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N-CoR is required for patterning the anterior–posterior axis of zebrafish hindbrain by actively repressing retinoid signaling

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ABSTRACT

Active repression of gene expression mediated by unliganded nuclear receptors plays crucial roles in early development of vertebrates. N-CoR (*n*uclear receptor *co*-repressor) is the first identified co-repressor that can repress retinoic acid (RA) inducible gene transcription in the absence of RA. Previously, N-CoR was reported to be required for late-stage organogenesis in mouse but whether N-CoR can affect RA-responsive early embryonic patterning is unknown. In this study, we report molecular cloning of zebrafish orthologue of N-CoR and its wide distribution pattern during zebrafish early development. Knocking down *n-cor* elevates endogenous RA signaling in zebrafish embryos and posteriorizes the neural ectoderm. Overexpressing or knocking down *n-cor* in zebrafish embryos alters the length of hindbrain in a manner similar to decreasing or increasing RA signaling in embryos, respectively. Taken together, our results demonstrate that N-CoR is essential for early hindbrain patterning by actively repressing retinoid signaling.

1. Introduction

Retinoid signaling plays crucial roles in vertebrate embryogenesis. It is transduced through the ligand, retinoic acid (RA), which binds to the heterodimers of retinoic acid receptors (RARs) and retinoid-X-receptors (RXRs) to regulate transcription of target genes containing *RA response elements* (RAREs) (Niederreither and Dollé, 2008). The DNA-bound receptors recruit co-repressors to actively repress transcription of RA-inducible genes in the absence of RA (Altucci and Gronemeyer, 2001; Niederreither and Dollé, 2008). N-CoR (*n*uclear receptor *co*-repressor, also known as N-CoR1) is the first co-repressor identified from cell cultures that can repress RA-inducible gene transcription by selectively interacting with DNA-bound RAR/RXR in the absence of RA (Horlein et al., 1995). SMRT (silencing *m*ediator of *r*etinoic and *t*hyroid

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hormone receptors, also known as N-CoR2) is a close relative of N-CoR, and it also serves as a co-repressor of RARs (Ordentlich et al., 1999; Park et al., 1999). Both N-CoR and SMRT mediate gene silencing through their conserved nuclear receptor interaction *d*omains (IDs) that bind to nuclear receptors and their independent *r*epression *d*omains (RDs) that interact with histone deacetylases (HDACs). The interaction of the co-repressor complex with HDAC leads to chromatin condensation and hence repression of gene transcription. Upon binding of ligands to receptors, the co-repressors are released from DNA-associated heterodimers, leading to activation of gene transcription (Horlein et al., 1995; Ordentlich et al., 1999; Park et al., 1999; Baniahmad, 2005).

By mediating active repression through nuclear receptors, the co-repressors play crucial roles in a variety of biological processes including cell proliferation, development, and homeostasis (Altucci and Gronemeyer, 2001; Baniahmad, 2005). Inappropriate gain of N-CoR function, mostly due to lack of proper dissociation of N-CoR from nuclear receptors, leads to repression of transcription despite the presence of ligand and causes a variety of diseases, including the human acute promyelocytic leukemia (APL) (Altucci and Gronemeyer, 2001; Jepsen and Rosenfeld, 2002). In most APL patients, RARα gene is fused with the promyelocytic leukemia (PML) gene due to abnormal chromosomal translocation. The resulting fusion protein occupies RARE-containing promoters and recruits N-CoR or SMRT along with HDACs, which leads to local chromatin condensation. Under normal concentration of RA, the complex is unable to de-condense chromatin to activate gene transcription, causing a block in myeloid differentiation (Lin et al., 2001; Qiu et al., 2007). On the other hand, mouse with knock out deletion of *N-CoR* generally dies around embryonic day E16, exhibiting defects in erythrocyte differentiation and development of the thymocyte and central nervous system (Jepsen et al., 2000). *In vitro* transcription assay using cells derived from *N-CoR*$^{-/-}$ mouse embryos shows that re-introduction of N-CoR expression can block the ligand-independent activation of the reporter gene containing a RARE in its promoter (Jepsen et al., 2000). Overexpression of dominant negative N-CoR in primary cultures of mouse limb mesenchyme de-represses unliganded RAR-mediated gene expression that is required for mouse skeletal progenitor differentiation (Weston et al., 2002). *SMRT* null mice die before E16.5 due to a lethal heart defect. Analysis of *SMRT*$^{-/-}$ mice reveals that SMRT is required for the RA dependent forebrain development. Results from cultured *SMRT*$^{-/-}$ cells show that SMRT is critical for preventing RAR dependent induction of neuronal differentiation in the absence of RA (Jepsen et al., 2007). In *Xenopus*, embryos overexpressing dominant negative SMRT exhibit reduction of anterior structures such as forebrain and cement gland, phenotypes that are similar to those treated by RA (Koide et al., 2001). Taken together, these observations suggest that active repression of RA signaling performed by co-repressors is essential for vertebrate growth and development. However, whether N-CoR plays roles in RA-responsive early embryonic patterning is unknown. In this study, we report molecular cloning of the zebrafish orthologue of N-CoR. Whole mount *in situ* hybridization reveals that *n-cor* message is widely present in zebrafish embryos at early development. Both overexpression and knock-down analyses of N-CoR in zebrafish embryos demonstrate that N-CoR is essential for vertebrate hindbrain patterning by actively repressing retinoid signaling.

2. Results

2.1. *Zebrafish n-cor encodes a highly conserved zebrafish orthologue of N-CoR*

Employing RACE-PCR and end–end PCR strategies, we obtain a full-length cDNA of zebrafish *n-cor* with a length of 9591 bp (EF016488). Sequence analysis reveal that the *n-cor* cDNA consists of a 412 nucleotide (nt) 5′-UTR (untranslated region), a 7230 nt coding region and a 1949 nt 3′-UTR including a presumptive polyadenylation signal (AATAAA) located at 22 nucleotides upstream of the predicted transcription terminator site. The *n-cor* cDNA is deduced to encode a protein comprising 2409 amino acids (Fig. 1A). The protein exhibits 56.2–60.3% amino acid identity with the N-CoRs of *Tetraodon nigroviridis* (CAF91071), African clawed frog (NP_001082225), red jungle fowl (XP_415843), mouse (EDL10343), rat (EDM04703) and human (NP_006302), 33.6–35.7% identity with the SMRTs of *Tetraodon nigroviridis* (CAF98508), zebrafish (XP_689998), African clawed frog (NP_001084492), red jungle fowl (XP_415107), mouse (NP_035554), rat (NP_001101804) and human (EAW98453). Protein sequence analysis indicates that the protein contains three repression domains (RDI, RDII and RDIII) and two interaction domains (IDI and IDII) that are conserved in other N-CoR orthologues (Fig. 1A and B). Within the interaction domains, there are three conserved CoRNR boxes (Fig. 1A and B). The functional domains of the predicted protein each shares high identities in amino acids with those of other vertebrate N-CoRs (data not shown). Phylogenetic analysis shows that the predicted protein is clustered into N-CoR family (Supplementary Fig. S1).

