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Input overload: contributions of retinoic acid signaling feedback mechanisms to heart development and teratogenesis

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For the better part of a century, extensive research has established that retinoic acid (RA), the major metabolic derivative of vitamin A, plays essential roles in early embryonic patterning and organogenesis in vertebrates. During vertebrate development, the levels of embryonic RA need to be tightly regulated, as congenital malformations in many organs result from excessive or decreased embryonic RA levels. Many recent reviews have extensively covered RA signaling components and their functions during development (Bastien and Rochette-Egly, 2004; Diez del Corral and Storey, 2004; Mark et al., 2006; Pan and Baker, 2007; Duester, 2008; Niederreither and Dolle, 2008; Pares et al., 2008; Mark et al., 2009; Pennimpede et al., 2010; Clagett-Dame and Knutson, 2011; Rhinn and Dolle, 2012; Samarut and Rochette-Egly, 2012; Schilling et al., 2012; Duester, 2013; Kedishvili, 2013; Das et al., 2014; Samarut et al., 2014). Therefore, the focus of this commentary is to highlight the importance of conserved canonical RA signaling feedback mechanisms to heart development and how new insights into improper RA signaling feedback may be cause for re-evaluation of congenital malformations due to aberrant canonical RA signaling. Although numerous recent studies have been insightful in elucidating non-canonical modes of RA signaling (reviewed in Al Tanoury et al., 2013), how these interesting modes of RA signaling impact heart development is currently not clear and is beyond the scope of this commentary. Here, we will first briefly review canonical RA signaling components and the progression of hypotheses regarding the role of RA signaling in early patterning of the vertebrate cardiac progenitors. We will then discuss the points of RA signaling feedback within the pathway and the consequences of improper feedback mechanisms to our understanding of normal development.

Components of the canonical RA signaling pathway

RA is the most active metabolic product of vitamin A. Vitamin A/retinol is provided in two dietary forms: preformed vitamin A, usually found in animal products, and carotenoids, the vitamin A precursor that is found primarily in plants (Bendich and Langseth, 1989). In

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amniotes, retinol is supplied from the mother through the circulation (D'Ambrosio et al., 2011). Retinol is stored in the liver through esterification by lecithin retinol acyltransferase (LRAT) (O'Byrne et al., 2005; Isken et al., 2007; Isken et al., 2008; Kim et al., 2008; Amengual et al., 2012), transported through the blood by Retinol binding protein 4 (RBP4) (Quadro et al., 2005; Isken et al., 2008; Amengual et al., 2014), and taken up into cells via Stimulated by retinoic acid-6 (STRA6) receptor (Fig. 1) (Blaner, 2007; Kawaguchi et al., 2007; Isken et al., 2008; Berry et al., 2013; Amengual et al., 2014). (NOTE: When referring to conserved genes and proteins from multiple species, we will follow the human nomenclature rules. However, when we refer to experiments from specific species, we will use the nomenclature rules for that organism.) In anamniotes, retinol is supplied to the embryo through the yolk (Albalat, 2009). In zebrafish *lratb*, *stra6*, and *rbp4* and *rbp4l* are all expressed in the yolk syncytial layer and have specific expression within the early embryo (Isken et al., 2007; Isken et al., 2008; zfin.org). Despite the different maternal sources of retinol in the embryos, the retinol storage and production machinery is conserved in amniotes and anamniotes. Upon entering the cells of the target tissue cells, retinol is bound by cellular retinol-binding proteins (also referred to as RBPs, although we will use the more common acronym CRBPs to distinguish from the plasma RBP4) (Dolle et al., 1990; Ruberte et al., 1991; Ghyselinck et al., 1999). During development, retinol is oxidized into retinal primarily through microsomal short-chain dehydrogenases/reductases (SDRs) (Pares et al., 2008; Kumar et al., 2012; Kedishvili, 2013). Specifically, in vertebrates, a SDR retinol dehydrogenase 10 (RDH10) promotes retinal formation, while a related SDR, called DHRS3, converts retinal back to retinol for eventual storage (Fig. 1). Although it was previously thought that the production of retinal was essentially ubiquitous within the vertebrate embryo, we now understand that DHRS3 and RDH10 have specific embryonic expression patterns and are the major enzymes involved in embryonic retinol storage and retinal production (Sandell et al., 2007; Feng et al., 2010; Sandell et al., 2012; Billings et al., 2013). Following the production of retinal, it is then oxidized into RA by retinaldehyde dehydrogenases (ALDH1As; Fig. 1), members of the aldehyde dehydrogenase family of enzymes (Niederreither et al., 1997; Niederreither et al., 1999; Marchitti et al., 2008; Napoli, 2012). Upon the production of RA, it is bound by cellular retinoic acid binding proteins (CRABPs), which facilitate the distribution of RA to the retinoic acid receptors (RARs) and the RA degradation machinery (Dolle et al., 1990; Ruberte et al., 1991; Durand et al., 1992; Romand et al., 2000; Cai et al., 2012). Within the target tissue, RARs and retinoid X receptors (RXRs), members of the nuclear hormone receptor family, mediate the transcriptional actions of RA (Bastien and Rochette-Egly, 2004; Samarut and Rochette-Egly, 2012). RAR/RXR heterodimers bind to retinoic acid response elements (RAREs), which commonly consist of direct repeats of canonical 6 nucleotide motifs separated by 1, 2, or 5 nucleotides (Bastien and Rochette-Egly, 2004; Delacroix et al., 2010; Mahony et al., 2011; Samarut and Rochette-Egly, 2012). However, recent chromatin immunoprecipitation-sequencing (ChIP-seq) data suggests that there is likely considerably more flexibility of RAR/RXRs, which bind to a much greater range of RARE motifs than previously appreciated (Moutier et al., 2012). It is generally thought that upon binding of RA to RARs, the RAR/RXR heterodimers shed transcriptional repressive machinery and recruit transcriptional activation machinery to active target gene expression (Bastien and Rochette-Egly, 2004; Samarut and Rochette-Egly, 2012). Members of the cytochrome P450

superfamily (CYP26s) oxidize RA, which facilitates its degradation (Fig. 1) (Abu-Abed et al., 1998; Abu-Abed et al., 2001; Sakai et al., 2001; Niederreither et al., 2002; Pennimpede et al., 2010).

