Retinoic Acid and Histone Deacetylases Regulate Epigenetic Changes in Embryonic Stem Cells*

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Background: Retinoic acid (RA) and RA receptors (RARs) are major regulators of transcription in stem cells.

Results: Histone deacetylase (HDAC) 1, 2, and 3 bind different RAR target gene regulatory regions blocking histone acetylation, whereas RA treatment removes HDACs and increases histone acetylation levels.

Conclusion: HDACs differentially repress RA target genes.

Significance: Identification of new repression mechanisms for RA target genes.

All-trans-retinoic acid (RA) is a vitamin A metabolite that plays major roles in regulating stem cell differentiation and development. RA is the ligand of the retinoic acid receptor (RAR) family of transcription factors, which interact with retinoic acid response elements (RAREs) within target gene proximal promoters and enhancers. Although RA-mediated gene activation is well understood, less is known about the mechanisms for repression at RA-regulated genes. Using chromatin immunoprecipitation experiments, we show that in embryonic stem cells in the absence of RA, histone deacetylases (HDACs) differentially bind to various RAREs in proximal promoters or enhancer regions of RA-regulated genes; HDAC1, HDAC2, and HDAC3 bind at RAREs in the Hoxa1 and Cyp26a1 gene regulatory regions, whereas only HDAC1 binds at the RARβ2 RARE. shRNA knockdown of HDAC1, HDAC2, or HDAC3 differentially increases the deposition of the histone 3 lysine 27 acetylation (H3K27ac) epigenetic mark associated with increases in these three transcripts. Importantly, RA treatment differentially mediates the removal of HDACs from the Hoxa1, Cyp26a1, and RARβ2 genes and promotes the deposition of the H3K27ac mark at these genes. Overall, we show that HDACs differentially bind to RA-regulated genes to control key epigenetic marks involved in stem cell differentiation.

Epigenetic modification of the genome is a major regulator of embryonic stem cell (ESC) differentiation and development. Stem cells have the ability to self-renew and to differentiate along multiple cell lineages, and epigenetic regulation of gene expression is essential for proper stem cell maintenance and differentiation (1, 2). Examination of these specific epigenetic changes and their modulators has unraveled a complex circuitry that finely regulates stem cell differentiation.

Genes are transcriptionally regulated in part at promoter regions where proteins involved in transcription, including RNA polymerase II, bind to initiate transcription. However, genes are also regulated by cis-regulatory elements termed enhancers, located upstream or downstream of transcription initiation sites (3–6). Epigenetic regulation at these enhancer regions is required for gene expression (5, 6). At both promoter and enhancer regions, histone tails are targets for extensive post-transcriptional modifications, such as acetylation and methylation, that regulate many aspects of gene transcription (7). Methylation of histone tails, especially trimethylation of histone 3 lysine 27 (H3K27me3) and histone 3 lysine 9 (H3K9me3), is generally associated with gene repression. Conversely, methylation of H3K4 generally marks active enhancer regions and acetylation of H3K4, H3K9, and H3K27 is highly correlated with transcriptional activation (8–10). The presence of the H3K27ac mark distinguishes active enhancers as compared with poised enhancers in ESCs, suggesting that enhancers with the H3K27ac marks are a better indicator of genes that play major roles during different stages of development (3, 11). Removal of histone methylation and acetylation is mediated by demethylases (KDMs/PRDMs) and histone deacetylases (HDACs), respectively (12).

All-trans-retinoic acid (RA) is a vitamin A metabolite that plays a major role in regulating ESC differentiation by acting as the ligand for the retinoic acid receptor (RAR) family of transcription factors (13). The RARα, β, and γ receptors bind retinoic acid response elements (RAREs) within target gene promoters as heterodimers with the retinoid X receptor family, and we have shown that RAR-γ specifically interacts with a subgroup of RAREs that are located at either the promoters or enhancers of many target genes in ESCs (14, 15). RARs regulate the deposition of both activating and repressive epigenetic marks on histones at target gene promoters. We have shown that upon RA exposure at RAR-regulated genes, the H3K27me3 repressive marks decrease, whereas the H3K4/9ac activation marks increase, allowing for transcriptional activation of target gene
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expression (14, 15, 17, 18). Although our lab and others have performed extensive studies focused on understanding how RARs recruit co-activators to promote gene expression, the corepressors that interact with RARs and how RA regulates them are not as well understood.

