

Accessibility of Different Histone H3-Binding Domains of UHRF1 Is Allosterically Regulated by Phosphatidylinositol 5-Phosphate

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SUMMARY

UHRF1 is a multidomain protein crucially linking histone H3 modification states and DNA methylation. While the interaction properties of its specific domains are well characterized, little is known about the regulation of these functionalities. We show that UHRF1 exists in distinct active states, binding either unmodified H3 or the H3 lysine 9 trimethylation (H3K9me3) modification. A polybasic region (PBR) in the C terminus blocks interaction of a tandem tudor domain (TTD) with H3K9me3 by occupying an essential peptide-binding groove. In this state the plant homeodomain (PHD) mediates interaction with the extreme N terminus of the unmodified H3 tail. Binding of the phosphatidylinositol phosphate PI5P to the PBR of UHRF1 results in a conformational rearrangement of the domains, allowing the TTD to bind H3K9me3. Our results define an allosteric mechanism controlling heterochromatin association of an essential regulatory protein of epigenetic states and identify a functional role for enigmatic nuclear phosphatidylinositol phosphates.

INTRODUCTION

A major concept that has emerged for the readout of histone modifications is the recruitment of proteins containing conserved domains that specifically interact with defined modifications. Systematic proteomic approaches have implied that individual chromatin modifications are usually recognized by multiple competing proteins (Patel and Wang, 2013). To understand the readout of chromatin marks, it is therefore imperative to dissect how the interplay between modifications and binding factors is regulated. The exact context appears to matter, as

neighboring modifications can influence interaction in a negative or positive manner. Posttranslational modifications of chromatin-associated proteins can directly affect the modification binding properties of chromatin proteins. Further, the expression of specific binding factors is modulated in cell differentiation and development (reviewed by Fischle, 2012).

Since transient changes in cellular states (e.g., nutrition levels) can have a lasting effect on gene expression patterns, it has been suggested that metabolites and small cellular molecules have an immediate impact on the condition of chromatin and the epigenetic state of cells (Lu and Thompson, 2012). Phosphatidylinositol phosphates (PIPs) are particularly interesting in the context of chromatin. A pool of these lipids exists in the cell nucleus, separately regulated from the cell membrane and cytoplasm by dedicated enzymes. However, the exact biochemical state of PIPs in this compartment is still unclear (Barlow et al., 2010; Fiume et al., 2012). The bulk of nuclear phospholipids copurifies with nonhistone chromosomal proteins (Manzoli et al., 1976). Interestingly, analysis of isolated chromatin fractions revealed that lipids associated with hetero- and euchromatin each showed distinct turnover rates (Rose and Frenster, 1965). Furthermore, hydrolysis of nuclear phospholipids by phospholipase C (PLC) was shown to change chromatin structure (Maraldi et al., 1984).

Few nuclear proteins have been found to associate with PIPs so far (Barlow et al., 2010; Fiume et al., 2012). PI4,5P₂ has been shown to activate and stabilize the chromatin remodeling complex BAF (Burgio et al., 2010). The H3K4me3-binding plant homeodomain (PHD) and adjacent polybasic region of ING2 and several related proteins associate with chromatin through a PI5P-mediated mechanism (Bua and Binda, 2009; Gozani et al., 2003; Jones et al., 2006). While this pathway is important in controlling ING2-dependent genes, especially in DNA damage pathways, interaction of H3K4me3 and PI5P are independent of each other (Bua et al., 2013). Binding of PI5P to a PHD domain causes ATX1 to detach from promoters and translocate from the nucleus to the cytosol (Ndamukong et al., 2010). Whereas major biochemical screens have been conducted for PIP-binding factors in the cytoplasmic fraction (Catimel et al., 2009;

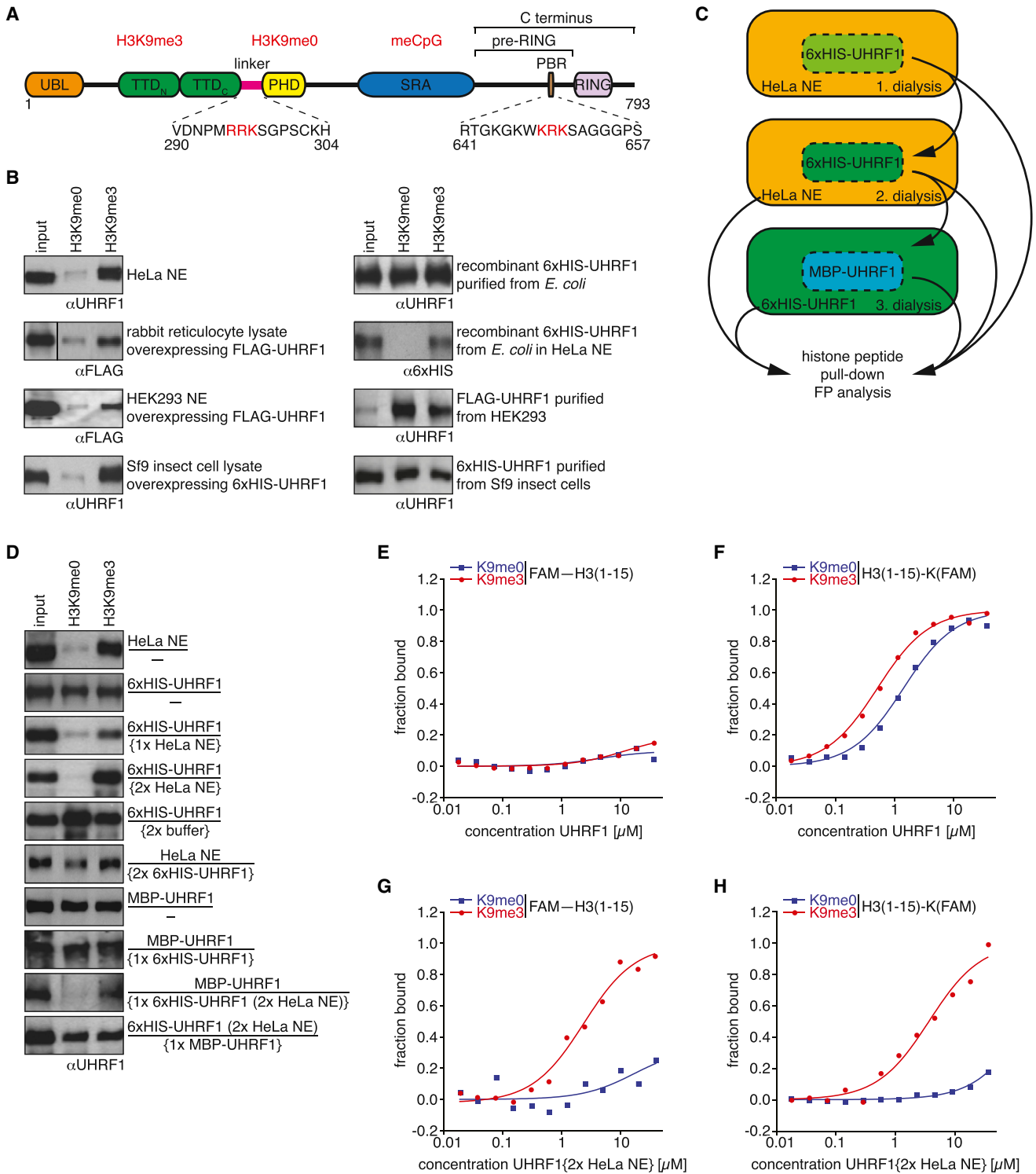


Figure 1. UHRF1 Binding to the Modified or Unmodified H3 Tail Is Regulated by Cellular Cofactors

(A) Domain structure of UHRF1. UBL, ubiquitin-like (aa 1–76); TTD, tandem tudor domain (aa 126–285); PHD, plant and homeodomain (aa 310–366); SRA, SET- and RING-associated (aa 435–586); PBR, polybasic region; RING, really interesting new gene (aa 724–763).

(B) Cellular lysates (NE, nuclear extract) and purified cellular or recombinant proteins were incubated with the specified biotinylated H3(1–20) peptides immobilized on streptavidin magnetic beads. Recovered material was analyzed by western blot. Input, 2%.

(C) Flow scheme for dialysis experiments as analyzed in (D)–(H).

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Pasquali et al., 2007), only one study attempted to define the nuclear interactome of a PIP, namely PI4,5P₂. There, lysine/arginine-rich patches with the consensus K/R-(X)_{n = 3-7}-K-X-K/R-K/R emerged as major interaction motifs (Lewis et al., 2011).

Ubiquitin-like with PHD and RING finger domains 1 (UHRF1), also known as inverted CCAAT box protein of 90 kDa (ICBP90) and NP95 in mouse, is a nuclear multidomain factor implicated in the maintenance of DNA methylation patterns during replication. Deletion of *UHRF1* in mice is embryonic lethal. The knockout embryonic stem cells show loss of DNA methylation, enhanced susceptibility to DNA replication arrest, increased sensitivity to DNA damaging agents, impaired maintenance of higher-order chromatin structure, and spurious transcription of repetitive DNA elements (Bostick et al., 2007; Sharif et al., 2007). *UHRF1* is upregulated in various cancer cells, including breast, prostate, and lung cancer, where it plays a key role in promoting proliferation (Bronner et al., 2010).

