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Supplemental Information

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

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SUPPLEMENTAL FIGURES

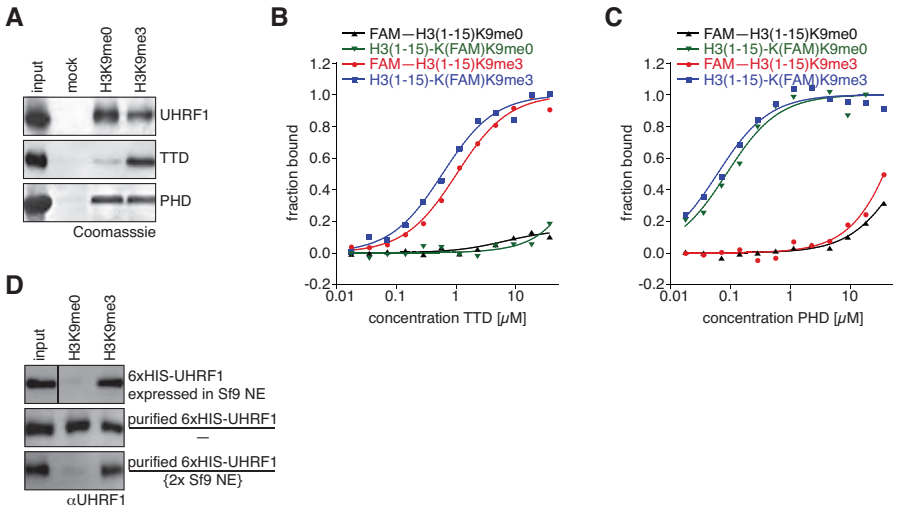

Figure S1. Different Functional States of the TTD and PHD Domains of UHRF1

Related to Figure 1

(A) UHRF1 or the isolated TTD or PHD domain were incubated with the specified biotinylated H3(1-20) peptides immobilized on streptavidin magnetic beads. Mock represents pull-down with beads only. Recovered material was analyzed by Coomassie staining. Input, 2%.

(B) and (C) Fluorescence polarization binding experiments of the TTD (B) or PHD (C) domain of UHRF1 using H3 peptides carrying the specified modification status on lysine 9 (K9). H3 peptides were either linked to fluorescein at the N terminus (FAM–H3[1-15]) via an amide bond or a non-natural lysine at the C terminus was added and labeled with amide-linked fluorescein at the ε-amino group (H3[1-15]-K[FAM]).

(D) Pull-down reactions using the indicated H3 peptides were performed using extracts from Sf9 cells expressing 6xHIS-UHRF1, 6xHIS-UHRF1 purified from this source after dialysis against buffer (–) or Sf9 cell nuclear extract (NE). Recovered material was analyzed by western blot. Input, 2%.

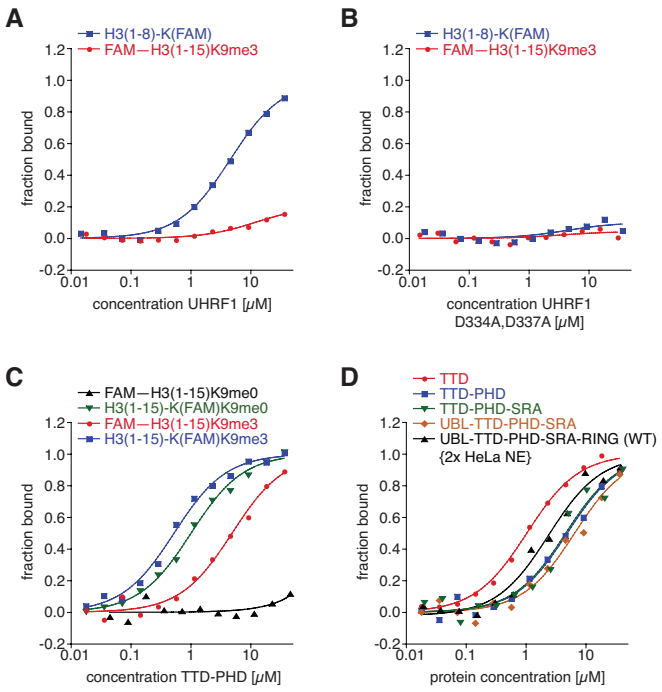

Figure S2. Fluorescence Polarization Binding Experiments of Different UHRF1 Wild-Type and Mutant Proteins

Related to Figure 2

(A)-(C) The specified proteins were analyzed in fluorescence polarization binding experiments using the indicated fluorescein-labeled peptides.

(D) The indicated recombinant protein fragments of UHRF1 or the wild-type (WT, UBL-TTD-PHD-SRA-RING) protein dialyzed twice against HeLa NE were analyzed in fluorescence polarization binding experiments using a FAM–H3(1-15)K9me3 peptide.

