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Dynamic regulation of ubiquitin-dependent cell cycle control

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Recent work revealed that cullin-RING ligases and the anaphase-promoting complex, two classes of ubiquitin ligases that are essential for cell division in all eukaryotes, are regulated in a highly dynamic manner. Here, we describe mechanisms that establish the dynamic regulation of these crucial ubiquitylation enzymes and discuss the functional consequences for cell division control.

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Introduction

Mitotic cells undergo dramatic changes in their composition and shape: organelles are dismantled upon entering the division stage and re-assembled upon completion of mitosis; chromosomes are packaged into units that facilitate their distribution into the emerging daughter cells; and interphase microtubules are taken apart and replaced by the mitotic spindle, a dynamic apparatus that exhibits continuous microtubule assembly at one end, and disassembly at the other. As exemplified by the chemotherapeutic paclitaxel, a molecule that disrupts microtubule turnover and triggers cell cycle arrest and death, interfering with the dynamics of mitotic processes can have disastrous consequences.

To ensure that the fundamental changes occurring during mitosis result in two identical daughter cells, cell division needs to be tightly regulated, and posttranslational modification of cell cycle regulators with ubiquitin plays an essential role in fulfilling this task. At all times during cell division, enzymes referred to as E3 ubiquitin ligases recruit specific substrates to promote attachment of single ubiquitin molecules or polymeric ubiquitin chains, reactions that can be completed within seconds [1–3]. Depending on the topology of this modification, these events alter the target's stability, localization, or activity [3]. Ubiquitin-dependent signaling can later be terminated by deubiquitylating enzymes, which rapidly disassemble ubiquitin marks [4]. Its rapid kinetics and reversibility predestine ubiquitylation as a modification that is well suited to control dynamic processes, such as cell division.

The best understood E3s with roles in cell division control are the cullin-RING-ligases (CRLs) and their relative, the anaphase-promoting complex (APC/C). These E3s are built around a cullin or cullin-like scaffold, which engages distinct substrate receptors and a catalytic RING-domain subunit [5–9]. CRLs and APC/C ubiquitylate key cell cycle regulators, such as modulators of cyclin-dependent kinase activity (e.g. the cyclins A, B, and E; Cdk-inhibitory kinase Wee1; Cdc25 phosphatase), components of cell cycle checkpoints (e.g. Cdk-inhibitors p21 and p27; XPC; histones), mitotic kinases (e.g. Plk1, Aurora A, Aurora B) or members of signaling pathways that coordinate cell division with conditions in the environment (e.g. the transcription factors c-myc or c-jun; components of the mTORC kinase complex) [10–16].

Befitting their central role in cell cycle control, ubiquitylation reactions catalyzed by CRLs and the APC/C have to occur at the right time and place, which relies on an intricate interplay between these E3s and their substrates. Many CRL-substrates require prior modifications, such as phosphorylation, glycosylation or hydroxylation, for their delivery to the E3 [5], whereas binding partners promote or inhibit substrate recognition by the APC/C [17–19]. In addition, the E3s themselves are subject to tight control, and suited for their function in orchestrating cell division, this occurs in a highly dynamic fashion: CRLs can rapidly exchange substrate receptors in response to altered biological need, and the mitotic APC/C cycles between an inhibited and partially active state, thus priming cells for rapid initiation of sister chromatid separation. In this perspective, we will focus on the mechanisms that establish dynamic CRL-regulation or APC/C-regulation and discuss the functional consequences for cell division.

Dynamic control of substrate-targeting to cullin-RING ligases

CRLs recruit substrates through a large number of dedicated receptors: for example, human CRL1 relies on 69 human F-box proteins, CRL3 depends on ~100 BTB proteins, and CRL4 exploits ~60 WD40-containing DCAFs [20••,21]. As observed for recombinant CRL1, substrate receptors can bind the cullin scaffold very tightly, with in an *in vitro* half-life of the complex of

about one week [22••], and most cullins are saturated by substrate receptors in cells [20••]. To control dynamic processes such as cell division, CRLs must therefore be capable of rapidly exchanging substrate receptors depending on biological need, and recent studies point to the Cand1 protein as a mediator of this activity [22••,23•,24•].

