Histone chaperone CAF-1 mediates repressive histone modifications to protect preimplantation mouse embryos from endogenous retrotransposons

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Edited by Mark Groudine, Fred Hutchinson Cancer Research Center, Seattle, WA, and approved October 13, 2015 (received for review June 30, 2015)

Substantial proportions of mammalian genomes comprise repetitive elements including endogenous retrotransposons. Although these play diverse roles during development, their appropriate silencing is critically important in maintaining genomic integrity in the host cells. The major mechanism for retrotransposon silencing is DNA methylation, but the wave of global DNA demethylation that occurs after fertilization renders preimplantation embryos exceptionally hypomethylated. Here, we show that hypomethylated preimplantation mouse embryos are protected from retrotransposons by repressive histone modifications mediated by the histone chaperone chromatin assembly factor 1 (CAF-1). We found that knockdown of CAF-1 with specific siRNA injections resulted in significant up-regulation of the retrotransposons long interspersed nuclear element 1, short interspersed nuclear element B2, and intracisternal A particle at the morula stage. Concomitantly, increased histone H2AX phosphorylation and developmental arrest of the majority (>95%) of embryos were observed. The latter was caused at least in part by derepression of retrotransposons, as treatment with reverse transcriptase inhibitors rescued some embryos. Importantly, ChIP analysis revealed that CAF-1 mediated the replacement of H3.3 with H3.1/3.2 at the retrotransposon regions. This replacement was associated with deposition of repressive histone marks, including trimethylation of histone H3 on lysine 9 (H3K9me3), H3K9me2, H3K27me3, and H4K20me3. Among them, H4K20me3 and H3K9me3 seemed to play predominant roles in retrotransposon silencing, as assessed by knockdown of specific histone methyltransferases and forced expression of unmethylatable mutants of H3.1K9 and H4K20. Our data thus indicate that CAF-1 is an essential guardian of the genome in preimplantation mouse embryos by deposition of repressive histone modifications via histone variant replacement.

CAF-1 | histone variant | retrotransposon | mouse | embryo

Histone chaperones are the key regulators of nucleosome assembly and play diverse roles in the maintenance of genome stability and epigenetic information. A subset of defined histone chaperones is known to deposit specific histones into the nucleosomes and promote their maturation. Those for histone H3–H4 dimers are especially important for many developmental processes, because H3 variants and the histone tail modifications of H3 and H4 are intimately associated with chromatin dynamics and serve as transcription regulators during development (1). For example, HIRA, one of the most studied histone chaperons that deposit H3.3–H4, is known to play indispensable roles during fertilization and early embryonic development (2, 3). H3.3 is enriched in nucleosomes at the transcription start sites of genes, enhancers, and gene bodies of actively transcribed genes (4). However, we know very little about the roles of another histone H3 variant, H3.1, and its chaperone chromatin assembly factor 1 (CAF-1) during development. CAF-1 promotes the specific deposition of H3.1–H4 dimers onto newly synthesized DNA (1). Histone H3.1 is a mammal-specific histone variant diverged from H3.2, a canonical H3 variant found throughout animals, and is enriched in lysine dimethylation, a modification associated with gene silencing (5, 6). Therefore, it has been assumed that H3.1–H4 and its chaperon CAF-1 would be responsible for maintaining the silenced status of the specific genomic regions during development.

During early mouse development, H3.1 becomes first detectable in blastomere nuclei at the morula stage and is involved in establishment of the heterochromatin areas at the periphery of nuclei (7). This process is thought to be dependent on CAF-1 because CAF-1–depleted embryos lost H3.1 and increased the incorporation of H3.3 (8). Intriguingly, CAF-1–deficient embryos failed to reach the blastocyst stage (8, 9), which might have been caused by the ectopic expression of some genes in the absence of H3.1-induced heterochromatin status. In this study, we first sought to identify the underlying mechanism for this developmental failure, and found that CAF-1 depletion led to

Significance

Retrotransposons constitute substantial proportions of mammalian genomes and can be harmful when activated ectopically. DNA methylation is the major mechanism for retrotransposon silencing, but we do not know how late preimplantation embryos, which are exceptionally hypomethylated, are protected from retrotransposons. Knockdown of the histone chaperone chromatin assembly factor 1 (CAF-1) resulted in significant up-regulation of retrotransposons (e.g., long interspersed element 1) and mouse embryonic death at morula stage. CAF-1 was responsible for deposition of histone variant H3.1/3.2 and repressive histone marks, including trimethylation of histone H4 on lysine 20 (H4K20me3) and H3K9me3, at retrotransposon regions. Depletion of H4K20me3 or H3K9me3 by knockdown of specific histone methyltransferases resulted in up-regulation of retrotransposons in morulae. Thus, hypomethylated preimplantation mouse embryos are protected by repressive histone modifications mediated by CAF-1.