HDACs are typically present within larger, co-repressor multiprotein complexes, where they mediate the removal of an acetyl group from lysines on histone tails (19). There are several subgroups of HDACs, and HDAC1, HDAC2, and HDAC3 are all members of the class I subfamily based upon their homology with the founding member in budding yeast, Rpd3 (12, 20). HDAC3 is a deacetylase present in complexes that contain co-repressors that have been reported to interact with the RARs, the nuclear receptor co-repressor (NCor1) and the silencing mediator of retinoic acid and thyroid hormone receptor (SMRT, also known as NCOr2) (19). HDAC1 and HDAC2 are deacetylase components of the nucleosome remodeling and deacetylation (NuRD), CoREST, and Sin3 co-repressor complexes (21).

To date, the specific interactions of various HDACs and their effects on histone acetylation at different RAR target gene promoters have not been examined. Additionally, whether RARs and their interacting co-activators and co-repressors can regulate H3K27ac levels at RA-regulated enhancer regions is unknown.

Here we determined which HDACs bind to RA-regulated genes and the role of RA in regulating HDAC interactions. We also examined HDAC regulation of H3K27ac mark deposition at known RA target gene promoters and enhancers and found that different HDACs bind and regulate histone acetylation in a gene-specific manner. Overall, these studies highlight the complex regulation of RA-mediated gene transcription during stem cell differentiation.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Antibodies**—Cells were treated with a final concentration of 1 \( \mu M \) all-trans-retinoic acid (Sigma), which was dissolved in 100% ethanol vehicle under dim light and kept at 4 °C (the final concentration of ethanol was <0.1%). The HDAC1 specific inhibitor JN1-26481585 was purchased from Selleck Chemicals (S1096, Houston, TX), and the HDAC3 specific inhibitor pimelic diphenylamide 106 was a kind gift from Dr. Joel Gottesfeld (26, 27). Both inhibitors were dissolved in 100% DMSO to a final concentration of 0.11 or 0.22 nM (for JN1-26481585), and 1, 2, or 5 \( \mu M \) (for 106 inhibitor) as indicated. These concentrations were optimized to achieve histone acetylation. HDAC1 (06-720; Millipore, Billerica, MA), HDAC2 (H-54; Santa Cruz Biotechnology, Santa Cruz, CA) (for Westerns), HDAC2 (7029; Abcam, Cambridge, MA) (for ChIP), HDAC3 (Ab7030; Abcam), H3K27ac (Ab4729, Abcam, Cambridge, MA), H3K4/19ac (06-599; Millipore), and actin (MAB1501; Millipore) antibodies were used.

**Derivation and Culture of the Murine ESC Lines and Lentiviral shRNA Infection**—CCE WT ESCs were cultured as previously described (28). HEK293T cells were transfected with 2 \( \mu g \) of PLKO.1 TRC cloning vectors with shRNA plasmids directed specifically toward murine HDAC1 (TRCN0000039402; Sigma), HDAC2 (TRCN00000393950; Sigma), or HDAC3 (TRCN00000393890; Sigma), 1 \( \mu g \) of pSV5-G envelope plasmid, and 1 \( \mu g \) of pCMVΔR8.9 packaging plasmid using Lipofectamine 2000 (Invitrogen). Lentiviral particles were collected from the supernatant. CCE WT ESCs were infected with shRNA lentiviral particles that were treated with polybrene at a 4 \( \mu g/ml \) final concentration. After 24 h, cells were selected with 1 \( \mu g/ml \) puromycin for an additional 3 days. Cells lines were screened for efficient knockdown by Western blotting.

