Dhrs3 Protein Attenuates Retinoic Acid Signaling and Is Required for Early Embryonic Patterning*§

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Background: Function of Dhrs3 and importance of the upstream metabolism of retinoic acid are not well understood in early embryonic development.
Results: Dhrs3 attenuates retinoic acid synthesis and is required for embryonic patterning.
Conclusion: dhrs3 is involved in maintaining balance of retinoic acid signaling and therefore regulates body axis formation.
Significance: This is the first functional study of Xenopus dhrs3 in embryonic development.

All-trans-retinoic acid (atRA) is an important morphogen involved in many developmental processes, including neural differentiation, body axis formation, and organogenesis. During early embryonic development, atRA is synthesized from all-trans-retinal (atRAL) in an irreversible reaction mainly catalyzed by retinal dehydrogenase 2 (aldh1a2), whereas atRAL is converted from all-trans-retinol via reversible oxidation by retinol dehydrogenases, members of the short-chain dehydrogenase/reductase family. atRA is degraded by cytochrome P450 family 26 (cyp26). We have previously identified a short-chain dehydrogenase/reductase 3 (dhrs3), which showed differential expression patterns in Xenopus embryos. We show here that the expression of dhrs3 was induced by atRA treatment and overexpression of Xenopus nodal related 1 (xnr1) in animal cap assay. Overexpression of dhrs3 enhanced the phenotype of excessive cyp26a1. In embryos overexpressing aldh1a2 or retinal dehydrogenase 10 (rdh10) in the presence of their respective substrates, Dhrs3 counteracted the action of Aldh1a2 or Rdh10, indicating that retinoic acid signaling is attenuated. Knockdown of Dhrs3 by antisense morpholino oligonucleotides resulted in a phenotype of shortened anteroposterior axis, reduced head structure, and perturbed somitogenesis, which were also found in embryos treated with an excess of atRA. Examination of the expression of brachyury, not, goosecoid, and papc indicated that convergent extension movement was defective in Dhrs3 morphants. Taken together, these studies suggest that dhrs3 participates in atRA metabolism by reducing atRAL levels and is required for proper anteroposterior axis formation, neuroectoderm patterning, and somitogenesis.

All-trans-retinoic acid (atRA)§ is a morphogen playing essential roles in various early developmental events, including neural differentiation, mesoderm and endoderm formation, anteroposterior (AP) patterning, and left-right symmetry of axis formation. It has been implicated that RA signaling is also involved in later organogenesis of the heart (1–3), lung (4, 5), kidney (6, 7), and pancreas (8). Deficiency or an excess of atRA induces developmental defects in the limb, branchial arches, central nervous system, and internal organs, indicating that a balanced RA concentration is essential for normal embryonic development (9, 10).

atRA is synthesized by two groups of dehydrogenases mainly through two enzymatic steps. All-trans-retinol (atROL) is first reversibly oxidized into all-trans-retinal (atRAL) by retinol dehydrogenases, members of the short-chain dehydrogenase/reductase family. The reaction is followed by irreversible oxidation of atRAL to atRA, mediated by aldehyde dehydrogenases. At least three retinoic acid-related aldehyde dehydrogenases have been identified in the human, mouse, zebrafish, and Xenopus with different physiological functions (11). atRA directly regulates transcription of target genes via interaction with the heterodimer of retinoic acid receptor and retinoid X receptor (12). The degradation of atRA is mainly carried out by cytochrome P450, family 26 (cyp26) (9). All these atRA metabolic enzymes coordinate with each other in developing embryos to maintain a proper gradient of atRA along the axes (13). Mis-

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regulation of these atRA metabolic enzymes, and thus atRA, results in severe defects of vertebrate embryonic development (14–16). For example, knockdown of rdh10 in Xenopus embryos down-regulated organizer gene expression and caused a ventralized phenotype and anteriorization of the brain. Over-expression of aldh1a2 inhibited the formation of the anterior axis, a phenotype that was also found in atRA-treated embryos. Mice lacking all three CYP26 genes showed duplication of the body axis because of the expansion of the Nodal expression domain (17).

We have previously identified a short-chain dehydrogenase/reductase 3 (dhrs3) through a gene profiling assay on different regions of early Xenopus gastrula, and we found that dhrs3 displayed differential expression in developing embryos (18). It was reported that human DHR53 (previously also called retSDR1) is inducible by atRA and also acts as a retinal reductase that increased concentrations of retinyl ester by converting retinol to retinol in a neuroblastoma cell line (19). Zebrafish dhrs3 was also found to be essential for neural ectoderm patterning in zebrafish embryos (20).

