Faithful propagation of DNA methylation patterns during DNA replication is critical for maintaining cellular phenotypes of individual differentiated cells. Although it is well established that Uhrf1 ubiquitin-like with PHD and RING finger domains and has an essential role in maintenance of DNA methylation by recruiting Dnmt1 to hemi-methylated DNA sites, the mechanism by which Uhrf1 coordinates the maintenance of DNA methylation and DNA replication is largely unknown. Here we show that Uhrf1-dependent histone H3 ubiquitylation has a prerequisite role in the maintenance DNA methylation. Using Xenopus egg extracts, we successfully reproduce maintenance DNA methylation in vitro. Dnmt1 depletion results in a marked accumulation of Uhrf1-dependent ubiquitylation of histone H3 at lysine 23. Dnmt1 preferentially maintains histone H3 ubiquitylation.

Several lines of evidence showing the ability of Uhrf1 to bind modified histone H3 (refs 12–16) and the inability of Dnmt1 to catalyse its conversion of hemi-methylated DNA to fully methylated DNA suggest that there may be an intermediate that connects Uhrf1 with Dnmt1 at DNA replication sites. Although direct interaction between Uhrf1 and Dnmt1 has been proposed as a means of recruiting Dnmt1 to hemi-methylated DNA sites (Supplementary Fig. 2a, b), we failed to detect a stable interaction between Uhrf1 and Dnmt1 (Supplementary Fig. 2a, b). Therefore, to elucidate the mechanism underlying cooperation of maintenance DNA methylation and DNA replication by Uhrf1, we first reconstituted maintenance DNA methylation using Xenopus interphase egg extracts. After DNA replication, genomic DNA purified from sperm chromatin showed a marked incorporation of a radiolabelled methyl group when S-[methyl-3H]-adenosyl-l-methionine was used as a donor, indicating that the sperm DNA was methylated (Fig. 1a). The addition of known replication inhibitors, such as aphidicolin, p27 or gemcitabine, almost completely abolished DNA methylation as well as DNA replication (Fig. 1a, Supplementary Fig. 3a, and data not shown), indicating that DNA-replication-dependent DNA methylation can be reproduced in this in vitro cell-free system.

Although neither (x)Uhrf1 nor (x)Dnmt1 bound to mitotic chromatin, both began binding to chromatin with the kinetics similar to that of xPcna after mitotic extracts were stimulated to initiate DNA replication, suggesting a strong correlation between the chromatin loadings of xUhrf1 and xDnmt1 and DNA replication (Supplementary Fig. 3b, c). Treatment with aphidicolin and p27 abolished the chromatin loading of xDnmt1 and xUhrf1, although a slight loading and DNA-replication-independent binding of xUhrf1 to chromatin was still detectable. Anti-xUhrf1 serum efficiently depleted xUhrf1 protein in the egg extract (Supplementary Fig. 4a). Depletion of xUhrf1 severely impaired xDnmt1 recruitment to the chromatin and DNA-replication-dependent DNA methylation, although xUhrf1 depletion did not affect DNA replication efficiency and timing when compared with mock depletion (Fig. 1b and Supplementary Fig. 4b, c). Add-back of recombinant human (rh)Uhrf1 partly rescued the recruitment of xDnmt1 to the chromatin and DNA-replication-dependent DNA methylation in xUhrf1-depleted extracts (Supplementary Fig. 4b and 5a). It should be noted that the depletion of xUhrf1 or xDnmt1 from the egg extract as well as mock depletion delayed DNA replication timing (see Supplementary Figs 3a and 4c, d), presumably owing to the dilution of interphase egg extracts by the immunodepletion procedure, which is usually observed in this in vitro cell-free system. However, it did not affect the efficiency of DNA replication and chromatin loading of xPcna, indicating that neither xUhrf1 nor xDnmt1 was essential for DNA replication under our experimental conditions (Fig. 1b and Supplementary Fig. 4c, d).

