# Lsh controls silencing of the imprinted *Cdkn1c* gene

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## Summary

**Epigenetic regulation, such as DNA methylation plays an important role in the control of imprinting. Lsh, a member of the SNF2 family of chromatin remodeling proteins, controls DNA methylation in mice. To investigate whether Lsh affects imprinting, we examined CpG methylation and allelic expression of individual genes in Lsh-deficient embryos. We report here that loss of Lsh specifically alters expression of the *Cdkn1c* gene (also known as *p57$^{(Kip2)}$*) but does not interfere with maintenance of imprints at the *H19*, *Igf2*, *Igf2r*, *Zac1* and *Meg9* genes. The reactivation of the silenced paternal *Cdkn1c* allele correlates closely with a loss of CpG methylation at the 5′ DMR at the *Cdkn1c* promoter, whereas KvDMR1 and DMRs of other imprinted genes were not significantly changed. Chromatin immunoprecipitations demonstrate a direct association of Lsh with the 5′ DMR at the *Cdkn1c* promoter, but not with Kv DMR1 or other imprinted loci. These data suggest that methylation of the 5′ DMR plays an important role in the imprinting of the *Cdkn1c* gene. Furthermore, it suggests that Lsh is not required for maintenance of imprinting marks in general, but is only crucial for imprinting at distinct genomic sites.**

Key words: Chromatin structure, DNA methylation, Gene imprinting, Lsh

## Introduction

Imprinting is an epigenetic mechanism that regulates mono-allelic expression in a parent-of-origin-dependent manner (Bartolomei and Tilghman, 1997; Reik and Walter, 2001; Feinberg et al., 2002; Delaval and Feil, 2004). Epigenetic modifications of chromatin label imprinted loci in a heritable fashion to ensure expression of either the maternal or paternal allele of the gene. Loss of imprinting leads to bi-allelic expression or complete silencing of a gene. As a consequence of aberrant gene expression, abnormal embryogenesis can occur that is frequently associated with overgrowth or undersizing of fetal or extra-embryonic tissue. Loss of imprinting also plays an important role in a number of human diseases (such as Beckwith-Wiedemann, Prader-Willi or Angelman syndrome) and is thought to play a role in cancer development (Paulsen and Ferguson-Smith, 2001; Feinberg et al., 2002).

Chromatin modifications that ensure the inheritance of the imprinting pattern are first erased during gametocyte development and then gender-specifically established either at late foetal stages or also after birth in female germ cells (Constancia et al., 1998; Verona et al., 2003; Delaval and Feil, 2004). Epigenetic modifications are based on DNA methylation or histone tail modifications that regulate the accessibility of chromatin. A great deal of evidences points to CpG methylation as a major epigenetic modification that commands the parental identity. Imprinted genes are frequently associated with differentially methylated regions (DMRs) that are established during gametocyte development and maintained with a high fidelity throughout embryogenesis (Constancia et al., 1998; Mann et al., 2000). Mutational analysis of DMRs has supported their crucial role in the mechanism of imprinting (Elson and Bartolomei, 1997; Wutz et al., 1997). CpG methylation, for example, can shape chromatin structure by influencing histone modifications such as acetylation or methylation levels (Bird, 2002). This in turn can alter the accessibility of transcription factors to their appropriate binding site and thus control gene expression. CpG methylation may also directly interfere with the recruitment of DNA binding factors to their target sites. For example, CpG methylation abolishes binding of CTCF to the ICR (imprinting control center) of H19, a differentially methylated region that controls *H19* and *Igf2* (insulin growth factor 2) gene expression (Burgess-Beusse et al., 2002). The ICR is located about 2 to 4 kb upstream of the *H19* gene (Tremblay et al., 1997). Binding of CTCF in turn affects the organization of a ‘chromatin boundary’ that blocks the interaction of a downstream enhancer with the *Igf2* promoter (Simpson et al., 2002). Ultimately, CTCF binding controls *H19* gene expression and suppresses *Igf2* gene transcription. The importance of CpG methylation for imprinting has also been demonstrated in *Dnmt1$^{-/-}$* embryos that display reduced CpG methylation and loss of imprinting at multiple genomic loci (Li et al., 1993; Caspary et al., 1998).

Lsh (lymphoid specific helicase), a member of the SNF2/helicase family of chromatin remodeling proteins is an epigenetic regulator in mice (Jarvis et al., 1996; Geiman et al., 1998; Yan et al., 2003a; Huang et al., 2004). Since Lsh regulates DNA methylation (Dennis et al., 2001), we tested whether changes in CpG methylation levels in *Lsh$^{-/-}$* mice can

influence the mechanisms of gene imprinting. As *Lsh*$^{-/-}$ mice die at birth (Geiman et al., 2001), we examined the maintenance of gene imprints in *Lsh*$^{-/-}$ embryos at day 17.5 of gestation. Among six analyzed imprinted loci, mono-allelic expression was only disturbed in the *Cdkn1c* (*p57*$^{(Kip2)}$) gene by deletion of *Lsh*. The bi-allelic expression of the *Cdkn1c* gene was accompanied by a substantial reduction of CpG methylation at the 5′ DMR of the *Cdkn1c* gene. Furthermore, Lsh is specifically associated with the promoter of *Cdkn1c* in wild-type cells. These results imply independent control mechanisms for imprinted genes and suggest a crucial role for Lsh and DNA methylation to execute the imprinting epigenetic program in a locus-specific manner.

## Materials and methods

### Mice

To generate DNA polymorphisms at imprinted loci, *Lsh*$^{+/-}$ *129/SvEv* (Geiman et al., 2001) mice were crossed with *CzechII/Ei* mice to produce *Lsh*$^{+/-}$ F1 hybrid mice. The *Lsh*$^{+/-}$ F1 hybrid mice were backcrossed with *Lsh*$^{+/-}$ *129/SvEv* mice to produce F2 hybrid embryos that were analyzed for allele polymorphism at imprinting loci. To investigate *Zac1* gene imprinting, *Zac1/lacZ* knock-in mice were used. Generation of *Zac1/lacZ* knock-in animals expressing the bacterial β-galactosidase cDNA under the transcription control of the endogenous *Zac1* promoter has been performed by conventional gene targeting methodology and will be described elsewhere (S.V.K. and C.L.S., unpublished). The mutant allele has been kept on a *129/SvEv* mouse background and proven to maintain the imprinting status both *in vivo* and in cultured primary MEFs (S.V.K. and C.L.S., unpublished). We crossed *Lsh*$^{+/-}$ mice and *Zac1/lacZ* mice to produce *Lsh*$^{+/-}$*lacZ*$^{+/-}$ F1 mice. These were backcrossed with *Lsh*$^{+/-}$ mice. The F2 hybrid offsprings were examined for β-galactosidase activity. *Lsh* genotyping was done as reported before (Geiman et al., 2001), while the presence of the *lacZ* gene was determined by using primers as indicated in Table 1A. Cycling conditions were 3 minutes at 94°C, then 30 seconds at 94°C, 30 seconds at 58°C and 1 minute at 72°C for 30 cycles followed by a final 7-minute extension step at 72°C.

