Coordination of Cell Cycle Events

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry and Molecular Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Acknowledgements

Graduate school is a long difficult journey and having a great support system is a key to success. At the top of the list are my parents. Since I was young, they encouraged me to work hard and pursue a degree from a university. Although surely they were not expecting me to obtain a Ph.D. in Biochemistry, they were non the less extremely supportive. Without their help and support I never would have made it to this point in my career and for that I am eternally grateful.

Dave Morgan is the biggest reason why I completed my Ph.D. Not only has he helped me grow as a scientist but also as a person. His knowledge, experience, and patience were key parts to my development and success. Through all my ups and downs during graduate school, Dave's door was always open. He always made time to talk, listen, and give advice. As my final days approach, I look back upon all the great memories and experiences I have shared in Dave's lab and am truly thankful for all that he has done for me.

Finding time outside of lab is an important part to maintain proper mental health while in graduate school. I spent almost all my free time with family or friends. My sisters, cousins, childhood friends, and new friends from UCSF, have been such an important part to my success. There are too many people to name but I would like to make a special note to thank Michael Johnston. Michael is one of my classmates and we bonded from the get go. He is an amazing person and I am very lucky to have him as a friend. I could not have survived graduate school without all your positive energy.

Lastly, I want to thank by best friend and wife, Natalie. As you know, the day-to-day grind of graduate school can be overwhelming, but through all of it you have been there for me

with a smile on your face and all the love in the world. You are my better half and I am so lucky to have found you. Thank you for all that you do and continue to do for me. I love you.

Abstract

Establishment of sister chromatid cohesion is essential for cell division. In budding yeast, establishment depends on acetylation of cohesin by Eco1, an acetyltransferase whose levels are high during S phase and then decline when DNA replication is complete. Interestingly, three different kinases act sequentially to generate a phosphodegron motif that triggers Eco1 destruction after S phase. Phosphorylation by Cdk1 in early S phase primes an adjacent serine for phosphorylation by the Dbf4-Cdc7 kinase. This second phosphorylation primes a third site for phosphorylation by the GSK-3 homolog, Mck1. Together, these phosphorylation sites create a degron with the proper spacing to be recognized by the ubiquitin ligase SCF^{Cdc4}. Although all three kinases are active during S phase, Eco1 is not degraded until S phase is complete. Here, we show that the activity of Dbf4-Cdc7 toward Eco1 is delayed until after S phase. Overproduction of Dbf4 results in early Eco1 degradation, arguing that Dbf4-Cdc7 activity is normally limited to provide enough kinase activity to fire replication origins but not enough to target Eco1. The Mcm replicative helicase complex is known to be a major target of Dbf4-Cdc7, and we tested the hypothesis that the Mcm complex at licensed origins sequesters Dbf4-Cdc7, thereby preventing Eco1 phosphorylation until all origins have fired. Consistent with this hypothesis, we found that Eco1 was degraded early when we blocked Mcm loading at origins by repressing *CDC6* expression in G1. Conversely, Eco1 degradation was delayed when we repressed *CDC45* expression to maintain Mcm complexes at origins. Forcing Mcm to remain in the nucleus did not affect Eco1 degradation timing, suggesting that soluble Mcm complexes do not sequester Dbf4-Cdc7. Together, these results suggest that the Mcm complex at unfired replication origins sequesters Dbf4-Cdc7 during S phase, and only after Mcm activation is the kinase freed to

phosphorylate Eco1. This mechanism allows Eco1 to establish cohesion during S phase before it is degraded.

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Chapter 1.

Introduction

Introduction

All living organisms persist as a result of cellular division. On a macroscopic scale, one cell dividing to become two identical cells appears trivial. However, the mechanisms that control the proper timing of events during each division are incredibly complex; it is therefore essential that this process occur repeatedly, without error, and the mechanisms that control this process must be robust, full of fail-safes, and have many built-in redundancies. If errors do occur, the outcomes can be catastrophic, sometimes resulting in fatal diseases including cancer.

The cellular division cycle is divided into four parts: G1, S, G2, and M phases (Figure 1). During G1, cells determine whether they have sufficient nutrients to commit to division. S phase is when the chromosomes are duplicated to form two identical copies or sister chromatids. After replication is complete, cells enter G2. Here, cells repair errors or damage as a result of replication. M phase begins with mitosis, when the duplicated chromosomes are condensed to form highly ordered structures (prophase). Once condensed, the chromosomes are aligned on the mitotic spindle (metaphase). The chromosomes are then segregated to opposite sides of the cell (anaphase), and repackaged in separate daughter nuclei (telophase). Finally, the cell membranes are pinched off to form two daughter cells (cytokinesis). Each of these phases is incredibly complex and involves numerous mechanisms that regulate the timing of events.

The work described in Chapter 2 involves a regulation mechanism that occurs during S phase. When chromosomes are replicated to form sister chromatids, they must be held together to prevent premature separation. This is achieved by a protein complex called cohesin, a large ring structure that encircles both sister chromatids after replication. These rings must remain closed around the chromosomes until anaphase. Anaphase is triggered by the release of a protein

called separase. Separase cleaves cohesin rings, almost simultaneously, allowing sister chromatids to segregate to opposite poles of the cell. Therefore, the regulation of ring opening and closing is a key step in ensuring faithful chromosome segregation.

Eco1 (Establishment of COhesion 1) is an essential protein whose levels oscillate during the cell cycle. Its primary function is to ensure that sister chromatids do not separate before anaphase, by locking cohesin rings in the closed position around the sisters. Eco1 modifies cohesin rings by enzymatically adding an acetyl group to one subunit of the cohesin complex (Smc3). This activity must occur during S phase as the chromosomes are being duplicated. Without the acetylation of Smc3, sister chromatids will separate prematurely, which can lead to chromosome loss and ultimately cell death. Additionally, if Eco1 is present throughout M phase, cohesin rings stay closed longer, thereby disrupting the cell's ability to separate chromosomes properly. Thus, the timing for when Eco1 is present and active is a key step in the regulation of chromosome segregation.

