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Reciprocal Regulation between 53BP1 and the Anaphase-Promoting Complex/Cyclosome Is Required for Genomic Stability during Mitotic Stress

Graphical Abstract

Highlights

- 53BP1 is an anaphase-promoting complex/cyclosome substrate in early mitosis
- 53BP1 interacts with the APC/C co-activators via its KEN boxes and tBRCT domain
- 53BP1 inhibits the APC/C in vivo and in vitro
- 53BP1 silencing results in extreme sensitivity to mitotic poisons

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In Brief

Kucharski et al. find that 53BP1 is a mitotic APC/C substrate and an interphase inhibitor. 53BP1 silencing leads to a hyperactive APC/C, resulting in cell cycle defects and sensitivity to mitotic poisons. This helps to explain observations that 53BP1 null mice display aneuploidy and cancer susceptibility.
Reciprocal Regulation between 53BP1 and the Anaphase-Promoting Complex/Cyclosome Is Required for Genomic Stability during Mitotic Stress

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SUMMARY

The anaphase-promoting complex/cyclosome (APC/C) is an E3 ubiquitin ligase that targets substrates for degradation to promote mitotic progression. Here, we show that the DNA damage response protein 53BP1 contains conserved KEN boxes that are required for APC/C-dependent degradation in early mitosis. Mutation of the 53BP1 KEN boxes stabilized the protein and extended mitotic duration, whereas 53BP1 knockdown resulted in a shorter and delayed mitosis. Loss of 53BP1 increased APC/C activity, and we show that 53BP1 is a direct APC/C inhibitor. Although 53BP1 function is not absolutely required for normal cell cycle progression, knockdown was highly toxic in combination with mitotic spindle poisons. Moreover, chemical inhibition of the APC/C was able to rescue the lethality of 53BP1 loss. Our findings reveal a reciprocal regulation between 53BP1 and APC/C that is required for response to mitotic stress and may contribute to the tumor-suppressor functions of 53BP1.

INTRODUCTION

The prevention of aneuploidy is of paramount importance for the maintenance of genome stability. Transgenic mice that are heterozygous for genes that control mitotic progression such as CENP-E, Mad2, and BubR1 are more susceptible to low levels of numerical chromosomal instability (CIN) and cancer (Janssen et al., 2009; Michel et al., 2001; Silk et al., 2013). However, inducing high levels of CIN by ablating the expression of genes important for mitosis represses cancer progression by increasing the proportion of cells that die during mitosis.

The anaphase-promoting complex/cyclosome (APC/C) is required for eukaryotic cell division. It is an E3 ubiquitin ligase that ubiquitinates regulators of mitosis, thus causing their degradation and allowing mitosis to proceed (Pines, 2011). Premature activation of the APC/C can cause mis-segregation of chromosomes and thus lead to aneuploidy and cancer. Critical substrates of the APC/C include the mitotic cyclins and Securin. The APC/C has two protein co-activators, Cdc20 and Cdh1, which control the initiation of anaphase and exit of mitosis, respectively. Many proteins have been discovered that regulate APC/C activity including Emi1, RASSF1A, TIF1γ, and MDC1 (Reimann et al., 2001; Sedgwick et al., 2013; Song et al., 2004; Townsend et al., 2009). Additionally, the APC/C has an inhibitory complex known as the spindle assembly checkpoint (SAC) that restrains the APC/C until the chromosomes have been properly aligned at metaphase. Although there are many anti-mitotic drugs that ultimately act on the APC/C by activating the SAC (Smolders and Teodoro, 2011), it has been recently demonstrated that directly inhibiting the APC/C might be an effective mechanism for killing cancer cells (Sackton et al., 2014).

Several studies have suggested that there is considerable crosstalk between DNA damage response (DDR) signaling and the APC/C. The Cdh1 inhibitor Mad2L2 was recently shown to participate in DNA repair (Boersma et al., 2015; Xu et al., 2015). A large-scale proteomic study also found that the APC/C associated with three members of the tandem BRCA1 repeat BRCT1 C-terminal (tBRCT) family including MDC1, PAXIP1, and 53BP1 (Woods et al., 2012). Although these studies suggest a close relationship between the APC/C and DDR, the mechanism and biological significance are poorly understood.

53BP1 is a well-characterized member of the tBRCT family. It contains several functional domains including an oligomerization domain, a tandem tudor domain, and a tBRCT domain, all contained in the C-terminal third of the protein (FitzGerald et al., 2009). In contrast, little is known about the N-terminal region of 53BP1 other than it is rich in ATM consensus sites and it is important for DNA repair functions (Di Virgilio et al., 2013; Zimmermann et al., 2013). 53BP1 has been extensively studied in its role in the DDR, which is to promote non-homologous end joining DNA repair (Panier and Boulton, 2014).

