SUPPLEMENTARY INFORMATION

GLP-catalyzed H4K16me1 promotes 53BP1 recruitment to permit DNA damage repair and cell survival

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Supplementary Information

Supplementary Figures S1 to S8
Supplementary Table S1
Figure S1

A. 

| H4K16me0 | H4K16me1 | H4K16me2 | H4K16me3 |
|----------|----------|----------|----------|
| 4 ng     | 16 ng    | 64 ng    | 4 ng     |

B. 

H4K16me1

16 ng 64 ng

C. 

H4K16me1

D. 

H4K16me1

H4

E. 

IR release

| Ctr | 1 h | 3 h |
|-----|-----|-----|
| H4K16me1 | H2AX | H4 |
| H4K16me1 | H2AX | H4 |

F. 

IR release

| Ctr | 1 h | 3 h |
|-----|-----|-----|
| H4K16me1 | H2AX | H4 |
| H4K16me1 | H2AX | H4 |

G. 

etoposide 40 μM

H. 

DAPI  H4K20me2  53BP1  Merge
Figure S1. H4K16me1 increases in response to DNA damage.

(A) Slot blot assay showing the high specificity of the H4K16me1 antibody. Cross reactivity with peptides carrying other modifications was not detected.

(B) Peptide competition assay showing that only pre-incubation of H4K16me1 peptide with H4K16me1 antibody efficiently blocks H4K16me1 epitope recognition.

(C) H4K16me1 expression was detected in HEK293 cells. Recombinant Histone H4 (rH4) was used as a negative control. The entire western blot membranes probed in parallel with H4K16me1 and H4 were shown. The blots in the frames were used in Figure 1B.

(D) H4K16me1 expression in different mammalian cell lines.

(E, F) HeLa and LoVo cells were exposed to 10 Gy X-ray irradiation and then re-cultured for 1 h or 3 h in fresh medium. Histones were extracted for western blotting.

(G) HCT116 cells were treated with 40 μM etoposide for the indicated time. Histones were extracted for western blotting.

(H) HeLa cells were micro-irradiated as previously described. After 10 min, cells were washed, fixed and stained with anti-H4K20me2 and anti-53BP1 antibodies. Similar results were obtained from three independent experiments. Scale bars: 10 μm.
Figure S2

A

B

GST  GST-SET8
+ + H3  + + H3
+ + H4  + + H4

H4K16me1  H4K16me1
H4  H4
H3K9me3  H3K27me3
H3  H3

GST  GST-SET8
+ + H3  + + H4
+ + H4  + + H4

H4K16me1  H4K16me1
H4  H4
H3K9me3  H3K36me3
H3  H3

GST  GST-SET8
+ + H3  + + H4
+ + H4  + + H4

H4K16me1  H4K16me1
H4  H4
H3K79me1  H4

GST  GST-SET8
+ + H3  + + H4
+ + H4  + + H4

H4K16me1  H4K16me1
H4  H4
H4K20me1  H4

B

GST  GST-GLP
+ + CBB
+ + CBB
+ + CBB
+ + CBB

C

GST  GST-GLP
+ + 0 5 10 20 50 μM Bix-01294
+ + 0 5 10 20 50 μM Bix-01294

H4K16me1  H4K16me1
H4  H4

H3K9me2  H3K9me2

C

D

C

G9a KO
+ + 0 5 10 20 50 μM Bix-01294
+ + 0 5 10 20 50 μM Bix-01294

H4K16me1  H4K16me1
H4  H4

G9a  G9a
Figure S2. GLP catalyzes H4K16me1 both *in vitro* and *in vivo*.

(A) *In vitro* methylation assays were performed using different methyltransferases. Recombinant histones H3 and H4 were used as substrates.

(B) Coomassie brilliant blue staining of the methyltransferases used in Figure 2B, indicated with “#”.

(C) Recombinant H4 (2 μg) was incubated with GST or GST-GLP in methylation reaction buffer with increasing concentrations of Bix-01294. Samples were then analyzed by western blotting.

