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Novel retinoic acid generating activities in the neural tube and heart identified by conditional rescue of *Raldh2* null mutant mice

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SUMMARY

Retinoid control of vertebrate development depends upon tissue-specific metabolism of retinol to retinoic acid (RA). The RA biosynthetic enzyme RALDH2 catalyzes much, but not all, RA production in mouse embryos, as revealed here with *Raldh2* null mutants carrying an RA-responsive transgene. Targeted disruption of *Raldh2* arrests development at midgestation and eliminates all RA synthesis except that associated with *Raldh3* expression in the surface ectoderm of the eye field. Conditional rescue of *Raldh2*$^{-/-}$ embryos by limited maternal RA administration allows development to proceed and results in the establishment of additional sites of RA synthesis linked to *Raldh1* expression in the dorsal retina and to *Raldh3* expression in the ventral retina, olfactory pit and urinary tract. Unexpectedly, conditionally rescued *Raldh2*$^{-/-}$ embryos also possess novel sites of RA synthesis in the neural tube and heart that do not correspond to expression of *Raldh1-3*. RA synthesis in the mutant neural tube was localized in the spinal cord, posterior hindbrain and portions of the midbrain and forebrain, whereas activity in the mutant heart was localized in the conotruncus and sinus venosa. In the posterior hindbrain, this novel RA-generating activity was expressed during establishment of rhombomeric boundaries. In the spinal cord, the novel activity was localized in the floorplate plus in the intermediate region where retinoid-dependent interneurons develop. These novel RA-generating activities in the neural tube and heart fill gaps in our knowledge of how RA is generated spatiotemporally and may, along with *Raldh1* and *Raldh3*, contribute to rescue of *Raldh2*$^{-/-}$ embryos by producing RA locally.

Key words: Aldehyde dehydrogenase, Retinaldehyde dehydrogenase, RALDH1, RALDH2, RALDH3, Retinoic acid, Spinal cord, Hindbrain, Heart, Retina, Olfactory, Urinary tract

INTRODUCTION

The role of vitamin A in higher animals is unique in that it functions not only in light absorption for vision (Wald, 1951), but also in gene regulation during development, where it influences pattern formation in several organs including the hindbrain (Maden et al., 1996; Gould et al., 1998; Niederreither et al., 2000; White et al., 2000), spinal cord (Sockanathan and Jessell, 1998; Pierani et al., 1999), eye (Wagner et al., 2000), heart (Dersch and Zile, 1993; Niederreither et al., 2001), kidney (Batourina et al., 2001), lung (Malpel et al., 2000) and limb buds (Stratford et al., 1999; Power et al., 1999). The ability of vitamin A (retinol) to influence development is made possible by a battery of enzymes controlling a two-step metabolic pathway in which retinol is oxidized to produce retinal followed by further oxidation of retinal to produce retinoic acid (RA) (reviewed by Duester, 2000). RA serves as a ligand for nuclear receptors that function in transcriptional regulation (reviewed by Mangelsdorf et al., 1994; Kastner et al., 1994). Mice carrying knockout mutations of RA receptors display phenotypes that resemble those seen during gestational vitamin A deficiency (reviewed by Kastner et al., 1995).

Genetic analysis of enzymes regulating RA synthesis indicates that retinol is ubiquitously oxidized to retinal by alcohol dehydrogenase (Molotkov et al., 2002), whereas retinal is tissue-specifically oxidized to RA by aldehyde dehydrogenase (Niederreither et al., 1999). Thus, RA is not produced by all cells of the body at all stages of development, but is instead generated in a unique spatiotemporal pattern. Metabolism of retinol to RA occurs at relatively low levels, but RA has been detected in embryos using sensitive reporter assays. Both a transgenic RA-reporter mouse strain containing *lacZ* linked to a RA response element (RARE) as well as a mouse embryo explant bioassay using *RARE-lacZ* reporter cells have shown that RA is undetectable in mouse embryos at 6.5 days of embryonic development (E6.5), but that RA is detectable at E7.5 onwards (Rossant et al., 1991; Ang et al., 1996). RA detected by these assays is initially found in the trunk, then by E8.5 it is additionally found in the posterior hindbrain and eye. The observation that endogenous RA synthesis initiates in the mouse at E7.5 is supported by studies indicating that retinaldehyde dehydrogenase 2 (RALDH2), capable of oxidizing retinal to RA, is first expressed at E7.5 in trunk

mesoderm, with additional expression by E8.5 in the optic vesicles (Niederreither et al., 1997; Haselbeck et al., 1999; Ulven et al., 2000). Targeted disruption of *Raldh2* results in embryonic lethality with a failure to develop beyond E8.75, indicating that this enzyme is essential for development (Niederreither et al., 1999). *Raldh2*$^{-/-}$ embryos lack all RA detection except for a reduced level in the eye field (Niederreither et al., 1999). Thus, an additional RA-generating enzyme must be at work in the eye, possibly RALDH1 or RALDH3 which, like RALDH2, are members of the aldehyde dehydrogenase (ALDH) family able to metabolize retinal to RA (Mic et al., 2000; Li et al., 2000; Grün et al., 2000; Suzuki et al., 2000).

Raldh2$^{-/-}$ embryos can be rescued by continuous maternal RA administration, which results in growth to at least E10.5, albeit with a truncated forelimb bud (Niederreither et al., 1999), as well as greatly improved hindbrain (Niederreither et al., 2000) and heart (Niederreither et al., 2001) development. However, it is unclear how much RA is needed for rescue and it is unknown whether additional RA-generating enzymes are expressed in *Raldh2*$^{-/-}$ embryos during the rescue. Such endogenous activity may provide local sources of RA complementing the systemic RA derived from maternal administration. As mentioned above, there is an RA-generating activity in the eye of *Raldh2*$^{-/-}$ embryos prior to rescue, and this plus other activities may exist in the rescued mutants. *Raldh1*, *Raldh2* and *Raldh3* have distinct embryonic expression patterns both in the eye and in other organs, suggesting that all three genes play important roles in RA generation throughout the embryo (Mic et al., 2000). However, it is not yet clear whether these three genes can account for all RA generation in mouse embryos. In particular, the spinal cord has been reported to be a site of RA synthesis at early embryonic stages prior to motor neuron formation (McCaffery and Dräger, 1994; Maden et al., 1998), and although *Raldh2* is expressed transiently in the dorsal spinal cord plus the somites (Zhao et al., 1996) it is unclear if this is the source of RA for the intermediate region where ventral interneurons develop (Pierani et al., 1999). Later in development, *Raldh2* is expressed in spinal cord motor neurons in mouse (Zhao et al., 1996; Haselbeck et al., 1999) and chick (Sockanathan and Jessell, 1998), and RA has been detected in the motor neurons using a binary mouse embryo reporter system (Solomin et al., 1998). The issue of RA generation for hindbrain development is also unclear. In *RARE-lacZ* reporter mice, *lacZ* expression is observed in the posterior hindbrain plus adjacent somites (Rossant et al., 1991). As *Raldh2* is expressed in somites, but not the hindbrain neuroepithelium itself, it has been suggested that RA produced in the somites may be transported to the hindbrain where it regulates development of posterior hindbrain (Gould et al., 1998; Swindell et al., 1999). However, the hindbrain might also possess an endogenous RA generating activity.

In order to examine the extent of retinoid signaling remaining in *Raldh2*$^{-/-}$ embryos after RA rescue, we describe here conditional RA rescue studies on an *Raldh2* null mutant mouse line carrying the *RARE-lacZ* reporter gene. In this case 'conditional' means that RA was provided only for certain lengths of time, with embryos then being allowed to develop beyond the last treatment. This has allowed us to use the *RARE-lacZ* reporter to examine endogenous RA production in rescued embryos during a period of time following clearance of the administered RA.

MATERIALS AND METHODS

Generation of *Raldh2* null mutant mice

The methodology for production of null mutant mice has been described previously (Joyner, 1993; Deltour et al., 1999). Briefly, a mouse *Raldh2* cDNA (Haselbeck et al., 1999) was used as a probe to isolate a 16.2 kb *Raldh2* genomic clone containing exons 2-5 derived from a mouse 129/SvJ genomic library prepared in lambda FIX-II (Stratagene) using conventional procedures (Sambrook et al., 1989). This DNA was used to construct a gene targeting vector in which exons 3 and 4 were deleted and replaced by a PGK-hygromycin selectable marker in the same transcriptional orientation as *Raldh2*, thus preventing translation beyond residue 67. This results in deletion of the entire catalytic domain (residues 271-484) including the catalytic cysteine at residue 301 (Lamb and Newcomer, 1999). The upstream homology consisted of a 2.3 kb fragment including exon 2, whereas the downstream homology was a 4.5 kb fragment within intron 4. Both fragments were generated by PCR amplification of DNA from the genomic clone using Pfu polymerase; a *Sac*I restriction site was also engineered into the downstream fragment such that it would lie just downstream of PGK-hygromycin after subcloning. The *Raldh2* gene targeting vector was introduced by electroporation into mouse embryonic stem (ES) cells (R1 cells from 129/Sv strain). We identified two targeted ES cell lines (clones 37 and 102) by Southern blot analysis using a 3′-flanking probe external to the targeting vector (i.e. a 1 kb *Hin*dIII-*Sac*I fragment containing exon 5) that detected both a wild-type 9.5 kb *Sac*I fragment and a mutant 6.5 kb *Sac*I fragment in which exons 3-4 were deleted. Both clones were microinjected into C57Bl/6 blastocysts and chimeric mice for each clone were produced. Matings of chimeric mice with C57Bl/6 mice produced *Raldh2*$^{+/-}$ heterozygous mutant mice from each clone, which were then mated to generate homozygous mice. As each clone resulted in the same embryonic lethal phenotype, all further experiments were performed on clone 102. All genotyping was performed by Southern blotting.

Detection of retinoic acid using a *RARE-lacZ* reporter gene

RARE-lacZ mice carry a *lacZ* transgene controlled by a retinoic acid response element (RARE), which is quite useful for identifying embryonic tissues containing RA by in situ staining for *lacZ* expression as previously described (Rossant et al., 1991). *Raldh2*$^{-/+}$ mice were mated to *RARE-lacZ* mice to generate a strain of *Raldh2*$^{-/+}$ mice carrying *RARE-lacZ*, identified by Southern blot analysis. Embryos were stained 6 hours for *lacZ* expression (β-galactosidase activity) using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as substrate (Hogan et al., 1994). Stained embryos were embedded in 3% agarose and sectioned at 100 μm with a vibratome as described (Mic et al., 2000).

