Investigation on the mechanistic roles of Tom1 and Dia2 in DNA replication and Cell cycle

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Dong-Hwan Kim

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Deanna M. Koepp, Ph.D.
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ABSTRACT

As cells duplicate and divide, the inheritance of accurate genetic information is critical to maintaining cell viability and preventing the generation of cancer-causing mutations. Thus, DNA replication is important and limited to once per cell cycle. The replication protein Cdc6 is required to assemble pre-replicative (pre-RC) complexes on origins of replication and is degraded after it has completed its function. The mechanisms of Cdc6 degradation in budding yeast are complex and not completely understood. I have identified a novel pathway that targets Cdc6 for degradation during G1 and at the G1 to S phase transition. The Hect E3 ligase Tom1 and the F-box protein Dia2 function co-operatively but independently of the SCF$^{\text{Cdc4}}$ complex. When this pathway is disrupted, the assembly of Cdc6 and Mcm4 on chromatin and replication origins is aberrant. Strikingly, defects in Tom1 or Cdc4 lead to increased DNA content in cells sensitized to re-replication. Therefore, multiple degradation pathways that limit Cdc6 protein levels cooperate to regulate pre-RC assembly and may act to prevent DNA re-replication.

Preserving genomic stability is crucial to cell viability and proliferation. The budding yeast F-box protein Dia2 is required for genomic stability and is targeted for ubiquitin-dependent degradation in a cell cycle-dependent manner, but the identity of the ubiquitination pathway is unknown. I have uncovered that the Hect-domain E3 ligase Tom1 targets Dia2 for ubiquitin-dependent degradation during G1 and G2/M. Tom1 binding to Dia2 is switched during the cell cycle. Tom1 recognizes specific positively charged residues in a Dia2 degradation/NLS domain. Defects in Dia2 proteolysis such as the loss of positively charged residues and deletion of TOM1 cause a delay in G1 to S-phase progression. Together, these results suggest that Tom1 is required for degradation of Dia2 during the cell cycle and that Dia2 protein degradation contributes to G1 to S-phase progression.
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CHAPTER II: Cdc6 degradation by a Tom1 and Dia2-dependent pathway regulates pre-RC assembly during the cell cycle

Dong-Hwan Kim: performed all experiments and generated figures and tables.
Wei Zhang: generated Cdc6, ΔN214 Dia2, and TPR Dia2 baculoviruses.
Deanna Koepp: generated fractionated yeast extracts

CHAPTER III: The Hect E3 ubiquitin ligase Tom1 controls Dia2 degradation during the cell cycle

Dong-Hwan Kim: performed all experiments and generated figures and tables.
CHAPTER I
INTRODUCTION

1. Ubiquitin-Proteasome System (UPS)

Ubiquitination

Ubiquitination plays a role in a variety of cellular processes including proliferation, cell cycle, signal transduction, transcription, DNA damage, and DNA repair (38, 41, 50, 65, 85). Ubiquitin is a small protein with 76 amino acids. Ubiquitination is a process in which the C-terminal glycine of ubiquitin is covalently attached onto a lysine of a substrate protein through a cascade of enzymatic reactions. Moreover, ubiquitins are themselves covalently linked to each other through one of seven lysines found on ubiquitin, leading to a chain. Ubiquitination determines the fate of substrate proteins, depending on the number of ubiquitin molecules or the type of ubiquitin-linked chains (49). Polyubiquitination (at least four ubiquitin molecules linked through the lysine 48) targets a protein for degradation via the 26S proteasome, whereas monoubiquitination or polyubiquitination through the lysine 63 or other lysines alters protein interaction, protein localization, intracellular trafficking, signal transduction, and DNA repair (38, 39, 41, 87).

Ubiquitination is a series of enzymatic reactions consisting of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3) (77, 78). Ubiquitin is activated by an E1 enzyme through a thioester bond in an ATP-dependent manner and transferred to an E2 enzyme. When the E2 binds to an E3 ubiquitin ligase,
ubiquitin is directly or indirectly attached onto a lysine of a substrate, which further targets the substrate for degradation via the 26S proteasome (Figure 1). The substrate can be monoubiquitinated and dissociated from the E3 ligase or polyubiquitinated after multiple rounds of these reactions. The human genome encodes 2 potential E1s, about 30 E2s, and more than 600 E3s (78), suggesting that an enormous number of substrates are recognized and targeted by those E2 and E3 enzymes.

**RING domain E3 ubiquitin ligases**

There are two major groups of E3 ubiquitin ligases - the RING domain E3 ligases and the Hect domain E3 ligases. RING domain E3 ligase family constitutes a large proportion of E3 ligases found in humans. RING E3s are either a single polypeptide or multi-subunit complexes. These RING E3 ligases contain a ring domain characterized by a Cys-X2-Cys-X(9-39)-Cys-X(1-3)-His-X(2-3)-Cys-X2-Cys-X(4-48)-Cys-X2-Cys (where X is any amino acid) sequence (16). The SCF (Skp1/Cdc53/F-box proteins) complexes are modular E3 ubiquitin ligases and their structures have been extensively studied as a multi-subunit RING domain E3. The SCF complexes are also highly conserved from yeast to humans (16). Each component of the SCF complex has a specific role in ubiquitination of a substrate. For example, an F-box protein acts as an adaptor and binds to a substrate. There are approximately 70 different F-box proteins in humans and 20 F-box proteins in yeast (11, 98). In addition, Skp1 interacts with an F-box protein and tethers it to the rest of an SCF complex. Cul1, also known as Cdc53, is a scaffold protein and functions as a landing pad for the formation of an SCF complex. Rbx1 (Roc1) is a
RING domain protein and recruits an E2 enzyme charged with ubiquitin. When a substrate is bound to an SCF complex, the SCF complex brings the substrate in close proximity to the E2-ubiquitin and ubiquitin is directly transferred from E2 to the substrate (Figure 2).

**Hect domain E3 ubiquitin ligases**

The other type of E3 ligases is Hect (homologous to E6-AP carboxyTerminus) E3 ligases. Hect family E3 is a single polypeptide protein and contains a Hect domain of 350 amino acids found at the C-terminus of the protein. The Hect domain was first described from human papilloma virus (HPV) E6-associated protein (E6AP) (81). The Hect E3s contain at least one or more protein-protein interaction domains located at the N-terminus of the proteins. However, the important function of the E3s such as E2 binding and ubiquitin transfer resides in the Hect domain. Unlike the SCF complexes described above, the Hect domain E3s have a distinct mechanism in that ubiquitin is transiently transferred from E2 to a catalytic cysteine of the protein and subsequently attached to a lysine of a substrate (Figure 3).

How this function is regulated is an emerging research topic. Several reports suggest that an intramolecular interaction between protein-protein interaction domains located at the N-terminus of the protein prevents the Hect E3s from being autoubiquitinated. Moreover, the binding of an adaptor protein to the Hect E3s via the protein interaction domains at the N-terminus alters the localization of the Hect E3s (81). The detailed
mechanisms by which the activity or function of the Hect E3s is regulated remain to be understood.

26S proteasome

The 26S proteasome is the core of proteolytic machinery in which ubiquitinated proteins are degraded. The 26S proteasome that is highly conserved in eukaryotes consists of the 20S core particle and the 19S regulatory particle (46, 63).

The 20S core particle is a barrel-shaped cylindrical structure that contains two identical inner β-rings consisting of seven β-subunits and two outer α-rings carrying seven α-subunits. The protease sites are located in the surface of the inner rings (46, 63). Thus, ubiquitinated proteins should enter the core particle to be degraded by the proteases.

By contrast, the 19S regulatory particle is divided into lid and base complexes. The base is composed of six different ATPase subunits and a few non-ATPase subunits, whereas the lid contains more than 10 Rpn subunits (46, 63). The role of the 19S regulatory particle is to recognize and guide ubiquitinated proteins to the 20S core particle of the 26S proteasome. When a ubiquitinated protein binds to the regulatory subunit, it is deubiquitinated, unfolded, and translocated into the core particle where it is degraded by the action of proteases (46, 63).

2. Cell cycle
Cells divide and undergo the cell cycle in response to external stimuli including nutrients, growth factors, and mitogens. In order to faithfully replicate DNA once per cell cycle, cell cycle is largely divided into the mitotic phase and interphase which includes G1, S, and G2. In late G1, a cell senses the availability of nutrients and growth factors and decides whether or not to enter the S phase. If the environmental stimuli signal to the cell not to go into the S phase, it maintains the quiescent state until it is ready to resume cell division. Once the cell enters the S phase, it will complete the cell cycle. During the S phase, DNA replication occurs and chromosomes are duplicated. After the S phase, the cell enters the G2 phase of the cell cycle and prepares for the mitosis. During the mitotic phase, duplicated chromosomes are segregated into two daughter cells and the cell cycle is completed. When errors and mistakes are generated as a cell replicates DNA and chromosomes are attached to the microtubules, the cell cycle is halted and various checkpoints and repair proteins fix these problems before the cell cycle is resumed or completed.

*Cell cycle control by ubiquitin-dependent degradation*

The degradation of cell cycle regulators by the ubiquitin-proteasome system is important to control the cell cycle. The cell cycle is tightly controlled by cell cycle regulators such as cyclins and Cdk-inhibitors (CKIs) (10, 25, 74). Cyclin-dependent kinase (Cdk) is a major engine for the cell cycle and its activity is periodically regulated throughout the cell cycle not only by the fluctuation of cyclin synthesis but also by ubiquitin-dependent degradation of cyclins or CKIs. For instance, Sic1, a CKI, inhibits
the activity of the S phase Clb-CdkS to maintain the Cdk activity low during G1. In late G1, Sic1 is phosphorylated by Cln-Cdk complex and targeted for ubiquitin-dependent degradation by the SCF^{Cdc4} E3 ligase complex (25). This allows a cell to enter the S phase from the G1 of the cell cycle (Figure 4).

In addition, the degradation of Pds1, also known as securin, (15) and mitotic cyclin (96) by the APC E3 ligase during the mitotic phase is required for the proper segregation of chromosomes and mitotic exit. During the metaphase, duplicated chromosomes are aligned and held together by cohesins. When sister chromatids are accurately attached to the microtubules through the kinetochores and bi-oriented, Cdc14 dephosphorylates the Pds1 and the dephosphorylated Pds1 is subsequently recognized by the APC^{Cdc20} E3 ligase for ubiquitin-mediated degradation. This leads to the activation of Esp1, separase, and separation of sister chromatids during the anaphase (15, 93). In order for a cell to exit mitosis, the activity of mitotic Clb-Cdks needs to be inhibited. Mitotic exit is also triggered by the action of the APC^{Cdc20} E3 ligase that targets mitotic Clb for ubiquitin-mediated degradation (Figure 4) (96).

S-phase checkpoint

The regulation of the cell cycle is critical to maintaining cell viability and genome stability. The integrity of cell cycle progression is monitored by multiple checkpoints including the S phase checkpoint and spindle checkpoint.

When a cell encounters DNA lesions or stalled replication forks in response to replication stress, the S phase checkpoint is activated and DNA replication is halted until
the obstacles are fixed or overcome. Two major kinases that govern the S phase checkpoint are the ATM/Tel1 and ATR/Mec1. The ATM/Tel1 kinase is primarily activated by DNA double-strand breaks (DSBs), whereas the activity of the ATR/Mec1 kinase is triggered by various DNA damage including replication stress, base adducts, UV-induced damage, and DSBs (26, 58). The uncoupling of helicase complexes and polymerases generates a region of single-strand DNA (ssDNA) which is further coated by replication protein A (RPA), resulting in the recruitment and activation of Mec1. In addition, the 9-1-1 checkpoint clamp consisting of Rad17-Mec3-Ddc1, a heterotrimeric structure related to PCNA, also senses the RPA-coated ssDNA and activates the Mec1 kinase. The activated Mec1 kinase phosphorylates downstream kinases such as Mrc1 and Rad9, which subsequently activate the effector kinases, Rad53 and Chk1. This signal cascade leads to the inhibition of the S phase progression, prevention of late origin firing, stabilization of replication fork, upregulation of repair genes, and transcriptional repression of cyclins (Figure 5) (26, 34, 58, 66).

**Spindle checkpoint**

The spindle checkpoint ensures that duplicated chromosomes are correctly attached to the mitotic spindles via the kinetochores at the metaphase. Failure to attach the microtubules to the kinetochores on each sister chromatid or bi-orient chromosomes prevents the onset of anaphase until all sister chromatids are properly attached to the microtubules.
The mitotic checkpoint complex (MCC) consisting of Mad2, BubR1, Bub3, and Cdc20 monitors the attachment between the kinetochores and microtubules (64). The unattached kinetochores cause the MCC complex to inhibit Cdc20 so that the APC\textsuperscript{Cdc20} E3 ligase activity is blocked, resulting in the inhibition of sister chromatid separation (Figure 6). The spindle assembly checkpoint (SAC) also plays an important role in maintaining kinetochore attachment (64). Mad2 senses the lack of tension and unattached kinetochores. Mad2 is highly concentrated at the kinetochores and its level is reduced as the kinetochores are correctly attached to the microtubules and tension is created between two sister chromatids. Aurora-B/Ipl1 also functions to correct improper attachments such as merotelic attachments in which one sister kinetochore is attached to the microtubules from opposite spindle poles (64). When the microtubules are precisely attached to the kinetochores of sister chromatids and chromosomes are bi-oriented by proper tension, Cdc20 is released from the MCC and forms the active APC\textsuperscript{Cdc20} complex, which further targets Pds1 for degradation, resulting in separation of sister chromatids (Figure 6). In addition, the SAC activity is also extinguished to allow the metaphase-anaphase transition to occur.

3. DNA Replication

Pre-replicative complex (pre-RC) assembly

Accurate duplication of chromosomes is essential for cell proliferation and genomic stability. There are about 400 replication origins in budding yeast. Those origins are fired during S phase of the cell cycle and all of chromosomes are precisely duplicated. In order
to initiate DNA replication, sites at which DNA replication takes place called origins need to be specified during the G1 phase of the cell cycle.

The autonomously replicating sequence (ARS) was originally identified in budding yeast, *Saccharomyces cerevisiae*. This block of the genome contains the A, B1, B2, and B3 elements. The A element carries the 11 bp AT-rich ARS consensus sequence (ACS) conserved in budding yeast chromosomes (44, 84). This conserved sequence (A element) and B1 element are the site recognized by the origin recognition complex (ORC) which is composed of ORC1-6 (43, 44). Once the ORC complex binds to origins of replication, it recruits additional pre-RC components, Cdc6 and Cdt1 and this process requires ATP hydrolysis.

Cdc6 is a member of AAA+ ATPases and required for DNA replication initiation (43, 44). In support of this, overexpression of Cdc18, a homolog of fission yeast, leads to re-replication (8, 70). However, overexpression of Cdc6 does not result in overduplication of genome in budding yeast (8), suggesting that other inhibitory mechanisms such as Cdk-mediated Mcm export and Orc inhibition are critical to prevent re-replication in budding yeast.

Cdt1 is also an important initiation protein that recruits the Mcm complex to the origins of replication. Recent study showed that two Cdt1 proteins bind to Orc6 via the two Cdt1 binding sites on Orc6 and bring a double hexamer of MCM complexes to the origins (Figure 7) (13).

Mcm2-7 complex, a helicase, plays an essential role in DNA replication initiation. In most eukaryotic organisms, Mcm2-7 complex is localized in the nucleus throughout the
cell cycle (44). Mcm2-7 complex associates with origins in G1 but dissociates from the origins as cells enter the S phase (44). Unlike the Mcm2-7 complexes in eukaryotic organisms, budding yeast Mcm2-7 complex is localized in the nucleus in G1 and exported to the cytoplasm during S and G2/M (8, 44, 67). This different localization of the Mcm2-7 complex in budding yeast helps to prevent origins from being fired more than once in a single cell cycle. When the Mcm2-7 complex is loaded onto origins, initiator proteins Cdt1 and Cdc6, are dissociated from the origins and not reassembled into the origins until the next G1 phase of the cell cycle.

