

H19 lncRNA controls gene expression of the Imprinted Gene Network by recruiting MBD1

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The *H19* gene controls the expression of several genes within the Imprinted Gene Network (IGN), involved in growth control of the embryo. However, the underlying mechanisms of this control remain elusive. Here, we identified the methyl-CpG-binding domain protein 1 MBD1 as a physical and functional partner of the *H19* long noncoding RNA (lncRNA). The *H19* lncRNA-MBD1 complex is required for the control of five genes of the IGN. For three of these genes—*Igf2* (insulin-like growth factor 2), *Slc38a4* (solute carrier family 38 member 4), and *Peg1* (paternally expressed gene 1)—both MBD1 and H3K9me3 binding were detected on their differentially methylated regions. The *H19* lncRNA-MBD1 complex, through its interaction with histone lysine methyltransferases, therefore acts by bringing repressive histone marks on the differentially methylated regions of these three direct targets of the *H19* gene. Our data suggest that, besides the differential DNA methylation found on the differentially methylated regions of imprinted genes, an additional fine tuning of the expressed allele is achieved by a modulation of the H3K9me3 marks, mediated by the association of the *H19* lncRNA with chromatin-modifying complexes, such as MBD1. This results in a precise control of the level of expression of growth factors in the embryo.

genomic imprinting | embryonic growth | long noncoding RNA partner | *Dlk1* | *Cdkn1c*

The imprinted *H19* locus belongs to a conserved gene cluster on chromosome 7 in the mouse and 11p15.5 in human, and it plays an important role in embryonic development and growth control. The cluster contains the insulin-like growth factor 2 (*Igf2*) gene, located 90 kb away from the *H19* gene, and both genes are coordinately regulated by an intergenic differentially methylated region (DMR) also called imprinting control region (ICR) and by downstream enhancers, with *H19* being expressed from the maternal and *Igf2* from the paternal allele (1, 2). The *Igf2* gene is under the additional control of somatic DMRs 1 and 2 in the embryo. Both genes are strongly expressed during embryogenesis and down-regulated after birth, with *H19* remaining expressed in adult skeletal muscle and heart.

The *H19* gene produces a 2.3 kb spliced, capped, and polyadenylated long noncoding RNA (lncRNA) (3). The *H19* locus also produces a microRNA (miR) from a highly conserved region in the first exon. This miR-675 plays a role in controlling placental growth at the end of gestation by regulating the expression of the *Igf1r* gene (4).

The targeted deletion of the gene (*H19*^{Δ3}) induces an overgrowth phenotype (+ 8% compared with WT mice), which can be rescued by transgenic reexpression of *H19* (5, 6). Expression of the *Igf2* gene is affected by the deletion of the *H19* gene, and it becomes biallelically expressed, with a 35% level of expression from the usually silent maternal allele. Similarly, eight other genes belonging to an Imprinted Gene Network (IGN) (7) also show an increased expression level in the absence of *H19*, which is restored to a normal level by transgenic reexpression. These data suggest that *H19* acts in *trans* to regulate the expression of these genes and to control growth of the embryo (6). Whether

this control is transcriptional or posttranscriptional and whether these nine targets are direct or indirect targets remain elusive.

Several lncRNAs interact with chromatin-modifying complexes and appear to exert a transcriptional control by targeting local chromatin modifications at discrete genomic regions (8, 9). In the case of imprinted clusters, the DMRs controlling the expression of imprinted genes exhibit parent-of-origin epigenetic modifications (DNA methylation and histone modifications) that govern the imprinting of the locus. In some of these clusters, lncRNAs control in *cis* the transcription of adjacent genes. For example, the *Kenq1ot1* lncRNA associates with the lysine methyltransferase G9a and the Polycomb Repressive Complex (PRC2) to regulate the expression of other genes of the locus in the placenta (10, 11). Similarly, in the context of X-inactivation, the *Xist* lncRNA associates with PRC2 and creates domains of repressive control on the inactive X chromosome (8). Alternatively, some lncRNAs, termed macroRNAs, such as *Aim* and *Nespas*, act by silencing promoters and enhancers by transcriptional overlap (12, 13). Finally, in other nonimprinted regions, several lncRNAs, such as *HOTAIR*, seem to exert their functional role by recruiting chromatin-modifying complexes for transregulation (14, 15).

To elucidate the mechanism of action of the *H19* lncRNA on the genes of the IGN, we performed RNA immunoprecipitation (RNA-IP) with specific proteins and discovered that *H19* RNA binds the methyl-CpG-binding domain protein 1 (MBD1). MBD1 belongs to the family of the methyl-CpG-binding domain proteins, such as MBD2, MBD3, MBD4, and MECP2 (methyl-CpG-binding protein 2) (16). MeCP2, MBD1, and MBD2 proteins bind to methylated DNA and recruit different histone

Significance

The *H19* imprinted gene produces a long noncoding RNA (lncRNA) exclusively expressed from the maternal allele. It is involved in the control of embryonic growth and regulates nine genes of an Imprinted Gene Network (IGN). Our goal was to decipher the molecular mechanisms that drive this control of the IGN. We show that this lncRNA represses several target genes through interaction with the methyl-CpG-binding domain protein 1 MBD1. This protein is involved in the maintenance of repressive H3K9me3 histone marks. The *H19* RNA is required for the recruitment of MBD1 to some of its targets, including the adjacent insulin-like growth factor 2 gene, and acts by a fine-tuned regulation on the expression levels of these growth-controlling genes of the IGN.

