

## **Supplemental Methods and Figures**

### **Methods**

#### **RNAi treatment and overexpression**

The siRNA oligonucleotides targeting mouse Bid (SI00929103) were purchased from Qiagen Inc. Mouse Bid siRNA target sequence is CACAGAAGATTCCATATCAAA. For MPCs,  $10 \times 10^6$  cells was washed once with PBS, resuspended in 100  $\mu$ l Mouse ES Cell Nucleofector Solution (Amaxa) containing 100 pmol siRNA, and transfected twice by Nucleofector Program A-30. After 72 hrs transfection, the transfected MPCs were treated as indicated in the figure legends.

#### **Cell cycle analysis**

BrdU incorporation analysis was performed according to BrdU Flow Kits (BD Pharmingen). Briefly, MPCs ( $0.5-1 \times 10^6$  cells/ml) and U2OS cells (20-40% confluence) were pulse labeled with 10  $\mu$ M BrdU for 45 min, and 2 hrs, respectively. Cells were fixed and permeabilized with BD Cytotfix/Cytoperm Buffer, and incubated with BD Cytoperm Plus Buffer followed by an additional short fixation with BD Cytotfix/Cytoperm Buffer. The incorporated BrdU was exposed by treatment with 30  $\mu$ g DNase for 1 hr at 37°C and probed with FITC-conjugated anti-BrdU antibody (BD Pharmingen) for 20 mins at room temperature. The total DNA content was stained immediately prior to flow cytometric analysis with 20  $\mu$ l of 7-aminoacridine (7-AAD) solution (BD Pharmingen).

#### **Intracellular staining**

Bid +/+ and/or Bid -/- MPCs were fixed and permeabilized with BD Cytotfix/Cytoperm Buffer, and incubated with BD Cytoperm Plus Buffer followed by an additional short fixation with BD Cytotfix/Cytoperm Buffer. For pChk1 staining, cells were stained with anti-pChk1 (S345) antibody (Cell signaling, #2348) and then Alexa Fluor 488 conjugated Goat anti-Rabbit IgG antibody (Invitrogen). DNA was stained by 7-AAD. Then cells were analyzed by flow cytometry.

**Quantitative Real-time PCR** The total RNA of  $10 \times 10^6$  cells were extracted and purified by

Trizol (Invitrogen) and 5 µgs of RNA were subjected to reverse-transcription (Invitrogen) to obtain cDNA. Quantitative RT-PCR was performed (SYBR Green Jumpstart Taq Ready-Mix (Sigma)) on the iQ5 Real-Time PCR Detection System (Bio-Rad). Sequences of the primers that were used: *actin* (5'-GGCTGTATCCCCTCCATCG-3', 5'-CCAGTTGGTAACAATGCCATGT-3'), *Chk1* (5'-GTTAAGCCACGAGAATGTAGTGA-3', 5'-GATACTGGATATGGCCTTCCCT-3'), *chk2* (5'-CTCGGCTATGGGCTCTTCAG, 5'-CTTCTCAACAGTGGTCCATCG-3'), *cdc25A* (5'- ACAGCAGTCTACAGAGAATGGG-3', 5'-GATGAGGTGAAAGGTGTCTTGG-3'), *p21* (5'-CCTGGTGATGTCCGACCTG-3', 5'-CCATGAGCGCATCGCAATC-3'), and *Noxa* (5'-GCAGAGCTACCACCTGAGTTC-3', 5'-CTTTTGC GACTTCCCAGGCA-3').

### **Antibodies**

The following antibodies were used in the supplemental figures. anti-phospho-Atm (S1981) monoclonal antibody (Cell signaling, #4526), anti-Atm monoclonal antibody (Cell signaling, #2873), anti-Mre11 polyclonal antibody (Millipore, 07-638), anti-Rad9 monoclonal antibody (Santa Cruz, M-389).

## Figures

### Figure S1. Cell cycle reentry is limited in Bid-deficient cells.

(A) The quantitative analysis of the arrested G1/early S phase cells following HU withdrawal in Fig 1C. Data were collected from three independent experiments. \*,  $p < 0.05$ .

