Depletion of Retinoic Acid Receptors Initiates a Novel Positive Feedback Mechanism that Promotes Teratogenic Increases in Retinoic Acid

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Abstract

Normal embryonic development and tissue homeostasis require precise levels of retinoic acid (RA) signaling. Despite the importance of appropriate embryonic RA signaling levels, the mechanisms underlying congenital defects due to perturbations of RA signaling are not completely understood. Here, we report that zebrafish embryos deficient for RA receptor \( \beta b1 \) (RAR\( \beta b1 \)), a conserved RAR splice variant, have enlarged hearts with increased cardiomyocyte (CM) specification, which are surprisingly the consequence of increased RA signaling. Importantly, depletion of RAR\( \beta b2 \) or concurrent depletion of RAR\( \beta b1 \) and RAR\( \beta b2 \) also results in increased RA signaling, suggesting this effect is a broader consequence of RAR depletion. Concurrent depletion of RAR\( \beta b1 \) and Cyp26a1, an enzyme that facilitates degradation of RA, and employment of a novel transgenic RA sensor line support the hypothesis that the increases in RA signaling in RAR deficient embryos are the result of increased embryonic RA coupled with compensatory RAR expression. Our results support an intriguing novel mechanism by which depletion of RARs elicits a previously unrecognized positive feedback loop that can result in developmental defects due to teratogenic increases in embryonic RA.

Introduction

Improper retinoic acid (RA) signaling during development can cause congenital malformations that affect the forelimbs, ocular, cardiovascular, respiratory, urogenital and nervous systems [1–4]. Despite almost a century of investigation, the mechanisms underlying many congenital defects due to fluctuations in RA signaling are still not understood. RA acts as a ligand for RA receptors (RARs), members of the nuclear hormone family of transcription factors [5]. Work using disparate embryonic models has provided critical insight into the molecular mechanisms and developmental requirements of RA function in vertebrate embryos [6–12]. In addition, RA deficiency and inappropriate RA signaling are associated with numerous types of cancers [13]. In the majority of cases, the mechanism by which loss of RARs promote tumorigenesis is not understood. Therefore, understanding the roles of RARs during development will help elucidate the mechanisms underlying congenital defects, and possibly cancers, caused by inappropriate RA signaling [3,4].

RA signaling employs a number of feedback mechanisms in order to maintain appropriate levels in the embryo and tissues. The best characterized feedback mechanism is through regulation of the RA producing [retinol dehydrogenases (RDHs) and retinaldehyde dehydrogenases (Aldh1a)] and degrading (Cyp26) enzymes. Specifically, increased RA signaling inhibits the expression of the RA producing enzymes, while promoting Cyp26a1 expression. Conversely, decreased RA signaling promotes expression of the RA producing enzymes, while inhibiting Cyp26a1 expression [14–18]. While modulation of RA signaling also affects the expression of other factors that control RA signaling [5,19], less well understood are feedback mechanisms that may influence RAR expression. RA response elements (RAREs) have been found in murine RAR\( \alpha \)2 and RAR\( \beta \)2 promoters and RAR\( \beta \)2 has been shown to be RA responsive [20–22]. However, if decreases in RA signaling, in particular due to loss of RAR expression, lead to compensatory expression of other RARs is less clear. While initial studies of mouse RAR KO mice suggested that there was not compensatory RAR expression in RAR deficient mice [11,12], more recent studies using siRNA to deplete RAR\( \alpha \)2 have challenged this model and suggested that there may be compensatory RAR expression in RAR\( \alpha \) deficient embryos [23]. Therefore, if there are RA feedback mechanisms that influence RAR expression and how the employment of these feedback mechanisms impact embryonic development are not well understood.

Here, we find that depletion of RAR\( \beta b1 \), a previously unrecognized yet conserved zebrafish RAR\( \beta b1 \) splice variant, causes an increase in CM specification and heart size, which is due to the triggering of a feedback mechanism that surprisingly promotes increased RA signaling from surplus embryonic RA and compensatory RAR expression. Our results provide insight into a newly recognized positive feedback mechanism that we posit resists...
fluctuations in RA signaling due to perturbation in RAR expression. However, if improperly maintained, the positive feedback can result in RA induced congenital defects. Altogether, the results from this study significantly enhance our understanding of the feedback mechanisms that are used to maintain appropriate RA signaling levels and previously unexplored mechanisms that potentially underlie congenital defects.

Results

RARb1 deficient embryos have enlarged hearts and increased CM specification

In contrast to the studies of RARs in mice [9–12], depletion of RARs has not been able to recapitulate all of the consequences of loss of RA signaling in zebrafish [8], which prompted us to determine if additional conserved RAR variants exist in zebrafish beyond what has already been reported [24]. We cloned a previously unrecognized RARz splice variant that is orthologous to human, mouse and Xenopus RARz1 termed RARzb1 (Figure 1A–1C). The previously cloned zebrafish RARz homologs RARza and RARzb are teleost specific paralogs and both are orthologous to the splice variant 2 found in tetrapods (Figure 1B, 1D) [24]. Both rarzb1 and rarzb2 are expressed maternally and zygotically (Figure 1E), with ubiquitous expression until the tailbud stage (Figure S1A–S1I). After the tailbud stage, their expression patterns deviate (Figure 1F–1H and Figure S1J–S1O).

