Supplementary Information

Methods

Supplementary Table 1 (Excel Spreadsheet)

Supplementary Figures and Legends

Methods

Plasmids and antibodies

cDNA for human Hmmr, HURP, NuSAP, Bard1, Brca1 and Ube2S was purchased from Open Biosystems. Importin-α, importin-β, Ran, Rcc1, RanBP1, and RanGAP were kindly provided by Drs. Rebecca Heald, Karsten Weis, and Petr Kalab. The cDNAs were used to construct vectors for IVT/T and expression in cells (pCS2; pCS2-HA; pCS2-myc), and for purification in *E. coli* (pET28; pMAL), using FseI/AscI restriction enzymes. Plasmids encoding truncation mutants were generated by PCR; and point mutants were generated by site-directed mutagenesis (Qiagen). Polyclonal antibodies against importin-α and importin-β were kindly provided by Karsten Weis. The following commercially available antibodies were used: αUbcH10 (polyclonal, Boston Biochem); αUbe2S (polyclonal, Strategic Diagnostics); αCdc27 [AF3.1], αCdc20 [E-9], αc-myc [9E10] [(monoclonal, Santa Cruz); αcyclin A, αcyclin B1, αp27, αHmmr [H-90], αBrca1 [C-20], αSkp1, αHA [Y-11], αHis [G18] (polyclonal; Santa Cruz); αHURP, αBard1 (polyclonal; Bethyl Laboratories); αNuSAP (polyclonal; ProteinTech Group); αCdh1, α-αtubulin (monoclonal, Sigma); αPlk1 (Upstate), α-βactin (Abcam); αEmi1 (Invitrogen); Cy3-conjugated anti-β-tubulin (Sigma). A detailed list of all constructs, proteins, antibodies, and RNAi sequences is found in Supp. Table 1.

Recombinant proteins

All recombinant proteins were expressed as N-terminal MBP or 6xHis-tagged fusions in BL21/DE3(RIL) (^{MBP}Brca1 1-600; ^{His}importin α1; ^{His}importin α1-CT) or Rosetta/DE3 (^{MBP}importin β; ^{His}importin β; ^{His}importin βΔN; ^{His}RanQ69L). Bacteria were grown to an $O.D._{600}$ of 0.4~0.6, and protein expression was induced by 0.5 mM IPTG. After grown for 14h at room temperature, bacteria were harvested, and lysates were prepared in lysis buffer supplemented with 0.2 mg/ml lysozyme (lysis buffer A, for 6xHis-tagged proteins: 50mM NaH_2PO_4, pH 8, 500mM NaCl, 10mM imidazole; lysis buffer B, for MBP-fusions: 20mM Tris base, pH 7.5, 500mM NaCl, 1mM DTT). After sonication, lysates were cleared and recombinant proteins were purified using

Ni-NTA agarose (6xHis-tagged proteins) or amylose resin (MBP-fusions). Beads were washed twice with lysis buffer supplemented with 0.1% Trition X-100, then twice with lysis buffer. MBP-fusions were eluted in lysis buffer B supplemented with 10mM maltose. 6xHis-tagged proteins were eluted in elution buffer containing 50mM NaH_2PO_4, 500mM NaCl and 250mM imidazole. All proteins were dialyzed in 1 x PBS with 2mM DTT. Aliquotes were frozen in liquid nitrogen and stored at -80 °C. The purification of Ube2S is described in the accompanying paper (Williamson et al., submitted). UbcH10, UbcH10^{C114S}, UbcH5c, E1, securin, securin mutants, and Emi1 were purified from BL21/DE3 (RIL) or SF9 cells (E1) using His_6-tags as described before (Jin et al., 2008). Purified ubiquitin and ubiquitin mutants were obtained from Boston Biochem.

***In vitro* degradation assays**

For degradation assays, extracts of HeLa S3 cells synchronized in mitosis by thymidine/nocodazole-arrest, in early G1 phase by release from thymidine/nocodazole-arrest for 2h, and in S phase by thymidine arrest, were prepared as described before (Reddy et al., 2007; Jin et al., 2008). Extracts of asynchronous HeLa S3 cells were prepared using the same protocol. When indicated, importin-β was depleted by Ran^{Q69L}-affinity columns as described before (Ribbeck et al., 1998). Candidate substrates were synthesized by *in vitro*-transcription/translation (IVT/T) using reticulocyte lysate pre-mix (Promega) in the presence of ^{35}S-Met/Cys (TransLabel; ICN). Extracts were supplemented with energy-mix and ubiquitin as described, and ^{35}S-labeled substrates were added (Jin et al., 2008). Where indicated, recombinant proteins were added, and reactions were incubated for 2h at room temperature (for mitotic extracts) or 30°C (for G1-extracts). Reactions were stopped by addition of gel loading buffer, and analyzed by SDS-PAGE and autoradiography. The degradation of endogenous HURP and NuSAP in mitotic extracts was also analyzed in extracts. To this end, mitotic extracts were activated by addition of UbcH10, p31^{comet}, ubiquitin, and energy mix. Reactions were mixed on ice and aliquoted prior to transferring them to 25°C. At the indicated

time points, the degradation reaction in an aliquot was stopped by adding of gel loading buffer. Reactions were analyzed by SDS-PAGE and Western blotting using specific antibodies against HURP, NuSAP, cyclin B1, or β-actin.

***In vivo* degradation assays**

293T cells were transfected using TransIT-293 (Mirus), and HeLa cells were transfected using TransIT-LT1. Cells were co-transfected with candidate substrate and Cdh1 or Cdc20 in a ratio substrate/Cdh1 1:3. When indicated, ubiquitin, ubiquitin-mutants or Emi1 were transfected as well in 4:1 excess of substrate; importin-$\beta^{\Delta N}$ was used in a 3:1 ratio to substrate (amount of DNA). The amount of transfected DNA was held constant by transfection of empty pCS2, if needed. Cells were harvested 48h after transfection, and processed for Western blotting. For time-resolved degradation assays, HeLa cells were transfected with HA-tagged wild-type or mutant HURP, and synchronized at mitosis by a thymidine/nocodazole synchronization protocol. Cells were released from the nocodazole arrest by transferring them to fresh medium. During the next three hours, cells were harvested at the indicated time points and dissolved in gel loading buffer. The levels of ^{HA}HURP or the respective mutants was analyzed by Western bloting using αHA antibodies.

