Corepressor SMRT is required to maintain Hox transcriptional memory during somitogenesis

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Nuclear hormone receptors (NRs), such as retinoic acid receptors (RARs), play critical roles in vertebrate development and homeostasis by regulating target gene transcription. Their activity is controlled by ligand-dependent release of corepressors and subsequent recruitment of coactivators, but how these individual receptor modes contribute to development are unknown. Here, we show that mice carrying targeted knockin mutations in the corepressor Silencing Mediator of Retinoid and Thyroid hormone receptor (SMRT) that specifically disable SMRT function in NR signaling (SMRT<sup>ΔR</sup>), display defects in cranial neural crest cell-derived structures and posterior homeotic transformations of axial vertebrae. SMRT<sup>ΔR</sup> embryos show enhanced transcription of RAR targets including Hox loci, resulting in respecification of vertebral identities. Up-regulated histone acetylation and decreased H3K27 methylation are evident in the Hox loci whose somitic expression boundaries are rostrally shifted. Furthermore, enhanced recruitment of super elongation complex is evident in rapidly induced non-Pol II-paused targets in SMRT<sup>ΔR</sup> embryonic stem cells. These results demonstrate that SMRT-dependent repression of RAR is critical to establish and maintain the somitic Hox code and segmental identity during fetal development via epigenetic marking of target loci.

Significance

Retinoic acid (RA) is an important transcriptional regulator during both vertebrate and invertebrate body patterning. The Homeobox (Hox) gene family is activated by a gradient of RA formed along the length of the embryo at specific time points during fetal development. Generation of a genetically modified mouse harboring mutations in the SMRT repressor demonstrated that SMRT-dependent repression of retinoic acid receptor (RAR) is critical to establish and maintain the somitic Hox code and segmental identity during fetal development via epigenetic marking of target loci.


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repression in RA-dependent pattern formation has been that the complete SMRT knockout is embryonic lethal, in part due to its association with numerous transcription factors, including p53, Myc, MyoD, PLZF, RelA/p65, and Stat5 (18, 19).

Previously, it has been demonstrated in mouse ES cells that RA also rapidly recruits super elongation complex (SEC) components to nonpaused target genes such as RA-degrading enzyme, Cyp26a1 (20). This class of genes is unique in comparison with embryos with RA-induced Hox gene clusters which possess paused-Pol II near their transcription start sites. Currently, it is unknown how these SEC components are rapidly recruited and regulated (20).

In the present study, by combining molecular, cellular, and gene targeting approaches, we demonstrated that the corepressor SMRT interactions with RARs are critical for specification and maintenance of axial Hox codes. We also show that repressive histone acetylation and methylation marks in Hox loci are established by histone deacetylases (HDACs) and Polycomb repressive complex (PRC) that are recruited by SMRT. Finally, we show that RA availability during development is regulated by SMRT-dependent transcriptional repression of the RA-degrading enzyme, Cyp26a1. These findings highlight unexpected roles for corepressor SMRT and importance of NR-dependent repression in murine axial development.

Results

SMRT\textsuperscript{NRID} Mutant Mouse Phenotype. Importantly, newborn SMRT\textsuperscript{NRID} mice are born at expected Mendelian ratios and appear grossly normal except for a kinked tail, potential neural tube closure defect, as well as a 10% reduction in body size and weight (SI Appendix, Fig. S1 A and B). This smaller body size and weight reduction persist until weaning. Postnatal metabolic phenotypes of SMRT\textsuperscript{NRID} mice have been discussed (21).

Normal Hindbrain Segmentation and Defective Cranial Neural Crest-Derived Structures. In contrast to reported RAR-mediated forebrain and neural stem cell phenotypes of SMRT null mice (19), we did not observe any major defects in the SMRT\textsuperscript{NRID} cortex (21). As RAR signaling plays critical roles in hindbrain patterning (16), we examined rhombomere segmentation in E8.5–10.5 SMRT\textsuperscript{NRID} embryos (SI Appendix, Fig. S1 C and D). Although we did not observe abnormal expression of rhombomere marker genes during hindbrain segmentation in SMRT\textsuperscript{NRID} animals, 2 of 10 embryos exhibited fused branches of the 9th and 10th cranial ganglia (SI Appendix, Fig. S1E). Furthermore, we observed malformations in the retrotympanic processes of the squamous temporal bone and thyroid cartilage with 100% penetrance (SI Appendix, Fig. S1 F and G). Interestingly, the spinal processes of the first and second cervical vertebrae were dramatically reduced in SMRT\textsuperscript{NRID} animals (SI Appendix, Fig. S2A). In addition, cervical spine bifida was prevalent in SMRT\textsuperscript{NRID} animals (SI Appendix, Fig. S2B). As these structures are formed by cells of cranial neural crest origin, we speculate that SMRT\textsuperscript{NRID} mice might suffer from NCC defects by either cell-autonomous or surrounding mesodermal RA de-repression (22). Together, these findings demonstrate that NR-dependent SMRT-directed repression is critical for the normal formation of NCC-derived skeletal elements rather than forebrain and neural progenitor developments as in whole-body SMRT null animals (19). Interestingly, the malformation of NCC-derived structures seen in SMRT\textsuperscript{NRID} mice was highly reminiscent of findings in Rara\textsuperscript{-/-} mutants, suggesting that a SMRT/RAR pathway may direct other developmental programs (17).

