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Transcriptional and post-transcriptional regulation of Cdc20 during the spindle assembly checkpoint in *S. cerevisiae*

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Abstract

The anaphase-promoting complex (APC) is a ubiquitin ligase responsible for promoting the degradation of many cell cycle regulators. One of the activators and substrate-binding proteins for the APC is Cdc20. It has been shown previously that Cdc20 can promote its own degradation by the APC in normal cycling cells mainly through a cis-degradation mode (i.e. via an intramolecular mechanism). However, how Cdc20 is degraded during the spindle assembly checkpoint (SAC) is still not fully clear. In this study, we used a dual-Cdc20 system to investigate this issue and found that the cis-degradation mode is also the major pathway responsible for Cdc20 degradation during the SAC. In addition, we found that there is an inverse relationship between APC^{Cdc20} activity and the transcriptional activity of the *CDC20* promoter, which likely occurs through feedback regulation by APC^{Cdc20} substrates, such as the cyclins Clb2 and Clb5. These findings contribute to our understanding of how the inhibition of APC^{Cdc20} activity and enhanced Cdc20 degradation are required for proper spindle checkpoint arrest.

Keywords

protein degradation; spindle assembly checkpoint; Cdc20; post-transcriptional regulation; Anaphase-Promoting Complex; ubiquitination

1. Introduction

The process of protein ubiquitination is catalyzed by the sequential action of a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). By targeting key proteins for degradation by the 26S proteasome, ubiquitin-mediated proteolysis plays an essential role in various cellular processes, including cell growth, morphogenesis, and cell cycle progression. Two classes of E3s play particularly prominent

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roles in cell cycle progression: the anaphase-promoting complex/cyclosome (APC/C) and the Skp1-cullin-F-box protein complex (SCF) [1].

The APC/C is a multi-subunit complex composed of at least 13 distinct subunits in budding yeast [2], whose activity is regulated in vegetative cells by association with substrate-binding coactivator proteins, Cdc20 or Cdh1 [3, 4]. The activity of the APC/C is coordinated with cell cycle progression, with APC^{Cdc20} being active early in mitosis and APC^{Cdh1} active from late mitosis through G1 phase [3]. Both Cdc20 and Cdh1 interact with substrates via their C-terminal WD-40 domain, which recognizes two typical classes of degradation motifs, the destruction box (D-box) and the KEN box [5, 6]. In addition, Cdc20 and Cdh1 contain two conserved sequences important for their activity, an 8-residue motif in the N-terminal half of the protein called the C-box [7], and an Ile-Arg (IR) motif at the very C-terminus of each protein [8]. The C-box is important for binding to and modulating the activity of the APC [7, 9]. While the IR motif of Cdh1, which contributes to its binding to the APC, is important for Cdh1 activity *in vivo* [10], there is still some controversy over the role of the IR motif in Cdc20. It has been reported that mutation of the IR motif in Cdc20 severely reduces Cdc20 activity *in vitro*, but does not have much effect on cell viability [11].

The APC is responsible for the degradation of substrates important for many aspects of cell cycle progression, including the metaphase-to-anaphase transition, exit from mitosis, and the preparation for the next round of DNA replication. However, the only essential APC targets (which if not degraded cause a permanent cell cycle arrest) are B-type cyclins and securin (Pds1 in *Saccharomyces cerevisiae*) [12]. The cyclins are activators of the major cyclin-dependent protein kinase in yeast (Cdc28/Cdk1). Securin/Pds1 is an inhibitor of anaphase initiation. Prior to anaphase, sister chromatids are held together by the cohesin protein complex [13]. Once the kinetochores of the sister chromatids achieve proper attachment to the mitotic spindle, the cohesin complex is cleaved by separase and chromosomes segregate to opposite poles [14]. Pds1 binds to and inhibits separase; APC^{Cdc20}-mediated degradation of Pds1 releases this inhibition and allows anaphase to commence [14–17]. Incorrect or incomplete attachment of chromosomes to the mitotic spindle activates the spindle assembly checkpoint (SAC) and blocks anaphase onset by inhibiting APC^{Cdc20}-mediated Pds1 degradation. In this process, Cdc20 forms mitotic checkpoint complexes (MCCs) with several other molecules, including Mad2, Mad3 and Bub3, resulting in inhibition of APC^{Cdc20} and cell cycle arrest at the metaphase-to-anaphase transition [18–22]. In addition, the degradation of Cdc20 is elevated during checkpoint arrest [23, 24]. Both enhanced Cdc20 degradation and the inhibition of APC^{Cdc20} activity are required for proper activation of mitotic checkpoint arrest, whereas blocking either process impairs the arrest [23–25].

Cdc20 protein levels fluctuate during both yeast and mammalian cell cycles, rising in S phase, peaking in mitosis, and declining upon exit from mitosis [26–28]. This pattern can be partially explained at the transcriptional level, which shows similar oscillations [27, 29, 30]. In addition, Cdc20 is unstable throughout the yeast cell cycle and its degradation depends on the APC [27]. It is generally believed that APC^{Cdh1} is responsible for Cdc20 degradation from anaphase to G1 phase [6, 31], whereas the mechanism for Cdc20 degradation in other cell cycle phases is less clear. Cdc20 can be ubiquitinated in both cis- and trans-modes [11]. In the trans-mode, one Cdc20 (or Cdh1) molecule triggers the degradation of a second

Cdc20 molecule. In the cis mode, one Cdc20 molecule functions as both the substrate and the APC activator, and is ubiquitinated by the APC. In an unperturbed cell cycle, the cis-degradation mode is the primary pathway for Cdc20 degradation [11]. Reconstitution of the MCC with the APC *in vitro* demonstrated that Mad3-Bub3 synergizes with Mad2 to stimulate Cdc20 autoubiquitination, while inhibiting the ubiquitination of substrates [32]. Though these results suggested that Cdc20 was also ubiquitinated during the SAC via a cis-mode of autoubiquitination, these studies did not conclusively exclude the operation of a trans-mode of autoubiquitination. In addition, this *in vitro* SAC system represents a simplified system and may not have faithfully mimicked the situation *in vivo*.

In this study, we utilize a dual Cdc20 system to further examine the mechanism of Cdc20 degradation during the SAC. We confirmed that Cdc20 is degraded *in vivo* during the SAC via the APC mainly through a cis mode. Although the C-box of Cdc20 is not required for this degradation, the IR motif is important for both rapid degradation of Cdc20 during the SAC and for full APC^{Cdc20} activity. Interestingly, we found that APC^{Cdc20} inhibition during metaphase leads to elevated transcriptional activity of the *CDC20* promoter, likely through a feedback mechanism involving increased levels of APC^{Cdc20} substrates.

