RDH10 Oxidation of Vitamin A Is a Critical Control Step in Synthesis of Retinoic Acid during Mouse Embryogenesis

Lisa L. Sandell1,2, Megan L. Lynn1, Kimberly E. Inman1, William McDowell1, Paul A. Trainor1,3*

1 Stowers Institute for Medical Research, Kansas City, Missouri, United States of America, 2 Department of Molecular, Cellular and Craniofacial Biology, University of Louisville, Louisville, Kentucky, United States of America, 3 Department of Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, Kansas, United States of America

Abstract
Retinoic Acid (RA) is a small lipophilic signaling molecule essential for embryonic development and adult tissue maintenance. Both an excess of RA and a deficiency of RA can cause pathogenic anomalies, hence it is critical to understand the mechanisms controlling the spatial and temporal distribution of RA. However, our current understanding of these processes remains incomplete. Vitamin A is metabolized to RA via two sequential enzymatic reactions. The first requires retinol dehydrogenase (RDH) activity to oxidize Vitamin A (retinol) to retinal, and the second requires retinaldehyde activity (RALDH) to oxidize retinal into RA. The first reaction has previously been attributed to the alcohol dehydrogenase (ADH) family, whose genes are ubiquitously or redundantly expressed. Consequently, the specificity of RA synthesis was thought to reside exclusively at the level of the second reaction. To better understand the metabolism of Vitamin A into RA during embryogenesis, we generated new mouse models that disrupt this process. Here we describe a new targeted knockout of Rdh10 in which RA synthesis is severely impaired, particularly at critical early embryonic stages. We also introduce a new mutant allele of Aldh1a2. Both mutations produce similar developmental defects resulting in lethality around embryonic day 10.5 (E10.5). The severity of the Rdh10 null phenotype demonstrates that embryonic oxidation of retinol is carried out primarily by RDH10 and that neither ADHs nor other enzymes contribute significantly to this reaction. We also show that reduced RA production results in upregulation of Rdh10. These data demonstrate that RDH10 plays a critical role in mediating the rate limiting RDH step of Vitamin A metabolism and functions as a nodal point in feedback regulation of RA synthesis. Moreover, RDH10-mediated oxidation of retinol plays as important a role in the control and regulation of RA production during embryogenesis as does the subsequent RALDH-mediated reaction.

Introduction
Retinoic acid (RA) is a derivative of Vitamin A (retinol) that plays an essential role in many vertebrate biological processes including energy metabolism, brain function, immune response, reproduction and embryonic development [reviewed in 1,2,3,4,5,6]. The processes of embryonic growth and patterning are particularly dependent on this small molecule, as can be appreciated by the striking abnormalities and midgestation lethality that occur when RA metabolism is limited or disrupted during development in mice [7,8,9,10] or other mammals [11,12,13,14]. The defects arising from RA perturbation occur because RA controls the transcription of a variety of essential developmental genes by virtue of its function as a ligand that binds to retinoic acid receptors (RAR). The RAR, which form heterodimers with retinoid receptors (RXR), activate or repress gene activity when RA is bound [reviewed in [15]]. Owing to the importance of RA in regulating essential developmental processes, it is critical to understand the mechanisms that regulate the metabolism of this key compound.

Unlike many signaling molecules whose function is modulated by changes in RNA transcription or protein activity, RA is a small molecule whose level and tissue distribution are regulated by biochemical synthesis and by degradation. It has been known for some time that synthesis of RA from the dietary precursor retinol occurs via two sequential enzymatic reactions - first retinol is oxidized to form retinal and, second, retinal is oxidized to form RA [16]. The first reaction is facilitated by enzymes with retinol dehydrogenase (RDH) activity and the second reaction is carried out by enzymes with retinaldehyde dehydrogenase activity (RALDH).

The RALDH second step of RA synthesis is carried out by RALDH1, RALDH2 and RALDH3, which are encoded by Aldh1a1, Aldh1a2 and Aldh1a3, respectively [7,8,9,10]. RALDH2 is the primary enzyme responsible for conversion of retinal to RA during early embryogenesis, mediating the major part of RA synthesis within the early developing embryo [8]. Loss of RALDH2 function eliminates RA signaling within the trunk region and results in severe developmental abnormalities, including shortened anteroposterior axis, defects in embryo turning, dilated and un-looped hearts, small somites, absence of forelimb buds and lethality by embryonic day 10.5 (E10.5). Loss of RALDH3 disrupts RA signaling in the embryonic head, resulting in defects in formation of the nose and eye and lethality at birth.


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* E-mail: pat@stowers.org (PAT); lisa.sandell@louisville.edu (LLS)
The severe phenotypes of embryos lacking RALDH2 or RALDH3 clearly demonstrate the critical nature of the RALDH reaction in embryonic RA synthesis.

In contrast to the well-established importance of the RALDH second reaction, the RDH first reaction of RA synthesis has received less attention due to the misperception that it occurs ubiquitously throughout embryogenesis. Two types of molecule are capable of RDH activity: *in vitro*, members of the alcohol dehydrogenase (ADH) family, which are cytosolic medium chain dehydrogenases (MDR), and members of the RDH family, which are membrane-bound short chain dehydrogenase/reductases (SDR) (reviewed in [17]). ADH enzymes have the ability to catalyze the conversion of retinol to retinal *in vitro* [18]. Owing to this biochemical activity of ADH enzymes, together with an overlapping or ubiquitous pattern of *Adh* gene expression during embryogenesis [19], it was initially proposed that the embryonic oxidation of retinol to retinal was carried out by ADH enzymes within the cytosol [20,21,22]. However, despite their biochemical activity and their widespread *in vivo* expression pattern, disruption of single and compound *Adh* genes produced no embryonic defects [21], a lack of effect that was speculatively attributed to potential redundancy within the gene family. The failure to identify an *Adh* mutant embryonic phenotype led to the prevailing view that the first step of embryonic Vitamin A metabolism is mediated by ubiquitous or redundant enzymes and plays a minimal role in the spatiotemporal regulation of RA synthesis.