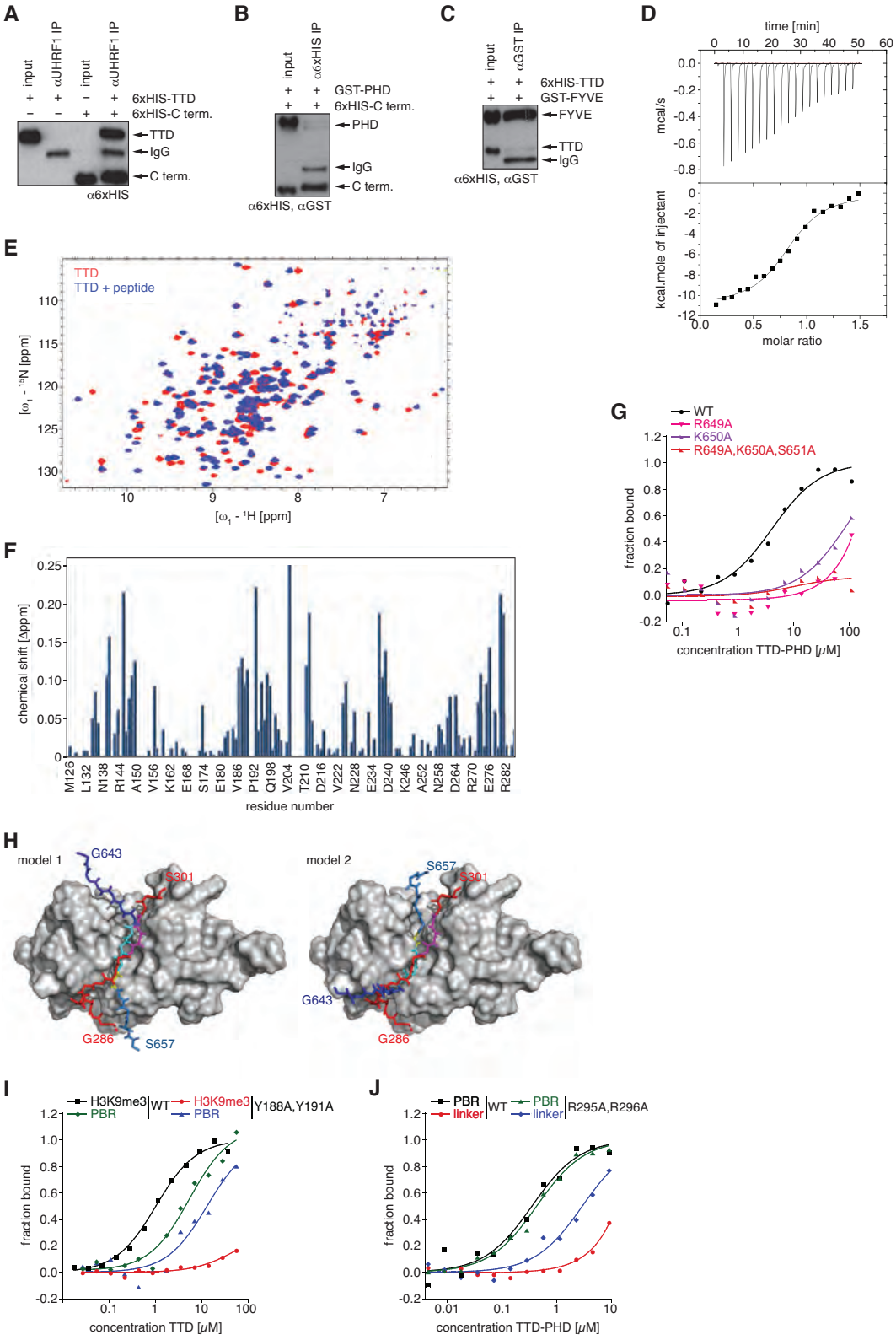
A
input
αUHRF1 IP
input
αUHRF1 IP
6xHIS-TTD
6xHIS-C term.
TTD
IgG
C term.
α6xHIS
B
input
α6xHIS IP
GST-PHD
6xHIS-C term.
PHD
IgG
C term.
α6xHIS, αGST
C
input
αGST IP
6xHIS-TTD
GST-FYVE
FYVE
TTD
IgG
α6xHIS, αGST
D
time [min]
mcal/s
kcal.mole of injectant
molar ratio
E
TTD
TTD + peptide
[ω1 - 15N [ppm]
[ω1 - 1H [ppm]
F
chemical shift [Δppm]
M126 L132 N138 R144 A150 V156 K162 E168 S174 E180 V186 P192 Q198 V204 T210 D216 V222 N228 E234 D240 K246 A252 N258 D264 R270 E276 R282
residue number
G
WT
R649A
K650A
R649A,K650A,S651A
fraction bound
concentration TTD-PHD [μM]
H
model 1
G643
S301
G286
S657
model 2
S657
S301
G643
G286
I
H3K9me3
PBR
WT
H3K9me3
PBR
Y188A,Y191A
fraction bound
concentration TTD [μM]
J
PBR
linker
WT
PBR
linker
R295A,R296A
fraction bound
concentration TTD-PHD [μM]

Figure S3. A PBR Region within the C Terminus of UHRF1 Interacts with the TTD

Related to Figure 3

(A)-(C) Immunoprecipitation experiments of the indicated recombinant protein domains. The 6xHIS-C terminus of UHRF1 was recovered by anti-UHRF1 antibodies in presence of 6xHIS-TTD (A) or GST-PHD (B). (C) The GST-tagged FYVE domain of Eea1 was precipitated by anti-GST antibodies in presence of 6xHIS-TTD of 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) Interaction of the TTD with the C terminus was analyzed by isothermal titration calorimetry. A representative titration is shown.

(E) HSQC spectra showing overlay of signals of ^{15}N-TTD in absence and presence of PBR peptide at 1:10 molar ratio.

(F) Histogram showing chemical shift changes of the ^{15}N-TTD upon titration with the PBR peptide at a 1:10 molar ratio.

(G) The TTD-PHD fragment of UHRF1 was analyzed in fluorescence polarization binding experiments using fluorescein-labeled wild-type (WT) or mutant PBR peptides.

(H) Overlay of the positioning of the TTD-PHD linker (red, R295 and R296 residues in purple) as determined in the TTD-PHD/H3K9me3 complex (pdb: 3ASK) and the PBR sequence in model 1 and model 2 (color code as in figure 3E). Images were generated by PyMol.

(I) Wild-type TTD domain or TTD domain mutated in the aromatic cage residues Y188 and Y191 was analyzed in fluorescence polarization binding experiments using FAM–H3(1-15)K9me3 or FAM–PBR peptides.

(J) Wild-type TTD-PHD domain (WT) or TTD-PHD domain mutated in the linker residues R295 and R296 (MT) was analyzed in fluorescence polarization binding experiments using FAM–H3(1-15)K9me3 or FAM–PBR peptides.

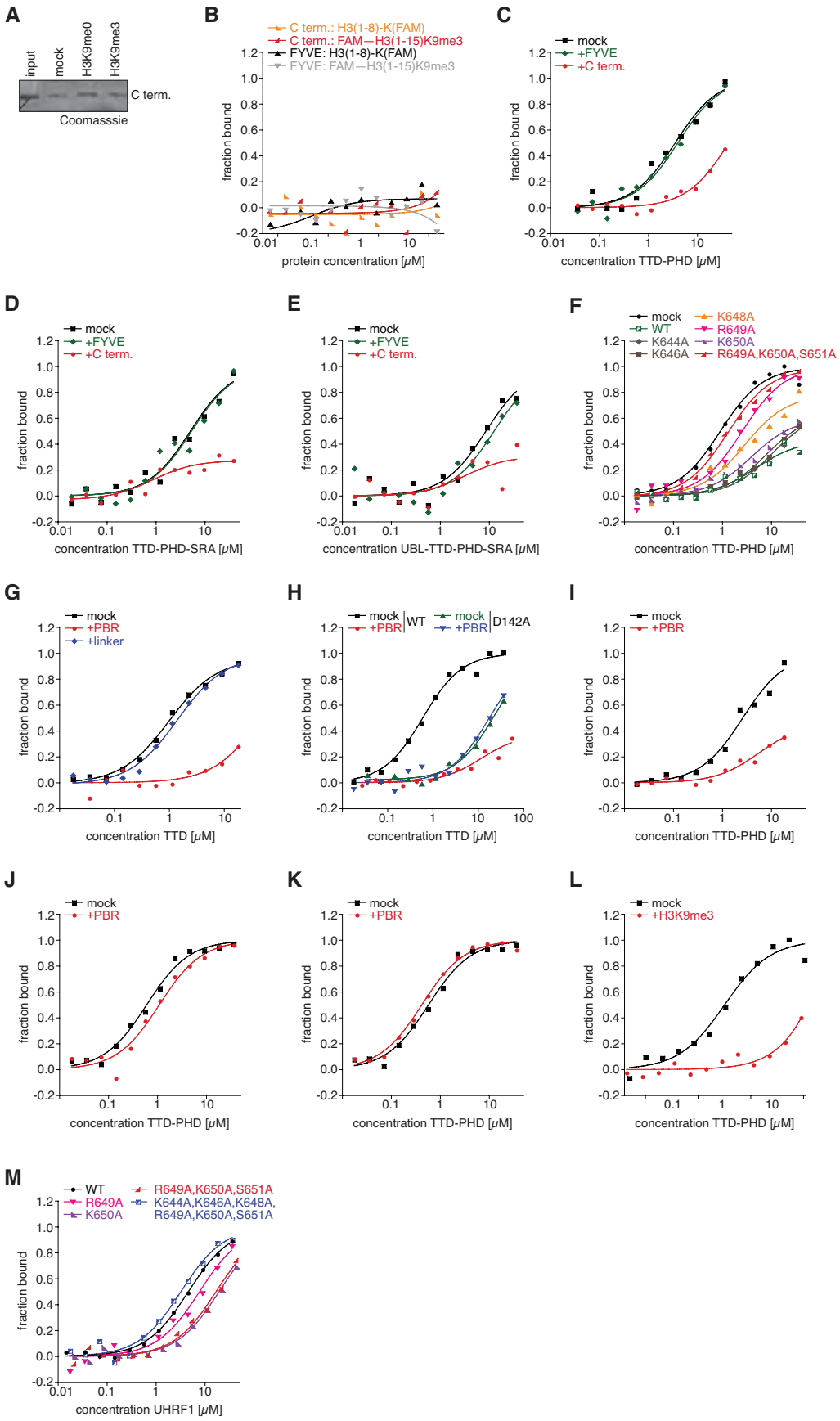
A
input
mock
H3K9me0
H3K9me3
C term.
Coomasssie
B
C term.: H3(1-8)-K(FAM)
C term.: FAM—H3(1-15)K9me3
FYVE: H3(1-8)-K(FAM)
FYVE: FAM—H3(1-15)K9me3
fraction bound
protein concentration [μM]
C
mock
+FYVE
+C term.
fraction bound
concentration TTD-PHD [μM]
D
mock
+FYVE
+C term.
fraction bound
concentration TTD-PHD-SRA [μM]
E
mock
+FYVE
+C term.
fraction bound
concentration UBL-TTD-PHD-SRA [μM]
F
mock
WT
K644A
K646A
K648A
R649A
K650A
R649A,K650A,S651A
fraction bound
concentration TTD-PHD [μM]
G
mock
+PBR
+linker
fraction bound
concentration TTD [μM]
H
mock | WT
+PBR
mock | D142A
+PBR
fraction bound
concentration TTD [μM]
I
mock
+PBR
fraction bound
concentration TTD-PHD [μM]
J
mock
+PBR
fraction bound
concentration TTD-PHD [μM]
K
mock
+PBR
fraction bound
concentration TTD-PHD [μM]
L
mock
+H3K9me3
fraction bound
concentration TTD-PHD [μM]
M
WT
R649A
K650A
R649A,K650A,S651A
K644A,K646A,K648A,
R649A,K650A,S651A
fraction bound
concentration UHRF1 [μM]