2. Materials and Methods

2.1. Yeast strains and plasmid constructions

The strains used in this study are listed in Table 1. Except for those used in the yeast two-hybrid assays, most strains used are derived from YJB14 [24], which was derived from W303 (*MATa bar1*Δ); or from the *MET3-CDC20* strain (*MATa, MET3-CDC20::URA3, CDC14-3HA*) [33], which was provided by Angelika Amon (MIT, Cambridge, MA). Yeast two-hybrid strains (PJ69-4a and PJ69-4) were provided by Stan Fields (University of Washington, Seattle, WA) [34]. To delete *MAD3*, the 5-untranslated region and 3′ region containing the C-terminal coding sequence were subcloned into pRS303 and then cut between these two regions and transformed into yeast to replace the given gene with *HIS3* by homologous recombination. Deletion of *MAD3* was confirmed by PCR analysis.

The plasmids used in this study are listed in Table 2. Most plasmids are derivatives of vectors provided by Akio Sugino [35]. To construct *CDC20* promoter-driven expression vectors, the endogenous *CDC20* promoter (430 bp) was amplified by PCR using primers MSO3143 (5′-GCG GAA TTC CCA CAA AGA ATG TGT CGT TC-3′) and MSO3118 (5′-GCG GGA TCC TAG TCT TCT TTG TAA TAC TTG-3′) and ligated into YIp204 or YCp22 between the BamH1 and EcoR1 sites. 8MYC-*CDC20* expressed Cdc20 with 8 N-terminal repeats of the Myc epitope. Codons 144–150 of Cdc20 were deleted to remove the C-box of *CDC20*. To append a 3×Flag epitope to the C-termini of Ndd1, Yox1, Mcm1, Fkh1 and Fkh2, truncated versions of the corresponding genes were inserted into pRS303 [36] inframe with a C-terminal 3×Flag tag, linearized within the genes, and transformed into cells. The pAS2 (bait) and pACTII (prey) plasmids used in yeast two-hybrid assays were gifts from Steven Elledge (Harvard Medical School, Boston, MA).

2.2. Cell culture conditions

Yeast cells were grown in YPD or selective minimal medium as described previously [37]. For M phase arrest, cells were grown in medium containing 50 μg/ml benomyl (Dupont) for 2 hours.

For protein stability analysis during the SAC, *MET3p-CDC20* strains were grown in raffinose-containing medium to mid-exponential phase (OD_{600} of ~0.3–0.5). 2% galactose was added to induce the expression of Pds1-mDB for 90 min, followed by addition of 2% dextrose with or without 1.25 mM methionine for 90 min. The cells were collected and transferred to benomyl-containing medium (with or without methionine) for an additional 2 hours, and then 500 μg/ml cycloheximide (Acros Organics) was added. Cells were removed at the indicated times, washed once with H_2O, and frozen in liquid nitrogen.

2.3. Yeast two-hybrid analysis

Briefly, PJ69-4α cells containing the bait plasmids (pAS2-Cdc20ΔC or its derivatives) were mated with PJ69-4a cells containing prey plasmids of pACTII-Mad2, Mad3 or Hsl1 to generate diploid cells that contained both the bait and prey plasmids. Potential interactions between bait and prey proteins were determined by growth on selective medium (CM-Trp-Leu-His-Ade).

2.4. Yeast extract preparation and immunoblot analysis

Cell pellets from 35 ml cultures were suspended in three volumes of lysis buffer (6.7% sodium dodecyl sulfate (SDS), 75 mM Tris/Cl (pH 7.5), 27% glycerol, 100 mM dithiothreitol (DTT)). Cells were broken by shaking the suspension together with 0.45 g glass beads (300 μl volume) for 3 min in a bead beater, and then incubating at 95°C for 10 min. Glass beads and cell debris were removed by centrifugation at 14,000 rpm for 10 min. The supernatant was further clarified by centrifugation at 65,000 rpm in a TLA 100.2 rotor (Beckman) for 10 min at 15°C. Protein extracts were separated on a protein gel containing 8% polyacrylamide and transferred to an Immobilon-P membrane (Millipore). The membranes were incubated with 5% non-fat dried milk/TBST (10 mM Tris-Cl [pH 7.5], 150 mM NaCl, 0.05% Tween 20) for 2 h followed by incubation with primary and secondary antibodies. Myc tags were detected with 9E10 monoclonal antibodies (Covance Research Products). The Flag tag was detected with anti-Flag M2 monoclonal antibody (Sigma-Aldrich). Cdc28 was detected with anti-PSTAIRE antibodies.

2.5. Assay of β-galactosidase activity

The assays were performed as described previously with minor modifications [38]. Cells were grown to mid-exponential phase (OD_{600} of ~0.3–0.5). Triplicate (1.5 ml) samples of the culture were collected by centrifugation and resuspended in 0.9 ml Z buffer (10 mM KCl, 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mM $MgSO_4$, 0.27% 2-Mercaptoethanol). The cells were permeabilized with 100 μl chloroform and 50 μl 0.1% SDS followed by brief votexing. 0.2 ml onitrophenyl-β-D-galactoside (ONPG, 4 mg/ml) was added and the mixtures were incubated at 30°C. After sufficient yellow color had developed, the reactions were stopped by addition of 0.5 ml Na_2CO3 (1 M) followed by centrifugation at maximal speed in a microfuge for 5 min. The optical densities of the supernatants at 420 nm and at

550 nm were recorded and the β-galactosidase activity was calculated as: 1000 × (OD_{420}–1.75 × OD_{550})/(OD_{600} of assayed culture × time × volume assayed).

3. Results

3.1. Cdc20 cis-degradation during the SAC

Cdc20 is degraded by the APC during the SAC [23, 25]. Since Cdc20 is the only APC activator active in mitosis prior to anaphase, Cdc20 must be autoubiquitinated by the APC through either a trans- or a cis-mode. To differentiate between these possibilities, we created a strain expressing two different forms of Cdc20, a wild-type form that can serve both as an APC activator and as a substrate, and a mutant form that can only serve as an APC substrate. The wild-type Cdc20 was under the control of the methionine-repressible *MET3* promoter (*MET3-CDC20*). Note that unlike regulation by other common promoters such as *GAL*, genes controlled by the *MET3* promoter are turned off in the presence of the regulator, methionine. The other version was a Myc-tagged form of Cdc20, Myc-Cdc20ΔC, whose C-box was deleted so that it could no longer bind to the APC as an activator. Expression of Cdc20ΔC was controlled by the endogenous *CDC20* promoter (Fig. 1A). Since Cdc20ΔC cannot bind to the APC as an activator, it cannot promote its own degradation via a trans-mode. By studying the degradation of Cdc20ΔC in the presence and absence of wild-type Cdc20, we were able to differentiate between cis- and trans-modes of degradation during mitotic checkpoint arrest.