Robust progression through the steps of the cell division cycle depends on the precisely ordered phosphorylation of hundreds of different proteins. Many of these phosphorylation events are catalyzed by the cyclin-dependent kinases (Cdks), which associate with a series of cyclin regulatory subunits to form stage-specific cyclin-Cdk complexes. The order of Cdk substrate phosphorylation, and thus the order of cell cycle events, is likely to depend on rising Cdk activity, coupled with variations in substrate affinities for different Cdk-cyclin complexes and the opposing phosphatases [1-4]

Dbf4-dependent kinase (Ddk) is a second major cell cycle kinase. Like Cdk, Ddk is an essential protein kinase composed of a catalytic subunit, Cdc7, and an activator subunit, Dbf4. Dbf4 levels, and thus Cdc7 activity, rise in late G1 and decline in anaphase due to ubiquitination

by the Anaphase-Promoting Complex/Cyclosome (APC/C^{Cdc20}) [5-7]. Ddk helps initiate DNA replication in S phase by phosphorylating the Mcm2-7 replicative helicase [8-13]. This phosphorylation depends on docking interactions between Ddk and the Mcm2-7 complex. Numerous lines of evidence suggest that Ddk docks primarily to the double-hexamer Mcm2-7 complex that is loaded at unfired origins of replication, and Ddk does not interact productively with Mcm2-7 complexes in solution or with Mcm single hexamers after origin firing [14,15]

In the budding yeast, *Saccharomyces cerevisiae*, the cohesin acetyltransferase, Eco1, is a substrate of both Cdk1 and Ddk. These phosphorylations are part of an unusually complex mechanism that promotes degradation of Eco1 via the ubiquitin ligase SCF^{Cdc4} [1-4,16,17]. To create a diphosphodegron motif that is recognized by the Cdc4 subunit of SCF^{Cdc4}, Eco1 is phosphorylated in sequence by 3 different protein kinases. First, Cdk1 phosphorylates serine 99. This primes the protein for phosphorylation at an adjacent site (S98) by Ddk, which primes Eco1 for phosphorylation at T94 by a third kinase, Mck1 (a homolog of GSK-3 in *S. cerevisiae*). Phosphorylation by Mck1 results in a diphosphorylated sequence (pT94-x-x-x-pS98) that is recognized by Cdc4, resulting in ubiquitination by SCF^{Cdc4} and destruction by the proteasome (Figure 2A) [5-7,16,17].

Eco1 is degraded in late S phase, despite the fact that all the kinases that regulate it are active at the beginning of S phase. Previous work [8-13,17] demonstrated that Cdk-dependent phosphorylation of S99 occurs in late G1 or early S phase, but subsequent phosphorylation by Ddk is delayed until late S phase. A mutation in Eco1 (ΔN97) that allows degradation in the absence of Ddk leads to its degradation in early S phase. Thus Ddk, despite being active in early S phase, does not target Eco1 until late S phase.

In Chapter 2 we explore the mechanism that delay Eco1 phosphorylation by Ddk. Our results support a model in which Ddk is sequestered during S phase by docking to Mcm complexes at unfired origins, preventing Ddk from phosphorylating Eco1 until most origins have fired and Ddk is thereby liberated. These results reveal an important mechanism in the timing and ordering of protein phosphorylation and degradation during progression through the cell cycle.

From **The Cell Cycle: Principles of Control** by David O Morgan

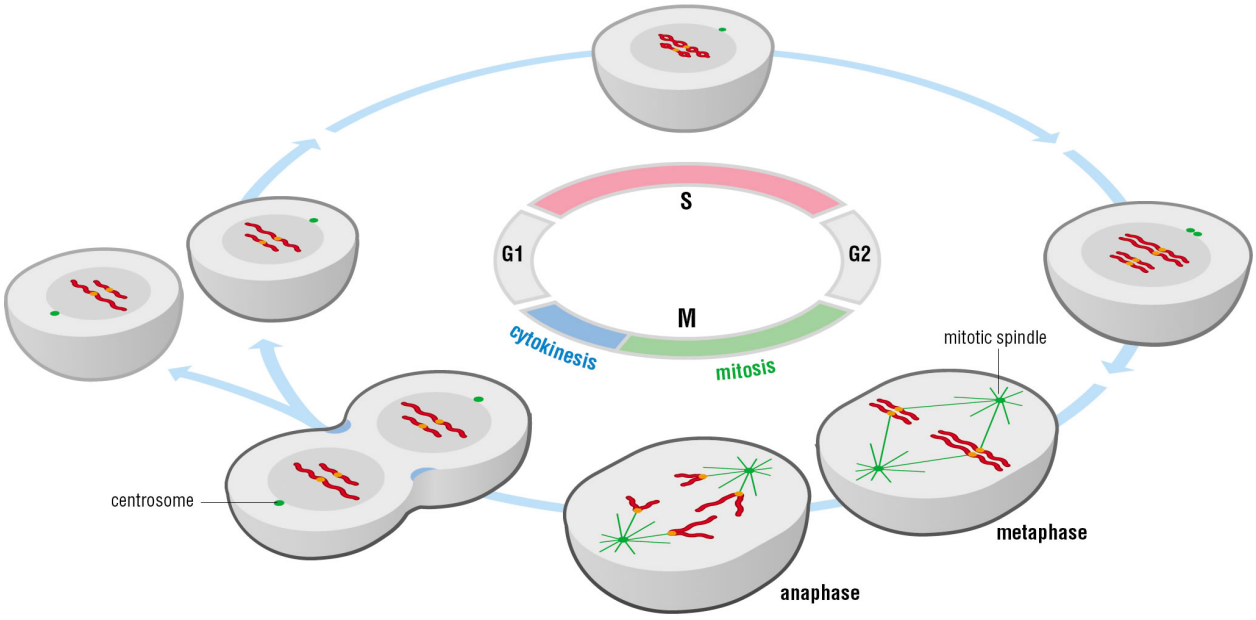


Figure 1. The cellular division cycle. The cell cycle begins in late G1, when cells commit to division if sufficient nutrients are present. S phase is when chromosomes are duplicated. M phase (mitosis and cytokinesis) completes the duplication of cellular organelles, segregates the duplicated chromosomes, and finally divides the cell into two daughter cells.

Chapter 2.

Completion of S phase frees Dbf4-Cdc7 to target Eco1 for destruction

Summary

Robust progression through the cell-division cycle depends on the precisely ordered phosphorylation of hundreds of different proteins by cyclin-dependent kinases (Cdks) and other kinases. The order of Cdk substrate phosphorylation depends on rising Cdk activity, coupled with variations in substrate affinities for different Cdk-cyclin complexes and the opposing phosphatases [1-4,14,15]. Here we address the ordering of substrate phosphorylation by a second major cell-cycle kinase, Cdc7-Dbf4 or Dbf4-dependent kinase (Ddk). The primary function of Ddk is to initiate DNA replication by phosphorylating the Mcm2-7 replicative helicase [12,13,18]. Ddk also phosphorylates the cohesin acetyltransferase, Eco1 [17]. Sequential phosphorylations of Eco1 by Cdk, Ddk, and Mck1 create a phosphodegron that is recognized by the ubiquitin ligase SCF^{Cdc4}. Ddk, despite being activated in early S phase, does not phosphorylate Eco1 to trigger its degradation until late S phase [17]. Ddk associates with docking sites on loaded Mcm double hexamers at unfired replication origins [14,15]. We hypothesized that these docking interactions sequester limiting amounts of Ddk, delaying Eco1 phosphorylation by Ddk until replication is complete. Consistent with this hypothesis, we find that overproduction of Ddk leads to premature Eco1 degradation. Eco1 degradation also occurs prematurely if Mcm complex loading at origins is prevented by depletion of Cdc6, and Eco1 is stabilized if loaded Mcm complexes are prevented from firing by a Cdc45 mutant. We propose that the timing of Eco1 phosphorylation, and potentially that of other Ddk substrates, is determined in part by sequestration of Ddk at unfired replication origins during S phase.

