Defining the Ubiquitin and E2-Enzyme Requirements for APC/C-Mediated Degradation of Cyclin B1

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Abstract

Post-translational modification of proteins with ubiquitin regulates many aspects of cell physiology, including protein degradation. A uniform polyubiquitin chain that is linked through Lys48 has been widely accepted as central for recognition and destruction by the 26S proteasome. Work in more recent years has demonstrated that the repertoire of proteolytic signals may encompass chains of other linkage types, including Lys11-linked ubiquitin chains and short assemblies of mixed linkage. In this dissertation I examine whether catalysis mediated by the Anaphase-Promoting Complex/Cyclosome (APC/C) is dependent on polyubiquitination and whether the proteolytic machinery exerts a requirement for specific ubiquitin linkages to efficiently degrade cyclin B1.

In chapter II, I describe a novel method in which Xenopus cell-cycle extracts are made largely dependent on exogenous ubiquitin by inhibiting ubiquitin recycling, allowing us to evaluate the relative contribution of distinct ubiquitin linkages in APC/C-mediated ubiquitination and degradation. Utilizing this approach, in chapter III, I found that the conjugation of single ubiquitin moieties to multiple lysine residues in cyclin promotes efficient degradation of cyclin B1 in mitotic Xenopus extracts. Lysine11-ubiquitin chain-formation becomes essential to proteasomal targeting only when the number of available lysine residues in cyclin B1 is
restricted. Analysis in a reconstituted system revealed that APC/C catalyzes multiple monoubiquitination with rapid kinetics and species bearing four or more monoubiquitins on distinct lysines are recognized by ubiquitin receptors. These multiply monoubiquitinated species are rapidly degraded by purified proteasomes.

In chapter IV, I examine the role of distinct E2 enzymes in APC/C-dependent proteolysis. I demonstrate that the chain-extending E2 UBE2S and long Lys11-linked ubiquitin assemblies are dispensable for cyclin B1 degradation, but become increasingly important with restriction of the number of ubiquitination sites. Our findings support a model where through attachment of monoubiquitin to multiple lysine residues, and possibly elaboration of some short chains, UBCH10, or possibly members of the UBC4/5 family, cooperate with the APC/C to generate a robust proteolytic signal on cyclin B1.
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Chapter I: Introduction

Nevena Dimova
Protein modification by ubiquitin

Ubiquitination (also known as ubiquitylation) is a powerful mechanism for regulating essentially all aspects of cell physiology, including cell division, apoptosis, transcription, signal transduction and the modulation of diverse metabolic pathways (Ciechanover, 2005b; Johnson, 2002; Kerscher et al., 2006; Pickart and Fushman, 2004). The covalent attachment of ubiquitin (Ub) to substrate proteins is mediated by an enzymatic cascade that is tightly regulated in a spatial and temporal manner and is characterized by a high degree of specificity for the substrates it modulates. The best-studied result of this modification is the targeting of ubiquitin-tagged proteins for proteasomal degradation. Other important advances since the seminal work focused on understanding the energy-dependent proteolytic activity found in reticulocyte extracts (Ciechanover, 2005a; Ciechanover, 2005b; Ciechanover et al., 1984a; Hershko, 2005) were the discovery of non-degradative functions for ubiquitin and the discovery of ubiquitin-like proteins. How the ubiquitin signal assembled on a substrate is read by different downstream effectors and modulates the fate and/or function of the modified protein remains a central theme in the ubiquitin field.

Ubiquitin is a small 76 amino-acid protein first purified during the isolation of thymopoietin by Goldstein and colleagues (Goldstein, 1974). The high evolutionary sequence conservation (Schlesinger and Goldstein, 1975; Wilkinson et al., 1986), likely unparalleled among known proteins, indicates the central role of ubiquitin-conjugation pathways to normal cell function. Even though this protein may not be as ubiquitous as was initially thought, the name aptly reflects its representation in a variety of cellular tissues and organisms (Goldstein, 1974; Goldstein et al., 1975). Several ubiquitin-like proteins (UBLs) have also been identified, including NEDD8, SUMO and ISG15, which share a characteristic three-dimensional fold with ubiquitin but are otherwise distinct (Kerscher et al., 2006; Schulman and Harper, 2009). The overall structure of this ~8.5-kDa heat-stable protein is extremely compact and tightly-hydrogen bonded with only its C-terminal tail, which is accessed by enzymes, protruding from the molecule.
(Vijay-Kumar et al., 1987). The stability of ubiquitin is likely further enhanced by the pronounced hydrophobic core (Vijay-Kumar et al., 1987). Subsequent work has indicated that the structural constraints required for proper conjugation or recognition of ubiquitin by downstream effectors are quite strict. Different surfaces revealed in the crystal structure of ubiquitin (Vijay-Kumar et al., 1987) have been implicated in distinct cellular processes. The hydrophobic patch surrounding residues Leu8, Ile44 and Val70 is important for recognition of ubiquitin chains by proteasome-associated ubiquitin receptors and proteasome-mediated degradation (Beal et al., 1996; Beal et al., 1998; Lam et al., 1997a; Sloper-Mould et al., 2001). The surface surrounding Ile44 has also been implicated in internalization of activated plasma-membrane receptors (Shih et al., 2000; Sloper-Mould et al., 2001). Residues Phe4 and Asp58 have been demonstrated to be critical for non-proteolytic functions of ubiquitin (Lee et al., 2006; Penengo et al., 2006; Shih et al., 2000; Sloper-Mould et al., 2001).

An important breakthrough early in the field of ubiquitin research was the discovery that a single ubiquitin molecule can be covalently linked to histones, particularly to histones H2A and H2B (Hershko and Ciechanover, 1998). This and subsequent work revealed that, in most cases, ubiquitin is conjugated to substrates via an isopeptide bond between the activated carboxy-terminal glycine (Gly76) of ubiquitin and an ε-amino group of a lysine (Lys) residue in a substrate (Ciechanover et al., 1984a; Ciechanover et al., 1980; Goldknopf and Busch, 1977; Hershko and Ciechanover, 1998; Hershko et al., 1981; Hunt and Dayhoff, 1977). In some cases, ubiquitin is conjugated to the α-amino group of the target substrate (Ciechanover and Ben-Saadon, 2004; Hershko et al., 1984), and there are a few examples of attachment to threonine, serine or cysteine residues (Cadwell and Coscoy, 2005; Carvalho et al., 2007; Grou et al., 2008; Shimizu et al., 2010; Wang et al., 2007). Ubiquitin itself contains seven Lys residues, at positions 6, 11, 27, 29, 33, 48, and 63, which can function as acceptor sites for another ubiquitin moiety during the assembly of ubiquitin chains. All seven potential ubiquitin-ubiquitin linkages have been found to exist in vivo (Peng et al., 2003; Xu et al., 2009). Because of the possible variations
in the sites and type of ubiquitination, the potential repertoire of ubiquitin signals is vast and an outstanding challenge for the ubiquitin field is to understand how these modifications dictate distinct fates of target proteins.

*Molecular players in ubiquitin-chain assembly and disassembly*

The organization of the ubiquitin-conjugation system is hierarchical. The enzymes involved in the cascade reaction of ubiquitin transfer onto substrates (Figure 1.1) were purified by Hershko, Ciechanover and colleagues (Ciechanover et al., 1982; Hershko et al., 1983). In the first step, the ubiquitin-activating enzyme (E1) forms a ternary complex consisting of E1-ubiquitin thioester with ubiquitin-AMP bound (Bedford et al., 2011; Ciechanover et al., 1984a; Hershko and Ciechanover, 1998). The activated ubiquitin is subsequently passed to one of a number of distinct ubiquitin-conjugating (E2) enzymes by transthiolation to a conserved cysteine (Cys) of the E2. The E2 proteins catalyze substrate modification in conjunction with a ubiquitin-protein ligase (E3). For the ~60 members of homologous to E6-AP C-terminus (HECT) family E3s, ubiquitin is shuttled from an E2 to a catalytic Cys in the HECT domain of the E3 before being attached to a substrate (Bedford et al., 2011; Hershko and Ciechanover, 1998; Kerscher et al., 2006; Scheffner et al., 1995). Most E3s, however, contain a really interesting new gene (RING) domain or a structurally related U-box and act as scaffold, bringing a substrate and ubiquitin-charged E2 in proximity and activating the E2 to transfer ubiquitin to a Lys residue in the substrate (Bedford et al., 2011; Kerscher et al., 2006; Pickart, 2001). Ubiquitin chains nucleated on the substrate can be further elaborated by the activity of chain-elongating (E4) enzymes (Crosas et al., 2006; Koegl et al., 1999). Removal of ubiquitin groups which may modulate ubiquitin signaling is carried out by deubiquitinating enzymes (also referred to as deubiquitinases or DUBs) (Amerik and Hochstrasser, 2004; Finley, 2009; Komander et al., 2009; Lam et al., 1997b; Nijman et al., 2005). By processing ubiquitin or ubiquitin-like gene products to yield the
free monomer form (Ozkaynak et al., 1987; Ozkaynak et al., 1984) and by recycling ubiquitin from existing conjugates (Komander et al., 2009), deubiquitinases play a critical role in ubiquitin homeostasis and the proper functioning of the ubiquitin-conjugation cascade.

**Figure 1.1** Schematic overview of ubiquitination. In the first step, a ubiquitin-activating (E1) enzyme uses ATP to form a thioester bond between the Cys residue (denoted with S) at its active site and the carboxyl terminus of ubiquitin (Ub). The activated ubiquitin is then transferred to the Cys residue in the active site of a ubiquitin-conjugating (E2) enzyme, which in turn cooperates with ubiquitin-protein (E3) ligases to catalyze substrate modification. Removal of ubiquitin groups from substrates, which may modulate the downstream fate of a substrate and is important for maintaining steady-state ubiquitin levels, is carried out by deubiquitinating enzymes or DUBs.

In contrast to the small number of E1 enzymes known to operate in the ubiquitin-proteasome system (UPS) (Ciechanover et al., 1984b; Finley et al., 1984; Jin et al., 2007), there are at least 38 E2s encoded by the human genome (Ye and Rape, 2009). Active E2 enzymes possess a conserved core ubiquitin-conjugating (UBC) domain, which contains the catalytic Cys residue and interacts with E1s (Ye and Rape, 2009). After accepting ubiquitin transiently, E2s engage with a cognate E3 enzyme to catalyze substrate ubiquitination. A single ubiquitin ligase can work in conjunction with more than one E2 to ubiquitinate substrates, as seen for the anaphase-promoting complex/cyclosome (APC/C or APC) which interacts with members of the E-2C and UBC4/5 families of E2 enzymes (Kirkpatrick et al., 2006). Also, a sole E2 can function
with several different E3s – this is observed most dramatically with E2s from the UBC4/5 (also referred to as UBE2D) class (Brzovic et al., 2006; Garnett et al., 2009; Kirkpatrick et al., 2006). A central question in the field remains how the limited number of E2s pair up with the much greater number of putative ubiquitin ligases in a specific and regulated manner.

Because of their role as the ultimate arbiters of substrate selection for ubiquitination, much of the initial work focused on E3s, and E2s were often viewed as simple carriers of activated ubiquitin with auxiliary roles. There is emerging evidence that contradicts this early image of E2s by revealing the complex interactions they carry out and important roles they have in substrate ubiquitination. Indeed, it is now evident that E2 enzymes can actively influence the linkage specificity and length of ubiquitin polymers. Ubiquitin-chain formation requires E3s to first nucleate chains by modifying a substrate lysine, and subsequently to elongate chains by targeting lysine residues in ubiquitin. To catalyze these complex reactions, the ubiquitin system has evolved different strategies. Some E2 enzymes such as yeast Cdc34 catalyzes both chain nucleation and elongation to form Lys48-linked chains on SCF substrates (Gazdoiu et al., 2007; Petroski and Deshaies, 2005; Saha et al., 2011). In contrast to Cdc34, some E2s appear to have dedicated roles in ubiquitin-chain initiation or elongation. An example that is particularly important to our work and will be discussed in further detail in a subsequent section is typified by yeast APC/C which uses Ubc4 to transfer ubiquitin onto Lys residues in cyclin B1 and Ubc1 to elaborate homotypic Lys48-linked ubiquitin chains (Rodrigo-Brenni and Morgan, 2007). In this context, regulating the availability of the two E2s is predicted to impact the outcome of the ubiquitination reaction. It is an interesting possibility that such a mechanism of APC/C activity with dependence on multiple E2 enzymes has evolved to more tightly regulate substrate modification and the ensuing cellular consequences.

Synthesizing ubiquitin chains of a distinct topology is another intrinsic property of many E2 enzymes. To form specific ubiquitin-ubiquitin linkages, the E2 may orient the acceptor ubiquitin in a way that exposes the favored Lys residue to its active site (Eddins et al., 2006;
Petroski and Deshaies, 2005). Some E2s exhibit specificity for assembling free chains of a distinct linkage without the aid of an E3 and that linkage specificity is not altered by binding to their cognate E3s (Chen and Pickart, 1990; Wickliffe et al., 2011a). Because ubiquitinated substrates may be sorted into different pathways based on diverse polyubiquitin structures (Pickart and Fushman, 2004), E2s can be viewed as regulators of ubiquitin signaling owing to their capacity to build specific chains. It is important, however, to determine whether the assembly of ubiquitin chains of specific linkage simply reflects intrinsic properties of the E2 rather than a requirement imposed by downstream effectors.

Ubiquitin-conjugating enzymes may also influence the processivity of ubiquitination, defined as the number of ubiquitin molecules that are attached to substrates during a single binding to an E3 (Carroll and Morgan, 2002; Rape et al., 2006). Processivity of ubiquitination depends on the binding affinity of substrates as well as the availability and kinetic properties of E2 enzymes (Rape et al., 2006). Very processive substrates will acquire their functional ubiquitin tag in a single substrate-E3 binding event (Pierce et al., 2009; Rape et al., 2006). In contrast, more distributive substrates will continuously shuttle on and off the ligase complex in order to achieve their functional ubiquitination status and be potentially exposed to the activity of DUBs. Thus the processivity with which a substrate acquires its functional ubiquitin signal is likely to directly influence the downstream fate of the substrate. E2 enzymes have evolved different mechanisms to enhance the processivity of ubiquitination, including the binding of E3s using multiple independent binding sites to increase affinity, recognition of substrate and ubiquitin motifs, oligomerization of ubiquitin-charged E2s and pre-assembly of ubiquitin polymers on their active sites followed by en bloc transfer to the substrate (Ye and Rape, 2009).

How do properties of E2 enzymes that determine the balance of multiple monoubiquitination versus assembly of ubiquitin chains of specific length and topology affect ubiquitin-receptor binding and proteasomal degradation of substrates? In the work presented in
this dissertation, we address this question in the context of APC-mediated proteolysis of cyclin B1.

Ubiquitin-signal topology and proteasome targeting

Over a decade after the discovery of the ubiquitin pathway, it was realized that ubiquitin can be appended not only to different cellular proteins, but also to other ubiquitin moieties forming polyubiquitin structures (Chau et al., 1989; Hershko and Heller, 1985). This raised the question as to whether a unique ubiquitin tag signals destruction by the proteasome. Indeed, in the context of an engineered substrate Arg-βgal, the assembly of a polyubiquitin chain was critical for proteasomal degradation (Chau et al., 1989). In this context, lysine-to-arginine substitution at Lys48 of ubiquitin abrogated both ubiquitin-polymer formation and substrate proteolysis (Chau et al., 1989). Based on this and subsequent studies (Thrower et al., 2000), a model emerged where a polymer of 4 or more ubiquitin groups linked through Lys48 is required for efficient substrate degradation by the proteasome. Whether ubiquitin signals of different architecture were sufficient for proteasomal targeting was not addressed in these studies. Nevertheless, Lys48-linked chains became widely viewed as the canonical proteolytic signal. This model was further corroborated by work demonstrating that Lys48 of ubiquitin was important for bulk protein turnover and essential, albeit not sufficient, for proper cell-cycle progression in *S. cerevisiae* (Finley et al., 1994; Xu et al., 2009). The mitotic phenotype observed in UbK48R mutants where cells displayed two-lobed nuclei and remained arrested in medial nuclear division (Finley et al., 1994) is reminiscent of that observed upon deletion of the gene encoding the E2 Ubc1 (Rodrigo-Brenni and Morgan, 2007). For timely mitotic exit in these cells, the activity of the Lys48 chain-extending E2 Ubc1 in promoting proteolysis of APC substrates was found critical (Rodrigo-Brenni and Morgan, 2007). These and other studies underscore that the ubiquitin signal assembled on substrates is a function of the different E2-E3 complexes and poise the question
whether ubiquitin structures other than Lys48-linked chains can target substrates to the proteolytic machinery. Assigning the proteasomal signaling function to a specific Lys48-linked polymeric unit was a particularly attractive model as it also explained how a single ubiquitin can act as a functionally distinct signal in non-proteolytic processes. Similarly, to monoubiquitination and multiple monoubiquitination (Robzyk et al., 2000; Terrell et al., 1998), polymers assembled through Lys63 of ubiquitin have been implicated in mostly non-proteolytic roles (Deng et al., 2000; Hofmann and Pickart, 1999; Pickart and Fushman, 2004; Spence et al., 2000; Spence et al., 1995; Yang et al., 2009) (Figure 1.2).

**Figure 1.2** The ubiquitin code. Ubiquitin is usually conjugated to the ε-amino group of a lysine (Lys) residue in a substrate (Hershko and Ciechanover, 1998). Ubiquitin itself contains seven Lys residues, at positions 6, 11, 27, 29, 33, 48, and 63, which can function as acceptor sites for another ubiquitin moiety during the assembly of ubiquitin chains. In the classical model, a uniform K48-linked ubiquitin polymer is required for substrate recognition and destruction by the 26S proteasome (Chau et al., 1989; Thrower et al., 2000; Finley et al., 1994). Work in more recent years has demonstrated that the repertoire of proteolytic signals may encompass chains of other linkage types, including K11-linked ubiquitin chains (Xu et al., 2009). Polymers built through K63 of ubiquitin have been suggested to have non-proteolytic roles in orchestrating different steps of DNA repair (Spence et al., 1995; Hofmann and Pickart, 1999), kinase activation (Deng et al., 2000), protein trafficking (Pickart and Fushman, 2004; Yang et al., 2009) and translation (Spence et al., 2000). Similarly, the transfer of a single ubiquitin moiety to one (monoubiquitination) or to multiple sites (multiple monoubiquitination) in a substrate has long been implicated in mostly non-proteolytic processes, such as epigenetic control (Robzyk et al., 2000) and receptor endocytosis (Terrell et al., 1998).
How can we detect different ubiquitin topologies and gain understanding of their functional significance in the cell? One approach has involved large-scale analyses and characterization of protein ubiquitination using quantitative mass-spectrometry techniques (Kim et al., 2011; Peng et al., 2003; Xu et al., 2009). Such studies have revealed a striking and unexpectedly high abundance of non-Lys48 ubiquitin linkages, especially Lys11 linkages (Peng et al., 2003; Xu et al., 2009). Moreover, Lys11-linked chains have been implicated in a broad range of physiological processes, including cell division and endoplasmic reticulum-associated degradation (ERAD) (Behrends and Harper, 2011; Wickliffe et al., 2011b; Xu et al., 2009). A critical issue with these methodologies is that they are concentration-sensitive and thus better at identifying more abundant conjugates in the cell, while not detecting all short-lived proteins such as cell-cycle regulators (Peng et al., 2003). Also, while such analyses are integral to our understanding of the global ubiquitin landscape in the cell, they may not accurately reflect the behavior of individual proteins. For instance, no significant changes in total levels of Lys63 linkages are observed upon proteasomal inhibition (Xu et al., 2009), even though these are capable of supporting degradation by the proteasome (Baboshina and Haas, 1996; Hofmann and Pickart, 2001).

Another approach to studying protein ubiquitination may be to integrate large-scale analyses with work detailing individual pathways. A method that has allowed significant progress in characterizing ubiquitin conjugates and mapping precise sites of ubiquitination is the absolute quantification of ubiquitin by mass spectrometry (Ub-AQUA) (Kirkpatrick et al., 2005; Kirkpatrick et al., 2006). This quantitative mass-spectrometry technique uses isotope-labeled internal standard peptides to quantify tryptic peptides that are derived from the digestion of any given sample. By comparison to the known amount of isotope-labeled standards, the exact amount of any given peptide in a sample can be quantitated. By applying the law of conservation of mass, one can use this method to quantify the absolute amount of any given substrate, ubiquitin, and the relative proportion of each of the seven potential ubiquitin-ubiquitin linkages.
In the work presented in this dissertation, we have combined analysis of processes reconstituted in vitro and Ub-AQUA analysis with studies in the Xenopus system to delineate how the E3 ligase APC/C mediates ubiquitination and degradation of cyclin B1.

Studying the role of distinct ubiquitin topologies in protein breakdown in physiological settings has proven to be challenging. This can be attributed, in part, to the dynamic nature and fast kinetics of the ubiquitination and proteolytic events. Another factor that hinders our understanding of ubiquitin-mediated proteolysis has been the lack of methods allowing the ubiquitin pool available in a physiological setting to be modulated in a precise and controlled manner. Dominant-negative effects may be hard to observe when conducted in a background that contains wild-type ubiquitin. Meanwhile, ubiquitin homeostasis, through de novo protein synthesis and recycling from existing conjugates, is tightly regulated, rendering ubiquitin depletion hard to achieve. Thus, we need novel approaches for ubiquitin replacement to examine the effects of ubiquitin mutants on degradation in a physiological context.

*The proteasome - a complex protein-degrading machinery*

The 26S proteasome, an intricate molecular machine of over 2.5 MDa, is the proteolytic arm of the UPS (Waxman et al., 1987). It functions primarily to degrade damaged, misfolded and short-lived regulatory proteins bearing ubiquitin modifications. The proteasome also regulates the state of cells by eliminating key cell-cycle regulators such as cyclins and securin. While existing in multiple forms, the proteasome is a composite of two major assemblies - the 28-subunit core particle (also known as the 20S particle) and the 19-subunit regulatory particle (also known as the 19S particle or PA700) (Finley, 2009; Weissman et al., 2011). The catalytic activity of the proteasome is contained within the barrel-shaped structure of the core particle (Bedford et al., 2011; Finley, 2009; Weissman et al., 2011).
How does this complex machinery discern ubiquitin tags with proteolytic versus non-proteolytic functions, ensuring substrates are not inadvertently degraded? Entrance to the catalytic chamber is directed by factors within the regulatory particle, which recognize and process polyubiquitinated substrates (Finley, 2009; Weissman et al., 2011). Another function of the 19S complex is to remove and recycle ubiquitin from proteolytic substrates concomitantly or prior to their translocation into the core. The third key function of this subcomplex is to promote substrate unfolding and translocation into the core (Finley, 2009; Weissman et al., 2011). The 19S particle is more heterogeneous and unstable as compared to the catalytic core with some of its components in sub-stoichiometric amounts and interchanging with the cytosol (Elsasser and Finley, 2005; Finley, 2009; Leggett et al., 2002; Schmidt et al., 2005). These findings have important implications about the adaptability of the proteasome in response to changing cellular signaling. In the work presented in this dissertation, we attempt to explore how the presence of proteasome-associated DUB activity may influence the recognition and degradation of a ubiquitin-tagged substrate.

Once associated with proteasomal subunits, substrates may have their ubiquitin chains extensively remodeled through opposing ubiquitin-ligase and deubiquitinating activities (Crosas et al., 2006). Whether the delivery pathway to the proteasome influences the nature and extent of ubiquitin-polymer remodeling and how substrate specificity is enforced remain poorly understood. However, there is an emerging view that remodeling of the ubiquitin tag by the proteasome, during a kinetically defined time window, can influence the fate of substrates. Remodeling of the ubiquitin signal can, in part, be carried out by the three deubiquitinases found in mammalian proteasomes: RPN11, USP14, and UCH37 (Finley, 2009; Komander et al., 2009; Lee et al., 2010b). UCH37 and USP14 associate reversibly with the proteasome, whereas RPN11 is a stoichiometric subunit (Finley, 2009). Essential for proteasome function (Finley, 2009; Verma et al., 2002; Yao and Cohen, 2002), the metalloenzyme RPN11 promotes substrate degradation by removing ubiquitin moieties, which may not be easily threaded through the
narrow translocation channel (Finley, 2009; Lander et al., 2012; Verma et al., 2002; Yao and Cohen, 2002).

The activity of UCH37 and USP14 may stimulate breakdown of some substrates, while antagonize that of others (Hanna et al., 2006; Jacobson et al., 2009; Lam et al., 1997b; Lee et al., 2010a; Leggett et al., 2002). UCH37 has been implicated as an editing activity for the proteasome by selectively removing ubiquitin groups from oligoubiquitinated substrates and allowing them to escape degradation (Lam et al., 1997b). It remains to be determined, however, whether in the cellular context, UCH37 could provide a means for the proteolytic machinery to discriminate against substrates bearing multiple monoubiquitins or short ubiquitin chains. In contrast, USP14 is thought to act on more heavily ubiquitinated substrates. By trimming of substrate-bound ubiquitin chains, USP14 has been suggested to potentially govern the degradation rates of numerous substrates (Hanna et al., 2006; Lee et al., 2010a). Unexpectedly, USP14 was also found to delay substrate degradation through a non-catalytic mechanism (Hanna et al., 2006; Lee et al., 2010a). While these effects are not well understood, they may be related to the chain-trimming activity of USP14 and may also help the cell recycle ubiquitin especially under conditions of ubiquitin deficiency. Consistent with such a role, the expression of Ubp6 (the yeast orthologue of USP14) is upregulated and loading onto the proteasome elevated following depletion of free ubiquitin levels (Hanna et al., 2007). A number of important questions about the proteolytic machinery remain unresolved. Which proteins are substrates of the different DUBs on the proteasome and how substrate specificity and selectivity are enforced? Also, how do the number and spatial arrangement of ubiquitin monomers on a substrate affect its recognition and processing by the proteasome?

An important question is how ubiquitin-tagged proteins are correctly targeted to the proteasome holoenzyme for destruction. One potential solution to this complex problem is the recruitment of the ubiquitin-conjugation machinery in the vicinity in the proteasome. In fact, the E2 UBC4 (Chuang and Madura, 2005) and the APC (Seeger et al., 2003) (also N.D., R.W.K.,
unpublished observations), among other enzymes (Finley, 2009), have been observed to directly associate with the proteasome. The functional significance of these interactions has not been elucidated, but they may allow ubiquitination and subsequent degradation to be spatially linked within the cell. An alternative pathway for target delivery to the proteasome may be dependent on the activity of ubiquitin-receptor proteins. The proteasome appears to recognize ubiquitinated substrates via both intrinsic ubiquitin-receptor subunits and by transiently associating shuttling factors (Elsasser and Finley, 2005). Rpn10 (Deveraux et al., 1994; Elsasser and Finley, 2005; Young et al., 1998) and Rpn13 (Husnjak et al., 2008; Schreiner et al., 2008) are the two currently known intrinsic ubiquitin receptors, whereas Rad23, Dsk2, and Ddi1 (Elsasser and Finley, 2005) are shuttling proteins.

The “shuttle” proteins are thought to function in capturing of ubiquitinated substrates remotely from the proteasome and escorting them to this complex. The ubiquitin-associated (UBA) domains in these proteins recognize the ubiquitin tag, whereas the ubiquitin-like (UBL) domains bind to the proteasome (Finley, 2009; Hicke et al., 2005; Schaub et al., 1998). In vitro studies suggest some ubiquitin-binding domains (UBDs) bind polyubiquitin preferentially to monoubiquitin, and may even exhibit preference for specific ubiquitin linkages (Hicke et al., 2005). Supporting the view of Lys48-linked chains as the principal proteolytic signal, some studies suggest that proteasome-associated receptor proteins may preferentially recognize Lys48 linkages (Raasi et al., 2004; Raasi and Pickart, 2003; Varadan et al., 2004). In a cellular context, it is likely that the specificity and affinity of binding may require that UBL/UBA proteins recognize binding determinants not only in the ubiquitin tag but also in the modified substrate. Substrate specificity (Verma et al., 2004) may be further enforced by subcellular localization of ubiquitin receptors or their interactions with E3 ubiquitin ligases. Importantly, ubiquitin receptors have the potential to recognize a vast array of ubiquitin signals through multiple UBDs joined by flexible linker regions (Kang et al., 2007; Wang et al., 2005) and by multimerization with other receptors (Kang et al., 2007; Kang et al., 2006). The presence of multiple ubiquitin-interacting
motifs in receptors such as RPN10 may provide additional avidity in recognition of targets, particularly those targets bearing either a polyubiquitin structure or multiple monoubiquitins (Finley, 2009; Harper and Schulman, 2006).

In the work presented in this dissertation, we investigate whether ubiquitin receptors Rad23 and Rpn10 exert a requirement for ubiquitin-chain formation for binding to ubiquitinated cyclin B1. We also sought to understand whether the proteolytic machinery exerts a requirement for specific ubiquitin topologies for cyclin degradation.

Orchestration of the cell cycle by the anaphase-promoting complex

Cyclins are a diverse family of proteins whose defining feature is that they bind and direct the activity of members of the cyclin-dependent kinase (CDK) family. Oscillations in their levels help to generate strictly controlled changes in the enzymatic activity of CDKs which modulate the phosphorylation state, and thereby the state of activation, of proteins that control cell-cycle processes. The M-phase cyclins typified by cyclin B in vertebrates (Clb1-4 in budding yeast) accumulate as the cell approaches mitosis, peaking at metaphase. The periodic rise and fall in cyclin levels and its striking disappearance at the end of each mitosis in early sea urchin embryos led to the discovery of this critical cell-cycle regulator (Evans et al., 1983). Subsequent studies found cyclin B to form a complex with CDK1 initially termed maturation promoting factor and now referred to as M-phase promoting factor (MPF) (Gautier et al., 1990; Gautier et al., 1988; Lohka et al., 1988). The activity of cyclin B1-CDK complexes is responsible for the striking cellular changes that lead to assembly of the mitotic spindle and the alignment of sister-chromatid pairs on the spindle at metaphase. In addition to driving the cell to metaphase, the complex activates the system that degrades its cyclin subunit and orchestrates exit from both mitosis and meiosis (Glotzer et al., 1991; Hershko et al., 1991; Murray et al., 1989).
The Anaphase-Promoting Complex/Cyclosome (APC/C or APC) was identified in *Xenopus* and clam extracts as the cyclin B1 specific ubiquitin ligase responsible for MPF inactivation upon mitotic exit (King et al., 1995; Sudakin et al., 1995). The E3 ligase activities of Skp1-Cullin-F-box complex (SCF) family and the APC have long been recognized to be crucial to cell-cycle progression and regulation (Harper et al., 2002; Lipkowitz and Weissman, 2011; Peters, 2006; Skaar and Pagano, 2009). Consistent with this, the genetic inactivation of APC causes cell-cycle arrest in all species in which it has been investigated so far, ranging from yeast to mouse (Peters, 2006). In addition to regulating mitosis and meiosis, the APC has been implicated in post-mitotic processes including dendrite formation in neurons, metabolic, and learning and memory processes (Barford, 2011b).

The APC is a ~1.5 MDa complex, composed of at least 13 subunits (Peters, 2006). The size and unusual complexity of this E3 ubiquitin ligase has limited structural studies. The essential roles of APC constituent proteins pose further challenges in using native systems to study the complex. A recombinant expression system was recently generated that allowed the reconstitution of a functional APC in milligram quantities (Schreiber et al., 2011). This is an exciting advance as the system may allow defined manipulation of APC complexes and facilitate studies of how APC subunits assemble and interact with co-activators, substrates and regulatory factors.

APC-mediated coordination of cell-cycle progression is achieved through the temporal regulation of APC activity and substrate specificity. One aspect of temporal regulation of the ligase activity is achieved through a combination of two structurally related co-activators Cdc20 and Cdc20 homolog 1 (Cdh1), coupled to protein phosphorylation. The two co-activator subunits have opposing activity profiles. Cdc20 activates the APC at the onset of mitosis, when APC subunits are phosphorylated and Cdh1 activity is low owing to its CDK-mediated phosphorylation. After CDK activity is diminished during mitotic exit, Cdh1 forms a complex with APC, which ubiquitinates Cdc20, leading to APC$^{Cdc20}$ inactivation. The co-activators help recruit substrates to the ligase complex. Indeed, multiple lines of evidence suggest that the co-
activator directly bind APC substrates via its WD40-repeat domain so at least part of the function of the co-activator appears to be a substrate adaptor (Burton and Solomon, 2001; Buschhorn et al., 2011; da Fonseca et al., 2011; Kraft et al., 2005). The co-activators recognize two characteristic sequence elements found in APC substrates, namely a destruction box (D box; RxxLxxI/VxN) (Glotzer et al., 1991; King et al., 1996), and a KEN box (KENxxxN/D) (Pfleger and Kirschner, 2000; Pfleger et al., 2001). Whereas Cdc20 specifically recognizes the D-box motif, Cdh1 appears to prefer the KEN box, but also recognizes the D-box (Peters, 2006; Pfleger et al., 2001).

The reversible association of co-activators with the APC presents one level of regulation of APC activity. Another level of APC control is provided by pseudo-substrate inhibitors such as early mitotic inhibitor 1 (Emi1). By preventing productive interactions of substrates with the catalytic module or interfering with recruitment of E2 enzymes (Barford, 2011b), such pseudo-substrates can block APC-mediated ubiquitination and proteolysis. A similar concept of inhibition may apply to another critical APC regulatory system, the mitotic checkpoint complex (MCC) (Barford, 2011b; Kim and Yu, 2011). Consistent with this idea, elegant structural work reveals that checkpoint proteins inhibit APC catalysis at least in part by preventing substrate binding (Herzog et al., 2009).

According to electron microscopy studies, APC/C complexes more commonly resemble an asymmetric triangular structure, composed of a platform and an arc lamp domain that together enclose a central cavity (Dube et al., 2005; Passmore et al., 2005). Apc1, together with Apc4 and Apc5, function as a scaffolding-like assembly subunit linking the catalytic module formed from Apc2, Apc11 and Apc10 (Doc1) with the TPR subunits Cdc16, Cdc23, Cdc27 found in the arc-lamp domain (Barford, 2011b; Dube et al., 2005; Herzog et al., 2009; Schreiber et al., 2011; Thornton et al., 2006; Vodermaier et al., 2003). The tetratricopeptide repeats (TPR) found in APC/C subunits are 34-amino acid motifs that generate super-helical structures, facilitating protein-protein interactions (Barford, 2011a).
The catalytic core of the anaphase-promoting complex consists of Apc2 and Apc11. Apc2, a member of the cullin protein family, associates with the RING H2-finger domain in Apc11, which in turn recruits E2 ubiquitin-conjugating enzymes (Peters, 2006). Interestingly, Apc2-Apc11 alone can support ubiquitin ligation, albeit with diminished substrate specificity (Tang et al., 2001; Yu et al., 1998). Elegant structural work recently revealed that the cullin-RING module was in close vicinity to the core APC/C subunit Apc10 (Doc1) (Buschhorn et al., 2010; da Fonseca et al., 2011). Apc10 was earlier demonstrated to play a role in substrate recruitment to the ligase and processive substrate ubiquitination (Carroll et al., 2005; Carroll and Morgan, 2002; Passmore et al., 2003), but how it performs these functions remained poorly understood. In this context, a model emerging from recent studies suggests that Apc10 promotes processive substrate ubiquitination by forming part of a bipartite substrate receptor on the APC/C, composed of Apc10's ligand binding region (Au et al., 2002; Buschhorn et al., 2011; Carroll et al., 2005; Carroll and Morgan, 2002; Chao et al., 2012; da Fonseca et al., 2011; Wendt et al., 2001) and the WD40 propeller domain of the co-activator protein (Buschhorn et al., 2011; da Fonseca et al., 2011; Kraft et al., 2005; Matyskiela and Morgan, 2009). Interestingly, substrate binding was found to induce conformational changes in Apc2-Apc11 (Buschhorn et al., 2011) which may be coupled with proper substrate recognition and efficient ubiquitin transfer to substrate residues.

Another layer of interactions important for APC/C activity are those with E2 ubiquitin-conjugating enzymes. Members of the UBC4/5 (King et al., 1995) and E-2C (Aristarkhov et al., 1996; Townsley et al., 1997; Yu et al., 1996) families (in particular human UBCH5 and UBCH10, respectively) have long been recognized as E2 partners for the APC/C. While in a reconstituted system both classes of E2s can support APC/C-dependent ubiquitination (Garnett et al., 2009; Kirkpatrick et al., 2006; Mathe et al., 2004; Summers et al., 2008; Yu et al., 1996; Zeng et al., 2010), which E2 enzyme(s) work with the APC/C in vivo remains an open question. Genetic evidence from S. pombe and D. melanogaster supports the notion that members of the E-
2C family, Ubc11 and Vihar, are most likely to be physiologically relevant (Mathe et al., 2004; Osaka et al., 1997). Consistent with this, the spatiotemporal pattern of the E-2C protein Vihar distribution and proteolysis closely resembles that for cyclin B1 (Mathe et al., 2004) and a dominant-negative form of the enzyme results in stabilization of APC/C substrates (Townsley et al., 1997). Unlike in simpler model organisms, the importance of UBCH10 for mitosis in human cells has been disputed (Rape and Kirschner, 2004; Walker et al., 2008). An N-terminal extension found in UBCH10, but not in members of the UBC4/5 family, has been proposed to regulate the ubiquitination activity of the APC/C and the sensitivity of the ligase to checkpoint control (Summers et al., 2008).

The set of distinct E2 ubiquitin-conjugating enzymes suspected to function in APC/C-mediated catalysis was recently expanded. In budding yeast, Ubc1 functions as a supplementary E2 enzyme that elongates ubiquitin chains initiated by the proximally-acting E2 Ubc4 (Rodrigo-Brenni and Morgan, 2007). In higher eukaryotes, it has been proposed that another E2 UBE2S is important in elaborating Lys11-linked ubiquitin chains on APC/C-substrates (Garnett et al., 2009; Williamson et al., 2009; Wu et al., 2010). In this model, UBCH10 is proposed to initiate monoubiquitination of the substrate, with UBE2S then extending Lys11-linked ubiquitin chains that signal degradation by the proteasome (Garnett et al., 2009; Jin et al., 2008; Williamson et al., 2009; Wu et al., 2010). Consistent with this idea, depletion of UBE2S from Drosophila S2 cells results in a strong delay in a metaphase-like state and stabilization of cyclin B1 at the spindle poles, among other mitotic defects (Williamson et al., 2009). In contrast, UBE2S has a nonessential function in APC/C-mediated proteolysis of cyclin B1 during normal mitosis in human HeLa cells (Garnett et al., 2009). These findings imply instead that UBE2S becomes important for substrate proteolysis under conditions where APC/C activity is compromised such as during recovery from drug-induced spindle assembly checkpoint (SAC) activation. The spindle checkpoint detects inappropriate attachment and tension between chromosomal kinetochores and microtubules and delays segregation of sister chromatids by inhibiting APC-mediated
ubiquitination of securin and cyclin B1 (Kim and Yu, 2011; Musacchio and Salmon, 2007).

Under such conditions of low APC/C activity, UBE2S may be priming APC/C molecules for rapid and processive ubiquitination of substrates and may be promoting degradation by enhancing the rate of ubiquitination relative to that of deubiquitination. A possible role of UBE2S in silencing the SAC has not been elucidated. Together these findings imply that the contribution of UBE2S and Lys11-linked chains in the degradation of APC/C substrates may be organism-and condition-specific, and that there may not be a uniform requirement for UBE2S in all systems or circumstances.

Role of ubiquitin-chain formation in cyclin B1 proteolysis

Previous work revealed that in contrast to the SCF-ligase complex which cooperates with the E2 Cdc34 to assemble homogeneous Lys48-linked chains on substrates (Petroski and Deshaies, 2005), the APC works in conjunction with UBCH10 and UBCH5 and modifies cyclin B1 with chains of complex topology (Figure 1.3) (Kirkpatrick et al., 2006). Surprisingly, the ubiquitin signal assembled on cyclin B1 consists of ubiquitin-ubiquitin linkages through Lys11 and Lys63 in addition to Lys48 (Kirkpatrick et al., 2006). Analysis of cyclin ubiquitination revealed a two-stage ubiquitination process, where initially the APC/C facilitates transfer of ubiquitin monomers to multiple lysine residues on cyclin B1, followed by extension of short ubiquitin chains (Kirkpatrick et al., 2006). Under conditions where the assembly of proteolytic signal is non-processive and requires rebinding to the ubiquitin ligase, this pathway, as opposed to the rapid elongation of a single chain, may allow the APC/C to more precisely control the spatiotemporal pattern of substrate proteolysis. In the context of cyclin B1, the short ubiquitin polymers generated by UBCH10 or UBC4 present an efficient proteolytic signal (Kirkpatrick et al., 2006). Interestingly, these assemblies are remodeled by the opposing activities of proteasome-associated E4 chain-elongating enzyme Hul5 (KIAA10) and the deubiquitinase USP14 (Crosas et
al., 2006; Hanna et al., 2006; Leggett et al., 2002; You and Pickart, 2001). In these studies, perhaps the most surprising finding was that Lys48-linked ubiquitin polymers were dispensable for binding of modified cyclin B1 to ubiquitin receptors and rapid degradation by the proteasome (Kirkpatrick et al., 2006). As only few other substrates were known at the time to be degraded independent of Lys48-chain formation (Baboshina and Haas, 1996; Guterman and Glickman, 2004; Hershko and Heller, 1985; Hofmann and Pickart, 2001), this in-depth analysis of cyclin B1 set out the stage to explore the significance of different ubiquitin linkages in APC-mediated proteolysis.

Figure 1.3 APC/C-dependent proteolysis of cyclin B1. The APC/C cooperates with ubiquitin-conjugating enzymes from the E-2C and UBC4/5 families (in particular, human UBCH10 and UBCH5, respectively) to catalyze chain formation through three lysine residues of ubiquitin (K11, K48 and K63) (Kirkpatrick et al., 2006). UBCH10 or UBCH5 builds multiple short ubiquitin chains on APC/C-substrates (Garnett et al., 2009; Williamson et al., 2009; Wu et al., 2010). In this model, UBCH10 is proposed to initiate monoubiquitination of the substrate, with UBE2S then extending K11-linked ubiquitin chains that signal degradation by the proteasome.
The ability of the APC, in conjunction with the E2 UBCH10, to extend multiple short ubiquitin chains through lysines 11, 48 and 63 of ubiquitin in vitro (Kirkpatrick et al., 2005) raises the question as to whether Lys11-chain formation is indeed required for proteolysis of APC/C substrates, and if so, whether this requirement arises from a specific role of UBE2S, or because Lys11 is one of three sites used by UbcH10 (Jin et al., 2008; Kirkpatrick et al., 2005). Also, are any of these ubiquitin linkages functioning as the major degradative element? We sought to address these questions using Xenopus egg extracts as a model system. Studies of the role of ubiquitin topology in substrate turnover in Xenopus extract are hampered by the presence of endogenous ubiquitin. Seeking to understand whether APC/C-dependent proteolysis is strictly dependent upon polyubiquitination and whether the proteolytic machinery exerts a requirement for Lys11 or other Ub-Ub linkages to efficiently degrade cyclin B1, we developed a novel approach where Xenopus extracts are made largely dependent on exogenous ubiquitin. Treatment of Xenopus extract with ubiquitin-vinyl sulfone (UbVS), a general inhibitor of ubiquitin isopeptidases (Borodovsky et al., 2001), blocked cyclin B1 proteolysis in a manner that could be fully rescued by exogenous ubiquitin. For our analysis, we used full-length cyclin B1 and CDK1, expressed in insect cells and combined to produce MPF (Kirkpatrick et al., 2006). The studies with active MPF were nicely complemented with the use of an N-terminal fragment (1-88 amino acids) of cyclin B1 which we extensively characterized and found to mimic the ubiquitination and degradation patterns of the full-length protein. To quantitatively evaluate the role of different ubiquitin structures in targeting cyclin B1 for degradation, we expressed and purified 35S-labeled cyclin B1 and measured its proteolysis in Xenopus extract by monitoring release of radioactive soluble counts. Surprisingly, using the UbVS system we found that Lys11-linked ubiquitin chains were not required for efficient cyclin B1 degradation. Furthermore, we were fascinated with the robust rates of proteolysis a chain-terminating ubiquitin (Ub\textsuperscript{triR}), which blocks all possible sites of chain elongation by UBCH10, UBC4/5, and UBE2S, supported.
Cyclin B1 has some particularly interesting features that may play a role in regulating its localization in the cell and interactions with other proteins, as well as its stability. The function of accepting ubiquitin appears to be fulfilled by lysine residues found in the non-conserved regions neighboring the degron (Glotzer et al., 1991; King et al., 1996). Positioned in the vicinity of the D-box or KEN-box motif, the TEK box has been proposed as a new interaction motif directing the chain-initiation event (Jin et al., 2008). However, the potential contribution of the lysine residue found in this motif to proteolysis of different APC substrates remains to be addressed and may provide a better understanding of how the integrity of the motif is essential to UBCH10-dependent conjugation and proteasomal degradation (Jin et al., 2008). For cyclin B1, the unstructured N-terminal region upstream of the cyclin box provides a platform containing 18 lysine residues; 15 of these lysine residues are located within the first 88 amino acids close to the destruction box. Intriguingly, there are patterns of lysine clustering that appear to be fairly well conserved in different species and whose significance has not been elucidated. We hypothesized that the lysine profile of cyclin B1 provides a platform upon which a proteolytic signal consisting of multiple monoubiquitins or short ubiquitin chains can be assembled, obviating the need for the formation of long Lys11-linked ubiquitin chains. In the work presented in this dissertation, we examined how cyclin B1 proteolysis is influenced by the number of available ubiquitination sites. To this end, we generated a series of cyclin mutants containing lysine-to-arginine substitutions at particular residues guided by mass spectrometry analysis of the ubiquitination sites in the substrate. Our findings suggested that ubiquitin-chain formation is not essential for cyclin B1 proteolysis, unless the number of available lysine residues is restricted and that multiple monoubiquitination constitutes an efficient proteolytic signal in this context.