History of RA in heart development

In humans, excess embryonic RA, from hypervitaminosis A and RA used in therapies of mothers (Lammer et al., 1985; Rizzo et al., 1991; Pan and Baker, 2007), can result in RA embryopathy, while RA deficiency can result from dietary maternal vitamin A deficiency, which is still common in developing countries (West and Mehra, 2010; Christian et al., 2013). Both excess and deficiency of RA result in a spectrum of development malformations in humans, including congenital heart defects. Animal models have demonstrated that the spectrum of congenital defects caused by inappropriate RA signaling is similar in all vertebrates (Heine et al., 1985; Stainier and Fishman, 1992; Lohnes et al., 1993; Lohnes et al., 1994; Mendelsohn et al., 1994; Yutzey et al., 1994; Niederreither et al., 1999; Hernandez et al., 2004; Emoto et al., 2005; Hernandez et al., 2007; Linville et al., 2009; Waxman and Yelon, 2009). Proper RA signaling is required for multiple aspects of vertebrate heart development, in particular the establishment of the cardiac progenitor fields (Pan and Baker, 2007; Duester, 2008; Niederreither and Dolle, 2008; Liu and Stainier, 2012; Schilling et al., 2012). Our understanding of how RA signaling affects early cardiac patterning has evolved considerably since the first studies to recognize the importance of proper RA signaling in normal heart development in the 1940s and '50s by Wilson and Warkany. These pioneering studies found that vitamin A deficient (VAD) rat embryos, achieved through limiting the maternal dietary supply of vitamin A, had pleiotropic developmental malformations, including ventricular outflow tract and aortic arch defects (Wilson and Warkany, 1949; Wilson and Warkany, 1950a; Wilson and Warkany, 1950b). Some time later, similar and actually more severe cardiac defects were found in VAD quail embryos, which had cardia bifida and hearts with a single enlarged ventricular chamber (Heine et al., 1985). Shortly after Warkany et al.'s initial studies, they also demonstrated that hypervitaminosis A, caused from excess dietary vitamin A, caused outflow tract and septal defects in mice (Kalter and Warkany, 1961), which are also found from hypervitaminosis A and RA embryopathy in humans (Pan and Baker, 2007).

While the experiments modulating vitamin A illustrated the importance of appropriate embryonic RA levels to heart development, it was an understanding that RA signaling is necessary and sufficient for proper anterior-posterior (A-P) patterning of the vertebrate embryo (Durston et al., 1989; Sive et al., 1990) that led to the hypothesis that the earliest requirement for RA signaling in heart development is to promote atrial cell identity at the expense of ventricular cell identity. The ventricular-atrial patterning hypothesis was based on the proposed relative anterior-posterior (A-P) positions, respectively, of the ventricular and atrial progenitors and the interpretations of some of the first experiments performed in chick and zebrafish embryos using exogenous RA (Osmond et al., 1991; Stainier and Fishman, 1992; Yutzey et al., 1994). Exogenous treatment of chick embryos with RA produces a variety of concentration dependent malformations, including smaller ventricles, enlarged atria, and expansion of cells expressing *atrial myosin heavy chain* (Osmond et al., 1991; Yutzey et al., 1994). Similarly, zebrafish treated with RA were reported to have

smaller ventricles (Stainier and Fishman, 1992). Furthermore, the ventricular-atrial hypothesis gained greater traction due to the location of *ALDH1A2* expression, the major embryonic RA producing enzyme, being posterior and adjacent to the cardiac progenitors in mouse and chick embryos and loss of function experiments using an RAR antagonist to inhibit RA signaling in chick embryos, which indicated that these embryos overtly had larger ventricles with smaller atria (Hochgreb et al., 2003).

Although the A-P model of atrio-ventricular patterning was initially an attractive model, the interpretations were based largely on cardiac chamber morphology, which is not a reliable indicator of cardiac progenitor specification and cardiomyocyte number in any model. Furthermore, the A-P model of cardiac patterning was derived prior to our understanding of the differential temporal contributions of progenitors from the first heart field (FHF) and second heart field (SHF), the availability of numerous tools and markers to assess chamber progenitor number, and a better understanding of the location of the atrial and ventricular progenitors within the anterior lateral plate mesoderm (ALPM). The first indication that RA signaling may not promote atrial specification at the expense of ventricular specification, despite its established role in A-P patterning, came from studies in zebrafish. A series of studies from the Yelon lab revealed, first, that RA signaling is required to limit the size of the cardiac progenitor field (Keegan et al., 2005) and, second, that RA signaling limits the specification of both atrial and ventricular cells (Waxman et al., 2008). In contrast to the interpretations of the previous studies, which was primarily based on morphological analysis, these experiments combined morphological analysis, counting individual cardiomyocytes using transgenic zebrafish embryos, and lineage tracing of the ALPM, which together revealed that RA signaling is required to limit the posterior boundary of both cardiac progenitors within the ALPM (Keegan et al., 2005; Waxman et al., 2008). Subsequent studies in *Xenopus* and mouse revealed similar observations. In *Xenopus*, RA signaling restricts cardiac specification, although the effects on chamber cell number were not examined (Collop et al., 2006). Analysis of *Aldh1a2* knockout (KO) mice also revealed that RA signaling restricts the posterior limit of cardiac progenitors (Ryckebusch et al., 2008; Sirbu et al., 2008).

