Ubiquitin Ligases and Cell Cycle Control

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APC/C, cell cycle checkpoint, cyclin-dependent kinase, mitosis, SCF, ubiquitylation

Abstract

The ubiquitin-proteasome system plays a pivotal role in the sequence of events leading to cell division known as the cell cycle. Not only does ubiquitin-mediated proteolysis constitute a critical component of the core oscillator that drives the cell cycle in all eukaryotes, it is also central to the mechanisms that ensure that the integrity of the genome is maintained. These functions are primarily carried out by two families of E3 ubiquitin ligases, the Skp/cullin/F-box-containing and anaphase-promoting complex/cyclosome complexes. However, beyond those functions associated with regulation of central cell cycle events, many peripheral cell cycle–related processes rely on ubiquitylation for signaling, homeostasis, and dynamicity, involving additional types of ubiquitin ligases and regulators. We are only beginning to understand the diversity and complexity of this regulation.

Contents

Cyclin-dependent kinase (CDK): a protein kinase that controls cell cycle progression through phosphorylation of numerous substrates in association with cyclins

Cyclin: the positive regulatory subunit of CDK; cyclin levels oscillate during the cell cycle, controlling CDK activity and phase transitions

INTRODUCTION

Cell Cycle Control and Ubiquitin-Mediated Proteolysis

The cell cycle can be characterized as a highly regulated sequence of events in which chromosomes and other cellular components are duplicated and divided into daughter cells in a unidirectional and irreversible manner. Many cellular proteins are dedicated to controlling progression through the four cell cycle phases: G1, S (when chromosome duplication occurs), G2, and M (when replicated chromosomes, among other cellular components, divide). These transitions are driven primarily by phosphorylation of many target proteins by a family of protein kinases known as cyclin-dependent kinases (CDKs). However, CDK levels are constant throughout the cell cycle. CDK activity is modulated by association with positive regulatory subunits known as cyclins, which, unlike CDKs, are expressed periodically during the cell cycle, thus driving phase transitions through regulation of CDK activity. Different cyclin-CDK complexes form at different stages of the cell cycle to phosphorylate key substrates involved in chromosome duplication, mitotic spindle

assembly, and chromosome segregation. The activity of cyclin-CDK complexes is further controlled by the periodic expression of negative regulators, known as CDK inhibitors (CKIs). The orchestrated synthesis and degradation of these proteins, and of other cell cycle regulators, are at the core of cell cycle control and ultimately dictate the unidirectionality of cell cycle progression (1–4).

Selective and programmed protein degradation provides direction, order, and appropriate timing of cell cycle events. Ubiquitylation is a posttranslational modification that can regulate the stability, localization, and function of target substrates. Although ubiquitin-mediated proteolysis plays numerous roles in cell cycle control and progression, as discussed below, perhaps the two most critical functions carried out by this system are the elimination by degradation of CKIs to potentiate the G1-S transition and the degradation of the anaphase inhibitor securin and the mitotic cyclin, cyclin B, to allow chromosome separation and mitotic exit. These degradation processes guarantee timely duplication of the genetic material and its equal distribution to daughter cells, maintaining genome integrity and cell viability. Deregulation of protein ubiquitylation or degradation processes can lead to aberrant cell proliferation and cancer (5–9).

Ubiquitylation and the Ubiquitin-Proteasome System

Ubiquitin-mediated proteolysis is carried out by the ubiquitin-proteasome system (UPS), which mediates the decoration of target substrates with multiple ubiquitin molecules (ubiquitylation) and induces their degradation through the 26S proteasome complex. Ubiquitin is a small (8 kDa), highly conserved protein that is covalently attached to substrates through a cascade of enzymatic reactions. Initially, the ubiquitin molecule is linked to a ubiquitin-activating enzyme (E1) in an ATP-dependent manner. Subsequently, the activated ubiquitin is transferred to a ubiquitin-conjugating enzyme (E2), and in collaboration with an E3 ubiquitin ligase, ubiquitin is finally linked to a specific lysine residue on the target protein. E3 ligases are able to recruit distinct sets of substrates and are the primary source of substrate specificity in the ubiquitylation process. Once the target substrate becomes polyubiquitylated, the 26S proteasome complex recognizes and degrades it in an ATP-dependent manner (10–12).

E3 ubiquitin ligases can mediate the addition of one ubiquitin molecule to a particular lysine residue on the substrate (monoubiquitylation), single ubiquitin molecules to different lysine residues on the substrate (multimonoubiquitylation), or ubiquitin chains extending from a particular lysine residue (polyubiquitylation). These chains may be linked through any of the seven lysine residues found on ubiquitin molecules, as well as the N-terminal methionine (Met1), but the most abundant ubiquitin linkages in cell cycle regulators that are recognized and degraded by the 26S proteasome complex are Lys11- and Lys48-linked chains. More recently, investigators showed that multimonoubiquitylation can be sufficient to target specific substrates for proteasomal degradation (13). The ubiquitylation process may also be modulated by deubiquitinating enzymes, a large group of proteases that hydrolyze ubiquitin-protein peptide bonds, reversing ubiquitylation of target proteins and recycling ubiquitin molecules. Alternatively, single ubiquitins and short ubiquitin chains can regulate target protein function (14–16).

E3 Ligases in Cell Cycle Control: The APC/C and SCF Complexes

E3 ubiquitin ligases facilitate the transfer of ubiquitin molecules from E2 enzymes to target substrates. Most E3 ligases contain a RING (really interesting new gene)-finger domain, which is responsible for interaction with an E2. Also, E3 ligases recognize a large number of substrates through adaptor proteins, providing specificity and versatility to the UPS. Conserved from yeast to humans, RING-finger E3 ligases not only serve as platforms to assemble

Ubiquitin: a 76-amino-acid polypeptide that is covalently linked to target substrates, which are usually directed for degradation by the proteasome

Proteasome: a large multisubunit protease complex that directs ubiquitylated proteins for degradation

E3 ubiquitin ligase: an enzyme complex that facilitates the transfer of ubiquitin from E2 ubiquitin-conjugating enzymes to target substrates

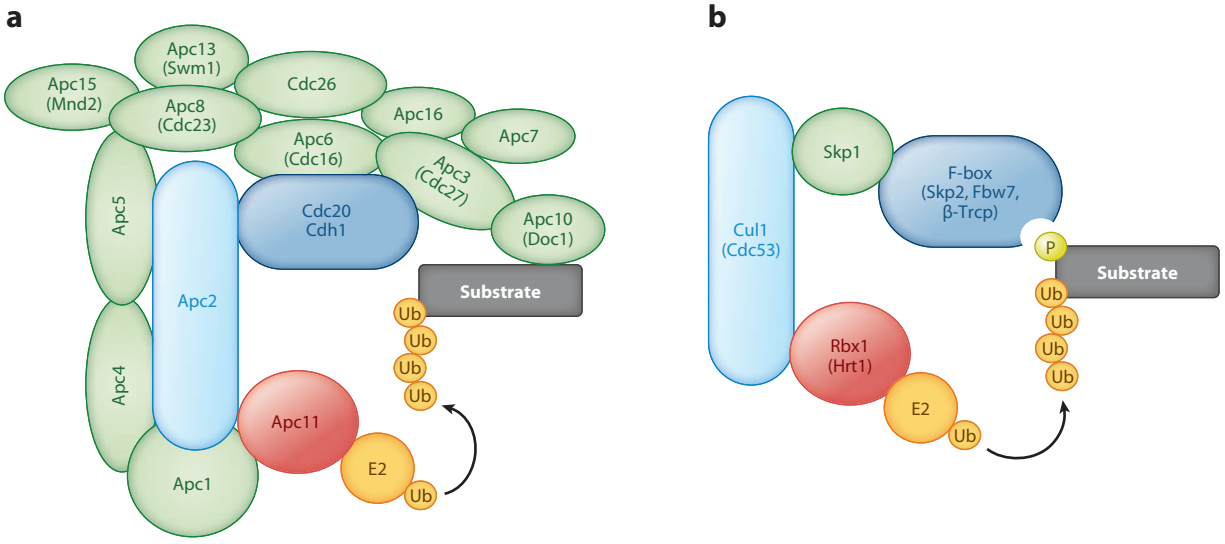


Figure 1

Schematic representation of the anaphase-promoting complex/cyclosome (APC/C) and Skp/cullin/F-box-containing (SCF) complexes. Both E3 ubiquitin ligases are composed of catalytic cores with similar structures, consisting of a cullin-like protein (Apc2 in APC/C and Cul1 in SCF; *light blue*) that serves as the complex scaffold, and a really interesting new gene (RING)-finger protein (Apc11 in APC/C and Rbx1 in SCF; *purple*) that recruits an E2 enzyme for substrate ubiquitylation (*orange and red*). (*a*) In APC/C complexes, substrate binding and specificity are provided by the alternative adaptors Cdc20 and Cdh1, which direct ubiquitin-mediated proteolysis of target substrates. APC/C is composed of many other highly conserved proteins that provide molecular scaffold support (*green*). Nomenclatures of human proteins are indicated with respective *Saccharomyces cerevisiae* correlates in parentheses. (*b*) SCF ligases have a variable component F-box protein (Skp2, Fbw7, β-Trcp) that recognizes specific phosphorylated sequences on target substrates (phosphodegrons), triggering their ubiquitylation and degradation. The adaptor protein Skp1 recruits F-box proteins to the SCF core. Abbreviation: P, phosphate (*yellow*).

