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Supplemental Data

Structural Basis for the Methylation State-Specific Recognition of Histone H4-K20 by 53BP1 and Crb2 in DNA Repair

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Supplemental Experimental Procedures

Preparation of Proteins and Peptides

All histone H3-K79 and H4-K20 peptides with different lysine methylation states as well as dimethylated H3-K9 and H3-K27 were synthesized by N-9-fluorenyl methylcarbonyl chemistry and purified by reversed phase HPLC (purity > 98%) at the Mayo Clinic peptide synthesis facility. Correct peptide masses were verified by mass spectrometry.

53BP1, JMJD2A and Crb2 constructs encompassing amino acids 1484-1603, 897-1011 and 358-507, respectively, were cloned into a modified pET15b vector (Novagen, Madison, WI) encoding an N-terminal His_6-tag and a TEV protease cleavage site. 53BP1 point mutants were generated with QuickChange (Stratagene, La Jolla, Ca).

Nonlabeled 53BP1 was prepared for ITC experiments and crystal trials while uniformly ^{15}N- and $^{15}N/^{13}C$-labeled proteins were used for NMR spectroscopy. Both non- and selenomethionine-labeled Crb2 were produced to grow crystals and an ^{15}N-labeled Crb2 sample was prepared for NMR titration. JMJD2A was produced nonlabeled.

In a typical nonlabeled protein production the plasmid was transformed in *E. coli* BL21(DE3) cells that were grown in Luria-Bertani (LB) broth at 37°C to an A_{600} of ~0.8,

transferred to an 18°C shaker incubator and after 1 hr induced by the addition of 1 mM isopropyl β-D-thiogalactoside. The cells were harvested 16 h later by centrifugation at 4000g for 15 mins. Pelleted cells were resuspended in 50 mM sodium phosphate buffer, pH 7.5 and 300 mM NaCl (bind buffer) with 5 mM imidazole, lyzed with a high-pressure microfluidizer Emulsiflex C-5 (Avestin Inc., Ottawa, Canada) and centrifuged at 20000g for 30 mins. The supernatant was then passed through a Ni^{2+}-loaded NTA column (Qiagen, Valencia, CA) and the protein eluted with 500 mM imidazole in bind buffer after washing the column with 20mM imidazole in bind buffer. The His_6-tag was cleaved by overnight incubation with TEV protease at room temperature. The protein was further purified by size exclusion chromatography using a preparative Superdex 75 column (GE Healthcare, Piscataway, NJ) and concentrated using 3 kDa centricon (Millipore, Billerica, MA) centrifugal filter units.

The procedure for preparing ^{15}N- and $^{15}N/^{13}C$-labeled proteins was similar as above except that M9 media with $^{15}NH_4Cl$ and $^{13}C_6$-glucose, supplemented with 0.1% w/v or 10% v/v of isotopically enriched ISOGRO (Isotec, St. Louis, MO) or Silantes OD2 (CIL, Andover, MA), respectively, replaced the LB media. For the production of selenomethionine-labeled Crb2 protein, auxotrophic *E. coli* B834(DE3) cells (Novagen, Madison, WI) and M9 media containing selenomethionine, nucleosides and all 20 amino acids except methionine were used (Botuyan et al., 2004).

X-Ray Crystallography

X-ray diffraction data for 53BP1 and Crb2 were collected at the X12-C beamline, Brookhaven National Laboratory (BNL), NY and 19-ID beamline (SBC-CAT) in the Advanced Photon Source (APS), Argonne National Laboratory, IL, respectively.

Crystals of free 53BP1 tandem tudor domains were grown at 22°C by vapor diffusion of hanging drops by mixing 1 μL of ~3 mM protein in 50 mM Tris/HCl, pH 7.0, 100 mM NaCl with 1 μL of reservoir solution (solution 1) containing 100 mM HEPES/Na, pH 7.0, 2% PEG 400 and 2 M ammonium sulfate. Crystals grew in 1 to 3 weeks. The space group of the crystals is C2 with one molecule per asymmetric unit. The structure of the 53BP1/H4-K20me2 complex was obtained by soaking crystals of the free protein for 1 to 6 days in solution 1 containing the peptide (residues 16-25) at concentrations ranging from 5 to 10 mM. Crystals were transferred into a cryoprotectant solution consisting of 30% glucose in solution 1, and were quick-frozen in a cryoloop (Hampton Research, Aliso Viejo, CA) with liquid nitrogen.