Mice lacking either one or both 53BP1 alleles were shown to be cancer-prone, suggesting that the protein is a bona fide tumor
suppressor. However, in addition to structural chromosomal aberrations, there was a notable increase in aneuploidy both in tumors and in primary splenocytes, showing that 53BP1 knockout results in CIN, which is inconsistent with the known functions of 53BP1; for this reason it has been proposed that 53BP1 has additional functions in suppressing CIN (Morales et al., 2006; Ward et al., 2005). Recently, three studies have suggested that 53BP1 has a role in the response to mitotic stress induced through the centrosome; however, the mechanistic details are still lacking (Fong et al., 2016; Lambrus et al., 2016; Mettig et al., 2016).

In the current study we demonstrate that 53BP1 is degraded early in mitosis through an APC/C-dependent mechanism requiring evolutionarily conserved KEN boxes on 53BP1. We observed that 53BP1 interacts with the APC/C using the KEN boxes and the tBRCT domain. Cells expressing a stable mutant of 53BP1 took longer to progress through mitosis. Conversely, cells lacking 53BP1 took shorter times to progress through mitosis and were extremely sensitive to spindle poisons. Additionally, 53BP1 inhibited the APC/C both in vivo and in vitro. Taken together, our results suggest the tumor-suppressor properties of 53BP1 are due in part to its ability to inhibit the APC/C, thus ensuring the proper progression of mitosis.

**RESULTS**

**53BP1 Levels Are Decreased during Mitosis in an APC/C- and Proteasome-Dependent Manner**

Inspection of the amino acid sequence of human 53BP1 shows that the protein contains three KEN boxes, at positions 54, 85, and 1136. Interestingly, comparison of amino acid sequences of 53BP1 across species shows that the presence of at least one KEN box is conserved from yeast to humans. Importantly, all three KEN boxes are present in disordered regions of 53BP1. Therefore, it is likely that 53BP1 is an APC/C substrate. To test this hypothesis, we synchronized HeLa cells in late G2 with the cyclin-dependent kinase-1 (CDK1) inhibitor RO3306, released them into mitosis, and monitored 53BP1 levels by western blot. Following release into mitosis, 53BP1 levels decreased independently of Cdc20 (Di Fiore and Pines, 2010; Hayes et al., 2006). Taken together, our results suggest that 53BP1 is an APC/C substrate, we blotted the same samples with an anti-K11 chain antibody. Western blot evidence that 53BP1 is an APC/C substrate, we blotted the same samples with an anti-K11 chain antibody. Western blot

**APC/CCdh1.**

To verify that 53BP1 levels decrease via the proteasome, HeLa cells were released into mitosis in the presence of the proteasome inhibitor MG132. 53BP1 levels were reduced in control cells but were rescued in cells treated with MG132 (Figure 1C). Cyclin B1 levels were similarly elevated following treatment with MG132. To further show that the APC/C targets 53BP1 for degradation during mitosis, we utilized an inhibitor of the APC/C, proTAME (tosyl arginine methyl ester) (Zeng and King, 2012). HeLa cells were synchronized using RO3306 and then released into media containing proTAME. Figure 1D shows that 53BP1 protein levels remain elevated in proTAME-treated cells, as does cyclin B1.

To determine which APC/C co-activator recognizes 53BP1 during mitosis, we knocked down Cdc20, Cdh1, or a core APC/C subunit, APC3, using small interfering RNAs (siRNAs). HeLa cells were synchronized in G2 with RO3306, released into mitosis, and harvested. Surprisingly, knockdown of both co-activators resulted in the partial rescue of 53BP1 protein levels, compared with the complete loss of 53BP1 protein levels in non-silencing controls (NSCs), and the full rescue of 53BP1 in APC3 knockdown cells (Figure 1E). Taken together, these data show that 53BP1 is a substrate of both APC/C^Cdh1^ and APC/C^Cdc20^.

**53BP1 Is Degraded during Mitosis Independently of the SAC**

In order to examine the relationship between the SAC and 53BP1 degradation, we synchronized HeLa cells via thymidine-nocodazole. Mitotic cells were collected by shake-off and released into either fresh media or media containing Taxol. 53BP1 protein levels remained extremely low in Taxol-treated cells and did not increase until the cells had begun to exit mitosis via slippage (Figure 2A). In contrast, cyclin B1 and Securin levels were stabilized in Taxol-treated cells, as is expected when the SAC is engaged. The APC/C substrates cyclin A2 and Nek2A are insensitive to SAC inhibition because they bind to the APC/C independently of Cdc20 (Di Fiore and Pines, 2010; Hayes et al., 2006). We similarly observed that cyclin A2 and 53BP1 were low in the presence of Taxol, indicating that 53BP1 can also be degraded while the SAC is engaged (Figure 2A). We also tested cells released from thymidine arrest to see whether 53BP1 levels decrease upon mitotic entry. We observed that 53BP1 levels increased in G2 and then declined as the cells entered mitosis. In cells released from thymidine to RO3306, the levels of 53BP1 did not decrease because the cells did not enter mitosis (Figure 2B).

