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Microtubule-Dependent Regulation of Mitotic Protein Degradation

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SUMMARY

Accurate cell division depends on tightly regulated ubiquitylation events catalyzed by the anaphase-promoting complex (APC/C). Among its many substrates, the APC/C triggers the degradation of proteins that stabilize the mitotic spindle, and loss or accumulation of such spindle assembly factors can result in aneuploidy and cancer. Although critical for cell division, it has remained poorly understood how the timing of spindle assembly factor degradation is established during mitosis. Here, we report that active spindle assembly factors are protected from APC/C-dependent degradation by microtubules. In contrast, those molecules that are not bound to microtubules are highly susceptible to proteolysis and turned over immediately after APC/C activation. The correct timing of spindle assembly factor degradation, as achieved by this regulatory circuit, is required for accurate spindle structure and function. We propose that the localized stabilization of APC/C substrates provides a mechanism for the selective disposal of cell-cycle regulators that have fulfilled their mitotic roles.

INTRODUCTION

Posttranslational modification with ubiquitin is widely used to regulate protein stability or activity, and it is essential for cell division in all eukaryotes (Komander and Rape, 2012). Substrates are selected for ubiquitylation by E3 ligases, many of which play important roles in cell-cycle control (Deshaies and Joazeiro, 2009). Among the ~600 human E3s, the anaphase-promoting complex (APC/C) is of particular interest as it controls essential steps in mitosis (Peters, 2006).

The APC/C was originally identified based on its role in triggering the degradation of cyclin B1, an activating subunit of Cdk1 (reviewed in Peters [2006]). It is now known to ubiquitylate a large number of cell-cycle regulators, thereby orchestrating progression of cells through mitosis and G1. Substrates are delivered to the APC/C by the WD40-repeat proteins Cdc20 and Cdh1, which recognize degron motifs referred to as D or KEN boxes (Buschhorn et al., 2011; da Fonseca et al., 2011; Tian et al., 2012; Visintin et al., 1997). Following substrate recognition, the APC/C-specific E2s Ube2C and Ube2S synthesize ubiquitin chains that are recognized by the proteasome (Garnett et al., 2009; Rape and Kirschner, 2004; Williamson et al., 2009; Wu et al., 2010; Yu et al., 1996). Depletion of Cdc20 or Ube2C and Ube2S stabilizes APC/C substrates and arrests cells prior to anaphase (Manchado et al., 2010; Williamson et al., 2009; Wolthuis et al., 2008).

As full activation of the APC/C leads to sister chromatid separation, it has to be delayed until all chromosomes have achieved bipolar attachment to the mitotic spindle. To this end, the APC/C is inhibited during spindle formation by a signaling network referred to as the spindle checkpoint (Kim and Yu, 2011; Musacchio and Salmon, 2007). The checkpoint components Mad2, BubR1, and Bub3 associate with Cdc20 to form the mitotic checkpoint complex, which binds the APC/C and blocks recognition of most of its substrates (Chao et al., 2012; Herzog et al., 2009; Schreiber et al., 2011). Once the spindle has been built and all chromosomes have been aligned at the metaphase plate, checkpoint complexes are disassembled, APC/C^{Cdc20} is activated, and sister chromatid separation ensues (Foster and Morgan, 2012; Reddy et al., 2007; Uzunova et al., 2012; Varetti et al., 2011). Thus, by virtue of the checkpoint, the spindle indirectly controls the stability of most APC/C substrates.

Spindle formation is a highly dynamic process that depends on a large number of proteins referred to as spindle assembly factors. Several of these factors, such as HURP, NuSAP, or Tpx2, associate with and stabilize microtubules, the main constituents of the spindle apparatus (Gruss et al., 2001; Koffa et al., 2006; Ribbeck et al., 2006; Silljé et al., 2006; Wittmann et al., 2000; Wong and Fang, 2006). Spindle assembly factors also recruit regulators of spindle formation: Tpx2, for example, promotes the spindle association of Aurora A and Eg5, thus controlling spindle pole integrity or spindle bipolarity (Gable et al., 2012; Kufer et al., 2002). After their activation in prometaphase, spindle assembly factors continue to play important roles during anaphase and telophase, when they stabilize kinetochore fibers, drive spindle elongation, or establish a microtubule-dense spindle midzone (Goshima et al., 2007; Uehara and Goshima, 2010).

Befitting their role in cell division, complete loss of spindle assembly factors leads to embryonic lethality or female infertility (Aguirre-Portolés et al., 2012; Tsai et al., 2008), whereas their overexpression can result in tumorigenesis (Aguirre-Portolés

et al., 2012; Gulzar et al., 2013; Hu et al., 2012; Tsou et al., 2003). These observations imply that cells need to control the abundance of spindle assembly factors, and indeed, HURP, NuSAP, and Tpx2 are turned over during mitosis by APC/C-dependent ubiquitylation and proteasomal degradation (Song and Rape, 2010; Stewart and Fang, 2005). HURP, NuSAP, and Tpx2 are also regulated by proteins of the importin family, which restrict their association with microtubules and prevent their recognition by the APC/C (Gruss et al., 2001, 2002; Koffa et al., 2006; Kufer et al., 2002; Ribbeck et al., 2006; Silljé et al., 2006; Song and Rape, 2010). A consequence of this dual regulation, the dissociation of importin β by GTP-bound Ran not only activates spindle assembly factors but also allows their APC/C-dependent inactivation. However, despite the importance of spindle formation for cell division, it has remained unclear how cells can sufficiently delay the degradation of HURP, NuSAP, and Tpx2 to allow these proteins to fulfill their critical roles in regulating spindle structure and function.

Here, we have discovered that spindle microtubules protect HURP, NuSAP, and Tpx2 from APC/C-dependent degradation. By contrast, those spindle assembly factor molecules that are not associated with microtubules are highly susceptible to proteolysis and degraded instantaneously after APC/C activation in early anaphase. The proper timing of spindle assembly factor degradation, as achieved by this regulatory circuit, is critical for mitosis, and inappropriate stabilization of spindle assembly factors disrupts spindle structure and function. Our findings suggest that localized stabilization of APC/C substrates enables the selective disposal of cell-cycle regulators that have fulfilled their mitotic roles, thus coupling the activity and stability of important spindle assembly factors.

RESULTS

Microtubules Protect Spindle Assembly Factors from Degradation

As most spindle assembly factors accumulate on the spindle during mitosis, we wished to determine whether their localization affected their stability. To this end, we tested whether microtubules, the main constituents of the spindle, influence the efficiency of spindle assembly factor degradation by the APC/C. We first generated extracts of prometaphase HeLa cells that contained inactive APC/C and soluble tubulin. We then supplemented these extracts with taxol to induce microtubule formation, GTP-charged Ran^{Q69L} to release spindle assembly factors from their inhibitors of the importin family, and $p31^{comet}$ to activate the APC/C. Without microtubules, these extracts supported the efficient degradation of spindle assembly factors and other known APC/C substrates, and depletion of the essential APC/C activator Cdc20 or addition of the APC/C^{Cdc20}-specific inhibitor Mad2 underscored the specificity of these reactions (Figures 1A, S1A, and S1B available online). By contrast, when microtubules were present, HURP, NuSAP, and Tpx2 were protected from degradation, whereas soluble APC/C substrates remained unstable (Figure 1A). The effects of microtubules on the stability of spindle assembly factors were reversible, as HURP was rapidly turned over once microtubules had been depolymerized with nocodazole (Figure 1B). Thus, microtubules protect multiple spindle assembly factors from degradation through APC/C^{Cdc20} in early mitotic extracts.

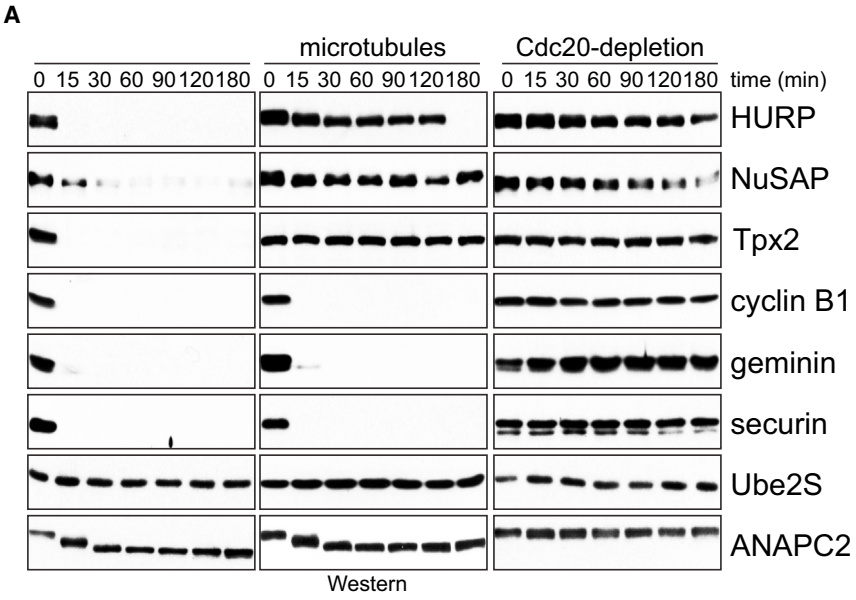
During cytokinesis and G1, the APC/C is activated by Cdh1 (Visintin et al., 1997). To determine whether microtubules stabilize spindle assembly factors against APC/C^{Cdh1}, we generated extracts of HeLa cells that were synchronized in G1. We supplemented these extracts with taxol and Ran^{Q69L} and then measured the stability of radiolabeled APC/C substrates. As we had seen for APC/C^{Cdc20}, microtubules protected the spindle assembly factor HURP, but not soluble cyclin B1, from APC/C-dependent degradation (Figure 1C). Soluble HURP was stabilized by the APC/C^{Cdh1} inhibitor Emi1, which attests to the specificity of these reactions.