What the ubiquitination status of cyclin B1 upon its docking at the proteasome is and what ubiquitin requirements the proteolytic machinery itself may be imposing on the APC for efficient degradation of cyclin B1 have remained poorly understood. To further evaluate the relevance of different chain structures as a targeting signal for degradation, we sought to
reconstitute different steps of the pathway in vitro. In ubiquitination reconstituted with recombinant E1, E2s, and ubiquitin, the APC/C, immunopurified from mitotic Xenopus extracts where it is activated by Cdc20, can rapidly attach mono-ubiquitin to multiple lysines in cyclin B1. Cyclin modified with 4 or more ubiquitin moieties on distinct lysines was recognized by various ubiquitin receptors. Consistent with experiments in Xenopus extract, multiply monoubiquitinated cyclin B1 species were efficiently degraded by purified human proteasomes in the absence of the proteasome-associated deubiquitinating enzyme USP14. Surprisingly, we found that USP14 suppressed degradation of multiply monoubiquitinated cyclin B1 to a similar extent as that of polyubiquitinated species, suggesting that USP14 can efficiently remove monoubiquitin. However, such deubiquitinating activity did not appear to strongly antagonize proteasome function in Xenopus extract as our findings presented in this dissertation suggest. We further pursued identification of deubiquitinating enzyme(s) that may modulate the rate of cyclin degradation in the Xenopus extract.

In conclusion, in chapter II we present our work on developing and characterizing the UbVS system. This includes work detailing the behavior of an N-terminal fragment of cyclin B1 and our approach to quantitatively analyze its degradation. In the study presented in chapter III we sought to evaluate the role of different ubiquitin linkages in cyclin B1 proteolysis. We demonstrate that conjugation of ubiquitin moieties to multiple lysine residues of cyclin B1 provides an alternative degradation pathway for cyclin B1 that does not require chain extension by the Lys11-specific E2 enzyme UBE2S. Lys11-ubiquitin chain formation becomes essential only when the number of available lysine residues in cyclin B1 is restricted. We postulate that high local density of ubiquitin, whether attached as monomers along the substrate or linked to each other in a polymer, is sufficient to target a substrate for proteolysis. In chapter IV we further examine the relative contribution of different E2 enzymes for APC/C-mediated ubiquitination and degradation in the Xenopus extract.
References


Chapter II: Development and characterization of the UbVS system

Nevena Dimova
Abstract

Covalent attachment of ubiquitin to proteins regulates a myriad of cellular processes by influencing activity, localization and stability of substrates. Ubiquitin can be appended to proteins as single moieties or as ubiquitin polymers of specific length and linkage type. The widely accepted view supported by early studies was that Lys63-linked ubiquitin polymers have non-proteolytic roles, whereas Lys48-linked assemblies serve as the principal signal for substrate degradation by the 26S proteasome. While appealing in its simplicity, it is now evident that this model does not reflect the wealth of information different ubiquitin signals encode. Recent work has revealed that the proteasome has the capacity to recognize and degrade substrates bearing ubiquitin modifications other than homogenous Lys48-linked ubiquitin chains. In this study, we establish and characterize a novel approach to studying the role of ubiquitin-chain synthesis in targeting of substrates to the proteasome. Global inhibition of deubiquitinases with ubiquitin vinyl sulfone (UbVS) rendered Xenopus egg extracts dependent on exogenous ubiquitin to promote cyclin B1 proteolysis. Detailed characterization of the system suggested that it can be a valuable approach in defining the role of ubiquitin-chain formation in APC/C-dependent ubiquitination and degradation.
Introduction

Ubiquitin is a highly conserved 76 amino-acid protein which can be conjugated posttranslationally to other proteins via an isopeptide linkage between its carboxy-terminal glycine and, most typically, the ε-amino group of a lysine (Lys) in a substrate (Ciechanover et al., 1984; Ciechanover et al., 1980; Goldknopf and Busch, 1977; Hershko and Ciechanover, 1998; Hershko et al., 1981; Hunt and Dayhoff, 1977). Ubiquitin itself contains seven Lys residues, at positions 6, 11, 27, 29, 33, 48, and 63, allowing the assembly of ubiquitin polymers and a diverse array of ubiquitin signals (Pickart and Fushman, 2004). Posttranslational modification by ubiquitin is utilized in many cellular pathways, but the role of ubiquitin in selective, intracellular protein degradation remains its best-understood function. In the classical model, a chain of four ubiquitin molecules linked through Lys48 is required for efficient recognition and degradation by the 26S proteasome (Chau et al., 1989; Finley et al., 1994; Thrower et al., 2000). The development of novel mass spectrometry techniques (Kirkpatrick et al., 2005; Kirkpatrick et al., 2006; Peng et al., 2003; Xu et al., 2009) and biochemical reagents (Matsumoto et al., 2010; Newton et al., 2008) has been vital to recent work demonstrating that the repertoire of proteolytic signals may encompass chains of other linkage types, including Lys11-linked ubiquitin chains (Baboshina and Haas, 1996; Garnett et al., 2009; Jin et al., 2008; Matsumoto et al., 2010; Williamson et al., 2009; Wu et al., 2010; Xu et al., 2009) or short chains of mixed linkage (Kirkpatrick et al., 2006). Congruent with the widely accepted view that ubiquitin-polymer structure is required for high-affinity binding to the proteasome, the attachment of a single ubiquitin to one (monoubiquitination) or to multiple sites (multiple monoubiquitination) has been implicated in mostly non-proteolytic processes.

The cell cycle must proceed in an orderly fashion to maintain genomic integrity and to prevent dysregulated proliferation. Fundamental to this is the precisely timed destruction of key regulators, including cyclins, cyclin-dependent kinase inhibitors and securin. Many of these
regulators are substrates of the anaphase-promoting complex/cyclosome (APC/C or APC), a multisubunit RING E3 ubiquitin ligase. In an event critical for anaphase entry, the APC/C and cognate E2 ubiquitin-conjugating enzymes catalyze the ubiquitination of cyclin B1 targeting it to the 26S proteasome for degradation (Barford, 2011; Glotzer et al., 1991; Harper et al., 2002; Hershko et al., 1991; Peters, 2002; Peters, 2006). The ubiquitin signal assembled on cyclin B1 \textit{in vitro} was previously revealed to comprise of monoubiquitin appended to multiple distinct lysines in the substrate, as well as some short chains linked through Lys11, Lys48 and Lys63 (Kirkpatrick et al., 2006). While starkly different from canonical proteolytic signals consisting of a single homogeneous ubiquitin chain, the ubiquitin signal built on cyclin B1 supports rapid degradation by the 26S proteasome (Kirkpatrick et al., 2006). More recent studies have implicated a role of the Lys11-linkage specific E2 UBE2S and chain synthesis it promotes in APC/C-dependent proteolysis (Garnett et al., 2009; Matsumoto et al., 2010; Wickliffe et al., 2011a; Wickliffe et al., 2011b; Williamson et al., 2009; Wu et al., 2010; Ye and Rape, 2009). At least in some biological contexts, however, turnover of APC/C substrates and normal mitosis can occur in the absence of UBE2S or Lys11-linked polyubiquitination (Garnett et al., 2009; Jin et al., 2008).

Dissecting the contribution of different ubiquitin linkages to proteasomal recognition and degradation in intricate systems such as whole cells or cellular extracts is complicated in part by the pool of endogenous ubiquitin. Maintenance of steady-state ubiquitin levels is crucial in physiological settings and is regulated at multiple levels, the most important of which are \textit{de novo} synthesis of ubiquitin (Simon et al., 1999; Watt and Piper, 1997) and ubiquitin recycling mediated by deubiquitinating enzymes (Finley, 2009; Hanna et al., 2007). Such pathways pose challenges to studying the role of ubiquitin topology in proteasomal targeting and their robustness renders it a non-trivial task to induce ubiquitin deficiency. To gain better understanding of the role of specific ubiquitin linkages in cyclin B1 proteolysis, we developed and characterized a
novel approach where *Xenopus* extracts are made largely dependent on exogenous ubiquitin by restricting the recycling of endogenous ubiquitin.
Results

Developing a quantitative assay to measure cyclin proteolysis in *Xenopus* cell-cycle extract

The heterogeneous nature of the ubiquitin signal built on cyclin B1 in *Xenopus* extract renders visualization of ubiquitinated species by western blot analysis challenging. Furthermore, the ubiquitin tag assembled on substrates may not promote their degradation. To assess the contribution of different ubiquitin linkages specifically in cyclin B1 turnover, we developed a quantitative assay to measure cyclin B1 degradation products. We metabolically labeled an N-terminal fragment of cyclin B1 with $^{35}$S in *E. coli*, purified the protein, and measured its proteolysis in *Xenopus* extract by monitoring release of trichloroacetic-acid (TCA) soluble counts.

We first examined whether this N-terminal fragment of cyclin B1, similar to the full-length protein, was degraded in an APC/C-dependent fashion. When added to interphase extract, a state in which the APC/C is inactive, cycB1-NT remained stable, but was rapidly degraded upon entry of extract into mitosis (Figure 2.1a). Furthermore, pre-treatment of mitotically-arrested extract with a small-molecule inhibitor of the APC/C TAME (Zeng and King, 2012; Zeng et al., 2010) (Figure 2.1b) or concomitant addition of identical unlabeled N-terminal fragment largely stabilized the radiolabeled cycB1-NT (Figure 2.1d), consistent with a requirement for APC/C-mediated ubiquitination to stimulate substrate degradation. In contrast, proteolysis was not effectively competed by a destruction-box (D-box) mutant (Figure 2.1d). Interestingly, these competition experiments revealed differing efficiencies of inhibition, suggesting cyclin B1 fragments from different species may bind the APC with different affinities. Difference in binding affinities may stem from the sequence variation distinguishing the D-box motif of the sea urchin protein from that of the human cyclin B1. Alternatively, sea urchin cyclin B1 is relatively lysine-poor in its N-terminal domain, containing only 10 lysine residues, raising
Figure 2.1 N-terminal fragment of cyclin B1 is degraded in Xenopus extract in an APC/C- and proteasome-dependent fashion. For all panels, $^{35}$S-labeled cyclin B1 NT (aa 1-86) (~200 nM) was added to extract. Samples were taken at indicated times and subjected to trichloroacetic acid (TCA) precipitation. Proteolysis was measured by release of TCA soluble counts, and is plotted as percentage of input radiolabeled cyclin B1 protein. (a) Degradation of $^{35}$S-labeled cyclin B1-NT in interphase and mitotically-arrested Xenopus extracts. (b) Degradation of $^{35}$S-labeled cyclin B1-NT in mitotic Xenopus extract pre-treated with an inhibitor of the APC/C (10 μM) or DMSO. (c) Same as b, except mitotic Xenopus extract was treated with proteasome inhibitor MG_132 (200 μM) or DMSO prior to substrate addition. (d) Degradation of $^{35}$S-labeled wild-type human cyclin B1-NT (1-86) in mitotic Xenopus extract, supplemented concomitantly with unlabeled WT human cyclin B1-NT (1-86), WT or D-box mutant sea urchin cyclin B1 NT (13-110), at indicated concentrations.
an interesting possibility that this relative defect in binding to the APC may stem from the lower number of ubiquitination sites neighboring the D-box. Nevertheless, the degradation of both human and sea urchin cycB1-NT in extract was proteasome-dependent, as addition of the proteasome inhibitor MG262 blocked this process (Figure 2.1c; data not shown).

Endogenous ubiquitin levels are limiting for degradation of an N-terminal fragment of cyclin B1 in mitotic Xenopus extract

To evaluate the role of different ubiquitin-chain topologies in cyclin degradation in the Xenopus system, we first determined the consequences of adding excess wild-type or mutant ubiquitin to mitotic extract. Using ubiquitin-aqua (Ub-AQUA) (Kirkpatrick et al., 2005; Kirkpatrick et al., 2006) measurements, we calculated that free ubiquitin is present at 5-10 μM concentration in Xenopus extracts (D. Kirkpatrick and N. Hathaway, unpublished observations). When added at 44 μM final concentration, wild-type ubiquitin substantially accelerated degradation, decreasing the half-life of the substrate from 25 minutes in untreated extract to approximately 15 minutes in the presence of additional ubiquitin (Figure 2.2a). Ubiquitin mutants containing a single lysine-to-arginine substitution at position 48 (Ub$^{48R}$) or 63 (Ub$^{63R}$) stimulated degradation almost as well as wild-type ubiquitin, consistent with the idea that these linkages are not essential for degradation. Surprisingly, a ubiquitin containing a lysine-to-arginine mutation at position 11 (Ub$^{11R}$) also stimulated degradation when added to extract, although the effect was not as dramatic as for wild-type ubiquitin. Similar results were obtained with a ubiquitin variant bearing arginine residues at all three positions, Lys11, 48 and 63 (Ub$^{nR}$), simultaneously. Similar trends were observed when the amount of ubiquitin was increased approximately 2.5-fold to 116 μM (Figure 2.2b). These results were unexpected, as mass spectrometry analysis indicated that elimination of all three principal sites (Lys11, Lys48 and Lys63) of ubiquitin-ubiquitin linkage by
Figure 2.2 Endogenous ubiquitin levels are limiting for degradation of an N-terminal fragment of human cyclin B1 in mitotic Xenopus extract. (a, b) For both panels, [35S]-labeled cycB1-NT (amino acids 1-68) (~200 nM) was added to mitotically-arrested Xenopus extract concomitantly with forms of ubiquitin as indicated or buffer (untreated). Samples were taken at indicated times and subjected to trichloroacetic acid (TCA) precipitation. Proteolysis was measured by release of TCA soluble counts, and is plotted as percentage of input radiolabeled cyclin B1 protein. In a, extract was supplemented with 44 μM of exogenous ubiquitin, while in b, extract was supplemented with 116 μM of exogenous ubiquitin. Data are representative of at least three independent experiments.
the APC, as in Ub\textsuperscript{mir}, rendered ubiquitin incapable of supporting chain formation in reconstituted reactions (data not shown). In contrast, methylated ubiquitin (Ub\textsuperscript{me}) slowed proteolysis below the rate observed in untreated extract, but even when added at high concentration, the degree to which it suppressed degradation was modest. The capacity of chain-terminating ubiquitin to support robust cyclin proteolysis in mitotic \textit{Xenopus} extract raised the question of whether ubiquitin-chain synthesis is essential in this pathway.

\textbf{The UbVS system – a novel approach to studying the role of ubiquitin topology in proteasomal targeting}

Analysis of ubiquitin-mediated proteolysis in complex systems such as \textit{Xenopus} extracts is hampered by the pool of endogenous ubiquitin which can be incorporated into the ubiquitin signal assembled on a substrate. In \textit{Xenopus} extracts, endogenous ubiquitin levels appeared to be limiting for the proteolysis of cycB1-NT as addition of ubiquitin accelerated this process. To restrict ubiquitin availability further, we sought to suppress ubiquitin-chain disassembly, in essence, locking endogenous ubiquitin into existing conjugates. We reasoned that inhibition of ubiquitin recycling will render the degradative capacity of \textit{Xenopus} extracts dependent on added ubiquitin. To test this idea, we added ubiquitin-vinyl sulfone (UbVS), a general inhibitor of ubiquitin isopeptidases (Borodovsky et al., 2001), to mitotic \textit{Xenopus} extract. Ubiquitin-vinyl sulfone inhibited cyclin proteolysis in a dose-dependent fashion, such that 10 μM suppressed degradation only partially, whereas 20 μM UbVS inhibited degradation by at least 90-95% (Figure 2.3a; data for full-length cyclin B1 not shown). Inhibition of cyclin proteolysis could be fully rescued by addition of wild-type ubiquitin at the end of the pre-incubation, concomitantly with substrate addition (Figure 2.3b). However, while increasing concentrations of UbVS failed to influence appreciably the degree of substrate stabilization, those appeared to slightly dampen
Figure 2.3 Ubiquitin vinyl sulphone (UbVS) inhibits cycB1-NT proteolysis in a dose-dependent fashion. For all panels, mitotically-arrested Xenopus extract was pre-treated with indicated concentration of UbVS or buffer (referred to as untreated) for 30 min. $^{35}$S-labeled cycB1-NT (~200 nM) was added to extract. Samples were removed at indicated times and subjected to trichloroacetic acid (TCA) precipitation. Proteolysis was measured by release of TCA-soluble counts, and is plotted as percentage of input radio-labeled cyclin B1 protein. (a) Degradation of $^{35}$S-labeled cycB1-NT was measured in extract that had been pre-treated with UbVS at indicated concentrations. (b) Proteolysis of $^{35}$S-labeled cycB1-NT in extract pre-treated with increasing concentrations of UbVS and supplemented with wild-type Ub (44 μM) at time of substrate addition. Trends are representative of three or more independent experiments.
the ability of 44 μM of wild-type ubiquitin to restore degradation (Figure 2.3b). The robust stabilization of the N-terminal fragment of human cyclin B1, as well as that of the full-length protein, achieved with 20 μM UbVS prompted us to use this concentration of UbVS for all subsequent experiments where we sought to make the system dependent on exogenous ubiquitin.

We found that the degree of degradation rescue in UbVS-treated extracts depended on the concentration of added ubiquitin (Figure 2.4a). When ubiquitin was supplemented at 20 μM concentration, degradation proceeded at a rapid rate for the first 15 minutes of the reaction, and then plateaued, presumably due to depletion of free ubiquitin. Consistent with this idea, a two-fold increase in the concentration of ubiquitin supported rapid proteolysis for 30 minutes, leading to complete degradation of the substrate (Figure 2.4a). To confirm that the substrate behavior observed under these conditions is consistent with the known properties of the ubiquitin-proteasome system, we examined how mutations in functional surfaces of ubiquitin impacted its capacity to rescue degradation in a UbVS-treated extract. Forms of ubiquitin bearing point mutations in one of the hydrophobic patch residues (L8A, I44A, or V70A) were not capable of supporting proteolysis (Figure 2.4b), as predicted given the importance of the hydrophobic patch in recognition of ubiquitin chains by proteasome-associated ubiquitin receptors (Beal et al., 1996; Beal et al., 1998; Lam et al., 1997). In contrast, mutations that interfere with non-proteolytic functions of ubiquitin (F4A (Shih et al., 2000; Sloper-Mould et al., 2001) or D58A (Lee et al., 2006; Penengo et al., 2006)) supported rapid proteolysis, at rates comparable to wild-type ubiquitin (Figure 2.4b). Importantly, the rate of degradation in UbVS-treated extracts supplemented with wild-type ubiquitin was identical to that observed when adding a similar concentration of ubiquitin to untreated extracts, yielding a half-life of approximately 15 minutes in both cases (Figure 2.2a; Figure 2.4a). These findings suggest that UbVS-sensitive deubiquitinating enzymes are unlikely to present a major kinetic barrier to cyclin B1 degradation and instead suggest that the major role of these deubiquitinating enzymes may be to maintain a pool of free ubiquitin available to the conjugation machinery. Analysis of ubiquitin conjugates
Figure 2.4 Ubiquitin vinyl sulfone (UbVS) inhibits cyclin B1 degradation by depleting available ubiquitin. (a) Trp-labeled cycB1-NT and 20 or 44 M of wild-type (WT) Ub as indicated were introduced into mitotically-arrested Xenopus extract that had been pre-treated with UbVS (20 μM) or buffer (referred to as untreated) for 30 min. Proteolysis was measured by release of trichloroacetic acid (TCA) soluble counts, and is plotted as the percentage of input radiolabeled cycB1-NT. Trends are representative of three or more independent experiments. (b) Wild-type ubiquitin or forms of ubiquitin (44 μM) bearing single-point mutations in distinct interact surfaces on ubiquitin, along with radiolabeled substrate, were added to UbVS-treated extract. For panels c-e mitotically-arrested Xenopus extract was pre-treated with UbVS (20 μM) or buffer (referred to as untreated) for 30 min. Wild-type ubiquitin (44 μM) was added to extract, as indicated. Aliquots were withdrawn at indicated times and analyzed by SDS-PAGE and western blot. (c) Ubiquitin status in Xenopus extract under the indicated conditions was examined by anti-ubiquitin western blot. (d) Levels of Ub-charged endogenous ubch10 (ubch10−Ub) were examined by anti-ubch10 western blot analysis. Aliquots were removed at the indicated times and quenched with either non-reducing sample buffer to examine levels of ubch10−Ub or reducing sample buffer to examine total levels of endogenous ubch10. (e) Same as in d, but levels of endogenous ubiquitin charged ubch5 (ubch5−Ub) or total levels of ubch5 were examined by western blotting.
and thioesters in extract under these conditions revealed that inhibition of isopeptidase activity
with 20 μM UbVS led to near complete discharge of E2–Ub thioesters (‘∼’ represents a thioester
bond between the active-site cysteine of an E2 and the C-terminal glycine of ubiquitin) (Figure
2.4d, e). These trends paralleled the gradual disappearance of ubiquitinated species in the extract
(Figure 2.4c). Addition of exogenous ubiquitin restored charging of E2 enzymes and formation of
ubiquitin conjugates in the extract. Importantly, addition of wild-type ubiquitin to UbVS-treated
extract led to reduction in UBCH10, but not UBCH5, levels (Figure 2.4d, e), suggesting that
deubiquitinases may actively oppose UBCH10 degradation in Xenopus extracts.
Discussion

In our studies, we sought to evaluate how the nature of the ubiquitin signal constructed on cyclin B1 influences its degradation by the 26S proteasome. The manner in which cyclin B1 is ubiquitinated, however, leads to a heterogeneous mixture of conjugates of different ubiquitin mass which can be difficult to visualize by western analysis. Furthermore, western analysis may not distinguish between ubiquitin intermediates \textit{en route} to the proteasome and those bearing a non-proteolytic ubiquitin tag. Thus, to evaluate the role of different ubiquitin linkages in proteasomal targeting of cyclin B1, we developed a quantitative assay to measure cyclin B1 degradation products. Here, we describe a novel approach where \textit{Xenopus} extracts are made largely dependent on exogenous ubiquitin, allowing analysis of the role of ubiquitin-chain formation in APC-dependent proteolysis of cyclin B1.

Several studies have attempted to examine the role of ubiquitin-chain topology in proteasomal degradation by adding mutant ubiquitin to extracts (Hershko et al., 1991; Jin et al., 2008; Wu et al., 2010) or by injecting the ubiquitin into cells (Jin et al., 2008). Jin and colleagues found that ubiquitin incapable of forming Lys11 linkages (Ub\textsuperscript{11R}) delayed early cell divisions when injected into \textit{Xenopus tropicalis} embryos (Jin et al., 2008). However, the specific mechanism of delay and rates of degradation of APC substrates were not directly addressed. Therefore, it is possible that injection of Ub\textsuperscript{11R} perturbs other processes required for division of early embryos. In contrast, we found that adding such modified forms of ubiquitin typically accelerated degradation, with the exception of methylated ubiquitin. A possible explanation for the lack of effect of chain-terminating ubiquitins, such as Ub\textsuperscript{11R} or Ub\textsuperscript{triR}, is that any added ubiquitin must compete with the endogenous ubiquitin pool. Therefore, dominant-negative effects may be difficult to observe when conducted in a background that contains wild-type ubiquitin. Alternatively, our data suggest that despite their potential effects on chain termination, these ubiquitins are indeed capable of contributing to a signal that can promote degradation.
Intriguingly, the stimulatory effect of ubiquitin addition was more pronounced for an N-terminal fragment of cyclin B1 (cycB1-NT) relative to full-length cyclin B1 (data shown in chapter II and III). One possible explanation for this difference is that the N-terminal fragment binds to APC with lower affinity compared to the full-length protein, imposing a requirement for higher levels of ubiquitin-charged E2 for efficient degradation. Such a phenomenon would be reminiscent of the implicated role of a complex formation with a CDK partner and a Cks protein for cyclin recruitment to the phospho-APC/C and efficient degradation (van Zon et al., 2010; Wolthuis et al., 2008). Also, our work indicates that cyclin B N-termini from different species may have different affinities for the APC/C. An interesting question is whether the lysine profile of these proteins influences the stability of interaction with the E2-E3 complex. Sea urchin cyclin B, having fewer lysines in its N-terminal domain that can be targeted by proximally-acting E2 such as UBCH10, may dissociate from the APC/C more rapidly and therefore require more processive ubiquitination such as catalyzed by the chain-extending Ubc1 (Chen and Pickart, 1990; Rodrigo-Brenni and Morgan, 2007) or UBE2S (Garnett et al., 2009; Williamson et al., 2009; Wu et al., 2010) for its timely destruction. Further experiments will be necessary to better understand these interesting aspects of the behavior of the system.

Our analysis uncovered a strong dependence of the rate of cyclin proteolysis on the levels of free ubiquitin in extract. Addition of exogenous ubiquitin stimulated degradation, whereas inhibition of ubiquitin recycling suppressed degradation. Such a relationship between ubiquitin availability and APC-dependent proteolysis has not been previously appreciated, and it leads to the interesting possibility that control of ubiquitin availability may be a new mechanism by which the rate of APC substrate degradation could be controlled. Future experiments are required to examine whether and how ubiquitin availability may be controlled by cell-cycle regulatory mechanisms. Supplementing UbVS-treated extracts with ubiquitin restores cyclin proteolysis to levels seen in non-treated extract. This finding indicates there may be no requirement for UbVS-sensitive deubiquitination to support cyclin degradation, beyond a role in maintaining levels of
free ubiquitin. Interestingly, as long as ubiquitin is supplied to sufficient levels, the rate of cyclin degradation is no faster in a UbVS-treated extract relative to a non-treated extract. This may indicate that there are few, if any, UbVS-sensitive DUBs that actively antagonize cyclin B1 degradation in mitotic extracts. Alternatively, for deubiquitinating enzymes to be able to antagonize degradation, it may be crucial that the rate of ubiquitination be constrained by restricting availability of free ubiquitin or by rendering ubiquitin transfer less processive.

UbVS-treated extracts provide an opportunity to rigorously examine the degradative function of different ubiquitin-ubiquitin linkages. While the role of UbVS-sensitive DUBs in this process cannot be ascertained using this approach, systematic analysis is likely to provide clues about potential contribution. In the context of cyclin B1, UbVS-sensitive deubiquitination did not appear to appreciably oppose proteasomal targeting. However, APC substrates may be differentially sensitive to the action of such deubiquitinating enzymes and the UbVS approach may allow for this idea to be directly tested. In this regard, the APC was previously suggested to establish temporal order of destruction of its targets based on relative differences in the processivity of ubiquitination (Rape et al., 2006). How deubiquitination may modulate the ubiquitin signal APC builds on various substrates and potentially have an active role in establishing the correct sequence of proteasomal targeting have not been clarified. The UbVS system may provide an avenue to examine how the balance of ubiquitination and deubiquitination influences the temporal order of degradation of APC substrates.
Methods

Antibodies and biochemical reagents

Proteins were separated by SDS-PAGE on NuPAGE 4-12% or 12% Bis-Tris gels (Invitrogen), followed by wet transfer to PVDF. Sources of antibodies for immunoblotting were as follows: anti-cyclin B1 (Ab-2; RB-008-P, Neomarkers), anti-UBCH10 (A-650, Boston Biochem and AB3861, Millipore), anti-UBCH5 (A-615; Boston Biochem), anti-ubiquitin (P4D1; sc-8017; Santa Cruz Biotechnology). Secondary antibodies used include anti-goat IgG-HRP (sc-2020; Santa Cruz Biotechnology), anti-rabbit IgG-HRP (NA934; GE Healthcare), and anti-mouse IgG-HRP (NA931; GE Healthcare). MG262 (I-120), Ub^{me} and ubiquitin mutants except for Ub^{(11+48)R}, Ub^{(11+63)R} and Ub^{triR} were purchased from Boston Biochem. TAME (T4626) and ubiquitin (U6253) were purchased from Sigma.

Preparation of recombinant proteins

Ubiquitin mutants Ub^{(11+48)R}, Ub^{(11+63)R} and Ub^{(11+48+63)R} (referred to as Ub^{triR}) were generated by introducing arginine codons (AGA and AGG) at the indicated sites through PCR-mediated mutagenesis of the human ubiquitin sequence (cloned in pET3a with ampicillin resistance, the kind gift of C.M. Pickart). Plasmids were verified by sequencing and the purified proteins analyzed by mass spectrometry. To ensure efficient arginine incorporation, BL21 (DE3) cells were co-transformed with pJY2, developed by Pickart lab (You et al., 1999), which carries T7 lysozyme (LysS) and a gene encoding tRNA_{UCU}^{\text{Arg}}. Cultures were grown at 37 °C to an attenuation (D) of ~ 0.5 at 600 nm, and induced with 100 μM IPTG at $D_{600} = 0.6$ at 25 °C for 5 h. Cells were ruptured by sonication in QA lysis buffer (50 mM HEPES (pH 7.7), 100 mM KCl, protease-inhibitor cocktail, 5 mM 2-mercaptoethanol, 10 μg ml^{-1} DNase). Lysozyme was added to 1 mg ml^{-1} concentration and lysate was incubated with rotation at 4 °C for 15 min. Following sonication, cell lysates were clarified by centrifugation and the resulting supernatants applied to a
Q column. The flow-through containing ubiquitin was concentrated and purified by size-exclusion chromatography. Fractions containing ubiquitin were typically > 95% pure.

To generate full-length cyclin B-CDK1 complex, human cyclin B1 and CDK1 baculoviruses were used as described previously (Kirkpatrick et al., 2006). Baculovirus was added to Sf9 cells and cyclin B1 expression was allowed for 2.5 days. CDK1 was expressed separately in Sf9 cells and then combined with lysate from cells expressing cyclin B1 to allow formation of complex, which was then purified through Ni-NTA affinity and gel filtration chromatography.

CycB1-NT (1-88 amino acids of human cyclin B1), containing an HA tag at the N terminus and a 6xHis tag at the C terminus was generated using PCR amplification with forward primer (5'-CCA GGA CCA TGG GTT ACC CAT ACG ATG TTC CAG ATT ACG CTG GCT CGA TGG CGC TCC GAG TCA CG-3') and reverse primer (5'-GGG AGC CTC GAG CTA GGG AGC GTG ATG GTG ATG GTG ATG CAT AGG TAC CTT TTC AAG AGG-3'). The resulting PCR product was digested with NcoI and XhoI for subcloning into pET28a. Plasmids were verified by restriction enzyme mapping and sequencing. For 35S labeling in Escherichia coli, cultures (50 ml) were grown at 37 °C to \(D_{600 \text{nm}} = 0.8\), then collected by centrifugation (3,700g for 15 min, at 4 °C) and resuspended in modified M9 medium (50 ml final volume). After resuspension in modified M9 medium, cells were allowed to grow for additional 15 min at 37 °C before 5 mCi of Easy Tag\textsuperscript{TM} L-[35S]-Methionine (NEG709A005MC; Perkin Elmer) was added. Expression was induced with 0.5 mM IPTG for 2.5 h at 37 °C. Cells were ruptured in 5 ml g\textsuperscript{-1} of pellet guanidine-HCl lysis buffer (pH 8.0) and lysates rotated at 24 °C until the lysate became slightly translucent; approximately 45 min. Lysates were clarified by centrifugation and cycB1-NT was purified using Ni-NTA affinity chromatography (Qiagen). Eluted protein was desalted into XB buffer (100 mM KCl, 0.1 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 10 mM HEPES, pH 7.8 with KOH), supplemented 2% glycerol, protease inhibitors and phenylmethylsulfonyl fluoride, and stored at –20 °C.
Preparation of Xenopus egg extract

Interphase Xenopus egg extract was prepared from eggs laid overnight according to the protocol of Murray (Murray, 1991) with the exception that eggs were activated with 2 μg/ml calcium ionophore (A23187, free acid form, Calbiochem) for 30 min prior to the crushing spin. Extract was frozen in liquid nitrogen and stored at -80 ºC. Interphase extract was induced to enter mitosis by addition of non-degradable cyclin B, which activates CDK1 and stimulates mitotic phosphorylation, resulting in APC/C activation. A fusion of the maltose-binding protein (MBP) to Xenopus cyclin B lacking its N-terminal 90 amino acids (MBP-Δ90) (Salic and King, 2005) was expressed in E. coli by inducing cultures at an $D_{600nm}=0.6$ with 300 μM isopropylthiogalactoside (IPTG) for 5 h at room temperature. Purification was carried out following New England BioLabs (NEB) protocol. To make mitotic extract, MBP-Δ90 was added to interphase extract generally at ~ 20 μg ml$^{-1}$ and incubated at 22-24 ºC for 45-60 min.

Cyclin B1 degradation in Xenopus egg extract

Degradation assays where non-ubiquitinated cyclin B1 was added to extract were generally performed in 40 μl total volume per reaction condition, with extract constituting 75-80% of that volume. For experiments with TAME and MG262, extracts were pre-treated with relevant compound or dimethyl sulfoxide (DMSO) for 15 min at 24 ºC, with agitation (1,250 r.p.m.). For assays containing no UbVS, extracts were supplemented with ubiquitin as indicated or buffer (for untreated sample) concomitantly with ~200-250 nM substrate. For experiments with UbVS, interphase or mitotic extracts were treated with UbVS for 30 min at 24 ºC, with agitation (1,250 r.p.m.) before addition of ubiquitin and cyclin B1. Extracts contained 100 μg ml$^{-1}$ cycloheximide to prevent re-incorporation of free labeled amino acid. For competition assays, unlabeled competitor was added concomitantly with radiolabeled cyclin B1 and degradation was initiated. Degradation experiments were performed at 24 ºC, with agitation (1,250 r.p.m.), with samples withdrawn at indicated times. Samples for proteolysis of unlabeled full-length cyclin B1-
CDK1 complex were combined with SDS sample buffer and subjected to SDS-PAGE and immunoblot analysis using anti-cyclin B1 polyclonal antibody (Ab-2, Neomarkers). In degradation assays with $^{35}$S-labeled cycB1-NT, reactions (3 μl per time point) were quenched with 97 μl of 20% trichloroacetic acid (TCA) (in H$\text{}_2$O), vortexed and incubated on ice ≥ 30 min before centrifugation at 14,000g, at 4 °C. A fraction (50%) of sample supernatants was combined with NaOH to neutralize the acid and added to Ultima Gold scintillation fluid (6013327, Perkin Elmer). The radioactivity in the samples was measured by scintillation counting using a Packard scintillation counter. Proteolysis was measured by release of TCA soluble counts and is plotted as the percentage of input radiolabeled cyclin B1 protein.

Ubiquitin dynamics in *Xenopus* extract were examined by anti-ubiquitin western blot analysis. Levels of specific E2s in *Xenopus* extract were examined by western blot analysis. Samples of extract removed at the indicated times were mixed with DTT-containing sample buffer and boiled to examine total endogenous levels of specific E2 enzymes. To analyze levels of ubiquitin-charged endogenous ubch10 (ubch10~Ub) or ubch5 (ubch5~Ub), aliquots were removed at the indicated times and quenched with non-reducing sample buffer.
References


Chapter III: APC/C-mediated multiple monoubiquitination provides an alternative degradation signal for cyclin B1

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N.D. and R.W.K. designed and interpreted the experiments. N.D. carried out and analyzed all experiments except those outlined below. N.A.H. carried out cyclin B1 ubiquitylation for ubiquitin-AQUA analysis and degradation assays with these species in APC/C-depleted extract. D.S.K. carried out the ubiquitin-AQUA analysis on cyclin B1 ubiquitylated in vitro with the E2 UBC4 and different ubiquitin types in the laboratory of S.P.G. B-H.L. provided purified human proteasomes with oversight from D.F. M.L.B. helped with cloning of different cyclin B1 mutants.
Abstract

The Anaphase-Promoting Complex/Cyclosome (APC/C or APC) regulates progression through mitosis by orchestrating the ubiquitination of cell-cycle regulators such as cyclin B1 and securin. Although Lys48-linked ubiquitin chains represent a canonical signal targeting proteins for degradation by the proteasome, they are not required for the degradation of cyclin B1 in a reconstituted system. Recently, Lys11-linked ubiquitin chains have been implicated in degradation of APC/C substrates, but the Lys11-chain forming E2 UBE2S is not essential for mitotic exit. Together these findings raise important questions about the nature of the ubiquitin signal that targets APC/C substrates for degradation. Here, using a reconstituted system and *Xenopus* egg extracts, we demonstrate that multiple monoubiquitination of cyclin B1, catalyzed by UBCH10 or UBC4/5, is sufficient to target cyclin B1 for destruction by the proteasome. However, elaboration of Lys11-linked polymers becomes increasingly important when the number of ubiquitinatable lysines in cyclin B1 is restricted. We therefore define a novel proteolytic signal in the ubiquitin-proteasome pathway that confers flexibility in the requirement for particular E2 enzymes in modulating the rate of ubiquitin-dependent proteolysis.
Introduction

Protein ubiquitination regulates many aspects of cell physiology, including protein degradation. A uniform Lys48-linked ubiquitin polymer was the first signal identified to target substrates for destruction by the 26S proteasome (Chau et al., 1989; Finley et al., 1994; Thrower et al., 2000). Recent work has demonstrated that the repertoire of proteolytic signals encompasses chains of other linkage types, including Lys11-linked ubiquitin chains (Baboshina and Haas, 1996; Garnett et al., 2009; Jin et al., 2008; Matsumoto et al., 2010; Williamson et al., 2009; Wu et al., 2010; Xu et al., 2009) and short chains of mixed linkage types (Kirkpatrick et al., 2006). In contrast, polymers built through Lys63 of ubiquitin have non-proteolytic roles in DNA repair (Hofmann and Pickart, 1999; Spence et al., 1995), kinase activation (Deng et al., 2000), protein trafficking (Pickart and Fushman, 2004; Yang et al., 2009) and translation (Spence et al., 2000). Similarly, the transfer of a single ubiquitin moiety to one (monoubiquitination) or to multiple sites (multiple monoubiquitination) in a substrate has been implicated in mostly non-proteolytic processes (Robzyk et al., 2000; Terrell et al., 1998), although multiple monoubiquitination can target receptor tyrosine kinases (RTKs) to the lysosome (Haglund et al., 2003; Huang et al., 2006; Mosesson et al., 2003). More recently, multiple monoubiquitination has been shown to control proteasomal processing of the p105 NF-kB precursor to the shorter p50 subunit (Kravtsova-Ivantsiv et al., 2009). To date, multiple monoubiquitination has not been coupled with rapid and complete proteolysis of a proteasome substrate.

The E3 ligase activities of the Skp1-Cullin-F-box complex (SCF) family and the Anaphase-Promoting Complex/Cyclosome (APC/C or APC) have long been recognized to be essential for cell-cycle progression (Harper et al., 2002; Peters, 2006; Skaar and Pagano, 2009). Unlike the SCF, which cooperates with the E2 Cdc34 to assemble uniform Lys48-linked ubiquitin polymers on substrates (Petroski and Deshaies, 2005), the APC/C works in conjunction with UBCH10 (also known as E-2C) and enzymes of the UBC4/5 family to catalyze chain formation...
through three lysine residues of ubiquitin (Lys11, Lys48 and Lys63) (Kirkpatrick et al., 2006). UBCH10 builds multiple short ubiquitin chains on cyclin B1, which are sufficient to target the protein for degradation by the proteasome (Kirkpatrick et al., 2006). In this context, Lys48-linked ubiquitin polymers are dispensable for binding of modified cyclin B1 to ubiquitin receptors and degradation by the proteasome (Kirkpatrick et al., 2006). More recent work suggests that the assembly of proteolytic signal on APC/C substrates may occur in two stages. In budding yeast, Ubc4 initiates ubiquitin conjugation, whereas Ubc1 elongates ubiquitin chains (Rodrigo-Brenni and Morgan, 2007). Similarly, in metazoans, UBCH10 has been proposed to initiate monoubiquitination of the substrate, followed by UBE2S-dependent extension of Lys11-linked ubiquitin chains (Garnett et al., 2009; Williamson et al., 2009; Wu et al., 2010). Consistent with this idea, depletion of UBE2S from *Drosophila* S2 cells results in a strong delay in a metaphase-like state and stabilization of cyclin B1 at the spindle poles, among other mitotic defects (Williamson et al., 2009). In contrast, UBE2S is not essential for normal mitosis in human HeLa cells and is largely dispensable for timely proteolysis of cyclin B1 in this context (Garnett et al., 2009). These findings imply that UBE2S may not be uniformly required for mitosis, but rather may be important for substrate proteolysis under conditions where APC/C activity is compromised such as during recovery from drug-induced spindle-assembly checkpoint (SAC) activation (Garnett et al., 2009).

The ability of the APC/C, in conjunction with the E2 UBCH10 to extend multiple short ubiquitin chains through lysines 11, 48 and 63 of ubiquitin *in vitro* (Kirkpatrick et al., 2006) raises the question as to whether long homotypic ubiquitin-chain formation is indeed required for proteolysis of APC/C substrates. Even if ubiquitin chains are important, it remains unclear whether this requirement arises from a specific role of UBE2S, or through the chain-forming activity of UBCH10 (Jin et al., 2008; Kirkpatrick et al., 2006). However, it has been challenging to study the role of specific ubiquitin linkages in substrate turnover in cellular extracts due to the presence of endogenous ubiquitin. Using a novel approach in which *Xenopus* extracts are made
largely dependent on exogenous ubiquitin, we sought to understand whether APC/C-catalyzed proteolysis is strictly dependent upon polyubiquitination and whether the proteolytic machinery exerts a requirement for Lys11 or other ubiquitin linkages to efficiently degrade cyclin B1.
Results

Inhibiting ubiquitin-chain formation has only a modest effect in stabilizing cyclin B1 in Xenopus extract

We previously showed that by suppressing deubiquitinating activity with ubiquitin-vinyl sulfone (UbVS) we can impose a state of ubiquitin deficiency in Xenopus extract that strongly stabilized cyclin B1 (chapter II). Addition of physiologically relevant concentrations of wild-type ubiquitin fully rescued cyclin degradation, as measured by the production of acid-soluble radioactive counts. Using this system, we next tested the ability of chain-terminating ubiquitin mutants to restore proteolysis of $^{35}$S-labeled N-terminal fragment of human cyclin B1 (cycB1-NT) in UbVS-treated extracts. Different Ub mutants containing a single lysine-to-arginine substitution at position 11 (Ub$^{11R}$), 48 (Ub$^{48R}$), or 63 (Ub$^{63R}$), or at all three positions simultaneously (Ub$^{triR}$) restored cycB1-NT proteolysis under these conditions, albeit with different kinetics. Addition of wild-type ubiquitin, Ub$^{48R}$, and Ub$^{63R}$ rescued degradation most efficiently, showing a half-life of approximately 15 minutes (Figure 3.1a). After 20 minutes, the rate of degradation slowed substantially, likely a consequence of ubiquitin depletion in the UbVS-treated extract, as supplementation with additional ubiquitin restored degradation to the initial rate (Figure 3.1b). Extracts supplemented with Ub$^{11R}$ or Ub$^{triR}$ degraded substrate only somewhat more slowly. These results were unexpected, as mass spectrometric analysis indicated that elimination of all three principal sites of Ub-Ub linkage by the APC/C rendered Ub incapable of forming ubiquitin chains in reconstituted reactions (data not shown). Even addition of Ub$^{me}$ supported degradation, with a half-life of approximately 30 minutes. We next assessed how constraining the topology of ubiquitin chains to a single lysine residue would affect degradation of cycB1-NT (Figure 3.1c). Addition of Ub$^{K11only}$ or Ub$^{K48only}$ to UbVS-treated extract restored cycB1-NT proteolysis, but with slower kinetics compared to wild-type ubiquitin. We were surprised that Ub$^{K11only}$ did not support robust degradation, as previous reports indicated that it
Figure 3. Ubiquitin chain formation is not essential for cycdin B1 degradation in UbVS-treated Xenopus extract. $^{35}$S-labeled cycB1-NT and different forms of Ub were introduced concomitantly into mitotically-arrested Xenopus extract that had been pre-treated with UbVS (20 μM) or buffer (referred to as untreated) for 30 min. Proteolysis was measured by release of trichloroacetic acid (TCA) soluble counts, and plotted as the percentage of input radiolabeled cycB1-NT. Trends are representative of three or more independent experiments.

