DNA methyltransferases are complementary in maintaining DNA methylation in embryonic stem cells

**Highlights**

- **ZFP57 maintains DNA methylation at the ICR of most imprinted regions in ES cells**
- **TET proteins may not be essential for maintaining most ICR DNA methylation in ES cells**
- **DNMT3 is required for the maintenance of DNA methylation at a subset of ICRs in ES cells**
- **Maintenance functions of DNMT1 and DNMT3 are complementary at repeats and genic regions**

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Liu et al., iScience 25, 105003 September 16, 2022 © 2022 The Author(s).

https://doi.org/10.1016/j.isci.2022.105003
Genomic imprinting is essential for normal development in mammals (Li and Li, 2020; Li et al., 2019a; Li, 2012). It is characterized by parent-of-origin-dependent mono-allelic expression of a few hundred imprinted genes (Barlow and Bartolomei, 2014; Bartolomei et al., 2020; Khamlichi and Feil, 2018; Li et al., 2019b). Most of the known imprinted genes are clustered in over 20 imprinted regions in the mouse genome. These clustered imprinting genes are controlled by cis-acting imprinting control regions (ICRs) in each imprinted region (Barlow and Bartolomei, 2014). Each ICR harbors a germline- and differentiation-methylated region (DMR) that modulates the expression of the imprinted genes. Differential DNA methylation at the ICRs is reset in germ cells, with the removal of original DNA methylation followed by the re-establishment of new DNA methylation at the ICRs (Barlow and Bartolomei, 2014). The patterns of differential DNA methylation at the ICRs are reconstituted again in the zygote and stably maintained thereafter in the somatic cells of the progeny.

ZFP57 is a KRAZ zinc finger protein required for the maintenance of DNA methylation at most known ICRs in mouse embryos (Hirasawa and Feil, 2008; Jiang et al., 2021; Li et al., 2008). Human ZFP57 has similar functions to mouse ZFP57 that it maintains genomic imprinting in humans and it can also substitute for mouse ZFP57 in maintaining DNA methylation in mouse ES cells (Mackay et al., 2008; Takikawa et al., 2013). ZFP57 can recognize the six-nucleotide consensus motif TGCCGC that is present at almost all known ICRs, with a much higher binding affinity for the ICR sequences with methylated DNA (Liu et al., 2012, 2013; Quenneville et al., 2011; Strogantsev et al., 2015). It binds to the cofactor KAP1/TRIM28 via its KRAZ box which in turn may recruit DNA methyltransferases to maintain DNA methylation at the ICRs (Li et al., 2008; Quenneville et al., 2011; Zuo et al., 2012). ZFP5445 is another KRAZ zinc finger protein that has also been shown to regulate genomic imprinting, with more prominent roles in humans than in mice (Jian and Bartolomei, 2019; Takahashi et al., 2019). Most DNA methylation occurs at the CpG sites in mammals, with a methyl group attached to the fifth position of the cytosine pyrimidine ring (5mC) (Zeng and Chen, 2019). DNA methyltransferases (DNMTs) are the enzymes that catalyze the methylation of cytosine by transferring a methyl group from the donor
S-adenosyl-L-methionine (SAM) (Li and Zhang, 2014). There are three well-known DNMTs in mammals, i.e. DNMT1, DNMT3A, and DNMT3B, with each containing an active DNA methyltransferase catalytic domain (Chen and Zhang, 2020). DNMT3L has sequence homology to these three DNMTs but does not have the active catalytic domain, although it can bind to DNMT3A and DNMT3B to enhance their catalytic activities. Passive loss of DNA methylation may occur during DNA replication if there is no maintenance methylation. Usually DNMT1 is considered to be the maintenance DNMT, whereas DNMT3A and DNMT3B participate in de novo methylation. UHRF1 has a functional domain for binding to hemi-methylated DNA and it helps DNA replication (Wu and Zhang, 2014). Alternatively, they may be excised by TDG and other DNA repair enzymes through the base excision repair (BER) pathway, which results in DNA demethylation (Hu et al., 2014).

Besides replication-dependent passive demethylation, DNA methylation at the CpG sites may be removed through the oxidative demethylation process mediated by Ten-Eleven Translocation (TET) proteins (Wang et al., 2020; Xu and Bochtler, 2020). There are three TET proteins (TET1, TET2, and TET3) in mammals (Wu and Zhang, 2017). TET can convert 5-methyl cytosine (5mC) to 5-hydroxymethyl cytosine (5hmC) first, which can then be further oxidized by TET to give rise to 5-formlycytosine (5fC) and subsequently 5-carboxylcytosine (5caC) (Bochtler et al., 2017). These oxidized cytosine derivatives cannot be recognized by DNMTs and thereby DNA demethylation occurs when they are serially diluted during multiple rounds of DNA replication (Wu and Zhang, 2014). Alternatively, they may be excised by TDG and other DNA repair enzymes through the base excision repair (BER) pathway, which results in DNA demethylation (Hu et al., 2014).

Embryonic stem (ES) cells are pluripotent stem cells that have the potential to give rise to different cell types. Nuclear target-derived ES (ntES) cells and induced pluripotent stem (iPS) cells also have multi-potential properties and they are good candidates for cell replacement therapies (Bar and Benvenisty, 2020). However, there are a number of studies indicating that genomic imprinting, including the germline-derived differential DNA methylation at the ICRs, is variably lost in ntES and iPS cells (Bar and Benvenisty, 2019; Li et al., 2019b). As the dysregulation of genomic imprinting causes a number of human diseases including cancer and diabetes, it is critical to examine how it is stably maintained in ES cells (Monk et al., 2019). This may help to derive therapeutically suitable ntES cells and iPS cells in the future with relatively stable DNA methylation at the ICRs.

Mouse ES cells are usually derived from the inner cell mass of the blastocysts. In a previous study, we derived a wild-type 129/DBA hybrid ES cell line called D1911 from the blastocyst derived from the timed mating between a 129S6/SvEvTac female mouse and a DBA/2J male mouse (Lau et al., 2016b). To analyze the functions of ZFP57 and ZFP445 in ES cells, we generated multiple deletion mutant ES clones using a CRISPR-based approach developed in our lab and then examined DNA methylation at the ICRs in the mutant ES cells (Liu et al., 2022). We also obtained multiple deletion mutant ES clones that lack one or two or three DNMTs from D1911 with the same CRISPR approach (Liu et al., 2022). Similarly, we also analyzed DNA methylation at the ICRs and other genomic regions including the somatic DMRs and repeats. We took advantage of SNPs present in the 129/DBA hybrid ES cell line and determined the allelic DNA methylation at the ICRs and DMRs harboring SNPs in these mutant ES cells.

**RESULTS**

**ZFP57 maintained most DNA methylation imprint in mouse embryonic stem cells**

The mutant ES clones with deletions at Zfp57, Zfp445, or both were generated by CRISPR-Cas9 from the parental wild-type (WT) 129/DBA hybrid ES cell line D1911 (Tables S1 and S2) (Figures S1A and S1B) (Lau et al., 2016b; Liu et al., 2022). No wild-type ZFP57 was detected in these Zfp57 mutant ES clones based on Western blot with affinity-purified rabbit polyclonal antibodies against ZFP57 (Figure S2A) (Li et al., 2008). Genomic DNA samples isolated from these mutant ES clones and the WT control ES cells were subjected to COBRA analysis to examine DNA methylation at the ICRs of some known imprinted regions (Figure 1). We found DNA methylation was lost at the ICRs of the Snrpn, Zac1, Peg3, and Peg13 in Zfp57/−/− and Zfp57/−/−; Zfp445/−/− (DKO) mutant ES clones compared with the WT ES clone (Figure 1). Complete loss of DNA methylation was observed at the IG-DMR of the Dlk1-Dio3 imprinted region in Zfp57/−/−; Zfp445/−/− (DKO) mutant ES clones compared with the WT ES clone (Figure 1). DNA methylation was also partially lost at Peg1, Kcnq1ot1, Gpr1, and Peg5 ICRs in Zfp57/−/− and Zfp57/−/−; Zfp445/−/− (DKO) mutant ES clones compared with the WT ES clone (Figure 1). There was no loss of DNA methylation at these ICRs in Zfp445/−/− mutant ES clones except for the partial loss of DNA methylation at the Peg5 ICR in 1 out of 2 Zfp445/−/− mutant ES clones (Figure 1). DNA methylation remained intact at the Slc38a4 ICR, but it was largely lost at the Gnas1A
ICR in Zfp445−/−, Zfp57−/− or Zfp445−/−; Zfp57−/− (DKO) mutant ES clones compared with the WT ES clone (Figure 1). Interestingly, DNA methylation was not lost at H19 ICR in Zfp57−/− mutant ES clones, but it seemed to be increased at H19 ICR in Zfp445−/− mutant ES clones compared with the WT ES clone (Figure 1). It was partially lost at H19 ICR in Zfp57−/−; Zfp445−/− (DKO) mutant ES clones compared with the WT ES clone (Figure 1). Taken together, ZFP57 but not ZFP445 is required for the maintenance of DNA methylation at most examined ICRs in mouse ES cells.

TET proteins may not be essential for the maintenance of DNA methylation imprint in mouse embryonic stem cells

To test if loss of DNA methylation at the ICRs caused by loss of ZFP57 could be prevented in mouse ES cells when TET proteins are absent, we eliminated ZFP57 in Tet TKO ES cells (Zfp57−/− & Tet TKO, Lane...
Figure 2. Loss of TET proteins does not prevent loss of DNA methylation imprint caused by loss of ZFP57 in mouse embryonic stem cells based on COBRA analysis

Genomic DNA samples were isolated from the Zfp57<sup>−/−</sup> mutant ES clones and the parental control Tet TKO ES clone. Then they were subjected to COBRA analysis to examine DNA methylation at some known imprinted regions. Lane 1, the parental Tet TKO ES clone lacking TET1, TET2, and TET3. Lane 2-3, two Zfp57<sup>−/−</sup> in Tet TKO (Zfp57<sup>−/−</sup> & Tet TKO) ES clones with the deletion mutation at Zfp57 (Zfp57 KO-3 and Zfp57 KO-4). Lane 4-6, three Zfp57<sup>−/−</sup>, Zfp445<sup>−/−</sup> in Tet TKO (Zfp57<sup>−/−</sup>, Zfp445<sup>−/−</sup> & Tet TKO) ES clones with the deletion mutations at both Zfp57 and Zfp445 (ZFP DKO-3, ZFP DKO-4, and ZFP DKO-5). U, unmethylated product after COBRA. M, methylated product after COBRA. The restriction enzymes used for COBRA are as follows: Snrpn, HhaI; Zac1, TaqαI; Peg1, TaqαI; Peg3, TaqαI; Peg13, TaqαI; Kcnq1ot1, TaqαI; H19, ClaI; IG-DMR, TaqαI; Gpr1, TaqαI; Slc38a4, TaqαI; Peg5, HhaI; Gnas1A, ClaI.

