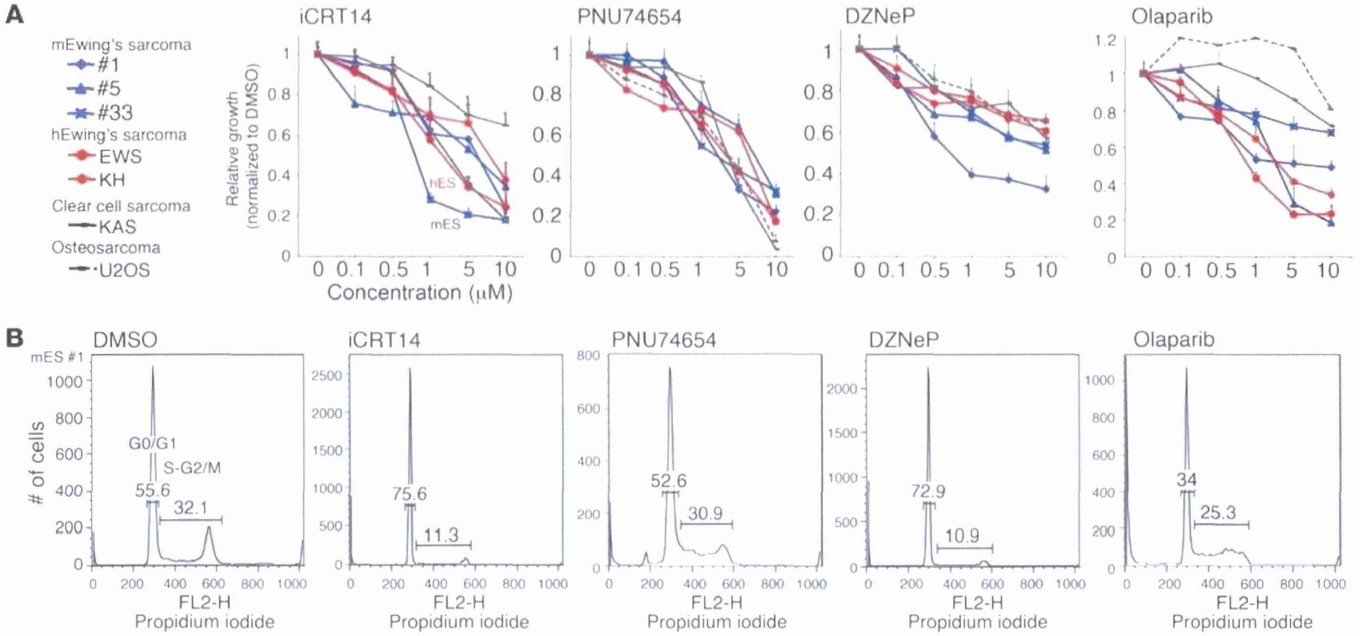

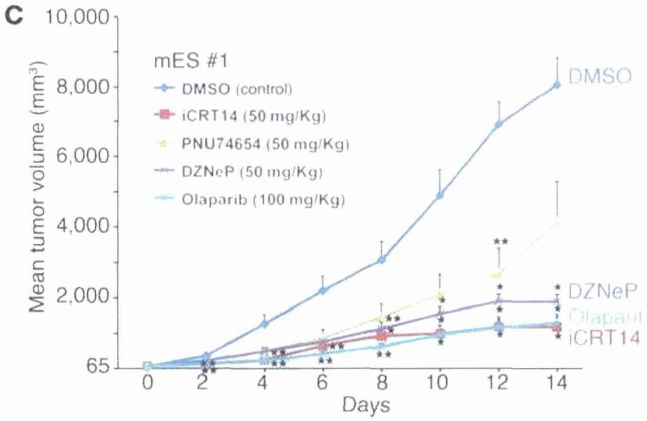

Figure 7

Effects of small molecule inhibitors on Ewing's sarcoma. (**A**) In vitro growth inhibition of mouse and human Ewing's sarcoma, human clear cell sarcoma, and human osteosarcoma cell lines by iCRT14, PNU74654, DZNeP, and olaparib. Cells were treated with each reagent at the indicated concentration for 48 hours. The experiment was performed in triplicate, and average suppression rates with standard errors are indicated. (**B**) Cell cycle analyses of mES #1 cells treated with 1 μM of each reagent (i.e., DMSO, iCRT14, PNU74854, DZNep, or Olaparib) for 48 hours. Frequencies (percentages) of G_1 and G_2/M are indicated. (**C**) Growth inhibitory effects of small molecules for mouse Ewing's sarcoma in vivo. mES #1 cells were transplanted subcutaneously into nude mice, and tumor volume was measured every other day. Mean tumor volumes ± SD for 5 mice of each group are plotted. $^*P < 0.01$; $^{**}P < 0.03$.

targeting exact cell types. The plasticity of precursor cells as well as their oncogenic potency due to chimeric transcription factors can be evaluated by the present approach and constitutes a useful tool for clarifying oncogenic mechanisms of childhood cancer.

Methods

Purification of eSZ cells. Femoral and humeral bones of BALB/c mouse embryos were removed aseptically on 18.5 dpc, and they were microdissected into eSZ, eGP, and eSyR under a stereomicroscope (Zeiss Stemi 2000-C, Carl Zeiss MicroImaging). Embryonic mesenchymal cells of the head or trunk were also prepared from the same embryos during each experiment. Each region was minced and gently digested with 2 mg/ml collagenase (Wako Pure Chemical) at 37°C for 2 hours. They were cultured in growth medium composed of Iscove's Modified Dulbecco's Medium (Invitrogen) supplemented with 15% fetal bovine serum and subjected immediately to retroviral infection. Fractionation of $PTHLH^+$ and $PTHLH^-$ eSZ populations was achieved using a rabbit anti-PTHLH (Abcam) and a CELLection Biotin Binder Kit (Dynal) according to the manufacturer's protocol. The frequency of the $PTHLH^+$ cells reached 8.3% of total eSZ cells (12-fold enrichment).

