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Brittney Marie Davidge
Portland State University, brittney.davidge7@gmail.com

Katia de Oliveira Rebola
Portland State University

Larry N. Agbor
University of Iowa

Curt D. Sigmund
University of Iowa

Jeffrey D. Singer
Portland State University, jsinger@pdx.edu

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RESEARCH ARTICLE

Cul3 regulates cyclin E1 protein abundance via a degron located within the N-terminal region of cyclin E

Brittney Davidge^{1,†}, Katia Graziella de Oliveira Rebola¹, Larry N. Agbor^{2,*}, Curt D. Sigmund² and Jeffrey D. Singer^{1,§}

ABSTRACT

Cyclin E and its binding partner Cdk2 control the G₁/S transition in mammalian cells. Increased levels of cyclin E are found in some cancers. Additionally, proteolytic removal of the cyclin E N-terminus occurs in some cancers and is associated with increased cyclin E–Cdk2 activity and poor clinical prognosis. Cyclin E levels are tightly regulated and controlled in part through ubiquitin-mediated degradation initiated by one of two E3 ligases, Cul1 and Cul3. Cul1 ubiquitylates phosphorylated cyclin E, but the mechanism through which Cul3 ubiquitylates cyclin E is poorly understood. In experiments to ascertain how Cul3 mediates cyclin E destruction, we identified a degron on cyclin E that Cul3 targets for ubiquitylation. Recognition of the degron and binding of Cul3 does not require a BTB domain-containing adaptor protein. Additionally, this degron is lacking in N-terminally truncated cyclin E. Our results describe a mechanism whereby N-terminally truncated cyclin E can avoid the Cul3-mediated degradation pathway. This mechanism helps to explain the increased activity that is associated with the truncated cyclin E variants that occurs in some cancers.

KEY WORDS: Cul3, Cyclin E, Cancer, G₁/S transition, Ubiquitin

INTRODUCTION

Cyclin E and its binding partner Cdk2 regulate the transition from G₁ to S phase and release from quiescence in mammalian cells (Koff et al., 1991; Geng et al., 2003). Thus, it is not surprising that cell cycle errors are associated with alterations in cyclin E function and/or abundance; fibroblasts lacking both cyclin E genes, cyclin E1 and cyclin E2, are unable to exit from quiescence (Geng et al., 2003). In contrast, overexpression of cyclin E is associated with cancer and tumorigenesis (Said and Medina, 1995). Analysis of the cyclin E protein has revealed several functional domains, including a central cyclin homology domain, which interacts with Cdk2, a unique N-terminal region, and a C-terminal PEST sequence (residues 385–401), which is commonly found in proteins that get degraded through the ubiquitin system (Lew et al., 1991; Rogers and Rechsteiner, 1986; Rogers et al., 1986; Richardson et al., 1993;

Honda et al., 2005; Rath and Senapati, 2014). In certain cancers, including breast, ovarian and melanoma, cyclin E (50 kDa) is known to be cleaved by proteases resulting in N-terminally truncated low molecular mass (LMM) forms ranging in size from 33 to 45 kDa (Scuderi et al., 1996; Porter and Keyomarsi, 2000; Porter et al., 2001; Harwell et al., 2000; Wang et al., 2003; Libertini et al., 2005). LMM cyclin E activates Cdk2 and demonstrates increased cyclin E–Cdk2 activity (Porter et al., 2001). These forms of cyclin E are associated with poor clinical prognosis in cancer patients (Porter et al., 2001; Harwell et al., 2000; Duong et al., 2012).

Cyclin E expression is restricted to the G₁/S transition by two distinct E3 ubiquitin ligases, which are responsible for the degradation of cyclin E: Cul1 and Cul3 (Clurman et al., 1996; Singer et al., 1999; Strohmaier et al., 2001; Welcker et al., 2003). Both Cul1 and Cul3 are members of the cullin-RING family of ubiquitin ligases. Cul1 SCF-based (SCF denotes an Skp1, Cul1 and F-box protein complex) ligases use Fbxw7 (as the F-box protein component of the SCF complex) as a substrate adaptor to recognize cyclin E (Strohmaier et al., 2001; Koepf et al., 2001; Hao et al., 2007). Cul1 mediated-degradation requires phosphorylation of cyclin E at T77 and T395 in order for ubiquitylation of cyclin E to occur (Loeb et al., 2005; Welcker et al., 2003; Clurman et al., 1996; Minella et al., 2008). Cul1-mediated degradation of cyclin E occurs at ~4 h following release from a thymidine block (S phase) (Bhaskaran et al., 2013).

In contrast to the Cul1 pathway, the mechanistic details of Cul3-mediated destruction of cyclin E remain largely uncharacterized. Similar to Cul1 complexes, Cul3 ubiquitin ligase complexes consist of a substrate adaptor [a ‘*bric-a-brac*; *tramtrack*, *broad-complex*’ (BTB) domain-containing protein] that binds near the Cul3 N-terminus to recruit substrates, and a C-terminal region that binds to the RING finger protein Rbx1 (Tyers and Jorgensen, 2000; Jin and Harper, 2002; Duda et al., 2008), which in turn, recruits an E2 ubiquitin-conjugating enzyme (Petroski and Deshaies, 2005). Previous studies from our laboratory have shown that Cul3 degrades cyclin E that is not bound to Cdk2 and regulation of cyclin E by Cul3 is necessary for the maintenance of quiescence in the liver (Singer et al., 1999; McEvoy et al., 2007).


Despite the advances, many details regarding the mechanism utilized by Cul3 for cyclin E ubiquitylation remain uncharacterized, including the location of the degron that Cul3 uses to recognize and ubiquitylate cyclin E. A degron is a sequence or structural motif that is required for a protein to be recognized and degraded in a ubiquitin-dependent manner and can include both the ubiquitylated lysine residue as well as other features required for ubiquitylation, such as a binding site (Laney and Hochstrasser, 1999). Here, we identify the degron targeted by Cul3 near the N-terminus of cyclin E, and a unique mechanism for Cul3-mediated cyclin E destruction is proposed.

¹Department of Biology, Portland State University, Portland, OR 97201, USA.

²Department of Physiology, Medical College of Wisconsin, Milwaukee, WI 53226-0509, USA.

[†]Present address: Center for Global Infectious Disease Research, Seattle Children’s Research Institute, Seattle, WA 98109, USA. *Present address: Danish Myograph Technology (DMT), Ann Arbor, MI 48108, USA.

[§]Author for correspondence (jsinger@pdx.edu)

 K.G.d.O.R., 0000-0001-5446-3922; C.D.S., 0000-0002-1453-0921; J.D.S., 0000-0002-0654-3036

RESULTS

Cyclin E binds directly to Cul3 independently of BTB domain-containing proteins

Cul3 has been shown to require a BTB domain-containing protein to bind substrates (Pintard et al., 2003; Pintard et al., 2004; Kwon et al., 2006; Geyer et al., 2003; Cummings et al., 2009; Choi et al., 2016; Chen et al., 2009), but previous work from our laboratory has shown that Cul3 binds cyclin E in a yeast two-hybrid screen, indicating that these proteins may interact directly with each other (Singer et al., 1999). In order to determine the nature of the interaction between Cul3 and cyclin E, and whether the two proteins interact directly with each other, several Cul3 mutants were co-transfected with cyclin E and their binding was measured through immunoprecipitation assays. The mutants represented disruptions of the major functional regions of Cul3: the BTB domain interaction region (residues 51–67; Cul3 Δ 51–67), the Ned8 modification site (K712R), and the gain of function 403–459 deletion (Wimuttisuk et al., 2014; McCormick et al., 2014; Wimuttisuk and Singer, 2007; Zheng et al., 2002; Boyden et al., 2012). Cyclin E bound to all Cul3 mutants tested, including the Cul3 Δ 51–67 mutant, which cannot bind BTB proteins (Fig. 1A, lane 3). This finding is consistent with cyclin E being able to bind directly to Cul3 without the aid of a BTB protein.

In order to further delineate the interaction between Cul3 and cyclin E, Cul3 and cyclin E binding was examined in the presence of SPOP, a BTB protein that targets other (non-cyclin E) Cul3 substrates for ubiquitylation (Kwon et al., 2006; Zhang et al., 2014). First, we compared the ability of cyclin E to bind SPOP in comparison to RhoBTB3 as a control, because RhoBTB3 has been shown to bind cyclin E (Lu and Pfeffer, 2013) (Fig. 1B). We determined that cyclin E associates with SPOP only in the presence of Cul3, whereas cyclin E binds to RhoBTB3 under all circumstances tested (Fig. 1B, compare lanes 3 and 5 to lanes 2 and 4). These data suggest that the observed interaction between cyclin E and SPOP can only occur if it is mediated by Cul3 (Fig. 1B). To further analyze this relationship, cyclin E and SPOP were co-transfected with wild-type (WT) Cul3 and we observed, as shown in Fig. 1C, that cyclin E can co-immunoprecipitate SPOP; however, cyclin E was not able to co-immunoprecipitate SPOP when the Cul3 mutant Cul3 Δ 51–67, which cannot bind BTB domain-containing proteins, was used (Fig. 1C, lane 2 compared to lane 3). These data demonstrate that Cul3 can link cyclin E to SPOP as immunoprecipitation of SPOP by cyclin E occurs only when SPOP is bound to Cul3. These data support the hypothesis that a direct interaction occurs between cyclin E and Cul3 and also indicate that the interaction between Cul3 and cyclin E occurs outside of the BTB-binding region on Cul3.