Cullin: a family of proteins that serves as a scaffold for E3 ubiquitin ligases and binds RING-finger proteins through conserved cullin domains

CRL: cullin-RING ligase

Anaphase-promoting complex/cyclosome (APC/C): a ubiquitin ligase complex that promotes mitotic progression through ubiquitylation and degradation of numerous cell cycle regulators

E2 enzymes and substrates in close proximity, but also stimulate E2 catalytic activity and mediate transfer of ubiquitin from E2 to substrates, thus favoring the process of protein ubiquitylation (17, 18). One large subfamily of RING-finger E3 ligases comprises the cullin-RING ligases (CRLs), which include two structurally similar enzymes involved in the proteolysis of key cell cycle–regulatory proteins: the anaphase-promoting complex/cyclosome (APC/C) (**Figure 1*a***) and Skp/cullin/F-box-containing (SCF) (**Figure 1*b***) complexes.

The APC/C controls progression through mitosis and the subsequent G1 interval by ubiquitylating many important cell cycle regulators, including mitotic cyclins, anaphase regulators, spindle assembly factors (SAFs), and DNA replication proteins (19–22). In humans, the APC/C core is composed of at least 14 different proteins, including the Apc11 RING-finger protein that interacts with an E2 enzyme, and the Apc2 cullin-like subunit that serves as a scaffold. Ube2S is the vertebrate E2 enzyme responsible for elongating ubiquitin chains on APC/C substrates, once initiated by other E2s, and it also defines the specificity of ubiquitin chain linkage. APC/C activation is achieved through further association with one of two coactivator subunits, which also serve as substrate adaptors: cell division cycle protein (Cdc)20 (also known as Slp1 and Fzy) and Cdh1 (Cdc20 homolog 1, also known as Hct1, Ste9, and Fzr) (19–22). Both adaptors recognize short destruction motifs (degrons) on target substrates through C-terminal domains composed of WD40 repeats. The canonical destruction motifs recognized by APC/C are the D-box (consensus sequence RXXLXXXXN) and the

KEN-box (consensus sequence KENXXXN) (23, 24). Whereas Cdc20 preferentially recognizes D-box motifs, Cdh1 recognizes both D-box and KEN-box motifs. Additionally, in collaboration with Cdc20 and Cdh1 adaptors, the Apc10 core protein also contributes to D-box recognition and processive substrate ubiquitylation by the APC/C (25–28). In yeast, the APC/C forms Lys48-linked ubiquitin chains on substrates, targeting them for degradation by the 26S proteasome (29). In contrast, the vertebrate APC/C preferentially assembles Lys11-linked chains on substrates (30). Temporal regulation of APC/C activity and its substrate selectivity are controlled not only by association with the specific adaptors Cdc20 and Cdh1, but also through binding of APC/C inhibitors and phosphorylation of the APC/C core (19–22). In addition, initiation motifs on APC/C substrates, distinct from the D-box and KEN-box sequences described above, determine the efficiency of ubiquitin chain initiation by cognate E2 enzymes, thereby specifying the order in which various substrates are degraded during mitosis (31).

The SCF complexes are RING-finger E3 ligases that play several central roles in cell cycle regulation, including controlling S-phase entry and mitotic entry by ubiquitylating CKIs, G1- and S-phase cyclins, and mitotic inhibitors (32–34). SCF ligases contain three invariable components: Skp1 (S-phase kinase-associated protein 1), Cul1 (also known as Cdc53), and Rbx1 (also known as Roc1 and Hrt1). The cullin subunit Cul1 serves as a scaffold that binds both the RING-finger protein Rbx1, which recruits the E2 enzyme, and the adaptor protein Skp1, which recruits the variable component F-box protein. F-box proteins bind to Skp1 through a conserved N-terminal F-box motif and recruit substrates through C-terminal protein-protein interaction domains, composed of, for example, WD40 repeat–based β-propellers or leucine-rich repeats (LRRs), thus conferring substrate specificity to the system. Phosphorylation of specific sequences on target substrates, known as phosphodegrons, is required for recognition by most of the best-characterized F-box proteins (35, 36), leading to formation of Lys48-linked ubiquitin chains and degradation (37). The SCF ligases that have the most dominant roles in cell cycle control contain the F-box proteins Skp2 (S-phase kinase-associated protein 2, also known as Fbl1), Fbw7 (F-box WD40 repeat–containing protein 7, also known as Cdc4, Sel-10, and Archipelago), and β-Trcp (β-transducin repeat–containing protein, also known as Slimb and Fbw1) (32–34). **Figure 2** illustrates the primary targets of the SCF and APC/C ubiquitin ligases and the points in the cell cycle in which they function.

THE CORE CELL CYCLE OSCILLATOR

Ubiquitin-Mediated Proteolysis and the Minimal Cell Cycle

Minimally, cells need to accomplish only two things to proliferate: duplication of the genetic material and division into two daughter cells. For eukaryotic organisms, both these processes have been highly conserved through evolution, and as discussed above, ubiquitin-mediated proteolysis plays critical roles for each. Central to the division process are the mitotic cyclins and the APC/C that promotes their degradation as cells undergo mitosis. For DNA replication to occur, an inhibitor known as geminin must be targeted for degradation, also by the APC/C. In most cell types, numerous regulatory constraints must be imposed on these processes to coordinate cell division with growth, cellular functions, and external signals, leading to a high level of complexity. The exception is the large cleavage embryos of amphibians, fish, and many marine invertebrates, such as sea urchins, starfish, and clams. To produce many cells from a zygote extremely rapidly, these organisms have opted for a stripped-down cell cycle truly devoid of most regulation, i.e., a free-running oscillator.

Skp/cullin/F-box-containing (SCF) complex: a ubiquitin ligase complex that recruits target substrates for ubiquitin-mediated degradation through different F-box proteins

SAF: spindle assembly factor

CDC: cell division cycle

Phosphodegron/degron: a protein motif on target substrates (phosphorylated or not) that is recognized and required for degradation by E3 ubiquitin ligases

Skp: S-phase kinase-associated protein

F-box protein: the variable component of SCF ligases that recognizes and directs target substrates to ubiquitin-mediated proteolysis