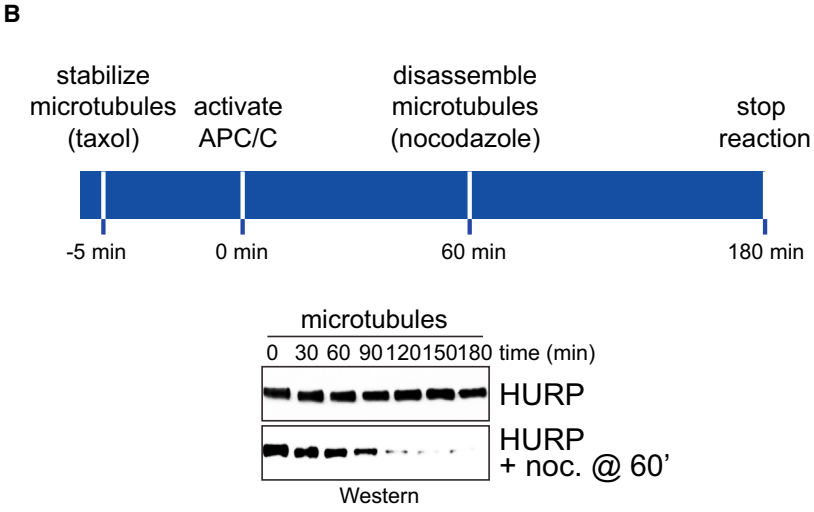
Prompted by these observations, we tested whether microtubules interfered with the ubiquitylation of spindle assembly factors by the APC/C. Indeed, the APC/C^{Cdc20}- and APC/C^{Cdh1}-dependent ubiquitylation of HURP, NuSAP, and Tpx2 was blocked by microtubules (Figures 2A, 2B, S1C, and S1D), with an efficiency comparable to that of Mad2, an established APC/C^{Cdc20} inhibitor (Figure S1E). Microtubules also ablated the ubiquitylation of a bacterially purified HURP domain that contained all APC/C degrons and microtubule-binding domains (Figure 2C), whereas unpolymerized tubulin did not impede these reactions (Figure S1F). In contrast to their effects on spindle assembly factors, microtubules neither affected the modification of soluble APC/C substrates, such as cyclin B1 (Figure 2D), nor did they interfere with the ubiquitylation of substrates of other E3s (Figure S1G). We conclude that microtubules protect spindle assembly factors from APC/C-dependent ubiquitylation and degradation in vitro.

HURP Contains Two Microtubule-Binding Domains

Multiple lines of evidence suggest that HURP, NuSAP, and Tpx2 are regulated by similar mechanisms: all proteins localize to a subset of spindle microtubules, the kinetochore fibers; their association with microtubules is inhibited by importins (Kalab and Heald, 2008); and their microtubule-regulated degradation is dependent upon the APC/C, which targets these spindle assembly factors at similar times during mitosis (Song and Rape, 2010; Stewart and Fang, 2005). Unfortunately, for none of these proteins were microtubule-binding domains sufficiently characterized to generate inactive mutants, a prerequisite for understanding how their degradation is controlled. Given the similarities in regulation, we decided to use HURP as a model substrate to define its microtubule-binding domains. We then introduced inactivating mutations into the respective domains to gain insight into the mechanism of microtubule-dependent APC/C regulation.

Previous work had located a microtubule-binding domain within the first 280 residues of HURP (Wong et al., 2008). By testing fragments that covered this region, we found a smaller motif, the MBD1, which associated with microtubules with similar affinity as the full-length protein (Figures 3A and 3B) (Wong and Fang, 2006). The interaction between microtubules and the MBD1 was disrupted by mutation of positively charged residues in the MBD1 or treatment of microtubules with subtilisin, which removes the negatively charged C terminus of tubulin. Thus, the MBD1 is a microtubule-binding motif that likely recognizes the C-terminal tail of tubulin.





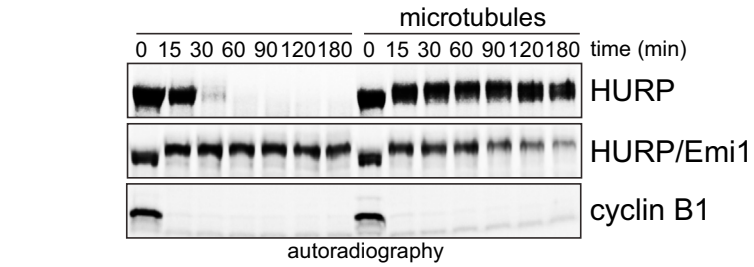


Figure 1. Microtubules Stabilize Spindle Assembly Factors

(A) Microtubules protect spindle assembly factors from APC/C^{Cdc20}-dependent degradation in extracts. Extracts of prometaphase HeLa cells were subjected to $p31^{comet}$ to activate APC/C^{Cdc20} and Ran^{Q69L} to release spindle assembly factors from importin inhibitors. As indicated, extracts were treated with taxol to stabilize microtubules. Control extracts were depleted of Cdc20, and the stability of endogenous substrates was monitored by immunoblotting.

(B) Microtubule-dependent stabilization of spindle assembly factors is reversible. Mitotic extracts were treated with taxol and Ran^{Q69L}. As indicated, nocodazole was added after 60 min to depolymerize microtubules.

(C) Microtubules prevent APC/C^{Cdh1}-dependent degradation of spindle assembly factors. ^{35}S-labeled HURP or cyclin B1 was added to G1 extracts of HeLa cells with active APC/C^{Cdh1} and treated with taxol and Ran^{Q69L}. As control, the APC/C^{Cdh1} inhibitor Emi1 was added, and substrate stability was monitored by autoradiography. See also Figure S1.

To test whether the MBD1 was essential for the binding of HURP to microtubules, we generated Oregon-green-labeled $HURP^{MBD1*}$, in which positively charged residues of the MBD1 were replaced with alanine. We incubated $HURP^{MBD1*}$ with rhodamine-labeled microtubules and monitored the interaction between the two partners by fluorescence microscopy. In these assays, mutation of the MBD1 strongly reduced the capacity of HURP to bundle, but not to bind, microtubules (Figures 3C and 3D), suggesting that a second domain provides an additional interaction surface. Indeed, a motif that we refer to as the MBD2 and that overlaps with the importin-β-binding site in HURP was enriched in positively charged residues, a common feature of microtubule-binding domains. When these charged residues were mutated in the background of $HURP^{MBD1*}$ ($HURP^{MBD1/2*}$), all microtubule binding of HURP was lost (Figures 3C and 3D). The MBD2, but not the MBD1, also bound importin β in vitro and in cells (Figures S2A and S2B), and accordingly, importin β inhibited the capacity of the MBD2 to associate with microtubules (Figure S2C). Thus, the MBD1 provides a constitutive high-affinity binding site for microtubules, whereas the weaker interaction between the MBD2 and microtubules is regulated by importin β.

