G9a Histone Methyltransferase Contributes to Imprinting in the Mouse Placenta

Alexandre Wagschal,1 Heidi G. Sutherland,2 Kathryn Woodfine,3 Amandine Henckel,1 Karim Chebli,1 Reiner Schulz,3 Rebecca J. Oakey,3 Wendy A. Bickmore,2 and Robert Feil1

Institute of Molecular Genetics, CNRS and University of Montpellier, Montpellier, France; 1 MRC Human Genetics Unit, Western General Hospital, Edinburgh, United Kingdom; 2 Department of Medical and Molecular Genetics, King’s College, London, United Kingdom

Received 22 June 2007/Returned for modification 17 August 2007/Accepted 5 November 2007

Whereas DNA methylation is essential for genomic imprinting, the importance of histone methylation in the allelic expression of imprinted genes is unclear. Imprinting control regions (ICRs), however, are marked by histone H3-K9 methylation on their DNA-methylated allele. In the placenta, the paternal silencing along the Kcnq1 domain on distal chromosome 7 also correlates with the presence of H3-K9 methylation, but imprinted repression at these genes is maintained independently of DNA methylation. To explore which histone methyltransferase (HMT) could mediate the allelic H3-K9 methylation on distal chromosome 7, and at ICRs, we generated mouse conceptuses deficient for the SET domain protein G9a. We found that in the embryo and placenta, the differential DNA methylation at ICRs and imprinted genes is maintained in the absence of G9a. Accordingly, in embryos, imprinted gene expression was unchanged at the domains analyzed, in spite of a global loss of H3-K9 dimethylation (H3K9me2). In contrast, the placenta-specific imprinting of genes on distal chromosome 7 is impaired in the absence of G9a, and this correlates with reduced levels of H3K9me2 and H3K9me3. These findings provide the first evidence for the involvement of an HMT and suggest that histone methylation contributes to imprinted gene repression in the trophoblast.

More than 80 mammalian genes undergo parent-of-origin-dependent expression. Most of these are clustered in domains, which are broadly conserved between mice and humans (33). The allelic expression along imprinted domains is regulated by “imprinting control regions” (ICRs) (7, 9, 54). DNA methylation is essential for the mechanism of imprinting (34), and all known ICRs are marked by DNA methylation on their maternally, or their paternally, inherited allele. The germ line establishment of these methylation imprints requires the DNA methyltransferase Dnmt3a (1, 20) and the related protein Dnmt3L (1–3, 15, 20). The somatic maintenance of methylation requires the maintenance methyltransferase Dnmt1 (17, 28). In the embryo, and after birth, ICRs are marked by parental allele-specific histone methylation as well. Specifically, together with other histone modifications, ICRs are consistently enriched in histone H3–lysine-9 methylation on their DNA-methylated allele (6, 40, 53, 56, 58). It is unknown which histone methyltransferase (HMT) mediates this H3–lysine-9 methylation and to what extent this epigenetic modification is involved in the maintenance of the allelic chromatin organization at ICRs.

During embryonic development, ICRs bring about parental allele-specific gene expression (7, 26). At some imprinted gene clusters this process involves the establishment of allele-specific histone modifications. Imprinted expression along the Kcnq1 domain on mouse distal chromosome 7 is mediated by a noncoding RNA (31) transcribed from the ICR, and chromatin on the domain’s repressed paternal chromosome is enriched in H3–lysine-9 dimethylation (H3K9me2) and H3–lysine-27 trimethylation (H3K27me3). This was observed most extensively in the placenta, in which the majority of the genes in this >800-kb domain are paternally repressed (25, 53). Genetic and biochemical studies have suggested that the Polycomb repressive complex PRC2 regulates H3K27me3 along the Kcnq1 domain (30, 53). It is unknown, however, which HMT could mediate the H3K9me2 on the repressed paternal chromosome. Several SET domain proteins have been found to specifically transfer methyl groups onto lysine-9 of histone H3 (18). Some of these HMTs bring about H3K9me2 preferentially, whereas others mediate H3K9me3. The HMT G9a was shown to be essential for genome-wide levels of H3K9me2, and fluorescence studies suggest that it mediates H3K9me2 at regions other than at the pericentric heterochromatin (39, 41, 48). The G9a protein forms a functional heterodimer with a closely related protein called Glp (G9a-like protein), also called EuHMTase1 in humans (35). Also this SET domain protein is essential for H3K9me2 at euchromatic regions (49).