Homoetic Transformation of Axial Vertebrae. Next, we examined the expression pattern of SMRT in developing mouse embryos. Using whole mount in situ hybridization (WISH), the expression of SMRT was ubiquitously detected in the developing somites, with highest signals occurring in the tail somites (SI Appendix, Fig. S2C). qPCR analyses of the somite regions confirmed the SMRT WISH results (SI Appendix, Fig. S2D). The expression of the related corepressor NCoR in the somites closely resembled the somitic expression pattern for SMRT at E10.5 (SI Appendix, Fig. S2D). These results suggested that SMRT and/or NCoR could play an important role in axial development.

An examination of embryos at multiple stages revealed a striking posterior homeotic transformation of C5–7 cervical vertebrae and anterior transformation of L1 lumbar vertebrae in SMRT\textsuperscript{NRID} mice, similar to those found in embryos exposed to a high dose of systemic RA at E10.5–11 (Fig. 1 A–C). Tuberculi anterior (TA) flanges, which should normally be unique to C6, appeared on both C5 and C6 vertebrae (Fig. 1B). In some cases, C6 lost the foramen transversarium (FT) which, in the upper six vertebrae, provides passage to a plexus of sympathetic nerves and vertebral blood vessels (SI Appendix, Fig. S2E). In addition, we found the frequent formation of either one or two incomplete ribs on C7, which are fused to the first thoracic rib (SI Appendix, Fig. S2 F and G). In 18% of SMRT\textsuperscript{NRID} mutants, the first lumbar vertebra (L1) acquires a thoracic identity with acquisition of a supernumerary 14th rib (Fig. 1C). The aforementioned pattern is highly reminiscent of that observed in Cyp26a1 null mice (23). No significant defects in somite size or overall number were observed in SMRT\textsuperscript{NRID} embryos as shown by WISH with somite markers, Myogenin and Unc5c.1 (SI Appendix, Fig. S2H), indicating that the homeotic shifts are not due to the global defects in somitogenesis. The above findings indicate that SMRT/NR-directed repression is an integral component in axial somite specification and that this repression cannot be compensated by another NR corepressor, NCoR.
Rescue of Homoeotic Transformation by RAR Antagonist at E10.5.

These homeotic effects demonstrate that SMRT is a required mechanistic component to accurately translate the chemical RA agonist gradient into an appropriate somitic Hox code during somitogenesis. Indeed, the axial phenotype of SMRTmRID mice is virtually identical to the previously described vertebral transformation seen with ectopic all-trans RA exposure, which is thought to disrupt axial positional information at late somitogenesis (24). This led us to wonder whether loss of repression in SMRTmRID embryos could be rescued with a chemical retinoid antagonist such as BMS 493 (a pan-RAR antagonist). We chose the lower BMS 493 dose that is not teratogenic in wild-type (WT) embryos. Remarkably, this antagonist treatment at E10.5 led to a near complete reversal of posterior cervical transformations back into a WT pattern (Fig. 1D and SI Appendix, Table S1), indicating that a mechanism (chemical or genetic) to oppose RAR activation is critical for establishing or maintaining positional information. Together these results provide genetic evidence that somitic identity is dependent on genetic repression mediated by SMRT/RAR corepressor complexes during the E10.5–11 time window. Nonetheless, we could not rule out possible involvement of other corepressors such as NCoR or inhibition of coactivator recruitments upon RAR antagonist treatment. In addition, lack of phenotypic changes in WT control with BMS 493 treatment indicates that SMRT-dependent repression is required for stable RA-signaling gradient during late somitogenesis.