Fbw7: F-box WD40 repeat–containing protein 7

β-Trcp: β-transducin repeat–containing protein

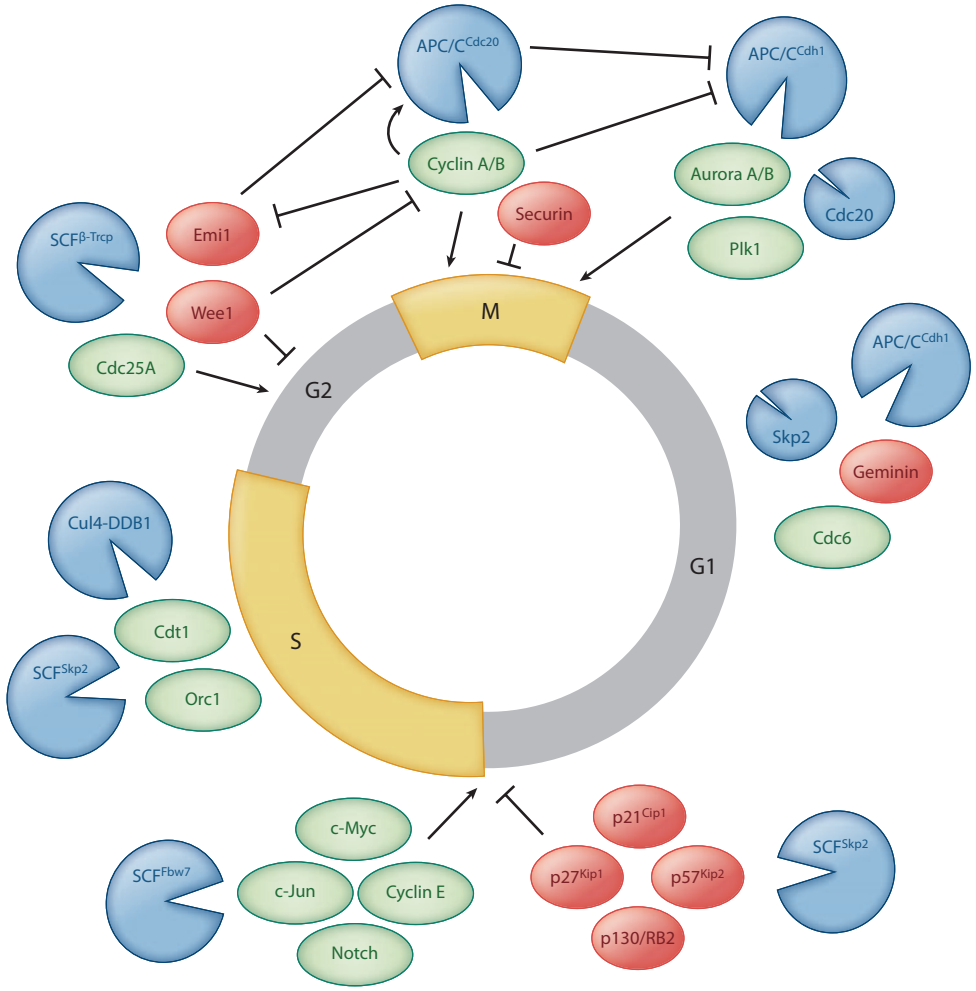


Figure 2

Ubiquitin-mediated degradation of key cell cycle regulators by the APC/C and SCF complexes. E3 ubiquitin ligases (*blue circles*) trigger the ubiquitylation and proteolysis of cell cycle activators (*green ovals*) and inhibitors (*red ovals*) at specific cell cycle phases: G1, S, G2, and M. APC/C ubiquitylates target substrates from the middle of M phase to the end of G1 phase, whereas SCF ligases are active from late G1 to early M phase. Cul4-DDB1 shows ubiquitin ligase activity in S phase.

Cleavage Embryos and the Cyclin B–APC/C Oscillator

As stated above, the early embryos of amphibians, fish, and many marine invertebrates go through rapid, simplified cell cycles. The study of these cell cycles has provided many important insights concerning the machinery and organization central to cell division in all eukaryotes, including the role of ubiquitin-mediated proteolysis. Although historically the first observations with respect to periodic accumulation and destruction of cyclins were made in the sea urchin (38), by far the bulk of modern research along these lines has been carried out using the oocytes of the frog *Xenopus laevis*. Therefore, we largely limit this discussion to the *Xenopus* system. *Xenopus* eggs, once fertilized, undergo 12 synchronous divisions without

any transcription (39, 40). Each cell cycle occurs in under an hour. To do this, the oocyte is preloaded with all the proteins and mRNAs required until zygotic transcription. Because no growth can occur in the absence of transcription and in such a short time interval, repeated cleavage converts one large cell (the zygote) to 4,000 or so small cells that can then be utilized for the developmental process after the midblastula transition. To produce this stockpile of cells, most cell cycle regulatory safeguards have been jettisoned. Another useful feature of the *Xenopus* oocyte system is that virtually all the phenomena of the embryonic cell cycle can be recapitulated in cell-free extracts, which can be produced in quantity and are extremely amenable to biochemical manipulation (41).

The process of cell division in the early *Xenopus* embryo is driven by the accumulation and destruction of primarily cyclin B1, the principal activator of Cdk1 (42, 43). Once cells or extracts have exited from mitosis, cyclin B1 accumulates continuously based on translation of stockpiled mRNA. Cyclin B1–Cdk1 complexes are maintained in an inactive state primarily by negative regulatory phosphorylation of Cdk1 on tyrosine 15 by the kinase Wee1 (44, 45). Once a threshold level of cyclin B1–Cdk1 is achieved, as determined by feedback relationships linking cyclin B1–Cdk1 to Wee1 and the tyrosine 15 phosphatase, Cdc25C (46, 47), rapid and complete Cdk1 activation occurs, driving the mitotic state. Cyclin B1–Cdk1 then phosphorylates many proteins, including subunits of the APC/C ubiquitin ligase, promoting its activation (48, 49). This constitutes a negative feedback loop, as the APC/C efficiently targets cyclin B1 itself and leads to its ubiquitin-mediated proteolysis. The degradation of cyclin B1 and concomitant inactivation of Cdk1 reverse the mitotic state and reset interphase. Recurrent cycles of cyclin B1 accumulation, Cdk1 activation, and cyclin B1 degradation can run in *Xenopus* oocyte extracts devoid of nuclei or other organelles (50). The relationship between cyclin B1 and the APC/C constitutes the basis of the autonomous oscillator that lies at the core of the primitive early embryonic cell cycle.

Bistability and Why Cyclin B1 Needs to Be Degraded

Although cyclin B1 is synthesized at a constant rate during interphase in the cleavage embryo cell cycle, cyclin B–Cdk1 activity accumulates explosively only during a short interval prior to mitosis (50, 51). The reason for this lies in the relationships between Cdk1 and its regulators Wee1 and Cdc25C. Wee1 and cyclin B1–Cdk1 exist in a double-negative feedback loop: Phosphorylation of cyclin B1–Cdk by Wee1 is inhibitory, as is phosphorylation of Wee1 by cyclin B1–Cdk1 (52). Therefore, at low concentrations of cyclin B1–Cdk1, Cdk1 activity is low because Wee1 is in excess. However, above a threshold level of cyclin B1–Cdk1, Cdk1 activity is high, overwhelming the inhibitory ability of a fixed amount of Wee1. Superimposed on these negative feedback relationships is a positive feedback relationship between cyclin B1–Cdk1 and its activator, Cdc25C. Cdk1 kinase activates Cdc25C, which then further activates Cdk1 (53, 54). This regulatory environment dictates that Cdk1 activity can be only low or high and that intermediate activity levels constitute unstable states. Researchers have demonstrated this by titrating different amounts of a nondegradable mutant cyclin B1 into either interphase or mitotic extracts (55, 56). As expected, low concentrations of cyclin B1 produced low levels of activity, whereas high concentrations produced high levels. However, intermediate concentrations produced either low or high levels of activity, but never intermediate levels. Most importantly, the feedback loops that drive and stabilize high Cdk1 activity when cyclin B1 levels are high explain the necessity for total degradation of cyclin B1 to inactivate Cdk1 and reverse the mitotic state.

The Role of Ubiquitin-Mediated Proteolysis in DNA Replication

In the early *Xenopus* embryonic cell cycle, cyclin E–Cdk2 triggers DNA replication. Unlike cyclins A and B, cyclin E is not regulated by ubiquitin-mediated proteolysis in these

cell cycles (as it is in somatic cells). However, like that of cyclins A and B, the concentration of cyclin E needs to change dramatically at different points in the cell cycle: high during S phase and low immediately after mitosis to allow prereplication-complex assembly (origin licensing). This concentration is accomplished by dilution rather than degradation (57). Upon nuclear envelope breakdown, cyclin E concentrated in the nucleus disperses in the extremely large cellular volumes associated with the early cleavage embryo. Once nuclei reassemble after mitosis, cyclin E–Cdk2 is actively transported and reconcentrated in the small nuclear volume, with replication initiating once a threshold concentration is achieved. However, although proteolysis does not figure centrally in the process of replication initiation in the early *Xenopus* embryo, it is crucial for insuring that replication origins fire only once per cell cycle, an aspect of regulation indispensable for genomic integrity. One mechanism alluded to above is the process of origin licensing and the role of cyclin E–Cdk2. As long as cyclin E–Cdk2 activity in the nucleus is high, the MCM (minichromosome maintenance) replication helicase is inhibited from loading onto chromatin. However, in the *Xenopus* early embryo, the more critical mechanism for limiting origin initiation to once per cell cycle is sequential proteolysis of the prereplication-complex assembly protein, Cdt1, and its inhibitor, geminin. Once DNA replication initiates, Cdt1 is ubiquitylated by the Cul4-DDB1 ubiquitin ligase and degraded (58, 59). To couple degradation of Cdt1 to ongoing replication, binding of Cdt1 to proliferating cell nuclear antigen, the polymerase processivity factor, is essential for activation of Cul4-DDB1. Nevertheless, Cul4-dependent degradation of chromatin-bound Cdt1 is not sufficient to prevent rereplication in oocyte extracts. An inhibitory Cdt1-binding protein, geminin (60, 61), is also required. Depletion of geminin from extracts allows unscheduled DNA replication to occur (62). Geminin is stable during interphase but is targeted by the APC/C for proteolysis during mitosis (60). This establishes a postmitotic geminin-free environment conducive to Cdt1 accumulation and activity, and therefore permissive for prereplication-complex assembly. Thus, in the early *Xenopus* embryonic cell cycle, the APC/C controls resetting of both the mitotic and replicative processes.

