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LSH Cooperates with DNA Methyltransferases To Repress Transcription^{∇†}

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LSH, a protein related to the SNF2 family of chromatin-remodeling ATPases, is required for efficient DNA methylation in mammals. How LSH functions to support DNA methylation and whether it associates with a large protein complex containing DNA methyltransferase (DNMT) enzymes is currently unclear. Here we show that, unlike many other chromatin-remodeling ATPases, native LSH is present mostly as a monomeric protein in nuclear extracts of mammalian cells and cannot be detected in a large multisubunit complex. However, when targeted to a promoter of a reporter gene, LSH acts as an efficient transcriptional repressor. Using this as an assay to identify proteins that are required for LSH-mediated repression we found that LSH cooperates with the DNMTs DNMT1 and DNMT3B and with the histone deacetylases (HDACs) HDAC1 and HDAC2 to silence transcription. We show that transcriptional repression by LSH and interactions with HDACs are lost in DNMT1 and DNMT3B knockout cells but that the enzymatic activities of DNMTs are not required for LSH-mediated silencing. Our data suggest that LSH serves as a recruiting factor for DNMTs and HDACs to establish transcriptionally repressive chromatin which is perhaps further stabilized by DNA methylation at targeted loci.

In vertebrate genomes, DNA methylation patterns are established during gametogenesis, embryo development, and cell differentiation by enzymes of the DNA cytosine methyltransferase family, which includes the maintenance DNA methyltransferase (DNMT) DNMT1 and the de novo methyltransferases DNMT3A and DNMT3B (1, 24, 45). DNMT1 binds to PCNA and functions primarily during S phase to restore fully methylated CpGs on hemimethylated daughter DNA strands generated during DNA replication (3, 4). DNMT3A and -3B are able to methylate unmethylated DNA and in mouse embryogenesis are required during gastrulation, when DNA methylation patterns are established in differentiating cell lineages of the embryo (24). Mice lacking DNMT proteins die early during embryogenesis and display aberrant expression of retrotransposons and various imprinted and nonimprinted genes (14, 18, 24). Genetic studies with plants and mammals have revealed that additional factors besides DNMTs are required for the establishment of DNA methylation patterns in vivo. Loss-of-function mutations in SNF2 family-related putative chromatin-remodeling ATPases such as the *Arabidopsis thaliana* DDM1 (decrease in DNA methylation 1) protein and its murine homolog Lsh (lymphoid-specific helicase) lead to dramatic hypomethylation of the genome in *Arabidopsis thaliana* and mice, respectively (5, 16). Unlike animals that are null for DNMTs, Lsh-deficient mice develop to term but die soon after birth with symptoms of renal failure (5). Interestingly,

mice expressing a hypomorph allele of Lsh with targeted disruption of the SNF2 domain survive much longer and display modest hypomethylation of DNA and premature aging (39). Collectively, these studies indicate that the low levels of DNA methylation (~30 to 35% of the wild-type level) in Lsh-deficient mice are compatible with embryonic development.

It is largely unknown how DDM1 and Lsh function to assist DNA methylation. Given the homology of DDM1 and Lsh with chromatin-remodeling ATPases of the SNF2 family, it has been suggested that chromatin remodeling by DDM1 and Lsh may facilitate the processivity of DNMT enzymes on nucleosomal DNA templates (20, 28). In agreement with the requirement for SNF2-like proteins to support DNA methylation in vivo, several studies have demonstrated that in vitro DNMT enzymes methylate DNA assembled into chromatin 10- to 20-fold less efficiently than naked DNA (11, 25, 31). Whether DDM1 or Lsh is able to stimulate DNA methylation on nucleosomal templates in vitro has not been demonstrated. However, it was reported that DDM1 exhibits features common to the SNF2 family of chromatin-remodeling proteins, such as ATP hydrolysis upon stimulation by DNA or nucleosomes and active sliding of mononucleosomes on DNA templates in vitro (2).

The biochemical properties of mammalian Lsh protein have not been investigated in detail, nor has it been determined whether, similar to many other chromatin-remodeling ATPases, Lsh associates with a large protein complex (22). It is also unclear whether Lsh directly interacts with DNMTs and actively recruits them to chromatin. A recent study has found that Lsh coimmunoprecipitates with de novo DNMTs Dnmt3a and Dnmt3b and stimulates methylation of nonmethylated episomal plasmids introduced into mouse embryonic fibroblasts (45). No interaction between Lsh and maintenance methyltransferase Dnmt1 was detected in these assays, suggesting

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that Lsh is involved primarily in de novo DNA methylation of nonmethylated sequences (45).

To address some of these questions, we focused on the properties of human Lsh protein, also known as HELLS (for *helicase lymphoid-specific*) or PASG (for *proliferation-associated SNF2-like gene*). For convenience we refer to this protein as LSH throughout this paper. Using size exclusion chromatography and sedimentation in sucrose gradients, we demonstrate that native LSH can be detected mostly as a free monomeric protein in nuclear extracts of human cells. Thus, if LSH associates with other proteins, such complexes are either unstable or not very abundant. Nevertheless, when targeted to GAL4 binding sites upstream of a reporter gene promoter, LSH acts as an efficient histone deacetylase (HDAC)-dependent transcriptional repressor. The region of LSH required for transcriptional silencing maps to the N-terminal coiled-coil motif, suggesting that transcriptional silencing by LSH is independent of its putative chromatin-remodeling activity. We further demonstrate that LSH coimmunoprecipitates with the HDACs HDAC1 and HDAC2 and that the DNMTs DNMT1 and DNMT3B interact with LSH in vitro and are essential for recruitment of HDACs to LSH in vivo. In cells expressing N-terminally truncated DNMT1 or null for DNMT3B, HDACs do not efficiently coimmunoprecipitate with LSH, suggesting that stable association of HDACs with the rest of the LSH complex requires both DNMTs to be present simultaneously. Taken together, our data demonstrate that LSH forms a transient or not very abundant protein complex in vivo and directly recruits DNMT1, DNMT3B, and HDACs to establish transcriptionally inactive chromatin. Transcriptional silencing by the LSH complex does not immediately result in methylation of DNA, but LSH-mediated increase in the local concentration of DNMTs on chromatin may eventually lead to DNA methylation and further stabilize a silenced chromatin state.

MATERIALS AND METHODS

Size exclusion chromatography and sucrose gradients. A Superose 6/300GL gel filtration column was calibrated with gel filtration standards thyroglobulin (669 kDa; Stokes radius [R_s] = 8.5), apoferritin (443 kDa; R_s = 6.1), alcohol dehydrogenase (150 kDa; R_s = 4.55), bovine serum albumin (66 kDa; R_s = 3.55), and carbonic anhydrase (29 kDa; R_s = 2). One milligram of HeLa nuclear extract or 1 μ g of purified recombinant LSH was loaded onto a column pre-equilibrated with buffer GF150 (20 mM HEPES-KOH [pH 7.9], 3 mM MgCl₂, 10% glycerol, and 150 mM KCl). Fractions (0.5 ml) were collected, trichloroacetic acid precipitated, and separated on 7% or 10% polyacrylamide gels. Native and recombinant LSH and native BRG1 were detected by Western blotting using anti-LSH (sc-46665) and anti-BRG1 (sc-H88) antibodies (Santa Cruz). The R_s of LSH was derived from the plotted standards. For sucrose gradients, 50 μ g of carbonic anhydrase (2.8S), 50 μ g of bovine serum albumin (4.3S) 50 μ g of alcohol dehydrogenase (7.4S), and 30 μ g of β -amylase (9S) were loaded as standards through a 2-ml linear 5 to 20% sucrose gradient made in buffer NE2 without glycerol (20 mM HEPES-KOH [pH 7.0], 10 mM KCl, 1 mM MgCl₂, 0.5 mM dithiothreitol, 0.1% Triton X-100, 420 mM NaCl, and protease inhibitor mix [Sigma P8340]). The protein standard gradient together with an identical gradient loaded with 500 μ g HeLa nuclear extract or 1 μ g recombinant LSH was spun for 4 h at 50,000 rpm in a Beckman TLS-55 rotor at 4°C. Fractions (100 μ l) were taken from the top of the gradient and run on 10% polyacrylamide gels. Protein standards were detected by Coomassie blue staining, and LSH was detected by Western blotting. The sedimentation coefficient of LSH was derived from the plotted standards. Calculations to determine the molecular weight of native LSH were applied as described previously (35) using the equation $M_r = 6\pi\eta_{20,w} \cdot s_{20,w} \cdot R_s \cdot N / (1 - \rho_{20,w}v)$, where R_s is the Stoke's radius (nm), $s_{20,w}$ is the sedimentation velocity ($S \times 10^{-13}$), $\eta_{20,w}$ is the viscosity of water at 20°C

(0.01002 g \cdot s⁻¹ cm⁻¹), N is Avogadro's number ($6.022 \times 10^{23} \cdot$ molecules⁻¹), $\rho_{20,w}$ is the density of water at 20°C (0.9981 g \cdot cm³), and v is the partial specific volume of protein (used 0.725 cm³/g).