Results

Eco1 degradation coincides with the rise in Dbf4 levels

In the budding yeast, *Saccharomyces cerevisiae*, Eco1 is regulated by an unusually complex mechanism that promotes Eco1 degradation via the ubiquitin ligase SCF^{Cdc4} [16,17]. To create a diphosphodegron motif that is recognized by the Cdc4 subunit of SCF^{Cdc4}, Eco1 is phosphorylated in sequence by 3 different protein kinases. First, Cdk1 phosphorylates serine 99. This primes the protein for phosphorylation at an adjacent site (S98) by Ddk, which primes Eco1 for phosphorylation at T94 by a third kinase, Mck1 (a homolog of GSK-3 in *S. cerevisiae*). Phosphorylation by Mck1 results in a diphosphorylated sequence (pT94-x-x-x-pS98) that is recognized by Cdc4, resulting in ubiquitination by SCF^{Cdc4} and destruction by the proteasome (Figure 1A) [16,17].

Eco1 is degraded in late S phase, despite the fact that all the kinases that regulate it are active at the beginning of S phase. Previous work [17] demonstrated that Cdk-dependent phosphorylation of S99 occurs in late G1 or early S phase, but subsequent phosphorylation by Ddk is delayed until late S phase. A mutation in Eco1 (ΔN97) that allows degradation in the absence of Ddk leads to its degradation in early S phase. Thus Ddk, despite being active in early S phase, does not target Eco1 until late S phase.

In our previous work [16,17], the timing of Eco1 degradation in the cell cycle was estimated by western blotting of lysates from cells released from a G1 arrest. Here, we extended this analysis to determine the timing of Eco1 degradation relative to that of the Ddk activator, Dbf4. A yeast strain expressing Eco1-TAP and Dbf4-6HA at endogenous loci was arrested in G1 with the mating pheromone α-factor and then released (Figure 1B). As seen previously, Eco1 levels rose as the cell entered the cell cycle (as indicated by budding) and peaked about 30

minutes after release, after which its levels declined gradually before rising again after mitosis. Df4 levels began to rise at cell-cycle entry and peaked after 60 minutes.

To more precisely determine the timing of Eco1 degradation, we used a single-cell method to monitor Eco1 levels during a normal cell cycle. For comparison, we analyzed Dbf4 levels as in our previous work [7]. Eco1 or Dbf4 were each tagged at the endogenous locus with a C-terminal eGFP. Analysis of eGFP-tagged proteins provides an accurate measure of the timing of degradation; however, because eGFP requires 15-30 minutes to fold, the rise in fluorescence signal from these proteins is delayed relative to the synthesis of the protein [19,20]. A spindle pole body (SPB) component, Spc42, was tagged in these strains with a C-terminal mCherry. SPB behavior provides a way to track the timing of events during the cell cycle: the SPB is duplicated in early S phase, and the two SPBs separate to initiate spindle assembly in early mitosis. The SPBs then separate further as the spindle elongates at anaphase, which coincides with segregation of sister chromatids [7,21-23]. Initial SPB separation occurs at about the time that S phase is completed [24].

Our single-cell analysis revealed that Eco1-GFP reached peak levels about 10 minutes before SPB separation and began to decline at the same time as SPB separation (Figure 1C and 1D). Thus, the onset of Eco1 degradation coincides with the completion of S phase and initiation of mitosis. As seen in our previous work [7], Dbf4-GFP levels peaked after SPB separation and then declined rapidly 30 minutes after SPB separation (Figure 1C and 1D), due to its APC/C^{Cdc20}-mediated degradation. Thus, Eco1 levels declined at roughly the time that Dbf4 reached maximal levels. Note that in the single-cell assay, Eco1 levels dropped gradually relative to the steep decline of the APC/C substrate Dbf4.

High levels of Dbf4 cause Ddk to target Eco1 more efficiently

The delay in Eco1 degradation until late S phase could be due to a combination of factors: Dbf4 levels are initially low in early S phase, and Ddk could be sequestered by the more abundant Mcm double hexamers loaded at licensed origins. To directly test whether Dbf4 is indeed limiting during S phase, we analyzed Eco1 degradation timing in cells overexpressing a stabilized Dbf4 mutant lacking its amino-terminal APC/C recognition sequences (ΔN2-64) [25].

We expressed *3HA-ΔN-DBF4* from the galactose-inducible *GAL1* promoter and analyzed Eco1 levels in cells released from a G1 arrest in galactose-containing medium. In wild-type cells, growth in galactose reduced the amount of Eco1 in G1-arrested cells, and also delayed cell cycle entry, such that Eco1 levels peaked 40-50 minutes after release (Figure 2, top panel). In cells with overexpressed Dbf4, Eco1 levels were very low and peaked about 30 minutes after release from G1, at about the time of early S phase (Figure 2, bottom panel). Overall, the levels of Eco1 were significantly reduced throughout the cell cycle. These results suggest that phosphorylation of Eco1 is normally restrained by limiting Dbf4 levels in S phase.

Eco1 degradation is not dependent on nuclear export of Mcms or disassembly of the replication complex

Although Dbf4 is limiting for Eco1 phosphorylation during early S phase, there is clearly sufficient kinase activity to target Mcm2-7 complexes at early replication origins. A potential explanation is that the small amount of Ddk is docked on loaded Mcm double hexamers at replication origins, thereby preventing Ddk from phosphorylating Eco1. It is also possible that Ddk interacts with Mcm2-7 complexes that exist in solution. We tested this possibility by

measuring the timing of Eco1 degradation in cells with abnormally high levels of Mcm2-7 complexes in the nucleus.