### Polymorphisms analysis

DNA was isolated from whole embryos after removal of the head and the internal organs. Parental origin of alleles of heterozygous individuals was discriminated by exploiting polymorphisms between two mouse strains. PCR primers were used as indicated in Table 1B. PCR products were purified with the QIAquick PCR purification kit (Qiagen), and then digested with the indicated restriction enzymes. The fragments were separated on 0.8-2.0% agarose gels for RFLP analysis to identify the parental origin of the alleles. Undigested PCR products and cleaved products derived from *129/SvEv* and *CzechII/Ei* mice were used as controls.

### RT-PCR analysis

Total RNA was isolated from day 17.5 embryo body tissues using RNAzol (Tel-Test) and the expression patterns of individual genes were analyzed and compared between *Lsh*$^{+/+}$ and *Lsh*$^{-/-}$ embryos. Residual genomic DNA was removed from total RNA with a DNase I (Invitrogen) treatment. Reverse transcription was performed on 2 μg of total RNA using the Mu-MLV reverse transcriptase kit (Ambion). Control reactions were prepared in parallel without reverse transcriptase. The different transcripts were amplified from 1/40 of the reverse transcription reaction in the presence of each of the specific primers. RFLP analysis was done as described above.

### Histochemical staining for β-galactosidase activity

We investigated β-galactosidase gene expression by X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining. Day 13.5 embryos were removed and rinsed with PBS. Embryos were fixed in fixative (0.2% (v/v) glutaraldehyde, 1.5% (v/v) formaldehyde, 5 mM EGTA (pH 8.0), 2 mM $MgCl_2$, in PBS) for 30 minutes. The samples were washed three times in buffer (2 mM $MgCl_2$, 0.01% (w/v) sodium deoxycholate, 0.02% (v/v) Nonidet P-40, in PBS). Staining was carried out at 30°C in the dark for 16 hours in the following buffer (1 mg/ml X-Gal, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, in PBS). For staining of tissue sections, embryos were immediately fixed in liquid nitrogen; 5 μm thick coronal sections were mounted on a set of gelatin-coated glass slides such that serial sections could be used for X-Gal stains.

### Bisulphite mutagenesis assay

An aliquot of 1 μg of genomic DNA was subjected to bisulphite treatment using CpGenome DNA modification kit (Intergen Co) according to the manufacturer's instructions. Primers were generated to match the DMRs of the imprinting gene *H19*, *Igf2r*, *Zac1* and *Cdkn1c*. The converted DNA was subjected to methylation-specific nested PCR, using the following primers (Table 1C) and conditions: for *H19* ICR (Tremblay et al., 1997); for the *Igf2r* gene (Lucifero et al., 2002); for *Cdkn1c* (Yatsuki et al., 2002). For outside primers the following PCR conditions were used: cycling conditions were 3 minutes at 94°C, then 30 seconds at 94°C, 30 seconds at 58°C and 1 minute at 72°C for 35 cycles followed by a final 7-minute extension step at 72°C. For use of inside primers, 30 cycles were used. The PCR products were separated in 1.5% agarose gels and purified using the QIAEX II gel extraction kit (Qiagen). Amplified fragments were subcloned into the pGEMT-Easy vector (Promega). Independent clones for each fragment were sequenced by using the T7 primer.

### Chromatin immunoprecipitation assay

In order to investigate the interaction between Lsh protein and chromatin, we performed chromatin immunoprecipitation assays using a stable transfected 3T3 cell that expressed Flag-tagged Lsh under an inducible promoter (Yan et al., 2003b). Zeocin-resistant cells were induced with 100 pM mifepristone for induction of the Lsh protein. Following cross-linking with culture medium containing 1% formaldehyde at 37°C for 10 minutes and washing twice with ice-cold PBS containing protease inhibitors (1 mM phenylmethyl sulfonyl fluoride (PMSF), 1 μg/ml aprotinin and 1 μg/ml peptatin A) cells were scraped off the dishes and pelleted. The cells were resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1) with protease inhibitors for 10 minutes on ice and sonicated four times for 30 seconds each at a power setting of 3.0 with the Sonicator 3000 (MISONIX) to get 100-1000 bp DNA fragments. The sample was centrifuged to remove cell debris and diluted ten-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM, Tris-HCl pH 8.1, 167 mM NaCl, protease inhibitors). The supernatants were pre-cleared with 80 μl of a mixture of salmon sperm DNA-protein A agarose slurry (Upstate Biotechnology). The slurry solution was centrifuged and the supernatants were incubated with 2 μl of Flag M2 antibody (Sigma) or murine IgG1 as isotype control with rotation overnight at 4°C. Then, 80 μl of salmon sperm DNA-protein A agarose slurry was added and incubated for 1 hour. The beads were washed several times, and the attached immune complexes were eluted with a buffer containing 1% SDS and 0.1 M $NaHCO_3$, followed by reverse-crosslinking at 65°C for 4 hours. DNA was purified by proteinase K digestion, phenol-chloroform extraction and ethanol precipitation. DNA was resuspended in 100 μl of 1×TE and 4 μl were used for PCR analysis. The amplification profile was designed for 30 cycles using the same primers described in the DMR polymorphism analysis (Table 1D).