***In vitro* ubiquitination reactions**

For ~10 ubiquitination reactions, human APC/C was purified from 1.5ml concentrated extracts of cells synchronized in G1 by immunoprecipitation using 75μl αCdc27-antibodies and 100 μl Protein G-agarose (Roche), as described before (Jin et al., 2008). When indicated, APC/C^{Cdc20} was purified from checkpoint-extracts, and p31^{comet} was added during the ubiquitination (Reddy et al., 2007). The washed beads were incubated with human 50 nM E1, E2 (concentrations and E2 as indicated; usually 50nM UbcH10 and 100nM Ube2S), 1 $^{mg}/_{ml}$ ubiquitin or ubiquitin mutants, energy mix (15mM creatine phosphate; 2mM ATP; 2mM $MgCl_2$; 0.2mM EGTA;

pH7.5), 1 mM DTT at room temperature for 30 min. When indicated recombinant ubiquitin mutants (Boston Biochem) were added. ^{35}S-labeled substrates were synthesized by IVT/T and added to the reactions, which were analyzed by SDS-gel electrophoresis and autoradiography.

Immunoprecipitation reactions

The co-immunoprecipitation of HURP-mutants with ^{myc}importin-β, ^{myc}Tpx2, or ^{myc}Aurora A, was performed in asynchronous 293T cells, or in HeLa cells synchronized in mitosis by thymidine/nocodazole treatment. Cell lysate was prepared in swelling buffer (20mM HEPES, pH 7.5; 5mM KCl; 1.5mM $MgCl_2$; 150mM NaCl) supplemented with 1 x EDTA-free protease inhibitor cocktail (Roche) and 0.1% Triton X-100, and added to α-myc affinity beads (Santa Cruz). Incubation was carried out at 4 ° C overnight with continuous mixing. After washed three times in swelling buffer with 0.1% Triton X-100, then twice in swelling buffer, resin-bound proteins were eluted in 2 x gel loading buffer. Samples were analyzed by 10% SDS-PAGE and Western blotting.

To analyze the interaction between HURP and importin β during mitotic exit, Flp-In T-REx U-2OS cells that stably express Flag-tagged importin β or $\beta^{\Delta N}$ were prepared following manufacturer's manual (Invitrogen) and induced with 0.2 μg/ml doxycyclin (Sigma) for 48 hours. 24 hours post induction, cells were treated with 100ng/ml nocodazole for 24 hours. Mitotic cells were collected by shake-off and released into fresh medium without nocodazole. Cells were harvested at indicated time points. Cell lysates were prepared in lysis buffer (20mM HEPES, pH 7.5; 5mM KCl; 1.5mM $MgCl_2$; 1xprotease inhibitor cocktail; 150mM NaCl; 0.1% Triton X-100), and incubated with αFLAG M2 affinity gel (Sigma). Beads were carefully washed, and bound proteins were eluted with gel loading buffer. The precipitating proteins were analyzed by Western blotting using specific antibodies. To test for the interaction between HURP and importin-β in prometaphase cells, the immunoprecipitation experiment was performed in the same cell lines arrested by nocodazole treatment.

To probe for the interaction between importin-β and HURP in reticulocyte lysate, ^{35}S-HURP was expressed by IVT/T in reticulocyte lysate, and incubated with increasing concentration of Ran^{GTP} on ice for 20 minutes. The reactions were diluted 20x in dilution buffer (20mM HEPES, pH 7.5; 5mM KCl; 1.5mM $MgCl_2$; 150mM NaCl; 0.1 mg/ml BSA; 0.1% Tween20), and incubated with Protein A-agarose (Roche) charged with an importin β antibody (Bethyl Laboratories) to precipitate endogenous importin β present in the reticulocyte lysate. After an incubation at 4 ˚C for 2h, the beads were washed three times in dilution buffer, and twice in wash buffer 2 (20mM HEPES, pH 7.5; 5mM KCl; 1.5mM $MgCl_2$). Bound proteins were eluted by gel loading buffer, and analyzed by gel electrophoresis and autoradiography.

MBP pull-down assays

Amylose resin was charged with MBP fusions in MBP coupling buffer (20mM HEPES, pH 7.5; 150mM NaCl; 5mM KCl; 1.5mM $MgCl_2$; 0.1mg/ml BSA; 0.1% Tween20). The charged resin was then incubated with either *in vitro* synthesized, radioactively-labeled proteins or recombinant proteins in MBP coupling buffer for 3~4 hours at 4 ° C with continuous mixing. After washed three times in wash buffer 1 (20mM HEPES, pH 7.5; 5mM KCl; 1.5mM $MgCl_2$; 0.1% Tween20), and twice with wash buffer 2 (20mM HEPES, pH 7.5; 5mM KCl; 1.5mM $MgCl_2$), resin-bound proteins were eluted in 2 x gel loading buffer.

RNAi, immunofluorescence and quantification

The siRNA-depletion against Emi1, Ube2S, and UbcH10 was performed as described (Williamson et al., submitted). The siRNA construct against HURP targets the 3'-UTR of HURP. The target sequence is 5'-CCTTCATATTATCAATGCT-3'. HeLa cells were transfected with 100nM siRNA using oligofectamine (Invitrogen). 24 hours post transfection, HeLa cells were transfected again with 3μg empty vector or plasmids encoding HA-tagged wild-type or mutant HURP using TransIT-LT1 (Mirus). Cells were synchronized by 2mM thymidine for 24 hours, and then arrested by 100 ng/ml nocodazole for 12 hours. After release into fresh growth

medium for 2 hours, cells were fixed for immunofluorescence. Mitotic stages were determined by DNA and spindle morphology. For rescue experiments, only cells with characteristic staining for wild-type, KR2 and KR2/3 HURP were counted.

For immunofluorescence analysis of HURP localization in human cells, HeLa cells were grown to 80% confluency on glass coverslips. When indicated, cells were treated for 4min with 45mM HEPES, 45mM Pipes, 50mM EGTA, 5mM $MgCl_2$, 1% Triton X-100, pH 7.5, before fixation. Cells were fixed with formaldehyde, and incubated with primary αHURP-antibody, or αHA-antibody, followed by secondary goat-anti rabbit antibody coupled to Alexa488 (Molecular Probes). Tubulin was stained with Cy3-β-tubulin-antibody (Sigma). Cells were visualized using 60x magnification on an Olympus IX71 microscope. Pictures were analyzed using ImageJ software.

