Supporting Information

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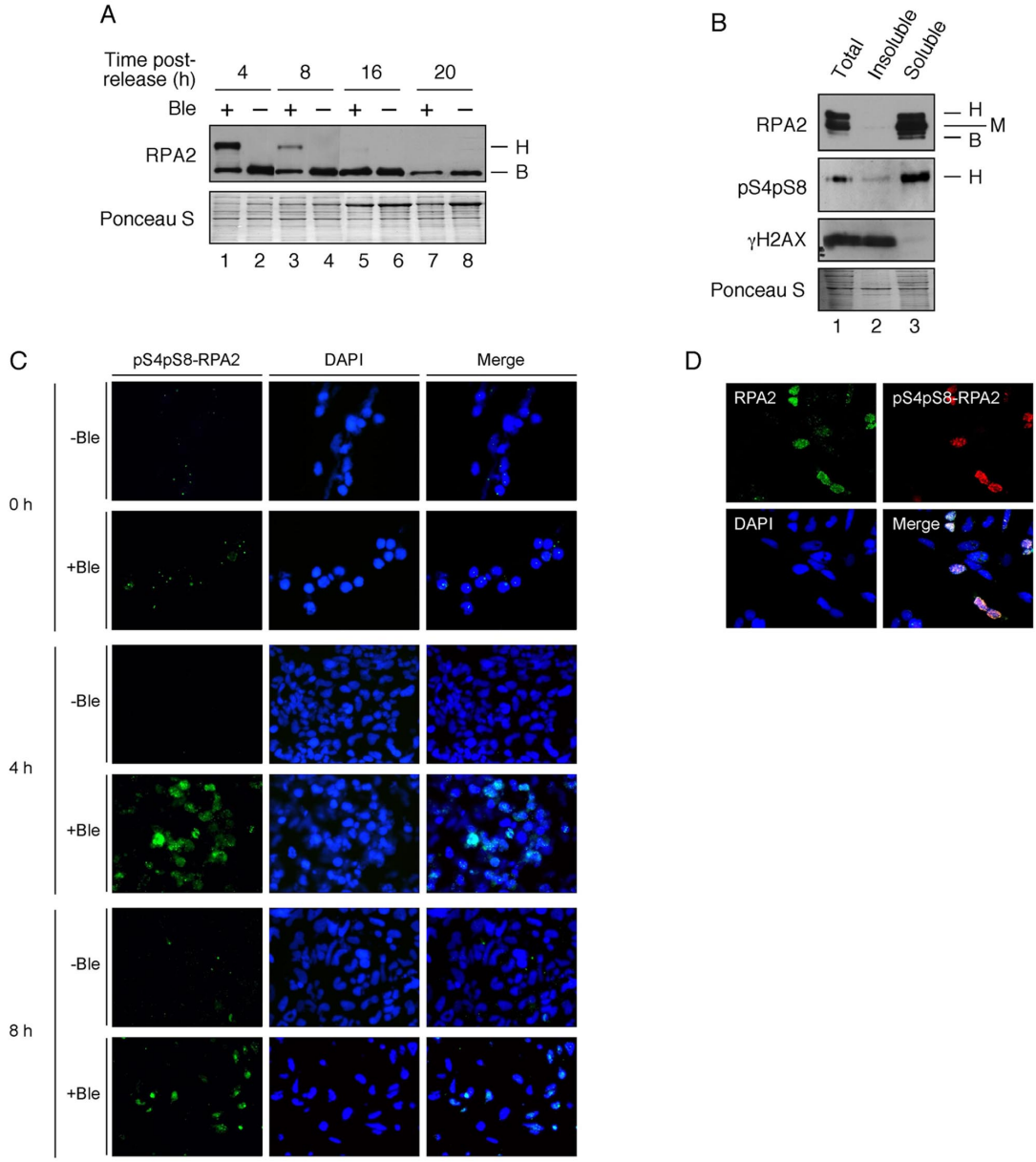