A second indication that RA signaling may not promote atrial specification at the expense of ventricular specification has come from gain of function experiments, also from the Yelon lab in zebrafish (Waxman and Yelon, 2009). Treating zebrafish embryos with different concentrations of RA, which cause various degrees of posteriorization, combined with counting the cardiomyocytes and chamber marker analysis indicated that there are dramatically different effects on cardiac progenitor specification that occur with increasing levels of embryonic RA (Waxman and Yelon, 2009). Specifically, treatment with high concentrations of RA, which strongly posteriorize embryos, can eliminate all cardiomyocyte specification. Importantly, RA treatment can eliminate cardiomyocytes in chicken embryos (Osmond et al., 1991; Yutzey et al., 1994), suggesting this effect on cardiomyocyte specification with high concentrations of RA is likely conserved in vertebrates. However, intermediate increases in RA, which retain cardiac specification, can produce independent effects on atrial and ventricular specification. Specifically, concentrations of RA causing a higher degree of posteriorization reduce ventricular specification without promoting atrial

specification, while concentrations of RA causing a comparatively lower degree of posteriorization can promote atrial specification without affecting ventricular specification. Interestingly, more recently we found that treatment with very low increases in RA that cause a minimal degree of posteriorization can actually promote atrial and ventricular specification (D'Aniello et al., 2013). How might these different increases in embryonic RA cause such a great variety of different cardiac phenotypes? Given the correlation between the RA-induced degree of posteriorization and observed cardiac defects, we hypothesize that the differential effects on atrial and ventricular progenitor specification are due to a progressive flattening of the RA signaling gradient. Similar non-intuitive, differential effects on progenitor territories are observed with the modulation of other morphogens in early development (Dorsky et al., 2003; Tucker et al., 2008).

A final indicator that RA may not promote atrial specification at the expense of ventricular specification is that fate maps have revealed there is not a relative anterior-posterior relationship of the ventricular and atrial progenitors. In zebrafish embryos, lineage tracing indicated that within the blastula and the anterior lateral plate mesoderm (ALPM) the atrial and ventricular progenitors lie in medio-lateral orientation relative to each other (Keegan et al., 2004; Schoenebeck et al., 2007). Furthermore, a more medio-lateral orientation of ventricular and atrial progenitors has also been reported in a newer fate map of chick embryos that incorporate the SHF, the later differentiating cardiomyocytes (Abu-Issa and Kirby, 2008). Altogether, detailed cardiomyocyte counting, analysis of additional markers, and lineage tracing experiments, support a hypothesis that at the extremes of excess and deficiency of RA signaling it is necessary and sufficient for restricting both atrial and ventricular specification in vertebrates. Importantly, intermediate increases in RA signaling, through inducing varying degrees of posteriorization, can differentially and independently affect atrial and ventricular specification.

Requirements of RA pathway machinery on heart development

Within the vertebrate embryo, it is clear that an appropriate concentration of RA is critical during early heart development. Therefore, it is not surprising that animals with mutations, or deficiency, in many of the genes that control embryonic RA levels and signaling result in cardiac defects (Table 1). Beginning with the uptake of retinol in the embryo, mutations in *STRA6* underlie Matthew-Wood syndrome (MWS) in humans, which includes a spectrum of congenital defects, including outflow tract, atrial and ventricular septal defects (Golzio et al., 2007; Pasutto et al., 2007). While mouse *Stra6* KOs only have mild ocular defects (Amengual et al., 2014), Stra6 deficient zebrafish embryos have complex phenotypes indicative of decreases and increases in RA signaling (Isken et al., 2008). However, although the effects on heart development have not been analyzed in detail, overtly the cardiac phenotypes are reminiscent of increased RA signaling in zebrafish (Stainier and Fishman, 1992; Waxman and Yelon, 2009). Similarly, Lratb deficient zebrafish embryos have elevated embryonic retinoic acid and phenotypes consistent with increased RA signaling (Isken et al., 2007), although analysis of the hearts was not performed. While *Lrat* and *Rbp4* mouse KO models do not have overt developmental defects (Quadro et al., 1999; Batten et al., 2004), interestingly *Rbp4* KO and double *Lrat; Rbp4* KO mutants are sensitive to modulation of vitamin A in their diets (Quadro et al., 2005; Kim et al., 2008), suggesting

these proteins are necessary to help buffer RA signaling levels during development, which is discussed more below. However, *Rbp4* KO mice do have subtle, transient cardiac developmental defects that include precocious cardiomyocyte differentiation and extracellular matrix deposition (Wendler et al., 2003). Of the enzymes required for retinol uptake and storage, DHRS3 seems to be the most important for development. Both *Dhrs3* KO mice and Dhrs3a deficient zebrafish embryos have elevated levels of RA (Feng et al., 2010; Billings et al., 2013), while the *Dhrs3* KO mice were also shown to have decreased retinyl esters, supporting their role in promoting RA storage. Importantly, *Dhrs3* KO mice have ouflow tract and septal defects, reminiscent of RA treatment (Billings et al., 2013). Heart development was not examined in Dhrs3a deficient zebrafish embryos (Feng et al., 2010). However, compared to the *Dhrs3* null mice, the overt phenotypes are not as strong, which could be due to redundancy with its paralog Dhrs3b (ZDB-GENE-041010-172; zfin.org).