Retroviral infection and transplantation. N-terminal FLAG-tagged *EWS-FLI1* and *EWS-ERG* were introduced into the pMYs-IRES-GFP or pMYs-IRES-Neo vectors. The full-length *EWS-FLI1* cDNA was a gift from Susanne Baker (St. Jude Children's Research Hospital, Memphis, Tennessee, USA), and *EWS-ERG* was cloned from a human Ewing's sarcoma case. Retroviral infections of eSZ, eGP, or shaft cells were performed as described previously (53). Infection efficiency was examined using a FACSCalibur flow cytometer (Becton Dickinson). After 48 hours of spin infection, the cells were mixed with growth factor–reduced Matrigel (Becton Dickinson) and were transplanted subcutaneously to BALB/c nude mice. The mice were observed daily to check for tumor formation and general condition. Tumors were resected and subjected to further examination when subcutaneous masses reached 15 mm in diameter. Some tumors (1×10^6 cells) were serially transplanted subcutaneously or injected into the tail veins (1×10^6 cells) of nude mice to confirm tumorigenicity and metastatic activities.

Histopathology and immunohistochemistry. Formaldehyde- or paraformaldehyde-fixed tumor tissues were embedded in paraffin, and sections were stained with hematoxylin and eosin using standard techniques. Bromodeoxyuridine (BrdU) labeling was achieved by intraperitoneal injection of 1 mg/ml BrdU 30 minutes before sacrifice. eSZ cells were cultured on chamber slides and were fixed with 100% methanol. EWS-FLI1 and EWS-ERG antigens were detected using a polyclonal rabbit anti-FLAG antibody (Sigma-Aldrich) in conjunction with the VECTOR M.O.M. Immunodetection Kit (Vector Laboratories) or FITC-conjugated anti-rabbit immunoglobulin. The following primary antibodies were used: anti-BrdU (Molecular