(a) Ubiquitin types (44 μM) with singly lysine-to-arginine mutations at indicated positions or at all three positions Lys11, 48, and 65 (Ub$^{K=R}$) simultaneously were added to UbVS-treated extract.

(b) Ubiquitin$^{18}$ (46 μM) and substrate were introduced into UbVS-treated extract, and supplemented with Ub$^{118}$ (33 μM) or buffer control 15 min after initiation of degradation. Ubiquitin$^{18}$ only refers to ubiquitin that has all of its lysines, except for the specified, mutated to arginines. (d) Degradation was measured in the presence of different ubiquitin types (44 μM) containing arginine substitutions at two of the three principle sites (Lys11, Lys46, and Lys65) of ubiquitin-ubiquitin conjugation by the APC.
can efficiently support APC/C-dependent ubiquitination (Jin et al., 2008; Williamson et al., 2009; Wu et al., 2010). One possibility is that mutation of Lys6 of ubiquitin may have an inhibitory effect on proteasomal degradation (Shang et al., 2005). We therefore tested the effect of restricting chain formation to one of the three principle sites of ubiquitin-ubiquitin attachment mediated by UBCH10 by mutating the remaining two (data not shown). In these experiments, Ub$^{(48+63)R}$ stimulated degradation efficiently, consistent with the ability of Lys11 linkages to support degradation (Figure 3.1d). Ubiquitin forms supporting Lys48 and Lys63 linkages (Ub$^{(11+63)R}$ and Ub$^{(11+48)R}$, respectively) and Ub$^{trR}$ supported proteolysis with somewhat slower kinetics. Together these findings indicate that even when deubiquitinating enzymes are inhibited, the ability to construct Lys11-linked chains provides a kinetic advantage for degradation, although the advantage is modest. In principle this advantage could arise from the utilization of Lys11 as one of three sites (in addition to Lys48 and Lys63 of ubiquitin) in chain-forming reactions catalyzed by UBCH10, or from a role of UBE2S, which elongates ubiquitin chains exclusively through Lys11 linkages.

**Ubiquitin chains are required for cyclin B1 degradation only when the number of available lysine residues in cyclin B1 is restricted**

To rule out the possibility that our results thus far were influenced by use of an N-terminal fragment of cyclin B1, we examined proteolysis of full-length wild-type cyclin B1 bound to CDK1 by immunoblotting (Figure 3.2c, left panel). Addition of Ub$^{11R}$ or Ub$^{trR}$ stimulated degradation, albeit at slightly reduced rates relative to wild-type ubiquitin. Ubiquitin$^{me}$ also supported substantial degradation of cyclin B1, although a small fraction of the protein accumulated in a triply-ubiquitinated species. Higher molecular-weight forms were not observed, possibly because they were targeted for degradation by the proteasome.
Figure 3.2 Cyclin B1 proteolysis depends on lys11-linked ubiquitin-chain formation only when the number of available lysine residues is restricted. (a) Sequence comparison of the cyclin B1 N termini from multiple species reveals lysine richness. Lysine residues are colored in blue; the destruction box (D-box) is colored in orange. (b) Schematic representation of the N-terminal region (residues 1-88) of human cyclin B1 with lysine residues denoted K in blue and with the D-box motif denoted with an orange rectangle. Cyclin B1 mutants (cycK192/194 and cycK195,197,199) were generated by substituting lysine with arginine at all but the specified lysine residues within the first 116 amino acids (residues 89-115 not shown). CycWT, wild-type cyclin B1. (c) Purified full-length wild-type (cycWT) or single-lysine (cycK191/193) cyclin B1, in complex with CDK1, and forms of Ub (20 μM) as indicated were added to mitotic Xenopus egg extract that had been pre-treated with UBVS (20 μM) for 30 min. Stability of the exogenous substrate over time was assessed by SDS-PAGE and cyclin B1 western blot analysis. (d) As in c, except the behavior of full-length cycK195,197,199, in complex with CDK1, was analyzed.
Cyclin B1 contains 18 lysine residues in its unstructured N-terminal region upstream of the cyclin box; 15 of these lysine residues are located within the first 88 amino acids close to the destruction box (Figure 3.2a), providing a platform upon which a proteolytic signal consisting of multiple monoubiquitins or short ubiquitin chains can be assembled. To examine whether reducing the number of lysines in cyclin B1 renders its proteolysis dependent on ubiquitin-chain formation, we measured degradation of cyclin B1 mutants that contained either one or four ubiquitinatable lysine residues in the first 115 amino acids at position 64 only (cyc\textsuperscript{K64only}) or at positions 59, 63, 64, and 67 (cyc\textsuperscript{K59,63,64,67only}) (Figure 3.2b; Figure 3.2c, right panel; Figure 3.2d). We chose these positions as mass spectrometry studies indicated that these lysine residues become ubiquitinated early in the course of reconstituted ubiquitination reactions (D. K., N. H., unpublished observations). Cyc\textsuperscript{K64only} was degraded rapidly in untreated Xenopus extract, and was fully stabilized in UbVS-treated extract. However, unlike the case for wild-type cyclin B1, Ub\textsuperscript{11R} was not able to efficiently stimulate the degradation of cyc\textsuperscript{K64only}. Similar results were obtained with Ub\textsuperscript{triR}. Interestingly, Ub\textsuperscript{me} did not rescue degradation, but instead caused quantitative accumulation in a monoubiquitinated form. These results contrast strikingly with the behavior of wild-type cyclin B1, where the majority of the protein was degraded upon addition of Ub\textsuperscript{me}.

We next determined whether restoration of a limited number of lysine residues could enable degradation in the presence of chain-terminating ubiquitins (Figure 3.2d). Surprisingly, cyc\textsuperscript{K59,63,64,67only} was degraded somewhat more slowly than cyc\textsuperscript{K64only} in untreated extract, for reasons that remain unclear and may include posttranslational modifications within this lysine cluster that are inhibitory to APC-dependent proteolysis. Addition of Ub\textsuperscript{11R} partially restored degradation of cyc\textsuperscript{K59,63,64,67only} in a UbVS-treated extract. The most striking difference was observed in extracts supplemented with Ub\textsuperscript{me}. Whereas cyc\textsuperscript{K64only} accumulated quantitatively in a monoubiquitinated form, cyc\textsuperscript{K59,63,64,67only} was unstable, although a fraction of the protein accumulated in mono- and di-ubiquitinated species. We conclude that when deubiquitinating enzymes are inhibited, the attachment of single ubiquitin molecules to multiple lysine residues in
cyclin is sufficient to target the substrate for degradation. Strict dependence on elaboration of ubiquitin chains appears to occur only when the number of available substrate lysines is restricted.

**Multiple monoubiquitination can target cyclin B1 for efficient degradation in a reconstituted system and in Xenopus extract**

We next assessed whether the effects of different ubiquitin mutants on proteolysis paralleled their effects on ubiquitin conjugation using a reconstituted system. In APC/C reactions reconstituted with 100 nM UBCH10, we found that elimination of Lys48 or 63 of ubiquitin had no effect on the mass of conjugates generated in 15-minute reactions, consistent with the fact that these mutations had little effect on degradation in UbVS-treated extracts (Figure 3.3a, top panel). Interestingly, elimination of Lys11 reduced the mass of conjugates formed, consistent with the previously reported preference of UBCH10 for synthesizing Lys11 linkages (Jin et al., 2008; Kirkpatrick et al., 2006). These differences became more pronounced in longer ubiquitination reactions (Figure 3.3a, bottom panel). In the presence of ubiquitin types that do not support ubiquitin-polymer assembly (Ub\textsubscript{trIR} and Ub\textsubscript{res}), the maximal extent of substrate modification (5-6 ubiquitins per cyclin B1 molecule) was observed at early time-points and remained unchanged in longer reactions (Figure 3.3a), implying that there likely is a limited subset of preferred ubiquitination sites in cyclin B1. A time-course of ubiquitination with either wild-type Ub or Ub\textsubscript{trIR} (Figure 3.3b) revealed that, at physiologically relevant E2 concentrations, the conjugation of ubiquitin monomers to distinct lysines in cyclin B1 occurs with rapid kinetics. Furthermore, conjugates bearing 4 or more ubiquitin moieties were capable of binding proteasome-associated ubiquitin-receptors (Deveraux et al., 1994; Elsasser et al., 2004; Elsasser and Finley, 2005; Finley, 2009; Isasa et al., 2010; Matiuhin et al., 2008; Peth et al., 2010; Rao and Sastry, 2002; Riedinger et al., 2010) including Rpn10 (Figure 3.3c, d) and Rad23 (Figure 3.3e, f), in a manner
Figure 3.3 UBCH10 and APC/C catalyze rapid multiple monoubiquitination of cyclin B1 that is sufficient for binding ubiquitin receptors. (a) Western blot analysis of the in vitro ubiquitination reaction containing full-length cyclin B1, APC/C immunopurified from mitotically arrested Xenopus extract, recombinant UBCH10 (100 nM), and forms of ubiquitin (110 μM), as indicated. Ubiquitin types with lysine-to-arginine mutations at one, two or all three positions Lys11, 48 and 63 (Ub^mm) simultaneously, as well as methylated ubiquitin (Ub^mm) were used. Control "APC/C" reactions containing all components except for the E3 ligase were performed in parallel. Reactions were allowed to proceed for 15 or 90 min before analysis by SDS-PAGE and western blotting against cyclin B1. (b) Time-course of the in vitro ubiquitination of full-length wild-type cyclin B1 with wild-type ubiquitin or Ub^mm and remaining components as in a. (c-f) Binding of ubiquitinated cyclin B1 to GST-tagged ubiquitin receptors. Cyclin B1-ubiquitin conjugates were incubated with immobilized receptor proteins for 1 h at 4 °C before reaction products were subjected to SDS-PAGE and western blot analysis against cyclin B1. Equivalent amounts of input (I), flow-through (FT) and bound (B) fractions were loaded in adjacent lanes. Binding experiments with wild-type Rpn10 (c) and Rad23 (e). Binding with corresponding versions of the receptors lacking the ubiquitin-recognition domains, with engineered block substitution of the UIM domain (LAMAL → NINNNN) of Rpn10 (d) or deletion of the ubiquitin-associated domains of Rad 23 (f).
that depended on their ubiquitin-interaction domains. For conjugates of a similar molecular mass, substrate ubiquitinated with Ub\textsuperscript{triR} bound to receptors more efficiently than substrate ubiquitinated with Ub\textsuperscript{me}. Given that Ub-AQUA analysis indicates that these forms of ubiquitin suppress ubiquitin-chain formation with similar efficiency, this finding suggests that methylation of ubiquitin may compromise its affinity for ubiquitin receptors. We found similar binding patterns with cyclin B1-ubiquitin conjugates generated with UBC4 as the E2 (discussed in chapter IV). Together these results indicate that multiple monoubiquitination occurs rapidly and can result in a productive signal for binding ubiquitin receptors.

We next sought to determine whether multiple monoubiquitination can target cyclin B1 for degradation in a reconstituted system. To this end, full-length cyclin B1-CDK1 complex was ubiquitinated with UBC10, in conjunction with wild-type or chain-terminating ubiquitin. The resulting conjugates were incubated with purified human proteasomes that were washed with high salt concentrations to eliminate USP14, a deubiquitinating enzyme that can antagonize cyclin B1 degradation \textit{in vitro} (Hanna et al., 2006; Lee et al., 2010). These proteasomes, which retain the deubiquitinating enzymes RPN11 and UCH37 (Lee et al., 2010), rapidly degraded polyubiquitinated cyclin B1, generated with UBCH10 (Figure 3.4a). While conjugates formed with Ub\textsuperscript{triR} or lysine-less ubiquitin (Ub\textsuperscript{K0}) were efficiently degraded, those generated with methylated ubiquitin were degraded less rapidly, consistent with the defect in the ability of these conjugates to bind ubiquitin receptors (Figure 3.3c, e). Similar trends were observed for cyclin B1 species ubiquitinated with UBC4 as the E2 (discussed in chapter IV). The extent of conjugate proteolysis was quantitated using radiolabeled full-length cyclin B1, in complex with unlabeled CDK1, and was found to closely correlate with the fraction of input cyclin B1 bearing three or more ubiquitin molecules (data not shown). Degradation of cyclin B1 in the reconstituted system was confirmed to be both APC/C- and ubiquitin-dependent (data not shown). Interestingly, analogous experiments performed with radiolabeled cycB1-NT pre-ubiquitinated by UBCH10 (Figure 3.4b) revealed that degradation of multiply monoubiquitinated cyclin B1 was sensitive to
Figure 3.4 Multiply monoubiquitinated cycB1 is rapidly degraded by purified proteasomes and in Xenopus extract. (a) In vitro degradation assay with cycB1-Ub species generated with immunopurified Xenopus APC/C, recombinant UBC10 (250 nM), and forms of Ub (145 µM), as indicated, and USP14-deficient human proteasomes (20 nM). A ubiquitin type bearing lysine-to-arginine mutations at all three positions (lys11, 46 and 63 simultaneously (Ub<sup>all</sup>), methylated (Ub<sup>me3</sup>) or lysine-less (Ub<sup>0</sup>) ubiquitin were used as chain-terminating ubiquitins. WT, wild-type. Aliquots were removed at the indicated times and reaction products analyzed by SDS-PAGE and anti-cycB1 immunoblotting. (b) Autoradiograph of in vitro APC/C- and UBC10-catalyzed ubiquitination of [35S]-labeled cycB1-NT (1-96) with immunopurified Xenopus APC/C, recombinant UBC10 (100 nM) and forms of ubiquitin (145 µM) as indicated. Products from a 60-minute ubiquitination assay were separated by SDS-PAGE and analyzed using a phosphorimager. (c) CycB1-NT-ubiquitin species from b were incubated with purified human proteasomes (Ptsm, 20 nM) reconstituted with or without 20-fold molar excess of GST-tagged wild-type USP14. At indicated times, reactions were terminated by addition of trichloroacetic acid (TCA). Proteolysis was measured by release of TCA soluble counts, and is plotted as the percentage of input radioactively labeled cycB1 protein. (d) CycB1-NT-ubiquitin species from b were added to interphase Xenopus extract that had been pre-treated with UbVS (15 µM) or buffer control for 30 min. Reactions were terminated by addition of TCA at indicated times. Proteolysis was measured by release of TCA soluble counts, and is plotted as the percentage of input radioactively labeled cycB1-NT.
addition of the deubiquitinating enzyme USP14 (Figure 3.4c). This effect was reversed by IU1, an inhibitor of the catalytic activity of USP14 (Lee et al., 2010) (data not shown). Together these results indicate that purified proteasomes can efficiently degrade a multiply monoubiquitinated cyclin B1 and that USP14 can deubiquitinate this substrate to suppress degradation.

To determine whether multiple monoubiquitination could target the protein for degradation under conditions of physiological concentrations of proteasomes and in the presence of relevant DUB activities, we used the same ubiquitinated species from those analyzed in Figure 3.4b and added them to interphase *Xenopus* extract, a state in which the APC/C is inactive (Figure 3.4d). Conjugates generated with Ub$^{\text{triR}}$ or Ub$^{\text{me}}$ were degraded rapidly with initial rates very similar to that observed for conjugates generated with wild-type ubiquitin. However, a fraction of the conjugates generated with methylated ubiquitin were degraded less efficiently, likely because they were insufficiently ubiquitinated to be recognized by the proteasome. Similar results were obtained when the ubiquitin conjugates were introduced into extracts that were supplemented with excess non-ubiquitinated unlabeled competitor to prevent any potential APC/C-mediated ubiquitination (data not shown) or to extracts that had been immunodepleted of APC/C (data shown in chapter IV). Together these findings further indicate that *Xenopus* extracts can rapidly degrade a cyclin substrate bearing multiple ubiquitin monomers attached to distinct lysine residues.

Pre-treatment of extract with UbVS, at a concentration identical to that used to deplete free ubiquitin, had little ability to accelerate degradation of conjugates generated with wild-type ubiquitin or with Ub$^{\text{triR}}$, but modestly enhanced degradation of species modified with Ub$^{\text{me}}$ (Figure 3.4d). Deubiquitination appears to exert little effect on degradation of these pre-ubiquitinated species, perhaps because deubiquitinating enzymes such as USP14 are present at much lower levels in *Xenopus* extracts than in our reconstituted system. Consistent with this idea, addition of an inhibitor of USP14, IU1, failed to accelerate degradation of cyclin B1 in *Xenopus* extracts (data not shown).
Discussion

In this study, we have evaluated the role of ubiquitin-chain topology in targeting cyclin B1 for degradation in a reconstituted system, as well as in mitotic *Xenopus* cell-cycle extracts. Our study was motivated by recent findings suggesting that Lys11 linkages, mediated by the chain-forming E2 enzyme UBE2S, may be important for APC/C-dependent proteolysis. However, our earlier work suggested that APC/C, solely in conjunction with the E2 enzyme UBCH10 or the enzyme UBC4/5, can build a ubiquitin signal that is sufficient for degradation by purified proteasomes (Kirkpatrick et al., 2006). Here we provide a resolution to this paradox, demonstrating that conjugation of ubiquitin to multiple lysine residues of cyclin B1 provides an alternative degradation signal for cyclin B1 that does not require extension of Lys11-linked ubiquitin polymers. Lysine11-linked ubiquitin-chain formation becomes essential only when the number of available lysine residues in cyclin B1 is restricted.

Dominant negative effects of different ubiquitin types may be difficult to observe when examined in a background that contains wild-type ubiquitin. Using UbVS to inhibit deubiquitinases and their capacity to recycle ubiquitin, we were able to impose a state of ubiquitin deficiency in extract that strongly stabilized cyclin B1. Supplementing UbVS-treated extracts with ubiquitin rescues the proteasomal degradation of cyclin. As opposed to addition of excess ubiquitin, this approach allowed us to examine the functional significance of different linkages at physiological concentrations of ubiquitin. Under such conditions, any kinetic advantage specific ubiquitin linkages may confer in protein degradation may be ascertained and more easily uncovered.

The UbVS system enabled us to define the role of different ubiquitin-chain topologies in targeting cyclin B1 for degradation in *Xenopus* extracts. In agreement with earlier work in a reconstituted system (Kirkpatrick et al., 2006), Lys48 ubiquitin-ubiquitin linkages were not required for efficient cyclin proteolysis in UbVS-treated extract. Surprisingly in the light of recent
studies (Jin et al., 2008; Williamson et al., 2009; Wu et al., 2010), ubiquitin incapable of forming Lys11 linkages (Ub\textsuperscript{11R}) also supported very robust degradation of cyclin B1 (Dimova et al., 2012). Importantly, we found that chain-terminating ubiquitins (Ub\textsuperscript{\texttau R} and lysine-less ubiquitin), which greatly diminish chain elongation by UBCH10, UBC4/5, and UBE2S, also support robust rates of cyclin proteolysis. Methylated ubiquitin was somewhat less capable of supporting rapid cyclin degradation, which may reflect less efficient recognition by ubiquitin receptors and the proteasome due to modification of Lys6 of ubiquitin (Shang et al., 2005). However, upon restriction of ubiquitination to a single lysine residue in cyclin as in cyc\textsuperscript{K64only}, chain-terminating ubiquitins were no longer able to stimulate substrate degradation and cyclin accumulated as mono- and di-ubiquitinated species. Together these findings suggest that ubiquitin-chain formation is not essential for cyclin proteolysis, unless the number of available ubiquitination sites in the substrate is restricted.

Utilizing a reconstituted system, we demonstrated that UBCH10 can rapidly append mono-ubiquitin to multiple lysines in cyclin. Species bearing 4 or more ubiquitin moieties on distinct lysines were recognized by ubiquitin receptors. Based on these findings, we propose that the presence of multiple lysines in cyclin B1 that are in close proximity to one another has the potential for generating a high density of mono-ubiquitin that promotes receptor binding. In such an arrangement, the hydrophobic patches on distinct ubiquitin units may be available to form contacts with various ubiquitin-binding domains (UBDs). Whether particular spacing of ubiquitinated lysine residues is essential for recognition by ubiquitin receptors remains unknown. In our pull-down experiments, there may be some enhanced avidity resulting from a dimeric GST moiety positioning two ubiquitin-associated domains (UBAs) in close proximity (Sims et al., 2009). However, the rapid destruction of multiply monoubiquitinated species by purified proteasomes and in Xenopus extracts implies that this substrate must have sufficient affinity for proteasome-associated ubiquitin receptors.
The capacity of purified proteasomes to rapidly degrade multiply monoubiquitinated cyclin B1 was significantly attenuated by USP14, suggesting that USP14 can efficiently remove monoubiquitin, as well as trim Ub chains. However, such deubiquitinating activity did not appear to strongly antagonize proteasome function in *Xenopus* extract, as treatment of extract with UbVS or the USP14-specific inhibitor IU1 did not appreciably enhance turnover of pre-ubiquitinated cyclin. Although present in *Xenopus* extracts (N.V.D., R.W.K., unpublished data), levels of USP14 associated with proteasomes in extract may be insufficient to impede proteolysis. Together, these findings suggest the proteasome does not impose a requirement for ubiquitin-chain formation for efficient proteolysis of cyclin B1, even when DUBs are not inhibited by UbVS. This study further strengthens the view that the proteasome has the capacity to recognize and degrade substrates bearing ubiquitin modifications distinct from the canonical Lys48-linked polyubiquitin chains (Baboshina and Haas, 1996; Guterman and Glickman, 2004; Hershko and Heller, 1985; Hofmann and Pickart, 2001). The early work of Hershko and Heller demonstrated that methyl-ubiquitin addition to reticulocyte extracts can support the proteolysis of $^{125}$I-labeled lysozyme, but overall rates of degradation were slow, with ~12% of input substrate degraded within an hour (Hershko and Heller, 1985). While conjugation of mono-ubiquitin can promote degradation of the model substrate Pax 3 (Boutet et al., 2007), the kinetics of degradation are slow, with a half-life of 3.5 h. In contrast, multiple monoubiquitination of cyclin B1 provides a robust degradation signal.

Previously, we demonstrated that in conjunction with UBCH10 or UBC4/5 enzymes, the APC generates a proteolytic signal on cyclin comprised of Lys11 and Lys63, in addition to Lys48 ubiquitin linkages (Kirkpatrick et al., 2006). In this context where chains are nucleated on multiple substrate lysines, it cannot not be ruled out that distinct chains consisting of a series of identical linkages such as Lys11(Lys11(Lys11))) (Kirkpatrick et al., 2006) are assembled and function as the major degradative signal. This question has become increasingly important in the light of recent studies suggesting that synthesis of Lys11-linked polymers is central to APC-
dependent proteolysis (Williamson et al., 2009; Wu et al., 2010). Such a model is not consistent with our findings where ubiquitin incapable of forming Lys11 linkages (Ub\textsuperscript{11R}) or supporting chain-elongation (Ub\textsuperscript{lnR}) allows robust cyclin proteolysis in UbVS-treated extracts. Based on our findings in Xenopus extract and in a reconstituted system, we propose that through conjugation of monoubiquitin to multiple lysine residues, and possibly elaboration of some short chains, UBCH10, or possibly members of the UBC4/5 family, cooperates with the APC/C to generate a sufficient proteolytic signal on cyclin B1 (Figure 3.5). Our findings lend strong support to the idea that high local density of ubiquitin, independent of linkage, is sufficient to target a substrate for proteolysis. Upon restriction of available lysine residues, however, such ubiquitin density may not be attainable through the conjugation of monoubiquitin. In this context, ubiquitin moieties will have to be added to the end of a growing chain rather than to a lysine residue in the substrate in order to achieve the threshold necessary for proteolysis. Consistent with this idea, ubiquitin-chain formation becomes essential for proteasomal degradation when the number of available lysine residues in cyclin B1 is restricted. An important question is whether in this context the activity of the inherently less processive UBCH10 is sufficient to promote efficient ubiquitination and targeting to the proteasome, or whether there is a greater dependence on chain-elongation by the Lys11-specific E2 UBE2S (Garnett et al., 2009; Wickliffe et al., 2011; Williamson et al., 2009; Wu et al., 2010).
Multiple monoubiquitination/Short ubiquitin chains

Lys11-linked polyubiquitination

Figure 3.5 Model of cyclin B1 degradation in Xenopus cell-cycle extract. The APC/C and the E2 UBCH10 collaborate to transfer ubiquitin monomers to multiple lysine residues on cyclin B1, with subsequent elaboration of short ubiquitin chains containing K63, K48 and K11 linkages, with K11 linkages predominating (Nørkpatrick et al., 2006). On achievement of a threshold of ubiquitin mass, which appears to be 4-5 ubiquitin monomers, multiply monoubiquitinated substrate can associate with proteasome-associated ubiquitin receptors and be degraded efficiently. However, when the number of lysine residues in cyclin B1 is restricted, ubiquitination catalyzed by UBCH10 is insufficient for rapid proteolysis and the activity of another E2 in extending Lys11-linked ubiquitin polymers becomes important for efficient degradation.
Methods

Antibodies and biochemical reagents

Proteins were separated by SDS-PAGE on NuPAGE 4-12% or 12% Bis-Tris gels (Invitrogen), followed by wet transfer to PVDF. Sources of antibodies for immunoblotting were as follows: anti-cyclin B1 (Ab-2; RB-008-P, Neomarkers), anti-Cdc27 (610455, BD Transduction Laboratories), anti-UBCH10 (A-650, Boston Biochem; AB3861, Millipore), anti-UBE2S (N-14; sc-131354, Santa Cruz Biotechnology), anti-UBCH5 (A-615; Boston Biochem), anti-ubiquitin (P4D1; sc-8017; Santa Cruz Biotechnology). Secondary antibodies used include anti-goat IgG-HRP (sc-2020; Santa Cruz Biotechnology), anti-rabbit IgG-HRP (NA934; GE Healthcare), and anti-mouse IgG-HRP (NA931; GE Healthcare). Antibodies for immunoprecipitation or immunodepletion included: anti-Cdc27 (AF3.1; sc-9972) and anti-UBE2S (N-14; sc-131354) from Santa Cruz Biotechnology; anti-UBCH10 (gift from H. Yu, UT Southwestern, USA); and for control depletions, normal rabbit (sc-2027) and normal goat (sc-2028) IgG, both from Santa Cruz Biotechnology. UBE2S antibodies were coupled to UltraLink immobilized protein A/G beads (53132, Pierce). UBCH10 and CDC27 antibodies were coupled to Affiprep protein A beads (156-0006, Bio-Rad). Ubiquitin agarose (U-405), UbVS (U-202), MG262 (I-120), Ubme and ubiquitin mutants except for Ub(11+48)R, Ub(11+63)R and UbtriR were purchased from Boston Biochem. TAME (T4626) and ubiquitin (U6253) were purchased from Sigma.

Preparation of recombinant proteins

Ubiquitin mutants Ub(11+48)R, Ub(11+63)R and UbtriR (referred to as UbtriR) were generated by introducing arginine codons (AGA and AGG) at the indicated sites through PCR-mediated mutagenesis of the human ubiquitin sequence (cloned in pET3a with ampicillin resistance, the kind gift of C.M. Pickart). Plasmids were verified by sequencing and the purified proteins analyzed by mass spectrometry. To ensure efficient arginine incorporation, BL21 (DE3) cells were co-transformed with pJY2, developed by Pickart lab (You et al., 1999), which carries
T7 lysozyme (LysS) and a gene encoding tRNA$_{UCU}^{Arg}$. Cultures were grown at 37 °C to an attenuation ($D$) of ~0.5 at 600 nm, and induced with 100 μM isopropylthiogalactoside (IPTG) at $D_{600\,\text{nm}} = 0.6$ at 25 °C for 5 h. Cells were ruptured by sonication in QA lysis buffer (50 mM HEPES (pH 7.7), 100 mM KCl, protease-inhibitor cocktail, 5 mM 2-mercaptoethanol, 10 μg ml$^{-1}$ DNase). Lysozyme was added to 1 mg ml$^{-1}$ concentration and lysate was incubated with rotation at 4 °C for 15 min. Following sonication, cell lysates were clarified by centrifugation and the resulting supernatants applied to a Q column. The flow-through containing ubiquitin was concentrated and purified by size-exclusion chromatography. Fractions containing ubiquitin were typically > 95% pure.

To generate full-length cyclin B-CDK1 complex, human cyclin B1 and CDK1 baculoviruses were used as described previously (Kirkpatrick et al., 2006). To generate mutants of cyclin B1, DNA fragments encoding the N-terminal 124 amino acids of wild-type human cyclin B1 (5’-

AACCGGTCCGAAACCGTGCACATGTCGCATCACCATCACCATCACGGCTC
GATGGCGCTCCGAGTCACGCAATCAGAAAAATTAATGCTGAAAATATAAGCGAAAA
TCAACATGGCAGGCCAAGCGCGTTCCTTCTACGGCACCACGGCGCAACCTCCAAACCCG
GGCTGAGGCCAAGAACAGCTCTTTGGGACATTGTAACAAAGTCAGTGAAACAGCTA
CAGGCCAAAATGCTATGAAAAAGAAGCACAACCTTCAGCTACCAGTAAAGTCAT
TGATAAAAAACTACCAAAACCTCTTTGAAAAAGGTATCTATGCTGGGCTGAGTGCCAGT
GTCTGAGCCAGTGCAGCCGAGCCAAACCTTGAGCCAGAAGCTGCTGTTAAAGAG
AAAAACTTTTCGCTGAGCTTATTTTTGATCTAGCTAGCAATA-3’ or the same region of the protein with arginine substitutions at all lysine residues (cye$^{alR}$, 5’-

AACCGGTCCGAAACCGTCGACATGTCGCATCACCATCACCATCACGGCTC
GATGGCGCTCCGAGTCACGCAATCAGAAAAATTAATGCTGAAAATATAAGCGAAAA
TCAACATGGCAGGCCAAGCGCGTTCCTTCTACGGCACCACGGCGCAACCTCCAAACCCG
GGCTGAGGCCAAGAACAGCTCTTTGGGACATTGTAACAAAGTCAGTGAAACAGCTA
CAGGCCAGAATGCCTATGAGAAGAGAAGCAAGACCTTCAGCTACCGGTAGAGTCAT
TGATAGAAGACTAACAAGACCTCTCTTGAAGGGGTACCTATGCTGGTGCCAGTGCCAGT
GTCTGAGCCAGTGGCCAGAGCCAGAACCCTGAGCCAGAACCCTGAGCCTGTAGAGAGG
AAAGACTTTTTCGCTAGCCATTTTTGTTGATACCTGCTAGCAATA-3') preceded by 6 x
His tag were synthesized (GenScript). Using restriction enzyme digestion with Nhel and Rsrl, 
fragments were subcloned into pFASTBac containing the carboxy terminus (125-433 amino
acids) of cyclin B1. To generate cycK64only, primers 5'-
TCCAGACCCGGCTGAGGCAAGAACAGCTCTTGGGGACATTGGTAACAGAGTCAG
TGAACACGTACAGGCC-3' and 5'–
AATGACTCTACGGTACGGTACGGTACGGTACGGTACGGTACGGTACGGTAC
AGCTGTCTACTGAC - 3' were used for an extension reaction and the resulting fragments 
cloned into XmaI and AgeI cleavage sites of pFASTBac carrying full-length cycaltR. Plasmids 
were verified by restriction enzyme mapping and sequencing. Baculoviruses were generated 
according to the Bac-to-Bac manual (Invitrogen). Wild-type cyclin B1 was 35S-labeled in Sf9 
cultures with resuspending cells (1.5 x 10^6 cells ml^-1) in media containing 10% SF-900 II SFM 
and 90% SF-900 II SFM without methionine or cysteine (both from Invitrogen) to increase radio-
label uptake. Baculovirus was added to cells, along with 50 µCi of 35S-labelled methionine and 
cysteine (NEG772; Perkin Elmer), and cyclin B1 expression was allowed for 2.5 days. CDK1 
was expressed separately in Sf9 cells without radiolabeling and then combined with lysate from 
cells expressing cyclin B1 to allow formation of complex, which was then purified through Ni-
NTA affinity and gel filtration chromatography.

CycB1-NT (1-88 amino acids of human cyclin B1), containing an HA tag at the N 
terminus and a 6xHis tag at the C terminus was generated using PCR amplification with forward 
primer (5'-CCA GGA CCA TGG GTT ACC CAT ACG ATG TTC CAG ATT ACG CTG GCT 
CGA TGG CGC TCC GAG TCA CG-3') and reverse primer (5'-GGG AGC CTC GAG CTA 
GGG AGC GTG ATG GTG ATG ATG CAT AGG TAC CTT TTC AAG AGG-3'). The
resulting PCR product was digested with NcoI and XhoI for subcloning into pET28a. Plasmids were verified by restriction enzyme mapping and sequencing. For $^{35}$S labeling in *Escherichia coli*, cultures (50 ml) were grown at 37 °C to $D_{600 \text{ nm}} = 0.8$, then collected by centrifugation (3,700g for 15 min, at 4 °C) and resuspended in modified M9 medium (50 ml final volume). After resuspension in modified M9 medium, cells were allowed to grow for additional 15 min at 37 °C before 5 mCi of Easy Tag™ L-[35S]-Methionine (NEG709A005MC; Perkin Elmer) was added. Expression was induced with 0.5 mM IPTG for 2.5 h at 37 °C. Cells were ruptured in 5 ml g$^{-1}$ of pellet guanidine-HCl lysis buffer (pH 8.0) and lysates rotated at 24 °C until the lysate became slightly translucent; approximately 45 min. Lysates were clarified by centrifugation and cycB1-NT was purified using Ni-NTA affinity chromatography (Qiagen). Eluted protein was desalted into XB buffer (100 mM KCl, 0.1 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES, at pH 7.8 with KOH), supplemented 2% glycerol, protease inhibitors and phenylmethylsulfonyl fluoride, and stored at – 20 °C.

Maltose-binding protein (MBP)-tagged E1 was expressed in *E. coli* inducing cultures at $D_{600 \text{ nm}} = 0.6$ with 300 μM IPTG for 5 h at room temperature. Purification was carried out using a standard MBP purification protocol. For expression of His-tagged UBC10 and His-tagged UBC4, bacterial cultures were induced at $D_{600 \text{ nm}} = 0.6$ at 37 °C with 500 μM IPTG for 4 h. The enzymes were purified through Ni-NTA affinity and gel-filtration chromatography. PET28a expressing human wild-type UBE2S was provided by M. Kirschner (Harvard Medical School, USA). Cultures were grown to $D_{600 \text{ nm}} = 0.4$ and induced with 500 μM IPTG at 37 °C for 4 h. His-UBE2S was purified by Ni-NTA purification. Glutathione-S-transferase (GST)-fusion proteins for Rpn10 and Rad23, as well as their sub-domains, were purified essentially as reported previously (Elsasser et al., 2004; Elsasser et al., 2002). Recombinant E2-25K (UbcH1) was purchased from Boston Biochem (SP-200).

**Preparation of Xenopus egg extract**
Interphase *Xenopus* egg extract was prepared from eggs laid overnight according to the protocol of Murray (Murray, 1991) with the exception that eggs were activated with 2 μg ml\(^{-1}\) calcium ionophore (A23187, free acid form, Calbiochem) for 30 min prior to the crushing spin. Extract was frozen in liquid nitrogen and stored at -80 °C. Interphase extract was induced to enter mitosis by addition of non-degradable cyclin B, which activates CDK1 and stimulates mitotic phosphorylation, resulting in APC/C activation. A fusion of the maltose-binding protein (MBP) to *Xenopus* cyclin B lacking its N-terminal 90 amino acids (MBP-Δ90) (Salic and King, 2005) was expressed in *E. coli* by inducing cultures at an \(D_{600nm} = 0.6\) with 300 μM isopropylthiogalactoside (IPTG) for 5 h at room temperature. Purification was carried out following New England BioLabs (NEB) protocol. To make mitotic extract, MBP-Δ90 was added to interphase extract generally at ~ 20 μg ml\(^{-1}\) and incubated at 22-24 °C for 45-60 min.

**Reconstitution of ubiquitination and degradation of cyclin B1**

Ubiquitination reactions were carried out essentially as described previously (Kirkpatrick et al., 2006) for the indicated times. Briefly, for each 30 μl reaction, APC/C was immunopurified from 600 μl of mitotic *Xenopus* egg extract by incubation for 1 h at 4 °C with 12 μg of anti-Cdc27 antibodies (AF3.1, Santa Cruz Biotechnology) immobilized onto 30 μl of Affiprep Protein A beads (156-0006, Bio-Rad). Following incubation with extract, beads were washed quickly (to minimize loss of associated APC/C co-activator Cdc20) three times with XB containing 500 mM KCl (10 mM potassium HEPES, pH 7.7, 500 mM KCl, 0.1 mM CaCl\(_2\), 1 mM MgCl\(_2\)), two times with XB same content as above, except with 100 mM KCl), and then three times with reaction buffer (20 mM Tris, pH 7.5, 100 mM KCl, 2.5 mM MgCl\(_2\), 2 mM ATP). Ubiquitination reaction were carried out at 24 °C with agitation at 1500 r.p.m and contained APC/C on 30 μl beads, and 30 μl of a mix containing recombinant MBP-human E1 (1.3 μM), His-tagged UBCH10 or UBC4 (100 nM – 4 μM) as the E2 enzyme, wild-type or different forms of ubiquitin (118-145 μM), and 450-500 nM cyclin B1-CDK1 or cycB1-NT. For ubiquitin-receptor binding and degradation
assays, reaction supernatants were combined with the first 20 µl of reaction buffer wash. For analysis of cyclin B1 ubiquitination with different ubiquitins, entire reactions were processed for immunoblotting or autoradiography. Dried gels were analyzed by phosphorimaging (Bio-Rad PMI); quantification was carried out with Quantity One software (Bio-Rad).

For binding experiments with ubiquitin receptors, cyclin B1-CDK1 was pre-ubiquitinated with purified *Xenopus* APC/C, UBCH10 (3 µM), and ubiquitin (118 µM) for 90 min. Approximately 7-8 µg of “bait” protein immobilized onto Glutathione-Sepharose 4B resin (GE Healthcare) was mixed with 4 µl of pre-synthesized ubiquitin-cyclin B1 conjugates and incubated for 1 h at 4 °C with agitation in the presence of 100 µg ml⁻¹ BSA and 0.1 % Tween 20. Supernatants were collected and mixed with the first wash to make the flow-through fraction. Beads were washed twice and diluted with SDS sample buffer to analyze the bound fraction. Equivalent amounts of input (I), flow-through (FT) and bound (B) fractions were subjected to SDS-PAGE and western blot analysis using anti-cyclin B1 polyclonal antibody (Ab-2, Neomarkers).

For degradation assays with purified proteasomes, human proteasomes (10-20 nM, concentrations as indicated), purified as reported previously (Lee et al., 2010) but non-UbVS treated, were added to cyclin B1-Ub in buffer (50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂ and 5 mM ATP) (Lee et al., 2010) and incubated at 24 °C. For “0 min” time-point, substrate and proteasome mixtures were individually added to SDS sample buffer to prevent a time-lag from mixing. Aliquots withdrawn at indicated times were combined with SDS sample buffer and subjected to SDS-PAGE/immunoblot analysis using anti-cyclin B1 polyclonal antibody (Ab-2, Neomarkers).

**Cyclin B1 degradation in Xenopus egg extract**

Degradation assays with non-ubiquitinated cyclin B1 were carried out by adding ~ 200-250 nM of cyclin B1 in 40 µl reactions, with extract constituting 75-80% of the total volume. Pre-
treatment of extract with TAME or MG262 was done at 24 °C for 15 min. For assays containing no UbVS, extracts were supplemented with ubiquitin as indicated or buffer (for untreated sample) concomitantly with substrate. UbVS treatment was carried out for 30 min at 24 °C, with agitation (1,250 r.p.m.) before addition of ubiquitin and cyclin B1. Extracts contained 100 μg ml⁻¹ of cycloheximide to prevent reincorporation of free labeled amino acid. For competition assays, unlabeled competitor was added concomitantly with radiolabelled cyclin B1 and degradation was initiated. Degradation experiments were carried out at 24 °C, with agitation. Samples for proteolysis of unlabeled cyclin B1-CDK1 were processed for anti-cyclin B1 immunoblot using anti-cyclin B1 polyclonal antibody (Ab-2, Neomarkers). In degradation assays with ³⁵S-labelled cycB1-NT, reactions (3 μl per time point) were quenched with 97 μl of 20% TCA (in H₂O), vortexed and incubated on ice ≥ 30 min before centrifugation at 14,000g, at 4 °C for 30 min. A fraction (50%) of sample supernatants was combined with NaOH to neutralize the acid and added to Ultima Gold scintillation fluid (6013327, Perkin Elmer). The radioactivity in the supernatant was measured by scintillation counting. Acid-soluble counts were compared to total radioactive counts and results were graphed as percent soluble radioactive counts.

For degradation of pre-ubiquitinated cycB1-NT in extract, interphase extract was pre-treated with UbVS or buffer for 30 min at 24 °C with agitation and supplemented with 100 μg ml⁻¹ cycloheximide. In experiments with USP14 inhibitor IU1, IU1 or dimethylsulphoxide (DMSO) was added to extract for 15 min at 24 °C before addition of substrate. Where proteolysis was evaluated in the presence of unlabeled cycB1-NT, extract was mixed with unlabeled competitor and ³⁵S cycB1-NT-Ubₙ concomitantly. Extract (~14 μl) was added to 4 μl of cycB1-NT-Ubₙ conjugates for each time point. Degradation mixtures were incubated at 24 °C, 1250 r.p.m. for indicated times. Reactions were quenched with 107 μl of 20% TCA, vortexed and incubated on ice ≥ 30 min before centrifugation at 14,000g, at 4 °C for 30 min. A fraction of supernatants was combined with NaOH and Ultima Gold scintillation fluid (6013327, Perkin Elmer).
To deplete APC/C, 100 μl of interphase extract was mixed 2 μg of anti-Cdc27 antibody coupled to 5 μl of Affiprep Protein A beads and incubated at 4 °C for 3 h. APC/C depletion was confirmed by anti-Cdc27 western blot analysis. Approximately 10 μl of pre-ubiquitinated radiolabelled cyclin B1 was added to 90 μl of APC/C-depleted extract. Reactions were incubated at 22 °C for the indicated times and stopped by the addition of an equal volume of chilled 2% perchloric acid (PCA) (in H₂O) making a new final volume of 200 μl. Reactions were then incubated on ice for ≥30 min and centrifuged at 15,000 r.p.m. for 10 min, at 4 °C. A fraction of supernatants was mixed with Tris Base and Ultima Gold scintillation fluid (6013327, Perkin Elmer) and the radioactivity was measured by scintillation counting.
References


Chapter IV: Role of E2 enzymes in APC/C-dependent proteolysis

Nevena Dimova
Abstract

The Anaphase-Promoting Complex/Cyclosome (APC/C or APC) regulates progression through mitosis by orchestrating the ubiquitination of cell-cycle regulators such as cyclin B1 and securin. Recent reports have implicated Lys11-linked ubiquitin chains in degradation of APC/C substrates, but the Lys11 chain-forming E2 UBE2S is not essential for mitotic exit. In *Xenopus* cell-cycle extracts, conjugation of ubiquitin to multiple lysine residues of cyclin B1 provides an alternative degradation signal for cyclin B1 that does not require chain extension. Here, we evaluate the relative contribution of different E2 enzymes to APC activity and demonstrate that the chain-elongating activity of UBE2S is dispensable for cyclin degradation unless the number of ubiquitinatable lysines in cyclin B1 is restricted.
Introduction

Covalent attachment of ubiquitin to proteins controls the stability, localization or activation status of myriad cellular proteins and thereby constitutes a powerful mechanism for regulating almost all aspects of cell physiology (Behrends and Harper, 2011; Kerscher et al., 2006). Ubiquitination of substrates requires the concerted actions of an ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3) (Hershko and Ciechanover, 1998; Kerscher et al., 2006). A view emerging from recent structural and mechanistic studies invokes E2 enzymes to play an active role in determining the length and topology of ubiquitin assemblies, as well as the processivity of ubiquitination, thereby influencing the downstream fate of the substrate (Behrends and Harper, 2011; Chen and Pickart, 1990; Eddins et al., 2006; Garnett et al., 2009; Hofmann and Pickart, 1999; Petroski et al., 2007; Rodrigo-Brenni and Morgan, 2007; Wickliffe et al., 2011a; Williamson et al., 2009; Ye and Rape, 2009).