Plasmids and reporter assays. Full-length human LSH (kindly provided by Robert Arceci, The Johns Hopkins University School of Medicine) or LSH fragments corresponding to amino acids 1 to 226 and 227 to 838 as well as the GAL4 DNA binding domain were PCR amplified and cloned into the pCDNA 3.1 vector (Invitrogen). DNMT3B and DNMT1 were cloned into the pEGFP vector (Clontech). The catalytically inactive DNMT1^{c/w} point mutant was previously described (34) and was provided by Heinrich Leonhardt. DNMT3B^{c/s} was generated by site-directed mutagenic PCR. In reporter assays, GAL4-TK-LUC (500 ng) and pact- β geo (500 ng) plasmids were cotransfected with 500 ng of the indicated effector plasmids into 2.5×10^5 HCT116 cells or DNMT knockout (KO), cells kindly provided by Bert Vogelstein, using JetPEI reagent (Autogen Bioclear). To test whether LSH-mediated repression was sensitive to HDAC inhibitors, trichostatin A (TSA) was added to the tissue culture medium after transfection to a 100 nM final concentration 24 h prior to lysing the cells. For rescue experiments with HCT116 KO cell lines, 500 ng of DNMT1-green fluorescent protein (GFP) or DNMT3B-GFP plasmid was cotransfected with the reporter plasmids in the presence or absence of GAL4-LSH or GAL4-LSH(1–226). All transfection experiments were performed in triplicate. At 48 h post-transfection, cells were harvested using reporter lysis buffer (Promega), and detection of luciferase activity was carried out according to the manufacturer's guidelines. Luminescence was measured in a TD20/20 luminometer (Turner Designs). Detection of β -galactosidase activity in the same lysates (as described above) was carried out as described previously (44).

Coimmunoprecipitation experiments. Immunoprecipitations were performed from HCT116 nuclear extracts (400 μ g of total nuclear protein) with 4 μ g anti-LSH (sc-46665), anti-HDAC1 (sc-7872), anti-HDAC2 (sc-7899), polyclonal anti-GFP antibody (a kind gift from K. Sawin, Wellcome Trust Centre for Cell Biology, Edinburgh), anti-Myc (CRUK), or mock control antihemagglutinin (anti-HA) (CRUK) antibodies. Immunoprecipitated complexes bound to protein G beads were washed with NE150 buffer (20 mM HEPES-KOH [pH 7.0], 10 mM KCl, 1 mM MgCl₂, 0.5 mM dithiothreitol, 0.1% Triton X-100, 150 mM NaCl, and protease inhibitors) and run on 7% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gels were transferred to nitrocellulose membranes and proteins detected by appropriate primary antibodies (as described above), secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Sigma), and ECL reagent (Amersham). The antibodies used for the detection of DNMTs were anti-DNMT3B (PA1-884) and anti-DNMT1 (sc-20701).

In vitro pull-down assays. LSH fragments corresponding to amino acids 1 to 503 and 248 to 883 were cloned into the pGEX 4T1 plasmid in frame with glutathione *S*-transferase (GST) tag and designated GST-LSH-N and LSH-C, respectively. pGEX-HDAC1, -2, and -3 were provided by Ronald Evans (Salk Institute, La Jolla, CA) and GST-GFP by Ken Sawin. All GST fusion proteins were expressed in *Escherichia coli* BL21(DE3) and purified on glutathione-Sepharose by standard methods. Full-length DNMT3B and a fragment of DNMT1 corresponding to amino acids 1 to 1125 were cloned into pGAD-T7Rec (Clontech) in frame with an HA tag. Full-length LSH was cloned into pGBK-T7 (Clontech) in frame with a Myc tag. One microgram of pGADT7-DNMT3B, pGADT7-DNMT1, or pGBK-LSH was translated in rabbit reticulocyte lysates (TnT T7 kit; Promega). One hundred nanograms of each GST fusion protein immobilized on glutathione-Sepharose beads and 5 μ l of the translation reaction mixtures were used in pull-down experiments performed in 100 μ l of NE2 buffer for 1 h at 4°C on a rotating wheel. The beads were washed four times with 700 μ l NE2 buffer, and LSH-bound DNMT proteins were detected on Western blots with appropriate antibodies. To detect interactions of LSH with DNMT1 in the presence of DNMT3B, 0.5 or 1 μ g of baculovirus-produced DNMT3B was added to the pull-down assay mixtures. LSH and DNMT3B were expressed in insect cells and purified as described previously (2, 38).

Chromatin immunoprecipitations. Chromatin immunoprecipitation assays were carried out as previously described (13). Briefly, HEK293 cells containing a stably integrated 5 \times GAL4-TK-LUC construct (13) were transiently transfected with 10 μ g of GAL4-LSH 226 or 10 μ g of pCDNA3.1 GALYBD control plasmid. At 48 h posttransfection, the cells were cross-linked with formaldehyde, sheared by sonication, and immunoprecipitated for 2 h at 4°C with the following antibodies: anti-HA control (CRUK), anti-H3 (Upstate; 05-499, lot 25198), anti-H3K9ac (Upstate; 07-352, lot 28741), anti-H4 (Upstate; 07-108, lot 25296), and anti-H4K12ac (Upstate; 07-595, lot 28885). Immunoprecipitated complexes were then captured on protein G Dynabeads (Invitrogen). Following extensive washing, DNA was recovered and 1 μ l of chromatin-immunoprecipitated DNA was used in 30 cycles of PCR. Primers for the 5 \times GAL4-TK promoter were

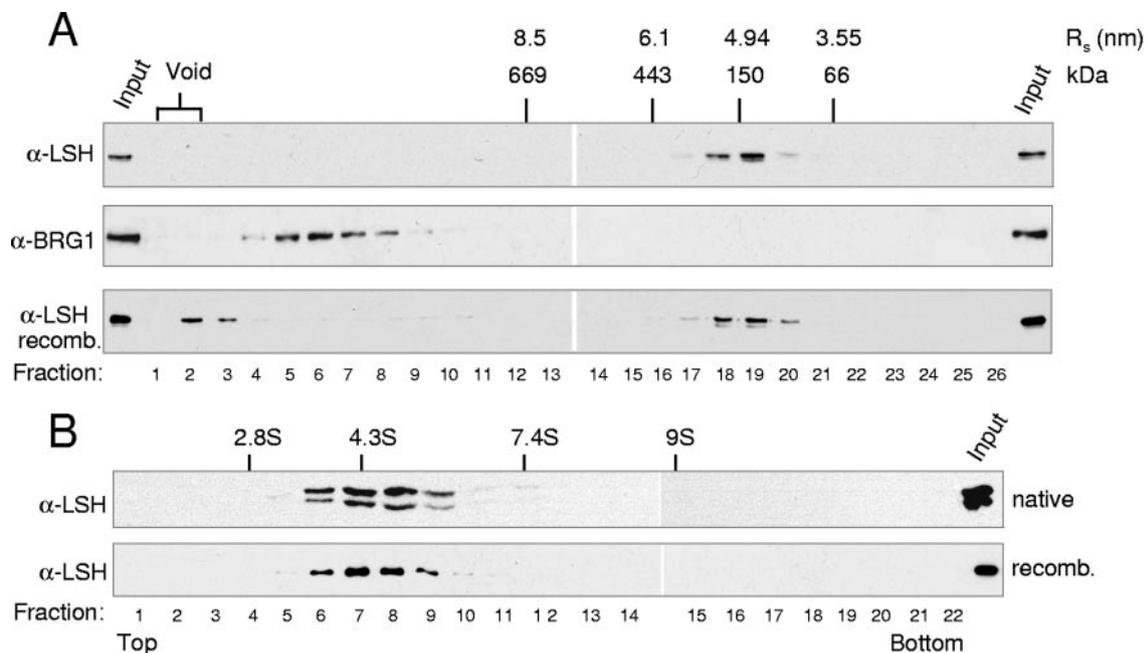


FIG. 1. Native LSH is a monomer in nuclear extracts of human cells. (A) HeLa nuclear extracts and recombinant LSH purified from insect cells were fractionated on a Superose 6 size exclusion column and run on sodium dodecyl sulfate-polyacrylamide gels. LSH and BRG1 proteins were detected in the collected fractions by Western blotting with appropriate antibodies. The molecular masses and Stokes radii (R_s) of marker proteins used to calibrate the column are indicated at the top. (B) HeLa nuclear extracts and recombinant LSH were fractionated in 5 to 20% sucrose gradients. LSH was detected in gradient fractions on Western blots with anti-LSH antibodies. Sedimentation coefficients of marker proteins are indicated above the blot.

