SMRTe Inhibits MEF2C Transcriptional Activation by Targeting HDAC4 and 5 to Nuclear Domains

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The silencing mediator for retinoic acid and thyroid hormone receptors (SMRT) mediates transcriptional repression by recruiting histone deacetylases (HDACs) to the DNA-bound nuclear receptor complex. The full-length SMRT (SMRTe) contains an N-terminal sequence that is highly conserved to the nuclear receptor corepressor N-CoR. To date, little is known about the activity and function of the full-length SMRTe protein, despite extensive studies on separated receptor interaction and transcriptional repression domains. Here we show that SMRTe inhibits MEF2C transcriptional activation by targeting selective HDACs to unique subnuclear domains. Indirect immunofluorescence studies with anti-SMRTe antibody reveal discrete cytoplasmic and nuclear speckles, which contain RARs in an RA-sensitive manner. Formation of the SMRTe nuclear speckles results in recruitment of several class I and II HDACs to these subnuclear domains in a process depending on HDAC enzymatic activity. Intriguingly, although HDAC4 is located primarily in the cytoplasm, coexpression of SMRTe dramatically translocates HDAC4 from the cytoplasm into the nucleus, where HDAC4 prevents MEF2C from activating muscle differentiation. SMRTe also translocates HDAC5 from diffusive nucleoplasm into discrete nuclear domains. Accordingly, SMRTe synergizes with HDAC4 and 5 to inhibit MEF2C transcription activation of target promoter, suggesting that nuclear domain targeting of HDAC4/5 may be important in preventing muscle cell differentiation. These results highlight an unexpected new function of the nuclear receptor corepressor SMRTe for its role in regulating cellular trafficking of nuclear receptor and selective HDACs that may play an important role in regulation of cell growth and differentiation.

The steroid/nuclear hormone receptors constitute an important superfamily of transcriptional regulators that control gene expression both in the absence and presence of hormones. The transcriptional activity of these receptors is regulated by interactions with coactivators and corepressors, depending on the presence or absence of ligands, respectively. In the absence of ligand, retinoic acid (RA)1 and thyroid hormone receptors (RARs and TRs) interact with the corepressors SMRT and N-CoR (1, 2). Upon ligand binding, corepressors dissociate from the liganded receptors due to a conformational change in the receptors. The liganded receptors bind the coactivators through a hydrophobic pocket on the surface of the receptor's ligand-binding domain (3). Prominent among the coactivators is the steroid receptor coactivator (SRC) family that includes SRC-1, TIF2/GRIP1, and RAC3/ACTR/AIB1 (3). It is believed that the exchange of corepressor with coactivator in response to ligand underlies a basic principle of transcriptional regulation by steroid/nuclear hormone receptors.

SMRT and N-CoR are two distinct, but highly related proteins that share similar domain structure and function (1, 2). We have previously reported the cloning of a full-length SMRT, designated SMRTe (SMRT-extended). SMRTe contains an N-terminal sequence that is highly related to N-CoR, including a SANT domain (4). Currently, the biological function of this N-terminal domain remains unknown. cSMRT represents a dominant negative mutant that contains only the receptor-interacting domain (5), similar to the dominant negative N-CoRi (6). SMRT and N-CoR also interact with other transcriptional regulators, such as Pit-1 (7), CBF-1/RBP-J (8), and PLZF (9). Furthermore, SMRT and N-CoR have been shown to interact with mSin3A and several members of histone deacetylase (HDAC) family (10–14). HDACs can be subdivided into two classes based on size and sequence considerations (15). The first class (I) contains HDAC1, 2, and 3, which are smaller in size and related in sequence to the yeast RPD3. The second class (II) contains HDAC4, 5, 6, and 7, which are larger in size and related in sequence to the yeast HDA1. HDAC catalyzes removal of acetyl-groups from hyperacetylated histone, and this deacetylation process leads to modification of nucleosome structure, resulting in transcriptional silencing (16).

To understand the activity and function of the full-length SMRTe, we have analyzed the expression, cellular distribution, and in vivo association of SMRTe with RARs and HDACs. SMRTe is differentially expressed in cancer cells and appears to be down-regulated by all-trans-RA. Indirect immunofluorescence analysis reveals localization of SMRTe in discrete cytoplasmic and nuclear speckles. In contrast, the dominant negative cSMRT is diffusely located in the cytoplasm, partly explaining its dominant negative activity. Double-label immunofluorescence reveals in vivo association of SMRTe with RARs.