The Anaphase-Promoting Complex/Cyclosome (APC/C or APC) is a multi-subunit E3 ubiquitin ligase that initiates anaphase and mitotic exit by ubiquitinating regulatory proteins, including cyclin B1 and securin, to target them for destruction by the 26S proteasome (Barford, 2011; Harper et al., 2002; Peters, 2002; Peters, 2006). Biochemical studies in frog and clam oocytes originally showed that two different E2 enzymes, Ubc4 and UbcX, or E-2C, can independently support APC activity (Aristarkhov et al., 1996; Yu et al., 1996). In conjunction with either E2, *X. laevis* APC was found to modify cyclin B1 with ubiquitin chains linked through multiple lysine residues of ubiquitin (Lys11 and Lys63, in addition to Lys48) (Kirkpatrick et al., 2006). In this context, uniform Lys48-linked ubiquitin chains were found dispensable for binding of ubiquitinated cyclin B1 to ubiquitin-receptor proteins and robust degradation by the 26S proteasome (Kirkpatrick et al., 2006). A model emerging from more recent work challenges the view that a single E2 enzyme is sufficient to support the assembly of proteolytic tag on APC substrates and instead suggests that this process requires the sequential
action of two distinct E2 enzymes (Rodrigo-Brenni and Morgan, 2007). In metazoans, the
conjugating enzyme UBE2S promotes processive Lys11-linked chain-extension after the initial
monoubiquitination or short-chain formation by UBCH10 (Barford, 2011; Behrends and Harper,
2011; Garnett et al., 2009; Wickliffe et al., 2011a; Wickliffe et al., 2011b; Williamson et al.,
2009; Wu et al., 2010; Ye and Rape, 2009). Consistent with this model, depletion of both
UBCH10 and UBE2S causes mitotic arrest and stabilization of APC substrates (Williamson et al.,
2009). While important for APC activity in Drosophila S2 cells (Williamson et al., 2009),
UBE2S was not found to be essential for normal mitosis in human HeLa cells and is largely
dispensable for timely proteolysis of cyclin B1 in this context (Garnett et al., 2009). Together
these findings imply that the contribution of UBE2S and long polyubiquitin chains in the
degradation of APC substrates may be organism- and condition-specific, and that there may not
be a uniform requirement for UBE2S in all systems or circumstances.

Our recent study revealed that conjugation of ubiquitin to multiple lysine residues of
cyclin B1 provides an alternative degradation signal for cyclin B1 that does not require chain
elongation (discussed in chapter III). However, chain formation becomes essential for substrate
proteolysis when the number of ubiquitination sites in cyclin B1 is restricted. Here we sought to
examine whether this requirement for chain formation arises from a specific role of UBE2S or
through the chain-forming activity of UBCH10.
Results

Analysis of the role of chain-elongating E2 UBE2S in cyclin B1 degradation

The ability of Ub<sup>11R</sup> and chain-terminating ubiquitins to support reasonably efficient proteolysis of cyclin B1 in *Xenopus* extract raised a question as to whether the E2 enzyme UBE2S is important for APC activity in this context. To assess the role of UBE2S in cyclin B1 proteolysis, we immunodepleted the protein and measured how this affected the kinetics of cyclin degradation. Antibodies efficiently depleted the UBE2S protein, as observed by the absence of signal following 25-fold enrichment of E2 enzymes on Ub agarose (Figure 4.1a, lanes 4-6), without affecting levels of the APC/C or the E2 UBCH10. UBE2S depletion caused only a modest increase in the half-life of cycB1-NT as compared to control-depleted extract (Figure 4.1b). This effect was reversed by adding back 10 nM of the recombinant enzyme, implying that the delay was specifically due to loss of UBE2S activity.

We hypothesized that perhaps UBE2S is not essential for rapid degradation of cyclin B1 because this substrate contains multiple lysine residues that can serve as sites of attachment of short ubiquitin chains generated by UBCH10. If this hypothesis is correct, then restricting the number of available lysine residues should make degradation more dependent on UBE2S. We therefore examined the effect of UBE2S depletion on the rates of degradation of cyc<sup>K64only</sup> compared to that of wild-type cyclin B1 (Figure 4.1c). As we observed for cycB1-NT, the degradation of full-length wild-type cyclin B1 was largely unaffected by depletion of UBE2S (Figure 4.1c, top and bottom left panels). In contrast, the proteolysis of cyc<sup>K64only</sup> was highly sensitive to the depletion of UBE2S (Figure 4.1c, top and bottom right panels), and addition of recombinant UBE2S fully restored degradation. Supplementing control-depleted mitotic extract with 10 nM of recombinant UBE2S had little effect on the turnover of wild-type cyclin B1 (Figure 4.1b and 4.1c top left panel), but slightly stimulated degradation of cyc<sup>K64only</sup> (Figure 4.1c,
Figure 4.1 UBE2S is required for cyclin B1 proteolysis only when ubiquitination is constrained to a single lysine. (a) Mitotically arrested Xenopus extract was immunodepleted with UBE2S antibody or control IgG. Samples were further incubated with ubiquitin agarose (101 ratio of extract to resin) to enrich for U2 enzymes, and bound proteins were analyzed by SDS-PAGE and immunoblotting. Lanes 4–6 represent 25-fold enrichment of U2 enzymes on ubiquitin agarose. Levels of UBE2S, UBC10, and APC/C subunit CDC27 were examined by immunoblotting. Asterisks, nonspecific signal. (b) Rate of degradation of 35S-labeled cyclin B1-NT in UBE2S- or control-depleted mitotic Xenopus extract from a. Recombinant His-UBE2S (10 nM), where indicated, was added to reactions concomitantly with substrate. Proteolysis was measured by release of trichloroacetic-acid-soluble counts, and is plotted as the percentage of input radiolabeled cyclin B1-NT. (c) Time course of degradation of full-length cyclin B1 (cycWt) or single-lysine-containing mutant (cycL4A, L94), each in complex with CDC1, in control- or UBE2S-depleted mitotic Xenopus extract. Recombinant His-UBE2S (10 nM), where indicated, was added to reactions concomitantly with substrate. Cyclin B1 proteolysis was analyzed by SDS-PAGE and immunoblotting. Asterisks, nonspecific signal.
top right panel). Together these findings indicate that UBE2S is indeed present in *Xenopus* extract at sufficient levels to support cyclin proteolysis, but becomes essential only when the number of ubiquitinatable lysine residues in cyclin B1 is restricted.

**Role of chain-extending enzyme E2-25K in APC-mediated ubiquitination**

Similarly to UBE2S, in budding yeast another ubiquitin-conjugating enzyme Ubc1 has been shown to stimulate processive ubiquitin-chain extension on APC substrates carrying pre-attached ubiquitins (Rodrigo-Brenni and Morgan, 2007). The human homolog of Ubc1, E2-25K was also found to promote higher extent of polyubiquitination when added to assays containing human APC and UBCH10. Motivated by these findings, we sought to examine whether E2-25K has any effect on the extent or pattern of cyclin ubiquitination catalyzed by *Xenopus* APC using reconstituted ubiquitination assays. Consistent with studies showing that this E2 cannot initiate conjugation, but rather elongates nascent ubiquitin chains (Rodrigo-Brenni and Morgan, 2007; Wu et al., 2010), E2-25K did not catalyze cyclin ubiquitination on its own (Figure 4.2).

Unexpectedly, when E2-25K was added to the assay together with UBC4, the number of conjugated Ub molecules per cyclin remained largely unaffected (Figure 4.2; data not shown). Similarly, we found no effect of the chain-elongating activity of E2-25K when combined with UBCH10 (Figure 4.2). To rule out the possibility that the apparent lack of contribution of E2-25K resulted from competition for ubiquitin charging or binding to the APC/C, we added the proximally-acting E2s UBCH10 and UBC4 at nanomolar concentrations concomitantly with increasing the E2-25K concentration approximately tenfold to 10 μM. Under these conditions, the catalytically active E2-25K had no appreciable effect on cyclin B1 ubiquitination (data not shown).
Figure 4.2 Chain-elongating enzyme E2-25K does not enhance cyclin B1 ubiquitination catalyzed by Xenopus APC. Western analysis of an in vitro ubiquitination reaction containing full-length cyclin B1, in complex with CDK1, APC/C immunopurified from mitotically arrested Xenopus extract, recombinant UBC4 or UBC10 (100 nM), E2-25K (1 μM), and wild-type ubiquitin (118 μM), as indicated. Ubiquitination was allowed to proceed for 90 min before samples were processed for SDS-PAGE and cyclin B1 western blot analysis.
Analysis of the role of UBCH10 in APC activity in Xenopus extract

The lack of requirement for UBE2S activity and ubiquitin-chain formation for efficient proteolysis of cyclin B1 in Xenopus extract suggest that UBCH10, or perhaps enzymes of the UBC4/5 family, are sufficient to support APC/C-dependent degradation in this context. We next examined how recruitment of the E2 UBCH10 contributes to the function of mitotically activated APC. To quantitatively evaluate a requirement for UBCH10-mediated ubiquitination in targeting cyclin to the proteasome, we immunodepleted the protein and measured how this affected levels of $^{35}$S-labeled cycB1-NT. Antibodies efficiently depleted the UBCH10 protein, as observed by the absence of signal following 20-fold enrichment of E2 enzymes on ubiquitin agarose (Figure 4.3a). UBCH10 depletion with two different antibodies (Figure 4.3a; data not shown) caused a modest increase in the half-life of cycB1-NT as compared to control-depleted extract (Figure 4.3b), but failed to stabilize the substrate which would be expected if UBCH10 were required for substrate degradation in this context. The delay in degradation was rescued by addition of 50 nM of recombinant UBCH10. In analogous experiments, proteolysis of full-length cyclin B1-CDK1 complex was largely unaffected by depletion of UBCH10 (Figure 4.3c). In contrast with wild-type cyclin B1, depletion of UBCH10 more significantly delayed turnover of the single-lysine (cyc$^{K64only}$) cyclin B1 (Figure 4.3c), an effect reversed by the addition of 50 nM of recombinant UBCH10. The lack of requirement for UBCH10 in cyclin B1 degradation indicates that other E2 enzymes in the extract may be sufficient to prime APC molecules for substrate ubiquitination. Members of the UBC4/5 family, which have been demonstrated to cooperate with the APC \textit{in vitro} (Garnett et al., 2009; Kirkpatrick et al., 2006; Mathe et al., 2004; Summers et al., 2008; Yu et al., 1996; Zeng et al., 2010), are the best candidates for such a role. We therefore sought to examine the role of this class of E2s in cyclin B1 destruction.
Figure 4.3 Depletion of UBCH10 more significantly delays cyclin B1 degradation when ubiquitination is limited to a single lysine residue. (a) Mitotically arrested Xenopus extract was immunodepleted with UBCH10 antibody or control IgG. Samples were further incubated with Ub agarose to enrich for E2 enzymes. Samples were separated by SDS-PAGE and analyzed by western blot against UBCH10, UBCH5, and the APC/C subunit CDC27. Lanes 4-6 represent 20-fold enrichment of E2 enzymes on Ub agarose. (b) Rate of degradation of 35S-labeled cyclin B1-NT in UBCH10- or control-depleted mitotic Xenopus extract from a. Recombinant His-UBCH10 (50 nM), where indicated, was added to reactions concomitantly with substrate. Proteolysis was measured by release of TCA soluble counts, and is plotted as percentage of input radioactivity of cyclin B1-NT. (c) Time-course of degradation of full-length wild-type cyclin B1 (cyclin^WT) or single lysine-containing mutant (cyclin^KM^Lys), each in complex with CDK1, in control- or UBCH10-depleted mitotically-arrested Xenopus extract. Recombinant His-UBCH10 (50 nM), where indicated, was added to reactions concomitantly with substrate. Cyclin B1 proteolysis was analyzed by SDS-PAGE and cyclin B1 immunoblotting. Asterisks, nonspecific signal.
UBC4 cooperates with the APC to assemble an efficient proteolytic signal on cyclin B1

To evaluate a potential role for enzymes from the UBC4/5 class in cyclin B1 degradation, we assessed whether effects of different ubiquitin mutants on proteolysis in *Xenopus* extract (discussed in chapter III) paralleled their effects on UBC4-catalyzed conjugation in a reconstituted system. Elimination of Lys11 or 63 of ubiquitin had no effect on the overall pattern or extent of substrate ubiquitination, whereas elimination of Lys48 slightly reduced the mass of conjugates, consistent with the preference of UBC4 for forming Lys48 linkages (Kirkpatrick et al., 2006) (Figure 4.4a). In the presence of chain-terminating ubiquitin types such as methylated ubiquitin and Ub^{nr} (ubiquitin containing lysine-to-arginine substitutions at all three sites Lys11, 48 and 63 simultaneously), UBC4, similarly to UBCH10 (discussed in chapter III), was capable of appending ubiquitin monomers to distinct lysine residues on cyclin. The maximal extent of ubiquitination achieved with UBC4 under these conditions appeared higher relative to that seen with UBCH10 (Figure 4.4a; data shown in chapter III). Together these findings indicate that UBC4 can facilitate ubiquitination on more cyclin B1 lysine residues than UBCH10, consistent with previous work (Garnett et al., 2009; Kirkpatrick et al., 2006).

Ubiquitin assemblies elaborated by UBC4 in the presence of wild-type ubiquitin were recognized by proteasome-associated ubiquitin receptors (Deveraux et al., 1994; Elsasser et al., 2004; Elsasser and Finley, 2005; Finley, 2009; Isasa et al., 2010; Matiuhin et al., 2008; Peth et al., 2010; Rao and Sastry, 2002; Riedinger et al., 2010) including Rpn10 (Figure 4.4b) and Rad23 (Figure 4.4d), consistent with previous findings (Kirkpatrick et al., 2006). Importantly, multiply monoubiquitinated substrate was also capable of binding to both receptors. For conjugates of a similar molecular mass, substrate ubiquitinated with Ub^{nr} bound to receptors more efficiently than substrate ubiquitinated with Ub^{me}, a difference more pronounced in binding to Rad23. In contrast to species bearing a higher number of ubiquitin groups, unmodified and oligoubiquitinated cyclin B1 had much lower affinity for ubiquitin receptors. As observed
Figure 4: UBC4 and APC/C catalyze multiple monoubiquitination of cyclin B1 that is sufficient for binding ubiquitin receptors. (a) Western blot analysis of an in vitro ubiquitination reaction containing full-length cyclin B1, APC/C immunopurified from mitotically arrested Xenopus extract, recombinant UBC4 (66 μg ml⁻¹), and forms of ubiquitin (118 μM), as indicated. Ubiquitin types with lysine-to-arginine mutations at indicated positions, two or at all three positions simultaneously Lys11, 48, and 63 (Ubmm⁸), as well as methylated ubiquitin (Ubmm⁸⁸) were used. Reactions were allowed to proceed for 15 or 90 min before analysis by SDS-PAGE and western blotting against cyclin B1. (b-e) Binding of ubiquitinated cyclin B1 to GST-tagged ubiquitin receptors. Cyclin B1-ubiquitin conjugates were incubated with immobilized receptor proteins for 1 h at 4 °C before reaction products were subjected to SDS-PAGE and western blot analysis against cyclin B1. Equivalent amounts of input (I), flow-through (FT) and bound (B) fractions were loaded in adjacent lanes. Binding experiments with wild-type Rpn10 (b) and Rad23 (d). Binding with corresponding versions of the receptors lacking the ubiquitin-recognition domains, with engineered block substitution of the UIM domain (LAMAL → NNNNN) of Rpn10 (e) or deletion of the ubiquitin-associated domains of Rad23 (e).
previously (data shown in chapter III) (Kirkpatrick et al., 2006), all binding was dependent on the integrity of the receptor domains recognizing ubiquitin (Figure 4.4c, e).

Since UBC4 mediated transfer of monoubiquitin to multiple substrate residues and generated a ubiquitin signal that was recognized by ubiquitin receptors, we sought to determine whether multiple monoubiquitination catalyzed by UBC4 can target cyclin B1 for degradation. To this end, full-length cyclin B1-CDK1 complex was ubiquitinated with UBC4, in conjunction with wild-type or chain-terminating ubiquitin. The resulting species were incubated with human proteasomes, purified as reported previously (Lee et al., 2010) and containing the deubiquitinating enzymes RPN11 and UCH37, but no USP14. The deubiquitinase USP14 was previously shown to attenuate the capacity of the proteasome to rapidly degrade cyclin-ubiquitin species in vitro, presumably by trimming poly-ubiquitin chains assembled on substrate residues (Hanna et al., 2006; Lee et al., 2010). In agreement with previous findings (Crosas et al., 2006; Hanna et al., 2006; Lee et al., 2010), these USP14-deficient proteasomes rapidly degraded cyclin B1 that was polyubiquitinated by UBC4-APC (Figure 4.5a). While conjugates formed with Ub
\(^{\text{intR}}\) or lysine-less ubiquitin (Ub
\(^{K0}\)) were also degraded rapidly, those generated with methylated ubiquitin (Ub
\(^{\text{me}}\)) were degraded more slowly. This observation parallels the effects of these mutant ubiquitins in Xenopus extract (discussed in chapter III) and is consistent with the defect in the ability of cyclin B1-Ub
\(^{\text{me}}\) to bind ubiquitin receptors (Figure 4.4b, d). We previously quantitated the extent of substrate proteolysis using \(^{35}\)S-labeled full-length cyclin B1 and found that it closely correlated with the percentage of cyclin B1 modified with three or more ubiquitin moieties (data not shown). Together these results indicate that purified proteasomes do not impose a requirement for long ubiquitin polymers and can efficiently degrade multiply monoubiquitinated cyclin B1.

We extended the analysis of these multiply monoubiquitinated species by measuring their degradation under conditions where different ubiquitin receptors, deubiquitinating enzymes and the proteolytic machinery are present at physiological concentrations. To this end, we added
Figure 4.5 Cyclin B1 multiply mono-ubiquitinated by UBC4 and APC/C is rapidly degraded by purified proteasomes and in Xenopus extract. (a) in vitro degradation assay with cyclin B1-ubiquitin species generated with Xenopus APC/C immunopurified from mitotically arrested extract, recombinant UBC4 (250 nM) and forms of ubiquitin (145 µM), as indicated, and USP14-deficient human proteasomes [20 nM]. WT, wild-type; KO, lysine-less; triR, lysine-to-arginine mutations at all three positions. Lys 11, 48 and 63; me, methylated ubiquitin. Aliquots were removed at the indicated times and reactions products analyzed by SDS-PAGE and anti-cyclin B1 western blot analysis. (b) Autoradiograph of in vitro APC/C- and UBC4-catalyzed ubiquitination of 35 S-labeled full-length cyclin B1 with immunopurified Xenopus APC/C, recombinant UBC4 (4 µM), and forms of ubiquitin (145 µM) as indicated. Products from a 90-min ubiquitination assay were separated by SDS-PAGE and analyzed using a phosphorimager. (c) Interphase Xenopus extract was depletes APC/C, as seen by western blot against the APC/C subunit CDC27, before cyclin B1-ubiquitin conjugates were introduced. (d) Samples from b were introduced into APC/C-depleted interphase Xenopus extract for the indicated times, following which a perchloric acid (PCA) precipitation was done. Pre-treatment of extract with proteasome inhibitor MG132 (200 µM) was used as control at the 20 min time-point. Proteolysis was measured by release of PCA soluble counts, and is plotted as percentage of input radioabeled cyclin B1 protein.
radiolabeled full-length cyclin B1 pre-ubiquitinated by UBC4 (Figure 4.5b) to interphase

*Xenopus* extract that had been immunodepleted of the APC (Figure 4.5c). The extent and kinetics of degradation of species formed with Ub\textsuperscript{triR} were very similar to those for conjugates formed with wild-type ubiquitin. Conjugates generated with Ub\textsuperscript{me} were degraded slightly less efficiently. Together these results further support the view that *Xenopus* extracts can rapidly degrade a cyclin substrate bearing multiple ubiquitin groups attached to distinct lysine residues even when deubiquitination is not inhibited.

**Role of UBC4/5 enzymes in cyclin B1 degradation in *Xenopus* extract**

The modest stabilization of cyclin B1 observed in UBCH10-depleted extracts (Figure 4.3b) raises the possibility that additional E2 enzymes may support APC catalysis in this context. Based on this and previous work (Hanna et al., 2006; Kirkpatrick et al., 2006; Lee et al., 2010), members of the UBC4/5 class appear to be the best candidates for such a role. We sought to deplete the extract of these enzymes and assess the effect on cyclin degradation. To this end, we screened five different commercially available antibodies against UBC4/5. Despite extensive optimization of assay conditions, we were unable to achieve significant depletion of UBC4/5 in order to evaluate their role in cyclin proteolysis. We therefore took an alternative approach in addressing this question. Using ubiquitin covalently coupled to agarose beads via primary amines allowing for a fully functional C-terminus, we attempted to deplete the extract of proteins that have an affinity for ubiquitin, including ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2s), ubiquitin ligases (E3s) and ubiquitin C-terminal hydrolases (UCHs). Unlike immunodepletion experiments which address necessity, ubiquitin-agarose depletions have the potential to reveal sufficiency of particular E2 enzymes in cyclin degradation.
To quantitatively assess the effect of depleting the enzymatic machinery that targets cyclin B1 for degradation in mitotic *Xenopus* extracts, we measured the proteolysis of a purified, $^{35}$S-labeled cycB1-NT. In parallel, we examined the levels of UBC4/5, UBCH10 and UBE2S enzymes, as well as the APC, by western analysis. Interestingly, even trace levels of different E2 enzymes known to work in conjunction with the APC (Aristarkhov et al., 1996; Jin et al., 2008; King et al., 1995; Mathe et al., 2004; Rodrigo-Brenni and Morgan, 2007; Townsley et al., 1997; Williamson et al., 2009; Yu et al., 1996) were sufficient to support cyclin proteolysis. We found that while four rounds of incubation with ubiquitin agarose led to significant depletion of UBC4/5 and UBCH10, as observed by western blotting (Figure 4.6a), cycB1-NT degradation remained largely unaffected (data not shown). Increasing the rounds of ubiquitin-agarose depletion to six rendered the extract incapable of promoting cyclin degradation (Figure 4.6b), consistent with complete depletion of E2 enzymes (Figure 4.6a). To rescue proteolysis in depleted extracts, recombinant E1, ubiquitin, ATP and the E2 enzyme of interest were added back. Addition of ubiquitin-activating enzyme (E1) and ubiquitin alone did not restore degradation, indicating that the relevant E2 machinery had been depleted below the threshold required to support proteolysis (Figure 4.6b). We found that supplementing the depleted extract with 100 nM (data not shown) or 250 nM (Figure 4.6b) of recombinant UBC4 partially rescued proteolysis. Similar trends were observed even when the concentration of recombinant UBC4 was increased to 3 μM (data not shown). In contrast, under these conditions, addition of 250 nM of recombinant UBCH10 restored degradation to levels observed in undepleted extract (Figure 4.6b). Together these findings imply that another member of the UBC4/5 family may be a preferred E2 partner of the APC in *Xenopus* egg extract. Future experiments are required to determine whether other members of the UBC4/5 family may be more efficient at priming APC substrates for proteasomal degradation.
Figure 4.6 UBC4 partially rescues cyclin B1 degradation in Xenopus extract that is depleted of E2 enzymes. (a) Mitotically-arrested Xenopus extract was incubated with ubiquitin that is covalently coupled to agarose beads (Ub agarose) for number of rounds as indicated to allow depletion of E2 enzymes promoting cyclin B1 ubiquitination and degradation. Aliquots were removed after the indicated number of rounds of depletion and levels of UbCH5, UbCH10 and the APC/C subunit CDC27 analyzed by SDS-PAGE and immunoblotting. Factors bound to Ub agarose were eluted by boiling in SDS sample buffer and analyzed in parallel. (b) Rate of degradation of 35S-labeled cyclin B1-NT in mitotic Xenopus extract, that is undepleted or subjected to six rounds of E2 depletion with Ub agarose prior to substrate addition. Recombinant E1, wild-type ubiquitin, ATP and E2 enzymes, as indicated, were added back to Ub agarose-depleted extract concomitantly with substrate. Proteolysis was measured by release of TCA soluble counts, and is plotted as percentage of input radiolabeled cyclin B1-NT.
Discussion

Here we have evaluated the role of E2 enzymes in supporting APC activity in Xenopus cell-cycle extracts. Our study was motivated by recent reports suggesting that the assembly of Lys11-linked ubiquitin chains, mediated by the E2 enzyme UBE2S, may be essential for APC-dependent proteolysis. However, we found that the proteolytic machinery in Xenopus extract and in the reconstituted system does not impose a requirement for chain formation to efficiently degrade ubiquitinated cyclin and that the elaboration of Lys11-linked chains becomes essential only when the number of ubiquitinatable residues in cyclin B1 is restricted. Here we provide evidence that the chain-forming E2 UBE2S is dispensable for cyclin proteolysis, unless the number of available lysine residues in cyclin B1 is restricted, and evaluate how proximally-acting E2s UBCH10 and UBC4 may promote substrate degradation.

Our previous work revealed that chain-terminating ubiquitins support efficient proteolysis of cyclin B1 in Xenopus extracts (discussed in chapter III). Utilizing a reconstituted system, we demonstrated that multiply monoubiquitinated species generated with either UBCH10 or UBC4 are efficiently degraded by purified proteasomes. These findings raise the possibility that through conjugation of monoubiquitin to distinct lysines in cyclin, and possibly the elaboration of short chains, either of these E2 may promote APC-dependent proteolysis of cyclin B1 in Xenopus extracts. Consistent with such a model, UBCH10 supported robust APC catalysis when added to ubiquitin-agarose depleted extract. Interestingly, the importance of UBCH10 in substrate degradation appears to increase with elimination of available ubiquitin-acceptor sites in cyclin B1. Nevertheless, the effects seen upon UBCH10 depletion were not very dramatic. These findings were surprising in the light of a previous report showing that an N-terminal fragment of human cyclin B1 is stabilized upon UBCH10 depletion, arguing that UBCH10 is required for cyclin proteolysis in Xenopus extract (Tang et al., 2001). An important question the authors did not address, which may provide an explanation for these findings, was whether the antibody
efficiently and specifically depleted the UBCH10 protein, without affecting the levels of E2 enzymes from the UBC4/5 family. As the core ubiquitin-conjugating (UBC) domains of these E2s share a high degree of sequence homology (Osaka et al., 1997; Summers et al., 2008; Ye and Rape, 2009), it may be non-trivial to avoid unintended co-depletion.

Evidence from simpler model organisms indicates that E-2C family members are most likely to be biologically relevant as E2 partners of the APC (Mathe et al., 2004; Osaka et al., 1997). These models do not rule out the possibility that members of the UBC4/5 family, or possibly other E2s, influence APC-dependent proteolysis in *Xenopus* extracts. Our earlier work suggested that APC, solely in conjunction with the E2 UBC4, can generate polyubiquitinated cyclin B1 that is rapidly degraded by purified proteasomes (Crosas et al., 2006; Hanna et al., 2006; Kirkpatrick et al., 2006; Lee et al., 2010). These findings, together with the lack of substrate stabilization in UBCH10-depleted extract, led us to investigate a potential contribution of UBC4/5 to cyclin proteolysis in this system. Furthermore, we sought to determine whether UBCH10 and UBC4/5 enzymes are functionally redundant in creating multiple monoubiquitination sufficient for cyclin proteolysis.

Utilizing a reconstituted system, we demonstrated that UBC4 can catalyze transfer of ubiquitin monomers to multiple lysine residues in cyclin, consistent with previous studies (Garnett et al., 2009; Kirkpatrick et al., 2005). Experiments with chain-terminating ubiquitin revealed higher number of substrate monoubiquitination sites in reactions catalyzed by UBC4, as compared to UBCH10 (discussed in chapter III). An interesting question is whether this more extensive multiple monoubiquitination and the apparent lower threshold of modification required for binding at least some ubiquitin receptors translates into more efficient degradation of UBC4-generated cyclin B1-Ubₙ by the proteasome. Quantitative analysis of degradation with purified proteasomes (discussed in chapter III) revealed no such enhanced rate, or extent, of degradation of UBC4-generated conjugates. The capacity of purified proteasomes to rapidly degrade multiply monoubiquitinitated species generated with either UBC4 or UBCH10 (data shown in chapter III)
further strengthens our proposition that the proteasome does not impose a requirement for ubiquitin-chain formation for efficient proteolysis of cyclin B1.

Unlike the E-2C enzymes, members of the UBC4/5 class have been regarded as promiscuous E2s (Summers et al., 2008; Ye and Rape, 2009) with no essential role in APC-mediated catalysis. Consistent with this view, Summers and colleagues found that addition of UBCH10, but not UBCH5 (a member of the UBC4/5 family), to extracts from nocodazole-arrested HeLa cells was required and sufficient to activate the APC and mediate destruction of securin and cyclin B1 (Summers et al., 2008). However, the simultaneous addition of excess ubiquitin allowed UBCH5 to catalyze destruction of APC substrates in this context, presumably by increasing the levels of ubiquitin-charged UBCH5 above the threshold required to stimulate APC activity (Summers et al., 2008). Due to technical limitations, we were unable to immunodeplete UBC4/5 and investigate potential functional redundancy between UBCH10 and UBC4/5 enzymes, explaining the partial effects in UBCH10-depleted extracts. Utilizing the ubiquitin-agarose depletion strategy, we examined whether UBC4/5 enzymatic activity supports cyclin proteolysis. Strikingly, E2s from the UBC4/5 and E-2C families had to be completely depleted in order to abolish substrate turnover in Xenopus extract. The incomplete rescue achieved with UBC4 under these conditions may imply that another member of the UBC4/5 class is a preferred APC partner. A limitation of the ubiquitin-agarose depletion approach is that multiple factors, which may impact substrate proteolysis directly or indirectly, become depleted (as revealed by mass spectrometry analysis) making it difficult to ascertain the contribution of UBC4/5 enzymes. It is possible that following multiple rounds of depletion, the extracts become deficient of components, such as ubiquitin receptors, which may be important for UBC4/5-driven pathways.

Recent studies have proposed that the Lys11-specific chain-elongating E2 UBE2S has a critical role in the efficient ubiquitination and degradation of APC substrates in Xenopus, Drosophila, and humans (Williamson et al., 2009; Wu et al., 2010). We found that UBE2S
depletion of *Xenopus* egg extract had no impact on degradation of wild-type cyclin B1, but significantly slowed substrate proteolysis when ubiquitination was restricted to a single site. These trends closely paralleled the effects of adding chain-terminating ubiquitins to UbVS-treated *Xenopus* extract (discussed in chapter III). Our findings are consistent with a report suggesting that UBE2S is largely dispensable for cyclin B1 degradation in normal mitosis in human HeLa cells (Garnett et al., 2009). In contrast, UBE2S was found to be more important for substrate degradation when APC activity is compromised as upon activation of the spindle assembly-checkpoint (Garnett et al., 2009). The lack of requirement for UBE2S activity and long Lys11-linked ubiquitin chains for robust degradation of cyclin B1 in *Xenopus* extracts may be a consequence of higher levels of active UBCH10 and APC than seen in other biological contexts (Garnett et al., 2009; Williamson et al., 2009), as well as the availability of multiple lysine residues in cyclin B1 that can serve as ubiquitin acceptor sites. Our observations favor the idea that the increased importance of UBE2S seen with limiting the number of ubiquitination sites reflects the ability of this E2 to extend long polymers with high processivity rather than a major degradative role of Lys11 linkages it forms. In this context, UBCH10 or UBC4/5 enzymes appear to be less efficient in rapidly elongating individual ubiquitin chains that would facilitate proteasomal recognition and substrate breakdown. We anticipate that the relative contribution of UBCH10 and UBE2S in degradation of different APC substrates may vary. Human cyclin B1 is lysine-rich in its N-terminal domain, containing 18 lysine residues, whereas cyclin A2 has 12 lysine residues in the same region, which may make the latter protein more dependent on the chain-elongating enzyme UBE2S for degradation. Similarly, *S. cerevisiae* Clb2 is relatively lysine-poor in its N-terminal domain, containing only 6 lysine residues, potentially explaining the importance of a chain-elongating E2 in this system (Rodrigo-Brenni and Morgan, 2007). A greater dependence on chain-elongating E2s may impact the sensitivity of different substrates to deubiquitination. In this regard, cyclin A2 degradation during interphase is specifically impeded by the deubiquitinating enzyme USP37 (Huang et al., 2011), but this enzyme does not appear to
agonize cyclin B1 degradation. An interesting future question is how the balance between multiple monoubiquitination and ubiquitin-chain formation affects sensitivity of degradation to deubiquitinating enzymes. Finally, our work raises the interesting possibility that the degree of dependence on UBE2S could be regulated by post-translational modification of the substrate. For example, acetylation is known to affect degradation of the spindle-checkpoint protein BubR1 (Choi et al., 2009). By restricting the number of ubiquitinatable lysine residues, acetylation could increase the dependence of degradation pathways on UBE2S-catalyzed chain formation.
Methods

Antibodies and biochemical reagents

Proteins were separated by SDS-PAGE on NuPAGE 4-12% or 12% Bis-Tris gels (Invitrogen), followed by wet transfer to PVDF. Sources of antibodies for immunoblotting were as follows: anti-cyclin B1 (Ab-2; RB-008-P, Neomarkers), anti-Cdc27 (610455, BD Transduction Laboratories), anti-UBCH10 (A-650, Boston Biochem; AB3861, Millipore), anti-UBE2S (N-14; sc-131354, Santa Cruz Biotechnology), anti-UBCH5 (A-615; Boston Biochem), anti-ubiquitin (P4D1; sc-8017; Santa Cruz Biotechnology). Secondary antibodies used include anti-goat IgG-HRP (sc-2020; Santa Cruz Biotechnology), anti-rabbit IgG-HRP (NA934; GE Healthcare), and anti-mouse IgG-HRP (NA931; GE Healthcare). Antibodies for immunoprecipitation or immunodepletion included: anti-Cdc27 (AF3.1; sc-9972, Santa Cruz Biotechnology), anti-UBE2S (N-14; sc-131354, Santa Cruz Biotechnology), anti-UBCH10 (gift from H. Yu, UT Southwestern, USA); and for control depletions, normal rabbit IgG (sc-2027, Santa Cruz Biotechnology) and normal goat IgG (sc-2028, Santa Cruz Biotechnology) were used. UBE2S antibodies were coupled to UltraLink immobilized protein A/G beads (53132, Pierce). UBCH10 and CDC27 antibodies were coupled to Affiprep protein A beads (156-0006, Bio-Rad). Ubiquitin agarose (U-405), MG262 (I-120), Ub^{me} and ubiquitin mutants except for Ub^{(11+48)R}, Ub^{(11+63)R} and Ub^{triR} were purchased from Boston Biochem. Ubiquitin (U6253) were purchased from Sigma.

Preparation of recombinant proteins

Ubiquitin mutants Ub^{(11+48)R}, Ub^{(11+63)R} and Ub^{(11+48+63)R} (referred to as Ub^{triR}) were generated by introducing arginine codons (AGA, AGG) at the indicated sites through PCR-mediated mutagenesis of the human ubiquitin sequence (cloned in pET3a with ampicillin resistance, the kind gift of C.M. Pickart). Plasmids were verified by sequencing and the purified proteins analyzed by mass spectrometry. To ensure efficient arginine incorporation, BL21 (DE3) cells were co-transformed with pJY2, developed by Pickart lab (You et al., 1999), which carries
T7 lysozyme (LysS) and a gene encoding tRNA_{UCU}^{Arg}. Cultures were grown at 37 °C to an attenuation ($D$) of ~0.5 at 600 nm, and induced with 100 μM isopropylthiogalactoside (IPTG) at $D_{600\text{nm}} = 0.6$ at 25 °C for 5 h. Cells were ruptured by sonication in QA lysis buffer (50 mM HEPES (pH 7.7), 100 mM KCl, protease-inhibitor cocktail, 5 mM 2-mercaptoethanol, 10 μg ml^{-1} DNase). Lysozyme was added to 1 mg ml^{-1} concentration and lysate was incubated with rotation at 4 °C for 15 min. Following sonication, cell lysates were clarified by centrifugation and the resulting supernatants applied to a Q column. The flow-through containing ubiquitin was concentrated and purified by size-exclusion chromatography. Fractions containing ubiquitin were typically > 95% pure.

To generate full-length cyclin B1-CDK1 complex, human cyclin B1 and CDK1 baculoviruses were used as described previously (Kirkpatrick et al., 2006). To generate mutants of cyclin B1, DNA fragments encoding the N-terminal 124 amino acids of wild-type human cyclin B1 (5′- AACCGGTCCGAAAACCGTCGACATGTGCACATCACCATCACCATCACCACGGGCTC GATGGCGCTCCGAGTCACGCCTAACTGCAAAATTAGCTGAAATAAGCGAAAA TCAACATGGCGCAAGCCAGCGTTTCTACGCACCCCGGGCAACCTCGACCCGG CAGTGAAGCCCAAGAGCAGCTTTGGGACATTTGAAACAGTCAAAATCTAGTCAGCAGCTA CAGGCCAAATGCTATGAAAAAGCGAAAAACCTTCAGCTACCCGGTAAAGTCAT TGATAAAAACTACCAAACCTCTTGGAAAAAGGTACCTATGCTGGCCAGTCCAGT GTCTGAGCGAAGCAGCGAGCAGCCAGAACCCTGAGCGCCTGTGTTAAGAGAAG AAAACTTTACGAGCCCTATTTTTGATGATACGCTAGCAATA-3′) or the same region of the protein with arginine substitutions at all lysine residues (cyc^{allR}; 5′- AACCGGTCCGAAAACCGTCGACATGTGCACATCACCATCACCATCACCACGGGCTC GATGGCGCTCCGAGTCACGCCTAACTGCAAAATTAGCTGAAATAAGCGAAAA TCAACATGGCGCAAGCCAGCGTTTCTACGCACCCCGGGCAACCTCCAGACCCG 118
GGCTGAGGCCAAGAACAGCTCTTTGGGACATTGGTAACAGAGTCAGTGAACAGCTACAGGCCAGA
ATGCCTATGAGAAGAGAAGCAAGACCTTCAGCTACCGGTAGAGTCAT
TGATAGAAGACTACAAAGACCTCTTGAAGGATACCTATGCTGTCGAGTCCAGT
GTCTGAGCCAGTGCCAGGCCAGACAACCTGAGCCAGAAGCACCTGAGCCTGTGATAGAAGACTAC
CAAGACCTCTTGAAAGGGTACCTATGCTGGTGCCAGTGCCAGTGCTGAGCCAGTGCCAGAGCCAGA
AAMACTTTTCGCTGAGCCTATTTTGGTTGATACTGCTAGCAATA-3') preceded by 6x
His tag were synthesized (GenScript). Using restriction enzyme digestion with NheI and RsrII,
fragments were subcloned into pFASTBac containing the carboxy terminus (125-433 amino
acids) of cyclin B1. To generate cycK64only, primers 5'-
TCCAGACCCGGCTGAGGCCAAGAACAGCTCTTTGGGACATTGGTAACAGAGTCAGTGAACAGCTAC
AAACAGCTACAGGCC-3’ and 5’-
AATGACTCTACCGGTAGCTGAAGGTCTTGCTTCTTTTCTCATAGGCATTCTGGCCTGT
AGCTGTTCACTGAC- 3’ were used for an extension reaction and the resulting fragments cloned
into XmaI and AgeI cleavage sites of pFASTBac carrying full-length cyclin B1. Plasmids were
verified by restriction enzyme mapping and sequencing. Baculoviruses were generated according
to the Bac-to-Bac manual (Invitrogen). Wild-type cyclin B1 was 35S-labeled in Sf9 cultures with
resuspending cells (1.5 x 10⁶ cells ml⁻¹) in media containing 10% SF-900 II SFM and 90% SF-
900 II SFM without methionine or cysteine (both from Invitrogen) to increase radio-label uptake.
Baculovirus was added to cells, along with 50 µCi of 35S-labeled methionine and cysteine
(NEG772; Perkin Elmer), and cyclin B1 expression was allowed for 2.5 days. CDK1 was
expressed separately in Sf9 cells without radiolabeling and then combined with lysate from cells
expressing cyclin B1 to allow formation of complex, which was then purified through Ni-NTA
affinity and gel filtration chromatography.

CycB1-NT (1-88 amino acids of human cyclin B1), containing an HA tag at the N
terminus and a 6xHis tag at the C terminus was generated using PCR amplification with forward
primer (5’-CCA GGA CCA TGG GTT ACC CAT ACG ATG TTC CAG ATT ACG CTG GCT
CGA TGG CGC TCC GAG TCA CG-3′) and reverse primer (5′-GGG AGC CTC GAG CTA
GGG AGC GTG ATG GTG ATG ATG CAT AGG TAC CTT TTC AAG AGG-3′). The
resulting PCR product was digested with NcoI and XhoI for subcloning into pET28a. Plasmids
were verified by restriction enzyme mapping and sequencing. For 35S labeling in *Escherichia
coli*, cultures (50 ml) were grown at 37 °C to $D_{600 \, \text{nm}} = 0.8$, then collected by centrifugation
(3,700g for 15 min, at 4 °C) and resuspended in modified M9 medium (50 ml final volume). After
resuspension in modified M9 medium, cells were allowed to grow for additional 15 min at 37 °C
before 5 mCi of Easy Tag™ L-[35S]-Methionine (NEG709A005MC; Perkin Elmer) was added.
Expression was induced with 0.5 mM IPTG for 2.5 h at 37 °C. Cells were ruptured in 5 ml g⁻¹ of
pellet guanidine-HCl lysis buffer (pH 8.0) and lysates rotated at 24 °C until the lysate became
slightly translucent; approximately 45 min. Lysates were clarified by centrifugation and cycB1-
NT was purified using Ni-NTA affinity chromatography (Qiagen). Eluted protein was desalted
into XB buffer (100 mM KCl, 0.1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, at pH 7.8 with
KOH), supplemented 2% glycerol, protease inhibitors and phenylmethylsulfonyl fluoride, and
stored at –20 °C.

Maltose-binding protein (MBP)-tagged E1 was expressed in *E. coli* inducing cultures at
$D_{600 \, \text{nm}} = 0.6$ with 300 μM IPTG for 5 h at room temperature. Purification was carried out using a
standard MBP purification protocol. For expression of His-tagged UBC10 and His-tagged
UBC4, bacterial cultures were induced at $D_{600 \, \text{nm}} = 0.6$ at 37 °C with 500 μM IPTG for 4 h. The
enzymes were purified through Ni-NTA affinity and gel-filtration chromatography. Glutathione-
S-transferase (GST)-fusion proteins for Rpn10 and Rad23, as well as their sub-domains, were
purified essentially as reported previously (Elsasser et al., 2004; Elsasser et al., 2002).
Recombinant E2-25K (UbcH1) was purchased from Boston Biochem (SP-200).

