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Supplementary Information for

Histone H3 tail binds a unique sensing pocket in EZH2 to activate the PRC2 methyltransferase

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This PDF file includes:

Supporting information table of contents

Supporting Figures

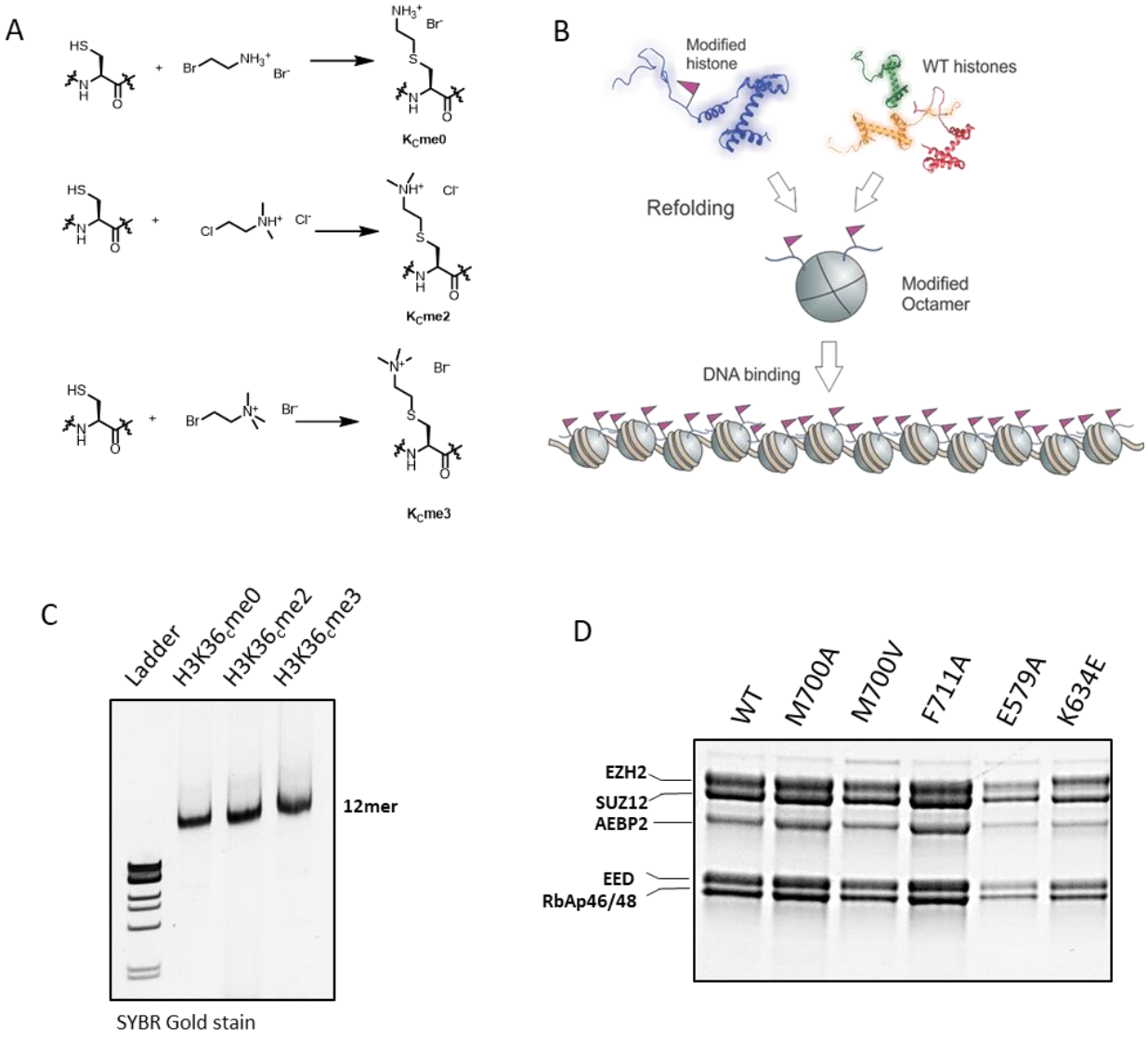

Figure S1. Chromatin substrates and enzyme complexes used in this study. **A-B.** Schematic depicting how H3 histones modified using cysteine alkylation with methyl-lysine analogs are combined with other histones to form modified octamers and homogeneously modified 12mer array chromatin substrates. **C.** Native APAGE gels stained for DNA using SYBR Gold showing 12mer array substrates containing octamers with the indicated H3K36 side chain. **D.** Ruby stained SDS-PAGE analysis of PRC2 complexes containing WT or mutant EZH2 subunits.

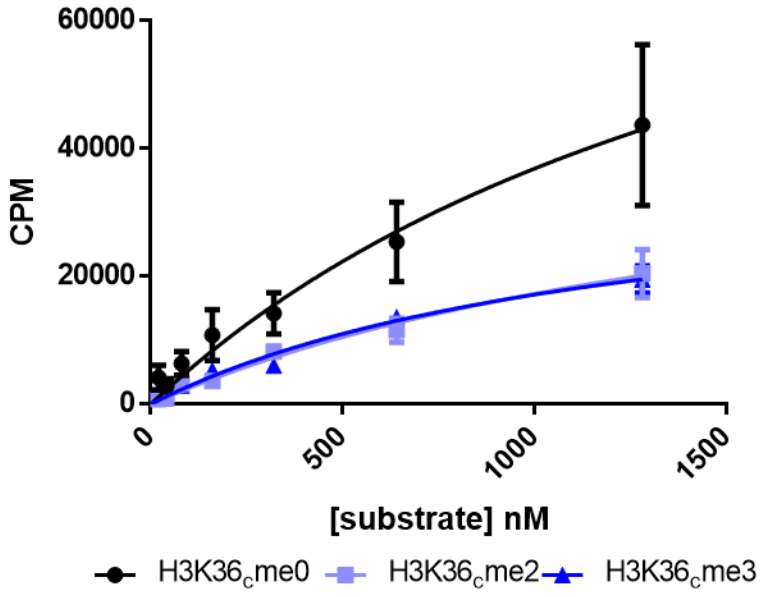

Figure S2. PRC2 HMT activity on mononucleosome substrates. Attempted kinetic analysis of WT PRC2 activity using 25 nM enzyme on increasing concentrations of mononucleosome substrates which are either modified or unmodified at H3K36. Saturation was not achieved even at very high concentrations of substrate. Error bars represent SEM (n=3).

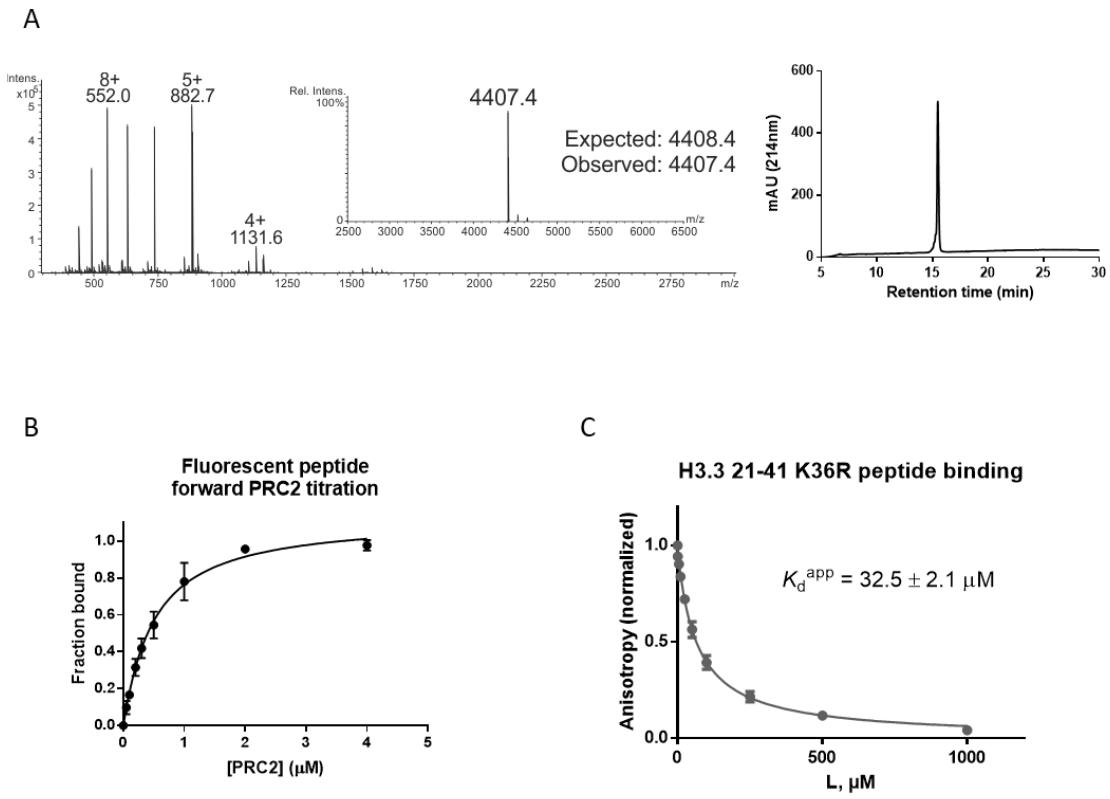

Figure S3. PRC2 binding to H3 peptide substrates measured by fluorescence anisotropy. **A.** Analytical data for H3 fluorescent peptide used in binding studies (ESI-MS and RP-HPLC). **B.** Forward titration of PRC2 with fluorescent H3 peptide consisting of residues 1-37 containing norvaline at K27 and C-terminal fluorescein used in determination of K_d calculations (K_d of reference fluorescent peptide = 465 ± 82 nM). **C**. Binding between PRC2 core and H3 peptide (residues 21-41) containing a K36R mutation. Shown is the change in steady-state anisotropy of a fluorescein-labeled H3 peptide upon being displaced from PRC2 core by the added unlabeled substrate peptide. Error bars represent SEM (n=3). The apparent K_d of the K36R mutation-containing peptide was determined to be 32.5 ± 2.1 μM).