2-3 of Figure 2) using CRISPR-Cas9 (Table S2) (Figure S1A) (Hu et al., 2014). There was no detectable wild-type ZFP57 in these Zfp57 mutant ES clones on Western blot (Figure S2A). We also removed both ZFP57 and ZFP445 in Tet TKO ES cells (Zfp57<sup>−/−</sup>; Zfp445<sup>−/−</sup> & Tet TKO, Lane 4-6 of Figure 2) using CRISPR-Cas9 (Table S2) (Figures S1A and S1B). Tet TKO ES cells lack all three TET proteins (TET1, TET2, and TET3) involved in active DNA demethylation (Hu et al., 2014). We found DNA methylation was still lost at the ICRs of Snrpn, Zac1 and Peg3 imprinted regions in Zfp57<sup>−/−</sup> or Zfp57<sup>−/−</sup>; Zfp445<sup>−/−</sup> mutant Tet TKO ES clones even in the absence of all three TET proteins (Figure 2). DNA methylation was partially lost at the ICRs of Peg1, Kcnq1ot1, IG-DMR of Dlk1-Dio3 imprinting region, Gpr1, Peg5, and Gnas1A in these mutant ES clones compared with the parental Tet TKO ES cells (Figure 2). At Peg13 ICR, it was partially lost in Zfp57<sup>−/−</sup> mutant Tet TKO ES clones (Lane 2-3 of Figure 2) but completely lost in...
Zfp445+/-; Zfp445-/- mutant Tet TKO ES clones (Lane 4-6 of Figure 2). DNA methylation was not lost at H19 ICR in Zfp57+/-; Zfp445-/- mutant Tet TKO ES clones (Lane 2-3 of Figure 2), but largely lost in Zfp57+/-; Zfp445-/- mutant Tet TKO ES clones (Lane 4-6 of Figure 2). There was no loss of DNA methylation at the Slc38a4 ICR in Zfp57+/- or Zfp57-/-; Zfp445-/- mutant Tet TKO ES clones, similar to Zfp57+/-; Zfp445+/-; Zfp445-/- (DKO) mutant ES clones (Figures 1 and 2). Based on these results, it seems that loss of TET proteins could not prevent loss of DNA methylation at the ICRs caused by loss of ZFP57 or loss of both ZFP57 and ZFP445 in mouse ES cells. Therefore, we think TET proteins may not play significant roles in the stable maintenance of DNA methylation at the ICRs in mouse ES cells.

Generation of mutant embryonic stem cell clones lacking DNA methyltransferases in mouse embryonic stem cells

We have proposed that ZFP57 may recruit DNA methyltransferases through its cofactor KAP1/TRIM28 to maintain DNA methylation at the imprinted regions in ES cells in a previous study (Zuo et al., 2012). As TET proteins do not seem to be essential for the maintenance of DNA methylation imprint in mouse ES cells, we wonder if ZFP57-mediated recruitment of DNA methyltransferases may be the primary pathway in maintaining DNA methylation at the ICRs in mouse ES cells. Thus, we generated a series of Dnmt mutant ES clones using CRISPR-Cas9, with 2 sgRNA constructs per target gene, in the 129/DBA hybrid D1911 ES cell line (Tables S1 and S3). These include Dnmt1-/-; Dnmt3a-/-; Dnmt3b-/- (DKO), and Dnmt1-/-; Dnmt3a-/-; Dnmt3b-/- (TKO) mutant ES clones. As UHRF1 has been shown to be a major partner for DNMT1 in maintaining DNA methylation, we also created Uhrf1-/- mutant (Uhrf1 KO) ES clones to test if it functions in the same pathway as DNMT1 in maintaining DNA methylation in ES cells (Tables S1 and S4) (Figure S1F). There was no detectable Dnmt1, Uhrf1, Dnmt3a, or Dnmt3b in the corresponding mutant ES clones on Western blots (Figure S2). As controls, two WT ES clones (WT-1 and WT-2) were also individually picked from the ES cell culture transfected with the empty pX330 vector. High levels of OCT4 and NANOG were detected in the ES culture of these mutant ES clones and the WT ES clones by iScience 25, 105003, September 16, 2022 5
more in-depth analyses of DNA methylation at the ICRs as well as the DMRs, repeats, and other genomic regions as illustrated later in discussion (Tables S6–S9).

DNMT1 played major role in maintaining DNA methylation at most known imprinting control regions in mouse embryonic stem cells according to whole genome bisulfite sequencing.

To confirm the COBRA results for Dnmt and Uhrf1 mutant ES clones, we analyzed WGBS data of two independent ES clones of Dnmt and Uhrf1 mutant ES cells as well as two WT ES clones at P4 to examine DNA methylation imprinting.
methylation at all known imprinted regions (Tables S6 and S7) (Figure 4). Indeed, we found DNA methylation was almost completely lost at the ICRs of the Zac1, Nespas, Grb10, Nap115, Inpp5f, Snrpn, Peg1, Kcnq1ot1, Peg3, Rasgrf1, Lgf2r and Peg13 in the Dnmt1 KO or Uhrf1 KO ES clones, similar to Dnmt TKO ES clones (Figure 4A) (Table 1). There was some DNA methylation remaining at the AK008011 ICR in the Dnmt1 KO or Uhrf1 KO ES clones, compared with Dnmt TKO ES clones (Figure 4A) (Table 1). By contrast, there was no significant loss of DNA methylation at these ICRs in the Dnmt3 DKO mutant ES clones compared with two WT ES clones (Figure 4A) (Table 1). There was little DNA methylation at the Zrsr1 and Peg10 ICRs in mouse ES cells, and therefore it is difficult to assess how DNA methylation may be maintained at these two ICRs in the Dnmt1 and wild-type control mouse ES cells.

DNA methyltransferases are differentially required for the maintenance of DNA methylation imprint at the known imprinted regions in mouse ES cells according to WGBS analysis (Figure 4). Genomic DNA samples were isolated from the Dnmt and Uhrf1 mutant ES clones as well as the wild-type (WT) control ES clones at early passage (P4). Then they were subjected to whole genome bisulfite sequencing (WGBS) analysis to examine DNA methylation at the known imprinted regions and other genomic regions. The percentages (%) of DNA methylation were calculated for the ICRs of the known imprinted regions. WT, wild-type ES clone (gray). Dnmt1 KO, Dnmt1 mutant ES clone (orange red). Uhrf1 KO, Uhrf1 mutant ES clone (blue). Dnmt3 DKO, Dnmt3a−/−; Dnmt3b−/− DKO mutant ES clone (green). Dnmt TKO, Dnmt TKO mutant ES clone (black) with deletion mutations at Dnmt1, Dnmt3a, and Dnmt3b. Two-way ANOVA was carried out in statistical analysis, with Dunnett multiple comparison test, for DNA methylation at the ICRs comparing two ES clones of each Dnmt or Uhrf1 mutant ES cells with two WT ES clones. The data were presented as mean ± SEM with the following statistical significance values: *p < 0.05; **p < 0.01; ***p < 0.001.

(A) Dnmt1 maintained DNA methylation imprint at most imprinting control regions (ICRs) in mouse ES cells. These include Zac1, Nespas, Grb10, Nap115, Inpp5f, Snrpn, Peg1, Kcnq1ot1, Peg3, Rasgrf1, Lgf2r, Peg13, and AK008011.

(B) There was little DNA methylation at the Zrsr1 and Peg10 ICRs in the Dnmt1 mutant and wild-type control mouse ES cells.

(C) Dnmt1 played major roles in maintaining DNA methylation imprint at five ICRs in mouse ES cells, whereas there was a significant loss of DNA methylation at these five ICRs in the Dnmt3 DKO mutant ES cells. These ICRs include Cdh15, Gpr1, IG-DMR of the Dlk1-Dio3 imprinted region, H19 and Impact.

(D) Dnmt3A and Dnmt3B played equally or more important roles than Dnmt1 in the maintenance of DNA methylation imprint at the Slc38a4, Mcts2, Peg5, and Gnas1A ICRs in mouse ES cells.
DNMT3A and DNMT3B contributed to the maintenance of DNA methylation at a subset of imprinting control regions in mouse embryonic stem cells according to whole-genome bisulfite sequencing

Despite that DNMT1 played major roles in maintaining DNA methylation at most known ICRs in mouse ES cells, loss of DNMT3A and DNMT3B caused significant loss of DNA methylation at Cdh15, Gpr1, IG-DMR of the Dlk1-Dio3 imprinted region, H19 and Impact ICRs in mouse ES cells (Figure 4C) (Table 1). Furthermore, they were also required for the maintenance of DNA methylation at the Slc38a4, Mcts2, Peg5, and Gnas1A ICRs in mouse ES cells (Figure 4D) (Table 1). Interestingly, DNMT3A and DNMT3B appeared to play no less important roles than DNMT1 in maintaining DNA methylation at these ICRs because DNA methylation was partially or severely lost at Slc38a4, Mcts2, Peg5, and Gnas1A ICRs in the Dnmt1 DKO mutant ES clones than in the Dnmt1 KO or Uhrf1 KO ES clones, in comparison to two Dnmt TKO and two WT ES clones (Figure 4D) (Table 1). These results are easily visible by the DNA methylation IGV plots at these ICRs in these ES clones (Figure 5). A similar loss of DNA methylation at the Slc38a4, Peg5 and Gnas1A ICRs was found in the COBRA analysis of Dnmt and Uhrf1 mutant ES clones compared with two WT ES clones (Figure 3). Therefore, DNMT3A and DNMT3B are essential for maintaining DNA methylation at a subset of ICRs in mouse ES cells.

