Supplemental Data

Mitotic Checkpoint Slippage in Humans Occurs via Cyclin B Destruction in the Presence of an Active Checkpoint

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Supplemental Experimental Procedures

Cell Culture and Drug Treatment

Human telomerase-immortalized retinal pigment epithelial (hTERT-RPE1) cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). Rat kangaroo kidney (PtK1) cells (American Type Culture Collection, Rockville, MD) were maintained in modified EMEM (Eagle's minimum essential medium containing Earle's balanced salt solution with nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, and 18 mM sodium bicarbonate) supplemented with 10% FBS. PtK2 YFP-Mad2-expressing cells (a gift from Dr. E.D. Salmon), were grown in modified EMEM supplemented with 15% FBS. All cells were maintained in a humidified incubator at 37ºC in a 5% CO_2 atmosphere. Prior to experiments, cells were removed from stock flasks and plated onto 25 mm^2 coverslips residing in the bottom of plastic dishes. These were then incubated until they were mitotically active.

5 hr before imaging, 200 nM or 3.2 μM nocodazole with or without 5 μM MG132 (for RPE1 cells) or 400 nM or 10 μM nocodazole with or without 20 μM MG132 (for PtK cells) were added to the media. Both of these drugs were purchased from Calbiochem. For filming, coverslip cultures were mounted in Rose chambers [S1, S2] in phenol-free L15 medium supplemented with 10% FBS (for RPE1 and PtK1 cells) or 15% FBS (for PtK2 YFP-Mad2 cells). Recordings were made on microscopes housed either in a 37ºC warm room or inside custom-built temperature-controlled incubators.

Cyclin B1-GFP Transfection and Expression

RPE1 cells growing on glass coverslips were transfected with either pCMX cyclin B1-GFP (a gift from Dr. J. Pines [S3]) or pEGFP cyclin B-Δ85 (a gift from Dr. N. Xu [S4]). For transfection, we used the FuGene 6 Transfection Reagent (Roche) according to the manufacturer's protocol. In brief, a mix of Fugene 6 and FBS-free medium (6 μl and 100 μl, respectively, per coverslip) was incubated for 5 min at 23ºC, followed by the addition of 2 μg of DNA. After a 20 min incubation at 23ºC, the medium surrounding the cells was replaced with FBS-free DMEM and the DNA/Fugene 6 mixture was added. Next, the cultures were incubated at 37ºC for 3 hr, after which FBS was added to a final concentration of 10%. 24 hr later, the transfected coverslip cultures were mounted in Rose chambers and examined at 37ºC with a microscope.

Live-Cell Microscopy

Low-magnification time-lapse phase-contrast recordings of RPE1 and Ptk1 cells were generated in a 37ºC room by a Nikon TMS microscope equipped with a video camera. Time-lapse recordings of transfected RPE1 cells were acquired at low magnification by a 20 × 0.5 PlanFluor or 40 × 0.75 PlanFluor objective mounted on a Nikon Eclipse TE2000-U. This microscope was equipped with an ORCA-ER cooled-CCD camera (Hamamatsu, Japan) and fast Uni-Blitz shutters (Vincent Associates, Rochester, NY). The hardware was driven by Image-Pro Plus 5.1.020 (Media Cybernetics, Silver Spring, MD). For the cyclin B immunostaining, RPE1 cells were incubated with 200 nM nocodazole for 5 hr, and before filming was initiated, the position of a mitotic cell was marked on the coverslip with a diamond-tip scribe (Carl Zeiss MicroImaging, Inc.) to allow the reidentification of the same cell after fixation.

Images of PtK2 YFP-Mad2-expressing cells were recorded in a near simultaneous phase-contrast (or DIC)/fluorescence mode, every 5 or 15 min, with a 60 × 1.4 PlanApo lens, at 37ºC. Each fluorescence image was collected as a Z-series with Nikon Eclipse inverted microscopes (TE200 or TE300) equipped with electronically controlled filter wheels (Ludl), piezo Z-positioners (Physik Instrumente, Germany), and shutters (Vincent Associates). Images were captured either with a CoolSnap HQ (Photometrics, Tucson, AZ) or Orca II (Hamamatsu) CCD camera. Each time point comprised 9 or 11 optical planes separated by 0.5 or 1 μm, respectively, with exposure times varying between 300 and 500 ms. These microscopes were operated by Isee software (Isee Imaging, Raleigh, NC). Time-lapse data sets were deconvolved as described below, image sequences were compiled with ImageJ 1.35c (NIH), and contrast-adjustment was conducted with Photoshop CS2 (Adobe Systems).

