Molecular Cell
Article

Regulated Degradation of Spindle Assembly Factors by the Anaphase-Promoting Complex

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DOI 10.1016/j.molcel.2010.02.038

SUMMARY

The ubiquitin ligase anaphase-promoting complex (APC/C) is essential for cell division in all eukaryotes. Loss of APC/C activity arrests cells at metaphase and results in severe aberrations of the mitotic spindle, but how the APC/C regulates spindle formation is not understood. Here, we report that the APC/C promotes the ubiquitination and degradation of four proteins required for Ran-dependent spindle assembly: Bard1, Hmmr, HURP, and NuSAP. Among these substrates, HURP and NuSAP can be degraded during spindle formation when the spindle checkpoint is active. Their degradation requires additional layers of regulation, and both SAFs are only degraded after being released from their inhibitor importin β by Ran^{GTP}. Our findings reveal a tightly regulated mechanism by which the APC/C and the GTPase Ran control the abundance of active spindle assembly factors to achieve the accurate formation of the mitotic spindle.

INTRODUCTION

Faithful chromosome segregation depends on the robust assembly of the mitotic spindle. In dividing cells of higher eukaryotes, the microtubules comprising the spindle originate from centrosomes and chromosomes. Whereas centrosomes act as microtubule-organizing centers, from where microtubules grow until being captured by kinetochores (Kirschner and Mitchison, 1986), the chromosomes promote growth of microtubules by regulating a conserved GTPase, Ran (Clarke and Zhang, 2008; Kalab and Heald, 2008).

Similar to most GTPases, Ran is active when bound to GTP (Ran^{GTP}) but inactive when loaded with GDP (Ran^{GDP}). The charging of Ran with GTP requires the guanine nucleotide exchange factor Rcc1, which accumulates on mitotic chromosomes (reviewed in Kalab and Heald, 2008). Conversely, cytoplasmic RanGAP and RanBP1 increase the GTPase activity of Ran to produce Ran^{GDP}. The spatial separation of Ran GTP binding and hydrolysis results in a gradient of Ran^{GTP}, with its highest concentration around chromatin (Kalab et al., 2002, 2006; Caudron et al., 2005).

Ran^{GTP} exerts its function in spindle formation by triggering the dissociation of spindle assembly factors (SAFs) from nuclear transport receptors of the importin β family (Gruss et al., 2001; Nachury et al., 2001; Wiese et al., 2001). The importins are inhibitors of Ran-dependent SAFs; for example, importin α/β interferes with the capacity of Tpx2 to promote microtubule polymerization and Aurora A activation (Gruss et al., 2001), and importin β inhibits the activity of the SAFs HURP and NuSAP to nucleate and crosslink spindle microtubules (Ribbeck et al., 2007; Koffa et al., 2006; Silljé et al., 2006). The local Ran-dependent release of SAFs from importins provides a molecular explanation for microtubule nucleation in the vicinity of mitotic chromosomes.

Although importins are highly abundant in human cells (Ribbeck et al., 1998), their regulatory capacity can be overwhelmed, and the concentration of SAFs during mitosis has to be tightly controlled. Increasing the levels of Ran-dependent SAFs leads to defective spindle formation and chromosome missegregation (Stewart and Fang, 2005; Li et al., 2007; Wong et al., 2008). As a consequence, the aberrant expression of Ran pathway components, such as the tumor suppressor Brca1-Bard1 or the oncogene Hmmr, has been linked to the development of tumors with abnormal spindle structures (Joukov et al., 2006; Pujana et al., 2007). Surprisingly, except for Tpx2, mechanisms regulating the abundance of Ran-dependent SAFs during mitosis are not known.

Most eukaryotic cell-cycle regulators are controlled by ubiquitin-dependent proteolysis, which depends on the recognition of substrates by E3 enzymes (Deshaies and Joazeiro, 2009; Wickliffe et al., 2009). Among the ~600 human E3s, the anaphase-promoting complex (APC/C) is an attractive candidate for regulating the turnover of SAFs. The APC/C and its physiological E2s UbcH10 and Ube2S are required for progression of cells through mitosis, when spindle formation takes place (Peters, 2006; Williamson et al., 2009). In addition, the APC/C localizes to the poles of a growing spindle (Tugendreich et al., 1995; Kraft et al., 2003), and depletion of APC/C subunits, codepletion of UbcH10 and Ube2S, or expression of APC/C inhibitors result in spindle defects (Ban et al., 2007; Goshima et al., 2007; Somma et al., 2008; Williamson et al., 2009). Moreover, the APC/C triggers the degradation of Tpx2 after completion of spindle assembly (Stewart and Fang, 2005). However, whether the APC/C controls SAFs during spindle formation has not been determined, and its role in regulating spindle assembly is not well understood.

Here, we report that the APC/C is responsible for the degradation of four Ran-dependent SAFs: Bard1, Hmmr, HURP, and NuSAP. The proteolysis of HURP and NuSAP can occur during spindle formation and is regulated by a mechanism centered

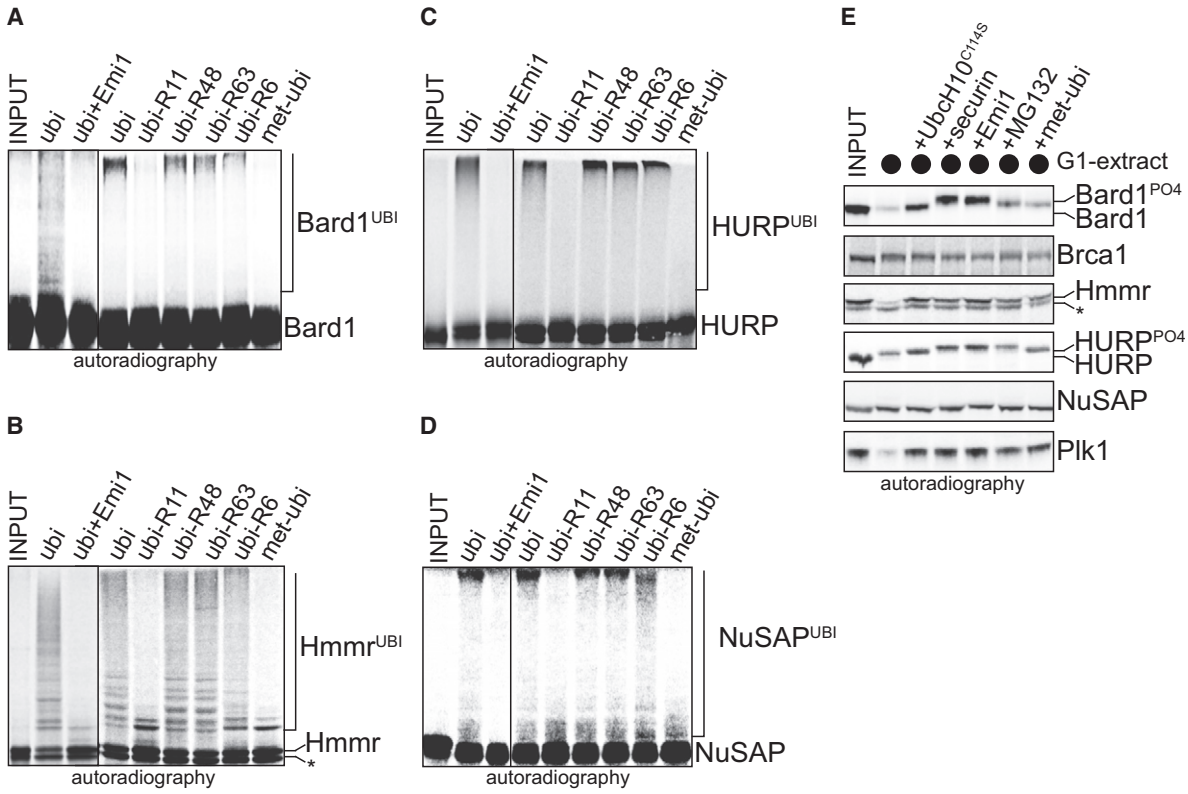

Figure 1. Bard1, Hmmr, HURP, and NuSAP Are APC/C Substrates In Vitro

(A) Bard1 is ubiquitinated by APC/C. ^{35}S-Bard was synthesized by IVT/T and added to APC/C^{Cdc20}, UbcH10, Ube2S, and p31^{comet}. As indicated, reactions were supplemented with Emi1 or ubiquitin mutants (ubi-R11: Lys11 of ubiquitin is changed to Arg). Reaction products were analyzed by autoradiography.

(B) Hmmr is ubiquitinated by APC/C. The ubiquitination of ^{35}S-Hmmr by APC/C^{Cdh1}, UbcH10, and Ube2S was analyzed as described above. The asterisk marks a truncation product of the Hmmr-IVT.

(C) HURP is ubiquitinated by APC/C. The ubiquitination of ^{35}S-HURP by APC/C^{Cdh1}, UbcH10, and Ube2S was analyzed as described above.

(D) NuSAP is ubiquitinated by APC/C. The ubiquitination of ^{35}S-NuSAP by APC/C^{Cdh1}, UbcH10, and Ube2S was analyzed as described above.