Because both 53BP1 and cyclin A2 are targeted for APC/C-Cdc20-mediated degradation early in mitosis, we asked whether 53BP1 binds to Cdc20 and whether this interaction occurs prior to the onset of mitosis. HeLa cells were synchronized in G2 with RO3306 or in mitosis with nocodazole. Cdc20 was then immunoprecipitated, separated on SDS-PAGE, and immunoblotted for 53BP1 and cyclin A2. As expected, binding of cyclin A2 to Cdc20 was detected only in G2 cells (Figure 2C) (Wolthuis et al., 2008). Interestingly, we also detected binding of 53BP1 to Cdc20 in cells under similar conditions. In slight contrast with cyclin A2, we also detected binding of 53BP1 by Cdc20 in asynchronous cells. These data suggest that 53BP1 is pre-bound to the APC/C in a similar manner as cyclin A2.

If 53BP1 is indeed a mitotic APC/C substrate that is ubiquitinated independently of SAC activity, then it should be ubiquitinated by the APC/C in the presence of nocodazole. To verify that this is the case, we performed an in vivo ubiquitination assay according to established protocols (Bloom and Pagano, 2005). We treated the cells with nocodazole and MG132, and examined the levels of ubiquitination on 53BP1. Levels of 53BP1 were normalized between the asynchronous and nocodazole-treated samples. We observed that the 53BP1 from mitotic cells is markedly more ubiquitinated compared with 53BP1 from asynchronous cells (Figure 2D). It has been demonstrated that the APC/C forms both K11- and K48-linked ubiquitin chains (Matsumoto et al., 2010; Wickliffe et al., 2011). To provide additional evidence that 53BP1 is an APC/C substrate, we blotted the same samples with an anti-K11 chain antibody. Western blot
analysis revealed that in the presence of nocodazole, 53BP1 was ubiquitinated with K11-linked chains, which was enhanced when cells were treated with MG132 (Figure 2D).

To confirm that 53BP1 ubiquitinated species are K11 linked, we utilized a ubiquitin mutant lacking all available lysines except for K11. If 53BP1 is ubiquitinated by the APC/C, then this ubiquitin mutant should be incorporated into ubiquitin chains. Therefore, we compared the incorporation of wild-type (WT) ubiquitin with K11-only ubiquitin. Levels of 53BP1 expression were normalized across the samples. We found that the K11 mutant ubiquitin is efficiently ligated to 53BP1 (Figure S1A). This effect was not observed using a control substrate, caspase 1 (Figure S1B), which is regulated by K63-linked ubiquitin chains (Labbé et al., 2011). Taken together, these results establish that 53BP1 interacts with Cdc20 and is ubiquitinated by the APC/C during mitosis even in the presence of an active SAC.

53BP1 Requires KEN Boxes for Cdc20 Interaction and Degradation during Mitosis

To determine whether the KEN boxes present in 53BP1 are required for degradation during mitosis, we derived FLAG-tagged mutants in which all the KEN boxes of 53BP1 were...
mutated to alanine residues (KEN-AAA). HeLa cells were transfected with the WT and mutant 53BP1 constructs, synchronized in mitosis, and then harvested. Western blot showed that whereas levels of WT 53BP1 protein were reduced in mitotic cells, the protein levels of the 53BP1 KEN box mutant remained constant (Figure 3A).

Because the KEN box mutant is more stable compared with WT 53BP1, it follows that it should also have lower affinity for Cdc20. To test this, we immunoprecipitated Cdc20 from 293T cells transfected with 3XFLAG-53BP1 and HA-ubiquitin. They were then treated with nocodazole for 20 hr, then MG132 for 4 hr. The cells were then harvested and 3XFLAG-53BP1 was immunoprecipitated in RIPA buffer. The proteins were then eluted, separated by SDS-PAGE, and analyzed for ubiquitination by immunoblot. 53BP1 levels were normalized prior to gel loading.

Figure 2. 53BP1 Is Degraded during Mitosis Independently of the SAC
(A) HeLa cells were synchronized by thymidine-nocodazole. Mitotic shake-off cells were collected, washed twice with PBS, and then released into fresh medium or medium containing Taxol. Cells were then harvested at various time points as shown. The cells were then processed and protein levels monitored by immunoblot.
(B) HeLa cells were synchronized by double-thymidine block and then released into fresh media. Five hours post release, nocodazole or RO3306 was added. The cells were harvested at time points as indicated. The cells were then processed as in (A).
(C) HeLa cells were plated and synchronized as indicated. Cdc20 was then immunoprecipitated and then analyzed together with interacting proteins by immunoblot. Asterisk (*) indicates the true band.
(D) 293T cells were co-transfected with 3XFLAG-53BP1 and HA-ubiquitin. They were then treated with nocodazole for 20 hr, then MG132 for 4 hr. The cells were then harvested and 3XFLAG-53BP1 was immunoprecipitated in RIPA buffer. The proteins were then eluted, separated by SDS-PAGE, and analyzed for ubiquitination by immunoblot. 53BP1 levels were normalized prior to gel loading.

See also Figure S1.
are required for binding to Cdc20 and 53BP1 degradation during mitosis.

