

## Lsh is involved in *de novo* methylation of DNA

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**Deletion of Lsh perturbs DNA methylation patterns in mice yet it is unknown whether Lsh plays a direct role in the methylation process. Two types of methylation pathways have been distinguished: maintenance methylation by Dnmt1 occurring at the replication fork, and *de novo* methylation established by the methyltransferases Dnmt3a and Dnmt3b. Using an episomal vector in Lsh<sup>-/-</sup> embryonic fibroblasts, we demonstrate that the acquisition of DNA methylation depends on the presence of Lsh. In contrast, maintenance of previously methylated episomes does not require Lsh, implying a functional role for Lsh in the establishment of novel methylation patterns. Lsh affects Dnmt3a as well as Dnmt3b directed methylation suggesting that Lsh can cooperate with both enzymatic activities. Furthermore, we demonstrate that embryonic stem cells with reduced Lsh protein levels show a decreased ability to silence retroviral vector or to methylate endogenous genes. Finally, we demonstrate that Lsh associates with Dnmt3a or Dnmt3b but not with Dnmt1 in embryonic cells. These results suggest that the epigenetic regulator, Lsh, is directly involved in the control of *de novo* methylation of DNA.**

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### Introduction

DNA methylation regulates a number of biological processes, including genomic imprinting, X chromosome inactivation, silencing of tumor suppressor genes, and repression of retroviral elements (Bird, 2002; Li, 2002). Loss of methylation in mice results in severe developmental defects and early embryonic lethality (Li *et al*, 1992; Okano *et al*, 1999; Dennis *et al*, 2001). A number of human inherited diseases linked to

faulty methylation pathways and exhibiting abnormal development include Rett, ICF, and ATRX syndromes (Amir *et al*, 1999; Okano *et al*, 1999; Gibbons *et al*, 2000). Moreover, aberrant methylation patterns are thought to be involved in tumorigenesis (Jones and Baylin, 2002; Chen *et al*, 2004; Yu *et al*, 2005) causing genomic instability, abnormal imprinting, and deregulated expression of oncogenes or tumor suppressor genes.

Two types of methylation pathways are functionally distinct: maintenance versus *de novo* methylation. During early mammalian embryogenesis, DNA methylation patterns are largely erased and re-established shortly after implantation in a wave of *de novo* methylation (Reik *et al*, 2001; Bird, 2002; Li, 2002). These newly established patterns are then thought to be faithfully copied after each round of replication onto the newly synthesized DNA strand. In contrast, genomic imprints, which are largely dependent on DNA methylation, are mostly established in germ cells and preserved throughout embryogenesis. Thus, maintenance activity is found in all somatic cells while the highest *de novo* methylation activity is found in embryonic cell lines, germ cells, or in postimplantation embryos.

Several DNA cytosine methyltransferases have been identified in mammalian cells (Chen and Li, 2004; Goll and Bestor, 2005). Dnmt1 is primarily responsible for maintenance methylation since Dnmt1 shows high affinity for hemimethylated substrates and is present at replication forks via its association with PCNA (Leonhardt *et al*, 1992; Chuang *et al*, 1997; Okano *et al*, 1998; Pradhan *et al*, 1999). *De novo* methylation activity is primarily dependent on Dnmt3a and Dnmt3b, two partially redundant Dnmt family members (Okano *et al*, 1999). Dnmt3a plays an additional crucial role in *de novo* methylation of imprinted sites in germ cells together with Dnmt3L, a Dnmt family member lacking catalytic activity (Hata *et al*, 2000; Bourc'his and Bestor, 2004; Kaneda *et al*, 2004). Although the enzymes responsible for methylation patterns have been identified, the precise molecular mechanisms including cofactors that lead to recruitment and efficient targeting of the enzymatic machinery to their appropriate sites are unknown.

We have previously reported that Lsh controls genomic methylation patterns in mice (Dennis *et al*, 2001, Muegge, 2005). Lsh belongs to the SNF2 family of proteins (Jarvis *et al*, 1996; Geiman *et al*, 1998), whose members participate in chromatin remodeling (Fyodorov and Kadonaga, 2001; Langst and Becker, 2004). Targeted disruption of Lsh in mice leads to developmental defects and early lethality (Geiman and Muegge, 2000; Dennis *et al*, 2001; Geiman *et al*, 2001; Fan *et al*, 2003; Sun *et al*, 2004). Lsh controls normal heterochromatin structure and function in mice, and upon deletion a number of epigenetic modifications are perturbed. For example, Lsh-deficient cells show genome wide CpG hypomethylation, altered histone H3 methylation, and increased acetylation levels for histone H3 and histone H4 (Dennis *et al*, 2001; Yan *et al*, 2003a, b; Huang *et al*, 2004; Sun *et al*, 2004). Since epigenetic modifications are closely

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linked, it remains unclear which one is the initial epigenetic modification targeted by Lsh. In this report we attempt to determine the functional role of Lsh in either maintenance or *de novo* methylation, and to characterize the role of Lsh in the establishment of epigenetic modifications. We provide evidence that Lsh is required for *de novo* methylation of DNA and that it is directly involved in the methylation process.

## Results

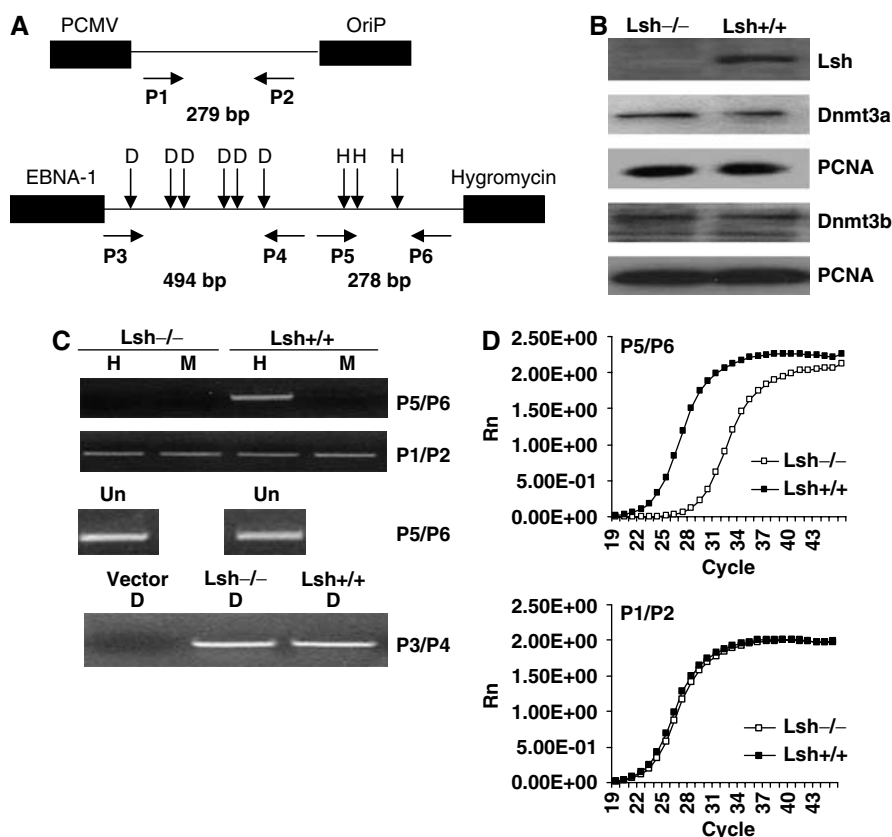
### Lsh is required for methylation of episomal DNA in MEF cells

Lsh is a global regulator of DNA methylation in mice. To investigate further the functional role of Lsh in methylation, we used an episomal vector system (Figure 1A) that allows for the discrimination between *de novo* and maintenance of genomic methylation. The episomal vector can be transfected into mammalian cells to serve as a target for *de novo* methylation (Hsieh, 1999). The presence of novel CpG methylated sites can be determined using methylation-sensitive PCR once the episomal construct is recovered and completely digested with a methylation-sensitive restriction enzyme such as *HpaII*. Figure 1A illustrates the design of PCR

primers to amplify regions of the episomal construct, pCEP4. Primer pair P5/6 surrounds multiple *HpaII* sites and can detect successful methylation. In contrast, the product of primer pair P1/P2 contains no *HpaII* sites, thus serving as an internal control for the amount of recovered episomal DNA. Primer pair P3/P4 amplifies a fragment that contains multiple *DpnI* sites and therefore is used as control to indicate successful replication in mammalian cells (*DpnI* can only digest bacterial Dam methylated DNA, and thus fails to cleave DNA that has replicated in mammalian cells).

