Molecular basis for H3K36me3 recognition by the Tudor domain of PHF1

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The PHD finger protein 1 (PHF1) is essential in epigenetic regulation and genome maintenance. Here we show that the Tudor domain of human PHF1 binds to histone H3 trimethylated at Lys36 (H3K36me3). We report a 1.9-Å resolution crystal structure of the Tudor domain in complex with H3K36me3 and describe the molecular mechanism of H3K36me3 recognition using NMR. Binding of PHF1 to H3K36me3 inhibits the ability of the Polycomb PRC2 complex to methylate Lys27 of histone H3 in vitro and in vivo. Laser microirradiation data show that PHF1 is transiently recruited to DNA double-strand breaks, and PHF1 mutants impaired in the H3K36me3 interaction exhibit reduced retention at double-strand break sites. Together, our findings suggest that PHF1 can mediate deposition of the repressive H3K27me3 mark and acts as a cofactor in early DNA-damage response.

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example of a robust reader of this post-translational modification (PTM). It is also the first single-Tudor module capable of recognizing methylated lysine. We found that interaction of Tudor with H3K36me3 inhibits PRC2-mediated H3K27 methylation. This is in line with recent reports that H3K36me3 antagonizes H3K27 methylation by PRC224–26. Furthermore, our results demonstrate Tudor-dependent accumulation of PHF1 at irradiation-induced DNA-damage sites, suggesting a previously unrecognized role of this interaction in DNA repair.

RESULTS
Structure of the H3K36me3-bound Tudor domain of PHF1

To elucidate the molecular mechanism of the H3K36me3 recognition, we obtained a 1.9-Å resolution crystal structure of the PHF1 Tudor domain in complex with an H3K36me3 peptide and established the determinants of specificity toward this epigenetic mark (Fig. 1 and Table 1). In the complex, the PHF1 Tudor domain folds into a five-stranded β-barrel, whereas the H3K36me3 peptide adopts an extended conformation (Fig. 1). The peptide is bound across one of the open edges of the β-barrel, formed by the twisted β2 and β4 strands. Overall, the binding interface is extensive, with 14 residues of the Tudor domain and 9 residues of the peptide (Thr32–Arg40) involved in direct contacts. The complex buries an accessible surface area of 437 Å² in the protein and 535 Å² in the peptide. The H3K36me3-binding site consists of three well-defined regions: a central aromatic cage, a hydrophobic patch and an acidic groove (brown, green and blue, respectively; Fig. 1).

The extended side chain of trimethylated Lys36 of the peptide occupies the aromatic cage formed by the Tyr47, Trp41, Phe65 and Phe71 residues of the Tudor domain. The aromatic moieties of Tyr47, Trp41 and Phe65 are positioned orthogonally to each other and are engaged in cation-π and hydrophobic interactions with the trimethylammonium group of Lys36. The aromatic moiety of Phe71 is slightly rotated, probably contributing more to the hydrophobic contact and less to the cation-π interaction. A similar mode of trimethylated lysine recognition via an aromatic cage has been found in other histone-binding modules, including the chromodomain, the PHD finger and the TTD (reviewed in refs. 27,28). In contrast to many of these modules, the aromatic cage of PHF1 Tudor is not pre-formed in the free state, even though the secondary-structure elements of PHF1 Tudor in complex with the peptide and in the apo state (PDB 2E5P) superimpose well (r.m.s. deviation of 1 Å), indicating that binding induces conformational changes in the aromatic cage.

The hydrophobic side chain of Pro38 and the neutral side chain of His39 of the peptide are bound in the hydrophobic patch, which is composed of four solvent-exposed leucine residues of the PHF1 Tudor domain: Leu38, Leu45, Leu46 and Leu48. Additionally, the intermolecular hydrogen bond formed between the backbone NH group of His39 and the carbonyl group of Leu46 restrains His39. The Thr32, Gly33 and Gly34 residues of the peptide lie in a shallow acidic groove, in which the backbone HN of Val35 is hydrogen bonded to one of the oxygen atoms of the carboxyl moiety of Glu66 of the protein. Another oxygen atom of this carboxyl group makes a hydrogen bond and a salt bridge with the ammonium group of Lys37.