The notion that the initial oxidative conversion of retinol occurs in an unregulated fashion mediated by ADHs has been challenged by recent discoveries highlighting a critical role for the SDR RDH10 in tissue specific synthesis of RA within the vertebrate embryo. The first indication that RDH10 is important for embryonic synthesis of RA came from characterization of mice with a point mutation termed *Rdh10*<sup>trans</sup> [23]. Embryos homozygous for this mutation exhibit a dramatic reduction in RA synthesis and severe defects in embryonic development including limb, craniofacial and organ defects that result in lethality between E10.5–14.5. Further evidence of a role for RDH10 came from *Xenopus*, in which XRDH10 was shown to be critical for RA synthesis and to be controlled by RA negative feedback inhibition [24]. In zebrafish, the *rdh10a* gene was found to be subject to positive and negative feedback regulation by RA, as was, inversely, *dhrs3a*, whose product catalyzes the reverse reaction converting retinal back into retinol [25]. In chick embryos, expression of *Rdh10* is regulated spatially and temporally in a pattern overlapping with the retinol transporter *Strab*, but does not appear to be regulated by feedback from excessive or reduced levels of RA [26]. These recent findings raise questions of the specific contribution of RDH10 versus ADHs and of the true mechanistic importance of the first oxidation step of Vitamin A metabolism in controlling embryonic RA synthesis.

The embryonic abnormalities and RA reduction observed in *Rdh10*<sup>trans</sup> mutant mice are dramatic, but generally not as severe as those caused by loss of RALDH2. Embryos lacking RALDH2 do not survive past E10.5 [8,27], while most homozygous *Rdh10*<sup>trans</sup> embryos remain viable through E10.5–E11.5 and embryos have been identified surviving as late as E14.5 [23,28]. The residual production of RA and the less severe phenotype of *Rdh10*<sup>trans</sup> embryos compared to *Aldh1a2* mutant embryos indicate that some RDH activity remains intact in the *Rdh10*<sup>trans</sup> embryos, mediated possibly by hypomorphic activity of the *Rdh10*<sup>trans</sup> point mutant enzyme, by other RDH enzymes, or by ADH enzymes. In order to further our understanding of the regulation of RA synthesis during embryonic development, it is important to know which enzymes catalyze the RDH reaction and whether the relevant enzymes are redundant and ubiquitous within the embryo or if they are instead expressed in a regulated fashion that may have bearing on the control of production of this important signaling molecule.

To clarify the enzymatic regulation of Vitamin A metabolism and RA synthesis during embryogenesis, we have generated new mouse models that disrupt this process and comprise a graded series of RA deficiency. Here we describe a novel targeted knockout allele of *Rdh10*. We show that eliminating RDH10 activity results in severe embryonic abnormalities, more extreme than the phenotype of the original *Rdh10*<sup>trans</sup> point mutant. In this report we also introduce a new point mutant allele of *Aldh1a2*, generated in a chemical mutagenesis screen, which disrupts the RALDH reaction and exhibits a phenotype similar to previously described targeted knockout alleles of *Aldh1a2*. Both the *Rdh10* knock out embryos and the *Aldh1a2* point mutant embryos exhibit similar shortened anteroposterior axes, defects in embryo turning, dilated and un-looped hearts, small somites, and forelimb bud agenesis. Each results in embryo death by ~E10.5. Using the RARElacZ reporter transgene, we show that RA synthesis in *Rdh10* null embryos is severely reduced at E9.5 and is almost completely eliminated at the earlier critical E8.0–E8.5 stage of development. The severity of the *Rdh10* null phenotype indicates that the RDH mediated oxidation of retinol to retinal during Vitamin A metabolism is carried out primarily by RDH10 and that neither ADH family members nor other enzymes contribute significantly to the RDH step of RA synthesis during early embryogenesis. In this report we also find that *Rdh10* gene expression is elevated when RA levels are reduced, providing evidence that *Rdh10* functions as a control point for feedback regulation of the RA synthesis pathway in mammals. These data demonstrate that the initial RDH reaction of Vitamin A metabolism, mediated primarily by RDH10, plays as critical a role in embryogenesis and is as important in the control and regulation of RA production as is the subsequent RALDH reaction.