We next tested whether mutation of the 53BP1 KEN boxes results in altered protein function. We first tested the ability of KEN-AAA to form foci at sites of DNA damage in response to ionizing radiation. Wild-type or mutant 53BP1 were expressed in 53BP1/C0/C0 HeLa cells generated by CRISPR (Feng et al., 2015) and displayed no apparent differences in either the ability to form foci or their persistence following 5 Gy of radiation (Figure S2B). We then tested the effect of expressing the KEN box mutant on mitotic progression. Compared with WT 53BP1, expression of the KEN-AAA resulted in mitosis 30% longer on average (Figures 3D and 3E). We also monitored the expression of various APC/C targets during mitosis. Consistent with the time-lapse experiments, we found a delay in the degradation of APC/C substrates, suggesting delayed mitotic exit in cells expressing the KEN box mutant (Figure S2A).

Cdc20 Binds to the N Terminus and tBRCT Domains of 53BP1, Whereas Cdh1 Binds Exclusively to 53BP1 tBRCT Domains

In order to better understand the mechanism of 53BP1 association with the APC/C, we performed a structure-function analysis. A series of deletion mutants of 53BP1 were derived, which are...
depicted in Figure 4E. 293T cells were co-transfected with Flag-tagged WT or deletion mutants of 53BP1 and hemagglutinin (HA)-tagged Cdc20. 53BP1 complexes were immunoprecipitated and tested for the presence of HA-Cdc20 by immunoblot. As expected, Figure 4A shows that the 53BP1 fragments containing KEN boxes efficiently immunoprecipitated Cdc20. Surprisingly, the tBRCT domains also associated with Cdc20. Because 53BP1 appears to interact with Cdc20 through two separate domains, we asked whether the binding between 53BP1 and Cdc20 is cell cycle dependent. Interestingly, whereas the 1–609 KEN-box-containing fragment of 53BP1 bound to Cdc20 in both interphase and mitotic cells, the 1220–1972 tBRCT-domain-containing fragment bound to Cdc20 only in interphase cells (Figure S3A). Thus, the binding mode of 53BP1 to Cdc20 appears to depend upon cell cycle state.

To determine the regions of Cdc20 required for association with 53BP1, we derived a series of Cdc20 deletion mutants (Figure 4F). Analysis of these mutants showed that 53BP1 associates with the disordered N terminus of Cdc20 and to a lesser extent with the WD40 domain (Figure 4B). Knockdown of Cdh1 also resulted in stabilization of 53BP1 (Figure 1E), suggesting both the Cdc20 and Cdh1 APC/C co-activators may bind 53BP1. In Figure 4C we show that exogenous Cdh1 does co-immunoprecipitation (IP) with 53BP1. In contrast with Cdc20, however, Cdh1 exclusively associated with the tBRCT domain of 53BP1. We similarly mapped the region on Cdh1 required for binding to 53BP1 and found that the C-terminal WD40 domain was essential (Figure 4D). To test whether the tBRCT domain of 53BP1 binds to a phosphorylated site on Cdh1, we generated a point mutant of 53BP1 (K1814M) that cannot bind to phosphorylated proteins (Kleiner et al., 2015). Compared with wild-type 53BP1, this mutant is defective in binding to Cdh1, suggesting that the interaction between the 53BP1 tBRCT domain and Cdh1 is regulated by phosphorylation (Figure S3D).
Because the interaction between 53BP1 and both APC/C co-activators required the tBRCT domain of 53BP1, it is possible that this interaction is also required for the degradation and ubiquitination of 53BP1. We tested the ability of the 53BP1 fragments to be degraded in mitosis. We found that in small fragments of 53BP1, the presence of a KEN box is sufficient to mediate the degradation of the protein. However, in a large fragment of 53BP1, the tBRCT domain is also required (Figure S3B). We then performed an in vivo ubiquitination experiment using the panel of 53BP1 fragments (Figure S3C). Consistent with the degradation experiment, a 53BP1 mutant lacking the tBRCT domain was poorly poly-ubiquitinated, whereas a 53BP1 deletion mutant comprised of only the 53BP1 C-terminal region was extensively ubiquitinated, indicating that the tBRCT domain is required for 53BP1 to be efficiently ubiquitinated in vivo.