Conditional retinoic acid rescue of *Raldh2*$^{-/-}$ embryos

Raldh2$^{-/-}$ embryos were treated with RA to rescue development, similar to that described previously (Niederreither et al., 1999), but with the modifications described here to provide a conditional approach. Following timed matings of heterozygous mutant parents, pregnant mice were orally administered all-*trans*-RA (Sigma) at a dose of 2.5 mg/kg body mass dissolved in 0.2 ml corn oil at 12 hour intervals from E6.75 to E8.25. In some cases treatment was ended at E7.25 or E7.75. Embryos were examined at stages ranging from E8.5 to E10.5, in some cases up to 60 hours following the last RA dose. In studies involving *Raldh2*$^{-/-}$ embryos carrying *RARE-lacZ*, only one parent carried the *RARE-lacZ* transgene in order to avoid gene dosage effects in embryos staining positive for *lacZ* expression.

Whole-mount in situ hybridization and immunohistochemistry

Mouse embryos were subjected to whole-mount in situ hybridization as described using antisense RNA probes (Wilkinson, 1992). The mouse cDNAs used to prepare probes included *Raldh1* (Hsu et al., 1999), *Raldh2* (Haselbeck et al., 1999), *Raldh3* (Mic et al., 2000), *Tbx5* (Chapman et al., 1996) and *Pax2* (Dressler et al., 1990). Whole-mount immunohistochemistry was performed as described previously using an antibody generated against mouse RALDH2 (Haselbeck et al., 1999).

RESULTS

Targeted disruption of *Raldh2* results in embryonic lethality

Following targeted disruption of *Raldh2*, all heterozygous mutant mice appeared normal. From eight litters of mice resulting from matings of *Raldh2*$^{-/+}$ mice we obtained 54 offspring with the following genotypes: 0 –/– (0%), 35 +/– (65%) and 19 +/+ (35%). The absence of surviving *Raldh2*$^{-/-}$ mice at birth indicated that the *Raldh2* mutation leads to an embryonic lethal phenotype. Timed matings revealed that *Raldh2*$^{-/-}$ embryos survive until E10.5, but that at E11.5 they are resorbed. *Raldh2*$^{-/-}$ embryos were distinguished from wild-type or *Raldh2*$^{-/+}$ embryos by a failure to develop beyond E8.75. A null mutant phenotype was confirmed by demonstrating that RALDH2 protein was undetectable in *Raldh2*$^{-/-}$ embryos, using whole-mount immunohistochemistry (data not shown). All *Raldh2*$^{-/-}$ embryos develop relatively normally until E8.5-E8.75, at which point they fail to undergo axial rotation and cease further growth. Thus, the mutation leads to a completely penetrant lethal phenotype, with defects essentially the same as those described for another *Raldh2* null mutant described previously (Niederreither et al., 1999). Defects include small somites and trunk, a lack of heart looping and chamber morphogenesis, small otic vesicles, and a lack of branchial arches 2 and 3.

Retinoic acid generation in *Raldh2*$^{-/-}$ embryos prior to rescue

RA was localized in *Raldh2*$^{-/-}$ embryos carrying the *RARE-lacZ* reporter gene. *Raldh2*$^{-/-}$ embryos have eliminated *lacZ* expression in the trunk and a large reduction in the eye field at E8.75 compared to wild-type mice (Fig. 1A,B). *Raldh2*$^{-/-}$ embryos do not show significant growth after E8.75 but do survive until E10.5. At E9.5-E10.5, *lacZ* expression is still absent from the trunk of mutants, but expression in the eye field has increased over that seen earlier (Fig. 1C-F). These findings indicate that RALDH2 is needed to establish essentially all RA biosynthesis in the trunk and most in the eye field, but that additional activity is present in the eye field as early as E8.75 and increases by E9.5. The remaining RA detected in the mutant eye field is localized in the surface ectoderm overlying the optic vesicle, but not in the optic vesicle itself, whereas in wild-type embryos there is a large amount of RA detection in both the optic vesicle and surface ectoderm (Fig. 1G,H).

The expression pattern of *Raldh2* mRNA from E8.25-E10.5 demonstrates that many sites of RA detection in *RARE-lacZ* wild-type embryos are sites of *Raldh2* expression. At E8.25, *Raldh2* is expressed at a high level in the optic vesicles and somites, both sites of *lacZ* expression (Fig. 1I,J). Optic vesicle expression of *Raldh2* is transient as it is missing by E8.75 and instead a nearby *Raldh2* mRNA signal occurs in the maxillary process, which persists to E10.5, when it is recognized as the maxillo-mandibular cleft (Fig. 1K-M). During these later stages, *Raldh2* is also expressed in a number of other tissues including the somites, nephric system, lung bud and heart, as previously detailed (Niederreither et al., 1997; Haselbeck et al., 1999). It can be seen that *lacZ* expression is detected at a high level in the trunk of wild-type embryos plus in the maxillo-mandibular cleft at E10.5 (Fig. 1F). However, at E10.5 it is clear that *lacZ* expression is detected in several locations where *Raldh2* mRNA was not detected including the olfactory pit, forebrain, midbrain and hindbrain, as well as in the eye where *Raldh2* mRNA is no longer detected (Fig. 1F). Previous whole-mount immunohistochemical studies have shown that RALDH2 protein is also not detected in any of those locations from E8.5-E10.5 except for the eye, where residual protein is detected only until E9.5 (Haselbeck et al., 1999). As accurate temporal assessment of the presence of RA in this reporter assay is dependent upon the turnover of β-galactosidase, some of the activity detected in the wild-type eye at E10.5 may be due to residual β-galactosidase produced earlier. However, as RALDH1 and RALDH3 are expressed at high levels in the wild-type eye by E10.5 (Mic et al., 2000), they are likely to be responsible for most *lacZ* expression detected in the eye by that stage. It should also be pointed out that the assay measures the presence of RA, but not necessarily its site of production, as RA produced in one location may diffuse to another. However, we show below that the *lacZ* expression detected in the olfactory pit, forebrain, midbrain and hindbrain are not due to diffusion from tissues expressing *Raldh2*.

RALDH3 is responsible for RA synthesis in eye of *Raldh2*$^{-/-}$ embryos

Previous studies have shown that *Raldh1* is initially expressed in the dorsal retina, whereas *Raldh3* is initially expressed in the surface ectoderm over the eye field and later in the ventral retina (Mic et al., 2000; Li et al., 2000). We found that *Raldh1* mRNA was expressed at E9.5 in the dorsal retina of wild-type embryos, but was absent in *Raldh2*$^{-/-}$ embryos (Fig. 2A,B). In contrast, *Tbx5* mRNA was expressed in the dorsal retina of both mutant and wild-type, thus verifying that dorsal retina tissue had in fact developed in the mutant (Fig. 2C,D). *Raldh3* mRNA was expressed in the surface ectoderm over the eye field in both *Raldh2*$^{-/-}$ embryos and wild-type at E8.75 (Fig. 2E,F) and at E9.5 (data not shown), but not in the ventral retina or other eye tissues at these stages. As a marker of ventral eye development, we found that *Pax2* mRNA was expressed ventrally in both mutant and wild-type (Fig. 2G,H). These findings suggest that *Raldh2* is not required for establishment of dorsoventral patterning in the eye, nor for initial *Raldh3* expression in the eye field surface ectoderm, but leave open the possibility that *Raldh2* may be needed to establish *Raldh1* and *Raldh3* expression in the dorsal and ventral retina, respectively. As *Raldh3* is expressed in the surface ectoderm over the eye field of the mutant, it is likely to be responsible for the RA (*RARE-lacZ* expression) detected in that location in *Raldh2*$^{-/-}$ embryos (Fig. 1G). Notice that both *RARE-lacZ* and *Raldh3* expression first appear in a patchy pattern over the surface of the eye in *Raldh2*$^{-/-}$ embryos (Fig. 1A, Fig. 2F).