**Results**

**Graded retinoid deficiency phenotypes: *Rdh10*<sup>trans</sup> vs. *Aldh1a2*<sup>mut</sup>**

The previously reported mouse *Rdh10*<sup>trans</sup> point mutation disrupts RDH10 function and causes numerous embryonic defects culminating in embryo death between E10.5–14.5 [23]. In *Rdh10*<sup>trans</sup> vs. *Aldh1a2*<sup>mut</sup> mutant embryos RA synthesis is reduced but not completely eliminated, as residual RA signaling is detected in the trunk neural tube. This raised the possibility that *Rdh10*<sup>trans</sup> embryos at E9.5 and E10.5 (Fig. 1A,B,D,E) may be a severe hypomorph rather than a complete null. For comparison with other mutants in the present study, we examined *Rdh10*<sup>trans</sup> vs. *Aldh1a2*<sup>mut</sup> embryos at E9.5 and E10.5 (Fig. 1A,B,D,E). Although the *Rdh10*<sup>trans</sup> phenotype is somewhat variable at E9.5, all embryos have numerous discernible abnormalities. First and second pharyngeal arches form in most *Rdh10*<sup>trans</sup> mutant embryos, but third and more posterior pharyngeal arches are absent or fused to the second arch (Fig. 1B). Otic vesicles are usually small, duplicated and displaced posteriorly (Fig. 1B). Mutant embryos exhibit cardiac abnormalities, including heart edema and looping defects (Fig. 1B). At E10.5 a reduction in size of the forelimb bud and absence of posterior pharyngeal arches are clearly evident (Fig. 1E). This constellation of defects observed in the *Rdh10*<sup>trans</sup> embryos is consistent with retinoid deficiency but is less severe than that observed in embryos in which RALDH2 function has been eliminated.

As part of the same chemical mutagenesis screen that yielded the *Rdh10*<sup>trans</sup> mutation, we also generated a mutant originally...
named “grimace”[29] that exhibited interesting abnormalities in formation of the pharyngeal arches (Fig. 1C). Mapping, cloning and sequence analysis were used to identify the grimace mutation, which was determined to be a single base change within the Aldh1a2 locus. We therefore henceforth refer to this mutation as Aldh1a2gri. Sequence analysis of genomic DNA reveals that the Aldh1a2gri mutation alters the 5' splice consensus sequence at the boundary of exon 4 and intron 4–5, changing the second intronic nucleotide from a conserved T to a C (Fig. 1G). The altered splice consensus sequence disrupts splicing of Aldh1a2 mRNA and leads to a reduction in Aldh1a2 transcript level. Quantitative RT-PCR reveals that Aldh1a2 transcript in homozygous Aldh1a2gri/gri mutants is reduced to less than 1% of that of wild type embryos (Fig. 1H). We therefore interpret the new Aldh1a2gri mutation is either a null allele of Aldh1a2 or an extreme hypomorph. Consistent with the loss of Aldh1a2 transcript, we observe the
phenotype of homozygous Aldh1a2<sup>geo/geo</sup> mutants to be very similar to that of established Aldh1a2 knockout alleles, each exemplifying the abnormalities associated with severe retinoid deficiency (Fig. 1C,F). Mutant Aldh1a2<sup>geo/geo</sup> embryos die by E10.5 exhibiting a number of striking defects including cessation of growth at ~E9.5–E9.0, defects in axial turning, dilated unlooped hearts, small somites and tiny otic vesicles. Whereas established knockout alleles of Aldh1a2 exhibit no axial turning, many Aldh1a2<sup>geo/geo</sup> mutants display a slightly milder phenotype with a small degree of embryo turning. The slightly milder turning defect may represent a difference of strain background on the axial turning phenotype or may indicate that the Aldh1a2<sup>geo</sup> allele is not an absolute null.

Comparison of the Rdh10<sup>geo</sup> versus the Aldh1a2<sup>geo</sup> phenotypes illustrates the less extreme defects elicited by Rdh10<sup>geo</sup> mutation relative to that of Aldh1a2<sup>geo</sup> (compare Fig. 1 E, F). At E9.5 Aldh1a2<sup>geo/geo</sup> mutants have extreme defects in extension and turning of the body axis, with some embryos completely unturned and others with an abnormal half-turned “J” shaped body axis when viewed from the front. In contrast, nearly all E9.5 Rdh10<sup>geo/geo</sup> embryos are completely turned. Aldh1a2<sup>geo/geo</sup> mutant embryos lack all branchial arches posterior to the first arch, whereas many Rdh10<sup>geo/geo</sup> embryos form second arches and, in some cases, rudimentary third arches. The milder phenotype of the Rdh10<sup>geo/geo</sup> embryos relative to the severe phenotype of Aldh1a2<sup>geo/geo</sup> mutants, together with the presence of residual RA activity detected using RARElac<sub>Z</sub> reporter mice [23], implies the presence of residual RDH function within Rdh10<sup>geo/geo</sup> embryos. The residual RA in these embryos could result from a small amount of RDH activity remaining for the mutant RDH10 protein, or to the function of unidentified RDH or ADH enzymes.

Generation of Rdh10 knockout mice

In order to directly evaluate the contribution of RDH10 in embryonic RA synthesis we sought to completely eliminate RDH10 function from developing mouse embryos. To that end, we generated mice null for RDH10 activity utilizing ES cells produced by the Chori-Sanger-UC Davis (CSD) arm of the international Knockout Mouse Project (KOMP) consortium. The CSD KOMP knockout construct is designed to create a series of alleles that can be generated by sequential deletion through exposure to Cre or FLP recombinase (Fig. 2A). The initial targeted knock-in construct inserts a splice acceptor-lacZ gene trap cassette between exon 1 and exon 2 of the Rdh10 gene, an allele we call Rdh10<sup>geo</sup>. The gene trap insertion is expected to produce a null allele by disrupting the endogenous Rdh10 transcript and producing instead a reporter lac<sub>Z</sub> fusion transcript. Exposure of the gene trap allele to FLP-recombinase can then be used to excise the disruptive splice acceptor cassette (flanked by FRT sites). FLP-mediated excision of the gene trap cassette creates a functionally wild type/conditional knockout allele of Rdh10 with loxP sites flanking exon 2, an allele we call Rdh10<sup>geo</sup>loxP. Cre-mediated recombination can then be used to excise the loxP-flanked exon 2, thereby producing a null allele we call Rdh10<sup>geo</sup>loxP. Exon 2 was chosen for deletion because its absence is predicted to create a frameshift mutation thereby truncating any resulting protein product and targeting the transcript for nonsense mediated decay. Prior to distribution, correct targeting of the gene trap construct to the Rdh10 genomic locus in ES cells was assessed by 3' and 3' PCR junction fragment assays at the KOMP production and distribution centers. The quality control tests included long range PCR assays pairing generic targeting cassette primers with Rdh10 locus-specific genomic primers homologous to the sequences upstream and downstream of the target site.