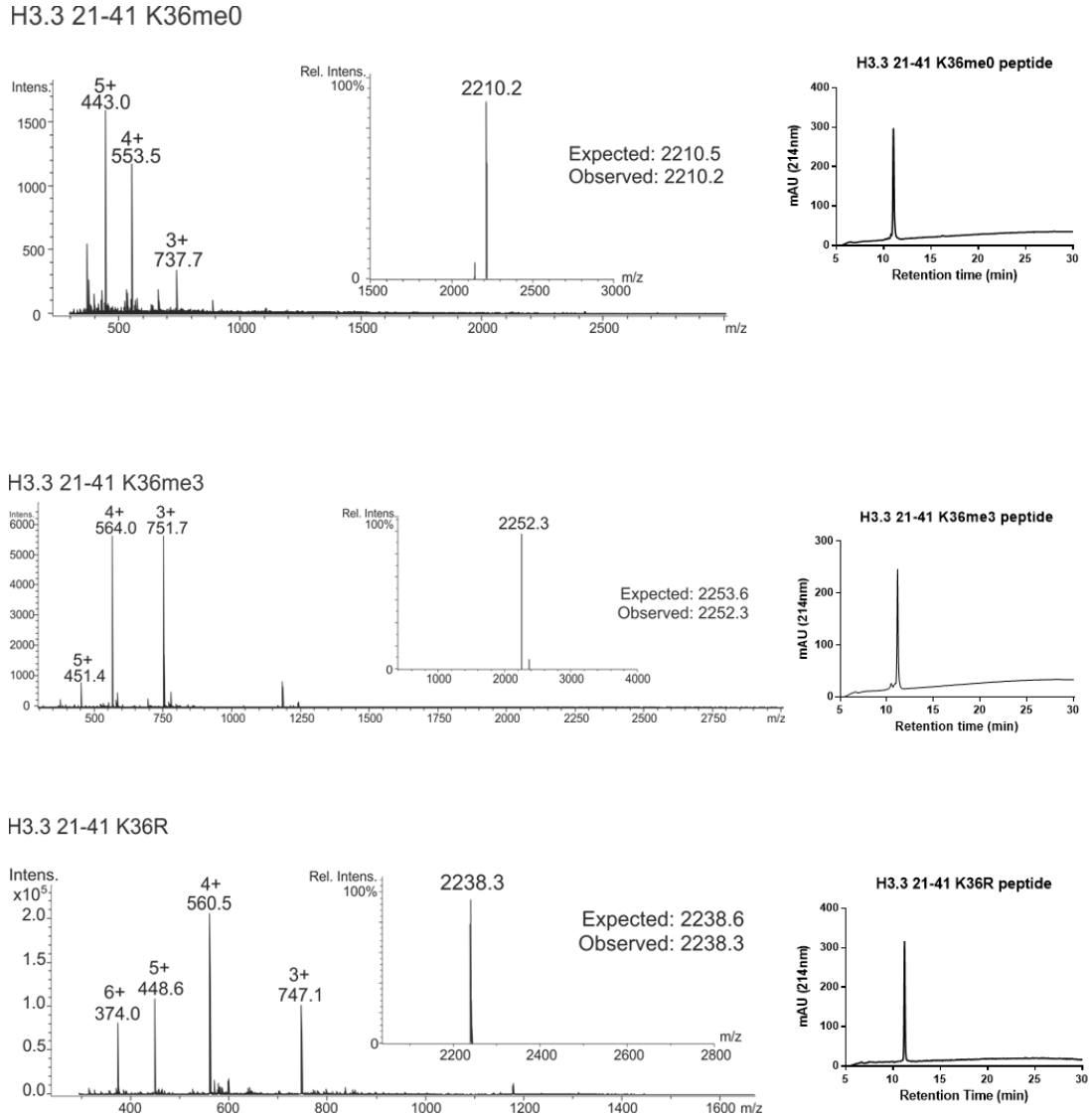

Figure S4. Analytical data for H3.3 peptides used in fluorescence anisotropy binding studies (ESI-MS and RP-HPLC).

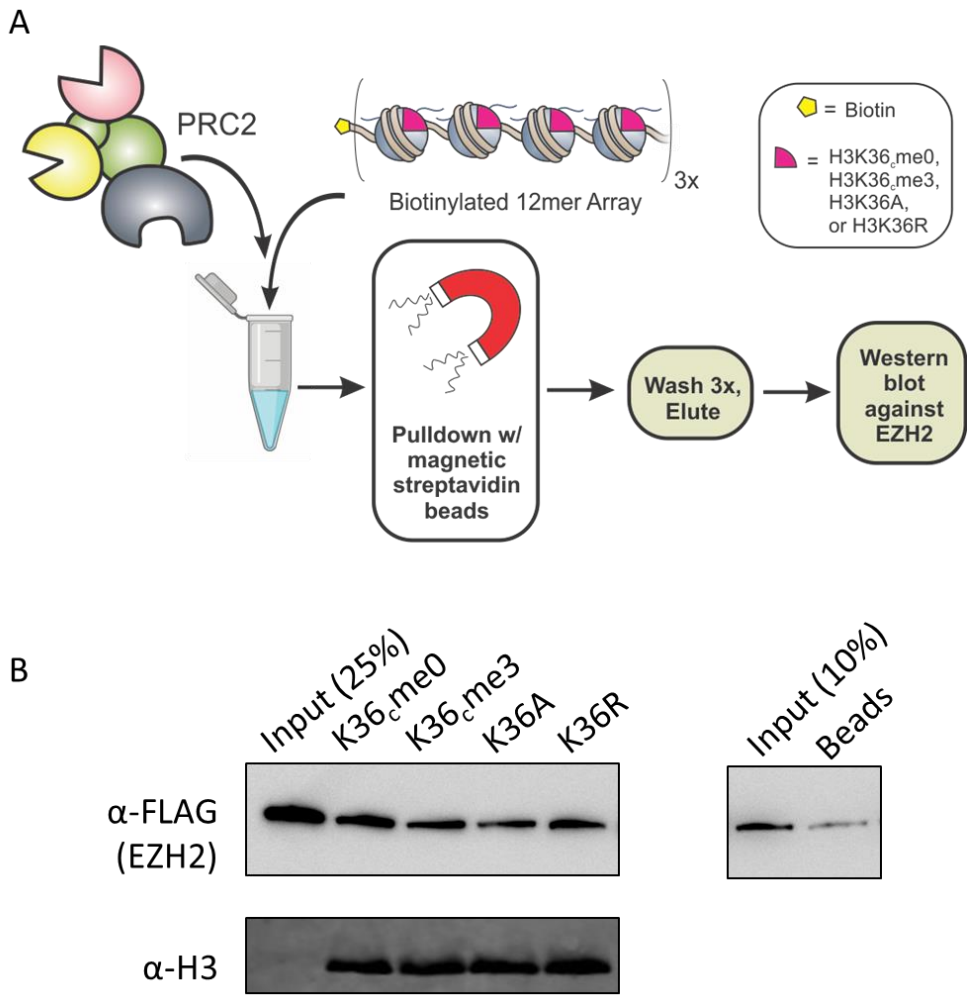

Figure S5: Pulldown experiments examining the effect of the H3K36 side chain on PRC2 binding. A. Schematic depicting experimental workflow. PRC2 complex (containing FLAG-tagged EZH2, EED, RbAp46/48, Suz12, and AEBP2 subunits) is incubated with biotinylated 12mer array substrates with octamers made with various versions of histone H3K36. Approximately 100 nM PRC2 was incubated with ~450 nM 601 sites in a 10 µL reaction containing binding buffer. After binding, pulldown was performed with magnetic streptavidin-coated beads. Samples were washed and eluted, then analyzed by western blot B. Western blots depicting results of pulldown binding experiments with PRC2 and 12mer arrays bearing H3K36cme0, H3K36cme3, H3K36A, or H3K36R octamers using an antibody against FLAG to measure levels of EZH2 pulled down. Input lanes represent PRC2 alone as a control. The lane labeled "Beads" represents a condition in which PRC2 was incubated with magnetic streptavidin-coated beads alone, without arrays, used as a measurement of background binding. H3 was used as a loading control.

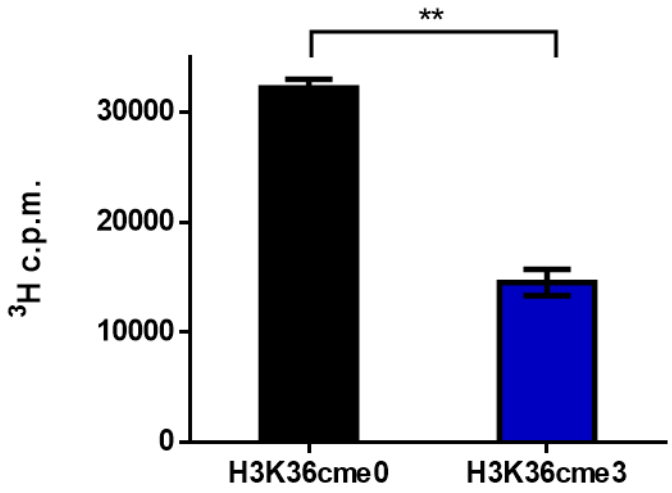

Figure S6. PRC2 (with Jarid2 subunit) HMT activity. PRC2 containing Jarid2 retains sensitivity to H3K36me3. Activity of Jarid2-containing PRC2 complex (25 nM) on 12mer arrays (400 nM 601 sites) modified or unmodified at H3K36. Mean PRC2 activity on H3K36$_c$me3 substrate is 45% of activity measured on unmodified substrate. Error bars represent SEM (n=3). $^{**}P \leq 0.01$.

