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How Proteolysis Drives the Cell Cycle

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Oscillations in the activity of cyclin-dependent kinases (CDKs) promote progression through the eukaryotic cell cycle. This review examines how proteolysis regulates CDK activity—by degrading CDK activators or inhibitors—and also how proteolysis may directly trigger the transition from metaphase to anaphase. Proteolysis during the cell cycle is mediated by two distinct ubiquitin-conjugation pathways. One pathway, requiring CDC34, initiates DNA replication by degrading a CDK inhibitor. The second pathway, involving a large protein complex called the anaphase-promoting complex or cyclosome, initiates chromosome segregation and exit from mitosis by degrading anaphase inhibitors and mitotic cyclins. Proteolysis therefore drives cell cycle progression not only by regulating CDK activity, but by directly influencing chromosome and spindle dynamics.

The periodicity of DNA replication and mitosis in eukaryotes contrasts with the continuous nature of most metabolic reactions that produce cellular growth. The eukaryotic chromosome cycle is composed of an ordered series of discrete events; the periods of replication and chromosome segregation do not overlap as they do in prokaryotes. Interposition of a chromosome-alignment step between replication and segregation completes the set of events that constitute the basic eukaryotic chromosome cycle. The steps in this cycle are initiated in sequence by the cell cycle regulatory machinery, which also controls centrosome duplication and cell division (cytokinesis), and coordinates these discontinuous events with cell growth. In this review, we explore how specific protein degradation provides direction, order, and proper timing to the key events of the chromosome cycle.

Biologists have long grappled with the problem of how cell division is controlled. Early models postulated the existence of an initiator that would accumulate during the cell cycle, inducing DNA replication (*1*) or mitosis (*2*) when it reached a critical concentration. The process of mitosis would then abruptly inactivate the initiator, resetting the cycle. This model proved to be remarkably prescient, for today we know these initiators include the mitotic cyclins, which accumulate during interphase to drive entrance into mitosis and are degraded at the end of mitosis to reset the cycle (*3–5*). Subsequent work has shown that proteolysis has a pervasive role in regulating cell cycle progression: Protein degradation is required for multiple processes in mitosis and also for the onset of DNA replication (Fig. 1).

To understand how proteolysis regulates transitions through the cell cycle, we must explain how proteolytic activity is controlled and how substrate specificity is achieved. Although there are many proteolytic processes inside and outside of cells, the ones known to be important for cell cycle progression rely on the assembly of a ubiquitin chain on the substrate, which targets it for degradation by the 26S proteasome (*6*). Ubiquitin, a small, highly conserved protein, is first activated at its COOH-terminus by formation of a thioester bond with the ubiquitin-activating enzyme, E1. Ubiquitin is subsequently transesterified to one member of a family of E2 or UBC (ubiquitin-conjugating) enzymes, for which there are 13 genes in the budding yeast genome (*7*). Finally, ubiquitin is transferred from the E2 to a lysine residue of the target protein, either directly or with the assistance of a ubiquitin-protein ligase (E3). An E3 is generally required for the formation of multiubiquitin chains on the substrate, a step that facilitates efficient recognition of the substrate by the proteasome. The rate and specificity of ubiquitin-mediated proteolysis may also be controlled by the disassembly of ubiquitin chains, which is catalyzed by a large and poorly characterized family of deubiquitinating enzymes (UBPs). There are more genes for UBPs (16) than for E2s (13) in budding yeast (*7*), and perturbations to deubiquitinating enzyme activity can profoundly alter cell physiology (*8*).

The enzymes of the ubiquitin system were first defined as eluates from a ubiquitin-affinity column (hence the letter E in their name). Whereas E1 and E2 enzymes formed covalent bonds with the ubiquitin column, the first E3 characterized could be eluted with high concentrations of salt or increased pH (*9*). An E3 was functionally defined as an activity that was both necessary and sufficient for the transfer of ubiquitin to the substrate in the presence of a ubiquitin-charged E2 enzyme, indicating that it participated in the final step of ubiquitination (*9*). In addition to facilitating multiubiquitination of substrates, E3s appear to be the primary source of substrate specificity in the ubiquitination cascade, as some E3s have been shown to directly bind substrates (*10*, *11*). Two E3s, E6-AP (*12*) and UBR1 (*11*), may function catalytically, forming a thioester with ubiquitin as an intermediate in the transfer reaction (*13*, *14*). Despite the similarity in reaction mechanism, UBR1 and E6-AP do not share significant sequence similarity. As relatively few E3s have been mechanistically characterized, it remains to be seen whether all

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will share a common reaction mechanism. For the purposes of this review, we therefore rely on the original E3 definition, as a component that is both necessary and sufficient for substrate ubiquitination in the presence of a ubiquitin-charged E2.

Two distinct proteolytic pathways are directly required for cell duplication (Fig. 1). The first pathway, which has been best characterized in budding yeast, promotes progression from G1 to S phase of the cell cycle and utilizes an E2 called CDC34. The second pathway initiates the onset of anaphase and exit from mitosis and uses a distinct set of E2s in conjunction with an E3-containing particle called the cyclosome or anaphase-promoting complex (APC). Although both pathways govern key steps in the chromosome cycle, their activities appear to be regulated differently.

Proteolysis at the G1-S Transition

Progression through the eukaryotic cell cycle requires the activity of a set of distinct cyclin–cyclin-dependent kinase (CDK) complexes. In budding yeast, the G1 cyclins CLN1, CLN2, and CLN3 drive cells through G1 by activating the kinase CDC28 (a homolog of fission yeast CDC2). Distinct cyclins promote S phase (DNA synthesis) (CLB5 and CLB6) and mitosis (CLB1 through CLB4). Higher eukaryotes contain functional homologs that act at similar stages of the cell cycle, with cyclins D and E functioning during G1, cyclins E and A during S phase, and cyclins A and B during mitosis. To simplify the nomenclature in this review, we will refer to cyclins as either G1 cyclins, S-phase cyclins, or mitotic cyclins, and the corresponding active kinase complexes as G1 CDKs, S-phase CDKs, or mitotic CDKs.