In this study, we addressed the question of how Dhrs3 acted in atRA metabolism during embryonic development, and how Dhrs3 regulated embryonic patterning. We found that the expression of dhrs3 was induced by atRA treatment and over-expression of Xenopus nodal related 1 (xnr1). Its spatial expression showed a complementary pattern to aldh1a2 at the neurula stage. In embryos overexpressing aldh1a2 or rdh10 in the presence of their respective substrates, Dhrs3 counteracted the action of Aldh1a2 or Rdh10, suggesting that RA signaling was attenuated. Knockdown of Dhrs3 by antisense morpholino oligonucleotides indicated that Dhrs3 was required for proper anteroposterior axis formation, neuroectoderm patterning, and somitogenesis and that Dhrs3 played an important role in convergent extension movement.

EXPERIMENTAL PROCEDURES

Maintenance of Frogs, Embryo Collection, and Fixation—Adult frogs (Xenopus laevis) were purchased from NASCO. Embryos were staged according to Nieuwkoop and Faber (21), and fixed in 1× HEMFA (0.1 M HEPES, pH 7.4, 2 mM EGTA, 1 mM MgSO4, 3.7% formaldehyde) for whole mount in situ hybridization.

Whole Mount in Situ Hybridization—The procedure was performed as described by Harland (22). Antisense RNA probes were synthesized using DIG RNA labeling mix (Roche Applied Science) with T7, T3, or SP6 RNA polymerase (Promega). Lineage tracing was performed by lacZ staining as described previously (23).

RT-PCR—Total RNA from sample (5 whole embryos or 20 animal caps) was extracted using TRIzol reagent (Invitrogen) and reverse-transcribed by Superscript III reverse transcriptase (Invitrogen) following the manufacturers’ protocol. PCR was performed by using GoTaq® Flexi DNA polymerase (Promega). The sequence of primers used for RT-PCR is listed in Table 1.

Animal Cap Assay—Animal caps were excised from the animal pole of embryos at stage 8.5, followed by culturing in animal cap culture solution (67% L-15 medium, 7.5 mM Tris/HCl, pH 7.5, 1 mg/ml BSA). For retinoid treatments, culture solution was supplemented with retinoid dissolved in DMSO.

Morpholino and mRNA Injection—A translation-blocking morpholino targeting Dhrs3 was used to knock down Dhrs3 (Dhrs3 MO-GCGTTTGTCAAGCCTGTCACTCAT). Anti-sense morpholino was purchased from Gene Tools, LLC (Philomath, OR). Capped mRNA was synthesized from linearized plasmids by using mMESSAGE mMACHINE SP6 kit (Ambion).

In Vitro Translation of Protein, SDS–PAGE, and Western Blot Analysis—TnT® SP6-coupled reticulocyte lysate system (L4600, Promega) was used to translate dhrs3 in vitro. FLAG-tagged full-length dhrs3 construct was translated in vitro with Dhrs3 morpholino according to the manufacturer’s instruction. The reaction product was separated by SDS–PAGE and detected by Western blot using anti-FLAG M2 antibody (Sigma). The signals were detected and analyzed by using LI-COR® Odyssey® infrared imaging system (LI-COR® Biosciences).

Quantitative PCR—Gene expression level of en2, krox20, hoxb3, myoD, fgf8, and pax3 was assessed using quantitative PCR. Briefly, total RNA from embryos was extracted and reverse-transcribed to synthesize cDNA. PCRs were performed by using SYBR Green PCR Master Mix (Takara) and specific primers with ABI 7900HT fast real time PCR system (Applied Biosystem). Expression level of odc was used for normalization. All quantitative PCRs were performed in triplicate.

Comparative Measurement of Endogenous atRA Using Cell-based Luciferase Assay—The atRA concentration in Xenopus embryos was measured using the method described elsewhere (24). Briefly, the endogenous atRA was extracted by first homogenizing embryos in distilled water, followed by liquid-liquid extraction using a mixture of hexane and isopropyl alcohol (65:15 v/v). The organic phase was collected, vacuum-dried, and resuspended in DMSO. The embryo extracts containing endogenous atRA were diluted 500-fold in DMEM before applying to HEK293T cells transiently transfected with pRL- Renilla reporter plasmid and retinoic acid-responsive element-driven luciferase reporter plasmid by Lipofectamine (Invitrogen). After 48 h of atRA treatment, the transfected cells were subjected to Dual-Luciferase® reporter assay system according to the manufacturer’s protocol (Promega). Serial dilutions of atRA were used to generate standard curve of log-log plot showing the relationship between the atRA standard and the corresponding luciferase activity. atRA concentration in Xenopus embryos was determined by using this standard curve. The atRA concentration of the injected embryos was normalized to