Table 3
IC_{50} values of inhibitors

	Inhibitors			
Tumor cells	**iCRT14 (μM)**	**PNU74654 (μM)**	**DZNeP (μM)**	**Olaparib (μM)**
mES #1	5.90	2.08	0.68	7.07
mES #5	5.61	6.79	10.30	2.36
mES #33	0.76	1.96	10.95	17.50
hES_EWS	1.71	2.98	13.46	0.86
hES_KH	7.41	6.05	15.87	2.70
hCCS_KAS	2.16	3.16	16.58	28.85
hOS_U2OS	14.79	3.42	19.33	40.42

Probes), anti-mouse CD99 (a gift of Dietmar Vestweber, Max Planck Institute for Molecular Biomedicine, Muenster, Germany), anti-COL2A (Millipore), anti-S100 (Dako), anti-COL10 (SLS), anti-CD57 (Sigma-Aldrich), anti-NGFR (Millipore), anti–β-catenin (Becton Dickinson), anti-nestin (Chemicon), and anti-myosin (Nichirei). Immunofluorescent images were photographed with a Zeiss LSM 710 laser scanning microscope with a ×40 objective (Zeiss) and LSM Software ZEN 2009 (Zeiss).

Western blotting. Western blot analysis was performed using lysates of whole tumor tissues as described previously (54).

RT-PCR and real-time quantitative RT-PCR. Total RNA extraction, reverse transcription, and RNA quantification were performed according to methods described previously (54). Conventional RT-PCR and real-time quantitative RT-PCR were performed by using a Gene Amp 9700 thermal cycler (Applied Biosystems) and a 7500 Fast Real-Time PCR System (Applied Biosystems), respectively. The sequences of the oligonucleotide primers are shown in Supplemental Excel File 6.

Luciferase assay. A 1,340-bp genomic DNA fragment upstream from the murine *Gdf5* exon 1 was amplified by PCR using the following primers: forward (5′-TTCTATAATCCTACTCTGTAG-3′) and reverse (5′-CTGAAAATAACTCGTTCTTG-3′). The fragment was inserted into the pGL4.10 vector (Promega) and transfected into eSZ, eGP, eSyR, or trunk cells using Lipofectamine 2000 (Invitrogen). Luciferase assays were performed as described previously (54).

In vitro differentiation assay. Cells were plated at 2×10^5 cells per well in 6-well plates and cultured in growth medium. Adipogenic, chondrogenic, osteogenic, myogenic, and neurogenic differentiation assays were conducted according to the methods previously described (55–57).

Microarray analysis. GeneChip analysis was conducted to determine gene expression profiles. A per cell normalization method was applied to eSZ and eGP samples (58). Briefly, cellular lysates were prepared with RLT buffer (QIAGEN). After RNA cocktails were added to the cell lysates according to the amount of DNA, total RNA was extracted using the RNeasy Mini Kit (QIAGEN). The murine Genome 430 2.0 Array (Affymetrix) was hybridized with aRNA probes generated from eSZ and eGP cells and murine Ewing's sarcoma tissue. After staining with streptavidin-phycoerythrin conjugates, arrays were scanned using an Affymetrix GeneChip Scanner 3000 and analyzed using Affymetrix GeneChip Command Console Software (Affymetrix) and GeneSpring GX 11.0.2 (Agilent Technologies) as described previously (59). The expression data for eSZ and eGP cells were converted to mRNA copy numbers per cell by the Percellome method, quality controlled, and analyzed using Percellome software (58). GSEA was performed using GSEA-P 2.0 software (60).