The PHF1 Tudor domain is specific for H3K36me3

We used three approaches to define the physiological ligand of the Tudor domain of PHF1 (Figs. 2 and 3, and Supplementary Fig. 1). Initially, we carried out a pull-down experiment with biotinylated histone peptides corresponding to unmodified H3 and singly modified H3K4me1, H3K4me2 and H3K4me3; H3K9me1, H3K9me2 and H3K9me3; H3K27me1, H3K27me2 and H3K27me3; and H3K36me1, H3K36me2 and H3K36me3. We used a yeast two-hybrid assay to monitor intermolecular hydrogen bonding and hydrophobic forces. Finally, we performed a pyridylboronic acid (PBA) competition assay. The results of all these experiments strongly indicate that PHF1 Tudor is specific for the trimethylated lysine on H3K36.

Table 1 Data collection and refinement statistics

| Data collection | PHF1 Tudor–H3K36me3 |
|-----------------|---------------------|
| Space group     | C2                  |
| Cell dimensions | 88.03, 29.67, 62.00 |
| α, β, γ (°)     | 90, 116.41, 90      |
| Resolution (Å)  | 23.41–1.85 (1.92–1.85) |
| Rmerge          | 0.024 (0.13)        |
| I / σI          | 19.4 (4.3)          |
| Completeness (%)| 96.4 (81.1)         |
| Redundancy      | 2.2 (1.7)           |

Refinement

| Resolution (Å) | 1.85 |
| No. reflections | 12,073 |
| Rwork / Rfree  | 0.2038 / 0.2439 |
| No. atoms       | 467               |
| Protein         | 76                |
| Ligand/ion      | 161               |
| Water           | 39.6              |
| R.m.s. deviations | Bond lengths (Å) | 0.007 |
| Bond angles (°) | 1.06             |

A single crystal was used for solving the structure. Values in parentheses are for highest-resolution shell.
Supplementary Fig. 1a). The histone peptides trimethylated at other lysine residues, including H4K20me3, H3K4me3, H3K9me3 and H3K27me3, induced differences in the chemical shifts observed in the corresponding spectra in Supplementary Fig. 2c. H3K36me2 and H3K36me3 caused resonance perturbations in the aromatic cage (Fig. 2b). H3K36me2 and H3K36me3 peptides caused smaller resonance perturbations and were bound to the aromatic cage residues of the Tudor domain. The aromatic cage residues of the Tudor domain are conserved in only PHF19. The aromatic cage residues across the Tudor domain from PHF1, MTF2 and PHF19, the three human homologs of 53BP1, shows that all four aromatic residues in the cage are conserved in only PHF19, suggesting that it also recognizes H3K36me3 (Fig. 3). Conversely, the 53BP1 Pcl Tudor domain, which lacks the two aromatic residues corresponding to Trp41 and Phe71 of PHF1, does not bind methylated histone peptides. The importance of the aromatic residues in PHF1 was underscored by the fact that substitution of either Trp41 or Tyr47 with an alanine disrupted binding to H3K36me3 even as the structure of the protein remained intact (Fig. 3a and Supplementary Fig. 3).