BLASTing the zebrafish genome database (http://www.ncbi.nlm.nih.gov) with the sequence of cloned cDNA, we determine that zebrafish *n-cor* gene is mapped to chromosome 5. The NCBI Map Viewer shows that the *pigl* gene (phosphatidylinositol glycan anchor biosynthesis, class L Gene) is immediately adjacent to the *n-cor* gene. After BLASTing the human and mouse genomes, we find that both human and mouse *Pigl* genes are also syntenic with N-CoR, and they are located right next to *N-CoR* on human chromosome 17 and mouse chromosome 11, respectively. Moreover, we do not find a duplicated 'b' copy for *n-cor* gene, as often occurs for other genes in zebrafish after mining the zebrafish genome database (Sun et al., 2005).

Taken together, these results suggest that our cloned gene encodes a zebrafish orthologue of N-CoR.

2.2. *Zebrafish n-cor message is widely expressed during zebrafish early development*

To understand the role of N-CoR in zebrafish early development, we first performed whole mount *in situ* hybridization analysis on zebrafish embryos. Consistent with the previous report from the Thisse Lab (Bertrand et al., 2007), our results reveal that zebrafish N-CoR mRNAs are widely present in zebrafish embryos from cleavage stage to 24 hpf (hours post

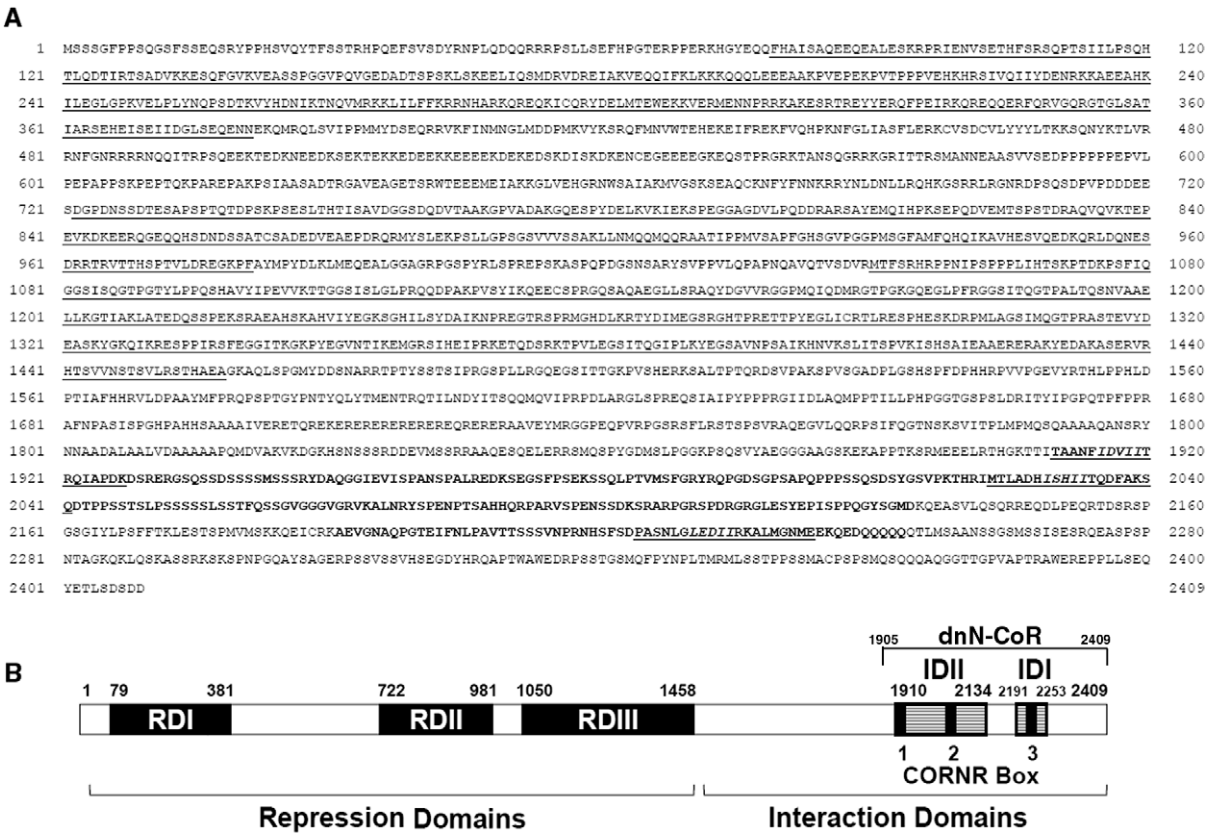

Fig. 1 – Complete deduced amino acid sequence and schematic structure of zebrafish N-CoR. (A) The complete deduced amino acid sequence of zebrafish N-CoR. The amino acid sequences representing the predicted three repression domains are underlined, those representing the predicted two interaction domains are bolded and those representing the predicted three CoRNR boxes are bolded and underlined. (B) Schematic diagram showing the conserved function domains in zebrafish N-CoR. RDI, RDII and RDIII denote the predicted repression domain I, II and III, respectively. IDI and IDII denote the predicted interaction domain I and II, respectively. The number above the domains stands for the amino acid position of zebrafish N-CoR protein. The protein fragment of aa1905 to aa2409 comprises the presumptive dominant negative form of N-CoR (dnN-CoR).

fertilization) (Supplementary Fig. S2). N-CoR mRNA is evenly distributed in all embryonic cells at 16-cell stage (1.5 hpf) (Supplementary Fig. S2A), suggesting that N-CoR mRNA is maternally stored in zebrafish embryos. The message remains uniformly distributed through gastrulation (Supplementary Fig. S2B and D). At bud stage, the *n-cor* expression in anterior part of the embryos is stronger than that of posterior part (Supplementary Fig. S2E). By 18-somite stage (18 hpf), the *n-cor* mRNA level in the somite region is clearly reduced while it is strongly present in the whole presumptive head region (Supplementary Fig. S2F). This similar pattern is maintained at 24 hpf (Supplementary Fig. S2G). Flat mount analysis on 24 hpf embryos reveals that the *n-cor* is expressed mainly in the ventricles of forebrain, midbrain and hindbrain, and also in the retina (Supplementary Fig. S2H).

