Supplemental Information

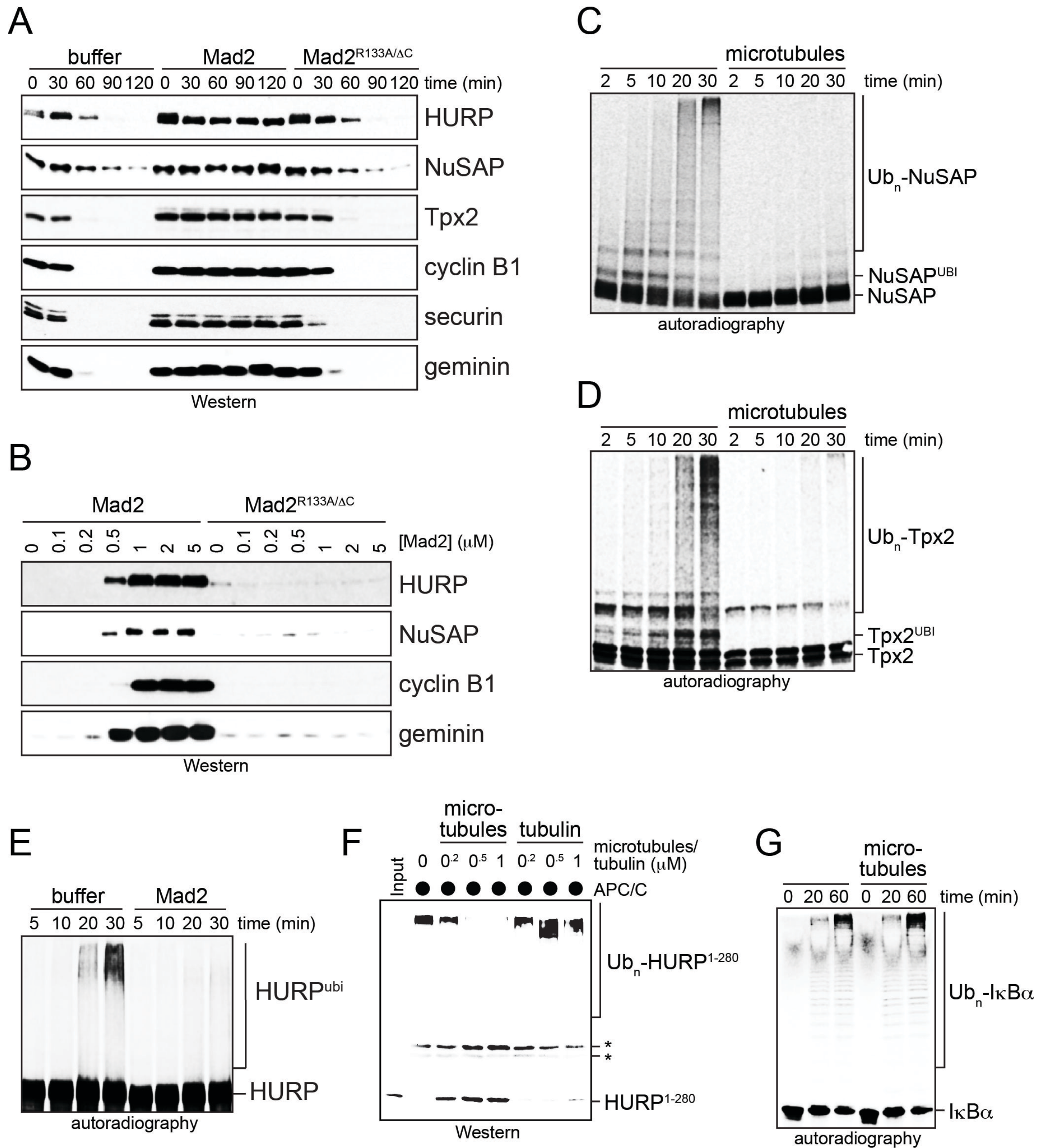
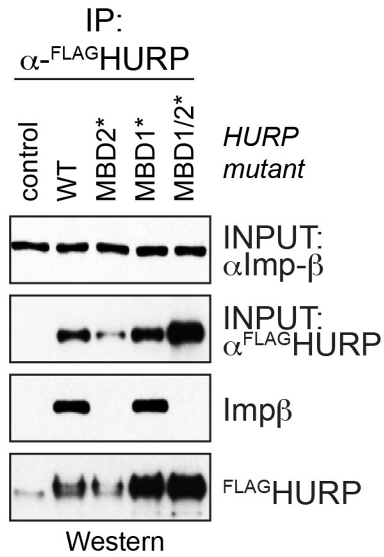
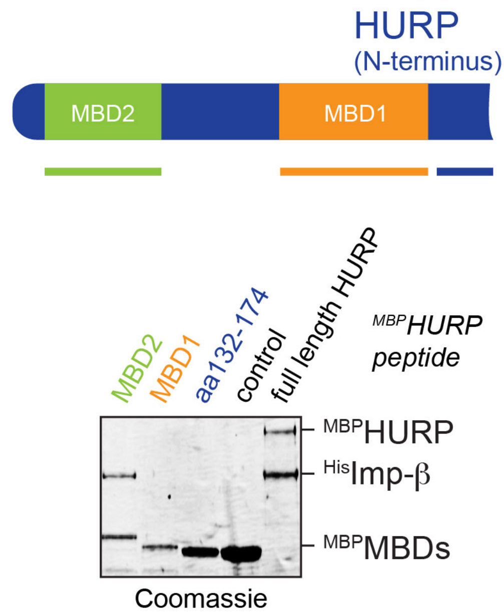


