The Hect-domain E3 ligase Tom1 and the F-box protein Dia2 control Cdc6 Degradation in G1*

Dong-Hwan Kim1, Wei Zhang1,2 and Deanna M. Koepp1

1From the Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN 55455

2Current address: Department of Experimental and Clinical Pharmacology, University of Minnesota, Minneapolis, MN 55455

*Running Title: Tom1 and Dia2 Regulate Cdc6 Degradation

To whom correspondence should be addressed: Deanna M. Koepp, Department of Genetics, Cell Biology and Development, University of Minnesota, 6-160 Jackson Hall, 321 Church St. SE, Minneapolis, MN 55455, Tel.: (612) 624 4201, Email: koepp015@umn.edu

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Background: DNA replication complex assembly is regulated to maintain genomic integrity.

Results: Proteolysis of a replication protein is dependent on a new ubiquitination pathway, which alters replication complex assembly.

Conclusion: A novel mechanism for degrading a replication protein is described.

Significance: This new pathway may contribute to regulation of DNA replication and genomic integrity.

SUMMARY
The accurate replication of genetic information is critical to maintaining chromosomal integrity. Cdc6 functions in the assembly of pre-replicative complexes and is specifically required to load the Mcm2-7 replicative helicase complex at replication origins. 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(Cdc64) (Skp1/Cdc53/F-box protein)3 complex. Notably, Cdc6 is unstable during the G1 phase of the cell cycle, but the ubiquitination pathway has not been previously identified. Using a genetic approach, we 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 phase of the cell cycle and act separately from SCF(Cdc64). Ubiquitination of Cdc6 is significantly reduced in dia2Δ and tom1Δ cells. Tom1 and Dia2 each independently immunoprecipitate Cdc6, binding to a C-terminal region of the protein. Tom1 and Dia2 cannot compensate for each other in Cdc6 degradation. Cdc6 and Mcm4 chromatin association is aberrant in tom1Δ and dia2Δ cells in G1 phase. Together, these results present evidence for a novel degradation pathway that controls Cdc6 turnover in G1 that may regulate pre-RC assembly.

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 (1)). 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 (2)).

Cdc6 is an AAA+-ATPase required for pre-RC assembly (3-6). 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 (7-11). Budding yeast Cdc6 is a highly unstable protein subject to multiple modes of degradation (12-14). In the best-understood degradation pathway, cyclin-dependent kinase (Cdk) phosphorylation targets Cdc6 for ubiquitination and degradation via the ubiquitin ligase complex...
SCF<sup>Cdc4</sup> at the G1/S phase transition and during G2/M (13-17). However, Cdc6 is also unstable during G1 and the degradation pathway responsible has not been identified (14).

To identify other pathways that might target Cdc6 for ubiquitination and degradation, we 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 (18). The human homolog of Tom1, Huwe1, targets human Cdc6 for degradation during the DNA damage checkpoint response (19,20). This function appears to be conserved in <i>S. cerevisiae</i> (19), although the contribution of Tom1 to Cdc6 degradation in an unperturbed cell cycle has not been investigated. The SCF<sup>Daub</sup> ubiquitin ligase complex regulates DNA replication and is required for genomic stability (21-24). Potential targets of SCF<sup>Daub</sup> include two proteins that travel with replication complexes at the replication fork, Mrc1 and Ctf4, although the physiological role of their degradation is unclear (25).

Here we show that both Tom1 and Dia2 contribute to Cdc6 degradation during G1. This work describes a new degradation pathway for Cdc6 and identifies a novel target for both the Tom1 and Dia2 ubiquitin ligase components.

