Non-germ Line Restoration of Genomic Imprinting for a Small Subset of Imprinted Genes in Ubiquitin-like PHD and RING Finger Domain-Containing 1 (Uhrf1) Null Mouse Embryonic Stem Cells*

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Background: Once erased, DNA methylation in imprinted genes was shown previously to be re-established only through germ line passage.
Results: UHRF1 re-expression in Uhrf1−/− mouse embryonic stem (ES) cells restores DNA methylation for a few imprinted genes.
Conclusion: DNA methylation in a few imprinted genes can be restored without germ line passage.
Significance: ES cells can be a useful model for studying DNA methylation establishment in imprinted genes.

The underlying mechanism for the establishment and maintenance of differential DNA methylation in imprinted genes is largely unknown. Previous studies using Dnmt1 knock-out embryonic stem (ES) cells demonstrated that, although re-expression of Dnmt1 restored DNA methylation in the non-imprinted regions, the methylation patterns of imprinted genes could be restored only through germ line passage. Knock-out of Uhrf1, an accessory factor essential for Dnmt1-mediated DNA methylation, in mouse ES cells also led to impaired global DNA methylation and loss of genomic imprinting. Here, we demonstrate that, although re-expression of UHRF1 in Uhrf1−/− ES cells restored DNA methylation for the bulk genome but not for most of the imprinted genes, it did rescue DNA methylation for the imprinted H19, Nnat, and Dlk1 genes. Analysis of histone modifications at the differentially methylated regions of the imprinted genes by ChIP assays revealed that for the imprinted genes whose DNA methylation could be restored upon re-expression of UHRF1, the active histone markers (especially H3K4me3) were maintained at considerably low levels, and low levels were maintained even in Uhrf1−/− ES cells. In contrast, for the imprinted genes whose DNA methylation could not be restored upon UHRF1 re-expression, the active histone markers (especially H3K4me3) were relatively high and became even higher in Uhrf1−/− ES cells. Our study thus supports a role for histone modifications in determining the establishment of imprinting-related DNA methylation and demonstrates that mouse ES cells can be a valuable model for mechanistic study of the establishment and maintenance of differential DNA methylation in imprinted genes.

In mammals, most genes are expressed from both parental alleles. However, a small subset of genes known as imprinted genes is monoallelically expressed in a parent of origin-specific manner. This phenomenon, termed genomic imprinting, is observed in eutherian mammals, marsupials, and flowering plants (1, 2). About 150 imprinted genes have been identified so far in the human and mouse genomes (MouseBook Imprinting Resource). Genomic imprinting plays an important role in mammalian embryonic development, fetal growth, metabolism, and behavior, and its disruption can cause various developmental defects and diseases, such as Angelman syndrome, Prader-Willi syndrome, Beckwith-Wiedemann syndrome, diabetes, and cancer (1–5).

In a genome, most imprinted genes are closely located in clusters. Genes in each cluster are regulated by an imprinting control region. Within each imprinting control region, there is a differentially methylated region (DMR), which is methylated on either the maternal or paternal allele (1, 6). The establishment of DMR DNA methylation occurs during male and female gametogenesis (1, 6). Once the genomic imprint is established, DMR methylation is stably maintained following fertilization and throughout development (1, 6).

As a fascinating paradigm for epigenetic regulation (1), extensive efforts have been devoted to study the underlying mechanisms of genomic imprinting. Differential DNA methyl-