**Chromatin Immunoprecipitation (ChIP)**—Experiments were performed as previously described (28, 29). Briefly, 2.5 \( \times 10^6 \) CCE WT ESCs were cultured with or without 1 \( \mu M \) RA and/or 106 inhibitor for 1, 8, or 24 h as indicated. Sonicated and pre-cleared lysates containing 15–45 \( \mu g \) of DNA were immunoprecipitated using 0.5–1.0 \( \mu g \) of antibodies specific for HDAC1, HDAC2, HDAC3, H3K27ac, or IgG (negative control). 3 \( \mu l \) of purified DNA was used for qPCR analysis using primers specific for designated gene promoter and enhancer regions. 3 \( \mu g \) of input DNA was used to normalize immunoprecipitated DNA, and all values were normalized to the IgG negative control, which in each experimental plot was set to 1.

**Western Blotting**—CCE WT and shRNA knockdown ESCs were harvested in SDS lysis buffer, boiled, and resolved on SDS-PAGE gels, followed by Western blotting using antibodies specific for HDAC1 (1:2000), HDAC2 (1:1000), HDAC3 (1:10000), H3K27ac (1:5000), H3K4/19ac (1:5000), or actin (1:10000).

**RNA Isolation and Reverse Transcription**—RNA was extracted using TRIzol (Invitrogen) according to the manufacturer’s instructions. Quantified RNA was reverse transcribed using the qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD).

**Real Time qPCR Analysis**—Reactions were carried out using SYBR Green quantitative PCR master mix and a Bio-Rad iCycler. Primers specific for each proximal promoter (pp), RARE, or gene are shown (Table 1). For mRNA quantitation, 36B4 was used as an internal control mRNA to which all values were normalized.

**Statistical Analysis**—Statistical analysis was performed on at least three separate, independent experiments (\( n = 3 \) or >3) using the Graph Pad Prism 6.0 software. The means ± S.E. were determined, and Student’s t test was used to compare two independent populations, where \( p < 0.05 \) was considered statistically significant.

**RESULTS**

HDACs Differentially Bind to the Promoter and Enhancer Regions of RA-inducible Genes in the Absence of RA—Because HDAC1, HDAC2, and HDAC3 play major roles in embryonic development, we first addressed their roles at RA-regulated genes in murine ESCs. We asked which HDACs interact at the enhancer and/or pp regions of the RA-regulated genes, including Hoxa1, Cyp26a1, and RARβ2, in ESCs cultured without RA. A schematic of the gene promoter and enhancer regions is shown in Fig. 1A. The Hoxa1 gene has an enhancer region with an RARE located ~4.6 kb downstream of the Hoxa1 pp (30).
The Cyp26a1 gene possesses two RAREs, one in the pp and a second in an enhancer ~2 kb upstream from the Cyp26a1 pp (31, 32). Finally, the RARβ2 gene possesses an RARE within its pp (33).

We performed ChIP experiments using antibodies specific for HDAC1, HDAC2, or HDAC3 to assess HDAC binding. By qPCR, we detected HDAC1 binding at the Hoxa1 RARE, Cyp26a1 pp/RARE1, Cyp26a1 RARE2, and the RARβ2 pp/RARE (Fig. 1B). HDAC2 also bound at the Hoxa1 RARE, Cyp26a1 pp/RARE1, and Cyp26a1 RARE2, but not at the RARβ2 pp/RARE (Fig. 1C). In contrast, we found that HDAC3 only bound at the Hoxa1 RARE and Cyp26a1 RARE2 regions.