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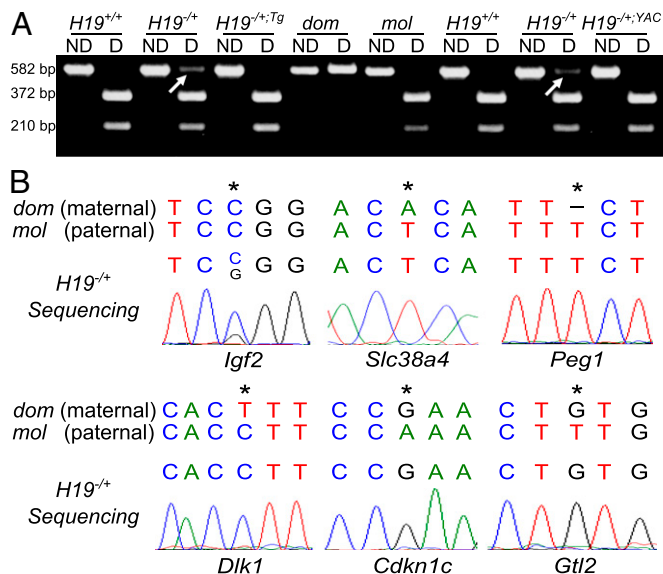


Fig. 1. Allele-specific expression of the IGF2 gene. (A) Allele-specific expression analysis of the *Igf2* gene detected by RT-PCR followed by *MspI* digestion in E14.5 limb muscle samples. *Molossinus* (*mol*, paternal) allele presents an *MspI* restriction site absent in the *domesticus* (*dom*, maternal) allele. Nondigested (ND) and digested (D) RT-PCR products are presented. On the left, results obtained with a *H19*^{-/-;Tg} × JF1 mating. On the right, results obtained with a *H19*^{-/-;YAC} × JF1 mating. Arrows show the maternal *Igf2* allele. (B) Allele-specific expression analysis of other imprinted genes of the network detected by RT-PCR and sequencing in *H19*^{-/-} E14.5 limb muscle samples. Maternal (*dom*, *domesticus*) and paternal (*mol*, *molossinus*) sequences are indicated. Stars indicate polymorphisms between the two alleles.

deacetylase (HDACs)- and histone lysine methyltransferase (KMT)-containing complexes that control chromatin compaction and gene silencing (17). In particular, MBD1 associates with the KMTs SETDB1 and SUV39H1, responsible for H3K9 methylation (18, 19). MBD1 actually binds both to methylated and unmethylated DNA sequences, and its role is still unclear (20). Interestingly, it was shown that MBD1, as well as other MBD proteins, can bind RNA in vitro (21), but this was never explored in vivo.

In the current study, we show that the *H19* lncRNA is one of the partners of MBD1. The *H19* lncRNA-MBD1 complex participates in the control of several genes of the IG, by modifying the repressive histone marks on DMR regions controlling their expression.

Results

The *H19* RNA Controls the Imprint of *Igf2* in Vivo. Because several imprinted genes were up-regulated in the absence of a functional *H19* gene (6), we investigated if this overexpression was due to loss of imprinting of these genes.

The *H19*^{Δ3} mice harbor a 3 kb deletion of the transcription unit. We established an *H19* transgenic line (*H19*^{Tg}) carrying the *H19* transcription unit under the control of the *neclin* gene promoter and used the previously described YZ8 one copy YAC line (*H19*^{YAC}) (22). These transgenic lines were bred onto an *H19*^{Δ3} background. *H19*^{-/-;Tg} and *H19*^{-/-;YAC} females were crossed with JF1 WT (*Mus musculus molossinus* or *mol*) males, to distinguish the parental origin of the alleles. Using restriction length polymorphism on cDNA samples from E14.5 muscle, we confirmed that the *Igf2* gene loses its imprinted status in *H19*^{-/-} embryos (Fig. 1A). Interestingly, we observed that *Igf2* imprint was rescued upon reintroduction of an *H19* transgene, as *Igf2* was exclusively expressed from the paternal allele in *H19*^{-/-;Tg} and *H19*^{-/-;YAC} embryos (Fig. 1A). These data suggest that the

H19 RNA represses the maternal expression of the nearby *Igf2* gene in *trans* and thus regulates *Igf2* gene levels in embryonic limb muscle.

We then focused on the other imprinted genes whose expression was increased in absence of the *H19* gene. In contrast to the *Igf2* gene, RT-PCR followed by sequencing indicated that the other *H19* targets of the IG (such as *Slc38a4*, *Peg1*, *Dlk1*, *Cdkn1c*, and *Gtl2*) remained monoallelically expressed in *H19*^{-/-} embryonic limb muscle (Fig. 1B). *Dcn* is biallelically expressed, however sequencing in WT embryonic limb muscle showed that this gene is actually not imprinted in this tissue (Fig. S1).