xDnmt1 depletion in extracts did not inhibit but rather enhanced xUhrf1 binding to chromatin, presumably owing to the increase in the hemi-methylated DNA region (Fig. 1c). Intriguingly, we detected slower-migrating forms of Xenopus histone H3 (xH3) in the xDnmt1-depleted chromatin fraction. These slower-migrating forms were hardly detectable in mock-depleted extracts, presumably owing to the rapid conversion of hemi-methylated DNA to fully methylated DNA by xDnmt1 (see also Fig. 1f). Immunodepletion of xUhrf1 from the extracts completely abolished the appearance of these slower-migrating forms in the xDnmt1-depleted extract (Fig. 1c), although DNA replication was not affected by double depletion of xDnmt1 and xUhrf1 (Supplementary Fig. 4d). Add-back of rhUhrf1 partially rescued the appearance of these slower-migrating forms of xH3 (Supplementary Fig. 5b). A band ladder with a higher molecular weight was detected in anti-H3 immunoprecipitates by anti-ubiquitin antibodies (Fig. 1d). In addition, we added an extreme excess of recombinant His-tagged ubiquitin to the egg extracts. In this case, we expected an upshift of the modified bands of xH3. As shown in Supplementary Fig. 6, an upshift of these bands was indeed apparent. Taken together, the slower-migrating forms that appeared after xDnmt1 depletion were ubiquitylated xH3 (xUbH3). Given that xUhrf1 is required for ubiquitylation of xH3 and that it possesses a RING finger domain (see Fig. 1c and Supplementary Fig. 5b), xUhrf1 is most likely to be an E3 ubiquitin ligase for xH3. In agreement with this, xUhrf1 was reported to be able to ubiquitylate H3 in vitro.

Mass spectrometry analyses for xUbH3 in xDnmt1-depleted extracts were performed (Supplementary Fig. 7a). Tandem mass spectrometry (MS/MS) database searches of xUbH3 found one potentially modified
Chromatin was incubated with interphase egg extracts containing radiolabelled the presence of p27 or aphidicolin, indicating that ubiquitylation of but detectable amounts of xUhrf1 were detected on the chromatin in addition of p27 or aphidicolin (Supplementary Fig. 8), although trace fractions was inhibited when DNA replication was blocked by the loss of xUbH3, suggesting that the molecular coupling of deubiquitylation of xUbH3 and rmDnmt1 release from chromatin. These results indicate that recruitment of rmDnmt1 to chromatin per se is insufficient for prevention of xH3 ubiquitylation (Fig. 1f).

Anti-xH3 immunoprecipitates from xDnmt1-depleted extracts specifically bound to xDnmt1, but not to xUhrf1 (Fig. 2a and Supplementary Fig. 9a). The reciprocal experiment using immune-purified xDnmt1, xUhrf1 and xUsp7, a known Dnmt1-binding protein, also revealed that xDnmt1, but not xUhrf1 or xUsp7, bound to ubiquitylated xH3 (Supplementary Fig. 9b and 10a). We then examined whether the interaction of xDnmt1 with xUbH3 was direct. Anti-xH3 immunoprecipitates from xDnmt1-depleted chromatin bound more rmDnmt1 than those from mock-depleted chromatin (Fig. 2b). Specific interaction between rmDnmt1 and xUbH3 was also observed when rmDnmt1 protein was precipitated using anti-Flag antibodies (Fig. 2c). We then performed Far-western blotting analysis using rmDnmt1 protein as a probe. rmDnmt1 specifically bound to xUbH3, but not to other ubiquitylated proteins (see Fig. 2d, middle panel) or polyubiquitin chain per se, showing a preference for diubiquitylated xH3 (Fig. 2d), further confirming the direct and specific binding of Dnmt1 to UbH3. Although xDnmt1 immunoprecipitates bound to nucleosomal xH3 and xUbH3 (Supplementary Fig. 10a), rmDnmt1 preferentially bound to nucleosomal xUbH3 (Fig. 2c) and to monomeric di- or tri-ubiquitylated xH3 on the membrane (Fig. 2d). Although the structural basis underlying Dnmt1 binding to UbH3 is largely unknown, these somewhat distinctive observations might be explained by the possibility that xDnmt1 complexes contain proteins capable of binding to non-ubiquitylated H3 and by the distinct forms of H3, such as nucleosomes, core histones or histone monomer.

The targeting sequence in mouse (m)Dnmt1 that is required for association with replication foci has been previously identified11. Wild-type rmDnmt1 effectively bound to xUbH3, whereas a mutant rmDnmt1 lacking association with replication foci (∆Rep; rmDnmt1(∆S35–425)) failed to do so (Fig. 2e). Far-western analysis further confirmed the specific binding of wild-type, but not ∆Rep, rmDnmt1 to xUbH3 (Supplementary Fig. 10b).