Data comparisons and clustering between murine and human microarray data sets. The microarray data from 10 murine Ewing's sarcoma samples were compared with human microarray data sets. Data from the ONCOMINE database (https://www.oncomine.org/) were accessed in June 2011. Five microarray studies containing 117 tumor samples that were analyzed using Human Genome U133A Array (Affymetrix) were queried for gene expression. CEL files from E-MEXP-353 (61), E-MEXP-1142 (62), GSE6481 (63), GSE7529 (64), GSE21122 (65), GSE6461 (66), GSE42548 (67), GSE23972 (68), GSE20196 (69), and GSE10172 (70) were downloaded. The probe sets of the human U133A array were translated into 23,860 murine 430 2.0 arrays by the translation function of GeneSpring using Entrez Gene ID to make a novel common platform. Hierarchical clustering was achieved using log-transformed data and the following procedure. For the initial statistical analysis, 13,026 genes that showed a "present" or "marginal" call in at least 24 of a total of 32 human Ewing's sarcoma samples were selected. Then, 12,340 probes were selected by 1-way ANOVA ($P < 0.05$) analysis. Finally, 1,819 probes that showed >2-fold differences of expression in at least 3 tumor types were selected. With these 1,819 probes, hierarchical clustering was performed using the average linkage method and the Pearson's centered measurements. In addition, a probe set consisting of the 2,000 sequences that were the most altered in expression in human and mouse round cell tumors (Ewing's sarcoma, neuroblastoma, poorly differentiated synovial sarcoma, and malignant lymphoma) was used to distinguish each tumor from the other 3 using a fold-change analysis. Then, the frequencies of these 2,000 probes were compared between mouse Ewing's sarcoma and 4 human tumor types and between human Ewing's sarcoma and 4 mouse tumor types to find the closest tumor type using similar entities from GeneSpring.

ChIP. A total of 5×10^6 cells per immunoprecipitation were cross-linked with 10% formaldehyde for 10 minutes at room temperature. Histone immunoprecipitation was performed with anti-histone antibodies targeted against H3K9/K14Ac, H3K4/me3, H3K27/me3, total H3 (Cell Signaling Technologies), or H3K9/me3 (Millipore) preconjugated to protein G magnetic beads. Immunoprecipitated DNA was amplified with primers specific for each region. Sequences are shown in Supplemental Excel File 6.

Cre/loxP-mediated gene silencing. eSZ cells were transduced with a floxed *EWS-FLI1* retrovirus, and Ewing's sarcoma cells were obtained from a subcutaneous tumor developed in a nude mouse. Tumor cells were transduced with pMSCV-Cre-puro retrovirus in vitro. Senescence-associated β-galactosidase expression was detected using a Senescence Detection Kit (Biovision) 4 days after transduction of the retrovirus.

siRNA interference studies. For knockdown of *FLI1*, *Dkk2*, *Catnb*, *Prkcb1*, *Ezh2*, *Igf1*, *Dkk1*, and *Erg*, siRNAs were purchased from QIAGEN. The list of siRNAs is shown in Supplemental Excel File 7. siRNAs were introduced into mouse Ewing's sarcoma cells according to the manufacturer's protocol. Knockdown efficiencies were confirmed by Western blotting using anti-FLAG (Sigma-Aldrich), anti-ERG and anti–PKC β1 (Santa Cruz Biotechnology), anti–β-catenin (Becton Dickinson), and anti-EZH2 (Cell Signaling Technologies) or RT-PCR.

Pharmacological experiments with specific inhibitors. Mouse Ewing's sarcoma cells were treated with MEK1 inhibitor U0126 (Cell Signaling Technologies) in vitro. Both mouse and human Ewing's sarcoma cell lines were treated with WNT/β-catenin inhibitors, iCRT14 and PNU74654 (Tocris Bioscience); an EZH2 inhibitor, DZNeP (Cayman Chemical); or a PARP1 inhibitor, olaparib (Selleckchem), both in vitro and in vivo. Inhibition of ERG phosphorylation was examined by Western blotting using anti-P-ERK1/2 and anti-ERK1/2 (Cell Signaling

Technologies). For in vivo experiments, 1×10^6 tumor cells were transplanted subcutaneously into nude mice, and the mice were treated with specific inhibitors when the tumor diameter reached 5 mm. All the inhibitors were dissolved in 0.2% DMSO, and they were administered by intraperitoneal injection 3 times per week.

