Regulation of Nuclear Hormone Receptors by Corepressors and Coactivators: a Dissertation

Xiaoyang Wu
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REGULATION OF NUCLEAR HORMONE RECEPTORS BY COREPRESSORS AND COACTIVATORS

A DISSERTATION PRESENTED

BY

XIAOYANG WU

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
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December 14, 2001
REGULATION OF NUCLEAR HORMONE RECEPTORS BY COREPRESSORS AND COACTIVATORS

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REGULATION OF NUCLEAR HORMONE RECEPTORS BY COACTIVATORS AND COREPRESSORS

Abstract

Nuclear hormone receptors (NHR) constitute a superfamily of ligand inducible transcriptional activators that enable an organism to regulate development and homeostasis through switching on or off target genes in response to stimuli reflecting changes in environment as well as endocrine. NHRs include classical steroid hormone receptors (GR, AR, ER and MR) and retinoid, thyroid hormone receptors. One long-term goal of our lab is to understand the molecular mechanisms through which the transcriptional activity of NHRs is regulated.

Extensive studies in the past few years have revealed that in addition to the dependence on ligand availability, the transcriptional activity of NHRs is also regulated by two types of proteins: coactivators and corepressors. In the absence of ligand, many NHRs, including TR and RAR can actively repress target gene transcription with the help of corepressors, proteins that physically interact with both NHRs and histone deacetylases (HDACs). Functional interactions between NHRs and corepressors therefore lead to tightly compact and transcriptionally non-permissive chromatin structures after the removal of obstructive acetyl groups from histone tails by HDACs. On the other hand, ligand binding stabilizes NHRs in a conformation that favors interaction with proteins other than corepressors; many of these proteins are able to potentiate the transcriptional activity of NHRs through various mechanisms, such as histone acetylation, chromatin remodeling and recruitment of basal transcription machinery and are collectively termed coactivators.

Two highly related corepressors, SMRT (silencing mediator of retinoid and thyroid hormone receptors) and N-CoR (nuclear receptor corepressor), have been cloned. This research in corepressor SMRT started by a systematic study of its subcellular
localization. We found that SMRT predominantly forms a specific nuclear punctuate structure that does not appear to overlap with any other well-known subnuclear domains/speckles. Although our searching for specific sequence signals that may determine the specific speckle localization of SMRT did not yield conclusive results, we discovered the colocalization of unliganded RAR and certain HDACs, including HDAC1, 3, 4 and 5, in the SMRT nuclear speckles. Moreover, SMRT is likely to be the organizer of such speckles since it appears to be able to recruit other proteins into these speckles.

The presence of HDAC1 in the SMRT speckles suggests a direct association between these two proteins, which has not been detected by previous biochemical analyses. Interestingly, HDAC1 point mutants that are completely defective in deacetylase activity failed to locate to SMRT nuclear speckles, while another partially active mutant maintained the colocalization. These discoveries may indicate SMRT nuclear speckles as novel nuclear domains involved in transcriptional repression. More physiologically relevant support for this hypothesis arises from study of HDAC4 and 5. HDAC4 and 5 are potent inhibitors of transcriptional activator MEF2C. Nuclear presence of HDAC4/5 can block the activation of MEF2C, which is required during muscle differentiation. Normally, HDAC4 is predominantly located in cytoplasm. However, we found that in the presence of SMRT overexpression, HDAC4 was found mostly in SMRT nuclear speckles. This accumulation enhanced HDAC4 mediated inhibition on MEF2C transcriptional activity in a transient transfection assay. SMRT overexpression also resulted in accumulation of HDAC5 in the SMRT nuclear speckles compared to the nuclear diffuse distribution in the absence of SMRT. Again, this accumulation of HDAC5 in nuclear speckles correlated with enhanced inhibition of MEF2C. Taken together, our study suggested that instead of being merely a corepressor for NHRs, SMRT might function as an organizer of a nuclear repression domain, which may be involved in a broad array of cellular processes.

In contrast to the limited number of corepressors, numerous coactivators have been identified; the SRC (or p160) family is relatively well studied. This family includes three highly related members, SRC-1, TIF2/GRIP1, RAC3/AIB1/ACTR/p/CIP. Similar domain structures are shared among these factors, with the most highly conserved region,
the bHLH-PAS domain found within the N terminal ~400 amino acid residues. This study of RAC3 aims to identify the function of the highly conserved N terminal bHLH-PAS domain by isolating interacting proteins through yeast two-hybrid screening. One candidate gene isolated encodes the C terminal fragment of the human homologue of the yeast protein MMS19. Functional studies of this small fragment revealed that it specifically interacted with human estrogen receptors (ERs) and inhibited ligand induced transcriptional activity of ERs in the transient transfection assay. Then we cloned the full-length human MMS19 cDNA and characterized the hMMS19 as a weak coactivator for estrogen receptors in the transient transfection assay. Furthermore, when tested on separate AF-1 or AF-2 of ERs, hMMS19 specifically enhanced AF-1 but had no effect on AF-2. These results identified hMMS19 as a specific coactivator for ER AF-1.
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<td>Activator of thyroid receptor</td>
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<tr>
<td>AD</td>
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<tr>
<td>AF-1 or 2</td>
<td>Activation function 1 or 2</td>
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<tr>
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<td>ARNT</td>
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<td>atRA</td>
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<td>Calcium/calmodulin-dependent protein kinase</td>
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<td>Mitogen-activated protein kinase</td>
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<td>Myogenic enhancing factor 2</td>
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<td>Methyl methanesulfonate</td>
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<td>MR</td>
<td>Mineralcorticoid receptor</td>
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<td>N-CoR</td>
<td>Nuclear receptor corepressor</td>
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<td>Nuclear receptor binding box</td>
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<tr>
<td>PAS</td>
<td>Period, Arnt, Sim</td>
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<td>p300/CBP associated factor</td>
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<td>p300/CBP interacting protein</td>
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<td>Silencing mediator of retinoid and thyroid hormone receptor</td>
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<td>SRA</td>
<td>Steroid receptor RNA activator</td>
</tr>
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<td>Description</td>
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<tr>
<td>SRC-1</td>
<td>Steroid receptor coactivator-1</td>
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<td>Transcription intermediary factor 2</td>
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<tr>
<td>TR</td>
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<td>Thyroid hormone receptor activator molecule-1</td>
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<td>TSA</td>
<td>Trichostatin A</td>
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<td>VDR</td>
<td>Vitamin D receptor</td>
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CHAPTER I

BACKGROUND

Higher organisms including humans respond to changes in environment or endocrine signaling by rapidly altering gene transcriptions. Steroid/nuclear receptors are a family of transcriptional activators whose activity is regulated by the binding of their cognate ligands, usually small lipophilic molecules including steroid hormones, thyroid hormone and the active forms of vitamin A and D[1-3]. In the absence of ligand binding, most steroid/nuclear receptors are inactive, and some, most notably retinoic acid receptor (RAR) and thyroid hormone receptor (TR), actively repress transcriptions of their target gene. Ligand binding triggers the activation of receptors, so that they will now bind to specific DNA sequence on the target gene promoters, the so-called hormone response elements (HREs), and activate the transcription. Most cellular actions of steroid/nuclear receptors occur in this way and are thus restricted in the nucleus, although cytoplasmic signaling pathways of steroid/nuclear receptors do exist and may account for those extremely rapid cellular effects mediated by these receptors[4, 5].

During the last decade, our knowledge of steroid/nuclear receptors exploded in three different areas. First, a plethora of molecules effecting the activation or repression of target genes have been identified, including corepressors and coactivators. Second, a mechanistic understanding of how steroid/nuclear receptors are activated by ligand binding has been established through determination of crystal structures of receptors and receptor-coregulator complexes. Third, targeted deletion in mouse has generated useful
models to unambiguously define the specific functions of individual receptors in a mammalian organism. Combined together, these advances have offered significant insights into steroid/nuclear receptor regulation of a broad array of physiological processes. In this work, I will first give a brief introduction of our current knowledge of steroid/nuclear receptors and their two important coregulators, corepressors and coactivators. I will then use estrogen receptor (ER) as a model to explain how the function of a receptor is regulated at different levels in vivo, with focus on the regulation exerted through corepressors and coactivators, introducing questions that I address in my thesis.

1. Steroid/nuclear receptors

The first steroid receptor, glucocorticoid receptor (GR) was cloned about 15 years ago. Cloning of a few other steroid receptors and TR revealed an extensive homology and led to the realization that these receptors form one family and the rapid expansion of this family by homology-based cloning of new members with the similar structure. As a result, many receptors were cloned without identified ligands and these receptors have been called “orphan receptors”. A total of about 40 mammalian receptors (including isoforms for the same receptor) have been identified. Based on their dimerization and DNA binding properties, these receptors can be divided into four sub classes[2]. Class I includes those steroid receptors, ER, androgen receptor (AR), progesterone receptor (PR), GR and mineralcorticoid receptor (MR), which function in the form of ligand induced homodimers and bind to HREs with inverted repeats of two half-sites. Class II includes TR, RAR, vitamin D receptor (VDR) and peroxisome proliferator activator receptor (PPAR), which form heterodimers with retinoid X receptor (RXR) and bind to HREs
with direct repeats of two half-sites. Class III include RXR and orphan receptors such as hepatocyte nuclear factor-4 (HNF-4), which bind to direct repeats as homodimers. Finally, Class IV includes all the other orphan receptors binding to extended core sites as monomers.

Like most transcriptional activators, steroid/nuclear receptors are modular proteins, consisting of a functionally separable DNA binding domain and transcriptional activating domains. Furthermore, based on sequence and structure similarity, about six modulatory regions can be found in a typical receptor, denoted as A, B, C, D, E and F from N to C-terminus (Fig. 1). A/B is the most variable region among steroid/nuclear receptors in terms of both size and sequences. Region C represents the highly conserved DNA-binding domain (DBD), while region E overlaps with the ligand-binding domain (LBD), which also contain residues responsible for dimerization. D is a small linker domain between the DBD (C) and the LBD (E) called “hinge region”, which usually harbors a nuclear localization signal and also functions to provide some structure flexibility to the overall receptor conformation. Two transcriptional activating domains have been identified in most receptors, namely AF-1 and AF-2 (for activation function 1 or 2) respectively. AF-1 is constitutively active and locates in the A/B region; AF-2 resides in the E/F and is active only when the receptor is bound with ligand. Both AF-1 and AF-2 activate transcription probably by recruiting coactivators, proteins that interact with and enhance the transcriptional activity of steroid/nuclear receptors. While many AF-2 specific coactivators have been isolated, very few AF-1 specific coactivators are known. Since AF-2 from different receptors appears to share some common coactivators, there may be a common theme underlying how AF-2 activates gene transcription, while
the mechanisms of AF-1 activation could be more divergent considering the sequence variation of A/B regions among different receptors. For example, the A/B region from GR contains an acidic activating region[6, 7] typically found in many transcriptional activators including GAL4[8], GCN4[9] and VP16 [10, 11], suggesting a similar activating mechanism to that of those activators. However, A/B region from ERs does not contain any acidic region[12], suggesting that a totally different mechanism may be used by ER AF-1.

The transcriptional activity of steroid/nuclear receptors is attributable to their interaction with corepressors and coactivators in the absence and presence of ligand, respectively (Fig. 2). Next I will focus on our current understanding of how corepressors and coactivators regulate the functions of steroid/nuclear receptors.

2. Steroid/nuclear receptor corepressors

It was long realized that unliganded TR and its viral oncogenic counterpart v-ErbA as well as RAR actively repress transcription. Later, the repression domains in TR and RAR were mapped to the LBD of each receptor[13]. In an in vivo competition assay, overexpression of the repression domain from TR/RAR reversed TR/RAR mediated silencing, suggesting the existence of limited cellular silencing mediator(s) or corepressor(s)[14, 15]. Furthermore, overexpression of full-length TR/RAR inhibited the transcriptional repression mediated by the respective repression domain fused with a heterologous DNA binding domain. This inhibition occurred in the absence, but not in the presence of ligand, suggesting that the corepressor(s) must dissociate from the receptors upon ligand binding[15]. By yeast two-hybrid screening, two related
corepressors, SMRT and N-CoR were soon cloned[16, 17]. Both proteins interact with unliganded RAR/TR as well as a number of orphan receptors, including COUP-TF [18, 19] and RevErb[20, 21], and all have been shown to be able to repress transcription. As expected for a corepressor, SMRT/N-CoR was released from receptors upon ligand binding.

Several lines of evidence indicate that SMRT and N-CoR are *bona fide* corepressors for TR/RAR. First of all, mutations introduced into RAR/TR that disrupted the interaction with SMRT/N-CoR also abolished the RAR/TR mediated transcriptional repression[22]; on the other hand, single cell injection of antibodies against SMRT/N-CoR relieved receptor-mediated repression[23, 24]. Furthermore, when overexpressed, SMRT C-terminus containing the receptor interacting domain but lacking any transcriptional repression domain acts as a dominant negative to inhibit receptor-mediated repression[22]. Similar experiments also established SMRT/N-CoR as corepressors for transcriptional factors Pit-1[25], PLZF[26, 27], MyoD[28] as well as antagonist-bound steroid receptors ER and PR[29-32], suggesting a general role of SMRT/N-CoR in mediating transcriptional repression. This is confirmed by genetic studies in mouse. Targeted knock-out of N-CoR gene in mouse results in early embryonic lethality[33]. The knock-out mice exhibit impaired definitive erythropoiesis, arrested T cell development at the double-negative stage and defects in neuronal development. Further studies indicated aberrant transcriptional regulation by TR as well as other transcription factors in the affected tissues, confirming the role of N-CoR as a corepressor for genes under the control of specific DNA-binding repressors and the repression
mediated by specific steroid/nuclear receptors and several other classes of DNA binding transcription factors.

SMRT and N-CoR share a similar domain structure (Fig. 3), suggesting functional similarity between these two proteins. N-CoR has approximately 1,000 amino acid residues at the N-terminus not found in the original SMRT; however, the recent cloning of the full-length SMRT, SMRTe/SMRTα[34, 35] revealed that SMRTe and N-CoR are similar in size and homologous to each other throughout the peptide sequence. As summarized in Figure 3, there are 3 or 4 autonomous repression domains present in N-CoR/SMRTe respectively. In addition, both proteins have a highly conserved SANT domain at the N-terminus and two adjacent receptor interacting domains (RIDs) at the C-terminus. Each SANT domain (SW13, ADA2, N-CoR, and TFIIB) consists of two repeats and is very likely a protein-protein interacting domain[36] although no interacting partners have been identified so far.

Recent work identified molecular determinants in corepressor RIDs for receptor interaction. Each RID contains one short peptide motif, or “CoRNR box”[37-39], with a consensus sequence of LXXI/H IXXX I/L[39] where L is Leucine, I is Isoleucine, H is Histidine and X can be any amino acid residue. Site-directed mutagenesis of the key hydrophobic amino acid residues in CoRNR boxes abolished the interaction with receptors, and synthetic peptides containing CoRNR box blocked the receptor-corepressor interaction, indicating that CoRNR boxes are sufficient and necessary in mediating receptor binding. However, despite the conservation of CoRNR boxes, SMRT and N-CoR do not bind receptors with equal affinity. For example, N-CoR prefers TR and RevErb, while SMRT interacts preferentially with RAR[40, 41]. Furthermore, the
two CoRNR boxes (CoRNR1 and CoRNR2) within a single corepressor do not contribute equally to the binding of a specific receptor[40]. For example, RAR interacts more strongly with CoRNR1 than with CoRNR2, while RXR interacts exclusively with CoRNR2. This may imply that the interaction between a corepressor and RAR/RXR dimer can be coordinated by the simultaneous binding of CoRNR1 and CoRNR2 to RAR and RXR, respectively. These results also suggest that the flanking sequences of CoRNR boxes may determine the interaction specificity with receptors[40, 42]. For example, Cohen et al found an additional LxxLL motif in front of CoRNR box 1 of N-CoR, which is not present in SMRT and appears to mediate the TR-specific interaction[42]. The structural basis for these corepressor-receptor interactions were studied by X-ray crystallography and will be discussed later.

Two possible mechanisms have been proposed to explain how SMRT and N-CoR repress gene transcription[43, 44]. First of all, SMRT and N-CoR may function to recruit histone deacetylases (HDACs), enzymes that remove acetyl groups from histone tails, resulting in more tightly compacted chromatin structure that is inhibitory to transcriptional activation. SMRT and N-CoR associate with the mSin3A-HDAC1/2 complex through a direct interaction with mSin3A[23, 45, 46], the same mSin3A-HDAC1/2 complex had been reported as corepressors for Mad-Max and Mxi-Max heterodimers[47, 48]. The mSin3A interacting domain in SMRT corresponds to one of the autonomous repression domains[45]. Furthermore, HDAC-specific inhibitor trichostatin A (TSA) relieved SMRT/N-CoR mediated repression, indicating that HDAC activity is involved in SMRT/N-CoR mediated transcriptional repression. In mammals, multiple HDACs have been cloned[49]. Based on sequence homology, these HDACs can
be divided into three classes. Class I includes HDAC 1, 2, 3 and 8, which resemble yeast Rpd3p; Class II includes HDAC 4, 5, 6, 7, 9 and 10[50], which are related to yeast Hda1p; Class III has a single member Sir2, which is NAD⁺ dependent and insensitive to the Class I and II inhibitor TSA[51]. Interestingly, each repression domain of SMRT/N-CoR associates with a distinct HDACs. SMRT repression domain 1 (RD1) indirectly associate with HDAC1/2; RD2 directly interacts with HDAC3, while RD3 and RD4 binds HDAC4, 5 and 7[52, 53]. However, only HDAC3 was found in the core SMRT/N-CoR complex biochemically purified from nuclear extracts[54, 55], raising the possibility that only HDAC3 is essential for SMRT/N-CoR function, while other HDACs may be required in a gene specific manner. Taken together, these data suggest that SMRT/N-CoR recruit multiple HDACs to repress transcription. Interestingly, recent data revealed that SMRT/N-CoR is also necessary to activate the enzymatic activity of HDAC3[56], suggesting a more complicated role of SMRT/N-CoR in mediating transcriptional repression instead of merely serving as a scaffold protein.