Figure S1 (related to Figure 1 and 2): Soluble spindle assembly factors can be targeted by APC/C^{Cdc20}. A. Extracts were prepared from prometaphase HeLa cells,

treated with Ran^{Q69L} to dissociate spindle assembly factors from importins, and incubated with p31^{comet} to activate the APC/C. Where indicated, the APC/C^{Cdc20}-specific inhibitor Mad2 or an inactive variant of Mad2 (Mad2$^{R133A/\Delta C}$) were added. The stability of endogenous APC/C-substrates in the extracts was monitored over time by Western blotting. **B.** Degradation assays were performed in the presence of increasing concentrations of Mad2 or inactive Mad2$^{R133A/\Delta C}$ and the abundance of APC/C-substrates after 120min of incubation was analyzed by Western blotting. **C.** Microtubules inhibit the APC/C^{Cdc20}-dependent ubiquitylation of NuSAP. ^{35}S-labeled NuSAP was purified and incubated with APC/C^{Cdc2} and a ubiquitylation cocktail. As indicated, microtubules were added prior to the ubiquitylation. Reactions were analyzed by autoradiography. **D.** Microtubules prevent APC/C^{Cdc20}-dependent ubiquitylation of Tpx2. The ubiquitylation of purified ^{35}S-labeled Tpx2 by APC/C^{Cdc20} was performed as described. **E.** Mad2 inhibits the ubiquitylation of HURP by APC/C^{Cdc20}. ^{35}S-labeled HURP was synthesized *in vitro* and incubated with APC/C^{Cdc20} purified from early mitotic cells in the presence or absence of recombinant Mad2. Reactions were analyzed by autoradiography. **F.** Tubulin polymerization is required for effects on APC/C-dependent ubiquitylation. Equal concentrations of taxol-stabilized microtubules or tubulin were incubated with bacterially purified, recombinant HURP$^{1-280}$, APC/C, and a ubiquitylation cocktail. Reactions were analyzed by Western blotting of recombinant HURP. **G.** Microtubules do not inhibit SCF-dependent ubiquitylation. SCF$^{\beta TrCP}$ purified from 293T cells was incubated with radiolabeled, phosphorylated, and purified IκBα. Ubiquitylation reactions contained microtubules where indicated. The reactions were analyzed by autoradiography.