Crystallization and cryoprotection of Crb2 follow the same protocol except that solution 1 was changed to 0.1 M HEPES/Na, pH 7.5, 0.8 M NaH_2PO_4 and 0.8 M KH_2PO_4. The trigonal crystals have a space group of $P3_221$, with three molecules of Crb2 in each asymmetric unit.

shRNA-Mediated Stable Inhibition of Dot1 Expression in HeLa and A549 Cell Lines

A plasmid encoding a short hairpin RNA (shRNA) targeting the *Dot1* gene was prepared as previously described (Trushin, et al., 2003). Complementary oligonucleotides were designed as follows: each oligonucleotide contains a 5'-BglII and 3'-HindIII restriction

sites, an RNA polymerase III start and termination sequence, and 19 nucleotides of *Dot1* specific sequence separated by a 9-nucleotide loop. The sense strand is 5'-GATCCCCGCACCTCTGAACTTCAGAATTCAAGAGATTCTGAAGTTCAGAGGTGTTTTTGGAAA-3' and the anti-sense strand is 5'-AGCTTTTCCAAAAAGCACCTCTGAACTTCAGAATCTCTTGAATTCTGAAGTTCAGAGGTGCGGG-3'. The oligonucleotides were purchased from Dharmacon, Lafayette, CO.

Because there is no antibody available to detect endogenous Dot1, the efficiency of shRNA against Dot1 was tested by co-transfecting the cells with a vector encoding Flag-tagged full length Dot1 (Feng et al., 2002) and detecting Dot1 by western blot using a Flag-directed antibody. HeLa and A549 stable cell lines knocking down Dot1 expression were generated.

The pFRT-H1P plasmid for RNA targeting and Flag-Dot1 construct were kindly provided by Dr. D. Billadeau and Dr. Y. Zhang, respectively.

Downregulation of Histone H4 Methylation at Lysine 20

HeLa cells were tansfected with 3 rounds of control siRNA (5'-UUCAAUAAAUUCUUGAGGUdTdT-3') or various siRNA directed against PR-Set7 (Smartpool, #1 (5'-ACCCGUGGCUGAAGCAUUAUUdTdT-3'), #2 (5'-GCAACUAGAGAGACAAAUCUUdTdT-3'). The oligonucleotides were purchased from Dharmacon, Lafayette, CO. 96 h after the first transfection, the cells were analyzed by immunofluorescent staining and immunoblotting.

Immunofluorescence and Western Blotting

After exposure to ionizing radiations, the cells were treated with paraformaldehyde for fixation using a standard protocol. In the case of phosphorylated-ATM staining, a 4 min detergent extraction step preceded fixation. The following antibodies were used for immunostaining: rabbit anti-53BP1 E6 (Rappold et al., 2001), rabbit anti-phosphorylated-H2AX (Ser139) (Ward and Chen, 2001), mouse anti-phosphorylated-ATM kinase (Ser1981) (Rockland, Gilbertsville, PA), rabbit anti-Dot1 (raised against a mix of N-terminal and C-terminal Dot1 peptides). For immunoblotting, cells were lysed in NETN buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris/HCl, pH 8.0, 0.1% NP-40) prior to histone extraction in a buffer containing 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1.5 mM Pefabloc and 0.2 M HCl. Extracted histones were neutralized by adding one-fifth volume of 1.5 M Tris/HCl, pH 8.8. The NETN fraction was used for PR-Set7 Western blotting.

Supplemental References

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Table S1. X-Ray Data Collection and Refinement Statistics

Data Collection		
	53BP1 Free	53BP1 + H4-K20me2
Space Goup	C2	C2
Cell Dimensions		
a, *b*, *c* (Å)	55.90, 77.83, 36.35	56.37, 78.54, 36.45
α, β, γ (°)	90.00, 121.13, 90.00	90.00, 121.20, 90.00
Resolution (Å)	50 – 1.25 (1.29 – 1.25)	50 – 1.70 (1.76 – 1.70)
R_{sym} or R_{merge}	0.029 (0.293)	0.084 (0.223)
Completeness (%)	93.2 (61.9)	82.8 (32.2)
Redundancy	7.0 (4.8)	6.4 (3.4)
Refinement		
Resolution (Å)	50 – 1.25	50 – 1.70
Number of Reflections	32860	11776
R_{work} / R_{free}	0.194(0.369)/0.241(0.336)	0.176(0.197)/0.251(0.212)
Number of Atoms	1396	1316
Protein	1184	1098
Ligand/ion	5	28
Water	207	180
B-factors	19.9	33.5
Protein	17.7	30.8
Ligand/ion	26.6	56.5/96.3
Water	32.6	44.4
R.m.s. deviations		
Bond lengths (Å)	0.015	0.024
Bond angles (°)	1.736	2.223
Ramachandran Plot Statistics		
Most Favorable Regions	93.1%	91.2%
Additional Allowed Regions	5.9%	8.8%
Generously Allowed Regions	1.0%	0.0%
Disallowed Regions	0.0%	0.0%

The highest resolution shell is shown in parentheses.