The second possible silencing mechanism of SMRT/N-CoR is to disrupt the transcriptional pre-initiation complex through direct interaction with general transcriptional machinery. SMRT/N-CoR was found to directly interact with general transcriptional factor TFIIB[57, 58]. The TFIIB interacting region of SMRT/N-CoR also corresponds to one of the autonomous repression domains. More importantly, N-CoR disrupted the functional interaction between TFIIB and TAF₃₂[58], which is important in forming a pre-initiation complex[59, 60]. Notably, the two mechanisms outlined above are not mutually exclusive and may function simultaneously.
3. Steroid/nuclear receptor coactivators: SRC family

The existence of steroid/nuclear receptor coactivators was proposed about a
decade ago based on the experiment of “squelching”, where activated steroid/nuclear
receptors can interfere with or inhibit transcriptional activity of other steroid/nuclear
receptors. This phenomenon was explained by competition among activated
steroid/nuclear receptors for a limited pool of cellular factors[61], which led to extensive
search for such kind of factors based on the assumption that these factors should
physically bind to steroid/nuclear receptor in the presence of ligand. As a result,
numerous factors that can bind to various steroid/nuclear receptors in the ligand-
dependent manner have been isolated; many of them are able to enhance the
transcriptional activity of the binding receptor and are therefore termed coactivators[62,
63]. Probably because of the approach used to clone these factors, nearly all of them are
AF-2 specific and only three AF-1 specific coactivators, the p68/p72 RNA helicase[64]
and the SRA, a RNA coactivator for steroid receptors[65], have been reported. Relatively
well studied among these coactivators is the SRC (steroid receptor coactivator)
family[66], also called p160 family to indicate that it contains homologous members with
a molecular weight of 160 kilodaltons.

The SRC family includes three related members, SRC-1[67], GRIP1/TIF2[68-70]
and RAC3/pCIP/AIB1/ACTR/TRAM-1[71-75]. GRIP1 and pCIP are the murine
orthologs of human TIF2 and RAC3, respectively, while AIB1, ACTR and TRAM-1 are
human polymorphic clones of RAC3. Most of these factors (SRC-1, GRIP1, RAC3 and
ACTR) were cloned by yeast two-hybrid screening using receptor LBDs as baits. For
example, SRC-1 was cloned in a yeast two-hybrid screen for human proteins specifically
bound to PR-LBD. Others were isolated in *in vitro* assays looking for proteins associated with immobilized receptor LBD in the presence of ligand. All of these proteins have been shown to bind to multiple steroid/nuclear receptors in a ligand dependent manner and enhance their AF-2 activity. For example, SRC-1 interacts ligand-dependently with PR, ER, TR, RXR, GR and PPAR, and enhances AF-2 activity of these receptors.

Interestingly, recent evidence supports that SRC-1 and TIF2 also functionally interact with N-terminus of steroid receptors and enhance the activity of AF-1[76-78]. SRC factors may also function to coactivate other transcriptional activators, such as NF-κB, SMAD3, AP-1, TEF and MEF2[79-83], suggesting that they participate in multiple cellular signaling pathways.

Through modulation of steroid/nuclear receptor activity, SRC coactivators play important roles in growth and reproduction, development and differentiation. For example, RAC3/pCIP knockout in mouse resulted in retarded growth, delayed puberty and reduced female reproduction[84]. In addition, the RAC3 (AIB1) gene is frequently amplified in ER positive breast cancer cells, and an *in vivo* association between RAC3 and ER was detected in breast cancer cell line MCF-7[71, 85], suggesting a pathological role of RAC3 in breast cancer.

The SRC family of coactivators shares a common domain structure (Fig. 4). From N- to C-terminus, each member contains a highly conserved bHLH-PAS domain, a receptor-interacting domain (RID) and a transcriptional activating domain (AD). The N terminal bHLH-PAS domain is the most conserved region among SRC family member. The bHLH (basic helix-loop-helix) domain is found in many transcriptional activators where it functions as a DNA-binding or dimerization surface[86]. The PAS (Per, Arnt,
Sim) domain was initially identified in Drosophila protein Period (Per) and Single-minded (Sim) as well as a mammalian protein ARNT (AHR nuclear translocator)[87]. It is typically comprised of 250-300 amino acids and contains a pair of highly degenerate 50 amino acid subdomains termed the A and B repeats. Similar domains were also found in many important regulators of circadian rhythm and neural development[88]. The function of PAS domain in those proteins is believed to act as a regulatory domain by mediating protein-protein interaction. In the case of AHR, the PAS domain also serves as a binding surface for small-molecule ligands such as dioxin[89-91]. However, functions for bHLH-PAS domains of SRC family members remain largely unknown, other than it is likely to mediate protein-protein interactions. Two recent reports demonstrated that the GRIP1 bHLH-PAS domain interacts with MEF2C, and the SRC-1 bHLH-PAS domain interacts with TEF[82, 83]. In both cases, the bHLH-PAS domains recruited GRIP1 and SRC-1 as coactivators to the transcriptional activators.

The RID of SRC coactivators contains key determinants for receptor interaction, namely NR boxes i, ii, iii, with the consensus sequence of LXXLL[92]. Crystallographic studies have detailed the molecular basis of interaction between liganded receptors and coactivator peptide containing LXXLL motifs[93, 94]. Briefly, receptor LBD is comprised of 12 helices and coactivator LXXLL peptide binds to a hydrophobic groove on the receptor surface, usually made up of helix 3, 4, 5, and 12. Helix 12 undergoes a dramatic change in orientation upon ligand binding to generate the hydrophobic groove for coactivator binding and, therefore, is the key determinant of the ligand-dependent recruitment of coactivators. Biochemical and biophysical studies have shown that a receptor dimer recruits only one coactivator[94-96], suggesting a coordinate binding of
distinct LXXLL motifs from the coactivator to the two receptor monomers. Interestingly, each receptor shows preferential binding of distinct LXXLL motifs within a single SRC coactivator. For example, in the case of TIF2, motif I preferentially binds RXRβ, while motif ii prefers PPARα[97]. The molecular basis of such selectivity appears to be provided by the flanking sequences of a specific LXXLL motif. The interacting abilities of each LXXLL motif and their particular arrangement in SRC coactivators determines the interactions of individual coactivators to different receptors, and consequently, the functional diversity among SRC coactivators. This diversity is obvious when considering the distinct phenotypes of SRC-1 and RAC3 knockout mice[84, 98].

One major mechanism by which SRC coactivators help to activate gene transcription is histone acetylation. Just as histone deacetylation is linked to transcriptional repression, increased acetylation of lysines in histone tails correlates with transcriptional activation[99, 100]. For example, yeast transcriptional activator GCN5 is a histone acetyl transferase (HAT)[101]. The human GCN5 homologue, P/CAF (p300/CBP associated factor) and its interacting protein CBP (CREB Binding Protein) both possess HAT activity with differential substrate specificity[102, 103]—P/CAF selectively acetylates H3 and H4, while p300/CBP acetylates all core histones in mononucleosomes. Both P/CAF and p300/CBP are known coactivators for steroid/nuclear receptors[104, 105]. Furthermore, mutations that disrupt the HAT activity of p300 or CBP abrogate the ability of these coactivators to enhance transcription mediated by ER[106] or TR-RXR[107] on reconstituted chromatin templates in vitro, suggesting that HAT activity is necessary for their ability to coactivate steroid/nuclear receptors. Interestingly, SRC coactivators, SRC-1 and ACTR possess inherent HAT activity on H3 and H4 in
mononucleosomes as well[74, 108]. Therefore, interaction of SRC with steroid/nuclear receptors may lead to histone acetylation through HAT activities inherent to either SRC coactivators or CBP/p300 and/or P/CAF that are recruited to receptors by SRC coactivators. This also implies that steroid/nuclear receptors may employ multiple HAT activities to activate target gene or distinct HAT activities may be required for activating specific genes[109].

The C-terminal transactivation domain of SRC coactivators overlaps with the CBP interacting domain, suggesting a central role of CBP in mediating SRC coactivators’ transcriptional activity. In addition to its HAT activity, CBP also facilitates gene transcription by recruiting general transcriptional machinery through direct interaction with TFIIB[110]. Furthermore, SRC-1 also binds to general transcriptional factors TBP and TFIIB[111]. Although the functional consequences of these interactions are not clear, it’s likely that SRC coactivators can activate transcription through recruiting or stabilizing the preinitiation complex. Indeed, SRC-1 was able to potentiate PR transactivation with non-chromatin templates in an in vitro system[112], suggesting an activating mechanism independent on histone acetylation. In addition, a weak transactivating domain on SRC coactivators at the far C-terminal end interacts with an arginine methyltransferase that further potentiates receptor-mediated gene activation[113]. Taken together, these results support SRC coactivators activate transcription through multiple mechanisms.

4. Regulation of steroid/nuclear receptors by coactivators and corepressors:

ER as a model
In mammals, there are two estrogen receptors encoded by distinct genes and referred to as ERα and ERβ; they share structural and functional similarity but display different tissue distributions\[114\]. As other members of the steroid/nuclear receptor superfamily, ERs are ligand-dependent transcriptional activators. In the absence of their cognate ligand, 17β-estradiol, ERs are inactively complexed with heat-shock protein hsp90\[115-117\]. Ligand binding causes the dissociation of the complex and formation of receptor homodimers that activate target genes by binding to EREs, DNA sequences consist of inverted repeats of half site GGTCA with a one-nucleotide spacer. Mouse genetics have demonstrated the central role of ERs in the pleiotropic effects of estrogen on growth, development and diseases. ERα knock-out (ERKO) mice are infertile. Female mice do not ovulate and display defects in the development of ovaries, uteri and mammary glands\[118, 119\]. Male mice show compromised sexual behavior and testes degeneration at puberty\[120, 121\]. ERβ knock-out mice (BERKO) display less severe phenotypes but also have defects in fertility and reproductive tissue development\[122\].

**SRC coactivators regulate both ER AF-1 and AF-2**

Like many steroid/nuclear receptors, ERs contain two transcriptional activating domains, AF-1 in the N terminal domain and AF-2 in the C terminal ligand-binding domain. AF-2 activity is dependent on the binding of estrogen and can be blocked by estrogen antagonist while AF-1 is constitutively active, which probably accounts for the partial agonistic effect of some anti-estrogens including tamoxifen\[123\]. Furthermore, the activities of AF-1 and AF-2 are dependent on the target gene promoter context and tissue type\[12, 124, 125\], thus sometimes one activating domain accounts for most of the
transcriptional activity exhibited by the full-length receptor. While in other cases, both AF-1 and AF-2 need to act synergistically to achieve the full activity of the intact receptor[76, 124]. This promoter context and tissue type specificity may be due to the spatial availability of specific coactivators for AF-1 and AF-2, respectively, on individual promoters. However, while AF-2 activity can be largely accounted for by ligand dependent recruitment of coactivators, including SRC coactivators, the precise molecular mechanism through which AF-1 participates in transcriptional activation remains to be determined. Recent studies of SRC coactivators demonstrate that these factors directly bind to ER AF-1 using regions other than the receptor interacting domain or NR boxes[76-78, 126]. In the case of SRC-1, the AF-1 interacting domain was mapped to the middle region, amino acid residues 361-1139[77], while the C terminal Glutamine-rich region seems to be mediating the GRIP1 interaction with ER AF-1[78]. Both SRC-1 and GRIP1 have been shown to enhance the activity of ER AF-1 and mediate the functional interaction between ER AF-1 and AF-2[77, 78, 127], providing new insights into the molecular basis of AF-1 activity as well as its synergy with AF-2. Nonetheless, the exact molecular mechanism of how SRC coactivators synergistically activate ER AF-1 and AF-2 is unknown.

**SRC coactivators are primary coactivators for ERs**

As discussed previously, numerous AF-2 specific coactivators have been isolated for steroid/nuclear receptors. In the case of ERs, those coactivators include SRC coactivators, p300/CBP, P/CAF. A recently isolated multiprotein complex involved in thyroid hormone receptor (TR) and vitamin D receptor (VDR) signaling (DRIP/TRAP
complex)[128, 129] has also been implicated in ER function through the interaction of its PBP/TRAp220/DRIP205 subunit with ER[130]. The big question is then how these coactivators coordinate to modulate ER function. Models of both combinatorial and sequential recruitment of these coactivators to ER have been proposed[131]. Recent studies present a body of evidence supporting a sequential recruitment model where different coactivators are recruited in an orderly fashion to the promoters of endogenous ER target genes following estrogen stimulation[132-134]. Using the technique of chromatin immunoprecipitation, Shang et al found that distinct coactivators appear sequentially on ER target gene promoter in MCF-7 cells following estradiol treatment[132]. Interestingly, SRC coactivators are among the first coactivators that appear at the promoter and more importantly, recruitment of SRC coactivators appear to be sufficient to induce assembly of an ERα complex capable of gene activation. In an in vitro system, Kim et al found that recruitment of SRC functional domains to gene promoters by heterologous DNA binding domain is sufficient to assemble a coactivator complex to activate transcription, while recruitment of CBP fragments capable of interacting with SRC coactivators failed to activate transcription, suggesting that SRC coactivators have to be recruited prior to the recruitment of CBP/p300[133]. Similarly, Liu et al found in vitro, synergistic enhancement of PR transcriptional activity by SRC-1 and p300 requires pre-incubation of PR-bound chromatin templates with SRC-1 before the addition of p300, but not vice versa, confirming a sequential recruitment of SRC-1 prior to p300[134]. Taken together, these data clearly reveal that SRC coactivators are primary assembling factors in forming the coactivator complex necessary for ER transcriptional activity.
SRC coactivators integrate multiple events regulating ER activation

Activation of ERs is a multiple-step process regulated not only by the ligand binding and the subsequent recruitment of coactivators including SRC coactivators, but also by a number of other events, including receptor dimerization[135], phosphorylation [136-138] and DNA binding[139]. Interestingly, recruitment of SRC coactivators appears to be the step that integrates these regulatory events during receptor activation, supporting again an essential role of SRC coactivators in regulating ER transcriptional activity. For example, MAPK phosphorylation of ERβ at Ser114 activates ERβ and this activation arises from stronger interaction of the phosphorylated ERβ with SRC-1[126]. Furthermore, protein kinase signaling pathways also result in phosphorylation of SRC coactivators[140-142]. Increased interaction between phosphorylated AIB1 and p300 has been observed which accounts for the enhanced transcriptional activity of ER upon activation of MAPK pathway, suggesting that AIB1 is the conduit through which signals from activated protein kinases are relayed to ERs[141]. Receptor dimerization may also affect transcriptional activity by interfering with coactivator binding. ERα and ERβ form functional homodimers as well as heterodimers[143], the differential transcriptional activity of these dimers may be caused by their differential affinity with SRC coactivator[144]. Finally, individual EREs mediate differential ER transcriptional activity[139], which is probably because individual ERE sequences induce changes in conformation of the DNA-bound receptor ERβ and influence AIB1/TIF2 recruitment[145]. Taken together, these studies indicate that the highly regulated recruitment of SRC coactivators is the critical step in ER activation.
The coactivator and corepressors exchange on receptors

ERs usually do not bind corepressor SMRT/N-CoR except in the presence of certain antagonists[29, 30]. This discovery indicates that corepressors are also important regulators of ER activity. Binding of SMRT/N-CoR to tamoxifen-bound ER may prevent the partial agonistic effect of tamoxifen. Indeed, the ER antagonist 4-hydroxy-tamoxifen (4-OHT) failed to activate ER-dependent transcription in wild-type mouse embryo fibroblasts (MEFs) but acted as a full agonist in N-CoR−/− MEFs[33]. In addition, a tamoxifen responsive ER-D351Y mutant exhibits reduced interaction with SMRT/N-CoR[146].

From the above discussions, the most important molecular event in steroid/nuclear receptor signaling following ligand binding is probably the switch from corepressor binding to coactivator binding. Such a ligand dependent switch is the key to transform a receptor from a transcriptional repressor to an activator and therefore, understanding the molecular basis of this switch will provide invaluable insights into how functions of steroid/nuclear receptors are regulated in vivo.

It is interesting to note that coactivators and corepressors use quite similar motifs to interact with receptor LBDs. Actually, the consensus sequence LXXI/H IXXX I/L of CoRNR boxes from corepressors represents an extended helix compared to LXXLL, the sequence found in NR boxes for coactivators. The similarity between CoRNR box and NR box suggests that corepressors and coactivators may utilize overlapping interaction surfaces on the receptors. This idea is supported by the observation that point mutations of certain residues in helix 3, 5 and 6 on receptor LBD structure disrupt interactions with SRC coactivators as well as with corepressors[39]. Then how can ligand binding
differentially affect corepressor binding compared to coactivator binding? Crystallographic studies of receptor LBD-NR box peptide have provided a compelling model to explain this[93, 94]. As mentioned before, ligand binding to LBD induces a shift in position of helix 12, which together with helix 3, 4 and 5, forms a hydrophobic pocket on the receptor surface where LXXLL helix fits in. Due to the extended size of the CoRNR box, it will not fit into the hydrophobic pocket because of the consequent molecular clash between the extended helix and helix 12. Therefore, corepressor cannot bind to liganded receptors. This model predicts a critical role of helix 12 in recruiting coactivators as well as in releasing corepressors, both have been supported by biochemical studies. Deletion of helix 12 in LBD domain usually abolishes its recruitment of SRC coactivators[70, 147], while helix 12 of RXR is inhibitory to corepressor binding and deletion of it enhanced the ability of RXR to repress transcription[148]. Furthermore, the crystal structure of antagonist bound ER reveals that helix 12 oriented in a position different from that of ligand bound ER, where it blocks the coactivator LXXLL helix binding[93]. Interestingly, while steroid receptors generally do not interact with corepressors, antagonist bound steroid receptors have been reported to be able to recruit SMRT/N-CoR, probably because of that the relocated helix 12 will no longer occlude their binding.

Summary and thesis goals

Corepressors SMRT/N-CoR and SRC coactivators are key regulators of the transcriptional activity of steroid/nuclear receptors. Both classes of cofactors are modular proteins containing receptor interacting domains and other domains functioning to recruit
either repression or activation complexes, respectively. Their RIDs use similar core motifs to interact with receptors on overlapping surfaces whose accessibility is reversibly regulated by ligand binding, making it possible for a receptor to switch between a transcriptional activator and repressor. It is clear now that both corepressors and coactivators function in large complexes. Understanding the molecular composition of each complex and the functional interactions among subunits of each complex holds the key to elucidate how the transcriptional activity of steroid/nuclear receptors is regulated. The focus of my thesis research is to study the functional interactions between SMRT and its associated proteins and try to isolate and characterize proteins that interact with RAC3 and participate in modulating receptor activity.

