Stable inheritance of DNA methylation is critical for maintaining differentiated phenotypes in multicellular organisms. We have recently identified dual mono-ubiquitylation of histone H3 (H3Ub2) by UHRF1 as an essential mechanism to recruit DNMT1 to chromatin. Here, we show that PCNA-associated factor 15 (PAF15) undergoes UHRF1-dependent dual mono-ubiquitylation (PAF15Ub2) on chromatin in a DNA replication-coupled manner. This event will, in turn, recruit DNMT1. During early S-phase, UHRF1 preferentially ubiquitylates PAF15, whereas H3Ub2 predominates during late S-phase. H3Ub2 is enhanced under PAF15 compromised conditions, suggesting that H3Ub2 serves as a backup for PAF15Ub2. In mouse ES cells, loss of PAF15Ub2 results in DNA hypomethylation at early replicating domains. Together, our results suggest that there are two distinct mechanisms underlying replication timing-dependent recruitment of DNMT1 through PAF15Ub2 and H3Ub2, both of which are prerequisite for high fidelity DNA methylation inheritance.
DNA cytosine methylation is a conserved epigenetic modification essential for embryonic development, transcriptional regulation, and genome stability. In higher eukaryotes, individual differentiated cells possess unique DNA methylation patterns that determine their cellular phenotypes. Therefore, the DNA methylation pattern must be precisely maintained in coordination with DNA replication during S phase. DNA methyltransferase 1 (DNMT1) contains multiple functional domains, including a replication focus targeting sequence (RFTS), an unmethylated CpG DNA-binding CXXC domain, two bromo-adjacent homology domains, and a C-terminal catalytic domain. The RFTS domain is not only critical for DNMT1 recruitment to DNA methylation sites but also functions as an auto-inhibitory domain of DNMT1.

The recruitment of DNMT1 to DNA methylation sites requires UHRF1, an E3 ubiquitin ligase. UHRF1 recognizes specific epigenetic modifications on DNA strands and histone H3 tails through its SET- and RING-associated (SRA) domain and tandem Tudor domain (TTD). The TTD domain of UHRF1 recognizes specific DNA motifs and is enriched in coordination with DNA replication during S phase. DNA methylation patterns that determine their cellular phenotypes. Individual differentiated cells possess unique DNA methylation sites. The RFTS domain is not only critical for DNMT1 recruitment to DNA methylation sites but also functions as an auto-inhibitory domain of DNMT1.

Results
Ubiquitylated PAF15 specifically binds replicating chromatin. Given that the ubiquitin ligase activity of UHRF1 and the ubiquitin binding activity of DNMT1 are essential for the recruitment of DNMT1 to hemi-methylated DNA, we speculated that factors associated with the DNA replication machinery also utilize ubiquitin signals to recruit DNMT1. To identify factors capable of binding DNMT1 in a ubiquitin signal-dependent manner, we used ubiquitin vinyl sulfone (UbVS) treatment, a pan-deubiquitylation enzyme inhibitor, to specifically enrich for ubiquitylated proteins in cell-free Xenopus extracts. In brief, pretreatment of egg extracts with UbVS inhibits ubiquitin turnover and results in an almost complete depletion of free ubiquitin, leading to the inhibition of both ubiquitylation and deubiquitylation pathways. Thus, the addition of recombinant ubiquitin to UbVS-treated extracts specifically enhanced ubiquitin signals, including UHRF1-mediated histone H3 ubiquitylation. Chromatin lysates from UbVS-treated extracts in the presence (UbVS+Ub) or absence (UbVS) of free ubiquitin were subjected to a pull-down experiment using recombinant Flag-tagged wild-type Xenopus DNMT1 (rxDNMT1WT) purified from insect cells (Supplementary Fig. 1a–c). As reported previously, rxDNMT1WT specifically interacted with H3Ub2 in denatured chromatins (Supplementary Fig. 1d, +sodium dodecyl sulfate (+SDS)). In native chromatins, rxDNMT1WT interacted with H3Ub2 as well as with unmodified and mono-ubiquitylated histone H3 (Supplementary Fig. 1d, −SDS), suggesting that indirect binding is also preserved under this condition.

We next subjected the pull-downs of rxDNMT1WT or endogenous xDNMT1 from native chromatins to mass spectrometric analysis. We identified 2840 unique peptides (including 26 ubiquitylated and 17 phosphorylated peptides), which mapped to 303 protein groups in chromatins from UbVS-treated extracts in the presence (UbVS+Ub) or absence (UbVS) of free ubiquitin (Supplementary Data 1). Of these xDNMT1-interacting chromatins, 24 were highly enriched in the xDNMT1 pull-downs in response to the addition of ubiquitin to UbVS-treated extracts (log-fold-change >2, p value < 0.05; Fig. 1a, Supplementary Data 1). We also found an enrichment of eight ubiquitylated and two phosphorylated peptides in the data set (Supplementary Data 2 and 3). Histone H3 variants were identified, together with other histone proteins, validating the interactors (Fig. 1a and Supplementary Data 1). Among the identified proteins, we focused on PAF15, one of the most highly enriched proteins (log-fold-change = 4.75), because it was reported to be associated with both proliferating cell nuclear antigen (PCNA) and DNMT1, and was targeted for dual mono-ubiquitylation at its H3-like N-terminal domain during S phase in human cells (see also Supplementary Fig. 1e). Suggesting that this interaction is conserved among vertebrates and is regulated in a ubiquitin signal-dependent manner.

The addition of sperm chromatin to cell-free Xenopus interphase egg extracts induces the assembly of replication-competent nuclei and a single round of DNA replication. Under these conditions, DNA replication typically begins approximately 40 min after sperm addition. After the completion of DNA replication and maintenance of DNA methylation, many chromosomal replication regulators including DNMT1 and UHRF1 dissociate from the chromatin. Using interphase egg extracts, we first examined the S-phase chromatin binding and ubiquitylation of xPAF15 along with the proteins involved in maintenance of DNA methylation. We found that slow migrating forms of xPAF15 bound to chromatin (Fig. 1b) in line with results using human cells. When an excess amount of recombinant His6-tagged ubiquitin was added to the egg extracts (Supplementary Fig. 1f), the slowly migrating xPAF15 bands were further upshifted (Fig. 1b), suggesting that these slow forms correspond to ubiquitylated xPAF15 (Fig. 1b). The binding kinetics of xPAF15 were generally similar to those of xDNMT1 or xUSP7 over the same time course (Fig. 1b). The chromatin binding of xPAF15, as well as that of xDNMT1 and xUSP7, were lost in the presence of aphidicolin (Fig. 1c), a DNA polymerase inhibitor, suggesting that the chromatin binding of these proteins requires ongoing DNA synthesis.

In order to explore the role of xPAF15 ubiquitylation in xDNMT1 recruitment, we first identified ubiquitylation sites in xPAF15. We determined that the highly conserved lysine residues, K15 and K24 of human PAF15 (hPAF15), correspond to K18 and K27 of xPAF15 (Supplementary Fig. 1e). Interphase egg extracts depleted of endogenous xPAF15 were supplemented with recombinant xPAF15-Flag, purified from insect cells, then sperm chromatin...
was added. Wild-type recombinant xPAF15 (rxPAF15WT), as well as the endogenous xPAF15, underwent ubiquitylation and bound to chromatin during DNA replication (Fig. 1d). In contrast, mutant xPAF15 with a substitution of lysine to arginine at both K18 and K27 (KRKR) failed to do so (Fig. 1d). Single xPAF15 mutants with the substitution at either site (K18R or K27R) underwent monoubiquitylation and retained the chromatin-binding activity (Fig. 1d).