Immunofluorescence

For BubR1, Mad1, Mad2, and double Mad2-MT staining, RPE1 cells were briefly rinsed, after a 9 or 15 hr incubation with 200 nM or 3.2 μM nocodazole, in PEM buffer (100 mM PIPES, 1 mM EGTA, 5 mM Mg^{2+} [pH 6.9]), permeabilized with 0.5% Triton-X 100 for 5 min and fixed for 20 min in 4% paraformaldehyde. Blocking was done in 10% goat serum for 45 min, followed by 45 min incubation each with primary and secondary antibodies. For Tpx2 staining, RPE1 cells were rinsed in PEM buffer, fixed for 10 min in 4% paraformaldehyde, and permeabilized with 0.2% Triton-X 100 for 5 min. Staining was for 45 min in PBS/3% BSA. For MT staining, cells were rinsed in PEM, permeabilized with 1% Triton X-100 for 1 min, and fixed with 1% glutaraldehyde for 5 min. Free aldehyde groups were then reduced with two 10 min rinses in 1 mg/ml $NaBH_4$. Staining was for 30 min at 37ºC. With both methods, cells were washed in PBST (containing 0.05% Tween-20) after fixing, and before and after incubation with secondary antibodies. For cyclin B staining, cells were rinsed in cytoskeleton buffer (137 mM NaCl, 5 mM KCl, 1.1 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 2 mM $MgCl_2$, 2 mM EGTA, 5.5 mM glucose, 5 mM PIPES [pH 6.1]), fixed for 10 min in 4% paraformaldehyde, and permeabilized with 0.3% Triton-X 100 for 10 min. Blocking was done in 10% FBS/0.1% Tween-20 for 15 min, followed by incubation with primary (40 min) and secondary (20 min) antibodies. Cells were washed in PBST (containing 0.1% Tween-20) before blocking, and before and after incubation with secondary antibodies. Except where stated, all steps were performed at room temperature. The primary antibodies used were mouse anti-α-tubulin (1:100 dilution, DM1α, Sigma), rabbit anti-BubR1 (1:2000 dilution, a gift from Dr. T. Yen), mouse anti-cyclin B (1:100 dilution, Ab-4 clone GNS11,

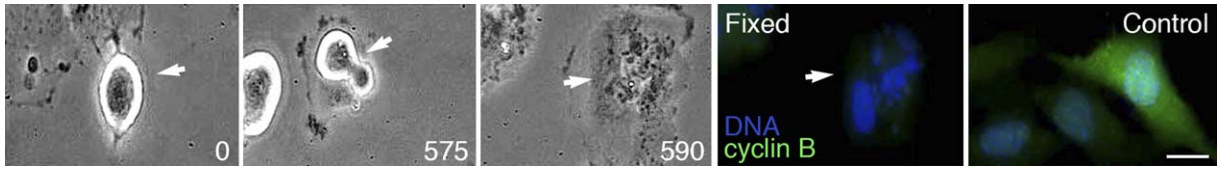

Figure S1. Cyclin B Is Absent from RPE1 Cells Shortly after They Exit Mitosis in the Presence of an Unsatisfied SAC

Three selected phase-contrast frames from a time-lapse recording of an RPE1 cell progressing through mitosis in the presence of 200 nM nocodazole. This cell (arrow) began to exit mitosis 575 min after nuclear envelope breakdown (0 min) and was fixed 15 min after it reflattened. It was then stained for DNA and cyclin B (fourth panel). The fifth panel shows a G2 control in the same culture, photographed with the same exposure as the experimental cell. See text for details. Scale bar equals 5 μm.