Maintenance of germline-derived imprinting control region methylation by DNMT1 as well as DNMT3A and DNMT3B in mouse embryonic stem cells

It appeared that DNMT3A and DNMT3B, together with DNMT1, were involved in maintaining DNA methylation at the ICRs in mouse ES cells. We wondered if germline-derived ICR methylation was, indeed,

| ICR            | Dnmt1 KO ES (DNMT1 function) | Uhrf1 KO ES (UHRF1 function) | Dnmt3 DKO ES (DNMT3A/3B function) | Dnmt TKO |
|----------------|-------------------------------|-------------------------------|-----------------------------------|----------|
| Zac1           | Lost (Yes)                    | Lost (Yes)                    | No loss (No)                      | Lost     |
| Nespas         | Lost (Yes)                    | Lost (Yes)                    | No loss (No)                      | Lost     |
| Grb10          | Lost (Yes)                    | Lost (Yes)                    | No loss (No)                      | Lost     |
| Nap115         | Lost (Yes)                    | Lost (Yes)                    | No loss (No)                      | Lost     |
| Inpp5f         | Lost (Yes)                    | Lost (Yes)                    | No loss (No)                      | Lost     |
| Snrpn          | Lost (Yes)                    | Lost (Yes)                    | No loss (No)                      | Lost     |
| Peg1           | Lost (Yes)                    | Lost (Yes)                    | No loss (No)                      | Lost     |
| Kcnq1ot1       | Lost (Yes)                    | Lost (Yes)                    | No loss (No)                      | Lost     |
| Peg3           | Lost (Yes)                    | Lost (Yes)                    | No loss (No)                      | Lost     |
| Rasgrf1        | Lost (Yes)                    | Lost (Yes)                    | No loss (No)                      | Lost     |
| Igl2r          | Lost (Yes)                    | Lost (Yes)                    | No loss (No)                      | Lost     |
| Peg13          | Lost (Yes)                    | Lost (Yes)                    | No loss (No)                      | Lost     |
| AK008011       | Mostly lost (Yes)             | Mostly lost (Yes)             | No loss (Minor)                   | Lost     |
| Cdh15          | Lost (Yes)                    | Mostly lost (Yes)             | Partial loss (Yes)                | Lost     |
| Gpr1           | Lost (Yes)                    | Lost (Yes)                    | Partial loss (Yes)                | Lost     |
| IG-DMR         | Lost (Yes)                    | Lost (Yes)                    | Partial loss (Yes)                | Lost     |
| H19            | Mostly lost (Yes)             | Mostly lost (Yes)             | Partial loss (Yes)                | Lost     |
| Impact         | Lost (Yes)                    | Lost (Yes)                    | Partial loss (Yes)                | Lost     |
| Zrsr1          | Low (NA)                      | Low (NA)                      | Low (NA)                          | Low (NA) |
| Peg10          | Low (NA)                      | Low (NA)                      | Low (NA)                          | Low (NA) |
| Slc38a4        | Partial loss (Yes)            | Partial loss (Yes)            | Lost (Yes)                        | Lost     |
| Mcts2          | Lost (Yes)                    | Lost (Yes)                    | Lost (Yes)                        | Lost     |
| Peg5           | Partial loss (Yes)            | Partial loss (Yes)            | Lost (Yes)                        | Lost     |
| Gnas1A         | Mostly lost (Yes)             | Mostly lost (Yes)             | Lost (Yes)                        | Lost     |

Note: NA, not applicable.

*methylation was very low (around 5%) at this ICR in WT ES cells.

methylation was relatively low (<20%) at this ICR in WT ES cells.

DNMT3A and DNMT3B appeared to have minor roles at the AK008011 ICR as DNA methylation was partially lost in the Dnmt1 KO or Uhrf1 KO ES clones but complete missing in the Dnmt TKO ES clones (Figure 4A).

Table 1. The effects of DNMTs or UHRF1 on ICR DNA methylation in ES cells
maintained by three DNA methyltransferases in mouse ES cells. For this, we took advantage of the SNPs present in the parental D1911 ES clone that was derived from the timed mating between a 129S6/SvEvTac (129) female mouse and a DBA2/J (DBA) male mouse (Lau et al., 2016b). Allelic DNA methylation analysis was performed to examine how germline-derived DNA methylation imprint is maintained at the ICRs with a maternal 129 allele and a paternal DBA allele in mouse ES cells (Table S9) (Figure 6).

DNA methylation imprint at the ICRs of \( \text{H19} \), \( \text{Rasgrf1} \), and IG-DMR of the \( \text{Dlk1-Dio3} \) imprinted region is established during spermatogenesis and stably maintained on the paternal chromosomes in somatic cells after fertilization. For other known ICRs, DNA methylation imprint is reset during oogenesis and stably maintained on the maternal chromosomes. In the WT ES cells of this study, DNA methylation imprint is located at the ICRs of \( \text{H19} \), \( \text{Impact} \), \( \text{Slc38a4} \), \( \text{Mcts2} \), \( \text{Peg5} \), and \( \text{Gnas1A} \). WT, wild-type ES clone (gray). \( \text{Dnmt1} \) KO, \( \text{Dnmt1} \) mutant ES clone (orange red). \( \text{Uhrf1} \) KO, \( \text{Uhrf1} \) mutant ES clone (blue). \( \text{Dnmt3a} \) /\( \text{Dnmt3b} \) DKO mutant ES clone (green). \( \text{Dnmt3} \) DKO, \( \text{Dnmt3} \) DKO mutant ES clone (black) with deletion mutations at \( \text{Dnmt1, Dnmt3a, and Dnmt3b} \). All methylation IGV plots have the same scale (0-100) in this figure.

Figure 5. Loss of DNA methylation imprint was confirmed at the known ICRs in \( \text{Dnmt} \) mutant ES clones based on the methylation IGV plot

Genomic DNA samples isolated from two independent ES clones (1 and 2) of \( \text{Dnmt} \) mutant, \( \text{Uhrf1} \) mutant, and wild-type (WT) control ES cells at P4 were subjected to whole genome bisulfite sequencing (WGBS) analysis. IGV methylation plots were obtained to examine DNA methylation at 24 known ICRs that include \( \text{Zac1, Nespas, Grb10, Nap115, Inpp5f, Snrpn, Peg1, Kcnq1ot1, Peg3, Rasgrf1, Igfl2r, Peg13, AK008011, Zrsr1, Peg10, Cdh15, Gpr1, IG-DMR of the Dlk1-Dio3 imprinted region, H19, Impact, Slc38a4, Mcts2, Peg5, and Gnas1A} \). WT, wild-type ES clone (gray). \( \text{Dnmt1} \) KO, \( \text{Dnmt1} \) mutant ES clone (orange red). \( \text{Uhrf1} \) KO, \( \text{Uhrf1} \) mutant ES clone (blue). \( \text{Dnmt3} \) DKO, \( \text{Dnmt3a} \)/\( \text{Dnmt3b} \) DKO mutant ES clone (green). \( \text{Dnmt3} \) DKO, \( \text{Dnmt3} \) DKO mutant ES clone (black) with deletion mutations at \( \text{Dnmt1, Dnmt3a, and Dnmt3b} \). All methylation IGV plots have the same scale (0-100) in this figure.

We found germline-derived ICR methylation was almost completely lost at the ICRs of \( \text{Zac1, Peg13, Nespas, Snrpn, and Inpp5f} \) on the maternal 129 chromosomes in the \( \text{Dnmt1} \) KO or \( \text{Uhrf1} \) KO ES clones, similar to \( \text{Dnmt1} \) KO ES clones (Figure 6A) (Table 2). In contrast, there was no significant loss of DNA methylation at these ICRs in the \( \text{Dnmt3} \) DKO mutant ES clones in comparison to two WT control ES clones (Figure 6A) (Table 2). DNA methylation was lost at the \( \text{Impact} \) and \( \text{Cdh15} \) ICRs on the maternal 129 chromosomes in the \( \text{Dnmt1} \) KO or \( \text{Uhrf1} \) KO ES clones. Nevertheless, there was also a significant loss of DNA methylation at these two ICRs in the \( \text{Dnmt3} \) DKO mutant ES clones compared with two WT ES clones (Figure 6B) (Table 2).

Surprisingly, high level of DNA methylation was observed at the \( \text{Cdh15} \) ICR on the paternal DBA chromosome in two WT ES clones that were largely lost in the \( \text{Dnmt1} \) KO or \( \text{Uhrf1} \) KO ES clones but also significantly reduced in the \( \text{Dnmt3} \) DKO mutant ES clones compared with two WT ES clones (Figure 6B) (Table 2). As expected, germline-derived ICR methylation was completely lost at these ICRs on the maternal 129
chromosomes in the Dnmt TKO ES cells (Figures 6A and 6B) (Table 2). Taken together, DNMT1 played major roles in maintaining germline-derived DNA methylation at these ICRs on the maternal chromosomes in mouse ES cells. Interestingly, DNMT3A and DNMT3B contributed to the maintenance of germline-derived DNA methylation at the Impact and Cdh15 ICRs on the paternal 129 chromosomes, which is consistent with their functions in maintaining overall DNA methylation at these two ICRs in mouse ES cells.

DNA methylation imprint was present at the Rasgrf1 ICR on the paternal DBA chromosome in the WT or Dnmt3 DKO ES cells (Figure 6A) (Table 2). There was no detectable DNA methylation at the Rasgrf1 ICR on the paternal DBA chromosome in the Dnmt1 KO or Uhrf1 KO or Dnmt TKO ES cells (Figure 6A). Low level of DNA methylation was similarly observed at the Rasgrf1 ICR on the maternal 129 chromosome in the WT, Dnmt1 KO, Dnmt3 DKO, and Dnmt TKO ES cells (Figure 6A). It seemed that DNMT1 but not DNMT3A and DNMT3B were required for maintaining DNA methylation imprint at the Rasgrf1 ICR on the paternal chromosome in mouse ES cells.

