Retinoic Acid Controls Heart Anteroposterior Patterning by Downregulating *Isl1* through the *Fgf8* Pathway

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

1 *Burnham Institute for Medical Research, Development and Aging Program, 10901 North Torrey Pines Road, La Jolla, California 92037, USA*

2 *Ulm University, Biochemistry and Molecular Biology Institute, 11 Albert Einstein Allee, 89801 Ulm, Germany*

Abstract

Distinct progenitor cell populations exist in cardiac mesoderm important for patterning of the heart. During heart tube formation in mouse, *Tbx5* is expressed in progenitors located more laterally, whereas *Isl1* and *Fgf8* are expressed in progenitors located more medially. Signals that drive mesodermal progenitors into various cardiac lineages include *Fgf8* which functions to induce *Isl1*. Studies in chick and zebrafish have shown that retinoic acid restricts the number of cardiac progenitors, but its role in mammalian cardiac development is unclear. Here, we demonstrate that *Raldh2*$^{-/-}$ mouse embryos lacking retinoic acid signaling exhibit a posterior expansion of the cardiac *Fgf8* expression domain as well as an expansion of *Isl1* expression into mesoderm lying posterior to the cardiac field. We provide evidence that retinoic acid acts specifically in the posterior-medial region of the cardiac field to establish the heart posterior boundary potentially by reducing *Fgf8* expression which restricts the *Isl1* domain.

Keywords

heart field; cardiac splanchnic mesoderm; retinoic acid signaling; FGF signaling; *Isl1*; *Raldh2*; *Fgf8*; *Tbx5*; mouse

INTRODUCTION

During early heart organogenesis, studies in vertebrate embryos have identified distinct progenitor cell populations in the splanchnic lateral plate mesoderm of the cardiac field that express unique combinations of transcription factors essential for heart development (Mjaatvedt et al., 2001; Waldo et al., 2001; Buckingham et al., 2005; Brade et al., 2007). Prior to heart tube formation in mouse it has been reported that *Isl1* is expressed widely in cardiac progenitors (Prall et al., 2007), but later during heart tube formation *Isl1* is expressed more medially (Cai et al., 2003) whereas *Tbx5* is expressed more laterally in the cardiac crescent (Hochgreb et al., 2003; Ryckebusch et al., 2008). Genetic studies in mouse embryos indicate that a loss of *Isl1* expression results in defects in the outflow tract, right ventricle, atria, and inflow tract (Cai et al., 2003), whereas a loss of *Tbx5* expression results in sinoatrial defects and hypoplastic left ventricle (Bruneau et al., 2001). Thus, *Tbx5* is important primarily for posterior heart development, whereas *Isl1* seems to function all along the heart anteroposterior axis with the possible exception of the left ventricle. As mutations in cardiac transcription

---

*Correspondence to: Gregg Duester, Development and Aging Program, Burnham Institute for Medical Research, 10901 North Torrey Pines Road, La Jolla, California 9203. E-mail: duester@burnham.org.

factors are associated with human congenital heart disease (Olson, 2006; Srivastava, 2006; Bruneau, 2008), further knowledge of how these genes are regulated during cardiac development may be important therapeutically. Also, multipotent progenitors expressing *Isl1* have been observed in postnatal as well as embryonic heart, suggesting that *Isl1*^{+} cells may be useful in regenerative stem cell therapies for heart disease (Moretti et al., 2006; Laugwitz et al., 2008).

Early in the process of heart development, decisions are made that drive cardiac mesodermal progenitors down distinct pathways. Various overlapping heart fields (first, second, anterior) have recently been described marked by expression of various transcription factors such as *Tbx5* (first heart field) or *Isl1* and *Fgf8* (second heart field); to avoid confusion it has been recommended that one refer to heart regions by the marker gene analyzed such as *Tbx5*^{+} or *Isl1*^{+} domains (Abu-Issa and Kirby, 2007). Factors that drive mesoderm to a cardiac fate are not well defined. However, recent studies on mouse embryos indicate that *Fgf8* is required to drive splanchnic mesoderm and ventral pharyngeal mesodermal progenitors toward an *Isl1*^{+} fate, placing *Fgf8* near the top of a cardiac signaling hierarchy (Ilagan et al., 2006; Park et al., 2006). As the vitamin A derivative retinoic acid (RA) has been found to repress *Fgf8* expression (Del Corral et al., 2003; Sirbu and Duester, 2006) this suggested to us that RA may play a role in limiting the number of mesodermal progenitors achieving an *Isl1*^{+} cardiac fate via *Fgf8* repression. Studies in zebrafish have demonstrated that RA limits the overall number of cardiac progenitor cells (Keegan et al., 2005) and chick studies demonstrate that RA limits the location of ventricular progenitors (Hochgreb et al., 2003), but the mechanism of RA action is unclear. RA directly controls gene expression by acting as a ligand for nuclear retinoic acid receptors that bind DNA regulatory elements (Mic et al., 2003). Support for *Fgf8* as an RA target gene has come from the identification of a functional retinoic acid response element upstream of the *Fgf8* promoter (Brondani et al., 2002), plus this regulatory element is conserved in the mouse, rat, and human *Fgf8* genes (Balmer and Blomhoff, 2005).

An RA requirement for anteroposterior patterning of the heart tube has been demonstrated in vitamin A deficient quail embryos (Heine et al., 1985; Dersch and Zile, 1993) as well as mouse embryos unable to convert vitamin A to RA (Niederreither et al., 2001). Loss of RA synthesis in mouse embryos leads to a reduction posteriorly in the atria/inflow tract domain, plus the outflow tract/ventricular domain located more anteriorly forms an abnormal medial distended cavity rather than undergoing rightward looping and septation into right and left ventricles (Niederreither et al., 2001). During early organogenesis the first step of RA synthesis (oxidation of retinol to retinaldehyde) is catalyzed throughout the embryo by overlapping alcohol/retinol dehydrogenases encoded by *Adh3*, *Adh4*, and *Rdh10* (Molotkov et al., 2002; Sandell et al., 2007), whereas the second step of RA synthesis (oxidation of retinaldehyde to RA) is catalyzed exclusively in trunk mesoderm by the tissue-specific retinaldehyde dehydrogenase encoded by *Raldh2* (*Aldh1a2*) (Sirbu et al., 2005; Sirbu and Duester, 2006). In both mouse and chick embryos, *Raldh2* is first expressed in the presomitic mesoderm, and then during the early somite stages a caudorostral wave of *Raldh2* expression occurs in the lateral plate mesoderm up to a location just posterior to the cardiac field (Hochgreb et al., 2003). Although *Raldh2* is likely to function in regulation of cardiac gene expression, the mechanism for how RA acts during early mouse heart development remains unclear as no target genes have been identified. In the studies reported here, we demonstrate that RA is required to limit the posterior extent of *Isl1* expression in the cardiac field, thus helping to establish the posterior boundary of the heart. We provide evidence that RA performs this function through RA repression of *Fgf8* in the posterior region of the cardiac field, thus limiting the action a potent inducer of *Isl1*.