Genetic evidence had indicated that Cand1 is required for CRL-function [25–27], yet when analyzed biochemically, it was found to wrap around the cullin and block the recruitment of substrate receptors [28,29]. Thus, while one approach pointed to Cand1 as a CRL-activator, another suggested that it inhibits the same enzymes. This conundrum was resolved by the discovery that Cand1 destabilizes CRL-receptor complexes by a million-fold [22••], thereby allowing the CRL backbone to team up with a distinct receptor to target another group of substrates (Figure 1a). Indeed, in the absence of Cand1, CRLs fail to exchange receptors, and they are impaired in their capacity to ubiquitylate substrates that need to be turned over in response to altered cellular conditions [22••,23•,24•].

The ability of Cand1 to exchange substrate receptors needs to be coordinated with ongoing ubiquitylation reactions and the abundance of remaining substrates for a given CRL. This function is in part fulfilled by the reversible modification of cullins with the ubiquitin-like protein Nedd8. Similar to ubiquitylation, neddylation requires an E3 that is comprised of the CRL's RING-subunit and an accessory factor, DCN1 [30••]. Nedd8 can be rapidly removed from cullins by the JAMM-domain containing CSN5, a component of the eight-subunit COP9-signalosome (CSN) [31••,32,33]. Neddylation induces conformational changes in the cullin that increase the catalytic activity of the CRL, but it also impedes turnover of CRL-receptor complexes by Cand1 [34,35]. As the CSN does not efficiently recognize CRLs that are bound to substrates and engaged in catalyzing their ubiquitylation [36,37••], CRLs are protected from deneddylation and Cand1 is blocked from exchanging receptors until the reaction cycle has been completed (Figure 1b).

Even if a substrate has been ubiquitylated and degraded, it could be problematic to immediately dismantle the CRL-receptor complex, especially if more substrates of this CRL await their ubiquitylation. In such cases, the CSN can regulate CRLs by means that require its binding to the E3, yet do not depend on its activity to remove Nedd8. Indeed, ~30% of all CRLs are engaged with CSN in human cells [20••]. When bound to CRLs, the CSN blocks both the substrate-binding site on the receptor as well as the catalytic RING-domain, thereby inhibiting the activity of CRLs and impairing the exchange of substrate receptors by Cand1 (Figure 1c) [36,37••,38••]. If substrates of this CRL are still abundant, they can displace the CSN, thereby activating the CRL to promote their own ubiquitylation (Figure 1d) [38••]. The CSN, therefore, helps to preserve a reservoir of primed CRL-complexes, allowing these enzymes to complete their task in turning over important substrates.

Collectively, the recent findings revealed that CRLs are dynamic assemblies that can rapidly adjust their activity to the availability of substrates (summarized in Figure 1): when bound to a given receptor, neddylated CRLs ubiquitylate specific substrates, a reaction that can promote the degradation of cell cycle regulators or change the activity of signaling pathways that are critical for cell division. Upon processing of the modified protein, the CSN inhibits the CRLs by removing Nedd8 or blocking catalytic domains. Remaining substrates of this CRL can release the CSN, thereby activating the CRL to trigger their own ubiquitylation. Conversely, if substrates of other CRLs need to be turned over, Cand1 can destabilize existing CRL-receptor complexes. This activity of Cand1 allows new CRL-receptor pairs to assemble that can then enter another ubiquitylation cycle.

