Supplementary information

Materials and Methods

Cell culture and synchronization

HeLa and HEK293 cells were maintained in Dulbecco modified Eagle medium (DMEM) medium supplemented with 10% (V/V) fetal bovine serum, 100 units ml^{-1} penicillin and 100 units ml^{-1} streptomycin at 37°C in 8% CO_2. To synchronize cells at the G1/S boundary, they were treated with 2.5 mM thymidine for 16 hours, released for 8 hours, and then treated with second thymidine for 16 hours. After two washes with phosphate-buffered saline (PBS), cells were cultured for different times as indicated in each experiment and harvested. Based on our experience, cells reach a peak of mitosis after 10 hours release, and exit from mitosis at 14 hours post-release. To obtain mitotic synchronized cells, cells were treated with 200 ng ml^{-1} nocodazole for 14 hours. After floating cells were gently washed away with PBS, mitotic cells were mechanically shaken off the culture dishes, washed three times with cold DMEM over a 1-hour period and then re-seeded onto dishes or polylysine-coated coverslips in prewarmed DMEM containing 10% FBS. To obtain S phase-enriched cells, HeLa cells were treated with 4 mM hydroxyurea for 40 hours. To block cells at G2 phase, cells were released from the double thymidine block for 6 hours, then incubated for 2 hours in the presence of 1 μM roscovitine, an inhibitor of Cdc2.

Transfections

For phenotype analysis of p38α and MK2 depletion using vector-based RNAi, HeLa cells were cotransfected with pBS/U6-p38α or pBS/U6-MK2 and pBabe-puro at a ratio of 9:1 using GenePorter reagents (Genlantis) according to manufacture's instructions. In brief, HeLa cells were seeded at 70% confluence in 10-cm dishes on the day before transfection. 10 μg plasmid DNA was diluted with 0.5 ml serum-free DMEM and mixed with 0.5 ml diluted GenePorter reagent. The mixture was incubated at room temperature for 30 minutes and then resuspended in 4 ml DMEM. After the cells were incubated in this mixture for 4 hours, 5 ml DMEM containing 20% serum was added. At 1 day post-transfection, 2 μg ml^{-1} puromycin was added to select transfection-positive cells for 2

days. After floating cells were washed away with PBS, the attached cells were incubated until harvesting for phenotype analysis.

To deplete proteins of interest using the siRNAs, HeLa cells were transfected with TransMessenger reagents (Qiagen) using the protocol provided by the manufacturer. Briefly, HeLa cells were seeded in 6-well plates at a density of 5 x 10^5 cells per well on the day before transfection. After 4 µl Enhancer R was diluted in Buffer EC-R, 2 µg RNA was added to the mixture and incubated at room temperature for 5 minutes, followed by the addition of 8 µl TransMessenger transfection reagent. The samples were incubated for 10 minutes at room temperature to allow transfection complex formation. Finally, the transfection complex was diluted with 900 µl DMEM without serum, and added drop-wise onto the cells. After a 3-hours incubation, medium was changed to DMEM containing 10% serum.

To express GFP-fusion Plk1 constructs, HeLa cells were transfected with PolyFect transfection reagents (Qiagen). In brief, cells were seeded on coverslips in 6-well plates on the day before transfection. Plasmid DNA, diluted into serum-free DMEM, was mixed with PolyFect reagent and vortexed for 10 seconds. After an 8-minutes incubation at room temperature, the transfection complex was diluted with serum-free DMEM and immediately transferred dropwise onto the cells in DMEM containing 10% serum. Flag-tagged Plk1 constructs were transfected into cells using GenePorter reagent as described above.

Immunoprecipitation (IP) and Immunoblotting (IB)

Cells were lysed in TBSN buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1.5 mM EDTA, 5 mM EGTA, 0.5% Nonidet P-40, 0.5 mM sodium vanadate) supplemented with phosphatase and proteinase inhibitors (20 mM *p*-nitrophenyl phosphate, 1 mM pefabloc, 10 µg ml^{-1} pepstatin A, 10 µg ml^{-1} leupeptin, 5 µg ml^{-1} aprotinin), and the lysates were clarified by centrifugation at 15,000 x g for 30 minutes. Lysates (1 mg) were incubated with either anti-phospho-Plk1 (15 µl), anti-Plk1 (1 µg) or anti-Myc (6 µl) antibody for 1.5 hours at 4°C, followed by a 1-hour incubation with Protein A-Sepharose beads. After immunocomplexes were washed with TBSN buffer four times and resolved by sodium

dodecyl sulfate-polyacrylamide gel electrophoresis, proteins of interest were detected by Western blot using antibodies as indicated in each specific experiment.

Immunofluorescence (IF) staining

Cells growing on coverslips were fixed with 4% paraformaldehyde for 10 minutes, and permeabilized with cold methanol for 2 minutes. After two washes with 0.1% Triton X-100/PBS for 5 minutes, cells were incubated for an additional 1 hour in PBST containing 3% bovine serum albumin for blocking, followed by a 1-hour incubation with various antibodies as indicated in each specific experiment. Finally, DNA was stained with 4',6'-diamidino-2-phenylinodole (DAPI). Rabbit phospho-histone H3 antibody, rabbit phospho-MK2 antibody, mouse Plk1 antibody, rabbit phospho-p38 and rabbit anti-p38 antibody were used at 1:100 dilutions. Rabbit phospho-Plk1 antibody and mouse γ-tubulin antibody were used at 1:1000 dilutions. After slides were processed under a Leica fluorescence microscope, representative images were taken by a Hamamatsu digital camera (ORCA-ER) using a Openlab software.

In vitro kinase assays

Purified MK2 (10 ng) was incubated with purified GST-Plk1 fragments (5 μg) in TBMD buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 2 mM EGTA, 0.5 mM sodium vanadate, 20 mM *p*-nitrophenyl phosphate) supplemented with 25 μM ATP and 10 μCi of [γ-^{32}P]ATP. The reaction mixtures were incubated at 30°C for 30 minutes and resolved by SDS-PAGE. The gels were stained with Coomassie brilliant blue, dried and subjected to autoradiography.

Recombinant Plk1 purification

Various domains of Plk1 were amplified by PCR, subcloned into pGEX-KG vector and overexpressed in *E. coli* BL21(DE3). Expression was induced by 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 37ºC for 5 hours after the cell density had reached 0.5 at 600 nm. To express Plk1-KM, Hi5 insect cells cultured in Ex-Cell 401 medium were infected with baculovirus encoding glutathione-S-transferase (GST)-Plk1-KM, and harvested 36 hours post-infection. Recombinant GST-fusion proteins were affinity

purified by incubation with glutathione-agarose beads followed by elution with soluble glutathione (30 mg ml^{-1}).

Fluorescence-activated cell sorter (FACS) analysis

Cells were harvested by trypsin digestion, washed with cold PBS, and resuspended in 75% ethanol at 4ºC for at least 8 hours. The fixed cells were collected by brief centrifugation and resuspended in PBS containing 200 μg ml^{-1} RNase A and 15 μg ml^{-1} propidium iodide. After incubation for 30 minutes at room temperature, samples were subjected to FACS.