UHRF1 is composed of at least five domains: an N-terminal ubiquitin-like domain (UBL), followed by a tandem tudor domain (TTD), a plant homeodomain (PHD), a SET and RING-associated (SRA) domain, and a C-terminal really interesting new gene (RING) domain (Figure 1A). The SRA domain preferentially binds to hemimethylated CpG, which during semiconservative replication of DNA recruits DNMT1 to copy the methylation pattern onto the daughter strand (Berkurek et al., 2014; Rottach et al., 2010; Sharif and Koseki, 2011). This process appears to also involve H3K23 ubiquitylation by the RING (Nishiyama et al., 2013). The isolated TTD binds H3K9me3 (Nady et al., 2011), while the isolated PHD recognizes the unmodified extreme H3 N terminus (Rajakumara et al., 2011). Functional cooperation by these modules was recently suggested by structural and functional studies on isolated TTD-PHD fragments (Arita et al., 2012; Cheng et al., 2013; Rothbart et al., 2013). Both DNA methylation and H3K9me3 are hallmarks of pericentric heterochromatin where UHRF1 is preferentially localized (Liu et al., 2013). UHRF1 is also found in euchromatin and regulates gene expression, particularly the silencing of tumor suppressor genes, possibly through affecting DNA methylation and histone modifications (Bronner et al., 2010; Wang et al., 2012). Posttranslational modification of UHRF1 has been implicated in regulating its stability and possibly directing its interaction with chromatin components (Arita et al., 2012; Ma et al., 2012).

While the interactions of many histone modification-binding domains with their target sites are well studied, it is not always clear if and how the findings on the isolated modules pertain to the proteins and complexes containing them. Here, we analyzed the histone modification binding properties of intact, full-length UHRF1. We found that the protein is allosterically regulated for

interaction with unmodified H3 versus H3K9me3, principally by PI5P, which controls access to the TTD and PHD domains. Our results imply that this mechanism contributes to directing UHRF1 heterochromatin localization and function.

RESULTS

UHRF1 Exists in Different Functional States

To obtain insights into the recognition of histone marks by UHRF1, we performed histone peptide pull-down experiments using the lysate or purified form of the FLAG- or 6×HIS-tagged protein from different expression systems. Endogenous UHRF1 in HeLa cell nuclear extract (NE) exhibited preference for H3K9me3 over the unmodified form (H3K9me0) (Figure 1B). Similar binding specificity was observed for UHRF1-FLAG in vitro translated in rabbit reticulocyte extract or in NE prepared from HEK293 cells overexpressing the fusion protein and for 6×HIS-UHRF1 in total lysate of Sf9 insect cells programmed for expression. In contrast, recombinant 6×HIS-UHRF1 from *E. coli* bound the H3K9me0 and H3K9me3 states equally well. Similarly, the UHRF1-FLAG or 6×HIS-UHRF1 proteins affinity purified from the overexpressing human embryonic kidney 293 (HEK293) or Sf9 cell extracts, respectively, displayed comparable interaction with both H3 tail peptides. Bacterial, recombinant 6×HIS-UHRF1 preferentially bound H3K9me3 when put into HeLa NE. As we observed distinct behavior in multiple expression systems and with different affinity tags at the N and C terminus, we concluded that the proteins in the NE and those that are affinity purified likely exist in different functional states.

UHRF1 Interaction with the Modified or Unmodified H3 Tail Is Regulated by Cellular Cofactors

Similar to previous findings (Nady et al., 2011), we observed that the isolated TTD of UHRF1 specifically binds to H3K9me3 (Figure S1A available online). In contrast, the PHD domain interacted with the extreme H3 tail irrespective of the modification status of the K9 site (Hu et al., 2011; Lallous et al., 2011; Rajakumara et al., 2011; Wang et al., 2011). This behavior was reflected in quantitative fluorescence polarization binding experiments (FP). We used two sets of peptides corresponding to residues 1–15 of the H3 tail with fluorescein attached to either the N or C terminus. Both types of peptides carrying the K9me3 mark bound to the TTD with similar affinity, while no interaction with the unmodified counterpart was observed (Figures S1B and S1C; see Tables S1 and S2 for a listing of all K_D values measured in this study). In contrast, the PHD only bound the C-terminally labeled peptides irrespective of the modification status of K9, but not the

(D) Cellular lysates or recombinant proteins (top) were dialyzed against the indicated sources (bottom) and analyzed by histone peptide pull-down as in (B).

(E) Fluorescence polarization binding experiment using purified recombinant 6×HIS-UHRF1 directly. H3 peptides were linked to fluorescein at the N terminus (FAM-H3[1–15]) via an amide bond.

(F) Fluorescence polarization binding experiment using purified recombinant 6×HIS-UHRF1 directly. A non-natural lysine at the C terminus was added and labeled with fluorescein at the ε-amino group (H3[1–15]-K[FAM]).

(G) Fluorescence polarization binding experiment using purified recombinant 6×HIS-UHRF1 after dialysis against HeLa NE. H3 peptides were linked to fluorescein at the N terminus (FAM-H3[1–15]) via an amide bond.

(H) Fluorescence polarization binding experiment using purified recombinant 6×HIS-UHRF1 after dialysis against HeLa NE. A non-natural lysine at the C terminus was added and labeled with fluorescein at the ε-amino group (H3[1–15]-K[FAM]). See also Figure S1 and Tables S1 and S2.

N-terminally labeled forms. Our findings provided an experimental approach to quantitatively define the interaction properties of UHRF1 and to dissect the contributions of the TTD and PHD within the full-length protein.

Since the observed H3 methylation-specific interaction of UHRF1 in the cell extract resembled that of the TTD, while the methylation nonspecific interaction of purified UHRF1 corresponded to that of the PHD, we reasoned that cellular cofactors direct differential binding modes relying on one or the other module. To test this, we set up a dialysis experiment, consecutively exposing recombinant 6×HIS-UHRF1 purified from *E. coli* to HeLa NE, but separated by a membrane with a very low (3 kDa) molecular weight cutoff (Figure 1C). After one round of dialysis, interaction with H3K9me0 was significantly decreased, while binding to H3K9me3 appeared unaffected (Figure 1D). After two rounds of dialysis, the interaction of the recombinant, dialyzed protein fully resembled that of the endogenous UHRF1 in HeLa NE. In parallel, the endogenous UHRF1 protein in the HeLa NE used for dialysis changed its properties, now binding H3K9me0 and H3K9me3 peptides equally well. Similar behavior was observed when NE from Sf9 insect cells instead of HeLa NE was used, indicating a general phenomenon (Figure S1D).

Quantitative FP measurements confirmed this switch in binding behavior. Purified, recombinant 6×HIS-UHRF1 did not bind N-terminally labeled H3K9me0 or H3K9me3 peptides (Figure 1E). Conversely, the protein interacted with C-terminally labeled H3 peptides with a slight preference for the K9me3 over the K9me0 state (Figure 1F). After two rounds of dialysis against HeLa NE, the properties changed completely. Interaction with H3K9me0 peptides irrespective of labeling at the N or C terminus was lost, while the H3K9me3 mark on both substrates was recognized, indicating that the TTD was responsible for the interaction and that any PHD binding contribution was removed (Figures 1G and 1H). We then performed consecutive dialysis experiments using UHRF1 with different tags (Figure 1D). Recombinant, bacterially expressed MBP-UHRF1, which bound equally well to H3K9me0 and H3K9me3 peptides, was dialyzed against 6×HIS-UHRF1 that had been already dialyzed twice against HeLa NE. Pull-down experiments showed that MBP-UHRF1 could be activated for specific H3K9me3 interaction using this scheme. Overall, the results indicated that small cofactors are transferred from HeLa NE to a recombinant protein, which can also be transferred to another recombinant protein.

Different H3 Tail Binding Modes of UHRF1 Are Mediated by the TTD and PHD Domains

We reasoned that the differential behavior of UHRF1 might be the consequence of allosteric regulation inducing conformational changes of the protein. Therefore, we compared the hydrodynamic properties of purified recombinant UHRF1 before and after dialysis against HeLa NE by analytical ultracentrifugation (Figure 2A). UHRF1 in a matched buffer control had a sedimentation coefficient around 3.7 S. The fit in the continuous c(s, ff0) model suggested that the majority of molecules displayed a frictional coefficient around 1.66 (Schuck and Rossman, 2000). After dialysis against HeLa NE, this distribution was significantly shifted toward lower frictional coefficients, demonstrating that the protein adopted a different overall conformation.

To gain further insights into the regulation of UHRF1, we analyzed a series of mutant recombinant proteins (Figure 2B). Deletion of the TTD had no effect on the interaction of UHRF1 with H3K9me0 and H3K9me3. Deletion of the PHD or mutagenesis of two key residues therein abolished H3 tail binding in pull-down (Figure 2C) and FP experiments (Figures S2A and S2B). The same mutations, however, had no effect on the interaction of the protein dialyzed against HeLa NE with H3K9me3. The results were consistent with the PHD mediating H3K9me0/H3K9me3 interaction in the form of the recombinant protein and the TTD mediating H3K9me3 binding after dialysis against HeLa NE. They also implied that the TTD is unavailable for histone binding in the state of the pure recombinant protein.