The episomal construct pCEP4 was stably transfected into MEFs derived from *Lsh*<sup>-/-</sup> or *Lsh*<sup>+/+</sup> embryos. Since the episomal construct serves as a target for *de novo* methylation by Dnmt3a and Dnmt3b, we first examined protein expression levels by Western blot analysis using nuclear extracts of stably transfected cell lines. As expected, Lsh protein levels were not detectable in *Lsh*<sup>-/-</sup> MEFs (Figure 1B). In contrast, DNA methyltransferases Dnmt3a and 3b were both expressed equally well in the presence or absence of Lsh, suggesting a comparable *de novo* methylation activity in both cell lines.

To examine the methylation status of the recovered episome, methylation-sensitive PCR analysis was performed as shown in Figure 1C. After quantification of recovered episome,



**Figure 1** Lsh is required for methylation of an episomal vector in MEF cells. (A) Map of the episomal vector pCEP4 illustrating the location of *HpaII*/*MspI* and *DpnI* sites as well as the position of the primers used for methylation-sensitive PCR analysis. Primer pairs P5/P6 are designed for detection of methylation, P3/P4 for detection of successful replication, and P1/P2 as an internal control. The length of the expected PCR fragment is indicated in base pairs (bp). (B) Western blot analysis using nuclear extracts derived from *Lsh*<sup>-/-</sup> and *Lsh*<sup>+/+</sup> embryonal fibroblasts (MEF) and specific antibodies against Lsh, Dnmt3a, Dnmt3b and PCNA (serving as control). (C) Methylation-sensitive PCR (upper panel). Episomal DNA derived from stably transfected *Lsh*<sup>-/-</sup> and *Lsh*<sup>+/+</sup> MEF cells was digested with *DpnI* and then either with the methylation-sensitive enzyme *HpaII* (H) or the methylation independent enzyme *MspI* (M) followed by PCR analysis with indicated primers. Replication of the episomal vector was confirmed using *DpnI* (lower panel). *DpnI* cuts only DNA that has been methylated in bacteria by the dam methylase. The replicated episomal DNA in mouse cells should be *DpnI* resistant. For adjustment of input undigested DNA (Un) was used before digestion. (D) Episomal DNA was derived as in C. and subjected to real time-PCR analysis using methylation-sensitive primer pair P5/P6 (upper panel) or the internal control P1/P2 (lower panel).

mal DNA to adjust for equal input using primers P5/P6 (undigested samples), the DNA was digested with the methylation-sensitive restriction enzyme *HpaII*. The successful amplification, using P5/P6 primers, of Lsh<sup>+/+</sup> derived DNA after treatment with *HpaII* indicated the presence of methylated CpG sites. Digestion with *MspI* (which cleaves DNA independent of methylation) served as control. In contrast, the P5/P6 PCR fragment was not detectable using DNA derived from Lsh<sup>-/-</sup> MEFs, suggesting an impaired gain of methylation in the absence of Lsh. To ensure that equal amounts of DNA were indeed present in wild type and Lsh<sup>-/-</sup> samples after digestion (since digestion and further handling can lead to unavoidable loss of DNA), the control primers P1/P2 were used which do not surround *HpaII* sites (Figure 1C). Using these internal control primers, the amplification was indistinguishable between Lsh wild type and Lsh<sup>-/-</sup> DNA. In order to quantify the appearance of methylation in the episome sequence, real-time PCR was performed. As shown in Figure 1D, the use of the methylation-sensitive primer set P5/P6 revealed a significant difference of methylation comparing wild type and Lsh<sup>-/-</sup> samples, whereas the internal control primers confirmed equal amounts of DNA after digestion. In an attempt to quantify methylation levels, a standard curve for PCR amplifications using known concentrations of the episomal DNA was performed and used to calculate the copy numbers using primers P5/P6 before and after digestion (Supplementary Figure 1). Wild-type samples were completely methylated at the examined sites of pCEP4. In contrast, Lsh<sup>-/-</sup> samples were only methylated about 1% by this calculation. These observations demonstrated that Lsh is essential for the acquisition of methylation on episomal DNA in MEFs.

#### **Lsh does not play a role in maintenance of methylation in MEF cells**

Gain of methylation on the episomal vector pCEP4 requires *de novo* methylation activity as well as the ability to maintain newly acquired methylation patterns. To differentiate between these two processes, we tested the ability of Lsh<sup>-/-</sup> cells to maintain methylation on previously *in vitro* methylated DNA. The episomal vector pCEP4 was treated with SssI methyltransferase and the degree of methylation was determined by digestion with the methylation-sensitive restriction enzyme, *HpaII*. Resistance to *HpaII* digestion indicated the successful methylation of the vector (Figure 2A).

The methylated episomal DNA was stably transfected into Lsh<sup>-/-</sup> and Lsh<sup>+/+</sup> MEFs and methylation-sensitive PCR performed after recovery of the episome. Episomal DNA derived from wild-type MEFs showed the expected PCR fragment using primer pair P5/P6 after *HpaII* digestion, indicating the presence of methylated sites on the episome (Figure 2B). Using episomal DNA derived from Lsh<sup>-/-</sup> MEFs, a comparable PCR amplification was observed suggesting a similar degree of methylation in the absence of Lsh (Figure 2B). These results were further confirmed using real-time PCR analysis. As shown in Figure 2C, there were no detectable differences in the amplification using primer pair P5/6 or the internal controls P1/P2 when comparing wild type and Lsh deficient cells indicating a similar ability to preserve methylation patterns. As summarized in Figure 2D, the calculated copy numbers which were amplified from premethylated plasmids were indistinguishable between

Lsh<sup>+/+</sup> and Lsh<sup>-/-</sup> cells. Thus, methylation patterns are faithfully maintained in the absence of Lsh, while the acquisition of a novel methylation mark depends on Lsh. We therefore conclude that Lsh plays a role in *de novo* methylation rather than in maintenance methylation.