Fig. S1. Pattern of RPA2 hyperphosphorylation following mitotic DNA damage. (*A*) Mitotically arrested U2-OS cells were treated with Ble (2 μg/ml for 1 h) and then were released into medium lacking nocodazole and Ble. Lysates were prepared from these cells at the indicated times, and the lysates were analyzed by Western blot for total RPA. (*B*) Hyperphosphorylated RPA2 is mostly soluble in mitotic cells subjected to DNA damage. Mitotically arrested cells were treated with 2 μg/ml Ble for 1 h in the presence of nocodazole, followed by incubation of cells in CSK buffer containing 0.5% (vol/vol) Triton X-100 for 5 min on ice. These permeabilized cells were centrifuged at 4°C at 800 $\times$ *g* for 5 min in a bench-top centrifuge. The supernatant was collected as the soluble fraction. The pellet was washed once with CSK buffer, yielding the insoluble fraction. The 2 fractions, as well as an aliquot of the total cellular lysate, were subjected to Western blot analysis with the indicated antibodies. For *A* and *B*, RPA2 migration position is indicated as follows: B = basal (nonphosphorylated); H = hyperphosphorylated; M = mitotic. Protein loading is indicated using an image of a portion of the Ponceau S–stained transfer membrane. (*C*) Association of RPA2 with chromatin following release from mitotic DNA damage. Mitotically arrested cells were mock treated or treated with Ble (2 μg/ml for 1 h) followed by release into medium devoid of nocodazole and Ble. Samples were extracted and fixed as described by Vassin, *et al.* (18), were stained for DAPI and with an antibody against pS4pS8-RPA2, and then were imaged by immunofluorescence microscopy. (*D*) Colocalization of total RPA2 and pS4pS8-RPA2. Samples prepared as in *C* were analyzed with the indicated antibodies to confirm colocalization and authenticity of the antibodies used.