The results of our biochemical analyses were confirmed in vivo: when expressed during interphase, HURP and $HURP^{MBD2*}$ colocalized with microtubules, indicative of a functional interaction (Figures 3E and 3F). This association was strongly reduced by mutation of the MBD1 or simultaneous inactivation of the MBD1 and MBD2. These findings show that during interphase, the MBD1 is the dominant microtubule-binding domain in HURP. As described later, the MBD2 contributes to

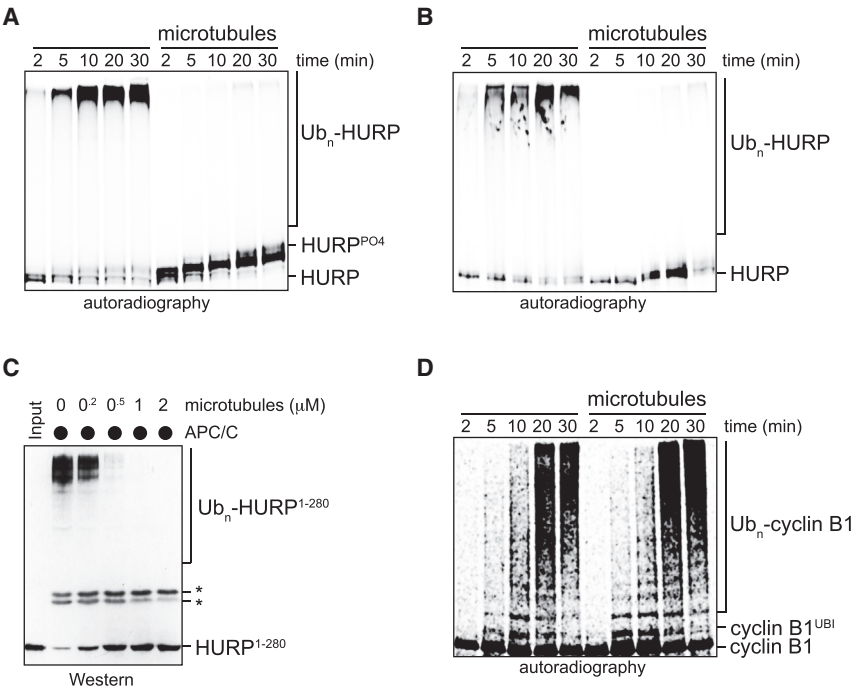


Figure 2. Microtubules Prevent Ubiquitylation of Spindle Assembly Factors by the APC/C

(A) Microtubules inhibit ubiquitylation of HURP by APC/C^{Cdc20}. ^{35}S-labeled HURP was synthesized in vitro, purified, incubated with Ran^{Q69L}, and subjected to ubiquitylation by APC/C^{Cdc20}. As indicated, HURP was prebound to microtubules. Reactions were monitored by autoradiography.

(B) Microtubules inhibit ubiquitylation of ^{35}S-labeled HURP by APC/C^{Cdh1} isolated from HeLa cells synchronized in G1. Reactions were analyzed in the presence of microtubules, as described.

(C) Microtubules prevent the ubiquitylation of bacterially expressed HURP. HURP$^{1-280}$, which contains all degrons and microtubule-binding domains, was incubated with microtubules and subjected to APC/C-dependent ubiquitylation. Reactions were analyzed by western blot. The asterisks mark cross-reactive bands.

(D) Microtubules do not affect the ubiquitylation of ^{35}S-labeled cyclin B1 by APC/C^{Cdc20}. See also Figure S1.

microtubule binding of HURP during mitosis (see below), indicating that both the MBD1 and MBD2 can mediate an interaction of the spindle assembly factor HURP to microtubules (Figure 3G).

Binding to Microtubules Is Necessary and Sufficient for HURP Stabilization

Having mapped HURP's microtubule-binding motifs, we tested whether their integrity was required for the stabilization of APC/C substrates by microtubules. We supplemented extracts containing APC/C^{Cdc20}, taxol, and Ran^{Q69L} with the respective HURP mutants and monitored the degradation of these substrates over time. While microtubules stabilized HURP and HURP^{MBD2*}, this effect was lost if the constitutive binding motif MBD1 was mutated (Figure 4A). The degradation of HURP^{MBD1*} and HURP$^{MBD1/2*}$ was dependent upon the APC/C, as both proteins were stabilized by depletion of Cdc20 (Figure S3A), addition of Mad2 (Figure S3B), or mutation of APC/C degrons (Figure 4A). Accordingly, in contrast to wild-type (WT) HURP (Figure 2A), microtubules did not inhibit the ubiquitylation of HURP^{MBD1*} and HURP$^{MBD1/2*}$ by the APC/C (Figures 4B and S3C). We conclude that the MBD1, but not the importin-β-regulated MBD2, is required for the stabilization of HURP by microtubules.

To test whether the MBD1 is sufficient to impose this regulatory mechanism onto APC/C substrates, we fused it to the N-terminal half of geminin, a protein that is turned over by the APC/C in the presence of microtubules. The fusion geminin^{MBD1}, but not WT geminin, was targeted to microtubules in vitro and to the spindle in cells, confirming that the MBD1 mediates an interaction with microtubules during mitosis (Figures 4C and 4D). Importantly, degradation of geminin^{MBD1} in extracts and its ubiquitylation by the APC/C were strongly inhibited by microtubules (Figures 4E and 4F). Similar results were obtained in assays that monitored the stability of MBD1 fusions to various other APC/C substrates. The mutation of positively charged residues within the MBD1 disrupted the spindle binding of geminin^{MBD1} and ablated the regulation of its ubiquitylation and degradation by microtubules (Figures 4C, 4E, and S3D). Thus, the MBD1 is not only required but also sufficient for the microtubule-dependent stabilization of HURP.

It is possible that any stable interaction might prevent the degradation of APC/C substrates, or, alternatively, this might be a function of the microtubule-binding domains present in spindle assembly factors. To address this issue, we fused geminin to the N-terminal microtubule-binding domain of tau, a protein that interacts with microtubules in interphase but does not act as a spindle assembly factor during mitosis (Cleveland et al., 1977). Although this domain afforded a similar affinity to microtubules as HURP's MBD1 (Figure 4D), geminin^{TAU} was not protected from APC/C-dependent degradation by microtubules (Figure 4E). Geminin^{TAU} was also not stabilized if the MBD1 was added in *trans*, showing that the MBD1 needs to be in the same peptide as the APC/C degrons. Moreover, locking geminin into dimeric complexes by fusing it to the leucine zippers of the transcription factor GCN4 did not prevent its APC/C-dependent degradation (Figure 4E). Together, these experiments indicate that stable interactions are not sufficient to protect HURP. Instead, a specific property of the MBD1, such as its capacity to bundle microtubules, might be required for its role in stabilizing HURP on microtubules.

Microtubules Stabilize HURP during Mitosis

We next wished to determine whether microtubules stabilize spindle assembly factors in vivo. To this end, we expressed low levels of GFP-tagged HURP or its microtubule-binding-deficient

mutants using lentiviruses (Figure S4A) and monitored the abundance of these variants in HeLa cells by video microscopy. In agreement with analyses of endogenous HURP (Koffa et al., 2006; Silljé et al., 2006; Wong and Fang, 2006), GFP-tagged HURP accumulated on the spindle until telophase and was completely turned over only after all sister chromatids had been distributed into the two daughter cells (Figure 5A). By contrast, HURP^{MBD1*} bound the spindle with lower efficiency than WT HURP, and HURP$^{MBD1/2*}$ did not show significant enrichment at the spindle. Moreover, the degradation of HURP^{MBD1*} and HURP$^{MBD1/2*}$ started much earlier than that of the WT protein, and both mutants were degraded beyond our detection limit shortly after sister chromatid separation had been initiated. Mutation of all APC/C degrons stabilized HURP^{MBD1*} and HURP$^{MBD1/2*}$ until G1, demonstrating that the premature degradation of these spindle assembly factor variants was carried out by the APC/C.

To independently assess whether microtubules protect spindle assembly factors from degradation, we measured the levels of microtubule-binding-deficient mutants by immunofluorescence microscopy. As seen before, inactivation of the MBD1 or both the MBD1 and MBD2 caused a strong decrease in the abundance of HURP that was especially apparent shortly before anaphase (Figures 5B, S4B, and S4C). By contrast, HURP, HURP^{MBD1*}, and HURP$^{MBD1/2*}$ were present at comparable levels in interphase when the APC/C was inactive (Figure S4B), or during mitosis, if the proteasome was inhibited, the APC/C^{Cdc20}-specific inhibitor Mad2 was overexpressed, or APC/C degrons were mutated (Figures 5B, S4C, and S4D). In agreement with the premature degradation of soluble HURP, stable HURP$^{\Delta APC/MBD1*}$, but not HURP^{MBD1*}, was detected on metaphase chromosomes, an observation that we also made for the GFP-tagged protein by live-cell analysis (Figures 5A and 5B). Based on these findings, we conclude that microtubules prevent the premature degradation of HURP during mitosis. If this regulation is lost, the spindle assembly factor is highly susceptible to proteolysis and depleted from cells almost instantaneously after the APC/C has been activated.