Consistent with previous findings, cells expressing Cdc20ΔC as their only form of Cdc20 could not proliferate and no colonies formed when expression of *MET3-CDC20* was repressed in methionine-containing rich medium (Fig. 1B). Introduction of wild-type Cdc20 allowed the cells to grow normally. Both wild-type Cdc20 and Cdc20ΔC were rapidly degraded during the SAC (Fig. 1C, upper). This degradation was partially Mad3 dependent, as Cdc20ΔC became more stable when Mad3 was deleted. When methionine was added to repress the expression of wild-type Cdc20, Cdc20ΔC still underwent rapid degradation during the SAC, and this degradation was similarly dependent on Mad3 (Fig. 1C, bottom). These results confirmed that Cdc20 is primarily degraded through the cis mode during the SAC. In addition, these experiments indicated that the C-box of Cdc20 is not required for the rapid degradation of Cdc20 during the SAC, suggesting that under these conditions Cdc20 may associate with the APC as part of the MCC (see Discussion).

3.2. The fourth D-box of Cdc20 is important for Cdc20 degradation by the APC during the SAC

In analyzing the sequence of Cdc20, we identified 4 potential D-boxes (DBs; “RxxL”) (Fig. 2A), two of which are in the C-terminal half of Cdc20 and have not been analyzed before, possibly due to their location in the WD40 domain required for binding to substrates [10]. We individually mutated these D-boxes to “AxxA” and investigated their potential roles in Cdc20 degradation by the APC. We first checked the interaction of these mutants with Mad2, Mad3 and the Cdc20 substrate, Hsl1 using the yeast two-hybrid assay to verify that the mutant proteins were functional in binding these proteins. We also removed the C-box from all Cdc20 constructs to reduce the toxicity of Cdc20 overexpression. Cdc20 mutated

for DB1, DB2 and DB3 maintained a similar interaction pattern as the parental Cdc20ΔC, whereas mutation of DB4 eliminated the interaction with both Hsl1 and Mad3 (Fig. 2B). In all of these D-box mutations, we changed the positively-charged arginine in the first position to the neutral alanine. To determine the role of the charge of DB4 in the interaction of Cdc20 with Hsl1 and Mad3, we mutated this "RxxL" motif to "KxxL" or "KxxA." These mutations fully or partially restored the interaction of Cdc20 with Hsl1 and Mad3 (Fig. 2C), suggesting that the positive charge of DB4 is important for maintaining the proper structure of Cdc20 or that it is necessary for the interaction of Cdc20 with its substrates.

Further analysis of these Cdc20ΔC mutants (mDB1, mDB2 and mDB3) demonstrated that they were all degraded at a similar rate as the parental Cdc20ΔC (Fig. 2D), indicating that individually each of these D-boxes plays little role in Cdc20 degradation during the SAC. Consistent with its inability to associate with Mad3, the mDB4 mutant was significantly stabilized.

3.3. The IR motif of Cdc20 is required for optimal Cdc20 activity and for its degradation during the SAC

Due to inconclusive reports, we revisited the role of the C-terminal IR motif in regulating Cdc20 activity. As in Fig. 1, we tested whether Cdc20ΔIR could rescue cell growth when the expression of wild-type Cdc20 was repressed. Although cells containing Cdc20ΔIR grew when it was the only form of Cdc20 expressed, growth was slower than that of cells containing wild-type Cdc20 (Fig. 3A). Thus, while non-essential, the IR motif is required for optimal activity of Cdc20 *in vivo*.

We further tested the influence of the IR motif on the stability of Cdc20 during the SAC. We found that Cdc20Δ(C+IR) was quite stable during the SAC, independent of the presence or absence of Mad3 (Fig. 3B). Thus, the IR motif appears to be important for Cdc20 degradation during the SAC. The stabilization of Cdc20Δ(C+IR) even in the presence of WT Cdc20 further argues against any contribution of trans degradation.

3.4. Inhibition of APC^{Cdc20} enhances Cdc20 promoter activity

Using the system in Fig. 1, we noticed that repression of wild-type *CDC20* expression resulted in a modest increase in the amount of Cdc20ΔC protein expressed from its endogenous promoter (Fig. 4A). This increase in level appears to be due to reduced APC^{Cdc20} activity, rather than activation of the SAC, since it still occurred in the absence of Mad3. One possible explanation for this finding would be trans-degradation of Cdc20ΔC by wild-type Cdc20 in a DB1-dependent manner [11]. However, the levels of Cdc20ΔC-mDB1 still increased upon depletion of wild-type Cdc20 (Fig. 4B), suggesting the involvement of a second mechanism. Another possibility is that reduced APC^{Cdc20} activity might lead to up-regulation of the transcriptional activity of the *CDC20* promoter. To test this hypothesis, we constructed a reporter system in which *lacZ* was fused to the *CDC20* promoter to monitor its activity. The results showed that the transcriptional activity of the *CDC20* promoter increased about 35% upon depletion of wild-type Cdc20 (Fig. 4C). This increase was not due to the addition of methionine to the medium, since methionine addition did not have any obvious effect on *CDC20* promoter activity in control cells not containing *MET3-CDC20*

(data not shown). These results indicate the involvement of Cdc20 activity in regulating *CDC20* promoter activity.

We next investigated whether the reduced APC^{Cdc20} activity during the SAC would also up-regulate the activity of the *CDC20* promoter. We found that *CDC20* promoter activity was similarly increased when metaphase-arrested cells were treated with the microtubule-destabilizing drug benomyl to induce the SAC (Fig. 4D), thus confirming that there is an inverse relationship between the activity of the *CDC20* promoter and that of APC^{Cdc20}.