2.3. *Knocking down n-cor results in elevated endogenous RA signaling*

The wide expression pattern of *n-cor* in zebrafish embryos suggests that N-CoR is involved in the zebrafish early development. Because N-CoR can repress retinoid signaling in mammalian cell cultures, we hypothesize that knocking down *n-cor* would elevate RA signaling in zebrafish embryos. Previously, it was reported that the homeostasis of RA signaling in embryos are maintained by the coordinate expression of *aldh1a2* (for RA synthesis) and *cyp26a1* (for RA metabolism) (Dobbs-McAuliffe et al., 2004). If the embryos are challenged to an elevated RA signaling, the expression of *cyp26a1* is up-regulated whereas the expression of *aldh1a2* is down-regulated (Dobbs-McAuliffe et al., 2004). To test whether N-CoR represses RA signaling in zebrafish embryos, we compare the expressions of *cyp26a1* and *aldh1a2* in the N-CoR deficient embryos (20 hpf) with those in the control embryos without any treatment or with those in the embryos treated with 10 nM RA. As shown in Fig. 2, *cyp26a1* is mainly expressed in the anterior epidermis, dorsal anterior spinal cord, somites and tail bud in the wild-type control embryos (Fig. 2A). Embryos microinjected with control MO display an almost identical *cyp26a1* expression pattern (Fig. 2B) with the wild-type embryos (Fig. 2A). However, when knocked down by *n-cor* MO that can specifically inhibit the translation of *n-cor* mRNA, the embryos exhibit strongly up-regulated expression of *cyp26a1* in the anterior epidermis and tail bud (Fig. 2C;

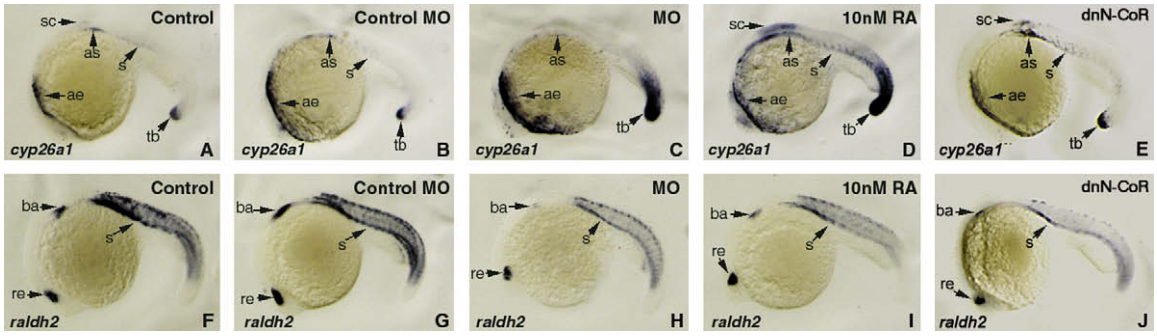

Fig. 2 – Knocking down *n-cor* resulting in elevation of endogenous RA signaling. Embryos at 1- to 2-cell stages are microinjected with *n-cor* MO (C and H) to knock-down *n-cor* expression or with dnN-CoR mRNA to reduce the function of N-CoR (E and J) and treated with 10 nM RA (D and I) to increase RA signaling. All treated embryos (C–E and H–J) together with wild-type control embryos (A and F) and control MO microinjected embryos (B and G) are grown to 20 hpf and examined for the expression of *cyp26a1* (A–E) and *aldh1a2* (also known as *raldh2*) (F–J), respectively, by whole mount *in situ* hybridization. All embryos are positioned anterior left and viewed laterally. *cyp26a1* is mainly expressed at anterior epidermis, dorsal anterior spinal cord, somites and tail buds (A), while *aldh1a2* is expressed at retina, branchial arches and somites (F) in the wild-type control embryo. Embryos microinjected with control MO display similar expression levels of *cyp26a1* (B) and *aldh1a2* (G) to the control embryos (A and F). Knocking down *n-cor* causes up-regulated expression of *cyp26a1* in anterior epidermis and tail bud (C) and down-regulated expression of *aldh1a2* in the regions of retina, branchial arches and somites (H). The expression changes also occur in the embryos treated with 10 nM RA (D and I) except that a strongly up-regulated expression of *cyp26a1* in dorsal anterior spinal cord and somites in RA treated embryos (D). Overexpression of dnN-CoR causes up-regulated expression of *cyp26a1* in dorsal anterior spinal cord and somites (E) and down-regulated expression of *aldh1a2* in the regions of retina, branchial arches and somites (J). ae, anterior epidermis; as, anterior somites; ba, branchial arches; re, retina; s, somites; sc, dorsal anterior spinal cord; tb, tail bud.

$n = 68/86$, compared with the controls in Fig. 2A and B), a phenotype that is also observed in the embryos treated with 10 nM RA (Fig. 2D). Additionally, elevated *cyp26a1* expression is also detected in the dorsal anterior spinal cord and somites in embryos treated with 10 nM RA but not in *n-cor* deficient embryos. *aldh1a2* is normally expressed in retina, branchial arches and somites (Fig. 2F). The embryos microinjected with control MO display the same *aldh1a2* expression pattern (Fig. 2G) as the wild-type controls (Fig. 2F). *n-cor* MO treatment results in a significant reduction in *aldh1a2* expression in the regions of retina, branchial arches and somites (Fig. 2H; $n = 64/86$), as compared with the control embryos (Fig. 2F and G). The down-regulation of *aldh1a2* (Fig. 2H) is similar to that shown in the 10 nM RA treated embryos (Fig. 2I). In summary, these results suggest that N-CoR is involved in the repression of RA signaling during zebrafish early development.

According to the primary structure of zebrafish N-CoR, the protein fragment of aa1905 to aa2409 comprises the presumptive dominant negative N-CoR (dnN-CoR) (Fig. 1). To investigate whether overexpression of dnN-CoR produces similar phenotypes to knocking down *n-cor*, we microinjected dnN-CoR mRNA into zebrafish embryos at 1- to 2-cell stage and then examined the expressions of *cyp26a1* and *aldh1a2* in the embryos at 20 hpf. Similar to the N-CoR deficient embryos (Fig. 2H), the dnN-CoR expressing embryos exhibit significant down-regulation of *aldh1a2* expression in retina, branchial arches and somites (Fig. 2J; $n = 16/32$). However, unlike the N-CoR deficient embryos (Fig. 2C), the dnN-CoR expressing embryos display a clearly increased level of *cyp26a1* expression in dorsal anterior spinal cord and somites (Fig. 2E; $n = 25/49$), which coincides with the regions of *smrt* expression (Bertrand et al., 2007).