Author contributions: Y.H. and A.O. designed research; Y.H., K.I., M.O., S.K., N.O., and A.O. performed research; Y.H., K.I., M.O., E.N.K., Y.O., Y.-i.T., and A.O. analyzed data; and Y.H. and A.O. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE69260).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1512775112/-/DCSupplemental.

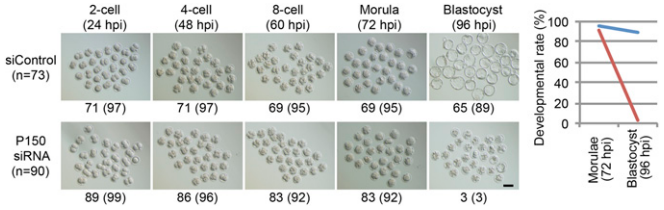
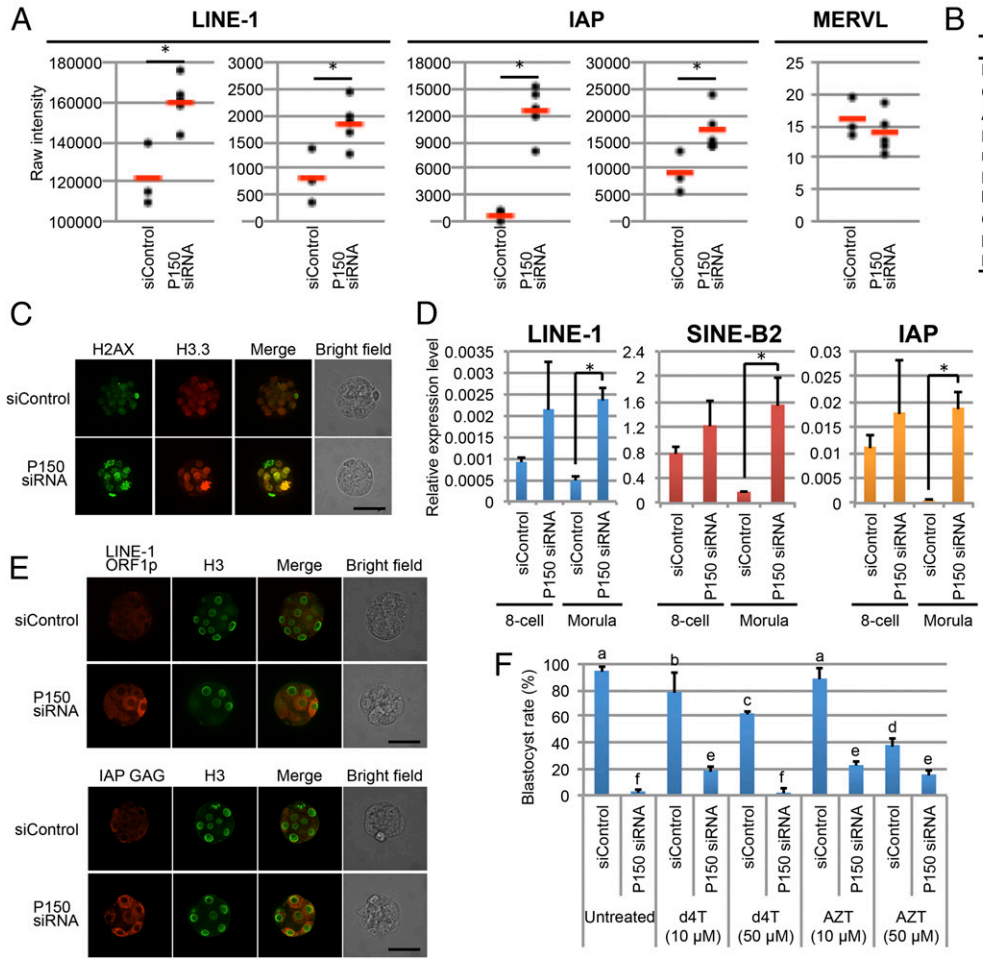


Fig. 1. P150 knockdown results in embryonic lethality at the morula stage. The effect of P150 knockdown on the development of preimplantation embryos. (*Left*) Representative photos of siControl- and P150-knockdown embryos through the preimplantation stage. The numbers at the bottom indicate the numbers (percentages) of surviving embryos. (Scale bar: 100 μm.) (*Right*) Blastocyst rate of siControl- (blue line) and P150-knockdown (red line) embryos. Results are from three replicate experiments.

derepression of multiple classes of retrotransposons in morula embryos. This finding indicates that CAF-1–dependent repressive histone modifications are responsible for retrotransposon silencing in late preimplantation mouse embryos, which carry hypomethylated DNA.