The strongest congenital heart defects are observed in mouse KOs for the major embryonic proteins that promote the production of RA: RDH10 and ALDH1A2. *Rdh10* mouse mutants were initially identified from a mutagenesis screen by Sandell et al (2007). While subsequent *Rdh10* KO analysis revealed that the severity of the phenotype can vary with the genetic background (Rhinn et al., 2011; Sandell et al., 2012; Chatzi et al., 2013), the most severe developmental defects in the *Rdh10* KO mice overtly resemble *Aldh1a2* KO mice, including linear, dilated and unlooped hearts (Sandell et al., 2012). Detailed analysis of *Aldh1a2* mutants, as mentioned above, has helped to support the hypothesis that a posterior source of RA signaling is necessary to restrict cardiac progenitor specification in vertebrates (Ryckebusch et al., 2008; Sirbu et al., 2008). Mouse *Aldh1a2* KOs and zebrafish *alh1a2* mutants both have overtly enlarged hearts (Niederreither et al., 1999; Keegan et al., 2005). The enlarged hearts in zebrafish *aldh1a2* mutants are due to a surplus of atrial and ventricular cardiomyocyte specification (Keegan et al., 2005; Waxman et al., 2008). For the mouse *Aldh1a2* KOs, although the cardiac phenotypes were initially interpreted as supporting the atrial-ventricular patterning model from cardiac morphology, revisiting the analysis of the cardiac defects with additional cardiac progenitor markers revealed that these mutants also display a posterior expansion of the cardiac progenitors (Ryckebusch et al., 2008; Sirbu et al., 2008). Studies initially in zebrafish and later in mice have suggested that the posterior expansion of the cardiac progenitors is potentially at the expense of neighboring forelimbs progenitors (Waxman et al., 2008; Zhao et al., 2009; Sorrell and Waxman, 2011; Cunningham et al., 2013). Despite the genetic data supporting a conserved requirement for RA in restricting cardiomyocyte specification, there are differences in interpretation as to whether or not there is strictly an expansion of the FHF, the earlier differentiating population of cardiomyocytes, and/or the second SHF, a later differentiating population of cardiomyocytes (Ryckebusch et al., 2008; Sirbu et al., 2008).

The transcriptional output of RA is mediated through RARs. In tetrapods, there are three RARs - α, β, and γ (Lohnes et al., 1994; Mendelsohn et al., 1994; Bastien and Rochette-Egly, 2004), while in zebrafish, there are four RARs-two α (RARαa and RARαb) and two γs (RARγa and RARγb), but no RARβ (Waxman and Yelon, 2007; Linville et al., 2009). Although numerous isoforms have been identified for each receptor in mice (Bastien and

Rochette-Egly, 2004), only two additional isoforms have been identified in zebrafish (D'Aniello et al., 2013). There is considerable redundancy with respect to receptor function in mice, as the single mutants do not have significant cardiac defects. However, double KOs of *RARα1*$^{-/-}$ with *RARβ*$^{-/-}$ or -$\gamma^{-/-}$ have outflow tract defects (Mendelsohn et al., 1994). More recent studies indicate the outflow tract defects are due to inappropriate SHF specification (Li et al., 2010). Even though the double KOs do recapitulate defects observed with VAD in humans (Mendelsohn et al., 1994), it is interesting that they do not recapitulate the severity of the enlarged hearts with looping defects found in *Rdh10* and *Aldh1a2* KOs (Niederreither et al., 1999; Sandell et al., 2012), which are indicative of strong early patterning defects. One explanation for the differences between these phenotypes likely reflects that RA signaling plays multiple roles during cardiogenesis, with the *RAR* KOs revealing later requirements of these receptors. In contrast to the redundancy revealed in mice, avian embryos appear to have distinct and non-redundant roles during cardiac development: RARγ specifically regulates the asymmetric orientation and the normal looping morphogenesis of the heart, while RARα2 regulates cardiac inflow tract formation (Romeih et al., 2003). Using zebrafish, we have recently found that depletion of zebrafish RARαb1 causes enlarged hearts with increased cardiomyocyte specification. Surprisingly, the enlarged hearts appear to be due to effects of excessive RA feedback (D'Aniello et al., 2013), which will be discussed below.

Significant work has examined the function CYP26 genes during development. In vertebrates, there are three CYP26 genes: CYP26a1, -b1, and-c1. CYP26A1 is the most prevalent RA degrading enzyme in the vertebrate embryo, expressed primarily in the anterior of the embryo during early development (Abu-Abed et al., 2001; Kudoh et al., 2002; Dobbs-McAuliffe et al., 2004; Emoto et al., 2005). In humans, loss of *CYP26A1* is associated with DiGeorge and Klipplel-Feil syndromes (Pennimpede et al., 2010), which both include outflow tract defects. While loss of *CYP26A1* alone in mice and zebrafish results in a spectrum of RA signaling defects indicative of increased RA signaling (Abu-Abed et al., 2001; Sakai et al., 2001; Niederreither et al., 2002; Emoto et al., 2005; Hernandez et al., 2007; Uehara et al., 2007), the defects are somewhat mild compared to treatment with exogenous RA, suggesting that increases in embryonic RA that can occur from failure to degrade endogenous sources of RA are somewhat minimal. However, *Cyp26a1* KO mouse and *cyp26a1* mutant zebrafish embryos are extremely hypersensitive to treatment with RA (Hernandez et al., 2007; Ribes et al., 2007), indicating they are necessary to buffer embryonic RA levels against excessive RA. With respect to the heart, *Cyp26a1* KO mice have looping defects (Abu-Abed et al., 2001). There is functional redundancy in the embryonic requirements for the Cyp26 enzymes during early development. Although *Cyp26c1* KO mice do not have significant defects, the double *Cyp26a1* and *Cyp26c1* mutants are more posteriorized than the Cyp26a1 KOs alone, with more severe cardiac looping defects (Uehara et al., 2007).