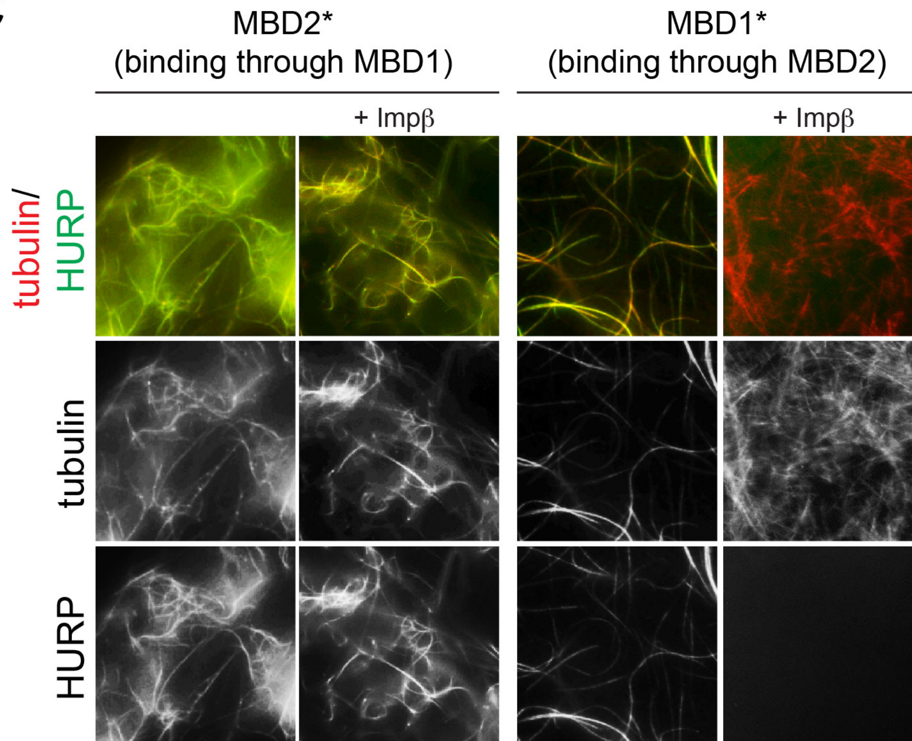


Figure S2

Figure S2 (related to Figure 3): Importin-β recognizes the MBD2 of HURP. A. Mutation of the MBD2 abolishes binding of importin-β to HURP in cells. 293T cells were transfected with FLAG-tagged HURP, mutants of its MBD1 or MBD2 (positively charged residues were changed to alanine), or a mutant that lacked both MBDs. Interphase cells were harvested and analyzed for interactions between HURP and importin-β by FLAG-affinity purification and Western blotting. **B.** Importin-β binds the MBD2 of HURP. MBP-fusions to HURP, the MBD1, or the MBD2, were immobilized on amylose beads and incubated with recombinant importin-β. Following extensive washes, binding reactions were analyzed by Coomassie staining. **C.** Importin-β blocks the microtubule-binding of the MBD2. Rhodamine-labeled microtubules were incubated with recombinant Oregon 488-labeled $HURP^{MBD1*}$ (functional MBD2) or $HURP^{MBD2*}$ (functional MBD1). Where indicated, a two-fold molar excess of importin-β was added. Binding of labeled HURP-proteins to microtubules was visualized by fluorescence microscopy.

