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Direct binding of CoREST1 to SUMO-2/3 contributes to gene-specific repression by the LSD1/CoREST1/HDAC complex

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Abstract

Post-translational modification of transcription factors by the small ubiquitin related modifier SUMO is associated with transcriptional repression, but the underlying mechanisms remain incompletely described. We have identified binding of the LSD1/CoREST1/HDAC co-repressor complex to SUMO-2. Here we show that CoREST1 binds directly and non-covalently to SUMO-2, but not SUMO-1, and CoREST1 bridges binding of the histone demethylase LSD1 to SUMO-2. Depletion of SUMO-2/3 conjugates led to transcriptional de-repression, reduced occupancy of CoREST1 and LSD1 and changes in histone methylation and acetylation at some, but not all, LSD1/CoREST1/HDAC target genes. We have identified a non-consensus SUMO-interaction motif (SIM) in CoREST1 required for SUMO-2 binding and we show that mutation of the CoREST1 SIM disrupted SUMO-2 binding and transcriptional repression of some neuronal specific genes in non-neuronal cells. Our results reveal that direct interactions between CoREST1 and SUMO-2 mediate SUMO-dependent changes in chromatin structure and transcription important for cell type-specific gene expression.

Introduction

Transcription repression mechanisms contribute to cell type-specific gene expression programs important for cell fate determination. Post-translational modification of histones plays a central role in regulating chromatin structure and gene transcription (Jenuwein and Allis, 2001). The first histone lysine specific demethylase identified, LSD1 (KDM1), regulates both gene activation and repression programs important for mammalian organogenesis (Shi et al., 2004; Wang et al., 2007). Transcriptional repression by LSD1 has been correlated with enzymatic removal of mono- and di-methyl groups from histone H3 Lys4 (Shi et al., 2004). LSD1 is a component of several multiprotein complexes where it has been found tightly associated with CoREST1 and histone deacetylases (HDAC) 1 and 2, (Hakimi et al., 2003; Humphrey et al., 2001; Shi et al., 2003; Shi et al., 2005; You et al., 2001). CoREST1 binds directly to LSD1 and is required for LSD1-mediated demethylation of nucleosomal substrates

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(Lee et al., 2005; Shi et al., 2005; Yang et al., 2006). The presence of LSD1 and HDACs in a common complex supports coordinated deacetylation and demethylation of histone tails to generate a repressive chromatin structure (Lan et al., 2008; Shi et al., 2003).

In addition to modulating LSD1 enzymatic activity, CoREST1 and other components of the LSD1 complex are key regulators of LSD1 recruitment to specific genes. CoREST1 was initially identified as a co-repressor for the DNA binding protein REST (also known as NRSF); binding of CoREST1 to REST recruits the CoREST1/LSD1 complex to silence neuronal genes in non-neuronal cells (Ballas et al., 2001; Ballas et al., 2005; Hakimi et al., 2002; Shi et al., 2004; Shi et al., 2005). The LSD1/CoREST1/HDAC core complex is also associated with the co-repressor CtBP Cwhich interacts directly with many DNA-binding transcription factors (Chinnadurai, 2002). CoREST1 binds directly to CtBP and this interaction may contribute to repression of some CtBP target genes (Cowger et al., 2007; Kuppuswamy et al., 2008). Recent studies of promoter-specific repressors such as Gfi-1 and Gfi-1b as well as genome-wide analyses have led to the identification of a large number of genes regulated by LSD1 (Garcia-Bassets et al., 2007; Saleque et al., 2007). In most cases, however, the protein-protein interactions that mediate recruitment of LSD1 to specific promoters have not been described.

Post-translational modification by the small ubiquitin related modifier SUMO regulates diverse cellular processes including cell cycle progression, genomic stability, intracellular trafficking and transcription (Geiss-Friedlander and Melchior, 2007; Gill, 2004; Hay, 2005; Johnson, 2004). In most cases, SUMO conjugation alters localization and/or activity of the substrate by providing a new protein-protein interaction interface. SUMO interaction motifs (SIMs) that mediate non-covalent binding to SUMO have been described. SIMs in several proteins, such as the DNA repair enzyme TDG and the tumor supressor PML, have been shown to be important for biological activity (Baba et al., 2005; Shen et al., 2006; Takahashi et al., 2005). In mammals, three SUMO paralogs are widely expressed: SUMO-2 and SUMO-3 which are 96% identical and SUMO-1 which is 45% identical to SUMO-2. Growing evidence suggests that SUMO-2/3 and SUMO-1 have some unique biological functions (Ayaydin and Dasso, 2004; Saitoh and Hinchey, 2000; Vertegaal et al., 2006). Although proteins that bind preferentially to SUMO-2/3 or SUMO-1 may contribute to distinct functions of the SUMO paralogs, with only a single type of SIM described to date, it is not clear how paralog-specific interactions are determined.

SUMO modification of transcription factors and cofactors has generally been correlated with transcriptional repression (Geiss-Friedlander and Melchior, 2007; Gill, 2004; Hay, 2005). Investigations of the molecular mechanisms underlying SUMO-dependent repression largely support the hypothesis that covalent attachment of SUMO provides a new interaction interface which mediates recruitment of transcriptional co-repressors (Ivanov et al., 2007; Lin et al., 2006; Ross et al., 2002; Yang and Sharrocks, 2004). Several chromatin modifying enzymes and chromatin binding proteins have been implicated as effectors of SUMO-mediated repression. For example, SUMO-modification of the transcription factor Elk-1 promotes recruitment of HDAC2, associated with histone deacetylation and transcriptional repression of the c-fos promoter (Yang and Sharrocks, 2004). Previous affinity chromatography studies revealed an association between LSD1 and SUMO-2 (Rosendorff et al., 2006). In that study, although repression by Gal4-SUMO-2 correlated with de-methylation of histone H3K4, LSD1 was not essential for repression of the synthetic reporter gene analysed. Thus, the molecular basis for LSD1 association with SUMO-2 and the functional significance of this association for LSD1 and/or SUMO-dependent repression of endogenous genes has not been described.

Here we show that CoREST1 binds directly and non-covalently to SUMO-2, but not SUMO-1, and bridges LSD1 binding to SUMO-2 *in vitro*. We identify a non-consensus SIM required for SUMO-2 binding by CoREST1 and establish that this motif also mediates SUMO-2-specific

binding in at least two other proteins. We found that de-conjugation of SUMO-2/3 led to de-repression, loss of promoter occupancy by CoREST1 and LSD1, increased H3K4 methylation, and increased H3 acetylation of the SCN1A and SCN3A genes, but did not affect another CoREST1/LSD1 target gene, SCN2A2. Furthermore, we show that mutation of the CoREST1 SIM disrupted binding of SUMO-2/3 and led to loss of CoREST1 occupancy and transcriptional repression of specific LSD1/CoREST1 target genes. Our findings reveal a role for SUMO-2/3 in gene-specific recruitment and activity of the LSD1/CoREST1/HDAC co-repressor complex, supporting a role for SUMOylation in regulation of histone modifications and transcriptional silencing of some neuronal specific genes in non-neuronal cells.

Results

The LSD1/CoREST1/HDAC co-repressor complex is associated with SUMO-2

A prevailing hypothesis to explain SUMO-mediated transcriptional repression is that SUMO provides a protein-protein interaction interface that recruits transcriptional co-repressors. We have therefore used an affinity purification strategy to identify nuclear SUMO binding proteins. We mixed soluble HeLa nuclear extracts with a non-conjugatable form of GST-SUMO-2 (GST-SUMO-2-GA) or control GST affinity resins and identified the bound proteins by mass spectrometry. Among the proteins retained specifically by GST-SUMO-2 (Supplemental Table 1), we have been particularly interested in a group of proteins including LSD1, CoREST1, HDAC1 and HDAC2, which were previously reported as components of the LSD1/CoREST1/HDAC co-repressor complex (Hakimi et al., 2003; Humphrey et al., 2001; Shi et al., 2003; Shi et al., 2005; You et al., 2001). Previous studies using whole cell extracts also revealed LSD1 association with GST-SUMO-2 (Rosendorff et al., 2006).