The Early *Drosophila* Embryo

Like the frog, the fruit fly *Drosophila* produces a large egg loaded with sufficient maternal material to sustain many rapid cell cycles in the absence of zygotic transcription and growth. However, the strategy employed is somewhat different. The first 13 mitotic cycles take place in a syncytial cytoplasm. They are extremely rapid, lasting approximately 9 min each, but not completely synchronous, as is the case with *Xenopus*. Also, very unlike *Xenopus*, there are no obvious oscillations of cyclin levels or of Cdk1 activity when the entire embryo is assayed (63). But injection of a nondestructible cyclin B prevents mitotic exit, suggesting that cyclin B proteolysis is essential. This apparent paradox is resolved by demonstrating that very local degradation of cyclin B, specifically at centrosomes and kinetochores, is sufficient to allow mitotic exit of syncytial nuclei without affecting the greater cytoplasmic pool (63). The implication of this is that the APC/C is tightly regulated not only temporally but also spatially, creating many local oscillators. Presumably, this adaptation allows numerous asynchronous nuclei to undergo rapid, autonomous cell cycles in a syncytial cytoplasm.

REGULATORY COMPLEXITY OF SOMATIC CELL CYCLES

Somatic Cell Cycles: From Yeast to Mammals

Owing to the need to respond to numerous internal and external signaling inputs, somatic cell cycles exhibit many additional levels of control compared with the simplified early divisions of *Xenopus* and *Drosophila* embryos. As a result, in most cases, the length of the cell

cycle has been extended, and phases in which growth occurs and regulatory signals are integrated, such as the G1 and G2 intervals, have been added to the core oscillator composed of only the S and M phases. Increased complexity in the role of ubiquitin-mediated proteolysis of cell cycle regulators reflects these changes. As we discuss in this section, the APC/C and SCF ligases control a multitude of events in somatic cell cycles from yeast to higher eukaryotes, including critical phase transitions, such as the G1-S and the metaphase-anaphase transitions, as well as essential regulatory checkpoints, such as the DNA damage and the spindle assembly checkpoints (SACs). However, the selective and programmed ubiquitin-mediated degradation of target substrates is also modulated by cellular responses to external signals, fluctuations in gene expression, and phosphorylation-dependent protein modifications.

The APC/C in Somatic Cells

As described above, the APC/C dictates sequential cycles of mitosis and DNA replication in early embryos with the ubiquitin-mediated destruction of mitotic cyclins and geminin, respectively. However, in somatic cells, APC/C regulates the progression not only through mitosis, but also through the subsequent G1 interval, which is largely controlled by association of APC/C with the alternative coactivator Cdh1 (not present in *Xenopus* and *Drosophila* embryonic cycles). Furthermore, in somatic cell cycles, APC/C activity is modulated by additional mechanisms, such as binding of APC/C inhibitors and transcriptional and post-translational regulation of substrate adaptors and the APC/C core (19–22).

APC/C^{Cdc20} is primarily responsible for promoting sister chromatid separation during anaphase and initiating exit from mitosis by triggering ubiquitin-mediated proteolysis of securin (also known as Pds1 and Cut2 in yeast) and cyclins A and B, respectively (64). Transcriptional regulation of the Cdc20 substrate adaptor adds one layer of complexity to the APC/C system in yeast and somatic metazoan cells. Although Cdc20 protein levels begin to accumulate in S phase, APC/C^{Cdc20} does not become fully active until the metaphase-anaphase transition (65, 66). APC/C^{Cdc20} inhibition during this interval allows the accumulation of mitotic cyclins, thereby potentiating the burst of Cdk1 activity necessary to drive progression through mitosis. During S and G2 phases, APC/C^{Cdc20} is inhibited by Emi1 (early mitotic inhibitor 1, also known as Fbx5), which prevents substrate binding to Cdc20 (67, 68); in early mitosis, APC/C^{Cdc20} is inhibited by the SAC, which we discuss in detail below (see The Role of Ubiquitylation in Regulation of Cell Cycle Checkpoints, below). In early mitosis, both polo-like kinases (PLKs) and cyclin B–Cdk1 phosphorylate Emi1, directing its ubiquitylation by $SCF^{\beta\text{-}Trcp}$ (see below) (69–72). Once Emi1 is degraded and the SAC is satisfied, APC/C^{Cdc20} is directly phosphorylated and activated by cyclin B–Cdk1, initiating the ubiquitin-mediated degradation of cyclin B in a negative feedback loop (73, 74) and mitotic exit. Targeting of securin, the other critical substrate of APC/C^{Cdc20}, leads to activation of the cysteine protease separase (also known as Esp1 and Cut1 in yeast), which in turn cleaves the cohesin subunit Scc1, allowing sister chromatids to separate (75–78). In late mitosis, several mechanisms inactivate APC/C^{Cdc20}, including downregulation of Cdc20 expression and Cdc20 degradation by APC/C^{Cdh1} (65, 66). However, in yeast, most ubiquitin-mediated proteolysis of Cdc20 occurs through *cis* autoubiquitylation during anaphase (79).

Another somatic cell–specific addition to the APC/C system is the alternative activator Cdh1. APC/C^{Cdh1} is responsible for completion of mitotic exit and progression through the subsequent G1 phase by maintaining low levels of mitotic proteins (80, 81). During S, G2, and early M phases, CDK-mediated phosphorylation prevents Cdh1 association with the APC/C core (82–84). Unlike APC/C^{Cdc20}, which is active at high levels of Cdk activity, APC/C^{Cdh1} is active at low levels of Cdk activity, mediating the continued degradation of mitotic cyclins as well as non-Cdk mitotic kinases (e.g., aurora A

SAC: spindle assembly checkpoint

and B and Plk1) and DNA replication factors during the G1 interval (85–91). In vertebrate cell cycles, APC/C^{Cdh1} is further inactivated by Emi1, which works as a pseudosubstrate inhibitor that competes with and prevents substrate binding to Cdh1 (68, 92, 93). Researchers have described a similar mechanism in yeast, in which APC/C^{Cdh1} activity is repressed by Acm1 (APC/C^{Cdh1} modulator 1), a specific inhibitor of Cdh1 substrate binding (58, 94–96). At the end of mitosis, APC/C^{Cdh1} is activated as a consequence of APC/C^{Cdc20}-mediated degradation of mitotic cyclins and concomitant inactivation of Cdk1, and in yeast, also through Cdh1 dephosphorylation by the Cdc14 phosphatase (83, 97). APC/C^{Cdh1} also promotes the G1 accumulation of the CKIs $p21^{Cip1}$ and $p27^{Kip1}$ through degradation of Skp2, the SCF substrate adapter that targets these proteins (see below) (98, 99). The low CDK environment during the G1 interval provides a stable period for cellular growth and integration of external and internal regulatory signals. Furthermore, low CDK activity enforced by APC/C^{Cdh1} is essential for the assembly of prereplication complexes at origins in preparation for DNA replication (88, 100, 101). In this same context, APC/C^{Cdh1} also triggers the ubiquitin-mediated proteolysis of geminin (60), releasing Cdt1 to carry out prereplication-complex assembly functions (102, 103). As cells approach the G1-S transition, transcription of S-phase cyclins leads to increased CDK activity, Cdh1 phosphorylation, and its subsequent dissociation from the APC/C core (82–84). APC/C^{Cdh1} is further inactivated by Cdh1 autoubiquitylation and degradation (104), as well as autoubiquitylation and degradation of its E2 ubiquitin-conjugating enzyme UbcH10 (105). In addition, the core SCF complex appears to regulate Cdh1 degradation through an unknown mechanism at the G1-S boundary (106). All these mechanisms contribute to maintaining APC/C in an inactive state until the next G2-M transition.

The SCF Complex in Somatic Cells

With the introduction of intervals dedicated to growth and regulation (G1 and G2 phases), the SCF ubiquitin ligases assume numerous important roles. SCF ligases regulate S-phase entry and mitotic onset by targeting various key regulatory proteins for degradation. Several of these SCF substrates are directly involved in modulating CDK activity. As discussed below, primarily three F-box protein adapters are responsible for cell cycle–relevant SCF activities in somatic cells (32–34).

Skp2 levels accumulate during the G1-S transition as a consequence of APC/C^{Cdh1} inactivation (98, 99). SCF^{Skp2} activity primarily mediates the degradation of the CKIs $p27^{Kip1}$, $p21^{Cip1}$, and $p57^{Kip2}$ and the pocket protein p130/RB2, increasing S-phase cyclin/CDK activity and allowing cells to progress through S and G2 phases (8, 33). Skp2 binds to target substrates through a C-terminal LRR domain and, at least in some cases, requires the additional binding of a small highly conserved cofactor, Cks1 (cyclin-dependent kinase subunit 1, also known as Suc1 in fission yeast) (107–110), which forms part of the substrate-binding surface (111). One such example is the SCF^{Skp2}-mediated degradation of the CKI $p27^{Kip1}$, a negative regulator of the G1-S transition. During late G1 phase, p27 is phosphorylated by cyclin E/Cdk2 on Thr187, triggering $SCF^{Skp2/Cks1}$-mediated recognition and ubiquitylation (107, 108). While cells progress through S and G2 phases, SCF^{Skp2} also induces the ubiquitin-mediated proteolysis of the origin-licensing factors Orc1 and Cdt1 (112, 113), preventing replicated origins from becoming relicensed and thus rereplicated (102, 103), although another CRL, Cul4-DDB1, may be more important for degradation of Cdt1 (58, 59). Finally, elimination of SCF^{Skp2} activity occurs during mitotic exit by APC/C^{Cdh1}-mediated degradation of Skp2, as mentioned above (98, 99).