(B) Hydroxyurea treatment does not result in a significant increase of cell death in U2OS cells. U2OS cells transfected with control siRNA or Bid siRNA for 72 hrs were treated with 10 mM hydroxyurea overnight. Then, cells were released to fresh medium containing nocodazole. Cells were fixed and DNA content was detected by 7-AAD. Quantitative analysis of the percentage of cell with less than 2N DNA content was obtained from three independent experiments. Error bar=90% confidence interval.

### Figure S2. Expression of Bid results in neither Atr activation in an *in vitro* system nor significant changes of cell death and sensor levels in U2OS cells.

(A) Bid does not facilitate TopBP1 activation of Atr *in vitro*. HA-Atrip and myc-Atr were overexpressed in 293T cells, and Atr/Atrip was isolated from whole cell extracts by immunoprecipitation with anti-HA conjugated agarose beads. Immunoprecipitated Atr/Atrip was incubated with the activating domain of TopBP 1 (TopBP1 AAD), the Atr substrate MCM2 peptide, and  $\gamma$ -<sup>32</sup>P-ATP. Reactions were separated on SDS-PAGE, stained with Coomassie Blue, and exposed to film. Duplicate samples were separated on SDS-PAGE, and immuno-blotted with anti-Atr and anti-Atrip.

(B) The protein levels of various DNA damage sensors are not significantly changed in Bid-deficient U2OS cells. U2OS cells were transfected with control siRNA or Bid siRNA for 72 hrs. Then the control knockdown and Bid knockdown cells were treated with 10 mM hydroxyurea for 2 hrs and various DNA damage sensors in Atm/Atr pathways were detected by immunoblots.

(C) Bid +/+ and Bid -/- MPCs were treated with 10 mM hydroxyurea for 2 hrs and various DNA damage sensors in Atr pathways were detected by immunoblots.

(D) TNF $\alpha$ /CHX treatment but not hydroxyurea treatment significantly induces apoptosis in U2OS cells. U2OS cells were treated with 10 mM hydroxyurea or 10 ng/ml TNF $\alpha$ +25  $\mu$ g/ml CHX for 5hrs. Cells were fixed and stained with anti-activated caspase 3 (Asp175) polyclonal antibody (Cell signaling, #9661). DNA was stained by Hoechst.

(E) Expression of wild-type or mutated Bid does not cause significant apoptosis in U2OS cells. U2OS cells transfected with control siRNA, Bid siRNA, wild-type or Helix 4 mutated Bid were treated with 10 mM hydroxyurea for 5 hrs. No obvious apoptotic cells were detected by activated caspase 3. DNA was stained by Hoechst.

**Figure S3. Chk1 phosphorylation is diminished in Bid  $-/-$  MPCs following DNA damage treatments.**

(A) Phosphorylated mChk1 present as a shifted band in immunoblots. Bid $+/+$  MPCs were treated with 10 mM hydroxyurea for 2 hrs. Cells were lysed in RIPA buffer with or without phosphatase inhibitors. The indicated protein phosphatases (PP1, protein phosphatase type; PP2A, protein phosphatase types 2A; CIAP, calf intestine alkaline phosphatase) were added to 400  $\mu$ g cell lysate and incubated at 30 $^{\circ}$ C for 30 minutes. Protein extracts were resolved by SDS-PAGE and immunoblotted with anti-Chk1 antibody. The solid arrow denotes the mobility of the shifted band corresponding to phosphorylated Chk1, and the dashed arrow denotes the mobility of the unshifted band corresponding to Chk1.

(B) Bid  $+/+$  and Bid  $-/-$  MPCs were treated 25  $\mu$ M etoposide for the indicated times. Total cell lysate was evaluated by SDS-PAGE followed by immunoblotting with the indicated antibodies. The quantitative analysis of the ratio of pChk1 to total Chk1 from three independent experiments.

(C) The quantitative analysis of the ratio of pChk1 to total Chk1 in Fig 2B from three independent experiments.