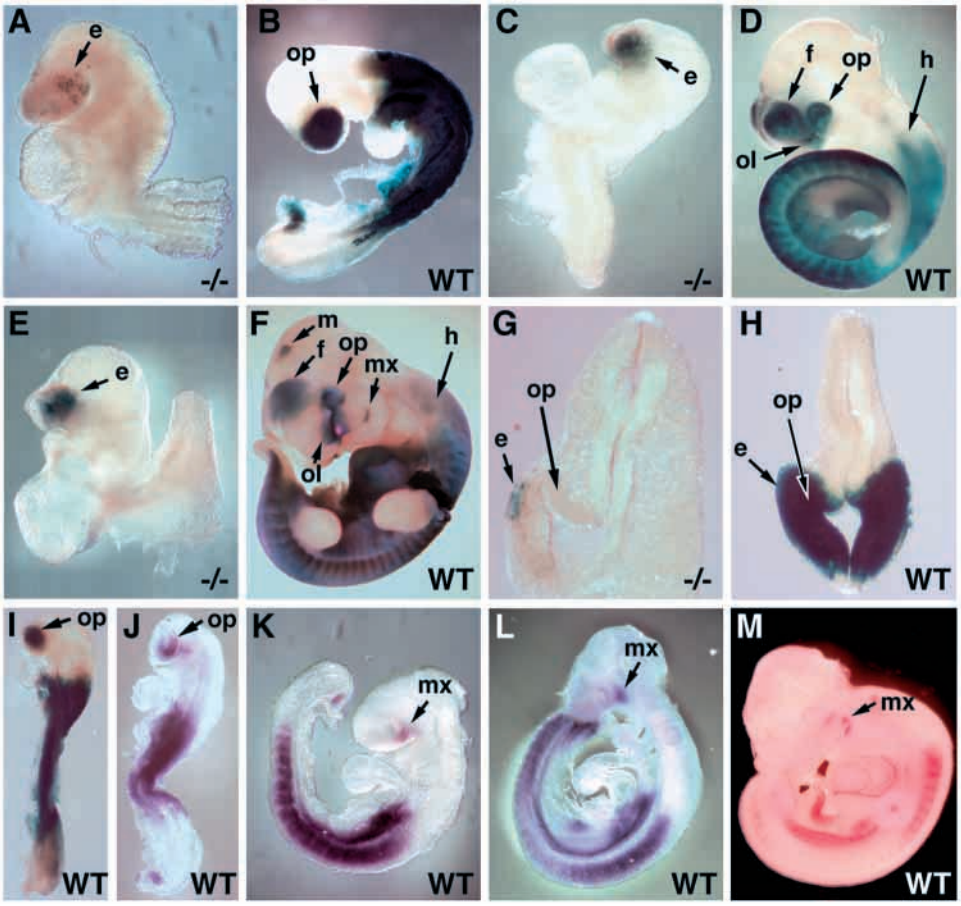

Fig. 1. RA localization in *Raldh2*$^{-/-}$ embryos carrying the *RARE-lacZ* RA-reporter gene. (A-F) Whole-mount staining for expression of *RARE-lacZ* was performed on *Raldh2*$^{-/-}$ (A,C,E) and wild-type (B,D,F) embryos at E8.75 (A,B), E9.5 (C,D) and E10.5 (E,F). (G,H) Frontal sections of *Raldh2*$^{-/-}$ (G) and wild-type (H) embryos at E8.75 show a large difference in *RARE-lacZ* expression in the eye field. (I,J) The correspondence between *Raldh2* expression and RA generation in the eye field and trunk of E8.25 wild-type embryos is demonstrated by *RARE-lacZ* expression (I) and detection of *Raldh2* mRNA by whole-mount in situ hybridization (J). (K-M) *Raldh2* mRNA detection is also shown in wild-type embryos at E8.75 (K), E9.5 (L) and E10.5 (M), where it is seen that the eye signal is now gone, but is replaced by nearby maxillary expression. e, eye field surface ectoderm; f, forebrain; h, hindbrain; m, midbrain; mx, maxillary process; ol, olfactory pit; op, optic vesicle.

Retinoic acid detection during conditional rescue of *Raldh2*$^{-/-}$ embryos

Maternal RA administration from E6.75-E10.25 was previously shown to provide near-complete rescue of *Raldh2*$^{-/-}$ embryos examined at E10.5 (Niederreither et al., 1999). In order to examine whether continuous RA treatment was needed for this rescue, we performed a conditional rescue by providing maternal RA administration at 12 hour intervals from E6.75-E8.25, then examining embryos at E10.5. We found that 13 out of 15 *Raldh2*$^{-/-}$ embryos examined at E10.5 (from 7 litters) had undergone considerable development and were externally indistinguishable from treated wild-type littermates except for the existence of severely truncated forelimb buds, as reported previously for embryos treated continuously to E10.25 (compare mutant and wild-type in Fig. 4C,D). Of the remaining two mutants that were not considered rescued, one was arrested in development, similar to an untreated mutant, and one had undergone abnormal embryonic turning, also similar to that described previously (Niederreither et al., 1999). Thus, our results demonstrate that administration of RA conditionally to only E8.25 provides a similar degree of rescue to that seen when RA was provided to E10.25.

We took advantage of this conditional RA rescue approach to examine endogenous RA generation in rescued *Raldh2*$^{-/-}$ embryos carrying *RARE-lacZ* by examining *RARE-lacZ* expression in embryos during a period of time following

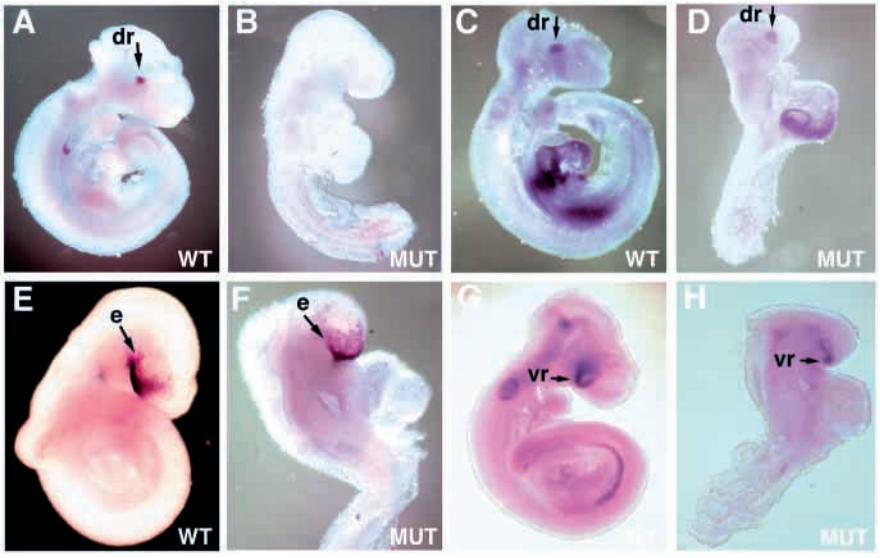

Fig. 2. Gene expression in the eye of *Raldh2*$^{-/-}$ embryos. Whole-mount in situ hybridization was performed in *Raldh2*$^{-/-}$ and wild-type embryos to examine expression of *Raldh1* at E9.5 (A,B), *Tbx5* at E9.5 (C,D), *Raldh3* at E8.75 (E,F) and *Pax2* at E9.5 (G,H). dr, dorsal retina; e, eye field surface ectoderm; vr, ventral retina.

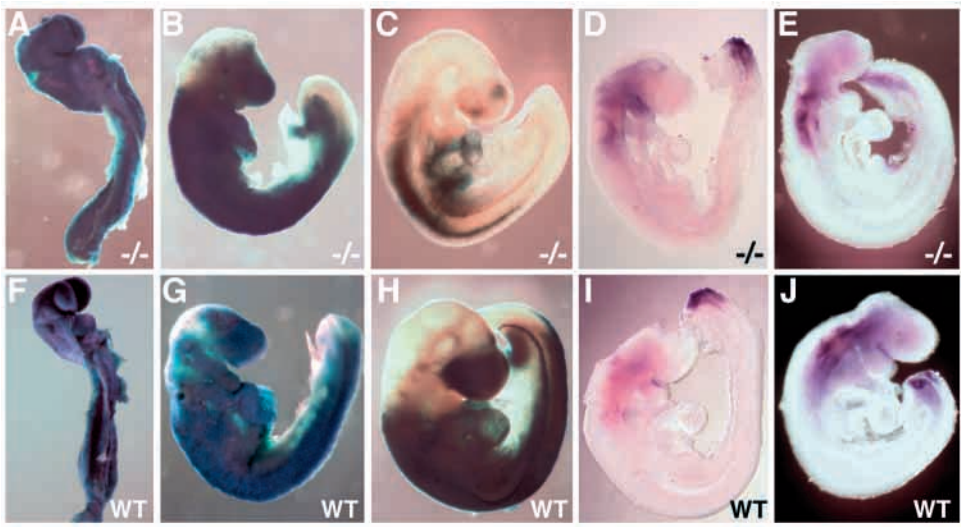

Fig. 3. Clearance of maternally administered RA in rescued *Raldh2*$^{-/-}$ embryos carrying *RARE-lacZ*. The time course of *RARE-lacZ* induction was examined in *Raldh2*$^{-/-}$ and wild-type embryos following RA administration at 12-hour intervals from E6.75-E8.25. β-galactosidase activity is shown at E8.5, 6 hours after the last RA dose (A,F); E8.75, 12 hours after the last RA dose (B,G); and E9.25, 24 hours after the last RA dose (C,H). For comparison, *RARE-lacZ* expression in untreated embryos of the same stages can be seen in Fig. 1. Expression of *Cyp26A1* during RA clearance was monitored in *Raldh2*$^{-/-}$ and wild-type embryos by whole-mount in situ hybridization at E8.75, 12 hours after the last RA dose (D,I); and E9.25, 24 hours after the last RA dose (E,J).

clearance of the administered RA. RA has a very quick turnover in mice as the plasma half-life is only 30-60 minutes (Armstrong et al., 1994) and approximately 99% clearance occurs by 8 hours in both plasma and E11 embryos (Collins et al., 1992). However, activity of the β-galactosidase reporter may persist longer than this. Thus, we observed *RARE-lacZ* expression in *Raldh2*$^{-/-}$ and wild-type embryos at various time points after the last RA dose (given at E8.25) to determine how long the β-galactosidase signal induced by the administered RA persists. At E8.5 (6 hours after the last RA dose) we found that *RARE-lacZ* was expressed uniformly throughout both *Raldh2*$^{-/-}$ and wild-type embryos (Fig. 3A,F). At E8.75 (12 hours after the last RA dose) we found that there was no longer uniform *RARE-lacZ* expression in either *Raldh2*$^{-/-}$ or wild-type embryos, as the head and tailbud regions were showing some clearance of the signal (Fig. 3B,G). By E9.25 (24 hours after the last RA dose) *Raldh2*$^{-/-}$ and wild-type embryos exhibited virtually complete clearance of β-galactosidase activity from head and tail regions that normally do not have activity (Fig. 3C,H); i.e. the RA-treated wild-type embryo shown in Fig. 3H appears similar to an untreated embryo shown in Fig. 1D. It can be seen that *Raldh2*$^{-/-}$ embryos at 24 hours post-treatment have less RA than wild-type embryos due to the loss of endogenous RA production by RALDH2, but a significant amount of RA is still detectable in specific regions of the neural tube, heart and eye (Fig. 3C,H). These clearance studies provide evidence that, at time points beyond 24 hours following the last RA dose, *RARE-lacZ* expression in *Raldh2*$^{-/-}$ embryos is a marker of endogenously produced embryonic RA rather than administered RA.