A similar human (h)Uhrf1-dependent ubiquitylation of hH3 was observed in HeLa cells. When hDnmt1 was depleted, ladders of slow-migrating bands of hH3 were readily detected (Fig. 3a). This ladder was also detected by anti-ubiquitin antibodies (Fig. 3b). However, this hUbH3 was not detected in hDnmt1 and hUhrf1 double-depleted cells (Fig. 3a). As with Xenopus egg extract (Supplementary Fig. 8), a ubiquitylation ladder of hH3 was specifically detected in S phase, but not in G1/S phase, chromatin (Supplementary Fig. 11). Notably, the ubiquitylation ladder of hH3 was detected even in the presence of hDnmt1 during normal S phase when hH3 immunoprecipitated from acid-extracted histones was used for immunoblotting (Fig. 3c). When hDnmt1 was knocked down, the ectopically expressed hUbH3 was detectable by anti-Flag antibody in acid-extracted chromatin proteins from cells expressing wild-type, but not the K23R mutant, hH3 (Fig. 3d), confirming that the ubiquitylation in mammalian cells occurred at the same site as that observed in Xenopus extracts. We then examined whether lysine 23 ubiquitylation is prerequisite for Dnmt1 binding to nucleosomes. Ectopically expressed wild-type or K23R hH3 proteins in cells depleted of hDnmt1 were immunoprecipitated and incubated with purified rmDnmt1. The ubiquitylation ladder was only detected in wild-type hH3 precipitates that specifically interacted with rmDnmt1 (Fig. 3e). Taken together with the results using Xenopus extracts, Uhrf1-dependent ubiquitylation of H3 at

Either recombinant wild-type or catalytic mutant (C1229S) mouse Dnmt1 (rmDnmt1) proteins26,27, which are highly homologous to xDnmt1 (91% similarity), were reintroduced in xDnmt1-depleted extracts. Wild-type rmDnmt1 suppressed ubiquitylation of xH3, whereas C1229S failed to do so. Interestingly, wild-type, but not C1229S mutant, rmDnmt1 disappeared on chromatin at 180 min, concomitant with the loss of xUbH3, suggesting that the molecular coupling of deubiquitylation of xUbH3 and rmDnmt1 release from chromatin. These results indicate that recruitment of rmDnmt1 to chromatin per se is insufficient for prevention of xH3 ubiquitylation (Fig. 1f).
lysine 23 seems to be an essential and a general histone mark for recruitment of Dnmt1 in eukaryotes.

Finally, we determined the physiological significance of the E3 ubiquitin ligase activity of Uhrf1. We generated HeLa cells allowing a conditional replacement of endogenous hUhrf1 with either wild-type, SRA (D474G/R489A) or RING finger (C713A/C715A/C716A) mutants of mUhrf1. Wild-type mUhrf1 effectively restored the ability to ubiquitylate hH3 in cells knocked down for endogenous hUhrf1, whereas SRA and RING mutants failed to do so (Fig. 4a). Immunohistochemical analysis using Uhrf1(Δ/Δ) mouse embryonic fibroblasts ectopically expressing wild-type mUhrf1, SRA and RING mutants revealed that both mDnmt1 and mUhrf1 formed nuclear foci during S phase (Fig. 4b). These foci co-localized well with those of mPcna (Supplementary Fig. 12). Again, wild-type mUhrf1 restored the ability of mDnmt1 to form nuclear foci and co-localize with mPcna foci in cells in which endogenous mUhrf1 was deleted. By contrast, although the RING mutant itself formed nuclear foci (Fig. 4b and Supplementary Fig. 12), it failed to restore the ability of mDnmt1 to do so. As expected, the SRA