In contrast to Skp2, the F-box protein Fbw7 primarily mediates the ubiquitylation of cell cycle activators in metazoans, such as cyclin E, c-Myc, c-Jun, and Notch (8, 34, 114). Also unlike Skp2, Fbw7 binds to substrate phosphodegrons through a domain composed of eight WD40 repeats that form an eight-bladed β-propeller

(115). In addition, Fbw7 can dimerize, increasing the efficiency of substrate binding and ubiquitylation of some substrates. Fbw7 levels are constant through the cell cycle, such that regulation of SCF^{Fbw7} activity is mostly mediated at the level of substrate phosphorylation. However, SCF^{Fbw7} activity may also be modulated by glomulin, a CRL inhibitor that binds directly to the RING-finger protein Rbx1, blocking its association with the E2 enzyme Cdc34 and thereby causing the accumulation of the Fbw7 targets cyclin E and c-Myc (116, 117). In budding yeast, one of the critical targets of the yeast ortholog of SCF^{Fbw7}, SCF^{Cdc4}, is the CKI Sic1. Investigators originally proposed that SCF^{Cdc4} interaction with Sic1 occurs only after progressive phosphorylation of at least six residues on Sic1 (35, 118), achieved only after maximal accumulation of G1 cyclins Cln1 and Cln2 and correspondingly high levels of Cdk1 activity (119, 120). SCF^{Cdc4}-mediated ubiquitylation and degradation of Sic1 release S-phase CDK from inhibition, promoting initiation of DNA replication (121, 122). The coupling of Sic1 degradation to phosphorylation on numerous low-efficiency sites may present a barrier to premature entry into S phase (35). However, more recently, researchers presented data supporting an alternative model, in which Sic1 destruction depends on the sequential phosphorylation of a small number of specific phosphodegrons by both Cln2- and Clb5-associated Cdk1 activity. These two kinase complexes collaborate in defined processive multiphosphorylation cascades, leading to Sic1 ubiquitylation by SCF^{Cdc4}, degradation, and the G1-S transition (123). In mammals and other metazoans, degradation of the prototypical substrate cyclin E1 by SCF^{Fbw7} also requires prior phosphorylation of specific phosphodegron sites (124–126). But unlike yeast Sic1, activation of a single high-affinity phosphodegron by autophosphorylation is sufficient to mediate efficient cyclin E ubiquitylation and degradation (124–128). The coupling of cyclin E–Cdk2 activation to cyclin E degradation establishes a negative feedback loop that limits maximal cyclin E–dependent Cdk2 activity to a narrow interval of the cell cycle.

$SCF^{\beta\text{-}Trcp}$ primarily regulates progression into mitosis through ubiquitin-mediated degradation of cell cycle inhibitors Wee1 and Emi1 (8, 33). Like Fbw7, β-Trcp binds to substrate phosphodegrons through a C-terminal domain consisting of WD40 repeats. During S and G2 phases, Wee1 kinase inhibits M-phase CDK activity by direct phosphorylation, preventing premature initiation of mitotic events (44, 129–131). As cells approach mitosis, $SCF^{\beta\text{-}Trcp}$ mediates Wee1 ubiquitylation and degradation, releasing M-phase CDK from inhibition, thereby initiating mitotic onset in somatic cells (132). Of note, in *Xenopus* eggs, an alternative SCF complex termed $SCF^{Tome\text{-}1}$ targets Wee1, also facilitating mitotic entry in embryonic cell cycles (133). As discussed above, $SCF^{\beta\text{-}Trcp}$ further contributes to mitotic progression through targeting of the APC/C inhibitor Emi1 during early mitosis, allowing APC/C^{Cdc20} activation and the metaphase-anaphase transition (69, 70).

AUXILIARY FUNCTIONS OF UBIQUITIN LIGASES IN ASSEMBLY AND MAINTENANCE OF THE CELL CYCLE MACHINERY

Roles for Ubiquitylation Beyond Driving the Major Cell Cycle Oscillations

As discussed above, ubiquitin-dependent protein degradation drives the cycles that determine DNA replication and cell division. Cyclin-dependent activation of CDKs and the closely linked ubiquitin-dependent proteolysis of cyclins constitute the core of the cell cycle oscillator. However, ubiquitylation and ubiquitin-dependent proteolysis of many other proteins are required for proper regulation of cell cycle progression and effective execution of cell division. Although an exhaustive list of cell cycle functions regulated by ubiquitin-mediated proteolysis is beyond the scope of this review, some of the critical ones are spindle

assembly; kinetochore, centrosome, and spindle pole function; chromosome disjunction; and cytokinesis.

Roles of Ubiquitylation in Assembly and Maintenance of the Mitotic Spindle

Chromosome separation, one of the essential elements of cell division, requires the de novo assembly of an elaborate microtubule-based machine known as the mitotic spindle. For the spindle to be assembled and function properly, the dynamicity, length, and attachments of its microtubules must be highly regulated. For many proteins that regulate microtubule function, ubiquitylation and ubiquitin-mediated proteolysis are critical for proper function. Logic would dictate that proteins that promote spindle formation and function, such as microtubule motors (e.g., kinesins), should be degraded by APC/C^{Cdh1} during mitotic exit after execution of their very specialized functions. This appears to be the case for vertebrate kinesins CENP-E and Kid, involved in various aspects of microtubule dynamics related to chromosome positioning and movement (134, 135). An interesting exception is the *Xenopus* egg/early embryo ortholog of Kid (Xkid), which needs to be degraded earlier at anaphase onset by APC/C^{Cdc20} for chromosome segregation to occur (136). Presumably the function of Kid can be regulated in somatic cells so that degradation at anaphase is not required. In yeast, kinesins of the BimC family, Cin8p and Kip1p, required for proper assembly of the mitotic spindle, are degraded by APC/C^{Cdh1} at mitotic exit (137, 138). However, the vertebrate ortholog Eg5 (kinesin 5) appears to be regulated functionally by reversible phosphorylation rather than by degradation. A large group of proteins involved in vertebrate spindle assembly has been designated SAFs. Not surprisingly, the APC/C degrades some SAFs and other proteins that regulate them (139). Bard1 and Hmmr are involved in spindle pole formation and function. Bard1, which forms a heterodimer with Brca1 (Brca1/Bard1), is necessary for recruiting the SAF TPX2 to spindle poles. TPX2 nucleates polar microtubules during spindle formation. Bard1, Hmmr, and TPX2 are all degraded by the APC/C beginning at anaphase onset when polar assembly functions are no longer needed and might be deleterious (139). Interestingly, Brca1/Bard1 has ubiquitin ligase activity that is essential for its spindle assembly functions (140). Although the relevant target is not known, Brca1/Bard1 ubiquitylates γ-tubulin, regulating its microtubule-nucleating functions (141, 142). However, we do not know whether γ-tubulin is the relevant target mediating TPX2 localization to the spindle pole. The aurora kinases A and B have multiple functions in spindle assembly, maintenance, and attachment to chromosomes. Like the proteins described above, the APC/C targets them upon mitotic exit (85, 89–91). Aurora B also has functions late in mitosis requiring redistribution from the centromeric regions of chromosomes to the spindle midzone. This dynamic behavior requires ubiquitylation by a Cul3-based CRL in conjunction with the substrate adapters KLHL9 and KLH13 (143). Ubiquitylation of aurora B by Cul3-associated ligase activity apparently promotes removal from chromosomes instead of significant proteosomal degradation. The extractase Cdc48/p97 may recognize ubiquitylated aurora B and mediate this process (143).

The Ran-importin β system regulates many SAFs (144). Specifically, they are sequestered in an inactive state by binding to nuclear transport receptors of the importin β family. The GTPase Ran mediates release and concomitant activation in response to mitotic chromatin condensation, thus coordinating spindle assembly with chromosome condensation. HURP and NuSAP are importin β/RAN-GTP–regulated SAFs that function near kinetochores, where they nucleate and cross-link microtubules. Unlike Bard1, Hmmr, and TPX2, APC/C^{Cdc20} targets HURP and NuSAP in early mitosis prior to anaphase (139). This is a phase when the APC/C is inactive for most but not all substrates (see below) and implies a

specialized mode of recognition by the APC/C. Although targeted, sufficient steady-state levels of HURP and NuSAP are maintained to carry out spindle assembly functions. The explanation appears to be that the pool of importin β–bound HURP and NuSAP is shielded from degradation because the importin β–interacting motifs and the APC/C degrons on both proteins physically overlap. Once released by the action of Ran-GTP, molecules are active in spindle assembly functions but also targeted for degradation. That protein activation and degradation can be coupled processes (e.g., cyclin E/Cdk2) is not unusual (127, 128). In this case, the coupling likely insures that steady levels of the SAFs are tightly regulated during spindle assembly. Indeed, evidence shows that either excessive or inadequate degradation of HURP or NuSAP during this period leads to aberrant spindle assembly (139).