5'-AATTGCTCAACAGTATGAACATTTC and 3'-CAATTGTTTTGTACGATCAAAGGA. Primers for the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) promoter were 5'-GAGGCTGTGAGCTGGCTGTC and 3'-CAGAGCAGAGTAGCAAGAGCAAGG.

RESULTS

Native LSH cannot be detected in a large protein complex.

Within the SNF2 family of ATPases, LSH is most closely related to the ISWI subfamily of chromatin-remodeling factors, which have diverse functions in transcriptional regulation (16, 20). Most SNF2 family members, including ISWI proteins, have intrinsic ATPase activity but usually associate with more than one additional polypeptide *in vivo* (8, 22). These additional subunits of chromatin-remodeling complexes either modulate the activity of SNF2 ATPases or target them to specific chromatin regions (22). Given the possibility that LSH may act as an accessibility factor for DNMTs and that mouse Lsh coimmunoprecipitates with Dnmt3a and Dnmt3b, we investigated whether LSH in human cells can be detected in a large protein complex. We fractionated HeLa nuclear extract by size exclusion chromatography and analyzed the elution profile of LSH by Western blotting. LSH appeared in four of the eluted fractions (fractions 17 to 20) with a peak corresponding to a molecular mass of about 150 kDa (Fig. 1A, top). In comparison, another chromatin-remodeling protein, BRG1, which is usually present within a 2-MDa complex (26, 36), eluted at a much higher molecular mass as expected (fractions 5 to 11) (Fig. 1A, middle). Since LSH eluted at an apparent molecular mass slightly greater than its theoretical mass of ~97 kDa, we considered the possibility that it may associate with a

relatively small protein(s). However, in a similar experiment recombinant LSH purified from insect cells eluted with a profile very similar to that of the native LSH, suggesting that the vast majority of LSH in HeLa nuclear extracts does not associate with additional proteins (Fig. 1A, bottom). Nuclear extracts from other human cell lines, cells synchronized in different stages of the cell cycle (see Fig. S1 in the supplemental material), and mouse embryonic stem (ES) cells, as well as extracts prepared from nuclear pellets, containing most of the insoluble chromatin, showed identical elution of LSH at ~150 kDa (not shown).

The molecular mass of a native protein or a protein complex can be determined accurately by an equation derived by Siegel and Monty, which combines the Stokes radius (a hydrodynamic radius of a molecule freely tumbling in solution) calculated from size exclusion chromatography with the sedimentation coefficient determined by separation in sucrose gradient (35). Based on the elution profile of protein standards from size exclusion chromatography we calculated the Stokes radius of LSH to be 4.94 nm. To establish the sedimentation coefficient of LSH, we fractionated HeLa and mouse ES cell nuclear extracts on 5 to 20% sucrose gradients and detected LSH in the gradient fractions by Western blots (Fig. 1B). In these experiments the sedimentation coefficient of both human and mouse LSH was calculated to be 4.5S, relative to protein standards. Applying the calculated Stokes radius and sedimentation coefficient of LSH in the Siegel-Monty equation, we derived a molecular mass of 91.5 kDa. The calculated molecular mass of native LSH is close to the predicted theoretical mass of LSH monomer (97 kDa), confirming that the vast majority of LSH

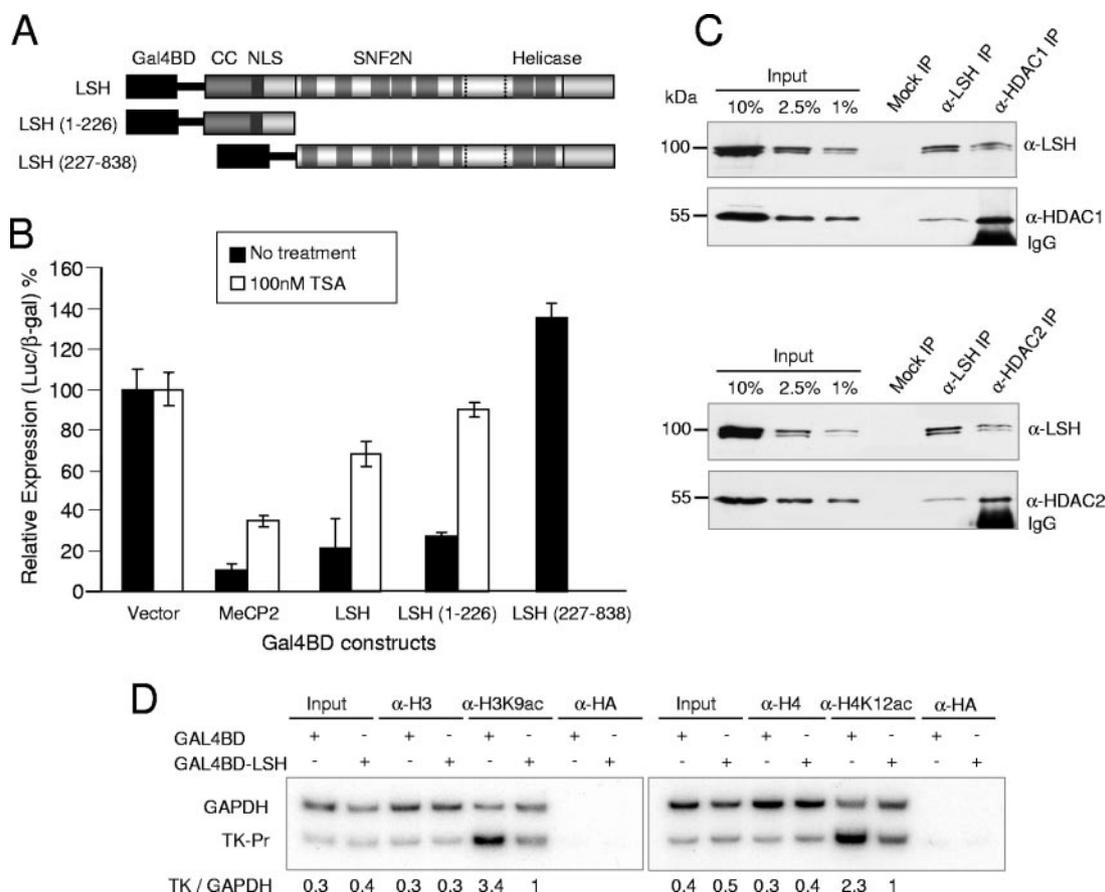


FIG. 2. LSH functions as an HDAC-dependent transcriptional repressor in vivo. (A) Schematic drawing of the full-length LSH and truncated LSH proteins fused to a GAL4BD. The functional domains of LSH, such as the coiled-coil domain (CC), the nuclear localization signal (NLS), and the eight conserved SNF2 motifs in the SNF2N and helicase domains, are indicated. (B) GAL4BD fusions of LSH were cotransfected into HCT116 cells with a luciferase reporter plasmid carrying GAL4 binding sites upstream of TK promoter and a control plasmid expressing β -galactosidase from an actin promoter. The relative expression of the reporters represents the ratio of luciferase to β -galactosidase products. MeCP2 was used as a control. The white bars represent experiments carried out in the presence of 100 nM TSA, which partially alleviates LSH- and MeCP2-mediated repression of the luciferase reporter. The error bars represent standard deviations of the means. (C) Coimmunoprecipitation experiments with anti-LSH, anti-HDAC1, and anti-HDAC2 antibodies from HCT116 nuclear extracts. Anti-HA antibodies were used in mock immunoprecipitations (IP) to control for nonspecific protein binding. (D) Chromatin immunoprecipitation with antibodies against acetylated H3-K9 and H4-K12. The TK promoter in cells transfected with GAL4BD-LSH is hypoacetylated compared to the GAPDH promoter used as an internal control. Anti-HA is a nonspecific control antibody.

protein in nuclear extracts of mouse and human cells is present as a free monomeric peptide.