The amazing ability of SMRT/N-CoR to interact with a large number of HDACs and help various transcriptional factors to repress gene transcription raised the possibility that SMRT/N-CoR may act as scaffold protein in the nucleus. However, current evidence supporting in vivo interaction between SMRT/N-CoR and HDACs arises primarily from biochemical analyses of cellular extracts, and it's somewhat controversial. For example, although co-immunoprecipitation revealed the association of HDAC1/2 and Class II HDACs, HDAC4, 5, 7 in the SMRT/N-CoR complex, they are not present in the purified SMRT/N-CoR complex. Clarification of the interaction between these HDACs and SMRT/N-CoR may provide important insight into how SMRT/N-CoR mediates transcriptional repression by steroid/nuclear receptors. Therefore, one specific aim of this thesis research is to study the molecular interactions between SMRT and other proteins that have been reported in the corepressor complex by immunofluorescence. During this course of study, we found that SMRT predominantly forms a specific nuclear dot
structure that does not appear to overlap with any other well-known subnuclear domains/speckles. We observed the colocalization of unliganded RAR and certain HDACs, including HDAC1, 3, 4 and 5, in the SMRT nuclear speckles. The presence of HDAC1 in the SMRT speckles suggests a direct in vivo association between these two proteins that has not been detected by previous biochemical analysis. However, HDAC1 point mutants defective in deacetylase activity lost colocalization with SMRT nuclear speckles, suggesting that HDAC activity of HDAC1 is required for its association with SMRT. Interestingly, association with SMRT resulted in nuclear accumulation of HDAC4, which correlated with the enhanced inhibition of MEF2C by HDAC4 in a transient transfection assay. These discoveries revealed an unexpected functional aspect of the interaction between SMRT and other proteins of the corepressors complex, which is to recruit these proteins into a novel subnuclear domain important in transcriptional repression.

The central role of SRC coactivators in steroid receptor activation attracts much interest in these proteins. Nevertheless, the N-terminal bHLH-PAS is the most highly conserved domain but does not have a clearly defined function in steroid/nuclear receptors signaling. It's very likely that this domain mediates interactions with additional components of the coactivator complex. Therefore, identification of interacting partners for bHLH-PAS domain may help to elucidate the composition as well as function of the coactivator complex. The second focus of this thesis research is to identify proteins that interact with the RAC3 bHLH-PAS domain by yeast two-hybrid screening. During this study, we cloned the human homologue of yeast protein MMS19 as a protein that specifically interacts with RAC3 bHLH-PAS domain. Unexpectedly, hMMS19 also
interacts directly with and functions as a specific coactivator for ERs. Unlike most coactivators, hMMS19 enhanced the AF-1, but not AF-2 of ERs. So we concluded that hMMS19 is a specific coactivator for human estrogen receptors through enhancement of AF-1 activity.

The third specific aim is to try to understand the molecular mechanism by which hMMS19 enhances the transcriptional activity of ER AF-1. First we studied whether this enhancement involves TFIIH mediated phosphorylation of ERα Ser118 since hMMS19 interacts with TFIIH and it has been previously reported that TFIIH was able to enhance the transcriptional activity of ERα AF-1 by phosphorylating Ser118. We found that hMMS19 activates mutant ER AF-1 where Ser118 has been changed to Ala, suggesting that the recruitment of TFIIH and subsequent phosphorylation of Ser118 by TFIIH is not involved. We demonstrated that hMMS19 may enhance ER AF-1 through cooperation with RAC3, whose ability in stimulating AF-1 has been reported. Interestingly, we found a novel region in RAC3 that binds directly to the N-terminus of ER as well as some other nuclear receptors. Based on these discoveries, a model integrating functions of each individual RAC3 domain was proposed to explain its ability to coactivate certain nuclear receptors such as ER. Taken together, these studies contribute to our understanding of coactivator complex by identifying maybe a new member and providing new insight into how coactivator complex mediate the synergistic activation of AF-1 and AF-2, the two transcriptional activating domains of steroid/nuclear receptors.
Chapter II

Materials and Experimental procedures
SMRTe Plasmids and Antibodies

The mammalian expression vector of SMRTe, pCMX-hSMRTe was made by taking the Asp718/XhoI fragment from pCMX-Gal-hSMRT(1-1111)[34] and cloning into pCMX-SMRT[16]. pCMX-N-CoR was a gift from Dr. M. Rosenfeld, pBJ5-HDAC1, 4, 6 and pBJ5-HDAC4mut(S246/467/632A) were gifts from Dr. S. Shreiber. The MEF2 3X Luciferase reporter was a gift from Dr. E. Olson. pCMX-HA-HDAC3 was created by taking the EcoRI fragment from pGEX-HDAC3 (a gift from Dr. W-M Yang) and cloning into pCMX with an in-frame HA tag at the N-terminus. Rabbit polyclonal antibodies against SMRT were described in Park et al[34]. The mouse anti-SMRT monoclonal antibody was obtained from GeneTex (San Antonio, Texas). Monoclonal antibodies against Flag (Anti-FLAG M2) and HA epitope were from KODAK and Santa Cruz. The fluorescein-conjugated goat anti-rabbit IgG was purchased from Rockland Inc and the rhodamine-conjugated goat anti-mouse IgG was purchased from Chemicon.

Yeast two-hybrid screening

The yeast two-hybrid screening was carried out as described previously[147]. Briefly, the pGBT-RAC3 (1-408) was used to screen a human placenta cDNA library in pACT2 vector (CLONTECH) for RAC3 interacting proteins. After primary selection on synthetic dropout media without tryptophan, leucine or histidine and supplemented with 50mM 3-aminotriazole, 25 positive clones were isolated, which were further tested for β-galactosidase expression by the liquid assay as described. The library plasmids from
positive clones were rescued and retransformed into yeast cells, together with the original bait and other constructs, for further confirming the specificity of protein-protein interaction.

**Rapid Amplification of cDNA 5’ ends (RACE)**

An hMMS19 gene specific 3’ primer: \( 5’\) CAT AAG ATA GGA GAT CTG GCT GGG CAC CCA AGA CTG TC \( 3’\), which contains the endogenous Bg/II restriction site was used together with an adaptor from the manufacturer to amplify the extreme 5’-end of hMMS19 from the Hela cell Marathon\textsuperscript{TM} ready cDNA (CLONTECH). Resulted product was re-amplified using the same 3’ primer and an internal adaptor from the manufacturer, which has a NorI restriction site. Purified products were subcloned into pBluescript vector utilizing the NorI and Bg/II site. Plasmids from 30 independent clones were purified and sequenced.

**Construction of hMMS19 expression plasmids**

The original pACT2-RS2.1 isolated by yeast two-hybrid screening was cut with SalI/BglII, the 700bp fragment released was assembled into SalI/BamHI digested pCMX vector containing an in-frame HA tag. This results in pCMX-HA-RS2.1. pCMX-HA-hMMS19\(\beta\) was constructed by assembling the SalI/BglII fragment from RACE and the BglII/NsiI fragment from AF007151 (Research Genetics) together into SalI/NsiI digested pCMX-HA-RS2.1 vector. pCMX-HA-hMMS19\(\alpha\) was obtained by replacing the Dra3/NsiI fragment of pCMX-HA-hMMS19\(\beta\) with the same fragment from EST clone.
BE206052 (IMAGE 3010660) (Incyte Genomic). All constructs were confirmed by sequencing.

**Northern Blot**

MTN\textsuperscript{TM} blots (CLONTECH) of either total mRNAs from various cancer cell lines or multiple human tissues were probed with \textsuperscript{32}P-labeled random-primed DNA corresponding to the \textit{NsiI/BgII} restricted 1.5kb fragment from the cloned hMMS19. Hybridization was performed using the ExpressHyb solution (CLONTECH) as recommended by the manufacturer. The blot was washed twice for 20 min in 2x SSC /0.1% SDS at room temperature and subjected to autoradiography at -70\textdegree C.

**GST-pull down assay**

GST fusion proteins were expressed in \textit{E. coli} BL-21 cells and purified by standard glutathione agarose bead according to manufacturer’s recommendation (Pharmacia). \textsuperscript{35}S labeled proteins were made by the coupled in vitro transcription and translation reactions using the T7-Quick reticulocyte lysate system (Promega). For the GST pull down assay, 5 \textmu g of glutathione agarose bead-conjugated fusion protein were incubated with 5 \textmu l of in vitro translated \textsuperscript{35}S-labeled protein with moderate shaking at 4\textdegree C overnight in binding buffer[149]. The pellet was washed 4 times with the binding buffer. Supernatant was carefully removed each time and in the end, bound protein was eluted in SDS sample buffer by boiling 5 min and then subjected to SDS-PAGE and autoradiography.
Site directed mutagenesis

ER Ser118 mutants were generated with the Quick-change site-directed mutagenesis system (Stratagene). Mutagenesis utilized a supercoiled, double stranded DNA template with the insert of interest and two, synthetic, complementary oligonucleotide primers containing the desired mutation. The primers extend during temperature cycling by means of pfuTurbo DNA polymerase. Then the parental DNA template is digested away with DpnI, which is specific for methylated DNA, thus selecting for mutation-containing synthesized DNA. The sequences of mutant constructs were confirmed by dideoxynucleotide chain-termination sequencing reactions using the T7 Sequenase protocol (USB).

Cell culture and transient transfection assay

HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO). One day before transfection, cells were seeded in 12-well plates at a density of 50,000 cells per well in phenol-red free DMEM supplemented with 10% charcoal resin-stripped FBS. Transfection was performed using a standard calcium/phosphate method as described before [149]. After ~10 hours, cells were washed with phosphate-buffered saline (PBS) and re-fed with fresh medium containing either vehicle alone or vehicle plus ligands. Cells were lysed 24 hrs after treatment for luciferase assay and β-galactosidase assays as previously described.
Indirect immunofluorescence

Hela cells were grown on coverglasses (no. 1, Erie Scientific, Portsmouth) in 12-well plates. Immunocytochemistry was performed as described[149]. Briefly, cells were washed twice with PBS before fixing for 1 minute in pre-chilled methanol/acetone (1:1) on dry ice. Cells were blocked with 2% normal goat serum. After immunostaining, the nuclei were counterstained 5 minutes with 1 μg /ml DAPI (4’, 6-diamidino-2-phenylindole dihydrochloride hydr. Sigma). The coverglasses were then mounted on Super Up-Rite microscope slides with ProLong Antifade Kit (Molecular Probes, Eugene, Oregon). The samples were analyzed using a Nikon immunofluorescence microscope. The 2-D images were acquired using a cooled CCD camera and the MetaMorph program. The images were false-colored with “Color Encode” command and processed using PhotoShop or Canvas to adjust the displaying levels.
Chapter III

Results
Chapter III-1.

SMRTe INHIBITS MEF2C TRANSCRIPTIONAL ACTIVITY BY TARGETING HDAC4 AND 5 TO NUCLEAR DOMAINS

A. Subcellular localization of SMRTe and N-CoR in culture cells

The steroid/nuclear receptor corepressors SMRTe and N-CoR mediate transcriptional repression by unliganded retinoic acid and thyroid hormone receptors. To provide insight into the function of SMRTe and N-CoR in cells, we analyzed the cellular localization of three isoforms of SMRT and N-CoR in mammalian cells using transient expression and immunofluorescence microscopy.

Expression of SMRTe in Hela cells revealed that a majority of transfected cells (80-90%) displayed exclusive nuclear speckle staining (Fig. 5, a), while about 10-20% of transfected cells displayed overall speckle pattern in both cytoplasmic and nuclear compartments (b). Similar distribution patterns were observed with monoclonal and polyclonal antibodies, and in other cell types (data not shown). Also, immunostaining of endogenous SMRTe showed nuclear speckle pattern (c), suggesting that such nuclear speckles are not artifacts of protein overexpression. The existence of endogenous cytoplasmic SMRTe is not conclusive due to high background of cytoplasmic staining with the available antibodies.

Because the structure and function of N-CoR is highly related to SMRTe, we also analyzed cellular distribution of N-CoR. We found N-CoR exclusively in nuclei (>99%
of transfected cells) forming similar SMRTe speckles (Fig. 5, d). Because the expression of SMRTe and N-CoR were controlled by the same promoter and staining intensity was similar, it is unlikely that the difference in distribution is due to protein levels. We suggest that the difference is intrinsic to SMRTe and N-CoR and distinct mechanisms may control SMRTe and N-CoR distribution.

B. Determinants of nuclear localization and speckle distribution of SMRTe

We then wished to determine the domain that determines SMRTe speckle localization by analyzing a series of SMRTe deletion mutants. The N-terminal extended domain between amino acids 1 to 1109 was located exclusively in the nucleus in a diffuse pattern (Fig. 6, panel b) suggesting that this domain contains nuclear localization signal but lacks the speckle signal. Interestingly, the highly conserved SNC-SANT domain was located mainly in the cytoplasm (panel c), indicating that nuclear localization signal is located outside of the SNC-SANT region. The original SMRT protein without the N-terminal extended domain distributed mainly in nuclear speckles in a manner that is similar to SMRTe (panel d) and consistent with an intact corepressors function. Furthermore, the repression domain 3 (RD3) (formally SRD1) was located exclusively in the nucleus with speckles (panel e), indicating that this domain contains a separated nuclear localization signal and potent speckle localization signal. In contrast, the RD4 domain (formally SRD2) was located exclusively in the cytoplasm with pronounced speckle pattern (panel f), suggesting that the RD4 contains a cytoplasmic speckle signal.
Interestingly, the steroid/nuclear receptor interacting domain alone (cSMRT) displayed a diffuse, mostly cytoplasmic staining (panel g), indicating that it lacks a nuclear localization signal. These data suggest that SMRTe contains multiple sequences for regulating its precise subcellular localization.

C. SMRT nuclear domain is unique among other nuclear structures

The speckle distribution of SMRTe prompted us to investigate if SMRTe might overlap with any known nuclear domains. Double immunofluorescence analysis of the SMRT nuclear speckles with the splicing factor SC35 showed no colocalization (Fig. 7, panel a-d). Similarly, SMRTe did not colocalize with PML oncogenic domains (panel e-h). In addition, SMRTe did not colocalize with the nucleolus, condensed chromatin or centromeres (data not shown). These data suggest that the SMRTe nuclear domains represent a novel class of nuclear structures.

D. Association of SMRTe with RARα \textit{in vivo}

To provide evidence for a role of SMRTe in RA signaling, we examined the association of SMRTe with RARα \textit{in vivo} in the absence or presence of RA. As previously reported, RARα alone distributed diffusely in nucleoplasm (data not shown). Interestingly, co-expression with SMRTe redistributed RARα into both the cytoplasmic
and nuclear speckles in the absence of ligand (Fig. 8, panel a-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 (panel d-f). This observation is consistent with previous *in vitro* evidence that atRA induces dissociation of SMRT from the receptor. 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 steroid/nuclear receptor interaction domain, antagonizes transcriptional repression by Gal4-RARα fusion protein[22]. Because cSMRT is located primarily in the cytoplasm, we speculated that this cytoplasmic cSMRT might either sequester unliganded Gal-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. As previously reported, Gal4-RARα alone was located exclusively in the nucleus (data not shown). Remarkably, cSMRT sequestered the Gal4-RARα to the cytoplasm in the absence of ligand (Fig. 9, panel a-c), and all-trans RA treatment partially released this sequestration, rendering accumulated nuclear localization of Gal4-RARα (panel 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 cellular compartmentalization may contribute to the dominant effect of cSMRT.

**E. Colocalization of SMRT nuclear domains with other interacting proteins**
In addition to unliganded RAR, SMRT also interacts with several other proteins involved in diverse signaling pathways. These include the promyelocytic leukemia zinc finger protein PLZF[26], ETO, the fusion partner of AML with t(8;21) translocation [150], the mammalian homologue of the Drosophila Suppressor of Hairless CBF1[151], the mammalian homologue of Drosophila chromatin binding protein BX42 (or Skip) [152].

As expected, we found that PLZF and ETO colocalized precisely with the SMRT nuclear domains (Fig. 10, panel a-c, d-j). However, we found no evidence of association between SMRT and CBF1 (not shown). The distribution of CBF1 is nearly uniformly located in the nucleus despite the presence or absence of SMRT overexpression. Furthermore, in cells that contain cytoplasmic SMRT, CBF1 remained exclusively in the nucleus uniformly. These data confirmed the in vivo interaction of SMRT with PLZF and AML1, but not with CBF1.

F. Interaction of SMRTe with class I HDACs

Biochemical evidence have suggested that HDAC1/2 and mSin3A form a stable protein complex [153-155]. It has also been reported that SMRT and N-CoR form a corepressor complex with mSin3A and HDAC1 [23, 45, 46]. However, the purified mSin3A-HDAC complex lacks SMRT and N-CoR[155]. To help understand this complexity and also to characterize potential SMRTe interactions with class I HDACs,
we analyzed colocalization between SMRT and HDACs and mSin3A/B. In the absence of SMRTe, HDAC1, 2 and 3 were located diffusely in the nucleoplasm (Fig. 11, panel a, e, f and m). Interestingly, co-expression of SMRTe caused a distribution of HDAC1 and HDAC3 (panel b-d, j-l), but not HDAC2 (panel f-h) or mSin3A (panel n-p) to distinctive SMRTe nuclear speckles. We noted that HDAC3 co-expression appeared to partially distort the structure of the SMRTe nuclear speckles. Because SMRTe colocalized with HDAC1 and HDAC3 in the absence of mSin3A, and because SMRTe does not colocalize with mSin3A, these data suggest an mSin3A-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[156]. The HDAC enzymatic activity depends on conserved residues within the catalytic domain, and point mutation of these residues disrupts HDAC activity[156]. 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. We found that only the partially defective mutant H141A (Fig. 12, panel a-c) remained associated with SMRTe nuclear speckles in this assay, while both H199A (panel d-f) and D176N (panel 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.

G. Interaction of SMRTe with class II HDACs
Recently, members of class II HDACs, HDAC4, 5, 7 were identified as SMRT/N-CoR interacting proteins[52, 54]. We sought to confirm these interactions in vivo in the double immunofluorescence assay. Consistent with a previous report, HDAC4 alone was located primarily in the cytoplasm (Fig. 13A, panel a)[157]. Strikingly, cotransfection with SMRTe enhanced translocation of HDAC4 from the cytoplasm into the nucleus, where it colocalized precisely with SMRTe nuclear speckles (panel b-d). We estimated that in the absence of SMRTe, about 70% of the transfected cells contained dormant cytoplasmic HDAC4, and 30% contained almost equal HDAC4 staining in the cytoplasm and nucleus (Fig. 13B). Strikingly, co-expression of SMRTe translocated HDAC4 into nuclear speckles in about 92% transfected cells, suggesting a mechanism of SMRTe-dependent nuclear import and nuclear domain targeting of HDAC4. This SMRTe-mediated nuclear import of wild type HDAC4 was even more pronounced than the HDAC4 mutation S246/467/632A that abolishes 14-3-3 binding in the cytoplasm[157]. The HDAC4 S246/467/632A 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. 13A, panel e). There were only about 4% of HDAC5 transfected cells contained HDAC5 dominant cytoplasmic staining (Fig. 13B). Strikingly, co-expression of SMRTe translocated HDAC5 almost completely from diffuse nucleoplasm staining into nuclear speckles (Fig. 13A, panel f-h and Fig. 13B), 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. 13A, panel 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.