| Name | Sequence (5’ to 3’) | Ref. |
|------|--------------------|-----|
| cdx4 Fw | GGATCACCAGGGGAGGAATG | 39 |
| cdx4 Re | TAAGAGCCCCCGGCCGTCGTGG | 39 |
| hoxa1 Fw | CAGCCGGATATCAATATATGG | 39 |
| hoxa1 Re | CCGGGGAGCCAGTTTGT | 39 |
| gbx2 Fw | CCCCCAAACTACAAACCTCTTAA | 55 |
| gbx2 Re | TGGCTCCGTATGGCAGAAACCTATT | |
| lhx1 Fw | AGGGACCTCAGGACCACTACAT | 56 |
| lhx1 Re | CTCTTTGGATCGGTCTTGTCGAACCA | |
| dhrs3 Fw | TTTCCTAATCGACCTGCAA | 18 |
| dhrs3 Re | AGGATGAAATGACCTGGAAGA | |
that of control embryos to generate relative atRA concentration. All assays were performed in triplicate.

**Measurement of Endogenous atRA with Liquid Chromatography-Mass Spectrometry (LC-MS)**—Fifty stage 10 *Xenopus laevis* embryos were homogenized in 100 μl of 0.9% w/v sodium chloride solution, followed by liquid/liquid extraction using a mixture of hexane and isopropyl alcohol (65:15 v/v). The mixture was vortexed for 1 min, followed by centrifugation at 1000 × g for 1 min. The supernatant containing atRA was recovered, dried in vacuo, and resuspended in 25 μl of methanol containing 5 mM ammonium formate and 0.1% acetic acid.

For the LC-MS analysis, 2 μl of atRA sample was separated on a C18 column (Agilent Zorbax, 2.1 × 150 mm, 1.8 μm) using ultra-HPLC system (Agilent 1290) with a flow rate of 0.35 ml/min, in a linear gradient of 10% buffer A (0.1% acetic acid) to 95% buffer B (100% ACN containing 0.1% acetic acid). The eluate was sprayed using Agilent Dual Jet Stream source into a Q-TOF type mass spectrometer, operating at negative mode (Agilent 6550 Q-TOF MS). The relative amount of atRA was quantified by logarithmic transformation of the ion chromatogram.

**RESULTS**

**dhrs3 Expression Was Up-regulated by All-trans-retinoic Acid**—Previously, we have reported that *dhrs3* was expressed in the neural plate and pronephros during early embryonic development (18), which prompted us to use the animal cap assay to examine the expression of *dhrs3* after atRA treatments and overexpression of chordin. Both atRA and chordin are morphogens involved in the neuroectoderm patterning in *Xenopus* (10, 25). atRA is also an essential factor for pronephros development (7). We found that animal caps exposed to exogenous atRA showed an elevated *dhrs3* expression, indicating that *dhrs3* was atRA-responsive (Fig. 1A, lane 3). This observation is in line with previous research findings in retSDR1, the human homolog of DHR3 (19). Moreover, the *dhrs3* expression domain in neurulae was also intensified and expanded, covering almost the entire neural plate after the atRA treatment. Similarly, expanded expression domains of *cyp26a1* were also observed after atRA treatment (Fig. 1B). By contrast, the expression of *dhrs3* was not induced by injection of *chordin* mRNA in animal cap cells (Fig. 1A, lane 4).

We also found that during gastrulation *dhrs3* expression was enriched in the dorsal blastopore lip, and its expression was localized in the involuting mesoderm during gastrulation (18). Various signaling pathways have been found to be involved in mesoderm formation during gastrulation, including Nodal, FGF, and Wnt signaling pathways (26–29). We therefore activated these mesoderm-related signaling pathways in animal caps and investigated their effects on *dhrs3* expression. We found that overexpression of *xnr1*, a ligand of nodal signaling pathway, induced *dhrs3* expression (Fig. 1A, lane 6). The induction of *dhrs3* by *xnr1* was not due to the mesoderm induction as *dhrs3* expression domain in *brachyury* expression was not elicited by injection of *chordin* mRNA (Fig. 1A, lane 4). To demonstrate that these signaling pathways were indeed activated, we examined an array of markers, including *hoxd1*, for atRA and Wnt signaling (Fig. 1A, lanes 3 and 5) (32, 33), *sox2* for *chordin* and FGF signaling (Fig. 1A, lanes 4 and 7) (34, 35), *siamois* for Wnt signaling (Fig. 1A, lane 5) (36), and *brachyury* for FGF and Nodal signaling (Fig. 1A, lanes 6 and 7) (28). As expected, all of these marker genes were up-regulated accordingly.

