Supporting Information

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SI Materials and Methods

Cell Culture and Synchronization. HeLa cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum in the presence of antibiotics in a humidified 5% CO_2 incubator at 37 °C. HeLa cells were synchronized with 2.5 mM thymidine (Sigma) for 16 h, released with fresh medium for 8 h, retreated with thymidine for 16 h, released with fresh medium from the double thymidine block, and then samples were prepared at various times. To block cells in S phase, cells were treated with 2 mM hydroxyurea for 12 h. To block cells in M phase, cells were treated with 100 ng/mL nocodazole for 12 h.

Fluorescence-Activated Cell Sorting (FACS) Analysis. For determination of the cell cycle phase, cells were collected by trypsinization and fixed in 75% ethanol, stained with 500 μL of 50 μg/mL propidium iodide solution, and subjected to FACS analysis. Cells were sorted by the Becton Dickinson FACScan machine and analyzed by CellQuest software (S1).

Immunoblot Analysis. Cells were collected and extracted in lysis buffer [0.5% Triton X-100, 20 mM Tris, pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 50 mM β-glycerophosphate, 25 mM NaF, 1 mM Na vanadate, 100 μg/mL PMSF, and protease inhibitor cocktail (Roche)]. After adjusting the protein concentration, proteins were resolved by SDS-PAGE and analyzed by Western blot analysis with specific antibodies. Anti-origin replication complex 2 (Orc2), anti-Erk2, anticyclinA, anti-cyclin-depnendent kinase (Cdk) 2, anti-Cdk1, and anti-GST polyclonal antibodies and anti-minichromosome maintenance 7 (Mcm7), anti-cell division cycle 6 (Cdc6), and anticyclin B1 monoclonal antibodies were from Santa Cruz. Anti-polo-like kinase 1 (Plk1) monoclonal from Upstate, anti-Cdt1 polyclonal from Bethyl Lab, anti-p-histone H3 polyclonal antibody from Cell Signaling, anti-β-tubulin and anti-FLAG antibody from Sigma, and anti-Cohesin/Rad21 antibody from Novus were used. Immune complexes were revealed with Amersham ECL™ Western blotting detection reagents (GE Healthcare).

GST Pull-Down Assay. GST-fused Cdc6 was expressed in *Escherichia coli* strain BL21 and purified using glutathione-sepharose 4B beads (GE Healthcare) according to the manufacturer's instructions. GST-Cdc6 bound to glutathione-sepharose 4B beads were incubated with the lysate of HEK 293T cells that had been expressed with FLAG-tagged Plk1 for 2 h at 4 °C. The resins were washed four times with lysis buffer [0.5% Triton X-100, 20 mM Tris, pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 50 mM β-glycerophosphate, 25 mM NaF, 1 mM Na vanadate, 100 μg/mL PMSF, and protease inhibitor cocktail (Roche)] and subjected to SDS-PAGE and Western blotting with anti-GST (Santa Cruz) and anti-FLAG (Sigma) antibodies.

Kinase Assay. For Cdk assays, cyclin A was immunoprecipitated from lysates from cells treated with hydroxyurea for 12 h and then released for 2 h, and cyclin B was immunoprecipitated from lysates treated with nocodazole for 12 h. Lysates with anticyclin A or an anticyclin B1 polyclonal antibody were incubated for 4 h at 4 °C with end-over-end mixing, followed by incubation with protein A agarose (Upstate) for 2 h at 4 °C. Immunoprecipitates were separated from supernatants by centrifugation and washed with lysis buffer. Activity was assayed with 1 μg histone H1 as substrate in reaction buffer [50 mM Tris pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 50 mM β-glycerophosphate, 0.1 mM sodium vanadate, 25 mM sodium fluoride, and protease inhibitor cocktail (Roche)] containing immunoprecipitated cyclin A- or cyclin B-associated Cdk, 20 μM ATP, and 10 μCi [γ-^{32}P] ATP at 30 °C for 30 min. Plk1 activity was assayed with purified His-tagged full-length Cdc6, GST-tagged regions of Cdc6, or 2 μg casein as substrate in reaction buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 2 mM EGTA, 0.5 mM sodium vanadate, 20 mM para-nitrophenyl phosphate) containing immunoprecipitated Plk1 protein, 25 μM ATP, and 25 μCi [γ-^{32}P] ATP at 30 °C for 30 min. The samples were suspended in SDS loading buffer, resolved by SDS-PAGE, and detected by autoradiography (S2).

