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A Conserved 12-Amino Acid Motif in Sall1 Recruits the Nucleosome Remodeling and Deacetylase Corepressor Complex*

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Sall1 is a multi-zinc finger transcription factor that represses gene expression and regulates organogenesis. In this report, we further characterize the domain of Sall1 necessary for repression. We show that endogenous Sall1 binds to the nucleosome remodeling and deacetylase corepressor complex (NuRD) and confirm the functionality of the Sall1-associating macromolecular complex by showing that the complex possesses HDAC activity. NuRD is involved in global transcriptional repression and regulation of specific developmental processes. The mechanism by which sequence-specific DNA-binding proteins associate with NuRD is not well understood. We have identified a highly conserved 12-amino acid motif in the transcription factor Sall1 that is sufficient for the recruitment of NuRD. Single amino acid substitutions defined the critical amino acid peptide motif as RRKQ*X*K-P*XX*F. This motif probably exhibits a more general role in regulating gene expression, since other proteins containing this domain, including all Sall family members and an unrelated zinc finger protein Ebfaz, mediate transcriptional repression and associate with NuRD. These results also have important implications for the pathogenesis of Townes-Brocks, a syndrome caused by SALL1 mutations.

It is well established that changes in chromatin structure are associated with activation and silencing of gene expression. The packaging of DNA in the nucleosome acts to inhibit the accessibility of DNA to transcriptional regulators and the molecular machinery required for gene expression. Gaining accessibility to DNA relies on two major mechanisms that include ATP-dependent chromatin remodeling and multiple types of modifications of nucleosomal histones (1–3). Two well described macromoleclar complexes, NuRD[2] and Sin3 (reviewed in Ref. 4), are instrumental in facilitating these enzymatic activities to establish transcriptional repression.

The NuRD complex is distinguished by its ability to exhibit both histone deacetylase and ATP-dependent nucleosome remodeling activity. NuRD has been purified from mammalian and *Xenopus* cells and is ~2 MDa in size (5–9). The mammalian complex is composed of at least eight polypeptides. The histone deacetylase proteins, HDAC1 and HDAC2, and two associated proteins, RbAp46 and RbAp48, are core components that are common to both the NuRD and Sin3 repression complexes. In addition to histone deacetylase activity, the NuRD complex has ATP-dependent nucleosome remodeling activity because of its association with the ATPase, Mi-2. The biochemical functions of the remaining NuRD-specific components, the methyl-CpG-binding protein, MBD3, and MTA1 and MTA2 are not well defined (reviewed in Ref. 4).

NuRD is widely conserved across the animal and plant kingdoms and has been shown to play a critical role in regulating gene expression during embryonic development (reviewed in Ref. 4). NuRD has been shown to inhibit Ras signaling during vulval development through effects on both the synMuvA and synMuvB pathways in *Caenorhabditis elegans* (10–15). In *Drosophila*, the sequence-specific transcription factor, Hunchback, has been found to interact with dMi-2 and, together with polycomb proteins, mediates repression of homeotic genes (16). In mammals, the Hunchback ortholog, Ikaros, recruits NuRD and regulates lymphocyte differentiation (17). Genetic studies in *Drosophila* indicate that NuRD modulates signaling in two other vital developmental pathways, *wingless* and *ecdysone* (18–21). These defined roles of NuRD indicate the importance of this complex in facilitating transcriptional repression through its association with sequence-specific DNA-binding proteins. However, the mechanism by which transcription factors recruit NuRD to regulate gene expression is not well understood.

Members of the *spalt* (S*al*) gene family encode for zinc finger transcription factors that repress gene expression and regulate organogenesis. Genetic evidence in *Drosophila* suggests that *spalt* and *spalt-related* repress downstream genes, *trachaeless* (*trh*), *ventral veinless* (*vvl*), and *knirps* (*kni*) in the developing trachea (22), *rhodopsin 1* (*rh1*) in differentiating photoreceptors (23), and *iriquois* (*iro*) and *knirps* (*kni*) during development of longitudinal veins (24). This genetic evidence is further augmented in *C. elegans*, where the Spalt ortholog, Sem-4, was found to mediate direct repression of the *Hox* gene, *egl-5*, and the LIM homeobox gene, *mec-3*, thereby regulating touch cell fate (25). S*al* genes are also critical for mammalian organ devel-

opment, since mutations in human *SALL1* cause Townes-Brocks syndrome (TBS) and lead to multiple birth defects, including hearing loss, imperforate anus, limb defects, hypoplastic kidneys, and cardiac anomalies (26). A mouse model expressing a truncated Sall1 N-terminal protein recapitulates all of the TBS abnormalities (27). In contrast, a *Sall1* null allele exhibits only recessive renal anomalies (28). The similarities between mice carrying the S*all1ΔZn²⁻¹⁰* allele and TBS phenotypes indicate that these developmental abnormalities are caused by the expression of a truncated N-terminal Sall1 protein (27).

Functional analysis of the Sall1 N terminus has suggested possible molecular mechanisms for the dominant pathogenesis of TBS due to expression of a truncated Sall1 protein. The truncated Sall1 protein produced by the *Sall1ΔZn²⁻¹⁰* mutant allele has been shown to repress transcription via a histone deacetylase (HDAC) chromatin-remodeling complex. We have previously shown that the repression domain is contained within the first 136 amino acids of the N terminus (29). All SALL1 truncated mutant proteins that would be expressed from documented TBS patient mutations are postulated to contain this identified minimal repression domain. Therefore, the role of the Sall1 N-terminal repression domain has potential implications for understanding the pathogenesis of TBS.

In this report, we demonstrate the association of endogenous Sall1 with the NuRD complex, one of the major transcriptional corepressor complexes in mammalian cells. We identify a conserved 12-amino acid peptide motif in Sall1 that functions as an independent repressor module and is sufficient for the recruitment of all NuRD components and its associated histone deacetylase activity. We have revealed the importance of this motif for the repression function of the Sall family members and an unrelated C_2H_2 zinc finger transcription factor, Ebfaz, suggesting a potential mechanism by which the NuRD corepressor complex is recruited to target genes by sequence-specific DNA-binding proteins.