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4 The abbreviations used are: DMR, differentially methylated region; ES, embryonic stem; 5-mC, 5-methylcytosine; qPCR, quantitative PCR; COBRA, combined bisulfite restriction analysis.
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... is believed to be the primary factor that controls genomic imprinting (7, 8). In support of this notion, the de novo DNA methyltransferase Dnmt3a and its non-enzymatic partner Dnmt3L are critically important for establishment of imprinting in both male and female germ cells (9–11). Once established, DNMT1 is required to maintain the methylation of imprinted genes following fertilization and throughout development (12, 13). In fact, the methylation in imprinted genes is protected against the genome-wide DNA demethylation that occurs in pre-implantation embryos. In further support for the role of DNA methylation in genomic imprinting, ZFP57, a Krüppel-associated box-containing zinc-finger protein that has been shown to be required for establishment of DMR methylation at the Surprn gene in oocytes and for maintenance of DMR methylation of several maternal and paternal imprinted genes in embryonic stem (ES) cells, turns out to be a methylated DNA-binding protein (14, 15). In addition, PGC7/Dppa3/Stella has been shown to protect maternal imprinting by inhibiting the conversion of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine by Tet3 in early embryos (16, 17). However, what makes DMR methylation in imprinted genes so unique is that, although re-expression of DNMT1 in Dnmt1-null ES cells rescues bulk DNA methylation in non-imprinted regions, the methylation patterns of imprinted genes can be restored only through germ line passage (18). This finding highlights a major obstacle for investigating the underlying mechanism of genomic imprinting because the establishment of genomic imprinting occurs only through gametogenesis, and currently there is no suitable cellular model for study.

In addition to DNA methylation, histone modifications have been linked to both establishment and maintenance of genomic imprinting (1, 6, 19). For example, repressive histone markers such as H3K9me2/3, H4K20me3, and H4R3me2 are associated with methylated DMRs, whereas H3K4me2/3 and H3/4ac are associated with non-methylated DMRs (20–23). However, a study with mouse primordial germ cells demonstrated that H3K9me3 and H4K20me3 are depleted at the imprinted genes before the establishment of imprinted DNA methylation, indicating that these repressive histone markers are unlikely to specify subsequent DNA methylation (21). On the other hand, in male primordial germ cells, H3K4me3 is biallelically enriched at the imprinting control regions of maternal imprinted genes, suggesting a role for this histone marker in antagonizing DNA methylation (21). Consistent with a role of H3K4 methylation in antagonizing de novo DNA methylation by Dnmt3a/b, the histone H3K4 demethylase KDM1B/AOF1/LSD2 is required for establishment of differential DNA methylation at several maternal DMRs, including Mest, Grb10, Plag1, and Impact in oocytes (24). Finally, histone variants and noncoding RNAs have also been implicated in the establishment and maintenance of genomic imprinting (25, 26). Thus, the mechanisms that lead to establishment of imprinted DNA methylation at the DMRs seem to be complex and may vary from one imprinted gene to another.

UHRF1, a multifunctional protein, is required for DNA maintenance methylation due to its essential role in targeting Dnmt1 to DNA replication forks during the S phase of cell cycle (27–30). Consequently, DNA methylation in Uhrf1−/− ES cells is severely impaired, and a previous study has shown loss of genomic imprinting for imprinted H19, Kcnq1ot1, and Gtl2 genes in Uhrf1−/− ES cells (29). Here, we re-expressed UHRF1 in Uhrf1−/− ES cells and examined the effect on the expression and DNA methylation of the imprinted genes. Surprisingly, we observed that, although re-expression of UHRF1 in Uhrf1−/− ES cells failed to rescue the patterns of expression and DNA methylation for most of the imprinted genes, it did for the imprinted H19, Nnat, and Dlk1 genes. We provide evidence that the histone modification status at the DMRs of imprinted genes may dictate whether DNA methylation could be restored upon UHRF1 re-expression. Our study also shows that mouse ES cells can be a useful model for dissecting the underlying mechanisms for establishment of differential DNA methylation in imprinted genes.

Experimental Procedures

Plasmids and Antibodies—All expression constructs were generated by a PCR-based cloning strategy and verified by DNA sequencing. UHRF1 was cloned into the pPYCAGIP vector containing a FLAG tag. the antibodies used were anti-UHRF1 (homemade); anti-H3K4me2, anti-H3K4me3, anti-H3K9me3, anti-H4K20me3, and anti-ZFP57 (Abcam); anti-H3ac (AbmART); anti-actin (Sigma-Aldrich) and anti-FLAG M2 (Sigma-Aldrich); and anti-mC (Eurogentec).