We used a translation blocking morpholino (MO) to examine the function of RARzb1 (Figure 1B). By 48 hours post-fertilization (hpf), RARzb1 deficient embryos had enlarged hearts with increased CM number and expression of CM marker genes myl7, vmhc and amhc (Figure 2A, 2B, 2M, 2N and Figure S2A–S2D). Similar increases in CM number were also found at 55 hpf (Figure S3A–S3C), suggesting the major addition of surplus CMs occurs during earlier stages of development. Consistent with this idea, we observed an expansion of CM differentiation (myl7, vmhc, and amhc) and progenitor (shk2.5 and hand2) marker expression in RARzb1 deficient embryos at earlier stages via in situ hybridization (ISH) and quantitative real-time PCR (qPCR; Figure 2C–2L, 2O–2Q). Injecting the RARzb1 MO along with rarzb1 mRNA that lacks the 5’UTR MO binding sequence is able to rescue the increased heart size, supporting the specificity of the phenotype (Figure S4A–S4D). Together, these results suggest that RARzb1 deficient embryos have increased CM specification, number and heart size.

Depletion of zebrafish RARzb paralogs promotes RA signaling

The increased atrial and ventricular CM number in RARzb1 deficient embryos are reminiscent of RA signaling deficient embryos [25,26]. Therefore, we examined hoxb5b expression, which functions downstream of RA signaling to restrict atrial CM number [26] and is likely a direct target of RARs (Figure S5A–S5D). Unexpectedly, we found that hoxb5b expression was increased in RARzb1 deficient embryos (Figure 3A–3C). While this was initially perplexing, our recent studies showed that Hoxb5b overexpression is able to mimic many of the teratogenic effects of RA treatment [27]. Therefore, we asked if the increases in hoxb5b expression in RARzb1 deficient embryos could be a cause of the enlarged hearts. While depletion of hoxb5b alone using a low concentration of hoxb5b MO does not affect CM number (Figure S6A–S6C), we found that concurrent depletion of RARzb1 and Hoxb5b largely restored heart morphology, CM differentiation marker expression, and CM number relative to the RARzb1 deficient embryos (Figure 3F–3N), suggesting that the increased CM number in RARzb1 deficient embryos is in part a consequence of the increased hoxb5b expression.

We next examined the expression of additional RA signaling responsive genes. Similar to hoxb5b, we found that the expression of additional RA signaling responsive genes, including cyph26a1, dhsx3a, hoxb6b and hoxb5a, was increased in RARzb1 deficient embryos (Figure 3A). Comparing RA responsive gene expression in RA treated and RARzb1 deficient embryos, we found that the trends were similar, but that RA treatment typically induced a greater increase in expression (Figure 3A). Conversely, treatment with DEAB, an antagonist of the RA producing enzyme Aldh1a, inhibited RA responsive gene expression (Figure 3A). These findings indicate that RARzb1 depletion paradoxically results in increased expression of RA signaling responsive genes.

We next wanted to determine if increases in RA signaling responsive genes were specific to RARzb1 depletion, so we examined RA responsive gene expression in RARzb2 deficient embryos. Previous studies found that RARzb2 deficient embryos lack forelimbs (pectoral fins) and dhsx5a expression [8,28], which we confirmed (Figure S7A, S7C, S7D, S7F, S7H, S7I). However, similar to RARzb1 depletion (Figure 3A and Figure 4A), RARzb2 deficient embryos had increased expression of RA signaling responsive genes (Figure 4A). While the previous studies found a loss of forelimbs, defects in heart development were not reported. Despite the loss of forelimbs and increase in RA signaling responsive genes, we did not observe an increase in heart size, CM number or CM gene expression (Figure S8A–S8D). Therefore, although eliciting similar increases in RA signaling responsive gene expression, individual depletion of RARzb1 and RARzb2 results in distinct defects.

To determine the functional consequences of concurrent RARzb1 and RARzb2 depletion, we co-injected a suboptimal dose of each MO. Unfortunately, co-injection of an optimal dose of each MO resulted in significant non-specific toxicity even when injected along with p53 MO. However, concurrent depletion of the RARzb2 using suboptimal MO doses resulted in a dramatic increase in RA signaling responsive genes, above what was seen with depletion of RARzb1 and RARzb2 alone using the optimum
MO doses (Figure 4A). Additionally, there was an anterior shift of hoxb5a expression in the spinal cord of RAR\(\alpha\)b\(1+2\) deficient embryos, suggesting the spinal cords are posteriorized (Figure S9A–S9E). Increased RA signaling inhibits aldh1a2 expression through a negative feedback mechanism [16–18]. Although aldh1a2 expression in individual RAR\(\alpha\)b\(1\) and RAR\(\alpha\)b\(2\) deficient embryos was not suppressed (Figure 4B), aldh1a2 expression was decreased in embryos depleted for both RAR\(\alpha\)b variants (Figure 4B). To corroborate the increases in endogenous RA signaling responsive genes, we used the RA signaling reporter line Tg(12XRARE-ef1a:EGFP)sk72 [29]. Again, co-depletion of both RAR\(\alpha\)bs resulted in a greater expansion of egfp expression, compared to the individual depletion of each RARb (Figure 4D–4H). Therefore, these experiments support the hypothesis that the RAR\(\alpha\)b\(1+2\) deficient embryos are sensing more significant increases in RA signaling than embryos deficient for either RAR\(\alpha\)b variant alone.

