Cell Reports
Article

Antagonism between Retinoic Acid and Fibroblast Growth Factor Signaling during Limb Development

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http://dx.doi.org/10.1016/j.celrep.2013.03.036

SUMMARY

The vitamin A metabolite retinoic acid (RA) provides patterning information during vertebrate embryogenesis, but the mechanism through which RA influences limb development is unclear. During patterning of the limb proximodistal axis (upper limb to digits), avian studies suggest that a proximal RA signal generated in the trunk antagonizes a distal fibroblast growth factor (FGF) signal. However, mouse and zebrafish genetic studies suggest that loss of RA suppresses forelimb initiation. Here, using genetic and pharmacological approaches, we demonstrate that limb proximodistal patterning is not RA dependent, thus indicating that RA-FGF antagonism does not occur along the proximodistal axis of the limb. Instead, our studies show that RA-FGF antagonism acts prior to limb budding along the anteroposterior axis of the trunk lateral plate mesoderm to provide a patterning cue that guides formation of the forelimb field. These findings reconcile disparate ideas regarding RA-FGF antagonism and provide insight into how endogenous RA programs the early embryo.

INTRODUCTION

Investigation of the signaling mechanisms underlying limb development has served as a paradigm for understanding general principles that govern embryogenesis. Many different signaling molecules converge to generate new tissues during growth and patterning of the limb (Rabinowitz and Vokes, 2012). Retinoic acid (RA), the active metabolite of vitamin A (retinol), is generated in specific tissues during embryogenesis and regulates many developmental processes, including limb formation, by serving as a ligand for nuclear RA receptors (RARs) (Duester, 2008). The mechanism through which RA controls limb development has been a topic of considerable debate ever since it was first reported that RA treatment of chick limbs could alter limb anteroposterior patterning (Tickle et al., 1982). In this case, RA treatment was found to merely mimic the action of sonic hedgehog (*Shh*) and did not provide the endogenous cue for anteroposterior patterning as originally suggested (Noji et al., 1991; Wanek et al., 1991; Litingtung et al., 2002).

Thinking has evolved over the past few years to a model in which RA antagonism of fibroblast growth factor (FGF) signaling has been proposed to control limb proximodistal patterning (Tabin and Wolpert, 2007). Patterning along the proximodistal axis of the vertebrate limb has been suggested to be controlled by opposing diffusible signals, with RA functioning as a proximal signal and FGF acting as a distal signal (Mercader et al., 2000). Chick forelimbs or hindlimbs ectopically exposed to RA or FGF8, or to antagonists of RAR or FGF receptor, display proximodistal limb patterning fate changes that either expand or contract expression of the proximal limb markers *Meis1* and *Meis2* (Mercader et al., 2000). Although *Meis* genes have not yet been demonstrated to be necessary for proximodistal patterning of vertebrate limbs, the *Drosophila* homolog is required for proximodistal patterning of fly limbs (Mercader et al., 1999). During normal limb proximodistal patterning, mouse genetic loss-of-function studies have verified a requirement for FGF8 or other distal FGF signals from the apical ectodermal ridge (AER) to control cell fate specification and survival to drive normal limb outgrowth and to restrict *Meis1* and *Meis2* expression to the proximal limb (Mariani et al., 2008). In contrast, mouse *Rdh10* mutants (Sandell et al., 2007) and *Raldh2* (*Aldh1a2*) mutants (Zhao et al., 2009) lacking RA synthesis have been unable to verify that endogenous RA functions as a proximal limb signal. However, mouse and zebrafish RA loss-of-function mutations disrupt initiation of forelimb development, although hindlimb development is not affected (Begemann et al., 2001; Grandel et al., 2002; Sandell et al., 2007; Zhao et al., 2009; Cunningham et al., 2011a).

Recent studies using recombinant heterotopic chick limb transplantations provided a further indication that RA may be needed for proximodistal patterning of both forelimbs and hindlimbs (Cooper et al., 2011; Roselló-Díez et al., 2011). While these studies report the ability of RA treatment to reprogram distal limb mesenchyme within a short time window to a proximal fate (Roselló-Díez et al., 2011) and to maintain early limb mesenchyme in a *Meis1*+ proximal fate alongside Wnt and FGF treatment (Cooper et al., 2011), they do not address a requirement for endogenous RA in proximal limb mesenchyme. RA gain-of-function effects on *Meis1/2* could represent disruption to

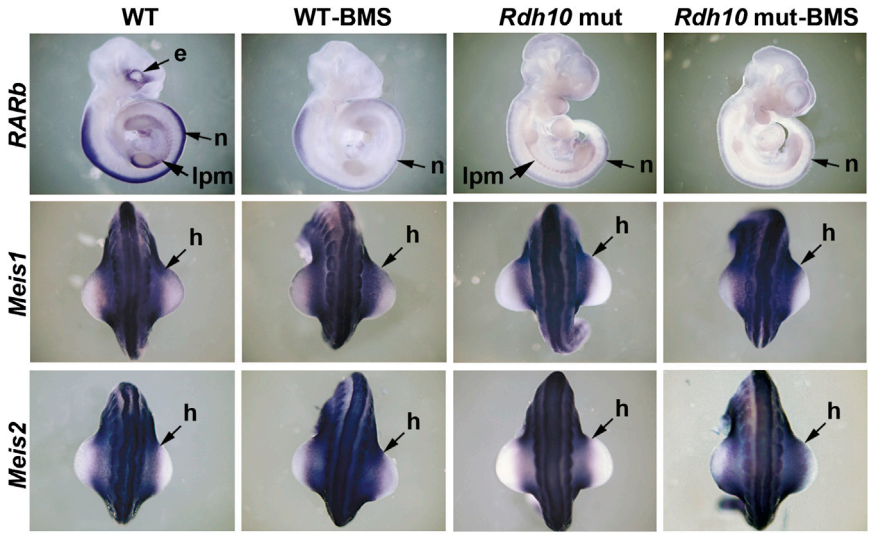

Figure 1. *Meis1/2* Expression Is Maintained During Limb Proximodistal Patterning following Loss of RA Signaling

Shown are in situ hybridization results for *Meis1*, *Meis2,* and *RARb* expression in E10.25 wild-type (WT) and *Rdh10* mutant (*Rdh10* mut) embryos cultured for 6 hr in DMEM/F-12 medium ± 10 μM BMS493; eye (e), neural (n), lateral plate mesoderm (lpm), and proximal hindlimb bud (h) expression are indicated. See also Figure S1.

specific FGF signaling functions that are already known to restrict *Meis1/2* expression in the distal limb from mouse genetic studies (Mariani et al., 2008), a conclusion not previously accounted for by the chick studies (Cooper et al., 2011; Roselló-Díez et al., 2011).

Here, we take a comprehensive genetic and complementary pharmacological approach to analyze limb development through initiation and patterning phases to consolidate conflicting data concerning the function of RA during limb development. We show that RA signaling is not required for limb proximodistal patterning, thus calling into question a role for RA-FGF antagonism during limb development. However, we provide genetic evidence that RA-FGF antagonism does occur during limb development but only along the trunk lateral plate mesoderm prior to forelimb budding to permit correct spatiotemporal induction of *Tbx5* needed for forelimb initiation. Thus, our studies show that RA controls limb development in a manner much different than that originally envisioned, and we provide insights into the biological function of RA-FGF antagonism.