The *H19* lncRNA Represses the IG in MEFs. To study the molecular mechanisms that drive the *H19*-mediated repression of the IG in the embryo, we chose to use primary mouse embryo fibroblasts (MEFs) as a model system. We measured IG expression levels in WT, *H19*^{-/-}, and *H19*^{-/-;Tg} MEFs. We observed, as previously shown in limb muscles, that *Igf2*, *Slc38a4*, *Dcn*, *Dlk1*, *Peg1*, *Gtl2*, *Cdkn1c*, and *Igf2r* are overexpressed in cells lacking *H19* RNA expression (Fig. 2A and Table S1). In MEFs that ectopically express the *H19* RNA in an *H19*^{-/-} background (*H19*^{-/-;Tg}), the expression of these genes is restored to WT level. Thus, both in MEFs and in embryonic limb muscle, the *H19* gene negatively regulates several genes of the IG. The level of expression of these target genes was often higher in MEFs compared with that in embryonic muscle samples. This suggests that a strict control of the expression of growth-controlling genes is exerted in vivo, whereas this control is more flexible in an in vitro culture system.

We also evaluated the levels of expression of the primary transcripts using primers in introns of target genes (Fig. 2B). For *Igf2*, *Slc38a4*, and *Peg1* genes, an increase in the level of primary transcript expression was detected in *H19*^{-/-} compared with WT MEFs. The primary transcripts are further down-regulated in the presence of the *H19* transgene, suggesting a possible transcriptional effect on the levels of expression of these three genes. However, we cannot fully exclude a posttranscriptional effect, as

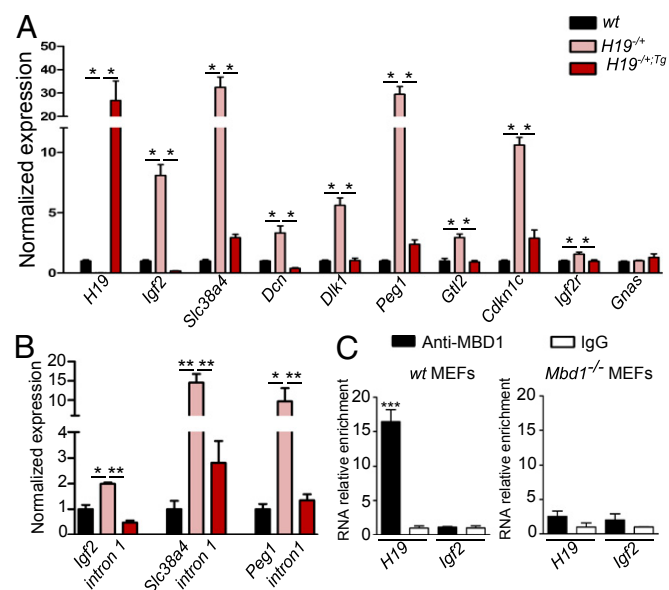


Fig. 2. *H19* modulates the IG in MEFs and interacts with the MBD1 protein. (A) Expression levels in E14.5 primary MEF samples were detected by RT-qPCR. The expression level of WT MEFs was set to 1, and histograms show modifications relative to this level ($n = 4$ for each genotype). (B) Primary transcript levels were detected using primers located in introns of the genes. (C) RIP with an antibody to MBD1 indicates binding to *H19* in WT MEFs. The enrichment of RNA over a random IgG is shown. *Igf2* mRNA was used as a negative control. The specificity of the antibody was tested by performing the experiment in *Mbd1*^{-/-} MEFs.

alternative splicing or nuclear degradation of transcripts containing introns could also occur.

***H19* lncRNA Interacts with the MBD1 Protein.** Because the level of expression of several imprinted genes was restored to WT levels in *H19*^{-/+Tg} cells, we hypothesized that the *H19* lncRNA may interact with proteins involved in epigenetic modifications. RNA immunoprecipitation assays (RIPs) were performed on WT MEFs. Interestingly, we observed that the *H19* RNA significantly coimmunoprecipitated with the MBD1 protein, previously shown to have a high affinity for RNA (Fig. 2C). The *Igf2* mRNA was used as a control for binding specificity, and the RIP experiment was performed in *Mbd1*^{-/-} MEFs to confirm the specificity of the MBD1 antibody. No interaction of the *H19* RNA was detected with either EZH2 or SUZ12, components of the PRC2 complex (Fig. S2). This result led us to hypothesize that *H19* could possibly control IGN expression through its interaction with the MBD1 protein.

MBD1 Is a Repressor of the IGN. To test if the MBD1 protein was involved in the *H19*-mediated repression of the IGN, we first investigated the expression level of the IGN in *Mbd1*^{-/-} MEFs (Fig. 3A). Five genes of the IGN (*Igf2*, *Slc38a4*, *Dcn*, *Dlk1*, and *Peg1*) are overexpressed in *Mbd1*^{-/-} compared with WT MEFs. This indicates that the repression of these genes is not only dependent on the presence of *H19* (Fig. 2A), but also on the presence of the MBD1 protein. We also performed *Mbd1* siRNA-mediated knockdown experiments. In cells treated with a *Mbd1* siRNA, these five genes were up-regulated compared with cells treated with a nonsilencing control siRNA (Fig. 3B). This confirms that MBD1 is necessary for the maintenance of IGN repression in MEFs. *Gtl2*, *Cdkn1c*, and *Igf2r* are not overexpressed in *Mbd1*^{-/-} MEFs compared with WT MEFs, suggesting that their repression is dependent on *H19* but does not require MBD1. Together, these results show that five out of nine genes of the IGN are common targets of both *H19* and MBD1, suggesting that this protein may be involved in the function of *H19* lncRNA as a repressor of this network.