Cell Culture, Transient Transfection, and Stable Cell Line Generation—E14 and Uhrf1−/− ES cells were routinely cultured with mouse embryonic fibroblast feeder cells in DMEM supplemented with 10% FBS, 1000 units/ml leukemia inhibitory factor, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 μM nonessential amino acids, 55 μM β-mercaptoethanol, 50 units/ml penicillin, and 50 mg/ml streptomycin (all from Invitrogen) in a 37 °C incubator at 5% CO2. Transient transfection was performed with Lipofectamine 2000 (Invitrogen) essentially according to the manufacturer’s instructions. For generation of FLAG-UHRF1-expressing stable Uhrf1−/− ES cell lines, the Uhrf1−/− ES cells were first transfected with the pPYCAGIP-FLAG-UHRF1 expression plasmid using Lipofectamine 2000. Two days after transfection, the cells were replaced with standard medium with the addition of 5 μg/ml puromycin (Invitrogen). After selection for 1–2 weeks with puromycin, single colonies were picked and cultured with 0.5 μg/ml puromycin for maintenance.

Immunostaining for DNA Methylation—Immunostaining for 5-mC was performed essentially as described (31). Briefly, the cells were washed with 1× PBS prior to fixation in 4% paraformaldehyde at room temperature for 20 min, treated with 4 N HCl at room temperature for 30 min, and neutralized with sodium borate (pH 9.0) for 20 min. After blocking with 5% BSA at 37 °C for 20 min, anti-5-mC antibody was added at a dilution of 1:500 and incubated for 2 h at 37 °C or overnight at 4 °C before the addition of and incubation with secondary antibodies for 1 h at 37 °C. Images were acquired with an Olympus TH4-20D microscope system.

Microarray and Quantitative PCR (qPCR)—Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Microarray experiments were performed on mouse genome 430A 2.0 arrays (Affymetrix).
qPCR was performed on an Mx3005P qPCR system (Stratagene). Relative gene expression was analyzed by quantitative RT-PCR, normalized to actin, and calculated with the ∆∆Ct method. The qPCR primers used were as follows: Ascl2, GC-CTATTGCTGGAGAGAA (forward) and CCAACTGTTGGAGAGTA (reverse); H19, TTGACTAATGTTGGTTGGT (forward) and AGGAACCTCCGAGATTTA (reverse); Dlk1, CATAGAGTGTGGTGTTGAG (forward) and GTGATGTGGCTGTGAAAT (reverse); Igf2r, AGAAGAGGAGGGAGGAGGA (forward) and TGTTGGTTTGTGTTATGTG (reverse); Impact, GAAGAAAACCTGAGGAGG (forward) and GGTTTGGTTTTTTTTATGTTATGAGT (reverse); Peg3, TTTTTAGATTTTTTGTTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1, TGGTTTTTGTTTTTTTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1, TGGTTTTTGTTTTTTTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-1, GGTGTTTTTTTTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-2, GATTGTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-1, GGTGTTTTTTTTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-2, GATTGTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-1, GGTGTTTTTTTTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-2, GATTGTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-1, GGTGTTTTTTTTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-2, GATTGTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-1, GGTGTTTTTTTTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-2, GATTGTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-1, GGTGTTTTTTTTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-2, GATTGTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-1, GGTGTTTTTTTTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-2, GATTGTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-1, GGTGTTTTTTTTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-2, GATTGTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-1, GGTGTTTTTTTTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-2, GATTGTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-1, GGTGTTTTTTTTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-2, GATTGTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-1, GGTGTTTTTTTTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-2, GATTGTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-1, GGTGTTTTTTTTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-2, GATTGTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-1, GGTGTTTTTTTTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-2, GATTGTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-1, GGTGTTTTTTTTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-2, GATTGTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-1, GGTGTTTTTTTTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-2, GATTGTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-1, GGTGTTTTTTTTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-2, GATTGTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-1, GGTGTTTTTTTTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse); Dlk1-2, GATTGTGTTTATGTTATGAGT (forward) and ATGATGTGGCTGTGTTGAG (reverse); Peg3, TTGTGGTTTGTGTTATGTG (forward) and TACCTGGTACAGTCTG (reverse).
CTCTTAGATACCG (reverse); and H19, AAGGAACATGC-TACATTCCAC (forward) and CTGAGATAGCTCTGAGAAC (reverse).