Intriguingly, DNA methylation was largely lost at the Mcts2 ICR and completely lost at the Gnas1A ICR on the maternal 129 chromosomes in the Dnmt1 KO or Uhrf1 KO or Dnmt3 DKO ES cells, compared with the WT ES cells (Figure 6C) (Table 2). There was low level of DNA methylation at the Gnas1A ICR on the paternal DBA
chromosome in the WT ES cells that were lost in the Dnmt1 KO or Uhrf1 KO or Dnmt3 DKO ES clones (Figure 6C) (Table 2). It appeared that DNMT3A and DNMT3B, together with DNMT1, maintained germline-derived DNA methylation at the Mcts2 and Gnas1A ICRs on the maternal 129 chromosomes in mouse ES cells. These are also consistent with their maintenance functions in overall DNA methylation at these two ICRs (Figures 4Da n d6C).

Similar observations were obtained from the allelic methylation IGV plots of these ICRs (Figure S5). Therefore, we conclude that DNMT3A and DNMT3B are required for the maintenance of germline-derived ICR methylation at a subset of ICRs in mouse ES cells although DNMT1 is the major DNAmethyltransferase for maintaining germline-derived DNA methylation imprint at most ICRs in mouse ES cells.

DNA methylation at repeats and other genomic regions in mouse embryonic stem cells

Besides the ICRs and DMRs, there are many other genomic regions that can be methylated in ES cells. Therefore, we examined DNA methylation at repeats and genic regions in the mutant ES cells in comparison to the WT ES cells (Table S8) (Figure S6). Overall, roughly 70% of the CpG sites of the entire genome were methylated in two WT ES clones (Figure S6A). A bit over 20% of CpG sites remained methylated in the Dnmt1 KO or Uhrf1 KO ES clones, whereas close to 40% of CpG sites were methylated in the Dnmt3 DKO mutant ES clones (Figure S6A). As expected, almost all CpG sites lost methylation in the Dnmt TKO ES clones (Figure S6A). These results suggest that all three DNA methyltransferases are required for maintaining germline-derived DNA methylation imprint at most ICRs in mouse ES cells.

Similar levels of DNA methylation (about 70-75%) were observed in the gene body, intron and intergenic regions in the WT ES cells (Figure S6B). 20-30% of CpG sites remained methylated in the gene body, intron and intergenic regions in the Dnmt1 KO or Uhrf1 KO ES cells while 30-40% of CpG sites were methylated in these regions in the Dnmt3 DKO mutant ES clones (Figure S6B). It appeared that three DNA methyltransferases were necessary for maintaining DNA methylation in the gene body, intron and intergenic regions in mouse ES cells and DNMT1 played a bit more important roles than DNMT3A and DNMT3B in these regions. About 60% of the CpG sites located in the exons of the genic regions and 20-30% of the CpG sites at the promoters of the genic regions were methylated in the WT ES cells (Figure S6B). Interestingly, partial loss of DNA methylation was similarly observed at the exonic and promoter regions in Dnmt1 KO or Uhrf1 KO or Dnmt3 DKO ES cells (Figure S6B). Therefore, DNMT3A and DNMT3B seemed to be as important as DNMT1 in maintaining DNA methylation in these regions in mouse ES cells.

Approximately 80% of the CpG sites located in the DNA, LINE, LTR, and SINE repeats were found to be methylated in the WT ES cells (Figure S6C). Slightly less DNA methylation was observed at the CpG sites

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**Table 2. Allelic DNA methylation at the ICRs in the WT and mutant ES cells**

| ICR         | WT ES | Dnmt1 KO | Uhrf1 KO | Dnmt3 DKO | Dnmt TKO |
|-------------|-------|----------|----------|-----------|---------|
| Zac1 (M)    | Me (M) | Me lost  | Me lost  | No Loss (M) | Me lost |
| Peg13 (M)   | Me (M) | Me lost  | Me lost  | No Loss (M) | Me lost |
| Nesnas (M)  | Me (M) | Me lost  | Me lost  | No Loss (M) | Me lost |
| Snrpn (M)   | Me (M) | Me lost  | Me lost  | No Loss (M) | Me lost |
| Inpp5f (M)  | Me (M) | Me lost  | Me lost  | No Loss (M) | Me lost |
| Rasgrf1 (P) | Me (P) | Low Me? (M) | Me lost (P) | Low Me? (M) | Low Me? (M) |
| Impact (M)  | Me (M) | Me lost  | Me lost  | Partial Loss (M) | Me lost |
| Cdh15 (M)*  | Me (M) | Me lost (M) | Me lost (M) | Partial Loss (M) | Me lost (M) |
| Mcts2 (M)   | Me (M) | Partial Loss (M) | Partial Loss (M) | Partial Loss (M) | Me lost |
| Gnas1A (M)* | Me (M) | Me lost (M) | Me lost (M) | Me lost (M) | Me lost (M) |
| Low Me (P)  | Me (P) | Me lost (P) | Me lost (P) | Me lost (P) | Me lost (P) |

M, maternal allele. P, paternal allele. Me, methylation. All ICRs in this table were reported to contain germline-derived DNA methylation on the maternal chromosomes except that Rasgrf1 ICR is reported to be methylated on the paternal chromosome.

*both maternal and paternal ICRs were similarly methylated in the WT ES cells.

*maternal and paternal ICRs had different levels of methylation in the WT ES cells.
located in the simple repeat regions (roughly 60%) in the WT ES cells (Figure S6C). DNA methylation was almost completely absent at all repeat regions in Dnmt TKO ES cells. Close to 30% of CpG sites at the SINE repeats were methylated in Dnmt1 KO or Uhrf1 KO or Dnmt3 DKO ES cells (Figure S6C). DNA methylation was similarly lost at the DNA, LINE, LTR, and simple repeat regions in the Dnmt1 KO or Uhrf1 KO ES cells, with 20-30% of the CpG sites methylated in the absence of DNMT1 or UHRF1 (Figure S6C). However, more than 40% of the CpG sites were still methylated at the DNA, LINE, LTR, and simple repeat regions in Dnmt3 DKO ES cells (Figure S6C). These results indicate that DNMT3A and DNMT3B are only slightly less crucial than DNMT1 and UHRF1 in maintaining DNA methylation at these repeat regions.

Fewer than 10% of the CpG sites were methylated in all CpG islands (CGIs) in the WT ES clones (Figure S6D). About 5% of the CpG sites in CGIs were still methylated in the Dnmt1 KO or Uhrf1 KO ES clones. But there were even fewer CpG sites (less than 5%) that remained methylated in the Dnmt3 DKO or Dnmt TKO ES clones. It seemed that DNMT3A and DNMT3B, together with DNMT1, are required for maintaining DNA methylation at the CGIs in mouse ES cells.

**DNA methylation at somatic differentially methylated regions in mouse embryonic stem cells**

We also examined DNA methylation at somatic DMRs in mouse ES cells (Figure S7) (Table S7). As expected, DNA methylation was almost completely absent at these somatic DMRs in Dnmt TKO ES cells (Figure S7). There were about 80% and 90% methylation at the Smoc2 DMR and Igf2-DMR0, respectively, in the WT ES cells (Figure S7A). Only 20-30% of CpG sites were methylated at Smoc2 DMR and approximately 40% of CpG sites were methylated at Igf2-DMR0 in the Dnmt1 KO or Uhrf1 KO ES clones (Figure S7A). Although there was no significant loss of DNA methylation at these two DMRs in the Dnmt3 DKO mutant ES clones compared with two WT control ES clones, DNMT3A and DNMT3B contributed to the maintenance of DNA methylation at both Smoc2 DMR and Igf2-DMR0 because little DNA methylation was observed at these two DMRs in the Dnmt TKO ES clones while there were 20-40% methylation at both DMRs in the Dnmt1 KO or Uhrf1 KO ES clones (Figure S7A).

Except for the Meg3 DMR with around 50% methylation in the WT ES cells and little methylation in the Dnmt1 KO or Uhrf1 KO or Dnmt TKO ES clones, 70-80% of the CpG sites were methylated at the Meg3-Intron, Pde10a, Park2 and Sloc2a2 DMRs in the WT ES cells and partial loss of DNA methylation occurred in these DMRs in the Dnmt1 KO or Uhrf1 KO ES clones in comparison to the WT and Dnmt TKO ES clones (Figure S7B). DNA methylation was also significantly lost at these five somatic DMRs in the Dnmt3 DKO mutant ES cells (Figure S7B). Therefore, DNMT3A and DNMT3B were required for maintaining DNA methylation at these five DMRs although DNMT1 was the major maintenance DNMT for them.

About 50-80% of the CpG sites were methylated at the Zdbf2, AC185554.1, and Gab1 DMRs in the WT ES cells (Figure S7C). Interestingly, loss of DNA methylation appeared to be more severe at these three DMRs in the Dnmt3 DKO mutant ES clones compared with the Dnmt1 KO or Uhrf1 KO ES clones (Figure S7C). DNMT3A and DNMT3B played more important roles than DNMT1 in maintaining DNA methylation at these three DMRs in ES cells.

Only 10-30% of the CpG sites were methylated at the Rian, Nesp, Igf2r-TSS, and Jade1 DMRs in the WT ES cells (Figure S7D). Partial loss of DNA methylation was observed at these DMRs in the Dnmt1 KO ES clones in comparison to the WT ES clones, whereas DNA methylation was similarly lost in the Dnmt3 DKO and Dnmt TKO ES clones (Figure S7D). Surprisingly, there was no loss of DNA methylation at the Igf2r-TSS DMR in the Uhrf1 KO ES clones although the partial loss of DNA methylation was observed at the Nesp and Jade1 DMRs in the Uhrf1 KO ES clones. Twice of CpG sites were methylated at the Rian DMR in the Uhrf1 KO ES clones compared with the Dnmt1 KO ES clones (Figure S7D). These results indicated that three DNMTs were necessary for maintaining DNA methylation at these DMRs with low levels of methylation. DNMT1 might not always require UHRF1 for its maintenance methylation.