**FIGURE 1. dhrs3 expression is induced by atRA.** A, expression of *dhrs3* in whole embryos and animal caps was induced in response to a 4-h treatment of 1 μM atRA or *xnr1* overexpression. **WE,** whole embryo; **AC,** animal cap. **B,** expression patterns of *dhrs3* and *cyp26a1* in stage 12 embryos in response to a 4-h treatment of 1 μM atRA. Dorsal view. **C,** expression patterns of *dhrs3*, *cyp26a1*, and *aldh1a2*, in stage 13 (dorsal view) and stage 35 embryos (lateral view).
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**FIGURE 2. Overexpression of dhrs3 counteracts the action of Aldh1a2.** A, morphology of stage 35 embryos injected with 2 ng of aldh1a2 alone or in combination with 2 ng of dhrs3 at the two-cell stage, followed by treatment with either DMSO or 0.5 μM atRAL during gastrulation. Arrows indicate the measure of the head diameter. B, quantification of the relative embryo head diameter of uninjected and injected embryos treated with atRAL. C, expression of atRA-responsive genes hoxd1, gbx2, cdx4, and lhx1 in animal caps overexpressing aldh1a2 alone or in combination with dhrs3, DMSO, or atRAL treatment. D, proposed role of dhrs3 in the atRA metabolism. Dashed arrow indicates the induced expression of dhrs3 by atRA. WE, whole embryos; AC, animal caps.

Because we observed that dhrs3 was up-regulated by atRA in animal cap assay, we next compared the expression pattern of dhrs3 with those of aldh1a2 and cyp26a1, respectively. At stage 13, dhrs3 was localized in the dorsal midline, which corresponds to the notochord region, and two bilateral patches in the neural plate (18). cyp26a1 encoding an enzyme for atRA degradation was localized in the anterior-most region and the region surrounding the posterior blastopore. aldh1a2 encoding a major enzyme for atRA synthesis was expressed in presumptive presomitic and lateral plate mesoderm. At the tadpole stage (stage 35), dhrs3 expression pattern was mainly localized in the pronephros and central nervous system, whereas aldh1a2 was expressed in the branchial arches and the tail region, and aldh1a2 expression was mainly found in the pronephros region, a region where dhrs3 was also expressed (Fig. 1C). Considering the facts that 1) atRA is an important signaling molecule during neural tube patterning and pronephros formation, 2) two important atRA metabolizing enzymes, cyp26a1 and aldh1a2, were localized to these regions, and 3) human homolog of DHRS3 exerts functions in atRA metabolism, we therefore suggested that dhrs3 also played a role in atRA metabolism during embryonic development.

**Dhrs3 Attenuated RA Signaling by Counteracting the Action of Aldh1a2 and Rdh10—**We further questioned how dhrs3 regulates RA signaling. Because human DHRS3 acts as a retinal reductase that increases concentrations of retinyl ester by converting retinal to retinol in a neuroblastoma cell line (19), we then studied the effects of dhrs3 on the embryos overexpressing aldh1a2. As a major atRA-producing enzyme in embryos, Aldh1a2 can oxidize atRAL to atRA (37). It has been shown that overexpression of aldh1a2 together with an exogenous supply of atRAL can elevate atRA levels, resulting in phenotypes of repression of head and anterior axis similar to those induced directly by atRA treatment (38). Here, two-cell stage embryos were injected with aldh1a2 mRNA alone or in combination with dhrs3 mRNA, which was followed by the treatment with either atRAL or DMSO during gastrulation stages. Without atRAL, overexpression with 2 ng of aldh1a2 alone or in combination with dhrs3 did not induce obvious morphological effects on the embryos (Fig. 2A). With the presence of 0.5 μM atRAL, there were no visible abnormalities in the uninjected embryos (Fig. 2A, left panel). However, embryos overexpressing aldh1a2 exhibited a shortened body axis and a smaller head structure (Fig. 2A, middle panel), which are typical phenotypes induced by atRA treatment (Fig. 5D). This result suggested that atRAL was oxidized by aldh1a2 into atRA. Interestingly, these phenotypes were partially rescued by co-overexpression with dhrs3 (Fig. 2A, right panel). To assess the severity of the observed phenotypes, we quantified the relative head diameter of the embryos by measuring the distance between the cement gland and the anterior-most somite as the head diameter. As shown in Fig. 2B, in the presence of atRAL, overexpression of aldh1a2 alone induced about 20% reduction in the relative head diameter, which was significantly rescued by co-overexpression with dhrs3. This result suggested that Dhrs3 counteracted the action of aldh1a2 possibly through reducing atRA levels.