Mutations in the N-terminal region of cyclin E prevent degradation by the Cul3 complex

To more precisely map the region of cyclin E that binds to Cul3, two C-terminally truncated cyclin E mutants were analyzed for binding to Cul3 and compared to full-length cyclin E. These consisted of the N-terminal 200 amino acids (STOP 200) of cyclin E and the N-terminal 300 amino acids (STOP 300) of cyclin E (Fig. 2A). A STOP 100 truncation of cyclin E was also transfected, but it was found to be unstable and therefore not used for further experimentation (Fig. 2A). Cul3 bound to both cyclin E truncation mutants as well as WT cyclin E (Fig. 2B, compare lanes 2 and 4 to lane 6), indicating that Cul3 interacts with the N-terminal half of cyclin E. To further pinpoint the binding site, we examined the potential binding of several cyclin E alanine-scanning mutants to Cul3 (Kelly et al., 1998). Each mutant in this set contains a charged

amino acid sequence that has been mutated to alanine residues (Fig. 2C). Among the set of mutants, one alanine-scanning mutant (DPDEE \rightarrow AAAAA; amino acids 41–45), which is located near the N-terminus, showed decreased binding to Cul3 (Fig. 2D, first panel lane 4 in comparison to lanes 2, 6 and 8).

After determining that Cul3 only requires the N-terminal portion of cyclin E for binding, we sought to ascertain whether this region contains all the necessary signals for Cul3-mediated degradation to occur. To efficiently identify the precise interaction region, we utilized a novel transfection assay that we developed using cells that are deficient for Cul3. Cul3 is responsible for the differences in cyclin E protein abundance between WT mouse embryonic fibroblasts (MEFs) and cells deficient for Cul3, as we have previously shown that both cell types express similar levels of cyclin E mRNA (McEvoy et al., 2007). It was observed that in Cul3 hypomorphic (floxed) MEFs, transfected cyclin E was more abundant than in WT MEFs, similar to the endogenous levels of cyclin E (McEvoy et al., 2007). When transfected into the same cells, controls that are not Cul3 substrates express at the same levels in these cell types. The same regulation of cyclin E was observed when comparing WT (Cul3 containing) and Cul3 knockout (KO) HEK293 cells (hereafter denoted 293 cells) (Ibeawuchi et al., 2015) (Fig. 3A, lanes 1 and 2). We reasoned that if a mutant cyclin E that lacked the Cul3 degron was transfected into these two genotypes, we would not see a difference in levels of cyclin E protein. By contrast, transfected cyclin E mutants that are Cul3 substrates would be expected to express at higher levels in the Cul3 KO cells than in WT 293 cells.

Two controls, WT cyclin E and lysine-lacking cyclin E, were transfected into the two cell types (WT and Cul3 KO 293 cells). As mentioned above, we observed that when transfected with equal amounts of WT cyclin E, the protein was detected at higher levels in the KO 293 cells than the WT cells (Fig. 3A, compare lanes 1 and 2). In contrast, lysine-lacking cyclin E was expressed evenly in both cell types (Fig. 3A, lanes 3 and 4), indicating the utility of this assay as a measure of the substrate being recognized for degradation instead of merely binding. To demonstrate that loss of degradation causes an increase in transfected cyclin E protein abundance, WT cyclin E was transfected in the presence and absence of the proteasome inhibitor MG132 (Fig. S1). We observed that the level of transfected cyclin E in the WT 293 cells increases in the presence of MG132 (Fig. S1).

The relative steady-state levels of the cyclin E truncations were examined by transfection into WT and Cul3 KO 293 cells. We observed that both the STOP 200 and STOP 300 cyclin E truncations are more abundant in the KO cells, implying that the degron recognized by Cul3 is within the first half of the cyclin E protein and that the C-terminal PEST sequence (residues 385–401) is not necessary for Cul3-mediated degradation to occur (Fig. 3B, compare lane 1 to lane 2 and compare lane 3 to lane 4). Taken together, these results demonstrate that the Cul3 degron resides in the N-terminal half of the cyclin.

To further establish the important role of the cyclin E N-terminal domain (residues 1–86) in cyclin E ubiquitylation by Cul3, a Flag-tagged fusion protein containing residues 2–86 of cyclin E was fused to YFP. We observed that the fusion protein is only ubiquitylated when both Cul3 and ubiquitin are present (Fig. 3C, compare lane 4 to lanes 1 through 3). Conversely, WT YFP is not ubiquitylated in this assay (Fig. 3C, lanes 5 through 8), thereby demonstrating the specificity of the interaction between Cul3 and the N-terminal portion of cyclin E, while clearly indicating that the cyclin E N-terminal region alone contains the signals necessary for Cul3-mediated ubiquitylation to occur.

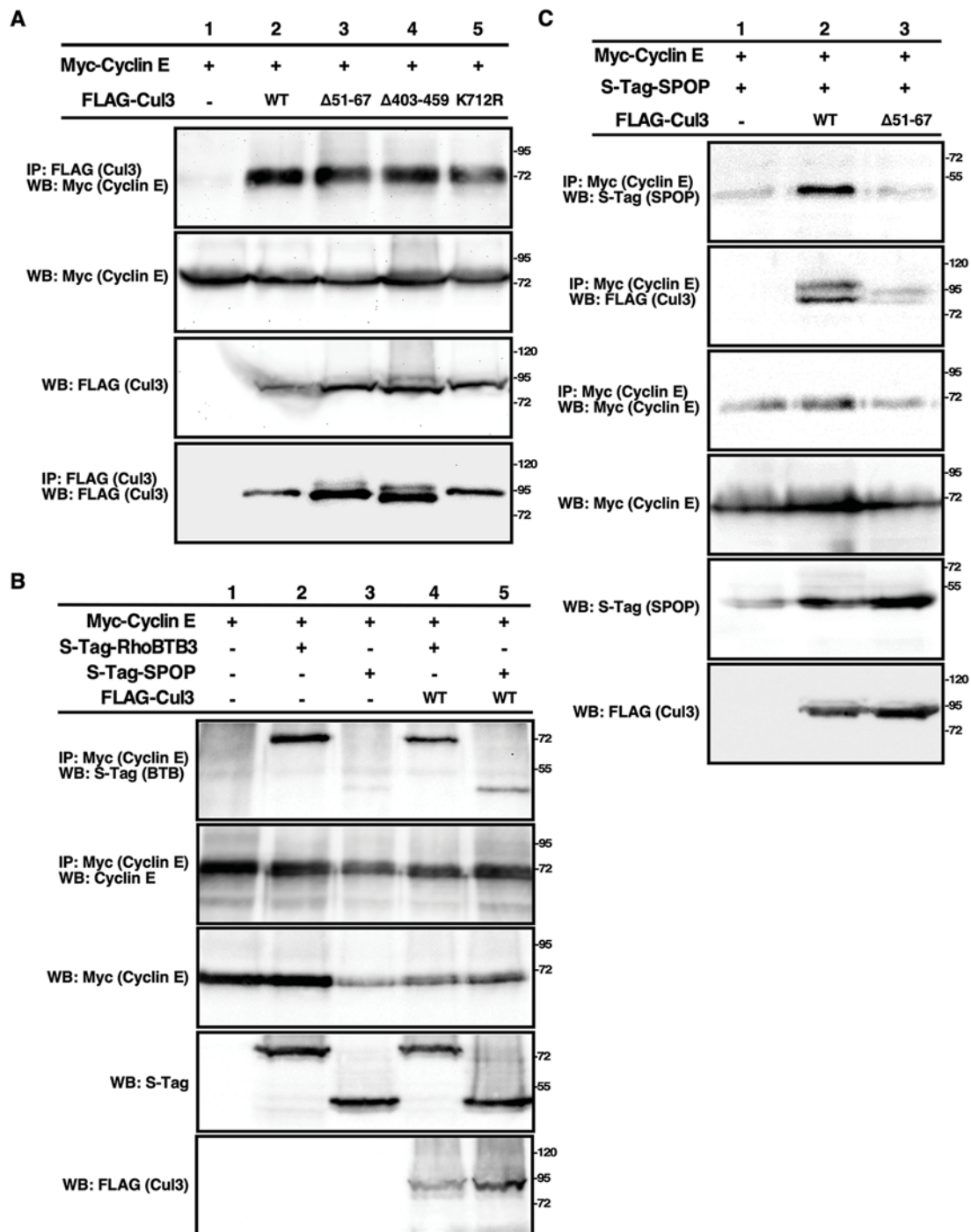


Fig. 1. Cul3 directly binds cyclin E. (A) HEK293 cells were co-transfected with Myc-cyclin E1 and different Flag-tagged WT Cul3 or Cul3 mutants, including Cul3 Δ 51-67, Cul3 Δ 403-459 and Cul3K712R or no construct (-). Immunoprecipitations (IPs) were performed using a Flag antibody. A western blot (WB) using anti-cyclin E antibody was performed to detect transfected cyclin E (top panel). Controls for the original protein levels in the lysates are shown in the middle two panels. (B) Myc-cyclin E was co-transfected with S-tag-RhoBTB3 (lanes 2 and 4) or S-tag-SPOP (lanes 3 and 5), with Flag-Cul3 in lanes 4 and 5. IPs were performed using a polyclonal Myc antibody. IP results are shown in the top panel. The lower three panels show the original protein levels in the lysates. (C) HEK293 cells were co-transfected with Flag-Cul3 or Flag-Cul3 Δ 51-67 and Myc-cyclin E, HA-ubiquitin and S-tag-SPOP. An IP for Myc-cyclin E was performed followed by western blotting to detect S-SPOP (C, top panel) and Flag-Cul3 (C, second panel). The lower three panels show the original protein levels of the lysates prior to IP. For all western blots, the migration of size markers is indicated on the sides of the gel images (kDa).

