© 2014. Published by The Company of Biologists Ltd | Development (2014) 141, 2260-2270 doi:10.1242/dev.103705

RESEARCH ARTICLE

Active repression by RARγ signaling is required for vertebrate axial elongation

Amanda Janesick[1], Tuyen T. L. Nguyen[1], Ken-ichi Aisaki[2], Katsuhide Igarashi[2], Satoshi Kitajima[2], Roshantha A. S. Chandraratna[3], Jun Kanno[2] and Bruce Blumberg[1,4,*]

ABSTRACT

Retinoic acid receptor gamma 2 (RARγ2) is the major RAR isoform expressed throughout the caudal axial progenitor domain in vertebrates. During a microarray screen to identify RAR targets, we identified a subset of genes that pattern caudal structures or promote axial elongation and are upregulated by increased RAR-mediated repression. Previous studies have suggested that RAR is present in the caudal domain, but is quiescent until its activation in late stage embryos terminates axial elongation. By contrast, we show here that RARγ2 is engaged in all stages of axial elongation, not solely as a terminator of axial growth. In the absence of RA, RARγ2 represses transcriptional activity *in vivo* and maintains the pool of caudal progenitor cells and presomitic mesoderm. In the presence of RA, RARγ2 serves as an activator, facilitating somite differentiation. Treatment with an RARγ-selective inverse agonist (NRX205099) or overexpression of dominant-negative RARγ increases the expression of posterior Hox genes and that of marker genes for presomitic mesoderm and the chordoneural hinge. Conversely, when RAR-mediated repression is reduced by overexpressing a dominant-negative co-repressor (c-SMRT), a constitutively active RAR (VP16-RARγ2), or by treatment with an RARγ-selective agonist (NRX204647), expression of caudal genes is diminished and extension of the body axis is prematurely terminated. Hence, gene repression mediated by the unliganded RARγ2–co-repressor complex constitutes a novel mechanism to regulate and facilitate the correct expression levels and spatial restriction of key genes that maintain the caudal progenitor pool during axial elongation in *Xenopus* embryos.

KEY WORDS: Active repression, Axial elongation, Chordoneural hinge, Posterior Hox, Presomitic mesoderm, Retinoic acid receptor

INTRODUCTION

Repression mediated through unliganded retinoic acid receptors (RARs) is an important yet understudied function exhibited by nuclear receptors (reviewed by Weston et al., 2003). Although RA plays a major role in patterning the hindbrain, retina, placodes and somites, its absence is crucial for the development of structures found at the head and tail of the embryo. RARs exhibit basal repression in the absence of ligand, binding constitutively to their targets, recruiting co-repressors, and actively repressing the basal transcriptional machinery (Chen and Evans, 1995). When ligand is present, co-repressors are replaced by co-activators and target genes are transcribed (Chakravarti et al., 1996).

We previously demonstrated that repression mediated through unliganded RARs was important for anterior neural patterning, establishing a novel role for RAR as a repressor *in vivo* (Koide et al., 2001). Overexpression of a dominant-negative RARα expanded anterior and midbrain markers caudally and shifted somitomeres rostrally (Blumberg et al., 1997; Moreno and Kintner, 2004). Exogenous RA, constitutively active RARα or derepression of RARα produced the opposite effect: severe anterior truncations, diminished anterior markers, and anteriorly shifted midbrain and hindbrain markers. Stabilization of co-repressors resulted in enhanced anterior neural structures and posteriorly shifted mid/hindbrain markers (Koide et al., 2001).

Axial elongation requires continual replenishing of bipotential caudal progenitor cells (maintained by Wnt and FGF signaling, but inhibited by RA) that give rise to notochord, neural tube and somites (Cambray and Wilson, 2002; Davis and Kirschner, 2000). The most stem-like cells are located in the chordoneural hinge (CNH), where the posterior neural plate overlies the caudal notochord (Beck and Slack, 1998). Cells from the CNH contribute to presomitic mesoderm (PSM), which supplies committed somitic precursor cells to the rostral determination wavefront (reviewed by Dequeant and Pourquie, 2008). PSM is initially homogenous and unorganized [expressing *Mesogenin1* (*Msgn1*) and *Tbx6*], then becomes patterned into somitomeres marked by *Thylacine2* (*Thyl2*) and *Ripply2* (reviewed by Dahmann et al., 2011). Epithelialization of presomitic domains results in mature somites (Nakaya et al., 2004).

RA is well known to function in the trunk, where it promotes differentiation of PSM into somitomeres (Moreno and Kintner, 2004). By contrast, RA is actively metabolized and cleared by CYP26A1 in the caudal region (Fujii et al., 1997). Treatment with RA leads to loss of posterior structures (Sive et al., 1990); $Cyp26a1^{-/-}$ mice exhibit posterior truncations and homeotic vertebral transformations (Abu-Abed et al., 2001; Sakai et al., 2001). Exposing embryos to RA inhibits proliferation of axial progenitor cells in CNH and PSM, leading to axial truncation from premature exhaustion of the progenitor pool (Gomez and Pourquie, 2009). Therefore, RA is normally excluded from unsegmented mesenchyme in PSM and the CNH. RARγ is expressed at high levels throughout the entire caudal region, including CNH and PSM (Mollard et al., 2000; Pfeffer and De Robertis, 1994), yet, based on *Cyp26a1* expression, RA is absent (de Roos et al., 1999). The physiological significance of RARγ expression in the embryonic posterior is uncertain. RARγ might function to terminate the body axis at late stages by inducing apoptosis (Olivera-Martinez et al., 2012), but that model would not explain the strong expression of RARγ observed at neurula, continuing through tailbud stages, despite the apparent absence of RA.

[1]Department of Developmental and Cell Biology, 2011 Biological Sciences 3, University of California, Irvine, CA 92697-2300, USA. [2]Division of Cellular and Molecular Toxicology, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. [3]IO Therapeutics, Santa Ana, CA 92705-5851, USA. [4]Department of Pharmaceutical Sciences, University of California, Irvine, CA 92697-2300, USA.

*Author for correspondence (blumberg@uci.edu)

Received 29 September 2013; Accepted 27 March 2014

Development (2014) 141, 2260-2270 doi:10.1242/dev.103705

Rarγ2 skirts the posterior edge of the determination wavefront and is co-expressed with PSM, CNH and posterior Hox markers. We hypothesized that *Rarγ2* serves a dual function: as an activator in somite differentiation but a repressor in the maintenance of PSM and the caudal progenitor pool. Loss of RARγ2 severely shortens the embryo body axis and inhibits somitogenesis. Loss of RARγ2 expands the anterior border of PSM expression near the wavefront (where activation is lost), but diminishes the expression domain of caudal PSM and posterior Hox genes (where repression is lost). Increasing RAR-mediated repression expands the expression of posterior Hox, PSM and CNH markers, creating smaller somitomere domains via an indirect, 'repressing a repressor' mechanism. Relief of repression results in a truncated body axis with decreased PSM and CNH markers. Axial extension and segmentation in vertebrates relies on the maintenance of unsegmented PSM mesenchyme and replenishing of caudal progenitor cells. Our data show that RARγ2 plays a crucial role in this process, repressing target genes to maintain PSM and caudal progenitors in the absence of RA, while activating others to promote somitogenesis in the presence of RA.

RESULTS

Posterior Hox, PSM and CNH genes are upregulated by RAR inverse agonist

We showed previously that active repression of RAR target genes by unliganded RAR is required for head formation (Koide et al., 2001). Treatment with the pan-RAR inverse agonist AGN193109 increased the expression of genes involved in patterning anterior neural structures, whereas treatment with pan-RAR agonist TTNPB decreased the expression of anterior marker and cement gland-specific genes (Koide et al., 2001), revealing a set of genes specifically upregulated/downregulated by TTNPB (Arima et al., 2005). Validation studies identified a subset upregulated by AGN193109. We hypothesized that active repression by unliganded RARs is biologically important and designed an experiment to identify genes upregulated or downregulated by modulating repression. Percellome analysis (Kanno et al., 2006) quantified the copy number per embryo of all genes represented on Affymetrix *Xenopus* microarray v1.0. Among these we identified a collection of genes linked to the maintenance of caudal axial progenitors that were downregulated by TTNPB and upregulated by AGN193109 (Table 1). RAR-mediated repression upregulates the steady-state expression of posterior Hox paralogs 9-13 and genes found in both unsegmented PSM and CNH. Thus, we hypothesized that RAR is a repressor required for axial elongation.

Xenopus RARs repress basal transcription in the absence of ligand

The ability of unliganded RARs to behave as repressors is well documented, although not all human receptor subtypes can recruit co-repressors (e.g. SMRT) in the absence of ligand (Wong and Privalsky, 1998). We tested the ability of *Xenopus* RAR (xRAR) subtypes to repress basal activity of a luciferase-dependent reporter using the GAL4-RAR system (supplementary material Fig. S1D-F) (Blumberg et al., 1996). *Xenopus* RARα, RARβ and RARγ suppressed basal activity *in vitro* and *in vivo* (supplementary material Fig. S1A,C), whereas human RARβ and RARγ did not (supplementary material Fig. S1B). Thus, xRARs can function as repressors in the absence of ligand.

Rarγ2 is expressed in the PSM and CNH but is mostly absent from the trunk

Whole-mount *in situ* hybridization (WISH) revealed that *Rarγ2* is the predominant isoform expressed in the *Xenopus* embryonic posterior (supplementary material Fig. S2A). In late neurula and early tailbud stage embryos, *Rarγ2* is strongly expressed in the anterior and posterior, but almost undetectable in the trunk. *Rarγ2* expression later becomes pronounced in the tail and head, particularly in hyoid, branchial and mandibular neural crest. *Rarγ1* is expressed similarly. QPCR analysis revealed that *Rarγ2* is 1000- to 4000-fold more abundant than *Rarγ1* at stages 10-22, and 100- to 400-fold more abundant at all other stages analyzed (supplementary material Fig. S2B). Subsequent experiments utilized *Rarγ2*-selective reagents. We conclude that *Rarγ2* is the predominant isoform expressed in the posterior region of embryos.

