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Identification and characterization of a functional zebrafish smrt corepressor (ncor2)

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Abstract

The retinoic acid receptors (RARs or rars) and the thyroid hormone receptors are members of the steroid receptor superfamily that interact with their DNA response elements (for RARs: retinoic acid response elements or RAREs) in the regulatory regions of promoters in the absence of their ligand. In this ligand minus configuration, it has been suggested that the RAR provides a binding site for a corepressor (SMRT or N-CoR) that also brings in other proteins to repress the gene. In the presence of the ligand, the receptor goes through an allosteric change eliminating the corepressor binding site and providing a coactivator binding site. In this manuscript we describe the isolation of the zebrafish corepressor, smrt. We show that its association with the zebrafish rar aa is sensitive to retinoic acid and that the corepressor mRNA is present in 8 cell zebrafish embryos – a time at which the embryonic genome is not active. We suggest that this rar–corepressor complex may be part of an embryonic, epigenetic switch that keeps retinoic acid responsive genes off before retinoic becomes available to the embryo.

Keywords

Retinoic acid receptor; Thyroid hormone receptor; N-CoR

1. Introduction

The retinoic acid receptors (RARs) are members of the steroid superfamily of ligand-activated nuclear receptors. They belong to a subfamily of receptors (that includes RARs and the thyroid hormone receptors) that bind to their DNA response elements (retinoic acid response elements, RAREs for RARs) in the absence of their ligand. Through RAR heteromeric binding with a retinoid X receptor (RXR) on RAREs, the RAR provides a binding site for a corepressor (for reviews see Gronemeyer et al., 2004; Privalsky, 2004; Germain et al., 2006; Perissi and Rosenfeld, 2005; Perissi et al., 2010; Germain et al., 2009). The two characterized vertebrate retinoic acid receptor and thyroid hormone receptor binding corepressors are N-CoR and SMRT (sometimes named N-CoR2). In the absence of ligand, an extended beta-strand in the carboxyl region of the RAR forms an anti-parallel beta-sheet with specific regions in the corepressor. In the presence of ligand, a beta-strand to alpha-helix change occurs in the RAR that removes the corepressor from the receptor and provides a binding region for transcriptional coactivators (le Maire et al., 2010). During this process, protein or proteins associated with the corepressor or coactivator can functionally change the chromatin structure. The corepressor brings in a histone deacetylase that will

condense chromatin and when it leaves, as retinoic acid produces an allosteric change to the RAR, the coactivator brings in a histone transacetylase that can open up the chromatin. A model of these changes is illustrated in Fig. 1, however it should be noted that the corepressor has several different association sites for other proteins and has been isolated within a corepressor complex of many proteins (Li et al., 2000)(Guenther et al., 2000) so that this model is most likely a modest interpretation of what might be going on.

Historically, the SMRT co-repressor (silencing mediator for retinoic and thyroid) and N-CoR (for nuclear co-repressor) were isolated from mammalian cells (Chen and Evans, 1995) (Horlein et al., 1995). In many cases, the isolation of the genes or parts of genes of the N-CoR and SMRT corepressors involved the use of the yeast two-hybrid selection procedure for isolating proteins that interact with each other—using a part of the receptor as “bait” and then selecting in yeast for cDNA products that interacted with the “bait”. Further characterization of the proteins indicated that these co-repressors were large, multi-domain proteins with binding affinities for several other proteins. In this study, we used this approach for isolating the zebrafish SMRT corepressor (smrt).

The discovery of repressor molecules that interact with unliganded retinoid receptors expanded the role of retinoid receptors in regulating gene expression during embryogenesis. Historically, deprivation of embryos from retinoids or exposing them to vitamin A (retinol) was shown to create a wide variety of teratological complications. In 1967 it was shown that the acid form of vitamin A, retinoic acid, was a much more potent teratogen than vitamin A (retinol) (Kocher, 1967). Developmental studies have shown that genes involved in producing retinoic acid from retinol (vitamin A) or beta-carotene and genes involved in oxidizing retinoic acid to a compound that does not effectively activate RARs (the cyp26s) are carefully controlled during development to portion out retinoic acid at different developmental times and anatomical locations to produce a normal embryo (Abu-Abed et al., 2001) (Blentic et al., 2003) (Dobbs-McAuliffe et al., 2004) (Haselbeck et al., 1999) (Hernandez et al., 2007) (Hu et al., 2008) (Nelson, 1999) (Niederreither et al., 2002) (Reijntjes et al., 2004) (Sakai et al., 2001) (White et al., 1996) (White et al., 2007) (Zhao et al., 2005) (Grandel et al., 2002) (Begemann et al., 2001).