**EXPERIMENTAL PROCEDURES**

*Plasmids and strains*- Yeast strains and oligonucleotides are described in Tables 1 and 2 (Supplemental Information). To generate the <i>CDC6</i>-3HA strain (DKY856), the 3HA-<i>TRP1</i> fragment containing the 3' end of <i>CDC6</i> open reading frame and the 3' UTR of <i>CDC6</i> was amplified with primers DHK9 and 10 using pFA6a-3HA-<i>TRP1</i> as a template (26) and integrated into strain DKY153 via homologous recombination. The HA-<i>CDC6</i> <i>TRP1</i> plasmid using a <i>GAL1,10</i> promoter (pDHK1) was constructed via PCR amplification of <i>CDC6</i> from genomic DNA with primers DHK6 and 7 and then inserted into <i>Sall</i> and <i>BamHI</i> sites of the p1216 plasmid. To generate the GST-<i>CDC6</i> baculovirus construct (pZHW073), the <i>CDC6</i> open reading frame was amplified with primers DHK25 and 26 using yeast genomic DNA and cloned into the pUNI-10 vector with <i>Ndel</i> and <i>BamHI</i> sites (pZHW072). Plasmid pZHW072 was then recombined with p1212 (27) 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 <i>NotI</i> and <i>BamHI</i> sites to construct the plasmid pDHK4. Plasmid pDHK4 was then recombined with p1214 (27) using cre-lox recombination to generate the Flag-TOM1 HECT baculovirus construct (pDHK5). The pUNI-10-<i>CDC6ΔN</i>, -<i>DIA2 TRP</i>, and -<i>DIA2-ΔN214</i> plasmids (pDHK7, pZHW094, pZHW079) were constructed by amplifying the <i>CDC6ΔN</i>, <i>DIA2 TRP</i>, and <i>DIA2-ΔN214</i> 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 <i>Xhol</i> and <i>NotI</i> sites, the <i>Ndel</i> and <i>Sall</i> sites, and the <i>Ndel</i> and <i>BamHI</i> sites of the pUNI-10 plasmid, respectively. The GST-<i>CDC6ΔN</i>, FLAG-<i>DIA2 TRP</i> and Flag-<i>DIA2-ΔN214</i> baculovirus constructs (pDHK8, pZHW095 and pZHW080) were also generated via cre-lox recombination using p1212 and p1214 (27). To construct the <i>cdc6ΔN-3HA</i> strain (DKY875), the <i>CDC6-3HA-TRP1</i> fragment was cloned into pBlueScript SK+ plasmid with <i>Xhol</i> and <i>NotI</i> sites to generate plasmid (pDHK2). The N-terminal 2-47 amino acid was then deleted by amplifying the <i>CDC6ΔN</i> fragment using the plasmid pDHK2 as a template with primers DHK46, 82, 81, and 48 and cloned into the <i>Xhol</i> and <i>NotI</i> sites of the plasmid pDHK2 to construct the plasmid <i>CDC6ΔN-3HA</i> (pDHK6). The <i>CDC6ΔN-3HA</i> fragment cut with the <i>Xhol</i> and <i>NotI</i> restriction enzymes was integrated into strain DKY 153 via homologous recombination. To generate the galactose-inducible HA-tagged TOM1 HECT domain plasmid (pDHK15), the HECT domain was amplified via PCR with primers DHK122 and 123 and then inserted into <i>BamHI</i> and <i>NotI</i> sites of the p1220 plasmid.

Reverse transcription PCR (RT-PCR)- Cultures were grown to mid-log phase (2 x 10<sup>7</sup> 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)<sub>30</sub> primer. The cDNA was amplified with primers DHK11 and 12 or ACT1-5 and ACT1-3.

*In vitro binding assays* – 1 μg 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 10^7 cells/ml were arrested with 40 µg/ml αF for 3 h. 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 total protein was 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 10^7 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 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 the 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.

In vivo and In vitro Ubiquitination assays - Cells were grown to mid-log phase and treated with DMSO or 50 µM MG132 for 2 h. 2 mg 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 anti-HA.11 antibodies (Covance). Image J software was used to quantify ubiquitinated Cdc6. For in vitro ubiquitination, 20 µg of crude yeast extracts isolated from wildtype, tom1Δ, and dia2Δ strains were incubated with 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).