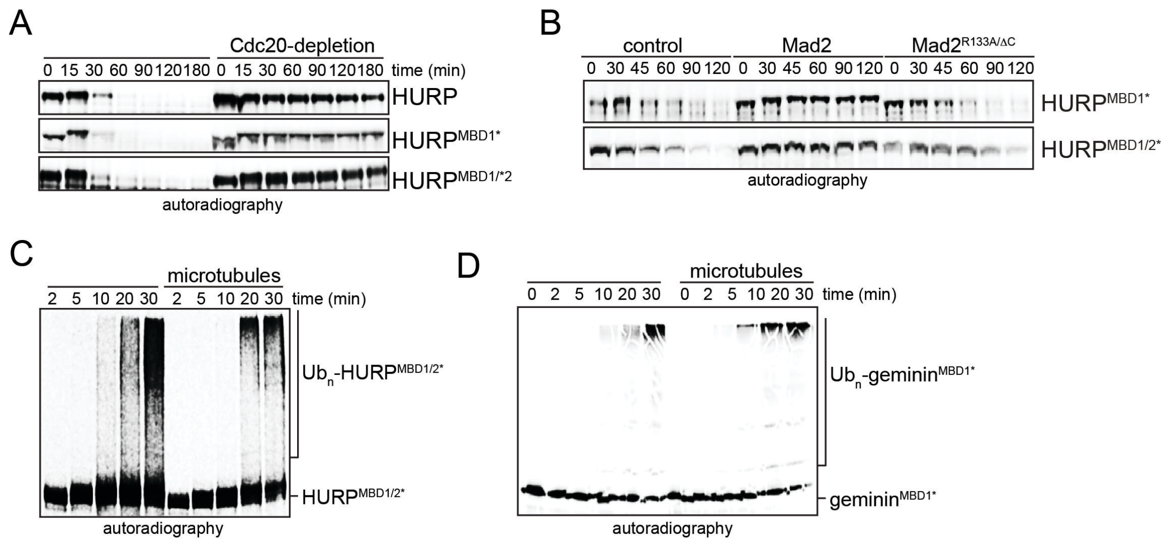


Figure S3

Figure S3 (related to Figure 4): Characterization of the effects of mutants and fusions of the MBD1 on APC/C-substrate ubiquitylation and degradation. A. Mutants in the MBD1 of HURP are degraded in an APC/C-dependent manner. Extracts of prometaphase HeLa cells were treated with Ran^{Q69L} to dissociate HURP from importin-β and supplemented with p31^{comet} to activate the APC/C. As indicated, Cdc20 was depleted from extracts using specific antibodies. The degradation of ^{35}S-labeled HURP, HURP^{MBD1*}, or HURP$^{MBD1/2*}$ was monitored by autoradiography. **B.** Mutants in the MBD1 of HURP are stabilized by the APC/C^{Cdc20}-inhibitor Mad2. ^{35}S-labeled HURP^{MBD1*} or HURP$^{MBD1/2*}$ were added to mitotic extract with active APC/C either in the presence of recombinant Mad2 or an inactive variant, Mad2$^{R133A/\Delta C}$. Substrate stability was monitored by autoradiography. **C.** Loss of microtubule-binding ablates any effects of microtubules upon HURP-ubiquitylation. ^{35}S-labeled ZZ/TEV-HURP$^{MBD1/2*}$ was purified after *in vitro* transcription/translation over IgG-sepharose, eluted with TEV, and incubated with active APC/C either in the presence or absence of microtubules. Reactions were analyzed by autoradiography. **D.** Mutations in the MBD1 obliterate its effect on the ubiquitylation of a heterologous APC/C-substrate. A fusion between the first 101 residues of geminin and a

mutant MBD1 of HURP was synthesized as a ^{35}S-labeled protein, purified over IgG-sepharose, eluted with TEV, and incubated with active APC/C either in the presence or absence of microtubules. Reactions were analyzed by autoradiography.

