SUPPLEMENTARY INFORMATION

MATERIALS AND METHODS

Cell Culture

Cos7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) plus 1% glutamine, penicillin, streptomycin and 10% fetal bovine serum. Cells were synchronized at the G_1/S boundary using a double thymidine block (Quintyne & Schroer, 2002). G_1 cells were harvested 17 h after removal of thymidine. Mitotic cells were obtained by nocodazole treatment (1.65 μM nocodazole, 14 h) followed by shake off. For 2D blot analysis of GFP-p27, cells were transfected with cDNA, replated after 24 h and then synchronized.

Two-dimensional immunoblotting

Synchronized cells (1.5×10^6 cells, 10 cm dish) were solubilized with lysis buffer (30 mM HEPES, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.2% Triton X-100, 2 mM NaF, 2 mM Na_3VO_4, 2 mM $Na_4O_7P_2$, pH 7.4) containing protease inhibitors at 4°C for 30 min. Cell lysates were centrifuged at 20,800 × *g* in a Eppendorf 5417 rotor for 15 min, and the supernatants were subjected to two-dimensional electrophoresis according to the carrier ampholine method of isoelectric focusing (pH 3.5-10) by Kendrick Laboratories (Madison, WI). Proteins in the 2nd dimension SDS gel were transferred onto PVDF membranes and analyzed by immunoblotting.

RNAi

Dynactin subunit and control siRNAs were purchased from Ambion ($p150^{Glued}$: 5'- GGTATCTGACACGCTCCTA-3'; p27: 5'- CCACCTAAAGAAGACTATG-3' and Arp11: 5'-GCGCGTACTTGCTTTGTAA-3'). The biochemical effects of p27, Arp11 and $p150^{Glued}$ knockdown on dynactin stability are described elsewhere (Yeh et al, 2012). Spindly siRNA (5'-GAAAGGGTCTCAAACTGAA-3') was synthesized by Thermo Scientific as previously described (Gassmann et al, 2010).

For siRNA transfection, 10^7 cells were resuspended in 0.5 ml OPTI-MEM and electroporated at 240 V using a BTX electrocell manipulator 600 with a mixture of 1.33 nM siRNA and 5 μg pCAGIG either lacking an insert ((Matsuda & Cepko, 2004); control) or containing wild type mouse p27 or the T186A variant (see below), or mRFP-C1 (Clontech) plasmid DNAs. In some experiments, a plasmid encoding histone 2B-mCherry (engineered by replacing GFP with mCherry in pBOS-H2BGFP-N1 as in Kanda et al, 1998; courtesy of Dr. Q. Vong, Carnegie Institution) was included. Cells were plated on gelatin-coated coverslips 72 hours prior to analysis.

To generate the wild type and T186A variant p27 rescue vectors, the full length open reading frame of mouse p27 was amplified from an EST clone (AA073653) and subcloned into *EcoR*I- and *Xho*I-digested pCAGIG to yield pCAGIG–p27-WT. Site-directed mutagenesis (ACC to GCC; via QuikChange; Stratagene) was performed on pCAGIG–p27-WT to yield pCAGIG-p27-T186A. To generate pEGFP-p27-WT and -T186A for 2D blot analysis the open reading frames of p27-WT and T186A were in-frame ligated into the *EcoR*I- and *Sal*I-cut pEGFP-C1 vector (Clontech).