3.5. Increased level of APC^{Cdc20} substrates stimulates CDC20 promoter activity

We sought to determine the underlying mechanism for the elevated transcriptional activity of the *CDC20* promoter when APC^{Cdc20} activity is reduced. A simple explanation could be that APC^{Cdc20} promotes the degradation of a transcription factor acting positively on the *CDC20* promoter. Inhibition of APC^{Cdc20} would result in increased levels of this transcription factor and increased expression of *CDC20*. Based on published data and analysis of the *CDC20* promoter, five transcription factors are likely to play major roles in regulating the activity of the *CDC20* promoter: Ndd1, Fkh1, Fkh2, Mcm1 and Yox1 [39–43]. While Ndd1, Fkh1, Fkh2 and Mcm1 positively regulate the *CDC20* promoter, Yox1 is a negative regulator that binds to and inhibits Mcm1 [43]. We first monitored the protein levels of these transcription factors in the presence and absence of Cdc20. While the levels of Ndd1, Fkh1 and Mcm1 appeared not to vary with changes of APC^{Cdc20} activity, the level of Fkh2 increased and that of Yox1 was greatly reduced when APC^{Cdc20} activity was repressed (Fig. 5A). Thus, APC^{Cdc20} could regulate *CDC20* promoter activity by influencing the protein levels of the transcription factors acting on the *CDC20* promoter.

Because Fkh2 levels increase upon APC^{Cdc20} inhibition, we wondered whether Fkh2 might be regulated directly by APC^{Cc20}. However, we found that Fkh2 is a quite stable protein (Fig. 5B), making it unlikely to be a substrate of APC^{Cdc20}. Thus, APC^{Cdc20} likely regulates Fkh2 levels indirectly, potentially through transcriptional regulation.

The stability and activity of many proteins is regulated through protein phosphorylation. In this context, it has been reported that both Fkh2 and Yox1 are substrates of Cdk1 (Cdc28)/cyclins [44, 45]. In addition, we noticed that upon repression of APC^{Cdc20} activity, there is a small increase in the amount of an electrophoretically-retarded form of Ndd1, which is often indicative of protein phosphorylation. In analyzing the sequence of Ndd1, we identified more than a dozen of SP/TP sites that could be potential phosphorylation sites for Cdk1/cyclins, suggesting that Ndd1 might be a substrate of Cdk1. Given the above, we speculated that phosphorylation of one or more of these transcription factors by Cdk1 could play a role in regulating *CDC20* promoter activity. Cyclins Clb2 and Clb5 are substrates of APC^{Cdc20} [17, 46, 47], raising the possibility that APC^{Cdc20} might regulate *CDC20* promoter activity through regulation of cyclin levels. We confirmed that reduced APC^{Cdc20} activity led to increased levels of Clb2 and Clb5 (Fig. 5C). We further tested whether increased levels of Clb2 and Clb5 could up-regulate the promoter activity of *CDC20*. Metaphase-arrested cells were switched into methionine-free medium to induce *MET3-CLB2* or *MET3-CLB5* expression. We found that moderate induction of either *CLB2* or *CLB5* from the *MET3* promoter up-regulated *CDC20* promoter activity about 25% (Fig. 5D), In contrast, upon the

addition of methionine to metaphase-arrested cells, the repression of either *CLB2* or *CLB5* from the *MET3* promoter downregulated *CDC20* promoter to a similar level (Fig. 5E), thus confirming the positive effects of the APC^{Cdc20} substrates Clb2 and Clb5 on *CDC20* promoter activity.

4. Discussion

One of the first APC substrates to be identified was its own activator, Cdc20. Cdc20 is degraded via the APC both during normal cycling conditions and during mitotic spindle checkpoint arrest. During normal cycling conditions, Cdc20 is degraded by the APC through an intramolecular (cis) mechanism [11]. Subsequent analysis of Cdc20 autoubiquitination *in vitro* using purified components demonstrated a synergistic effect of Mad2 and Mad3-Bub3 in promoting Cdc20 autoubiquitination and the involvement of the APC/C subunit Mnd2/APC15 in this process [32]. We have expanded these findings and demonstrated that Cdc20 undergoes similar cis-degradation during the SAC, independent of the C-box. The C-box is required for Cdc20 binding to and activation of the APC. The dispensability of the C-box for degradation of Cdc20 by the APC during the SAC suggests that Cdc20 may bind to the APC via different motifs when it acts as a substrate during the SAC than when it binds as an APC activator when the SAC is inactive. In addition, it raises the possibility that MCC proteins may participate in the recruitment of Cdc20 to the APC. One such candidate is Mad3, which can exist in a complex with the APC [25].

The role of the IR motif in Cdc20 function has been somewhat unclear. While the IR motif was shown to be important for both Cdc20 autoubiquitination and APC^{Cdc20} activity *in vitro*, no obvious growth phenotype was observed for cells in which the IR motif was mutated [11]. Using a more quantitative analysis, we found that deletion of the IR motif leads to a small reduction in growth rate compared to wild-type Cdc20. The discrepancy between the strong role for the IR motif *in vitro* and its modest role *in vivo* may reflect the following factors: *in vivo*, reduced autoubiquitination of Cdc20ΔIR results in increased levels of Cdc20ΔIR (data not shown), which in turn partially counteracts the effects of reduced activity of Cdc20ΔIR. Consistent with a recent paper showing that the IR motif is important for the SAC [48], we found that the IR motif is required for SAC-induced Cdc20 degradation. We noticed that the Cdc20ΔIR mutant was used in the previous study to demonstrate the synergistic effects of Mad2 and Mad3-Bub3 on promoting Cdc20 autoubiquitination [32]. Based on the results that the IR motif is important for proper SAC function, the use of the Cdc20ΔIR mutant with purified MCC components to study Cdc20 autoubiquitination could fail to faithfully mimic the reactions during the SAC *in vivo*.

It has been unclear why the proper function of the SAC requires both the inhibition of APC^{Cdc20} activity and the rapid degradation of Cdc20 during the SAC[23]. In this study, we found that inhibition of APC^{Cdc20} activity during the SAC increases the transcription of *CDC20*. If Cdc20 were not degraded during the SAC, then its increased transcription would lead to an increase in Cdc20 levels, partially counteracting inhibition of APC^{Cdc20} and impairing the SAC. Thus, our finding that APC inhibition leads to increased *CDC20* transcription contributes to a mechanistic explanation for why Cdc20 degradation is critical for checkpoint maintenance. In addition, the increased transcription of *CDC20* and

subsequent increased protein synthesis could prime cells for efficient exit from the SAC once all the chromosomes have been properly attached to the mitotic spindle.