To verify our observation, we used the animal cap assay with a similar experimental design to examine the expression level of four atRA-responsive genes, including hoxd1, gbx2, cdx4, and lhx1 (39). The animal caps overexpressing aldh1a2 alone or in combination with dhrs3 were cultured in 0.25 μM atRAL or DMSO and were subsequently collected for RT-PCR analysis. In the absence of exogenous atRAL, we found that ectopic aldh1a2 alone only slightly induced the expression of the atRA-
responsive genes (Fig. 2C, lane 4), possibly by oxidizing endogenous atRAL into atRA, although co-overexpression with dhrs3 suppressed such induction (Fig. 2C, lane 5). In the presence of atRAL, all atRA-responsive genes examined were slightly induced (Fig. 2C, lanes 2 and 6), possibly due to oxidation by endogenous enzymes. Overexpression of aldhl1a2 further induced the expression of atRA-responsive genes (Fig. 2C, lane 7), implicating increased production of atRA by aldhl1a2, although co-overexpression with dhrs3 attenuated this induction (Fig. 2C, lane 8, compare with lane 7). These results demonstrated that dhrs3 suppressed the expression of atRA-responsive genes induced by the combined effects of aldhl1a2 and atRAL. This also suggested that Dhrs3 was involved in the reduction of atRA levels, possibly by competing with Aldh1a2 for the same substrate, atRAL (Fig. 2D).

To further test our hypothesis that Dhrs3 reduces the level of atRA oxidized from atRAL, we studied the effect of Dhrs3 on the embryos overexpressing rdh10, which catalyzes the oxidation of atRAL into atRA. Animal cap assay indicated that in the presence of atROL, ectopic rdh10 strongly induced the expression of atRA-responsive genes, including hoxd1, gbx2, and cdx4 (Fig. 3A, lane 5), whereas co-overexpression with dhrs3 reduced the expression of these four genes (Fig. 3A, lane 6). These data suggested that Dhrs3 antagonized the action of Rdh10, resulting in a lower level of atRAL and thus atRA. This was further supported by a rescue experiment, in which the phenotypes induced by dhrs3 overexpression were reversed by the treatment with a sub-teratogenic dose of 0.5 μM atRAL during gastrulation (Fig. 3, B and C). Quantification of relative embryo length of embryos exposed to atRAL with or without dhrs3 overexpression (C). *** analysis of variance, p < 0.0005. D and E, dhrs3 overexpression enhanced the phenotypes induced by ectopic cyp26a1. Lateral view of stage 35 embryos exhibited shortened trunk of AP axis when overexpressing dhrs3, cyp26a1, or both (D). Two-cell stage embryos were injected with 4 ng of dhrs3, 2 ng of cyp26a1 mRNA, or both. Quantification of relative embryo length is shown in E. F, overexpression of dhrs3 caused a posterior shift of the expression domain of en2, krox20, and hoxb3. At the four-cell stage, one dorsal blastomere of the embryo was injected with 4 ng of dhrs3 mRNA together with 100 pg of lacZ mRNA as lineage tracer. Injected side is on the right (asterisk). Overexpression of dhrs3 caused a posterior shift of the expression domain of the examined neural markers. Images of en2, krox20, and hoxb3 are the frontal view of the embryos. Percentages of embryos showing the phenotype is marked in the figure.
FIGURE 4. dhrs3 negatively regulates the synthesis of endogenous atRA. A, log-log plot showing the relationship between different concentrations of atRA standard applied to HEK293T cells and the respective luciferase activity. B, relative concentration of embryonic atRA under different treatment conditions compared with control as measured by cell-based luciferase assay. Overexpression of cyp26a1 and dhrs3 reduced the endogenous atRA level, whereas overexpression of aldhlα2, rdhl10, or knockdown of dhrs3 strongly increased atRA concentration in the embryos. The measured concentration of atRA in cell culture is indicated in the plot as mean ± S.D. in nM. Readings were taken in triplicate. C, relative concentration of embryonic atRA in aldhlα2-overexpressing embryos and Dhrs3 knockdown embryos, as measured by LC-MS. Relative level of atRA is represented as fold change compared with control reading. Injection dose of each condition per embryo is as follows: 4 ng for cyp26a1; 4 ng for dhrs3 ORF; 30 ng for Dhrs3 MO; 4 ng for aldhlα2; 4 ng for rdhl10.

ment through antagonizing Aldhlα2 (Fig. 2) and Rdhl10 (Fig. 3, A and B).

We also found that overexpression of dhrs3 resulted in a dose-dependent posterior truncation, which was similar to that induced by cyp26a1 overexpression (Fig. 3, D and E). Moreover, overexpression of dhrs3 enhanced the phenotype of excessive cyp26a1 (Fig. 3, D and E). We next examined the expression of en2, krox20, and lhx3, which are midbrain and hindbrain markers of neuroectoderm patterning and are regulated by RA signaling (9). We found that overexpression of dhrs3 caused a posterior shift of the expression domain of these genes on the injection side of embryos (Fig. 3F), which was also similar to the effects exerted by the ectopic cyp26a1 (supplemental Fig. 1). Taken together, these data suggested that Dhrs3 negatively regulates RA signaling.