Next, we examined how depletion of free ubiquitin from egg extracts affects xPAF15 chromatin binding. Pretreatment of egg extracts with UbVS completely suppressed the chromatin loading of both xPAF15 and xDNMT1, whereas xUHRF1 chromatin binding was maintained (Supplementary Fig. 1g, h). Addition of free
ubiquitin to UbVS-treated extracts efficiently restored PAF15 chromatin binding (Supplementary Fig. 1h). These results demonstrate that the mono-ubiquitylation of xPAF15 at K18 and/or K27 is important for stable xPAF15 chromatin association.

We then examined the role of xPCNA binding in xPAF15 chromatin loading. xPAF15 formed a stable complex with xPCNA in the egg extracts (Supplementary Fig. 1i) as it did in human cells\(^{32,33}\). Substitution of phenylalanine with alanine at two conserved residues within the PCNA interacting peptide motif (PIP-box) of glutathione S-transferase (GST)-xPAF15 (FF/AA) abolished its interaction with xPCNA (Supplementary Fig. 1j). Although the WT rxPAF15 bound to the chromatin (Fig. 1e), the rxPAF15\(^{FF/AA}\) mutant failed to do so, as did the rxPAF15\(^{K18R/K27R}\) mutant (Fig. 1e). These results suggest that xPAF15 chromatin loading requires interaction with xPCNA.

**UHRF1 recognizes the N-terminal H3-like sequence of PAF15.** We next examined the requirement of the E3 ubiquitin ligase xUHRF1 for xPAF15 ubiquitylation and chromatin loading. As we demonstrated previously, immunodepletion of UHRF1 from egg extracts inhibited DNMT1 recruitment and chromatin association of xPAF15 (Fig. 2a, Supplementary Fig. 2a). Addition of recombinant WT xUHRF1 (rxUHRF1\(^{WT}\)) purified from insect cells to UHRF1-depleted extracts rescued the chromatin loading of xPAF15 (Fig. 2a, Supplementary Fig. 2a). We also tested the effect of recombinant xUHRF1 containing D333A/D336A point mutations in the PHD finger that are expected to cause a loss of interaction with the histone H3 tail\(^{13,14}\). Strikingly, rxUHRF1\(^{D333A/D336A}\) did not support xPAF15 ubiquitylation and chromatin loading (Fig. 2a, Supplementary Fig. 2a), suggesting that UHRF1-PHD has a crucial role in the regulation of PAF15. In contrast, xDNMT1 depletion resulted in the accumulation of xUHRF1 and ubiquitylation of histone H3 on the chromatin (Supplementary Fig. 2b, c). Dual mono-ubiquitylated xPAF15 (xPAF15Ub2) also accumulated on the chromatin (Supplementary Fig. 2b, c). These effects were restored by the addition of rxDNMT1 (Supplementary Fig. 2b, c). Our results indicate that both xPAF15 ubiquitylation and its chromatin recruitment are xUHRF1 dependent.

The PHD finger of hUHRF1 (hPHD) has been shown to bind to N-terminal \(^1\)ARTK\(^1\) residues of histone H3\(^{13,14}\). Given that the N-terminal portion of PAF15 shares significant homology with the N-terminal tail of histone H3 (Fig. 2b), we reasoned that hPHD likely to bind the N-terminal portion of PAF15. Isothermal titration calorimetry (ITC) demonstrated that hPHD bound to human PAF15\(_{2-11}\) with \(K_d = 2.2 \pm 0.3 \mu M\). This value is comparable to that for the N-terminal histone H3 peptide (\(K_d = 1.5 \pm 0.1 \mu M\)) (Fig. 2b, Supplementary Fig. 2d). In order to determine the binding mode of hPHD to PAF15, the crystal structure of hPHD bound to hPAF15\(_{2-11}\) was determined at 1.7 Å resolution (Table 1). The structure showed that the hPAF15\(_{2-11}\) peptide bound to the acidic surface of hPHD (Fig. 2c, left), with the hPHD-TK\(^1\) sequence of hPAF15\(_{2-11}\) being recognized in a manner similar to that of the \(^1\)ARTK\(^1\) of histone H3 (Supplementary Fig. 2e, f). The N-terminus of hPAF15\(_{2-11}\) formed a hydrogen bond with hPHD-E355, and hPAF15\(_{2-11}\)-V2 was surrounded by the hydrophobic residues V352, P353, and W358 of the hPHD (Fig. 2c right). hPAF15\(_{2-11}\)-R3 and -K5 formed an electrostatic interaction with hPHD-D334 and -D337 and a hydrogen bond with hPHD-C316, respectively (Fig. 2c right). The importance of the above interactions in the complex formation was further validated by mutation analysis. ITC data demonstrated that hPHD-D334A/D337A failed to bind to WT hPAF15\(_{2-11}\) while the WT hPHD was unable to bind to hPAF15\(_{2-11}\)-R3A or -T4D (Fig. 2b). Consistently, rxPAF15\(^{R3A}\) and rxPAF15\(^{T4D}\) failed to bind to chromatin during S phase in xPAF15-depleted extracts (Fig. 2d). Highlighting the importance of the hPHD recognition of hPAF15, in vitro ubiquitylation assays revealed that UHRF1 D334A/D337A and PAF15 R3A or T4D mutations significantly decreased the ubiquitylation of hPAF15, as well as the UHRF1 H741A mutation that disrupted E3 activity (Fig. 2e, f). The PAF15 K5A mutation had only a small effect on chromatin binding, interaction with hPHD, and ubiquitylation (Fig. 2b, d, e). Together, these findings suggest that the PHD finger of UHRF1 is responsible for the association with the N-terminal end of PAF15 with a binding mode similar to that of histone H3 and plays a critical role in PAF15 ubiquitylation by UHRF1.

**PAF15Ub2 forms a complex with DNMT1.** We recently reported that DNMT1 specifically binds to H3Ub2 via the RFTS domain\(^{23}\). Given the similarity of PAF15 to the H3 tail and its ability to be dual mono-ubiquitylated, we asked whether PAF15 is also specifically recognized by the RFTS domain of DNMT1. Immunoprecipitation (IP)–western blotting analysis using solubilized chromatin revealed that the majority of xPAF15Ub2 bound to xDNMT1 (Fig. 3a, Supplementary Fig. 3a). Although xDNMT1 bound to H3Ub2, xPAF15Ub2 failed to do so (Fig. 3a), suggesting that xDNMT1-H3Ub2 and xDNMT1-xPAF15Ub2 complexes are mutually exclusive. Similar results were obtained using UbVS+Ub-treated egg extracts (Supplementary Fig. 3b). Next, we examined the DNMT1 binding of ubiquitylation-deficient xPAF15 mutants on chromatin. rxPAF15\(^{K18R/K27R}\) failed to bind to chromatin as described above (Fig. 3b, see also Fig. 1d). Although rxPAF15\(^{K18R}\) or rxPAF15\(^{K27R}\) mutants bound to xPCNA on the chromatin as effectively as had rxPAF15\(^{WT}\), they failed to bind to xDNMT1 (Fig. 3b). These results suggest that the binding of xPAF15 to xDNMT1 requires dual mono-ubiquitylation of PAF15 and that single mono-ubiquitylation of xPAF15 is not sufficient for the complex formation. This may also explain the apparently strong chromatin interaction of single mono-ubiquitylation of PAF15 K18R or K27R compared to dual mono-ubiquitylation of PAF15\(^{WT}\), likely due to defective recruitment of DNMT1/USP7 complex.
To further analyze the interaction between the RFTS of human DNMT1 (hRFTS) and PAF15Ub2, we prepared ubiquitylated hPAF15 (residues 2–30) analogs, in which G76C Ub was linked to K15C and/or K24C of hPAF15 by disulfide bonds (hPAF152–30Ub2, hPAF15K15ub, and hPAF15K24Ub, Supplementary Fig. 3c, see “Methods”). The ITC experiment using hRFTS and hPAF152–30Ub2 was performed under a condition with higher c value (c = n[titrand]/K_d: 10,000) than that with an optimal value (1 < c < 1000)
Fig. 2 UHRF1 recognizes and ubiquitylates the N-terminal H3-like sequence of PAF15. a Mock-depleted or UHRF1-depleted extracts were supplemented with the indicated recombinant proteins (wt/D333A/D336A xUHRF1; see “Methods”) and chromatin was isolated. Chromatin-bound proteins were analyzed by immunoblotting using the indicated antibodies. For the protein levels of each protein in the extracts, see Supplementary Fig. 2a. b Comparison of the N-terminal sequence of PAF15 and histone H3 across different species. Residues mutated in the PAF15 mutants used in this study are shaded. Superimposition of plots of enthalpy changes in the interaction between hPHD and hPAF152-11 peptides by ITC measurement. c Recognition of the N-terminus of hPAF15 by hPHD. The left panel shows the crystal structure of PHD in complex with hPAF15, hPHD as a surface model with electrostatic potential (red, negative; blue, positive). The right panel shows recognition of PAF15 N-terminus (green stick model) by hPHD (pink stick model). Hydrogen bonds and water molecules are shown as black lines and balls, respectively. 

