimer partners for at least 10 nuclear receptors other than RARs, and disulfiram inhibits the enzymatic activity of many if not all 19 members of the aldehyde dehydrogenase family to which RALDH2 belongs. By removing RALDH2 genetically our findings suggest that endogenous RA action is not required to induce genes needed for limb anteroposterior or proximodistal patterning, but that RA action is required at an earlier stage when forelimb induction occurs.

RA Inhibits FGF Signaling in the Body Axis Near the Forelimb Field

Our studies suggest RA does not play an instructive role in forelimb development, but rather a permissive role through action in the body axis near the forelimb field at the 8-somite stage when forelimb induction occurs [25]. During the 1-10 somite stages, RA functions permissively during development of the body axis by repressing *Fgf8* posteriorly at the neuroectoderm/epiblast junction to prevent *Fgf8* expression from extending anteriorly into neuroectoderm [3,4], and by repressing *Fgf8* anteriorly in cardiac lateral plate mesoderm to prevent *Fgf8* expression from extending posteriorly into trunk lateral plate mesoderm [5]. By limiting the cardiac and epiblast *Fgf8* domains, we suggest that RA permits an Fgf8-free zone to develop in between needed for proper development of the trunk including the forelimb fields. This hypothesis is supported by previous studies demonstrating that expression of a constitutively active FGF receptor (Fgfr1) in zebrafish results in expansion of the heart field and loss or reduction of pectoral fins [27]. To further test this hypothesis, we examined the effect of the FGF receptor antagonist SU5402 on pectoral fin development in RA-deficient zebrafish embryos using the RA synthesis inhibitor DEAB as previously described [15]. Zebrafish embryos treated with DEAB to inhibit RA synthesis from the bud stage (~9.5-10 hpf) to somite 12-13 (~15 hpf) were found to always lack pectoral fins when observed at 96 hpf (n = 0/8 fin positive with 4 μM DEAB; n = 0/7 fin positive with 5 μM DEAB; n = 17/17 fin positive with 0.1% DMSO vehicle). Treatment during the same time period with 3 μM SU5402 resulted in yolk sac edema, but pectoral fins always developed (n = 14/14 fin positive). Importantly, treatment with both DEAB and SU5402 during this time period often rescued pectoral fin development (n = 6/17 fin positive with 4 μM DEAB + 3 μM SU5402; n = 3/8 fin positive with 5 μM DEAB + 3 μM SU5402). Rescued pectoral fins were smaller than those present in vehicle-treated embryos (Fig. S5). These findings suggest that loss of RA synthesis results in an increase in FGF signaling which inhibits pectoral fin development. Treatment with both inhibitors also resulted in less yolk sac edema in most embryos (including all those that were fin positive), suggesting that DEAB may be reducing the toxicity of SU5402, consistent with a loss of RA leading to increased *Fgf* expression as previously reported in mouse.

In order to determine if excessive *Fgf8* expression observed in *Raldh2*$^{-/-}$ mouse embryos results in excessive FGF signaling, we examined expression of *Sprouty2* (*Spry2*) which is induced by FGF signaling and functions to regulate transmission of the FGF signal [28]. Whereas wild-type embryos exhibited anterior and posterior domains of *Spry2* mRNA separated by a large negative region in the trunk, *Raldh2*$^{-/-}$ embryos at 6-8 somites exhibited ectopic *Spry2* mRNA encroaching into the trunk where the forelimb field normally develops (Figure 4C; n = 3/3). Thus, a loss of RA signaling leads to a large increase in trunk FGF signaling.

Previous studies on chick embryos suggested that *Fgf8* expressed in intermediate mesoderm might be needed for limb initiation due to the ability of FGF-beads to induce extra limbs in the interlimb flank [29]. However, studies on mouse embryos have shown that *Tbx5* expression in the forelimb field precedes *Fgf8* expression in the intermediate mesoderm by about 18 hours [25], and conditional mutagenesis has demonstrated that *Fgf8* expression in the intermediate mesoderm is unnecessary for limb induction although it is required at later stages for kidney development [30]. Also, further studies on chick embryos demonstrated that ablation of the intermediate mesoderm [31] or neuroectoderm [32] does not affect limb initiation. Thus, we futher pursued the hypothesis that *Fgf8* expression may normally inhibit rather than stimulate forelimb induction. Here, we found that wild-type mouse embryos at 10-13s have no *Fgf8* expression detectable in the intermediate mesoderm (Figure 4A; n = 7/7). However, unrescued *Raldh2*$^{-/-}$ embryos at 12-13s always exhibited an abnormal domain of *Fgf8* expression in the intermediate mesoderm adjacent to the region where the forelimb field has failed to develop (Figure 4A; n = 4/4). Interestingly, *Raldh2*$^{-/-}$ embryos at 11-13s rescued by brief RA treatment still exhibited ectopic *Fgf8* expression in the intermediate mesoderm although this domain was