Only limited studies have been conducted on the transcriptional regulation of *CDC20*. The major transcription factors appear to be those with known functions in S/M phase. APC^{Cdc20} could negatively regulate *CDC20* transcription either directly or indirectly. One direct way would be to target a potential transcription factor for degradation. Alternatively, indirect regulation could occur through other APC substrates, such as cyclins Clb2 and Clb5. By regulating Cdk1 activity, APC^{Cdc20} can influence many aspects of cellular activity. Published data and protein sequence analysis indicate that all of the major transcriptional regulators of *CDC20* contain potential phosphorylation sites for Cdk1/cyclins. In support of such indirect regulation, we found that enhanced expression of *CLB2* and *CLB5* leads to up-regulation of the *CDC20* promoter. Interestingly, we found that decreased APC^{Cdc20} activity led to an increase of Fkh2 protein level and to a great reduction of Yox1 protein level. We do not currently know the underlying mechanisms for the change of Yox1 protein level. It is possible, for instance, that enhanced Cdk1 activity triggers the phosphorylation-dependent degradation of Yox1 by an SCF complex.

Although we did not address the potential regulation of *CDC20* transcription by APC^{Cdh1}, it seems likely that Cdh1, like Cdc20, could also participate in this process via degradation of cyclins in anaphase and G1 phase. Indeed, the APC activity correlates inversely with the transcriptional profile of *CDC20* during the cell cycle, in which *CDC20* mRNA levels are lowest in G1 phase, gradually increase during S phase, and peak in M phase [27]. By regulating the degradation of important cyclins, APC coordinates CDK activity with the cell cycle. Conversely, CDK activity tightly regulates APC activity at both transcriptional and post-translational levels. These interconnected regulatory schemes coordinate and contribute to proper cell cycle progression.

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Abbreviations

APC/C	the anaphase-promoting complex/cyclosome
SCF	the Skp1-cullin-F-box protein complex
SAC	the spindle assembly checkpoint
MCCs	mitotic checkpoint complexes

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Highlights

- Cdc20 is primarily degraded via a *cis* mechanism during the spindle assembly checkpoint.
- The C-terminal IR motif is required for Cdc20 *cis*-degradation.
- Inhibition of APC^{Cdc20} enhances Cdc20 promoter activity.

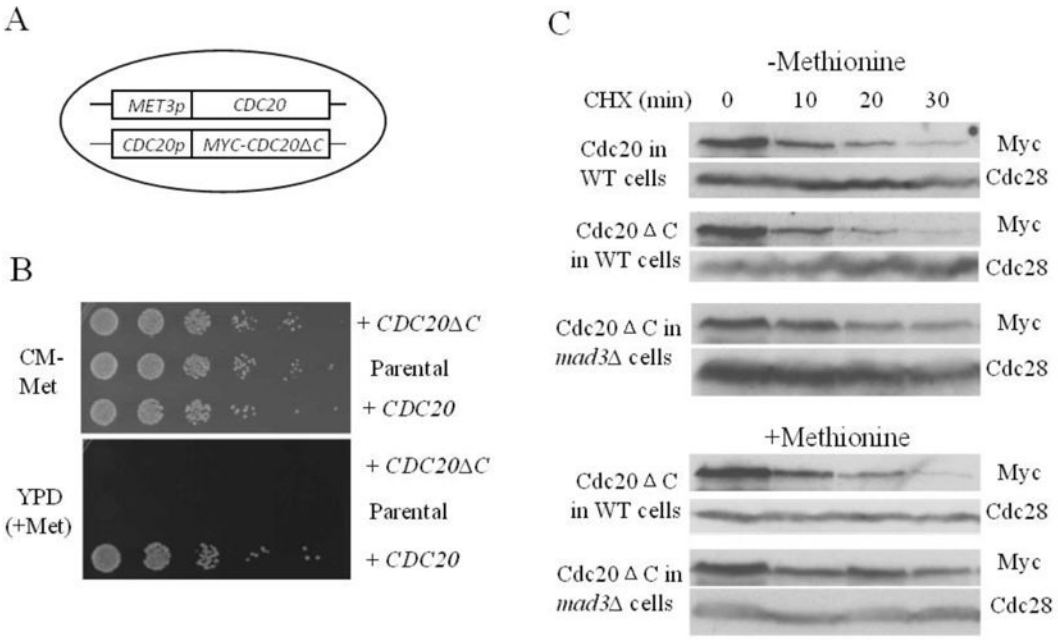

Figure 1.

Cis-degradation of Cdc20 during the SAC. (A) Experimental design. Many of the strains in this study contained a mutant version of Myc-tagged *CDC20ΔC* (or *CDC20*) under its endogenous promoter and *MET3p-CDC20*, in which wild-type *CDC20* is under the control of the methionine-repressible *MET3* promoter. Many of the strains also contained a galactose-inducible non-degradable form of Pds1 (GAL-Pds1-mDB), whose expression arrests cells in metaphase. (B) Wild-type *CDC20* or a *CDC20ΔC* mutant expressed from the endogenous *CDC20* promoter was introduced into the parental strain carrying *MET3-CDC20*. The strains were tested for growth on a YPD plate (which contains methionine) or on a synthetic minimal medium plate lacking methionine. (C) Half-lives of Cdc20ΔC during the SAC. Metaphase arrested cells were treated with benomyl to induce the SAC. Sample were collected at the indicated times following addition of cycloheximide (CHX) and analyzed by immunoblotting against the Myc tag. In the indicated panels, 1.25 mM methionine was added before adding benomyl to repress the expression of wild-type *CDC20* from the *MET3* promoter. Further details can be found in “Materials and Methods.”