**Table 1. Oligonucleotides used in this study**

| Gene name | Accession number | Primers | Polymorphism(129 →Czech) | Restriction enzyme (129→Czech) |
|---|---|---|---|---|
| A Genotyping | | | | |
| *lacZ* | L08936 | Forward 5′-gtctcgttgctgcataaacc-3′<br>Reverse 5′-tcgtctgctcatccatgacc-3′ | | |
| B Polymorphisms between 129Sv and Czech mice | | | | |
| *H19* | NM_02123 | Forward 5′-caaagcacccgtgactctgtttcc-3′<br>Reverse 5′-gggcatgttgaacactttatgatggaac-3′ | 1631A→G | No site→*Bgl*I |
| *Igf2* | NM_010514 | Forward 5′-tcagtaatcgatatggggatcccagtggggaa-3′<br>Reverse 5′-ccagtcatcgatatctcactgatggttgctggac-3′ | 1225C→T | *Dde*I→no site |
| *Igf2r* | L22109 | Forward 5′-ttacactgatggtgatgactgtggcagtg-3′<br>Reverse 5′-tggcaggcccccgagtttgactgac-3′ | 41T→C | *Bgl*II→no site |
| *Cdkn1c* | AF160190 | Forward 5′-aggagccgtccatcaccaatcag-3′<br>Reverse 5′-cagagacctgctcagggacctgttc-3′ | 1684A→G | No site→*Tfi*I |
| *Meg9* | AK013406 | Forward 5′-caggtgacaacgctgaattgg-3′<br>Reverse 5′-ggtgtggacagtcctctcagg-3′ | 124A→T | *Nhe*I→no site |
| C Primers for bisulfite sequencing | | | | |
| *H19* | U19619 | Outside forward 5′-gagtatttaggaggtataagaatt-3′<br>Outside reverse 5′-atcaaaaactaacataaaaccct-3′<br>Inside forward 5′-gtaaggagattatgtttatttttgg-3′<br>Inside reverse 5′-cctcattaatcccataactat-3′ | | |
| *Igf2r* | L06446 | Outside forward 5′-ttagtggggtatttttatttgtatgg-3′<br>Inside forward 5′-gtgtggtatttttatgtatagttagg-3′<br>Reverse 5′-aaatatcctaaaaatacaaactacac-3′ | | |
| *Cdkn1c* | AP001293 | Forward 5′-aggatttagttggtagtagt-3′<br>Inside reverse 5′-ttttcaatttcaacaacacc-3′<br>Outside reverse 5′-tatcctatccaacttaaacc-3′ | | |
| *KvDMR1(ICG8a)* | AP001295 | Outside forward 5′-ggtttagttaggaagggatg-3′<br>Inside forward 5′-ggatgaggaaggtaggtttt-3′<br>Reverse 5′-ctaactaatataacctcacc-3′ | | |
| *KvDMR1(ICG8b)* | AP001295 | Outside forward 5′-gtgtgattttatttggagag-3′<br>Inside forward 5′-taaggtgagtggtttaggat-3′<br>Reverse 5′-aatcccccacacctaaattc-3′ | | |
| *Zac1* | AJ308559 | Forward 5′-atttgttatttagtttgggttggg-3′<br>Inside reverse 5′-cccaaattcaaaatttatcacctc-3′<br>Outside reverse 5′-attctcccaaaaattcttaaaaatc-3′ | | |
| D Primers for polymorphism analysis of DMR and/or chip assay | | | | |
| *H19* | U19619 | Forward 5′-aaggaacatgctacattcac-3′<br>Reverse 5′-ctgagatagctcttgagaac-3′ | | |
| *Igf2r* | L06446 | Forward 5′-gtgtggcaccctcatgcatag-3′<br>Reverse 5′-aggtatcctgagggtgcaaac-3′ | | |
| *Cdkn1c* | AP001293 | Forward 5′-cagccacggtactgccaggac-3′<br>Reverse 5′-cgcggcctcctcacgattagc-3′ | | |
| *KvDMR1(ICG8a)* | AP001295 | Forward 5′-agggatgaggaaggtaggcc-3′<br>Reverse 5′-ctggctgatatgacctctcc-3′ | | |
| *KvDMR1(ICG8b)* | AP001295 | Forward 5′-caaggtgagtggcctaggac-3′<br>Reverse 5′-cacctgaattccgagtcggc-3′ | | |
| *Zac1* | AJ308559 | Forward 5′-atttgttatttagtttgggttggg-3′<br>Reverse 5′-cccaaattcaaaatttatcacctc-3′ | | |

## Results

### The Zac1 imprinting pattern is maintained in the absence of Lsh

Previously, we reported a global loss of CpG methylation in all tissues derived from *Lsh*$^{-/-}$ mice (Dennis et al., 2001). The overall decrease of DNA methylation in Lsh-deficient tissue is about 50% in comparison to wild-type DNA. In order to determine whether Lsh can affect gene imprinting, we used the *Zac1/lacZ* transgenic mouse model. The *Zac1* (*Plagl1* – Mouse Genome Informatics) gene encodes a zinc finger transcription factor and is paternally expressed (Spengler et al., 1997). In the *Zac1/lacZ* knock-in strain of mice, one allele of the imprinted *Zac1* gene had been deleted by homologous recombination and replaced with the *lacZ* gene. Thus breeding of *Lsh* heterozygous mice with the *Zac1/lacZ* heterozygous mice allowed distinguishing between the parental alleles at the *Zac1* locus. If the *lacZ* allele is inherited from the paternal site, the *lacZ* gene is expressed, whereas propagation from the maternal site silences the *lacZ* gene. Since the *Zac1* gene is expressed in multiple adult and embryonic tissues (Piras et al., 2000), this transgenic mouse model allows examination of aberrant imprinting control in different tissues using histochemical staining for β-galactosidase activity.

As shown in Fig. 1A, β-galactosidase activity was detected in the tail of wild-type and Lsh heterozygous mice, when the *lacZ* allele was propagated from the paternal side. Similarly, the *Lsh*$^{-/-}$ embryos displayed normal paternal expression pattern of the *lacZ* allele. When the *lacZ* allele was propagated from the maternal side, *lacZ* activity was completely suppressed in wild-type tails as well as in tissue derived from *Lsh*$^{-/-}$ embryos. Thus silencing of the maternal allele was not relieved in the absence of Lsh. In order to determine if Lsh deletion possibly resulted in a ‘leaky’ phenotype and could

affect imprinting in selected tissues, whole embryo stains for β-galactosidase activity were performed. As shown in Fig. 1B, several wild-type tissues expressed the paternal *lacZ* allele (such as the neural tube, somites, sympathetic ganglia, distal second brachial arch, and telencephalic vesicles, skeletal muscle, kidney, liver, lung, brain, and heart), and tissues from the $Lsh^{-/-}$ embryo showed an indistinguishable staining pattern. In contrast, *lacZ*-tagged alleles that were maternally inherited exhibited complete suppression of the *lacZ* gene in all tissues examined. Similarly, $Lsh^{-/-}$ embryos sustained an exclusively paternal expression pattern of the *Zac1* locus, and did not show any signs of reactivation of the maternal allele in any tissue. Thus, despite global DNA hypomethylation in $Lsh^{-/-}$ tissues, imprinting was not in general affected by the loss of Lsh.