Deletion of the C-terminal region resulted in a recombinant UHRF1 protein that specifically recognized the H3K9me3 peptide in pull-down (Figure 2B) as well as FP (Figure 2D) experiments, reminiscent of the wild-type recombinant protein after HeLa NE dialysis. In this construct, the TTD is clearly available for binding the H3K9me3 tail. While a fragment containing only the TTD, PHD, and SRA domains showed similar preference for H3K9me3, the isolated TTD-PHD fragment displayed intermediate behavior. It bound to H3K9me3 and, somewhat weaker, to H3K9me0. FP analysis indicated that this fragment could interact with the C-terminally labeled H3K9me0 and H3K9me3 peptides and also with the N-terminally labeled H3K9me3 peptide, but not with the N-terminally labeled H3K9me0 peptide (Figure S2C). Binding to the C-terminally labeled peptides was overall stronger compared to the N-terminally labeled peptide, and there was a slight preference for the H3K9me3 over the H3K9me0 state. Recent work has suggested a cooperative mode of interaction involving simultaneous recognition of the extreme N terminus of H3 by the PHD and of the K9me3 mark by the TTD in this context (Arita et al., 2012; Cheng et al., 2013; Rothbart et al., 2013; Xie et al., 2012). However, since the full-length protein dialyzed against HeLa NE did not bind the C-terminally labeled H3K9me0 peptide (Figure 1H), meaning that the PHD is not contributing to H3 interaction, but showed binding to the N-terminally labeled H3K9me3 peptide similar to the TTD (Figure S2D), we deduced that the behavior of the isolated TTD-PHD could not explain the binding properties of the full-length protein.

A PBR Sequence in the C-Terminal Domain of UHRF1 Binds to a Peptide-Binding Groove on the Surface of the TTD

Because of the altered methylation specificity of the UHRF1 C-terminal deleted protein, we further investigated the role of this region in regulating H3 tail binding. In immunoprecipitation experiments, the isolated C terminus bound to the isolated TTD (Figure S3A), but not the PHD domains of UHRF1 (Figure S3B) or the FYVE domain of the Eea1 protein (Figure S3C), which belongs to the RING superfamily. Isothermal titration calorimetry deduced a binding strength of the isolated C terminus to the TTD of 4 μ M (Figure S3D).

Additional mapping identified a short region within the pre-RING region of the C terminus that was sufficient for binding the TTD in pull-down experiments (Figure 3A). Due to its high content of basic amino acids, we termed this stretch a polybasic

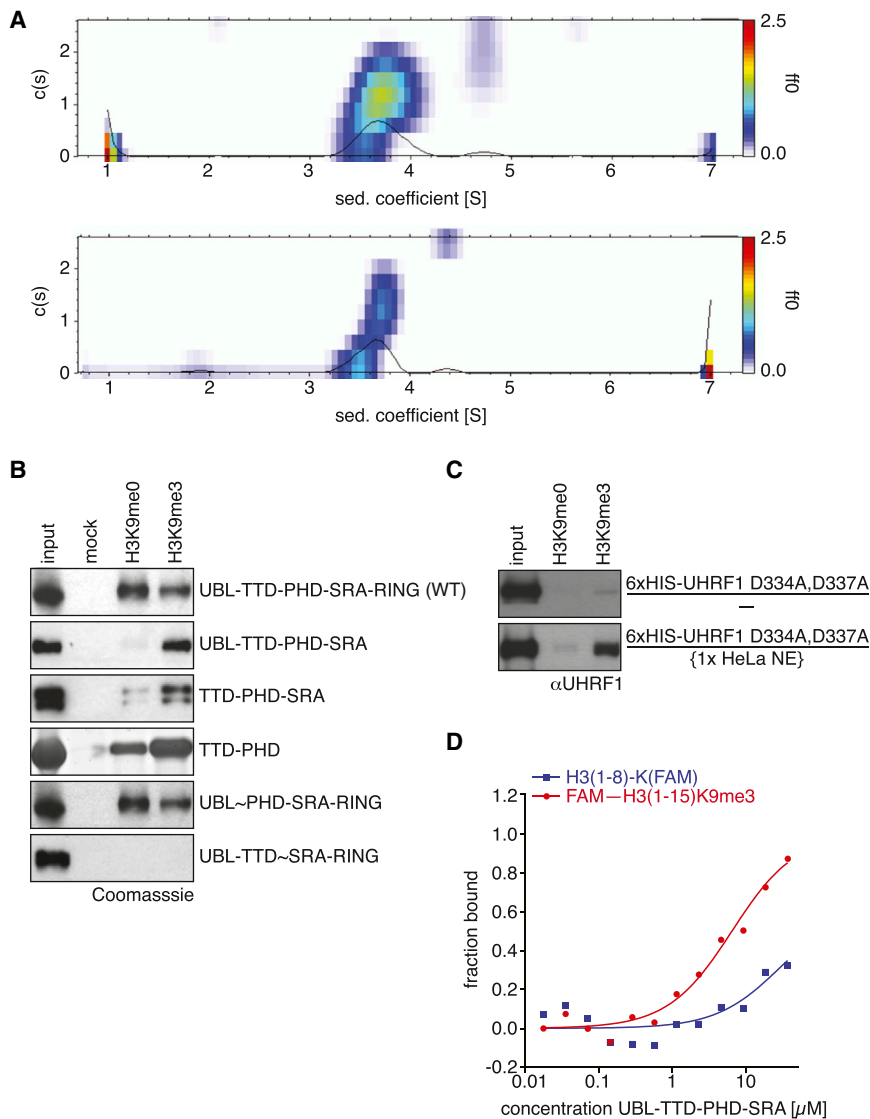


Figure 2. The C Terminus Blocks UHRF1 H3K9me3 Interaction

(A) Recombinant 6xHIS-UHRF1 either dialyzed against buffer (top) or against HeLa NE (bottom) was analyzed by analytical ultracentrifugation. Distribution of sedimentation coefficient (S, x axis), concentration (c[S]; left y axis), and frictional ratio (f/f_0 ; right y axis; intensity of the corresponding sedimentation coefficient is indicated by superposition of the heatmap).

(B) Recombinant proteins were incubated with the specified biotinylated H3 (1–20) peptides immobilized on streptavidin magnetic beads. Recovered material was analyzed by western blot. Input, 2%.

(C) Mutant recombinant UHRF1 protein was either analyzed directly or after dialysis against HeLa NE by histone pull-down experiment as in (B).

(D) Recombinant UHRF1 lacking the C terminus was analyzed in fluorescence polarization binding experiments. See also Figure S2 and Tables S1 and S2.

region (PBR) (Figure 1A). Previous analyses of the cooperative binding mode of the UHRF1 TTD-PHD fragment have implicated the short linker region between these domains in directing the synergistic recognition of H3K9me3 (Arita et al., 2012; Cheng et al., 2013; Rothbart et al., 2013; Xie et al., 2012). A peptide binding groove on the isolated TTD was found to bind to H3 tail peptides with low μM affinity (Nady et al., 2011) as well as to the TTD-PHD linker with relatively weak affinity ($K_D > 100 \mu\text{M}$). Interestingly, the PBR, the TTD-PHD linker region, and the H3 tail all share an RKS sequence motif (Figure 3B). We measured the K_D for binding of the TTD to the PBR at 4 μM , which was much lower than that for the free TTD-PHD linker peptide (Figure 3C) (Rothbart et al., 2013).

In order to further characterize the intramolecular interaction, we performed a nuclear magnetic resonance (NMR) titration of increasing concentrations of the PBR peptide into ^{15}N -labeled TTD. We observed significant chemical shift changes indicative of an interaction in the micromolar range (Figure S3E). Chemical

the histone H3 peptide and the TTD-PHD linker, the observed chemical shift perturbations were more extensive, involving extra surface residues such as K187, E193, L225, I211, and D275 (Figures 3D and S3F).

To better understand how the PBR interacts with the TTD, we performed docking calculations with HADDOCK, which uses NMR chemical shift changes to guide the simulations (de Vries et al., 2007; Dominguez et al., 2003). Two plausible models of the docked PBR were deduced. These differed in the direction of the bound PBR peptide (Figure 3E). Model 1 suggested an important contribution of PBR residues R649 and K650, while model 2 implicated residues K648 and R649 for binding to the TTD. Mutagenesis analysis of PBR peptides by FP indicated an essential contribution of R649 (Figure 3F). Mutagenesis of K648 had a stronger effect compared to mutagenesis of K650. In contrast, mutagenesis of residues K644 and K646 had no effect on the interaction. Similar results were obtained for the TTD-PHD fragment (Figure S3G). While we were not able to

shift changes larger than 0.094 ppm were concentrated around the groove formed at the interface between the two tudor domains (Figure S3F). This region was previously shown to bind the sequence N-terminal to the methylated lysine in the complex of the TTD with H3K9me3 (Nady et al., 2011). It also accommodates the linker in the crystal structure of the H3K9me3/TTD-PHD complex, thereby directing the cooperative binding mode engaging both modules (Arita et al., 2012; Cheng et al., 2013; Rothbart et al., 2013; Xie et al., 2012). We therefore refer to this region of the TTD as the peptide-binding groove (Figure 3D). Although the PBR can bind to the same TTD peptide-binding groove as