Results

CAF-1 Is Responsible for the Repression of Endogenous Retrotransposons in Preimplantation Mouse Embryos. First, we injected siRNA targeting *P150* (*Caf1a*), the gene encoding the large subunit of CAF-1, into zygotes and examined whether their development was compromised. Although most embryos injected with *P150*-siRNA developed to the morula stage, only a few (4%) developed into blastocysts (Fig. 1). This was in marked contrast to embryos injected with control siRNA, 94% of which reached the blastocyst stage. This finding implied that CAF-1 is critically important for embryonic development during the morula-to-blastocyst transition. To determine the mechanisms underlying this arrest of embryos at the morula stage, we analyzed the gene expression profiles of P150-knockdown morulae by microarray. Of the 50,682 genes expressed significantly, 3,308 were identified as differentially expressed genes (DEGs) between P150-knockdown and control embryos. Among



Upregulated

Term	Count	%	P-Value
Programmed cell death	26	3.10	7.83E-06
Cell death	26	3.10	9.00E-06
Apoptosis	25	2.98	2.04E-05
Macromolecule metabolic process	46	5.48	2.68E-05
Macromolecule biosynthetic process	39	4.64	9.61E-05
Cellular biosynthetic process	40	4.76	1.06E-04
Biosynthetic process	40	4.76	1.29E-04

Downregulated

Term	Count	%	P-Value
cell division	49	3.98	2.77E-11
cell cycle	73	5.93	1.69E-08
organelle fission	32	2.60	6.14E-07
cell cycle phase	43	3.49	2.14E-06
mitosis	30	2.44	2.65E-06
nuclear division	30	2.44	2.65E-06
cofactor metabolic process	29	2.36	3.36E-06

Fig. 2. P150 knockdown resulted in derepression of retrotransposons. (*A*) Expression levels of LINE-1, IAP, and MERVL regions in morula embryos analyzed by microarray analysis. Each dot represents a single embryo. All probes for LINE-1 and IAP showed significant up-regulation of retrotransposons (*$P < 0.05$). MERVL (also known as MuERV-L), a retrotransposon transiently expressed at the two-cell stage (10), was not affected by P150 knockdown. Red bars indicate the mean value. (*B*) GO analysis of up-regulated (*Left*) and down-regulated (*Right*) genes in P150-knockdown embryos. The data indicate that P150 knockdown leads to cell death and cell cycle arrest. (*C*) Up-regulated histone H2AX phosphorylation, a hallmark of DNA damage, in the nuclei of P150-knockdown morulae. (*D*) qPCR analysis of different classes of retrotransposons (LINE-1, SINE-B2, and IAP) in eight-cell and morula embryos. The level of β-actin was set as 1.0. Results are from three replicate experiments. Each experiment was performed with 10–15 zygotes (*$P < 0.05$, significant difference). Bars show SEM. (*E*) Detection of LINE-1 ORF1p and IAP GAG proteins in P150-knockdown morulae. (Scale bar: 50 μm.) (*F*) Rescue of P150-knockdown embryos by treatment with reverse transcriptase inhibitors. Different characters indicate significant differences ($P < 0.05$). Bars show SEM ($n = 3$). (*G*) The blastocyst development rate of P150-knockdown embryos under treatment with reverse transcriptase inhibitors, with the rate of corresponding control siRNA embryos set at 100%. Different characters indicate significant differences at $P < 0.05$.

them, we noted that all four probes for the endogenous retrotransposons, long interspersed nuclear element (LINE)-1 and intracisternal A particle (IAP), covered by the Agilent Mouse GE 8 × 60K microarray were up-regulated in P150-knockdown embryos (Fig. 2*A*). Gene Ontology (GO) analysis of DEGs revealed that "programmed cell death" and "cell death" were up-regulated whereas "cell division" and "cell cycle" were down-regulated, indicating that P150 knockdown leads to cell death and cell-cycle arrest (Fig. 2*B*). Consistent with this, P150-knockdown morulae showed frequent histone H2AX phosphorylation, a hallmark of DNA damage, in the nuclei (Fig. 2*C*). As it is known that hyperactivation of retrotransposons causes cell-cycle arrest and apoptosis of the host cells (11), we assumed that this ectopic up-regulation of retrotransposons caused by P150 knockdown might be one of the causes of the developmental arrest of embryos at the morula stage. We then analyzed the expression levels of several retrotransposon classes in preimplantation embryos with or without P150 knockdown by quantitative RT-PCR (qPCR). It is known that the majority of mammalian retrotransposons are LINEs or short interspersed nuclear elements (SINEs) whereas approximately one tenth of them are LTR elements, such as IAPs (Mouse Genome Sequencing Consortium, 2002; www.genome.gov/10001859). Therefore, we analyzed LINE-1, SINE-B2, and IAP motifs as representatives of each retrotransposon class. Interestingly, down-regulation of P150 resulted in significant elevations of all retrotransposons analyzed at the morula stage, indicating that these retrotransposons were repressed via CAF-1 (Fig. 2*D*). We further confirmed translation of retrotransposon mRNAs by localization of LINE-1 open reading frame-1 (ORF1p) and IAP group-specific antigen (GAG) proteins in P150-siRNA embryos (Fig. 2*E*).