The instability of microtubule-binding-deficient HURP implied that the removal of spindle assembly factors contributes to robust spindle function. To test this hypothesis, we filmed cells that expressed HURP$^{\Delta APC}$, a stable mutant that lacks all APC/C degrons (Song and Rape, 2010), but retained its capacity to bind microtubules. Confirming APC/C's role in targeting HURP, GFP-HURP$^{\Delta APC}$ was not degraded during any stage of mitosis, allowing it to persistently bind microtubules throughout mitosis (Figure 6A). The stabilization of HURP resulted in strong mitotic defects: HURP$^{\Delta APC}$, but not WT-HURP or inactive HURP$^{\Delta APC/MBD1/2*}$, impaired the assembly of a metaphase plate with completely aligned chromosomes, induced formation of multiple spindle poles, and delayed anaphase onset (Figures 6A–6D). Immunofluorescence analysis against γ-tubulin and centrin showed that expression of HURP$^{\Delta APC}$ triggered spindle pole fragmentation, rather than centrosome amplification (Figures S5A and S6B). In agreement with these results, inducible overexpression of HURP, but not inactive HURP$^{MBD1/2*}$, led to spindle defects, such as spindle elongation (Figure 6E). Thus, while losing its microtubule-dependent regulation caused premature degradation of HURP, the inappropriate stabilization of this spindle assembly factor interfered with accurate spindle function and, consequently, faithful cell division.

The APC/C and Importin β Cooperate to Ensure Spindle Structure and Function

Contrary to the expression of HURP$^{\Delta APC}$, inhibition of the APC/C or the proteasome had little effect on spindle formation and only led to spindle breakdown after a prolonged metaphase arrest (Daum et al., 2011; Stevens et al., 2011; Zeng et al., 2010). The APC/C-dependent degradation of spindle assembly factors thus likely cooperates with another mechanism to ensure spindle function, and indeed, all microtubule-regulated APC/C substrates studied here are also inhibited by members of the importin family. As HURP$^{\Delta APC}$ was neither recognized by the APC/C nor importin β (Figure S5C), the latter two regulators might cooperate in ensuring spindle function. To test this hypothesis, we inhibited either the APC/C by depleting its activator Cdc20 (Figure S6A); importin β by treating cells with importazole, a molecule that inactivates importin β without stabilizing spindle assembly factors (Soderholm et al., 2011); or APC/C and importin β by subjecting cells to siRNAs against Cdc20 and importazole at the same time. We then monitored cells that stably expressed ^{mCherry}histone H2B and ^{GFP}tubulin by video microscopy.

In agreement with earlier reports (Daum et al., 2011; Stevens et al., 2011; Zeng et al., 2010), inhibition of the APC/C had no apparent effect on spindle formation, but often caused a metaphase arrest followed by cohesion fatigue and chromosome scattering, spindle elongation, and spindle rotation (Figures 7A and 7B). In other cases, cells depleted of Cdc20 were able to proceed into anaphase, albeit after a delay, which is consistent with reports that very low levels of Cdc20 are sufficient to support anaphase onset (Wolthuis et al., 2008). Also confirming published results (Soderholm et al., 2011), the inhibition of importin β slightly delayed chromosome congression, yet cells eventually completed spindle assembly and initiated sister chromatid separation (Figures 7A and 7B). By contrast, when cells were concurrently treated with siRNAs against Cdc20 and importazole, a much larger fraction of cells arrested prior to anaphase, a metaphase plate with completely aligned chromosomes was rarely observed, and arrested cells died without visible chromosome scattering or spindle rotation (Figures 7A and 7B). Similar phenotypes were observed when importazole was combined with TAME (Figures 7C, S6B, andS6C), a compound that interferes with the activity of APC/C^{Cdc20} (Zeng et al., 2010), or MG132, a molecule that blocks proteasomal degradation (Figure S6D). These findings were independent of the reporter used to monitor cell division, as we made similar observations when the APC/C and importin β were inhibited in cells that stably expressed the kinetochore marker $^{LAP/GFP}$CenpA (Figure S6E). Our experiments, therefore, confirm that neither the APC/C nor importin β is essential for spindle formation. However, spindle function is highly compromised when the APC/C and importin β are inhibited at the same time, a condition that simultaneously removes two players that restrict the activity of spindle assembly factors. We conclude that the rapid degradation of spindle assembly factors, a

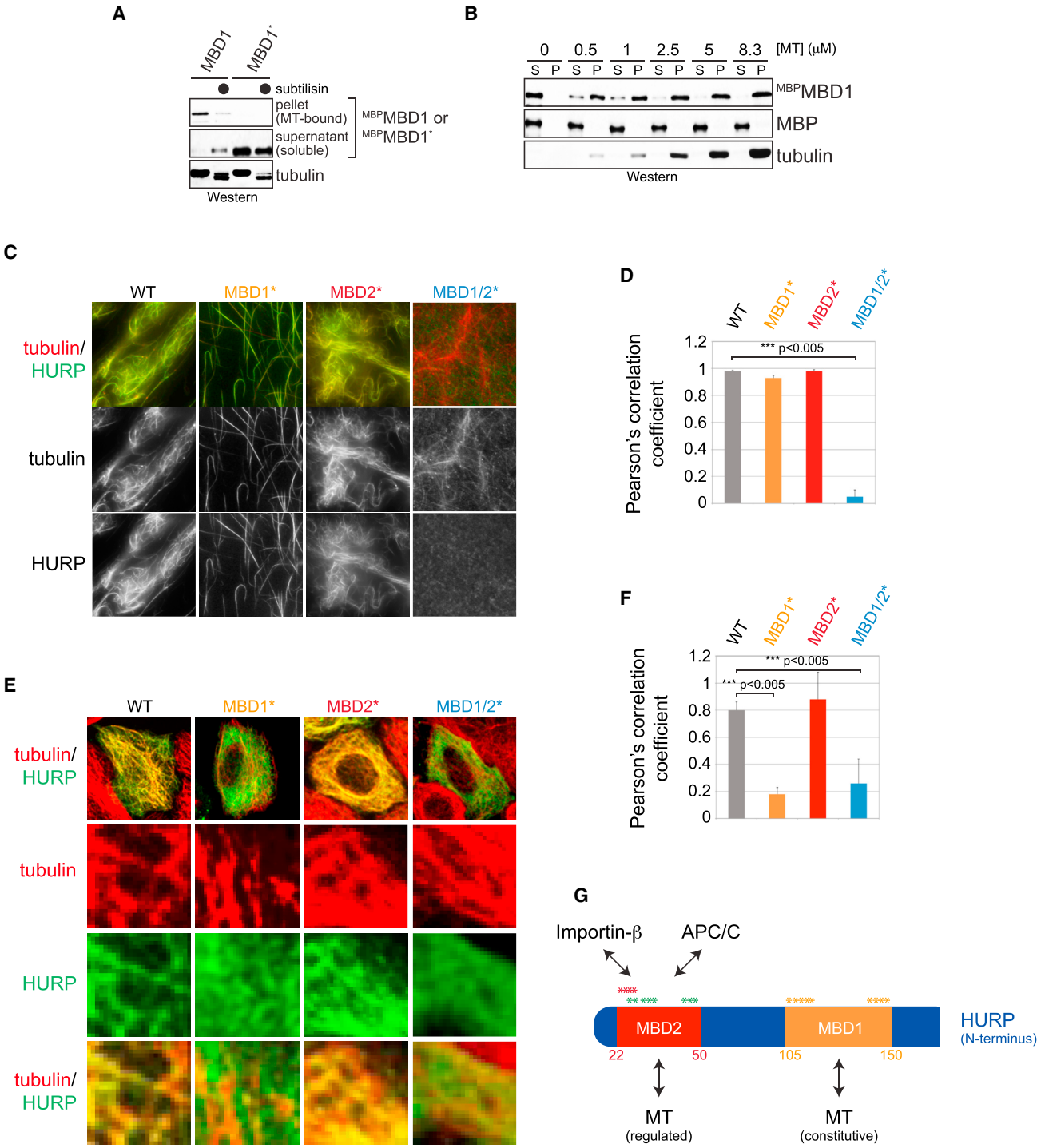


Figure 3. HURP Contains Two Microtubule-Binding Motifs

(A) Taxol-stabilized microtubules were incubated with ^{MBP}MBD1 or mutant ^{MBP}MBD1* in which positively charged residues were exchanged to alanine. As indicated, microtubules were treated with subtilisin. Binding reactions were centrifuged through a sucrose cushion, and microtubule-bound and soluble fractions were analyzed by western blot.

(B) The MBD1 binds microtubules with similar affinity as full-length HURP. ^{MBP}MBD1 was incubated with taxol-stabilized microtubules and subjected to sucrose gradient centrifugation.

(C) The MBD1 and MBD2 both mediate microtubule binding of HURP. Recombinant full-length HURP or mutants in its MBD1 or MBD2 were labeled with Oregon green and incubated with rhodamine-labeled microtubules. Binding was analyzed by fluorescence microscopy.

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reaction that is tightly controlled by microtubules and importin β, is a fundamental component of the control mechanisms that ensure the robustness and accuracy of metazoan cell division.