Given its substrate specificity and its global effects, G9a could potentially regulate the allelic H3K9 methylation at the imprinted Kcnq1 domain and that observed at ICRs. To test this hypothesis, a gene trap approach was used to generate G9a-deficient mouse conceptuses. This allowed us to perform studies on placentas and embryos, rather than on cells in culture, which can sometimes give rise to aberrant epigenetic effects on imprinted genes (5, 59). Our in vivo approach did not provide evidence for G9a to be essential in the allelic regulation of DNA methylation at the different ICRs analyzed, al-
though moderate reductions in H3K9 methylation were observed. Interestingly, however, we found that the absence of G9a has pronounced effects on the paternal repression along the Kcnq1 domain in the placenta. In particular, G9a deficiency affected genes that are imprinted in the trophoblast only and which are not dependent on DNA methylation for the somatic maintenance of their allelic silencing. This provides the first in vivo evidence for the involvement of a SET domain protein in genomic imprinting and emphasizes the relative importance of histone methylation in placenta-specific imprinting.

MATERIALS AND METHODS

G9a-deficient conceptuses. The G9a gene was trapped by insertion of a β-galactosidase-neomycin phosphotransferase (βgeo) construct comprising a splice acceptor and a polyadenylation insert and the endogenous G9a gene. At the imprinted locus analyzed, C57BL/6 mice. Concomitantly, a second line was derived by back-crossing to a M2 Mus spretus) congenic mouse line, SDP711. G9a-deficient embryos and placentas were obtained by intercrossing these two lines. Genotyping was performed by PCR against the βgeo-int and the endogenous G9a gene. For the analysis described elsewhere (53), we used different antisera against G9a (Upstate 07-441 as antibody A and Abcam 1186 as antibody B), and H3K4me3 (Upstate 07-442 as antibody A and Abcam 1186 as antibody B), and H3K4me2 (Upstate 07-030). As a negative control (mock precipitation), we used a rabbit antiserum directed against chicken immunoglobulin G (IgG; C2288; Sigma). Precipitation levels were determined by real-time PCR, using a SYBR Green PCR kit (Qiagen). Each PCR was run in triplicate, and results are presented as the average value of the precipitated material corrected for the average value of the corresponding mock precipitation. ChIP was also performed on placentas at 9.5 days postcoitum (dpc) after cross-linking with 1% formaldehyde (10 min at 20°C) using antisera against G9a (Upstate 07-551) and RNA polymerase II (Abcam 5131). As a negative control, a rabbit antiserum to chicken IgG (C2288; Sigma) was used.

Analysis of DNA methylation. A 200-ng aliquot of genomic DNA was digested in a volume of 20 μl with appropriate restriction enzymes. Aliquots were taken for PCR in the presence of [32P]dCTP (1% of total dCTP) during all cycles for single-strand conformation (see Table S3 in the supplemental material) were performed in the presence of [32P]dCTP (1% of total dCTP) during all cycles for single-strand conformation polymorphism (SSCP) analysis or only during the last cycle of reamplification (hot-stop PCR) (52). Relative band intensities were determined using ImageQuantTL imaging software (Amersham Biosciences). Allelic ratios were compared between wild-type (WT) and G9a-/- placentas by using the Student t test.

Microarray analysis. Per genotype, three total RNA samples were pooled and then quantified on an Agilent Bioanalyzer. Three μg of pooled RNA sample was used to synthesize double-stranded cDNA using the SuperScript II (Invitrogen) using random primers. All reverse transcription-PCR (RT-PCR) amplifications (see Table S3 in the supplemental material) were performed in the presence of [32P]dCTP (1% of total dCTP) during all cycles for single-strand conformation polymorphism (SSCP) analysis or only during the last cycle of reamplification (hot-stop PCR) (52). Relative band intensities were determined using Image-QuantTL imaging software (Amersham Biosciences). Allelic ratios were compared between wild-type (WT) and G9a-/- placentas by using the Student t test.

Microarray data. Microarray analysis of the G9a HMT were deposited in the GEO repository and are accessible at https://atlas.genetics.kcl.ac.uk/.