K48 on cyclin E is located in a newly identified degron and serves as a ubiquitylation site for Cul3

To pinpoint specific residues on cyclin E that may be involved in degradation, cyclin E alanine-scanning mutants were transfected

into the Cul3 WT and KO 293 cells (Kelly et al., 1998). Three alanine-scanning mutants, DPDEE \rightarrow AAAAA (residues 41–45), KIDR \rightarrow AIAA (residues 48–51), and DKED \rightarrow AAAA (residues 79–82), showed similar levels of protein expression in the WT cells

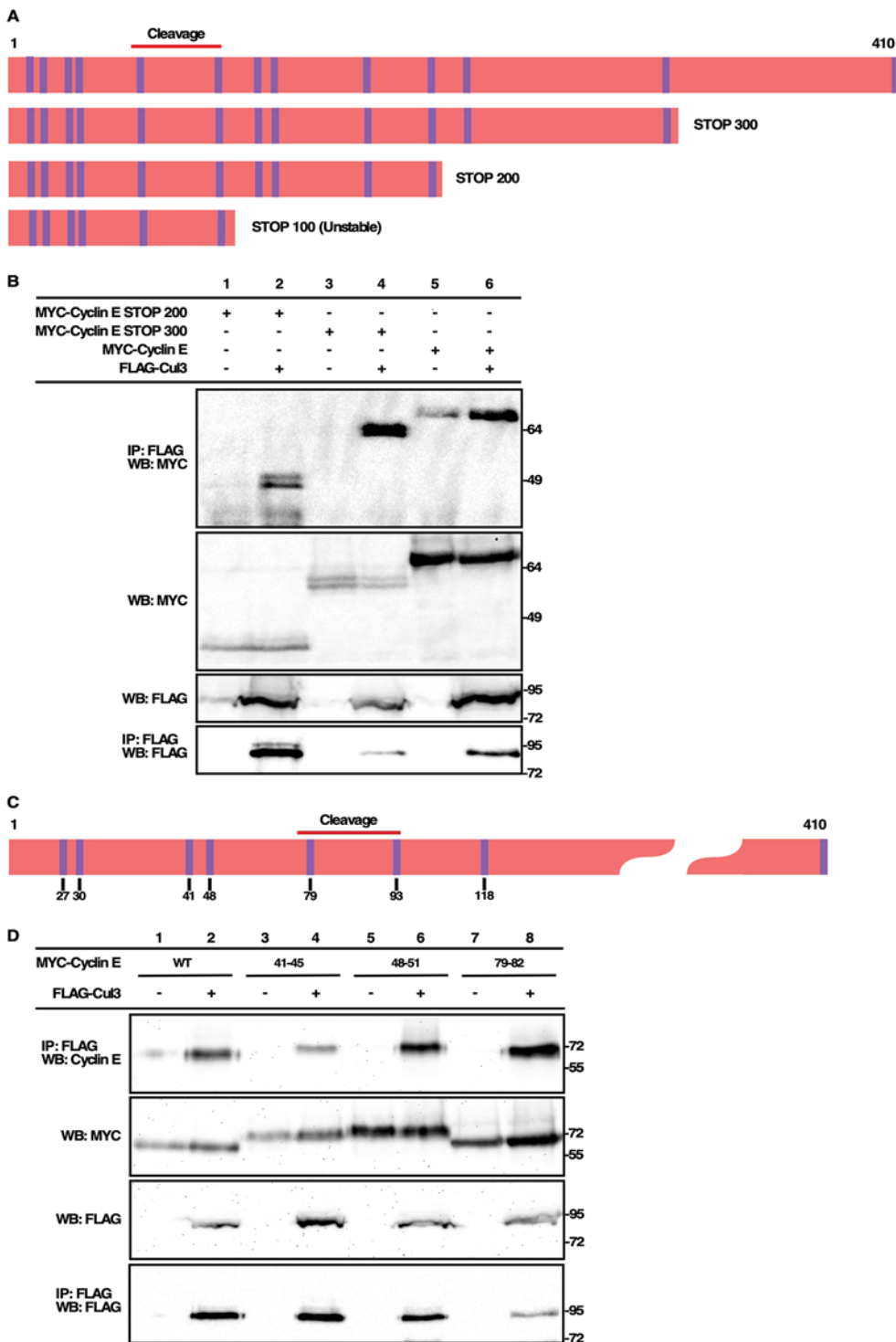


Fig. 2. The N-terminal domain of cyclin E is required for binding to Cul3.

(A) A cartoon showing full-length WT cyclin E (top) and three truncation mutants of the protein, STOP 100, STOP 200 and STOP 300. (B) HEK293 cells were transfected with constructs as indicated. Upper blot, immunoprecipitation (IP) for Flag-Cul3 and western blot (WB) for Myc-cyclin E showing binding. Middle two blots show relative levels of transfected protein in cell extracts and the lower panel shows the Flag blot of the Flag IP. (C) Cartoon depicting the locations of point mutants and alanine-scanning mutants (designated here by the first mutated amino acid number) on cyclin E. The cleavage site which produces LMM cyclin E is shown in red. (D) HEK293 cells were transfected with constructs as indicated. Upper blot, IP for Flag-Cul3 and western blot for Myc-cyclin E showing binding of three N-terminally located alanine-scanning mutants (lanes 4, 6, and 8) in comparison to WT cyclin E (lane 2). The middle two blots show levels of transfected protein in the cell extracts. The lower panel shows the Flag blot of the Flag IP. For all western blots, the migration of size markers is indicated on the sides of the gel images (kDa).

compared to Cul3 KO cells, which suggests that they are not as easily targeted for degradation by Cul3 as WT cyclin E (Fig. 3D, DPDEE is shown in lanes 1 and 2, KIDR in lanes 3 and 4, and DKED in lanes 5 and 6; WT cyclin E is shown in Fig. 3A lanes 1 and 2 for comparison). All three of these mutants are located near the N-terminus of cyclin E. In order to further characterize the importance of this region for Cul3-mediated degradation to occur, two Myc-tagged constructs containing deletions in this region were created (Fig. 3E). The first construct, cyclin E Δ 31-82, is missing the region encompassing the three alanine-scanning mutants that

demonstrated increased abundance. The second cyclin E deletion construct, cyclin E Δ 2-86, was designed to resemble the LMM cyclin E found in some cancer cells and is missing the entire N-terminal region (Fig. 3E). These mutants both expressed at similar levels in WT cells and Cul3 KO cells (Fig. 3F, compare lane 1 to 2, and lane 3 to lane 4), suggesting that residues 31 through 82 on cyclin E are necessary for Cul3-mediated degradation of cyclin E to occur.

Changes in abundance exhibited by mutant proteins can often be explained by changes in the location of the protein within the cell. In order to determine whether the phenotypes of the three stable