Table 1 Data collection and refinement statistics.

| PHD:PAF15 (PDB: 6I1W) |
|------------------------|
| **Data collection**     |
| Beam line              | PF-BL17A |
| Wavelength (Å)         | 0.98     |
| Space group            | P622     |
| Cell dimensions a, b, c (Å) | 36.7, 37.6, 220.2 |
| Resolution (Å)         | 44.03–1.70 (1.73–1.70) |
| R_p (Å)                | 36.69–1.70 |
| No. of reflections      | 10,653   |
| R_work/R_ref (%)       | 17.6/18.9 |
| No. of atoms           | 522      |
| Average B factors (Å²) | 26.6     |
| PHD                    | 526      |
| PAF15                  | 26.4     |
| Ion                    | 19.4     |
| Water                  | 38.4     |
| R.m.s. deviations      | 0.005    |
| Bond lengths (Å)       | 0.928    |

Values in parentheses are for the highest-resolution shell.

Table 1 Data collection and refinement statistics.

- **PHD:PAF15 (PDB: 6I1W)**
  - **Data collection**
    - Beam line: PF-BL17A
    - Wavelength: 0.98 Å
    - Space group: P622
    - Cell dimensions: a, b, c (Å) = 36.7, 37.6, 220.2
    - Resolution (Å): 44.03–1.70 (1.73–1.70)
    - R_p (Å): 36.69–1.70
    - No. of reflections: 10,653
    - R_work/R_ref (%): 17.6/18.9
    - No. of atoms: PHD = 522, PAF15 = 51, Ion = 4, Water = 96
    - Average B factors (Å²): PHD = 26.6, PAF15 = 26.4, Ion = 19.4, Water = 38.4
    - R.m.s. deviations: Bond lengths (Å) = 0.005, Bond angles (°) = 0.928

The table provides data collection and refinement statistics for the PHD:PAF15 complex, with key parameters such as the beam line, wavelength, space group, cell dimensions, resolution, and number of reflections. This information is crucial for validating the structural integrity and quality of the X-ray crystallographic data.

because the measurement using lower concentrations of proteins (even 1/4 of the original) resulted in an insufficient calorimetical reaction for the reliable detection. Nevertheless, the results indicated that hRFTS binds to the hPAF152–30Ub2 with high affinity (K_d = 1.4 ± 0.7 nM) in a 1:1 stoichiometric complex, which is comparable to that of hRFTS bound to H3Ub2.23 In contrast, the binding affinity of hRFTS to hPAF152–30K15Ub was much lower (K_d = 1.2 ± 0.8 μM) than that of hRFTS to hPAF15Ub2. Interaction of hRFTS with hPAF152–30K24Ub resulted in a complex thermodynamic curve showing both exothermic and endothermic responses, which makes it difficult to determine its precise binding constant (K_d = n.d.). In addition, stoichiometric binding of 1:1 was abrogated in hRFTS:PAF15Ub1 at K15 or K24. These results indicate that dual mono-ubiquitylation of PAF15 is important for specific interaction with hRFTS. We then performed size-exclusion chromatography in line with small-angle X-ray scattering (SEC-SAXS) of hRFTS, hRFTS-hPAF152–30Ub2, or hRFTS–H31–37WUb2 on chromatin in mock-depleted extracts, whereas the level of H3Ub2 in the DNMT1 complex was partially suppressed compared to the control (Fig. 4a). Very importantly, although xH3Ub2 was hardly detected in the mock-depleted chromatin in clear contrast to xPAF15Ub2 (Fig. 4b, lanes 1–3), xH3Ub2 was drastically enhanced when xPAF15 was depleted (Fig. 4b, lanes 4–6). Addition of rPAF15WT to the endogenous xPAF15-depleted extracts suppressed the enhanced xH3Ub2, whereas that of rPAF15K18R/K27R failed to do so (Fig. 4b, Supplementary Fig. 4b), suggesting that depletion of xPAF15 was complemented by xH3Ub2. Interestingly, the kinetics of xDNMT1 chromatin loading appeared to correlate with the dual mono-ubiquitylation of either xPAF15 or xH3. Consistent with this, DNMT1 predominantly interacted with PAF15Ub2, not with H3Ub2, on chromatin in mock-depleted extracts, whereas the level of H3Ub2 in the DNMT1 complex significantly increased in the absence of PAF15 (Fig. 4c). Taken together, our results reveal an essential role for PAF15Ub2 in maintenance of DNA methylation, which can only be partially compensated for by H3Ub2.

Complementation of xDNMT1 recruitment by xH3Ub2 in the xPAF15Ub2 is predominantly utilized for xDNMT1 recruitment. We next examined the role of xPAF15Ub2 in the recruitment of xDNMT1 and subsequent maintenance of DNA methylation in Xenopus egg extracts. When xPAF15 was almost completely depleted from the extracts (Supplementary Fig. 4a), DNA replication-dependent DNA methylation of sperm DNA was partially suppressed compared to the control (Fig. 4a). Very importantly, although xH3Ub2 was hardly detected in the mock-depleted chromatin in clear contrast to xPAF15Ub2 (Fig. 4b, lanes 1–3), xH3Ub2 was drastically enhanced when xPAF15 was depleted (Fig. 4b, lanes 4–6). Addition of rPAF15WT to the endogenous xPAF15-depleted extracts suppressed the enhanced xH3Ub2, whereas that of rPAF15K18R/K27R failed to do so (Fig. 4b, Supplementary Fig. 4b), suggesting that depletion of xPAF15 was complemented by xH3Ub2. Interestingly, the kinetics of xDNMT1 chromatin loading appeared to correlate with the dual mono-ubiquitylation of either xPAF15 or xH3. Consistent with this, DNMT1 predominantly interacted with PAF15Ub2, not with H3Ub2, on chromatin in mock-depleted extracts, whereas the level of H3Ub2 in the DNMT1 complex significantly increased in the absence of PAF15 (Fig. 4c). Taken together, our results reveal an essential role for PAF15Ub2 in maintenance of DNA methylation, which can only be partially compensated for by H3Ub2.
The C-terminal region of DNMT1 largely increased the binding of the RFTS domain to unmodified H3 and H3Ub2\textsuperscript{35}, it might be possible to preferentially suppress the xH3Ub2 pathway in extracts by supplying an optimal amount of recombinant hRFTS. We estimated that Xenopus interphase egg extracts contained ~0.1 μM of xDNMT1. Addition of 0.6 μM of hRFTS to the extracts resulted in the persistent presence of H3Ub2 on chromatin over the duration of S phase (Supplementary Fig. 4c), presumably due to suppression of xDNMT1 binding to xH3Ub2 and its deubiquitylation by xDNMT1-bound USP7\textsuperscript{25}. Under these conditions, xPAF15Ub2 was also upregulated, but the effect appeared to be transient and much weaker than in the case of xH3Ub2 (Supplementary Fig. 4c). These results suggest that the addition of an optimal amount of RFTS selectively inhibited the xH3Ub2 pathway in terms of its ability to recruit xDNMT1 (Supplementary Fig. 4c), apparently without affecting DNA replication and maintenance of DNA methylation. Importantly, the addition of 0.6 μM of hRFTS and the concomitant depletion of PAF15 dramatically reduced DNA methylation in the egg extracts (Fig. 4d). Consistent with this, chromatin loading of both xPAF15Ub2 and xDNMT1 was readily detectable in extracts in the presence of hRFTS, whereas that of xDNMT1 was not when xPAF15 was depleted (Fig. 4e). Taken together, our results indicate that UHRF1 primarily ubiquitylates PAF15 during S phase, promoting DNMT1 recruitment and subsequent maintenance of DNA methylation in Xenopus egg extracts.