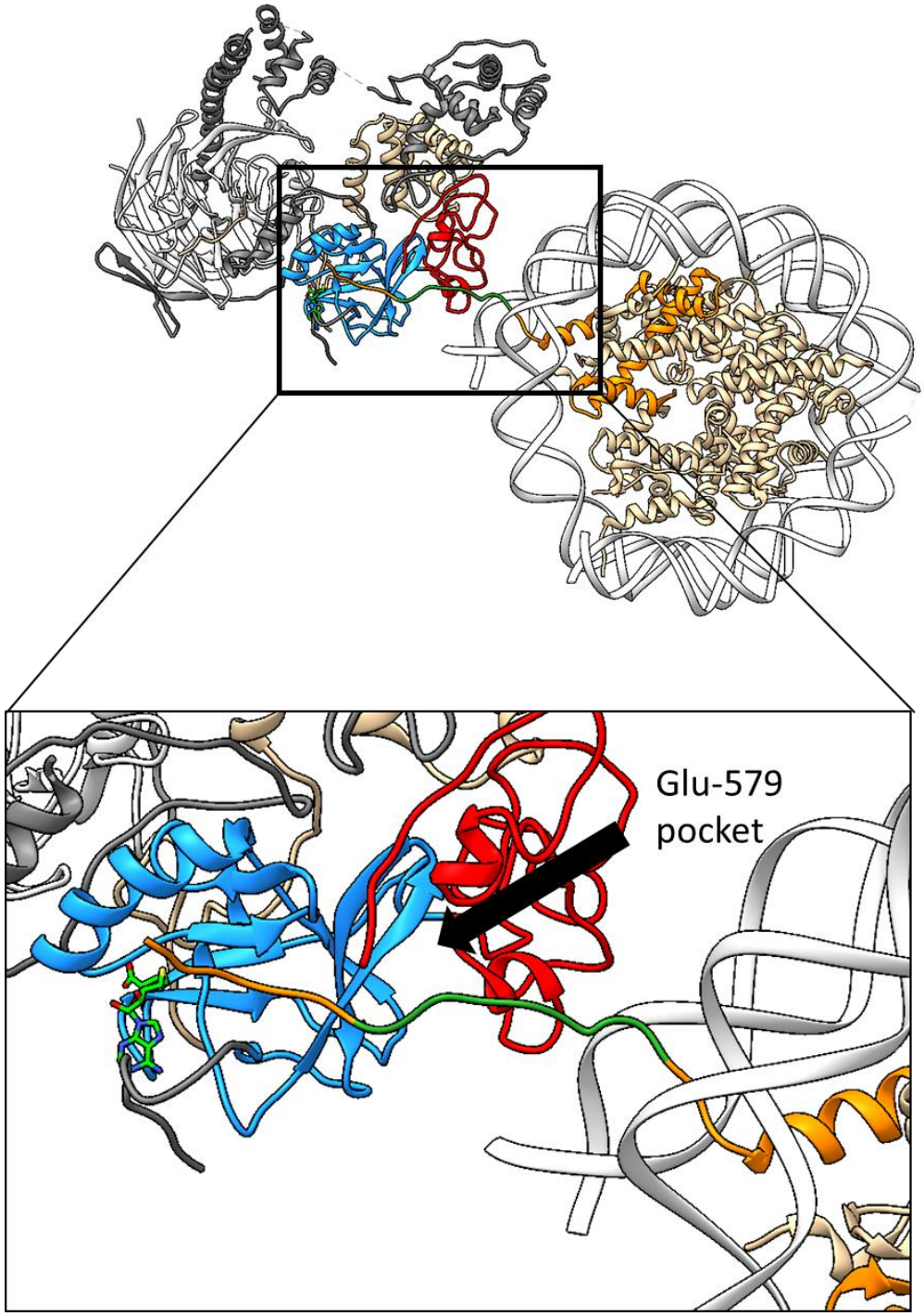

Figure S7. Structural model of EZH2 interaction with the H3 tail on a nucleosome substrate. Figure generated using the supplementary dataset from a recently-published cryo-EM structural model of PRC2 engaged with its substrate (1). The H3 substrate is depicted in orange, with density unavailable between residues 30-38 (modeled in green). The CXC and SET domains of EZH2 are depicted in red and blue, respectively, while the rest of EZH2 is depicted in gray.

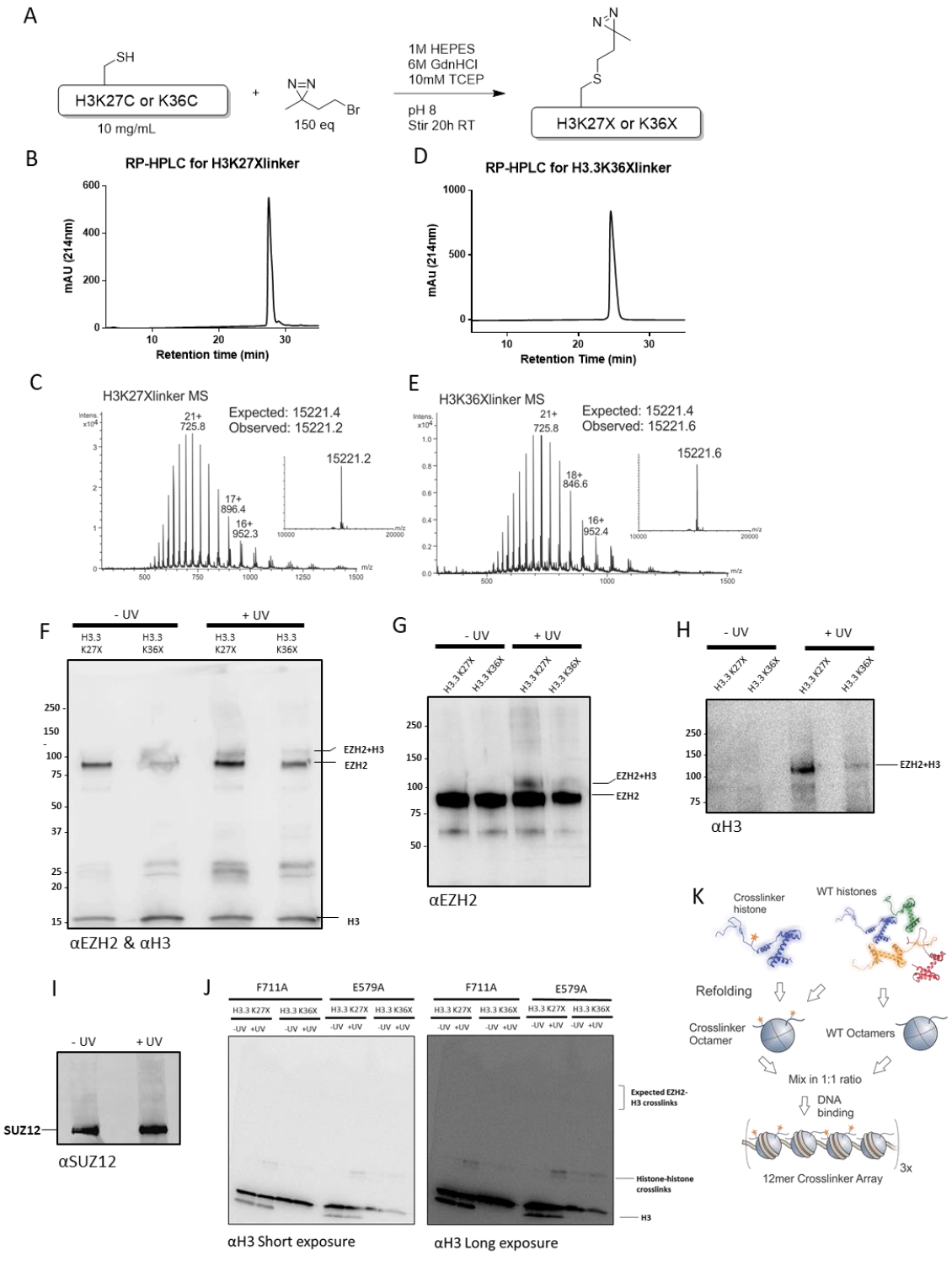

Figure S8. Photocrosslinker-containing nucleosome arrays reveal an interaction between EZH2 and H3K36. **A.** Cysteine alkylation strategy used to generate histones with a diazirine photocrosslinker at position 27 or 36 of H3. **B.** RP-HPLC chromatogram of purified H3 containing a diazirine at position 27. **C.** ESI-MS spectrum of purified H3 containing a diazirine at position 27. **D.** RP-HPLC chromatogram of purified H3 containing

a diazirine at position 36. **E.** ESI-MS spectrum of purified H3 containing a diazirine at position 36. **F.** The crosslinking reaction was analyzed by Western blot using antibodies against both H3 and EZH2, with UV-dependent bands appearing at ~100 kDa. **G.** The crosslinking reaction was analyzed using an antibody against EZH2. **H.** Western blot was performed using an antibody against the C terminus of H3. A band at ~100kDa was observed. **I.** The reaction, employing H3 containing a diazirine at position 36, was also analyzed using an antibody specific for Suz12, but no bands corresponding to a higher molecular weight were observed. **J.** Crosslinking was performed with 12mers containing crosslinkers as above in the presence of PRC2 containing mutations at F711A or E579A in EZH2. Higher-order bands expected to correspond to crosslinked mutant EZH2 were not observed. **K.** Schematic illustrating how the histone with pre-installed photocrosslinker either at the K27 or K36 position is combined with unmodified histones to form crosslinker or WT octamers, which are then combined in equimolar ratios to generate stochastic arrays containing 50% crosslinker octamers. Crosslinker arrays were combined with PRC2 enzyme and irradiated to generate covalent crosslinks. The reaction was separated on a denaturing gel using SDS-PAGE and analyzed by western blot. Note, H3 containing a diazirine at position 27 is known to crosslink to EZH2 (2) and so acts as a positive control.

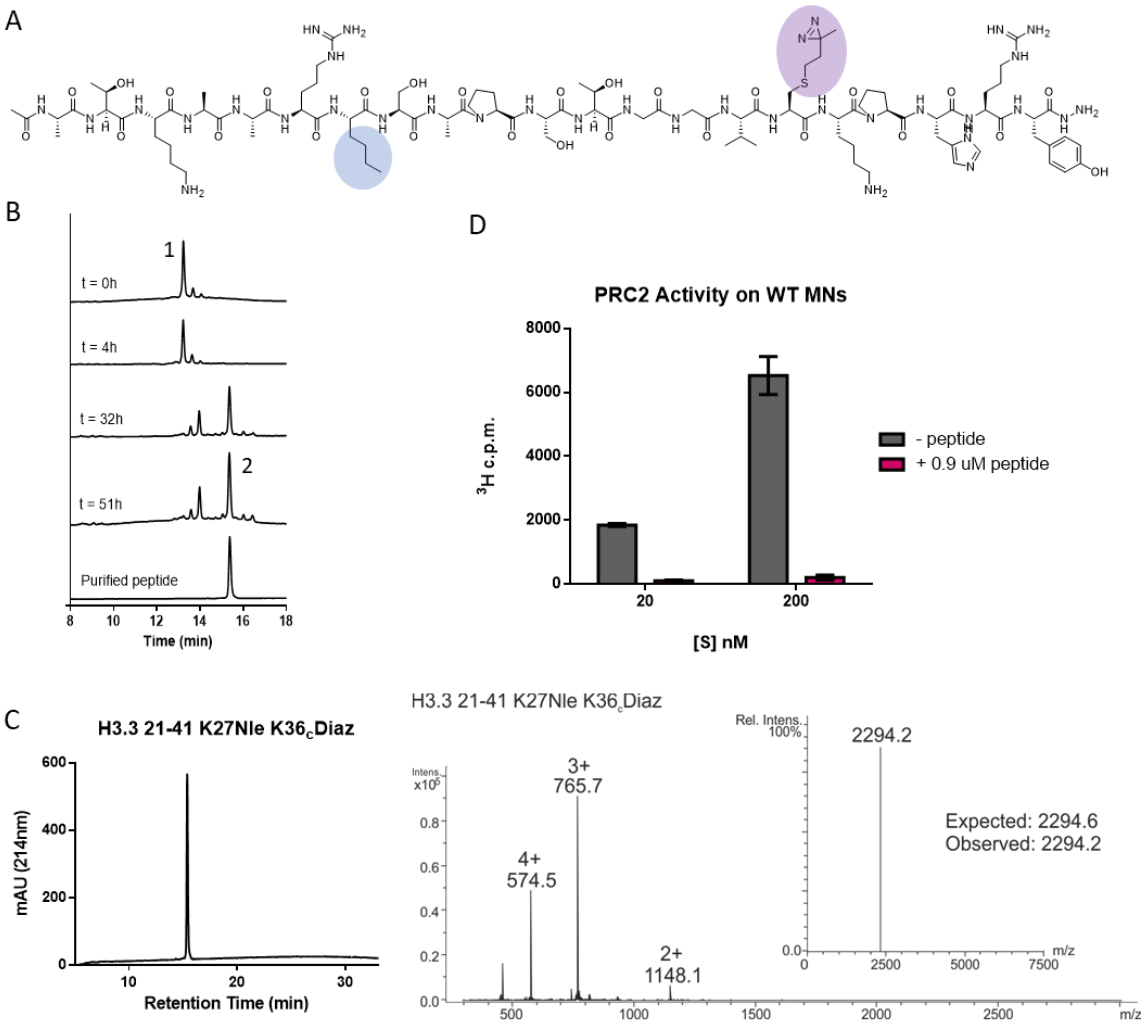

Figure S9. Design and synthesis of H3 photocrosslinker peptide probe. **A.** Chemical structure of photocrosslinker peptide probe employed in photocrosslinking-MS studies. The peptide corresponds to residues 21-41 of the histone H3.3 tail, with a norleucine anchor incorporated at position H3K27 (blue) and a photoactivatable diazirine installed using cysteine alkylation at position H3K36 (purple). **B.** RP-HPLC analysis of reaction progression using a gradient of 40-60%B showing peptide starting material (1) containing H3K27Nle-K36C alkylated with a diazirine-containing small molecule to generate product (2). **C.** Characterization of purified photocrosslinker peptide probe; Left, RP-HPLC using a gradient of 0-73%B and, right, ESI-MS. **D.** PRC2 activity measured on two concentrations of unmodified mononucleosome substrates, without or in the presence of the norleucine-containing photocrosslinker probe inhibitor. Error bars represent SEM (n=3).