LSH is an HDAC-dependent transcriptional repressor. Given that in fractionation experiments we failed to detect LSH within a large protein complex, we decided to explore whether LSH could interact transiently with other proteins. We initially intended to use a full-length GAL4 binding domain (GAL4BD)-tagged LSH in a yeast two-hybrid screen. However, we found that GAL4BD-LSH strongly repressed adenine and histidine genes in yeast reporter strains. The *Saccharomyces cerevisiae* genome encodes a protein, YFR038W, which shares 39% identity and 57% similarity with human LSH. Therefore, it was possible that the mammalian protein could mimic some of the protein-protein interactions of YFR038W that facilitate transcriptional repression. To investigate further whether LSH could act as a transcriptional repressor in mammalian cells, we cotransfected colorectal HCT116 cells with a plasmid expressing full-length LSH fused

to GAL4BD and two reporter plasmids. The first reporter plasmid carried five GAL4 binding sites upstream of a thymidine kinase (TK) promoter driving the expression of the firefly luciferase gene, while the other control plasmid lacked GAL4 binding sites and expressed β -galactosidase from an actin promoter. The effect of LSH on transcription from the targeted and nontargeted reporter was measured as a ratio of luciferase to β -galactosidase expression. A GAL4BD-tagged transcriptional repression domain (TRD) of methyl-CpG binding protein MeCP2, which is known to strongly repress transcription in such assays, served as a control (21). In these experiments the full-length GAL4BD-LSH as well as GAL4BD-MeCP2 consistently reduced the expression of the luciferase reporter to about 20 to 25% and 10%, respectively, of the levels observed in cells transfected with an empty vector (Fig. 2B). This suggests that, like MeCP2, LSH can function as an efficient transcriptional repressor when targeted to a promoter of a reporter gene in human cells.

To investigate whether a specific domain of LSH was responsible for transcriptional silencing, we tested several LSH deletion constructs in the luciferase reporter assay. Interestingly, the N-terminal portion of LSH (amino acids 1 to 226), containing a predicted coiled-coil motif, was sufficient to repress the reporter gene to levels comparable to those observed with full-length LSH. A polypeptide corresponding to the SNF2 and helicase domains of LSH (amino acids 226 to 238), did not significantly affect the expression of the luciferase reporter (Fig. 2A and B). These experiments indicate that the 226-amino-acid coiled-coil region in the N terminus of LSH functions as a TRD and is sufficient for silencing of the luciferase reporter.

Since coiled-coil regions of many proteins are engaged in protein-protein interactions, we asked whether the TRD of LSH functionally cooperates with corepressor proteins such as HDACs. Consistent with this hypothesis, transcriptional repression by MeCP2, full-length LSH, and LSH(1–226) was partially alleviated when we repeated the luciferase reporter assays in the presence of the deacetylase inhibitor TSA (Fig. 2B). This indicates that transcriptional repression by LSH may operate through an interaction between the coiled-coil domain and HDACs. To explore this further, we asked whether HDACs coimmunoprecipitate with the endogenous LSH from HCT116 nuclear extracts. Antibodies against LSH but not control anti-GFP antibodies (mock immunoprecipitation) immunoprecipitated LSH and coimmunoprecipitated HDAC1 and HDAC2 (Fig. 2C). We could also detect LSH in reciprocal immunoprecipitations with anti-HDAC1 and anti-HDAC2 antibodies (Fig. 2C). Additionally, chromatin immunoprecipitations from cells carrying stably integrated copies of the luciferase reporter showed a threefold decrease of acetylated lysine 9 of histone H3 and a twofold decrease of acetylated lysine 12 of H4 at the TK promoter after the cells were transfected with GAL4BD-LSH (Fig. 2D). Further RNA interference knock-down experiments with HDAC1 and HDAC2 in HCT116 cells revealed that depletion of these two HDACs, but not HDAC3, could, similarly to TSA treatment, alleviate the repression of luciferase reporter (see Fig. S2 in the supplemental material). This indicates that HDAC1 and HDAC2 are essential for LSH-mediated repression.

Transcriptional repression by LSH requires DNMT1 and DNMT3B. Zhu et al. have recently reported that mouse Lsh coimmunoprecipitates with the de novo DNMTs Dnmt3a and Dnmt3b, but not with the maintenance DNMT Dnmt1, from extracts of mouse embryonic fibroblasts (45). Dnmt1, Dnmt3b, and Dnmt3a have also been shown to interact with each other and to coimmunoprecipitate and copurify with HDAC1 and HDAC2 (Fig. 3A) (9, 17, 31–33). Given all these complex interactions, we next investigated whether DNMTs contribute to LSH-mediated transcriptional repression. KO HCT116 cell lines that are genetically null for DNMT1, DNMT3B, DNMT1 and DNMT3B, or DNMT3A and DNMT3B have been generated by homologous recombination (15, 29, 30). Subsequently it was found that most DNMT1 KO cell lines, including the DNMT1/DNMT3B double-KO (DKO) line, express a truncated DNMT1 protein missing 150 amino acids of the N terminus, including the regions essential for binding to DNMT3A, DNMT3B, and PCNA (Fig. 3A; see Fig. 6A) (6, 37). To our surprise, neither GAL4BD-LSH nor GAL4BD-

LSH(1–226) repressed the reporter luciferase gene in DNMT1 KO and DNMT3B KO HCT116 cells (Fig. 3B and C). The same was observed in DNMT1/DNMT3B DKO and DNMT3A/DNMT3B DKO cells (not shown). However, when we cotransfected Dnmt1-GFP with either GAL4BD-LSH or GAL4BD-LSH(1–226) and the reporter plasmids into DNMT1 KO cells, luciferase expression was reduced to levels comparable to those observed in wild-type HCT116 cells (Fig. 3B). Interestingly, a catalytically inactive Dnmt1 carrying a single point mutation, C1229W (34), also rescued LSH-mediated repression in DNMT1 KO cells as well as wild-type Dnmt1. This indicates that the enzymatic activity of DNMT1 is not required for transcriptional silencing of the luciferase reporter.

To determine which region of DNMT1 is required for LSH-mediated repression, we tested whether shorter DNMT1 constructs could restore the repression of the luciferase reporter in DNMT1 KO cells (Fig. 3A). DNMT1(1–1125)-GFP, which contains the known interaction sites with DNMT3A, DNMT3B, and HDACs but is lacking the C-terminal catalytic domain, could partially restore the repression by GAL4BD-LSH and GAL4BD-LSH(1–226), while the shorter proteins DNMT1(1–701)-GFP and DNMT1(1–250)-GFP did not (Fig. 3B). This suggests that a relatively large portion of the DNMT1 N terminus and perhaps some of the C-terminal amino acids are involved in protein-protein interactions that are crucial for LSH-mediated repression. Cotransfection of DNMT3B-GFP with GAL4BD-LSH or GAL4BD-LSH(1–226) into DNMT1 KO cells also led to a modest (~30%) decrease of transcription from the reporter gene (Fig. 3B). This indicates that elevated levels of DNMT3B are not sufficient to restore LSH-mediated repression in cells expressing low levels of truncated DNMT1.

We next attempted to rescue the LSH-mediated repression of the luciferase reporter in DNMT3B KO cells. As in the case of DNMT1, when we cotransfected either GAL4BD-LSH or GAL4BD-LSH(1–226) and the reporter plasmids with either wild-type GFP-DNMT3B or catalytically inactive GFP-DNMT3B^{C640S}, the repression of the luciferase gene was largely restored (Fig. 3C). Interestingly, cotransfection of GAL4BD-LSH with DNMT1-GFP into DNMT3B KO cells could also reduce the expression of the luciferase reporter by approximately 45 to 50%, indicating that DNMT1, when overexpressed, could (although not very efficiently) compensate for the lack of DNMT3B (Fig. 3C). Collectively, these experiments demonstrate that both DNMT1 and DNMT3B are required for effective GAL4-LSH-mediated transcriptional silencing.