Rarγ2 is expressed where RA is probably absent (owing to CYP26A1 expression). Key posterior genes were upregulated by AGN193109. We hypothesized that RARγ2 posterior to the wavefront is a repressor, maintaining unsegmented PSM and the progenitor cell pool required for axial elongation. We used double WISH to compare the expression of *Rarγ2* with that of *Hoxc10*, an important member of the Abd-B Hox gene family promoting caudal development over thorax (Lamka et al., 1992). *Rarγ2* expression completely overlaps caudal *Hoxc10* expression (Fig. 1E,H) but not the anteriormost neural or lateral plate expression of *Hoxc10* (Fig. 1E,H). These data position

Table 1. Percellome analysis reveals that posterior Hox, PSM and CNH markers are upregulated by RAR inverse agonist

Unigene	109 (fold)	*P*	TTN (fold)	*P*	Symbol	Gene name	Cat
Xl.72193	3.57	2.11×10^{-3}	0.19	5.77×10^{-4}	*Hoxc13*	Homeobox C13	PP
Xl.266	3.47	4.26×10^{-3}	0.12	2.26×10^{-4}	*Hoxa11*	Homeobox A11	PP
Xl.21864	3.15	2.03×10^{-3}	0.22	2.68×10^{-4}	*Hoxc10*	Homeobox C10	PP
Xl.72292	3.02	7.32×10^{-3}	0.16	1.62×10^{-4}	*Hoxd9*	Homeobox D9	PP
Xl.9560	2.73	9.74×10^{-4}	0.40	5.98×10^{-3}	*Hoxa9*	Homeobox A9	PP
Xl.12067	2.80	8.05×10^{-3}	0.18	2.51×10^{-5}	*Esr2*	Enhancer of Split related 2	PSM
Xl.29033	2.79	9.31×10^{-4}	0.26	1.62×10^{-5}	*Esr9*	Enhancer of Split related 9	PSM
Xl.78953	2.90	4.29×10^{-4}	0.37	2.68×10^{-3}	*Tbx6*	T-box gene Tbx6	PSM
Xl.483	2.53	4.18×10^{-3}	0.17	3.36×10^{-8}	*Msgn1*	Mesogenin 1	PSM
Xl.14524	2.32	2.76×10^{-2}	0.42	1.46×10^{-2}	*Esr5*	Enhancer of Split related 5	PSM
Xl.933	2.49	4.46×10^{-2}	0.40	2.73×10^{-2}	*xBra3*	T2, Brachyury homolog	CNH
Xl.1066	2.44	4.31×10^{-2}	0.34	2.09×10^{-3}	*xNot*	Notochord homeobox	CNH
Xl.457	3.10	1.37×10^{-3}	0.02	2.81×10^{-7}	*Derriere*	Growth differentiation factor 3	NC
Xl.16206	2.43	7.64×10^{-3}	0.27	2.35×10^{-6}	*Pnp*	Purine nucleoside phosphorylase	NC

Blastula stage embryos were soaked in 1 µM RAR agonist TTNPB (TTN), 1 µM RAR inverse agonist AGN193109 (109) or vehicle control (0.1% ethanol) until harvesting at stage 18. Cat, expression category: PP, posterior patterning; PSM, presomitic mesoderm; CNH, chordoneural hinge; NC, expression not characterized. Fold induction or reduction is relative to control vehicle. *P*-values were generated using CyberT.

Development (2014) 141, 2260-2270 doi:10.1242/dev.103705

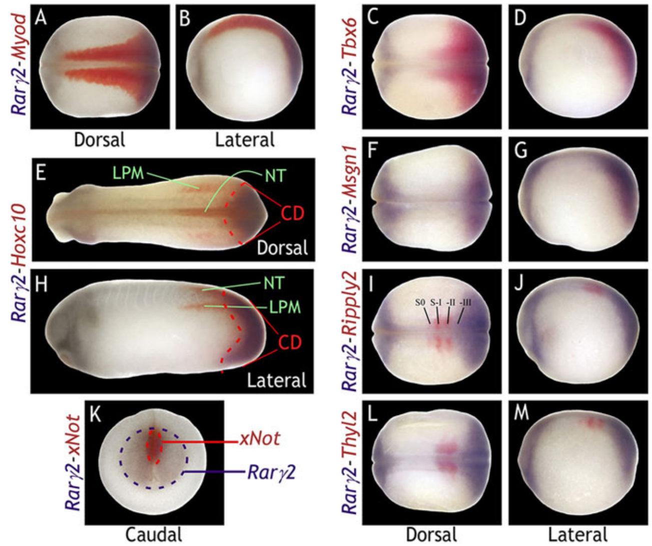

Fig. 1. Double WISH reveals the spatial relationship between *Rarγ2* and posterior Hox, PSM and CNH genes. (A-M) *Rarγ2* is stained with BM Purple and the other genes are stained with Fast Red. *Rarγ2* is caudal to *Myod* and *Tbx6* (A-D), but synexpressed with *Msgn1* (F,G) in neurula stage *Xenopus* embryos. (E,H) *Rarγ2* is synexpressed with the caudal domain (CD) of *Hoxc10* but not with neural tube (NT) or lateral plate mesoderm (LPM) of *Hoxc10* in tailbud stage embryos. *Rarγ2* overlaps with S–III domains of *Ripply2* (I,J) and *Thyl2* (L,M) expression, but not with more anterior somitomeres (S–II, S–I, S0). (K) *Rarγ2* overlaps with *xNot* expression in neurula stage embryos. Dorsal and lateral views shown with anterior to the left, except in K (caudal view with dorsal at top).

Rarγ2 as a potential regulator of posterior Hox genes and the caudal body plan.

We next defined the anterior limit of *Rarγ2* expression relative to the determination wavefront. *Myod* is a general muscle marker abutting and partially overlapping *Rarγ2* expression (Fig. 1A,B). *Thyl2* and *Ripply2* mark somitomeres, which are prepatterned PSM domains containing non-epithelialized, immature somites (Tam et al., 2000). *Thyl2* and *Ripply2* are only expressed in newly forming somitomeres and are assigned negative Roman numerals (S–I, S–II, etc.) versus mature somites (SI, SII, etc.) (Pourquie and Tam, 2001). *Msgn1* (Buchberger et al., 2000) is expressed caudal to *Thyl2* and *Ripply2*, marking non-patterned PSM-containing cells committed to the somitic fate (Nowotschin et al., 2012). *Tbx6* is also expressed in PSM, but unlike *Msgn1* its expression domain overlaps with somitomeres (Hitachi et al., 2008). *Rarγ2* and *Msgn1* are synexpressed at neurula (Fig. 1F,G) and tailbud (supplementary material Fig. S3) stages; *Tbx6* expression overlaps *Rarγ2* but extends rostrally beyond the *Rarγ2* domain (Fig. 1C,D; supplementary material Fig. S3). Anterior expression of *Rarγ2* mRNA ends at an RA-responsive region (supplementary material Fig. S4), coinciding with the most posterior somitomere domain (S–III) of *Thyl2* or *Ripply2* (Fig. 1I-M), thus skirting the posterior edge of the wavefront.

xNot, a notochord marker that regulates trunk and tail development, is concentrated in the extreme posterior notochord and floor plate by late neurula (von Dassow et al., 1993) and is often employed as a CNH marker in *Xenopus* (Beck and Slack, 1998) to reveal the location of bipotential stem cells (Cambray and Wilson, 2007; Takemoto et al., 2011). *xNot* is co-expressed with *Rarγ2* (Fig. 1K), agreeing with data suggesting that *Rarγ2* is present in CNH (Pfeffer and De Robertis, 1994). The double WISH data are consistent with *Rarγ2* functioning as an activator near where RA is present at the wavefront, yet as a repressor where it coincides with *Msgn1*, *xNot* and *Cyp26a1*.

RARγ-selective chemicals modulate activation or repression by RARγ

To separate the effects of RARγ in the posterior from RARα in the trunk, we characterized RARγ-selective agonist NRX204647 (4647) (Shimono et al., 2011; Thacher et al., 2000) and RARγ-selective inverse agonist NRX205099 (5099) (Tsang et al., 2003) in *Xenopus* embryos. Like AGN193109, 5099 is an inverse agonist, reducing RARγ signaling activity below basal levels by stabilizing the co-repressor complex bound to RARγ. Embryos treated with 1 μM agonist 4647 become primarily trunk (no head or tail structure), while 0.1 μM perturbs axial elongation (supplementary material Fig. S5), producing anterior truncations characteristic of RAR activators (Sive et al., 1990). Inverse agonist 5099 at 1 μM delayed development, producing enlarged heads and shortened trunks; half the dose elicited similar but weaker phenotypes, with effects absent at 0.1 μM (supplementary material Fig. S5). Treating neurula embryos significantly reduced severity but did not eliminate the phenotype (supplementary material Fig. S5).

To test the effects of these chemicals *in vivo* without interference from endogenous RARs, we mutated the DNA-binding specificity of a full-length RAR, $RAR^{EGCKG \rightarrow GSCKV}$. The mutant receptor recognizes a mutant TK-luc reporter, $(RXRE^{1/2}\text{-}GRE^{1/2})\times 4$ TK-luc, to which endogenous RARs do not bind (Klein et al., 1996). In transient transfection assays, 4647 selectively activated RARγ at doses below 0.1 μM (supplementary material Fig. S6A). Similarly, 5099 selectively antagonized 10 nM 9-cis RA activation of RARγ below 0.1 μM (supplementary material Fig. S6B). We conclude that 4647 and 5099 behave as subtype-selective ligands to activate or repress RARγ.

RARγ-selective chemicals affect posterior Hox genes, PSM and somitomeres

We hypothesized that 4647 treatment of embryos would decrease posterior Hox gene expression and markers of PSM, whereas 5099 would produce the opposite effect. Microarray analysis (Table 1) revealed that *Hoxc13* and *Hoxc10* expression was upregulated by inverse agonist AGN193109 and downregulated by agonist TTNPB. We infer that increased expression of *Hoxc13* and *Hoxc10* results from RAR repressing the expression of a repressor of their expression. The expression pattern of *Hoxc13* (supplementary material Fig. S7) was not previously characterized.

