Cell, Vol. 97, 431–434, May 14, 1999, Copyright ©1999 by Cell Press

How the Cyclin Became a Cyclin: Regulated Proteolysis in the Cell Cycle

Minireview

Deanna M. Koepp,*† J. Wade Harper,† and Stephen J. Elledge*†‡§
*Howard Hughes Medical Institute
†Department of Biochemistry
‡Department of Molecular and Human Genetics
Baylor College of Medicine
Houston, Texas 77030

One hundred years from now when the dust of the present has long settled and the wizened eye of history levels its gaze at the field of cell cycle regulation, what discovery will it view as the most seminal? With some debate, it is likely to be the discovery of cyclins and the cyclin-dependent kinases (Cdks) they regulate. Cyclins are key regulators of cell cycle transitions whose abundance varies through a cell cycle. Not only did the union of cyclins and Cdks unravel the long-standing mystery of mitotic entry and oocyte maturation and lead to the discovery of the Cdk inhibitors (CKI), but the very nature of cyclin periodicity held within itself the seeds of an equally significant discovery; the role of ubiquitin-mediated proteolysis in cell cycle control. It is now widely understood that cyclin/Cdks work hand-in-hand with ubiquitin-mediated proteolysis to provide the logical framework for cell cycle regulation. Not only are the levels of cyclins regulated by ubiquitination, but so are the levels of a host of other key cell cycle regulators.

To duplicate, cells must generally double their contents but precisely solve two specific problems: they must replicate their DNA once and only once per cell cycle, and they must segregate their chromosomes precisely to daughter cells. These are biochemically incompatible processes that are partitioned into temporally distinct cell cycle "states." The general strategy employed to prevent improper transitions between these states is the use of inhibitory barriers that must be overcome in order for the transition to occur. Often, the same molecule is used both to promote one transition and to inhibit a subsequent transition. For example, in *S. cerevisiae* Sic1 promotes exit from mitosis by inhibiting Clb/Cdc28 kinases but acts as a barrier to S phase entry that must be overcome. Likewise, S phase cyclins promote initiation of DNA synthesis but prevent the reestablishment of new competent origins thus preventing rereplication, while mitotic cyclins promote entry into mitosis but inhibit mitotic exit. By coupling positive and negative regulators, the cell cycle ensures the maintenance of a single "state" that carries out one defined set of processes at a time. Once the "state" has accomplished its task, events are set in motion that overcome the inhibitory barrier to allow the transition to the next state.

The transitions between these states are controlled by ubiquitin-mediated proteolysis. The formation of ubiquitin–protein conjugates requires three components: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a specificity factor (E3) that functions in substrate recognition. Polyubiquitinated proteins are degraded by the 26S proteasome. How and when specific proteins are ubiquitinated is the critical issue concerning the cell cycle, and E3 complexes provide the key to regulated proteolysis. Two E3 complexes were discovered through studies of cell cycle regulation: the cyclosome/anaphase-promoting complex (APC) and the SCF complex.

Classes of Ubiquitination Substrates

Ubiquitination substrates relevant to the cell cycle fall into two broad categories: those whose destruction is required for cell cycle progression (e.g., Sic1, Pds1, and B-type cyclins), and those whose destruction is not essential but is important for cellular homeostasis (e.g., Cdc6, Cdc20, and G1 cyclins) (Figure 1A). As mentioned above, several proteins have temporally distinct positive and negative roles and their destruction relieves the negative barriers they impose on cell division. In contrast, destruction of strictly positively acting factors