Results

Microarray Analysis of Imprinted Genes in Uhrf1<sup>−/−</sup> ES Cells—As UHRF1 is required to mediate DNA methylation by DNMT1, deletion of Uhrf1 in mouse ES cells has been shown to result in substantial reduction of global DNA methylation and DNA methylation in a few imprinted genes tested (28–30). We thus wished to analyze the effect of Uhrf1 deletion on the expression of the majority of imprinted genes. By Western blot analysis, we confirmed the absence of Uhrf1 proteins in Uhrf1<sup>−/−</sup> ES cells compared with wild-type E14 cells (Fig. 1A). Cellular RNAs were then prepared from wild-type and Uhrf1<sup>−/−</sup> ES cells and subjected to expression analysis using mouse genome 430A 2.0 arrays. Among a total of 102 imprinted genes that were spotted in the array, we found that 25 genes were either up- or down-regulated in Uhrf1<sup>−/−</sup> ES cells with a cutoff of >1.5-fold change, whereas 77 genes remained unaffected. The relative expression levels of the 25 affected genes are shown in the heat map in Fig. 1B. Considering that many imprinted genes may not express or do not show imprinted expression in ES cells (18), our microarray analysis suggested a widespread loss of genomic imprinting in Uhrf1<sup>−/−</sup> ES cells.

Re-expression of UHRF1 Restores Global DNA Methylation in Non-imprinted Regions—To investigate whether re-expression of UHRF1 in Uhrf1<sup>−/−</sup> ES cells could affect the expression and DNA methylation of imprinted genes, we transfected Uhrf1<sup>−/−</sup> ES cells with the expression plasmid pYCAC-GIP-UHRF1 and used puromycin to select stably expressing cell lines. We first screened the isolated stable ES cells for restoration of DNA methylation by immunofluorescence staining using anti-5mC antibody. We then analyzed the levels of FLAG-UHRF1 expression using an UHRF1-specific antibody that recognizes both mouse and human UHRF1. We found that all cell lines with restored DNA methylation exhibited a relatively high level of FLAG-UHRF1 expression (>2-fold compared with endogenous UHRF1 in E14 ES cells) (data not shown). Although the observed high-level expression of FLAG-UHRF1 could be due to the intrinsic property of the antibody used (raised against human UHRF1), a stable cell line with the lowest FLAG-UHRF1 expression was selected for all subsequent experiments. As shown by Western blot analysis using anti-human UHRF1 antibody, this stable cell line expressed ~2-fold more UHRF1 compared with the wild-type ES cells (Fig. 2A). Immunofluorescence staining analysis using anti-5mC antibody revealed uniformly increased DNA methylation for essentially all cells in this stable cell line (Fig. 2B). Furthermore, quantitative HPLC analysis of the global level of DNA methylation demonstrated that UHRF1 re-expression restored the global level of DNA methylation to that of the wild-type cells (Fig. 2C). Together, these data demonstrate that UHRF1 re-expression rescues the global DNA methylation defect in Uhrf1<sup>−/−</sup> ES cells.

We next attempted to confirm that UHRF1 re-expression led to the global restoration of DNA methylation in non-imprinted regions using the COBRA assay (33). We randomly selected six TCGA sites in mouse genome promoters that are known to be methylated in wild-type ES cells (34). The genomic DNA was first treated with sodium bisulfite and then subjected to PCR amplifications using paired primers specific for these six sites. The PCR products were recovered and digested with the TaqI restriction enzyme, and the degrees of TaqI digestion represent the extent of CpG methylation within the TCGA sites. As shown in Fig. 2D, COBRA assay confirmed that all six sites were methylated in the wild-type E14 ES cells, and their levels of DNA methylation were substantially reduced in Uhrf1<sup>−/−</sup> ES cells. Importantly, UHRF1 re-expression restored the levels of TaqI digestion essentially to those in wild-type samples. We concluded that the DNA methylation in non-imprinted regions is recovered upon re-expression of UHRF1, in agreement with the global restoration of DNA methylation upon UHRF1 re-expression.

