

# Active repression by unliganded retinoid receptors in development: less is sometimes more

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**The retinoid receptors have major roles throughout development, even in the absence of ligand. Here, we summarize an emerging theme whereby gene repression, mediated by unliganded retinoid receptors, can dictate cell fate. In addition to activating transcription, retinoid receptors actively repress gene transcription by recruiting cofactors that promote chromatin compaction. Two developmental processes for which gene silencing by the retinoid receptors is essential are head formation in *Xenopus* and skeletal development in the mouse. Inappropriate repression, by oncogenic retinoic acid (RA)\* receptor (RAR) fusion proteins, blocks myeloid differentiation leading to a rare form of leukemia. Our current understanding of the developmental role of retinoid repression and future perspectives in this field are discussed.**

The importance of retinoids and their receptors in development has been recognized for years and is the topic of a number of comprehensive reviews (Mark et al., 1999; Sucov and Evans, 1995; Wolf, 1984; Zile, 1998, 2001). Much less dated is our knowledge of the receptors as factors that mediate chromatin compaction and decompaction (for review see Glass and Rosenfeld, 2000). This, combined with the realization that chromosomal architecture and stability profoundly influence cell differentiation, has intensified interest in the developmental role of these and other nuclear receptors. Although traditionally thought of solely as ligand transducers, unliganded retinoid receptors are emerging as major players in many

cellular processes, through their role as gene repressors. Here, we concentrate on repression by unliganded retinoid receptors as a mechanism underlying important embryonic events.

## The retinoid receptors: recruiters of chromatin remodeling factors

The retinoids include natural forms of vitamin A such as retinol, and its derivatives such as retinal acid and RA. RA is the natural ligand for a class of nuclear receptors comprised of two subfamilies: the RARs and the retinoid X receptors (RXRs), each containing three members:  $\alpha$ ,  $\beta$ , and  $\gamma$  (Leid et al., 1992). Several isoforms, with distinct amino termini, expression patterns, and functional properties, have also been identified. The pleiotropic effects of retinoids are mediated through RAR-RXR heterodimers or RXR homodimers bound to bipartite response elements (RAREs) typically located upstream of target genes.

Like other nuclear receptors, RAR and RXR dimers modulate transcription by recruiting coactivators and corepressors. When ligand is present, conformational changes in the receptor reorient the ligand-binding domain, exposing a binding site for coactivators. These coactivators, including CBP/p300, p300/CBP-associated factor, and p160 family members (such as RIP140, and SRC), interact with the receptors through a motif containing the sequence LXXLL. In turn, they recruit proteins, many of which possess histone acetyltransferase (HAT) activity. In the absence of ligand, RARs bind to DNA and interact with corepressors such as nuclear receptor corepressor (NCoR)-1 and NCoR-2/SMRT (silencing mediator of RAR and thyroid hormone receptor) (Chen et al., 1996; Horlein et al., 1995), also through LXXLL motifs. NCoRs are among the factors that recruit mSin3A and its associated histone deacetylases (HDACs), although both corepressors can also interact directly with HDACs in an mSin3A-independent manner (Huang et al., 2000). Acetylation of the lysine residues of histone tails promotes a relaxed chromatin conformation that enables transcriptional activity, whereas histone deacetylation reverses these effects, maintaining chromatin in the condensed state that typifies transcriptional repression.

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\*Abbreviations used in this paper: APL, acute promyelocytic leukemia; dnRAR $\alpha$ , dominant-negative version of the RAR $\alpha$ ; E, embryonic age; HDAC, histone deacetylase; LCoR, ligand-dependent corepressor; NCoR, nuclear receptor corepressor; RA, retinoic acid; RAR, RA receptor; RARE, RXR homodimer bound to bipartite response element; RXR, retinoid X receptor; TSA, trichostatin A; CYP26, cytochrome p450, 26; TR, thyroid hormone receptor.