Therefore, an explanation for the apparent contradiction of both the necessity for retinoic acid in the normal embryo and its strong teratological potential needed to be reconciled. The RAR co-repressors help to at least partially explain this. With their presence in the very early embryo, development in the vertebrate starts off with that subset of genes with RARE regulatory elements being repressed through the RAR-corepressor-HDAC mechanism and presumably remaining repressed until cells access retinoic acid. An attempt to visualize and identify those developmental windows and anatomical regions that have access to retinoic acid was made through the generation of retinoic acid indicator transgenic mice by this laboratory and others (Rossant et al., 1991) (Reynolds et al., 1991) (Mendelsohn et al., 1991) (Balkan et al., 1992a) and replicated in zebrafish by this laboratory (Perz-Edwards et al., 2001). Ectopic exposure of the early embryo to retinoic acid would then release genes from repression that were not meant to be expressed creating considerable developmental chaos.

With the recent isolation and description of the zebrafish N-CoR corepressor (ncor) (Xu et al., 2009), our functional isolation and description of the zebrafish SMRT (smrt) corepressor allows one to fully examine the role of these important regulatory cofactors in the early zebrafish embryo.

2. Material and methods

2.1. Fish care

Our care and breeding of zebrafish was performed under approved IACUC protocols.

2.2. cDNA cloning

The original smrt cDNA clone, selected via interaction with the DEF domain of rar aa via the two-hybrid Clontech Matchmaker system was from an accompanying Clontech zebrafish cDNA library from 28 day old zebrafish. This cDNA was used to screen a phage cDNA library to obtain sequences 5' to the original clone. 5'-RACE was performed on cDNA from adult female fish. The composite cDNA is represented on the UCSC browser as 45 exons covering a genomic region of over 197,000 bp.

2.3. DNA sequencing

DNA sequencing was performed by the Duke Comprehensive Cancer Center DNA Sequencing facility.

2.4. Yeast two-hybrid procedures

The initial, partial, smrt cDNA was isolated using the Clontech MATCHMAKER Two-hybrid system. With the additional genome duplication that occurred with teleosts, some genes in zebrafish remain duplicated. For zebrafish retinoic acid receptors, there are two retinoic acid receptor alphas: rar aa and rar ab; two retinoic acid receptor gammas: rar ga and rar gb; and no retinoic acid receptor betas. We cloned the z rar aa DEF cDNA domain region in fusion with the yeast gal4 DNA binding domain vector, pGBT9 as “bait” and screened a Clontech two-hybrid zebrafish cDNA library for a rar interaction with a cDNA that was cloned in frame with the gal4 activation domain. After the isolation of the full cDNA clone of smrt, clones were made with different regions of smrt for studies on interaction and ligand susceptibility (Fig. 3). To examine the ability of smrt to interact with the zebrafish thyroid hormone alpha-A1 receptor (Essner et al., 1997), its DEF domain region was cloned into the pAS-2 two-hybrid gal4 DNA binding domain vector. These interaction studies were measured through the production of two-hybrid complexes activating beta-galactosidase activity.

The *Saccharomyces cerevisiae* strain, SFY526 (genotype: MATa, ura3-52, his 3-200, ade 2-101, lys 2-101, trp 1-901, leu 2-3, 112, can^r, gal4-542, gal80-538, URA::GAL1-lacZ) was used as the reporter host using selection procedures provided by the Clontech MATCHMAKER instructions. Transformations, beta-galactosidase activity measurements and other experiments were performed as per the Matchmaker instructions.

2.5. In situ hybridization

8 cell zebrafish embryos were fixed and processed in accordance with standard procedures (Thisse and Thisse, 2008). Anti-sense transcripts with digoxigenin labeled UTP were hybridized to the embryos and alkaline phosphatase labeled anti-digoxigenin Fab fragments were adsorbed to embryos, washed and reacted with BM Purple (Roche) substrate. Confirmation of the presence of z smrt and z ncor RNA in 8 cell embryos was made through qRT-PCR identification of their presence.