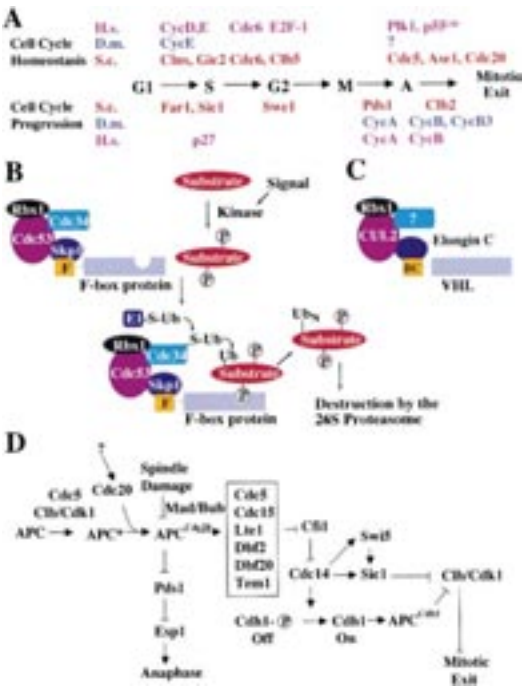

Figure 1. Ubiquitination Pathways in the Cell Cycle

(A) A diagram showing ubiquitination substrates from *H. sapiens*, *D. melanogaster*, and *S. cerevisiae* and the time of the cell cycle in which they are degraded.

(B) A model for phosphorylation-driven ubiquitination through the SCF pathway.

(C) Composition of the VHL-Elongin complex using the same color as in (B) to denote SCF homologs.

(D) A diagram of the pathway for activation of the APC leading to anaphase and mitotic exit.

§To whom correspondence should be addressed (e-mail: selledge@bcm.tmc.edu).

such as G1 cyclins is important for resetting the balance of regulatory factors for the next cell cycle such that the timing of subsequent transitions are not inappropriately advanced.

Ubiquitination Machines Used in the Cell Cycle

SCF. Investigation of the pathway responsible for Cdc34-dependent degradation of Sic1 and G1 cyclins in yeast set the foundation for discovery of the SCF ubiquitin ligase system (Schwob et al., 1994; Bai et al., 1996; Feldman et al., 1997; Skowyra et al., 1997). The SCF is the general name for a large collection of modular E3s that are responsible for the ubiquitination of many proteins, including several important for the cell cycle. The SCF is named for three of its core components, Skp1, Cdc53/cullin, and an F box–containing protein (Figure 1B). Skp1 and F box proteins interact through the F box motif. Cdc53 functions as a bridging molecule to bind the Skp1/F box complex to the E2 Cdc34.

There are over 400 F box proteins currently in the database, with 20 in *S. cerevisiae* and over 100 in *C. elegans*. Many F box proteins also bear obvious protein–protein interaction domains like WD40 or leucine-rich repeats (LRRs). The idea that F box proteins are substrate-specific receptors for ubiquitination substrates came from the finding that the F box proteins Cdc4 and Grr1 are independently involved in Sic1 and Cln ubiquitination, respectively, while other components of the genetic pathway are required for destruction of both of these substrates (Bai et al., 1996; see Table 1). This idea has been borne out by biochemical reconstitution of the Sic1 and Cln1 ubiquitination pathways via SCF^{Cdc4} and SCF^{Grr1} complexes, respectively (Feldman et al., 1997; Skowyra, et al., 1997, 1999) (Figure 1B). In both cases, substrate recognition by the F box protein requires that the substrate be phosphorylated. With Sic1, which inhibits Cdks required for S phase entry, this is achieved by Cln/Cdc28-mediated phosphorylation that allows nutrient and cell size information to be coupled to initiation of DNA replication. Association of Grr1 with Clns is mediated by autophosphorylation that allows activation of Cln kinases to eventually turn themselves off to reset the balance for the next G1. F box proteins are also regulated. They are regulated transcriptionally (Zhang et al., 1995; Chu et al., 1998) and by proteolysis (Zhou and Howley, 1998), and the association of F box proteins with Skp1 can also be regulated (Li and Johnston, 1997).

In mammalian cells, the levels of CKI p27 and the G1 cyclins D1 and E are controlled by phosphorylation-dependent ubiquitination (Elledge and Harper, 1998 and references therein). Given the conservation of regulatory pathways, it is likely that destruction of these and other cell cycle proteins will also involve SCF complexes. Recently is was found that the cell cycle–regulated transcription factor E2F1 binds to SCF^{Skp2} and is likely to be ubiquitinated by this complex (Marti et al., 1999). The SCF pathway may be the central pathway through which protein kinases control the stability of substrate proteins. It is likely that the SCF will also control the stability of certain nonphosphorylated proteins, although this awaits experimental verification.