EXPERIMENTAL PROCEDURES

Plasmids—The GAL4DB and eukaryotic GST fusion expression plasmids have been described previously (29). The full-length Sall1 fusion (residues 1–1322) proteins were constructed using PCR and the following primers: 5′-AACGGATCCTCGCG-GAGGAAGCAAGCG-3′ with 5′-CGTGCGGCCGCTTAG-CTTGTGACGATCTCTTT-3′ or 5′-CGTTCTAGATTAGCT-TGTGACGATCTCTTT-3′ for GST and BXG fusions, respectively. The GAL4DB expression constructs for N-terminal Sall1 mutants were generated using the following primer: N-(2–435) (5′-GCGTCTAGATTAGACATTTGGTGGCTTGCTTTT and 5′-ATAGGATCCTATCGCGGAGGAAGCAAGCGAAG). The other N-terminal Sall1 mutants were created in the context of the minimal repression domain N-(1–136) and were created using the following primers: 5′-TAATTCTAGAGTGGTGGTGGTG-GTGGCAACTGGG with N-(1–136) (5′-ATAGGATCCTA-TCGCGGAGGAAGCAAGCGAAG), N- (12–136) (5′-ATAGG-ATCCCAATCCGACCCCGAAGTGGCC), and Sall2Alt-Sall1 chimera (2–12 Sall2Alt + 13–136 Sall1) (5′-AATGGATCCGCG-CAGGAAACCGGGAGCAGCTCTCGACTCGGAGGTTGA-TCCTGAAGTGGCCTCGCTTCCTCGGCGAGATGGTGAC-

ACA). A BXG-Sall1 construct consisting of the first 12 amino acids of Sall1 was created with the following primer: N-(1–12) (5′-GATCCTCGCGGAGGAAGCAAGCGAAGCCTCAAC-ATTTCCAAT and 5′-CTAGATTGGAAATGTTGAGGCTT-CGCTTGCTTCCTCCGCGAG). The same construct was created in pEBG using primer N-(1–12) (5′-GATCCTCGCGGA-GGAAGCAAGCGAAGCCTCAACATTTCCAAGC and 5′-GGCCGCTTGGAAATGTTGAGGCTTCGCTTGCTTCCT-CCGCGAG). The resulting PCR fragments were digested with BamHI and XbaI or BamHI and NotI and inserted into BXG or pEBG, respectively. The Sall1 site mutants were created in BXG and pEBG using the Stratagene QuikChange site-directed mutagenesis kit. The following mutagenic oligonucleotide primers were designed to substitute an alanine residue for amino acid residues 2–12 found in the N-terminal motif of Sall1: S2A (5′-CCAGGGGGATCCGCACGGAGGAAGCAA-GCGAAG and 5′-CTTCGCTTGCTTCCTCCGTGCGGATC-CCCCTGG), R3A (5′-CCGCGTGGATCCTCGGCCAGGAA-GCAAGCGAAG and 5′-CTTCGCTTGCTTCCTGGCCGA-GGATCCACGCGG), R4A (5′-GGATCCTCGCGGGCCAA-GCAAGCGAAG and 5′-CTTCGCTTGCTTGGCCCGCGA-GGATCC), K5A (5′-GGATCCTCGCGGAGGGCCCAAGC-GAAGCCTCAACATTTC and 5′-GAAATGTTGAGGCTT-CGCTTGGGCCCTCCGCGAGGATCC), Q6A (5′-GGATC-CTCGCGGAGGAAGGCGGCGAAGCCTCAACATTTC and 5′-GAAATGTTGAGGCTTCGCGCGCTTCCTCCGCGAG-GATCC), K8A (5′-CGGAGGAAGCAAGCGGCCCCTCAA-CATTTC and 5′-GAAATGTTGAGGGGCCGCTTGCTTC-CTCCG), P9A (5′-CGGAGGAAGCAAGCGAAGGCGCAA-CATTTCCAATCC and 5′-GGATTGGAAATGTTGGCGCT-TCGCTTGCTTCCTCCG), H11A (5′-CGAAGCCTCAAGC-CTTCCAATCCGACCCC and 5′-GGGGTCGGATTGGAA-GGCTTGAGGCTTCGC), F12A (5′-GCGAAGCCTCAACA-TGCGCAATCCGACCCC and 5′-GGGGTCGGATTGGCG-ATGTTGAGGCTTCGC). The N-terminal Ebfaz-(1–435) construct was made as a GAL4 fusion protein using the following primers: 5′-AATGGATCCTCCAGGAGAAAGCAGGC-GAAGCCACGAAGTGTG and 5′-ATTTCTAGATTAATG-AATCTCTAGCACAGCCAGGCTGGTAAAGTCCCG. The same N-terminal domain of Ebfaz was also made as a GST fusion protein using the same forward primer and the reverse primer 5′-ATTGCGGCCGCTTAATGAATCTCTAGCACA-GCCAGGCTGGTAAAGTCCCG. All constructs were verified by DNA sequencing and detection of the expressed fusion proteins using anti-GAL4DB or anti-GST Western blotting.

Reporter Assays—COS-1 cells were plated in 6-well plates at a density of 1×10^5 cells/well and transfected using FuGENE (Roche Applied Science) according to the manufacturer's directions. The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. For reporter assays, cells were transiently transfected with 1 μg of GAL4DB fusion plasmid, 2 μg of luciferase reporter plasmid, and 0.05 μg of cytomegalovirus-β-galactosidase control plasmid. Cells were harvested at 48 h in reporter lysis buffer (Promega). Lysates were assayed for luciferase and β-galactosidase activity using a Turner Biosystems luminometer according to the manufacturer's protocols with 15 and 2.5% of the total lysate, respectively (luciferase (BD

PharMingen); β-galactosidase (PE Biosystems)). Luciferase activity was normalized to β-galactosidase activity and divided by the average obtained for GAL4DB fusion plasmid alone to obtain -fold repression. Statistical significance of the site mutations was determined by an independent samples *t* test with a probability value of <0.05 taken to indicate significance.

Protein Interaction Assays—Transfected COS-1 cells were allowed to express GST-Sall1 fusion proteins or GST-Ebfaz-(1–435) for 48 h, washed with phosphate-buffered saline, and incubated for 1 h on ice in lysis buffer (1% Triton X-100, 200 mM sucrose, 50 mM Tris (pH 7.4), 150 mM NaCl, and protease inhibitors (1 µg/ml leupeptin, 2 µg/ml antipain, 10 µg/ml benzamidine, 1 µg/ml chymostatin, 1 µg/ml pepstatin, 24 µg/ml Pefabloc, 20 mM NaF, and 2 mM sodium molybdate). The cell suspension was disrupted (3 × 20 s) with a Fisher sonic dismembrator model 500 at 37% amplitude. The GST-Sall1 and GST-Ebfaz fusions and associated protein complexes were isolated by precipitation of 50 µg of total protein (or 100 µg for the Sall1-N-(1–12) GST fusion) with glutathione-Sepharose beads (Amersham Biosciences) for 2 h at 4 °C. GST fusions and Sall1-interacting proteins were separated by SDS-PAGE.

HDAC Activity Assays—Complexes associating with GST-Sall1 N-terminal fusions (1–435, 1–136, 1–12, Sall2AltSall1 chimera, and 11–136) were isolated as described above and were assayed for HDAC activity using the HDAC fluorescent activity assay/drug discovery kit (BIOMOL). This assay system allows detection of a flurophore upon deacetylation of a substrate with a histone-like sequence containing acetylated lysine side chains. GST-Sall1 fusions and associated protein complexes isolated on glutathione-Sepharose beads were incubated with 100 µM acetylated substrate in 100 µl of assay buffer. The reactions were incubated at 37 °C for 30 min. Aliquots (30 µl) were withdrawn and mixed with 20 µl of assay buffer and 50 µl of developer. The fluorescence was measured on a fluorometric plate reader with excitation set at 360 nm and emission detection set at 465 nm. HDAC activity was expressed as arbitrary fluorescence units. For inhibitor assays, the reactions were carried out in the presence of 100 nm trichostatin A (TSA).