A

Lac Z staining

| | ♂(Lsh+/-LacZ+/-) X ♀(Lsh+/-) | ♂(Lsh+/-) X ♀(Lsh+/-LacZ+/-) |
|---|---|---|
| Lsh+/+ LacZ+ | + (n=6) | - (n=5) |
| Lsh+/+ LacZ- | - (n=3) | - (n=4) |
| Lsh-/- LacZ+ | + (n=5) | - (n=4) |
| Lsh-/- LacZ- | - (n=4) | - (n=6) |
| Lsh+/- LacZ+ | + (n=12) | - (n=9) |
| Lsh+/- LacZ- | - (n=10) | - (n=13) |

B

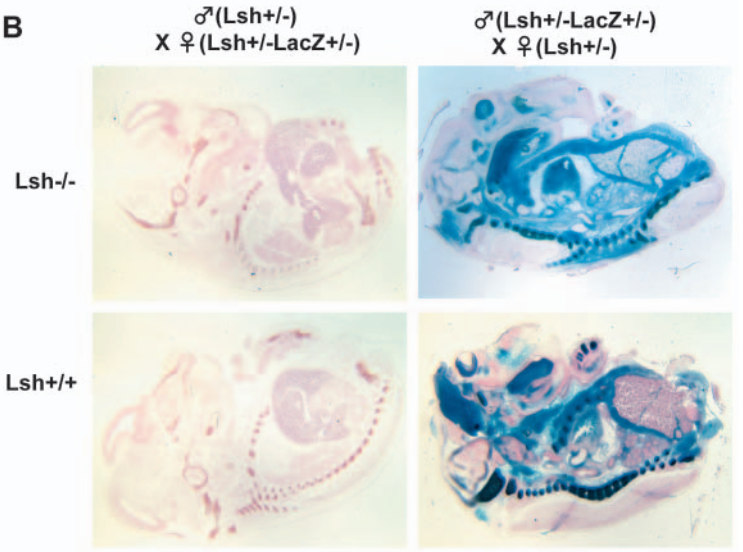


**Fig. 1.** Detection of *lacZ* gene expression in mouse embryos. (A) Summary of the staining for β-galactosidase activity in embryos with different genotypes. (B) Representative staining for β-galactosidase activity in day 13.5 embryos. Neither $Lsh^{-/-}$ nor *Zac1* mutants show any overt morphological abnormalities (Geiman et al., 2001) (C.L.S. and S.V.K., unpublished). *lacZ* gene expression was widely distributed in $Lsh^{+/+}$ and $Lsh^{-/-}$ embryos when the *lacZ* gene was inherited from the paternal side. No expression was observed when the *lacZ* gene was propagated from the maternal side.

## Lsh deficiency leads to bi-allelic expression of the *Cdkn1c* gene

In order to investigate whether Lsh deficiency could affect the expression pattern of other imprinted genes, we introduced allele-specific polymorphisms into the $Lsh^{-/-}$ background (Fig. 2A). $Lsh^{+/-}$ mice were crossed with the wild-type *Czech* strain to generate F1 hybrid mice. These were backcrossed with $Lsh^{+/-}$ mice (F2 hybrids) and genetic analysis performed to distinguish the parental alleles at distinct imprinted loci. Since genetic polymorphisms of hybrid mice created unique restriction enzyme sites (see Table 1B), genomic DNA was amplified by PCR, and RFLP analysis performed (Fig. 2B). $Lsh^{-/-}$ and $Lsh^{+/+}$ mice were selected that had two distinguishable parental alleles (e.g. only F2 hybrid mouse #2 but not embryo #1 showed polymorphisms at the maternally expressed *H19*, *Igf2r*, *Meg9*, and *Cdkn1c* genes and at the paternally expressed *Igf2* gene). Next, RT-PCR analysis of total RNA from selected $Lsh^{+/+}$ and $Lsh^{-/-}$ hybrid embryos derived from day 17.5 of gestation was performed. As shown in Fig. 3, transcripts of all imprinted loci were readily detectable, indicating that none of the examined loci were silenced in the absence of Lsh. Next, we used RFLP analysis to determine the parental origin of each transcript. In wild-type embryos, *H19*, *Igf2r*, *Cdkn1c* and *Meg9* were expressed only from the maternal allele and *Igf2* was expressed from the paternal allele (Fig. 3). Similarly, in $Lsh^{-/-}$ embryos, *H19*, *Igf2*, *Igf2r*, and *Meg9* retained the correct imprinting pattern and displayed monoallelic expression. In contrast, the *Cdkn1c* gene was bi-allelically expressed in $Lsh^{-/-}$ samples indicating a loss of imprinting due to the absence of Lsh (Fig. 3). These results suggest that Lsh controls imprinting only at specific loci, such as the *Cdkn1c* gene, leading to de-repression of the silenced paternal allele.

## Reduced methylation status at the DMR of the *Cdkn1c* gene

To address the question why Lsh affected only the *Cdkn1c* gene, we examined allele-specific methylation differences comparing different imprinted loci. Using a bisulphite mutagenesis assay, we performed methylation analysis at distinct DMRs. This technique allows precise determination of the methylation state at each single CpG dinucleotide, independently of the sequence context. The DMRs have been confirmed to play an important role in the control of imprinted genes and frequently consist of CpG islands that are methylated depending on their parental origin. Genomic DNA derived from F2 hybrids, $Lsh^{-/-}$ embryos, or littermate wild-type controls was bisulphite treated and amplified and subcloned PCR products were subjected to sequence analysis. The parental origin of each sequence could be determined based on genetic polymorphisms within the amplified region.

First, we investigated part of the 3.7 kb-long DMR2 located within intron 2 of the *Igf2r* gene (Wutz et al., 1997). Methylation of the DMR2 is maternally inherited and is thought to control imprinting. In wild-type embryos, maternally derived clones were almost completely methylated at all seven CpG sites within the examined site, whereas the paternal allele was completely unmethylated (Fig. 4). This DNA methylation pattern was entirely preserved in the absence

