A Phosphomimetic Mutation in the Sall1 Repression Motif Disrupts Recruitment of the Nucleosome Remodeling and Deacetylase Complex and Repression of Gbx2*

Shannon M. Lauberth†, Amy C. Bilyeu†, Beth A. Firulli§, Kristen L. Kroll¶, and Michael Rauchman†**

From the †Departments of Biochemistry and Molecular Biology, Saint Louis University, and ‡Veterans Affairs Medical Center, †Department of Molecular Biology and Pharmacology, Washington University, St. Louis, Missouri 63106, and §Herman B Wells Center for Pediatric Research, Indiana Medical School, Indianapolis, Indiana 46202-5225

The multizinc finger transcription factor Sall1 is a critical developmental regulator that mediates repression through the recruitment of the nucleosome remodeling and deacetylase (NuRD) complex. Although a short conserved peptide motif in Sall1 is sufficient to recruit NuRD, its ability to regulate native Sall1 target genes in vivo has not been demonstrated. In this report, we demonstrate an in vivo role for the Sall1 repression motif and describe a novel direct target gene of Sall1, Gbx2, that is directly repressed in a NuRD-dependent fashion. The ability of Sall1 to repress Gbx2 was impaired in Xenopus embryos expressing mutant forms of Sall1 that are defective for NuRD binding. Finally, we demonstrate that protein kinase C phosphorylates serine 2 of the Sall1 repression motif and reveal that a phosphomimetic mutation of serine 2 disrupts the ability of Sall1 to repress Gbx2 in cell culture and Xenopus embryos. Together, these studies establish that Sall1 recruits NuRD via the Sall1 repression motif to mediate repression of a native target gene and suggest a model in which dynamic control of gene expression by Sall1 is modulated by serine phosphorylation of the Sall1 repression motif.

Sall proteins are transcription factors encoded by evolutionarily conserved genes found in species as diverse as Drosophila, Caenorhabditis elegans, and vertebrate species. Sall genes regulate diverse developmental processes in several model organisms. Mutations in the human SALL1 and SALL4 genes have been linked to the autosomal dominant inherited diseases, Townes-Brock and Okihiro syndromes, respectively. Both syndromes are characterized by multiple developmental defects, including a combination of anal, renal, limb, and ear abnormalities (1–6), demonstrating the critical role of Sall genes in organ development.

The members of the spalt (sal) gene family have been shown to function as transcriptional repressors. Genetic evidence in Drosophila has demonstrated a cell autonomous role for spalt as a transcriptional repressor (7–9). Similarly, in recent work we revealed that Sall proteins contain an N-terminal repression domain that recruits the nucleosome remodeling and deacetylase (NuRD) complex, revealing a strong correlation between repression and NuRD complex interaction (10). We reported a peptide motif (SRM) that is necessary and sufficient for Sall1-mediated repression and NuRD recruitment (10). The NuRD complex possesses ATP-dependent and histone deacetylase chromatin-remodeling activities and consists of several subunits, including HDAC1, HDAC2, RbAp46, RbAp48, Mi-2β, MBD3, MTA1, and MTA2 (reviewed in Ref. 11). The recruitment of NuRD to specific promoters by transcription factors is thought to play an essential role during transcriptional repression (12–14). However, the requirement of NuRD binding by the Sall1 protein to mediate repression at chromatin has not been directly assessed, because no endogenous target genes of Sall1 have been identified. Thus, the in vivo significance of the association between Sall1 and NuRD has not yet been determined.

It has been suggested that Sall1 may facilitate dual transcriptional regulatory roles. In contrast to transcriptional repression mediated by Sall proteins, it has been shown that Sall proteins can function as transcriptional activators in some contexts (8, 15). The ability of Sall1 to function as a transcriptional activator, despite a strong interaction with the NuRD corepressor complex, suggests the possibility that a molecular switch likely coordinates the transcriptional repression and activation activities of Sall1. Post-translational modifications of histones and non-histone transcription factors are important regulators of gene expression. Previous studies have shown that methylation of lysine 4, a modification associated with active gene transcription, disrupts NuRD binding to histone H3 (16, 17) suggesting the possibility that post-translational modifications of Sall1 may similarly impair the recruitment of NuRD at genes that are activated by Sall1. However, it is not known if post-translational modifications of Sall1 itself also modulate its ability to control gene expression.

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† To whom correspondence should be addressed: St. Louis Veterans Affairs Medical Center, 657/1118-JC, 915 North Grand Blvd., St. Louis, MO 63106. Tel.: 314-289-6485; Fax: 314-289-7012; E-mail: rauchman@slu.edu.

‡ The abbreviations used are: NuRD, nucleosome remodeling and deacetylase; SRM, Sall repression motif; PKC, protein kinase C; MOPS, 4-morpholinepropanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; ChIP, chromatin immunoprecipitation; qPCR, quantitative PCR; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.
In the present study, we reveal a biological role for the Sall repression motif (SRM) in vivo. We provide the first direct evidence that together Sall1 and NuRD mediate direct repression of a bona fide target gene of Sall1. We show that Sall1 is phosphorylated on serine 2 of the SRM by protein kinase C (PKC). Most importantly, a phosphomimetic mutation of serine 2 (S2E) disrupts NuRD recruitment thereby preventing Gbx2 repression by Sall1 in Xenopus embryos.

**EXPERIMENTAL PROCEDURES**

**Microinjection Manipulation**—For Xenopus embryo injections, capped mRNA was prepared by linearizing pCS2 plus XsalF with NotI, a gracious gift provided by Y. Sasai (Kyoto University) followed by transcription with SP6 RNA polymerase using the mMessage Machine kit (Ambion) according to the manufacturer’s instructions. Embryos produced by in vitro fertilization were injected at the 8-cell stage with 400 pg of XsalF, XsalFΔ12, XsalFS2A, or XsalFS2E and 50 pg of β-galactosidase mRNA into two left animal blastomeres in a volume of 10 nl. Embryos were cultured in 0.2X MMR plus gentamycin (100 µg/ml) to neurula stage (stage 15) then fixed in MEMFA (0.1M MOPS (pH 7.4), 2 mM EGTA, 1 mM MgSO4, 3.7% formaldehyde) for 1 h, stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, fixed in MEMFA for an additional hour, and stored in ethanol at −20 °C. Embryos were staged according to a previous study (18).