Table S1. List of unique crosslinks with score >100 identified between H3 photocrosslinking peptide probe and PRC2 complex using StavroX analysis software. All observed crosslinks occurred to the same tryptic peptide belonging to the EZH2 subunit of PRC2. Calculated M+H+: 1643.891. L* = norleucine, Z = diazirine photocrosslinker

Score	Protein a	Sequence a	Site (a)	Protein b	Sequence b	Site (b)	m/z	z	M+H+
117	EZH2	DPVQK	P631	H3_peptide	L*SAPSTGGVZK	Z36	411.723	+4	1643.871
115	EZH2	DPVQK	K634	H3_peptide	L*SAPSTGGVZK	Z36	548.627	+3	1643.868
108	EZH2	DPVQK	K634	H3_peptide	L*SAPSTGGVZK	Z36	548.627	+3	1643.868
105	EZH2	DPVQK	D630	H3_peptide	L*SAPSTGGVZK	Z36	411.723	+4	1643.870

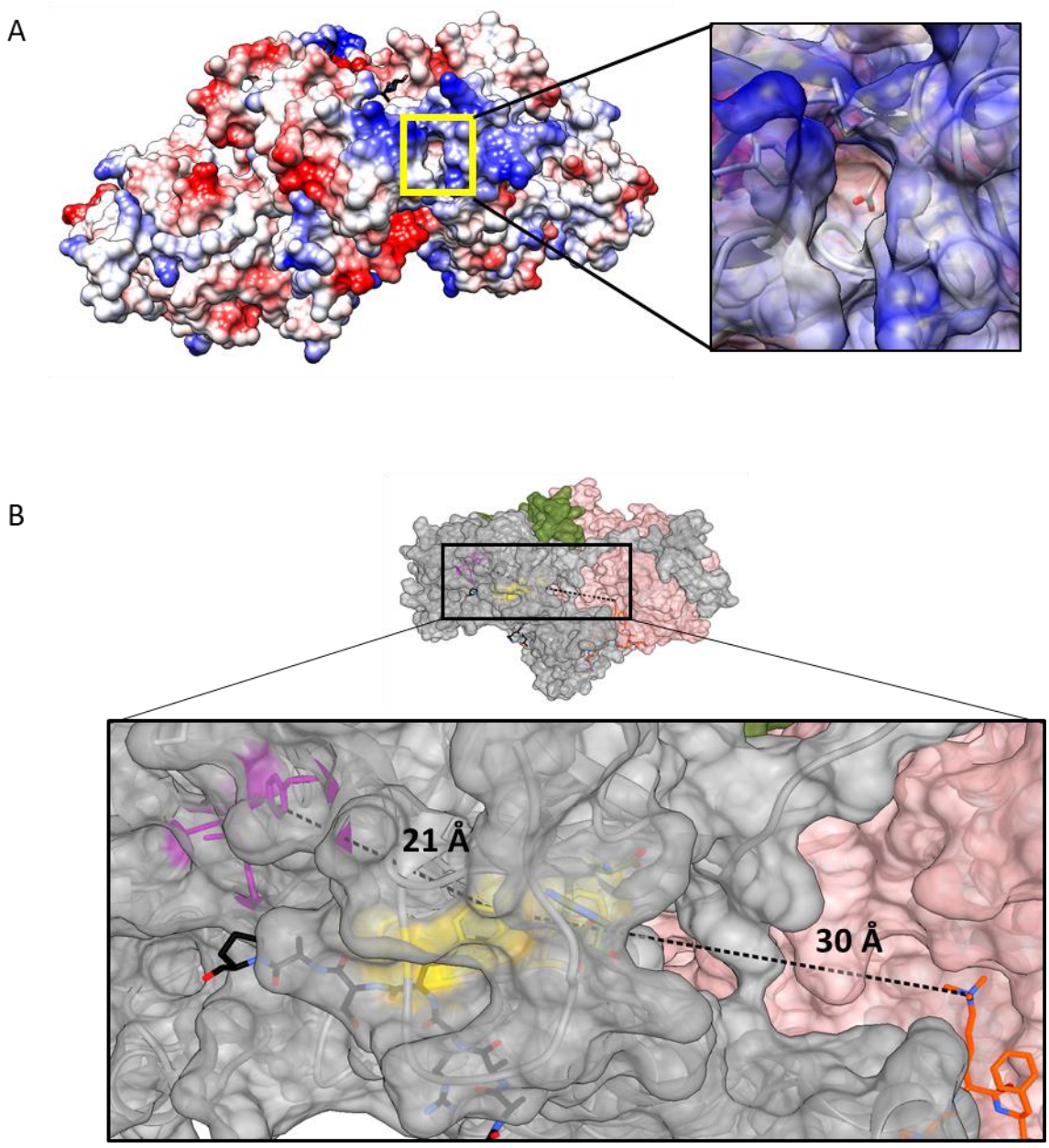

Figure S10. Structural views of PRC2 and the putative H3K36 sensor region. **A.** View of PRC2 structure (PDB ID: 5HYN) rendered with Coulombic surface coloring. The putative H3K36 sensor pocket is boxed in yellow. *Inset:* Zoom in to Glu-579 pocket; residue Glu579 is depicted at the base of pocket in sticks. **B.** Depiction of the same structure indicating the distance separating the putative H3K36 sensor pocket (purple) from the enzyme active site (yellow) compared to the distance between the active site and EED binding site (with trimethylated lysine bound to EED depicted in orange), which mediates a known allosteric activation event.

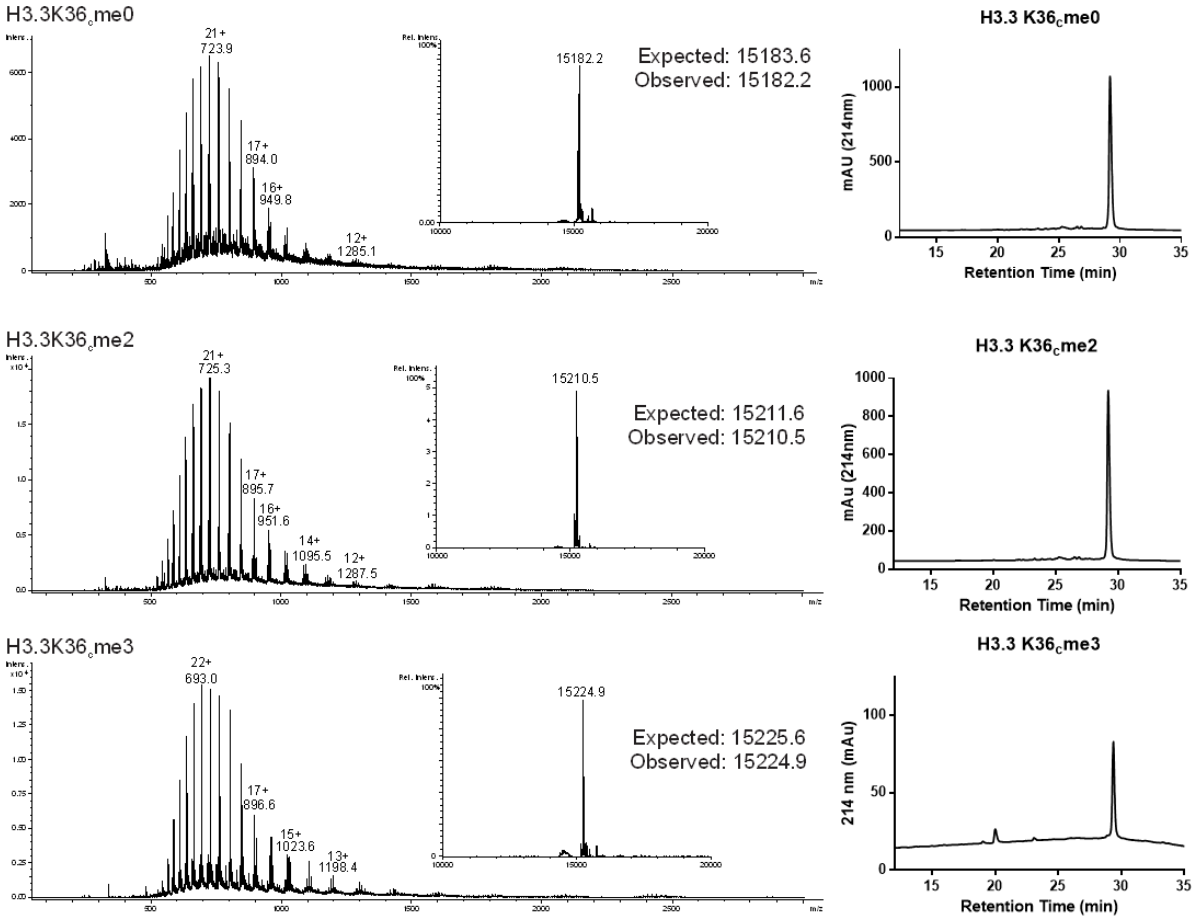

Figure S11. Analytical data for H3.3 histones alkylated with methyl-lysine analogs used in PRC2 HMT activity assays (ESI-MS and RP-HPLC).