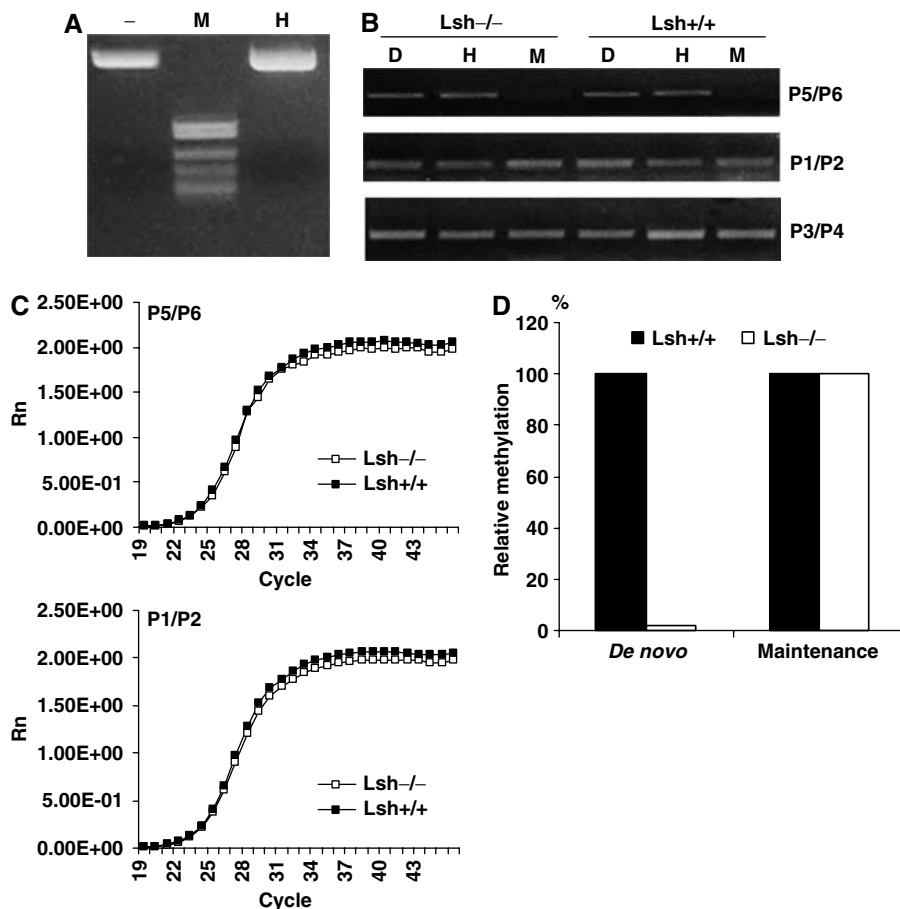
#### **Lsh is required for Dnmt3a or Dnmt3b mediated silencing of a retroviral transgene**

*De novo* methylation is thought to be conferred by either Dnmt3a or Dnmt3b activity in mice (Hsieh, 1999; Okano *et al*, 1999). In order to understand whether Lsh is involved in the methylation process mediated by either DNA methyltransferase, we stably overexpressed both proteins in Lsh<sup>-/-</sup> and Lsh<sup>+/+</sup> MEFs. By Western blot analysis, an increase in Dnmt3a or Dnmt3b proteins over endogenous methyltransferase levels was observed using nuclear extracts from Lsh<sup>-/-</sup> MEFs and Lsh<sup>+/+</sup> MEF controls (Figure 3A). To investigate the silencing function caused by methylation, the retroviral reporter vector pMSCV-hGFP was used (kind gift of Dr Jonathan Keller, NCI, Frederick). Upon methylation, expression of the green fluorescence protein (GFP) is suppressed and can be monitored by either fluorescence microscopy or FACS analysis. After infection of Lsh<sup>-/-</sup> and Lsh<sup>+/+</sup> MEFs with the retroviral vector pMSCV-hGFP, GFP expression was monitored by microscopy (Figure 3B). Lsh wild-type cells that were either stably transfected with Dnmt3a, Dnmt3b, or both showed a decrease in GFP expression within 8 days after infection. In contrast, Lsh deficient cells stably expressing Dnmt3a, Dnmt3b, or both were unable to significantly reduce GFP expression. Quantitative measurement of fluorescence expression by FACS analysis revealed a reduction of GFP intensity of about 25–30% when comparing wild-type cells with Lsh<sup>-/-</sup> MEFs, suggesting an impairment of reporter silencing in the absence of Lsh (Figure 3C). In contrast, untransfected MEFs (Un) that did not overexpress the Dnmt3 proteins were not able to silence the integrated reporter gene and thus, GFP expression levels were indistinguishable between wild type and Lsh deficient cells (Figure 3C). Lsh, therefore, plays an important role in Dnmt3a or Dnmt3b mediated silencing of retrovirus directed protein expression.

#### **Lsh functionally cooperates with *de novo* methylation mediated by either Dnmt3a or Dnmt3b**

Since Lsh plays an important role in the downregulation of GFP expression, we tested whether CpG hypermethylation was directly involved in gene silencing of the retroviral transgene using methylation-sensitive PCR. For detection of methylation, primers were designed around *HpaII*/*MspI* sites in the GFP gene (P9/P10) and the 5'-LTR region (P11/P12) of the vector (Figure 3D). The control primers P7/P8 did not surround *HpaII* sites thus serving as internal control.

After the undigested genomic DNA was quantified to adjust for equal input using primers P11/12 (Figure 4A), restriction enzyme digestion with the methylation-sensitive enzyme *HpaII* was performed. Lsh wild-type samples were successfully amplified and generated PCR fragments in the 5'LTR (primer P11/P12) and the GFP coding region (P9/P10), suggesting methylation within the amplified regions (Figure 4A). In contrast, Lsh<sup>-/-</sup> samples did not generate the expected PCR fragments indicating reduced methylation (Figure 4A). This decrease of methylation occurred in Dnmt3a, Dnmt3b, or Dnmt3a/b overexpressing Lsh<sup>-/-</sup>



**Figure 2** Lsh does not play a role in the maintenance of methylation in MEF cells. (A) Ethidium bromide stain of *in vitro* methylated episomal vector pCEP4 that has been digested with *HpaII* (H) and *MspI* (M). Resistance to *HpaII* digestion indicates that the episome has been fully methylated. (B) Methylation-sensitive PCR. Methylated episomal DNA was stably transfected into Lsh<sup>-/-</sup> and Lsh<sup>+/+</sup> MEF cells and the recovered DNA was first digested with *DpnI*. Then the DNA was either digested with the methylation-sensitive enzyme *HpaII* (H) or the methylation independent enzyme *MspI* (M) followed by PCR analysis with the indicated primers as shown in Figure 1A. (C) Real-time PCR analysis. Episomal DNA was derived as described in (B), and subjected to real time-PCR analysis using the methylation-sensitive primer pair P5/P6 (upper panel) or the internal control primer pair P1/P2 (lower panel). (D) The relative copy numbers for real time-PCR products of *HpaII* digested episomal DNA were calculated based on the standard curve equation. Results of Figure 1 are represented as 'de novo' and results from Figure 2 as 'maintenance' methylation.

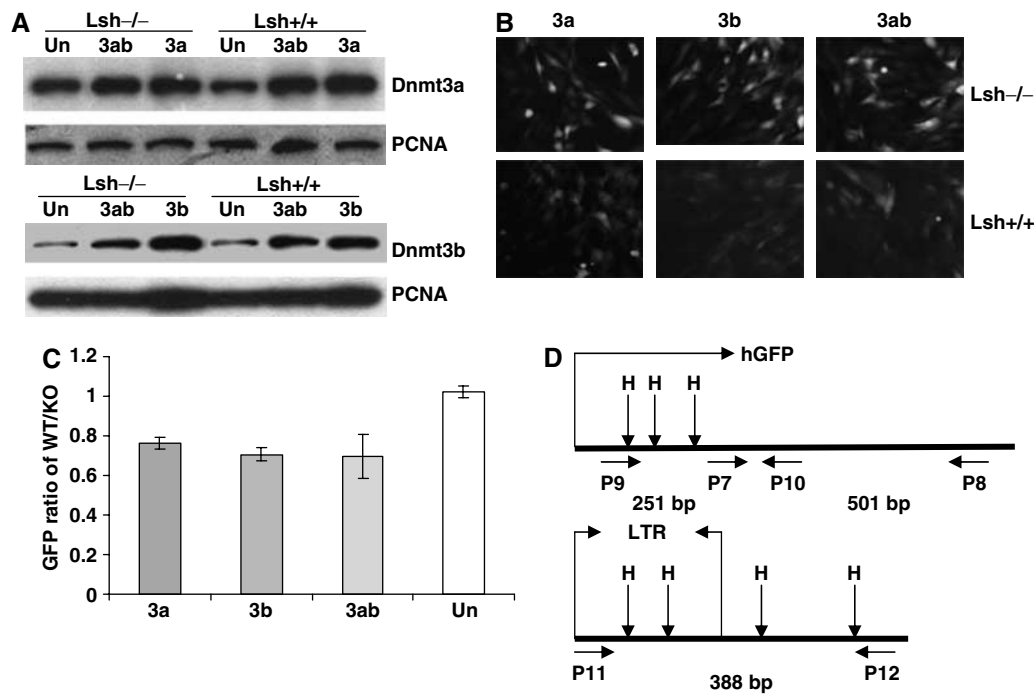
MEFs, suggesting that Lsh is required for methylation induced by either Dnmt3 protein. As control, to ensure equal amounts of DNA after digestion, the internal methylation independent primers were used (P7/P8). Distinct methylation levels when comparing wild type and Lsh<sup>-/-</sup> samples were further confirmed using real-time PCR (Figure 4B-D). Whereas the use of primer pair P7/P8 verified equal loading of DNA after digestion, use of primer pair P11/12 indicated significant differences in methylation at the 5'LTR region in wild type versus Lsh deficient samples. Without overexpression of the Dnmt3 proteins, Lsh<sup>+/+</sup> and Lsh<sup>-/-</sup> samples were indistinguishable since methylation was undetectable (data not shown). In an attempt to quantify methylation levels, a standard curve for PCR amplifications using known concentrations of the template was performed and used to calculate the copy numbers using primers P11/12 before and after digestion (Supplementary Figure 2). Although the frequency of having all four sites methylated in wild-type cells was low, the deletion of Lsh revealed consistently a 10-fold reduction in Dnmt3a, Dnmt3b or Dnmt3a,b transfected cells. Possibly, the low methylation