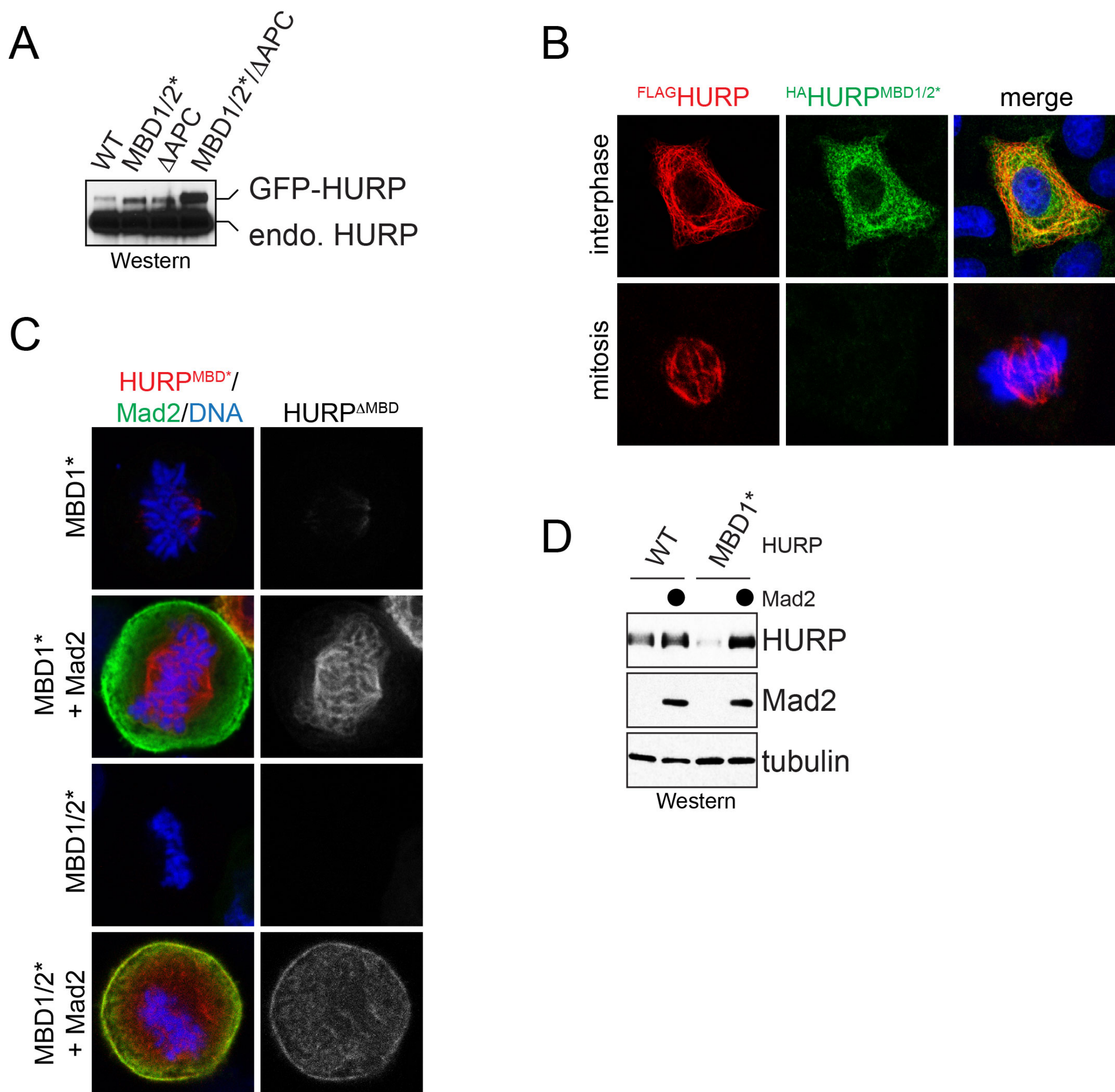


Figure S4 (related to Figure 5): Microtubules protect HURP from degradation. A. Levels of lentivirus-dependent expression of GFP-tagged HURP or microtubule-binding mutants. **B.** Loss of microtubule-binding reduces HURP-levels in mitosis. HeLa cells were simultaneously transfected with ^{FLAG}HURP and ^{HA}HURP$^{MBD1/2*}$ and analyzed for HURP-expression at different cell cycle stages by immunofluorescence. **C.**

Overexpression of the Cdc20-inhibitor Mad2 stabilizes microtubule-binding deficient HURP-mutants in late metaphase/early anaphase. HeLa cells were transfected with $^{FLAG}HURP^{MBD1*}$ or $^{FLAG}HURP^{MBD1/2*}$ in the presence or absence of Mad2-overexpression. The levels of HURP proteins were detected by immunofluorescence microscopy (HURP: red; Mad2: green; DNA: blue). **D.** Microtubule-binding deficient HURP is stabilized in mitosis by Mad2-overexpression. Mitotic HeLa cells expressing HURP or $HURP^{MBD1*}$ were harvested by shake-off and analyzed for HURP levels in the presence or absence of Mad2-overexpression by Western blotting.