(D) Bid  $+/+$  MPCs were treated with Bid-specific siRNA or control siRNA using nucleofectin and incubated for 72 hours. Bid knockdown and control knockdown cells were treated with 10

mM hydroxyurea for 2 hrs, and total cell lysate was resolved by SDS-PAGE followed by immunoblotting with the indicated antibodies. The quantitative analysis of the ratio of pChk1 to total Chk1 from three independent experiments. \*,  $p < 0.05$ .

(E) U2OS cells were treated with 10 mM hydroxyurea for 2 hrs. Then, Chk1 was immunoprecipitated from total cell lysate. Chk1 and phosphorylated Chk1 were detected in immunoblots. The asterisk indicates the IgG heavy chain band.

(F) U2OS cells were transfected with control siRNA or *Bid* siRNA for 72 hrs and then treated with 10 mM hydroxyurea for 2 hrs. Then, Chk1 was immunoprecipitated from total cell lysate. The immunoprecipitated Chk1 and phosphorylated Chk1 were detected in immunoblots. The exposure time of anti-pS317/Chk1 was increased from upper to lower panel.

**Figure S4. p53 phosphorylation is diminished in Bid  $-/-$  MPCs following DNA damage treatments.**

(A) The quantitative analysis of the ratio of pS15/p53 to total p53 in Fig 2E.

(B) The transcription level of p53-target genes is diminished in Bid  $-/-$  MPCs following replicative stress. Bid  $+/+$  and Bid  $-/-$  MPCs were treated 10 mM hydroxyurea for 3 hours. , total RNA was extracted and real-time PCR performed to detect the relative transcription levels of *p21* and *Noxa*. *Actin* was used as the reference. (The bars indicate the s.d., N=3)

(C) Atm auto-phosphorylation is unaffected in the absence of Bid following etoposide treatment. U2OS cells were treated with Bid-specific siRNA No.7, Bid-specific siRNA No.8 or control siRNA for 72 hrs. Bid knockdown and control knockdown cells were treated with 10 mM hydroxyurea or 25  $\mu$ M etoposide for 2 hrs, and total cell lysate was resolved by SDS-PAGE followed by immunoblot with the indicated antibodies.

(D) U2OS cells were transfected with control siRNA, Bid siRNA and/or Atm siRNA for 72 hrs. Then cells were treated with 10 mM hydroxyurea for 2 hrs and Chk1 phosphorylation was detected by immunoblots.

### **Figure S5. Chk1 phosphorylation is diminished in Bid<sup>-/-</sup> MPCs by flow cytometry**

(A) The cell cycle profile is similar between Bid<sup>+/+</sup> and Bid<sup>-/-</sup> MPCs. Bid<sup>+/+</sup> and Bid<sup>-/-</sup> MPCs were labeled with BrdU for 45 min. Then cells were fixed and permeabilized with BD Cytotfix/Cytoperm Buffer. BrdU was detected by intracellular staining with FITC-conjugated anti-BrdU antibody (BD Pharmingen). DNA was stained by 7-AAD. Then cells were analyzed by flow cytometry. Quantitative analysis of the cell cycle data was obtained from eight independent experiments. Error bar=90% confidence interval. *p* value is calculated by student's t-test.

(B) U2OS cells transfected with control siRNA or Bid siRNA were labeled with BrdU for 2 hrs and analyzed by flow cytometry. Quantitative analysis of the cell cycle data was obtained from six independent experiments. Error bar=90% confidence interval. *p* value is calculated by student's t-test.

(C) Bid<sup>+/+</sup> and Bid<sup>-/-</sup> MPCs were treated with 10 mM hydroxyurea for 2 hrs. Then cells were fixed and permeabilized with BD Cytotfix/Cytoperm Buffer. Chk1 phosphorylation was detected by intracellular staining with anti-pChk1 (S345) antibody (Cell signaling, #2348) and Alexa Fluor 488 conjugated Goat anti-Rabbit IgG antibody (Invitrogen). DNA was stained by 7-AAD. pChk1-positive cells were gated as black rectangle.

