# Report

# APC/C- and Mad2-mediated degradation of Cdc20 during spindle checkpoint activation

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Abbreviations: APC/C, anaphase promoting complex/cyclosome; SAC, spindle assembly checkpoint; MCC, mitotic checkpoint complex

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The spindle assembly checkpoint (SAC) is an important mechanism that prevents the separation of sister chromatids until the microtubules radiating from the spindle poles are correctly attached to the kinetochores. Cdc20, an activator of the Anaphase Promoting Complex/Cyclosome (APC/C), is known as a major downstream target for inhibition by the SAC through the binding of mitotic checkpoint proteins, such as Mad2 and BubR1. Here, we report that the SAC negatively regulates the stability of Cdc20 by targeting it for proteasome-dependent degradation. Once the checkpoint is activated by spindle poisons, a major population of Cdc20 is degraded via APC/C, an event that requires the binding of Cdc20 to Mad2. We propose that the degradation of Cdc20 represents a critical control mechanism to ensure inactivation of APC/C<sup>Cdc20</sup> in response to the SAC.

## Introduction

The Spindle Assembly Checkpoint (SAC) is elicited by improperly aligned chromosomes during prometaphase. A single unattached or tensionless kinetochore can activate the SAC and prevent the onset of anaphase.<sup>1</sup> Mechanistically, this inhibition is achieved through the delayed degradation of securin and Cyclin B by APC/C<sup>Cdc20</sup>.<sup>2</sup> APC/CCdc20 is a multi-subunit E3 ubiquitin ligase that targets many regulatory proteins for proteasome-dependent degradation during mitosis, allowing first the metaphase-anaphase transition and then mitotic exit (the latter in collaboration with APC/C<sup>Cdh1</sup>).<sup>3</sup> As the activator of APC/C in mitosis, Cdc20 is the essential target of the SAC,<sup>4,5</sup> but how Cdc20 is inhibited by the SAC remains unclear. Our current understanding of the SAC suggests that Mad2, as part of the Mitotic Checkpoint Complex (MCC; containing Mad2, Mad3/BubR1, and Bub3), binds Cdc20, inhibiting its ability to activate APC/C, impeding the transition to anaphase.<sup>2</sup> The SAC also dampens APC/C activity through inhibitory phosphorylation

Previously published online as a *Cell Cycle* E-publication: www.landesbioscience.com/journals/cc/article/7606 of multiple Serine/Threonine residues on Cdc20 by Cdk1 and the spindle checkpoint protein Bub1,<sup>6,7</sup> although it is unclear how phosphorylation reduces the ability of Cdc20 to activate APC/C. In *Saccharomyces cerevisiae*, SAC activation leads to the targeted ubiquitination of Cdc20 by APC/C, with consequent degradation by the proteasome.<sup>8</sup> Failure to reduce the level of Cdc20 during SAC activation causes cells to slip through the mitotic checkpoint, resulting in aneuploidy. Here, we investigated the regulation of Cdc20 stability during SAC activation in mammalian cells and explored the contribution of spindle checkpoint proteins to Cdc20 stability.

# **Results and Discussion**

The level of Cdc20 protein is regulated by the cell cycle. Beginning with accumulation in S phase, the level of Cdc20 peaks in mitosis before decreasing in the late stages of mitosis after the degradation of Cyclin B and securin, reaching its nadir in G<sub>1</sub>.<sup>3</sup> Upon performing a nocodazole synchronization, shake-off, and release (Fig. 1A), we surprisingly found that the Cdc20 level of cells in prometaphase was not dramatically higher than that of G1 cells and was much lower than that of G<sub>2</sub> cells, which remain attached following nocodazole treatment and shakeoff (Fig. S1 and S2). To further investigate this observation, we lysed prometaphase and G2 cells from both HeLa and U-2 OS cells, and the differences in the levels of Cdc20 and several other mitotic proteins, including Cyclin A, Cyclin B, Plk1 and Cdh1, were analyzed by immunoblotting (Fig. 1B). As expected, the levels of Cyclin B, Plk1 and Cdh1 did not change in nocodazole-arrested mitotic cells, and Cyclin A was degraded in a spindle checkpoint-independent manner, as previously described.<sup>9</sup> Notably, Cdc20 levels were reproducibly lower in the nocodazolearrested mitotic cells in comparison to the G2 cells. We reasoned that this downregulation of Cdc20 in prometaphase could be a specific response to mitotic spindle checkpoint activation by nocodazole and that Cdc20 levels would not decrease in normal mitotic cells without spindle poison treatment. To test this hypothesis, we released U-2 OS cells from a thymidine arrest (S phase) and collected mitotic cells by shake-off 13 hours after release. As expected, these normal (i.e., not spindle-poisoned) mitotic cells had a similar amount of Cdc20 compared to the attached, G2 cells (Fig. 1C). To exclude the possibility that Cdc20 downregulation was due to prolonged (16 hours) treatment with nocodazole, we released cells from a