efficiency in the retroviral transgene in comparison to the full methylation of the episomal vector was due to distinct retroviral target sequences, the integration of the transgene or a difference in the experimental time frame. Taken together, the difference in CpG methylation levels between wild type and Lsh deficient cells correlated with the difference in silencing of GFP expression. These results suggest, therefore, that Lsh cooperates with either Dnmt3a or Dnmt3b for *de novo* methylation.

#### **Silencing of Lsh in embryonal stem cells results in loss of *de novo* methylation**

Embryonal stem (ES) cells are used for *de novo* methylation assays since they are rich in endogenous Dnmt3a/b proteins in contrast to somatic cells and readily methylate newly integrated retroviral DNA (Okano *et al*, 1999; Chen *et al*, 2002). To further investigate the molecular mechanism of Lsh in methylation, we established ES cell lines with low levels of the Lsh protein using RNA interference.

Stably expressing Lsh hairpin siRNA in ES cell lines (siLsh#1 and siLsh#2) significantly decreased Lsh protein



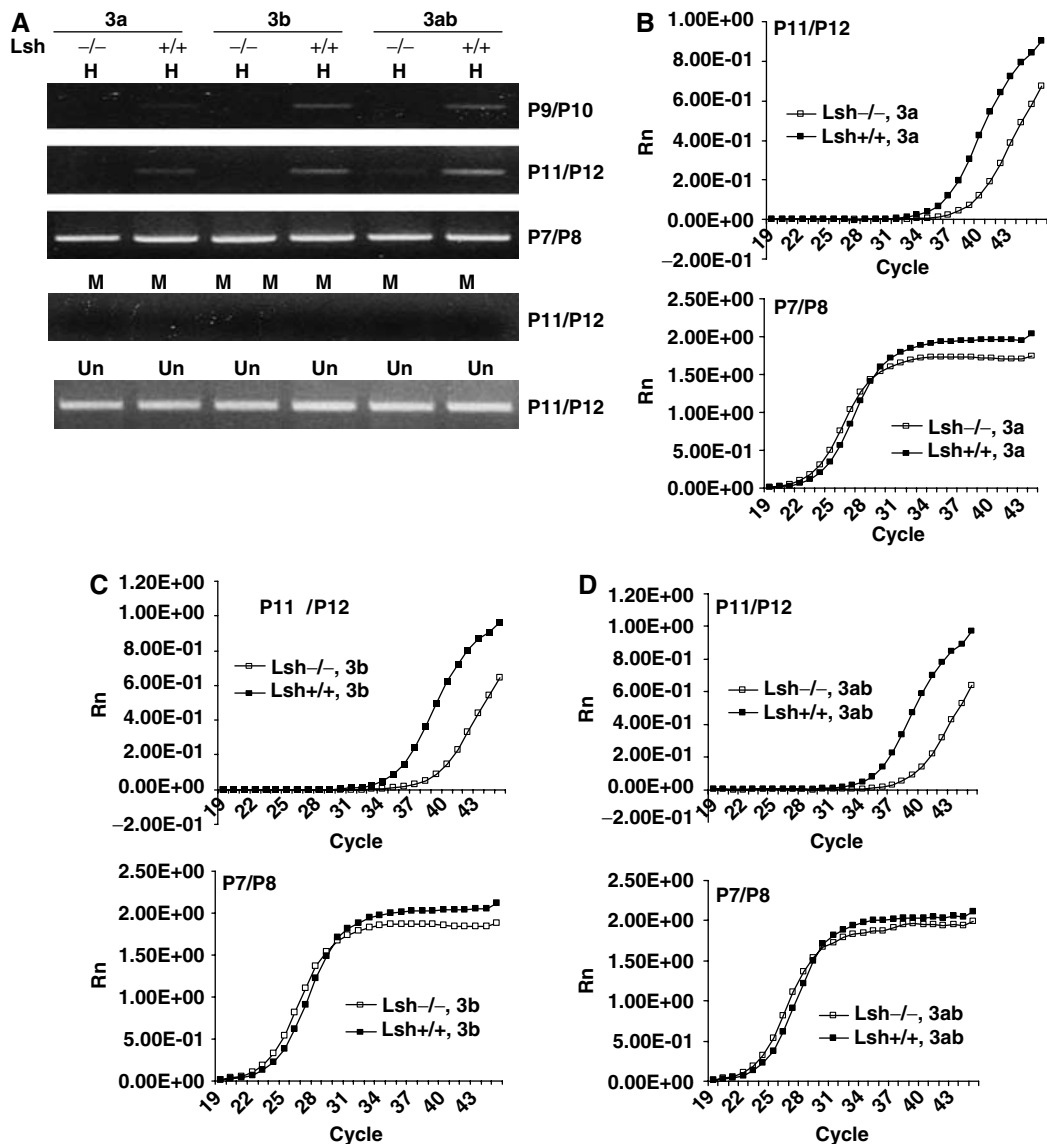
**Figure 3** Lsh is required for silencing of Dnmt3a or Dnmt3b mediated silencing of a retroviral transgene. (A) Western blot analysis using nuclear extracts derived from Lsh<sup>-/-</sup> and Lsh<sup>+/+</sup> mouse embryonal fibroblasts stably expressing Dnmt3a, Dnmt3b, Dnmt3a/Dnmt3b, or untransfected (Un) MEFs. For detection, specific antibodies were used against Dnmt3a, Dnmt3b, or PCNA as control. (B) Fluorescence analysis. Lsh<sup>+/+</sup> and Lsh<sup>-/-</sup> MEFs that were stably expressing Dnmt3a, Dnmt3b, or Dnmt3a/Dnmt3b were infected with pMSCV-hGFP and examined after 8 days for GFP expression using a fluorescence microscope. (C) FACS analysis. The GFP intensity of Lsh<sup>+/+</sup> and Lsh<sup>-/-</sup> MEF cells expressing Dnmt3a, Dnmt3b, Dnmt3a/Dnmt3b, or untransfected (Un) was measured by FACS analysis 8 days after retroviral infection. The difference in fluorescence intensity was expressed as GFP ratio of Lsh<sup>+/+</sup> over Lsh<sup>-/-</sup>. (D) Map of the retroviral vector pMSCV-hGFP indicating the location of *HpaII/MspI* sites and the position of the primers used for methylation-sensitive PCR analysis. Primer pair P9/P10 detects methylation within the GFP region. Primer pair P7/P8 serves as internal control. Primer pair P11/P12 detects methylation in the 5'-LTR and the adjacent region. The length of the expected PCR fragments is indicated in base pairs (bp).