1 The abbreviations used are: RA, retinoic acid; RAR, retinoic acid receptor; TR, thyroid hormone receptor; SMRT, silencing mediator for retinoid and thyroid hormone receptors; SMRTe, SMRT-extended; HDAC, histone deacetylase; MEF2, myocyte enhancer factor 2; SNC, SMRT-N-CoR conserved domain; HA, hemagglutinin; kb, kilobase(s); aa, amino acid(s); RD3, 4, repression domains 3 and 4; aTRA, all-trans RA; SANT, SWI3/ADA2/N-CoR/TFIIB domain; cSMRT, c-terminal domain of SMRT.

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and HDAC1, 3, 4, and 5. We show that the association of SMRTe with RARα is atRA-sensitive, whereas the association with HDAC1 depends on HDAC enzymatic activity. Remarkably, SMRTe also mediates nuclear translocation of HDAC4 from the cytoplasm and nuclear domain targeting of both HDAC4 and HDAC5 to SMRTe nuclear speckles. Cotransfection experiments demonstrate a synergistic inhibition by SMRTe and HDAC4/5 on MEF2C-mediated transcriptional activation. Therefore, these data suggest a new function of SMRTe in cellular trafficking and a potentially novel mechanism for SMRTe-mediated transcriptional inhibition.

**MATERIALS AND METHODS**

**Plasmids and Antibodies**—The full-length human SMRTe was constructed by assembling the N-terminal-extended sequence (4) with the original SMRT (1) into the pCMX vector, creating pCMX-SMRTe. The class I and class II HDACs and the HDAC mutants (17), the MEF2c and MEF2Cc3-luciferase reporter (18), the rabbit (4) mouse anti-SMRTe (GeneTex, San Antonio, TX), and the mouse anti-SC35 and the rabbit anti-Daxx antibodies (19) have been described before. The anti-FLAG M2 and the anti-HA antibodies were purchased from Kodak and/or Santa Cruz Biotechnology. The fluorescein- or rhodamine-coupled goat secondary antibodies were purchased from Rockland and Chemicon.

**In Vitro Transcription/Translation**—The in vitro transcription/translation reactions were conducted by the T7-Quick reticulocyte lysate system (Promega, Inc.). N-CoR, SMRT, SMRTe, and cSMRT are all in the pCMX vector under the regulation of T7 and cytomegalovirus promoters. The reactions were incubated at 30 °C for 90 min according to the manufacturer’s instruction.

**Northern and Western Blot**—A Western blot was conducted using the ECL kit (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. The anti-SMRTe monoclonal antibodies were provided by GeneTex (San Antonio, TX). Total cell lysate were prepared by lysing cells directly in SDS-sample buffer and analyzed on 6% SDS-polyacrylamide gel. After blotting and incubation with primary and secondary antibodies, the blot was developed by ECL reagents and subjected to autoradiography. For Northern blotting, the cancer cell line cotransfected with the N-terminal-extended sequence (4) with the original SMRT protein without the N-terminal extended domain alone (cSMRT) displayed a diffuse, mostly cytoplasmic staining with the available antibodies.

To analyze the SMRTe mRNA levels in cancer cells, we conducted Northern blotting of various cancer cells with three different regions of SMRTe as probes (Fig. 1E). All probes detected a 9.5-kb band. This SMRTe message was expressed most abundantly in HeLa and SW480 cells, consistent with the Western blot data. Interestingly, the SMRTe probe covering the N-terminal conserved SNC-SANT domain detected an additional band of 7.5 kb. This 7.5-kb message was also expressed abundantly in HeLa and SW480 cells but was absent in MOLT-4 and A549 cells. By contrast, N-CoR was expressed as an 8.5-kb message that migrated between these two SMRTe bands. N-CoR expression was also most abundant in HeLa and SW480 cells, suggesting coordinated regulation of SMRTe and N-CoR expression is possible.