To measure the abundance of endogenous, spindle-bound HURP in pre-anaphase HeLa cells, cells were simultaneously transfected with siRNA constructs targeting UbcH10, Ube2S and $p31^{comet}$ using Lipofectamine2000 (Invitrogen). Where indicated, cells were treated 48h post transfection with 20μM MG132 (Boston Biochem) for 4 to 6 hours. Cells were extracted with 1% Triton X-100, fixed with formaldehyde, and incubated with αHURP-antibody followed by goat anti-rabbit Alexa488 (Molecular Probes). The fluorescence intensity of spindle-bound HURP was quantified by ImageJ software with background subtracted from each analyzed image. Images of ~150 metaphase cells from three independent experiments were analyzed.

Synchronization

To analyze the levels of HURP, Hmmr, and Bard1 during exit from mitosis, HeLa cells were treated with 2mM thymidine for 24h, released for 3h, and treated with 100ng/ml nocodazole for 11h. Cells were arrested in mitosis in the presence of nocodazole for ~2h. Cells were released by extensive washing with prewarmed medium, and plated into fresh DMEM, 10%FBS. Samples were taken at the indicated time points and processed for Western blot analysis. For

synchronization in quiescence, T24 cells were serum-starved in the presence of 0.1% FBS for 72h, and released into fresh DMEM, 10% FBS. Samples were taken at the indicated time points and processed for Western blot analysis as described before.

Supplementary Figures

Supplementary Figure 1: Analysis of Bard1, Hmmr, and HURP-degradation in extracts. A. Bard1 and Hmmr, are degraded in mitotic extracts by APC/C^{Cdc20}. The ^{35}S-labeled candidate substrates were added to extracts of checkpoint-arrested cells (mitotic extracts). APC/C was activated in mitotic extracts by addition of UbcH10 and $p31^{comet}$. APC/C-inhibitors were added to test for specificity. The reactions were analyzed by autoradiography. Bard1 and HURP are phosphorylated by CDKs in mitotic extracts, if APC/C is strongly inhibited. The asterisk marks a stable truncation product of the Hmmr-IVT/T. **B.** Bard1, Hmmr and HURP are stable in extracts with inactive APC/C. ^{35}S-labeled Bard1, Hmmr, and HURP were incubated in extracts of asynchronous cells (upper panel), or in extracts of cells synchronized in S phase (lower panel), and detected by autoradiography. In both extracts, APC/C is inactive. **C.** Phosphorylation of Bard1 is not required for its APC/C-dependent degradation. The known CDK-phosphorylation sites in Bard1 (Choudhury et al., 2005) were mutated to generate $Bard1^{\Delta CDK}$. The degradation of ^{35}S-Bard1 and ^{35}S-$Bard1^{\Delta CDK}$ was analyzed in mitotic extracts with active APC/C^{Cdc20}, using autoradiography.

Supplementary Figure 2: Analysis of SAF degradation in vivo. A. Bard1 is degraded when bound to Brca1. Formation of the Bard1/Brca1-complex results in autoubiquitination of both proteins, which can be used as an indirect readout for complex-formation. Bard1 and ^{HA}Brca1(1-400) were co-expressed with ^{His}ubiquitin and the APC/C-activators Cdc20/Cdh1 or the APC/C-inhibitor Emi1. Autoubiquitinated Bard1 and Brca1 were purified by NiNTA-agarose under denaturing conditions, and levels were detected by Western blotting. **B.** Depletion of Emi1 results in APC/C-dependent degradation of SAFs. HeLa cells were treated with siRNAs against Emi1 or Emi1, UbcH10, Ube2S, as indicated. The co-depletion of UbcH10 and Ube2S results in inactivation of the APC/C (Williamson et al., 2009), leading to an increase in substrate levels compared to Emi1-depleted cells. The levels of the potential APC/C-substrates were

determined by Western blotting using specific antibodies. **C.** Bard1, Hmmr, HURP, and NuSAP are co-regulated with APC/C-substrates upon re-entry of quiescent cells into the cell cycle. T24 cells were synchronized in quiescence, and allowed to re-enter the cell cycle by serum addition. Samples were taken at the indicated time points, and protein levels determined by Western blotting.

Supplementary Figure 3: Identification of domains required for the APC/C-dependent degradation of SAFs. A. Deletion analysis of Bard1. ^{35}S-Bard1 and the indicated truncation mutants were synthesized by IVT/T and incubated for 2h in mitotic extracts with active APC/C^{Cdc20} (APC/C^{Cdc20} was activated by addition of p31^{comet} and UbcH10). When indicated, the APC/C-inhibitor Emi1 was added, and degradation products were analyzed by autoradiography (left panel). The middle and right panels show a summary of the truncation analysis, including domains present in the various mutants and their sensitivity towards APC/C (+: degradation; -: stable). **B.** Deletion analysis if Hmmr. ^{35}S-Hmmr and the indicated truncation mutants were tested for degradation as described above. **C.** Deletion analysis of HURP. ^{35}S-HURP and the indicated truncation mutants were tested for degradation as described above. HURP was incubated in Ran^{Q69L}-supplemented G1-extract containing active APC/C^{Cdh1}. **D.** Deletion analysis of NuSAP. ^{35}S-NuSAP and the indicated truncation mutants were tested for degradation as described above. NuSAP was incubated in mitotic extract containing active APC/C^{Cdc20}. Activated extract has been supplemented with Ran^{Q69L}, which causes the dissociation of the importin-β inhibitor, as described later in the manuscript.

Supplementary Figure 4: Regulation of APC/C-activity by importin-β and Ran. A. Importins do not affect the ubiquitination of cyclin B1 by the APC/C. ^{35}S-cyclin B1 was incubated with APC/C^{Cdh1}, UbcH10 and Ube2S in the presence of the indicated importin proteins. The ubiquitination products were analyzed by autoradiography. **B.** Importin-β and Ran^{GTP} do not regulate the ubiquitination of Plk1. ^{35}S-Plk1 was incubated with APC/C^{Cdh1},