Figure S4. The PBR Sequence within the C Terminus Blocks TTD/H3K9me3 Interaction

Related to Figure 4

(A) The C terminus of UHRF1 was incubated with the specified biotinylated H3(1-20) peptides immobilized on streptavidin magnetic beads. Mock represents pull-down with beads only. Recovered material was analyzed by Coomassie staining. Input, 2%.

(B) The C terminus of UHRF1 or the FYVE domain of Eea1 were analyzed in fluorescence polarization binding experiments using the indicated fluorescein-labeled peptides.

(C)-(E) The specified fragments of UHRF1 alone (mock) or in presence of the C terminus of UHRF1 or the FYVE domain of Eea1 (1:2 molar ratio) were analyzed in fluorescence polarization binding experiments with a FAM–H3(1-15)K9me3 peptide.

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

(G) The TTD alone (mock) or in presence of PBR or linker peptides (1:5 molar ratio) was analyzed in fluorescence polarization binding experiments with a FAM–H3(1-15)K9me3 peptide.

(H) Wild-type TTD (WT) and TTD mutated in the D142 residue within the peptide-binding groove (MT) alone or together with the PBR peptide (1:5 molar ratio) were analyzed in fluorescence polarization binding experiments using a FAM–H3(1-15)K9me3 peptide.

(I)-(K) The TTD-PHD alone (mock) or in presence of the PBR peptide (1:5 molar ratio) was analyzed in fluorescence polarization binding experiments using FAM–H3(1-15)K9me3 (I), H3(1-15)-K(FAM)K9me3 (J) or H3(1-15)-K(FAM)K9me0 (K) peptides.

(L) TTD-PHD alone (mock) or in presence of the H3(1-20)K9me3 peptide (1:5 molar ratio) was analyzed in fluorescence polarization binding experiments using fluorescein labeled PBR peptide.

(M) Wild-type UHRF1 (WT) or UHRF1 carrying the indicated mutations were analyzed in fluorescence polarization binding experiments with a H3(1-8)-K(FAM) peptide.

