Stella protein facilitates DNA demethylation by disrupting the chromatin association of the RING finger–type E3 ubiquitin ligase UHRF1

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Stella is a maternal gene required for oogenesis and early embryogenesis. Stella overexpression in somatic cells causes global demethylation. As we have recently shown, Stella sequesters nuclear ubiquitin–like with PHD and RING finger domains 1 (UHRF1), a RING finger–type E3 ubiquitin ligase essential for DNA methylation mediated by DNA methyltransferase 1 and triggers global demethylation. Here, we report an overexpressed mutant Stella protein without nuclear export activity surprisingly retained its ability to cause global demethylation. By combining biochemical interaction assays, isothermal titration calorimetry, immunostaining, and live-cell imaging with fluorescence recovery after photobleaching, we found that Stella disrupts UHRF1’s association with chromatin by directly binding to the plant homeodomain of UHRF1 and competing for the interaction between UHRF1 and the histone H3 tail. Consistently, overexpression of Stella mutants that do not directly interact with UHRF1 fails to cause genome-wide demethylation. In the presence of nuclear Stella, UHRF1 could not bind to chromatin and exhibited increased dynamics in the nucleus. Our results indicate that Stella employs a multilayered mechanism to achieve robust UHRF1 inhibition, which involves the dissociation from chromatin and cytoplasmic sequestration of UHRF1.

Stella (also known as Dppa3/PGC7) is a gene that is specifically expressed in primordial germ cells (PGCs), oocytes, and preimplantation embryos (1–3). Genetic ablation of Stella does not affect PGC development or the fertility of male mice, but substantially impairs the fertility of females (4–6). Interestingly, Stella interacts with ubiquitin–like with plant homeodomain (PHD) and RING finger domains 1 (UHRF1) (7, 8), a factor essential for DNA methylation (9, 10), and overexpression of Stella in somatic cells causes genome-wide demethylation (7, 8). As shown in our previous study, Stella is critical for safeguarding the oocyte-specific DNA methylation pattern (8), which was confirmed by a recent report (11).

Mechanistically, Stella sequesters nuclear UHRF1 in growing oocytes and prevents excessive de novo methylation mediated by UHRF1 and its partner protein DNA methyltransferase 1 (DNMT1) (8). Similarly, in human HEK293 cells, overexpression of Stella sequesters UHRF1 and impairs the maintenance of DNA methylation (8). Stella sequesters UHRF1 from the oocyte nucleus through an Exportin1-mediated nuclear export mechanism. Nuclear exclusion of UHRF1 is affected by mutations within Stella that either abolish its nuclear export or disrupt its interaction with UHRF1 (8).

Notably, although ectopic overexpression of Stella also impairs the maintenance of DNA methylation in mouse fibroblast NIH3T3 cells, no prominent cytoplasmic enrichment of UHRF1 was observed under these conditions (7). Thus, in addition to the role for Stella in sequestering UHRF1, other molecular mechanisms that negatively regulate UHRF1 function likely exist.

UHRF1 contains five known functional domains, including a ubiquitin–like (UBL) domain, tandem tudor (TTD) domain, PHD domain, SET and RING-associated (SRA) domain, and a RING–finger E3 ligase domain. The SRA domain recognizes hemimethylated DNA generated during DNA replication (12–14), and the TTD-PhD domains cooperatively recognize the histone H3 tail methylated at lysine 9 (H3K9me2/3) (15–17), both of which enhance the nucleosomal binding of UHRF1 (18, 19). The multivalent engagement of H3K9me2/3 involves the anchoring of the N terminus of the H3 tail to the UHRF1 PHD domain, and the recognition of the methylated K9 by the TTD domain (16–20). In addition to their roles in the chromatin recruitment of UHRF1, both nucleosomal DNA and H3K9
methylation activate the E3 ligase activity of UHRF1 toward histone H3 at a number of lysine residues, including K14, K18, and K23 (21–23). The ubiquitinated H3 is then recognized by DNMT1 and releases the autoinhibition of DNMT1 (21, 22, 24, 25). Consistent with a regulatory model mediated by reciprocal positive allosteric activation, mutations within the PHD or SRA domains dramatically disrupt the chromatin association of UHRF1 and impairs its role in maintaining DNA methylation (15, 16, 18, 19, 23).