(E) Bard1 and Hmmr are degraded in an APC/C-dependent manner. The turnover of ^{35}S-labeled proteins was analyzed in G1 extracts supplemented with UbcH10. UbcH10^{C114S} (dominant-negative E2), securin (competitive inhibitor), Emi1 (APC/C inhibitor), MG132 (proteasome inhibitor), and methylubiquitin (chain formation inhibitor) were added as indicated. Reactions were incubated for 2 hr and analyzed by autoradiography. In extracts, Bard1 and HURP were phosphorylated by cyclin CDKs when endogenous cyclin B1 was stabilized due to strong APC/C-inhibition. We ensured that CDK-dependent phosphorylation is not responsible for stabilization of Bard1 in the absence of APC/C (Figure S1C). The asterisk marks a truncation product of the Hmmr-IVT/T.

on their inhibitor importin β and Ran^{GTP}. Our results suggest that spindle formation relies on the proper activation and correctly timed degradation of SAFs, which is brought about by a unique interplay between the GTPase Ran and the APC/C.

RESULTS

Bard1, Hmmr, HURP, and NuSAP Are Substrates of the APC/C

To identify substrates of the APC/C required for spindle assembly, we performed an in vitro expression cloning screen. We synthesized ~40 spindle-binding proteins by in vitro transcription/translation (IVT/T) and monitored their ubiquitination by the APC/C and its E2s UbcH10 and Ube2S (Williamson et al., 2009). To test for specificity, we ensured that the ubiquitination of potential substrates was blocked by the APC/C inhibitor Emi1 or by a ubiquitin mutant lacking Lys11, as expected for human APC/C (Jin et al., 2008). Using this approach, we identified Bard1, Hmmr, HURP, and NuSAP as candidate substrates, which were modified with K11-linked ubiquitin chains in an APC/C-dependent manner (Figures 1A–1D). Bard1, a subunit of the Brca1-Bard1 tumor suppressor, acts in spindle pole formation (Joukov et al., 2006); Hmmr regulates the localization of Tpx2 at the spindle pole (Groen et al., 2004); and HURP and NuSAP nucleate and crosslink microtubules in the vicinity of chromatin (Raemaekers et al., 2003; Koffa et al., 2006; Silljé et al., 2006; Wong and Fang, 2006).

To test whether these substrates are degraded in an APC/C-dependent manner, we monitored their stability in human extracts. Bard1 and Hmmr were turned over in G1 extracts with active APC/C^{Cdh1}, but not if APC/C was inhibited by a dominant-negative mutant of the APC/C-E2 UbcH10 (UbcH10^{C114S}), the APC/C-inhibitor Emi1, an excess of a competing APC/C-substrate, or the proteasome inhibitor MG132 (Figure 1E). Bard1 and Hmmr were also degraded in mitotic extracts with active APC/C^{Cdc20}, but both proteins were stable in S phase

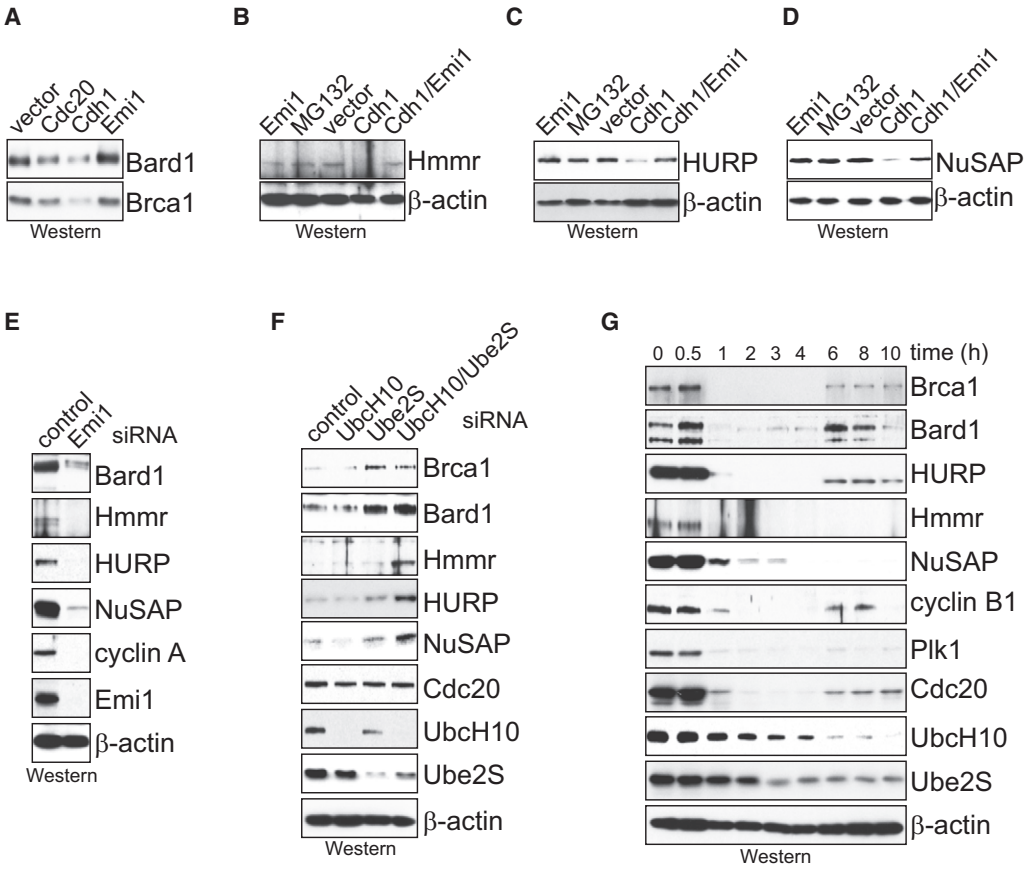

Figure 2. Bard1, Hmmr, HURP, and NuSAP are APC/C Substrates In Vivo

(A) Bard1 is degraded by APC/C in vivo. 293T cells were transfected with Bard1, ^{HA}Brca1 (amino acids 1–400), and either the APC/C activators Cdc20/Cdh1 or the APC/C inhibitor Emi1. The levels of Bard1 and Brca1 were determined by western blot using αBard1 and αHA antibodies.

(B) Hmmr is degraded by APC/C in vivo. Hmmr was transfected with Cdh1 and Emi1 as indicated, and levels of Hmmr were analyzed by western blot using αHmmr antibodies.

(C) HURP is degraded by APC/C in vivo. 293T cells were transfected with HURP, Cdh1, and Emi1 as indicated, and the levels of HURP were analyzed by western blot using αHURP antibodies.

(D) ^{HA}NuSAP is degraded by the APC/C in vivo. NuSAP was transfected with Cdh1 and Emi1 as described above, and levels of NuSAP were analyzed by western blot using αHA antibodies.

(E) APC/C activation depletes Bard1, Hmmr, HURP, and NuSAP from cells. Emi1 was depleted from HeLa cells using a characterized siRNA, and the abundance of indicated proteins was analyzed by western blot.

(F) Bard1, Hmmr, HURP, and NuSAP levels increase in HeLa cells upon APC/C inhibition caused by depletion of UbcH10 and Ube2S. The levels of indicated proteins were analyzed by western blot.

(G) Bard1, Hmmr, HURP, and NuSAP are degraded upon exit from mitosis. HeLa cells arrested in mitosis by thymidine/nocodazole were released into fresh medium. Samples were taken at different time points, and the levels of indicated proteins were determined by western blot.

extracts or in extracts of asynchronous cells with inactive APC/C (Figures S1A and S1B available online). Surprisingly, despite being strongly ubiquitinated by purified APC/C, HURP and NuSAP were only incompletely degraded in G1 or mitotic extracts with active APC/C, suggesting that their turnover is subject to additional layers of regulation, as discussed below (Figures 1E and S1A).

To determine whether the APC/C is able to promote the degradation of these substrates in vivo, we overexpressed the APC/C activators Cdc20 or Cdh1 in 293T cells. As previously observed for other APC/C substrates, this treatment triggered the degradation of Bard1 (Figure 2A), and further experiments implied that Bard1 was recognized by the APC/C when bound to its partner Brca1 (Figure S2A). Brca1 was also degraded following APC/C activation in cells. However, we did not observe Brca1 degradation in extracts (Figure 1E) nor its ubiquitination by purified APC/C (data not shown), suggesting that it might not be a direct APC/C substrate. In addition, increased levels of Cdh1 also resulted in the degradation of Hmmr, HURP, and NuSAP, which could be blocked by parallel expression of the APC/C inhibitor Emi1 (Figures 2B and 2D). Thus, the APC/C is able to trigger the proteolysis of Bard1, Hmmr, HURP, and NuSAP in cells.

For the analysis of endogenous proteins, we altered the APC/C activity in HeLa cells by depleting APC/C regulators. To activate the APC/C, we decreased the levels of its inhibitor Emi1

using a characterized siRNA (Williamson et al., 2009). This treatment markedly reduced the abundance of Bard1, Hmmr, HURP, and NuSAP, as well as that of known APC/C substrates (Figure 2E). If the APC/C-E2s UbcH10 and Ube2S were depleted in conjunction with Emi1, no degradation was observed (Figure S2B). Conversely, the inhibition of the APC/C by depletion of UbcH10 and Ube2S increased the levels of Bard1, Hmmr, HURP, and NuSAP (Figure 2F), strongly suggesting that the endogenous SAFs are degraded by the APC/C.

We next tested whether the candidate substrates are regulated during cell-cycle progression in parallel with known APC/C substrates. Similar to most substrates of the APC/C, Bard1, Hmmr, HURP, and NuSAP accumulated in HeLa cells arrested in mitosis with nocodazole, and they were degraded upon exit from mitosis (Figure 2G). All candidate substrates were also absent in quiescent cells with active APC/C, but they were coexpressed with APC/C substrates upon cell-cycle entry (Figure S2C). Thus, Bard1, Hmmr, HURP, and NuSAP are coregulated with known APC/C substrates during cell-cycle progression. Based on these experiments, we conclude that Bard1, Hmmr, HURP, and NuSAP are substrates of the APC/C.