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Figure 1. Cdc20 levels decrease in SAC-activated mitotic cells, but not in normal mitotic cells. (A) HeLa cells were synchronized in prometaphase by nocodazole treatment for 16 hours. Round, prometaphase cells (M) were then collected by gentle shake-off and replated in fresh medium for the indicated times. The remaining, attached cells (predominantly in  $G_2$  as shown in Fig. S1;  $G_2$ ) were also harvested. Protein extracts were analyzed by immunoblotting with antibodies against the indicated proteins. (B) HeLa cells and U-2 OS cells were synchronized in prometaphase by nocodazole treatment for 16 hours. Round, prometaphase cells (M) were then collected by gentle shake-off, and the remaining, attached cells in  $G_2$  ( $G_2$ ) were also harvested. Protein extracts were analyzed by immunoblotting with antibodies against the indicated proteins. (C) U-2 OS cells were treated with thymidine for 24 hours to arrest them in S phase. Thymidine was then washed away to allow the cells to progress through the cell cycle for an additional 13 hours, after which time the majority of cells were in mitosis. These normal mitotic cells (M) were then collected by gentle shake-off, and the remaining, attached, non-mitotic cells ( $G_2$ ) were also harvested. Protein extracts were analyzed by immunoblotting with antibodies against the indicated proteins. (D) The decrease in Cdc20 in nocodazole-arrested (NZ) mitotic cells is not due to adaptation to the SAC. HeLa cells were treated with thymidine for 24 hours to arrest cells in S phase. Thymidine was then washed away to allow the cell cycle for three hours before nocodazole was added for an additional eight hours (first two lanes). In parallel, HeLa cells were synchronized in prometaphase by nocodazole treatment for 16 hours (last two lanes). Round, prometaphase cells (M) were then collected by gentle shake-off, and the remaining attached cells ( $G_2$ ) were also harvested. Protein extracts were analyzed by immunoblotting with antibodies against the indicated proteins. (D) The decrease i

thymidine block into medium containing nocodazole for only eight hours. As shown in Figure 1D, Cdc20 was still degraded in the mitotic cells exposed to nocodazole for the shorter time period, suggesting that the downregulation of Cdc20 is not due to mitotic adaptation.

Next we tested whether inhibition of the proteasome could rescue the Cdc20 level in nocodazole-arrested mitotic cells. Adding MG132 to nocodazole-arrested cells restored Cdc20 to a level comparable to  $G_2$  cells (Fig. 2). The increase in Cyclin A demonstrated the effectiveness of MG132 in inhibiting the proteasome. To confirm that the degradation of Cdc20 in mitosis was specific to spindle checkpoint activation, we treated cells with taxol, another microtubule poison, or a taxol and MG132 combination, and results similar to nocodazole treatment were observed (Fig. S3). Therefore, we concluded that the degradation of Cdc20 is a specific response to SAC activation, and this degradation is proteasome dependent.

The ubiquitin ligase required for Cdc20 degradation during SAC activation was unknown. The only ubiquitin ligase known to induce Cdc20 degradation is APC/C<sup>Cdh1</sup>, which ubiquitinates Cdc20 during the  $G_1$  phase of the cell cycle. In mammalian cells,



Figure 2. Proteasome inhibition rescues Cdc20 levels in nocodazole-arrested mitotic cells. (A) HeLa cells were synchronized in prometaphase by nocodazole treatment for 16 hours. Either DMSO or MG132 was added to cells six hours before collection. Round, prometaphase cells (M) and attached cells ( $G_2$ ) were lysed and protein extracts were analyzed by immunoblotting with antibodies against the indicated proteins. (B) U-2 OS cells were synchronized in prometaphase by nocodazole treatment for 16 hours. Either DMSO or MG132 was added to cells six hours before collection. Round, prometaphase cells (M) and attached cells ( $G_2$ ) were lysed, and protein extracts were analyzed by immunoblotting with antibodies against the indicated cells ( $G_2$ ) were lysed, and protein extracts were analyzed by immunoblotting with antibodies against the indicated proteins.