H. SMRT 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 over-expression of HDAC4 and 5[158]. Because SMRTe enhanced 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. 14). Consistent with previous findings[159, 160], we found that MEF2C alone strongly activated reporter gene expression 40-100 folds and that HDAC4 and 5 inhibited this activation by 2-4 folds, respectively. In contrast, HDAC1, 2, 3 and 6 did not have much effect on MEF2C function (Fig. 14A). On the other hand, SMRTe alone also had only minor effect on the
MEF2C activity (Fig. 14B). However, cotransfection of SMRTe and HDAC4 synergistically inhibited MEF2C activity by 20 folds. Similar effect was observed by co-expression 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.

**Summary**

The corepressor SMRT and N-CoR interact with DNA-bound steroid/nuclear receptor to mediate transcriptional repression by a mechanism involving histone deacetylation. In this study, we have analyzed the subcellular localization of 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, HDAC1, 3, 4 and 5 to these nuclear domains. 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[54, 55, 161], and HDAC3 can be coprecipitated with HDAC4 and 5[162]. 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[23, 45]. Consistent with our findings, mSin3A does not colocalize well with N-CoR[163]. Therefore, we
suggest that HDAC1 may interact with SMRTe in vivo in an mSin3A-independent manner. Perhaps protein modification or a yet-to-be-identified protein might stabilize this in vivo interaction between HDAC1 and SMRTe.

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.
A. Identification of hMMS19 as a RAC3-interacting protein by yeast two hybrid screen

To identify proteins that interact with RAC3 N-terminal bHLH-PAS domain, we conducted the yeast two-hybrid screening, using the RAC3 N-terminal aa 1-408 as bait to screen a human placenta cDNA library. More than $2 \times 10^6$ clones were screened and from which we isolated 25 positive colonies. Among the potential positive clones, one clone designated RS2.1 (RAC3 screening clone #2.1) showed specific interaction with the bait in yeast two-hybrid assay after retransformation of the rescued library plasmid (Fig. 15A). As controls, RS2.1 did not interact with RAR, p53 or the bHLH-PAS domain of mSim. When cotransformed into yeast cells with Gal4 DBD fused different regions of the RAC3 N-terminus, RS2.1 interacted with only the PAS-A/B domain and the interaction is stronger than that with the bait (Fig. 15B). None of the separate bHLH, PAS-A or PAS-B domain interacted with RS2.1. These data suggest that both PAS-A and PAS-B are required for RS2.1 interaction.

The RS2.1 sequence matched a partial cDNA sequence in the Genbank (Accession #: AF007151), whose function is unknown. However, the putative open reading frame of AF007151 ends at about 260bp in front of where RS2.1 starts.
Interestingly, the peptide sequence encoded by RS2.1 shares significant homology with the C-terminus of the yeast protein Mms19, a regulator of yeast TFIIH with functions in both DNA repair and transcription [164]. Further alignment of AF007151 and yMms19 indicated significant homology between these two proteins, suggesting that the full-length RS2.1 and AF007151 may encode the same protein. To clarify this, we blasted both DNA sequences against dbEST database. Five EST clones that include the stop codon region of AF007151 were found, and four of them have a single G insertion between #937 and #938 nucleotide of AF00715. This G insertion will switch the reading frame to the same as RS2.1. These results were later confirmed by sequencing AF007151 and one of the EST clones. Therefore, we concluded that RS2.1 and AF007151 are partial cDNA sequences of the same gene transcript, and AF007151 may represent a natural mutant of this gene.

The full-length RS2.1 cDNA was then cloned by rapid amplification of the 5' cDNA end (RACE). This resulted in a cDNA with a 3090 bp open reading frame encoding a protein with 1030 amino acid residues. The original RS2.1 encodes only the C terminal 164 amino acid residues, which thus appears to be the RAC3 interacting domain. On the contrary, the AF007151 sequence predicts a truncated protein at amino acid residue 746 of hMMS19 followed by a 33 aa sequence. Comparison with yeast Mms19 demonstrated a 42% overall similarity between RS2.1 and yMms19. Similar proteins were also predicted from the genomic sequences of D. melanogaster, C. elegans and A. thaliana. Multiple-sequence alignment using ClustalW program (Fig. 16) indicates that these proteins from different species can be roughly divided into three domains: the highly conserved N and C termini and the more divergent central region. From now on,
we will refer to the full-length hMMS19 containing the AF007151 mutation as hMMS19-mt.

**B. Expression of human Mms19**

Northern blot of various cancer cell lines and human tissues using the central 1.5 kb of hMMS19 as the probe detected a single band at about 4.0 kb (Fig. 17). This band is ubiquitously expressed in all cell lines and tissues analyzed. The transcript amount appeared to be higher in brain, placenta, skeletal muscle and kidney than in heart, lung or liver, while the amount looked quite even in cancer cell lines. By blast search against the high-throughput human genomic sequences, we found that human Mms19 gene maps to chromosome 10q23.3 region.

To determine the subcellular localization of hMMS19, we transiently transfected HeLa cells with an expression plasmid carrying HA-tagged hMMS19 gene. By indirect immunofluorescence using antibodies against HA epitope, we found that the HA-tagged hMMS19 protein is located exclusively in the cell nucleus (Fig. 18). This localization is consistent with the presumed roles of MMS19 in DNA repair and transcriptional regulation through its functional interaction with TFIIH.

**C. In vitro interaction of RAC3 and hMMS19**
To confirm the physical interaction between RAC3 and hMMS19, we performed GST pull down assays using GST-RAC3(1-407) and various GST-RAC3 fusion proteins to pull down in vitro translated $^{35}$S-labeled full-length hMMS19, the C-terminal truncated hMMS19-mt and the C-terminal RS2.1 fragment. As shown in Fig. 19A, significant amount of in vitro translated, $^{35}$S labeled hMMS19 and RS2.1 were pulled down by GST-RAC3 (1-407). In contrast, only residual amount of hMMS19-mt were pulled down from the input in the same experiment, suggesting the C terminal 164aa RS2.1 fragment is the RAC3-interacting domain.

Yeast two-hybrid assay has suggested that the RAC3 region responsible for the interaction with hMMS19 is the combined PAS-A and PAS-B domain. This was again confirmed by GST pull down (Fig. 19B). In this experiment, GST-RAC3-bHLH failed to pull down any $^{35}$S labeled hMMS19α. In contrast, GST-PAS-A or GST-PAS-B alone was able to pull down a small amount of hMMS19, suggesting a weak interaction between hMMS19 and PAS-A or PAS-B separately. Strikingly, GST-PAS-A/B pulled down $^{35}$S-labeled hMMS19 substantially. Similarly, GST-RAC3 (1-407) also pulled down a substantial amount of hMMS19. These data confirmed the results from yeast two-hybrid assay, suggesting that both PAS-A and PAS-B are involved in the interaction with hMMS19.

D. The hMMS19 C-terminal RS2.1 fragment inhibits ER activation
Because RAC3 plays an important role in modulating ER activity \textit{in vivo}, we asked if RS2.1 also has effects on ER activity. To test this possibility, HEK293 cells were transiently transfected with estrogen receptor $\alpha$ (hER$\alpha$) and a luciferase reporter gene driven by estrogen responsive element (ERE). After transfection, 17$\beta$-estradiol induced a strong activation of the reporter gene (Fig. 20A). Intriguingly, co-transfection of the RS2.1 fragment inhibited the ligand-dependent activation of hER$\alpha$ by about 4 fold (Fig. 20A). The inhibition is specifically mediated by ER since no inhibition was observed when the reporter gene lacks the ERE (data not shown). Interestingly, the ligand dependent activity of either RAR$\alpha$ or TR$\beta$ was not affected by RS2.1 (Fig 20A), suggesting that this inhibition is receptor specific.

To confirm the inhibition by RS2.1 on ER activity, we transiently transfected either hER$\alpha$ or hER$\beta$ with increasing amount of RS2.1. A dose dependent inhibition was observed on both hER$\alpha$ and hER$\beta$ and the maximum repression were up to 9 and 6 fold, respectively (Fig 20B). These data suggest that MMS19 may selectively affect both ER$\alpha$ and ER$\beta$ activity.

Because RS2.1 represents the small C-terminal fragment of hMMS19 that interacts with both ER and RAC3, RS2.1 may inhibit ER activation as a dominant negative mutant, disrupting the functional interaction between ER and RAC3. If this were true, then overexpression of RAC3 in the presence of RS2.1 may reverse the inhibition of ER activity. So we examined the effect of RS2.1 on ER$\alpha$ in the presence of RAC3 overexpression. Indeed, as shown in Fig. 20C, cotransfection of RAC3 and RS2.1 relieved RS2.1 mediated inhibition of ER$\alpha$ in the transient transfection assay.
E. hMMS19 interacts with ER

The inhibition of ER activity by RS2.1 prompted us to test if RS2.1 interacts with ER directly. GST-RS2.1 was incubated with $^{35}$S-labeled hER$\alpha$ or hER$\beta$ in the absence of ligand. In both cases, GST-RS2.1 pulled down a significant portion of each $^{35}$S labeled protein (Fig. 21A), indicating an interaction between RS2.1 and ERs. Interestingly, in the same experiment, only a slightly more than background level of $^{35}$S labeled RAR$\alpha$ or TR$\beta$ were pulled down by GST-RS2.1, consistent with the transient transfection data showing that RS2.1 had no effect on either receptor's activity. We next examined whether ligand could effect this interaction. The GST pull down experiment was repeated in the presence of 1 $\mu$M 17$\beta$-estradiol or tamoxifen, a partial antagonist of ER. Neither ligand was able detectably change this interaction (Fig. 21B). Taken together, these data suggest that the Mms19 interacts directly with ER in a ligand-independent manner.

F. hMMS19 enhances AF-1 activity of ER

Since RS2.1 acts as a dominant negative to disrupt the functional interaction between RAC3 and ER and inhibit the transcriptional activity of ER, we then asked whether full-length hMMS19 has any effect on ER activity. So we tested the effect of full-length Mms19 on ER activation by a transient transfection assay. We found that full-length Mms19 weakly, but consistently, enhanced the ligand-induced activity of hER$\alpha$ (Fig. 22). In contrast, hMMS19mt, which lacks the C-terminal RS2.1 fragment, failed to
enhance ER activity, suggesting that the ability of MMS19 to modulate ER activity depends on ER-MMS19 interaction.

To dissect whether hMMS19 coactivation is mediated through either the AF-1 region or AF-2 region of hERα, we subcloned hERα A/B-C and CD-E/F regions separately into the same mammalian expression vector. In 293 cells, both isolated domains can activate the reporter in the presence of ligand, although to a lesser extent compared to full-length ER. As expected, the N-terminus is also active in the absence of ligand. Strikingly, the activity of isolated ERα-N was enhanced 6-fold after co-transfection of hMMS19 in the presence or absence of E2. On the other hand, AF-2 activity was not affected by co-transfection of Mms19 (Fig. 23). These findings suggest that Mms19 may function as an AF-1 specific coactivator of ERs.

Summary

In this chapter, we have identified and characterized the human homologue of the yeast DNA repair and TFIIH regulator MMS19 as a RAC3/ER-interacting protein. The human MMS19 interacts with the N-terminal PAS-A/B domain of RAC3 through an evolutionarily conserved C-terminal domain. Overexpression of hMMS19 modulates ER-mediated transcriptional activation by enhancing the AF-1 function of ER. These data reveal a novel function of hMMS19 as an AF-1 specific transcriptional coactivator of estrogen receptor.
A. hMMS19 coactivation is independent on ERα Ser118 phosphorylation

The apparent association between hMMS19 and TFIIH and the ability of TFIIH to enhance ER activity by phosphorylating Ser118 suggest a simple model where hMMS19 stimulates ER activity through enhanced Ser118 phosphorylation by recruiting TFIIH to ER. This model predicts that the ability of hMMS19 to enhance ER activity is dependent on the presence of TFIIH kinase activity as well as Ser118 phosphorylation. To test this model, we first mutated Ser118 to either Ala or Glu and tested whether hMMS19 was still able to enhance AF-1 activity of either mutant. In transient transfection assay, it has been shown that compared to wild-type ER, ER S118A has a much reduced ligand-dependent transcriptional activity consistent with its inability to be phosphorylated by TFIIH at Ser118, while ER S118E is more active probably because it mimics the constitutive phosphorylation state. Surprisingly, we found that ER N-terminus harboring either mutation activates target gene slightly better than the wild type (Fig. 24), suggesting that both mutations somehow weakly enhanced the AF-1 activity. Furthermore, coexpression of hMMS19 was able to enhance the transcriptional activity of either mutated ER N-terminus (Fig. 24). These results argues for a Ser118
phosphorylation independent pathway to activate at least the isolated ER AF-1, which likely involves hMMS19.

As a further support that hMMS19 enhances ER AF-1 in a TFIIH kinase activity-independent manner, overexpression of CDK7 and MAT1, the two subunits of TFIIH responsible for phosphorylating ERα, failed to further enhance ER AF-1 activity when cotransfected with hMMS19 (Fig. 25). Previous results have shown that CDK7 and MAT1 overexpression is sufficient to enhance ligand-induced transcriptional activity of full-length ERα.

B. hMMS19 cooperates with RAC3 to enhance ER transcriptional activity

hMMS19-interacting domain in ERα was mapped to the hinge region (D domain) (Hui Li, unpublished data), indicating a lack of direct interaction between hMMS19 and ERα N-terminus used in the previous experiments, which is comprised of A/B-C regions of ERα. Therefore, the observed enhancement on ERα AF-1 activity by hMMS19 is very likely mediated by some other factors. Because hMMS19 interacts with RAC3 and RAC3 coactivates both AF-1 and AF-2, we investigated whether RAC3 could be mediating functional interaction between ERα AF-1 and hMMS19.

Firstly, we checked if RAC3 and hMMS19 together could activate ERα transcriptional activity better than either factor alone. 293 cells were transiently transfected with the ERE driven luciferase reporter gene and hERα or the separate ER-N or ER-C, containing AF-1 or AF-2 respectively. As expected, hMMS19 coexpression
enhanced the activity of AF-1 as well as the full-length hERα in the presence of ligand, but had no effect on AF-2 alone (Fig. 26), while RAC3 coexpression enhanced the transcriptional activity of all three activators. Interestingly, coexpression of hMMS19 and RAC3 together further enhanced the AF-1 activity as well as the ligand induced hERα activity (Fig. 26). Furthermore, the RAC3 truncation mutant, RAC3ΔN which lost the N-terminal bHLH-PAS domain and therefore the ability to interact with hMMS19, failed to stimulate ER AF-1 activity when transfected alone or together with hMMS19 (Fig. 27). These results suggest that the functional interaction between hMMS19 and RAC3 is necessary to coactivate ER AF-1.

C. Selective interaction between RAC3 and ER N-terminus

Direct interaction between ERα AF1 and SRC-1/GRIP1 has been shown by in vitro GST pull down assay and further, the ERα AF-1-interacting domain in GRIP1 was mapped to the extreme C-terminus. We sought to test the direct interaction between ERα AF-1 and RAC3 and also map the region in RAC3 responsible for the interaction. GST fused various domains of RAC3 were incubated with in vitro translated, 35S labeled ERα N-terminus consisting of A/B-C regions of ERα. GST-RAC3 (342-646) pulled down a significant amount of 35S-ER-N from the input, while other fusion proteins as well as GST alone failed to do so (Fig. 28A, left panel), suggesting a specific interaction between RAC3 (342-646) and ERα AF-1. To further confirm this interaction, we asked whether RAC3 (342-646) interacts with full-length ER. GST-RAC3 (342-646) was incubated with
in vitro translated, $^{35}$S-labeled full-length ERα and GST pull-down was performed in the absence of ligand or in the presence of either estradiol or tamoxifen. Interaction was detected in all three conditions, with strongest interaction detected in the presence of estradiol (Fig. 28A, right panel), which is likely caused by the additional interaction between ER LBD and the first LXXLL motif present within aa 612-646 of RAC3 under this condition. Our data thus support that the aa 342-646 of RAC3 contain another ER-interacting surface that binds specifically to ER-N.

We then tried to fine map the region of RAC3 (342-646). RAC3 (342-646) was further divided into smaller domains and GST fusion proteins of each domain were made and incubated with in vitro translated, $^{35}$S labeled ER-N again. Fig. 28B shows that the N terminal half of RAC3 (342-646) mediates the interaction with ER-N, however, this half region binds to ER-N much more weakly than the intact RAC3(342-646), suggesting that sequence in the C terminal half may be also involved.

**D. RAC3 (342-646) selectively interacts with nuclear receptors N terminus**

We then asked whether RAC3 (342-646) only interacts with ERα or it also interacts with N-terminus of other nuclear receptors. N-terminal A/B-C regions of ERβ, GRα and RARα were generated and labeled with $^{35}$S-methionine by coupled in vitro transcription and translation. Interestingly, we observed specific interactions of GST-RAC3 (342-646) with N-terminus from ERβ and RARα, but not from GRα (Fig. 29A), suggesting selective interactions between RAC3 and distinct receptors’ N-terminus.
While the two ends of RAC3 (342-646) overlap with the highly conserved PAS-B and RID domain, the middle part amino acid residues 400-612 is relatively less conserved in TIF2 and SRC-1, so we investigated whether the corresponding regions in SRC-1 and TIF2 also directly interact with ER-N. In the GST pull down assay, GST-TIF2 (343-664) was able to pull down the in vitro translated, $^{35}$S-labeled ER-N, while GST-SRC-1(334-656) couldn’t (Fig. 29B), suggesting a selective interaction with ER-N among SRC coactivators. Furthermore, GST-TIF2 (343-664) also interacts with N-terminus from ER$\beta$ and RAR$\alpha$, but not that from GR$\alpha$, indicating a similar selection pattern as RAC3 (342-646).

**Summary**

In this chapter, we found that hMMS19 enhances the transcriptional activity of both ER-N mutants in which Ser118, the phosphorylation target of TFIH, is changed to either Ala or Glu. Furthermore, overexpression of CDK7 and MAT1 failed to further enhance the transcriptional activity of ER AF-1 in the presence of hMMS19, suggesting that hMMS19 enhances ER AF-1 through a Ser118 phosphorylation-independent pathway. However, hMMS19 must coactivate ER AF-1 indirectly considering the lack of physical interaction between hMMS19 and ER-N. RAC3 may mediate the functional interaction between hMMS19 and ER-N because it directly binds to both peptides. Interestingly, we found a novel region in RAC3 that mediates the direct interaction with ER-N. This region is less conserved in SRC coactivators, its counterpart in TIF2, but not
SRC-1 also binds to ER-N, suggesting a selective interaction with ER AF-1 among SRC coactivators.
Chapter IV

Discussion
IV-1. FUNCTIONAL IMPLICATION OF SMRTe NUCLEAR SPECKLES

SMRTe as an organizer of a novel nuclear domain

Cell nucleus has a well-organized, yet dynamic substructure that represents a novel regulatory level of many molecular events[165]. Remarkably, regulation of both transcriptional activation and repression by nuclear compartmentalization has been reported. Using indirect immunofluorescence and transiently transfected cells, we found that SMRTe distributes in discrete nuclear and cytoplasmic speckles. We believe that these speckles are not an artifact of protein overexpression because: 1. Endogenous SMRTe exhibits a similar nuclear speckle pattern although lacks cytoplasmic staining, probably because of the weak signal; 2. In parallel experiments, cells transfected with N-CoR in the same expression vector as SMRTe exhibits exclusive nuclear speckles, indicating that the cytoplasmic staining of SMRTe is unlikely caused by overexpression.