Ube2S, UbcH10, and importin-β/α and Ran^{Q69L} as indicated, and reactions were analyzed by autoradiography. **C.** Ran^{GTP} promotes SAF-dissociation from importin-β and ubiquitination by APC/C. ^{35}S-HURP was synthesized in reticulocyte lysate. One half of the reaction was incubated with increasing concentrations of Ran^{Q69L} and APC/C^{Cdh1}, and the ubiquitination of HURP was analyzed by autoradiography, as described before. The other half of the reaction was incubated with increasing concentrations of Ran^{Q69L}. Then, importin-β, which is abundant in reticulocyte lysate, was immunoprecipitated with specific antibodies. IPs were washed and co-IPed HURP was detected by autoradiography. The control reaction of the IP (input) was incubation with non-specific IgG. **D.** Ran^{GTP} triggers degradation of HURP, and less efficiently, NuSAP in G1-extracts. ^{35}S-HURP and ^{35}S-NuSAP were incubated in G1-extracts supplemented with GTP-bound Ran^{Q69L}. Various APC/C- and proteasome inhibitors were added as indicated, and the turnover of the candidate substrates was analyzed by autoradiography. **E.** Ran^{GTP} promotes the degradation of HURP and NuSAP by APC/C^{Cdc20} in mitotic extracts. APC/C^{Cdc20} was activated in mitotic extracts by addition of UbcH10 and $p31^{comet}$. Then, GTP-bound Ran^{Q69L} was added, and the turnover of ^{35}S-HURP and ^{35}S-NuSAP was analyzed as described above. **F.** Ran and importins do not affect the degradation of cyclin B1 in mitotic extracts. APC/C^{Cdc20} was added in mitotic extracts by addition of UbcH10 and $p31^{comet}$, and the degradation of endogenous cyclin B1 was analyzed over time by Western blot. As indicated on the left, different Ran and importins were added before the degradation reaction was started. **G.** Ran and importins do not affect the degradation of cyclin B1 in G1-extracts. The degradation of ^{35}S-cyclin B1 was analyzed over time in G1-extracts by autoradiography. Ran and importins were added as indicated on the left.

Supplementary Figure 5: Identification and characterization of importin-β binding sites in HURP. A. Importin-β recognizes basic amino acids in the amino-terminus of HURP. MBP or ^{MBP}importin-β were immobilized on amylose resin, and incubated with ^{35}S-$HURP^{117}$ or $HURP^{117}$-mutants, in which stretches of basic residues were exchanged with alanine. Beads were

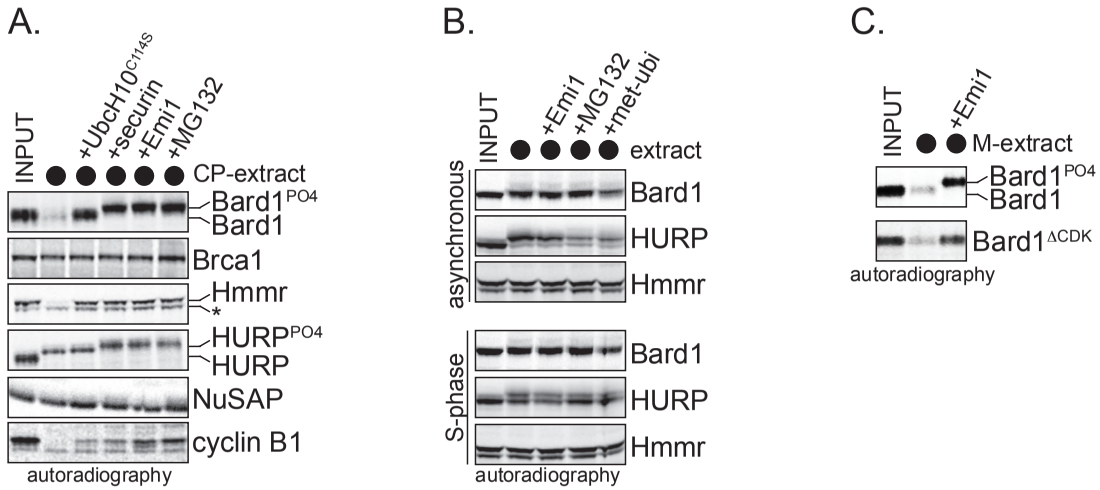
washed, and bound proteins were detected by autoradiography. **B.** Mutation of KR2 or KR2/3 does not affect binding of HURP to Tpx2. 293T cells were co-transfected with ^{HA}HURP, ^{HA}HURP^{KR2}, or ^{HA}HURP$^{KR2/3}$, and ^{myc}Tpx2. Tpx2 was precipitated by αmyc-beads, and co-purifying HURP proteins were analyzed by Western blotting using αHA-antibodies. **C.** Its importin-β binding site is not required for the interaction of HURP with Aurora A. 293T cells were co-transfected with ^{HA}HURP, ^{HA}HURP^{KR2}, or ^{HA}HURP$^{KR2/3}$, and ^{myc}Aurora A. Aurora A was precipitated by αmyc-beads, and co-purifying HURP proteins were analyzed by Western blotting using αHA-antibodies.

Supplementary Figure 6: Importin-β and the APC/C bind to overlapping motifs in the amino-terminus of HURP. A. Sequence of the N-terminal 60 residues of HURP. The location of the importin-binding motifs KR2 and KR3 is shown above, while the APC/C-binding motifs, the D-, KEN-, and TEK-boxes, are depicted below. **B.** Mutation of KR2/3 mostly ablates ubiquitination of HURP by the APC/C. Mitotic APC/C^{Cdc20} was purified from mitotic extracts and activated by p31^{comet}/UbcH10. The *in vitro* ubiquitination reactions also included Ube2S. HURP or HURP$^{KR2/3}$ were synthesized by IVT, and their ubiquitination by APC/C^{Cdc20} was analyzed by autoradiography. Importin-β was added as indicated. **C.** Degradation of HURP^{KR2} and HURP$^{KR2/3}$ in G1-extracts. HURP, HURP^{KR2}, and HURP$^{KR2/3}$ were synthesized by IVT, and incubated in G1-extracts in the presence of Ran^{GTP} or Ran^{GTP} and importin-β$^{\Delta N}$. When indicated, Emi1 was added. The degradation products were analyzed by autoradiography. **D.** Mutation of KR2/3 stabilizes HURP *in vivo*. ^{HA}HURP and ^{HA}HURP$^{KR2/3}$ were co-expressed with Cdh1 in 293T cells, where indicated, and the levels of the HURP-mutants was measured by Western blotting against the HA-epitope.