CoREST1 binds to SUMO-2 directly and non-covalently

Identification of multiple LSD1/CoREST1/HDAC co-repressor complex subunits ined on the GST-SUMO-2 affinity resin. We suggested that a multiprotein complex was reta therefore investigated which subunits bound directly to SUMO-2. Using purified recombinant proteins, we found that $(His)_6$-CoREST1 bound efficiently to GST-SUMO-2 (Figure 1A). SUMO-2 possesses stronger intrinsic transcriptional repression activity than SUMO-1 in reporter gene assays and residues K33 and K35 contribute to the transcriptional repression activity of SUMO-2 (Chupreta et al., 2005; Rosendorff et al., 2006). As shown in Figure 1A, we found that $(His)_6$-CoREST1 bound to GST-SUMO-2, but almost no binding was observed to GST-SUMO-1 or GST-SUMO-2-K33,35A. Mutation of SUMO-2 K33, K35 did not affect binding to UBC9 (Rosendorff et al., 2006) or SENP5 (Ouyang and Gill, unpublished data), indicating that these mutations do not disturb the overall conformation of SUMO-2. Thus, CoREST1 binds directly and non-covalently to SUMO-2 *in vitro* and this binding correlates with transcriptional repression.

We then examined CoREST1 association with SUMO-2/3 *in vivo*. As shown in Figure 1B, 3xFLAG-CoREST1 co-immunoprecipitated endogenous SUMO-2/3 conjugates from cell lysates. Notably, almost no SUMO-1 conjugates co-immunoprecipitated with CoREST1, consistent with the preference for SUMO-2 binding observed *in vitro*. The SUMO-2/3 conjugates co-immunoprecipitated with CoREST1 are not SUMO-2/3-modified forms of CoREST1 because they were not immunoreactive to anti-FLAG or anti-CoREST1 antibody (data not shown). In reciprocal assays, CoREST1, as well as LSD1 and HDACs 1 and 2, co-immunoprecipitated with HA-SUMO-2-GG *in vivo* (Supplemental Figure 1), further confirming that the LSD1/CoREST1/HDAC co-repressor core complex associates with SUMO-2/3 conjugates in cells.

CoREST1 bridges LSD1 binding to SUMO-2

In contrast to our findings with CoREST1, we did not observe binding of purified LSD1 to SUMO-2 (Figure 2). We therefore reasoned that LSD1 was retained on the GST-SUMO-2 affinity resin through an indirect mechanism. Since CoREST1 binds directly to both LSD1 and SUMO-2, we asked if CoREST1 can bridge recruitment of LSD1 by SUMO-2. To this end, we mixed purified recombinant $(His)_6$-LSD1 with purified GST-SUMO-2 in the absence or presence of purified $(His)_6$-CoREST1. As shown in Figure 2, LSD1 did not bind to SUMO-2 directly. However, LSD1 was retained by the GST-SUMO-2 resin in the presence of CoREST1. These data suggest that SUMO-2 binding by CoREST1 could be the mechanism underlying the observed association of LSD1 with SUMO-2 *in vivo* (Supplemental Figure 1).

Mapping the SUMO-2 binding domain in CoREST1

In order to map the SUMO-2 binding domain in CoREST1, we generated a series of CoREST1 truncation mutants (Figure 3A). Results from *in vitro* binding assays performed with recombinant $(His)_6$-tagged CoREST1 deletions and GST-SUMO-2 indicated that the region between amino acids 241-300 of CoREST1, which includes part of the first coiled-coil region, is important for binding to SUMO-2 (Figure 3B and Supplemental Figure 2). As shown in Figure 3B, an amino terminal fragment of CoREST1, residues 1-300, bound SUMO-2 as well as wild type (WT) CoREST1, whereas truncations 1-240 or 301-482 did not bind to GST-SUMO-2 although the C-terminal fragment 301-482 bound to GST-LSD1 perfectly well. As summarized in Figure 3A, all of the truncated CoREST1 derivatives lacking amino acids 241-300 failed to bind to GST-SUMO-2 *in vitro.* A small fragment encompassing this region, 237-300, was not sufficient to bind SUMO-2 under these conditions. Truncated forms of CoREST1 that failed to bind to GST-SUMO-2 *in vitro* also had decreased binding to SUMO-2/3 conjugates *in vivo* (data not shown). Based on these results, we conclude that CoREST1 residues 241-300 are necessary, but not sufficient, for binding SUMO-2.

To further localize the SUMO-2 interaction motif in CoREST1, we made internal deletions of CoREST1 within the region essential for binding, 241-300. The consensus SUMO interaction motif (SIM) has key hydrophobic residues often flanked by acidic residues (Hecker et al., 2006; Song et al., 2004). Although there are predicted consensus SIMs in the C-terminus of CoREST1, our deletion analyses revealed that these are not important for SUMO-2 binding. Nonetheless, guided by previous studies, we made small deletions within the region 241-300, including deletions of an acidic stretch (aa 255-263), a hydrophobic patch (aa 270-275) and the sequence in between (aa 264-270) (Figure 4B). Individual deletion of these small regions significantly reduced, but did not eliminate, CoREST1 binding to SUMO-2 *in vitro* (Figure 3C). Strikingly, deletion of all three regions together (d255-275) led to complete abrogation of SUMO-2 binding activity of CoREST1, although binding to GST-LSD1 was not affected. Thus, deletion analysis reveals that CoREST1 amino acids 255-275 are required for binding to SUMO-2 (Figure 3C).

An unusual SUMO-2-specific interaction motif in CoREST1

A close homolog of CoREST1, CoREST3, is also a component of the LSD1/CoREST1/HDAC complex (Shi et al., 2005) and CoREST3 was associated with GST-SUMO-2 in our affinity chromatography assays (Supplemental Table 1). Interestingly, we found that CoREST3 did not bind to SUMO-2 *in vitro*, although CoREST3 bound well to GST-LSD1 (Figure 4A). These findings raise the possibility that CoREST3, like CoREST1, may regulate LSD1 demethylase activity; differential binding to SUMO-2, however, supports distinct biological functions of CoREST1 and CoREST3.

We compared the sequences of CoREST1 and CoREST3 in the region required for SUMO-2 binding of CoREST1 (amino acids 255-275). As shown in Figure 4B, the alignment reveals

that CoREST3 lacks hydrophobic residues conserved in CoREST1. Structural studies have revealed that the hydrophobic core (I/V-X-I/V-I/V or I/V-I/V-X-I/V) in the consensus SIM makes direct contact with SUMO (Baba et al., 2005; Reverter and Lima, 2005). Although the hydrophobic core in the CoREST1 SUMO-2 interaction motif is notably different from the consensus SIM, largely due to absence of a hydrophobic residue at position 4, we nonetheless reasoned that the hydrophobic residues could be important for direct binding of CoREST1 to SUMO-2. As shown in Figure 4C, mutation of three hydrophobic residues in CoREST1 (IIV) into alanines (AAA) resulted in very weak to no binding to SUMO-2 *in vitro*. Also, when the hydrophobic residues were replaced with the CoREST3 counterparts (NSY), binding to SUMO-2 was almost completely abolished. Neither the AAA nor NSY mutations affected binding to LSD1, consistent with the fact that the hydrophobic patch is distinct from the region of CoREST1 binding to LSD1 (Shi et al., 2005; Yang et al., 2006). These studies define a hydrophobic core in the CoREST1 SIM that is required for high affinity binding to SUMO-2.