Because the mechanisms underlying the cardiac defects were not really understood in vertebrates, we recently examined the roles of Cyp26 enzymes in zebrafish (Rydeen and Waxman, 2014). We found that while there was not evidence for significant early cardiac patterning defects in zebrafish deficient Cyp26a1 or Cyp26c1 alone, depletion of Cyp26a1

and Cyp26c1 together promotes an increase in atrial specification, due to an anterior shift in the cardiac progenitor field within the ALPM. Importantly, lineage tracing and marker analysis of the ALPM in the Cyp26 deficient embryos revealed that the increase in atrial specification was at the expense of the more anterior endothelial progenitors, and not at the expense of the ventricular progenitors (Rydeen and Waxman, 2014). These results suggest that endogenous increases in RA signaling, due to Cyp26a1 and Cyp26c1 loss, cause remarkably similar cardiac specification defects to what we observed with low-intermediate levels of posteriorization from treatments with exogenous RA (Waxman and Yelon, 2009). Moreover, these results provide additional evidence that RA signaling does not promote an increase in atrial cells at the expense of ventricular cells.

Feedback on RA pathway machinery

Within the early embryo, RA functions as a morphogen with sources and sinks (White et al., 2007; Cai et al., 2012; Schilling et al., 2012). Like other morphogens, RA signaling incorporates feedback to ensure robustness of the A-P RA signaling gradient, which is necessary for proper embryonic patterning. One of the most intriguing aspects of RA signaling is the thoroughness by which it incorporates positive and negative regulation of the RA pathway machinery (Fig. 1). The manner by which RA signaling regulates its own production machinery is somewhat intuitive. Within the embryo, RA positively regulates the expression of enzymes involved in retinol storage or RA degradation, while it negatively regulates the expression enzymes involved in the production of RA (Fig. 1). Thus, excess RA promotes the expression of *STRA6*, *LRAT*, *DHRS3*, *CRBP1*, *CRABP2*, and *CYP26A1*, while repressing *RBP4*, *RDH10* and *ALDH1A2* expression (Fig. 1) (Smith et al., 1991; Chazaud et al., 1996; Niederreither et al., 1997; Begemann et al., 2001; Cerignoli et al., 2002; Emoto et al., 2005; Strate et al., 2009; Feng et al., 2010; Sandell et al., 2012; Billings et al., 2013; D'Aniello et al., 2013; Kashyap et al., 2013; Amengual et al., 2014). Deficiency in RA promotes the opposite responses in these genes. The regulation at the transcript level is a conserved aspect of RA signaling in vertebrates, as these feedback responses are found in zebrafish through mammals. Currently, we do not understand many aspects of the conserved feedback, including its evolutionary origin. From an evolutionary perspective, *in silico* genomic studies and functional studies in mollusks support the hypothesis that retinoic acid (RA) signaling is an ancient molecular pathway that predates the origins of the bilaterian body plan (Albalat, 2009; Andre et al., 2014). Therefore, it would be particularly interesting if the mechanisms underlying RA feedback regulation were ancient, predating the common bilaterian ancestor. While the intracellular production and degradation components are predicted to be conserved in protostomes and invertebrate deuterostomes (Albalat, 2009; Albalat and Canestro, 2009; Andre et al., 2014), if RA signaling regulates the expression of these genes in a manner similar to that found in vertebrates has not been examined. However, it is known that RA positively regulates *Cyp26* expression in chordate *Ciona*, but does not significantly affect *Aldh1a* expression (Ishibashi et al., 2003; Ishibashi et al., 2005), suggesting that at least the positive regulation of *Cyp26* by RA predates a common chordate ancestor.

Although the conserved feedback on production and degradation machinery in vertebrates is clear, the level and conservation of RA signaling feedback on RAR expression is still poorly

understood. Recent studies have indicated that RA signaling promotes *RAR* and *RXR* expression in mouse and zebrafish embryos (Begemann et al., 2001; Feng et al., 2010; Kashyap et al., 2013). However, we currently do not understand the mechanisms underlying this transcriptional activation for the majority of these genes. Of course, the explosion of ChIP-seq technology and the ever increasing ability to efficiently analyze cis-regulatory modules at a systems level will enhance the ability to assess the directness of RA regulation of these genes. It has long been recognized that there are canonical RAREs in the promoters of multiple RARs, with the mouse *RARβ2* being responsive to RA, suggesting it is a direct target (de The et al., 1990; Loudig et al., 2000). However, RA regulation of the *RARβ2* isoforms is likely not highly conserved in vertebrates as zebrafish do not have an *RARβ2* and RA signaling does not regulate the Ciona *RAR* (Ishibashi et al., 2003; Ishibashi et al., 2005; Waxman and Yelon, 2007). Despite it becoming apparent that RA signaling promotes the expression of its own receptors, regardless of the directness, the purpose of the positive regulation of RARs by RA signaling in development is also not understood and necessitates investigation.