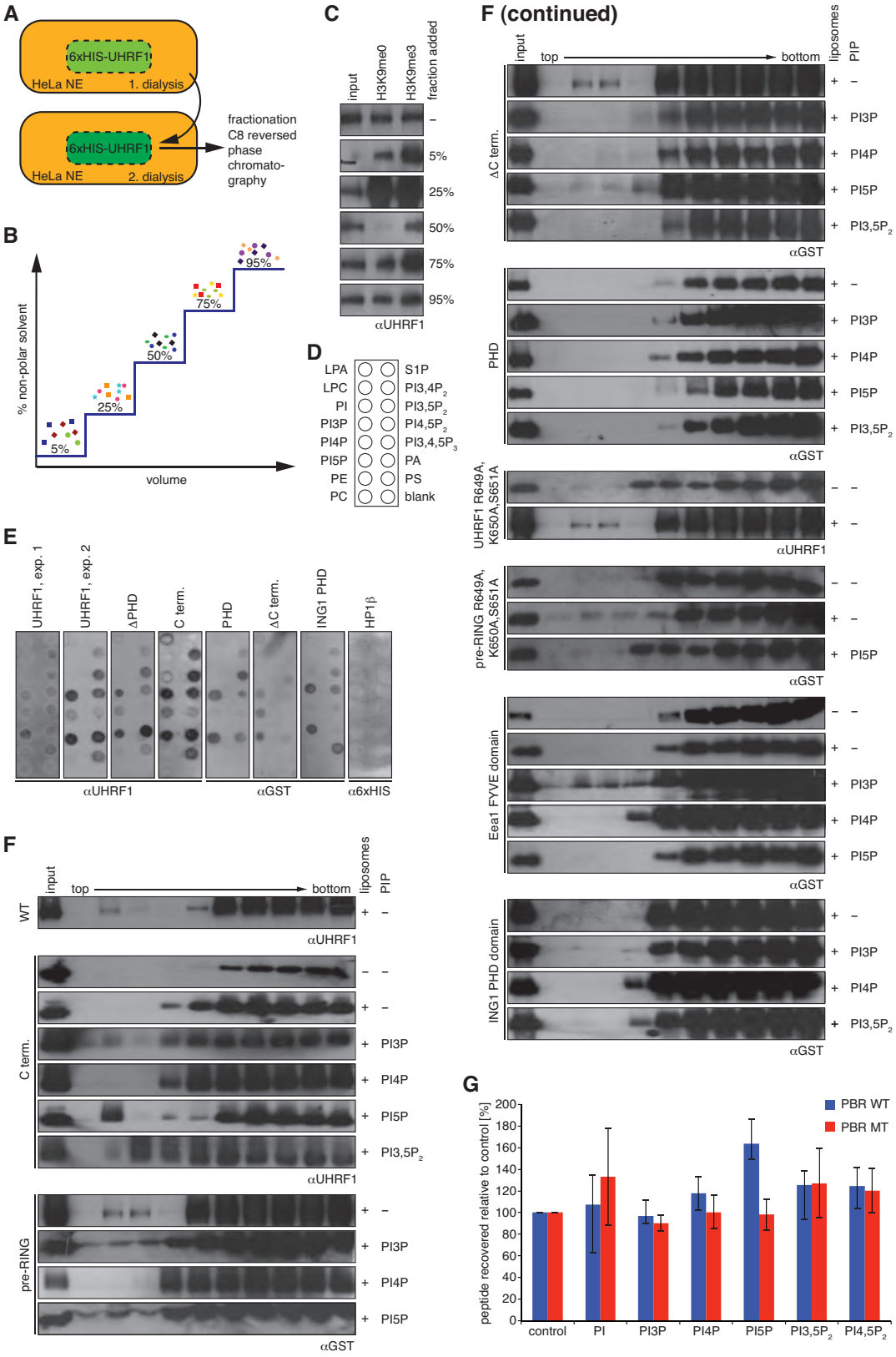
A
6xHIS-UHRF1
HeLa NE
1. dialysis
6xHIS-UHRF1
HeLa NE
2. dialysis
fractionation
C8 reversed
phase
chromato-
graphy
B
% non-polar solvent
volume
5%
25%
50%
75%
95%
C
input
H3K9me0
H3K9me3
fraction added
–
5%
25%
50%
75%
95%
αUHRF1
D
LPA
LPC
PI
PI3P
PI4P
PI5P
PE
PC
S1P
PI3,4P2
PI3,5P2
PI4,5P2
PI3,4,5P3
PA
PS
blank
E
UHRF1, exp. 1
UHRF1, exp. 2
ΔPHD
C term.
PHD
ΔC term.
ING1 PHD
HP1β
αUHRF1
αGST
α6xHIS
F
input
top
bottom
liposomes
PIP
WT
αUHRF1
C term.
PI3P
PI4P
PI5P
PI3,5P2
αUHRF1
pre-RING
αGST
F (continued)
ΔC term.
PHD
αGST
UHRF1 R649A, K650A,S651A
αUHRF1
pre-RING R649A, K650A,S651A
Eea1 FYVE domain
ING1 PHD domain
G
PBR WT
PBR MT
peptide recovered relative to control [%]
control
PI
PI3P
PI4P
PI5P
PI3,5P2
PI4,5P2

Figure S5. The PBR Sequence of UHRF1 Interacts with PI5P

Related to Figure 5

(A) Flow scheme of the dialysis and purification experiment for enrichment of the cellular cofactors of UHRF1.

(B) Fractionation scheme of the cellular components enriched with UHRF1 protein or UHRF1 fragments after dialysis against HeLa NE on a C8 reversed phase column.

(C) Fractions from the dialysis and purification scheme depicted in (A) and (B) using wild-type UHRF1 protein were lyophilized. Recombinant UHRF1 incubated with the different fractions was analyzed in pull-down experiments using the specified biotinylated H3(1-20) peptides immobilized on streptavidin magnetic beads. Recovered material was analyzed by western blot. Input, 2%.

(D) Layout of the lipid dot blot. LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; PI, phosphatidylinositol; PI3P, phosphatidylinositol 3-phosphate; PI4P, phosphatidylinositol 4-phosphate; PI5P, phosphatidylinositol 5-phosphate; PE, phosphatidylethanolamine; PC, phosphatidylcholin; S1P, shingosin 1-phosphate; P3,4P_2, phosphatidylinositol 3,4-bisphosphate; P3,5P_2, phosphatidylinositol 3,5-bisphosphate; P4,5P_2, phosphatidylinositol 4,5-bisphosphate; P3,4,5P_3, phosphatidylinositol 3,4,5-trisphosphate; PA, phosphatidic acid; PS, phosphatidylserine.

(E) The indicated proteins were incubated with lipids spotted on a nitrocellulose membrane according to the scheme in (E). Retained material was analyzed by western blot. ΔPHD and ΔC terminus correspond to the UHRF1 UBL-TTD~SRA-RING and UBL-TTD-PHD-SRA fragments, respectively. The PHD domain of ING1 and HP1β protein served as controls.

(F) The indicated wild-type proteins and protein fragments or mutant proteins and protein fragments were incubated with liposomes containing the indicated PIPs (see scheme of Figure 5E). Fractions of the liposome floatation assay after centrifugation were analyzed by western blot. The FYFE domain of Eea1 and the PHD domain of ING1 served as control. WT, wild-type UHRF; ΔC terminus, UBL-TTD-PHD-SRA; input, 5%.

(G) Fluorescein labeled wild-type (WT) or R649A,K650A,S651 mutant (MT) PBR peptides were incubated with the indicated biotinylated PIPs immobilized on agarose beads. Fluorescence signals after washing were recorded in 96-well plates and normalized to control without lipids. Error bars correspond to SD.

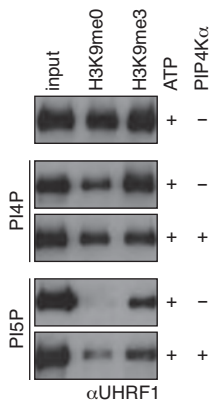

Figure S6. PIP4Kα Inhibits the Allosteric Regulation of UHRF1 by PI5P

Related to Figure 6

UHRF1 alone or together with PI4P or PI5P was incubated with the indicated combinations of PIP4Kα and ATP. The protein was then analyzed in pull-down reactions with the specified biotinylated H3(1-20) peptides immobilized on streptavidin magnetic beads. Recovered material was analyzed by western blot. Input, 2%.