***H19* lncRNA Requires MBD1 to Repress Its Targets.** The next challenge was to test if *H19* lncRNA and MBD1 act together to repress the IGN, or if they act through independent pathways. We performed siRNA-mediated knockdown of *H19* lncRNA in *Mbd1*^{-/-} MEFs to test if *H19* requires the MBD1 protein to repress the five genes of the IGN that show *H19* lncRNA and MBD1 dependence. We observed that *Gtl2*, a target of *H19* but not of MBD1, was overexpressed in cells treated with an *H19* siRNA compared with cells treated with a control siRNA, as expected (Fig. 3C). In contrast, the expression of common targets of *H19* and MBD1, such as *Slc38a4*, *Dlk1*, and *Peg1*, was not affected by the down-regulation of *H19* lncRNA in *Mbd1*^{-/-} MEFs, whereas *Igf2* and *Dcn* mRNA levels were strongly down-regulated (50% reduction) (Fig. 3C). These data indicate that *H19* lncRNA requires the MBD1 protein to repress *H19*-MBD1 common targets. This also suggests that in the absence of MBD1, *H19* lncRNA could act as an activator of *Igf2* and *Dcn* rather than a repressor.

In summary, these data demonstrate that the *H19* lncRNA represses five genes of the IGN (*Igf2*, *Slc38a4*, *Dcn*, *Dlk1*, and *Peg1*) in a manner dependent on the MBD1 protein.

Direct Targets of the *H19* lncRNA-MBD1 Complex. The MBD1 protein is a DNA-methylation-dependent transcriptional repressor that also binds unmethylated CpG islands via its CXXC domain (20, 23). To test if genes of the network modulated by the *H19* lncRNA-MBD1 complex are direct or indirect targets, we performed MBD1 ChIP-quantitative PCR (qPCR) experiments in MEFs. MBD1 binds to the DMR1 region of *Igf2* and to the DMRs of *Slc38a4* and *Peg1* in WT MEFs (Fig. 4A). The *Slc38a4* and *Peg1* paternally expressed genes harbor maternally methylated gametic DMRs. The *Igf2* somatic DMR1 is an unusual DMR, as it displays DNA hypermethylation on the paternal expressed allele and is thought to bind a repressor protein on the maternal allele, which results in a silencer effect on this allele (24). Interestingly, further sequencing of the immunoprecipitated DMRs using JF1 polymorphisms showed that the MBD1 protein binds to both the paternal and the maternal alleles (Fig. 4C and Fig. S3). We next performed MBD1 ChIP experiments in *H19*^{-/+} MEFs to test if the *H19* lncRNA was required for the