2.4. *Knocking down n-cor posteriorizes anterior–posterior axis of zebrafish embryos in ways similar to increasing RA signaling*

RA has been shown to play critical roles in the patterning of anterior–posterior axis in the developing nervous system of vertebrates (Niederreither et al., 1999; Kudoh et al., 2002). Administering teratogenic RA causes the expression expansion of the posterior gene *hoxb1b* and expression reduction of the anterior gene *otx2* (Kudoh et al., 2002). Since our results show that knocking down *n-cor* up-regulates endogenous RA signaling, we then examine whether the expressions of *otx2* and *hoxb1b* are affected in the N-CoR deficient embryos. Normally, *otx2* is expressed anteriorly (Fig. 3A and A′) whereas *hoxb1b* is expressed posteriorly (Fig. 3E) in the embryos during 80–90% epiboly stages. Microinjection of control MO does not change the expression patterns (Fig. 3A, B, A′, B′, E and F). However, when microinjected with the *n-cor* MO, the embryos display reduced expression of *otx2* (Fig. 3C and C′; $n = 27/40$) and expanded expression of *hoxb1b* anteriorly (Fig. 3G; $n = 15/40$), as compared with control embryos (Fig. 3A, A′, B, B′, E and F). This reduced expression pattern of *otx2* (Fig. 3C and C′) is similar to that of embryos treated with 50 nM RA (Fig. 3D and D′), while the expanded expression pattern of *hoxb1b* (Fig. 3G) is similar to that of embryos treated with 10 nM RA (Fig. 3H). Previously, it was reported that 10 nM RA has no effect on *otx2* expression whereas 50 nM RA treatment reduces *otx2* expression significantly (Hans and Westerfield,

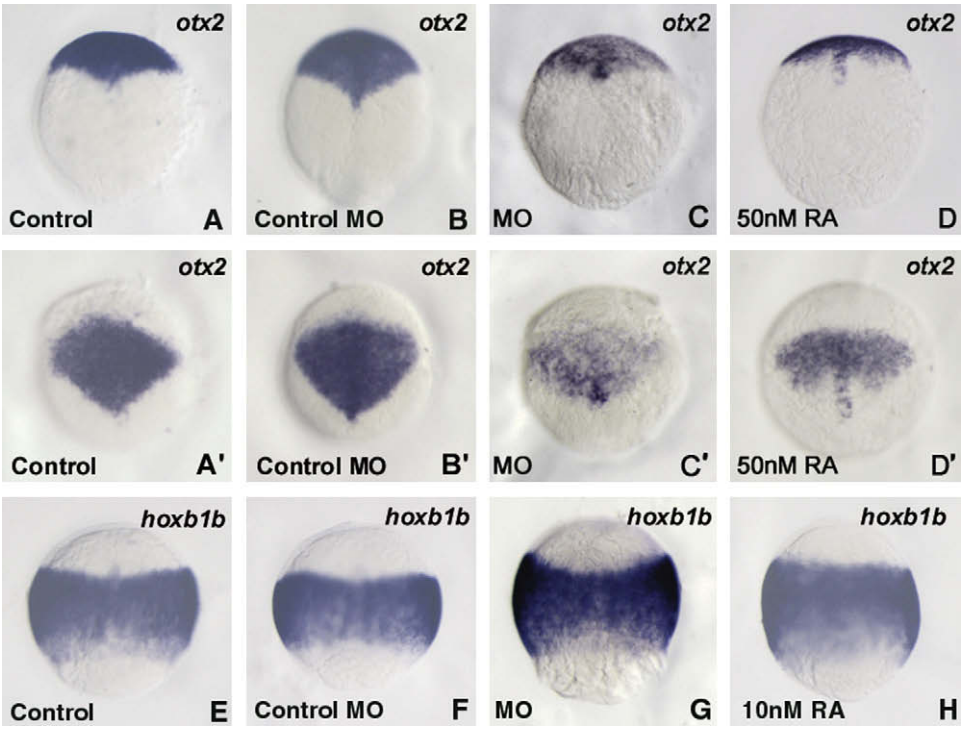

Fig. 3 – Knocking down *n-cor* posteriorizing anterior–posterior axis of zebrafish embryos in ways similar to increasing RA signaling. Embryos at 1- to 2-cell stages are microinjected with *n-cor* MO (C, C′ and G) to knock-down *n-cor* expression or treated with 50 nM RA (D and D′) or 10 nM RA (H) to increase RA signaling. All treated embryos (C, C′, D, D′, G and H) together with wild-type control embryos (A, A′ and E) and the control MO microinjected embryos (B, B′ and F) are grown to 80–90% epiboly and examined for the expression of *otx2* (A–D and A′–D′) and *hoxb1b* (E–H) by whole mount *in situ* hybridization. The embryos of (A)–(H) are positioned anterior top and viewed dorsally. The embryos of A′–D′ are the same ones of A–D but positioned anterior front, respectively. *otx2* is expressed anteriorly (A and A′) whereas *hoxb1b* is expressed posteriorly in the embryos (E). Microinjection of control MO does not change the expression patterns (B, B′ and F). Knocking down *n-cor* causes anterior reduction of *otx2* expression (C and C′) and anterior expansion of *hoxb1b* expression (G), respectively. Embryos treated with 10 nM RA (H) or 50 nM RA (D and D′) display expanded expression of *hoxb1b* (H) and reduced expression of *otx2* (D and D′), respectively.

2007). Taken together, the results suggest that N-CoR is essential for the normal patterning of anterior–posterior axis in the developing central nervous system by repressing RA signaling.

2.5. *Altering N-CoR expression changes the anterior–posterior axis patterning of hindbrain in ways similar to altering RA signaling in zebrafish embryos*

It is known that retinoid signaling patterns the anterior–posterior axis of hindbrain. Increasing RA signaling results in reduction of anterior part and expansion of posterior part of hindbrain whereas reducing RA signaling results in an opposite phenotype. Therefore, if N-CoR is involved in repressing retinoid signaling, enhancing or reducing N-CoR expression would alter anterior–posterior axis of hindbrain in ways similar to those resulting from decreasing or increasing RA signaling, respectively. To test this, we compare the lengths of hindbrain segments in the N-CoR overexpressed or depleted embryos with those in the 1 μM DEAB or 1 nM RA treated embryos.