(D) pChk1-positive cells were gated based on the hydroxyurea-induced population. The peak of pChk1-positive Bid<sup>+/+</sup> and Bid<sup>-/-</sup> cells was shown as yellow and red dash line, respectively.

(E) Quantitative analysis of the percentage of pChk1-positive cells from three independent experiments. Error bar, S.E.M. *p* value is calculated by student's t-test. \*\*, *p*<0.01.

(F) Gating on pChk1-positive cells, Bid<sup>+/+</sup> MPCs significantly increased pChk1-positive population following hydroxyurea treatment.

(G) Quantitative analysis of the relative mean fluorescence intensity (MFI) increase of pChk1 signal by hydroxyurea in pChk1-positive cells from three independent experiments. The HU-induced MFI increase of pChk1 signal from pChk1-positive Bid<sup>+/+</sup> MPCs was set as 100 arbitrarily. Error bar, S.E.M. *p* value is calculated by student's t-test. \*, *p*<0.05.

(H) Chk1 phosphorylation is diminished in Bid  $-/-$  MPCs with high DNA content. Bid  $+/+$  and Bid  $-/-$  MPCs were treated with 10 mM hydroxyurea for 2 hrs. Then cells were fixed and permeabilized with BD Cytofix/Cytoperm Buffer. Chk1 phosphorylation was detected by intracellular staining with anti-pChk1 (S345) antibody (Cell signaling, #2348) and Alexa Fluor 488 conjugated Goat anti-Rabbit IgG antibody (Invitrogen). DNA was stained by 7-AAD. Late S/G2/M phase cells (high DNA content) were gated. pChk1-positive cells were gated as black rectangle.

(I) pChk1-positive cells were gated based on the hydroxyurea-induced population. The peak of pChk1-positive Bid  $+/+$  and Bid  $-/-$  cells was shown as blue and yellow dash line, respectively.

(J) Quantitative analysis of the percentage of pChk1-positive cells in late S/G2/M phase cells (high DNA content) following hydroxyurea treatments from three independent experiments. Error bar, S.E.M.  $p$  value is calculated by student's t-test. \*\*,  $p < 0.01$ .

(K) Quantitative analysis of the relative mean fluorescence intensity (MFI) of pChk1 signal in late S/G2/M phase cells (high DNA content) following hydroxyurea treatments from three independent experiments. The MFI of pChk1 signal from Bid  $+/+$  MPCs was set as 100 arbitrarily. Error bar, S.E.M.  $p$  value is calculated by student's t-test. \*,  $p < 0.05$ .

**Figure S6. Bid interacts with RPA *in vitro*.**

(A) Purified Bid and RPA was incubated in the binding buffer (20 mM HEPES, 100 mM KCl, 5 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 0.1% NP40, pH7.4) at room temperature for 30 min. Then, Bid was immunoprecipitated using biotin-conjugated anti-Bid antibody and streptavidin-agarose beads. Samples were analyzed using SDS-PAGE followed by immunoblotting with the indicated antibodies.

(B) Individual staining of RPA and Bid in Fig 3F.

(C) U2OS cells overexpressing HA-tagged wild-type or Helix 4 mutated hBid was transfected with Bid siRNA for 72 hrs. Silent mutations were introduced in the Bid siRNA-target region so that only endogenous Bid was knocked down by Bid siRNA. Then, cells were treated with

hydroxyurea for 24 hrs and released into fresh media for 8 hrs. The apoptotic cells were detected by Annexin V-FITC Apoptosis Detection Kit (BioVision).

(D) RPA32 foci are diminished in the absence of Bid following replicative stress. U2OS cells were transfected with control siRNA or Bid siRNA for 72 hrs and then treated with 10 mM hydroxyurea for 4 hrs. After soluble proteins were pre-extracted by cytoskeleton buffer containing 0.5% Triton X-100, the cells were fixed and stained with anti-RPA32 antibody.

(E) The cells containing more than 5 visible RPA foci were counted from 5 independent experiments. \*,  $p < 0.05$ .