Molecular cloning of *CDC34*, a gene required for the G1-S transition in budding yeast, revealed that a ubiquitin conjugation step was required just before the initiation of DNA replication. *CDC34* encodes a ubiquitin conjugating enzyme (*15*) that participates in the destruction of multiple proteins, including the G1 cyclins CLN2 and CLN3 (*16*, *17*), as well as proteins not directly related to cell cycle control (*18*). However, accumulation of these substrates does not account for the cell cycle arrest of *cdc34*^{ts} mutants. The nature of the crucial target of CDC34 at the G1-S transition was first implied by genetic studies. A strain deficient in all S-phase and mitotic cyclins recapitulated the *cdc34*^{ts} mutant phenotype, suggesting that the CDC34 pathway might be required for generating S-phase CDK activity (*19*). Extracts made from *cdc34*^{ts} mutants inhibit S-phase CDKs, implying that *CDC34* may be required for the degradation of a CDK inhibitor. A candidate for this activity was SIC1, a tight-binding S-phase CDK inhibitor (*20*, *21*). SIC1 is normally degraded as wild-type cells enter S phase, but accumulates in *cdc34*^{ts} mutants. SIC1 appears to be the crucial substrate blocking progression from G1 to S phase in *cdc34*^{ts} mutants, because *cdc34*^{ts} *sic1*Δ double mutants initiate DNA replication at the nonpermissive temperature (*19*). As predicted by these findings, expression of a non-degradable form of SIC1 in wild-type strains blocks cell division at the G1-S transition (*22*). Ubiquitin-dependent proteolysis of a CDK inhibitor is therefore a crucial mechanism by which the onset of S phase is controlled.

Besides *CDC34*, three other genes are required for the G1-S transition in budding yeast: *CDC4* (*23*), *CDC53* (*24*), and *SKP1* (*25*). Cells with temperature-sensitive mutations in any of these genes exhibit phenotypes similar to that of *cdc34*^{ts} mutants, and in each case deletion of *SIC1* enables these mutants to replicate their DNA. Both *CDC53* (*24*) and *SKP1* (*25*) are members of conserved, multigene families, but there is little information about their biochemical functions. *CDC4* contains two recognizable sequence motifs that are found in many unrelated proteins: an F box, which serves as an interaction domain for SKP1 (*25*), and eight WD-40 repeats (*26*), which may serve as a platform for protein-protein interaction (*27*). Insect cell lysates expressing CDC53, CDC4, and SKP1 (and supplemented with CDC34, ubiquitin, and E1) can sustain ubiquitination of SIC1, suggesting that one of these components functions as an E3 (*28*). CDC53 may recognize other substrates of the CDC34 pathway such as G1 cyclins, although the interaction with substrates may not be direct (*29*). The functions of CDC4 and SKP1 remain obscure, and there is no apparent sequence similarity between any of these proteins and known E3s (*11*, *30*).

Although physical interactions between components of the CDC34 pathway have yet to be defined, genetic and functional evidence suggests that a dynamic population of E3 complexes may exist. For example, distinct mutant alleles of *SKP1* differentially affect the stability of CDC34 pathway substrates (*25*), suggesting that there may be multiple SKP1-containing complexes involved in ubiquitin-mediated proteolysis. Besides CDC4, many proteins contain potential SKP1-binding F boxes, including GRR1 (*25*), which is required for catabolite repression. Mutant *grr1* cells fail to degrade the CDC34 substrate CLN2, but proceed rapidly into S phase, suggesting that SIC1 is destroyed on schedule (*31*). In addition to its roles in the CDC34 pathway, SKP1 is an essential subunit of the centromere-binding CBF3 complex, suggesting that it participates in diverse cellular functions (*32*).

Regulation of the CDC34 pathway. The substrates of the CDC34 pathway differ in how their stability is influenced by cell cycle stage. For example, G1 cyclins in budding yeast are turned over rapidly throughout the cell cycle (*29*). Although stabilized versions of G1 cyclins can accelerate passage through G1 (*33*), there is little evidence to suggest that the rate of G1 cyclin proteolysis is modulated to control progression through G1. Instead, rapid constitutive turnover may simply entrain G1 cyclin abundance to the rate of transcription. A similar situation may hold for the regulation of the abundance of cyclin E in animal cells (*34*). In contrast, SIC1 is stable throughout G1 phase, but becomes unstable as cells enter S phase (*19*, *35*).

Differential regulation of the stability of CDC34 pathway substrates is achieved through substrate-specific phosphorylation, which appears to serve as the trigger for

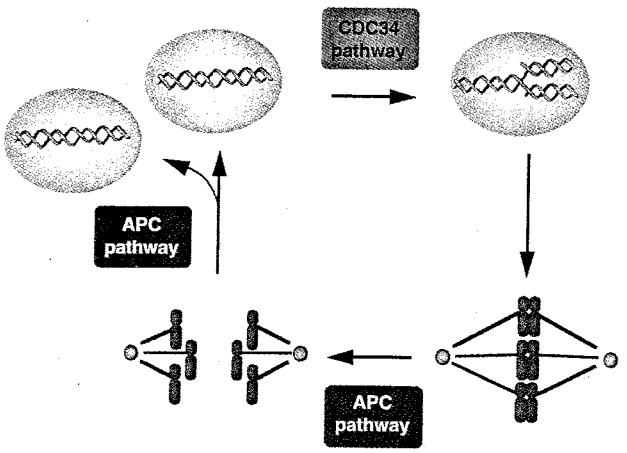

Fig. 1. Two distinct proteolytic pathways that participate in the regulation of the chromosome cycle. The nuclear cycle is depicted with a G1 phase nucleus in the upper left quadrant and an S phase nucleus on the right; mitotic spindles representing metaphase (right) and anaphase (left) are shown below. The CDC34 pathway promotes passage from G1 to S phase by degrading a CDK inhibitor. After chromosomes align at the metaphase plate, the APC pathway promotes the transition from metaphase to anaphase by degrading anaphase inhibitors, and subsequently promotes exit from mitosis by degrading mitotic cyclins.