Roles of Ubiquitylation in Chromosome Structure and Condensation

The processes of chromatin condensation and decondensation are critical for entry and exit from mitosis, respectively. Chromatin condensation during mitosis is mediated in part by phosphorylation of nucleosomal histone H3 on serine 10 by aurora B and, to a lesser extent, aurora A kinase. Phosphorylation of histone H3 then recruits condensin I, a five-protein complex that mediates chromatin compaction. The monoubiquitylation of histone H2A that occurs during interphase and is mediated by the RING1B ubiquitin ligase (145–147) impairs association of aurora B with chromatin and phosphorylation of histone H3 serine 10, thereby preventing efficient chromatin condensation that would occur at the onset of mitosis (148). However, a specific histone-deubiquitylating enzyme, Ubp-M (USP16), is recruited to nucleosomes at the G2-M boundary to remove ubiquitin from histone H2A and allow phosphorylation of histone H3 serine 10 and subsequent chromatin condensation (148). Upon mitotic exit, this process must be reversed. The APC/C-dependent proteolysis of aurora A and aurora B described above and concomitant loss of histone H3 serine 10 phosphorylation promote chromatin decondensation and restoration of the interphase state.

Another aspect of chromosome structure and function is the maintenance of a centromere, which serves as the attachment site for the mitotic spindle. All eukaryotes utilize a specialized histone H3 variant to form nucleosomes at centromeres (CENP-A in metazoans and Cse4 in yeast). Because centromeric function must be limited to the centromere, preventing ectopic association of CENP-A/Cse4 with noncentromeric chromosomal sites is critical. All eukaryotes appear to have mechanisms for eliminating ectopic centromeric histone H3 variants. In yeast, a ubiquitin ligase Psh1 specifically targets ectopic Cse4 for proteosomal degradation (149, 150). A Cse4-specific chaperone, Scm3, binds specifically to centromeric Cse4p, protecting it from Psh1-mediated ubiquitylation, thus maintaining centromeric function. In *Drosophila*, the CENP-A ortholog CID is regulated in a similar fashion but is targeted by an SCF ubiquitin ligase containing the F-box protein Ppa (partner of paired) as the substrate adapter (151). The ubiquitin ligase carrying out this function in vertebrates has not yet been identified. However, an interesting parallel may exist in the targeting of CENP-H and CENP-I by the STUbL [small ubiquitin-like modifier (SUMO)-targeted ubiquitin ligase] Rnf4. CENP-H and CENP-I are inner kinetochore proteins that, in part, function to promote loading of newly synthesized CENP-A onto centromeres. Normally the ubiquitin-like protein SUMO modifies CENP-H and CENP-I as part of their normal function (152). However, excessive SUMOylation leads to ubiquitylation by the STUbL Rnf4 and proteosomal degradation (152). Whereas this pathway does not appear to function in regulating CENP-H and CENP-I in the context of the normal kinetochore, it may be a mechanism to prevent ectopic association of CENP-H and CENP-I with noncentromeric chromatin, an association

that risks the ectopic recruitment of CENP-A. Another aspect of centromere regulation is coordination with the cell cycle. Normally, CENP-A is recruited to centromeric DNA at the end of mitosis or in early G1 phase. In *Drosophila*, RCA1 (an ortholog of Emi1) and cyclin A are required during G2 phase through the metaphase-anaphase transition for recruitment of CID and proper assembly of centromeres during anaphase (153). The critical function of both these proteins in this context is to inhibit APC/C^{Cdh1}. The simplest interpretation of these findings is that premature degradation of an APC/C^{Cdh1} substrate (or substrates), the identity of which is uncertain, interferes with centromere specification and propagation during anaphase.

Role of Ubiquitylation in Regulating Cytokinesis and Mitotic Exit

As stated above, APC/C-dependent proteolysis clears from the cell many proteins that carry out mitosis-specific functions as cells complete mitosis and enter interphase. Analysis of the specific role(s) of protein degradation is complicated in that many mitotic proteins have multiple and diverse functions as cells progress through mitosis. In addition, functional redundancy may obscure the importance of degradation for any particular protein. However, activity of the APC/C is clearly required for cytokinesis beyond its role in the degradation of cyclin B. Sea urchin embryos can be manipulated so that a cleavage furrow forms in the presence of a nondegradable cyclin B. However, direct inhibition of the APC/C blocks cleavage-furrow formation, indicating that another or several APC/C targets must be degraded to permit cytokinesis (154). Plk1, a possible candidate, is a protein kinase with multiple roles in mitosis. However, Plk1 degradation beginning at anaphase is essential for cytokinesis. Understanding the basis for this is complicated because Plk1 has both positive and negative functions in cytokinesis. One possible explanation for the requirement for Plk1 proteolysis is the role of Plk1 as a negative regulator of the antiparallel microtubule–bundling protein PRC1 (155). PRC1 activity is key to forming the spindle midzone complex, which organizes the cleavage furrow at the end of anaphase. Therefore, the requirement for degradation of Plk1 may be explained by the need to activate PRC1. Interestingly, both PRC1 and Ase1, its yeast ortholog, are APC/C substrates targeted for degradation at the end of mitosis when the spindle must be disassembled (156–158).

Aurora B is another protein kinase with multiple roles in mitosis, including cytokinesis, that is targeted by the APC/C (89, 90). Although degradation of aurora B does not appear to be necessary for cytokinesis, ubiquitylation is involved in late mitotic functions of aurora B. As stated above, ubiquitylation of aurora B by Cul3 in conjunction with substrate adapters KLH9 and KLH13 is required for removing aurora B from centromeric regions of chromosomes. However, ubiquitylation by Cul3 with a different substrate adapter, KLH21, appears to be involved in recruiting aurora B to the spindle midzone, where its cytokinesis functions occur prior to degradation by the APC/C (159).

Regulation of the Centrosome Cycle by Ubiquitylation

One centrosome organizes each pole of a bipolar spindle. To maintain genomic integrity, centrosome duplication must occur only once per cell cycle. As a consequence, proteins that promote centrosome duplication must be restricted, often by ubiquitin-mediated proteolysis. Plk4 promotes centrosome duplication, and its overexpression leads to excessive centrosome numbers (160–162). $SCF^{\beta\text{-}Trcp}$ targets Plk4 for proteolysis (163, 164), controlling its levels. Plk4 in turn negatively regulates another SCF ligase containing the substrate specificity factor Fbw5 (165, 166). SCF^{Fbw5} promotes ubiquitylation and degradation of the procentriolar organizing protein HsSAS-6 (165, 166). As with overexpression of Plk4, overexpression of HsSAS-6 promotes overproduction of centrosomes. Therefore, a regulated cascade containing a protein kinase and two

SCF ubiquitin ligases controls centrosome homeostasis. Two other SCF ligases (containing F-box proteins Fbw7 and cyclin F) have also been implicated in regulation of the centrosome cycle (167, 168). SCF^{Fbw7}-mediated targeting of cyclin E is necessary for restricting centrosome duplication. Cyclin F, which behaves like other cyclins in terms of cell cycle periodicity, peaking in G2 phase, is required for targeting the centrosomal protein CP110 for ubiquitin-mediated proteolysis. Failure to degrade CP110 in G2 phase leads to spindle abnormalities and mitotic errors. Finally, ubiquitylation of γ-tubulin by Brca1/Bard1 also prevents centrosome amplification (142).