RESULTS

Effects of G9a deficiency on the embryo and placenta. To explore the role of G9a, we derived embryos and placentas deficient for this HMT. This was achieved as part of a gene trap targeting approach on ES cells, using a promoterless β-geo construct containing a splice acceptor and a polyadenylation signal. Insertion of this construct into a gene’s intron leads to a chimeric splice product and, consequently, the production of a lacZ fusion protein that lacks the protein sequence encoded by exons of the trapped gene that are 3’ of the gene-trap insertion. Sequence analyses of insertions into mouse genes encoding nuclear proteins (47) identified one ES line in which the construct had inserted in intron 11 of the G9a gene (Fig. 1A). Heterozygous mice were derived by making chimeric animals using the targeted ES cells, followed by germ line transmission. Heterozygous mice were intercrossed to generate G9a-/- conceptuses in which the site of gene-trap insertion was confirmed by PCR amplification and DNA sequencing. No transcription was detected from the 3’-half of the G9a gene (Fig. 1B). The G9a-β-geo fusion protein lacks the ankyrin repeats and, most importantly, the catalytic SET domain of the wild-type protein and so is likely to be functional null. Accordingly, Western blotting showed that in the G9a-/- conceptuses there was a strong reduction of global H3K9me2 (Fig. 1C).

G9a-/- embryos were viable and present at the expected frequency up to 10 dpc. In agreement with an earlier study (48), at later stages we observed embryonic death and resorption. Development of the embryos was grossly abnormal at 8.5 to 9.5 dpc. The ectoderm showed a consistent nonclosure of the neural groove, and the G9a-/- embryos were about half the size of WT embryos (Fig. 1D). The placenta, in contrast, did not show gross developmental abnormalities, with a normal
Reverse transcriptase followed by duplex PCR of centas. Reverse transcription was performed with (+) or without (-)/H11001.

The morphology of the maternal decidua and the three embryonic layers: labyrinthine trophoblast, spongiosotrophoblast, and the giant cell layer (Fig. 1D). However, size measurements on several G9a−/− versus WT placentas showed an ~10% reduction in placental diameter. To assess trophoblastic differentiation and cell death, we counted the polyploid giant cells on sequential sections of G9a−/− and WT placentas. There was an 18% reduction in the number of giant cells in the G9a−/− placentas, and these showed a twofold increase in cell death compared to WT (see Fig. S1 in the supplemental material). Giant cell reduction was higher than expected given the size reduction of these placentas, indicating that G9a deficiency had a moderate effect on trophoblastic differentiation.

G9a deficiency causes loss of imprinting in the placenta but not the embryo. To be able to distinguish the parental chromosomes in our gene expression studies, we crossed the G9a line onto a congenic mouse line (SDP711) in which distal chromosome 7 and proximal chromosome 11 were derived from Mus spretus on an otherwise C57BL/J6J (Mus musculus) background. The original line (C57BL/6J background) and the newly derived G9a heterozygous line (congenic SDP711 background) were intercrossed to generate G9a−/− placentas and embryos. Single nucleotide polymorphisms were used to distinguish the maternal and paternal transcripts of imprinted genes. This was done by RT-PCR followed by electrophoretic detection of SCSPs or by hot-stop PCR (52, 53). WT and G9a−/− placentas and embryos (data not shown) were compared at 9.5 dpc. The carefully dissected embryonic portions of WT placentas showed maternal expression of Osbp5, Phlda2, Cdkn1c, Cd81, and Ascl2 at the Kcnq1 domain. Expression of the noncoding RNA Kcnq1ot1, which is transcribed from the domain’s ICR (called KvDMR1) (46), was from the paternal chromosome exclusively (Fig. 2A).

Cdkn1c and Phlda2, located in the central portion of the domain, faithfully maintained their paternal repression in the absence of G9a. Furthermore, the Kcnq1ot1 noncoding RNA remained expressed from the paternal allele only. However, altered imprinted expression was detected at the proximal and distal portions of the domain (Fig. 2A; see also Fig. S2 in the supplemental material). Loss of imprinting was defined as detection of an allelic ratio between the maternal and the paternal allele (maternal/paternal ratio [M/P]), which was below that observed in the cohort of all WT placentas (Fig. 2C; see also Table S1 in the supplemental material). In several of the G9a−/− placentas, there was clear derepression of the paternal alleles of the Osbp5, Ascl2, and Cd81 genes. However, Osbp5, Ascl2, and Cd81 did not show loss of paternal repression in concert. In one placenta there was loss of imprinting at Ascl2 only, whereas in three others, there was loss of imprinting at both Ascl2 and Osbp5 (Fig. 2A; see also Fig. S1A in the supplemental material). Morphologically, these placentas appeared comparable to the other G9a−/− placentas.