Since CoREST1 binds specifically to SUMO-2 dependent on a region of CoREST1 that does not match the current SIM consensus, we wondered if this unusual SIM contributes to SUMO-2-specific binding in other proteins. We searched for proteins containing a small sequence pattern: I/V/L-D/E-I/V/L-D/E-I/V/L with N-terminal acidic residues. We tested 11 out of approximately 50 matches for direct binding to SUMO-2 *in vitro*. Using this approach we have identified three proteins, including FIP1L1 and RBBP4, that bound directly to SUMO-2 but not SUMO-1 *in vitro* (Figure 4D and 4E), although binding was notably weaker than for CoREST1. In contrast, UBC9 bound to SUMO-1 and SUMO-2 equally well (Figure 4E). Furthermore, when the hydrophobic residues in the predicted SIM were mutated to alanines, FIP1L1 and RBBP4 binding to SUMO-2 was greatly reduced (Figure 4F). Thus, these studies reveal an unusual type of SIM that mediates SUMO-2-specific binding in several proteins.

SUMO-2/3 de-conjugation leads to de-repression of specific LSD1/CoREST1 target genes

Based on our identification of direct CoREST1/SUMO-2 interactions, we hypothesized that repression of some CoREST1 target genes is SUMO-dependent. We therefore examined expression of known CoREST1/LSD1 target genes upon depletion of CoREST1, LSD1 or SUMO-2/3 conjugates. We first generated CoREST1 stable knockdown HeLa cells (Figure 5A). Consistent with what has been reported previously (Shi et al., 2005), LSD1 protein levels also decreased upon CoREST1 knockdown. We then confirmed by RT-qPCR (Figure 5B), that the known CoREST1/LSD1 target genes SCN1A, SCN2A2 and SCN3A were up-regulated in both CoREST1 and LSD1 stable knockdown cells.

We next determined if expression of these CoREST1/LSD1 target genes was sensitive to depletion of SUMO-2/3 conjugates. To this end, we co-transfected cells with a plasmid expressing a truncated form of the SUMO-2/3-specific protease SENP3 (3xFLAG-SENP3-dN), a catalytically inactive mutant SENP3 (3xFLAG-SENP3-dN-C/S) or control vector. Overexpression of active SENP3 led to a decrease of bulk SUMO-2/3 conjugates, while levels of SUMO-1 conjugates were barely affected (Figure 5C). Examination of mRNA levels by RT-qPCR revealed that both SCN1A and SCN3A transcripts were up-regulated when SENP3 was overexpressed (Figure 5D). In contrast, SCN2A2 transcript level was not affected by SENP3 overexpression. These data reveal that de-conjugation of SUMO-2/3 led to transcriptional activation of some, but not all, CoREST1/LSD1 target genes indicating that SUMO-2/3 and the CoREST1/LSD1 complex are both required for transcriptional repression in some contexts.

De-conjugation of SUMO-2/3 reduces CoREST1 and LSD1 occupancy of specific promoters

We then analysed occupancy of the SCN1A, SCN2A, and SCN3A promoters by chromatin immunoprecipitation (ChIP). Under repressed conditions, all three promoters were occupied by both CoREST1 and LSD1, as expected. SUMO-2/3 was also found to ChIP to the SCN1A and SCN3A promoters, while the SCN2A2 promoter was not occupied by SUMO-2/3 (Figure 6A and 6B). When SENP3 was overexpressed, SUMO-2/3 occupancy was decreased on the SCN1A and SCN3A promoters, consistent with the removal of SUMO-2/3 conjugates. Notably, overexpression of SENP3 led to reduced binding of CoREST1 and LSD1 at the SCN1A and SCN3A promoters, although binding of these factors to the SCN2A2 promoter was unaffected (Figure 6C). Moreover, when SENP3 was overexpressed, there was an increase in both AcH3 and diMeH3K4 levels on the SCN1A and SCN3A, but not the SCN2A2, promoters (Figure 6C). Thus, there is a strong correlation between SUMO-2/3 occupancy and transcriptional repression of the SCN1A and SCN3A, promoters. Furthermore, SUMO-2/3 deconjugation was associated with loss of CoREST1/LSD1 occupancy and reduced activities of both HDAC and LSD1 at these promoters. Importantly, overexpression of SENP3 did not affect integrity of the LSD1/CoREST1/HDAC core complex (Supplemental Figure 4). Thus, SUMO contributes to stable binding and activity of the LSD1/CoREST1/HDAC complex on specific promoters.

The SUMO-2 interaction motif in CoREST1 is essential for repression of specific target genes

Our finding that SUMO-2/3 and the LSD1/CoREST1/HDAC complex together contribute to transcriptional repression in some contexts, prompted us to address whether the SUMO-2 interaction motif in CoREST1 is required for transcriptional repression and promoter binding of SCN1A and SCN3A. We generated RNAi-rescue constructs resistant to the CoREST1 RNAi (Supplemental Figure 5), encoding either CoREST1 WT or the AAA mutant that failed to bind SUMO-2 *in vitro* (Figure 4). A schematic of the CoREST1 rescue constructs is shown in Figure 7A. As expected, knockdown of endogenous CoREST1 led to de-repression and expression of CoREST1-WT-res restored repression of SCN1A, SCN2A2 and SCN3A (Figure 7B). CoREST1-AAA-res also restored repression of SCN2A2, but the CoREST1-AAA mutant failed to repress transcription of SCN1A or SCN3A (Figure 7B). ChIP studies revealed reduced binding of CoREST1-AAA to the SCN1A and SCN3A promoters, although the SCN2A2 promoter was fully bound (Figure 7C). These data indicate that stable binding of CoREST1 and transcriptional repression of the SCN1A and SCCN3A genes are dependent on an intact CoREST1 SIM. The findings presented here support a model (Figure 7D, right panel) in which recruitment of the LSD1/CoREST1/HDAC co-repressor complex to some promoters depends on direct binding of the CoREST1 SIM to SUMO-2/3 conjugated to a transcription factor (TF) or other protein at the promoter. Thus, SUMO contributes to promoter-specific occupancy and repression by the LSD1/CoREST1/HDAC complex and this co-repressor complex contributes to SUMO-dependent repression of some target genes.

Discussion

Our studies have revealed that SUMO-2/3 contributes to gene-specific binding and activity of the LSD1/CoREST1/HDAC repressor complex. We have identified a direct, non-covalent interaction between CoREST1 and SUMO-2/3. Notably, our studies have revealed an unusual non-consensus SUMO interaction motif (SIM) required for SUMO-2-specific binding by CoREST1 and at least two other proteins. De-conjugation of SUMO-2/3 or mutation in the CoREST1 SIM led to loss of promoter occupancy and transcriptional de-repression of SCN1A and SCN3A. These effects were specific as promoter binding and repression of another CoREST1/LSD1 target gene, SCN2A2 was not dependent on SUMO-2/3 conjugation or the CoREST1 SIM. Furthermore, consistent with previous biochemical studies, the integrity of the LSD1/CoREST1/HDAC co-repressor core complex was unaffected by SUMO-2/3

deconjugation (Supplemental Figure 4). Thus, we have identified a direct interaction with SUMO-2/3 that supports SUMO-dependent changes in chromatin structure and gene expression important for silencing neuronal genes in non-neuronal cells.