Immunoprecipitation Assay. Cells were extracted in lysis buffer [0.5% Triton X-100, 20 mM Tris, pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 50 mM β-glycerophosphate, 25 mM NaF, 1 mM Na vanadate, 100 μg/mL PMSF, and protease inhibitor cocktail (Roche)]. Lysates were incubated with anti-Plk1 or anti-GFP polyclonal antibodies or anti-Cdc6 monoclonal antibodies (Santa Cruz) for 4 h at 4 °C with end-over-end mixing, followed by incubation with protein A/G agarose (Santa Cruz) for 2 h at 4 °C. Immunoprecipitates were separated from supernatants by centrifugation and washed four times with lysis buffer. Proteins were resolved by SDS-PAGE and analyzed by Western blot analysis with anti-Cdc6, anti-Plk1, anti-Cdk1, and anti-Erk2 antibodies.

Immunofluorescence. For immunofluorescence, cells were fixed, permeabilized, incubated in 0.1% Triton X-100-PBS containing 3% BSA for blocking, incubated with antipolyclonal Plk1 (Santa Cruz) and anti-Cdc6 (Santa Cruz) antibodies. Cells were washed three times with 0.1% TritonX-100-PBS and then incubated with CyTM3-conjugated anti-mouse secondary antibody and FITC-conjugated anti-rabbit secondary antibody (Jackson Immuno Research Laboratories) and DAPI (Sigma) for staining nuclear DNA. Images were collected and analyzed by the Z series of Applied Precision Deconvolution Microscope and Deltavision software.

1. Yim H, Erikson RL (2009) Polo-like kinase 1 depletion induces DNA damage in early S prior to caspase activation. *Mol Cell Biol* 29:2609–2261.

2. Zhou T, Aumais JP, Liu X, Yu-Lee LY, Erikson RL (2003) A role for Plk1 phosphorylation of NudC in cytokinesis. *Dev Cell* 5:127–138.

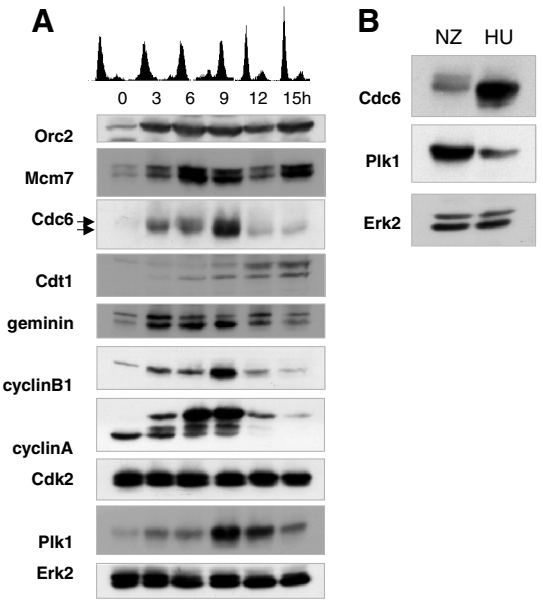

Fig. S1. Cdc6 is highly phosphorylated during mitosis in HeLa cells. (*A*) HeLa cells were synchronized with the double thymidine block as described in *Cell Culture and Synchronization*. Whole cell extracts were subjected to immunoblot analysis in a time course with anti-Orc2, anti-Mcm7, anti-Cdc6, anti-Cdt1, antigeminin, anticyclin B1, anticyclin A, anti-Cdk2, anti-Plk1, and anti-Erk2 antibodies. FACS analysis of cell cycle progression was performed. (*B*) HeLa cells were synchronized with hydroxyurea or nocodazole and whole cell extracts were subjected to immunoblot analysis with anti-Cdc6, anti-Plk1, and anti-Erk2 antibodies.

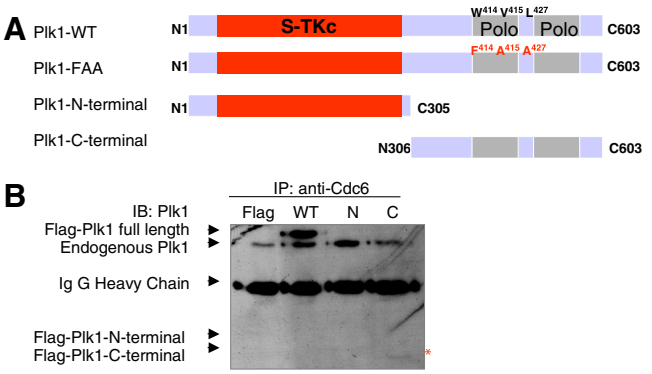

Fig. S2. Cdc6 associates with Plk1 through C terminus. (*A*) FLAG-tagged Plk1 constructs used in the transfection experiment. Plk1 is composed of kinase domain (residues 53–305) and polo-box domain (residues 410–440) as shown in the figure. And polo-box mutant is substituted at W414F, V415A, and L427A (FAA). (*B*) FLAG, FLAG-tagged wild-type Plk1 (WT), and N-terminal and C-terminal deletion mutant were expressed in HEK293T cells for 48 h. Cell extracts were immunoprecipitated with anti-Cdc6 antibody and then immunoblot analysis was performed with anti-Plk1 antibody.