Inhibition of Retrotransposon Activity Partially Rescues P150-Knockdown Embryos. To test whether the derepression of retrotransposons was responsible for the arrest of embryos at the morula stage, we treated P150-knockdown embryos with the reverse transcriptase inhibitors 3′-azido-3′-deoythymidine (AZT) or 2′, 3′-didehydro-3′-deoxythymidine (d4T). The blastocyst formation rate per morula was increased significantly, to 23% and 19%, by treatment with 10 μM of AZT and d4T, respectively, compared with nontreated control embryos (4%; $P < 0.0005$, Fisher exact test; Fig. 2*F* and Fig. S1). This indicated that up-regulation of retrotransposons was one of the causes of developmental arrest in P150–down-regulated embryos. In these experiments, restoration of the embryo viability was moderate, probably because AZT and d4T are strongly toxic to embryos (12, 13) and should be used at limited concentrations and times. However, it was clear that AZT and d4T had positive effects on the development of P150-knockdown embryos when control siRNA embryos were used as controls (Fig. 2*G*).

CAF-1 is Essential for Histone H3.1/3.2 Deposition on Retrotransposons in Preimplantation Embryos. Next, we examined the changes in histone variants in embryos at the morula-to-blastocyst transition. For immunocytochemistry, we used two antibodies that recognized H3.1/3.2 and H3 variants. The variants H3.1 and H3.2 were indistinguishable because they have only a single amino acid difference (6). Immunocytochemistry revealed that strong H3.3 staining in nuclei at the morula stage [embryonic day (E) 3.5] diminished rapidly at the blastocyst stage on E4.5, whereas H3.1/3.2 was stably localized in nuclei throughout this period (Fig. 3*A*). Notably, the embryos treated with *P150* siRNA showed more intense H3.3 staining than control siRNA-treated embryos at the blastocyst stage (Fig. 3*A*). These findings indicate that H3.3 was replaced with H3.1/3.2 by CAF-1 at the morula-to-blastocyst transition. We then examined whether this conversion mediated by CAF-1 occurred at the retrotransposon regions in morula embryos by ChIP analysis. Following P150 knockdown, significant decreases in the H3.1/3.2 levels were

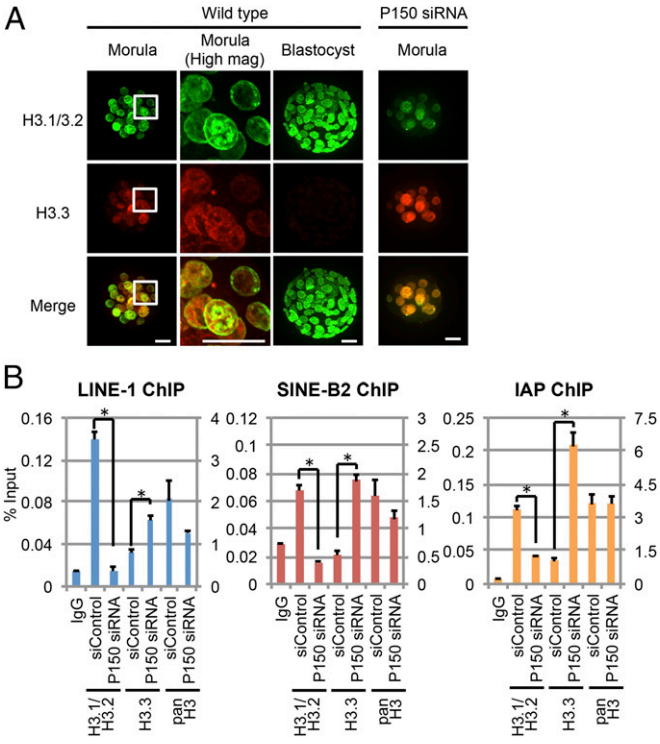


Fig. 3. P150 knockdown impeded incorporation of H3.1 into the nucleus and retrotransposon regions. (*A*) Immunocytochemical analysis of histone H3.1/3.2 (green) and H3.3 (red) in morulae and blastocysts. The intensity of H3.3 immunostaining was diminished in the blastocysts. The P150-knockdown morula showed a higher intensity of H3.3 immunostaining than of H3.1/3.2. (Scale bar: 25 μm.) (*B*) ChIP analysis of H3.1/3.2, H3.3, and pan-H3 on retrotransposons in siControl- and P150-knockdown embryos at the morula stage. The values for pan-H3 are shown by the right *y* axis. Changes in the values for pan-H3 produced by P150 siRNA treatment were used for normalization of the values of H3.1.3.2 and H3.3. Normal rabbit IgG was used as a negative control. Different characters indicate statistical significance ($P < 0.05$). Bars show SEM ($n = 3$).

observed for all retrotransposon regions examined (LINE-1, SINE-B2, and IAP), whereas the H3.3 levels increased significantly at these regions (Fig. 3*B*). These findings suggested that CAF-1 was responsible for the replacement of H3.3 with H3.1/3.2 in the retrotransposon regions at the morula stage.