now well-separated from the *Fgf8* expression domain observed posteriorly at the neuroectoderm/epiblast junction which has retracted posteriorly compared to the unrescued mutant (Figure 4A; $n = 3/3$). We compared *Fgf8* expression in *Raldh2*$^{-/-}$ embryos at 10-11s treated with either brief RA treatment (0.1 mg RA per g food from E6.75-E8.25) or extended RA treatment (similar to brief treatment except 0.25 mg RA per g of food from E7.75-E8.5), and found that extended RA treatment eliminated the ectopic domain of *Fgf8* expression in the intermediate mesoderm parallel to somites 6-10 (Figure 4B; $n = 4/4$). Extended RA treatment resulted in *RARE-lacZ* expression (RA activity) not only in the neuroectoderm (as found for brief treatment) but also in the somitic, intermediate, and lateral plate mesoderm of *Raldh2*$^{-/-}$ embryos (Figure 4B). Extended RA treatment of E8.5 *Raldh2*$^{-/-}$ embryos resulted in a significant increase in the size of the forelimb field marked by *Tbx5* expression (Figure 4B; $n = 2/2$) compared to brief RA treatment (Figure 3H). Previous RA rescue studies of *Raldh2*$^{-/-}$ embryos demonstrated that extended RA treatment increases the size of the forelimb bud at E10.5, in some cases close to normal size [8,16]. Taken together, these findings suggest that brief RA treatment eliminates excessive FGF8 signaling emanating from the neuroectoderm/epiblast junction which may allow the forelimb field to initiate, but that the field may be delayed and undersized due to excessive FGF8 signaling emanating from the intermediate mesoderm which requires higher levels of RA to repress *Fgf8*.

Support for a direct role of RA in regulation of *Fgf8* comes from studies suggesting that a nearby RARE represses the major isoform (Fgf8b) when RAR and RA are both present, but allows Fgf8b expression when RAR is unliganded [33]. We provide further evidence that RA regulation of *Fgf8* is direct using chromatin immunoprecipitation (ChIP). We show that a conserved RARE near the *Fgf8* promoter binds all three RAR isoforms (RARa, RARb, and RARg) using ChIP with RAR antibodies and chromatin from 5-somite mouse embryos (Figure S6). This finding is important since it shows that the mouse *Fgf8* gene can bind RAR in vivo just prior to induction of *Tbx5* in the forelimb field.

Conclusion

Previous studies of limb RA action proposed that RA acts instructively in both forelimb and hindlimb bud mesoderm to induce genes needed for limb anteroposterior and proximodistal patterning such as *Shh, Hoxb8, Hand2*, and *Meis1/2*. However, our findings indicate that RA signaling in limb mesoderm is dispensable for expression of these patterning genes in their normal locations and at normal levels, and our RA-reporter data using *RARE-lacZ, Cdx1*, and RARβ strongly support this critical point by demonstrating that RA activity is absent. We point out that our hypothesis is based upon the assumption that these are indeed very good markers of RA activity with well-characterized RAREs. Although previous studies indicate administration of excess RA can lead to induction of *Shh, Hoxb8, Hand2*, and *Meis1/2*, we suggest this is an abnormal response to a teratogenic dose of RA and does not reflect the normal function of RA; these genes have not been reported to possess bona fide RAREs but may contain DNA sequences loosely resembling a RARE that bind RARs weakly and function only during excess RA treatment.

Although our studies suggest that hindlimb patterning does not require RA, other studies on vitamin A deficient quails suggested that hindlimb patterning was affected [34]; we suggest that the quail findings were due to a general effect on embryonic health (maybe secondary to cardiac defects that occur in those embryos) as it is clear that a complete loss of RA in mouse *Raldh2* mutants leads to severe cardiac and body axis defects that prevent embryos from developing to the stage when hindlimbs arise. *Cyp26b1* expression in forelimb and hindlimb bud mesenchyme stimulates RA degradation and prevents distal limb teratogenesis which was suggested to be important for proximodistal patterning of the limb buds [14]. Our findings suggest that the function of *Cyp26b1* is not to provide a gradient of RA needed for patterning,

but to eliminate an RA signal unnecessary for limb patterning that is teratogenic for distal limb patterning; in the fetal testis, the function of *Cyp26b1* is simply to eliminate RA so that meiosis does not occur until after birth [35].