Overexpression of dhrs3 Reduced Concentration of atRA in Embryos—To assess the direct effect of dhrs3 on atRA metabolism, embryonic atRA was quantitated by using HEK293T cells transfected with the retinoic acid-responsive element-driven luciferase vector (24). In this measurement method, the elevation of the luciferase activity is directly correlated with the expression of atRA but not retinol (data not shown) (24). We were able to measure the changes within the range from 10⁻⁷ to 10⁻¹¹ M of atRA (Fig. 4A). The atRA concentration in embryos overexpressing atRA-degrading enzyme cyp26a1 was lower compared with control (Fig. 4B), as we expected. Similarly, overexpression of dhrs3 also reduced the level of endogenous atRA (Fig. 4B). In contrast, overexpression of aldhlα or rdhl10, both of which can enhance atRA synthesis, caused a significant increase of the atRA concentration in embryos compared with that in control embryos. We have observed a similar trend when we analyzed the content of endogenous atRA in embryos using the LC-MS method (Fig. 4C and supplemental Fig. 3), albeit less profound. Embryos overexpressing aldhlα2, or injected with Dhrs3 MO, showed a 2.02- or 4.01-fold increase of atRA compared with the control, respectively. This demonstrated that dhrs3 negatively regulates RA signaling by suppressing atRA synthesis.

dhrs3 Was Required for AP Axis Formation and Neuroectoderm Patterning—RA signaling pathway has been implicated in various developmental processes, including axis formation and neuroectoderm patterning (37, 40). Using an antisense MO targeting dhrs3 5’-UTR region, we carried out a loss-of-function study to further investigate the roles of dhrs3 during early embryonic development (Fig. 5A). The specificity of the Dhrs3 MO was confirmed by the inhibition of in vitro translation (Fig. 5B) of the full-length dhrs3-FLAG construct. In addition, when Dhrs3 MO and dhrs3-FLAG mRNA were co-injected into the Xenopus embryos at the two-cell stage, the Dhrs3 MO strongly suppressed the translation of Dhrs3-FLAG in vitro, further supporting the specificity of Dhrs3 MO (Fig. 5C). Embryos injected with Dhrs3 MO at the two-cell stage showed a severely shortened anteroposterior (AP) axis and reduced head structure when compared with the uninjected control embryos at stage 35 (Fig. 5D). This abnormal morphology resembled the phenotype of embryos exposed to 1 μM atRA at gastrulation stage, which also displayed a shortened AP axis and reduced head structure (Fig. 5D). Increasing injection doses of Dhrs3 MO at the two-cell stage induced more severe reduction of the head structure as revealed by the reduction of the relative head diameter (Fig. 5E), whereas co-injection with dhrs3 mRNA rescued the phenotype with the head diameter statistically larger than
that of Dhrs3 MO alone (Fig. 5, D and E). We also tested the effect of Dhrs3 MO on atRA synthesis using cell-based luciferase assay. Knockdown of Dhrs3 increased the concentration of atRA in embryos, similar to the effects of overexpression of aldhlA2 and rdh10, but in a less significant manner (Fig. 4 B). Together, these results confirmed the specificity of Dhrs3 MO and indicated that dhrs3 was required for the formation of the AP axis and head structure.

Because overexpression of Dhrs3 caused posterior shift of hindbrain markers, next we used whole mount in situ hybridization to examine the effects of overexpression of aldhlA2 and rdh10, but in a less significant manner (Fig. 4B). Together, these results confirmed the specificity of Dhrs3 MO and indicated that dhrs3 was required for the formation of the AP axis and head structure.

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Because the AP axis of developing embryos undergoes dramatic extension during gastrula and neurula stages, which is driven at least in part through cell movements called convergent extension (CE) (41), we also examined the expression patterns of the mesoderm markers brachyury, notochord homeobox (not), and goosecoid, which are expressed during the normal CE movements at the neurula stages (27). Compared with the corresponding control embryos (Fig. 7, A–C), the expression domains of brachyury and not in Dhrs3 morphants were clearly shortened along the dorsal midline (Fig. 7, E and F), indicating a defect of the CE movement at the neurula stages, and simultaneously the expression of goosecoid was reduced (Fig. 7G). The observed alterations of not and goosecoid expression in Dhrs3 morphants were consistent with those induced by atRA (Fig. 7, N and O), which also showed a reduction of brachyury expression (Fig. 7M). However, overexpression of dhrs3 had little effect on the expression of these three marker genes (Fig. 7, I–K). These data suggested that dhrs3 regulated the AP axis development through modulating the CE movement.