We next addressed whether UHRF1 differentially ubiquitylates PAF15 and histone H3 during S-phase progression. To this end, we depleted endogenous xUHRF1 from interphase egg extracts and supplemented the reaction mixture with rxUHRF1\textsuperscript{WT} at various time points after the addition of sperm chromatin. Subsequently, chromatin fractions were isolated at the indicated time points, and the ubiquitylation of both PAF15 and H3 on the chromatin was assessed (Fig. 4f). rxUHRF1\textsuperscript{WT} added to UHRF1-depleted extracts before the start of DNA replication \((t = 0\) min, Supplementary Fig. 4d, e) did not effectively ubiquitylate PAF15 or histone H3 (Fig. 4g, lanes 1–2). In contrast, when rxUHRF1 was added back in early S phase \((t = 30 \text{ or } 60 \text{ min}, \text{Fig. } 4g, \text{lanes } 3–6, \text{Supplementary Fig. 4d, e})

Fig. 3 PAF15Ub2 forms a complex with DNMT1. A Reciprocal immunoprecipitation of PAF15 and DNMT1 from chromatin lysates. IP was performed with control (Mock), anti-xDNMT1 (DNMT1), or anti-xPAF15 (PAF15) antibody from chromatin lysates. Supernatants after immunoprecipitation (IP-sup) or immunoprecipitates (IP-ppt) were analyzed by immunoblotting using the indicated antibodies. B Sperm chromatin was replicated in interphase egg extracts containing xPAF15-Flag\textsubscript{3} (wild-type, K18R, K27R, or K18RK27R (KRKR)). Isolated and solubilized chromatin proteins were subjected to immunoprecipitation using anti-Flag antibodies. The resultant immunoprecipitates were analyzed by immunoblotting using the indicated antibodies. C Superimposition of plots of enthalpy changes in the interaction between hRFTS and hPAF15\textsubscript{2–30} or its ubiquitylated analogs by ITC measurement. D Pull-down of ubiquitylated PAF15 from denatured chromatin extracts using recombinant wild-type xDNMT1-Flag\textsubscript{3} and its ubiquitin-binding mutants (P253A/L256A or I317AI362A).
Fig. 4 xPAF15Ub2 promotes recruitment of xDNMT1 and maintenance of DNA methylation. a, d Sperm chromatin was added to either mock- or xPAF15-depleted extracts containing radiolabeled S-[methyl-3H]-adenosyl-L-methionine in the absence (a) or presence of 0.6 μM hRFTS (e). The efficiency of DNA methylation was measured at the time points indicated. Bar graphs depict the quantification of incorporated SAM into genomic DNA with mean and SEM from three independent experiments. Statistical significance was determined using Student’s t test. b, e Sperm chromatin was added to mock- or xPAF15-depleted interphase extracts in the absence (b) or presence (f) of hRFTS. PAF15-depleted extracts were supplemented with either buffer alone (lanes 4–6), purified wild-type xPAF15-Flag3 or K18R/K27R(KRKR) mutant xPAF15-Flag3 (320 nM final concentration, lanes 7–9) or 10–12, respectively) in the experiment described in b. At the indicated time points, chromatin fractions were isolated and subjected to immunoblotting using the antibodies indicated. For the PAF15 levels in extracts, see Supplementary Fig. 2a. c Sperm chromatin was replicated in mock- or PAF15-depleted interphase egg extracts. Isolated and solubilized chromatin proteins were subjected to immunoprecipitation using an anti-xDNMT1 antibody. The resultant immunoprecipitates were analyzed by immunoblotting using the indicated antibodies. Asterisks, non-specifically detected proteins. f Schematic of experimental approach to test the differential regulation through UHRF1 during the progression of S phase. g Sperm chromatin was added to xUHRF1-depleted extracts and incubated for 0, 30, 60, 90, 120, or 150 min. Extracts were then supplemented with recombinant xUHRF1-Flag3 and further incubated for 7.5 or 15 min. Chromatin fractions were isolated and chromatin-bound proteins were analyzed by immunoblotting using the antibodies indicated. Source data are provided as a Source Data file.
Fig. 4d, e), it restored PAF15 ubiquitylation and chromatin recruitment. However, the addition of rxUHRF1 WT at later time points (t = 90–150 min, lanes 7–12, Supplementary Fig. 4d, e) failed to restore PAF15 ubiquitylation and instead induced significant histone H3 ubiquitylation. Notably, unlike rUHRF1 WT, the addition of rxUHRF1 D33A/D36A failed to induce histone H3 ubiquitylation under these conditions (Supplementary Fig. 4f). Consistent with the recruitment of DNMT1 via both PAF15 and histone H3 ubiquitylation, we found that DNMT1 loading was restored by the addition of rUHRF1 in both early and late S phase (Fig. 4g). UHRF1 therefore efficiently promotes PAF15 ubiquitylation during early S phase but prefers histone H3 as its substrate in late S phase for DNMT1 chromatin recruitment.