Supplementary Figure 7: Cell-cycle dependent regulation of HURP by importin-β. A. Importin-β-binding is not required for HURP-stabilization during interphase, when APC/C is inactive. The levels of ^{HA}HURP and ^{HA}HURP^{KR2} were analyzed in interphase HeLa cells by

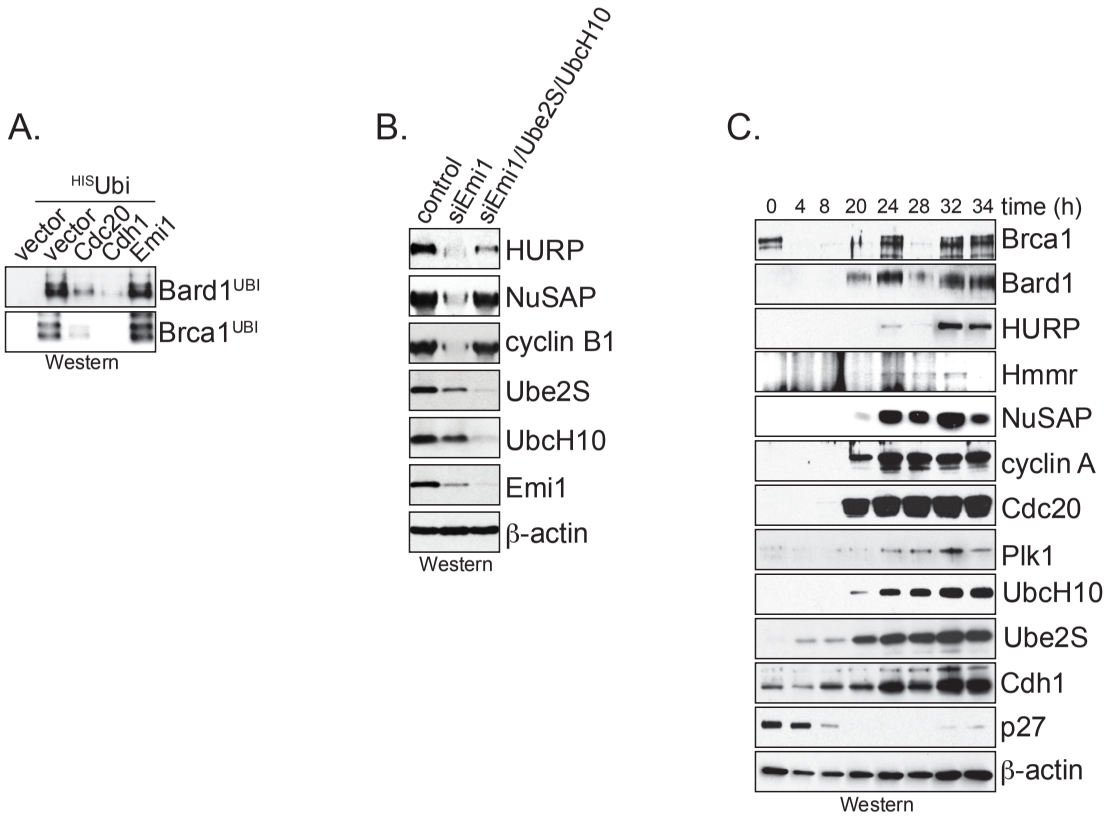
fluorescence microscopy as described above. The difference in localization is due to loss of importin-β binding. We did observe some cells with HURP also in the cytoplasm, in agreement with previous observations that HURP is shuttling between the nucleus and the cytoplasm (Sillje et al., 2006). **B.** Loss of importin-β-binding results in degradation of HURP during prometaphase. HeLa cells were transfected with ^{HA}HURP, ^{HA}HURP^{KR2}, or ^{HA}HURP$^{KR2/3}$, and arrested in mitosis by treatment with nocodazole (upper panel), or S phase by treatment with thymidine (lower panel). Where indicated, the proteasome inhibitor MG132 (20μM) was added for 5h. The levels of HURP mutants were analyzed by Western blotting using αHA-antibodies. As a loading control, levels of β-actin were determined by Western blot. **C.** Importin-β binds HURP and NuSAP during prometaphase. U2OS cells stably expressing ^{FLAG}importin-β or ^{FLAG}importin-β$^{\Delta N}$ were synchronized in prometaphase with nocodazole, and importin-complexes were purified on FLAG-agarose. Were indicated, the expression of importin-β was stimulated by doxycycline treatment; however, both cell lines showed expression of epitope tagged importin-β-constructs in the absence of additional doxycycline (not shown). Co-purifying proteins were analyzed by Western blot using specific antibodies. Note, that expression of importin-β$^{\Delta N}$, even at low concentrations, increases the abundance of HURP and NuSAP. **D.** Mitotic importin-β/SAF complexes are dissociated by Ran^{GTP}. ^{FLAG}importin-β or ^{FLAG}importin-β$^{\Delta N}$ were stably expressed in U2OS cells and affinity-purified after synchronization of cells in mitosis by nocodazole-treatment. The complexes were then incubated with buffer, GTP-bound Ran^{Q69L}, or GDP-bound Ran^{T24N}, respectively. Proteins binding to the importins were detected by Western blotting using specific antibodies. **E.** The majority of HURP and NuSAP associates with importins during prometaphase. Extracts of HeLa cells arrested in prometaphase with nocodazole were incubated with immobilized MBP or ^{MBP}importin-β$^{\Delta N}$ (which is insensitive to Ran^{GTP}). As indicated, the mitotic extract was pre-incubated with GTP-bound Ran^{Q69L} to dissociate endogenous SAF-importin complexes. HURP and most of NuSAP only binds MBPimportin-β$^{\Delta N}$, if complexes to endogenous importins were dissociated by the Ran^{Q69L}-treatment. **F.** Endogenous HURP and NuSAP are degraded in cells with an active spindle

checkpoint. HeLa cells were either grown asynchronously, or they were synchronized in mitosis by activation of the spindle checkpoint through treatment with nocodazole (noc) or taxol (tax). The cells were treated with proteasome inhibitor MG132 for 4h, were indicated. The abundance of SAFs, Plk1 (which is not degraded in checkpoint-arrested cells) and cyclin A (which is known to be degraded in checkpoint-arrested cells) was analyzed by Western blot using specific antibodies. The mitotic index of the experiments is shown in the panel below. **G.** Overexpression of NuSAP results in strong spindle assembly defects in almost all mitotic cells. Two examples of mitotic cells expressing NuSAP or the stabilized NuSAP$^{\Delta DK}$ are shown (green: NuSAP; red: tubulin; blue: DNA).