We began soaking embryos in RARγ-selective doses of 4647, 5099 or vehicle control after gastrulation (stage 12.5) to focus on axial elongation. Treatment with 10 nM 4647 resulted in diminished caudal structures at stage 40 (supplementary material Fig. S5), reducing expression domains of *Hoxc10*, *Hoxd10* and *Hoxc13* (Fig. 2A-C). Conversely, treatment with 0.5 μM 5099 expanded their neural and lateral domains (Fig. 2A-C). To determine short-term effects of chemical treatments, we soaked embryos for 1 h at various stages and evaluated *Hoxc10* expression (supplementary material Fig. S8) and that of *Tbx6* (not shown) at stage 22. Repression by 5099 is required at early neurula, whereas activation by 4647 is required at mid- and late neurula stages for expected expansion and reduction, respectively, of *Hoxc10* expression (supplementary material Fig. S8). Higher, non-receptor-selective doses exacerbated effects on posterior Hox genes (supplementary material Fig. S9), suggesting that RARγ2 is the primary mediator. *Hoxc10* nearly abuts *Krox20*, demonstrating trunk shortening in 5099-treated embryos (supplementary material Fig. S9G,H). High

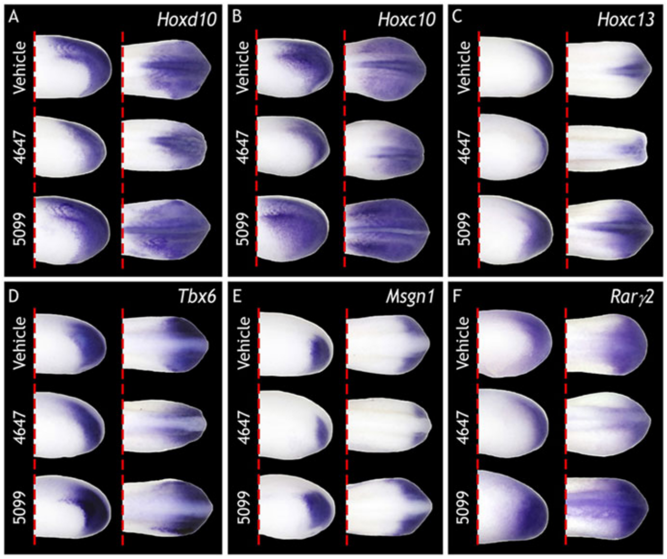

Fig. 2. Posterior Hox and PSM markers are reduced by RARγ-selective agonist and expanded by RARγ-selective inverse agonist. (A-F) WISH from embryos treated post-gastrulation (stage 12.5) with 10 nM 4647, 0.5 µM 5099 or vehicle (0.1% ethanol). Dashed red line represents half the embryo axis. 4647 diminishes and 5099 expands the expression of (A) *Hoxd10* (4647, 16/16; 5099, 17/17 embryos), (B) *Hoxc10* (4647, 14/14; 5099, 21/21), (C) *Hoxc13* (4647, 12/12; 5099, 16/16), (D) *Tbx6* (4647, 11/12; 5099, 17/17), (E) *Msgn1* (4647, 15/15; 5099, 14/14), and (F) *Rarγ2* (4647, 15/15; 5099, 9/9) relative to control vehicle. Embryos shown in lateral or dorsal view at tailbud stage, anterior to left.

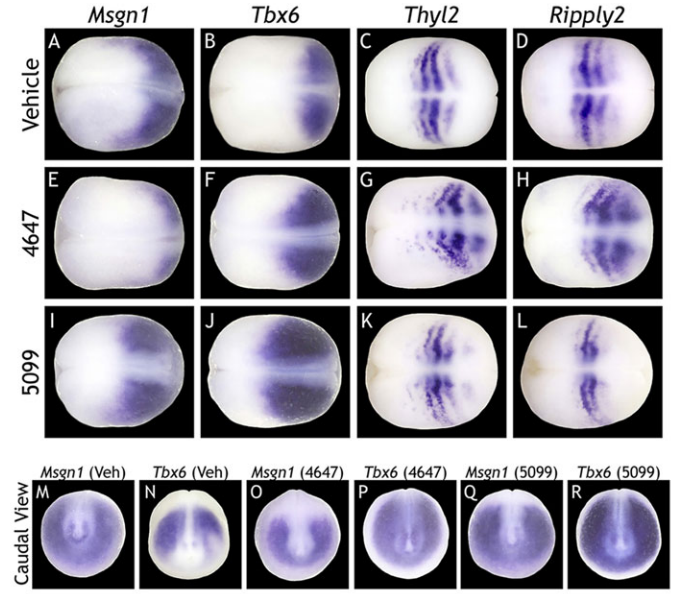

Fig. 3. PSM markers are modulated by RARγ-selective agonist and inverse agonist. (A-R) WISH from embryos treated post-gastrulation (stage 12.5) with 10 nM 4647, 0.5 µM 5099 or vehicle (0.1% ethanol). (A-D) Control expression of *Msgn1*, *Tbx6*, *Thyl2* and *Ripply2*. (E) *Msgn1* expression diminished by 4647 treatment (17/17 embryos). (F) *Tbx6* expression expanded by 4647 treatment (22/22). (G,H) Somitomere domains of *Thyl2* (19/19) and *Ripply2* (17/17) are thicker and posteriorly expanded. (I,J) *Msgn1* (17/17) and *Tbx6* (13/13) expression expanded by 5099 treatment. (K,L) Somitomere domains of *Thyl2* (15/17) and *Ripply2* (26/26) are fewer and thinner. Embryos are shown in dorsal view at neurula stage, anterior to left. (M-R) Caudal views of *Msgn1* and *Tbx6*.

doses of 4647 create embryos lacking anterior and posterior structures, as indicated by the absence of mid/hindbrain markers *En2* and *Krox20* and of posterior gene *Hoxc10* (supplementary material Fig. S9C-F).

Msgn1 and *Tbx6* were upregulated by inverse agonist and downregulated by agonist in the microarray analysis (Table 1). *Msgn1* and *Tbx6* domains were reduced at tailbud stages by post-gastrulation treatment of embryos with 4647, whereas expression was expanded in embryos treated with inverse agonist 5099 (Fig. 2D,E). However, in neurula stage embryos, 4647 reduced *Msgn1* expression while *Tbx6* expression was expanded (Fig. 3E,F,O,P). Expression of *Tbx6* and *Msgn1* was expanded by 5099 (Fig. 3I,J,Q,R), an effect that was more pronounced at higher doses (supplementary material Fig. S10I,J,Q,R). Somitomere markers *Thyl2* and *Ripply2* showed thicker domains; S–III expanded to the posteriormost edge of the embryo where somites are not found in controls (Fig. 3G,H). At non-receptor-selective doses, 4647 exacerbated the phenotypes of *Msgn1*, *Tbx6* and *Ripply2* (supplementary material Fig. S10E,F,H,O,P) and promoted ectopic expression of *Thyl2* in the midline, with somitomeres occupying nearly the entire anteroposterior axis (supplementary material Fig. S10G). By contrast, 5099 treatment produced fewer, thinner somitomeres (Fig. 3K,L), an effect more pronounced at higher doses (supplementary material Fig. S10K,L).

Since *Rarγ2* is co-expressed with *Msgn1*, we expected that 4647 would reduce and 5099 would expand *Rarγ2* expression. *Rarγ2* expression was expanded by inverse agonist and reduced by agonist (Fig. 2F) as verified by QPCR (supplementary material Fig. S11), which is surprising given that other receptor subtypes (RARα2 and RARβ2) are induced by agonist (Leroy et al., 1991; Sucov et al., 1990). The data indicate that 5099 enhances repression by RARγ, increasing caudal gene expression, whereas 4647 relieves repression by RARγ, diminishing caudal gene expression.

Relief of repression reduces domains of posterior Hox and PSM markers

Treatment with 4647 activates RARγ and removes repressors from RARγ targets, creating posterior truncations. We hypothesized that loss of RARγ2 would phenocopy 4647 treatment once RARγ2-mediated repression was lost. We designed AUG MOs to capture both pseudoalleles of *Rarγ2*. Knockdown of RARγ2.1/2.2 resulted in loss of *Hoxc10*, *Hoxd10*, *Hoxa11* and *Hoxc13* expression, together with severe curvature and reduction of the injected side (Fig. 4A-D). Microinjection of splice-blocking MO capturing both pseudoalleles of *Rarγ2* reduced the expression of *Rarγ2* as measured by QPCR, phenocopying the AUG MOs (supplementary material Fig. S12). We demonstrated that axial truncation on the injected side was not due to developmental delay (supplementary material Fig. S13). To establish that RARγ2 is solely responsible for the axial truncations and reduction in posterior Hox and PSM domains, we showed that *Rarγ2* MO can only be rescued with *Rarγ2*, but not *Rarα2* or *Rarβ2*, mRNA (Fig. 5). RARγ2 knockdown reduced and shifted the expression of *Msgn1* and *Tbx6* anteriorly along the midline (Fig. 4E,F,I-J′) and caused an anterior shift in the paraxial domains of *Thyl2* and *Ripply2*, while obliterating lateral expression (Fig. 4G,H). The complexity of the *Rarγ2* MO phenotype is likely to be due to the fact that RARγ2 knockdown both disrupts its repressive function in the absence of ligand and its activation in the presence of ligand, particularly near the determination wavefront.

When the dominant-negative co-repressor c-SMRT is overexpressed, it binds RAR and blocks recruitment of co-repressors (Chen et al., 1996). We identified several c-SMRT isoforms from *Xenopus*, selecting that most similar to human c-SMRT that we used previously. Microinjection of *Xenopus laevis* (Xl) *c-smrt* mRNA relieved repression by GAL4-xRARγ in whole embryos (supplementary material Fig. S14). This effect was potentiated by addition of 1 μM TTNPB (supplementary material Fig. S14). Overexpression of Xl *c-smrt* mRNA caused significant reductions in the neural and lateral domains of *Hoxc10* and *Hoxd10* (Fig. 6B,D). Xl *c-smrt* also reduced *Hoxc13*, *Tbx6*, *Msgn1* and *xNot* (Fig. 6F,H,H′,J,J′,L). Similar to *Rarγ2* MO, moderate truncation of injected axes was observed in 70% of embryos, but the midline, rostral shifting of *Tbx6* and *Msgn1* (as in *Rarγ2* MO embryos) was minimal. We conclude that Xl c-SMRT relieves repression of *Rarγ2*, causing loss of progenitor and PSM cells and posterior Hox gene expression.

Another method for relieving repression is overexpression of constitutively active VP16-RARγ2 (RARγ2 fused to the VP16 activation domain). Microinjection of VP16-*Rarγ2* mRNA led to a truncated axis on the injected side in 100% of embryos and loss of *Hoxc10*, *Hoxd10*, *Msgn1* and *Tbx6* expression (Fig. 7). These embryos were less curved than *Rarγ2* MO-injected or *c-smrt*-injected embryos, but rostral expansion of neural/midline and lateral domains was consistently observed, similar to *Rarγ2* MO embryos.