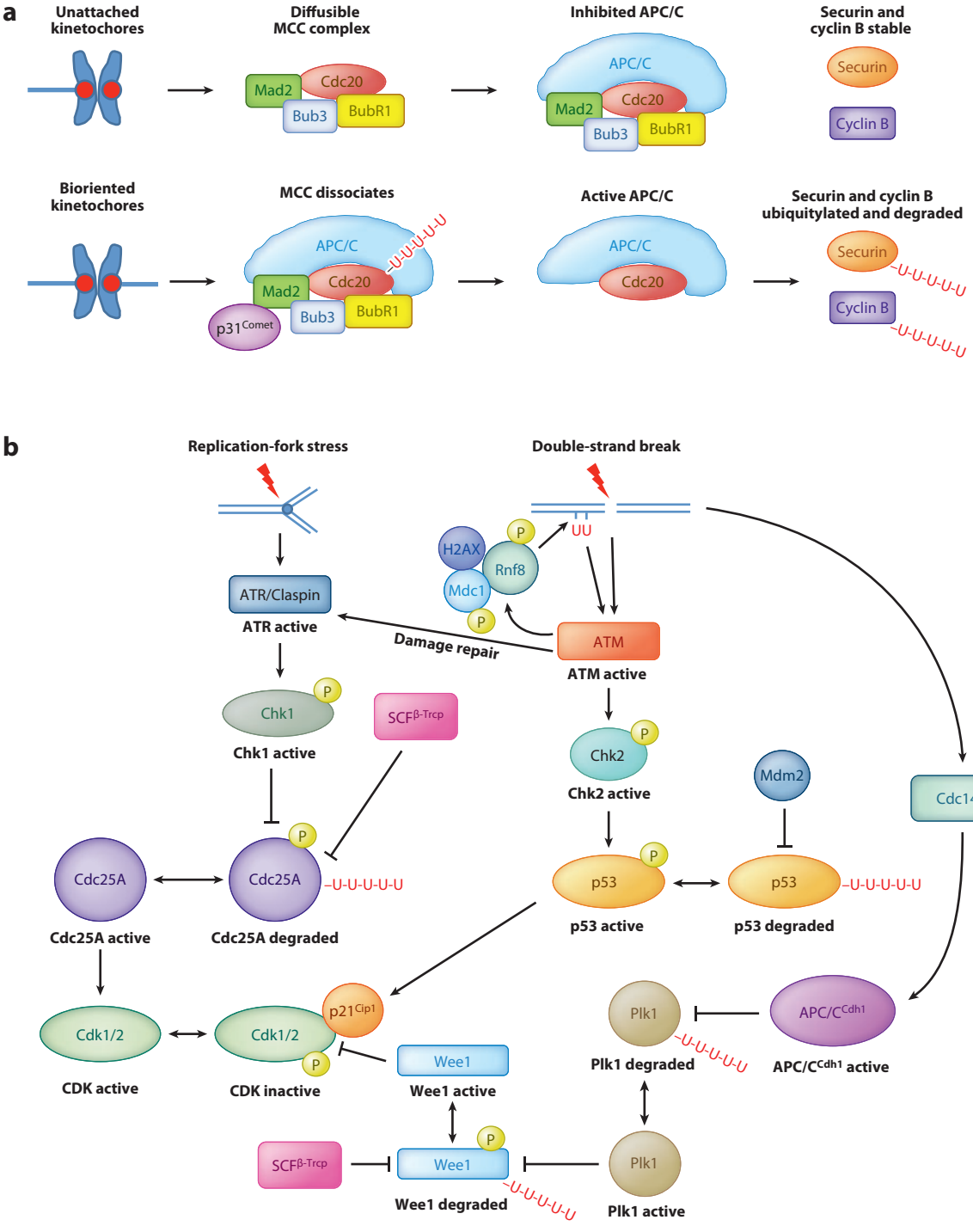
THE ROLE OF UBIQUITYLATION IN REGULATION OF CELL CYCLE CHECKPOINTS

Cell Cycle Checkpoints

Cell cycle checkpoints provide two critical functions for all eukaryotic cells. First, they halt or delay cell cycle progression when a cell sustains any of various types of damage, thus preventing irreparable harm, especially loss or mutation of genetic material. Second, they enforce an appropriate order of cell cycle events, again to ensure the integrity of the genome. An example of this second type of checkpoint is the SAC, which blocks anaphase until all pairs of chromosomes are bioriented on the mitotic spindle. Most, if not all, checkpoints utilize ubiquitylation to carry out signaling functions.

The Spindle Assembly Checkpoint

The SAC blocks anaphase until the kinetochores corresponding to every chromosome are properly attached to a bipolar mitotic spindle (**Figure 3*a***). Release from this checkpoint requires both attachment and tension owing to biorientation (kinetochores from each pair of sister chromatids associated with microtubules from opposite spindle poles). Microtubule poisons, which prevent spindle formation and function, also trigger this checkpoint. The SAC functions by generating a diffusible inhibitor of the APC/C at kinetochores that do not satisfy the conditions described above (169, 170). One unattached kinetochore is sufficient to completely inhibit the APC/C with respect to its two critical anaphase substrates, securin and cyclin B1. But proper attachment of the last kinetochore is sufficient to rapidly reverse SAC-mediated APC/C inhibition. The inhibitor generated by unattached kinetochores, known as the mitotic checkpoint complex (MCC), contains three checkpoint-specific proteins (Mad2, BubR1/Mad3, and Bub3) as well as the APC/C substrate adaptor/activator Cdc20 (170). Both Mad2 and BubR1 can inhibit the APC/C individually in vitro (171, 172), but the combination is much more effective both in vitro and in vivo (173). Mad2 and BubR1 are loaded onto Cdc20 at kinetochores. The ability of an unattached kinetochore to generate sufficient MCC to completely inhibit the APC/C depends on the existence of Mad2 in two distinct conformational states and an autocatalytic mode of Mad2 conversion to the active state. Kinetochore-bound Mad1 binds and converts Mad2 to the closed state, which can then bind additional Mad2 molecules in the open state, converting them to the closed state (58, 174–177). Only the closed form of Mad2 is incorporated into the MCC, which then diffuses and binds to APC/C complexes (169, 178). BubR1 contains a canonical KEN-box and inhibits the APC/C in part by serving as a pseudosubstrate (179, 180). In addition, structural studies indicate that Cdc20 bound to Mad2 and BubR1 interacts with the APC/C differently than does non-checkpoint-inhibited Cdc20 (178, 181). Specifically, Cdc20 is displaced from its normal position so that it cannot form a bipartite D-box receptor with the APC/C subunit Apc10, thus blocking recruitment of D-box-containing substrates. Although Cdc20 itself is an anaphase target of APC/C^{Cdh1}, Cdc20 is autoubiquitylated during the SAC in the absence of Cdh1 (182–184), a process that may be facilitated by the modified position of Cdc20 when presented to the APC/C in the context of the MCC (178). APC/C-mediated



degradation of active Cdc20 helps maintain the SAC (185). However, ubiquitylation of Cdc20 is also important for inactivation of the SAC in that ubiquitylation causes dissociation of Mad2 and BubR1 from inhibited Cdc20. Presumably, when SAC requirements are met and the production of the MCC ceases, ubiquitylated Cdc20 is either deubiquitylated to form active APC/C or degraded by the proteasome to be replaced by active Cdc20. However, the deubiquitylating enzyme USP44 prevents excessive ubiquitylation of Cdc20 inhibitor complexes prior to meeting SAC requirements (186). Another protein, $p31^{Comet}$, that cooperates with Cdc20 ubiquitylation by binding and displacing Cdc20-bound Mad2, thereby neutralizing its inhibitory effect, contributes to reactivation of the APC/C once the checkpoint signal ceases (184, 187). Presumably this occurs because $p31^{Comet}$ binds to the same surface of Mad2 as does BubR1 (178, 188).

As stated above, the critical targets of APC/C^{Cdc20} cannot be ubiquitylated while the SAC is active. However, at least two other APC/C^{Cdc20} targets, cyclin A and NEK2A, are actively ubiquitylated by SAC-inhibited APC/C (189, 190). Cyclin A has functions in S phase and the G2-M transition, and NEK2A is a protein kinase with early mitotic functions at the kinetochore and the centriole. The key to the ability of these substrates to evade the SAC appears to be that they interact directly with subunits of the core APC/C (NEK2A interacts directly, and cyclin A interacts via the small phosphate-binding adapters Cks1 and Cks2 that associate with its CDK partner) and therefore do not depend on Cdc20 as an adaptor; they also have an extremely high affinity for Cdc20 (191, 192). The role of Cdc20 bound to these substrates is simply as an APC/C activator requiring interactions with the APC/C that are not blocked during SAC. These observations are consistent with structural studies showing that Cdc20 interacts differently with the APC/C during SAC. We do not yet understand why these particular substrates are designed to be degraded at prometaphase. Expression of a nontargeted allele of cyclin A prevents anaphase but shows no obvious earlier phenotypes (193), and expression of a comparable allele of NEK2A shows no overt phenotype, although subtle alterations in kinetochore function might be difficult to detect in real time.