To verify that the partial loss of imprinting was not linked to the parental backgrounds used, we performed the crosses between the G9a heterozygous mouse lines in the reciprocal orientation. This resulted in the same phenotype, with frequent relaxation of imprinting at Osbp5, Cd81, and Ascl2, which in one of the placentas was observed at all three genes (Fig. 2B). As in the initial cross, no loss of imprinting was observed at Cdkn1c and Kcnq1ot1 (data not shown).

Since we had detected a moderate reduction in the number of giant cells in the G9a−/− placentas, the observed loss of imprinting could have been related to a less-advanced trophoblastic development. We excluded this possibility by analyzing WT placentas 1 day earlier in development, at 8.5 dpc. This showed that Osbp5, Cd81, and Ascl2 were expressed from the maternal chromosome at this earlier developmental stage as well (see Fig. S3 in the supplemental material). Ascl2, Osbp5, and Cd81 are imprinted in the trophoblast only. The Cdkn1c, Phlda2, and Kcnq1ot1 genes, in contrast, are also imprinted in the embryo (38, 53). We therefore studied these genes in embryos as well and found that their allelic expression is not altered in the absence of G9a (data not shown). Together, these findings indicate that, in the absence of G9a, there is normal paternal repression at these genes in the central part of the Kcnq1 domain, but that the establishment or the maintenance of silencing is affected at
the distal and proximal genes, which are imprinted in the trophectoderm only.

At the neighboring Igf2-H19 domain (54), no allelic changes in gene expression were observed. Maternal (M) and paternal (P) specific bands are indicated. Asterisks indicate placentas in which the M/P ratio was different from that observed in the cohorts of WT placentas. In the Phlda2 analysis, the black dot indicates a secondary, maternal-specific band. (B) Loss of imprinting (asterisks) at Osbp5, Cd81, and Ascl2 was also observed in (SDP711 × C57BL6)F1 placentas. (C) M/P band intensity ratios. In each of the panels, the average ratio for all WT placentas analyzed is shown to the left. Examples of G9a−/− placentas that were below this range for Ascl2, Osbp5, or Cd81 are shown to the right. In the G9a−/− placentas, M/P ratios were significantly lower than in WT placentas for Ascl2 (mean, 2.3 versus 5.2; P < 0.0001) and Osbp5 (mean, 2.3 versus 5.2; P < 0.00001). For Cd81, the means were not different between the WT and G9a−/− mice (5.2 versus 8.4; P = 0.014). (D) Unaltered allelic expression of the Igf2 and H19 genes in G9a−/− placentas. (E) Unaltered paternal expression of the U2af1-rs1 gene.

FIG. 2. Altered imprinted gene expression in G9a−/− placentas. (A) RT-PCR in the presence (+) or absence (-) of reverse transcriptase on (C57BL6 × SDP711)F1 placentas at 9.5 dpc (WT and G9a−/−). In all panels, the first two lanes show control amplifications from C57BL6 (Dom) and SDP711 WT placentas, respectively. Maternal (M) and paternal (P) specific bands are indicated. Asterisks indicate placentas in which the M/P ratio was different from that observed in the cohorts of WT placentas. In the Phlda2 analysis, the black dot indicates a secondary, maternal-specific band. (B) Loss of imprinting (asterisks) at Osbp5, Cd81, and Ascl2 was also observed in (SDP711 × C57BL6)F1 placentas. (C) M/P band intensity ratios. In each of the panels, the average ratio for all WT placentas analyzed is shown to the left. Examples of G9a−/− placentas that were below this range for Ascl2, Osbp5, or Cd81 are shown to the right. In the G9a−/− placentas, M/P ratios were significantly lower than in WT placentas for Ascl2 (mean, 2.3 versus 5.2; P < 0.0001) and Osbp5 (mean, 2.3 versus 5.2; P < 0.00001). For Cd81, the means were not different between the WT and G9a−/− mice (5.2 versus 8.4; P = 0.014). (D) Unaltered allelic expression of the Igf2 and H19 genes in G9a−/− placentas. (E) Unaltered paternal expression of the U2af1-rs1 gene.