PAF15 is important for maintenance of DNA methylation in mouse embryonic stem cells (mESCs). We had previously shown that murine UHRF1 (mUHRF1) ubiquitylates two neighboring lysines at the N-terminus of mPAF15 (K15 and K24) with a similar spacing as in histone H3, suggesting a similar role in the recruitment of DNMT1 and the maintenance of DNA methylation in murine cells. To investigate the interaction between murine DNMT1 (mDNMT1) and mPAF15 and the role of ubiquitylation, we used CRISPR/Cas9-based gene editing to introduce K15R, K24R, or both K15R/K24R (KRKR) mutations into mPAF15 (Fig. 5d, e). We next co-expressed GFP-mDNMT1 in WT mPAF15 (Fig. 5d, e). We next co-expressed GFP-mDNMT1 in WT mPAF15 and mutant mPAF15 KRKR, we titrated pre-

To assess how mPAF15Ub2 shapes the methylome of mESCs at single-nucleotide resolution, we performed reduced representation bisulphite sequencing (RRBS) on WT and Paf15 KRKR mESCs (Supplementary Table 2). Consistent with our immunofluorescence measurements, RRBS analysis revealed a significant loss of global DNA methylation in Paf15 KRKR mESCs compared to WT mESCs (Fig. 6a, c, Supplementary Fig. 6d). Furthermore, we observed a significant decrease in DNA methylation levels at all genomic regions examined, including repetitive elements, gene bodies, promoters, and CpG islands in Paf15 KRKR mESCs (Fig. 6b). To determine whether mPAF15Ub2-dependent methylation is associated with particular chromatin features, we analyzed the levels of several histone modifications (H3K9me2, H3K9me3, and H3K14ac) at regions differentially methylated in Paf15 KRKR mESCs (p < 0.05 and methylation difference >25%). However, we found neither active nor repressive histone modifications to be enriched at hypomethylated regions resulting from mPAF15Ub2 loss (Supplementary Fig. 6f). We then analyzed how the loss of mPAF15Ub2 affects DNA methylation levels of lamina-associated, late-replicating regions found to be hypomethylated in a multitude of cancer types. These hypomethylated regions, referred to as partially methylated domains (PMDs), differ from the heavily methylated domains (HMDs) comprising the bulk of the remaining genome. Our RRBS analysis demonstrated a stark reduction in DNA methylation at both PMDs and HMDs in Paf15 KRKR mESCs (Supplementary Fig. 6e), suggesting that mPAF15Ub2 contributes to the maintenance of DNA methylation at both PMDs and HMDs in mESCs.

To investigate the relationship between replication timing and mPAF15Ub2-dependent maintenance of methylation, we compared our Paf15 KRKR methylome data with Repli-seq maps from ESCs. Remarkably, regions hypomethylated in Paf15 KRKR ESCs were associated with a significantly earlier replication timing than regions of unchanged DNA methylation, which on average tended to replicate later in S phase (Fig. 6d). These results indicate that mPAF15Ub2 has an essential role in the maintenance of DNA methylation, especially at early replicating sequences, and imply that mH3Ub2 is sufficient to sustain DNA methylation at late replicating regions in the absence of mPAF15Ub2. In contrast to Paf15 KRKR ESCs, the average replication timing of hypomethylated regions in Uhrf1 KO and Dnmt1 KO ESCs was essentially identical to that of regions of...
unchanged DNA methylation (Fig. 6c, f). These results indicate that the complete disruption of maintenance of DNA methylation by genetic ablation of DNMT1 or UHRF1, which abolishes both mPAF15Ub2 and mH3Ub2, leads to genome-wide hypomethylation irrespective of replication timing. Together, these data show that mPAF15Ub2 and mH3Ub2 constitute two distinct pathways of mDNMT1 recruitment that together accomplish complete maintenance of DNA methylation throughout every cell cycle (Fig. 6g).

**Discussion**

Our current study provides clear evidence that PAF15 within DNA replication machinery complexes plays a pivotal role in the maintenance of DNA methylation. We have recently reported that
UHRF1-mediated H3Ub2 recruits DNMT1 to DNA methylation sites, which likely functions independently of DNA replication fork progression. PAF15 in a complex with PCNA also undergoes UHRF1-mediated dual mono-ubiquitylation, which is essential for DNMT1 recruitment, and subsequent maintenance of DNA methylation. Thus, our results suggest that dual mono-ubiquitylation at two lysine residues spaced by 4–9 amino acids (mH3 K14–K18–K23 and mPAF15 K15–K24) in the flexible region of the proteins serves as a specific code for the maintenance of DNA methylation. This notion is supported by the finding that the recognition of PAF15Ub2 by RFTS was very similar to that of H3Ub2.

The fact that UHRF1 targets two distinct proteins, histone H3 and PAF15, for generating a specific code is consistent with the previous report that there are two modes of maintenance of DNA methylation. As to why UHRF1 would have two modes of usage, our results strongly suggest that PAF15Ub2 and H3Ub2 function in different contexts depending on the replication timing, as PAF15 ubiquitylation occurs only during early S phase, whereas histone H3 ubiquitylation can be induced in late S phase. It has been reported that DNA methylation levels are different between early and late replicating domains, with the former containing a much higher degree of DNA methylation than the latter. The enrichment of DNA methylation sites in early replicating domains would explain why cells expressing mPAF15-K15R/K24R have a substantial loss of DNA methylation as observed in mESCs, as well as in Xenopus egg extracts. Although H3Ub2 is markedly increased when PAF15Ub2 is perturbed, it might occur with less efficiency in early replicating domains.

H3Ub2 might also play a dominant role in the recruitment of DNMT1 under particular conditions in which PAF15 is not functional. For example, the replication block induced by ultraviolet (UV) irradiation leads to PAF15 poly-ubiquitylation and subsequent proteasomal degradation. Therefore, replication fork stalling across heterochromatin at late replicating domains might induce PAF15 degradation, which might then be compensated for by H3 ubiquitylation. Alternatively, H3 ubiquitylation could function as a proofreader for the failure of DNA methylation by PAF15Ub2-dependent DNMT1 recruitment. Consistent with this idea, the level of xH3Ub2 on chromatin as well as in complex with xDNMT1 increased upon xPAF15 depletion and the masking of H3Ub2 by RFTS in the absence of PAF15 resulted in an almost complete loss of DNA methylation. Whereas deletion of Dnmt1 or Uhrf1 causes embryonic lethality, it is noteworthy that Paf15 knockout mice remain viable despite abnormal hematopoietic stem cell function. These observations suggest that loss of PAF15 function in the recruitment of DNMT1 could partly be compensated for by histone H3, ensuring the stable inheritance of DNA methylation.

We found that both the interaction with PCNA and dual mono-ubiquitylation by UHRF1 are essential for PAF15 function in the maintenance of DNA methylation. PAF15 is an intrinsically disordered protein and binds to trimeric PCNA via the PIP-box motif at the front face and its N-terminus interacts with the inner ring of PCNA and exits the clamp from the back face, suggesting that the ubiquitylation sites of PAF15 could locate near the nascent strand where a methyl group does not yet exist. Thus, PAF15Ub2 could directly recruit DNMT1 to the back face of PCNA, facilitating the processivity of DNMT1-mediated DNA methylation on the nascent DNA (Fig. 6g). These structural features are also consistent with the fact that early replicating domains contain a much higher degree of DNA methylation at which time PAF15Ub2 is predominantly recruiting DNMT1. We also note that, during the revision of our manuscript, a recent study has also shown that full-length hPAF15Ub2 binds DNMT1 in vitro.

In conclusion, we propose that maintenance of DNA methylation is coordinated with S-phase progression via UHRF1-dependent dual mono-ubiquitylation of two distinct proteins, PAF15 and histone H3, which may contribute to the robustness of DNA maintenance methylation by ensuring the recruitment and activation of DNMT1 (Fig. 6g). Further research is required to clarify how the different modes of DNMT1 recruitment are chosen and to identify potential additional factors contributing to the dual mono-ubiquitylation signaling of DNA maintenance methylation.