CDC34-dependent ubiquitination (Fig. 2). CLN2 and CLN3 are phosphorylated by CDC28 as a prelude to CDC34-dependent ubiquitination (*16*, *17*). Phosphorylation appears to be necessary for proteolysis, because non-phosphorylatable mutants of CLN2 and CLN3 are stable in vivo (*17*, *36*). Constitutive proteolysis of CLNs is therefore a result of phosphorylation of CLNs by the associated CDC28 subunit.

A similar mechanism appears to regulate the stability of SIC1. G1 CDK activity is required for the assembly of multiubiquitin chains on SIC1 in vitro, and mutation of CDK consensus phosphorylation sites in SIC1 both reduces SIC1 ubiquitination in vitro and stabilizes SIC1 in vivo (*22*). Phosphorylation of SIC1 appears to be the only step in its destruction that is regulated by cell cycle position or CDK activity, because purified, phosphorylated SIC1 can be ubiquitinated by the CDC34 pathway in the absence of G1 cyclin–CDK activity (*37*). Thus, because G1 cyclins become dispensable when *SIC1* is deleted (*38*), a crucial function of G1 cyclins must be to prime SIC1 for CDC34-dependent ubiquitination. The cell cycle dependence of SIC1 stability is therefore a direct reflection of the activity of the G1 CDK.

Although phosphorylation renders substrates susceptible to the action of the CDC34 pathway, little is known about the underlying mechanism. Phosphorylation may activate binding of substrates to the putative CDC53-containing E3 complex (*29*) by creating an epitope that is directly recognized by the ubiquitination machinery, much like tyrosine phosphorylation enhances protein-protein interactions mediated by Src-homology 2 (SH2) domains. Alternatively, phosphorylation may perturb the conformation of proto-substrates, revealing a cryptic ubiquitination signal peptide. Whereas PEST sequences (rich in proline, glutamate, serine, and threonine) (*39*) have been implicated in the proteolysis of CDC34 pathway substrates (*17*, *40*), they remain insufficiently well defined to serve as an accurate prognosticator of targeting to this pathway, because approximately one-third of all the open reading frames in the budding yeast genome contain PEST regions (*41*).

Conservation of the CDC34 pathway. Vertebrate cells express structural and functional homologs of *CDC34* (*42*), *CDC53* (*43*, *44*), and *SKP1* (*25*, *45*). The CDK inhibitor p27 is degraded in part through a CDC34 pathway in human cells (*46*), and in *Xenopus* eggs, the CDK-dependent initiation of DNA replication requires a CDC34 homolog (*47*). By analogy to budding yeast, these data suggest that the CDC34 pathway may trigger DNA synthesis in metazoans by degrading a CDK inhibitor. In contrast to budding yeast which require *CDC53* function to divide, one nematode homolog of *CDC53*, known as *cul-1*, is required to limit the number of cell divisions during embryonic development (*44*). *CDC53*-like genes (*cullins*) may therefore also target the destruction of proteins that positively regulate cell division, such as G1 cyclins (*29*).

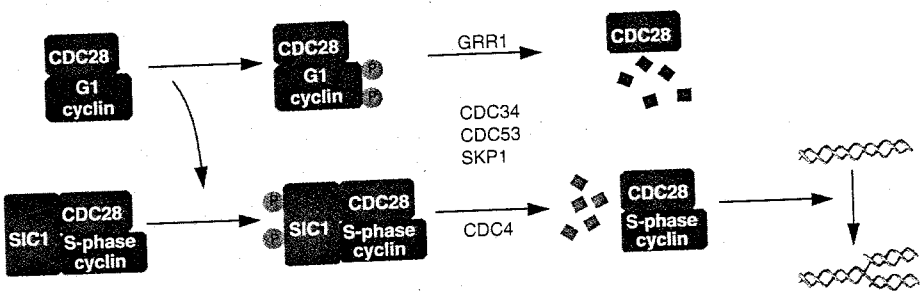

Fig. 2. A model for the regulated proteolysis of G1 cyclins and the CDK inhibitor SIC1 by the CDC34 pathway in budding yeast. G1 cyclins bind to CDC28 to produce an active G1 CDK complex. Phosphorylation of the G1 cyclin by CDC28 enables recognition of the G1 cyclin by components of the CDC34 pathway that ubiquitinate the protein and target it for degradation. The autocatalytic nature of the phosphorylation reaction makes G1 cyclins constitutively unstable. In contrast, the destruction of the CDK inhibitor SIC1 is cell-cycle dependent. SIC1 is stable during early G1 phase and prevents the precocious activation of S-phase CDKs. G1 CDKs assembled in late G1 phase phosphorylate SIC1, enabling its recognition and ubiquitination by components of the CDC34 pathway. The active S-phase CDK then initiates DNA replication by phosphorylating key substrates that remain to be identified. Wheras CDC34, CDC53, and SKP1 are required for degradation of both G1 cyclins and SIC1, GRR1 and CDC4 may be substrate-specific components of the destruction pathway (*25*).

