Supporting Material

Experimental Procedures:

Cell culture and antibodies

All cell lines were maintained in RPMI 1640 medium with 10% fetal calf serum at 37°C in 5% CO₂ (v/v). For HCC 1937-BRCA1 cells and 293FT cells, cell culture medium was supplemented with 500 µg/ml G418. For cell cycle analyses, cells were treated with 1 µg/ml nocodazole for 24 hours. Arrested cells were released into cell cycle by removal of drug and addition of fresh cell culture medium, and cells were harvested at the timepoints indicated.

Rabbit anti-BRCA1 (C23) and anti-BARD1 (C20) antibodies were generous gifts from Dr. David Livingston. Rabbit anti-myc, V5, and BACH1 were raised by immunizing rabbits with peptides EQKLISEEDI, GKPIPNPLLGLDST, and NFKPSPSKNKGMFPGFK, respectively. Rabbit polyclonal antibody against phospho-Serine 990 of BACH1 was raised against phospho-peptide CIVISRSTS*PTFNKQT and affinity-purified. Rabbit anti-phospho Histone H3 antibody was purchased from Upstate Biotechnology Inc. Rabbit anti-E2F1 (C-20) and anti-RNA pol II (N-20) were from Santa Cruz Biotechnology Inc.

Cell lysis and protein dephosphorylation

Cells were lysed in NETN buffer (0.5% NP-40, 1 mM EDTA, 50 mM Tris-HCl, pH 8.0, 0.15 M NaCl). To dephosphorylate proteins, 200 units λ, protein phosphatase
(New England Biolabs) was added into whole cell lysates, and incubated at 30 °C for two hours. To protect proteins from dephosphorylation, 10 mM NaF and 50 mM β-glycerophosphate were included in the lysis buffer.

**GST-BRCT fusion proteins and pull down assay**

A DNA fragment encoding the BRCA1 BRCT domain (residues 1599 to 1863) was cloned into the pGEX-4T1 vector. Constructs of GST-BARD1-BRCT containing residues 554 to 777 of BARD1 and GST-53BP1-BRCT containing residues 1687 to 2973 of 53BP1 were generated similarly. TopBP1 BRCT-6 (residue 795 to 954), Fcp1 BRCT domain (residue 587 to 785), *S. cerevisiae* RAD9 BRCT domain (residue 962 to 1309), MDC1 BRCT domain (residue 2727 to 3089), *S. cerevisiae* REV1 BRCT domain (residue 1 to 318), TopBP1 BRCT1,2 (residue 1 to 330), TDT BRCT domain (residue 6 to 162), DNA ligase III BRCT domain (residue 814 to 922), Ect2 BRCT domain (residue 113 to 342), *S. pombe* Crb2 BRCT domain (residue 478 to 778), DNA ligase IV BRCT domain (residue 618 to 911), *S. pombe* Rad4 BRCT 1,2 (residue 1 to 227) and BRCT 3,4 (residue 228 to 520) were all cloned into pGEX-4T1. Purification of GST fusion proteins and pull down assays were performed as described previously (10).

The BRCA BRCT domain (residue 1646 to 1859) was also cloned into pET43a-NUS-His-TEV (from Dr. C. Ward). Recombinant BRCA1 BRCT was purified and tags were removed by TEV cleavage.

We obtained Fcp1 cDNA form Dr. Danny Reinberg, *S. cerevisiae* Rad9 cDNA from Dr. David Stern, DNA ligase III cDNA from Dr. Alan Tomkison, DNA ligase IV cDNA from Dr. David Chen, ECT2 cDNA from Dr. Toru Miki, *S. pombe* Rad4 and Crb2
cDNA from Dr. Mitsuhiro Yanagida, and TDT cDNA from ATCC (IMAGE clone 4509721).

**Generation of BACH1 mutants, cell transfection, immunoprecipitation and immunoblotting**

Full-length BACH1 cDNA inserted into pCDNA3.1/myc-his B (Invitrogen) was obtained from Dr. David Livingston. All mutants of BACH1 were generated using the Quickchange site-directed mutagenesis (Invitrogen, inc). For transfection, 5 µg plasmid with 15 µl FuGENE 6 were used according to the manufacturer’s protocol (Roche Diagnostics, inc). Immunoprecipitation and immunoblotting were performed as described previously (10).