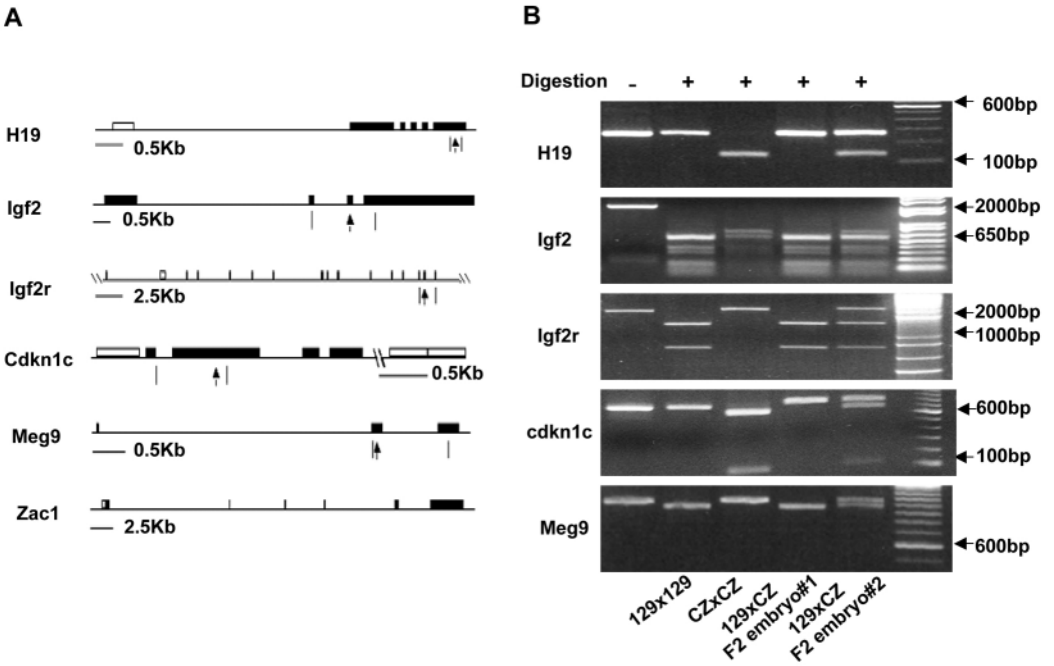


**Fig. 2.** Genomic DNA polymorphism analysis. (A) Schematic representation indicating the distinct imprinted genes examined. The filled boxes represent exons. The open boxes represent the position of differentially methylated regions as examined in this study. The lines under the graph indicate the position of the primers used for genomic DNA polymorphism analysis. The arrows indicate the position of the genomic polymorphisms. (B) Genomic DNA from F2 hybrids was amplified at specific regions using primers presented in Table 1B. PCR products were digested with *Bgl*I for *H19*, *Dde*I for *Igf2*, *Bgl*II for *Igf2r*, *Nhe*I for *Meg9*, and *Tfi*I for *Cdkn1c*. Parent-of-origin alleles are distinguished by the size of the DNA fragments generated by digestion and visualized by ethidium bromide stain after agarose gel electrophoresis. Mice that were homozygous for 129 alleles and homozygous for Czech alleles served as controls. Only embryo #2 but not embryo #1 showed appropriate polymorphisms at the maternally expressed *H19*, *Igf2r*, *Meg9*, and *Cdkn1c* genes and at the paternally expressed *Igf2* gene.

of Lsh. Thus neither did the DMR2 of the *Igf2r* gene exhibit loss of DNA methylation, nor was any change in the *Igf2r* gene expression pattern detectable, suggesting that this imprinted locus was not affected by Lsh deletion.

Next, we characterized the methylation status of the 5′ DMR of the *Zac1* gene. The 5′ DMR, which is about 1580 bp in length, contains a 420 bp-long CpG island that spans part of the promoter region and the first exon of the *Zac1* gene (Smith et al., 2002). This island shows differential methylation on the maternal allele. In wild-type embryos, we detected clones that were fully methylated at the DMR or completely unmethylated in the ratio 1:1, representing the maternal and paternal alleles (Fig. 4). In $Lsh^{-/-}$ samples, a few unmethylated CpG sites sporadically occurred without any recognizable pattern. Since the *Zac1* gene remained imprinted in all examined tissues of the $Lsh^{-/-}$ mice, the data suggested that limited demethylation was not sufficient to activate the silent allele.

The mouse *Igf2* and *H19* genes are located 70 kb apart on chromosome 7, and demonstrate reciprocal imprinting status (Fig. 2A). Only the paternal allele of the *Igf2* gene and only the maternal allele of the *H19* gene are expressed. A DMR upstream of the *H19* gene is essential for their parental allele-specific expression (Tremblay et al., 1997). This region contains conserved CTCF-binding sites involved in the establishment of a 'chromatin boundary' that regulates the imprinted expression of *Igf2* and *H19*. Recently, we studied DNA methylation of the H19 locus at a 3.8 kb region comprising the ICR, examining about ten methylation-

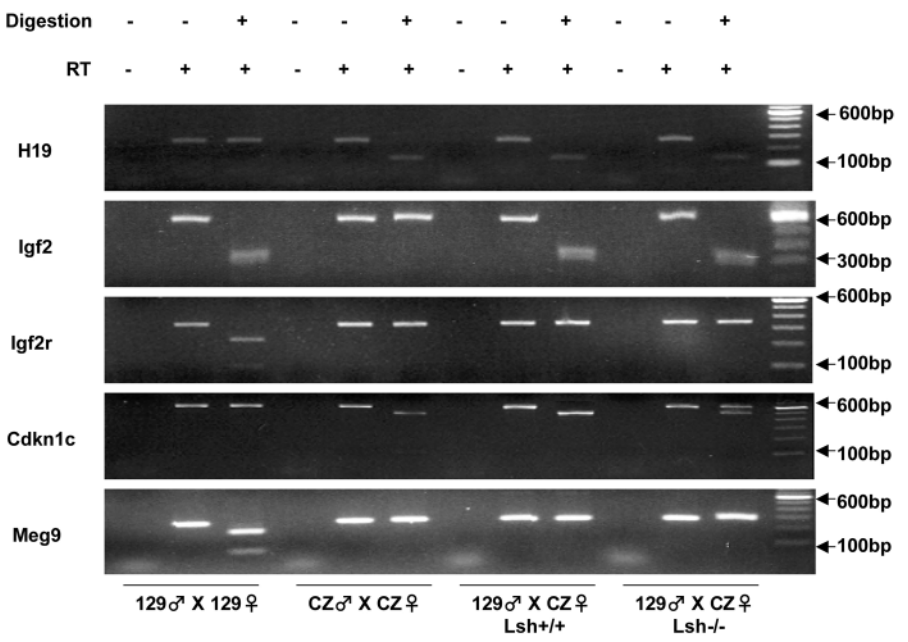


**Fig. 3.** Effect of Lsh on expression of different imprinted genes. RT-PCR analysis for the indicated genes was performed using total RNA derived from F2 hybrid offspring. PCR products were subjected to RFLP analysis by using restriction enzymes listed in Table 1. Omission of reverse transcriptase and uncut PCR products served as controls.