**In Situ Hybridization**—In situ hybridizations were performed as described (19) with modifications. Vitelline envelopes were not removed from embryos, and archenterons were not punctured prior to fixation in MEMFA. Embryos were rehydrated through ethanol washes followed by a single wash with acetic acid/ethanol. Following hybridization, embryos were washed three times with 2X SSC and twice with 0.2X SSC without CHAPS. RNase treatment was omitted. Probes were generated by linearization of the plasmid template, pCRII-TOPO plus Gbx2 with NotI (a gracious gift provided by Y. Sasai, Kyoto University) and transcribed with the SP6 RNA polymerase in the presence of digoxigenin-11-UTP (Roche Applied Science). Hybridization signals were detected using alkaline phosphatase-conjugated anti-digoxigenin antibodies (Roche Applied Science) and images were captured with a Photometrics color camera.

**Chromatin Immunoprecipitation**—ChIP assays were performed as described in the Upstate protocol with minor modifications. Briefly, chromatin was prepared from −4.5 × 10⁷ P19 cells for each condition. The cells were reversibly cross-linked in 1% formaldehyde (Sigma) for 10 min at room temperature. Cross-linking was stopped by adding glycine to a final concentration of 125 mM. Isolated nuclei were sonicated on ice (15 times for 20 s) at 25% amplitude, yielding 0.2- to 1-kb fragments of DNA, and sonication efficiency was verified in each experiment by running the samples on a 1% agarose gel. The sheared chromatin was precleared with Protein G salmon sperm-coupled agarose (Upstate), and 10 µl of the precleared chromatin was removed to use as an input control. For immunoprecipitation, equal amounts of chromatin were incubated with a monoclonal antibody to Sall1 previously described (10), MBD3 antibody (Abcam no. ab3755), or nonspecific IgG (Santa Cruz Biotechnology no. sc-2027) overnight at 4 °C. Following sequential washes and elution (1% SDS, 0.1 M NaHCO₃), cross-links were reversed at 65 °C overnight, and the samples were treated with proteinase K for 2 h at 45 °C. The samples were purified, and quantitative PCR (qPCR) was performed to measure the relative amounts of DNA.

**Quantitative Real-time PCR**—qPCR analyses of purified immunoprecipitated DNA fragments from all ChIP experiments were analyzed in real-time using the ABI 7300 Real-Time PCR Systems (Applied Biosystems) and SYBR Green I methodology. Sequence-specific PCR primer sets were designed by Primer Express 3.0 (PE Applied Biosystems) to amplify specific regions of mouse Gbx2 and Gapdh. The thermal cycling parameters were as follows: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. The dissociation curve for each primer pair confirmed a single reaction product. Reactions were performed in triplicate using samples from three independent cell harvests. The amount of DNA from each amplification product was determined relative to a standard curve of input chromatin. Data are shown as the % input, which was calculated by determining the apparent immunoprecipitation efficiency at the Gbx2 and Gapdh gene loci as a ratio of the amount of immunoprecipitated DNA to that of the normalized starting material (% of input DNA). The following primer sets were used for qPCR analysis: Gbx2 (−0.7 kb, 5'-GCCAGGGACTCTAGGG-TCTGA-3' and 5'-CCGGCCGGAACAACTTAA-3'), Gbx2 (−4.6 kb, 5'-CCCTCTGCGGTCCGTAGA-3' and 5'-CTCCA-AGGCGCAAGACTGTGA-3'), Gbx2 (−5.4 kb, 5'-CTAGGCG-CCCGATTAGC-3' and 5'-CTCCTGCGCATACGCGC-ATT-3'), and Gapdh (5'-TTCATCCCGCTCTGTTGCG-3' and 5'-TCAGGGTCCCCATAAGCTTGGT-3').