Most SCF substrates are degraded in the early parts of the cell cycle (Table 1). However, two reports indicate that the SCF is also required for the degradation of a key mitotic regulator, Wee1 (Swe1 in *S. cerevisiae*). Wee1-like kinases inhibit Cdk function by tyrosine phosphorylation and prevent entry into mitosis. Kaiser et al. (1998) demonstrated that Swe1 binds to the F box protein Met30, and *met30* and SCF mutants are defective in Swe1 polyubiquitination, indicating that SCF^{Met30} controls Swe1 ubiquitination. In addition, Michael and Newport (1998) demonstrate that *Xenopus* Wee1 is degraded in a Cdc34-dependent manner and find that this proteolysis is required for mitotic entry and is coupled to the completion of DNA replication. Genetic evidence exists for a second SCF substrate required for mitosis. Mutants in *CDC4* arrest in G1 because they fail to degrade Sic1. However, *cdc4sic1* double mutants arrest in G2 suggesting the existence of an unknown inhibitor of mitotic entry (Schwob et al., 1994).

Skp1, Cdc53/cullin and F box proteins are not the only

Table 1. Substrates of the APC and SCF

SCF		
Substrate	Function	F Box Protein
Cln1[a]	G1 cyclin	Grr1
Cln2[a]	G1 cyclin	Grr1
Cln3[a]	G1 cyclin	?
Clb5[a]	S phase cyclin	?
Sic1[a]	CDK inhibitor	Cdc4
Far1[a]	CDK inhibitor	Cdc4
Rum1[b]	CDK inhibitor	Pop1 + Pop2
Swe1[a]/Wee1[c]	Mitotic inhibitory kinase	Met30/?
Cdc6[a]/Cdc18[b]	DNA replication factor	Cdc4/?
Gcn4[a]	Transcription factor for amino acid regulation	Cdc4
Armadillo[d]/β-catenin[e]	Wnt/Wg-activated transcription factor	Slimb/β-TRCP
Cubitus interruptus (Ci)[d]	Hedgehog-activated transcription factor	Slimb
E2F-1[e]	Cell cycle transcription factor	Skp2
Gic2[a]	Polarized bud growth regulator	Grr1
IκB[e]	NF-κB inhibitor	β-TRCP
CD4[e] (via HIV Vpu)	T_H cell HIV receptor	β-TRCP
Putative Substrates		
Cyclin D1[e]	G1 cyclin	
Cyclin E[e]	G1 cyclin	
p21[e]	CDK inhibitor	
p27[e]	CDK inhibitor	
p57[e]	CDK inhibitor	

APC	
Substrate	Function
Clb2[a]	Mitotic cyclin
Cyclin A[e,f]	Mitotic cyclin
Cyclin B[c,e,f]	Mitotic cyclin
Cdc20[a]/p55Cdc[e]	APC regulator
Cdc5[a]	Mitotic kinase and APC regulator
Pds1[a]/Cut2[b]	Anaphase inhibitor
Geminin[c]	DNA replication inhibitor
Ase1[a]	Spindle protein
NIMA[g]	Mitotic kinase

Superscripts refer to organism: [a], *S. cerevisiae*; [b], *S. pombe*; [c], *X. laevis*; [d], *D. melanogaster*; [e], *H. sapiens*; [f], *S. solidissima*; and [g], *A. nidulans.*

essential components of SCF complexes. The recently identified protein Rbx1/Roc1, which contains a Ring-H2 finger domain termed the R box, is also an essential component of the SCF (Kamura et al., 1999; Ohta et al., 1999; Skowyra et al., 1999). Mammalian Rbx1/Roc1 was discovered as a protein that bound the von Hippel Lindau (VHL) tumor suppressor complex (Kamura et al., 1999) and as a protein that interacts with Cul4a in the two-hybrid system (Ohta et al., 1999) and that associates with the SCF (Tan et al., 1999). The VHL complex also contains a Skp1 homolog, Elongin C, and a Cdc53 homolog, Cul2, and is therefore structurally analogous to SCF complexes (Figure 1C). VHL binds Elongin B/C through a short BC box motif related to the F box (Stebbins et al., 1999). Additional BC box–containing proteins can bind to Elongin B/C in place of VHL and may confer different functions upon the complex, much like F box proteins do to the SCF (Kamura et al., 1998).