**DNA replication initiation**

After the Mcm2-7 double hexamer is loaded onto origins of replication, Cdc45 helicase-activating protein and Sld3 are recruited to the Mcm2-7 complex by Dbf4-dependent Cdc7 kinase (DDK)-mediated Mcm2-7 phosphorylation (35). Then, the S phase cyclin-dependent kinase (S-Cdk) phosphorylates Sld2 and Sld3 and the phosphorylated Sld2 binds to Dpb11 which contains two tandem phosphopeptide-binding domains, also known as BRCT domains (28). The N-terminal BRCT domain recruits phosphorylated Sld3, whereas the C-terminal pair of the BRCT domains binds to phosphorylated Sld2. When phosphorylated Sld2 interacts with Dpb11, Sld2-Dpb11 complex interacts with polymerase ε-GINS complex. In this complex, polymerase ε mediates the interaction between GINS and Sld2 since GINS alone does not have a high affinity for Sld2 binding in vitro (2).
Once the pre-loading complex (pre-LC) including Sld2, Dpb11, GINS, and polymerase ε is formed, the pre-LC is subsequently recruited to the Mcm2-7, Sld3, and Cdc45 complex via the interaction between phosphorylated Sld3 and Dpb11. Thus, the function of the pre-LC complex is to bring the polymerase ε-GINS to the pre-RC complex to form the Cdc45-Mcm-GINS (CMG) complex, an active helicase. This leads to the formation of DNA replication initiation complex (Figure 8, DNA replication initiation I).

After pre-LC complex binds to the pre-RC complex to form the CMG complex, Dpb11, Sld2, and Sld3 are released from the complex. Then, the CMG complex containing polymerase ε unwinds the origin DNA and moves along the replication fork as DNA is synthesized (Figure 9, DNA replication initiation II).

There are multiple early origins that need to be fired during the S phase of the cell cycle. Therefore, the role of Sld3 turnover is critical to initiate the firing of other early origins. When the pre-LC is loaded onto the first replication origin, Sld3 is dissociated and binds to the second origin located next to the first origin. This also forms a complex with new Cdc45 onto the second origin and prepares for subsequent loading of the pre-LC complex to form the CMG complex (2).

*DNA replication elongation*

Eukaryotic organisms require three DNA polymerases including polymerase α, δ, and ε for DNA replication (2, 51). Polymerase α forms a complex with primase which synthesizes a short RNA primer. The polymerase α extends the DNA using this RNA
primer. The RFC, clamp loader, recognizes the 3’end of the DNA and brings the PCNA sliding clamp onto the double-strand DNA. This short stretched DNA is further extended by either polymerase δ or polymerase ε. The polymerase δ primarily extends the lagging strand, whereas polymerase ε synthesizes the DNA on the leading strand.

Polymerase ε is made of four subunits, Pol2, Dpb2, Dpb3, and Dpb4 (2). The N-terminal half of Pol2 has the catalytic activity and is dispensable for cell viability. However, the C-terminal half of Pol2 is necessary for cell growth.

On the lagging strand, the okazaki fragments formed by Pol α/primase and Pol δ are further processed by the action of Pol δ and flap endonuclease FEN1 to remove and replace the RNA primer with DNA (4, 42). The gaps made by this process are sealed and joined together by DNA ligase.

4. Biological roles of Tom1, Dia2, and Cdc6

Hect E3 ligase Tom1

Tom1 is a Hect family E3 ubiquitin ligase in budding yeast. It contains a conserved Hect domain at the C-terminus, two E3 ubiquitin ligase domains of which function is not known and a potential protein-protein interaction domain at the N-terminus of the protein. Tom1 plays a role in a wide variety of cellular processes including transcriptional activation, mRNA export, and DNA damage response (32, 40, 82, 86). In particular, Tom1 targets histone H3 for ubiquitin-dependent degradation in response to activation of the S-phase checkpoint via Rad53-mediated histone H3 phosphorylation (86). In addition, DNA replication initiator protein Cdc6 is also targeted for degradation by Tom1 in
response to DNA damage (32). These reports indicate that Tom1 has a role in DNA damage response, although more potential Tom1 substrates involved in this process remain to be identified.

Huwe1, a human homolog of Tom1, has been shown to be important for cell proliferation and growth suppression. Under normal cell growth conditions, Huwe1 polyubiquitinates c-Myc via the lysine 63-linked chains, leading to transcriptional activation of downstream target genes. Huwe1 also targets p53 for ubiquitin-dependent degradation (1, 12) during cell proliferation. By contrast, Huwe1 switches its targets during DNA damage. For example, replication initiator Cdc6 and anti-apoptotic protein Mcl-1 are targeted for degradation to inhibit the assembly of pre-RC complex and promote apoptosis in response to DNA damage, respectively (32, 100). These findings suggest that some aspect of Huwe1 function appears to be conserved in budding yeast, Saccharomyces cerevisiae.

In addition to its role in DNA damage response, Tom1 also plays a role in mRNA export. Yra1/REF, a component of mRNA ribonucleoprotein (mRNP) complex, is ubiquitinated by Tom1 and dissociated from mRNP complex before mRNA export (40). Intriguingly, Yra1 has also been known to interact with and recruit Dia2 to replication origins (90), implying that Tom1 may also directly or indirectly bind Dia2.

**F-box protein Dia2**

The budding yeast F-box protein Dia2 assembles a functional SCF complex with Cdc53, Skp1, and Rbx1 and recruits a substrate to the SCF complex for ubiquitin-
mediated degradation (36, 47, 48, 52). Dia2 also has a role in maintaining genomic stability and DNA replication. For example, the deletion of DIA2 renders cells sensitive to DNA damage, increases chromosome loss and rearrangement, and accumulates DNA damage foci (5, 47, 71). In addition, our previous work showed that Dia2 is a chromatin bound protein and associates with origins of DNA replication (47). In support of the idea that Dia2 plays a role in DNA replication, Dia2 has been shown to assemble with replisome components and control the progression of replication forks (60, 62). Moreover, Dia2 protein is stabilized during S phase or in response to activation of the S-phase checkpoint (45), suggesting that stabilized Dia2 may form the SCF^{Dia2} complex to target S-phase or replication proteins for degradation. Two potential Dia2 substrates, Mrc1 and Ctf4, that travel along with replication fork have been identified, although the biological significance of their ubiquitination remains to be understood (60).

The rate of Dia2 degradation varies during the cell cycle. Dia2 is unstable during G1 and G2/M phases but stable in S phase. There are two conflicting reports about the pathway responsible for Dia2 degradation. One report suggests that Dia2 is degraded via an auto-ubiquitination mechanism (60), as shown in other F-box proteins (27, 101). In contrast, our previous work shows that Dia2 degradation is not dependent on an auto-ubiquitination mechanism (45), as deletion of the F-box domain required for assembly of an SCF complex does not stabilize Dia2, indicating that a non-SCF E3 ligase might control Dia2 degradation. However, the identity of E3 ligase responsible for this pathway is not known.
Cdc6 degradation and its role in re-replication control

Cdc6 is a replication initiation protein required for assembly of pre-RC complex during the G1 phase of the cell cycle. Cdc6 is targeted for ubiquitin-dependent degradation during the cell cycle and is subject to multiple modes of degradation in budding yeast (20, 21, 73, 75).

Mode 1 represents the degradation of Cdc6 during G1 phase and does not require either the anaphase-promoting complex (APC/C) or SCF E3 ligase complex. Mode 1 Cdc6 degradation is relatively rapid and independent of Cdk-mediated phosphorylation (20). More importantly, the E3 ubiquitin ligase responsible for this pathway is not known.

Mode 2 Cdc6 degradation is dependent on the SCF\textsuperscript{Cdc4} E3 ligase complex at the G1 to S-phase transition. The turnover of Cdc6 protein at this stage is extremely rapid and requires Cdk-mediated phosphorylation for the SCF\textsuperscript{Cdc4} complex to recognize Cdc6 for ubiquitin-dependent degradation. This phosphorylation event is dependent on G1-cyclins but not S-cyclins. Cdc6 contains eight Cdk consensus sites and the phosphorylation of the first four consensus sites located at the N-terminus of the protein is important for Mode 2 degradation (20, 21, 73).

During G2/M phases of the cell cycle, Cdc6 is subject to Mode 3 degradation. This pathway is also governed by activity of the SCF\textsuperscript{Cdc4} complex and requires phosphorylation of two Cdk consensus sites found at the C-terminus of Cdc6 (73).

As described above, Cdc6 turnover rate varies during the cell cycle and only a single E3 ubiquitin ligase for Cdc6 degradation has been identified, the SCF\textsuperscript{Cdc4} complex.
Moreover, the turnover rate of Cdc6 at the G1 to S phase transition (Mode 2) is very rapid compared to those of Mode 1 and 3, suggesting that multiple E3 ligases may be required for Mode 2 Cdc6 degradation.

Cdc6 degradation is an important pathway to inhibit DNA re-replication. In budding yeast, multiple regulatory mechanisms including Cdc6 degradation, nuclear export of Mcm2-7 complex, and ORC inhibition are critical to prevent the re-assembly of pre-RC complex after S phase until the G1 phase of the next cell cycle (3, 6, 68). First, Cdk-dependent Cdc6 phosphorylation marks it for ubiquitination by the SCF\(^\text{Cdc4}\) complex, resulting in degradation via the 26S proteasome. In addition, phosphorylation of Cdc6 at the N-terminal domain (amino acids 2-47) also induces its association with Clb2-Cdk complex such that Cdc6 activity for pre-RC assembly is blocked (61). Second, Cdk inhibits the activity of ORC through phosphorylation of Orc2 and Orc6, leading to inhibition of Cdt1/Mcm2-7 complex loading onto origins. Moreover, binding of S-phase cyclin Clb5 to Orc6 also inhibits the recruitment of Cdt1/Mcm2-7 complex to ORC (13). Third, Mcm2-7 complex is exported to the cytoplasm to avoid re-loading of Mcm2-7 complex onto origins during S or G2/M phases (67). When these multiple inhibitory mechanisms are compromised, re-replication is observed in budding yeast (3, 68). For example, mutations of Orc2 and Orc6 phosphorylation sites along with constitutively nuclear localization of Mcm2-7 complex and overexpression of stable Cdc6\(\Delta N\) mutant are simultaneously required to induce re-replication in budding yeast.

To precisely duplicate the genome, origins in eukaryotic cells are fired during S phase only once per cell cycle. However, disruption of the above regulatory mechanisms in
budding yeast causes some origins to be fired more than once during S or G2/M phases, resulting in over-duplication of portions of the genome (Figure 10). Thus, failure to control re-replication leads to genomic instability such as gene amplification, chromosome loss and rearrangement. Although multiple overlapping mechanisms are required to control re-replication in budding yeast, overexpression of Cdc18, a fission yeast homolog of Cdc6, is sufficient to induce re-replication in fission yeast (70). In addition, human Cdc6 is often overexpressed in various cancers (8), suggesting that regulation of Cdc6 expression is crucial to prevent genomic instability and tumorigenesis.
**Figure 1. Ubiquitin-proteasome system.**

E1 ubiquitin-activating enzyme is charged with ubiquitin in an ATP-dependent manner.

Ubiquitin is then transferred to E2 ubiquitin-conjugating enzyme through a thioester bond.

With the help of E3 ubiquitin ligase, ubiquitin is directly or indirectly attached onto a lysine of a substrate. After multiple rounds of this reaction, polyubiquitinated substrate is targeted for degradation via the 26S proteasome.
Figure 2. SCF E3 ligase complex.

Multi-subunit SCF E3 ligase complexes consist of Cdc53 (Cul1), Rbx1(Roc1), Skp1, and F-box protein. When an F-box protein binds a substrate, ubiquitin is directly transferred from an E2 to a lysine of the substrate. Rbx1 interacts with the E2 through the RING domain. The F-box protein interacts with the substrate via the WD40 repeats located at the C-terminus of the protein. The SCF$^{Cdc4}$ E3 ligase complex is shown here as an example.
Figure 3. Major E3 ligase types.

RING E3 ligases contain the RING and substrate binding domains. When a RING E3 binds an E2 and a substrate, ubiquitin is directly transferred from the E2 to a lysine of the substrate. By contrast, a HECT E3 ligase transiently accepts ubiquitin from an E2 via a catalytic cysteine in the HECT domain. Ubiquitin is then subsequently attached onto a lysine of the substrate.
In late G1, Cdk inhibitor (CKI) such as Sic1 is targeted for ubiquitin-mediated degradation by the SCF$^{Cdc4}$ complex to promote the G1 to S phase transition. During mitosis, the APC$^{Cdc20}$ complex targets mitotic cyclin for ubiquitin-dependent degradation to allow a cell to exit mitosis.
Figure 5. Activation of the S-phase checkpoint.

Upon DNA damage or replication stress (i.e. DNA lesion shown here), Mec1 is recruited to RPA (shown in green) and activated. This leads to activation of downstream effector kinase Rad53 via phosphorylation of Mrc1. Activation of Rad53 inhibits S-phase progression, stabilizes the replication fork, and inhibits late origin firing. In addition, activated Rad53 also blocks homologous recombination and mitosis.
Figure 6. Role of the Spindle checkpoint in chromosome separation.

The inaccurate attachment of microtubules from spindle poles (shown in green circle) to the kinetochore (i.e. monotelic attachment) is monitored by the MCC complex and leads to the inhibition of the APC<sup>Cdc20</sup> complex. When sister chromatids are correctly attached to the microtubules, this results in activation of the APC<sup>Cdc20</sup> complex which in turn targets Pds1 for ubiquitin-dependent degradation. Esp1, separase, is then released from Pds1 and cleaves the cohesin to separate sister chromatids.
Figure 7. pre-RC assembly in G1.

ORC complex binds to replication origin and recruits Cdc6 which further brings Cdt1/Mcm2-7 complex to the origin of replication to assemble the pre-RC complex.
Figure 8. DNA replication initiation I.

Dbf4-dependent Cdc7 Kinase (DDK)-mediated Mcm2-7 phosphorylation (not shown here) causes Cdc45 and Sld3 to bind to the Mcm2-7 complex. S-phase Cyclin-dependent Kinase (S-Cdk) then phosphorylates Sld2 and Sld3. Once phosphorylated Sld2 binds to Dpb11, GINS-polymerase ε complex is also recruited to the Sld2-Dpb11 complex to form the pre-loading complex (pre-LC), which subsequently interacts with the pre-RC complex via the interaction between Sld3 and Dpb11.
Figure 9. DNA replication initiation II.

When the Cdc45-Mcm-GINS (CMG) complex is formed next to the origin, Sld2, Sld3, and Dpb11 are dissociated from the complex. The active CMG complex containing polymerase ε then unwinds DNA and establishes replication forks. Polymerase α/primase complex (Primase is not depicted here) later joins the CMG complex to initiate DNA replication on both strands. Polymerase ε and polymerase δ (not shown in the diagram) then elongate the leading and lagging strands, respectively. DNA elongation process is not shown in the diagram.
Figure 10. Re-replication.

Multiple inhibitory mechanisms including Cdc6 degradation, Mcm2-7 export, and ORC inhibition play a crucial role in preventing origins from being fired more than once within a single cell cycle in budding yeast, *Saccharomyces Cerevisiae*. In re-replication condition, one of the origins is re-fired during S or G2 phase, leading to the portion of chromosome over-duplicated.
CHAPTER II

Cdc6 degradation by a Tom1 and Dia2-dependent pathway regulates pre-RC assembly during the cell cycle

Cdc6 functions in the assembly of pre-replicative complexes at replication origins. This activity is controlled to limit DNA replication to once per cell cycle. Cdc6 is targeted for protein degradation by multiple mechanisms in *Saccharomyces cerevisiae*, although only a single pathway and E3 ubiquitin ligase for Cdc6 has been identified, the SCF<sup>Cdc4</sup> complex. Using a genetic approach, I identified two additional E3 ubiquitin ligase components required for Cdc6 degradation, the F-box protein Dia2 and the Hect-domain E3 Tom1. Both Dia2 and Tom1 control Cdc6 turnover during G1, a cell cycle stage when Cdc4 does not recognize Cdc6. Surprisingly, Dia2 and Tom1 also contribute to Cdc6 protein turnover at the G1/S transition when SCF<sup>Cdc4</sup> acts. Genetic epistasis analysis indicates that Dia2 and Tom1 function cooperatively but act separately from SCF<sup>Cdc4</sup>. A mutant defective in all three ubiquitin ligase activities exhibits exacerbated Cdc6 protein stabilization and sensitivity to replication stress. Cdc6 and Mcm4 chromatin association is aberrant in *tom1Δ* and *dia2Δ* cells after S phase has been completed. Strikingly, defects in Tom1 or Cdc4 lead to increased DNA content in cells sensitized to re-replication. Thus, multiple degradation pathways that limit Cdc6 protein levels cooperate to regulate pre-RC assembly and may act to prevent DNA re-replication.
**Introduction**

DNA replication is a highly coordinated process to precisely duplicate chromosomes during S phase of the cell cycle. Initiation of DNA synthesis requires assembly of pre-replicative complexes (pre-RCs) at replication origins (reviewed in (84)). Regulation of pre-RC assembly is critical to prevent over duplication of genetic material. Pre-RC assembly occurs during the G1 phase of the cell cycle and re-assembly is blocked for the remainder of the cycle (reviewed in (7)).