RESULTS

Limb Proximal Identity Is Maintained following Combined Genetic and Pharmacologic Abrogation of RA Signaling

Our genetic analysis incorporates use of two key RA-signaling mouse mutants. The *Raldh2*$^{-/-}$ knockout lacks RA activity in both mesoderm and neuroectoderm (based upon the *RARE-lacZ* RA-reporter transgene), resulting in a failure to develop beyond embryonic day 8.75 (E8.75), but this mutant can be used to examine axial patterning of lateral plate mesoderm that gives rise to the forelimb field at E8.5 (Zhao et al., 2009). The ENU-induced *Rdh10*$^{trex/trex}$ mutant survives through limb-patterning stages from E10.5 to E14.5. *trex* contains a point mutation that generates a form of RDH10 lacking detectable enzyme activity, and *trex* embryos lack detectable RA activity (*RARE-lacZ* expression) in limb mesoderm although RA activity remains in posterior neuroectoderm, likely due to another retinol-metabolizing enzyme; thus, *trex* is essentially a null mutant (Sandell et al., 2007; Cunningham et al., 2011a). The *Rdh10*$^{-/-}$ knockout mouse phenotype was suggested be more severe than *trex* with less survival beyond E10.5 (Rhinn et al., 2011; Sandell et al., 2012), but this may be explained by strain differences (Rhinn et al., 2011). Here, we used *trex* mutants referred to as *Rdh10* mutants.

We analyzed expression of the proximal limb markers *Meis1* and *Meis2* in *Rdh10* mutants lacking *RARE-lacZ* expression in limb mesoderm and found no deficit in proximal limb expression at E10.5 for either gene (Figure S1). To eliminate the possibility that *Meis1/2* genes are induced by residual RA signaling that may still persist in limb field mesoderm of *Rdh10* mutants, we cultured E10.25 wild-type and *Rdh10* mutant embryos for 6 hr with the pan-RAR antagonist BMS493 at 10 μM (Germain et al., 2002). Previous studies in chick embryos treated with RAR antagonist-loaded beads (including BMS493) were performed at an equivalent stage (HH20) and culture time, although at much higher concentration (6 mM), leading to the conclusion that RA is actively needed at this stage for proximal limb expression of *Meis1/2* (Mercader et al., 2000; Roselló-Díez et al., 2011). We utilized BMS493 at 10 μM as this has previously been shown to downregulate RA signaling in mouse embryos (Wendling et al., 2000; Chen et al., 2007), and this concentration was nontoxic compared to 100 μM BMS493, which caused tissue necrosis during culture (data not shown). *RARb,* an established RA target gene (Mendelsohn et al., 1991), was already strongly downregulated in control *Rdh10* mutants versus control wild-type embryos (n = 2/2), but *Meis1/2* expression was unchanged (n = 3/3 for *Meis1*; n = 3/3 for *Meis2*) (Figure 1). BMS493 treatment of wild-type embryos resulted in strong *RARb* downregulation in lateral plate mesoderm, neural tube, and eye (n = 3/3), but *Meis1/2* expression remained normal in lateral plate mesoderm and proximal limb (n = 2/2 for *Meis1*; n = 5/5 for *Meis2*). Without BMS493 treatment, *Rdh10* mutant embryos displayed residual *RARb* expression in the interlimb lateral plate mesoderm (n = 2/2), but treatment with BMS493 eliminated this residual expression (n = 3/3). In contrast, *Rdh10* mutants treated with BMS493 maintained normal expression of *Meis1/2* in the proximal limb (n = 2/2 for *Meis1*; n = 3/3 for *Meis2*) (Figure 1). Thus, BMS493 nullification of RAR nuclear receptors in *Rdh10* mutants that already have a severe loss of RA synthesis provides conclusive evidence that RA signaling is unnecessary to control *Meis1/2* limb expression.

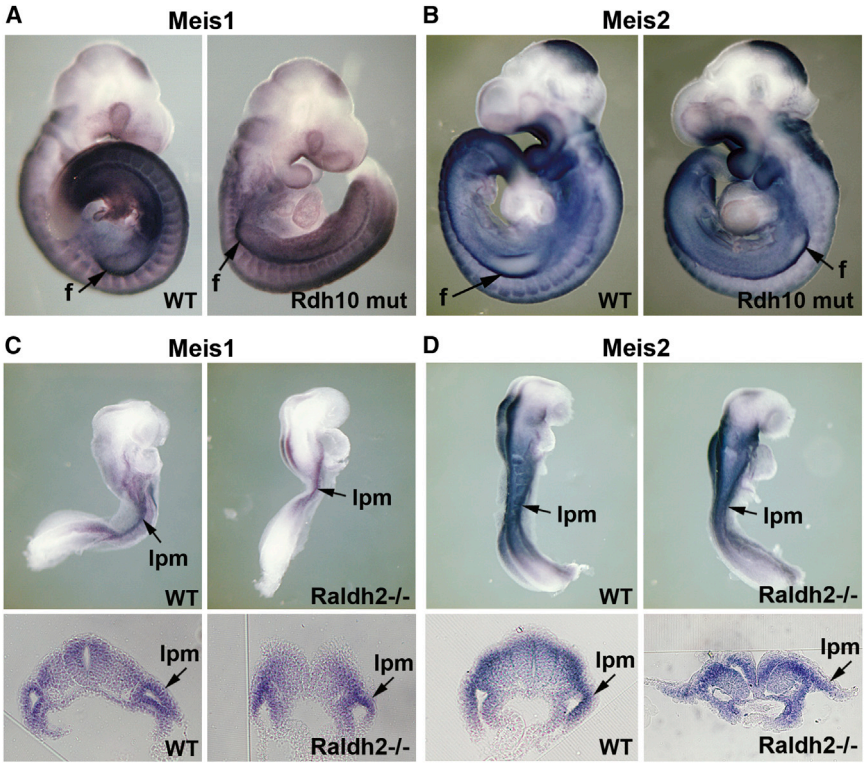

Figure 2. *Meis1/2* Expression Is Established during Limb Field Prepatterning in the Absence of RA Signaling

(A–D) In situ hybridization for *Meis1* and *Meis2* prior to and during limb development.

(A and B) *Meis1* and *Meis2* expression at E9.5 in wild-type and *Rdh10* mutant embryos; forelimb bud (f) expression is indicated.

(C and D) *Meis1* and *Meis2* expression prior to budding at E8.5 in wild-type and *Raldh2*$^{-/-}$ embryos. Lower panels represent transverse sections through the presumptive forelimb field revealing the lateral plate mesoderm (lpm).

See also Figure S2.

RA Signaling Is Not Required to Establish *Meis1/2* Expression during Limb Field Prepatterning

RA signaling is first observed in lateral plate mesoderm at E7.5 when *RARE-lacZ* expression is first detected (Rossant et al., 1991). As *Rdh10* mutants lack RA activity (*RARE-lacZ* expression) in lateral plate mesoderm at early stages prior to limb budding (Cunningham et al., 2011a), we also analyzed *Meis1/2* at E9.5 to look for a potential role in RA controlling the earliest stages of limb proximodistal patterning. *Meis1/2* expression was maintained similar to wild-type in E9.5 *Rdh10* mutant proximal forelimbs shortly after budding (n = 9/9) (Figures 2A and 2B) and in lateral plate mesoderm of the prebudding hindlimb field (n = 9/9) (Figure S2). In order to examine *Meis1/2* expression at E8.5, we utilized *Raldh2*$^{-/-}$ embryos lacking *RARE-lacZ* expression in all trunk and caudal tissues; *Meis1/2* expression was expressed robustly throughout the lateral plate mesoderm of *Raldh2*$^{-/-}$ embryos similar to wild-type (n = 13/13) (Figures 2C and 2D). Thus, in the absence of regulation by RA, we postulate that *Meis1/2* expression in lateral plate mesoderm from its onset prior to limb development until its appearance in the proximal limb represents a default proximal limb state.