Figure S1

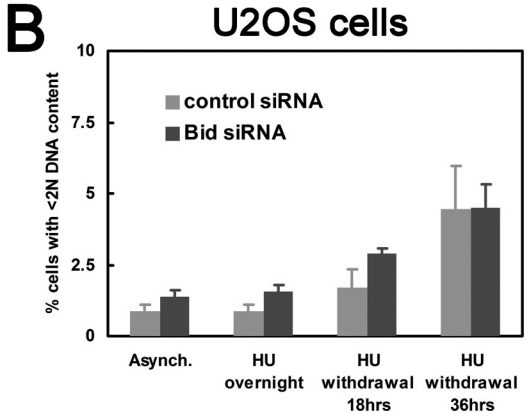
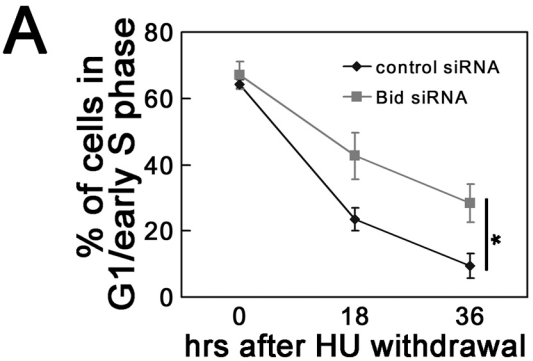