A recent report by Downes et al has demonstrated the similar dot pattern of endogenous SMRTe, and they suggest that the presence of SMRTe in these dots/speckles is dependent on the deacetylase activity of class II HDACs[166]. However, based on the ability of SMRTe to recruit unliganded RAR as well as selective HDACs from the diffuse nuclear distribution to discrete nuclear speckles, we conclude that SMRTe is the organizer of those nuclear speckles. Our data also directly argues against that any HDACs could be the organizer of the nuclear speckles. For example, SMRT RD2 domain is the region that interacts with HDAC3, but it did not show nuclear dot structure, and the
SMRT RD3 fragment that contains the HDAC4/5 interacting domain locates constitutively in the cytoplasm, while we would expect RD3 to be in the nuclear speckles if HDAC4/5 was the organizer. The discrepancy between our and Downes et al’s results probably arises from the fact that Downes et al were looking at the distribution of endogenous SMRTe staining in the presence of overexpressed HDAC5, which as one would expect, may overwhelm the endogenous SMRTe and disrupt its natural localization. Nonetheless, consistent with our observation that the deacetylase activity of HDAC1 is required for its localization in the SMRT nuclear speckles, Downes et al found that the deacetylase activity of class II HDACs also appears necessary for their distribution to the nuclear speckles.

**Dynamic property of the SMRTe nuclear speckles**

First of all, SMRTe nuclear speckles have dynamic composition. Our data revealed the in vivo association between SMRTe and unliganded receptor as well as HDAC1, 3, 4 and 5. However, biochemical analysis so far has failed to isolate a single SMRTe complex containing so many components. Immuno-affinity chromatography purified SMRTe/N-CoR complex from nuclear extract identified only HDAC3 tightly associated with SMRTe/N-CoR. Two explanations could account for the discrepancy between biochemical analysis and immunofluorescence result as pointed out by Downes et al. First, some components of SMRTe complex may not be soluble, therefore will not be present in the nuclear extract. Downes et al have shown that some components may be
associated with nuclear matrix and therefore will be lost during preparation of nuclear extract. Second, the association of some components may be dynamic and not stable enough to sustain the chromatography. Indeed, dynamic association of SMRT/N-CoR with different subsets of corepressor complex to mediate transcriptional repression by specific repressors has been observed. Laherty et al reported that SAP30, a component of the mSin3 corepressor complex was required for N-CoR-mediated repression by antagonist-bound ER and the homeodomain protein Rpx, but not for N-CoR-mediated repression by unliganded RAR or TR[167]. It should be noticed that our immunofluorescence approach tends to detect steady-state interaction because cells are fixed and most proteins are overexpressed in our experiments. It’s likely that the SMRTe nuclear speckles are more dynamic in terms of that their composition may change depending on the cell type and cell growth and differentiation. For example, class II HDACs are differentially expressed in selective tissues, so we may expect different types of HDACs in SMRTe nuclear speckles in different cell types. Furthermore, as we have shown that recruitment of HDAC4 and 5 into SMRTe nuclear speckles enhanced their inhibition of MEF2C activity, recruitment of HDAC4 and 5 must be tightly regulated in order to timely activate MEF2C. Therefore, the dynamic composition of SMRTe nuclear speckles may be an important aspect of their function to bring distinct corepressor complex to specific transcriptional repressors.

In addition, SMRTe itself displays a dynamic subcellular distribution. Although most transfected cells display exclusive nuclear speckles, many cells have both cytoplasmic and nuclear speckles. 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 addition to nuclear localization, RD3 also appears to contribute to speckled nuclear localization. In contrast, RD4 is responsible for speckled cytoplasmic localization, and cSMRT appears to be lacking both nuclear and speckle localization signals. Recently, phosphorylation by mitogen-activated protein kinases (MAPK) has been shown to cause cytoplasmic localization of SMRT[168], suggesting a complex signaling pathway that regulate SMRTe localization.

Despite the obvious similarity of SMRT and N-CoR in both domain structure and function, we found that N-CoR was detected exclusively in the nucleus. Whether this difference in subcellular distribution reflects any significant functional difference between SMRTe and N-CoR is not clear, however, recent data suggest that SMRT and N-CoR may function differentially in vivo. Targeted ablation of N-CoR gene in mouse with wild type SMRT results in embryonic lethality, indicating that SMRT and N-CoR are not functionally redundant proteins[33].

**Biological functions of the SMRTe speckles**

Possible functions of SMRTe speckles include:

*Transcriptional repression* 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 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, SMRTε can recruit the interacting HDACs into discrete nuclear speckles. The colocalization of HDAC3, 4 and 5 with SMRTε is consistent with previous biochemical findings. In particular, HDAC3 has been shown to form a stable complex with SMRT and N-CoR[54, 55, 161], and HDAC3 can be coprecipitated with HDAC4 and 5[162]. Supports for an active role of SMRTε in regulating histone deacetylation come from observations that localization of HDAC1 to SMRTε 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 deacetylase inhibitor trichostatin A and sodium butyrate, can disrupt colocalization of HDAC5 and 7 with the isoform of SMRTε, SMRTα[166]. Together, these data suggest an important role for SMRTε in organizing a novel nuclear domain that may be implicated in histone deacetylation and transcriptional repression.

**B. nuclear-cytoplasmic shuttling**

The colocalization of unliganded RARα with SMRTε in the cytoplasmic and nuclear speckles confirms the reported interaction of these two proteins in vivo. The dissociation of RARα from the SMRTε cytoplasmic speckles upon atRA treatment confirms the proposed theory of ligand-dependent dissociation of RARα from the corepressor. It appears that the presumed "SMRTε-free" RARα can be readily transported into the nucleus. However, it is not clear whether the presence of cytoplasmic RARα is due to nuclear export or regulated import by SMRTε. It is intriguing to note that TRβ also undergoes a T3-enhanced nuclear translocation[169]. Based on these findings it is reasonable to speculate that SMRTε might be responsible for regulating nuclear-cytoplasmic shuttling of unliganded TRβ and RARα. In addition, we
found that cSMRT is primarily located in the cytoplasm, where it can block nuclear localization of Gal4-RARα, suggesting that cellular compartmentalization may in part contribute to the dominant negative activity of cSMRT.

The functional interaction between HDAC4 and SMRTe also suggest a new role for SMRTe in regulating cytoplasmic-nuclear trafficking of HDAC4, 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[157]. 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[159, 160]. Indeed, coexpression of SMRTe and HDAC4 abolishes MEF2C transcriptional activity. Remarkably, 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 (CaMK)[170]. Thereby, phosphorylation of HDAC4 and 5 and/or SMRTe by CaMK 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. After myoblast fusion, HDAC4 is relocated from cytoplasm to nucleus[171]. While 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 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.
Indeed, recent findings confirm that although HDAC4 contains intrinsic nuclear export as well as import signal, it appears that interaction with other proteins such as MEF2 is necessary for its nuclear import[172]. Consistent with the possible role of SMRT in muscle differentiation, N-CoR interacts with MyoD and inhibits muscle cell differentiation[28]. Therefore, it is reasonable to speculate that steroid/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 the SMRTe-interacting proteins, and it now poses the challenge to further define the exact mechanism of gene regulation by the SMRTe nuclear domains.
MMS19 was initially identified in a yeast genetic screen for mutations that render cells sensitive to the alkylating agent methyl methanesulfonate (MMS) and later to UV and DNA cross-linking agents[164]. Analysis of MMS19 mutation in yeast suggests that it plays a role in nucleotide excision repair and transcriptional regulation by affecting TFIIH activity. Recently, two groups isolated human MMS19 based on its homology to the yeast protein[173, 174]. Seroz et al reported a sequence that is almost identical to our sequence except a difference over a stretch of 39 amino acid residues in the central part of the protein. They found that this isoform of hMMS19 failed to complement the yeast mms19 mutant although it interacts directly with two subunits of human TFIIH complex, XPB and XPD, suggesting a possible conserved function in regulating TFIIH. While Queimado et al reported a sequence completely identical to ours and interestingly, they found this isoform of hMMS19 was able to rescue the yeast mms19 deletion mutant phenotype, so they concluded that this isoform of hMMS19 is the human ortholog of yeast MMS19. However, the functions of MMS19 remain elusive. In this study, we isolated the human MMS19 as a RAC3-interacting protein. We also found that hMMS19 selectively interacts with ER and functions as an AF-1 specific transcriptional coactivator of ER, which may help to reveal the in vivo function of hMMS19. On the other hand, functions of bHLH-PAS domain of SRC coactivators and molecular mechanism of ER

AF-1 are poorly understood, our findings provide an unexpected molecular link between these two events.

Interaction of hMMS19 with RAC3 and functional significance of RAC3

bHLH-PAS domain

The bHLH-PAS domain is the most conserved region in the SRC coactivators; however, the function of this domain is largely unknown. The isolation of hMMS19 as a bHLH-PAS domain-interacting protein supports our hypothesis that this domain may be involved in protein-protein interaction during the assembly of coactivator complex. Consistent with this notion, we found that deletion of bHLH-PAS domain in RAC3 impaired its ability to enhance transcriptional activity of ER AF-1. Although previous transient transfection experiments in mammalian cells suggest that bHLH-PAS domain of SRC coactivators is dispensable for their activity[67, 72], recent data demonstrate that deletion of bHLH-PAS domain in SRC-1 reduced by 50% its enhancement of PR activity on chromatin templates in an in vitro system[134], confirming the importance of bHLH-PAS domain in coactivator function. We thus propose that at least one important function of bHLH-PAS domain of RAC3 is to recruit hMm19 and maybe, a subsequent coactivator complex. Further analysis of the hMMS19-RAC3 interactions reveals that interaction is mediated through the PAS-A/B region. Interestingly, strong interaction occur only in the presence of both PAS-A and PAS-B, suggesting that there may be two interacting surfaces that together stabilize the interaction between hMMS19 and RAC3.
Since the PAS domain is highly conserved among SRC coactivators, hMMS19 might also interact with SRC-1 and/or TIF2, but this remains to be tested. However, despite the conservation of PAS domain in different proteins, hMMS19 does not interact with the PAS domain of Sim, suggesting that different PAS domains may be involved with different interacting protein. The bHLH-PAS domains from SRC coactivators may also function differentially, as recently reported that PASB of N-CoA1 (SRC-1), but not TIF2 or RAC3 interacts with STAT6[175].

The RAC3-interacting surface in hMMS19 maps to its C-terminal conserved domain. Structural prediction suggests that this region contains multiple potential amphipathic helices, which might mediate the interaction with the PAS domain of RAC3. Remarkably, during their cloning of hMMS19, Queimado et al identified highly conserved HEAT repeat domains with 4 repeats within the C terminal 170 amino acid residues of hMMS19, which overlaps with the RAC3 interacting domain. HEAT-repeat domains have been found in a variety of cytoplasmic and nuclear proteins including huntingtin, β-importin and several proteins involved in transcriptional regulation. Crystal structure of β-importin reveals that a typical HEAT repeat unit consists of 30-34 amino acid residues and forms two α-helices separated by a short loop[176]. Arrays of HEAT repeats form tandemly arranged bi-helical structures that can serve as flexible scaffolds where other proteins assemble to form a complex[176-178]. It’s likely that these HEAT repeats directly mediate the interaction with bHLH-PAS domain of RAC3.

Interaction of hMMS19 with ER
Analysis of the interactions of hMMS19 with steroid/nuclear receptors reveals strong binding with ERα and ERβ in a ligand-independent manner, while under the same condition, almost no interaction was detected between hMMS19 and other receptors. The ER binding domain in hMMS19 was mapped to the C-terminus, the same region where RAC3 binds. Although we don’t know whether RAC3 and ER interact with same amino acid residues or different regions in hMMS19 C-terminus, from above discussions, it’s possible that RAC3 and ER would utilize distinct HEAT repeat units of hMMS19. Therefore, hMMS19 HEAT repeats may serve as a scaffold structure for assembling the ER-coactivator complex as these repeats do in other complexes.

The hMMS19 interacting region in ERα was mapped to the hinge region (D) (Hui Li, unpublished data). D region is less well characterized and poorly conserved among nuclear hormone receptors, however, this region has been found involved in association with the molecular chaperone heat shock protein 90 (hsp90) and therefore is capable of mediating protein-protein interaction. Recent data also demonstrate that D region mediates interactions of ER with coactivators L7/SPA[29] and PGC-1[179]. More interestingly, specific lysine residues in this region can be acetylated by p300, which leads to reduced hormone sensitivity of ERα[180]. These data indicate that D region may be an important co-regulator-binding surface for ER. Sequence alignment illustrates a significant similarity in D region between ERα and ERβ but not with other steroid/nuclear receptors, suggesting a molecular basis for the selective interaction between hMMS19 and ERs, although the interaction of hMMS19 with ERβ D region has not been examined. In addition, hMMS19 C-terminus contains several LXXLL motifs
within HEAT repeat I and II, whether these motifs contribute to the interaction with ER in the presence of ligand remains to be determined.

Because hMMS19 interacts with both ER and RAC3 and ER also interacts with RAC3, several possibilities may explain the relevance of these interactions. For instance, hMMS19 may interact with ER first and in the absence of ligand. This interaction may modulate, for example, the phosphorylation states of ER by targeting TFIIH activity to ER or this interaction may serve to recruit RAC3 or stabilize the ligand-independent interaction between ER AF-1 and RAC3. Upon ligand binding, ER undergoes a conformational change that allows the receptor to bind the LXXLL motifs of RAC3 via a coactivator-binding pocket in the LBD. RAC3 then interacts with hMMS19 via the N-terminal PAS domain to stabilize the complex or to signal a subsequent event in transcription. Alternatively, since it is clear that RAC3 uses separate domains for interactions with hMMS19 and liganded ER, RAC3 may form a complex with hMMS19 first, which is then recruited to the liganded receptor and the additional interaction with ER provided by hMMS19 would serve to stabilize the coactivator complex. Future experiments using chromatin immunoprecipitation may be necessary to distinguish between these possibilities by examining whether hMMS19 and RAC3 are sequentially or simultaneously recruited to ER target gene promoter. Nonetheless, whichever scenario it is, Our data support that hMMS19 functions as a key factor in assembling the ER-coactivator complex through the multiple interacting surfaces on its C-terminus, as we have shown that the C-terminus of hMMS19 blocked the ligand-dependent ER transcriptional activity probably through disrupting the functional interaction between ER and RAC3 (Fig.20C).
Interaction of RAC3 with ER AF-1

Our data confirm the previous reports that SRC coactivators interact with both ER-N and C-terminal LBD. In the case of GRIP1, Webb et al has mapped the ER interacting domain to the C terminal Glutamine-rich region and because the homology in this region among all three SRC coactivators, they proposed that this region is a conserved ER AF-1 interacting domain[78]. However, we detected only a very weak interaction between the corresponding region in RAC3 and ER-N. Instead, aa 342-646 of RAC3 interacts strongly with ER-N. The corresponding region of TIF2, aa 343-664 also interacts significantly with ER-N, which was not detected by Webb et al. Surprisingly but consistent with sequence alignment of the corresponding regions among SRC coactivators, which shows a stronger similarity between RAC3 and TIF2, the same region from SRC-1, aa 334-656 does not interact with ER-N in our experiment. Furthermore, the same region of RAC3 or TIF2 interacts with the N-terminus of ERβ, RARα, but not GRα, while the corresponding region of SRC-1 does not interact with the N-terminus of any of these receptors. Interestingly, the same region of SRC-1 has been found to be able to interact with progesterone receptor (PR) N-terminus[77]. Therefore, it’s possible that RAC3 (342-646) and its corresponding regions in TIF2 and SRC-1 selectively interact with a subset of receptor N-terminus, which may represent yet another level of functional differentiation among SRC coactivators.

Amino acid 342-646 of RAC3 contains a part of the PAS-B domain and RID of RAC3 at the N- and C-terminal ends respectively. Although our data indicate that either
PAS-B or RID alone does not interact with ER-N, removal of either N or C-terminal end of RAC3 342-646 almost abolished its interaction with ER-N. Secondary structure prediction reveals that the middle region of RAC3 342-646 consists primarily of random coils, it’s thus possible that the two ends are required for the correct folding of the middle region, which function either alone or together with the two helical ends to form the interacting surface for ER-N.

Other AF-1 specific coactivators

Two types of coactivators that specifically enhance the AF-1 function but not AF-2 function of ERα have been identified. The first one is the RNA coactivator for steroid receptors, SRA[65]; the second type includes p72/p68[64, 181], two members of the RNA-binding DEAD-box protein family. Interestingly, functional interaction between SRA and p72/p68 has been detected, suggesting they are components of a same coactivator complex that is required for ERα AF-1 activity.