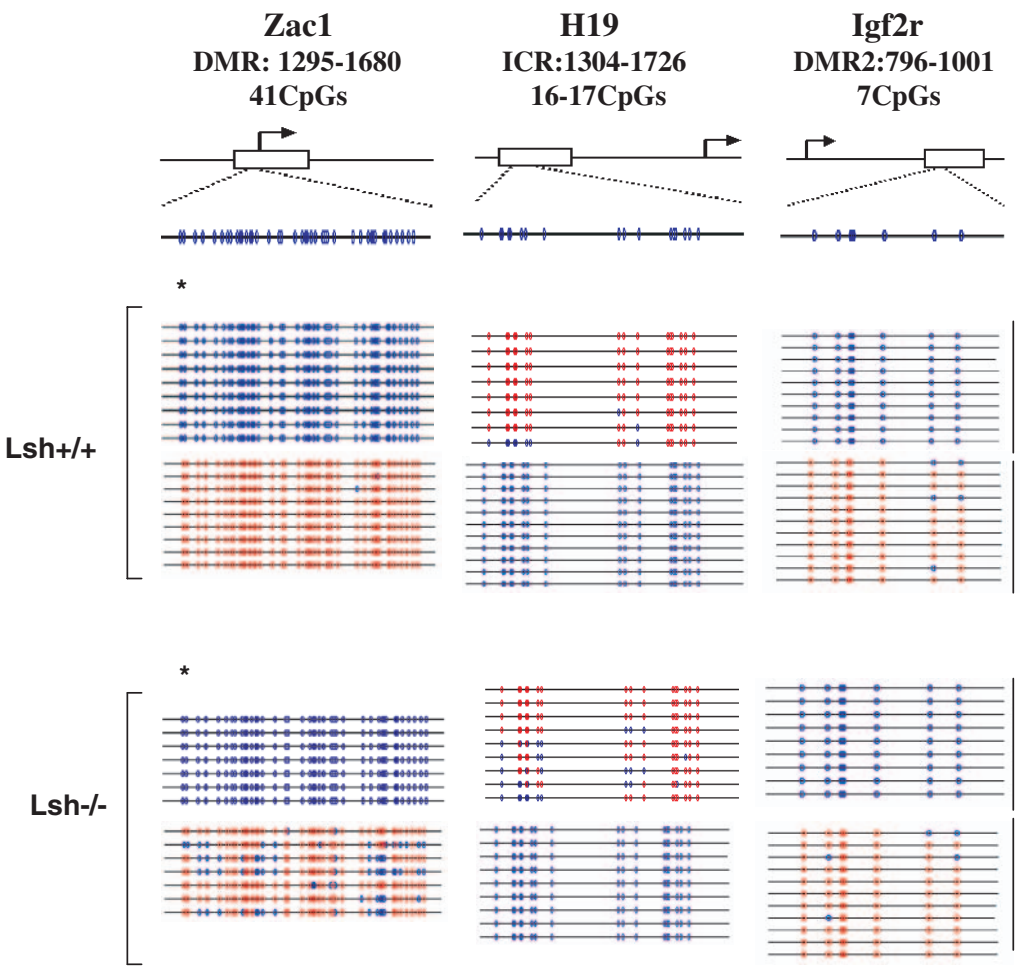


**Fig. 4.** Methylation analysis of DMRs at different imprinted loci in the absence of Lsh. The methylation status of individual DNA alleles derived from $Lsh^{+/+}$ and $Lsh^{-/-}$ embryos are represented in the graph. The gene fragments were amplified for *H19*, *Igf2r*, *Zac1* DMRs from sodium bisulphite-modified DNA and subjected to sequence analysis. (Top) Open boxes correspond to DMRs of each gene. The arrow indicates the transcription start site. Each row corresponds to an individual sequenced strand of DNA, and each circle represents a CpG on the strand, red circles and blue circles indicate methylated and unmethylated sites, respectively. Maternal (M) and paternal (P) alleles were distinguished by a DNA polymorphism in the DMR sequence (*129/Czech*: Igf2,1654A/G, U19619; Igf2r, 851C/G, L06446). *Parent-of-origin determined by the methylation pattern.

sensitive HhaI sites by Southern analysis. Although hypomethylation of some HhaI sites was observed, their precise location could not be determined based on the high density of HhaI sites. Therefore a conclusion as to whether CpG hypomethylation was indeed affecting CTCF binding sites was not possible. Thus we examined in this study a 400 bp-long region of the ICR that comprised two CTCF binding sites. (Note: Previous Southern analysis had not shown any evidence of hypomethylation at the single HhaI site located within this region.) Almost all 16 CpG sites were methylated on the paternal allele, whereas the maternal allele was completely unmethylated (Fig. 4). No significant differences in the methylation pattern of the paternal allele comparing wild-type and $Lsh^{-/-}$ tissues were found. Thus the similar methylation pattern in Lsh wild-type and Lsh-deleted samples corresponded with the lack of expression changes in the *Igf2* and *H19* genes.

Next, we examined the methylation changes at the *Cdkn1c* gene that is maternally expressed (Hatada and Mukai, 1995) (Fig. 5). DNA methylation analysis had identified several DMRs in the locus (Yatsuki et al., 2002). The DMR (KvDMR1) of the *Kcnq1ot1 (Lit1)* promoter is located about 150 kb downstream of the *Cdkn1c* gene. The KvDMR1 gets methylated in oocytes and is unmethylated in sperm, and may represent an imprinting mark in this domain. Loss of methylation and silencing of the *Cdkn1c* gene has been implicated in patients with Beckman-Wiedeman syndrome (Diaz-Meyer et al., 2003). A recent study reported that deletion of the KvDMR1 in mice results in reactivation of the silenced paternal *Cdkn1c* allele (Fitzpatrick et al., 2002). Two sites within the KvDMR1 were analyzed by bisulphite sequencing (known as ICG8a and ICG8b) (Yatsuki et al., 2002), but no significant methylation differences were detectable comparing $Lsh^{+/+}$ and $Lsh^{-/-}$ samples. In contrast, the CpG methylation pattern of the ICG5 site (Yatsuki et al., 2002) was greatly altered in the absence of Lsh (Fig. 5). The ICG5 site is contained within a 5′ DMR located in the promoter upstream of the *Cdkn1c* gene and is largely methylated on the paternal allele in wild-type controls. However, $Lsh^{-/-}$ samples had lost dramatically more than half of the cytosine methylation. Thus the substantial decrease of CpG methylation in the 5′ DMR at the paternal allele is closely associated with loss of paternal silencing and bi-allelic expression of the *Cdkn1c* gene.

### Lsh is physically associated with the DMR of the *Cdkn1* gene

In order to understand why Lsh dramatically affects the methylation state of the *Cdkn1c* gene, we investigated the possibility that Lsh directly interacts with specific sites of the *Cdkn1c* gene. Using the chromatin immunoprecipitation assay (ChIPs), we examined binding of Lsh to the distinct differentially methylated regions that we had previously examined for CpG methylation by bisulphite mutagenesis. Nuclei were prepared from stably transfected 3T3 cells that