We observed SUMO-2/3-dependent changes in histone acetylation and methylation at the SCN1A and SCN3A promoters suggesting that both histone modification enzymes, HDAC and LSD1, are recruited in a SUMO-dependent manner (Figure 6). We have shown that CoREST1 can bridge LSD1 to SUMO-2 *in vitro* (Figure 2) and LSD1 was recruited to SCN1A and SCN3A promoters in a SUMO-dependent manner *in vivo* (Figure 6). We have not observed direct binding of recombinant HDAC1 to SUMO-2 and we were unable to establish direct binding of HDAC1 to CoREST1 *in vitro* (data not shown), although it is known that these proteins associate *in vivo* (Supplemental Figure 4 and Gu et al., 2005). Other factors in the LSD1/CoREST1/HDAC co-repressor complex may bridge HDAC1 to SUMO either directly or via CoREST1. In this regard, it is interesting to note that two other components of the LSD1/CoREST1/HDAC complex, RBBP4 and ZnF198, also bind non-covalently to SUMO (Figure 4 and Hecker et al., 2006). A role for ZNF198 binding to SUMO-modified HDAC1 in regulation of LSD1/CoREST1/HDAC complex activity has been suggested (Gocke and Yu, 2008). RBBP4 is a component of the core HDAC1,2 complex (Humphrey et al., 2001) and our data raise the possibility that RBBP4/SUMO-2 interactions may regulate HDAC activity or localization beyond the LSD1/CoREST1/HDAC complex.

SUMO-2/3 dependent and independent recruitment of CoREST1/LSD1 co-repressor complex

Our studies have revealed a novel mechanism that contributes to promoter-specific recruitment and activity of the LSD1/CoREST1/HDAC repressor complex. In the most well understood case, direct interactions between CoREST1 and REST support recruitment of LSD1/CoREST1/HDAC to multiple REST target genes such as SYN1 (Hakimi et al., 2002; Lan et al., 2007) and SCN2A2 (Ballas et al., 2005; Lunyak et al., 2002; Shi et al., 2004; Shi et al., 2005), both of which contain REST binding sites in their promoters. This recruitment leads to H3K4 demethylation, histone deacetylation and transcriptional repression in non-neuronal cells, where REST is expressed. Our data indicate that CoREST1/LSD1 occupancy and repression of SCN2A2 was not SUMO-2-dependent (Figures 5 and 6), consistent with the view that REST-dependent recruitment of the LSD1/CoREST1/HDAC complex is largely SUMO-2/3 independent.

In contrast, we have found that promoter occupancy and transcriptional repression of the CoREST1/LSD1 target genes SCN1A and SCN3A are SUMO-2/3 modification dependent. Overexpression of the SUMO-specific protease SENP3 led to reduced binding of CoREST1 and LSD1, increased histone H3K4 methylation and increased H3 acetylation at the SCN1A and SCN3A promoters (Figure 6). Although SCN1A and SCN3A are neuronal specific sodium channel genes, these genes are not known to be Pregulated by REST and lack REST binding sites in their promoters (Bruce et al., 2004). Interestingly, the histone demethylase SMCX is required for repression of SCN2A2, but not SCN1A or SCN3A, further indicating that distinct repression mechanisms are in play despite the fact that th ese are all CoREST1/LSD1 target genes (Tahiliani et al., 2007). Thus, SUMO-dependent and SUMO-independent mechanisms exist for recruitment of the LSD1/CoREST1/HDAC co-repressor complex to specific genes (Figure 7D).

Our data support the model that SUMO-2/3-modification of a factor, distinct from REST, is required for CoREST1 binding and subsequent repression at promoters such as SCN1A and SCN3A (Figure 7). SUMO-2/3 modified proteins that contribute to recruitment of CoREST1 at specific promoters are currently unknown. Many transcription factors, co-factors and chromatin associated proteins have been shown to be post-translationally modified by SUMO. Notably, several subunits of the LSD1/CoREST1/HDAC complex including HDAC1, LSD1,

ZnF198 and CtBP have been reported to be SUMOylated (David et al., 2002;Gocke et al., 2005;Kagey et al., 2003;Kunapuli et al., 2006). We have not observed SUMOylation of CoREST1 in our studies, although a recent report indicated SUMO modification of CoREST1 when co-expressed with SUMO-1 (Muraoka et al., 2008). SUMO-modification of LSD1/CoREST1/HDAC complex components is unlikely to affect integrity of the complex (Supplemental Figure 4) but could contribute to promoter-specific repression. An important feature of SUMOylation is that it is reversible and therefore SUMO-dependent recruitment of LSD1/CoREST1/HDAC provides a possible mechanism for signal-dependent regulation of specific gene expression programs.

An unusual SIM mediates SUMO-paralog specific binding of CoREST1

SUMO exerts many of its biological functions through non-covalent interactions with proteins bearing SIMs. Although many structurally distinct ubiquitin binding motifs have been identified, only one type of SIM has been described to date. The consensus SIM consists of a hydrophobic core (I/V-X-I/V-I/V or I/V-I/V-X-I/V) generally flanked by acidic residues and makes direct contact with a small surface in SUMO (Baba et al., 2005; Hecker et al., 2006; Reverter and Lima, 2005; Song et al., 2004). SUMO-1 and SUMO-2/3 which are about 45% identical, have distinct as well as overlapping functions (Ayaydin and Dasso, 2004; Saitoh and Hinchey, 2000; Vertegaal et al., 2006). SUMO binding proteins that bind preferentially to SUMO-1 or SUMO-2/3 have been described and could contribute to functional differences between the SUMO paralogs. With only a single type of SIM described to date, however, it is not clear how SUMO binding proteins discriminate between SUMO paralogs, although acidic residues flanking the hydrophobic core have been suggested to contribute to the affinity and/or SUMO-paralog specificity of binding (Hecker et al., 2006; Meulmeester et al., 2008).

We have found that CoREST1 binds to SUMO-2 *in vitro* and SUMO-2/3 conjugates *in vivo*, but no binding to SUMO-1 was detected. We have mapped a SUMO-2 interaction motif in CoREST1 to a small region (255-275), distinct from the CoREST1 regions implicated in binding REST, BAF57, LSD1, or HDAC1 (Andres et al., 1999; Battaglioli et al., 2002; Gu et al., 2005; Shi et al., 2005; Yang et al., 2006). This SUMO-2 interaction motif has a hydrophobic core IDIEV, which is notably different from the current consensus, in particular due to the absence of a hydrophobic residue at position 4. Point mutations of hydrophobic residues in the SIM disrupted CoREST1 binding to SUMO-2, indicating that the hydrophobic core is an essential component of the SIM. In fact, we have used this motif to identify additional SUMO-2 binding proteins. Of 11 proteins with a motif similar to the CoREST1 SIM (I/V/L-D/E-I/V/L-D/E-I/V/L with N-terminal acidic residues) that were tested, 3, FIP1L1, RBBP4 and SND1, bound directly to SUMO-2 but not SUMO-1 *in vitro* (Figure 4E and Supplemental Figure 3), while the remainder did not bind to either SUMO-1 or SUMO-2. Mutational studies confirmed that SUMO-2 binding by FIP1L1 and RBBP4 required the region of homology to CoREST1 (Figure 4F) although binding by SND1 did not (Supplemental Figure 3). Although we have identified residues in the CoREST1 SIM required for SUMO-2 binding, small peptide fragments of CoREST1 containing the SIM were not sufficient to bind to SUMO-2 (Figure 3A and Supplemental Figure 2). Further studies will provide insights into the structural basis for SUMO-2/3-specific binding by the unusual SIM in CoREST1 and other proteins.