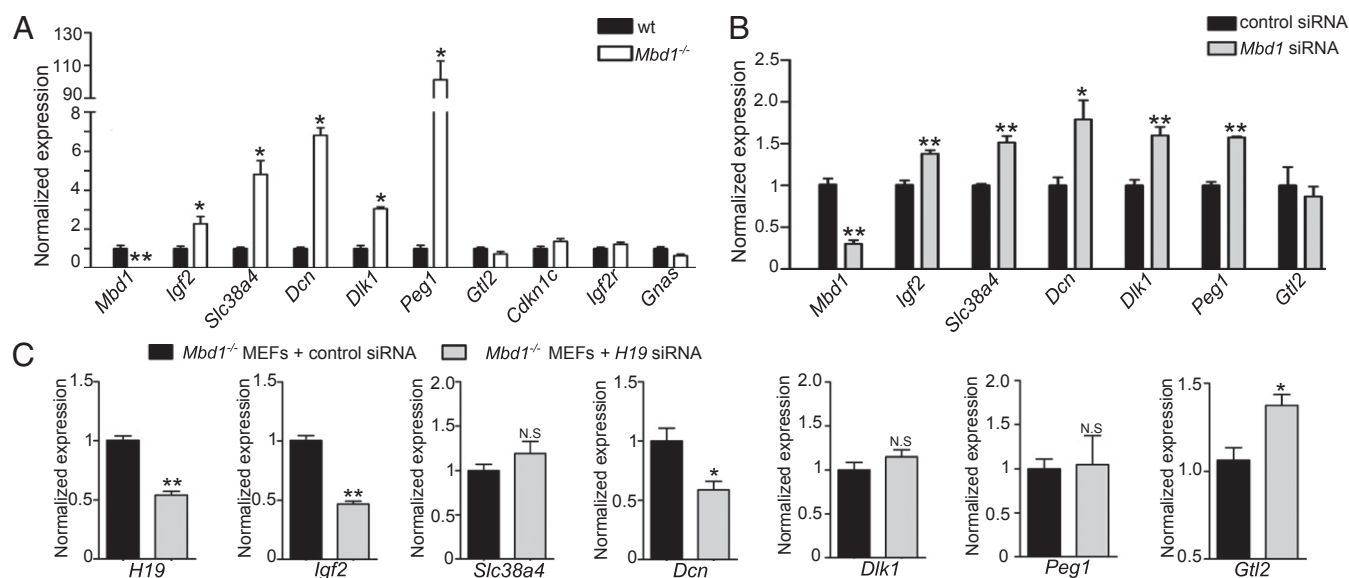


Fig. 3. *H19* represses its targets via the MBD1 protein. (A) Expression levels of the IGN in *Mbd1*^{-/-} samples were detected by RT-qPCR. The expression level in WT MEFs was set at 1, and histograms show modifications relative to this level ($n = 4$). (B) siRNA-mediated knockdown experiments of MBD1 in WT MEFs. The expression level in MEFs treated with a nonsilencing control was set at 1, and histograms show modifications relative to this level ($n = 6$). (C) siRNA-mediated knockdown experiments of *H19* in *Mbd1*^{-/-} MEFs. The expression level in MEFs treated with a nonsilencing control was set at 1, and histograms show modifications relative to this level ($n = 6$).

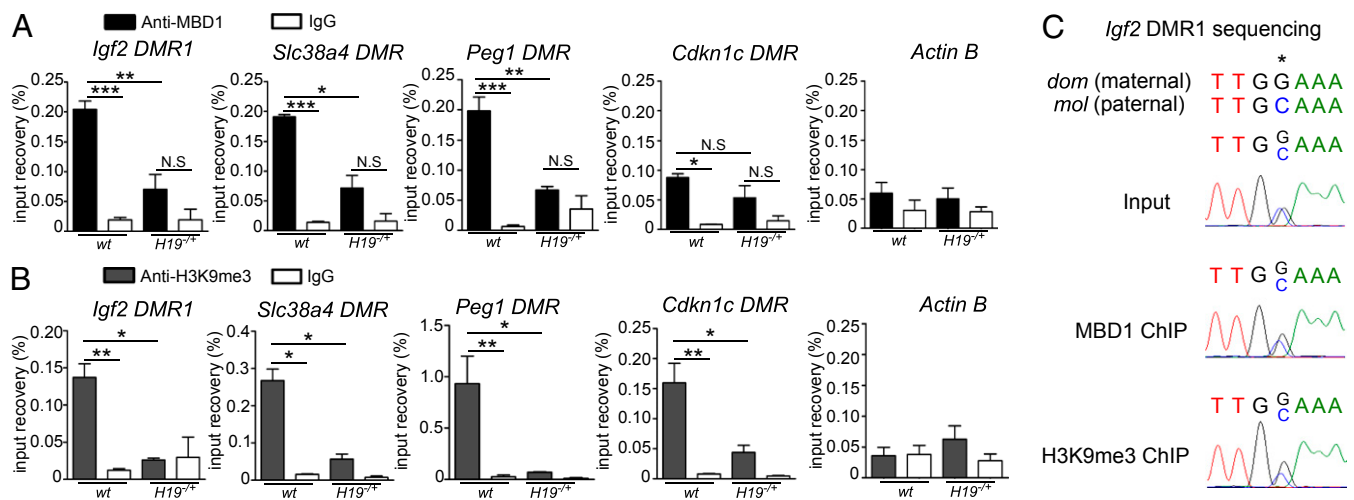


Fig. 4. *H19* is necessary for recruitment of MBD1 and H3K9me3 to the *Igf2* DMR1 and *Slc38a4* and *Peg1* DMRs. (A) Chromatin immunoprecipitation with an antibody to MBD1 in WT and *H19*^{-/-} MEFs (n = 4). Histograms represent the ratio of immunoprecipitated DNA relative to the input sample. *Igf2* DMR1, *Slc38a4*, *Peg1*, and *Cdkn1c* DMR regions were analyzed. *Actin B* was included as a negative control. (B) Chromatin immunoprecipitation with an antibody to H3K9me3 in WT and *H19*^{-/-} MEFs (n = 3). (C) Sequencing of immunoprecipitated *Igf2* DMR1 in WT MEFs. Maternal (*dom*, *domesticus*) and paternal (*mol*, *molossinus*) sequences are indicated. Star shows polymorphism between the two alleles.

binding of MBD1 to the *Igf2* DMR1 and *Slc38a4* and *Peg1* DMRs observed in WT MEFs. As expected, we found that MBD1 binding was lost in the absence of *H19* lncRNA (Fig. 4A). The *Cdkn1c* DMR, a target of *H19* lncRNA but not of MBD1, was used as a control and no difference in the binding of MBD1 to this region was observed (Fig. 4A). Taken together, these results suggest that *H19* lncRNA directly represses *Igf2*, *Slc38a4*, and *Peg1* by recruiting the MBD1 protein to the DMRs of these three imprinted genes.