Chromatin fractionation - Chromatin fractionation assay was performed as described (28). 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 immunoprecipitation as previously described (29). PCR was performed using primers for early-firing origins ARS1 and ARS305 and non-origin region ACF2.

RESULTS

Tom1 and Dia2 regulate Cdc6 degradation - To determine whether other ubiquitin ligases might have a role in Cdc6 protein turnover, we examined whether overexpression of CDC6 caused a growth defect in ligase mutants that had known roles in DNA replication or the cell cycle. Two ligases that fit this profile include the F-box protein Dia2 and the Hect-domain E3 Tom1 (20-23). Previous work has shown that Tom1 can target Cdc6 for degradation in response to DNA damage (20), 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 (30,31), but we observed an exacerbated growth phenotype in both dia2Δ and tom1Δ cells (Figure 1A). Intriguingly, the Cdc6 overexpression phenotype in the tom1Δdia2Δ double mutant mimicked the phenotype of the single mutants.

We 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 1B, Cdc6 was partially stabilized in both dia2Δ and tom1Δ cells. No changes in CDC6 mRNA abundance were detected in these strains (Figure 1C), consistent with the possibility that Dia2 and Tom1 might control Cdc6 ubiquitination. 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 1D).

Cdc6 binds Dia2 and Tom1 - We examined whether Dia2 and Tom1 interact with Cdc6 using two different approaches. First, we 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 2). When either the Flag-tagged Hect domain of Tom1 or Myc-tagged Dia2 was immunoprecipitated, we observed substantial co-purification of GST-Cdc6 (Figure 2A). We also observed reciprocal co-purification when GST-Cdc6 was purified from the same cells (Figure 2B). Next we tested whether endogenously expressed HA-tagged Cdc6 can co-immunoprecipitate endogenously expressed Myc-tagged Dia2 or Flag-tagged Tom1 (Figure 2C). 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, we considered the possibility that Dia2 interacted with Cdc6 indirectly by virtue of both proteins binding to chromatin, but we still observed co-precipitation when lysates were treated with DNase I (Figure 2D). We conclude that both Tom1 and Dia2 form a complex with Cdc6, although we 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 (32). Our results with insect cell-expressed protein indicate that the Tom1 Hect domain interacts with Cdc6 (Figure 2A, B). Dia2 is an F-box protein that contains TPR repeats in the N-terminus and an LRR domain in the C-terminus (22,23). Since F-box proteins typically associate with substrate proteins via their C-terminal repeat domains, we asked whether a truncated Dia2 protein lacking the N-terminus still bound Cdc6. We 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 with ΔN214 Dia2 under these conditions (Figure 2E). Together, these results suggest that the N-termini of Tom1 and Dia2 are dispensable for binding Cdc6.

**Tom1 and Dia2 control Cdc6 degradation in G1 phase** - Previous work from the Diffley laboratory has shown that there are three modes of Cdc6 degradation (14). 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 (14). 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 (14). We 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 (14), Cdc6 is still unstable in cdc6-1 cells under these conditions (Figure 3A). Strikingly, we found that Cdc6 was stabilized in both tom1Δ and dia2Δ strains (Figure 3A). The observation that Dia2 is involved in Cdc6 turnover in G1 was surprising, as Cdc6 is not stabilized in scf mutants in G1 (14).

The most straightforward explanation for our results is that Cdc6 may be a direct ubiquitination target of both Tom1 and Dia2. To test this hypothesis, we 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, which use a catalytic cysteine in the transfer of ubiquitin to substrate proteins (32). Cdc6 was stabilized in G1 in a Tom1 mutant that had this cysteine replaced with alanine (Figure 3B). Likewise, Dia2 contains an F-box domain required for assembly with the other SCF components to form a functional E3 ligase complex (22,23). Cdc6 is stabilized in a dia2 ΔF-box mutant in cells arrested in G1 (Figure 3B). 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 (14). We verified that skp1-11, cdc53-1 and cdc34-2 mutants do not stabilize Cdc6 in G1-arrested cells (data not shown). However, when we examined Cdc6 turnover in a Dia2 mutant lacking both the F-box domain and the C-terminal LRR region (TPR mutant), we also observed Cdc6 stabilization (Figure 3C). Our results suggest that Dia2 contributes to Cdc6 turnover in an F-box dependent manner but that this function is likely independent of a traditional SCF complex.