levels by more than 90% as shown by Western blot analysis, whereas Dnmt3a and Dnmt3b levels remained unchanged (Figure 5A). Using Southern blot analysis, genomic DNA was examined for DNA hypomethylation at minor satellite repeat sequences (Figure 5B). Whereas control ES cells were fully methylated at these repeats, low levels of Lsh protein were associated with a small degree of hypomethylation after several passages *in vitro*. The delay in hypomethylation mimicked a similar time course generated by conditional deletion of Dnmt3b in ES cells, which suggests ongoing *de novo* methylation in ES cells at minor satellite sequences (Chen *et al*, 2003). In contrast to embryonic stem cells, 3T3 fibroblasts represent somatic cells that have generally lower levels of Dnmt3a or Dnmt3b proteins and *de novo* methylation activity (Chen *et al*, 2002). Silencing of Lsh in 3T3 cells using the same siLsh vector had no detectable effect on CpG methylation at minor satellite sequences (Supplementary Figure 3A,B) consistent with the idea of low or absent *de novo* methylation activity at pericentric sequences in somatic cells (Chen *et al*, 2002). To determine the effect of Lsh reduction on silencing of retroviral genes, siLsh ES cells were infected with the retroviral vector pMSCV-hGFP that controls expression of the green fluorescence protein (GFP). Within 48–72 h after infection, GFP mRNA levels were notably reduced in wild-type ES cells in contrast to siLsh ES cells, which maintained GFP mRNA levels as shown by RT-PCR analysis (Figure 5C). FACS analysis revealed a decrease in GFP protein of about 80% when comparing control ES cells to

Lsh deficient ES cells, suggesting that successful silencing of a retroviral vector requires the presence of Lsh (Figure 5D). Using methylation-sensitive PCR analysis, the genomic DNA derived from control ES cells 72 h after infection demonstrated *HpaII* resistance indicating the acquisition of methylation (Figure 5E). In contrast, siLsh ES cells showed no detectable CpG methylation at the GFP gene (P9/P10) or the 5'-LTR (P11/P12) regions as demonstrated by sensitivity to *HpaII* digestion. Real-time PCR analysis confirmed the differences in methylation (P11/P12) seen in the presence (control ES) or absence of Lsh (siLsh) (Figure 5F). In contrast, the control PCR reactions (P7/P8) remained indistinguishable comparing control or siLsh derived DNA. In summary, the use of retroviral target sequences in Lsh deficient embryonic stem (ES) cells confirms a role for Lsh in *de novo* methylation.

#### Lsh is involved in *de novo* methylation of endogenous genes

Since Lsh participates in *de novo* methylation of episomal constructs and retroviral transgenes, we also wanted to test whether Lsh deletion in ES cells effects *de novo* methylation of endogenous genes such as the Oct-4 gene (Gidekel and Bergman, 2002). Using methylation-sensitive PCR at two distinct sites in the promoter region of the Oct-4 gene (Figure 6A), we had previously noticed that Lsh<sup>-/-</sup> MEFs have decreased methylation levels at site 2 in comparison with Lsh<sup>+/+</sup> (Figure 6B) but show a similar degree of



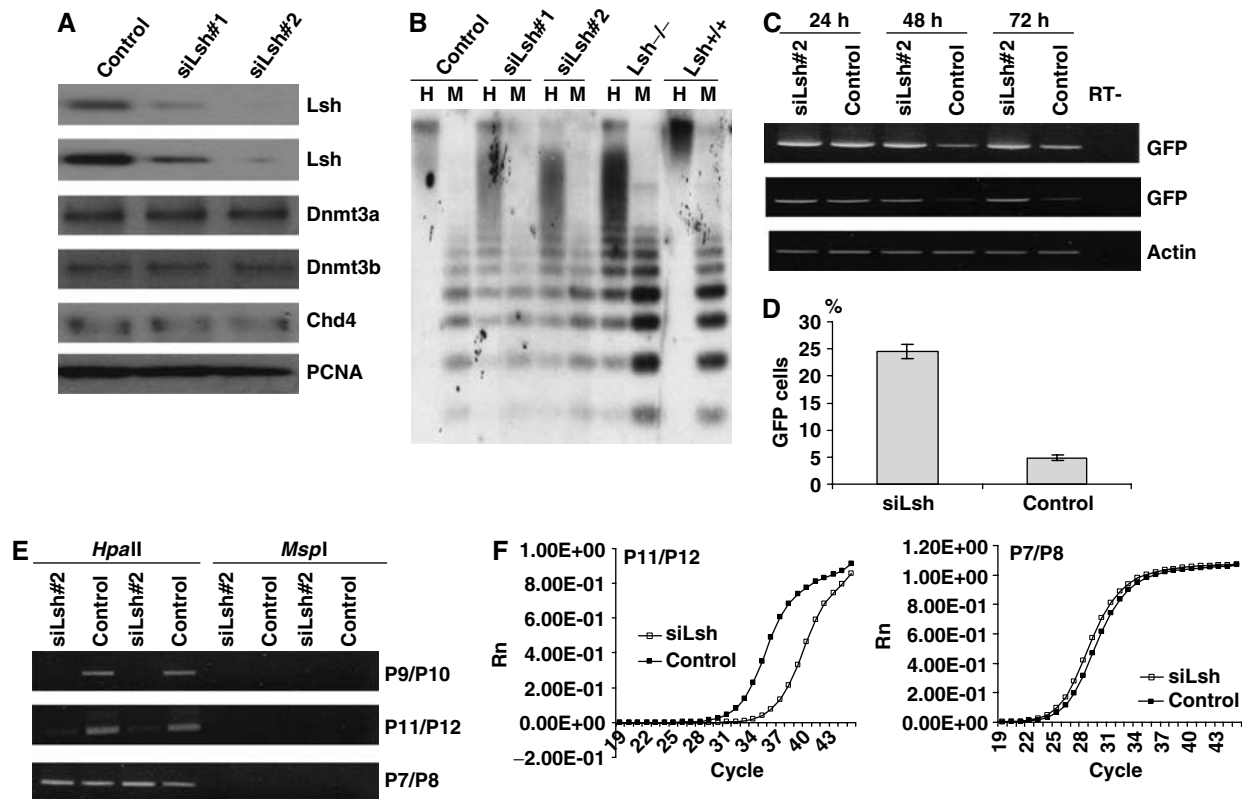
**Figure 4** Lsh functionally cooperates with *de novo* methylation mediated by either Dnmt3a or Dnmt3b. (A) Methylation-sensitive PCR. At 8 days after the retroviral infection, genomic DNA from Lsh<sup>-/-</sup> and Lsh<sup>+/+</sup> MEF cells stably expressing Dnmt3a, Dnmt3b, or Dnmt3a/Dnmt3b was extracted, digested with *HpaII* (H) or *MspI* (M) and subjected to PCR with the indicated primer pairs. For adjustment of DNA undigested DNA (Un) was used before digestion. (B–D). Real-time PCR analysis of *HpaII* digested DNA using primer pair P11/P12. Control primers P7/P8 are used in the lower panel of the graphs.

methylation at site 1 (data not shown). This suggested that Lsh played a role in methylation at selected sites of the Oct-4 gene. It has been reported that differentiation of ES cells *in vitro* is accompanied by CpG methylation at the endogenous Oct-4 gene (Lee *et al*, 2004). Using siLsh ES cells and control ES cells, we compared methylation levels at site 1 and site 2 before (0 day) and after differentiation (6 days) using treatment with retinoic acid. After quantification using undigested genomic DNA to ensure equal input (Figure 6C) digestion with methylation-sensitive restriction enzymes was performed. Site 1 and site 2 are both largely unmethylated in undifferentiated control ES cells or siLsh ES cells (0 day) (Figure 6C and D). On differentiation of control ES cells, site 1 and site 2 gain resistance to restriction enzyme digestion with HpyCh4IV (Hy) or *HpaII* (H) indicating *de novo* methylation (6 Days). In contrast, siLsh ES cells showed a decrease in methylation at site 2 consistent with the observed methyla-

tion defect in Lsh<sup>-/-</sup> MEFs. (Note that amplification of site 2 serves as internal control after digestion to guarantee equal amounts of DNA during the amplification reaction using methylation-sensitive primers for site 1 and *vice versa*.) These results suggested that Lsh deletion in ES cells leads to an impairment in the establishing methylation pattern at endogenous sequences, supporting its role in *de novo* methylation.