**Subcellular Localizations of Full-length SMRTe and SMRTe Fragments**—To help understand the function of SMRTe, we analyzed the subcellular localizations of full-length SMRTe and several SMRTe fragments in mammalian cells by indirect immunofluorescence (Fig. 2). We found that a majority of full-length SMRTe-transfected cells (80–90%) displayed exclusive nuclear speckle staining (Fig. 2a), whereas about 10–20% of transfected cells displayed overall speckle pattern in both cytoplasmic and nuclear compartments (Fig. 3A, a). Similarly, immunostaining of endogenous SMRTe also revealed nuclear speckle pattern, suggesting that such nuclear speckles are not an artifact of protein overexpression. The existence of endogenous cytoplasmic SMRTe is not conclusive due to high background of cytoplasmic staining with the available antibodies.

We then wished to determine the domain that determines SMRTe speckle localization by analyzing a series of SMRTe deletion mutants (Fig. 1A). The N-terminal extended domain between amino acids 1 and 1109 was located exclusively in the nucleus in a diffuse pattern, suggesting that this domain contains the nuclear localization signal and lacks the speckle signal. Interestingly, the highly conserved SNC-SANT domain was located mainly in the cytoplasm, indicating that nuclear localization signal is located outside of the SNC-SANT region. The original SMRT protein without the N-terminal extended domain was distributed mainly in nuclear speckles in a manner that is similar to SMRTe and consistent with an intact corepressor function. Furthermore, the repression domain 3 (RD3, formally SRD1 (21)) was located exclusively in the nucleus with speckles, indicating that this domain contains a separated nuclear localization signal and a potential speckle localization signal. In contrast, the RD4 domain (formally SRD2 (21)) was located exclusively in the cytoplasm with a pronounced speckle pattern, suggesting that the RD4 contains a cytoplasmic speckle signal. Interestingly, the nuclear receptor interacting domain alone (cSMRT) displayed a diffuse, mostly cytoplasmic staining, indicating that it lacks a nuclear localization signal.

The speckle localization pattern of SMRTe prompted us to investigate whether the SMRTe nuclear speckles correspond to any known nuclear structures. We found no evidence of colo-
calization between SMRTe and the splicing factor SC35, nor did SMRTe colocalize with the promyelocyte oncogenic domains revealed by anti-Daxx staining (19). SMRTe staining was also not observed in the nucleoli, condensed chromatin, or centromeres (data not shown), suggesting that these SMRTe nuclear speckles represent a novel class of nuclear structures. These data also suggest that SMRTe contains multiple sequences for regulating its precise subcellular localization.

**Association of SMRTe with RARα in Vivo**—To provide evidence for a role of SMRTe in RA signaling, we examined the association of SMRTe with RARα in vivo in the absence of RA (Fig. 3A). As previously reported, RARα alone was distributed diffusely in nucleoplasm (data not shown). Interestingly, coexpression with SMRTe redistributed RARα into both the cytoplasmic and nuclear speckles in the absence of ligand (α–c), demonstrating an in vivo interaction between full-length SMRTe and unliganded RARα. Interestingly, atRA treatment abolished this colocalization, leading to exclusive nuclear staining of RARα without affecting the SMRTe localization (d–f). This observation is consistent with previous in vitro evidence that atRA induces dissociation of SMRT from the receptor (1). As a control, no cytoplasmic colocalization between SMRTe and RXRα was observed (data not shown), indicating specificity of this assay.

The dominant negative mutant cSMRT, which only contains the nuclear receptor interaction domain, antagonizes transcriptional repression by Gal4-RARα fusion protein (1). Because cSMRT is located primarily in the cytoplasm, we speculated that this cytoplasmic cSMRT might either sequester unliganded Gal4-RARα in the cytoplasm or be translocated into the nucleus by Gal4-RARα. This was tested by double immunofluorescence staining of cSMRT and Gal4-RARα (Fig. 3B). As previously reported, Gal4-RARα alone was located exclusively in the nucleus (data not shown). Remarkably, cSMRT seques-