(D) H4K16me1, H3K9me2, G9a and histone H4 expression levels in G9a knockout (KO) HCT116 cells.
Figure S3

A

| Time post micro-irradiation (s) | Ctr 20 40 60 120 180 240 300 |
|---------------------------------|--------------------------------|
| mCherry-GLP WT                 | mCherry-GLP ΔSET              |
| GFP-G9a WT                     | mCherry-GLP WT               |

B

| Time post micro-irradiation (s) | Relative intensity |
|---------------------------------|--------------------|
| mCherry-GLP WT                 | mCherry-GLP ΔSET   |
| GFP-G9a WT                     | mCherry-GLP WT    |

C

| Time post micro-irradiation (s) | mCherry-GLP         |
|---------------------------------|---------------------|
| Ctr 20 40 60 120 180 240 300    | WT                  |
| G9a KO                          |                     |

D

| Relative intensity |
|--------------------|
| Time post micro-irradiation (s) |
| WT                  |
| G9a KO              |

E

| Relative intensity |
|--------------------|
| Time post micro-irradiation (s) |
| WT                  |
| G9a KO              |

F

| Input | IgG | IP: Flag |
|-------|-----|----------|
|       |     | etoposide |
| DAPI  | GLP | γH2AX    |

G

| Input | IgG | IP: G9a |
|-------|-----|---------|
|       |     | etoposide |
| DAPI  | GLP | G9a     |

H

| Input | IgG | IP: G9a |
|-------|-----|---------|
|       |     | etoposide |
| DAPI  | GLP | G9a     |

I

| Input | IgG | IP: GLP |
|-------|-----|---------|
|       |     | etoposide |
| DAPI  | GLP | RPA32   |

J

| Input | IgG | IP: G9a |
|-------|-----|---------|
|       |     | etoposide |
| DAPI  | GLP | G9a     |
Figure S3. GLP has different roles to G9a in the DNA damage response.

(A) Dynamics of mCherry-GLP (WT), mCherry-GLP (ΔSET) or GFP-G9a at micro-irradiated sites (indicated by a broken white line) in HeLa cells. Consistent results were observed in three different experiments. Scale bars: 10 μm.

(B) Relative intensities of mCherry-GLP (WT), mCherry-GLP (ΔSET) or GFP-G9a at micro-irradiated sites in the experiment described in A. The data represent the means ±SD.

(C) Dynamics of mCherry-GLP at micro-irradiated sites (indicated by a broken white line) in HCT116 WT and HCT116 G9a KO cells. Consistent results were observed in three different experiments. Scale bars: 10 μm.

(D) Relative intensities of mCherry-GLP at micro-irradiated sites in the experiment described in C. The data represent the means ±SD.

(E) HCT116 WT and HCT116 G9a KO cells were micro-irradiated and fixed 10 min later for immunostaining with the indicated antibodies. Scale bars: 10 μm.

(F) Soluble (Dt) and chromatin fractions (Chr) extracted from HCT116 cells were immunoprecipitated with anti-Flag M2 beads and probed with the indicated antibodies.

(G, H) HCT116 cells were treated with 40 μM etoposide for 30 min before drug withdrawal. After re-culture for 30 min, cells were collected and the Dt fraction was used for anti-G9a immunoprecipitation. For (G), anti-G9a (Sigma) and anti-GLP (NOVUS) antibodies were used; for (H), anti-G9a (Sigma) and anti-GLP (MBL) antibodies were used.

(I) shCtr and shGLP HeLa cells were mixed in culture to control for background staining and then treated with 40 μM etoposide for 30 min or left untreated. The cells were fixed and RPA32 foci formation was detected by confocal microscopy. Arrows indicate shGLP cells. Ctr, no etoposide treatment. Scale bars: 10 μm.
Figure S4

A

|   | NC | GLP &2 |
|---|----|--------|
|   | -  | +      |
|   | +  | -      |
| +  | +  | +      |
| IR|    |        |

B

|   | Ctrl | G9a KO |
|---|------|--------|
| H4K16me1| -  | +      |
| H3K9me2 | +  | -      |
| H2AX    | +  | -      |
| G9a     | -  | +      |
| H4      | -  | -      |

