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Report

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 of the limb field in the body axis during forelimb induction but that RA is unnecessary at later stages when hindlimb budding and patterning occur. 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 an 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 an 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 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 that appear relatively normal [8, 16]; given that 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

Given that *Raldh2*$^{-/-}$ embryos fail to grow beyond E8.5, mutants were rescued with 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 to E8.25) and then, analyzed at E10.5, display RA activity in the neural tube and in the mesonephros adjoining the proximal hindlimb bud (Figures 1A–1D; 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 (Figures 1E and 1F). *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. Given that *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 to E8.25. Hindlimb buds of a normal size were

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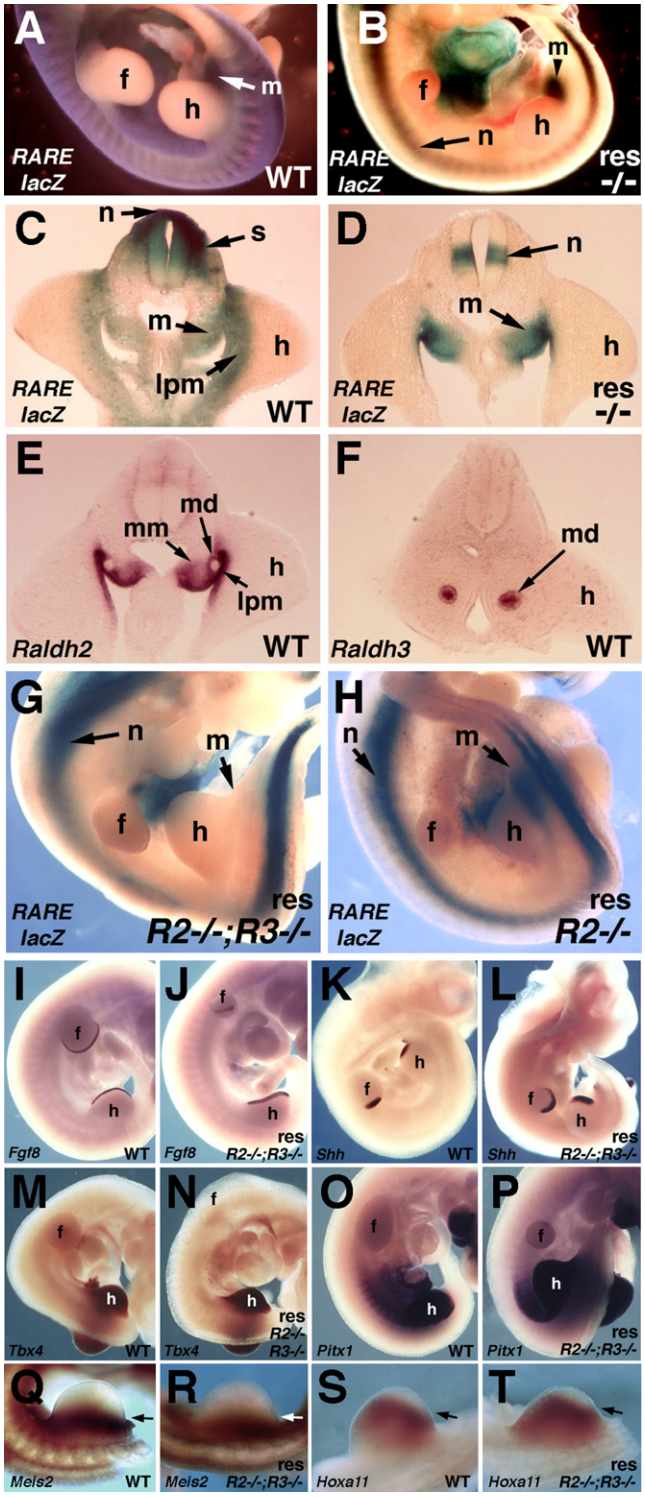

Figure 1. RA Is Unnecessary for Hindlimb Budding and Patterning

(A and 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 and D) Transverse sections through the hindlimbs of the embryos shown in (A) and (B).

(E and F) *Raldh2* and *Raldh3* mRNA in transverse sections through wild-type hindlimbs demonstrates that *Raldh3* colocalizes with mesonephric RA activity.