Soluble Mcm2-7 complexes are gradually exported from the nucleus during S phase, greatly reducing their nuclear concentration. To artificially maintain Mcm2-7 complex levels in the nucleus, we used a previously described Mcm2 subunit carrying two extra nuclear localization sequences in its C-terminal region. This mutation makes all Mcm proteins constitutively nuclear throughout S phase [26]. We used fluorescence microscopy to track single cells (as in Figure 1C, D) containing Eco1-GFP with or without the Mcm2-2xNLS mutation. We also analyzed an Mcm2-GFP-2xNLS-expressing strain to verify that this mutant protein remains in the nucleus throughout the cell cycle (data not shown). The timing of Eco1 degradation was not affected by high levels of Mcm2-7 complexes in the nucleus, arguing that high levels of soluble Mcm2-7 complexes do not prevent Ddk from targeting Eco1 (Figure 3A).

We next tested whether Mcm2-7 single hexamers on DNA sequester Ddk activity and thereby delay Eco1 phosphorylation. We depleted cells of the F-box protein Dia2, which is required for the disassembly of the replication fork CMG (Cdc45-Mcm-GINS) complex when replication is completed [27,28]. In the absence of Dia2, Mcm2-7 single hexamers and other CMG components remain associated with chromatin. We found that depletion of Dia2 had no effect on Eco1 levels or degradation timing (Figure 3B). These results suggest that Ddk is not sequestered away from Eco1 by interactions with soluble Mcm2-7 or with single hexamers at the replication fork.

Preventing Mcm2-7 loading allows premature Eco1 degradation

Numerous lines of evidence suggest that Ddk docks primarily to the double-hexamer Mcm2-7 complex that is loaded at unfired origins of replication, and Ddk does not interact productively with Mcm2-7 complexes in solution or with Mcm single hexamers after origin firing [14,15]. Further evidence [8,14,29-33] supports the idea that Ddk is targeted to licensed origins by interacting with a compound docking site that is formed by the double Mcm2-7 hexamer. Since the majority of origins in yeast do not fire until the middle of S phase [34], we hypothesized that Eco1 is not phosphorylated until late S phase because Ddk is restrained before then by its docking interactions at origins. We tested this hypothesis by disrupting the timing of Mcm complex loading and origin firing.

The protein Cdc6 collaborates with the Origin Recognition Complex (ORC) during G1 to load Mcm complexes on origin DNA [35-37]. In the absence of Cdc6, Mcm complexes are not loaded, and the cell enters the cell cycle but fails to initiate DNA replication. We reasoned that the absence of loaded Mcm complexes would prevent sequestration of Ddk, allowing it to phosphorylate Eco1 and thereby trigger premature Eco1 degradation. We depleted yeast cells of Cdc6 by placing *CDC6* under the control of the *GAL1* promoter and shutting off transcription by growth in glucose medium prior to G1 arrest [38]. Strikingly, in cells depleted of Cdc6, Eco1 levels remained very low and peaked early, 20 minutes after G1 release (Figure 4A). Thus, when the Mcm complex is not loaded at replication origins, Ddk is able to target Eco1 upon entry into S phase.

We devised a complementary experiment in a strain in which the Mcm complex is loaded but held in the inactive double-hexamer form at origins when the cell enters S phase. If these complexes sequester Ddk away from Eco1, then preventing Mcm activation should block Eco1 degradation. For this purpose, we depleted cells of Cdc45, which is required for the recruitment

of Sld3, GINS, and ultimately for the firing of origins [39-42]. *CDC45* was placed under the control of the *GAL1* promoter, and its expression was repressed by growth in glucose medium prior to G1 arrest. Upon release from G1 in cells lacking Cdc45, Eco1 rose to abnormally high levels, and its degradation was greatly delayed (Figure 4B). These results are consistent with our hypothesis that in wild-type cells, Ddk is sequestered during S phase by unfired origins and is therefore unable to target Eco1.

Discussion

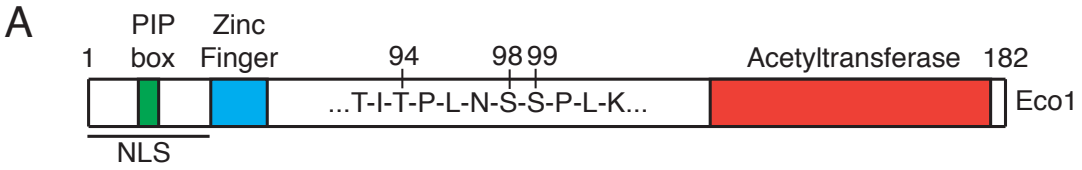
For a cell to divide successfully, a series of carefully orchestrated events must occur at the correct time and in the correct sequence. The timing of cell-cycle events depends in large part on the timing of phosphorylation of numerous proteins that govern those events. Here we provide evidence for a mechanism by which substrate competition determines the unusual late S-phase timing of Eco1 phosphorylation by Ddk. Our results argue that sequestration of Ddk by unfired replication origins limits Ddk activity toward Eco1 until late S phase.

The essential function of Eco1 is to acetylate the cohesin subunit Smc3 during S phase to ensure proper sister-chromatid cohesion [43,44]. Premature Eco1 degradation can cause lethal defects in chromosome segregation, whereas stabilization of Eco1 beyond S phase leads to levels of cohesion that can delay anaphase progression [16]. Thus, there is an optimal S-phase window during which Eco1 must act. However, because Eco1 degradation is triggered by the same kinase that triggers DNA replication, achieving the optimal window of Eco1 function requires a mechanism to keep the kinase away from Eco1 until replication is complete. Our results suggest a likely mechanism.

This mechanism depends on quantitative features of the system. There are an estimated 700 origins of replication in *S. cerevisiae* [34,45-49], resulting in several hundred loaded double Mcm hexamers in early S phase. Recent global studies suggest that there are roughly 25 molecules/cell of Dbf4 and 66 molecules/cell of Eco1 [50]. Western blotting also suggests that Dbf4 amounts are far lower than the number of origins [51]. Thus, Mcm double hexamers greatly outnumber Ddk complexes. In addition, docking interactions between Ddk and the Mcm double hexamer enhance binding and kinase activity [31,33]. Considering these factors, it seems likely that Dbf4-Cdc7 is occupied primarily with origin Mcm complexes during S phase. Once S phase nears completion and the number of loaded Mcm double hexamers declines, rising levels of Dbf4 (and Ddk activity) would then be free to target Eco1.

Eco1 is thought to interact with the clamp loader PCNA at the replication fork [52]. It is conceivable that this interaction, which depends on a PCNA-Interacting-Protein box (PIP box), places Eco1 within range of Ddk during early S phase. However, we found that mutation of the PIP box does not affect the timing of Eco1 degradation (data not shown).