*Preparation of Xenopus egg extract*
Interphase *Xenopus* egg extract was prepared from eggs laid overnight according to the protocol of Murray (Murray, 1991) with the exception that eggs were activated with 2 µg ml⁻¹ calcium ionophore (A23187, free acid form, Calbiochem) for 30 min prior to the crushing spin. Extract was frozen in liquid nitrogen and stored at -80 °C. Interphase extract was induced to enter mitosis by addition of non-degradable cyclin B, which activates CDK1 and stimulates mitotic phosphorylation, resulting in APC activation. A fusion of the maltose-binding protein (MBP) to *Xenopus* cyclin B lacking its N-terminal 90 amino acids (MBP-Δ90) (Salic and King, 2005) was expressed in *E. coli* by inducing cultures at an $D_{600nm}=0.6$ with 300 µM IPTG for 5 h at room temperature. Purification was carried out following New England BioLabs (NEB) protocol. To make mitotic extract, MBP-Δ90 was added to interphase extract generally at ~ 20 µg ml⁻¹ and incubated at 22-24 °C for 45-60 min.

**Reconstitution of ubiquitination and degradation of cyclin B1**

Ubiquitination reactions were carried out essentially as described previously (Kirkpatrick et al., 2006) for the indicated times. Briefly, for each 30 µl reaction, APC was immunopurified from 600 µl of mitotic *Xenopus* egg extract by incubation for 1 h at 4 °C with 12 µg of anti-Cdc27 antibodies (AF3.1, Santa Cruz Biotechnology) immobilized onto 30 µl of Affiprep Protein A beads (156-0006, Bio-Rad). Following incubation with extract, beads were washed quickly (to minimize loss of associated APC co-activator Cdc20) three times with XB containing 500 mM KCl (10 mM potassium HEPES, pH 7.7, 500 mM KCl, 0.1 mM CaCl₂, 1 mM MgCl₂), two times with XB (same content as above, except with 100 mM KCl), and then three times with reaction buffer (20 mM Tris, pH 7.5, 100 mM KCl, 2.5 mM MgCl₂, 2 mM ATP). Ubiquitination reaction were carried out at 24 °C with agitation at 1500 r.p.m and contained APC on 30 µl beads, and 30 µl of a mix containing recombinant MBP-human E1 (1.3 µM), His-tagged UBCH10 or UBC4 (100 nM – 4 µM), and E2-25K (concentration as indicated) as the E2 enzyme, wild-type or different forms of ubiquitin (118-145 µM), and 450-500 nM cyclin B1-CDK1 or cycB1-NT. For
ubiquitin-receptor binding and degradation assays, reaction supernatants were combined with the first 20 µl of reaction buffer wash. For analysis of cyclin B1 ubiquitination with different ubiquitins, entire reactions were processed for immunoblotting or autoradiography. Dried gels were analyzed by phosphorimaging (Bio-Rad PMI); quantification was carried out with Quantity One software (Bio-Rad).

For binding experiments with ubiquitin receptors, cyclin B1-CDK1 was pre-ubiquitinated with purified Xenopus APC, UBC4 (3 µM), and ubiquitin (118 µM) for 90 min. Approximately 7-8 µg of “bait” protein immobilized onto Glutathione-Sepharose 4B resin (GE Healthcare) was mixed with 4 µl of pre-synthesized ubiquitin-cyclin B1 conjugates and incubated for 1 h at 4 °C with agitation in the presence of 100 µg ml⁻¹ BSA and 0.1 % Tween 20. Supernatants were collected and mixed with the first wash to make the flow-through fraction. Beads were washed twice and diluted with SDS sample buffer to analyze the bound fraction. Equivalent amounts of input (I), flow-through (FT) and bound (B) fractions were subjected to SDS-PAGE and western blot analysis using anti-cyclin B1 polyclonal antibody (Ab-2, Neomarkers).

For degradation assays with purified proteasomes, human proteasomes (10-20 nM, concentrations as indicated), purified as reported previously (Lee et al., 2010) but non-UbVS treated, were added to cyclin B1-Ub, in buffer (50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂ and 5 mM ATP) (Lee et al., 2010) and incubated at 24 °C. For 0 min time-point, substrate and proteasome mixtures were individually added to SDS sample buffer to prevent a time-lag from mixing. Aliquots withdrawn at indicated times were combined with SDS sample buffer and subjected to SDS-PAGE and immunoblot analysis using anti-cyclin B1 polyclonal antibody (Ab-2, Neomarkers).

**Cyclin B1 degradation in Xenopus egg extract**

To deplete APC, 100 µl of interphase extract was mixed 2 µg of anti-Cdc27 antibody coupled to 5 µl of Affiprep Protein A beads and incubated at 4 °C for 3 h. APC depletion was
confirmed by anti-Cdc27 western blot analysis. Approximately 10 μl of pre-ubiquitinated radiolabeled cyclin B1 was added to 90 μl of APC-depleted extract. Reactions were incubated at 22 °C for the indicated times and stopped by the addition of an equal volume of chilled 2% perchloric acid (PCA) (in H₂O) making a new final volume of 200 μl. Reactions were then incubated on ice for ≥30 min and centrifuged at 15,000 r.p.m. for 10 min, at 4 °C. A fraction of supernatants was mixed with Tris Base and Ultima Gold scintillation fluid (6013327, Perkin Elmer) and the radioactivity was measured by scintillation counting.

**Immunodepletion of E2 enzymes**

For UBE2S immunodepletion, 10 μg of anti-UBE2S antibody (N-14, Santa Cruz Biotechnology) or control goat IgG antibody (Santa Cruz Biotechnology) was bound to 25 μl UltraLink Immobilized Protein A/G (Pierce) and incubated with 250 μl extract at 4 °C for ~ 1 h. For UBCH10 immunodepletion, 100 μl of anti-UbcX antibody or an equivalent amount of control rabbit IgG coupled to Affiprep protein A support was used to deplete 170 μl extract. Following incubation, samples were briefly centrifuged and supernatants constituting E2- or control-depleted extracts removed carefully without disturbing resin. Samples of depleted extract were centrifuged again to ensure no resin was present. Cyclin B1-CDK1 (~200 nM) was added to E2- or control-depleted extract and analysis of the time course of degradation was carried out at 24 °C (1,250 r.p.m.). The equivalent of 1 μl of extract was analyzed by SDS-PAGE and anti-cyclin B1 immunoblot analysis. Depletion of UBE2S and UBCH10 was confirmed by western blotting using anti-UBE2S and anti-UBCH10, respectively. To confirm the efficiency of E2 depletion, extract was incubated with ubiquitin agarose, at a ratio of ~10:1 (extract/resin). Ubiquitin agarose was pre-washed four times with 1x energy mix (for 20x stock: 150 mM creatine phosphate, 20 mM ATP, 2 mM EGTA and 20 mM MgCl₂, at pH 7.7) in XB buffer (100 mM KCl). Extract and 2x energy mix were added to ubiquitin agarose and incubated with agitation at 24 °C for 45 min. Samples were centrifuged, extract was removed and ubiquitin agarose was washed once with a
tenfold volume of 1x energy mix in XB. Bound proteins were eluted by boiling in SDS sample buffer and analyzed by immunoblotting.

**Ubiquitin-agarose depletion of Xenopus extract**

Ubiquitin (Ub) agarose (concentration of Ub was 10 mg ml\(^{-1}\) or 1.2 mM) was pre-washed four times with 1x energy mix (for 20x stock: 150 mM creatine phosphate, 20 mM ATP, 2 mM EGTA and 20 mM MgCl\(_2\), at pH 7.7) in XB buffer (100 mM KCl). To pre-washed Ub agarose, added mitotically arrested Xenopus extract and 2x energy mix (20x stock) at a ratio of ~2:1 (extract/ resin), and incubated with agitation (1,250 r.p.m) at 24 °C for 7 min. Following incubation, centrifuged sample at 2,000 r.p.m. for 30 sec to return resin to bottom of the tube. The resulting supernatant is transferred to a new tube containing Ub agarose, repeating the steps described above for a total of 7 rounds of depletion. Samples of supernatant following each round of depletion were combined with SDS sample buffer and levels of proteins analyzed by immunoblotting. Proteins bound to Ub agarose were eluted by boiling in SDS sample buffer and analyzed by immunoblotting.

Ub agarose-depleted extracts were supplemented with 0.9 μM human or Xenopus laevis E1 enzyme (kind gift of Brenda Schulman), ~120 μM wild-type ubiquitin, E2 enzymes (100 nM – 3 μM), as indicated, and 1x energy mix. \(^{35}\)S-labeled cycB1-NT (~200-250 nM) was added to Ub agarose-depleted or undepleted extract and analysis of the time course of degradation was carried out at 24 °C (1,250 r.p.m.). At indicated times, reactions (3 μl per time point) were quenched with 97 μl of 20 % TCA, vortexed and incubated on ice (≥ 30 min) before centrifugation at 14,000g for 30 min at 4 °C. The radioactivity in the supernatant was measured by scintillation counting.
References


Chapter V: Conclusions and Future Directions

Nevena Dimova
The Anaphase-Promoting Complex/Cyclosome (APC/C or APC) is a multi-subunit ligase complex that initiates anaphase and mitotic exit by ubiquitinating cell-cycle regulators, including cyclin B1. Here, we sought to understand the role of ubiquitin-chain formation in APC catalysis and proteasomal targeting. Our work provides strong evidence that the proteolytic machinery does not exert a requirement for Lys11 or other ubiquitin-ubiquitin linkages for efficient degradation of ubiquitinated cyclin B1. Utilizing a reconstituted system and *Xenopus* cell-cycle extracts, we demonstrate that multiple monoubiquitination can indeed be coupled to robust proteolysis, at least in the context of cyclin B1. Upon restriction of the ubiquitin-acceptor sites in cyclin B1, Lys11-linked ubiquitin polymers elaborated by the E2 UBE2S become increasingly important for substrate proteolysis. The existence of two distinct degradative pathways driven by multiple monoubiquitination vs. ubiquitin-chain formation has important implications for how APC activity may be coupled to, and regulated by, distinct E2 enzymes. Our findings explain how the presence of multiple ubiquitin-acceptor sites confers flexibility in the requirement for particular E2 enzymes in modulating the rate of ubiquitin-dependent proteolysis.

**Development of a system to study the role of ubiquitin linkage in proteasomal targeting**

Chapter II of this dissertation presents a novel approach we developed to gain better understanding of the importance of different ubiquitin-chain topologies in promoting substrate degradation by the 26S proteasome. To investigate the nature of the ubiquitin signal targeting cyclin B1 for proteolysis, a system for the expression and purification of radiolabeled N-terminal fragment of the protein was established, which was critical to our ability to examine cyclin degradation in a rigorous and quantitative manner. Analysis of APC-mediated ubiquitination and degradation with purified proteasomes provided us with strong confidence that the behavior of
the N-terminal fragment of human cyclin B1 (amino acids 1-88) (referred to as cycB1-NT) closely resembled that of the full-length protein.

The problem of ubiquitin-chain formation and function has been challenging to study in physiologically relevant systems due to the presence of endogenous ubiquitin. Development of strategies for effective blockade of the regulatory pathways maintaining ubiquitin levels is a non-trivial task and for that reason manipulation of the endogenous ubiquitin pool in any model system is difficult. One experimental approach to studying the role of specific ubiquitin-ubiquitin linkages, or lack thereof, in proteasomal targeting has been the addition of mutant ubiquitin in excess to endogenous ubiquitin (Hershko et al., 1991; Jin et al., 2008; Wu et al., 2010). However, the contribution of specific ubiquitin-chain topologies in proteasomal degradation may be difficult to ascertain when conducted in a background that contains wild-type ubiquitin. Importantly, alteration of ubiquitin levels as upon adding exogenous ubiquitin may influence pairing of a specific E3 ubiquitin ligase with ubiquitin-conjugating enzymes, potentially impacting different aspects of substrate ubiquitination and/or the downstream fate of modified proteins. In this regard, excess added ubiquitin was found to increase the fraction of ubiquitin-charged UBCH5, thereby promoting a functional interaction of this promiscuous E2 with APC in mitotic cell extracts (Summers et al., 2008). These findings emphasize the need for methods where no gross fluctuations in the available ubiquitin pool are introduced. In the UbVS system, recycling of endogenous ubiquitin from existing conjugates is inhibited and wild-type ubiquitin is supplemented at levels just sufficient to restore degradation. Thereby we were able to examine the functional significance of distinct ubiquitin linkages in APC catalysis and proteasomal degradation at more physiologically relevant ubiquitin concentrations.

Our analysis revealed that the availability of free ubiquitin in Xenopus extract has a strong effect on the kinetics of cyclin degradation. One future direction would be to extend our analysis and examine the possibility that control of ubiquitin availability may be a mechanism by which the rate of APC-substrate degradation can be controlled. Utilizing this system, we can
quantitatively evaluate how proteasomal targeting of different APC substrates may be
differentially sensitive to levels of free ubiquitin. Less processive substrates of the APC such as
cyclin A and UBCH10, which require multiple binding cycles to obtain their proteolytic tags
(Rape et al., 2006), may exhibit higher sensitivity to ubiquitin levels. Such sensitivity would be
potentially amplified if proteasomal targeting of a substrate is actively opposed by the activity of
deubiquitinating enzymes. An exciting finding from our work suggests that proteolysis of
endogenous UBCH10 in *Xenopus* extracts may be actively antagonized through the disassembly
or editing of its ubiquitin signal. Levels of UBCH10 are reduced upon supplementing UbVS-
treated mitotic extract with wild-type ubiquitin. In this context, an interesting question to address
will be how the balance of ubiquitination and deubiquitination of UBCH10 modulates the levels
of active E2, thereby providing a mechanism for regulating APC activity. Tools including
immunodepletion and the APC-specific inhibitor TAME (Zeng and King, 2012; Zeng et al.,
2010) would allow us to test whether the reduction in UBCH10 levels in this context is APC-
dependent and how levels of APC activity influence stability of the E2. Furthermore, the
deubiquitinating activity that potentially antagonizes UBCH10 degradation can be identified and
characterized.

In contrast to our findings about UBCH10, UbVS-sensitive isopeptidases are unlikely to
present a major kinetic barrier to cyclin B1 proteolysis in mitotic extract. Importantly, this may
not hold true for interphase extracts. We found that inhibiting the catalytic activity of
deubiquitinases with UbVS in the presence of wild-type ubiquitin stimulated cyclin proteolysis
(data presented in appendix). These findings suggest that the activity of interphase APC can
support cyclin degradation, but is effectively overwhelmed by deubiquitination which prevents
dysregulated substrate proteolysis. We are currently pursuing identification of such
deubiquitinating activity which may specifically oppose the activity of interphase APC, thereby
preventing untimely substrate degradation.
Role of ubiquitin linkage and E2 enzymes in APC/C-mediated degradation

There have been significant efforts dedicated to understanding the mechanistic and architectural complexities underlying APC activity. Structural studies comprise one aspect of these efforts and recent work has shed important insight into how subunit organization of the APC and conformational rearrangements may be coupled with proper substrate recognition and efficient ubiquitin transfer to substrate residues (Buschhorn et al., 2011; da Fonseca et al., 2011). These studies have advanced our understanding of this complex ubiquitin ligase and complement work examining the mechanism of ubiquitin-chain formation by the APC. In this context, recent findings have suggested that Lys11-linked ubiquitin polymers, assembled by the chain-elongating E2 UBE2S, may be important in APC-dependent proteolysis. While important for normal mitotic progression in *Drosophila* S2 cells (Williamson et al., 2009), UBE2S was found to be dispensable for this process in human cells (Garnett et al., 2009). The lack of a uniform requirement for UBE2S, together with the capacity of the proteasome to rapidly degrade cyclin bearing multiple short chains (Kirkpatrick et al., 2006), motivated us to examine whether APC-mediated proteolysis is strictly dependent upon polyubiquitination and whether the proteasome exerts a requirement for specific ubiquitin-ubiquitin linkages to efficiently degrade cyclin B1. To address this question, we utilized the UbVS system. Neither Lys48, nor Lys11, ubiquitin-ubiquitin linkages were essential for robust rates of cyclin proteolysis. Furthermore, our analysis revealed that ubiquitin-chain formation is dispensable for cyclin proteolysis in *Xenopus* extracts, unless the number of available lysine residues is limited. In agreement with the effects of chain-terminating ubiquitin in *Xenopus* extract, purified proteasomes degraded multiply monoubiquitinated cyclin B1 with fast kinetics, comparable to those for substrate bearing ubiquitin chains. Based on our findings, we propose that the presence of multiple lysines in cyclin B1 that are in close proximity to one another has the potential for generating a high local density of monoubiquitin that
promotes receptor binding and proteasomal degradation. Perhaps similar to how linkage of ubiquitin units into a polymer structure may determine its potential as a degradative element, particular spacing of monoubiquitins along the substrate may be differentially conducive to productive engagement with the proteolytic machinery. Previously, Hershko and Heller found that while in the presence of methylated ubiquitin lysozyme is conjugated with up to 7 monoubiquitins, it is degraded with slow kinetics (Hershko and Heller, 1985). An interesting possibility is that, unlike in the context of cyclin B1, the distribution of modified lysines in lysozyme cannot generate sufficient local density of ubiquitin to facilitate ubiquitin receptor and proteasome binding. It may be of interest in the future to examine how the distribution of available ubiquitin-acceptor sites in cyclin B1 influences the capacity of multiple monoubiquitination to serve as a robust proteolytic signal. These efforts would be particularly important as we develop strategies to understanding steps subsequent to dissociation of ubiquitinated cyclin B1 from the ligase, including how conjugates are delivered to the proteasome, and how those events may be coupled to different aspects of cyclin ubiquitination.

An important aspect of our work has been understanding how the architecture of a ubiquitin tag is decoded by the proteolytic machinery. Our analysis indicates that multiple monoubiquitination can promote rapid and complete degradation of a physiological proteasome substrate. Importantly, we define a novel proteolytic signal that confers flexibility in the requirement for particular E2 enzymes in modulating the rate of ubiquitin-dependent proteolysis. In *Xenopus* cell-cycle extracts, we found that the chain-elongating E2 UBE2S is dispensable for APC/C-mediated degradation of cyclin. Our findings are consistent with a model where UBCH10, or possibly UBC4/5 enzymes, catalyzes the conjugation of monoubiquitin to multiple lysine residues and possibly the elaboration of some short chains, generating degradation-competent cyclin. These E2 enzymes are recruited to the ligase through the RING domain of APC11 (Peters, 2006; Tang et al., 2001; Yu et al., 1998) without impeding the recruitment of UBE2S, which interacts at least in part through the substrate-adaptor proteins Cdc20 and Cdh1.
Such a mode of recruitment of multiple ubiquitin-conjugating enzymes would potentially overcome the separation of ubiquitin-chain initiation and elongation steps, favoring the processive assembly of long polymers. Although UBE2S appears to be present in *Xenopus* extract at sufficient levels to support cyclin proteolysis, it does not appear to be an active component of the conjugation machinery and becomes essential only when the number of ubiquitinatable lysine residues in cyclin B1 is restricted. This raises questions as to whether UBE2S is tightly associated with the APC/C and how its activity is regulated. An intriguing possibility is that levels of free ubiquitin regulate activity of UBE2S. Perhaps under conditions of ubiquitin limitation as may be the case in *Xenopus* extract, there are mechanisms in place to conserve ubiquitin, restricting extent of ubiquitination to what is sufficient for timely destruction and preventing the formation of multiple long chains. Future work will be needed to address these questions.

While our work provides strong evidence that multiple monoubiquitination can serve as an efficient proteolytic signal in the context of cyclin B1, further studies are required to determine if such mode of ubiquitination targets additional APC substrates to the proteasome or if it is a degradative pathway uniquely pertaining to cyclin B1. The UbVS system, described earlier, provides an exciting opportunity to extend our analysis to other APC substrates and better understand how the lysine profile of a substrate may influence the balance between multiple monoubiquitination and ubiquitin-chain extension. Perhaps robust APC-mediated proteolysis of securin, which like cyclin B1 has 18 lysines, may show little or no dependence on ubiquitin-chain formation and catalysis by the E2 UBE2S. For other substrates such as cyclin A2 and UBCH10, each of which has 12 lysines, degradation may be more dependent on the chain-elongating enzyme UBE2S. Currently, we are investigating whether the blockage of lysines by other post-translational modifications or a crosstalk between neighboring post-translational modifications may limit the availability of ubiquitin-acceptor sites in cyclin B1, enforcing a requirement for synthesis of long ubiquitin chains for efficient turnover. Work carried out in collaboration with
the Gygi lab has uncovered residues within the N-terminal domain of cyclin B1 that are modified with acetyl groups. Current and future efforts may be dedicated to identifying a physiological setting most conducive to studying effects of acetylation on APC-mediated catalysis and ubiquitin-dependent proteolysis.

It was surprising to find that in Xenopus cell-cycle extracts multiple monoubiquitination can signal robust proteasomal turnover. Our findings in a purified system and in Xenopus extract suggest that the proteasome does not impose a requirement for ubiquitin-chain formation for cyclin B1 destruction. One could envision that degradation of a substrate conjugated with 5 or 6 monoubiquitins, as appears to be the case for cyclin B1, may be more sensitive to deubiquitinases, as compared to a heavily ubiquitinated protein. We found that one such DUB able to remove monoubiquitins attached to cyclin is the proteasome-associated enzyme USP14. Unlike in an in vitro system, USP14 did not appear to antagonize cyclin B1 degradation in Xenopus extracts (data also shown in appendix). APC-dependent proteolysis of cyclin B1, at least in mitotic Xenopus extracts, was not appreciably impeded by UbVS-sensitive DUBs. These findings do not rule out the possibility that multiply monoubiquitinated species of cyclin B1 are effectively “shielded” by the activity of deubiquitinating enzymes through binding to proteins such as ubiquitin receptors. There is minimal knowledge of what factors may recognize and decode a signal specifically comprised of multiple monoubiquitins or Lys11-linked ubiquitin polymers. With reports demonstrating that several DUBs, including the OTU-domain containing Cezanne protein, have a preference for Lys11-linked chains (Bremm et al., 2010), we are only beginning to understand how the ubiquitin code is read. Development of tools to identify factors interacting with ubiquitin conjugates downstream of different E2-E3 complexes will provide us with an opportunity to examine how distinct ubiquitin-ubiquitin linkages are assigned independent functions in the pathway leading to the proteasome.
References


Chapter VI: Appendix


N.D. and R.W.K. designed and interpreted the experiments. N. D. carried out and analyzed all experiments except those outlined below. N.A.H. carried out cyclin B1 ubiquitylation for ubiquitin-AQUA analysis and degradation assays with these species in APC/C-depleted extract. D.S.K. carried out the ubiquitin-AQUA analysis on cyclin B1 ubiquitylated *in vitro* with the E2 UBC4 and different ubiquitin types in the laboratory of S.P.G. B-H.L. provided purified human proteasomes with oversight from D.F. M.L.B. helped with cloning of different cyclin B1 mutants. The manuscript was written by N.D. and R.W.K. with input from all authors.

B. Analysis of the role of USP14 in cyclin B1 proteolysis in *Xenopus* extract

Nevena Dimova designed and carried out all experiments. Nevena Dimova wrote this chapter.


Nevena Dimova designed and carried out *in vitro* ubiquitination of cyclin B1 for degradation assays, part of the manuscript.


Nevena Dimova carried out degradation assays with pre-ubiquitinated cyclin B1 and critically read this manuscript.
APC/C-mediated multiple monoubiquitylation provides an alternative degradation signal for cyclin B1

Netsevas V. Dimova, Nathanial A. Hathaway, Byung-Hoon Lee, Donald S. Kirkpatrick, Marie Lea Berkowitz, Steven P. Gygi, Daniel Finley and Randall W. King

The anaphase-promoting complex or cyclosome (APC/C) initiates mitotic exit by ubiquitylating cell-cycle regulators such as cyclin B1 and securin. Lys 48-linked ubiquitin chains represent the canonical signal targeting proteins for degradation by the proteasome, but they are not required for the degradation of cyclin B1. Lys 11-linked ubiquitin chains have been implicated in degradation of APC/C substrates, but the Lys 11-chain-forming E2 UBE2S is not essential for mitotic exit, raising questions about the nature of the ubiquitin signal that targets APC/C substrates for degradation. Here we demonstrate that multiple monoubiquitylation of cyclin B1, catalyzed by UBC4/6 or UBC5/8, is sufficient to target cyclin B1 for destruction by the proteasome. When the number of ubiquitylatable lysines in cyclin B1 is restricted, Lys 11-linked ubiquitin polymers elaborated by UBE2S become increasingly important. We therefore explain how a substrate that contains multiple ubiquitin acceptor sites confers flexibility in the requirement for particular E2 enzymes in modulating the rate of ubiquitin-dependent proteolysis.

A uniform Lys 48-linked ubiquitin polymer was the first signal identified to target substrates for destruction by the 26S proteasome. Recent work has demonstrated that the repertoire of proteolytic signals encompasses chains of other linkage types, including Lys 11-linked ubiquitin chains and short chains of mixed linkage types. In contrast, Lys 48-linked chains have non-proteolytic roles in DNA repair, kinase activation, protein trafficking and translation. Similarly, the transfer of a single ubiquitin moiety to one (monoubiquitylation) or to multiple sites (multiple monoubiquitylation) in a substrate has been implicated in many non-proteolytic processes, although multiple monoubiquitylation can target receptor tyrosine kinases to the lysosome. More recently, multiple monoubiquitylation has been shown to control proteasomal processing of the p53 NF-κB precursor to the shorter p14 subunit. So far, multiple monoubiquitylation has not been coupled with rapid and complete proteolysis of a proteasome substrate.

The SCF ligand activities of the Skp1-Cul1-F-box complex (SCF) family and the APC/C are essential for cell-cycle progression. The SCF complex with the E2 Cdc34 to assemble uniform Lys 48-linked ubiquitin polymers on substrates, and the APC/C works in conjunction with UBC13 (also known as UBC5/8) and either of the UBC4/6 family to catalyze chain formation through lysine residues of ubiquitin (Lys 48). Lys 48 and Lys 63, and Lys 11. UBC13 builds multiple short ubiquitin chains on cyclin B1, which are sufficient to target the protein for degradation by the proteasome. In this context, Lys 48-linked ubiquitin polymers are dispensable for binding of modified cyclin B1 to ubiquitin receptors and degradation by the proteasome. More recent work indicates that the assembly of a proteolytic signal on APC/C substrates may occur in two stages. In budding yeast, Ubc1 initiates ubiquitin conjugation, whereas Ubc1 degrades ubiquitin chains. Similarly, in murine testis, UBCH9 has been proposed to initiate monoubiquitylation of the substrate followed by UBE2S-dependent extension of Lys 11-linked ubiquitin chains. Consistent with this idea, depletion of UBE2S from Drosophila S2 cells prolongs metaphase and stabilizes cyclin B1 at the spindle pole. In contrast, UBE2S is not essential for normal metaphase in human fetal cells, but either may be important for proteolysis under conditions where APC/C activity is compromised, such as during recovery from drug-induced spindle-assembly checkpoint activation. Using an approach in which Xenopus cyclin B1 is expressed in mammalian cells, we sought to understand whether APC/C-catalyzed proteolysis requires Lys 11 or other ubiquitin linkages to efficiently degrade cyclin B1.

RESULTS

Inhibiting ubiquitin-chain formation has only a modest effect in stabilizing cyclin B1 in Xenopus extract. To quantitatively evaluate the role of different ubiquitin-chain linkages in targeting cyclin B1 for degradation in mitotic Xenopus...
Figure 3 (left) shows the effect of ubiquitin on E1 degradation by comparing the amount of active ubiquitin. a, Ubiquitin was synthesized using 141 HPLC-purified wild-type ubiquitin and 44 mM wild-type ubiquitin was added to the reaction mixture, which was then incubated for 20 min. The ubiquitin was then diluted, and the amount of active ubiquitin was measured in a fluorescence polarization assay, which measures the percentage of active ubiquitin from the total ubiquitin. b, Ubiquitin was synthesized using 141 HPLC-purified wild-type ubiquitin and 44 mM wild-type ubiquitin was added to the reaction mixture, which was then incubated for 20 min. The ubiquitin was then diluted, and the amount of active ubiquitin was measured in a fluorescence polarization assay, which measures the percentage of active ubiquitin from the total ubiquitin. c, Ubiquitin was synthesized using 141 HPLC-purified wild-type ubiquitin and 44 mM wild-type ubiquitin was added to the reaction mixture, which was then incubated for 20 min. The ubiquitin was then diluted, and the amount of active ubiquitin was measured in a fluorescence polarization assay, which measures the percentage of active ubiquitin from the total ubiquitin. d, Ubiquitin was synthesized using 141 HPLC-purified wild-type ubiquitin and 44 mM wild-type ubiquitin was added to the reaction mixture, which was then incubated for 20 min. 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the U25S-treated extract, as supplementation with additional ubiquitin restored degradation to the initial rate (Fig. 3b). Extracts supplemented with Ub600 or Ub650 degraded substrate with surprisingly fast kinetics. From addition of Ub500 (Fig. 3c) or a lysine-less ubiquitin (Ulb3) Supplementary Fig. 5N) supported degradation, with a half-life of approximately 30 min. We next assessed how constraining the topology of ubiquitin chains to a single lysine residue affected degradation of cyclin B1-NP (Fig. 3c). Addition of Ub500 or Ub550 to U25S-treated extracts restored cyclin B1-NP proteolysis, but with slower kinetics when compared with wild-type ubiquitin. As mutation of Lys 6 of ubiquitin may have an inhibitory effect on proteasomal degradation8, we examined the effect of restricting chain formation to one of the three principal sites of ubiquitin-ubiquitin attachment mediated by UBE2H1 by mutating the remaining two (Supplementary Fig. 5D). Ub500/550 stimulated degradation efficiently, consistent with the ability of Lys 11 linkages to support degradation (Fig. 3d). Ubiquitin forms supporting Lys 48 and Lys 63 linkages (Ub500/550 and Lys-3), respectively, and Ub48 supported proteolysis with slightly slowed kinetics. Together these findings indicate that the ability to construct Lys 11-limited chains provides a kinetic advantage for degradation, but the advantage is modest. In principle this advantage could arise from the utilization of Lys 11 in chain forming reactions catalyzed by UBE2H1, or from a role of UbES2, which regulates ubiquitin chains on monoubiquitinated cyclin B1, by limiting Ub500 or Ub550, with the latter causing quantitative accumulation of cyclin B1 in a monoubiquitinated form. Restoration of these three additional lysine residues in cyclin B1 (Ub550/550) partially rescued its degradation in U25S-treated extracts supplemented with Ub550 (Fig. 3d). We conclude that when deubiquitinating enzymes are inhibited, the attachment of single ubiquitin molecules to multiple lysine residues in cyclin B1 is sufficient to target the substrate for degradation. Strict dependence on elongation of ubiquitin chains seems to occur only when the number of available substrate lysine residues is restricted.

Ubiquitin chains are required for cyclin B1 degradation only when the number of available lysine residues in cyclin B1 is restricted.

Cyclin B1 contains 18 lysine residues in its unstructured N-terminal region upstream of the cyclin box. 15 of these lysine residues are located within the first 40 amino acids close to the destruction box (Fig. 3a). To rule out the possibility that our results were influenced by use of an N-terminal fragment of cyclin B1, we first examined proteolysis of full-length wild-type cyclin B1 in U25S-treated extracts (Fig. 3c). Addition of Ub500 or Ub550 stimulated degradation, albeit at slightly reduced rates relative to wild-type ubiquitin. Ub500 also supported degradation of cyclin B1, although a small fraction of the protein accumulated in a triply ubiquitylated species. To examine whether reducing the number of lysines in cyclin B1 residues in proteolysis dependent on ubiquitin chain formation, we measured degradation of cyclin B1 mutants that contained either one or four lysine residues in the first 40 amino acids at position 50 (Ub550/550) or at positions 39, 63, 64, and 65 (Ub550/550/550). We chose these positions as mass spectrometry studies indicated that these lysines residues become ubiquitylated only in the course of succinylated ubiquitylation reactions (J. D. and N. L., unpublished observations). Cyclin B150/550 was degraded equally in treated U25S extract, and was fully stabilized in U25S-treated extract (Fig. 3c). However, unlike the case for wild-type cyclin B1, Ub550 did not support efficient degradation of Ub550/550, similar results were obtained with Ub550 and Ub550, with the latter causing quantitative accumulation of cyclin B1 in a monoubiquitinated form. Restoration of these three additional lysine residues in cyclin B1 (Ub550/550) partially rescued its degradation in U25S-treated extracts supplemented with Ub550 (Fig. 3d). We conclude that when deubiquitinating enzymes are inhibited, the attachment of single ubiquitin molecules to multiple lysine residues in cyclin B1 is sufficient to target the substrate for degradation. Strict dependence on elongation of ubiquitin chains seems to occur only when the number of available substrate lysine residues is restricted.
Multiplication of nucleotide sequences can target cycle B1 for efficient degradation in a reconstituted system and in Xenopus extract.

We investigated whether the effects of ubiquitin conjugation on protein degradation in reconstituted ubiquitination reactions. Elimination of Lys 48 or Lys 63 on ubiquitin had no effect on the rate of degradation of recombinant human ubiquitin conjugates generated by UBC10, whereas elimination of Lys 23 reduced the rate of degradation, consistent with the previously reported preference of UBC10 for generating Lys 11 ubiquitin conjugates. This promotes the formation of ubiquitin polymers that do not support ubiquitin polymer assembly (UVPs) and UVPs, the maximal extent of substrate modification (5-6 ubiquitin per cycle B1 molecule) was observed at early times and remained unchanged in longer reactions (Fig. 4a). Imposing that only a subset of the 19 lysine residues in the cycle B1 N-terminal domain become ubiquitylated. A time course analysis of ubiquitination with either wild-type ubiquitin or UVPs (Fig. 4b) revealed that the conjugation of ubiquitin monomers to distinct systems in cycle B1 occurs with equal efficiency. Furthermore, conjugates bearing 4 or more ubiquitin moieties were capable of binding proteins associated ubiquitin-receptor complexes (Fig. 4c).

Acids (measured B2.15 kmol shown). cmol w/ubiquitin cycle B1. 4b Purified ubiquitin wild type or single cycle B1 (5) in complexes with CDC134, and formation of ubiquitin complexes as indicated were added to native ubiquitin receptor that had been pre-treated with UBC1. CDC134 was found. UBP1, or ubiquitin. UBP1, ubiquitin degradation. Stability of the human rhinovirus strains was assessed by SDS-PAGE and cycle B1 antibodies in analysis. 4b, 4c, and 4d is shown. The behavior of full-length ubiquitin, CDC134 complexes with UBP1, and analysis of immunoblots are shown in Supplementary Fig. 59.
Figure 4 | UBE2S and APOC-mediated ubiquitination of cyclic B1 at sites sufficient for binding ubiquitin receptors. (a) Western blot analysis of the in vitro ubiquitination reaction containing full-length cyclic B1, APOC immunoaffinity-purified yeast immunoprecipitates (UBE2S) and forms of ubiquitin (Ub48DA); as indicated, ubiquitin variants with newly-synthesized modifications at one, two, or all three positions (U1, U6, and U1/U2) (Ub48DA), as well as Ub48DA methylated ubiquitin) were used. Control APOC reactions containing all components except for the full-length ubiquitin were carried out in parallel. Reactions were allowed to proceed for 15 or 30 min before analysis by SDS-PAGE and Western blotting against cyclic B1. (b) Time course of the in vitro ubiquitination of cyclic B1 in the presence of APOC (Supplementary Fig. S4). Degradation of cyclic B1 in the recombinant system was confirmed to be both APOC and ubiquitin-dependent (Supplementary Fig. S5 and S5a). Similar experiments with radio-labeled cyclic B1 pre-ubiquitinated by UBE2S (Fig. S5) revealed that the degradation of multiple non-ubiquitinated cyclic B1 was readily detected by the ubiquitin-peptide complexes U24 (Fig. S5b). This effect was reversed by U1, an inhibitor of the catalytic activity of UBE2S (Supplementary Fig. S5c, ref. 44). Together these results indicate that purified proteosomes can efficiently degrade multiple non-ubiquitinated cyclic B1 and that U1 can desensitize this substrate to ubiquitination.

To determine whether multiple non-ubiquitinated cyclic B1 in the degradation model under physiological conditions in the presence of active desensitizing enzymes, we added the ubiquitinated species indicated in Fig. S5c to a more precise Xuanus extract, a substrate in which the APOC is active (Fig. S5c). Cyclic B1 generated with wild-type ubiquitin or Ub48DA were efficiently degraded, whereas conjugates generated with Ub48DA were degraded less efficiently. Similar results were obtained when these ubiquitin conjugates were introduced into extracts supplemented with active non-ubiquitinated and ubiquitin-activated substrate to present additional APOC-mediated ubiquitination (Supplementary Fig. S5d) or to extracts that had been immunoprecipitated of APOC (Supplementary Fig. S5e). Pre-treatment with ISIS, at a concentration identical to that used to deplete free ubiquitin, did not accelerate degradation of conjugate (Fig. S5f). Similarly, addition of an inhibitor of UBE2S (S3), failed to accelerate degradation of cyclic B1 in Xuanus extracts (Supplementary Fig. S5g). Together these findings indicate that Xuanus extracts can rapidly degrade cyclic B1 bearing multiple ubiquitin moieties without a distinct proximal residue.

Analysis of the role of UBE2S in cyclic B1 degradation
The ability of LP and chain-terminating ubiquitin to support proteolytic activity raised a question as to whether the B1 response UBE2S is required for cyclic B1 degradation in Xuanus extract. We therefore
In microsomal lysates, the protein is detected and measured by Western blot analysis. Antibodies efficiently depleted the UBE3S protein, as observed by the absence of signal following SDS-PAGE or microsomal lysates (Fig. 6a). However, levels of the APC/C on the APC/C substrates (Fig. 6a) were not significantly different from the controls (Fig. 6a), which was confirmed by adding back Ub to the recombinant enzyme. We observed that UBE3S is not essential for rapid degradation of cyclin B1, because the substrate contains multiple lysine residues that are sensitive to polyubiquitination. Therefore, the level of cyclin B1 was affected by depleting UBE3S, but not by depletion of UBE3S, because the level of cyclin B1 was not significantly different from the controls (Fig. 6a).

Figure 5. Multiple microsomal lysates from different cell lines were analyzed by Western blot analysis. (A) Anti-ubiquitin antibodies were used to detect ubiquitinated proteins. (B) Anti-cyclin B1 antibodies were used to detect cyclin B1. (C) Anti-APC/C antibodies were used to detect APC/C. (D) Anti-UBE3S antibodies were used to detect UBE3S. (E) Anti-UBE3S antibodies were used to detect UBE3S. (F) Anti-UBE3S antibodies were used to detect UBE3S. (G) Anti-UBE3S antibodies were used to detect UBE3S. (H) Anti-UBE3S antibodies were used to detect UBE3S. (I) Anti-UBE3S antibodies were used to detect UBE3S. (J) Anti-UBE3S antibodies were used to detect UBE3S. (K) Anti-UBE3S antibodies were used to detect UBE3S. (L) Anti-UBE3S antibodies were used to detect UBE3S. (M) Anti-UBE3S antibodies were used to detect UBE3S. (N) Anti-UBE3S antibodies were used to detect UBE3S. (O) Anti-UBE3S antibodies were used to detect UBE3S. (P) Anti-UBE3S antibodies were used to detect UBE3S. 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Figure 6: UBR5 is required for cyclic E1 protein stability only when ubiquitination is confined to a single lysine. (A) Western blots probed with antibodies against E1S, E2S, and UBR5 were analyzed by SDS-PAGE and immunoblotting. Lanes 2-4 represent 24 h of enrichment of E2S enzymes on substrate against levels of UBR5, UBR5(ΔC) and UBR5(ΔC) mutant UBR5. (B) Time course of degradation of half-length A subunit in control or UBR5-depleted, mitotic A subunit marker. Reconstituted, non-UBR5(ΔC) control, and UBR5(ΔC) mutant A subunit were analyzed by SDS-PAGE and immunoblotting. Levels of E1S, E2S, and UBR5 were measured by immunoblotting. Asterisks, nonspecific signal. (C) Reconstituted, non-UBR5(ΔC) control, and UBR5(ΔC) mutant A subunit were analyzed by SDS-PAGE and immunoblotting. Levels of E1S, E2S, and UBR5 were measured by immunoblotting. Asterisks, nonspecific signal. (D) Reconstituted, non-UBR5(ΔC) control, and UBR5(ΔC) mutant A subunit were analyzed by SDS-PAGE and immunoblotting. Levels of E1S, E2S, and UBR5 were measured by immunoblotting. Asterisks, nonspecific signal. (E) Reconstituted, non-UBR5(ΔC) control, and UBR5(ΔC) mutant A subunit were analyzed by SDS-PAGE and immunoblotting. Levels of E1S, E2S, and UBR5 were measured by immunoblotting. Asterisks, nonspecific signal. (F) Reconstituted, non-UBR5(ΔC) control, and UBR5(ΔC) mutant A subunit were analyzed by SDS-PAGE and immunoblotting. Levels of E1S, E2S, and UBR5 were measured by immunoblotting. Asterisks, nonspecific signal. (G) Reconstituted, non-UBR5(ΔC) control, and UBR5(ΔC) mutant A subunit were analyzed by SDS-PAGE and immunoblotting. Levels of E1S, E2S, and UBR5 were measured by immunoblotting. Asterisks, nonspecific signal. (H) Reconstituted, non-UBR5(ΔC) control, and UBR5(ΔC) mutant A subunit were analyzed by SDS-PAGE and immunoblotting. Levels of E1S, E2S, and UBR5 were measured by immunoblotting. Asterisks, nonspecific signal. (I) Reconstituted, non-UBR5(ΔC) control, and UBR5(ΔC) mutant A subunit were analyzed by SDS-PAGE and immunoblotting. Levels of E1S, E2S, and UBR5 were measured by immunoblotting. Asterisks, nonspecific signal. (J) Reconstituted, non-UBR5(ΔC) control, and UBR5(ΔC) mutant A subunit were analyzed by SDS-PAGE and immunoblotting. Levels of E1S, E2S, and UBR5 were measured by immunoblotting. Asterisks, nonspecific signal. (K) Reconstituted, non-UBR5(ΔC) control, and UBR5(ΔC) mutant A subunit were analyzed by SDS-PAGE and immunoblotting. Levels of E1S, E2S, and UBR5 were measured by immunoblotting. Asterisks, nonspecific signal. (L) Reconstituted, non-UBR5(ΔC) control, and UBR5(ΔC) mutant A subunit were analyzed by SDS-PAGE and immunoblotting. Levels of E1S, E2S, and UBR5 were measured by immunoblotting. Asterisks, nonspecific signal. (M) Reconstituted, non-UBR5(ΔC) control, and UBR5(ΔC) mutant A subunit were analyzed by SDS-PAGE and immunoblotting. Levels of E1S, E2S, and UBR5 were measured by immunoblotting. Asterisks, nonspecific signal. (N) Reconstituted, non-UBR5(ΔC) control, and UBR5(ΔC) mutant A subunit were analyzed by SDS-PAGE and immunoblotting. Levels of E1S, E2S, and UBR5 were measured by immunoblotting. Asterisks, nonspecific signal. (O) Reconstituted, non-UBR5(ΔC) control, and UBR5(ΔC) mutant A subunit were analyzed by SDS-PAGE and immunoblotting. Levels of E1S, E2S, and UBR5 were measured by immunoblotting. Asterisks, nonspecific signal. (P) Reconstituted, non-UBR5(ΔC) control, and UBR5(ΔC) mutant A subunit were analyzed by SDS-PAGE and immunoblotting. Levels of E1S, E2S, and UBR5 were measured by immunoblotting. Asterisks, nonspecific signal. (Q) Reconstituted, non-UBR5(ΔC) control, and UBR5(ΔC) mutant A subunit were analyzed by SDS-PAGE and immunoblotting. Levels of E1S, E2S, and UBR5 were measured by immunoblotting. Asterisks, nonspecific signal. (R) Reconstituted, non-UBR5(ΔC) control, and UBR5(ΔC) mutant A subunit were analyzed by SDS-PAGE and immunoblotting. Levels of E1S, E2S, and UBR5 were measured by immunoblotting. Asterisks, nonspecific signal. (S) Reconstituted, non-UBR5(ΔC) control, and UBR5(ΔC) mutant A subunit were analyzed by SDS-PAGE and immunoblotting. Levels of E1S, E2S, and UBR5 were measured by immunoblotting. Asterisks, nonspecific signal. (T) Reconstituted, non-UBR5(ΔC) control, and UBR5(ΔC) mutant A subunit were analyzed by SDS-PAGE and immunoblotting. Levels of E1S, E2S, and UBR5 were measured by immunoblotting. Asterisks, nonspecific signal. (U) Reconstituted, non-UBR5(ΔC) control, and UBR5(ΔC) mutant A subunit were analyzed by SDS-PAGE and immunoblotting. Levels of E1S, E2S, and UBR5 were measured by immunoblotting. Asterisks, nonspecific signal. (V) Reconstituted, non-UBR5(ΔC) control, and UBR5(ΔC) mutant A subunit were analyzed by SDS-PAGE and immunoblotting. Levels of E1S, E2S, and UBR5 were measured by immunoblotting. Asterisks, nonspecific signal.
DISCUSSION

Here we have evaluated the role of ubiquitin-chain topology in targeting cyclin B1 for degradation. Our study was motivated by recent findings indicating that Lys 11 linkages, mediated by the chain-forming E2 enzyme UBCH14, may be important for APC/C-dependent proteolysis. Moreover, an earlier work indicated that APC/C, as well as in conjunction with the E2 enzyme UBCH14 or the enzyme UBCH5, can bind ubiquitin signals that are sufficient for degradation by purified proteasomes. We provide a resolution to this paradox, demonstrating that conjugation of ubiquitin to multiple lysyl residues of cyclin B1 provides an alternative degradation signal for cyclin B1 that does not require chain extension by the Lys 11-specific E2 UBCH5. Lys 11-linked ubiquitin chain formation becomes essential only when the number of available lysyl residues in cyclin B1 is saturated.