**Plasmid Construction**—A 960 bp DNA fragment of the Gbx2 gene locus was amplified from mouse genomic DNA using the following primer sequences corresponding to (−660 bp to −1620 bp) (5'-ATGTGACTACCTTGCAGAAAGACGCCTAG-3' and 5'-TACTCGAATTCACCCGTCGC-3'). The PCR product was digested with KpnI and XhoI and cloned upstream of a heterologous promoter driving luciferase (pGL3SV40-luc, Promega). Mutations of the AT recognition sites in the context of the Gbx2 reporter were introduced using sited-directed mutagenesis with the following primer sets: ATmut1 (5'-CTCTCTGGAAATATTTCG-3' and 5'-TACTCGAGATTCACCC-3'), ATmut2 (5'-CAGATTTAAAGACTCTGTTATATTCTC-3' and 5'-CAGATTTAAAGACTCTGTTATATTCTC-3'). The Fl-tagged mutants of full-length Sall1, including Sall2Alt-Sall1 chimera (2–12 Sall2Alt plus 13–1322 Sall1) and Sall1Δ12 were generated by excising a 5'-fragment of the respective Sall1 N-terminal mutants previously described in the context of Sall1 (2–136) (10) with BamHI (5'-end of cDNA) and KpnI (nucleotide 501). The DNA sequence was then ligated into pcDNA3 (Invitrogen) containing full-length Sall1 that was also BamHI-KpnI digested to excise the 5'-fragment for replacement. Using pCS2-XsalF as a template, XsalFΔ12 was created by PCR amplification using: 5'-CGATTGCGATTTCA-
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TTTCTGACTGTGATAAATCTCTACC-3' and 5'-ATGCTTACTCTTACC-3' and 5'-ATGCTTATCTCCTCAGAGCGCAGGAAGC-3'. The PCR product was digested with EcoRI (5'-end of cDNA) and Bpl (nucleotide 1106) and ligated into pCS2-XsAlF that was also EcoRI-Bpl-digested. The GAL4DB and eukaryotic GST expression constructs for N-terminal Sall1 (1-200) and Sall1 (1-200); GAL4DB-S2A (5'-GGGGGAATTCCATGGCCGCGCGGAGGAAGC-3') and 5'-GGCTTCTCCGCGCCATGGATCC-3'. GAL4DB-S2E (5'-GGGGGAATTCCATGGCCGCGGAGGAAGC-3') and 5'-GGCTTCTCCGCGCCATGGATCC-3'). The serine point mutants were also created in the context of XsAlF using pCS2+ XsAlF as the template. The following oligonucleotides were used for the mutagenesis of XsAlF: S2A (5'-GGGGGAATTCCATGGCCGCGGAGGAAGC-3' and 5'-GGCTTCTCCGCGCCATGGATCC-3'). The sense and antisense synthetic oligonucleotides from mouse Gaet were synthesized by Invitrogen and were annealed by heating the oligonucleotides at 95 °C for 5 min and then cooled gradually at room temperature. The annealed oligonucleotides were 32P-end labeled [γ-32P]ATP (3000 Ci/ml) using T4 polynucleotide kinase, and unincorporated nucleotides were removed using Sephadex G-25 columns (Roche Applied Science). The DNA sequences for the Gbx2 gel shift probes are as follows: Wild-type Gbx2-5' -CAATCCAGAGGCGTCGCAACCCCCCCCCCCCATTCCTCCCTGAAGATGGAGCAGATTTTAATTATCGTGGCTGCTTCCTCCGCGCCATTCGTGGCAGGACGAAGCAGGAGGAAAGCAGGAAGC-3' and 5'-CCGCAATCATCGGCGCGGGAAGCGCAACCCGt3' and 5'-GCTTCCGCGCCATGGATCCGTAATCTCCTCCGCGCCATGGATCCGTAATCTCCTCCGCGCCATGGATCCGTAATCTCCTCCGCGCCATGGATCCGTGGCAGGACGAAGCAGGAGGAAAGCAGGAAGC-3'. Mutations were verified by DNA sequencing.

Reporter Assays—COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. The cells were plated in 6-well plates at a density of 1 × 10^5 cells/well and transfected using FuGENE HD (Roche Applied Science) according to the manufacturer's directions. For reporter assays, cells were transiently transfected with 1 μg of Flu or GAL4DB fusion plasmid, 2 μg of luciferase reporter plasmid, and 0.05 μg of cytomegalovirus-β-galactosidase. Lysates were prepared 48 h after transfection and assayed for luciferase and β-galactosidase activity using a Turner Biosystems luminometer according to the manufacturer's protocols with 12 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 or Flu 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.

Electrophoretic Mobility Gel Shift Assays—The gel-shift assays were performed according to the gel-shift assay system (Promega) with minor modifications. Nuclear extracts were prepared by transiently transfecting COS-1 cells with 1 μg of Flu or Sall1-Flu as described. After 48 h, nuclear protein was extracted using the NE-PER Nuclear and Cytoplasmic Extraction kit according to the manufacturer's instructions (Pierce). The sense and antisense synthetic oligonucleotides from mouse Gbx2 were synthesized by Invitrogen and were annealed by heating the oligonucleotides at 95 °C for 5 min and then cooled gradually at room temperature. The annealed oligonucleotides were 32P-end labeled [γ-32P]ATP (3000 Ci/ml) using T4 polynucleotide kinase, and unincorporated nucleotides were removed using Sephadex G-25 columns (Roche Applied Science). The DNA sequences for the Gbx2 gel shift probes are as follows: Wild-type Gbx2-5' -CAATCCAGAGGCGTCGCAACCCCCCCCCCCCATTCCTCCCTGAAGATGGAGCAGATTTTAATTATCGTGGCTGCTTCCTCCGCGCCATTCGTGGCAGGACGAAGCAGGAGGAAAGCAGGAAGC-3' and 5'-CCGCAATCATCGGCGCGGGAAGCGCAACCCGt3' and 5'-GCTTCCGCGCCATGGATCCGTAATCTCCTCCGCGCCATGGATCCGTAATCTCCTCCGCGCCATGGATCCGTGGCAGGACGAAGCAGGAGGAAAGCAGGAAGC-3'. Mutations were verified by DNA sequencing.

Immunoprecipitations—P19 cells were maintained in a-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% confluency in 1 ml per 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). COS-1 cells were maintained as described above. 500 µg of total protein lysate was precleared for 1 h at 4°C using Protein G-Sepharose beads (Sigma). Precleared lysate was mixed with 500 µl of Nonidet P-40 lysis buffer and 5 µg of mouse monoclonal antibody against Sall1 or anti-FLAG. 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 (Santa Cruz Biotechnology), and RbAp46/48 (15G12, Genetex); Miß2 (CHD4) was graciously provided by D. Murray (NIA, National Institutes of Health) protein kinase C recognizing the conventional isoforms, α and β (Sigma), and a phosphoserine antibody (Qiagen). A purified monoclonal Sall1 antibody previously described (10) was used for the immunoprecipitation of Sall1.

**Identification of Phosphorylated Sall1-Flu Using Pro-Q Diamond Phosphoprotein in Gel Stain**—Phosphoproteins were detected after separation on 5% SDS-PAGE gel by following the instructions from the manufacturer (Molecular Probes). In brief, SDS-PAGE gels were fixed in a solution containing 50% methanol and 10% acetic acid, washed with several changes of water to remove SDS, and stained with the Pro-Q Diamond dye. After destaining, the gel images were analyzed using a 2UV Transilluminator BioDoc-It System (UVP). After destaining, the gel images were analyzed using a 2UV Transilluminator BioDoc-It System (UVP).