#### Lsh interacts with Dnmt3a and Dnmt3b in ES cell

Since Lsh participates in the process of *de novo* methylation, we tested the idea of whether Lsh directly associates with the known *de novo* methyltransferases Dnmt3a or Dnmt3b. Using nuclear extracts from ES cells or from the embryonal cell line P19, we precipitated Lsh with affinity purified antisera directed against a C-terminal peptide of Lsh (Lsh#1) or directed against Lsh recombinant protein (Lsh#2). As shown by



**Figure 5** Silencing of Lsh in embryonic stem cells results in loss of *de novo* methylation. (A) Western analysis: nuclear extracts from two ES cell lines (#1 and #2 both received the same construct) stably expressing a silencing vector for Lsh (siLsh) were examined by Western analysis using specific antibodies against Lsh, Dnmt3a, Dnmt3b, Chd4 or PCNA as control. A scrambled sequence serves as the siRNA vector control. (B) Southern blot analysis: genomic DNA derived from two siLsh ES cell lines, an ES cell control, Lsh<sup>-/-</sup> MEFs, and Lsh<sup>+/+</sup> MEF cells was digested with *HpaII* (H) or *MspI* (M), blotted, and probed for minor satellite sequence using the probe MR150. (C) RT-PCR analysis: siLsh ES cells were infected with the retroviral vector pMSCV-hGFP and after the indicated time points (24, 48, 72 h) RNA was extracted, reverse transcribed and analyzed by PCR for expression of GFP.  $\beta$ -Actin serves as a control. (D) FACS analysis: 72 h after retroviral infection of siLsh and control ES cells GFP expression was measured by FACS analysis. (E) Methylation-sensitive PCR: genomic DNA derived from the siLsh and control ES cells 3 days after retroviral infection was digested with *HpaII* or *MspI* and subjected to PCR analysis with the indicated primer pairs. (F) Real time-PCR analysis: as in (E), *HpaII* digested genomic DNA was subjected to real time-PCR using P11/P12. The right panel shows the internal control reaction with primers P7/P8.

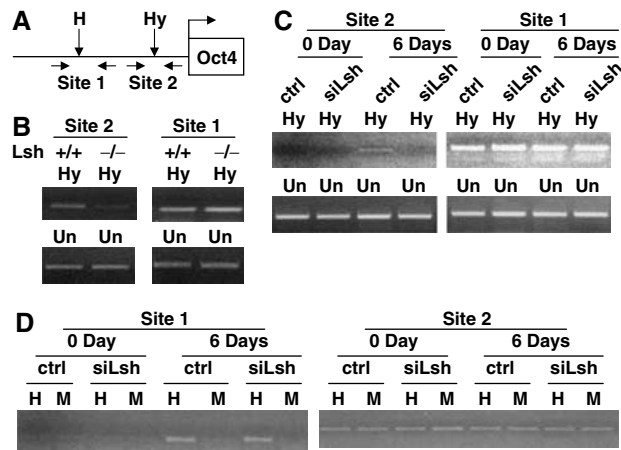
Western blot analysis, Dnmt3a as well as two isoforms of Dnmt3b could be co-immunoprecipitated in the pluripotent embryonal carcinoma cell line P19 (Figure 7A) or ES cells (not shown) using either anti-Lsh antibody, while control rabbit serum served as a control. In contrast, the maintenance methyltransferase Dnmt1 was not precipitated together with Lsh under the same conditions (Figure 7A). In order to test for the reciprocal precipitation, specific antisera suitable for immunoprecipitation of either Dnmt3a or Dnmt3b were applied using nuclear extracts of the embryonal cell line P19. As shown in Figure 7B, either anti-Dnmt3a or anti-Dnmt3b antisera were able to precipitate endogenous Lsh. In contrast, a specific antiserum against Dnmt1 failed to precipitate Lsh, although it was suitable to precipitate endogenous Dnmt1. To further support the specific interaction of Lsh with Dnmt3a or Dnmt3b, the embryonal cell line P19 was stably transfected with plasmids expressing either Dnmt3a or Dnmt3b as myc-tagged fusion proteins. As expected, specific precipitation of Lsh with affinity purified antisera directed against a C-terminal peptide of Lsh (Lsh#1) or directed against Lsh recombinant protein (Lsh#2), but not a control serum was able to detect the Dnmt3a or Dnmt3b fusion proteins by Western analysis using specific anti-myc antibodies (Figure 7C). In a

reciprocal approach, using specific anti-myc antibodies for immunoprecipitation, we were able to detect Lsh in the precipitate (Figure 7D). In summary, these results suggest that Lsh specifically interacts with Dnmt3a or Dnmt3b, but not Dnmt1 in embryonal stem cells. This supports further the idea that Lsh is involved in *de novo* methylation in contrast to maintenance of methylation and furthermore suggests a direct role for Lsh in the process.

## Discussion

Here we present evidence that Lsh is involved in the process of *de novo* methylation at retroviral genes, episomal targets and endogenous sequences and that Lsh interacts with Dnmt3a and Dnmt3b.

We have previously demonstrated that Lsh deletion in mice leads to multiple epigenetic modifications including genomic hypomethylation, alterations in the pattern of histone methylation, and changes in acetylation levels for histone H3 and histone H4 (Muegge, 2005). Since cross talk exists between different types of histone modifications and DNA methylation, it was unclear which epigenetic modifications were altered first by Lsh. The association with Dnmt3a

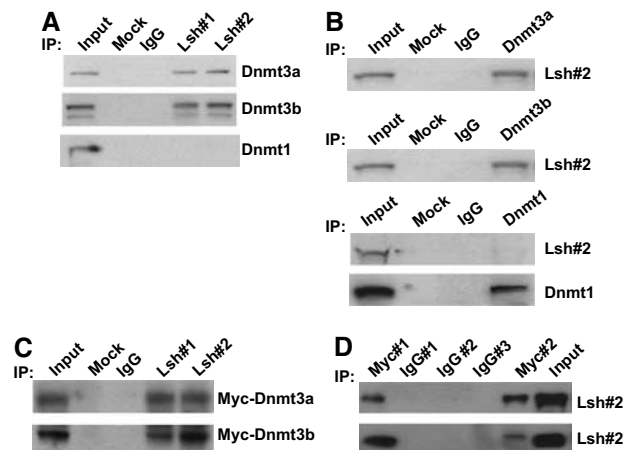


**Figure 6** Lsh is involved in *de novo* methylation at the Oct4 gene. (A) Map of murine Oct4 gene and its promoter indicating the location of *HpaII/MspI* site (H in Site 1) and *HpyCh4 IV* (Hy in Site 2) and the position of the primers used for methylation-sensitive PCR analysis. Due to different recognition sequences of *HpaII* and *HpyCh4 IV*, site 1 and site 2 can be used as internal control for each other in methylation-sensitive PCR. (B) Methylation-sensitive PCR. Genomic DNA derived from the Lsh wild type and Lsh<sup>-/-</sup> MEF cells was digested with *HpyCh4 IV* and subjected to PCR analysis with the indicated primer pairs. Site 1 served as internal control for site 2. Undigested DNA (Un) was used for adjustment of DNA input before digestion. (C) Methylation-sensitive PCR. Genomic DNA derived from the siLsh and control ES cells at 0 day and 6 days after differentiation was digested with *HpaCh4 IV* and subjected to PCR analysis with the indicated primer pairs. Site 1 served as internal control for site 2. Undigested DNA (Un) was used for adjustment of DNA input before digestion. (D) Methylation-sensitive PCR. Genomic DNA derived from the siLsh and control ES cells at 0 day and 6 days after differentiation was digested with *HpaII* and *MspI* and subjected to PCR analysis with the indicated primer pairs. Site 2 served as internal control for site 1.

and b suggests that methylation changes are the primary molecular effect of Lsh.