Cdc6 is an AAA+-ATPase required for pre-RC assembly (14, 54, 72, 97). Cdc6 binds to the Origin Recognition Complex (ORC) along with another pre-RC protein, Cdt1, to recruit the MCM2-7 replicative helicase complex to replication origins (17, 18, 57, 69, 92). Budding yeast Cdc6 is a highly unstable protein subject to multiple modes of degradation (20, 21, 75). In the best-understood degradation pathway, cyclin-dependent kinase (Cdk) phosphorylation targets Cdc6 for ubiquitination and degradation via the ubiquitin ligase complex SCF$^{Cdc4}$ at the G1/S phase transition and during G2/M (20-22, 73, 83). However, Cdc6 is also unstable during G1 and the degradation pathway responsible has not been identified (21). Moreover, the rate of Cdc6 degradation fluctuates during the cell cycle (21), an observation not fully explained by the activity of a single ubiquitin ligase.

To identify other pathways that might target Cdc6 for ubiquitination and degradation, I assayed other E3 ubiquitin ligases that function in DNA replication or during S phase. Tom1 is a Hect-domain E3 ubiquitin ligase that targets excess histone H3 for DNA replication checkpoint-dependent degradation (86). The human homolog of Tom1,
Huwe1, targets human Cdc6 for degradation during the DNA damage checkpoint response (32, 33). This function appears to be conserved in S. cerevisiae (32), although the contribution of Tom1 to Cdc6 degradation in an unperturbed cell cycle has not been investigated. The SCF$^{\text{Dia2}}$ ubiquitin ligase complex regulates DNA replication and is required for genomic stability (5, 47, 62, 71). Potential targets of SCF$^{\text{Dia2}}$ include two proteins that travel with replication complexes at the replication fork, Mrc1 and Ctf4, although the physiological role of their degradation is unclear (60).

Here, I show that both Tom1 and Dia2 contribute to Cdc6 degradation during G1 and surprisingly, also during the G1 to S phase transition. This work describes new degradation pathways for Cdc6 and identifies a novel target for both the Tom1 and Dia2 ubiquitin ligase components.

Tom1 and Dia2 target Cdc6 for degradation

To determine whether other ubiquitin ligases might have a role in Cdc6 protein turnover, I examined whether overexpression of CDC6 caused a growth defect in ligase mutants that had known roles in DNA replication or cell cycle. Two ligases that fit this profile include the F-box protein Dia2 and the Hect-domain E3 Tom1 (5, 33, 47, 71). Previous work has shown that Tom1 can target Cdc6 for degradation in response to DNA damage (33), but a role for Tom1 in Cdc6 turnover in an unperturbed cell cycle has not been examined. Overexpression of CDC6 causes a growth defect in wildtype yeast cells (9, 23), but I observed an exacerbated growth phenotype in both dia2Δ and tom1Δ cells (Figure 11A).
I then performed a protein stability assay using HA-tagged Cdc6 expressed from the endogenous locus in wildtype, *tom1Δ* and *dia2Δ* cells. Log phase cultures were treated with cycloheximide to inhibit protein synthesis and Cdc6 protein levels examined over time. As shown in Figure 11B, Cdc6 was partially stabilized in both *dia2Δ* and *tom1Δ* cells. Another unstable cell cycle protein, Clb5, was not stabilized in *dia2Δ* or *tom1Δ* cells, indicating that the results with Cdc6 were not due to non-specific defects in protein degradation (Figure 12). In addition, no changes in *CDC6* RNA abundance was detected in these strains (Figure 11C), consistent with the possibility that Dia2 and Tom1 might control Cdc6 ubiquitination.

**Cdc6 binds Dia2 and Tom1**

I examined whether Dia2 and Tom1 interact with Cdc6 using two different approaches. First, I tested whether GST-Cdc6 co-purified with either Myc-tagged Dia2 or the Flag-tagged Hect domain of Tom1 when expressed in baculovirus-infected insect cells (Figure 13). When either Myc-tagged Dia2 or the Flag-tagged Hect domain of Tom1 was immunoprecipitated, I observed substantial co-purification of GST-Cdc6 (Figure 13A). I also observed reciprocal co-purification when GST-Cdc6 was purified from the same cells (Figure 13B). Next, I tested whether endogenously expressed HA-tagged Cdc6 can co-immunoprecipitate endogenously expressed Myc-tagged Dia2 or Flag-tagged Tom1 (Figure 13C). Immunoprecipitation of Cdc6 resulted in Tom1 and Dia2 co-purification, indicating that these proteins interact under physiological conditions. GST-tagged Cdc6 purified from baculovirus-infected insect cells also
interacted with endogenously expressed Flag-tagged Tom1 and Myc-tagged Dia2 in yeast extracts (data not shown). Since Dia2 is a chromatin-bound protein, I considered the possibility that Dia2 interacted with Cdc6 indirectly by virtue of both proteins binding to chromatin, but I still observed co-precipitation when lysates were treated with DNase I (Figure 14A). I also observe no change in Cdc6 association with either Tom1 or Dia2 when the other is missing, indicating that both Tom1 and Dia2 can bind Cdc6 independently of each other (Figure 14B). I conclude that both Tom1 and Dia2 form a complex with Cdc6, although I cannot determine whether all three proteins assemble into the same complex from these results.

Tom1 is a large protein with multiple domains, including the conserved Hect domain found in the C-terminus of the protein that contains the catalytic cysteine (82). My results with insect cell-expressed protein indicate that the Tom1 Hect domain interacts with Cdc6 (Figure 13A, B). Dia2 is an F-box protein that contains TPR repeats in the N-terminus and an LRR domain in the C-terminus (5, 47). Since F-box proteins typically associate with substrate proteins via their C-terminal repeat domains, I asked whether a truncated Dia2 protein lacking the N-terminus still bound Cdc6. I co-expressed ΔN214 Dia2, which lacks the N-terminal TPR repeats but still contains the F-box domain and LRR region, with GST-Cdc6 in insect cells. GST-Cdc6 co-purified ΔN214 Dia2 under these conditions (Figure 13D). Together, these results suggest that the N-termini of Tom1 and Dia2 are dispensable for binding Cdc6.
Tom1 and Dia2 mediate degradation of Cdc6 at the G1/S transition

Cdc6 protein turnover is regulated throughout the cell cycle (20, 75). Thus, I investigated whether Tom1- and Dia2-dependent turnover of Cdc6 changed during the cell cycle. I first examined the abundance of the Cdc6 protein as cells progressed from a late G1 arrest into G2. Cells were arrested with alpha factor and then released into media containing nocodazole. In wildtype cells, there is a burst of Cdc6 expression in late G1 (9, 20, 75), but the protein disappears as cells enter and proceed through S phase (Figure 15A). In both tom1Δ and dia2Δ, Cdc6 protein abundance decreases as cells enter S phase but does not disappear altogether as it does in wildtype cells. These results were somewhat surprising, as it has previously been shown that SCF\(^{Cdc4}\) targets Cdc6 at the G1/S transition (20, 21). My results suggest that Tom1 and Dia2 may also control Cdc6 degradation at the G1/S transition. To test protein turnover in early S phase, I performed cycloheximide protein stability assays on cells that had been arrested in G1 with alpha factor and then released into hydroxyurea-containing media. In wildtype cells, Cdc6 has a half-life of less than 5 minutes and in cdc4-1 cells, the protein is partially, but significantly, stabilized as previously reported (20). Importantly, I also observed significant stabilization of Cdc6 in both tom1Δ and dia2Δ cells in early S phase (Figure 15B).

I next asked whether Tom1 and Dia2 function in the same pathway as Cdc4 in the turnover of Cdc6. I generated cdc4-1 tom1Δ, cdc4-1 dia2Δ and tom1Δ dia2Δ double mutants and assessed the stability of Cdc6 in early S phase. Strikingly, Cdc6 was strongly stabilized in both the cdc4-1 tom1Δ and cdc4-1 dia2Δ strains relative to the
single mutants (Figure 15B), indicating that the Cdc4 pathway functions independently of Tom1 and Dia2. However, stabilization of Cdc6 in the \textit{tom1Δ dia2Δ} mutant was not significantly different than in the \textit{dia2Δ} and \textit{tom1Δ} single mutants (Figure 15B, C). Similar results were observed in cells arrested directly with hydroxyurea (data not shown). To examine this further, I compared the growth phenotypes of the single and double ligase mutants on rich media in the presence and absence of hydroxyurea or MMS to induce replication stress (Figure 16A). The \textit{cdc4-1 tom1Δ} and \textit{cdc4-1 dia2Δ} mutant exhibited enhanced growth defects compared to the respective single mutants. Strikingly, the \textit{tom1Δ dia2Δ} mutant phenotype was very mild relative to the other double mutants. These results suggest either that Dia2 and Tom1 function in the same pathway or that they mutually reinforce each other’s activity. Nevertheless, these data indicate that Cdc4 acts independently of Dia2 and Tom1.

If Dia2 and Tom1 were in the same pathway, I would expect the triple ligase mutant to show the equivalent growth phenotype to either of the \textit{cdc4-1 dia2Δ} or \textit{cdc4-1 tom1Δ} double mutants. However, in all conditions the \textit{cdc4-1 tom1Δ dia2Δ} triple mutant showed the most severe phenotype, although the difference between the triple mutant and the \textit{cdc4-1 tom1Δ} and \textit{cdc4-1 dia2Δ} double mutants was subtle. I also compared the stability of Cdc6 in the double mutants to the triple mutant using a longer time course (Figure 16B). Here, I observed that the triple ligase mutant exhibited substantially greater stabilization of Cdc6 than the double mutants. Thus, the genetic relationship among these three ligases is more complex than initially suggested.
Is the Tom1/Dia2 pathway responsible for Cdc6 degradation Mode 1?

Previous work from the Diffley laboratory has shown that there are three modes of Cdc6 degradation (21). Mode 1 functions prior to Start during G1 and is Cdk-independent, whereas Modes 2 and 3 are Cdk- and Cdc4-dependent and function in early S and G2/M, respectively (21). The identity of the E3 ligase in Mode 1 was not determined, although genetic evidence suggested that neither the APC nor SCF complexes were responsible (21). I tested whether Tom1 or Dia2 might contribute to the turnover of Cdc6 in G1 using the cycloheximide stability assay in cells arrested with alpha factor. As has been previously shown (21), Cdc6 is still unstable in cdc4-1 cells under these conditions (Figure 17). Strikingly, I found that Cdc6 was stabilized in both tom1Δ and dia2Δ strains (Figure 17). The observation that Dia2 is involved in Cdc6 turnover in G1 was surprising, as Cdc6 is not stabilized in scf mutants in G1 (21). I next asked whether Tom1 and Dia2 function in the same pathway in G1 by examining Cdc6 turnover in the tom1Δ dia2Δ double mutant arrested in G1 with alpha factor. I found that the rate of turnover in the double mutant was equivalent to the turnover in tom1Δ (Figure 17), indicating that Tom1 and Dia2 contribute to Cdc6 degradation in G1, again either in the same pathway or acting cooperatively.
Tom1 and Dia2 are required for Cdc6 ubiquitination

The most straightforward explanation for these results is that Cdc6 may be a direct ubiquitination target of both Tom1 and Dia2. To test this hypothesis, I examined whether the ubiquitin ligase activity of Tom1 or Dia2 was required for Cdc6 protein turnover. Tom1 is a member of the Hect-domain E3 ubiquitin ligase family and so uses a catalytic cysteine in the transfer of ubiquitin to substrate proteins (82). Cdc6 was stabilized in a tom1 mutant that had this cysteine replaced with alanine (Figure 18A). These assays were performed in G1-arrested cells, but similar results were observed in S-phase arrested cells (data not shown). Likewise, Dia2 contains an F-box domain required for assembly with the other SCF components to form a functional E3 ligase complex (5, 47). Cdc6 is stabilized in a dia2 ΔF-box mutant in cells arrested in G1 (Figure 18A) or early S phase (data not shown). This result suggests a requirement for the F-box domain of Dia2, but it is at odds with previous work indicating that scf mutants do not stabilize Cdc6 in G1 (21). I verified that skp1-11, cdc53-1 and cdc34-2 mutants do not stabilize Cdc6 in G1-arrested cells (Figure 19A). Moreover, I did not observe any Skp1 associated with Cdc6 in G1 cells (Figure 19B). However, when I examined Cdc6 turnover in a Dia2 mutant lacking both the F-box domain and the C-terminal LRR region (TPR mutant), I also observed Cdc6 stabilization (Figure 18B). These results suggest that Dia2 contributes to Cdc6 turnover in an F-box dependent manner but that this function may be independent of a traditional SCF complex.

I next asked whether Tom1 and Dia2 are required for Cdc6 ubiquitination. I immunoprecipitated HA-tagged Cdc6 from wildtype, tom1Δ and dia2Δ cells that had
been incubated with the proteasome inhibitor MG132. I probed the immunoprecipitates with anti-ubiquitin antibodies. Under these conditions, I observed modified forms of Cdc6 in proteasome-inhibited cells, but these forms were moderately reduced in both \textit{tom1}\(\Delta\) and \textit{dia2}\(\Delta\) cells, even in the presence of MG132 (Figure 18C). In addition, I developed an \textit{in vitro} ubiquitination assay using GST-Cdc6 purified from baculovirus-infected insect cells. When GST-Cdc6 was incubated with fractionated yeast extracts, E1, E2, ubiquitin and an ATP regeneration system, I observed Cdc6 ubiquitin conjugates (Figure 18D, lanes 1-4). As a negative control, I performed the same assay using fractionated yeast extracts from the \textit{cdc4}\(\text{1}\) strain and observed a significant decrease in ubiquitination of Cdc6 (Figure 18D, lanes 5 & 6). I repeated these assays using extracts from \textit{tom1}\(\Delta\) and \textit{dia2}\(\Delta\) strains and also observed decreased ubiquitination with these extracts (Figure 18D, lanes 7-14). The loss of ubiquitinated Cdc6 appeared more complete in \textit{tom1}\(\Delta\) extracts than in \textit{dia2}\(\Delta\) extracts. Together, these results indicate that Cdc6 ubiquitination and protein stability are dependent on both Tom1 and Dia2.

\textit{Tom1 and Dia2 still recognize a Cdc6 N-terminal mutant}

Deletion of an N-terminal region in Cdc6 has been shown to lead to a stabilized protein (20). The region encompassing amino acids 2-47 contains 4 CDK consensus sites that are important for SCF\textsuperscript{Cdc4}\(-\)-mediated degradation of Cdc6 at the G1 to S phase transition. However, this N-terminal truncation mutant of Cdc6 (Cdc6\(\Delta\)N) is also stabilized in G1 cells, a time when Cdc4 does not control Cdc6 proteolysis (20, 21). Thus, I sought to determine whether the Tom1 and Dia2-dependent pathway was
responsible for stabilization of this mutant in G1. I generated strains that endogenously expressed HA-tagged Cdc6ΔN in wildtype, \textit{tom1Δ} and \textit{dia2Δ} backgrounds and performed protein stability assays. As previously reported, Cdc6ΔN is partially stabilized in G1-arrested cells (20). Surprisingly, Cdc6ΔN stability is increased in \textit{tom1Δ} and \textit{dia2Δ} cells, indicating that Tom1 and Dia2 likely interact with a different region of the Cdc6 protein (Figure 20A). Similar results are observed when the stability assay is performed in cells arrested in early S phase (Figure 20B). To test whether Tom1 and Dia2 associate with a Cdc6 protein lacking the N-terminus, I co-incubated GST-tagged Cdc6ΔN, Flag-tagged Tom1 Hect domain and Myc-tagged Dia2 expressed from baculovirus-infected insect cells and performed immunoprecipitation assays. When either the Tom1 Hect domain or Dia2 was immunoprecipitated, Cdc6ΔN co-purified (Figure 20C), indicating that these proteins are able to form a complex. I conclude that Dia2 and Tom1 recognize a domain in Cdc6 downstream of amino acid 47.