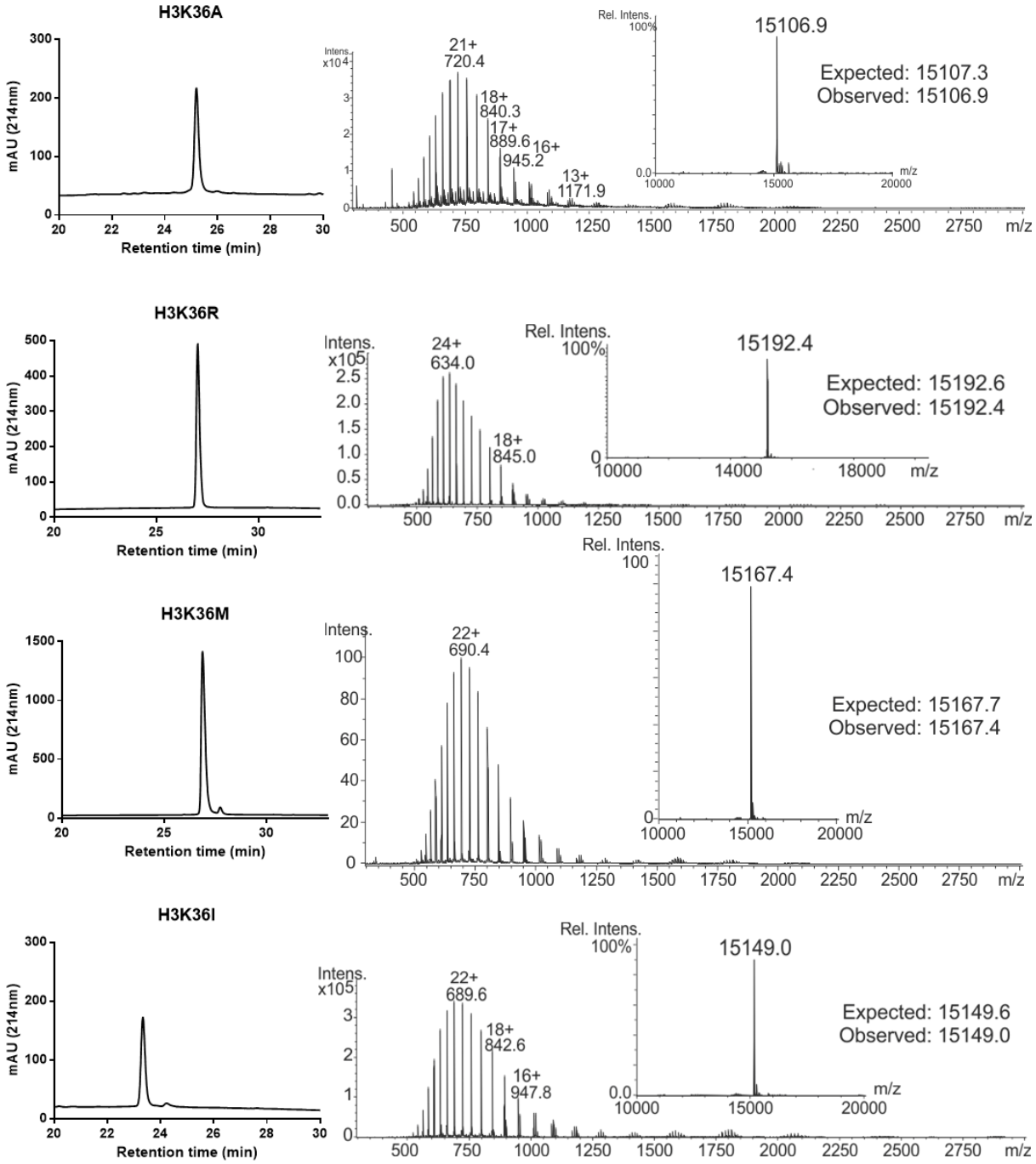

Figure S12. Analytical data for H3.3 mutant histones (H3K36A, H3K36R, H3K36M, H3K36I) used in PRC2 HMT activity assays (ESI-MS and RP-HPLC).

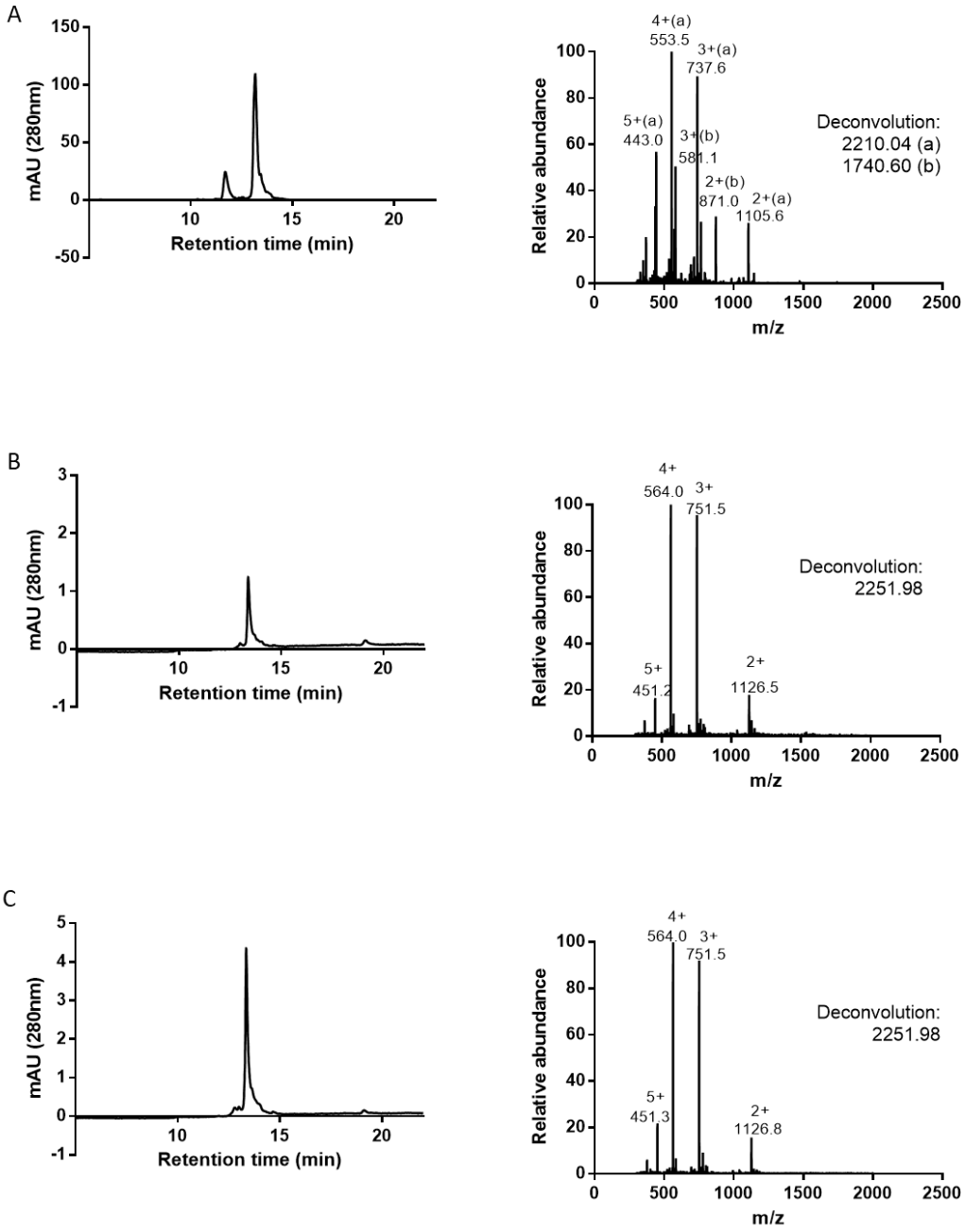

Figure S13. A. RP-HPLC and ESI-MS of peptides incubated with PRC2 under conditions identical to those employed for binding measurements in fluorescence anisotropy assays. Peptides identified by MS include H3 21-41 unmodified substrate peptide (peptide a) and H3K27me3-containing stimulating peptide with residues 23-37 (peptide b). No methylated substrate was detected by MS after incubation of these peptides together with 400 nM PRC2 and 5 μM SAM in binding buffer at 20 degrees C for ten minutes. **B.** RP-HPLC and ESI-MS of H3 21-41 K27me3 at a concentration equal to 1% trimethylated product peptide. **C.** RP-HPLC and ESI-MS of H3 21-41 K27me3 peptide at a concentration equal to 5% trimethylated product peptide.

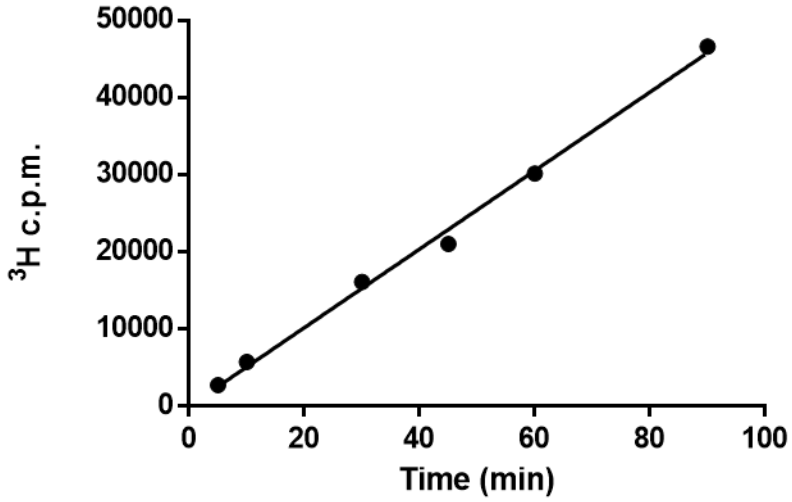

Figure S14. Time course of WT PRC2 activity using approximately 25 nM enzyme complex on 400nM on H3K36$_c$me0 12mer array substrates.

Supporting Materials and Methods

General Laboratory Materials

Amino acids, amino acid derivatives, and coupling reagents were purchased from Novabiochem (Darmstadt, Germany). Dimethylformamide (DMF), dichloromethane (DCM), triisopropylsilane (TIS) were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was purchased from Thermo Scientific (Waltham, MA).

2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) was purchased from Genscript (Piscataway, NJ). Trifluoroacetic acid (TFA) was purchased from Halocarbon (Peachtree Corners, GA). N,N-diisopropylethylamine (DIPEA) was purchased from Acros Organics (Morris Plains, NJ). Chemical reagents and solvents were generally purchased from Sigma-Aldrich or Fisher Scientific (Pittsburgh, PA). Restriction enzymes and T4 DNA ligase were obtained from New England BioLabs (Ipswich, MA). Primers were synthesized by and purchased from Integrated DNA Technologies (Coralville, IA) and gene sequencing was performed by Genewiz (South Plainfield, NJ).

Gene mutagenesis was achieved using the QuickChange II Site-Directed Mutagenesis Kit from Agilent (Santa Clara, CA) and PCR purification was performed using kits from Qiagen (Hilden, Germany). Slide-A-Lyzer dialysis cassettes and mini-dialysis units were purchased from Thermo Fisher Scientific. Size-exclusion chromatography was performed on an AKTA FPLC system from GE Healthcare (Chicago, IL) equipped with a P-920 pump and an UPC-900 monitor.