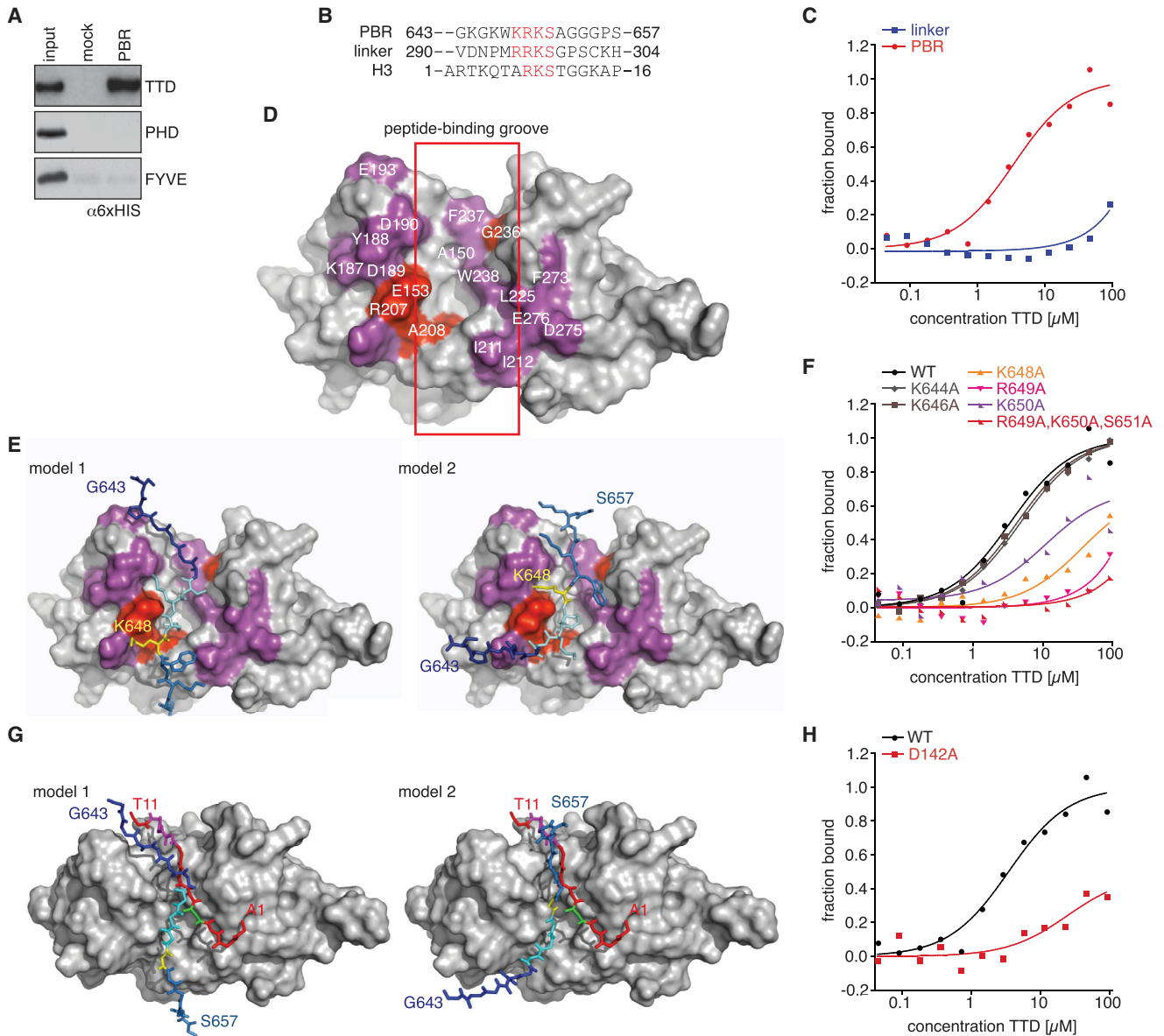


Figure 3. The PBR Sequence of the Pre-RING Region Binds a Peptide Groove on the Surface of the TTD

(A) TTD or PHD domains of UHRF1 or the FYVE domain of Eea1 were incubated with a biotinylated PBR peptide immobilized on streptavidin magnetic beads or beads alone (mock). Recovered material was analyzed by western blot. Input, 2%.

(B) Sequence comparison of the UHRF1 PBR and linker regions with the H3 tail.

(C) TTD of UHRF1 was analyzed in fluorescence polarization binding experiments with peptides corresponding to the UHRF1 linker or PBR (FAM at the N terminus).

(D) Surface model of the TTD (Protein Data Bank ID [PDB] 2L3R). Residues with chemical shift changes >0.094 ppm upon titration of the PBR peptide are shown in purple; those lining the TTD peptide-binding groove (red box), which have disappeared upon titration of the PBR peptide, are shown in red.

(E) HADDOCK models of likely positions of the PBR peptide in the TTD peptide-binding groove. K648 is highlighted in yellow as a visual guide of the orientation of the peptide; R649, K650, S651 is shown in cyan; the N and C termini of the peptide are shown in blue and dark blue, respectively. Color codes of residues showing major changes upon addition of the PBR peptide are as in (D).

(F) TTD of UHRF1 was analyzed in fluorescence polarization binding experiments with the indicated wild-type (WT) or mutant FAM-PBR peptides.

(G) Overlay of the positioning of the H3 tail (red; K4 residue in green, K9 residue in purple) as determined in the TTD/H3K9me3 complex (PDB 2L3R) and the PBR sequence in model 1 and model 2 (color code as in E). Images were generated by PyMOL.

(H) Wild-type (WT) or D142A mutant TTD of UHRF1 was analyzed in fluorescence polarization binding experiments with WT FAM-PBR peptide. See also Figure S3 and Tables S1 and S2.

unambiguously discriminate between the two binding modes for the TTD/PBR interaction, the mutagenesis results favored model 2. Comparison of the TTD/PBR complex with the TTD/H3K9me3 (Figure 3G) or the TTD/linker (Figure S3H) complexes indicated a mutually exclusive binding mode.

While methylated K9 of H3 within the RKS motif binds in a pocket formed in the TTD, the RKS sequence of the PBR does not interact in the same region. Instead, the UHRF1 PBR is centrally located in the TTD peptide-binding groove. Indeed, mutagenesis of two caging aromatic acid residues implicated in methyl-lysine binding in the TTD/H3K9me3 complex had only a very limited effect on TTD/PBR interaction (Figure S3I). In both NMR models, R649 engages in hydrogen bonding with D142, and mutagenesis of this residue significantly impaired binding of the PBR to the TTD (Figure 3H). In model 2, K648 engages in hydrogen bonding with D189 and D190. These are critical residues in the TTD peptide-binding groove that interact with R296 of the anchored TTD-PHD linker and with T6 of the histone H3K9me3 peptide, respectively (Arita et al., 2012; Nady et al., 2011). While mutagenesis of R295 and R296 of the linker within the TTD-PHD fragment had no effect on PBR interaction, it resulted in a slight increase in binding to free linker peptide (Figure S3J).

The PBR Sequence Is Sufficient for Blocking H3K9me3 Binding of the TTD

Next, we investigated the role of the C-terminal domain of UHRF1 in regulating H3K9me3 binding of the TTD. While the C terminus did not show specific interaction with H3K9me0 or H3K9me3 peptides (Figures S4A and S4B), it severely reduced recovery of the TTD on H3K9me3 beads when titrated into histone peptide pull-down experiments (Figure 4A). Likewise, the C-terminal domain of UHRF1, but not the FYVE domain of Eea1, blocked TTD/H3K9me3 interaction in FP experiments (Figure 4B). Similar results were obtained for the TTD-PHD, TTD-PHD-SRA, and UBL-TTD-PHD-SRA recombinant fragments of UHRF1 (Figures S4C–S4E).

Titration of H3K9me3, but not H3K9me0 peptide, into an immunoprecipitation of the C terminus resulted in significantly reduced recovery of the TTD (Figure 4C). These results were confirmed using only the PBR region of the C-terminal domain. In FP experiments, the PBR peptide blocked binding of the TTD to the H3K9me3 peptide (Figure 4D). Mutagenesis of the K644 and K646 residues that were not implicated in the binding of the PBR to the TTD peptide-binding groove had only a limited effect. While mutagenesis of the K650 residue somewhat reduced the blocking effect of the PBR peptide, changing the individual K648 or R649, or the combined R649, K650, and S651 sites, resulted in severe loss of blocking. Similar results were obtained for the TTD-PHD (Figure S4F).

Only the PBR, but not the much weaker binding linker peptide, blocked interaction of the TTD with H3K9me3 (Figure S4G). This effect was abrogated in the D142A mutant (Figure S4H). Moreover, the PBR peptide competed with the N-terminally labeled H3K9me3, but not the C-terminally labeled H3K9me3 or H3K9me0 peptides, for binding to the TTD-PHD (Figures S4I–S4K). The results supported the idea that binding of either the H3K9me3 or PBR peptides to the TTD are mutually exclusive

and independent of linker function as well as PHD binding to the extreme N terminus of H3. Presence of an H3K9me3 peptide compromised the interaction of the TTD (Figure 4E) or TTD-PHD (Figure S4L) with the PBR, while a H3K9me0 peptide had a much reduced effect.

Next, we designed multiple UHRF1 protein constructs with mutations in the PBR region. While mutagenesis of K650 had no effect, the R649A mutant showed increased binding to H3K9me3 in FP experiments (Figure 4F). This effect was stronger when, in addition, the K650 and S651 or the K644, K646, K648, K650, and S651 residues were also mutated. The same mutations had only little effect on the interaction of UHRF1 with an H3K9me0 peptide (Figure S4M). Similarly, we found that the UHRF1 K644A, K646A, K648A, R649A, K650A, S651A mutant protein had increased binding to H3K9me3 in pull-down experiments (Figure 4G). While the results potentially implied that there are additional interfaces between the TTD and C terminus, they supported a blocking effect of the PBR in the context of the full-length UHRF1 protein.