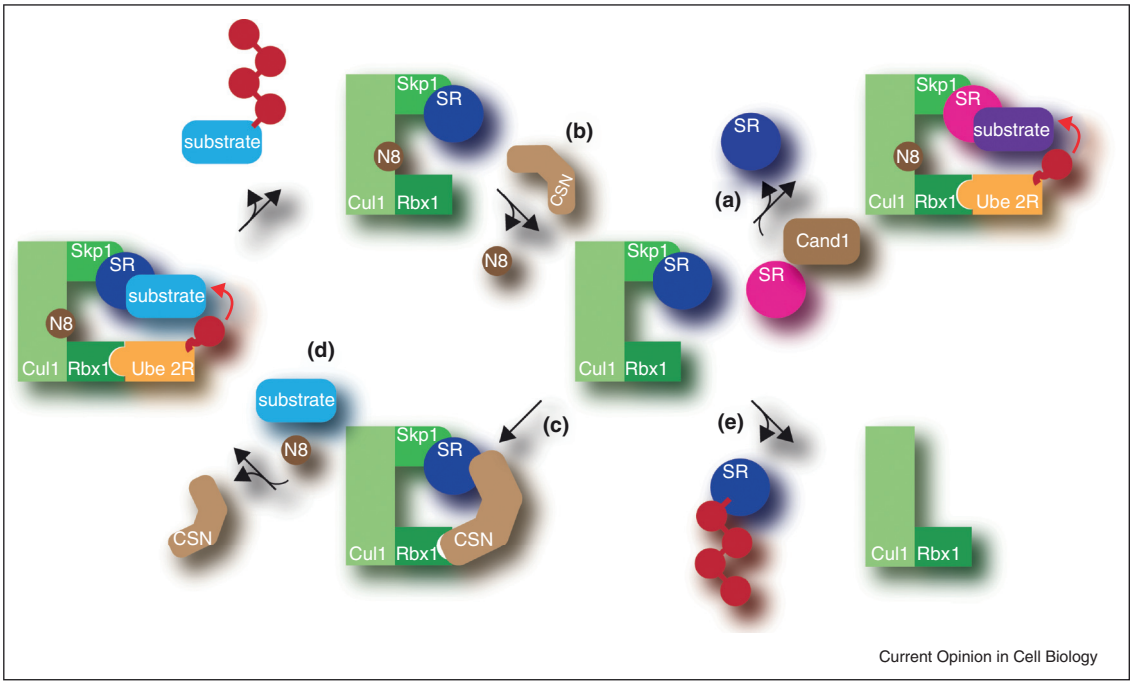
Dynamic control of APC/C-activity by the spindle assembly checkpoint

A variation on the theme of dynamic E3-control is seen at the metaphase-anaphase transition, a key event in the cell cycle that depends on the APC/C. Because the APC/C promotes sister chromatid separation, its full activation needs to be delayed until all chromosomes have achieved bipolar attachment to the spindle, or otherwise, daughter cells would not be guaranteed to obtain an identical chromosome set. The task of inhibiting the APC/C during early mitosis is delegated to an intricate signaling network referred to as the spindle assembly checkpoint [39,40]. Once chromosome alignment has been completed, cells rapidly shut down this mitotic checkpoint, activate the APC/C, and initiate sister chromatid separation.

When the spindle checkpoint is active, a Mitotic Checkpoint Complex (MCC) composed of BubR1, Mad2, Bub3, and Cdc20, binds to the APC/C (Figure 2a) [39,41••,42••]. Within this APC/C^{MCC}, the position of Cdc20 is distinct from its location in APC/C^{Cdc20}, the form of the APC/C that drives sister chromatid separation: Cdc20 is displaced from APC10, which disrupts recognition of D-box degrons at the interface between Cdc20 and APC10 [41••,42••,43]. In addition, BubR1 inserts a KEN-box into the central cavity of Cdc20, thereby blocking the binding of Cdc20 to KEN-box degrons in substrates [41••,44]. Through these means, the spindle checkpoint impairs the ability of the APC/C to recruit substrates, such as cyclin B1 or securin, whose stabilization prevents sister chromatid separation.