RESULTS

RA Functions Specifically in the Posterior-Medial Region of the Heart Field

The early expression pattern of *Raldh2* is consistent with secreted RA reaching only the posterior heart domain (atria/inflow tract) (Moss et al., 1998; Xavier-Neto et al., 2000; Hochgreb et al., 2003). Here, we confirm that *Raldh2* expression in a wild-type mouse embryo (E8.25, 6-somite stage) is limited to somitic and lateral plate mesoderm lying just posterior to the cardiac crescent (Fig. 3F). Analysis of wild-type and *Raldh2*$^{-/-}$ mouse embryos carrying the *RARE-lacZ* RA-reporter transgene has shown that *Raldh2* is responsible for all RA signaling in the heart at E8.75 (15-somite stage) (Mic et al., 2002), but analysis of RA signaling in wild-type embryos at earlier stages is necessary to determine where RA first acts during cardiac development. Here, we demonstrate that RA signaling at the cardiac crescent stage (6-somite stage) is limited to the posterior portion of the cardiac field as shown by *RARE-lacZ* expression in a wild-type embryo (Fig. 1C). These findings demonstrate that early cardiac RA activity is normally limited posteriorly to the atria/inflow tract region (thus absent anteriorly in the outflow tract/ventricular region), and that a loss of *Raldh2* eliminates this RA activity.

A comparison of E9.5 wild-type and *Raldh2*$^{-/-}$ embryos stained for *Raldh2* mRNA demonstrates a complete absence of *Raldh2* expression in the mutant which exhibits a heart tube with an abnormal medial distended cavity (Fig. 1A–B); this abnormal *Raldh2*$^{-/-}$ heart cavity has previously been demonstrated to exhibit a significantly reduced atria/inflow tract domain plus an enlarged outflow tract/ventricular domain that fails to undergo rightward looping and fails to septate into right and left ventricles (Niederreither et al., 2001). Maternal administration of RA at a low dose is known to rescue these *Raldh2*$^{-/-}$ cardiac patterning defects (Niederreither et al., 2001; Mic et al., 2002); this treatment provides less RA activity than that normally observed in wild-type embryos (Mic et al., 2003). We previously reported that low-dose maternal dietary RA administration from E6.75–E8.25 is sufficient to rescue early somite and cardiac defects observed in *Raldh2*$^{-/-}$ embryos, plus analysis of rescued mutants carrying *RARE-lacZ* can be used to identify where RA signaling acts during embryonic rescue (Sirbu and Duester, 2006). In those studies, we found that RA activity in rescued *Raldh2*$^{-/-}$ embryos at the 1–6 somite stages occurs in the posterior neuroectoderm, node ectoderm, and anterior mesoderm (near the cardiac field), but not in the somitic or presomitic mesoderm where *Raldh2* is normally expressed indicating that some tissues respond to dietary RA whereas others do not (we provided evidence for RA-binding proteins that sequester RA at critical target sites). We have now further characterized RA activity in anterior mesoderm and report that RA-rescued *Raldh2*$^{-/-}$ embryos at the 1–6 somite stages exhibit *RARE-lacZ* expression in the posterior region of the cardiac crescent but limited to a medial domain (Fig. 1C; $n = 6/6$). Thus, RA activity in rescued *Raldh2*$^{-/-}$ embryos was not observed throughout the lateral-medial extent of the posterior cardiac crescent (as seen in a 6-somite wild-type embryo; Fig. 1C), but was instead focused on the medial region where the previously described *Isl1*$^{+}$ domain exists (Cai et al., 2003). Transverse sections of a rescued 6-somite embryo confirmed that cardiac RA activity was confined to the medial splanchnic mesoderm (Fig. 1D–E). Wild-type embryos as early as the 1-somite stage have previously been shown to exhibit an anterior boundary of RA activity (*RARE-lacZ* expression) at the level of the posterior cardiac field (Sirbu et al., 2005). RA activity in a 1-somite stage RA-rescued *Raldh2*$^{-/-}$ embryo was also observed in the posterior heart field, and transverse sections revealed that RA was limited to the medial splanchnic mesoderm (Fig. 1F–G). The RA activity we detect in RA-rescued *Raldh2*$^{-/-}$ embryos at 1–6 somites has very little if any overlap with the lateral cardiac domain where *Tbx5* is expressed (Ryckebusch et al., 2008). Our mutant rescue studies indicate that RA signaling activity in posterior-medial cardiac mesoderm is sufficient for heart tube patterning.

Loss of RA Signaling Expands the *Isl1*^{+} Cardiac Domain Posteriorly

As our RA rescue studies indicate that cardiac RA signaling is required medially where *Isl1*^{+} progenitors exist, we examined *Raldh2*^{−/−} embryos to determine whether the absence of RA affected *Isl1* expression in the cardiac crescent. *Raldh2*^{−/−} embryos exhibit a marked increase in *Isl1* expression in the cardiac field which begins to be detectable at the 3-somite stage and becomes increasingly evident by the 5-somite stage (Fig. 2A–D; *n* = 5/5). The observed expansion in *Raldh2*^{−/−} cardiac *Isl1* expression occurs in the posterior region of the developing heart tube, and extends posteriorly into splanchnic mesoderm not normally marked by *Isl1* expression. Examination of *Isl1* expression in *Raldh2*^{−/−} embryos at 6-somites revealed further posterior expansion of *Isl1* expression into non-cardiac mesoderm that does not express *Isl1* in wild-type embryos (Fig. 2G–H; *n* = 2/2); also see transverse sections (Fig. 2K–N). These findings indicate that RA signaling is required to limit expansion of the cardiac *Isl1*^{+} domain along the anteroposterior axis.