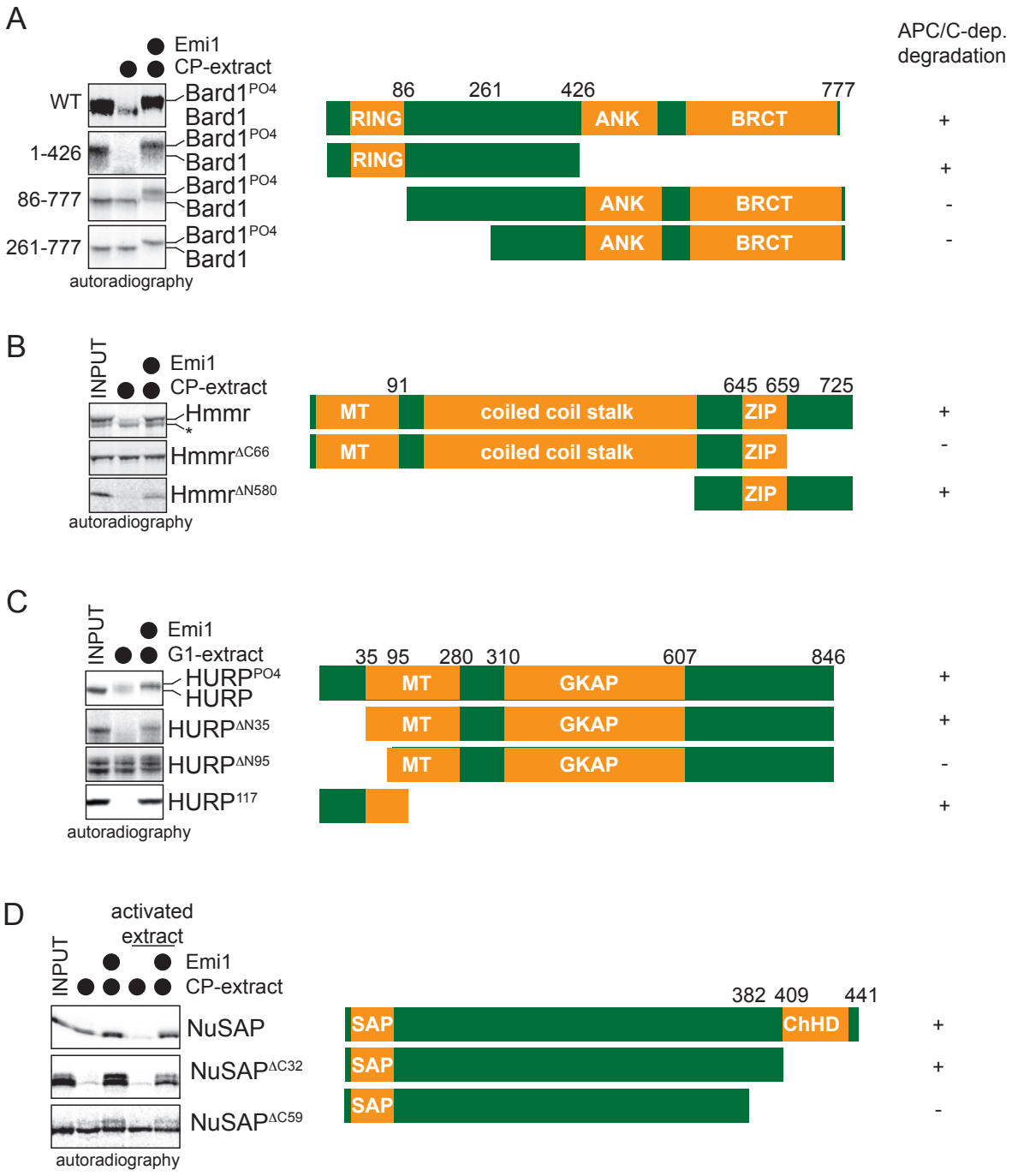


Supp. Figure 1

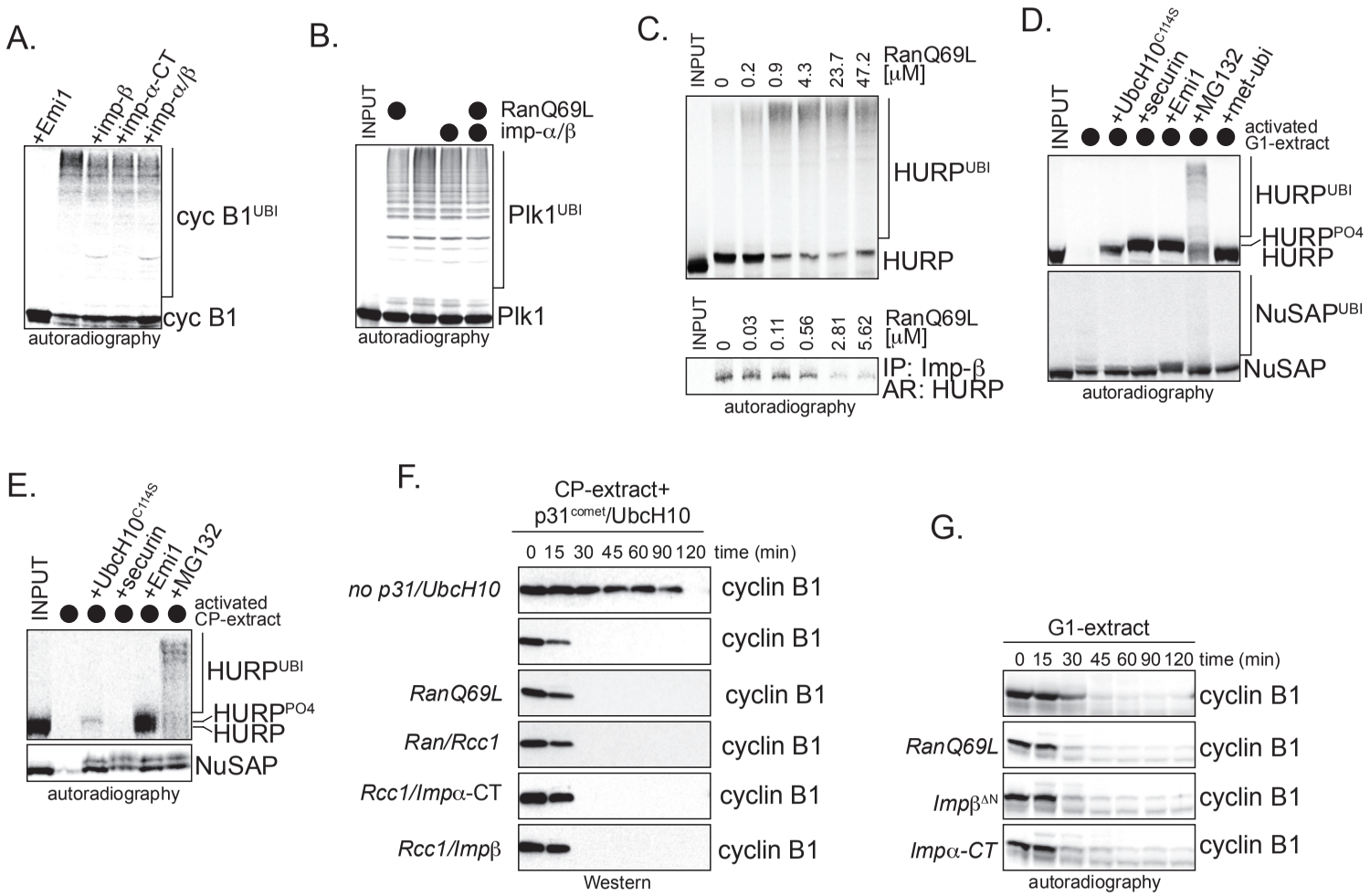
Supplemental Text and Figures



Supplementary Figure 2

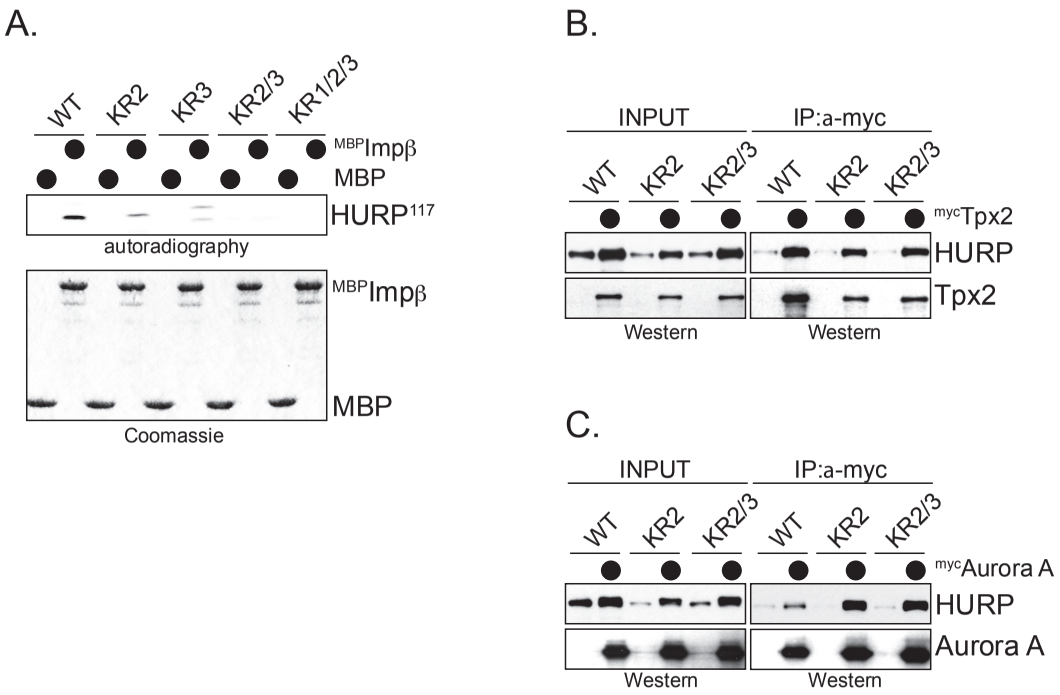


Supp. Figure 3

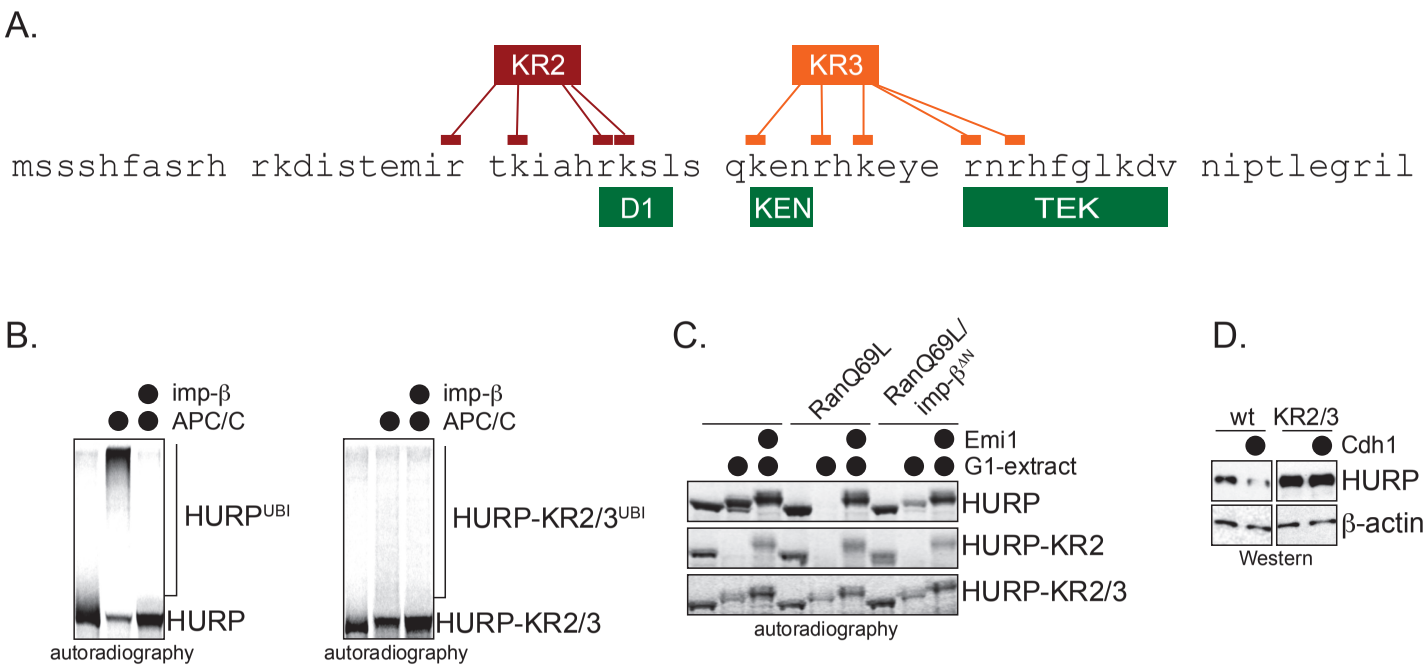


Supp. Fig. 4

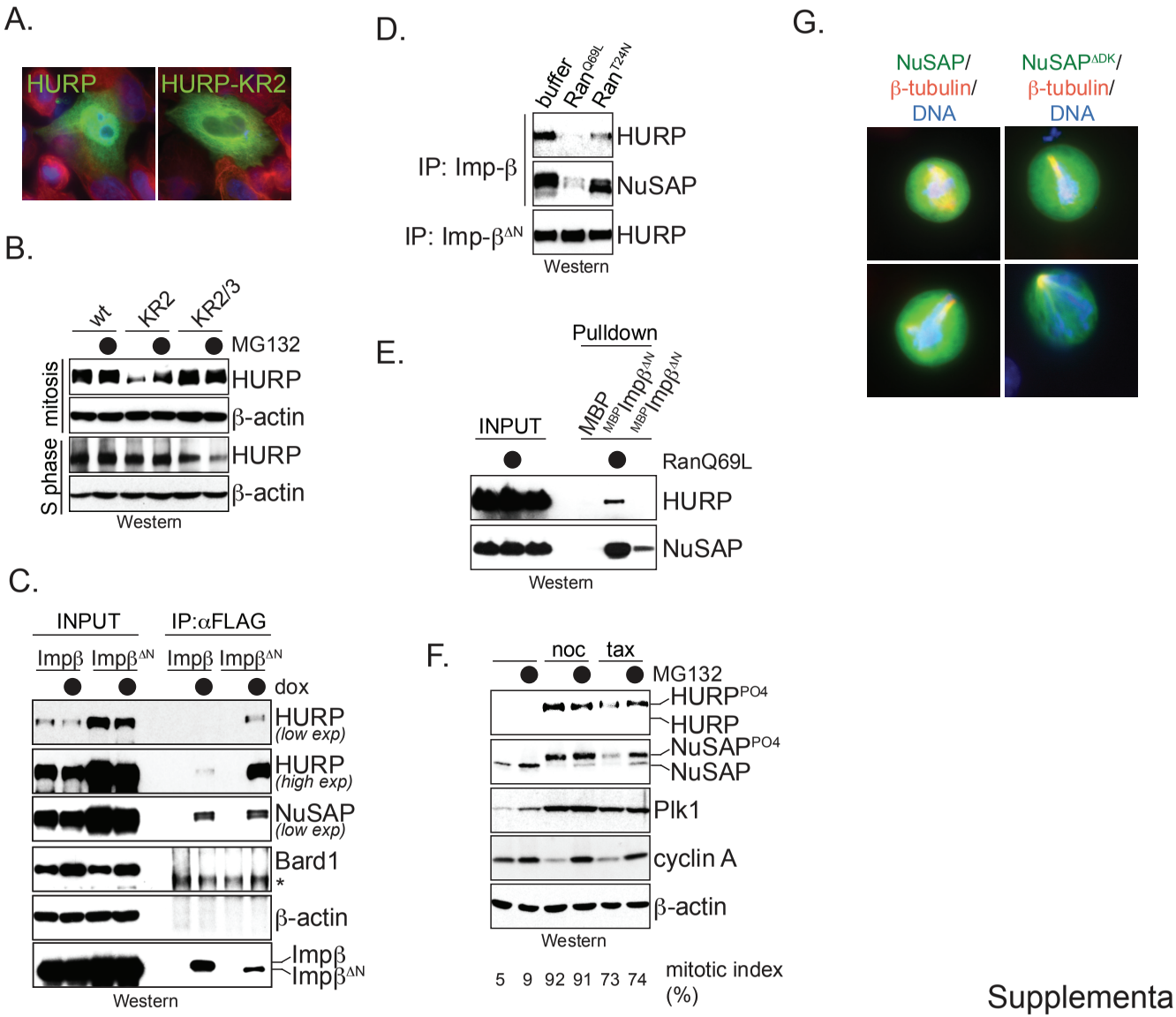
Supplemental Text and Figures



Supp. Figure 5



Supp. Figure 6



Supplementary Figure 7

Supplemental Text and Figures

construct		
IVT	SAFs, APC substrates & importins	full-length
	Bard1 1-426	truncation
	Bard1 86-777	truncation
	Bard1 261-777	truncation
	Bard1 DD1	full-length