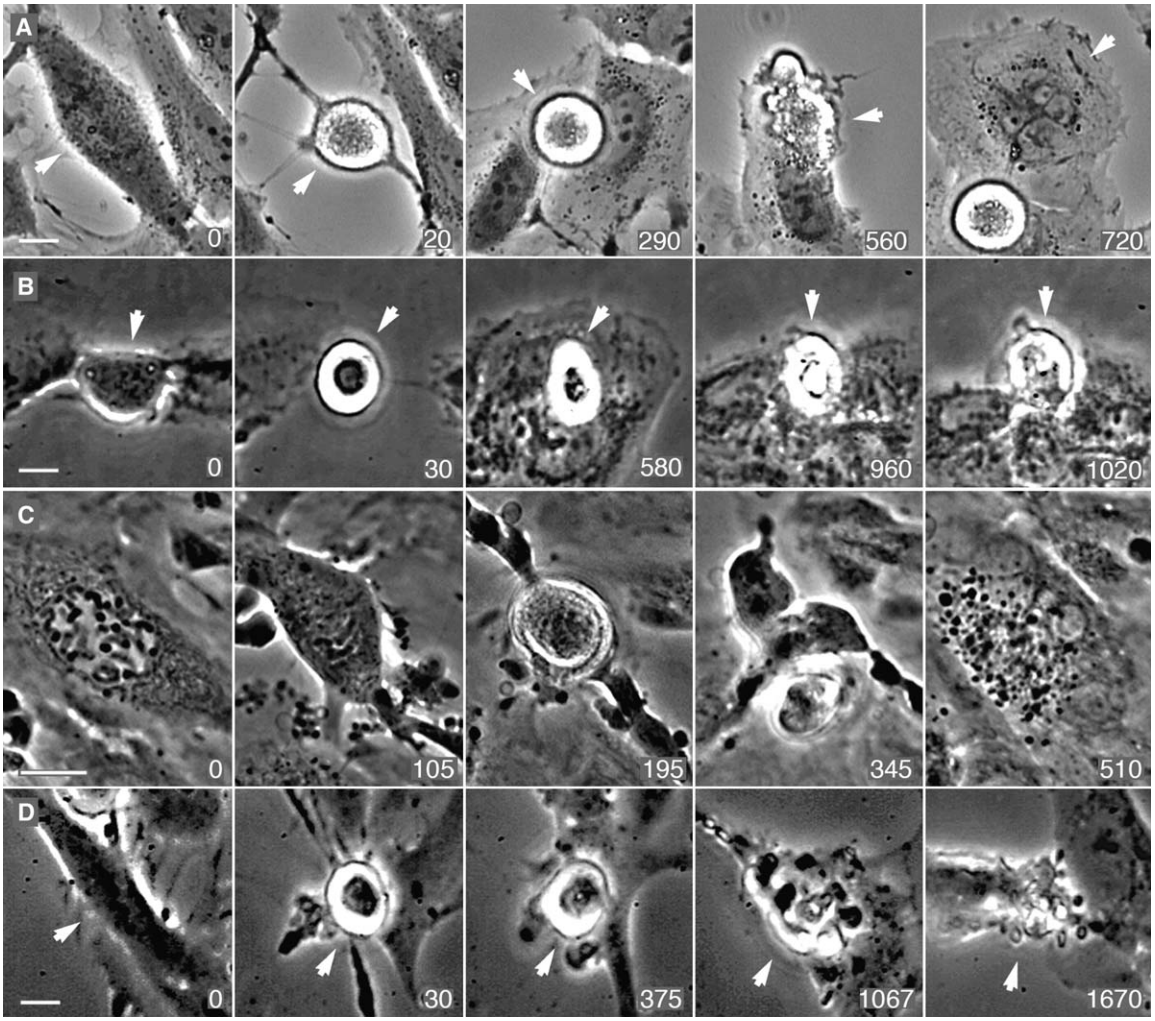

Figure S2. RPE1 and PtK1 Cells Require Active Proteasomes to Exit Mitosis in the Presence of an Unsatisfied SAC

Selected frames from phase-contrast video recordings of RPE1 (A, B) and PtK1 (C, D) cells incubated with 200 nM (A) or 10 μM nocodazole (C), and 200 nM nocodazole plus 5 μM MG132 (B) or 10 μM nocodazole plus 20 μM MG132 (D). Arrows mark the cells of interest. Note that, relative to controls that escape the block and enter the next G1 with multiple micronuclei (A, C), cells treated with nocodazole and MG132 fail to exit mitosis and die after a prolonged arrest (B, D). Time is shown in minutes. Scale bars equal 5 μm.

Lab Vision), mouse anti-Mad1 (1:100 dilution, clone 9B10, Novus Biologicals), rabbit anti-Mad2 (1:100 dilution, a gift from Dr. E. Salmon), and mouse anti-Tpx2 (1:200 dilution, clone 18D5, BioLegend). AlexaFluor 488 and 546 (Molecular Probes) were used as secondary antibodies (1:100 dilutions). DNA was counterstained with Hoechst 33342 (Molecular Probes).

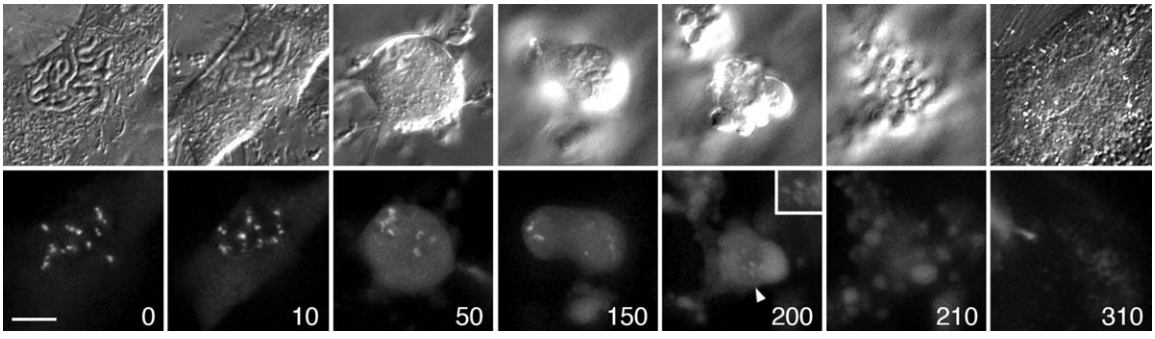

Figure S3. Mad2 Remains Associated with Kinetochores in PtK2 Cells as They Exit Mitosis in the Presence of an Unsatisfied SAC