Figure 3. Cdc20 degradation in nocodazole-arrested mitotic cells is dependent on APC/C. (A) HeLa cells were transfected with siRNAs directed against *LacZ*, *Cdh1*, *Cdc27*, or *APC2*. Asynchronous cells (AS) without siRNA transfection served as a control. Forty-eight hours after transfection, the cells were synchronized in prometaphase by nocodazole treatment for 16 hours. Round, prometaphase cells (M) were then collected by gentle shake-off, and the remaining attached cells (G2) were also harvested. Protein extracts were analyzed by immunoblotting with antibodies against the indicated proteins. (B) U-2 OS cells were transfected with siRNAs directed against *LacZ*, *APC2* or *Cdc27*. Forty-eight hours after transfection, the cells were synchronized in prometaphase by nocodazole treatment for 16 hours. Round, prometaphase cells (M) were then collected by gentle shake-off, and the remaining attached cells (G2) were also harvested. Protein extracts were analyzed by immunoblotting with antibodies against the indicated proteins.

Cdh1 recognizes the KEN box motif at the N-terminus of Cdc20 to target it for degradation.<sup>10</sup> Recently, another degron motif, the CRY box, was also reported to be partially responsible for the APC/C<sup>Cdh1</sup>mediated degradation of Cdc20 in mouse oocytes.<sup>11</sup> However, in mitosis, Cdh1 is largely inactive due to phosphorylation by mitotic kinases,<sup>3</sup> making it unlikely that APC/C<sup>Cdh1</sup> is activated by the SAC to induce degradation of Cdc20. Alternatively, the binding of spindle checkpoint proteins, such as Mad2 or BubR1, to Cdc20 in response to SAC activation could induce degradation by the APC/C core complex. Studies in S. cerevisiae suggest that the SAC regulates Cdc20 stability in mitosis, and the degradation of Cdc20 induced by SAC activation does not depend on Cdh1, instead requiring Mad2 and the core components of the APC/C complex.<sup>8</sup> To test this possibility in mammalian cells, we performed siRNA-mediated knockdowns of Cdh1 and two core components of the APC/C, Cdc27 and APC2 (Fig. 3). In accordance with the findings in budding yeast, knockdown of *Cdh1* did not rescue the Cdc20 protein level, while knockdown of either Cdc27 or APC2 increased the level of Cdc20. Thus, the core APC/C is required for the destruction of Cdc20 during SAC activation in mammalian cells, and it remains unknown whether a novel, unidentified adapter is required for this process.

Finally, we tried to identify the motifs required for Cdc20 degradation during SAC activation in mitosis. To this end, we constructed a panel of characterized<sup>6,10-15</sup> and novel Cdc20 mutants (Fig. 4A and B and Fig. S4), focusing on functional domains required for Cdh1-dependent degradation, Cdc27 binding (as a readout of APC/C binding), Cdk1 phosphorylation, Mad2 binding and BubR1 binding. We generated HeLa cells stably expressing the Cdc20 mutants at relatively low level by retroviral transduction and assessed the stability of these mutants in nocodazole-arrested mitosis in comparison to the remaining, attached G<sub>2</sub> cells (Fig. 4C). In accordance with our siRNA results, the 166-499 and 1-470 mutants showed increased stability, likely because they failed to bind APC/C.<sup>13</sup> Some mutants, such as 1-479, 1-489, IRAA and  $\Delta$ C-Box, were still unstable in mitosis, despite slightly reduced binding to APC/C (ref. 13 and data not shown). Notably, R132A, which retains robust binding to both APC/C and BubR1 but lacks Mad2 binding (Fig. 4B), is more stable than wild type Cdc20, though not as stable as the two APC binding mutants, suggesting that Mad2 is involved in the APC/C-dependent degradation of Cdc20 during SAC activation.