Our findings reported here suggest a different model of limb RA action that does not involve RA induction of limb genes, but which incorporates the concept of RA antagonism of FGF signaling. In our model, RA acts outside the forelimb field in the body axis in neuroectoderm [4], cardiac mesoderm [5], and intermediate mesoderm (reported here) to repress expression of *Fgf8* in order to prevent excessive secretion of FGF8 that enters the forelimb field and inhibits forelimb induction (Figure 4D). Thus, instead of RA counteracting distal FGF signaling emanating from the AER after forelimb budding has occurred, as originally proposed [11], our findings suggest that RA acts prior to forelimb induction to reduce FGF signaling in the developing trunk, thus providing an environment permissive for induction of forelimbs. This conclusion is firmly supported by our zebrafish studies demonstrating that an FGF receptor antagonist can rescue pectoral fin development in RA-deficient embryos. Further support comes from studies on the *Fgf8* promoter where we have demonstrated the existence of a RARE in vivo by ChIP analysis of mouse embryo chromatin, and where others have shown with cell line assays that the *Fgf8* RARE represses the major isoform (Fgf8b) when RAR and RA are both present [33]. We further suggest that limb proximodistal patterning either does not require a proximal signal or uses one distinct from RA.

Our findings also support a model in which RA signaling is required for induction of forelimbs but not hindlimbs based upon the observation that embryos lacking limb bud RA activity exhibit relatively normal hindlimb buds but small forelimb buds (Figure 4D). This temporal model is supported by previous studies demonstrating that *Raldh2* expression during somite stages 1-10 is present in presomitic mesoderm located just anterior to the border of *Fgf8* expression at the epiblast/neuroectoderm junction, but that *Raldh2* expression later retracts anteriorly (lost in presomitic mesoderm and newly formed somites) such that by the time hindlimb induction is occurring (23-28 somite stage) RA signaling does not reach the tailbud/neuroectoderm junction and is no longer required for posterior *Fgf8* regulation or somitogenesis [4]. We suggest that the role of RA in forelimb induction is part of a more fundamental event in which RA repression of *Fgf8* helps establish the trunk as a distinct region during a brief period of body axis extension when the primitive streak still exists. This regulatory event does not continue when the primitive streak gives way to the tailbud and the hindlimbs arise; in the tailbud another mechanism presumably exists to limit caudal *Fgf8* expression. Future studies on this unique temporal action of RA should shed more light on the signaling mechanisms underlying early organogenesis.

Experimental Procedures

Generation of *Raldh* Null Mutant Embryos

Raldh2$^{-/-}$ embryos [10] and *Raldh2*$^{-/-}$;*Raldh3*$^{-/-}$ double homozygous embryos [19] were previously described. All mouse studies conformed to the regulatory standards adopted by the Animal Research Committee at the Burnham Institute for Medical Research.

Rescue with a Physiological Dose of RA

Rescue of embryos by maternal dietary RA supplementation was performed as previously described [4] with low RA doses demonstrated to provide embryos an amount of RA in the normal physiological range [17]. For brief treatment, the final RA concentration was 0.1 mg/g food and treatment was from E6.75-E8.25. For extended treatment, an RA concentration of 0.1 mg/g food was used from E6.75-E7.75 followed by an RA concentration of 0.25 mg/g food

from E7.75-E8.5. For embryos analyzed at time points after RA treatment was ended, mice were returned to standard mouse chow until the point of analysis.

In situ Hybridization and Retinoic Acid Detection

Whole-mount in situ hybridization was performed as described previously; wild-type and mutant embryos were treated identically and stained for the same length of time [10]. The *RARE-lacZ* RA-reporter transgene, which places *lacZ* (encoding β-galactosidase) under the control of a RARE, was used to detect RA activity in embryos [18]; wild-type and mutant embryos were stained for the same length of time. Stained embryos were embedded in 3% agarose and sectioned at 30 μm with a vibratome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Tabin C, Wolpert L. Rethinking the proximodistal axis of the vertebrate limb in the molecular era. Genes & Development 2007;21:1433–1442. [PubMed: 17575045]
2. Duester G. Retinoic acid synthesis and signaling during early organogenesis. Cell 2008;134:921–931. [PubMed: 18805086]
3. Del Corral RD, Olivera-Martinez I, Goriely A, Gale E, Maden M, Storey K. Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension. Neuron 2003;40:65–79. [PubMed: 14527434]
4. Sirbu IO, Duester G. Retinoic acid signaling in node ectoderm and posterior neural plate directs left-right patterning of somitic mesoderm. Nature Cell Biol 2006;8:271–277. [PubMed: 16489341]
5. Sirbu IO, Zhao X, Duester G. Retinoic acid controls heart anteroposterior patterning by down-regulating *Isl1* through the *Fgf8* pathway. Dev Dyn 2008;237:1627–1635. [PubMed: 18498088]
6. Lu HC, Revelli JP, Goering L, Thaller C, Eichele G. Retinoid signaling is required for the establishment of a ZPA and for the expression of *Hoxb-8*, a mediator of ZPA formation. Development 1997;124:1643–1651. [PubMed: 9165113]
7. Stratford T, Horton C, Maden M. Retinoic acid is required for the initiation of outgrowth in the chick limb bud. Curr Biol 1996;6:1124–1133. [PubMed: 8805369]
8. Mic FA, Sirbu IO, Duester G. Retinoic acid synthesis controlled by *Raldh2* is required early for limb bud initiation and then later as a proximodistal signal during apical ectodermal ridge formation. J Biol Chem 2004;279:26698–26706. [PubMed: 15069081]
9. 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]
10. 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]
11. Mercader N, Leonardo E, Piedra ME, Martínez-A C, Ros MA, Torres M. Opposing RA and FGF signals control proximodistal vertebrate limb development through regulation of Meis genes. Development 2000;127:3961–3970. [PubMed: 10952894]