The interaction of LSH with HDAC1 and HDAC2 is lost in DNMT KO cells. Given that the repression by LSH was sensitive to TSA and that HDAC1 and HDAC2 coimmunoprecipitated with LSH from nuclear extracts of wild-type HCT116 cells, we next examined whether these interactions remain intact in DNMT KO cells, where GAL4BD-LSH was unable to silence the reporter gene. When we immunoprecipitated the endogenous LSH from either DNMT1 KO or DNMT3B KO extracts, we could not detect HDAC1 and HDAC2 in anti-LSH immunoprecipitations (Fig. 4A, B, C, and D, top panels; compare with Fig. 2C). Consistently, anti-HDAC1 and anti-HDAC2 antibodies efficiently immunoprecipitated HDACs but failed to coimmunoprecipitate LSH from extracts of DNMT1 and DNMT3B KO cells (Fig. 4A, B, C, and D, bottom

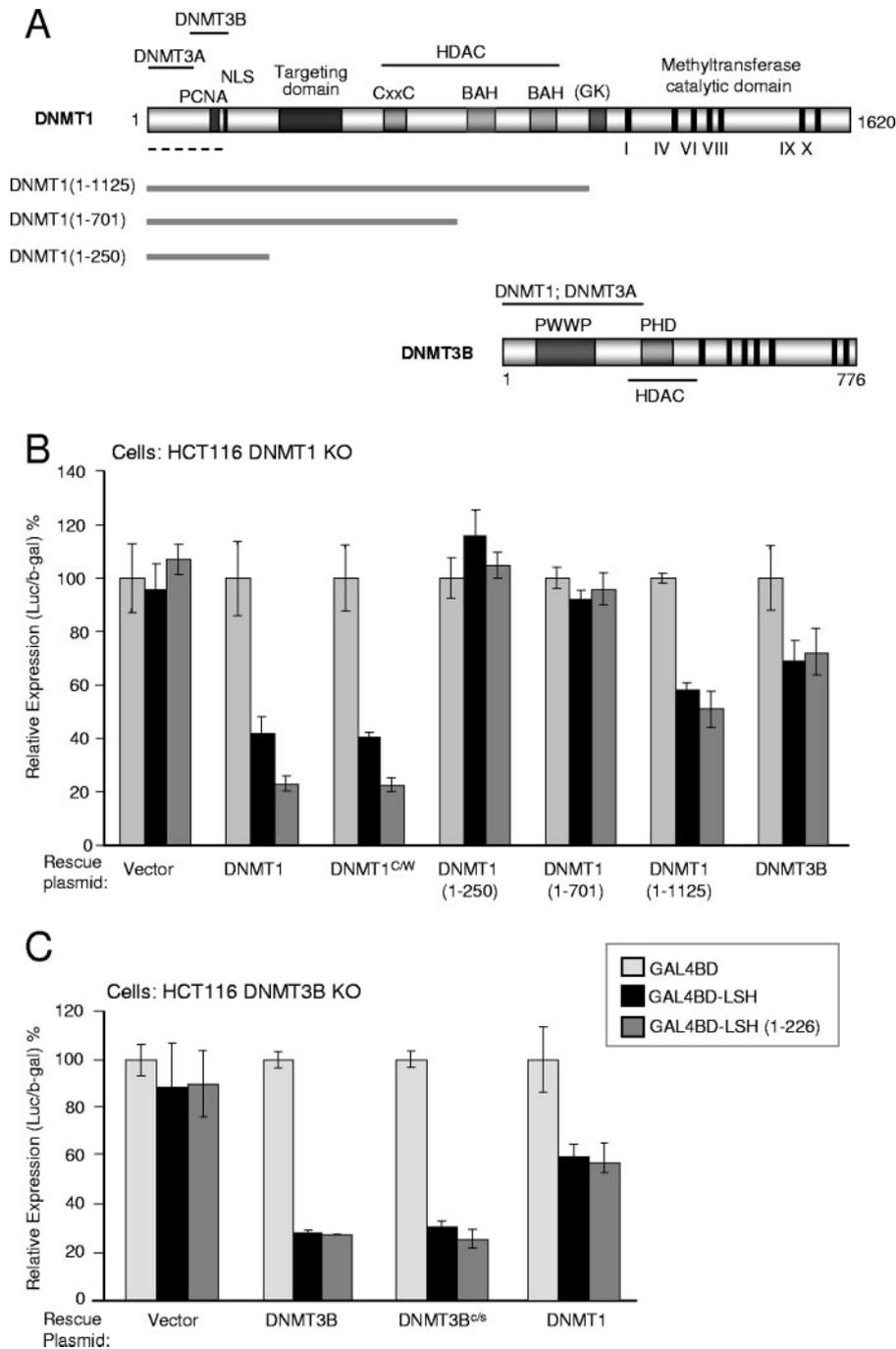


FIG. 3. Transcriptional repression by LSH requires DNMT3B and the N-terminal portion of DNMT1. (A) Schematic representation of DNMT1 and DNMT3B proteins with their functional domains. The cysteine-rich putative DNA binding CxxC domain, bromo-adjacent homeobox motifs (BAH), GK-rich repeats, the domain involved in targeting to replication foci, and the catalytic part of DNMT1 are indicated. Mapped interactions with DNMT3A, DNMT3B, PCNA, and HDAC1 and -2 are shown above the diagram. The dashed line indicates the portion of DNMT1 that has been spliced out in DNMT1 KO HCT116 cells with targeted disruption of the DNMT1 gene (6, 37). The DNMT3B protein contains a DNA binding PWWP motif, a PHD, domain and a conserved catalytic DNMT domain. The portions of DNMT3B interacting with DNMT1 and DNMT3A are indicated. (B) Neither full-length GAL4BD-LSH nor the TRD of LSH, GAL4-LSH(1-226), could silence the luciferase reporter in DNMT1 KO cells. Cotransfection of GAL4BD-LSH proteins together with wild-type GFP-tagged DNMT1, catalytically inactive DNMT1^{C/W}, and the N-terminal portion of DNMT1(1-1125) can rescue the repression of luciferase reporter in DNMT1 KO cells. Shorter N-terminal DNMT1 proteins [DNMT1(1-250) and DNMT1(1-701)] did not rescue the repression of the luciferase reporter gene. DNMT3B was used as an additional control. Error bars indicate standard deviations. (C) GAL4BD-LSH and GAL4BD-LSH(1-226) did not repress the luciferase reporter in DNMT3B KO cells. Cotransfection of LSH proteins with either GFP-DNMT3B or a catalytically inactive GFP-DNMT3B^{C/S} restored the repression of the reporter to levels observed in wild-type HCT116 cells. Cotransfection of GAL4BD-LSH with GFP-DNMT1 also reduced the repression of luciferase in DNMT3B KO cells, although not as efficiently as DNMT3B.

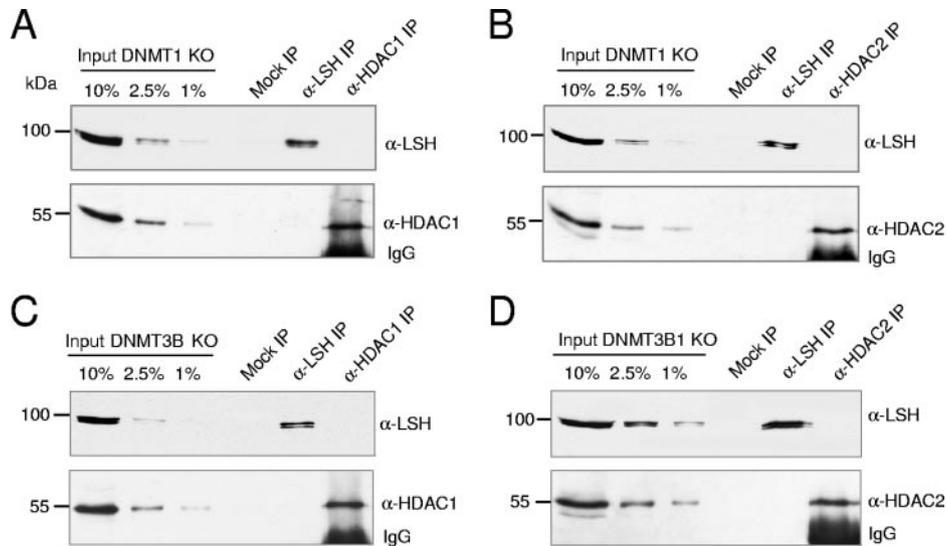


FIG. 4. The interactions of LSH with HDACs are lost in DNMT KO cells. (A and B) Endogenous LSH, HDAC1, and HDAC2 could be efficiently immunoprecipitated (IP) from extracts of DNMT1 KO cells. However, LSH could not be detected in HDAC immunoprecipitations, nor was HDAC1 or HDAC2 detected in LSH immunoprecipitations. IgG, immunoglobulin G. (C and D) LSH, HDAC1 and HDAC2 do not coimmunoprecipitate from extracts of DNMT3B KO cells. Anti-GFP antibodies were used as a control for nonspecific interactions.

panels). As the protein levels of LSH, HDAC1, and HDAC2 in KO cells did not differ significantly from those in the wild-type HCT116 cells, these experiments indicate that DNMT1 and/or DNMT3B could either directly or indirectly recruit HDAC1 and HDAC2 to LSH. Notably, the presence of both DNMTs is required to promote stable interactions of HDACs with LSH.