Figure 3

The role of ubiquitylation in major cell cycle checkpoints. (*a*) The spindle assembly checkpoint. Kinetochores unattached to a mitotic spindle produce a diffusible inhibitor of the APC/C. This complex is termed the mitotic checkpoint complex (MCC) and consists of the APC/C adaptor and activator Cdc20, Mad2, BubR1, and Bub3. Under these conditions, the critical anaphase targets, securin and cyclin B, are stable. When chromatid pairs achieve biorientation on the mitotic spindle, causing kinetochores to experience tension, MCC is no longer produced, and MCC bound to the APC/C is disassembled as a result of Cdc20 autoubiquitylation and the cooperative action of $p31^{Comet}$. With APC/C available to uninhibited Cdc20, securin and cyclin B are targeted and degraded. Anaphase then ensues. (*b*) DNA damage checkpoints. Two of the more prevalent DNA damage checkpoints are illustrated. In the case of replication-fork stress, ATR (ataxia telangiectasia mutated related) is activated in response to exposure of single-strand DNA. ATR, with its cofactor claspin, activates the kinase Chk1 by phosphorylation. Chk1 in turn phosphorylates the cyclin-dependent kinase (CDK)-activating phosphatase Cdc25A, priming it for further phosphorylation and creating a phosphodegron for $SCF^{\beta\text{-}Trcp}$, leading to Cdc25A ubiquitylation and degradation. In the absence of Cdc25A, Cdk1 and Cdk2 are inhibited by Tyr15 phosphorylation owing to the prevailing activity of Wee1. In response to double-strand breaks, ATM (ataxia telangiectasia mutated) is recruited and activated. This depends on basal ubiquitylation of histone H2A by the ligase Rnf8, which renders chromatin accessible to ATM. ATM locally phosphorylates the histone variant H2AX, leading to the recruitment of the mediator Mdc1, which is also phosphorylated by ATM and, as a result, recruits more Rnf8 and ATM, creating amplification through a positive feedback loop. As a result, DNA repair initiates and the kinase Chk2 is activated by phosphorylation. In the process of strand-break repair, end resection exposes single-strand DNA, indirectly activating the ATR pathway. Phosphorylation of p53 by activated Chk2 confers resistance to Mdm2-mediated ubiquitylation and degradation, leading to expression of the CDK inhibitor $p21^{Cip1}$, which inhibits Cdk1 and Cdk2. DNA damage also promotes the delocalization of the phosphatase Cdc14B from the nucleolus in an ATM-independent manner, dephosphorylating Cdh1 and thereby activating APC/C^{Cdh1}. As a result, the kinase Plk1 is ubiquitylated and degraded, thus stabilizing the $Plk1/SCF^{\beta\text{-}Trcp}$ targets Wee1 and claspin (the latter not shown in the figure) and further inhibiting Cdk1 and Cdk2.

DNA Damage Checkpoints

Both replication stress and various forms of DNA damage trigger cell cycle checkpoints that block cell cycle progression to avoid further damage or to prevent mutation or loss of genetic material (**Figure 3*b***). In the best case scenario for a cell, checkpoint restraint on cell cycle progression allows time for repair of damage, after which the cell is released from the checkpoint. Ubiquitylation plays important roles both in DNA damage signal amplification and in regulating the checkpoint machinery. At double-strand breaks, efficient recruitment of the upstream signaling kinase ATM (ataxia telangiectasia mutated) depends on the ubiquitin ligase RNF8 (194). RNF8 mono- or diubiquitylates histone H2A at a basal level, leading to chromatin remodeling necessary for ATM association of chromatin near the break. ATM then phosphorylates a histone variant known as H2AX, leading to binding of an adapter, Mdc1, which ATM also phosphorylates. Phosphorylated Mdc1 binds both additional ATM and RNF8 (195–198), creating a positive feedback loop necessary to produce a strong damage signal received by both the downstream DNA repair machinery and DNA damage checkpoint machinery. A parallel process takes place at sites of photodamage requiring nucleotide excision repair (NER). The DDB1/DDB2 adapter heterodimer, also known as UV-DDB, recognizes UV-induced DNA lesions and recruits the Cul4A CRL to locally monoubiquitylate histone H2A, a process that leads to chromatin remodeling essential for providing access to the NER machinery (199, 200).

Checkpoints associated with both replicative stress and DNA damage utilize ubiquitin-mediated proteolysis for signal transduction and modulation. Replicative stress caused by impairment of fork movement leads to exposure of single-strand DNA, which is recognized by the ATR/ATRIP (ataxia telangiectasia mutated related/ATR-interacting protein) complex. This triggers the intra–S phase checkpoint, which blocks new replication origin firing to minimize DNA damage caused by collapsed replication forks. Activation of the ATR kinase and association of a cofactor claspin lead to phosphorylation and activation of the checkpoint kinase Chk1. The principal target of Chk1 is the CDK-activating phosphatase Cdc25A, which must be inactivated to block origin firing. Phosphorylation of Cdc25A by Chk1 primes it for phosphorylation by additional kinases, creating a phosphodegron for $SCF^{\beta\text{-}Trcp}$ and leading to its ubiquitin-mediated degradation (201–203). Loss of Cdc25A shifts the pool of Cdk2 to the inactive tyrosine 15–phosphorylated state, thus preventing further origin firing. Other forms of DNA damage that require DNA resection for repair, such as double-strand breaks, indirectly trigger the ATR-Chk1-Cdc25A axis owing to the exposure of single-strand DNA during the repair process (204). However, the primary sensing kinase activated by double-strand breaks is ATM, which signals downstream directly to the alternative checkpoint kinase Chk2 (205). Although Chk2 activation promotes G2 arrest by inhibitory phosphorylation of CDK, activating phosphatases Cdc25B and C, it also promotes both G1 and G2 arrest by phosphorylation of p53 (206), protecting p53 from ubiquitylation by the ubiquitin ligase Mdm2 and subsequent degradation (207, 208). Stabilization and accumulation of p53 then lead to expression of the CKI $p21^{Cip1}$, causing cell cycle arrest. One final important mechanism of G2 arrest in response to DNA damage is the ubiquitin-mediated degradation of the promitotic kinase Plk1. Normally, Plk1 is active at the G2-M transition, during which it phosphorylates mitotic inhibitors Wee1 and Emi1, promoting their ubiquitylation by $SCF^{\beta\text{-}Trcp}$ and subsequent proteasomal degradation (70–72, 132). Plk1 also targets claspin, the cofactor for Chk1 activation by the same pathway, promoting maximal CDK activation during the unrestrained cell cycle (209, 210). However, upon DNA damage, the phosphatase Cdc14B is released from the nucleolus and dephosphorylates the APC/C cofactor Cdh1, which is normally held inactive during G2 phase by CDK-mediated

phosphorylation. The activation of APC/C^{Cdh1} then promotes the ubiquitin-mediated degradation of Plk1, allowing the accumulation of the CKI Wee1 and the activation of Chk1 via stabilization of claspin (211). The reversal of these events, i.e., the stabilization of Plk1 and the degradation of Wee1 and claspin, is critical for checkpoint recovery (209, 210, 212).

SUMMARY POINTS

1. Ubiquitin-mediated proteolysis plays a central role as a component of the core cell cycle oscillator that drives cell division as well as in numerous signaling pathways that regulate cell cycle phases.
2. The principal ubiquitin ligase families involved in cell cycle functions are the SCF and APC/C CRLs.
3. Ubiquitin-mediated proteolysis of several key regulatory proteins renders cell cycle phase transitions unidirectional and irreversible, whereas cell cycle degradation of many other proteins that have phase-specific functions simply clears them from the cell when their functions are no longer required or might be deleterious.
4. Whereas the simple cell cycles of lower vertebrates and marine invertebrates rely primarily on an oscillator composed of cyclin B–Cdk1 and the APC/C ubiquitin ligase, the increased regulatory requirements of somatic cells expand the roles for ubiquitin ligases, particularly those of the SCF family.
5. Ubiquitin-mediated proteolysis plays a role in many peripheral cell cycle–linked processes beyond regulating major cell cycle phase transitions, such as maintenance and assembly of the mitotic spindle, chromosome structure, and condensation; cytokinesis; and centrosome duplication.
6. Ubiquitylation in the context of cell cycle control is not always linked to protein degradation but may specify protein relocalization, chromatin remodeling, and regulation of function.

FUTURE ISSUES

1. Although the activation of cyclin B–Cdk1 in the cell cycle has properties consistent with bistability, we know less about the nature of S-phase CDK activation at the G1-S boundary. This is in part owing to the inherent difficulties in establishing cell-free systems from somatic cells as compared with oocytes.
2. Although much is understood concerning the roles and regulation of ubiquitin ligases in the cell cycle, much less information is available about the contribution of deubiquitylating enzymes. This is clearly an area for future research.
3. For some mitotic substrates of the APC/C, e.g., cyclin A and Nek2A, ubiquitylation and degradation begin at prophase prior to satisfying the SAC. But whether there are deleterious consequences of persistence of these proteins is unknown, at least until anaphase. Because these proteins have evolved novel ways of interacting with the APC/C to evade the SAC, a functional reason for this likely exists.

4. Although the APC/C targets many substrates during mitosis and mitotic exit, there is significant variation to the timing and kinetics of degradation. We understand this for a few substrates, such as cyclin A and Nek2A, but for the most part we do not know why some substrates are targeted earlier than others, even though they interact with the same substrate adapters.
5. Regulation of histone ubiquitylation appears to be important both for DNA condensation during mitosis and for chromatin remodeling in response to DNA damage. Further investigation is clearly required to understand how histone ubiquitylation is regulated both globally and locally in response to genotoxic damage.

DISCLOSURE STATEMENT

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