Tbx5[+] Cardiac Domain is Reduced in the Absence of RA Signaling

Previous molecular examination of mouse *Raldh2*^{−/−} cardiac development (performed prior to reports of early cardiac *Isl1* expression) revealed that expression of *Nkx2.5*, *Mef2c*, *Hand2*, and *Gata4* are not significantly altered, but that a reduction in *Tbx5* expression was noticed at E8.5–E9.0 (Niederreither et al., 2001). As our results suggest that RA signaling acts earlier than E8.5 (10 somites), we examined E8.0 embryos for the effect of a loss of RA on the *Tbx5*^{+} domain which is normally limited to a lateral location during heart tube formation (Hochgreb et al., 2003; Ryckebusch et al., 2008). *Tbx5* expression in *Raldh2*^{−/−} embryos ranging from 3–6 somites (E8.0–E8.25) was observed to be greatly reduced in the posterior-lateral region of the cardiac crescent (Fig. 3A–D; *n* = 4/4). Double-staining for *Tbx5* and *Raldh2* mRNAs at the 6-somite stage demonstrated a complete loss of *Raldh2* mRNA in *Raldh2*^{−/−} embryos along with a reduction in *Tbx5* mRNA in the posterior-lateral region of the cardiac crescent (Fig. 3E). Comparison of a double-stained wild-type embryo (Fig. 3E) with an embryo stained for only *Raldh2* mRNA (Fig. 3F), demonstrates that expression of *Tbx5* and *Raldh2* in the splanchnic mesoderm overlaps only slightly. A similar slight overlap of *Tbx5* and *Raldh2* expression was previously reported in sections of double-stained wild-type embryos which demonstrated that these two expression domains are largely distinct (Hochgreb et al., 2003; Ryckebusch et al., 2008). Also, we find that *Raldh2* mRNA in paraxial mesoderm reaches a location no further anterior than the first somite which is located posterior to the cardiac crescent (Fig. 3E–F). These findings provide evidence that RA synthesized by RALDH2 in the splanchnic and somitic mesoderm located posterior to the heart field diffuses into the posterior heart field where it is needed to maintain high levels of *Tbx5* expression. However, our RA rescue studies indicate that RA activity is not required in lateral cardiac mesoderm where *Tbx5* is expressed, but only in medial cardiac mesoderm (Fig. 1C). Also, since *Tbx5* is still expressed in the anterior portion of the *Raldh2*^{−/−} cardiac crescent, it is unlikely that RA is needed instructively to induce *Tbx5* expression. Thus, we suggest that RA is functioning in posterior-medial mesoderm as a permissive factor for posterior-lateral cardiac *Tbx5* expression, potentially by regulating expression of another secreted factor.

RA is Required to Limit *Fgf8* Expression and FGF Signaling in the Posterior Cardiac Field

Recent studies indicate that *Fgf8* is expressed at high levels in an early cardiac domain overlapping the *Isl1*^{+} domain, and that *Fgf8* is required for induction of cardiac *Isl1* expression (Ilagan et al., 2006; Park et al., 2006). We found that *Raldh2*^{−/−} embryos exhibited a marked posterior expansion of *Fgf8* expression in the developing heart at the 1-somite stage (Fig. 4A–B; *n* = 4/4); this is in addition to an anterior expansion of *Fgf8* expression in the primitive streak region that we previously reported (Sirbu and Duester, 2006). A ventral view of 1-somite wild-type and *Raldh2*^{−/−} embryos demonstrated that *Fgf8* is normally expressed most highly in

posterior-medial cardiac mesoderm (with decreasing expression as one proceeds anteriorly), and that the expanded cardiac *Fgf8* expression domain in the mutant is a posterior extension of its normal domain in the cardiac crescent (Fig. 4C). At the 3–5 somite stages, the cardiac *Fgf8* expression domain was still expanded posteriorly, although the overall level of expression was now much lower (Fig. 4D–E; $n = 5/5$). Transverse sections of 1-somite embryos through the posterior region of the heart demonstrated that *Fgf8* mRNA in the heart field is normally localized to the medial splanchnic mesoderm and that *Raldh2*$^{-/-}$ embryos exhibit expanded *Fgf8* mRNA in the splanchnic mesoderm relative to wild-type embryos at the same axial level (Fig. 4F–G). Thus, the expanded domain of *Fgf8* expression observed in the absence of RA is located within the same posterior splanchnic mesodermal domain where RA was found to act during rescue of *Raldh2*$^{-/-}$ embryos (compare Fig. 1G and 4G).

As *Fgf8* overexpression expands the cardiac field (Reifers et al., 2000; Alsan and Schultheiss, 2002) and *Fgf8* loss-of-function reduces the mouse cardiac field and reduces *Isl1* expression (Ilagan et al., 2006; Park et al., 2006), our studies suggest that RA may limit the size of the *Isl1*$^{+}$ domain by limiting the amount of FGF8 signaling. To determine whether our observed increase in *Fgf8* mRNA in the posterior region of the heart results in excessive FGF signaling, we examined expression of *Sprouty2* (*Spry2*) which is induced in an Erk-dependent fashion by FGF signaling and functions to limit transmission of the signal resulting in phosphorylation of Erk1/2 (Minowada et al., 1999). *Raldh2*$^{-/-}$ embryos at the 3–5 somite stages exhibited a marked increase in *Spry2* expression in the posterior heart field, overlapping the region where expanded expression of *Fgf8* and *Isl1* is observed (Fig. 2E–F; $n = 3/3$); transverse sections show increased *Spry2* expression in the splanchnic mesoderm and neuroectoderm (Fig. 2I–J). *Spry2* mRNA was also upregulated at the posterior end (primitive streak region) of *Raldh2*$^{-/-}$ embryos (Fig. 2E–F), consistent with the increase in *Fgf8* expression in that region noted here (Fig. 4A–B) and reported previously (Sirbu and Duester, 2006). These results indicate that a loss of RA signaling leads to an increase in cardiac FGF8 signaling through the MAPK-Erk1/2 pathway, thus supporting a model in which expansion of the *Isl1*$^{+}$ domain in *Raldh2*$^{-/-}$ embryos is due to increased FGF8 signaling.

RA Treatment Rescues Aberrant Expression of *Fgf8* and *Isl1* in *Raldh2*$^{-/-}$ Embryos

The studies detailed above suggest that RA treatment of *Raldh2*$^{-/-}$ embryos should repress *Fgf8* and *Isl1* expression. *Raldh2*$^{-/-}$ and wild-type embryos were collected from pregnant mice treated with a low dose dietary RA supplement from E6.75–E7.75 or E6.75–E8.25 or no RA as a control. At the 1-somite stage, untreated *Raldh2*$^{-/-}$ embryos exhibited a posterior expansion of *Fgf8* mRNA compared to wild-type, whereas this posterior expansion was prevented in RA-treated *Raldh2*$^{-/-}$ embryos (Fig. 5A–C). Likewise, RA treatment of *Raldh2*$^{-/-}$ embryos at the 6-somite stage prevented the posterior expansion of *Isl1* mRNA observed in untreated *Raldh2*$^{-/-}$ embryos (Fig. 5D–F).