These results, combined with previous studies in budding yeast, recommend a model in which the proteasome-dependent degradation of Cdc20 is the initial response to checkpoint activation, preventing premature chromosome segregation. In such a model, upon SAC activation, immediate, Mad2- and APC/C-mediated degradation of Cdc20 is required to enact acute inhibition of APC/C<sup>Cdc20</sup> activity by reducing Cdc20 levels before a sustained checkpoint response can be implemented. Without this early step in checkpoint establishment, the existing levels of Cdc20 could produce the threshold APC/C activity for

overriding early attempts to implement the checkpoint, causing some cells to progress through mitosis and become aneuploid. Notably, this scenario appears to occur in yeast, where mild overexpression of Cdc20 can abrogate the SAC.<sup>8</sup>

The observed Mad2 binding dependence for Cdc20 degradation is also supported by studies in yeast demonstrating that Mad2 binding mutants of Cdc20 can bypass the SAC.<sup>4</sup> The potential roles of other spindle checkpoint proteins, such as BubR1, in this process remain unknown, but it is likely that they also contribute to Cdc20 degradation during SAC activation. While this manuscript was in preparation, it was reported that Cdc20 preferentially binds to BubR1 and Bub3 in mitotic cells arrested with spindle poisons and that Mad2 is more responsible for initiating the formation of this Cdc20, BubR1 and Bub3 complex.<sup>16</sup> Other proteins, such as a SAC-specific APC/C activator, may also be required for Cdc20 degradation in response to SAC activation. Finally, it remains unclear why a small population of Cdc20 is spared from Mad2- and APC/Cmediated degradation. Perhaps this Cdc20 is sequestered in another complex or it may remain stable due to deubiquitinating enzyme activity, as with other APC/C substrates.<sup>17</sup> Clearly, further research into the coordination of spindle checkpoint protein activity for the induction of Cdc20 degradation and checkpoint establishment is required.

#### **Materials and Methods**

Cell synchronization and drug treatment. HeLa and U-2 OS cells were arrested in S phase with 2.5 mM thymidine for 24 hours or synchronized in prometaphase by treatment with 100 ng/ml nocodazole or 100 nM taxol for up to 16 hours. For proteasome inhibition, cells were treated with 10  $\mu$ M MG132 for six hours.

Biochemical methods. Extract preparation, immunoprecipitation, and immunoblotting were previously described.<sup>18,19</sup> Rabbit



Figure 4. Cdc20 mutants impaired in their binding to APC/C or Mad2 are more stable in nocodazole-arrested mitotic cells. (A) Summary of the characteristics of wild type Cdc20 and ten Cdc20 mutants. (B) Cdc20 R132A does not bind Mad2 but still binds Cdc27. HEK293T cells were transfected with constructs encoding the indicated FLAG-tagged Cdc20 mutants. Exogenous proteins were immunoprecipitated (IP) from whole cell extracts with an anti-FLAG resin, and the immunocomplexes were probed with antibodies to the indicated proteins. (C) HeLa cells were infected with retroviruses expressing HA-tagged wild type Cdc20 or the indicated Cdc20 mutants and selected with puromycin treatment. The selected cells were synchronized in prometaphase by nocodazole treatment for 16 hours. Round, prometaphase cells (M) were then collected by gentle shake-off, and the remaining attached cells ( $G_2$ ) were also harvested. Protein extracts were analyzed by immunoblotting with antibodies against the indicated proteins.

polyclonal antibodies were from Santa Cruz Biotechnology (Cdc20 and FLAG) and Millipore (phospho-histone H3). Mouse monoclonal antibodies were from Sigma-Aldrich (Cdc27), Lab Vision (Cdh1), Zymed/Invitrogen (Cul1, Skp1), and Covance (HA). The rabbit polyclonal antibodies against Cyclin A and Cyclin B were previously described.<sup>20,21</sup>

**RNA interference.** Cells were transfected using HiPerFect transfection reagent (Qiagen) according to the manufacturer's protocol. A siRNA to *LacZ* mRNA (CGUACGCGGAAUACUUCGA) served as a control. The siRNA corresponding to *Cdh1* mRNA (UGAGAAGUCUCCCAGUCAG) was previously validated.<sup>22</sup> The siRNA pools targeting *Cdc27* or *APC2* were purchased from Dharmacon.

Plasmids. Cdc20 point mutants were generated using the QuickChange Site-directed Mutagenesis kit (Stratagene). All deletion mutants were generated by a PCR-based approach. All constructs were confirmed by sequencing.

Transient transfections and retrovirus-mediated gene transfer. For retrovirus production, GP-293 (Clontech) cells were transfected with FuGENE-6 according to the manufacturer's instructions. At 48 hours post-transfection, the virus-containing medium was collected and supplemented with 8  $\mu$ g/ml polybrene (Sigma). Cells were then infected by replacing the cell culture medium with the viral supernatant for six hours.

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