Figure 3 | hUhrf1- and S-phase-dependent ubiquitylation of hH3 at lysine 23 in HeLa cells. a. Extracts from asynchronous HeLa cells knocked down of control (−), hDnmt1, hUhrf1 or hDnmt1 and hUhrf1 by their specific shRNAs were subjected to immunoblotting using the indicated antibodies. b. Acid-extracted histones from asynchronous HeLa cells knocked down for control (−) or hDnmt1 were immunoprecipitated with anti-H3 antibodies. The resultant immunoprecipitates were subjected to immunoblotting using anti-H3 (left) and anti-Ub (right) antibodies. Asterisk indicates nonspecific bands. c. HeLa cells were synchronized at G1/S and released into S phase. Acid-extracted histones were subjected to immunoblotting using anti-H3 antibody. Immunoprecipitates were analysed by immunoblotting using anti-H3 (left) and anti-Ub (right) antibodies. Asterisk indicates nonspecific bands. d. hDnmt1 was knocked down in HeLa cells ectopically expressing either wild-type or K23R mutant Flag-tagged hH3. Cells were collected at 72 h after depletion and histone proteins were isolated by an acid extraction. Cell extracts (bottom) and extracted histones (top) were subjected to immunoblotting using anti-Dnmt1 antibodies and anti-Flag antibodies, respectively. e. Ubiquitylation of hH3 at lysine 23 is required for nucleosomal binding of Dnmt1. HeLa cells were transfected with the indicated plasmids and the infected with lentiviruses expressing sh-hDnmt1. Cells were collected 72 h after infection and the extracts were immunoprecipitated with anti-Flag antibodies according to the experimental protocols from Abcam as described in Methods. After extensive wash, the immunoprecipitates were incubated with Hisab-rmDnmt1 for 1 h at 4°C. The final precipitates were washed four times and subjected to immunoblotting using the indicated antibodies (right) as well as the extracts (left) and Flag–immunoprecipitates (middle).
The level of DNA methylation in mouse embryonic stem cells was examined by DNA-methylation-sensitive Southern blotting using intra-cisternal A-type particle (IAP) and murine leukemia virus (MLV) regions of the endogenous retrotransposon as probes. Wild-type mUhrfl or SRA or RING mutants were introduced into mUhrfl<sup>*f</sup> embryonic stem cells with their comparable expressions (Fig. 4c, top panels). The untreated embryonic stem cells showed a high level of DNA methylation in both IAP and MLV regions of the endogenous retrotransposon. When endogenous mUhrfl was deleted, their methylation levels were significantly reduced, showing decreased amounts of undigested DNA by HpaII (Fig. 4c). Wild-type mUhrfl effectively restored the level of global DNA methylation, whereas the RING mutant or the SRA mutant failed to do so. A bisulphite DNA sequencing analysis of the IAP Gag region of the endogenous retrotransposon and minor satellite repeats in mouse embryonic stem cells also demonstrated that wild-type mUhrfl, but not the RING mutant or the SRA mutant, restored the level of DNA methylation (Fig. 4d). Taken together, the results indicate that the RING finger domain of mUhrfl and consequent ubiquitination of mH3 at lysine 23 appear to be required for maintaining the level of DNA methylation in mouse embryonic stem cells.

In conclusion, our present results strongly suggest that Uhrf1-dependent ubiquitination of H3 acts as a platform for the recruitment of Dnmt1 to DNA replication sites (Supplementary Fig. 1). The rapid turnover of H3 ubiquitination during normal S phase has suggested the presence of a deubiquitylation mechanism coupled with Dnmt1-dependent DNA methylation rather than its proteasomal degradation. In fact, the Dnmt1 complex showed deubiquitylation activity towards ubiquitylated H3 (Supplementary Fig. 14a, b). Thus, it will be of great interest to elucidate the molecular basis underlying the coupling of this deubiquitylation- and Dnmt1-dependent DNA methylation.

### METHODS SUMMARY

**Xenopus egg extracts.** Cytosstatic factor (CSF)-arrested and interphase egg extracts were prepared as described<sup>36</sup>, except that metaphase II-arrested eggs were washed in extraction buffer (EB; 5 mM MgCl₂, 100 mM KCl, 20 mM HEPES-KOH, pH 7.5) instead of in 1× Marc’s modified Ringers (MMR). Incubation of extracts was carried out at 22 °C.

**Immunodepletion.** For immunodepletion of Xenopus egg extracts, 250 μl of antiserum against xUhrf1 and xDnmt1 were conjugated to 75 μl of protein A agarose (Roche). Twenty-five microliters of antibody beads were mixed with 100 μl interphase egg extracts and incubated at 4 °C for 1 h. This was repeated three times. For add-back experiments, mUhrfl wild-type or the C1229S mutant was added to xDnmt1-depleted extracts at 10 ng μl⁻¹.

**Monitoring DNA methylation and replication in Xenopus egg extracts.** DNA methylation was monitored by the incorporation of S-[methyl-<sup>3</sup>H]-adenosyl-l-methionine. Genomic DNA was purified using a Wizard Genomic DNA purification kit (Promega) according to the manufacturer’s instructions. The DNA replication efficiency was determined as described previously<sup>10</sup>.

**Wild-type and mutant Uhrf1-expressing mouse embryonic stem cell cloning.** Six micrograms of linearized pCAG-IRE-PURO plasmid containing either a Myc-tagged wild-type allele, an SRA domain mutant (D474G, R489A) or a RING domain mutant (C713A, C715A, C716A) Uhrf1 were co-transfected into mUhrfl<sup>-/-</sup> embryonic stem cells expressing various Myc-tagged mUhrfl mutants by Cre recombinase expression. Cells were immunostained 72 h after deletion of mUhrfl(WT) or mUhrfl(SRA) or mUhrfl(RING) mutant failed to form nuclear foci or to restore the ability of mDnmt1 to form them. These results suggest that the RING finger domain of mUhrfl and consequent ubiquitination of mH3 at lysine 23 are required for the recruitment of mDnmt1 to DNA replication sites. Similar results were also observed when mUhrfl mutants in the conserved amino acids required for the putative interaction with E2 enzymes were used (Supplementary Fig. 13a–c)<sup>28</sup>.