Another level of RAR feedback in development is compensatory expression of RARs, which we hypothesize could be a relatively unappreciated feedback response to loss of RA signaling. However, initial reports of *RAR* KO mice suggested that when RARs are lost there was not increased expression of the remaining RARs (Lohnes et al., 1993; Lufkin et al., 1993). Contradictory to these early experiments, more recent reports in mouse and zebrafish found instead that when RARs are depleted, compensatory expression of the remaining RARs is initiated (Manshouri et al., 1997; D'Aniello et al., 2013). The different results in these studies may be due to experimental differences, depletion of the RARs vs. the KOs. However, given there are more sensitive assays available now to assess expression compared to what was available at the initial time analysis of the mouse *RAR* KOs, it is likely worthwhile revisiting if there is compensatory RAR expression in these models. Although we do not yet understand the mechanisms by which compensative RAR expression is achieved, it would be interesting if transcriptional de-repression is one way it is initiated. It has been proposed that active repression of RARs is required for some developmental processes (Koide et al., 2001; Weston et al., 2003; Janesick et al., 2014). Therefore, perhaps RAREs in the promoter of RARs that promote their expression are also used to facilitate de-repression of their expression when other RARs are depleted. Alternatively, the compensatory expression of RARs could be activated through RAREs bound by the remaining, but depleted RARs, as suggested by Manshouri et al. (1997).

Feedback through direct transcriptional activation

One of the mechanisms by which RA positively regulates pathway components during development is through direct transcriptional regulation. A number of the positively regulated RA signaling pathway components, in addition to the aforementioned *RARs*, have RAREs in their promoters. Pathway components that have RAREs in their promoters besides RARs, include *CYP26A1*, *CRBP1*, and *CRABP2* (Smith et al., 1991; Durand et al., 1992; Loudig et al., 2000; Loudig et al., 2005; Hu et al., 2008; Li et al., 2012). Of these, the *CYP26A1* promoter has been the best studied, in part because of its responsiveness to RA in many developmental contexts. The responsiveness of the *CYP26A1* promoter to RA is

mediated by multiple direct repeat 5 (DR5) RAREs in humans, mice, rats, and zebrafish, with at least two of them being almost absolutely conserved (Loudig et al., 2000; Loudig et al., 2005; Hu et al., 2008; Zhang et al., 2010; Li et al., 2012). In rats, mice, and zebrafish, the multiple RAREs in the promoters have been shown to interact to enhance the responsiveness to RA. Although the RAREs for *CRBP1*and *CRABP2* have been shown to be responsive to RA *in vitro* (Smith et al., 1991; Durand et al., 1992), if these RAREs mediate the *crabp2a* responsiveness to RA in zebrafish embryos has not been shown (Cai et al., 2012). Furthermore, if RAREs mediate the conserved RA responsiveness of *LRAT* and *DHRS3* during development has not been shown (Cerignoli et al., 2002; Feng et al., 2010; D'Aniello et al., 2013; Kashyap et al., 2013). In addition to promoting the storage and degradation of RA, RA represses the expression of the major embryonic production enzymes *RDH10* and *ALDH1A2* (Niederreither et al., 1997; Emoto et al., 2005; Strate et al., 2009; Feng et al., 2010; Sandell et al., 2012; D'Aniello et al., 2013). How this conserved negative response to RA takes places has not been investigated. Thus, despite some inroads, we currently do not have a thorough understanding of the transcriptional mechanisms underlying the conserved feedback responses to RA signaling during development. A direct transcriptional mechanism of regulation for all these genes, or at least the positively regulated RA responsive genes, would seem to be the most efficient feedback mechanism, and one that we hypothesize might be favored evolutionarily. We expect that direct transcriptional regulation would allow for the fastest response to fluctuations in RA signaling. An even more intriguing question is what are the transcriptional mechanisms of RA negatively regulated genes? As increased RA is typically associated with transcriptional activation, one hypothesis would be that the regulation of these genes would have to be indirect. However, a fascinating hypothesis is that these might also be direct targets and repression of expression would occur through ligand-induced transcriptional inhibition at RAREs (Hu et al., 2004; Epping et al., 2005; Kumar and Duester, 2014).

Buffering the RA signaling gradient

A combination of elegant mathematical modeling studies and *in vivo* analysis in zebrafish has illustrated that self-induced negative feedback is necessary for robustness of the RA morphogen gradient and helps explain how RA signaling can maintain an appropriate gradient even with >20 fold fluctuations in RA (White et al., 2007; Schilling et al., 2012). Specifically, in zebrafish, experimental work from the Schilling lab has demonstrated that Cyp26a1 and Crabp2a are necessary for the robustness of the signaling gradient (White et al., 2007; Cai et al., 2012). While extremely informative, the mathematical models have not yet incorporated the majority of RA responsive machinery. We speculate, however, that all the levels of RA positive and negative feedback contribute to the robustness of the RA signaling gradient in vertebrate embryos. Incorporating what is known about the sensitivity of mouse and zebrafish models to modulation of RA signaling, it could be already argued that these components are necessary for the robustness. For instance, the lack of overt developmental defects in *Lrat* and *Rbp4* KO mice suggests that in vitamin A and RA sufficient conditions these enzymes are largely dispensable for embryonic development (Quadro et al., 1999; Batten et al., 2004). However, *Rbp4* KOs alone or *Lrat;Rbp4* double KOs are sensitive to modulation of vitamin A (Quadro et al., 2005; Kim et al., 2008),

suggesting that they are indispensible for embryonic development in RA insufficient conditions. Specifically, when mothers of *Rbp4* KO mice are kept on a vitamin A deficient diet, the *Rbp4* KO embryos can resemble *Aldh1a2* KO embryos (Quadro et al., 2005). Furthermore, double *Lrat; Rbp4* KO mice are even more sensitive to vitamin A deficiency, resulting in more severely affected embryos compared to *Rbp4* KO mice alone (Kim et al., 2008). The sensitively of *Rbp4* KO mice to vitamin A deficiency, and similarity of these phenotypes in mice to individuals with MWS, has led to the hypothesis that the severity of MWS defects, which harbor mutations in STRA6, may be due to nutritional deficiencies of the mothers (Quadro et al., 2005; Amengual et al., 2014).