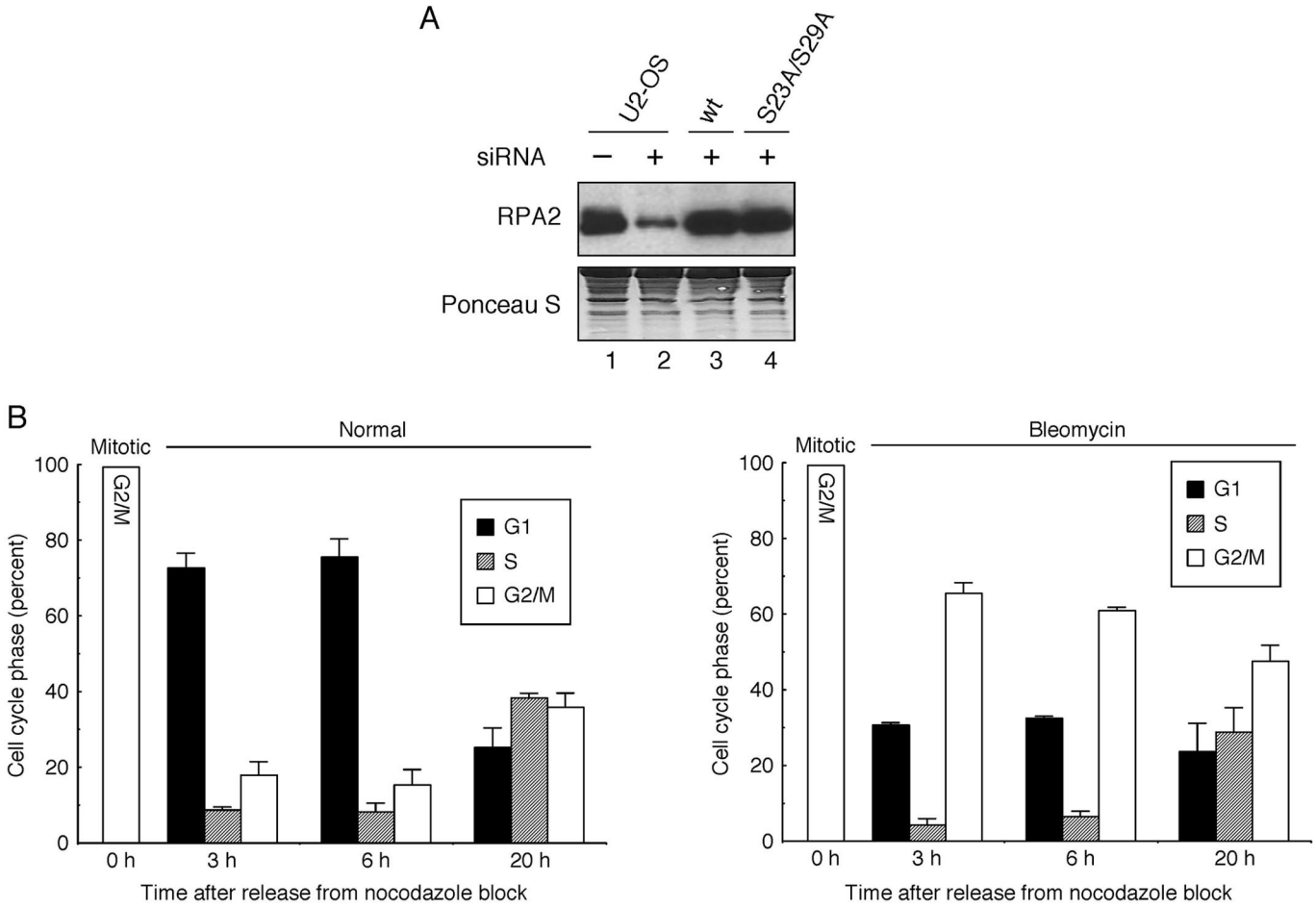

Fig. S2. Effect of mitotic DNA damage on cell-cycle progression. (*A*) RPA2 levels in silenced and replaced U2-OS cells. The parental U2-OS cell line (lanes 1 and 2) and representative U2-OS clones that support inducible expression of ectopic WT-RPA2 (lane 3) or S23A/S29A-RPA2 (lane 4) were tested. To induce ectopic RPA2 expression, clones were incubated in the absence of doxycycline for 48 h before silencing. The U2-OS and clonal cell lines then were subjected to siRNA-mediated silencing of RPA2 for 72 h (lanes 2–4). Lysates were prepared and subjected to Western blot analysis to detect RPA2. Nonsilenced U2-OS cells were used as a control for determination of silencing efficiency and the level of ectopic RPA2 expression (lane 1). The Ponceau S–stained transfer membrane is shown as a protein-loading control. (*B*) The parental U2-OS cell line was treated with nocodazole for 12 h, and the mitotic cells were isolated by mechanical shake-off. The mitotic cells were mock treated or treated with Ble (2 μg/ml Ble for 1 h) and then were released into medium lacking nocodazole and Ble. Cells were collected 3, 6, and 20 h after release and were analyzed for DNA content by FACS.