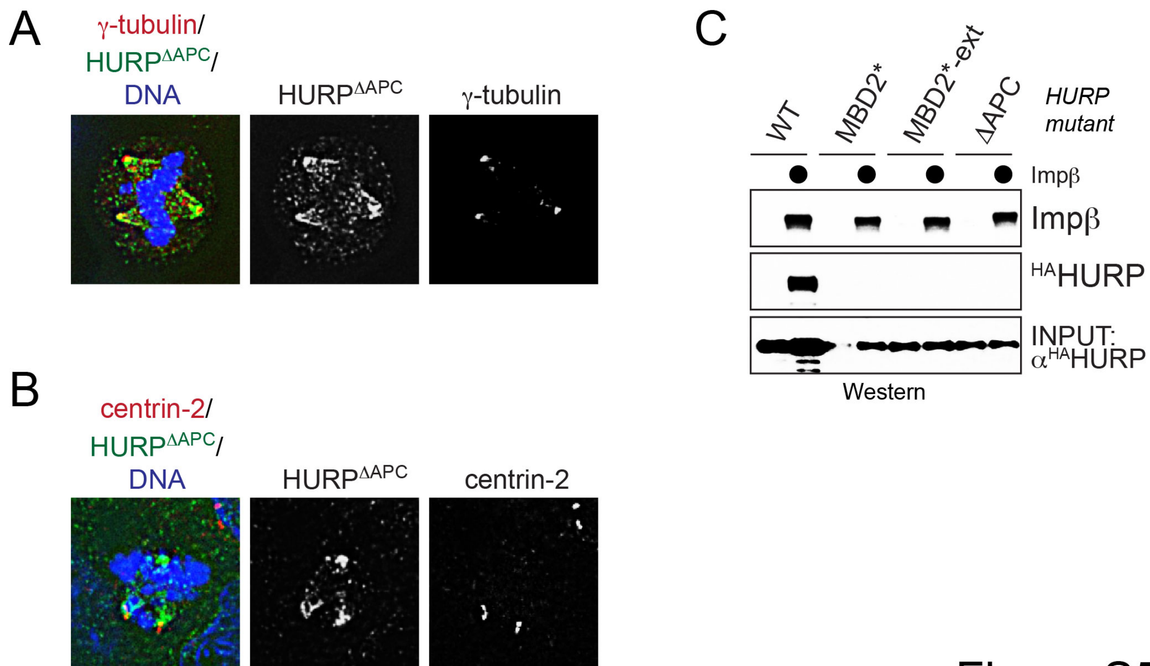


Figure S5

Figure S5 (related to Figure 6): Stabilization of HURP causes spindle pole fragmentation. A. HeLa cells expressing GFP-tagged HURP$^{\Delta APC}$ and having multipolar spindles were stained for GFP (green), γ-tubulin (red), and DNA (blue). **B.** HeLa cells expressing GFP-tagged HURP$^{\Delta APC}$ and having multipolar spindles were stained for GFP (green), centrin-2 (red), and DNA (blue). **C.** HURP$^{\Delta APC}$ is neither recognized by importin-β nor the APC/C. Lysates of HeLa cells expressing ^{myc}importin-β, ^{HA}HURP or the indicated mutants were subjected to αmyc-affinity purification, and co-precipitating HURP was detected by Western blotting using specific antibodies.