**Methods**

**Primers.** All oligonucleotide sequences are listed in Supplementary Table 3.

**Xenopus egg extracts.** Xenopus laevis was purchased from Kato-S Kagaku and handled according to the animal care regulations at the University of Tokyo. Preparation of interphase egg extracts, chromatin isolations, immunodepletions, and UbVS reactions was performed as described previously with minor modifications. Briefly, all extracts were supplemented with energy regeneration mix (2 mM ATP, 20 mM phosphocreatine, and 5 μg/ml creatine kinase). Demembranated sperm nuclei (3000–4000 sperm/μl in the final reaction) were added to egg extracts and incubated at 22 °C. For chromatin spin-down from the egg extracts, sperm nuclei were incubated in 15–25 μl of the extract preparation. The extracts were diluted with ten volumes of ice-cold chromatin purification buffer (CPB; 50 mM KCl, 5 mM MgCl2, 20 mM HEPES-KOH, pH 7.7) containing 2% sucrose, 0.1% NP-40, and 2 mM N-ethylmaleimide (NEM) and kept on ice for 5 min. Diluted extracts were underlayered with 1.5 ml of a 30% sucrose cushion in CPB and centrifuged at 13,000 × g for 10 min at 4 °C using a swing-bucket rotor. The pellets were resuspended in Laemmli sample buffer. For xPAF15 depletion, 290 μl of antisera were coupled to 50 μl of recombinant protein A-sepharose (rPAS, GE Healthcare). Antibody beads were washed three times in phosphate-buffered saline (PBS) and added with 5 μl fresh rPAS. Beads were washed twice in
CPB, split into three portions, and 100 μl extracts were depleted in three rounds at 4 °C, each for 1 h. For xUHRF1 depletion, 170 μl of antiserum were coupled to 35 μl of rPAS. Antibody beads were washed three times in PBS and added with 4 μl fresh rPAS. Beads were washed twice in CPB, split into two portions, and 100 μl extracts were depleted in two rounds at 4 °C, each for 1 h. For xDNMT1 depletion, 250 μl of antiserum were coupled to 50 μl of rPAS. Antibody beads were washed three times in PBS and added with 5 μl fresh rPAS. Beads were washed twice in CPB, split into three portions, and 100 μl extracts were depleted in three rounds at 4 °C, each for 1 h. For add-back experiments, recombinant xPAF15 was added to xPAF15-depleted extracts at 320 nM, recombinant xUHRF1 was added to xUHRF1-depleted extracts at 110 nM, and recombinant xDNMT1 was added to xDNMT1-depleted extracts at 85 nM.

For UbVS reactions, egg extracts were incubated with 20 μM UbVS (Boston Biochem, Cambridge, MA, USA) for 30 min at 22 °C. Sperm nuclei were then added to egg extracts with or without 58 μM ubiquitin (Boston Biochem). For quantification of PAF15Ub2 on chromatin, immunoblot films from three
Fig. 6 mPaf15Ub2 is required for the proper maintenance of DNA methylation in mouse ESCs. a, b DNA methylation levels (%) as measured by RRBS in wild-type (WT) and Paf15f1/K15f2 (KRKR) double mutant ESCs. a Global DNA methylation levels and b CpG methylation levels at CpG islands, promoters, genes, and repeats in wt and KRKR ESCs. p Values based on ANOVA with post hoc Tukey's test. c Density plot depicting the distribution of DNA methylation levels of individual CpG sites in wt and KRKR ESCs. d-f Replication timing of hypomethylated vs. unchanged tiles in Paf15f1 KRKR ESCs. d, f Model of the two pathways of dual mono-ubiquitylation facilitating maintenance of DNA methylation. Both requiring UHRF1, Paf15Ub5 and H3U2b preferentially contribute to the DNMT1-mediated mediation of DNA methylation of early and late replicating regions, respectively. For the boxplots in a, b, d, f, the horizontal black lines within boxes represent median values, boxes indicate the i.5–interquartile range. Source data are provided as a Source Data file.

Recombinant Xenopus proteins. The X. laevis Paf15 cDNA was amplified by PCR from a X. laevis cDNA library using primers 3621 and 3622 and ligated into pT2A vector. For GST–xPaf15 expression, the amplified xPaf15 genes with primers 3667 and 3668 were inserted into linearized pGEX-4T1 vector. Recombinant xPaf15 was expressed in E. coli (Ko11 strain) according to the manufacture's instructions. Protein expression in Xenopus oocytes was induced by the injection of 0.5 mg of recombinant protein immobilized on a nitrocellulose membrane (1:500 dilution for western blots). Rabbit polyclonal antibodies raised against Xenopus DNM1T and UHRF1 have been previously described12,15 (1:500 dilution for western blots). Rabbit polyclonal antibody against human xPCNA (Novus) was affinity-purified with Image J, and the average intensity normalized to UHRF1 was selected as variable modifications. Peptide identification was filtered at a false discovery rate <0.1. Non-label protein quantification was performed using the Precursor Ions Quantifier node in Proteome Discoverer 2.2. The RAW files have been deposited to the ProteomeXchange Consortium51,52.

Pull-down of DNMT-interacting proteins from chromatin. MNase-digested chromatin fractions were prepared as described previously18,19. Briefly, the chromatin was sheared and digested with MNase, and the resulting digestion products were analyzed by SDS-PAGE (10% HEPES-KOH, pH 7.5, 100 mM KCl, 2.5 mM MgCl2, 0.1 mM CaCl2). Two independent experiments were scanned. The pixel intensity of protein bands was then quantified with Image J, and the average intensity normalized to UHRF1 was calculated for each set of conditions. Antibodies against xPaf15 were raised in rabbits by immunization with a GST-tagged recombinant full-length xPaf15. Antisera were further affinity-purified with the recombinant protein immobilized on a nitrocellulose membrane (1:500 dilution for western blots). Rabbit polyclonal antibodies raised against Xenopus DNM1T and UHRF1 have been previously described12 (1:500 dilution for western blots). Rabbit polyclonal antibody against human xPCNA (Novus) was affinity-purified with Image J, and the average intensity normalized to UHRF1 was selected as variable modifications. Peptide identification was filtered at a false discovery rate <0.1. Non-label protein quantification was performed using the Precursor Ions Quantifier node in Proteome Discoverer 2.2. The RAW files have been deposited to the ProteomeXchange Consortium51,52.
containing sperm chromatin. The reaction was stopped by adding 1% SDS, 40 mM EDTA and spotted onto Whatman glass microfiber filters followed by tri-chloroacetic acid (TCA) precipitation with 5% TCA containing 2% phosphotungstate. Filters were washed in ethanol, dried, and TCA-precipitated radioactivity was counted in scintillation liquid.

**Structure of the UHRF1 PHD finger bound to PAFl52-11.** The PHD finger of human UHRF1 (residues 292–366) was expressed as a fusion protein with GST and small ubiquitin-like modifier-1 (SUMO-1) at its N-terminus. Cell culture and purification were performed according to our previous report14,15. Briefly, hPHD was expressed in E. coli Rosetta® (DE3) (Novagen) as a six histidine-tag fusion protein. The protein was further purified by anion-exchange chromatography using a HiTrap Q HP column and SEC using HiLoad 26/60 Superdex75 column (GE Healthcare). The GST-SUMO-1 fusion hPHD was eluted with reduced glutathione at 200 mM NaCl, and 2 mM DTT for 12–24 h at room temperature for completing autocleavage of Npro. The protein was further purified using HiTrap SP HP and HiLoad 16/60 Superdex 75 (GE Healthcare) following previous reports of purification methods.