Identification of APC/C Recognition Motifs

As a first step toward dissecting the mechanism underlying the degradation of these SAFs, we determined their APC/C recognition motifs, such as D, KEN, or TEK boxes (Peters, 2006; Jin et al., 2008). We first identified truncation mutants of the candidate substrates, which showed resistance against APC/C-dependent degradation in extracts (Figure S3). Subsequently, we introduced point mutations into the SAF domains required for degradation to disrupt D, KEN, and TEK boxes and then analyzed the turnover of the mutant SAFs in extracts and cells.

This analysis identified specific APC/C recognition motifs, whose mutation led to stabilization of each substrate. We found two D boxes in the N terminus of Bard1 (Figure 3A–3C); D, KEN, and TEK boxes in the C terminus of Hmmr (Figures 3D–3F); D, KEN, and TEK boxes in the N terminus of HURP (Figures 3G–3I); and a D and KEN box in the C terminus of NuSAP (Figures 3J–3L). For all SAFs, multiple APC/C recognition motifs had to be mutated in combination to stabilize the protein in extracts and cells. The importance of degrons recognized by the APC/C further supports our conclusion that Bard1, Hmmr, HURP, and NuSAP are substrates of this ubiquitin ligase.

Of interest, all APC/C recognition motifs are in close proximity to SAF domains required for spindle assembly. The D boxes in Bard1 flank the RING domain required for it to function at the spindle pole (Joukov et al., 2006); the degrons of Hmmr are at its C terminus, which plays key roles during spindle pole maturation (Joukov et al., 2006); and the D, KEN, and TEK boxes of HURP and NuSAP are in their respective microtubule-binding domains (Raemaekers et al., 2003; Wong et al., 2008).

Importin β and Ran^{GTP} Regulate the Ubiquitination and Degradation of SAFs

The proximity of degrons to functionally important domains suggested that activity and stability of SAFs might be coregulated. HURP and NuSAP, two Ran-dependent SAFs, are inhibited by importins and activated by Ran^{GTP} (Silljé et al., 2006; Koffa et al., 2006; Ribbeck et al., 2006). Strikingly, despite being efficiently ubiquitinated by purified APC/C, these two SAFs are stable in extracts, which contain high levels of importins. By contrast, Bard1 and Hmmr, which are not known to bind importins, are effectively degraded in these extracts. These observations suggested that importins and Ran^{GTP} might not only control the activation, but also the degradation of HURP and NuSAP.

To test this hypothesis, we added recombinant importins to the APC/C-dependent ubiquitination of HURP and NuSAP. Strikingly, importin β, but not α, blocked the ubiquitination of both SAFs in a dose-dependent manner (Figures 4A–4C). The mutants importin β^{3W} and importin β^{462}, which bind substrates with low affinity (Moore et al., 1999; Cingolani et al., 2002), did not significantly impair HURP ubiquitination (Figure 4D). In contrast to the SAFs, importins did not affect the ubiquitination of Plk1 or cyclin B1 (Figures S4A and S4B). When synthesized by IVT/T, HURP is bound to importin β, which is abundant in reticulocyte lysate (Figure S4C). Ran^{GTP} dissociated HURP from importin β and promoted its APC/C-dependent ubiquitination (Figures 4C and S4C). Moreover, Ran^{GTP} allowed the APC/C to ubiquitinate HURP and NuSAP in the presence of excess importin β (Figures 4E and 4G). Ran^{GTP} promoted the ubiquitination of HURP slightly more efficiently than its dissociation from importin β, suggesting that ubiquitination might interfere with the rebinding of HURP to importin β. Thus, importin β and Ran^{GTP} are able to regulate the APC/C-dependent ubiquitination of HURP and NuSAP.

We next tested whether higher concentrations of Ran^{GTP} trigger the degradation of HURP and NuSAP in extracts, as suggested by the in vitro ubiquitination studies. We added constitutively GTP-bound Ran^{Q69L}, Ran and its GEF Rcc1, or Rcc1 (to activate endogenous Ran) to G1 extracts with APC/C^{Cdh1} or to mitotic extracts with APC/C^{Cdc20} and monitored the stability of HURP and NuSAP. Indeed, Ran^{GTP} strongly accelerated the degradation of both SAFs, whereas it had no effects on the degradation of other APC/C substrates, such as cyclin B (Figures 5A–5C and S4D–S4G). Ran^{GTP} induced the degradation of HURP at concentrations that also promoted its ubiquitination by the APC/C and its dissociation from importin β (Figure 4C). Ran^{T24N}, which cannot be charged with GTP, was unable to promote SAF degradation (Figures 5A–5C), and increasing the rate of GTP hydrolysis by adding RanGAP/RanBP1 stabilized HURP and NuSAP even in the presence of Ran/Rcc1. Thus, Ran^{GTP} strongly promotes the degradation of HURP and NuSAP in extracts, consistent with importin β stabilizing these SAFs.

We addressed the role of importin β in stabilizing SAFs by altering its abundance in extracts and cells. Consistent with the assays described above, HURP and NuSAP, but not cyclin B1, were stabilized in Ran^{GTP}-treated extracts by the Ran-insensitive importin $\beta^{\Delta N}$ or by an excess of importin β over Ran^{GTP} (Figures 5A–5C, S4F, and S4G). By contrast, the SAFs were not stabilized by importin α or by the importin β^{462} mutant defective in cargo binding (Figures 5A–5C). Moreover, if importin β was depleted from G1 extracts, HURP was turned over in an APC/C-dependent manner even without additional Ran^{GTP} (Figure 5D). The results from extracts were reproduced in 293T cells, in which importin $\beta^{\Delta N}$ protected HURP against APC/C-dependent degradation caused by Cdh1 overexpression (Figure 5E). Binding to

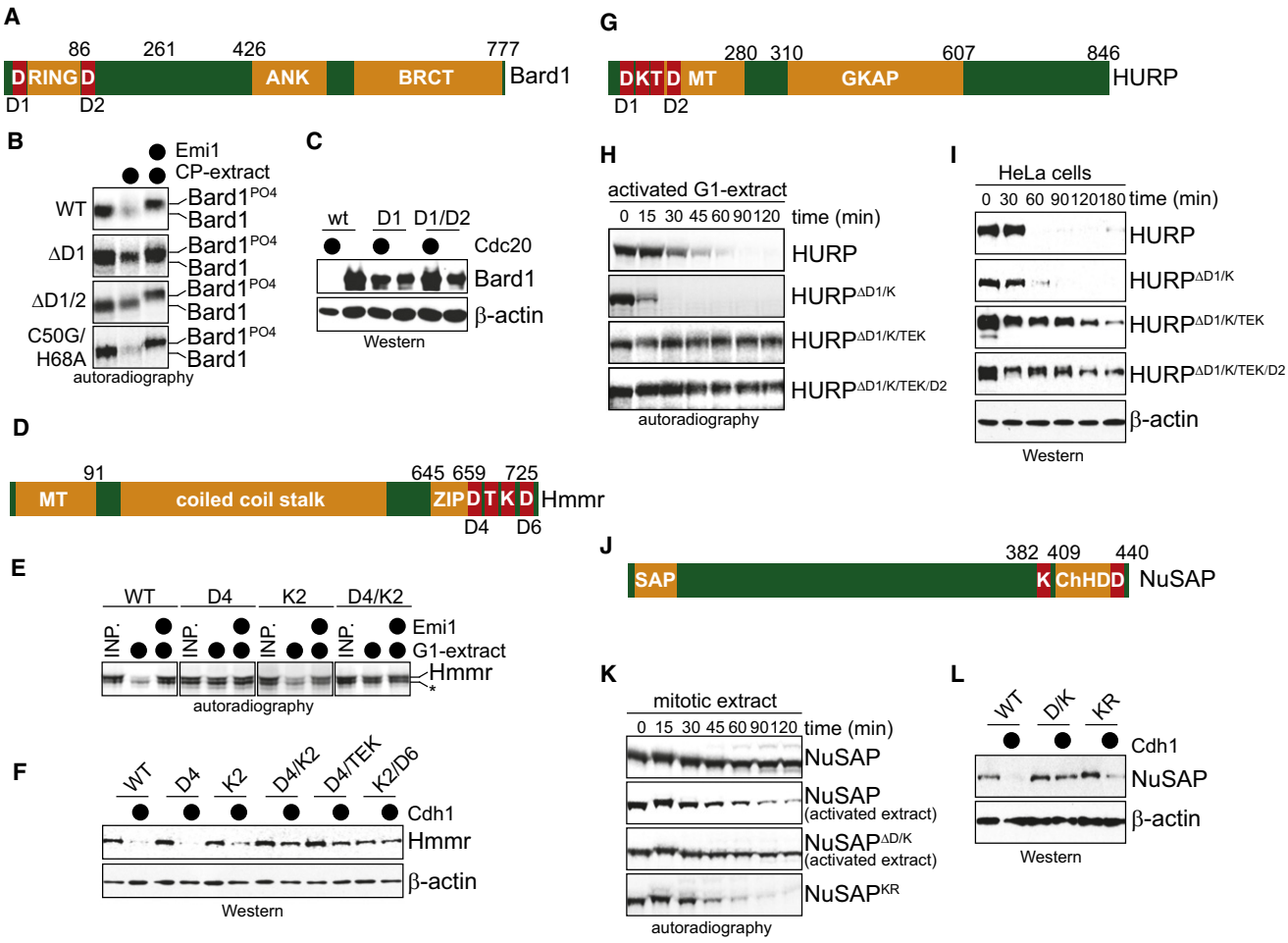

Figure 3. The SAF Degrons Are Adjacent to Domains Required for Spindle Assembly

(A) Schematic overview of Bard1. RING, RING domain; ANK, ankyrin repeats; BRCT, Brct domain; D, D box.