We next examined the consequences of this functional interaction on heart development. We found that the hearts of RAR\(\alpha\)b\(1+2\) deficient embryos had increased atrial size, CM number, and a dramatic increase in amhc expression (Figure 4I, 4L–4N and Figure S10A–S10D). Significant effects on CM number or heart size were not found when using a suboptimal dose of either RAR\(\alpha\)b\(1\) or RAR\(\alpha\)b\(2\) MO alone (Figure 4I–4K, 4M), though we did find a modest increase in CM marker gene expression in the RAR\(\alpha\)b\(1\) deficient embryos (Figure 4N). Interestingly, in RAR\(\alpha\)b\(1+2\) deficient embryos we found more significant increases in atrial CM number and amhc expression (Figure 4M, 4N), which were remarkably similar to the consequences of modest increases in RA signaling due to RA treatment [27]. Increased RA signaling can also inhibit forelimb development [17] and RAR\(\alpha\)b deficient embryos also have smaller forelimbs and a modest reduction of tbx5a expression (Figure S7A, S7B, S7D, S7F, S7G, S7I). A functional interaction...
with the RAR\textsubscript{a} variants that resulted in loss of forelimbs was also observed (Figure S7D, S7E). Therefore, concurrent depletion of RAR\textsubscript{a} variants elicits increases in RA signaling with heart and forelimb phenotypes that are strikingly similar to increases in RA signaling caused from RA treatment.

RAR\textsubscript{a}b deficient embryos have increased embryonic RA

We sought to understand the mechanism underlying the increase in RA signaling in RAR\textsubscript{a}b deficient embryos. In the absence of RA, RARs are thought to interact with transcriptional co-repressors, while binding of RA converts the RARs to transcriptional activators [1,5]. A previous study in Xenopus suggested that RARs are required as transcriptional repressors in some developmental contexts [6]. However, our gain-of-function analysis did not support that these zebrafish RARs function as transcriptional repressors (Figure S11A–S11L), consistent with what we have reported previously [29]. However, Manshouri et al. [23] found a compensatory increase in the expression of other RARs when using siRNA to deplete RAR\textsubscript{a} in mice. Similarly, we found that the expression of other zebrafish RARs [24] was...
increased in RARβ-deficient embryos (Figure 4C and Figure S12A–S12L), suggesting that compensatory RAR expression is a conserved response to depletion of RARα homologs in vertebrates. Although Manshouri et al. [23] proposed the compensatory RAR expression was RA signaling dependent, our results suggest that the expression of most RARs is potentially regulated independent of RA signaling (Figure 4C), because the effects on RAR expression did not parallel modulation of RA signaling using RA and DEAB. While we observed compensatory expression of other RARs in RARαβ-deficient embryos, it is difficult to conclude that increased RAR expression is the sole cause of the increase in RA signaling since overexpression of RARs in zebrafish embryos does not produce significant positive or negative effects on RA responsive gene expression (Figure S11A–S11J) [29]. Nevertheless, our results suggest that when depleting RARαβs in zebrafish embryos compensatory RARs are present that can mediate RA signaling.

Because we did not have evidence that RARs act as transcriptional repressors or that the increased expression of RARs alone contributes to the increases in RA signaling in RARαβ-deficient embryos, we hypothesized that the depletion of RARs may trigger an increase in embryonic RA. Although aldhl1a2 expression was suppressed in RARαβ1 and RARαβ1+2 deficient embryos similar to when embryos sense increases in RA signaling (Figure 4B) [16–18], the expression of rdh10a and rdh10b, which control a limiting step in RA production in vertebrates by generating retinal from retinol [14,15], was increased in RARαβ1 and RARαβ1+2 depleted embryos (Figure 4B and Fig. S13A–S13C). Interestingly, rdh10b expression, which was not sensitive to modulation of RA signaling, was increased in RARαβ deficient embryos (Figure 4B). Therefore, our results suggest that depletion of RARαβs triggers an increase in RA through promoting rdh10 expression.
In addition to inhibiting aldhl2 expression, increased RA signaling promotes a negative feedback mechanism that limits RA levels by positively regulating Cyp26a1 expression [16–18]. Since we observe an increase in cyp26a1 expression in RARa b1 deficient embryos (Figure 3A, 3D, 3E and Figure 4A), which was also consistent with the hypothesis that there is increased embryonic RA, we postulated that the increased Cyp26a1 may be protecting the RARa b1 deficient embryos from teratogenic increases in embryonic RA. Therefore, we concurrently depleted RARa b1 and Cyp26a1 to determine if there was a functional interaction indicative of increased embryonic RA. For these experiments, a suboptimal dose of cyp26a1 MOs (Figure S14A–S14E) was used to more easily discern a functional interaction. In either the RARa b1 or Cyp26a1 deficient embryos alone, we never observed absence of the MHB or defects in tail elongation (Figure 5A–5C, 5E–5G). However, co-depletion of RARa b1 and Cyp26a1 resulted in a loss of the MHB and truncated tails (Figure 5D, 5H), similar to increases in RA signaling [17,19,29,30]. Furthermore, we found that RARa b1+Cyp26a1 deficient embryos had dismorphic hearts with a specific reduction in ventricular CM number compared to controls embryos hearts (Figure 5I–5L, 5Q), which interestingly resembles the trend we previously found in embryos with intermediate increases in RA signaling [27].