DISCUSSION

The findings reported here demonstrate that a lack of RA in mouse embryos changes the fate of mesodermal progenitors such that cardiac *Isl1*$^{+}$ progenitors are expanded posteriorly by the 3-somite stage. Expansion of *Isl1* expression in *Raldh2*$^{-/-}$ embryos is accompanied by an even earlier increase in cardiac *Fgf8* expression by the 1-somite stage. Repression of *Fgf8* is possibly the earliest role of RA signaling in cardiac development as it occurs by E7.75 (1-somite stage) just 6 hours after first onset of *Raldh2* expression and RA signaling which occurs at E7.5 (Sirbu et al., 2005). Previous studies demonstrated that RA deficiency results in a decrease in posterior cardiac *Tbx5*$^{+}$ progenitors observed at the 10-somite stage and later (Niederreither et al., 2001) and we demonstrate here that loss of posterior *Tbx5* expression occurs by the 3-somite stage. Gene disruption studies in mouse have demonstrated crucial roles for *Isl1* (Cai

et al., 2003) and *Tbx5* (Bruneau et al., 2001) in heart tube morphogenesis along the anteroposterior axis. Thus, the early changes in *Isl1* and *Tbx5* expression we observe in *Raldh2*$^{-/-}$ embryos could lead to significant alterations in anteroposterior patterning of the heart tube. Such changes in *Raldh2*$^{-/-}$ embryos may result in the previously observed cardiac malformations which include a smaller sinoatrial domain posteriorly (likely due to decreased *Tbx5* expression) and an aberrant medial distended cavity located anteriorly where the ventricular/outflow tract domain normally resides (Niederreither et al., 2001). We suggest that the medial distended outflow tract/ventricular domain observed in *Raldh2*$^{-/-}$ embryos may be due to aberrant cardiac morphogenesis in response to an expanded population of *Isl1*$^+$ progenitors following increased FGF8 signaling. After submission of this paper another article reported that *Raldh2*$^{-/-}$ embryos exhibit altered second heart field formation resulting in expanded expression of *Isl1* and other second heart field markers, but that this expanded *Isl1*$^+$ progenitor population was not able to differentiate into cardiomyocytes and likely contributed to the abnormal heart morphology observed (Ryckebusch et al., 2008).

Our studies have provided evidence that RA action during early heart patterning is normally limited to the posterior cardiac region, and we have demonstrated that a posterior-medial cardiac mesodermal cell population overlapping the *Fgf8*$^+$ and *Isl1*$^+$ domains (but not the lateral *Tbx5*$^+$ domain) is the only mesoderm exhibiting RA activity during our low-dose RA rescue of *Raldh2*$^{-/-}$ cardiac patterning. These findings suggest that this posterior-medial domain represents the RA target tissue during early cardiac development and that it is required for proper anteroposterior patterning of the heart tube. Also, we provide evidence that *Fgf8* expression in the posterior-medial cardiac field is an early target of RA signaling during heart development, and that excessive FGF8 signaling following a loss of RA signaling is associated with posterior expansion of the *Isl1*$^+$ cardiac domain. Cardiac *Fgf8* function is conserved as it is required for induction and expansion of the zebrafish heart (Reifers et al., 2000), and mouse genetic studies have uncovered an essential role for *Fgf8* in upregulating *Isl1* expression (Ilagan et al., 2006; Park et al., 2006). Previous studies in RA-deficient zebrafish embryos demonstrated that a loss of RA transforms non-cardiac mesoderm into cardiac mesoderm (Keegan et al., 2005). We propose that the general principle of RA limiting the allocation of mesoderm to a cardiac cell fate has been conserved from fish to mammal, with mammalian heart development employing RA signaling to limit the posterior extent of *Isl1*$^+$ progenitors in lateral plate mesoderm. Our findings thus suggest that RA limits the number of cells achieving an *Isl1*$^+$ fate by restricting expression of *Fgf8*, thus placing RA near the top of an important cardiac signaling hierarchy: RA –| FGF8 → Erk1/2 → Isl1. As our findings suggest that RA signaling does not function directly in the posterior-lateral cells expressing *Tbx5*, the ability of RA to stimulate *Tbx5* expression may function permissively through RA downregulation of *Fgf8* in posterior-medial cardiac mesoderm which may affect posterior-lateral cardiac mesoderm in a non-cell autonomous fashion. Further experiments are needed to determine if *Tbx5* expression is repressed by FGF8 signaling.

There is no evidence that RA is required to induce expression of genes involved in early cardiac development (Niederreither et al., 2001). Instead, our studies indicate that RA plays a repressive role during cardiac development, by limiting the amount of FGF8 signaling posteriorly. The primary action of RA signaling during early heart tube formation may thus be downregulation of *Fgf8* leading to reduced *Isl1* expression needed to define the posterior limit of the cardiac field. This conclusion is supported by studies demonstrating the existence of a functional retinoic acid response element upstream of the *Fgf8* promoter (Brondani et al., 2002). Also, other studies have demonstrated the existence of a mutual RA-FGF8 antagonism during posterior body axis extension (Del Corral et al., 2003), with RA generated by *Raldh2* repressing *Fgf8* in the epiblast (Sirbu and Duester, 2006) and FGF8 repressing *Raldh2* expression in the presomitic mesoderm (Del Corral et al., 2003). Additionally, a reduction in FGF8 signaling through mutation of *Tbx1* and *Crkl* leads to aortic arch and thymic defects in

a mouse model of human del22q11 (DiGeorge) syndrome, and this loss results in higher *Raldh2* expression and increased RA signaling; these defects can be rescued by a partial loss of *Raldh2* function (*Raldh2*$^{-/+}$) suggesting that *Fgf8* normally functions to repress *Raldh2* (Guris et al., 2006; Moon et al., 2006). As our findings indicate that *Raldh2* represses *Fgf8* in the posterior cardiac field, it appears that a mutual antagonism may exist between RA and FGF8 signaling during various stages of cardiac development. A disruption in RA-FGF8 antagonism during heart tube formation may thus represent the underlying molecular defect in some types of congenital heart disease.