Supplementary Information is available in the online version of the paper.

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Author Contributions M.N. and A.N. planned studies and interpreted the data. A.N. and L.Y. performed most of the Xenopus studies. J.S. and K.H. validated the mH3K9 mutant lines and performed bisulphite DNA sequencing analysis. Y.J. and K.N. performed most of the mammalian studies. T.Kaw and T.Kod performed LC/MS/MS analysis. S.S. and F.I. generated purified recombinant mDnmt1 proteins. K.A. generated the purified recombinant hUHRF1 protein. M.N. and A.N. wrote the paper.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.N. (mkh-naka@med.nagoya-cu.ac.jp) or A.N. (enishiyama@med.nagoya-cu.ac.jp).
METHODS
Plasmids and proteins. Full-length xUhrf1 was isolated from Xenopus total egg cDNA by PCR. The cDNA fragment of xUhrf1 that encodes amino acids 1–650 was cloned into pET16b to be expressed as His-tagged protein. The expression constructs for His-tagged geminin and GST-tagged p27 were gifts from T. S. Takahashi. His- and GST-tagged fusion proteins were expressed in Escherichia coli BL21-CodonPlus-RIL cells (Stratagene), purified using standard molecular biology techniques, and stored in aliquots at −80 °C until use. Recombinant His-tagged ubiquitin and its mutant proteins were purchased from Boston Biochem.

Immunodepletion. Diluted 20-fold with SDS sample buffer and boiled for 5 min.

pH 7.7, and 2% sucrose) up to 300 extracts were prepared as described, except that metaphase II-arrested eggs were used. DNA methylation and replication in Xenopus egg extracts.

KOH, pH 7.5) instead of in 1

Monitoring DNA methylation and replication in Xenopus egg extracts.

tagged ubiquitin and its mutant proteins were purchased from Boston Biochem.

and was diluted tenfold with lysis buffer (1% Triton X-100, 1 mM EDTA, 150 mM NaCl, 3,350 entries) assuming the presence of the digestion enzyme trypsin.

For immunodepletion of Xenopus egg extracts, 250 μl of anti-serum against xUhrf1 and xDnmt1 were conjugated to 75 μl of protein A agarose (Roche). Twenty-five microliters of antibody beads were mixed with 100 μl interphase egg extracts and incubated at 4 °C for 1 h. This was repeated three times. For add-back experiments, xDnmt1 wild-type or the C1229S mutant was added to xDnmt1-depleted extracts at 10 ng μl

Pulldown of xUbH3 by immunopurified xDnmt1. Immunoprecipitation of xDnmt1 was performed as described above. Immunopurified xDnmt1 bound to protein A agarose was incubated with 40 μl of the MNase-digested chromatin fraction isolated from either mock- or xDnmt1-depleted extracts. After incubation on ice for 1 h, beads were washed with CPB buffer containing 0.1% NP-40. Bound proteins were analysed by bound degradation.

Purification of rhUhrf1. A DNA fragment encoding full-length hUhrf1 was amplified by PCR and cloned into pCAGGS-EF1α (GE Healthcare) and pCS-RfA-ETHygro vector with Gateway LR clonase (Invitrogen). The recombinant protein was expressed in the E. coli strain Rosetta (DE3). Cells were grown at 37 °C in Luria-Bertani medium, induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when they reached an optical density of 0.5–0.6 at 600 nm and were then incubated at 15 °C overnight.

The cells were collected, released in suspension buffer (50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl, 1 mM dithiothreitol (DTT), 30 μM ZnOAc, 10% glycerol and 0.2 mM phenylmethylsulphonyl fluoride (PMSF)) and disrupted by sonication on ice. After centrifugation, the supernatant was applied to a GST affinity column of glutathione Sepharose 4 Fast Flow (GE Healthcare Bio-Sciences) equilibrated with PMSF-free lysis buffer and eluted by digestion using PreScission protease. The protein was further purified by anion-exchange chromatography using a HiTrap Q HP column, by DNA binding protein affinity chromatography using a HiTrap Heparin column and by size-exclusion chromatography using a HiLoad 26/60 Superdex200 column (GE Healthcare Bio-Sciences).