Immunoprecipitations—P19 cells were maintained in α-modified Eagles' medium containing 7.5% bovine calf serum and 2.5% fetal bovine serum. P19 cells were plated in T-175 flasks, washed in phosphate-buffered saline, and harvested at ~90% confluence in 1 ml/10^7 cells in Nonidet P-40 lysis buffer (1% Triton X-100, 300 mM NaCl, 20 mM Tris, pH 7.4, 2 mM EDTA, 2 mM EGTA, 0.4 mM sodium vanadate, 0.4 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40). 500 µg of total protein lysate was precleared for 30 min at 4 °C using Protein G-Sepharose beads (Sigma). Precleared lysate was mixed with 500 µl of Nonidet P-40 lysis buffer as described above and 5 µg of monoclonal antibody against an N-terminal epitope of Sall1. The sample was then incubated at 4 °C for 1 h, and immune complexes were precipitated with Protein G-Sepharose beads. The bound proteins were analyzed by Western blotting.

Antibodies—Protein interaction assays and immunoprecipitations were performed with antibodies against HDAC1 (Sigma), HDAC2, mSin3A, RbAp46, MTA1, MTA2, MBD3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), RbAp46/48 (15G12; Genetex), MBD2a (Abcam), p66 (Upstate), and Mi2β (CHD4) (gra-

ciously provided by D. Murray (NIAIRP, National Institutes of Health)). A purified monoclonal Sall1 antibody derived from mouse ascites was used for the immunoprecipitation of Sall1. This anti-Sall1 antibody is directed against the same N-terminal epitope as polyclonal antisera previously described (27, 29).

RESULTS

NuRD Associates with Endogenous Sall1 in Cell Culture—Previously, we demonstrated that the N terminus of Sall1 mediates repression by recruitment of an HDAC-containing complex (29). Two well characterized HDAC-containing complexes, NuRD and Sin3, are known to associate with sequence-specific transcription factors (17, 30) and thereby mediate repression. Our results suggested that a multiprotein complex, possibly NuRD, could be responsible for Sall1 repression of target genes (29). To determine if Sall1 associates with NuRD under physiological conditions, extracts prepared from P19 cells, embryonic carcinoma cells that endogenously express Sall1, were immunoprecipitated with a monoclonal antibody against an N-terminal epitope of Sall1. As shown in Fig. 1, endogenous Sall1 associates with all components of the NuRD complex (5, 7–9). HDAC1, HDAC2, RbAp46, RbAp48, MTA-1, MTA-2, Mi-2β, and p66 were identified by Western blotting of Sall1 immunoprecipitates. In contrast, Sin3A, a Sin3-specific factor, does not associate with endogenous Sall1, further indicating that the corepressor complex in association with Sall1 is NuRD (Fig. 1). One NuRD component, MBD3, was not identified endogenously, since it comigrates with IgG light chain (~32 kDa), but its presence was confirmed by its ability to associate with GST fusions of Sall1 (Figs. 2*C* and 5*B*). Furthermore, the methylated DNA-binding protein MBD-2 was not found to associate with endogenous Sall1, suggesting that Sall1 recruits NuRD but not MeCP1, a complex that contains all NuRD components plus MBD-2 (data not shown) (6, 31, 32). These results demonstrate that Sall1 recruits the NuRD complex and suggest that Sall1 mediates repression through histone-deacetylase activity and ATP-dependent nucleosome remodeling.

A Highly Conserved N-terminal Motif Mediates Potent Transcriptional Repression through an Association with NuRD—Our previous work identified a potent repression domain that maps to the first 136 amino acids in the N terminus of Sall1 (29). To further characterize the N-terminal repression domain, we aligned the N termini of the Sall family members, Sall1 to -4. This alignment revealed a conserved 12-amino acid motif found at the extreme N terminus of the Sall proteins (Fig. 2*A*). Based on our alignment, we performed a BLAST search and found other C_2H_2 zinc finger transcription factors that contain the conserved motif, including friend of GATA (FOG1 and FOG2) (33) and chicken ovalbumin upstream promoter transcription factor-interacting protein 1 (CTIP1) and 2 (CTIP2) (34) that are known to mediate transcriptional repression. We also identified this highly conserved motif in Sall1 orthologs in other vertebrate species, including *Homo sapiens*, *Bos taurus*, *Rattus norvegicus*, *Gallus*, *Xenopus laevis*, *Danio rerio*, *and M. musculus*. The motif is also present in the *C. elegans Sal* ortholog, Sem-4 and *Xenopus* XsalF (Fig. 2*A*). The motif is not present in *Drosophila* spalt-major, spalt-related, or in the FOG ortholog, u-shaped. The high degree of evolutionary conserva-
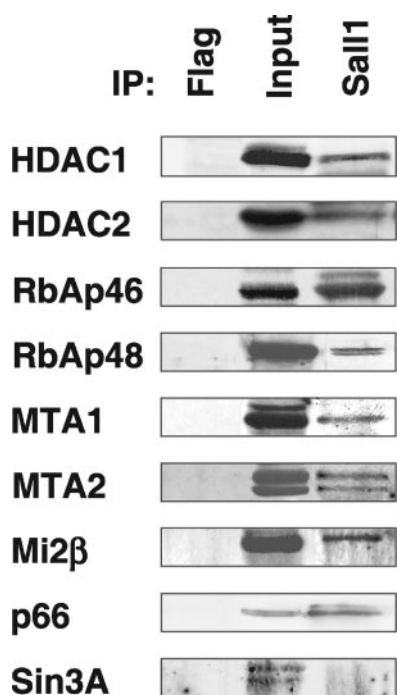
FIGURE 1. NuRD associates with Sall1 *in vivo*. P19 cell extracts were immunoprecipitated (*IP*) with a control FLAG or monoclonal Sall1 antibody followed by Western blotting with antibodies to the NuRD complex.

FIGURE 2. Conserved N-terminal motif found in Sall proteins. *A*, alignment of the extreme N termini of mammalian Sall family members, Sall1–4, and the *Sal* orthologs, Sem-4 (*C. elegans*) and XsalF (*Xenopus*), revealed sequence homology within the first 12 amino acids of the N terminus. The conserved residues are shown in **boldface type**. An alternative splice form of Sall2 (Sall2Alt) encodes a different 5′ exon that leads to a dissimilar N terminus. Sequences were obtained from the NCBI data base and have the following accession numbers: *M. musculus*, NP_056587 Sall; *M. musculus* SallII, Q9QX96; *M. musculus* SallII, Q9ER75; *M. musculus* Sal4, NP_958797; *C. elegans* Sem-4, AABO3333; *M. musculus* Sall2Alt, AJ007396; *X. laevis XsalF*, AA579483. *B*, GAL4DB fusion proteins of full-length Sall1, Sall2, and Sall2Alt were coexpressed with a reporter in which luciferase is under the control of the SV40 promoter and five upstream GAL4 binding sites (G5SV40Luc). -Fold repression was calculated by dividing the normalized luciferase activity of cells expressing GAL4DB alone by the activity of the GAL4-Sall fusion proteins. Values are plotted as the mean ± S.D. of triplicate transfections from three independent experiments and demonstrate that the conserved motif present in Sall1 and Sall2 and absent in Sall2 Alt is important for repression. *C*, Sall2Alt is missing the conserved N-terminal motif and does not interact with HDAC1, HDAC2, RbAp46, RbAp48, MTA1, MTA2, and MBD3 as compared with Sall1 and Sall2, which contain the motif. Whole cell extracts were prepared from COS-1 cells transfected with GST alone or GST fusions identical to the GAL4DB fusions (Fig. 2*B*) and precipitated with glutathione-Sepharose. Eluates were analyzed for the presence of endogenous NuRD complex components by Western blotting using the indicated antibodies.

tion and the absence of this motif in a native Sall2 alternative splice form (Fig. 2*A*) that differs only in the first 24 N-terminal amino acids (35) suggests its biological importance.