Although, deletion of either the PHD domain or SRA domain of UHRF1 impairs its interaction with Stella (7). The detailed biochemical characterization of the Stella-UHRF1 intermolecular interaction and the potential regulatory mechanisms remain to be elucidated. Here, the UHRF1 PHD domain and the C-terminal region of Stella are responsible for the direct Stella-UHRF1 interaction. Moreover, the binding of Stella to the UHRF1 PHD domain disrupts the engagement of both the unmodified and H3K9me3 histone tail by UHRF1 in vitro, as well as UHRF1 chromatin association in vivo. Consequently, Stella is capable of impairing the DNA methylation maintenance function of UHRF1 independent of its nuclear sequestering activity. We propose a multi-layered working mechanism by which Stella regulates UHRF1 function.

Results

A Stella mutant deficient in nuclear export induces global DNA demethylation

We and other researchers have reported that overexpression of Stella in somatic cells induces global passive DNA demethylation (7, 8). In addition, in our previous study, Stella sequestered UHRF1 from nuclei in native mouse oocytes and in HEK293 cells upon ectopic overexpression through an Exportin1-dependent nuclear export mechanism (8). We mutated the nuclear export signal (NES) sequence (L44A/L46A) within Stella and termed this mutant Stella(NESmut) (4, 8); we then overexpressed the mutant in B2-17 cells, a cell line derived from HEK293 cells with a GFP cassette randomly integrated into the genome that is silenced by DNA methylation, to explore whether the nuclear export of UHRF1 is essential for the DNA demethylation process triggered by Stella overexpression (26). Surprisingly, the Stella(NESmut) protein also robustly activated the silenced GFP reporter (Fig. 1A). We extracted genomic DNA from the transfected cells and quantified the level of 5-methylcytosine (5mC) using a UHPLC-MS/MS analysis to determine whether the Stella(NESmut) protein also induced global demethylation (27). Similar to the WT Stella protein, ectopic overexpression of the Stella(NESmut) protein resulted in a marked decrease in the global 5mC level (Fig. 1, B and C). In addition, 5mC immunostaining analysis further confirmed that FLAG-Stella(NESmut) induced a marked reduction of DNA methylation (Fig. 1D). Consistent with our previous report that UHRF1 displayed a prominently nuclear localization pattern in HEK293 cells overexpressing the Stella(NESmut) protein that remained stably bound with UHRF1 (8), our observations here suggested that the Stella protein impaired the function of nuclear UHRF1 independent of the nuclear-sequestering mechanism (8).

We next sought to determine whether the interaction between Stella(NESmut) and UHRF1 was essential for Stella(NESmut)-mediated demethylation. We introduced point mutations into the Stella(NESmut) protein that are known to attenuate its interaction with UHRF1 (8) and analyzed the changes in global 5mC levels in B2-17 cells following overexpression of these mutants. Among the Stella mutants we analyzed, the KRR mutant (K85E/R86E/R87E) and R107E mutant were more potent at disrupting the Stella(NESmut)-UHRF1 interaction than the KR (K113E/R114E) mutant (8). Notably, the capacity of the Stella(NESmut) mutants to induce global demethylation was positively correlated with their binding affinity for UHRF1 (Fig. 1C) (8): the 5mC levels in B2-17 cells expressing the Stella(NESmut) or the KR mutant were markedly decreased, whereas global demethylation was significantly impared in cells expressing the KRR and R107E mutants (Fig. 1C). Consistent with these findings, a Western blot analysis revealed a positive correlation between the level of the GFP reporter in B2-17 cells and the extent of global demethylation (Fig. 1C). Collectively, the interaction between Stella and UHRF1 is required for the DNA demethylation induced by Stella(NESmut).