SRA[65]

SRA was fortuitously cloned in a yeast two-hybrid screening of a human B-lymphocyte cDNA library for PR AF-1 interacting protein. It has multiple splicing isoforms, with one of them expressed at significantly higher levels in the breast cancer cell lines MCF-7 and T-47D. In the transient transfection assay, all the isoforms were able to specifically enhance the transcriptional activity of steroid receptors, such as PR, GR, AR and ER, but had no effect on TR, RAR, RXR or PPAR, suggesting that SRA is a steroid receptor specific coactivator. Moreover, truncation of the A/B domain of PR significantly reduced
coactivation by SRA, while transcription activation by PR lacking the LBD was fully responsive to SRA, indicating that SRA mediates transcriptional activation of steroid receptors through AF-1. Most surprisingly, SRA is a RNA coactivator as supported by a few lines of evidence. First, attempts to translate the SRA clones in vitro or in vivo all failed. Second, an affinity column containing a mouse monoclonal antibody raised against putative SRA open reading frame failed to detect endogenous SRA in various cell lines. Third, extensive mutagenesis of SRA, introducing multiple translational stop codons in all reading frames, did not affect the activity of SRA. Finally, SRA retained its ability to coactivate a reporter gene in transfection experiments in the presence of cycloheximide. SRA is unlikely to directly interact with steroid receptors AF-1 because of the lack of sequence homology in this region among these receptors. Rather, SRA may be indirectly associated with receptors as a component of a ribonucleoprotein complex. Indeed, biochemical analyses demonstrated that SRA and SRC-1 were eluted in the same fraction from whole cell extracts of human T-47D cells. Taken together, SRA may function as an adaptor molecule in a coactivator complex specific for steroid steroid/nuclear receptors.

p72/p68[64, 181]

p72 and p68 are two closely related members of the DEAD-box-containing RNA binding protein family and are presumably involved in multiple facets of cell functions. A possible role of p68 in ER signaling was first revealed by the affinity purification of p68 from MCF-7 cell extracts by GST fused ERα A/B-C region. Both in vivo and in vitro studies demonstrated direct interaction between p68 and ERα A/B region, which can be potentiated by the Ser118 phosphorylation of ER. Surprisingly, p68 does not interact with
ERβ or any other steroid/nuclear receptors. Consistently, p68 overexpression enhanced transcriptional activity of ERα through coactivating AF-1, while it has no effect on functions of ERβ or any other steroid/nuclear receptors. These results established p68 as an ERα AF-1 specific coactivator. On the other hand, p72 was also related to ER signaling since it was isolated in a yeast two-hybrid screening with the activation domain at the carboxyl terminus of AIB1 being the bait. As expected, p68 also interacts with the same domain of SRC coactivators, whereas p72 also specifically interacts with ERα A/B region. Moreover, p68/p72 also interacts directly with SRA through one of their conserved DEAD-box, box viii. Furthermore, p68 elutes together with SRA-SRC-1 complex in the in vitro fractionation. Interestingly, deletion of box viii in both proteins abrogated their interaction with SRA as well as their ability to enhance the transcriptional activation of a ER target gene in vivo, suggesting that interaction with SRA is required for p68/p72 to function as ERα coactivator.

Role of hMMS19 in ER signaling

The physiological relevance of hMMS19 in ER signaling is demonstrated by overexpression of the C-terminal RS2.1 fragment and the full-length hMMS19, each of which significantly affect transcriptional activity of ER in transfection assays. Overexpression of RS2.1 inhibits ligand-dependent transcriptional activation of an ER reporter gene probably due to a dominant negative effect. Meanwhile, overexpression of RS2.1 has no effect on basal promoter activity in the absence of ligand, consistent with the observation that ER binds to chromatin in response to ligand. As expected, the full-
length hMMS19 enhances ligand-dependent transcription of the ER reporter gene, whereas the truncation mutant lacking the RAC3/ER-interacting domain has no effect on ER activity. Intriguingly, hMMS19 strongly enhances the AF-1 activity of ER in both the absence and presence of ligand and has no effect on the activity of a separated AF-2 domain. Therefore, we suggest that hMMS19 functions as an AF-1 specific coactivator of ER. Currently, only a few potential AF-1 specific coactivators were reported, such as p68/p72 RNA helicase[64, 181], and the mechanism by which AF-1 activate transcription and synergizes with AF-2 are largely unknown. The human MMS19 interacts with both ERs and RAC3, suggesting that hMMS19 may bridge the functions of AF-1 and AF-2.

Because hMMS19 regulates AF-1 activity of ER without directly interacting with ER-N, it’s intriguing to ask what factor(s) mediate the function of hMMS19. This led us to discover that the interaction between RAC3 and hMMS19 is necessary for enhancing AF-1 activity. Furthermore, since hMMS19 interacts with the PAS domain of RAC3, we have demonstrated that the loss of PAS domain abolished the cooperation between hMMS19 and RAC3 to activate ER AF-1. However, how hMMS19 functions to activate transcriptional activity of AF-1 as well as the full-length ER is not clear, two possibilities exist:

A. **hMMS19 may function to facilitate hTFIIH mediated ER phosphorylation.**

Biochemical and molecular biological studies in yeast suggest that yeast MMS19 function as a positive regulator of TFIIH[164]. Deletion of MMS19 in yeast results in general defecation in transcription. Interestingly, hMMS19 is able to complement the yeast mms19 deletion mutant when introduced into yeast cells, suggesting that the function of MMS19 in regulating TFIIH is conserved during evolution[173]. Since hMMS19 directly
interacts with two subunits of human TFIIH complex, XPD and XPB[174], it’s likely that hMMS19 may be able to recruit TFIIH to estrogen receptors, which leads to ER phosphorylation and subsequent activation of ER transcriptional activity. Our data from the phosphorylation site mutated ER-N suggest that this may not be the mechanism for hMMS19 to activate at least the isolated ER AF-1, although we are not sure whether this can account at least partially for how hMMS19 activate the full-length ER. Also, our data are obtained from transient transfection assay in 293 cells using a specific promoter-driven reporter gene. Given the cell type and promoter context specificity of ER activity, we cannot rule out that the stimulated coactivation by phosphorylation may occur for some genes in certain cell types.

**B. hMMS19 may function to recruit/stabilize coactivator complex and/or general transcriptional machinery.** From previous discussions, hMMS19 may function as a scaffold protein to facilitate complex assembly. Furthermore, since hMMS19 selectively interacts with ER, it may function as an adapter to recruit specific coactivator complex to ER. Such a role is consistent with its structure property with multiple HEAT repeats at the C-terminus which has been identified as the RAC3- and ER- interacting domain. It is also consistent with the fact that hMMS19 cannot activate gene transcription by itself when recruited to a gene promoter by a heterologous DNA binding domain fusion (our unpublished data). Interestingly, the idea of “adapters” or “integrators” has been proposed to explain the function of certain members of coactivator complex, such as SRA[65] and CBP[105], considering their ability to interact with other coactivators in
addition to receptors. Therefore, the ability of certain coactivators to act as scaffold proteins may be a common theme in the assembly of receptor-coactivator complex.

In addition to assembling coactivator complex, hMMS19 may also be able to recruit/stabilize general transcriptional machinery to ER in light of its association with TFIIH. As a general transcriptional factor, TFIIH plays important roles in transcription initiation[182, 183]. Through its inherent protein kinase and DNA helicase activity, it is involved in phosphorylation of the carboxyl terminal domain (CTD) of the RNA polymerase II, promoter melting and promoter clearance, all of which are crucial for efficient transcription initiation[184]. Interestingly, hMMS19 binds to the two TFIIH subunits, XPB and XPD[174], which possess the DNA helicase activity and are responsible for, at least, the promoter melting. Thus it’s likely that hMMS19 would recruit and stabilize TFIIH at the promoter region and facilitate the transcription initiation. In addition, recent data demonstrate that TFIIH is also an important member of the transcription reinitiation intermediate that is essential for sustained high level transcription from a given promoter[185]. Intriguingly, in vitro studies of ER activated transcription has revealed dual functions of ER in both transcription initiation and reinitiation[186]. We suspect that by bridging TFIIH and ER, hMMS19 can be an important factor involved in ER mediated transcription reinitiation.

Based on the above discussion, we propose a model of how hMMS19 and RAC3 cooperate to activate ER transcriptional activity (Fig. 30). In this model, in addition to the interaction between liganded hERα LBD and RAC3 LXXLL motifs in the receptor interacting domain, the region immediately before RID of RAC3 also contacts the hERα AF-1 region. Both interactions may be required to stabilize the receptor-coactivator
complex. Furthermore, the two protruding ends of RAC3 function to recruit other factors required for the maximum transcriptional activity, e.g. the N-terminus bHLH-PAS domain recruits MMS19 which also binds directly to the hinge region of ER and therefore adds another stabilization force to the overall complex; while the C-terminus is necessary to recruit CBP/PCAF complex which also directly binds the receptor. Both complexes at the N or C-terminus may synergistically activate ER transcriptional activity in multiple ways: 1. through phosphorylating ER by kinase activity from TFIH-hMMS19 complex; 2. through histone acetylation by the HAT activity from RAC3 or CBP/p300 or pCAF; 3. through recruiting the basal transcriptional machinery due to the recruitment of TFIH by hMMS19 and recruitment of TFIIB by CBP. This model predicts a central role of RAC3 and hMMS19 in assembling the coactivator complex, although we don’t know if they interact with ER simultaneously or sequentially, future experiments are necessary to clarify this.
Chapter V

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CHAPTER VI

FIGURES
Fig. 1 Schematic representation of a nuclear hormone receptor domain structure. A typical nuclear receptor consists of A, B, C, D, E and F six regions. The variable N-terminal region A/B contains the ligand-independent transcriptional activation domain AF-1. C is the conserved DNA binding domain, followed by the hinge region D. E/F contains the ligand binding domain as well as the ligand-dependent transcriptional activation domain AF-2. (Adapted from Aranda A. and Pascual A[3].)
**Fig. 2 Current model of transcriptional regulation by nuclear hormone receptors: the role of coactivators and corepressors.** In the absence of ligand, corepressors SMRT/N-CoR will recruit a corepressors complex containing HDAC activity to the receptors and actively repress transcription through deacetylating histones or some other protein. Ligand binding induces the release of corepressors and recruitment of coactivator complex containing HAT activity, resulting in transcriptional activation. Both corepressor and coactivator complex may also affect transcription through functional interaction with basal transcriptional machinery (Adapted from Glass CK and Rosenfeld MG, *Genes Dev.*(2000) Vol 14(2), 121-141).
Fig. 3 Schematic representation of the N-CoR/SMRTe domain structure as well as SMRT, cSMRT, and hemagglutinin (HA)-tagged deletion constructs used in this study. *RD1-4*, transcriptional repression domains; *ID1-2*, nuclear receptor interacting domains; *LX*, LXXLL-like motif; *SNC*, SMRTe/N-CoR conserved domain; *SANT*, SWI3/ADA2/N-CoR/TFIIBB 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 GenBankTM accession number AF125672.
Fig. 4 Schematic diagram of the domain structures of full-length human RAC3, TIF2, and SRC-1. The starting and ending residues of indicated domains are shown. The RAR-binding and p300-binding domains defined in mSRC-1 are also indicated. The numbers at the right are the length of individual proteins and the percentage similarity. The pairwise similarities were calculated to be 65% between RAC3 and TIF2, 64% between TIF2 and SRC1, and 59% between RAC3 and SRC1 (Adapted from Li et al. Proc Natl Acad Sci USA (1997) Vol 94, 8479-8484).
Fig. 5 Subcellular localization of full-length SMRTe and N-CoR. HeLa cells were transiently transfected with SMRTe or HA-tagged N-CoR Immunohistochemistry was conducted with mouse anti-SMRTe monoclonal antibodies (a-c), or mouse anti-HA monoclonal antibodies (d). The mouse primary antibodies were detected by goat anti-mouse rhodamine-coupled secondary antibody (red). The endogenous SMRTe speckle staining is also shown (c).
Fig. 6 Subcellular localization of full-length SMRTe and SMRTe fragments. HeLa cells were transiently transfected with SMRTe, SMRT, cSMRT, or HA-tagged deletion mutants shown in lower right (RD3=SRD1, RD4=SRD2).
Fig. 7 Co-staining of SMRTe and SC35 or Daxx. HeLa cells were transiently transfected with SMRTe and stained with rabbit anti-SMRTe polyclonal antibodies and mouse anti-SC35 antibodies (a-c), or mouse anti-SMRTe monoclonal antibodies (d-f) and rabbit anti-Daxx antibodies. The rabbit primary antibodies were detected by fluorescein-coupled goat anti-rabbit secondary antibodies (green). The mouse primary antibodies were detected by goat anti-mouse rhodamine-coupled secondary antibody (red). No colocalization was observed in the double labeling of SMRTe and the SC35-labeled splicing speckle (c), or SMRTe and the Daxx-labeled promyelocyte nuclear domain (f).
Fig. 8 Ligand-independent colocalization of RAR with SMRTe. SMRTe sequesters RAR in the cytoplasmic speckles in the absence (-) (a-c), but not the presence (+) (d-f), of atRA. SMRTe and HA-tagged hRAR were double-labeled with rabbit anti-SMRTe and mouse anti-HA antibodies, followed by fluorescein (green) or rhodamine (red)-coupled secondary antibodies, respectively. HeLa cells were transiently transfected with pCMX-hSMRTe and pCMX-hRAR followed by $10^6$ M atRA treatment for 24 h before immunostaining.
Fig. 9 cSMRT sequesters Gal4-RAR in the cytoplasm in an RA-sensitive manner. pCMX-cSMRT and pCMX-Gal4-RAR were cotransfected in HeLa cells. Gal4-RAR was detected by mouse anti-Gal4 DNA binding domain monoclonal antibodies (red) and the cSMRT with rabbit anti-SMRTe polyclonal antibodies (green). (a-c) show that in the absence of atRA, Gal-RAR is sequestered in the cytoplasm; (d-f) show that atRA treatment relocate Gal-RAR to the nucleus; (g-i) show that as a control, cSMRT cannot sequester Gal in the cytoplasm.
Fig. 10 SMRTe colocalizes with PLZF and ETO. HeLa cells were transiently transfected with SMRTe and PLZF or ETO-Flag and stained with rabbit anti-SMRTe polyclonal antibodies and mouse anti-PLZF antibodies (a-c), or mouse anti-Flag monoclonal antibodies (d-f). The rabbit primary antibodies were detected by fluorescein-coupled goat anti-rabbit secondary antibodies (green). The mouse primary antibodies were detected by goat anti-mouse rhodamine-coupled secondary antibody (red).
Fig. 11 SMRTe recruits HDAC1 and HDAC3 into nuclear speckles but not HDAC2 or mSin3A. 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 m 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 (m). Cotransfection with SMRTe recruits HDAC1 (b-d) and HDAC3 (j-l), but not HDAC2 (f-h) or mSin3A (n-p) into nuclear speckles.
Fig. 12 Histone deacetylase activity is required for localization of HDAC1 to SMRTe nuclear speckles. SMRTe and each HDAC1 mutant were cotransfected and analyzed as in Fig.11. 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.
Fig. 13A Association of SMRTe with class II HDACs. Class II HDACs, HDAC4, 5, or 6 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).
Fig. 13B 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.
Fig. 14 SMRTe enhances HDAC-mediated transcriptional inhibition on MEF2C. A, HEK293T cells were transfected with MEF2C (0.2 µg/well) and a MEF2C-dependent luciferase reporter (0.4 µg/well) together with each indicated HDAC or empty vector (0.2 µg/well). Note that only HDAC4 and 5 show significant inhibition on MEF2C transcriptional activation. Coexpression of SMRTe and HDAC4 and 5 synergistically inhibits MEF2C-mediated transcriptional activation. Transfection was conducted as in A with the addition of SMRTe where indicated at a concentration of 0.2 µg/well. SMRTe alone has little effect on MEF2C activity, whereas cotransfection of SMRTe and HDAC4 or HDAC5 synergistically inhibits MEF2C activation by 20-fold.
Fig. 15 Interactions of RS2.1 with RAC3 in vivo.

**A**, yeast two-hybrid interaction of RS2.1 with RAC3-N. RS2.1 is expressed in the pACT2 vector as a Gal4 activation domain fusion, whereas the baits are expressed in pGBT vector as Gal4 DNA binding domain fusion. Vector alone without insert is marked as "-". Sim-N contains the bHLH-PAS domain of the Sim.

**B**, yeast two-hybrid interaction of RS2.1 with various regions of the RAC3 N-terminal domains, conducted as described in **A**.
Fig. 16 Multiple sequence alignment of human MMS19 with related sequences of different species. Multiple sequences were aligned by ClustalW and plotted using ESPript program. Consensus amino acids are boxed and displayed in white against red background. Highly conserved residues are also boxed and displayed in red. hMMS19 and hMMS19mt are indicated as well as the starting residue encoded by the yeast two-hybrid clone RS2.1. Amino acid sequences of related proteins in *C.elegans, A.thaliana* and *S.cerevisiae* are derived from the Genbank accession numbers: T33176, BAB11076 and NP_012138, respectively. Amino acid sequence of the related protein in *D. melanogaster* is predicted by GENSCAN from *Drosophila* genomic sequence AE003603.
Fig. 17 Ubiquitous expression of hMMS19 in human tissues and cancer cells. Total mRNA samples from various human tissues and cancer cell lines were probed by a $^{32}$P-labeled random-primed DNA corresponding to the NsiI/BglII restricted 1.5kb fragment from the cloned hMMS19. A unique band of hMMS19 transcript in the size of about 4 kilobase was detected in all the samples examined; β-Actin is shown as a control.
Fig. 18 Nuclear localization of hMMS19. Indirect immunofluorescence analysis of HeLa cells transfected with HA-hMMS19 and detected by an anti-HA monoclonal antibody followed by a rhodamine-coupled secondary antibody. The right panel shows nuclear staining with 4’, 6-diamidino-2-phenyl-indole (DAPI) of the same cells.
Fig. 19 Interactions of $^{35}$S-hMMS19 with GST-RAC3-N in GST pull-down assay.

**A.** The hMMS19 C-terminus (RS2.1), hMMS19-mt and full-length hMMS19 were translated in reticulocyte lysate and labeled with $^{35}$S-methionine and incubated with glutathione agarose beads coupled with GST or GST-RAC3-N, bound proteins were eluted and analyzed by SDS-PAGE and autoradiography. While hMMS19C and hMMS19 can be pulled down, very few amount of hMMS19-mt was pulled down.

**B.** Interaction of full-length hMMS19 with RAC3 in GST pull-down assay, conducted as described in **A.** except that different GST-RAC3 fusion proteins are used in this experiment. Only GST-RAC3-PASAB and the bait can pull down significant amount hMMS19.
Fig. 20 The RS2.1 fragment modulates transcriptional activity of ER. 