3. Results

3.1. cDNA size, structure, alignment with Ensembl assembly

Knowing the co-repressor interaction domain with the RAR to be within the carboxyl half of the RAR for identified mammalian RAR/TR corepressors, and because of reports by others suggesting that the mammalian RAR- α interacted most effectively with the SMRT corepressor (Farboud et al., 2003), the zebrafish rar aa (GenBank ID: NM_131406) DEF domain was used as “bait” in the yeast two hybrid selection procedure. This allowed us to isolate a clone coding for a region of smrt that interacted with the DEF rar aa expression clone. From this clone a series of 3 additional steps that included screening a phage cDNA library and 5'-RACE allowed us to reconstruct a 9958 base pair cDNA (with 22 bp of polyA) coding for 2262 amino acids and including a 651 base 5' untranslated region and a 2522 base untranslated 3' region. The conceptual amino acid sequence is presented in Fig. 2 (GenBank ID: JF828767). There is an algorithm predicted smrt (named ncor2, 8447 bp, GenBank ID: XM_68906) in the Sanger zebrafish assembly most of whose exons do cover part of what we have mapped as smrt (ncor2). The smrt cDNA that we reconstructed is 9958 bp in length. Alignment on the UCSC browser of the zebrafish assembly indicated concurrence with 45 exons, and 9936 bp of genomic sequence suggested a polyA tail of 22 bp. The exons for the smrt cDNA cover over 197,000 bp on chromosome 8 of the assembly. Amino acid homologies of z smrt to mouse and human N-CoR and SMRT are consistent with z smrt being a SMRT corepressor (i.e. comparison of domains of z smrt with those of hSMRT and hN-CoR show a higher homology of z smrt to hSMRT—a specific comparison of the receptor interaction domains are discussed in Section 3.3). A zebrafish ncor has been mapped to chromosome 5 (Xu et al., 2009).

3.2. Binding to z raraa and z thr alpha a1

To examine the functionality of the protein encoded by the cDNA, we tested the ligand sensitivity of the carboxyl end region of the z smrt using the yeast two-hybrid technology. Based upon the mammalian model it would be predicted that in the presence of ligand the SMRT corepressor would dissociate from the RAR or the thyroid hormone receptor (TR). Association with either “bait” construct would recreate a gal4 transcription factor within a yeast strain whose β -galactosidase could be activated by gal4. By measuring beta-galactosidase, one could measure the functional interaction between the two proteins. In Fig. 3A we illustrate the subclone of smrt (delta smrt) that we introduced into yeast as gal4 activation domain fusion plasmids. These were co-introduced with either a DEF rar aa or a DEF TR gal 4 DNA binding domain fusion proteins. Therefore, the production of beta-galactosidase would indicate the association of the DEF domains with the respective Smrt clones. In Fig. 3B it is shown that smrt interacts with both of the receptor clones to produce β -galactosidase activity and their respective associations were ligand specific to each receptor (retinoic acid and triac, a synthetic thyroid hormone). Within the context of this assay, the disassociation of the rar aa DEF to the smrt clone is more ligand sensitive. As one can see there is also a dose dependency, though this may be a function of several factors, including permeability of the respective ligands into the yeast cells or a difference in the dissociation effect that might be present if the natural rxr heterodimer partner is present under normal conditions.

3.3. Homology of the two zebrafish corepressor association receptor association sites

There is a difference in the number of corepressor receptor association sites (*CoRNRs*, *CoRepressor Nuclear Receptor boxes*); in the mammalian N-CoR versus SMRT, with N-CoR having 3 and SMRT having 2. There are also studies that suggest the binding of SMRT to RAR α is stronger than to thyroid hormone receptors. With the zebrafish ncor corepressor isolated and identified by others (Xu et al., 2009), we compared the homologies

of the regions of the two common human Ncor and Smrt *CoRNRs* (*CoRepressor Nuclear Receptor* boxes) with the zebrafish ncor and smrt *CoRNRs* in Fig. 4. This figure is modeled after the results of le Maire et al., 2010 where they identify the β 1 helix (see shaded area) in CoRNRI as being important for RAR binding to SMRT (see shaded area in figure), its absence in CoRNRII and the differences in the α 1 helices of the CoRNRI and the CoRNRII (bold and underlined). One can see the conservation of these sequences between human SMRT and zebrafish smrt.