This notion was further supported by examining the expression of paraxial protocadherin (papc) in Dhrs3 morphants. papc is known to regulate the CE movement and axis formation (42, 43). Compared with the control (Fig. 7D), although dhrs3 overexpression had little effect on papc expression (Fig. 7L), depletion of Dhrs3 resulted in more diffuse papc signals in the dorsal midline region (Fig. 7H). These diffuse signals were not observed in control embryos (Fig. 7D). Similarly, atRA treatment also led to diffused and even more suppressed papc expression (Fig. 7P).

Because the Wnt/PCP signaling pathway has been implicated in the regulation of the CE movement, we also examined the expression of molecules involved in Wnt/PCP signaling, including wnt5a, wnt11, dishevelled, and frizzled. However, we did not find obvious changes at the mRNA level of these genes in Dhrs3 morphants (data not shown). Taken together, these data indicated that Dhrs3 was required for neuroectoderm patterning, normal formation of AP axis during gastrulation, as well as AP axis extension during the CE movement.

dhrs3 Was Required for Somitogenesis—Apart from the CE movement, somitogenesis is another mechanism that extends the AP axis by forming sequential somite blocks along the AP direction. Therefore, we examined whether dhrs3 was necessary for somitogenesis. Immunohistochemical staining of the somites at stage 28 with antibody 12/101 showed that the formation of somite was disrupted in Dhrs3 morphants (Fig. 8B). The 12/101 signals in Dhrs3 morphants lacked the segmentation pattern compared with the control (Fig. 8, compare B with A). In addition, the staining of the somites was reduced (Fig. 8B’). Next, we examined expression of genes involved in the somite formation, including myoD, pax3, and fgf8 in Dhrs3 morphants (44–46). In the control embryos (Fig. 8, C–E), the segmentation of somites was clearly shown by expression of myoD, papc, and fgf8 (Fig. 8, C’–E’, arrow); however, in Dhrs3 morphants, this characteristic segmentation pattern was lost, and expressions of myoD, papc, and fgf8 became diffuse (Fig. 8, F–H and F’–H’). Real time PCR indicated their expression levels were also suppressed compared with those of the control.
Inhibition of aldh1a2 and dhrs3 that presents the substrate to Dhrs3, which increases concentrations of retinyl ester by converting retinal to retinol in a neuroblastoma cell line (19), our own observations strongly suggest that Dhrs3 suppresses the effects of ectopic Aldh1a2 by competing with Aldh1a2 for the same substrate atRAL, the precursor of atRA, and that it reverses the effects of ectopic rdh10 by simply consuming the reaction product of rdh10, which is also atRAL. This is further supported by the finding that the effects of overexpression of dhrs3 were rescued by a sub-teratogenic dose of atRAL (Fig. 3). Fourth, knockdown of Dhrs3 by antisense morpholino oligonucleotides caused an increase of the atRA concentration in Dhrs3 morphants (Fig. 4). In line with this, knockdown of Dhrs3 induced an expansion of lhx1 in pronephros, whereas overexpression of dhrs3 inhibited the expression of lhx1 in the same region (supplemental Fig. 2). It has been known that the pronephros development is regulated by RA signaling (37, 47), and many genes related to the pronephros development are also induced by RA signaling, including lhx1, wt-1, and pteg (7, 48–50). It has also been reported that an increase of RA signaling results in an expansion of the pronephric domain, and inhibition of RA signaling suppresses the formation of the pronephros (7). These data provided strong evidence that dhrs3 negatively regulated RA signaling by modulating RA metabolism. Further biochemical characterization of Dhrs3 on retinoid metabolism is required to determine the enzymatic specificity during embryonic development. It is also interesting to identify the protein that presents the substrate to Dhrs3, which also determines the substrate specificity of Dhrs3.