Intriguingly, even though APC/C^{MCC} is unable to ubiquitylate its key substrates, it subjects the

Figure 1



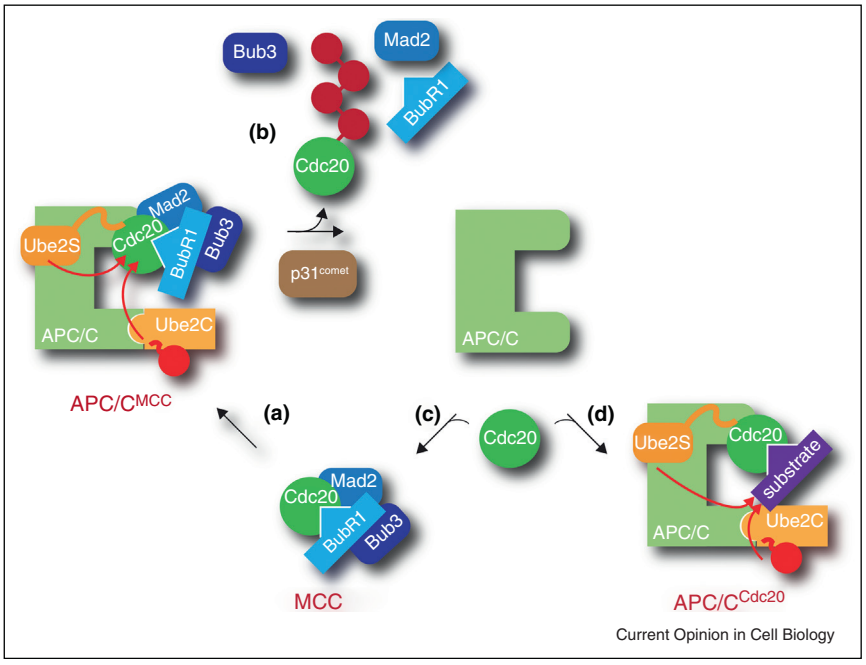
Dynamic assembly of CRL-substrate receptor complexes. As shown with CRL1 as an example, CRLs form dynamic complexes with many substrate receptors (SR). Substrate receptors are recruited to Cul1 by the Skp1 adaptor protein. When modified with Nedd8 (N8), Cul1-SR complexes recruit specific substrates and catalyze their ubiquitylation. After processing of the ubiquitylated substrate, the CRL-SR is deneddylated by the large CSN complex **(b)**, reducing its catalytic activity. Subsequently, the CRL-SR can **(a)** be subjected to Cand1-dependent exchange of substrate receptors, allowing other substrates to be ubiquitylated; **(c)** CSN can remain bound to stabilize an inactive CRL-SR complex; **(d)** substrates can displace CSN to promote their own ubiquitylation; or **(e)** the SR can be targeted for CRL-dependent ubiquitylation and proteasomal degradation. CRLs are therefore dynamic assemblies that adapt their composition to biological need.

Cdc20-molecule within MCC to ubiquitylation and degradation, a reaction that leads to the disassembly of spindle checkpoint complexes (Figure 2b) [45••,46•,47••,48,49••,50••,51••,52,53]. As a result of this reaction, the overexpression of APC/C-E2s leads to spindle checkpoint bypass and division errors in cultured cells or mice [45••,54–56], while inhibiting the APC/C stabilizes the checkpoint [47••,51••,57]. Thus, there is a balance of powers: while the spindle checkpoint inhibits the APC/C, the APC/C can strike back and turn off the checkpoint. The significance of this dynamic relationship was initially challenged, as expression of a Cdc20-variant that lacked all Lys residues did not arrest cells with a permanently active checkpoint [58]. However, mutating all lysines in Cdc20 impaired binding to Mad2 [47••], inappropriately increased the affinity of Cdc20 to APC/C [57], and in yeast, interfered with its capacity to support substrate ubiquitylation [49••], suggesting that this mutant fails to recapitulate the dynamic behavior of the wild-type protein.