Much like TTD of 53BP1, which is specific for a dimethylated lysine residue, the single PHF1 Tudor domain contains an acidic residue in close proximity to the aromatic cage. In the 53BP1 complex, the aspartate forms a hydrogen bond and an ionic contact with the dimethylammonium group, and these interactions account for the preference for dimethylated over trimethylated lysine. A much longer distance between the carboxylic group of Asp66 and the K36me3 group in the PHF1 complex (5.6 Å in PHF1 compared to 2.8 Å in 53BP1) suggests that the residues adjacent to Lys36 are necessary for the interaction. A comparison of the amino acid sequences of the histone peptides shows that only H3K36me3 contains a basic residue (Lys37) followed by a proline and a histidine residue C-terminal to methylated Lys36, and a Gly33-Gly34 tandem N-terminal to methylated Lys36 (Supplementary Fig. 2c).
Figure 4  Recognition of H3K36me3 by PHF1 inhibits PRC2 methylation transferase activity. (a) Western analysis of wild-type and mutated Flag-PHF1 and EZH2 in PRC2 complexes. (b) HKMT assays with PHF1–PRC2 complexes purified from HEK293T cells on native wild-type chromatin (WT SON, blue) and chromatin lacking the H3K36me mark (∆set2 SON, red). Error bars represent s.d. based on three experiments. (c) Western analysis of whole-cell extract from HEK293T cells 48 h after transfection with wild-type HA-PHF1 or W41A or Y47A mutants. Empty vector served as a control. (d) Western analysis of K562 cells stably expressing Flag-PHF1.

The PHF1 Tudor–H3K36me3 interaction inhibits PRC2 activity

We purified human PHF1–PRC2 complexes from HEK293T cells using wild-type or mutated Flag-PHF1. Mutations that disrupt the aromatic cage of the PHF1 Tudor domain had no apparent effect on the association with EZH2, in agreement with previous observations that the PHD fingers region of PHF1 (ref. 12), but not the Tudor domain, is responsible for the interaction with EZH2 (Fig. 4a). We tested the enzymatic activity of PHF1–PRC2 on purified native short oligonucleosomes (SONs) by histone methyltransferase assays (Fig. 4b). We used native chromatin from the yeast *Saccharomyces cerevisiae* because it contains a high level of H3K36me3 owing to its high transcription activity and gene density30. When compared to the wild-type PHF1–PRC2 complex, the PRC2 complexes containing PHF1 mutants impaired in H3K36me3 binding (W41A or Y47A) showed a substantial increase in methyltransferase activity (Fig. 4b, blue columns). Notably, we detected a similar marked increase in the methyltransferase activity of wild-type PHF1–PRC2 on chromatin purified from ∆set2 mutant cells, which completely lack H3K36 methylation (Set2 is the sole H3K36-specific methyltransferase in yeast31) (Fig. 4b, blue and red columns on the left-hand side). As evidenced by liquid counts and autoradiogram analysis (Supplementary Fig. 5), loss of the H3K36me mark on chromatin resulted in more robust catalytic activity of wild-type PHF1–PRC2. In contrast to normal chromatin, the PRC2 complexes harboring PHF1 mutants defective in H3K36me3 binding did not show increased methyltransferase activity compared to wild-type PHF1–PRC2 when using ∆set2 chromatin (Fig. 4b, compare red columns). These results confirm that H3K36me3 is inhibitory to PRC2 (refs. 24–26) and now clearly link PHF1 binding to this functional cross-talk, as both wild-type PHF1 and H3K36me3 are required to block PRC2. Taken together, these data indicate that recognition of H3K36me3 by the PHF1 Tudor domain negatively regulates the enzymatic activity of PRC2.

Binding of the PHF1 Tudor domain to H3K36me3 decreases H3K27me3 levels in vivo. To examine the effect of this interaction, we overexpressed wild-type or mutated hemagglutinin (HA)-tagged PHF1 in HEK293T cells and assessed the global levels of H3K27me3 and H3K36me3 by western blot analysis (Fig. 4c). Overexpression of wild-type PHF1 led to a decrease in the global level of H3K27me3.
as compared to the endogenous level of this mark seen in the empty vector control. In contrast, exogenous PHF1 W41A or Y47A mutants had no effect on the level of H3K27me3. Notably, the levels of EZH2, total H3 and H3K36me3 were equal regardless of whether we used wild-type or mutated PHF1. Furthermore, stable overexpression of wild-type PHF1 in K562 cells resulted in a similar reduction in H3K27me3 levels (Fig. 4d). These data support the idea that PHF1 inhibits PRC2 methyltransferase activity in response to H3K36me3, as overexpression of PHF1 oversensitizes PRC2 to H3K36me3, causing a decrease in H3K27me3 levels, an effect that is not seen upon overexpression of mutants unable to bind H3K36me3.