EXPERIMENTAL PROCEDURES

Generation of *Raldh2*$^{-/-}$ Embryos and Conditional RA Rescue

A line of *Raldh2*$^{+/-}$ adult mice was described previously (Mic et al., 2002). Embryos derived from timed matings of *Raldh2*$^{+/-}$ mice were genotyped by PCR analysis of yolk sac DNA to identify *Raldh2*$^{-/-}$ embryos. Following mating, noon on the day of vaginal plug detection was considered embryonic day 0.5 (E0.5). Embryos were staged according to somite number. Rescue of *Raldh2*$^{-/-}$ embryos by maternal dietary RA supplementation was performed as previously described (Sirbu and Duester, 2006); the RA dose employed has been demonstrated to be in the normal physiological range (Mic et al., 2003). Briefly, all-*trans*-RA (Sigma Chemical Co.) was dissolved in corn oil and mixed with powdered mouse chow to provide a final concentration of 0.1 mg/g for treatment from E6.75–E8.25. Such food was prepared fresh each morning and evening and provided *ad libitum*. Embryos were analyzed at E8.0 and E8.25 when the mother was still on the RA-supplemented diet. All mouse studies conformed to the regulatory standards adopted by the Animal Research Committee at the Burnham Institute for Medical Research.

In situ Hybridization and Retinoic Acid Detection

Whole-mount in situ hybridization was performed as described previously to detect mRNA (Mic et al., 2002); wild-type and *Raldh2*$^{-/-}$ embryos were treated under identical hybridization conditions and stained for the same length of time. For double-labeling, two antisense RNA probes were used and detection was performed simultaneously. RA activity was detected by examining embryos carrying the *RARE-lacZ* RA-reporter transgene which places *lacZ* (encoding β-galactosidase) under the transcriptional control of a retinoic acid response element (RARE) (Rossant et al., 1991). β-galactosidase activity was detected using staining with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as previously described (Sirbu and Duester, 2006). Stained embryos were embedded in 3% agarose and sectioned at 30 mm with a vibratome.

Acknowledgements

References

Abu-Issa R, Kirby ML. Heart field: from mesoderm to heart tube. Annu Rev Cell Dev Biol 2007;23:45–68. [PubMed: 17456019]

Alsan BH, Schultheiss TM. Regulation of avian cardiogenesis by Fgf8 signaling. Development 2002;129:1935–1943. [PubMed: 11934859]

Balmer JE, Blomhoff R. A robust characterization of retinoic acid response elements based on a comparison of sites in three species. J Steroid Biochem Mol Biol 2005;96:347–354. [PubMed: 16081280]

Brade T, Gessert SMKh, Pandur P. The amphibian second heart field: Xenopus islet-1 is required for cardiovascular development. Dev Biol 2007;311:297–310. [PubMed: 17900553]

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]

Bruneau BG. The developmental genetics of congenital heart disease. Nature 2008;451:943–948. [PubMed: 18288184]

Bruneau BG, Nemer G, Schmitt JP, Charron F, Robitaille L, Caron S, Conner DA, Gessler M, Nemer M, Seidman CE, Seidman JG. A murine model of Holt-Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. Cell 2001;106:709–721. [PubMed: 11572777]

Buckingham M, Meilhac S, Zaffran S. Building the mammalian heart from two sources of myocardial cells. Nature Rev Genet 2005;6:826–835. [PubMed: 16304598]

Cai CL, Liang X, Shi Y, Chu PH, Pfaff SL, Chen J, Evans S. Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. Dev Cell 2003;5:877–889. [PubMed: 14667410]

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]

Dersch H, Zile MH. Induction of normal cardiovascular development in the vitamin A-deprived quail embryo by natural retinoids. Dev Biol 1993;160:424–433. [PubMed: 8253275]

Guris DL, Duester G, Papaioannou VE, Imamoto A. Dose-dependent interaction of Tbx1 and Crkl and locally aberrant RA signaling in a model of del22q11 syndrome. Dev Cell 2006;10:81–92. [PubMed: 16399080]

Heine UI, Roberts AB, Munoz EF, Roche NS, Sporn MB. Effects of retinoid deficiency on the development of the heart and vascular system of the quail embryo. Virchows Arch B Cell Path 1985;50:135–152.

Hochgreb T, Linhares VL, Menezes DC, Sampaio AC, Yan CYI, Cardoso WV, Rosenthal N, Xavier-Neto J. A caudorostral wave of RALDH2 conveys anteroposterior information to the cardiac field. Development 2003;130:5363–5374. [PubMed: 13129847]

Ilagan R, Abu-Issa R, Brown D, Yang YP, Jiao K, Schwartz RJ, Klingensmith J, Meyers EN. Fgf8 is required for anterior heart field development. Development 2006;133:2435–2445. [PubMed: 16720880]

Keegan BR, Feldman JL, Begemann G, Ingham PW, Yelon D. Retinoic acid signaling restricts the cardiac progenitor pool. Science 2005;307:247–249. [PubMed: 15653502]

Laugwitz KL, Moretti A, Caron L, Nakano A, Chien KR. Islet1 cardiovascular progenitors: a single source for heart lineages? Development 2008;135:193–205. [PubMed: 18156162]

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]

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]

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]

Mjaatvedt CH, Nakaoka T, Moreno-Rodriguez R, Norris RA, Kern MJ, Eisenberg CA, Turner D, Markwald RR. The outflow tract of the heart is recruited from a novel heart-forming field. Dev Biol 2001;238:97–109. [PubMed: 11783996]

Molotkov A, Fan X, Deltour L, Foglio MH, Martras S, Farrés J, Parés X, Duester G. Stimulation of retinoic acid production and growth by ubiquitously-expressed alcohol dehydrogenase *Adh3*. Proc Natl Acad Sci USA 2002;99:5337–5342. [PubMed: 11959987]

Moon AM, Guris DL, Seo JH, Li L, Hammond J, Talbot A, Imamoto A. Crkl deficiency disrupts Fgf8 signaling in a mouse model of 22q11 deletion syndromes. Dev Cell 2006;10:71–80. [PubMed: 16399079]

Moretti A, Caron L, Nakano A, Lam JT, Bernshausen A, Chen Y, Qyang Y, Bu L, Sasaki M, Martin-Puig S, Sun Y, Evans SM, Laugwitz KL, Chien KR. Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. Cell 2006;127:1151–1165. [PubMed: 17123592]

Moss JB, Xavier-Neto J, Shapiro MD, Nayeem SM, McCaffery P, Dräger UC, Rosenthal N. Dynamic patterns of retinoic acid synthesis and response in the developing mammalian heart. Dev Biol 1998;199:55–71. [PubMed: 9676192]