53BP1 Is Required for Normal Cell Cycle Progression, and Reduced Expression Sensitizes Cells to Mitotic Poisons

Because we observed that expressing the 53BP1 KEN box mutant resulted in slow mitotic progression, we asked whether reducing 53BP1 levels might also have an effect. 53BP1 was knocked down in HeLa cells using siRNAs, which were then synchronized with thymidine-nocodazole, and cell cycle analysis was performed. Figure 5A shows that when 53BP1 was knocked down in asynchronous cells there was a 50% reduction in the percentage of cells in mitosis as determined by measuring pSer28 histone H3-positive cells. The phenotype of 53BP1 knockdown was far more apparent in synchronized cells. Figure 5A shows that whereas cells transfected with NSC siRNAs contained mostly cells arrested in G2/M (71.3%), 53BP1 knockdown cells displayed a similar phenotype as cells transfected with siRNAs (Figure S3C and data not shown). Examples are provided in Figure S4A. Interestingly, we observed a long delay in the ability of 53BP1 knockdown cells, relative to NSCs, to commit to mitotic entry, consistent with the flow cytometry analysis. A similar pattern was observed in cells treated with Taxol (Figure 6D; Movies S5 and S6). These effects were not limited to HeLa cells and were also observed in other cell lines including H1299, 293T, and PC3. These effects were not restricted to cancer cells because similar results were obtained using BJ fibroblasts, suggesting that the replication stress common in cancer cells is not required for these phenotypes (Figure S4B). Additionally, similar results were obtained without using thymidine to pre-synchronize the cells, ruling out possible DNA damage effects (Figure S4C). Moreover, 53BP1−/− HeLa cells displayed a similar phenotype as cells transfected with siRNA (Figure S5C and data not shown).

Because cells lacking 53BP1 expression take less time to complete mitosis compared with the NSC cells, we asked whether the 53BP1 knockdown cells would also have a higher incidence of anaphase defects. To address this question, we used the cell line HCT116 because of its stable, near-diploid karyotype. We therefore knocked down 53BP1 in this cell line and 48 hr later fixed the cells and stained them with DAPI. We then examined the cells in anaphase for the presence of lagging chromosomes, anaphase bridges, and multi-polar spindles. Examples are

Finally, we asked whether an RNAi-resistant 53BP1 cDNA could rescue the effects of 53BP1 knockdown. Indeed, transfection of the 53BP1-expressing cDNA reduced the number of cells with sub-2N DNA content as well as increasing numbers of cells in G2/M (Figures 5D and 5E), confirming that our observations were due to 53BP1 knockdown.

To further investigate the phenotypes of 53BP1 knockdown, we utilized time-lapse microscopy and single-cell analysis. HeLa cells were transfected with siRNAs to knock down 53BP1, synchronized with thymidine, and released. Figure 6A shows that in contrast with cells expressing the stable 53BP1 KEN box mutant, knockdown cells spent less time in mitosis. On average, NSC cells spent 124.4 min in mitosis compared with 69.2 min (si53BP1-1) and 45.2 min (si53BP1-2). Despite transiting through mitosis more rapidly, 53BP1 knockdown cells were often delayed in mitotic entry and in many cases did not enter mitosis (Figure 6B; Movies S1 and S2).

Release of thymidine synchronized 53BP1 knockdown cells into nocodazole resulted in a more dramatic effect. The combination of 53BP1 knockdown and nocodazole treatment was highly toxic; only 8% (si53BP1-1) and 35% (si53BP1-2) of cells underwent mitotic slippage and entered into G1 compared with 73% of NSC cells (Figure 6C; Movies S3 and S4). Examples are provided in Figure S4A. Interestingly, we observed a long delay in the ability of 53BP1 knockdown cells, relative to NSCs, to commit to mitotic entry, consistent with the flow cytometry analysis. A similar pattern was observed in cells treated with Taxol (Figure 6D; Movies S5 and S6). These effects were not limited to HeLa cells and were also observed in other cell lines including H1299, 293T, and PC3. These effects were not restricted to cancer cells because similar results were obtained using BJ fibroblasts, suggesting that the replication stress common in cancer cells is not required for these phenotypes (Figure S4B). Additionally, similar results were obtained without using thymidine to pre-synchronize the cells, ruling out possible DNA damage effects (Figure S4C). Moreover, 53BP1−/− HeLa cells displayed a similar phenotype as cells transfected with siRNA (Figure S5C and data not shown).

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Figure 5. 53BP1 Is Critical for Normal Cell Cycle Progression

(A) HeLa cells were transfected with siRNAs as indicated. Twenty-four hours following transfection, the cells were synchronized by thymidine over 20 hr, released for 4 hr, and treated with nocodazole for 16 hr. Following treatment the cells were harvested and analyzed by flow cytometry for DNA content and pSer28 histone H3.

(B) HeLa cells were transfected as in (A), but processed for SDS-PAGE. Equal quantities of total cell extract were separated, and the protein levels were monitored by immunoblot.

(C) HeLa cells were transfected with siRNA as indicated. They were then treated with thymidine for 20 hr, harvested, and analyzed by flow cytometry for DNA content.

(D) HeLa cells were transfected with siRNA and DNA as indicated. They were then treated and processed as in (A).

(E) HeLa cells were treated as in (C), but processed for SDS-PAGE as in (B).
shown in Figure S4D. We found that the 53BP1 knockdown cells had 1.9-fold (si53BP1-1) and 2.1-fold (si53BP1-2) more defects overall compared with the NSC cells (Figure S4B). Taken together, the results demonstrate that cells lacking 53BP1 have a number of cell cycle defects: they struggle to enter mitosis and transit through mitosis faster, resulting in anaphase defects.