(B) Mutation of D boxes stabilizes Bard1 in mitotic extracts. ^{35}S-labeled mutants were tested for APC/C-dependent degradation in mitotic extracts with active APC/C^{Cdc20}. The C50G/H68A mutant ablates the function of the RING domain but has no effect on APC/C-dependent degradation.

(C) Mutation of D boxes stabilizes Bard1 in vivo. 293T cells were cotransfected with Bard1 mutants and Cdc20, and Bard1 levels were determined by western blot.

(D) Schematic overview of Hmmr. MT, microtubule-binding domain; ZIP, leucine zipper; T, TEK box; K, KEN box.

(E) Mutation of a D and KEN box stabilizes Hmmr in G1 extracts. The indicated ^{35}S-labeled point mutants were tested for APC/C-dependent degradation in G1 extracts as described above. Reaction products were analyzed by autoradiography.

(F) Mutation of D, KEN, and TEK boxes stabilizes Hmmr in vivo. ^{myc}Hmmr or the indicated mutants were coexpressed in 293T cells with Cdh1, and protein levels were analyzed by western blot using αmyc antibodies.

(G) Schematic overview of HURP. MT, microtubule-binding domain; GKAP, GKAP domain).

(H) The degradation of HURP in G1 extracts depends on D, KEN, and TEK boxes. To ensure degradation of WT HURP, G1 extracts were treated with Ran^{Q69L} (see later). The indicated ^{35}S-labeled mutants were incubated in G1 extracts before being analyzed by autoradiography.

(I) Degradation of ^{HA}HURP and its mutants in HeLa cells after release from nocodazole arrest. Samples were taken at the indicated times and analyzed for HURP levels by western blot.

(J) Schematic overview of NuSAP. The SAP and ChHD domains were defined by Raemaekers et al. (2003).

(K) Degradation of NuSAP in mitotic extracts depends on D and KEN boxes. If indicated, the mitotic extract was activated with Ran^{GTP}. ^{35}S-labeled mutants were incubated in the extracts for different times before being analyzed by autoradiography. In NuSAP^{KR}, a stretch of Lys residues has been changed to alanines, leading to its degradation in the absence of Ran^{GTP}.

(L) The degradation of NuSAP in cells depends on D and KEN boxes. The respective ^{HA}NuSAP mutants (WT; DK, mutation of D and KEN box; KR, mutation of a Lys-rich cluster) were coexpressed with Cdh1 in 293T cells, as indicated. The levels of NuSAP and its mutants were detected by western blot.

importin $\beta^{\Delta N}$ also delayed the APC/C-dependent degradation of endogenous HURP upon exit of U2OS cells from mitosis (Figure 5F). We conclude that Ran^{GTP} and importin β control the ubiquitination and degradation of HURP and NuSAP.

Importin β Is a Substrate-Specific APC/C Inhibitor

Importin β controls the degradation of HURP and NuSAP, but not cyclin B1, and thus likely regulates the SAFs rather than the APC/C. If this were the case, mutating the importin-binding

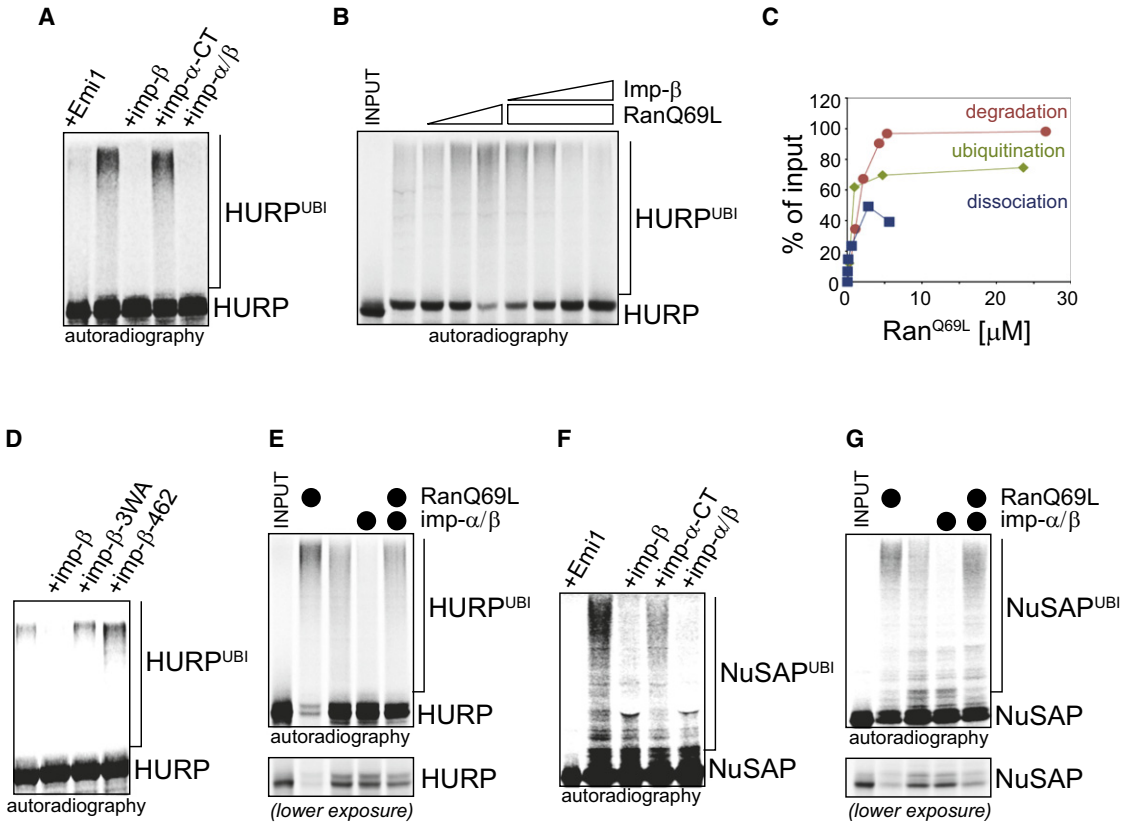

Figure 4. Importin β and Ran^{GTP} Regulate the Ubiquitination of HURP and NuSAP

(A) Importin β blocks the APC/C-dependent ubiquitination of HURP. ^{35}S-HURP was incubated with APC/C^{Cdh1}, Ube2S, and UbcH10. As indicated, Emi1, importin β, importin α-CT, or importin α/β were added. Reactions were analyzed by autoradiography.

(B) Importin β inhibits HURP ubiquitination in a dose-dependent manner. ^{35}S-HURP was incubated with APC/C^{Cdh1}, increasing concentrations of Ran^{Q69L}, and importin β, as indicated.

(C) Quantification of effects of Ran^{GTP} on HURP degradation in mitotic extracts (red), HURP ubiquitination by APC/C^{Cdh1} (green), and HURP dissociation from importin β (blue).

(D) An intact cargo-binding domain is required for importin β to act as APC/C inhibitor. Importin β and two mutants with reduced cargo affinity (importin β^{3WA}; importin-β^{462}) were analyzed for their effect on ubiquitination of HURP by APC/C as described above.

(E) Ran^{GTP} allows HURP ubiquitination in the presence of importin β. ^{35}S-HURP was incubated with APC/C^{Cdh1}, Ube2S, and UbcH10. Importin β/α and GTP-bound Ran^{Q69L} were added as indicated. Reactions were analyzed by autoradiography.

(F) Importin β blocks the APC/C-dependent ubiquitination of NuSAP. ^{35}S-NuSAP was analyzed for APC/C-dependent ubiquitination as described above.

(G) Ran^{GTP} allows NuSAP ubiquitination in the presence of importin β. ^{35}S-NuSAP was incubated with APC/C^{Cdh1}, Ube2S, and UbcH10. Importin β/α and Ran^{Q69L} were added as indicated, and reactions were analyzed by autoradiography.

sites of HURP should result in its APC/C-dependent degradation in the absence of Ran^{GTP}. Deletion analysis showed that the N-terminal 117 residues of HURP (HURP^{117}) were sufficient to mediate importin β binding in vitro (Figure S5A). The mutation of two stretches of basic amino acids (KR2 and KR3) to alanine residues impaired the binding of HURP^{117} to importin β, and the simultaneous mutation of both (KR2/3) abrogated this interaction (Figure S5A). As expected for loss of importin binding, mutation of KR2 or KR2/3 ablated the nuclear accumulation of HURP^{117} in HeLa cells (Figure 6A). In 293T or HeLa cells, the mutation of KR2 or KR2/3 in full-length HURP dramatically reduced its binding to importin β, but not to Tpx2 or Aurora A (Figures 6B, S5B, and S5C). Thus, KR2 and KR3 are crucial importin-β-binding sites in HURP.

KR2 and KR3 overlap with the APC/C-binding motifs in HURP (Figure S6A). Accordingly, the mutation of both KR2 and KR3 not only abrogated the association of HURP with importin β, but also its APC/C-dependent ubiquitination and degradation (Figures S6B–S6D). Similarly, loss of APC/C recognition motifs (HURP$^{\Delta DKT}$) interfered with HURP binding to importin β (Figure 6B). These findings imply that importin β and APC/C compete for access to HURP, and indeed, addition of importin β blocked the interaction between HURP and the APC/C substrate targeting subunit Cdh1 (Figure 6C). This strongly suggests that importin β stabilizes HURP by shielding its APC/C-binding sites.