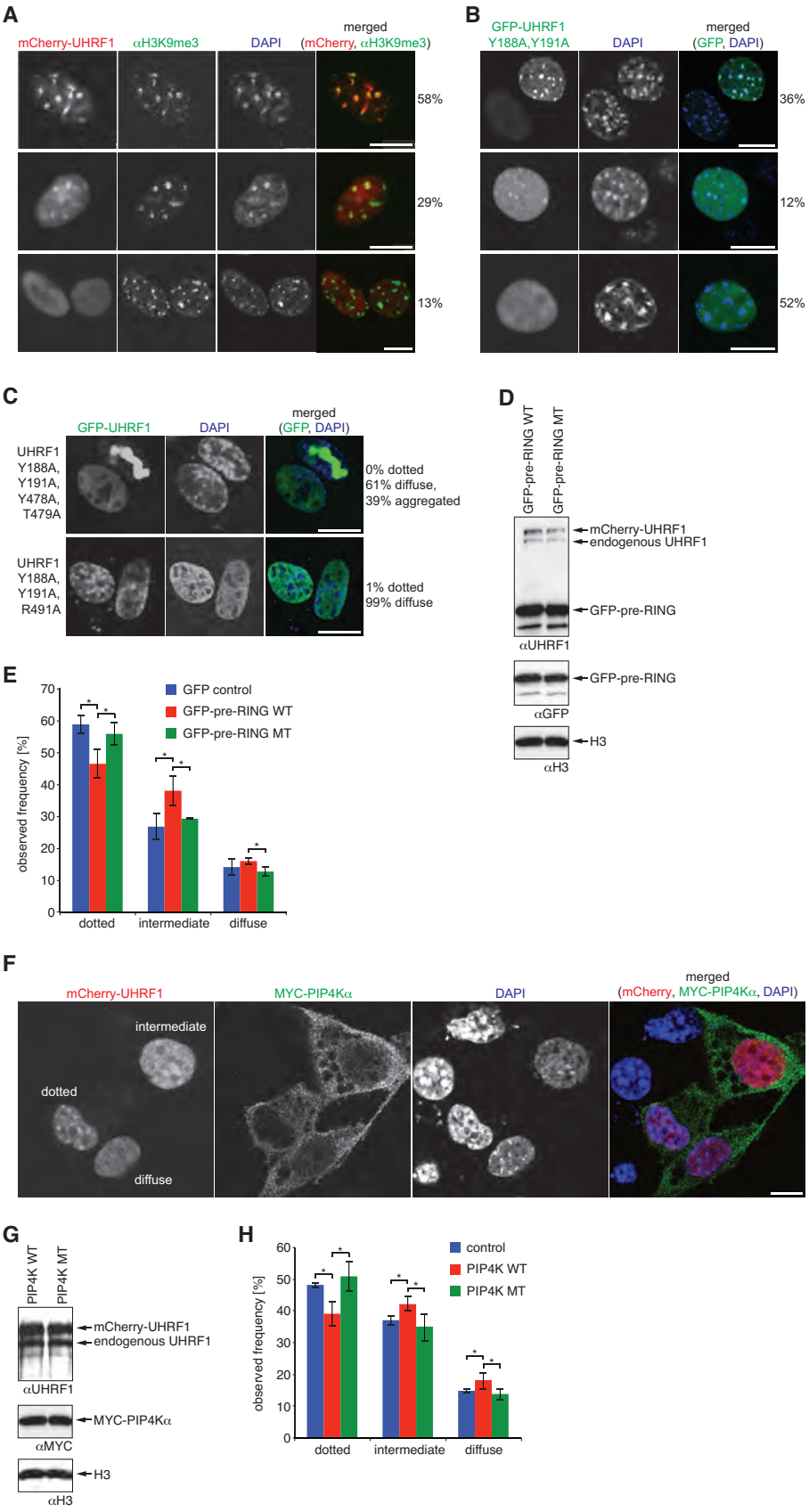

Figure S7. Nuclear Localization of UHRF1 is Regulated by the PBR and PI5P

Related to Figure 7

(A) mCherry and anti-H3K9me3 immunofluorescence signals of NIH3T3 cells transiently expressing mCherry-UHRF1. Images of representative cells of different phenotypes observed at the indicated frequency ($n > 500$) are shown. DAPI was used to stain DNA. Scale bar, 10 μm.

(B) and (C) NIH3T3 cells were transfected with the indicated constructs. Fluorescence signals were analyzed by confocal microscopy. Images of representative cells of different phenotypes observed at the indicated frequency ($n > 500$) are shown. DAPI was used to stain DNA. Scale bar, 10 μm.

(D) NIH3T3 cells were transfected with mCherry-UHRF1 together with GFP-pre-RING wild-type (WT) or GFP-pre-RING K644A,K646A,K648A,R649A,K650A,S651A (MT) constructs. Total cell lysates were analyzed by western blotting.

(E) Frequencies of different nuclear distribution of mCherry-UHRF1 coexpressed with GFP control, GFP-pre-RING wild-type (WT) or GFP-pre-RING K644A,K646A,K648A,R649A,K650A,S651A (MT) in NIH3T3 cells ($n > 500$). Error bars represent SD of three independent experiments. Asterisks indicate significance intervals of $p < 0.05$ determined by a two-tailed Student's t-test.

(F) mCherry and anti-MYC immunofluorescence signals of NIH3T3 cells transiently expressing mCherry-UHRF1, MYC-PIP4Kα and untagged PIP4Kβ. Cells with different distribution of the mCherry signal are indicated. DAPI was used to stain DNA. Scale bar, 10 μm.

(G) NIH3T3 cells were transfected with mCherry-UHRF1 together with MYC-PIP4Kα and untagged PIP4Kβ (PIP4K WT) or MYC-PIP4Kα G131L,Y138F and untagged PIP4Kβ D278A (PIP4K MT). Total cell lysates were analyzed by western blotting.

(H) Frequencies of different nuclear distribution of mCherry-UHRF1 coexpressed with MYC-PIP4Kα and untagged PIP4Kβ (PIP4K WT) or MYC-PIP4Kα G131L,Y138F and untagged PIP4Kβ D278A (PIP4K MT) in NIH3T3 cells ($n > 500$). Error bars represent SD of three independent experiments. Asterisks indicate significance intervals of $p < 0.05$ determined by a two-tailed Student's t-test.

SUPPORTING TABLES

Table S1. Apparent Dissociation Constants of Wild-Type and Mutant UHRF1 Proteins Determined by Fluorescence Polarization Measurements

Related to Figures 1-5

	FAM–H3(1-15)		H3(1-15)-K(FAM)		H3(1-8)-K(FAM)
	K9me0	K9me3	K9me0	K9me3	
TTD	nb	1.0 ± 0.2	nb	0.6 ± 0.2	–
TTD Y188A,Y191A	nb	nb	–	–	
PHD	nb	nb	0.1 ± 0.1	0.1 ± 0.05	2.3 ± 0.3
TTD-PHD	nb	4.9 ± 1.0	1.0 ± 0.3	0.5 ± 0.2	–
TTD-PHD-SRA	nb	4.6 ± 3.0	–	–	–
UBL-TTD-PHD-SRA	–	6.5 ± 3.5	–	–	>100
UHRF1 (UBL-TTD-PHD-SRA-RING)	nb	nb	1.5 ± 0.3	0.5 ± 0.2	4.5 ± 2.0
UHRF1 {2x HeLa NE}	nb	2.6 ± 1.7	>100	4.1 ± 2.0	–
UHRF1 D334A,D337A	–	–	–	nb	nb
UHRF1 R649A	–	44 ± 11	–	–	7.7 ± 3.0
UHRF1 K650A	–	nb	–	–	19 + 5
UHRF1 R649A,K650A,S651A	–	27 ± 8	–	–	17 ± 7
UHRF1 K644A,K646A,K648A, R649A,K650A,S651A	–	8.0 ± 2.2	–	–	3.2 ± 1.2
C terminus	–	nb	–	–	nb

All values given in μM; nb, not binding (curves did not even get near inflection point); >100, faithful determination of K_D not possible since curves barely reach inflection point; –, not measured.