LSH coimmunoprecipitates with DNMT1 and DNMT3B. Our experiments thus far suggested that LSH participates in a protein complex (or complexes) that contains DNMT1, DNMT3B, HDAC1, and/or HDAC2. To investigate further

whether we could detect an interaction between LSH and DNMT1, we cotransfected DNMT1 KO cells with GAL4DB-LSH and DNMT1-GFP and used anti-GAL4 and anti-GFP antibodies for immunoprecipitation experiments. Anti-GFP antibodies detected DNMT1-GFP in anti-GAL4 immunoprecipitations but not in control anti-HA immunoprecipitations, suggesting that GAL4BD-LSH and DNMT1-GFP interact with each other (Fig. 5A, top panel). In similar experiments we found that GAL4DB-LSH and DNMT3B-GFP cotransfected into DNMT3B KO cells also coimmunoprecipitate (Fig. 5A,

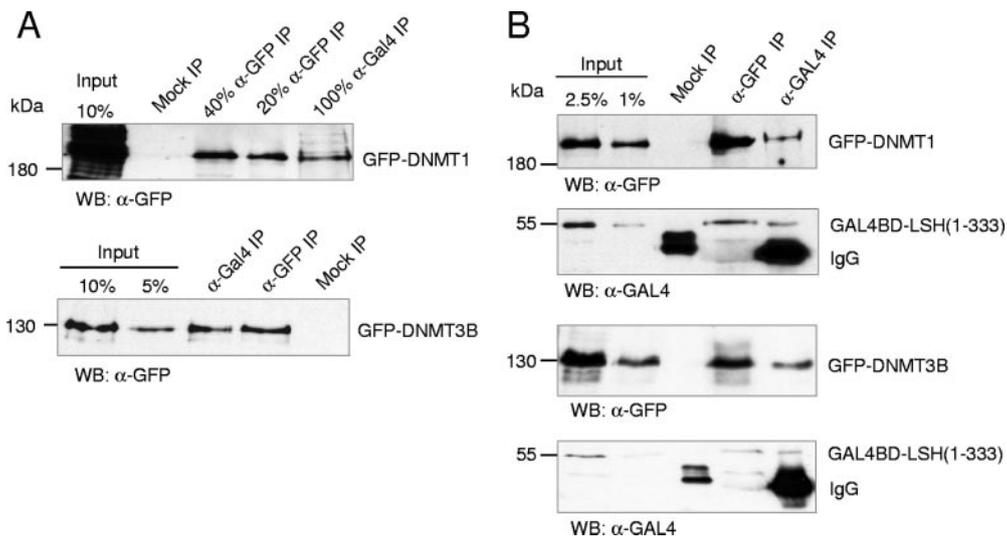


FIG. 5. The coiled-coil TRD domain of LSH interacts with DNMTs. (A) GAL4BD-LSH coimmunoprecipitated with GFP-DNMT1 when both proteins were coexpressed in DNMT1 KO cells. We could detect only about 20% of GFP-DNMT1 in the immunoprecipitations (IP) with anti-GAL4BD antibodies. GAL4BD-LSH coimmunoprecipitated more efficiently with GFP-DNMT3B when both proteins were coexpressed in DNMT3B KO cells. WB, Western blot. (B) GAL4BD-LSH(1-333) protein, containing the coiled-coil TRD domain of LSH, coimmunoprecipitates with GFP-DNMT1 and GFP-DNMT3B from extracts of DNMT1 and DNMT3B KO cells, respectively. Anti-HA antibodies (mock immunoprecipitation) were used as a control.

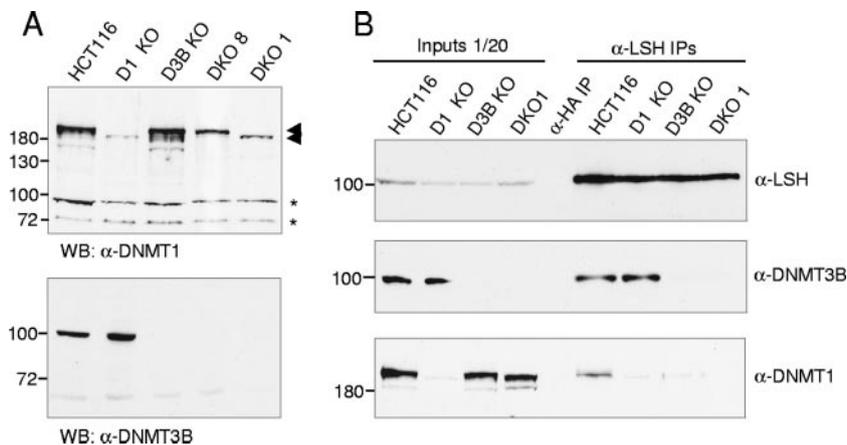


FIG. 6. The interaction of LSH with DNMT1 in vivo requires DNMT3B. (A) Western blots (WB) with antibodies against the C terminus of DNMT1 and the N terminus of DNMT3B on nuclear extracts of HCT116 and KO cell lines. Note that a truncated form of DNMT1 is detectable in DNMT1 KO (D1 KO) cells as well as in DNMT1/DNMT3B DKO (DKO1) cells, while a second DKO cell line (DKO8) expresses full-length DNMT1. DNMT3B is detectable only in HCT116 and DNMT1 KO2 cells. In the top panel, the full-length and the truncated DNMT1s are indicated with arrowheads. The asterisks indicate nonspecific bands that serve as loading controls. (B) Anti-LSH antibodies efficiently immunoprecipitate LSH from nuclear extracts of HCT116 and KO cells. In identical anti-LSH immunoprecipitations (IP), DNMT3B immunoprecipitates with LSH from HCT116 and DNMT1 KO extracts, indicating that the N terminus of DNMT1 is not required for the interaction of DNMT3B with LSH. In contrast, DNMT1 coimmunoprecipitates with LSH only from HCT116 cells, suggesting that the presence of DNMT3B mediates the recruitment of DNMT1 to LSH.

bottom panel). Consistent with our reporter assays, when we cotransfected the cells with the coiled-coil TRD domain of LSH and either DNMT1-GFP or DNMT3B-GFP, we could detect LSH(1–333) coimmunoprecipitating with each of the two DNMTs (Fig. 5B). These experiments clearly demonstrate that the TRD of LSH is necessary and sufficient for the interaction of LSH with DNMT1 and DNMT3B in vivo.

DNMT3B is required for the recruitment of DNMT1 to LSH. As the luciferase reporter assays and coimmunoprecipitation experiments described above relied on overexpression of tagged proteins, we next examined whether the endogenous LSH interacts with DNMTs in HCT116, DNMT1, and DNMT3B KO cells. In agreement with other studies (6, 37), an antibody against the C terminus of DNMT1 detected a truncated DNMT1 protein in nuclear extracts of DNMT1 KO and DNMT1/DNMT3B DKO1 cells compared to the wild-type HCT116 and DNMT1/DNMT3B DKO8 cells (Fig. 6A, top panel). As observed by others, the truncated DNMT1 protein was significantly more abundant in one of the DKO cell lines (DKO1) than in the KO cell line, where DNMT1 was barely detectable (Fig. 6A, top panel). We did not detect DNMT3B protein either in DNMT3B KO or in any of the two DKO cell lines (Fig. 6A, bottom panel). We further used extracts from four of these cell lines, HCT116, DNMT1 KO, DNMT3B KO, and DKO1, to immunoprecipitate LSH and asked whether DNMT1 and DNMT3B could be detected in LSH immunoprecipitations (Fig. 6B, top panel). DNMT3B was present in anti-LSH immunoprecipitations from HCT116 cells, as expected, but it was also detectable in anti-LSH immunoprecipitations from DNMT1 KO cells (Fig. 6B, middle panel). However, DNMT1 coimmunoprecipitated with LSH only from extracts of wild-type HCT116 cells and not from extracts of DNMT3B KO or DKO1 cells (Fig. 6B, bottom panel). These results indicate that DNMT1 does not efficiently interact with LSH in the absence of DNMT3B (Fig. 6A). On the other hand,

the presence of DNMT1 may not be required for the interaction of DNMT3B with LSH, given that approximately equal amounts of DNMT3B coimmunoprecipitate with LSH from HCT116 and DNMT1 KO cells expressing a truncated DNMT1 that does not contain the DNMT3B interaction domain (Fig. 3A and 6A). Taken together with the reporter luciferase assays, these immunoprecipitation experiments suggest that LSH may exist in a complex with DNMT3B with or without DNMT1. However, an LSH complex containing DNMT1, HDAC1, and HDAC2 must also include DNMT3B.