Increased repression expands posterior Hox and PSM markers

Treatment with 4647 or microinjection of *c-smrt* or VP16-*Rarγ2* mRNA relieved repression by RARγ, increasing RAR signaling, decreasing posterior Hox and PSM markers. Decreasing RAR signaling should produce the opposite effect. We microinjected mRNA overexpressing the RA catabolic enzyme CYP26A1 and observed rostral shifts in the lateral and neural expression domains of *Hoxc10* and *Hoxd10* (supplementary material Fig. S15). Microinjection of dominant-negative (DN)-RARγ2 should phenocopy 5099 treatment because co-repressors would be retained on RARγ2 targets, leading to repression. Overexpression of DN-RARγ2 increased the expression of *Msgn1* and *Tbx6* in both lateral and paraxial domains, and shifted *xNot* expression rostrally

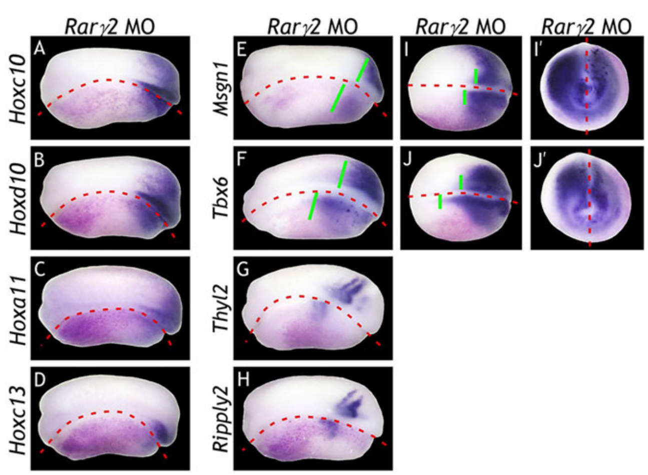

Fig. 4. RARγ2 knockdown alters expression of posterior Hox and PSM markers. (A-J′) Embryos were injected unilaterally at the 2- or 4-cell stage with 7.5 ng *Rarγ2.1* MO+7.5 ng *Rarγ2.2* MO. Injected side is indicated by magenta β-gal lineage tracer. *Rarγ2.1*/*2.2* MO decreases expression of (A) *Hoxc10* (18/18 embryos), (B) *Hoxd10* (12/12), (C) *Hoxa11* (9/9) and (D) *Hoxc13* (16/16) in tailbud stage embryos. *Rarγ2.1*/*2.2* MO decreases lateral, but expands midline, expression (green lines) of (E) *Msgn1* (10/13) and (F) *Tbx6* (8/11), knocking down and shifting expression rostrally of (G) *Thyl2* (13/15) and (H) *Ripply2* (13/14) in tailbud stage embryos. *Rarγ2.1*/*2.2* MO decreases lateral, but expands midline, expression (green lines) of (I) *Msgn1* (35/36) and (J) *Tbx6* (20/20) in neurula stage embryos. Embryos shown in dorsal view with anterior on left. (I′,J′) Caudal views of I and J.

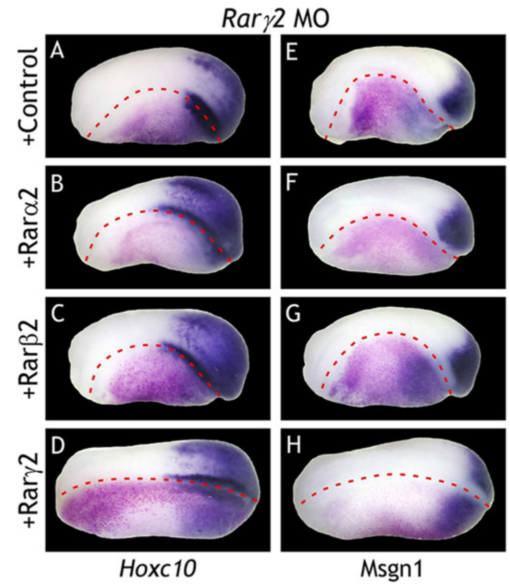

Fig. 5. *Rarγ2* mRNA rescues posterior Hox and PSM expression in *Rarγ2* MO embryos. (A-H) Embryos injected unilaterally at 2- or 4-cell stage. Injected side is indicated by magenta β-gal lineage tracer. (A,E) 5 ng *Rarγ2.1* MO+5 ng *Rarγ2.2* MO+control (*mCherry*) mRNA diminishes *Hoxc10* and *Msgn1* expression, curving the embryo axis in 100% of embryos (*Hoxc10*, 23/23; *Msgn1*, 13/13). (B,C,F,G) Co-injection of *Rarγ2* MO and 1 ng *Rarα2* mRNA or 1 ng *Rarβ2* does not rescue the phenotype; however, (D,H) 1 ng *Rarγ2* mRNA partially rescues axial curvature and *Hoxc10* (18/23) and *Msgn1* (23/35) expression. Tailbud embryos shown in dorsal view with anterior to left.

(Fig. 8B,D,F). DN-RARγ2 phenocopied the effects of *Cyp26a1* mRNA (Moreno and Kintner, 2004) on somitomere markers *Thyl2* and *Ripply2;* rostral shifting and knockdown of somitomere expression was the phenotype that we observed (Fig. 8H,J,K).

Microinjection of *Rarγ2* MO alone resulted in knockdown of *Hoxc10* and axial truncation (Fig. 9A,B,E). We hypothesized that this phenotype was due to loss of repression, reasoning that the phenotype should be rescued with DN-RARγ2. Axial defects and lateral knockdown of *Hoxc10* expression were partially recovered with DN-*Rarγ2* mRNA (Fig. 9C,D,E). The neural domain of *Hoxc10* expression was rescued in nearly all embryos and a rostral shift often observed. We conclude that increasing repression with DN-RARγ2 or overexpressing CYP26A1 (removing ligand) promotes caudal gene expression, similar to chemical treatment with 5099. Moreover, loss of caudal structures and gene expression due to *Rarγ2* MO are rescued by restoring repression with DN-RARγ2.

DISCUSSION

RARγ repression in caudal development

Most studies consider only one aspect of RAR signaling, namely its role as a ligand-activated transcription factor promoting the expression of target genes. In developmental biology, RA signaling has been studied extensively for its ability to promote differentiation and establish boundaries in somitogenesis, neurogenesis and rhombomere segmentation (reviewed by Rhinn and Dolle, 2012). Liganded RAR has been predicted to function passively in the caudal region until required to facilitate body axis cessation (Olivera-Martinez et al., 2012), when somitogenesis is nearing completion because the determination wavefront, moving the RA source caudally, has exhausted the progenitor cell pool (Gomez and Pourquie, 2009). Here, liganded RARγ would function as an activator promoting apoptosis (Shum et al., 1999) at terminal tailbud stage. However, this does not address why RARγ2 would be highly expressed where RA is

Development (2014) 141, 2260-2270 doi:10.1242/dev.103705

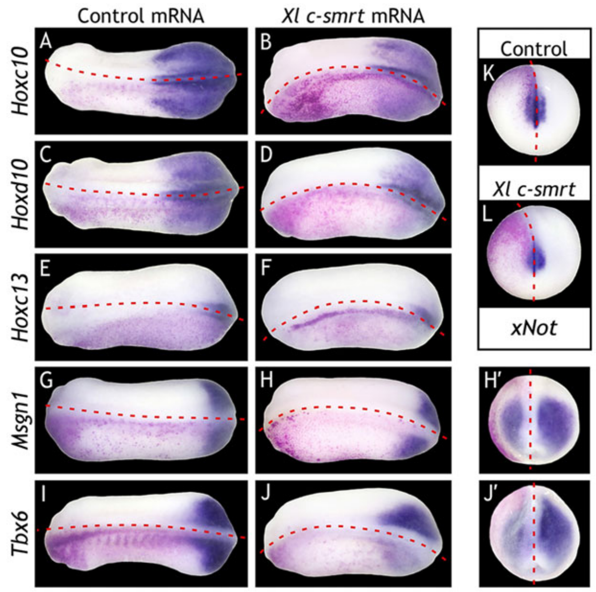

Fig. 6. c-SMRT overexpression knocks down posterior Hox, PSM and CNH markers. Embryos injected unilaterally at 2- or 4-cell stage with 4 ng *c-smrt* mRNA or control (*mCherry*) mRNA. Injected side indicated by magenta β-gal lineage tracer. (A,C,E,G,I,K) Control expression of *Hoxc10*, *Hoxd10*, *Hoxc13*, *Msgn1*, *Tbx6* and *xNot*. (B,D,F,H,J,L) *c-smrt* overexpression shortens the axis on injected side in 70% of embryos. (B) *c-smrt* mRNA results in lateral knockdown (13/23 embryos), neural knockdown (7/23) or neural rostral shift (7/23) in *Hoxc10* expression. (D) *c-smrt* mRNA produces neural and lateral knockdown (15/19) or lateral knockdown alone (4/19) of *Hoxd10* expression. (F,H,J) *c-smrt* mRNA knocks down expression of *Hoxc13* (14/18), *Msgn1* (12/14) and *Tbx6* (15/15). Tailbud embryos shown with anterior to left. (H′,J′) Caudal views of H and J. (L) *c-smrt* mRNA knocks down *xNot* (12/15) expression in neurula stage embryos (caudal view, dorsal to top).

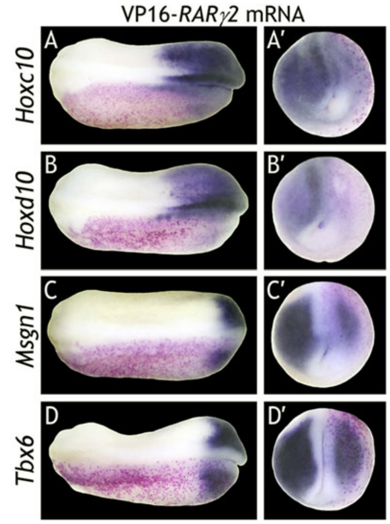

Fig. 7. VP16-RARγ2 overexpression knocks down posterior Hox and PSM marker expression. Embryos injected unilaterally at 2- or 4-cell stage with 0.3 ng VP16-*Rarγ2* mRNA or control (*mCherry*) mRNA. Injected side is indicated by magenta β-gal lineage tracer. Control expression of *Hoxc10*, *Hoxd10*, *Msgn1* and *Tbx6* is shown in Fig. 6A,C,G,I. (A-D) VP16-*Rarγ2* overexpression shortens the axis on injected side in 100% of embryos. (A,B) VP16-*Rarγ2* mRNA results in neural/midline rostral shift and lateral knockdown in *Hoxc10* (9/13 embryos) and *Hoxd10* (7/13) expression. Neural/midline knockdown is also observed (*Hoxc10*, 4/13; *Hoxd10*, 7/13). (C,D) VP16-*Rarγ2* mRNA rostrally shifts and/or knocks down *Msgn1* (12/12) and *Tbx6* (13/13) expression. Tailbud embryos shown with anterior to left. (A′-D′) Caudal views of A-D.

presumed absent due to CYP26A1 expression. Here we show that RARγ is engaged in all stages of caudal development, not solely as a terminator of the body axis. RARγ functions as an unliganded repressor required for the maintenance of the posterior PSM and progenitor cell population that allows axial elongation (Fig. 10). RARγ acts as a liganded activator in the anterior, segmented PSM to facilitate somite differentiation (Fig. 10). Repression mediated by the unliganded receptor–co-repressor complex constitutes a novel mechanism by which posterior markers are upregulated during axial elongation in *Xenopus* embryos.