Demonstration of *RARE-lacZ* Sensitivity to Subnanomolar RA Levels

Previous studies on *Rdh10* and *Raldh2* mutants indicated a lack of RA activity in lateral plate mesoderm based upon the absence of *RARE-lacZ* RA-reporter transgene expression (Sandell et al., 2007; Zhao et al., 2009). However, the sensitivity of *RARE-lacZ* has been questioned. To quantify the sensitivity of *RARE-lacZ*, we cultured wild-type and *Rdh10* E8.5 embryos in a series of low RA concentrations. Treatment of *Rdh10* mutants with as little as 0.25 nM induced *RARE-lacZ* in lateral plate mesoderm, intermediate mesoderm, somites, and neural tube (n = 2/2), whereas treatment with 1 nM or 2.5 nM showed additional *RARE-lacZ* induction in the head (n = 3/3) (Figure 3; Figure S3). The RA activity we detect at 0.25 nM is at the level of the dissociation constant (k_D) of RA with the three RA receptors (0.2–0.7 nM) (Allenby et al., 1993), demonstrating very high sensitivity of the transgene. In comparison, the endogenous RA concentration in mouse E10.5 proximal hindlimbs is reported to be 30 nM (Horton and Maden, 1995). Control *Rdh10* mutant embryos displayed no *RARE-lacZ* activity in the lateral plate mesoderm, typical of uncultured mutants (Figure 3; Figure S3). This demonstration of substantial (if not complete) loss of limb field RA signaling in *Rdh10* mutants, which we additionally nullified with an RAR antagonist (Figure 1), would be expected to yield significant observable reductions of *Meis1/2* expression if RA was an endogenous proximal signal in limb proximodistal patterning.

Defective Forelimb Initiation in *Rdh10* Mutants Relative to Loss of RA-FGF Antagonism in the Adjacent Second Heart Field

Next, we turned to the investigation of forelimb hypoplasticity reported for *Rdh10* mutants (Sandell et al., 2007). *Tbx5* expression in lateral plate mesoderm of the forelimb field, normally induced at the eight-somite stage and essential for forelimb specification (Agarwal et al., 2003), totally fails in *Raldh2*$^{-/-}$ mutants that display no sign of forelimb initiation (Zhao et al., 2009). Here, *Rdh10* mutants at 10–15 somites similarly displayed no forelimb *Tbx5* expression (Figure 4A; n = 5/5). However, at 16–19 somites we did observe *Tbx5* induction (Figure 4A; n = 4/4). Furthermore, in *Rdh10* mutants, the anterior-posterior length of the *Tbx5* domain was greatly reduced relative to somite position (Figure 4A). Since *Tbx5* demarcates the domain destined for *Fgf10* upregulation and forelimb budding (Agarwal et al., 2003), the observed reduction in *Tbx5* expression correlates with the consequent stunted forelimb, which is reduced in size in terms of both its anteroposterior and proximodistal length.

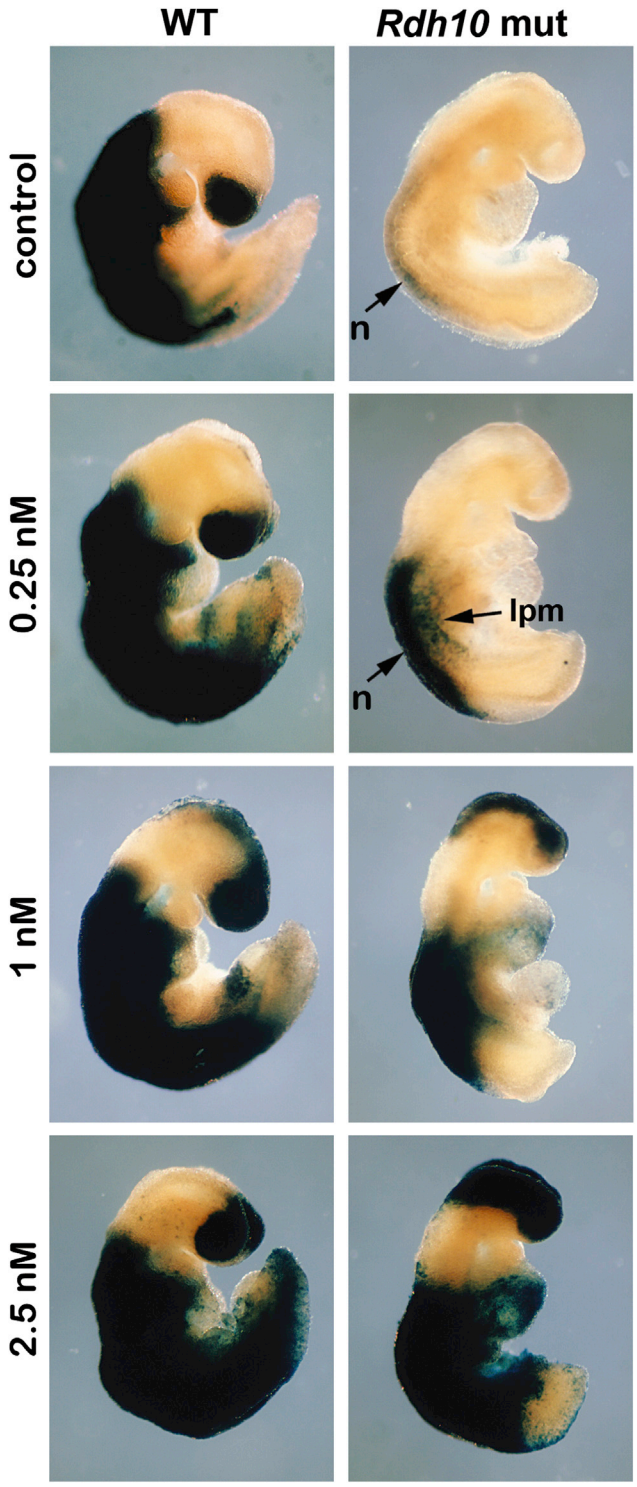

Figure 3. The *RARE-lacZ* Reporter Transgene Is Sensitive to RA Signaling Down to 0.25 nM RA

Wild-type (WT) and *Rdh10* mutant (*Rdh10* mut) E8.5 embryos stained with X-gal to detect *RARE-lacZ* RA signaling activity following culture for 12 hr in DMEM/F-12 medium (control) or DMEM/F-12 plus 0.25 nM, 1 nM, or 2.5 nM RA. Neural (n) and lateral plate mesoderm (lpm) expression is indicated. See also Figure S3.

We suggest this reduction in anteroposterior length precludes and impedes sufficient proximodistal growth and patterning due to the inherently smaller AER. Similarly, *TBX5* haploinsufficiency in humans (Holt-Oram syndrome) causes both forearm shortening and subsequent patterning defects, and left-right forelimb asymmetry (left smaller than right) is observed in Holt-Oram individuals (Bruneau et al., 2001) and here in mouse *Rdh10* mutants (n = 17/20; also see Figure S1). Therefore, the observed *Tbx5* deficiency in *Rdh10* mutants is a sufficient and likely explanation for the stunted forelimb and consequential patterning defects.