Our evidence supports a novel mechanism for timing phosphorylation events at the end of S phase. This cell cycle transition is not governed by a major regulatory switch like those that trigger progression through Start, the G2/M transition, or the metaphase-anaphase transition. This mechanism might provide a way to time the phosphorylation of other Ddk targets involved in later cell-cycle functions. There is evidence that Ddk activity helps control other cell-cycle processes, including homologous recombination in meiosis [53-55] and rDNA segregation in mitosis [25]. We speculate that the timing of these and other Ddk-dependent processes might be governed, at least in part, by the mechanisms we describe.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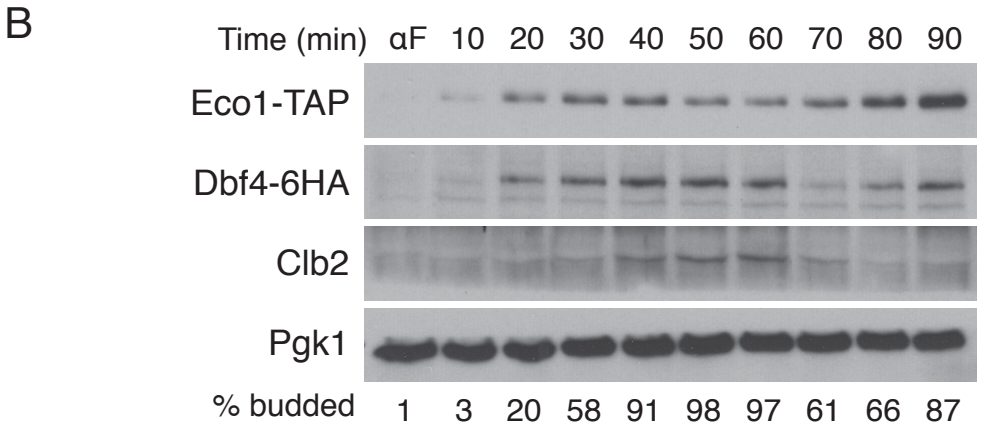
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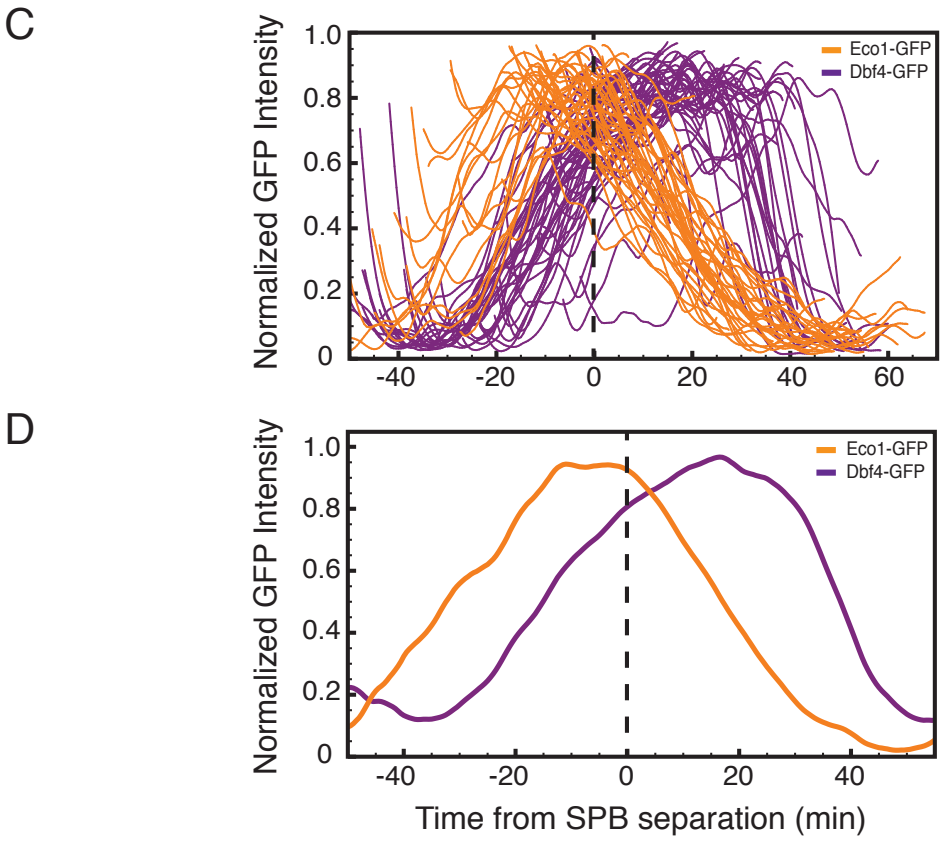
C
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D
Normalized GFP Intensity
1.0
0.8
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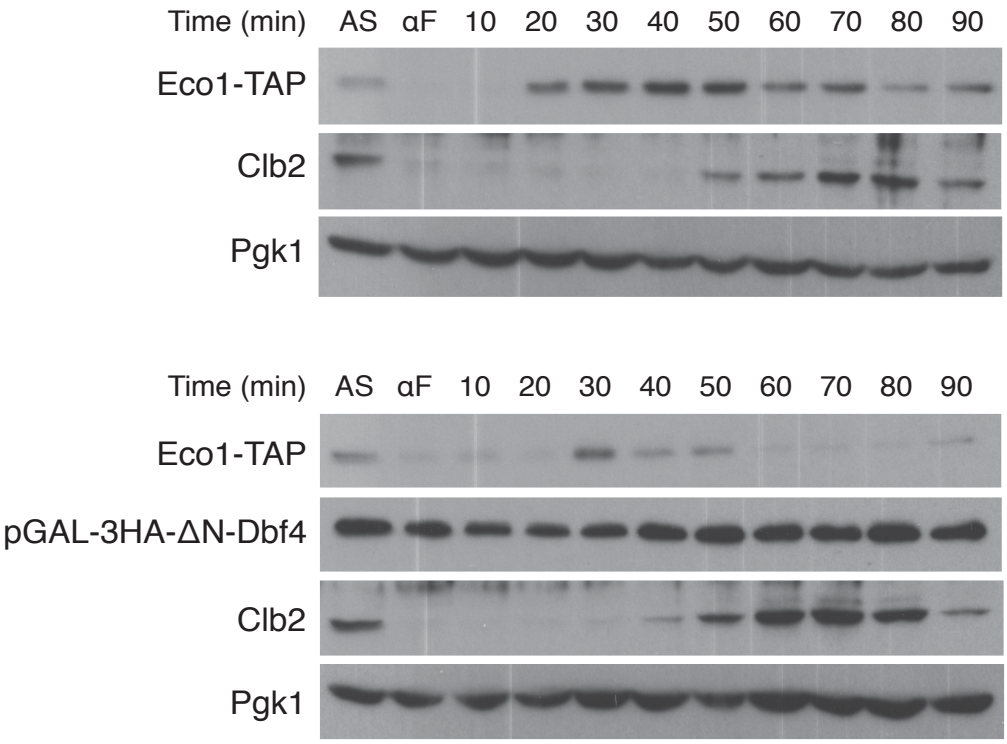
0.4
0.2
0
-40
-20
0
20
40
Time from SPB separation (min)
Eco1-GFP
Dbf4-GFP

