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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