To test whether the highly conserved motif is important for repression function, we compared the repression activity of the Sall family members, Sall1 and Sall2, to the naturally occurring splice variant, Sall2Alt. The ability of the N-terminal motif to affect transcription was analyzed by fusing the GAL4 DNA binding domain (GAL4DB) to full-length Sall1, Sall2, and Sall2Alt. These GAL4 fusion proteins were transfected with a reporter plasmid containing the luciferase gene under the control of a modified SV40 promoter with five copies of the GAL4 DNA binding element. We have successfully used this reporter gene assay to assess the repression function of Sall1, since there are currently no known target genes for Sall1. We have also shown that Sall1 repression of luciferase activity depends on DNA binding (29). Using this same reporter gene assay, we revealed that Sall1 and Sall2 mediate strong transcriptional repression, whereas Sall2Alt significantly reduced the ability to repress transcription from over 100-fold to only 4-fold (Fig. 2*B*). We also tested Sall2Alt for a physical interaction with components of the NuRD corepressor complex using full-length Sall1, Sall2, and Sall2Alt GST fusion proteins. The GST-Sall proteins were isolated on glutathione-Sepharose beads and analyzed by Western blotting. Whereas the Sall proteins were expressed at similar levels, in contrast to Sall1 and Sall2, Sall2Alt failed to recruit NuRD (Fig. 2*C*). Sall family members Sall3 and Sall4 were also found to mediate repression and associate with the NuRD complex (data not shown).

Our analysis revealed that Sall proteins contain an N-terminal repression domain that recruits NuRD, and the extreme N-terminal amino acids are important for this function. We
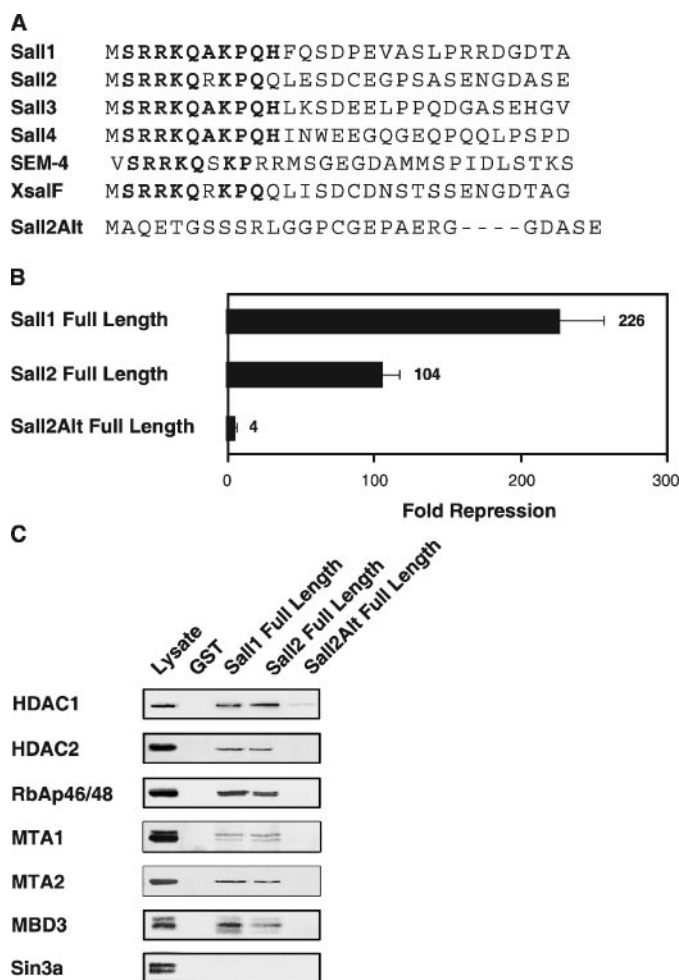
suspected that other zinc finger transcription factors containing the motif probably mediate repression through a recruitment of NuRD. Recent analysis has revealed that NuRD recruitment is important for the repression function of FOG1 and CTIP2, both of which contain the motif (36, 37). To test the importance of this motif in a non-Sal-related zinc finger transcription factor, we analyzed the repression function of zinc
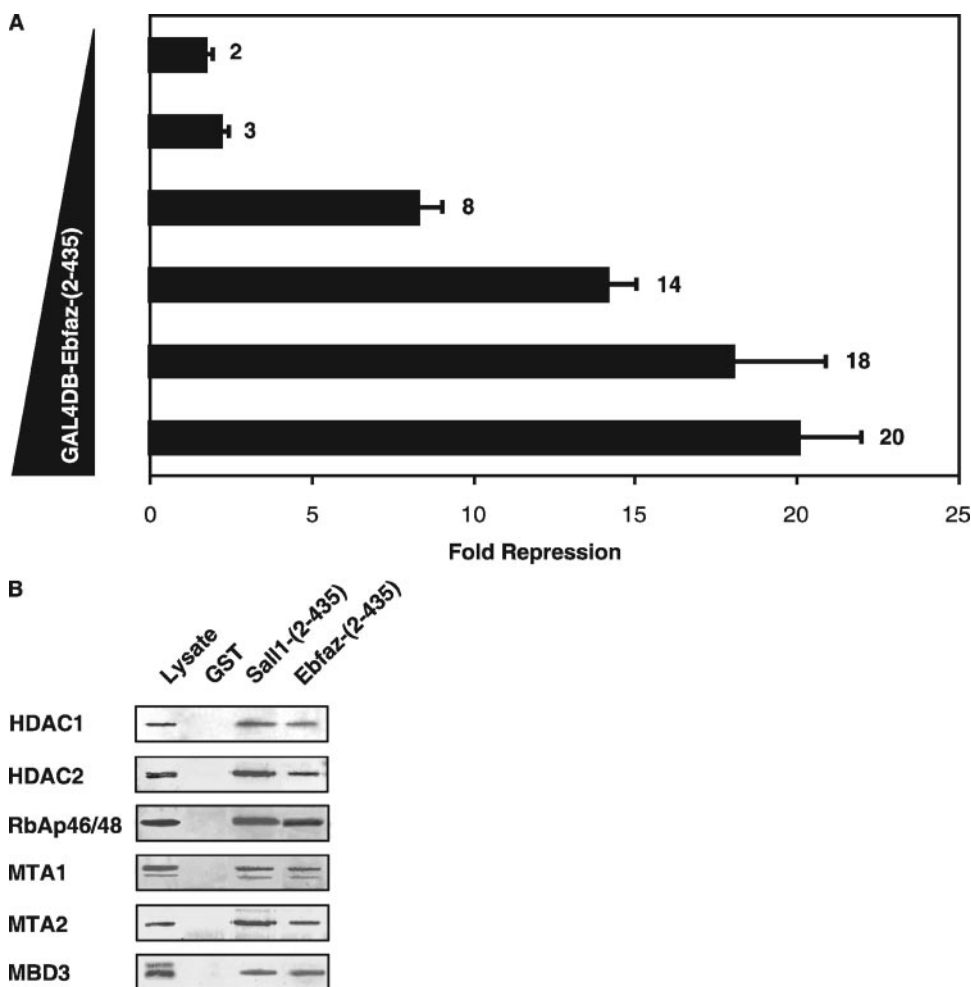
FIGURE 3. **Transcriptional repression is mediated by the N terminus of Ebfaz.** *A*, an N-terminal construct of the C_2H_2 zinc finger transcription factor, Ebfaz-(1–435), was expressed as a GAL4 fusion protein and tested for its ability to mediate repression of the luciferase reporter described in Fig. 2*A*. Increasing doses of GAL4DB-Ebfaz-(1–435) or GAL4DB alone (0.010, 0.1, .25, 1.6, 3.0, 6.5, or 13.0 μg) was expressed with the luciferase reporter, and -fold repression was calculated as in Fig. 2*B*. Values are plotted as mean ± S.D. of triplicate transfections from three independent experiments and demonstrate a dose-dependent repression of luciferase activity. *B*, whole cell extracts prepared from COS-1 cells transfected with GST alone or the GST-Ebfaz-(1–435) construct were incubated with glutathione-Sepharose and analyzed for endogenous NuRD components as described in the legend to Fig. 2*C*. HDAC1, HDAC2, RbAp46, RbAp48, MTA1, MTA2, and MBD3 were found to associate with the N-terminal domain of Ebfaz.