DISCUSSION

In this study, we discovered localized stabilization as a mechanism that tightly controls the abundance of critical spindle assembly factors. At the heart of this regulatory circuit are microtubules, the main constituents of the mitotic spindle and direct binding partners of spindle assembly factors. When bound to microtubules, HURP, NuSAP, and Tpx2 are protected from APC/C-dependent degradation. By contrast, if these substrates are not associated with the spindle, they are highly susceptible to degradation and turned over shortly after the APC/C has been fully activated at the metaphase-anaphase transition. It is well established that spindle defects indirectly result in APC/C inhibition by triggering the spindle assembly checkpoint, a signaling network that impedes the capacity of APC/C^{Cdc20} to recognize its substrates. Our current work suggests that the spindle also directly inhibits the turnover of specific APC/C substrates, a mechanism that is important for accurate cell division.

Based on our findings, we propose a framework for the temporal and spatial regulation of spindle assembly factors (Figure 7D): HURP, NuSAP, or Tpx2 are initially sequestered by importins, which inhibit and stabilize Ran-dependent spindle assembly factors (Kalab and Heald, 2008; Song and Rape, 2010). As importin β does not block HURP's high-affinity microtubule-binding domain, spindle assembly factors could be loaded onto the spindle in their inactive, importin-bound states. In contrast to surgical mutations in microtubule- and importin-β-binding sites, expression of dominant-negative importin or Ran variants displaces HURP from the spindle (Silljé et al., 2006), suggesting that the global interference with the Ran gradient also has secondary effects upon spindle assembly factor targeting. In proximity to chromatin, Ran^{GTP} dissociates HURP from importin β, thereby activating the spindle assembly factor by allowing its MBD2 to engage microtubules. Importantly, as the MBD1 is already bound to microtubules, the active spindle assembly factor remains protected from recognition by the APC/C. When HURP is no longer required, it will be released from the spindle, and— as suggested by the instantaneous degradation of soluble HURP upon APC/C activation—rapidly degraded. In this model, only spindle assembly factors that have fulfilled their mitotic role are turned over: inactive spindle assembly factors are stabilized by importin β, whereas those molecules that are engaged in spindle formation are protected by microtubules. The microtubule-dependent regulation of APC/C substrate degradation, therefore, effectively couples the activity and stability of critical cell-cycle proteins.

How microtubules stabilize specific APC/C substrates requires further analyses, but our results indicate that a direct interaction with spindle assembly factors plays an important role: whereas microtubules stabilized HURP, NuSAP and Tpx2, they had no effects on soluble APC/C substrates. Moreover, the mutation of HURP's MBD1 allowed the degradation of this spindle assembly factor in the presence of microtubules, while the transfer of the MBD1 to soluble APC/C substrates was sufficient to impose this control mechanism. By associating with spindle assembly factors, microtubules could impede the recognition of substrates by the APC/C or interfere with rate-limiting steps of the ubiquitylation reaction, such as chain initiation (Williamson et al., 2011). Potentially pointing toward the latter mechanism, the MBD1 does not overlap with the D box, KEN box, or initiation motif in HURP (Song and Rape, 2010), and its transfer to other substrates allowed their stabilization even though these proteins contained degrons at different positions than HURP. These findings make it seem unlikely that microtubules simply shield a critical degron from recognition by the APC/C. Instead, we favor the idea that the MBD1 confers a property onto microtubules, such as bundling, that is not conducive to the buildup of ubiquitin chains by the APC/C. Indeed, all spindle assembly factors subject to this regulatory mechanism bundle microtubules, and HURP requires the MBD1 for this function. Moreover, a microtubule-binding domain that does not bundle microtubules failed to impose microtubule-dependent stabilization, even though it could target APC/C substrates to microtubules with similar efficiency as the MBD1.

As microtubule bundlers often accumulate on kinetochore fibers, but not astral microtubules, this mechanism of stabilization might be of particular relevance for the critical subset of spindle microtubules that mediate chromosome attachment and sister chromatid separation. Supporting this notion, the stabilization of HURP impaired the assembly of a metaphase plate with completely aligned chromosomes. This result is consistent with the finding that overexpression of spindle assembly factors can impair cell division and lead to tumorigenesis and that it has been inversely correlated with therapeutic outcome (Aguirre-Portolés et al., 2012; Gulzar et al., 2013; Pérez de Castro and Malumbres, 2012; Tsou et al., 2003). Without proper APC/C activity, spindle assembly factors might accumulate over multiple cell cycles and exert their effects due to a gradual increase in their abundance. It is also possible that a fraction of spindle assembly factor molecules are degraded

(D) Quantification of HURP and microtubule colocalization in vitro. Green (HURP) and red (tubulin) signals were correlated using ImageJ and JACoP, resulting in a Pearson's correlation coefficient. Error bars represent the SD of three independent experiments.
(E) MBD1 mediates microtubule binding of HURP in interphase. HeLa cells were transfected with ^{FLAG}HURP or mutants in its MBDs and analyzed by immunofluorescence microscopy (green indicates HURP; red indicates β tubulin).
(F) Pearson's correlation coefficient to quantify the colocalization between HURP mutants and microtubules in interphase cells. Error bars represent the SD of three independent experiments.
(G) Overview of binding motifs in the N-terminal domain of HURP. The MBD1 provides a constitutive binding site for microtubules; MBD2 overlaps with APC/C degrons and the importin-β-binding site (Song and Rape, 2010). The asterisks mark residues mutated to alanine in order to ablate the functions of the MBD1 (orange), MBD2 (red), or APC/C degrons (green). See also Figure S2.

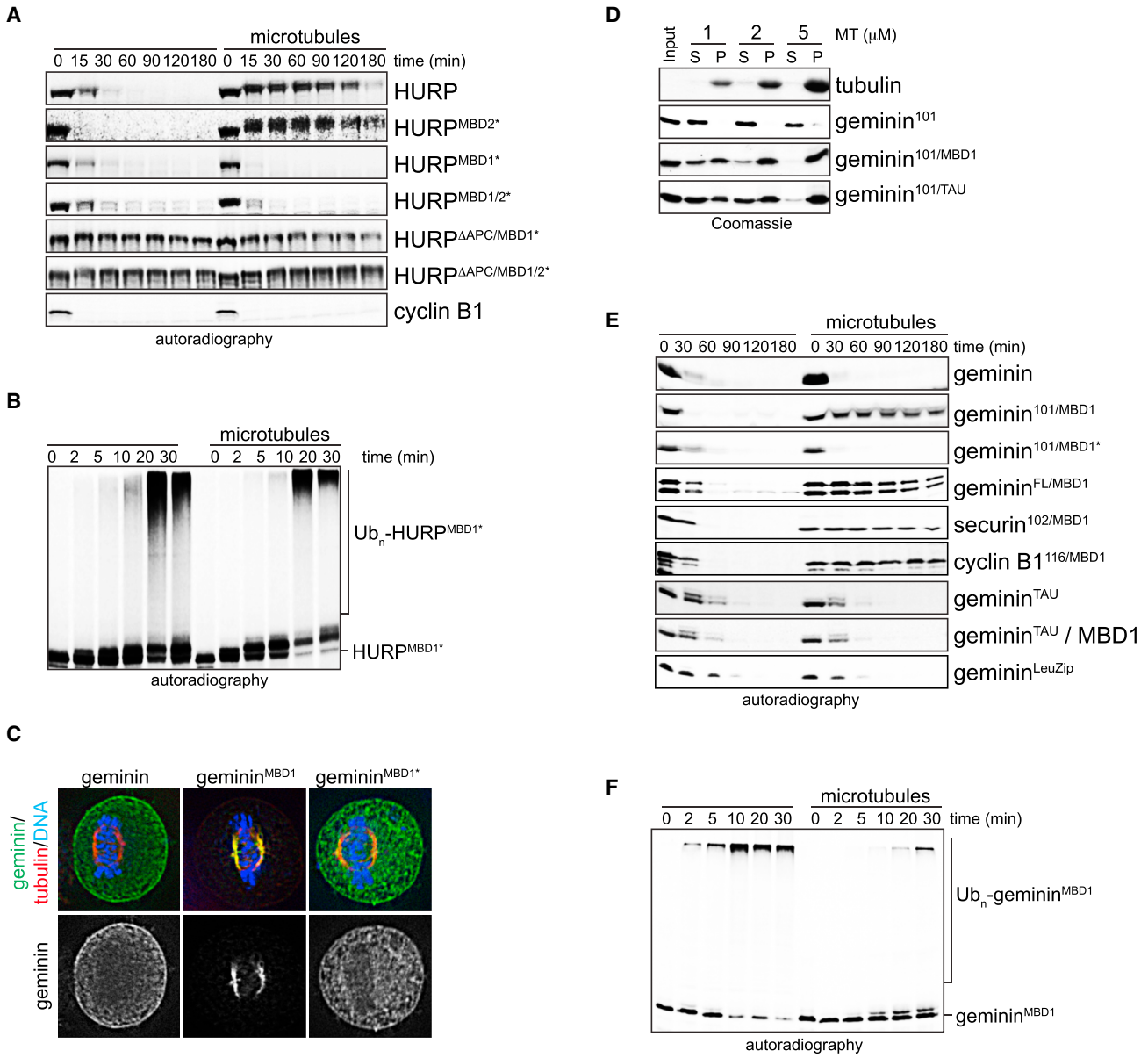

Figure 4. The MBD1 Is Required and Sufficient for the Regulation of HURP Stability by Microtubules

(A) MBD1-mutation allows degradation of HURP in the presence of microtubules. Mitotic extracts with APC/C^{Cdc20} and Ran^{Q69L} were supplemented with ^{35}S-labeled HURP, HURP mutants (MBD1*, MBD2*, or ΔAPC), or cyclin B1, and degradation was monitored by autoradiography. As indicated, microtubules were stabilized by taxol.