We next examined FGF8 signaling in *Rdh10* mutant embryos, since *Raldh2*$^{-/-}$ embryos that completely lack RA activity display two fronts of ectopic *Fgf8* expression that encroach into the forelimb field, one emanating from the heart (Ryckebusch et al., 2008; Sirbu et al., 2008) and another from the caudal progenitor zone (CPZ) (Zhao et al., 2009). Immediately prior to *Tbx5* induction and during early lateral plate *Tbx5* expression (six-, eight-, and ten-somite stages), *Rdh10* mutants displayed only a single front of ectopic *Fgf8* expression, from the heart alone, which moved closer to the anterior portion of the forelimb domain (Figure 4B; n = 7/7). *Rdh10* mutants from six somites onward retained a normal CPZ *Fgf8* expression domain and anterior boundary relative to the node (Figure 4B; n = 7/7). Simultaneously, *Spry2*, a negative regulator of FGF signaling induced by FGF8-ERK activity (Minowada et al., 1999), was found to be expressed ectopically in the trunk and forelimb field posterior to the heart in eight- to nine-somite *Rdh10* mutants (Figure 4C; n = 3/3). This intriguing difference in ectopic *Fgf8* expansion between *Raldh2*$^{-/-}$ and *Rdh10* mutants led us to more closely examine the distribution of RA activity at this time. *Rdh10* mutant *RARE-lacZ* expression was initially restricted to the posterior neural tube at six- to seven-somite stages (and intermediate mesoderm at 10- to 15-somite stages) reaching down to the CPZ *Fgf8* expression domain, but *RARE-lacZ* was absent from the dorsal heart where it is normally present (Figures 4D and 4E; n = 10/10). Thus, compared to *Raldh2*$^{-/-}$ embryos, *Rdh10* mutants display only one front of ectopic *Fgf8* expression (emanating from the heart) and concomitantly display a less severe disruption to forelimb *Tbx5* expression (delayed and reduced versus absent).

RA Controls Forelimb Initiation and Second Heart Field Expansion Independently

Isl1, which is required for proliferation of cardiac progenitors, is a highly conserved marker of second heart field progenitors (Cai et al., 2003; Stolfi et al., 2010). FGF8 is required for *Isl1* expression and second heart field expansion in mammals (Ilagan et al., 2006; Park et al., 2006), and FGF signaling also induces *Isl1* in primitive chordates (Davidson et al., 2006). *Rdh10* mutants, which exhibit ectopic cardiac *Fgf8* expression as shown above, displayed ectopic posterior expansion of *Isl1* extending from cardiac lateral plate mesoderm into the forelimb lateral plate domain (Figure 5A; n = 6/6). These results are similar to previously analyzed *Raldh2*$^{-/-}$ embryos that display ectopic cardiac *Isl1* expression and a distended heart phenotype (Ryckebusch et al., 2008; Sirbu et al., 2008). To address the possibility that an enlarged cardiac domain caused by loss of RA signaling may influence the balance between cardiac and forelimb progenitors in the lateral plate mesoderm, as first suggested in

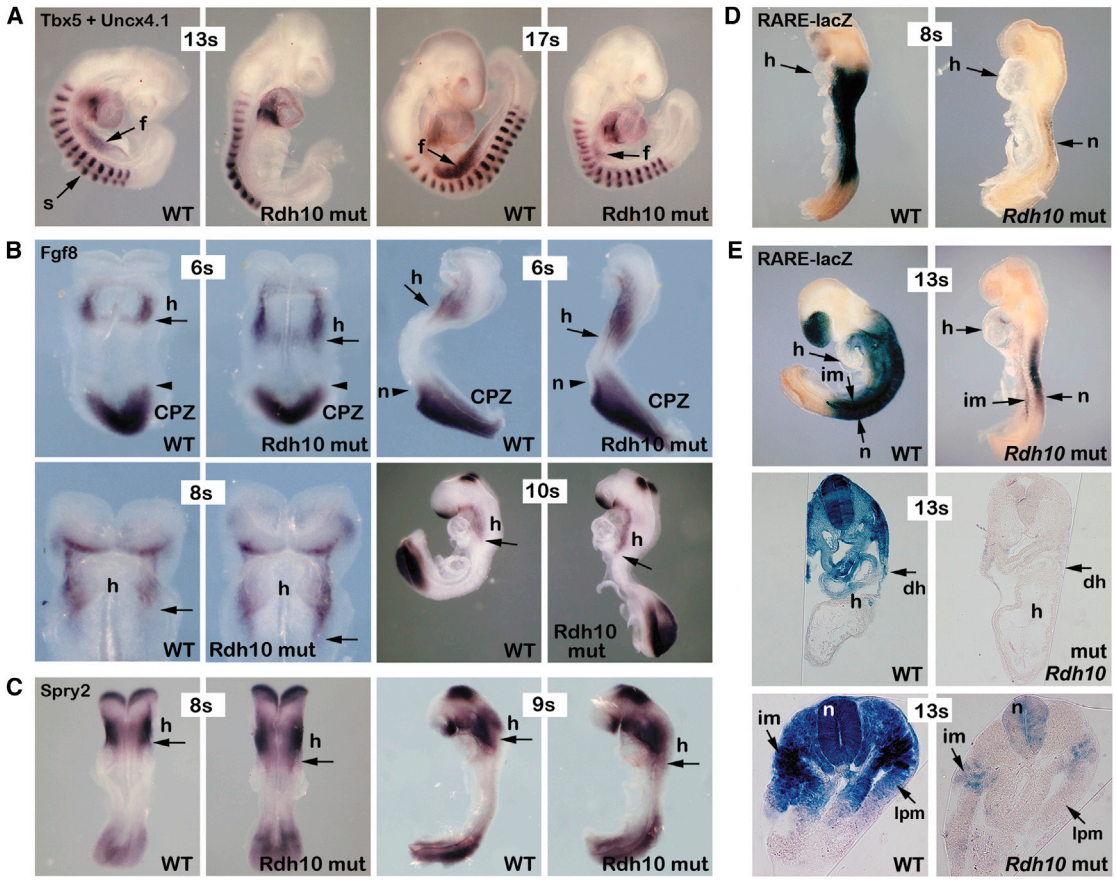

Figure 4. Spatiotemporal *Tbx5* Deficiency Is Concomitant with Expanded Heart FGF Signaling and Reduced Heart RA Signaling

(A–C) In situ hybridization at E8.5 in wild-type (WT) and *Rdh10* mutant (*Rdh10* mut) embryos (lateral view unless stated).

(A) Shown is *Tbx5* expression in the presumptive forelimb field (f) in 13-somite (13s) and 17-somite (17s) embryos; embryos were costained for *Uncx4.1* expression, which labels somites as previously described (Zhao et al., 2009).

(B) *Fgf8* expression preceding and during normal *Tbx5* initiation at six-somite (6s; dorsal and lateral views), eight-somite (8s; ventral view), and ten-somite (10s) stages. Arrows represent the posterior boundary of the heart (h); arrowheads represent the anterior boundary of the caudal progenitor zone (CPZ) and node (n).

(C) *Spry2* expression during normal *Tbx5* initiation at 8s (dorsal view) and nine-somite (9s) stages. Arrows represent the posterior boundary of the heart (h).