\textit{Cdc6 remains associated with chromatin in ubiquitin ligase mutants}

Cdc6 associates with chromatin in late M and early G1 to facilitate origin licensing and pre-RC formation (14, 18, 54). The degradation of Cdc6 as cells finish G1 and enter S phase is thought to contribute to preventing origins from firing more than once per cell cycle (37, 68). Cdc6 mutants that exhibit impaired degradation have been linked to inappropriate re-loading of Cdc6 on chromatin during G2/M and even re-replication under specific conditions (19, 37, 68). I examined the chromatin association of Cdc6 during S and G2/M in \textit{tom1Δ} and \textit{dia2Δ} cells. I used wildtype cells as a negative control.
and the \textit{cdc4-1 sic1\Delta} strain as a positive control. Cells were arrested in G1 with alpha factor and then released into media containing nocodazole to arrest in M phase. Samples were collected at 0, 45, and 90 minutes after release from G1 for chromatin fractionation. The abundance of Cdc6 is significantly increased in the whole cell extracts from \textit{tom1\Delta}, \textit{dia2\Delta} and \textit{cdc4-1 sic1\Delta} cells relative to wildtype, consistent with impaired degradation in these strains (Figure 21A). Wildtype cells exhibited Cdc6 chromatin association at time 0 that subsequently dropped and stayed low throughout the time course. In the \textit{cdc4-1 sic1\Delta} positive control, Cdc6 chromatin association was somewhat reduced relative to wildtype cells at time 0 but showed considerable chromatin association throughout the time course. The chromatin association of Cdc6 in the \textit{dia2\Delta} and \textit{tom1\Delta} strains was higher at time 0 than wildtype and remained higher at 45 minutes for both strains, with association decreasing in \textit{tom1\Delta} cells at 90 minutes but remaining higher in \textit{dia2\Delta} cells at 90 minutes. The \textit{dia2\Delta} and \textit{tom1\Delta} strains did not achieve a complete G1 arrest, which may have contributed to greater Cdc6 chromatin association early in the time course, although cell cycle profiles later in the time course were comparable to wildtype. Overall, these results suggest that a fraction of Cdc6 remains associated with chromatin from late G1 through S phase and into G2/M in \textit{dia2\Delta} and \textit{tom1\Delta} cells as ubiquitination of Cdc6 is impaired.

To examine whether the aberrant Cdc6 chromatin association led to increased association of the MCM2-7 complex with replication origins, I used chromatin immunoprecipitation. Wildtype, \textit{tom1\Delta} and \textit{dia2\Delta} cells expressing HA-tagged Mcm4 were arrested in G1 and then released into nocodazole. Mcm4 chromatin association at
two early firing origins (ARS1 and ARS305) and one non-origin region (ACF2) was
assayed at 30-minute intervals (Figure 21B). All strains exhibited only negligible Mcm4-
binding at the non-origin region ACF2 throughout the time course. For both origins, the
association of Mcm4 dropped to low levels at the 60 and 90-minute timepoints in
wildtype cells. By contrast, Mcm4 association at ARS1 and ARS305 remained higher
than wildtype in tomlΔ cells throughout the experiment and was especially pronounced at
the 60 and 90-minute timepoints. In dia2Δ cells, Mcm4 was strongly associated with
ARS1 during the entire time course, but the effect at ARS305 was less obvious. Mcm4
association with ARS305 was reduced in dia2Δ cells relative to wildtype at the 30-minute
timepoint, but remained at this level at the 60 and 90-minute timepoints as the association
in wildtype cells dropped. Overall, the trend suggests that increased Cdc6 abundance and
chromatin association in both the tomlΔ and dia2Δ strains may lead to increased
association of Mcm proteins at origins.

Failure to clear Cdc6 from chromatin has the potential to lead to firing origins more
than once per cell cycle. In budding yeast, there are three key pathways that prevent re-
replication: destruction of Cdc6, nuclear export of the Mcm2-7 complex, and inactivation
of ORC by phosphorylation (3, 6, 68). When all three of these pathways are
compromised, re-replication can be observed in a single cell cycle (68). To investigate
whether the degradation of Cdc6 dependent on Tom1 and Dia2 may contribute to re-
replication control, I generated dia2Δ, tomlΔ, cdc4-1 mutants in a strain background
sensitized to re-replication, orc2-6A orc6-4A mcm7-2NLS (hereafter referred to as orc*
mcm*) (68). I used the cdc6ΔN allele as a positive control. Cells were arrested with 2C
DNA content using nocodazole and then shifted to 37°C, as \textit{cdc4-1} and \textit{tom1Δ} are sensitive to high temperature. The \textit{dia2Δ} strain was tested at both 25°C and 37°C, as this strain is sensitive to low temperatures, but no discernible differences were observed. I used flow cytometry to measure DNA content in each strain prior to nocodazole arrest (asy), after nocodazole arrest (time 0) and 1, 2 and 3 hours after arrest at the elevated temperature. The percentage of cells above 2C DNA content in the \textit{orc* mcm*} control strain remained relatively constant throughout the experiment. By contrast, a large fraction of the \textit{cdc6ΔN orc* mcm*} population shifted above 2C DNA content at the 1, 2 and 3 h timepoints, suggesting increased DNA levels (Figure 22). Strikingly, the \textit{tom1Δ orc* mcm*} and \textit{cdc4-1 orc* mcm*} mutants also had large populations above 2C DNA content, whereas the \textit{dia2Δ orc* mcm*} mutant resembled the negative control \textit{orc*mcm*} strain. To determine whether the observed increase in DNA content was the result of nuclear DNA replication and not mitochondrial DNA replication, I generated rho\textsuperscript{0} versions of all strains and repeated the re-replication assays (76). Even in the absence of mitochondrial DNA, I observed a significant increase in the population of cells exhibiting greater than 2C DNA content in the \textit{cdc4-1}, \textit{tom1Δ} and \textit{cdc6ΔN} strains (data not shown). I note that all of the strains, with the exception of \textit{cdc4-1 orc* mcm*}, did not achieve a complete nocodazole arrest at the start of the experiment, suggesting that some cells were in G1 or S-phase at the time of the shift to 37°C. Increased Cdc6 protein levels in these stages could conceivably lead to increased loading of Mcm complexes that contribute to DNA over-replication. However, the \textit{orc* mcm*} control strain did not exhibit significant increases in >2C DNA content despite an incomplete nocodazole arrest.
Discussion

Altogether these data suggest a model in which the Hect-domain E3 ligase Tom1 and the F-box protein Dia2 cooperate to target Cdc6 for ubiquitin-mediated destruction during G1 and the G1/S transition. The activity of Tom1 and Dia2 is distinct from \( SCF^{Cdc4} \) during the G1/S transition. Failure to target Cdc6 for destruction via Tom1 leads to accumulation of chromatin-bound Cdc6 and Mcm4 during S and G2/M. These pathways may contribute to the control of DNA replication as increased nuclear DNA was observed when regulation of ORC and the MCM complex are also compromised.

Based on the genetic data, the relationship between Dia2 and Tom1 is complex and is not fully explained by the suggestion that they function in the same pathway. One explanation that accounts for all observed phenotypes is that Dia2 and Tom1 cooperate to target Cdc6 and that the Cdc4 pathway inhibits the activity of Dia2/Tom1. In this scenario, Dia2 and Tom1 might function in the same pathway, although they could not be strictly dependent on each other because defects in Cdc6 turnover are observed in the single mutants. Alternatively, Dia2 and Tom1 might function separately but promote each other’s activity. For example, Dia2 may enhance the function of Tom1, as the Cdc6 degradation and ubiquitin phenotypes are more severe in the \( tom1\Delta \) mutant than \( dia2\Delta \). Alternatively, it is possible that Tom1 and Dia2 form a larger complex, which is required for efficient association with Cdc6. It is not clear how Cdc4 inhibition of Dia2 or Tom1 might be manifested. It is apparent that Dia2 functions outside the context of an SCF complex in this pathway, as Skp1 is not associated with Cdc6 during G1 or Cdc6 is still turned over in \( scf \) mutants in G1, similar to previous studies (20, 21). A non-SCF role for
Dia2 has not been previously described, but other F-box proteins have been shown to function outside of traditional SCF complexes (24, 29, 91, 99).

It is surprising that three E3 ubiquitin ligases are required to target a single protein for degradation at the same point in the cell cycle. However, previous work has shown that the half-life of Cdc6 is significantly longer in G1 and G2/M than it is at the G1 to S transition (20, 21). It may be that the convergence of three ubiquitin ligase activities at this point in the cell cycle is responsible for the accelerated turnover rate. This leads to the obvious question of how specificity is achieved. My results suggest that Tom1 and Dia2 can bind to a region of Cdc6 that also contains one of the Cdc4-interacting domains, but it seems unlikely that the domain recognized by Cdc4 will precisely match the region recognized by Tom1 and Dia2. Cdc4 has been shown to recognize particular Cdk phosphorylation sites on Cdc6 (21), but it is not clear whether Cdk phosphorylation is important to Cdc6 protein turnover mediated by Tom1 and Dia2. Indeed, it seems unlikely that Cdk phosphorylation will be critical in this pathway, as the degradation also occurs during G1 when Cdk activity levels are low. I look forward to future studies that address the nature of specificity for these ubiquitin ligases.

My results support the idea that the Tom1 and Dia2 might also account for a fraction of the G1-specific degradation of Cdc6. The observation that they act separately from the Cdc4 pathway is consistent with Mode 1 degradation, but investigation of whether Cdk activity affects the activity of Tom1 or Dia2 will be necessary to resolve this question. One possibility is that Cdc6 is targeted for degradation after DNA damage, as Tom1 has been shown to have a role in such a pathway (32) and dia2Δ cells exhibit endogenous
DNA damage (5, 47, 71). However, I think this is unlikely during G1 phase, as dia2Δ cells only accumulate DNA damage foci during S and G2/M (5, 47). Alternatively, it is possible that Tom1 and Dia2 might represent a novel mode of Cdc6 degradation. Indeed, the observation that Cdc6ΔN stability is enhanced in G1 in the absence of Tom1 and Dia2 suggests that there may be still another degradation pathway for Cdc6 in G1.

Failure to clear Cdc6 from origins as cells progress into S phase is likely to have deleterious consequences. In vitro studies have shown that the extent of Mcm2-7 loading onto origins is linked to the amount of Cdc6 present (79). I also observe increased Mcm4 association with two early firing origins in mutants that partially stabilize Cdc6. In addition, inappropriate re-loading of Cdc6 onto origins in G2/M correlates with re-replication when the DNA replication initiation factor Dpb11 is also overexpressed (37), suggesting that origins may be firing more than once per cell cycle. Recent in vitro studies suggest that Cdc6 dissociates from origins in a late pre-RC assembly step that requires ATP hydrolysis (94). My in vivo studies cannot distinguish between the possibility that Cdc6 is rapidly dissociating and re-associating with chromatin in the ubiquitin ligase mutants versus a constant amount of Cdc6 remaining associated with chromatin during S and G2. The Cdc4 and Tom1/Dia2 pathways may provide redundancy to ensure that Cdc6 protein levels are reduced sufficiently to prevent replication defects. Consistent with this, the cdc4-1 and tom1Δ mutants exhibit increased nuclear DNA synthesis in a strain background sensitized to re-replication. It is not clear why the dia2Δ mutant does not exhibit any significant increase in the percentage of cells with greater than 2C DNA content in these assays, but I note that the dia2Δ mutant
exhibits much weaker defects in Cdc6 ubiquitination, degradation and chromatin
association than either $tom1\Delta$ or $cdc4\Delta$. Thus, there may be a threshold level of Cdc6
chromatin association required to observe increased DNA synthesis. Alternatively, $dia2\Delta$
mutants may exhibit only limited DNA synthesis at a subset of origins, which is difficult
to detect by measuring bulk DNA content by flow cytometry.

Future directions

I have identified two E3 ubiquitin ligase components required for Cdc6 degradation
during G1 and at the G1 to S phase transition, the Hect E3 ligase Tom1 and the F-box
protein Dia2. The observation that Cdc6 is stabilized in $dia2\Delta F$-box mutant but not in
$scf$ mutants in G1 suggests that the F-box protein Dia2 might form a non-traditional SCF
complex via the F-box domain. Consistent with this idea, I also observed that Dia2 does
not bind to Skp1 in G1. Budding yeast has three Cullins (Cdc53 (Cul1), Rtt101 (Cul8),
and Cul3). Of these Cullin proteins, both Rtt101 and Cul3 do not require Skp1 as an F-
box protein to form a functional SCF complex (59, 80). Interestingly, $rtt101\Delta$ mutant has
similar phenotypes to $dia2\Delta$ mutant such as the sensitivity of DNA damaging agents or
replication stress, suggesting that Rtt101 might be a promising candidate. To test the
possibility that Dia2 forms an SCF complex with Rtt101, a co-immunoprecipitation assay
would be required to determine whether Rtt101 interacts with Dia2 during G1. In
addition, Cdc6 stability also needs to be examined in $rtt101\Delta$ mutant in G1.

I showed that both Tom1 and Dia2 regulate Cdc6 turnover at the G1 to S phase
transition independently of the SCF$^{Cdc4}$ complex. When hydroxyurea is used to arrest
cells in early S phase, it also activates the S-phase checkpoint since hydroxyurea
treatment lowers dNTP pools, which induces replication stress. To rule out the possibility
that Tom1 and Dia2 target Cdc6 for degradation in response to activation of the S-phase
checkpoint, I examined Cdc6 abundance in tomlΔ and dia2Δ mutants when cells were
released from a G1 arrest into medium. These results show that Cdc6 abundance is
increased in tomlΔ and dia2Δ mutants as cells enter the S phase from the G1 phase of the
cell cycle. To further confirm that Tom1- and Dia2-mediated Cdc6 degradation is distinct
from the SCF<sup>Cdc4</sup> pathway at the G1 to S phase transition, Cdc6 abundance also needs to
be observed in cdc4<sup>-1</sup>tom1Δ and cdc4<sup>-1</sup>dia2Δ mutants when cells are released from a
G1-arrest to rich medium.

My data suggest that both Tom1 and Dia2 bind a domain in Cdc6 downstream of
amino acid 47. This domain contains two Cdk-phosphorylation sites that are recognized
by the SCF<sup>Cdc4</sup> complex. In order to map the binding sites of Tom1 and Dia2 on Cdc6, a
series of Cdc6 truncation mutants need to be generated and tested to identify the
minimum domain required for Tom1 and Dia2 binding using in vitro binding assays.

An important question that needs to be answered in the future is to determine the
specificity of three E3 ubiquitin ligases for Cdc6 degradation. I do not know whether
Tom1 and Dia2 require Cdk-dependent phosphorylation for Cdc6 degradation or not.
However, it is unlikely that Cdk phosphorylation is required for this pathway as Tom1
and Dia2 also control Cdc6 degradation during G1 when Cdk activity is low.
Nevertheless, the requirement of Cdk phosphorylation for Tom1- and Dia2- mediated
pathway needs to be determined by examining the stability of cdc6 phosphorylation

46
mutants carrying various Cdk consensus site mutations in *tom1Δ* and *dia2Δ* strains. Moreover, in vitro binding assays would also be necessary to determine whether Tom1 and Dia2 still bind to Cdc6 phosphorylation mutants.