We next asked whether Tom1 and Dia2 are required for Cdc6 ubiquitination. We immunoprecipitated HA-tagged Cdc6 from wildtype, tom1Δ and dia2Δ cells that had been incubated with the proteasome inhibitor MG132. We probed the immunoprecipitates with anti-ubiquitin antibodies. Under these conditions, we observed modified forms of Cdc6 in proteasome-inhibited cells, but these forms were reduced to approximately 60% of wildtype in tom1Δ cells and
to 40% of wildtype in dia2Δ cells (Figure 4A). In addition, we developed an in vitro ubiquitination assay using GST-Cdc6 purified from baculovirus-infected insect cells. When GST-Cdc6 was incubated with crude yeast extracts, we observed Cdc6 ubiquitin conjugates (Figure 4B). We performed this assay using crude extracts from tom1Δ and dia2Δ strains and observed decreased ubiquitination (Figure 4B). Together, our results indicate that Cdc6 ubiquitination and protein stability are dependent on both Tom1 and Dia2.

Deletion of an N-terminal region in Cdc6 has been shown to lead to a stabilized protein (13). The region encompassing amino acids 2-47 contains 4 CDK consensus sites that are important for SCF-Cdc4-mediated degradation of Cdc6 at the G1 to S-phase transition. However, this N-terminal truncation mutant of Cdc6 (Cdc6ΔN) is still stabilized in G1 cells, even though Cdc4 does not control Cdc6 proteolysis in this phase (13,14). Thus, we sought to determine whether the Tom1 or Dia2 was responsible for stabilization of this mutant in G1. We generated strains that endogenously expressed HA-tagged Cdc6ΔN in wildtype, tom1Δ, and dia2Δ strains and performed protein stability assays. As previously reported, Cdc6ΔN is partially stabilized in G1-arrested cells (13). Surprisingly, Cdc6ΔN stability is increased in tom1Δ and dia2Δ cells, indicating that Tom1 and Dia2 likely interact with a different region of the Cdc6 protein (Figure 4C). To test whether Tom1 and Dia2 associate with a Cdc6 protein lacking the N-terminus, we 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 4D), indicating that these proteins are able to form a complex. We conclude that Dia2 and Tom1 recognize a domain in Cdc6 downstream of amino acid 47.

Our results suggested that Tom1 and Dia2 are required for Cdc6 ubiquitination and degradation, but it was not clear whether Tom1 and Dia2 work together or independently, although the CDC6 overexpression results from Figure 1 suggested Tom1 and Dia2 did not act in redundant, separate pathways. To investigate this further, we examined Cdc6 protein stability in a tom1Δ dia2Δ double mutant arrested in G1 with alpha factor. When we plotted the rate of turnover in the double mutant compared to wildtype and the single mutants, the rate of turnover in the double mutant was indistinguishable from the turnover in tom1Δ cells (Figure 5A). This is consistent with our previous results that suggested Tom1 and Dia2 are not redundant.