**TABLE 1**

| Target          | Forward (5' → 3')                                      | Reverse (5' → 3')                                      | Product size |
|-----------------|-------------------------------------------------------|-------------------------------------------------------|--------------|
| Hoxa1 RARE      | TCTTGCCTGACGTGCGTGACATTAACTCAGTGGACGCTA            | GAGCTCAGAAGAAACTCAGGTGCACCTG                         | 268 bp       |
| Hoxa1 pp        | AATTGCCTGAGAAGTCGTCCATCGAGCCT                            | CACGTCGCAAGCTGGATGACGCTA                             | 276 bp       |
| Cyp26a1 RARE1   | CCGAATCCCAATATTAAAGATGA                                | CTTTATAAGCCCTCCAGGTTAC                                 | 87 bp        |
| Cyp26a1 RARE2   | TTTCTGAGTGTGTCAGCCGTCCTCCACAAAC                       | TTTCTGAGTGTGTCAGCCGTCCTCCACAAAC                       | 64 bp        |
| RARβ RARE       | TTGCTGTGTGACTGGACAGCTG                                 | CCCCGCTTTTGGAGGAAAGATAGA                              | 284 bp       |
| Hoxa1 mRNA      | TTTCCACACGAGGACGTGGGAGCTCAAAC                        | TTTCCACACGAGGACGTGGGAGCTCAAAC                        | 220 bp       |
| Cyp26a1 mRNA    | GAAACCTGAGACTGGAGCTCAAAC                              | GAAACCTGAGACTGGAGCTCAAAC                              | 272 bp       |
| RARβ mRNA       | GATCCCTCTGAGTTTCCCAACCAG                             | GATCCCTCTGAGTTTCCCAACCAG                             | 247 bp       |
| 36B4 mRNA       | AGAACTAACCCAGCCTTGGAGATAGA                            | AGAACCTAACCCAGCCTTGGAGATAGA                           | 448 bp       |
findings that HDAC1, HDAC2, and HDAC3 all bind to the RARs as the HDAC component of the NCoR/SMRT complex (34), we detected HDAC1 rather than HDAC3 at the RARβ2 pp/RARE and Cyp26a1 pp/RARE1 (Fig. 1B). HDAC2 and HDAC3 were not bound at the RARβ2 pp/RARE (Fig. 1, C and D).

**HDACs Differentially Regulate the Deposition of the H3K27ac Mark in the Absence of RA**—The H3K27ac epigenetic mark identifies active enhancers in ESCs (3). We next determined whether HDAC1, HDAC2, or HDAC3 specifically regulated H3K27ac deposition by using shRNAs specific for each HDAC to generate ESC lines with stable knockdowns (k.d.) of HDAC1, HDAC2, or HDAC3 (Fig. 2, a–c). As previously reported (35), we observed a compensatory increase in HDAC2 and HDAC1 proteins in HDAC1 k.d. or HDAC2 k.d., respectively (Fig. 2, a and b). We found that global H3K27 and H3K9/14 acetylation increased in all HDAC knockdown lines (Fig. 2, d and e), demonstrating that all three HDACs participate in regulating histone acetylation in ESCs.

We next performed ChIP experiments in the HDAC k.d. cell lines using an antibody for H3K27ac. If a specific HDAC prevents the deposition of the H3K27ac mark, we would expect to observe an increase in H3K27ac levels when that HDAC was knocked down. First, we detected no significant increase in H3K27ac levels at the Hoxa1 pp (Fig. 3aA), which is expected because we showed that there are no HDACs at this pp region (Fig. 1, B–D). Second, we observed an ~3-fold increase in H3K27ac level at the Hoxa1 RARE when HDAC1, HDAC2, or HDAC3 was knocked down as compared with WT or “empty vector” (E.V.) cells (Fig. 3aB). This result is in line with our findings that HDAC1, HDAC2, and HDAC3 all bind to the region with the Hoxa1 RARE (Fig. 1, B–D). Additionally, knockdown of HDAC1, HDAC2, or HDAC3 caused a 2.5-fold increase in Hoxa1 mRNA levels (Fig. 3bA).

We detected no significant changes in H3K27ac levels at the Cyp26a1 pp/RARE1 in the HDAC k.d. lines (Fig. 3aC), but knockdown of HDAC1 or HDAC3 was associated with a 4-fold increase, and knockdown of HDAC2 was associated with a 2-fold increase in H3K27ac at the Cyp26a1 RARE2 as compared with the parental WT cells (Fig. 3aD). Consistent with this result, knockdown of HDAC1, HDAC2, and HDAC3 resulted in 4-fold increases in Cyp26a1 mRNA levels (Fig. 3bB). Thus, HDAC1, HDAC2, and HDAC3 all bind near the Cyp26a1 RARE and negatively regulate Cyp26a1 mRNA levels in ESCs.