Table S2. Apparent Dissociation Constants of Wild-Type and Mutant TTD and TTD-PHD Fragments Determined by Fluorescence Polarization Measurements

Related to Figures 1-5

	TTD WT	TTD D142A	TTD Y188A, Y191A	TTD-PHD WT	TTD-PHD R295A, R296A
PBR WT	3.5 ± 1.9	nb	13 ± 4	3.5± 1.6	4.3 ±1.8
PBR K644A	4.9 ± 1.6	–	–	–	–
PBR K646A	4.3 ± 1.4	–	–	–	–
PBR K648A	61 ± 8	–	–	–	–
PBR K649A	nb	–	–	nb	–
PBR K650A	19 ± 5	–	–	92 ± 21	–
PBR R649A, K650A,S651A	nb	–	–	nb	–
linker	nb	–	–	>100	30 ± 9

All values given in μM; nb, not binding (curves did not even get near inflection point); >100, faithful determination of K_D not possible since curves barely reach inflection point; –, not measured.

EXTENDED EXPERIMENTAL PROCEDURES

Plasmids

All cloning was done according to standard procedures. Mutagenesis was performed using the QuickChange protocol (Stratagene). Details are available upon request. All clones are based on human UHRF1 cDNA (NM_001048201). For expression in *E. coli* : pETM13 UHRF1(aa 1-793)-6xHIS; petM40 MBP-UHRF1(aa1-793)-6xHIS; pET16 10xHIS-TTD(aa 126-285), pGEX4T3 GST-PHD(aa298-366)-10xHIS; pET16b 10xHIS-TTD-PHD(aa 126-376); pETM13 TTD-PHD-SRA(aa 126-605)-6xHIS; pETM13 UBL-TTD-PHD-SRA(aa 1-619)-6xHIS; pETM13 C terminus(aa 605-793)-6xHIS; pETM13 preRING(aa 605-675)-6xHIS, pETM13 UBL-TTD~SRA-RING(aa 1-309,371-793)-6xHIS; pETM13 UBL-TTD~SRA(aa 1-309,371-619)-6xHIS. For expression in Sf9 cells: pFastbac1 6xHIS-UHRF(aa1-793). For expression in mammalian cells: pcDNA3.1 UHRF1(aa1-793)-2xHA2xFLAG, pEGFP-C1 UHRF1(aa1-793), pEGFP-C1 UHRF1(aa1-793) R491A, pEGFP-C1 UHRF1(aa1-793) Y188A,Y191A, pmCherry UHRF1(aa1-793), pmCherry UHRF1(aa1-793) R491A, pmCherry UHRF1(aa1-793) Y188A,Y191A, pEGFP-C1 pre RING (aa 605-675), pEGFP-C1 pre RING (aa 605-675) K644A,K646A,K648A,R649A,K650A,S651A. pcDNA3.1 MYC-PIP4Kα and pcDNA3.1 PIP4Kβ have been described (Bultsma et al., 2010). Mutant kinases were PIP4Kα G131L,Y138F and PIP4Kβ D278A in the same backbone. The plasmid for expression of GST-FYVE domain of Eea1 was a gift of Harald Stenmark (Oslo University), the plasmid for expression of 6xHIS-PHD domain of ING1 was a gift of Tatiana Kutateladze (University of Colorado).

Peptides

Peptides were synthesized using Fmoc chemistry on an Intavis Respep XL synthesizer. TentaGel R RAM resin (cap.: 0.18 mmol/g) served as solid support and the amino acid side-chains were protected as follows: Arg(Pbf), Asn(Trt), Asp(OtBu), Cys (Trt), Gln (Trt), His(Trt), Lys(Boc), Ser(tBu), Thr(tBu), Trp(Boc), Lys(Me_3) and Lys(Mtt) for orthogonal deprotection of the ε-amino group. Coupling reactions

were performed with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU) as coupling reagent and N-methylmorpholine (NMM) in DMF/NMP as base. Lys(me3) was introduced manually with PyOxim (Ethyl cyano(hydroxyimino)acetato-O^2]tri-1-pyrrolidinylphosphonium hexafluorophosphate) at the indicated positions. N-terminal labeling of H3-derived peptides was performed with fluorescein using 5-Carboxyfluorescein, Succinimidyl Ester. 5,6-Carboxyfluorescein was coupled at the N-terminal amino group of peptides derived from PBR with PyOxim on solid support. 5,6-Carboxyfluorescein was coupled at the ε-amino group of C-terminal Lys residues using HBTU/ Hydroxybenzotriazole (HOBt) and NMM in DMF. Pseudoproline dipeptides were used for efficient synthesis of H3 and PBR-derived peptides. Peptides were cleaved off the resin with TFA:Phenol:Triisopropylsilane:H_2O (85:5:5:5) for four hrs. All peptides were purified by reversed phase C18 HPLC and verified by ESI- and MALDI-MS. Peptide backbones; methylated residues are indicated in bold: H3(1-8)-K(FAM) = ARTKQTAR-K(ε-amino-FAM); FAM–H3(1-15) = α-amino-FAM-ARTKQTAR**K**STGGKA; H3(1-15)-K(FAM) = ARTKQTAR**K**STGGKA-K(ε-amino-FAM); H3(1-20)-K(biotin) = ARTKQTAR**K**STGGKAPRKQL-K(ε-amino-biotin); FAM–PBR WT = α-amino-FAM-GKGKWKRKSAGGGPS; FAM–PBR K644A = α-amino-FAM-GAGKWKRKSAGGGPS; FAM–PBR K646A = α-amino-FAM-GKGAWKRKSAGGGPS; FAM–PBR K648A = α-amino-FAM-GKGKWARKSAGGGPS; FAM–PBR R649A = α-amino-FAM-GKGKWKAKSAGGGPS; FAM–PBR 650A = α-amino-FAM-GKGKWKRASAGGGPS; FAM–PBR R649A,K650A,S651A = α-amino-FAM-GKGKWKRKSAGGGPS; FAM-linker = α-amino-FAM-VDNPMRRKSGPSCKH; PBR-K(biotin) = GKGKWKRKSAGGGPS-K(ε-amino-biotin); linker-K(biotin) = VDNPMRRKSGPSCKH -K(ε-amino-biotin). For competition experiments biotinylated peptides were used.

Antibodies

Western blotting: anti-UHRF1 (Santa Cruz sc-373750, 1:5000 and sc-100606, 1:1000), anti-GST (Santa Cruz sc-138, 1:1000), anti-6xHIS (Santa Cruz, sc-57598 1:500), anti-FLAG (Sigma F3165, 1:1000 and Santa Cruz sc-807, 1:1000), anti-MYC (Abcam ab-9106, 1:5000), anti-H3 (Abcam ab-1791,

1:10000), anti-GFP (Santa Cruz sc-9996, 1:2000).

Immunoprecipitation: anti-UHRF1 (Santa Cruz sc-373750, 1:1000), anti-GST (Santa Cruz, sc-138, 1:500).

Immunofluorescence: anti-MYC (Abcam ab9103, 1:500) anti-H3K9me3 (Active Motif 39161, 1:1000), anti-rabbit IgG Alexa 488 (Invitrogen A21206, 1:500).