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Although the retinoid receptors are typically thought to interact with corepressors primarily in the absence of ligand, there are exceptions. RAREs that are spaced 1 bp apart bind RAR/RXR heterodimers in a polarity that is opposite to those separated by 5 bp, resulting in decreased ability of NCoR to dissociate from receptors upon ligand binding (Kurokawa et al., 1995). Surprisingly, at these response elements, NCoR binding in the presence of ligand results in gene activation through recruitment of HDAC3 (Jepsen et al., 2000). Therefore, NCoR or HDAC recruitment does not necessarily imply repression, and is not limited to unliganded receptors, rather the role of NCoR recruitment to the retinoid receptors may be encrypted in the response elements within promoters. The importance of RARE configuration in dictating receptor action is further revealed by the identification of a completely novel type of response element composed of two half-binding sites separated by 87 nucleotides. Surprisingly, this RARE seems to be a target of an unliganded phosphorylated RAR $\alpha$  homodimer, never described previously (Brondani et al., 2002). As researchers continue to characterize the response elements, more complex roles for these receptors will likely be uncovered. Finally, the recent discovery of a ligand-dependent corepressor (LCoR), which causes gene repression through liganded nuclear hormone receptors including RARs, further highlights the level of complexity of receptor function, as liganded receptors have traditionally been considered to activate transcription (Fernandes et al., 2003). LCoR possesses an LXXLL motif (similar to the other cofactors described) through which it interacts with the receptors, and can evoke repression through an HDAC-dependent or -independent manner (Fernandes et al., 2003).

Receptor-mediated repression seems as important as activation throughout various embryonic processes (Mannervik et al., 1999). NCoR-deficient embryos exhibit abnormalities in erythrocyte, thymocyte, and neural development and generally die around embryonic age (E)16 (Jepsen et al., 2000). Moreover, NcoR has been distinguished as a principle regulator of neural stem cell fate, in that its activity promotes self-renewal of these cells, repressing their differentiation into astrocytes (Hermanson et al., 2002). The absence of HDAC1 is also detrimental, resulting in embryonic lethality before E10.5, attributed to major proliferation defects and developmental retardation (Lagger et al., 2002). This is consistent with the effects of knocking out other components of repressor complexes in invertebrates (for review see Ahringer, 2000), and with the block in metamorphosis seen in tadpoles treated with the HDAC inhibitor, trichostatin A (Sachs et al., 2001). More specifically, NCoR recruitment by unliganded thyroid receptors represses expression of genes in preparation for tadpole metamorphosis (Sachs et al., 2002). Together, these findings support a prominent role for gene silencing in development. This is further emphasized by the widespread expression of LCoR in fetal tissues, including high levels in two-cell embryos (Fernandes et al., 2003). Here, we discuss the developmental role of repression by the retinoid receptors, highlighting two systems that are quite distinct from one another, but surprisingly similar in their requirement for RAR-mediated repression.

### Head formation in *Xenopus*

Studies showing a requirement for RAR-mediated repression during head formation in *Xenopus* were the first to uncover a major developmental role for active repression by retinoid receptors. Neural induction and anteroposterior (A-P) patterning of the neuroectoderm is largely regulated by signals secreted from the dorsal blastopore region, or “Spemann’s organizer,” and requires the inhibition of factors that promote epidermal fate and formation of more posterior structures such as the trunk (for review see Sasai and De Robertis, 1997). In response to treatment of early embryos with RA, or microinjection of constitutively active RARs, there is a posteriorization of otherwise anterior neural tissue, or even anterior truncations (for review see Blumberg et al., 1997). In contrast, expression of anterior markers is enhanced by microinjection of mRNA encoding a dominant-negative version of the RAR $\alpha$  (dnRAR $\alpha$ ) (Blumberg et al., 1997). These findings imply that RA must be absent or available at very low levels for appropriate patterning of anterior structures—an implication that is consistent with the expression of enzymes that regulate RA concentration. RALDH2 (retinaldehyde dehydrogenase 2) is important for RA production, and cytochrome p450, 26 (CYP26) metabolizes RA. In *Xenopus*, Cyp26 is expressed in future anterior CNS structures, whereas Raldh2 is restricted to the posterior region, suggesting a gradient of RA, with the lowest levels in the anterior regions during neural induction. Interestingly, RAR $\alpha$  and RAR $\gamma$  are expressed in prospective anterior tissues, despite the apparent absence of RA in these regions, and despite the sensitivity of these tissues to exogenous RA treatment (Ellinger-Ziegelbauer and Dreyer, 1991, 1993; Pfeffer and De Robertis, 1994). A convincing explanation for this paradox is that the repressive function of unliganded RARs, rather than simply an absence of RA, is essential for proper head formation (Koide et al., 2001).