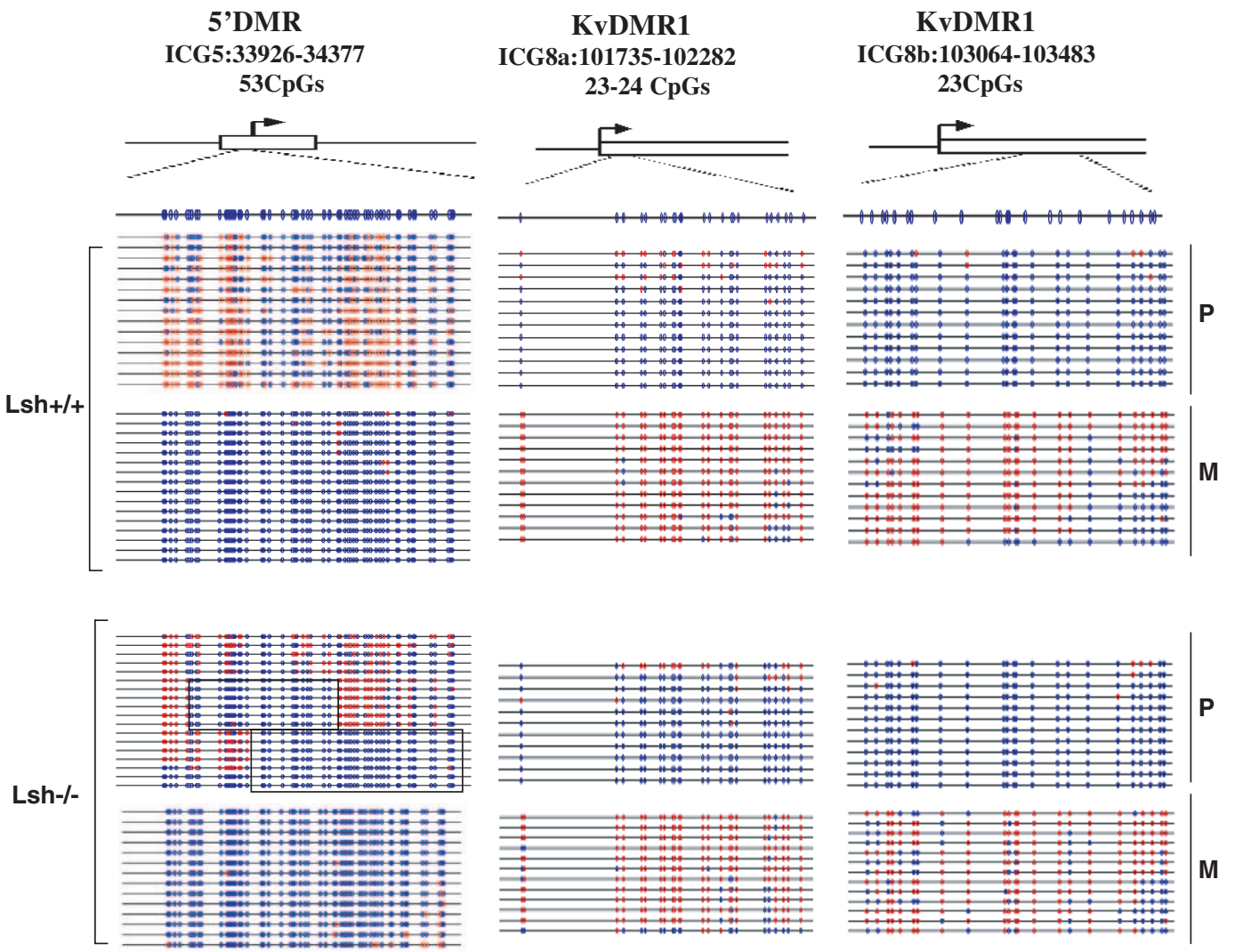


**Fig. 5.** Methylation analysis at three DMRs of the *Cdkn1c* locus in the absence of Lsh. The methylation status was determined as outlined in Fig. 4. The arrow indicates the transcription start site. Each row corresponds to an individual sequenced strand of DNA, and each circle represents a CpG on the strand, red circles and blue circles indicate methylated and unmethylated sites, respectively. Maternal (M) and paternal (P) alleles were distinguished by a DNA polymorphism in the DMR sequences (129/Czech: 5′ DMR *Cdkn1c* (ICG5) 34012 T/G; KvDMR1: ICG8a 101803 A/G and ICG8b 103181 G/C).

could be induced to express high levels of Lsh as a Flag-tagged protein (Yan et al., 2003b). Chromatin was precipitated with anti-Flag antibody and examined by PCR amplification for the presence of DMRs of the *H19*, *Igf2r*, *Zac1* or the *Cdkn1c* gene. As shown in Fig. 6, only sequences from the DMR of *Cdkn1c* were detected in the Flag-tagged precipitates. Furthermore, only the 5′ DMR of the *Cdkn1c* promoter region, but not the KvDMR1 were associated with Lsh.

These data suggest a direct role for Lsh in DNA methylation and chromatin structure in the regulation of imprinting at specific sites such as the *Cdkn1c* gene.

## Discussion

Epigenetic modifications such as DNA methylation are important mechanisms for control of gene imprinting. In this study we have evaluated the role of Lsh, a major regulator of CpG methylation levels in mice, for its effect on gene imprinting. We found that Lsh affects only selected genes such as the maternally expressed *Cdkn1c* gene but had no impact on the majority of imprinted loci analyzed (the paternally expressed *Zac1*, *Igf2* or maternally expressed *H19*, *Igf2r* and *Meg9* genes). Thus independent control mechanisms for genomic imprinting exist and Lsh participates in the control in a locus-specific manner. Since *Cdkn1c* is a cell cycle inhibitor, bi-allelic expression may be in part responsible for the growth retardation observed in the whole $Lsh^{-/-}$ embryo, cultured lymphocytes and embryonal fibroblasts derived from $Lsh^{-/-}$ embryos (Geiman and Muegge, 2000; Geiman et al., 2001; Fan et al., 2003).

Various evidence points to DNA methylation as an important mechanism in genomic imprinting. For example treatment of cell cultures with the demethylating drug azacytidine abolishes the imprint of several genes (El Kharroubi et al., 2001). In addition, deletion of the major 'maintenance' methyltransferase gene *Dnmt1* leads to global demethylation and a loss of imprinting at

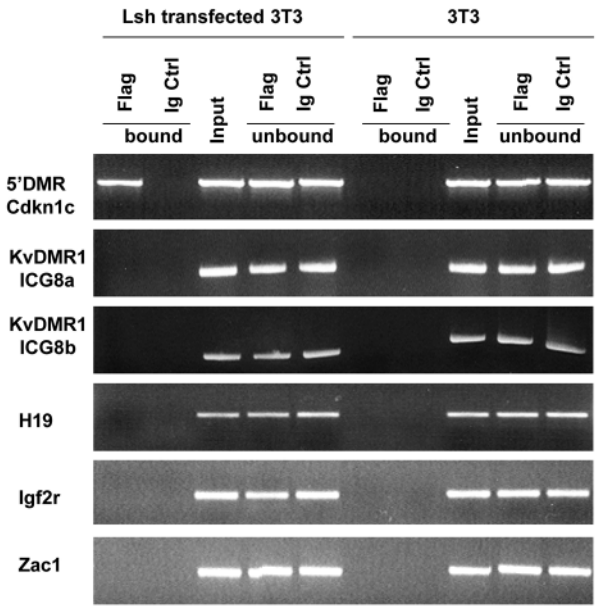


**Fig. 6.** Binding of Lsh to the DMR of *Cdkn1c*. Chromatin immunoprecipitation assays were performed in 3T3 cells that had stably integrated the Flag-Lsh expression plamids. Specific antibodies directed against the Flag-tagged form of Lsh and murine control antibodies of the same isotype were used. Aliquots of chromatin taken before immunoprecipitation were used as 'Input' controls. The immunoprecipitates obtained were PCR amplified using the primers specific to the DMRs of indicated genes.

many loci (Li et al., 1993; Caspary et al., 1998). The close association of CpG hypomethylation at the 5′ DMR of the promoter region, and the de-repression of the silent paternal *Cdkn1c* gene as reported in this study give further support for a functional role of DNA methylation in imprinting.