Re-expression of UHRF1 Restores the Expression of Only a Small Subset of Imprinted Genes—To test whether UHRF1 re-expression can also restore DNA methylation in imprinted genes, we performed RT-qPCR analysis to determine whether UHRF1 re-expression rescues the expression defect of the 25 imprinted genes identified above by microarray analysis. As shown in Fig. 3A, H19 transcription was significantly up-regulated in Uhrf1<sup>−/−</sup> ES cells, and UHRF1 re-expression suppressed H19 transcription to a level equivalent to that in the wild-type ES cells. Consistent with the notion that the
imprinted Igf2 gene is controlled by the same imprinting control region as $H19$ and that its transcription is regulated reciprocally to that of $H19$ (35), we found that Igf2 transcription was reduced in the Uhrf1$^{-/-}$ ES cells, and UHRF1 re-expression enhanced Igf2 transcription to that in the wild-type cells (Fig. 3A).

Similarly, we found that UHRF1 re-expression restored the transcription of the imprinted Nnat and Dlk1 genes to the levels in wild-type cells. In contrast to these four genes, we observed no restoration of transcription for the other 21 imprinted genes. Thus, re-expression of UHRF1 in Uhrf1$^{-/-}$ ES cells restores transcription of only a small subset of imprinted genes.

Re-expression of UHRF1 Restores DNA Methylation in Only a Small Subset of Imprinted Genes—We next asked if UHRF1 re-expression is able to rescue the lost DNA methylation in the imprinted genes. We first examined DNA methylation in DMRs for genes whose expression was restored upon UHRF1 re-expression. COBRA assay with TaqI demonstrated that UHRF1 re-expression restored DMR DNA methylation in the imprinted $H19$ and Dlk1 genes (Fig. 3B). As DNMT1 re-expression in Dnmt1$^{-/-}$ ES cells fails to restore DNA methylation in imprinted genes (18), we were surprised by this observation. To confirm this unexpected result, we tested DNA methylation at a different site in the $H19$ DMR by COBRA assay using the restriction enzyme BstUI, which recognizes the CGCG sequence. This analysis again confirmed that UHRF1 re-expression led to restoration of DNA methylation (Fig. 3B). Similarly, COBRA assay using the restriction enzyme HpyCH4IV, which digests ACGT sites, confirmed restoration of DNA methylation to an ACGT site within the Nnat DMR and an ACGT site within the Nnat DMR (Fig. 3B). Thus, for all three imprinted clusters ($H19$/Igf2, Dlk1, and Nnat) whose expression was restored upon re-expression of UHRF1, DNA methylation in their DMRs appeared to also be restored. We found no restoration of DMR DNA methylation for the Peg3, Igf2r, and Snrpn
imprinted genes (used as controls), whose expression was not restored upon UHRF1 re-expression (Fig. 3B). Our transcription and DNA methylation analyses demonstrate a correlation between restoration of DNA methylation and restoration of transcription. Furthermore, our data demonstrate that UHRF1 re-expression leads to restoration of expression and DNA methylation for only a small subset of imprinted genes.

Confirmation of Restored DMR DNA Methylation by Bisulfite Sequencing—To confirm the COBRA results that UHRF1 re-expression leads to restoration of DNA methylation for a small subset of imprinted genes, we carried out DNA methylation analysis by bisulfite sequencing (36). In the wild-type ES cells, bisulfite sequencing revealed that approximately half of the DMR sequences were heavily methylated, and the rest were essentially not methylated for H19, Nnat, Dlk1, Igf2r, and Snrpn (Fig. 4). These data are consistent with the notion that the imprinted genes are differentially methylated on either the paternal or maternal alleles, but not both. Uhrf1 knock-out resulted in the loss of this differential DNA methylation, as DNA methylation in all DMR sequences from H19, Nnat, Igf2r, Peg3, and Snrpn was significantly reduced in Uhrf1−/− ES cells (Fig. 4). Importantly, UHRF1 re-expression restored DNA methylation in ~30–40% of the DMR sequences from the H19 and Nnat genes. Thus, consistent with the COBRA assay results, bisulfite sequencing analysis demonstrated that UHRF1 re-expression led to restoration of DMR DNA methylation in H19 and Nnat. Also consistent with the COBRA assay data, bisulfite sequencing analysis demonstrated that UHRF1 re-expression did not recover the lost DNA methylation in the DMRs of the Igf2r, Peg3, and Snrpn genes. It is noteworthy that Uhrf1 knock-out only moderately reduced DNA methylation in the Dlk1 DMR, although UHRF1 re-expression did restore the level of DNA methylation to that of wild-type cells. The results from bisulfite sequencing analysis provide compelling evidence that UHRF1 re-expression rescues the DNA methylation defect in a small subset of imprinted genes.