3.4. Presence of the corepressors in the embryo before the genome is transcriptionally active

The importance of the RAR-corepressor complex to the repression of genes in the early embryo is implicated by the presence of both corepressors at the 8 cell stage of the embryo. While the developmental expression of these genes is described in the supplementary material of Bertrand et al.(2007), their localization before the embryonic genome turns on was not presented. The developmental expression of zebrafish ncor has also been described in the supplementary material of Xu et al.(2009). In Fig. 5 we illustrate the presence of two zebrafish corepressor mRNAs in the 8 cell embryo suggesting that this repressive mechanism is available at this early time. Independent and additional studies of ours show that rar aa, rar ab, hdac3, rxr ab, and rxr ga are present in 8 cell embryos and oocytes suggesting that the basic components of a repression complex are present before the embryonic genome is turned on.

4. Discussion

In this study we have isolated and characterized the “large form” of zebrafish smrt. Its sequences match some of the sequences of a smaller, algorithm derived z smrt sequence but exceed it in size. Its sequence is also consistent with the genomic assembly of the zebrafish genome. Functionally, both by two-hybrid selection of the original 3' DEF region of the rar aa cDNA as “bait” in our initial isolation of a smrt cDNA and through interactive experiments in yeast with 3' regions of the zebrafish retinoic acid aa and thyroid hormone alpha a1 receptor regions, we have shown that the corepressor–receptor interaction is ligand sensitive (Figs. 3A and B), further confirming its identity and consistent with its predicted function. In situ localization of both zebrafish smrt and zebrafish ncor in 8 cell embryos (Fig. 5), at a time when the embryonic genome is still inactive, suggests that this corepressive function may play a role in keeping a subset of genes repressed at fertilization and until retinoic acid becomes selectively available to specific target tissue.

There has been a tendency for gene regulation studies to focus upon the activation of genes, however, in multicellular organisms it is clear that each cell type might have larger clusters of genes that are being repressed during development. The restriction of expression of certain genes is necessary for the many decisions that are made during cellular diversification and organogenesis. As a laboratory with extensive experience in both producing and studying transgenic lines of vertebrates (Balkan et al., 1992a) (Balkan et al., 1992b) (LaMantia et al., 1993) and zebrafish (Linney et al., 1999) (Linney and Udvadia, 2004) (Hu et al., 2008) (Lassiter and Linney, 2007) (Perz-Edwards et al., 2001) (Udvadia and Linney, 2003) we are interested not only what sequences drive specific expression for differential gene expression, but what sequences and mechanisms keep a regulatory region from being expressed in different cell types. Attention to the issue of repression has grown and as the area of stem cell biology grows, what and how genes are repressed to maintain the pluripotent phenotype of the embryonic stem cell is important to both maintaining and unleashing its potential. There is a history of stem cell responsiveness to retinoic acid. The conceptual precursors to mouse embryonic stem cells (ES cells), embryonal carcinoma cells (EC cells), were isolated from normally occurring tumors that had differentiated

components. As the stem cell of these tumors, different EC cell lines were isolated. At least two of the mouse EC lines themselves could be induced to differentiate via retinoic acid exposure: F9 EC cells (Strickland and Mahdavi, 1978) and P19 EC cells (McBurney et al., 1982). Therefore, identifying and examining how corepressors are organized and function is of growing concern.

A knockout of the mouse SMRT gene implicates it in the development of the heart and in neuronal function (Jepsen et al., 2007) including the derepression of the *jmjD3* demethylase gene that can play a role in epigenetic processes. However, the interaction of either SMRT or N-CoR is not unique to either the RARs or the THR and there are associated sites for several other proteins that can assemble as a repression complex (Li et al., 2000)(Guenther et al., 2000). Therefore any knockout or knockdown of one or both corepressors could produce phenotypes independent of their interaction with RAR or THR.