Figure 4. Concurrent depletion of RARa b1 and RARa b2 promotes increased RA signaling and atrial CM number. qPCR for (A) RA signaling responsive gene, (B) RA metabolizing gene, and (C) zebrafish rar expression in control sibling, RARa b1 deficient, RARa b2 deficient, RARa b1+RARa b2 (suboptimal doses) deficient, RA treated, and DEAB treated embryos at the 8 s stage. (D–G) ISH for egfp expression in Tg(12XRARE-et1a:EGFP) embryos. Brackets indicate the length of egfp expression in the spinal cord. (H) Measurements of the length in arbitrary units (AU) of egfp expression in the spinal cord of Tg(12XRARE-et1a:EGFP) embryos. (I–L) Hearts from control and RARa b depleted Tg(-5.1myl7:DsRed-NLS)f2 embryos. Images are frontal views. Red indicates ventricle. Green indicates atrium. (M) Mean CM number from Tg(-5.1myl7:DsRed-NLS)f2 hearts at 48 hpf. (N) qPCR for CM marker gene expression at 48 hpf. While modest increases in vmhc expression in RARa b1+RARa b2 deficient embryos were observed relative to RARa b1 (suboptimal dose) deficient embryos, corresponding increases in ventricular CM number were not observed.

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Although one interpretation of the functional interaction of RARαb1 and Cyp26a1 depletion is that there is increased embryonic RA levels in these embryos, we wanted to further test this hypothesis using additional assays. First, we sought to use a distinct readout of embryonic RA, so we made a novel stable transgenic RA sensor line which incorporated the RARαb ligand binding domain (RLBD) fused to the Gal4 DNA binding domain (GDBD) expressed under the β-actin promoter (Figure S15A–S15G) [31]. Previous studies have found that similar GDBD fusions with nuclear hormone receptor LBDs create an effective reporter of nuclear hormone activity [6,32,33]. We observed a dramatic increase in reporter expression when RARαb1 and Cyp26a1 were depleted together in Tg(β-actin:GDBD-RLBD)−/−; Tg(UAS:EGFP) embryos. Images are frontal views. Red indicates ventricle. Green indicates atrium. (M–P) ISH for egfp in Tg(β-actin:GDBD-RLBD)−/−; Tg(UAS:EGFP) embryos. Lateral views with dorsal right and anterior up. (Q) Mean CM number at 48 hpf and (R) qPCR for egfp expression at 15 s in control sibling, RARαb1 deficient, Cyp26a1 deficient, and RARαb1+Cyp26a1 deficient embryos. Double asterisks in Q indicate a statistically significant difference relative to control and RARαb1 deficient embryos. Pound sign in Q indicates a statistically significant difference relative to RARαb1 deficient embryos.

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Figure 5. Concurrent depletion of RARαb1 and Cyp26a1 results in phenotypes resembling RA treatment. (A–D) Control sibling, RARαb1 deficient, Cyp26a1 deficient, and RARαb1+Cyp26a1 deficient embryos. A suboptimal dose of the cyp26a1 MOs was used that did not cause ostensible defects for these experiments. In D, arrow indicates loss of the MHB and line indicates shortened tail. Images are lateral views with dorsal right and anterior up. (E–H) ISH for eng2a, which marks the MHB. 100% of (E) control sibling (n = 11), (F) RARαb1 deficient (n = 7), and (G) Cyp26a1 deficient (n = 7) had eng2a expression. 85% of (H) RARαb1+Cyp26a1 deficient embryos (n = 7) had a complete absence of eng2a expression (arrow in H). Equivalent results were obtained using pax2a, which also marks the MHB (data not shown). (I–L) Hearts from control sibling, RARαb1 deficient, Cyp26a1 deficient, and RARαb1+Cyp26a1 deficient embryos. (M–P) ISH for egfp in Tg(β-actin:GDBD-RLBD)−/−; Tg(UAS:EGFP) embryos. Lateral views with dorsal right and anterior up. (Q) Mean CM number at 48 hpf and (R) qPCR for egfp expression at 15 s in control sibling, RARαb1 deficient, Cyp26a1 deficient, and RARαb1+Cyp26a1 deficient embryos. Double asterisks in Q indicate a statistically significant difference relative to control and RARαb1 deficient embryos. Pound sign in Q indicates a statistically significant difference relative to RARαb1 deficient embryos.