To determine the role of H3K36me3 recognition by PHF1 in the PHF1–PRC2 HKMT activity at target loci, we tested the occupancy of HA-PHF1 (wild type and mutants) and the levels of H3K27me3 and H3K36me3 at the promoter of MYT1 (ref. 32) in HEK293T cells (Fig. 5). We performed chromatin immunoprecipitation (ChIP) analyses on chromatin that was sheared to ~500 bp after fixing. We used primer probes specific for the MYT1 gene, spanning from −2,000 bp to +1,200 bp relative to the transcription start site. At all locations tested, the occupancy of exogenous wild-type PHF1 was considerably higher than was the occupancy of the PHF1 W41A or Y47A mutants impaired in H3K36me3 binding, whereas H3K36me3 levels remained unchanged (Fig. 5a). These results indicate that binding of the Tudor domain to H3K36me3 is important for targeting of PHF1 to the MYT1 gene. However, as expected, H3K27me3 levels were substantially reduced upon overexpression of wild-type PHF1. Mutation of the PHF1 Tudor domain residues that are crucial for H3K36me3 binding partially rescued this effect and restored the methyltransferase activity of PRC2. The inverse correlation between H3K36me3-dependent PHF1 occupancy and the H3K27me3 level reinforces the idea that PHF1 inhibits PRC2 activity in response to H3K36me3.

To investigate the importance of the PHF1–H3K36me3 interaction in PRC2-mediated deposition of H3K27me3 in a more physiological context, we generated mouse embryonic stem (mES) cells stably expressing Flag-PHF1. Flag immunoprecipitation and MS analysis, that transduced Flag-PHF1 was assembled in a normal PRC2 complex in mES cells (data not shown). We performed ChIP assays using primers for genes known to be either transcriptionally active or silenced in mES cells. For example, the Hoxa4 and Hoxa11 clusters (Fig. 5b). In contrast, transcriptionally active genes Oct4 and Nanog, which typically contain a high level of H3K36me3, showed a reproducible decrease of the H3K27me3 signal in cells expressing Flag-PHF1. These results suggest that PHF1 Tudor-mediated inhibition of the HKMT activity of PRC2 is specific to the genomic sites that contain H3K36me3.

Functional Tudor is required for localization of PHF1 at DSBs

After irradiation of cells, H3K36me2 levels increase rapidly at DNA-damage sites, and then decrease34. Analysis of histone methylation reveals that generation of this PTM is the main immediate methylation event at DSBs, which also correlates with DNA repair efficiency34. H3K36me2 recruits and stabilizes DNA repair components including Ku70, Ku80 and NBS1 at DSBs, enhancing NHEJ repair34. Recently, PHF1 was shown to localize to sites of DNA damage and interact directly with Ku70–Ku80 (ref. 11). Although the PHF1 Tudor domain is not involved in the interaction with Ku70–Ku80, it has a role in targeting of PHF1 to DSBs31. We examined whether the PHF1 localization at DSBs depends on the ability of the Tudor domain to recognize methylated H3K36. We used a 405-nm laser to microirradiate U2OS cells expressing GFP-labeled PHF1—wild type, W41A or Y47A, mutants impaired in binding to H3K36me3—to induce DNA DSBs. We then used fluorescence microscopy to visualize the GFP-tagged proteins. Wild-type GFP-PHF1 was recruited to the DSB sites 1 min after irradiation and dissociated within 6 min (Fig. 6, Supplementary Fig. 6a and Supplementary Video 1). In contrast, the and W41A and Y47A mutants defective in H3K36me3 binding had substantially shorter retention times at the DSB sites, dissociating immediately after the initial accumulation at the DSBs. Accumulation of wild-type and mutant proteins was completely abrogated in the presence of a Poly (ADP-ribose) polymerase 1 (PARP1) inhibitor and reduced by an ataxia telangiectasia mutated (ATM)/ataxia telangiectasia and Rad3 related (ATR) kinase inhibitor (Supplementary Fig. 6b–d and Supplementary Video 2). This result indicates that the recruitment of PHF1 to DNA lesions strongly depends on activation of PARP1 in addition to requiring Ku70–Ku80 (ref. 11). Together, these data suggest a role for the recognition of methylated H3K36 by the Tudor domain in the stabilization of PHF1 at DSB sites in the PARP1- and Ku70–Ku80-initiated DNA-damage response pathways.
DISCUSSION