CAF-1 Mediates the Deposition of Multiple Repressive Histone Modifications Onto Retrotransposons. It is known that H3.1 is enriched with repressive methylation histone marks at the lysine residues, such as H3K9 (14). Therefore, we next analyzed what types of histone modifications contributed to the repression of retrotransposons in morulae. As shown in Fig. 4*A*, our ChIP experiments using specific antibodies indicated significant enrichments of all four repressive histone marks we analyzed (H3K9me2, H3K9me3, H3K27me3, and H4K20me3) at the retrotransposon regions by CAF-1 (Fig. 4*A*). It has been reported that IAPs are relatively resistant to demethylation during preimplantation development, in contrast to LINE-1 regions that undergo substantial demethylation (62% vs. 15–27% CpG methylation at the blastocyst stage, respectively) (15). This IAP hypermethylation might be contradictory to its overexpression upon P150 knockdown, so we examined whether this would affect its methylation status. Interestingly, IAPs showed remarkable demethylation following P150 knockdown (58.9% demethylation vs. 13.3% in controls) whereas LINE-1 regions remained hypomethylated irrespective of siRNA treatment (9.2% vs. 6.7%; Fig. 4*B*). Thus, it is possible that removal of the repressive histone modifications

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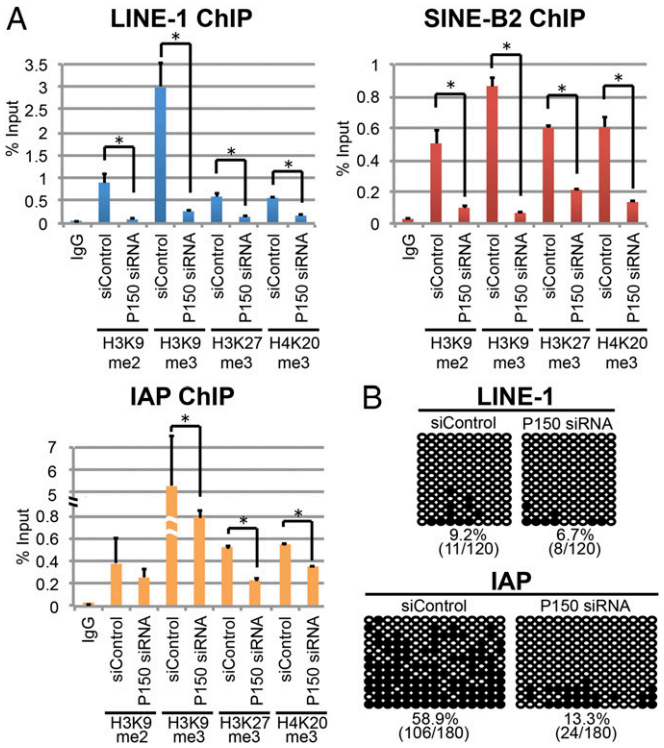

Fig. 4. CAF-1 is responsible for the deposition of repressive histone marks on retrotransposons. (*A*) ChIP analysis of retrotransposons using antibodies for H3K9me2, H3K9me3, H3K27me3, and H4K20me3 in siControl- and P150-knockdown morulae. Normal rabbit IgG was used as a negative control. As the H3K9me3 level was still high after P150 siRNA treatment in IAP regions, we confirmed the specificity of the antibody by using ES cells (Fig. S2). Bars show SEM ($n = 3$; $^*P < 0.05$). (*B*) Bisulfite sequencing analysis of LINE-1 and IAP regions in P150-knockdown embryos at the morula stage.

and DNA demethylation concomitantly occurred at IAP regions, leading to its derepression in P150-knockdown embryos.

As mentioned earlier, CAF-1 is responsible for the deposition of four types of repressive histone marks. Therefore, we next sought to identify which histone mark played the predominant role in retrotransposon silencing. For this purpose, we reduced these histone marks by knockdown of the responsible lysine methyltransferases (or their associated proteins) and then checked for any derepression of the retrotransposons. When single histone marks were depleted with specific siRNAs (Fig. S3 illustrates the specificity of each siRNA), the highest expression levels of LINE-1, SINE-B2, and IAP regions were observed by down-regulation of H4K20me3 (methyltransferase Suv420h1/2; Fig. 5*A*). By contrast, reduction of H3K9me2 (G9a), H3K9me3 (by ESET or Suv39h1/2), or H3K27me3 (by Suz12, Eed, and Ezh2) did not increase the retrotransposon expression levels except for LINE-1 regions, which were also derepressed by down-regulation of H3K9me3 by *Suv39h1/2* siRNA (Fig. 5*A*). We next examined the combinational effects of siRNAs, but no synergistic effect was observed (Fig. 5*A*). Additionally, we examined the effect of ESET knockdown by siRNA injection in immature germinal vesicle (GV)-stage oocytes because it was possible that the maternal ESET protein might persist to the morula or blastocyst stages (16), which could not be reduced by siRNA injection into zygotes. By using this knockdown method, all retrotransposons were up-regulated, with significant increases for LINE-1 and IAP regions (Fig. 5*B*), whereas a slight elevation of SINE-B2 might indicate inefficient knockdown and/or the presence of residual maternal ESET. Perhaps the effects of Suv420h1/2 knockdown might have also been improved by siRNA injection into oocytes. Thus, it is likely that H4K20me3 and H3K9me3 are the histone marks that play predominant roles in retrotransposon silencing at the morula-to-blastocyst transition. We confirmed that P150 knockdown did not affect the total mRNA levels of lysine methyltransferases or the total amounts of histone modifications (Fig. S4). Histone methyltransferases might have functions other than histone methylation: for example, G9a can bring about de novo DNA methylation through its ankyrin domain (17). Therefore, we examined the essential roles of H3K9me3 and H4K20me3 for retrotransposon silencing by forced expressions of unmethylatable histone mutants. As expected, forced expressions of H3.1K9R (lysine 9 was replaced