finger protein 423 (Ebfaz). The Ebfaz gene expresses the highly conserved motif and has been shown to mediate negative regulation of early B-cell factor (EBF; also known as olfactory-1, OLF1), a basic helix-loop-helix transcription factor (38); however, the functional domain(s) of Ebfaz required for this negative regulation have not been defined. An N-terminal GAL4 fusion of Ebfaz-(1–435) was analyzed for its ability to repress the GAL4-responsive SV40 reporter. This Ebfaz fusion protein repressed luciferase activity in a dose-responsive manner (Fig. 3*A*). The same N-terminal domain of Ebfaz expressed as a GST fusion protein revealed that repression of Ebfaz correlates with recruitment of NuRD (Fig. 3*B*). These results are consistent with our analysis of the conserved motif in the Sall family members and strongly suggest that the conserved motif probably exhibits a more general role in regulating gene expression.

The N-terminal 12-Amino Acid Motif of Sall1 Is Required for Transcriptional Repression and Binding of NuRD—To determine if the conserved motif is required for repression, we took advantage of the naturally occurring splice variant of Sall2, Sall2Alt, by creating a Sall2Alt-Sall1 chimeric construct to replace the nucleotides encoding the first 12 amino acids of Sall1 with those of the first 12 amino acids of Sall2Alt. This construct was created within the context of the shorter repression domain N-(1–136), which has been shown to be both necessary and sufficient for transcriptional repression by Sall1 (29). As depicted in Fig. 4*A*, the chimeric (Sall2Alt-Sall1) construct and a construct deleting the extreme N-terminal 10 amino acids of Sall1, Sall1-N-(11–136), were expressed as GAL4DB fusions. A GAL4 fusion consisting of the full N terminus, Sall1-N-(1–435), was also analyzed, because this region corresponds to both a mutation shown to cause TBS (1277–1278GA) and our mouse model Sall1-ΔZn^{2-10} that faithfully mimics TBS (27, 39). Consistent with our previous findings (29), both Sall1-N-(1–136) and -(1–435) display a comparable level of repression of the GAL4 reporter. The chimeric construct was expressed equivalently to its wild-type counterpart Sall1 N-(2–136), yet it did not mediate repression of the reporter (Fig. 3*B* and data not shown). Furthermore, removal of the extreme N-terminal 10 amino acids, Sall1-N-(11–136), also abrogates repression (Fig. 3*B*). To examine whether NuRD binding correlates with transcriptional repression by the N-terminal motif of Sall1, the same N-terminal constructs were expressed as GST fusion proteins and purified on glutathione-Sepharose. Only the Sall1 constructs containing the conserved N-terminal 12-amino acid motif were capable of associating with components of the NuRD complex, including HDAC1, HDAC2, RbAp46, RbAp48, MTA1, and MTA2 (Fig. 3*C*). Thus, the first 12 amino acids of Sall1 are required for repression through interaction with components of the NuRD repressor complex.

Conserved 12-Amino Acid Motif Functions as Independent Repression Module—Because the conserved region of Sall1 was shown to be required for potent transcriptional repression and NuRD association, we next asked whether this motif was also sufficient. We expressed the 12-amino acid motif as a GAL4DB fusion and assayed its ability to repress the GAL4-responsive reporter. Increasing amounts of the N-terminal motif GAL4DB-Sall1-N-(1–12) revealed a dose-dependent repression of luciferase activity (Fig. 5*A*). These results are consistent
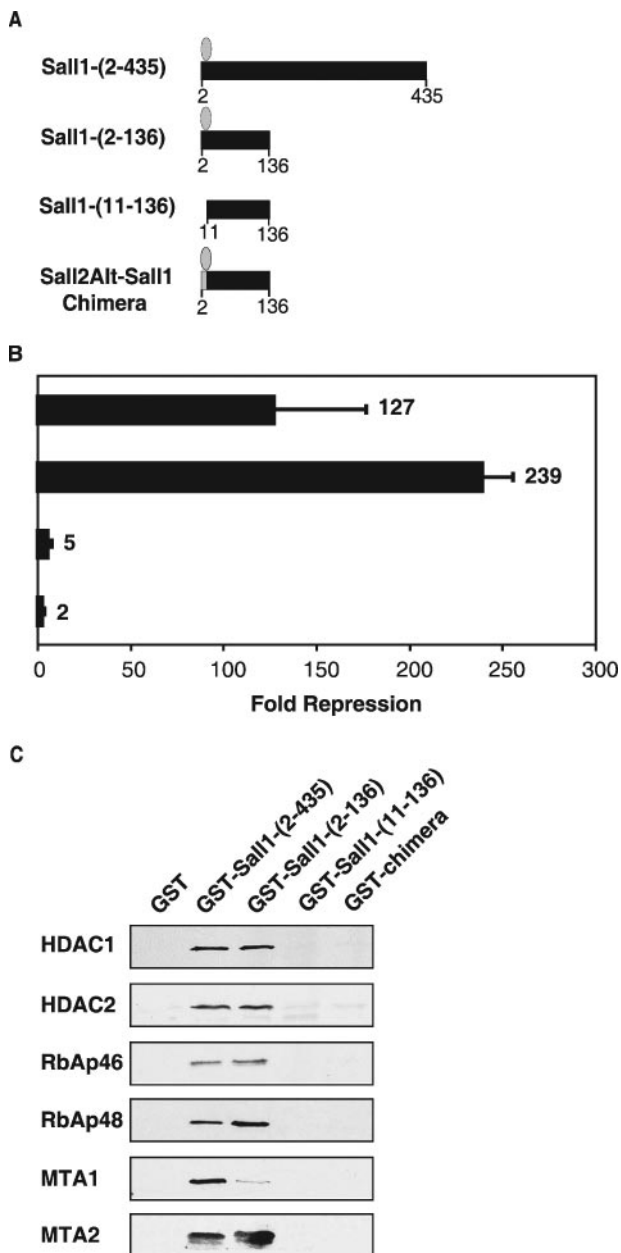
GAL4DB fusion. Thus, the 12-amino acid motif can repress luciferase activity when fused at either the N or C terminus, further indicating that these 12 amino acid residues can function as an independent repressor module. We conclude that the first 12 amino acids of Sall1 are both necessary and sufficient for transcriptional repression.