We also examined RA-treated embryos for expression of *Cyp26A1* encoding a P450 enzyme known to play an important role in RA degradation (Abu-Abed et al., 2001; Sakai et al., 2001). *Cyp26A1* is normally expressed in the hindbrain and tailbud from E8.5-E9.5, and upon RA treatment expression in the head domain is expanded both anteriorly and posteriorly (Fujii et al., 1997; MacLean et al., 2001). *Raldh2*$^{-/-}$ and wild-type embryos examined at either 12 hours (Fig. 3D,I) or 24 hours (Fig. 3E,J) after RA treatment exhibited similar *Cyp26A1* expression, indicating no dependence upon RALDH2 for expression. Expression in the head and tailbud domains was observed as previously reported including expanded expression in the head due to RA treatment (Fujii et al., 1997). As *Cyp26A1* expression is found in the head and tailbud domains where *RARE-lacZ* expression first clears (compare Fig. 3B and 3D), this is consistent with it functioning to clear some of the administered RA in those regions. However, RA clearance in most of the embryo may depend upon other enzymes or upon maternal metabolism via maternal-fetal circulation. Thus, there is

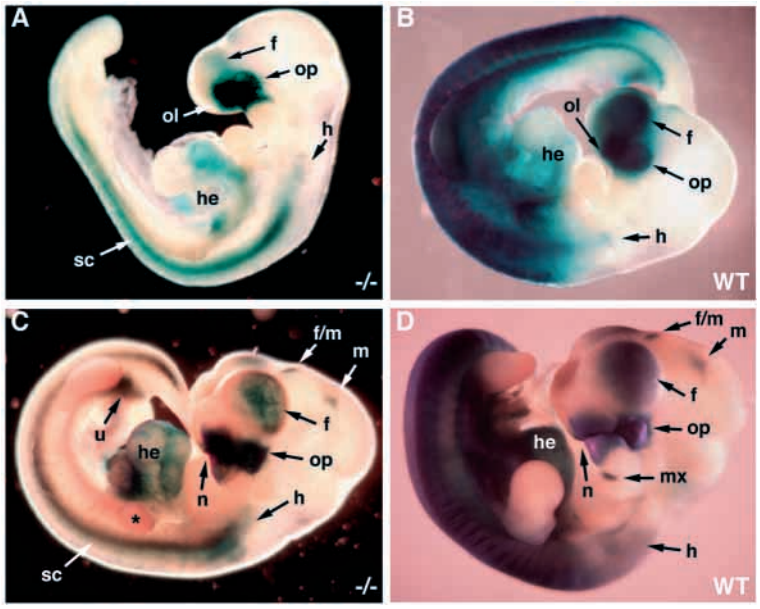

Fig. 4. Endogenous RA localization in conditionally rescued *Raldh2*$^{-/-}$ embryos carrying *RARE-lacZ*. Staining for expression of *RARE-lacZ* was performed on *Raldh2*$^{-/-}$ and wild-type embryos treated with RA every 12 hours from E6.75-E8.25, then examined at E9.5 (A,B) or E10.5 (C,D). f, forebrain; f/m, forebrain/midbrain junction; h, hindbrain; he, heart; m, midbrain; mx, maxillary process; n, nasolacrimal groove; ol, olfactory pit; op, optic vesicle; sc, spinal cord; u, urinary tract; *, truncated forelimb bud.

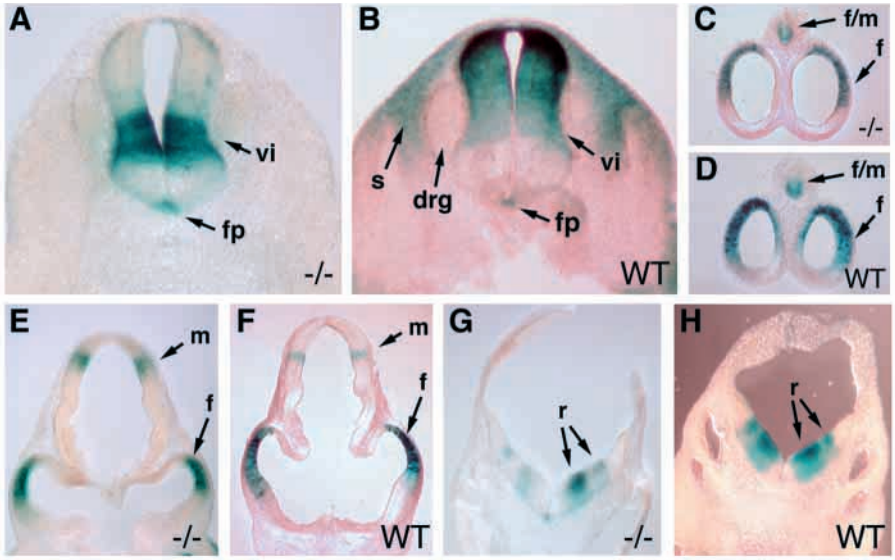

Fig. 5. Novel RA-generating activity in neural tissues. Shown are vibratome sections of E10.5 *Raldh2*$^{-/-}$ and wild-type embryos treated with RA to E8.25 showing localization of *RARE-lacZ* expression. (A,B) Transverse sections of the posterior spinal cord; the lower level of β-galactosidase activity observed in the intermediate region (vi) of the wild-type relative to the corresponding region of the mutant may be due to sequestration of the substrate (X-gal) by the high level of β-galactosidase present dorsally and in the somites, thus reducing the amount of substrate able to diffuse into the intermediate region during staining of whole-mount embryos; alternatively, the mutant may have higher expression of the novel enzyme. (C-H) Frontal sections of forebrain (C,D); frontal sections of midbrain (E,F); and oblique transverse sections through the hindbrain just rostral to the occipital somites (G,H). drg, dorsal root ganglia; f, forebrain; f/m, forebrain/midbrain junction; fp, floorplate; m, midbrain; r, rhombomere; s, somite; vi, ventral interneurons.

no evidence that *RARE-lacZ* expression observed in specific regions of the neural tube, heart and eye in *Raldh2*$^{-/-}$ embryos 24 hours after RA treatment (Fig. 3C) is due to changes in expression of *Cyp26A1* that would allow the administered RA to persist in specific regions. In fact, some *RARE-lacZ* expression remains in the mutant posterior hindbrain, despite expression of *Cyp26A1* there (Fig. 3C,E), suggesting de novo RA synthesis. Importantly, *RARE-lacZ* expression in the mutant continues well beyond 24 hours post-treatment as discussed below.

Endogenous retinoic acid generation in conditionally rescued *Raldh2*$^{-/-}$ embryos

We examined endogenous RA at E9.5 and E10.5 in rescued *Raldh2*$^{-/-}$ embryos carrying *RARE-lacZ*. This is 30-54 hours following the last dose of RA, thus well beyond the point when the β-galactosidase signal produced by the administered RA had cleared. Rescued *Raldh2*$^{-/-}$ embryos examined at E9.5 (30 hours post-treatment) exhibited considerable *RARE-lacZ* expression (Fig. 4A,B), in fact much more than the amount expected from the RA that would be produced by expression of *Raldh1* and *Raldh3*, on the basis of their known expression patterns (Mic et al., 2000). In *Raldh2*$^{-/-}$ embryos examined at E10.5 (54 hours post-treatment), even more *RARE-lacZ* expression was evident, indicating de novo RA synthesis by enzymes other than RALDH2 (Fig. 4C,D). The eye and olfactory regions of rescued mutants expressed high levels of *RARE-lacZ*, but this was expected since these are sites of *Raldh1* and *Raldh3* expression (Mic et al., 2000). However, *RARE-lacZ* expression in the spinal cord, posterior hindbrain, heart and posterior urinary tract of rescued *Raldh2*$^{-/-}$ embryos was unexpected as the expression pattern of *Raldh2* had suggested that it might be responsible for this RA, either by local production (through expression directly in heart and urinary tract) or by paracrine distribution (through expression in mesoderm adjacent to the neural tube) (Niederreither et al., 1997; Haselbeck et al., 1999). In addition, *Raldh1* and *Raldh3* are not expressed in the spinal cord, posterior hindbrain and heart in mouse embryos (Haselbeck et al., 1999; Mic et al., 2000).

It can also be seen that E10.5 *Raldh2*$^{-/-}$ embryos (Fig. 4C) express *RARE-lacZ* in portions of the forebrain and midbrain similar to the treated wild-type control (Fig. 4D) as well as the untreated wild-type control described above (Fig. 1F). These sites of *RARE-lacZ* expression in the forebrain and midbrain do not coincide with expression of *Raldh1*, *Raldh2*, or *Raldh3* (Mic et al., 2000), and their presence in untreated wild-type embryos indicates that they are not the result of RA treatment. Also, their existence in *Raldh2*$^{-/-}$ embryos indicates that in wild-type embryos they are not due to RA produced by RALDH2, either locally or in nearby tissues. This indicates the existence of a novel neural RA-generating enzyme distinct from the three known RALDHs.

Novel sites of retinoic acid generation in neural tube of *Raldh2*$^{-/-}$ embryos

Histological sections throughout the neural tube demonstrated morphologically normal development of the brain and spinal cord in E10.5 conditionally rescued *Raldh2*$^{-/-}$ embryos carrying *RARE-lacZ* (Fig. 5). Transverse sections of the posterior spinal cord indicated that *RARE-lacZ* expression in the mutant was limited to the intermediate region (where ventral interneurons develop) and the floorplate, whereas wild type had this plus additional *RARE-lacZ* expression throughout the dorsal neural tube and somites (Fig. 5A,B). As *Raldh2* is expressed in somites and in the dorsal neural tube itself at E10.0 (Zhao et al., 1996; Niederreither et al., 1997; Haselbeck et al., 1999), and as RA is now missing in the null mutant, this indicates that RALDH2 normally produces the RA that is detected in the dorsal spinal cord. RA in the intermediate region and floorplate, however, is evidently derived from novel enzymatic activity not yet described, and it is possible that the RA-generating enzyme in the intermediate region is not the same as that observed in the floorplate. In the intermediate region of the spinal cord, the novel RA-generating enzyme is localized at an axial level where certain ventral interneurons develop (Pierani et al., 1999). At E10.5 it is clear that no RA generation is taking place in the region destined to develop later into motor neurons located

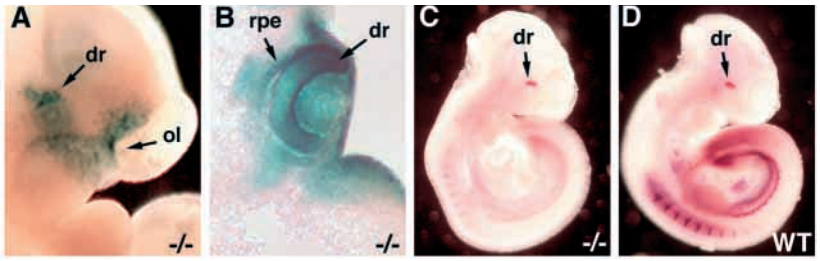

Fig. 6. Induction of *Raldh1* in dorsal retina of *Raldh2*$^{-/-}$ embryos by conditional RA rescue. (A,B) *RARE-lacZ* expression in the eye of E10.5 *Raldh2*$^{-/-}$ embryos treated with RA to E8.25 is shown (A) as a whole-mount stained for only 2 hours and (B) as a frontal section to depict the high level of expression in dorsal retina. (C,D) Combined detection of *Raldh1*+*Raldh2* mRNAs by whole-mount in situ hybridization at E9.75, using both probes simultaneously, for *Raldh2*$^{-/-}$ (C) and wild-type (D) embryos treated with RA to E8.25; this shows that the rescued mutant, which has very low *Raldh2* mRNA detection relative to wild type, now expresses *Raldh1* in the dorsal retina. dr, dorsal retina; ol, olfactory pit; rpe, retinal pigment epithelium.

between the floorplate and the intermediate region (Fig. 5A,B); however, at E12.5 *Raldh2* expression has been demonstrated in motor neurons (Zhao et al., 1996; Haselbeck et al., 1999), suggesting that RALDH2 provides RA for motor neuron development at that point in development.