We detected a 3-fold increase in H3K27ac mark deposition at the RARβ2 pp/RARE in the HDAC1 k.d. cells (Fig. 3aE) and an increase in RARβ2 mRNA level (Fig. 3bC). Strikingly, neither HDAC2 nor HDAC3 affected the deposition of the H3K27ac mark at the RARβ2 pp/RARE (Fig. 3aE).

We also assessed the expression of genes important for proper ESC differentiation that are regulated by Hoxa1 or RARβ2. Wnt3a is a major regulator of the developing nervous system and is essential for many facets of neuronal differentiation (36). Our lab has shown that Wnt3a is regulated by Hoxa1, because Wnt3a expression is greatly reduced in Hoxa1−/− ESCs (37). Interestingly, we found that Wnt3a transcripts increased in HDAC1, HDAC2, and HDAC3 k.d. cells as compared with control (Fig. 3cA). This is consistent with our finding that HDAC1, HDAC2, or HDAC3 k.d. increased Hoxa1 mRNA levels (Fig. 3bA). The Sprouty family of genes are regulators of neuronal and endothelial cell differentiation (38), and we observed a 2-fold decrease in Spry2 expression in RARβ2−/− ESCs. HDAC1 k.d. induces ~6-fold increase in Spry2 mRNA levels (Fig. 3cB), which is again in line with our finding that RARβ2 transcriptional activation is primarily regulated by HDAC1 (Fig. 3cB).

Overall, these experiments show that different HDACs regulate the deposition of the H3K27ac epigenetic mark at different genes. HDAC1, HDAC2, and HDAC3 have roles at the Hoxa1 RARE and Cyp26a1 RARE2, whereas only HDAC1 plays a role at the RARβ2 pp/RARE. These findings have implications for proper stem cell differentiation because downstream targets of Hoxa1 and RARβ2 that are important for specific lineage differentiation are also differentially affected by specific HDAC knockdown.

**Pharmacological Inhibition of HDAC1 or HDAC3 Regulates H3K27ac Deposition in the Absence of RA**—We treated WT ESCs with the HDAC1 specific inhibitor JNJ-26481585 (39) at various concentrations and performed ChIP experiments to assess the levels of the H3K27ac mark. We found that JNJ-26481585 treatment did not cause changes in the deposition of the H3K27ac at the Hoxa1 pp (Fig. 4aA) but caused significant increases in H3K27ac levels at the Hoxa1 RARE, Cyp26a1 pp/RARE1, Cyp26a1 RARE2, and the RARβ2 pp/RARE (Fig. 4a, B–E). This is in line with our finding that HDAC1 does not bind to the Hoxa1 pp but interacts at the other gene regulatory

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regions to regulate H3K27ac levels (Fig. 1B). When either HDAC1 or HDAC2 was knocked down, we did not observe a significant increase in the H3K27ac levels at the Cyp26a1 pp/RARE1 (Fig. 3A). Our result using the JNJ-26481585 inhibitor suggests that the increase in HDAC2 protein level in HDAC1 k.d. cells (Fig. 2A) can partially compensate for HDAC1 at this promoter region.

Similarly, we treated WT ESCs with the HDAC3 specific inhibitor pimelic diphenylamide 106 (26, 27) and performed ChIP experiments. We found that 106 treatment did not change H3K27ac levels at the Hoxa1 pp (Fig. 4A), but 106 inhibitor increased the levels of H3K27ac by 3-fold compared with the untreated cells, at the Hoxa1 RARE (Fig. 4B). Similarly, 106 treatment did not cause changes in the H3K27ac mark at the Cyp26a1 pp/RARE1 (Fig. 4C) but resulted in increased levels of H3K27ac near the Cyp26a1 RARE2 (Fig. 4D). In contrast, we did not observe any change in the level of the H3K27ac mark at the RARβ2 pp/RARE after 106 treatment (Fig. 4E). These results demonstrate that H3K27ac is a substrate of HDAC3 and that HDAC3 levels are inversely associated with H3K27ac levels at the Hoxa1 RARE and Cyp26a1 RARE2, regions where we showed binding of HDAC3 (Fig. 1D).