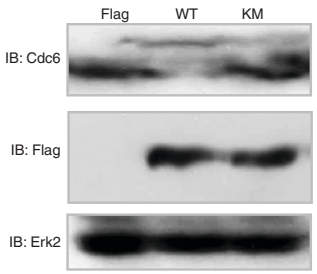

Fig. S3. Cdc6 is phosphorylated in cells expressing wild-type of Plk1 but not kinase defective mutant of Plk1. FLAG, FLAG-tagged wild-type of Plk1 (WT), and kinase defective mutant of Plk1 (KM) were expressed in HeLa cells for 48 h. Extracts were immunoblotted with anti-Cdc6, anti-Plk1, and anti-Erk2 antibodies.

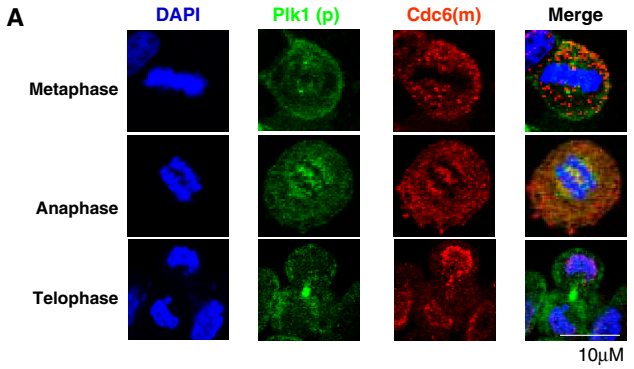

Fig. S4. Cdc6 colocalizes with Plk1 in the central spindle. HeLa cells grown on coverslips were synchronized with the double thymidine block. Nine hours after release, the cells were fixed with 4% paraformaldehyde. Cells were stained with antipolyclonal Plk1 (Santa Cruz; green) and antimonoclonal Cdc6 (Santa Cruz; red). Nuclear DNA was stained by DAPI. (Scale bar: 10 μm.)