Our future interest in this gene focuses upon its potential role in an early, embryonic epigenetic switch involving the zebrafish *rar* alpha gene products. We suggest that a corepressive complex is present at fertilization and represses a subset of important genes, and that these genes are derepressed by the precise allocation of retinoic acid through retinaldehyde dehydrogenases and the limitation or restriction of retinoic acid distribution by the precise expression of the *cyp26a1*, *cyp26b1* and *cyp26c1* genes that oxidize retinoic acid to product that cannot “throw this epigenetic switch” by interacting with the *rar* so that the corepressor–histone deacetylase complex leaves the *rar/rxr* heterodimer on the RAREs of selective genes. Through these selective interactions we suggest that cascades of gene expression and repression are initiated and associated with at least some of the steps involved in cellular phenotype diversification.

While we are in the process of using both in situ hybridization, and qRT-PCR to examine the presence of many retinoic acid related genes at fertilization and during early development, the absence of a complete array of techniques or reagents (e.g. antibodies) to identify the presence of the functional proteins will require additional approaches. These will include transgenic lines that are retinoic acid responsive early in development (Hu et al., 2008) and modified, constitutively active retinoic acid receptors (Balkan et al., 1992b) (Cash et al., 1997) that we previously have described. While this epigenetic switch is not a new thought (Weston et al., 2003), the system may be a paradigm for a reversible mechanism for what has been described as the bookmarking of the genome (Zaidi et al., 2010), at least for cells that still have mitotic potential. This could explain the apparent paradox of retinoic acid being both an essential compound for normal development and a potent teratogen—through indiscriminate ectopic exposure of retinoic acid, switches are thrown in cells that are not meant to see retinoic acid.

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Abbreviations

SMRT	smrt-silencing mediator for retinoic acid and thyroid hormone receptors
N-CoR	ncor-nuclear corepressor
RAR	rar-retinoic acid receptor
THR	thr-thyroid hormone receptor

References

- Abu-Abed S, Dolle P, Metzger D, Beckett B, Chambon P, Petkovich M. Theretinoic acid-metabolizing enzyme, CYP26A1, is essential for normal hindbrain patterning, vertebral identity, and development of posterior structures. *Genes Dev.* 2001; 15:226–240. [PubMed: 11157778]
- Balkan W, Colbert M, Bock C, Linney E. Transgenic indicator mice for studying activated retinoic acid receptors during development. *Proc. Natl Acad. Sci. USA.* 1992a; 89:3347–3351. [PubMed: 1314386]
- Balkan W, Klintworth GK, Bock CB, Linney E. Transgenic mice expressing a constitutively active retinoic acid receptor in the lens exhibit ocular defects. *Dev. Biol.* 1992b; 151:622–625. [PubMed: 1318236]
- Begemann G, Schilling TF, Rauch GJ, Geisler R, Ingham PW. The zebrafish neckless mutation reveals a requirement for *raldh2* in mesodermal signals that pattern the hindbrain. *Development.* 2001; 128:3081–3094. [PubMed: 11688558]
- Bertrand S, et al. Unexpected novel relational links uncovered by extensive developmental profiling of nuclear receptor expression. *PLoS Genet.* 2007; 3:e188. [PubMed: 17997606]
- Blentic A, Gale E, Maden M. Retinoic acid signalling centres in the avian embryo identified by sites of expression of synthesising and catabolising enzymes. *Dev. Dyn.* 2003; 227:114–127. [PubMed: 12701104]
- Cash DE, Bock CB, Schughart K, Linney E, Underhill TM. Retinoic acid receptor alpha function in vertebrate limb skeletogenesis: a modulator of chondrogenesis. *J. Cell Biol.* 1997; 136:445–457. [PubMed: 9015314]
- Chen JD, Evans RM. A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature.* 1995; 377:454–457. [PubMed: 7566127]
- Dobbs-McAuliffe B, Zhao Q, Linney E. Feedback mechanisms regulate retinoic acid production and degradation in the zebrafish embryo. *Mech. Dev.* 2004; 121:339–350. [PubMed: 15110044]
- Essner JJ, Breuer JJ, Essner RD, Fahrenkrug SC, Hackett PB Jr. The zebrafish thyroid hormone receptor alpha 1 is expressed during early embryogenesis and can function in transcriptional repression. *Differentiation.* 1997; 62:107–117. [PubMed: 9447705]
- Farboud B, Hauksdottir H, Wu Y, Privalsky ML. Isotype-restricted corepressor recruitment: a constitutively closed helix 12 conformation in retinoic acid receptors beta and gamma interferes with corepressor recruitment and prevents transcriptional repression. *Mol. Cell. Biol.* 2003; 23:2844–2858. [PubMed: 12665583]
- Germain P, et al. International Union of Pharmacology. LXIII. Retinoid X receptors. *Pharmacol. Rev.* 2006; 58:760–772. [PubMed: 17132853]
- Germain P, et al. Differential action on coregulator interaction defines inverse retinoid agonists and neutral antagonists. *Chem. Biol.* 2009; 16:479–489. [PubMed: 19477412]
- Grandel H, et al. Retinoic acid signalling in the zebrafish embryo is necessary during pre-segmentation stages to pattern the anterior–posterior axis of the CNS and to induce a pectoral fin bud. *Development.* 2002; 129:2851–2865. [PubMed: 12050134]
- Gronemeyer H, Gustafsson JA, Laudet V. Principles for modulation of the nuclear receptor superfamily. *Nat. Rev. Drug Discov.* 2004; 3:950–964. [PubMed: 15520817]
- Guenther MG, Lane WS, Fischle W, Verdin E, Lazar MA, Shiekhhattar R. A core SMRT corepressor complex containing HDAC3 and TBL1, a WD40-repeat protein linked to deafness. *Genes Dev.* 2000; 14:1048–1057. [PubMed: 10809664]
- Haselbeck RJ, Hoffmann I, Duester G. Distinct functions for *Aldh1* and *Raldh2* in the control of ligand production for embryonic retinoid signaling pathways. *Dev. Genet.* 1999; 25:353–364. [PubMed: 10570467]
- Hernandez RE, Putzke AP, Myers JP, Margaretha L, Moens CB. Cyp26 enzymes generate the retinoic acid response pattern necessary for hindbrain development. *Development.* 2007; 134:177–187. [PubMed: 17164423]
- Horlein AJ, et al. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature.* 1995; 377:397–404. [PubMed: 7566114]