**In vitro ubiquitylation assay.** Protein expression in E. coli and purification of mouse UBA1 (E1), human UHRF1 (WT and its mutants), and ubiquitin were performed according to the previous reports16,17. E1 enzyme was expressed in E. coli Rosetta 2 (DE3) (Novagen) as a six histidine-tag fusion protein. The protein was purified using TALON™ (Clontech), HiTrap Q HP column, and SEC using HiLoad 26/60 Superdex200 column (GE Healthcare). hiUHRF1 WT and mutants, D334A/D337A and H741A, were expressed in E. coli Rosetta 2 (DE3) as a GST-fusion protein. The protein was purified GST-affinity chromatography of 254 g/mL of human PAFl52 harboring HA-tag at the C-terminus was cloned into the modified pET21b vector, pET-Npr vector18. The Npr-550 fused PAFl52 was purified from the pellet fraction. The inclusion body was then solubilized in buffer containing 8 M urea, 50 mM Tris-HCl (pH 7.5), and 25 mM DTT by stirring overnight at 4 °C. Then the denatured fusion proteins were purified by Ni Sepharose 6 Fast Flow (GE Healthcare). The eluates were dialyzed in a step-wise manner to gradually remove the solution. The urea was additionally incubated with a buffer containing 100 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 2 mM DTT for 12–24 h at room temperature for completing autodegradation of Npr. The protein was further purified using HiTrap SP HP and HiLoad 16/60 Superdex 75 (GE Healthcare).

Standard ubiquitylation reaction mixtures contained 116 μM ubiquitin, 200 mM E1, 65 μM E2, 3 μM E3, 5 mM ATP, and 30 μM PAFl52-HA as a substrate in ubiquitylation reaction buffer (50 mM HEPES [pH 8.0], 150 mM NaCl, 5 mM MgCl2, 0.1% Triton X-100, 2 mM DTT). The mixture was incubated at 30 °C for 30 min and the reaction was stopped by adding 3x SDS loading buffer. The reaction was analyzed by SDS-PAGE, followed by western blotting using 1/2000 diluted anti-HA antibody (MBL, #M180-3).

**SEC-SAXS data collection, processing, and interpretation.** SAXS data were collected on Photon Factory BL-10C using a UPLC ACQUITY (Waters) interfaced with 1200 Series II HPLC system (Agilent Technologies) at a flow rate of 0.25 mL/min at 4 °C. The flow rate was reduced to 0.025 mL/min at an elution volume of 1.63–2.30 mL. X-ray scattering was collected every 20 s on a PILATUS3 2 M detector over an angular range of g = 0.27889 Å–1. UV spectra were recorded every 10 s. Circular averaged SAXS data were carried out using the program SAngler58 to obtain one-dimensional scattering data I(q) as a function of q = (4π/λ)sin(θ/2), where λ is the scattering angle and λ is the X-ray wavelength 1.5 Å). The scattering intensity was normalized on an absolute scale using the scattering intensity of water59. The multiple concentrations of the scattering data around the peak at A280 and I(0) were extrapolated to zero-concentration using a Serial Analyzer60. The molecular weights of samples were calculated from the I(q) data of Ovalbumin (Sigma) at the highest values of A280 and I(0). The radius of gyration Rg and the forward scattering intensity I(0) were estimated from the Guinier plot of I(q) in the smaller angle region of q < 1.3. The distance distribution function P(r) of the sample at the highest peak of A280 and I(0) was calculated using the program GNOM61, where the experimental I(q) data were used in a q-range of 0.00885–0.17670 Å−1. The maximum particle dimension Dmax was estimated from the P(r) function as the distance r for which P(r) = 0. The molecular weight of the sample was estimated by comparing the I(0)/c (where c is the protein concentration) of the sample to that of Ovalbumin.

**Cell culture.** The mESc line J1 was originally provided by the laboratory of Dr. Rudolf Jaenisch (Whitehead Institute). Dmtn1 KO mESCs were described in ref. 62 and Uhrf1 KO mESCs were described in ref. 63. All mESc lines were maintained on 0.2% gelatin-coated dishes in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 16% fetal bovine serum (FBS, Sigma), 0.1 mM b-mercaptoethanol (Invitrogen), 2 mM L-glutamine (Sigma), 1× Minimum Essential Medium non-essential amino acids (Sigma), 100 U/mL penicillin, 100 mg/mL streptomycin (Sigma), recombinant LIF (ESGRO, Millipore), and 2% (1 mM D3P3253 and 1 mM CHIR99021 (Axon Medchem, Netherlands)). Baby hamster kidney (BHK) cells contained a stably integrated lac operator (lacO) array used for the F3H assay were kindly provided by the laboratory of Dr. David L. Spector64. BHK cells were grown in a humidified atmosphere at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 1 mM Gentamycin (Serva GmbH) and 10% FBS (Sigma). All cell lines were regularly tested for mycoplasma contamination.

**CRISPR/Cas9 gene editing and excision.** For generation of Pafl5 K15R and K24R mutant mESCs, specific primers for each desired mutation were cloned into a modified version of the SpCas9-T2A-Puroycin/gRNA vector (pX55965, Addgene plasmid #62988), in which SpCas9 is fused to truncated human Gemini (hGem) to preferentially generate double-strand breaks when homology-directed repair is active66. To generate targeted donors for each desired mutation, single-stranded oligodeoxynucleotides harboring either the K15R or K24R substitution and ~100 bp homologous to the respective genomic locus were synthesized (IDT, Coralville, IA, USA). Cells were transfected with a 4:1 ratio of donor oligo and Cas9/gRNA construct. RNA vector was obtained via cut-ligation. Two days after transfection, cells were plated at clonal density and subjected to a transient puromycin selection (4 μg/mL for 48 h after transfection). Cell lysis in 96-well plates, PCRs of lysates, and restriction digestion were performed as previously described67. Successful insertion of Pafl5 K15R and K24R mutations was confirmed by Sanger sequencing. For generation of the Pafl5 K15R/K24R double-mutant ESC lines, three characterized Pafl5 K24R single mutants were subjected to a second round of gene editing to achieve the K15R substitution as described above.

**Quantitative real-time PCR (qRT-PCR) analysis.** Total RNA was isolated using a NucleoSpin Triprep Kit (Macherey-Nagel) according to the manufacturer’s instructions. cDNA synthesis was performed with a High-Capacity cDNA Reversetranscription Kit (with RNase Inhibitor; Applied Biosystems) using 2 μg of total RNA as input. qRT-PCR assays with the oligonucleotides listed in Supplementary Table 3 were performed in 8 μL reaction volumes with 5 μg of gDNA-free input. For SYBR green detection, FastStart Universal SYBR Green Master Mix (Roche) was used. The reactions were run on a LightCycler480 (Roche).

**Co-IP and western blotting of mouse samples.** For co-IP of Dmtn1, 1.5 × 106 of mESCs were lysed in 250 μL of lysis buffer (10 mM Tris/Cl pH 7.5, 150 mM NaCl, 0.5 mM EIDTA, 0.5% NP40, 1.5 mM MgCl2, 0.5 μg/mL Benzonase (Sigma-Aldrich), 1× Complete Protease Inhibitor Cocktail (Roche), 1× Complete Proteinase Inhibitor Cocktail (Sigma-NEM) at 4 °C for 30 min. Lysates were cleared by centrifugation at 20,000 × g for 15 min at 4 °C, and the protein concentration was measured using Pierce 660 nm

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Protein Assay Reagent according to the manufacturer’s instructions. For DNM1T1 IP, we used an anti-DNM1T1 nanobody (commercial name: DNM1T1-Trap, ChromoTek) which binds to a domain derived from the amino chain of an alpha-antibody raised against DNM1T1. Equal amounts of protein extracts were incubated with 25 µl of DNM1T1 (unlabeled) for 2 h at 4 °C under constant rotation. Beads were washed three times with washing buffer (10 mM Tris/Cl pH 7.5, 150 mM NaCl, 0.5 mM EDTA) and boiled in Laemmli buffer at 95 °C for 10 min. Bound fractions were separated and visualized as a western blot.