Thus, loss of importin binding should trigger HURP degradation in the absence of Ran^{GTP}. We were able to test this

hypothesis because mutation of KR2 strongly reduced the affinity of HURP to importin β but still allowed its ubiquitination by APC/C. HURP^{KR2} was quickly degraded in G1 extracts without additional Ran^{GTP} (Figure 6D), and importin β$^{\Delta N}$ failed to stabilize HURP^{KR2} under these conditions (Figure S6C). The degradation of HURP^{KR2} required APC/C and the proteasome, as HURP^{KR2} was stabilized by addition of APC/C- and proteasome inhibitors or by mutation of its APC/C recognition sites (HURP$^{KR2/3}$ and HURP$^{KR2\Delta DKT}$) (Figure 6D). Similar degradation kinetics as in extracts were observed when the stability of HURP mutants was analyzed in cells (Figure 6E). NuSAP appears to be regulated in a similar manner, as deletion of a Lys-rich region adjacent to its KEN box led to NuSAP degradation in the absence of Ran^{GTP} (Figure 3K). Thus, importin β stabilizes HURP, and most likely NuSAP, by direct binding to the SAFs, rather than by regulating the APC/C or the proteasome.

The above findings also implied that importin β needs to bind to sites overlapping with the APC/C recognition motifs of HURP to stabilize this SAF. In agreement with this observation, HURP^{KR2} was not stabilized in extracts or cells if an importin-binding motif was fused to the N terminus of HURP^{KR2} (NLS-KR2) (Figure 6D), even though this fusion rescued nuclear import of HURP$^{117/KR2}$ in cells (Figure 6A). Thus, importin β acts as a substrate-specific APC/C inhibitor, which masks the APC/C recognition motifs of SAFs.

Importin β Stabilizes SAFs during Mitosis

When in the cell cycle does importin β regulate the stability of SAFs? To address this question, we compared the abundance of HURP to that of HURP^{KR2}, which has lost regulation by importin β yet is still recognized by the APC/C. Though HURP and HURP^{KR2} were expressed at similar levels in interphase HeLa cells (Figure S7A and S7B), we found strong differences in their abundance during mitosis. Consistent with previous reports (Wong and Fang, 2006; Silljé et al., 2006), HURP accumulated in prometaphase cells on spindle microtubules (Figure 7A). By contrast, only low amounts of the importin binding-deficient HURP^{KR2} were detected under these conditions, and in the majority of cells, HURP^{KR2} was absent from spindle microtubules. In addition, only low concentrations of HURP^{KR2} were observed in HeLa cells synchronized in prometaphase with nocodazole, as detected by western blotting (Figures 7B and S7B). HURP^{KR2} reaccumulated under these conditions when cells were treated with the proteasome inhibitor MG132 (Figures 7A and S7B). These findings suggest that importin β is able to protect HURP from proteasomal degradation during early stages of mitosis.

To test whether the APC/C is responsible for the reduced levels of HURP^{KR2}, we mutated the APC/C recognition motifs of HURP in addition to its KR2 site (HURP$^{KR2\Delta DKT}$; HURP$^{KR2/3}$). Both mutants were stabilized in comparison to HURP^{KR2} and detected at high levels on spindle microtubules (Figure 7A). Accordingly, HURP$^{KR2/3}$ was abundant in lysates of synchronized HeLa cells, as measured by western blotting (Figure S7B). In addition, HURP^{KR2} was stabilized during prometaphase by coexpression of Emi1 or a dominant-negative version of the APC/C-specific E2 UbcH10 (UbcH10^{C114S}) (Figure 7B). Confirming our mechanistic analysis in extracts, the fusion of an importin-binding site to the N terminus of HURP^{KR2} did not inhibit its degradation (Figure 7A); this also shows that the premature degradation of HURP^{KR2} in cells did not result from nuclear exclusion prior to mitosis. Our findings, therefore, provide evidence that importin β protects HURP from APC/C-dependent degradation during early mitosis.

Consistent with these results, endogenous HURP and NuSAP bind importin β in U2-OS cells synchronized in prometaphase (Figures S7C and S7D). Moreover, immobilized importin β$^{\Delta N}$ efficiently captured HURP and NuSAP from lysates of prometaphase cells, only if these lysates were pretreated with Ran^{GTP} to dissociate endogenous SAF-importin complexes, suggesting that most of HURP and NuSAP is bound by importins at this time of mitosis (Figure S7E). However, as Ran^{GTP} dissociates HURP and NuSAP from importin β in the vicinity of chromatin, a fraction of the endogenous SAFs should be degraded in an APC/C-dependent manner. To test this assumption, we inhibited the APC/C by depleting UbcH10, Ube2S, and the APC/C-activator p31^{comet}, as reported previously (Williamson et al., 2009), and then analyzed the abundance of endogenous, spindle-bound HURP in pre-anaphase cells by fluorescence microscopy. Importantly, the levels of HURP in pre-anaphase cells strongly increased upon APC/C inhibition, which was accompanied by spindle defects (Figure 7C). To corroborate these findings, we synchronized HeLa cells in prometaphase using nocodazole or taxol and inhibited the proteasome with MG132 (Figure S7F). In taxol-treated cells, HURP and NuSAP accumulated upon addition of MG132. HURP and NuSAP were more stable in nocodazole-treated cells, as seen before, suggesting that microtubules might regulate the degradation of SAFs. Together, these findings show that importin β is able to protect HURP and NuSAP from APC/C-dependent degradation during prometaphase, which is the same time when it also regulates the activation of both SAFs.

The Regulated Degradation of SAFs Is Important for Mitosis

We finally determined whether the regulated degradation of SAFs is important for cell-cycle progression. As loss of importin β binding leads to the degradation of HURP before anaphase, the mutation of the KR2 sites in HURP should interfere with its role in spindle formation. We depleted HURP in prometaphase cells with spindles disassembled with nocodazole, released the cells into fresh medium to allow spindle formation, and then counted the number of cells entering anaphase as a measure of successful spindle formation. Confirming earlier reports (Wong and Fang, 2006), the loss of HURP by siRNAs delayed anaphase entry, which could be rescued by expression of siRNA-resistant HURP (Figure 7D). By contrast, HURP^{KR2}, which does not bind importin β yet is recognized by the APC/C, did not rescue the phenotypes caused by depletion of endogenous HURP, suggesting that the stabilization of HURP by importin β is required for efficient spindle formation.

To determine the importance of the SAF degradation, we analyzed spindle structures in cells expressing HURP or NuSAP at higher levels. The expression of the stable HURP$^{\Delta D1\Delta K\Delta TEK\Delta D2}$ increased the number of prometaphase cells with incomplete chromosome alignment, suggesting that stabilization of