DNMT3B directly binds to LSH, while DNMT1 and HDACs do not. To explore whether any of the proteins that coimmunoprecipitate with LSH bind to LSH directly, we expressed and purified from *E. coli* two GST-tagged recombinant LSH polypeptides, designated LSH-N and LSH-C (Fig. 7A). LSH-N contains amino acids 1 to 503 of LSH and includes the N-terminal coiled-coil and the SNF2 domain. LSH-C corresponds to amino acids 248 to 883 and includes the SNF2 domain and the remainder of the LSH C terminus. We used these two proteins bound to glutathione-Sepharose beads or a control GST-GFP protein to pull down HA-tagged DNMT1 (amino acids 1 to 1125) and full-length DNMT3B in vitro translated in rabbit reticulocyte lysate. Neither LSH-N nor LSH-C could bind DNMT1 in these assays (Fig. 7B). In contrast, LSH-N but not LSH-C or GFP efficiently pulled down DNMT3B (Fig. 7B). As DNMT1 and DNMT3B are known to bind each other (17) and LSH did not coimmunoprecipitate with DNMT1 from extracts of DNMT3B-deficient cells, we asked whether LSH-N would be able to pull down DNMT1 when DNMT3B was present. To investigate this, we added increasing amounts of recombinant DNMT3B expressed and purified from insect cells to reticulocyte lysate containing in vitro-translated DNMT1 (Fig. 7C, bottom panel). We could detect increasing amounts of DNMT1 being pulled down by GST-LSH-N only when the baculovirus-produced DNMT3B

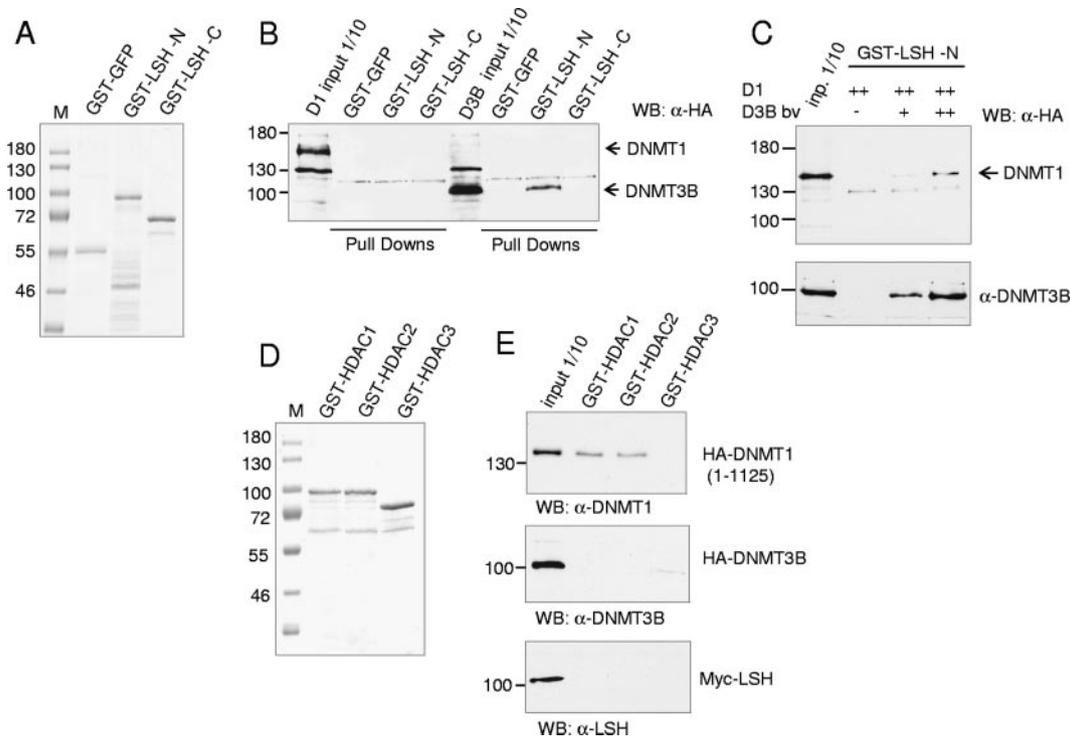


FIG. 7. LSH directly interacts with DNMT3B but not with DNMT1 or HDACs. (A) A Coomassie blue-stained gel shows GST-tagged purified GFP, LSH-N, and LSH-C proteins used in the pull-down assays (B and C). (B) The N terminus of LSH binds in vitro-translated DNMT3B. Neither the N terminus nor the C terminus of LSH pulls down in vitro-translated DNMT1 (amino acids 1 to 1125). WB, Western blot. (C) GST-LSH-N can pull down DNMT1 in the presence of recombinant DNMT3B (0.5 and 1 μ g). (D) A Coomassie blue-stained gel shows purified GST-HDAC1, HDAC2, and HDAC3. (E) GST-HDAC1 and GST-HDAC2 pull down in vitro-translated DNMT1 but not DNMT3B or LSH.

was present (Fig. 7C). Consistent with our coimmunoprecipitation assays, these experiments imply that DNMT3B directly binds to the N terminus of LSH, while the interaction of DNMT1 with LSH in vitro and in vivo requires the presence of DNMT3B.

In order to examine whether HDAC1 and HDAC2 could directly bind to LSH, DNMT1, or DNMT3B, we expressed GST-tagged full-length HDAC1, HDAC2, and, as a control, HDAC3 in *E. coli* and bound them to glutathione-Sepharose (Fig. 7D). We next used the Sepharose-bound HDAC proteins to pull down in vitro-translated HA-tagged DNMT1(1-1125), full-length DNMT3B, and Myc-tagged LSH (Fig. 7E). In agreement with previous reports, we could detect DNMT1 bound to HDAC1 and HDAC2 but not to HDAC3 (Fig. 7E, top panel). However, neither DNMT3B nor LSH was pulled down by GST-HDAC1, -2, or -3 (Fig. 7E, middle and bottom panels). These in vitro experiments are consistent with our reporter assays and coimmunoprecipitation results suggesting that DNMT1 recruits HDAC1 and HDAC2 to the LSH-bound DNMT3B (Fig. 8).

DISCUSSION

The plant SNF2 family protein DDM1 and its mammalian homolog LSH were initially identified as proteins essential for the establishment of DNA methylation in vivo (5, 16). DDM1-deficient plants and mice with targeted disruption of the *Lsh* gene develop with dramatically reduced levels of methylated

cytosine within their genomes and are defective in silencing of various transposable elements and a few specific genes (7, 12, 19). In mice, lack of *Lsh* is not essential for embryonic development but leads to postnatal death (5). Cytological studies

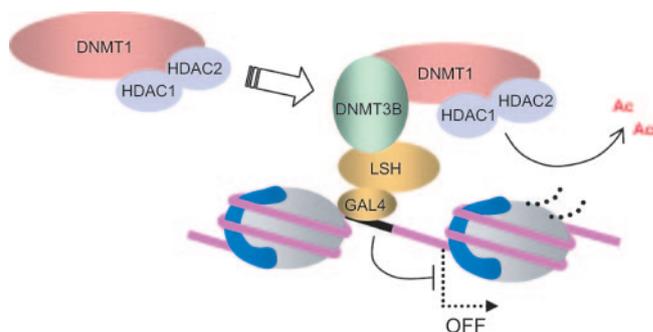


FIG. 8. A model of how the LSH-associated protein complex acts to repress transcription. Our experiments are consistent with a model where DNA-bound LSH recruits a complex that includes DNMT3B, DNMT1, HDAC1, and HDAC2. LSH-associated HDACs remove acetyl groups (Ac) from histone tails, generating deacetylated chromatin incompatible with transcriptional activation. LSH does not directly interact with DNMT1 and HDACs but requires DNMT3B for the assembly of the repressive complex. On the other hand, HDAC1 and HDAC2 require both DNMT1 and DNMT3B for association with the LSH complex. The order of these interactions explains why in cells expressing N-terminally truncated DNMT1, which does not bind DNMT3B, or in cells lacking DNMT3B, LSH-mediated repression is disrupted.

and experiments with ES cells have shown that although Lsh is present together with DNMT1 at replication foci during late S phase, Lsh is dispensable for maintenance of DNA methylation on replicating episomal plasmids (42, 45). It was further found that Lsh coimmunoprecipitates with de novo methyltransferases Dnmt3a and Dnmt3b and that cells treated with Lsh small interfering RNA have reduced de novo DNA methylation (45). Taken together, these studies led to the conclusion that Lsh is involved primarily in de novo DNA methylation but is dispensable for the maintenance of DNA methylation during DNA replication.