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 (Figures 1G and 1H). 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 available online 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 (Figures 1I, 1J, and S2) and normal expression of *Shh* in the zone of polarizing activity (ZPA) needed for anteroposterior patterning (Figures 1K, 1L, and S2). We also detected normal hindlimb expression of *Tbx4* and *Pitx1*, which function in hindlimb induction (Figures 1M–1P). 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 (Figures 1Q–1T). Moreover, although *Meis2* expression is proposed to be dependent on RA from the flank [11], our data do not support this hypothesis. Given that 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 in the Limb Mesoderm

Although we find no requirement for RA during hindlimb budding or patterning, forelimb buds do appear to require RA

(G and 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 and J), *Shh* (K and L), *Tbx4* (M and N), *Pitx1* (O and P); *Meis2* in hindlimbs (Q and R); and *Hoxa11* in hindlimbs (S and 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.

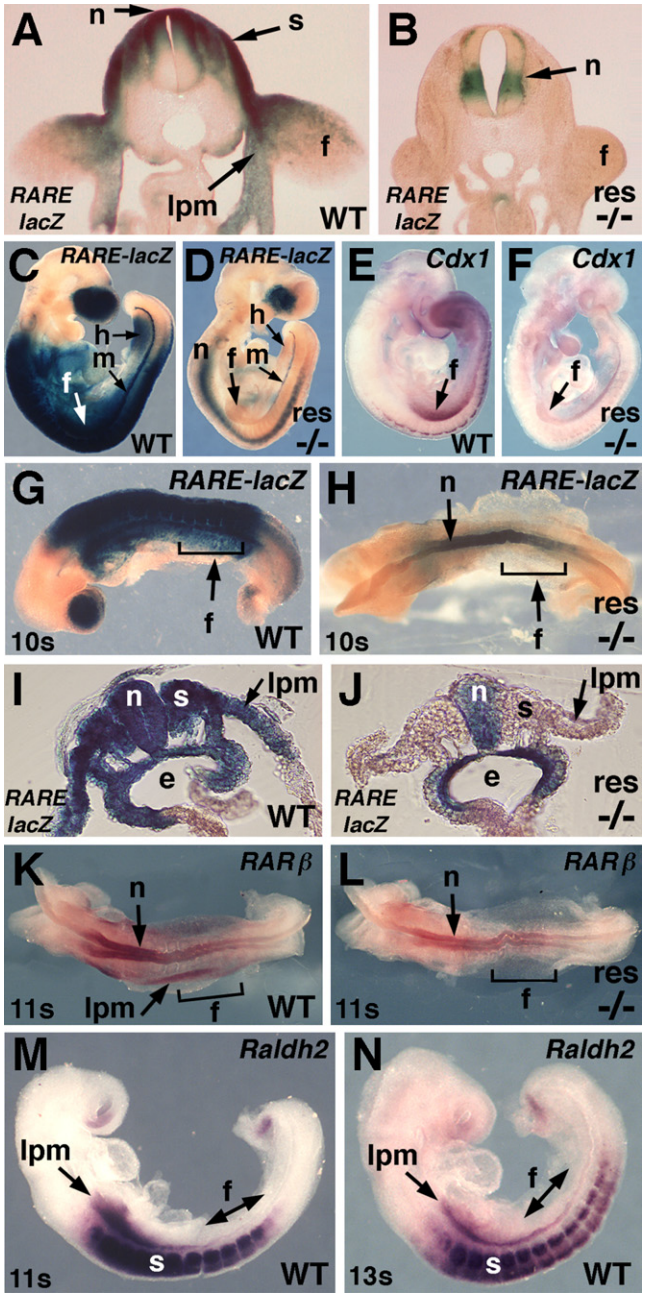

Figure 2. Forelimb Induction Requires RA Signaling in the Body Axis, but Not in the Limb Mesoderm

(A and B) Transverse sections through the forelimbs of the E10.5 embryos shown in Figures 1A and 1B.

(C–F) *RARE-lacZ* expression (C and D) and RA-responsive *Cdx1* mRNA (E and 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 and 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 and J) Transverse section through forelimb field of embryos shown in (G) and (H), showing no RA activity in limb field lpm of mutant.