In this study, we show that the Tudor domain of PHF1 binds strongly and specifically to H3K36me3, and that this interaction is required for the multifunctioned activity of PHF1. We report that recognition of H3K36me3 by PHF1 inhibits the ability of the PRC2 complex to methylate H3K27. Binding of PHF1 to H3K36me3 may represent a mechanism for regulation of PRC2, which can be particularly important for delineating transcriptionally active and repressed regions. H3K27me3 and H3K36me3 are known to be antagonistic marks. Previous studies have demonstrated the inhibition of PRC2 by H3K36me3 (refs. 24–26), and it has been proposed that this PTM acts as a barrier to prevent deposition of H3K27 methylation at transcriptionally active genes (refs. 24,25). Knockdown of the mouse homolog of PHF1 results in downregulation of several Hox genes in NIH 3T3 cells. Our data show that it is the PHF1 component of the PRC2 complex that is responsible for reading H3K36me3 and inhibiting PRC2 activity in the presence of this PTM.

Binding of the PHF1 Tudor domain to H3K36me3 may sterically preclude EZH2 priming at H3K27 (Fig. 5c); however, further work is needed to define the precise mechanism of PRC2 inhibition and to explore the effects of additional components that modulate PRC2 function. EED and JARID2, two other subunits of the PRC2 complex, can both activate and reduce PRC2 activity,35–41. The WD40 repeat of EED increases methyltransferase activity in response to H3K27me3 but decreases activity in response to H1K26me3 (ref. 40). Furthermore, in the absence of H3K36me3, PHF1 can increase the methyltransferase activity of PRC2 (refs. 8,9), which could be due to possible interactions of the PHD fingers of PHF1 with other PTMs (for example, unmodified histone H3 or H3K4me3, which cause no steric collision with H3K27me3). These results support the idea that PHF1 senses the local epigenetic environment through distinct modules. The presence of H3K36me3 and its recognition by the Tudor domain is required to inhibit PRC2 activity, and distinctive distributions of this PTM in different cells may lead to opposite outcomes, for example, as seen in the regulation of Hox genes in NIH 3T3 and GC1Spg cells. Thus, the exact composition of the PRC2 complex and the level and distribution of PTMs, which are known to fluctuate in transient localization of PHF1 at DNA-damage sites. PHF1 has been shown to interact and colocalize with Ku70–Ku80 at DSBs, and two of the regions necessary for targeting were identified as the Tudor domain and a region C-terminal to the PHD fingers of PHF1 (ref. 11). PHF1 can use both the Ku70–Ku80-dependent and/or PARP1-dependent mechanisms for accumulation, which may differentially contribute to anchoring and retention of PHF1. Rapid accumulation and stabilization of PHF1 could be essential in the early response to DNA damage, which involves not only Ku70–Ku80 and PARP1 but also PRC2, as other components of PRC2, such as EZH2, are found to colocalize with damaged DNA.43,44 A dynamic regulation of these complexes through variation in their composition could lead to greater sensitivity to the cellular environment and response to extrinsic stimuli, providing a mechanism for differentiating local epigenetic states.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Protein Data Bank: Atomic coordinates for the PHF1 Tudor domain in complex with an H3K36me3 peptide have been deposited with accession code 4HC2.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