The PBR of UHRF1 Interacts with PI5P

To isolate the small cellular cofactor of UHRF1, we set up a chromatographic purification scheme (Figures S5A and S5B). Dialyzed, recombinant UHRF1 and the associated factors from the HeLa NE (all presumably <3 kDa) were separated by reversed-phase chromatography. Individual fractions were lyophilized and then tested for their ability to induce H3K9me3-specific binding of full-length recombinant UHRF1. The activating factor(s) could be separated by step-wise elution at 50% acetonitrile from a C8 column (Figure S5C). While it was possible to further refine the purification scheme and to combine different chromatography methods, several attempts to identify the activating factors by mass spectrometry failed.

In order to gain insights into the nature of the compounds from another direction, we defined the region of UHRF1 binding the NE factor(s). To this end, different domains of UHRF1 were subjected to dialysis against HeLa NE. Material eluting at 50% acetonitrile in our purification scheme was analyzed in histone tail pull-down experiments with recombinant UHRF1. The fragments of UHRF1 containing the C-terminal domain (WT, UBL-TTD~SRA-RING, C terminus), but not those without the C terminus (UBL-TTD~SRA, UBL-TTD-PHD~SRA), were able to recruit factor(s) that induced H3K9me3-specific binding (Figure 5A). Indeed, dialysis of the C terminus against HeLa NE relieved its inhibition of the interaction between the TTD and H3K9me3 (Figure 5B), while dialysis of the TTD itself had no effect (Figure 5C).

Upon a closer look at the PBR sequence within the C terminus, we realized that it contains a motif (K/R-(X)_{n=3-7}-K-X-K/R-K/R) that had been implied in binding PIPs (Lewis et al., 2011). Indeed, treatment with phospholipase A2 (PLA2) that hydrolyzes PIPs abolished the capability of HeLa NE to activate H3K9me3-specific binding of recombinant UHRF1 (Figure 5D). We then tested recombinant UHRF1 for interaction with different PIPs, its precursors, and related compounds in standard lipid dot blot assays (Figures S5D and S5E). Similar to the PHD domain of ING1, UHRF1 bound PI5P in these assays (Bua and Binda, 2009).

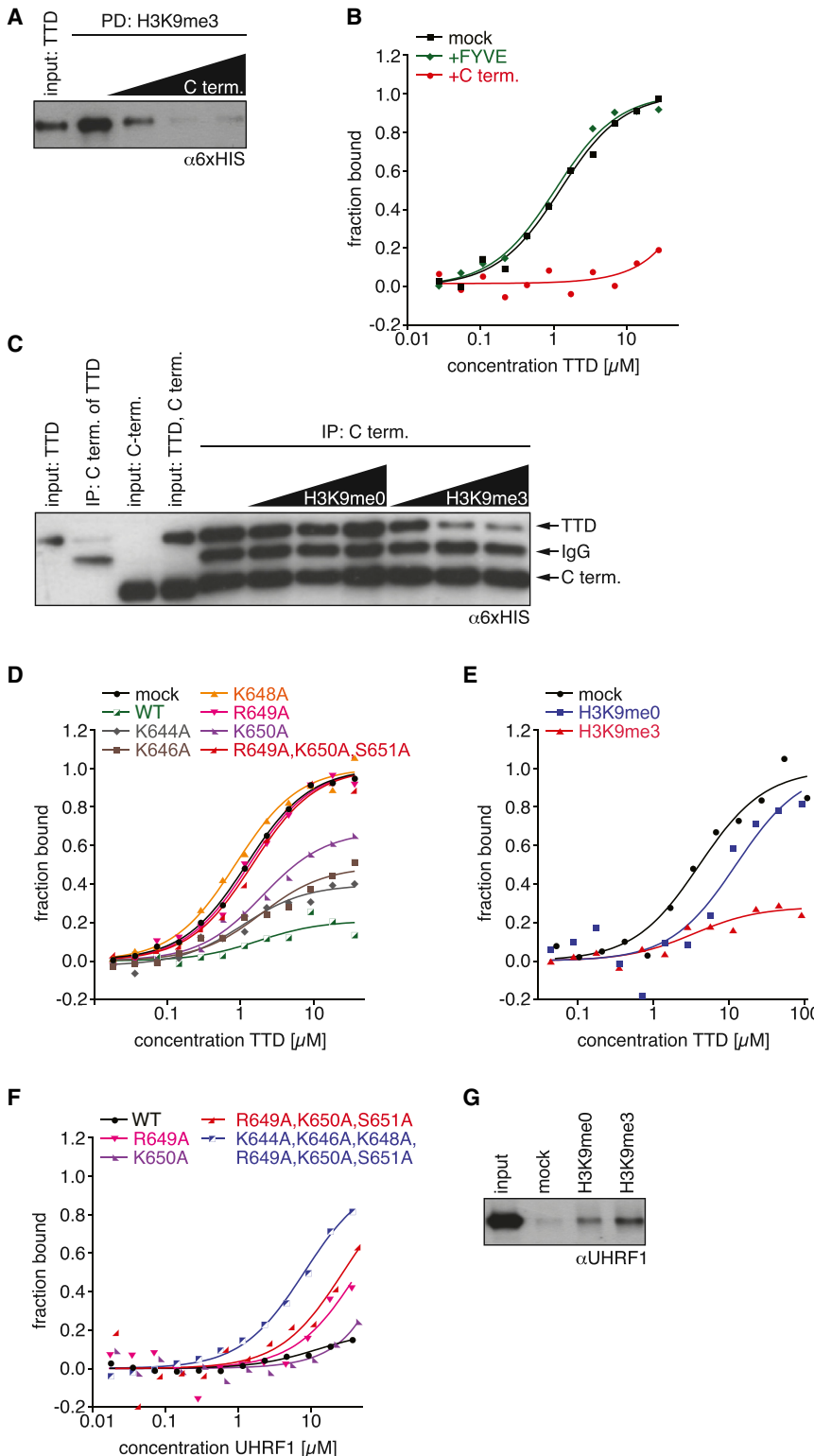


Figure 4. The PBR Blocks H3K9me3 Binding of the TTD

(A) TTD was incubated with increasing concentrations of the C terminus (molar ratio TTD:C term. = 2:1, 1:1, 1:1.5) and analyzed in pull-down (PD) experiments using an immobilized H3K9me3 peptide. Recovered material was analyzed by western blot. Input, 2%.

(B) TTD alone (mock) or in the presence of the C terminus of UHRF1 or the FYVE domain of Eea1 (1:2 molar ratio) was analyzed in fluorescence polarization binding experiments with FAM-H3(1–15)K9me3 peptide.

(C) C terminus was incubated with the TTD in the presence of increasing concentrations of the indicated H3(1–20) peptides (molar ratio TTD:C term.:peptide = 1:1:1, 1:1:2.5, 1:1:5, 1:1:7.5) and immunoprecipitated using antibodies against UHRF1. Recovered material was analyzed by western blot. Running positions of proteins recognized by the primary and/or secondary antibodies are indicated. Input, 5%.

(D) TTD alone (mock) or in the presence of the indicated PBR wild-type (WT) or mutant peptides (1:5 molar ratio) was analyzed in fluorescence polarization binding experiments with FAM-H3(1–15)K9me3 peptide.

(E) TTD alone (mock) or in the presence of the indicated H3(1–20) peptides (1:5 molar ratio) was analyzed in fluorescence polarization binding experiments with FAM-H3(1–15)K9me3 peptide.

(F) Wild-type (WT) UHRF1 or UHRF1 carrying the indicated mutations was analyzed in fluorescence polarization binding experiments with FAM-H3(1–15)K9me3 peptide.

(G) Mutant UHRF1 K644A,K646A,K648A,R649A, K650A,S651A was analyzed in a pull-down experiment using the specified biotinylated H3(1–20) peptides immobilized on streptavidin magnetic beads or beads alone (mock). Recovered material was analyzed by western blot. Input, 2%. See also Figure S4 and Tables S1 and S2.

As with ING1, additional interactions with other lipids (PI5P > PI3P = PA > PI3,5P₂ = PI4,5P₂ > PS) were also observed. As anticipated, no interaction of the H3K9me3-binding HP1β protein with any of the lipids was seen.

tained on liposomes containing PI5P. No binding to liposomes containing PI3P, PI4P, PI3,5P₂, or PI4,5P₂ was observed.

Mapping of the interaction using lipid dot blots as well as liposome flotation assays defined the C terminus and, within that,

the PBR-containing pre-RING region as the PI5P binding domain of UHRF1 (Figures 5G, S5E, and S5F). Mutagenesis of R649, K650, and S651 within the PBR was sufficient to severely reduce binding of full-length UHRF1 as well as the pre-RING region to PI5P. Lastly, we compared binding of fluorescently labeled wild-type and R649A,K650A,S651A mutant PBR peptides to biotinylated PIPs immobilized on streptavidin beads (Figures 5H and S5G). A clear preference of the wild-type PBR for PI5P over PI, PI3P, PI4P, PI3,5P₂, and PI4,5P₂ was observed.

PI5P Regulates UHRF1 Interaction with the Modified or Unmodified H3 Tail

When titrated into immunoprecipitations of the C-terminal domain with the TTD, PI5P, but not PI4P, blocked the interaction (Figure 6A). Similarly, PI5P, but not PI4P, reduced the recovery of the TTD on the PBR peptide in pull-down experiments (Figure 6B).

We then asked whether PI5P could modulate H3 tail binding of UHRF1. In histone pull-down experiments, PI5P induced H3K9me3-specific binding of recombinant UHRF1 (Figure 6C). While PI3P also showed some activation, neither PA, PI, PI4P, PI3,5P₂, nor PI4,5P₂ had an effect. All attempts with PI5P containing different acyl chain lengths to induce H3K9me3-specific binding of UHRF1 in the FP-based experiments in solution failed. Also, the isolated inositolphosphate head groups of the PIPs had no effect (not shown).