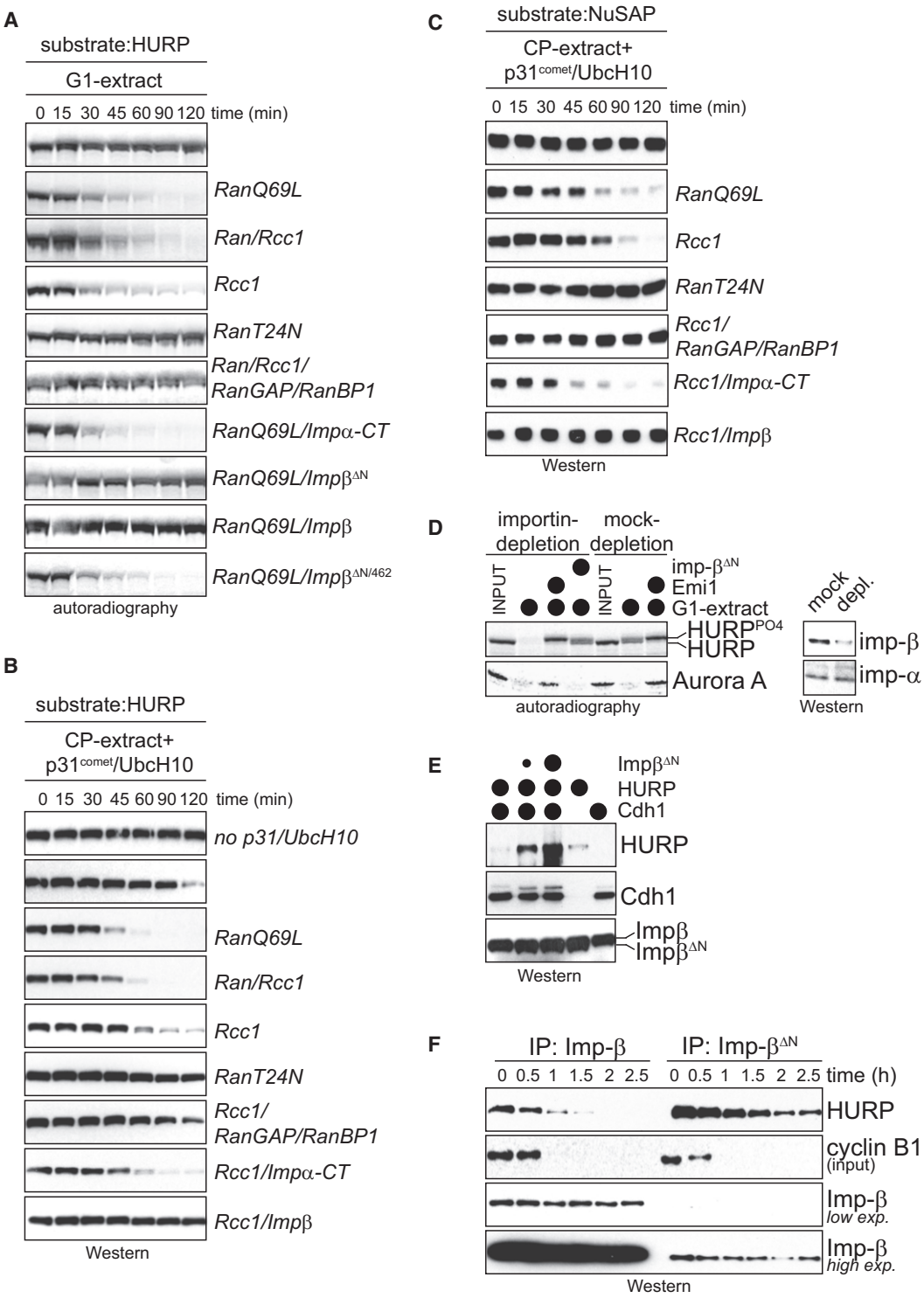

Figure 5. Importins and Ran^{GTP} Control the Degradation of HURP and NuSAP

(A) Importin β and Ran^{GTP} control the stability of HURP in G1 extracts. The degradation of ^{35}S-HURP in G1 extracts was analyzed by autoradiography. When indicated, extracts were supplemented with GTP-bound Ran^{Q69L}, Ran and its GEF Rcc1, Rcc1 to activate endogenous Ran, nucleotide-free Ran^{T24N}, RanGAP/RanBP1 to generate Ran^{GDP}, importin α-CT, importin-β$^{\Delta N}$, importin β, and the cargo-binding-deficient importin β$^{\Delta N/462}$.

(B) Importin β and Ran^{GTP} control the stability of HURP in mitotic extracts. APC/C^{Cdc20} was activated in mitotic extracts by UbcH10 and p31^{comet}, and degradation of endogenous HURP was monitored by western blot using αHURP antibodies. Ran or importin proteins were added as described above.

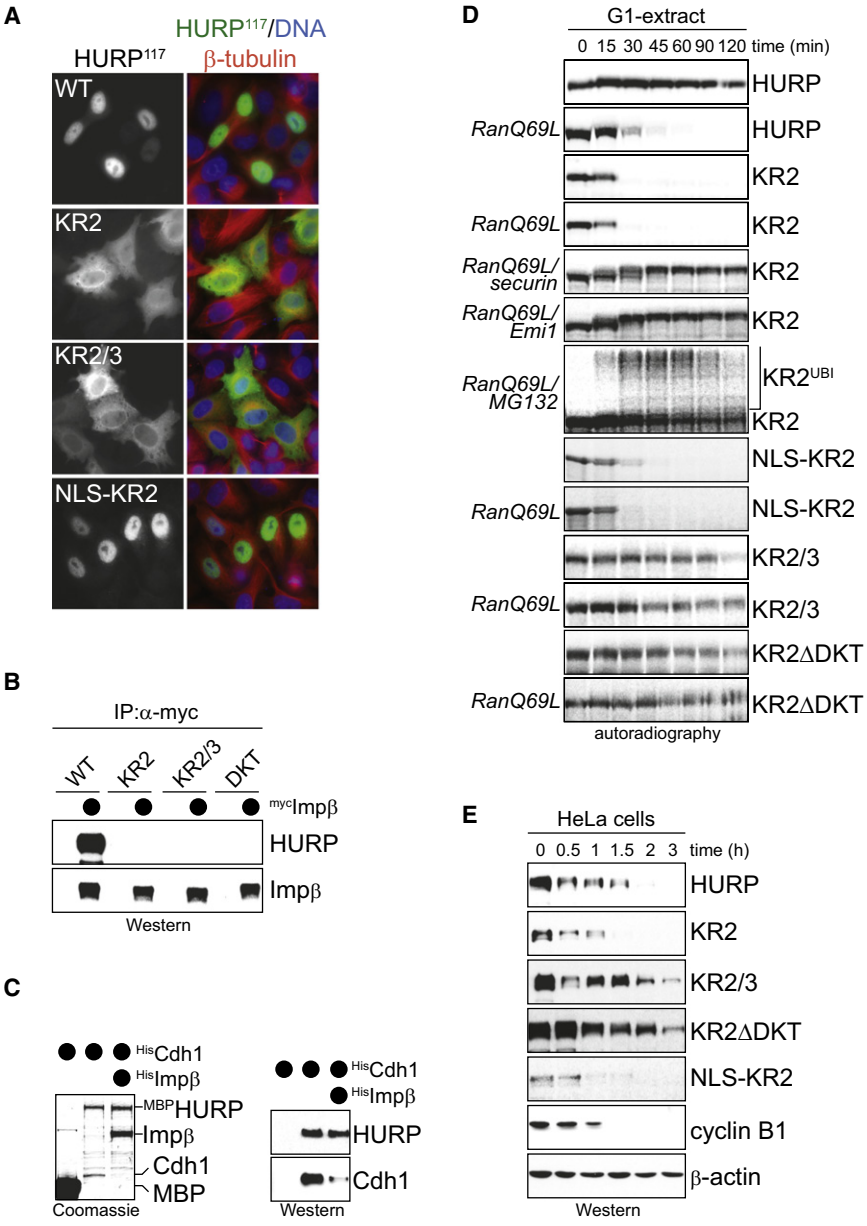

Figure 6. Importin β Stabilizes HURP by Direct Binding

(A) Mutation of KR2 or KR2/3 ablates the nuclear accumulation of HURP^{117}. HeLa cells transfected with ^{HA}HURP^{117} or indicated mutants were analyzed by fluorescence microscopy using αHA antibodies. In NLS-KR2, the SV40-NLS was fused to the N terminus of HURP^{117}-KR2. The right panel shows staining of HURP (green), tubulin (red), and DNA (DAPI; blue).

(B) Mutation of KR2 or KR2/3 abrogates binding of HURP to importin β. HeLa cells were transfected with ^{HA}HURP, ^{HA}HURP^{KR2}, ^{HA}HURP$^{KR2/3}$, or ^{HA}HURP$^{\Delta DKT}$ (lacking D, KEN, and TEK boxes) and ^{myc}importin-β and were synchronized in mitosis with nocodazole. Importin β was precipitated by αmyc beads, and copurifying HURP was detected by western blot using αHA antibodies.

(C) Importin β and APC/C compete for access to HURP. MBP or ^{MBP}HURP were bound to amylose resin. As indicated, ^{MBP}HURP was preincubated with ^{His}importin β. ^{His}Cdh1 was added to all reactions, and the beads were analyzed for bound ^{His}Cdh1 using Coomassie (left) and western blot (right).

(D) Mutation of its importin-β-binding site triggers APC/C-dependent degradation of HURP in the absence of Ran^{GTP}. The degradation of ^{35}S-labeled HURP mutants in G1 extracts was monitored by autoradiography. As indicated, Ran^{Q69L}, securin as competitive APC/C inhibitor, Emi1, or MG132 were added.

(E) Degradation of importin-β-binding mutants of HURP in cells. HeLa cells expressing ^{HA}HURP or indicated mutants were released from a nocodazole-dependent mitotic arrest, and degradation of HURP was analyzed by western blot.

HURP delayed the establishment of a metaphase plate (Figure 7E). In addition, almost all spindles in cells expressing HURP$^{\Delta D1\Delta K\Delta TEK\Delta D2}$ were highly aberrant. We often observed short spindles with broadened spindle poles and highly unstructured spindle microtubules, which is reminiscent of spindle aberrations observed upon overexpression of the APC/C-inhibitor Emi1 (Ban et al., 2007). These effects were more pronounced with NuSAP, in which overexpression of the wild-type protein was sufficient to induce strong aberrations in the structure of the mitotic spindle (Figure S7G). We conclude that the regulated degradation of HURP and NuSAP is important for cells to achieve accurate spindle assembly.

(C) Importin β and Ran^{GTP} control the stability of NuSAP in mitotic extracts. The degradation of endogenous NuSAP was monitored in mitotic extracts with active APC/C by western blot using αNuSAP antibodies. As indicated, Ran or importin proteins were added.

(D) Depletion of importin-β-like proteins promotes degradation of HURP in G1 extracts. Importin-β-like proteins were depleted from G1 extracts by a Ran affinity column, as described (Ribbeck et al., 1998). The extent of depletion is shown by western blot (right). ^{35}S-HURP or ^{35}S-Aurora A were added to importin- and mock-depleted extracts and were analyzed for APC/C-dependent degradation after 2 hr by autoradiography. Extracts were supplemented with importin β$^{\Delta N}$ or Emi1, as indicated.

(E) Importin β stabilizes HURP in vivo. The APC/C-dependent degradation of HURP was induced in 293T cells by Cdh1 expression. As indicated, increasing amounts of an importin β$^{\Delta N}$ plasmid were cotransfected, which stabilized HURP in a dose-dependent manner.

(F) Importin β stabilizes HURP upon exit from mitosis. U2OS cells stably expressing ^{FLAG}importin β (left) or ^{FLAG}importin β$^{\Delta N}$ (right) were released from a nocodazole-induced mitotic arrest into G1. As FLAG-tagged importins are expressed well below the level of the endogenous importin β, we monitored the stability of HURP bound to affinity-purified ^{FLAG}importins. HURP was detected by western blot.