(D and E) Wild-type (WT) and *Rdh10* mutant embryos stained with X-gal to detect *RARE-lacZ* RA signaling activity preceding and immediately following normal *Tbx5* initiation at 6s and 13s stages. (E) Transverse sections through the heart (middle panels) and presumptive forelimb field (lower panels) are shown. Activity in heart (h), neural tube (n), intermediate mesoderm (im), dorsal heart (dh), and lateral plate mesoderm (lpm) is indicated.

studies on zebrafish *raldh2* mutants (Keegan et al., 2005), we crossed *Raldh2*$^{+/-}$ mice with *Isl1-nlacZ*$^{+/-}$ knockin mice (Sun et al., 2007) to generate embryos that have lost both RA activity and *Isl1*. *Raldh2*$^{-/-}$ embryos were used in this experiment due to their total absence of *Tbx5* expression in the forelimb field lateral plate mesoderm (Zhao et al., 2009) and the appearance of a distended heart phenotype (Ryckebusch et al., 2008; Sirbu et al., 2008). In *Raldh2*$^{-/-}$;*Isl1-nlacZ*$^{+/-}$ embryos, we observed ectopic posterior expansion of *lacZ* expression (Figure 5B; Figure S4; n = 8/8) indicating that the *Isl1-nlacZ* knockin transgene recapitulates the ectopic expansion of *Isl1* messenger RNA (mRNA) following loss of RA activity. *Raldh2*$^{-/-}$;*Isl1-nlacZ*$^{-/-}$ embryos (that lack *Isl1* function) displayed a loss of the distended heart phenotype observed in *Raldh2*$^{-/-}$ embryos (n = 5/5), but they did not display forelimb *Tbx5* expression (n = 0/5), which was absent in *Raldh2*$^{-/-}$ embryos (n = 0/8) but maintained in *Isl1-nlacZ*$^{-/-}$ embryos (n = 6/6) (Figure 5C; Figure S5). Thus, while we provide evidence that the distended heart phenotype of *Raldh2*$^{-/-}$ embryos is dependent upon ectopic *Isl1* expression downstream of ectopic *Fgf8* expression, ectopic *Fgf8* expression disrupts forelimb initiation through a pathway that does not require *Isl1*. As restriction of the heart field is insufficient to rescue forelimb initiation, our results genetically uncouple RA loss-of-function forelimb defects from second heart field expansion due to ectopic heart *Fgf8* expression.

RA-FGF Antagonism Is Required to Program the Forelimb Field

To test the sufficiency of FGF8 to inhibit *Tbx5* induction in mouse, we cultured wild-type embryos in recombinant FGF8 protein during the period when *Tbx5* expression normally initiates in the forelimb field (i.e., seven-somite embryos were cultured for 12 hr to generate 12-somite embryos). Control cultured embryos yielded normal *Tbx5* induction in the forelimb field (n = 4/4), while those

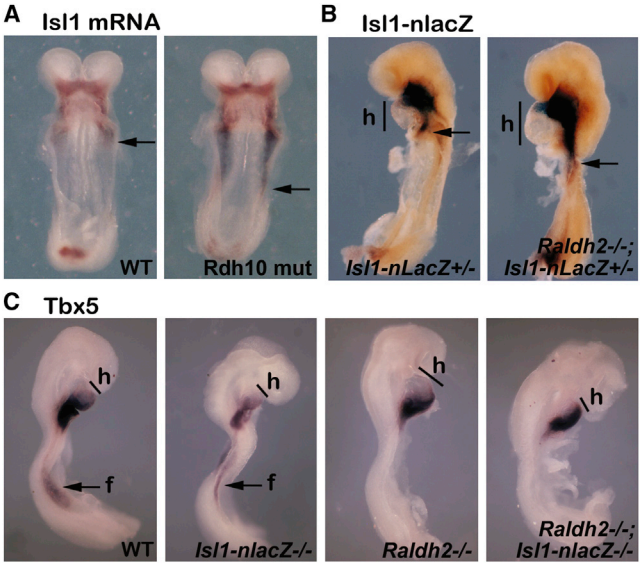

Figure 5. RA Controls Limb *Tbx5* Independently of Heart *Isl1*

(A–C) E8.5 mouse embryos analyzed by in situ hybridization (A and C) or stained with X-gal to detect *Isl1-nlacZ* expression (B).

(A) *Isl1* mRNA in *Rdh10* mutant (*Rdh10* mut) versus wild-type (WT) embryos (ventral view). Arrows represent the posterior boundary of the heart field.

(B) *Isl1-nlacZ* expression in $Raldh2^{-/-}$;$Isl1\text{-}nlacZ^{+/-}$ versus $Isl1\text{-}nlacZ^{+/-}$ embryos. Arrows represent the posterior boundary of the heart field (h).

(C) Assessment of heart expansion (h) and forelimb field *Tbx5* expression (f) in wild-type, $Isl1\text{-}nlacZ^{-/-}$, $Raldh2^{-/-}$, and $Raldh2^{-/-}$;$Isl1\text{-}nlacZ^{-/-}$ embryos. See also Figures S4 and S5.

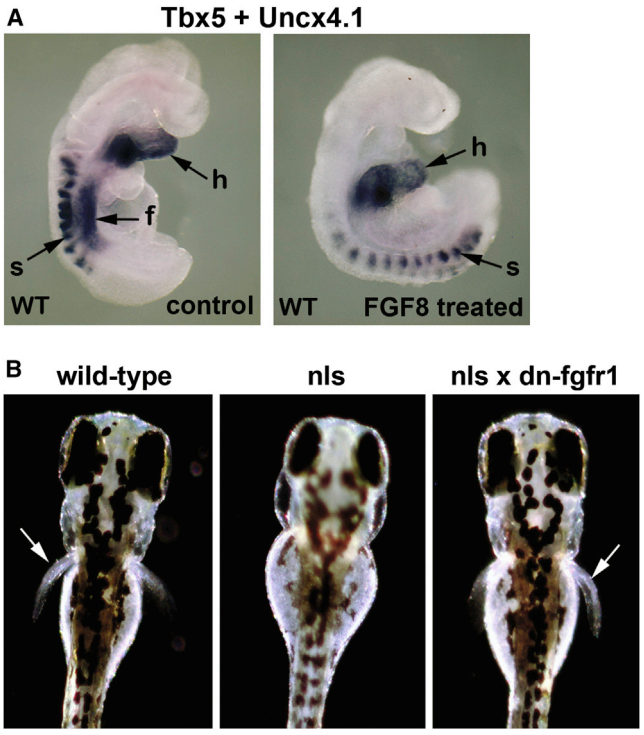

Figure 6. RA-FGF Antagonism Is Required to Generate the Forelimb Field

(A) Assessment of forelimb field *Tbx5* expression (f) in control versus FGF8-treated wild-type (WT) embryos cultured in vitro for 12 hr. Heart *Tbx5* mRNA (h) and somite *Uncx4.1* mRNA (s) are also indicated.

(B) Rescue of missing pectoral fins in *raldh2* (*nls*) mutant zebrafish by crossing to heat-shock-inducible dn-fgfr1 transgenic fish (heat shock at 37°C; 8–10 hpf). Arrows indicate pectoral fins.

See also Figure S6.

cultured with FGF8 (10 μg/ml) had no sign of a forelimb *Tbx5* domain (n = 0/5), although heart *Tbx5* expression was unaffected (n = 5/5) (Figure 6A). Culturing embryos in recombinant FGF10 protein (10 μg/ml) did not noticeably change limb *Tbx5* expression (n = 6/6), revealing a distinction between FGF8 and FGF10 function (Figure S6). We postulate that in the absence of RA signaling, ectopic *Fgf8* expression negatively impacts axial patterning of the forelimb field, resulting in loss of *Tbx5* induction. Therefore, axial RA-FGF antagonism near the early heart has two distinct functions: one to restrict the second heart field via *Isl1* repression, and another to permit correct axial patterning of forelimb field lateral plate mesoderm through a mechanism independent of *Isl1*.