C.A.M. and T.G.K. designed the study. C.A.M., N.A., R.W., C.G.A., M.-E.L., S.R. and J.K.N. performed experiments and together with Z.H., C.A., J.N., C.A.K., A.Y., J.C. and T.G.K. analyzed the data. T.G.K. and C.A.M. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

DNA constructs, mutagenesis, and protein expression and purification. Wild-type PHF1 Tudor domain (residues 14–87 and residues 28–87) was cloned from full-length human PHF1 (Open Biosystems). Point mutants (W41A and Y47A) were generated by site-directed mutagenesis using the Stratagene QuickChange XL kit. Wild-type and mutant proteins were expressed in Escherichia coli BL21(DE3) pLysS cells grown in LB medium and in M9 minimal medium supplemented with 13NH4Cl or with 13NH4Cl and 13C6glucose. Expression was induced with 0.5 mM IPTG, and the bacteria were harvested by centrifugation and lysed by sonication. The unlabeled, 15N-labeled and 15N and 13C-labeled GST-fusion proteins were purified using glutathione–Sepharose 4B beads (GE Healthcare). The GST tag was either cleaved with PreScission protease, or left for the purposes of western blot analysis, in which case the GST-fusion protein was eluted off the glutathione-Sepharose beads using 0.05 M reduced 1-glutathione (Sigma-Aldrich). Proteins were further purified by size-exclusion chromatography.

X-ray crystallography. A solution of 3 mg ml−1 PHF1 Tudor (residues 28–87) was incubated overnight with H3K36me3 peptide (residues 31–40) in a 1:1.5 molar ratio before crystallization. Crystals of the complex were grown using the sitting-drop vapor-diffusion method at 4 °C by mixing 1 µl of the protein-peptide solution with 1 µl of precipitant solution containing 0.1 M HEPES, pH 7.5, 20% PEG 10000. Crystals grew in a monoclinic space group C2 with two molecules per asymmetric unit. The complete data sets were collected at 100 K on a home source Rigaku/MSC Ru-H3 X-ray generator and Raxis IV++ area detectors system. The data were processed with D*TREK42. The molecular-replacement solution was generated using the program Phaser46 and the solution structure of PHF1 (PDB 2E5P) as a search model. The initial models were built with COOT and refined using the program Phenix47. Ramachandran plot statistics showed 96.8% of residues in the allowed regions, 3.2% in the generously allowed regions and none in the disallowed regions. Remaining statistics are shown in Table 1.

Pull-down assays. GST-fusion PHF1 Tudor (residues 14–87) was incubated with C-terminally biotinylated peptides (Upstate Biotechnology) corresponding to the unmodified H3 (residues 1–21) and singly modified (H3K4me1, H3K4me2 and H3K4me3 (residues 1–21); H3K27me1, H3K27me2 and H3K27me3 (residues 21–44); and H3K36me1, H3K36me2 and H3K36me3 (residues 21–44)) histone tails in the presence of streptavidin-Sepharose beads (GE Healthcare) in binding buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl and 0.05% Nonidet P-40. The beads were collected by centrifugation and washed five times with the peptide binding buffer. Bound protein was detected by western blotting using anti-GST HRP conjugate monoclonal antibodies (GE Healthcare). A negative control using GST-labeled Tudor in the absence of the peptides was run in parallel to ensure that the protein did not bind to the streptavidin beads.

Nuclear magnetic resonance spectroscopy. NMR experiments were collected on a Varian INOVA 600-MHz spectrometer equipped with a cryogenic probe. Backbone assignments were obtained through analysis of the triple-resonance HNACAB and CBCA(CO)NH experiments carried out on uniformly 15N- and 13C-labeled PHF1 Tudor (residues 14–87) in 20 mM Tris, pH 6.8, and 150 mM NaCl. Initial assignments were obtained using the PINE program and were verified and completed in CcpNMR.