Immunofluorescence microscopy and image analysis

For kinetochore labeling of dynein, dynactin and Plk1, cells were pre-extracted with 0.5% Triton X-100 in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 4 mM Mg_2SO_4, pH 7.0) for 1 minute at room temperature prior to fixation in -20°C methanol for 10 min. For Mad1 staining, cells were pre-extracted for 1 minute, and for ZW10 and Spindly staining, cells were pre-extracted for 5 min, prior to fixation in 4% paraformaldehyde in PHEM buffer for 20 min at room temperature. 3F3/2, Bub1, Bub3, MPS1 and BubR1 were immunolocalized as described (Campbell & Gorbsky, 1995; Taylor et al, 2001). In a subset of experiments (as indicated), cells were treated with 0.66 μM nocodazole for 30 min at 37°C prior to extraction and fixation. Coverslips were incubated with primary antibodies for 1 hour and secondary antibodies for 30 min. Z-series (0.2 μm) were collected on a Zeiss Axiovert 200 microscope (Plan-Apochromat 100X, 1.4 NA objective) using a Cooke SensiCam camera (ASI) binned 1×1. The fluorescence intensity of kinetochore proteins was quantified as described (Hoffman et al, 2001; Sivaram et al, 2009); data were exported from SlideBook and analyzed using NIH Image J software. For quantification, ACA labeling was used to determine kinetochore position and the ratio of the integrated signal intensity of different kinetochore proteins was normalized to the ACA intensity for each kinetochore. The mean intensities relative to ACA were determined and compared to controls (100%).

To quantify Plk1 intensity at spindle poles and in intracellular cytokinetic bridges, cells processed as above were co-stained for Plk1 and tubulin. The region corresponding

to each spindle pole or cytokinetic bridge was outlined based on the tubulin signal and the integrated Plk1 density in this region was normalized to the tubulin intensity.

Expression and purification of free human p27

Human p27 was subcloned into pDESTHisMBP (Nallamsetty et al, 2005). The protein was expressed in *E.coli* strain BL21(DE3)RIPL cells. Cultures (6 mL) were grown at 37˚C shaking at 220 rpm to an $OD_{600,}$ of 1.0 and then added to 650 ml of fresh LB medium with ampicillin and chloramphenicol. Growth was continued at 37˚C shaking at 250 rpm for ~6 h to an OD_{600} of ≈ 1.0. The temperature was then reduced to 13˚C and expression was induced using 1 mM IPTG. The culture was grown overnight and the cells were harvested by centrifugation. The cell pellet was resuspended in 50 mM Tris-HCl, 100-200 mM NaCl, 5 mM imidazole, pH 7.5, containing 1 mg/ml lysozyme, incubated on ice for 30 min, and sonicated. Cell debris was collected by centrifugation at 35,000 rpm for 40 min. The supernatant was loaded onto a Ni-NTA resin column equilibrated with washing buffer (50 mM Tris-HCl, 200 mM NaCl, 1 mM β-ME, 5 mM imidazole, pH 7.5), and incubated with the resin for 1 hour at 4˚C, after which the flow-thorough was collected. The column was washed with 1 L of washing buffer, followed by wash buffer containing 20 mM imidazole. The column was eluted using 50 mM Tris-HCl, 200 mM NaCl, 1mM β-ME, 300 mM imidazole, pH 7.5. Fractions were pooled, rTEV protease (Tropea et al, 2009) was added, and the solution was dialyzed against 4 L of 50 mM Tris-HCl, 50 mM NaCl, 1 mM DTT, 0.5 EDTA, pH 8.0, at 4 ˚C overnight. The sample was then dialyzed against the same buffer without EDTA and DTT. The cleaved MBP-6xHis-tag was removed using Ni-NTA resin. The column flow-through

was concentrated using VivaSpin 20 ml concentrators and subjected to gel filtration on a Superdex75 column equilibrated with 50 mM Tris-HCl, 150 mM NaCl, 2.5 mM β-ME, 0.25 mM EDTA, pH 8.0. Fractions were collected and concentrated to ~10 mg/mL for crystallization.

Crystal structure determination of p27

The p27 protein was crystallized by mixing 200 nL of the protein solution (~10 mg/mL) in 50 mM Tris-HCl, 150 mM NaCl, 2.5 mM β-ME, 0.25 mM EDTA, pH 8.0, with 200 nL of the precipitant, 19% PEG 2000, 0.15 M KH_2PO_4. Crystallization was carried out at room temperature. In an effort to improve crystal quality and the resolution limit, we also attempted in situ proteolysis (Dong et al, 2007). The crystals that were ultimately used for diffraction data collection were grown from samples containing 0.2 μg/mL of α-chymotrypsin, but they were isomorphous with those obtained without the protease. Single crystals were flash-frozen in liquid nitrogen using 30% glycerol as the cryo-protectant and 100 mM vitamin C as a free-radical scavenger to alleviate secondary radiation damage. Diffraction data were collected at the Argonne National Laboratory, South-Eastern Collaborative Access Team (SER-CAT), beamline 22-ID, and processed using HKL2000 (Otwinowski Z & Minor, 1997). The structure was solved by an automated molecular replacement pipeline, as implemented in the BALBES server (Long et al, 2008), with default settings. The system used fragments of structures with the LβH fold, but otherwise low amino-acid sequence similarity; iterative rounds of refinement and automated model rebuilding produced a useful model. Final steps were carried out by Arp/wARP (Morris et al, 2003) and the model was refined using REFMAC5 (Murshudov