In order to generate mice bearing an Rdh10 knockout allele, we obtained from the KOMP consortium ES cell clone Rdh10_D08, which is heterozygous for the Rdh10 gene trap allele. These ES cells were injected into C57BL/6J blastocysts to produce chimeric mice that were identified by PCR amplification of the flgeo gene from tail DNA samples. Male mice chimeric for the Rdh10 gene trap allele were then mated to identify individual animals transmitting the targeted allele. Mice carrying the Rdh10<sup>geo</sup> gene trap allele were crossed with mice carrying ubiquitously expressed FLP recombinase [30] to generate a conditional Rdh10<sup>geo</sup>loxP allele. Exposure of the gene trap allele to FLP recombinase resulted in excision of the gene trap/flgeo portion of the targeting cassette and leaving a pair of loxP sites flanking exon 2 to create the conditional allele. Rdh10<sup>geo</sup>loxP mice were then crossed with mice bearing a female germline expressed Cre recombinase [31]. The resulting exposure

Figure 2. Targeted Rdh10 gene trap and knockout allele. (A) Schematic diagram of Rdh10 knockout allele obtained as ES cells from KOMP consortium and derivative alleles. For Rdh10<sup>geo/geo</sup> allele, a gene-trap splice acceptor flgeo cassette is introduced between exon 1 and exon 2 of Rdh10 and loxP sites are introduced surrounding exon 2. For the Rdh10<sup>geo</sup> allele, the gene trap cassette is excised returning function, conditionally, to the Rdh10 gene. For the Rdh10<sup>geo/loxP</sup> allele, exon 2 is deleted by Cre excision of the DNA between the loxP sites. (B) Rdh10 RNA in situ hybridization of E9.5 wild type embryo reveals expression of Rdh10 in specific tissues. Rdh10 RNA staining pattern n>10 embryos. (C) Staining E9.5 Rdh10<sup>geo/geo</sup> embryofor β-galactosidase activity (arrow) reveals gene trap transcript expressed in pattern similar to Rdh10 RNA expression. Rdh10 β-galactosidase staining pattern n>10 embryos. ba, branchial arch; ht, heart; lb, limb bud; op, optic vesicle; psm, presomitic mesoderm; s, somite.

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of the Rdh10\(^{geo}\) allele to Cre recombinase caused excision of the loxp-flanked exon 2 thereby generating Rdh10\(^{geo/delta}\) allele.

Embryos heterozygous for the Rdh10\(^{geo}\) gene trap alleles were assessed for expression of the reporter \(\beta\text{-galactosidase}\) transcript by staining for \(\beta\)-galactosidase activity. In these embryos \(\beta\)-galactosidase activity was very weak but the Rdh10\(^{geo}\) gene trap transcript could be detected in a pattern similar to that of the endogenous Rdh10 transcript (Fig. 2 B–C). Both the endogenous Rdh10 mRNA, detected by RNA in situ hybridization and the Rdh10\(^{geo}\) gene trap reporter are present in the posterior pharyngeal region and the lateral plate mesoderm between the fore- and hind-limb buds. Expression of Rdh10 mRNA and the Rdh10\(^{geo}\) gene trap reporter are also present in the isthmus of the midbrain and in the dorsal somites. These sites correspond to the previously reported expression domains for Rdh10 [23,32].

**Rdh10 gene trap allele causes severe retinoid deficiency phenotype**

Animals heterozygous for the Rdh10\(^{geo}\) allele are viable and fertile, with no overt abnormalities. Homozygous Rdh10\(^{geo/geo}\) embryos, however, exhibit all the hallmarks of extreme retinoid deficiency, with a phenotype remarkably similar to embryos lacking Aldha2. At E9.5, Rdh10\(^{geo/geo}\) embryos display defects in embryo growth and axial turning (Fig. 3A–B). Posterior pharyngeal somites. These sites correspond to the previously reported are also present in the isthmus of the midbrain and in the dorsal embryos. First arches are small and malformed, while arches posterior to the first are absent altogether in most embryos. Forelimb buds appear to be caught in an abnormal leftward “J” shape or, occasionally, a rightward “L” body shape when viewed from the front (Fig. 4 B). Some embryos have a milder phenotype in which the tailbud is positioned in front of the embryo, indicating that axial turning is complete. The growth defect in Rdh10\(^{geo/delta}\) mutants is most obvious at E10.5. For wild type embryos, overall embryo size increases substantially between E9.5 and E10.5 (Fig. 4 A, C). Rdh10\(^{geo/delta}\) embryos, in contrast, appear to cease growth and axis extension around E9.25. Rdh10\(^{geo/delta}\) embryos have abnormalities in many tissues and structures affected by retinoid deficiency including the heart, otic vesicle, pharyngeal arches and forelimb buds. Cardiac defects are observed in all Rdh10\(^{geo/delta}\) embryos. Hearts fail to undergo normal looping and chamber formation, remaining, instead, simple tubes oriented vertically and enlarged by edema. Otic vesicles are present, but are tiny and occasionally duplicated. Pharyngeal arch defects are also apparent in Rdh10\(^{geo/delta}\) embryos. First arches are small and malformed, while arches posterior to the first are absent altogether in most embryos. Forelimb buds are reduced in size or absent.