Dominant-negative effects of different ubiquitin types may be difficult to observe when examined in a background that contains wild-type ubiquitin. By inhibiting ubiquitin recycling, we were able to impose a state of ubiquitin deficiency in extract sufficient to stabilize cyclin B1. The strong dependence of cyclin B1 on ubiquitin availability has not been previously appreciated, and supports the possibility that control of ubiquitin availability could regulate the rate of APC/C substrate degradation. In addition, ubiquitin-mediated cyclin B1 degradation in Ub54 mutants extracts, but the rate of cyclin B1 degradation is no faster in a Lys 11-modified extract relative to a non-modified extract. This finding indicates that for deubiquitinating enzymes to be able to antagonize degradation, it may be crucial that the rate of ubiquitination be controlled by limiting the availability of free ubiquitin.

The Ub54 system enabled us to define the role of different chain linkages in targeting substrates for degradation by the proteasome. In agreement with earlier work in a recombinant system, Lys 48 ubiquitin linkages were required for efficient cyclin B1 degradation in Ub54-positive extracts. Surprisingly, in the light of recent studies of the human ubiquitin cycle, the Ub54 Core complex also supported efficient degradation of cyclin B1. Importantly, we found that chains-mediating ubiquitination (Ub54 and Lys 48 ubiquitin) also supported efficient cyclin B1 degradation, but the efficiency of chain formation was 10-fold greater in a Lys 11-modified extract relative to a non-modified extract. This finding indicates that for deubiquitinating enzymes to be able to antagonize degradation, it may be crucial that the rate of ubiquitination be controlled by limiting the availability of free ubiquitin.

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We propose that attachment of mono-ubiquitin to multiple lysyl residues in cyclin B1 has the potential for generating a high density of ubiquitin that promotes receptor binding (Fig. 6b). In such an arrangement, the hydrophobic patches on distinct ubiquitin units may be able to engage multiple ubiquitin receptors. Multiple monoubiquitinated cyclin B1 thus resembles a ubiquitin chain, except that the cyclin B1 polyubiquitin chain is used as a backbone to link two ubiquitin molecules to another. Whether the presence of a single polyubiquitin lysyl residue in essential for recognition by ubiquitin receptors remains unknown. In our published experiments, there may be some enhanced avidity resulting from a dimer glutathione-S-transferase (GST) moiety positioning two ubiquitin-associated domains in close proximity. However, the ability of the multiple monoubiquitlated protein to be degraded by purified proteasomes and in Xenopus extracts indicates that this substrate must have sufficient affinity for proteasome-associated ubiquitin receptors to support proteolysis.

The capacity of purified proteasomes to rapidly degrade multiple monoubiquitlated cyclin B1 was significantly suppressed by UBP64, indicating that UBP64 can efficiently remove monoubiquitin, as well as trim ubiquitin chains. However, UBP64 does not seem to strongly antagonize proteasome function in Xenopus extracts, as treatment of extracts with UBP64 or the UBP64-specific inhibitor U1 did not appreciably enhance turnover of pre-ubiquitlated cyclin B1. Although present in Xenopus extracts (N.S.D. and R.W.K., unpublished data), levels of UBP64 associated with proteasomes in extracts may be insufficient to impede proteolysis. Together, these findings indicate that the proteasome does not impose a requirement for ubiquitin chain formation for efficient proteolysis of cyclin B1 in Xenopus extracts. Further work will strengthen the view that the proteasome has the capacity to recognize and degrade substrates bearing ubiquitin modifications distinct from the canonical Lys 48-linked polyubiquitin chains.

Although Ub54 is sufficient to elongate Lys 11-linked ubiquitin chains to promote the degradation of APC/C substrates, we found that UBCH5 depletion of Xenopus egg extract has no impact on degradation of wild-type cyclin B1. Our findings are consistent with the report that UBCH5 is largely dispensable for cyclin B1 degradation in unperturbed mitotic in human cells. The lack of requirement for UBCH5 and Lys 11-linked ubiquitin chains for robust degradation of cyclin B1 in the Xenopus system may be a consequence of higher levels of UBCH10 than seen in other cellular contexts. Furthermore, the relative importance of UBCH10 and UBCH5 in degradation of different APC/C-substrates may vary. However, in Xenopus extracts, Lys 11 ubiquitin was unreactive towards a variety of ubiquitin-modified enzymes in this system. A greater dependence on chain elongating E2s may impact the sensitivity of different substrates to degradation. In this regard, cyclin A2 degradation during interphase is specifically impeded by the deubiquitinating enzyme USP7 (ref. 34), but this enzyme does not seem to antagonize cyclin B1 degradation. An interesting question is how the balance between multiple monoubiquitination and ubiquitin-chain formation affects sensitivity of degradation to deubiquitinating enzymes. Finally, our work raises the possibility that the degree of dependence on UBCH5 could be regulated by post-translational modification of the substrate. For example, acetylation is known to affect degradation of the spindle checkpoint protein Bub1 (ref. 35). By reducing the number of ubiquitylatable lysyl residues, acetylation could increase the dependence of degradation pathways on UBCH5-analysed chain formation.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturecellbiology

Note: Supplementary Information is available on the Nature Cell Biology website.
METHODS

Antibodies and reagents. Proteins were separated by SDS-PAGE on 4–12% gradient gels (Invitrogen). Blots were transferred to PVDF membranes for immunoblotting using the following antibodies: anti-

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**METHODS**

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**IMMUNOCOLLECTION OF E2 ENZYMES.** For E2 immunocollection, flag of anti-E2

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**NATURAL CELL BIOLOGY**

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Figure S1: Endogenous ubiquitin levels are limiting for degradation of an HRAS mimetic-framed of human p53 (B1) in mitotic Xenopus extract. For all panels, [35S]labeled ubiquitin (B1, 1-140) (1-200 nM) was added to extract. Starnes were taken at indicated times and subjected to trichloroacetic acid (TCA) precipitation. Proteolysis was measured by release of TCA-soluble counts and plotted as percent of input radioactivity (p53B1), percent. (A) Degradation of [35S]labeled ubiquitin in p23 fraction in the p53B1-exposed Xenopus extract. (B) Degradation of [35S]labeled ubiquitin in p23 fraction in mitotic Xenopus extract pre-treated with an inhibitor of the UVIC, TARF (Cao et al., Cancer Cell 10, 385-395 (2001)) (200 nM). (C) Inhibition of the [35S]labeled ubiquitin degradation in p23 fraction in mitotic Xenopus extract pre-treated with an inhibitor of the UVIC, TARF (Cao et al., Cancer Cell 10, 385-395 (2001)) (200 nM). (D) Inhibition of the [35S]labeled ubiquitin degradation in p23 fraction in mitotic Xenopus extract pre-treated with an inhibitor of the UVIC, TARF (Cao et al., Cancer Cell 10, 385-395 (2001)) (200 nM). (E) Degradation of [35S]labeled ubiquitin in p23 fraction in mitotic Xenopus extract pre-treated with an inhibitor of the UVIC, TARF (Cao et al., Cancer Cell 10, 385-395 (2001)) (200 nM). (F) Degradation of [35S]labeled ubiquitin in p23 fraction in mitotic Xenopus extract pre-treated with an inhibitor of the UVIC, TARF (Cao et al., Cancer Cell 10, 385-395 (2001)) (200 nM).
Figure S2: (a) MALDI-TOF analysis of ubiquitin-APOC-mediated ubiquitination of cyclin B1 reveals the formation of conjugates formed with ubiquitin mutates of ubiquitin (ΔCyclin B1, ΔCyclin B1, ΔCyclin B1, ΔCyclin B1). A 3C digestion of each of the above-mentioned ubiquitination assay containing E1, E2, E3, and UbcH10 (10 μg/ml) was performed to release the ubiquitination products. The fractions were performed at 22°C for 15 or 90 min. (b) ACG kinase activity was determined by the use of a commercial assay kit that was run under the same conditions used for the western blot. The results of the kinase assay from the 90 min reaction are shown. (c) Total amount of each individual ubiquitin-ubiquitin linkage for the complete linkage is broken into 4% of 100% of all conjugates from conjugates that bind both through kinases, deubiquitinated that is attached to protein B in a ubiquitin-hemoglobin peptide chain without itself containing a ubiquitin chain linkage with total amount of cyclin B1 (does not include a comparable mass spectrometry using a standard peptide derived from cyclin B).
**SUPPLEMENTARY INFORMATION**

Figure S3: (a) Phenotypic analysis of cell lines. (b) Graphs showing the release of soluble counts (% of Total) over time for different treatments. (c) Additional graphs for further analysis.

**Figure Details:**
- Graph (a) shows the release of soluble counts (% of Total) over time for different treatments.
- Graph (b) displays the release of soluble counts (% of Total) for various conditions.
- Graph (c) provides additional data points for comparison.

**Experimental Details:**
- The phenotypic analysis was conducted on cell lines treated with different substances.
- The release of soluble counts was measured over time for each condition.
- Additional graphs provide further insights into the experimental results.

**References:**
- The supplementary information is representative of three or more independent experiments.

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SUPPLEMENTARY INFORMATION

Figure S6: Multiple protein bands linked to cyclin B3 are rapidly degraded by purified p27Kip1. **A** Autoradiograph of an in vitro APOC activity assay. In the presence of p27Kip1, U2OS cells (106 cells/ml) APOC was prepared from Xenopus extract. The amounts of cyclin B3 activity in the supernatant were determined as total amount of cyclin B3 activity in the supernatant in the sample. In the sample degradation assay with cyclin B3-4 species from a and USP14 deficient human Prokinesis (10 nM). At indicated times, samples were subjected to p27Kip1 and APOC precipitation and proteases were treated with 0.5 M TCA in a cold bath. Samples were plotted as percent of total and total percent of cyclin B3-1 species from a and USP14 deficient human Prokinesis (10 nM).
Figure S3: USP14-mediated inhibition of proteolysis is dependent on the catalytic activity of USP14 as it is reversed by USP14 inhibitor 1011. A) Autoradiograph of in vitro PDCD4-UBD deubiquitination of p38 MAPK, same as in Fig. 5c. b) GST-USP14 (1400 M) or 200 nM mKRevase overexpressed in HeLa cells was pre-incubated with 10 nM (U111023) or DMSO control prior to allowing association with p38 MAPK. p38-USP14 (Ub) species from an in vitro incubation of p38 MAPK with proteins were isolated with proten-A agarose. Western blots were treated as described before the addition of trichloroacetic acid (TCA). Proteolysis was measured by release of TCA-soluble counts, and plotted as percent of input radiolabeled cyclin E2 protein. GST-ubiquitinated unmodified and ubiquitinated p38 MAPK from in vivo ubiquitination assays shown as autoradiograph in Fig. 5c were included as controls. 100 nM USP14 (1400 M) alone, or with purified human USP14 (1400 M) or GST-USP14 (1400 M) in the absence or presence of 100 nM USP14 (1400 M) for 20 min at 24 °C. Before reactions were terminated by the addition of TCA, cell lysates were incubated with GST-USP14 (1400 M) alone.
SUPPLEMENTARY INFORMATION

Figure S6: Degradation of pre-ubiquitinated cyclin E1 in Xenopus extract does not require ubiquitination by endogenous APC/C. a, Autoradiograph of X. laevis APC/C (10 ng) ubiquitination of Myc-APC/C (3 pg) performed as in Fig. 6c (b) by E1 (2 pg) co-incubation from Xenopus extract centrifuged and spin-purified as in Fig. 6a. (c) (b) (WT) in vitro co-precipitation with ubiquitinated pre-ubiquitinated cyclin E1 (3 pg). Time course of degradation was performed at 37 °C and reactions were terminated at indicated times by addition of trichloroacetic acid (TCA). Proteins were measured by kinase of total soluble extracts, and plotted as percent of input radiolabeled cyclin E1 protein. As in a, except that cyclin E1 was introduced into mitogen-activated Xenopus extract. b, Autoradiograph of endogenous APC/C (10 pg) ubiquitination of full-length cyclin E1. Reaction mix (E1, 3 pg), APC/C (1 µg), and Myc-APC/C (0.5 µg) were incubated with mitogen-activated X. laevis extract, and cyclin E1 was labeled with [14C]lysine. The reaction was performed at 37 °C for 30 min. Samples were loaded onto 10% SDS-PAGE and visualized by autoradiography. Proteins were measured by co-precipitation of Myc-APC/C (3 pg) and plotted as percent of input radiolabeled cyclin E1 protein. 

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Figure 6. (a) does not accelerate degradation of cyclin B3-GFP conjugates added to Xenopus extract. In (b), both cyclin A and cyclin B3-GFP were preincubated with U19, U13, or U13B and subjected to microinjection into the extract. The microinjected extract that had been preincubated with U13 (40 nM) for 1 hr (200 µl) or U19 control for 10 min at 24°C. Degradation was performed at 24°C with reactions terminated at indicated times by the addition of 10% trichloroacetic acid (TCA). Proteolysis was measured by release of TCA-soluble counts, and depicted as percent of input trichloroacetic acid (TCA) protein. Bottom panel (b) is an enlargement of the graph for 0.2 min of the time course shown in (a).
Figure S8: Deposition of UBC15 more significantly impacts ubiquitin B1 degradation when ubiquitination is limited to a single ubiquitin nucleo.
(a) Samples from control, control, and UBC15-depleted extracts were processed for SDS-PAGE and western analysis. Equal volumes of these extracts were incubated with 10 μg of anti-ubiquitin for 2 hours and then washed three times. Samples were separated by SDS-PAGE and analyzed by western blot against UBC15, UBC10, and the APC/C substrate CDC27. Lanes 4-6 represent 20-fold enrichment of U2 enzymes or 10 μg of anti-ubiquitin for 2.5 hours.
(b) Time-course of deubiquitination of two ubiquitin nucleos of B1 ubiquitin (UBC15) or single ubiquitin-containing mutants (UBC15), batch in control or UBC15-depleted reconstituted reconstituted samples. Reconstituted H4 ubiquitin (0.5 μM) was added to reactions containing mutant ubiquitin and B1 microsomes. Reactions were analyzed by SDS-PAGE and Western blotting.
Figure 1D

Western blot analysis of endogenous proteins from the indicated cell lines. (A) Normalizing SDS-PAGE. (B) Reducing SDS-PAGE.

Figure 1E

Western blot analysis of endogenous proteins from the indicated cell lines. (A) Normalizing SDS-PAGE. (B) Reducing SDS-PAGE.

Figure 3C

Figure 3D

Supplementary Figure 5

Unwashed
UBVS
UBVS + Ubi
UBVS + Ubi + HEPES

Ubb
UBVS + Ubi + HEPES

Supplementary Figure 5: Unwashed samples of immuneblots.
SUPPLEMENTARY INFORMATION

Supplementary Figure 5A

Supplementary Figure 5B

cyclin B1, Control depletion

Supplementary Figure 6B

cyclin B1, UBC10 depletion

Figure S9 continued
Analysis of the role of USP14 in cyclin B1 degradation in *Xenopus* extract
Results

Analysis of the role of USP14 in cyclin B1 degradation in *Xenopus* extracts

Our previous findings indicated that as long as ubiquitin (Ub) availability is high enough, deubiquitination mediated by UbVS-sensitive DUBs is unlikely to present a major kinetic barrier to cyclin B1 degradation in mitotic *Xenopus* extract (Dimova et al., 2012). While the analysis we carried out could not ascertain a role for DUBs, which are not inhibited by UbVS, our findings suggested that there are few, if any, UbVS-senstitive deubiquitinases that actively antagonize proteasomal targeting of cyclin B1 in mitotic extracts. Alternatively, our findings suggested that for such activity to oppose degradation, it may be crucial that the rate of ubiquitination be constrained by limiting activity of the ubiquitination machinery or by limiting availability of free ubiquitin.

Activity of the APC in mitotic *Xenopus* extracts is dependent on the reversible association with the activator Cdc20, which helps recruit substrates to the APC and may also directly stimulate the catalytic activity of the ligase (Kimata et al., 2008). Surprisingly, work from our lab uncovered that in *Xenopus* extracts Cdc20 also interacted with interphase APC, albeit with different dynamics relative to the mitotically phosphorylated ligase (Xing Zeng, unpublished observations). We hypothesized that the catalytic capacity of interphase APC$^{\text{Cdc20}}$, while sufficient to promote ubiquitin-dependent proteolysis, is strongly opposed by the activity of deubiquitinases. To test this idea, we took advantage of the UbVS system (described in chapter II) (Dimova et al., 2012) and a purified $^{35}$S-labeled N-terminal fragment (1-88 amino acid residues) of human cyclin B1 (referred to as cycB1-NT). When added to interphase extract, cycB1-NT, which is incapable of binding endogenous CDK1 and phosphorylating the APC, remained largely stable for the duration of the experiment. Low levels of degradation, observed at later times, were presumably resulting from partial activation of the ubiquitination machinery.
Addition of 110 μM of wild-type ubiquitin, which accelerates substrate degradation in mitotic extracts (Dimova et al., 2012), had no appreciable impact on cyclin stability in interphase extract. Upon pre-treatment with 20 μM UbVS, basal proteolysis was slightly inhibited in extracts, likely due to ubiquitin deficiency. Surprisingly, wild-type ubiquitin stimulated cyclin B1 degradation in UbVS-treated extract (Figure S1b). The degree of stimulation depended on the concentration of added ubiquitin (data not shown). Under these conditions, no phosphorylation of Cdc27 was observed by western blotting (Figure S1a), suggesting that the observed degradation was unlikely due to mitotic entry. These findings indicate that UbVS-sensitive deubiquitination may oppose proteasomal targeting of cyclin B1 in interphase extract.

Previous work, carried out in collaboration with the Finley lab, revealed that in a reconstituted system, the proteasome-associated DUB USP14 strongly attenuated the capacity of proteasomes to rapidly degrade polyubiquitinated cyclin B1 (Hanna et al., 2006; Lee et al., 2010b), presumably by trimming ubiquitin chains. Unexpectedly, USP14 was comparably efficient at removing substrate-linked ubiquitin, suppressing the turnover of multiply monoubiquitinated cyclin B1 (Dimova et al., 2012). These findings motivated us to examine a potential role of USP14 in cyclin proteolysis in Xenopus extract. To this end, we first wanted to test whether USP14 was required for cyclin turnover. USP14 levels in total extracts were hard to detect by western analysis (Figure S2a). To confirm its presence in this physiological context, we sought to enrich for USP14 by isolating endogenous proteasomes from Xenopus extract using the UBl-domain of ubiquitin receptor Rad23 (Figure S2a). Mass spectrometry (Mike Aguiar, Gygi lab) and western blot analyses of isolated proteasomes indicated that levels of proteasome-bound USP14 in interphase extracts are comparable to those in mitotic extracts. Importantly, species in both extracts were similarly UbVS-reactive (Borodovsky et al., 2001), suggesting proper proteasomal association and activation of USP14 (Figure S2a).

We next examined how inhibiting the activity of USP14 will impact cyclin B1 degradation in Xenopus extract. Pre-treatment of mitotically arrested Xenopus extract with IU1
Figure S1. Ubiquitin vinyl sulfone (UbVS) stimulates cyclin proteolysis in interphase Xenopus extract in a ubiquitin-dependent manner. (a) Aliquots were removed from interphase Xenopus extract before or after treatment with UbVS (20 μM) or buffer for 30 min, as well as during the time course of cycB1-NT degradation. Phosphorylation of APC subunit Cdc27 was examined by immunoblotting. Last lane represent phosphorylation of Cdc27 in mitotically arrested Xenopus extract. (b) 35 S-labeled cycB1-NT and 110 μM of wild-type ubiquitin, where indicated, were introduced concomitantly into interphase Xenopus extract that had been pre-treated with UbVS (20 μM) or buffer (untreated) for 30 min. Proteolysis was measured by release of TCA soluble counts and is plotted as percentage of input radio-labeled cycB1-NT.
Figure S2 USP14-specific inhibitors, IU1 and IU2, do not impact cyclin B1 proteolysis in Xenopus extract. (a) Proteasomes, affinity purified from interphase or mitotically arrested Xenopus extract, were incubated with ubiquitin vinyl sulfone, (UVS) where indicated. Levels of USP14, unmodified or as covalent-adduct form with UBSV, were examined by western blot against USP14. In parallel, USP14 levels in total extract were analyzed. (b) Mitotically arrested Xenopus extract was pre-treated with USP14-specific inhibitors, IU1 or IU2 (200 µM), for 20 min at 24 ºC before cycB1-NT (~ 200 nM) and wild-type ubiquitin (44 µM) were added concomitantly, as indicated. Proteolysis was measured by release of TCA soluble counts and is plotted as percentage of input radiolabeled cycB1-NT. (c) Mitotically arrested Xenopus extract was treated with APC inhibitor TAME (200 µM), prior to addition of USP14 inhibitors, IU1 or IU2 (200 µM) for 20 min at 24 ºC. CycB1 NT (200 nM) was added to extract. Proteolysis was measured by release of TCA soluble counts and is plotted as percentage of input radiolabeled cycB1-NT. (d) Mitotically arrested Xenopus extract was treated with proteasome inhibitor MG132 or DMSO, as indicated, prior to addition of USP14 inhibitor IU1 (100 µM). CycB1-NT (200 nM) and wild-type ubiquitin (44 µM), where indicated, were added concomitantly. Proteolysis was measured by release of TCA soluble counts and is plotted as percentage of input radiolabeled cycB1-NT.
Figure S2 (Continued)
or IU2, two different small-molecule inhibitors of the catalytic activity of USP14, had a slightly inhibitory effect on cycB1-NT degradation (Figure S2b). To compensate for the ubiquitin deficiency potentially imposed by USP14 inhibition, we supplemented extracts with wild-type ubiquitin. In the presence of 44 μM of added ubiquitin, the rate of cyclin degradation was no faster in IU1- or IU2-treated extract relative to a non-treated extract. Similar trends were observed in interphase *Xenopus* extracts (data not shown). The failure of the USP14 inhibitors to stimulate cyclin turnover could result from inefficiency of USP14 in opposing the processive ubiquitination and proteasomal targeting, mediated by the APC. We therefore sought to examine whether partial inhibition of APC activity may reveal an effect of USP14 on cyclin degradation. To this end, we used TAME which was suggested to stabilize APC substrates by terminating ubiquitination prior to assembly of an efficient proteolytic signal, favoring deubiquitination rather than degradation (Zeng and King, 2012). We hypothesized that if USP14 acts on such under-ubiquitinated species, then attenuating its isopeptidase activity with IU1 or IU2 may rescue substrate degradation in TAME-treated extracts. As expected, the proteolysis of cycB1-NT was highly sensitive to TAME addition (Zeng and King, 2012; Zeng et al., 2010) (Figure S2c). Concomitant treatment of extract with USP14 inhibitors, IU1 or IU2, failed to rescue the TAME effect on substrate stability. These findings suggest that USP14 may not actively oppose APC-mediated proteolysis by deubiquitinating modified intermediates.

We next sought to determine whether a partial inhibition of the 26S proteasome may better reveal an effect of USP14 on cyclin degradation. When the catalytic activity of the proteasome is attenuated, ubiquitin conjugates may remain associated with the 19S sub-complex longer, allowing potentially for more extensive deubiquitination by USP14. At final concentrations higher than 100 μM, the proteasome inhibitor MG262 largely blocked cyclin turnover (data not shown). In contrast, pre-treatment of extract with 25 μM MG262 delayed degradation, extending substrate half-life from 30 minutes in untreated extract to 55 minutes (Figure S2d). Following treatment with the proteasome inhibitor, extract was incubated with IU1
for 15 minutes prior to substrate addition. The kinetics of cyclin degradation observed in MG262-treated extracts were largely unaffected by the addition of the USP14 inhibitor IU1. A possible explanation for the lack of effect of IU1 is that the catalytic activity of USP14 does not modulate the rate of cyclin degradation in a major way. Alternatively, our data indicate that in spite of the sequence conservation between the human and frog USP14, IU1 fails to efficiently inhibit the activity of the enzyme in *Xenopus* extract.

We next attempted to rule out the possibility that our results thus far were influenced by failure of the small-molecule inhibitors to bind *Xenopus* USP14. To this end, we examined the ability of IU1 to interact with the enzyme’s catalytic site, preventing labeling with UbVS. When isolated *Xenopus* proteasomes were briefly incubated with UbVS, a significant fraction of the present USP14 was found as a covalent adduct with the ubiquitin derivative (Figure S3a). At 20 μM final concentration, IU1 was not capable of inhibiting the formation of USP14-UbVS adducts, as compared to untreated or negative-control IU1C-treated proteasomes (Figure S3a). When the concentration of IU1 was increased 5-fold to 100 μM, labeling with UbVS was partially diminished, as suggested by reduced levels of the slower migrating form of USP14. IU1-47 exhibited higher affinity for the *Xenopus* USP14 than the parental compound. Even at 20 μM final concentration, IU1-47 significantly reduced extent of UbVS labeling, whereas at 100 μM, it completely abolished this interaction (Figure S3a). Surprisingly, this more potent inhibitor IU1-47 had no appreciable effect on the kinetics of cyclin degradation in mitotic *Xenopus* extracts, supplemented with 44 μM of wild-type ubiquitin (Figure S3b). Similar results were obtained in interphase extracts (data not shown).

Degradation of cyclin conjugates generated with wild-type or chain-terminating ubiquitin was largely unaffected by pre-treatment of interphase *Xenopus* extract with the USP14-specific inhibitor IU1-47 (Figure S3c). This is very similar to what we observed in analogous experiments performed with IU1 (Dimova et al., 2012). The efficiency of cyclin degradation in *Xenopus* extracts suggested, surprisingly, that USP14 activity is unlike to strongly impact the proteasomal
**Figure S3** USP14-specific inhibitor IUI-47 has no effect on cyclin B1 proteolysis in *Xenopus* extract. (a) *Xenopus* proteasomes, affinity purified from mitotically arrested *Xenopus* extract were incubated with USP14-specific inhibitor IUI or IUI-47, or a specificity control IUC-2, at the indicated concentrations, for 15 min at 24 °C prior to chase with 2 μM ubiquitin vinyl sulfone (UVVS). Reaction species were processed for SDS-PAGE and anti-USP14 western analysis. (b) Degradation of 35S-labeled cyclin B1-NT in mitotically arrested *Xenopus* extract pre-treated with an USP14-inhibitor IUI-47, concentrations as indicated. Cyclin B1-NT (~200 nM) was added to extract. Proteolysis was measured by release of TCA soluble counts and is plotted as percentage of input radiolabeled cyclin B1-NT. (c) 35S-labeled cyclin B1-NT was preubiquitinated with UbWT, UbK48 or UbK63 and UBCH10. Resulting cyclin B1-NT ubiquitin species were introduced into interphase *Xenopus* extract that had been pre-treated with USP14-inhibitor IUI-47 (200 μM) or DMSO control for 15 min at 24 °C. Degradation was performed at 24 °C with reactions terminated at indicated times by the addition of trichloroacetic acid (TCA). Proteolysis was measured by release of TCA soluble counts, and is plotted as percentage of input radiolabeled cyclin B1 protein. Bottom panel is an enlargement of the graph for 0-5 min of the time-course shown in top panel.
Figure S3 (Continued)
targeting of cyclin B1. A possible explanation for these observations is that deubiquitinating enzymes such as USP14 are present at much lower levels in *Xenopus* extracts than in our *in vitro* reconstituted reactions (Dimova et al., 2012).

The Ub-AMC hydrolyzing activity of USP14 was reported to increase 800-fold upon association with the proteasome over that of isolated protein (Lee et al., 2010a). Thus, the deubiquitinating activity of USP14 is strongly enhanced by proteasomes (Borodovsky et al., 2001; Lee et al., 2010a; Leggett et al., 2002). Our findings raised the possibility that levels of USP14 associated with proteasomes in extracts may be insufficient to impede cyclin turnover. To test this idea, we sought to increase loading of proteasomes with USP14 by adding recombinant protein to extracts. Based on previous reports (Borodovsky et al., 2001; Lee et al., 2010a; Leggett et al., 2002), we reasoned that exogenous USP14 would be specifically activated in the context of the proteasome and perhaps render kinetics of substrate turnover comparable to those in a purified system reconstituted with the deubiquitinase (Crosas et al., 2006; Dimova et al., 2012; Hanna et al., 2006; Lee et al., 2010a). When added to mitotically arrested extract, GST-tagged human USP14, but not GST alone, delayed cyclin proteolysis in a dose-dependent fashion (Figure S4a; data not shown). Surprisingly, we found that co-treatment of these extracts with 200 μM IU1-47 failed to accelerate degradation (Figure S4b).

Previous work demonstrated that USP14 delays the breakdown of conjugates by the proteasome and that a major component of its inhibitory effect is non-catalytic in nature (Hanna et al., 2006). These findings prompted us to examine the possibility that in *Xenopus* extracts USP14 utilizes mostly non-catalytic functions to modulate the activity of the proteasome. We found that an active-site mutant of USP14, USP14 (C114A), inhibited cyclin degradation, albeit to a lesser degree than the wild-type protein (Figure S4b). The kinetics of degradation in extracts supplemented with USP14 (C114A) were not altered by the addition of IU1-47, consistent with resistance of USP14 non-catalytic functions to IU1 treatment (Lee et al., 2010a). Thus, our analysis may indicate a contribution of USP14, independent of its catalytic function, even though
Figure 54 Exogenous USP14 delays proteolysis of cyclin B1 in Xenopus extract. (a) Mitotically arrested Xenopus extract was incubated with recombinant GST-tagged USP14 at concentrations, as indicated for 15 min prior to addition of [35S]-labeled cycB1-NT. Time course of degradation was carried at 24 °C and reactions terminated at indicated times by the addition of trichloroacetic (TCA) acid. Proteolysis was measured by release of TCA soluble counts and is plotted as percentage of input radiolabeled cycB1-NT. (b) Recombinant GST-tagged wild-type (WT) or catalytically inactive variant (CA) (C114A) of USP14 (6 μM) were added to mitotically arrested Xenopus extract for 15 min, prior to treatment with USP14-specific inhibitor IU1-47 (200 μM) or DMSO for 15 min. Time course of degradation was initiated with substrate addition (200 nM). Proteolysis of radiolabeled cycB1-NT was measured as in (a).
further work will be required to address this possibility. Together our findings suggest that the abundance of proteasomes containing USP14 in *Xenopus* extract may be below levels necessary to influence cyclin proteolysis. Alternatively, the ubiquitin signal assembled on cyclin B1 may be primarily modulated by activities upstream of the proteasome. Even following a recent identification of USP37 as a DUB antagonizing ubiquitination of cyclin A2 by APC<sup>Cdh1</sup> (Huang et al., 2011), it remains to be determined if there is a ubiquitin isopeptidase specific for cyclin B1. We set out, therefore, to search for such activity that may influence the rate of cyclin B1 proteolysis and perhaps that of other APC substrates.

**Deubiquitinating activity antagonizing APC-dependent proteolysis in *Xenopus* extracts**

We sought to broaden our approach and look for APC-associating DUBs which could potentially influence APC-mediated proteolysis. Based on our findings in interphase extract, we initially examined potential association of the ligase with UbVS-sensitive isopeptidases. We immunopurified interphase or mitotic APC from *Xenopus* egg extract and treated the isolated material with hemagglutinin (HA)-tagged UbVS to label any co-purifying deubiquitinases. Following incubation with HA-UbVS, covalently labeled species were analyzed by western blotting against the HA epitope tag. When material isolated on anti-Cdc27 resin was subjected to less stringent washes, multiple HA immunoreactive species were evident (Figure S5a). We reasoned that any deubiquitinase that may specifically modulate the ubiquitin signal built on substrates by the APC is likely to be recruited to the ligase module in the presence of substrate. Thus, extract was supplemented with 10 μM N-terminal fragment of sea urchin cyclin B1 (13-110 amino acid residues) during immunoprecipitation. Isolated APC was washed with excess low-salt buffer and incubated with 10 μM HA-UbVS for 30 minutes. We found that the patterns of HA-reactive species for the Cdc27- and control IgG-immunopurification were largely identical.
Interestingly, one band in the high M₉ region of the gel appeared specific for the Cdc27 immunopurification (Figure S5b). The intensity of the band remained largely unaffected by the addition of excess substrate (Figure S5b; data not shown).

To examine the pattern of isopeptidase activities co-purifying with interphase Xenopus APC in an independent manner, we carried out labeling with a different active-site probe. To this end, we utilized ubiquitin-vinyl methylester (Ub-VME) where vinyl methylester is used as the thiol-reactive functional group at the carboxy terminus. As the vinyl sulfone substitution may hinder interaction or not be reactive with the active sites of some deubiquitinases, such proteins will be refractory to labeling. Thus, a ubiquitin probe in which the vinyl sulfone moiety is replaced with a different group such as vinyl methylester can potentially allow for additional DUBs to be visualized. Consistent with this idea, we found the overall pattern of labeling with Ub-VME to be different from that in assays containing UbVS (data not shown). Intriguingly, far fewer species form covalent adducts with Ub-VME, suggesting differential affinity for the ubiquitin probe (data not shown). Interestingly, we found a Ub-VME-reactive species that migrated similarly to the APC-IP specific band seen in UbVS experiments. Using this approach, we were unable to detect any DUB/DUBs associating with mitotic APC (data not shown). We found no evidence of differential labeling with UbVS even when extracts were supplemented with cyclin B1 and wild-type ubiquitin (data not shown).

We next tested whether the UbVS labeling we observed was indeed reflecting specific association of particular deubiquitinases with the APC. To this end, we immunopurified APC from interphase extract that had been pre-depleted with either a control IgG or an antibody against the APC subunit Cdc27 and incubated the resulting material with HA-UbVS. Surprisingly, western analysis revealed that the appearance of labeled species remained unchanged in extracts pre-depleted of the APC, as compared to control-depleted extracts (Figure S5b). These results suggest that the UbVS-reactive species may not be specifically binding to the
Figure S5. UbVS-labeling of deubiquitinases co-purifying with Xenopus APC. (a) Xenopus APC was immunopurified from interphase or mitotic extract, as indicated, for 1 h before material bound to resin was washed and incubated with 15 μM HA-tagged UbVS at 24°C for 20 min. Entire reactions were mixed with sample buffer and samples were analyzed by SDS-PAGE/anti-HA western blotting. Cdc27, an APC subunit. (b) Xenopus APC was immunopurified from interphase extract that had been supplemented with 10 μM of sea urchin N-terminal fragment of cyclin B1 (cycB1-NT) and pre-depleted with Cdc27 or control IgG. Following incubation for 1 h at 4°C, immunopurified material was subjected to quick washes with low-salt buffer and incubated with 10 μM of HA-UbVS at 24°C for 30 min. Entire reactions were mixed with sample buffer and reactions were analyzed by SDS-PAGE/anti-HA western blotting. Red asterisk denotes band specific for immunopurification with CDC27 IgG.
ubiquitin ligase. Together our findings may indicate that there are few, if any, UbVS-reactive DUBs that form a stable association with the APC in *Xenopus* extract.
Discussion

Here we have evaluated a potential role of USP14 in proteasomal targeting of cyclin B1 in *Xenopus* cell-cycle extracts. Our study was motivated by previous work (Dimova et al., 2012; Hanna et al., 2006; Lee et al., 2010a) indicating that the proteasome-associated deubiquitinase USP14 or its yeast orthologue Ubp6 strongly reduces the efficiency of cyclin B1 degradation by purified proteasomes. Although, USP14 has been found to modulate the stability of a number of cellular proteins *in vivo* (Hanna et al., 2006; Lee et al., 2010a), to date, its activity has not been linked to APC-mediated proteolysis. In the context of *Xenopus* egg extracts, we conclude that USP14 is unlikely to regulate the magnitude of proteasome activity against ubiquitinated cyclin B1.

Our previous work showed that in a reconstituted system USP14 can antagonize the degradation of cyclin B1-Ubₙ species (Dimova et al., 2012; Lee et al., 2010a). At the basis of this inhibition was the capacity of USP14 to trim polyubiquitin assemblies (Dimova et al., 2012; Lee et al., 2010b), as well as remove substrate-attached monoubiquitins (Dimova et al., 2012). Cyclin turnover *in vitro* was markedly stimulated by USP14-specific inhibitor IU1, whereas degradation of pre-ubiquitinated cyclin B1 in *Xenopus* extract was largely insensitive to IU1 treatment (Dimova et al., 2012). We sought to resolve this paradox by examining a potential role of USP14 in the context of *Xenopus* proteasomes. Although USP14 is present in the *Xenopus* system and associates with the proteasome in both interphase and mitotic extract, our analysis suggested that it may be of low abundance. This raises an interesting possibility that the limited ubiquitin availability in extracts may be accounted, at least in part, by lower levels of proteasome-bound USP14. Consistent with this idea, treatment with the USP14-specific inhibitor IU1 caused a slight delay in cyclin proteolysis, an effect rescued by exogenous ubiquitin.

The magnitude of proteasome inhibition by USP14 that has been observed in a reconstituted system (Dimova et al., 2012; Hanna et al., 2006; Lee et al., 2010a) and in some
physiological contexts (Hanna et al., 2006; Lee et al., 2010a) does not appear to parallel that in *Xenopus* egg extracts. Perhaps this reflects that the deubiquitinating enzyme USP14 is present at much lower levels on *Xenopus* proteasomes than in our *in vitro* reconstituted reactions. Consistent with this idea, upon increasing the levels of USP14 by adding recombinant protein, we observed a significant delay in cyclin turnover. Interestingly, in *Xenopus* extracts a major component of this inhibitory effect may be noncatalytic in nature. Similar observations have been reported for the yeast orthologue Ubp6 (Hanna et al., 2006). This may provide a possible explanation for the lack of effect of USP14-specific inhibitors, IU1 and IU2, on the kinetics of degradation of cyclin-ubiquitin conjugates introduced in extract. It is unlikely that these findings reflect some artifact of *in vitro* ubiquitination, rendering conjugates insensitive to deubiquitinating activity in extract.

When allowed to be ubiquitinated by the endogenous machinery, cyclin was efficiently degraded in IU1- or IU2-treated extracts, with rates largely identical to those in untreated extract. These findings are in agreement with our previous work indicating that few, if any, UbVS-sensitive DUBs such as USP14 may be strongly modulating cyclin degradation in mitotic *Xenopus* extracts.

Importantly, exogenous ubiquitin was found to stimulate cyclin proteolysis in interphase extracts in a manner dependent on DUB inhibition with UbVS. Under these conditions, there is no detectable phosphorylation of Cdc27 that would indicate mitotic entry of extracts. Intriguingly, both interphase and mitotic APC appear to associate with proteasomes in *Xenopus* extract. Future work will be required to elucidate the functional significance of such interactions. Such an association, however, raises an important question as to how ubiquitination and degradation may be coupled, and how this may influence sensitivity to deubiquitination. Our analysis thus far has not yielded any UbVS-sensitive deubiquitinases that form stable interaction with the APC. These findings do not rule out the possibility that there are isopeptidases antagonizing APC activity or modulating the ubiquitin signal it assembles on target proteins. Such deubiquitinating enzymes would not necessarily associate with the E3 ligase to exert their effect.
Methods

Antibodies and biochemical reagents

Protein samples were resolved by SDS-PAGE on NuPAGE 4-12% Bis-Tris or 12% Bis-Tris gels (Invitrogen), followed by wet transfer to PVDF and immunoblotting against the indicated proteins. Sources of commercial antibodies used for western analysis are as follows: anti-cyclin B1 (Ab-2; RB-008-P, Neomarkers), anti-Cdc27 (610455, BD Transduction Laboratories™), Secondary antibodies used include anti-goat IgG-HRP (sc2020; Santa Cruz Biotechnology), anti-rabbit IgG-HRP (NA934; GE Healthcare), and anti-mouse IgG-HRP (NA931; GE Healthcare). For APC/C immunopurification from Xenopus extract, anti-Cdc27 (AF3.1; sc-9972, Santa Cruz Biotechnology) was used. Other reagents utilized include ubiquitin vinyl sulfone (UbVS) (U-202, Boston Biochem), TAME (T4626, Sigma), Mg262 (I-120, Boston Biochem), methylated ubiquitin (U-502, Boston Biochem), ubiquitin mutants except for Ub11,48R, Ub11,63R, and UbtriR were purchased from Boston Biochem. Wild-type ubiquitin was purchased from Sigma-Aldrich (U6253-25MG) and Boston Biochem.

Preparation of recombinant proteins

CycB1-NT (1-88 amino acids of human cyclin B1) , containing an HA tag at the N terminus and a 6xHis tag at the C terminus was generated using PCR amplification with forward primer (5’-CCA GGA CCA TGG GTT ACC CAT ACG ATG TTC CAG ATT ACG CTG GCT CGA TGG CGC TCC GAG TCA CG-3’) and reverse primer (5’-GGG AGC CTC GAG CTA GGG AGC GTG ATG GTG ATG CTG CAT AGG TAC CTT TTC AAG AGG-3’). The resulting PCR product was digested with NeoI and XhoI for subcloning into pET28a. Plasmids were verified by restriction enzyme mapping and sequencing. For 35S labeling in Escherichia coli, cultures (50 ml) were grown at 37 °C to $D_{600\text{ nm}} = 0.8$, then collected by centrifugation (3,700g for 15 min, at 4 °C) and resuspended in modified M9 medium (50 ml final volume). After
resuspension in modified M9 medium, cells were allowed to grow for additional 15 min at 37 °C before 5 mCi of Easy Tag™ L-[35S]-Methionine (NEG709A005MC; Perkin Elmer) was added. Expression was induced with 0.5 mM IPTG for 2.5 h at 37 °C. Cells were ruptured in 5 ml g⁻¹ of pellet guanidine-HCl lysis buffer (pH 8.0) and lysates rotated at 24 °C until the lysate became slightly translucent; approximately 45 min. Lysates were clarified by centrifugation and cycB1-NT was purified using Ni-NTA affinity chromatography (Qiagen). Eluted protein was desalted into XB buffer (100 mM KCl, 0.1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, at pH 7.8 with KOH), supplemented 2% glycerol, protease inhibitors and phenylmethysulfonfyl fluoride, and stored at –20 °C.

Maltose-binding protein (MBP)-tagged E1 was expressed in E. coli inducing cultures at $D_{600\text{nm}} = 0.6$ with 300 μM IPTG for 5 h at room temperature. Purification was carried out using a standard MBP purification protocol. For expression of His-tagged UBCH10 and His-tagged UBC4, bacterial cultures were induced at $D_{600\text{nm}} = 0.6$ at 37 °C with 500 μM IPTG for 4 h. The enzymes were purified through Ni-NTA affinity and gel-filtration chromatography. Glutathione S-transferase (GST)-tagged USP14, wild-type or catalytically inactive variant C114A, were expressed and purified as reported previously (Lee et al., 2010a).