Cell cycle assay. Single-cell suspensions were permeabilized with 0.1% Triton X-100 in PBS, and 50 mg/ml propidium iodide and 1 mg/ml RNAse A were added. The cell suspensions were then analyzed by using a FACSCalibur flow cytometer and ModFit software (Becton Dickinson).

Cloning retroviral integration sites. Retroviral integration sites of individual mouse Ewing's sarcoma were isolated by inverse PCR, sequenced, and mapped as described previously (71).

Accession numbers. The microarray data sets are accessible through the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo), with accession numbers GSE32615 and GSE32618.

Statistics. Continuous distributions were compared with 2-tailed Student's *t* test. Survival analysis was performed using the Kaplan-Meier life table method, and survival between groups was compared with the log-rank test. The 2-proportion *z* test was used to evaluate the significance of differences in the matched probe sets between 2 tumor types. All *P* values were 2 sided, and a *P* value of less than 0.05 was considered significant.

Study approval. Animals were handled in accordance with the guidelines of the animal care committee at the Japanese Foundation for Cancer Research, which gave ethical approval for these studies.

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RESEARCH ARTICLE

Active repression by RARγ signaling is required for vertebrate axial elongation

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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.

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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.

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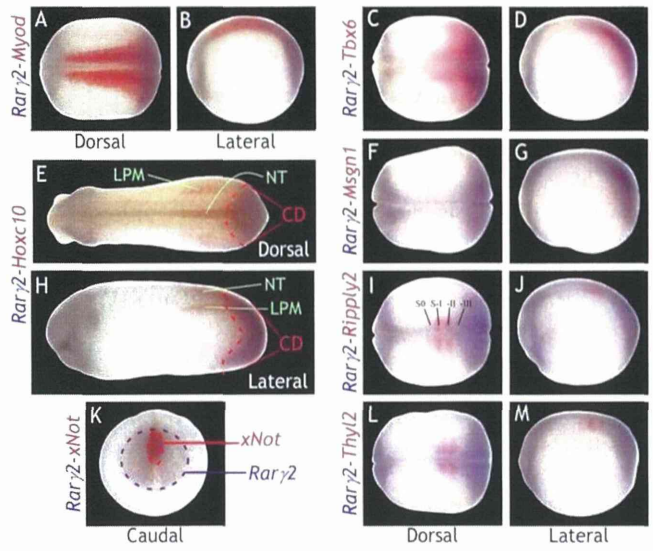

Fig. 1. Double WISH reveals the spatial relationship between *Rarγ2* and posterior Hox, PSM and CNH genes. (A-M) *Rarγ2* is stained with BM Purple and the other genes are stained with Fast Red. *Rarγ2* is caudal to *Myod* and *Tbx6* (A-D), but synexpressed with *Msgn1* (F,G) in neurula stage *Xenopus* 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, $(RXRE^{1/2}\text{-}GRE^{1/2})\times4$ 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

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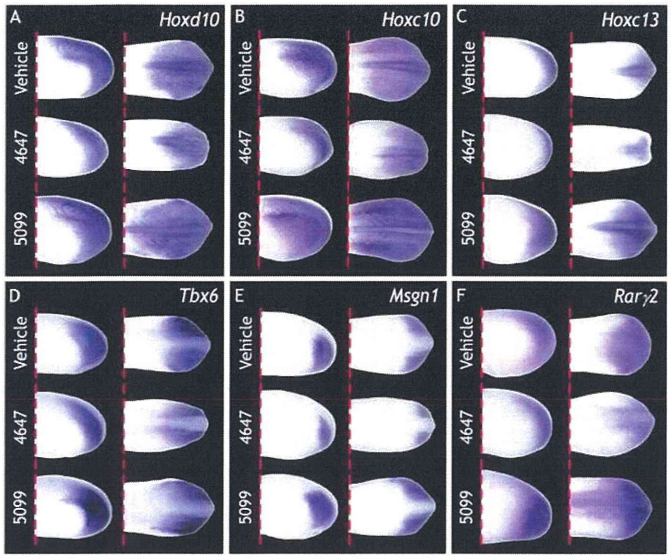