The distal and proximal genes, which are imprinted in the trophectoderm only.

To globally assess levels of gene expression, we performed microarray (Affymetrix) analyses on WT versus G9a−/− placentas. Embryos were not included in this study, given the gross developmental abnormalities induced by the absence of G9a. 39,000 transcripts were analyzed in two independent
experiments on pools of G9a−/− and WT placentas at 9.5 dpc.

G9a itself was readily expressed in WT placenta and, as expected, not in G9a−/− placentas. Sixty genes were altered fourfold or more in their expression levels. Of these, 27 showed a >10-fold change in G9a−/− placentas (see Table S2 in the supplemental material). The latter is a negative cell cycle regulator, which had been proposed earlier to be controlled by G9a (8). Its upregulation could explain the moderate reduction in size of the G9a−/− placentas. The derepression of the Mage-a gene family extends recent in vitro studies on the involvement of G9a and Glp in the silencing of the Mage-a2 gene (23, 48, 49). Of the 65 imprinted genes that were included in the microarray study, 9 showed a significant change in their expression levels, of two- to fourfold. Significantly altered expression was not detected for Ascl2, Cd81, or Osbpl5 though, agreeing with our finding that the relaxation of imprinting is partial and not detected in all the placentas (see Table S2 in the supplemental material). This finding was confirmed by real-time PCR amplification for four of the G9a−/− placentas (see Fig. S1B in the supplemental material).

G9a recruitment regulates H3-K9 methylation. The loss of imprinting at Ascl2, Cd81, and Osbpl5 in the G9a−/− placentas suggested that these genes could be marked by H3-K9 methylation on their repressed paternal alleles. To address this question in more detail, we performed ChIP on nonfixed chromatin extracted from 25 WT placentas at 9.5 dpc, using two antisera directed against H3K9me2 and two against H3K9me3 (Fig. 3). Enrichment of both H3K9me2 and H3K9me3 on the repressed alleles of Ascl2 and Cd81. Also, at the Cdkn1c gene and at KvDMR1, there was H3K9me2 and H3K9me3 enrichment on the repressed allele. In contrast to Ascl2 and Cd81, these regions have DNA methylation on their repressed parental allele as well (25, 46). Although only little chromatin was precipitated, levels of H3K9me2 were higher
than background. For H3K9me3, the highest levels of precipitation were detected at the KvDMR1. These data indicate that in WT placenta, Ascl2, Cdkn1c, and KvDMR1 are enriched both in H3K9me2 and H3K9me3 on their repressed alleles.

Next, we analyzed by ChIP a small number of available G9a–/– placentas (3) versus WT placentas (6). In the absence of G9a, no allelic enrichment of H3K9me2 and H3K9me3 was observed at Ascl2, Cdkn1c, or Cdkn1c, and precipitation levels were considerably reduced compared to WT placentas (Fig. 4A; see also Table S1 in the supplemental material). This points to a reduction in H3K9me2/me3 at these imprinted genes and extends the recent finding that G9a regulates specific gene loci and controls local levels of both H3K9me2 and H3K9me3 (23). The KvDMR1, in contrast, retained high levels of H3K9me2 but showed decreased precipitation of H3K9me3. Major satellite DNA at pericentric heterochromatin retained high levels of H3K9me3 in the absence of G9a (Fig. 4B). At the ICR upstream of the H19 gene, precipitation levels of both H3K9me2 and H3K9me3 were lower than at the KvDMR1, and only the latter modification was reduced in the absence of G9a.

Given the reduction in histone methylation at the Kcnq1 domain genes in the G9a–/– placentas, we explored whether G9a could be bound to these genes in WT placenta. Cross-linked chromatin was precipitated with an antibody directed against the N-terminal domain of G9a. At Ascl2, G9a was preferentially precipitated on the repressed paternal allele of Ascl2 (Fig. 4C). As a control we used an antiserum against the serine-5 phosphorylated form of RNA polymerase II (Pol II), which was detected predominantly on the active maternal allele of the Ascl2 gene. Thus, histone modifications (including H3-K9 methylation) on the silenced paternal alleles could prevent binding of Pol II. Under the experimental conditions used, however, we had little precipitation above background at Cdkn1c and Cdk81, and so we could not determine whether at these genes the paternal allele also binds G9a (data not shown).