Figure 2. Oscillations of Eco1 and Dbf4 during the cell cycle. (A) Diagram of Eco1 primary structure, depicting the three phosphorylation sites that govern Eco1 degradation. (B) Cells carrying epitope-tagged Eco1 and Dbf4 were arrested in G1 with alpha factor and released. At the indicated times after release, budding index was determined and cell lysates were analyzed by western blotting. Clb2 was used as a cell cycle marker and Pgk1 was used as a loading control. (C) Asynchronous cells, expressing either Eco1-GFP or Dbf4-GFP, were analyzed by spinning disk confocal microscopy. Images were taken every 30 seconds for 70 minutes. Maximum intensity projections were used to create single cell traces of Eco1-GFP or Dbf4-GFP, aligned in silico to SPB separation. Fluorescence signals were normalized to maximum intensity. Before normalization, peak intensity of Dbf4-GFP was 2.5 fold greater than that of Eco1-GFP. (D) Averaged traces of the single-cell data in (C).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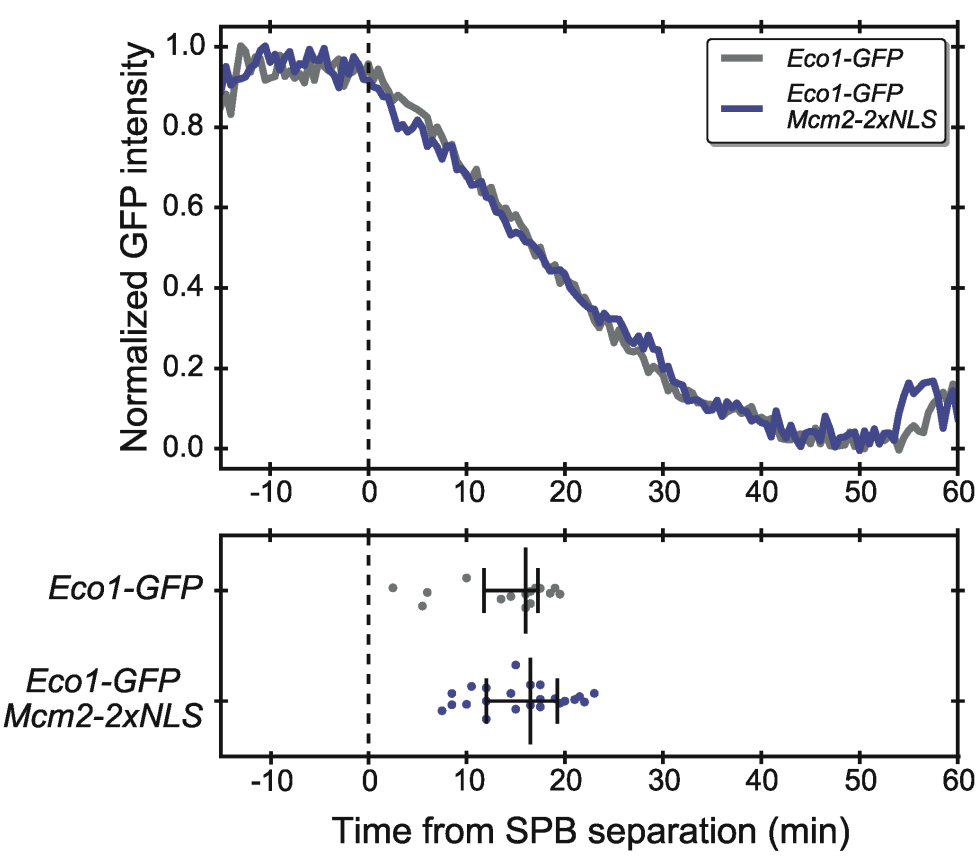
Eco1-TAP
Clb2
Pgk1
Time (min) AS αF 10 20 30 40 50 60 70 80 90
Eco1-TAP
pGAL-3HA-ΔN-Dbf4
Clb2
Pgk1

Figure 3. Overexpression of Dbf4 causes premature Eco1 degradation. (Top) A wild-type strain containing Eco1-TAP was grown in galactose-containing medium and arrested in G1 with alpha factor. Following release from G1, cells were sampled at the indicated times for analysis by western blotting. (Bottom) A strain containing *ΔN-DBF4* under the control of the *GAL1* promoter was released from a G1 arrest in galactose medium, and cells were sampled at the indicated times for analysis by western blotting. Clb2 was used as a cell cycle marker and Pgk1 was used as a loading control. This experiment is representative of results from 3 separate experiments.

A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60
Eco1-GFP
Eco1-GFP
Mcm2-2xNLS
Time from SPB separation (min)

B

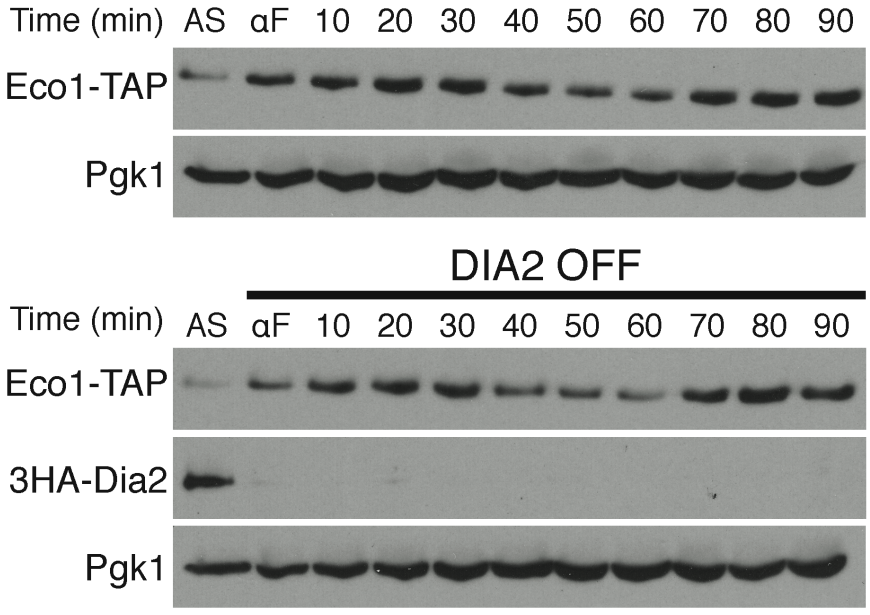
Time (min) AS αF 10 20 30 40 50 60 70 80 90
Eco1-TAP
Pgk1
DIA2 OFF
Time (min) AS αF 10 20 30 40 50 60 70 80 90
Eco1-TAP
3HA-Dia2
Pgk1