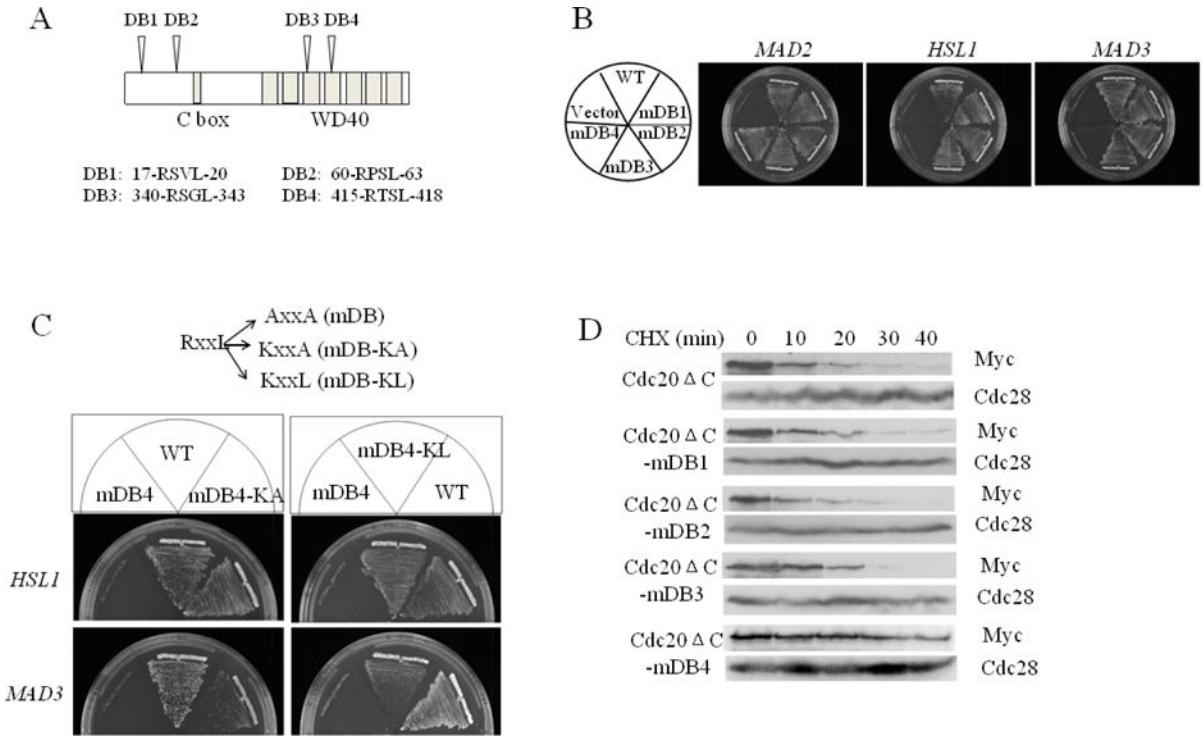

Figure 2.

DB4 of Cdc20 is required for Cdc20 degradation by the APC during the SAC. (A) Diagram of Cdc20 with the C-box and potential D-boxes indicated. (B) Interactions of Cdc20ΔC and its D-box (DB) mutants with Hsl1, Mad2 and Mad3 by yeast two-hybrid analysis. Cdc20ΔC was used in the analysis to reduce the toxicity of Cdc20 over-expression. (C) Interaction of Cdc20ΔC DB4 mutants with Hsl1 and Mad3. (D) The half-lives of various Cdc20ΔC mutants were analyzed during the SAC as in Fig. 1D. Methionine was added to the medium to repress the expression of *MET3-CDC20*.

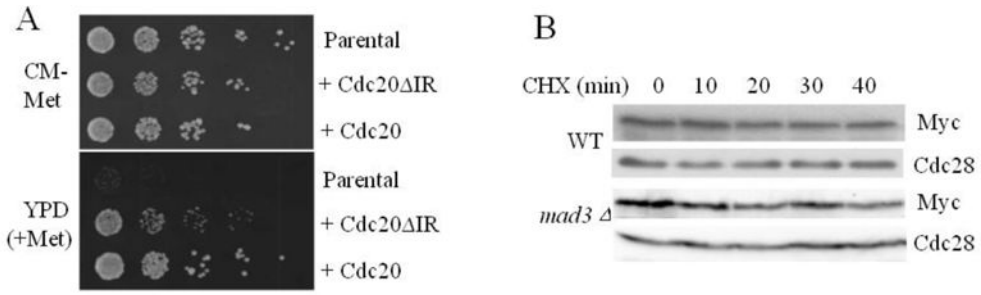

Figure 3.

The role of the C-terminal IR motif in Cdc20 degradation during the SAC. (A) *CDC20* or *CDC20ΔIR* expressed from the endogenous *CDC20* promoter was introduced into *MET3-CDC20* cells. The strains were tested for growth on a YPD plate (which contains methionine) or on a synthetic minimal medium plate without methionine. (B) The half-lives of Cdc20Δ(C+IR) during the SAC in *MET3-CDC20* and *MET3-CDC20 MAD3*Δ strains were determined as in Fig. 1C.