Table S2. X-Ray Data Collection and Refinement Statistics	
Data Collection (SAD)	*Peak*
	Crb2 (SeMet)
Space Goup	$P3_221$
Cell Dimensions	
a, *b*, *c* (Å)	116.92, 116.92, 87.02
α, β, γ (°)	90.00, 90.00, 120.00
Wavelength	0.97956
Resolution (Å)	50 – 2.40 (2.49 – 2.40)
R_{sym} or R_{merge}	0.111 (0.499)
$I/\sigma I$	46.8 (7.4)
Completeness (%)	99.9 (100)
Redundancy	13.8 (14.3)
Refinement	
Resolution (Å)	50 – 2.40
Number of Reflections	25798
R_{work} / R_{free}	0.192 (0.214)/0.256 (0.316)
Number of Atoms	3718
Protein	3496
Ligand/ion	55
Water	167
B-factors	
Protein	32.2
Ligand/ion	82.8
Water	39.8
R.m.s. deviations	
Bond lengths (Å)	0.029
Bond angles (°)	2.619
Ramachandran Plot Statistics	
Most Favorable Regions	83.9%
Additional Allowed Regions	14.1%
Generously Allowed Regions	1.9%
Disallowed Regions	0.0%

The highest resolution shell is shown in parentheses.

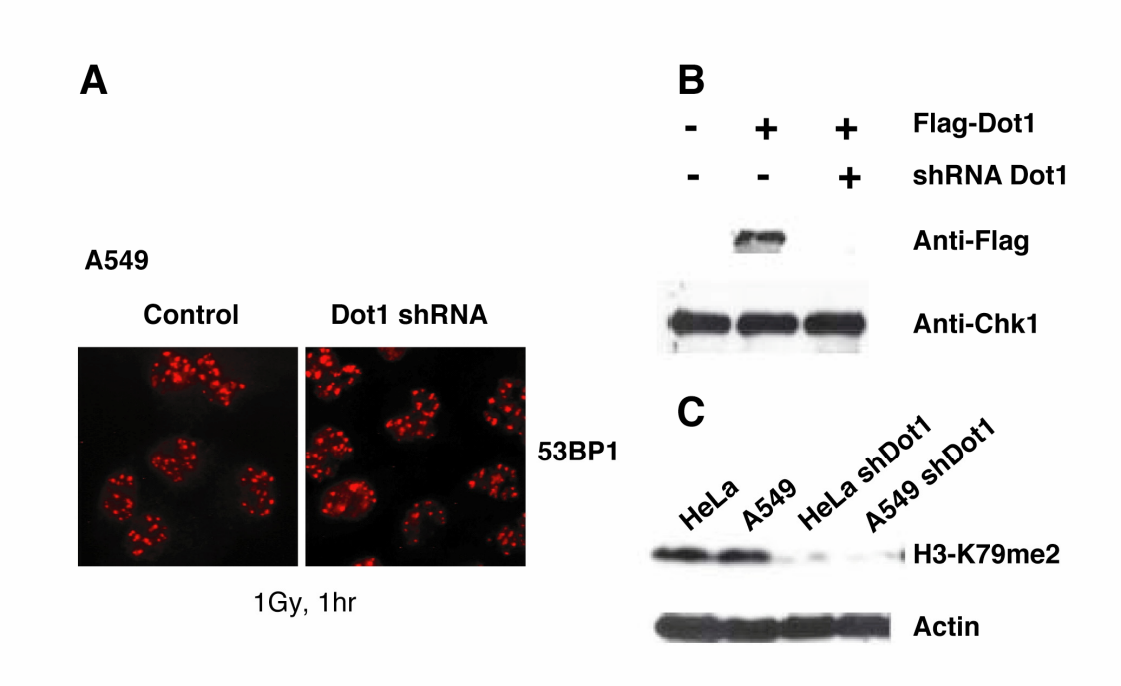

Figure S1. shRNA-Mediated Knockdown of Dot1

(A) A549 and HeLa stable shRNA Dot1 knockout cell lines were immuno-analyzed for 53BP1 localization (53BP1) in response to 1 Gy of ionizing radiation. The results for A549 cells are shown.

(B) The efficiency of shRNA against Dot1 (shRNA Dot1) was tested by co-transfection with Flag-tagged full length Dot1 (Flag-Dot1) and a western blot against the Flag tag (Anti-Flag) because there is no antibody available to detect endogenous Dot1.

(C) Aliquot of the shRNA-treated stable A549 (A549 shDot1) and HeLa cell lines (HeLa shDot1) were examined for dimethylated histone H3-K79 (H3-K79me2) levels by immunoblotting.