The interaction between UHRF1 and Stella is mediated by the PHD domain of UHRF1

Although the interaction between Stella and UHRF1 was reported in several studies (7, 8), detailed sequences that directly contribute to this interaction have not been completely characterized. We determined the regions within UHRF1 and Stella that mediate their interaction to obtain further insights into the nuclear export-independent mechanism by which Stella regulates UHRF1. We generated different truncated Stella and UHRF1 proteins to map regions responsible for their interaction (Fig. S1A). We performed glutathione S-transferase pulldown experiments using glutathione S-transferase-tagged recombinant full-length or truncated forms of UHRF1. We observed direct interactions between recombinant truncated Stella and UHRF1 proteins containing the PHD domain after extensive washes with 150 mM NaCl (Fig. S1B) or 500 mM NaCl (Fig. S2A). Both the PHD and SRA domains of UHRF1 interact with Stella (7), but our results clearly indicated that the PHD domain, but not the SRA domain, is responsible for the direct interaction between Stella and UHRF1 (Fig. S1B).

We also generated FLAG-tagged Stella and its truncated forms (Fig. S1A) and then performed pulldown experiments with antibodies against FLAG. We validated the direct interaction between Stella and UHRF1 and identified that the C terminus of Stella was uniquely responsible for this direct interaction (Fig. S2, B–D), consistent with a previous report using cell extracts as the input material for pulldown experiments (7). In addition, we confirmed that mutations in the nuclear export signal did not affect the direct interaction between Stella and UHRF1 (Fig. S2, B–D). Collectively, these results supported the hypothesis that the PHD domain of UHRF1 (UHRF1-PHD) is the main region responsible for its robust interaction with Stella.
Stella disrupts the binding between the UHRF1-PHD and the histone H3(1–18) K9me0 peptide

According to several studies, the PHD domain of UHRF1 is required for its recruitment to chromatin and its role in maintaining genomic DNA methylation (16, 23, 28–30). The UHRF1-PHD was proven to interact with the N-terminal tail of histone H3, with a preference for unmethylated H3R2 (28).

Based on the above reports and our observation of the direct interaction between UHRF1-PHD and Stella (Fig. S1B), we hypothesized that Stella might compete for the binding between the UHRF1-PHD and the histone H3 tail.

To quantitatively compare the strength of the UHRF1-Stella and UHRF1-H3 tail interactions, we performed isothermal titration calorimetry (ITC) experiments to measure the $K_d$ of these interactions. Under 300 mM NaCl concentration, UHRF1-TPS bound Stella with a $K_d$ of 1.70 nM (Fig. 2A), whereas it bound the H3(1–18)K9me0 peptide with a $K_d$ of 15.01 nM (Fig. 2B). These results indicated that UHRF1 had a much higher affinity to Stella than to the unmethylated histone H3 tail.

We then devised two sets of competition assays to test this hypothesis: the blocking assay (a mixture of the H3 tail, truncated UHRF1 proteins, and Stella and an incubation to allow them compete) and the dissociation assay (preform the complex of the H3 tail and truncated UHRF1 proteins, and then compete with Stella) (Fig. 2C). Because the UHRF1-PHD binds the very N terminus of the H3 tail, we synthesized H3(1–18) K9me0/2/3-bio peptides that were biotinylated at the side chain of K18.
Stella dissociates UHRF1 from chromatin

A

Time (min)

0 10 20 30 40

µcal/sec

0 -0.1 -0.3 -0.5

Stella

Kd = 1.70 ± 0.47 µM

Molar Ratio

0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5

B

Time (min)