Decrease of H3K9me3 in the Absence of *H19* lncRNA. MBD1 is involved in the maintenance of H3K9me3 through cell division, especially by recruiting KMTs (18). We therefore hypothesized that *H19* lncRNA, because it interacts with MBD1, could be involved in the establishment of this histone mark to repress its targets. To address this, we investigated H3K9me3 levels at DMRs by ChIP and qPCR. H3K9me3 was indeed present at the *Igf2* DMR1 and *Slc38a4* and *Peg1* DMRs in WT MEFs (Fig. 4B). Further sequencing of the immunoprecipitated *Igf2* DMR1 and *Peg1* and *Slc38a4* DMRs, using JF1 polymorphisms, also showed that this mark was present on both alleles, even if it is clearly more important on the maternal allele for the *Igf2* gene (Fig. 4C and Fig. S3). In *H19*^{-/-} MEFs, we observed a loss of H3K9me3 on the *Igf2*, *Slc38a4*, and *Peg1* DMRs. H3K9me3 was also lost on the *Cdkn1c* DMR, despite the fact that this gene is not dependent on MBD1.

This shows that *H19* is important for the maintenance of the H3K9me3 transcriptional repressive mark, which is concordant with a control of *Igf2*, *Slc38a4*, and *Peg1* through an interaction with MBD1 and the H3K9 KMTs.

Discussion

The *H19* locus has been shown to be involved both in embryonic growth control and tumorigenesis (5, 25, 26). *H19* and *Igf2* belong to the IGN, first described in 2006 (7). Using loss- and gain-of-function mouse mutants, we showed that *H19* itself has several targets among the IGN and is capable of repressing the expression of these genes in E14.5 embryonic muscle (6). This results in a fine-tuned regulation of embryonic growth mediated by the *H19* gene. Identification of the underlying molecular mechanisms through which it controls its targets is an important issue.

Recent studies have shown that this locus could act through the production of the miR-675 to control placental growth (4)

and that the full-length *H19* RNA could interact with the EZH2 protein and inhibit E-cadherin expression in bladder cancer metastasis (27). Therefore, the *H19* locus appears to have multiple functions by acting on specific genes through distinct molecular mechanisms, depending on the biological and spatiotemporal contexts during development and disease states.

Here we show that in primary MEFs produced from mid-gestation embryos, the methyl-CpG-binding domain protein MBD1 is a partner of the *H19* lncRNA. This *H19*-MBD1 complex induces the H3K9me3 histone tail modifications on DMRs of target genes, such as *Igf2*, *Slc38a4*, and *Peg1*. This results in the repression of these genes in the presence of *H19* lncRNA. Interestingly, the modulation of expression of the IGN is found neither in neonate or adult muscle nor in placenta (6). Therefore, we suspect that this *H19* lncRNA interaction with MBD1 is specific to embryonic stages. This is probably linked with the establishment of the histone marks at a specific moment in early development. This is then maintained throughout further cell divisions by MBD1-dependent association with chromatin assembly factor CAF1 at the replication forks (18).

Interestingly, MBD1 has been described as having affinity for RNA by in vitro experiments (21). Here we identify *H19* as one of the RNA partners of MBD1. The *H19* gene was thought to be essentially found in the cytoplasm, as it associates with polyosomes (28). Our experiments now also provide evidence for a nuclear role for this lncRNA.

Finally, MBD1 has been described as having affinity both for methylated and unmethylated DNA sequences (23, 29). Recent data have suggested that a mutant of MBD1 lacking the MBD domain but conserving the CXXC region shows preferential binding for unmethylated sequences (20). It is possible that the binding of *H19* RNA to the MBD1 protein modifies the binding capacities of MBD1 and directs it to both methylated and unmethylated DMRs.

In our study, we identified five genes as being targets of both *H19* lncRNA and MBD1. The double deletion experiment using siRNA against *H19* in *Mbd1*^{-/-} MEFs confirmed that MBD1 is required for the function of *H19* lncRNA to repress these genes. Interestingly, these five common targets of *H19* and MBD1 are all paternally expressed genes (except for *Dcn*, which is not imprinted at this stage of development). This observation could reinforce the parental conflict theory, in the sense that the maternally expressed *H19* gene controls growth by repressing paternally expressed genes.

In contrast, four other genes (*Gtl2*, *Cdkn1c*, *Igf2r*, and *Gnas*) are regulated by *H19* lncRNA but are not affected by the absence of MBD1. For example, *Cdkn1c* is a target of *H19* but not of MBD1, and H3K9 methylation is reduced on its DMR. This therefore suggests that *H19* could repress these other genes through other chromatin-modifying partners. The control of expression of the IGN genes can therefore be mediated by an epigenetic effect via the *H19* lncRNA. Alternatively, these genes could be controlled by posttranscriptional effects, although an effect of the miR-675 can be excluded, as it is not expressed in the embryo (4).

In addition, an indirect effect of *H19* could be mediated by direct targets acting on the other IGN genes. For example, *Igf2r* is a negative regulator of the insulin and IGF signaling pathway. Therefore, in *H19*^{-/-} mice, increased *Igf2* expression may be compensated by *Igf2r* overexpression as a response to maintain overall homeostasis.