et al) and PHENIX with manual corrections using COOT (Emsley & Cowtan, 2004). Crystallographic details are provided in Table I. The atomic coordinates of the LβH domain of human p27 were deposited in the Protein Data Bank under the code 3TV0. Figures were prepared using PYMOL (www.pymol.org).

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SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Biochemical analysis of p27 rescue. Cos7 cells were cotransfected with control or p27 siRNAs plus pCAGIG-based rescue plasmids endoding wildtype p27 or the T186A variant and harvested after 72 h. Detergent lysates were (A) immunoblotted directly or (B) subjected to velocity sedimentation into a 5-20% sucrose gradient and then immunoblotted for the dynactin subunits indicated (or a control, dynein intermediate chain (IC), in A).

Supplemental Figure 2. Mitotic indices of Cos7 cell populations transfected with dynactin subunit siRNAs following nocodazole treatment (mean ± SD, two experiments, n >600 cells). 58 hours after RNAi transfection, cells were treated with 150 nM nocodazole for 14 hours prior to fixation, then stained for phospho-histone H3 to identify mitotic figures.

Supplemental Figure 3. Localization of p27 in dynactin subunit-depleted cells. Low level expression of wildtype p27 and the T186A variant was driven by pCAGIG-based rescue vectors. Cos7 cells were cotransfected with dynactin siRNAs plus pCAGIG plasmid (vector) or p27 rescue plasmids, then fixed and stained for p27 (green). The figure shows representative prometaphase cells in the asynchronous population. The insets (2×) show merged images with anti-centromere antigen (red). Bar = 5 μm.

Supplemental Figure 4. Representative images of prometaphase kinetochores in asynchronous Cos7 populations depleted of dynactin subunits, as quantified in Figure 2C. The insets (2×) show merged images with anti-centromere antigen (red). Bar = 5 μm. (A) siRNA-treated Cos7 populations were fixed and stained for ZW10, Bub1, BubR1, Spindly, MPS1, or Bub3 (green). (B) PBIP1 and NudC labeling (green) in control and p27/p25-depleted cells.

Supplemental Figure 5. Effects of Spindly depletion on dynactin, dynein, Plk1 and Mad1 localization to kinetochores. (A) Representative images of prometaphase cells in asynchronous populations. Cos7 cells were fixed and stained for the proteins indicated (green). The insets (2×) show merged images with anti-centromere antigen (ACA; red). DIC: dynein intermediate chain. Bar = 5 μm. (B) Quantitative analysis of fluorescence intensities at kinetochores as in (A) were normalized to ACA pixel values and expressed as percent of controls (mean ± S.E.M., n > 600 kinetochores, 15 cells).