Trypsin digestion and LC–MS/MS. The method for trypsin digestion of protein has been described previously. LC–MS/MS analysis was performed using an LTQ orbitrap Elite ETD mass spectrometer (Thermo Fisher Scientific). The methods used for LC–MS/MS were slightly modified from those described previously. The mass spectrometer was operated in data-dependent acquisition mode in which MS acquisition with a mass range of m/z 350–1,600 was automatically switched to MS/MS acquisition under the control of Xcalibur software. The top three precursor ions in the MS scan were selected by Orbitrap, with a resolution of R = 240,000, and those in subsequent MS/MS scans, with an ion trap in automated gain control (AGC) mode where AGC values were 1 × 10⁶ and 1.00 × 10⁴ for full MS and MS/MS, respectively. For fragmentation, collision-induced dissociation and electron transfer dissociation were used.

Database searching. Tandem mass (MS/MS) spectra were extracted using Proteome Discoverer version 1.3. All MS/MS samples were analysed using Mascot (Matrix Science; version 2.4). Mascot was set up to search Sprot_2012_08.fasta (selected for Xenopus laevis, 3,350 entries) assuming the presence of the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 3.0 p.p.m. The maximum number of missed cleavage sites was set at 3. The carbamidomethyl of cysteine was specified in Mascot as a fixed modification. Amidase of arginine, methyls of lysine and arginine, dimethyls of lysine and arginine, acetyl of lysine, trimethyl of lysine, phosphos of serine, threono and tyrosine, GlyGly of lysine and LeuArgGlyGly of lysine, all of which have a trypsin-digested ubiquitinated protein signature, were specified in Mascot as variable modifications.

Criteria for protein identification. Scaffold (version Scaffold_3.6.3, Proteome Software) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least three identifiable peptides. Protein probabilities were assigned by the Protein Prophet algorithm. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped together to satisfy the principle of parsimony.

Plasmid construction. To generate lentivirus-based sh-hUhrf1 or sh-hDnmt1, the plasmid DNA was isolated from E. coli C, and the reaction was stopped by the addition of chromatin purification buffer (CPB, 50 mM KCl, 5 mM MgCl₂, 20 mM HEPES-KOH, pH 7.7, and 2% sucrose) up to 300 μl of Hi₃-geminin, GST-p27 or aphidicolin (150 μM) was added to the extracts 10 min before the addition of sperm DNA. Genomic DNA was purified using a Wizard Genomic DNA purification kit (Promega) according to the manufacturer’s instructions. The DNA replication efficiency was determined as described previously. Isolation of chromatin fractions from Xenopus egg extracts. Sperm nuclei were incubated in extracts at a concentration of 3,000 nuclei per μl.

Monitoring DNA methylation and replication in Xenopus egg extracts. DNA methylation was monitored by the incorporation of S-[(methyl-³H)]-adenosyl-L-methionine. Demembranated sperm nuclei (3,000 nuclei per μl) were added to an egg extract containing S-[(methyl-³H)]-adenosyl-L-methionine, incubated at room temperature (22–25 °C), and the reaction was stopped by the addition of chromatin purification buffer (CPB, 50 mM KCl, 5 mM MgCl₂, 20 mM HEPES-KOH, pH 7.7, and 2% sucrose) up to 300 μl. Histone proteins, chromatin fractions were washed in 300 mM NaCl and acid-extracted.

Immunoprecipitation. Two micrograms of the antibody for immunoprecipitation were incubated with protein A agarose beads for 1 h at room temperature and washed three times with 400 μl of CPB buffer. For a pulldown experiment, immunoprecipitated histone H3 was incubated with egg extract at 4 °C for 1 h. Beads were washed four times with lysis buffer and bound proteins were finally eluted with SDS sample buffer and analysed by SDS–PAGE.

For immunodepletion of Xenopus egg extracts, 250 μl of anti-serum against xUhrf1 and xDnmt1 were conjugated to 75 μl of protein A agarose (Roche). Twenty-five microliters of antibody beads were mixed with 100 μl interphase egg extracts and incubated at 4 °C for 1 h. This was repeated three times. For add-back experiments, xDnmt1 wild-type or the C1229S mutant was added to xDnmt1-depleted extracts at 10 ng μl⁻¹.

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Immunoprecipitation. Two micrograms of the antibody for immunoprecipitation were incubated with protein A-agarose beads for 1 h at room temperature and washed in CPB buffer. Xenopus egg extracts were diluted fivefold in CPB buffer and incubated with the antibody-bound beads for 1 h at 4 °C. Beads were then washed twice with CPB, once with CPB containing 0.1% NP-40, and twice with CPB alone. Proteins were finally eluted with SDS sample buffer and analysed by SDS–PAGE.