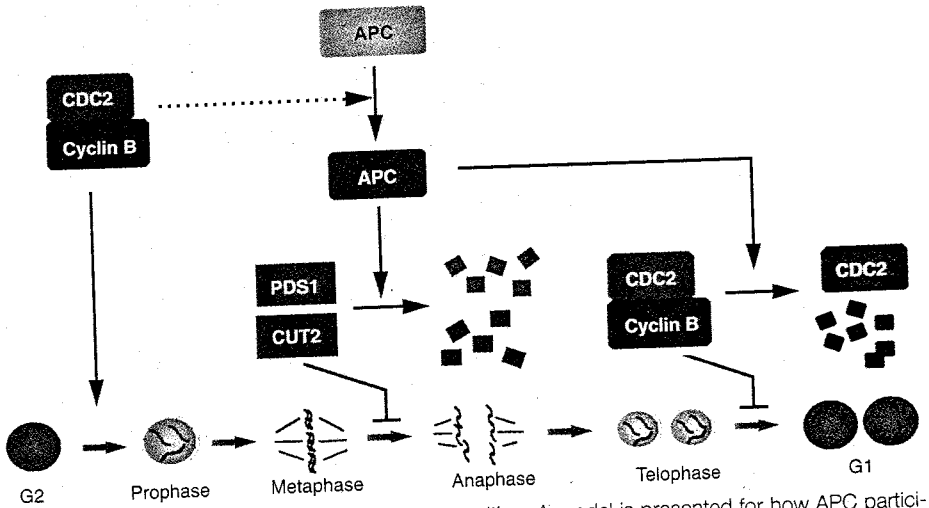

Fig. 3. Proteolysis at the metaphase-anaphase transition. A model is presented for how APC participates in the degradation of both mitotic cyclins and anaphase inhibitors. The model summarizes findings from various organisms. Although some substrates have been identified in only a single organism, the APC appears to have a universal role in mitotic proteolysis in eukaryotes. In this model, cyclin B accumulates during interphase to activate CDC2, producing an active mitotic CDK that triggers entry into prophase, resulting in the eventual formation of the mitotic spindle and metaphase plate. The mitotic CDK also leads to the activation of the APC by an undefined pathway. Once the APC is activated, it catalyzes the ubiquitination of several substrates, including the anaphase inhibitors PDS1 (budding yeast) and CUT2 (fission yeast). The APC also mediates proteolysis of cyclin B, which is universally required for exit from telophase.

Proteolysis in Mitosis

Mitosis in all organisms is initiated by the mitotic CDK composed of cyclin B and CDC2, which is historically known as maturation promoting factor (MPF). Cyclin B accumulates during interphase, culminating in the activation of MPF and entry into mitosis. After a lag period, MPF then induces its own demise by activating the mitotic cyclin destruction system. The degradation of cyclin B is required for the exit from telophase into the subsequent interphase, whereas the degradation of other substrates is important for earlier steps in anaphase progression (Fig. 3). In early em-

bryos in which cyclins are synthesized continuously, it is the periodic activation of the mitotic cylin destruction machinery that ultimately drives cell cycle progression.

The NH_2-terminal domain of mitotic cyclins contains a conserved 9-amino acid motif called the destruction box (D box), that is necessary for cyclin ubiquitination and subsequent degradation (*48–53*). Because deletion of the NH_2-terminus does not interfere with the capacity of mitotic cyclins to activate CDC2 and drive the cell into mitosis, these mutants dominantly arrest cell division in telophase (*5, 54–57*). If the D-box and neighboring sequences from cyclin B are grafted onto otherwise stable proteins, those proteins become unstable in mitosis (*48, 58–60*). The requirements for the destruction of A-type cyclins and some B-type cyclins may be more complex, perhaps involving sequence determinants outside the NH_2-terminus (*51, 52, 61*).

In contrast to the regulation of substrates of the CDC34 pathway, phosphorylation of cyclin B does not appear to be required for its degradation (*62*). Instead, it is the activity of the E3 complex that fluctuates during the cell cycle. The mitotic cyclin ubiquitination machinery utilizes two E2 enzymes: UBC4 (*63*), which participates in other proteolytic processes, and a newly discovered E2, called UBCx (*64*) or E2-C (*65*), which may be specific to the mitotic cyclin degradation pathway. Genetic studies have implicated an additional E2, UBC9, in the proteolysis of mitotic cyclins in budding yeast (*66*). It appears unlikely, however, that this E2 participates in D box–dependent turnover (*53, 63, 67*). The regulated component of the mitotic cyclin ubiquitination system, activated at the metaphase-to-anaphase transition, is a large E3 complex, known as the cyclosome (*68*) or the anaphase-promoting complex (APC) (*63*). The latter name derives from the finding that components of the APC are essential for anaphase progression in multiple organisms.

Characterization of the APC has benefited from the convergence of a genetic screen in budding yeast, which identified several genes required for both anaphase progression and mitotic cyclin degradation (*67*), and a biochemical approach in clam and frog egg extracts, in which mitotic cyclin ubiquitination was reconstituted with purified components (*63, 68, 69*). The APC isolated from *Xenopus* is composed of eight subunits [Table 1; (*70*)], four of which contain tetratricopeptide repeats (*71, 72*), which are thought to mediate protein-protein interactions (*73*). Three of these proteins, CDC16, CDC23, and CDC27, form a complex required for anaphase progression in budding yeast (*74*); similar genes are required for anaphase in other fungi (*75–77*) and mammalian cells [(*78*); Table 1]. *CDC26*, which is also required for proteolysis of mitotic cyclins, physically interacts with other APC components in budding yeast (*79*), but does not copurify with the *Xenopus* complex (*70*). Another subunit, found in both yeast and *Xenopus* complexes (*70, 79*), is similar to BIME from *Aspergillus*, a protein that is also necessary for anaphase progression (*80, 81*). Although the APC meets the functional criteria for an E3 enzyme, it is not yet clear how this complex recognizes substrates containing a D box. None of the eight *Xenopus* APC subunits identified to date show sequence similarity to UBR1 (*11*) or the E6-AP family of proteins (*30*), and no ubiquitin thioesters have been detected with any of the subunits (*63*). Thus, the APC may primarily serve to bring together ubiquitin-charged E2s and D box–containing substrates, rather than act as an intermediate covalent carrier of ubiquitin.