**RA Differentially Regulates HDAC Removal**—We next hypothesized that the addition of RA would result in the
removal of HDACs from these regulatory regions. We again performed ChIP experiments using HDAC antibodies in extracts from WT ESCs cultured in the presence or absence of RA. We found that RA had no effect on HDAC1 binding at the Hoxa1 RARE and the Cyp26a1 pp/RARE1, but the addition of RA caused the loss of HDAC1 at the Cyp26a1 RARE2 and the RARβ2 pp/RARE (Fig. 5A). Interestingly, whereas HDAC2 bound to the Hoxa1 RARE, Cyp26a1 pp/RARE1, and Cyp26a1 RARE2, RA had no effect on its binding (Fig. 5B). We also showed that RA caused a loss of HDAC3 binding at the Hoxa1 RARE and Cyp26a1 RARE2 (Fig. 5C). These results demonstrate that RA regulates the binding of HDAC1 and HDAC3 but not HDAC2.

RA Increases H3K27ac Levels at Both Proximal Promoters and RAREs—Because H3K27ac was recently identified as a specific mark of active enhancers (3), we next determined whether RA could affect the deposition of the H3K27ac mark at the RAREs (Fig. 1A). We found that in untreated WT ESCs, the H3K27ac mark was present at all pp regions and RAREs compared with the IgG negative controls (Fig. 6A–E). After RA treatment, the increase in the H3K27ac mark at the Hoxa1 pp was 3-fold (Fig. 6A), whereas it was 5-fold at the Hoxa1 RARE (Fig. 6B). RA resulted in an 8-fold increase in the H3K27ac level at the Cyp26a1 pp/RARE1 (Fig. 6C) and a 7-fold increase at the Cyp26a1 RARE2 (Fig. 6D). At the RARβ2 pp/RARE, increases were
Additionally, RA treatment caused 40-, 200-, and 10-fold increases in Hoxa1, Cyp26a1, and RARβ2 mRNA levels, respectively (Fig. 6a–C). Overall, these data demonstrate that RA regulates changes in the H3K27ac mark, thus identifying another mechanism by which RA regulates ESC differentiation.

**DISCUSSION**

Although prior publications (24, 25) have shown that RARs can interact with HDAC3, we have found that different HDACs bind to the promoter and enhancer regions of RA-regulated genes. We show here that in addition to HDAC3, HDAC1 and HDAC2 play major roles in regulating transcriptional repres-
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FIGURE 6. RA increases H3K27ac levels at RAREs in WT ESCs. a, ChIP experiments were performed at various time points (1, 8, and 24 h) with or without treatment with 1 μM RA using an antibody specific for H3K27ac or IgG (negative control). qPCR was performed using primers specific for Hoxa1 pp (A), Hoxa1 RARE (B), Cyp26a1 pp/RARE1 (C), Cyp26a1 RARE2 (D), or RARβ2 pp/RARE (E). Fold change represents immunoprecipitated DNA relative to the IgG negative control set to 1 after normalized to pre-IP input DNA. b, RT-PCR analysis was used to determine mRNA levels compared with 36B4 internal control mRNA levels of Hoxa1 (A), Cyp26a1 (B), and RARβ2 (C). Error bars represent standard error of independent experiments where n = 4 for biological repeats. *, p < 0.05 comparing RA treatment with untreated control samples.

sion of RA-regulated genes (Figs. 1 and 3). Importantly, we have found that the RARβ2 pp/RARE is regulated primarily by HDAC1 (Fig. 1B). We also show that in the absence of RA, k.d. of HDAC1, HDAC2, or HDAC3 causes a specific increase in the H3K27ac mark at RAREs, proving that H3K27 is a substrate for HDAC1, HDAC2, and/or HDAC3 deacetylation, depending on the gene (Fig. 3a, A–E). Furthermore, we have found that RA regulates HDAC binding in a gene-specific manner. A summary of these findings is depicted in a model (Fig. 7).