Figure S2

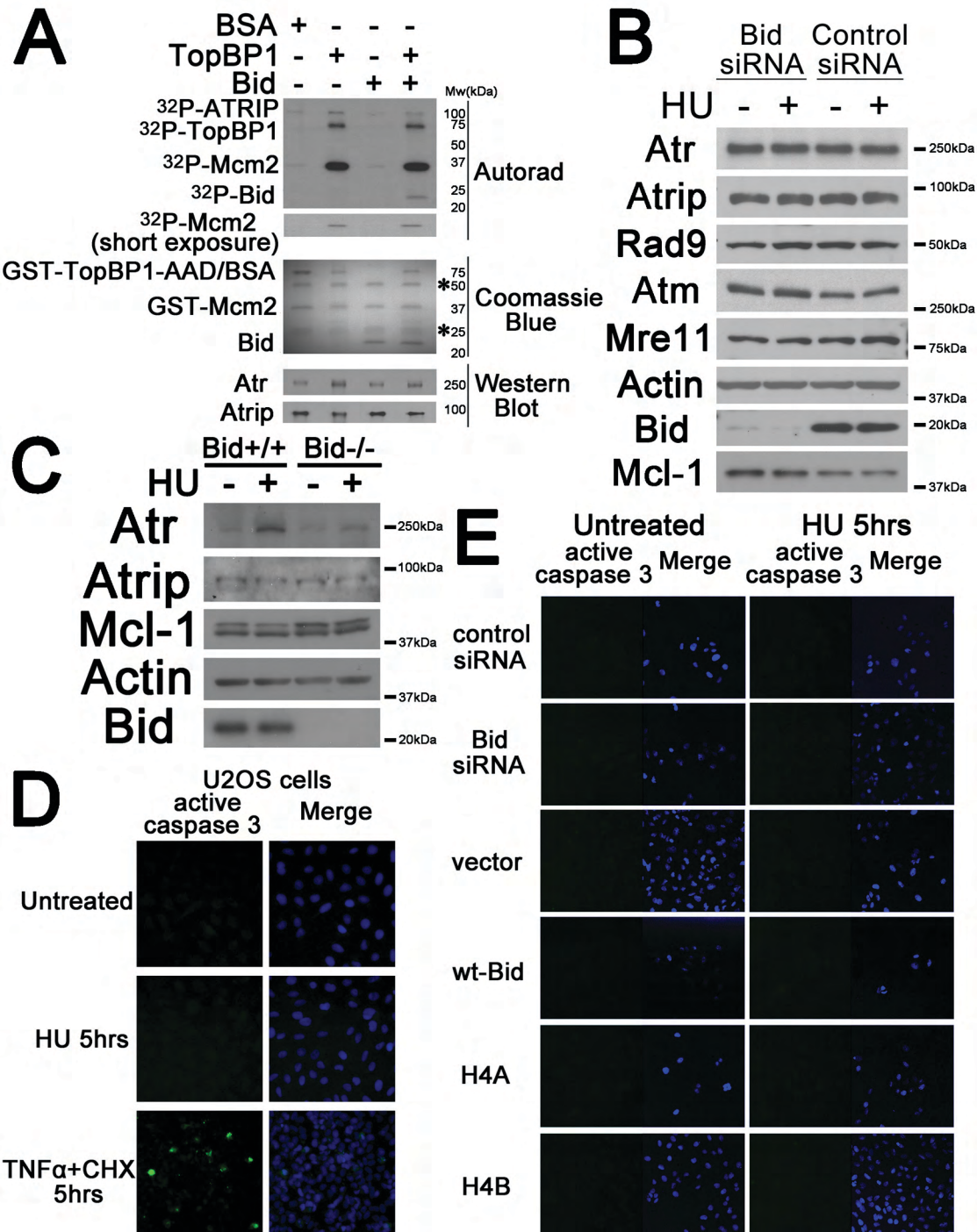


Figure S3

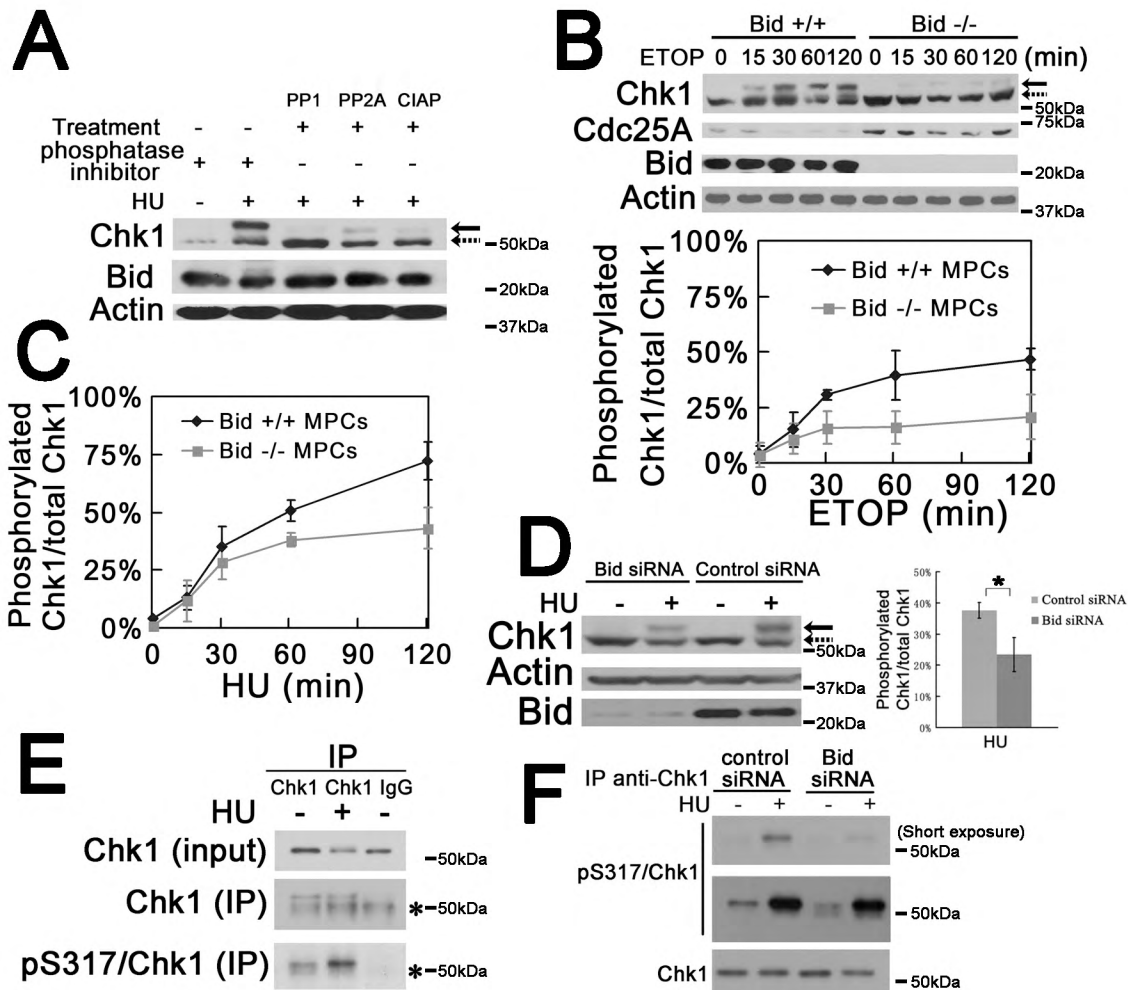


Figure S4

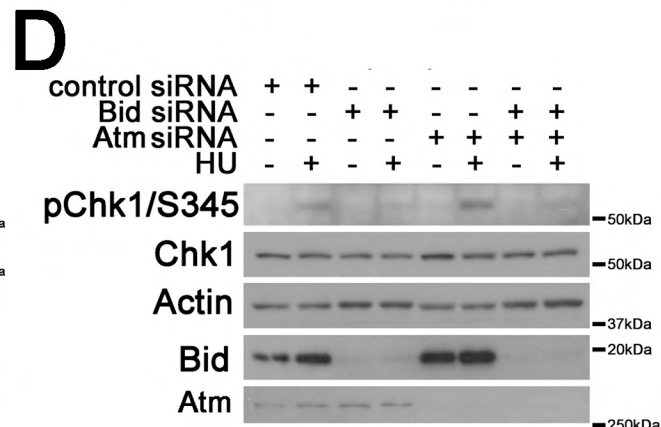