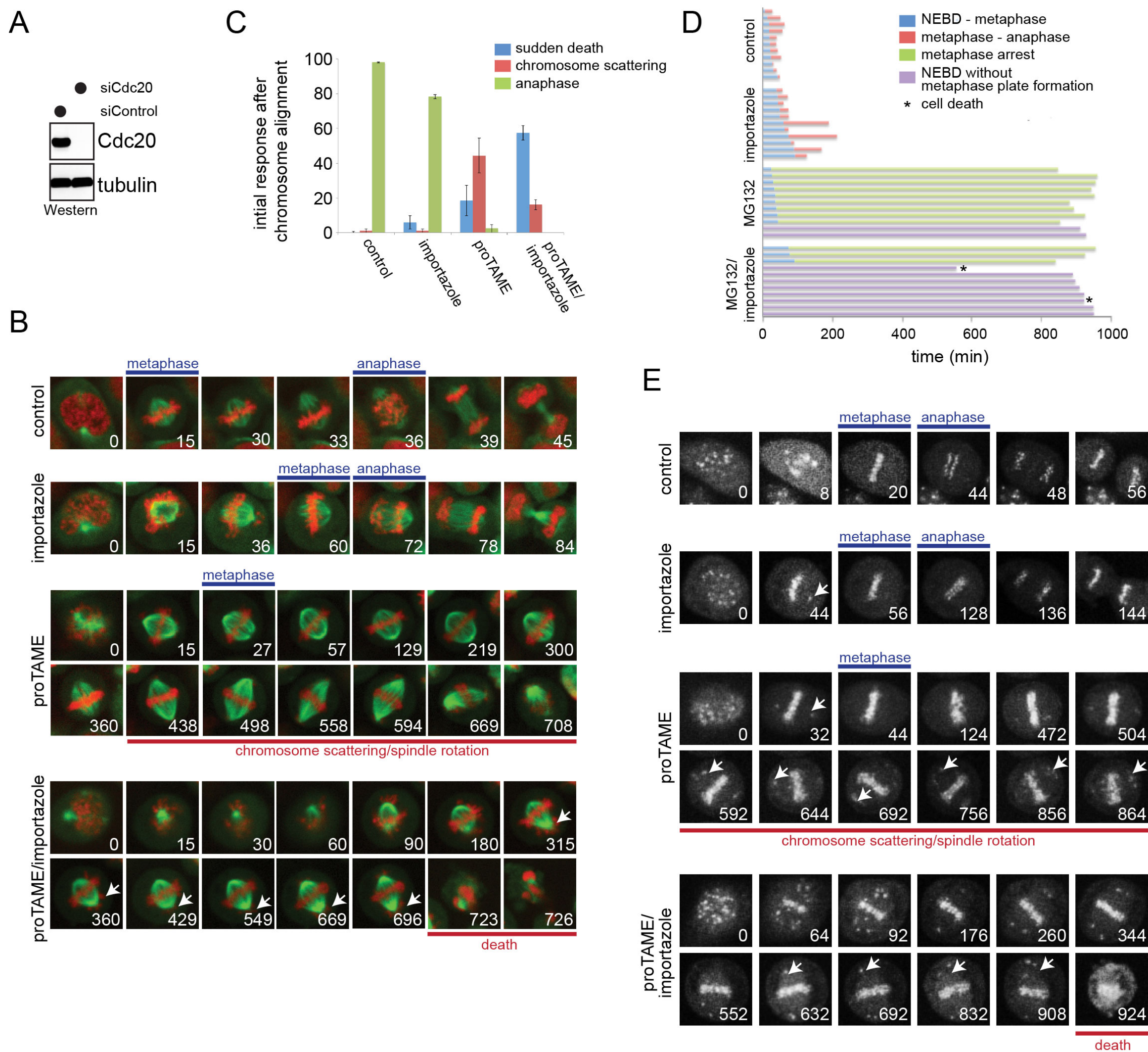


Figure S6

Figure S6 (related to Figure 7): APC/C and importin-β together regulate spindle structure and function. A. Cdc20-siRNAs efficiently deplete Cdc20 protein in cells. HeLa cells were transfected with characterized Cdc20-siRNAs (Williamson et al., 2009), and protein levels were measured with Western blotting. **B.** Small-molecule inhibition of the APC/C (with proTAME) and importin-β (with importazole) produces synergistic effects on spindle formation and cell division. HeLa cells expressing ^{mCherry}histone H2B and ^{GFP}tubulin were treated with proTAME, importazole, or both, and their progression through mitosis was monitored with video microscopy. **C.** Quantification of cell division

defects observed upon APC/C- and/or importin-β-inhibition. Results represent three independent experiments; n>50 per condition. **D.** Proteasome-inhibition synergizes with loss of importin-β-activity during mitosis. HeLa cells stably expressing ^{mCherry}histone H2B and ^{GFP}tubulin were treated with proteasome inhibitor MG132, importazole, or both, and their progression through mitosis was monitored with video microscopy. Cells were analyzed for the time taken from nuclear envelope breakdown to completion of chromosome congression (blue); the time between metaphase and anaphase initiation (red); the time for metaphase arrest (green); and the time of pre-anaphase arrest without ever establishing a metaphase plate (purple). The asterisk denotes cells that underwent cell death during the time course of the experiment. **E.** Synergistic effects of APC/C- and importin-β-inhibition during mitosis can also be observed in cells expressing a kinetochore marker. HeLa cells stably expressing ^{GFP}CenpA were treated with proTAME, importazole, or both, and their progression through mitosis was monitored by video microscopy.