Our microarray results suggest that axial elongation is regulated by RAR-mediated repression. Enhancing repression with AGN193109 upregulated, and activation of RAR by TTNPB downregulated, many posterior Hox, PSM and CNH genes in neurula stage embryos. We identified AGN193109-upregulated genes expressed in PSM (Table 1) that are mostly absent from regions of somite maturation (Blewitt, 2009; Yoon et al., 2000). The CNH markers *xBra3* and *xNot* were also upregulated by AGN193109, thus both PSM and CNH markers were upregulated by enhancing RAR repression and downregulated by increasing RAR activation. Current literature suggests the existence of a negative-feedback loop between these two populations of cells: *Msgn1* is induced by *Brachyury* and *Wnt8* in CNH but represses their expression to promote PSM fates (Fior et al., 2012; Yabe and Takada, 2012). Our results support a novel role of RAR repression in the maintenance of cells in both unsegmented PSM and stem-like CNH.

We showed that *X. laevis* RARα, RARβ and RARγ can repress basal transcriptional activity in the absence of RA and examined whether this repression is physiologically relevant in caudal development. *Rarγ2* is expressed in embryonic regions where it might actively repress genes involved in axial elongation. *Rarγ2* is synexpressed with the PSM marker *Msgn1* and overlaps with *Tbx6*, *Hoxc10*, the S–III domains of *Thyl2* and *Ripply2*, and the CNH marker *xNot*. By contrast, *Rarγ2* is expressed at low levels in trunk (where *Myod* and *Rarα* are expressed) and in the anterior, segmented PSM expression domains of *Thyl2* and *Ripply2*. Since absence of RA is required for the proliferation and/or survival of caudal PSM and CNH cells, the presence of RARγ in posterior tissue would be contradictory if it functioned as an activator. We infer that RARγ acts as a repressor throughout unsegmented PSM and CNH where RA is absent, but as an activator of somitomere markers near the differentiation wavefront where *Rarγ2* overlaps with S–III and where *Raldh2* expression indicates the presence of RA. It remains unknown what repressors RARγ targets to indirectly upregulate caudal genes. One possibility is that RARγ represses *Ripply2*, which functions to repress *Tbx6* (reviewed by Dahmann et al., 2011), as supported by the observation that increasing activation with 4647 expands *Ripply2* posteriorly. Hence, RARγ would normally function in the posterior to repress *Ripply2*, therefore promoting expression of *Tbx6*.

RARγ repression promotes the maintenance of unsegmented PSM and CNH

Since high doses of 4647 result in embryos consisting largely of trunk, it is predictable that nearly the entire embryo differentiated into somitomeres (with thicker boundaries). At lower, RARγ-selective 4647 doses, somitomeres were shifted posteriorly and thickened. This phenotype, which is also seen with RA treatment or FGF inhibition by SU5402, was attributed to increased numbers of cells allocated to somitomeres and a decreased progenitor pool (Dubrulle et al., 2001; Moreno and Kintner, 2004). 5099 upregulates both *Tbx6* and *Msgn1*, indicating that unsegmented PSM is expanded by increased RAR

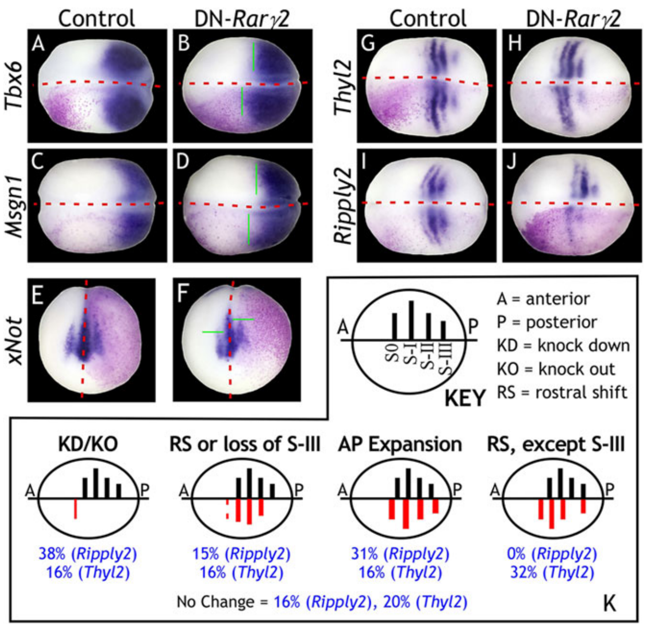

Fig. 8. Overexpression of DN-*Rarγ2* mRNA expands expression of PSM and CNH markers, shifting or knocking down somitomere markers *Thyl2* and *Ripply2*. (A-J) Embryos injected unilaterally at 2- or 4-cell stage. Injected side is indicated by magenta β-gal lineage tracer. (A,C,E,G,I) Control (*mCherry*) mRNA does not alter expression of *Tbx6*, *Msgn1*, *Thyl2*, *Ripply2* or *xNot*. (B,D,F) 2 ng DN-*Rarγ2* mRNA expands expression of *Msgn1* (8/11) and *Tbx6* (15/23) (green lines) and rostrally shifts *xNot* (8/10). (H,J) DN-*Rarγ2* overexpression produces multiple phenotypes of *Thyl2* and *Ripply2* expression, as characterized and scored in K. Neurula embryos shown in dorsal view with anterior to left.

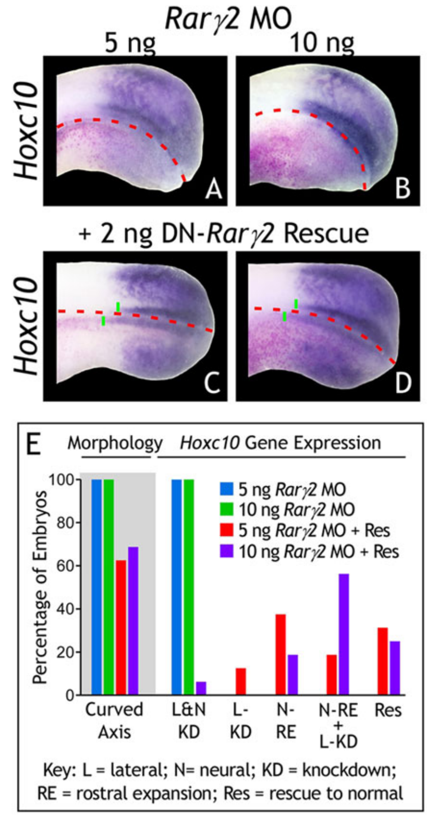

Fig. 9. DN-*Rarγ2* mRNA rescues posterior Hox expression in *Rarγ2* MO embryos. (A-D) Embryos injected unilaterally at 2- or 4-cell stage. Injected side is indicated by magenta β-gal lineage tracer. (A) 2.5 ng *Rarγ2.1* MO +2.5 ng *Rarγ2.2* MO or (B) 5 ng *Rarγ2.1* MO+5 ng *Rarγ2.2* MO diminishes *Hoxc10* expression and curves the embryo axis. (C,D) 2 ng DN-*Rarγ2* mRNA partially rescues this effect and expands neural expression of *Hoxc10*. Tailbud embryos shown in dorsal view with anterior to left. (E) Detailed scoring of the rescue experiment.

repression. However, we note distinct differences in the effects of 4647 on *Tbx6* versus *Msgn1*. *Tbx6* is upregulated by 4647 at early stages but downregulated at later stages, as also observed for the T-box gene *Tbx1* (Janesick et al., 2012). Unlike *Msgn1*, *Tbx6* plays a dual role in the unsegmented PSM and the determination front where it controls the anteroposterior patterning of somitomeres via *Ripply2* (Hitachi et al., 2008).

Msgn1 expression does not overlap somitomeres and functions to maintain unsegmented PSM by encouraging the differentiation of caudal stem cells. Loss of *Msgn1* expression leads to smaller somitomeres owing to the accumulation of bipotential progenitor cells that have not received signals to commit to PSM fate (Fior et al., 2012; Yabe and Takada, 2012). Treatment with 4647 also leads to loss of *Msgn1* and thus somitomeres should be smaller; however, they are larger. Despite such divergent early stage phenotypes, *Msgn1*$^{-/-}$ embryos (Yoon and Wold, 2000) and 4647 embryos both display fewer somites and reduced caudal structures at late stages. Caudal progenitors cannot be instructed to become somites in *Msgn1*$^{-/-}$ embryos. In 4647-treated embryos, the pool is expeditiously transformed into thickened somitomeres early, but the progenitor supply is exhausted before axial elongation is complete, reducing somitomere numbers. That 4647 can differentiate somitomeres at all without *Msgn1* is intriguing. Either *Tbx6* compensates for *Msgn1* knockdown, or 4647 can induce uncommitted, non-PSM progenitor cells to differentiate into somitomeres.

Relief of RARγ repression suppresses PSM and CNH marker gene expression

If RARγ2 functions solely as a repressor, then RARγ2 knockdown should induce a loss of repression phenotype. *Rarγ2* MO microinjection resulted in severely truncated body axes with caudal PSM and posterior Hox markers significantly reduced at tailbud stages, similar to 4647 treatment. This phenotype was attributed to axial defects, not merely developmental delay. We noted three differences between 4647-treated and *Rarγ2* MO-injected embryos. First, axes of *Rarγ2* MO embryos were significantly curved, which was attributed to imbalance/dominance of the uninjected side versus the truncated injected side. Second, caudal PSM markers, while qualitatively reduced with *Rarγ2* MO, also expanded rostrally, even when accounting for shortened axes on injected sides. Third, thickened, posteriorly expanded somitomeres were not seen with *Rarγ2* MO. RARγ2 acting as an activator near the somitogenesis front where RA is present would explain some discrepancies. RA functions in the determination wavefront to antagonize proliferating PSM and promote somitomere differentiation (Moreno and Kintner, 2004). If RA acts through RARγ2 in the wavefront, then loss of *Rarγ2* should expand unsegmented PSM and reduce somitomere expression, exactly as observed.