(B) The MBD1 is required for the inhibition of HURP ubiquitylation by microtubules. ^{35}S-labeled $HURP^{MBD1*}$ was incubated with buffer or microtubules before being subjected to ubiquitylation by APC/C^{Cdc20}. Reactions were followed by autoradiography.

(C) The MBD1 targets a soluble APC/C substrate to the spindle. HeLa cells were transfected with geminin, $geminin^{MBD1}$ (residues 1–101 of geminin fused to the MBD1 of HURP), or $geminin^{MBD1*}$ (charged residues in the MBD1 mutated to alanine), and localization was determined by immunofluorescence microscopy (green shows geminin; red shows tubulin; blue shows DNA/DAPI). The bottom panel shows the localization of the geminin proteins alone.

(D) Fusions of HURP's MBD1 or the N-terminal microtubule-binding domain of tau induce microtubule binding of soluble APC/C substrates with similar efficiency. Binding of recombinant proteins to microtubules was analyzed by sucrose gradient centrifugation (S indicates soluble fraction; P indicates microtubule-bound fraction).

(E) The MBD1 imposes microtubule-dependent regulation of degradation. ^{35}S-labeled substrates were added to extracts with APC/C^{Cdc20} and Ran^{Q69L} (MBD1indicates HURP's MBD1; TAU indicates N-terminal microtubule-binding domain of tau; LeuZip indicates leucine zippers of GCN4 transcription factor). As indicated, microtubules were stabilized by taxol or the ^{MBP}MBD1 was added to induce microtubule bundling. Reactions were monitored by autoradiography.

(F) Microtubules inhibit the ubiquitylation of $geminin^{MBD1}$. ^{35}S-labeled $geminin^{MBD1}$ was incubated with buffer or microtubules and subjected to ubiquitylation by APC/C^{Cdc20}. Reactions were monitored by autoradiography. See also Figure S3.

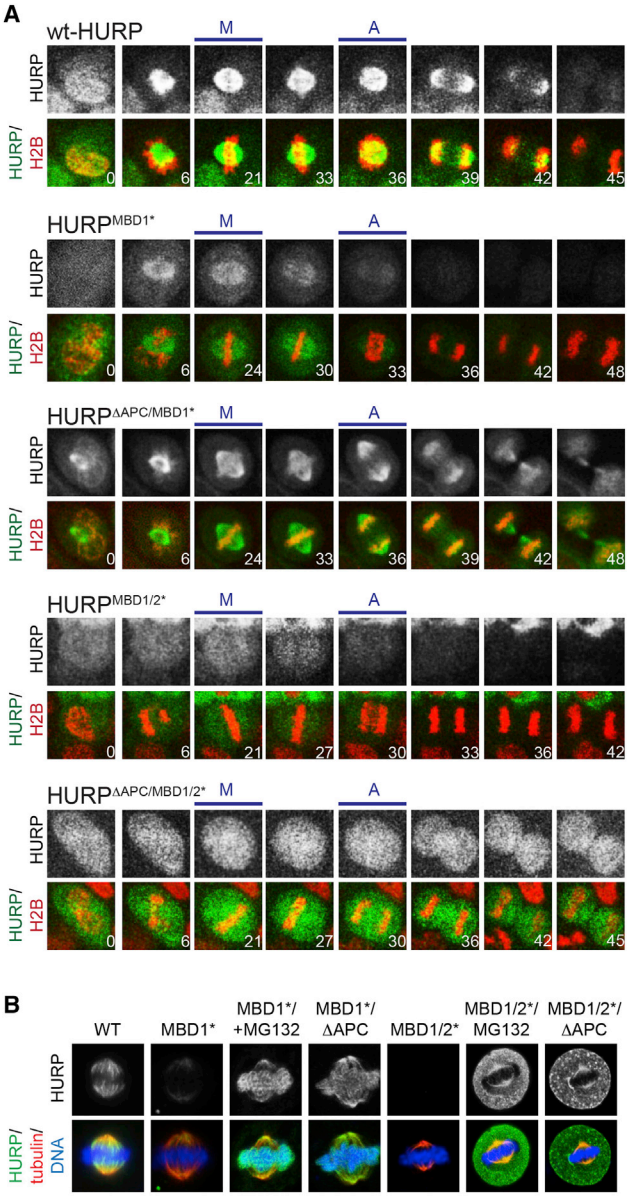

Figure 5. Microtubule Binding Stabilizes HURP during Mitosis

(A) HeLa cells were transduced with lentiviruses expressing ^{mCherry}histone H2B and GFP-tagged HURP, HURP^{MBD1*}, HURP$^{\Delta APC/MBD1/2*}$, HURP$^{MBD1/2*}$, or HURP$^{\Delta APC/MBD1/2*}$ (MBD1/2* indicates mutation of microtubule-binding motifs; ΔAPC indicates mutation of all degrons). Progression through mitosis was monitored by live cell imaging. The upper panels show the levels of the GFP-tagged HURP variant, whereas lower panels display GFP-HURP (green), mCherry-histone H2B (red), and the time post-nuclear envelope breakdown. M indicates first frame with metaphase chromosome alignment; A indicates first frame with sister chromatid separation.

(B) HeLa cells were transfected with ^{FLAG}HURP, ^{FLAG}HURP^{MBD1*}, or ^{FLAG}HURP$^{MBD1/2*}$; where indicated, APC/C degrons were mutated (^{FLAG}HURP$^{\Delta APC/MBD1*}$; ^{FLAG}HURP$^{\Delta APC/MBD1/2*}$) or cells were treated with MG132. Cells were analyzed by immunofluorescence microscopy. Upper panel shows HURP proteins; lower panel shows HURP (green), tubulin (red), and DNA (blue). See also Figure S4.

immediately after the last kinetochore has been attached to the spindle, a hypothesis that is consistent with the high processivity of HURP ubiquitylation (Rape et al., 2006; Song and Rape, 2010). Alternatively, spindle assembly factor molecules that were released from microtubules might need to be turned over even during spindle formation and checkpoint signaling, potentially to ensure microtubule dynamics. In agreement with this hypothesis, proteasome inhibition stabilized the microtubule-binding-deficient HURP in mitotic cells that had retained high levels of kinetochore-bound Mad1 and BubR1 (data not shown), a condition that usually signals an active spindle checkpoint (Kim and Yu, 2011; Musacchio and Salmon, 2007).

Our work underscores the notion that two essential cell-cycle regulators, the APC/C and Ran, cooperate in establishing a robust spindle that can effectively separate sister chromatids during mitosis. Similar to the misregulation of spindle assembly factors, aberrant activity of the APC/C or Ran has been linked to tumorigenesis (García-Higuera et al., 2008; Jung et al., 2006; Kalab and Heald, 2008; Manchado et al., 2010; van Ree et al., 2010; Wagner et al., 2004). Understanding how ubiquitin-dependent proteolysis is integrated with other mitotic regulators, such as Ran, to allow faithful cell division and how this interplay between multiple levels of cell-cycle control is disrupted in disease will be an important avenue for future work.

EXPERIMENTAL PROCEDURES

Plasmids, siRNAs, Antibodies, and Proteins

cDNAs encoding APC/C substrates were cloned into pCS2 for IVT/T and into pCS2-HA and pcDNA5/FRT/TO (Invitrogen) for expression in cells and stable cell-line generation. An N-terminal Flag tag was introduced into pcDNA5/FRT/TO by PCR. MBD1 consisted of residues 65–174 of HURP, and MBD2 consisted of the first 69 residues. HURP^{MBD1*} and HURP^{MBD2*} were generated by replacing the following residues with alanine: for MBD1*, K105, K107, R110, K112, K114, and R115; for MBD2*, R20, K22, R26, and K27. HURP$^{\Delta APC}$ was prepared by mutating D, KEN, and TEK boxes in HURP, as described (Song and Rape, 2010). The geminin^{MBD1} fusion was generated by fusing the first 101 residues of geminin to the MBD1 by hybrid PCR. For recombinant proteins, cDNAs were cloned into pMAL and pET28 for expression in *E.coli*, and the purification procedures for MBP-tagged and 6×His-tagged proteins were as described (Song and Rape, 2010). ON-TARGETplus Human Cdc20 siRNA-SMARTpool was purchased from Dharmacon.