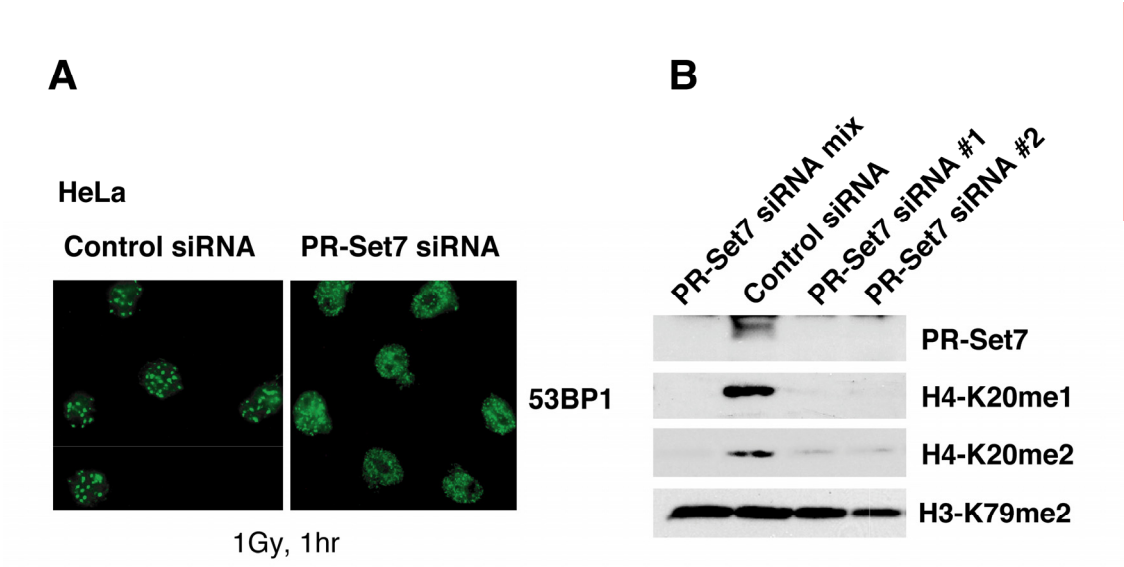

Figure S2. siRNA-Mediated Knockdown of PR-Set7/Set8

(A) HeLa cells, treated 3 consecutive times with control (Control siRNA) or PR-Set7-specific siRNA (PR-Set7 siRNA), were immuno-analyzed for 53BP1 localization (53BP1) in response to 1 Gy of ionizing radiation.

(B) An aliquot of the siRNA-treated cells was collected and examined for PR-Set7 (PR-Set7) as well as monomethylated and dimethylated histone H4-K20 levels (H4-K20me1 and H4-K20me2) by immunoblotting. Expression levels of dimethylated histone H3-K79 (H3-K79me2) were used as loading control.

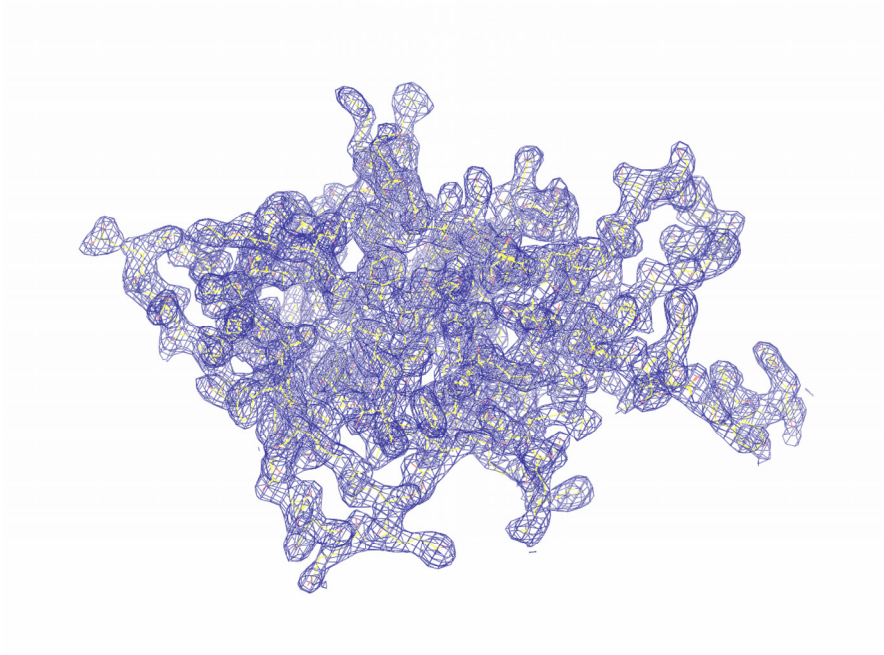

Figure S3. Sample of Crb2 Tandem Tudor Domains Electron Density Map

The electron density map of one of the three Crb2 molecules in the asymmetric unit is shown.