Analytical reversed-phase HPLC (RP-HPLC) was performed on an Agilent 1200 series instrument with a Vydac C18 column (5 micron, 4 × 150 mm), using 0.1% trifluoroacetic acid (TFA) in water (HPLC solvent A), and 90% acetonitrile, 0.1% TFA in water (HPLC solvent B), as the mobile phases. Analytical gradients were 0–73% HPLC buffer B over 30 minutes at a flow rate of 1 mL/min unless stated otherwise. Preparative scale purifications were conducted on a Waters prep LC system comprised of a Waters 2545 Binary Gradient Module and a Waters 2489 UV detector. A Vydac C18 preparative column (15–20 micron, 20 × 250 mm) or a semi-preparative column (12 micron, 10 mm × 250 mm) was employed at a flow rate of 18 ml/min or 4 ml/min, respectively.

ESI–MS analysis was conducted on a MicrOTOF-Q II ESI-Qq-TOF mass spectrometer (Bruker Daltonics). UV spectrometry was performed on an Agilent 8453 UV-Visible spectrophotometer. Anti-mouse and anti-rabbit HRP secondary antibodies were purchased from Bio-Rad (Hercules, CA). The rabbit α-H3K27me1 primary antibody and mouse α-H4 antibody were purchased from Millipore (Burlington, MA). Rabbit α-H3 (C-terminus) primary antibody was purchased from Abcam (Cambridge, UK). Rabbit α-Suz12 and α-EZH2 primary antibodies were purchased from Active Motif (Carlsbad, CA). All gel and western blot images were acquired with an ImageQuant LAS 4000 instrument (GE Healthcare).

Molecular graphics and analyses were performed with the UCSF Chimera package (Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco).

Statistical Methods

Error bars depicted represent the standard error of the mean. Where applicable, normalized values for independent replicates were used. T-tests were performed to determine P values where applicable. In the case of calculating errors associated with the ratio of k_{cat}/K_m, standard propagation of error was used.

Peptide Synthesis

Peptides were synthesized on Rink Amide-ChemMatrix resin (PCAS Biomatrix) using manual addition of reagents and a stream of dry nitrogen gas to agitate the reaction mixture. Typical cycles were: (i) FMOC deprotection with 2mL of 20% piperidine in DMF (1 x 1 min; 1 x 8 min) and (ii) coupling of 5 eq. amino acid to the peptide chain with 4.9 eq HATU and 10 eq DIPEA (2 x 20 min). The resin was washed thoroughly with DMF between each step. Once synthesis was complete, peptides were deprotected and cleaved from the resin using 95% TFA, 2.5% TIS and 2.5% H2O. Crude peptides were precipitated using diethyl ether, dissolved in water with 0.1% TFA, and analyzed via RP-HPLC. The peptide was subsequently purified by preparative RP-HPLC, lyophilized, and stored at -20 °C.

Synthesis of photocrosslinker peptide

A histone peptide corresponding to residues 21-31 of the H3.3 tail was synthesized using Fmoc-SPPS as described above containing norleucine at the position corresponding to H3K27 and cysteine at the position corresponding to H3K36. Cysteine alkylation was used to generate the diazirine photocrosslinker peptide. The crude cysteine-containing peptide was dissolved in ~3.3mL of alkylation buffer (1 M HEPES, 6 M guanidinium chloride, 20 mM TCEP, pH 7.8) to a final concentration of ~7 mM and transferred to a round-bottom flask. 150 equivalents of 3-(2-bromoethyl)-3-methyl-3H-diazirine (Enamine Chemicals;

Monmouth Jct, NJ) were added to the peptide solution. The alkylation reaction was protected from light in a dark hood. The reaction was performed under nitrogen and was monitored for ~50 hours until it went to completion. The product was purified by preparative RP-HPLC and the identity of the product was verified by ESI-MS.

Expression of recombinant histones

Unmodified recombinant human histones H2A, H2B, H3.3 (C110A) and H4 were expressed in *E. coli* and purified from inclusion bodies by flash chromatography using Biotage Isolera (Uppsala, Sweden). The purified histones were analyzed by RP-HPLC and ESI-MS. The following mutant histones were generated by Quik Change Site-Directed Mutagenesis (Agilent) using an *E. coli* expression system and pET30a vector containing Kanamycin resistance: H3.3K36A, H3.3K36R, H3.3K36M, H3.3K36I, H3.3K27C, H3.3K36C. The proteins were produced analogously to the wild type. Briefly, expression plasmids were transformed into Rosetta(DE3) cells (Millipore; Burlington, MA) and 1L of culture per histone was grown at 37 °C to OD_{600} = 0.6. Expression was induced by addition of 0.5mM IPTG (Gold Biotechnology; St. Louis, MO) for 2-3 hours. Cells were lysed by sonication in lysis buffer (50 mM Tris pH 7.6, 100 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol) for 5 minutes in cycles of 30s ON, 30s OFF at 40% amplitude at 4°C. The lysate was then spun at 4°C for 30 minutes at 15,000 RPM. The supernatant was decanted and the pellet was washed in 10 mL lysis buffer + 1% v/v Triton-X, then centrifuged at 4°C for 15 minutes at 15,000 RPM. The supernatant was again decanted and another Triton wash was performed in 10mL lysis buffer + 1% v/v Triton-X. The sample was centrifuged at 4°C for 15 minutes at 15,000 RPM. The supernatant was decanted and a final wash was performed without detergent in 10mL of lysis buffer. The sample was

spun at 4°C for 15 minutes at 15,000 RPM. The supernatant was decanted and the resulting pellet containing histone protein in inclusion bodies was resuspended in buffer containing 6M guanidine, 20mM Tris, 1mM EDTA, 100mM NaCl, 1mM DTT. The resuspension buffer was added to the pellet and nutated in the cold room at 4°C for 2 hours, then centrifuged for 30 min at 30,000g. The supernatant was removed, acidified with TFA, filtered, and injected onto a flash chromatography system (Biotage Isolera) equipped with a 10g Biotage SNAP Ultra flash chromatography cartridge and purified using a gradient of 0-73%B (solvents as described above for preparative HPLC, p. S19). Fractions were analyzed by LC-MS. Pure fractions were pooled and the pooled protein was analyzed then lyophilized and stored at -20 °C.

Histone cysteine alkylation with methyl-lysine analogs

In order to mimic lysine side chains with different methylation states, H3.3 containing C110A and K36C mutations was alkylated with methyl-lysine analogs as previously described (3, 4) to generate analog substrates with 0, 2, or 3 methyl groups at those positions. Analogs used were (2-bromoethyl)-trimethylammonium bromide (Sigma Aldrich), (2-chloroethyl)-dimethylammonium chloride (Sigma Aldrich), and (2-bromoethyl)-ammonium bromide (Fischer Scientific). Histone cysteine alkylation reactions were typically performed at a 0.66 μmol scale.

Synthesis of photocrosslinker histone

Cysteine mutations were introduced into histone H3.3 using site-directed mutagenesis as described above, either at position H3K27 or H3K36. Cysteine alkylation was used to generate the diazirine photocrosslinker histone protein, typically on a 2-5 μmol scale. The crude cysteine-containing protein was dissolved in alkylation buffer (1M HEPES, 6M guanidinium chloride, 20mM TCEP, pH 7.8) to a final concentration of 10 mg/mL and transferred to a round-bottom flask. 150 equivalents of 3-(2-bromoethyl)-3-methyl-3H-diazirine (Enamine Chemicals) were added to the protein solution. The alkylation reaction was performed under nitrogen using a balloon and was constantly agitated using a stir bar. The reaction was monitored for ~24 hours until it went to completion. The product was purified by preparative RP-HPLC and the identity of the product was verified by ESI-MS.

DNA Preparation

Dodecameric or monomeric repeats of the 601 sequence (5) separated by 30-bp linkers were produced from pWM530 by EcoRV digestion and PEG-6000 precipitation as previously described (6). The purified DNA was dissolved in TE buffer and stored at −20 °C in aliquots.

Octamer Assembly

Octamers containing the desired unmodified or alkylated histone variants were prepared as previously described (7). Briefly, histones were dissolved in histone unfolding buffer (20 mM Tris-HCl, 6 M GdnCl, 0.5 mM DTT, pH 7.5), combined (1.05 eq. H2A, 1.05 eq. H2B, 1.0 eq. H3, 1 eq. H4), and the total histone concentration was adjusted to 1 mg/mL. Octamers were assembled by dialysis at 4 °C against 3 × 1 L of octamer refolding buffer (10 mM Tris-HCl, 2 M NaCl, 0.5 mM EDTA, 1 mM DTT, pH 7.5) and subsequently

purified by size exclusion chromatography on a Superdex S200 10/300 column. Fractions containing octamers were combined, concentrated, diluted with glycerol to a final concentration of 50% (v/v) and stored at −20 °C.

Nucleosome array reconstitution

Dodecameric arrays or mononucleosomes were typically assembled on a 50-200 pmol scale from purified octamers and recombinant DNA in the presence of buffer DNA by salt gradient dialysis as previously described (8).

Cloning, expression, and purification of PRC2

Recombinant 5-component PRC2 was generated by expressing EZH2 (mutant or WT) containing an N-terminal FLAG tag with N-terminally His-tagged wild-type AEBP2, EED, SUZ12, and RbAp48 in Spodoptera frugiperda (Sf9) cells using a MultiBac baculovirus expression system (9). The N-terminal FLAG tag on EZH2 was used to purify active PRC2 complex from cell lysates, then further purified using size exclusion chromatography (Superose 6; GE Healthcare Lifesciences) or ion exchange (monoQ; GE Healthcare Lifesciences). The purity of the final PRC2 preparations was assessed by SDS-PAGE with Ruby staining. Pelleted cell lysates were resuspended in 40 µL of HEGN600 buffer (25 mM HEPES pH 7.0, 600 mM NaCl, 1mM EDTA, 10% glycerol, 0.02% NP-40) per mL of cells. The 1L pellet was suspended in a total volume of 40 mL using HEGN600. The cell suspension was lysed by dounce homogenization followed by sonication: 5 rounds of 5 sec on/off for 30 seconds at 35% power. Lysate was transferred to 40mL centrifuge tubes, and centrifuged at 17,000xg for 30 minutes at 4 °C. The lysate was diluted with HEGN0 (25 mM HEPES pH 7.0, 0 mM NaCl, 1 mM EDTA, 10% glycerol, 0.02% NP-40) to a final concentration of 350 mM NaCl. The lysate was then spun down again at 17,000xg for 30

minutes at 4 °C. 1 mL of 50% FLAG bead slurry (Genscript G1 a-DYKDDDDK) was resuspended for 500 µL of beads (0.5 µL beads/mL culture) and washed 2x with 10 bead volumes (5mL) HEGN200 buffer (25 mM HEPES pH 7.0, 200 mM NaCl, 1 mM EDTA, 10% glycerol, 0.02% NP-40) by resuspending and then spinning at 1,000xg for 3 minutes. The supernatant and FLAG resin were nutated at 4 °C together for 3 hours in two 50 mL Falcon tubes. After 3 hours, the beads were pelleted by spinning at 1,000xg for 3 minutes. The beads were washed 3 x 10 minutes by nutating at 4 °C with 10-15 mL of HEGN350 buffer (25 mM HEPES pH 7.0, 350 mM NaCl, 1 mM EDTA, 10% glycerol, 0.02% NP-40). Finally, they were washed with 15 mL of HEGN350 buffer + 3 mM $MgCl_2$ + 0.2 mM PMSF. Beads were pelleted and resuspended in a minimal amount of HEGN350 buffer + 3 mM $MgCl_2$ + 0.2 mM PMSF buffer and transferred to a 1.5 mL Eppendorf tube. 5 mg/mL of FLAG peptide stock was diluted to 0.25 mg/mL using HEGN350 buffer + 3 mM $MgCl_2$ + 0.2 mM PMSF. Beads were resuspended in 1 bead volume (500 µL) of diluted FLAG peptide and incubated with end-over-end rotation for 10 minutes at 4 °C. The sample was centrifuged and supernatant was removed for the first elution (E1) sample. This elution process was repeated two more times to obtain E2 and E3 samples.