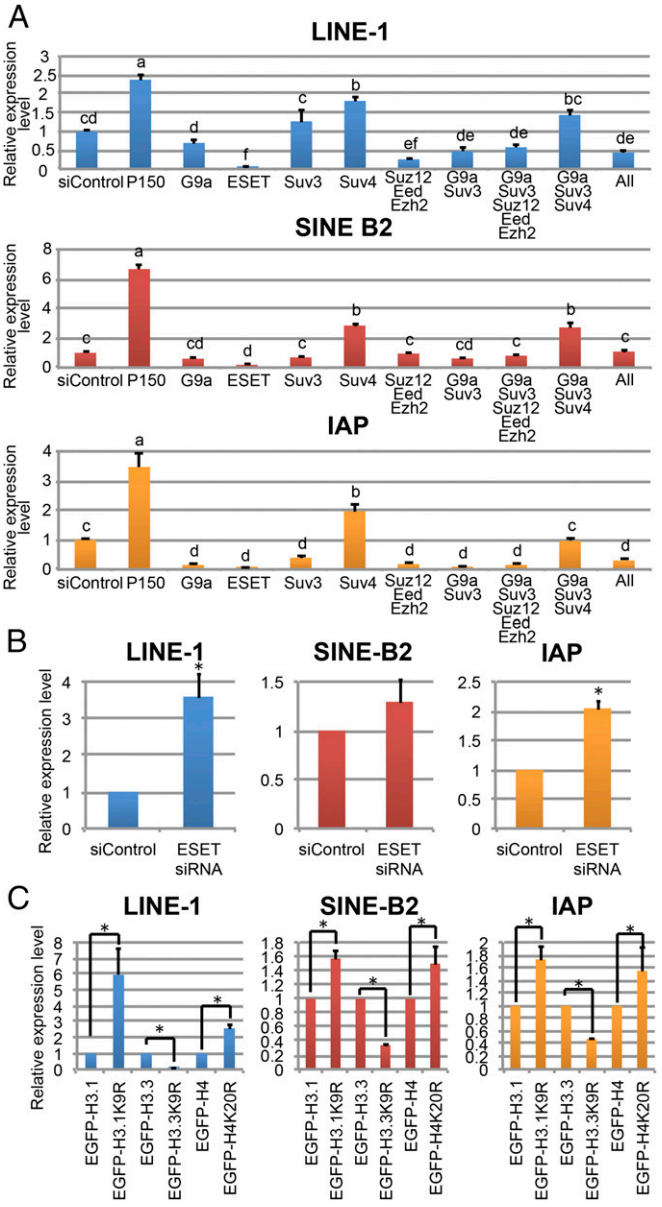

Fig. 5. The effects of knockdown of histone methyltransferases or dominant-negative mutations of H3.1K9, H3.3K9, and H4K20 on retrotransposon expressions in morulae. (*A* and *B*) Knockdown of histone methyltransferases. siRNA was injected into zygotes (*A*) or immature oocytes (*B*). The level of siControl was set as 1.0. Each experiment was performed with 10–15 embryos. Suv3, Suv39h1/2; Suv4, Suv420h1/2. Different characters or asterisk numbers indicate significant differences at $P < 0.05$. (*C*) Mutations of H3.1K9, H3.3K9, and H4K20 ($^*P < 0.05$). Bars show SEM ($n = 3$).

with arginine 9) and H4K20R up-regulated retrotransposons in morulae whereas forced expression of H3.3K9R did not (Fig. 5*C* and Fig. S5 *A–D*). Additional ChIP analysis revealed that intact H3.1 and mutant H3.1K9R were localized equally to the retrotransposon regions, indicating that repressive histones were not always methylated before nucleosomal assembly at these target regions (Fig. S5*E*).

Notably, the derepression of these retrotransposons was consistent with the developmental rates of embryos treated with the same single siRNAs or their combinations (Fig. 6). The rate of development to blastocysts was significantly decreased, from ~90% to <60%, when the siRNA treatment contained Suv420h1/2-siRNAs (Fig. 6 and Fig. S6). These data further supported our notion that derepression of retrotransposons caused death of morulae. We could not obtain comparative data for ESET knockdown by GV-stage siRNA injection because the resultant embryos frequently showed developmental arrest at the four-cell to eight-cell stage as a result of the irreversible damage to GV-stage oocytes caused by injection (as confirmed by control siRNA injection).