- Hu P, et al. Retinoid regulation of the zebrafish *cyp26a1* promoter. *Dev. Dyn.* 2008; 237:3798–3808. [PubMed: 19035346]
- Jepsen K, et al. SMRT-mediated repression of an H3K27 demethylase in progression from neural stem cell to neuron. *Nature.* 2007; 450:415–419. [PubMed: 17928865]
- Kochar D. Teratogenic activity of retinoic acid. *Acta Pathol. Microbiol. Scand.* 1967; 70:398–404. [PubMed: 4867280]
- LaMantia AS, Colbert MC, Linney E. Retinoic acid induction and regional differentiation prefigure olfactory pathway formation in the mammalian forebrain. *Neuron.* 1993; 10:1035–1048. [PubMed: 8318228]
- Lassiter CS, Linney E. Embryonic expression and steroid regulation of brain aromatase *cyp19a1b* in zebrafish (*Danio rerio*). *Zebrafish.* 2007; 4:49–57. [PubMed: 18041942]
- le Maire A, et al. A unique secondary-structure switch controls constitutive gene repression by retinoic acid receptor. *Nat. Struct. Mol. Biol.* 2010; 17:801–807. [PubMed: 20543827]
- Li J, Wang J, Nawaz Z, Liu JM, Qin J, Wong J. Both corepressor proteins SMRT and N-CoR exist in large protein complexes containing HDAC3. *EMBO J.* 2000; 19:4342–4350. [PubMed: 10944117]
- Linney E, Udvadia AJ. Construction and detection of fluorescent, germline transgenic zebrafish. *Meth. Mol. Biol.* 2004; 254:271–288.
- Linney E, Hardison NL, Lonze BE, Lyons S, DiNapoli L. Transgene expression in zebrafish: a comparison of retroviral-vector and DNA-injection approaches. *Dev. Biol.* 1999; 213:207–216. [PubMed: 10452858]
- McBurney MW, Jones-Villeneuve EM, Edwards MK, Anderson PJ. Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line. *Nature.* 1982; 299:165–167. [PubMed: 7110336]
- Mendelsohn C, Ruberte E, LeMeur M, Morriss-Kay G, Chambon P. Developmental analysis of the retinoic acid-inducible RAR-beta 2 promoter in transgenic animals. *Development.* 1991; 113:723–734. [PubMed: 1668276]
- Nelson DR. A second CYP26 P450 in humans and zebrafish: CYP26B1. *Arch. Biochem. Biophys.* 1999; 371:345–347. [PubMed: 10545224]
- Niederreither K, Abu-Abed S, Schuhbaur B, Petkovich M, Chambon P, Dolle P. Genetic evidence that oxidative derivatives of retinoic acid are not involved in retinoid signaling during mouse development. *Nat. Genet.* 2002; 31:84–88. [PubMed: 11953746]
- Perissi V, Rosenfeld MG. Controlling nuclear receptors: the circular logic of cofactor cycles. *Nat. Rev. Mol. Cell Biol.* 2005; 6:542–554. [PubMed: 15957004]
- Perissi V, Jepsen K, Glass CK, Rosenfeld MG. Deconstructing repression: evolving models of co-repressor action. *Nat. Rev. Genet.* 2010; 11:109–123. [PubMed: 20084085]
- Perz-Edwards A, Hardison NL, Linney E. Retinoic acid-mediated gene expression in transgenic reporter zebrafish. *Dev. Biol.* 2001; 229:89–101. [PubMed: 11133156]
- Privalsky ML. The role of corepressors in transcriptional regulation by nuclear hormone receptors. *Annu. Rev. Physiol.* 2004; 66:315–360. [PubMed: 14977406]
- Reijntjes S, Gale E, Maden M. Generating gradients of retinoic acid in the chick embryo: Cyp26C1 expression and a comparative analysis of the Cyp26 enzymes. *Dev. Dyn.* 2004; 230:509–517. [PubMed: 15188435]
- Reynolds K, Mezey E, Zimmer A. Activity of the beta-retinoic acid receptor promoter in transgenic mice. *Mech. Dev.* 1991; 36:15–29. [PubMed: 1664231]
- Rossant J, Zirngibl R, Cado D, Shago M, Giguere V. Expression of a retinoic acid response element-hsplaZ transgene defines specific domains of transcriptional activity during mouse embryogenesis. *Genes Dev.* 1991; 5:1333–1344. [PubMed: 1907940]
- Sakai Y, et al. The retinoic acid-inactivating enzyme CYP26 is essential for establishing an uneven distribution of retinoic acid along the antero-posterior axis within the mouse embryo. *Genes Dev.* 2001; 15:213–225. [PubMed: 11157777]
- Strickland S, Mahdavi V. The induction of differentiation in teratocarcinoma stem cells by retinoic acid. *Cell.* 1978; 15:393–403. [PubMed: 214238]