53BP1 Regulates APC/C Activity

Because we observed that 53BP1 interacts with APC/C co-activators and modulation of 53BP1 affected mitotic progression, we therefore asked whether these effects were due to changes in APC/C activity. We first determined the effect of 53BP1 knockdown on the levels of known APC/C substrates. 53BP1 expression was silenced and levels of APC/C substrates were determined by immunoblot. Figure 7A shows that the APC/C substrates BubR1, Cdc20, cyclin B1, cyclin A2, Securin, PLK1, and Cdc6 were all reduced in 53BP1 knockdown cells relative to NSCs. This phenotype was also present in 53BP1−/− HeLa cells (Figure S5A). Consistent with a role for 53BP1 in specifically regulating APC/C activity, the levels of cyclin E, which is not an APC/C substrate, were unaffected by the depletion of 53BP1 in asynchronous cells (Figure 7A). Differences in the levels of APC/C substrates between NSC and si53BP1 knockdown in asynchronous cells cannot be attributed to differences in cell cycle state because the cell cycle profiles are very similar in the two populations (Figure 5A). To confirm that reduction of APC/C substrates was due to 53BP1 knockdown, we tested whether siRNA-resistant cDNA could rescue APC/C substrate levels. The levels of cyclin B1, Cdc20, and BubR1 were all restored to normal following the exogenous expression of 53BP1 (Figure 7B). Because the levels of the APC/C substrates are low after depletion of 53BP1, these data suggest that 53BP1 may function as an APC/C inhibitor.

If the reduced levels of APC/C substrates in 53BP1 knockdown cells were due to high APC/C activity, then substrates would be predicted to be less stable. To test this idea, we performed a cycloheximide chase experiment. Figure S6A shows that the APC/C substrates had a decreased half-life in 53BP1 knockdown cells compared with NSCs. Consistent with a role for proteasome-mediated degradation in the reduction in the levels of APC/C substrates, the levels of these proteins were restored in the presence of MG132 (Figure S6B). To further demonstrate that 53BP1 negatively regulates APC/C activity, we tested the ability of cell extracts prepared from cells treated with either NSCs or si53BP1 to ubiquitinate cyclin B1. Consistent with a model where 53BP1 is a repressor of APC/C function, cyclin B1 was more efficiently ubiquitinated by the extracts of 53BP1 knockdown cells (Figure 7C).

During mitosis the SAC acts as an essential APC/C inhibitor. To rule out the possibility that 53BP1 may be inhibiting APC/C through the SAC, we determined whether 53BP1 associates with the SAC. 293T cells were transfected with FLAG-tagged 53BP1 or Cdc20. Immunoprecipitation of the FLAG-tagged proteins was performed and tested for the presence of the SAC components Bub1, BubR1, and Mad2. Although Cdc20 interacted robustly with these proteins, no interaction was observed between 53BP1 and the SAC (Figure S6C). Therefore, 53BP1 appears to inhibit the APC/C independently of the SAC.

As a final demonstration that 53BP1 can function as a direct APC/C inhibitor, we produced recombinant 53BP1 (r53BP1) in E. coli (Figure S6D) and tested its ability to inhibit the APC/C in vitro. Figure 7D shows that r53BP1 inhibited the APC/C-dependent ubiquitination of cyclin B1 with comparable efficiency as Emi1 at concentrations shown previously to inhibit the APC/C (Wang and Kirschner, 2013). The ability of 53BP1 truncation mutants to inhibit APC/C-directed ubiquitination of cyclin B1 was also assessed. Figure 7E shows that when used individually, 53BP1 fragments failed to block cyclin B1 ubiquitination. Because we found that both the N and C termini of 53BP1 can independently bind to the APC/C, we reasoned that both of these regions might be required to block APC/C activity. Therefore, we tested the ability of the 1–609 and 1220–1972 fragments to block the ubiquitination reaction in trans. Indeed, the combination caused a modest decrease in ubiquitination of cyclin B1. We next tested whether the 53BP1 KEN boxes are required for the effective inhibition of the APC/C. We found no difference in inhibition of the APC/C between WT and KEN box mutant 53BP1 (Figure S6E), indicating that the KEN boxes on 53BP1 are dispensable for APC/C inhibition. Importantly, this result indicates that 53BP1 is not acting as a pseudosubstrate inhibitor by saturating the KEN box binding sites of the APC/C co-activators.

Given that 53BP1 functions as an APC/C inhibitor, we asked whether chemical inhibition of the APC/C using proTAME could rescue the deleterious effects of 53BP1 knockdown. Figure 7F shows that whereas proTAME had no effect on the ability of cells transfected with the NSC siRNA to enter mitosis, it largely rescued the phenotypes of 53BP1 knockdown both with respect to the lag in mitotic entry and the lethality of nocodazole (Figure 7F; Movies S7 and S8). The rescue of the 53BP1 silencing phenotypes by addition of proTAME indicates that the source of the cell cycle defects was due to excess APC/C activity. Taken together, our data suggest that 53BP1-mediated inhibition of the
APC/C is required for cell cycle progression and the cellular responses to mitotic stress.