In contrast to the sensitivity of *Rbp4* mutants to vitamin A deficiency, experiments demonstrate that CRABP2 and CYP26A1 enzymes act as buffers against increased embryonic RA signaling. Zebrafish Crabp2a deficient embryos have relatively minor hindbrain defects (Cai et al., 2012). However, increases and decreases in *crabp2a* expression, which transports RA to Cyp26a1 (Kedishvili, 2013), leads to hypersensitivity to RA treatment and less sensitivity to loss of RA signaling in zebrafish (Cai et al., 2012). Although CYP26A1 mutants in zebrafish and mice have comparatively stronger phenotypes than deficiency of the other aforementioned enzymes (Abu-Abed et al., 2001; Sakai et al., 2001; Hernandez et al., 2007; Ribes et al., 2007; Uehara et al., 2007), as mentioned above, the CYP26A1 mouse and zebrafish mutants are hypersensitive to RA treatment, with RA treatment using sub-optimal concentrations that do not affect wild-type animals causing strong posteriorization of CYP26A1 mutant embryos (Hernandez et al., 2007; Ribes et al., 2007). Therefore, while phenotypes in the RA signaling pathway may be relatively mild in vitamin A/RA sufficient embryonic contexts, these molecules are necessary to buffer against RA deficiency or excess.

Consequences of inappropriate RA feedback

Incorporating our understanding of the known RA feedback mechanisms and embryonic phenotypes, it would seem intuitive that deficiency or excess of RA would rapidly initiate feedback mechanisms that are necessary to restore a balance in RA signaling and a proper signaling gradient in early vertebrate development. However, one conundrum with our current understanding of defects from improper RA signaling is that excess or deficiency of RA signaling in embryos affects similar organs and can even cause overtly similar phenotypes. Multiple recent studies have found that inappropriate embryonic RA signaling can cause seemingly contradictory phenotypes indicative of both increases and decreases of RA signaling. For instance, increases of embryonic RA in mice can induce, paradoxically, longer-term induction of RA deficiency and perturb renal development (Lee et al., 2012). This study shows that after the initial RA treatment there is maintained deficiency of embryonic RA levels, which is correlated with decreased *Aldh1a2* and *Rdh10* and increased *Cyp26a1* and *Cyp26b1* expression (Lee et al., 2012). The renal defects can be partially rescued with RA treatment (Lee et al., 2012), supporting a hypothesis that the renal phenotypes are due to the suppression of embryonic RA after the initial excess of RA. In light of these results in mice, we have observed similar phenotypes in zebrafish using a hyper-active RAR, which induced both brain defects associated with increased RA signaling and loss of RA responsive gene expression, including *hoxb5b* and *dhrs3a* (Waxman and

Yelon, 2011). However, we did not assess other components of the RA machinery or the effect on cardiogenesis. Therefore, although we currently do not understand if the subsequent loss of RA responsive targets in this context is due to inappropriate maintenance of depressed RA signaling as in mice, we hypothesize this is the case.

In contrast to RA-induced loss of RA signaling, we have also found evidence for the opposite: RA deficiency-induced gain of RA signaling. We found that depletion of RARαb1 caused larger hearts with increased cardiac specification, but this was unexpectedly induced from increased RA signaling (D'Aniello et al., 2013). Furthermore, we found that *hox* gene and *cyp26a1* expression were increased in RARαb1 deficient embryos, while *aldh1a2* was downregulated, supporting the hypothesis that an increase in RA was triggered in the RARαb1 depleted embryos (D'Aniello et al., 2013). Importantly, depletion of RARαb2, the second RARαb isoform in zebrafish, or concurrent depletion of RARαb1 and RARαb2 also resulted in increased RA signaling, suggesting this effect was not specific to RARαb1 (D'Aniello et al., 2013). Additionally, we found that the expression of the other zebrafish *RARs* was increased when the RARαb isoforms were depleted. We also found that treatment with low concentrations of RA were able to mimic this effect, supporting that low levels of RA can also promote increased cardiomyocyte specification (D'Aniello et al., 2013). Therefore, in contrast to increases in RA signaling, modest decreases in RA can lead to inappropriate positive feedback that results in teratogenic levels of RA signaling. Moreover, dramatic losses of RA signaling, as occur in *Aldh1a2* KO mice (Niederreither et al., 1999), would not be predicted to elicit phenotypes due to inappropriate feedback, as the lack of RA would cause a breakdown of the feedback loop.