0 10 20 30 40

µcal/sec

0 -0.1 -0.2 -0.3 -0.4 -0.5

H3(1-18)K9me0

Kd = 15.01 ± 6.40 µM

Molar Ratio

0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5

C Blocking assay

Binding mixture

add in the following order

Stella (with or w/o)
Buffer
Peptide-biotin
BSA
UHRF1
Streptactin-beads

Wash

150 mM, 0.5 % NP40 or 500 mM, 0.5 % NP40

D Dissociation assay

Binding mixture

Peptide-biotin
Buffer
BSA
UHRF1
Streptactin-beads

Wash out unbound
add Stella compete

150 mM, 0.5 % NP40 or 500 mM, 0.5 % NP40

E

150 mM Blocking assay

Biotinylated-peptides pull down

H3(1-18)K9me0-bio
UHRF1-TPS
Stella WT
Stella(NESmut)
Stella N(1-84)
Stella C(85-150)

F

Blocking assay

Biotinylated-peptides pull down

Input

150 mM
500 mM

H3(1-18)K9me0-bio
H3(1-18)K9me3-bio
UHRF1-full length
Stella WT
First, we confirmed that all the aforementioned H3 tail peptides were bound by the UHRF1-TPS (tudor-PHD-SRA) protein, but not Stella (Fig. S3A). Although Stella was reported to bind to H3K9me2 (31), our direct interaction assay did not support this interaction (Fig. S3A), consistent with our previous results (8). We also confirmed that UHRF1-TPS associated weakly with the H3(1–18) peptide that was unmethylated at K9 and strongly with the H3(1–18) peptides methylated at K9, particularly the trimethylated form (Fig. S3A), consistent with the literature (16, 20, 28, 32, 33).

Then, we performed the blocking assay to compare the binding between the H3(1–18) K9me0-bio peptide and UHRF1-PHD in the presence or absence of Stella. The addition of WT Stella, Stella(NESmut), or Stella C(85–150), but not the Stella N(1–84) control, markedly reduced the interaction of the UHRF1-PHD and the H3(1–18) K9me0-bio peptide (Fig. 2D). Similar effects were observed when UHRF1-TPS or full-length UHRF1 were used in the blocking assay (Fig. 2, E and F).

Because the UHRF1-PHD binds the very N terminus of the H3 tail and the tudor domain of UHRF1 (UHRF1-TTD) binds methylated H3K9, both UHRF1-PHD and UHRF1-TTD were able to bind the H3(1–18) K9me3-bio peptide (Fig. S3B). Interestingly, Stella competed for the binding between the H3(1–18) K9me3-bio peptide and UHRF1-PHD, but not UHRF1-TTD (Fig. S3B), consistent with the direct interaction of Stella with the PHD domain, but not the tudor domain, of UHRF1 (Fig. S1B).

We also performed a dissociation assay to examine whether Stella dissociated the preformed complexes between truncated UHRF1 proteins and the H3(1–18) K9me0-bio peptide. As expected, the preformed complexes between the H3(1–18) K9me0-bio peptide and various forms of UHRF1 (UHRF1-PHD, UHRF1-TPS, and full-length UHRF1) were disrupted by the addition of Stella (Fig. 3A and Fig. S4, A and B). Together, these results supported the hypothesis that Stella disrupted the binding of the UHRF1-PHD to the histone H3(1–18) K9me0-bio peptide.

**Stella disrupts the binding between UHRF1 and the histone H3(1–18) K9me3 peptide**

The interaction between UHRF1 and the histone H3 tail is multivalent. UHRF1 also interacts with methylated H3K9 via its TTD domain; notably, the PHD domain of UHRF1 is required multivalently. UHRF1 also interacts with methylated H3K9 via its TTD domain; notably, the PHD domain of UHRF1 is required multivalently. UHRF1 also interacts with methylated H3K9 via its PHD domain, but not the tudor domain, of UHRF1.

We then performed a blocking assay and observed a clear reduction in the binding of the full-length UHRF1 protein or UHRF1-TPS to the H3(1–18) K9me3-bio peptide in the presence of 150 mM NaCl following the addition of Stella, and the effect became even more prominent in the presence of 500 mM NaCl (Fig. 2F and Fig. S4C). Again, this finding was due to direct competitive binding because the interaction between UHRF1-TPS and the H3(1–18) K9me3-bio peptide was disrupted by WT Stella, Stella(NESmut), and Stella C(85–150), but not by Stella N(1–84) (Fig. 3B).