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**FIG. 1.** Expression of SMRTe in vitro and in vivo. A, schematic representation of SMRTe, SMRT, cSMRT, and hemagglutinin (HA)-tagged deletion constructs used in this study. RD1–4, transcriptional repression domains; ID1–2, nuclear receptor interacting domains; LX, LXXL-like motif; SNC, SMRTe/N-CoR conserved domain; SANT, SWI3/ADA2/N-CoR/TFIIH domains; Q, poly-glutamine repeat; EK/ER, alternating acidic/basic regions; SG, alternating serine/glycine region. The numerical numbers are amino acid residues based on GenBank® accession number AF125672. B, expression of SMRTe in vitro and in vivo. Lanes 1–4 show an autoradiography of the [35S]methionine labeled in vitro translated proteins. Lanes 5–7 show a Western blot of 293T cells transfected with empty vector or pCMX-SMRTe at two different concentrations, followed by Western blot analysis with SMRTe monoclonal antibody. C, Western blot analysis of endogenous SMRTe in indicated mammalian culture cells. Equal amounts of lysate were loaded in each well as determined by Coomassie Blue staining (not shown). D, down-regulation of SMRTe protein levels by RA in NB4 acute promyelocytic leukemia cells. NB4 cells were treated with 1 μM all-trans-RA, and cell lysate was prepared and analyzed by Western blot. Coomassie Blue staining indicates equal protein loading. E, cancer cell Northern blot for SMRTe and N-CoR. Northern blot was conducted by sequential hybridization with the SMRTe N terminus probe (aa 1–165), the cSMRT probe, the SNC-SANT domain probe, the human N-CoR probe, and the β-actin probe as control.
tered the Gal4-RARα to the cytoplasm in the absence of ligand (a–c), and all-trans-RA treatment partially released this sequestration, rendering accumulated nuclear localization of Gal4-RARα (d–f). These data suggest that SMRTe can interact with unliganded RARα in vivo and that atRA can disrupt this interaction. These data also suggest that cellular compartmentalization may contribute to the dominant negative effect of cSMRT.

Interactions of SMRTe with Class I HDACs—Transcriptional repression by the unliganded nuclear receptors involves the recruitment of HDACs to target promoter by the corepressors. Biochemical evidence suggests that HDAC1 and 2 and mSin3A form a complex in vivo (22–24). SMRT and N-CoR also form a complex with mSin3A and HDAC1 and 2 (10–12). Nonetheless, the purified complexes mSin3A-HDAC1 and mSin3A-HDAC2 do not contain SMRTe or N-CoR (24), and SMRTe and N-CoR complexes contain only HDAC3 (25–27). To understand this complexity and also to characterize potential SMRTe interactions with class I HDACs, we analyzed their association in vivo (Fig. 4A). In the absence of SMRTe, HDACs 1, 2, and 3 were located diffusely in the nucleoplasm (a, e, i). Interestingly, coexpression of SMRTe caused a distribution of HDAC1 (b–d) and HDAC3 (f–j), but not HDAC2 (f–h) or mSin3A (n–p) to distinctive SMRTe nuclear speckles. We noted that HDAC3 coexpression appeared to partially distort the structure of the SMRTe nuclear speckles. Because SMRTe colocalizes with HDAC1 and HDAC3 in the absence of mSin3A, and because SMRTe does not colocalize with mSin3A, these data suggest a mSin3-independent mechanism of interaction between SMRTe and HDAC1 and 3 in vivo.

To further analyze the interaction between HDAC1 and SMRTe, we tested this colocalization with three HDAC1 point mutants whose HDAC enzymatic activities have been disrupted to various degrees (15). The HDAC enzymatic activity depends on conserved residues within the catalytic domain, and point mutation of these residues disrupts HDAC activity (15). Three HDAC1 point mutants, H141A, H199A, and D176N that are either partially defective (H141A) or completely void (H199A and D176N) of HDAC activity were analyzed (Fig. 4B). We found that only the partially defective mutant H141A remained associated with SMRTe nuclear speckles in this assay (a–c), whereas both H199A (d–f) and D176N (g–i) mutants, which lack HDAC activity completely, failed to interact with SMRTe. These data indicate that the enzymatic activity of HDAC1 may be critical in the recruitment of HDAC1 to SMRTe nuclear speckles.