To further investigate whether PI5P is indeed the cellular molecule regulating UHRF1, we made use of a specific kinase. PIP4K α transduces together with PIP4K β to the cell nucleus and phosphorylates PI5P on the 4-position, thereby yielding PI4,5P₂ (Bultsma et al., 2010). Addition of PIP4K α and ATP to an H3 tail pull-down reaction of recombinant UHRF1 abolished the activating function of PI5P (Figure S6). ATP alone had no effect, and the kinase did not alter the outcome with PI4P in this assay. We then incubated HeLa NE or a buffer control with PIP4K α and either ATP or the nonhydrolysable ATP γ S. UHRF1 was dialyzed against the treated lysate or buffer and analyzed in H3 tail pull-down experiments. Pretreatment of the HeLa NE with ATP and PIP4K α abolished the activating effect of the lysate (Figure 6D). Incubation of the HeLa NE with ATP alone or PIP4K α and ATP γ S also slightly induced some H3K9me3-specific binding of UHRF1. In contrast, pretreatment with ATP γ S had no effect. We think ATP and PIP kinase(s) present in the NE might account for these observations. No effect was observed with the buffer control, and there was no differential binding of the recombinant UHRF1 to H3K9me0 and H3K9me3. Altogether, the results indicated that PI5P is a cellular coactivator of UHRF1, allosterically regulating binding to different modification states of the H3 tail.

PI5P Directs TTD-Dependent UHRF1 Localization to Heterochromatin

Previous studies have found both strong and limited localization of UHRF1 to H3K9me3-enriched heterochromatin (Karagianni et al., 2008; Nady et al., 2011; Papait et al., 2007). We hypothesized that these discrepancies might be due to different cellular systems and conditions analyzed. We found that a large fraction (58%) of NIH 3T3 cells transiently expressing

mCherry-UHRF1 showed colocalization of the fusion protein with DAPI-dense and H3K9me3-positive foci of pericentromeric heterochromatin. Lower enrichment at these regions (intermediate) was seen for 29% of cells, while 13% of cells displayed diffuse nuclear distribution (Figure S7A). Mutation of two residues in the aromatic cage implicated in methyl-lysine binding in the TTD/H3K9me3 complex resulted in significantly more cells (64%) showing intermediate and diffuse phenotypes, while 36% of cells showed dotted UHRF1 Y188A,Y191A distribution (Figure S7B). The results suggested that other domains of the protein besides the TTD are also involved in heterochromatin localization. Indeed and in agreement with recent findings (Liu et al., 2013), after mutation of both the TTD and SRA domains, no heterochromatin localization of mCherry-UHRF1 was detected (Figure S7C). While the exact regulation of UHRF1 subnuclear and chromatin association is unclear, the protein might rely on different domains for target binding, and there could be a cell-cycle dependency (Papait et al., 2007).

To determine the regulatory role of the PBR, we coexpressed GFP-tagged wild-type or mutant (K644A,K646A,K648A,R649A,K650A,S651A) pre-RING domain together with mCherry-UHRF1 in NIH 3T3 cells (Figures 7A and S7D). We thought this region might exert dual effects by simultaneously interfering with TTD/H3K9me3 binding as well as by titrating endogenous PI5P. Quantification of the different phenotypes indicated that an average of 12% of cells exhibited a shift in their mCherry-UHRF1 distribution from dotted to intermediate or diffuse appearance upon expression of wild-type GFP-pre-RING. This effect was not seen for coexpression of mCherry-UHRF1 with the mutant GFP-pre-RING (Figures 7B and S7E).

To further establish a regulatory role of PI5P for UHRF1, we made use of the PIP4K kinases to manipulate nuclear levels of the cofactor. Consistent with the phenotypes of pre-RING overexpression, coexpression with MYC-PIP4K α and PIP4K β (PIP4K WT) relative to coexpression of empty vector resulted in an average of 9% fewer cells displaying the nuclear dotted pattern of mCherry-UHRF1. In contrast, kinase mutant PIP4Ks (PIP4K α G131L,Y138F and PIP4K β D278A) had no such effect (Figures 7C and S7F–S7H). Given that only a fraction of cells in the population (on average 22%) relied on the TTD for localization to pericentric heterochromatin, we deduced from these experiments that PI5P binding to the PBR has a marked impact (~40%) on heterochromatin association of this UHRF1 subpopulation.

DISCUSSION

Regulation of UHRF1 H3 Tail Binding by Conformational Rearrangements Allosterically Induced by PI5P

By identifying a mode of regulation of UHRF1 by a cellular cofactor, our findings clarify discrepancies in the literature regarding the binding activities and domain usage of this important epigenetic regulator. Various reports used different experimental systems (i.e., protein expression in bacteria, which do not have PIPs, versus expression in insect cells, reticulocyte lysate, or mammalian cells, which all contain PIPs) and investigated full-length, deletion mutants, or point mutants of UHRF1 proteins. Distinct domains were implicated in binding to

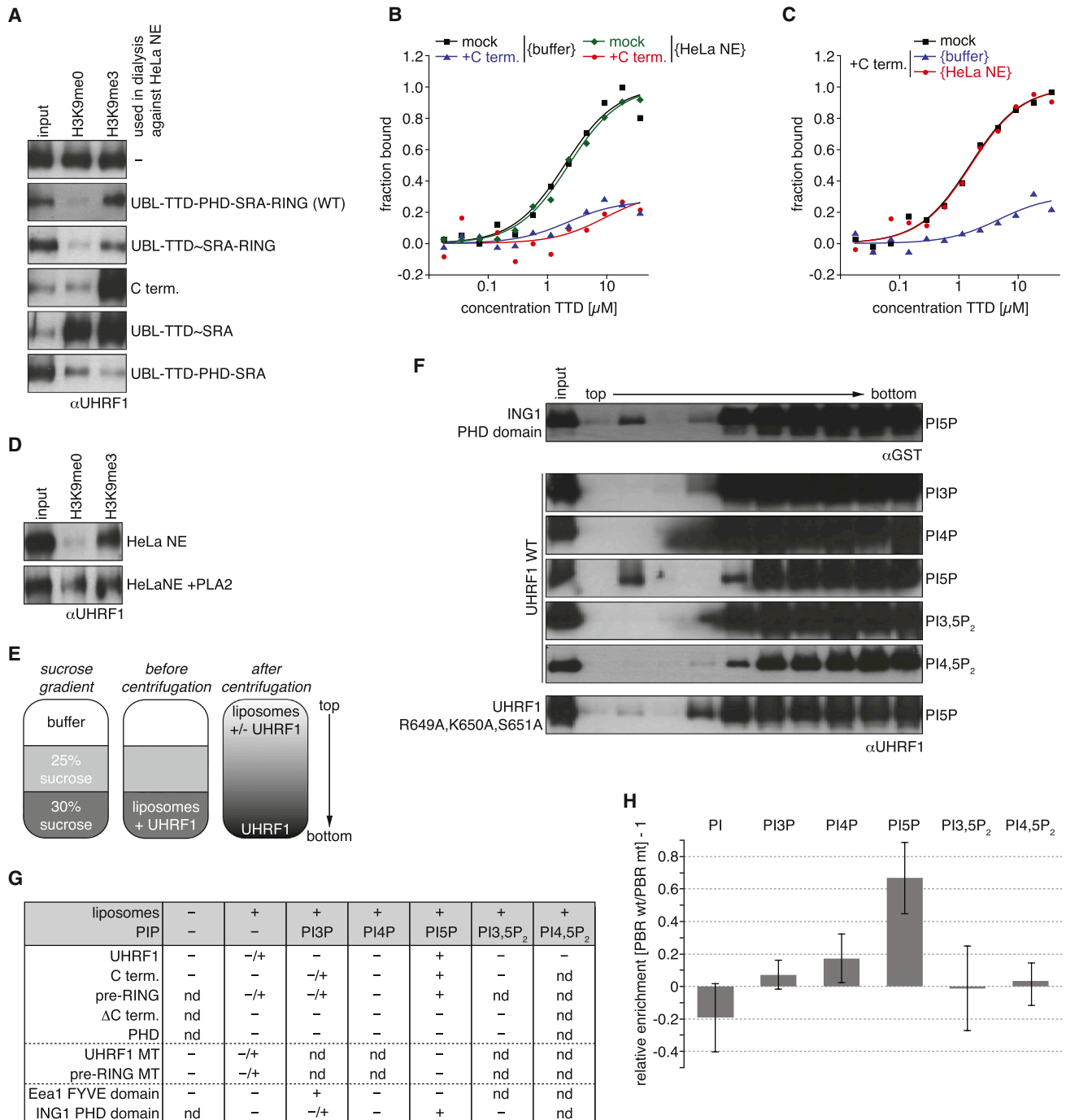


Figure 5. The PBR of UHRF1 Interacts with PI5P

(A) The indicated fragments of UHRF1 were dialyzed against HeLa NE. Proteins and cellular factors inside the dialysis membrane were separated on a C8 reversed-phase column. Recombinant 6×HIS-UHRF1 was incubated with the material eluting at 50% acetonitrile and probed in a histone peptide pull-down experiment. Recovered material was analyzed by western blot. Input, 2%.

(B) TTD alone (mock) or incubated with the C terminus dialyzed against HeLa NE or buffer control (molar ratio TTD:C term. = 1:2) was analyzed in fluorescence polarization binding experiments using FAM-H3(1–15)K9me3 peptide.