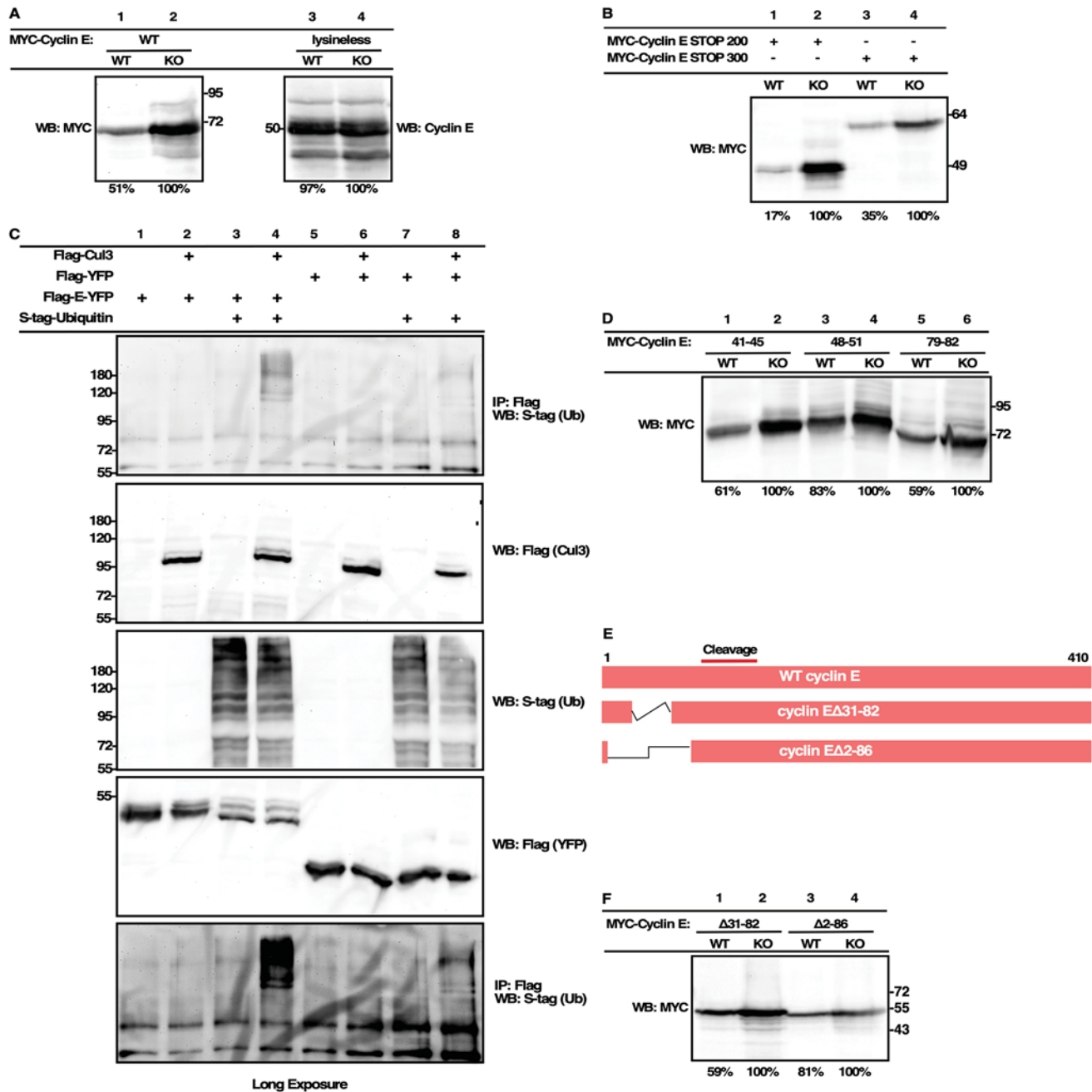


Fig. 3. The cyclin E N-terminal domain regulates cyclin E stability in the presence of Cul3. (A) Myc-tagged WT cyclin E was transfected into Cul3 KO and WT HEK293 cells. There is a difference in the level of cyclin E between these two cell types, as indicated by western blotting (WB; left panel). Levels of a transfected cyclin E mutant without lysine residues (lysineless), which cannot be ubiquitylated, are shown in the right panel. (B) Myc-tagged STOP 200 and STOP 300 truncations of cyclin E were transfected into WT and Cul3 KO 293 cells and their expression level was assessed using a Myc (cyclin E) antibody. (C) Ubiquitylation assay for the cyclin E residues 2–86 and YFP fusion protein. Cells were transfected with either Flag-tagged cyclin E–YFP (Flag-E-YFP) or Flag-tagged YFP in the presence and absence of S-tag–ubiquitin and Flag–Cul3. Lysates were harvested and analyzed via immunoprecipitation (IP; Flag) and western blotting. Top panel: results of the ubiquitylation assay. The cyclin E–YFP fusion protein is shown in lanes 1 through 4 and WT YFP is shown in lanes 5 through 8. Middle three panels, western blots showing the total amount of protein in each lysate prior to the IPs. Lower panel, long exposure of the ubiquitylation assay. (D) Three Myc-tagged alanine-scanning mutants, DPDEE→AAAAA (residues 41–45, lanes 1 and 2), KIDR→AIAA (amino acids 48–51, lanes 3 and 4) and DKED→AAAA (amino acids 79–82, lanes 5 and 6), were transfected into both WT and Cul3 KO HEK293 cells and their abundance was measured using an anti-Myc (cyclin E) antibody. (E) Diagram showing two mutants in which portions of the N-terminal domain had been deleted. (F) Expression of transfected Myc–cyclin E Δ 31-82 and Myc–cyclin E Δ 2-86 are shown in both the WT and KO cells (lanes 1 through 4). For A, B, D and E, quantification of the presented western blot (WB) is listed below each lane as a percentage relative to the sample in the KO lane for each pair. For all western blots, the migration of size markers is indicated on the sides of the gel images (kDa). The blots shown are representative of $n=2$ (A,B) or $n=3$ (D,E).

alanine-scanning mutants are the result of localization changes, HeLa cells were transfected with each construct and visualized using immunofluorescence microscopy. The cellular localization of

the three mutants was found to be predominantly nuclear, similar to WT cyclin E, indicating that their increased abundance is not caused by mis-localization (Fig. S2).

The DPDEE→AAAAA (residues 41–45) mutant cannot bind Cul3 as well as other cyclin E constructs (Fig. 2D), which could provide a partial explanation for its increased protein levels in our assay. The other two mutants, KIDR→AIAA (residues 48–51) and DKED→AAAA (residues 79–82) both contain lysine residues (Fig. 3D). The increased protein levels of these alanine-scanning mutants as well as cyclin EΔ31–82 imply that both lysine residues K48 and K80 are potential ubiquitylation sites, and the degron that is recognized by Cul3 likely resides within the region spanned by the cyclin EΔ31–82 deletion.

To establish whether ubiquitylation on K48 or K80 of cyclin E regulates its relative expression levels, we determined whether the alanine-scanning mutants were stabilized by a dominant-negative mutation in ubiquitin that prevents K48 branching (K48R), a type of branching that leads to degradation (Raasi and Pickart, 2005). We have shown that this ubiquitin mutation results in stabilization of cyclin E whereas a mutation in another important lysine in ubiquitin, K63, has no effect (Fig. S3). To examine this, all of the alanine-scanning mutants within the first 200 amino acids of cyclin E were transfected into both cell types in the presence or absence of a mutant K48R ubiquitin. We observed that, unlike WT cyclin E, which was stabilized in WT cells by the addition of the dominant-negative K48R ubiquitin mutant (Fig. 4A, compare lanes 1 and 2 to lanes 3 and 4), the KIDR (48–51)→AIAA mutant was unaffected, indicating it is no longer a substrate for ubiquitylation-dependent degradation (Fig. 4A, lanes 13–16). The DPDEE (41–45)→AAAAA (Fig. 4A, lanes 9–12) mutant behaved similarly to the KIDR (48–51)→AIAA mutant. The DKED (79–82)→AAAA mutant (Fig. 4A, lanes 17–20) appears similar to WT (Fig. 4A, top left panel, lanes 1–4) in this assay, suggesting K48 (the KIDR lysine) and not K80 (the DKED lysine) is the ubiquitylation site for Cul3. As a second control, cyclin E T395A, which cannot be degraded by Cul1, was examined, and we observed that it is a substrate of Cul3, which demonstrates the specificity of our assay (Fig. 4A, lanes 5–8). In order to confirm that K48 is a ubiquitylation site on cyclin E, a point mutant, cyclin E K48R, was constructed for use in the ubiquitylation assay. Like the KIDR (48–51) alanine-scanning mutant, cyclin E K48R is more stable in WT cells than WT cyclin E (Fig. 4A, lanes 21 through 24), suggesting that K48 on cyclin E is indeed a ubiquitylation site utilized by Cul3 *in vivo*. These data suggest the presence of a Cul3 degron on cyclin E located near the cyclin E N-terminus. The residues DPDEE (41–45) and KIDR (48–51) constitute the Cul3 binding site (DPDEE) and the ubiquitylation site (KIDR) utilized by Cul3 to target cyclin E for ubiquitin-mediated proteolysis.

Previous work from our laboratory has shown that Cul3 regulates endogenous cyclin E, as WT cyclin E has a longer half-life in cells that are deficient for Cul3 and this longer half-life is not associated with changes in mRNA levels of either cyclin E1 or cyclin E2 (McEvoy et al., 2007). To determine whether cyclin E K48R is differentially degraded by Cul3 as compared to WT cyclin E, we measured the half-life of these proteins in cells of both genotypes (Cul3 WT and KO). Cells were transfected with full-length Myc-tagged WT cyclin E or Myc-tagged cyclin E K48R. Following addition of cycloheximide (CHX) the cells were harvested every 2 h for 8 h. We observed that WT cyclin E has a short half-life in the WT cell line but a dramatically increased half-life in the KO cells, demonstrating that loss of Cul3 increases the half-life of cyclin E [Fig. 4B, compare WT cyclin E in WT cells (top left), and WT cyclin E in KO cells (bottom left)]. Next, we measured the half-life of the cyclin E mutant K48R, the putative ubiquitylation site. Cyclin E K48R had a half-life longer than WT

cyclin E in both WT and Cul3 KO cells, demonstrating that K48 on cyclin E is required for Cul3-mediated degradation to occur [Fig. 4B,C, compare the half-life of WT cyclin E (Fig. 4B) to cyclin E K48R (Fig. 4C)].