Fewer than 10% of the CpG sites were methylated at the Cd81, Ascl2, Kcnq1-Intergenic1, Ndn, Tssc4, Kcnq1-Intergenic2, Sfmb12, and Cdkn1c DMRs in mouse ES cells (Figure S7D). Therefore, it is difficult to assess if DNA methylation may be lost at these DMRs in the Dnmt or Uhrf1 mutant ES cells.

In summary, DNMT3A and DNMT3B, together with DNMT1, maintained DNA methylation at the examined somatic DMRs in mouse ES cells. They appeared to play more important roles in maintaining DNA methylation at about half of the somatic DMRs than DNMT1. UHRF1 may not always work with DNMT1 in the maintenance of DNA methylation at the DMRs although they seemed to function similarly in most DMRs.
DNA methylation at the imprinting control regions in mouse embryonic stem cells after extended culture

Based on the COBRA and WGBS results of the mouse ES clones at P4, the earliest passage of ES cell culture we obtained for DNA methylation analysis, DNMT1 was the major DNA methyltransferase involved in the maintenance of DNA methylation at most known ICRs. Intriguingly, we found DNMT3A and DNMT3B, together with DNMT1, were also required for the maintenance of DNA methylation at a subset of known ICRs in mouse ES cells at P4. To test if the ES cell culture condition may cause DNA methylation to be lost at the ICRs, COBRA analysis was performed for some ICRs in two independent ES clones of Dnmt1 KO, Uhrf1 KO, Dnmt3 DKO, Dnmt TKO as well as the WT control ES cells after they had been cultured on the feeder cells for a total of 10 passages (P10) and 20 passages (P20), respectively, as the last Lipofectamine-mediated transfection (see STAR Methods) (Figures S8 and S9). Except for the slight change at the Kcnq1ot1 ICR described later in discussion, there was no obvious difference in DNA methylation at the ICRs of the Snrpn, Zac1, Peg1, Peg3, Peg13, H19, IG-DMR of Dlk1-Dio3 imprinted region, Gpr1, Slc38a4, Peg5 and Gnas1A in the WT ES clones at P10 or P20 compared with those at P4 (Figures 3, S8, and S9). Similar to the COBRA results obtained from these Dnmt and Uhrf1 mutant ES clones at P4 (Figure 3), DNA methylation was absent at the ICRs of the Snrpn, Zac1, Peg1, Peg3, and Peg13 in the Dnmt1 KO and Uhrf1 KO mutant ES clones at P10 and P20, in comparison to the WT and Dnmt TKO ES clones at P10 and P20 (Figures S8 and S9). However, DNA methylation was still intact at these ICRs in the Dnmt3 DKO mutant ES clones at P10 and P20 (Figures S8 and S9). Surprisingly, there seemed to be a slight increase in DNA methylation at the Kcnq1ot1 ICR in all Dnmt and Uhrf1 mutant ES clones as well as in the WT ES clones. Nevertheless, DNA methylation was still largely lost at the Kcnq1ot1 ICR in the Dnmt1 KO and Uhrf1 KO mutant ES clones at P10 and P20, but mostly retained in the Dnmt3 DKO mutant ES clones at P10 and P20, in comparison to the WT and Dnmt TKO ES clones at P10 and P20 (Figures S8 and S9). DNA methylation was lost at the H19 ICR in the Dnmt1 KO, Uhrf1 KO and Dnmt TKO mutant ES clones at P10, whereas it was retained at the H19 ICR in the Dnmt3 DKO mutant ES clones at P10 (Figure S8). Similar results were obtained at the H19 ICR in the Dnmt1 KO, Uhrf1 KO and Dnmt3 DKO mutant ES clones at P20 (Figure S9). Intriguingly, H19 ICR was slightly methylated in both Dnmt TKO mutant ES clones at P20 (Figure S9).

Similar to what had been observed at P4, DNA methylation was partially lost at the IG-DMR of the Dlk1-Dio3 imprinted region as well as the ICRs of Gpr1, Slc38a4, Peg5 and Gnas1A in the Dnmt1 KO, Uhrf1 KO, and Dnmt3 DKO mutant ES clones at P10 and P20 (Figures 3, S8, and S9). Loss of DNA methylation seemed to be more severe at the IG-DMR and Gpr1 ICR in two Dnmt1 KO mutant ES clones at P4, P10, and P20 than in two Dnmt3 DKO mutant ES clones at these passages, whereas there was a more severe loss of DNA methylation at the Peg5 and Gnas1A ICRs in two Dnmt3 DKO mutant ES clones at P4, P10 and P20 than in two Dnmt1 KO mutant ES clones at the same passages (Figures 3, S8, and S9). Interestingly, there was still a residual amount of DNA methylation at IG-DMR, Slc38a4, Peg5, and Gnas1A ICRs in both Dnmt TKO mutant ES clones at P10 and P20 (Figures S8 and S9).

Taken together, DNA methylation was largely similar at the examined ICRs in the WT and mutant ES clones at P10 and P20 compared with those at P4. There was no further loss or significant gain of DNA methylation at the ICRs in the WT or Dnmt1 KO or Dnmt3 DKO mutant ES clones after the extended culture of ES cells. These results suggest that it is the maintenance DNA methylation, rather than de novo DNA methylation, that DNMT3A and DNMT3B play in regulating steady-state DNA methylation level at a subset of ICRs in mouse ES cells, which is functionally similar to DNMT1.

DISCUSSION

ZFP57 maintains DNA methylation at most known ICRs in mouse embryos (Jiang et al., 2021; Takahashi et al., 2015, 2019). It has been shown to maintain DNA methylation at multiple ICRs in our previous studies using the Zfp57 mutant ES cells generated by homologous recombination or directly derived from the blastocysts (Lau et al., 2016a; Takikawa et al., 2013; Zuo et al., 2012). Similar findings were reported in other studies (Anvar et al., 2016; Coluccio et al., 2018; Quenneville et al., 2011; Riso et al., 2016). Consistent with the observed results in Zfp57 maternal-zygotic mutant (M−Z) embryos, we found DNA methylation was lost at most known ICRs in the mutant ES cells lacking ZFP57 (Figure 1) (Jiang et al., 2021; Li et al., 2008; Takahashi et al., 2019). Indeed, DNA methylation was similarly lost at multiple examined ICRs in the blastocyst-derived Zfp57 mutant ES clones either from Zfp57 zygotic mutant (M−Z−) embryos or Zfp57 maternal-zygotic mutant embryos because maternal Zfp57 was likely depleted in the Zfp57 mutant ES clones derived from Zfp57 zygotic mutant blastocysts (Lau et al., 2016a).
Although the loss of ZFP445 did not affect DNA methylation at most examined ICRs, DNA methylation at the H19 ICR was significantly reduced in Zfp57\(^{-/-}\) Zfp445\(^{-/-}\) mutant ES cells (Figure 1). These results are similar to what was reported in Zfp445 zygotic mutant embryos as well as in the mutant embryos lacking both zygotic Zfp57 and zygotic Zfp445, although there were no reported data for the Zfp57 maternal-zygotic mutant embryos lacking zygotic Zfp445 (Takahashi et al., 2019). Therefore, ZFP57 and ZFP445 may be partially redundant in maintaining DNA methylation at the H19 ICR and this could be further examined in future research. It is unclear why DNA methylation at the H19 ICR was somewhat increased in Zfp445\(^{-/-}\) mutant ES cells (Figure 1).

There are a few previous studies suggesting the roles of TET proteins in genomic imprinting in mice (Dawlaty et al., 2013; Yamaguchi et al., 2013; Zhang et al., 2016). Except for the H19 ICR, we did not observe a significant increase in DNA methylation at multiple ICRs examined in ES cells before (Liu et al., 2015). Nevertheless, we tested the functions of TET proteins again by removing Zfp57 and Zfp445 using CRISPR-Cas9 in the Tet TKO ES cells lacking all three TET proteins. DNA methylation at the examined ICRs including the H19 ICR was still lost upon loss of ZFP57 and ZFP445 even in the absence of TET proteins (Figure 2). A similar loss of DNA methylation occurred to the ICRs in the Tet TKO ES cells upon loss of ZFP57, with the exception that there was only partial loss of DNA methylation at the Peg13 ICR and no loss of DNA methylation was observed at the H19 ICR in absence of both ZFP57 and TET proteins (Figure 2). These results further support the notion that ZFP57 is the master regulator in genomic imprinting in mouse ES cells and it may be partially redundant with ZFP445 at a small subset of the ICRs such as H19 ICR. These also suggest that TET proteins may not be critical for stable maintenance of DNA methylation at the ICRs in mouse ES cells. We had proposed in a previous study that ZFP57 recruited DNA methyltransferases via KAP1/TRIM28 to maintain DNA methylation at the ICRs (Zuo et al., 2012). Therefore, ZFP57-mediated recruitment of DNA methyltransferases may be the key mechanism underlying the maintenance of ICR DNA methylation.

In this study, we found that DNMT1 is the primary DNA methyltransferase in maintaining DNA methylation at most known ICRs in mouse ES cells, whereas DNMT3A and DNMT3B contribute to the maintenance of DNA methylation at a subset of ICRs (Figures 4 and 7A). According to WGBS, DNMT3A and DNMT3B were required for maintaining DNA methylation at five ICRs including Cdh15, Gpr1, IG-DMR of the Dlk1-Dio3 imprinted region, H19, and Impact although DNMT1 played much more important roles in maintaining DNA methylation at these five ICRs in mouse ES cells (Figure 4C). There was no obvious reduction in DNA methylation at the H19 ICR in the Dnmt3 DKO mutant ES clones at P4, P10, and P20 based on COBRA though (Figures 3, S8, and S9). This discrepancy could be owing to a small reduction in DNA methylation in the Dnmt3 DKO mutant ES clones that were recognized by WGBS, but it could not be easily detected by COBRA that is based on gel analysis of the product after restriction enzyme digestion. In addition, only one CpG site of the H19 ICR was analyzed by COBRA while many CpG sites were measured in WGBS, which may result in some difference if not all CpG sites of this ICR were uniform in DNA methylation level.