Furthermore, the above results suggest that SMRTmRID mice may be generally more sensitive to exogenous RA exposure than wild-type littermates. While “standard” teratogenic doses of RA are 100 mg/kg (Fig. 1E and F), in SMRTmRID mice “non-teratogenic” doses of 5 mg/kg at E10.5 provoked cervical homeotic transformations (SI Appendix, Table S1). This lack of protection against low-dose RA suggests that SMRT repression enhances the robustness of the gene expression gradient by shielding it from exposure to potential external perturbance such as fluctuations in RA concentrations (SI Appendix, Table S1) during late somitogenesis. This enhanced ability of RA to change somatic identities in already specified somites suggests that SMRT, as a platform for chromatin modifiers, could be required to maintain the chromatin modification status of relevant RAR targets such as Hox genes, at least for the duration of their patterning functions.

Shifted Hox Gene Expression Boundary in SMRTmRID Mice. Posterior cervical transformations are a classic example in which anterior expansion of Hox gene expression results in specification of anterior segments to more posterior ones (25–27). As retinoids are known to activate the Hox cluster, we assessed the expression boundaries of posterior Hox genes normally associated with cervical regions in SMRTmRID embryos (Fig. 2A and B and SI Appendix, Fig. S3A and B). Interestingly, the increased expression of Hoxc5 was detected in the anterior dorsal axis by qPCR (Fig. 2A). Notably, in situ hybridization assays confirmed that the anterior boundary of Hoxc5 expression was shifted forward by one segment in SMRTmRID embryos (Fig. 2B–F). Additionally, after antagonist treatment at E10.5, the Hoxc5 expression boundary was normalized to WT levels (Fig. 2G). This demonstrates that in addition to vertebral patterning, SMRT-dependent repression is required to maintain the Hox expression boundary for somite identity at late somitogenesis.

Epigenetic Spatial Control of Hox Genes in SMRTmRID Mice. SMRT has been identified as a component of histone deacetylase-containing protein complexes (28, 29). We wished to examine whether SMRT complexes play a role in the epigenetic control of Hox C loci in developing embryos at E10.5. We examined spatially distinct somite regions designated as front (F), middle (M), and rear (R) (Fig. 3B and SI Appendix, Fig. S4A) in ChIP assays. In the mouse Hox C loci, RAREs are located both 3′ and 5′ to the Hoxc4 locus. We first examined the occupancy of RAR and HDAC3, a component of SMRT complexes in the 5′ RARE (Fig. 3A). Occupancy of RAR was slightly reduced in all three somite regions of SMRTmRID embryos. It has been suggested in prior ChIP studies that activation of RAR leads to reduction in its occupancy to its binding sites in certain target genes (30, 31). It is possible that SMRT derepression mimics receptor activation by decreasing RAR promoter occupancy. Occupancy of a SMRT complex component, HDAC3, was dramatically reduced in SMRTmRID somites especially in M and R regions where most of the axial defects were identified. It is tempting to speculate that in the F region, other corepressors could compensate for SMRT in RAR-mediated repression as we did not find major defects in hindbrain specification.

Next, we looked at three chromatin marks (AcH3, H3K4me3, and H3K27me3) in exon 1 of each Hox C locus in spatially distinct somites of E10.5 embryos (Fig. 3B). Front somite regions (F), which are specified earlier than middle regions (M), had higher enrichments of AcH3 and H3K4me3 in the proximal Hox C loci. While H3K27me3 repressive marks were reduced in proximal Hox genes, they were increased in distal Hox C loci. Middle regions, which are specified earlier than rear regions (R) but later than front regions, displayed higher enrichment of activation marks in more distal Hox genes. At the same time, enrichment of repressive marks was not observed proximal to middle Hox genes. In distal Hox C-expressing rear regions, enrichment of repressive marks and absence of activation marks in proximal Hox were evident. Thus, spatial epigenetic marks...
closely resemble the known Hox C loci expression pattern along the rostral-to-caudal somites. Recently, it has been reported that NCoR occupancy was low to absent in Hox C loci during RA activation of ES cells (32). It is possible that Hox C loci are predominantly under the control of the SMRT/RAR complex.