Niederreither K, Vermot J, Messaddeq N, Schuhbaur B, Chambon P, Dollé P. Embryonic retinoic acid synthesis is essential for heart morphogenesis in the mouse. Development 2001;128:1019–1031. [PubMed: 11245568]

Olson EN. Gene regulatory networks in the evolution and development of the heart. Science 2006;313:1922–1927. [PubMed: 17008524]

Park EJ, Ogden LA, Talbot A, Evans S, Cai CL, Black BL, Frank DU, Moon AM. Required, tissue-specific roles for Fgf8 in outflow tract formation and remodeling. Development 2006;133:2419–2433. [PubMed: 16720879]

Prall OW, Menon MK, Solloway MJ, Watanabe Y, Zaffran S, Bajolle F, Biben C, McBride JJ, Robertson BR, Chaulet H, Stennard FA, Wise N, Schaft D, Wolstein O, Furtado MB, Shiratori H, Chien KR, Hamada H, Black BL, Saga Y, Robertson EJ, Buckingham ME, Harvey RP. An nkx2–5/bmp2/smad1 negative feedback loop controls heart progenitor specification and proliferation. Cell 2007;128:947–959. [PubMed: 17350578]

Reifers F, Walsh EC, Leger S, Stainier DY, Brand M. Induction and differentiation of the zebrafish heart requires fibroblast growth factor 8 (fgf8/acerebellar). Development 2000;127:225–235. [PubMed: 10603341]

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]

Ryckebusch L, Wang Z, Bertrand N, Lin S-C, Chi X, Schwartz R, Zaffran S, Niederreither K. Retinoic acid deficiency alters second heart field formation. Proc Natl Acad Sci USA 2008;105:2913–2918. [PubMed: 18287057]

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 Dev 2007;21:1113–1124. [PubMed: 17473173]

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]

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

Srivastava D. Making or breaking the heart: from lineage determination to morphogenesis. Cell 2006;126:1037–1048. [PubMed: 16990131]

Waldo KL, Kumiski DH, Wallis KT, Stadt HA, Hutson MR, Platt DH, Kirby ML. Conotruncal myocardium arises from a secondary heart field. Development 2001;128:3179–3188. [PubMed: 11688566]

Xavier-Neto J, Shapiro MD, Houghton L, Rosenthal N. Sequential programs of retinoic acid synthesis in the myocardial and epicardial layers of the developing avian heart. Dev Biol 2000;219:129–141. [PubMed: 10677260]
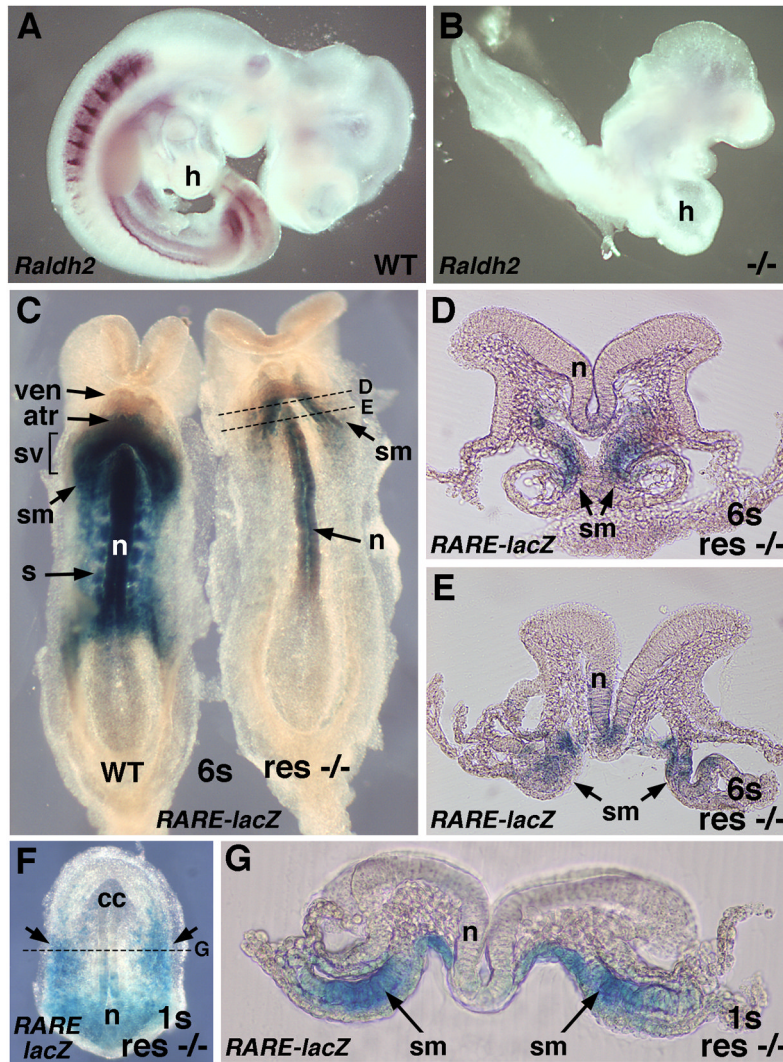
Fig. 1.
RA rescue targets the posterior-medial region of the heart field during heart tube formation.
(A–B) Detection of *Raldh2* mRNA in E9.5 wild-type (WT) and *Raldh2*$^{-/-}$ embryos; mutant
lacks *Raldh2* mRNA and exhibits a medial distended cardiac cavity. (C) RA activity in 6-somite
wild-type and *Raldh2*$^{-/-}$ embryos carrying *RARE-lacZ* RA-reporter transgene following rescue
by maternal dietary RA administration (ventral view); mutant exhibits RA activity only in the
neuroectoderm and posterior-medial region of the heart field. (D–E) Serial transverse sections
through the RA-rescued *Raldh2*$^{-/-}$ embryo indicated in panel C. (F) RA activity in 1-somite
Raldh2$^{-/-}$ embryo rescued by maternal dietary RA administration (ventral view); RA activity
is observed in the posterior region of the heart field and in the posterior neuroectoderm. (G)
Transverse section through the RA-rescued *Raldh2*$^{-/-}$ embryo indicated in panel F. atr, atrial
progenitor; cc, cardiac crescent; h, heart; n, neuroectoderm; s, somite; sm, splanchnic
mesoderm (component of lateral plate mesoderm); sv, sinus venosa (inflow tract); ven,
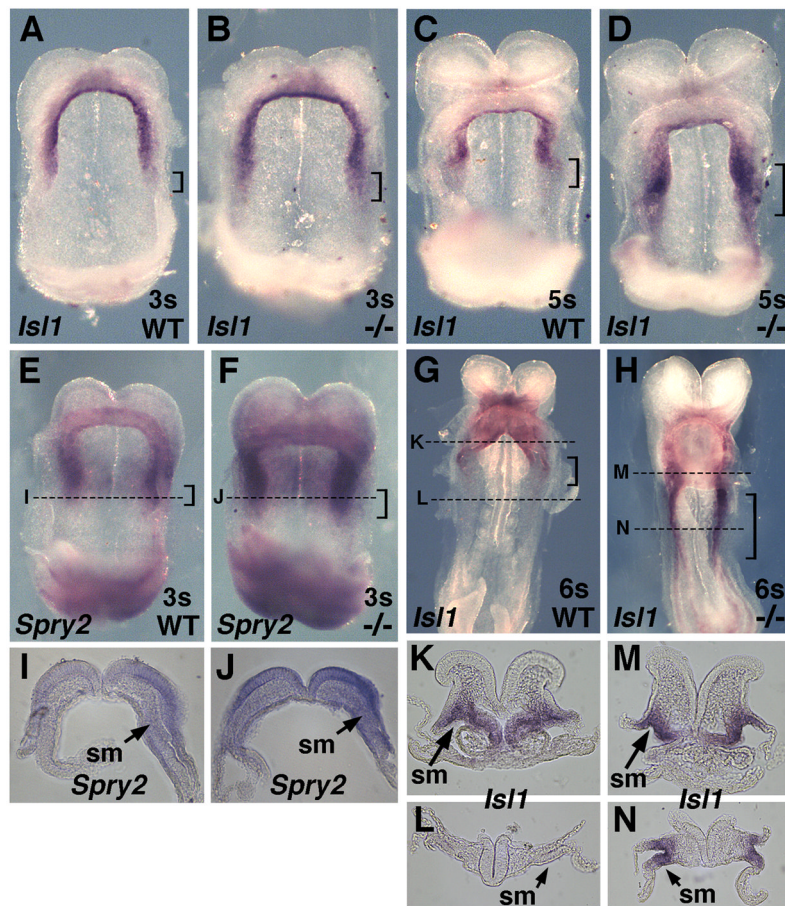ventricular progenitor.