Multiple co-repressors contribute to SUMO-dependent regulation of chromatin structure and transcription

Our studies have identified the LSD1/CoREST1/HDAC co-repressor complex as an effector of SUMO-dependent repression. Our findings add to a growing body of work indicating that SUMOylation regulates gene expression by promoting changes in chromatin structure. Recent studies have identified multiple chromatin regulators that are recruited to promoters by SUMO-dependent mechanisms including HDAC2, the histone methyl transferases SETDB1 and

SUV4-20H, the ATP-dependent remodeler Mi2, and chromatin associated proteins HP1 and L3MBTL1 and 2 (Ivanov et al., 2007; Stielow et al., 2008a; Stielow et al., 2008b; Yang and Sharrocks, 2004). We have previously analysed repression by a Gal4-SUMO-2 fusion and although we observed SUMO-2-dependent H3K4 demethylation, neither LSD1 nor CoREST1 was required for repression of a reporter gene by GAL4-SUMO-2 (Rosendorff et al., 2006 and data not shown). Thus, we have proposed that in the context of the GAL4-SUMO-2 reporter gene assay, many different co-repressors may be recruited to the promoter, such that depleting any one of them has only subtle effects on gene expression (Valin and Gill, 2007). Studies of repression by SUMOylated Sp3 also suggest functional redundancy in SUMO-dependent repression mechanisms (Stielow et al., 2008a; Stielow et al., 2008b). Nonetheless, depletion of LSD1 or CoREST1 or mutation of the SUMO-2 binding motif in CoREST1 was sufficient to de-repress the SUMO-dependent SCN1A and SCN3A genes (Figures 5 and 7 and Shi et al., 2004; Shi et al., 2005). Thus, on endogenous promoters, SUMO-dependent regulation of co-repressor recruitment and/or function can be very specific. The recent identification of multiple effectors of SUMO-dependent repression highlights the need for further studies to understand the basis for context-dependent regulation of co-repressor activity by SUMO. Additional insights into SUMO-dependent repression mechanisms will illuminate the complex transcriptional mechanisms that support cell type-specific gene expression programs.

Experimental procedures

Cloning and DNA constructs

Coding sequences were cloned in bacterial and/or mammalian expression vectors. Gene truncations or point mutations were generated by PCR methods. See supplemental material for details.

Protein expression and *in vitro* protein binding assays

GST or 6xHis tagged proteins were expressed in BL21(DE3) and purified with Glutathione Sepharose-4B or Ni-NTA affinity beads, respectively. 1 or 5μg GST tagged proteins were mixed with 1μg $(His)_6$ tagged proteins in 0.5mL GST Pulldown Buffer (20mM Tris-HCl pH7.5, 100mM NaCl, 0.1mM EDTA, 0.1% Igepal CA-630, 5% Glycerol, 2mM DTT) and rotated at 4°C for 2 hour. After centrifugation, the supernatant was transferred to a new tube and Glutathione Sepharose-4B beads were added. After rotating at 4°C for 1 hour, the beads were washed extensively and the bound proteins were eluted with SDS sample buffer and subjected to Western Blot analysis. The PVDF membrane was then stained with Coomassie.

Cell culture, transfection, stable cell generation and immunoprecipitation

HeLa and HEK293T cells were transfected with Lipofectamine-2000 (Invitrogen). Retrovirus produced in HEK293T cells was used to infect HeLa cells to generate cells stably expressing the indicated shRNAs and/or cDNAs. NEM was included in the buffers for cell lysis and immunoprecipitation. See supplemental material for details.

Reverse transcription, quantitative PCR and Chromatin Immunoprecipation Assay

Total RNA was extracted from cultured cells using RNeasy Mini Kit (Qiagen). 5μg total RNA was used for reverse transcription using SuperScript® III Reverse Transcriptase (Invitrogen). Relative mRNA levels were determined by SYBR Green based quantitative PCR and normalized to GAPDH. Chromatin Immunoprecipation (ChIP) was performed as described previously with small modifications (Shi et al., 2004). Details and primer sequences can be found in supplemental material.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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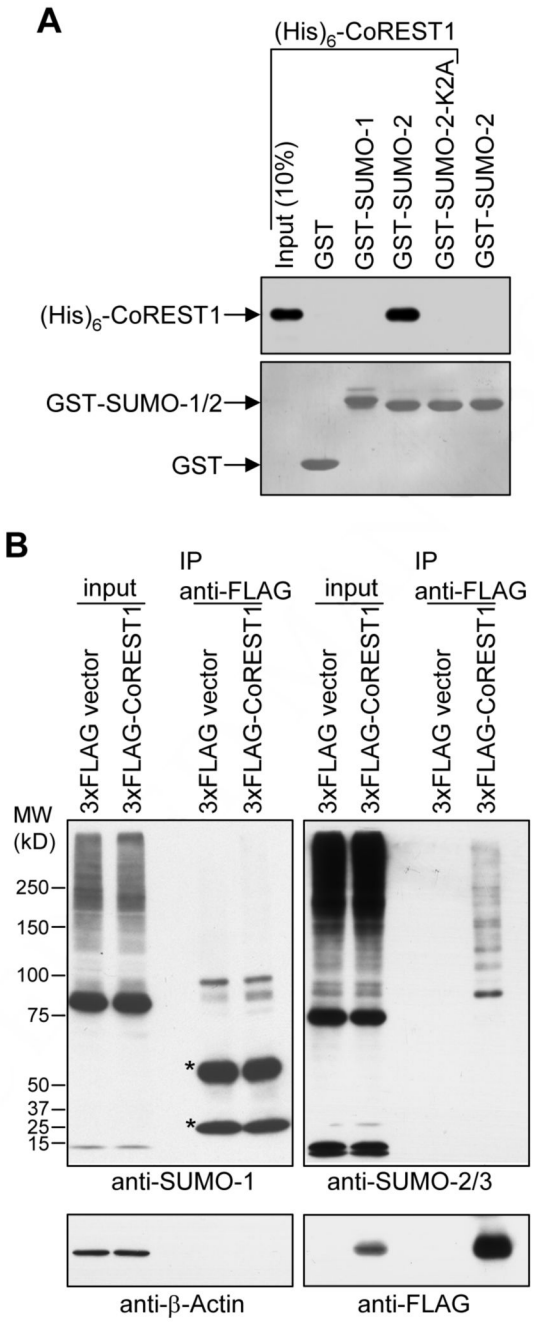

Figure 1.

CoREST1 binds to SUMO-2

(A) CoREST1 binds to SUMO-2 directly and non-covalently *in vitro*.

Purified recombinant $(His)_6$-CoREST1 was mixed with GST, GST-SUMO-1, GST-SUMO-2, or GST-SUMO-2-K2A (K33A, K35A). Proteins retained on Glutathione Sepharose beads were visualized using anti-6xHis antibody to detect $(His)_6$-CoREST1 or Coomassie staining to detect GST or GST tagged SUMO variants.

(B) Cell lysates from HEK293T cells transfected with 3xFLAG-CoREST1 or 3xFLAG vector were immunoprecipitated with anti-FLAG Agarose beads. Bound proteins were subjected to Western Blot (WB) analyses with antisera to SUMO-1 or SUMO-2/3, as indicated. Anti-FLAG

WB shows the expression of 3xFLAG-CoREST1 and the anti-β-Actin WB indicates equal loading. Asterisks indicate positions of IgG heavy and light chains. 4% of input material was loaded.