(K and L) RARβ is not expressed in lateral plate mesoderm of E8.5 (11 somite) rescued *Raldh2*$^{-/-}$ embryo.

(M and N) *Raldh2* expression at 11–13 somite stages.

e, endoderm; f, forelimb field; lpm, lateral plate mesoderm; n, neural tube; s, somite.

for normal development. We further investigated RA signaling during budding of rescued forelimbs by 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 (Figures 2A and 2B; n = 6/6); RA activity was also not present in the small forelimb bud at E9.25, when it is first morphologically detectable (Figures 2C, 2D, and 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 that they lack RA activity (Figures 2E, 2F, and 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 (Figures 2G–2J; 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 in lateral plate mesoderm (Figures 2K and 2L; 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 because 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 (Figures 2M and 2N). Thus, even though RA generated by *Raldh2* in wild-type embryos (1) is normally present in somites and lateral plate mesoderm fated to become limb and (2) can induce *Cdx1* in limb mesoderm, RA is not acting there to initiate forelimb development; 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 (13 s) stage (Figures 3A and 3B; n = 2/2). Rescued *Raldh2*$^{-/-}$ embryos exhibited *Tbx5* expression at 18 s, although the size of the forelimb field was much smaller than normal (Figures 3C and 3D; 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 10 s (Figures 3E and 3F; n = 4/4) but that a small domain of *Tbx5* expression was observed at 13 s (Figures 3G and 3H; n = 3/3). These

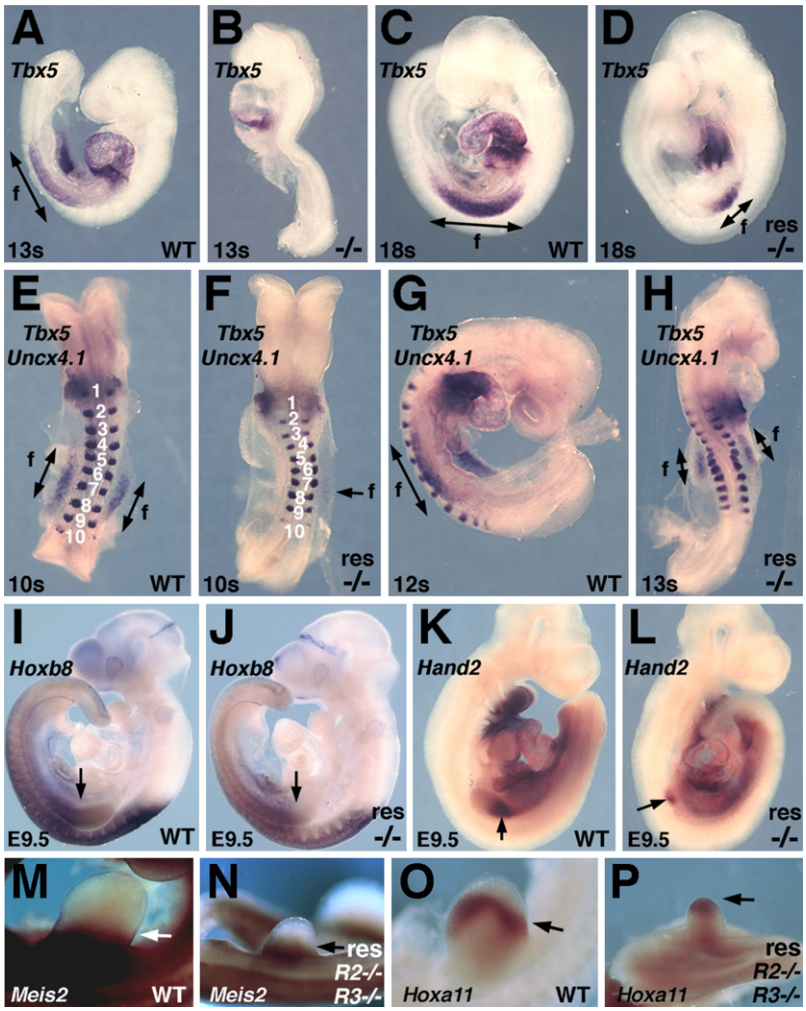

Figure 3. RA Is Required for Induction, but Not A-P or P-D Patterning, of Forelimb Buds

(A–D) *Tbx5* mRNA in *Raldh2*$^{-/-}$ embryos that are unrescued (A and B) or rescued with brief RA treatment (C and 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 and J) *Hoxb8* mRNA in rescued *Raldh2*$^{-/-}$ embryo; note similar anteroposterior boundary of expression in rescued mutant and wild-type.