The Rdh10\(^{geo}\) construct was designed to cause a null mutation via deletion of exon 2, generating a stop codon that should disrupt the open reading frame of the Rdh10 mRNA and target the transcript for nonsense mediated decay. In order to verify that the Rdh10\(^ {delta}\) transcript encodes a premature stop codon, we characterized Rdh10 cDNA from wild type and Rdh10\(^{delta/delta}\) mutant embryos. The wild type Rdh10 mRNA encodes an open reading frame that spans the 6 spliced exons of the transcript (Fig. 4 E). Amplification of Rdh10 cDNA from Rdh10\(^{delta/delta}\) embryos produced a distinct product 235 bp shorter than the wild type cDNA. Direct sequencing of the mutant Rdh10 cDNA revealed that exon 2 is absent and exon 1 is spliced directly to exon 3, producing a frame shift and generating a stop codon within the open reading frame in the third codon of exon 3 (Fig. 4 E). Protein produced from the Rdh10\(^{delta}\) transcript would be encoded by exon 1 only. Quantitative RT-PCR was performed to assess transcript levels of the Rdh10\(^{delta}\) transcript. Transcript from Rdh10\(^{delta/delta}\) embryos was detected at \(\sim 50\%\) the level produced by wild type embryos, suggesting that the Rdh10\(^ {delta}\) transcript is targeted for degradation by nonsense mediated decay (Fig. 4 F).

**Loss of Rdh10 or Aldha2 decreases RA production**

Rdh10 and RALDH2 play sequential roles in synthesis of RA. In order to assess the extent to which the new Rdh10\(^{delta}\) and Aldh1a2\(^{geo}\) mutations disrupt RA synthesis, we utilized the RARE-lacZ reporter transgene, which allows the level and distribution of RA signaling to be visualized by staining for \(\beta\)-galactosidase.

![Figure 3. Phenotype of Rdh10\(^{geo}\) gene trap allele.](Image 38x24 to 76x41)
Figure 4. Phenotype and characterization of transcript of \textit{Rdh10}\textsuperscript{delta} allele. (A, C) Wild type and (B, D) homozygous \textit{Rdh10}\textsuperscript{delta/delta} embryos were collected at E9.5 (A–B) and E10.5 (C–D. (E9.5 mutant embryos \( n = 34 \); rightward turn \( \gamma \) complete \( n = 33 \). E9.5 with leftward turn \( n = 1 \). E10.5 mutant embryos \( n = 5 \).) Formalin-fixed embryos were stained with DAPI and imaged by confocal microscopy. For each embryo, a Z-stack of confocal slices was collapsed to form a "pseudo-SEM" image. (E) Schematic diagram of \textit{Rdh10} spliced mRNA exon structure, along with coding sequence for \textit{Rdh10} wild-type (full length) and \textit{Rdh10}\textsuperscript{delta} mutant (truncated) as determined by direct sequencing of reverse transcribed \textit{Rdh10} mRNA. Yellow boxes indicate spliced exons of \textit{Rdh10} mRNA. Dark blue arrow represent coding sequence of spliced wild type \textit{Rdh10} mRNA, blue boxes beneath arrow symbolize truncated coding sequence resulting from splicing of exon 1 directly to exon 3. Nucleotide and protein sequence indicated beneath schematic diagram shows 3\' end of exon 1 spliced directly to 5\' end of exon 3, generating a premature stop codon. Pink box indicates exon 1 derived sequence, green box represents exon 3 derived sequence. (F) Histogram of elative level of \textit{Rdh10} mRNA as assessed by reverse transcription qPCR. doi:10.1371/journal.pone.0030698.g004
activity [33]. Loss of RALDH activity in Aldh1a2geo/geo mutant embryos causes a severe reduction in RA signaling (Fig. 5 A–B), as has been observed for previously reported knockout alleles of Aldh1a2 [8,27]. Whereas wild type littermate embryos have strong RA signaling throughout the trunk and within the forebrain, homozygous Aldh1a2geo/geo mutant embryos are almost completely devoid of RA signaling. A small residual amount of RA signaling can be detected in mutant embryos in the forebrain, consistent with the RA signaling in previously reported alleles [8,27]. In the mutants we examine here, there is also a very small amount of residual RA signaling detected within the trunk, specifically within the neural tube region at the level of the first few somites. These data demonstrate that the Aldh1a2geo mutation is either a null allele or extreme hypomorph. The residual RA signaling observed within the forebrain of mutant embryos corresponds to the signal from activity mediated by RALDH1 and RALDH3. The small amount of RA signaling detected in the trunk of Aldh1a2geo/geo mutant embryos, which has also been observed in one of the established Aldh1a2 knockout alleles [34], may result from a tiny fraction of wild type activity remaining in the Aldh1a2geo allele or from variation in the null phenotype on different strain backgrounds.

