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Supplemental Material to:

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Chk1-Mad2 interaction: A crosslink between the DNA damage checkpoint and the mitotic spindle checkpoint

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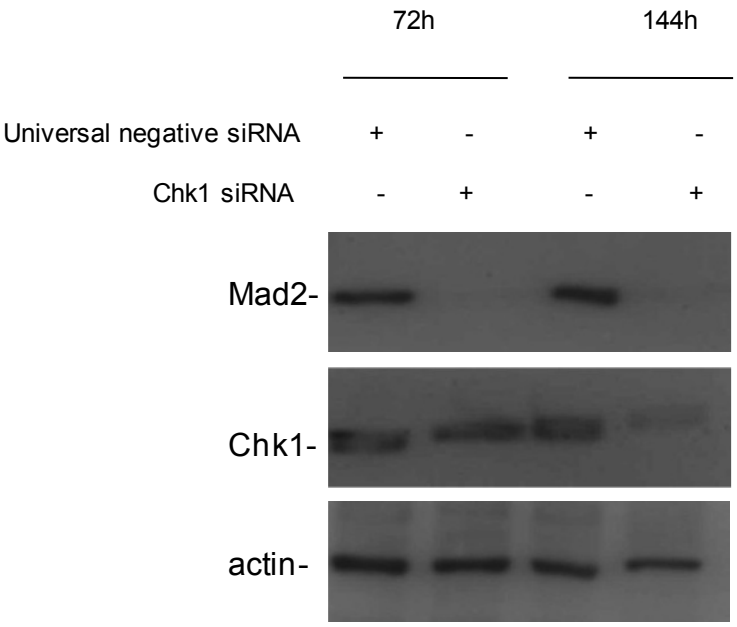