**BIAcore peptide binding assay**

Surface plasmon resonance measurements were performed on BIAcore 3000 (BIAcore) at 15 °C. Biotinylated peptides were passed over the surface of the streptavidin-coated chip at 10 µl/min to an equivalent of 250 resonance units. All proteins were dialyzed in buffer containing 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl and 1 mM PMSF before BIAcore analysis. Analytes were diluted with the same buffer to achieve concentrations from 1 nM to 3 µM. 90-150 µl analytes were injected at a speed of 30 µl/min. To regenerate the surface after each sample, 10 µl of 0.5% SDS in 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, and 1 mM PMSF was injected twice at a rate of 10 µl/min. For analysis phospho-BACH1 mutants, 3 µM recombinant BRCA1-BRCT proteins was passed over the chips. The results are expressed as the percentage of the resonance units.
measured on mutant phospho-BACH1 peptides compared with that on wild-type phospho-BACH1 peptide.

**Isothermal Titrator calorimetry**

Isothermal titration calorimetry (ITC) experiments were described previously (15). Briefly, experiments were recorded using a VP-ITC instrument (MicroCal), and acquired at 35°C on samples containing 17 µM BRCA1-BRCT (starting concentration), 50 mM sodium phosphate buffer, pH 7.5, and 300 mM NaCl. Incremental amounts of a 200 µM solution of BACH1 phosphopetide were added. All data analysis was performed using Origin 5.0 software (MicroCal). The titration data were fitted using nonlinear least squares analysis, giving a $K_d$ of 2.4 µM, a $\Delta H^0$ of −19.0 kcal.mol⁻¹, and a stoichiometry ($N$) of 1.03.

**BACH1 siRNA transfection and controls**

The siRNA specific for BACH1 was chemically synthesized (Dharmacon). The sequence of BACH1 siRNA is AGCUUACCCGUCACAGCUdTdT. 2 nmol siRNA with 5 µl oligofectamine were used for each transfection according to the manufacturer’s protocol (Invitrogen). To obtain the maximal RNA interference effect, cells were transfected with siRNA again 24 hours after the initial siRNA transfection. Cells were harvested for further analysis 48 hours after the second siRNA transfection. For reconstitution experiments, two siRNA transfections were carried out immediately after two lentivirus infections.
Lentivirus packaging and infection

A silent mutant of BACH1 (SM) was created by changing 4 nucleotides in the BACH1 siRNA targeting region (G69T, T70A, C71G, A72C substitutions). A BACH1 silent mutant construct containing a S990A mutation was also generated. Both BACH1 constructs were introduced into the pLenti6/V5-DEST vector using the Gateway recombination system (Invitrogen). Plasmids were transfected into the 293FT viral packaging cell line by lipofection according to the manufacturer’s protocol (Invitrogen). Cell culture medium was changed on the following day, and virus was harvested 48 hours after plasmid transfection. For virus infection, cells in 6-well plates were cultured with a mixture of 0.5 ml virus-containing medium, 1 ml fresh cell culture medium and 6 µg/ml polybrene (Sigma) overnight. Cells were harvested 48 hours after virus infection.

G2-M accumulation assay

Cells were exposed to 6 Gy γ-irradiation, allowed to recover for three hours, and then treated with nocodazole (1 µg/ml) for 20 hours or as indicated. Cells were fixed with 3% paraformaldehyde, and stained with rabbit anti-phospho-Histone H3 antibody, followed by incubation with Rhodamine conjugated goat anti-rabbit IgG secondary. The positive cells were examined by immunofluorescence microscopy or flow cytometry analysis.
Figure legends

**Fig. S1.** Lysates were prepared from HCC1937 cells and HCC1937 cells reconstituted with wild-type BRCA1. Cell lysates were immunoprecipitated and immunoblotted as indicated.