**In Vivo Labeling with [γ-32P]Orthophosphate**—COS-1 cells were cultured and transfected as described above. 48 h after transfection, the cells were washed with phosphate-free Dulbecco’s modified Eagle’s medium and incubated for 4 h with the same medium containing (0.3 mCi/well) [γ-32P]orthophosphate (carrier-free, PerkinElmer Life Sciences). Labelling was stopped by washing the cells twice with ice-cold phosphate-buffered saline. The cells were then incubated for 1 h on ice in Nonidet P-40 lysis buffer as described above. Incorporated [32P] was analyzed by autoradiography.

**Two-dimensional Phosphopeptide Mapping**—Phosphopeptide mapping was performed as described (20). Briefly, COS-1 cells were grown, transfected, labeled with [32P] and immunoprecipitated as described above. Phosphopeptide bands were identified by autoradiography and cut by aligning the image with radioactive marker spots. The bands were then rehydrated in 400 µl of 50 mM ammonium bicarbonate and digested with 30 µg of TPCK-treated trypsin (Worthington). The digested peptides were removed from the acrylamide, washed twice with 50 mM ammonium bicarbonate, and concentrated in a Speed-Vac. The peptides were washed four times with 1 ml of ddH₂O and twice in pH 1.9 buffer (2.8% formic acid, 7.8% glacial acetic acid). Equal counts of each sample were resuspended in 4 µl of pH 1.9 buffer, spotted onto cellulose TLC plates, and run in the first dimension in pH 1.9 buffer on a Hunter Thin Layer Electrophoresis apparatus (HTLE 7000, CBS Scientific, Inc.) for 35 min at 1300 V. Plates were dried and rotated 90 degrees and run in the second dimension in isobutyric acid buffer (62.5% isobutyric acid, 1.9% n-butanol, 4.8% pyridine, 2.9% glacial acetic acid) in a TLC tank. The plates were removed when the liquid phase migration was 1 cm from the top of the plates and exposed to a phosphorimagining screen for visualization and analysis.

**In Vitro Phosphorylation Assay**—COS-1 cells were transfected with Flu-Sall1 or Flu-Sall1S2A and immunoprecipitated as described above. The immunocomplexes were washed three times in Nonidet P-40 buffer and resuspended in 40 µl of ddH₂O. 10 µl of kinase buffer (120 mM Tris, pH 7.5, 60 mM MgCl₂, 6 mM CaCl₂) was added, followed by 5 µl of phosphatidyserine (1 mg/ml) and 2.0 µl (0.05 µg) of purified PKC (Pro-mega). The samples were briefly vortexed, 1 µl of γ-32P (10 mCi/ml) was added, and the mixture was incubated for 5 min at 30°C. Following the incubation, 1 µl of 1× unlabeled ATP was added, and the mixture was incubated for an additional 30 min. Samples were diluted with 2× SDS-sample buffer, boiled for 10 min, and resolved by SDS-PAGE. The gels were dried and analyzed by autoradiography.

**RESULTS**

**NuRD Recruitment Is Required for Sall1-mediated Repression in Vivo**—Our previous work identified a highly conserved 12-amino acid motif in Sall1 (SRM) that is necessary and sufficient for the recruitment of the NuRD complex (Fig. 1). Although our biochemical analysis revealed an important biological role for the peptide motif (10), the in vivo significance of the SRM remained unknown. To test the importance of the SRM in vivo, we performed microinjection manipulations in Xenopus embryos. Published studies revealed that the overexpression of XsalF, the Xenopus ortholog of Sall1, suppresses the expression of Gbx2, an anterior hindbrain gene in Xenopus embryos (15). Because XsalF contains the SRM (Fig. 1), we tested if the repression of Gbx2 by XsalF requires the NuRD recruitment motif. Injection of wild-type XsalF mRNA along with β-galactosidase as a lineage tracer into two unilateral animal blastomeres suppressed Gbx2 as previously described (82%, n = 62, Fig. 1B) (15). In contrast, the injection of mRNA encoding XsalF with the SRM deleted (XsalF12) resulted in a failure to repress Gbx2 expression (60.4%, n = 53, Fig. 1D) or resulted in significant unilateral expansion (32.1%, n = 53, Fig. 1C) of Gbx2 expression. The un.injected (right) side of the injected embryos reveals comparable Gbx2 expression when compared with the control uninjected embryos (Fig. 1A). Because the first cleavage in Xenopus embryos usually determines bilateral symmetry, RNAs are incorporated unilaterally into one side of the embryo, so injected and uninjected sides can be directly compared in the injected 8-cell embryos. 6% of the embryos could not be scored, and none of the XsalF12-injected embryos revealed the suppression of Gbx2 as shown in the wild-type XsalF-injected embryos. We thus conclude that the NuRD recruitment motif is required for repression of a Sall1 target in vivo. Furthermore, deletion of the SRM has dominant-negative or gain-of-function activity resulting in increased Gbx2 expression.

**Sall1 Regulates Gbx2 through the Recognition of Sall1 Binding Sites**—To determine if Sall1 binds to Gbx2 directly, electrophoretic mobility shift assays (EMSA) were performed using a DNA probe of Gbx2 sequence end-labeled with
Regulated Expression of Sall1 Targets

Sall1  **M**SRR**Q**K**R**PQ**H**F**
SEM-4  -SRR**Q**K**S**KP**R**RM
XsalF  **M**SRR**Q**K**R**P**Q**L
Sall2Alt  MAQETGSSRLG

 FIGURE 1. Expression of Gbx2 depends on NuRD recruitment. Alignment of the extreme N termini of mammalian Sall family members 1–4, and the Sall orthologs, Sem-4 (C. elegans) and XsalF ( Xenopus), reveals the sequence conservation within the first 12 amino acids of the N terminus. The conserved residues are shown in boldface type. *, denotes the critical residues in Sall1 that are required for repression and NuRD recruitment (10). Although amino acid 12 is not conserved, it is always hydrophobic and is required for Sall1-mediated repression. An alternative splice form of Sall2 (Sall2Alt) encodes a different 5’ exon, resulting in a dissimilar N terminus. Sequences were obtained from the NCBI data base and have the following accession numbers: M. musculus, NP_056587 Sall1; C. elegans, Sem-4; AAB03333; M. musculus Sall2Alt, AJ007396; and X. laevis XsalF, AA579483. 400 pg of capped RNA (XsalF or XsalFΔ12) was injected into two left animal blastomeres. Co-injection of β-galactosidase verified the site of injection, and embryos were harvested at the neurula stage (A–D). Whole mount in situ hybridization for Gbx2 revealed reduced expression in the XsalF-injected side (left) compared with the un.injected (right) side in 82% of embryos (B, 82%). Deletion of the SRM (XsalFΔ12) abolished this repression (D, 60%) or resulted in increased Gbx2 expression (C, 32%). 6% could not be scored. Control embryos were not injected (A, con). Arrows indicate the site of injection.