(K and L) *Hand2* mRNA in rescued *Raldh2*$^{-/-}$ embryo; note small expression domain in rescued mutant.

(M–P) (M and N) *Meis2* mRNA in forelimbs and (O and P) *Hoxa11* mRNA in forelimbs of E10.5 wild-type and rescued *Raldh2*$^{-/-}$;*Raldh3*$^{-/-}$ embryos.

f, forelimb field.

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, given that we do not detect RA in the cells where *Tbx5* is induced (Figures 2H, 2J, and 2L).

We find that forelimbs lacking RA activity still express *Shh* even though expression appears distally rather than posteriorly as normal (Figures 1L and 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 (Figures 3I, 3J, and 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 (Figures 3K, 3L, and 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 (Figures 3M–3P). Thus, taken together with our findings above, which demonstrate 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 of 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 that use 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 downregulation of *Shh* and *Hoxb8*. However, these chemicals may have nonspecific effects because RXRs are heterodimer partners for at least 10 nuclear receptors other than RARs, and disulfiram inhibits the enzymatic activity of many, if not all, of 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 that RA does not play an instructive role in forelimb development but, rather, plays 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 them, which is 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 by 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 (Figure 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 of 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. These findings suggest that the regulatory mechanisms for induction are conserved between zebrafish pectoral fin and tetrapod limb.

In order to determine whether 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 hr [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 further pursued the hypothesis that *Fgf8* expression may normally inhibit, rather than stimulate, forelimb induction. Here, we found that wild-type mouse embryos at 10–13 s have no *Fgf8* expression detectable in the intermediate mesoderm (Figure 4A; n = 7/7). However, unrescued *Raldh2*$^{-/-}$ embryos at 12–13 s 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 11–13 s 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 10–11 s 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 by using chromatin immunoprecipitation (ChIP). We show that a conserved RARE near the *Fgf8* promoter binds all three RAR isoforms (RARa, RARb, and RARg) by using ChIP with RAR antibodies and chromatin from 5 somite mouse embryos (Figure S6). This finding is important because it shows that the mouse *Fgf8* gene can bind RAR in vivo just prior to induction of *Tbx5* in the forelimb field.

Conclusions

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 induction of these patterning genes, and our RA reporter data from *RARE-lacZ*, *Cdx1*, and RARβ strongly support this critical point; 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 that administration of excess RA can lead to induction of *Shh*, *Hoxb8*, *Hand2*, and *Meis1/2*, we suggest that this is an abnormal response to a teratogenic dose of RA and does not reflect the normal function of RA. Other studies suggested that RA degradation stimulated by *Cyp26b1* expression in limbs may be important for proximodistal patterning of the limb buds [14], but 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 that is teratogenic for distal limb patterning. Thus, limb proximodistal patterning either does not require a proximal signal or uses one distinct from RA. Our findings reported here suggest a different model of limb RA action that does not involve RA induction of limb genes but that