Axial curvature and loss of *Hoxc10* and *Msgn1* expression in *Rarγ2* MO-injected embryos could be rescued by *Rarγ2*, but not *Rarα2* or *Rarβ2* mRNA. Therefore, *Rarγ2* is the sole receptor responsible for axial elongation, in agreement with *Rarγ2* as the only RAR expressed in caudal domains. *Rarβ2* is present only in trunk and pharyngeal arches (Escriva et al., 2006) and *Rarα2* is completely absent from the blastopore and surrounding area (see figure S1A,B in the supplementary material of Janesick et al.,

Development (2014) 141, 2260-2270 doi:10.1242/dev.103705

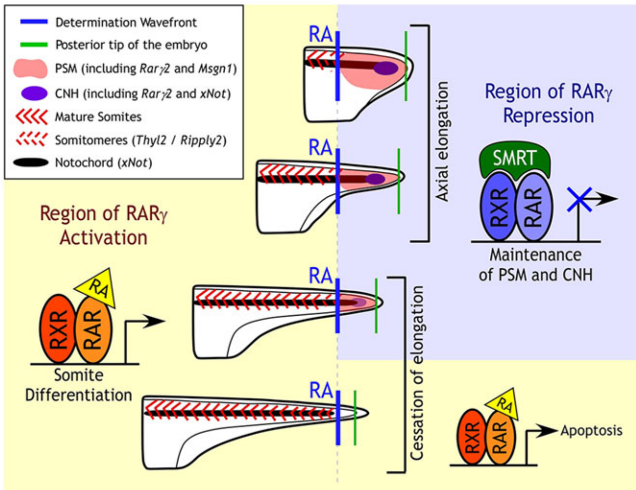

Fig. 10. RARγ functions as both transcriptional activator and repressor during somitogenesis and axial elongation. RARγ is activated by RA near the determination wavefront where PSM differentiates into somitomeres, then mature somites. The progenitor pool within the PSM and CNH domains, which is maintained by *Rarγ* repression, feeds into the wavefront until exhausted, as somitogenesis proceeds faster than progenitors are replenished (Gomez and Pourquie, 2009). As PSM and CNH domains diminish, the distance between RA/wavefront (blue line) and the posterior tip of the embryo (green line) becomes shorter. RA is able to enter the posterior, activating *Rarγ*, switching its function from repressor promoting growth to activator terminating growth. RXR, retinoid X receptor.

2013). *Hoxc10* expression could be rescued in *Rarγ2* MO-injected embryos by co-injecting DN-*Rarγ2* mRNA, definitively establishing that RARγ2 functions as a repressor in the caudal domain. DN-RARγ2 restored *Hoxc10* expression, especially in neural tube, where additional rostral expansion was often observed. DN-RARγ2 rescue restored curved axes only partially. We predict that axial curvature is a loss-of-activation effect inhibiting somitomere formation; therefore, the phenotype should not be rescued by DN-RARγ2, but rescued by wild-type RARγ2, as we observed.

Perhaps the most direct method for relieving repression of RARγ2 in caudal regions is overexpression of dominant-negative co-repressor c-SMRT, which binds RARγ2 preventing recruitment of co-repressors and thereby blocking repression. c-SMRT overexpression resulted in truncated axes with loss of posterior Hox, unsegmented PSM and CNH markers, but not rostral shifting of *Msgn1* and *Tbx6* as had been observed for *Rarγ2* MO embryos. This indicates that rostral shifting in *Rarγ2* MO embryos results from loss of activation rather than relief of repression. We previously showed that c-SMRT not only relieves repression of RAR but also potentiates ligand-mediated activation (Koide et al., 2001). Since c-SMRT was expressed ubiquitously, it could superactivate RARα or RARγ where RA is present. It should also be noted that c-SMRT can interact with other nuclear receptors and transcription factors. Therefore, we can only conclude that c-SMRT overexpression inhibits maintenance of the caudal PSM and progenitor pool (where RA is absent). We cannot draw conclusions about somitomere markers in c-SMRT overexpression embryos since their expression is controlled by RAR activation, which c-SMRT does not reduce.

RAR signaling and posterior Hox gene regulation

We identified a novel function for RARγ as a transcriptional repressor in the regulation of posterior Hox genes. Posterior Hox genes pattern caudal embryonic regions, promote axial elongation (Young et al., 2009) and are linked to cell cycle progression (Gabellini et al., 2003) and therefore proliferation. Axial elongation involves the addition of tissue, as cells must proliferate to contribute segments. Normally, FGF and RA signaling are mutually antagonistic, but we provide evidence that RARγ can support proliferative mechanisms in the absence of RA.

Hox gene expression was altered by 4647 and 5099 treatment, even post-gastrulation. Hence, although Hox gene expression is initiated collinearly during gastrulation, this temporal pattern is not immutable. In support of this model, axial progenitor cells transplanted to anterior locations do not retain their previous Hox identity (McGrew et al., 2008). Furthermore, manipulation of anteroposterior locations of PSM and the determination wavefront resulted in corresponding changes in Hox gene expression (Iimura et al., 2009; Wellik, 2007). We showed that 4647 treatment pushes determination fronts caudally and observed posterior regressions of *Hoxc10*, *Hoxd10* and *Hoxc13* expression. Conversely, rostral expansion in PSM by increasing RAR repression was accompanied by anterior shifts in posterior Hox expression. Owing to posterior prevalence, rostral shifts of *Hoxc10* or *Hoxd10* expression could indicate that thoracic segments will develop caudal structures at later stages. Similarly, rostral shifts in *Hoxc13* could drive lumbar segments to sacral morphology. Homeotic transformations from manipulating RAR repression deserve future study.

Conclusions

We conclude that the RAR-mediated repression of caudal genes is crucial for axial elongation, establishing another important role for active repression by nuclear receptors in body axis extension, as previously shown for head formation (Koide et al., 2001). RARγ2 is likely to function as an activator near the determination wavefront and a repressor to maintain axial progenitor pools in the PSM and CNH. As axial elongation nears completion, RARγ2 functions as an activator because the progenitor pool is exhausted and RA comes into close proximity to the caudal domain of RARγ2, where it can then promote apoptosis and terminate the body axis. This model is attractive because it utilizes the same protein to activate or repress target genes depending on the proximity to RA and explains the high levels of posterior RARγ2 expression. RARγ2 is likely to function in multiple steps of somitogenesis and axial elongation (Fig. 10): (1) preservation of undifferentiated states in the progenitor pools (marked by the CNH); (2) maintenance of PSM; (3) initiation of somitomere differentiation; and (4) axial termination. Future studies require RARγ target gene identification because very few ChIP studies have ascertained direct targets, and even fewer studies have explored subtype-selective RAR targets. In the case of inverse agonist-upregulated genes (the focal point of our study), identifying repressors of PSM and progenitors will be key, as these genes are likely to be targeted by unliganded RAR in a classic ‘repression of a repressor’ mechanism.

MATERIALS AND METHODS

Percellome microarray analysis

Xenopus laevis eggs from three different females were fertilized *in vitro* and embryos staged as described (Janesick et al., 2012). Stage 7 embryos were treated in groups of 25 in 60-mm Petri dishes with 10 ml 0.1× MBS containing 1 μM RAR agonist (TTNPB), 1 μM RAR inverse agonist (AGN193109) or vehicle control (0.1% ethanol). Three dishes per treatment per female were collected (27 dishes total: three technical replicates, three biological replicates per treatment). Each dish of embryos was harvested at stage 18 into 1.5 ml RNAlater (Invitrogen) and stored at 4°C. Samples were homogenized, RNA isolated and DNA quantitated (Kanno et al., 2006). Graded-dose spiked cocktail

Development (2014) 141, 2260-2270 doi:10.1242/dev.103705

(GSC) made of five *Bacillus subtilis* RNA sequences present on Affymetrix GeneChip arrays (AFFX-ThrX-3_at, AFFX-LysX-3_at, AFFX-PheX-3_at, AFFX-DapX-3_at, AFFX-TrpnX-3_at) was added to the sample homogenates in proportion to their DNA concentration (Kanno et al., 2006). GSC-spiked sample homogenates were processed and probes synthesized using standard Affymetrix protocols, applied to *Xenopus* microarray v1.0 GeneChips and analyzed using Percellome software (Kanno et al., 2006). Absolutized mRNA levels were expressed as copy number per cell for each probe set.

Percellome microarray data were analyzed using CyberT (Kayala and Baldi, 2012). We did not use low value thresholding/offsetting or log/VSN normalizations. Bayesian analysis used a sliding window of 101 and confidence value of 10. The *P*-values reported are Bonferroni corrected and Benjamini and Hochber corrected. The full microarray dataset is available at GEO under accession number GSE57352. Genes included in Table 1 comprise a subset upregulated by AGN193109/downregulated by TTNPB based on their regional expression in the posterior.

Embryo microinjection

Xenopus eggs were fertilized *in vitro* and embryos staged as described (Janesick et al., 2012). Embryos were injected bilaterally or unilaterally at the 2- or 4-cell stage with gene-specific morpholinos (MOs) (supplementary material Table S1) and/or mRNA together with 100 pg/embryo β-galactosidase (β-gal) mRNA. For all MO experiments, control embryos were injected with 10 ng standard control MO (GeneTools). Embryos were maintained in 0.1× MBS until appropriate stages. Embryos processed for WISH were fixed in MEMFA, stained with magenta-GAL (Biosynth), and then stored in 100% ethanol (Janesick et al., 2012).

pCDG1-DN-*xRarγ2* was constructed by cloning amino acids 1-393 (lacking the AF-2 domain) into the *Nco*I-*Bam*HI site of pCDG1 (Blumberg et al., 1998). pCDG1-VP16-*xRarγ2* was constructed by cloning the VP16 activation domain upstream of *xRarγ2* into pCDG1. pCDG1-*xRarα2*, pCMX-GAL4-*Rarα* and GAL4-*Rarγ* were from Blumberg et al. (Blumberg et al., 1996). *X. laevis Rarβ1* and *Rarβ2* sequences were found by aligning to the *X. tropicalis* sequences. pCDG1-*xRarβ2* and pCMX-GAL4-*xRarβ* cloning primers are listed in supplementary material Table S2. pCDG1-*xCyp26a1* and pCDG1-*c-smrt* were constructed by PCR amplification of *xCyp26a1* coding regions (Hollemann et al., 1998) or Xl *c-smrt* (37b−, 41+) (Chen et al., 1996; Malartre et al., 2004) and cloning into pCDG1.

xRarα1$^{\mathrm{EGCKG}\rightarrow\mathrm{GSCKV}}$, *xRarα2*$^{\mathrm{EGCKG}\rightarrow\mathrm{GSCKV}}$, *xRarβ2*$^{\mathrm{EGCKG}\rightarrow\mathrm{GSCKV}}$, *xRarγ*1$^{\mathrm{EGCKG}\rightarrow\mathrm{GSCKV}}$ and *xRarγ2*$^{\mathrm{EGCKG}\rightarrow\mathrm{GSCKV}}$ were designed according to Klein et al. (1996), constructed by two-fragment PCR, and cloned into pCDG1 (primer sequences are provided in supplementary material Table S3). Four copies of RXRE$^{1/2}$-GRE$^{1/2}$ (GGAAGGGTTCACCGAA-AGAACACTCGC) were cloned upstream of the TK-luciferase reporter. All pCDG1 plasmids were sequence verified, linearized with *Not*I, and mRNA transcribed using mMessage mMachine T7 (Ambion). pCS2-*mCherry* was linearized with *Not*I and transcribed from the SP6 promoter.