[32P]orthophosphate and containing recently described in vitro binding sites of Sall1 (ATAA(A/T)(A/T)) (21). Although Sall1 has been shown to associate with these sites in heterochromatin (21), the role of these DNA recognition sites in the recruitment of Sall1 to endogenous target genes is not known. We scanned Gbx2 sequence for the Sall1 binding sites and identified two that exactly match the consensus, ATAAAAA and ATAAATT, separated by only 16 bp (Fig. 2A). These Sall1 binding sites are absolutely conserved between Mus musculus and Homo sapiens and are contained within a 126-bp region that is 93% conserved between these species. As revealed in Fig. 2B, lane 3, a single prominent DNA-protein complex was identified with nuclear extracts prepared from COS-1 cells expressing Flu epitope-tagged Sall1 (Flu-Sall1). In contrast, the specific DNA-protein complex is not present in control COS-1 cells that are transfected with empty Flu expression plasmid (pcDNA3) (Fig. 2B, lane 2). Consistent with this result, two affinity-purified antibodies that recognize different epitopes of Sall1 supershifted the Sall1-bound DNA probe (Fig. 2B, lanes 4 and 5). To further test the specificity of this interaction, binding competition was performed with 10-fold molar excess of unlabeled probe. In the presence of cold competitor the specific DNA-protein complex is not detected (Fig. 2B, lane 6). We also tested the same Gbx2 probe containing point mutations in both Sall1 binding sites (Fig. 2A). The same amount of cold mutant probe competed less efficiently than wild-type probe indicating a reduced affinity of Sall1 for the mutated recognition sequences (Fig. 2B, lane 7). These results reveal that Sall1 specifically interacts with Gbx2 through binding of specific DNA recognition sites (ATAAAA and ATAAATT).

Sall1 Associates with NuRD to Directly Regulate Gbx2 in Vivo—To examine whether Sall1 and NuRD directly bind Gbx2 in vivo, we performed quantitative ChIP. This method involved formaldehyde cross-linking of P19 embryonic carcinoma cells that endogenously express Sall1 and all the NuRD components (10). Chromatin was sheared to 0.2- to 1-kb fragments, precipitated with nonspecific IgG or specific antibodies against Sall1 and the NuRD specific component, MBD3, and analyzed by qPCR. The qPCR primer sets were designed to scan 1 kb around the Sall1 binding sites that were bound in the EMSA experiments. The results of three independent ChIP experiments revealed significant enrichment of Sall1 binding at −0.7-kb upstream of the transcriptional start site and spanning −1 kb (−1620 bp to −660 bp). Notably, this region contains the AT recognition sequences that are bound by Sall1 in the EMSA experiments. The control IgG antibody did not enrich at this same domain further indicating the specificity of Sall1 binding (Fig. 3A). In comparison, the ChIP assay revealed significantly weaker or no enrichment of Sall1 at more distal regions located −4.6 and −5.4 kb upstream of the Gbx2 transcriptional start site. These regions do not contain any AT recognition sequences and are separated from the Sall1-enriched region by at least 1000 bp thereby correlating with the distribution of sonicated fragments in the ChIP reactions (200–1000 bp). As a negative control, Sall1 binding to Gapdh was comparable to the control IgG immunoprecipitation further indicating specificity (Fig. 3A). These results reveal that Gbx2 is specifically bound by Sall1 in vivo.

To examine whether NuRD binding is specific to the same Gbx2 sequence bound by Sall1, MBD3-precipitated DNA was also analyzed by qPCR. The MBD3 immunoprecipitation revealed that the NuRD-specific component is bound to the same −0.7 kb region of Gbx2 as Sall1. Similarly, MBD3 did not reveal significant enrichment for the more distal regions of Gbx2 or Gapdh as shown for Sall1 (Fig. 3A). Previously, we revealed that endogenous Sall1 and all NuRD components associate in P19 cells (10). Our ChIP analysis further reveals co-occupancy of endogenous Sall1 and a NuRD-specific component on chromatin.

To investigate whether Sall1 and NuRD have a direct role in repression at the Gbx2 locus, we cloned the ChIP-enriched sequence of Gbx2 (nucleotides −1620 to −660 bp) upstream of a heterologous promoter-driving luciferase (pGL3SV40-luc). Sall1-Flu was co-expressed with the Gbx2 reporter construct in
COS-1 cells and assayed for repression activity. Sall1 repressed the $Gbx2$-containing reporter by 12.5-fold. The repression activity was noticeably reduced to 2-fold when the reporter was co-expressed with a chimeric construct that was created by replacing the 12-amino acid motif of Sall1 with those of a naturally occurring splice variant of Sall2, Sall2Alt, that encodes for a dissimilar N terminus. Similarly, deletion of the SRM abrogated repression of the $Gbx2$ reporter (Sall1/H9004/H9012) to 1.8-fold (Fig. 3B). Both the chimeric and deletion construct resulted in a loss of repression that is statistically significant ($p < 0.05$). These results are consistent with our Xenopus experiments and further reveal a requirement for the NuRD recruitment motif to mediate the repression of $Gbx2$.