Chemical shift perturbation experiments were carried out using uniformly 15N-labeled wild-type or mutant Tudor (residues 14–87). 1H, 15N HSQC spectra were recorded in the presence of increasing concentrations of 12-mer histone tail peptides (synthesized by the University of Colorado Denver Biophysics Core Facility). Kd values were calculated by a nonlinear least-squares analysis in Kaleidagraph using the equation

\[
\Delta \delta = \Delta \delta_{\text{max}} \frac{(\langle L \rangle + [P] + K_d)}{(\langle L \rangle + [P] + K_d + 4\langle P \rangle [L]/2 \langle P \rangle)}
\]

where \(\langle L \rangle\) is the concentration of the peptide, \([P]\) is the concentration of the protein, \(\Delta \delta\) is the observed normalized chemical shift change and \(\Delta \delta_{\text{max}}\) is the normalized chemical shift change at saturation, calculated as

\[
\Delta \delta_{\text{max}} = \sqrt{(\Delta \delta^2 + \Delta \delta_{\text{set2}}^2)} + \Delta \delta_{\text{max}}^2
\]

where \(\delta\) is the chemical shift in p.p.m.

Isothermal titration calorimetry. ITC experiments were carried out at 25 °C on a VP-ITC calorimeter (MicroCal). Protein and peptide were kept in an identical buffer of 20 mM Tris, pH 6.8, and 150 mM NaCl. We injected 30 sequential injections of 10 µl of peptide (1.5–3 mM) into 1.7 ml of PHF1 Tudor (0.04 mM) at 180-s intervals. Control experiments measuring the heat of dilution were performed by injecting the peptide into buffer, and the values obtained were subtracted from the raw data before the fitting process. Binding isotherms were analyzed by nonlinear least-squares fitting of the data using Microcal ORIGIN software (Microcal) and a one-site binding model.

PHF1–PRC2 complex purification. Small amounts of plasmsids encoding wild-type or mutant 3×Flag-PHF1 were transfected into HEK293T cells using calcium phosphate. At 48 h after transfection, cells were harvested and whole-cell extracts were prepared. Flag-PHF1 was purified by incubating the extracts overnight with 3×Flag agarose resin (Sigma-Aldrich), which was then washed extensively. Complex composition was examined by SDS-PAGE and silver staining, and by western blotting with antibodies to Flag (Sigma-Aldrich) or EZH2 (Santa Cruz Biotechnology). PRC2 complexes were also purified similarly but after co-transfection of vectors expressing all subunits (HA-tagged EZH2, SUZ12, EED, RhAp48 and Flag-PHF1), with similar results.

Histone methyltransferase assays. Purified protein complexes were incubated with 500 ng of short oligonucleosomes (purified from yeast cells as described) or 300 ng of the indicated histone H3 peptides in a final volume of 15 µl using 1 µM of [3H]AdoMet (5-adenosyl-l-[methyl-3H]methylene, 80 Ci mmol−1; SAM) in HMT buffer (20 mM Tris-HCl pH 8, 50 mM KCl, 0.1 mM EDTA, 5% (v/v) glycerol, 1 mM DTT, 1 mM PMSF) for 45 min at 30 °C. Each reaction was spotted onto p81 filters, washed three times with 50 mM sodium carbonate, pH 9.2, and processed for scintillation counting, or loaded on a gel and processed for fluorography. In results from liquid counting, error bars represent s.d. of independent reactions. Oligonucleosomes were purified from yeast cells because of the high content of H3K36me3 and the possibility to use yeast Set2 mutant cells that completely lack this mark.