The APC and chromosome segregation. Anaphase and telophase were initially viewed as a reversal of prophase and metaphase that resulted from the inactivation of MPF (*82*). However, non-degradable forms of mitotic cyclin arrest the cell cycle in telophase rather than metaphase (as predicted by this hypothesis) in both *Xenopus* (*55*) and budding yeast (*56*). Therefore, inactivation of MPF cannot serve as the trigger for sister chromatid segregation. Nevertheless, inhibition of APC activity through either substrate competition in *Xenopus* extracts (*55*), antibody microinjection in tissue culture cells (*78*), or mutation in fungi (*67*) prevents chromosome segregation. These findings create a paradox: anaphase does not require degradation of mitotic cyclins, yet it remains dependent upon D box–mediated proteolysis catalyzed by APC. The simplest resolution of this dilemma is to postulate the existence of non-cyclin substrates that inhibit anaphase until they are degraded via APC-mediated proteolysis (*55*).

This hypothesis has recently been vindicated by the discovery of two non-cyclin proteins that are degraded during anaphase, CUT2 and PDS1. In fission yeast, CUT2 is an essential nuclear protein that decreases in abundance as cells undergo anaphase (*60*). CUT2 is normally not detectable in G1 arrested cells, but deletion of the CUT2 NH_2-terminus (which removes two D box–like sequences) enables the protein to accumulate (*60*). The wild-type protein also becomes detectable in G1-arrested cells containing a mutation of an APC subunit (CUT9, Table 1), suggesting that APC-mediated proteolysis is an important determinant of CUT2 stability. Importantly, overexpression of a non-degradable form of CUT2 blocks anaphase, but does not block exit from mitosis (*60*). A similar story holds for PDS1 from budding yeast, an unstable protein that was identified in a genetic screen for proteins that are required to maintain sister chromatid cohesion prior to anaphase (*83*). Degradation of PDS1 requires an intact D box and functional APC, and PDS1 is a substrate for purified *Xenopus* APC (*84*). Furthermore, overexpression of non-degradable mutants of PDS1 arrests cells in metaphase (*84*). Deletion of *PDS1*, in contrast, allows a large fraction of cells to undergo anaphase in the presence of mu-

Table 1. Subunits of the anaphase-promoting complex. Similar subunits from each organism are shown on the same row. NR indicates that a particular subunit has not been reported from that organism. TPR protein, tetratricopeptide-repeat proteins.

Organism					Required for anaphase?	Comments	References
X. laevis	*S. cerevisiae*	*S. pombe*	*A. nidulans*	Mammals			
APC1	APC1	NR	BIME	TSG24	Yes	Couples M phase to S phase	(*70, 79–81, 120*)
APC2	NR	NR	NR	NR	?	–	(*70*)
APC3	CDC27	NUC2	BIMA	CDC27Hs	Yes	TPR protein	(*53, 63, 74–76, 78*)
APC4	NR	NR	NR	NR	?	–	(*70*)
APC5	NR	NR	NR	NR	?	–	(*70*)
APC6	CDC16	CUT9	NR	CDC16Hs	Yes	TPR protein	(*53, 60, 63, 67, 74, 77, 78*)
APC7	CDC23	NR	NR	NR	Yes	TPR protein	(*53, 67, 70, 71, 74*)
APC8	NR	NR	NR	NR	?	TPR protein	(*70*)
NR	CDC26	NR	NR	NR	Yes	–	(*79*)

tant APC (85), suggesting that PDS1 is an important, although perhaps not the sole, target of APC during anaphase.

We currently do not understand how PDS1 and CUT2 inhibit anaphase. One hypothesis is that such proteins might function as a chromosomal glue that holds chromosomes together until the glue is dissolved at anaphase, releasing the chromatids and initiating other anaphase movements (55). Surprisingly, meiotic spindles that lack chromosomes still undergo anaphase spindle movements on schedule (86) indicating that chromosome separation itself cannot be the sole trigger of other anaphase events. We propose that normal anaphase spindle movements are triggered by the APC-dependent degradation of at least two different classes of proteins: one class that is involved in holding sister chromatids together (such as CUT2 and PDS1), and a second class that directly influences the behavior of the mitotic spindle, where a portion of APC appears to be located (78). However, budding yeast mutants that lack kinetochores still exhibit some types of anaphase movement when APC is also mutated (67) indicating that certain aspects of anaphase may be controlled by APC-independent mechanisms.

Regulation of APC activity. There is a temporal lag between the activation of MPF and the activation of the mitotic cyclin degradation system (87). Biologically, this lag makes sense: MPF must remain active long enough to induce the events of mitosis such as nuclear envelope breakdown, chromosome condensation, and alignment of chromosomes on the metaphase plate before the APC is activated and mitosis is extinguished. Although many of the components of the APC have now been identified, we still do not understand how its activity is regulated. Phosphorylation may positively regulate E3 activity, because several subunits of APC become phosphorylated during mitosis in *Xenopus* (63, 70) and *Aspergillus* (88), and phosphatases can inactivate the mitotic form of the clam cyclosome (89) and *Xenopus* APC in vitro (70). Candidate kinases include MPF itself, which can partially activate interphase cyclosome fractions with a lag phase (68), and protein kinase A, which is activated in mitosis and is essential for the activation of cyclin B degradation in *Xenopus* extracts (90). The budding yeast gene *CDC20* (91), which is similar to the *Drosophila fizzy* gene, may also encode a regulator of APC activation; mutations in both genes prevent anaphase and degradation of mitotic cyclins (92). Finally, protein dephosphorylation may also be an important mechanism for controlling mitotic cyclin stability, as protein phosphatase-1 (PP1) mutants in a variety of organisms arrest before anaphase with stable cyclins (93). However, it is unclear whether PP1 directly impinges on the APC, or whether it is needed to satisfy the requirements of a pre-anaphase checkpoint.