Similarly, Stella disrupted the preformed complex between the H3(1–18) K9me3-bio peptide and UHRF1 (full-length or UHRF1-TPS) (Fig. 3C, and Fig. S4, A and B). The data presented above collectively support a direct competition between Stella and the histone H3 tail, regardless of its K9 methylation state, for binding to UHRF1 in vitro.

**Overexpression of Stella(NESmut) leads to the dissociation of UHRF1 from chromatin in vivo**

We took advantage of the FRAP assay (fluorescence recovery after photobleaching) to determine whether the biochemical observations from the in vitro experiments would be supported in vivo. The chromatin-bound proteins generally display slower molecular mobility and recovery dynamics than the unbound proteins in the FRAP assay.

We established a HEK293-derived cell line stably expressing a GFP-tagged UHRF1 to obtain comparable results. Then, we packaged lentiviruses expressing mCherry-Stella(NESmut) or mCherry, and the GFP-UHRF1 HEK293 cells with these viruses in parallel. Consistent with previous studies (8), the mCherry-Stella(NESmut) and GFP-UHRF1 proteins were mainly located in the nucleus, and the mCherry protein was distributed throughout the cell (Fig. 4A).

FRAP data showed a marked difference in the recovery dynamics of GFP-UHRF1 in mCherry- and mCherry-Stella(NESmut)-expressing cells (Fig. 4, A and B). Strikingly, the GFP-UHRF1 signals in mCherry-Stella(NESmut)-expressing cells were nearly completely saturated at 7 s after bleaching, whereas GFP-UHRF1 signals had barely recovered in the mCherry-expressing cells (Fig. 4A). The fluorescence intensity of GFP-UHRF1 in the bleached region remained much lower than in unbleached regions of control cells expressing mCherry, even at 40 s after bleaching (Fig. 4A). Notably, we needed to bleach a very large area (~1/3 of the nucleus) because the GFP-UHRF1 signal in mCherry-Stella(NESmut)-expressing cells almost immediately recovered otherwise. The statistical analysis also revealed significantly different recovery dynamics of these two groups, as mCherry-expressing cells had a mean $t_{1/2}$ = 15.83 s compared with the $t_{1/2}$ = 1.80 s for the mCherry-Stella(NESmut)-expressing cells (Fig. 4B, $p < 0.0001$, two-tailed $t$ test). The remarkably different recovery dynamics of GFP-UHRF1 in

**Figure 2. Stella blocks the PHD domain and dissociates UHRF1 from H3(1–18) K9me0-bio peptide.** A, quantification of the binding affinity between the UHRF1-TPS and Stella protein ($K_\text{d} = 1.70 \pm 0.47 \mu\text{M}; N_{\text{mean}} = 0.8$) by ITC. B, quantification of the binding affinity between the UHRF1-TPS and H3(1–18) K9me0 ($K_\text{d} = 15.01 \pm 6.40 \mu\text{M}; N_{\text{mean}} = 1.16$) by ITC. All data shown are representative of 3 independent experiments. C, the blocking assay and dissociation assay were performed as shown in the streamlined schematic. D, the binding of UHRF1-PHD to H3(1–18) K9me0-bio peptides was blocked by the WT, NESmut, and C(85–150) Stella proteins, but not the N(1–84) Stella protein, in the presence of 150 mM NaCl. The molar ratios of Stella to UHRF1 were 0, ×1, and ×2. E, the binding of UHRF1-TPS, which mainly relies on the PHD, to H3(1–18) K9me0-bio peptides was also blocked by the WT, NESmut, and C(85–150) Stella proteins, but not the N(1–84) Stella protein, in the presence of 150 mM NaCl. The molar ratios of Stella to UHRF1 were 0, ×1, and ×3.
control cells and cells expressing mCherry-Stella(NESmut) confirmed that Stella overexpression induced the dissociation of UHRF1 from chromatin in vivo.