Fig. 2.
Isl1$^+$ cardiac domain expands posteriorly after loss of RA. *Isl1* mRNA in wild-type and *Raldh2*$^{-/-}$ embryos at (A–B) 3-somites, ventral view. and at (C–D) 5-somites, ventral view. Brackets indicate a marked increase in *Isl1* mRNA in the posterior cardiac field of mutants. (E–F) *Spry2* mRNA in 3-somite wild-type and *Raldh2*$^{-/-}$ embryos (ventral view) demonstrating a significant increase in posterior cardiac expression in the mutant (brackets); transverse sections shown in panels I and J. (G–H) *Isl1* mRNA in wild-type and *Raldh2*$^{-/-}$ embryos at 6-somites (ventral view) with brackets pointing out posterior expansion of *Isl1* mRNA in mutant; transverse sections shown in panels K–N. sm, splanchnic mesoderm.
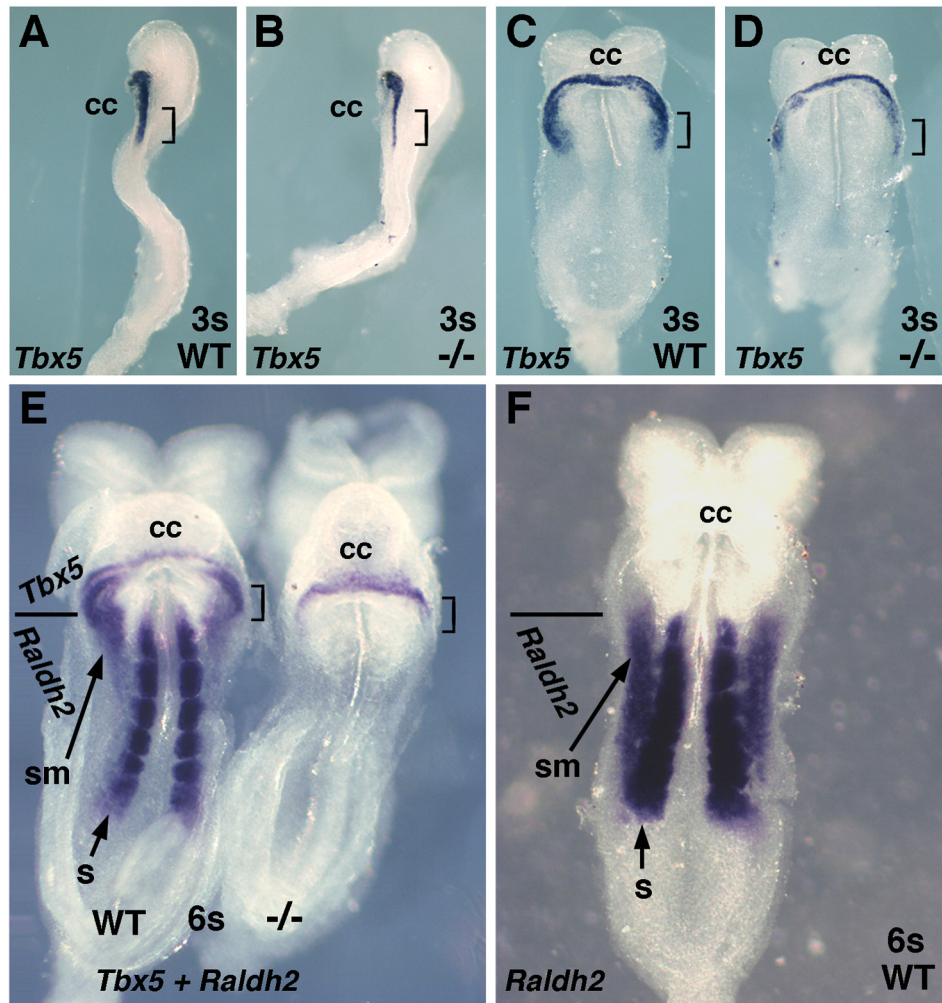
Fig. 3.
Loss of RA reduces the size of the *Tbx5*$^+$ cardiac domain. (A–B) *Tbx5* mRNA in 3-somite wild-type and *Raldh2*$^{-/-}$ embryos (lateral view); brackets point out reduced *Tbx5* mRNA in posterior region of cardiac crescent in mutant. (C–D) Ventral view of 3-somite embryos shown in panels A and B. (E) Double-labeling of *Tbx5* mRNA (anterior) and *Raldh2* mRNA (posterior) in 6-somite wild-type and *Raldh2*$^{-/-}$ embryos (ventral view); mutant lacks *Raldh2* mRNA; brackets identify reduction in posterior-lateral *Tbx5* domain in mutant. (F) Detection of *Raldh2* mRNA in a 6-somite wild-type embryo (compare to panel E). cc, cardiac crescent; s, somite; sm, splanchnic mesoderm.