RAREs are comprised of two direct repeats (DR) of the AGGTCA sequence with 5 base pairs (DR5) or two base pairs (DR2) between the repeats. Both the Hoxa1 and RARβ2 genes possess identical DR5 RAREs (30, 33). The fact that HDAC3 is recruited to the Hoxa1 RARE but not to the RARβ2 RARE is extremely interesting, because it suggests that despite similar binding patterns of the RARs and co-activators to these RAREs, other interacting transcriptional regulatory proteins at these two RAREs are different. A previous study found that RARs interact with 462 target loci in ESCs, whereas only 47 interact with the classical DR5 repeat, and only a subset of these are responsive to RA (40). Comparing these genes with those reported in a study that examined binding of HDAC1 in ESCs (41), we determined that only 7 out of 47 RAR-bound genes also interact with HDAC1. This would further suggest that the circuitry of epigenetic gene regulation that allows for precise control of stem cell differentiation is quite complex.

HDAC1, HDAC2, and HDAC3 are all class I HDACs and can sometimes act redundantly (12, 42–44). However, they have been shown to be functionally different, particularly during development (12, 35, 45). We found that HDAC1, HDAC2,
and HDAC3 exert different functions in terms of regulating H3K27ac levels on different genes (Fig. 3A–E) and are differentially regulated by RA in the context of different genes (Fig. 5, A–C). These results have two major implications. Our data indicate first that these HDACs are functionally different in terms of RA-regulated ESC differentiation and second that RARs may interact with various co-repressor complexes that contain these different HDACs.

Despite the fact that HDAC1 and HDAC2 are nearly identical in structure and sequence, our finding that they are functionally different in terms of RA- and RAR-regulated transcription is in line with other studies showing that HDAC1 and HDAC2 have different functions. Although germ line deletion of HDAC1 is early embryonic lethal, HDAC2 germ line deletions result in viable mice, but with low viability after birth (35, 42, 46–48). Using conditional knockouts of HDAC1 or HDAC2 in ESCs, Dovey et al. (35) showed that HDAC1, not HDAC2, is the major deacetylase that regulates both deposition of the H3K56ac mark and differentiation along specific lineages (12). Similarly, HDAC1 has different functions compared with HDAC3 during development (49). Maroni et al. (49) found that knockdown of HDAC1, and not HDAC3, caused an increase in expression of the Runx2 gene and increased alkaline phosphatase activity, indicative of early osteoblast maturation. Conversely, HDAC3, and not HDAC1, increased late osteogenic markers, including calcium and collagen deposition (49).

To date, RARs have been shown to interact with the NCoR/SMRT (NCoR1/NCoR2) co-repressor complex, and the HDAC associated with this complex is HDAC3 (24, 25). We detected HDAC1 and HDAC2 binding at various RAREs (Fig. 1, B and C), suggesting that RARs also interact with other co-repressor complexes such as NuRD, Sin3, and/or REST/CoREST. The NuRD co-repressor complex is comprised of multiple proteins that mediate repression, including HDAC1 and HDAC2, chromatin remodelers, and histone KDMs (19). Similarly, HDAC1 and HDAC2 are the deacetylases present in the Sin3 and REST/CoREST co-repressor complexes, and like the NuRD complex, the Sin3 and REST/CoREST complexes are essential for proper development (21, 50). That RARs interact with other co-repressor complexes in addition to NCoR/SMRT is supported by a
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RA and HDACs play an important role in the regulation of gene expression. HDACs are responsible for the deacetylation of histones, which can lead to the recruitment of repressive transcription factors and the repression of gene expression. RA, on the other hand, has been shown to promote the acetylation of histones, which can lead to the recruitment of activators and the activation of gene expression.

In this study, we have shown that HDACs and RA work in concert to regulate the expression of specific genes in stem cells. We have identified several mechanisms by which HDACs and RA interact to regulate gene expression. These mechanisms include the formation of repressor complexes, the recruitment of histone modifying enzymes, and the activation of transcription factors.

Our results suggest that HDACs are important regulators of gene expression in stem cells and that RA can modulate the activity of HDACs to promote differentiation. These findings have implications for the development of novel therapeutic strategies for stem cell and cancer therapies.

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