(C) TTD was dialyzed against buffer or HeLa NE. Protein alone (mock) or in the presence of the C terminus was analyzed in fluorescence polarization binding experiments using FAM-H3(1–15)K9me3 peptide.

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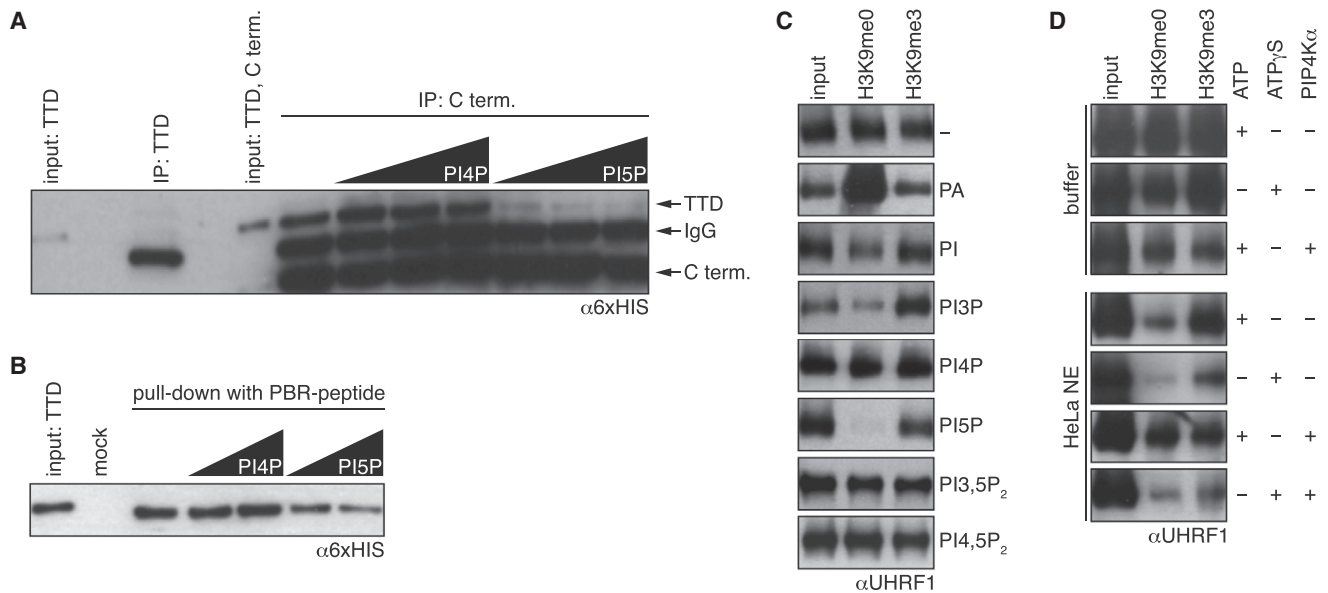


Figure 6. PI5P Releases Blocking of the TTD by the C Terminus

(A) C terminus was incubated with TTD in the presence of increasing concentrations of the indicated PIPs (molar ratio TTD:C term.:PIP = 1:1:5, 1:1:10, 1:1:20) and immunoprecipitated using antibodies against UHRF1. Recovered material was analyzed by western blot. Running positions of proteins recognized by the primary and/or secondary antibodies are indicated. Input, 5%.

(B) TTD was incubated with increasing concentrations of the indicated PIPs (molar ratio TTD:PIP = 1:5, 1:10) and analyzed in pull-down experiments using an immobilized PBR peptide. Recovered material was analyzed by western blot. Mock, pull-down with beads only; input, 2%.

(C) UHRF1 in the presence of the indicated lipids (2-fold molar excess) was incubated with the specified biotinylated H3(1–20) peptides immobilized on streptavidin magnetic beads. Recovered material was analyzed by western blot. Input, 2%.

(D) Recombinant UHRF1 was dialyzed against buffer or HeLa NE that was incubated with the indicated combinations of PIP4K α , ATP, or ATP γ S. After dialysis, the protein was probed in histone peptide pull-down reactions. Recovered material was analyzed by western blot. Input, 2%. See also [Figure S6](#).

unmodified and K9me3 H3, and different interaction specificities were observed (Hu et al., 2011; Karagianni et al., 2008; Liu et al., 2013; Rajakumara et al., 2011; Rottach et al., 2010; Wang et al., 2011). We propose that most of the differences can be explained on the basis of the following regulatory mechanism. In the ground state of the recombinant protein or highly purified cellular protein, the C-terminal region of UHRF1 is folded back onto the middle region with the PBR sequence bound to the peptide-binding groove of the TTD (Figure 7D, top). This prevents the interaction of the TTD-PHD linker and also the H3 N-terminal tail with the TTD (Figure 4) (Nady et al., 2011). In this state, the PHD domain is able to bind to the extreme unmodified N terminus of H3. In an intermediate state that is artificially stabilized by mutagenesis of the PBR, the blockage of the TTD is released (Figure 7D, middle). We think this conformation of UHRF1 reflects the cooperative mode that we and others have observed

for the isolated TTD-PHD fragment (Arita et al., 2012; Cheng et al., 2013; Rothbart et al., 2013; Xie et al., 2012). It is characterized by a slight preference for H3K9me3 over the unmodified state, as seen for C-terminally labeled peptides (H3[1–15]-K [FAM]). Here, the linker sequence occupies the peptide-binding groove of the TTD. The N terminus of the H3 tail can bind to the PHD, while the aromatic cage of the TTD can recognize K9me3. In an activated state, the PBR is bound by PI5P, further stabilizing the domain orientation, giving TTD access to H3K9me3. Large conformational rearrangements not only free the TTD for H3K9me3 binding, but also block the PHD from binding to the extreme unmodified N terminus of H3 (Figure 7D, bottom).

Further biophysical and structural studies will be needed to clarify the exact nature of the overall conformational rearrangements of the protein in the different states as well as the exact binding modes of the H3 tail and PI5P to UHRF1. The

(D) Recombinant UHRF1 was dialyzed against untreated HeLa NE or HeLa NE that was incubated with phospholipase A2. After dialysis, the protein was probed in a histone peptide pull-down experiment. Recovered material was analyzed by western blot. Input, 2%.

(E) Scheme of liposome flotation assay. The sucrose gradient was loaded with liposomes and UHRF1. After centrifugation, the gradient was fractionated and analyzed for localization of UHRF1.

(F) The indicated proteins were incubated with liposomes containing the indicated PIPs. Fractions of the liposome flotation assay after centrifugation were analyzed by western blot. WT, wild-type; input, 5%.

(G) Summary of multiple liposome flotation assays using different proteins. MT, mutation R649A,K650A,S651A. +, major protein signal at the top of the gradient; -/+, minor protein signal at the top of the gradient; nd, not determined.

(H) Fluorescein-labeled wild-type (WT) or R649A,K650A,S651A mutant (MT) PBR peptides were incubated with the indicated biotinylated PIPs immobilized in 96-well plates. Fluorescence signals after washing were recorded. Averaged ratio of enrichment of WT versus MT of four independent experiments is plotted. Error bars correspond to error propagation of SEM reflected in five independent experiments. See also [Figure S5](#) and [Tables S1](#) and [S2](#).