Both NCoR-1 and -2 are expressed in the developing CNS, overlapping with RAR $\alpha$  transcripts (Koide et al., 2001). Treatment with AGN193109, an inverse agonist that promotes the repressive function of RAR, reverses RA-induced head truncations, causes an enlargement of the head and reduction in the tail, and up-regulates the expression of anterior markers. Microinjection of c-SMRT (NCoR-2) or morpholino antisense oligonucleotides against either xRAR $\alpha$ 1, or xRAR $\alpha$ 2 alone, or in combination, gives rise to posteriorized embryos, decreases the expression of anterior markers, and causes a reduction or loss in anterior structures including the head and cement gland (Koide et al., 2001). These effects can be rescued by microinjection of dnRAR $\alpha$ . Together, these results strongly suggest that a loss of RAR-mediated repression underlies the head formation defects caused by RA, and that RARs are not only transcriptional activators that transduce an RA signal, but also function in their unliganded form as gene repressors. Accordingly, overexpression of CYP26 in *Xenopus* causes an expansion of the region expressing anterior markers (Holleman et al., 1998), perhaps due to increased repression by the RARs in cells normally exposed to higher levels of RA. Conversely, CYP26 mutant mice exhibit abnormal patterning of the anterior CNS (Abu-Abed et al., 2001). These effects are not surprising, as tight control of ligand concentration would intu-

itively be critical given the distinct functions reported for unliganded versus liganded receptors.

### Skeletal development

Similar to *Xenopus* head formation, the status of RAR activity largely influences the fate of skeletal progenitor cells in mice. Most of the vertebrate skeleton is formed on a cartilaginous template. In response to various cues, condensations prefiguring the cartilage skeleton form, followed by the differentiation of condensed cells into chondroblasts—the early matrix-producing cells of cartilage. Dramatic skeletal abnormalities are elicited in mice by excess RA treatment (for reviews see Underhill et al., 1995; Underhill and Weston, 1998), or by ectopic expression of a weak constitutively active RAR $\alpha$ 1 in the developing limbs (Cash et al., 1997). The underlying cause of these defects is a failure of condensed mesenchymal cells to differentiate into chondroblasts (Weston et al., 2000, 2002).

Primary cultures of mouse limb mesenchyme recapitulate the *in vivo* chondrogenic sequence, in that various condensations of mesenchymal cells appear within a day or two of culture initiation, followed by the differentiation of these cells into chondroblasts, producing detectable nodules of cartilage (Ahrens et al., 1977). Treatment of these cultures with RAR antagonists increases nodule formation and induces expression and activity of Sox9, a transcription factor required for chondroblast differentiation (Weston et al., 2002). In addition, Sox9 activity is dramatically induced by the introduction of dnRAR $\alpha$  or dnRXR $\alpha$  into the cultures, and is inhibited by transfection of constitutively active versions of these receptors. The antagonist-induced increase in cartilage formation is blocked by the HDAC inhibitor trichostatin A, whereas the presence of a dominant-negative NCoR-1, which is unable to recruit HDACs, attenuates the antagonist-induced increase in Sox9 activity (Weston et al., 2002). These results indicate that recruitment of HDACs by RARs is essential for chondroblast differentiation. Consistent with this, expression of CYP26A1 and B1 is elevated in chondroprogenitors (Abu-Abed et al., 2002), suggesting a decrease of RA in these cells, which in turn would support the recruitment of HDACs to unliganded RARs and thus promote differentiation. It is worth noting that the oxidized derivatives of RA do not appear to be involved in retinoid signaling (Niederreither et al., 2002). Together, there is compelling evidence for a requirement for RAR-mediated repression in differentiation of murine skeletal progenitors, which closely parallels that described for head formation in *Xenopus*.