- Thisse C, Thisse B. High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nat. Protoc.* 2008; 3:59–69. [PubMed: 18193022]
- Udvadia AJ, Linney E. Windows into development: historic, current, and future perspectives on transgenic zebrafish. *Dev. Biol.* 2003; 256:1–17. [PubMed: 12654288]
- Waxman JS, Yelon D. Comparison of the expression patterns of newly identified zebrafish retinoic acid and retinoid X receptors. *Dev. Dyn.* 2007; 236:587–595. [PubMed: 17195188]
- Weston AD, Blumberg B, Underhill TM. Active repression by unliganded retinoid receptors in development: less is sometimes more. *J. Cell Biol.* 2003; 161:223–228. [PubMed: 12719467]
- White JA, et al. Identification of the retinoic acid-inducible all-trans-retinoic acid 4-hydroxylase. *J. Biol. Chem.* 1996; 271:29922–29927. [PubMed: 8939936]
- White RJ, Nie Q, Lander AD, Schilling TF. Complex regulation of *cyp26a1* creates a robust retinoic acid gradient in the zebrafish embryo. *PLoS Biol.* 2007; 5:e304. [PubMed: 18031199]
- Xu F, et al. N-CoR is required for patterning the anterior–posterior axis of zebrafish hindbrain by actively repressing retinoid signaling. *Mech. Dev.* 2009; 126:771–780. [PubMed: 19735730]
- Zaidi SK, et al. Mitotic bookmarking of genes: a novel dimension to epigenetic control. *Nat. Rev. Genet.* 2010; 11:583–589. [PubMed: 20628351]
- Zhao Q, Dobbs-McAuliffe B, Linney E. Expression of *cyp26b1* during zebrafish early development. *Gene Expr. Patterns.* 2005; 5:363–369. [PubMed: 15661642]

Fig. 1.