Finally, I have found that Tom-mediated Cdc6 degradation pathway is important to control re-replication in a strain background sensitized to re-replication. To support this result, 2-D gel and microarray comparative genomic hybridization (CGH) analyses would be required to confirm the re-replication and determine what percentage of early origins are re-fired after the S phase within a single cell cycle in *tom1Δ* mutant sensitized to re-replication.
Figure 11

A

WT

dia2Δ

tom1Δ

vector Cdc6
vector Cdc6
vector Cdc6

B

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C

RT:

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Cdc6

ACT1

1  2  3  4

Percentage of Cdc6 protein

WT
tom1Δ
dia2Δ

0  5  10  20  30
Figure 11. Tom1 and Dia2 control Cdc6 proteolysis. (A) Over-expression of Cdc6 results in an exacerbated growth defect in both dia2Δ and tom1Δ strains. The 10-fold serial dilutions of wild-type, dia2Δ, and tom1Δ cells carrying empty vector or CDC6 under the control of GAL1,10 promoter were spotted onto minimal plates with 2% galactose. Plates were incubated at room temperature for 2-3 days. (B) Cdc6 is partially stabilized in tom1Δ and dia2Δ mutants. Wildtype, tom1Δ, and dia2Δ strains were grown to mid-log phase at 30°C. After cycloheximide treatment (100 μg/ml), samples were taken at the indicated times and immunoblotted with anti-HA and -Pgk1 antibodies. Pgk1 was used as a loading control. (C) Cdc6 mRNA level is not changed in both tom1Δ and dia2Δ mutants. RT-PCR assay was conducted to examine the level of Cdc6 transcript in wildtype, dia2Δ, and tom1Δ strains. ACT1 was used as a loading control. RT, reverse transcriptase.
Figure 12
Figure 12. Clb5 stability is not affected in *tom1Δ* and *dia2Δ* mutants. Wildtype, *tom1Δ*, and *dia2Δ* cells were grown to mid-log phase in minimal medium supplemented with 2% raffinose. Clb5 transcription was induced by addition of galactose for 1h and repressed by adding glucose in the medium. 100 μg/ml cycloheximide was also added to block protein translation. Samples were taken at the indicated times. The turnover of Clb5 protein was analyzed by performing stability assays.
Figure 13
**Figure 13. Tom1 and Dia2 interact with Cdc6.** (A and B) Tom1 Hect domain and Dia2 interact with Cdc6. Hi5 insect cells were co-infected with GST-Cdc6 and FLAG-Hect or Myc-Dia2 baculoviruses. FLAG-Hect, Myc-Dia2 or GST-Cdc6 protein was immunoprecipitated with anti-Flag, -Myc or -GST antibodies and analyzed by immunoblotting. (C) Endogenously expressed Tom1 and Dia2 co-precipitate with Cdc6. Total cell lysates of Tom1\(^{\text{FLAG}}\) Dia2\(^{\text{MYC}}\) or Tom1\(^{\text{FLAG}}\) Dia2\(^{\text{MYC}}\) Cdc6\(^{\text{HA}}\) were immunoprecipitated with affinity matrix anti-HA.11 and immunoblotted with anti-Flag, -Myc, and -HA antibodies. (D) The leucine rich repeat domain of Dia2 is required for its interaction with Cdc6. Total cell lysates from GST-Cdc6 and MYC-Dia2, FLAG-TPR, or FLAG-ΔN214 baculovirus-infected Hi5 insect cells were immunoprecipitated with anti-GST antibodies and analyzed by immunoblotting with anti-GST, -Myc, or -Flag antibodies.
Figure 14

A

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Figure 14. Tom1 and Dia2 bind independently to Cdc6. (A) Dia2 binding to Cdc6 is resistant to DNase I treatment. Cell lysate from the Dia2^{MYC} Cdc6^{HA} strain was treated with 20 units of DNase I (Promega) for 45 min on ice. Cdc6 was immunoprecipitated with anti-HA antibodies and immunoblotted with anti-HA and -Myc antibodies. (B) Tom1 and Dia2 do not require each other to interact with Cdc6 in S phase. The indicated strains were arrested with 200 mM hydroxyurea for 3 h. Cell lysates were used for immunoprecipitation assays with anti-HA antibodies and analyzed by immunoblotting with anti-HA, -Myc, and –Flag antibodies.
Figure 15. Tom1 and Dia2 control Cdc6 turnover at the G1/S transition. (A) Cdc6 abundance is increased in *tom1Δ* and *dia2Δ* mutants at the G1/S transition. Wildtype, *tom1Δ*, and *dia2Δ* strains were arrested with αF for 3 h and released into rich medium containing 15 μg/ml nocodazole. Samples were collected at the indicated times and immunoblotted with anti-HA antibodies. Pgk1 was used as a loading control. Flow cytometry analysis was performed to monitor the release from αF arrest. (B) Cdc6 is stabilized in both *tom1Δ* and *dia2Δ* mutants at the G1/S transition and Cdc6 stabilization is significantly increased in *cdc4-1 tom1Δ* and *cdc4-1 dia2Δ* mutants. The indicated strains were arrested with αF for 3 h and released into rich medium containing 200 mM hydroxyurea for 90 min. Cultures were shifted to 37 °C for 30 min and treated with cycloheximide (100 μg/ml). Samples were taken at the indicated times and analyzed by immunoblotting with anti-HA antibodies. Pgk1 was used as a loading control. Three independent experiments were used to quantify the rate of Cdc6 turnover. Error bars indicate standard deviations. (C) Cdc6 turnover rate in *tom1Δ dia2Δ* mutant is indistinguishable from the *tom1Δ* or *dia2Δ* mutant. The results of quantification of the *tom1Δ dia2Δ* mutant and *tom1Δ* and *dia2Δ* mutants are shown in the graph.
Figure 16

A

WT
cdc4-1
tom1Δ
dia2Δ
cdc4-1 tom1Δ
cdc4-1 dia2Δ
tom1Δ dia2Δ
cdc4-1 tom1Δ dia2Δ

150mM HU

0.003% MMS

B

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Cdc6^HA
Pgk1

HU
Asy
1C 2C
1C 2C
1C 2C
Figure 16. Cdc4, Tom1 and Dia2 all contribute to Cdc6 protein turnover. (A) Hydroxyurea- and MMS-sensitivity is exacerbated in *cdc4-1 tom1Δ, cdc4-1 dia2Δ*, and *cdc4-1 tom1Δ dia2Δ* mutants. Five-fold serially diluted cultures of the indicated strains were spotted onto rich medium containing 150 mM hydroxyurea or 0.003% Methyl methanesulfonate (MMS). (B) The *cdc4-1 tom1Δ dia2Δ* triple mutant exhibits highly stabilized Cdc6. The indicated mutants were arrested with 200 mM hydroxyurea for 3h and shifted to 37°C for 30 min. Samples were collected at the indicated times and prepared as in Figure 15. Error bars indicate standard deviations.
Figure 17

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Cdc6<sup>NA</sup> and Pgk1 expression over time for different genotypes.

Graph showing the percentage of Cdc6 protein over time for different genotypes.
Figure 17. Tom1 and Dia2 control Cdc6 proteolysis in G1. Cdc6 is stabilized in both

tom1Δ and dia2Δ mutants in G1. The indicated strains were arrested with αF for 3 h and
shifted to 37°C for 30 min. Samples were prepared for stability assays as in Figure 15.
Three independent experiments were used for quantification. Error bars indicate standard
deviations.
Figure 18

A

B

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Y

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Figure 18. Tom1 and Dia2 target Cdc6 for ubiquitin-dependent degradation. (A) The catalytic activity of Tom1 Hect domain and the F-box domain of Dia2 are required for Cdc6 turnover. Wildtype, \textit{tom1C3235A}, \textit{dia2ΔF} strains arrested with $\alpha F$ were used in protein stability assays. Samples were collected at indicated times after cycloheximide addition. Pgk1 was used as a loading control. Three independent experiments were used for quantification. (B) The TPR mutant stabilizes Cdc6. Samples from wildtype and TPR strains were prepared as in (A). The asterisk indicates a non-specific band. Error bars indicate standard deviations. (C) Cdc6 ubiquitination is dependent on Tom1 and Dia2. Wildtype, \textit{tom1Δ}, and \textit{dia2Δ} strains were cultured to mid-log phase and treated with DMSO or 50 $\mu$M MG132 for 2h. Cdc6 protein was immunoprecipitated with anti-HA antibodies and visualized by immunoblot assays with anti-ubiquitin antibodies. The asterisk indicates a non-specific band. (D) In vitro ubiquitination of Cdc6. GST-Cdc6 protein expressed from baculovirus-infected insect cells was purified using glutathione-sepharose 4B beads. GST-Cdc6 protein was incubated with ubiquitin, E1, E2, ATP, an ATP regeneration system, and fractionated yeast extracts purified from wildtype, \textit{cdc4-1}, \textit{tom1Δ}, or \textit{dia2Δ} strains at 30 ºC for 45min. Samples were run on 6% SDS-PAGE and immunoblotted with anti-GST antibodies.
Figure 19

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B

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Figure 19. Cdc6 degradation in G1 is not dependent on SCF. (A) Cdc6 is not stabilized in scf mutants in G1 phase. Wildtype, skp1-11, cdc53-1, and cdc34-2 cells were arrested with αF for 3h and stability assays were performed as in Figure 17. (B) Cdc6 does not bind to Skp1 in G1 phase. Strain DKY559 was arrested with αF for 3h and treated with 50μM MG132 for 2h. Cell lysates were immunoprecipitated with anti-HA antibodies and analyzed by immunoblotting with anti-HA, -Myc, -Skp1 antibodies. Cdc6 input was not detected in cell extract with a short exposure. Asterisk indicates the IgG heavy chain.
Figure 20

A

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Percentage of Cdc6 protein over time.
Figure 20. Tom1- or Dia2-mediated Cdc6 proteolysis does not require the N-terminal 47 amino acids of Cdc6. (A) Cdc6ΔN stabilization was increased in tom1Δ and dia2Δ mutants in G1. Wildtype, cdc6ΔN, cdc6ΔN tom1Δ, and cdc6ΔN dia2Δ strains arrested with αF were used for stability assays. Three independent experiments were used for quantification. (B) Cdc6ΔN was significantly stabilized in tom1Δ and dia2Δ mutants at G1/S. Wildtype, cdc6ΔN, cdc6ΔN tom1Δ, and cdc6ΔN dia2Δ strains were arrested in early S and stability assays were performed as shown in Figure 15B. The rate of full-length Cdc6 and Cdc6ΔN turnover was measured by quantifying three independent results. (C) Cdc6ΔN binds to Tom1 Hect domain and Dia2. Total lysates isolated from Hi5 insect cells infected with GST- Cdc6ΔN, FLAG-Hect, and Myc-Dia2 baculoviruses were incubated together as shown in the figure. FLAG-Hect or Myc-Dia2 was immunoprecipitated with anti-Flag, or -Myc antibodies and analyzed by immunoblotting.
**Figure 21. Failure to degrade chromatin-bound Cdc6 by a Tom1- and Dia2-dependent pathway enhances the association of Mcm4 with early origins.** (A)

Chromatin-bound Cdc6 is increased in *cdc4-1 sic1Δ, tom1Δ* and *dia2Δ* cells. The indicated strains were arrested with αF at 30°C for 2.5 h and shifted to 37°C for 30 min before release into rich medium containing 15μg/ml nocodazole at 37°C. Samples were taken at the indicated times and prepared for chromatin fractionation. Samples were analyzed by immunoblot assays with anti-HA, -Pgk1, and -Histone H3 antibodies. Flow cytometry was used to monitor cell cycle progression. WCE, whole cell extract. The ratio of chromatin-bound Cdc6 to Histone H3 was measured using Image J software. (B)

Mcm4 origin association is increased in *tom1Δ* and *dia2Δ* cells. The indicated strains were arrested and released as in (A). Samples were taken at the indicated times and prepared for chIP assays with HA-tagged Mcm4. *ARS1* and *ARS305* are early firing origins. *ACF2* is non-origin control. Three replicates of each experiment were quantified using Image J. Error bars indicate standard deviations.
Figure 22
Figure 22. Cells lacking Cdc4 and Tom1 exhibit aberrant DNA content. Increased DNA synthesis occurs in \textit{cdc4-1} and \textit{tom1}\(\Delta\) mutants. \textit{cdc4-1}, \textit{tom1}\(\Delta\), \textit{dia2}\(\Delta\), and \textit{cdc6}\(\Delta N\) mutants were generated in the strain with non-phosphorylatable ORC and the constitutive nuclear localization of the Mcm2-7 complex. Cells were arrested with 15µg/ml nocodazole for 3h and shifted to 37°C. Re-replication was analyzed by flow cytometry analysis. \textit{orc* mcm*} refers to \textit{orc2 6A orc6 4A MCM7-2NLS}. The percentage of the cells with more than 2C DNA content was measured using Flow Jo software. >2C indicates the percentage of cells containing more than 2C DNA content.
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**Table II. Oligonucleotides used in Chapter II**

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CHAPTER III

The Hect E3 ubiquitin ligase Tom1 controls Dia2 degradation during the cell cycle

The ubiquitin proteasome system plays a pivotal role in controlling the cell cycle. The budding yeast F-box protein Dia2 is required for genomic stability and is targeted for ubiquitin-dependent degradation in a cell cycle-dependent manner, but the identity of the ubiquitination pathway is unknown. I demonstrate that the Hect domain E3 ubiquitin ligase Tom1 is required for Dia2 protein degradation. Deletion of DIA2 partially suppresses the temperature-sensitive phenotype of tom1 mutants. Tom1 is required for Dia2 ubiquitination and degradation during G1 and G2/M phases of the cell cycle, whereas during S phase, the Dia2 protein is stabilized. I find that Tom1 binding to Dia2 is enhanced in G1 and reduced in S phase, suggesting a mechanism for this proteolytic switch. Tom1 recognizes specific positively charged residues in a Dia2 degradation/NLS domain. Loss of these residues blocks Tom1-mediated turnover of Dia2 and causes a delay in G1 to S-phase progression. Deletion of DIA2 rescues a delay in the G1 to S-phase transition in the tom1Δ mutant. Together, these results suggest that Tom1 targets Dia2 for degradation during the cell cycle by recognizing positively charged residues in the Dia2 degradation/NLS domain and that Dia2 protein degradation contributes to G1 to S-phase progression.
Introduction

The highly conserved ubiquitin proteasome system (UPS) plays a role in a number of cellular processes including cell cycle control, DNA replication, and DNA damage response (50, 65, 85). In budding yeast, the F-box protein Dia2 serves as an adaptor for a multicomponent SCF (Skp1/Cdc53/F-box protein) E3 ubiquitin ligase complex and functions to maintain genomic stability (5, 47, 71). Dia2 assembles with the components of the replisome complex and regulates the progression of the DNA replication fork (60, 62), suggesting a role in controlling the S-phase progression.

Dia2 itself is an unstable protein targeted for degradation by the UPS (45, 60). There are conflicting reports about the pathway responsible for Dia2 protein turnover. One study suggests that Dia2 degradation is the result of an autoubiquitination pathway (60), similar to other F-box proteins (52, 101). By contrast, our lab previously indicates that an SCF-independent pathway targets Dia2 for degradation, as deletion of the F-box domain required for binding the core SCF complex does not stabilize the protein. Rather, a 20-amino acid motif that overlaps with a nuclear localization sequence (NLS)-containing domain was found to be required for Dia2 protein turnover (45). However, the identity of the ubiquitin ligase responsible for targeting Dia2 for degradation via this domain was not determined.

The Dia2 protein turnover rate varies throughout the cell cycle. Dia2 is at its most unstable during G1 phase, is moderately unstable during G2/M and is substantially stabilized in S phase. Dia2 protein stabilization is dependent on the activation of the S-phase checkpoint pathway (45). One model that explains these observations is that Dia2
is stabilized after checkpoint activation so that the SCF\(^{\text{Dia2}}\) complex may assemble to
target S-phase specific proteins for degradation. Two identified targets include
replication proteins Mrc1 and Ctf4, although no physiological role for their degradation
has yet been established (60).

In this chapter, I identify the E3 ubiquitin ligase that targets Dia2 for ubiquitin-
mediated degradation and describe how Dia2 is recruited to the degradation pathway.
Moreover, I show that failure to degrade Dia2 leads to a cell cycle progression defect.

*Tom1 targets Dia2 for ubiquitin-dependent degradation*

To identify the E3 ubiquitin ligase that targets Dia2 for degradation, I carried out a
candidate approach. Based on the literature, the Hect-domain E3 ubiquitin ligase Tom1
was a promising candidate, as it has been shown to interact with Yra1 (40), a protein
required for Dia2 association with chromatin (90), and Rad53, a S-phase checkpoint
kinase (40). I first asked whether there was a genetic relationship between Tom1 and
Dia2 by examining the growth of wildtype, *tom1Δ*, *dia2Δ*, and *tom1Δ dia2Δ* strains at
30°C and 37°C. Interestingly, the temperature-sensitive phenotype of *tom1Δ* mutant was
partially suppressed by deletion of *DIA2* at 37°C (Figure 23A). The *dia2Δ* mutant also
partially rescued the temperature-sensitive phenotype of the *tom1C3235A* mutant in
which the catalytic cysteine is replaced with alanine. These results suggest that the
temperature-sensitive phenotype of *tom1Δ* and *tom1C3235A* mutants may be due in part
to aberrant accumulation of the Dia2 protein. If this were the case, I would expect that
overexpression of *DIA2* in *tom1Δ* cells would also lead to a growth defect more
significant than the mild defect observed when \textit{DIA2} is overexpressed in wildtype cells. To test this, \textit{DIA2} was overexpressed in wildtype, \textit{tom1\textDelta}, and \textit{tom1C3235A} mutants using the \textit{GAL1,10} galactose-inducible promoter. Under these conditions, overexpression of \textit{DIA2} caused a stronger growth defect in \textit{tom1\textDelta} and \textit{tom1C3235A} mutants than in wildtype cells (Figure 23B).

To determine whether Tom1 and Dia2 physically interact, I performed an \textit{in vitro} binding assay using the Flag-tagged Hect domain of Tom1 and Myc-tagged Dia2 expressed from insect cells. When the Flag-tagged Hect domain of Tom1 was purified, Myc-tagged Dia2 co-precipitated. Reciprocal co-purification of the Flag-tagged Hect domain of Tom1 was observed when Myc-tagged Dia2 was immunoprecipitated (Figure 23C).