How the APC/C can target Cdc20 without ubiquitylating most of its substrates is incompletely understood, but current evidence suggests that this process is taking place continuously during active checkpoint signaling. The ubiquitylation of Cdc20 and disassembly of checkpoint complexes are strongly promoted by $p31^{comet}$, a protein that adopts a Mad2-like conformation and interacts with both spindle checkpoint complexes at kinetochores and on the APC/C (Figure 2b) [45••,46•,48,52,59,60]. Depletion of $p31^{comet}$ prevents the dissociation of checkpoint components from APC/C even in the presence of spindle damage, suggesting that APC/C^{MCC}-complexes are constantly turned over during early mitosis [47••]. Similar observations have been made upon depletion of an APC/C-subunit, APC15, which is critical for Cdc20-autoubiquitylation, but does not play essential roles in targeting mitotic substrates [49••,50••,61]: loss of APC15 protects checkpoint complexes from disassembly, even if chromosome alignment had not been completed. APC15 is located close to the binding site of checkpoint complexes on APC/C, where it might help expose critical Lys residues in Cdc20 or directly affect the catalytic activity of the APC/C [50••]. Thus, rather than stably binding the APC/C and locking it in an inactive state, spindle checkpoint complexes are constantly turned over on this E3.

The APC/C requires the target of the checkpoint-dependent ubiquitylation event, Cdc20, not only for substrate delivery, but also for achieving its full catalytic

Figure 2



Dynamic regulation of the APC/C by the spindle assembly checkpoint. During spindle checkpoint signaling, the mitotic checkpoint complex (MCC) composed of BubR1, Mad2, Bub3, and Cdc20 binds the APC/C to form APC/C^{MCC} **(a)**. APC/C^{MCC} does not interact with most APC/C-substrates, but targets Cdc20 for autoubiquitylation and degradation **(b)**. This reaction is constantly promoted by p31^{comet}. The turnover of Cdc20 disassembles MCC and releases free APC/C. Mitotic cells synthesize new Cdc20 protein that can **(c)** team up with checkpoint effectors to generate new MCC, if the checkpoint is still turned on, or **(d)** associate with APC/C to form active APC/C^{Cdc20}, which can bind and ubiquitylate mitotic substrates, thereby triggering mitotic exit.

activity [62]. Thus, while the ubiquitylation and degradation of Cdc20 helps disassemble checkpoint-complexes, it would ultimately also shut down the APC/C. To counteract this unwanted inactivation, mitotic cells actively translate Cdc20 mRNA to produce new Cdc20 protein [51••,58,63]. If chromosome attachment has not been completed and the checkpoint is still turned on, newly synthesized Cdc20 engages Mad2, BubR1, and Bub3 to form new MCC (Figure 2c), a reaction that is required to maintain stable spindle checkpoint signaling [51••,58]. Conversely, if chromosome alignment has been completed, formation of new MCC-complexes will be blocked by several means [40], and the newly produced Cdc20 can rapidly generate active APC/C^{Cdc20} to drive sister chromatid separation (Figure 2d). Regulation of the APC/C by the spindle checkpoint is, therefore, a dynamic process that requires continuous production and disassembly of APC/C^{MCC}.

Functional consequences of dynamic E3 regulation for cell cycle control

Why are the E3s that are most critical for cell cycle control regulated in such a dynamic manner? As seen in other systems [64], the dynamic regulation of an enzymatic activity allows cells to rapidly respond to altered conditions in their environment, a prerequisite for successful cell division: cells need to integrate the fluctuating availability of nutrients into their division program, arrest the cell cycle in response to damage, or adapt to the dramatic structural changes during mitosis. Indeed, dynamic regulation of CRLs and the APC/C allows cells to quickly respond to altered cell cycle states: re-sculpting of CRL-adaptor complexes by Cand1 is essential when yeast cells restructure transcriptional programs in response to changes in their nutrient source [24•], or when plant cells are exposed to auxin, a hormone that controls cell division and morphology [26]. Similarly, the APC/C has to be dramatically activated within minutes of completing chromosome attachment to the spindle, and the continuous disassembly of checkpoint-complexes likely helps to drive the rapid exchange from APC/C^{MCC} to fully active APC/C^{Cdc20} [65].