To isolate cytoplasmic and nuclear fractions, 2 x 10^7 of mESCs were treated with 400 µl of hypotonic buffer (10 mM Tris-HCl pH 8, 10 mM KCl, 1.5 mM MgCl2, 1 mM DTT, 1 x Protease Inhibitor, 2 mM PMSE, 5 mM NEM, and 0.1% Triton X-100) for 20 min on ice. The cytoplasmic fraction was separated from nuclei by centrifugation at 1300 x g for 10 min at 4 °C, then supplemented with 150 mM NaCl and clarified by centrifugation at 20,000 x g for 15 min at 4 °C. Nuclei were lysed as described above. Anti-mPAF15 antibody (Santa Cruz, sc-390515, 2 µg) was added to the cytoplasmic and nuclear lysate and incubated for 2 h at 4 °C under constant rotation. To precipitate mPAF15-bound proteins, 20 µl of protein G beads (GE17968-06) was added to the lysate for an overnight incubation at 4 °C under constant rotation.

Western blots for mDNMT1 were performed as described previously using a monoclonal antibody (rat anti-DNMT1, 14F8, 1:10 dilution) and a polyclonal antibody (rabbit anti-DNMT1, Abcam, ab17654, 1:2500 dilution). Other antibodies used for detection were mouse anti-PAF15 antibody (Santa Cruz, sc-390515, 1:1500 dilution), polyclonal rabbit-anti-H3 (Abcam, ab1791, 1:5000 dilution), and a monoclonal mouse-anti-tubulin (Sigma, T9026, 1:2000 dilution). The following secondary antibodies conjugated to horseradish peroxidase were used: goat polyclonal anti-rabbit IgG (Sigma, A9044, 1:5000), for detection of horseradish peroxidase-conjugated antibodies, an ECL Plus reagent (GE Healthcare, Thermo Scientific) was used.

Reduced representation bisulfite sequencing. For RRBS, genomic DNA was isolated using a QIAamp DNA Mini Kit (Qiagen), after an overnight lysis and proteinase K treatment. Preparation of the RRBS library was carried out as described previously, with the following flowcharting bisulfite treatment was performed using an EZ DNA Methylation-Gold™ Kit (Zymo Research Corporation) according to the manufacturer’s protocol except that libraries were eluted in 2x 20 µL elution buffer. RRBS libraries were sequenced on an Illumina HiSeq 1500 in 50 bp paired-end mode.

RRBS alignment and analysis. Raw RRBS reads were first trimmed using Trim Galore (v.0.3.1) with the “--rrbs” parameter. Alignments were carried out with the mouse genome (mm10) using basmap (v.2.9.0) and the parameters “-s 12 -v 10 -r 2 -1 l.” Cpg-patent methylation cells were extracted from the mapping output using basmmap methratio.py. Analysis was restricted to Cpg with a coverage >10. A methylKit™ was used to identify differentially methylated regions between the respective contrasts for the following genomic features: (1) repeats identified by Repbase, (2) gene promoters (defined as gene start sites -2 kb/+2 kb), and (3) gene bodies (defined as the longest isoform per gene) and Cpg islands (as defined by ref. 17). Differentially methylated regions were identified as regions with p < 0.05 and a fold change in methylation between two groups >2.5.

Data processing and analysis. Chromatin IP–sequencing reads for H3K9me2,3, H3K4me2,3 and H3K27ac in ESCs and EpiCs were downloaded from GSE60204, GSE293468, and GSE131267, respectively. Reads were aligned to the mouse genome (mm10) with Bowtie (v.1.2.2) with parameters “-a -m 3 -n 3 -best-strata.” Peak calling and signal pile-up was performed using MACs2 callpeak with the parameters “-extsize 150-downsize 200.” Tag densities for 1 kb Tiled detected in RRBS were calculated using custom R scripts. Replication data for mouse ESCs (mm10) for replication timing analysis was downloaded from http://www.replicationdomain.org/69. The average replication timing ratio was calculated over 1 kb Tiled detected in RRBS using custom R scripts. Data of partially methylated domains and highly methylated domains (mm10) was downloaded from https://www.nichd.nih.gov/pmd/ and used to calculate average DNA methylation levels (RRBS) over these regions.

High-throughput immunofluorescence and image analysis. Ecs were grown in 96-well microplates (μClear, Greiner Bio-One), washed with PBS, and fixed with 3% formaldehyde. After three washing steps with PBST, cells were permeabilized (0.5% Triton-X100), treated with denaturing solution (2 N HCl) for 4 min, and incubated with denaturing solution (150 mM Tris-HCl, pH 8.5) for 20 min. Cells were then blocked in 2% bovine serum albumin for 1 h and incubated with primary antibody (mouse-anti 5mC, Diagenode 33D3) for 1 h at 37 °C. After washing three times with PBST, cells were incubated with secondary antibody (goat-anti-mouse coupled to Alexa647, Thermo Fisher) for 1 h at 37 °C. Cells were washed three times with PBST, counterstained with 200 ng/ml 4.6-diamidino-2-phenylindole (DAPI), and finally covered with PBS. Images were acquired by automation with an Operetta High-Content Image Analysis System (PerkinElmer, ×40 high NA objective) followed by analysis with the Harmony software (PerkinElmer). DAPI was used for the detection of single nuclei and 5mC modifications were measured in selected nuclei based on the antibody signal intensity.

F3H assay. The F3H assay was performed as described previously37. In brief, BHK cells containing multiple lac operator repeats were transiently transfected on coverslips using polyethyleneimine and fixed with 3% formaldehyde 24 h after transfection. For DNA counterstaining, coverslips were incubated in a solution of DAPI (200 ng/ml) in PBST and mounted in Vectashield. Cell images were collected using a Leica TCS SP5 confocal microscope. To quantify the interactions within the lac spot, the following intensity ratio was calculated for each cell: (mCherry spot – mCherrybackground)/(GFP spot – GFPbackground) in order to account for different expression levels. The following constructs used in the F3H assay have been described previously: pcAG-eGFP-mDNMT170, pcAG-eGFP-mDNMT1, and pcGFP-LacI17. To generate the mCherry-mPAF15 WT and KRRK expression constructs, the coding sequences of mPAF15 WT and KRRK were excised via Assis and NotI restriction digestion from the GFP-PAF15 WT and KRRK constructs38 and ligation into the pcAG-Cherry IB vector29.

Reporting summary. Further information on research design is available in the Nature Research Reporting linked to this article.

Data availability

The data that support this study are available from the corresponding authors upon reasonable request. The crystal structures of the human UHRF1 PBD in complex with PAF15(2-11) has been deposited in the Protein Data Bank under accession code 6IW7. Sequencing data reported in this paper (wt and PAF15KRKR RRBS) are available at ArrayExpress (EMBL-EBI) under accession number E-MTAB-7930. The mass spectrometric proteomics data have been deposited at the ProteinPilot database via the PRIDE partner repository with dataset identifier PXD015282. The source data under Figs. 1b–e, 2a, 3a, 3b, 4a–f, 5a, 6a, 6f, and Supplementary Figures 1b–d, f, g, a–c, b, d, e, 4a–f, 5c, d, and 6a–c are provided as Source Data files.
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Author contributions
A.N., K.A., H.L., and M.N. conceived the study and experimental design, analyzed the experimental results, and co-wrote the manuscript. A.N., Y.C., S.K., T.K., H.H., G.N., and H.A. performed most of the Xenopus studies. S.K., H.Y., and K.A. analyzed the structural basis of the hPHD:PAF15\_11 and hRFTS:PAF15\_K15Ub/K24Ub complex. A.E., Y.S., and K.T. performed the LC-MS/MS analysis. C.B.M., S.B., W.Q., C.T., and H.L. analyzed mPAF15 and mDNMT1 interaction and DNA methylation using mouse embryonic stem cells.

Competing interests
The authors declare no competing interests.

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