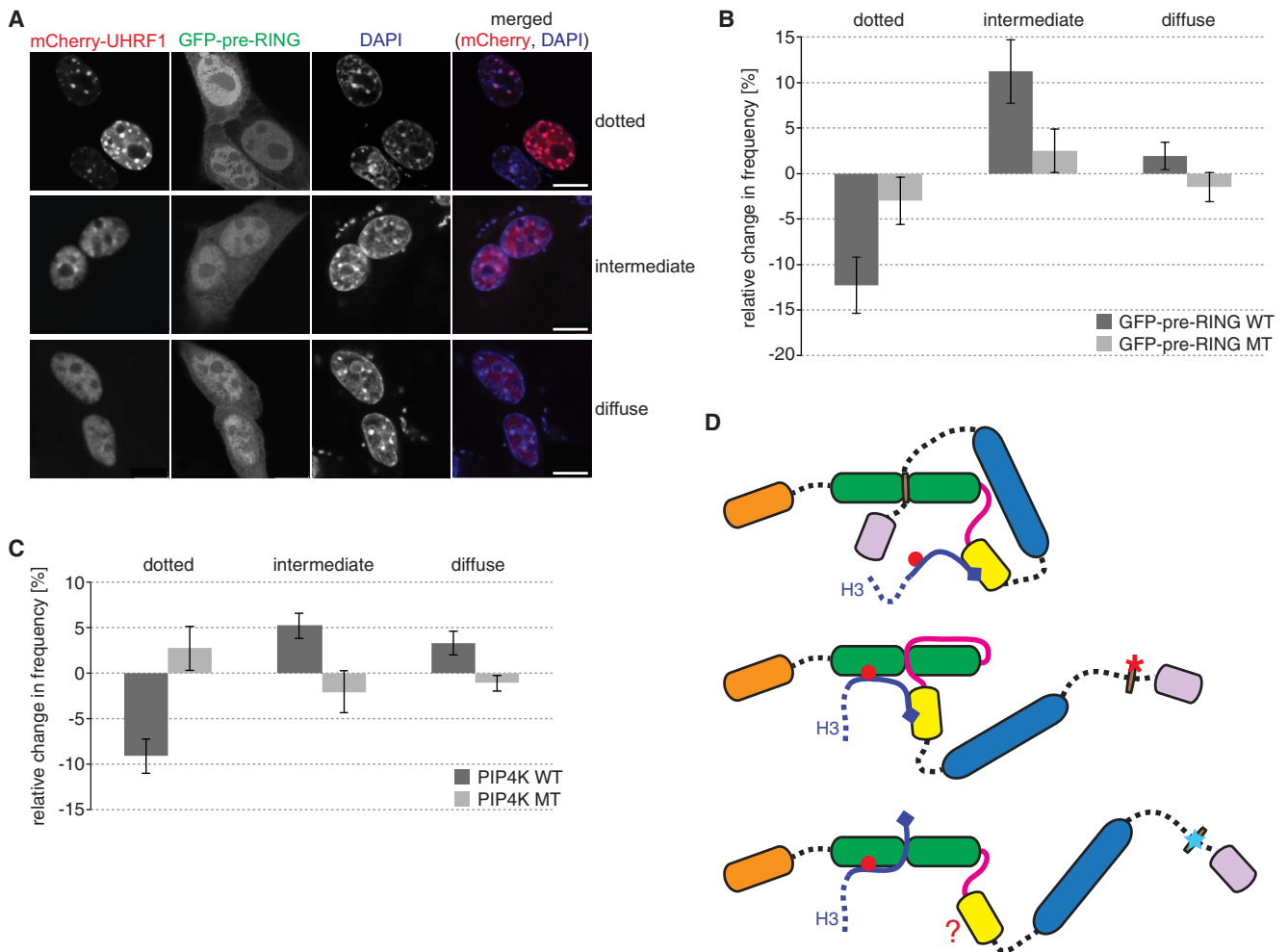


Figure 7. Nuclear PI5P Regulates Heterochromatin Association of UHRF1

(A) NIH 3T3 cells were transfected with mCherry-UHRF1 together with GFP-pre-RING. Fluorescence signals were analyzed by confocal microscopy. Images of representative cells of different phenotypes observed at the indicated frequencies ($n > 500$) are shown. DAPI was used to stain DNA. Scale bar, 10 μm .

(B) Histogram showing changes in frequencies of observed nuclear distribution of mCherry-UHRF1 when coexpressing GFP-pre-RING wild-type (WT) or GFP-pre-RING K644A,K646A,K648A,R649A,K650A,S651A (MT) relative to coexpression of a GFP control in NIH 3T3 cells as classified in (A) ($n > 500$). Error bars represent error propagation of SEM reflected in four independent experiments.

(C) Histograms showing changes in frequencies of observed nuclear distribution of mCherry-UHRF1 coexpressing MYC-PIP4K α and untagged PIP4K β (PIP4K WT) or MYC-PIP4K α G131L,Y138F and untagged PIP4K β D278A (PIP4K MT) relative to coexpression of empty vector in NIH 3T3 cells as classified in (A) ($n > 500$). Error bars represent error propagation of SEM reflected in four independent experiments.

(D) Different conformational states of UHRF1. Top: the PHD mediates binding to unmodified extreme N terminus of H3 in the absence of PI5P, and the TTD is blocked from binding H3K9me3 by the PBR. Middle: when the PBR is mutated (red asterisk), it is dissociated from the TTD. The linker can mediate cooperative binding of the H3 tail by the PHD and TTD domains. Bottom: PI5P (blue star) binding by the PBR results in conformational rearrangement. The TTD is free to bind the H3K9me3 tail. Unknown mechanisms (indicated by the question mark) block the PHD from binding the unmodified extreme N terminus of H3. See also Figure S7.

mechanism of PHD blocking in the activated state is particularly unclear at this point. Our data question whether the cooperative state seen for the TTD-PHD exists in the context of the full-length protein. It might be an artificial state of an isolated fragment. The increase seen in H3K9me3 binding of TTD-PHD over TTD varies from 2-fold (this study; Arita et al., 2012) to 5-fold (Cheng et al., 2013; Rothbart et al., 2013), which might be due to different experimental conditions. In all cases and likely due to the flexible linkage of the TTD and PHD, it is not sufficient to account for the large binding differences for the unmodified and K9me3 H3 tail observed for cellular UHRF1.

Whether the slight preference for the K9me3 over the unmodified H3 peptide with C-terminal fluorescein (H3[1–15]-K[FAM]; Figure 1 and Table S1) reflects cooperativity of TTD and PHD in the ground state remains to be seen.

How are the hydrophobic acyl chains of PI5P bound to UHRF1 outside of a lipid membrane or micellar structure? While mutagenesis of the PBR is sufficient to abolish interaction in the lipid flotation assay (Figure 5F), we think that other regions of the C terminus and/or additional unknown components are involved in PI5P binding. First, in the liposome flotation assay, only the polar head groups of the PIPs are exposed; the hydrophobic

acyl chains are embedded in the lipid layer. Nevertheless, inositol 1,5-bisphosphate is not sufficient to induce allosteric activation of UHRF1. Second, while addition of PI5P to UHRF1 activates the protein in pull-down assays, it does not have this effect in solution FP assays (data not shown). We speculate that the phospholipid might get artificially enriched on the surface of the beads used in the pull-down assays. In the nuclear extract, PI5P is bound by UHRF1 and likely other factors that might work as “sinks” for the PIP. For sufficient transfer across the dialysis membrane, PI5P might need other small molecules (e.g., ions, peptides, RNA, etc.) that stabilize it in solution.

Cellular Regulation of UHRF1

While our findings identify a role for PI5P in modulating UHRF1 function, regulation of the protein in the cellular context is likely more complicated. Recent work had reported partially overlapping and redundant roles of the TTD and SRA domains in UHRF localization, where the two regions appear to mediate crosstalk between H3K9 methylation and DNA methylation at the level of DNA methylation maintenance (Liu et al., 2013). In contrast, another report suggested that cooperative interplay of the TTD and PHD is required for this function (Rothbart et al., 2013). The fact that the concentration of PI5P in the nuclei of murine erythroleukemia cells increases 20-fold during G1 phase but drops after S phase (Clarke et al., 2001) could indicate that switching between the unmodified H3-binding ground state and the activated H3K9me3-binding state of UHRF1 is required for differential localization of the protein during the cell cycle (Papait et al., 2007). Further work will need to clarify the precise crosstalk of the chromatin modification binding TTD, PHD, and SRA domains of UHRF1 under defined cellular conditions. Regulation of H3K9me3 and unmodified H3 tail interaction by PI5P also likely has an impact on UHRF1 binding to and targeting of its enzymatic partners such as G9a, DNMT1, and HDAC1.

Another level of regulation of UHRF1 is provided by post-translational modifications. Several proteomics studies have defined serine phosphorylation events within the linker and C-terminal region. Also, acetylation of two lysine residues within the SRA was found (Choudhary et al., 2009; Dephoure et al., 2008; Olsen et al., 2010; Rigbolt et al., 2011). While the functions of most modifications are unknown, phosphorylation by CDK1 at S639 is involved in degradation of the protein (Ma et al., 2012). Phosphorylation of S298 within the linker by PKA interferes with TTD-PHD cooperativity in vitro (Arita et al., 2012). Based on our mutagenesis studies of the PBR, we speculate that phosphorylation of S651 (S651ph) might have a regulatory role in PI5P binding and in modulating H3 interaction of UHRF1 (Rigbolt et al., 2011).

We predict that PI5P, as well as other nuclear PIPs, might have more general, so far unrecognized roles in directly regulating the chromatin binding activity of different proteins. Consistent with a role in signaling, the amounts of certain nuclear PIPs are increased in mammalian cells by physiological ligands or processes as well as by cellular stresses (Shah et al., 2013). This might directly impact gene regulation. In general, nuclear phospholipids undergo changes in abundance that match the transcriptional activity during the cell cycle (Fraschini et al., 1999). Defining further chromatin proteins that are directly regulated

by PIPs or other phospholipids might establish new paradigms of signal transduction in the cell nucleus.

EXPERIMENTAL PROCEDURES

Reagents

Detailed listing of plasmids, peptides, antibodies, and proteins can be found in the [Supplemental Information](#).

Dialysis of UHRF1

Nuclear extracts were prepared as described (Dignam et al., 1983). Protein concentration ranged from 10–15 mg/ml. Proteins (5 mg in 400 μ l) were dialyzed against 10 ml HeLa NE or comparable buffer (in 50 ml tubes) overnight in dialysis cups (Pierce) with a 3,500 Da molecular weight cutoff at 4°C.

Pull-Downs, Immunofluorescence and Fluorescence Polarization, and Liposome Flotation Assay

Experiments were performed as previously described (Fischle et al., 2008; Rusten and Stenmark, 2006), with minor modifications as can be found in the [Supplemental Experimental Procedures](#).

Binding of PBR Peptides to PIP Beads

A 10 μ l slurry of PIP beads (Echelon Biosciences) was incubated with 20 ng of fluorescein-labeled PBR peptide. After washing, samples were transferred to black 96-well plates (Corning), and fluorescence intensity (excitation at 485 nm, emission at 535 nm) was measured in a HIDEX Chameleon II plate reader. Results from three successive reads were averaged, and recovery of the peptides was normalized relative to the input.

NMR Spectroscopy

Chemical shift changes of the residues of the TTD upon titration of unlabeled linker peptide were mapped using prior available chemical shift assignments deposited in the Biological Magnetic Resonance Bank (BMRB) (Nady et al., 2011).

Molecular Docking

Docking of the PBR peptide to the TDD was performed using HADDOCK v2.1 software (de Vries et al., 2007; Dominguez et al., 2003).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.molcel.2014.04.004>.

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