Figure 4. Increased Mcm complex in the nucleus or depletion of Dia2 has no impact on Eco1 degradation. (A) Strains containing Eco1-GFP or Eco1-GFP in an Mcm2-2xNLS background were grown under normal conditions and visualized with a spinning disk confocal microscope as in Figure 1C, D. (Top) Single cell traces were averaged, normalized, and aligned to SPB separation. (Bottom) Each dot represents the time at which the Eco1-GFP signal was reduced by 50% in a single cell. The central bar indicates the median, and error bars indicate the 25^{th} and 75^{th} percentiles. (B) (Top) A wild-type strain containing Eco1-TAP was grown in galactose medium, arrested with alpha factor and switched to dextrose medium before release from G1. Cells were sampled at the indicated times after release for analysis by western blotting. (Bottom) Cells containing Eco1-TAP and *DIA2* under the control of the *GAL1* promoter were arrested in alpha factor and switched to dextrose medium before release from G1. Switching media to YEP^{DEX} shut off Dia2 expression. Clb2 was used as a cell cycle marker and Pgk1 was used as a loading control. This experiment is representative of results from 3 separate experiments.

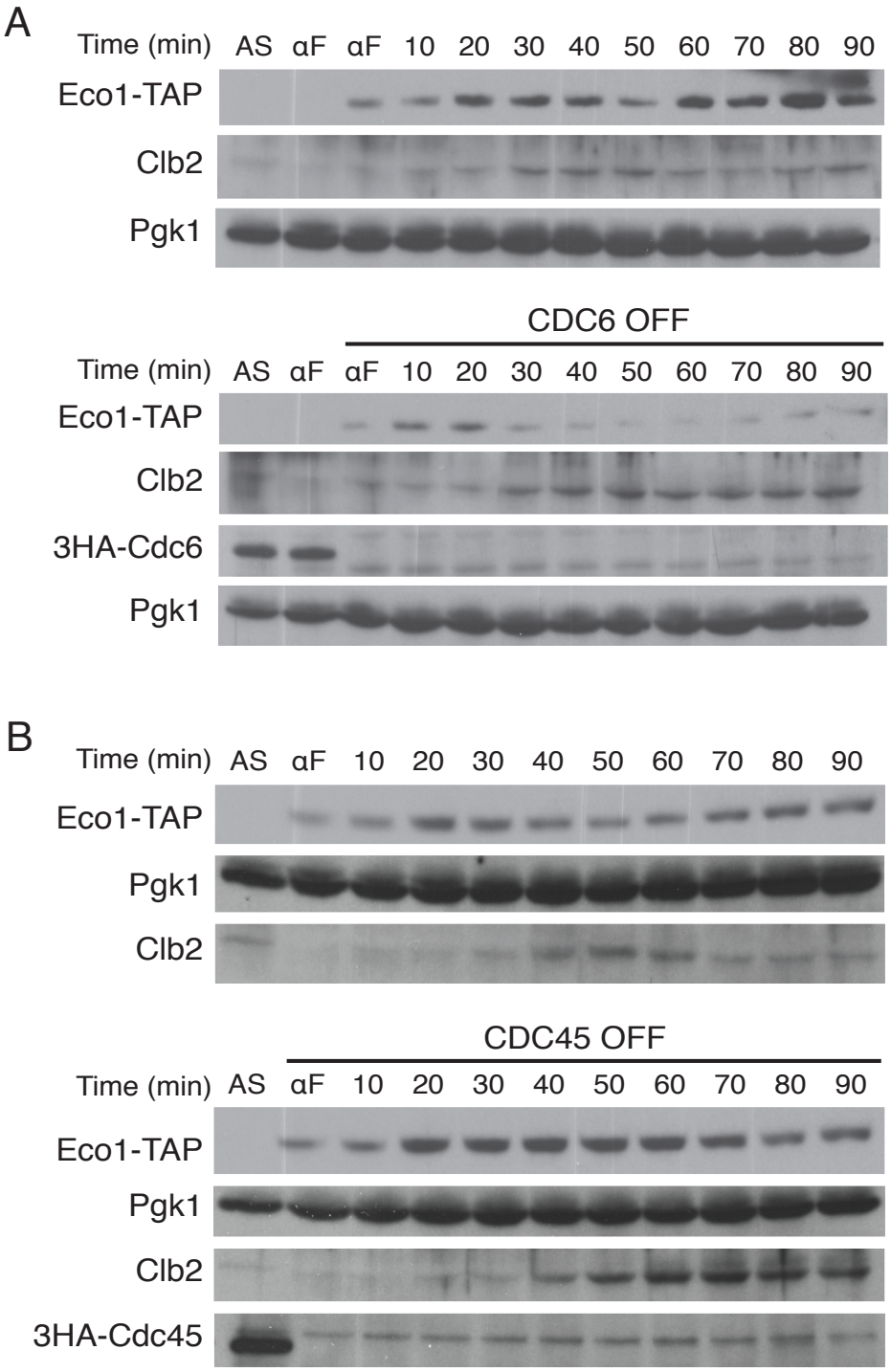
A
Time (min) AS αF αF 10 20 30 40 50 60 70 80 90
Eco1-TAP
Clb2
Pgk1
CDC6 OFF
Time (min) AS αF αF 10 20 30 40 50 60 70 80 90
Eco1-TAP
Clb2
3HA-Cdc6
Pgk1
B
Time (min) AS αF 10 20 30 40 50 60 70 80 90
Eco1-TAP
Pgk1
Clb2
CDC45 OFF
Time (min) AS αF 10 20 30 40 50 60 70 80 90
Eco1-TAP
Pgk1
Clb2
3HA-Cdc45

Figure 5. Defects in Mcm complex loading or activation alter the timing of Eco1 degradation. (A) (Top) A wild-type strain containing Eco1-TAP was arrested with alpha factor in galactose medium, released for 40 min and then rearrested with alpha factor. Following release, cells were sampled at the indicated times for analysis by western blotting. (Bottom) Cells containing *CDC6* under the control of the *GAL1* promoter were treated and analyzed as in the top panel. Clb2 was used as a cell cycle marker and Pgk1 was used as a loading control. (B) (Top) A wild-type strain containing Eco1-TAP was grown in galactose medium, arrested with alpha factor and switched to a dextrose medium. Cells were released and sampled at the indicated times for analysis by western blotting. (Bottom) Cells containing *CDC45* under the control of the *GAL1* promoter were treated and analyzed as in the top panel. Clb2 was used as a cell cycle marker and Pgk1 was used as a loading control. This experiment is representative of results from 3 separate experiments.