DNMT3A and DNMT3B were as equally important as DNMT1 in maintaining DNA methylation at four ICRs (Slc38a4, Mcts2, Peg5, and Gnas1A) with relatively low levels of DNA methylation in the WT ES clones (Figure 4D). Actually, they appeared to be more important than DNMT1 in the maintenance of DNA methylation at the Peg5 and Gnas1A ICRs. Based on allelic DNA methylation analysis, DNMT3A and DNMT3B, together with DNMT1, maintained germline-derived DNA methylation imprint at a subset of ICRs in mouse ES cells (Figure 5) (Figure 7A). Therefore, DNMT3A and DNMT3B may be partially redundant with DNMT1 in maintaining DNA methylation at a subset of ICRs in mouse ES cells even though DNMT1 is the major DNA methyltransferase in maintaining DNA methylation at most known ICRs.

It is interesting that DNMT3A and DNMT3B seem to be more important than DNMT1 in maintaining DNA methylation at a few ICRs, particularly the ones with relatively low levels of DNA methylation (Figure 4D). This indicates that these ICRs are more sensitive to loss of DNA methylation in ES cells. DNMT1 is insufficient to maintain DNA methylation at these sensitive ICRs. In this case, DNMT3A and DNMT3B can provide an alternative pathway for maintaining DNA methylation at these ICRs in ES cells at relatively low levels. By contrast, DNA methylation at most ICRs is relatively stable in ES cells and DNMT1 is sufficient to maintain DNA methylation at these ICRs by itself without the need for DNMT3A and DNMT3B. Indeed, DNA methylation was found to be very stable in a subset of ICRs that was only lost after a very long-term passage of ES cells lacking DNMT3A and DNMT3B (Chen et al., 2003). After the extended culture for 10 and 20 passages, there was no significant loss of DNA methylation at the examined ICRs in the WT ES clones according to COBRA (Figures 3, S8, and S9). In general, similar loss of DNA methylation at the ICRs was obtained in the Dnmt1 KO, Dnmt3 DKO, and
Dnmt TKO mutant ES clones at P4, P10, and P20. These results support our hypothesis that DNMT3A and DNMT3B are involved in maintaining DNA methylation at a subset of ICRs including IG-DMR and a few other ICRs as the loss of DNMT1 did not result in further loss of DNA methylation at these ICRs even after the extended culture of the Dnmt1 KO mutant ES clones. On the contrary, partial loss of DNA methylation persisted at these ICRs in the Dnmt1 KO and Dnmt3 DKO mutant ES clones at P10 and P20. De novo methylation mediated by DNMT3A and DNMT3B cannot compensate for partial loss of DNA methylation at these ICRs in mouse ES cells. Therefore, three DNA methyltransferases are all required but they are partially redundant for maintaining DNA methylation at the repeats, DMRs, and other genomic regions in mouse ES cells.

DNMT1 is known to be mainly involved in the maintenance of DNA methylation, whereas DNMT3A and DNMT3B are de novo DNA methyltransferases (Li and Zhang, 2014). DNMT1 and UHRF1 were reported to function in de novo DNA methylation in oocytes (Li et al., 2018; Maenohara et al., 2017). DNMT1 can cooperate with DNMT3A in de novo methylation (Fatemi et al., 2002). There are a few other studies, suggesting that DNMT3A and DNMT3B may participate in the maintenance methylation of repetitive elements and genic regions in mouse ES cells (Chen et al., 2003; Gujar et al., 2019; Liang et al., 2002). Consistent with these studies, DNMT3A and DNMT3B were as important as DNMT1 in maintaining DNA methylation at all repeats, most DMRs, and other genomic regions in mouse ES cells (Figures 7, S6, and S7). They were required for maintenance of DNA methylation not only at the promoters and CGIs with relatively low levels of DNA methylation but also in the highly methylated repeats, DMRs, gene bodies, and intergenic regions. Thus, three DNA methyltransferases are all required but they are partially redundant for maintaining DNA methylation at the repeats, DMRs, and other genomic regions in mouse ES cells.

Figure 7. Schematic diagrams are shown for DNA methyltransferases in maintaining DNA methylation in mouse embryonic stem cells

The arrows mean that three DNMT proteins act at the maintenance methylation of the ICRs, repeats, genic or intergenic regions (A–C). The thickness of the lines of these arrows represents the importance of their maintenance functions, with thin or thick lines indicating minor or major roles, respectively.

(A) Maintenance of DNA methylation at ICRs in mouse ES cells. DNMT1 maintains DNA methylation at 13 ICRs such as Zac1 and Snrpn. It is the major DNA methyltransferase that maintains DNA methylation at five other ICRs including H19 and IG-DMR of the Dlk1-Dio3 imprinted region. Interestingly, DNMT3A and DNMT3B contribute minor roles in the maintenance of DNA methylation at these five ICRs in mouse ES cells as well. In contrast, DNMT3A and DNMT3B, together with DNMT1, play major roles in maintaining DNA methylation at four ICRs including Peg5 and Gnas1A.

(B) Maintenance of DNA methylation at repeats in mouse ES cells. DNMT1 is the major DNA methyltransferase that maintains DNA methylation at most repeats including LINE and LTR, whereas DNMT3A and DNMT3B are also important for the maintenance of DNA methylation at these repeats. By contrast, DNMT3A and DNMT3B play equally important roles as DNMT1 in maintaining DNA methylation at SINE.

(C) Maintenance of DNA methylation at genic and intergenic regions in mouse ES cells. DNMT3A and DNMT3B play equally important roles as DNMT1 in maintaining DNA methylation at the exons, introns, and other genic regions. DNMT1 appears to be the major DNA methyltransferase for maintaining DNA methylation in the intergenic regions although DNMT3A and DNMT3B are also important for the maintenance of DNA methylation in the intergenic regions.
Limitations of the study

Most of the deletion mutant ES clones were generated by CRISPR from the 129/DBA hybrid wild-type ES clone D1911 (Lau et al., 2016b). However, Tet TKO mutant ES clone was used for the generation of Zfp57 and/or Zfp445 deletion mutations to examine if loss of three TET proteins could prevent loss of DNA methylation at the ICRs of the imprinted regions caused by loss of ZFP57 and ZFP445 in the ES cells (Hu et al., 2014). DNA methylation at the Peg13 ICR was partially lost in two Zfp57 deletion mutant ES clones derived from the Tet TKO ES cell line, whereas it was completely lost in two Zfp57 KO ES clones generated from D1911 (Figures 1 and 2). DNA methylation at the H19 ICR appeared to be slightly different when both ZFP57 and ZFP445 were lost in the DKO mutant ES clones derived from these two different ES cell lines. DNA methylation at other examined ICRs was similarly affected in these mutant ES clones (Figures 1 and 2). We suspect that small differences observed at the Peg13 and H19 ICRs could be owing to some inherent differences of two parental ES cell lines. This hypothesis may be tested in the future when the same ES cell line is used for the generation of Zfp57 and Zfp445 deletion mutations with or without TET proteins.

There are quite many SNPs that are present in the 129/DBA hybrid wild-type ES clone D1911 derived from the mating of a female mouse on the 129S6/SvEvTac genetic background and a male mouse on the DBA2/J genetic background. But only a subset of ICRs harbors an SNP that has allowed us to perform allelic analysis of DNA methylation at these ICRs in the WGBS analysis. Therefore, it is not possible to determine how germline-derived DNA methylation was lost at the H19, Peg5, and other ICRs in the absence of DNMT proteins, unlike a subset of ICRs such as Gnas1A and Impact in this study (Figure 6). In the future, a hybrid ES clone carrying more SNPs may be required to examine allelic DNA methylation at most ICRs upon loss of DNMT proteins.

STAR+METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105003.

ACKNOWLEDGMENTS

The work in the authors’ laboratories has been supported by the grants from Ministry of Science and Technology of the People’s Republic of China (Grant # 2018YFC1005004), and from Science and Technology Commission of Shanghai Municipality, China. The authors would like to thank Weijun Jiang and Fenghua Chen for their help with some experiments in this study. We also appreciate the help from the personnel in the animal facilities of ShanghaiTech University and the Protein Center of Shanghai Zhangjiang Laboratory, particularly Ms. Chaohua Zheng. Many thanks go to the Molecular and Cell Biology Core Facility (MCBCF),...
and Multi-Omics Core Facility (MOCF) at the School of Life Science and Technology in ShanghaiTech University for providing technical support.