To test for a physical interaction between the 12-amino acid motif of Sall1 and NuRD, we created a fusion protein consisting of amino acids 1–12 of Sall1 in frame with GST. The GST-Sall1 fusion protein was expressed in COS-1 cells, isolated on glutathione-Sepharose beads, and analyzed by Western blotting. All GST-Sall1 proteins were expressed at similar levels except for Sall1-N-(1–12), which was 2-fold less well expressed (data not shown) and required 2-fold more of the cell lysate to be used in the precipitation (see "Experimental Procedures"). Antibodies against endogenous NuRD components revealed an association of GST-Sall1-N-(1–12) with endogenously expressed members of the NuRD complex, including HDAC1, HDAC2, RbAp46/48, MTA-1, MTA-2, Mi-2β (CHD4), and MBD3. These associations were specific to Sall1, since they were not found for GST alone (Fig. 5B). These results reveal a correlation between strong transcriptional repression and NuRD complex interaction for the 12-amino acid motif of Sall1.

To test the functionality of the N-terminal Sall1-associated complexes, HDAC activity was measured. COS-1 cell lysates expressing GST-Sall1-N-terminal constructs (1–435, 1–136, and 112) were purified using glutathione-Sepharose, and the associating complexes were tested for their ability to deacetylate ϵ-acetylated lysine residues of a histone-like sequence using a fluorimetric assay. The full N terminus (residues 1–435) and the minimal repression domain (residues 1–136) of Sall1 exhibited comparable HDAC activity that was significantly higher than background. TSA significantly decreased the HDAC activity associated with these N-terminal constructs, further demonstrating that N-terminal associating complexes possess active TSA-sensitive HDAC activity. Alteration (Sall2AltSall1 chimera) or deletion (residues 11–136) of the 12-amino acid motif resulted in a reduction of HDAC activity to a level that was comparable with that associated with the GST control. This result indicates that the majority of HDAC activity associated with the N terminus of Sall1 requires the 12-amino acid motif. Moreover, HDAC activity associated with GST-(1–12) is similar to that seen with the longer N-terminal constructs GST-(1–136) and GST-(1–435) (Fig. 5C). This finding is consistent with our observation that amino acids 1–12 are sufficient to recruit NuRD components and indicates that this motif accounts for the majority of TSA-sensitive HDAC activity in the N terminus of Sall1. Together, our results indicate that a 12-amino acid motif found in the extreme N terminus of Sall1 is both necessary and sufficient for Sall1 repression, HDAC recruitment, and TSA-sensitive HDAC activity.

Fine Mapping of the Critical Residues in the N-terminal Repression Motif of Sall1—To further refine the critical amino acids in the repression domain, we generated expression constructs encoding alanine substitutions for individual residues 1–12 of Sall1 within the context of the previously characterized

FIGURE 4. **Transcriptional repression is mediated by amino acids 2–12 of Sall1.** *A*, schematic of GAL4-Sall1 N-terminal fusion proteins: full N terminus (residues 2–435), minimal repression domain (residues 2–136), N-terminal deletion construct (residues 11–136), and a Sall2Alt-Sall1 chimera replacing the first 12 amino acids of Sall1 with the first 12 amino acids of Sall2Alt (*shaded region*). The Sall1 N terminus contains a C$_2$HC zinc finger (*oval*) that we previously showed was not required for repression (27). GAL4DB was fused at the N terminus. *B*, the GAL4-Sall1 N-terminal fusion proteins were coexpressed with the luciferase reporter as described in the legend to Fig. 2*A*. Values are plotted as the mean ± S.D. of triplicate transfections from three independent experiments and demonstrate that the first 12 amino acids of Sall1 are required for repression. *C*, the same N-terminal constructs of Sall1 were expressed as GST fusions and analyzed for an association with NuRD. The N-terminal constructs of Sall1 that are missing the conserved N-terminal motif do not interact with HDAC1, HDAC2, RbAp46, RbAp48, MTA1, and MTA2.