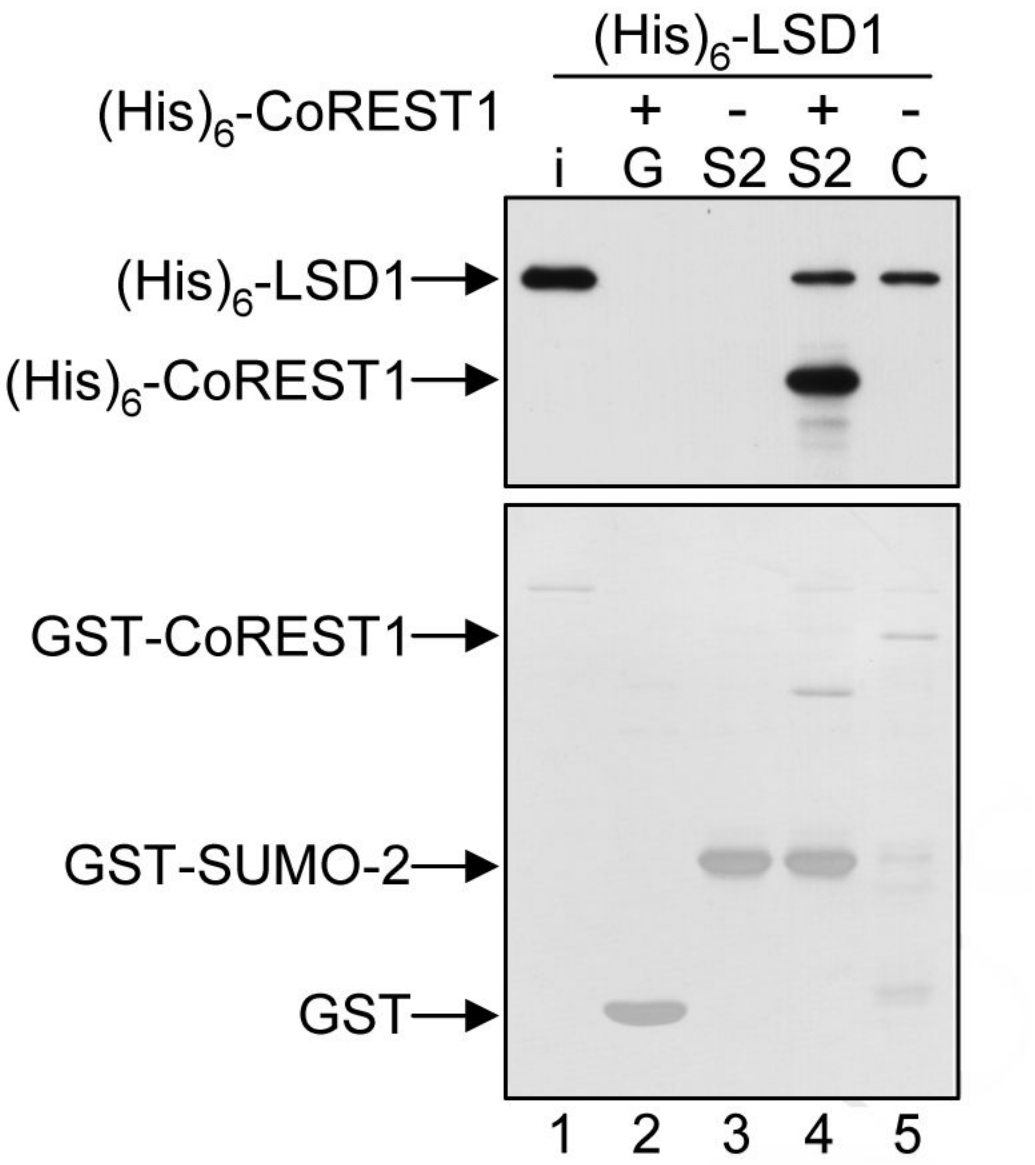

Figure 2.

CoREST1 bridges LSD1 binding to SUMO-2

Purified recombinant $(His)_6$-LSD1 was incubated with GST (G), GST-SUMO-2 (S2) or GST-CoREST1 (C). Purified recombinant $(His)_6$-CoREST1 was included in the binding reaction where indicated (lanes 2 and 4). Proteins retained on Glutathione Sepharose beads were visualized using anti-6xHis antibody to detect $(His)_6$-LSD1 and $(His)_6$-CoREST1 or Coomassie staining to detect GST ,GST-SUMO-2 or GST-CoREST1. Input=10%.

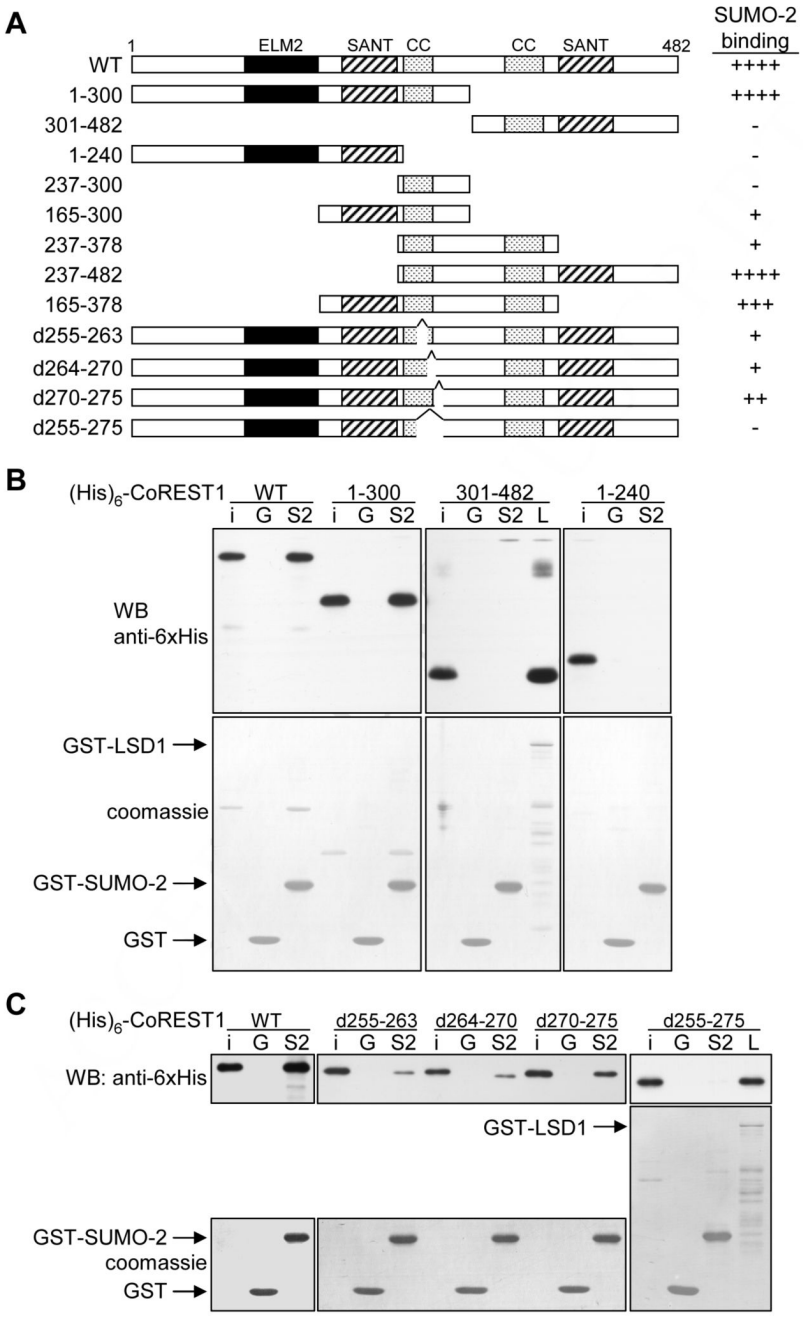

Figure 3.

Mapping the SUMO-2 interaction domain in CoREST1

(A) Summary of SUMO-2 binding by CoREST1 deletion mutants.

Relative affinity of SUMO-2 binding by CoREST1 deletion mutants is indicated on the right based on *in vitro* protein binding assays in Figures 3B, 3C and Supplemental Figure 2. The positions of indicated CoREST1 domains are: ELM2, 100-164; first SANT domain, 188-236; first Coiled Coil (CC), 241-268; second CC, 331-366; second SANT domain, 379-427.

(B) Representative binding of CoREST1 deletions to SUMO-2 *in vitro*.