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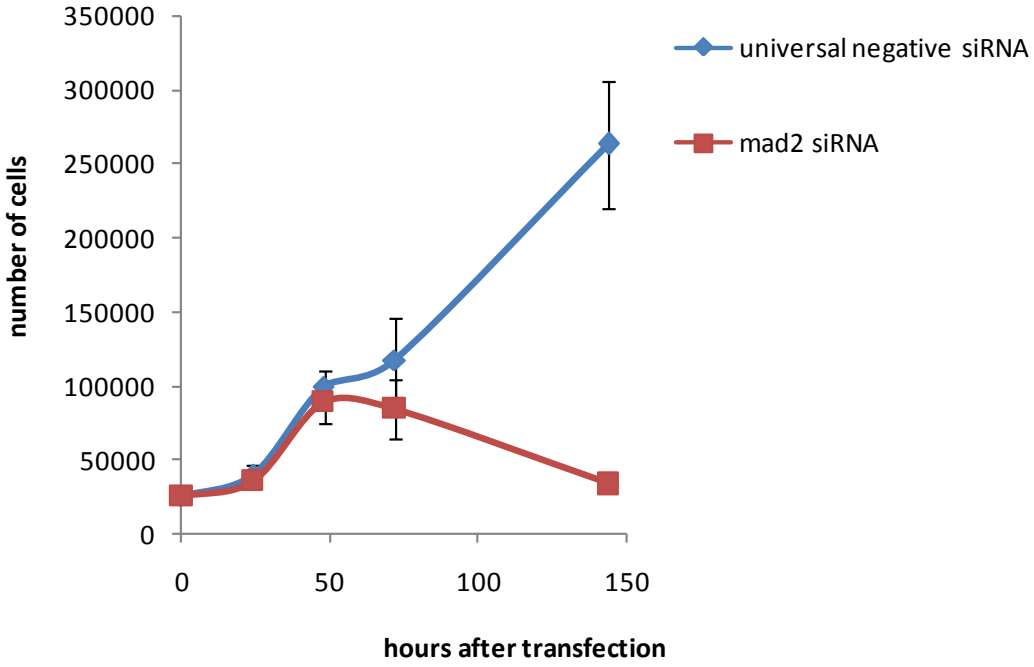
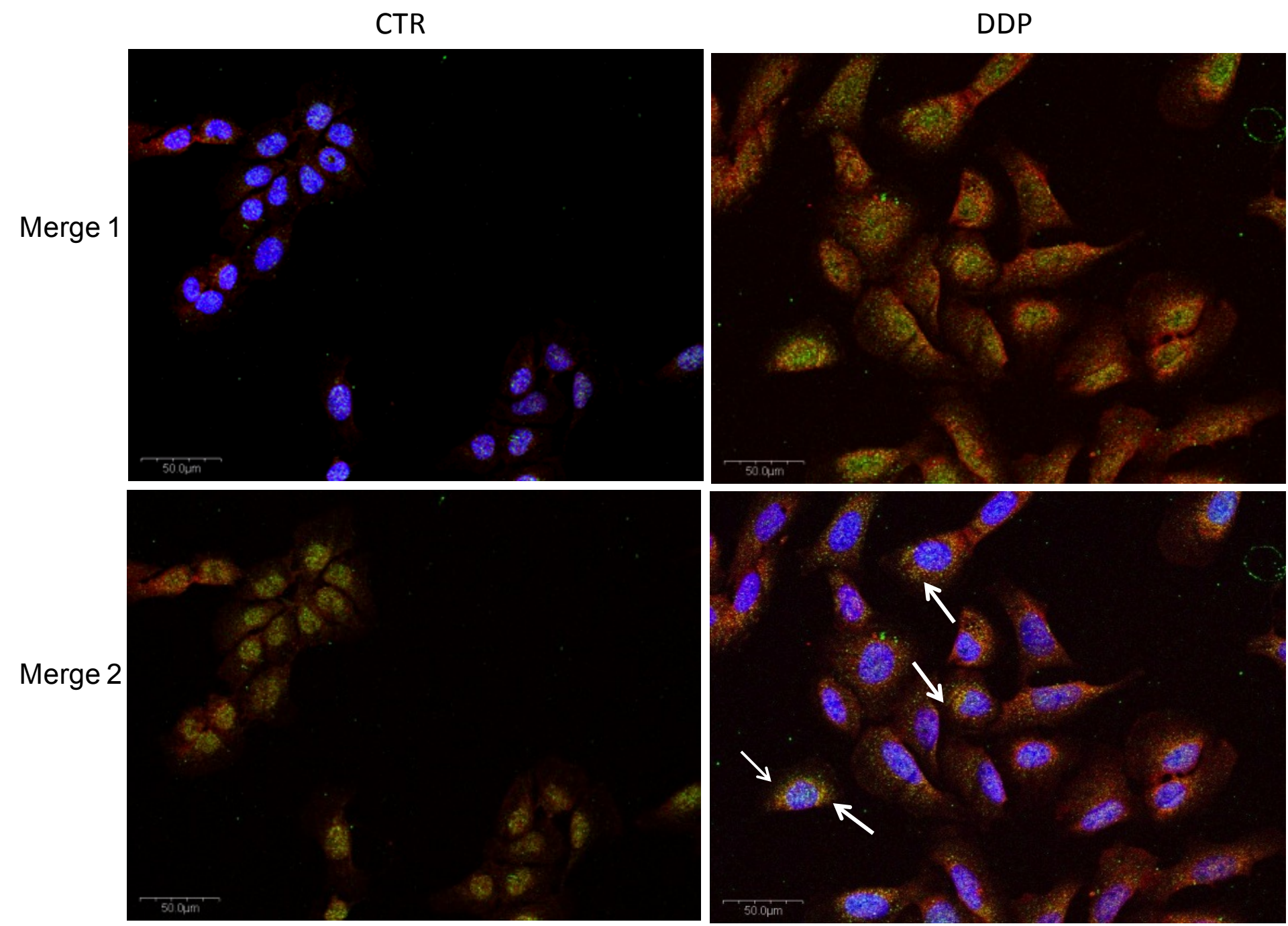