In addition, we performed a biochemical fractionation assay to extract the nuclear soluble fraction and chromatin-bound fraction in Stella(NESmut)-expressing T-REx-293 cells and control cells. First, we controlled Stella(NESmut) expression using a “Tet-On” system under the control of doxycycline (DOX). Then, we separated different cellular fractions and compared the levels of the UHRF1 protein in the nuclear soluble fraction and chromatin-bound fraction in the presence or absence of DOX. UHRF1 and DNMT1 were largely bound to chromatin before Stella(NESmut) expression was induced with DOX (Fig. 4C). Strikingly, UHRF1 shifted into the soluble fraction upon DOX induction (Fig. 4C). These results again supported the hypothesis that overexpression of the Stella(NESmut) protein dissociated UHRF1 from chromatin. Finally, the Stella(NESmut)-(KRR) protein that was mutated at both the NES- and UHRF1-interacting regions failed to dissociate chromatin-bound UHRF1 (Fig. 4D), further supporting the significance of the direct binding between Stella and UHRF1. Together, the FRAP results and fractionation data support a role for Stella in dissociating chromatin-bound UHRF1 in vivo.

Discussion

In our previous report, Stella safeguarded oocytes from excessive de novo DNA methylation by antagonizing UHRF1 activity (8). Moreover, Stella inhibited UHRF1 function by sequestering it from the nucleus (8). Here, we reveal a novel regulatory mechanism by which Stella dissociates UHRF1 from chromatin through direct competition for the interaction between UHRF1 and the histone H3 tail. Thus, Stella utilizes a double-layer regulatory mechanism involving UHRF1 chromatin dissociation and nuclear export to ensure the suppression of the UHRF1 nuclear function (Fig. 5).

Regarding the competition between Stella and the histone H3 tail, an interesting question is how does Stella effectively compete with the large amounts of histone H3 tails in the nucleus. The interaction between Stella and UHRF1 is relatively strong and is not disrupted by even extensive washes with 750 mM NaCl (Fig. S2D). Moreover, UHRF1 shows a higher binding
affinity to Stella than to H3 tail in our ITC analysis (Fig. 2, A and B). Thus, compared with H3K9me0-containing nucleosomes, Stella likely forms a more stable protein complex with UHRF1 in vivo. In addition, Stella exerts its function only at highly expressed conditions and its mRNA level in oocytes is ~40-fold higher than the UHRF1 mRNA (8).

Although Stella forms a relatively strong protein complex with UHRF1, we previously reported that Stella and UHRF1 are
Stella dissociates UHRF1 from chromatin

not completely co-localized during oogenesis (8). Thus, another unknown mechanism must exist that regulates the dynamics of the Stella-UHRF1 interaction. Importin-5 (IPO5) mediates the nuclear localization of Stella during development (4). Future investigations aiming to determine whether IPO5 or other mechanisms, such as potential posttranslational modifications of Stella or UHRF1, disrupt the interaction between Stella and UHRF1 will be very interesting.

Aberrant DNA methylation frequently occurs in many human cancers (34, 35) and neurological disorders (36–38). DNA methylation inhibitors, such as 5-aza-cytidine and 5-aza-2'-deoxycytidine, are FDA-approved anticancer drugs. However, these inhibitors suppress DNA methyltransferases through direct incorporation into genomic DNA, resulting in severe cytotoxicity, including myelosuppression (4). 5-aza-2'-deoxycytidine (5-aza-dC), which is FDA-approved as a treatment for myelodysplastic syndrome, is FDA-approved as a treatment for myelodysplastic syndrome.