Repressive mechanism for regulatory elements that RAR/RXR bind to in the promoters of genes. The RAR/RXR heterodimer binds to the RARE in the regulatory region of a gene in the absence of ligand. A binding site for the SMRT or N-CoR is provided by the RAR receptor and the corepressor associates with a histone deacetylase to aid in the condensation of the chromatin—(left) the OFF position in the absence of the retinoic acid ligand. In the present of retinoic acid, the binding site for the corepressor is eliminated through an allosteric change in the RAR that now provides a binding site for a transcriptional coactivator and an accompanying histone acetyl transferase (right)—the ON position of the switch.

Fig. 2.
(Upper figure) The exon arrangement of the zebrafish smrt on zebrafish maps to chromosome 8 as identified on the UCSC browser of the zebrafish genomic assembly. (Middle) The mRNA organization of the zebrafish smrt with the box region being the coding region of the mRNA. (Bottom) The conceptual amino acid sequence of zebrafish smrt. GenBank ID: JF828767.

Fig. 3.

Domain regions and ligand dissociation effects of the zebrafish smrt protein. A) Region (delta smrt) of zebrafish smrt that was assayed in yeast for interaction capability with the retinoic acid receptor and thyroid hormone receptor. Because mammalian SMRT has been shown to associate with both RAR alpha and with thyroid hormone receptors, constructs having the nuclear localization domain, ligand binding domain and corepressor/coactivator association sites (the DEF domains of the retinoic acid receptor, RAR, and the thyroid hormone receptor, TR) were constructed and independently introduced into yeast cells containing the receptor interactive domains of zebrafish smrt. Interactions with either the DEF domain region of zebrafish rar aa or the DEF domain region of the thyroid hormone alpha a1. B) Delta smrt was transfected alone, or in combination with DEF rar aa or DEF tralpha a1 (a zebrafish thyroid hormone receptor), to measure interactivity of smrt with either of the receptors. Axis to left is normalized beta-galactosidase activity that was activated through association. Ethanol (0.1%) was used as a control because this level of ethanol was introduced with retinoic acid and triac exposures. The activity shows that smrt interacts with both of the receptors in the absence of their ligands. With increasing concentration of either ligand (retinoic acid for rar and triac for tr) the interaction decreases with concomitant decrease in β -galactosidase activity. No significant activity could be detected in yeast containing either the smrt fragment alone or either receptor fragment alone.

RAR interaction domains of human and zebrafish smrt for CoRNRI

		helix b1	helix a1
human SMRT	CoRNRI	RVVT	<u>LAQHISEVITODY</u> TRHHPO
zebrafish smrt	CoRNRI	RVVT	<u>LAQHISEVITKDY</u> TRQSQQ
human N-CoR	CoRNRI	RLIT	<u>LADHICQIITODF</u> ARNQVS
zebrafish ncor	CoRNRI	RIMT	<u>LADHISHIITODF</u> AKSQDT
human SMRT	CoRNRII	EHAS	TNMG <u>LEAIIIRKAL</u> MGKYDQ
zebrafish smrt	CoRNRII	EASG	NTIG <u>LEAIIIRKAL</u> MGKYDE
human N-CoR	CoRNRII	ADPA	SNLG <u>LEDIIRKAL</u> MGSFDD
zebrafish ncor	CoRNRII	SDPA	SNLG <u>LEDIIRKAL</u> MGNMEE

Fig. 4.

RAR interaction domain homologies. The subdomains of the receptor interaction domains or CoRNRI of the z smrt and z ncor show considerable homology with the human subdomains, consistent with the results in Fig. 3. z smrt comparisons based upon information for human N-CoR and human SMRT indicated in le Maire et al.(2010). The zebrafish ncor sequence is based upon Xu et al.(2009).

Fig. 5.

In situ hybridization of zebrafish corepressors in 8 cell embryos. To the left is expression of smrt and to the right is expression of ncor. This is at a time before the embryonic genome is turned on. Similar results for rar aa, rar ab, hdac3, rxr ga and rxr ab for 8 cell embryos and oocytes indicating that these necessary components for repression are present at fertilization. These are consistent with results of others identifying early embryonic expression of these genes (Bertrand et al., 2007) (Xu et al., 2009) (Waxman and Yelon, 2007). Using quantitative RT-PCR we have confirmed the presence of z smrt and z ncor in cDNAs from four separate 1–8 cell RNA extractions.