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Discussion

Together, our study supports a novel paradigm whereby RARαb depletion elicits a positive feedback mechanism that can result in teratogenic increases in RA signaling. Importantly, our
work highlights that loss and gain of RA signaling can cause similar developmental defects. RA signaling is required to restrict CM specification [25,26], while high increases in RA signaling can eliminate CM specification (Figure 7A) [27]. However, our present findings suggest that low increases in RA signaling, achieved when treating embryos with mM concentrations of RA or through RARαb depletion, can also promote increases in both atrial and ventricular CM specification (Figure 7A). As we found previously, modest, but slightly higher increases of RA signaling can promote atrial CM specification without significantly affecting ventricular CM specification [27], which is strikingly similar to what we found with concurrent depletion of the RARβ variants here (Figure 7A). Moreover, intermediate increases in RA signaling can inhibit ventricular CM specification, which is similar what we observed when concurrently depleting RARβ1 and Cyp26a1 (Figure 7A). It also appears that modulation of Hox activity downstream of both gain and loss RA signaling is at least partially responsible for the increases in CM specification, suggesting the hypothesis that the similar effects on CM number are actually due to opposite perturbations of anterior-posterior patterning within the ALPM. Therefore, our analysis corroborates and extends previous observations that there are differential effects on atrial and ventricular CM populations as there is a progressive increase from low to intermediate levels of RA signaling in the early embryo.

It is interesting that depletion of RARα homologs using MOs in zebrafish, presented in this study, and Xenopus [6] elicit similar phenotypic responses. In Xenopus embryos, RARα depletion alone results in loss of the MHB [6]. While depletion of RARβ1 alone does not result in MHB defects in zebrafish embryos, we have found that RARβ1+Cyp26a1 deficient embryos completely lack the MHB. Taken together, these results suggest that the underlying consequences of increased RA signaling due to depletion of RARα homologs are likely conserved at least in Xenopus and zebrafish embryos, but that in Xenopus perhaps the role of Cyp26 enzymes in protecting the brain has been lost. Despite similarities in the phenotypes that both point to an increase in RA signaling in RARβ and RARβ1 deficient Xenopus and zebrafish embryos, our results contrast with the model proposed by Koide et al. [6], which concluded that RARs are required to function as transcriptional repressors. Importantly, the tools used in the previous study, including dominant-negative RARs, transcriptional co-repressors, and
inverse agonists, would not have allowed them to distinguish between a transcriptional de-repressive model and the positive feedback mechanism involving the production of excess RA supported here.

In addition to the phenotypic similarities when depleting RARα homologs in Xenopus and zebrafish, depletion of zebrafish RARαbs results in compensatory RAR expression similar to RARα depletion in mice [23], supporting the hypothesis that this feedback response to RARα deficiency is conserved in vertebrates. Importantly, the response to RAR depletion is likely different than complete ablation of RARs. RAR KO mice have not been reported to have compensatory increases in other RARs [11,12], suggesting that a complete loss of RAR expression may cause a breakdown of this feedback loop. However, when considering the probability that RAR expression would be completely lost vs. depleted, we postulate that insults resulting in depletion of RAR expression would be much more likely. Consistent with this idea, variable levels of RAR expression deficiency, which in the case of RARβ can be due to epigenetic silencing, is commonly observed in a variety of cancers [13].

Given the conserved feedback mechanisms already recognized that limit fluctuations in RA signaling in vertebrates [16,17,19,23], it seems logical that a conserved mechanism that senses RAR deficiency would also exist to prevent loss of RA signaling. We propose that this newly recognized positive feedback mechanism would be more suitable to prevent transient deficiency in RARs. As demonstrated here, persistent RARαb depletion can result in a hypervigilant response of RA signaling and RA-induced teratogenic defects. Overall, these data provide insight into a previously unappreciated RAR-dependent positive feedback mechanism (Figure 7B), which is active during development. Further elucidation of this RA signaling feedback mechanism may illuminate the etiology of poorly understood RA-insensitive cancers [13,23] and congenital defects [1,3].

Materials and Methods

Ethics Statement

All zebrafish husbandry and experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Cincinnati Children's Hospital Medical Center.

Zebrafish husbandry and transgenic lines

Zebrafish (Danio rerio) were raised and maintained as previously described [35]. The following transgenic lines were used: Tg(-5.1myl7:DsRed-NLS) [36], Tg(-5.1myl7:EGFP)pres26 [37], Tg(12XRARE-ef1a:EGFP)sk72 [29], Tg(β-actin:CDBD-RLBD)ch1 (was created using the Gateway/Tol2 system [38] and additional characterization is reported in [31]), Tg(UAS:EGFP) [34], and Tg(UAS:nfsB-mcherry) [39].

ISH

Whole-mount ISH was carried out using standard procedures [40]. All probes except rarαb1 (accession number: KF030797) and rarαb2 were reported previously. myl7 (formerly called omc2; ZDB-GENE-991019-3), amhc (ZDB-GENE-031112-1), vmyc (ZDB-GENE-991123-5), nkk2.5 (ZDB-GENE-980526-321), hand2 (ZDB-GENE-000511-1), hoxb5a (ZDB-GENE-980526-70), hoxb5b (ZDB-GENE-000823-6), dhrs3a (ZDB-GENE-040801-217), cyp26a1 (ZDB-GENE-990415-44), rarαb1/2 (which recognizes both isoforms and was formerly called rarαb [24]; ZDB-GENE-980526-72), rarαa (ZDB-GENE-980526-284), varga (ZDB-GENE-980526-331), vargb (ZDB-GENE-070314-1), rdh16a (ZDB-GENE-070112-2242), tbx5a (ZDB-GENE-030909-7), eng2a (ZDB-GENE-980526-167), egf2b (formerly called krox20; ZDB-GENE-980526-283), egfβ (accession number: JQ064510.1), and mcherry (accession number: JN795134.1).
MO and mRNA injections