The N-terminal domain of cyclin E is required for Cul3-mediated degradation

As LMM cyclin E can be found endogenously in some cells, we sought to determine whether loss of Cul3 affects the presence of these LMM forms. Overall, the Cul3 KO 293 cells have more endogenous cyclin E than the WT (Ibeawuchi et al., 2015). We observed that the 50 kDa endogenous cyclin E band increases upon inhibition of the proteasome in WT cells to equal the amount of the 50 kDa protein that is present in the KO cells prior to proteasome inhibition, demonstrating that the 50 kDa band is a substrate of Cul3 in 293 cells (Fig. 5A, lane 2 vs lane 3). Two LMM cyclin E bands are also detected, the smallest of which is ~43 kDa. These two LMM cyclin E bands are present in both WT and KO cells (Fig. 5A). The relative abundance of the LMM bands is elevated equally in both cell types upon proteasome inhibition (Fig. 5A, lanes 2 and 4), indicating that LMM cyclin E is targeted for ubiquitin-mediated proteolysis equally in both cell types.

Next, to determine whether the rate of degradation of LMM cyclin E is impacted by Cul3, Myc-tagged cyclin EΔ2–86, which lacks the N-terminal domain, was transfected into WT and Cul3 KO 293 cells. CHX was added to the samples and time points were collected every 2 h over the course of 10 h (Fig. 5B). The results of this experiment shows that cyclin EΔ2–86 has the same half-life in WT and KO cells, demonstrating that Cul3 is unable to regulate the abundance of cyclin E protein that lacks its N-terminal domain (Fig. 5B). To further test the role of this degron in Cul3-mediated degradation, we created a YFP fusion protein which contains residues 2 through 86 (the N-terminal region) of cyclin E fused to a Flag-tagged YFP. This cyclin E–YFP fusion protein has a shorter half-life than YFP alone in WT cells but not in the Cul3 KO cells, consistent with our previous conclusion that the N-terminal domain of cyclin E contains the signals necessary for degradation (Fig. S4A). In addition, Cul3 can bind the YFP-containing protein that contains the Cul3 degron but cannot bind YFP alone (Fig. S4B). These results are consistent with our above data demonstrating the importance of K48, which is located in the N-terminal region, for Cul3-mediated degradation of cyclin E. Together, these data suggest that Cul3 is unable to regulate the LMM truncated forms of cyclin E that occur *in vivo* and lack the Cul3 degron. LMM cyclin E has been shown to bind Cdk2 and results in increased Cdk2 activity (Porter et al., 2001; Wingate et al., 2005). Our data indicate that LMM cyclin E is not a Cul3 substrate, providing a possible mechanism that LMM cyclin E1 might employ in order to bypass ubiquitylation by the Cul3 pathway, thereby increasing its availability to bind Cdk2 (Fig. 5C).

Cyclin E2 lacks the Cul3 degron and is not targeted for degradation by Cul3

The structure of cyclin E bound to Cdk2 has been determined (Honda et al., 2005), but the published structure only includes amino acids 81–363 of cyclin E, omitting the N-terminal region. Recently, advanced modeling techniques have predicted a structure for the N-terminal region of cyclin E that shows that this portion of the protein is mostly disordered with the notable exception of a predicted α -helix encompassing the sequence DEEMAKID (residues 43–50, Fig. 6A) (Rath and Senapati, 2014). Coincidentally, the alanine scanning mutants located

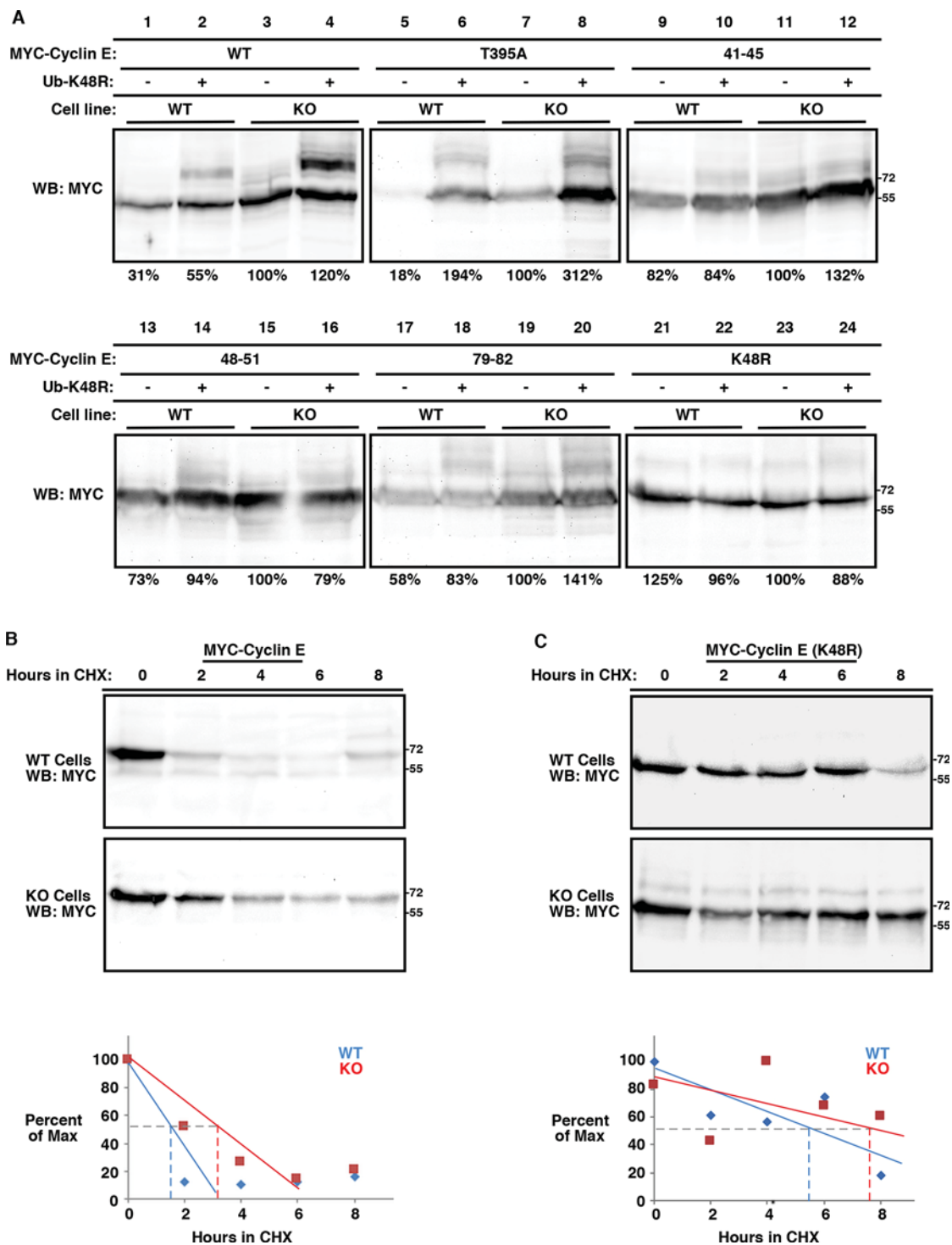


Fig. 4. Cul3-mediated ubiquitylation of cyclin E on K48 regulates cyclin E abundance. (A) Alanine-scanning and point mutants located within the first 200 amino acids of the cyclin E protein were transfected in Cul3 WT and KO 293 cells in the presence or absence of an S-tagged K48R ubiquitin construct. Wild-type cyclin E (lanes 1–4) and cyclin E T395A, which cannot be degraded by Cul1 (lanes 5–8), were included as controls. Three cyclin E alanine-scanning mutants are shown here, DPDEE→AAAAA (residues 41–45, lanes 9–12), KIDR→AIAA (residues 48–51, lanes 13–16), and DKED→AAAA (residues 79–82, lanes 17–20). The point mutant cyclin E K48R is also shown (lanes 21–24). The total amount of protein in each lane was quantified for the blot shown and is presented as a percentage relative to that in the knockout lane. (B,C) Cul3 WT and KO 293 cells were transfected with Myc-tagged cyclin E (B) or Myc-tagged cyclin E K48R (C). After 24 h, CHX was added, and cells were harvested at the indicated time points. Half-lives were determined via western blotting (top), and quantified (for the blot shown, bottom). For all western blots, the migration of size markers is indicated on the sides of the gel images (kDa). The blots shown are representative of $n=2$ (A, lanes 1–20, B,C) or $n=1$ (A, lanes 21–24).

within this proposed helix [DPDEE (41–45)→AAAAA and KIDR (48–51)→AIAA] exhibited the most notable phenotypes in our study as DPDEE (41–45) exhibited diminished binding to the Cul3

complex and KIDR (48–51)→AIAA cannot be degraded in a Cul3-dependent manner (Figs 2D, 4 and 6). Our data suggest that this structural motif may form part of the interface on cyclin E that is