with our past observations that the full N terminus of Sall1-N-(1–435) and the minimal repression domain, Sall1-N-(1–136) are capable of potent transcriptional repression (29). Whereas we use an N-terminal GAL4DB fusion for analysis of the 12-amino acid motif (Fig. 5), Lin *et al.* (40) used a C-terminal
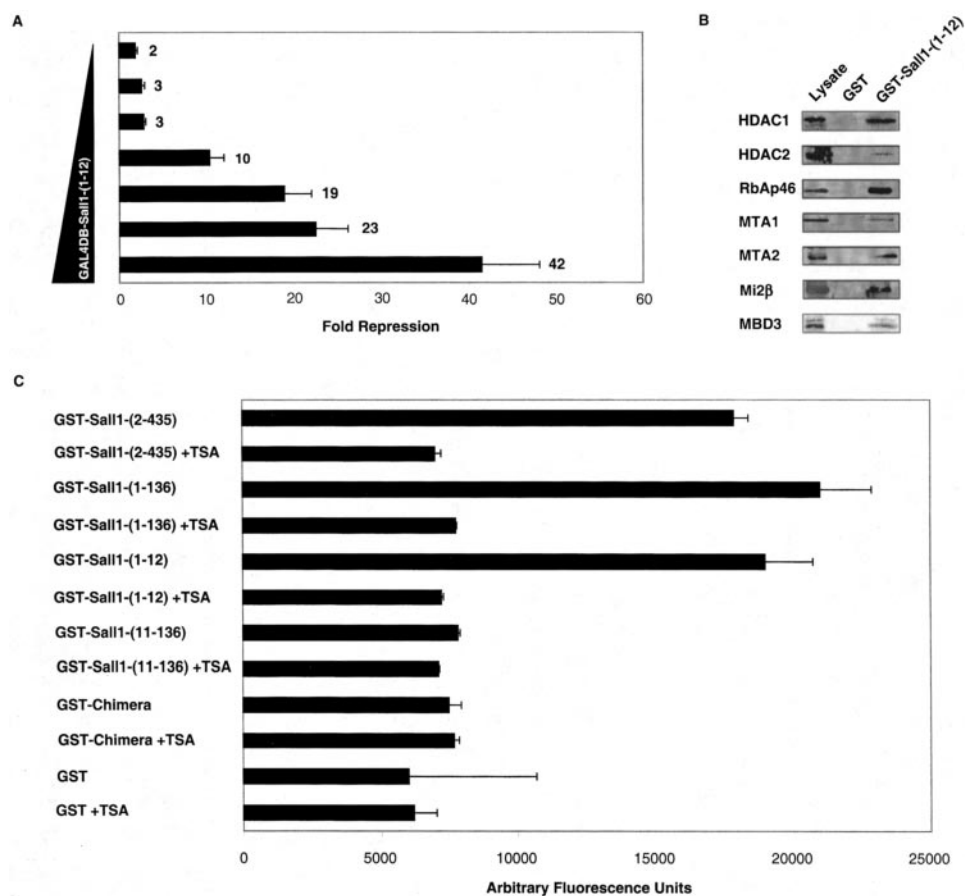
FIGURE 5. **The N-terminal 12-amino acid motif of Sall1 is sufficient for transcriptional repression, recruitment of NuRD, and HDAC activity.** *A*, increasing doses of GAL4DBSall1-(1–12) or GAL4DB alone (0.010, 0.1, .25, 1.6, 3.0, 6.5, and 13.0 μg) were expressed with the luciferase reporter, and -fold repression was calculated as in the legend to Fig. 2*B*. Values are plotted as mean ± S.D. of triplicate transfections from three independent experiments. *B*, whole cell extracts prepared from COS-1 cells transfected with GST alone or the GST construct consisting of a fusion to the first 12 amino acids of Sall1 were incubated with glutathione-Sepharose and analyzed for endogenous NuRD components as described in the legend to Fig. 2*C*. *C*, COS-1 cell lysates expressing GST-Sall1-N-terminal constructs (1–435, 1–136, 1–12, Sall2AltSall1 chimera, and 11–136) or GST alone were purified using glutathione-Sepharose and tested for deacetylase activity in the presence or absence of 100 nM TSA as described under "Experimental Procedures." The deacetylase activity is expressed as arbitrary fluorescence units. The quantifications represent mean fluorescence activities ± S.D. derived from two independent experiments.

we analyzed point mutations of the Sall1 repression motif for their ability to associate with NuRD. As shown in Fig. 6*B*, the mutations that completely abolished repression also led to a loss (R4A, K5A, and P9A) or a significant reduction (R3A) in binding of NuRD components. Thus, each of the mutations that led to an abolishment of repression also revealed significantly reduced or complete loss of binding of the NuRD components. In contrast, mutations that did not affect repression of the GAL4 reporter, S2A and H11A, also did not disrupt recruitment of NuRD (Fig. 4*B*). These findings strengthen the observed correlation between repression and NuRD recruitment by identifying point mutations that lead to total abrogation of repression and recruitment of NuRD and further support that NuRD functions as a Sall1 corepressor.

DISCUSSION

Our work provides the first direct evidence of a short conserved peptide motif that can recruit the NuRD complex. We show that the conserved motif of Sall1 is necessary and sufficient for the recruitment of NuRD, demonstrating a potential mechanism for Sall1-mediated transcriptional repression. The association of Sall1 with NuRD was maintained during high stringency immunoprecipitation experiments and binding assays and was entirely dependent on the extreme N-terminal 12 amino acids of Sall1. Furthermore, all TSA-sensitive HDAC activity is in association with this 12-amino acid motif. Here we localize the NuRD interaction domain to the conserved 12-amino acid motif present in a diverse group of transcriptional repressors.

Specific recruitment of the NuRD corepressor complex by transcription factors is thought to play an essential role in transcriptional repression. The discovery of this 12-amino acid motif that is sufficient for recruiting NuRD has potential implications for understanding the mechanism of repression mediated by a group of transcription factors. The motif is found in all four Sall family members, Sall1–4 as well as five other families of zinc finger transcription factors. Of those that have been examined, friend of GATA (FOG1 and FOG2), chicken ovalbumin upstream promoter transcription factor-interacting proteins (CTIP1 and CTIP2), and zinc finger protein 423 (Ebfaz) are known transcriptional repressors. Ebfaz represses B cell factor (38), and our studies suggest that this regulation may require NuRD. CTIP and Evi3 are implicated in the pathogen-

minimal repression region N-(1–136). GAL4 fusions of the Sall1 site-specific mutants were coexpressed with the GAL4-responsive SV40-luciferase reporter. Individual substitutions of amino acids 3 (R3A), 4 (R4A), 5 (K5A), and 9 (P9A) resulted in a complete loss of repression by Sall1, whereas substitutions of amino acids 2 (S2A) and 11 (H11A) did not have a significant effect on reporter activity ($p > 0.05$). The substitution of amino acids 6 (Q6A), 8 (K8A), and 12 (F12A) resulted in a partial loss of repression that is statistically significant ($p = 0.029$, $p = 0.035$, and $p = 0.032$, respectively) (Fig. 6*A*). Whereas amino acid 12 was not conserved, this residue is always hydrophobic and varies between a valine, phenylalanine, leucine, or isoleucine in the transcription factors containing the conserved motif (Fig. 2). These results define the critical repression motif in the N terminus of Sall1 as RRKQ*X*KP*XX*F. Since our data indicate that Sall1-mediated repression requires NuRD, point mutations that abrogate repression would be expected to disrupt recruitment of the co-repressor complex. To test this possibility,
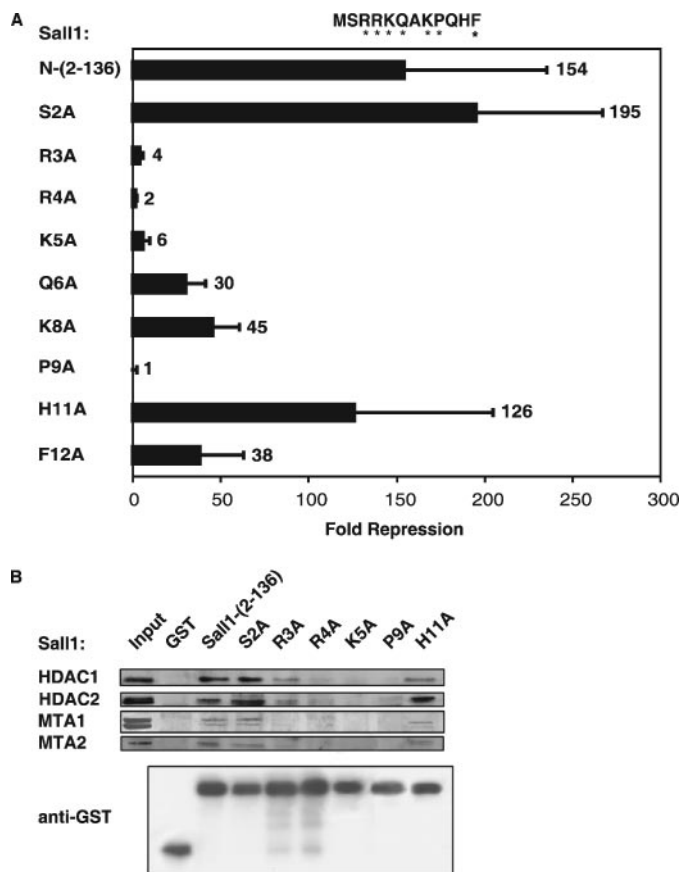
FIGURE 6. **Sall1-mediated repression and NuRD association is abolished by single amino acid mutations of the conserved repression motif.** *A,* site-specific mutations of the Sall1-N terminus were created by substituting individual amino acids 2–12 of Sall1 with alanine residues. The single amino acid mutations were created within the context of the minimal repression domain (residues 2–136). The minimal repression domain (residues 2–136) and site-specific mutations of Sall1 were expressed as GAL4DB fusions to test their ability to repress luciferase activity. -Fold repression was calculated as described in the legend to Fig. 2*B*. The results are reported as the mean ± S.D. of triplicate transfections from two independent experiments. *B,* point mutations of Sall1 S2 or H11 had no effect on repression activity (*A*) and were found to associate with the components of the NuRD complex. In comparison, Sall1 interaction with NuRD is significantly reduced (R3A) or lost (R4A and K5A) with single amino acid mutations that result in significantly reduced repression (*A*). Extracts were prepared from COS-1 cells expressing GST alone, GST-Sall1-N-(2–136), or GST-Sall1 N-terminal point mutations, S2A, R3A, R4A, K5A, P9A, and H11A. Glutathione-Sepharose precipitates from N-terminal GST fusions of Sall1-N-(2–136), and the Sall1 site mutants were analyzed for NuRD complex components as described in the legend to Fig. 2*C*.