As shown in Fig. 4, the distance between MHB and r7 (r1–r6) in the N-CoR overexpressed embryos (Fig. 4D; 440.5 ± 17.6, $n = 23$) is 4.3% longer ($p < 0.01$) than that in the wild-type controls (Fig. 4B; 422.4 ± 19.5, $n = 16$). r1–r6 lengthening (8.1% longer, $p < 0.01$) also occurs in the DEAB treated embryos (Fig. 4F; 456.7 ± 29.6, $n = 15$). The length of r1–r6 in the control MO embryos (Fig. 4H; 430.3 ± 17.1, $n = 20$) is similar ($p > 0.05$) to that in the wild-type controls (Fig. 4B). However, r1–r6 is 17.3% shorter ($p < 0.01$) in the *n-cor* MO embryos (Fig. 4J; 355.9 ± 23.2, $n = 19$) than that in the control MO embryos (Fig. 4H). r1–r6 shortening (9.4% shorter, $p < 0.01$) also occurs in the RA treated embryos (Fig. 4L; 382.7 ± 24.4, $n = 11$).

Within r1–r6 region, the length of r1–r2 in the N-CoR overexpressed embryos (Fig. 4D; 130.9 ± 9.7, $n = 23$) is 8.3% longer ($p < 0.01$) than that in the wild-type controls (Fig. 4B; 120.9 ± 9.9, $n = 16$). The lengthening (14.9% longer, $p < 0.01$) also occurs in the DEAB treated embryos (Fig. 4F; 138.9 ± 15.6, $n = 15$). However, the lengths of r3–r5 in the N-CoR overexpressed embryos (Fig. 4D; 252.9 ± 12.2, $n = 23$) and in the DEAB treated embryos (Fig. 4F; 260.5 ± 20.2, $n = 15$) are both slightly longer (2.7% and 5.8% longer, respectively, $p < 0.05$) than those in the wild-type controls (Fig. 4B; 246.2 ± 12.0, $n = 16$).

The lengths of r1–r2 and r3–r5 in the control MO embryos (Fig. 4H; 123.2 ± 8.0; 245.4 ± 13.8, $n = 20$) are both similar

($p > 0.05$) to those in the wild-type controls (Fig. 4B). The length of r1–r2 is 20.9% shorter ($p < 0.01$) in the *n-cor* MO embryos (Fig. 4J; 97.5 ± 14.2, $n = 19$) than that in the control MO embryos (Fig. 4H). The shortening (24.5% shorter, $p < 0.01$) also occurs in the RA treated embryos (Fig. 4L; 91.3 ± 17.0). However, the lengths of r3–r5 in the *n-cor* MO embryos (Fig. 4J; 226.1 ± 11.7, $n = 19$) and in the RA treated embryos (Fig. 4L; 239.3 ± 12.9, $n = 11$) are both slightly shorter (7.9% and 2.8% shorter, $p < 0.01$ or $p < 0.09$, respectively) than those in the controls (Fig. 4H and B).

Taken together, these results demonstrate that overexpressing N-CoR and reducing RA signaling produce a similar phenotype on hindbrain, namely expansion of r1–r6, whereas reducing N-CoR expression exhibits a similar effect to increasing RA signaling, namely reduction of r1–r6 and the length change of r1–r2 plays a bigger role than that of r3–r5 in the length change of r1–r6 in zebrafish embryos.

In addition to hindbrain length changes, the expression level of *hoxb4a* is altered in the embryos subjected to various treatments. The expression of *hoxb4a* is reduced significantly in the *n-cor* overexpressed embryos (Fig. 4C and D). This phenotype also occurs in the DEAB treated embryos (Fig. 4E and F). On the other hand, the expression of *hoxb4a* is increased in the *n-cor* depleted embryos (Fig. 4I and J). Likewise, the up-regulated expression of *hoxb4a* also occurs in the RA treated embryos (Fig. 4K and L). Previously, it was reported that *hoxb4a* transcription is directly regulated by RA signaling (Begemann et al., 2001; Emoto et al., 2005). Therefore, our observations suggest that N-CoR is involved in the regulation of *hoxb4a* expression by actively repressing RA signaling.

We next examine the length change between r6 and s1 at 11-somite stage. As shown in Fig. 5, the distance between r6 and s1 in the N-CoR overexpressed embryos (Fig. 5D; 101.8 ± 3.8, $n = 6$) is 13.8% shorter ($p < 0.01$) than that in the wild-type controls (Fig. 5B; 118.0 ± 8.1, $n = 21$) and the reduced distance (59.6% shorter, $p < 0.01$) also occurs in the DEAB treated embryos (Fig. 5F; 47.7 ± 17.0, $n = 7$). The length between r6 and s1 in the control MO embryos (Fig. 5H; 114.8 ± 7.3, $n = 15$) is similar ($p > 0.05$) to that of wild-type controls (Fig. 5B). However, the length between r6 and s1 in the *n-cor* MO embryos (Fig. 5J; 132.8 ± 7.9, $n = 5$) is 15.7% longer ($p < 0.01$) than that in the control MO embryos (Fig. 5H) and the lengthening phenotype (32.2% longer) is also observed ($p < 0.01$) in RA treated embryos (Fig. 5L; 156.0 ± 7.6, $n = 12$). The results demonstrate that overexpressing N-CoR and reducing RA signaling produce a similar phenotype on hindbrain, namely reduction of posterior most hindbrain, whereas reducing N-CoR expression exhibits a similar effect to increasing RA signaling, namely expansion of posterior most hindbrain.

In summary, our results suggest that *n-cor* is involved in hindbrain patterning by actively repressing RA signaling.