Analogous to the RA attenuation resulting from disruption of Aldh1a2geo, loss of RDH10 function in the Rdh10geo/geo mutants also elicits a dramatic reduction in RA signaling (Fig. 5 C–J). At E8.5 RA signaling in mutant embryos is essentially absent (Fig. 5 C–F). At E9.5 residual RA signaling can be detected in Rdh10geo/geo embryos within the trunk neural tube region, the extent and pattern being slightly broader than is seen for the Aldh1a2geo/geo mutants (Fig. 5 G–J). Residual RA signaling within the forebrain, which is observed in Aldh1a2geo/geo mutants, is completely abolished in Rdh10geo/geo embryos. For the Rdh10geo/geo mutant embryos, as with the Aldh1a2geo/geo mutant embryos, the residual RA signaling in the trunk region is localized to the neural tube. Rdh10geo/geo embryos also retain a small amount of residual signaling in the heart and tissue dorsal to the heart. Rdh10geo/geo embryos have variable residual RA signaling that is somewhat reflective of their variability in phenotype. These data indicate that, at the critical E8.0–E8.5 stage of development, RDH10 is essential for nearly all RA signaling within the early developing embryo. At E9.5 and later stages, the residual RA detected indicates that other enzymes can contribute a small amount of RDH activity to metabolism of Vitamin A into RA and the small amount of RA produced results in signaling concentrated within the trunk neural tube.

**Rdh10 transcription is upregulated by retinoid deficiency**

The severe phenotype of the Rdh10geo/geo and Rdh10geo/geo mutants and dramatic reduction in RA synthesis, indicate that RDH10 is responsible for most RDH activity within the early embryo and raises the possibility that the RDH reaction could function as a control point in converting Vitamin A to RA. We therefore sought to determine if embryo RA levels feed back to regulate transcription of the Rdh10 gene. To that end we evaluated Rdh10 expression in Aldh1a2geo/geo mutant embryos in which RA production is severely limited. Rdh10 expression levels were assessed by quantitative RT-PCR of E9.5 Aldh1a2geo/geo mutant embryos and their wild type and heterozygous littermates (Fig. 6 A). The RA deficient Aldh1a2geo/geo embryos have significantly elevated Rdh10 expression levels relative to wild type embryos, with expression levels in homozygous mutant embryos ranging between 1.3-fold and 1.8-fold higher than wild type and heterozygous littermates. The elevated Rdh10 transcript in Aldh1a2geo/geo mutant embryos was expressed in the normal spatial distribution within the embryo at E8.5 (Fig. 6B). These data showing elevation of Rdh10 expression in RA-deficient Aldh1a2geo/geo mutant embryos clearly demonstrate that Rdh10 transcription is regulated by feedback from the metabolic product RA in mice. Interestingly, Rdh10 contains a potential RAR-RXR (RARE) sequence within exon 5, however to date it is not known whether this is a functional motif nor if it acts in a negative or positive fashion in response to retinoid signaling as has been observed for other RAREs. Nonetheless, our results provide compelling evidence that the RDH reaction is an important control point in regulating metabolism of Vitamin A into RA in mammals and sets the stage for future examination of the complexities of feedback and feedforward enzymatic regulation during this process.
**Discussion**

**Importance of RDH reaction in RA production**

Vertebrate embryonic development depends upon precise spatial and temporal control of RA signaling. Two sequential enzymatic steps are required for metabolism of retinol into RA, the RDH first reaction and the RALDH second reaction (Fig. 6C). Biochemically, either step could function as a control point for regulating synthesis of RA. However, because the RDH first reaction was initially attributed to ubiquitous or redundant members of the ADH family and was thought to occur in an unregulated fashion, the initial step of Vitamin A metabolism was interpreted as being unimportant for the control of RA synthesis. Over time this led to acceptance of the view that that control of Vitamin A metabolism and RA synthesis was driven predominantly by the RALDH second step.

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**Figure 6. Rdh10 is up-regulated by reduction of RA.** (A) Histogram representing relative levels of Rdh10 mRNA from wild type, heterozygous Aldh1a2+/− and homozygous Aldh1a2−/− embryos, as assessed by quantitative RT-PCR, demonstrating elevated levels of Rdh10 transcript in homozygous Aldh1a2−/− mutants. (B) RNA in situ hybridization for expression of Rdh10 mRNA in E8.5 wild type and Aldh1a2−/− mutant embryos, demonstrating that the increased Rdh10 expression occurs in a pattern similar to the normal expression pattern for the gene (mutant embryos n = 5). (C) Model depicting that metabolic product RA feeds-back to reduce expression of membrane bound Rdh10 in mouse embryos. Pink lines represent that RDH10 and RDH reaction occur in membrane-bound compartment. Cytosolic ADH enzymes do not contribute to any physiologically relevant production of RA in the embryo. doi:10.1371/journal.pone.0030698.g006
The view that the RDH first reaction occurs in a ubiquitous and unregulated fashion was challenged by the identification of Rdh10<sup>trex</sup>, a mutation that dramatically impairs RA synthesis via disruption of RDH10, a spatially and temporally regulated membrane-bound SDR. However, because the RDH reaction had previously been thought to be carried out by redundant or ubiquitous ADH family members, and because the Rdh10<sup>trex</sup> mutant embryos exhibited a milder phenotype and survived longer than embryos lacking Aldha2, the RDH first reaction continued to be thought of as relatively unimportant for the spatiotemporal control of RA synthesis. The residual production of RA and the less severe phenotype of Rdh10<sup>trex</sup> embryos compared to Aldha2<sup>-/-</sup> mutant embryos indicated that some RDH activity remained intact in the Rdh10<sup>trex</sup> embryos, mediated possibly by remaining activity of the hypomorphic Rdh10<sup>trex</sup> point mutant enzyme, or by other RDH enzymes, or by ADH enzymes. Given that genetic or environmental perturbations causing excessive or insufficient levels of RA cause embryonic malformations, it is important to resolve the question of which enzymes catalyze the RDH reaction during embryogenesis and, further, to understand whether these enzymes function redundantly and ubiquitously or if their regulated expression may serve as a control point for production of RA.