Interaction of SMRTe with Class II HDACs—Recently, members of the class II HDACs were identified as proteins that also interact with SMRT (13, 14). We sought to confirm these interactions in vivo in the double immunofluorescence assay (Fig. 5A). Consistent with a previous report (17), HDAC4 alone was located primarily in the cytoplasm (a). Strikingly, cotransfection with SMRTe enhanced translocation of HDAC4 from the cytoplasm into the nucleus, where it colocalized precisely with the SMRTe nuclear speckles (b–d). We estimated that, in the absence of SMRTe, about 70% of the transfected cells contained dormant cytoplasmic HDAC4, and 30% contained approximately equal HDAC4 staining in the cytoplasm and nucleus.
Strikingly, coexpression of SMRTe translocated 92% of HDAC4 into nuclear speckles, suggesting a mechanism of SMRTe-dependent nuclear import and nuclear domain targeting of HDAC4. This SMRTe-mediated nuclear import of the wild type HDAC4 was even more pronounced than the HDAC4 mutation S246A/S467A/S632A that abolishes 14-3-3 binding in the cytoplasm (17). The HDAC4 S246A/S467A/S632A mutant also colocalized with SMRTe nuclear domain (data not shown), suggesting SMRTe may play a role in the cytoplasmic-nuclear domain trafficking of HDAC4.

Similarly, we analyzed the colocalization of HDAC5 and SMRTe by double immunofluorescence assay. HDAC5 alone was located diffusely in the nucleoplasm (Fig. 5A, a). There were only about 4% of HDAC5-transfected cells that contained dominant cytoplasmic staining (Fig. 5C). Strikingly, coexpression of SMRTe translocated HDAC5 almost completely from diffuse nucleoplasm staining into nuclear speckles (Fig. 5A, f–h, Fig. 5C), again suggesting a SMRTe-dependent nuclear domain targeting of HDAC5. We also tested the interaction of SMRTe with HDAC6 and found that HDAC6 was located exclusively in the cytoplasm and did not show interaction with SMRTe (Fig. 5A, i–l). Overall, it can be concluded that SMRTe selectively interacts with certain members of class II HDACs, and it may play an important role in regulating nuclear import of HDAC4, as well as nuclear domain targeting of HDAC4 and 5.

**SMRTe Enhances HDAC4/5-mediated Transcriptional Inhibition on MEF2C**—MEF2C-mediated transcriptional activation is required for muscle cell differentiation, and such transcriptional activation is inhibited by overexpression of HDAC4 and 5 (28). Because SMRTe enhances translocation of cytoplasmic HDAC4 into the nucleus, where HDAC4 can interact with MEF2C and inhibit its transcriptional activity, we speculated that SMRTe might facilitate the ability of HDAC4 to inhibit MEF2C function by promoting nuclear localization of HDAC4. This possibility was tested by cotransfection of SMRTe and HDAC4 to determine their effects on MEF2C-dependent activation of a luciferase reporter gene (Fig. 6). Consistent with previous findings (18, 29, 30), we found that MEF2C alone strongly activated reporter gene expression 40- to 100-fold and that HDAC4 and 5 inhibited this activation by 2- and 4-fold, respectively. In contrast, HDAC1, 2, 3, and 6 did not have much effect on MEF2C function. On the other hand, SMRTe alone also had only minor effect on the MEF2C activity (Fig. 6B). However, cotransfection of SMRTe and HDAC4 synergistically inhibited MEF2C activity by 20-fold. Similar effect was observed by coexpression of SMRTe and HDAC5, demonstrating that SMRTe may synergize with HDAC4 and 5 to inhibit MEF2C-dependent transcriptional activation of target promoter. Therefore, the inhibition of MEF2C transcriptional activation appears to correlate well with targeting of HDAC4 and 5 to SMRTe nuclear domains.

**DISCUSSION**

The corepressors SMRT and N-CoR interact with DNA-bound nuclear receptors to mediate transcriptional repression by a mechanism involving histone deacetylation. In this study, we have analyzed the subcellular localization and expression of full-length SMRTe and characterized the association of SMRTe with RARα and HDACs in *vivo*. We found that SMRTe is localized within discrete cytoplasmic and nuclear speckles, and such localization results in recruitment of unliganded RARα and selective HDACs to these nuclear domains. Recruitment of
HDAC1 to SMRTe speckles depends on its histone deacetylase activity, suggesting a potential role for SMRTe in regulating histone deacetylation. SMRTe actively translocates HDAC4 from cytoplasm into the nucleus, and HDAC5 from nucleoplasm into nuclear speckles. Accordingly, cotransfection of SMRTe with HDAC4 or 5 synergistically inhibits MEF2C transcriptional activation. These data suggest a novel mechanism by which SMRTe inhibits MEF2C transcriptional activity by recruiting HDAC4 and 5 to specific nuclear domains.