DISCUSSION

In this study we describe a mechanism by which the 53BP1 protein negatively regulates APC/C activity by binding through its tBRCT domains to the APC/C co-activators Cdc20 and CDH1. Early in mitosis the APC/C ubiquitinates 53BP1 in a KEN-box-dependent manner and thereby relieves the 53BP1-dependent inhibition of the APC/C. We found that 53BP1 also binds to Cdc20 through its tBRCT domain in interphase cells, but via the KEN boxes only during mitosis. Mutation of the 53BP1 KEN boxes resulted in cells with a slow transit through mitosis.
and conversely, knockdown of 53BP1 accelerated mitotic transit. Our findings suggest a reciprocal regulation of APC/C and 53BP1 that is important for cell cycle progression. APC/C<sup>CDc11</sup> maintains the G1 state. Therefore, we propose 53BP1 helps allow the transition to S phase and G2 by inhibiting the APC/C. Once the cell reaches mitosis, 53BP1 is ubiquitinated and degraded to allow mitotic progression.

Interestingly, the TBRCt domains of 53BP1, MDC1, MCPH1, and PAXIP1 (Coster et al., 2007; Singh et al., 2012; Townsend et al., 2009; Woods et al., 2012) have all been shown to interact with the APC/C, consistent with our observations. Interestingly, MDC1 appears to have the opposite effect of 53BP1 in regulating the activity of the APC/C, because knockdown of MDC1 causes mitotic arrest because of a lack of APC/C activity (Townsend et al., 2009). Therefore, a balance between several BRCT-containing proteins may act to properly regulate APC/C activity.

The DDR is largely inactivated during mitosis, leaving only apical DDR signaling by ATM and γH2AX to label DNA breaks for repair in G1 (Giunta et al., 2010; Nelson et al., 2009; Orthwein et al., 2014; van Vugt et al., 2010). Relevant to our study, DNA damage foci during mitosis have been shown to completely lack 53BP1, suggesting a strong negative regulation (Nelson et al., 2009). It is now clear that active DNA repair during mitosis is deleterious, because 53BP1 activity during mitosis increases genomic instability (Lee et al., 2014; Orthwein et al., 2014). Therefore, APC/C-dependent degradation of 53BP1 might be an additional mechanism by which the DDR is switched off in mitosis.

We found that the interaction between 53BP1 and the APC/C co-activators occurs through both the KEN boxes and TBRCt domain of 53BP1. This dual mode of binding creates a constitutive interaction between 53BP1 and the Cdc20, allowing 53BP1 to be both an APC/C substrate and an inhibitor. It is possible that the three KEN boxes of 53BP1 create an unusually high affinity between 53BP1 and the APC/C co-activators, which is consistent with its early degradation in mitosis, akin to cyclin A2 and Nek2A (Di Fiore and Pines, 2010; Hayes et al., 2006; Wolthuis et al., 2008). Our observations are in agreement with a recent study showing that 53BP1 is ubiquitinated in mitotic cells in a manner that is regulated by the deubiquitinase ubiquitin specific protease-7 (USP7) (Yim et al., 2016). The ubiquitin ligase was not identified in this study, but our data suggest that it is the APC/C.

Our data are consistent with several other studies that indicate a role for 53BP1 in the maintenance of genomic integrity and proper mitotic progression. Notably, 53BP1 was highlighted in a screen for proteins that suppress mitotic catastrophe in yeast (Xia et al., 2001). 53BP1 was also found to repress mitotic catastrophe elicited by the HIV Env protein in syncytia (Perfettini et al., 2010). Furthermore, 53BP1 knockout mice develop highly aneuploid tumors. More recently, it has been shown that 53BP1 is critical for inducing cell cycle arrest following the induction of mitotic stress (Fong et al., 2016; Lambros et al., 2016; Melting et al., 2016). Our data are largely in agreement with their findings and suggest that the cellular response to mitotic stress might involve inhibition of the APC/C by 53BP1. Clearly, 53BP1 is not required for normal mitotic progression because knockout animals are viable. However, these studies collectively implicate 53BP1 in the response to mitotic stresses, such as that induced by centrosome depletion or spindle poisons.

It has been previously suggested that an efficient way of killing CIN cells may be to increase the levels of CIN to a catastrophic threshold where a cell cannot successfully exit mitosis (Janssen et al., 2009; Silk et al., 2013). In this context, we determined that knockdown of 53BP1 can be tolerated in unstressed cells, but is lethal combined with spindle poisons. Given that it has been shown in principle that chemical inhibition of the APC/C is an effective way of killing tumor cells (Sackton et al., 2014), it would also be interesting to determine whether tumors with low 53BP1 expression are hypersensitive to such treatments.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Treatments**

Cells were maintained in DMEM (Wisent) supplemented with 10% fetal bovine serum (HyClone; Thermo Scientific) and 0.1% gentamicin (Wisent). RO3306 (Sigma-Aldrich) synchronization was performed by treating HeLa cells for 20 hr with 9 μM RO3306. Cells synchronized in mitosis were obtained by 20 hr treatment with 2.5 mM thymidine, 4 hr of release before adding 100 ng/ml nocodazole, proTAME (Boston Biochem) was used at 5-20 μM. MG132 was purchased from Sigma and used for 4 hr treatments. Cell irradiation was performed with a Radsource RS2000 irradiator at 160 kV and 25 mA.