Although speculative, the dynamic regulation of mitotic APC/C might also affect the timing of substrate degradation. Whereas most APC/C-substrates are stabilized by the spindle checkpoint, some proteins, such as cyclin A or Nek2A, are degraded despite incomplete chromosome attachment and ongoing spindle checkpoint signaling. These checkpoint-independent APC/C-substrates not only require classical degrons, but also additional motifs or binding partners that provide further interaction

surfaces with the APC/C [19,66]. It is possible that their increased affinity to the APC/C allows these substrates to kinetically compete with checkpoint effectors in binding newly synthesized Cdc20, thereby establishing their own degradation. It is interesting to note that an interphase inhibitor of the APC/C, Emi1, blocks APC/C's ability to bind substrates as well as its activity to assemble ubiquitin chains, and hence, does not allow APC/C-dependent turnover of inhibitor complexes [67•,68•]. These findings suggest that dynamic regulation of the APC/C is particularly important during mitosis, the time when certain substrates are turned over despite an urgent cellular need to keep the APC/C in check.

Dynamic control of an enzymatic machinery provides unique opportunities for small molecule interference, and this turned out to be no different for the CRLs and APC/C. Consistent with the CRL-cycle being regulated by reversible modification of cullins with Nedd8, acute inhibition of the Nedd8-E1 by the small molecule MLN4924 leads to the rapid loss of all modified cullins and brings the CRL-cycle and cell division to a halt [20••,69]. Similarly, dynamic regulation of the APC/C has been successfully targeted by the small molecule TAME, which mimics a C-terminal motif in Cdc20 that mediates its interactions with checkpoint-inhibited APC/C [51••,57]. TAME interferes with the ubiquitylation of Cdc20 by APC/C^{MCC}, thereby stabilizing spindle checkpoint complexes and triggering a persistent mitotic arrest. Prolonged interference with APC/C-activity ultimately leads to cohesion fatigue, a process during which the continuous strain on chromosomes attached to opposite spindle poles disrupts the mechanical link between sister chromatids [70,71], and it can result in cell death. As independent work had pointed to Cdc20-inhibition being a powerful strategy to inhibit cancer cell division [72], interfering with the dynamic interplay between the APC/C and the spindle checkpoint might indeed open up new avenues towards successful chemotherapy.

Outlook

While it is now clear that CRLs and APC/C are regulated in a highly dynamic manner, the progress in this field has raised many new questions. For example, not all CRL-substrate receptors appear to be responsive to Cand1, yet how Cand1 discriminates between different receptors is not fully understood [22••]. It is possible that Cand1-independent receptors are turned over by autoubiquitylation once they run out of substrates (Figure 1e) [73,74], a reaction that has also been documented for APC/C-specific E2 enzymes [46•,75]. The regulatory advantages of receptor turnover as opposed to Cand1-mediated exchange have not been fully explained. In addition, the neddylation efficiency of the seven human cullins appears to be significantly different from each other [20••], with one cullin being modified by a unique Nedd8-E2 [76] and some CRLs being neddylated at particular locations [77], yet how these properties of the neddylation pathway affect the CRL-cycle has not been systematically addressed. In a similar manner, crucial aspects of APC/C-dependent spindle checkpoint disassembly, such as the mechanism of $p31^{comet}$, how ubiquitylated Cdc20 is extracted from APC/C^{MCC}, or the particular role of Apc15, require further attention. It is likely that the mechanisms underlying the dynamic regulation of CRLs and APC/C will become clearer as researchers continue to develop methods for quantitative dissection of the rapid reactions that drive the cell cycle, similar to those that revealed the role of Cand1 as a substrate receptor exchange factor [22••]. Although it seems intuitive that a dynamic process, such as cell division, calls for highly dynamic regulators, such as the CRLs and APC/C, understanding their behavior and its consequences for cell cycle control will surely keep many scientists moving in the future.

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