SMRTe is a unique full-length corepressor that contains an extended N-terminal domain longer than the previously reported sequence (4). SMRTe is almost identical to SMRTβ (31), except that it lacks a 17-aa insert at residue 721, it contains an extra 8-aa insert at residue 1016, and it has a correct reading frame for 5 aa starting from nucleotides 2360, as well as several single aa polymorphisms. Our assembled SMRTe vector encodes a full-length protein in vitro and in vivo that is similar in size to in vitro translated N-CoR and the endogenous SMRTe.

We also identified a shorter SMRTe message that appears to contain the conserved SNC-SANT domain. However, it is currently unclear whether this message represents the reported SMRTβ (31) or other members of the SMRT/N-CoR family.

FIG. 4. SMRTe recruits HDAC1 and HDAC3 into nuclear speckles but not HDAC2 or mSin3A. A, SMRTe was transfected into HeLa cells together with HA-tagged HDAC2, HDAC3, or FLAG-tagged HDAC1 or mSin3A and double-labeled by rabbit anti-SMRTe and mouse anti-HA or anti-FLAG antibodies. Panels a, e, i, and n show transfections of each HDAC or mSin3A alone. We found that, without cotransfection with SMRTe, HDAC1, 2, and 3 were diffusely distributed in the nucleoplasm (a, e, i), whereas mSin3A alone displayed a fine speckled pattern (n). Cotransfection with SMRTe recruits HDAC1 (b–d) and HDAC3 (j–l), but not HDAC2 (f–h) or mSin3A (o–p) into nuclear speckles. B, histone deacetylase activity is required for localization of HDAC1 to SMRTe nuclear speckles. SMRTe and each HDAC1 mutant were cotransfected and analyzed as in A. The partially active H141A mutant colocalized well with SMRTe (a–c), whereas the H199A (d–f) and D176N (g–i) mutants, which are void of deacetylase activity, did not show colocalization with SMRTe.
Northern blot analysis reveals that the expression levels of SMRTe and N-CoR are coordinated in different cancer cells, suggesting that these two proteins may function together. Recent data also suggest that SMRTe is not sufficient to complement the function of N-CoR in mice, because “knock-out” of the N-CoR gene resulted in embryonic lethality (32), suggesting that these two corepressors are not functionally redundant.

By indirect immunofluorescence analysis we show that the full-length SMRTe protein is localized at discrete cytoplasmic and nuclear speckles. The identity and biological function of these speckles remains unknown. We found that the N-terminal extended domain and the RD3 of SMRTe are localized exclusively in the nucleus, suggesting that multiple nuclear localization signals may be involved in nuclear localization of SMRTe. In contrast, RD4 is responsible for speckled cytoplasmic localization, and cSMRT appears to lack both nuclear and speckle localization signals. Recently, phosphorylation by mitogen-activated protein kinases has been shown to cause cytoplasmic localization of

**Fig. 5. Association of SMRTe with class II HDACs.** A, double immunofluorescence staining of SMRTe and FLAG-tagged HDAC4, 5, or 6. These class II HDACs were transfected alone (a, e, i) or together with SMRTe. In the absence of SMRTe, HDAC4 is located primarily in the cytoplasm (a). Coexpression with SMRTe translocates HDAC4 from cytoplasm into the nucleus (b–d). SMRTe also recruits HDAC5 from diffuse nucleoplasmic staining (e) into nuclear speckles (f–h). SMRTe did not recruit the cytoplasmic HDAC6 into the nucleus (i–l). B, quantitation of the relative localization of HDAC4, HDAC5, and HDAC6 in the absence (−) or presence (+) of SMRTe coexpression. The localizations of each HDAC shown in A were quantitated by recording transfected cells with specific localization patterns. Cyto indicates cells with predominant cytoplasmic staining; Cyto/Nu indicates cells with both cytoplasmic and nuclear staining; Nu indicates cells with predominant nuclear staining. The nuclear staining with diffused or speckled localization is also indicated for HDAC5. The nuclear staining of HDAC4 in the presence of SMRTe is primarily in nuclear speckles. About 250 to 350 transfected cells were recorded in each experiment.
SMRTe Inhibits MEF2C Transcriptional Activation