**Preparation of Xenopus egg extract**

Interphase Xenopus egg extract was prepared from eggs laid overnight according to the protocol of Murray (Murray, 1991) with the exception that eggs were activated with 2 μg ml⁻¹ calcium ionophore (A23187, free acid form, Calbiochem) for 30 min prior to the crushing spin. Extract was frozen in liquid nitrogen and stored at -80 °C. Interphase extract was induced to enter mitosis by addition of non-degradable cyclin B, which activates CDK1 and stimulates mitotic phosphorylation, resulting in APC/C activation. A fusion of the maltose-binding protein (MBP) to Xenopus cyclin B lacking its N-terminal 90 amino acids (MBP-Δ90) (Salic and King, 2005) was expressed in E. coli by inducing cultures at an $D_{600\text{nm}}=0.6$ with 300 μM...
isopropylthiogalactoside (IPTG) for 5 h at room temperature. Purification was carried out following New England BioLabs (NEB) protocol. To make mitotic extract, MBP-Δ90 was added to interphase extract generally at ~20 μg ml⁻¹ and incubated at 22-24 °C for 45-60 min.

**APC/C purification and reconstituted ubiquitination of cyclin B1**

Reactions were performed essentially as described previously (Kirkpatrick et al., 2006) for the indicated times. Briefly, for each 30 µl reaction, APC/C was immunopurified from 600 µl of mitotic *Xenopus* egg extract by incubation for 1 h at 4 °C with 12 µg of anti-Cdc27 antibodies (AF3.1, Santa Cruz Biotechnology) immobilized onto 30 µl of Affiprep Protein A beads (156-0006, Bio-Rad). Following incubation with extract, beads were washed very quickly to minimize loss of associated APC/C co-activator Cdc20 three times with XB high salt (10 mM potassium HEPES, pH 7.7, 500 mM KCl, 0.1 mM CaCl₂, 1 mM MgCl₂), two times with XB (the same content as XB high salt, except with 100 mM KCl), and then three times with the reaction buffer (20 mM Tris, pH 7.5, 100 mM KCl, 2.5 mM MgCl₂, 2 mM ATP). Ubiquitination reactions were typically performed at 24 °C with agitation on a shaker at 1500 r.p.m.. Ubiquitination reactions contained immunoprecipitated APC/C on 30 µl beads, and 30 µl of a mix containing recombinant MBP-human E1 (1.3 µM), His-tagged UBCH10 (100 nM – 4 μM, concentrations as indicated) as the E2 enzyme, wild-type or different forms of ubiquitin (118-145 μM, concentrations as indicated), and 450-500 nM of HA-cyclin B1 NT(1-88)-His.

**Cyclin B1 degradation in Xenopus egg extract**

Degradation assays where nonubiquitinated cyclin B1 was added to extract were generally performed in 40 µl total volume per reaction condition with extract constituting 75-80% of that volume. For experiments with TAME and Mg262, extracts were pre-treated with relevant compound or DMSO control for 15 min at 24 °C, 1250 r.p.m.. Extracts contained 100 μg ml⁻¹ cycloheximide to prevent re-incorporation of free labeled amino acid. Degradation experiments
were performed at 24 °C, 1250 r.p.m., with samples taken at indicated times. In degradation assays with \(^{35}\)S-labeled cyclin B1 NT, reactions (3 μl per time-point) were terminated by addition of 97 μl of 20% TCA (in H\(_2\)O), vortexed and incubated on ice ≥ 30 min before centrifugation at 14,000g, 4 °C. A fraction (50%) of sample supernatants was combined with NaOH to neutralize the acid and added to Ultima Gold scintillation fluid (6013327, Perkin Elmer). A Packard scintillation counter was used to take all measurements. Acid-soluble counts were compared to total radioactive counts and results were graphed as percent soluble radioactive counts.

For degradation of preubiquitinated cycB1-NT in extract, interphase extract was pre-treated with USP14 inhibitor, as indicated, or DMSO for 15 min at 24 °C, 1250 r.p.m. and supplemented with 100 μg ml\(^{-1}\) cycloheximide prior to substrate addition. For degradation of \(^{35}\)S cyclin B1 NT-Ub conjugates, extract (14 μl) was added to 4 μl of cyclin B1 NT-Ub\(_n\) conjugates for each time-point. Degradation mixtures were incubated at 24 °C, 1250 r.p.m. for indicated times. Reactions were quenched by addition of 107 μl of 20% TCA (in H\(_2\)O), vortexed and incubated on ice ≥ 30 min before centrifugation at 14,000g, at 4 °C for 30 min. A fraction of supernatants was combined with NaOH and added to Ultima Gold scintillation fluid (6013327, Perkin Elmer).
References


complex induces a spindle checkpoint-dependent mitotic arrest in the absence of spindle damage. Cancer Cell 18, 382-395.
Enhancement of proteasome activity by a small-molecule inhibitor of USP14

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Proteasomes, the primary mediators of ubiquitin–protein conjugate degradation, are regulated through complex and poorly understood mechanisms. Here we show that USP14, a proteasome-associated deubiquitinating enzyme, can inhibit the degradation of ubiquitin–protein conjugates both in vitro and in cells. A catalytically inactive variant of USP14 has reduced inhibitory activity, indicating that inhibition is mediated by trapping of the ubiquitin chain on the substrate. A high-throughput screen identified a selective small-molecule inhibitor of the deubiquitinating activity of human USP14. Treatment of cultured cells with this compound enhanced degradation of several proteasome substrates that have been implicated in neurodegenerative disease. USP14 inhibition accelerated the degradation of oxidized proteins and enhanced resistance to oxidative stress. Enhancement of proteasome activity through inhibition of USP14 may offer a strategy to reduce the levels of aberrant proteins in cells under proteotoxic stress.

The proteasome is essential for life in eukaryotes and regulates many aspects of cell physiology. Proteasome substrates are targeted for proteasome clearance by ubiquitination, a conserved signal for proteasome-dependent degradation. The proteasome holoenzyme is composed of a 19-subunit regulatory particle (known as the 19S, 19S complex, or 19S) and a 20S subunit core particle (known as the 20S or 20S complex). The 19S subcomplex binds to the 20S, where it is degraded. The mechanisms regulating proteasome activity poorly understood, but involve numerous proteins that reversibly associate with some but not all 19S and deliver ubiquitinated conjugates to the proteasome, whereas others open the axial channel into the 20S. A third class of associated proteins, composed of ubiquitin ligases and deubiquitinating enzymes (DUBs), modulates proteasome-dependent ubiquitin chains. Ubiquitin chains vary in their linkage type and length, and longer variants interact more strongly with the proteasome. The extension and cleavage of ubiquitin chains at the proteasome vary under substrate degradation rates by changing substrate affinity for the proteasome.

Marinomonas proteus are associated with three USP14, UCHE and USP14 (cub 6–25). USP14 and USP14 associate reversibly with the proteasome, whereas USP14 is a static, ubiquitin-derived substrate. These enzymes reside in the 20S and remove ubiquitin from the substrate before substrate degradation. The release of ubiquitin squares it from degradation, minimizing fluctuations in ubiquitin pools. The activity of USP14 for free ubiquitin is thought to be catalyzed, and USP14 is committed to degrading the substrate. USP14 can also catalyze the deubiquitination of ubiquitin chains on the proteasome, but not all peptides can catalyze deubiquitination on the proteasome. USP14 can also catalyze deubiquitination of ubiquitin chains on the proteasome, but not all peptides can catalyze deubiquitination on the proteasome.

In contrast to USP14, USP14 and USP14 can catalyze ubiquitination independently of ubiquitin, catalytically activating USP14, and perhaps USP14, deubiquitinate the chains from their substrate-DUB activity, thus shortening chains rather than removing them on base. Little is known about such chain-trimming reactions. One model is that chain trimming increases the activity of proteasomes to discriminate between long and short multibiquitin chains. Here we show that a small-molecule inhibitor of deubiquitinating USP14 accelerates protein degradation in vitro and in cells. These findings reveal that, for those substrates tested, proteasome function is limited by USP14-dependent chain-trimming. Thus, otherwise competent substrates of the proteasome can be rejected when chain trimming is faster than competing steps leading to substrate degradation.

**USP14 inhibits the proteasome in vitro**

We have previously shown that USP14, the most abundant form of USP14, is a potent inhibitor of the proteasome. To test whether this is also true of USP13 from human, we first developed a purification procedure that results in proteasome activity detectable USP14 (modified from ref 23). Such proteasomes retain high levels of ubiquitin-7-amidase-alkaline phosphatase (U7A-AP) luciferase activity (data not shown), which is previously UCHE-dependent (Supplementary Fig. 1). This activity can be inhibited reversibly using ubiquitin-7-amidase-alkaline phosphatase (U7A-AP) luciferase activity. USP14, which forms an adduct with the active site of USP14 (by 20S of the 19S-20S complex, class A), with the USP14 was reconstituted with recombinant USP14 (Supplementary Fig. 1). US-AMC hydrolyzing activity was increased 80% over that of isolated USP14 (Fig. 1). Thus, the deubiquitinating activity of USP14 is inhibited by proteasome (see also ref 16, 11, 15, 14, 13, 13, 12, 11). Using the USP13 activity, the affinity of USP14 for the proteasome was found to be 10.4 nM (Supplementary Fig. 1).

Proteases reconstituted with a saturating amount of USP14 were challenged with a model protease substrate, ubiquitin.
inhibitory in Ag-GFP stabilization in comparison to USP14(ACE1A) (Fig. 18), the non-catalytic effect being dominant. Met-Glu7, a stable protein, was unaffected by USP14. It will be interesting to determine what substrate features underlie the differing sensitivities of these substrates to catalytic and non-catalytic inhibition of degradation.

The effect of USP14 on tau degradation was confirmed in HEK293 cells. As in MEFs, USP14 overexpression stabilized tau (Fig. 19). BrdU, obtained with USP14 controls differed from those obtained using MEFs, as expected, given that HEK293 cells express endogenous USP14, the expression of USP14(ACE1A) in U87MG had no effect on tau, whereas in HEK293 cells for U87MG(ACE1A) constant partial accelerated tau degradation (Fig. 19). These results presumably reflect the replacement of endogenous USP14 from the proteasome. As expected, deletion of the UBL domain attenuated the dominant-negative effect (Fig. 19). In contrast to USP14(MBD), the short N-terminal (SF) of USP14 expressed from a developmentally regulated RNA that lacks a V.2-class proteasomal event (see above) between the UBL and the catalytic domain—did not exhibit a dominant-negative effect (Fig. 19). This result indicated that USP14(SF) might bind proteasomes and counter the action of full-length USP14. Thus, USP14(SF) may be an endogenous inhibitor of USP14 activity. Consistent with this possibility, USP14(MBD) binds proteasomes, but it is not activated extrinsically by proteasome binding, as shown by its inability to interact with U87MG (Fig. 19). USP14(SF) also seems to lack non-catalytic proteosome inhibitory capacity, because its expression in U87MG(MBD) did not mobilize Ag-GFP (Supplementary Fig. 6).

A selective small-molecule inhibitor of USP14

The results above suggested that chain trimming at the proteasome cattergories for degradation of multiple substrates. Therefore, a small-molecule inhibitor of USP14 might enhance proteasome activity. We screened a library of compounds for the ability to ablate USP14 using V5 proteosomes reconstituted with USP14 and assayed with UBA-MC, identifying 115 as true USP14 inhibitors (details in Methods, Supplementary Table 1 and Supplementary Fig. 7). When the hits were counter-screened against a panel of 23, only three of the strong hits showed selectivity for USP14. We proceeded with more detailed studies of the strongest 1h (1h-1-propyl)-1-benzyl-1H-pyrazole-3,5-dicarbonitrile, 5-[(5-fluorophenyl)methyl]-3-pyridinecarboxamide, referred to hereafter as 115 (Fig. 2A). Its structure is suggestive of a kinase site-directed thiol proteasome inhibitor. The IC₅₀ for blocking 115 (IC₅₀ of 35) for USP14 in a 4-5 µM (Fig. 2B and Supplementary Fig. 8) failed to significantly inhibit the activity of USP14 (50 µM, Supplementary Figs 9 and 18) as well as UBA-MC, hydrazine by proteasomes lacking USP14, which is attributable to U377 (Supplementary Fig. 8). We also identified a compound that is closely related to 115 (115) but does not inhibit USP14(115)(Fig. 2C) and USP14(SF) (Fig. 2D) and used this as a specificity control in subsequent assays. In the absence of proteasome, USP14 is inactive in U1 (Supplementary Fig. 8), indicating that USP14 is specific to the catalytic form of USP14. USP14 could potentially inhibit USP14 by preventing its folding on the proteasome, but due to the symmetry of this process, it could equally be due to the presence of USP14, which is responsible for USP14 inhibition was rapidly established with the addition of 115 and rapidly reversed upon its removal (Fig. 2D and Supplementary Fig. 13).

We used 115 and USP14 to test whether 115 could inhibit the trim- ming of ubiquitin chains by the proteasomes. To separate chain trim- ming from submolecular degradation, three assays were done in the presence of proteasome inhibitors. When proteasomes lacking USP14 were tested, 115 had no effect on ubiquitin chain trimming (Fig. 3A), chain trimming was strongly inhibited by USP14, as apparent from the increased electrophoretic mobility of Uc-CB9 species. Addition of USP14 to the assay reversed this effect (Fig. 3A, see also Supplementary Fig. 14).

We next tested whether USP14 could enhance degradation. Proteasomal degradation of Uc-CB9 was indeed markedly stimulated by 115.

Figure 2 | 115 inhibitors have an USP14 specificity and reversibility. A. Chemical structure of 115 and USP14. Analytical data shown in Supplementary Fig. 6 B. Peptide: 10µM diglycyperamidylated USP-AMC hydrolysis by proteasomes formed USP14 (115) or AMC (115) in the presence of 115. AMC was detected by fluorescence. B. Peptide: 10µM diglycyperamidylated USP-AMC hydrolysis by proteasomes formed USP14 (115) or AMC (115) in the presence of 115. AMC was detected by fluorescence.
Figure 3 | a | b | c | d | e | f | g | h | i | j | k | l | m | n | o | p | q | r | s | t | u | v | w | x | y | z

**Figure 3** | This figure shows...
of UPF4's catalytic site and U1 treatment, as well as the observation that UPF4 is required for U1 to affect protein degradation, provide strong evidence for the importance of this enzyme in the UPF4-mediated degradation of ubiquitin-independent substrates. The results of these experiments are consistent with the idea that UPF4-dependent ubiquitin-independent degradation is a pathway that can be exploited by the cell to degrade misfolded or misfolded proteins that are resistant to the action of other ubiquitin-dependent proteasomal pathways. The identification of UPF4 as a novel ubiquitin-independent protease that can degrade misfolded proteins provides new insight into the cellular mechanisms of protein quality control and may have implications for understanding the pathogenesis of diseases associated with protein misfolding and degradation.
METHODS

The use of antibodies and the study of pharmacological agents in mammalian cells was used to block the effects of USPs on cell viability. In our experiments, we observed that USPs inhibited the growth of cell lines, and this inhibition was specific to USP expression. We found that USPs exerted their effects by binding to the promoters of genes involved in cell cycle progression, including those encoding cyclins and CDKs. These interactions were found to be mediated by a conserved motif present in the USP family. We also observed a dose-dependent inhibition of cell proliferation in response to USP expression, suggesting that these interactions are critical for the regulation of cell cycle progression. Furthermore, we observed that USPs exerted their effects by blocking the activity of CDKs, which are key regulators of cell cycle progression.

In conclusion, our results indicate that USPs play a critical role in regulating cell cycle progression and may represent a novel target for the development of cancer therapeutics. Further studies are needed to validate these findings and to determine the mechanisms by which USPs modulate CDK activity. These studies may provide insights into the development of new therapeutic strategies for the treatment of cancer.
is predominantly used to buffer (0.01 M TRIS-HCl, pH 7.4, 1 mM MgCl₂, and 1 mM ATP). Where indicated, purified recombinant USP1 was incubated with propos-
ted (at least 2-fold) in protease solution to ensure the reaction. To test the effect of USP1, 10 nM USP1 was pre-incubated with 100 nM ATP for 4 hr before addition.

Proteins that underwent 20-30% of chymotrypsin digestion were added 39 min in absence of cells to inhibit the activity of proteasomes. For staining purposes, vehicle or 10 nM USP1 was added to homogenate in absence of AMR, with the proteasome activity measured as the presence of AMR. Where indicated, purified recombinant USP1 was incubated with 100 nM AMR for 4 hr before addition.

Supplementary Fig. 1. Intracellularity of C5aR1 was assessed by the MT binding assay. Briefly, cells were grown in 96-well plates (1 × 10⁴) and were washed with 200 µl of PBS. After 10 min at room temperature, cells were fixed with 200 µl of 4% paraformaldehyde for 20 min and washed with 200 µl of PBS. Cell suspensions were then added to 96-well plates (1 × 10⁴) and 200 µl of biotinylated C5aR1 mAb were added for 1 hr at 4°C. After washing, 200 µl of streptavidin-alkaline phosphatase conjugate was added for 30 min at room temperature. The reaction was stopped by 200 µl of 1 N NaOH. The plates were read at 405 nm. A standard curve was established using serial dilutions of C5aR1. The IC₅₀ was determined using GraphPad Prism version 6.0 (GraphPad Software, Inc.). The cell suspensions were preincubated, cells were pre-treated with 100 nM USP1 for 2 hr, and 200 µl of C5aR1 mAb were added for 2 hr. Incubation was continued for 2 hr, and the reaction was stopped as described above.

1. Materials and Methods. 1.1. Protein preparation. HEK293 cells were grown in DMEM supplemented with 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin. The medium was changed every 2-3 days.

1.2. Cell culture. HEK293 cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 100 units/ml of penicillin and streptomycin. The medium was changed every 2-3 days.
Figure S1. A preparation of purified human proteasomes that lack endogenous USP14, but contain UCH37. a, b, Human proteasomes were affinity-purified from an iRPM111-frag1 cell line of HEP293T cells as described in Methods. Indicated amounts of proteasomes were analyzed by SDS-PAGE and immunoblotted with anti-USP14 antibody. Samples were compared to recombinant USP14 protein and lysate from wildtype and LuxN47 MEFs. Lanes 1 and 2 of panel a were loaded with 3.8 pmol of proteasome. c, Western blot analysis of purified human proteasomes (7 μg) pre-treated with Ub-V5 (VS) or untreated (VS) with Sup-LLVY-AMC staining. d, One dimensional SDS-PAGE and Coomassie Brilliant Blue (CBB) staining of human proteasomes (5 μg). This, together with c, suggests that there is essentially no change in proteasome integrity upon Ub-V5 treatment of human proteasomes. e, Immunoblot analysis with anti-UCH37 antibody demonstrates the presence of endogenous UCH37 in the purified proteasomes (1.5 μg). Reactivity with Ub-V5 indicates that the band represents active UCH37. Asterisks (∗) indicate nonspecific signal. Note that, in a separate experiment, the stoichiometry of UCH37 on proteasomes purified from iRPM111 cells was estimated using purified recombinant UCH37 as a standard. This visual assay allowed for a rough estimate of one UCH37 molecule to one proteasome (data not shown).
Figure S3. Reconstitution of USP14-proteasome complexes. a, Coomassie staining of purified recombinant proteins (0.5 μg per lane). b, Gel-shift assay of USP14-proteasome binding. Purified human proteasomes (5 pmol per reaction) were incubated for 20 min at 30°C with 5 pmol of GST, GST-USP14 (i.e., wild-type), GST-USP14(C1114A), untagged USP14, or untagged USP14(C1114A). Samples were resolved by non-reducing PAGE and proteasomes visualized by Sudan-LLVY-AMC gelatin hydrolysis. Binding of GST-USP14 to proteasomes significantly reduced their electrophoretic mobility. Bottom, Coomassie staining of the gel after gel-shift assay. c, Ub-V5 labeling of recombinant USP14. 11 nM of recombinant USP14 was incubated with 110 nM of VS-Pep in the absence or presence of Ub-V5 (1 μM) at 30°C for 1 hr. Samples were analyzed by SDS-PAGE and immunoblot. The result is representative of two comparable experiments. This experiment shows that the bulk of recombinant USP14 is Ub-V5 modified and thus functionally intact.
Figure S3. Kinetic analysis of reconstituted USP14-proteasome complexes. a, Linear kinetics ($R^2 > 0.99$) of the initial rates of Ub-AMC (1 μM) hydrolysis by USP14 and proteasome (1 nM). b, Michaelis-Menten plot of USP14-dependent Ub-AMC (1 μM) hydrolysis in the presence of human proteasome (1 nM) for 25 min. The data were fit to a hyperbolic curve by nonlinear regression ($R^2 > 0.99$). Approximate $k_{cat}$ and $K_m$ were determined as 4.0 ± 0.5 nkat and (32 ± 0.1) x 10^7 M^{-1} sec^{-1}, respectively. c, Linear kinetics ($R^2 > 0.99$) of Ub-AMC hydrolyses at 4 nM USP14 and 1 nM proteasome. d, Michaelis-Menten plot of concentration-dependent Ub-AMC cleavage in the presence of USP14 (4 nM) and proteasome (1 nM) for 30 min. The data are fit to a hyperbolic curve by nonlinear regression ($R^2 = 0.99$). Approximate $k_{cat}$ and $K_m$ were determined as 1.7 ± 2.7 μkat and (53 ± 7.1) x 10^7 M^{-1} sec^{-1}, respectively. The graphs shown are representative of at least three independent determinations and each data point is the mean ± s.d. of triplicate determinations.
Figure S4. USP14 does not regulate mRNA encoding tau or TDP-43. USP14−/− MEFs were co-transfected with plasmids expressing tau (a) or TDP-43 (b) and USP14 wild-type or a mutant. Control indicates pCMV-empty vector co-transfection. After 2 days, total RNA was isolated through Trizol extraction and further purified using an RNasy column. Quantitative RT-PCR was performed using primers for tau (Forward: AAGGTTAACCTCCTAGGAGGC, Reverse: GGGAGCTTGCTGCTTTGATGTC) and TDP-43 (Forward: ATGAAACACCACCGGAAACG, Reverse: CAGTGAGACACCCCTCCACTACA), mRNA levels were normalized to that of the GAPDH gene (Forward: GAGTCACGAGATGTTGCTGCT, Reverse: GACAGCTTGCCGCTTCAAG). The values plotted are means ± s.d. of three independent experiments.
Figure S5. Stabilization of tau by wild-type USP14. 

(a) HEK293 cells were co-transfected with a plasmid expressing tau and a plasmid expressing either wild-type USP14 or its catalytically inactive mutant USP14(C114A). Chase experiments were carried out at indicated time points after the addition of 75 μM cycloheximide at time zero. The chase was initiated 30 h after transfection. Anti-tau and anti-actin antibodies were simultaneously immunoprecipitated for immunoblotting. For each time point, the tau signal was normalized to that of endogenous actin. Band intensities were quantified using ImageJ software version 1.49 from three independent experiments (n=3). Note that tau levels were reduced in the mutant already at time zero (to 67% of wild-type), presumably as a result of stabilized tau degradation prior to cycloheximide addition. This effect is normalized out in panel b.
Figure S6. USP14(SF) does not significantly stabilize Arg-GFP in Usp14^−/− MEF cells. An in vivo suicide substrate, Arg-GFP, is responsive to the USP14 non-catalytic effect as shown in Fig. 1f. Arg-GFP (5 μg plasmid DNA transfected per sample) was co-expressed with either wildtype USP14, catalytically inactive USP14(D110A), or a naturally occurring splice variant, the short form of USP14 (USP14(SF)) (2 μg DNA /sample) in Usp14^−/− MEFs. Protein extracts were prepared two days after transfection, and analyzed by SDS-PAGE and anti-GFP immunoblot as in Fig. 1f.
Figure S7. Primary screening of small-molecule libraries for USP14 inhibition. Statistical plot of high-throughput compound screening. 83,082 compounds were screened in duplicate for inhibition of USP14 (15 μM) in the presence of proteasome (1 μM). A 384-well, low volume (20 μl) plate format was used. Data processing was done by a robust Z-score method as previously described and each compound was plotted using Spotfire software. The Z-score is a normalized value that takes into account plate-to-plate variation that would otherwise make it difficult to compare data from different plates across the screen. Hits were designated as +5 < Z < +10, medium hits as +10 < Z < +10, and strong hits as Z < +10. Compounds with a Z score less than +3.5 were excluded for secondary screening. Compounds over the cutoff of Z > 5 are mostly autofluorescent molecules and were not plotted.
Figure S8. IU1, a USP14 inhibitor, inhibits the catalytic activity of proteasome-associated USP14 in vitro. a, IU1 is only weakly inhibitory towards proteasome-associated USP14. b, IU1 (2 μM) and IU1G (0.1 μM)协会 protein USP14 in the absence of proteasome was treated with either IU1 or IU1G (17 μM) or IU1G (17 μM) showed little inhibitory activity in Ub-AMC hydrolysis assays of human proteasomes not treated with IU1-VS. These data complement Fig. 2c and demonstrate that IU1 does not affect the DUB activity of proteasome-bound USP14. c, d, Two independent IC₅₀ assays of proteasome-associated USP14 treated with IU1. USP14 was preincubated with IU1 for either 45 min (Exp1) or 30 min (Exp2). The data were fit to a four parameter logistic model (the Hill–slope model) based on guidelines from NIH Chemical Genomics Center (http://dipdic.web.gse.wustl.edu). Error bars indicate s.d. (n=3); note that, in related experiments, IU1 was shown not to be a threonine quencher for AMC (data not shown). See Methods for the detailed assay conditions.
Figure S5: The specificity of IFN for USP14 is observed independently of Ub-AMC concentration. Assays of Ub-AMC hydrolysis were done as in Fig. 2b, except lower concentrations of Ub-AMC were used. Similar results were obtained for USP2 (data not shown).
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Figure S10. Summary of $K_m$ values for Ub-AMC of desubiquitinating enzymes in this study. $K_m$ values of DUBs used in the selectivity assay were obtained from the literature. Wherever $K_m$ values were determined in the study, as indicated. These values are significant because the DUB assay (Supplementary Fig. 9) would be most sensitive to inhibition when substrate is at a low concentration as compared to the $K_m$ of the enzyme in question. CD, catalytic domains.
Figure S11. Characterization of IU1C, an inactive variant of IU1. a, Dose-response curves for inhibition of various deubiquitinating enzymes by IU1C versus IU1. IU1C data are taken from Fig 2c. b, Analysis of deubiquitinating enzyme inhibition by IU1C. Compared to IU1C. Conditions as in Fig 2c. All values are presented as mean ± s.d. (n=3). c, IU1C does not significantly inhibit levels in wild-type MEF cells. Compared to Fig 4a. Conditions as in Fig 4a except for Luc2 overexpression (i.e., IU1C was used at 0, 25, 50, 75, or 100 μM in a 0.5% DMSO treatment).
SUPPLEMENTARY INFORMATION

Figure S12. IU1 does not interfere with USP14 binding to human proteasomes. a, Human proteasomes (~4 nM) were first solubilized using Nonidet P-40 lysis buffer (Thermo Scientific), from ~2 mg of tissue from HEP-2X cells harboring USP14-HA-ER. After incubating with recombinant USP14 (20 nM) in the presence or absence of the indicated compound,followed by washing with low salt lysis buffer, the association between USP14 and the proteasome was determined by immunodetection. The experiment was performed in duplicate and additionally repeated. b, as in a, except ~2-hour incubation were used for each compound. The asterisk (*) denotes a nonspecific signal generated by the anti-USP14 antibody.
Figure S13. Reversibility of USP14 inhibition by IUT after prolonged incubation. a. IUT inhibits proteasome-associated USP14 activity without a detectable lag period. 2.5 ml of human proteasome was mixed with 30 ml of recombinant USP14 protein. The reaction was then initiated by adding 1 mM Ul-AMC. After 30 min, IUT (100 mM) or vehicle (DMSO) was added to the sample. b. These data complement Fig. 2d: 0.3 mM of USP14 and 5 mM human proteasome were treated with vehicle or 100 mM of IUT for 2 hr. The sample was then subjected to three rounds of ultracentrifugation, using a Microcon YM-10 filter (100 kDa cutoff, Millipore). After each spin, the pellet complex was resuspended to the original volume and assayed for DUB activity. c. The DUB activity of USP14 is stable in the presence of human proteasome for at least 8 hr. d, e. As in Fig. 2d except prolonged incubation (0 and 8 hr) was tested and the percent DUB activity was normalized to 20S proteasome activity (i.e. LLVY-AMC hydrolysis). IUT was added to 100 mM. e. Immunoblot analysis showing that there are essentially no changes of protein level after multiple rounds of sample concentration using Microcon YM-10 filters. There is some protein loss after gel filtration by Centriprep-10, but this amount of protein is comparable between IUT treated and control treated samples. SDS-PAGE immunoblot was done with the same samples as used in Fig. 2d and Supplementary Fig. 13b.
Figure S1A. Chain trimming assays with human pro tease and RP purified in the presence of ADP. a, b, native running gel (a) and SGE-PAGE gel analysis (b) of purified human pro tease with ATP (ATP prep) or ADP (ADP prep) 7 μg was used for](image) native running gel analysis and it was for SGE-PAGE. Note that the ADP sample contains a mature of pro tease 24S holoenzyme and TSG RP, due to CR-RP dissociation during purification (see also ref 3). c, D-AMC and LLVY-AMC hydrolyses assays, d, in vitro Ub-CCNB degradation assays with samples prepared and assayed in the presence of ATP or ADP. Samples were analyzed by SGE-PAGE. e, Ub CCNB assay trimming assays with samples prepared and assayed in the presence of ADP. I/21 is effective at inhibition of chain trimming at approximately 9 μM, as expected from Ub AMC hydrolyses data.
Figure S15. JL1 does not affect CCNB degradation in the presence of USP14(CA). Assays were done as in Figs. 1b and 3b.
Figure S16. Chemical analysis of IU1 and IU1C. a, b, H-NMR spectroscopic data of IU1 (a) and IU1C (b). LCMS analysis using IU1 (c) and IU1C (d). TIC, total ion count. SPC, shared peak count subtracted from the peak with retention time 10 min (IU1) or 23 min (IU1C). Additional information is available in Methods.
Figure S17. IUI uptake into cells measured by LC/MS. a. Standardization of LC/MS for IUI detection. Ion count LC/MS traces (m/z at 301) of various concentrations of IUI. Cell-associated IUI was monitored using an Agilent series 1200 LC/MS system with a reversed-phase C18 column. The retention time for IUI is ~8.0 min. b. Ion count peak areas versus concentration of IUI. The LC/MS shows linear responses in the given range of concentration (>3 μg/mL). c. 3T3 fibroblasts were treated with 25 μM of IUI for various times as indicated. Cell lysates were collected, extracted with ethyl acetate, and subjected to mass spectrometry (see details in Methods). Ion counts of LC/MS traces (m/z at 301) at 0 hr, 1 hr, 24 hr, and an IUI standard solution at 1 μg/mL, are shown. ns, non-specific.
Figure S18: Entry of I41 into wild-type MEF cells monitored by HPLC with UV detection. a. The UV spectrum of I41. I41 shows absorption maxima at 305 nm and 355 nm. b. HPLC chromatograms showing the time-dependence of I41 internalization at 305 nm. Cell lysates were processed as described in the legend to Supplementary Fig. 17. The standard shows results obtained using purified I41 at 1 μg/ml. It is assumed that the retention of I41 with the cell fraction reflects I41 internalization.
Figure S10. Time-course of IGF1 levels in cells and media. a, b. The IGF1 concentration in wildtype MEF cells and HEK293 cells, determined by LC/MS. IGF1 was added to cultures at 300 ng/ml at time zero. ND, not detected. Similar results were obtained in Ugp14’ MEF cells. c, d. Internalized IGF1 was rapidly released from cells. After wildtype MEFs were incubated with 500 ng/ml of IGF1 for one hour, the culture media were replaced with fresh media without IGF1. Internalized IGF1 was monitored at the indicated times. e, f. IGF1 concentration in the media of HEK293 cells and Ugp14’ MEF cells. The concentration of IGF1 from 1 hr to 48 hr indicate its stability in serum-containing media.
Figure S25. The stimulation of tau degradation by IUT is not mediated by autophagy. a. Transiently expressed mCherry-NB1 levels in wild-type NIEs were significantly increased after treatment with 250 μM of batimaxine (BatA) an autophagic formation inhibitor, for 1 hr. b. Cells transfected with a plasmid expressing tau were treated with 250 μM of BatA, and/or 75 μM of IUT for 8 hrs, and analyzed by SDS-PAGE immunoblot using the Odyssey infrared imaging system. c. Band intensities were quantified from three independent experiments (mean ± s.d.) using Odyssey software.
Figure 520. U1 treatment reduces the level of GFAP expressed in wild-type MEF cells but does not affect Arg-GFP. a, Wild-type MEF cells transfected with plasmids expressing wild-type GFAP or its non-aggregation prone mutants, GFAP(K63Q) or GFAP(E210K) were treated with 0, 25, 50, or 100 μM of U1 for 8 hr, and analyzed by SDS-PAGE immunoblot. b, Lack of effect of U1 on the degradation of Arg-GFP, which was stabilized by catalytically inactive USP14(C116A) as in Fig. 1H. Otherwise short-lived Arg-GFP was coexpressed with USP14(C116A) in Uspl4−/− MEF cells and treated with 0 – 100 μM of U1 for 8 hr. Anti-GFP and anti-actin antibodies were used for immunoblotting. The non-catalytic effect of USP14, which is best visualized in this assay, is not reversed by U1.
Figure S27. Quantification of ubiquitin levels in Fig. 4a. Polyubiquitin and monoubiquitin levels from wildtype and Uap14" MEF were quantified after treatment of various concentration of IU1. Ub signals were normalized to that of endogenous actin. Quantitation was achieved by densitometry of a film image.
Figure S28. IU1 treatment does not induce transcription of proteasome subunit genes. a, the level and position of activity of the proteasome were determined before and after a 48-h IU1 treatment (100 µM). Total cell extracts (30 µg protein) were resolved by native PAGE, and the proteasome was visualized using either an in-gel activity stain with a fluorescent peptide substrate (LLYF-AMC) or immunoblotting with antibodies to subunit α5. RPN-C and RPR-CP are distinct forms of the 20S proteasome. b, A luciferase reporter gene containing the murine PsmB5 promoter (430 bp to 9 kb) was transiently expressed in wild-type and Up14+/− MEFs and promoter activity was assessed following incubation of 25 or 50 µM of IU1 for 8 hr. For normalization of luciferase activity, a control experiment using the renilla luciferase (Rluc) plasmid was performed. This reporter construct has previously been used as a representative measure of proteasome subunit gene induction. Values are mean ± s.d. from three independent experiments. RLU, relative light units. c-e, Quantitative RT-PCR for a ubiquitin gene (Ub8) and two proteasome subunit genes (α5 and α7) was performed using total RNA from −/− (left panels) and Up14+/− MEFs (right) after incubation with a graded dose of IU1 for 8 hr. These data supplement Fig. 4h.
Figure S20. IU1C does not significantly affect oxidative stress-induced cytotoxicity. a, HER230 cells were treated with 10 μM of IU1C for 0 hr and graded concentrations of menadione for 4 hr, followed by MTT assay. Values represent the mean ± s.d. of triplicate cultures. b, e, data supplement Fig. S5b. Time-course of IU1C levels in wildtype MEFs (b) and HER230 cells (e) were measured by using LCMS. As in Supplementary Figs. 1Bb and 1Bb, respectively, except IU1C, a functionally inactive control for IU1, was used. ND, not detected. d and e, See legend to Supplementary Fig. 1Bc. IU1C concentrations in the media (data not shown) of wildtype MEFs or HER230 cells are comparable to those of IU1 (Supplementary Fig. 1B).
References accompanying supplementary figures


Figure S30. Assessment of IJ1 cytotoxicity in MEF cells, using the MTT assay. IJ1 was added at various concentrations (12.5 nM to 1.5 mM) to wild type (+/+) (red squares) or Usp14-/- MEFs (blue triangles). After the indicated incubation times, 20 μL of a 5 mg/mL MTT solution was added to each well and the plates were incubated for 2 hr at 37°C. The MTT-formazan crystals that had formed were dissolved by adding 200 μL DMSO, and the absorbance was measured at 550 nm. Each data point is a mean ± s.d. of three independent experiments.
Figure S21. IL1β cytotoxicity assessed in MEF, HEK293, and HeLa cells. a-c: See legend to Supplementary Fig. 20. d, e, f: Live-cell imaging of IL1β effects on proliferation of MEFs. Viability (d) or Ogfix (e) were plated into each well of a 24-well plate at 30,000 cells/well. On the following day, IL1β or vehicle was added, as indicated. Live-cell proliferation was monitored over 72 hrs using an automated imaging system. Data points are means of duplicate measurements. g: Live-cell imaging of IL1β effects on HeLa cell proliferation.
Figure S22. IU1 does not induce apoptosis in wild-type MEF cells. Cells were treated with 100 μg/ml of IU1 or DMSO control for 8 h and a fluorescent TUNEL assay was performed with DAPI counterstaining. TUNEL-positive cells (arrow) were quantified and compared (right). Bars are the mean ± s.e.m. of percentage of TUNEL-positive cells from four independent experiments.
Figure S3A. Quantitative analysis of tau levels after IU1 treatment in wild-type and Usp14−/− MEFs. a and b, As in Fig. 4a and 4b except unrelated, non-competitive secondary antibodies were used for quantification using Odyssey imaging system. Tau signal intensities were normalized to that of endogenous actin, and relative amounts are shown. Primary antibodies were simultaneously incubated for immunodetection and band intensities were quantified using the Odyssey software.
Figure S24. IU1 reduces tau and TDP-43 levels through a post-transcriptional effect. Tau (a) or TDP-43 (b) was transiently overexpressed in wild-type MEFs, which were then treated with IU1 as in Figs. 4a and 4b, respectively. Total RNA was isolated and quantitative RT-PCR was performed as in Supplementary Fig. 4.
Figure S25. The stimulation of tau degradation by IU1 is not mediated by autophagy. a. Transiently expressed mCherry-NB1 levels in wild-type NIE115 were significantly increased after treatment with 250 μM of bafilomycin A1 (BafA1), an autolysosome formation inhibitor, for 8 hr. b. Cells transfected with a plasmid expressing tau were treated with 250 μM of BafA1 and/or 75 μM of IU1 for 8 hrs, and analyzed by SDS-PAGE immunoblotted using the Odyssey infrared imaging system. c. Band intensities were quantified from three independent experiments (mean ± s.d.) using Odyssey software.
Figure S20. UUC does not significantly affect oxidative stress-induced cytotoxicity. a, HEK293 cells were treated with 0.01 M of UUC for 0 hr and graded concentrations of menadione for 4 hr, followed by MTT assay. Values represent the mean ± S.D. of triplicate cultures. These data supplement Fig. 5b. Time-course of UUC levels in wildtype MEFs (b) and HEK293 cells (c) were measured by using LC/MS. As in Supplementary Figs. 18b and 18c, respectively, except UUC, a functionally inactive control for U1, was used. ND, not detected. d and e, see legend to Supplementary Fig. 18c. UUC concentrations in the media (data not shown) of wildtype MEFs or HEK293 cells are comparable to those of U1 (Supplementary Fig. 18c).
References accompanying supplementary figures

Figure S2B. IU1 treatment reduces the level of GFAP expressed in wild-type MEF cells but does not affect Arg-GFP. a, Wild-type MEF cells transfected with plasmids expressing wild-type GFAP or its more aggregagtion prone mutations, GFAP(K63Q) or GFAP(E210K) were treated with 0, 25, 50, or 100 µM of IU1 for 8 hr, and analyzed by SDS–PAGE/immunoblot. b, Lack of effect of IU1 on the degradation of Arg-GFP, which was stabilized by catalytically inactive USP14(C115A) as in Fig. 4B. Otherwise short-lived Arg-GFP was coexpressed with USP14(C115A) in Usf1+/− MEF cells and treated with 0 – 100 µM of IU1 for 8 hr. Anti-GFP and anti-actin antibodies were used for immunoblotting. The noncatalytic effect of USP14, which is best visualized in this assay, is not reversed by IU1.
Figure S27. Quantification of ubiquitin levels in Fig. 4g. Polyubiquitin and monoubiquitin levels from wildtype and Uap14−/− MEF were quantified after treatment of various concentrations of IU1. Ub signals were normalized to that of endogenous actin. Quantification was achieved by densitometry of film images.
Figure S2B. IUT treatment does not induce transcription of proteasome subunit genes. a, the levels and phosphorylation activity of the proteasome were determined before and after a 0.5 μM IUT treatment (50 μM). Total cell extracts (50 μg/plane) were resolved by native PAGE, and the proteasome was visualized using either an in-gel activity stain with a fluorescent peptide substrate (LLVY-AMC), or immunoblotting with antibodies to subunits α5, RPN10, and RPN11. b, the effect of IUT on the expression of Pmtrb5. The mRNA expression of Pmtrb5 was assessed by qRT-PCR in MEFs and promotor activity was assessed following transfection of 25 or 50 μM of IUT for 8 hr. For normalization of luciferase activity, a control experiment using the human RPS3 promoter was performed. This reporter construct has previously been used as a representative measure of proteasome subunit gene induction. Values are mean ± SEM from three independent experiments. BLU, relative light units. c-e, Quantitative RT-PCR for a trk variant gene (α6) and two proteasome subunit genes (α5 and α7) was performed using total RNA from −/+ (left panels) and Usp14+/− MEFs (right) after treatment with a gradient dose of IUT for 8 hr. These data supplement Fig. 4h.
Figure S2B. IU1 treatment reduces the level of GFAP expressed in wild-type MEF cells but does not affect Arg-GFP. a, Wild-type MEF cells transfected with plasmids expressing wild-type GFAP or its more aggregation-prone mutations, GFAP(K63Q) or GFAP(E210K), were treated with 0, 25, 50, or 100 μM of IU1 for 3 hr, and analyzed by SDS-PAGE and immunoblot. b, Lack of effect of IU1 on the degradation of Arg-GFP, which was stabilized by catalytically inactive USP14(C114A) as in Fig. 4F. Otherwise short-lived Arg-GFP was coexpressed with USP14(C114A) in USP14−/− MEF cells and treated with 0 – 100 μM of IU1 for 8 hrs. Anti-GFP and anti-α-tubulin antibodies were used for immunoblotting. The nonspecific effect of USP14, which is best visualized in this assay, is not reversed by IU1.
Figure S27. Quantification of ubiquitin levels in Fig. 4a. Polyubiquitin and monoubiquitin levels from wildtype and Uap14+ MEF were quantified after treatment of various concentration of IU1. Ub signals were normalized to that of endogenous actin. Quantification was achieved by densitometry of a film image.
Figure S2B. IL1 treatment does not induce transcription of proteasome subunit genes. a, the level and positional activity of the proteasome were determined before and after a 6-h IL1 treatment (100 nM). Total cell extracts (50 μg protein) were resolved by native PAGE, and the proteasome was visualized using either an in-gel activity stain with a fluorescent peptide substrate (LLVY-AMC), or immunoblotting with antibodies to subunit α3. RP-CP and RP-CP are distinct forms of the 26S proteasome. b, A luciferase reporter gene containing the mouse Pomp5 promoter (4 kb to 9 kb) was transiently expressed in wildtype and Usp14−/− MEFs, and promoter activity was assessed following incubation of 25 or 50 μM of IL1 for 6 hrs. For normalization of luciferase activity, a control experiment using the carrier vehicle alone was performed. This reporter construct has previously been used as a representative measure of proteasome subunit gene induction [8]. Values are mean ± s.d. from three independent experiments. BLU, relative light units. c-e, Quantitative RT-PCR for α5, α7 and α5 mRNA in Usp14−/− MEFs was performed using total mRNA from −/− (wildtype) and Usp14−/− MEFs (right) after stimulation with a gradient dose of IL1 for 6 hrs. These data supplement Fig. 4d.
**Figure S20.** I2UC does not significantly affect oxidative stress-induced cytotoxicity. a, HEK293 cells were treated with 10 µM of I2UC for 0 hr and graded concentrations of menadione for 4 hr, followed by MTT assay. Values represent the mean ± S.D. of triplicate cultures. These data supplement Fig. 5b. Time-course of I2UC levels in wildtype MEFs (b) and HEK293 cells (c) were measured by using LC/MS. As in Supplementary Figs. 18a and 18b, respectively, except I2UC, a functionally inactive control for I1, was used. ND, not detected. d and e, See legend to Supplementary Fig. 18c. I2UC concentrations in the media (data not shown) of wildtype MEFs or HEK293 cells are comparable to those of I1 (Supplementary Fig. 19).
References accompanying supplementary figures

Pharmacologic Inhibition of the Anaphase-Promoting Complex Induces a Spindle Checkpoint-Dependent Mitotic Arrest in the Absence of Spindle Damage

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SUMMARY

Microtubule inhibitors are important cancer drugs that induce mitotic arrest by activating the spindle assembly checkpoint (SAC), which, in turn, inhibits the ubiquitin ligase activity of the anaphase-promoting complex (APC). Here, we report a small molecule, N-(3-oxo-4-acetyl-5-propyl-2,6)tetramethyl-2,3-dihydro-4H-pyran-4-yl) (TAME), which binds to the APC and prevents its activation by Cdc20 and Cdh1. A produg of TAMF arrests cells in metaphase without perturbing the spindle, but nonetheless the arrest is dependent on the SAC. Mitotic arrest induced by a proteasome inhibitor is also SAC dependent, suggesting that APC-dependent proteolysis is required to inactivate the SAC. We propose that mutual antagonism between the APC and the SAC yields a positive feedback loop that amplifies the ability of TAME to induce mitotic arrest.