Because the IGN genes seem to play an essential role in controlling the growth of the embryo, it would seem likely that a complex regulation involving several levels of control, including transcriptional, posttranscriptional, and indirect mechanisms, would be required for this crucial process. Our experiments show that *H19* lncRNA is required for direct binding of MBD1 to regulatory regions of *Igf2*, *Slc38a4*, and *Peg1* genes. Therefore, we postulate that *H19* could recruit the MBD1 protein to its DNA targets. How *H19* lncRNA recognizes the correct DNA regions remains unknown. The *H19* RNA could possibly produce a triplex structure with target DNA sequences, similar to what has been described for rDNA genes (30).

Our detailed study of the *Igf2* gene and the binding of MBD1 to the *Igf2* DMR1 shows that MBD1 targets both alleles of the *Igf2* gene. The H3K9me3 modification is therefore brought to both alleles (Fig. 5). The reduction of H3K9me3 on the *Igf2* DMR in the absence of *H19* is quite straightforward, as this reduction will lead to expression of the normally silent maternal *Igf2* allele. Although it is difficult to evaluate if the expression of the paternal allele is also increased, this is reminiscent of a previous observation in which the DNA methylation profiles of the *Igf2* locus were studied in *H19*^{Δ3} and *H19*^{Δ13} mice (31). DNA

methylation was acquired on the maternal *Igf2* allele (and resulted in loss of imprint) but was also lost on the paternal allele. The authors suggested a cross-talk between the two alleles with exchange of methylation from one allele to the other. Thus, this DMR1 region appears to be a flexible region with respect to epigenetic marks, such as DNA and histone methylation. This may reflect a specific property of the somatic DMRs.

Finally, we have provided evidence that *Slc38a4* and *Peg1* overexpression in the absence of *H19* lncRNA is linked to a decrease in H3K9me3 at their gametic DMR. In the WT samples, this repressive histone mark is present on both alleles, a situation that has also been previously described for the *Rasgf1* gene, another paternally expressed gene (32). Other marks independent of *H19*, such as H3K27me3 marks and/or DNA methylation, must act as an additional lock to prevent expression from the silent allele (33). The importance of controlling the level of expression of the *Slc38a4* gene (a system A amino acid transporter) was recently illustrated by the observation that human placentas from low birth weight children (with fetal macrosomia) showed an increase in *Slc38a4* expression (34). The control of *H19* on the DMR of this gene could be involved in maintaining low expression of *Slc38a4* in the embryo to obtain normal development.

In conclusion, we have identified the MBD1 protein as a partner of the *H19* full-length lncRNA. This complex brings H3K9me3 modifications to chromatin at DMRs of certain imprinted gene targets of *H19* (Fig. 5). The DMR regions could therefore represent areas in which repressive H3K9me3 histone marks are present on both alleles, even though they are clearly defined by differential DNA methylation. The effect of the *H19* RNA is to tether the MBD1 protein to these regions and to provide H3K9me3 modifications to finely control the level of expression of the normally expressed allele. This is an interesting observation, as it was thought up to now that imprinted genes were “on” or “off” depending on their parental origin. Our data suggest that even the expressed alleles are under a precise control to avoid overexpression of these genes controlling growth. Mediating this control through lncRNAs associated with chromatin-modifying complexes brings an additional level of fine tuning of embryonic growth.