To further examine the requirement of Sall1 binding to the $Gbx2$ promoter for repression activity, we tested the same $Gbx2$ minimal repression domain of Sall1-(2–136), because we previously showed that this domain is sufficient for repression and NuRD recruitment (10). The GAL4 fusion protein was 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 to assess the repression function of the serine point mutants S2A- and S2E-(2–136). In agreement with our previous results (10), mutating the serine to an alanine, Sall1-(2–136) S2A does not have a significant affect ($p > 0.05$) or modestly increased transcriptional repression. In contrast, the phosphomimetic substitution S2E significantly abolishes repression (Fig. 4A, $p < 0.05$). We tested an additional phosphomimetic substitution in which the serine was mutated to an aspartic acid (S2D). This point mutant also abolished repression (data not shown). We also tested the serine point

**FIGURE 2.** Sall1 requires DNA binding sites, ATAAAA and ATATT, for interaction with the $Gbx2$ locus. **Top,** $Gbx2$ wild-type and mutant probes used in EMSAs. The $Gbx2$ mutant probe contains point mutations in the Sall1 DNA binding sites which are underlined. **Bottom,** EMSA showing Sall1 binding to the $Gbx2$ probe. Lane 1 contained the designated wild-type $Gbx2$ probe alone, and lane 2 (mock) indicates the same probe with nuclear extract expressing empty Flu expression plasmid (pcDNA3) as a control. Lanes 3–7 indicate nuclear extract prepared from COS-1 cells transfected with full-length Flu-Sall1. Supershift assays were performed using affinity-purified antibodies that recognize different epitopes of Sall1 (lanes 4 and 5), and a cold competition assay was performed with 10-fold molar excess $Gbx2$ wild-type probe (lane 6) or 10-fold molar excess of $Gbx2$ mutant probe (lane 7).
Regulated Expression of Sall1 Targets

A

![Graph showing expression levels of Sall1, MBD3, and IgG.](Image)

Gbx2

-5.4 kb

-4.6 kb

-0.7 kb

| % INPUT |
|---------|
| 3.5     |
| 3.0     |
| 2.5     |
| 2.0     |
| 1.5     |
| 1.0     |
| 0.5     |
| 0.0     |

Gapdh

| % INPUT |
|---------|
| 3.5     |
| 3.0     |
| 2.5     |
| 2.0     |
| 1.5     |
| 1.0     |
| 0.5     |
| 0.0     |

B

![Graph showing fold repression of Gbx2-Luc and Sall1.](Image)

Sall1

12.5

Sall2/Sall1 Chimera

2.0

Sall1Δ12

1.8

ATmut1

1.9

ATmut2

0.8

Fold Repression

In Figure 3, Sall1 and NuRD directly bind Gbx2 in vivo to mediate repression. ChIP assays were performed on P19 cells with anti-Sall1 (gray bars), anti-MBD3 (horizontal lines), or a nonspecific IgG control antibody (black bars). A, immunoprecipitated DNA was analyzed by qPCR amplification. ChIP analysis revealed Sall1 and MBD3 binding to a Gbx2 region at −0.7 kb; the −4.6 kb and −5.4 kb regions of Gbx2 and Gapdh served as negative controls. The qRT-PCR results represent ±S.D. derived from three independent experiments. B, COS-1 cells were transfected with a pGL3-promoter reporter containing a 960-bp promoter region of Gbx2 (~1620 bp to −660 bp) and the indicated Flu-epitope tagged Sall1 constructs consisting of wild-type Sall1, Sall2Sall1 chimera where the first 12 amino acids are altered, and a Sall1 construct (Δ12) where the first 12 amino acids are deleted. COS-1 cells were also transfected with mutant pGL3-promoter reporters and Flu-Sall1. ATmut1 contains a mutation in the first AT recognition sequence (ATAAA to ACAAAA), and ATmut2 contains a mutation in the second AT recognition sequence (ATATT to ACAACC). The -fold repression was calculated by dividing the normalized luciferase activity of COS-1 cells expressing Flu alone by the activity of the Sall1 expression plasmids and is expressed as the mean ± S.D. for triplicate transfections of three independent experiments. Sall1 immunoblot reveals equal expression of each Sall1-flu fusion protein for each transfection condition.

Identification That Sall1 Is Phosphorylated at Serine 2—Because the phosphomimetic mutant, S2E, exhibited a significant effect on NuRD recruitment and repression activity, we examined the phosphorylation of Ser-2 by expressing the following GST-Sall1 fusion peptides in COS-1 cells: Sall1-(2–12) containing the SRM, Sall1-(2–12) S2A, and Sall1-(2–12) S2E. The cells were then radiolabeled with [32P]orthophosphate. The GST-Sall1 proteins were purified on glutathione- Sepharose and analyzed by SDS-PAGE followed by autoradiography for [32P]incorporation. We found that the SRM, consisting of only amino acids 2–12 fused to GST, but not GST alone, was labeled. The S2A and S2E point mutants within the context of the SRM were expressed equivalently to Sall1-(2–12) containing the SRM, Sall1-(2–12) S2A, and Sall1-(2–12) S2E. The cells were then radiolabeled with [32P]orthophosphate. The GST-Sall1 proteins were purified on glutathione-Sepharose and analyzed by SDS-PAGE followed by autoradiography for [32P]incorporation. We found that the SRM, consisting of only amino acids 2–12 fused to GST, but not GST alone, was labeled. The S2A and S2E point mutants within the context of the SRM were expressed equivalently to Sall1-(2–12), yet they both completely abolished [32P] incorporation (Fig. 5A). In the context of the SRM, Ser-2 is the only potential phosphoacceptor, thus the GST-Sall1-(2–12) constructs reveal that phosphorylation of the SRM occurs exclusively on Ser-2 when expressed as an SRM fusion peptide in cell culture.