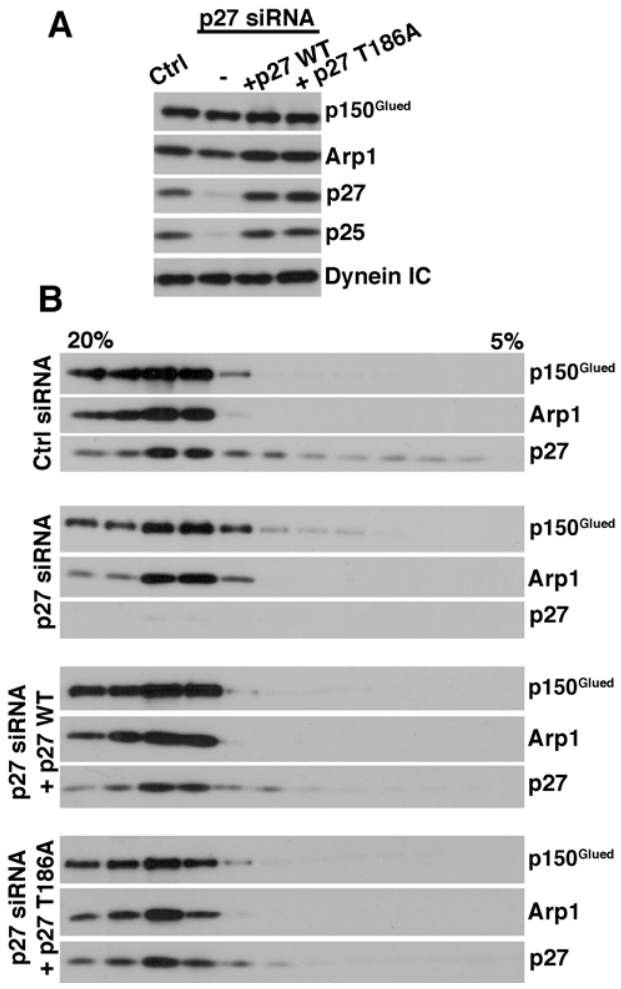
Supplemental Figure 6. Effects of expression of wildtype p27 and the T186A variant on kinetochore Plk1 and 3F3/2 labeling in control and p27/p25-depleted cells. Cos7 cells were cotransfected with control or p27 siRNAs plus pCAGIG plasmid (vector) or p27 rescue plasmids, then fixed and stained for Plk1 (green, left panels) and 3F3/2 (green, right panels) plus anti-centromere antigen (ACA; red). The figure shows representative prometaphase cells in the asynchronous population. Bar = 5 μm.

Supplemental Figure 7. Effects of low level p27 expression in Cos7 cells on mitotic progression. Low level expression of wildtype p27 and the T186A variant was driven by pCAGIG-based rescue vectors. (A) Transfected, asynchronous Cos7 cells were stained for phospho-histone H3 to identify mitotic figures. (A) The percent of cells cotransfected with control siRNA plus p27 rescue plasmids in specific mitotic stages, based on chromosome configuration (mean ± SD, three experiments, n>6000 cells). (B) The percent of mitotic cells in populations of cells (transfected as in B) (mean ± SD, three experiments, n >6000 cells).

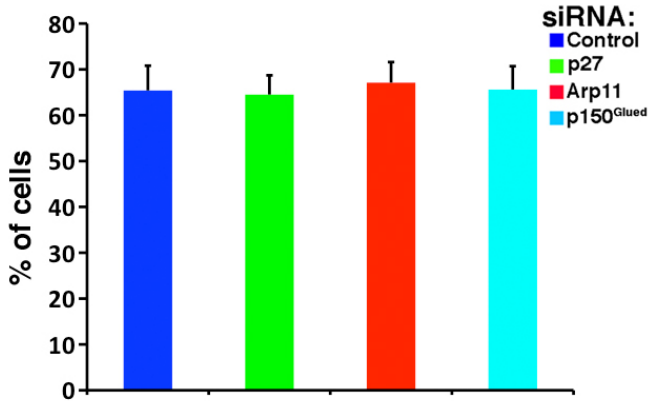
Movies 1 - 6. Representative time-lapse movies illustrating mitotic progression in cells expressing histone 2B-mCherry (as in Figure 1E). Cos7 cells were cotransfected with histone 2B-mCherry and (Movie 1) control siRNA, (Movie 2) p27 siRNA, (Movie 3) p27 siRNA plus wild-type p27 rescue plasmid, (Movie 4) p27 siRNA plus the p27 T186 variant, (Movie 5) Arp11 siRNA, or (Movie 6) $p150^{Glued}$ siRNA. The time stamp was set to 0:00 (minutes:seconds) upon nuclear envelope breakdown. Movies were made in ImageJ with Sorensen3 maximal compression. Playback is set at 600× real time.