Fig. 2. Posterior Hox and PSM markers are reduced by RARγ-selective agonist and expanded by RARγ-selective inverse agonist. (A-F) WISH from embryos treated post-gastrulation (stage 12.5) with 10 nM 4647, 0.5 μM 5099 or vehicle (0.1% ethanol). Dashed red line represents half the embryo axis. 4647 diminishes 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.

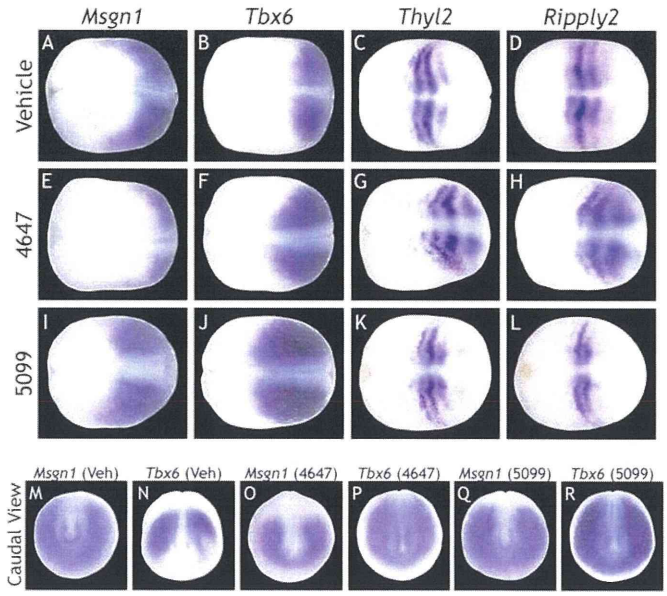

Fig. 3. PSM markers are modulated by RARγ-selective agonist and inverse agonist. (A-R) WISH from embryos treated post-gastrulation (stage 12.5) with 10 nM 4647, 0.5 μM 5099 or vehicle (0.1% ethanol). (A-D) Control expression of *Msgn1*, *Tbx6*, *Thyl2* and *Ripply2*. (E) *Msgn1* expression diminished 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

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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. 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).

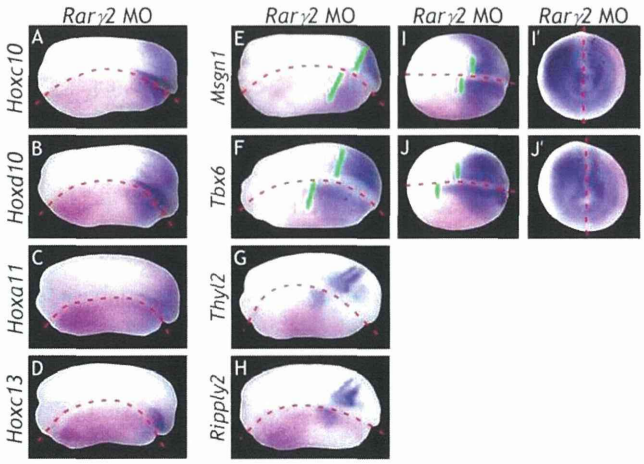

Fig. 4. RARγ2 knockdown alters expression of posterior Hox and PSM markers. (A-J′) Embryos were injected unilaterally at the 2- or 4-cell stage with 7.5 ng *Rarγ2.1* MO+7.5 ng *Rarγ2.2* MO. Injected side is indicated by magenta β-gal lineage tracer. *Rarγ2.1/2.2* MO decreases expression of (A) *Hoxc10* (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.