Supplementary figure 1



Supplementary figure 2

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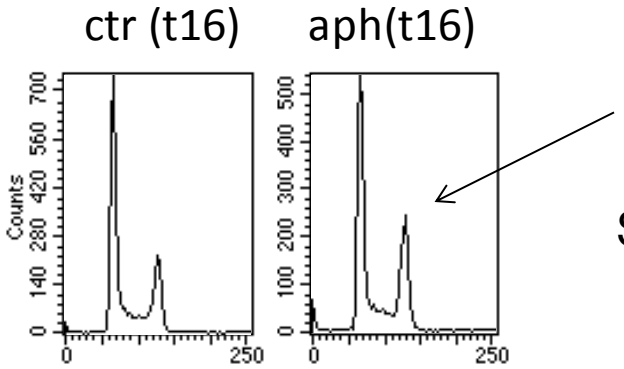
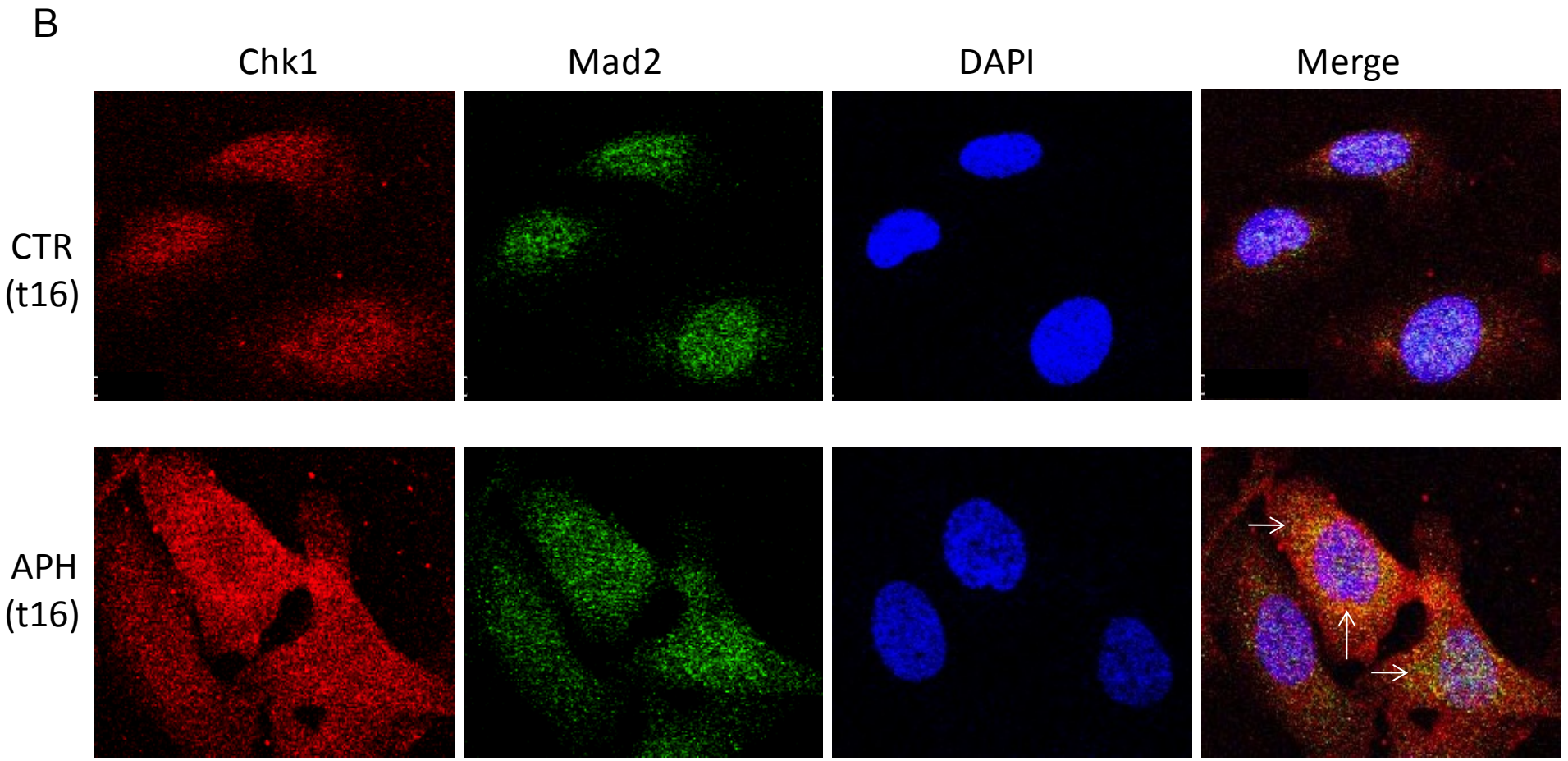