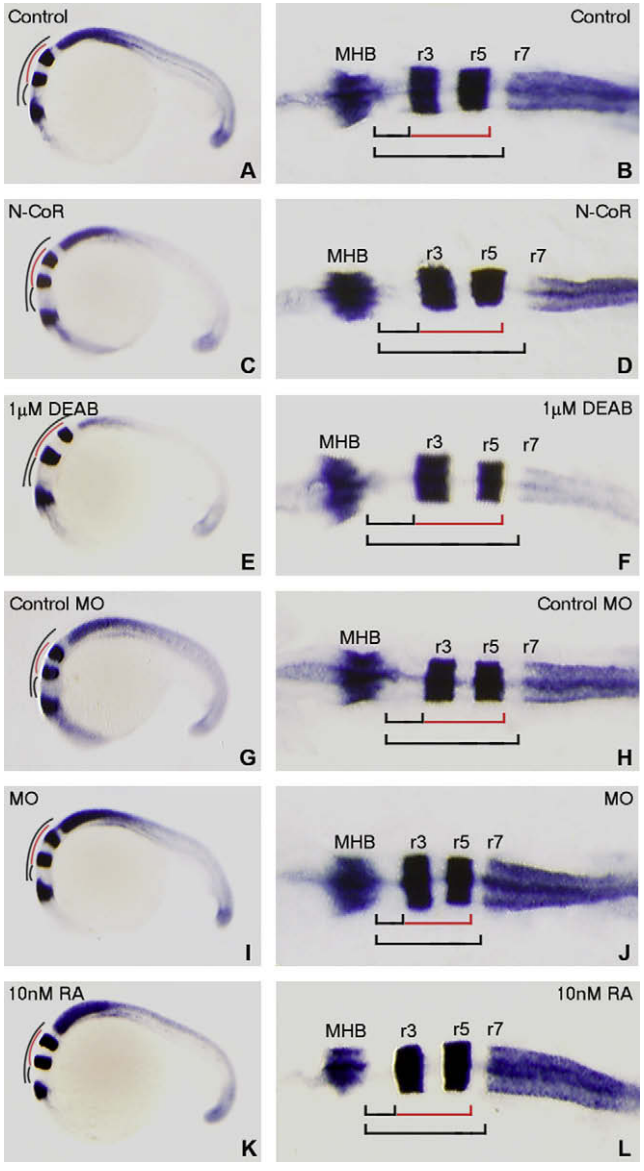

Fig. 4 – Alteration of N-CoR expression resulting in changes in r1–r6 hindbrain length that are similar to those caused by altering RA signaling in zebrafish embryos at 20 hpf. Embryos at 1- to 2-cell stages are microinjected with *n-cor* mRNA (C and D) or *n-cor* MO (I and J) to enhance or impair zebrafish N-CoR function, respectively. Alterations of RA signaling in embryos are performed by exogenous administration of 1 μM DEAB (E and F) or 10 nM RA (K and L) to reduce or enhance RA signaling, respectively. All treated embryos (C–F and I–L) together with wild-type control embryos (A and B) and control MO microinjected embryos (G and H) are grown to 20 hpf (22-somite stage) and then hybridized by RNA probes of *engrailed2a* (marking MHB), *krox20* (marking r3 and r5) and *hoxb4a* (marking anterior boundary of r7). Whole mounted embryos (A, C, E, G, I and K) are positioned anterior left and viewed laterally whereas flat-mounted embryos (B, D, F, H, J and L) are positioned anterior left and viewed dorsally. The r1–r2, r3–r5 and r1–r6 lengths of the embryos overexpressing N-CoR (D) or treated with DEAB (F) are longer than those of the control embryos (B), respectively. The control MO microinjected embryos (H) have similar lengths of r1–r2, r3–r5 and r1–r6 to the control embryos (B). The lengths of r1–r2, r3–r5 and r1–r6 in the *n-cor* knocked down (J) or RA treated embryos (L) are shorter than those in the control embryos (B and H). MHB, midbrain–hindbrain boundary; r, rhombmere.

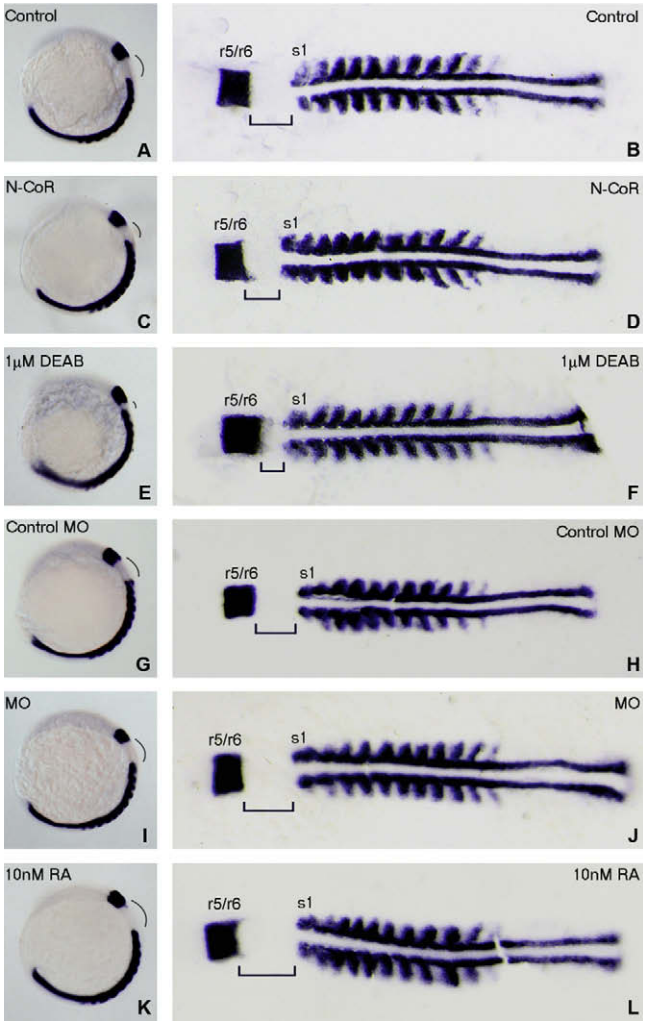

Fig. 5 – Alteration of N-CoR expression resulting in changes of hindbrain length between r6 and s1 similar to those changes caused by altering RA signaling in zebrafish embryos at 11-somites stage. Embryos at 1- to 2-cell stages are microinjected with N-CoR mRNA (C and D) or *n-cor* MO (I and J) to enhance or impair zebrafish N-CoR function, respectively. Alterations of RA signaling in embryos are performed by exogenous administration of 1 μM DEAB (E and F) or 10 nM RA (K and L) to reduce or enhance RA signaling, respectively. All treated embryos (C–F and I–L) together with wild-type control embryos (A and B) and control MO microinjected embryos (G and H) are grown to 11-somite stage and then hybridized by RNA probes of *val* (marking r5 and r6) and *myoD* (marking s1). Whole mounted embryos (A, C, E, G, I and K) are positioned anterior left and viewed laterally whereas flat-mounted embryos (B, D, F, H, J and L) are positioned anterior left and viewed dorsally. The lengths between r6 and s1 of the embryos overexpressing N-CoR (D) or treated with DEAB (F) are shorter than those of control embryos (B). The control MO microinjected embryo (H) has similar length between r6 and s1 to control embryos (B). The lengths between r6 and s1 of the *n-cor* knocked down (J) or RA treated embryos (L) are longer than those of the control embryos (B and H). r, rhombmere; s1, the first somite.