Restoration of Imprinted DNA Methylation Correlates to Large Extent with Binding of ZFP57—The finding that UHRF1 re-expression led to restoration of imprinted DNA methylation in H19, Nnat, and Dlk1 but not the other imprinted genes provided us an excellent opportunity to investigate the potential underlying mechanism(s). First, we asked if the restoration of imprinted DNA methylation is determined by the binding of UHRF1. Using anti-FLAG M2 antibody for ChIP assay of FLAG-UHRF1, we observed that FLAG-UHRF1 was associated with all tested DMRs regardless of whether DNA methylation...
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in DMRs was restored or not upon UHRF1 re-expression (Fig. 5A). This result indicates that the binding of UHRF1 is unlikely the determining factor for differential restoration of imprinted DNA methylation.

As a newly identified sequence-specific methylated CpG-binding protein, ZFP57 has been implicated in the establishment and especially the maintenance of differential DNA methylation in some imprinted genes (14, 15). We thus analyzed whether UHRF1 knock-out and re-expression affect the expression of ZFP57 by Western blot analysis. Fig. 5B shows that knock-out and re-expression of UHRF1 did not affect the levels of ZFP57 proteins in ES cells. We then analyzed the binding of ZFP57 by ChIP. The representative results (Fig. 5C) show that, for the three imprinted genes whose DNA methylation was restored upon UHRF1 re-expression, the binding of ZFP57 was restored for H19 and Nnat, but not for Dlk1. However, for all four genes whose DNA methylation was not restored upon UHRF1 re-expression, no restoration of ZFP57 binding was observed (Fig. 5C). Thus, our data reveal a correlation between the binding of ZFP57 and the restoration of imprinted DNA methylation, with the exception of Dlk1, consistent with the notion that ZFP57 is involved in the establishment or maintenance of imprinted DNA methylation in some but not all imprinted genes.

Histone Modifications May Determine Restoration of Imprinted DNA Methylation—As a methylated DNA-binding protein, ZFP57 is likely to play a role during and/or after restoration of DNA methylation. As demonstrated by both COBRA and bisulfite sequencing analyses, DNA methylation was nearly completely lost in Uhrf1−/− ES cells in most DMRs we analyzed, except for Dlk1. Thus, the restoration of imprinted DNA methylation likely requires a coordinated function of de novo and maintenance DNA methylation. As de novo DNA methylation by Dnmt3a/b is highly influenced by histone modification status, especially histone H3K4 methylation (37–39), we next compared the histone modification status for various DMRs in the wild-type, Uhrf1−/−, and UHRF1-re-expressing Uhrf1−/− ES cells by ChIP assay. As shown in Fig. 6A, the levels of H3 acetylation in the H19 and Nnat DMRs in the wild-type ES cells were considerably lower compared with the other DMRs as well as the promoter of the non-imprinted Gapdh gene. Although Uhrf1 knock-out led to increased levels of H3 acetylation for all DMRs and the Gapdh promoter, the magnitude of H3 acetylation was still substantially lower for H19 and Nnat compared with the others. Furthermore, re-expression of UHRF1 down-regulated H3 acetylation to the levels in the wild-type ES cells for only H19 and Nnat, but had no effect on H3 acetylation for the other DMRs. ChIP analysis revealed that Uhrf1 knock-out led to a 2-fold increase in H3K4me2 in the DMRs of H19, Dlk1, Peg3, Nnat, Igf2r, and Impact, and UHRF1 re-expression moderately down-regulated the levels of H3K4me2 in H19, but not in the other DMRs (Fig. 6B). More striking results were observed for H3K4me3. First, ChIP results revealed that the levels of H3K4me3 were markedly lower in the H19 and Nnat DMRs compared with the other DMRs and the Gapdh promoter (Fig. 6C). Second, Uhrf1 knock-out led to marked increases in H3K4me3 in all DMRs, except H19 and Nnat (Fig. 6C). Third, UHRF1 re-expression failed to restore the elevated H3K4me3 levels in the DMRs back to the levels in the wild-type ES cells. Thus, a striking difference between imprinted genes whose DNA methylation was restored upon UHRF1 re-expression (H19 and Nnat) and those whose DNA methylation was not restored (Peg3, Nnat, Igf2r, and Impact) is the persistent low level of H3K4me3 even in Uhrf1−/− ES cells. Although UHRF1 re-expression did not down-regulate the elevated level of H3K4me3 in Dlk1, the overall low level of H3K4me3 in this region may explain the DNA methylation restoration upon UHRF1 re-expression (see “Discussion”).