As the SMRT<sup>mRID</sup> mutation abrogates RAR-dependent recruitment of SMRT/HDAC co-repressor complexes, we predicted an increase in histone acetylation of Hox C loci in SMRT<sup>mRID</sup> embryos. Indeed, in SMRT<sup>mRID</sup> somites, increased H3K27ac3 marks were detected in certain Hox C loci in M and R regions (Fig. 3B). Unexpectedly, a reciprocal decrease in H3K27me3 was detected, a modification known to be mediated by EZH2 methyltransferase as a part of the PRC2 complex (33). These repressive marks can be removed by opposing demethylases such as Jmjd3 and UTX (34). Therefore, we examined Jmjd3 and Suz12 (a PRC2 component) recruitment to the Hox5 locus (Fig. 3C). Consistent with the reduced levels of H3K27me3, there was decreased occupancy of PRC2 methyltransferase complex (Suz12) only in SMRT<sup>mRID</sup> somites, suggesting SMRT plays a role in maintaining the H3K27 trimethylation status of this Hox locus. There was a trend in increased occupancy of Jmjd3 demethylase but did not reach statistical significance. Although, Jmjd3 has been suggested as a direct RAR target in neural stem cells, we did not detect derepression of Jmjd3 expression in SMRT<sup>mRID</sup> somites (SI Appendix, Fig. S4 B and C) (19).

We further examined the role of SMRT in Hox gene regulation in P19 embryonal carcinoma (EC) cells, a model system for RA-dependent activation of Hox genes (Fig. 3D). In transient transfection assays, overexpression of a SMRT<sup>mRID</sup> construct did not change either basal or RA-dependent activation of endogenous Hox5 expression, as expected from the lack of phenotype in heterozygous SMRT<sup>mRID</sup> mice. In contrast, expression of a C-terminal portion of SMRT-containing RID but lacking repression domain (SMRT-C) resulted in derepression and enhanced RA-dependent activation of the Hox C5 locus, suggesting SMRT recruitment is required for repression of the Hox C5 locus (Fig. 3D). Knockdown of SMRT expression via siRNA also resulted in derepression and enhanced activation of Hox5 genes (Fig. 3E). Furthermore, pharmacological inhibition of HDAC activity by trichostatin A (TSA) resulted in dramatic increase in Hox5 expression, providing evidence for the importance of histone deacetylation in Hox5 regulation. We further examined the role of Hox5 H3K27 trimethylation in vivo by knocking down Ezh2 methyltransferase by siRNA (Fig. 3E). Ezh2 knockdown resulted in derepression of Hox5 similar to SMRT knockdown. Interestingly, SMRT knockdown or overexpression of SMRT-C in P19 EC cells resulted in increased expression of NCoR and Ezh2. Ezh2 knockdown resulted in reciprocally enhanced expression of SMRT and NCoR, suggesting a dynamic link between SMRT and the polycomb repressive complex, REC2 (SI Appendix, Fig. S4 D and E). These results also implicate that SMRT is a preferential corepressor for RAR action in Hox5 gene expression.

Enhanced RAR Activity and RA Clearance in SMRT<sup>mRID</sup> Mice. To further examine derepression of RAR in this mouse model, we crossed the SMRT<sup>mRID</sup> mice with a transgenic reporter line (RARE-G (RARE-lacZ)) in which in vivo activation of RAR can be visualized by β-galactosidase (β-gal) activity (SMRT<sup>mRID</sup>-RARE) (35). In SMRT<sup>mRID</sup>-RARE embryos, higher β-gal activity was present all along the dorsal axis as well as the otic and optic vesicles (Fig. 4A). Interestingly, the caudal boundary of reporter expression along the dorsal axis was unchanged relative to WT, suggesting that retinoic acid degrading activity is diminished or potentially enhanced in the tailbud region of the presomitic mesoderm (Fig. 4B). Thus, despite presumptively increased RA signaling, a relatively normal embryonic development suggests that this process is not significantly perturbed in this region. This relatively undisturbed RARE-lacZ caudal boundary in the SMRT<sup>mRID</sup> could be due to the higher expression of retinoid degrading activities.

To the extent that a gene expression boundary is generated by the balance of a source and a sink (36), we wished to explore the impact of SMRT<sup>mRID</sup> on potential changes in RA synthetic and metabolizing enzymes. qPCR analysis revealed that the expression of the critical RA-generating enzyme, Aldh1a2, was unchanged in SMRT<sup>mRID</sup> embryos (SI Appendix, Fig. S4F) (37). In contrast, analysis of the Cyp26 genes encoding the RA-degrading enzymes (23; in the isolated somites revealed enhanced Cyp26a1 expression in E10.5 SMRT<sup>mRID</sup> embryos (Fig. 4 C–E). Cyp26a1 is known to control RA-mediated signaling by limiting RA levels in a spatiotemporal manner (38). Cyp26a1 deregulation was confirmed by WISH at different developmental stages (Fig. 4 C and E and SI Appendix, Fig. S4 G and H) and suggests that SMRT-dependent repression is required for normal