There is no evidence to date that Lsh directly binds to Dnmt3a or Dnmt3b. Instead, Lsh and Dnmt3 are likely part of a yet unidentified large complex including other protein or possibly RNA components involved in *de novo* methylation. Lsh, being a member of the SNF2 chromatin remodeling family, may facilitate access to some chromatin templates and thus promote targeting of Dnmt3a or 3b to specific genomic targets. Alternatively, Lsh may facilitate the stable association of Dnmt3s with their specific targets and promote the methylation efficiency of nucleosomal templates or condensed chromatin.

The finding that Lsh is not required for maintenance of methylation at episomal plasmids and does not interact with Dnmt1 is consistent with previous observations. For example, the biologic phenotype of Dnmt1<sup>-/-</sup> mice differ greatly from Lsh<sup>-/-</sup> mice: Dnmt1<sup>-/-</sup> mice die around day 9–11 of gestation showing severe developmental defects (Li *et al*, 1992), whereas Lsh<sup>-/-</sup> mice die as newborns and are grossly morphologically normal (with the exception of renal and lymphoid development) (Muegge, 2005). Dnmt1 deletion perturbs imprints (Li *et al*, 1993), while Lsh<sup>-/-</sup> mice generally maintain imprints (with the exception of *Cdkn1c*) (Fan *et al*, 2005). Also, Lsh is associated with pericentric heterochromatin and unlike Dnmt1 is not continuously associated with the replication fork (Yan *et al*, 2003a). Thus, our data



**Figure 7** Lsh interacts with Dnmt3a and Dnmt3b. (A) Western analysis for detection of Dnmt3a, Dnmt3b or Dnmt1 after immunoprecipitation (IP) with Lsh-specific antibodies (Ab): Lsh#1 (C-terminal peptide, affinity purified) and Lsh#2 (recombinant protein, affinity purified) using P19 nuclear extracts. The blots are probed with a monoclonal anti-Dnmt3a, Dnmt3b and Dnmt1 antibodies. The negative controls are normal rabbit IgG or omission of antibodies (mock). Nuclear extracts before IP serve as positive controls. (B) Western analysis for detection of Lsh and Dnmt1 after IP with specific antibodies against Dnmt3a, Dnmt3b or Dnmt1 using P19 nuclear extracts. The following antibodies were used for Western analysis: anti-Lsh#2 (recombinant protein, affinity purified) Dnmt1. The negative controls are normal mouse IgG or omission of antibodies (mock). (C) Western analysis for detection of Myc-Dnmt3a and Myc-Dnmt3b fusion proteins after IP with anti-Lsh#1 (C-terminal peptide) and Lsh#2 (recombinant protein). The nuclear extracts were prepared from P19 cells stably expressing Myc-Dnmt3a (upper panel) or Myc-Dnmt3b (lower panel). The negative controls are normal rabbit IgG or omission of antibodies (mock). (D) Western analysis for detection of Lsh after IP with anti-Myc antibodies using nuclear extracts derived from P19 cells stably expressing Myc-Dnmt3a or Myc-Dnmt3b. Two different anti-myc antibodies were used for IP. The negative controls are normal mouse IgG (IgG#1), mouse IgG1 (IgG#2) or normal rabbit IgG (IgG#3).

suggest that no functional link exists between Lsh and Dnmt1.

Also, the phenotypes of Dnmt3ab<sup>-/-</sup> mice differ somewhat from that of Lsh deleted mice (Okano *et al*, 1999; Dennis *et al*, 2001), suggesting that Lsh and Dnmt3s are not obligated to associate in one complex and can also have distinct biological functions. For example, loss of Dnmt3a and b, in contrast to Lsh deletion, affects the methylation pattern of imprinted genes (Chen *et al*, 2003). Since most methylation imprints are established in germ cells, it implies a specific role for Dnmt3s in the maintenance of methylation pattern at some imprinted loci. Lsh deletion, in contrast, does not in general affect imprinted genes with the exception of the paternal allele of the imprinted *Cdkn1c* (p57<sup>KIP2</sup>) gene. This site acquires *de novo* methylation during early embryogenesis and deletion of Lsh results in loss of methylation and silencing at the paternal allele and bi-allelic expression of the *Cdkn1c* gene (Fan *et al*, 2005). This finding is again consistent with a role for Lsh in *de novo* methylation versus maintenance of methylation.

Aberrant methylation patterns have been implicated in cancer development (Jones and Laird, 1999; Baylin *et al*, 2000). Loss of imprinting leading to overexpression of *Igf2* increases the rate of tumor formation in mice (Sakatani *et al*, 2005). Genomic hypomethylation, causing chromosomal seg-



regulation defects, results in aggressive lymphoma development and an enhanced rate of sarcoma development in *Nf1* and *p53* mutant mice (Eden *et al*, 2003; Gaudet *et al*, 2003). Thus, a defect in normal maintenance of methylation may be a crucial feature in tumorigenesis. On the other hand, *de novo* methylation of tumor suppressor genes may also be involved in the pathogenesis of some tumors (Chen *et al*, 2004; Yu *et al*, 2005). In these scenarios, inhibition of *de novo* methylation may decrease tumorigenesis. Thus, it is crucial to determine the components involved in either maintenance or *de novo* methylation pathways, which could serve as potential molecular targets of intervention in cancer. The finding that Lsh participates in *de novo* methylation may be useful for the generation of selective molecular tools for the prevention of or interference with tumorigenesis.

## Materials and methods

### Plasmids

pCEP4 (Invitrogen) is an Epstein-Barr virus (EBV)-based plasmid vector carrying the EGV replication origin (oriP) and nuclear antigen (encoded by the EBNA-1 gene) to allow for extrachromosomal replication and stable expression in mammalian cells (Einav *et al*, 2003). The replication of pCEP4 was confirmed by digestion with *DpnI* since *DpnI* restriction sites require methylation by the bacterial dam methylase. Thus, episomal DNA becomes resistant to *DpnI* after replication in mammalian cells. The expression vectors pcDNA-Dnmt3a and pcDNA-Dnmt3b were generated by subcloning the corresponding cDNAs into the *EcoRI* site of pcDNA6 (Chen *et al*, 2003). pcDN-Myc-Dnmt3a and pcDN-Myc-Dnmt3b were gift from Dr T Chen, Novartis Institute for Biomedical research, Inc. MA. pMSCV-hGFP (kind gift of Dr Jonathan Keller, NCI, Frederick) has an hGFP reporter gene driven by the murine stem cell virus long terminal repeat (LTR). For RNA interference, the following target site of Lsh was used: 5'-ATTCGGTAGCGGAGACTTC-3' and subcloned into pSEC-neo (Ambion). A scrambled DNA sequence served as negative control and was subcloned into pSEC-neo vector as well.

### Tissue culture and transfection

Lsh<sup>-/-</sup> and Lsh<sup>+/+</sup> mouse embryonic fibroblasts (MEF) were generated as described previously (Fan *et al*, 2003) and grown in DMEM (Invitrogen/GIBCO) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics (Invitrogen/GIBCO).