12. Azcoitia V, Araci LM, Martinez-A C, Torres M. The homeodomain protein Meis1 is essential for definitive hematopoiesis and vascular patterning in the mouse embryo. Dev Biol 2005;280:307–320. [PubMed: 15882575]
13. Mariani FV, Ahn CP, Martin GR. Genetic evidence that FGFs have an instructive role in limb proximal-distal patterning. Nature 2008;453:401–405. [PubMed: 18449196]
14. Yashiro K, Zhao X, Uehara M, Yamashita K, Nishijima M, Nishino J, Saijoh Y, Sakai Y, Hamada H. Regulation of retinoic acid distribution is required for proximodistal patterning and outgrowth of the developing limb. Dev Cell 2004;6:411–422. [PubMed: 15030763]
15. Gibert Y, Gajewski A, Meyer A, Begemann G. Induction and prepatterning of the zebrafish pectoral fin bud requires axial retinoic acid signaling. Development 2006;133:2649–2659. [PubMed: 16774994]
16. Niederreither K, Vermot J, Schuhbaur B, Chambon P, Dollé P. Embryonic retinoic acid synthesis is required for forelimb growth and anteroposterior patterning in the mouse. Development 2002;129:3563–3574. [PubMed: 12117807]
17. 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]
18. Rossant J, Zirngibl R, Cado D, Shago M, Giguère V. 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]
19. Molotkov A, Molotkova N, Duester G. Retinoic acid guides eye morphogenetic movements via paracrine signaling but is unnecessary for retinal dorsoventral patterning. Development 2006;133:1901–1910. [PubMed: 16611695]
20. Riddle RD, Johnson RL, Laufer E, Tabin C. *Sonic hedgehog* mediates the polarizing activity of the ZPA. Cell 1993;75:1401–1416. [PubMed: 8269518]
21. Logan M, Tabin CJ. Role of Pitx1 upstream of Tbx4 in specification of hindlimb identity. Science 1999;283:1736–1739. [PubMed: 10073939]
22. Sandell LL, Sanderson BW, Moiseyev G, Johnson T, Mushegian A, Young K, Rey JP, Ma JX, Staehling-Hampton K, Trainor PA. RDH10 is essential for synthesis of embryonic retinoic acid and is required for limb, craniofacial, and organ development. Genes & Development 2007;21:1113–1124. [PubMed: 17473173]
23. Subramanian V, Meyer BI, Gruss P. Disruption of the murine homeobox gene *Cdx1* affects skeletal identities by altering the mesodermal expression domains of *Hox* genes. Cell 1995;83:641–653. [PubMed: 7585967]
24. Houle M, Sylvestre JR, Lohnes D. Retinoic acid regulates a subset of Cdx1 function in vivo. Development 2003;130:6555–6567. [PubMed: 14660544]
25. Agarwal P, Wylie JN, Galceran J, Arkhitko O, Li C, Deng C, Grosschedl R, Bruneau BG. *Tbx5* is essential for forelimb bud initiation following patterning of the limb field in the mouse embryo. Development 2003;130:623–633. [PubMed: 12490567]
26. Mendelsohn C, Ruberte E, LeMeur M, Morriss-Kay G, Chambon P. Developmental analysis of the retinoic acid-inducible RAR-β2 promoter in transgenic animals. Development 1991;113:723–734. [PubMed: 1668276]
27. Marques SR, Lee Y, Poss KD, Yelon D. Reiterative roles for FGF signaling in the establishment of size and proportion of the zebrafish heart. Developmental Biology 2008;321:397–406. [PubMed: 18639539]
28. Minowada G, Jarvis LA, Chi CL, Neubuser A, Sun X, Hacohen N, Krasnow MA, Martin GR. Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed. Development 1999;126:4465–4475. [PubMed: 10498682]
29. Crossley PH, Minowada G, MacArthur CA, Martin GR. Roles for FGF8 in the induction, initiation, and maintenance of chick limb development. Cell 1996;84:127–136. [PubMed: 8548816]
30. Perantoni AO, Timofeeva O, Naillat F, Richman C, Pajni-Underwood S, Wilson C, Vainio S, Dove LF, Lewandoski M. Inactivation of FGF8 in early mesoderm reveals an essential role in kidney development. Development 2005;132:3859–3871. [PubMed: 16049111]

31. Fernandez-Teran M, Piedra ME, Simandl BK, Fallon JF, Ros MA. Limb initiation and development is normal in the absence of the mesonephros. Dev Biol 1997;189:246–255. [PubMed: 9299117]