The *rar* 
1 MO (5'-TGCAAGTCATGCATGTTGCTG-3') was designed to the 5' UTR of *rar*. Additional MOs targeting another region of the 5' UTR and the donor splice junction, which saturated the available MO target sites, were also tried. However, injection of these MOs resulted in significant toxicity and were not able to be used for analysis. Sequences to the *rar* MOs were reported previously [8,26]. The total amount of *rar* MO injected was 16 ng. The total amount of *rar* MO injected was 7 ng. The suboptimal doses used to test genetic interactions were half these concentrations. The amount of *hsa* MO used was 0.25 ng. A cocktail of 4 ng *cyp26a1* MO1 (5'-CTTATCATCCTTACCTTTTTG G-3') and 2 ng *cyp26a1* MO2 (5'-TACCTACCTAGTCTTCTTTTCTTG-3') produced a phenotype similar to *gr* mutants [17]. Suboptimal doses used in experiments were 0.9 ng (cyp26a1 MO1) and 0.45 ng of (cyp26a1 MO2). For all injection experiments, 3 ng of p35 MO were used to help suppress non-specific MO-induced cell death [41]. For experiments, the total amount of MO injected was always kept constant by equilibrating the concentrations with Standard Control MO (Gene Tools).

Capped mRNA was made using a Message Machine Kit (Ambion). 150 pg of mRNA was used for over-expression of all mRNAs in all experiments.

Cell culture and luciferase assay

Luciferase reporter assays were performed in HEK 293 cells as previously described [29].

Western blot analysis and ChIP

Western blots were performed as previously described [29]. Mouse monoclonal anti-myc antibody (Covance) was used for both Western blot analysis and ChIP experiments. The dynabeads (Invitrogen) ChIP protocol was adapted from the Dorsky Lab (University of Utah) ZFN Protocol. qPCR was used to quantify the enrichment of the fragment containing the RARE (DR5) in embryos injected with the *myc-rar* mRNA with respect to control un.injected embryos.

Comparison of genomic sequences

The genomic sequence flanking zebrafish *hsa* (−8 to +8 kb) was compared with the corresponding region for *Hoxb5* in mouse using mVista. NHR SCAN was used to identify binding sites for nuclear receptor.

Identification of *rar* and RT-PCR

*Rar* was identified by using BLAST against the zebrafish genome (Ensemble_V7) with the human and mouse RAR A domains. MacVector was used for sequences alignments. For RT-PCR, primer pairs were designed so that they specifically recognized *rar* and *rar* (Figure 1B). Primer sequences are available upon request.

Cloning

The full-length coding sequence for *rar* was cloned into pCS2+. The *rar* construct used for overexpression was reported previously [29]. The myc tagged *rar* was made using the pCS2+MT vector. For *rar* and *rar* probes, 536 base pairs (bps) of *rar* and 443 bps of *rar*, which include the 5' untranslated region (UTR) and the specific A domains with no overlap, were cloned (Figure 1B). These fragments were cloned into pGEM-T easy (Promega).

qPCR

Total RNA was isolated from 25 embryos, homogenized in TRIzol (Ambion) and collected using Pure link RNA Micro Kit (Invitrogen). 1 µg or 0.5 µg mRNA was used for cDNA synthesis using the Thermoscript Reverse Transcriptase kit (Invitrogen). Quantitative real time PCR (qPCR) for *mif*, *mif*, *mifs*, and *kin22*, *hoxb5a*, *hoxb5b*, *hoxb5a*, *cyp26a1*, *aldh1a2*, *rdh10a*, *rdh10b*, *xor*, *rar*, *rar*, *rar*, *egf* and *mcherry* was performed using standard PCR conditions in a Bio-Rad CFX PCR machine with Power SYBR Green PCR Master Mix (Applied Biosystems). Expression levels were standardized to *ef1a* expression and all the data were analyzed using the 2^(-ΔΔCt) Livak Method. All experiments were performed in a biological triplicate. Primer sequences are available upon request.

Area and length measurements

Areas of *mif*, *mif* and *mifs* expressing cells were measured using ImageJ and statistical analysis was performed as previously described [26]. Length of *egfp* expression and distance between *hsa* and *egfb* were measured also using ImageJ and statistical analysis was performed as previously described.

Imaging of zebrafish heart and cell counting

Immunohistochemistry, cell counting and statistical analysis were done as previously described [26].

RA and DEAB treatment

RA and DEAB, treatment of embryos was done as previously described [26,27]. Embryos that have been used for gene expression analysis at 8 somites were treated with 1 µM DEAB, an Aldh1a2 inhibitor, beginning at 40% epiboly or with 1 µM RA for 1 hr beginning at 40% epiboly. For analysis of the effects of low concentrations of RA on heart development, embryos were treated with 0.05 µM RA for 1 hr beginning at 40% epiboly and harvested at 48 hpf. For rescue experiments related to the heart phenotype of RAR deficient embryos, embryos were treated with 0.025 µM DEAB beginning at 40% epiboly until 24 hpf. For rescue experiments related to the MHB in RAR deficient embryos, embryos were treated with 0.25 µM DEAB.

Statistical analysis

To assess whether the means of two groups are statistically different from each other, we applied the Student's t-test. A p value of <0.05 was considered statistically significant.