$(His)_6$ tagged CoREST1 full length (WT) or truncated proteins bearing the indicated amino acids were incubated with GST (G), GST-SUMO-2 (S2), or GST-LSD1 (L). Bound proteins

were visualized by WB with anti-6xHis antibody (top) or Coomassie staining (bottom). Input=10%.

(C) CoREST1 residues 255-275 are required for binding to SUMO-2.

Deletions of CoREST1 acidic stretch (d255-263), linker (d264-270), hydrophobic core (d270-d275) or a combined deletion (d255-275) were assayed for binding to GST-SUMO-2 as described above.

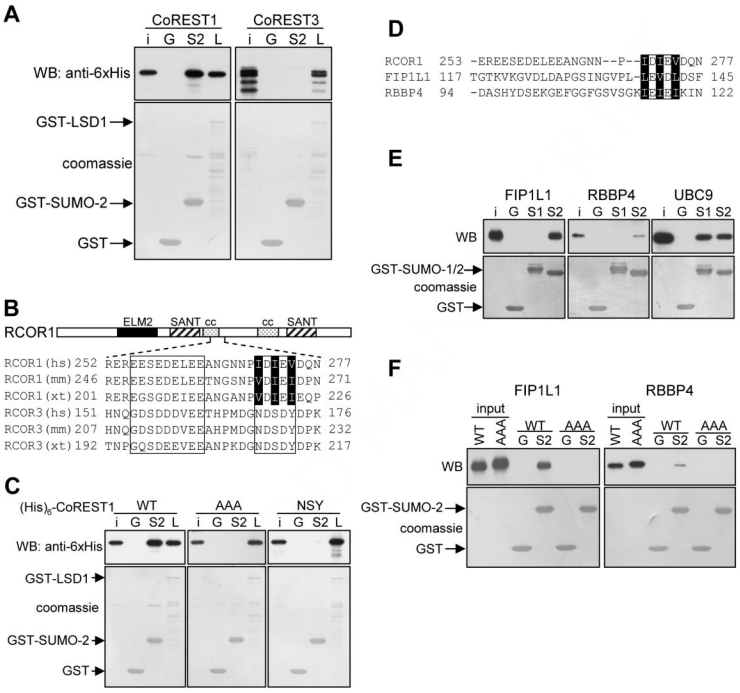

Figure 4.

An unusual SUMO-2 interaction motif in CoREST1

(A) CoREST3 binds to LSD1 but does not bind to SUMO-2 *in vitro*.

Purified recombinant $(His)_6$-CoREST1 or $(His)_6$-CoREST3 were assayed for binding to GST (G), GST-SUMO-2 (S2), or GST-LSD1 (L) as described in Figure 3.

(B) Sequence alignment of a region of CoREST1 (RCOR1) and CoREST3 (RCOR3) proteins from *Homo sapiens* (hs), *Mus musculus* (mm), and *Xenopus tropicalis* (xt). Acidic stretch and hydrophobic core of CoREST1 are boxed. CoREST1 hydrophobic residues (IIV) mutated in further assays are shaded.

(C) Purified recombinant wild type CoREST1 (WT) or the indicated mutants (IIV to AAA or IIV to NSY) were assayed for binding to GST (G), GST-SUMO-2 (S2), or GST-LSD1 (L) as described in Figure 3.

(D) Sequence alignment of the indicated regions of RCOR1, FIP1L1 and RBBP4. Hydrophobic residues mutated in further assays are shaded.

(E) Purified recombinant $(His)_6$-FIP1L1, $(His)_6$-RBBP4, and control $(His)_6$-UBC9 were assayed for binding to GST (G), GST-SUMO-1 (S1), or GST-SUMO-2 (S2) as described in Figure 1. Proteins were visualized by WB using anti-FIP1L1 or anti-6xHis antibodies (top) or Coomassie staining (bottom). Input=2% for FIP1L1 and RBBP4; 10% for UBC9.

(F) Purified recombinant $(His)_6$-FIP1L1 WT or AAA mutant (LVL to AAA), $(His)_6$-RBBP4 WT or AAA mutant (III to AAA) were assayed for binding to GST (G) or GST-SUMO-2 (S2) as described above. Input=2%.

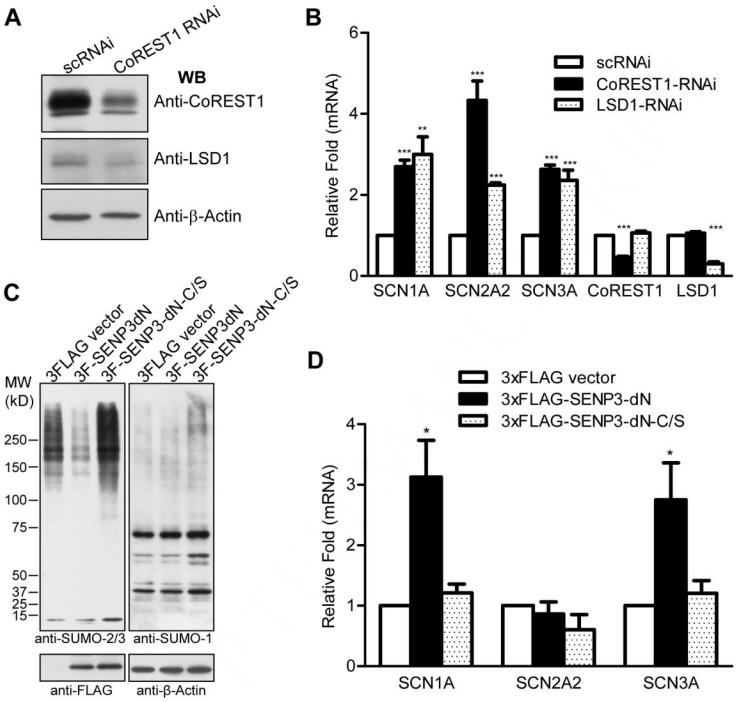

Figure 5.

Repression of some CoREST1 target genes is SUMO-2/3 dependent

(A) CoREST1 RNAi reduces CoREST1 levels.

Protein extracts prepared from HeLa cells stably expressing control scrambled RNAi (scRNAi) or CoREST1 RNAi were analyzed by WB with the indicated antibodies.

(B) De-repression of neuronal specific genes by RNAi-mediated knockdown of CoREST1 or LSD1.

RNA isolated from HeLa cells stably expressing control scRNAi, CoREST1 RNAi or LSD1 RNAi was subjected to RT-qPCR to examine the mRNAs levels of the CoREST1/LSD1 target genes SCN1A, SCN2A2 and SCN3A. Error bars of SEMs were generated from multiple experiments ($n \geq 2$). Asterisks denote statistically significant values relative to scRNAi (t test, $^{**}p<0.05$, $^{***}p<0.01$).

(C) SUMO-2/3 de-conjugation by SUMO-specific protease SENP3 overexpression.

Total protein lysates from HeLa cells transfected with 3xFLAG-SENP3-dN, catalytically inactive 3xFLAG-SENP3-dN-C/S or 3xFLAG vector were analyzed with antisera to SUMO-1 or SUMO-2/3, as indicated. Anti-FLAG WB shows the expression of 3xFLAG-SENP3 and the anti-β-Actin WB indicates equal loading.

(D) Depletion of SUMO-2/3 conjugates leads to de-repression of SCN1A and SCN3A but not SCN2A2.

SCN1A, SCN2A2, and SCN3A mRNA levels were determined by RT-qPCR from HeLa cells transfected with 3xFLAG-SENP3-dN, 3xFLAG-SENP3-dN-C/S or 3xFLAG vector. Error bars of SEMs were generated from multiple experiments ($n \geq 4$). One-way ANOVA analyses showed that the SCN1A and SCN3A transcript levels in 3xFLAG-SENP3-dN over-expression cells were significantly higher than those over-expressing catalytically inactive 3xFLAG-SENP3-dN-C/S or 3xFLAG vector ($^{*}p<0.1$).