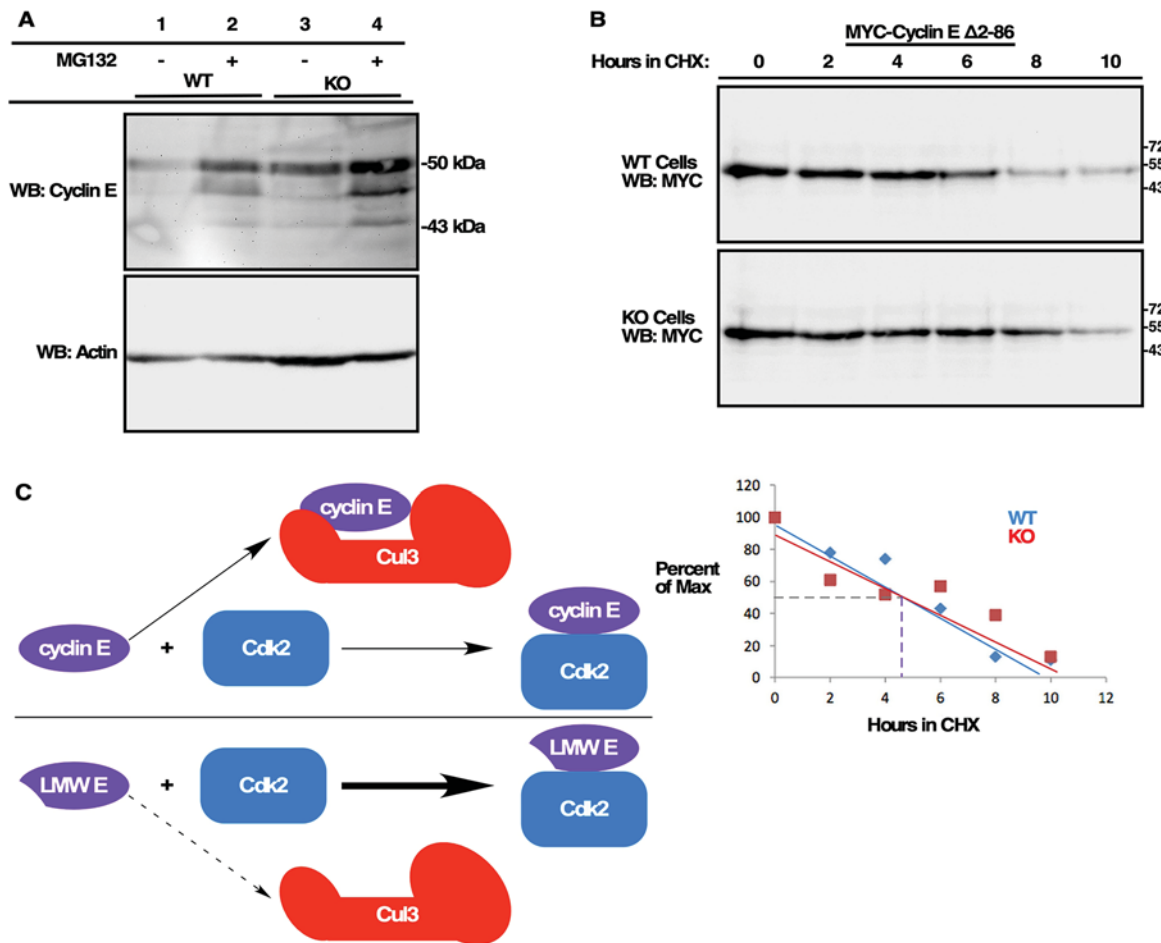


Fig. 5. LMM cyclin E is not degraded by Cul3. (A) The upper blot shows levels of endogenous cyclin E in WT and Cul3 KO 293 cells. The proteasome inhibitor MG132 has been added to the cells shown in lanes 2 and 4. The lower blot shows levels of actin in the same cells. (B) WT and Cul3 KO 293 cells were transfected with cyclin E Δ 2-86. CHX was added at 24 h post transfection and cells were harvested every 2 h following CHX addition. Top panel, western blots showing protein levels. Lower panel, quantification of results for blot shown (representative of $n=2$). (C) Model showing full-length cyclin E being ubiquitylated and degraded via a Cul3-mediated process during G₁, leaving some cyclin E available to activate Cdk2 (top). In cells containing LMM cyclin E, which lacks its N-terminal domain, correct ubiquitylation by Cul3 might not occur, possibly contributing to increased activation of Cdk2. For all western blots, the migration of size markers is indicated on the sides of the gel images (kDa).

responsible for Cul3 interaction and also functions as the Cul3 degnon on cyclin E (Fig. 6A). The putative helix comprising the Cul3 degnon, including the K48 ubiquitylation site, is conserved in rodents (Fig. 6B), which is notable as Cul3 also regulates cyclin E in mice (Singer et al., 1999; McEvoy et al., 2007).

Mammals contain two cyclin E-encoding genes, *CCNE1* (cyclin E1) and *CCNE2* (cyclin E2), which produce different proteins (Sherr and Roberts, 1999; Geng et al., 2003). The two cyclin E proteins share a high degree of similarity and many structural similarities, including the Cdk2-interacting domain (Zariwala et al., 1998). To determine whether both cyclin E proteins contain the proposed Cul3 degnon, we analyzed the net charge of the region by calculating the difference between the number of basic residues and the number of acidic residues and ascertained that the cyclin E1 degnon is acidic with a net charge of negative two. The corresponding region on cyclin E2, in contrast, is extremely basic with a net charge of plus four (Fig. 6C). A Chou–Fasman algorithm was used to predict possible structures of both cyclin E proteins in the degnon region (Chou and Fasman, 1975). This method predicts structural differences in the proposed degnon region between the two proteins, revealing what is possibly a

helical region in cyclin E1 is potentially an unstructured region in cyclin E2 (Fig. 6C, ‘h’ indicates helix and ‘n’ indicates no predicted structure). Taken together, this information suggests that the Cul3 degnon comprises a structural feature that is unique to cyclin E1.

Next, to determine whether the observed Cul3-mediated degradation of cyclin E is specific to cyclin E1, cyclin E2 was transfected into the WT and KO 293 cells. Cyclin E2 was expressed evenly in both cell types, indicating that it does not get degraded by a Cul3-dependent mechanism (Fig. 6D, lanes 1 and 3 compared to lanes 2 and 4), and unlike cyclin E1, cyclin E2 did not bind Cul3 in an immunoprecipitation assay (Fig. 6E). Finally, transfected cyclin E2 has a similar half-life in the WT and KO 293 cells, suggesting that it is not a Cul3 substrate (Fig. S5). Together, these data suggest that Cul3 selectively targets cyclin E1 for Cul3-mediated degradation.

Loss of Cul3 results in early accumulation of cyclin E after release from serum starvation

The Cul1 E3 ligase also targets cyclin E for degradation and does so in a distinct window in the cell cycle, beginning at ~4 h after the