Protein Expression and Purification

Proteins were expressed in BL21-RIL *E. coli* cells or Sf9 insect cells using standard procedures (details are available upon request). HIS-tagged proteins were purified on HisPur Cobalt resin (Pierce) and GST tagged-proteins were purified on glutathione sepharose (GE Healthcare) according to manufacturers' protocols. Eluates form the resins were dialyzed into 50 mM Tris-HCl pH 8, 150 mM NaCl, 10% v/v glycerol, 1 mM DTT and concentrated using ultracentrifugation (Amicon). HEK293 cells were grown in DMEM Glutamax II (supplemented with pen/strep, glutamine, and BGS) and transfected using a Calcium-Phosphate transfection kit (Clontech). After lysis FLAG-UHRF1 was purified on sheep-anti-mouse Dynabeads (Invitrogen) loaded with anti-FLAG antibody. After 2 hrs at 4C, beads were washed twice with SP buffer (50 mM Tris-HCl pH 8, 100 mM NaCl) and once with the same buffer but 500 mM NaCl. Protein was eluted twice in 15 µl FLAG peptide (Sigma, 5 µg/µl in SP buffer).

Peptide Pull-downs

40 µl streptavidin paramagnetic beads (MagneSphere, Promega) were washed three times with PBS in low-binding tubes. 10 µg biotin-labeled peptide or water was added for 2 hrs at 4°C with rotation followed by three washes with PBS. 10 µg recombinant proteins in 500 µl PD300 buffer (20 mM HEPES-NaOH pH 7.9, 300 mM KCl, 0.2% v/v Triton X-100, 20% v/v glycerol) were added and incubated three hrs at 4°C with rotation. Beads were washed six times with PD300 and recovered material was eluted in 30 µl PDelute buffer (50 mM Tris-HCl pH 8, 25% v/v glycerol, 0.25% w/v bromophenol blue, 1 mM EDTA, 2% w/v SDS, 1 mM TCEP) by boiling for 5 min. PIP suspension of the lyophilized diC16 forms

(Echelon Biosciences) were made by adding water. Protein was preincubated at 10-fold molar excess of PIPs in PD300 buffer for overnight at 4°C in a low-binding tube. Nuclear extracts of cells were prepared according to published procedures (Osborn et al., 1989) and used at 2 mg total protein per pull-down. Samples were pre-cleared by addition of Triton x-100 to 0.2% v/v, protease inhibitor (Roche complete), and 20 µl pre-washed beads for 2 hrs at 4°C with rotation. For pull-downs using HeLa NE pre-reacted with Phospholipase A2, 100 µg PLA2 (Sigma, bovine pancreas) was added to 1 ml HeLa NE supplemented with 1 mM $CaCl_2$ and reacted overnight at 4°C. PBR peptides were incubated with 10-fold molar excess of PIP (diC16, Echelon Biosciences) overnight at 4°C. PBR peptides were then applied to beads as above. TTD was loaded with 10-fold molar excess PIP for 3 hrs at 4°C with rotation.

Dialysis of UHRF1

HeLa cells were grown in fermenters, harvested, washed with PBS and flash frozen in liquid nitrogen. Nuclear extracts were prepared according to the Dignam protocol with final dialysis in buffer C (20 mM HEPES pH 7.9, 20% v/v glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT) (Dignam et al., 1983). Extracts were clarified by high speed centrifugation, aliquoted an stored at -80°C for further use. Protein concentration ranged from 10-15 mg/ml.

Proteins (5 mg in 400 µl) were dialyzed against 10 ml HeLa NE or comparable buffer (in 50 ml tubes) overnight in dialysis cups (Pierce) with 3500 Da molecular weight cut-off at 4°C. Solutions were clarified by high speed centrifugation and protein concentration was spectroscopically determined. For sequential dialysis MBP-UHRF1 was dialyzed against 6xHIS-UHRF1 that had been treated as described above.

For pull-downs using HeLa NE pre-reacted with PIP4Kalpha: HeLa NE was incubated with recombinant PIP4Kα supplemented with ATP or ATPγS, and $MgCl_2$ (all 1 mM final concentration), for 8 hrs at room temperature. Addition of ATP and $MgCl_2$ resulted in massive protein degradation in the extracts, preventing analysis of endogenous UHRF1. Therefore purified UHRF1 was dialyzed against the PIP4Kα-treated extracts.

Fluorescence Polarization

Fluorescence polarization (FP) experiments were performed and analyzed as described in FP buffer (25 mM Tris pH 7.5, 50 mM NaCl, 5% v/v glycerol) using a HIDEX Chameleon II plate reader at 4°C (Fischle et al., 2008). For UHRF1 dialyzed against HeLa NE or buffer: Full-length UHRF1 was dialyzed twice against 10 ml of HeLa nuclear extract or comparable buffer overnight at 4°C. Protein was used for fluorescence polarization measurements after concentration determination, without any further treatment. For competition FP experiments: Purified proteins were pre-incubated with non-labeled competitor peptide or protein domain at the indicated molar ratios for 30 min at 4°C. For all experiments shown signals from multiple measurements ($n > 3$) were normalized, averaged and plotted.

Analytical Ultracentrifugation

Double-sector charcoal filled epon cells were loaded with 412 µl of sample buffer or 392 µl protein sample at 0.5 OD_{280} of recombinant UHRF1, either dialyzed against HeLa NE or buffer. Samples were equilibrated in the Beckman XL-A analytical ultracentrifuge 2 hrs to 20°C under vacuum, prior to starting the run. During the run at 50,000 rpm, scans were continuously acquired until all material was at the bottom of the cell. Data analysis was performed with the SEDFIT software using a calculated partial specific volume of 0.722 ml/g for recombinant UHRF1 (Schuck and Rossmanith, 2000). Generally, after positioning the meniscus and the bottom, a simplex fit for the meniscus position was performed at a resolution of 200. The frictional ratio was fitted with the simplex algorithm. Initial values were further refined by fitting with the Marquard-Levenberg and simulated annealing algorithms until the root mean square deviation converged at a minimum. Final fitting was done with a resolution of 200 for the sedimentation coefficient. To address sedimentation coefficient and frictional ratio distribution of the sedimenting UHRF1 population, the continuous C(S)/ff0 distribution model was used at a resolution of 100 for C(S) and 10 for the frictional coefficient (range 1-2.5).

Co-immunoprecipitation

10 µl Dynabeads (Invitrogen) were washed once with PBS, twice with IP150 buffer (50 mM Tris pH 8, 150 mM NaCl, 5% v/v glycerol, 0.05% v/v Triton x-100), and resuspended in IP150 buffer supplemented with 5% w/v BSA. 0.22 nmoles protein were mixed with 1 µl anti-UHRF1 antibodies in 100 µl IP150 buffer, 5% w/v BSA. After 30 min incubation at 4°C, 10 µl washed beads were added and the reactions were rotated at 4°C for 2 hrs. 0.22 nmoles TTD was then added for three more hrs. Reactions were washed five times with IP150 buffer, and eluted using 30 µl PDelute buffer (see above). Samples were evaluated by SDS-PAGE and western blotting. For immunoprecipitations with added H3 competitor peptide, TTD was pre-incubated with H3 peptides at 1:2.5, 1:5, and 1:7.5 molar ratio for 30 min. For immunoprecipitations with added PIPs as competitor, the steps outlined above were followed, with two changes. C terminus and PIPs or water were incubated overnight at 4°C, at a molar ratio of 1:5, 1:10, and 1:20, before mixing with anti-UHRF1 antibody. The detergent was omitted in the IP150 buffer.