AUTHOR CONTRIBUTIONS

X.L. conceived and designed the study, wrote the article with the help of all authors. Y.L., Z.X, J.S., Y.Z., S.Y., Q.C., C.S., S.G., and Q. L. performed all experiments for this study. Z.X. and Y.Z. carried out WGS and WGBS sequence data analysis.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: June 18, 2022
Revised: July 15, 2022
Accepted: August 18, 2022
Published: September 16, 2022

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### Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit polyclonal anti-UHRF1 | Santa Cruz, USA | Cat. # SC-98817; RRID: AB_2214278 |
| Mouse monoclonal anti-DNMT1 | Abcam, USA | Cat. # ab13537; RRID: AB_300438 |
| Rabbit polyclonal anti-DNMT3A | Abcam, USA | Cat. # ab2850; RRID: AB_303355 |
| Mouse monoclonal anti-DNMT3B | Xu Lab, Chinese Academy of Sciences (Ge et al., 2004) | |
| Rabbit polyclonal anti-ZFP57 | Li Lab, ShanghaiTech University (Li et al., 2008) | |
| **Bacterial and virus strains** |        |            |
| T1 competent cells | Shanghai Weidi Biotechnology Co, Ltd | Cat. # DL10155 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Fetal bovine serum (FBS) | Sigma, USA | Cat. # F2442 |
| Penicillin/Streptomycin solution | Beyotime, China | Cat. # C0222 |
| Trypsin-EDTA solution | Sigma, USA | Cat. # T4049 |
| Leukemia inhibitory factor (LIF) or ESGRO | Millipore, USA | Cat. # ESG1107 |
| T4 DNA ligase | Takara, Japan | Cat. # 2011A |
| T4 polynucleotide kinase (PNK) (10 U/μL) | Takara, Japan | Cat. # 2021S |
| Bpf | Thermo Scientific, USA | Cat. # FD1014 |
| Gelatin | Sinopharm Chemical Reagent (SCR), China | Cat. # 9000-70-8 |
| Bacto-yeast extract | Sangon, China | Cat. # A515245-0500 |
| Bacto-tryptone | Sangon, China | Cat. # A505247-0500 |
| Agar | Macklin, China | Cat. # A800730 |
| Lipofectamine 2000 | Invitrogen, USA | Cat. # 11668019 |
| DMEM | Invitrogen, USA | Cat. # C1199500CP |
| Non-essential amino acid (NEAA) solution | Hyclone, USA | Cat. # SH30238.01 |
| Dithiothreitol (DTT) | Fisher Biotech, USA | Cat. # BP172-25 |
| Dimethyl sulfoxide (DMSO) | Amethyst, China | Cat. # 966629 |
| Carbenicillin | Yeasen, China | Cat. # 60202E508 |
| β-mercaptoethanol | Sigma, USA | Cat. # M3148 |
| Sodium chloride (NaCl) | Sangon, China | Cat. # A501218-0001 |
| PBS (20X) | Sangon, China | Cat. # BS48117-0500 |
| Protease K | Abcone, China | Cat. # P78893 |
| Tris | Beyotime Biotechnology, China | Cat. # S761 |
| SDS | Sigma, USA | Cat. # L4390 |
| Agarose | Abcone, China | Cat. # A88490 |
| 100bp Plus DNA Ladder | Monad, China | Cat. # ME40101M |
| PageRular Prestained Protein Ladder | Fermentas, USA | Cat. # 26617 |
| GeneGreen nucleic acid dye | Tiangen, China | Cat. # RT210 |
| EDTA | Amethyst,China | Cat. # 976151-500G |
| EcoRV | NEB, USA | Cat. # R0195S |
| HindIII | NEB, USA | Cat. # R0104S |
| TaqI | Monad, China | Cat. # MF02801S |
| Clal | Monad, China | Cat. # MF00501M |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| HhaI               | NEB, USA | Cat. #R0139V |
| BstUI              | NEB, USA | Cat. #R0518L |

**Critical commercial assays**

- AxyPrep DNA Gel Extraction Kit: Axygen of Corning Cat. # 35717KE1
- AxyPrep Endo-Free Plasmid Midiprep Kit: Axygen of Corning Cat. # 09318KA2
- EZ DNA Methylation-Gold™ Kit: ZYMO Research, USA Cat. #D5006
- Blood/Cell/Tissue Genomic DNA Extract Kit: TIANGEN, China Cat. # DP304-02

**Deposited data**

- Raw and analyzed WGBS Data and WGS data: This paper GEO: GSE208759

**Experimental models:** Cell lines

- D1911: Li Lab, ShanghaiTech (Lau et al., 2016b)
- Tet TKO: Xu Lab, Chinese Academy of Sciences (Hu et al., 2014)

**Oligonucleotides**

- CACCGAATAGGAATTTGTGACGTCC: Genewiz, Zfp445-sgRNA1-F
- AAACGGACGTCACAAATTCCTATTC: Genewiz, Zfp445-sgRNA1-R
- CACCGAGCTCAGCGCAATCTTTATC: Genewiz, Zfp445-sgRNA2-F
- AAACGATAAAGATTGCGCTGAGCTC: Genewiz, Zfp445-sgRNA2-R
- CACCGTGACATCTTCCGTTGCA: Genewiz, Uhrf1-sgRNA1-F
- AAACGTCCAGAGATATTGTCAC: Genewiz, Uhrf1-sgRNA1-R
- CACCGTGACTATACCGCAACATC: Genewiz, Uhrf1-sgRNA2-F
- AAACGATGTTGCCTATAGCTCAC: Genewiz, Uhrf1-sgRNA2-R
- CACCGCTAAATGAGCACGTCGGTGA: Genewiz, Zfp445-sgRNA1-F
- AAACACTCCGGACGTCATTTAGC: Genewiz, Zfp445-sgRNA1-R
- CACCGATACCTGAGGGGCGGCTTT: Genewiz, Zfp445-sgRNA2-F
- AAACAACGCCCGCCCTAAGATATC: Genewiz, Zfp445-sgRNA2-R
- CACCGATAGGGCTCTTCCGTTGCAT: Genewiz, Dnmt1-sgRNA1-F
- CACCGTAGGTACAGGCTCCTTGCAC: Genewiz, Dnmt1-sgRNA1-R
- CACCCGCAACGGTTGTCCGGCACA: Genewiz, Dnmt1-sgRNA2-F
- AAAACTGGGGGCACACGGTGTTGCC: Genewiz, Dnmt1-sgRNA2-R
- CACCGGTCCAGCCTCGGCTCATAA: Genewiz, Dnmt1-sgRNA3-F
- AACCTAGGACACCGAGGCTGGAAC: Genewiz, Dnmt1-sgRNA3-R
- CACCGCAGTACTTGGGCTGTGG: Genewiz, Dnmt1-sgRNA4-F
- AAACATGTACCGCAAAGCCATCTAC: Genewiz, Dnmt1-sgRNA4-R
- CACCGTGCTTGAAGTGAGCCGTGATC: Genewiz, Dnmt3a-sgRNA1-F
- CACCGGGACGTCAGCTGACCGTGA: Genewiz, Dnmt3a-sgRNA1-R
- CACCGTTCCAGCCCTCGGCTCCT: Genewiz, Dnmt3a-sgRNA2-F
- AAACCTGGGGCATTCAATCTTCT: Genewiz, Dnmt3a-sgRNA2-R
- CACCGTGCTTGGTACACCCGATA: Genewiz, Dnmt3b-sgRNA1-F
- CACCGGCTTCTCTCTTGATGT: Genewiz, Dnmt3b-sgRNA1-R
- CACCGCTTGGTACACCCGATA: Genewiz, Dnmt3b-sgRNA2-F
- AAAGCTGGGGCATTCAATCTTCT: Genewiz, Dnmt3b-sgRNA2-R

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Xiajun Li (lixj1@shanghaitech.edu.cn).

Material availability
All ES cell clones generated in this study are available with a completed Material Transfer Agreement upon request directed to the lead contact.

Data and code availability
- The WGBS data for the mouse ES clones used in this study have been deposited in the Gene Expression Omnibus (GEO). They can be accessed under the GEO number GSE208759. The WGS data for two 129S6/SvEvTac genomic DNA samples can also be accessed under the GEO number GSE208759.
- There is no original code developed in this study.
- Any additional information that is required to analyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

ES cell lines
The 129/DBA hybrid D1911 ES cell line was derived from the blastocyst in a previous study of our lab, which was generated from the mating between a female mouse on the 129S6/SvEvTac genetic background and a male mouse on the DBA2/J genetic background (Lau et al., 2016b). The Tet TKO ES cell line containing...
mutations in Tet1, Tet2 and Tet3 was generated in another study (Hu et al., 2014). These ES cell lines and their derived mutant ES clones were cultured on the irradiated SNL feeder cells with the ES cell growth medium containing 15% of heat inactivated fetal bovine serum (FBS) (Liu et al., 2022). They were frozen in liquid nitrogen for long-term storage in the freezing medium containing 25% FBS and 10% of dimethyl sulfoxide (DMSO). When they were needed again, the frozen ES clones were thawed in the water bath of 37°C and plated onto the SNL feeder cells after DMSO was washed away from the thawed ES cells with 5–10 mL of ES cell growth medium in a 15-mL conical tube (Liu et al., 2022).

METHODS DETAILS

Construction of sgRNA-expressing plasmids for generating deletion mutations by CRISPR-Cas9

Standard molecular cloning procedures were used to make the CRISPR-Cas9 sgRNA plasmid constructs targeting mouse Zfp57, Zfp445, Uhrf1, Dnmt1, Dnmt3a and Dnmt3b genes (Liu et al., 2022). Two complementary oligos for each sgRNA were annealed in vitro and cloned into pX330 with restriction enzyme digestion and ligation (Table S1). The resultant plasmids were sequenced to confirm the inserted DNA fragment encoding the sgRNA targeting the gene of the interest.

ES cell culture

Standard ES cell culture protocol was used for this study (Liu et al., 2022). The ES cell growth medium contained DMEM with glutamine and high glucose and 15% of heat inactivated fetal bovine serum (FBS), plus non-essential amino acids and β-mercaptoethanol. The cell culture plates were seeded with irradiated mouse embryonic fibroblast (MEF) or SNL feeder cells first before the resuspended ES cells were plated on top of the feeder cells. The medium was changed daily when there were enough ES cells growing to prevent spontaneous differentiation of ES cells. Then the ES cell culture was harvested by trypsin digestion and split again before it was too confluent. They were frozen in the freezing medium containing 25% FBS and 10% of DMSO for long-term storage until they were needed again.