The most straightforward explanation for these results is that Tom1 targets Dia2 for ubiquitin-dependent degradation. To ask whether Tom1 is a required for Dia2 protein degradation, I first assessed the turnover of the Dia2 protein in \textit{tom1} mutants using a cycloheximide stability assay. Wildtype, \textit{tom1\textDelta}, and \textit{tom1C3235A} cells were grown to mid-log phase and Dia2 protein abundance was measured over time. I observed that Dia2 was partially stabilized in both \textit{tom1\textDelta} and \textit{tom1C3235A} mutants compared to wildtype (Figure 24A). Notably, Dia2 mRNA abundance was not changed in the \textit{tom1\textDelta} mutant (Figure 24B), indicating that the change in Dia2 protein levels was not the result of transcriptional regulation. To examine Dia2 ubiquitination, I developed an \textit{in vitro} ubiquitination assay using fractionated yeast extracts and GST-Dia2 purified from insect cells. When extracts from wildtype cells were incubated with GST-Dia2 in the presence
of E1, an ATP regeneration system, and ubiquitin, Dia2 ubiquitin conjugates were observed (Figure 24C, lane 3). However, Dia2 ubiquitin conjugates were significantly reduced when extracts from the \textit{tom1}Δ strain were used (Figure 24C, lane 5), indicating that Dia2 ubiquitination is dependent on Tom1. Together, these results suggest that Tom1 targets Dia2 for ubiquitin-mediated degradation.

\textit{Tom1 controls Dia2 proteolysis during G1 and G2/M}

Dia2 is unstable during G1 and G2/M phases of the cell cycle but is stabilized during S phase or in response to activation of the S-phase checkpoint (45). I investigated when during the cell cycle Tom1 controls Dia2 turnover by performing stability assays in cells synchronized in G1 by alpha factor (αF), early S phase by hydroxyurea (HU), which also activates the S-phase checkpoint, or at metaphase with nocodazole. As shown in Figure 25A, Dia2 was turned over in wildtype but stabilized in \textit{tom1}Δ and \textit{tom1}C3235A mutants in G1. By contrast, no significant turnover of Dia2 was observed in \textit{tom1}Δ and \textit{tom1}C3235A mutants in cells arrested with HU (Figure 25B). However, I found that Dia2 was partially stabilized in \textit{tom1}Δ and \textit{tom1}C3235A mutants in cells arrested by nocodazole (Figure 25C), suggesting that Tom1 is required for Dia2 proteolysis during G2/M. I conclude that degradation of Dia2 during G1 and G2/M phases of the cell cycle is dependent on Tom1.

The mechanistic basis for how Dia2 is stabilized in response to initiation of S-phase or S-phase checkpoint activation is not known. With the identification of Tom1 as the E3 ubiquitin ligase that targets Dia2 for degradation, one possible explanation is that Tom1
and Dia2 no longer interact during S phase or in response to S-phase checkpoint activation. To test this idea, I conducted co-immunoprecipitation assays in synchronized cells (Figure 25D). Cells expressing Myc-tagged Dia2 and Flag-tagged Tom1 from their endogenous loci were arrested in late G1 with αF, early S phase with HU, and G2/M phase with nocodazole. When Flag-tagged Tom1 was immunoprecipitated from αF-arrested cells, I observed significant co-precipitation of Myc-tagged Dia2. However, this co-precipitation was almost completely absent in HU-arrested cells. By contrast, I observed a modest interaction between Tom1 and Dia2 in nocodazole-arrested cells. Thus, inhibition of the interaction between Tom1 and Dia2 may explain the proteolytic switch observed as cells enter S phase or when the S-phase checkpoint is activated.

Tom1 recognizes a stretch of positively charged residues in Dia2

Our lab has previously shown that a domain just upstream of the F-box in Dia2, which also contains two canonical nuclear localization sequences, was required for Dia2 protein turnover. Although nuclear localization is required for Dia2 degradation, this domain is also likely important for recognition by the degradation pathway as the addition of an exogenous NLS to a Dia2 mutant lacking this domain is still stabilized (45). I hypothesized that Tom1 may bind to this domain in Dia2 so I tested whether various truncated Dia2 mutants are able to co-immunoprecipitate Tom1 (Figure 26). I generated strains expressing epitope-tagged Tom1 and the Dia2 mutants expressed from their endogenous loci (Figure 26, upper left panel). As shown, Tom1 and full-length Dia2 co-immunoprecipitate. The Dia2 mutants used include a mutant containing only the
N-terminal TPR region (*TPR*), a mutant in which the F-box domain was deleted in frame (*ΔF-box*), a mutant that lacks the first 149 amino acids of the N-terminus (*ΔN149*), and a mutant with the NLS/degradation domain deleted in frame but containing a exogenous SV40 Tag NLS attached to the N-terminus (*SVΔNLS*). In the co-immunoprecipitation assay, all Dia2 proteins except the SVΔNLS mutant co-purify with Tom1, indicating that the NLS domain of Dia2 is required for its interaction with Tom1.

The domain in Dia2 required for degradation consists of two stretches of lysine residues interrupted by a nine-residue sequence. My co-immunoprecipitation results were unable to determine which sections of this domain were required for recruitment to Tom1. I speculated that there were three possibilities: 1) Tom1 may recognize the lysine residues, perhaps even use them as sites of ubiquitin conjugation, 2) Tom1 may recognize the nine-residue sequence in between the lysines, or 3) Tom1 recognizes positively charged residues. To distinguish between these possibilities, I generated three mutants, one in which the lysines were changed to arginines to conserve charge (Dia2-KR), one in which the lysines were mutated to alanines (Dia2-KA) and one in which the nine-residues middle sequence was deleted in frame (Dia2Δ185-193) (Figure 27A). For the Dia2-KA and Dia2Δ185-193 proteins, I also added the SV40 Tag NLS to the N-terminus to avoid altering the nuclear localization of Dia2 (SVDia2-KA, SVDia2Δ185-193). I then examined the stability of these proteins using the cycloheximide stability assay in G1-arrested cells. As shown in Figure 27B, the turnover rates of wildtype Dia2 and the Dia2-KR mutant were indistinguishable, indicating that exchanging the lysines with arginines has no effect on Dia2 protein turnover. Intriguingly, the SVDia2-KA mutant protein was
significantly stabilized in αF-arrested cells, whereas both the SVDia2 and SVDia2Δ185-193 proteins were turned over with similar rates (Figure 27C). These results indicate that it is not the lysine residues per se, but rather the positive charge of these residues in the Dia2 degradation domain that are necessary for protein turnover.

Based on the stability assay results, I predicted that the positively charged residues in the NLS/degradation domain would be required for Tom1 to bind Dia2. To test this, I carried out a co-immunoprecipitation assay using strains expressing epitope-tagged Dia2-KR or SVDia2-KA proteins and Flag-tagged Tom1. I observed that Flag-tagged Tom1 co-precipitated with Dia2-KR but not with the SVDia2-KA protein (Figure 27D). Together, these results suggest that Tom1 specifically recognizes the positive charge on the lysine residues in the Dia2 NLS/degradation domain.

*Defects in Dia2 protein turnover lead to a G1 to S phase progression delay*

If degradation of Dia2 via Tom1 is the major pathway for Dia2 degradation, I would expect that overexpression of a Dia2 mutant unable to bind Tom1 would produce a stronger overexpression phenotype than full-length Dia2 in wildtype cells. In addition, if Tom1 only recognizes the positively charged residues in the Dia2 degradation/NLS domain, I would also expect that overexpression of the *SV dia2-KA* mutant would be indistinguishable from the *DIA2* overexpression phenotype in *tom1Δ* cells. To test these predictions, I examined the overexpression phenotypes of full-length *DIA2, dia2-KR, SV dia2-KA*, and *SV dia2-Δ185-193* mutants in wildtype and *tom1Δ* cells (Figure 28A). I observed that both wildtype and *tom1Δ* cells overexpressing the *dia2-KR* mutant behaved
as the parental strains overexpressing full-length DIA2. By contrast, overexpression of the SV dia2-KA mutant impaired the growth of wildtype cells compared to cells overexpressing full-length SV DIA2 or SV dia2-ΔI85-193. Moreover, the growth defect of tom1Δ cells caused by DIA2 overexpression was indistinguishable from overexpression of the SV dia2-KA mutant and looked similar to the results when SV dia2-ΔI85-193 is overexpressed (Figure 28A). These results suggest that Tom1 is the primary E3 ubiquitin ligase that controls Dia2 protein turnover, recognizing specific positively charged residues in the degradation/NLS domain.

Our lab has previously reported that the stabilized forms of dia2 mutants such as SVΔ214 and SVNLS modestly increased the percentage of G1 cells in an asynchronous population (45). Therefore, I would expect a similar phenotype to be observed in the SV dia2-KA mutant. To test this, wildtype cells carrying overexpression constructs for SV DIA2 or SV dia2-KA were grown to mid-log phase and their cell cycle distributions were measured by flow cytometry (Figure 28B). Consistent with previous results, overexpression of SV DIA2 showed a modest increase in G1 cells relative to cells carrying an empty vector. Furthermore, cells expressing SV dia2-KA exhibited a significant increase in the G1 population. These results suggested that the dia2-KA mutant may transit more slowly through G1 phase into S phase than wildtype cells (Figure 28B). To test this possibility, the same strains were arrested with αF and released into medium containing nocodazole to monitor cell cycle progression from late G1 to metaphase (Figure 28B). Cells overexpressing the SV DIA2 exhibited a mild delay in the G1 to S phase transition as compared to the control cells. In addition, overexpression of
the SV dia2-KA mutant prolonged the delay in G1 to S phase progression (Figure 28B), suggesting that Dia2 protein turnover is important for a proper progression from G1 to S. I further asked whether the tom1Δ mutant also exhibits the same delay in the G1 to S phase transition as would be expected if Dia2 is a significant target of Tom1. Wildtype, tom1Δ, dia2Δ, and tom1Δ dia2Δ strains arrested with αF were released into rich medium containing nocodazole to monitor cell cycle progression from late G1 to metaphase (Figure 28C). Under these conditions, the tom1Δ mutant proceeded into S-phase from G1 phase with a 5-minute delay, compared to wildtype and the dia2Δ mutant. Interestingly, the delay in G1 to S-phase progression observed in the tom1Δ mutant was rescued by deletion of DIA2 (Figure 28C). These findings indicate that Tom1-mediated Dia2 turnover in G1 is required for an efficient G1 to S phase transition.

Discussion

Altogether, these results suggest that the Hect-domain E3 ubiquitin ligase Tom1 recruits Dia2 for degradation during G1 and G2/M phases of the cell cycle by recognizing specific positively charged residues just upstream of the F-box domain. Failure to efficiently degrade Dia2 leads to defects in cell cycle dynamics.

These data suggest that the proteolytic inhibition of Dia2 degradation that occurs as cells enter S phase or activate the S-phase checkpoint depends on reduced binding between Dia2 and Tom1. In principle, such an outcome could be the result of a conformational change in Dia2, such as those that accompany post-translational modifications, although there is currently no evidence for S-phase specific modification
of Dia2. Alternatively, Tom1 has additional substrates and interaction partners that may compete for binding to Tom1 more effectively during S phase or in response to activation of the S phase checkpoint. Interestingly, Tom1 targets excess histones for degradation when they are phosphorylated by the S-phase checkpoint kinase Rad53. Moreover, Tom1 physically interacts with Rad53 (86), suggesting that it is possible this role of Tom1 might influence its behavior during checkpoint activation. Future studies will be necessary to distinguish between these possibilities.

How Tom1 binds and recruits target proteins is an open question. My results suggest that a short stretch of positively charged amino acids in Dia2 are required to bind Tom1. This domain may serve as a degron for targeting substrates to Tom1. The two other known targets of Tom1 include histone H3 (86) and Yra1, an mRNA export factor (40). Both proteins contain short stretches of positively charged amino acids, although neither shows a similarly spaced arrangement of residues, with the intervening sequence, as observed in Dia2 (D.H. Kim and D.M. Koepp, unpublished observations). It is possible that a single stretch of 3-4 positively charged residues on either side of the intervening sequence is sufficient for interaction with Tom1 or that the spacing of the positive charges does not play a role in recognition. Indeed, deletion of the intervening sequence does not alter Dia2 degradation kinetics, consistent with the idea that spacing of the residues does not play a role in recognition. I look forward to future studies investigating whether the motif I have identified serves as a recognition domain for other Tom1 targets.
Previous work suggested a role for Dia2 degradation in cell cycle dynamics (45). The results presented here confirm and extend those observations, showing that impaired degradation of Dia2 leads to a delay of the G1/S phase transition. However, the mechanism by which accumulated Dia2 slows down the G1 to S transition is not clear. One explanation might be that Dia2 has an as yet unidentified ubiquitination target that promotes G1 progression or S-phase entry. In this scenario, excess Dia2 would lead to inappropriate degradation of this unknown target. Alternatively, excess Dia2 may simply lead to change in the balance of various SCF complexes by competing for core component proteins. For example, the relative concentration of those SCF complexes that have G1 ubiquitination substrates, such as SCF$^{Cdc4}$, which targets the Cdk inhibitor Sic1 and the DNA replication protein Cdc6 (20, 88, 95), may be reduced. Regardless of the mechanism, these observations suggest that Dia2 protein levels are modulated throughout the cell cycle to prevent adverse effects on cell cycle progression.

In summary, I have identified a novel degradation pathway for the F-box protein Dia2 involving the Hect domain E3 ligase Tom1. This study establishes a mechanistic basis for recognition of Dia2 by Tom1 and demonstrates a role for Dia2 degradation in cell cycle dynamics.

**Future directions**

I have shown that the Hect E3 ligase Tom1 targets Dia2 for ubiquitin-dependent degradation during G1 and G2/M. In vitro ubiquitination assay showed that Dia2 ubiquitination is dependent on Tom1. However, I could not identify the E2 responsible
for Dia2 ubiquitination. Since this assay used the fractionated yeast extracts containing multiple E2 proteins, the E2 enzyme required for Dia2 ubiquitination needs to be identified. In order to address this question, Dia2 stability needs to be examined in those E2 mutants. Once the E2 is identified, an in vitro ubiquitination assay using purified E1, E2, Tom1, Dia2, ubiquitin, and ATP needs to be conducted in the future. I also showed that Tom1 binding to Dia2 is reduced as cells enter the S phase or activate the S-phase checkpoint. As suggested in discussion, Tom1 physically interacts with Rad53 in response to replication stress (86), implying that Tom1 binding to Dia2 is decreased because Tom1 might have a high affinity for Rad53 binding in response to activation of the S-phase checkpoint. To test this possibility, a co-immunoprecipitation assay would be required to determine whether binding of Tom1 to Dia2 is increased in rad53-21 mutant during S phase or in response to activation of the S-phase checkpoint. Likewise, it is also important to examine whether the interaction between Tom1 and Rad53 is reduced by overexpression of Dia2 upon activation of the S-phase checkpoint.

My results suggest that the specific positively charged residues in the degradation/NLS domain of Dia2 are required for the interaction between Tom1 and Dia2. If this domain were critical for Tom1 to recognize its potential substrates, I would expect that this domain might serve as a Tom1 recognition site in other Tom1 substrates. Searching for proteins that contain a domain or motif similar to this would be critical to identify potential substrates of Tom1. Furthermore, identification of Tom1 targets involved in cell cycle progression will help better understand how cell cycle dynamics are regulated by Tom-mediated proteolysis.
I also observed that excess Dia2 in G1 causes a delay in the G1 to S-phase progression. If excess Dia2 alters the balance of SCF complexes in G1, the abundance of Sic1 which is targeted by the SCF$^{\text{Cdc4}}$ complex for degradation might be increased by overexpression of Dia2 or in $\text{tom1}^\Delta$ mutant during G1. To address this question, Sic1 abundance or stability needs to be observed when Dia2 is overexpressed or in $\text{tom1}^\Delta$ mutant in G1.