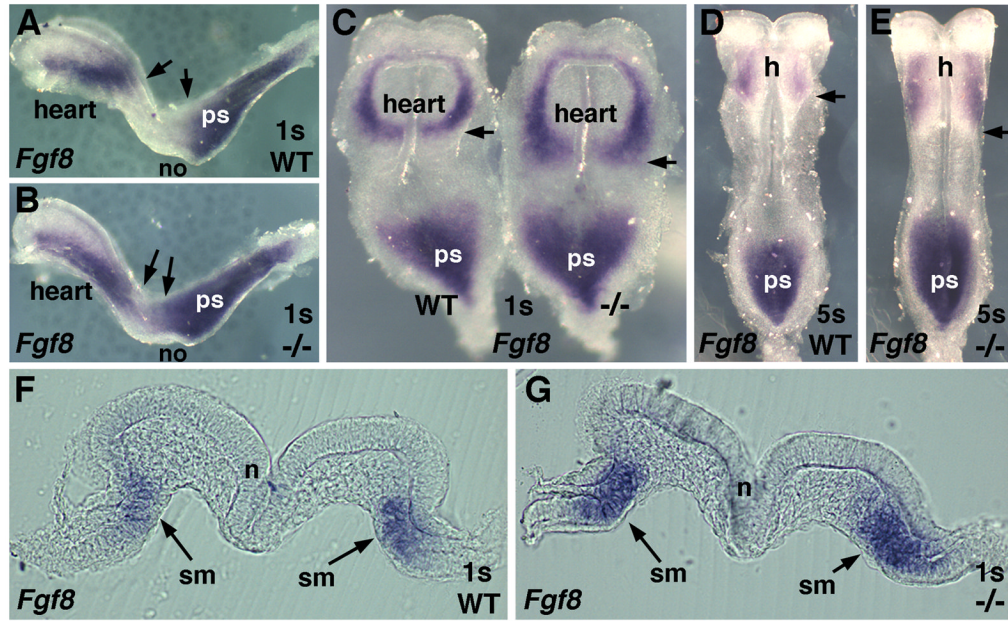
Fig. 4.
Posterior expansion of cardiac *Fgf8* expression following loss of RA signaling. (A–B)
Detection of *Fgf8* mRNA in 1-somite wild-type and *Raldh2*$^{-/-}$ embryos (lateral view); arrows
point out posterior expansion of the cardiac domain and anterior expansion of the primitive
streak domain in the mutant. (C) Ventral view of 1-somite embryos in panels A and B; note
posterior expansion of *Fgf8* mRNA in the heart field. (D–E) Detection of *Fgf8* mRNA in 5-
somite wild-type and *Raldh2*$^{-/-}$ embryos; arrows show posterior expansion in heart field. (F–
G) Transverse sections of 1-somite wild-type and *Raldh2*$^{-/-}$ embryos through posterior region
of *Fgf8*-positive cardiac domain (just below the label 'heart' in embryos shown in panel C). h,
heart; n, neural tube; no, node; ps, primitive streak; s, somite; sm, splanchnic mesoderm.
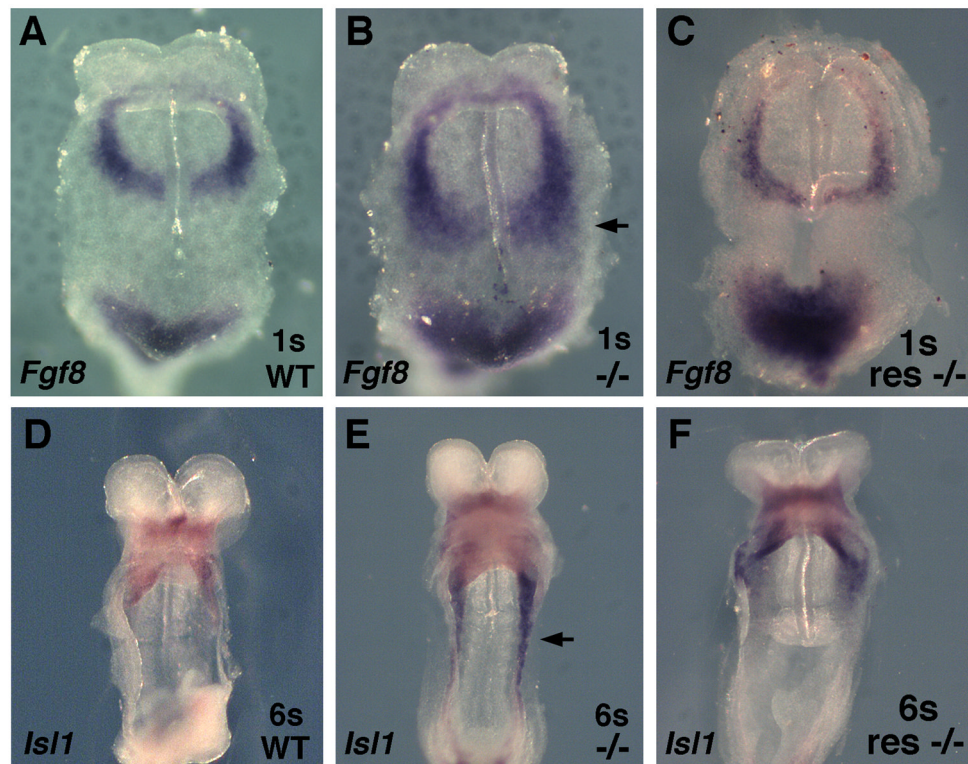
Fig. 5.
RA treatment represses *Fgf8* and *Isl1* expression. (A–C) *Fgf8* mRNA in 1-somite embryos, and (D–F) *Isl1* mRNA in 6-somite embryos; all embryos are displayed from a ventral view to observe the heart field. The arrows indicate posterior expansion of *Fgf8* or *Isl1* expression in untreated *Raldh2*^{−/−} embryos relative to wild-type, but this posterior expansion is prevented in *Raldh2*^{−/−} embryos treated with maternal dietary RA supplementation (res −/−).