To more rigorously test the hypothesis that forelimb initiation requires axial RA-FGF antagonism, we performed genetic rescue studies on zebrafish *raldh2* (*nls*) mutants lacking pectoral fins (forelimbs) (Begemann et al., 2001) crossed with *hsp70:dn-frgr1-EGFP* fish carrying a heat-shock-inducible dominant-negative FGF receptor transgene (Lee et al., 2005). Genetic abrogation of FGF signaling by heat shock resulted in pectoral fin rescue in *raldh2* mutants (Figure 6B; n = 10/39). These findings complement our mouse data, underpinning a requirement for RA-FGF antagonism to allow normal forelimb initiation.

DISCUSSION

Antagonism between RA and FGF was postulated as a mechanism controlling limb proximodistal patterning based upon a pharmacological analysis of chick embryos. Those studies suggested that proximal RA signaling induces *Meis1/2* in the proximal limb, thus opposing distal FGF signaling from the AER that represses *Meis1/2* in the distal limb, establishing the border between the stylopod and zeugopod (Mercader et al., 2000). Using genetic RA loss-of-function studies, we provide convincing evidence that RA is not required for limb patterning and RA-FGF antagonism does not occur along the limb proximodistal axis as originally proposed. Our findings instead demonstrate that RA-FGF antagonism along the anteroposterior axis of the trunk lateral plate mesoderm prior to budding is required to permit correct spatiotemporal induction of *Tbx5* for normal forelimb initiation (Figure 7). Thus, the gain-of-function studies performed in chick identified RA and FGF as important signaling agents for limb development, but the genetic loss-of-function studies reported here show that RA controls limb development in a much different manner than originally envisioned.

The early pharmacological studies suggesting antagonism between RA and FGF along the proximodistal axis of the limb (Mercader et al., 2000) were followed by studies employing heterotopic transplantation of intact or recombinant chick limbs to further support a role for endogenous RA as a proximalizing signal for limb patterning (Cooper et al., 2011; Roselló-Díez

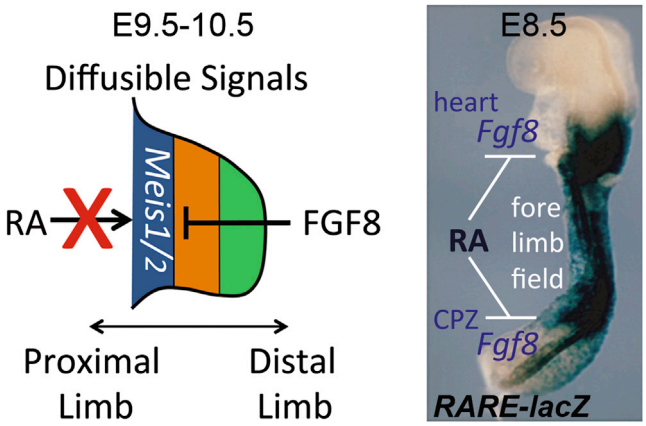

Figure 7. Model for Axial RA-FGF Antagonism prior to Limb Budding
Previous genetic studies support FGF8 repression of *Meis1/2* in distal limb elements, but our genetic findings show that RA signaling is not required for *Meis1/2* expression in the proximal limb. Thus, RA-FGF antagonism is not required along the limb proximodistal axis to provide *Meis1/2* expression proximally in the stylopod (blue) and prevent distal expression in the zeugopod (orange) and autopod (green). Instead, our studies support a model in which RA is required earlier for axial antagonization of *Fgf8* expression (in both the second heart field and caudal progenitor zone -CPZ) prior to forelimb budding for correct induction and positioning of *Tbx5* expression in the forelimb field.

et al., 2011). However, the ability of RA treatment to increase *Meis1/2* could represent abnormal disruption to FGF signaling that is known to repress *Meis1/2* in the distal limb (Mariani et al., 2008). Also, the apparent link between RA and limb pattern reprogramming in chick but not mouse may be explained by different experimental methodology in the use of RAR antagonists to eliminate RA signaling. In chick, high concentrations of RAR antagonists are typically used to soak beads prior to limb implantation. Beads soaked in very high millimolar concentrations of BMS493 (2 mg/ml = 6 mM) were used to demonstrate negative effects on *Meis1* (Roselló-Díez et al., 2011), contrasting with our results from BMS493-treated mouse embryos. Local concentrations at the implantation site in chick were likely to be far higher than the 10 μM BMS493 sufficient to suppress RA signaling used in our study and others (Germain et al., 2002; Chen et al., 2007). BMS493 functions as an inverse agonist and actively recruits corepressors to RARs bound at retinoic acid response elements (RAREs), thus silencing nearby genes below basal levels (Germain et al., 2002). Recently, chromatin immunoprecipitation analysis using a pan-RAR antibody revealed 13,385 RAREs in the mouse genome (Moutier et al., 2012), but most are unlikely to be associated with endogenous RA signaling. It is possible that very high RAR-antagonist concentrations silence numerous genes in the vicinity of these RAREs, including genes not normally regulated by RA.

Our findings demonstrate that both the initial expression of *Meis1* and *Meis2* in lateral plate mesoderm and their subsequent expression in the proximal limb bud do not require RA signaling. This indicates that some other mechanism is responsible for induction of trunk and proximal limb *Meis1/2* expression. Downregulation of *Meis1/2* expression in the distal limb requires FGF8 signaling derived from the AER, driving more distal fates (Mariani et al., 2008). We suggest that since AER *Fgf8* expression appears after limb bud formation (Crossley et al., 1996), the proximalmost limb domain is out of range of early AER FGF signals, leading to maintenance of proximal *Meis1/2* while AER FGF activity restricts *Meis1/2* expression as distal elements are formed. Autonomous mechanisms may also play a part in specifying *Meis1/2*-negative distal domains. Autonomous collinear expression along the *Hoxd* cluster has been shown to be evident in the developing limb via progressive shifting of individual *Hox* genes from association with transcriptionally silent to transcriptionally active *cis*-regulatory elements (Noordermeer et al., 2011). Considering the evidence against RA as a proximal signal in mouse and the general conservation of RA signaling components among vertebrate embryos (i.e., expression of synthetic enzymes, degradative enzymes, receptors, binding proteins), we favor a single diffusible signal model for proximodistal patterning of vertebrate embryos with distal *Fgf8* expression guiding autonomous mechanisms, much like how posterior *Shh* expression acts across the anteroposterior axis (Litingtung et al., 2002).