C

|   | NC | SETB1 |
|---|----|-------|
| H4K16me1| -  | +      |
| SETB1   | +  | -      |
| H2AX    | -  | +      |
| H4      | -  | -      |

D

|   | Input | IP-Flag |
|---|-------|---------|
| +  | -     | +       |
| -  | +     | -       |
| -  | -     | +       |
| etoposide | ATM | Flag-GLP |

E

|   | IP-Flag |
|---|---------|
| etoposide | P-S/T-Q | Flag-GLP |
| Flag-GLP | Flag-GLP |

F

|   | +  | +  | +  | Flag-GLP |
|---|----|----|----|---------|
| +  | -  | -  | +  | Flag-ATM |
| -  | +  | +  | +  | ATP     |
| -  | -  | +  | +  | Flag-GLP |

G

|   | Flag-GLP | ATM | ATP | His-G9a |
|---|-----------|-----|-----|---------|
| +  | -         | +   | +   | +       |
| pull down | 1st round | 2nd round | His pull down |

H

|   | NC | ATR Bi |
|---|----|--------|
| H4K16me1| -  | +      |
| ATR     | +  | -      |
| H2AX    | +  | -      |
| H4      | -  | -      |

I

|   | NC | VE-821 |
|---|----|--------|
| H4K16me1| -  | +      |
| ATR     | +  | -      |
| H2AX    | +  | -      |
| H4      | -  | -      |
Figure S4. GLP catalyzes H4K16me1 in response to DNA damage in an ATM-dependent manner.

(A) HCT116 cells were transfected with GLP siRNA#2 for 72 h before exposure to 10 Gy X-ray. The cells were then re-cultured for 30 min in fresh medium before protein extraction and western blotting with the indicated antibodies.

(B) G9a WT (Ctr) and G9a KO cells were treated with X-ray at 10 Gy. After 1 h, whole cell lysates and histones were extracted for western blotting.

(C) The same experiment described in (A) was performed with SET8 siRNA. #, non-specific bands.

(D) HCT116 cells were transfected with Flag-GLP for 2 d before 40 μM etoposide treatment for 30 min. Whole cell lysates were extracted and immunoprecipitated with anti-Flag M2 beads, and the interaction between GLP and ATM was assessed by western blotting.

(E) HCT116 cells were transfected with Flag-GLP for 2 d before 40 μM etoposide treatment for 30 min. Whole cell lysates were extracted and immunoprecipitated with an anti-Flag antibody, and the P-S/T-Q signal of GLP was assessed by western blotting.

(F) An in vitro phosphorylation assay was performed to check the activity of ATM towards GLP. Flag-GLP was purified from HCT116 cells, and ATM was immunoprecipitated from HCT116 cells and then incubated with GLP in phosphorylation reaction buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM MgCl2, 0.5 mM DTT, 30 μM ATP] at 30 °C for 1 h.

(G) Flag-GLP and Flag-ATM were purified separately from G9a KO HCT116 cells and an in vitro phosphorylation assay was performed. Un-phosphorylated GLP or phosphorylated GLP was incubated with His-G9a in PBS (0.5% Triton X-100) at 4 °C for 3 h before His pull-down and detection by western blotting.

(H) HCT116 cells were transfected with ATR siRNA for 2 d and irradiated with X-ray at 10 Gy. After 30 min, chromatin bound fraction were extracted and detected by western blotting.

(I) HCT116 cells were pre-treated with 10 μM VE-821 for 4 h and irradiated with X-ray at 10 Gy. After 30 min, histones were extracted and detected by western blotting.
Figure S5
Figure S5. GLP regulates NHEJ by targeting 53BP1.

(A) Representative images showing GLP expression and 53BP1 foci formation in the experiment described in Figure 4E. Similar results were observed in at least three independent experiments.

(B) HeLa cells were pre-treated with or without 2.5 μM Bix-01294 for 24 h before X-ray irradiation. Cells were fixed and stained with anti-H4K16me1 and anti-53BP1 antibodies (upper panels) or stained with anti-H3K9me2 and anti-53BP1 antibodies (lower panels).