Chapter 3.

Conclusions

Conclusions

Cellular division is a fundamental part of all living organisms. Over time cells have evolved elaborate and extensive mechanisms to ensure that events occur at the correct time and in the correct sequence. Failure to correct mistakes or deviation from a cells division program can have catastrophic consequences. Thus, producing identical cells without error is not only a monumental task but also paramount for the survival of all living species.

At the present time, much is still unknown about all the mechanisms that control cell division. To understand many of the fundamental processes required for division we used the budding yeast *Saccharomyces cerevisiae*. These single celled eukaryotes are tractable for several reasons: their division times are 90 minutes, are easily cultured, and can be genetically manipulated in a timely and efficient manner. Furthermore, budding yeast are a fantastic organism to apply the scientific method. You can make observations, propose questions, formulate hypotheses, and test them all in the span of a few years.

In this thesis I have identified a unique mechanism by which the cell cycle kinase DDK targets different substrates in an ordered and timely manner. DDK is essential and is required to initiate DNA replication. It phosphorylates the helicase (MCM complex) responsible for unwinding the DNA strands. This activity occurs throughout S phase leading to the sequential firing of origins of replication. While the DNA is being replicated, Eco1 is acetylating the cohesin complexes to keep them locked around the replicated chromosomes. Eco1's activity is required to prevent sister chromosomes from separating before anaphase. Previous work from our lab [17] demonstrated that DDK phosphorylates Eco1. Interestingly, it was shown that Eco1 phosphorylation by DDK was delayed until late S phase. Furthermore, this delay is required in

order to give Eco1 sufficient time to acetylate cohesin. The focus of this work was to understand the mechanism responsible for this delay.

Previous work from our lab [7] used a single cell tracking method to demonstrate that the DDK activator Dbf4 oscillated during the cell cycle. We decided to extend this technique to include Eco1 with the goal of understanding how Dbf4 and Eco1 levels fluctuate relative to each other. We discovered that Eco1 levels peaks 20 minutes before Dbf4. Curiously, Eco1 levels don't drop until Dbf4 reach maximum levels. This lead us to wonder where Dbf4 is limiting early on in S phase. Perhaps low levels of Dbf4 are sufficient to fire origins but cannot target Eco1 until later when Dbf4 levels are high. We tested this by overexpressing a non-degradable form of Dbf4 and looked at Eco1 turnover. Sure enough, increasing Dbf4 levels led to Eco1 being degraded earlier and more efficiently.

We next wondered if Dbf4 could be sequestered away from Eco1 during S phase. Previous evidence [8,14,29-31,33,56], supported the idea that Ddk interacts and binds unfired DNA replication origins. We attempted to demonstrate this using the awesome power of yeast genetics. First we depleted cells of a protein called Cdc6. This protein is essential and is required for loading of Mcm complex. With no Cdc6 present the complex cannot bind to DNA potentially freeing DDK to target Eco1. When we depleted cells of Cdc6 we saw Eco1 levels drop immediately upon entry into S phase and continue to stay low throughout that cell cycle. Next we depleted cells of Cdc45. Cdc45 is essential and is required for origins to fire. With no Cdc45 present Mmc complex loaded at origins should be stuck there and sequester any DDK. When we depleted cells of Cdc45 we saw that Eco1 degradation was delayed and not degraded. Combined these results point to DDK being sequestered by the Mcm complex loaded at unfired origins.

Lastly, we tested whether Mcm complex itself is capable of sequestering DDK. We envisioned two different scenarios: one where Mcm complex is kept on DNA post replication and the other increasing levels of free MCM in the nucleus. The first scenario we tested by depleting cells of Dia2. Dia2 is required for the disassembly of Mcm complex from DNA at the end of S phase. Without Dia2 Mcm complex would be stuck on DNA at non-origin sites for prolonged periods. When we depleted cells of Dia2 we saw no change in Eco1 degradation. This indicated that only Mcm complex loaded at unfired origins is capable of sequestering DDK. To test the last scenario we used a mutant of Mcm2 containing two copies of an NLS (nuclear localization sequence). This mutant prevents the Mcm complex from being exported out of the nucleus at the end of S phase. Using our single cell tracking method we discovered that maintaining Mcm complex in the nucleus throughout the cell cycle had no effect on when Eco1 was degraded. Together they results futher support the idea that Mcm complex sequesters DDK only when loaded at unfired origins.

All the results from this work paint the following picture: early in S phase low levels of Dbf4 activate sufficient DDK to phosphorylate the Mcm complex. This activity is required for origin firing. Meanwhile, Eco1 is busy acetylating cohesin to ensure that newly replicated sister chromatids stay together. If Eco1 is degraded early it has catastrophic effects on the cell cycle. Additionally, if it is around too long you get too much cohesion. So the window during which Eco1 must be active is critical.

As cells progress through S phase less and less Mcm complex remains at unfired origins. Meanwhile, Dbf4 levels are steadily rising increasing DDK activity. By the time S phase is completed, DDKs favorite substrate is off DNA and out of the nucleus. This leaves it free to target Eco1 upon completion of S phase.

How one kinase can target multiple substrates at different times during the cell cycle is incredibly interesting. We see it with Cdk1 and how it can target different substrates in a coordinated fashion [7]. Having a second cell cycle kinase use this mechanism opens several interesting possibilities. The first is whether other substrates of DDK that are targeted in this coordinated fashion. Currently, outside of the Mcm complex and Eco1 nothing is known about other Ddk targets during mitosis. Large-scale screens looking for targets of Ddk would allow us to determine whether other substrates follow this mechanism. . Lastly there is evidence that Ddk activity helps control other cell-cycle processes, including homologous recombination in meiosis [53-55] and rDNA segregation in mitosis [25]. We speculate that the timing of these and other Ddk-dependent processes might be governed, at least in part, by the mechanisms we describe.

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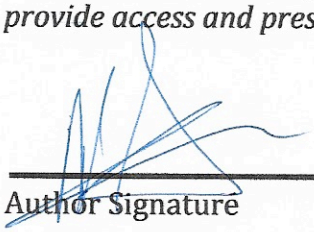

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