Although MPF is clearly an upstream regulator of the APC, mitotic CDK activity is not required for the maintenance of APC activity. In budding yeast and mammalian cells, the APC remains active during G1 phase until the G1 CDKs are activated (50, 59). Although the mechanism remains obscure, the ability of G1 kinases to inhibit degradation remains reversible until the *CDC4*-dependent step, after which APC is stably inactivated until the next mitosis (50, 94). G1-CDK activity is therefore particularly important in controlling proteolysis during the cell cycle, because it switches the APC pathway off and switches on proteolysis of SIC1.

In most cells the presence of unattached chromosomes or defects in spindle assembly activates an internal cellular signaling pathway, known as the spindle assembly checkpoint, that blocks the onset of anaphase and stabilizes APC substrates (95). The activity of this system is required for chromosome segregation with high fidelity; its loss may contribute to the aneuploidy that is characteristic of cancerous cells (96). Although several components of the spindle assembly checkpoint have been identified, their relation to the APC remains unclear. The checkpoint may inhibit activation of the APC, stabilizing all APC substrates. Alternatively, or additionally, substrates such as PDS1 or CUT2 may be modified to shield them from APC until all chromosomes are properly attached to the mitotic spindle. During normal mitotic cycles, the intrinsic lag period to APC activation may provide sufficient time for proper chromosome alignment; the checkpoint pathway may intervene only when necessary to enhance the fidelity of chromosome segregation.

A variation of this important cell cycle brake is engaged during the second meiotic division of vertebrate oocytes, in which cells naturally arrest in a metaphase-like state until fertilization occurs (97). This arrest is crucial because it synchronizes the cell cycle states of the female and male pronuclei, ensuring equal genetic contributions to the resulting zygote. Superficially this arrest is indistinguishable from that induced by the spindle-assembly checkpoint, because mitotic cyclins are stable and MPF activity is high. Although mitogen-activated protein (MAP) kinase appears to be required for both types of arrest (98, 99), it is unclear if the mechanisms of arrest are identical.

Proteolytic Pathways Compared

For both the CDC34 and APC proteolytic pathways, CDK activity serves as a trigger for destruction, either directly or indirectly, and destruction in turn regulates the amount of CDK activity by degrading a CDK activator or inhibitor. The view emerging from the study of the CDC34 pathway is that ubiquitination of substrates is controlled not by regulating the activity of the ubiquitinating enzymes, but by modulating the susceptibility of the substrates. Theoretically, substrates involved in diverse processes such as cell cycle arrest in response to mating factors (FAR1) (100), cell cycle progression (CLN2, SIC1), and regulation of amino acid biosynthesis (GCN4) (18) could be directed to the CDC34 pathway through the action of distinct, independently regulated kinases. Thus, the CDC34 pathway has the potential to initiate the destruction of specific substrates at different times during the cell cycle in response to various regulatory inputs. By linking CDC34-dependent ubiquitination of a substrate to the action of multiple kinases, a combinatorial response, reminiscent of that of transcriptional promoter elements, could be achieved.

In contrast, the APC pathway appears to be regulated at the level of the ubiquitination machinery, which may facilitate the coordinated destruction of multiple substrates at a single point in the cell cycle. However, a mechanism based solely on regulation of the APC may not provide the flexibility required for optimal cell cycle control. Positive regulation of APC activity may be combined with negative regulation of substrates to make the system sensitive to a wider variety of inputs. For example, whereas B-type cyclins are stabilized by the spindle-assembly checkpoint, A-type cyclins are not (99, 101, 102), suggesting that the degradation of cyclin B under these conditions may be inhibited at the substrate level. Furthermore, chromosomes segregate in a budding yeast *cdc15*^{ts} mutant (implying destruction of anaphase inhibitors such as PDS1), whereas a portion of cyclin B remains stable (67). CDC15 may be part of a regulatory circuit that protects cyclin B from the activity of APC until the anaphase spindle bisects the division plane of the cell (103).

Does proteolysis regulate other steps in the cell cycle? Just as chromosome segregation is initiated by the degradation of an inhibitor, there may be other proteins that must be degraded by the APC pathway before events of the next cell cycle can take place. A biochemical screen for proteins degraded in mitosis has identified a substrate of APC that bears no sequence similarity to cyclins

or the anaphase inhibitors described above (*104*). In budding yeast, the APC-dependent degradation of ASE1, a microtubule-binding protein, appears to be required for prompt disassembly of the mitotic spindle at the end of mitosis (*105*). Additional proteins involved in spindle morphogenesis and mitotic control, such as mammalian CENP-E (*106*), *Drosophila* PIMPLES (*107*), and *Aspergillus* NIMA (*108*), are degraded at the end of mitosis. However, it is unclear if these proteins are APC substrates, and it remains to be determined whether destruction of these proteins controls the execution of a specific step in the cell division program.

Besides their respective roles in late G1 and mitosis, the CDC34 and APC pathways may function at other steps in the cell division cycle. Analysis of *sic1* deletion mutants suggests there is a requirement for CDC34 function in G2-M phase (*19*), and mutations in APC subunits uncouple S phase from mitosis (*109*), or mitosis from S phase (*80, 110*). Although these findings imply interphase functions for the APC, there is no evidence that the ubiquitin ligase associated with the APC is active during this stage of the cell cycle, and no interphase-specific substrates of the APC have been reported. Besides the CDC34 and APC pathways, a host of distinct E1, E2, and E3 enzymes have been implicated in various cell cycle processes, including control of cell size in fission yeast (*111*), re-entry into the cell cycle in budding yeast (*112*), progression through G2 phase in hamster cells (*113*), progression through G2-M in budding and fission yeast (*66, 114*), and regulation of DNA replication in budding yeast (*115*).