	Bard1 DD1/2	full-length
	Bard1 C50G/H68A	full-length
	Bard1 DCDK	full-length
	Hmmr DC66	truncation
	Hmmr DN580	truncation
	Hmmr DD4	full-length
	Hmmr DK2	full-length
	Hmmr DD4/K2	full-length
	HURP DN35	truncation
	HURP DN95	truncation
	HURP KR2	full-length
	HURP KR2/3	full-length
	HURP NLS-KR2	full-length
	HURP KR2DDKT	full-length
	HURP DD1/K	full-length
	HURP DD1/K/TEK	full-length
	HURP DD1/K/TEK/D2	full-length
	HURP 1-117	truncation
	HURP 1-117 KR2	truncation
	HURP 1-117 KR3	truncation
	HURP 1-117 KR2/3	truncation
	HURP 1-117 KR1/2/3	truncation
	NuSAP DC32	truncation
	NuSAP DC59	truncation
	NuSAP DD/K	full-length
	NuSAP KR	full-length
in vivo expression	Bard1	full-length
	HA-Brca1 1-400	truncation
	Bard1 D1	full-length
	Bard1 D1D2	full-length
	Myc-Cdh1, Cdc20, Emi1	full-length
	HA-Cdh1	full-length
	His-Ub from Lingyan	full-length
	His-Ub from Lingyan	full-length
	Myc-Hmmr LR3	full-length
	Myc-Hmmr LR3 D4	full-length
	Myc-Hmmr LR3 K2	full-length
	Myc-Hmmr LR3 D4/K2	full-length
	Myc-Hmmr LR3/D4/TEK	full-length
	Myc-Hmmr LR3/K2/D6	full-length
	HA-HURP	full-length
	HA-HURP KR2	full-length
	HA-HURP KR2/3	full-length
	HA-HURP KR2DDKT	full-length