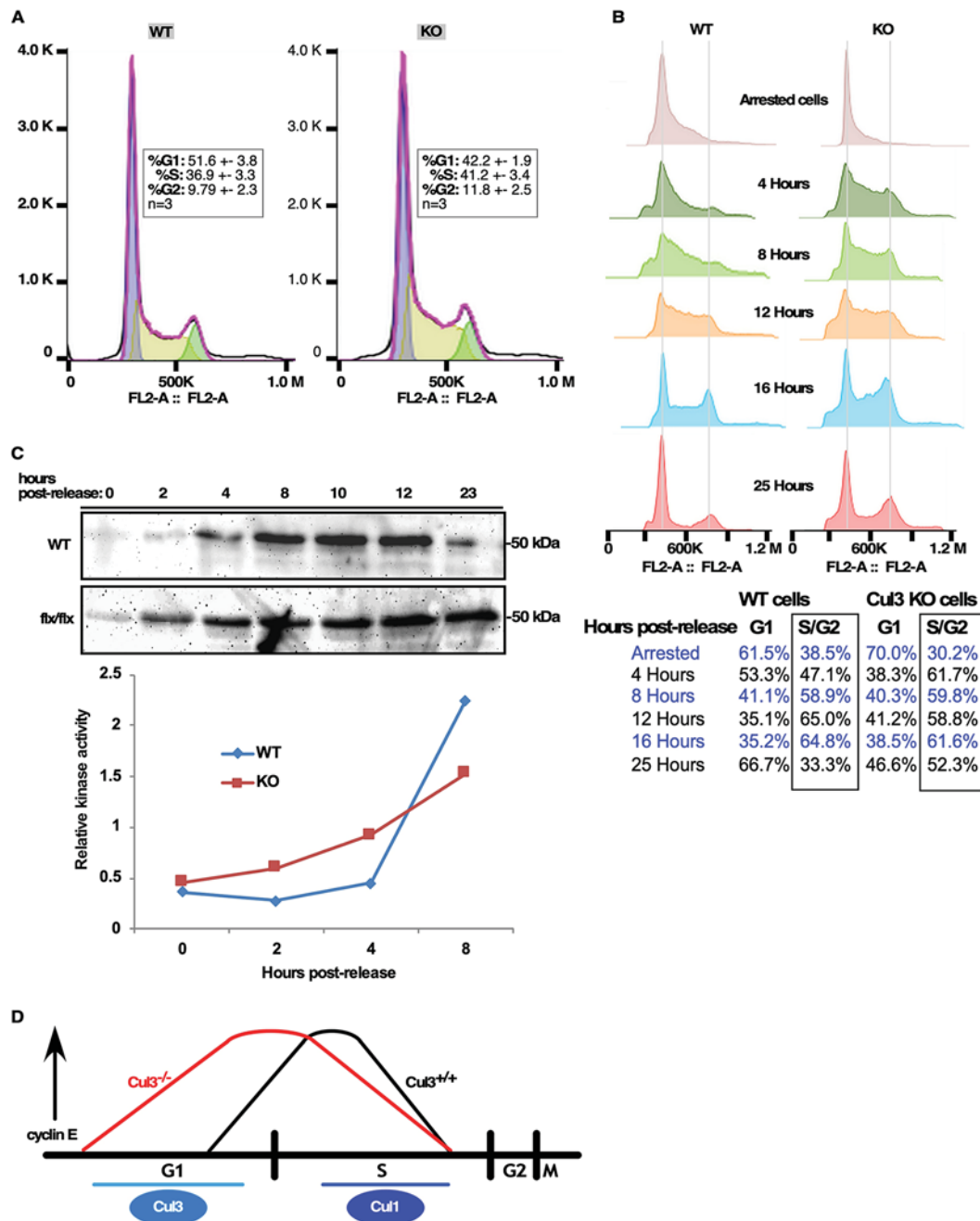


Fig. 7. Cul3 regulates cyclin E degradation early during G₁. (A) Flow cytometry analysis of proliferating 293 cells. WT cells are shown on the left and Cul3 KO cells are shown on the right. Cells in G₁ are shown in purple, S phase cells are shown in yellow, and G₂/M cells are shown in green. The quantification shown represents the mean \pm s.d. of three experiments. (B) Cells were serum-starved and released into G₁. Cells were harvested at 4-h intervals, stained with propidium iodide, and analyzed by flow cytometry. (C) Western blot showing levels of endogenous cyclin E in mouse embryonic fibroblasts (MEFs) that are WT (WT, western blot, top row) or deficient for Cul3 (flx/flx, western blot, bottom row). Results of a kinase assay for cyclin E–Cdk2 activity in synchronized HEK293 cells are shown in the graph beneath the western blot. Cells were synchronized in G₁ and then released and harvested at the indicated time points. Cyclin E and Cdk2 were purified from the lysates via immunoprecipitation and then used in a kinase assay which couples kinase activity to phosphate production. Cells that had been serum-starved and released were collected at 2-h intervals as indicated. The plot is derived from one dataset; $n=1$, except for the 0 hour time point where $n=2$. (D) These data are consistent with a model where Cul3 is responsible for maintaining cyclin E levels during G₁ in order to prevent early entrance into S phase. Cul1 is known to degrade cyclin E after S phase has begun.

E are associated with poor prognosis in cancer patients and overexpression of the LMM cyclin E has also been shown to cause cell cycle errors including chromosome mis-segregation (Akli et al., 2010; Duong et al., 2012; Bagheri-Yarmand et al., 2010). Others have shown that these errors result from the increased ability of LMM cyclin E to bind and activate Cdk2, which

increases Cdk2 kinase activity (Porter et al., 2001; Wingate et al., 2005). Healthy cells rely upon degradation of cyclin E protein by the ubiquitin–proteasome system in order to restrict levels of cyclin E. The Cul3 E3 ligase targets cyclin E for degradation, and our work sheds light upon the mechanism utilized by Cul3 to ubiquitylate cyclin E.

We have identified a Cul3 degron in cyclin E1 that spans the acidic region between residues 41 and 51 (DPDEEMAKIDR), located near the N-terminus of the cyclin. This degron consists of a region involved in Cul3 binding (residues 41–45) and a ubiquitylated lysine residue, K48. The N-terminal region containing the degron had not been shown to be part of the core cyclin E protein and is not necessary for Cdk2 activation to occur (Porter and Keyomarsi, 2000; Porter et al., 2001). Our analysis, as well as those from other investigators, suggest that this acidic region forms a structural motif within the disordered N-terminal domain on cyclin E1 (Fig. 6A; Rath and Senapati, 2014). In addition, we show that Cul3 uses this structural feature, which is conserved in mammals (Fig. 6B), to recognize cyclin E1 for ubiquitylation (Fig. 6). We demonstrate that the Cul3 degron is specific to cyclin E1, as the corresponding region in cyclin E2 carries a positive (basic) charge (Fig. 6C) and levels of cyclin E2 are not affected by deletion of Cul3 (Fig. 6D; Fig. S5).

In addition, and surprisingly, mutation of the Cul3 degron on cyclin E results in a stable cyclin E, implying that, in 293 cells, the Cul3 pathway is the predominant pathway involved in the degradation of cyclin E (Fig. 4A, lower right panel, Fig. 4C). Mutation of K48 in cyclin E to an arginine residue (K48R) results in stabilization of cyclin E, as this form has a longer half-life than WT cyclin E, implying that K48 is the principal ubiquitylation site on cyclin E utilized by Cul3 (Fig. 4A, lower right panel, Fig. 4C). The longer half-life of cyclin E K48R in comparison to WT cyclin E has been seen in multiple experiments ($n=4$), demonstrating the significance of this result. Similarly, deletion of the cyclin E N-terminus (cyclin E Δ 2–86) prevents Cul3-mediated degradation of cyclin E (Fig. 5B). The half-life of cyclin E1 that cannot be ubiquitylated by Cul3 is longer than the half-life of WT cyclin E, indicating the necessity for Cul3 in cyclin E regulation (Fig. 4B compared to Figs 4C and 5B).

LMM cyclin E, which results from proteolytic processing and often lacks portions of the N-terminal region, represents a particularly active form of cyclin E that is made in cancer cells and associated with tumorigenesis (Porter and Keyomarsi, 2000; Keyomarsi et al., 1995; Bagheri-Yarmand et al., 2010; Akli et al., 2004; Akli et al., 2010; Porter et al., 2001). Our data suggest that the loss of the newly identified Cul3 degron may contribute to the increased Cdk2 activity that is observed in cells containing LMM cyclin E (Fig. 5C). We constructed a cyclin E deletion mutant, cyclin E Δ 2–86, that is missing the N-terminal region, similar to the naturally occurring LMM cyclin E variants that lack the majority of the N-terminal domain (Fig. 3E). Like cyclin E K48R, cyclin E Δ 2–86 is also more stable, suggesting that cyclin E1 that lacks the degron cannot be targeted for degradation by Cul3 (Figs 3F and 5B). This finding suggests that removal of the N-terminal cyclin E degron allows for the bypassing of Cul3-mediated degradation of LMM cyclin E1, resulting in more cyclin E being available to interact with Cdk2 (Fig. 5C). Although LMM cyclin E1 is still a substrate of the ubiquitin system (Delk et al., 2009), our results suggest that it is unable to be degraded in a Cul3-dependent manner (Fig. 5A). Levels of endogenous LMM cyclin E are increased upon addition of MG132 in Cul3 KO 293 cells (Fig. 5A), which further supports the idea that cyclin E lacking its N-terminal region is not a Cul3 substrate (Fig. 5A–C).

In addition to clarifying the mechanistic details regarding Cul3-mediated degradation of cyclin E1, the work presented here also identifies the temporal window during which such degradation occurs. Previous work has shown that loss of Cul3 results in cyclin E accumulation and exit from quiescence in mice (McEvoy et al., 2007), leading us to hypothesize that loss of Cul3 results in earlier

increases in cyclin E and entry into S phase. Here, this hypothesis is supported, as cells that are hypomorphic for Cul3 both enter the cell cycle earlier than their WT counterparts and show increased levels of cyclin E and associated kinase activity earlier than WT cells following release from serum starvation (Fig. 7B,C). These data suggest that Cul3 is responsible for maintaining levels of cyclin E early in the cell cycle, preceding the start of S phase, which is in contrast to the Cul1 degradation pathway, which degrades cyclin E later during S phase (Bhaskaran et al., 2013). Taken together, our data suggest that during G₁, Cul3 functions to suppress levels of cyclin E via ubiquitylation of its N-terminal domain (Fig. 5B). Lack of cyclin E regulation by Cul3 during G₁ might contribute to the increased cyclin E–Cdk2 activity that has been observed in cancer cells containing LMM cyclin E (Figs 5C and 7D).