We also analyzed the status of the repressive histone markers H3K9me3 and H4K20me3, both of which have previously been shown to be enriched in the DMRs of imprinted genes (20–23). In contrast to H3K4me3, we found that H3K9me3 and H4K20me3 were enriched in each DMR compared with the control Gapdh promoter. Uhrf1 knock-out led to a substantial reduction in these markers in all DMRs (Fig. 6, D and E). UHRF1 re-expression led to complete or partial restoration of both repressive markers for H19 and Nnat, but not for the other DMRs. Thus, the status of H3K9me3 and H4K20me3 in the imprinted genes whose DNA methylation was not restored, suggesting that these histone markers are unlikely to be major determinants for DNA methylation restoration.

Discussion

The imprinted DNA methylation that differentially marks the paternal and maternal genomes is established during male and female gametogenesis and then stably maintained following fertilization and throughout development (1, 6). A seminal observation made by Tucker et al. (18) is that re-expression of DNMT1 in Dnmt1 knock-out mouse ES cells restored methylation of bulk DNA to normal levels, but did not restore the
methylolation of the imprinted genes H19 and Igf2r. Restoration of monoallelic DNA methylation and expression of the H19 and Igf2r imprinted genes was observed only after germ line transmission. The concept derived from this study is that the establishment of imprinted DNA methylation is dependent on the unique environments of male and female germ cells, and once erased, the imprinted DNA methylation cannot be restored in non-germ line cells. As an accessory factor required for recruiting DNMT1 to DNA replication forks, previous studies have documented a global reduction in bulk DNA methylation and loss of imprinted DNA methylation in Uhrf1 knock-out ES cells (28, 29). Consistent with the observation by Tucker et al. (18) that DNMT1 re-expression restored methylation of bulk DNA, we found that Uhrf1 re-expression also restored global DNA methylation (Fig. 2). Also consistent with the observation by Tucker et al., we found that, unlike DNA methylation in the bulk non-imprinted regions, DNA methylation in the majority of the imprinted genes was not restored upon Uhrf1 re-expression (Figs. 3 and 4). However, Uhrf1 re-expression did lead to restoration of DNA methylation in the imprinted genes H19, Nnat, and Dlk1 (Figs. 3 and 4). Paternally imprinted H19 was among the first identified genomic imprinted genes and encodes a long noncoding RNA that has been implicated in regulation of IGF2 expression (40). Maternally imprinted Nnat encodes the neuronatin protein, which is involved in brain development (41), whereas Dlk1 (atypical NOTCH ligand delta-like 1 homologue) encodes membrane-bound and secreted isoforms with functions in several developmental processes (42). Thus, the major unexpected finding in our study is that re-expression of Uhrf1 in Uhrf1−/− ES cells can restore imprinted DNA methylation in these three imprinted genes.