In both rescued mutant and wild-type embryos at E10.5, *RARE-lacZ* expression is found in large dorsolateral domains within the forebrain and at the forebrain/midbrain junction (Fig. 5C,D), and in small dorsolateral patches within the midbrain (Fig. 5E,F) as well as in the posterior hindbrain (Fig. 5G,H). Note that *RARE-lacZ* expression in the head is limited to neuroepithelia, with no expression in surrounding head mesoderm. In the brain it appears that the novel RA-generating activity (or activities) accounts for all *RARE-lacZ* expression, as the patterns are the same in rescued mutant and wild-type; furthermore, none of the three *Raldh* genes are expressed in those locations at E10.5 (Mic et al., 2000).

Induction of *Raldh1* during rescue of *Raldh2* mutants

Conditionally RA-rescued *Raldh2*$^{-/-}$ embryos have undergone morphologically normal eye development, with a level of *RARE-lacZ* expression in the eye and olfactory regions comparable to wild type, including a high level of expression in the dorsal retina (Fig. 6A,B). Conditionally rescued mutants also expressed *Raldh1* in the dorsal retina (Fig. 6C,D). Thus, whereas mutants normally do not express *Raldh1* in the dorsal retina, as shown above (Fig. 2B), limited RA treatment was able to induce *Raldh1* (directly or indirectly), which is likely responsible for *RARE-lacZ* expression observed in the dorsal retina. The expression pattern of *Raldh1* indicates that its role in RA synthesis may be limited to the dorsal retina.

Raldh3 expression in eye, olfactory pit and ureteric bud of *Raldh2* mutants

Whereas *Raldh1* can account for RA synthesis in the dorsal retina of conditionally rescued mutants, *RARE-lacZ* expression was also observed in the retinal pigment epithelium, ventral retina and olfactory pit of *Raldh2*$^{-/-}$ embryos, which appeared morphologically normal (Fig. 6B, Fig. 7A). Conditionally rescued *Raldh2*$^{-/-}$ mutants and wild-type embryos at E9.5-E10.5 were found to express *Raldh3* in all those locations (Fig. 7B-E). Although RA treatment was unnecessary to observe *Raldh3* expression in the surface ectoderm over the eye field (Fig. 2F), RA treatment was necessary to observe *Raldh3* expression in retinal tissues of the eye.

Note that *Raldh3* is not expressed in the forebrain or midbrain at E9.5-E10.5, thus does not account for *RARE-lacZ* expression in those tissues at these stages (Fig. 7A,D,E).

RARE-lacZ expression was found in the posterior urinary tract of E10.5 rescued *Raldh2*$^{-/-}$ embryos (Fig. 7F,G) as was expression of *Raldh3* (Fig. 7H). Examination of the developing

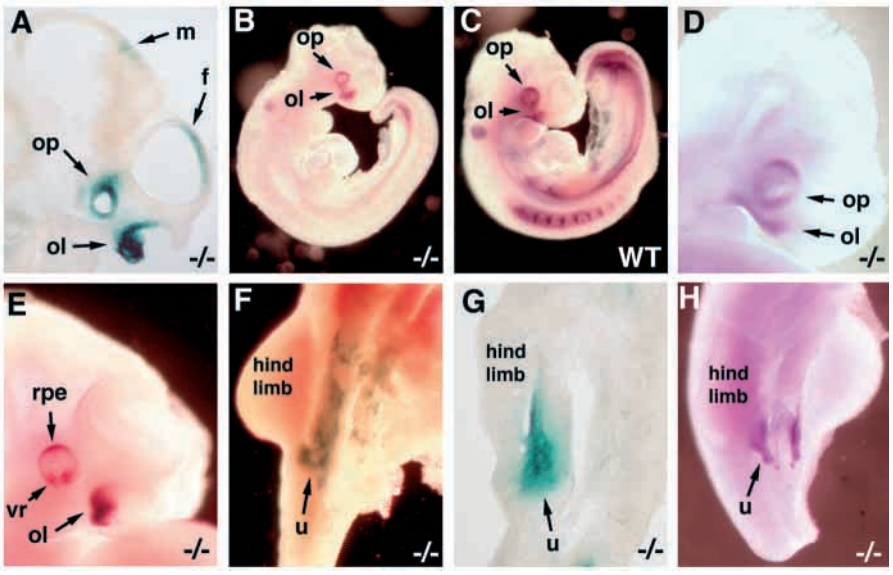

Fig. 7. *RARE-lacZ* and *Raldh3* expression in the eye, olfactory pit and ureteric bud of conditionally rescued *Raldh2*$^{-/-}$ embryos. (A) *RARE-lacZ* staining in the head of an E10.5 *Raldh2*$^{-/-}$ embryo treated with RA to E8.25 is shown in a sagittal section. (B,C) Combined detection of *Raldh3*+*Raldh2* mRNAs by whole-mount in situ hybridization at E9.5 using both probes simultaneously for E9.5 *Raldh2*$^{-/-}$ (B) and wild-type embryos treated with RA to E8.25 (C); the rescued mutant expresses *Raldh3* in the optic vesicle and olfactory pit. (D,E) Higher magnification views of *Raldh3* mRNA detection in the head of conditionally rescued *Raldh2*$^{-/-}$ embryos at E9.5 (D) and E10.5 (E). (F-H) Analysis of posterior urinary tract of E10.5 *Raldh2*$^{-/-}$ embryos treated with RA to E8.25 shows expression of *RARE-lacZ* in whole-mount and frontal section, respectively (F,G), plus expression of *Raldh3* mRNA in whole-mount (H). f, forebrain; m, midbrain; ol, olfactory pit; op, optic vesicle; rpe, retinal pigment epithelium; vr, ventral retina; u, urinary tract.

metanephros in wild-type embryos from E10.5 to E13.5 demonstrated colocalization of *RARE-lacZ* and *Raldh3* in the ureteric buds (data not shown).

Initiation of novel RA-generating activities in the neural tube and heart

In order to examine whether the novel RA-generating activities in the neural tube and heart were present at earlier stages, we performed conditional rescues of *Raldh2*$^{-/-}$ embryos in which RA was withdrawn at time points earlier than E8.25. When the last dose of RA was given at E7.25 and embryos allowed to develop until E8.5 (30 hours after the last dose) or E9.25 (48 hours after the last dose), we found that *RARE-lacZ* was expressed in the posterior hindbrain and spinal cord, with expression in the spinal cord increasing as the embryos developed from E8.5 to E9.25 (Fig. 8A,B). This time-dependent increase in spinal cord *RARE-lacZ* expression is consistent with de novo RA synthesis by a novel RA-generating activity.

When viewed dorsally it was observed that rescued *Raldh2*$^{-/-}$ embryos in contrast to wild-type had no *RARE-lacZ* expression in the somites, but rather expression was limited to neural tissue (Fig. 8C,D). Sections show that *RARE-lacZ* expression in the mutant is limited to the neuroepithelium of the spinal cord and posterior hindbrain extending anteriorly at least to the rhombomere 4/5 (r4/r5) boundary (Fig. 8E,F). In wild-type embryos the same neuroepithelial expression of *RARE-lacZ* was observed in the hindbrain, but the occipital somites adjacent to r7-r8 also expressed *RARE-lacZ* (Fig. 8G). Sections of mutant spinal cord at E9.25 (Fig. 8H) and E8.5 (data not shown) show that expression is already limited at early stages to the intermediate region. Thus, it is clear that the novel RA-generating activity is not in somitic mesoderm (where *Raldh2* is expressed) or other head mesoderm, but is present at least as early as E8.5 in hindbrain and spinal cord neuroepithelia of *Raldh2*$^{-/-}$ and wild-type embryos.

When RA was administered only until E7.25, *Raldh2*$^{-/-}$ embryos developed further than non-rescued mutants as they underwent embryonic turning; however, the hindbrain neuroepithelium was serrated and small otic vesicles persisted (compare mutant in Fig. 8E,F with wild-type in Fig. 8G). Also, embryonic turning was incomplete and although the heart began to express *RARE-lacZ* at E8.5, it failed to continue *RARE-lacZ* expression at E9.25 and displayed a medial distended cavity similar in appearance to non-rescued mutants (Fig. 8A,B). On the other hand, when one additional dose of RA was given at E7.75 we found that embryonic turning and heart development was much improved in mutants examined at E9.25 (36 hours after the last dose) (Fig. 8I). Under these conditions, however, development did not continue beyond E9.25 as embryos examined at E10.25 (60 hours after the last dose) were not further developed (Fig. 8J). It is useful, however, to note that neural tube and heart *RARE-lacZ* expression was still observed 60 hours after the last RA dose, providing yet more evidence that this is due to endogenous RA synthesis rather than the administered RA.

RARE-lacZ expression in the heart of E9.25 *Raldh2*$^{-/-}$ embryos following RA rescue to E7.75 was localized in the conotruncus and sinus venosa, whereas in wild type it was found in these locations as well as the atrium (Fig. 8K,L). Thus, it is clear that a novel RA-generating activity exists in the conotruncus and sinus venosa that does not correspond to the three RALDHs. As *Raldh2* expression is localized in the sinus venosa and atrium, but not in the conotruncus (Moss et al., 1998), the novel activity is unique for the conotruncus but overlaps with RALDH2 in the sinus venosa.

DISCUSSION

RALDH2 produces retinoic acid for embryonic development

Identification of enzymes with physiological roles in RA synthesis is a prerequisite to understanding how regulated ligand synthesis contributes to retinoid signaling. The genetic findings described here, together with those published earlier (Niederreither et al., 1999), indicate that *Raldh2* does indeed play a critical role in embryonic development, by providing the RA synthesis needed for several aspects of development. Whereas *Raldh2*$^{-/-}$ embryos fail to develop beyond E8.75 and have almost no detectable RA, conditional RA rescue stimulates additional RA synthesis throughout the embryo and development continues. At E10.5, 54 hours after maternal RA treatment, rescued embryos appear morphologically and histologically normal with the exception of truncated forelimb buds, and molecular analysis indicates that de novo RA synthesis is extensive (although less due to loss of RALDH2) plus marker genes are expressed properly in the eye, olfactory pit, urinary tract, hindbrain and tailbud. However, there may exist subtle defects in some tissues in addition to the overt forelimb bud defect observed.