Finally, this study suggests that the mode of recognition of cyclin E1 by Cul3 is unique, as it does not require a substrate adaptor. We show that Cul3 binds cyclin E independently and outside of the BTB-binding region (residues 51–67) on Cul3 (Fig. 1A,C), suggesting that Cul3 does not require a BTB protein in order to ubiquitylate cyclin E1. As other Cul3 substrates are known to require BTB proteins to enhance binding to the Cul3 E3 ligase complex (Zhang et al., 2004; Cummings et al., 2009), our finding that cyclin E can bind Cul3 directly suggests that cyclin E is a unique substrate of Cul3. A search of the literature revealed another Cul3 substrate, heat-shock factor 2 (HSF2), which is targeted for degradation upon binding directly to Cul3 via two PEST sequences (Xing et al., 2010). PEST sequences are defined as acidic regions rich in the amino acids D, E, P, S and T that are flanked by basic residues (Rogers et al., 1986). Our results show that the C-terminal PEST sequence in cyclin E is not required for Cul3-mediated degradation to occur (Fig. 3B); however, the degron that we identified near the cyclin E N-terminus lies within a region that also satisfies the definition of a PEST sequence (residues 31–51, RKANVTVFLQDPDEEMAKIDR). This observation suggests that a PEST motif facilitates ability of Cul3 to recognize cyclin E, demonstrating similarity to the PEST motifs in HSF2 which facilitate its ubiquitylation by Cul3 (Xing et al., 2010). Together, this information suggests a new mode of cullin substrate recognition whereby Cul3 binds some substrates without the aid of a BTB domain-containing protein. This idea will merit further study to determine what, if any, role BTB proteins might play in instances where Cul3 is responsible for substrate recognition. Others have suggested that the BTB protein RhoBTB3 is involved in cyclin E degradation and knockdown of RhoBTB3 resulted in elevated levels of cyclin E and increased proliferation (Lu and Pfeffer, 2013), which suggests to us that RhoBTB3 might play a non-canonical role in cyclin E ubiquitylation.

MATERIALS AND METHODS

Cell culture and transfections

Cells [HeLa, ATCC CCL-2; HEK 293, ATCC CRL-1573; MEFs, our laboratory source, McEvoy et al. (2007)] were maintained in DMEM supplemented with 10% fetal bovine serum and penicillin-streptomycin. HEK 293 and HeLa cells were split 1:20 for transfection in 6 cm dishes the night before transfection. Transfections were performed using the calcium phosphate precipitation method. For immunoprecipitations and expression level assays, cells were harvested 48 h post transfection. For HeLa and 293 cells, between 1 and 10 μ g of plasmid DNA was transfected into each plate. For experiments utilizing MG132 (Sigma-Aldrich), the drug was added ~18 h before harvest at a final concentration of 20 μ M. All transfections were harvested using RIPA buffer supplemented with protease inhibitors and then sonicated before being used for immunoprecipitations or western blotting.

Plasmids

All Cul3 mutants (Cul3 K712R, Cul3Δ51-67, and Cul3Δ403-459) were expressed using the same p3x-Flag vector (Sigma) as was WT Cul3. Cyclin E mutants were expressed using the CS2+ Myc-tagged expression vector, and a CS2+ S-tagged vector was used for the expression of SPOP. The pCMV3-tag-6 vector (Agilent) was used for construction of the cyclin E–YFP fusion protein shown in Fig. 3C and Fig. S4.

Western blotting and immunoprecipitations

Western blots and immunoprecipitations were conducted as previously described (Wimuttisuk et al., 2014). In short, a sonicated transfection lysate was added to the desired antibody in an Eppendorf tube and brought to a final volume of 500 μl. 40 μl of IPA–Sepharose beads were then added to the mixture, and the immunoprecipitations were placed on a rotator for 2 h at room temperature before being rinsed with RIPA buffer, heated in SDS-loading buffer, and run on an SDS-PAGE gel. The following antibodies were used for immunoprecipitations at 1–2 μg or at the indicated dilution for western blotting: monoclonal anti-FLAG (1:1000, cat. number F3165, Sigma-Aldrich), monoclonal anti-Myc (9E10) (1:500, c-Myc mouse anti-Human, cat. number MA1-980, Invitrogen), polyclonal anti-c-Myc (A14) (1:500, Santa Cruz Biotechnology), S-peptide monoclonal antibody (1:1000, clone 6.2, cat. number MA1-981, Invitrogen), monoclonal anti-HA.11 (16B12) (1:1000, cat. number: MMS-101P-200, BioLegend), polyclonal anti-HA (1:1000, cat. number PA1-985, Invitrogen), polyclonal anti-β actin (cat. number AM4302, Invitrogen), polyclonal anti-cyclin E (1:500 for endogenous cyclin E, 1:1000 for transfected samples, Singer et al., 1999), polyclonal anti-Cul3 (Singer et al., 1999; McEvoy et al., 2007), and monoclonal anti-cyclin E (HE12) (1:500 for endogenous protein, 1:100 for transfected samples, cat. number 32-160-0, Invitrogen).

Half-life determination

For experiments using CHX (Sigma-Aldrich), cells were transfected as described and the drug was added 24 h post-transfection at a final concentration of 50 μg/ml. Cells were then harvested at the time points indicated. Quantification of the western blots for all CHX experiments was done using the FluorChem SP software (Alpha Innotech) or ImageJ (National Institutes of Health) and graphs were generated using Microsoft Excel.

Immunofluorescence

HeLa cells were grown on coverslips, transfected, followed by incubation in 4% paraformaldehyde/PBS at room temperature for 10 min. Cells were then permeabilized using a solution of 1% Triton X-100 with 2 mM EGTA and 5 mM PIPES, followed by incubation in methanol at –20°C for 10 min. Cells were then rinsed with PBS and stained overnight with a polyclonal Myc antibody (1:600, A14, Santa Cruz Biotechnology). The next day, cells were rinsed and stained with an Alexa Fluor 488-conjugated secondary antibody (Abcam) followed by DAPI, rinsed in methanol, and mounted on coverslips for viewing. Microscopy was conducted using a Zeiss M2 microscope and AxioVision software.

Flow cytometry

Proliferating cells were harvested when they were 70% confluent. Cells were resuspended in 70% ethanol in PBS and stored at 4°C until analysis. Prior to analysis, fixed cells were stained in a solution of propidium iodide in PBS with RNase A at 37°C for a minimum of 30 min. Cells were then strained and analyzed on a BD Accuri C6 benchtop flow cytometer (BD). Three proliferating samples of each genotype were analyzed and 20,000–50,000 cells were counted for each sample. Analysis shown in Fig. 7A was completed using FlowJo software (TreeStar).

Kinase assays

Cells were harvested in RIPA and lysed. Proliferating cells were harvested between 50% and 75% confluence and synchronized cells were serum-starved for 24 h and then harvested at 2-h intervals following release into the cell cycle. Cell lysates were incubated in the polyclonal anti-cyclin E antibody (1:100) overnight on a rotator at 4°C. The next morning, Sepharose beads were added to the lysates and they were incubated for an additional

hour and a half at room temperature. Beads were precipitated on ice and the supernatant was removed, followed by two washes with RIPA and two washed with histone wash buffer (25 mM Tris-HCl pH 7.5, 70 mM NaCl, 10 mM MgCl₂ and 1 mM dithiothreitol). Kinase reactions were then completed in the microcentrifuge tubes containing the kinase and beads according to the protocol provided with the Universal Kinase Activity Kit (R&D Systems, product number EA004). Following the final incubation, the liquid contents of each tube were transferred to a 96-well plate and the absorbance was read at 630 nm on a BioTek Synergy HT plate reader. Data were analyzed using Microsoft Excel.

Construction of cyclin E mutants

The majority of the alanine-scanning mutants were a kind gift from Jim Roberts at the Fred Hutchinson Cancer Center (Kelly et al., 1998). The point mutants were made using site-directed mutagenesis and all point mutants were confirmed by sequencing. The sequence of alanine-scanning mutants that demonstrated a phenotype were also re-confirmed. Truncations were cloned by using mutagenesis to generate a stop codon at the designated location in the cyclin E protein. Both deletions were made using site-directed mutagenesis and were also confirmed by sequencing. Forward primer sequences for mutagenesis are as follows: cyclin EΔ2-86 5'-GGA-CCTGAATTCCATGGTTTACCCAAACTCAA-3', cyclin E KIDR point mutant (K48R) 5'-CGCCGTCCTGTCGATTCTGGCCATTCTTCAT-3', cyclin EΔ31-82 5'-GCTCGCTCCAGGAAGGATGACCGGGTTAC-3'. Primer sequences for the cyclin E truncations are as follows: Cyclin E STOP 200 5'-TCATCTTTATTTATTTGAGCCAAACTTGAGGAA-3', and STOP 300 5'-TTTCCTTATGGTATATGAGCTGCTTCGGCCTGG-3'. All reverse primers were reverse complements of the forward primers. The control YFP fusion protein construct was made by cloning YFP in frame with a Flag tag into the pCMV-3Tag-6 vector. The cyclin E–YFP fusion was then constructed by cloning residues 2–86 of cyclin E in between the Flag tag and YFP.

Chou–Fasman structure prediction

The Chou–Fasman algorithm was used for the structural prediction shown in Fig. 6C (Chou and Fasman, 1975).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: B.D., K.R.d.O.R., J.D.S.; Methodology: B.D., K.R.d.O.R., J.D.S.; Formal analysis: B.D., K.R.d.O.R., J.D.S.; Investigation: J.D.S.; Resources: L.N.A., C.D.S.; Writing - original draft: B.D., K.R.d.O.R., J.D.S.; Writing - review & editing: B.D., K.R.d.O.R., J.D.S.; Supervision: J.D.S.; Project administration: J.D.S.; Funding acquisition: J.D.S.

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Supplementary information

Supplementary information available online at <http://jcs.biologists.org/lookup/doi/10.1242/jcs.233049.supplemental>

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