We also tested whether Tom1 and Dia2 bind Cdc6 in a cooperative or competitive manner. Our binding assays using baculovirus-expressed protein shown in Figure 2 suggested that Tom1 and Dia2 can bind Cdc6 independently, as copurification is observed when Cdc6 and either ubiquitin ligase is co-expressed. In addition, endogenous Cdc6 still immunoprecipitated with either Tom1 or Dia2 in cells lacking the other ligase (data not shown). To test whether Tom1 and Dia2 might affect each other’s interaction with Cdc6, we compared the efficiency of copurification with Cdc6 when both Tom1 and Dia2 are present (Figure 5B). We incubated equal amounts of GST-Cdc6 with Myc-tagged Dia2, the Flag-tagged Hect domain of Tom1, or a mixture of Myc-Dia2 and Flag-Hect domain of Tom1 and then performed immunoprecipitations. Interestingly, we did not observe any enhancement or inhibition of Cdc6 co-purification with either Dia2 or Tom1 when all three proteins were present. We also examined whether Tom1 and Dia2 can compensate for each other’s function when overexpressed. We examined Cdc6 protein turnover in tom1Δ cells overexpressing DIA2 and in dia2Δ cells overexpressing the Tom1 Hect domain. In each case, the degradation of the Cdc6 protein was not significantly altered by overexpression of the other ubiquitin ligase (Figure 5C). Together, these results suggest that Tom1 and Dia2 bind Cdc6 independently of each other and that Tom1 and Dia2 cannot compensate for each other in Cdc6 protein turnover.
in late G1 in \textit{tom1Δ} and \textit{dia2Δ} 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, we used chromatin immunoprecipitation. Wildtype, \textit{tom1Δ} and \textit{dia2Δ} cells expressing HA-tagged Mcm4 were arrested in G1 and Mcm4 chromatin association at two early firing origins (\textit{ARS1} and \textit{ARS305}) and one non-origin region (\textit{ACF2}) was assayed (Figure 6B). All strains exhibited only negligible Mcm4-binding at the non-origin region \textit{ACF2}. Mcm4 association at \textit{ARS1} and \textit{ARS305} was substantially higher than wildtype in \textit{tom1Δ} cells. In \textit{dia2Δ} cells, Mcm4 was strongly associated with \textit{ARS1}, but the effect at \textit{ARS305} was less obvious. These results suggest that increased Cdc6 abundance and chromatin association in both the \textit{tom1Δ} and \textit{dia2Δ} strains may lead to increased association of Mcm proteins at origins.

**DISCUSSION**

Altogether our data indicate that the Hect-domain E3 ligase Tom1 and the F-box protein Dia2 are required to target Cdc6 for ubiquitin-mediated destruction during G1 phase of the cell cycle. Our results suggest that Tom1 and Dia2 bind Cdc6 independently of each other and cannot substitute for each other. We favor a model in which Tom1 and Dia2 function in the same pathway, as the degradation of Cdc6 is not enhanced in a \textit{tom1Δ dia2Δ} double mutant.

One possible explanation is that Dia2 and Tom1 act in a sequential pathway to target Cdc6 for degradation, although there is no evidence to indicate which ligase acts first or whether there are intervening steps in the pathway. It seems that Dia2 functions outside the context of an SCF complex in this pathway, as we do not observe any defect in Cdc6 degradation in \textit{scf} mutants, similar to previous studies (13,14). 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 (33-36). It is possible that additional factors are required to complex with Dia2 to contribute to the ubiquitination of Cdc6.

We recently determined that Tom1 also targets Dia2 for ubiquitin-mediated degradation during G1 (37). In principle, this pathway could affect Cdc6 degradation via Tom1. However, we did not find any evidence that Dia2 competes with Cdc6 for binding to Tom1. Moreover, if Dia2 competed with Cdc6, we would expect that Cdc6 degradation would be enhanced in \textit{dia2Δ} cells, rather than inhibited. It is possible that Dia2 degradation is a by-product of Cdc6 degradation. Such a scenario would suggest that Dia2 is an accessory factor for Tom1, but in this case we might expect cooperative binding, which we did not observe. Future studies will be necessary to delineate the precise mechanistic roles of Dia2 and Tom1 in targeting Cdc6 for degradation.

Our results support the idea that Tom1 and Dia2 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 I 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 (19) and \textit{dia2Δ} cells exhibit endogenous DNA damage (21-23). However, we think this is unlikely during G1 phase, as \textit{dia2Δ} cells only accumulate DNA damage foci during S and G2/M (22,23). Rather, we think it is likely that Tom1 and Dia2 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.