The following antibodies were used: αHURP and αimportin β (polyclonal; Bethyl Laboratories); αNuSAP (polyclonal; ProteinTech Group); αTpx2 (polyclonal; Novus); αCdc27 [AF3.1], αCdc20/p55CDC [E-7], and αC-Myc [9E10] (monoclonal; Santa Cruz Biotechnology); αcyclin B1, αgeminin, αsecurin, αGFP and αANAPC2 (polyclonal; Santa Cruz Biotechnology); αUbe2S (polyclonal; Novus); αMBP (monoclonal; NEB Biolab); αtubulin [DM1A] (monoclonal; Calbiochem); αFlag M2 (monoclonal), αFlag (polyclonal) and αγtubulin (monoclonal) (Sigma); αHA [C29F4] (monoclonal; Cell Signaling); αcentrin [20H5] (monoclonal; Millipore); αMad2 (monoclonal; BD Biosciences); αβ-tubulin (Developmental Studies Hybridoma Bank); Goat anti-rabbit Alexa488 (Invitrogen); and Donkey anti-mouse DyLight 549 and Goat anti-mouse DyLight 549 (Jackson Laboratories).

In Vitro Degradation Assays

In vitro degradation assays were performed as described (Song and Rape, 2010). To test microtubule-dependent stabilization of substrates, 20 μM paclitaxel/taxol (Sigma) was added to HeLa S3 extracts (prewarmed to 30°C) to promote microtubule polymerization; where indicated, 140 μM nocodazole (Sigma) was added to destabilize microtubules. To deplete Cdc20, 400 μl mitotic extract was depleted twice at 4°C for 1 hr with 2 μg monoclonal αCdc20 antibody.

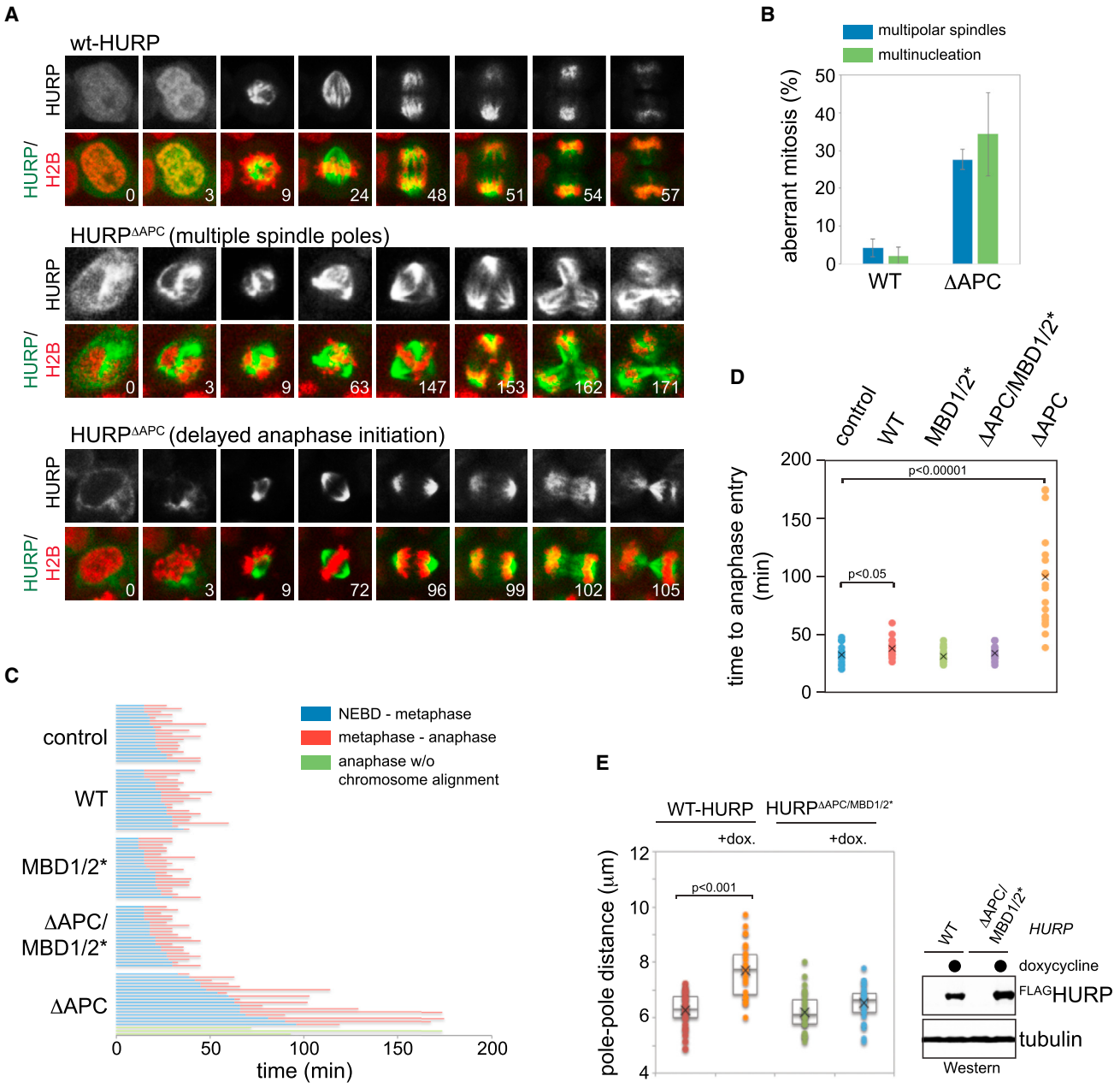

Figure 6. Stabilization of HURP Interferes with Spindle Structure and Function

(A) Expression of stabilized HURP in mitosis leads to persistent microtubule binding and mitotic defects. HeLa cells transduced with lentiviruses expressing ^{mCherry}histone H2B, and GFP-tagged HURP or HURP$^{\Delta APC}$, were filmed through mitosis. Upper panel shows HURP; lower panel shows GFP-tagged HURP (green), ^{mCherry}histone H2B (red), and the time post-nuclear envelope breakdown.

(B) Stabilization of HURP results in multiple spindle poles and multinucleation. Cells expressing GFP-HURP or GFP-HURP$^{\Delta APC}$ were filmed through mitosis and analyzed for >2 spindle poles or multinucleation. Results were from three independent experiments, including at least 50 dividing cells per condition and per experiment. Error bars represent the SD of three independent experiments.

(C) Expression of HURP$^{\Delta APC}$ delays the assembly of a metaphase plate with completely aligned chromosomes. HeLa cells expressing GFP-tagged HURP or mutants were filmed through mitosis. The time from nuclear envelope breakdown to assembly of a metaphase plate (blue) and from metaphase to anaphase initiation (red) are shown. Expression of HURP$^{\Delta APC}$ frequently caused anaphase initiation after a profound delay, even though a metaphase plate had never been established (green).

(D) Quantification of the time required for anaphase entry in the presence of HURP proteins.

(E) Increased levels of active HURP cause spindle defects. Stable 293T cell lines expressing HURP or HURP$^{\Delta APC/MBD1/2*}$ under an inducible promoter were treated with doxycycline, and spindle structures were analyzed by microscopy against α- and γ-tubulin. Left panel shows pole-pole distance in the presence or absence of HURP; right panel shows inducible expression of HURP variants. See also Figure S5.