Movies 7 – 10. Representative phase contrast time-lapse movies illustrating mitotic progression. Cos7 cells were cotransfected with (Movie 7) control siRNA, (Movie 8) p27 siRNA, (Movie 9) p27 siRNA plus wild-type p27 rescue plasmid, or (Movie 10) p27 siRNA plus the p27 T186 variant. The time stamp was set to 0:00 (minutes:seconds) upon nuclear envelope breakdown. Movies were made in ImageJ with Sorensen3 maximal compression. Playback is set at 630× real time.

Supplemental Figure 1



Supplemental Figure 2



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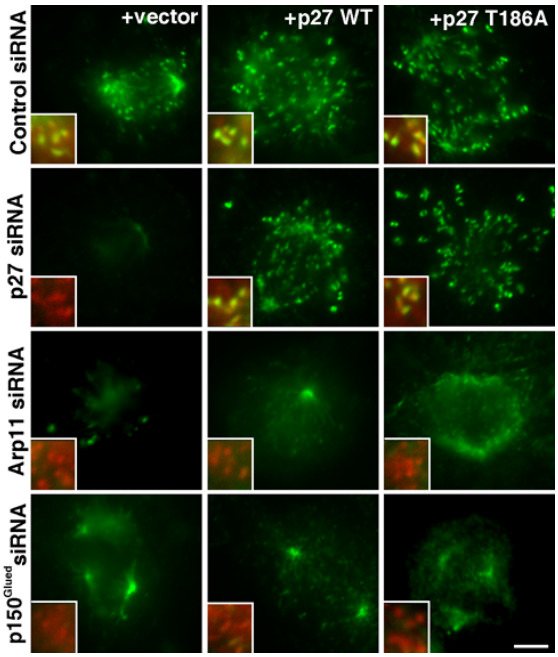

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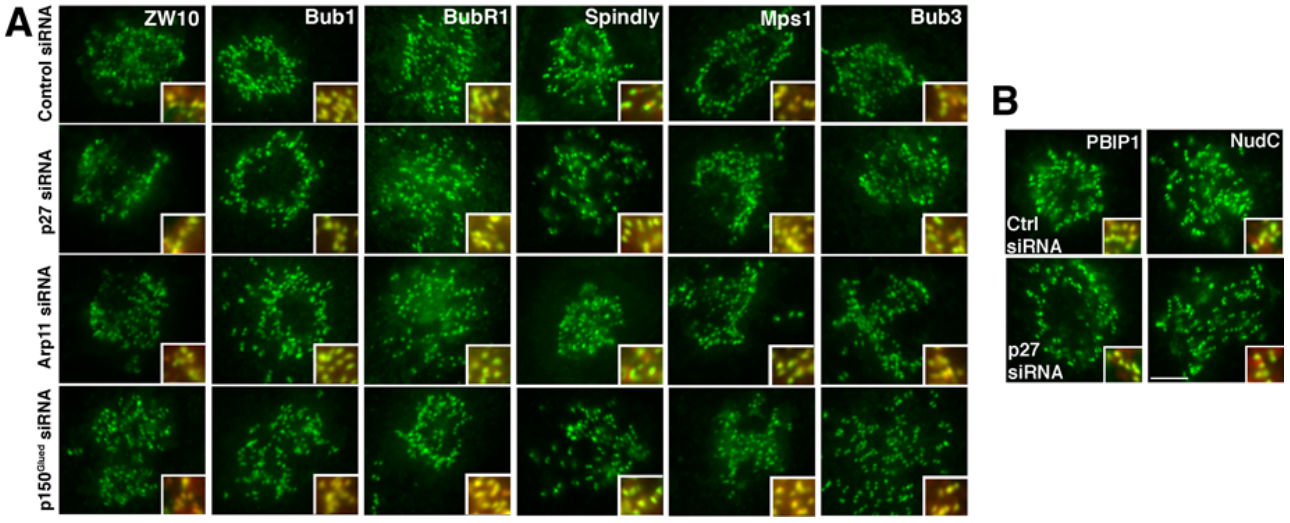