**Plasmids**

53BP1 constructs were cloned from N-Myc-53BP1 WT plPC-Puro Addgene 19836, which was a kind gift of Tita de Lange (Dimitrova et al., 2009), into p3XFLAG-myc CMV26, pGEX-6P1, or pCDNA3 HA, pCDNA3 HA-CDH1 and HA-CD20 were cloned by first producing cDNA from cells by RT-PCR and then conventional PCR cloning. GST-53BP1 constructs and the Emi1 APC/C inhibiting domain 300–447 were generated by cloning the sequences into the pGEX-6P1 vector. PKS5-Ubiquitin WT (17608; Addgene) and K11 only (22901; Addgene) were kind gifts of Ted Dawson and Sandra Weller (Lim et al., 2005; Livingston et al., 2009). All constructs were verified by restriction digestion and sequencing.

**Immunoprecipitation and Cell Extracts**

293T cells were seeded on 10-cm dishes and transfected overnight using calcium phosphate co-precipitation. Cells were lysed on ice in 1 mL of NP-40, EDTA, Tris, NaCl (NETN) (Townsend et al., 2009) or radio-immunoprecipitation assay (RIPA) buffer with protease inhibitor cocktail (Roche). Cell debris was pelleted by centrifugation at maximum speed for 15 min at 4°C. The supernatant was then incubated with 10 μL of packed EZview Red anti-FLAG beads (Sigma-Aldrich) or 10 μL of protein G agarose (EMD Millipore) with the indicated antibody for 2 hr at 4°C. The beads were then pelleted, washed five times with buffer, and then re-suspended in 1× sample buffer. Cell extracts were prepared by lysing cells as above in NETN buffer, followed by protein quantification via Bradford assay (Bio-Rad Laboratories).

**Ubiquitination Assays**

35S-labeled HA-53BP1, HA-cyclinA2, or HA-cyclin B1 were produced by incubating 30 μL of TnT Quick Coupled Transcription/Translation System (Promega) together with 1 μg of plasmid DNA and 2 μL of [35S]methionine per reaction (Perkin Elmer). The UBE1, UBCH10, and UBE2S enzymes and 10x Energy Regeneration System (ERS), as well as FLAG-ubiquitin, were purchased from Boston Biochem. In vitro ubiquitination was performed essentially as described previously (Kraft et al., 2006). For more details, see the Supplemental Experimental Procedures.

**Protein Purification**

Expression of the GST-fusion proteins was induced in BL21 E. coli for 4 hr at 37°C with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Recombinant proteins were purified on glutathione Sepharose 4B (GE Healthcare) and eluted with PreScission Protease (GE Healthcare) according to the manufacturer’s instructions. For more details, see the Supplemental Experimental Procedures.
Antibodies and Western Blotting
Western blotting was performed using standard protocols for SDS-PAGE and wet transfer for at least 24 hr at 30 V onto nitrocellulose membranes (Bio-Rad). The antibodies are listed in the Supplemental Information. Conditions for western blots were the use of 5% nonfat dry milk in TBS-T (50 mM Tris [pH 7.5], 150 mM NaCl, 0.5% Tween 20). The bands were visualized by enhanced chemiluminescence (Western Lightning [PerkinElmer] or SuperSignal West Femto [Thermo Fisher Scientific]) and exposure on film.

Time-Lapse Microscopy
HeLa cells were seeded on six-well plates and transfected as indicated. Synchronization was performed as described above. Phase-contrast imaging was performed on a Zeiss Axiovert microscope. Following the addition of nocodazole or Taxol, the cells were placed in an incubation chamber to maintain temperature and CO2 levels during acquisition. For analysis, at least 100 cells were followed for each condition and the outcome of mitosis recorded. The duration of mitosis is measured from nuclear envelope breakdown until cytokinesis, slippage, or death.

siRNA Transfection
HeLa cells were transfected using Lipofectamine 2000 (Invitrogen, Life Technologies). siRNA sequences and additional details are provided in the Supplemental Information.

Flow Cytometry
A Cell Lab Quanta SC flow cytometer (Beckman Coulter) was used to analyze samples stained either with DAPI to measure DNA content or phospho-Ser28 histone H3 content. The cells were stained and analyzed as previously described (Darzynkiewicz et al., 2001; Juan and Darzynkiewicz, 2004).

Statistical Methods
Error bars indicate mean ± SEM. Statistical significance was calculated using Student’s t test.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, six figures, and eight movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.01.080.

AUTHOR CONTRIBUTIONS

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