To gain insight into the enzymes and regulation of RA synthesis, we have generated a knockout mutation of Rdh10 and demonstrate that complete loss of the RDH10 enzyme results in embryonic death until E10.5. Rdh10<sup>delta/delta</sup> embryos have severe defects in growth, axial turning, and cardiac and pharyngeal development. The severe embryonic phenotypes of Rdh10<sup>delta/delta</sup> mutants unequivocally demonstrate that redundant or ubiquitous enzymes, including the members of the ADH family, do not contribute significantly to physiologically relevant production of RA during embryogenesis. Moreover, the severity of the phenotypes resulting from loss of an individual enzyme, RDH10, provides clear proof that the first RDH reaction of Vitamin A metabolism is as critical a control point in the synthesis of RA as the second RALDH reaction.

Although the phenotype of Rdh10<sup>delta/delta</sup> mutant embryos is very severe, it is not identical to the extreme phenotype of loss of Aldha2. Loss of RALDH2 function results in embryos that apparently cease to grow at around E8.5, whereas loss of Rdh10 results in embryos that apparently cease to grow at around E9.0. The slightly milder phenotype of the Rdh10<sup>delta/delta</sup> embryos is likely due to the small amount of residual RA produced at E9.5 within the trunk region of affected embryos. The identity of the enzymes responsible for the residual trunk RA at E9.5 is unknown. It is possible that the small amount of residual RA production in Rdh10<sup>delta/delta</sup> mutant embryos represents the contribution of other, as yet unidentified, SDR enzymes closely related to RDH10 or, alternatively, ADH enzymes. Recently, we demonstrated that a compound knockout of multiple Adh genes does not impair the residual RA synthesis in Rdh10<sup>trex</sup> embryos nor exacerbate the phenotype [35], a result which argues against a role for ADHs. Whichever enzymes are ultimately found to be responsible for the small amount of residual RA, it is clear that they are insufficient to produce physiologically relevant levels of RA and are unable to compensate for loss of Rdh10.

Concurrent with the present study, a distinct knockout allele of Rdh10, a deletion of Rdh10 exon 4, was reported in the literature [36]. The phenotypes of the Rdh10 exon 4 deletion allele (Rdh10<sup>flu/fl</sup>) are largely consistent with the Rdh10<sup>delta/delta</sup> allele we describe here. In both cases, the majority of embryos survive until around E10.5 and exhibit defects in growth and extension of the body axis, absence of posterior pharyngeal arches, compacted somites, and small or duplicated otic vesicles. RA signaling is also notably absent from the forebrain region in both knockout models at this stage. The primary difference is that we do not observe embryo survival beyond E10.5 in our Rdh10<sup>delta/delta</sup> mutants, whereas a small fraction (10%) of exon 4 deletion mutants survive until E12.5. This difference may be related to variation in strain background or the relative importance of the individual exons for Rdh10 activity that were deleted. We also examined RA signaling in Rdh10<sup>delta/delta</sup> embryos in a broader range of embryos and observe that between the 1–6 somite stages (Fig. 5C–F), RA signaling is not detected in Rdh10<sup>delta/delta</sup> embryos compared to the robust detection of reporter activity in control embryos. Taken together, these data indicate the important role of Rdh10 in RA signaling during the early critical E8.0–8.5 window of development.

**Feedback regulation on RA metabolism**

In addition to showing that RDH10 is the predominant enzyme responsible for embryonic conversion of retinol to retinal, the data presented here reveal that Rdh10 gene expression is modulated by presence or absence of RA, being upregulated by RA deficiency in Aldha2<sup>Tre/tre</sup> mutants. The upregulation of Rdh10 expression when RA synthesis is impaired demonstrates that Rdh10 is a point for feedback control in Vitamin A metabolism and RA synthesis in mammals (Fig. 6C). A similar mode of regulation has been observed in Xenopus and zebrafish, indicating that the mechanistic feedback control of Rdh10 to modulate the RA synthesis pathway is evolutionarily conserved [24, 25]. The evolutionary conservation of feedback regulation of the Rdh10 gene by RA lends further support to the concept that RDH10-mediated oxidation of retinol to retinal plays an important role in regulating the key process of embryonic Vitamin A metabolism and RA synthesis.

The conversion of retinol to retinal is a reversible reaction. Enzymes of the SDR family can mediate both the forward and the reverse directions. Transcriptional regulation of SDR enzymes to mediate RA homeostasis is observed in other contexts. In zebrafish embryos, excess RA induces expression of dhrs3a, an SDR that catalyzes the reduction of retinal into retinol [25]. In adult mouse liver, chronic exposure to ethanol causes complex changes in RA metabolism and is associated with transcriptional changes in RA metabolic genes including induction of the SDR Dhrs9 [37]. Together these data suggest that reversible interconversion of retinol to retinal by SDRs serve as a key control step in RA homeostasis. Consistent with this, biochemical analysis of Vitamin A metabolism in embryos has indicated that the first enzymatic reaction, the oxidation of retinol to retinal, is the rate-limiting step in the synthesis of retinoic acid [38].