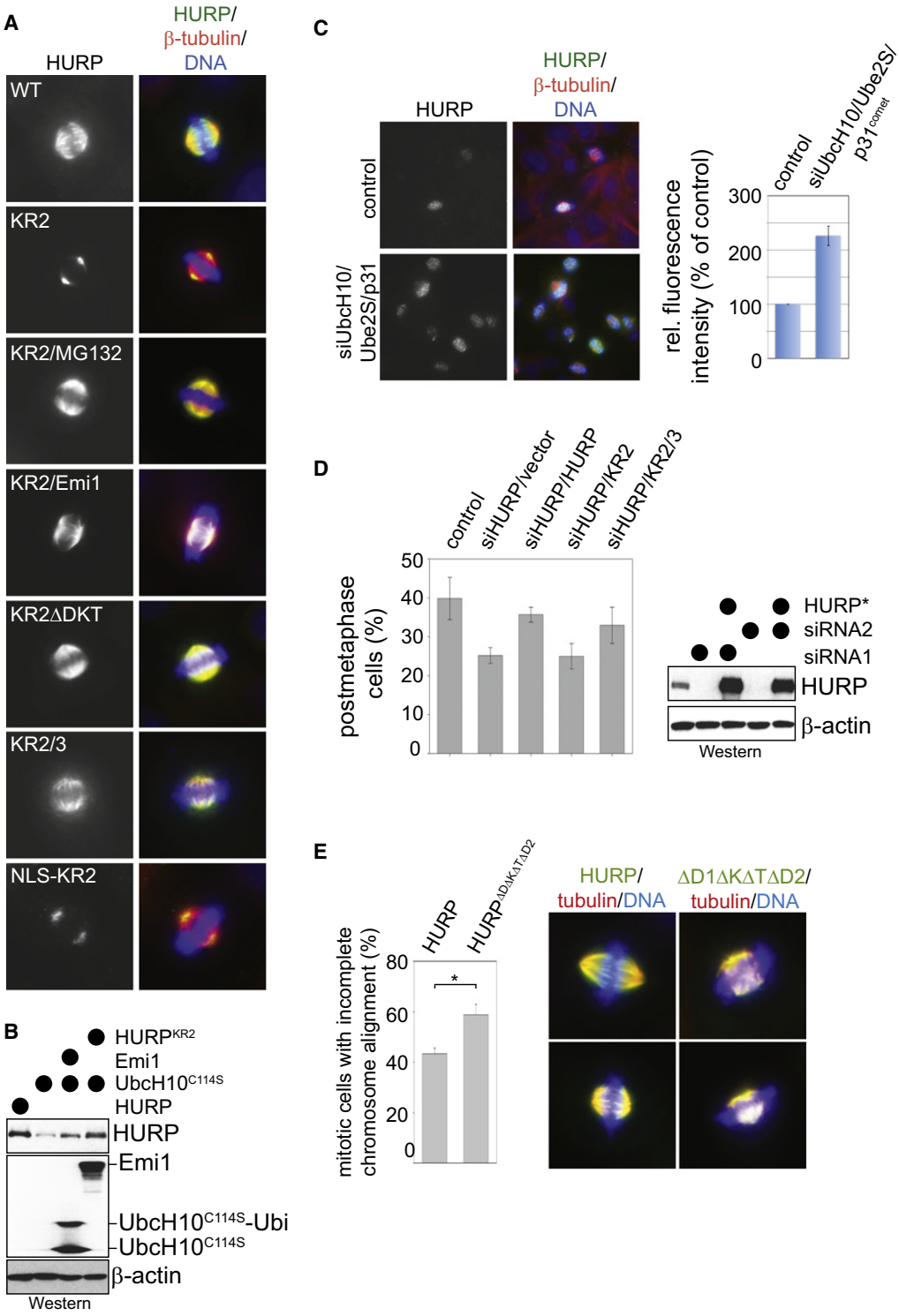

Figure 7. Importin β Stabilizes SAFs during Mitosis

(A) Stabilization by importin β is required for accumulation of HURP on spindle microtubules. ^{HA}HURP and mutants were expressed in HeLa cells, and their intracellular location was determined in pre-anaphase cells by immunofluorescence using αHA-antibodies (green). The spindle was stained with antibodies against β-tubulin (red), and DNA was detected by DAPI (blue). MG132 was added as indicated.

(B) APC/C inhibition stabilizes HURP^{KR2} in prometaphase. HeLa cells expressing ^{HA}HURP or ^{HA}HURP^{KR2} were synchronized in prometaphase by nocodazole treatment. As indicated, UbcH10^{C114S} or ^{myc}Emi1, which inhibit the APC/C, were coexpressed. The levels of HURP or HURP^{KR2} were determined by western blot.

DISCUSSION

Our search for APC/C substrates was motivated by the aberrant spindle assembly in the absence of APC/C and the sparse information on SAFs known to be regulated by this machine. Here, we report the identification of four APC/C substrates with roles in regulating spindle formation: Bard1, Hmmr, HURP, and NuSAP. Among these substrates, HURP and NuSAP can be degraded during spindle assembly, although the spindle checkpoint is active. Consequently, the degradation of these SAFs requires additional layers of regulation, and they are only degraded after being released from importin β by Ran^{GTP}. If this unique mechanism of regulation is lost, spindle formation is impaired. Our findings reveal a unique mode of regulation for APC/C-dependent ubiquitination and provide a molecular basis for the role of APC/C in spindle formation.

The APC/C Controls Ran-Dependent Spindle Assembly

All APC/C substrates identified in this study have functions in Ran-dependent spindle assembly. Bard1 and Hmmr localize to spindle poles, and loss of their activity results in unfocused poles or multipolar spindles (Groen et al., 2004; Maxwell et al., 2005; Joukov et al., 2006). HURP and NuSAP, which are enriched on kinetochore fibers, promote the nucleation and crosslinking of spindle microtubules (Silljé et al., 2006; Ribbeck et al., 2006). Aberrant levels of any of these SAFs cause spindle defects, which demonstrates that regulating their abundance is important for proper spindle formation (Figures 7E and S7G) (Raemaekers et al., 2003; Tsou et al., 2003; Maxwell et al., 2005; Silljé et al., 2006; Wong and Fang, 2006; Joukov et al., 2006; Li et al., 2007). As similar spindle abnormalities are observed upon depletion of APC/C subunits (Goshima et al., 2007; Somma et al., 2008), codepletion of UbcH10 and Ube2S (Williamson et al., 2009), or overexpression of APC/C inhibitors (Ban et al., 2007), the stabilization of these components of the Ran-pathway likely contributes to the spindle defects caused by APC/C inhibition.

What is the reason for the APC/C regulating so many substrates within the same pathway? One explanation might be found in the observation that many components of Ran-dependent spindle assembly function in dynamic protein interaction networks. For example, Bard1 binds Hmmr and Tpx2 to keep the activity of Hmmr in spindle assembly in check, and HURP, Tpx2, and Aurora A were suggested to be part of a complex in *X. laevis* extracts (Joukov et al., 2006; Pujana et al., 2007; Koffa et al., 2006). It is possible that degradation of a single complex subunit could disturb the balance between distinct activities. Consistent with this hypothesis, depleting either Brca1/Bard1 or Hmmr results in centrosome amplification, whereas codepleting these proteins has less dramatic effects (Pujana et al., 2007). Targeting multiple SAFs could also allow the APC/C to regulate spindle assembly more robustly than it would by degrading a single protein, and spindle formation might be less sensitive to the aberrant expression of a single SAF.

The APC/C thus targets active HURP and NuSAP for degradation during spindle assembly, and it removes Tpx2, Bard1, Hmmr, and the remaining HURP and NuSAP from cells once spindle formation has been completed, thereby resetting this pathway for a new round of cell division. We conclude that the APC/C is an important regulator of Ran-dependent spindle formation.

Regulation of APC/C-Dependent Degradation by Importin β and Ran^{GTP}

Our findings also reveal a reciprocal function of Ran in regulating APC/C-dependent degradation events. Similar to its role in spindle formation, Ran^{GTP} controls the turnover of SAFs by counteracting importin β. As we have shown in detail for HURP, importin β competitively inhibits the APC/C recognition of HURP by directly binding to its degrons, and our experiments with NuSAP suggest that it is regulated in a similar manner. By contrast, importin β interacts with but does not stabilize cyclin B1, and accordingly, the binding sites for importin β and APC/C localize to different regions of cyclin B1 (Hagting et al., 1999). We have tested multiple APC/C substrates without functions in spindle assembly, but none was stabilized by importin β (data not shown). Our results, therefore, identify importin β as a substrate-specific inhibitor and Ran^{GTP} as a substrate-specific activator of the APC/C.

Importin β and Ran^{GTP} likely regulate SAF degradation prior to anaphase, when spindle formation takes place. $HURP^{KR2}$, which does not associate with importin β yet is recognized by the APC/C, is degraded despite incomplete spindle assembly. As a consequence, the expression of $HURP^{KR2}$ in prometaphase cells failed to rescue the delay in anaphase entry caused by lack of endogenous HURP. Consistent with these observations, most of HURP and NuSAP appear to be bound by importins during prometaphase. To act in spindle formation, HURP and NuSAP have to be dissociated from importin β by Ran^{GTP} (Silljé et al., 2006; Ribbeck et al., 2006), which should expose their degrons. Accordingly, we observed that some endogenous HURP was

(C) Some endogenous HURP is degraded in an APC/C-dependent manner before metaphase. HeLa cells were treated with siRNA against UbcH10, Ube2S, and $p31^{comet}$, which inhibits the APC/C and arrests cells in prometaphase. Endogenous HURP was detected by fluorescence microscopy using αHURP antibodies (left). The fluorescence intensity in pre-anaphase cells was measured using ImageJ. The quantification of three independent experiments is shown on the right. At least 100 mitotic cells were counted per experiment to calculate the standard deviation.