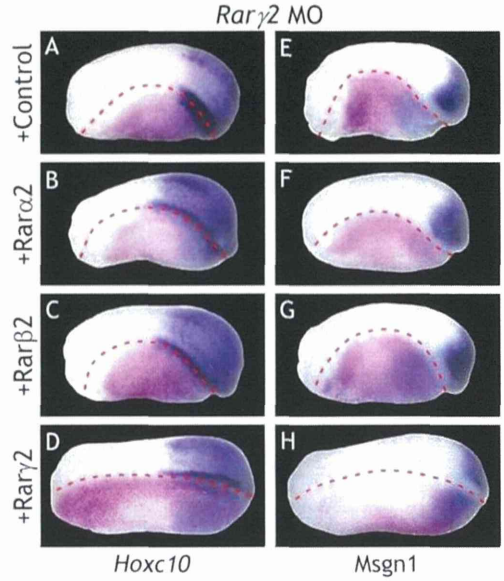

Fig. 5. *Rarγ2* mRNA rescues posterior Hox and PSM expression in *Rarγ2* MO embryos. (A-H) Embryos injected unilaterally at 2- or 4-cell stage. Injected side is indicated by magenta β-gal lineage tracer. (A,E) 5 ng *Rarγ2.1* MO+5 ng *Rarγ2.2* MO+control (*mCherry*) mRNA diminishes *Hoxc10* and *Msgn1* 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.

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

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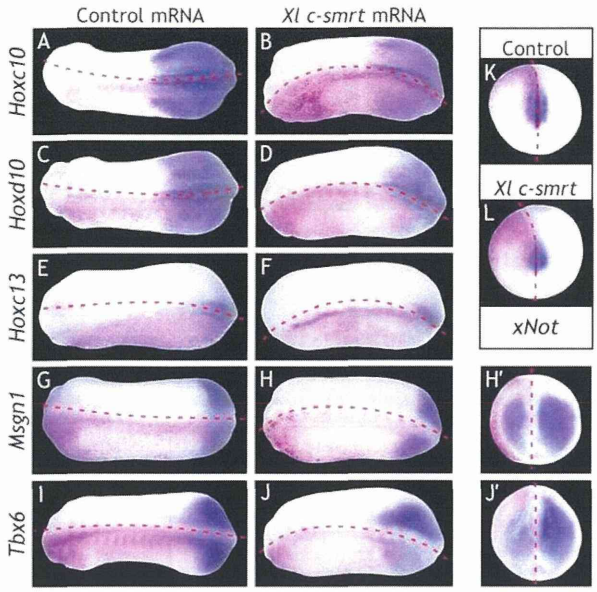

Fig. 6. c-SMRT overexpression knocks down posterior Hox, PSM and CNH markers. Embryos injected unilaterally at 2- or 4-cell stage with 4 ng *c-smrt* mRNA or control (*mCherry*) mRNA. Injected side indicated by magenta β-gal lineage tracer. (A,C,E,G,I,K) Control expression of *Hoxc10*, *Hoxd10*, *Hoxc13*, *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).

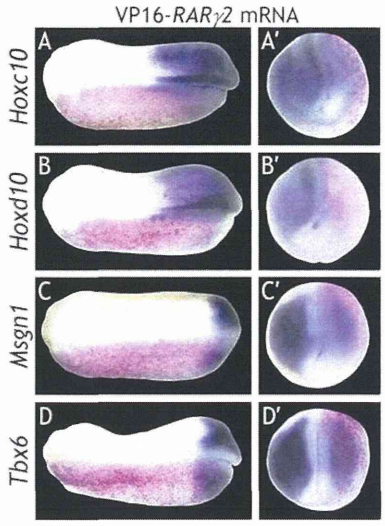

Fig. 7. VP16-RARγ2 overexpression knocks down posterior Hox and PSM marker expression. Embryos injected unilaterally at 2- or 4-cell stage with 0.3 ng VP16-*Rarγ2* mRNA or control (*mCherry*) mRNA. Injected side is indicated by magenta β-gal lineage tracer. Control expression of *Hoxc10*, *Hoxd10*, *Msgn1* 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