3. Discussion

Active repression of gene expression mediated by transcriptional repressors plays crucial roles in vertebrate embryogenesis (Niederreither and Dollé, 2008). Search for co-factors of repressors in inhibition of mouse gene transcription resulted in the discovery that N-CoR serves as a co-repressor by selectively interacting with DNA-bound RAR/RXR and thyroid hormone receptor (T_3R)/RXR heterodimers (Horlein et al., 1995) in the absence of their ligands, RA and T_3, respectively. N-CoR was then found to be conserved in other animals including human, rat and frog. In this study, we report molecular cloning of the zebrafish orthologue of N-CoR. The zebrafish N-CoR exhibits more than 56% amino acid identity with mammalian N-CoRs and the characteristic functional domains including three repression domains and two interaction domains are all well conserved. Phylogenetic analysis clusters zebrafish N-CoR into N-CoR family. BLASTing of the vertebrate genomes reveals that the *n-cor* genes in zebrafish, mouse and human are all linked with the *pigl* genes. The results suggest that *N-CoR* gene is highly conserved during evolution.

Like the expression of *N-CoR* in mouse embryos (Jepsen et al., 2000), the zebrafish *n-cor* mRNA is widely present in zebrafish embryos at early development (Bertrand et al., 2007). The wide distribution pattern of *n-cor* message implies that N-CoR plays critical roles in embryogenesis. Consistently, mouse with *n-cor* targeted deletion dies around E16, exhibiting developmental defects in central nervous system, erythrocyte and thymocyte (Jepsen et al., 2000). However, whether N-CoR affects the patterning of anterior–posterior axis of hindbrain remains unknown. In this study, we demonstrate that knocking down *n-cor* in zebrafish embryos results in a reduction of anterior hindbrain (r1–r6) and expansion of posterior most hindbrain (r7–r8), whereas overexpressing N-CoR results in expansion of anterior hindbrain and reduction of posterior most hindbrain. Our results reveal that N-CoR is required for patterning the anterior–posterior axis of zebrafish hindbrain.

It is well known that RA signaling plays critical roles in the patterning of anterior–posterior axis of vertebrate hindbrain (White and Schilling, 2008). The length of r1–r6 hindbrain in *aldh1a2* mutant zebrafish embryos is expanded while the distance between r5 and s1 (comprising r6, r7 and r8) is much shorter than that in wild-type embryos (Begemann et al., 2001; Grandel et al., 2002; Hernandez et al., 2007). DEAB treated embryos display similar phenotypes (Gu et al., 2006). On the other hand, *cyp26a1* null zebrafish embryos exhibit expanded rostral spinal cord territory at the expense of hindbrain region (Emoto et al., 2005). Knocking down all three *cyp26* genes results in expression of RA-responsive genes that are normally limited to the posterior hindbrain expanding abnormally to the entire hindbrain (Hernandez et al., 2007). Though *in vitro* data show that RAR interacts more strongly with SMRT than with N-CoR (Zamir et al., 1997), previous studies have demonstrated that N-CoR is involved in active repression of RA signaling *in vivo* (Jepsen et al., 2000; Weston et al., 2002). By knocking down *n-cor* in zebrafish embryos, we found that the expression of *cyp26a1* is up-regulated and that of *aldh1a2* is down-regulated. The result implicates that

N-CoR is involved in repressing RA signaling during zebrafish early development. Consistently, the developmental defects of the anterior–posterior hindbrain axis in the N-CoR overexpressed or N-CoR deficient embryos are similar to those in the embryos challenged with excess or deficiency of RA signaling, respectively. Taken together, our results demonstrate that N-CoR is required for patterning the anterior–posterior axis of zebrafish hindbrain by actively repressing retinoid signaling.

It is proposed that transcription repression by N-CoR is mediated by unliganded receptors such as TR/RXR and RAR/RXR. In the absence of RA, the RARE-bound RAR/RXR heterodimers recruit co-repressors, which then recruit HDAC to actively repress transcription. Binding of RA to the receptors leads to the release of the co-repressors and recruitment of co-activators to activate gene transcription (Niederreither and Dollé, 2008). Recently, multiple evidences have revealed that expressions of a number of target genes of RA signaling are de-repressed or activated when the function of either *N-CoR* or *SMRT* is blocked in the embryos (Jepsen et al., 2000, 2007; Koide et al., 2001; Weston et al., 2002). In this study, we report that the expressions of *cyp26a1* and *hoxb4a* are up-regulated in the N-CoR deficient embryos. These results suggest that ligand-independent RA-receptor activation occurs in the absence of N-CoR. However, whether the effect of knocking down N-CoR on transcription is ascribed to 'activation' by co-repressor-free, unliganded RAR, remains unclear. Previously, research in yeast revealed that TR constitutively activates transcription in the absence of T3 (Ohashi et al., 1991). Recent studies demonstrated that knocking down N-CoR has no effect on the targeting of TR, but rather results in the unliganded, co-repressor-free TR recruiting and interacting with a co-activator, p300, which leads to hyperacetylation of histone tails and transcriptional de-repression (Choi et al., 2008). The results suggest that unliganded RAR may use the similar mechanism to actively up-regulate transcription in the absence of co-repressors. Alternatively, the model of concentration-dependent competition between RA and N-CoR for access to RAR/RXR complex might explain the active transcription in the absence of the co-repressors.

Interestingly, our results show that N-CoR deficient embryos fail to show the phenotype of elevated *cyp26a1* expression in anterior spinal cord and somites whereas the expression of *cyp26a1* is strongly up-regulated in anterior dorsal spinal cord and somite in the dnN-CoR expressing embryos. Searching the zebrafish genome database (http://www.ncbi.nlm.nih.gov), we found that zebrafish has no duplicated 'b' copy for the *n-cor* gene in the genome but has SMRT, a close relative of N-CoR. Whole mount *in situ* hybridization analysis reveals that *smrt* message is present in posterior hindbrain, anterior spinal cord and somites during middle somitogenesis stage (Bertrand et al., 2007). Because previous studies have demonstrated that both SMRT and N-CoR can interact RAR/RXR through their interaction domains and that SMRT preferentially interacts with RAR (Horlein et al., 1995; Ordentlich et al., 1999; Park et al., 1999; Koide et al., 2001; Baniahmad, 2005; Jepsen et al., 2007), microinjected dnN-CoR in the embryos could compete with both endogenous N-CoR and SMRT for their binding to RAR/RXR receptors. Therefore, the phenotype resulting from expression of dnN-CoR could be due to functional disruption of both N-CoR and SMRT, and SMRT could have redundant function with N-CoR in repressing RA signaling during zebrafish early development.