Immunoprecipitation of chromatin-bound histone H3. Extracts containing sperm chromatin were diluted fivefold with CPB containing 0.1% NP-40, and were then incubated on ice for 5 min. The chromatin was isolated via centrifugation at 10,000g for 10 min through a 0.5-ml sucrose cushion of CPB with 30% sucrose, which was underlayered in the tube with 10 μl of 2 M sucrose. Removal of the supernatant by aspiration left 50 μl and an equal volume of nucleosome buffer (micrococcal nuclease) was added. The chromatin pellet was re-suspended by vortexing and incubated at 22 °C for 20 min. The reaction was stopped by the addition of 10 mM EDTA and the tube was centrifuged at 15,000 r.p.m. for 10 min. For H3 immunoprecipitation, the supernatant was supplemented with 1% SDS and was diluted tenfold with lysis buffer buffer (CPB, 150 mM NaCl, 15 mM Tris-HCl, pH 8.0). Diluted lysates were incubated with 2 μg of histone H3 antibody conjugated to 10 μl of protein A agarose. Beads were then washed three times with 400 μl of lysis buffer. For a pulldown experiment, immunoprecipitated histone H3 was incubated with egg extract at 4 °C for 1 h. Beads were washed four times with lysis buffer and bound proteins were finally eluted with SDS sample buffer and analysed by SDS–PAGE.
pOZ-C-hH3 (a gift of H. Tagami, Nagoya City University) was inserted into a pCAGGS vector digested with EcoRI (end-filled). The hH3(K23R) mutant plasmid was generated by PCR-based, site-directed mutagenesis for pCAGGS-hH3-Flag. To construct pCAG-puro-Myc-mUhrf1, a PCR fragment containing the full-length cDNA was sub-cloned into pCAG-puro-Myc vector. The pCAG-puro-Myc-mUhrf1 mutant plasmids were generated by PCR-based, site-directed mutagenesis for pCAG-puro-Myc-mUhrf1.

**Immunoprecipitation and immunoblotting analyses for mammalian cells.**

293T cells or HeLa cells transfected with the indicated constructs were lysed as quickly as possible in TBSN buffer (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.5% NP-40, 5 mM EGTA, 15 mM EDTA, 0.5 mM Na3VO4, 50 μM P-619 and 20 mM p-ribozyme phosphatase (PNPP)). The resulting lysates were clarified by centrifugation at 15,000g for 20 min at 4°C before immunoprecipitation with the specific antibody. Immunoprecipitated proteins were separated by SDS–PAGE, transferred to a polyvinylidene difluoride (Immobilon-P; Millipore) membrane and then detected by immunoblotting with the indicated antibodies using an enhanced chemiluminescence detection system.

**Cell culture, transfection and virus generation and infection.** Cell lines were cultured as recommended by American Type Culture Collection (Manassas). HeLa cells were trapped in S phase by treating them with 2.5 mM thymidine (Sigma) for 24 h, cultured as recommended by American Type Culture Collection (Manassas). HeLa cells infected with the viruses were treated with 10 μg/ml 3′-methyladenosine (Sigma) for 2–3 days. To generate mUhrf1-expressing cells, PvuI-linearized pCAG-puro-Myc-mUhrf1 plasmids were cut with PvuI and transfected into the cells by the calcium phosphate precipitation method. Cells transfected with the indicated plasmids were treated with 2 μg/ml−1 of puromycin (Sigma) for 5 days.

**Histone acid extraction.** Cell pellets were re-suspended in PBS with 0.5% Triton X-100 and protease inhibitors at a density of approximately 106 cells ml−1 and tubes were rotated at 4°C for 10 min to lyse the cells. The lysates were centrifuged at 4°C for 10 min at 2,000 r.p.m., and the pellets were rinsed once in the extraction buffer. The histones in the insoluble chromatin pellet were then extracted in 0.2 N HCl for 10 min at 4°C on a rotator. The lysates were centrifuged at 4°C for 10 min at 10,000 r.p.m., and the supernatants containing histones were collected and adjusted to pH 8.0 with 2 M Tris.

**Immunofluorescence analysis.** Mouse embryonic fibroblasts grown on glass slides were treated with 0.1% Triton X-100 in CSK buffer (100 mM NaCl, 300 mM sucrose,10 mM PIPES, pH 6.8, 3 mM MgCl2 and 1 mM EGTA) for 2 min at 4°C, fixed with 4% paraformaldehyde in PBS, and then treated with methanol for 20 min at −20°C. The cells were incubated in blocking solution (5% BSA in PBS) and incubated in detection solution containing primary antibodies (5% BSA in PBS). After four washes with PBS, the samples were incubated in detection solution containing the secondary antibodies. Images were collected using BZ-9000 (KEYENCE).