Retinoic acid synthesis in *Raldh2* mutants

Our use of conditional RA rescue enabled identification of several sites of RALDH2-independent RA synthesis assayed by *RARE-lacZ* expression. Previous RA rescue studies of *Raldh2*$^{-/-}$ embryos relied upon continuous treatment until the point of analysis (Niederreither et al., 1999; Niederreither et al., 2000; Niederreither et al., 2001), thus making it impossible to determine whether the administered RA or endogenous sources independent of RALDH2 contributed to survival. We demonstrated that maternally administered RA induced *RARE-lacZ* uniformly throughout the embryo at 6 hours after the last dose, but that by 24 hours this expression had been eliminated. Thus, *RARE-lacZ* expression observed from 30-60 hours following the last dose of RA reflects endogenous synthesis of RA. In conditionally RA-rescued *Raldh2*$^{-/-}$ embryos, endogenous RA synthesis was quite extensive and this may have contributed to the ability of the administered RA to perform the rescue, especially as the endogenous RA was produced locally where it is normally needed.

Our studies indicate that RA treatment to E8.25 is necessary and sufficient to obtain growth of *Raldh2*$^{-/-}$ embryos to E10.5, but that treatment to only E7.25 or E7.75 is insufficient, although this does allow growth to E9.25. Thus, RA treatments that terminate at E7.25, E7.75 or E8.25 progressively allow *Raldh2*$^{-/-}$ embryos to grow further into development. Our findings demonstrate that treatment to E8.25 provides enough RA to stimulate development of endogenous sources of RA generation in the eye, olfactory pit, brain, spinal cord, heart and urinary tract of E10.5 rescued *Raldh2*$^{-/-}$ embryos.

We demonstrated that some sites of endogenous RA

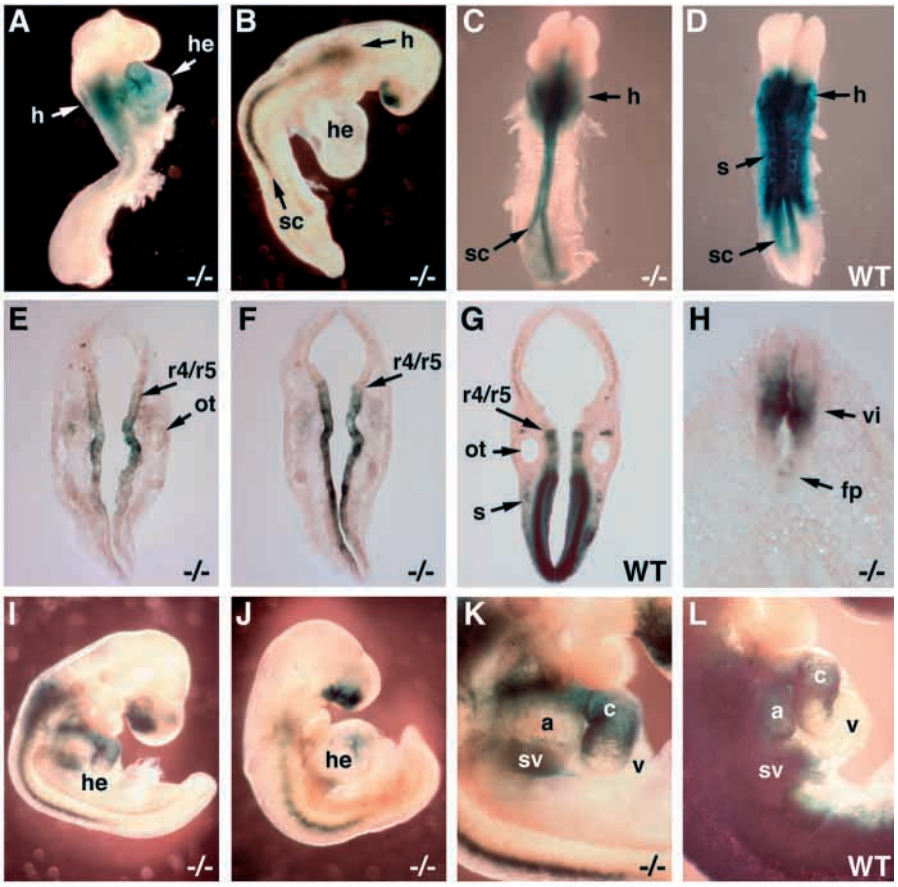

Fig. 8. Early expression of novel RA-generating activities in the neural tube and heart. Staining for *RARE-lacZ* expression is shown for *Raldh2*$^{-/-}$ and wild-type embryos treated with only two doses of RA at E6.75 and E7.25 (A-H) or three doses of RA at E6.75, E7.25 and E7.75 (I-L). For the E7.25 rescue: (A) E8.5 embryo shown 30 hours after the last dose; (B) E9.25 embryo shown 48 hours after the last dose; dorsal view of rescued *Raldh2*$^{-/-}$ embryo at E8.5 (C) and wild-type embryo (D). (E-G) Frontal sections through the hindbrain at E9.25; (H) transverse section through the spinal cord of an E9.25 rescued embryo. For the E7.75 rescue: (I) E9.25 embryo shown 36 hours after the last dose and (J) E10.25 embryo shown 60 hours after the last dose; also shown are higher magnification views at E9.25 of the heart for rescued *Raldh2*$^{-/-}$ embryo (K) and wild-type embryo (L). a, atrium, c, conotruncus; fp, floorplate; h, hindbrain; he, heart; ot, otic vesicle; r4/5, rhombomere 4/5 boundary; s, somite; sc, spinal cord; sv, sinus venosa; v, ventricle; vi, ventral interneurons.

synthesis in *Raldh2*$^{-/-}$ embryos could be related to other known RA-generating enzymes, RALDH1 or RALDH3. Endogenous RA synthesis in the eye and olfactory fields of *Raldh2*$^{-/-}$ embryos is likely due to *Raldh1* expressed in the dorsal eye as well as *Raldh3* expressed in the surface ectoderm over the eye field, retinal pigment epithelium, ventral retina and olfactory epithelium, as shown here and previously described (McCaffery et al., 1991; Haselbeck et al., 1999; Mic et al., 2000; Li et al., 2000; Grün et al., 2000; Suzuki et al., 2000). We also found that endogenous RA synthesis was occurring in the urinary tract of mutants and that *Raldh3* expression was localized in this same location. Thus, *Raldh3* may function in RA synthesis for the developing urinary tract in addition to *Raldh2*, which is expressed in the nephric stromal cells (Batourina et al., 2001). However, the additional sites of endogenous RA synthesis in the neural tube and heart of *Raldh2*$^{-/-}$ embryos could not be explained by the three known RALDHs. It is unclear whether these novel activities are catalyzed by one additional enzyme or more than one, or whether the enzyme(s) is a member of the ALDH family or a distinct enzyme family.

The existence of an RA-generating enzyme expressed in the brain distinct from RALDH1, RALDH2 and RALDH3 can also be proposed simply by examination of wild-type *RARE-lacZ* expression. As initially described (Rossant et al., 1991) and as shown here, *RARE-lacZ* is expressed in discrete regions of the forebrain, midbrain and hindbrain from E8.5-E10.5. A direct comparison with the expression patterns of the three *Raldh* genes shows that none are expressed in the brain from E8.5-E10.5 as shown here and as described previously (Mic et al., 2000), although later at E12.5 there is expression of *Raldh3* in the forebrain (Li et al., 2000). As for the trunk, novel RA-generating activity has now been observed through our analysis of *Raldh2*$^{-/-}$ embryos as this removed the activity of RALDH2, which produces a tremendous amount of RA in the trunk that may confound analysis of discrete RA sources. Now it can be seen very clearly that RALDH2 does not produce all the RA that is observed in the spinal cord, posterior hindbrain, heart and urinary tract.

Our results suggest that one function of *Raldh2* is to provide RA in order to directly or indirectly induce some of these additional RA-generating activities. The novel RA-generating activities in the neural tube and heart, as well as *Raldh1* and *Raldh3* in the optic vesicle, are not expressed in *Raldh2*$^{-/-}$ embryos prior to RA rescue. This is not due to an absence of these tissues, because development of the mutant does proceed far enough to produce all these tissues. As maternally administered RA can stimulate these additional RA-generating activities, it can be hypothesized that RA produced by RALDH2 is normally providing this function.

Sources of retinoic acid for spinal cord development

RA present in the spinal cord from E8.5-E10.5 is derived from at least two sources. Dorsal RA is derived from diffusion of RA from somites expressing *Raldh2* and/or from expression of *Raldh2* directly in the dorsal spinal cord (Zhao et al., 1996) as this RA is absent in *Raldh2*$^{-/-}$ embryos. As *Raldh2* expression in the wild-type dorsal spinal cord is transient (Zhao et al.,

1996), it is unclear if *RARE-lacZ* expression observed there is due to local RA synthesis or to somitic RA synthesis, which would require a surprisingly long diffusion range of RA from mesodermal tissues expressing *Raldh2*. As for RA observed more ventrally, E10.5 mutants still contain RA in the intermediate region of the spinal cord as well as the floorplate, indicating a second and possibly third source of RA directly in neuroectodermal tissues. Wild-type E10.5 embryos express all these activities, thus have RA dorsally and intermediately (which appears merged), as well as in the floorplate. Other studies have shown that isolated mouse spinal cord tissue from E9.0-E10.9 contains RA as well as RA-generating activity (McCaffery and Dräger, 1994). The RA-generating activity reported there cannot be accounted for simply by RALDH2, as this enzyme is missing in the null mutant, but instead must also include the novel activity we have detected in the neural tube. Our conditional RA rescue studies provide evidence that *Raldh2* expressed in the somites or paraxial mesoderm from E7.5-E8.5 may produce RA that induces the novel spinal cord RA-generating enzyme, as we did not observe this novel activity in *Raldh2*$^{-/-}$ embryos unless they were treated with RA during that time period.