### Comparison between two distinct developmental systems: novel insights

Similarities in the role of gene repression by unliganded RARs in *Xenopus* CNS and mouse skeletal development provide some useful insights. For instance, the dynamic, and often abundant expression of the retinoid receptors does not necessarily correspond to the spatial and temporal availability of RA during development. This is evident by the requisite expression of RARs during *Xenopus* head formation, in regions not only devoid of RA, but where RA has detrimental effects. Similarly, during skeletal development in mouse

limb buds, RAR $\alpha$  and RAR $\gamma$  are highly expressed in chondroprogenitors despite high levels of CYP26 in these cells. In fact, excess RA during cartilage formation causes profound skeletal anomalies. Therefore, it is reasonable to infer that these receptors have important functions beyond transducing a retinoid signal, and that the teratogenic effects of excess RA can be caused, at least in part, by the derepression of otherwise silenced genes.

A finding common to both analyses is that manipulation of a single receptor isoform (xRAR $\alpha$ 1 or -2 in the case of head formation and RAR $\alpha$ 1 for skeletal development) has profound phenotypic consequences. These findings are surprising given the presumed redundancy between RAR family members, inferred from mouse knockout studies in which ablation of multiple receptor subtypes is necessary to produce noticeable phenotypes (Lohnes et al., 1994; Mendelsohn et al., 1994). Similarly, animals in which thyroid hormone receptors have been knocked out develop a completely normal central nervous system, whereas mice containing a mutated thyroid hormone receptor  $\beta$ , in which the ligand binding function of the receptor subtype is abolished, exhibit severe neurological development and dysfunction (Hashimoto et al., 2001). The latter effects are more consistent with the effects of congenital hypothyroidism and thyroid hormone resistance syndrome, both characterized by severe CNS dysfunction. Such discrepancies may be due to the distinct approaches taken to manipulate receptor function. In knockout mice, receptors are absent and thus cannot activate or repress target genes, whereas the use of selective antagonists or dominant-negative versions of the receptors, inhibits transcriptional activation, but maintains or enhances receptor-mediated repression. A full explanation for the lack of severe phenotypes in some of the receptor-knockout mice must await further functional studies.

In both systems discussed, RAR-mediated repression leads to the activation of specific markers of the differentiated phenotype. To date, the direct target genes whose repression causes the onset of differentiation have not been identified. Obvious candidates are genes satisfying various conditions: (i) they are expressed in progenitors but down-regulated just before, or during, differentiation; (ii) they interfere with differentiation and/or promote proliferation of progenitors; (iii) they are transcriptionally regulated by RA; and (iv) they contain RAREs within their promoters. To date, there is little evidence to suggest that any genes satisfy all conditions. Ongoing studies in our laboratories and others, however, are focused on unraveling the mechanisms whereby RAR-mediated repression leads to cell differentiation in both the *Xenopus* model of head formation and the mouse model of skeletal development. To this end, *Crescent*, a gene whose overexpression interferes with anterior patterning (Pera and De Robertis, 2000), appears to be up-regulated by RA treatment according to microarray studies (unpublished data). *Crescent* is expressed in the head organizer region at the early gastrula stage in *Xenopus*, but down-regulated by the tailbud stage (Pera and De Robertis, 2000). Microinjection of *Crescent* mRNA leads to cyclopia and a reduction or even loss in the most anterior cement gland (Pera and De Robertis, 2000). Given the induction of *Crescent* by RA, this gene represents a logical candidate for repression by unliganded RARs in this system.

Potential direct targets of RAR-mediated repression during chondroblast differentiation in the mouse, include members of the *Fgf* and *Wnt* genes as well as members of the homeobox (*Hox*) genes, including *Msx1*. Members from all gene families figure prominently in skeletal development, and several are demonstrated targets of retinoid signaling. In addition, several of the *Hox* genes have been shown to possess RAREs in their promoters (for review see Ross et al., 2000) and RAREs have been identified upstream of one of the *Fgf* family members (Brondani et al., 2002). *Msx1* represents one of the best candidates, as this gene is expressed in proliferating chondroprogenitors but not in differentiated chondroblasts, during limb development, and its maintained expression has been associated with an inhibition in differentiation (for reviews see Davidson, 1995; Bendall and Abate-Shen, 2000). *Msx* misexpression studies implicate these genes as promoters of cell proliferation and inhibitors of differentiation during many processes including chondrogenesis (Hu et al., 2001; Mina et al., 1996). Moreover, RA induces *Msx1* expression in EC cells (Shen et al., 1994). Similarly, we have observed an expansion of *Msx1* expression in the limbs of transgenic mice ectopically expressing a constitutively active RAR $\alpha$  (unpublished data). Aside from those mentioned, evidence is currently too scarce to postulate additional genes that are direct targets of retinoid repression during both *Xenopus* head formation and mouse skeletal development. A more comprehensive list of DNA-binding targets for the nuclear receptors will undoubtedly come from ongoing microarray analyses and from studies coupling chromatin immunoprecipitation with microarrays of human and mouse intergenic sequences, as has been done in yeast (Lee et al., 2002; Ren et al., 2000).