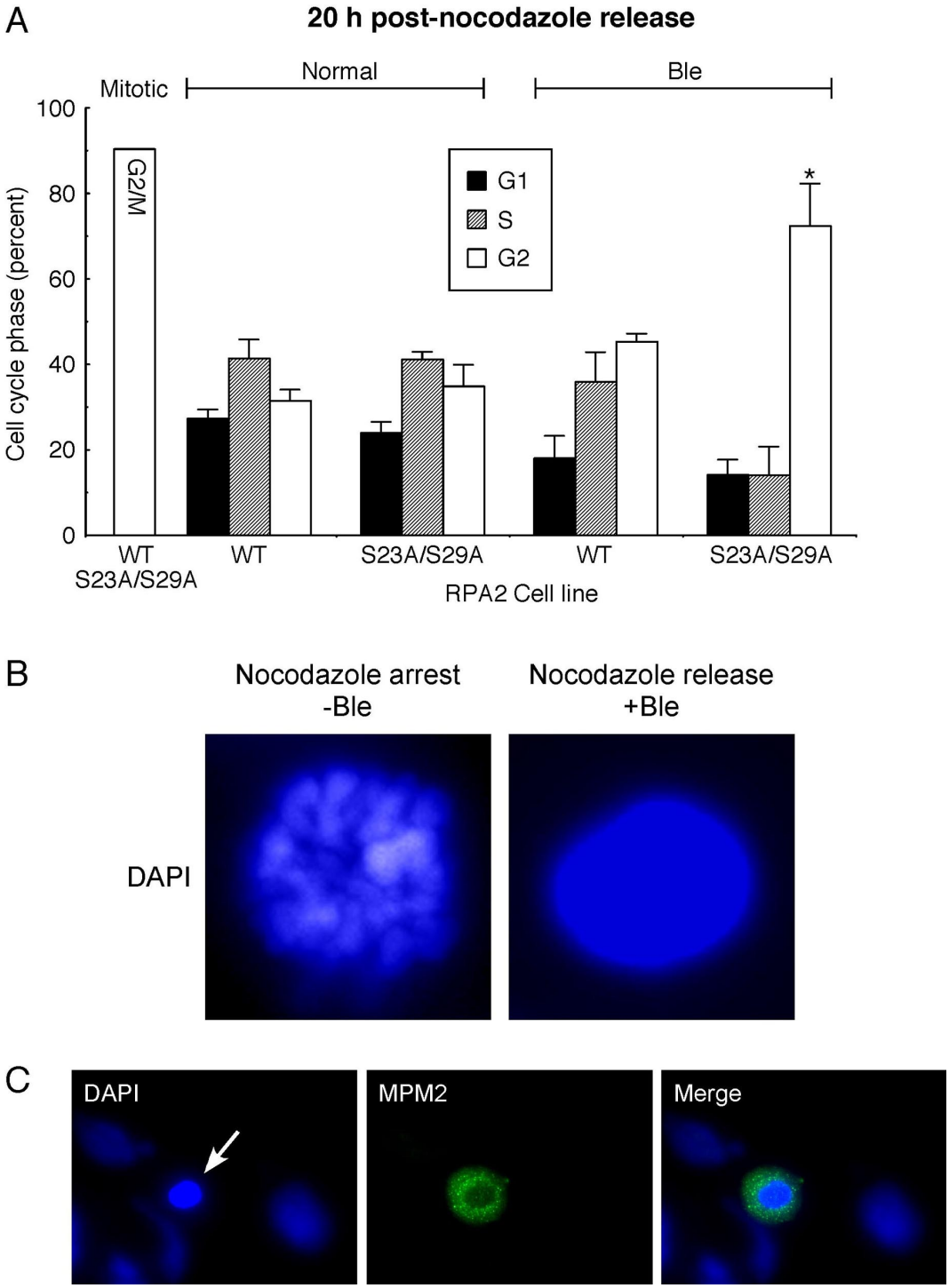

Fig. S3. Analysis of cells after release from mitotic DNA damage. (*A*) Cells obtained as described as in Fig. 2 *A* were analyzed by FACS for DNA content 20 h after release. (*B* and *C*) Cells released from mitotic DNA damage often have positive staining for the mitotic marker MPM2 and DNA that has a globular appearance. (*B*, *Left*) Normal prometaphase in a nocodazole-arrested cell. (*B*, *Right*) A Ble-treated mitotic cell 3 h after release from nocodazole and Ble. Such cells appear to have semi-decondensed DNA with a globular appearance. (*C*) Cells showing semi-decondensed DNA often are MPM2 positive after release from a damaged mitosis. Mitotic U2-OS cells were treated with Ble (2 μg/ml for 1 h) and then were released into fresh medium lacking nocodazole and Ble. Three hours after release, cells were fixed and stained with DAPI and anti-MPM2 antibodies and were imaged by immunofluorescence microscopy. Arrow indicates a DAPI-stained nucleus with an appearance similar to that shown in *B* (*Right*) and which stains positive for MPM2.