A key hypothesis made from chick studies speculates that departure from an RA signal and exposure to an FGF signal provide the patterning cue for distal limb fate specification (Cooper et al., 2011; Roselló-Díez et al., 2011). While freedom from RA is indeed necessary for normal distalization of limb mesenchyme, we show here that RA cannot provide an endogenous proximal cue since *Meis1/2* and limb patterning are not RA dependent. The presence of RA in the proximal limb is thus an unnecessary consequence of diffusion from the lateral plate mesoderm where RA action is needed, but RA is prevented from entering the distal limb bud by RA degradation controlled by distal limb expression of *Cyp26b1* (Yashiro et al., 2004). Another study utilizing RA-treated chick limbs displaying loss of AER *Fgf8* expression suggested that clearance of RA in the distal limb by *Cyp26b1* (which is induced by *Fgf8*) may be needed to achieve normal distal *Fgf8* expression in the AER (Probst et al., 2011). However, our previous studies on *Rdh10* mutants demonstrated that loss of limb RA activity does not expand distal limb *Fgf8* expression (Cunningham et al., 2011a), indicating that RA-FGF antagonism is not required along the limb proximodistal axis to control AER *Fgf8* expression. Our data are consistent with the idea that *Cyp26b1* functions to prevent RA from interfering with FGF-driven proximodistal patterning and growth (downstream effects of *Fgf8*) rather than instructively driving a proximal fate via RA compartmentalization to control either *Meis1/2* or *Fgf8* limb expression.

Instead of RA-FGF antagonism occurring along the limb proximodistal axis after budding has occurred, our findings demonstrate that axial RA-FGF antagonism in the trunk prior to budding helps program the forelimb field to permit induction of *Tbx5*. This action of RA prevents the appearance of FGF8 signaling in the region fated for the forelimb field and sets the stage for proper proximodistal patterning at a later point when distal limb FGF8 signaling initiates in the AER. Our studies demonstrate that models for limb patterning do not need to invoke proximodistal RA-FGF antagonism, but that models for forelimb initiation need to incorporate axial RA-FGF antagonism to more fully understand how lateral plate mesoderm progenitors exiting the primitive streak achieve a forelimb fate. The lack of an RA

requirement for hindlimb initiation is likely related to the observation that the RA requirement for caudal repression of *Fgf8* during body axis extension ends between E8.5 and E9.5 when the primitive streak has regressed (Cunningham et al., 2011b), thus prior to hindlimb initiation, which occurs at E9.5. Therefore, the role of RA in forelimb development is intimately intertwined with its role in controlling the late aspects of gastrulation (Duester, 2008). These findings advance our understanding of the regulatory logic that drives limb development, providing a basis to understand how limb deformities arise and how they may be treated or prevented.

EXPERIMENTAL PROCEDURES

Mutant Models

Raldh2$^{-/-}$ mice (germ-line null) have been described previously (Zhao et al., 2009). Genotyping was performed by PCR analysis of yolk sac DNA. *Rdh10*$^{trex/trex}$ mice (*Rdh10* mutants) generated via ethylnitrosourea mutagenesis have also been reported previously (Sandell et al., 2007). Mutants were identified by DNA sequencing of a PCR product overlapping the mutation from analysis of yolk sac DNA. Mice carrying the *RARE-lacZ* RA-reporter transgene (Rossant et al., 1991) were crossed with *Raldh2*$^{-/-}$ and *Rdh10* mutants. *Isl1-nlacZ* knockin mice, which express *lacZ* under control of the *Isl1* promoter and exhibit a homozygous null phenotype, were genotyped by PCR analysis of yolk sac DNA (Sun et al., 2007). Zebrafish *raldh2* mutants (*nls*) have been reported previously (Begemann et al., 2001), and zebrafish carrying the *hsp70:dn-frgr1-EGFP* transgene expressing a heat-shock-inducible dominant-negative FGF receptor have been described elsewhere (Lee et al., 2005). All mouse and fish studies conformed to the regulatory standards adopted by the Animal Research Committee at the Sanford-Burnham Medical Research Institute.

Mouse Embryo In Vitro Culture and Treatment

Wild-type, *Rdh10* mutant, and *Raldh2*$^{-/-}$ embryos at the six- to nine-somite stages or at E10.5 were cultured for 12 hr in serum-free (retinoid-free) Dulbecco's modified Eagle's medium with nutrient mixture F-12 (DMEM/F-12) culture media (GIBCO-Life Technologies) in Millicell culture plate inserts (Millipore) at 37°C in 5% CO_2. Retinoid treatments included all-*trans*-retinoic acid or BMS493 (both from Sigma Chemical) dissolved in DMSO vehicle, which was administered as a control. FGF treatments included FGF8 (eBioscience) or FGF10 (R&D Systems). After culture, embryos were processed for whole-mount in situ hybridization or stained with X-gal as described below.

Detection of RA and mRNA

RA activity was detected using mouse embryos carrying the *RARE-lacZ* transgene in which β-galactosidase, encoded by *lacZ*, is under the transcriptional control of a RARE; β-galactosidase activity was detected in embryos by staining 18 hr with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as previously described (Rossant et al., 1991). Detection of mRNA was performed by whole-mount in situ hybridization as previously described (Cunningham et al., 2011a). For tissue sectioning, stained embryos were incubated in a gelatin-BSA solution (0.5% gelatin, 30% BSA, 20% sucrose in 1× PBS; Sigma) for 1 hr and then embedded in fresh gelatin-BSA solution polymerized with 1.75% glutaraldehyde and sectioned at 30 μm with a vibratome.

Zebrafish Studies

Homozygous *raldh2* mutant zebrafish do not develop pectoral fins (Begemann et al., 2001). Heterozygous *raldh2* mutants were crossed with fish carrying the *hsp70:dn-frgr1-EGFP* transgene, which after heat shock expresses a dominant-negative form of the FGFR1 receptor (Lee et al., 2005). Crosses of heterozygous *raldh2;hsp70:dn-frgr1-EGFP* fish to heterozygous *raldh2* mutants generated embryos that were either heat shocked at 37°C for 2 hr prior to pectoral fin development or not subject to heat shock. Heat shock for 2 hr from 8–10 hr postfertilization resulted in 180 embryos carrying *hsp70:dn-frgr1-EGFP* (identified by enhanced GFP [EGFP] fluorescence) that were then examined at 3 days old. PCR genotyping identified 39 homozygous *raldh2* mutants and 141 fish that were either wild-type or heterozygous for the *raldh2* mutation. Among the 39 homozygous *raldh2* mutants carrying *hsp70:dn-frgr1-EGFP,* we found that about 25% displayed pectoral fins (n = 10/39). Similar analysis of control embryos (those not heat shocked or those heat shocked but not carrying *hsp70:dn-frgr1-EGFP*) demonstrated that all homozygous *raldh2* mutants failed to develop pectoral fins.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.03.036.

LICENSING INFORMATION

ACKNOWLEDGMENTS

We thank V. Prince for providing zebrafish *raldh2* mutants, K. Poss and P.D.S. Dong for providing zebrafish carrying the *hsp70:dn-frgr1-EGFP* transgene, and J. Rossant for providing *RARE-lacZ* mice. We also thank the following for mouse complementary DNAs used to prepare in situ hybridization probes: V. Giguere (*RARb*), P. Gruss (*Meis2*), J.C. Izpisua-Belmonte (*Meis1*), A. Mansouri (*Uncx4.1*), G. Martin (*Fgf8*, *Spry2*), P. Ruiz-Lozano (*Isl1*), and V. Papaioannou (*Tbx5*). We gratefully acknowledge the SBMRI Animal Facility and Zebrafish Facility for timed matings and animal care. This work was funded by National Institutes of Health grants GM062848 (G.D.) and DE016082 (P.T.) and by the Stowers Institute for Medical Research (P.T.). T.J.C., X.Z., and G.D. designed the study, analyzed the data, and wrote the paper. T.J.C. and X.Z. performed all the experiments. L.L.S., S.M.E., and P.A.T. provided valuable reagents for the study. All authors discussed the results and commented on the manuscript.