Linking the cycle together. Although we have discussed the CDC34 and APC pathways separately, the regulatory interactions that make up the cell cycle are in fact interdependent. Each regulatory transition appears to have two important functions: to initiate a chromosomal event, and to enable a downstream regulatory event (Fig. 4). This logic ensures that cell cycle events occur in the proper order. DNA replication is triggered by the activity of G1 cyclins, which induce degradation of the kinase inhibitor SIC1 through the CDC34 pathway. However, G1 CDK activity also enables mitosis by inactivating the APC and allowing mitotic cyclins to accumulate. Mitosis is then triggered by mitotic CDK activity, which induces cellular processes necessary for chromosome condensation and alignment, and also enables the activation of the APC. The activated APC then executes two important functions: it triggers chromosome segregation by degrading anaphase inhibitors, and it enables a new round of DNA replication by destroying mitotic CDK activity, which inhibits formation of DNA prereplication complexes and blocks expression of G1 cyclins (*116–118*). The primary external input in this logic circuit is the activation of G1 CDKs, which is responsive to environmetnal cues such as nutritional status in budding yeast, or the presence or absence of growth factors in metazoan cells. In other organisms such as fission yeast, nutritional controls also influence the activation of mitotic CDKs.

The Awesome Power of Proteolysis

The chemical irreversibility of proteolysis is exploited by the cell to provide directionality at critical steps of the cell cycle. However, proteolysis also has an important role in regulating the timing of cell cycle transitions. Furthermore, the interdependence of CDK activity and ubiquitin-dependent proteolysis ensures that cell cycle events occur in the proper order. Several features of ubiquitin-mediated proteolysis make it a useful regulatory mechanism that complements CDK function. First, the obliterative nature of proteasome-mediated degradation ensures the simultaneous inactivation of all functions of a multidomain regulatory protein such as cyclin. Second, ubiquitin-dependent proteolysis allows for subunit-selective remodeling of heteromeric regulatory complexes—a potentially important consideration given that CDKs may simultaneously assemble with cyclins, substrates, and inhibitors. Third, given sufficient time, the entire pool of a substrate can be completely inactivated by proteolysis, even if the substrate's affinity for the proteolytic machinery is moderate. This may allow for greater flexibility in the evolution of targeting signals, as appears to be the case in the analogous vectorial process of protein translocation across intracellular membranes (*119*). This constellation of features makes proteolysis uniquely suited for resetting a

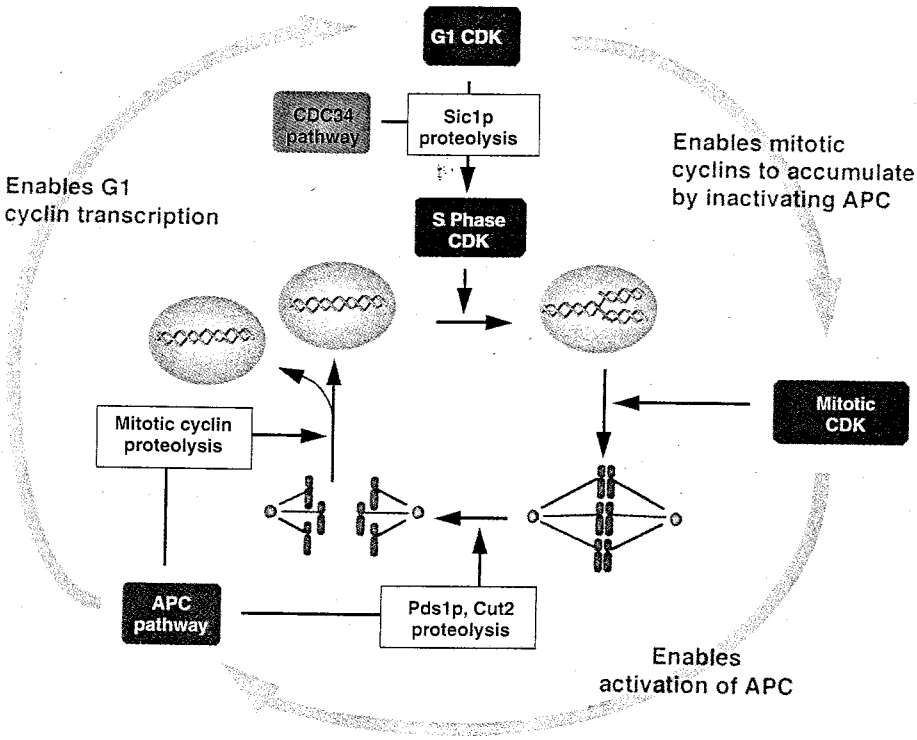

Fig. 4. How proteolysis drives the cell cycle. The model depicts a composite eukaryotic cell cycle and incorporates observations made in several different organisms. The chromosome cycle is depicted in the center of the figure, with interphase nuclei above and mitotic spindles below. The regulatory states of the cell cycle are interconnected by a series of dependencies. Each regulatory state has two functions: to trigger a chromosomal event such as replication, chromosome alignment, or segregation, and to enable the transition to a subsequent regulatory state (gray arrows). For example, G1 CDKs trigger DNA replication by activating S-phase CDKs through proteolysis and also enable mitotic cyclins to accumulate by inactivating the APC. Mitotic CDKs trigger chromosome condensation and spindle assembly, and also enable the activation of the APC. The active APC initiates anaphase by ubiquitinating anaphase inhibitors such as CUT2 and PDS1. The APC also catalyzes destruction of cyclin B, resulting in exit from mitosis, and enabling G1 cyclins to be resynthesized in the next cell cycle (*116*). The destruction of mitotic CDK activity is also required to allow formation of prereplication complexes (*117*), a prerequisite for DNA replication.

regulatory system to its ground state.