For further purification by size exclusion chromatography, a Superdex200 10/300 column (GE Healthcare Lifesciences) was equilibrated in HEGN350 buffer by running in buffer A (HEGN350) at 0.4 mL/min for two column volumes (~50 mL). The protein sample was concentrated to 1 mL and injected on a 1 mL loop. SEC was run with the following parameters: 0.4 mL/min, 1.5 column volumes, collecting 0.5 mL fractions. Fractions were analyzed by SDS-PAGE, loading 15 µL of each fraction with UV-Vis absorbance on a 7% Tris-acetate gel. The gel was visualized with Coomassie stain. Selected fractions were

pooled and concentrated to 1 mL, and a second round of SEC was run. Fractions were analyzed by SDS-PAGE again as described above. The gel was visualized with Coomassie stain. Selected fractions were pooled and concentrated to ~600 µL. The final sample was then aliquoted, flash frozen, and stored at -80 °C.

PRC2 subunit sequences

EZH2, Uniprot Q15910, 85.36 kDa

FLAG-EZH2

DYKDDDDKMGQTGKKSEKGPVCWRKRVKSEYMRLRQLKRFRRADEVKSMFSS
NRQKILERTEILNQEWKQRRIQPVHILTSVSSLRGTRECSVTSDLDFPTQVIPLKTL
NAVASVPIMYSWSPLQQNFMVEDETVLHNIPYMGDEVLDQDGTFIEELIKNYDG
KVHGDRECGFINDEIFVELVNALGQYNDDDDDDDGDDPEEREEKQKDLEDHRD
DKESRPPRKFPSDKIFEAISSMFPDKGTAEELKEKYKELTEQQLPGALPPECTPNID
GPNAKSVQREQSLHSFHTLFCRRCFKYDCFLHPFHATPNTYKRKNTETALDNKP
CGPQCYQHLEGAKEFAAALTAERIKTPPKRPGGRRRGRLPNNSSRPSTPTINVLES
KDTDSDREAGTETGGENNDKEEEEKKDETSSSSEANSRCQTPIKMKPNIEPPENV
EWSGAEASMFRVLIGTYYDNFCAIARLIGTKTCRQVYEFRVKESSIIAPAPAEDVD
TPPRKKKRKHRLWAAHCRKIQLKKDGSSNHVYNYQPCDHPRQPCDSSCPCVIAQ
NFCEKFCQCSSECQNRFPGCRCKAQCNTKQCPCYLAVRECDPDLCLTCGAADH
WDSKNVSCKNCSIQRGSKKHLLLAPSDVAGWGIFIKDPVQKNEFISEYCGEIISQD
EADRRGKVYDKYMCSFLFNLNNDFVVDATRKGNKIRFANHSVNPNCYAKVMM
VNGDHRIGIFAKRAIQTGE ELFFDYRYSQADALKYVGIEREMEIP

EED, Uniprot O75530, 50.2 kDa

MSEREVSTAPAGTDMPAAKKQKLSSDENSNPDLSGDENDDAVSIESGTNTERPD
TPTNTPNAPGRKSWGKGKWKSKKCKYSFKCVNSLKEDHNQPLFGVQFNWHSK
EGDPLVFATVGSNRVTLYECHSQGEIRLLQSYVDADADENFYTCAWTYDSNTSH
PLLAVAGSRGIIRIINPITMQCIKHYVGHGNAINELKFHPRDPNLLLSVSKDHALRL
WNIQTDTLVAIFGGVEGHRDEVLSADYDLLGEKIMSCGMDHSLKLWRINSKRM
MNAIKESYDYNPNKTNRPFISQKIHFPDFSTRDIHRNYVDCVRWLGDLILSKSCEN
AIVCWKPGKMEDDIDKIKPSESNVTILGRFDYSQCDIWYMRFSMDFWQKMLAL
GNQVGKLYVWDLEVEDPHKAKCTTLTHHKCGAAIRQTSFSRDSSILIAVCDDASI
WRWDRLR

SUZ12, Uniprot Q15022, 83.06 kDa

MAPQKHGGGGGGGSGPSAGSGGGGFGGSAAVAAATASGGKSGGGSCGGGGSY
SASSSSSAAAAAGAAVLPVKKPKMEHVQADHELFLQAFEKPTQIYRFLRTRNLIA
PIFLHRTLTYMSHRNSRTNIKRKTFKVDDMLSKVEKMKGEQESHSLSAHLQLTFT
GFFHKNDKPSPNSENEQNSVTLEVLLVKVCHKKRKDVSCPIRQVPTGKKQVPLN
PDLNQTKPGNFPSLAVSSNEFEPSNSHMVKSYSLLFRVTRPGRREFNGMINGETN
ENIDVNEELPARRKRNREDGEKTFVAQMTVFDKNRRLQLLDGEYEVAMQEMEE
CPISKKRATWETILDGKRLPPFETFSQGPTLQFTLRWTGETNDKSTAPIAKPLATR
NSESLHQENKPGSVKPTQTIAVKESLTTDLQTRKEKDTPNENRQKLRIFYQFLYN
NNTRQQTEARDDLHCPWCTLNCRKLYSLLKHLKLCHSRFIFNYVYHPKGARIDV
SINECYDGSYAGNPQDIHRQPGFAFSRNGPVKRTPITHILVCRPKRTKASMSEFLE
SEDGEVEQQRTYSSGHNRLYFHSDTCLPLRPQEMEVDSEDEKDPEWLREKTITQI

EEFSDVNEGEKEVMKLWNLHVMKHGFIADNQMNHACMLFVENYGQKIIKKNL
CRNFMLHLVSMHDFNLISIMSIDKAVTKLREMQQKLEKGESASPANEEITEEQNG
TANGFSEINSKEKALETDSVSGVSKQSKKQKL

RBBP4, Uniprot Q09028, 47.66 kDa

MADKEAAFDDAVEERVINEEYKIWKKNTPFLYDLVMTHALEWPSLTAQWLPDV
TRPEGKDFSIHRLVLGTHTSDEQNHLVIASVQLPNDDAQFDASHYDSEKGEFGGF
GSVSGKIEIEIKINHEGEVNRARYMPQNPCIIATKTPSSDVLVFDYTKHPSKPDPSG
ECNPDLRLRGHQKEGYGLSWNPNLSGHLLSASDDHTICLWDISAVPKEGKVVDA
KTIFTGHTAVVEDVSWHLLHESLFGSVADDQKLMIWDTRSNNTSKPSHSVDAHT
AEVNCLSFNPYSEFILATGSADKTVALWDLRNLKLKLHSFESHKDEIFQVQWSPH
NETILASSGTDRRLNVWDLSKIGEEQSPEDAEDGPPELLFIHGGHTAKISDFSWNP
NEPWVICSVSEDNIMQVWQMAENIYNDEDPEGSVDPEGQGS

AEBP2, Uniprot Q6ZN18, 54.47 kDa

MAAAITDMADLEELSRLSPLPPGSPGSAARGRAEPPEEEEEEEEEEEEAEAEAVA
ALLLNGGSGGGGGGGGGGVGGGEAETMSEPSPESASQAGEDEDEEEDDEEEEDE
SSSSGGGEEESSAESLVGSSGGSSSDETRSLSPGAASSSSGDGDGKEGLEEPKGPR
GSQGGGGGGSSSSSVVSSGGDEGYGTGGGGSSATSGGRRGSLEMSSDGEPLSRM
DSEDSISSTIMDVDSTISSGRSTPAMMNGQGSTTSSSKNIAYNCCWDQCQACFNS
SPDLADHIRSIHVDGQRGGVFVCLWKGCKVYNTPSTSQSWLQRHMLTHSGDKPF
KCVVGGCNASFASQGGLARHVPTHFSQQNSSKVSSQPKAKEESPSKAGMNKRR
KLKNKRRRSLPRPHDFFDAQTLDAIRHRAICFNLSAHIESLGKGHSVVFHSTVIAK

RKEDSGKIKLLLHWMPEDILPDVWVNESERHQLKTKVVHLSKLPKDTALLLDPN
IYRTMPQKRLKRTLIRKVFNLYLSKQ

JARID2, Uniprot Q92833, 138.73 kDa

MSKERPKRNIIQKKYDDSDGIPWSEERVVRKVLYLSLKEFKNSQKRQHAEGIAGS
LKTVNGLLGNDQSKGLGPASEQSENEKDDASQVSSTSNDVSSSDFEEGPSRKRPR
LQAQRKFAQSQPNSPSTTPVKIVEPLLPPPATQISDLSKRKPKTEDFLTFLCLRGSP
ALPNSMVYFGSSQDEEEVEEEDDETEDVKTATNNASSSCQSTPRKGKTHKHVHN
GHVFNGSSRSTREKEPVQKHKSKEATPAKEKHSDHRADSRREQASANHPAAAPS
TGSSAKGLAATHHHPPLHRSAQDLRKQVSKVNGVTRMSSLGAGVTSAKKMREV
RPSPSKTVKYTATVTKGAVTYTKAKRELVKDTKPNHHKPSSAVNHTISGKTESS
NAKTRKQVLSLGGASKSTGPAVNGLKVSGRLNPKSCTKEVGGRQLREGLQLRE
GLRNSKRRLEEAHQAEKPQSPPKKMKGAAGPAEGPGKKAPAERGLLNGHVKKE
VPERSLERNRPKRATAGKSTPGRQAHGKADSASCENRSTSQPESVHKPQDSGKA
EKGGGKAGWAAMDEIPVLRPSAKEFHDPLIYIESVRAQVEKFGMCRVIPPPDWR
PECKLNDEMRFVTQIQHIHKLGRRWGPNVQRLACIKKHLKSQGITMDELPLIGGC
ELDLACFFRLINEMGGMQQVTDLKKWNKLADMLRIPRTAQDRLAKLQEAYCQY
LLSYDSLSPEEHRRLEKEVLMEKEILEKRKGPLEGHTENDHHKFHPLPRFEPKNG
LIHGVAPRNGFRSKLKEVGQAQLKTGRRRLFAQEKEVVKEEEEDKGVLNDFHKC
IYKGRSVSLTTFYRTARNIMSMCFSKEPAPAEIEQEYWRLVEEKDCHVAVHCGK
VDTNTHGSGFPVGKSEPFSRHGWNLTVLPNNTGSILRHLGAVPGVTIPWLNIGM
VFSTSCWSRDQNHLPYIDYLHTGADCIWYCIPAEEENKLEDVVHTLLQANGTPG
LQMLESNVMISPEVLCKEGIKVHRTVQQSGQFVVCFPGSFVSKVC