Discussion

Retrotransposons are widely distributed in the mammalian genome and may be repressed primarily by DNA methylation. However, during the mammalian life cycle, two dynamic waves of DNA demethylation occur in a genome-wide manner. The first wave occurs during the development of primordial germ cells (PGCs) and reaches a most-hypomethylated state at approximately E12.5–13.5 in mice. During this period, LINE-1, IAP, and other endogenous retrovirus family retrotransposon regions are associated with abundant accumulation of the repressive histone mark H3K9me3 (18).

After fertilization, the second wave of DNA demethylation occurs by active and passive mechanisms (19, 20). It has been reported that retrotransposon silencing is achieved by loss of activating marks (H3K4me3) until the eight-cell stage rather than acquisition of conventional heterochromatic marks (21). Here, we identified an active silencing mechanism for retrotransposons in late preimplantation embryos, which have globally hypomethylated DNA. As shown in Fig. 2*D* [Control siRNA (siControl)], retrotransposons were more strongly repressed in morulae than in eight-cell embryos. Therefore, it is reasonable to suppose that the histone status at the eight-cell stage is repressive to some extent, but that it is further enriched with repressive marks by CAF-1 at the morula stage for more effective retrotransposon silencing. The retrotransposon regions at this stage were enriched with multiple types of repressive histone marks, including H3K9me3, H3K9me2, H3K27me3, and H4K20me3. However, their contributions to retrotransposon silencing seemed to be different, as H3K9me3 and H4K20me3 were most influential in the expression levels of all retrotransposons examined. This result was unexpected because, in PGCs and ES cells, H3K9me3 is reported to be the major repressive histone mark that silences retrotransposons whereas H4K20me3 plays a very minor role, if any (18, 22). It was reported that depletion of H4K20me3 in ES cells resulted in increased frequencies of telomere recombination associated with the loss of heterochromatic features (23). This indicates that H4K20me3 depletion can destabilize heterochromatin in some circumstances. According to previous studies, the retrotransposon silencing mechanisms in PGCs and ES cells are complicated. In PGCs, deletion of H3K9me3 by KO of the H3K9 methyltransferase ESET (also called Setdb1) resulted in widespread reactivation of IAP, but not LINE-1 (18). In ES cells, silencing of IAP was dependent on ESET, but silencing of LINE-1 was dependent on the other methyltransferase Suv39h1/2 (22, 24). Intriguingly, CAF-1 knockdown in ES cells resulted in efficient induction of the two-cell–like state and up-regulation of MERVL, but not non-LTR retrotransposons (25, 26). A genome-wide siRNA analysis revealed that CAF-1 is the major or the sole repressor of MERVL (26). Thus, it is likely that CAF-1 differentially represses retrotransposons in ES cells and preimplantation embryos. Our findings indicated that the retrotransposons in late preimplantation embryos were silenced by the mechanisms that were shared among different retrotransposon classes, because down-regulation of ESET or Suv420h1/2 consistently caused derepression of all retrotransposons analyzed, namely LINE-1, SINE-B2, and IAP.

The DNA hypomethylation status in the embryonic genome before implantation is replaced by hypermethylation via de novo DNA methylation after implantation. Therefore, it is reasonable to suppose that, to repress endogenous retrotransposons during the periimplantation stages, the mammalian embryonic genome acquires the repressive histone marks first and then de novo DNA methylation secondarily. In this regard, it is probable that methylated H3K9 marks (H3K9me2 and H3K9me3) at the retrotransposon regions might have another important role in that they could serve as epigenetic marks for future DNA methylation because HP1 proteins bind specifically to methylated H3K9 to recruit de novo DNA methyltransferases (27, 28). After implantation, the DNA methylation levels in many retrotransposons increase to the maximal levels by E6.5/7.5 (29), and DNA methylation alone is sufficient to suppress retrotransposons (22).

Our study demonstrated that the histone chaperone CAF-1 contributes to the incorporation of H3.1/3.2 into the nucleosomes, which may facilitate an efficient, large-scale conversion of histone modifications to protect the hypomethylated genome from retrotransposons. Although further studies are necessary, our ChIP analysis using histone mutants indicated that methylation of histones was not a prerequisite for their recruitment to nucleosomes at the target sites. Intriguingly, it has been reported that, in ES cells, H3.3 was responsible for the deposition of H3K9me3 at LTR-retrotransposon IAP and MusD regions by ATRX–DAXX histone chaperone complexes (30). The H3K9me3 marks were lost from the retrotransposon regions concomitantly with H3.3 loss upon differentiation into neuronal precursor cells (30). Our results showed that IAP regions and other retrotransposons were enriched with repressive histone modifications in association with H3.1/3.2 in morulae and blastocysts. Therefore, it is intriguing that retrotransposons are further enriched