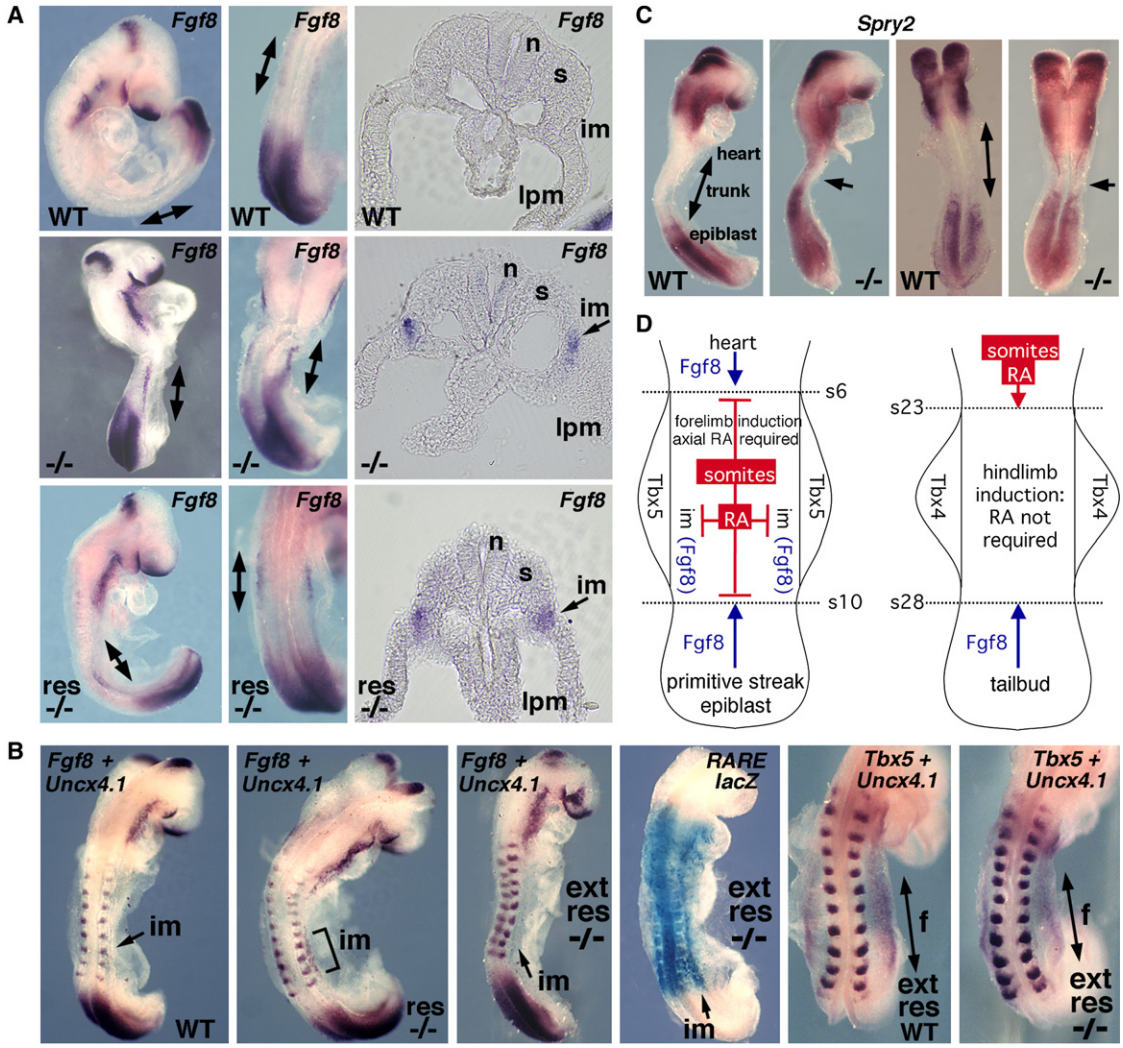

Figure 4. Ectopic *Fgf8* Expression Near the Forelimb Field following Loss of RA

(A) *Fgf8* mRNA in 13 somite embryos: wild-type (top), unrescued *Raldh2*$^{-/-}$ (middle), and rescued *Raldh2*$^{-/-}$ with brief RA treatment (bottom). In mutants, note the 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 the 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.

incorporates the concept of RA antagonism of FGF signaling in the developing trunk to provide 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. 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). Our temporal model is supported by previous studies demonstrating that *Raldh2* expression in presomitic mesoderm retracts anteriorly as development proceeds such that, by the time hindlimb induction is occurring, RA signaling does not reach the tail bud 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. 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 to E8.25. For extended treatment, an RA concentration of 0.1 mg/g food was used from E6.75 to E7.75 followed by an RA concentration of 0.25 mg/g food from E7.75 to 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.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and six figures and can be found with this article online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)01058-6.

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