Two differentially methylated regions have been identified in the *Cdkn1c* gene: KvDMR1 located in an intron within the *Kcnq1* gene (about 150 kb downstream of the *Cdkn1c* gene) and 5′ DMR located in the *Cdkn1c* promoter region. The KvDMR1 is methylated on the maternal allele, whereas the 5′ DMR is methylated on the silenced paternal allele. Furthermore the KvDMR1 site is critical for repression of the silenced paternal allele, as deletion of the unmethylated paternal KvDMR1 results in bi-allelic expression of the embryo (Fitzpatrick et al., 2002). In contrast, deletion of the methylated KvDMR1 on the maternal allele had no effect on *Cdkn1c* gene expression. However, CpG methylation must also play a role in *Cdkn1c* silencing, since genomic hypomethylation caused by deletion of the *Dnmt1* gene resulted in bi-allelic expression of *Cdkn1c* (Caspary et al., 1998). In this report we provide the first evidence that the methylation of the 5′ DMR is critical for paternal silencing, implying that two mechanisms (suppression by the methylated 5′ DMR as well as umethylated KvDMR1) are critical for silencing of the paternal *Cdkn1c* allele.

Although DNA methylation is an important mechanism in the control of imprinting, not all loci are equally affected by methylation and other chromatin-modifying mechanisms are involved, too. For example, in *Dnmt1*$^{-/-}$ mice, H19 and *Cdkn1c* are activated by DNA hypomethylation, whereas *Igf2*, *Igfr* and *Kvlqt1* are silenced and the *Mash2* gene appears unaffected (Li et al., 1993; Caspary et al., 1998). Likewise, uniparental murine embryonal fibroblasts that are treated with the demethylating drug azacytidine show loss of imprinting and de-repression of a few genes (*H19*, *Cdkn1c*, *Peg3*, *Zac1*), whereas other loci remain unaffected (*Grb10*, *Sgca*, *Snrpn*, *U2af1*) (El Kharroubi et al., 2001). Thus, distinct epigenetic mechanisms in addition to DNA methylation have been postulated in the control of imprinting. Furthermore, in some cases the establishment of imprinting may be independent of DNA methylation. For example, in *Dnmt3a/b*-deleted ES cells, CpG methylation is lost at several imprinted loci over time in culture (Chen et al., 2003). Re-introduction of *Dnmt3a/b* transgenes leads to proper remethylation of the paternal allele, but not the maternal allele. Therefore at least at some loci, the imprinted memory is apparently stored as epigenetic modification independent of CpG methylation. The 5′ DMR (ICG5) of the *Cdkn1c* is another example, since it is not methylated in germ cells, but obtains differential methylation in somatic cells before day 7.5 of gestation.

Since most DMRs such as the KvDMR1 and other DMRs analyzed in this study, obtain their methylation pattern already in germ cells, they require only maintenance of methylation during embryogenesis. Our results therefore suggest that Lsh does not play a general role in maintenance of methylation, since deletion of the major maintenance methyltransferase *Dnmt1*, in contrast to *Lsh* deletion, leads to loss of methylation and imprinting at many loci (Li et al., 1993; Caspary et al., 1998). Instead, the specific effect of Lsh on the *Cdkn1c* gene is consistent with the hypothesis that Lsh may play a role in de novo methylation because only the 5′ DMR of the *Cdkn1c* gene acquires methylation in the embryo and not in germ cells. Lsh may promote recruitment and association of de novo DNA methyltransferases (such as Dnmt3a) to specific sites in the genome, or alternatively may facilitate the DNA methyltransferase activity on nucleosomal targets by remodeling chromatin and giving greater accessibility to hidden CpG sites. The patchy loss of methylation at the 5′ DMR of the *Cdkn1c* gene (Fig. 5) may reflect the inaccessibility of DNA methyltransferase in the absence of Lsh, to the central portion of the nucleosomal DNA as opposed to the edges that show greater probability of Dnmt exposure in vitro (Okuwaki and Verreault, 2004). However, in order to test the hypothesis that Lsh plays an important role in de novo methylation, different experimental systems are required. To examine a general role in de novo methylation, the acquisition of methylation patterns in integrated retroviral sequences in embryonal stem cells or in episomal constructs should be tested (Okano et al., 1999; Hsieh, 1999).

The human and mouse promoter regions of *Cdkn1c* share high homology, however, only the murine CpG islands have been reported to show differential methylation. Thus either the human *CDKN1C* gene may be independent of methylation (and Lsh), or alternatively, Lsh may participate in the imprinting control of the human gene, but independently of CpG methylation. We have previously shown that Lsh also controls post-translational modifications such as histone acetylation or methylation levels (Yan et al., 2003; Huang et al., 2004). However, further analysis of genomic imprinting control at the human *CDKN1C* gene has to be performed in order to determine the role of Lsh.

Loss of imprinting has been implicated in the origin of sporadic cancers and human inherited syndromes that are cancer prone (Reik and Walter, 2001; Paulsen and Ferguson-Smith, 2001; Feinberg et al., 2002). A subset of patients with Beckwith-Wiedemann syndrome that are prone to childhood malignancies show a functional mutation in the *Cdkn1c* gene. We report here the hypomethylation at the *Cdkn1c* promoter correlated with bi-allelic expression. Since *Cdkn1c* is a cell cycle inhibitor its role has been largely implicated as a tumor suppressor gene whose loss of function promotes growth and tumor progression. However, a number of tumors have been reported that do not show silencing, but instead show overexpression of the *Cdkn1c* gene (Hartmann et al., 2000; Lai et al., 2000; Ito et al., 2002). For example, a subset of patients with head and neck cancers, or patients with hepatoblastoma exhibit an upregulation of *Cdkn1c* gene expression with reactivation of the paternal allele, and frequent loss of heterozygosity of the maternal gene. Furthermore, some patients with Wilms tumor show paternal expression of *Cdkn1c* and loss of heterozygosity of the maternal region. Though *Cdkn1c* is a cell cycle inhibitor, it interacts with transcription factors (such as MyoD) and proteins of the c-Jun/stress-activated kinase pathway (Chang et al., 2003; Reynaud et al., 2000). Thus inhibition of the UV-or stress-induced apoptotic pathway *Cdkn1c* may contribute to cancer progression or therapy resistance of some tumors.

Investigating Lsh's unique contribution to the epigenetic regulation at distinct imprinted loci should help our understanding of the multiple mechanisms that control imprinting, and their role in pathogenetic processes such as cancer.

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