Supplementary Figure 3

B



Supplementary material

Cell lines, transfections and drugs

Human U2OS osteosarcoma cells were grown in Dulbecco's modified Eagle medium and the ovarian cancer cell lines OVCAR-8, OVCA-432 , A2780 and OVCAR-5 were grown in RPMI. Both media were supplemented with 1% glutamine and 10% fetal bovine serum. Cells were cultured at 37°C in 5% CO_2. OVCAR-8 and OVCAR-5 were kindly supplied by Dr. Pommier (National Cancer Institute, Bethesda, MD), and A2780, OVCA-432 and U2OS were obtained from the American Type Culture Collection (ATCC). The authors have not authenticated the cell lines within the last six months. Scramble siRNA and Chk1 siRNA were synthesized by Sigma; the sequences used and the method of transfection (with Lipofectamine 2000) have been previously described (reference 13 of the manuscript). The Mad2 siRNA duplex target sequence used to deplete Mad2 expression in U2OS cells is: 5' GGAAGAGTCGGGACCACAGTT 3' and it was synthesized by Sigma. The Universal Negative Control siRNA was also purchased by Sigma. PF-00477736 was kindly provided by Pfizer and dissolved in DMSO in a stock solution of 10 mM, stored at -20°C. DDP at a final concentration of 30 μM (close to the IC50 for U2OS) was dissolved in medium just before use. Cyclohesimide (Sigma) is dissolved in dimethylsulphoxide (DMSO) at stock solution of 50 mg/ml and used as 30 μg/ml. Aphidicholin (Sigma) was also dissolved in DMSO and dissolved in medium at the working concentration of 2.4 μg/mL just before use. To test the sensitivity of cells transfected with scramble, and Chk1 siRNAs, three wells for each sample were counted with the Beckman Cell Coulter, 72 h after transfection and 72 and 144 h after transfection with Universal Negative Control or Mad2 siRNA.

Purification of GST-fusion proteins and protein kinase assay

Briefly, from a glycerol stock stored at -80°C, *E. coli* containing the pGEX–3X /GST-construct (GST Gene Fusion System, Pharmacia Biotech) of interest (Mad2, Mad2 mutants, Cdc25C aa 250-56) was streaked onto a LB medium agar plate in sterile conditions and incubated overnight at 37°C. One colony was inoculated into LB medium containing the antibiotic of selection then incubated for 12-15 h at 37°C with vigorous shaking. The culture was then diluted 1:100 into fresh, pre–warmed LB medium with the antibiotic and left to grow at 37°C with shaking until the A_{600} reached 0.5-0.6, then induced with 0.5 mM of IPTG to produce fusion protein with an overnight–incubation at 30°C. The bacterial culture was pelleted and lysed with a sonicator on ice and fusion proteins were dissolved by adding 1% Triton X–100 to the sonicated cells and gently rotated for 30

minutes at 4°C. The soluble fraction was mixed with Glutathione Sepharose 4B (GE Healthcare), carrying glutathione molecules which bind to the fusion protein through the GST-tag, and shaken for 2 h in the cold room. After washes to remove un-bound proteins, the fusion protein was eluted with 10 mM of GSH from the matrix. The eluted material was monitored for GST-fusions proteins by SDS-PAGE. The polyacrylamide gel was stained with Coomassie Blue (Instant Blue, Expedeon). The recombinant GST-Chk1 was in part kindly provided by Dr Yolanda Sanchez (Dartmouth Medical School, NH, USA) and in part obtained in the laboratory by using a variant protocol (induction at room temperature overnight; lysis of bacteria with lysozime 1 mg/mL; washing beads in NaCl 300 mM). The kinase reaction was run in 20 µL reaction containing approximately 1.5 µg of recombinant substrate proteins, 5 µCi of ^{32}P-γATP (Amersham), 1 µM ATP, 1 mM DTT, 50 mM Tris HCl pH7.5, 20 mM $MgCl_2$ and 20 mM of MnCl2. Reactions were incubated for 30 minutes at 30°C, then mixed with 2x loading buffer and heated at 90°C for 5 minutes, cooled on ice and loaded on 10% SDS-PAGE gels. The gel was run for about 3 h and then exposed to autoradiographic films to detect phosphorylated forms in the presence of ^{32}P-γATP.

Immunofluorescence

To co-stain with Mad2 and Chk1, cells, either untreated or 24 h after treatment with DDP were fixed with 4% paraformaldehyde (in PBS solution) for 20 minutes at room temperature, washed twice in PBS and stored at 4^{o}C. Just before staining cells were incubated with glycine 100 nM for 15 minutes then permeabilized for 5 minutes with Triton X-100 (0.1% in PBS), and blocked in Blocking Buffer (5% Fetal Bovine Serum in PBS) for 45 minutes. Cells were then incubated with rabbit polyclonal anti Mad2 (Covance PRB-452C) diluted 1:500 overnight at 4°C then washed three times in PBS following incubation with Anti Rabbit IgG Alexa Fluor 488 (Molecular Probes) as secondary antibody for 1 h. After three washes in PBS cells were incubated with monoclonal anti-Chk1 antibody (DCS-310, Sigma) diluted 1:500 for 1h at room temperature, followed by three washes in PBS and incubation with Anti mouse IgG Alexa Fluor 594 (Molecular Probes) diluted 1:500. After washing in PBS, cells were incubated with DAPI (final concentration 30 ng/mL in PBS) for 1 minute to stain nuclei, then mounted with 7 µL of Vectashield solution (VectorLab) and analyzed with the confocal microscope (IX81, Olympus).