Embryo treatments and reporter assays

Microinjected embryos were treated at stage 8 with the following chemicals (in 0.1× MBS): TTNPB (RAR agonist), NRX204647 (RARγ-selective agonist), NRX205099 (RARγ-selective inverse agonist) or 0.1% ethanol vehicle. Twenty-five embryos were treated in each 60-mm Petri dish containing 10 ml chemical. Treated embryos were fixed in MEMFA and processed for WISH, or separated into five-embryo aliquots at stage 10.5 for luciferase assays, or separated into five-embryo aliquots at neurula or tailbud stage for QPCR as described (Janesick et al., 2012). Each group of five embryos was considered one biological replicate (*n*=1).

WISH

Embryos were microinjected or treated with chemicals after the completion of gastrulation (stage 12.5). WISH was performed as previously described (Janesick et al., 2012). *Rarγ1*, *Rarγ2*, *Rarα* (Blumberg et al., 1992), *Hoxc10*, *Ripply2*, *Thyl2*, *Msgn1* (Klein et al., 2002), *Hoxd10* (Lombardo and Slack, 2001), *Tbx6* (Uchiyama et al., 2001), *Raldh2* (Glinka et al., 1996) and *Myod* (Hopwood et al., 1989) probes were prepared by PCR amplification of coding regions from cDNA with T7 promoter at the 3′ end and *in vitro* transcribed. *Hoxc13* sequence was derived from EST clone XL042b19. Relevant primers are listed in supplementary material Table S4. *Krox20* (Bradley et al., 1993) and *En2* (Bolce et al., 1992) probes were made using T7 and T3 polymerase from *Eco*RI and *Xba*I linearized plasmids, respectively. Probes were transcribed with MEGAscript T7 (Ambion) in the presence of digoxigenin-11-UTP (Roche). Double WISH was conducted as described (Janesick et al., 2012). DNP-*Rarγ2* was transcribed in the presence of dinitrophenol-11-UTP (PerkinElmer). *Hoxc10* expression was quantitated using MATLAB (MathWorks) (supplementary material Fig. S8). The number of purple pixels was calculated by thresholding individual RGB channels (R&B>170, G>120) and dividing by the total number of pixels occupied by the embryo.

Transfection

1 µg CMX-*Rar*$^{\mathrm{EGCKG}\rightarrow\mathrm{GSCKV}}$ effector plasmid was co-transfected with 5 µg tk-(RXRE$^{1/2}$-GRE$^{1/2}$)×4 luciferase reporter and 5 µg pCMX-β-galactosidase transfection control plasmids as previously described (Chamorro-Garcia et al., 2012). For activation assays, NRX204647 was tested from 10^{-11} M to 10^{-5} M. For antagonism assays, NRX205099 was tested from 10^{-10} M to 10^{-5} M against 10^{-8} M 9-cis RA. All transfections were performed in triplicate and reproduced in multiple experiments. Data are reported as normalized luciferase±s.e.m. or percentage reduction±s.e.m. using standard propagation of error (Bevington and Robinson, 2003).

Quantitative real-time reverse transcription PCR (QPCR)

Total RNA from five-embryo pools was DNase treated, LiCl precipitated, and reverse transcribed into cDNA (Janesick et al., 2012). First-strand cDNA was quantitated in a Light Cycler 480 System (Roche) using primer sets listed in supplementary material Table S5 and SYBR Green. Each primer set amplified a single band as determined by gel electrophoresis and melting curve analysis. QPCR data for supplementary material Figs S2 and S7 were analyzed by ΔCt relative to *Histone H4*, correcting for amplification efficiency between RARs (Pfaffl, 2001). QPCR data for supplementary material Figs S11 and S12 were analyzed by ΔΔCt relative to *Histone H4*, normalizing to control embryos. Error bars represent biological replicates calculated using standard propagation of error.

Acknowledgements

We thank Connie Chung for technical help during the early stages of this study and Dr Dennis Bittner for editorial assistance.

Competing interests

The authors declare no competing financial interests.

Author contributions

T.T.L.N. performed WISH. K.A., K.I., S.K. and J.K. executed the Percellome microarray experiment. R.A.S.C. provided 4647 and 5099 chemicals with advice on use. A.J. and B.B. designed, supervised and performed experiments, and wrote, edited and submitted the manuscript.

Funding

Supported by grants from the National Science Foundation (NSF) [IOS-0719576, IOS-1147236 to B.B.]. A.J. was a predoctoral trainee of NSF IGERT DGE 0549479. K.I., S.K. and J.K. were funded by Health Sciences Research Grant H15-kagaku-002 from the Ministry of Health, Labour and Welfare, Japan.

Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.103705/-/DC1

References

Abu-Abed, S., Dollé, P., Metzger, D., Beckett, B., Chambon, P. and Petkovich, M. (2001). The retinoic acid-metabolizing enzyme, CYP26A1, is essential for normal hindbrain patterning, vertebral identity, and development of posterior structures. *Genes Dev.* **15**, 226-240.

Arima, K., Shiotsugu, J., Niu, R., Khandpur, R., Martinez, M., Shin, Y., Koide, T., Cho, K. W. Y., Kitayama, A., Ueno, N. et al. (2005). Global analysis of RAR-responsive genes in the Xenopus neurula using cDNA microarrays. *Dev. Dyn.* **232**, 414-431.

Beck, C. W. and Slack, J. M. W. (1998). Analysis of the developing Xenopus tail bud reveals separate phases of gene expression during determination and outgrowth. *Mech. Dev.* **72**, 41-52.

Bevington, P. R. and Robinson, D. K. (2003). *Data Reduction and Error Analysis for the Physical Sciences*. New York: McGraw-Hill Education.

Blewitt, R. (2009). Enhancer of split-related-2 mRNA shows cyclic expression during somitogenesis in Xenopus laevis. *Biosci. Horizons* **2**, 22-31.

Blumberg, B., Mangelsdorf, D. J., Dyck, J. A., Bittner, D. A., Evans, R. M. and De Robertis, E. M. (1992). Multiple retinoid-responsive receptors in a single cell: families of retinoid "X" receptors and retinoic acid receptors in the Xenopus egg. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 2321-2325.

Blumberg, B., Bolado, J., Jr, Derguini, F., Craig, A. G., Moreno, T. A., Chakravarti, D., Heyman, R. A., Buck, J. and Evans, R. M. (1996). Novel retinoic acid receptor ligands in Xenopus embryos. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 4873-4878.

Blumberg, B., Bolado, J., Jr, Moreno, T. A., Kintner, C., Evans, R. M. and Papalopulu, N. (1997). An essential role for retinoid signaling in anteroposterior neural patterning. *Development* **124**, 373-379.

Blumberg, B., Kang, H., Bolado, J., Chen, H., Craig, A. G., Moreno, T. A., Umesono, K., Perlmann, T., De Robertis, E. M. and Evans, R. M. (1998). BXR, an embryonic orphan nuclear receptor activated by a novel class of endogenous benzoate metabolites. *Genes Dev.* **12**, 1269-1277.

Bolce, M. E., Hemmati-Brivanlou, A., Kushner, P. D. and Harland, R. M. (1992). Ventral ectoderm of Xenopus forms neural tissue, including hindbrain, in response to activin. *Development* **115**, 681-688.

Bradley, L. C., Snape, A., Bhatt, S. and Wilkinson, D. G. (1993). The structure and expression of the Xenopus Krox-20 gene: conserved and divergent patterns of expression in rhombomeres and neural crest. *Mech. Dev.* **40**, 73-84.

Buchberger, A., Bonneick, S. and Arnold, H.-H. (2000). Expression of the novel basic-helix-loop-helix transcription factor cMespo in presomitic mesoderm of chicken embryos. *Mech. Dev.* **97**, 223-226.

Cambray, N. and Wilson, V. (2002). Axial progenitors with extensive potency are localised to the mouse chordoneural hinge. *Development* **129**, 4855-4866.

Cambray, N. and Wilson, V. (2007). Two distinct sources for a population of maturing axial progenitors. *Development* **134**, 2829-2840.

Chakravarti, D., LaMorte, V. J., Nelson, M. C., Nakajima, T., Schulman, I. G., Juguilon, H., Montminy, M. and Evans, R. M. (1996). Role of CBP/P300 in nuclear receptor signalling. *Nature* **383**, 99-103.

Chamorro-García, R., Kirchner, S., Li, X., Janesick, A., Casey, S. C., Chow, C. and Blumberg, B. (2012). Bisphenol A diglycidyl ether induces adipogenic differentiation of multipotent stromal stem cells through a peroxisome proliferator-activated receptor gamma-independent mechanism. *Environ. Health Perspect.* **120**, 984-989.

Chen, J. D. and Evans, R. M. (1995). A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature* **377**, 454-457.

Chen, J. D., Umesono, K. and Evans, R. M. (1996). SMRT isoforms mediate repression and anti-repression of nuclear receptor heterodimers. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 7567-7571.

Dahmann, C., Oates, A. C. and Brand, M. (2011). Boundary formation and maintenance in tissue development. *Nat. Rev. Genet.* **12**, 43-55.

Davis, R. L. and Kirschner, M. W. (2000). The fate of cells in the tailbud of Xenopus laevis. *Development* **127**, 255-267.

de Roos, K., Sonneveld, E., Compaan, B., ten Berge, D., Durston, A. J. and van der Saag, P. T. (1999). Expression of retinoic acid 4-hydroxylase (CYP26) during mouse and Xenopus laevis embryogenesis. *Mech. Dev.* **82**, 205-211.

Dequéant, M.-L. and Pourquié, O. (2008). Segmental patterning of the vertebrate embryonic axis. *Nat. Rev. Genet.* **9**, 370-382.

Dubrulle, J., McGrew, M. J. and Pourquié, O. (2001). FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal Hox gene activation. *Cell* **106**, 219-232.

Escriva, H., Bertrand, S., Germain, P., Robinson-Rechavi, M., Umbhauer, M., Cartry, J., Duffraisse, M., Holland, L., Gronemeyer, H. and Laudet, V. (2006). Neofunctionalization in vertebrates: the example of retinoic acid receptors. *PLoS Genet.* **2**, e102.

Fior, R., Maxwell, A. A., Ma, T. P., Vezzaro, A., Moens, C. B., Amacher, S. L., Lewis, J. and Saude, L. (2012). The differentiation and movement of presomitic mesoderm progenitor cells are controlled by Mesogenin 1. *Development* **139**, 4656-4665.

Fujii, H., Sato, T., Kaneko, S., Gotoh, O., Fujii-Kuriyama, Y., Osawa, K., Kato, S. and Hamada, H. (1997). Metabolic inactivation of retinoic acid by a novel P450 differentially expressed in developing mouse embryos. *EMBO J.* **16**, 4163-4173.