Raldh3 expression has not been detected in the spinal cord of mouse embryos (Mic et al., 2000; Grün et al., 2000), but it has been detected in the posterior spinal cord of chick embryos, limited to the region adjacent to the hindlimb field and localized in two small lateral regions where interneurons develop (Grün et al., 2000). *Raldh3* expression in chick spinal cord is much more limited than expression of the novel intermediate region enzyme described here for mouse, which is detected all along the anteroposterior axis of the spinal cord and encompasses both medial and lateral portions of the intermediate region. *Raldh3* may thus have a chick-specific function as an RA-generating enzyme for a small region of the spinal cord adjacent to the hindlimb field.

Just prior to motor neuron growth, the ventral spinal cord can be divided into several domains along the dorsoventral axis (V0, V1, V2, MN, V3 and FP), each expressing a unique set of transcriptional regulators for development of several types of ventral interneurons (V0-V3), motor neurons (MN) or floorplate (FP) (Pierani et al., 1999). The pattern of *RARE-lacZ* expression we observe in rescued *Raldh2*$^{-/-}$ embryos appears to coincide with a region encompassing at least V0 and V1 plus a small domain representing FP, but no expression in MN. This is an important finding as it has been reported that RA signaling functions in vivo to generate V0 and V1 interneurons prior to motor neuron growth (Pierani et al., 1999). It was proposed in those studies that the RA needed for this may be derived from nearby somites where *Raldh2* is expressed, although a neural source was not ruled out. Our findings provide evidence that the RA needed for this signaling may be produced locally in V0 and V1 neural cells by the novel RA-generating activity discovered here, although an RA signal from the somites may also contribute to generation of these cells. Thus, the new activity described here fills a gap in our knowledge of how RA is generated spatiotemporally in the spinal cord for interneuron development.

Sources of retinoic acid for hindbrain development

Previous studies on hindbrain development in *Raldh2*$^{-/-}$ embryos demonstrated a reduction of the posterior hindbrain (r4-r8) and posterior expansion of anterior hindbrain (r1-r3) (Niederreither et al., 2000). In contrast, vitamin A-deficient quail embryos totally lack posterior hindbrain (Maden et al., 1996). Thus, it has been hypothesized that some residual RA signaling exists in *Raldh2*$^{-/-}$ embryos (Gavalas and Krumlauf, 2000). Our findings provide evidence of a novel RA-generating activity distinct from RALDH2 located in the posterior hindbrain neuroepithelium up to at least the r4/r5 boundary that could provide a local source of RA. This neural RA-generating activity is undetectable in *Raldh2*$^{-/-}$ embryos prior to RA rescue, but perhaps a low basal activity provides some residual RA signaling.

As *Raldh2* is not expressed in the posterior hindbrain neuroepithelium but is limited to adjacent somites, it has previously been proposed that RA produced in somites is somehow transported to the neuroepithelium where *Hox* gene expression is induced (Swindell et al., 1999; Niederreither et al., 2000). This would require a large amount of diffusion or transport in order to provide RA along the entire anteroposterior axis of the hindbrain. A further complication of this hypothesis is that, in addition to RA, a high molecular mass component from the somites is also required to initiate *Hoxb4* expression in the posterior portion of the hindbrain (r7-r8) lying adjacent to the occipital somites (Gould et al., 1998). This additional component could be a carrier protein needed to transport RA from the somite or a distinct factor needed in tandem with somite-derived RA to regulate gene expression in the neuroepithelium; the possibility that the neuroepithelium itself may produce RA as a downstream response to the somite factor has also been raised, but evidence for such an enzyme has been lacking (Gould et al., 1998). Based upon our discovery of RA-generating activity induced in the hindbrain neuroepithelium following RA treatment, we hypothesize that one function of *Raldh2* in anterior somites may be to produce RA that either alone, or with another secreted factor, induces expression of the novel RA-generating activity in r7-r8, with expression then spreading to at least the r4/r5 boundary to provide RA locally where it is needed.

Retinoic acid generation during eye development

Most of the RA detected in the eye field of wild-type embryos at E8.75 is generated by *Raldh2* expressed in the optic vesicle. A small amount of RA is still detectable at E8.75 in the eye field of *Raldh2*$^{-/-}$ embryos, as shown here and as described before (Niederreither et al., 1999). Our findings do not support a previous hypothesis suggesting that *Raldh1* may be responsible for this RA synthesis and that *Raldh2* may be needed to induce *Raldh3* expression (Wagner et al., 2000). Instead, our findings demonstrate that *Raldh3* is initially expressed independently of *Raldh2*, and that *Raldh1* requires *Raldh2* expression or maternal RA administration to be expressed. We report that *Raldh3* expression is observed in both *Raldh2*$^{-/-}$ and wild-type embryos at E8.75 as a region of staining in the surface epithelium over the eye field. On the other hand, *Raldh1* expression is not observed in the eye field of the mutant unless RA is administered, in which case it is observed in the dorsal retina as normal. Also, we find that even though initial *Raldh3* expression in the eye surface ectoderm of *Raldh2*$^{-/-}$ embryos does not require *Raldh2*, subsequent expression in the ventral retina and retinal pigment epithelium does require RA treatment.

A lack of *Raldh1* or *Raldh3* expression in retinal tissues of the mutant prior to rescue is not due to a lack of dorsoventral polarity in the optic vesicle, as we demonstrated that the mutant expresses *Tbx5* dorsally and *Pax2* ventrally in the optic vesicle. Our results thus provide evidence that *Raldh2* is not essential for establishing dorsoventral polarity in the optic vesicle, but functions downstream of this event. A transient RA signal is found initially throughout the optic vesicle, produced by a short burst of *Raldh2* expression in the optic vesicle at approximately E8.25-E8.5. We hypothesize that this relatively uniform RA signal may then function to create two independent domains of RA signaling in the already established dorsal and ventral regions of the optic vesicle by inducing expression of *Raldh1* and *Raldh3* in spatially restricted regions of the optic vesicle, which later give rise to the dorsal and ventral retina, respectively. Independently of these events it is seen that *Raldh3* initiates RA synthesis in surface ectoderm over the eye, which later differentiates into lens. Thus, retina and lens have independent mechanisms for generation of RA locally.

Retinoic acid synthesis in the developing heart

Previous studies on RA synthesis in the developing heart indicated that RALDH2 is expressed in the sinus venosa, atria and ventricles, but not in the conotruncus despite the presence of RA in all these tissues during heart development (Moss et al., 1998). Our studies have shown that during the early stages of rescue, the *Raldh2*$^{-/-}$ mutant heart expresses novel RA-generating activity in the sinus venosa and conotruncus distinct from RALDH2. This novel RA-generating activity may be important for development and eventual fusion of the different embryonic fields producing the inflow tract, the heart tube and the outflow tract, and may have contributed significantly to the ability of RA-rescued embryos to effectively grow to E10.5.

Raldh2 has been shown to be essential for heart development as null mutants fail to produce more than a simple heart tube without looping or chamber morphogenesis, and this likely contributes to embryonic lethality, as shown here and described previously (Niederreither et al., 1999). RA-rescued *Raldh2*$^{-/-}$ embryos treated continuously with RA survive past midgestation and heart development continues, but defects in cardiac neural crest populating the conotruncus ultimately cause lethality by E13.5 (Niederreither et al., 2001). In this case, continuous RA treatment may be contributing to the defect as neural crest cells are particularly sensitive targets of RA teratogenesis (Lammer et al., 1985). Thus, systemic RA treatment may allow certain developmental processes to occur in mutants such as ventricular cardiomyocyte differentiation and growth of the atria and sinus venosa, but RA provided systemically may damage developmental processes dependent upon cardiac neural crest migration such as formation of the outflow tract septation, which is still defective in RA-rescued *Raldh2*$^{-/-}$ embryos (Niederreither et al., 2001). This points out the importance of local production of RA for developmental processes as opposed to receiving it systemically.

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REFERENCES

Abu-Abed, S., Dollé, P., Metzger, D., Beckett, B., Chambon, P. and Petkovich, M. (2001). The retinoic acid-metabolizing enzyme, CYP26A1, is essential for normal hindbrain patterning, vertebral identity, and development of posterior structures. *Genes Dev.* **15**, 226-240.

Ang, H. L., Deltour, L., Hayamizu, T. F., Zgombic-Knight, M. and Duester, G. (1996). Retinoic acid synthesis in mouse embryos during gastrulation and craniofacial development linked to class IV alcohol dehydrogenase gene expression. *J. Biol. Chem.* **271**, 9526-9534.

Armstrong, R. B., Ashenfelter, K. O., Eckhoff, C., Levin, A. A. and Shapiro, S. S. (1994). General and reproductive toxicology of retinoids. In *The Retinoids: Biology, Chemistry and Medicine*, 2nd edition (ed. M. B. Sporn, A. B. Roberts and D. S. Goodman), pp. 545-572. New York: Raven Press, Ltd.

Batourina, E., Gim, S., Bello, N., Shy, M., Clagett-Dame, M., Srinivas, S., Costantini, F. and Mendelsohn, C. (2001). Vitamin A controls epithelial/mesenchymal interactions through *Ret* expression. *Nature Genet.* **27**, 74-78.

Chapman, D. L., Garvey, N., Hancock, S., Alexiou, M., Agulnik, S. I., Gibson-Brown, J. J., Cebra-Thomas, J., Bollag, R. J., Silver, L. M. and Papaioannou, V. E. (1996). Expression of the T-box family genes, *Tbx1-Tbx5*, during early mouse development. *Dev. Dyn.* **206**, 379-390.

Collins, M. D., Eckhoff, C., Chahoud, I., Bochert, G. and Nau, H. (1992). 4-methylpyrazole partially ameliorated the teratogenicity of retinol and reduced the metabolic formation of all-*trans*-retinoic acid in the mouse. *Arch. Toxicol.* **66**, 652-659.

Deltour, L., Foglio, M. H. and Duester, G. (1999). Impaired retinol utilization in *Adh4* alcohol dehydrogenase mutant mice. *Dev. Genet.* **25**, 1-10.

Dersch, H. and Zile, M. H. (1993). Induction of normal cardiovascular development in the vitamin A-deprived quail embryo by natural retinoids. *Dev. Biol.* **160**, 424-433.

Dressler, G. R., Deutsch, U., Chowdhury, K., Nornes, H. O. and Gruss, P. (1990). *Pax2*, a new murine paired-box-containing gene and its expression in the developing excretory system. *Development* **109**, 787-795.