Experimental procedures

Antibodies

Commercial antibodies used in this study include: anti-5-methylcytidine (Eurogentec, number BI-MECY-0500; 1:1000 for immunostaining), anti-FLAG (Beyotime, number AF0036; 1:100 for immunostaining), anti-hDNMT1 (Abmart, number M30106; 1:2000 for Western blotting), anti-UHRF1 (Santa Cruz, number sc-373750, H-8; 1:2000 for Western blotting), anti-FLAG (Abmart, number M20008; 1:2000 for Western blotting), anti-β Tubulin (Santa Cruz, number sc-58866; 1:2000 for Western blotting), anti-PCNA (Santa Cruz, number sc-25280, F-2; 1:1000 for Western blotting), anti-H3 (Protein-tech, number 17168-1-AP; 1:2000 for Western blotting), anti-GFP (Transgen, number HT801, 1:500 for Western blotting), and anti-RFP (MBL, number PM005; 1:1000 for Western blotting).

Cell culture

B2-17 (26), HEK293, and T-REx-293 cells were cultured in Dulbecco’s modified Eagle’s medium (high glucose, HyClone) supplemented with 10% fetal bovine serum (Biological Industries) and 1% penicillin-streptomycin. The T-REx-293 cell line expressing doxycycline-inducible Stella(NESmut) was generated using a lentiviral system as previously described (43).

Transfection and lentivirus infection

Transfections and lentivirus infections were performed using previously described methods (8). Generally, the lentiviral packaging vectors pMDL, pRev, and pVSVG (Invitrogen), together with the expression vector were transfected into 293FT cells growing in dishes coated with poly-D-lysine using VigoFect (Vigorous). The supernatant containing the viral particles was collected 48 h after transfection and then filtered through a 0.45-μm filter (Millipore) after centrifugation at 4,000 rpm for 30 min at 4 °C. The HEK293 cells stably expressing GFP-UHRF1 and cells expressing mCherry-Stella (WT or mutant sequences) were generated using previously reported methods (8).

UHPLC-MS/MS analysis

The UHPLC-MS/MS analysis was performed as previously described (44, 45). The stable isotope 5′-(methyl-d3)-2′-deoxycytidine ([2D3]5mC) was used as an internal standard for calibrating the quantitation of 5mC levels.

Peptide information

All peptides were purified using reverse-phase C18 HPLC (purity >85%) and verified by ESI- and MALDI-MS (Scilight-Peptide). The following peptide sequences were used: H3(1–18) K9me0-bio: ARTKQTARK(me0)STGGKAPRK-(ε-amino-biotin); H3(1–18) K9me2-bio: ARTKQTARK(me2)STGGKAPRK-(ε-amino-biotin); and H3(1–18) K9me3-bio: ARTKQTARK(me3)STGGKAPRK-(ε-amino-biotin).

ITC

UHRF1-TPS and Stella proteins were dialyzed against buffer containing 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 5% glycerol at 4 °C for more than 15 h. H3(1–18) K9me0 peptide was dissolved directly into the same buffer mentioned above. ITC assays were performed with a MicroCal ITC200 (Malvern) at 25 °C. The UHRF1-TPS protein was used at 200 μM to titrate with 14 μM Stella, and H3(1–18) K9me0 peptide at 600 μM was titrated into 30 μM UHRF1-TPS. Data points were normalized by dilution heat, and processed with MicroCal Origin software for curve fitting, dissociation constant, and molar ratio calculations. Data were analyzed by GraphPad Prism 7 Software.