In yeast, Rbx1/Roc1 is essential for viability and is required for Sic1 and Cln ubiquitination in vitro (Kamura et al., 1999; Ohta et al., 1999; Skowyra et al., 1999). Consistent with this, Rbx1/Roc1 greatly enhances the activity of insect cell–derived SCF^{Cdc4} complexes toward Sic1 (Kamura et al., 1999) and allows reconstitution of phosphorylation-dependent Cln1 ubiquitination by recombinant SCF^{Grr1} complexes (Skowyra et al., 1999). Rbx1/Roc1 is highly conserved and it is therefore likely that insect cell–derived Rbx1/Roc1 is responsible for the previously observed activity of SCF^{Cdc4} toward Sic1 (Feldman et al., 1997; Skowyra et al., 1997). The ability of Rbx1/Roc1 to activate the SCF appears to reflect its recruitment of Cdc34 into the SCF complex (Skowyra et al., 1999). Furthermore, association of Cdc34 with the Rbx1/SCF complex greatly stimulates its autopolyubiquitination activity, suggesting that the complex is an allosteric activator of Cdc34.

The APC also contains an Rbx1/Roc1-related protein, Apc11, which is required for Clb2 ubiquitination in vitro (Zachariae et al., 1998b). The finding that mammalian cullin Apc2 interacts with Apc11 in cotransfection experiments (Ohta et al., 1999) strengthens the mechanistic parallels between the APC and the SCF and suggests that the role of Apc11 involves E2 recruitment. Since Rbx1/Roc1 is a component of multiple cullin complexes, including the VHL complex, it seems likely that many cullin complexes will be involved in transfer of ubiquitin or ubiquitin-like proteins. In addition to Rbx1/Roc1 and Apc11, yeast contain several R box proteins, three of which (Hrd1, Rad18, and Ubr1) are also involved in ubiquitination pathways. It has been proposed that E3 complexes fall into two basic categories, the SCF-like complexes such as SCF, VHL-Elongin, and the APC, which utilize R box proteins, and the HECT domain proteins, which so far have not been linked to R box proteins (Skowyra et al., 1999).

APC. In mitosis, two events are subject to proteolytic control, sister chromosome separation and exit from telophase into G1. These ubiquitin-mediated proteolytic events depend upon the APC, an E3 composed of 12 subunits in budding yeast (Zachariae et al., 1998a) and at least 10 subunits in mammals and *Xenopus* (Yu et al., 1998). A central question has been how this E3 can differentially regulate the timing of substrate ubiquitination. Destruction of many APC targets requires the presence of a conserved 9-residue motif called the "destruction box." In *Drosophila*, expression of destruction box–deficient cyclin A leads to a metaphase delay while deletion of destruction boxes in cyclin B and B3 lead to early anaphase and late anaphase arrest, respectively (Sigrist et al., 1995). Similarly, deletion of the destruction boxes in negative regulators of sister chromosome separation, Pds1 in budding yeast and Cut2 in *S. pombe*, blocks cells in metaphase (Yanagida, 1998).

Timing of Substrate Destruction

Progression from metaphase to anaphase to mitoic exit is controlled by the sequential degradation of inhibitors by the APC. How does a cell know when and which substrate to ubiquitinate? Substrate specificity is accomplished in part by the regulated association of the APC with two classes of WD40 repeat–containing coactivator proteins, Cdc20/fizzy and Cdh1/Hct1/fizzy-related (Schwab et al., 1997; Visintin et al., 1997; Fang et al., 1998; Kramer et al., 1998). In budding yeast, Cdc20 controls ubiquitination of Pds1 at the metaphase-to-anaphase transition whereas its homolog Cdh1/Hct1 controls mitotic cyclin destruction and mitotic exit (Visintin et al., 1997; Schwab et al., 1997; Sigrist and Lehner, 1997). Cdc20 is expressed during G2 and binds the APC during metaphase. Association of Cdc20 with the APC allows ubiquitination of Pds1, thereby removing the block to anaphase, and expression of Cdc20 prior to mitosis can cause premature degradation of Pds1 but not mitotic cyclins (Visintin et al., 1997).

Several substrates may be ubiquitinated by the same form of the APC, but in a defined order. How this works is not clear. One possibility is that substrates of the APC are themselves specific inhibitors of the destruction of other substrates. Evidence supporting this model comes from *Drosophila* in which destruction of cyclin A is required for destruction of cyclin B, and destruction of cyclin B is required for the destruction of cyclin B3, whose destruction is required for mitotic exit (Sigrist et al., 1995).