Duester, G. (2000). Families of retinoid dehydrogenases regulating vitamin A function: production of visual pigment and retinoic acid. *Eur. J. Biochem.* **267**, 4315-4324.

Fujii, H., Sato, T., Kaneko, S., Gotoh, O., Fujii-Kuriyama, Y., Osawa, K., Kato, S. and Hamada, H. (1997). Metabolic inactivation of retinoic acid by a novel P450 differentially expressed in developing mouse embryos. *EMBO J.* **16**, 4163-4173.

Gavalas, A. and Krumlauf, R. (2000). Retinoid signalling and hindbrain patterning. *Curr. Opin. Genet. Dev.* **10**, 380-386.

Gould, A., Itasaki, N. and Krumlauf, R. (1998). Initiation of rhombomeric *Hoxb4* expression requires induction by somites and a retinoid pathway. *Neuron* **21**, 39-51.

Grün, F., Hirose, Y., Kawauchi, S., Ogura, T. and Umesono, K. (2000). Aldehyde dehydrogenase 6, a cytosolic retinaldehyde dehydrogenase prominently expressed in sensory neuroepithelia during development. *J. Biol. Chem.* **275**, 41210-41218.

Haselbeck, R. J., Hoffmann, I. and Duester, G. (1999). Distinct functions for *Aldh1* and *Raldh2* in the control of ligand production for embryonic retinoid signaling pathways. *Dev. Genet.* **25**, 353-364.

Hogan, B., Beddington, R., Costantini, F. and Lacy, E. (1994). *Manipulating the Mouse Embryo*, Second Edition. Cold Spring Harbor: Cold Spring Harbor Laboratory.

Hsu, L. C., Chang, W. C., Hoffmann, I. and Duester, G. (1999). Molecular analysis of two closely related mouse aldehyde dehydrogenase genes: identification of a role for *Aldh1*, but not *Aldh-pb*, in the biosynthesis of retinoic acid. *Biochem. J.* **339**, 387-395.

Joyner, A. L. (1993). *Gene Targeting: A Practical Approach*. Oxford: IRL Press.

Kastner, P., Chambon, P. and Leid, M. (1994). Role of nuclear retinoic acid receptors in the regulation of gene expression. In *Vitamin A in Health and Disease* (ed. R. Blomhoff), pp. 189-238. New York: Marcel Dekker, Inc.

Kastner, P., Mark, M. and Chambon, P. (1995). Nonsteroid nuclear receptors: What are genetic studies telling us about their role in real life? *Cell* **83**, 859-869.

Lamb, A. L. and Newcomer, M. E. (1999). The structure of retinal dehydrogenase type II at 2.7 Å resolution: Implications for retinal specificity. *Biochemistry* **38**, 6003-6011.

Lammer, G. J., Chen, D. T., Hoar, R. M., Agnish, N. D., Benke, P. J., Braun, J. T., Curry, C. J., Fernhoff, P. M., Grix, A. W., Lott, I. T., Richard, J. M. and Sun, S. C. (1985). Retinoic acid embryopathy. *N. Engl. J. Med.* **313**, 837-841.

Li, H., Wagner, E., McCaffery, P., Smith, D., Andreadis, A. and Dräger, U. C. (2000). A retinoic acid synthesizing enzyme in ventral retina and telencephalon of the embryonic mouse. *Mech. Dev.* **95**, 283-289.

MacLean, G., Abu-Abed, S., Dollé, P., Tahayato, A., Chambon, P. and Petkovich, M. (2001). Cloning of a novel retinoic-acid metabolizing cytochrome P450, *Cyp26B1*, and comparative expression analysis with *Cyp26A1* during early murine development. *Mech. Dev.* **107**, 195-201.

Maden, M., Gale, E., Kostetskii, I. and Zile, M. H. (1996). Vitamin A-deficient quail embryos have half a hindbrain and other neural defects. *Curr. Biol.* **6**, 417-426.

Maden, M., Sonneveld, E., Van der Saag, P. T. and Gale, E. (1998). The distribution of endogenous retinoic acid in the chick embryo: implications for developmental mechanisms. *Development* **125**, 4133-4144.

Malpel, S., Mendelsohn, C. and Cardoso, W. V. (2000). Regulation of retinoic acid signaling during lung morphogenesis. *Development* **127**, 3057-3067.

Mangelsdorf, D. J., Umesono, K. and Evans, R. M. (1994). The retinoid receptors. In *The Retinoids: Biology, Chemistry and Medicine*, 2nd Edition (ed. M. B. Sporn, A. B. Roberts and D. S. Goodman), pp. 319-349. New York: Raven Press, Ltd.

McCaffery, P., Tempst, P., Lara, G. and Dräger, U. C. (1991). Aldehyde dehydrogenase is a positional marker in the retina. *Development* **112**, 693-702.

McCaffery, P. and Dräger, U. C. (1994). Hot spots of retinoic acid synthesis in the developing spinal cord. *Proc. Natl. Acad. Sci. USA* **91**, 7194-7197.

Mic, F. A., Molotkov, A., Fan, X., Cuenca, A. E. and Duester, G. (2000). RALDH3, a retinaldehyde dehydrogenase that generates retinoic acid, is expressed in the ventral retina, otic vesicle and olfactory pit during mouse development. *Mech. Dev.* **97**, 227-230.

Molotkov, A., Fan, X., Deltour, L., Foglio, M. H., Martras, S., Farrés, J., Parés, X. and Duester, G. (2002). Stimulation of retinoic acid production and growth by ubiquitously-expressed alcohol dehydrogenase *Adh3*. *Proc. Natl. Acad. Sci. USA* (in press).

Moss, J. B., Xavier-Neto, J., Shapiro, M. D., Nayeem, S. M., McCaffery, P., Dräger, U. C. and Rosenthal, N. (1998). Dynamic patterns of retinoic acid synthesis and response in the developing mammalian heart. *Dev. Biol.* **199**, 55-71.

Niederreither, K., McCaffery, P., Dräger, U. C., Chambon, P. and Dollé, P. (1997). Restricted expression and retinoic acid-induced downregulation of the retinaldehyde dehydrogenase type 2 (RALDH-2) gene during mouse development. *Mech. Dev.* **62**, 67-78.

Niederreither, K., Subbarayan, V., Dollé, P. and Chambon, P. (1999). Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. *Nature Genet.* **21**, 444-448.

Niederreither, K., Vermot, J., Schuhbaur, B., Chambon, P. and Dollé, P. (2000). Retinoic acid synthesis and hindbrain patterning in the mouse embryo. *Development* **127**, 75-85.

Niederreither, K., Vermot, J., Messaddeq, N., Schuhbaur, B., Chambon, P. and Dollé, P. (2001). Embryonic retinoic acid synthesis is essential for heart morphogenesis in the mouse. *Development* **128**, 1019-1031.

Pierani, A., Brenner-Morton, S., Chiang, C. and Jessell, T. M. (1999). A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. *Cell* **97**, 903-915.

Power, S. C., Lancman, J. and Smith, S. M. (1999). Retinoic acid is essential for shh/hoxd signaling during rat limb outgrowth but not for limb initiation. *Dev. Dyn.* **216**, 469-480.

Rossant, J., Zirngibl, R., Cado, D., Shago, M. and Giguère, V. (1991). Expression of a retinoic acid response element-*hsplacZ* transgene defines specific domains of transcriptional activity during mouse embryogenesis. *Genes Dev.* **5**, 1333-1344.

Sakai, Y., Meno, C., Fujii, H., Nishino, J., Shiratori, H., Saijoh, Y., Rossant, J. and Hamada, H. (2001). The retinoic acid-inactivating enzyme CYP26 is essential for establishing an uneven distribution of retinoic acid along the anterio-posterior axis within the mouse embryo. *Genes Dev.* **15**, 213-225.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

Sockanathan, S. and Jessell, T. M. (1998). Motor neuron-derived retinoid signaling specifies the subtype identity of spinal motor neurons. *Cell* **94**, 503-514.

Solomin, L., Johansson, C. B., Zetterström, R. H., Bissonnette, R. P., Heyman, R. A., Olson, L., Lendahl, U., Frisén, J. and Perlmann, T. (1998). Retinoid-X receptor signalling in the developing spinal cord. *Nature* **395**, 398-402.

Stratford, T., Logan, C., Zile, M. and Maden, M. (1999). Abnormal anteroposterior and dorsoventral patterning of the limb bud in the absence of retinoids. *Mech. Dev.* **81**, 115-125.

Suzuki, R., Shintani, T., Sakuta, H., Kato, K., Ohkawara, T., Osumi, N. and Noda, M. (2000). Identification of RALDH-3, a novel retinaldehyde dehydrogenase, expressed in the ventral region of the retina. *Mech. Dev.* **98**, 37-50.

Swindell, E. C., Thaller, C., Sockanathan, S., Petkovich, M., Jessell, T. M. and Eichele, G. (1999). Complementary domains of retinoic acid production and degradation in the early chick embryo. *Dev. Biol.* **216**, 282-296.

Ulven, S. M., Gundersen, T. E., Weedon, M. S., Landaas, V. O., Sakhi, A. K., Fromm, S. H., Geronimo, B. A., Moskaug, J. O. and Blomhoff, R. (2000). Identification of endogenous retinoids, enzymes, binding proteins, and receptors during early postimplantation development in mouse: Important role of retinal dehydrogenase type 2 in synthesis of all-*trans*-retinoic acid. *Dev. Biol.* **220**, 379-391.

Wagner, E., McCaffery, P. and Dräger, U. C. (2000). Retinoic acid in the formation of the dorsoventral retina and its central projections. *Dev. Biol.* **222**, 460-470.

Wald, G. (1951). The chemistry of rod vision. *Science* **113**, 287-291.

White, J. C., Highland, M., Kaiser, M. and Clagett-Dame, M. (2000). Vitamin A deficiency results in the dose-dependent acquisition of anterior character and shortening of the caudal hindbrain of the rat embryo. *Dev. Biol.* **220**, 263-284.

Wilkinson, D. G. (1992). Whole mount *in situ* hybridization of vertebrate embryos. In *In Situ Hybridization: A Practical Approach* (ed. D. G. Wilkinson), pp. 75-83. Oxford: IRL Press.

Zhao, D., McCaffery, P., Ivins, K. J., Neve, R. L., Hogan, P., Chin, W. W. and Dräger, U. C. (1996). Molecular identification of a major retinoic-acid-synthesizing enzyme, a retinaldehyde-specific dehydrogenase. *Eur. J. Biochem.* **240**, 15-22.