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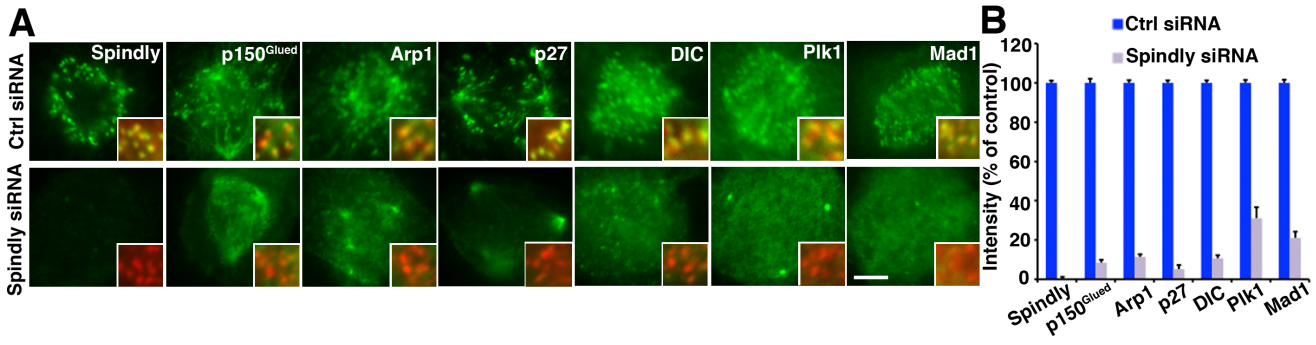
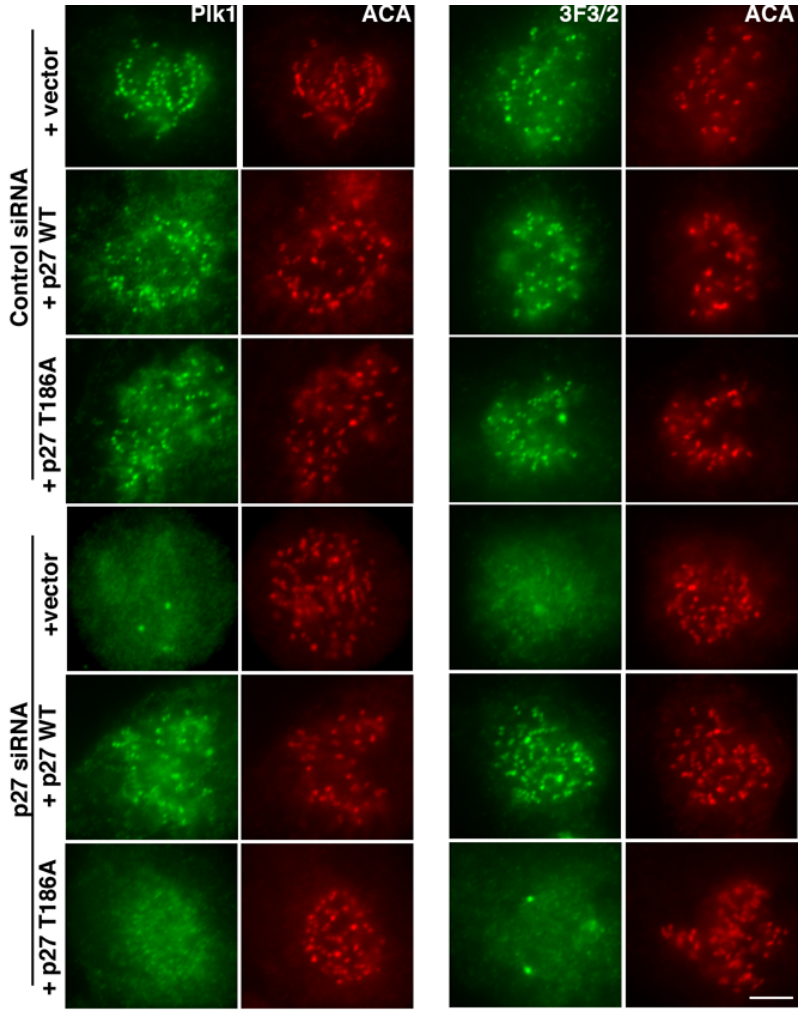


Supplemental Figure 6



Supplemental Figure 7