![Fig. 3. Epigenetic spatial control of Hox genes in SMRT<sup>mRID</sup> mice.](https://www.pnas.org/content/115/36/9056/F1.large.jpg)
RA metabolism. The enhanced expression of Cyp26a1 would result in more rapid degradation of RA and partially limit the teratogenic impact of derepressed RAR activity. Despite this compensatory RA clearance, the developmental phenotypes in SMRT<sup>mRID</sup> animals are still evident. Thus, SMRT plays a major role in retinoid homeostasis by repressing the RA-degradation enzyme Cyp26a1 in the livers of 10-d-old and 2-mo-old SMRT<sup>mRID</sup> mice (Fig. 4 H and I). To confirm that SMRT-dependent repression is a critical factor in Cyp26a1 regulation, we examined the SMRT occupancy and acetylation status of Cyp26a1 promoters in both WT and SMRT<sup>mRID</sup> livers under vitamin A deficiency (SI Appendix, Fig. S4I). In vitamin A-deficient WT livers, SMRT is enriched on the Cyp26a1 RARE while SMRT occupancy is compromised in SMRT<sup>mRID</sup> livers, suggesting that Cyp26a1 is a target of SMRT-dependent repression. Accordingly, in mutant mice, loss of SMRT recruitment to the Cyp26a1 RARE results in increased acetylated histone marks and strongly suggests that SMRT helps to prevent excessive retinoid metabolism, which in nutrient-deficient environments is potentially life sparing.

**Discussion**

Our observations are summarized in the model shown in Fig. 4 L and M. While in WT animals, the SMRT complex is recruited to RAR/RXR target promoters in the absence of hormone, in SMRT<sup>mRID</sup> mutants, derepression can enhance target gene activity in the absence of ligand and “superactivate” in the presence of ligand. Thus, loss of nuclear receptor-SMRT binding decouples the apo-receptor status and target silencing. While the depression affects many target genes at the transcriptional initiation steps,
certain classes of genes are additionally derepressed at the elongation steps, such as Cyp26a1. These derepressions affect the Hox code expression and ultimately alter the somite identity. Furthermore, we provide evidence that SMRT-dependent repression is critical for regulation of RA-metabolizing enzyme Cyp26a1 in both developing embryos and the postnatal liver.

Importantly, we demonstrated that SMRT has a nonredundant role with NCoR and that loss of SMRT interaction with nuclear receptors produces clear developmental phenotypes that can be rescued by the retinoid antagonist. Derepression in mutant mice sensitizes the RA-signaling axis, resulting in posterior homeotic transformation and enhanced teratogenic susceptibility to retinoic acid. This enhanced sensitivity to retinoid agonist and antagonist in already specified cervical somites at late somitogenesis in SMRT<sup>mRID</sup> indicates that SMRT repression is critical for refining and maintaining the spatial boundaries in the developing somites until the formation of sclerotomes.

During somitogenesis, once Hox codes are established for newly formed somites, Hox expression domains must be maintained at least for the duration of their patterning period. To mediate the necessary long-term repression of Hox genes, components of PRC2 trimethylate H3K27. Mutations in Polycomb genes result in aberrant Hox gene expression, increased sensitivity to the teratogenic effect of RA, and posterior homeotic transformations similar to that observed in SMRT<sup>mRID</sup> animals (40). The present study provides evidence that the SMRT corepressor complex is a critical component in the maintenance of this transcriptional cellular memory during late somitogenesis. Together these results lead us to propose that SMRT complex-dependent dynamic epigenetic antagonism is the mechanism through which the RA morphogenetic field is translated to an epigenetic and gene expression gradient during murine axial development.

**Methods**

**Animals.** SMRT<sup>mRID</sup> knockin mice were generated by standard homologous recombination using an insertedneo cassette for positive selection and a tk cassette for negative selection. Chimeric mice were generated by microinjection of two independently targeted ES clones into blastocysts. Two independent mouse mutant lines were established. SMRT<sup>mRID</sup> mice were backcrossed for at least four generations to sv129. Homozygous SMRT<sup>mRID</sup> mice are viable in sv129 or sv129/c57Bl6 mixed background, indicating that the previously described lethality of the SMRT<sup>mRID</sup> is background specific (41). All mice were fed a Labdiet 5002 containing 18 IU/g vitamin A. All experiments involving mice were approved by the Committee on Animal Care at the Salk Institute for Biological Studies.

Further details on materials and methods used in skeletal preparations, in situ hybridization, transient transfection, siRNA-mediated knockdown, gene expression analysis, chromatin immunoprecipitation assays, pharmacological treatments, and vitamin A deficiency study are included in **SI Appendix, Materials and Methods**.

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