It has been useful to view the cell cycle principally as a kinase cycle, with downstream events such as DNA replication occurring as a function of the activity of a particular CDK. The discovery that mitotic cyclins must be degraded for cells to exit mitosis revealed that proteolysis is critical in controlling CDK activity and driving progression through the cell cycle. The more recent finding that the same ubiquitination pathway triggers anaphase independently of changes in CDK activity indicates that proteolysis directly controls a step in the chromosome cycle, and is not used solely to drive the cell cycle oscillator. Just as CDKs initiate cell cycle transitions such as the onset of mitosis by phosphorylating key substrates, the APC pathway initiates chromosome segregation by ubiquitinating key substrates. Cell duplication can therefore no longer be viewed as a simple kinase cascade; instead, phosphorylation and proteolysis are interdependent partners that collaborate to effect the remarkable process of cell division.

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Cell Cycle Control of DNA Replication

Bruce Stillman

The initiation of DNA replication in eukaryotic cells is a highly regulated process that leads to the duplication of the genetic information for the next cell generation. This requires the ordered assembly of many proteins at the origins of DNA replication to form a competent, pre-replicative chromosomal state. In addition to this competent complex, at least two cell cycle regulated protein kinase pathways are required to affect a transition to a post-replicative chromosomal state. Protein kinases required to establish mitosis prevent re-replication of the DNA. As cells exit mitosis, the cell cycle is reset, allowing the establishment of a new, competent replication state.

The transmission of genetic information from one cell generation to the next requires the accurate duplication of the DNA during the S phase of the cell cycle and the faithful segregation of the resultant sister chromatids during mitosis. In most eukaryotic cells, these two events are normally dependent on each other and thus the replication of the genome and mitosis occur in alternative, oscillating cycles. The molecular mechanisms that determine how DNA replication is initiated, how it is restricted to S phase, and how replication occurs only once per cell cycle in most eukaryotic cells have become major areas of attention. In this review, recent progress in these exciting areas is discussed. More detailed reviews on these issues can be found elsewhere (*1*, *2*).

The groundwork for understanding the control of DNA replication came from cell fusion experiments (*2*, *3*). Cells were synchronized at various stages of the cell cycle, then fused, and the marked nuclei were maintained to direct DNA replication and mitosis. For example, when a cell in the G1 phase of the cell cycle was fused to a cell in S phase, the G1-derived nucleus immediately initiated DNA replication, much earlier than if the cell had not been fused (Fig. 1). Other cell fusions demonstrated that G2 cells could not activate G1 nuclei, nor could G2 nuclei initiate DNA replication when fused to S-phase cells.

The cell fusion experiments revealed three important phenomena. First, only chromosomes from G1 cells are competent to initiate DNA replication. Second, S-phase cells, but not cells in G1 or G2, contain an activator of initiation of DNA replication that can work on the competent (G1) chromosomal state. Third, G2 nuclei do not re-replicate DNA until they pass through mitosis. The key goals of current research are to understand the molecular nature of the competent state and how is it established; the nature of the activator or activators present in S-phase cells; what prevents G2 nuclei from re-replicating, and how the competent state is erased during mitosis. This review focuses on these issues, primarily through discoveries in yeast that have general relevance to control of DNA replication in all cells.

Initiation: Replicators and Initiators

A key starting point to understanding the cell cycle controls that are imposed on the process of DNA replication is the origin of DNA replication. In eukaryotes, just as in bacteria, the location of the origin of DNA replication is determined by cis-acting DNA sequences (the replicator element in the DNA) and a trans-acting protein (the initiator protein) that binds to the replicator (*4*–*6*). Eukaryotic chromosomes are too large to replicate from a single origin and so contain multiple origins, more than are actually needed to replicate each chromosome (*7*). Although best understood at the present time in the yeast *Saccharomyces cerevisiae*, replicators and potential initiator proteins are beginning to be characterized in a wide variety of eukaryotes (*4*, *7*, *8*–*18*). In *S. cerevisiae*, replicators consist of multiple functional DNA elements, only one of which is essential (A) (*19*–*22*). Adjacent to the essential element are two or three functionally conserved DNA elements (B1, B2, and B3) that, although not individually essential, are necessary for initiation and influence the frequency with which an origin is used (*19*–*22*). The A, B1, and B2 elements form the core of the replicator and bind essential DNA replication proteins, whereas the B3 element functions as a replicator enhancer by binding a protein called autonomously replicating sequence (ARS)–binding Factor 1 [Abf1p, (*23*)].

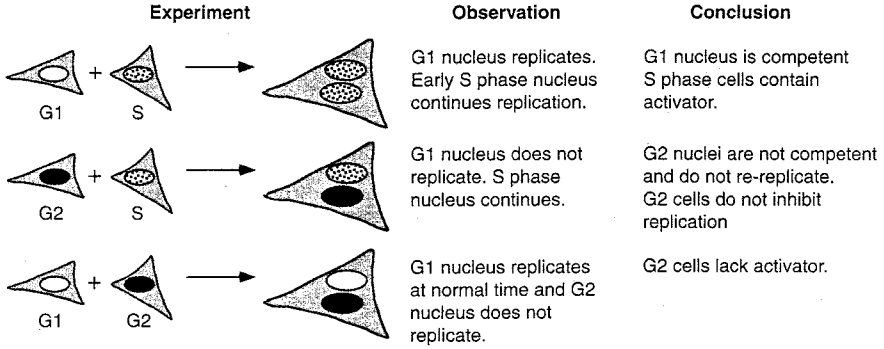

Fig. 1. Cell fusion experiments. Human HeLa cells that had been synchronized at different stages of the cell cycle were fused and the fate of the marked nuclei was followed. Data from (*3*).

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