Maintenance of imprinted DNA methylation. H3-K9 trimethylation is consistently associated with the DNA-methylated allele of ICRs (6, 40, 53, 56, 58; this study). It is enriched on the repressed allele of several imprinted gene promoters as well, including H19 and Cdkn1c. Furthermore, part of the cellular G9a is associated with Dnmt1, at replication foci (10), suggesting a link between the maintenance of H3-K9 and DNA methylation (29, 43). Indeed, the HMTs Suv39h1 and h2 are required for directing DNA methylation (29, 43). At the ICR upstream of the H19 gene, precipitation levels of both H3K9me2 and H3K9me3 were lower than at the KvDMR1, and only the latter modification was reduced in the absence of G9a.

Also in the G9a–/– placentas, levels of allelic DNA methylation were unaltered at the KvDMR1 and the H19 ICRs, the Igf2 DMR2, and at Cdkn1c and U2af1-rs1 (Fig. 5B).
DISCUSSION

The main finding from this study is that the HMT G9a contributes to the allelic repression of genes that are imprinted in the trophoblast only. This suggests that histone H3–lysine-9 methylation is one of the factors involved in placenta-specific imprinting. Importantly, no effects were observed on imprinting control regions, which stably maintained their allelic DNA methylation imprints in the absence of G9a, both in the placenta and in the embryo.

A variable degree of paternal derepression was observed at the Axl2, Cdb1, and Osphb5 genes in G9a−/− placentas. Why are these trophoblast-specific genes susceptible to loss of imprinting, whereas other genes that are imprinted more broadly appear unaffected? One distinction of these placenta-specific genes is that they do not acquire DNA methylation on their repressed paternal promoters during development and remain imprinted in the absence of Dnmt1 (25, 50). Their imprinting maintenance is thus independent of DNA methylation. As a consequence, these genes may rely more heavily on covalent histone modifications, including H3–lysine-9 and –lysine-27 methylation and histone H3 deacetylation (25, 53; this study).

The involvement of multiple layers of silencing explains the incomplete penetrance of the loss of imprinting that we observed in the G9a−/− placentas. Many transcriptional repressors act as part of a set of redundant silencing mechanisms. Such a multilayered silencing can cause partial gene derepression in a stochastic manner in case one of the mechanisms is deficient. For instance, studies on the silencing of genes by X chromosome inactivation show that this is controlled by multiple layers of silencing mechanisms (37), each of which reduces the change in each cell of gene reactivation occurring (4). At imprinted genes in the central portion of the Kcnq1 domain, as well as at the H19 and U2af1-rs1 genes, the allelic repression was unaltered in the absence of G9a. These genes, however, use the additional, firm layer of repression put into place by DNA methylation and would therefore not readily lose imprinting due to G9a deficiency. Moreover, the continued paternal repression of some genes of the central part of the Kcnq1 domain (including Cdkn1c) may be controlled by the KvDMR1. Recent studies indicate that, on its unmethylated allele, this intronic ICR binds the CTCF protein and could function as a chromatin boundary, thereby preventing promoter-enhancer interactions that are required for the expression of nearby genes (13, 14, 19, 26). The maternal DNA methylation at the KvDMR1 was not affected in the G9a−/− conceptuses, and G9a deficiency would therefore not have changed its allelic boundary function.

Our data do not exclude that G9a deficiency had stochastically affected the establishment of the allelic repression at the placenta-specific genes. It is technically challenging to determine when precisely during development the repressive H3–lysine-9 methylation becomes established. In case the chromatin repression is an early event, as suggested to be the case for some of the genes in the domain (27), maternally transmitted G9a protein could influence this process, and this might explain some of the differences between individual G9a−/− placentas. Imprinting establishment at the Kcnq1 domain requires the KvDMR1 (13) and, in particular, transcriptional elongation of the noncoding RNA Kcnq1ot1, which is expressed from the ICR (31). Presumably, during early development the full-length Kcnq1ot1 RNA mediates the local recruitment of chromatin-modifying complexes, including PRC2 proteins and G9a, but this remains to be demonstrated.