**Fig. S2.** Expression of BACH1 mutants. (A) Silent mutant of BACH1 (SM) and S990A silent mutation (SPM) were transiently expressed in 293T cells using a lentivirus expression system. Cell lysates were immunoprecipitated with anti-V5 antibody and blotted with anti-BACH1 antibody. A whole cell lysate (WCL) from un-transfected cells was included as a control. (B) A549 cells were infected with SM or SPM lentivirus, followed by transfection with BACH1 siRNA. Cell lysates were immunoblotted with anti-BACH1 or anti-BRCA1 antibodies. An unrelated siRNA was used as a negative control.

**Fig. S3.** Time course of BACH1 dependent G2-M checkpoint. A549 cells were transfected with BACH1 siRNA. G2-M checkpoint assays were performed after treatment with nocodazole as indicated.

**Fig. S4.** Isothermal titration for the interaction of BRCA1-BRCT with BACH1 phosphorylated peptide. Raw titration data plotted as microcalories per second versus time (minutes). The titration consisted of 57 injections of a 200 mM solution of phosphopeptide into a 1.9 ml starting solution of BRCA1-BRCT at an initial
concentration of 17 μM. Integrated heat measurement from raw titration data corrected for the dilution of BACH1 phosphopeptide into buffer. The data is plotted as kcal·mol⁻¹ of phosphopeptide injected into BRCA1-BRCT solution versus the molar ratio of phosphopeptide to BACH1-BRCT.

**Fig. S5.** Binding of Top BP1 BRCT-6 and Fcp1 BRCT domain to phospho-E2F1 and phospho-RNA pol II respectively. (A) 293T cell lysates were incubated with beads bound to either GST-TopBP1 BRCT-6, TopBP1 BRCT-6 W886R mutant, Fcp1-BRCT or Fcp1-BRCT W710R mutant as indicated. Proteins bound to the beads were eluted and blotted with anti-E2F1 or anti-RNA pol II antibodies (upper panels). The protein loading controls were shown in the middle and lower panels. (B) 293T cell lysates were incubated with or without λ ppase, followed by incubation with GST-TopBP1-BRCT-6 or GST-Fcp1-BRCT proteins immobilized on beads. Western blots were performed as described in (A). (C) 200 ng of GST fusion proteins were incubated with 20 ng of biotinylated phosphopeptides (biotin-RLLDSpSQIVI for TopBP1 BRCT#6; biotin-YpSPTpSPSYpSPTpSPS for Fcp1) or control unphosphorylated peptides. Peptide-protein complexes were retrieved and immunoblotted with anti-GST antibodies. The loading controls for GST fusion proteins are shown in lower panels.

**Fig. S6.** Preferential binding of BRCT domains to a phospho-peptide library. (A) Phospho-peptide library [ISRST(p)SXXXNKQTK-biotin, where X is varied as any amino acid] or unphosphorylated peptide library (ISRSTSPXXXNKQTK-biotin) were immobilized on biacore chip surfaces. 1 μM GST BARD1-BRCT, GST-53BP1-BRCT
domains and their mutants were passed over the chip surfaces. Resonance units (RU) were measured by BIAcore 3000. (B) 1 µM of each of the eight additional tandem GST-fused BRCT domains and (C) three single BRCT domains were used to examine their abilities to bind the phosphorylated versus unphosphorylated peptide libraries.
Figure S1
Figure S2

A

IP: anti-V5

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Blot: anti-BACH1

B

Con siRNA | BACH1 siRNA | SM+siRNA | SPM+siRNA

Blot: anti-BACH1

Blot: anti-BRCA1
Figure S3

Time course of Nocodazole treatment

PH3 positive fractions % of control

Control siRNA
BACH1 siRNA

14hr 16hr 18hr 20hr 22hr 24hr
Figure S5

A

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C

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Figure S6

A

BARD1-BRCT

53BP1-BRCT

BARD1-BRCT-W635R

53BP1-BRCT-W1830R

BARD1-BRCT-W762R

53BP1-BRCT-W1946R
Figure S6

C

- TDT-BRCT
  - RU
  - S: 0, pS: 200

- s.c. REV1-BRCT
  - RU
  - S: 0, pS: 180

- DNA ligase III-BRCT
  - RU
  - S: 30, pS: 180