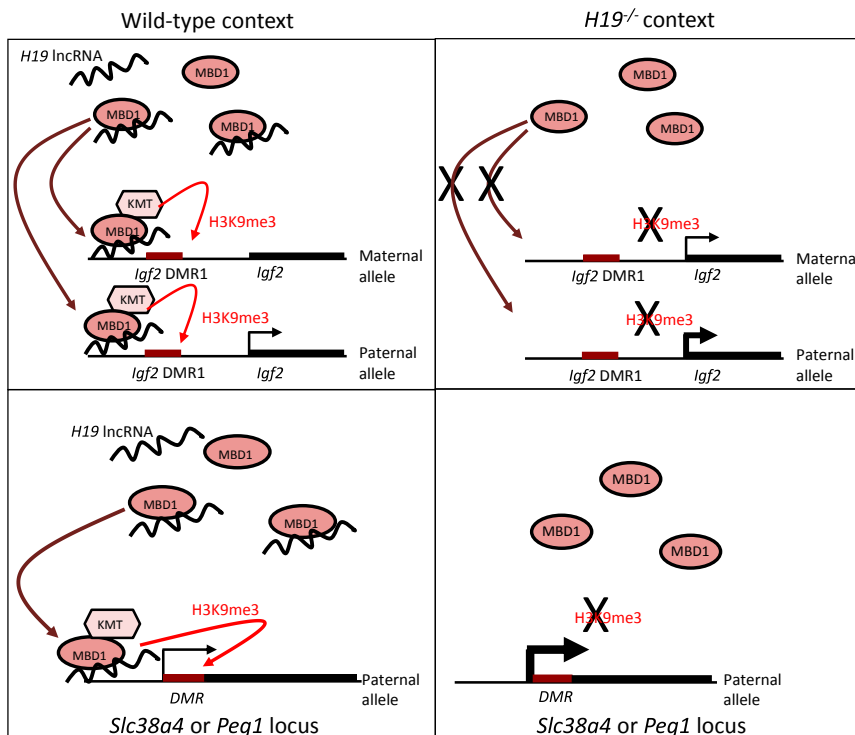


Fig. 5. Model of *H19*-mediated regulation of *Igf2*, *Slc38a4*, and *Peg1* genes. In WT cells, the *H19* lncRNA interacts with the MBD1 protein and recruits it to the *Igf2* DMR1, both on the maternal and paternal allele (Upper Left). This recruitment induces H3K9me3 on both alleles, probably via interaction with an H3K9 KMT. In *H19*^{-/-} cells, MBD1 cannot be recruited to the *Igf2* DMR1, leading to a loss of H3K9me3 (Upper Right). This results in an increase of *Igf2* transcription, concomitant with a loss of *Igf2* imprinting. On the *Slc38a4* and *Peg1* paternal DMRs, the *H19* lncRNA recruits MBD1 and induces H3K9me3 (Lower Left). In absence of *H19*, the lack of binding of MBD1 results in a loss of H3K9me3 and in overexpression of the paternal allele (Lower Right). Therefore, *H19* exerts a fine-tuned regulation of these genes, by modulating the presence of the repressive H3K9me3 histone mark on the active alleles.

Materials and Methods

Mouse Strains. All experimental designs and procedures were in agreement with the guidelines of the animal ethics committee of the Ministère de l'Agriculture (France). The *H19*^{+/+} (*H19*^{-/-}), *Tg*, and *YAC* line used in this work were previously described (6). *H19*^{Δ3/+;Tg} or *H19*^{Δ3/+;YAC} females were mated with WT JF1 males. *Wt*, *H19*^{-/+} and *H19*^{-/-;Tg}, or *H19*^{-/-;YAC} embryos (E14.5) were then dissected to collect limb muscle or to produce primary MEFs.

Allele-Specific Expression Analysis. Polymorphisms between *Mus musculus molossinus* (JF1) and *Mus musculus domesticus* were extracted from previous studies (35), or from the National Institute of Genetics (NIG) mouse genome database (<http://molossinus.lab.nig.ac.jp/msmdb/index.jsp>). Reverse transcription was performed using PrimeScript Reverse Transcriptase (Takara Bio Inc.) followed by PCR with GoTaq polymerase (Promega) according to the manufacturer's guidelines, with oligos that span an exon region that harbors a single defined base difference or a restriction fragment length polymorphism between the two mouse strains. Allele-specific expression was determined either by sequencing or digestion by *MspI* for the *Igf2* gene. Primers are listed in Table S2.

Gene Expression Analysis. Total RNAs were extracted using miRNeasy kit (Qiagen), and reverse transcription was performed as above. RT-qPCR was performed on 10 ng cDNA in 10 mL final volume with SYBR qPCR Premix Ex Taq (Takara Bio Inc.) in a LightCycler 2.0 apparatus (Roche). Gene expression levels were normalized to the geometric mean of the expression levels of *Sdha*, *Tfrc*, and *ActB* housekeeping genes with geNorm software (v3.4) (Table S1) (36).

RIP Experiments. RNA ChIP Kit (Active Motif) was used to perform RIP experiments, according to the manufacturer's instructions. Two million cells were used per IP. Cells were fixed in 1% formaldehyde solution (Sigma). Total chromatin and RNAs were sonicated into 200–800 bp fragments, using a Bioruptor (Diagenode). We used 1% of the chromatin/RNA to purify the input RNA. The remaining chromatin/RNA was immunoprecipitated with 2 μg of MBD1 antibody (Diagenode pAb-078-050), EZH2 antibody (Diagenode

pAb-039-050), SUZ12 antibody (Diagenode pAb-029-050), or 2 μg of non-specific rabbit IgG (negative control, Diagenode kch-504-250). RNA was purified using TRIzol LS reagent (Life Technologies), reverse-transcribed as above, and analyzed by RT-qPCR.

siRNA-Mediated Knockdown Experiments. RNAi-mediated knockdown was performed with Stealth RNAi siRNA against *Mbd1* (Life Technologies, mss206539) and by Silencer Select siRNA against *H19* (Life technologies, 4390771) or a non-targeting control (12935–400 and 4390843), at a final concentration of 50 pmol/mL. Transfection of oligos into cell lines was achieved using Lipofectamine RNAimax (Life Technologies). RNA was extracted 48 h later.

Chromatin Immunoprecipitation Experiments. ChIP experiments were performed using the HighCell ChIP kit (Diagenode), according to the manufacturer's instructions. One million cells were used per IP. Cells were grown to 80–90% confluency and then fixed with 1% formaldehyde solution (Sigma). Chromatin was sonicated into 200–800 bp fragments, and 1% of the chromatin was used to purify the input DNA. Chromatin was immunoprecipitated with 2 μg of MBD1 antibody, H3K9me3 antibody (Diagenode CS-056-100), or non-specific rabbit IgG. DNA was purified and analyzed by qPCR.

Statistical Analysis. Data are presented as the mean ± SEM. Statistical significance of the different experiments was determined using a Kruskal–Wallis test followed by post hoc paired comparisons, or a Mann–Withney test, using Prism software (v5.0a). Results were considered statistically significant when $P < 0.05$ compared with WT * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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