G9a deficiency led to a two-thirds reduction in global H3K9me2, indicating that this is not the only HMT regulating H3K9me2. That G9a is involved in the allelic repression of placenta-specific genes follows from the strongly reduced H3–lysine-9 methylation levels that we observed in the G9a-deficient placentas. Interestingly, the reduced histone methylation concerned both H3K9me2 and H3K9me3. This was rather unexpected given that, globally, G9a deficiency leads to a ma-
major reduction in H3K9me2 but not H3K9me3 (39, 41, 48). However, in a recent study (23) reduction of G9a led to reduced levels of both H3K9me2 and H3K9me3 at specific genes, including the Mage-a2 gene. Also, the embryonic repression of the Oct3/4 gene involves G9a-mediated acquisition of H3K9me3 (12). Most likely, other HMTs contribute to maintaining the allelic H3–lysine-9 methylation at imprinted genes and ICRs as well. Glp, for instance, is present in mammalian cells together with G9a and has an effect on global levels of H3K9me2 as well (49). One other candidate to be tested is SETDB1/ESET, an H3-K9-specific HMT which is associated with a methyl-CpG binding protein and with Dnmt1 (29, 43). This SET domain protein could be important for ICRs and for imprinted genes that acquire allelic DNA methylation during embryonic development (40).

Our finding of unaltered DNA methylation at ICRs extends an earlier study on G9a-deficient embryos which showed unchanged DNA methylation at the ICR controlling the Snrpn imprinted domain on central chromosome 7 (57). In this study, however, loss of Snrpn methylation was observed in G9a-deficient ES cells. One explanation for this discrepancy could be that G9a deficiency affects methylation maintenance more readily in cultured ES cells than in the embryo. Even in WT ES cells, in vitro culture can give rise to DNA methylation changes at ICRs (5, 59). Although we did detect reduced H3K9me2/3, the combined data indicate that G9a is not essential for the in vivo maintenance of DNA methylation at ICRs or at imprinted promoters that acquire their DNA methylation during embryogenesis. Also genes undergoing X chromosome inactivation acquire DNA methylation on their repressed promoters during early development (37), and X inactivation was reported to be unaffected by G9a deficiency (36).

In conclusion, the loss of imprinting in the placenta did not affect the genes along the Kcnq1 domain in concert but, rather, occurred in a stochastic manner. This implies that H3–lysine-9 methylation is not the only epigenetic modification that maintains the paternal silencing along this domain (Fig. 6). Removal of this layer of repression in the placenta induces a less-efficient maintenance of repression, particularly at genes that do not also use CpG methylation as part of their silencing mechanism. One further layer of repression is provided by H3K27me3, similar to that on the inactive X chromosome (30, 37, 53). It remains to be explored whether other mechanisms linked to G9a can explain the paternal silencing of genes at the Kcnq1 domain as well. For instance, recent studies indicate that G9a, via its partner protein Glp, can mediate the local recruitment of transcriptional corepressor molecules, such as CtBP (35, 51). Whatever G9a’s precise additional modes of action, our study provides the first in vivo evidence for involvement of an HMT in imprinted gene repression. It highlights the importance of histone methylation rather than DNA methylation in imprinting maintenance in the mouse trophoblast.

Intriguingly, several other imprinted mouse loci comprise genes that seem to be imprinted in the placenta only, without the involvement of promoter DNA methylation (55). It should now be interesting to determine whether G9a also plays a contributing role here. From a more general perspective, our data expand earlier work on several imprinted genes, showing that they have lower levels of DNA methylation in the placenta than in the embryo or are not methylated at all in this extraembryonic tissue (25, 44, 50). Concordantly, the maintenance of imprinted gene repression would be less tightly controlled in the trophoblast lineage than in the embryo proper. This could be particularly crucial during early development, given the finding that culture of preimplantation embryos leads to a preferential loss of imprinting in the placenta (32, 42).

ACKNOWLEDGMENTS

We thank Sheila Webb for blastocyst injections, Patricia Cavelier for histology, and Philippe Arnaud for discussion and comments.