To determine if Ser-2 is phosphorylated in the context of full-length Sall1, we performed phosphopeptide analyses. Flu-
Figures and Tables

Figure 4. S2E phosphomimetic mutation of the Sall1 repression motif prevents repression and NuRD recruitment. In A: Left panel, COS-1 cells were transfected with the indicated GAL4DB-Sall1 fusion proteins and a reporter with lucifase under control of the SV40 promoter and 5 upstream GAL4 binding sites. The fold repression was calculated as luciferase activity relative to GAL4DB alone and is expressed as the mean ± S.D. for triplicate transfections of three independent experiments. Right panel, COS-1 cells were also transfected with GST fusions corresponding to the GAL4 fusions of Sall1. GST pulldown of GST Sall1N-(2–136) or GST Sall1N-(2–136) with S2A and S2E point mutations were analyzed for association with selected NuRD components by Western blot using antibodies specific to HDAC1, HDAC2, RbAp46, RbAp48, and Mi-2. B, P19 cell extracts were immunoprecipitated (IP) with a control FLAG or monoclonal Sall1 antibody and separated by SDS-PAGE. The gel was then either transferred and probed with a polyclonal Sall1 or phosphospecific antibody or stained with Pro-Q Diamond Phosphoprotein in Gel Stain to reveal that Sall1 exists as a serine phosphoprotein in vivo.

The S2E Phosphomimetic Mutation Disrupts Sall1-mediated Repression of Gbx2 in Vivo—Our studies reveal that, in cell culture, serine 2 of the SRM is phosphorylated and a phosphomimetic substitution disrupts NuRD recruitment and Sall1-mediated repression. To determine if phosphorylation of the SRM regulates Sall1 targets in vivo, we tested the effect of the serine point mutants, S2A and S2E, on Gbx2 expression in Xenopus embryos. The serine point mutant, S2A, was expressed as capped RNA in the context of full-length XsalF and injected into two left animal blastomeres of 8-cell Xenopus embryos. The microinjection manipulations revealed that the S2A mutant is capable of repressing Gbx2 expression in the majority of injected embryos (53.85%, n = 52 Fig. 8A, panel I). In contrast, the phosphomimetic mutant, S2E, reveals a result similar to XsalFΔ12 with a failure to suppress Gbx2 expression (Fig. 8C). The S2E embryos showed no change (59.18%, n = 49).
To further examine the repression function of the phospho-
mimetic mutant (S2E), we compared the repression activity of
Sall1-Flu, S2A-Flu, and S2E-Flu. These Flu fusions were trans-
fected with the Gbx2-luciferase reporter in COS-1 cells and
assayed for repression activity. Sall1 repressed the Gbx2-con-
taining reporter by 4.3-fold, whereas the phosphomimetic, S2E,
significantly abolishes repression (p < 0.05). In comparison,
mutating the serine to an alanine did not have a significant
effect on transcriptional repression (p > 0.05) (Fig. 8B). These
results are consistent with our analysis of the serine point
mutants using the heterologous system (Fig. 4A) and strongly
suggest that the phosphorylation of Ser-2 plays a general role in
regulating Sall1-mediated repression of an endogenous target
gene.

**DISCUSSION**

Our work provides the first direct evidence that Sall1 func-
tions as a potent transcriptional repressor to directly regulate
target gene expression. We describe an in vivo role for the SRM
by demonstrating the requirement for NuRD recruitment in
mediating repression of Gbx2. We also demonstrate the impor-
tance of recently described DNA recognition sites in the bind-
ing of Sall1 to the Gbx2 locus to mediate transcriptional repres-
sion. Furthermore, we reveal that Sall1 interacts with and is
phosphorylated by PKC thereby uncovering a potential mech-
anism for the regulation of Sall1-mediated repression that is
driven by post-translational modification of the SRM.

Biochemical analysis of Sall1-mediated repression has relied
extensively on the use of a heterologous system, because direct
target genes of Sall1 had not been identified. To our knowledge,
our studies provide the first evidence of a bone fide Sall1 target
gene. Previous studies have identified the localization of Sall1 to
heterochromatin (21, 26–28) suggesting that Sall1 may func-
tion similarly to the C2H2 transcription factor, Ikaros, which
has been shown to redistribute target genes to heterochromatin
(29, 30). Thus, the discovery of a Sall1 target gene will allow
future elucidation of the mechanism of Sall1-mediated repres-
sion at the chromatin level.

Previous research has revealed that Sall1 and NuRD play a
role in the regulation of gene expression during development in
many species, including Drosophila, C. elegans, and mouse
(7–9, 31–34). These studies suggest the biological significance

**FIGURE 5. Phosphorylation of Sall1 at serine 2.** A, COS-1 cells were trans-
fected with GST-Sall1-(2–12) or GST-Sall1-(2–12) S2A or S2E point mutants.
The cells were radiolabeled with [32P]orthophosphate for 4 h prior to harvesting
the lysates. GST-Sall1 constructs were complexed to glutathione, sepa-
rated by SDS-PAGE, and analyzed by autoradiography to localize phospho-
rylation to Ser-2 of the SRM. B, phosphopeptide mapping of Sall1. COS-1 cells
were radiolabeled with [32P]orthophosphate after transient transfection with
Flu-tagged Sall1-(2–1322) or Flu-tagged Sall1S2A-(2–1322). Purified [32P]-la-
beled Sall1 was digested with trypsin, and the resulting phosphopeptides
were separated in two dimensions: by electrophoresis and by ascending
chromatography. The autoradiograms of the resulting phosphopeptide
maps are shown. The left panel shows a phosphopeptide map of full-length
Flu-Sall1-(2–1322) revealing five phosphopeptides (1–5). The right panel
shows that point mutagenesis of Ser-2 to alanine eliminates the phosphos-
phorylation of peptides 1–3 confirming this site as a phosphorylation site of Sall1.
This figure is representative of three independent experiments.