Isothermal Titration Calorimetry

TTD and C terminus were dialyzed two times together but in separate dialysis tubing against 1 l filtered (0.45 µm) DITC buffer (20 mM Tris pH 8.0, 100 mM NaCl). Used DITC buffer was de-gassed and retained for washing the ITC sample cell and for ITC control buffer/protein runs. Proteins were concentrated, filtered (0.45 µm) and used for ITC measurements in an ITC-200 (MicroCal) pre-equilibrated to 20°C. The C terminus was added to the sample cell at 50 µM, while the TTD was loaded in the syringe at 500 µM. ITC injection conditions were: 20 injections, 20°C, reference power = 7, 120 second initial delay, spinning = 700-1000, feedback = high. The first injection was 0.2 µl, 0.4 sec duration, 120 sec delay, and the following 19 injections were 2 µl, 4 sec duration, 150 sec delay. The first injection was excluded from the data analysis. Baselines of resulting spectra were adjusted to zero, with buffer/protein reference titrations (having negligible signal) subtracted from protein-protein curves. Curves were evaluated and fits were generated using the software associated with the MicroCal instrument.

PIP Dot Blots

PIP strips (Echelon Biosciences) were blocked with 5 mL PBS-B (PBS, 3% BSA (fatty acid free)) at room temperature for 1 hr. Strips were incubated overnight at 4°C with 5 µg of purified protein in 5 ml PBS-B followed by three washes, 10 min each in 10 ml PBS-T (PBS, 0.1% v/v Tween-20). Reactions were developed by incubation with primary antibody diluted in 5ml PBS-B for 1 hr at 4°C. Membranes were washed three times with 10 ml PBS-T, and then incubated for 1 hr in 5 ml PBS-B with secondary antibody. Strips were washed three times in 10 ml PBS-T, developed with ECL reagent (GE Healthcare) and exposed to film.

Liposome Floatation Assay

10 µl of Poly-piposomes (Echelon Biosciences) were incubated with 0.5 nmoles purified UHRF1, for 10 min on ice. The protein/piposome mixture was filled to 300 µl with cold flotation buffer (50 mM Tris-HCl pH 8, 150 mM NaCl), and mixed with 200 µl cold 75% sucrose in flotation buffer. The mixture (final concentrations 30% sucrose, 1 µM PIP, 1 µM UHRF1, 20 µM liposomes) was applied to a thick-wall polyallomer 11 x 34 mm centrifuge tube (Beckmann). The second layer of the gradient, 400 µl 25% sucrose in flotation buffer, was applied without disturbing the bottom layer. The top layer, 100 µl flotation buffer, was applied without disturbing the middle layer. Tubes were centrifuged for one 1 hr, 55,000 rpm at 4°C, in a TLS-55 swinging bucket rotor (Beckmann). 100 µl fractions were collected from the top of the tube using a pipette, and 15 µl of each fraction was analyzed by SDS-PAGE and western blotting.

Binding of PBR Peptides to PIP Beads

PBR peptides labeled with fluorescein, either WT or R649A-K650A-S651A mutant, were used for PIP bead pull-downs. 10 µl slurry of PIP beads (Echelon Biosciences, control/PI/PI3P/PI4P/PI5P/PI34P/PI35P/PI45P/PI345P) was washed in low-binding tubes twice with binding buffer (10 mM HEPES-NaOH pH 7.9, 150 mM NaCl, 0.5% NP40). 20 ng fluorescein labeled

WT or R494A,K650A,S651A PBR peptide in 50 µl binding buffer were added to the drained beads, and incubated for 2.5 hrs at 4°C in the dark. Beads were then washed four times, using 100 µl binding buffer each time. 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. Fluorescence intensities were averaged from three successive reads and recovery of the peptides was normalized relative to the input.

Reversed Phase Fractionation

Dialyzed proteins (~2-3mg) were filtered (0.45 µm) and applied (1 ml/min) to a preparatory C8 column (Grace Vydac) pre-equilibrated with 5% acetonitrile, 0.1% formic acid in H_20 at room temperature on a Shimadzu HPLC system. A step gradient of 15 min each at 5%-25%-50%-75%-95% acetonitrile in H_2O/formic acid was applied. Fractions corresponding to the different steps were lyophilized, resuspended in 40 µl dialysis buffer, and incubated overnight with purified full-length UHRF1 (30 µg) for further analysis using the peptide pull-down assay.

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 BMRB (Nady et al., 2011). ^{1}H-^{15}N HSQC spectra were collected at 25°C on a Bruker Avance 500MHz spectrometer. Composite chemical shift changes (**Δ**ppm) were calculated using the equation $((\Delta ppmN/6.5)^2 + (\Delta ppmHN^2))^{0.5}$. Histogram profiles were plotted with Microsoft Excel, and figures of TTD with residues corresponding to significant chemical shift changes were generated with PyMol (DeLano Scientific, US).

Molecular Docking

The docking of the PBR peptide to the TDD was performed using HADDOCK v2.1 software (de Vries et al., 2007; Dominguez et al., 2003). Chemical shift perturbation data were translated into

ambiguous interaction restraints (AIR) to drive the docking process. Residues having both high solvent accessibility and large chemical shift perturbation upon binding, were selected as active residues for AIR. These include residues Y140, E153, D190, E193, R207, I211, F237, W238, and E276. The semi-flexible regions of the TTD were defined automatically by HADDOCK, and all residues in the PBR peptide were set to be flexible. The starting structures for the docking experiment were an extended conformation of the PBR peptide generated with CNS (Brunger et al., 1998), and a conformation of the TTD derived from the TTD-PHD crystal structure (pdb: 3ASK; (Arita et al., 2012)). 5,000 docked structures were calculated, and the 200 best solutions were clustered using 6.5 Å rmsd cut-off. Two models, that represent two best clusters each, were selected for subsequent analysis. The selected models, 1 and 2, have similar HADDOCK-scores, of -109 and -108, respectively.

Immunofluorescence Analysis

NIH3T3 cells were grown on glass cover slips to 50% confluency for transfection. Cells were transfected using Lipofectamine LTX (Invitrogen). For competition experiments pmCherry UHRF1construct was co-transfected together with a 2-fold excess of pEGFP-C1 PBR constructs (i.e. 1.25µg and 2.5µg per 6-well dish). PIP4K constructs were cotransfected with pmCherry UHRF1 construct at 1:1:1 ratio. All expressions were carried out ON (14-16 hrs).

Cells were washed twice with PBS, fixed with 3.7% v/v formaldehyde in PBS for 10 min and permealized with 0.5% v/v Triton-X-100 in PBS for 5 min. Slides were blocked for 30 min in blocking buffer (2.5% w/v BSA, PBS, 0.05% v/v Tween-20) before incubation for 3 hrs with primary antibodies diluted in the same buffer. Slides were washed three times in wash buffer (PBS, 0.05% v/v Tween-20) and incubated for 1 hr with secondary antibodies. Slides were stained with DAPI (1 µg/ml in wash buffer) and mounted with Vectashield (Vector, #H-1000). Images were taken on a Leica SP5 confocal microscope.

SUPPLEMENTAL REFERENCES

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