**Spatial localization of RA production in tissue and cell**

The finding that the RDH1 reaction, mediated primarily by RDH10, is a major control point in RA synthesis has implications for understanding the tissue specificity and sub-cellular localization of the sequential steps of RA production. With respect to tissue distribution of Vitamin A metabolism and RA synthesis, it is notable that the expression pattern of Rdh10 within an embryo is very similar to, but not identical to that of Aldha1<sup>2</sup> [23, 32]. In chick, the expression pattern of Rdh10 has also been shown to overlap with that of the gene encoding the retinol transporter Stra6 [26]. Together, these data suggest that retinol is imported into cells, oxidized into retinal and subsequently into the product RA across the entire range of cells. We have previously shown that maternal administration of intermediate retinal, which exposes all cells of the embryo to the retinal intermediate, can rescue RDH10 deficient embryos to birth [39] and recently this has now been extended postnatally [36]. Although viable, these rescued embryos still exhibit some abnormalities including limb...
and behavioral defects. The defects remaining in retinal rescued animals indicate that while embryo viability is not dependent on spatially restricted retinal production, proper tissue patterning and development are.

With respect to sub-cellular localization of Vitamin A metabolism, the medium chain reductase ADH enzymes are known to function within the cytosol, while the SDR RDHs function in a membrane bound capacity. RDH10 is associated with the membrane-bound compartment [40] and its membrane association is necessary for its function [41]. Recently, we demonstrated that the RDH reaction occurs not in the cytosol where ADHs reside, but in a membrane-bound cellular compartment such as that occupied by RDH10 [39]. Within the cytosol, RBP1 binds to retinol and inhibits oxidation. The membrane localization of RDH10 is functionally important, presumably because it allows RDH10 to oxidize RBP1-free pools of retinol available in the membrane compartment (Fig. 6C). These findings suggest that ADHs do not contribute significantly to retinol oxidation because they cannot oxidize the RBP1-bound retinol in the cytosol and are unable to access RBP1-free retinol from membranes (Fig. 6C). They explain why the cytosolic ADHs, although widely expressed, cannot compensate for the loss of RDH10 during embryogenesis.

The lack of function of ADHs in embryonic retinol oxidation is consistent with studies of individual and compound Adh knockout mice, which suggest that ADHs function primarily in governing post-natal retinol homeostasis (reviewed in [35]). In adult mice, ADH1 provides considerable protection against vitamin A toxicity [42,43] whereas ADH4 promotes survival during vitamin A deficiency [44], a difference which illustrates distinct physiological functions for these two enzymes in retinol metabolism. In contrast, ADH3 appears to function redundantly in both the prevention of retinol toxicity and Vitamin A deficiency, a dual role consistent with its ubiquitous expression. Collectively, these biochemical and compound mutant analyses strongly argue against a significant role for ADHs in Vitamin A metabolism and RA synthesis during embryonic development.

Given that Rdh10 is the predominant enzyme oxidizing retinol to retinal and the mounting evidence that ADHs make little if any contribution, we conclude that the conversion of retinol to retinal and the mounting evidence that ADHs make little if any contribution, we conclude that the conversion of retinol to retinal and the mounting evidence that ADHs make little if any contribution to retinol oxidation is predominantly, if not completely, governed by RDH10 [39]. Within the cytosol, RBP1 binds to retinol and inhibits oxidation. The membrane localization of RDH10 is functionally important, presumably because it allows RDH10 to oxidize RBP1-free pools of retinol available in the membrane compartment (Fig. 6C). These findings suggest that ADHs do not contribute significantly to retinol oxidation because they cannot oxidize the RBP1-bound retinol in the cytosol and are unable to access RBP1-free retinol from membranes (Fig. 6C). They explain why the cytosolic ADHs, although widely expressed, cannot compensate for the loss of RDH10 during embryogenesis.

The Materials and Methods section provides detailed information on the experimental procedures used in this study, including methods for generating and characterizing mouse models, methods for assessing gene expression and protein localization, and methods for analyzing data. The materials and methods section is crucial for reproducibility and validation of the research findings.

The qPCR experiments were performed to analyze the expression levels of the Rdh10 gene in different mouse strains. qPCR is a powerful technique for quantifying gene expression and is widely used in biomedical research. The qPCR primers used in this study were designed to amplify a specific region of the Rdh10 gene, allowing for accurate quantification of gene expression. The results of the qPCR experiments provide important insights into the role of Rdh10 in Vitamin A and RA metabolism during embryonic development.

In conclusion, this study provides valuable insights into the function and role of the RDH10 enzyme in Vitamin A and RA metabolism during embryonic development. The findings have implications for understanding the mechanisms underlying retinal development and the role of RDH10 in retinal disease.
RNA in situ hybridization and pseudo-SEM imaging of whole mouse embryos

Whole mount in situ hybridization for Rdh10 was performed as in [23]. Embryo morphology was visualized by staining whole embryos with DAPI to label all cell nuclei and imaging fluorescent signal. Embryos were fixed in 4% formalin overnight at 4°C then stained with 2 ug/ml DAPI in PBS and imaged by confocal microscopy using an LSM5 Pascal confocal microscope. For each embryo, a Z-stack of confocal slices was collapsed to form a “pseudo-SEM” image. RA signaling was visualized by staining embryos carrying the RARE-lacZ transgene for β-galactosidase activity. A subset of the brightfield embryo images (Fig. 2D, Fig. 5 G-J, 6B) were processed with Helicon Image processing software to compile and render the focused regions of multiple focal planes as a single in-focus image. A minimum of 5 embryos were used for each parameter analysed unless otherwise stated.

Web addresses for reagents and technique resources
Transnetyx automated qPCR genotyping: www.Transnetyx.com

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