Selected DIC (top) and fluorescence (bottom) frames from a time-lapse video recording of an YFP-Mad2-expressing PtK2 cell exiting mitosis in the presence of 10 μM nocodazole. Inset shows a higher magnification view of kinetochore-associated Mad2 well after the violent blebbing, characteristic of cells exiting mitosis, was initiated (at time 150). Time is shown in minutes. Scale bar equals 5 μm.

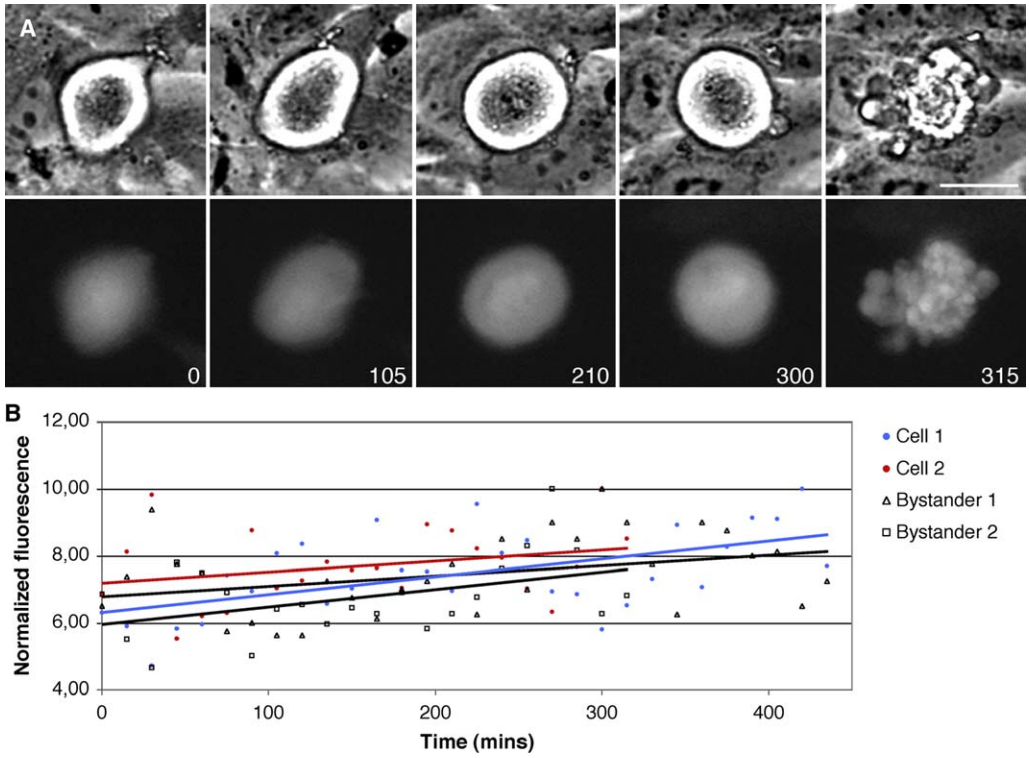

Figure S4. In the Presence of Nocodazole and MG132, GFP-cyclin B Degradation Requires Proteasome Activity

(A) Selected phase-contrast and fluorescence frames from a mitotic GFP-cyclin B expressing RPE1 cell treated prior to entering mitosis with 200 nM nocodazole and 5 μM MG132.

(B) Normalized fluorescence intensity (as calculated in Figure 3) of two experimental cells (red and blue lines), as well as bystander cells (black lines) in the same field, over a 5-7 hr period. The red line depicts the cell shown in (A). The data are typical of the five cells followed for this experiment, which reveals that in the absence of active proteasomes, GFP-cyclin B fluorescence does not decay. Time is shown in minutes. Scale bar equals 5 μm.

After staining, cells were imaged as a Z-series (300 nm apart) on an Olympus IX70 inverted microscope equipped with a CM350 camera (Photometrics). The images were then deblurred with the SoftWorx 2.5 deconvolution algorithm (Applied Precision, Issaquah, WA) and presented as maximal intensity projections.

Supplemental References

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S3. Hagting, A., Karlsson, C., Clute, P., Jackman, M., and Pines, J. (1998). MPF localization is controlled by nuclear export. EMBO J. *17*, 4127–4138.

S4. Chang, D.C., Xu, N., and Luo, K.Q. (2003). Degradation of cyclin B is required for the onset of anaphase on mammalian cells. J. Biol. Chem. *278*, 37865–37873.