32. Rong PM, Teillet MA, Ziller C, Le Douarin NM. The neural tube/notochord complex is necessary for vertebral but not limb and body wall striated muscle differentiation. Development 1992;115:657–672. [PubMed: 1425345]

33. Brondani V, Klimkait T, Egly JM, Hamy F. Promoter of FGF8 reveals a unique regulation by unliganded RARa. J Mol Biol 2002;319:715–728. [PubMed: 12054865]

34. Stratford T, Logan C, Zile M, Maden M. Abnormal anteroposterior and dorsoventral patterning of the limb bud in the absence of retinoids. Mech Dev 1999;81:115–125. [PubMed: 10330489]

35. Bowles J, Knight D, Smith C, Wilhelm D, Richman J, Mamiya S, Yashiro K, Chawengsaksophak K, Wilson MJ, Rossant J, Hamada H, Koopman P. Retinoid signaling determines germ cell fate in mice. Science 2006;312:596–600. [PubMed: 16574820]

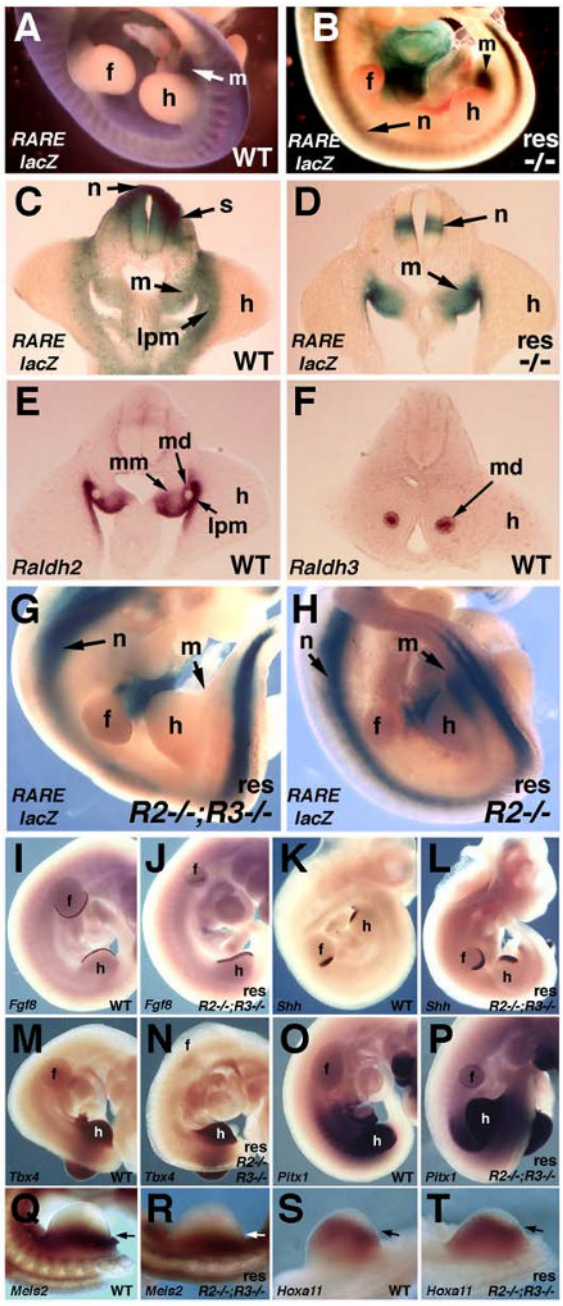

Figure 1.

RA is unnecessary for hindlimb budding and patterning. (A-B) *RARE-lacZ* expression in E10.5 wild-type (WT) and *Raldh2*$^{-/-}$ embryos rescued by brief RA treatment (res -/-); mutant forelimb is smaller than hindlimb which has RA activity nearby in mesonephros. (C-D), Transverse sections through the hindlimbs of the embryos shown in panels A and B. (E-F) *Raldh2* and *Raldh3* mRNA in transverse sections through wild-type hindlimbs demonstrates that *Raldh3* co-localizes with mesonephric RA activity. (G-H) *RARE-lacZ* expression in a rescued *Raldh2*$^{-/-}$;*Raldh3*$^{-/-}$ embryo compared to a rescued *Raldh2*$^{-/-}$ embryo demonstrates that the hindlimb develops without mesonephric RA. (I-T), E10.5 wild-type and rescued *Raldh2*$^{-/-}$;*Raldh3*$^{-/-}$ (*R2*-/-;*R3*-/-) embryos hybridized with probes for *Fgf8* (I-J), *Shh* (K-L),

Tbx4 (M-N), *Pitx1* (O-P); *Meis2* (Q-R); and *Hoxa11* (S-T); anterior to left, posterior to right. f, forelimb bud; h, hindlimb bud; lpm, lateral plate mesoderm; m, mesonephros; md, mesonephric duct; mm, mesonephric mesenchyme; n, neural tube; s, somite.