The loss-of-function assay indicated that dhrs3 was required for the AP axis formation. During gastrulation, the high expression of brachyury in the mesoderm was temporarily disrupted by dhrs3 knockdown, suggesting a regulatory role of Dhrs3 in the mesoderm formation that would subsequently affect the AP axis formation. However, at the neurula stages, the disrupted

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**DISCUSSION**

Retinoic acid is produced from retinol by two successive enzymatic reactions catalyzed by different sets of dehydrogenases (37, 40). The importance of the upstream metabolic pathway, including the redox reaction between atROL and atRAL, however, is less understood during the early embryonic development. Here, we have shown that Dhrs3 is inducible by atRA and plays an important role in attenuating RA synthesis and signaling. We also suggested that Dhrs3 regulated embryonic development by decreasing the atRAL supply and in turn reducing the atRA level. This suggestion is supported by the following observations. First, overexpression of dhrs3 induced similar phenotypes as did overexpression of cyp26a1, and the phenotypes induced by excessive cyp26a1 were further enhanced by dhrs3 overexpression (Fig. 3). Given that cyp26a1 negatively regulates RA signaling by degrading atRA, it is therefore very likely that dhrs3 also plays a negative role in the regulation of RA signaling. Second, in the cell-based luciferase assay, the endogenous atRA level in embryos was strongly reduced by dhrs3 overexpression, although the atRA level was elevated by Dhrs3 knockdown, as shown by LC-MS analysis (Fig. 4). Third, ectopic dhrs3 reduced the phenotypes of aldh1a2 overexpression in the presence of atRAL as well as rdh10 overexpression in the presence of atROL (Figs. 2 and 3). Although aldh1a2 and rdh10 catalyze different enzymatic reactions in the RA metabolism, both promote the synthesis of atRA (39). The reduction of aldh1a2- or rdh10-induced phenotype by dhrs3 suggested that dhrs3 functions by either removing atRAL or directly inhibiting aldh1a2 and/or rdh10. Considering that human DHRS3 has already been shown to act as a retinal reductase, which increases concentrations of retinyl ester by converting retinal to retinol in a neuroblastoma cell line (19), our own observations strongly suggest that Dhrs3 suppresses the effects of ectopic Aldh1a2 by competing with Aldh1a2 for the same substrate atRAL, the precursor of atRA, and that it reverses the effects of ectopic rdh10 by simply consuming the reaction product of rdh10, which is also atRAL. This is further supported by the finding that the effects of overexpression of dhrs3 were rescued by a sub-teratogenic dose of atRAL (Fig. 3). Fourth, knockdown of Dhrs3 by antisense morpholino oligonucleotides caused an increase of the atRA concentration in Dhrs3 morphants (Fig. 4). In line with this, knockdown of Dhrs3 induced an expansion of lhx1 in pronephros, whereas overexpression of dhrs3 inhibited the expression of lhx1 in the same region (supplemental Fig. 2). It has been known that the pronephros development is regulated by RA signaling (37, 47), and many genes related to the pronephros development are also induced by RA signaling, including lhx1, wt-1, and pteg (7, 48–50). It has also been reported that an increase of RA signaling results in an expansion of the pronephric domain, and inhibition of RA signaling suppresses the formation of the pronephros (7). These data provided strong evidence that dhrs3 negatively regulated RA signaling by modulating RA metabolism. Further biochemical characterization of Dhrs3 on retinoid metabolism is required to determine the enzymatic specificity during embryonic development. It is also interesting to identify the protein that presents the substrate to Dhrs3, which also determines the substrate specificity of Dhrs3.

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brachyury expression was partially recovered (Fig. 7), although the AP axis formation did not seem to catch up. These observations implicated that at this stage Dhrs3 may modulate the AP axis formation through interaction with other tissues. Indeed, at the early neurulation, we found that the notochord did not extend to its full length in Dhrs3 morphants, as shown by brachyury and not expression (Fig. 7). This phenotype was also observed in embryos with CE movement defects (27). Furthermore, Dhrs3 morphants also displayed changes in the expression domain and intensity of lhx1 and papc, both of which have been shown to participate in the regulation of the CE movement (51). These findings further supported that the shortened body axis was induced at least in part by the shortening of the notochord, which possibly resulted from the defects in the CE movement. Detailed molecular mechanisms underlying the defective CE movement when Dhrs3 is knocked down await further investigations. Thus far, information on how the CE movement is regulated in X. laevis is not yet completed. It has been reported that atRA can induce the expression of retinoic acid inducible genes, which then activate Rho small GTpase through binding to frizzled7 (52). Both Rho small GTpase and frizzled7 are required in the regulation of the CE movement (53). It is of special interest to determine how dhrs3 acts to regulate lhx1, papc, and the activity of CE effectors, namely Rho small GTpases.

RA signaling is also essential for somitogenesis. Studies have already shown that embryos with disrupted somitogenesis also display a shortened AP axis (54). In this study, abnormal somite formation was also observed after knockdown of dhrs3, suggesting that defective somitogenesis under the influence of altered RA signaling may also be attributed to the formation of the shortened body axis.

In summary, we have investigated the role of dhrs3 in RA signaling as well as in early embryonic development. We showed that dhrs3 attenuated RA signaling and that it was required for the normal anteroposterior axis formation through mediating mesoderm formation, convergent extension movement, and somitogenesis.

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