The functional significance of degrading Cdc6 during G1 is not entirely clear. Previous work from the Diffley laboratory has suggested that a greater fraction of Cdc6 is chromatin-bound during G1 (13). It is possible that degradation of Cdc6 may be used to regulate pre-RC assembly on replication origins in G1. The increased accumulation of chromatin-bound Cdc6 and Mcm4 observed in \textit{dia2Δ} and \textit{tom1Δ} cells is consistent with this hypothesis. Alternatively, it is possible that Dia2 and Tom1 target soluble Cdc6 for degradation. Future studies will be necessary to distinguish between these possibilities.

In summary, we have identified two novel E3 ubiquitin ligase components, Tom1 and Dia2, which contribute to Cdc6 ubiquitination and degradation during the G1 phase of the cell cycle. In the absence of Tom1 and Dia2, Cdc6 and Mcm4 chromatin association is altered. Altogether, our results indicate that multiple ubiquitin ligases control Cdc6 degradation, perhaps to regulate pre-RC assembly.
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FOOTNOTES
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\(^{1}\)To whom correspondence may be addressed: Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN 55455
\(^{2}\)Current address: Department of Experimental and Clinical Pharmacology, University of Minnesota, Minneapolis, MN 55455
\(^{3}\)The abbreviations used are: SCF, Skp1/Cdc53/F-box protein, pre-RC, pre-replicative complex, Cdk, cyclin-dependent kinase, \(\alpha F\), alpha factor, CHX, cycloheximide

FIGURE LEGENDS

\textbf{FIGURE 1.} Tom1 and Dia2 control Cdc6 proteolysis. (A) Overexpression of Cdc6 results in an exacerbated growth defect in \textit{tom1}\(^{\Delta}\), \textit{dia2}\(^{\Delta}\), and \textit{tom1}\(^{\Delta}\)\textit{dia2}\(^{\Delta}\) strains. The 10-fold serial dilutions of wild-type, \textit{tom1}\(^{\Delta}\), \textit{dia2}\(^{\Delta}\), and \textit{tom1}\(^{\Delta}\)\textit{dia2}\(^{\Delta}\) cells carrying empty vector or \textit{CDC6} under the control of \textit{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 \textit{tom1}\(^{\Delta}\) and \textit{dia2}\(^{\Delta}\) mutants. Wildtype, \textit{tom1}\(^{\Delta}\), and \textit{dia2}\(^{\Delta}\) strains were grown to mid-log phase at 30°C. After cycloheximide (CHX) treatment (100 \(\mu g/ml\)), 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 \textit{tom1}\(^{\Delta}\) and \textit{dia2}\(^{\Delta}\) mutants. RT-PCR assay was conducted to examine the level of Cdc6 transcript in wildtype, \textit{dia2}\(^{\Delta}\) and \textit{tom1}\(^{\Delta}\) strains. \textit{ACT1} was used as a loading control. RT, reverse transcriptase. (D) Clb5 stability is not affected in \textit{tom1}\(^{\Delta}\) and \textit{dia2}\(^{\Delta}\) mutants. Wildtype, \textit{tom1}\(^{\Delta}\), and \textit{dia2}\(^{\Delta}\) 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. 100 \(\mu g/ml\) cycloheximide (CHX) was added to block protein translation.

\textbf{FIGURE 2.} Tom1 and Dia2 interact with Cdc6. (A, 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 from strains expressing Flag-Tom1 and Myc-Dia2 or Flag-Tom1, Myc-Dia2 and HA-Cdc6 were immunoprecipitated with anti-HA.11 affinity matrix and immunoblotted with anti-Flag, -Myc, and - HA antibodies. (D) Dia2 binding to Cdc6 is resistant to DNase I treatment. Cell lysate from the strain expressing Myc-Dia2 and HA-Cdc6 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-Flag and -Myc antibodies. (E) 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-\(\Delta N214\) baculovirus-infected Hi5 insect cells were immunoprecipitated with anti-GST antibodies and analyzed by immunoblotting with anti-GST, -Myc, or -Flag antibodies.