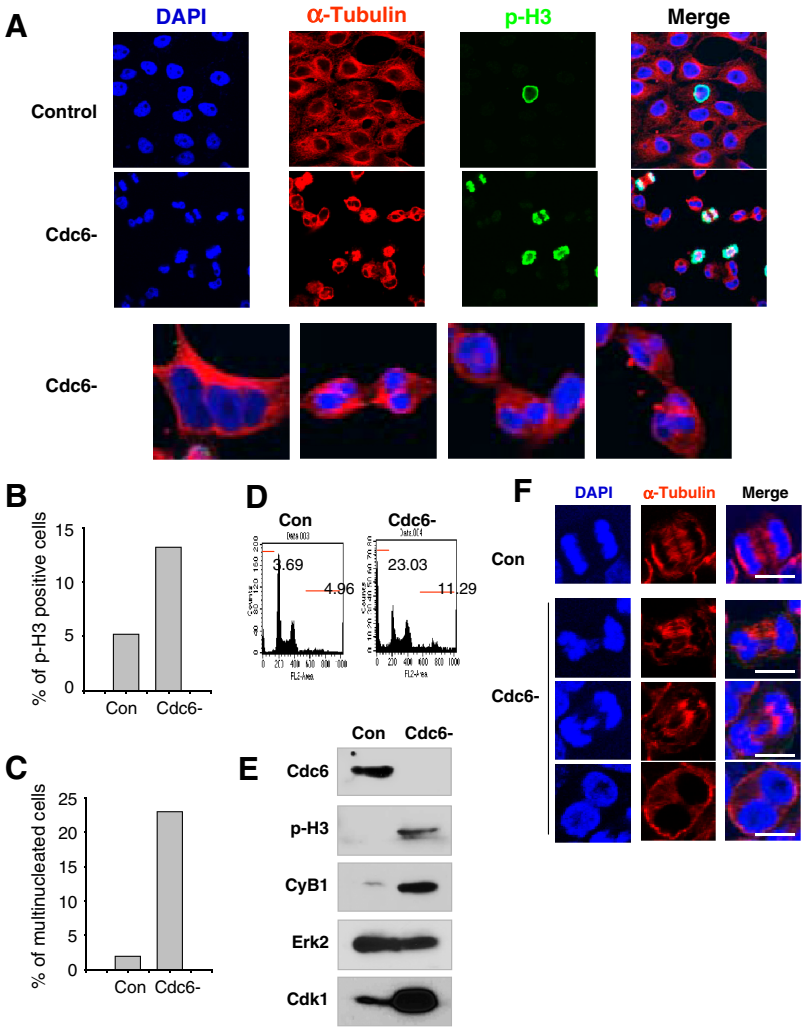

Fig. S5. Depletion of Cdc6 by siRNA induces cytokinetic defects and multinuclear cells. (*A*) HeLa cells were infected with lentivirus expressing human Cdc6-targeting shRNA (Cdc6-) or with control virus (Con) and selected with puromycin. Cells were fixed with 4% paraformaldehyde and stained with anti-α-tubulin, anti-p-Histone H3 (S10), and DAPI. (*B* and *C*) The populations of p-histone H3 positive cells and multinucleated cells were counted and quantified. (*D* and *E*) HeLa cells were infected with lentivirus expressing human Cdc6-targeting shRNA or with control virus and selected with puromycin for 2 d. FACS analysis was performed and the extracts were immunoblotted with anti-Cdc6, anti-Plk1, anti-Cdk1, anti-p-H3 (S10), and anti-Erk2 antibodies. (*F*) HeLa cells were infected with lentivirus and selected with puromycin. Cells were fixed with 4% paraformaldehyde and stained with anti-α-tubulin and DAPI. (Scale bar: 10 μm.)

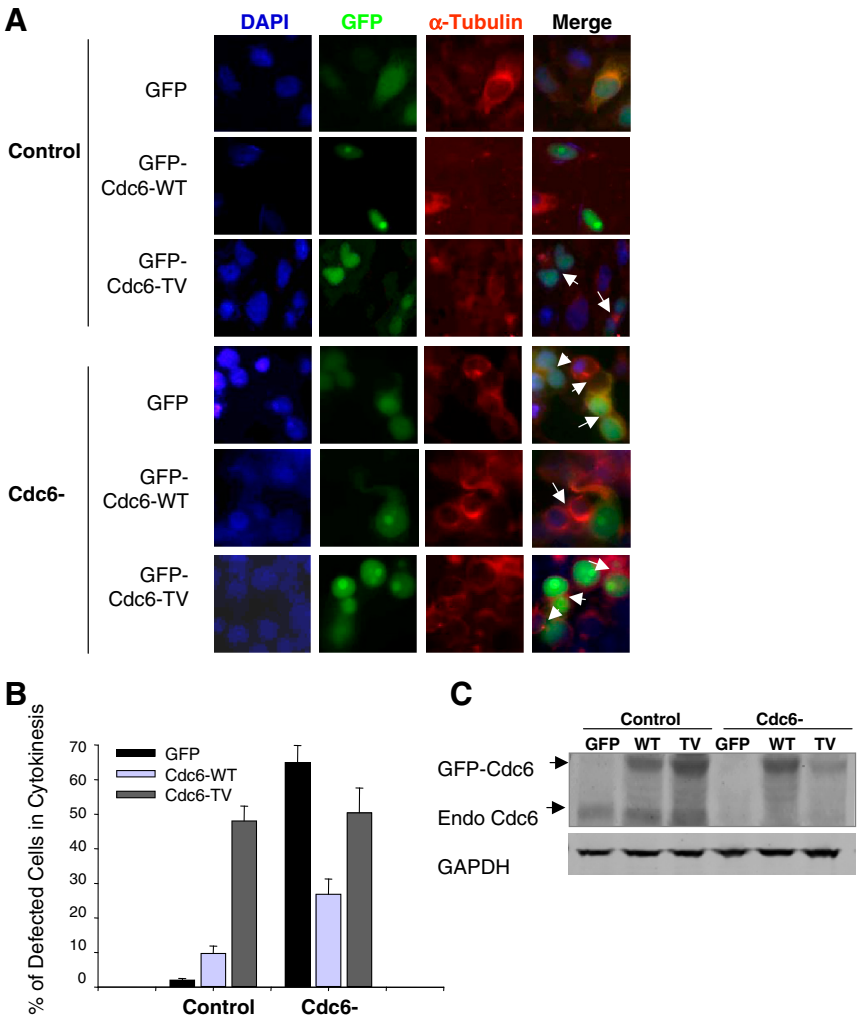

Fig. S6. Cytokinetic defects by Cdc6 depletion are reduced by the expression of wild-type Cdc6. HeLa cells were infected with lentivirus expressing human Cdc6-targeting shRNA or with control virus for 24 h, and cells were transfected with GFP, GFP-tagged Cdc6-WT, and Cdc6-TV for 2 d. (*A*) Cells fixed with 4% paraformaldehyde and stained with anti-α-tubulin, anti-GFP, and DAPI. (*B*) Cells showing incomplete segregation and cytokinesis were counted and quantified. (*C*) Extracts were immunoblotted with anti-Cdc6 and anti-GAPDH antibodies.