Immunostaining and high-content image analysis

Control T-REx 293 cells and doxycycline-inducible FLAG-Stella(NESmut)-expressing T-REx-293 cells were mixed and subjected to immunostaining after doxycycline treatment for 4 days. Cells were plated on 96-well imaging plates or sterile coverslips in 12-well plates after coated with poly-D-lysine for 3 h. Immunostaining experiments were performed at room temperature. Cells were fixed with 4% paraformaldehyde in PBS for 15 min and treated with 2 x HCl for 30 min, then neutralized with Tris-HCl (pH 8.0). After extensive washing and blocked with 1% BSA, cells were incubated with rabbit anti-FLAG polyclonal antibody and mouse anti-5mC antibody for 2 h and concurrently detected by dye-labeled secondary antibodies (Alexa Fluor 555-labeled anti-rabbit IgG or Alexa Fluor 488-labeled anti-mouse IgG). DNA was stained with Hoechst 33342 (Sigma). The immunostaining samples were analyzed by Olympus FY1000 laser scanning confocal microscope (×60 objective, zoom ×3). The intensity of 5mC signals in FLAG-Stella(NESmut) negative and positive cells were measured and analyzed with the Opera Phenix High-Content Screening System (PerkinElmer Life Sciences, ×60 objective). Data were analyzed by GraphPad Prism 7 Software.
Stella dissociates UHRF1 from chromatin

Pulldown, blocking, and dissociation assays

Proteins, peptides, and beads were incubated in buffer containing 150 mM NaCl (20 mM Tris-Cl, pH 8.0, 1 mM DTT, 5% glycerol, and 0.5% Nonidet P-40), and subjected to extensive washes with 150/500/750 mM NaCl. For protein pulldown assays, the wash buffer was supplemented with 0.5% Nonidet P-40 and 0.2% Triton X-100. For peptide pulldown, peptide blocking, and dissociation assays, the wash buffer was supplemented with 0.5% Nonidet P-40.

We typically used ~1 μM H3(1–18) K9me0/3-bio peptides and 0.2 μM UHRF1 proteins in our pulldown and competition experiments. The relative molar ratio of Stella to UHRF1 proteins were included in each figure legend. In the blocking experiments, reagents were added in the following order: Stella, buffer, peptide, BSA, UHRF1, and streptactin-beads. All the incubations were performed at 4 °C for 12–14 h.

Live-cell imaging and FRAP

Cells were plated onto 35-mm glass bottom dishes (In Vitro Scientific) coated with poly-D-lysine. Live-cell imaging and FRAP experiments were performed using an Olympus FV1200 laser scanning confocal microscope (×60 objective, zoom ×9) in a imaging chamber with a controlled temperature (37°C), CO₂ (5%), and humidity. The bleaching area (5.15 × 11.59 μm) and power were set to constant values to produce even bleach points between biological replicates. Fluorescence recovery was observed in 1-s intervals over 250 s using Olympus FV10-ASW software.

Each FRAP experiment started with a three-time line-average image (pre-bleach) of the cells followed by a bleach (start at 2 s, end at ~2.74 s) to 1/5 of the nucleus with the laser set to full power. The average GFP intensity before photobleaching was set to 1. Fluorescence recovery was measured after a time lapse acquisition after bleaching, and the image size was 256 × 256 pixels. FRAP curves of GFP-UHRF1 were plotted over 150 s. The half-life (t₁/₂) of recovery is termed as the time from the bleach start point (t₀) to the time point where the GFP intensity recovered to approximately half of the original fluorescence intensity. The fluorescence was fit to a curve using nonlinear regression and one-phase exponential association models (46) with GraphPad Prism 7 Software. The t₁/₂ and standard deviation for t₁/₂ were calculated using GraphPad software; p values for the comparison of t₁/₂ values between two groups were calculated using a two-tailed unpaired t test (47).

Cell fractionation

Cell fractionation was performed as previously described (48). Briefly, cells were incubated with buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.34 mM sucrose, 10% glycerol, 1 mM DTT, 0.1% Triton X-100, and 1× protease inhibitor mixture (Roche)) for 8 min on ice. The supernatant (S1) was collected after centrifugation, and the pellet was further incubated with buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, and 1× protease inhibitor mixture) for 30 min on ice. After centrifugation, supernatant S2 was combined with S1 and collected as the soluble fraction; the final chromatin fraction was washed and lysed in a solution containing 10 mM Tris (pH 8.0), 1% SDS, and 5 mM EDTA. The protein fractions were boiled with 2× Laemmli sample buffer for immunoblotting analyses.

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