What role Dia2 plays in during S phase or in response to activation of the S-phase checkpoint is an unanswered question. Given that Dia2 is stabilized during S phase or upon replication stress, Dia2 might have a role in stabilizing replication fork or controlling the progression of replication fork in response to replication stress by assembling the SCF$^{\text{Dia2}}$ complex and targeting replication or S-phase proteins for degradation. Therefore, identification of Dia2 substrates during S phase or in the presence of replication stress will help us better understand the mechanistic role of Dia2 in DNA replication or activation of the S-phase checkpoint.
Figure 23

A

30°C

37°C

WT
tom1Δ
dia2Δ
tom1Δ dia2Δ
tom1C3235A
tom1C3235A dia2Δ

B

Vector
DIA2
Vector
DIA2
Vector
DIA2

C

Dia2MYC

IP: FLAG INPUT

- + - + : Hect FLAG

Dia2 MYC Hect

1 2 3 4

Hect FLAG

1 2 3 4

IP: MYC INPUT

- + - + : Dia2 MYC

Dia2 MYC Hect

1 2 3 4
Figure 23. Tom1 genetically and physically interacts with Dia2. (A) The temperature-sensitive phenotype of $tom1\Delta$ and $tom1C3235A$ mutants is partially suppressed by deletion of $DIA2$. The indicated strains were grown to mid-log phase and spotted in 10-fold serial dilutions onto rich medium plates. The plates were incubated at 30°C and 37°C for 2 days. (B) Overexpression of $DIA2$ results in a growth defect in $tom1\Delta$ and $tom1C3235A$ mutants. The 10-fold serial dilutions of wild-type, $tom1\Delta$, and $tom1C3235A$ cells carrying empty vector or $DIA2$ under the control of $GAL1,10$ promoter were spotted onto minimal plates with 2% galactose. Plates were incubated at 30°C for 2-3 days. (C) The Hect domain of Tom1 binds to Dia2. Hi5 insect cells were co-infected with Flag-Tom1 Hect domain and Myc-Dia2 baculoviruses. Flag-Tom1 Hect domain or Myc-Dia2 protein was immunoprecipitated with anti-Flag or -Myc antibodies and analyzed by immunoblotting. (-) and (+) represent uninfected and infected with the indicated baculoviruses. Anti-Flag or –Myc antibodies were added to the indicated immunoprecipitation assays.
Figure 24

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Dia2

Pgk1

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DIA2

ACT1

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Dia2-Ubn

GST-Dia2
Figure 24. **Tom1 is required for ubiquitin-dependent Dia2 degradation.** (A) Dia2 is partially stabilized in $tom1\Delta$ and $tom1C3235A$ mutants. Wildtype, $tom1\Delta$ and $tom1C3235A$ cells were grown to mid-log phase and treated with cycloheximide (100 $\mu$g/ml). Samples were taken at the indicated times and processed for stability assay. Immunoblotting was performed with anti-Myc and -Pgk1 antibodies. Pgk1 was used as a loading control. (B) Dia2 mRNA levels are not changed in the $tom1\Delta$ mutant. RT-PCR was conducted to examine the level of Dia2 transcript in wildtype and $tom1\Delta$ strains. ACT1 was used as a loading control. RT, reverse transcriptase. (C) **In vitro** ubiquitination of Dia2. GST-Dia2 protein expressed from baculovirus-infected insect cells was purified using glutathione-sepharose 4B beads. GST-Dia2 protein was incubated with ubiquitin, E1, ATP, an ATP regeneration system, and fractionated yeast extracts purified from wildtype or $tom1\Delta$ strains at 30°C for 45min. Samples were run on 6% SDS-PAGE and immunoblotted with anti-GST antibodies.
Figure 25

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*Tom1* 

Bound Dia2 / IP'd Tom1

0.39  0.05  0.12

*Tom1* Flag

-  +  -  +  +  : TOM1 Flag

IP : FLAG

Tom1 Flag

Dia2 YFC

INPUT

Tom1 Flag

Dia2 YFC
Figure 25. **Tom1 regulates Dia2 turnover in G1 and G2/M.** (A) Dia2 stabilization in \textit{tom1\textDelta} and \textit{tom1C3235A} mutants in G1 phase. Wildtype, \textit{tom1\textDelta} and \textit{tom1C3235A} strains were arrested with $\alpha$F for 3 h. After cycloheximide treatment (100 $\mu$g/ml), samples were collected at the indicated times and immunoblotted with anti-Myc antibodies. Pgk1 was used as a loading control. Flow cytometry analysis was performed to monitor the $\alpha$F arrest. Dia2 protein turnover was quantified with three independent experiments. Error bars indicate standard deviations. (B) Dia2 is stable in S phase. The indicated strains were arrested with 200 mM hydroxyurea for 3 h. Samples were taken at the indicated times after cycloheximide treatment (100 $\mu$g/ml) and analyzed by immunoblotting with anti-Myc antibodies. Pgk1 was used as a loading control. Three independent results were used to quantify the rate of Dia2 turnover. Error bars indicate standard deviations. The HU arrest was monitored by flow cytometry. (C) Dia2 is stabilized in \textit{tom1\textDelta} and \textit{tom1C3235A} mutants in G2/M phase. Wildtype, \textit{tom1\textDelta} and \textit{tom1C3235A} cells were arrested with nocodazole (15$\mu$g/ml) for 3 h. Stability assays were performed as in (A and B). The results of quantification of Dia2 turnover in wildtype, \textit{tom1\textDelta} and \textit{tom1C3235A} strains are shown in the graph. Error bars indicate standard deviations. Flow cytometry was used to examine the nocodazole arrest. (D) Dia2 binding to Tom1 is regulated during the cell cycle. Dia2\textsuperscript{MYC} and Tom1\textsuperscript{FLAG} Dia2\textsuperscript{MYC} cells were arrested with $\alpha$F, hydroxyurea, and nocodazole for 3 h as in (A, B, and C), respectively. Total cell lysates (2mg) of the indicated strains were immunoprecipitated with anti-Flag antibodies and immunoblotted with anti-Flag and -Myc antibodies. The ratio of bound Dia2 to immunoprecipitated
Toml was measured using Image J software. Flow cytometry was conducted to monitor the arrests in G1, S, and G2/M.
Figure 26
**Figure 26. Tom1 binds to Dia2 through the degradation/NLS domain of Dia2.**

Endogenously expressed Tom1 and Dia2 co-precipitate each other via the degradation/NLS domain of Dia2. The indicated strains were grown to mid-log phase. 2 mg of total cell lysates were immunoprecipitated with anti-Flag antibodies and immunoblotted with anti-Flag and -Myc antibodies.
Figure 27

A

AKKKNNNVLSPPKKIKG  WT
AKRRRRNVLSPRRRIRG  KR
AAAAANNVLSPAAAIAAG  KA
AKKKK*********KKKIKG  Δ185-193

B

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Dia2 MYC

Pgk1

C

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Dia2 MYC

Pgk1

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<td>INPUT</td>
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</tr>
<tr>
<td>TOM1 FLAG</td>
<td>-</td>
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Tom1 FLAG

Dia2 KR MYC

Dia2 SV KA MYC
Figure 27. Tom1-mediated Dia2 proteolysis requires positively charged residues in the degradation domain of Dia2. (A) Domain containing the degradation/NLS region of Dia2. (B) Substitution of lysine for arginine does not stabilize Dia2 in G1. Wildtype and dia2-KR strains were arrested with αF for 3 h. Samples were taken at the indicated times and prepared for stability assay as described in Figure 3A. Three independent results were used for quantification. Error bars indicate standard deviations. (C) The SVDia2-KA mutant protein is stabilized in G1 phase. The indicated strains were arrested with αF for 3 h. Stability assay was performed as in (Figure 25A and 27B). SV indicates the SV40 Tag NLS. Quantification shows the rates of Dia2 turnover in the indicated strains. Error bars indicate standard deviations. (D) The SVDia2-KA mutant protein does not bind to Tom1. The indicated strains were grown to mid-log phase and used for a co-immunoprecipitation assay. Flag-tagged Tom1 was immunoprecipitated with anti-Flag antibodies. Immunoblot assay was conducted with anti-Flag and –Myc antibodies. See also Figure 26.
Figure 28

A

WT

Vector
DIA2
dia2-KR

Vector
DIA2
dia2-KR

tom1Δ

Vector
SV DIA2
SV dia2-KA
SV dia2-1185-193

Vector
SV DIA2
SV dia2-KA
SV dia2-1185-193

B

Time
(min)

Vector
1C 2C 1C 2C 1C 2C

SV DIA2
75 60 45 30 15

SV dia2-KA

C

Time
(min)

WT
tom1Δ
dia2Δ
tom1Δ dia2Δ

40 35 30 25 0

Asy

1C 2C 1C 2C 1C 2C 1C 2C
Figure 28. Tom1-mediated Dia2 turnover is required for efficient G1 to S phase progression. (A) Overexpression of the SVdia2-KA mutant leads to a growth defect in wildtype cells. Wildtype and tom1Δ strains carrying the indicated galactose-inducible vectors were spotted in 10-fold dilutions onto minimal media containing 2% galactose. Plates were incubated at 30°C for 2-3 days. SV indicates the SV40 Tag NLS. (B) Overexpression of the SVdia2-KA mutant causes a delay in G1 to S-phase progression. Wildtype cells carrying the indicated galactose-inducible plasmids were grown to mid-log phase in minimal media containing 2% galactose. Samples were processed for flow cytometry. Separately, cells were also arrested with αF for 3 h and released into minimal media containing 2% galactose and 15μg/ml nocodazole. Samples were taken at the indicated times and prepared for flow cytometry. (C) Suppression of G1 to S phase progression delay of tom1Δ mutant by deletion of DIA2. Wildtype, tom1Δ, dia2Δ, and tom1Δ dia2Δ cells were arrested with αF for 3 h and released into rich medium containing 15μg/ml nocodazole. Samples taken at the indicated times were processed for flow cytometry. Arrows indicate start of S-phase.
Table III. Yeast Strains used in Chapter III

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CHAPTER IV

Summary and Discussion

*Cdc6 degradation by a Tom1 and Dia2-dependent pathway regulates pre-RC assembly during the cell cycle*

I have identified that the Hect E3 ligase Tom1 and the F-box protein Dia2 are required for Cdc6 degradation during G1 and at the G1 to S phase transition. Tom1 and Dia2 act in the same pathway or co-operatively but function independently of the SCF\textsuperscript{Cdc4} complex. Consistent with this, *cdc4-1 tom1Δ* and *cdc4-1 dia2Δ* mutants exhibit greater Cdc6 stabilization than single mutants at the G1 to S phase transition. In addition, *tom1Δ dia2Δ* double mutant shows equivalent Cdc6 turnover to *tom1Δ* or *dia2Δ* single mutants in G1 and at the G1 to S phase transition. Tom1 and Dia2 recognize a Cdc6 N-terminal mutant which lacks amino acids 2-47. Defects in Tom1- or Dia2-mediated Cdc6 proteolysis lead to aberrant Cdc6 chromatin association and Mcm4 binding to early origins. More importantly, increased DNA content is observed in *tom1Δ* mutant when ORC phosphorylation and Mcm2-7 export to the cytoplasm are compromised, suggesting that Cdc6 degradation by a Tom1-mediated pathway may control re-replication along with other inhibitory mechanisms.

It is surprising that Cdc6 is stabilized in *dia2 ΔF-box* mutant but not in *scf* mutants during the G1 phase of the cell cycle because previous work showed that SCF complexes are not responsible for Cdc6 degradation in G1 (21). Although a non-SCF role of Dia2 has not been previously identified, Dia2 might play a role in Cdc6 degradation independently of a traditional SCF complex since other F-box proteins have been shown
to function outside of SCF complexes (24, 29, 91, 99). Identifying Dia2 interacting proteins in G1 will help to elucidate the non-SCF role of Dia2 in Cdc6 degradation during G1. For example, Dia2 might assemble a Cullin-based complex with Rtt101, also known as Cul8, to regulate Cdc6 degradation during G1. Future experiments will be necessary to test these possibilities. In addition, I cannot rule out the possibility that other pathways may also contribute to Cdc6 degradation during G1 phase. This possibility is supported by the observation that the Cdc6ΔN mutant is stabilized in G1 and its stability is significantly enhanced in tom1Δ and dia2Δ mutants during G1 phase. Thus, identification of another E3 ligase responsible for this pathway will provide insight into the mechanism of Cdc6 degradation during G1.

The biological significance of Tom1- or Dia2-mediated Cdc6 proteolysis in G1 still remains elusive. The Tom1- and Dia2-mediated pathway may be important to regulate the assembly of the pre-RC complex during G1. In support of this idea, I observed that Cdc6 chromatin association and Mcm4 binding to early origins are increased in tom1Δ and dia2Δ mutants during G1. Given that Cdc6 synthesis is increased in late G1 and only an individual Cdc6 protein is required for pre-RC assembly at a single origin, excess Cdc6 proteins may need to be targeted for degradation by the Tom1- and Dia2-mediated pathway. Future studies will be required to unravel the biological significance of Cdc6 degradation during G1.

My data suggest that multiple E3 ligases are required for Cdc6 degradation at the G1 to S phase transition. This raises a question about how specificity of these E3s is regulated. The SCF<sup>Cdc4</sup> complex requires Cdk-mediated phosphorylation for recognition
and degradation of Cdc6 (73). However, it is not clear whether Tom1 and Dia2 recognize phosphorylated Cdc6. The requirement of Cdk-dependent phosphorylation for Tom1- or Dia2-mediated Cdc6 degradation needs to be examined. If Tom1 and Dia2 bind phosphorylated Cdc6 for degradation, it is likely that they recognize Cdk consensus sites located in a domain in Cdc6 C-terminal to amino acid 47 since I have shown that Tom1 and Dia2 bind the Cdc6 N-terminal mutant. Among four consensus sites (T135, S354, T368, and S372) located in the domain described above, two sites T368 and S372 have been shown to be recognized by the SCF$^{Cdc4}$ complex for degradation during G2/M (73). If Cdk-mediated phosphorylation is important for Tom1 and Dia2 to target Cdc6 for degradation, phosphorylation at the other two consensus sites T135 and S354 might be required for Tom1 and Dia2 to recognize Cdc6, as a Tom1- and Dia2-mediated pathway is distinct from the SCF$^{Cdc4}$ complex. However, it is unlikely that the Tom1- and Dia2-mediated pathway requires Cdk-dependent phosphorylation for Cdc6 degradation, as Tom1 and Dia2 control Cdc6 degradation in G1 when Cdk activity is low.

I have suggested that Tom1-mediated Cdc6 degradation may be important to control re-replication in a strain sensitized to re-replication. Although flow cytometry analysis shows that DNA content above 2C is increased in $tom1\Delta$ mutant in a background sensitized to re-replication, I do not know what fraction of early origins are re-fired in that strain within a single cell cycle. Alternatively, flow cytometry assay might not be sensitive to detect a subset of origins that have been re-fired within a single cell cycle. Thus, microarray comparative genomic hybridization (CGH) assay will be needed to further analyze and answer this question.
Loss of re-replication control may contribute to tumorigenesis. Recent work in budding yeast has demonstrated that the absence of re-replication control is a potent inducer of gene amplifications (31), which are hallmarks of cancer cells. Intriguingly, the human homolog of Tom1, HUWE1/ARF-BP/Mule, has been proposed to contribute to tumorigenesis, but the mechanism by which this occurs is unclear. HUWE1/ARF-BP/Mule has multiple ubiquitination targets, several of which act in antagonistic pathways (1, 12, 100), making it difficult to sort out the contribution of HUWE1/ARF-BP/Mule to cancer development. No link to re-replication has yet been investigated, although increased Cdc6 abundance has been observed in HUWE1-depleted cells in the presence of DNA damage (32). This suggests that the role of HUWE1 in Cdc6 degradation may be to promote growth arrest in response to DNA damage, implying that HUWE1-mediated Cdc6 degradation may help to impede the growth of cancer cells. In this case, the loss of HUWE1 may contribute to tumorigenesis. However, future studies will be needed to determine the significance of this observation.

The Hect E3 ubiquitin ligase Toml controls Dia2 degradation during the cell cycle

I have found that the Hect-domain E3 ligase Tom1 targets Dia2 for ubiquitin-dependent degradation during G1 and G2/M phases by recognizing the specific positively charged residues in the degradation/NLS domain of Dia2. Deletion of DIA2 partially suppresses the temperature-sensitive growth phenotype of tom1Δ and tom1C3235A mutants. Moreover, overexpression of Dia2 in tom1Δ mutants causes a growth defect, suggesting that Tom1 controls Dia2 degradation. Tom1-mediated Dia2 proteolysis during
the cell cycle is regulated by a proteolytic switch mechanism. Tom1 binding to Dia2 is enhanced in G1 and reduced during S phase or in response to activation of the S-phase checkpoint. The positively charged residues in the degradation/NLS domain are required for Tom1 to recognize and target Dia2 for degradation. Defects in Dia2 degradation caused by loss of the positively charged residues or deletion of TOM1 result in a delay in G1 to S phase progression. In support of this, deletion of DIA2 rescues a delay in G1 to S phase progression of \textit{tom1}\textDelta mutant.

I showed that Dia2 ubiquitination is dependent on Tom1. However, I do not know the identity of E2 enzyme for Dia2 ubiquitination since the fractionated yeast extract used in the \textit{in vitro} assay contains multiple E2 proteins. Identifying the E2 protein responsible for this will be needed in the future.