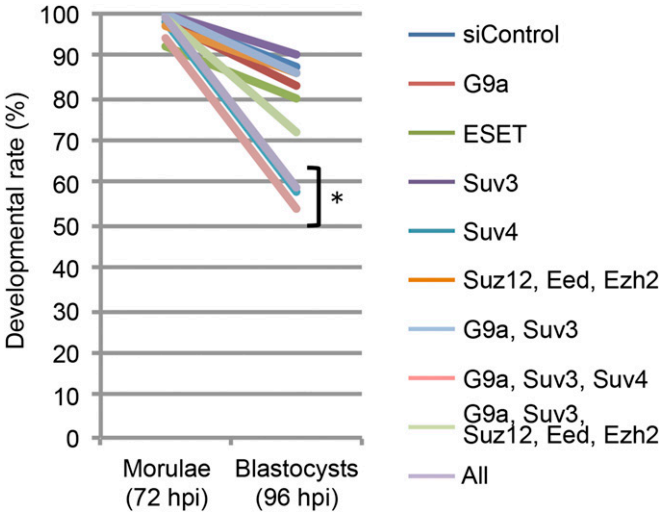

Fig. 6. The effect of knockdown of histone methyltransferases on the development from morulae to blastocysts. The blastocyst formation rates were lowest when Suv420h1/2 was down-regulated (asterisk). Suv3, Suv39h1/2; Suv4, Suv420h1/2.

with H3K9me3 by H3.3 in ES cells derived from blastocysts. It is possible that the deposition of H3.3-H3K9me3 at the IAP and MusD regions might be unique to ES cells, and does not reflect the in vivo condition.

Another important question remaining to be answered is which genomic regions other than retrotransposons are enriched with repressive histone modifications by CAF-1. This could represent the epigenetic transition from the embryonic-to-somatic cell status upon implantation, which might give clues to understanding the evolution of eutherian mammals at the molecular level. Such analysis would become possible with future ChIP technologies followed by next-generation sequencing (i.e., ChIP-seq) based on scarce material.

Materials and Methods

Animals. Six- to 10-wk-old (C57BL/6 × DBA/2) F1 (BDF1) female and 8- to 12-wk-old male ICR mice were used for the collection of oocytes and spermatozoa, respectively. The animals were provided with water and commercial laboratory mouse chow ad libitum and housed under controlled lighting conditions (daily light from 0700 h to 2100 h). They were maintained under specific pathogen-free conditions. All animal experiments described here were approved by the animal experimentation committee at the RIKEN Tsukuba Institute and were performed in accordance with the committee's guiding principles.

Collection of Oocytes, In Vitro Fertilization and Embryo Culture. Collection of spermatozoa, oocytes, and fertilized embryos was performed as described previously (31). In brief, spermatozoa were collected from the caudal epididymides of male mice. The sperm suspension was incubated for capacitation in human tubal fluid (HTF) medium for 1.5 h at 37 °C under 5% (vol/vol) CO_2 in humidified air. Oocytes were collected from the excised oviducts of female mice that had been superovulated with equine chorionic gonadotropin (CG) followed 48 h later with human CG. Cumulus–oocyte complexes were recovered into preequilibrated HTF medium. The sperm suspension was added to the oocyte cultures, and morphologically normal fertilized oocytes were collected 2 h after insemination. Fertilized embryos were cultured in potassium simplex optimized medium (KSOM) at 37 °C under 5% (vol/vol) CO_2 in humidified air until use.

RNA Preparation and Amplification. RNA preparation and amplification for microarray analysis was performed as described previously (19). In brief, total RNA was extracted with TRIzol (Invitrogen) from 10 morulae cultured for 72 h. This was subjected to two rounds of linear amplification using TargetAmp 2-Round Aminoallyl-aRNA Amplification Kits (Epicentre Technologies) according to the manufacturer's instructions. Amplified RNA was labeled with Cy3 dye (GE Healthcare) and hybridized to a whole mouse genome oligo DNA microarray (8 × 60K; Agilent Technologies) for 16 h at 65 °C, according to the manufacturer's instructions.

Primers and Antibodies. Primers and antibodies used in this study are listed in Tables S1 and S2, respectively.

CAF-1 Knockdown Experiments. For knockdown of CAF-1, we chose P150 as the targeting subunit based on previous studies (8, 9). As far as we tested, knockdown of the other two subunits, P60 and P50 (or P48), had no effect on the embryonic development (Fig. S7) (*SI Materials and Methods*).

ACKNOWLEDGMENTS. We thank Dr. Sandy Martin for providing an antibody against LINE-1 ORF1p and Dr. Bryan R. Cullen for providing an antibody against IAP GAG. This study was supported by Ministry of Education, Culture, Sports, Science and Technology of Japan Grants-in-Aid 25112009 (to A.O.), 23220011 (to A.O.), and 15K18545 (to Y.H.) and by the RIKEN Epigenetics Program Strategic Programs for R&D.

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