**FIGURE 6. Mutation of serine 2 of the SRM disrupts the incorporation of
32P.** A, COS-1 cells were radiolabeled with [32P]orthophosphate after tran-
sient transfection with Flu-tagged Sall1-(2–1322) or Flu-tagged Sall1S2A-(2–
1322). An anti-FLAG control immunoprecipitation was performed on a cell
lysate expressing Flu-tagged Sall1-(2–1322). Purified [32P]-labeled Sall1 and
S2A were separated on 5% SDS-PAGE and analyzed by autoradiography.
B, after the products were run on 5% SDS-PAGE and transferred to 3MM What-
man paper, the phosphorylated Sall1 and S2A bands were excised, and radio-
activity was measured by using a scintillation counter. The specific activity of
[γ-32P]ATP in the reaction mixtures was 2000 cpm/pmol ATP. The amounts of
Sall1 protein and incorporation of 32P into Sall1 and S2A are included in the
table.
and possible convergent roles of Sall1 and NuRD during organogenesis. In our ChIP assays, both Sall1 and NuRD directly bind an identical region of Gbx2, suggesting the possibility that they function together to repress transcription of a gene that is critical for hindbrain development. Because Sall1 and NuRD are expressed during neuronal development this raises the possibility that the NuRD complex may be implicated in the regulation of developmental targets by Sall1. Future studies will require linking these biochemical and in vivo findings to the regulation of neuronal development by Sall1 in vivo.

Sall1 and Gbx2 are well defined transcriptional regulators that are critical for embryonic development (7–9, 35–38). Gbx2 is a homeobox-containing transcription factor that is required for the proper determination of the mid-hindbrain boundary in the neural tube (36, 38). Gbx2 has also been shown to be required for the development of the inner ear (39) and for normal arch artery development (38). Mutations in SALL1 result in Townes-Brocks syndrome, which is characterized by multiple birth defects, including hearing loss, cardiac anomalies, and mental retardation (3). These phenotypes implicate Sall1 in the development of multiple developmental processes that appear to share remarkable similarity with the developmental roles of Gbx2. Thus, Sall1 and Gbx2 may function in a pathway to regulate similar developmental processes.

Phosphorylation is one of the major mechanisms regulating the activity of transcription factors (40). Our discovery that serine 2 of the SRM is phosphorylated by PKC has potential implications for understanding the regulated recruitment and or activity of NuRD at target genes. We previously identified that all four Sall family members, Sall1–4, contain the NuRD recruitment motif, SRM, that is also present in five other families of zinc finger transcription factors. Of those that have been

FIGURE 7. Sall1 is phosphorylated by PKC. A, P19 cell extracts were immunoprecipitated (IP) with a control FLAG or monoclonal Sall1 antibody followed by Western blotting with an antibody to PKC. B, purified Sall-Flu-(2–1322) and Sall1S2A-Flu-(2–1322) were expressed in COS-1 cells and immunopurified. An anti-FLAG control immunoprecipitation was performed on a cell lysate expressing Sall-Flu-(2–1322). The purified proteins were then incubated in the absence or presence of purified PKC for 5 min at 30 °C, followed by the addition of 1 μl of 1 mm dATP for 30 min at 30 °C. The products were then run on 5% SDS-PAGE and analyzed by autoradiogram.

FIGURE 8. Phosphorylation of the SRM disrupts Sall1-mediated repression of Gbx2 in vivo. A, the serine point mutants, S2A and S2E, were created in the context of XsalF and expressed as capped RNA for injection into eight-cell Xenopus embryos. The injections and in situ hybridization experiments were performed exactly as described in the legend for Fig. 1. The microinjection manipulations revealed that the S2A mutant does not disrupt XsalF-mediated repression, revealing either a decrease in Gbx2 expression (A, panel II, 53.85%, n/H11005 52) or equal expression (data not shown). In contrast, the phosphomimetic mutant, S2E, reveals a result similar to XsalF/H900412 where disruption of NuRD recruitment results in a failure to suppress Gbx2 expression. The S2E embryos showed no change (A, panel IV, 59.18%, n = 49) or unilateral expansion (A, panel III, 36.73%, n = 49) in Gbx2 expression on the injected side. Control embryos were not injected (A, panel I, con). Arrows indicate site of injection. B, COS-1 cells were transfected with the indicated Flu-Sall1 fusion proteins and the Gbx2 (H11002 1620 bp to H11002 660 bp) luciferase reporter. The -fold repression was calculated as luciferase activity relative to Flu alone and is expressed as the mean ± S.D. for triplicate transfections of three independent experiments. C, table showing total number of injected Xenopus embryos for each mRNA and the effects on Gbx2 expression. The representative images are displayed in Figs. 1 and 8. Wild-type XsalF and S2A were both capable of repressing Gbx2 expression. In contrast, XsalFA12- and S2E-injected embryos showed a loss of repression or increased levels of Gbx2 expression.
Regulated Expression of Sall1 Targets

examined, Sall1, Sall4, friend of GATA (FOG1 and FOG2), and chicken ovalbumin upstream promoter transcription factor-interacting proteins (CTIP1 and CTIP2) have been shown to mediate transcriptional repression and associate with NuRD (10, 12, 13). Despite a strong interaction with NuRD, these same transcriptional regulators have also been shown to mediate activation (41–44). Our studies demonstrate that Ser-2 of the SRM is phosphorylated by PKC, and a phosphomimetic mutation of this residue impairs Sall1-mediated repression and the binding of NuRD. Because Ser-2 is present in all of the transcription factors containing the SRM, our findings suggest a potential regulatory mechanism that could regulate the recruitment of NuRD binding at specific genes to determine whether they are activated or repressed. Thus, the role of this phosphorylation event may have broader implications for regulating gene expression.

In summary, we have identified a novel target gene of Sall1. Our studies demonstrate an in vivo role for the SRM, which is critical for the NuRD-dependent regulation of this Sall1 target gene. Furthermore, we have discovered that phosphorylation of the SRM by PKC may explain how gene expression can be regulated by Sall1 in a dynamic fashion. By uncovering a mechanism of regulated NuRD recruitment by a sequence-specific DNA binding factor, future studies should enable us to define in greater detail how NuRD modulates gene expression at specific targets.

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