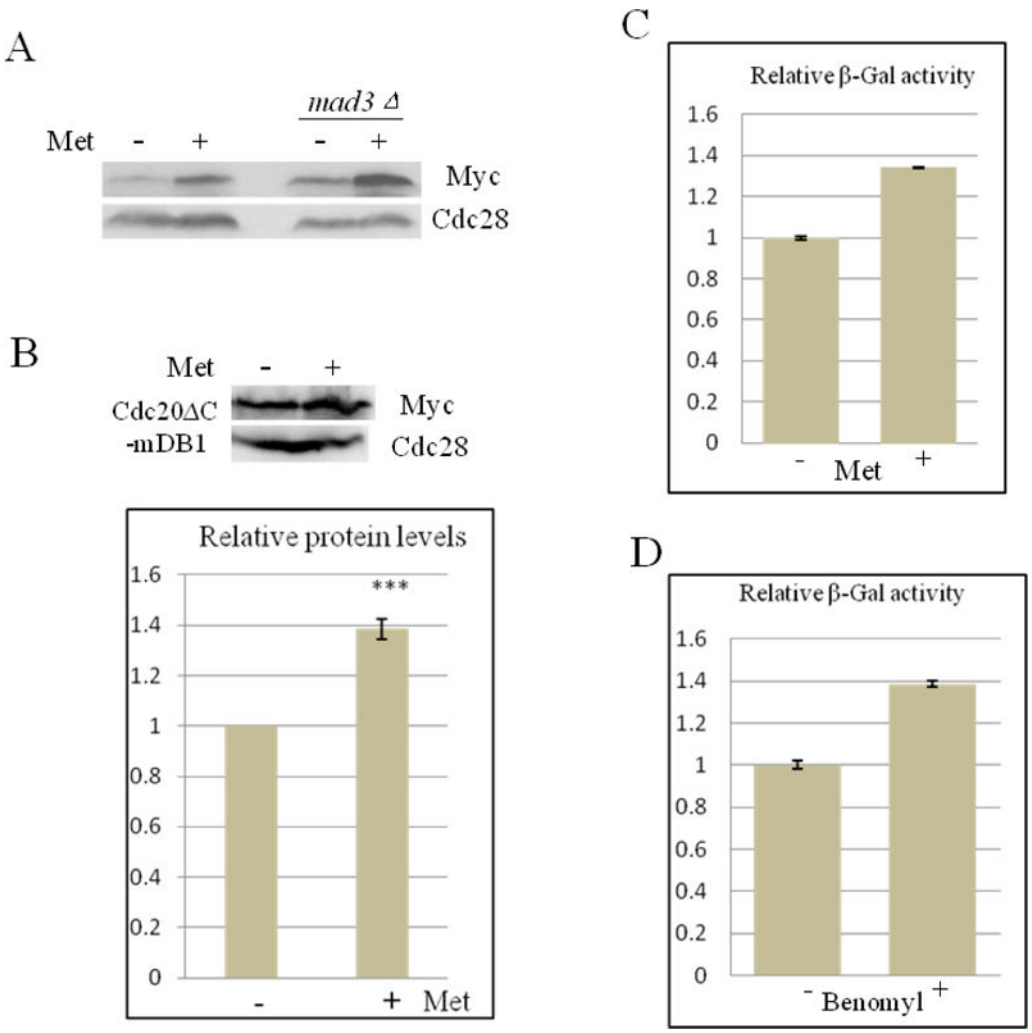

Figure 4.

Reduced APC^{Cdc20} activity increases *CDC20* promoter activity. (A) Cells (YRW0301111 or its *mad3Δ* derivative) were arrested in metaphase by *GAL-PDS1-mDB* induction followed by addition of glucose and methionine to repress wild-type *CDC20* expression. The levels of Myc-Cdc20ΔC expressed from the endogenous *CDC20* promoter were determined by immunoblotting. (B) The levels of Myc-Cdc20ΔC-mDB1 expressed from the endogenous *CDC20* promoter in *MET3-CDC20* cells were determined in the presence or absence of methionine as in (A). The relative protein levels were quantitated from three independent experiments using Image Lab software from Bio-Rad. The protein level of Cdc20ΔC-mDB1 in the absence of methionine was normalized to 1. (***: $p < 0.01$) (C) *lacZ* was expressed from the endogenous *CDC20* promoter as a reporter for promoter activity. Relative β-galactosidase activities were determined in *MET3-CDC20* cells in the presence or absence of methionine as in (A). The average activity in the absence of methionine was normalized to 1. (D) *CDC20* promoter reporter activity during the SAC. After arrest in metaphase by *GAL-PDS1-mDB* induction, W303 cells (YRW0211144) were further treated with benomyl to induce the SAC and the relative β-galactosidase activity driven by the endogenous *CDC20* promoter was determined. The average activity without benomyl treatment (DMSO

only) was normalized to 1. Both (C) and (D) show the means and standard deviations from three independent experiments. All data are representative of at least two independent experiments.

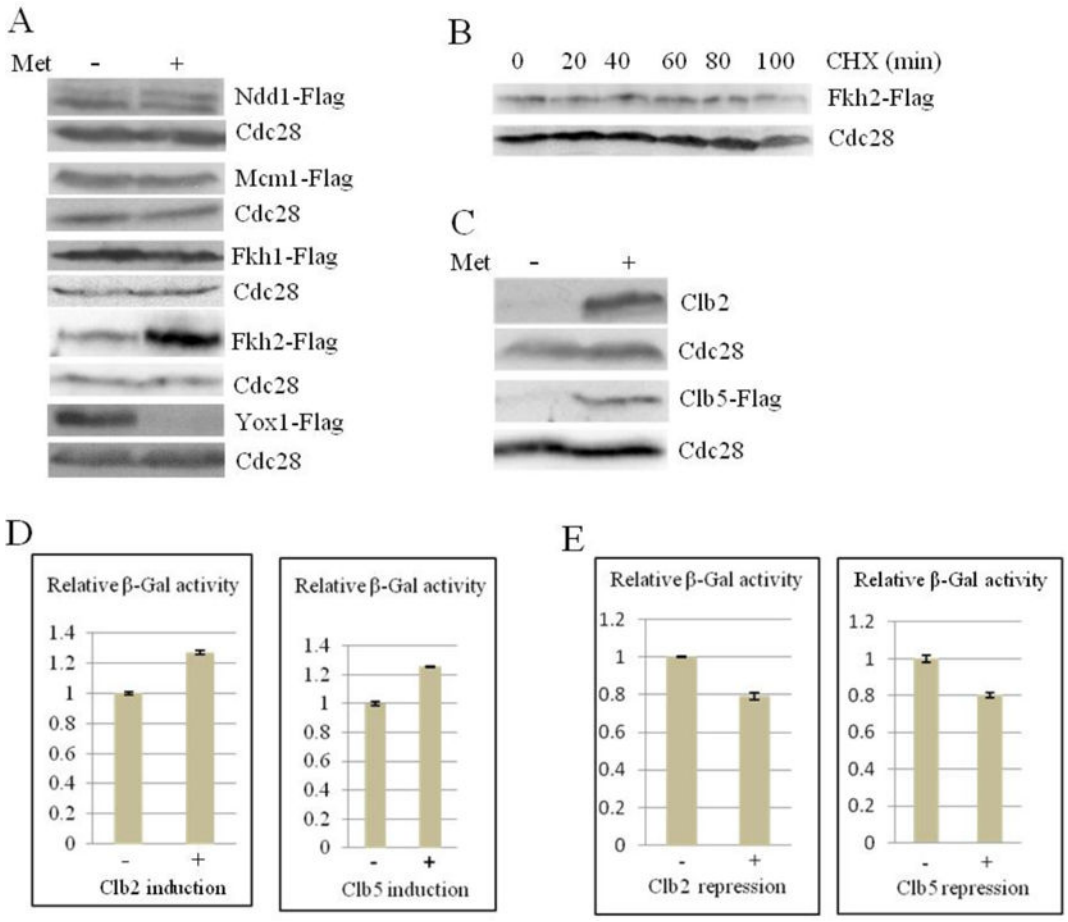

Figure 5.