Despite the important role of Lsh in supporting efficient DNA methylation in mammalian cells, very little is known about how this protein interacts with the DNA methylation machinery. This lack of information may be due to difficulties in identifying proteins that interact with Lsh. Indeed, based on the observed molecular mass of the native LSH calculated from biochemical fractionation experiments, we found that the vast majority of the nuclear pool of LSH in human and mouse cells appears to consist of free monomeric LSH. Therefore, purification of a low-abundance LSH complex by traditional chromatography methods would not have been feasible. Additionally, we observed that the full-length LSH had a strong repressive effect when recruited to the promoters of reporter genes in yeast, further complicating the use of yeast two-hybrid screens for identification of LSH-interacting partners. Taking advantage of our discovery that LSH behaved as a transcriptional repressor in mammalian cells as well as in yeast, we used luciferase reporter assays as a tool to determine which region of LSH mediates transcriptional repression and to identify proteins that interact with LSH *in vivo*. By recruiting truncated LSH polypeptides to GAL binding sites upstream of the luciferase reporter gene driven by the TK promoter, we found that the N-terminal coiled-coil domain of LSH spanning amino acids 1 to 226 was necessary and sufficient for transcriptional silencing of the reporter. Therefore, it seemed plausible that this region of LSH, which we designated the TRD, interacts with corepressor proteins that modify chromatin into a transcriptionally nonpermissive state.

As LSH-mediated silencing of the luciferase reporter was sensitive to the deacetylase inhibitor TSA, we performed coimmunoprecipitation assays and found that two HDACs, HDAC1 and HDAC2, interact with LSH *in vivo*. Further, *in vitro* experiments revealed that neither HDAC1 nor HDAC2 directly binds to LSH, suggesting that they are recruited to LSH by other proteins. Given the previous reports that LSH coimmunoprecipitates with de novo DNMTs from extracts of mouse cells, we examined whether transcriptional silencing of the luciferase reporter required the presence of DNMTs. In human colorectal carcinoma HCT116 cells with targeted disruption of the DNMT3B gene (DNMT3B KO1) as well as cells expressing low levels of N-terminally truncated DNMT1 protein (DNMT1 KO2), we observed a significant reduction of LSH-mediated repression, indicating that these two DNMTs could be involved in the recruitment of HDAC1 and HDAC2 to LSH. Our further experiments confirmed that LSH does not coimmunoprecipitate with HDACs from extracts of DNMT3B KO1 and DNMT1 KO2 cells. We also observed that the endogenous LSH as well as GAL4BD-tagged LSH coimmuno-

precipitated with DNMT1 and DNMT3B from extracts of HCT116 cells.

It has been previously reported that both DNMT1 and DNMT3B interact with HDAC1 and HDAC2 *in vitro* and *in vivo* (9, 10, 32). Therefore, it was possible that DNMT1 and DNMT3B bind to LSH independently of each other and promote the recruitment of HDACs to LSH-targeted chromatin. However, we found that although LSH coimmunoprecipitated with DNMT3B from DNMT1 KO cells, it did not interact with HDACs or the truncated DNMT1 in these cells and was unable to silence the luciferase reporter. Taken together, our data suggest that DNMT1-bound HDAC1 and HDAC2 are recruited to LSH indirectly via DNMT3B (Fig. 8). Our *in vitro* pull-down experiments further support the *in vivo* reporter assays and coimmunoprecipitation data. Interestingly, we observed that when DNMT1 was overexpressed in DNMT3B KO cells, it could, to some extent, rescue the transcriptional repression of luciferase reporter mediated by GAL4BD-LSH. As DNMT1 does not directly bind to LSH, it is possible that the recruitment of DNMT1 to LSH may occur via DNMT3A or some other protein. However, the first possibility seems unlikely, since we did not detect DNMT3A protein in any of the HCT116 wild-type or KO cell lines except DKO cells (not shown). Given that overexpression of DNMT3B in DNMT1 KO cells transfected with GAL4BD-LSH also reduced the expression of the luciferase reporter by about 30%, it is possible that DNMT3B can either function in transcriptional repression independently of the truncated DNMT1 or, to some extent but not very efficiently, interact with the C terminus of DNMT1. The second interpretation seems plausible, since the N-terminal portion of DNMT1(1–1125) did not fully rescue LSH-mediated repression in DNMT1 KO cells compared to the full-length DNMT1.

In summary, we have investigated whether and how LSH interacts with the DNA methylation machinery in human cells and found that the LSH-associated complex contains at least four proteins: DNMT3B, DNMT1, HDAC1, and HDAC2. We show that GAL4BD-mediated targeting of LSH to a TK promoter driving the expression of a reporter gene results in transcriptional silencing, which is dependent upon the recruitment of HDACs via DNMT3B and DNMT1. However, transcriptional repression and recruitment of DNMTs did not immediately result in DNA methylation, as bisulfite sequencing did not detect methylated CpGs at TK promoter sequences 4 days after GAL4BD-LSH and the reporter plasmid were co-transfected into cells (see Fig. S3 in the supplemental material). These observations are consistent with previous reports that DNA methylation follows rather than precedes transcriptional silencing established by negative transcriptional regulators, including chromatin-related mechanisms (23, 40). Thus, although LSH protein serves as a scaffold for assembly of the DNMT/HDAC complex, the primary function of the LSH complex may not be to methylate DNA but to establish deacetylated inactive chromatin. Transcriptional repression caused by deacetylation of histone tails by LSH-interacting HDAC1/2 can be viewed as an initial and, perhaps, reversible step in LSH-mediated heterochromatin formation. A longer-term association of LSH with specific loci and a persistently high local concentration of DNMT1 and DNMT3B may result in methylation of CpGs at these loci. The kinetics of DNA

methylation induced by the LSH-associated complex requires a more detailed investigation. Nevertheless, experiments with unmethylated episomal plasmids capable of replicating in mammalian cells indicate that Lsh-facilitated DNA methylation was observed weeks after the plasmids were introduced into these cells (45). Taken together, these data suggest that LSH cooperates with DNMTs and HDAC1/2 to act as a general transcriptional repressor in mammalian cells.

In mouse cells, Lsh as well as DNMT1 and DNMT3B are required for DNA methylation of pericentromeric major satellite repeats. Yan et al. have reported that in Lsh-deficient mouse embryonic fibroblasts, H3K4 dimethylation, a histone modification associated with transcriptionally permissive chromatin, accumulates at normally heterochromatic, Lsh-bound and H3K4-depleted pericentromeric sequences (43). Our experiments provide a mechanistic explanation for the observed increase of histone acetylation and other positive histone modifications at pericentric heterochromatin and other loci in Lsh^{-/-} cells (41, 43).

It is still unclear whether and how LSH and/or the LSH-associated DNMT complex is recruited to chromatin to establish transcriptionally silenced chromatin and DNA methylation at specific loci. One could also envision that LSH continuously scans chromatin for regions that challenge the processivity of DNMT enzymes. How LSH recruitment is related to functional states of chromatin and what makes certain loci preferred targets for LSH would be intriguing questions to answer. Based on observations in plants that DNA methylation of transposable elements and related tandem repeats is dependent on small interfering RNA and DDM1, it has been suggested that small interfering RNA may guide DDM1 to establish heterochromatin at specific genomic locations (19). However, this proposed model still awaits experimental proof in both plants and in animal cells.

Thus far our preliminary experiments indicate that LSH binds to DNA and to linker DNA regions of reconstituted chromatin more efficiently than either DNMT3B or DNMT1 (data not shown) (27, 31). It is possible to envision that the binding properties of the LSH complex as a whole are determined by the additive affinities of individual proteins, i.e., LSH, DNMTs, and HDACs, for DNA and/or histones. In vitro reconstitution of the LSH complex and further investigation of how this complex interacts with nucleosomal DNA in vitro and in vivo may provide interesting insights.

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