Cell culture, transfection and antibodies for western analysis. Full-length human PHF1 was obtained from Open Biosystems (Thermo Scientific) and cloned into the pIRE2-EGFP plasmid to produce PHF1 with a C-terminal HA tag. The mutant constructs W41A and Y47A were generated using the Stratagene QuikChange XL Site-Directed Mutagenesis kit according to the manufacturer’s instructions. HEK293T cells were propagated in DMEM containing 10% (v/v) FBS and penicillin-streptomycin, and PHF1 (wild type or mutant) or an empty plasmid (negative control) was transiently transfected using polyethylenimine. Cells were harvested 48 h after transfection and the whole-cell lysate was collected for western analysis. Alternatively, western analysis was performed on acid-extracted histones from stably transfected K562 cells. Antibodies used included a rabbit polyclonal to the HA tag (Abcam ab9110), a rabbit polyclonal to histone H3 (Abcam ab1791), a rabbit polyclonal to H3K27me3 (Millipore 17–622), a rabbit monoclonal to H3K36me3 (Cell Signaling D5A7), a rabbit monoclonal to EZH2 (Cell Signaling D2C9) and normal rabbit IgG (Abcam ab37415).

Chromatin immunoprecipitation. We fixed 1 × 106 transiently transfected cells with 1% (v/v) formaldehyde for 14 min at room temperature before collection. Chromatin was sonicated to fragments of ~500 bp. Immunoprecipitations were performed in 167 mM NaCl, 0.01% (w/v) SDS, 1.1% (v/v) Triton X-100, 16.7 mM Tris pH 8.1, 1 mM EDTA containing protease inhibitor cocktail with 4 µg antibody and protein A–coupled magnetic beads (Millipore). Samples were assayed by qPCR using seven primer sets for the Myt1 gene corresponding to positions −1680 to −1555 (1), −1280 to −1211 (2), −807 to −725 (3), −442 to −297 (4), −3 to 83 (5), 292 to 397 (6) and 761 to 866 (7) around the transcription start site, and a primer set for the non-target PCLB4 gene as a control. We assayed 1% of each of the input chromatin DNA and the ChIP DNA samples by qPCR in triplicate using a Roche LightCycler 480. Fold enrichments of histone marks were calculated as IgG-subtracted %Input divided by the IgG-subtracted %Input of the reference gene PCLB4. Data are shown as an average and s.d. over three biological replicates.

Mouse embryonic stem cells. mES cells (ww6) were cultured following standard protocol on fibroblasts or in the presence of recombiant leukemia inhibitory
factor. Clones expressing Flag-tagged PHF1 were selected for after transduction with virus containing the pRTF vector (empty or PHF1). ChIP analysis was performed using 500 µg of cross-linked chromatin and anti-H3 (Abcam) or anti-H3K27me3 (ActiveMotif). Primers used for qPCR are available upon request and are located in the proximity of the transcription start site. The mean values and s.e.m. were computed between two distinct ChIP experiments.

**Fluorescence microscopy and UVA laser irradiation.** Fluorescence images were obtained and processed using a confocal scanning laser microscopy system (FV-500, Olympus). UVA laser irradiation was used to induce DSBs in U2OS cells as described11. Briefly, U2OS cells in glass-bottomed dishes were microirradiated with a 405-nm pulse laser (Olympus) along a user-defined path. The laser was focused through a 40× objective lens and the treatment dose was controlled by the number of scans used. A single laser scan at full power delivers about 1,600 nW. Cells were treated with 0.1 µM 8-MOP. The irradiation dose was fixed in the experiments as 50 scans. Experiments with inhibitors were carried out by pre-treating cells with 1.0 µM PARP1 inhibitor (AZD2281/KU0059436) for 4 h or 1 mM ATM (ATR) kinase inhibitor (cat. no. 118501, Calbiochem) for 4 h before irradiation. The efficacy of the PARP1 inhibitor (AZD2281) was confirmed through the loss of accumulation of the PARP1 cofactor XRCC1 to single-stranded breaks (induced by low doses of radiation) as well as a decrease in poly(ADP-ribose) (PAR) polymer as detected by western analysis.

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