### In the wrong place at the wrong time: RAR-mediated repression and acute myeloid leukemia

The impact of RAR-mediated repression is underscored by the pathogenesis of acute promyelocytic leukemia (APL). APL is characterized by a block at various stages of hematopoietic progenitor cell (HPC) differentiation and, to date, has been associated with five translocation events, each fusing RAR $\alpha$  to another protein (for reviews see Lin et al., 2001; Zelent et al., 2001). RARs are critical during myeloid differentiation (Collins et al., 1990; Onodera et al., 1995; Tsai and Collins, 1993). Unlike head formation or skeletal development, where RAR $\alpha$ -mediated repression promotes differentiation, in the case of APL, RAR $\alpha$  fusion proteins prevent myeloid cell differentiation (Grignani et al., 1993; Rousselot et al., 1994), which is attributed to an inability of these fusion proteins to dissociate from repressor complexes in the presence of physiological levels of RA (for review see in Lin et al., 2001). The fusion proteins exhibit enhanced corepressor binding efficiency, presumably causing irreversible silencing of genes required for myeloid differentiation. Incidentally, included among these targets are perhaps genes not normally bound to RARs, but targeted nonetheless, by their novel fusion partners. Consequently, myeloid cells continue to proliferate, expanding the number of progenitors normally fated to undergo cell differentiation. Similarly, in mice treated with RAR panantagonists, granulocyte precursor numbers are substantially increased within all he-

matopoietic compartments, despite no change in cell death of mature granulocytes or progenitors (Walkley et al., 2002). Therefore, the fine balance between proliferation and differentiation during normal hematopoiesis seems to rely on appropriate gene repression by RARs.

### Summary and future directions

Head formation in *Xenopus* and skeletal development in mice are unique in that they involve differentiation events that are inhibited by RA, despite RA having a general role as an inducer of differentiation in many cell types. Given these opposite roles for RA in varying systems, it is plausible that receptor-mediated repression similarly has dual roles, being required both for induction of differentiation (in the case of neural development in *Xenopus* and skeletogenesis in mice), and for enhancing the proliferation of precursors, while inhibiting differentiation in other cells such as hematopoietic precursors. In this respect, the receptors can be thought of as molecular devices through which precise global control of gene transcription is achieved during development, through the combined action of liganded and unliganded receptors. The dynamic expression of receptors throughout development, irrespective of ligand availability, supports this idea.

As researchers unravel the mode of action for the nuclear receptors in more precise detail, a major challenge will be to place these molecular mechanisms into the context of the developmental processes affected by these receptors. This will require cataloguing of the genes directly acted upon by the receptors as well as a clear understanding of the developmental role of these genes. On a more molecular level, use of technologies such as fluorescence resonance energy transfer to monitor interactions between individual receptor subtypes and cofactors within live cells will be useful for relating cofactor exchange with specific developmental processes (Llopis et al., 2000). The notion of alternative HDACs being recruited may provide an additional level of complexity to the function of the receptors. It also remains to be shown how gene repression is influenced by posttranslational modifications, and by interactions with other (non-RXR) partners. Finally, an interesting possibility worth exploring is whether different receptor subtypes (or isoforms thereof) are distinct in their capacities to mediate gene repression. Indeed, recent findings suggest differences in cofactor stoichiometries and patterns of interaction among the distinct RAR subtypes (Germain et al., 2002), as RAR $\beta$  and RAR $\gamma$  were recently shown to be transcriptional activators even in the absence of ligand, contrasting the strong repressing activity of unliganded RAR $\alpha$  (Hauksdottir et al., 2003). Undoubtedly, the need for studies of this nature and the ongoing identification of novel roles for retinoid receptors will continue to fuel interest in the field of retinoid signaling and development.

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