(C) NHEJ efficiency following G9a, GLP or SET8 siRNA-mediated knockdown. A pool of GLP siRNA#1 and #2 was used. “NC” indicates the scrambled siRNA used as a negative control. The data represent the means ± SD, *p < 0.05, ***p < 0.001 (Student’s t-test).

(D) NHEJ efficiency following treatment with 2.5 μM Bix-01294 for 24 h. Three independent experiments were performed. The data represent the means ± SD, ***p < 0.001 (Student’s t-test).

(E) HR efficiency following GLP, G9a or BRCA1 siRNA-mediated knockdown in DR-U2OS cells. A pool of GLP siRNA#1 and #2 was used. “NC” indicates the scrambled siRNA used as a negative control. The data represent the means ± SD, n.s., not significant, **p < 0.01, ***p < 0.001 (Student’s t-test).

(F) Western blot analysis of H4K20me2, GLP and Actin in shCtr and shRGLP cells.

(G) mRNA levels of DNA repair factors in shCtr and shGLP cells.

(H) shGLP cells were transfected with reconstituted Flag-GLP (WT) or Flag-GLP (ΔSET) for 72 h and then treated with 40 μM etoposide for 30 min. The cells were then fixed and immunostained with anti-Flag and anti-53BP1 antibodies. Arrows indicate successfully transfected cells. Ctr, no etoposide treatment. Scale bars: 10 μm.
Figure S6

A

B

C

[Images of immunofluorescence staining for DAPI, GLP, RNFR, and Merge, with statistical graphs showing n.s. and (+).]
Figure S6. Foci formation of RNF8, RNF168 and FK2 in control and GLP-deficient cells

(A-C) shCtr and shGLP HeLa cells were mixed in culture to control for staining background and then treated with 40 μM etoposide for 30 min or left untreated. The cells were fixed and RNF8 (A), RNF168 (B) and FK2 (C) foci formation was detected by confocal microscopy. Arrows indicate the shGLP cells. (-), no etoposide treatment. (+), etoposide treatment. Scale bars: 10 μm. Representative images are shown on the left. Calculations from at least 200 cells are shown on the right. The data represent the means ± SD. n.s., not significant. Consistent results were obtained from at least three different experiments.
Figure S7

A
H4K16me1
H4K16ac
γH2AX
H4
etoposide release
Ctr 0 0.5 1 2 4 6 h

B
Marker I
0 2 4 μg

C
Input IP: 53BP1
H4K16me1
H4K20me2
53BP1
1 1.99
1 3.63

D
A-196

E
Ctr A-196
H4K16me1
H4K20me2
γH2AX
H4

F
Ctr A-196
IR
H4K16me1
H4K20me2
γH2AX
53BP1
Input

G
H4K16me1
H4K20me2
53BP1

H
53BP1 foci count

I
Beads
H4K16me1
H4K20me2
Input
His-Tudor
His-Tudor
Biotin
Pull down

WT
D1521A

WT
D1521A

WT
D1521A

WT
D1521A
Figure S7. H4K16me1 cooperates with H4K20me2 for 53BP1 recruitment in the DDR.

(A) HCT116 cells were treated with 40 μM etoposide for 30 min. The cells were then washed four times with PBS and re-cultured in fresh medium. Histones were then extracted at each release time (indicated) and analyzed by western blotting.

(B) The efficiency of mononucleosome extraction was assessed by electrophoresis of the DNA extracted from obtained mononucleosomes.

(C) HCT116 cells were treated with 10 Gy X-ray irradiation and re-cultured for 30 min. The chromatin fractions were extracted for anti-53BP1 immunoprecipitation. The interacted histones were analyzed by western blotting.

(D) HCT116 cells were treated with A-196 at indicated concentration for 2 d before histone extraction and western blotting.

(E) HCT116 cells were pre-treated with 2.5 μM A-196 for 2 d and irradiated with X-ray at 10 Gy. After 30 min, histones were extracted and analyzed by western blotting.