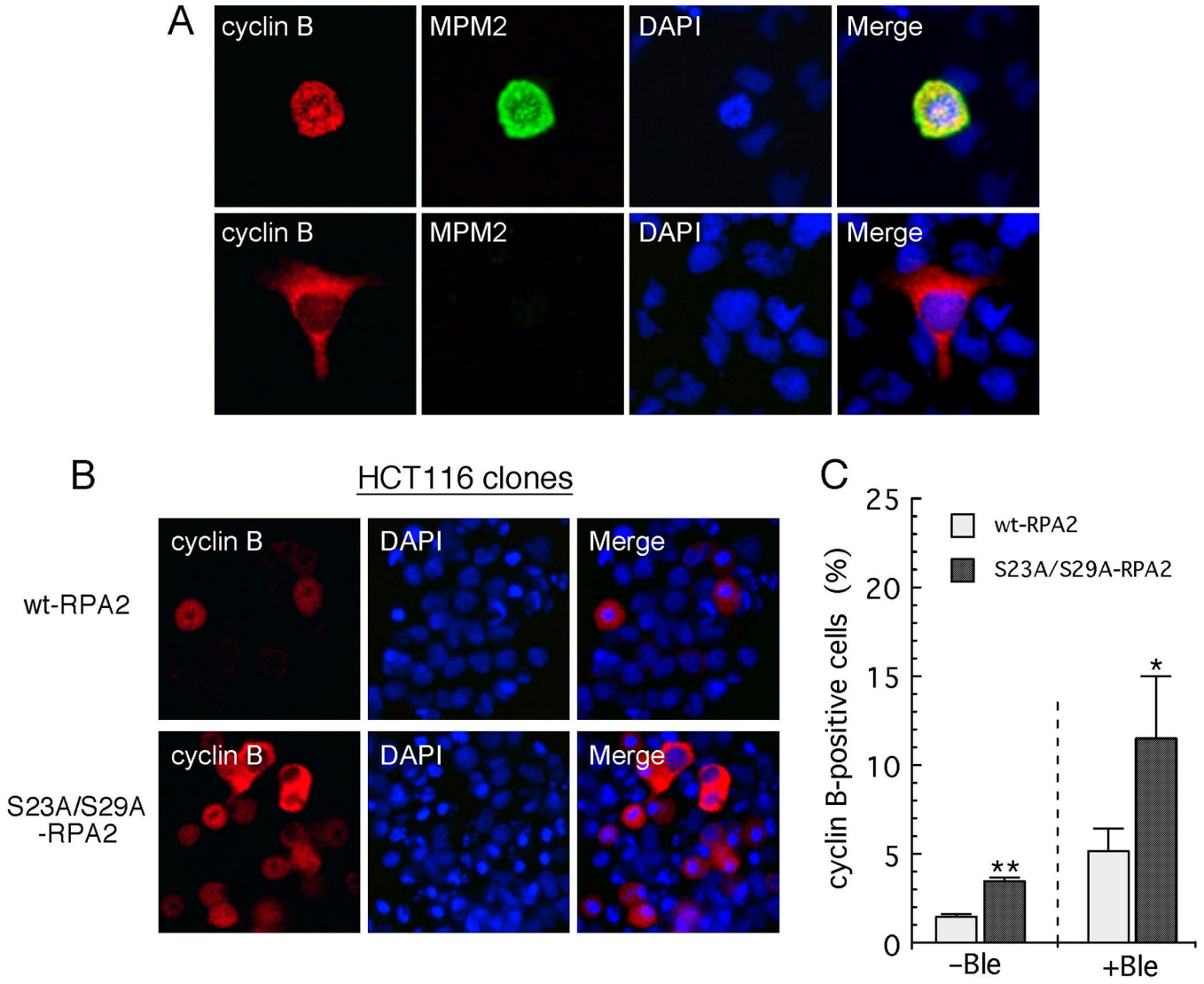

Fig. S4. Pattern of cyclin B–positive cells on release from mitotic DNA damage. (*A*) Cells released from a damaged mitosis often stain positive for both MPM2 and cyclin B (*Upper*). Cells also appear to have an interphase-like G2 state with flat nuclear morphology that stains positive for cyclin B and negative for MPM2 (*Lower*). (*B*) Effects of the S23A/S29A-RPA2 mutation also are seen in a different cell background. Mitotic HCT116 clones silenced for endogenous RPA2 and ectopically expressing mutant or WT-RPA2 were treated or untreated with 2 μg/ml Ble for 1 h in the presence of nocodazole followed by release into medium devoid of any drug. Three hours after release, cells were fixed and stained for cyclin B to determine the number of cells in G2 or mitosis. (*C*) Quantitation of cyclin B–positive cells imaged as explained in *B*.