(D) Stabilization by importin β is required for HURP function in early mitosis. HURP was depleted from HeLa cells by siRNA, and cells were arrested in mitosis by nocodazole. As indicated, depleted HeLa cells were transfected with siRNA-resistant HURP mutants. At 1 hr after release into new medium, the number of cells that had initiated anaphase was determined. At least 100 mitotic cells were counted per experiment to calculate the standard deviation. The right panel shows HURP depletion by siRNA and rescue by siRNA-resistant HURP.

(E) Degradation of HURP is important for spindle formation. $^{HA}HURP$ or $^{HA}HURP^{\Delta D1\Delta K\Delta T\Delta D2}$ were expressed in HeLa cells and detected by immunofluorescence against HA (green). The percentage of HURP-positive pre-anaphase cells with incomplete chromosome alignment was determined 48 hr post transfection in three independent experiments. At least 100 mitotic cells were counted per experiment to calculate the standard deviation ($p < 0.05$; left). Most spindles in cells expressing the stable $^{HA}HURP^{\Delta D1\Delta K\Delta T\Delta D2}$ were defective, examples for which are shown on the right.

degraded in an APC/C-dependent manner during prometaphase. Thus, activation and degradation of HURP can occur during prometaphase, and both events are under control of importin β and Ran^{GTP}.

The tight relationship between the Ran^{GTP}-dependent activation and degradation of SAFs is reminiscent of transcription factors, which are often simultaneously activated and marked for degradation by ubiquitin (Lipford and Deshaies, 2003). Similar to the temporal delay between the ubiquitin-dependent activation of transcription factors and their degradation, the proteolysis of HURP and NuSAP has to wait until the SAFs have fulfilled their role in spindle formation, or otherwise the cycle of activation and degradation would be futile. We speculate that several factors might contribute to the proper timing of SAF degradation during mitosis. It is possible that, in the presence of an active spindle checkpoint, the APC/C ubiquitinates HURP and NuSAP with slow kinetics. Spindle-bound deubiquitinating enzymes might also delay the degradation of SAFs, as suggested for APC/C regulation after DNA damage (Bassermann et al., 2008). However, we favor the hypothesis that the degradation of HURP and NuSAP during prometaphase requires microtubules or an unknown microtubule-dependent activity. As both HURP and NuSAP promote microtubule nucleation (Koffa et al., 2006; Ribbeck et al., 2006), their microtubule-dependent degradation would ensure that the SAFs had been sufficiently active before being turned over. Experiments are underway to test this hypothesis.

EXPERIMENTAL PROCEDURES

A detailed description of the used constructs, siRNAs, antibodies, and additional procedures can be found in the Supplemental Information.

In Vitro 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 2 hr, and in S phase by thymidine arrest were prepared as described before (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 using reticulocyte lysate premix (Promega) in the presence of ^{35}S-Met/Cys (TransLabel; ICN). Extracts were supplemented with energy mix and ubiquitin, and ^{35}S-labeled substrates were added (Jin et al., 2008). Where indicated, recombinant proteins were added, and reactions were incubated for 2 hr at room temperature (for mitotic extracts) or 30°C (for G1 extracts). Reactions were stopped by addition of gel loading buffer and analyzed by autoradiography. The degradation of endogenous HURP and NuSAP was also analyzed in mitotic extracts. 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, reactions were stopped by gel loading buffer and analyzed by western blotting using 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 cotransfected with candidate substrate and Cdh1 or Cdc20 in a substrate/Cdh1 ratio of 1:3. As indicated, ubiquitin, ubiquitin mutants, or Emi1 were transfected in 4:1 excess of substrate; importin $\beta^{\Delta N}$ was used in a 3:1 ratio to substrate. The amount of transfected DNA was held constant by transfection of pCS2 if needed. Cells were harvested 48 hr 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 by thymidine/nocodazole. Cells were released from the nocodazole arrest by transfer to fresh medium. Cells were harvested at the indicated time points, and the levels of ^{HA}HURP or respective mutants were analyzed by western blotting using αHA antibodies.

In Vitro Ubiquitination Reactions

For ~10 ubiquitination reactions, human APC/C was purified from 1.5 ml concentrated HeLa S3 extracts by immunoprecipitation using 75 μl αCdc27 antibodies and 100 μl Protein G agarose (Roche). APC/C^{Cdc20} was purified from mitotic extracts, and $p31^{comet}$ was added during the ubiquitination (Reddy et al., 2007). Washed beads were incubated with human 50 nM E1, E2 (usually 50 nM UbcH10 and 100 nM Ube2S), 1 $^{mg}/_{ml}$ ubiquitin or ubiquitin mutants, energy mix (15 mM creatine phosphate, 2 mM ATP, 2 mM $MgCl_2$, and 0.2 mM EGTA [pH 7.5]), 1 mM DTT, and ^{35}S-labeled substrates synthesized by IVT/T at room temperature for 30 min. Reactions were analyzed by autoradiography.

Immunoprecipitation

The coimmunoprecipitation 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. Cell lysate was prepared in IP buffer (20 mM HEPES [pH 7.5], 5 mM KCl, 1.5 mM $MgCl_2$, 150 mM NaCl, EDTA-free protease inhibitor cocktail (Roche), and 0.1% Triton X-100) and added to α-myc beads (Santa Cruz). Incubation was carried out at 4°C overnight with continuous mixing. After washing five times in IP buffer, resin-bound proteins were eluted in 2 × 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 hr. At 24 hr postinduction, cells were treated with 100 ng/ml nocodazole for 24 hr. Mitotic cells were collected by shake-off and released into fresh medium without nocodazole. Cell lysates were prepared at indicated time points in IP buffer (20 mM HEPES [pH 7.5], 5 mM KCl, 1.5 mM $MgCl_2$, 1 × protease inhibitor cocktail, 150 mM NaCl, and 0.1% Triton X-100) and incubated with αFLAG M2 affinity gel (Sigma). Beads were washed, and bound proteins were eluted with gel loading buffer. The precipitating proteins were analyzed by western blotting using specific antibodies.

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

MBP Pull-Down Assays

Amylose resin was charged with MBP fusions in MBP buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 5 mM KCl, 1.5 mM $MgCl_2$, 0.1 mg/ml BSA, and 0.1% Tween20) and then incubated with either in vitro synthesized, radioactively labeled proteins or recombinant proteins in MBP buffer for 3 hr at 4°C. After careful washes in WB buffer (20 mM HEPES [pH 7.5], 5 mM KCl, 1.5 mM $MgCl_2$, and 0.1% Tween20), resin-bound proteins were eluted in gel loading buffer and analyzed by Coomassie staining or western blotting.

RNAi, Immunofluorescence, and Quantification

The siRNA depletion against Emi1, Ube2S, and UbcH10 was performed as described (Williamson et al., 2009). The HURP siRNA targets its 3′UTR (CCTTCATATTATCAATGCT). HeLa cells were transfected with 100 nM siRNA using oligofectamine (Invitrogen). As indicated, at 24 hr posttransfection, HeLa cells were transfected again with 3 μg plasmids encoding HA-tagged wild-type

or mutant HURP using TransIT-LT1 (Mirus). Cells were synchronized by 2 mM thymidine for 24 hr and then arrested by 100 ng/ml nocodazole for 12 hr. After release into fresh growth medium for 2 hr, cells were fixed for immunofluorescence. Mitotic stages were determined by DNA and spindle morphology.

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

To measure the abundance of spindle-bound HURP in pre-anaphase HeLa cells, cells were simultaneously transfected with siRNA constructs targeting UbcH10, Ube2S, and $p31^{comet}$ using Lipofectamine2000 (Invitrogen). Cells were extracted, fixed with formaldehyde, and incubated with αHURP antibody followed by Alexa488-goat anti-rabbit. The fluorescence intensity of spindle-bound HURP was quantified by ImageJ software. Images of ~150 pre-anaphase cells from three independent experiments were analyzed.

Synchronization

HeLa cells were treated with 2mM thymidine for 24 hr, released for 3 hr, and treated with 100 ng/ml nocodazole for 11 hr. Cells were released by washing with prewarmed medium and plated into fresh DMEM, 10% FBS. Samples were taken at 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 72 hr and released into fresh DMEM, 10% FBS. Samples were taken at the indicated time points and processed for western blot analysis as described before.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, one table, and eight figures and can be found with this article online at doi:10.1016/j.molcel.2010.02.038.

ACKNOWLEDGMENTS

We are very grateful to Rebecca Heald, Karsten Weis, and Petr Kalab for reagents, invaluable advice, and critically reading the manuscript. We thank Julia Schaletzky for her insight throughout the project and for carefully reading the manuscript; Michelle Yasukawa and Ann Fischer for tissue culture support; and members of the Drubin and Barnes laboratories for help with fluorescence microscopy. We thank all members of the Rape lab for many discussions, suggestions, and encouragement. This work was funded by a grant from the NIH MR (RO1 GM083064) and an NIH Director New Innovator Award to M.R. M.R. is a Pew fellow.

Received: July 30, 2009
Revised: November 19, 2009
Accepted: February 19, 2010
Published: May 13, 2010

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