Although I have identified a domain, also known as degron, required for Dia2 to bind Tom1, the sites of ubiquitination in Dia2 are still not known. When the lysines located in the degradation domain were replaced with arginines, Dia2-KR was still turned over as fast as wildtype Dia2, indicating that those lysines do not serve as ubiquitination sites. However, it is possible that the TPR domain of Dia2 may contain the ubiquitination sites. One model that supports this idea is that the TPR Dia2 binding to Tom1 is weaker than full-length or \textDelta N149 Dia2, implying that the TPR domain may be quickly degraded as soon as Tom1 binds and ubiquitinates it. We look forward to identifying the ubiquitination sites on Dia2 in the future.

The biological significance of Tom1 in cellular processes remains to be further understood, although several reports have shown that Tom1 functions in DNA damage,
transcriptional activation, and mRNA export (32, 40, 82, 86). Thus, identifying Tom1 targets will be important to clarify the biological role of Tom1 in various cellular processes including DNA replication and DNA damage response. I have found that the specific positively charged residues in the degradation/NLS domain in Dia2 are required for Dia2 binding to Tom1. I was also able to identify a list of proteins that contain a stretch of positively charged residues similar to this using a BLAST search. If this domain serves as a degron for potential Tom1 targets, it will be important to test whether those candidate proteins are *bona fide* Tom1 substrates. One of those candidates I found using the BLAST search is Dpb11 which assembles the pre-LC complex with Sld2, GINS, and Polymerase ε. Given that abundance of Dpb11 is low and it is likely an unstable protein, it is tempting to speculate that Dpb11 may be a novel substrate of Tom1. Identifying Tom1 substrates that are involved in DNA replication or cell cycle will help us better understand the mechanistic role of Tom1 in these processes. In addition to the BLAST search, screening potential suppressors of the temperature-sensitive phenotype of *tom1* mutants may be a good alternative to identify Tom1 substrates.

Dia2 has been shown to play a role in DNA replication and activation of the S-phase checkpoint (5, 45, 47, 60, 62). However, the mechanistic role of Dia2 in these processes is not clear. Dia2 has two potential substrates, Mrc1 and Ctf4, but the biological significance of their ubiquitination is not known (60). Identification of Dia2 substrates involved in cell cycle, DNA replication, and activation of the S-phase checkpoint will be necessary to elucidate the functional significance and mechanistic role of Dia2 in those processes.
cellular processes. In addition to its role in activation of the S-phase checkpoint or DNA replication, I also showed that Tom1-mediated Dia2 turnover in G1 contributes to G1 to S phase progression, suggesting that Dia2 may have an additional role in controlling the G1 to S phase transition or have a potential target that promotes the entry into S phase from G1 phase. For example, defects in Dia2 turnover in G1 may alter the balance of SCF complexes such that the abundance of Cdk inhibitor Sic1, which is targeted by the SCF$^\text{Cdc4}$ complex for degradation in late G1, might be increased, leading to a defect in G1 to S-phase progression. Alternatively, Cdc6 could be that potential substrate of Dia2 in G1 as overexpression of Dia2 in G1 leads to a delay in G1 to S phase progression.

Altogether, I have found that Tom1 and Dia2 control Cdc6 degradation during G1 and at the G1 to S-phase transition and that Tom1 also targets Dia2 for degradation during G1 and G2/M. This raises an important question about the relationship among Tom1, Dia2, and Cdc6. How does Tom1-mediated Dia2 proteolysis affect the role of Dia2 in Cdc6 degradation? Given that $tom1\Delta$ mutant exhibits more severe defects or phenotypes such as Cdc6 ubiquitination, aberrant Cdc6 chromatin association, and enhanced Mcm4 binding to early origins than $dia2\Delta$ mutant, it is likely that Tom1-mediated Dia2 degradation may account for the difference of these defects or phenotypes. Why do Tom1 and Dia2 function in the same pathway to control Cdc6 degradation? How does Tom1-mediated Dia2 degradation contribute to regulating Cdc6 proteolysis during G1? One possible explanation is that if Tom1 did not control Dia2 degradation in G1, both Tom1 and stabilized Dia2 may function redundantly to target Cdc6 for degradation. As a result, Cdc6 levels may be too low in G1 by these two redundant pathways. This
may result in inhibition of pre-RC assembly at some origins in G1, leading to insufficient DNA replication initiation. In support of this idea, when the *SV dia2 KA* mutant which is not targeted for degradation by Tom1 was over-expressed in wildtype cells, it caused a significant delay in G1 to S phase progression, implying that the regulation of Cdc6 degradation in G1 may be important for a proper G1 to S phase progression. Future studies will be needed to clarify this relationship.
CHAPTER V

Materials and Methods

Plasmids and strains

Yeast strains and oligonucleotides used in the Chapter II are described in Tables I and II. To generate the CDC6-3HA strain (DKY546), the 3HA-TRP1 fragment containing the 3’ end of CDC6 open reading frame and the 3’ UTR of CDC6 was amplified with primers DHK9 and 10 using pFA6a-3HA-TRP1 as a template (56) and integrated into strain DKY153 via homologous recombination. The centromeric HA-CDC6 TRP1 plasmid using a GAL1,10 promoter (pDHK1) was constructed via PCR amplification of CDC6 from genomic DNA with primers DHK6 and 7 and then inserted into SalI and BamHI sites of the p1216 plasmid. To generate the GST-CDC6 baculovirus construct (pZHW073), the CDC6 open reading frame was amplified with primers DHK25 and 26 using yeast genomic DNA and cloned into the pUNI-10 vector with NdeI and BamHI sites (pZHW072). Plasmid pZHW072 was then recombined with p1212 (55) using cre-lox recombination to generate plasmid pZHW073. To generate the FLAG-TOM1 HECT baculovirus construct (pDHK5), the HECT domain was amplified using yeast genomic DNA with primers DHK84 and 85 and cloned into the pUNI-10 vector with XhoI and BamHI sites to construct the plasmid pDHK4. Plasmid pDHK4 was then recombined with p1214 (55) using cre-lox recombination to generate the FLAG-TOM1 HECT baculovirus construct (pDHK5). The pUNI-10-CDC6ΔN, -DIA2 TPR, and -DIA2-ΔN214 plasmids (pDHK7, pZHW094, pZHW079) were constructed by amplifying the
CDC6ΔN, DIA2 TPR, and DIA2-ΔN214 fragments using the plasmids pDHK6, pACK140, and pACK137 as a template with primers DHK104, 105, ZHW57, 54, 58, and AK43. The amplified fragments were then cloned into the XhoI and NotI, the NdeI and SalI, and the NdeI and BamHI sites of the pUNI-10 plasmid, respectively. The GST-CDC6ΔN, FLAG-DIA2 TPR and FLAG-DIA2-ΔN214 baculovirus constructs (pDHK8, pZHW095 and pZHW080) were also generated via cre-lox recombination using p1212 and p1214 (55). To construct the CDC6ΔN-3HA strain (DKY875), the CDC6-3HA-TRP1 fragment was cloned into pBluescript SK+ plasmid with XhoI and NotI sites to generate plasmid (pDHK2). The N-terminal 2-47 amino acid was then deleted by amplifying the CDC6ΔN fragment using the plasmid pDHK2 as a template with primers DHK46, 82, 81, and 48 and cloned into the XhoI and NotI sites of the plasmid pDHK2 to construct the plasmid CDC6ΔN-3HA (pDHK6). The CDC6ΔN-3HA fragment cut with the XhoI and NotI restriction enzymes was integrated into strain DKY 153 via homologous recombination. For generating the strains used in re-replication assay (Figure 21), the cdc4-1 allele was amplified using yeast genomic DNA isolated from the cdc4-1 strain (DKY173) with primers DHK88 and 89 and then inserted into pRS406 URA3 plasmid (pDHK3). Plasmid pDHK3 was linearized with the BpU10I restriction enzyme and transformed into YJL2068 (orc2 6A orc6 4A MCM7-2NLS from J. Li, UCSF, Nguyen et al., 2001). A loop-in/out gene replacement method was used to generate strain DKY891. The open reading frames of TOM1 and DIA2 were replaced with KanMX via homologous recombination in strains YJL2068 and DKY891 to construct strains DKY876 and 878, respectively. The CDC6ΔN-3HA fragment cut with XhoI and NotI restriction enzymes
was also integrated into strain YJL 2068 via homologous recombination to generate strain DKY896. For generating the mitochondrial DNA deficient rho\(^0\) strains (DKY955, 957, 959, 961, and 963), strains YJL2068, DKY891, 876, 878, and 896 were grown on rich medium plates containing 10\(\mu\)g/ml ethidium bromide. The loss of mitochondrial DNA was confirmed by testing individual colonies on YP plates containing 3\% glycerol.

Yeast strains, plasmids, and oligonucleotides used in the Chapter III are described in Tables III, IV, and V. To generate the tom1\(\Delta\) strain (DKY526), the open reading frame of TOM1 was replaced with KanMX via homologous recombination in strain DKY153. The TOM1-3FLAG strain (DKY538) was generated using the previously described method (30). The centromeric 9MYC-DIA2 KR, 9MYC-SV40-DIA2-KA, and 9MYC-SV40-\(\Delta185-193\) plasmids using a GAL1,10 promoter (pDHK9, 10, and 11, respectively) were constructed via the generation of the KR or KA substitution and the deletion of 185-193 fragment (\(\Delta185-193\)) by the PCR stitching method using the plasmids pACK135 or pACK176 with primers DHK107, 108, 109, 110 and 112, 113, 114, 115, 116, 117 and followed by the amplification of the KR, KA, and \(\Delta185-193\) fragments with primers DHK106 and 111. Then, these fragments were inserted into MluI and HpaI sites of the pACK135 or pACK176 plasmids. To construct the 9MYC-KR, 9MYC-SV40-KA, and 9MYC-SV40-\(\Delta185-193\) strains (DKY952, 968, and 969), the 9MYC-KR, 9MYC-SV40-KA, and 9MYC-SV40-\(\Delta185-193\) fragments were cloned into the plasmids pACK142 and pACK181 with MluI and HpaI sites to generate plasmids (pDHK12, 13, and 14,
respectively). The plasmids (pDHK12, pDHK13, and pDHK14) linearized with the BclI restriction enzyme were integrated into strain DKY194 via homologous recombination.

**Reverse transcription PCR (RT-PCR)**

Cultures were grown to mid-log phase (2 x 10⁷ cells/ml) at 30°C and total RNA was isolated with PURE LINK micro- to midi-Kit (Invitrogen) using the manufacturer’s protocol. After DNase I treatment, 3 µg of total RNA was reverse transcribed using Superscript II (Invitrogen) with oligo (dT)₅₀ primer. The cDNA was amplified with primers DHK11, 12, 16, and 17 or ACT1-5 and ACT1-3.

**In vitro binding assays**

1mg of total cell lysate isolated from baculovirus-infected Hi5 insect cells was immunoprecipitated with anti-Flag M2 monoclonal (Sigma), anti-Myc 9E10 monoclonal (Covance) or anti-GST polyclonal (Santa Cruz) antibodies and immunoblotted with anti-Flag M2, anti-Myc 9E10 and anti-GST (Santa Cruz) antibodies.

**Stability assays**

Cells at 1 x 10⁷ cells/ml were arrested with 40µg/ml αF, 200 mM hydroxyurea or 15µg/ml nocodazole for 3 h. To arrest cells in early S, cells were released from αF arrest into rich medium containing 200 mM hydroxyurea for 90 min. Cycloheximide was added at 100 µg/ml. Cell pellets were washed and lysed by vortexing with glass beads in 20% trichloroacetic acid (TCA) for 3 min. Lysed cell pellets were centrifuged at 3,000 rpm for
10 min and resuspended in Laemmli buffer. Precipitated proteins were neutralized with 1M Tris and boiled for 5 min. Protein concentration was quantified using the RC/DC protein assay kit (BioRad). 20 μg of proteins were run on 8% SDS-PAGE. Protein abundance was measured using the Image J software and normalized against a loading control.

**Immunoprecipitation**

Cells were cultured to mid-log phase (2 x 10⁷ cells/ml) and collected by centrifuging at 4,000 rpm for 2 min. For Cdc6 co-immunoprecipitation analysis, total cell lysate was isolated by vortexing the cells with glass beads in RIPA buffer (1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 20 mM Tris pH 7.5, 10% Glycerol) with protease inhibitor cocktail (Roche) for 40 min at 4°C. Protein concentration was determined using Bio-Rad protein assay (Bio-Rad). 1 mg of total lysate was incubated with affinity matrix anti-HA.11 (Covance) and Protein A/G agarose (Santa Cruz) for 3 h at 4°C. Samples were washed with RIPA buffer 4 times and boiled in Laemmli buffer for 5 min.

To perform Tom1 co-immunoprecipitation assays, cells were cultured to mid-log phase (2 x 10⁷ cells/ml) and collected by centrifuging at 4,000 rpm for 2 min. Total cell lysate was isolated by vortexing the cells with glass beads in 0.3% CHAPS buffer (0.3% CHAPS, 40 mM HEPES pH 7.4, 120 mM NaCl, 1mM EDTA, 50mM NaF, 10mM Glycerol 2-β) with protease inhibitor cocktail (Roche) for 40 min at 4°C. Protein concentration was determined using Bio-Rad protein assay (Bio-Rad). 2 mg of total lysate was incubated with anti-Flag M2 monoclonal (Sigma) antibodies and Protein A/G
agarose (Santa Cruz) for 3 h at 4°C. Samples were washed with CHAPS buffer 4 times
and boiled in Laemmli buffer for 5 min.

**In vivo and In vitro Ubiquitination assays**

For *in vivo* Cdc6 ubiquitination, cells were grown to mid-log phase and treated with
DMSO or 50 μM MG132 for 2h. 2mg of total protein was used for immunoprecipitation
with anti-HA.11 monoclonal antibodies (Covance). Proteins were analyzed by 8% SDS-
PAGE followed by an immunoblot assay with anti-P4D1 monoclonal ubiquitin and
- HA.11 antibodies (Covance).

For *in vitro* Cdc6 ubiquitination, 50 μg of fractionated yeast extracts were incubated with
50 μg ubiquitin, 50 nM E1, 1.5 μg Cdc34 (E2), 10 mM ATP, 60 mM creatine phosphate,
1 mM magnesium acetate, 150 μg/ml creatine kinase, and 40 μg GST-Cdc6 protein
bound to the Glutathione Sepharose 4B beads (GE Health care) at 30°C for 45 min.
Samples were run on 6% SDS-PAGE and immunoblotted with anti-GST polyclonal
antibodies (Santa Cruz).

To perform *in vitro* Dia2 ubiquitination assay, 50 μg of fractionated yeast extracts were
incubated with 50 μg ubiquitin, 50 nM E1, 10 mM ATP, 60 mM creatine phosphate, 1
mM magnesium acetate, 150 μg/ml creatine kinase, and 40 μg GST-Dia2 protein bound
to the Glutathione Sepharose 4B beads (GE Health care) at 30°C for 45 min. Samples
were run on 6% SDS-PAGE and immunoblotted with anti-GST polyclonal antibodies
(Santa Cruz).
**Chromatin fractionation**

Chromatin fractionation assay was performed as described (53). Protein concentration was quantified using the RC/DC protein assay kit (BioRad). 30 μg of whole cell extract and crude chromatin fraction were resolved on 8% and 15% SDS-PAGE, respectively and analyzed by immunoblotting with anti-HA.11 (Covance), anti-3-phosphoglycerate kinase 22C5 (anti-Pgk1, Invitrogen), and anti-Histone H3 (Abcam) antibodies. The ratio of chromatin-bound Cdc6 to Histone H3 was measured using the Image J software.

**Chromatin immunoprecipitation**

Total yeast cell lysates were prepared for chromatin immunoprecipitations as previously described (89). PCR was performed using primers for early-firing origins ARS1 and ARS305 and non-origin region ACF2.

**Flow cytometry**

For re-replication assays, cells were arrested with 15 μg/ml nocodazole for 3h and shifted to 37°C. When cells were shifted to 37°C, nocodazole was also added to prevent cells from breaking through the nocodazole arrest. Samples were fixed at the indicated times and stained with propidium iodide. DNA re-replication was analyzed using FACS Calibur 501 (University of Minnesota flow cytometry facility). Flow Jo software was used to measure the populations of the cells with more than 2C DNA content.
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APPENDIX

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MANUSCRIPT TITLE - The Hect E3 ubiquitin ligase Tom1 controls Dia2 degradation during the cell cycle

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