(F) HCT116 cells were pre-treated with 2.5 μM A-196 for 2 d and irradiated with X-ray at 10 Gy. Whole cell lysates were extracted for anti-53BP1 Co-IP. The interacted histones were analyzed by western blotting.

(G-H) Hela cells were pre-treated with 2.5 μM A-196 or 2.5 μM Bix-01294 respectively for 2 d, or pre-treated with a combination of 2.5 μM A-196 and 2.5 μM Bix-01294 for 2d and then subjected to 10 Gy X-ray irradiation. After 20 min, cells were fixed and stained with anti-53BP1 antibody. 53BP1 foci formation was detected by confocal microscopy. Consistent results were observed from three independent experiments. Representative confocal images are shown in (G). Calculations from at least 200 cells per sample are shown in (H). The data represent the means ± SD, ****p<0.0001 (Student’s t-test).

(I) A peptide pull-down assay was performed to monitor the interactions between His-Tudor WT or His-Tudor D1521A with the indicated peptides.
Figure S8
Figure S8. GLP-catalyzed H4K16me1 is important for cell survival after DNA damage.

(A) Western blots showing endogenous GLP expression in shCtr and shGLP cells and exogenously re-expressed GLP in shGLP cells.

(B) Western blots showing endogenous GLP knockdown in the experiment performed in Figure 6F.

(C) Representative images for the colony formation assay performed in Figure 6F. Consistent results were observed from three independent experiments.

(D) Western blots showing endogenous 53BP1 knockdown and Flag-GLP over-expression in the experiment performed in Figure 6G.

(E) Representative images for the colony formation assay performed in Figure 6G. Consistent results were observed from three independent experiments.
Table S1. The primers used in RT-PCR assays.

| Gene   | Primer Forward       | Primer Reverse       |
|--------|----------------------|----------------------|
| Ku70   | TTGCTTCTGCCTAGCGATACC | AAACCTGGATCATCAAACCGT |
| Ku70   | GACCCTGAAAAGGCGAGTT  | TTGACGAGAAATTCTTCGGG |
| RNF168 | GCACCTGACATAATCCCCCTTG | CCAAGGCAGACACTTTACCGAA |
| XRCC1  | TCAAATGTCTCAGCAAAATCAA | TCCAAACTTGAGGACACAGAG |
| Actin  | CCAAGGCGGATCAGAGTAGAG | ATCTGCTGCCACCACTAGAA |
| ATM    | GATCTCGAATCGGCTTTAAA | TCCCTGAATACAGTGCCCT |
| ATR    | TGGATGCCAATCTAAGTCCCA | CAATCTGAGGACGAATTGCC |
| DNAPK  | CCCGCTTTCTCCTCAGAG  | ACCTCGACCCATCTTC |
| RNF8   | GAACGTATGCAAATGCACAAAGA | ACCGTATGCAAATGCACAAAGA |
| RPA32  | GCCGCTTTCTCCTCAGAG  | ACCTCGACCCATCTTC |
| RPA32  | TCTTTTGGGCATAGGCAACA | ACCGTATGCAAATGCACAAAGA |
| RIF1   | GCCAAAAGGTACATTGCAGG  | AGCTGCTCCATTACAG |
| RIF1   | GGTCACAATAGGCAACAACA | ACCGTATGCAAATGCACAAAGA |
| NBS1   | CAGGAGGGAAATCTCCAGAAA | GAAGCAAAACCGGACTT |
| BRCA1  | CAGGAGGGAATCTCCAGAAA | GAAGCAAAACCGGACTT |
| BRCA1  | CTGCTCTTGGGACTCACTG | ACCGTATGCAAATGCACAAAGA |
| PARP1  | CTGCTCTTGGGACTCACTG | ACCGTATGCAAATGCACAAAGA |
| PARP1  | CTGCTCTTGGGACTCACTG | ACCGTATGCAAATGCACAAAGA |
| GLP    | CATGCAGCCATCTGTG | ACCGTATGCAAATGCACAAAGA |
| GLP    | CTGCTCTTGGGACTCACTG | ACCGTATGCAAATGCACAAAGA |