A. RS2.1 inhibited ligand-induced ER transcriptional activity, while it had no effect on transcriptional activity of hRAR or hTR on RARE-tk-Luc or TRE-tk-Luc reporter, respectively. B. dose-dependent inhibition of ligand-induced transcriptional activity of ERs by RS2.1. Increasing concentrations of RS2.1 (-, 0 ng; +, 100 ng; ++, 200 ng; +++ , 400 ng) were transfected into HEK293 cells together with 20 ng of ER and 0.5 μg of the ERE-E1A-Luc reporter. After transfection, cells were treated with solvent or 25 pM of E2, and relative luciferase activity was determined.
Fig. 21 The human MMS19 interacts with estrogen receptors. A, interaction of full-length hER with RS2.1 in GST pull-down assay in the absence or presence of 1 µM 17-estradiol (E2) or 1 µM 4-dihydroxytamoxifen (OHT). GST-RS2.1 pulls down a significant amount of 35S-ER in a ligand-independent manner. B, interactions of ER, hRAR, or hTR with GST-RS2.1 in GST pull-down assay conducted as described earlier. C, Coomassie Blue staining of GST and GST-RS2.1 protein used in the GST pull-down experiments.
Fig. 22 The full-length hMMS19, but not hMMS19-mt, consistently enhances ER-mediated transcriptional activation. 200 ng of hMMS19 or hMMS19-mt were transfected into HEK293 cells together with 20 ng of ER and 0.5 μg of the ERE-E1A-Luc reporter. Relative luciferase activity was determined as described before.
Fig. 23 The human MMS19 is an AF-1-specific transcriptional coactivator of ER.
The hMMS19 enhances transcriptional activation by the ER AF-1 domain in the absence
or presence of E2. The ER AF-1 contains the N-terminal A/B region and the DNA
binding domain of ER. The ER AF-2 contains the DNA binding domain and the D-F
region. Note that cotransfection of hMMS19 significantly enhances transcriptional
activation by ER AF-1 but has no effect on ER AF-2.
Fig. 24 ER N S118 mutants can still be activated by hMMS19. 293 cells were transiently transfected with ERE-E1a-Luc together with wild type ER-N or mutant ER-N where Ser118 was changed to either Ala or Glu. Both mutants are still transcriptionally active and the activity can still be enhanced by hMMS19 cotransfection.
Fig. 25 Coexpression of CDK7 and MAT1 failed to enhance the activation of hMMS19 on ER N. 293 cells were transiently transfected with ERE-E1a-Luc together with ER-N, cotransfection of hMMS19 stimulated ER-N mediated transcriptional activation, however, further expression of CDK7 and MAT1 together, two TFIIH subunits that are capable of phosphorylating Ser118, have no appreciable effect on ER-N transcriptional activity.
Fig. 26 Cooperative effect of hMMS19 and RAC3 on ER and ER AF1, but not AF2. 293 cells were transiently transfected with ERE-E1a-Luc and hERα or the separate ER-N or ER-C, containing AF-1 or AF-2 respectively. Cells were treated with or without β-estradiol to examine the ligand induced activity. Effect of RAC3, hMMS19 or combined RAC3 and hMMS19 were tested on the transcriptional activity of each activator. Cooperative enhancement by RAC3 and hMMS19 on the transcriptional activity mediated by hERα or ER-N was observed.
Fig. 27 RAC3 N-terminus required for its activation of ER-N. 293 cells were transiently transfected with ERE-E1a-Luc and the hERα N-terminus. While cotransfection of RAC3 enhanced the transcriptional activity of hERα N, the truncation mutant RAC3dN failed to do so. RAC3dN also lost the ability to further enhance the activity when co-transfected with hMMS19.
Fig. 28 RAC3 (342-646) directly binds ER N-terminus. A. ER N interacting domain mapping on RAC3. GST fused various domains, which, when combined together covered the full-length RAC3, were incubated with in vitro translated, $^{35}$S labeled ER-N. A strong interaction with the probe was observed for GST-RAC3 (342-646) only. Right panel shows that RAC3(342-646) binds to full length hERα also. The interaction with full-length hERα is independent on the presence of ligand. B. Further mapping of RAC3(342-646). RAC3(342-646) was further divided into small domains and GST fusion propteins of each domain were made and incubated with in vitro translated, $^{35}$S labeled ER-N again. The interacting domain was mapped to the N terminal half of RAC3(342-646).
Fig. 29A. RAC3(342-646) also binds RAR. ERβ, but not GR. Interactions between RAC3(342-646) and various nuclear receptor N terminus were examined by GST pull down assay. N terminus of rERβ, hRARα and hGRα were in vitro translated and labeled with $^{35}$S and then incubated with GST-RAC3(342-646). Selective interactions between GST-RAC3(342-646) and rERβ or hRARα were observed, while no interaction with hGRα can be detected.

B. TIF2, but not SRC1 corresponding region binds to ERs and RAR. The ability of SRC1 and TIF2 counterparts of RAC3(342-646) to interact with N terminus of various nuclear receptors were examined by GST pull down assay. GST-SRC1(334-656) failed to interact with any N terminus of those receptors, while GST-TIF2(343-664) also binds that of ERs as well as RAR, but not GR.
Fig. 30 A Model of how RAC3 activates ER. In addition to the interaction between liganded hERα ligand binding domain and RAC3 LXXLL motifs in the receptor interacting domain, the region immediately before RID of RAC3 also contacts the hERα AF-1 region. Both interactions may be required to stabilize the receptor-coactivator complex. Further, the two protruding ends of RAC3 may function to recruit other factors required for the maximum transcriptional activity, e.g. the N-terminus bHLH-PAS domain recruits MMS19 which also binds directly to the hinge region of ER and therefore adds another stabilization force to the overall complex; while the C-terminus is necessary to recruit CBP/P/CAF complex which also directly binds the receptor. Both complexes at the N or C-terminus may synergistically function to recruit the basal transcriptional machinery.
CHAPTER VII
REPRINTS

SMRTE inhibits MEF2C transcriptional activation by targeting HDAC4 and 5 to nuclear domains.

The human homologue of the yeast DNA repair and TFIIH regulator MMS19 is an AF-1-specific coactivator of estrogen receptor.
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 RARα in an RA-sensitive manner. Formation of the SMRTe nuclear speckles results in recruitment of several class I and class 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 transactivation 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 SMRT 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) and thyroid hormone receptors (RARα and TRβ) 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/ACTRAIB1 (8). 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 domain 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-CoR (6). SMRT and N-CoR also interact with other transcriptional regulators, such as Pit-1 (7), CBFI/RBP-Jc (8), and P/ZIP (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 HD1A. 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 RARα 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 RARα

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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 RARα 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 RARα
and HDAC1, 3, 4, and 5. We show that the association of SMRTe with RARa 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 MEF2Cx3-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 lystate system (Promega, Inc.). N-CoR, SMRTe, and cSMRTe 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 Blots—A Northern 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 lysates 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 Northern blot was purchased from CLONTECH. The blot was hybridized with indicated 32P-labeled DNA probes that were generated by random priming reactions (Ambion, Inc.).

Indirect Immunofluorescence—HeLa cells were seeded on coverglasses in 12-well plates 1 day prior to transfection. Twelve hours after transfection, cells were fixed in a methanol:acetic acid (1:1, v/v) mixture and processed for indirect immunofluorescence staining as previously described (19). Cell nuclei were stained with the DNA dye DAPI (4',6-diamidino-2-phenylindole dihydrochloride hydrate, Sigma Chemical Co.). Coverglasses were mounted with a ProLong Antifade kit (Molecular Probes). Standard epifluorescence microscopy was performed on an Olympus IX-70 deconvolution microscope equipped with a back-illuminated cool coupled device camera (Princeton Scientific Instruments), and the image was processed using the MetaMorph software (University Imaging Corp.).

Cell Culture and Transient Transfection—HeLa and 293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.). One day prior to transfection, cells were seeded in 12-well plates, and transient transfection was performed using a standard calcium phosphate precipitation method. Transfection was continued for 12 h, and the precipitates were removed by washing with phosphate-buffered saline. After transfection, cells were grown for 38-40 h and analyzed for luciferase and β-galactosidase activities as previously described (20). The luciferase activity was determined with an MLX plate luminometer (Dynex) and normalized with the cotransfected β-galactosidase activity.

RESULTS

Expression of SMRTe in Cancer Cells and Its Regulation by RA—The full-length human and mouse SMRTe (4) contains an N-terminal sequence of 1012 amino acids (aa) that is extended from the original SMRT sequence (1) (Fig. 1A). This full-length human SMRTe was assembled and transcribed/translated in vitro, and the 35S-labeled product was analyzed by SDS-polyacrylamide gel electrophoresis in relation to N-CoR, SMRTe, and cSMRT (Fig. 1B). The largest polypeptide migrated as a 270-kDa protein (lane 3), which is very similar in size to N-CoR and is much larger than SMRTe and cSMRTe. Transfection of SMRTe in mammalian cells also produced similar protein as detected by Western blotting using anti-SMRTe antibody (lanes 6 and 7). Similarly, the endogenous SMRTe was readily detectable in cell lysate prepared from HeLa, MCF7, T47D, and CV-1 cells, as well as from HBL100, 293T, and SWI-3 cells, albeit at slightly lower levels (Fig. 1C). Furthermore, we found that SMRTe expression appeared to be down-regulated by all-trans-RA in NB4 leukemic cells (Fig. 1D). These data indicate that SMRTe encodes a full-length protein of 270 kDa that is differentially expressed in cancer cells and negatively regulated by RA.

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 nuclear localization signal but 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 (R3, 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-
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; GX, LXXLL-like motif; SNC, SMRTe/N-CoR conserved domain; SANT, SWI3/ADA2/N-CoR/TFIID 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.

CoR probe

SMRTe/N-

SMRTe

CoR

SNC-

CoR conserved domain;

SANT domain probe, the human

N-CoR probe, and the β-actin probe as control.

SMRTe inhibition of MEF2C transcriptional activation

demonstrating an in vivo 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 Ga14-RXRα fusion proteins (1). Because cSMRT is located primarily in the cytoplasm, we speculated that this cytoplasmic cSMRT might either sequester unliganded Ga14-RXRα in the cytoplasm or be translocated into the nucleus by Ga14-RXRα. This was tested by double immunofluorescence staining of cSMRT and Ga14-RXRα (Fig. 3B).

Interestingly, atRA treatment abolished this colocalization, leading to exclusive nuclear staining of RXRα 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 Ga14-RXRα fusion protein (1). Because cSMRT is located primarily in the cytoplasm, we speculated that this cytoplasmic cSMRT might either sequester unliganded Ga14-RXRα in the cytoplasm or be translocated into the nucleus by Ga14-RXRα. This was tested by double immunofluorescence staining of cSMRT and Ga14-RXRα (Fig. 3B). As previously reported, Ga14-RXRα alone was located exclusively in the nucleus (data not shown). Remarkably, cSMRT seques-
Interactions of SMRTe with Class I HDACs—Transcriptional repression by the unliganded nuclear receptor 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 (j-l), 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 SMRTe (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.
SMRTe Inhibits MEF2C Transcriptional Activation

![Image of figure 3](image)

**Fig. 3. Ligand-independent colocalization of RARα with SMRTe.** A, SMRTe sequesters RARα in the cytoplasmic speckles in the absence (−)−(a−c), but not the presence (+), of atRA (d−f). SMRTe and HA-tagged hRARα were double-labeled with rabbit anti-SMRTe and mouse anti-HA antibodies, followed by fluorescein (green) or rhodamine (red)-coupled secondary antibodies, respectively. HeLa cells were transiently transfected with pCMX-hSMRTe and pCMX-HA-RARα in the cytoplasm in an RA-sensitive manner. pCMX-cSMRT and pCMX-Gal4-RARα were cotransfected in HeLa cells. Gal4-RARα was detected by mouse anti-Gal4 DNA binding domain monoclonal antibodies (red) and the cSMRT with rabbit anti-SMRTe polyclonal antibodies (green).

(Fig. 5B). 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, c). 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
SMRTe Inhibits MEF2C Transcriptional Activation

**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 m 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 (m). Cotransfection with SMRTe recruits HDAC1 (b–d) and HDAC3 (j–l), but not HDAC2 (f–h) or mSin3A (n–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.

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 SMRTα (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.
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**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.

**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 addition to nuclear localization, RD3 also appears to contribute to speckled nuclear localization. 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
SMRT (33), suggesting a complex signaling pathway that regulates SMRT localization.

The colocalization of unliganded RARα with SMRTε in the cytoplasmic and nuclear speckles confirms the reported interaction of these two proteins in vivo. Because RARα alone is diffusely distributed in the nucleoplasm, and because the speckled pattern of SMRTε is independent of RARα, these data suggest that SMRTε recruits RARα into these speckles. The dissociation of RARα from the SMRTε cytoplasmic speckles upon atRA treatment confirms the proposed theory of ligand-dependent dissociation of RARα from the corepressor. It appears that the presumed “SMRTε-free” RARα can be readily transported into the nucleus. However, it is not clear whether the presence of cytoplasmic RARα is due to nuclear export or regulated import by SMRTε. It is intriguing to note that TRβ also undergoes a thyroid hormone-enhanced nuclear translocation (34). Based on these findings it is reasonable to speculate that SMRTε might be responsible for regulating nuclear-cytoplasmic shuttling of unliganded TRβ and RARα. In addition, we found that cSMRT is primarily located in the cytoplasm, where it can block nuclear localization of Gal4-RARα, suggesting that cellular compartmentalization may in part contribute to the dominant negative activity of cSMRT.

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 SMRTε with HDAC1, 3, 4, and 5; however, we have found that SMRTε does not colocalize with HDAC2, HDAC6, and mSin3A. Interestingly, SMRTε can recruit the interacting HDACs into discrete nuclear speckles. The colocalization of HDAC3, 4, and 5 with SMRTε 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 SMRTε 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 SMRTε 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 SMRTε.

Support for an active role of SMRTε in regulating histone deacetylation comes from observations that localization of HDAC1 to SMRTε nuclear speckles depends on the histone deacetylase activity of HDAC1. Similarly, it was recently reported that HDAC3 and 7 mutants, when lacking HDAC activity, as well as the deacetylase inhibitor trichostatin A and sodium butyrate, can disrupt colocalization of HDAC5 and 7 with SMRTα (36). Together, these data suggest an important role for SMRTεα 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 SMRTε, suggesting a new role for SMRTε in regulating cytoplasmic-nuclear trafficking, which might be independent of its nuclear corepressor function. In the absence of SMRTε, HDAC4 is localized in the cytoplasm due to 14-3-3 binding (17). SMRTε 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 SMRTε 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 SMRTε, consistent with its stronger inhibitory effect on MEF2C activity. We have found that SMRTε also recruits HDAC5 into nuclear speckles, correlating with an enhancement of HDAC5-mediated transcriptional inhibition on MEF2C function by SMRTε. This observation suggests that HDAC5 localization to SMRTε nuclear domains might further suppress MEF2C activity. Because SMRTε also recruits HDAC4 into the nuclear speckles, it is conceivable that SMRTε 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 SMRTε by calcium/calmodulin-dependent protein kinases might disrupt HDAC-SMRTε interaction, leading to segregation and subsequent export of HDAC4 and 5. Alternatively, this signal-dependent phosphorylation event might trigger nuclear export of the entire SMRTε-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 nuclear 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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REFERENCES

The Human Homologue of the Yeast DNA Repair and TFIIH Regulator MMS19 Is an AF-1-specific Coactivator of Estrogen Receptor*

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Steroid/nuclear hormone receptors are ligand-dependent transcriptional regulators that control gene expression in a wide array of biological processes. The transcriptional activity of the receptors is mediated by an N-terminal ligand-independent transcriptional activation function AF-1 and a C-terminal ligand-dependent transcriptional activation function AF-2. The nuclear receptor coactivator RAC3 (also known as AIB1/ACTR/pCIP/TRAM-1/SRC-3) is amplified in breast cancer cells, where it forms a complex with estrogen receptor (ER) and enhances AF-2 activity of the receptor. Here, we identify a putative human homologue of the yeast DNA repair and transcriptional regulator MMS19 as a RAC3-interacting protein. The human MMS19 interacts with the N-terminal PAS-AB domain of RAC3 in vitro and in vivo through a conserved C-terminal domain. Interestingly, the human MMS19 also interacts with estrogen receptors in a ligand-independent manner but not with retinoic acid receptor or thyroid hormone receptor. Overexpression of the interacting domain of hMMS19 strongly inhibits ER-mediated transcriptional activation, indicating a dominant negative activity. In contrast, overexpression of the full-length hMMS19 enhances ER-mediated transcriptional activation. We find that hMMS19 stimulates the AF-1 activity of Erα, but not the AF-2 activity, suggesting that hMMS19 may be an AF-1-specific transcriptional coactivator of estrogen receptor.

Steroid/nuclear hormone receptors (SNR)² are ligand-dependent transcriptional regulators that control gene expression in a wide array of biological processes such as development and reproduction (1, 2). These receptors share a common domain structure: an N-terminal ligand-independent transcriptional activation function (AF-1, or A/B region), a centrally located DNA binding domain (or C region), and a C-terminal ligand-dependent transcriptional activation function (AF-2, LBD, or E/F domain). The AF-1 and AF-2 function together to regulate gene expression in a cell-type-specific and promoter-specific manner (3–6). However, the exact mechanism is currently unclear.

Several transcriptional coactivators for SNR have been identified, including the steroid receptor coactivator (SRC)/p160 family (9), which contains SRC-1 (10), GRIP1/TFII F (11–13), and RAC3/AIB1/ACTR/pCIP (14–18). The SRC/p160 family of coactivators binds to a hydrophobic pocket on the surface of the receptor LBD in a ligand-dependent manner (19, 20). The coactivator enhances transcriptional activation of the receptor via a mechanism involving histone acetylation and recruitment of additional coactivators such as CBP/p300 and P/CAF (21, 22). Genetic studies demonstrate that SRC-1 and RAC3 (pCIP) are involved in regulating hormonal responses in mice (23–25). Interestingly, the SRC/p160 coactivators also interact with the AF-1 domain of several SNRs in a ligand-independent manner (6, 26–30). For ERα, such interaction is regulated by phosphorylation of the AF-1 region, suggesting that SRC/p160 coactivators may be involved in cross-talk between AF-1 and AF-2 of the receptor (26–28). RAC3 (AIB1) is unique among the SRC/p160 family of coactivators, because it is amplified and overexpressed in a subset of breast cancers (16). It has been demonstrated that RAC3 (AIB1) activators form a stable complex with ERα in MCF-7 breast cancer cells (31), suggesting that RAC3 may play an important role in regulating ER function in vivo.

The SRC/p160 coactivators share a common domain structure, including a highly conserved N-terminal basic-helix-loop-helix (bHLH) and Per-Arnt-Sim (PAS) domains (9). The PAS domain can be subdivided into A and B regions. This domain is highly conserved among several Drosophila and mammalian proteins, including Per, Sim, and Arnt (32), as well as in many regulators of circadian rhythm and neural development (for a review, see Ref. 33). The bHLH-PAS domain of Drosophila proteins plays an important role in mediating protein-protein interaction (34–36). However, the function of the bHLH-PAS domain of the SRC/p160 coactivators remains largely unknown. Here, we report the identification of a human homologue of the yeast MMS19 as an RAC3-interacting protein isolated in a yeast two-hybrid screening with the bHLH-PAS domain of RAC3 as bait. Interestingly, hMMS19 also interacts with ERα in a ligand-independent manner. Our data suggest that hMMS19 may function as an AF-1-specific transcriptional coactivator of ER.