Immunoprecipitation

U2OS cells were first transiently transfected with plasmids over-expressing both FLAG-Chk1 and Mad2 using Lipofectamine 2000 (following the manufacturer's instructions). 24 h after

transfection they were either left untreated or treated with DDP 30 μM for 2 h, following wash and incubation with medium with no drug. 24 h after treatment cells were lysed with a buffer containing the following reagents at their final concentrations: Tris HCl 10 mM (pH 7.5), KCl 400 mM, EDTA 1 mM and a cocktail of protease inhibitors 100x (Sigma) in water. Cell lysis was completed by four cycle of thermal shock (37°C- dry-ice). One hundreds μg of cell lysates (adjusted for a final salt concentration of about 130 mM) were first precleared by incubation with an appropriate matrix (Preclearing Matrix F, Santa Cruz Biotechnology), for 1 h at 4°C while rotating. The precleared extracts were then incubated with an IP Matrix (Immuno Cruz Optima F IP Matrix, Santa Cruz Biotechnology), previously incubated in PBS to form a complex with either 1 μg of a Rabbit polyclonal Chk1 antibody (a300-298a, Bethyl Laboratories) or 1 μg of Rabbit IgG for 1 h at 4°C while rotating. After overnight incubation (at 4°C rotating) the precleared extracts with the IP antibody-IP matrix complex, were washed four times in Lysis Buffer (with a final salt concentration of 130 mM), resuspended in a loading Dye (containing SDS, Bromophenol, ß-mercaptoethanol), boiled and loaded on 10% SDS-Polyacrylamide gel. Western Blot Analysis was done as already described (reference 13 in the main text): Chk1 protein was detected by incubating with a monoclonal anti-Chk1 antibody (G4, Santa Cruz Biotechnology) and then with a Light Chain Specific secondary mouse monoclonal Antibody (ImmunoJackson Laboratories), while Mad2 was detected with a monoclonal anti-Mad2 antibody (17D10, Santa Cruz Biotechnology) and then with a secondary mouse monoclonal antibody specific to avoid recognition of immunoglobulins (Genetex, GTX221667-O1S). For Chk1 co-immunoprecipitation with Flag Mad2, U2OS cells were first transiently transfected with plasmids over-expressing both FLAG-Mad2 and HA-Chk1 and after 48 h proteins extracted as described above. Two μg of monoclonal Anti FLAG antibody (clone M2, Sigma) or 2 μg of Mouse IgG were used to immunoprecipitate 200 μg of cell lysates. The procedure followed for the immunoprecipitation was similar to the one above described but this time the Immuno Cruz IP/WB Optima E and the Preclearing Matrix E were used (Santa Cruz Biotechnology). Chk1 protein was detected by incubating with a polyclonal anti-Chk1 antibody (a300-298a, Bethyl Laboratories) while Mad2 was detected with a polyclonal anti-Mad2 antibody (FL-205, Santa Cruz Biotechnology). They were then both detected with a secondary rabbit antibody specific to avoid recognition of immunoglobulins (Genetex, GTX221666).

Legend to the Supplementary figures

Supplementary Figure 1

A. Western Blot Analysis of Chk1, Mad2 and Actin in U2OS cells 72 and 144 h after either Universal Negative Control or Mad2 siRNA transfection.
B. Cell growth curve of U2OS cells after either Universal Negative Control or Mad2 siRNA transfection. The data represent the mean ±SD of two independent experiments.

Supplementary Figure 2

Merge 1 and merge 2 figures enlargement of Chk1, Mad2 and DAPI staining in U2OS cells untreated and after treatment with DDP. White arrows show Chk1/Mad co-localization in the perinuclear region after DDP treatment.

Supplementary Figure 3

A. Analysis of DNA content by FACS in untreated cells and after 16 hrs from release with aphidicholin. The arrow points the slight accumulation of cells in G2.
B. Merge of Chk1, Mad2 and DAPI staining in U2OS cells either untreated or after 16 hrs of release from aphidicholin.