Obtaining deletion mutant ES clones by CRISPR-Cas9

An CRISPR-based approach developed in our lab was used to generate deletion mutant ES clones (Liu et al., 2022). First, D1911 ES cells were plated on the feeder cells made of the MEF cells derived from the mice expressing the puromycin-resistant gene. Then two pX330 constructs containing the inserted DNA fragment encoding the sgRNAs targeting the gene of interest were mixed in order to generate a deletion mutation for all mutant ES clones except for generation of the Zfp57 mutations in the Tet TKO ES cells (Table S2) (see below). This plasmid DNA mix was co-transfected with the plasmid expressing puromycin-resistant gene product into D1911 ES cells by Lipofectamine 2000. The transfected ES cells were subjected to the same Lipofectamine-mediated transfection experiment with the plasmid containing puromycin-resistant gene and the empty pX330 plasmid without expressing any sgRNA, which was also followed by 1 μg/mL of puromycin selection for 2 days. The mutant ES clones of Zfp57−/− and Zfp445−/− were generated by CRISPR-Cas9 through Lipofectamine-mediated transfection of D1911 ES cells with two sgRNA expression plasmids targeting Zfp57 or Zfp445, respectively (Table S2). The Zfp57−/−; Zfp445−/− mutant ES clones were obtained by co-transfection of four sgRNA expression plasmids into D1911 ES cells, with two of them targeting Zfp57 or Zfp445, respectively (Table S2). The Tet TKO ES clones carrying deletion mutations in the Zfp57 or Zfp445 or both genes were generated similarly when the Tet TKO ES cells were used for PCR-based screening and sequencing to identify the candidate ES clones containing deletion at the target gene. The PCR product spanning the deletion of the target gene was cloned into the pBluescript vector by restriction digestion and ligation, and the ligation mixture was transformed into competent bacterial cells afterwards. The resultant bacterial colonies were subjected to sequencing to identify the exact deleted region on the target gene. The mutant ES clones containing the deletion mutation that likely resulted in a null mutation of the target gene were cultured again to obtain the protein lysate samples for western blot and genomic DNA samples for DNA methylation analysis, respectively.

The control WT ES clones were similarly picked from the growing ES cell culture after D1911 ES cells were subjected to the same Lipofectamine-mediated transfection experiment with the plasmid containing puromycin-resistant gene and the empty pX330 plasmid without expressing any sgRNA, which was also followed by 1 μg/mL of puromycin selection for 2 days. The mutant ES clones of Zfp57−/− and Zfp445−/− were generated by CRISPR-Cas9 through Lipofectamine-mediated transfection of D1911 ES cells with two sgRNA expression plasmids targeting Zfp57 or Zfp445, respectively (Table S2). The Zfp57−/−; Zfp445−/− mutant ES clones were obtained by co-transfection of four sgRNA expression plasmids into D1911 ES cells, with two of them targeting Zfp57 or Zfp445, respectively (Table S2). The Tet TKO ES clones carrying deletion mutations in the Zfp57 or Zfp445 or both genes were generated similarly when the Tet TKO ES cells...
were used for CRISPR-Cas9 with two sgRNA expression plasmids for each target gene through Lipofectamine-mediated transfection, with the exception that only one sgRNA plasmid was used for generation of Zfp57 mutations in the Tet TKO ES cells (Table S2).

The Dnmt1 KO, Uhrf1 KO and some other mutant ES clones described below were obtained from the parental D1911 ES cells by CRISPR through Lipofectamine-mediated transfection of two sgRNA plasmids each targeting Dnmt1 or Uhrf1 or another gene of interest (Tables S3 and S4). An intermediate Dnmt3b KO mutant ES clone obtained from the D1911 ES cells by CRISPR was used for Lipofectamine-mediated transfection to target Dnmt3a in order to obtain the Dnmt3 DKO mutant ES clones carrying deletion mutations in both Dnmt3a and Dnmt3b (Table S3). To obtain the Dnmt TKO mutant ES clones, an intermediate mutant ES clone with deletion mutations in the Dnmt1 and Dnmt3a genes was first obtained from co-transfections of the sgRNA plasmids into the D1911 ES cells by CRISPR (Table S3). Then this intermediate mutant ES clone was subject to Lipofectamine-mediated transfection of two sgRNA expression plasmids targeting Dnmt3b in order to obtain the Dnmt TKO mutant ES clones containing deletion mutations in Dnmt1, Dnmt3a and Dnmt3b.

**Western blot analysis**

Total whole cell lysate samples were obtained from the ES cell culture on a well of 6-well plate. The standard protocol was used for western blot. The blot containing the mutant ES clones for the target gene was subject to immunoblot with the antibodies against the protein product of the target gene. Then the same blot was probed with the antibodies against GAPDH for sample loading assessment.

**Immunofluorescence of ES cells**

The ES cell culture on 24-well plate was fixed with 4% of paraformaldehyde (PFA) solution for 10 min at 20°C–25°C. Then it was incubated with the antibodies against OCT4 (Santa Cruz Biotechnology, Cat#sc-5279) or NANOG (Santa Cruz Biotechnology, Cat#sc-376915). The fluorescence images were taken under an inverted microscope.

**Genomic DNA sample preparation**

Genomic DNA samples were harvested from the ES cells after they had been cultured on gelatin-coated plates for one generation to remove almost all feeder cells in ES cell culture. For the genomic DNA samples at Passage 4 (P4) of the control WT, Dnmt1 KO and Uhrf1 KO ES clones, they had been cultured on the feeder cells for 4 passages since the parental D1911 ES cells were used for Lipofectamine-mediated transfection for isolation of the mutant and control WT ES clones. For the genomic DNA samples at Passage 4 (P4) of Dnmt3 DKO and Dnmt TKO mutant ES clones, they had been cultured on the feeder cells for 4 passages since the last Lipofectamine-mediated transfection for CRISPR-Cas9 in order to obtain the Dnmt3 DKO and Dnmt TKO mutant ES clones from the intermediate Dnmt3b−/− mutant and Dnmt1−/−; Dnmt3a−/− mutant ES clones, respectively. The mutant ES clones were used for genomic DNA preparation at P4 soon after they were confirmed to carry deletion mutations at the target genes. Genomic DNA samples at P10 and P20 were obtained from the ES clones after they had been cultured on the feeder cells for a total of 10 and 20 passages, respectively, since the last Lipofectamine transfection of the D1911 ES cells or the intermediate mutant ES cells.

**COBRA analysis of the ICRs in ES cells**

For COBRA analysis, 1 μg of purified genomic DNA was first subjected to bisulfite treatment with the EZ DNA methylation-Gold™ Kit (Zymo Research #D5006). A fraction of purified bisulfite mutagenized DNA sample was then used for PCR amplification of the ICRs. Usually two rounds of nested PCR reactions were carried out to obtain enough PCR product for restriction enzyme digestion and gel analysis. Then the relative amount of the originally methylated and unmethylated DNA at every tested ICR was estimated based on the band intensities of the digested product on the gels.

**Whole-genome bisulfite sequencing (WGBS) of ES samples**

Genomic DNA samples for Dnmt mutant ES cells and control WT ES cells were harvested from the ES cells devoid of feeder cells after being cultured on gelatin-coated plates for one generation. The purified genomic DNA samples were subjected to whole-genome bisulfite sequencing (WGBS) (Table S5). Low quality sequence reads were removed by Fastp v0.20.0 (https://github.com/OpenGene/fastp). Trim Galore
(v0.4.1, http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) was applied to trim the adaptor sequences from the raw reads so that the clean reads can be obtained for further analysis. Mapping was carried out for the clean reads by Bismark (v0.13.1; bowtie v2.2.9) (Krueger and Andrews, 2011). Duplicate aligned sequence reads were removed with duplicate_bismark, And Bismark methylation extractor was used to extract the DNA methylation data from the aligned sequence reads after filtering out the duplicate aligned reads. Then DNA methylation of the CpG sites was examined and quantified for all known ICRs as well as somatic DMRs (Table S6). Similar DNA methylation analyses were performed for the repeats and other genomic regions based on the sequence information available on the UCSC website. Please refer to Table S5 for the information regarding the sequenced reads, mapped reads and bisulfite conversion rates of these samples.

**Generation of the SNP data for the 129/DBA hybrid ES cells**

First, we performed whole-genome sequencing (WGS) for two 129S6/SvEvTac genomic DNA samples. Then the SNPs present in the parental D1911 hybrid ES clone were identified by comparing the 129S6/SvEvTac genomic DNA sequence with that of the DBA2/J genomic DNA sequence in the genome database. Specifically, two genomic DNA samples obtained from the tails of two 129 male mice were subjected to whole genome sequencing (WGS) analysis. The PCR duplicates were removed from the next-generation sequencing (NGS) data before the NGS data were mapped to the genome of DBA2/J mice stored in the UCSC databases using the Burrows-Wheeler Aligner (BWA) tool (v0.7.15-r1140) (Li and Durbin, 2009). Then the Genome Analysis Toolkit (GATK) software package was applied to generate the SNP table with default parameters (McKenna et al., 2010; Van der Auwera et al., 2013).

**Assignment of allelic DNA methylation reads**

After the SNP data were obtained with BWA and GATK for the parental 129/DBA hybrid ES cell line D1911 mentioned above, all WGBS reads of each ES sample were mapped to the N-masked mouse reference genome that was generated with BEDTools (Quinlan and Hall, 2010). The adaptor sequences were removed using Trim Galore (v0.4.1) as above. The obtained clean reads were further mapped by Bismark (v0.13.1) (Krueger and Andrews, 2011). Then the SNPsplit (v0.3.4) software was used to separate the allelic DNA methylation reads with default parameters (Krueger and Andrews, 2016). The PCR duplicates were removed and DNA methylation at each CpG site was extracted with Bismark (v0.13.1). For each CpG site, only those reads with the SNPs covered more than once were used for further allelic methylation analysis.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

In WGBS analysis, the percentage of DNA methylation was quantified for each CpG site of an ICR or DMR first with the number of the sequence reads containing methylated C divided by the total number of the sequence reads of this CpG site. Then the average methylation level was calculated for all CpG sites of this ICR or DMR. DNA methylation levels at the repeats, genic regions and whole genome were calculated similarly after the percentage of each CpG site methylation was quantified first and then average methylation level was obtained for all CpG sites afterwards.

Two independent ES clones were obtained for the the Dnmt1 KO, Uhrf1 KO, Dnmt3 DKO, Dnmt TKO and the wild-type ES cells. Then their genomic DNA samples were subjected to WGBS analysis thereafter. Statistical analysis was carried out with the R Statistical Software (R version 4.2.1). DNA methylation level at an ICR was compared among the Dnmt1 KO, Uhrf1 KO, Dnmt3 DKO, Dnmt TKO and the wild-type ES clones by using one-way or two-way ANOVA with Dunnett multiple comparison test. All statistical analyses are also described in detail in the figure legends as well as in the methods details section. The values in the graphs of the figures were presented as mean ± SEM with the following statistical significance: *p < 0.05; **p < 0.01; ***p < 0.001.