Transcriptional repression by DNA binding proteins involves a series of protein-protein interactions with corepressors such as SMRT, N-CoR, and HDACs. In this study, we have also confirmed the interactions of SMRTe with HDAC1, 3, 4, and 5; however, we have found that SMRTe does not colocalize with HDAC2, HDAC6, and mSin3A. Interestingly, SMRTe can recruit the interacting HDACs into discrete nuclear speckles. The colocalization of HDAC3, 4, and 5 with SMRTe is consistent with previous biochemical findings. In particular, HDAC3 has been shown to form a stable complex with SMRT and N-CoR (25–27), and HDAC3 can be coprecipitated with HDAC4 and 5 (15). The colocalization of SMRTe with HDAC1, but not HDAC2 or mSin3A, is somewhat unexpected, because HDAC1 has been reported to form a complex with SMRT indirectly via interaction with mSin3A (10, 12). Consistent with our findings, mSin3A does not colocalize well with N-CoR (35). Therefore, we suggest that HDAC1 may interact with SMRTe in vivo in an mSin3A-independent manner. Perhaps protein modifications or a yet-to-be-identified protein/factor might stabilize this in vivo interaction between HDAC1 and SMRTe.

Support for an active role of SMRTe in regulating histone deacetylation comes from observations that localization of HDAC1 to SMRTe nuclear speckles depends on the histone deacetylase activity of HDAC1. Similarly, it was recently reported that HDAC5 and 7 mutants, when lacking HDAC activity, as well as the deacytase inhibitor trichostatin A and sodium butyrate, can disrupt colocalization of HDAC5 and 7 with SMRTe (36). Together, these data suggest an important role for SMRTe in organizing a novel nuclear domain that may be implicated in histone deacetylation and transcriptional repression.

Finally, we have revealed a functional interaction between HDAC4 and SMRTe, suggesting a new role for SMRTe in regulating cytoplasmic-nuclear trafficking, which might be independent of its nuclear corepressor function. In the absence of SMRTe, HDAC4 is localized in the cytoplasm due to 14-3-3 binding (17). SMRTe induces a dramatic translocation of the cytoplasmic HDAC4 into the nucleus. In the nucleus, HDAC4 can interact with MEF2C and inhibit its transcriptional activity that is required for muscle cell differentiation (17). Indeed, coexpression of SMRTe and HDAC4 abolishes MEF2C transcriptional activity. This synergistic inhibition is likely due to enhanced accumulation of HDAC4 in the nucleus. Contrasting to HDAC4, HDAC5 is distributed diffusely in the nucleoplasm in the absence of SMRTe, consistent with its stronger inhibitory effect on MEF2C activity. We have found that SMRTe also recruits HDAC5 into nuclear speckles, correlating with an enhancement of HDAC5-mediated transcriptional inhibition on MEF2C function by SMRTe. This observation suggests that HDAC5 localization to SMRTe nuclear domains might further suppress MEF2C activity. Because SMRTe also recruits HDAC4 into the nuclear speckles, it is conceivable that SMRTe nuclear domains may play an active role in antagonizing transcriptional activation.

It was reported recently that HDAC4 and 5 are exported from the nucleus during muscle cell differentiation via a process controlled by calcium/calmodulin-dependent protein kinases (37). Thereby, phosphorylation of HDAC4 and 5 and/or SMRTe by calcium/calmodulin-dependent protein kinases might disrupt HDAC-SMRTe interaction, leading to segregation and subsequent export of HDAC4 and 5. Alternatively, this signal-dependent phosphorylation event might trigger nuclear export of the entire SMRTe-HDAC complex. Although it is clear that nuclear export of HDAC4 and 5 accompanies MEF2C activation and muscle cell differentiation, little is known about how HDAC4 and 5 are imported and maintained in the nu-
nucleus. Our data suggest, for the first time, a mechanism of SMRTe-mediated nuclear import of HDAC4 and nuclear domain targeting of HDAC4 and 5. We believe that this mechanism may provide an important piece of the puzzle for understanding signal-dependent muscle cell differentiation. Consistently, N-CoR interacts with MyoD and inhibits muscle cell differentiation (38). Therefore, it is reasonable to speculate that nuclear receptor corepressors may enhance transcriptional repression by targeting selective HDACs to specific nuclear domains, leading to long-term repression of muscle-specific genes. We speculate that this strategy may be prototypic for regulating the activity of other SMRTe-interacting proteins, and it now poses the challenge to further define the exact mechanism of gene regulation by the SMRTe nuclear domains.

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