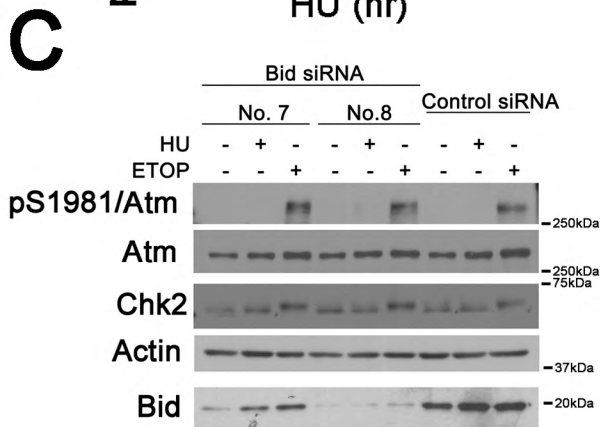
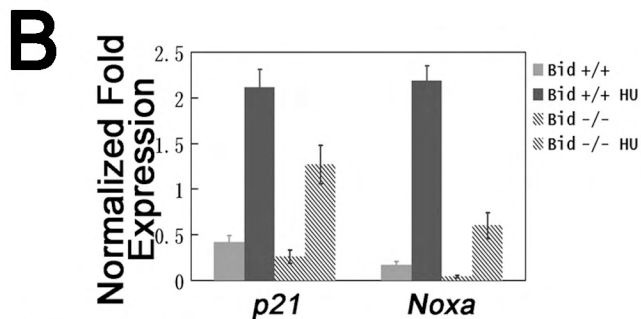
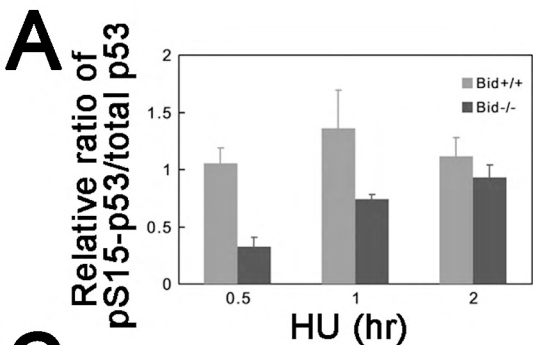