Supporting Information

**Figure S1** Comparison of RAR and RAR expression. (A, D, G, J, M) RAR expression. (B, E, H, K, N) RAR expression. (C, F, I, L, O) RAR expression. Arrow in C indicates hpd expression and all views are lateral. Arrows in M and N indicate hindbrain and anterior spinal cord expression. Arrowheads in K and L indicate hindbrain and anterior spinal cord expression. Arrows in M and N indicate differences in the expression of the developing tail. In A–O, all views are lateral. In D–O, dorsal is to the right.

**Figure S2** RAR deficient embryos have enlarged hearts at 72 hpf. (A) Control sibling Tg(-5.1myl7:GFP) embryo. (C) RAR deficient Tg(-5.1myl7:GFP) embryo. Arrow in C indicates pericardial edema with enlarged heart. (B, D) Higher magnification images of the fluorescent hearts of the Tg(-5.1myl7:GFP) control sibling and RAR deficient Tg(-5.1myl7:GFP) embryos in A and C. Images are lateral views with dorsal up and anterior right.
Figure S3  RARb1 deficient embryos have enlarged hearts with increased CM number at 48 hpf. (A, B) Hearts from control sibling and RARb1 deficient Tg(-5.1myl7:DsRed-NLS) embryos at 48 hpf. Images are frontal views. Red indicates ventricle. Green indicates atrium. (C) Mean CM number at 48 hpf. (TIF)

Figure S4  Specificity controls for the translation blocking rarb1 MO. (A–C) Control sibling, RARb1 deficient, and RARb1 deficient+rarb1 mRNA injected embryos. Images are lateral views with anterior right at 48 hpf. Red outline indicates ventricles. Green indicates atrium. Arrow indicates ecnode from control sibling. Arrow indicates a small heart from RARb1 deficient embryo. (D) qPCR for CM marker gene expression in 48 hpf control sibling, RARb1 deficient, RARb1 deficient embryos+rarb1 mRNA, and RARb1 deficient embryos+kaede (control) mRNA injected embryos at 48 hpf. Pound sign indicates a statistically significant difference compared to RARb1 deficient and RARb1 deficient embryos+kaede (control) mRNA injected embryos (p<0.05). (TIF)

Figure S5  RARs can directly bind the RA response element (RARE) in the zebrafish hoxb5b regulatory region. (A) mVista sequence alignment of mouse Hoxb5 and zebrafish hoxb5b genomic regions. Purple boxes represent exons. Light blue boxes indicates 5’ and 3’ UTR. Peaks represents levels of sequence identity in a 50 bp window. Purple peaks are conserved regions in exons. Light blue peaks are conserved regions in 5’ UTR. Pink peaks are conserved non-coding sequences. Arrow indicates the presence of a RARE in the conserved sequence between 4 kb and 4.5 kb identified previously [42], which we confirmed using the NHR Scan database. (B) Sequence conservation (red) between mouse and zebrafish DR5 RARE. (C) Western blot for myc-tagged RARb1. (D) ChIP from control sibling and myc-rarb1 mRNA injected embryos. Negative control amhc primers did not detect any enrichment (data not shown). (TIF)

Figure S6  A suboptimal dose of hoxb5b MO does not affect CM cell number at 48 hpf. (A, B) Hearts from control sibling and Hoxb5b deficient Tg(-5.1myl7:DsRed-NLS) embryos at 48 hpf. Images are frontal views. Red indicates ventricle. Green indicates atrium. (C) Mean CM number at 48 hpf. (TIF)

Figure S7  RARb1 and RARb2 function partially redundantly to promote proper heart development. (A–C) Control sibling, RARb1 deficient, and RARb2 deficient embryos. Images in A–C are dorsal views with anterior to the left. Arrows in B indicate smaller forelimbs. (D) Percentage of control sibling (n = 20), RARb1 deficient (n = 20), and RARb2 deficient (n = 20) embryos with normal, small or no forelimbs. An optimal dose of the rarb1 and rarb2 MOs was used for experiments in D. (E) Percentage of embryos by normal, small, or no forelimbs after injection with a suboptimal dose of rarb1 MO (n = 28), a suboptimal dose of rarb2 MO (n = 26), and co-injected with suboptimal doses of the rarb1 and rarb2 MOs (n = 17). (F–H)ISH of tbx5a, a forelimb marker, in control sibling, RARb1 deficient, RARb2 deficient embryos. Arrows in F–H indicate tbx5a expression the LPM. (I) Areas of the amount of cells expressing the tbx5a at 24 hpf. (TIF)

Figure S8  RARb2 deficient embryos do not have enlarged hearts. (A, B) Hearts from control sibling and RARb2 deficient Tg(-5.1myl7:DsRed-NLS) embryos at 48 hpf. Images are frontal views. Red indicates ventricle. Green indicates atrium. (C) Mean CM number from the hearts of control sibling and RARb2 deficient Tg(-5.1myl7:DsRed-NLS) embryos at 48 hpf. (D) qPCR for CM marker gene expression in control sibling and RARb2 deficient embryos at 48 hpf. We do find a modest decrease in CM number (C) and myl7 expression (D), which is likely due to a very modest amount of MO-induced toxicity. (TIF)