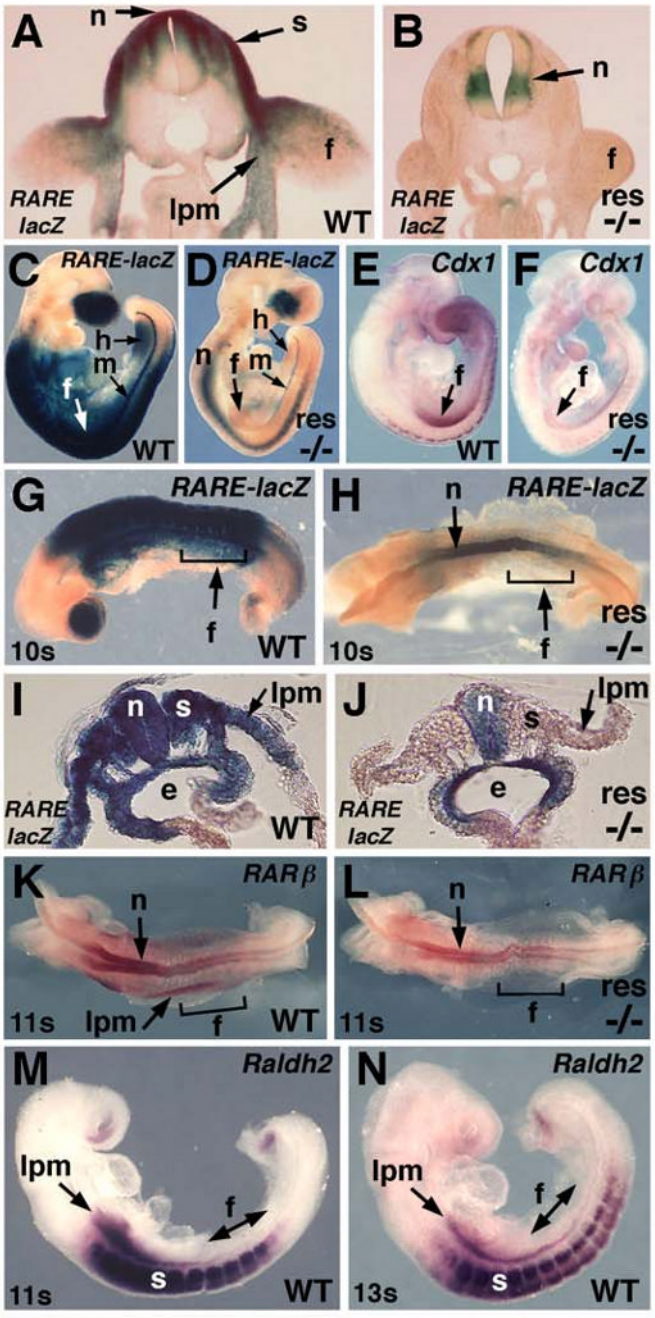

Figure 2.

Forelimb induction requires RA signaling in the body axis but not limb mesoderm. (A-B) Transverse sections through the forelimbs of the E10.5 embryos shown in Figure 1a-b. *RARE-lacZ* expression (C-D) and RA-responsive *Cdx1* mRNA (E-F) in E9.5 rescued *Raldh2*$^{-/-}$ embryos; wild-type and mutant embryos were stained for the same length of time here and in all other studies. (G-H) *RARE-lacZ* expression in E8.5 (10-somite) rescued *Raldh2*$^{-/-}$ embryo; in the mutant no RA activity is detected in somites or eye which normally express *Raldh2* but no other RA-synthesizing enzyme at this stage; brackets indicate the beginning of the forelimb field lying parallel to somites 6-10. (I-J) Transverse section through forelimb field of embryos shown in panels G and H showing no RA activity in limb-field lpm of mutant. (K-L) RARβ is

not expressed in lateral plate mesoderm of E8.5 (11-somite) rescued *Raldh2*$^{-/-}$ embryo. (M-N) *Raldh2* expression at 11-13 somite stages. e, endoderm; f, forelimb field; lpm, lateral plate mesoderm; n, neural tube; s, somite.