Why might gain or loss of RA signaling result in inappropriate signaling due to excessive feedback that causes congenital defects? We hypothesize that the RA feedback mechanism is designed to rapidly buffer embryonic RA levels within a certain range and length of time. Inappropriate increases or decreases in RA signaling outside of this range or sustained moderate increases or decreases may cause prolonged feedback that ultimately exceeds the intent of returning the RA signaling to a normal level. In light of these new revelations regarding the consequences of inappropriate RA signaling due to feedback and the variability of cardiac defects that can be induced by exogenous RA treatment, it may be worthwhile to revisit the cause of some RA induced congenital heart defects, as has been done with kidney development (Lee et al., 2012). Future studies elucidating the transcriptional mechanisms underlying RA signaling feedback and the scenarios where inappropriate RA signaling feedback occurs will enhance our understanding of the teratogenic mechanisms underlying RA induced congenital heart defects, as well as the broad spectrum of tissues affected by inappropriate RA signaling. Since RA is commonly used to treat acne and cancers (Soprano et al., 2004; Pan and Baker, 2007), a greater understanding of the mechanisms underlying inappropriate RA signaling feedback may lead to new, rather unexpected treatments for the prevention of congenital defects and unwanted side effects.

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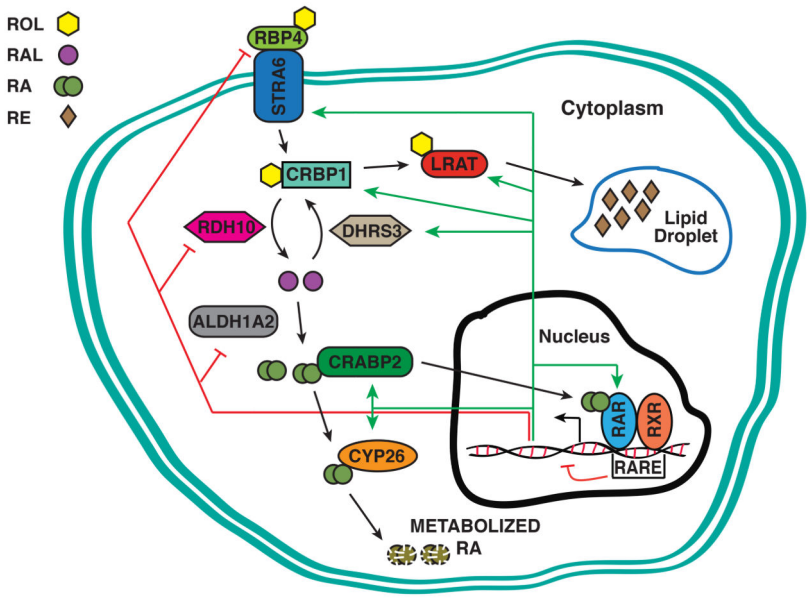

Figure 1. Schematic of feedback in the RA signaling pathway

Retinol (ROL), bound to RBP4 binds to its receptor STRA6 in the cell membrane. Upon transport into the cell, CRBP1 binds ROL and delivers it to LRAT for storage as Retinyl esters (RE) or to RDH10, which oxides it to retinal (RAL). ALDH1A oxidizes RAL to RA or RAL can be reduced back to ROL via DHRS3 for storage. Upon conversion to RA, it binds to CRABP2, which can distribute RA to RARs or CYP26 enzymes for oxidation and degradation. Green arrows indicate where positive transcriptional feedback has been observed, while red bars indicate where negative transcriptional feedback has been observed. Although we depict that both positive and negative feedback is initiated through transcriptional activation, this is only a hypothesis. One mechanism of negative feedback could be through ligand-induced negative feedback at RAREs, indicated by the red bar emanating from the RARE. Overall, there is only limited data supporting that direct activation of the positively regulated RA signaling pathway genes is through RAREs in their promoters, while there is currently no data describing how the negatively regulated RA signaling pathway genes are suppressed.

Table 1

Affected Genes	Species	Heart Phenotypes	References
STRA6	Human	Matthew-Wood sindrome: outflow tract-atrial and ventricular septal defects	(Golzio et al., 2007; Pasutto et al., 2007)
	Zebrafish	Aberrant heart-reminiscent of increased RA signaling	(Isken et al., 2008)
DHRS3	Mouse	Outflow tract and septal defects	(Billings et al., 2013)
RDH10	Mouse	Looping defects, enlarged hearts, and edema	(Sandell et al., 2012)
ALDH1A2	Mouse	Looping defects, enlarged hearts, edema, increased cardiomyocyte specification	(Niederreither et al., 1999; Ryckebusch et al., 2008; Sirbu et al., 2008)
	Zebrafish	Enlarged hearts due to increased cardiomyocyte specification	(Keegan et al., 2005)
CYP26A1	Human	DiGeorge and Klippel-feil; Outflow tract defects	(Pennimpede et al., 2010)
	Mouse	Gross aberrant cardiac defects including looping defects	(Abu et al., 2001; Emoto et al., 2005; Ribes et al., 2007)
CYP26A1; CYP26C1	Mouse	Gross aberrant cardiac defects including looping defects	(Uehara et al., 2007)
	Zebrafish	Abnormal heart with increased atrial CMs	(Rydeen et al., 2014)
RARa2	Chicken	Defects in cardiac inflow tract	(Romeih et al., 2003)
RARγ	Chicken	Defects in asymmetric orientation and looping morphogenesis	(Romeih et al., 2003)
RARal; RARβ	Mouse	Outflow tract and aortic arch abnormalities; persistent truncus arteriousus; dextroposed aorta; high ventricular septal defects; abnormal aortic arch pattern.	(Mendelsohn et al., 1994; Li et al., 2010)
RARal; RARγ			
RARab1	Zebrafish	Enlarged hearts with increased cardiomyocyte specification	(D'Aniello et al.,2013)