\textbf{FIGURE 3.} Tom1 and Dia2 control Cdc6 proteolysis in G1. (A) Cdc6 is stabilized in both \textit{tom1}\(^{\Delta}\) and \textit{dia2}\(^{\Delta}\) mutants in G1. The indicated strains were arrested with \(\alpha F\) for 3 h and shifted to 37°C for 30 min. prior to performing stability assays as in Figure 1B. Three independent experiments were used for quantification. Error bars indicate standard deviations. (B) The catalytic activity of Tom1 Hect domain and the F-box domain of Dia2 are required for Cdc6 turnover. Wildtype, \textit{tom1C3235A}, \textit{dia2}\(^{\Delta F}\) strains arrested with \(\alpha F\) were used in protein stability assays. Samples were collected at indicated times after cycloheximide (CHX) addition. Pgk1 was used as a loading control. Three independent experiments were
used for quantification. (C) 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.

FIGURE 4. Tom1 and Dia2 target Cdc6 for ubiquitin-dependent degradation and recognize a domain in Cdc6 C-terminal to amino acid 47. (A) Cdc6 ubiquitination is dependent on Tom1 and Dia2. Wildtype, tom1Δ, and dia2Δ strains were cultured to mid-log phase and treated with DMSO or 50 µM MG132 for 2 h. Cdc6 protein was immunoprecipitated with anti-HA antibodies and visualized by immunoblot assays with anti-ubiquitin antibodies. The asterisk indicates a non-specific band. Error bars indicate standard deviations. (B) 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 crude yeast extracts purified from wildtype, tom1Δ, or dia2Δ strains at 30 °C for 45 min. Samples were run on 6% SDS-PAGE and immunoblotted with anti-GST antibodies. (C) 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. (D) 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 5. Tom1 and Dia2 cannot substitute for each other in Cdc6 degradation. (A) Cdc6 turnover rate in tom1Δ dia2Δ mutant is indistinguishable from the tom1Δ or dia2Δ mutant. The results of quantification of wildtype, tom1Δ, dia2Δ, and tom1Δ dia2Δ mutants are shown in the graph. (B) Tom1 Hect domain and Dia2 bind Cdc6 independently of each other. Insect cell lysates expressing the indicated baculoviruses were incubated with anti-Flag or -Myc antibodies and analyzed by immunoblotting with anti-Flag, -Myc, or -GST antibodies. (C) Overexpression of the alternate ubiquitin ligase does not rescue the Cdc6 degradation defect in tom1Δ and dia2Δ mutants. Left panel, tom1Δ cells carrying empty vector or DIA2 under the control of GAL1,10 promoter were grown to mid-log phase and arrested with αF for 3 h in minimal medium supplemented with 2% raffinose. Expression of DIA2 was induced with addition of 2% galactose for 30 min. Stability assays were performed as in Figure 3. Flow cytometry was used to monitor the αF arrest. Right panel, the same experiment performed with dia2Δ cells expressing the Tom1 Hect domain. Error bars indicate standard deviations.

FIGURE 6. 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 tom1Δ and dia2Δ cells. The indicated strains were arrested with αF for 3 h and samples were prepared for chromatin fractionation. Samples were analyzed by immunoblot assays with anti-HA, -Pgtk1, and -histone H3 antibodies. Flow cytometry was used to monitor cell cycle arrest. 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 as in (A). Samples were prepared for chIP assays with HA-tagged Mcm4. ARS1 and ARS305 are early firing origins. ACF2 is non-origin control. The ratio of Mcm4 chromatin IP to input is shown in the graph. Three replicates of each experiment were quantified using Image J. Error bars indicate standard deviations.
Figure 3

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