4. Materials and methods

4.1. Cloning of zebrafish n-cor full-length cDNA

To clone the full-length zebrafish *n-cor* cDNA, we first used RACE (rapid amplification of cDNA ends) strategy to define the 5′- and 3′-ends of *n-cor* cDNA, respectively. BLASTing the zebrafish EST database (http://www.ncbi.nlm.nih.gov/) with human N-CoR (XM_037176) using TBLASTN tools, we identified a set of zebrafish ESTs that are candidate ESTs of zebrafish *n-cor*. To perform 5′-RACE, the 549 bp EST (AF154977), predicted to encode the N-terminal part of N-CoR, was selected to design the gene-specific primer (GSP) to amplify 5′-end of the gene. The GSP for 5′-RACE was 5′-TGCTCGACTCTTCTCTGGACTGCTCTGG-3′. To perform 3′-RACE, the 577 bp EST (BM035772), predicted to encode the C-terminal part of N-CoR, was selected to design the GSP to amplify 3′-end of the gene. The GSP for 3′-RACE was 5′-TGTCGCAAAGCAGAAGTCGGTAATGCTC-3′. RACE-PCR was conducted with SMART RACE cDNA Amplification Kit (Clontech, USA), using mRNA isolated from adult fish as template (Zhao et al., 2005).

The full-length coding sequence of zebrafish *n-cor* cDNA was obtained by end–end PCR with the high fidelity DNA polymerase (Clontech, USA), basing on the sequences obtained by 5′- and 3′-RACE. The sequences of primers were 5′-TCACAGCATGTCCAGTTCG-3′ (forward) and 5′- ACAGTGAAAGAAGGGCGATG-3′ (reverse). The conditions for the PCR were 94 °C for 1 min, 30 cycles of (94 °C for 30 s, 66 °C for 40 s and 68 °C for 6 min), followed by 68 °C for 6 min.

4.2. Whole mount in situ hybridizations and measurement of hindbrain length

Whole mount *in situ* hybridizations were performed as described previously (Zhao et al., 2005). The cDNA template for making antisense RNA probe of *n-cor* was a PCR amplified fragment, using primers of 5′-AGGTGATGAGGAAGAAGC-3′ (forward) and 5′-ATCTCCAGCTCCTCCTTC-3′ (reverse). The templates of other RNA probes including *aldh1a2* (Begemann et al., 2001), *cyp26a1* (Dobbs-McAuliffe et al., 2004), *engrailed2a* (Fjose et al., 1992), *krox20* (Oxtoby and Jowett, 1993), *hoxb4a* (Prince et al., 1998), *hoxb1b* and *otx2* (Kudoh et al., 2002), *myoD* (Weinberg et al., 1996) and *valentine* (Moens et al., 1998), were cloned as described in the literatures. The embryos were strictly staged as previously reported (Kimmel et al., 1995).

The lengths of hindbrain segments were measured in the flat-mounted embryos with the software UTHSCSA ImageTool (http://ddsdx.uthscsa.edu/dig/itdesc.html) and shown in mean ± standard derivation. The length unit was arbitrary. Results were subjected to Student's *t*-test.

4.3. In vitro synthesis of mRNA and microinjection of mRNAs into zebrafish embryos

To synthesize mRNA *in vitro*, full-length coding sequences of *n-cor* (EF016488) and dnN-CoR (cDNA was amplified with primers of 5′-GGAAGATCTACCACCATGGGAAAGACCACCATAT

CAGCT-3′ and 5′-CCGCTCGAGTCAGTCGTCGCTGTCTGAGA G-3′) were subcloned into pBluescript vector (Stratagene, USA) under T_7 promoter direction. *n-cor* mRNA was synthesized and capped *in vitro* using mMESSAGE mMACHINE Kit and tailed with Poly(A) Tailing Kit (Ambion, USA) while mRNA of dnN-CoR was synthesized, capped and tailed using mMESSAGE mMACHINE T7 Ultra Kit (Ambion, USA). About 2 nl of 0.25 μg/μl *n-cor* mRNA or dnN-CoR mRNA were microinjected into a zebrafish embryo at 1- to 2-cell stage to overexpress N-CoR or dnN-CoR, respectively. Embryos treated with 1 μM DEAB (4-diethylamino-benzaldehyde) or 10 nM RA (Sigma, USA) at 1- to 2-cell stages were used as controls of reduced or increased RA signaling.

4.4. *Morpholinos design and microinjection of morpholinos into zebrafish embryos*

Antisense morpholino (MO) of *n-cor* was designed by Gene Tools (http://www.gene-tools.com) against translation start site or 5′-UTR of zebrafish *n-cor* gene. The sequences of MOs were 5′-GACATGCTGTGACCTTTTGCTGGCC-3′ (MO1) and 5′-TGGAGCACAGCTACTCAATCACGGG-3′ (MO2), respectively. Control MO was the standard MO provided by Gene Tools. MOs were dissolved in nanopure water and approximately 1 nl of solution containing either 4 ng MO1 or 6 ng MO2 was injected into each embryo at 1- to 2-cell stage. Embryos treated with 10 nM RA (Sigma, USA) at 1- to 2-cell stages were used as controls of increased RA signaling.

The specificity and efficiency of MO2 on knocking down zebrafish *n-cor* were determined by *in vitro* transcription assay. We first made an expression construct by fusing the 5′-UTR of *n-cor* with the firefly luciferase reporter gene. We then placed the construct into cloning vector pBluescript SK (Stratagene, USA) under the control of T_7 promoter. The mRNA of the construct was then synthesized, capped and tailed using mMESSAGE mMACHINE T_7 Ultra Kit (Ambion, USA). One-hundred picograms mRNA plus 20 pg *Renilla* luciferase expression vector (inner control) and 6 ng MO2 or control MO were injected into each zebrafish embryo at 1- to 2-cell stage. Three pools of 15 injected embryos at the bud stage were collected to perform dual luciferase assay. Embryos were homogenized in 100 μl of 1× Passive Lysis Buffer (Promega, USA), followed by centrifugation at 12,000*g* for 10 min at room temperature to remove cell debris. The supernatant was used for dual luciferase activity assays according to the manufacturer's protocol (Promega, USA) and as previously described (Zhao et al., 2005). Experiments were repeated three times. Results were subjected to Student's *t*-test. The dual luciferase assay showed that MO2 blocks about 83% of the translation of the injected *n-cor*-luciferase mRNA. When microinjected into zebrafish embryos, *n-cor* MO1 (4 ng per embryo) gave the same phenotypes as *n-cor* MO2 (6 ng per embryo).

4.5. *DNA sequencing and construction of phylogenetic tree*

The coding sequences of the cDNAs were determined on both strands for accuracy. The sequence data were analyzed using the software Jellyfish (Zhao et al., 2005). The phylogenetic tree was constructed using Mega 4 (http://www.megasoftware.net/).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mod.2009.09.001.

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