**Matintenance of Uhrf1-fl/fl embryonic stem cells.** Uhrf1-fl/fl embryonic stem cells were cultured on mitomycin-C-treated non-proliferating feeder mouse embryonic fibroblast cells in standard embryonic stem cell medium supplemented with 20% FBS and LIF. For conditional knockout of the endogenous mUhrf1 gene, 800 nM 4-OH tamoxifen (4-OHT, SIGMA, no. H7904, dissolved in ethanol) was added to the medium. Ablation of the mUhrf1 transcript was confirmed by qPCR using the following primers specific for mUhrf1 exons 4 and 5, mUhrf1-RT-F01, ATGATTGCCAGGGGAGTTC; and mUhrf1-RT-R01, GTGTTGAGGGGAGTGAAAG. Decreases in mUhrf1 protein expression were assessed using an Uhrf1-specific antibody (Uhrf1-M-132 antibody, Santa Cruz Biotechnology, no. sc-98817). Expression of the Myc-tagged exogenous wild-type or mutant Uhrf1 proteins was detected with a mouse monoclonal anti-Myc tag antibody (Millipore, 05-724, clone 4A6).

**Wild-type and mutant Uhrf1-expressing mouse embryonic stem cell cloning.** Six micrograms of linearized pCAG-IRE-Pre-Puro vector containing either a Myc-tagged wild-type, a SRA domain mutant (D47A/R489A) or a RING domain mutant (C713A/C715A/C716A) mUhrf1 cDNA were transfection into Uhrf1-fl/fl embryonic stem cells using FuGENE HD transfection reagent (Roche Applied Science, no. 6366244). Stable clones were selected by puromycin (1 mg ml−1) treatment. Endogenous mUhrf1 genes are conditionally deleted by the addition of 4-hydroxytamoxifen from Uhrf1-fl/fl embryonic stem cells expressing ER-Cre.

**Global DNA methylation analysis by methylation-sensitive Southern blotting.** Endogenous mUhrf1 genes were conditionally deleted by the addition of 4-hydroxytamoxifen from Uhrf1-fl/fl embryonic stem cells expressing ER-Cre. Genomic DNA was purified from embryonic stem cells using an AllPrep DNA/RNA mini kit (Qiagen, no. 80204) according to the supplier’s protocol. One microgram of genomic DNA was digested with 20 U HpaII (New England BioLabs, no. R0171L) or 10 U MspI (New England BioLabs, no. R0106L) overnight at 37°C. Digests were separated with 0.8% agarose-gel, transferred to a nylon membrane and hybridized with specific probes generated with the following primers: 5′-AGTGCGGACAGTTATCTGCTG-3′; IAP-LTR-probeR, 5′-CAGAAGATGTCGTCGCTTGGT-3′; IAP-INTR-probeR, 5′-GAATCTCAGATGTAATCTCTC-3′; MLV_gag_probeF, 5′-TCAGAGATCGGACAGACC-3′ and MLV_gag_probeR, GGATTAAGG CAGGTTAAGG-3′. Relative DNA methylation was determined by counting radioactivity of the undigested DNA (tops of the gels) and represented as a percentage of DNA undigested by HpaII in Uhrf1-fl/fl mouse embryonic stem cells.

**Bisulphite DNA sequencing analysis.** Two micrograms of genomic DNA were treated with sodium bisulphite using an EpiTect bisulphite kit (Qiagen, no. 59104) following the manufacturer’s protocol. The Gag antigen of the IAP retrotransposons was amplified from the bisulphite-reated genomic DNA using the following primers: IAP-Gag-Bs-F01, 5′-ATTGATTGTTCTAGTTAAATTTATATATTTGGG-3′ and IAP-Gag-Bs-R01, 5′-AAGGGATCTCAGTTAAATTTATATATTGGG-3′; and MLV_gag_probeF, 5′-TCAGAGATCGGACAGACC-3′ and MLV_gag_probeR, GGGTAAGG CAGGTTAAGG-3′. PCR products were cloned into TOPO TA Cloning Dual Promoter vector (Life Technologies, no. K4600-01). Successful clones were sequenced using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, no. 4337454). Methylated CpGs are shown as closed circles and unmethylated ones are represented with open circles.

**In vitro deubiquitylation assays.** For the substrate, hUbH3 was acid-extracted from Hdmnt1-depleted HeLa cells which were released 4 h from the G1/S boundary by double thymidine treatment. The hUbH3 was mixed with immune-precipitated Xdnmt1 from Xenopus egg extract in reaction buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 2 mM EDTA, 2 mM DTT) for 4 h at 37°C. To block the reactions, Laemmli buffer was added to the reaction, and the proteins were analysed by immunoblotting. 31. Fujinoki, M. et al. Identification of 36-KDa flagellar phosphoproteins associated with hamster sperm motility. J. Biochem. 133, 361–369 (2003).

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