Regulation of *CDC20* promoter by APC^{Cdc20}. (A) Appropriate strains were grown as in Fig. 4A. The levels of the transcription factors Ndd1, Mcm1, Fkh1, Fkh2 and Yox1 were determined by immunoblotting in the presence and absence of methionine. (B) Half-lives of Fkh2-Flag in metaphase. After cells (YRW0527142) were arrested in metaphase by *GAL-PDS1-mDB* induction, samples were collected at the indicated times following addition of cycloheximide (CHX) and analyzed by immunoblotting against the Flag tag. (C) The protein levels of the APC^{Cdc20} substrates Clb2 and Clb5 were determined as in (A). (D) Cells (YRW0407161 and YRW0407162) were arrested in metaphase by *GAL-PDS1-mDB* induction, followed by spin-down and washing once with methionine-free medium. Cells were split and grown for 3 hours in methionine-free medium to induce *MET3-CLB2/CLB5* expression, or into methionine-containing medium to repress *CLB2* and *CLB5* expression. The relative β-galactosidase activity driven by the endogenous *CDC20* promoter was determined. The average activity without Clb2/Clb5 induction was normalized to 1 and the data show the means and standard deviations from three independent experiments. (E) Similar to (D), cells (YRW0101171 and YRW0109171) were arrested in metaphase by *GAL-PDS1-mDB* induction. Cells were split and half continued to grow for 2.5 hours in the presence of methionine to repress *MET3-CLB2/CLB5* expression. The relative β-

galactosidase activity driven by the endogenous *CDC20* promoter was determined. The average activity without Clb2/Clb5 repression was normalized to 1.

Table 1

Strains used in this study.

Strain	Relevant genotype	Source
MET-CDC20	*MATa,MET3-CDC20::URA3 CDC14-3HA*	33
YRW0124112	*MET-CDC20 LEU2:GAL-PDS1-mDB-HA*	This study
YRW0301111	YRW0124112 *TRP1:CDC20p-8Myc-Cdc20ΔC*	This study
YRW0413111	YRW0124112 *TRP1:CDC20p-8Myc-Cdc20*	This study
YRW0413113	YRW0301111 *Mad3Δ::HIS3*	This study
YRW1007111	YRW0124112 *TRP1:CDC20p-8Myc-Cdc20ΔIR*	This study
YRW0714111	YRW0124112 *TRP1:CDC20p-8Myc-Cdc20ΔC-mDB1*	This study
YRW0714114	YRW0124112 *TRP1:CDC20p-8Myc-Cdc20ΔC-mDB2*	This study
YRW0714116	YRW0124112 *TRP1:CDC20p-8Myc-Cdc20ΔC-mDB3*	This study
YRW1105161	YRW0124112 *TRP1:CDC20p-8Myc-Cdc20ΔC-mDB4*	This study
YRW0514115	YRW0124112 *TRP1:CDC20p-8Myc-Cdc20Δ(C+IR)*	This study
YRW0713113	YRW0124112 *NDD1::NDD1-Flag* [pRS303]	This study
YRW1007113	YRW0124112 *FKH1::FKH1-Flag* [pRS303]	This study
YRW1208111	YRW0124112 *MCM1::MCM1-Flag* [pRS303]	This study
YRW0527142	YRW0124112 *FKH2::FKH2-Flag* [pRS303]	This study
YRW0202124	YRW0124112 *YoX1::YoX1-Flag* [pRS303]	This study
YRW0612131	YRW0124112 *CLB5::CLB5-Flag* [pRS303]	This study
YRW1110111	*MET-CDC20 TRP1:CDC20p-8Myc-Cdc20ΔC*	This study
YRW1121132	YRW0124112/YCplac22-*CDC20p-lacZ*	This study
YJB15	*MATa bar1Δ*	24
YRW0108122	YJB15 *LEU2: LEU2:GAL-PDS1-mDB*	This study
YRW0211144	YRW0108122 *TRP1:CDC20p-lacZ*	This study
PJ69-4a	*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	34
PJ69-4α	*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	34
YRW0407161	YRW0211144/pRS313-*MET3p-CLB2-Flag*	This study
YRW0407163	YRW0211144/pRS313-*MET3p-CLB5-Flag*	This study
YRW0101171	YRW0211144 *CLB5::MET3p-8Myc-CLB5* [pRS303]	This study
YRW0109171	YRW0211144 CLB2::MET3p-8Myc-CLB2 [YIpac211]	This study

Table 2

Plasmids used in this study

Plasmid	Description
pRW0226117	YIp128-*GAL-PDS1-mDB*-HA
pRW0220112	YIp204-*CDC20p-8Myc-CDC20*
pRW0225111	YIp204-*CDC20p-8Myc-CDC20ΔC*
pRW0801103	pAS2-*CDC20ΔC*
pRW1218101	pAS2-*CDC20ΔC-mDB1*
pRW1207101	pAS2-*CDC20ΔC-mDB2*
pRW0110115	pAS2-*CDC20ΔC-mDB3*
pRW1207103	pAS2-*CDC20ΔC-mDB4*
pRW0319116	pAS2-*CDC20ΔC-mDB4-KA*
pRW0319117	pAS2-*CDC20ΔC-mDB4-KL*
pRW0716111	pAS2-*CDC20Δ(C+IR)*
pRW0319114	PACTII-*MAD3*
pRW0504101	PACTII-*MAD2*
pRW0504103	PACTII-*HSL1(667–872)*
pRW0331111	pRS303-*MAD3Δ*
pRW0226111	YIp204-*CDC20p-8Myc-CDC20-mDB1*
pRW0226113	YIp204-*CDC20p-8Myc-CDC20-mDB2*
pRW0226115	YIp204-*CDC20p-8Myc-CDC20-mDB3*
pRW0704111	YIp204-*CDC20p-8Myc-CDC20ΔC-mDB1*
pRW0704113	YIp204-*CDC20p-8Myc-CDC20ΔC-mDB2*
pRW0704115	YIp204-*CDC20p-8Myc-CDC20ΔC-mDB3*
pRW0510111	YIp204-*CDC20p-8Myc-CDC20Δ(C+IR)*
pRW1229114	YIp204-*CDC20p-lacZ*
pRW1025135	YCp22-*CDC20p-lacZ*
pRW0609113	pRS303-*NDD1(781 bp-end)-3×Flag*
pRW1125115	pRS303-*MCM1(301 bp-end)-3×Flag*
pRW0802111	pRS303-*FKH1(301 bp-end)-3×Flag*
pRW0504142	pRS303-*FKH2(1291 bp-end)-3×Flag*
pRW0118121	pRS303-*YoX1(301 bp-end)-3×Flag*
pRW0402161	pRS313-*MET3p-CLB2-3×Flag*
pRW0402162	pRS313-*MET3p-CLB5-3×Flag*
pRW1212163	pRS303-*MET3p-8Myc-CLB5(1-650bp)*
pRW1221161	YIp211-*MET3p-8Myc-CLB2(1-800bp)*