Figure S9  Patterning of the spinal cord is affected in the RARb1+RARb2 deficient embryos. (A–D) Hoxb3a (spinal cord) and egr2b (rhombomeres 3+5) expression in control (n = 32), RARb1 deficient (n = 23), RARb2 deficient (n = 16), and RARb1+RARb2 deficient embryos (n = 19). (E) Measurements of the distance in arbitrary units (AU) between hoxb5a and egr2b expression. Expression of hoxb3a in the spinal cord is expanded rostrally. The rostral expansion of hoxb3a in RARb1 deficient embryos trends similarly as RARb2 deficient and RARb1+RARb2 deficient embryos, but it is not statistically significant (p = 0.06). (TIF)

Figure S10  RARb1 and RARb2 function partially redundantly to promote proper heart development. (A–D) Control sibling, RARb1 deficient (suboptimal dose), RARb2 deficient (suboptimal dose), and RARb1+RARb2 (suboptimal doses) deficient embryos at the 72 hpf. Arrow in D indicates pericardial edema and the enlarged heart. (TIF)

Figure S11  Rarb1 and rarb2 mRNA overexpression do not significantly affect RA responsive genes. (A–J) ISH for the RA responsive genes cyp26a1, dbr3a, and hoxb5b at 8 s. (A, D, G) Control sibling, (B, E, H) rarb1 mRNA, and (C, F, I) rarb2 mRNA injected embryos. Injection of either rarb mRNA did not inhibit RA responsive gene expression. Images in A–C are lateral views with anterior up and dorsal right. Images in D–I are dorsal views with anterior up. (TIF)

Figure S12  RAR expression in RARb1, RARb2, or RARb1+2 deficient embryos. (A, B) ISH for rarb1 in RARb1+2 deficient embryos. (C, D) ISH for rarb2 in RARb1 deficient embryos. (E, F) ISH for rara2 in RARb1+2 deficient embryos. (G, H) ISH for rara1 in RARb1+2 deficient embryos. (I, L) ISH for rarb1 in RARb1+2 deficient embryos. RAR expression is often expanded in the tailbud region of embryos deficient for the other RAR homologs, while additional regions also appear to have increased or low levels of ectopic expression. All views are lateral with dorsal right at 8 s. Arrows in A–H indicate distance of expression in the tail. Arrowheads in F, H, L indicate regions of increased or ectopic expression. (TIF)

Figure S13  Rdhl10a expression in RARb1+2 deficient embryos. (A–D) ISH for rdhl10a in RARb1+2 deficient embryos at 8 somites. (A, B) Lateral views with dorsal right. (C, D) Dorsal views with anterior up. Brackets indicate expansion of rdhl10a in the ALPM. Arrow indicates increased expression in the somites. (TIF)
Figure S14 Characterization of cyp26a1 splice-blocking MOs used in experiments. (A) Schematic of the cyp26a1 locus and the intron-exon boundaries targeted by the different cyp26a1 MOs. Black bar indicates MO1, Red bar indicates MO2. MO1 primarily causes usage of two in-frame cryptic splice sites. Dashed lines indicate the alternate introns caused by the cryptic splices induced from MO1. MO2 causes the introduction of a premature stop codon (red X). (B) RT-PCR for the WT cyp26a1 transcripts and alternate transcripts induced from the different MOs. U and L indicate bands depicted in A. (C) Control sibling embryo. (D) Embryos injected with cocktail of cyp26a1 MO1+2. Co-injection of cyp26a1 MO1 and MO2 causes a phenotype equivalent to or stronger than the cyp26a1/giraffe (gir) mutant (E). Injection of the individual MOs causes the phenotypes consistent with cyp26a1 loss of function at low frequency (data not shown). A suboptimal dose of the cyp26a1 MO cocktail was used for functional interaction experiments with RARzb1 (Figure 4). Arrows in D and E indicate shortened tail. Views in C–E are lateral with anterior right.

(TIF)

Figure S15 Characterization of the novel transgenic RA sensor line. (A) Schematic of the RAR domains and the Gal4 DNA binding domain (GDBD)/RAR ligand binding domain (RLBD) fusion protein. Grey indicates the GDBD. Yellow indicates the RLBD. D is a linker domain and F is a domain with unknown function (as in Figure 1). (B, C) Schematics representing the GDBD-RLBD fusion acting on the Gal4-UAS:EGFP transgene. The GDBD-RLBD is expressed under the β-actin promoter. (B) In the absence of RA, egfp is not expressed. (C) In the presence of RA (red triangles), the GDBD-RLBD is able to promoted egfp (UAS responsive gene) transcription. (D–G) Tg(β-actin:GDBD-RLBD);Tg(UAS:fnf-B-mcherry) embryos are responsive to RA treatment. ISH for mcherry. Equivalent results were found when the Tg(β-actin:GDBD-RLBD) line was crossed to Tg(UAS:EGFP) fish (data not shown) as were used for experiments in Figure 5. More detailed characterization of the stable transgenic RA sensor lines is reported in [31]. (D, E) Lateral views with dorsal right. (F, G) Dorsal views. In images D–G anterior is up.

(TIF)

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Author Contributions
Conceived and designed the experiments: ED JSW. Performed the experiments: ED ABR JA AM JSW. Analyzed the data: ED ABR AM JSW. Contributed reagents/materials/analysis tools: ED ABR JA AM JSW. Wrote the paper: ED JSW.

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