It is not known whether Cdc20 or Cdh1 binds directly to substrates and whether, like their WD40 F box counterparts, substrate phosphorylation will play a role in substrate selection. Theoretically, phosphorylation could also control inhibitor binding to Cdc20 and Cdh1. The spindle assembly checkpoint controlled by the *MAD/BUB* genes prevents anaphase in part by regulated association of Mad2 with Cdc20 (Figure 1D). Whether this process involves phosphorylation remains to be determined although there are several protein kinases in the Mad/Bub pathway.

The Cdc14 Switch Controls Mitotic Exit

In contrast to Cdc20, Cdh1 is present constitutively but binds the APC only during mitotic exit and G1, points in the cell cycle where mitotic cyclins are unstable. Cdh1 overexpression causes degradation of ectopically expressed Clb2, and its homolog *fizzy-related* is required for destruction of mitotic cyclins in *Drosophila*. A recent flurry of papers has clarified how Cdh1 activity is regulated (Visintin et al., 1998; Zachariae et al., 1998a; Jaspersen et al., 1999). Not unexpectedly, Cdh1 regulation underlies the inverse relationship between Cdk activity and activation of B-type cyclin destruction. During

most of the cell cycle, Cdh1 is inhibited by Cdk phosphorylation (Figure 1D). Cdh1 is activated by the phosphatase Cdc14, which functions in three parallel processes that ensure precipitous destruction of B-type cyclins and mitotic exit. Cdc14 acts during telophase to dephosphorylate not only Cdh1, but also Swi5, a transcription factor held dormant in the cytoplasm by Cdk phosphorylation. Swi5 dephosphorylation activates Sic1 transcription. Cdc14 also protects Sic1 from SCF^{Cdc4} degradation by dephosphorylating Sic1. Increased levels of Sic1 block Clb/Cdc28 activity, which helps tip the balance of kinase and phosphatase activity toward Cdh1 dephosphorylation and mitotic exit (Figure 1D).

Regulated access of Cdc14 to its substrates is responsible for setting up the timing of mitotic exit (Shou et al., 1999; Visintin et al., 1999). During G1 through early mitosis, Cdc14 is in the nucleolus. As sister chromosomes separate, Cdc14 is released from the nucleolus and spreads throughout the nucleus and into the cytoplasm where it finds key substrates. Cdc14 is sequestered in the nucleolus through association with Cfi1/Net1, which shows constitutive nucleolar localization. Deletion of *CFI1/NET1* leads to dispersion of Cdc14 throughout the cell. Mitotic exit requires several genes (Jaspersen et al., 1998), including *TEM1*, *CDC15*, *DBF2*, *DBF20*, *LTE1*, and *CDC5*. Deletion of *CFI1/NET1* rescues temperature-sensitive *tem1*, *cdc5*, *cdc15*, and *dbf2* mutants, suggesting that the mitotic exit role of these proteins is to release Cdc14 from Cfi1/Net1. Given that several of these genes encode protein kinases, phosphorylation of Cdc14, Cfi1/Net1, or both is a likely mechanism for dissociation. A key unresolved issue is the nature of the *CDC20*-dependent signal that activates the mitotic exit pathway, for this ensures that anaphase preceeds mitotic exit.

Future Prospects for Proteolytic Control of the Cell Cycle

As is readily apparent, proteolysis plays a tremendously important and varied role in cell cycle regulation. While the SCF and APC are key regulators, other proteolytic systems may also contribute to cell cycle control. A well-documented case is the stabilization of p53 in response to DNA damage. In addition, a novel proteolyic system cabable of cleaving cyclin A was recently found to be activated by the presence of the CKI p27 in cell extracts (Bastians et al., 1998). Furthermore, the regulation of protein stability at the proteolytic step rather than the ubiquitination step is an interesting and unexplored avenue for cell cycle control. A possible example of this may come from the recent observation that the Cdk subunit Cks1 (Suc1 in *S. pombe*) associates with the proteasome and may regulate it (Kaiser et al., 1999). The significance of these new discoveries is not yet clear but they are certain to keep cell cycle research on the cutting edge.

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