Gabellini, D., Colaluca, I. N., Vodermaier, H. C., Biamonti, G., Giacca, M., Falaschi, A., Riva, S. and Peverali, F. A. (2003). Early mitotic degradation of the homeoprotein HOXC10 is potentially linked to cell cycle progression. *EMBO J.* **22**, 3715-3724.

Glinka, A., Delius, H., Blumenstock, C. and Niehrs, C. (1996). Combinatorial signalling by Xwnt-11 and Xnr3 in the organizer epithelium. *Mech. Dev.* **60**, 221-231.

Gomez, C. and Pourquié, O. (2009). Developmental control of segment numbers in vertebrates. *J. Exp. Zool. B Mol. Dev. Evol.* **312B**, 533-544.

Hitachi, K., Kondow, A., Danno, H., Inui, M., Uchiyama, H. and Asashima, M. (2008). Tbx6, Thylacine1, and E47 synergistically activate bowline expression in Xenopus somitogenesis. *Dev. Biol.* **313**, 816-828.

Hollemann, T., Chen, Y., Grunz, H. and Pieler, T. (1998). Regionalized metabolic activity establishes boundaries of retinoic acid signalling. *EMBO J.* **17**, 7361-7372.

Hopwood, N. D., Pluck, A. and Gurdon, J. B. (1989). MyoD expression in the forming somites is an early response to mesoderm induction in Xenopus embryos. *EMBO J.* **8**, 3409-3417.

Iimura, T., Denans, N. and Pourquié, O. (2009). Establishment of Hox vertebral identities in the embryonic spine precursors. *Curr. Top. Dev. Biol.* **88**, 201-234.

Janesick, A., Shiotsugu, J., Taketani, M. and Blumberg, B. (2012). RIPPLY3 is a retinoic acid-inducible repressor required for setting the borders of the pre-placodal ectoderm. *Development* **139**, 1213-1224.

Janesick, A., Abbey, R., Chung, C., Liu, S., Taketani, M. and Blumberg, B. (2013). ERF and ETV3L are retinoic acid-inducible repressors required for primary neurogenesis. *Development* **140**, 3095-3106.

Kanno, J., Aisaki, K.-i., Igarashi, K., Nakatsu, N., Ono, A., Kodama, Y. and Nagao, T. (2006). "Per cell" normalization method for mRNA measurement by quantitative PCR and microarrays. *BMC Genomics* **7**, 64.

Kayala, M. A. and Baldi, P. (2012). Cyber-T web server: differential analysis of high-throughput data. *Nucleic Acids Res.* **40**, W553-W559.

Klein, E. S., Pino, M. E., Johnson, A. T., Davies, P. J. A., Nagpal, S., Thacher, S. M., Krasinski, G. and Chandraratna, R. A. S. (1996). Identification and functional separation of retinoic acid receptor neutral antagonists and inverse agonists. *J. Biol. Chem.* **271**, 22692-22696.

Klein, S. L., Strausberg, R. L., Wagner, L., Pontius, J., Clifton, S. W. and Richardson, P. (2002). Genetic and genomic tools for Xenopus research: the NIH Xenopus initiative. *Dev. Dyn.* **225**, 384-391.

Koide, T., Downes, M., Chandraratna, R. A. S., Blumberg, B. and Umesono, K. (2001). Active repression of RAR signaling is required for head formation. *Genes Dev.* **15**, 2111-2121.

Lamka, M. L., Boulet, A. M. and Sakonju, S. (1992). Ectopic expression of UBX and ABD-B proteins during Drosophila embryogenesis: competition, not a functional hierarchy, explains phenotypic suppression. *Development* **116**, 841-854.

Leroy, P., Nakshatri, H. and Chambon, P. (1991). Mouse retinoic acid receptor alpha 2 isoform is transcribed from a promoter that contains a retinoic acid response element. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10138-10142.

Lombardo, A. and Slack, J. M. (2001). Abdominal B-type Hox gene expression in Xenopus laevis. *Mech. Dev.* **106**, 191-195.

Malartre, M., Short, S. and Sharpe, C. (2004). Alternative splicing generates multiple SMRT transcripts encoding conserved repressor domains linked to variable transcription factor interaction domains. *Nucleic Acids Res.* **32**, 4676-4686.

McGrew, M. J., Sherman, A., Lillico, S. G., Ellard, F. M., Radcliffe, P. A., Gilhooley, H. J., Mitrophanous, K. A., Cambray, N., Wilson, V. and Sang, H. (2008). Localised axial progenitor cell populations in the avian tail bud are not committed to a posterior Hox identity. *Development* **135**, 2289-2299.

Mollard, R., Viville, S., Ward, S. J., Décimo, D., Chambon, P. and Dollé, P. (2000). Tissue-specific expression of retinoic acid receptor isoform transcripts in the mouse embryo. *Mech. Dev.* **94**, 223-232.

Moreno, T. A. and Kintner, C. (2004). Regulation of segmental patterning by retinoic acid signaling during Xenopus somitogenesis. *Dev. Cell* **6**, 205-218.

Nakaya, Y., Kuroda, S., Katagiri, Y. T., Kaibuchi, K. and Takahashi, Y. (2004). Mesenchymal-epithelial transition during somitic segmentation is regulated by differential roles of Cdc42 and Rac1. *Dev. Cell* **7**, 425-438.

Nowotschin, S., Ferrer-Vaquer, A., Concepcion, D., Papaioannou, V. E. and Hadjantonakis, A.-K. (2012). Interaction of Wnt3a, Msgn1 and Tbx6 in neural versus paraxial mesoderm lineage commitment and paraxial mesoderm differentiation in the mouse embryo. *Dev. Biol.* **367**, 1-14.

Olivera-Martinez, I., Harada, H., Halley, P. A. and Storey, K. G. (2012). Loss of FGF-dependent mesoderm identity and rise of endogenous retinoid signalling determine cessation of body axis elongation. *PLoS Biol.* **10**, e1001415.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45.

Pfeffer, P. L. and De Robertis, E. M. (1994). Regional specificity of RAR gamma isoforms in Xenopus development. *Mech. Dev.* **45**, 147-153.

Pourquié, O. and Tam, P. P. L. (2001). A nomenclature for prospective somites and phases of cyclic gene expression in the presomitic mesoderm. *Dev. Cell* **1**, 619-620.

Rhinn, M. and Dolle, P. (2012). Retinoic acid signalling during development. *Development* **139**, 843-858.

Sakai, Y., Meno, C., Fujii, H., Nishino, J., Shiratori, H., Saijoh, Y., Rossant, J. and Hamada, H. (2001). The retinoic acid-inactivating enzyme CYP26 is essential for establishing an uneven distribution of retinoic acid along the anterio-posterior axis within the mouse embryo. *Genes Dev.* **15**, 213-225.

Shimono, K., Tung, W.-e., Macolino, C., Chi, A. H.-T., Didizian, J. H., Mundy, C., Chandraratna, R. A., Mishina, Y., Enomoto-Iwamoto, M., Pacifici, M. et al. (2011). Potent inhibition of heterotopic ossification by nuclear retinoic acid receptor-gamma agonists. *Nat. Med.* **17**, 454-460.

Shum, A. S. W., Poon, L. L. M., Tang, W. W. T., Koide, T., Chan, B. W. H., Leung, Y.-C. G., Shiroishi, T. and Copp, A. J. (1999). Retinoic acid induces down-regulation of Wnt-3a, apoptosis and diversion of tail bud cells to a neural fate in the mouse embryo. *Mech. Dev.* **84**, 17-30.

Sive, H. L., Draper, B. W., Harland, R. M. and Weintraub, H. (1990). Identification of a retinoic acid-sensitive period during primary axis formation in Xenopus laevis. *Genes Dev.* **4**, 932-942.

Sucov, H. M., Murakami, K. K. and Evans, R. M. (1990). Characterization of an autoregulated response element in the mouse retinoic acid receptor type beta gene. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5392-5396.

Takemoto, T., Uchikawa, M., Yoshida, M., Bell, D. M., Lovell-Badge, R., Papaioannou, V. E. and Kondoh, H. (2011). Tbx6-dependent Sox2 regulation determines neural or mesodermal fate in axial stem cells. *Nature* **470**, 394-398.

Tam, P. P. L., Goldman, D., Camus, A. and Schoenwolf, G. C. (2000). 1 Early events of somitogenesis in higher vertebrates: allocation of precursor cells during gastrulation and the organization of a meristic pattern in the paraxial mesoderm. *Curr. Top. Dev. Biol.* **47**, 1-32.

Thacher, S. M., Vasudevan, J. and Chandraratna, R. A. S. (2000). Therapeutic applications for ligands of retinoid receptors. *Curr. Pharm. Des.* **6**, 25-58.

Tsang, K. Y., Sinha, S., Liu, X., Bhat, S. and Chandraratna, R., A. (2003). Disubstituted Chalcone Oximes Having Rar(Gamma)-Retinoid Receptor Antagonist Activity. European Patent Office.

Uchiyama, H., Kobayashi, T., Yamashita, A., Ohno, S. and Yabe, S. (2001). Cloning and characterization of the T-box gene Tbx6 in Xenopus laevis. *Dev. Growth Differ.* **43**, 657-669.

von Dassow, G., Schmidt, J. E. and Kimelman, D. (1993). Induction of the Xenopus organizer: expression and regulation of Xnot, a novel FGF and activin-regulated homeo box gene. *Genes Dev.* **7**, 355-366.

Wellik, D. M. (2007). Hox patterning of the vertebrate axial skeleton. *Dev. Dyn.* **236**, 2454-2463.

Weston, A. D., Blumberg, B. and Underhill, T. M. (2003). Active repression by unliganded retinoid receptors in development: less is sometimes more. *J. Cell Biol.* **161**, 223-228.

Wong, C. W. and Privalsky, M. L. (1998). Transcriptional silencing is defined by isoform- and heterodimer-specific interactions between nuclear hormone receptors and corepressors. *Mol. Cell. Biol.* **18**, 5724-5733.

Yabe, T. and Takada, S. (2012). Mesogenin causes embryonic mesoderm progenitors to differentiate during development of zebrafish tail somites. *Dev. Biol.* **370**, 213-222.

Yoon, J. K. and Wold, B. (2000). The bHLH regulator pMesogenin1 is required for maturation and segmentation of paraxial mesoderm. *Genes Dev.* **14**, 3204-3214.

Yoon, J. K., Moon, R. T. and Wold, B. (2000). The bHLH class protein pMesogenin1 can specify paraxial mesoderm phenotypes. *Dev. Biol.* **222**, 376-391.

Young, T., Rowland, J. E., van de Ven, C., Bialecka, M., Novoa, A., Carapuco, M., van Nes, J., de Graaff, W., Duluc, I., Freund, J.-N. et al. (2009). Cdx and Hox genes differentially regulate posterior axial growth in mammalian embryos. *Dev. Cell* **17**, 516-526.