Multiple lines of evidence support the conclusion that Uhrf1 re-expression in Uhrf1−/− ES cells restored DNA methylation in the imprinted genes H19, Nnat, and Dlk1. First, gene expression analysis showed that Uhrf1 re-expression restored their expression to the levels in the wild-type cells (Fig. 2). Second, COBRA analysis showed that Uhrf1 re-expression restored DNA methylation in their representative sites within DMRs (Fig. 3B). Third, bisulfite sequencing analysis demonstrated that Uhrf1 re-expression restored the lost DNA methylation in Uhrf1−/− ES cells in their DMRs (Fig. 4). Fourth, ChIP analysis showed that Uhrf1 re-expression restored the binding of ZFP57 for both H19 and Nnat. Although this was not observed for Dlk1, the fact that nearly half of the sequenced H19, Nnat, and Dlk1 clones have fully restored DNA methylation and that the rest of the clones are unmethylated provides compelling evidence that Uhrf1 re-expression restores the imprinted monoallelic methylation for the DMRs of H19, Nnat, and Dlk1. However, due to the lack of nucleotide polymorphic sites that would allow us to distinguish in Uhrf1−/− ES cells the paternal and maternal alleles of H19, Nnat, and Dlk1, one caveat in our study is that we cannot be sure that Uhrf1 re-expression restored DNA methylation for only the imprinted paternal H19 and Dlk1 alleles and the maternal Nnat allele.
FIGURE 6. Potential correlation between histone modification status at imprinted DMRs and restoration of imprinted DNA methylation. The levels of H3ac (A), H3K4me2 (B), H3K4me3 (C), H3K9me3 (D), and H4K20me3 (E) in each DMR were assessed by ChIP-qPCR in E14, Uhrf1−/−, and UHRF1-expressing Uhrf1−/− (Uhrf1−/− + UHRF1) ES cells. The ChIP data are shown as the mean ± S.D. of the percentage of each PCR product in the immunoprecipitated sample compared with that in the input sample. Dark-gray bars, E14 ES cells; light-gray bars, Uhrf1−/− ES cells; gray bars, UHRF1-expressing Uhrf1−/− ES cells.
Our study raises the intriguing question as to why UHRF1 re-expression can lead to restoration of DNA methylation for a small subset of imprinted genes, but not for the majority. Because imprinted DNA methylation is established during gametogenesis, a simple explanation for the lack of DNA methylation restoration for the majority of imprinted genes is that, in Uhrf1−/− ES cells, these imprinted genes are protected against de novo DNA methylation. Consistent with this idea, we found that for all four representative imprinted genes whose DNA methylation was not restored upon Uhrf1 re-expression, their DMRs gained considerably higher levels of the active histone markers H3ac and H3K4me3 in Uhrf1−/− ES cells. In contrast, for the H19 and Nnat genes, whose DNA methylation could be restored upon UHRF1 re-expression, the levels of acetylation and H3K4me3 in their DMRs were maintained at substantially lower levels in Uhrf1−/− ES cells. Thus, it is tempting to suggest that, although Uhrf1 knock-out led to loss of imprinted DNA methylation in the H19 and Nnat genes, the histone modifications, especially low H3K4me3 (or possibly other factors such as noncoding RNAs), that differentially decorate the paternal and maternal alleles of these genes are likely to be maintained. The low level of H3K4me3 and perhaps additional histone marker(s) or unidentified factor(s) may work together to maintain the original imprinted alleles poised for de novo DNA methylation by Dnmt3a/b. Once UHRF1 is re-expressed, de novo DNA methylation and maintenance methylation would work together and lead to restoration of DNA methylation in the imprinted allele, but not the other one. For most of the imprinted genes, loss of imprinted DNA methylation led to significant increases in active histone markers, especially H3K4me3 (Fig. 6). The increased levels of active histone markers are likely to prevent de novo DNA methylation and thus the restoration of imprinted DNA even upon UHRF1 re-expression. Dlk1 appears to be an exception because UHRF1 re-expression restored its DNA methylation, but not its histone modifications and binding of ZFP57. It is noteworthy that, unlike H19 and Nnat, DNA methylation in the Dlk1 DMR was reduced but not entirely lost in Uhrf1−/− ES cells (Fig. 4). Thus, it is possible that the restoration of DNA methylation in the Dlk1 DMR upon UHRF1 re-expression is more dependent on maintenance methylation and less on de novo DNA methylation. This could explain why its DNA methylation could be restored even with a relatively high level of H3K4me3.

Our study demonstrates that mouse ES cells can be a valuable model not only for mechanistic study of genomic imprinting maintenance but also for genomic imprinting establishment. As genomic imprinting is established during gametogenesis, the previous studies of genomic imprinting establishment were essentially dependent on knock-outs, transgenic models, and male and female germ cells (1, 43, 44). The lack of a suitable cellular model hinders the mechanistic study of genomic imprinting. The findings in this study will allow us to further dissect the underlying mechanisms for establishment of differential DNA methylation in the imprinted genes H19, Nnat, and Dlk1. Furthermore, Uhrf1−/− ES cells could also serve as a model for investigating under what conditions UHRF1 re-expression could restore DNA methylation to other imprinted genes.

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