CGYSVSETVHFATTQWTSMGFETAKEMKRRHIAKPFSMEKLLYQIAQAEAKKE
NGPTLSTISALLDELRDTELRQRRQLFEAGLHSSARYGSHDGSSTVADGKKKPRK
WLQLETSERRCQICQHLCYLSMVVQENENVVFCLECALRHVEKQKSCRGLKLM
YRYDEEQIISLVNQICGKVSGKNGSIENCLSKPTPKRGPRKRATVDVPPSRLSASS
SSKSASSSS

EZH2 sequence highlighting residues mutated in this study

MGQTGKKSEKGPVCWRKRVKSEYMRLRQLKRFRRADEVKSMFSSNRQKILERT
EILNQEWKQRRIQPVHILTSVSSLRGTRECSVTSDLDFPTQVIPLKTLNAVASVPIM
YSWSPLQQNFMVEDETVLHNIPYMGDEVLDQDGTFIEELIKNYDGKVHGDRECG
FINDEIFVELVNALGQYNDDDDDDDGDDPEEREEKQKDLEDHRDDKESRPPRKF
PSDKIFEAISSMFPDKGTAEELKEKYKELTEQQLPGALPPECTPNIDGPNAKSVQR
EQSLHSFHTLFCRRCFKYDCFLHPFHATPNTYKRKNTETALDNKPCGPQCYQHL
EGAKEFAAALTAERIKTPPKRPGGRRRGRLPNNSSRPSTPTINVLESKDTDSDREA
GTETGGENNDKEEEEKKDETSSSSEANSRCQTPIKMKPNIEPPENVEWSGAEASM
FRVLIGTYYDNFCAIARLIGTKTCRQVYEFRVKESSIIAPAPAEDVDTPPRKKKRK
HRLWAAHCRKIQLKKDGSSNHVYNYQPCDHPRQPCDSSCPCVIAQNFCEKFCQC
SSECQNRFPGCRCKAQCNTKQCPCYLAVR**E**CDPDLCLTCGAADHWDSKNVSCK
NCSIQRGSKKHLLLAPSDVAGWGIFIKDPVQ**K**NEFISE**Y**CGEIISQDEADRRGKVY
DKYMCSFLFNLNNDFVVDATRKGNKIRFANHSVNPNCYAKV**M**MVNGDHRIGI**F**
AKRAIQTGEELFFDYRYSQADALKYVGIEREMEIP

Western Blot protocol

SDS-PAGE gels (4-20% Tris-glycine; Biorad) were transferred to PVDF membranes and blocked with TBST (50 mM Tris pH 7.5, 150 nM NaCl, 0.1% Tween-20) containing 3% milk for 1 hour. Membranes were then incubated in TBST with 3% milk containing 1:1,000 dilution of primary antibody for 1 hour, washed 3 x 5 minutes with TBST, and incubated in TBST containing 3% milk and 1:15,000 dilution of secondary HRP-conjugated antibody for 1 hour. Membranes were again washed 3 x 5 minutes with TBST and imaged.

HMT Assays

The methyltransferase activity of PRC2 and PRC2 mutants was measured using a scintillation-based assay. Typical assay conditions are as follows: mononucleosomes or 12mer arrays (up to 400 nM 601 sites, each containing 2 copies of histone H3) were incubated with approximately 25 nM PRC2 in 10 μL HMT buffer (50 mM HEPES pH 7.5, containing 0.5 mM $MgCl_2$, 1 mM DTT) in the presence of 1 μM [^{3}H]-SAM (Perkin Elmer) in a 10 μL reaction volume for 45 minutes, a time point determined to be within the linear range for measurement of initial velocity (see Fig. S13 for time course data and determination of linear range) at 30 °C. Reactions were quenched by spotting on P81 phosphocellulose filter paper (Reaction Biology). The filters were dried for 1 hr at RT, washed three times with 0.2 M $NaHCO_3$ pH 9, and dried on a gel dryer for 45 min at 40 °C. Scintillation counting was performed with 1 mL Ultima Gold scintillation cocktail on a MicroBeta2 scintillation counter (Perkin Elmer). Michaelis-Menten kinetic values were

obtained by plotting the data in GraphPad Prism 6.01 and performing nonlinear regression analysis.

For Western blot-based HMT assays, 600 nM of 601 sites of 12mer array substrates (containing H3K36$_c$me0/2/3 octamers) were incubated with 1 mM SAM and 120 nM PRC2 in a 30 µL reaction with the HMT buffer described above. The reaction was incubated at 30 °C for 1 hour and quenched with the addition of SDS-containing loading buffer and boiling at 95 °C for five minutes. 10 µL of each reaction were loaded onto an SDS-PAGE gel and analyzed by Western blot as described above.

Photocrosslinking-Mass spectrometry

1.2 nmoles of recombinant PRC2 was incubated with 47 nmoles of photocrosslinker peptide in a buffer containing 50 mM HEPES pH 7.5, 2 mM $MgCl_2$, 1 mM SAM and 0.002% Tween. The peptide and enzyme were allowed to bind for 20 minutes on ice before UV irradiation (λ = 365 nm) for 20 minutes in an open Eppendorf tube. The lamp was placed ~5cm from the tube. After UV irradiation, a FLAG pulldown was performed for the FLAG-tagged EZH2 subunit and excess peptide was washed away. Crosslinked EZH2 was eluted from the beads using 150 mM NH_4OH pH 11 then lyophilized overnight.

Residual proteins were resuspended in 200 µL of 50mM Ammonium Bicarbonate pH 8. TCEP was added to 5 mM final concentration and left to incubate at 60 °C for 20 min. 15 mM chloroacetamide was added and left to incubate in the dark at room temperature for a further 30 minutes. 1.5 µg of LysC-Trypsin Mix (Promega) was added to each sample and incubated end-over-end at 37 °C for 16 hours. 0.25 µg of Trypsin Gold was added and

incubated end-over-end at 37 °C for 3 hours. Samples were acidified by adding TFA to a final concentration of 0.2%. Samples were dried completely in a speedvac and resuspended with 20 µL of 0.1% formic acid pH 3. 5 µL was injected per run using an Easy-nLC 1000 UPLC system. Samples were loaded directly onto a 45cm long 75 µm inner diameter nano capillary column packed with 1.9 µm C18-AQ (Dr. Maisch, Germany) mated to metal emitter in-line with an Orbitrap Elite (Thermo Scientific, USA). The mass spectrometer was operated in data-dependent mode with the 120,000 resolution MS1 scan (400-1800 m/z) in the Orbitrap followed by up to 20 MS/MS scans with CID fragmentation in the ion trap. Dynamic exclusion list was invoked to exclude previously sequenced peptides for 120s if sequenced within the last 30s and a maximum cycle time of 3s was used.

Raw files were converted to mzXML format using MSconvert with default settings. mzXML files were analyzed using StavroX software. Photo-cystine was defined as an amino acid with composition $C_7H_{13}N_3OS$ and Cys-Diazirine was defined as the crosslinker (composition $-N_2$; Site 1: Z; Site 2: Any AA). All other settings were left as recommended. A FASTA file consisting of the sequences of H3 sequence and PRC2 subunits included in the experiment was utilized.

Sequence Alignment

Sequence alignment was performed using Clustal Omega (12).

PRC2 Pulldown Experiments

Approximately 100 nM PRC2 was incubated with ~450 nM 601 sites in a 10 µL reaction containing binding buffer (final concentration: 50 mM HEPES pH 7.0, 0.5 mM MgCl2, 0.002% Tween). The mixture was allowed to bind on ice for thirty minutes. After 30 minutes, magnetic streptavidin-coated beads (Dynabeads M-270, ThermoFisher Scientific) were added and the arrays were pulled down using a magnetic manifold. The supernatant was removed and the beads were washed 3x, then eluted in 1x SDS loading buffer by boiling at 95 °C for five minutes. The reactions were separated by SDS-PAGE using a 4-20% Tris-HCl gel (Biorad) and proteins were transferred to a PVDF membrane, then analyzed by western blotting.

Fluorescence Anisotropy Peptide Binding Assays

For the forward titration of PRC2 for reference peptide K_d determination with H3 1-37 K27Nva-fluorescein labeled peptide, the concentration of the reference fluorescent peptide was fixed at 100 nM with 5 µM SAM and 100 µM H3K27me3 stimulating peptide, with increasing concentrations of PRC2 in a buffer containing 50 mM HEPES pH 8, 50 mM NaCl, 2 mM $MgCl_2$, and 1 mM DTT. Data fitting for one-site binding was performed with Graphpad Prism 6.01 software and K_d of this reference peptide was determined.

For substrate peptide K_d determinations, this reference fluorescent peptide was competed away with substrate peptides unmodified or modified at H3K36. In these binding measurements, the same conditions were used except that PRC2 concentration was fixed at 400nM, and 100nM fluorescein-labeled peptide was competed away with increasing concentrations of H3.3 substrate peptide. For all conditions, the wavelengths λ_{ex} = 490 nm

and λ_{em} = 520 nm were used. Fluorescence polarization and anisotropy measurements were made at 20°C using a DM302 fluorimeter (Horiba). Data was normalized by rescaling from 0 to 1 such that zero represents unbound peptide (100% free) and one represents 100% bound peptide. Data was fitted using DynaFit software (10) in a 1:1 model. Equations relating to data fitting can be found within references (10, 11). Constraints employed are outlined below, in an example of a typical code used to fit the data with this software.

Example of Dynafit code used to determine data fitting curve

```
[task]
   task = fit
   data = equilibria
[mechanism]
   P + LL <==> P.LL : KdL dissociation
   P + UL <==> P.UL : KdU dissociation
[constants]
   KdL = 0.4 ; fixed !
   KdU = 1 ?

[concentrations]
   LL = 0.1
   P = 0.4
[responses]
   LL = 0 ?
   P.LL = 1 ?
[data]
   variable UL
   file ./PRC2/data/fit_k36me0.txt
[output]
   directory ./PRC2/output/fit-k36me0
```

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