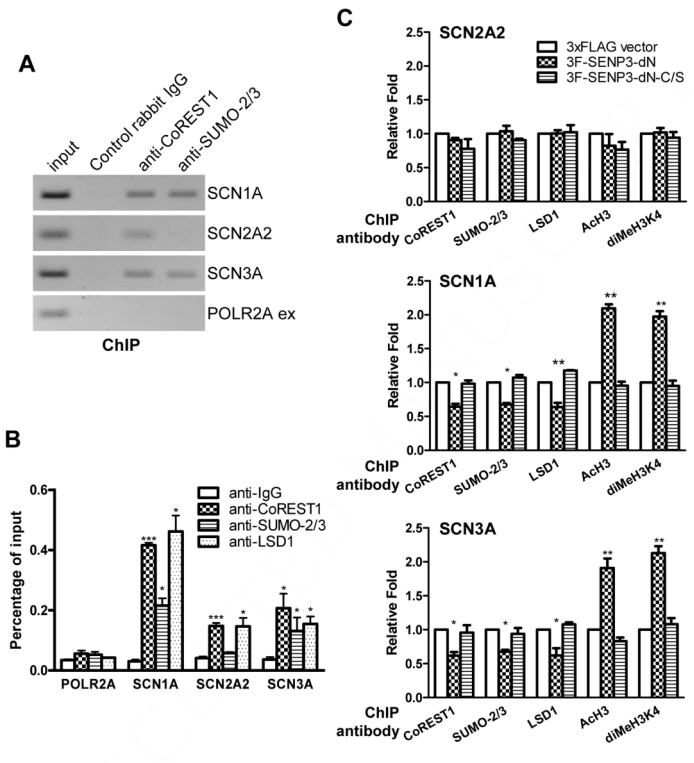

Figure 6.

SUMO-2/3 regulates CoREST1/LSD1 occupancy and histone modification status at specific promoters

(A) SCN1A and SCN3A promoters are occupied by both CoREST1 and SUMO-2/3 while SCN2A2 promoter is occupied by CoREST1 but not SUMO-2/3 in HeLa cells.

Chromatin immunoprecipitation (ChIP) assays were performed using anti-CoREST1, anti-SUMO-2/3 or control IgG and PCR was performed with primers specific for SCN1A, SCN2A2 and SCN3A promoters or RNA polymerase II polypeptide A exon (POLR2A ex). Input=1%.

(B) Quantification of CoREST1, LSD1 and SUMO-2/3 occupancy on the SCN1A, SCN2A2 and SCN3A promoters by ChIP-qPCR. Error bars of SEMs were generated from multiple experiments ($n \geq 2$). Asterisks denote statistically significant occupancy of the protein at tested promoters relative to occupancy at POLR2A exon (t test, *$p<0.1$, **$p<0.05$, ***$p<0.01$).

(C) Over-expression of 3xFLAG-SENP3-dN is coupled with dissociation of SUMO-2/3, CoREST1 and LSD1 and elevated levels of acetylated histone 3 (AcH3) and di-methylated histone 3 lysine 4 (diMeH3K4) at SCN1A and SCN3A.

HeLa cells transfected with 3xFLAG-SENP3-dN, 3xFLAG-SENP3-dN-C/S or 3xFLAG vector were subject to ChIP assays with the indicated antibodies. Relative fold values indicate occupancies relative to 3xFLAG vector set at 1. Error bars of SEMs were generated from duplicate experiments. Asterisks denote statistically significant values in 3xFLAG-SENP3-dN over-expression samples compared to 3xFLAG-SENP3-dN-C/S or 3xFLAG vector samples by one-way ANOVA analysis. (*$p<0.1$, **$p<0.05$, ***$p<0.01$).

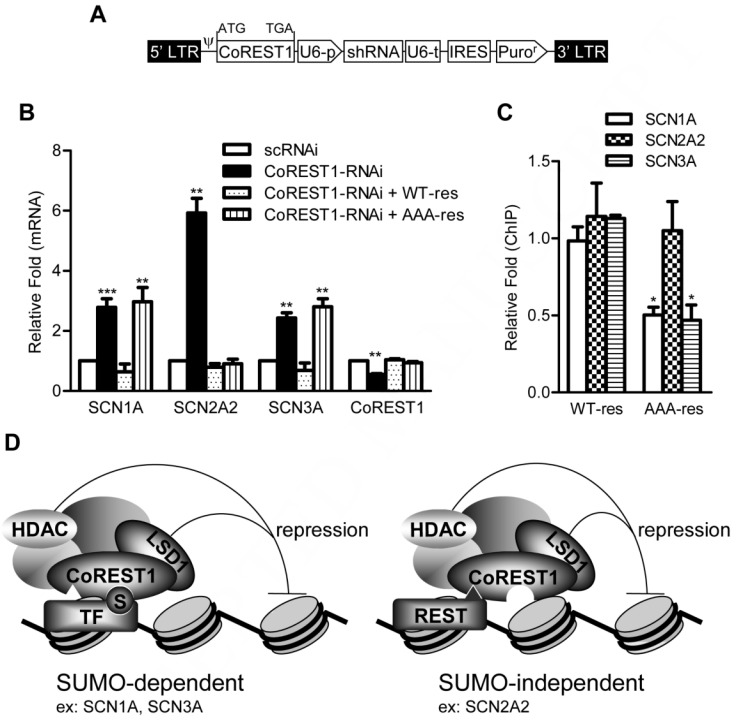

Figure 7.

SUMO-2 interacting motif in CoREST1 is required for transcriptional repression of SCN1A and SCN3A

(A) Schematic map of rescue plasmid construction.

cDNA encoding CoREST1-WT or CoREST1-AAA resistant to CoREST1-RNAi, and a U6 promoter (U6-p) driven CoREST1 shRNA cassette were cloned in a pMSCV based retroviral vector. Expression of puromycin resistance gene (Puro^r) was controlled by an internal ribosomal entry sequence (IRES). U6-t, RNA polym Perase III transcription terminator.

(B) CoREST1-mediated repression of SCN1A and SCN3A depends on its SUMO-2 interacting motif.

mRNA was isolated from HeLa cells stably expressing the indicated control (scRNAi) or CoREST1 RNAi alone or with CoREST1-RNAi resistant WT (WT-res) or AAA (AAA-res) and levels of the SCN1A, SCN2A2, SCN3A and CoREST1 transcripts were determined by RT-qPCR. Error bars of SEMs were generated from multiple experiments ($n \geq 3$). Asterisks denote statistically significant values relative to scRNAi (t test, $^{**}p<0.05$, $^{***}p<0.01$).

(C) Recruitment of CoREST1 to SCN1A and SCN3A, but not SCN2A2, promoters depends on its SUMO-2 interaction motif.

Anti-CoREST1 ChIP analyses were performed using CoREST1 WT rescue (WT-res) or mutant rescue (AAA-res) stable HeLa cells and quantitated by qPCR. Fold change relative to control HeLa cells is indicated. Error bars of SEMs were generated from duplicate experiments. Asterisks denote statistically significant values relative to WT-res (t test, $^{*}p<0.1$).

(D) Model illustrating SUMO-2/3-dependent (left panel) and SUMO-independent (right panel), promoter-specific transcriptional repression by the LSD1/CoREST1/HDAC co-repressor complex. On the left, the CoREST1 SUMO interaction motif binds a SUMO-2/3 (S) modified transcription factor (TF) or chromatin associated protein. On the right, a distinct region of CoREST1 is shown binding to REST, as an example of a SUMO-independent mechanism of recruitment.