esis of B cell lymphoma and lymphocyte development (41, 42). Two other unrelated proteins, zinc finger protein 64 and hypothetical protein LOC55565 that contain this motif have not been characterized.

Sall and FOG proteins have well defined roles in regulating gene expression during embryonic development (22–27, 43). A subset of GATA target genes are repressed through association with FOG (reviewed in Ref. 43). Recent studies demonstrate that FOG-1 recruits NuRD to GATA targets in the context of native chromatin via a 45-amino acid region in FOG-1 that contains the conserved repression motif (36, 44). Together with our data showing that this motif is necessary and sufficient for repression and NuRD recruitment, it is likely that Sall1 also recruits NuRD to chromatin in order to mediate repression.

The requirement of NuRD binding by Sall1 to mediate repression at chromatin has not been directly assessed, since there are currently no known endogenous target genes for Sall1. Future analysis will address the *in vivo* significance of this association between Sall1 and NuRD.

Notably, our sequence alignment identifies the 12-amino acid repression motif in Sem-4, the *C. elegans* Sall ortholog, indicating that it is not confined to vertebrates. Genetic evidence in *C. elegans* reveals that Sem-4 mediates direct repression of the *Hox* gene, *egl-5*, and the Lim homeobox gene, *mec-3*, controlling development of touch neurons (25). This analysis demonstrates that Sall1 acts as a direct repressor *in vivo* and reveals the importance of Sall proteins in the regulation of *Hox* genes. Moreover, studies in *C. elegans* demonstrate important roles for NuRD components, including the Sall-interacting proteins HDAC1/2, RbAp46/48, and MTA1/2 in vulva development. Sem-4 is also required for normal formation of the vulva and may interact genetically with the NuRD-specific component MTA1 (10, 11, 25). Together, these studies support a biological role for Sal as a transcriptional repressor and suggest that Sall1-mediated transcriptional repression at least in part relies on its association with NuRD.

Our studies reveal a strong correlation between repression and NuRD complex interaction facilitated by individual residues of the conserved 12-amino acid motif. We predict that individual residues of the motif probably contribute to transcriptional repression in various ways. We show that lysine 5 of the conserved motif is required for binding of NuRD. This result is consistent with Hong *et al.* (36). This residue may be a target for post-translational modifications, since the nucleosomal ATPases and deacetylases of NuRD remove the acetyl group from lysines located in both histones and transcription factors (reviewed in Ref. 4) (44). Our analysis reveals that the R3A mutation, which abrogates repression, reveals a partial affect on the recruitment of the NuRD components. This result is also in agreement with Hong *et al.* (36), who found that an R3G mutation significantly reduced repression, yet interaction with the NuRD components, MTA1, RbAp46, and RbAp48 was mostly preserved. Thus, arginine 3 does not appear to be absolutely required for binding of NuRD. This residue may, however, affect the stability or activity of the complex at target promoters *in vivo*. It is also possible that other, as yet unidentified, functionally important components of the Sall1-associated repressor complex require this residue for binding. Further analysis is being conducted to identify potential modification sites that probably contribute to the overall transcriptional repression facilitated by Sall1.

Modular effector domains that mediate transcriptional activation or repression often retain their activity when fused to other DNA binding proteins. The identification of this 12-residue repression motif thus has potential implications for studying normal gene expression and for intervening in cases of aberrant function, as has been shown for other modular domains. The 298-amino acid N terminus of the *Drosophila* Engrailed protein functions as a potent transcriptional repressor, and when fused to the DNA binding domain

of heterologous transcription factors, it can function as a dominant negative chimeric protein that efficiently reprograms targeted genes (46). Other effector domains, including the Kruppel-associated box, have also been used to elucidate biological functions of uncharacterized genes (47). The discovery of this small peptide that is sufficient for recruitment of the NuRD complex could prove to be a powerful tool for the understanding of gene regulation and possibly for drug development.

The role of the Sall1 conserved motif in transcriptional repression has potential implications for understanding TBS. Truncated proteins expressed from all of the documented TBS mutations correlate with the conserved repression motif, suggesting that the repression domain may contribute to a dominant negative or gain-of-function mechanism in the pathogenesis of this syndrome. Studies in *Xenopus* also suggest an important role for the N terminus of Sall1. Expression of an N-terminal truncation containing amino acids 1–57 of XsalF that includes the conserved repression motif has dominant negative properties and affects central nervous system development in *Xenopus* (48). One possibility for how the Sall1 mutant protein could affect gene expression and normal development is by the recruitment of NuRD to the truncated N-terminal Sall1 protein, thereby sequestering the NuRD components and preventing the complex from interacting with repressors, such as wild-type Sall proteins. Further analysis of the mechanism describing Sall1-mediated repression and regulation of its targets will be required to understand the role of Sall1 during normal development and the pathogenesis of TBS.

In summary, we have identified a conserved repression motif found in the N terminus of Sall1. Our studies demonstrate that this peptide motif can function as an independent repression module that is sufficient to recruit all components of the NuRD complex and can account for all functional HDAC activity. Discovery of this minimal repression domain and its association with NuRD should greatly enhance our understanding of regulated gene expression facilitated by a subset of zinc finger transcriptional repressors.

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A Conserved 12-Amino Acid Motif in Sall1 Recruits the Nucleosome Remodeling and Deacetylase Corepressor Complex

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