The mouse ES cells (CCE) were cultured on gelatin-coated dishes without feeder cells in Knockout DMEM (Invitrogen/GIBCO) supplemented with 15% Knockout Serum Replacement, 0.1 mM nonessential amino acid, 0.1 mM  $\beta$ -mercaptoethanol, 50 U/ml of penicillin, 50  $\mu$ g/ml of streptomycin (Invitrogen/GIBCO), and 1000 U/ml ESGRO (mouse leukemia inhibitor factor, Chemicon). For long-term culture, ES cells were trypsinized and passaged every other day. P19 mouse EC cells were grown in alpha-MEM with 10% fetal calf serum, with L-glutamine, and pen/strep. All transfections were performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instruction. Cells were subsequently selected in hygromycin (Invitrogen) for pCEP4, neomycin (Invitrogen) for pSEC-Lsh-neo, blasticidin for pcDNA-Myc-Dnmt3a or 3b, puromycin (Invitrogen) for co-transfection of *in vitro* methylated-pCEP4 and Dnmt3a or Dnmt3b with pBabepuro (ratio 1:10) conferring puromycin resistance. *In vitro* methylation was performed using CpG methylase Sss I (NEB) and successful methylation confirmed by *HpaII* and *MspI* digestion.

### Differentiation of ES

CCE mouse ES cells were grown on 0.1% gelatin-coated plates in knockout DMEM media (Invitrogen) with 15% knockout serum replacement (Invitrogen), L-glutamine, pen/strep, nonessential amino acids,  $\beta$ -mercaptoethanol, and 1000  $\mu$ /ml Esgr0 (LIF, Chemicon). For differentiation, cells were placed in Petri dishes for aggregation in the absence of LIF for 4 days. ES cells were then grown for 2 more days in 1  $\mu$ M all-*trans* retinoic acid (Sigma). Cells

were harvested for nuclear extract at day 0 and day 6 of differentiation as previously described (Geiman and Muegge, 2000).

### Immunoprecipitations

Nuclear extract was generated as previously described (Geiman and Muegge, 2000). Immunoprecipitations for Western blotting were performed by standard methods, in which the nuclear extract buffer was adjusted to a final concentration of 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 0.5% NP-40. In each case, 200  $\mu$ g of nuclear extract was precleared for 30 min with protein G agarose (Invitrogen), incubated with 20  $\mu$ l antibody for 2 h or overnight at 4°C with rotation. Washing was performed five times in 500  $\mu$ l buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 0.5% NP-40) at 4°C, 5 min each on a rotator. Samples were separated on a 6% Tris-glycine SDS-PAGE gel and blotted onto Immobilon P membrane (Millipore). Antibodies used for immunoprecipitation Western blots were species matched normal IgG and IgG1 (Santa Cruz Biotechnology), anti-Lsh C-terminal peptide affinity purified antibody (Lsh#1), anti-Lsh recombinant protein affinity purified antibody (Lsh#2), Dnmt3a and Dnmt3b antibodies (Imgenex), Myc antibody (Roche). Western blot detection was performed according to the manufacturers' instructions (Amersham).

### Western blot and antibodies

Nuclear protein extracts were prepared using Nuclear Pure kit (Sigma) and high salt buffer as described previously (Wang *et al*, 2004) (Figures 1, 3 and 5). The nuclear extracts were separated on 8% Tris-Glycine precast gels (Invitrogen) by electrophoresis and transferred onto PVDF membrane (Invitrogen). Western blotting was performed according to standard procedures using ECL detection reagents, according to the manufacturer's instructions (Amersham). The following primary antibodies were used: murine Dnmt3a (Imgenex); murine Dnmt3b (Imgenex); murine PCNA (Upstate); murine Chd4 (Gift from Dr W Wang, NIH/NIA/IRP); affinity purified rabbit antiserum against a C-terminal portion of Lsh (Lsh#1) (Geiman and Muegge, 2000); affinity purified rabbit antiserum against murine recombinant Lsh (Lsh#2). The following secondary antibodies were used: monoclonal mouse anti-rabbit HRP-conjugated IgG (Sigma), goat anti-mouse HRP-conjugated IgG (Upstate).

### Recovery of episomal and genomic DNA, and methylation-sensitive PCR

Episomal DNA was recovered using the Hirt method (Hirt, 1967) and genomic DNA was extracted using the DNeasy kit (Qiagen). DNA was completely digested with *HpaII*, *MspI*, *Hpych4IV*, or *DpnI*. In order to analyze the methylation status of the episomal DNA vector pCEP4, PCR primer pairs P1/2, P3/4, and P5/6 were used:

- P1 (forward, 5'ACGAACATAAACCCTGACTACGACAT-3');
- P2 (reverse, 5'GTAAGAGCTTCAGCCAAGATTACA-3');
- P3 (forward, 5'AGCCCTCCCGTATCGTAGTT-3');
- P4 (reverse, 5'GCAGAGCGAGGTATGTAGGC-3');
- P5 (forward, 5'-CTGCCAGTGGCGATAAGTCGTGTCTT-3');
- P6 (reverse, 5'CGACAGGACTATAAAGATACCAGGCG-3').

For analysis of the methylation status of the retroviral transgene pMSCV-hGFP, primer pairs P7/P8, P9/P10 and P11/P12 were used: P7 (forward, 5'GCCCACCCTCGTGACCACCCTGACC-3'); P8 (reverse, 5'-CGAAGCTCCAGCAGGACCATGTGATC-3'); P9 (forward, 5'-ATGAGC AAGGGCGAGGAGCTGTTAC-3'); P10 (reverse, 5'-CTTGAAGAAGTC GTGCTGCTTCATGTGGT-3'); P11 (forward, 5'CGCCAGTCCCTCCGAT AGACTGCGT-3'); P12 (reverse, 5'AGTCCCCTGGGACGTCTCCAGGG TT-3'). For analysis of the methylation status of Oct4, primer pairs were used: site 1, forward, AGAGGGTGCAGTGCCCAACAGGCTTTGT, reverse, CCCAGGAAGGCCTTCATTTTCAACCTT; site 2, forward, AAGGTTGAAAATGAAGGCCTTCTGGGG, reverse, TTTCACTCTC CTCCCCAATCCAC. PCR reactions were carried out as follows: 5 min at 94°C, 35 cycles of 60 s at 94°C, 30 s at 60°C, and 60 s at 72°C, and finally 5 min at 72°C. The PCR products were electrophoresed on 1% agarose gels, stained with ethidium bromide, and photographed. For real-time PCR analysis, the ABI PRISM 7900 machine (Applied Biosystems) and Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) were used. The PCR was initiated with one cycle of 50°C for 2 min, one cycle of 95°C for 5 min, followed by 45 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, and finally one cycle of 83°C for 10 s. The negative control without target was

carried for each PCR. For every methylation-sensitive PCR, we adjusted the starting amount of template using undigested DNA in the PCR reaction before enzymatic digestion. The digested DNA was further controlled by using a primer set that amplified an area that did not contain methylation-sensitive restriction enzyme sites. To quantify the amount of methylation on the template using real-time PCR data, standard titration experiments for each template and each primer set were performed and linear regression equation and the calculation for the log copy number were established using Prism 3.0 software (GraphPad Software, Inc.) and Microsoft Excell.

#### **Southern blot analysis**

Genomic DNA was extracted from ES cells, digested with *HpaII* and *MspI*, separated by electrophoresis on 1% agarose gels, and blotted onto Nytran Plus membranes (Scheicher and Schuell). The blots were hybridized for 2 h at 65°C in Rapid-hyb buffer (Amersham) with <sup>32</sup>P-labeled probes and washed twice in 2 × SSC/0.1% SDS at room temperature for 20 min and twice in 0.2 × SSC/0.1% SDS at 65°C for 20 min. The minor satellite repeat probe was a 66-mer oligonucleotide: 5'-GACTGAAAACACATTCGTTGGAAACGGGATTTGTAGAACAGTGTATATCAATGAGTTACAATGAG-3'.

#### **Reverse transcription-PCR**

For detection of GFP mRNA in ES cells transiently infected with pMSCV-hGFP (Jiang *et al*, 2004), the cells were harvested at 24, 48 and 72 h. Total RNA was prepared using RNeasy kit (Qiagen) and 1 µg of RNA was reverse transcribed using Omniscript Reverse Transcriptase (Qiagen). Omission of reverse transcriptase served as negative control. cDNA was amplified in a GeneAmp PCR system

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#### **Supplementary data**

Supplementary data are available at *The EMBO Journal* Online.

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