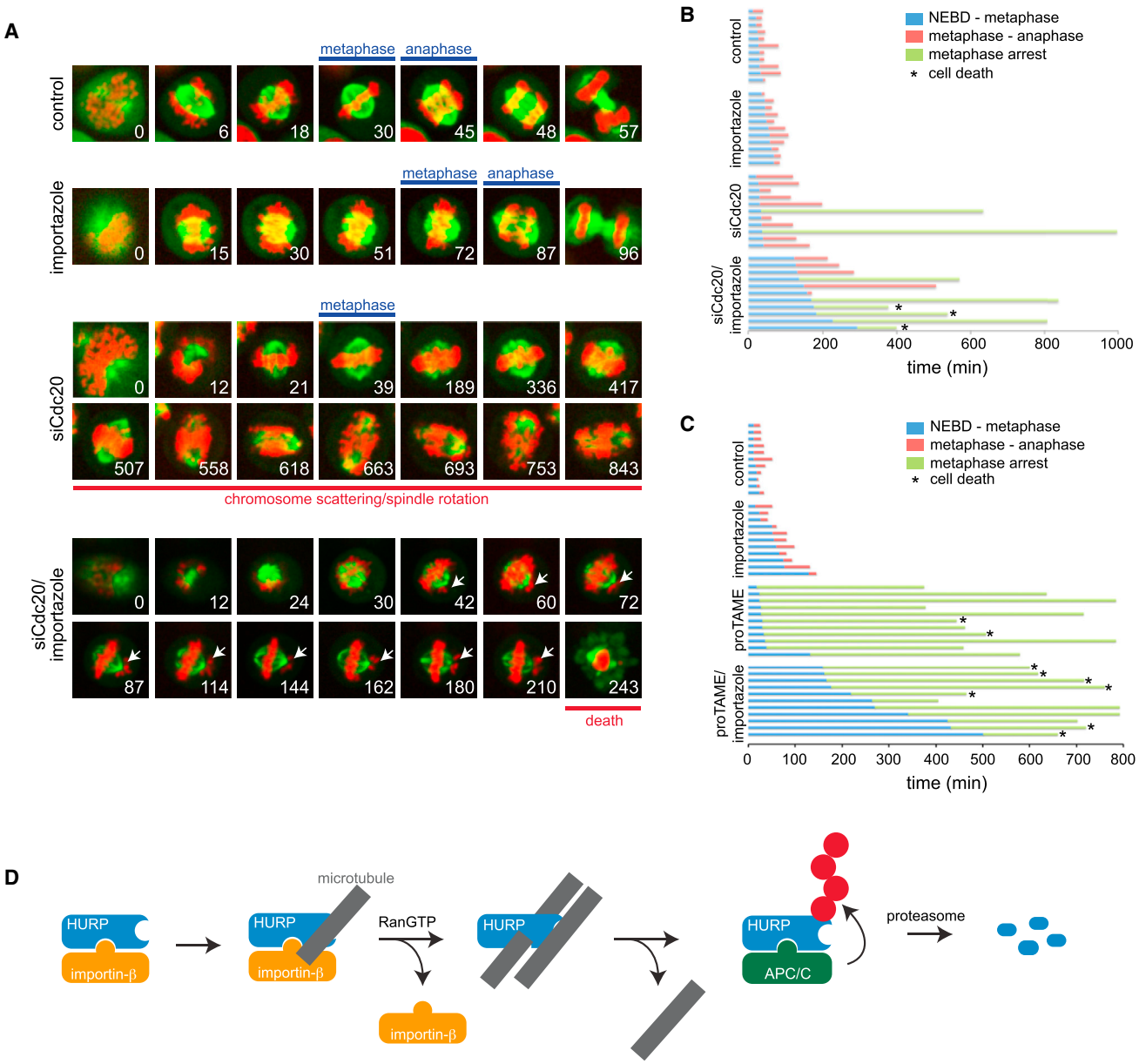

Figure 7. The APC/C and Importin β Regulate Spindle Function

(A) Simultaneous inhibition of the APC/C and importin β results in cooperative defects in spindle structure and function. HeLa cells stably expressing ^{GFP}tubulin and ^{mCherry}histone H2B were treated with siRNAs against Cdc20, importazole, or siRNAs against Cdc20 and importazole at the same time. Progression of cells through mitosis was monitored by video microscopy (tubulin shown in green; histone shown in red; arrows indicate misaligned chromosomes).

(B) Quantification of the mitotic timing for HeLa cells expressing ^{mCherry}histone H2B and ^{GFP}tubulin, after being treated with siRNAs against Cdc20 or importazole, as shown on the left. Blue indicates time from nuclear envelope breakdown to completion of chromosome alignment; red indicates time from metaphase to anaphase initiation; green indicates cells permanently arrested prior to anaphase. The asterisk marks cells undergoing cell death.

(C) The Cdc20 inhibitor proTAME displays similar functional interactions with importin β as Cdc20-siRNAs. HeLa cells stably expressing ^{mCherry}histone H2B and ^{GFP}tubulin were treated with proTAME, importazole, or both and filmed through mitosis. Experiments were quantified as described.

(D) Model of microtubule-dependent regulation of APC/C substrate degradation. Ran-dependent spindle assembly factors are inhibited by importin β. As seen for HURP, importin β does not ablate the microtubule binding of a constitutive motif, the MBD1, indicating that spindle assembly factors might be loaded onto the spindle in their inactive state. Ran^{GTP}-dependent dissociation of HURP allows the MBD2 to engage microtubules, thereby activating the spindle assembly factor. Once HURP is not required, its release from microtubules results in rapid ubiquitylation by the APC/C and proteasomal degradation, a reaction that is required for maintaining proper spindle structure and function. See also Figure S6.

In Vitro Ubiquitylation Reactions

APC/C- or $SCF^{\beta TrCP}$-dependent ubiquitylation of ^{35}S-labeled substrates was performed as described (Song and Rape, 2010; Wickliffe et al., 2011). As indicated, 1 μg microtubules were preincubated with purified substrates (Williamson et al., 2011) at 25°C for 5 min before being added to ubiquitylation reactions. For ubiquitylation of recombinant HURP, ~0.25 μM Flag-tagged $HURP^{1-280}$ was used.

HURP-Microtubule Interaction Assays

Taxol-stabilized microtubules were prepared by incubating prewarmed porcine brain tubulin with 80 μM taxol at 37°C for 10 min. Polymerized microtubules were stabilized by bringing the final concentration of taxol to 160 μM. Subtilisin-treated microtubules were prepared by incubating taxol-stabilized microtubules with 200 μg/ml subtilisin (a gift from Eva Nogales) at 37°C for 30 min. The reaction was stopped by 2 mM phenylmethanesulfonylfluoride. Microtubules were spun down at room temperature (RT) at 14,000 rpm for 20 min, and pellets were resuspended in binding buffer consisting of 1×BRB80 (80 mM PIPES, 1 mM MgCl2, and 1 mM EGTA [pH 6.8]), 1mM DTT, 5% sucrose, and 20 μM taxol.

For copelleting experiments, 250nM ^{MBP}MBD1 was incubated with control or subtilisin-treated microtubules in a 20 μl reaction for 10 min at RT. Reactions were spun at 14,000 rpm for 20 min at RT. The supernatant and pellet fractions were solubilized in 2× Laemmli buffer. A total of 10% of supernatant and pellet were subjected to αMBP immunoblot or Coomassie blue.

For monitoring the HURP-microtubule interaction by microscopy, ^{MBP}HURP was labeled with Oregon green 488 iodoacetamide (Invitrogen), cleaned by PD SpinTrap G-25 columns (GE Healthcare), and mixed with rhodamine-labeled microtubules. As indicated, 6×His importin β was preincubated with labeled HURP at equal molar ratio for 5 min. Images were taken using 60× magnification on an Olympus IX81 microscope, and processed using ImageJ.

Cell Culture, Transfection, and Immunofluorescence

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). For plasmid transfection, cells were plated on coverslips and transfected using TransIT-LT1 (Mirus). For siRNA, cells were transfected with 50 nM oligos using RNAiMAX (Invitrogen). Cells were fixed 48 hr posttransfection for immunofluorescence or subjected to live cell imaging 24 hr posttransfection. For drug treatment, cells were treated with 20 μM MG132, 20 μM proTAME (Boston Biochem; a gift from Randy King), or 20 μM importazole (a gift from Rebecca Heald) for 6–8 hr before fixation. Cells were fixed with cold methanol (−20°C) for 3 min or 3.7% formaldehyde for 20 min. Images were taken using Zeiss LSM 710 confocal microscope or Olympus IX81 microscope, deconvolved using Metamorph, and processed using ImageJ.

Flp-In T-REx 293 cells to stably express Flag-tagged HURP were generated following manufacturer's manual (Invitrogen). For immunofluorescence, cells were plated on coverslips coated with poly-D-lysine and induced with 0.5 μg/ml doxycycline for 48 hr before fixation.

Production of Lentiviruses and Transduction

eGFP-tagged HURP and mCherry-tagged histone H2B were cloned into pEF-1α/pENTR vector and recombined into pLenti X1 DEST by LR recombination to generate lentiviral expression constructs. Lentiviruses were produced in 293T cells by cotransfection of lentiviral constructs with packaging plasmids (Addgene) for 48–72 hr. Transduction was carried out by infecting ~50% confluent HeLa cells with lentiviruses in the presence of 6 μg/ml Polybrene (Sigma).

Live Cell Imaging

HeLa cells that stably express mCherry-H2B and GFP-tubulin (a gift from Rebecca Heald) or LAP-tagged CenpA (a gift from Iain Cheeseman) were maintained in DMEM-phenol-red media containing 10% FBS at 37°C with 5% CO_2 and imaged at a single focal plane every 3 min for the indicated time periods beginning 4 hr postdrug treatment. Images were taken with a 0.95 NA 40× objective on an Olympus Revolution XD spinning disk confocal microscope equipped with a charged-couple device camera (Andor technology) and a Yokogawa spinning disk. Movies were assembled using Metamorph and analyzed with Photoshop.

Cell Synchronization, Immunoprecipitation and Pull-down Assays

Cell synchronization, immunoprecipitation, and MBP pull-down assays were performed as previously described (Song and Rape, 2010).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2013.12.022.

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