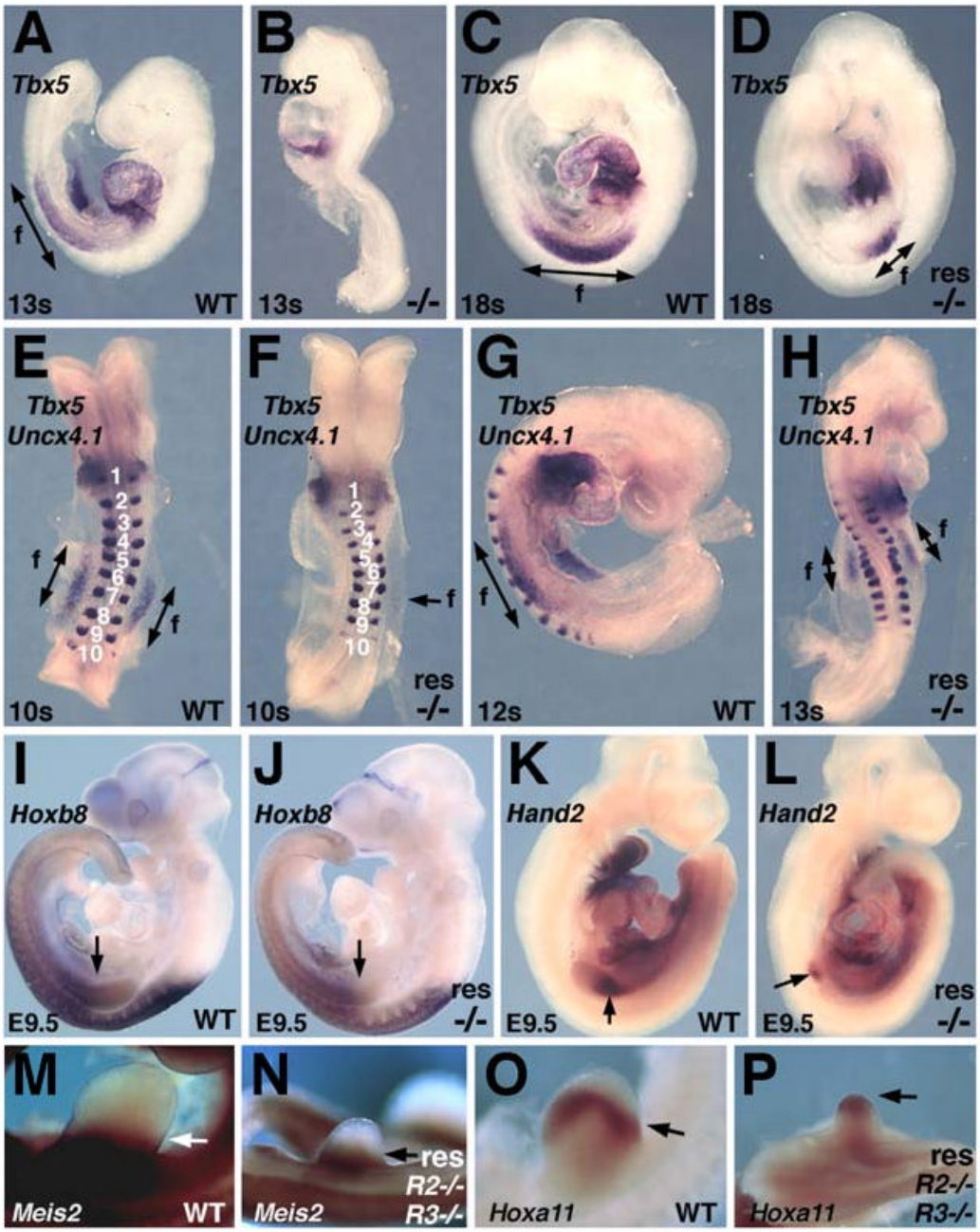

Figure 3.

RA is required for induction but not A-P patterning of forelimb buds. *Tbx5* mRNA in *Raldh2*$^{-/-}$ embryos that are unrescued (A-B) or rescued with brief RA treatment (C-D); note smaller forelimb field in rescued mutant compared to wild-type. (E-H) *Tbx5* and *Uncx4.1* mRNA double-staining in rescued *Raldh2*$^{-/-}$ embryos; note delay in *Tbx5* expression in rescued mutant. (I-J) *Hoxb8* mRNA in rescued *Raldh2*$^{-/-}$ embryo; note similar anteroposterior boundary of expression in rescued mutant and wild-type. (K-L) *Hand2* mRNA in rescued *Raldh2*$^{-/-}$ embryo; note small expression domain in rescued mutant. (M-N) *Meis2* mRNA and (O-P) *Hoxa11* mRNA in E10.5 wild-type and rescued *Raldh2*$^{-/-}$;*Raldh3*$^{-/-}$ embryos. f, forelimb field.