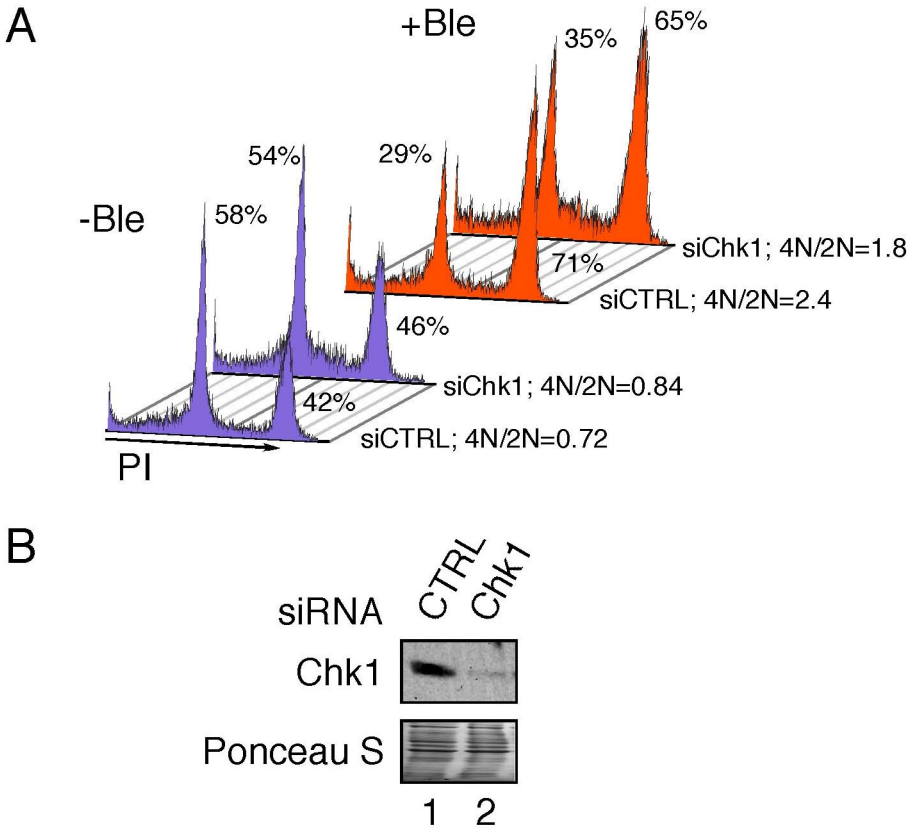

Fig. S5. Silencing of Chk1 leads to a decrease in cells arrested with a 4N DNA content on release from mitotic DNA damage. (*A*) Cells treated with Chk1 or control (*CTRL*) siRNAs were nocodazole arrested (12 h), and the mitotic cells were collected by shake-off. These mitotic cells then were mock treated or treated with Ble (0.05 μg/ml) for 1 h. DNA content was analyzed by FACS 3 after release. (*B*) Western blot analysis demonstrating silencing of Chk1.

Table S1. Quantitation of DNA synthesis in U2-OS cells following mitotic DNA damage

Time after nocodazole release (h)	Bleomycin treatment	Fraction of BrdU-positive cells (%)
4	−	3.3 ± 2.8
4	+	1.2 ± 0.3
20	−	24.9 ± 0.2
20	+	14.7 ± 0.6

Mitotic U2-OS cells were isolated and treated as described in the legend for Fig. S2. Cells then were released into medium lacking nocodazole and bleomycin. At 4 or 20 h after release, cells were incubated for 1 h with 10 μM bromodeoxyuridine (BrdU) (BD Biosciences) and then were treated for FACS as described in the manufacturer's protocol with the following exceptions: BrdU incorporation was detected using a rat anti-BrdU antibody (Harlan Sera-Lab) in combination with a FITC-conjugated anti-rat antibody. The fraction of BrdU-positive cells was determined by FACS.

Table S2. Quantitation of mitotic cells after release from a mitosis containing DNA damage

Time after nocodazole release (h)	Bleomycin treatment	Fraction of MPM2-positive cells (%)
0	−	84.2 ± 3.4
3	−	0.3 ± 0.1
3	+	5.2 ± 0.7
6	−	0.1 ± 0.2
6	+	1.0 ± 0.6
20	−	0.6 ± 0.2
20	+	0.5 ± 0.4

Mitotic U2-OS cells were isolated and treated as described in the legend of Fig. S2. The fraction of MPM2-positive cells was determined as described in *Materials and Methods*.