**MATERIALS AND METHODS**

**Plasmids**—The DNA fragments encoding various N-terminal domains of human RAC3 (14) were amplified by polymerase chain reaction.
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RESULTS

Isolation of a Putative Human MMS19 in Yeast Two-hybrid Screening—To identify proteins that interact with the bHLH-PAS domain of RAC3, we conducted yeast two-hybrid screening with the RAC3-N-terminal domain as bait. The RS2.1 clone showed strongest interaction. Sequence analysis and database search revealed that RS2.1 encodes a C-terminal 164-amino acid polypeptide that shares homology with the yeast MMS19 (38). The full-length cDNA of RS2.1 was obtained, and it predicts an open reading frame of 1030 amino acids (Fig. 1A). The full-length RS2.1 is named hMMS19 because it is related in size and sequence to the yeast MMS19. We also found a mutant cDNA clone in the GenBank® accession number AF007151, which contains a single deletion at nucleotide 2240, resulting in a truncation mutant at 779 amino acids. We designate this truncation mutant as hMMS19-mt. Comparison of hMMS19 with the yeast homologue shows a 36% overall similarity (or 26% identity). The homology is most striking at the N- and C-terminal regions with 61% or 57% similarities, respectively. Related sequences were also predicted from the genomes of Drosophila melanogaster, Caenorhabditis elegans, and Arabidopsis thaliana. Alignment of these sequences indicates that MMS19 is conserved through evolution at the N- and C-terminal domains, whereas the central region has diverged significantly. We also found that the hMMS19 gene resides in a genomic clone (RP11–445I23, accession number AL359388), which maps to chromosome 10q24. Interestingly, loss of heterozygosity in this region was observed in several human cancers, including endometrial and prostate carcinomas (39, 40).

Northern blot analysis of hMMS19 in various human cancer cells revealed a single transcript of about 4 kb (Fig. 1B, top). The size of this message is in agreement with the length of the hMMS19 cDNA. The hMMS19 transcript was detected in all cancer cell lines analyzed as well as in several human tissues such as heart, brain, placenta, liver, skeletal muscle, and kidney (data not shown), suggesting that hMMS19 is ubiquitously expressed. Furthermore, by indirect immunofluorescence, we found that hMMS19 is located in the cell nucleus (Fig. 1C). This nuclear localization is consistent with the hypothesis that MMS19 is involved in DNA repair and transcriptional regulation.

Interactions of hMMS19 with RAC3—To further analyze the interaction of hMMS19 with RAC3, we first tested the specificity of RS2.1 interactions with various baits in a yeast two-hybrid assay (Fig. 2A). As expected, RS2.1 alone did not activate reporter expression nor did the bait RAC3-N or other unrelated baits. Coexpression of RS2.1 with RAC3-N strongly activated reporter expression as expected. In contrast, RS2.1 did not interact with the bHLH-PAS domain of mouse Sim2 (Sim-N) nor did it interact with RARs or p53. These data confirm that RS2.1 interacts specifically with RAC3-N in the yeast two-hybrid assay.

We then mapped the RS2.1-interacting region within the bHLH-PAS domain of RAC3 (Fig. 2B). The bHLH-PAS domain of RAC3 was subdivided into bHLH, PAS-A, PAS-B, or PAS/A/B (Fig. 2F) and tested for interactions with RS2.1 in a similar assay. As expected, none of these baits alone activated reporter expression (data not shown). Coexpression of RS2.1 with PAS/A or PAS-C-N each strongly activated reporter expression, whereas coexpression with bHLH, PAS-A, or PAS-B did not activate reporter expression in this assay. These data suggest that the PAS-A and PAS-B domains are both required for strong interaction with RS2.1.

To confirm the interaction of RS2.1/hMMS19 with RAC3 in vitro, we performed a GST pull-down assay using GST fusion of various RAC3 N-terminal fragments to pull down the 35S-labeled RS2.1 fragment or the full-length hMMS19. The RS2.1 fragment and full-length hMMS19 were transcribed/translated and labeled with 35S-methionine in reticulocyte lysate. First, the 35S-RS2.1 probe was incubated with GST-RAC3-N, and the bound probe was eluted and analyzed by SDS-PAGE and autoradiography. As expected, GST-RAC3-N, but not GST alone, pulled down a significant amount of 35S-RS2.1 (Fig. 2C), confirming in vitro interaction of RS2.1 with RAC3-N. Second, 35S-hMMS19 was incubated with GST or GST fusion of bHLH, PAS-A, PAS-B, PAS-A/B, or the bHLH-PAS domain of RAC3. The bound hMMS19 probe was analyzed by SDS-PAGE and autoradiography (Fig. 2D). As expected, GST or bHLH did not bring down a detectable amount of 35S-hMMS19. In contrast, GST-PAS-A or GST-PAS-B each pulled down a noticeable amount of 35S-hMMS19, suggesting that hMMS19 can interact
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Fig. 1. Amino acid sequence of human MMS19 and its expression pattern and subcellular localization. A, multiple sequence alignment of hMMS19 with related sequences of different species by the ClustalW program. The aligned sequences were plotted using the ESPript program. Consensus amino acids are boxed and displayed in white against the red background. Highly conserved residues are also boxed and displayed in red. The ending sequence of hMMS19-mt mutant is indicated, as well as the starting amino acid of the yeast two-hybrid clone RS2.1. Amino acid sequences of related proteins in C. elegans, A. thaliana, and S. cerevisiae are derived from GenBank® accession numbers T33176, BAB11076, and NP_012135, respectively. The amino acid sequence of the related protein in D. melanogaster is predicted by GENSCAN from Drosophila genomic sequence AE009603. Four consensus motifs predicted by MEME software are shown under the aligned sequences. B, Northern blot analysis of the expression pattern of hMMS19 in human cancer cells. β-Actin is shown as a control. C, nuclear localization of hMMS19. Indirect immunofluorescence analysis of HeLa cells transfected with HA-hMMS19 and detected by an anti-HA monoclonal antibody followed by a rhodamine-coupled secondary antibody. The bottom panel shows nuclear staining with 4',6-diamidino-2-phenylindole (DAPI) of the same cells.

weakly with PAS-A and PAS-B separately. Interestingly, GST-PAS-A/B pulled down a much more significant amount of 35S-hMMS19, suggesting a synergistic effect by PAS-A and PAS-B for binding hMMS19. GST-RAC3-N pulled down an amount of 35S-hMMS19 similar to that of GST-PAS-A/B, confirming that the bHLH region is not involved in the interaction. The other
C-terminal RAC3 fragments, including the nuclear receptor-interacting domain and the transcriptional activation domain, showed no binding to hMMS19 (data not shown). Therefore, these data are consistent with the above yeast two-hybrid results in implicating the PAS-A/B domain of RAC3 as the binding surface for hMMS19.

**Human MMS19 Interacts with Estrogen Receptor**—Because RAC3 interacts with nuclear receptors, we tested whether hMMS19 could interact with nuclear receptor in a similar GST pull-down assay. Intriguingly, we found that GST-RS2.1 pulled down a significant fraction (about 20% of input) of ERα in a ligand-independent manner (Fig. 3A). Similarly, GST-RS2.1 also pulled down a significant amount of ERβ (Fig. 3B), suggesting that hMMS19 can interact with both ERα and ERβ. Similar results were obtained by GST fusion of full-length hMMS19 with RAC3 in GST pull-down assay, conducted as described in C except that different GST-RAC3 fusion proteins are used in this experiment (E). Coomassie Blue staining of GST and GST-RAC3 fusion proteins used in the GST pull-down experiments. F, schematic of the RAC3 N-terminal domain fragments used in this study.

The above interactions of hMMS19 with RAC3 and ERs prompted us to investigate whether hMMS19 could modulate transcriptional activity of ER. To test this possibility, we performed reporter gene assay by transient transfection in HEK293 cells. First, we analyzed the effects of overexpression of RS2.1 on ER-mediated transcriptional activation of a luciferase reporter gene driven by ER-responsive elements (Fig. 4A and B). As expected, both ERα and ERβ strongly activated luciferase expression in an E2-dependent manner. Cotransfection with RS2.1 significantly reduced the ligand-dependent transactivation in a dose-dependent manner without affecting reporter expression in the absence of E2. We found that, at the highest concentration of RS2.1, both ERα and ERβ activities were reduced about 8-fold. Importantly, coexpression of RAC3 can reverse the inhibitory effect of RS2.1 on ER transcriptional activity (Fig. 4C). The inhibition of ER activity by RS2.1 is specific, for RS2.1 had no effect on an otherwise identical reporter lacking ER binding sites (data not shown). Furthermore, overexpression of RS2.1 had no effect on ligand-dependent transcriptional activation of RARα or TRβ (Fig. 4D), suggesting that RS2.1 may selectively affect ER transcriptional activity. These data are consistent with the preferential interaction of RS2.1 with ERs but not with RARα or TRβ, implicating that hMMS19 may play a more important role in regulating ER activity than RAR or TR.

The inhibition of ER transcriptional activity by RS2.1 may be explained by the dominant negative effect of RS2.1, because this fragment contains the RAC3/ER-interacting domain. It is possible that RS2.1 interferes with the assembly of a functional ER-coactivator complex. If RS2.1 indeed acts as a dominant negative mutant, the full-length protein should have a positive effect on ER transcriptional activity. Indeed, overexpression of hMMS19 enhanced ER transcription activation in HEK293 cells (Fig. 5A). The moderate effect of this enhancement may be due to high levels of endogenous hMMS19 in these cells. In contrast, the hMMS19-mt mutant, which lacks the C-terminal interacting domain, failed to coactivate ER-mediated transcriptional activation.
Human Homologue of MMS19 Is a Coactivator of Estrogen Receptor

The above findings suggest that hMMS19 may function as a coactivator of estrogen receptors. Intriguingly, the yeast MMS19 has been shown to regulate transcription by influencing TFIH activity (38), and TFIH has been shown to regulate the AF-1 function of human ERα (41). To shed light on the mechanism of MMS19 function in regulating ER activity, we examined whether hMMS19 affects AF-1 or AF-2 activity of ERα. We subdivided ERα into the N-terminal AF-1 and the C-terminal AF-2 domains, each of which contains the DNA binding domain. The effect of overexpression of hMMS19 on the ERα AF-1 or ERα AF-2 was then tested by transient transfection (Fig. 5C). As expected, E2 strongly activated transcription mediated by the ERα AF-2 but to a lesser extent than the full-length ERα, suggesting that AF-1 is required for maximal transcriptional activity of ERα. Cotransfection of hMMS19 had no effect on this ligand-dependent transcriptional activity of ERα AF-2, suggesting that hMMS19 is not an AF-2-dependent coactivator. As expected, ERα AF-1 had a constitutive activity in the absence of ligand. Addition of E2 slightly enhanced the ERα AF-1 activity, presumably due to E2-mediated phosphorylation of the ERα AF-1 domain (28, 42). Interestingly, cotransfection of hMMS19 strongly enhanced AF-1 activation in both the absence and presence of E2 by 6.0- and 2.5-fold, respectively, suggesting that hMMS19 can regulate ER transcriptional activity by stimulating the N-terminal AF-1 function of ER.

DISCUSSION

MMS19 was initially identified in a yeast genetic screen for mutations that render cells sensitive to the alkylating agent methyl methanesulphonate (MMS) (43) and later to UV and DNA cross-linking agents (44, 45). Analysis of MMS19 mutation in yeast suggests that it plays a role in nucleotide excision repair and transcription regulation by affecting TFIH activity (38, 46, 47). Here, we isolated a human MMS19 homologue as a RAC3-interacting protein. Intriguingly, hMMS19 interacts with both the coactivator RAC3 and the receptor ER and functions as an AF-1-specific transcriptional coactivator of ER.

The bHLH-PAS domain is the most conserved region in the SRC/p160 coactivators (14); however, the function of this domain is largely unknown. The isolation of hMMS19 as a bHLH-PAS domain-interacting protein suggests that this domain may be involved in protein-protein interaction. Further analysis of the hMMS19-RAC3 interactions reveals that interaction is mediated through the PAS-A/B region. Interestingly, strong interaction occurs only in the presence of both PAS-A and PAS-B, suggesting that there may be two interacting interfaces that together stabilize the interaction between hMMS19 and RAC3. The involvement of PAS domain in protein-protein interaction can also be found in the Drosophila protein Period, which interacts with Timeless through its PAS domain (48). Because the PAS domain is highly conserved among the SRC/p160 coactivators, hMMS19 might also interact with SRC-1 and/or TIF2, but this remains to be tested. However, despite the conservation of PAS domains in different proteins, hMMS19 does not interact with the PAS domain of Sim, suggesting the different PAS domains may be involved with different interacting proteins. The interacting surface in hMMS19 maps to its C-terminal conserved domain. Structural prediction suggests that this region contains multiple potential amphipathic helices, which might mediate the interaction with the PAS domain of RAC3.

Analysis of the interactions of hMMS19 with nuclear receptors shows strong binding with ERα and ERβ in a ligand-independent manner. Because ER also interacts with RAC3 (49), several possibilities may explain the relevance of these interactions. For instance, hMMS19 may interact with ER first in the absence of ligand. This interaction may modulate, for instance, the phosphorylation states of ER by targeting TFIH activity to ER. Upon ligand binding, ER undergoes a conformational change that allows the receptor to bind the LXXLL motifs of RAC3 via a coactivator-binding pocket in the LBD (9). RAC3 then interacts with hMMS19 via the N-terminal PAS domain to stabilize the complex or to signal a subsequent event in transcription. Alternatively, RAC3 may form a complex with hMMS19 first, which is then recruited to the liganded receptor. It is clear that RAC3 uses separate domains for interactions with hMMS19 and liganded ER. However, it is currently unknown whether hMMS19 can interact with RAC3 and ER simultaneously. The fact that the C-terminal domain of hMMS19 interacts with both RAC3 and ER suggests that the interactions of hMMS19 with RAC3 and ERs may be mutually exclusive. However, it is equally possible that different motifs in the C-terminal domain of hMMS19 interact with RAC3 and ER independently.

The physiological relevance of hMMS19 in ER signaling is
**FIG. 4.** The human MMS19 modulates transcriptional activity of ER. A. Dose-dependent inhibition of ligand-induced transcriptional activity of ERα by RS2.1. Increasing concentrations of RS2.1 (−, 0 ng; +, 100 ng; ++, 200 ng; ++++, 400 ng) were transfected into HEK293 cells together with 20 ng of ERα and 0.5 μg of the ERE-E1A-Luc reporter. After transfection, cells were treated with solvent or 25 μM of E2, and relative luciferase activity was determined as described under "Materials and Methods." B. Dose-dependent inhibition of ligand-induced transcriptional activity of ERβ by RS2.1 was conducted as described in A except that ERβ is used. C. Coexpression of RAC3 reverses RS2.1-mediated repression of ERα transcriptional activity. D. RS2.1 had no effect on transcriptional activity of hRARα or hTRβ on β-RARE-tk-Luc or TRE-tk-Luc reporter, respectively.

**FIG. 5.** The human MMS19 is an AF-1-specific transcriptional coactivator of ER. A. The full-length hMMS19, but not hMMS19-mt, consistently enhances ERα-mediated transcriptional activation. B. Coexpression of hMMS19 and RAC3 further enhances transcriptional activation of ERα. C. The hMMS19 enhances transcriptional activation by the ERα AF-1 domain in the absence or presence of E2. The ERα AF-1 contains the N-terminal A/B region and the DNA binding domain of ERα. The ERα AF-2 contains the DNA binding domain and the D-F region. Note that cotransfection of hMMS19 significantly enhances transcriptional activation by ERα AF-1 but has no effect on ERα AF-2.

demonstrated by overexpression of the C-terminal RS2.1 fragment and the full-length hMMS19, each of which significantly affect transcriptional activity of ER in transfection assays. Overexpression of RS2.1 inhibits ligand-dependent transcriptional activation of an ER reporter gene due to a dominant negative effect. Overexpression of RS2.1 has no effect on basal promoter activity in the absence of ligand, consistent with the observation that ER binds to chromatin in response to ligand (50, 51). As expected, the full-length hMMS19 enhances ligand-dependent transcription of the ER reporter gene, whereas the truncation mutant lacking the RAC3/ER-interacting domain has no effect on ER activity. Intriguingly, hMMS19 strongly...
enhances the AF-1 activity of ER in both the absence and presence of ligand and has no effect on the activity of a separated AF-2 domain. Therefore, we suggest that hMMS19 functions as an AF-1-specific coactivator of ER. Currently, only a few potential AF-1 coactivators were reported, such as the p68 RNA helicase (7), and the mechanism of AF-1 coactivator is largely unknown. The human MMS19 is unique among other coactivators in that it interacts with both ERs and RAC3. Because hMMS19 interacts with the PAS domain of RAC3 and regulates AF-1 activity of ER, it is intriguing to speculate that the PAS domain of RAC3 may be important for regulating AF-1 function. However, the mechanisms by which AF-1 activates transcription and synergizes with AF-2 are largely unknown, and further study is required to fully understand the mechanism of transcriptional activation by nuclear receptors. Our finding that hMMS19 is an AF-1 coactivator that also interacts with AF-2 coactivator RAC3 suggests that hMMS19 may bridge the functions of AF-1 and AF-2.

During preparation of this manuscript, another group reported that hMMS19 interacts directly with the XPD and XPB subunits of TFIIH, confirming a regulatory role of hMMS19 on TFIIH activity (8). TFIIH is a multiprotein complex consisting of nine subunits, each participating in transcription and nucleotide excision repair pathways. TFIIH harbors several enzymatic activities such as a DNA-dependent ATPase linked to XPD and XPB and a cdk-activating protein kinase, a cyclin-dependent kinase that phosphorylates the polymerase II C-terminal tail domain. Recently, it was shown that the AF-1 domain of hERs is phosphorylated by the TFIIH cyclin-dependent kinase in a ligand-dependent manner (41). Furthermore, it was shown that phosphorylation of polymerase II C-terminal tail domain precedes the dissociation of ER/p160 coactivator complex from target promoters (50). Therefore, the identification of hMMS19 as an ER- and RAC3-interacting protein suggests a novel mechanism by which TFIIH may be recruited to ER target promoters. In light of the important role of TFIIH in regulating the transcriptional activity of ER (41), we suggest that hMMS19 might be involved in regulation of ER activity by bridging TFIIH with ER. Possibly, the interaction of hMMS19 with RAC3 might be involved in ligand-dependent recruitment of TFIIH to ER (41). Alternatively, hMMS19 might facilitate TFIIH-mediated phosphorylation of ER in specific promoters and cell types.

In summary, we have identified and characterized the human homologue of MMS19 as a coactivator of estrogen receptor. Currently, only a few potential AF-1 coactivators were reported, such as the p68 RNA helicase (7), and the mechanism of AF-1 coactivator is largely unknown. The human MMS19 is unique among other coactivators in that it interacts with both ERs and RAC3. Because hMMS19 interacts with the PAS domain of RAC3 and regulates AF-1 activity of ER, it is intriguing to speculate that the PAS domain of RAC3 may be important for regulating AF-1 function. However, the mechanisms by which AF-1 activates transcription and synergizes with AF-2 are largely unknown, and further study is required to fully understand the mechanism of transcriptional activation by nuclear receptors. Our finding that hMMS19 is an AF-1 coactivator that also interacts with AF-2 coactivator RAC3 suggests that hMMS19 may bridge the functions of AF-1 and AF-2.

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