Received: February 12, 2013
Revised: March 18, 2013
Accepted: March 22, 2013
Published: April 25, 2013

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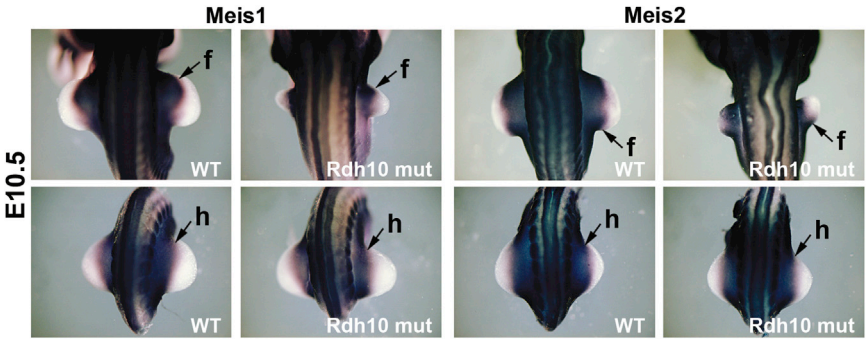

Figure S1. *Meis1/2* Expression in Budding Limbs, Related to Figure 1

In situ hybridization for *Meis1* and *Meis2* in forelimbs (f) and hindlimbs (h) of E10.5 *Rdh10* mutant (*Rdh10* mut) and wild-type (WT) mouse embryos.

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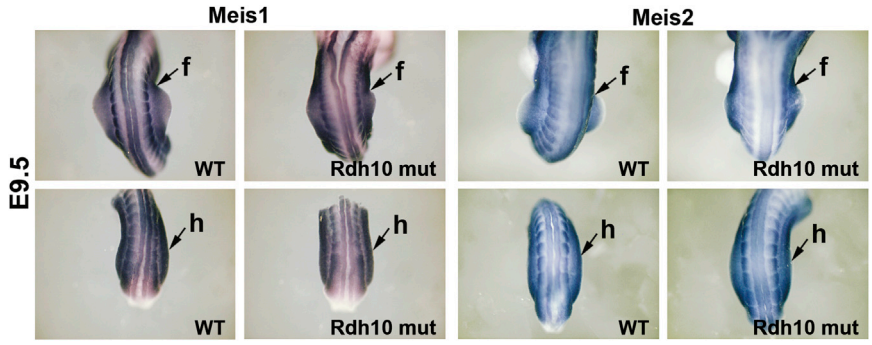

Figure S2. *Meis1/2* Expression in Early Limb Fields, Related to Figure 2

In situ hybridization for *Meis1* and *Meis2* in forelimbs (f) and pre-budding hindlimbs (h) of E9.5 *Rdh10* mutant (*Rdh10* mut) and wild-type (WT) mouse embryos.

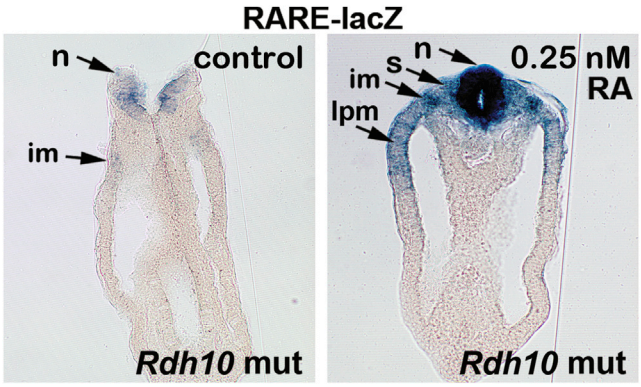

Figure S3. Validation of *RARE-lacZ* Sensitivity, Related to Figure 3

Transverse sections through the limb field of E8.5 *Rdh10* mutant (*Rdh10* mut) embryos stained with X-Gal to detect *RARE-lacZ* and RA signaling activity following culture for 12 hr in DMEM/F-12 medium (control) or DMEM/F-12 plus 0.25 nM RA. neural tube (n), somite (s), intermediate mesoderm (im), and lateral plate mesoderm (lpm) are indicated by arrows.

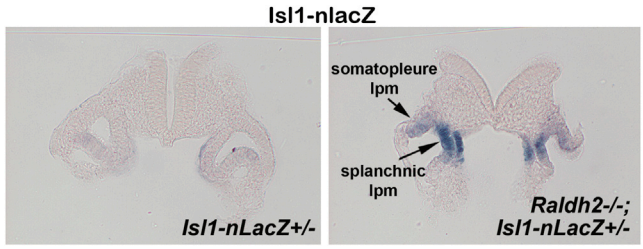

Figure S4. Ectopic *Isl1* Expression in *Raldh2*$^{-/-}$ Embryos, Related to Figure 5

Shown are transverse sections through the limb fields of *Isl1-nlacZ*$^{+/-}$ and *Raldh2*$^{-/-}$;*Isl1-nlacZ*$^{+/-}$ E8.5 embryos stained for *Isl1-nLacZ* expression with X-Gal. Lateral plate mesoderm (lpm) is indicated.

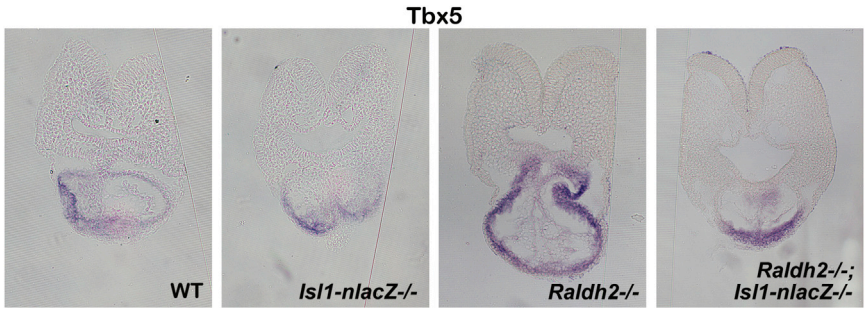

Figure S5. Rescue of Expanded Heart Phenotype, Related to Figure 5

Shown are transverse sections through the heart of E8.5 wild-type (WT), *Isl1-nlacZ*$^{-/-}$, *Raldh2*$^{-/-}$, and *Raldh2*$^{-/-}$;*Isl1-nlacZ*$^{-/-}$ embryos stained for *Tbx5* expression by in situ hybridization.

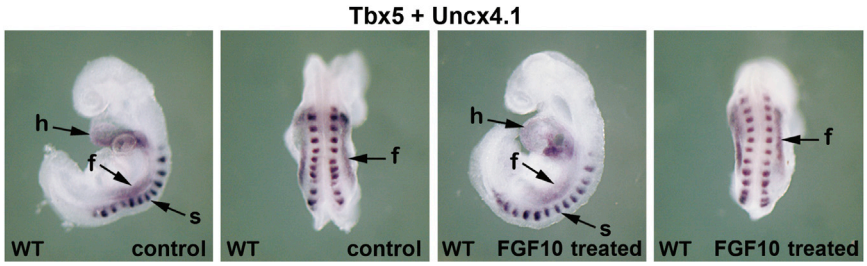

Figure S6. Effect of FGF10 on Initiation of *Tbx5* Expression in the Forelimb Field, Related to Figure 6

Assessment of forelimb field *Tbx5* expression (f) in control versus FGF10-treated E8.5 wild-type (WT) embryos cultured in vitro for 12 hr in DMEM/F-12 medium. Heart *Tbx5* expression (h) and somite *Uncx4.1* expression (s) are also indicated.