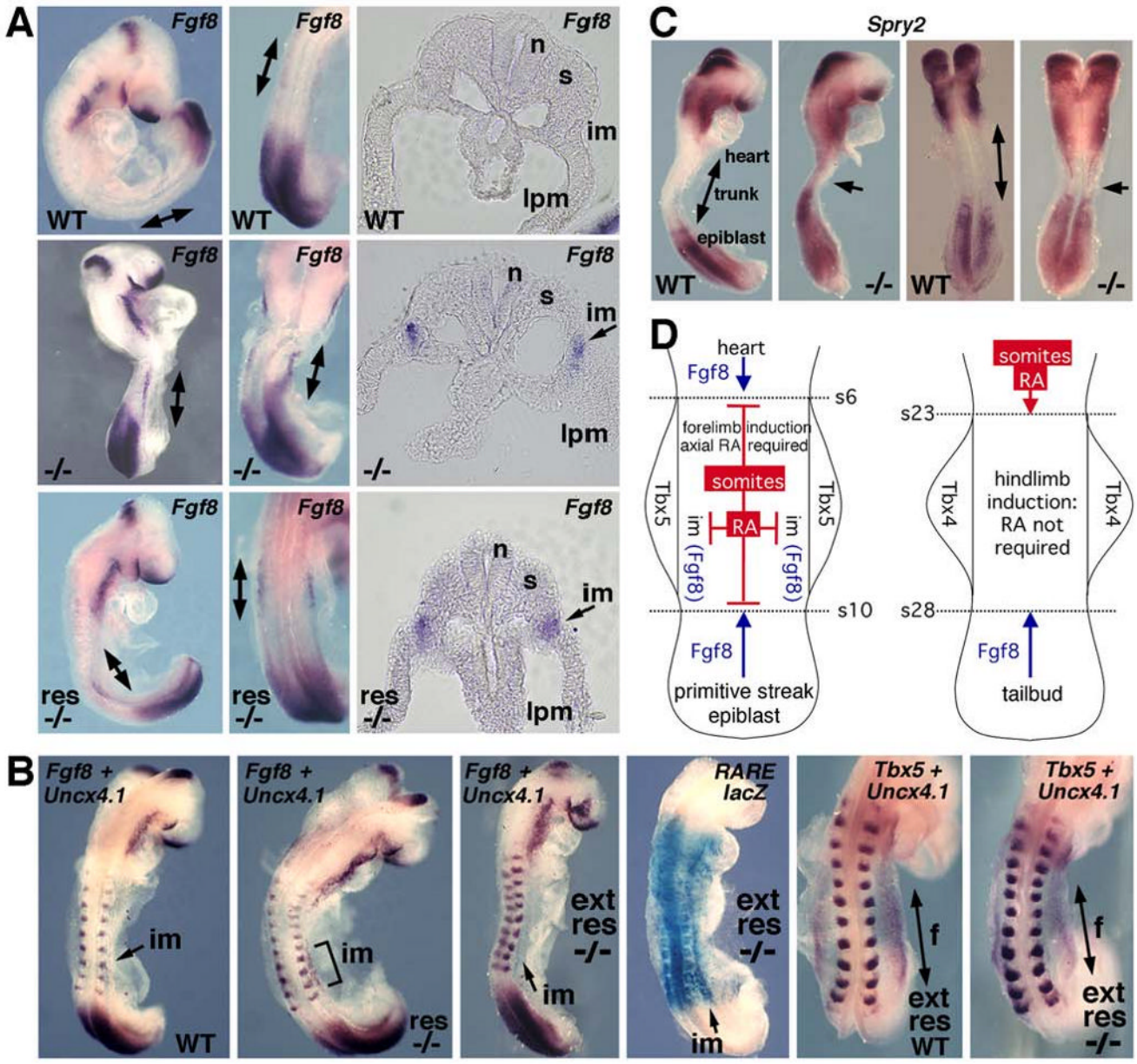

Figure 4.

Ectopic *Fgf8* expression near the forelimb field following loss of RA. (A) *Fgf8* mRNA in 13-somite embryos: wild-type (top panels), unrescued *Raldh2*$^{-/-}$ (middle panels), and rescued *Raldh2*$^{-/-}$ with brief RA treatment (bottom panels); in mutants note abnormal domain of *Fgf8* mRNA in the intermediate mesoderm adjacent to the forelimb field marked by double-arrow in whole-mount and arrow in transverse sections. (B) *Fgf8* and *Uncx4.1* (somite marker) expression in 10-somite *Raldh2*$^{-/-}$ embryos following brief RA rescue (res -/-) or extended RA rescue (ext res -/-); note loss of *Fgf8* mRNA in intermediate mesoderm following extended RA rescue and higher levels of *RARE-lacZ* expression showing that RA activity has been stimulated in intermediate mesoderm. Extended RA treatment also results in comparable *Tbx5* mRNA domains in the forelimb fields of 12-somite wild-type and *Raldh2*$^{-/-}$ embryos. (C) Expression of *Spry2* (a marker of FGF signaling) in 7-somite wild-type and unrescued

Raldh2$^{-/-}$ embryos; arrows in mutants point out expansion of FGF signaling into trunk domain where forelimbs develop. (D) Model for RA signaling based on studies presented here as well as previous findings [4,5] suggesting that RA acts in the body axis to repress *Fgf8* during the 1-10 somite stages to provide an environment permissive for forelimb induction; at the 23-28 somite stages RA signaling has retracted anteriorly and is not involved in hindlimb induction. e, endoderm; f, forelimb bud; h, hindlimb bud; im, intermediate mesoderm (mesonephros); lpm, lateral plate mesoderm; n, neural tube; s, somite.