Figure S5

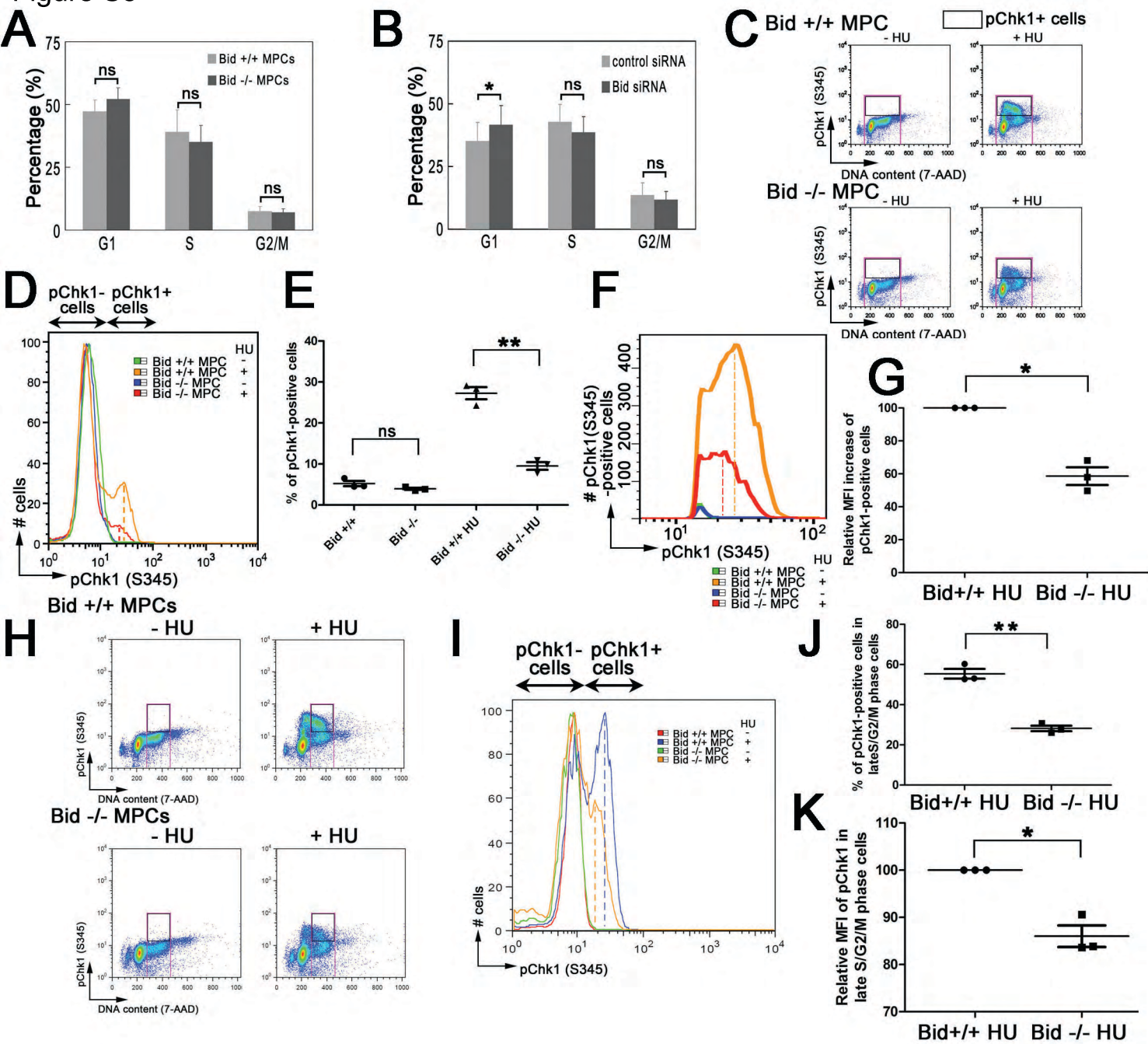


Figure S6