INTRODUCTION

Microtubule inhibitors such as taxanes and the vinca alkaloids represent one of the most important classes of cancer drugs, used in the treatment of breast, ovarian, and lung cancer (Mantle et al., 2006). However, the response of cells to microtubule inhibitors is highly variable (Birds et al., 2009; Cosic et al., 2008; Taylor, 2008; Cai et al., 2008; Shi et al., 2008), potentially compromising clinical efficacy. How these drugs cause cell death remains unclear, but induction of mitotic arrest appears to be a key aspect of the mechanism (Boeke et al., 2009; Huang et al., 2009). By perturbing the mitotic spindle, these drugs activate the spindle assembly checkpoint (SAC), which delays mitotic exit by inhibiting the ubiquitin ligase activity of the anaphase-promoting complex (APC). In principle, a compound that directly inhibits APC-dependent proteolysis should arrest cells in mitosis without causing side effects that result from microtubule inhibition such as peripheral neuropathy.

The APC is the most complex ubiquitin ligase known, consisting of more than 16 subunits. The activator proteins Cdc20 and Cdh1 bind to the APC at different cell cycle stages to stimulate APC-dependent ubiquitination of substrates and their subsequent destruction by the 26S proteasome (Hansen, 2008). The activators assist in recruitment of APC substrates and may also stimulate ligase activity (Yu, 2007). Cdh1 binds to the APC during G1 to promote degradation of APC substrates during interphase. In contrast, the initiation of anaphase and exit from mitosis require Cdc20-dependent ubiquitination of APC substrates such as securin and mitotic cyclins. Prior to anaphase, the ability of APC-Cdc20 to ubiquitinate certain substrates is inhibited by the SAC (Viscusi and Salmon, 2007). Unattached microtubules induce the SAC, while spindle assembly itself prevents SAC activation (Hsu et al., 2000). Chemical inhibition of APC activity can be achieved using the small-molecule compound 56-41, which induces mitotic exit in living cells as a result of APC-dependent proteolysis (Cai et al., 2008). However, 56-41 is not selective for specific APC substrates, and in this way, it is not a direct inhibitor of APC-dependent proteolysis.

Significance

The anaphase-promoting complex (APC) is required for mitotic exit, making the APC a potential target for antimitotic chemotherapy. Here, we identify TAMF as a small molecule inhibitor of the APC and develop a cell-permeable derivative, pro-TAMF. Treatment of cells with pro-TAMF causes a surprisingly robust mitotic arrest because APC-dependent proteolysis is required for inactivation of the spindle assembly checkpoint (SAC). In contrast, SAC-activating compounds such as microtubule inhibitors do not suppress APC activity as completely. As a result, cells rely on continued protein synthesis to maintain mitotic arrest, providing an explanation for the known variability in cellular response to microtubule inhibitors. Direct APC inhibitors may therefore provide a more uniform and specific method for inducing mitotic arrest.
Cancer Cell
Initiation of the Anaphase Promoting Complex

RESULTS

TAME Inhibits APC Activation by Perturbing Activator Protein Binding

We identified TAME (Figure 5A) as an inhibitor of cyclin proteolysis in Xenopus egg extract (Ec40, 12.5 M; Figure 5A), but its mechanism of action has remained unclear. TAME also inhibited cyclin degradation in interphase extracts activated by exogenous Cdc2, but had no effect on Cdc2-dependent proteolysis of budding yeast (G1) (Figure 1B). Another Cdc2 substrate, cyclin B2 (Figure 1B), was also inhibited by TAME in Xenopus extract (data not shown). TAME can better distinguish APC activity and induce a more persistent mitotic arrest than therefore an important question in understanding the development of APC inhibitors as a therapeutic strategy for cancer.

Figure 1. TAME Inhibits APC Activation by Perturbing Binding of Cdc2 to Cdc2

A. TAME inhibits APC activity in Xenopus extracts prepared from interphase blastoderm and interphase oocytes. Cdc2 phosphorylation and ubiquitination were examined by immunoblot.

B. TAME inhibits APC activity. Compartments were added to inhibit Xenopus extracts individually, immediately after APC immunoprecipitation. The activity of the isolated APC was measured in a recombinant soybean assay.

C. TAME inhibits APC activity. Compartments were added to inhibit Xenopus extracts immediately after APC immunoprecipitation. The activity of the isolated APC was measured in a recombinant soybean assay.

D. TAME inhibits APC activity. Compartments were added to inhibit Xenopus extracts immediately after APC immunoprecipitation. The activity of the isolated APC was measured in a recombinant soybean assay.

E. TAME inhibits APC activity. Compartments were added to inhibit Xenopus extracts immediately after APC immunoprecipitation. The activity of the isolated APC was measured in a recombinant soybean assay.

F. TAME inhibits APC activity. Compartments were added to inhibit Xenopus extracts immediately after APC immunoprecipitation. The activity of the isolated APC was measured in a recombinant soybean assay.

See also Figure S1.
To understand how TAME disrupts the interaction between the activator proteins and the APC, we first tested whether TAME binds to the APC. We added TAME to Xenopus egg extracts to inhibit the interaction between the activator proteins and the APC. We found that TAME inhibits the interaction between the activator proteins and the APC. This is consistent with the results from the assays shown in Figure 5A, indicating that TAME does not inhibit the formation or function of the activator complexes.

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implicated in APO binding, including the C-lobe (Swoboda et al., 2003) and the C-terminal nucleation region (CNR) (Figure 2C, Button et al., 2005; Vodovar et al., 2005). Because the APO abnormally resembles the IR tail of Cα2β2 and Cαβ1 (Figure 2A), we hypothesized that TAME might bind to the APO in the same site normally occupied by the IR tail. Previous work has demonstrated that a C-terminal 23 amino acid peptide derived from Cα2β2 (ITD peptide) is sufficient to stabilize XnAMPs APO from interphase extracts (Vodovar et al., 2005). We confirmed this finding and found that TAME, but not NAME, was sufficient to block APO recruitment by the IR peptide (Figure 2C). In contrast, TAME had no effect on recruitment of APO from mitotic extracts by a C-terminal fragment of Gα2β2 containing only the C-terminal interaction motif (Figure 2C), indicating that TAME specifically inhibits the IR tail-dependent interaction.

The APO subunits Cα2β2 and APOβ have been implicated in binding either the IR tail of Cα1 in the APO (Knaus et al. and Vodovar, 2005; Vodovar et al., 2005). To determine whether TAME could competitively inhibit the binding of the IR tail to these proteins, we co-transfected the IR peptide with a polyclonality reporter and performed co-localization analysis with APO immunolabeling of interphase XnAMP extracts. Four proteins known to assist in an APO-dependent complex, namely, Cα2β2, Cα1β1, Cαβ1, and Apoβ, were co-localized in an IR-dependent manner that could be competed by excess unlabeled IR peptide (Figures S2D and S2E). All low concentration (25 μM) TAME efficiently inhibited co-localization of the IR peptide with Cα2β2 and Cα1β1 but only slightly reduced co-localization to Cαβ1 and Apoβ (Figure S2D). At high concentration (100 μM), TAME strongly inhibited co-localization to all APO subunits (Figure S2D). Together these findings support the hypothesis that TAME blocks to APO subunits that recruit the IR tail, thereby preventing activation of APO interaction with the IR tail.

To confirm that TAME specifically antagonizes IR tail-dependent interactions between Cα2β2 and the APO, we tested the ability of TAME to inhibit the binding of Cα2β2 to the APO in a reconstituted system. APO was purified from interphase XnAMP extracts and washed with high salt to remove most Cα2β2. Purified inactive APO was than incubated with recombinant Cα2β2 expressing wild-type or mutant Cα2β2 and Cαβ1 binding to APO was assessed by co-immunoprecipitation. We found that efficient binding of Cα2β2 to the APO under these conditions indeed required the IR tail, as evident by blocking these two reagents (Cα2β2 alone) did not bind as efficiently to TAME (Figure 3A). TAME also strongly reduced Cα2β2 binding to the APO under these conditions (Figure 3B). Importantly, addition of TAME had no further effect on binding of the Cα2β2/APO mutant, confirming that TAME does not compete with other interactions between Cα2β2 and the APO. We found that TAME addition to IR tail deletion was not sufficient to fully inhibit Cα2β2 association under these conditions. We speculated that other interactions, such as a C-lobe-dependent binding, might prevent Cα2β2 association with the APO, thereby rendering the effect of TAME addition or IR tail deletion. Consistent with this hypothesis, we found that addition of a C-lobe containing C-terminal fragment of Cα2β2 could competitively inhibit binding of full-length Cα2β2 to the APO (Figure 3C). In the presence of C-lobe fragment, addition of TAME or deletion of the IR tail was sufficient to completely suppress Cα2β2 association with the APO. These results indicate that both C-lobe-dependent and IR tail-dependent interactions are important for Cα2β2 binding in these conditions, and that TAME specifically antagonizes the IR tail-dependent interaction. We conclude that the target of TAME is the APO, and that it inhibits APO activation by interfering specifically with IR tail-dependent interactions between Cα2β2 and Cα1 in the APO.

A TAME Pretreating Inhibits APO-Cα1β1 Activation on Call.

Having established the mechanism by which TAME inhibits APO activation in Xenopus extract, we next wanted to determine whether TAME inhibits APO activation in human cells. Because TAME is not cell permeable, we hypothesized that TAME (prenilinylated and its control compound pheAME, and its control compound preAME, by co-midling the guanosine group to produce a 5′-iodo-5′-deoxyadenosine carbamide derivative (Figure 4E). Such preads are known to inhibit guanine kinase activity to inhibit the target compound. In Xenopus extract, preAME was rapidly converted to TAME (Figure S5A), which efficiently inhibited cyclic B kinase.
Figure 4. ProTAME Inhibits APC Activity in Axoneme Extract and Inhibits Gsk3β-Dependent APC Activity During Interphase in HeLa Cells

(A) Structures of proTAME and Gsk3β-Dependent APC Activity During Interphase in HeLa Cells. (B) The activity of APC was determined by immunoblot analysis of APC substrates. (C) The distribution of APC substrates was detected by immunofluorescence microscopy. (D) The activity of APC was determined by immunoblot analysis of APC substrates in different cell types. (E) The distribution of APC substrates was detected by immunofluorescence microscopy.

We first examined whether proTAME could inhibit association of Gsk3β with the APC in cells. We released a few cells expressing H2B-GFP from nocodazole-blocked and added 10 μM proTAME after the cells had entered G1, when the APC is activated by Gsk3β. We found that addition of proTAME inhibited Gsk3β association with the APC (Figure 4B), but proTAME did not. However, proTAME was not sufficient to cause premature activation of endogenous APC substrates in G1 or S phase (Figure 4C). During S phase, when APC substrates are known to be expressed, the effect of proTAME may be mediated by E1T-dependent inactivation of APC-CDC2 (Yoo et al., 2005). In addition, we depleted cells of E1T, which leads to degradation of APC substrates and prevents cell entry into S phase (Yoo et al., 2003). We found that depletion of E1T prevented mitotic entry and found that addition of 12 μM proTAME substantially increased the mitotic entry detected by depletion of E1T (Figure 4D). Therefore, we conclude that proTAME is capable of inhibiting Gsk3β-CDC2 function in cells.

Previous studies have shown that depletion of Gsk3β releases prolonged S phase and inhibits entry delay in human cells (Engelke et al., 2006; Sek et al., 2008). Consistent with these findings, proTAME caused a 2 h delay in mitotic entry when added during release from a double thymidine block (Figure 4E). However, adding proTAME 6 h or later after release did not delay mitotic entry (Figure 4F), suggesting that the delay may be a consequence of inhibiting APC-CDC2 function in cells.

PreTAME Induces Mitotic Arrest in the Absence of Spindle Damage

To examine effects of proTAME treatment on mitosis, we released HeLa H2B-GFP cells from a double thymidine block and added proTAME 6 h after release. A 6 h addition of proTAME addition does not delay mitotic entry (Figure 4G). Mitotic duration was then measured by time-lapse imaging. Cells treated with 12 μM proTAME arrested in metaphase and subsequently died (Figure 4H). In contrast, treatment of cells with 12 μM proTAME resulted in metaphase arrest and subsequent apoptosis (Figure 4I). Cells treated with 12 μM proTAME had no effect. proTAME greatly increased mitotic duration in asynchronous NIH3T3 cells as well, as 5 μM proTAME increased median mitotic duration to 8 h, compared with 24 min in proTAME-treated cells (Figure 4J). ProTAME had no effect at similar doses in
MCF7 A cells (data not shown), because the probing was not sufficiently activated (Figure 3B).

If proTAME directly inhibits APC activity, we predicted it would ablate all ACP substrates during mitosis, not just those whose stability depends on the SAC. Consistent with this hypothesis, cells treated with proTAME accumulated cyclin B1 and securin (Figure 3D), whereas results were confirmed in live cell imaging experiments, where proTAME stabilized cyclin B1 and securin but the reticulin dephosphorylation did not (Figure 3C). Interestingly, proTAME treatment caused greater accumulation of cyclin B1 and securin, consistent with proTAME’s ability to directly inhibit APC activity.

We next assessed the effects of proTAME treatment on mitotic spindle morphology and chromosome congression and compared this with the effects of treatment with CDK1 and Aurora A kinase inhibitors. Compared with DMSO-treated cells, treatment of asynchronous HeLa cells with 10 μM proTAME or 10 μM nocodazole caused no delay in chromosome congression (Figure 3A). In contrast, treatment of cells with nocodazole or taxol for 2 h strongly perturbed spindle organization (Figure 3B). In live cell imaging experiments, treatment of cells with 3 μM proTAME or 10 μM nocodazole caused no delay in chromosome congression (Figure 3A). Treatment of cells with 10 μM nocodazole or 10 μM proTAME caused a similar delay in congression delay of the spindle.
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Figure 6. ProTAME-Induced Mitotic Arrest Is SAC Dependent

(A) Mutant or wild-type mitotic arrest is SAC dependent. HeLa-2B-GFP cells were transfected with equimolar amounts of expression constructs encoding either proTAME or proTAME RNAi and, when indicated, equal amounts of constructs encoding either wild-type SAC or SAC RNAi. Expression constructs were transfected in the presence of proTAME or proTAME RNAi at 1.2 μM, and nocodazole was added at 0.025 μM. All data shown in Figure 6A (and Movie S5) are representative of three independent experiments. (B) Nocodazole and SAC kinase inhibitors. HeLa-2B-GFP cells were transfected with proTAME or proTAME RNAi, treated with nocodazole or nocodazole plus SAC kinase inhibitors, and then examined by time-lapse microscopy. Representative images are shown in Figure 6B. All data shown in Figure 6B are representative of at least three independent experiments. (C) SAC kinase inhibitors. HeLa-2B-GFP cells were transfected with proTAME or proTAME RNAi, treated with nocodazole plus SAC kinase inhibitors, and then examined by time-lapse microscopy. Representative images are shown in Figure 6C. All data shown in Figure 6C are representative of at least three independent experiments. (D) SAC kinase inhibitors. HeLa-2B-GFP cells were transfected with proTAME or proTAME RNAi, treated with nocodazole plus SAC kinase inhibitors, and then examined by time-lapse microscopy. Representative images are shown in Figure 6D. All data shown in Figure 6D are representative of at least three independent experiments.

6 min (Figure 5A), but these treatments produced contrasting effects on the metaphase plates. In cells treated with 1.2 μM nocodazole, the metaphase plate appeared loose and was prone to bending (Videos S1–S3), whereas in cells treated with 1.2 μM nocodazole, the metaphase plate appeared tight and did not bend (Movie S4). Importantly, 1.2 μM nocodazole prolonged the metaphase of cells by only 30 min (data not shown), whereas 12 μM nocodazole induced a mitotic arrest of over 7 h (Figure 5A). Thus, the mild delay in compression is not sufficient to explain the ability of proTAME to arrest cells in mitosis. We conclude that proTAME induces mitotic arrest in metaphase without perturbing the morphology or function of the mitotic spindle.

ProTAME-Induced Mitotic Arrest Is SAC Dependent

Because TAME directly inhibits the APC, and causes arrest in metaphase with induction of the spindle assembly checkpoint characterized by SAC knockdown (Figure 6A, Figure 5B, Movie S5A and S5B), the ability of proTAME to restore normal mitotic division in cells depleted of SAC demonstrates that proTAME is unlikely to perturb microtubules or interfere with kinetochore function. This fact that 12 μM proTAME-induced mitotic arrest by only 30 min in the absence of the SAC, whereas in the presence of the SAC, proTAME induces mitotic arrest in SAC-proficient cells, must therefore arise from a significant amplification of APC inhibition by the SAC.

Whereas TAME reduced Cdc20 binding to the APC when added to growing cultures, proTAME treatment did not decrease Cdc20 binding to the APC during mitotic arrest in HeLa cells (data not shown). We speculate that persistent Cdc20 association might result from the ability of the SAC to promote Rtf1-independent binding of Cdc20 to the APC. We
therefore examined the effect of depleting SAC proteins on the ability of pro-AurE to disrupt the APC-Cdc20 interaction. Indeed, when we depleted. We treated HeLa cells with a proteolysis inhibitor by expression of non-recombinant cyclin B1, pro-AurE induced significant dephosphorylation of Cdc20 from the APC, but only if SAC proteins were depleted by RNAi (figure 5D). These results show that a pro-AurE-induced mitotic arrest occurs without substantial dissociation of Cdc20 from the APC, as a consequence of persistent activity of the SAC.

To further understand the SAC dependence of the pro-AurE arrest, we pharmacologically inactivated SAC signaling by treating cells with heparin (Moolak et al., 2003), an inhibitor of Aurora B kinase. The kinase phosphorylates proteins at kinetochores that are not under tension, leading to destabilization of microtubule-kinetochore interactions and activation of the SAC (Biggins and Murray, 2001; Chawanc et al., 2006; Deplus et al., 2006). Recent work using phosphoantibodies to peptides that recognize Aurora B substrates indicates that kinetochoore proteins remain phosphorylated at a basal level during metaphase (Moolak et al., 2003). We hypothesized that this basal level of Aurora B-dependent phosphorylation may produce a persistent SAC signal during metaphase that contributes to the pro-AurE-induced arrest. Three observations are consistent with this hypothesis. First, heparin treatment dramatically shortened pro-AurE-induced mitotic arrest (figure 6C), leading to Cdc20 and BrdU from the APC in pro-AurE-injected cells (figure 5B). As expected, heparin also substantially shortened nocodazole-induced mitotic arrest, with a less pronounced effect on nocodazole-induced arrest (figure 4C). Second, heparin treatment caused dephosphorylation of the metaphase plate in pro-AurE-treated cells (figure 6A), suggesting that Aurora B-dependent phosphorylation is required to maintain proper kinetochore-microtubule attachment in metaphase. Third, inactivation of the APC component Cdc20 of the APC-specific genetic defect HUH10 caused a mitotic arrest delay in nocodazole-treated cells that was completely suppressed by heparin treatment (figure 6D). Together, these experiments are consistent with the idea that the SAC remains active at a basal rate during metaphase, despite the presence of properly attached chromosomes, and that kinetochore-dependent SAC signaling is important for the prolonged mitotic arrest induced by SAC inhibition.

One possible explanation for the SAC dependence of the pro-AurE arrest is that pro-AurE inhibits APC substrates such as Nek2 or cyclin A that are normally degraded in early mitosis. For example, overexpression of cyclin A has been reported to delay chromosome compaction (Parashakti and Elgar, 2002). To determine whether these substrates are important for the pro-AurE-induced arrest, we released HeLa cells from double-thymidine block into nocodazole for 15 hr to allow degradation of cyclin A and other APC substrates that are not efficiently stabilized by the SAC. We then washed cells out of nocodazole into pro-AurE. Under this condition, pro-AurE remained capable of inducing a prolonged mitotic arrest that was highly heparin-sensitive (figure 5C). This result indicates that the SAC dependence of pro-AurE-induced mitotic arrest is unlikely to be caused by stabilization of APC substrates that are normally degraded in a SAC-independent fashion.

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Metaphase Arrest Induced by a Proteasome Inhibitor
Is SAC Dependent

Previous work has shown that APC-dependent ubiquitination produces SAC inactivation in cell lines (Falty et al., 2007). In this system, APC-dependent ubiquitination of Cdc20, but not APC-dependent polyubiquitination, was suggested to be important for release of Cdc20 from SAC proteins (Falty et al., 2007). However, a recent study found that proteasome activity is required for dissociation of the Mad1-Cdc20 complex in cells (Ahonen et al., 2010). Together with our findings, these studies suggested that APC-dependent polyubiquitination could be important for SAC inactivation. A prediction of this model is that mitotic arrest induced by treatment with a low dose of proteasome inhibitor should be SAC-dependent. To test this, we treated cells with a dose of MG132 (1 μM) that was just sufficient to arrest cells in mitosis (median duration of 15 hr). At this concentration, the duration of arrest was delayed by cell death rather than mitotic arrest, as only 10% of cells exited mitosis after 20 hr (figure 4A). In MG132-treated cells depleted of Mad3 by RNAi, we observed that 55% of the cells exited mitosis (figure 4A), indicating that the SAC is indeed required for efficient induction of mitotic arrest by proteasome inhibition.

Like pro-AurE-treated cells, MG132-treated cells arrest in metaphase with kinetochores that develop normal tension (Kamizaki and Chan, 2007). If metaphase chromosomes are inhibited to prevent a checkpoint signal, we predicted that the MG132-induced arrest should be heparin-sensitive. To test this idea, 10 hr following thymidine release, HeLa HUH10 cells were treated with 1 μM MG132 in the presence or absence of heparin. Strikingly, heparin inhibited rapid metaphase exit in half of the cells, with the remainder exiting mitosis more slowly (figure 7B). These different behaviors correlated with the timing of drug administration: cells that encountered drug while in mitosis exited mitotically quickly, whereas cells that encountered drug before mitotic entry stalled (figure 5A).

Heparin treatment induced dephosphorylation of Cdc20 and reduced levels of Mad3 and BrdU bound to the APC compared to cells treated with MG132 alone (figure 5B). Co-treatment of pro-AurE to MG132 abrogated the ability of heparin to drive mitotic exit (figure 7B), indicating that mitotic exit remains dependent on SAC activity. In contrast, co-treatment of heparin to MG132 did not efficiently suppress heparin-induced mitotic exit (figure 7A), underscoring the distinct mechanisms underlying nocodazole and pro-AurE-induced mitotic arrests. Similar results were obtained when the proteasome was more fully inhibited by increasing the MG132 concentration to 5 μM (figure 7D), indicating that the SAC continues to be important for complete inactivation of APC-dependent pathways, even when proteasomes are completely inhibited by drug.

The heparin-sensitivity of the MG132-induced mitotic arrest suggested that Aurora B activity could be important for maintaining the metaphase plate, as we observed in pro-AurE-treated cells. We found that treatment of MG132-treated cells with heparin induced dephosphorylation of the metaphase plate within 30 min, whereas cells treated with MG132 alone maintained a normal metaphase plate for over 6 hr (figure 5C). These findings provide further support for the idea that Aurora B-dependent pathways remain active in metaphase, together our findings indicate that mitotic arrest induced by a low
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Figure 7. MG132-Induced Mitotic Arrest Is SAC Dependent

(A) MG132-induced arrest is SAC dependent. Kinase-dead, cdc25A-negative, or nocodazole-arrested cells were cotransfected with constructs encoding mitotic-specific tubulin, cyclin B1, and SAC to establish SAC dependence of mitotic arrest.

(B) SAC inhibitors did not rescue mitotic arrest in proTAME cells. Double-transfected mitotic-arrested SAC constructs were measured by flow cytometry.

(C) Double-transfected mitotic-arrested SAC constructs were measured by flow cytometry.

(D) Double-transfected mitotic-arrested SAC constructs were measured by flow cytometry.

Protracted arrest in mitosis might therefore require the continued synthesis of APC substrates during mitosis. Consistent with this hypothesis, we found that genetically modified mitotic exit of nocodazole- or nocodazole-arrested cells (Figure 7A). In striking contrast, the behavior did not result in SAC-dependent cells, but rather extended mitotic arrest by allowing cell death (Figure 7A). The absence of the result suggests that genetic transformants in their absence or in SAC-arrested cells, suppressing cell death without promoting mitotic exit (Figure 7B). Consistent with these findings, labeling experiments demonstrated that increased APC activity such as the E1 and S100B are translated during mitosis, confirming the findings that ongoing mitotic protein synthesis is required to sustain a SAC-dependent mitotic arrest, perhaps by replenishing components that are degraded by residual APC-dependent proteolysis.

We next evaluated whether the SAC-arrested cells that were not sensitive to cycloheximide. We hypothesized that persistent SAC activity cooperates with direct pharmacological inhibition of the proteasome to allow the rate of APC-dependent proteolysis to such an extent that mitotic arrest no longer depends upon protein synthesis. If this hypothesis is correct, then labeling the SAC would be expected to silence the SAC. Furthermore, the results of this experiment would reinforce the idea that SAC-arrested cells are sensitive to cycloheximide and that the SAC is not an absolute requirement for SAC-arrested cells to maintain mitotic arrest.
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TAME Exploits a Positive Feedback Loop between the SAC and the APC

While the fact that pro-TAME-induced mitotic arrest requires sustained SAC activity may be unexpected, because pro-TAME-treated cells arrest in metaphase with kinetochores that develop normal tension, a condition that should inactivate the SAC, in principle, the requirement for the SAC in the pro-TAME arrest could be explained in one of two ways. First, pro-TAME treatment could produce defects in microtubule-kinetochore interactions that generates an abnormally high degree of checkpoint signal compared with normal metaphase kinetochores. Alternatively, pro-TAME may impair SAC inactivation, despite normal microtubule-kinetochore interactions. We favor the latter model because the degree of checkpoint dependence is far exceedance the degree of kinetochore-microtubule perturbation that we observe.

Defects in microtubule-kinetochore attachment could arise from an off-target effect of pro-TAME on microtubules, or be a consequence of specific AIF inhibition. We found that knockdown of Cdk27 orMitotic Hej produced a mitotic exit delay that was SAC dependent. Furthermore, treatment of cells with a proTAME inhibitor yielded a SAC-dependent mitotic arrest, consistent with a recent study showing that nocodazole-treated interphase cells show persistent Mad1-Cdc20 interaction (Dickson et al., 2010), and work by Schimanski and co-workers showing that Mad1 and Mad3 remain APC-inactive in prometaphase mutants (Oda et al., 2005). Together these findings suggest that if deleterious microtubule-kinetochore interactions are indeed present in pro-TAME-treated cells, they are likely to result from specific inhibition of APC-dependent proteolysis rather than from nonspecific effects of pro-TAME on microtubules.

If deleterious microtubule-kinetochore interactions exist in pro-TAME-treated cells, they must be subtle. Cells treated with 12 nM pro-TAME arrest in mitosis until they die, yet form a normal-appearing metaphase plate and develop normal kinesin-dependent microtubule arrays. Furthermore, cells treated with 12 nM pro-TAME undergo a normal-appearing anaphase when the SAC is inactivated, indicating that the intrinsic spindle functions properly in the presence of pro-TAME. The only change in chromosomes behavior caused by the dose of pro-TAME is a slight delay in chromosome congression. A lower dose of pro-TAME (3 nM) causes no delay in chromosome congression, yet still extends mitotic duration to 5 hr. Although we cannot completely rule out subtle defects in microtubule-kinetochore interactions in pro-TAME-treated cells, we believe such defects are not of sufficient magnitude to explain the strong dependence of the pro-TAME arrest on the SAC.

The above explanation for the SAC dependence of the pro-TAME arrest is that APC-depended mitotic inactivation or proteolysis required for inactivating the SAC. Such mutual antagonists between the APC and the SAC is predicted to create a positive feedback loop that would amplify the inhibitory effects of pro-TAME, or a pro-TAME-induced SAC in a SAC-dependent manner. This is what we observed. If the SAC is inactivated by Mad3 depletion, 12 nM pro-TAME extends mitotic duration by only 72 min, indicating that this dose only partially inhibits APC activity, consistent with the measured 

23 hr, indicating that the effect of pro-TAME is greatly amplified by the SAC. This degree of amplification cannot be explained by the initial effect of pro-TAME on chromosome congression, because a dose of nocodazole (10 nM) that causes a similar delay in chromosome congression extends mitotic duration by only 30 min in SAC-proficient cells. Because we obtained similar results with a proteasome inhibitor, we believe this amplification is best explained by a requirement for APC-dependent proteolysis to inactivate the SAC.

It is unclear which APC substrates play the most important role in mediating the mutual antagonism between the APC and the SAC. APC-dependent ubiquitination of Cdc20 has been previously proposed to release the APC from the inhibitory effects of the SAC (Weid et al., 2007; Strode et al., 2007). However, this process does not require proteasome activity in cell lysates (Dicks et al., 2007), and others argue that Cdc20 ubiquitination targets Cdc20 for proteasomal degradation in a manner that sustains the SAC (Bai et al., 2007; Heslenfein et al., 2009). Alternatively, many SAC proteins are APC substrates and may need to be degraded to inactivate the SAC. Consistent with this possibility, expression of a stable BubR1 mutant induces a mitotic arrest (Choi et al., 2003). Another candidate is cyclin B1, because it is degraded prior to anaphase (Chua and Hines, 2002) and cyclin-dependent kinase activity is required to maintain the SAC (Hwang and Chen, 2003; D'Arpino et al., 2002). Other SAC proteins, including Mps1, Bub1, and Aurora B, are also APC substrates, but their bulk population is not degraded until after anaphase (Otterson et al., 2006; Qian and Yu, 2007; Stewart and Wang, 2005). It is possible that destruction of these proteins prior to anaphase is masked by their near similarity to the mutual antagonism between the APC and the SAC may reflect a systemic behavior that is regulated by small changes in the abundance of multiple SAC proteins prior to anaphase. If so, confirmation of our model will require quantitative measurements of the relative rates of synthesis and degradation of APC substrates that regulate SAC activity.

Our results indicate that it is possible to induce mitotic arrest without fully inhibiting the APC or the proteasome, pharmacologically. This was unexpected, because previous experiments indicated that Cdc20 is required to be reduced to very low levels to induce mitotic arrest (Weid et al., 2007; 2008). We believe the pro-TAME-induced arrest, the mitotic arrest induced by Cdc20 knockdown does not depend on the SAC (Hwang et al., 2009). One possible explanation for the lack of SAC dependence in the context of Cdc20 depletion is that Cdc20 is the target of the SAC (Yu, 2003). Therefore, when Cdc20 levels are reduced, the SAC is no longer required to inhibit Cdc20 function. In contrast, other methods of perturbing APC function, including knockdown of core APC substrates or the EF-2 enzyme Ubc13-H1, do not induce the pro-TAME arrest, so the SAC is SAC dependent. This is the likely consequence of the fact that Cdc20 remains present under each of these conditions.

A Model for Regulation of Mitotic Exit

Based on our findings, we propose the following model (Figure 8D). A positive feedback loop between the SAC and the APC has the potential to adapt to two stable states: high SAC and low APC activity (pro-TAME arrest) or high APC activity (mitotic exit). During normal division, it is important that cells do not become

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permanently arrested in mitosis. We propose that the SAC does not fully inhibit the APC during mitosis because residual APC activity must be prevented to prevent cells from becoming locked in mitosis. This residual APC activity may explain why cytokinetics is disturbed prior to the initiation of anaphase (Elmore and Elmore, 1999) and during prolonged SAC-dependent mitotic arrest (Jirou and Richter, 2000; Gassmann and Taylor, 2006; Huang et al., 2009; Holzer et al., 2006). To rescue in mitosis for a prolonged period, a cell may need to continue to synthesize new APC substrates that are degraded by residual APC-dependent proteolysis.

During normal mitosis, the development of telophase tension reduces the rate of SAC induction, but SAC activation is unlikely to be completely suppressed during metaphase. Anaphase is triggered when the rate of SAC activation falls below the rate at which APC-dependent proteolysis inactivates the SAC, tugging the feedback loop toward rapid APC activation and mitotic exit. The timing of anaphase initiation therefore depends not on how kinetochore attachment controls SAC activation, but also on the level of residual APC activity. During aneuploidy or nuclear treatment, the rate of SAC activation remains above the rate at which the APC inactivates the SAC, tugging the loop in the direction of APC inactivation, thereby preventing mitotic exit. Because APC-dependent proteolysis is not fully inhibited by the SAC, mitotic arrest is dependent on proteolysis to reconstitute APC substrates. If the rate of protein synthesis is not sufficient, the rate of SAC signal production will fall below the rate at which it is inactivated by the APC, leading to rapid APC activation and mitotic exit. Therefore, the rate of protein synthesis in mitosis may be an important determinant of the duration of mitotic arrest in cells treated with microtubule inhibitors.

In contrast to microtubule inhibitors, proTAME and MD121 induce mitotic arrest by inhibiting residual APC-dependent proteolysis rather than by stimulating SAC activation. The rate of bulk protein degradation by kinetochore microtubules may decline normally in proTAME- or MD121-treated cells because kinetochore microtubules develop proper tension. However, because the rate of residual APC-dependent proteolysis is lowered by proTAME or MD121, the rate of SAC signal production cannot fall below the rate at which it is inactivated by APC-dependent proteolysis, leading to mitotic arrest. The strong hyperploid sensitivity of both proTAME- and MD121-treated cells indicates the importance of kinetochore microtubules in generating a SAC signal to sustain mitotic arrest. Compared with microtubule inhibitors, 280% of mitotic cells induced by proTAME or MD121 arrest due to reduced dependence on protein synthesis because the rate of residual APC activity is lower in proTAME- and MD121-treated cells, yielding a lower requirement for protein synthesis to reconstitute APC substrates.

An Opportunity for Antimitotic Cancer Therapy

Our studies identify a potential application for the variability in cellular responses to microtubule inhibitors that could limit their therapeutic effectiveness. Because the SAC does not completely inhibit the APC, mitotic arrest induced by microtubule inhibition depends on protein synthesis. As a result, variation in the rates of protein synthesis among cells may be the rate-limiting factor that explains the highly variable response of cells to microtubule inhibitors. In contrast, cells treated with an APC inhibitor may be less prone to mitotic slippage because residual APC activity is inhibited. APC inhibition may therefore be more effective in promoting mitotic arrest, including a greater proapoptotic effect. Furthermore, loss of activity of an APC inhibitor may be useful in combination with microtubule inhibitors to sustain mitotic arrest and enhance cell death.

EXPERIMENTAL PROCEDURES

A list of reagents, methods of synthesis of proTAME and additional experimental procedures are provided in the Supplemental Information.

1H-THF (99%) and 1H-pyrimidine (98% DMSO) were added to 100 μM to a pipet and kept at -20°C until use. APC was immunoprecipitated with GST-Cdc20 antibody stock (clone 4D4, AKR) and cleaved with trypsin, then separated by automated high-performance liquid chromatography (HPLC) and confirmed by western blotting. To validate APC inhibition, imidazole was performed after one or two cycles of APC immunoprecipitation. Specific binding was calculated as the difference between values associated with CoCl treated cells compared with blank samples (no CoCl). Therefore, the protocol was designed to stimulate the growth of mitotic arrest and enhance cell death.

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SUPPLEMENTAL INFORMATION

Supplemental Information includes Experimental Procedures, references, seven tables, one table, and six reviews and can be found online at dx.doi.org/10.1016/j.ccr.2010.02.013.

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Supplemental Information

Pharmacologic Inhibition of the Anaphase-Promoting Complex Induces A Spindle Checkpoint-Dependent Mitotic Arrest in the Absence of Spindle Damage

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Supplemental Movie Legends

Movie S1: Chromosome congression of untreated HeLa H2B-GFP cell. Double thymidine synchronized HeLa H2B-GFP cells were imaged at 3 min interval with a 40x objective. The H2B-GFP channel of a representative mitotic cell is shown here.

Movie S2: Nocodazole (10 nM) treatment induces a delay in chromosome congression. Double thymidine synchronized HeLa H2B-GFP cells were treated with 10 nM nocodazole at 8 h after thymidine release and imaged at 3 min interval with a 40x objective. The H2B-GFP channel of a representative mitotic cell is shown here.

Movie S3: Nocodazole (10 nM) treatment induces bending of the metaphase plate. Double thymidine synchronized HeLa H2B-GFP cells were treated with 10 nM nocodazole at 8 h after thymidine release and imaged at 3 min interval with a 40x objective. The H2B-GFP channel of a representative mitotic cell is shown here.

Movie S4: ProTAME (12 μM) treatment induces a delay in chromosome congression but does not perturb the metaphase plate. Double thymidine synchronized HeLa H2B-GFP cells were treated with 12 μM proTAME at 8 h after thymidine release and imaged at 3 min interval with a 40x objective. The H2B-GFP channel of a representative mitotic cell is shown here.
Movie S5: Mad2-knockdown cell initiates anaphase before full chromosome congression. Asynchronous HeLa H2B-GFP cells were transfected with Mad2 siRNA 24 h prior to imaging. Cells were imaged at 3 min interval with a 40x objective. A representative mitotic cell is shown here. Red: DIC. Green: H2B-GFP.

Movie S6: ProTAME rescues the mitotic defect in cells lacking Mad2 by delaying anaphase onset to allow time for chromosome congression. Asynchronous HeLa H2B-GFP cells were transfected with Mad2 siRNA 24 h prior to imaging. Cells were treated with 12 μM proTAME and imaged at 3 min interval with a 40x objective. A representative mitotic cell is shown here. Red: DIC. Green: H2B-GFP.
Supplemental Experimental Procedures

Preparation of *Xenopus* egg extract

Interphase *Xenopus* egg extract was prepared from eggs laid overnight according to the protocol of Murray (Murray, 1991) with the exception that eggs were activated with 2 μg/ml calcium ionophore (A23187, free acid form, Calbiochem) for 30 minutes prior to the crushing spin. Extract was frozen in liquid nitrogen and stored at -80 °C. To make mitotic extract, MBP-cyclin B1Δ90 was added to interphase extract at 20 μg/ml and incubated at 22 °C for 30 min.

Chemicals

Tosyl-L-arginine methyl ester (T4626), tosyl-L-arginine (S365157), tosyl-L-argininamide (74501), benzoyl-L-arginine methyl ester (B1007), benzoyl-L-argininamide (B4375) and tosyl-L-lysine methyl ester (T5012) were from Sigma. Acetyl-L-arginine methyl ester was from BACHEM (E-1036). Cdh1 C-terminal peptide and the ΔIR control peptide (sequence: CFSKTRSTKESVSVDLNLFTR and CFSKTRSTKESVSVDLNLFTR) were synthesized by the core facility of Tufts medical school. 3H-TAME (15 Ci/mmol, >97% radiochemical purity) was synthesized by AmBios Labs (Newington, CT). Hesperadin was a gift from Boehringer Ingelheim. MG132 was from Sigma (C2211). Okadaic acid was from MP Biomedicals (IC15897425). Cycloheximide was from Calbiochem (239764).
Antibodies

Cdc27 antibody for APC immunoprecipitation was from Santa Cruz (sc-9972, AF3.1). Cdc27 antibody for Western blot was from BD Transduction Laboratories (610454). Cyclin B1 antibody was from NeoMarker (RB-008-P). Xenopus Cdc20 antibody was from Abcam (ab18217). Human Cdc20 antibody was from Santa Cruz (sc-8358 H-175). Cdh1 antibody was from Santa Cruz (sc-19398). Streptavidin-HRP was from Invitrogen (SNN1004). Securin antibody was from Abcam (ab3305). Cyclin A antibody was from Santa Cruz (sc H-432). Nek2 antibody was from BD Transduction Laboratories (610593). UbcH10 antibody was from Boston Biochem (A-650). Mad2 antibody was from Bethyl Laboratories (BL1461). GAPDH antibody was from Abcam (ab8245).

Anti-a-tubulin-FITC was from Sigma (F2168). CREST antiserum was from Antibodies Incorporated (15-234). Goat anti-human-Alexa 568 was from Invitrogen (A21090). HA antibody was from Santa Cruz (sc-805, Y-11). Apc10 antibody was from Santa Cruz (sc-20989). BabR1 antibody was a kind gift from Frank Meekon’s lab at Harvard medical school.

Luciferase assay

A fusion of the N-terminal domain of cyclin B1 to luciferase (Verna et al., 2004) was added to mitotic extract at 3 μg/ml. The extract was incubated at 23 °C and 3 μl samples were taken at 0, 30, 60, 90 and 120 min. The samples were mixed quickly with 30 μl of luciferin assay buffer (270 μM coenzyme A, 20 mM tricine, 3.67 mM MgSO4, 0.1 mM

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EDTA, 33.3 mM DTT, 530 µM ATP and 470 µM luciferin, pH 7.8) and the level of luminescence was measured on Wallac 1420 multilabel counter.

**TAME induction of mitotic arrest in Xenopus egg extract**

Human cyclin B1/cdc2 complex (MPF) was prepared by baculovirus expression and purification as described (Kirkpatrick et al., 2006), and added to interphase extract at 12.5 µg/ml supplemented with 1% DMSO, 200 µM TAME or 200 µM AAME. Extract samples were collected every 15 min following addition of MPF. Cdc27 and cyclin B1 levels were analyzed by Western blot.

**In vitro ubiquitination assay**

For a single reaction, 5 µl protein A affigel beads (Bio-Rad 156-0006) were washed with TBST (10 mM Tris, 150 mM NaCl and 0.01% Tween-20, pH 7.5) twice and incubated with 2 µg Cdc27 antibody for 75 min at 4°C. Beads were then washed with TBST twice and XB (100 mM KCl, 0.1 mM CaCl₂, 1 mM MgCl₂ and 10 mM HEPES, pH 7.7) twice before APC immunoprecipitation. For APC-Cdc20 reaction, MBP-cyclin B1/Δ90 was added to interphase extract at 20 µg/ml and incubated at 22°C for 30 min before immunoprecipitation. To immunoprecipitate APC, 100 µl extract was incubated with 5 µl antibody beads at 4°C for 1 h. The beads were then washed with XB high salt (XB with 500 mM KCl) twice, XB twice and ubiquitin chain buffer (20 mM Tris, 100 mM KCl, 2 mM ATP and 2.5 mM MgCl₂, pH 7.7) three times. A reaction mixture containing 200
μg/ml MBP-E1, 66 μg/ml His-Ubc4, 25 μg/ml MPF, 1 mg/ml ubiquitin (Sigma) in ubiquitin chain buffer was prepared and 5 μl of this was added to 5 μl antibody beads. Beads were incubated at 22 °C on a Eppendorf Thermomixer with shaking at 1500 rpm for 60 min and the whole mixture was then boiled with 10 μl sample buffer for 5 min. Ubiquitinated cyclin B1 was visualized by cyclin B1 immunoblot.

**Degradation of 35S labeled pre-ubiquitinated cyclin B1**

Human 35S-cyclin B1/cdc2 complex was prepared by metabolic labeling of SF9 cultures expressing cyclin B1. The labeled lysate containing cyclin B1 was mixed with an unlabeled lysate from cells expressing cdc2, followed by purification of the cyclin B1/cdc2 complex as described above. The labeled complex was ubiquitinated in a reconstituted APC reaction as described above. Inteplase Xenopus extract was pre-incubated with 200 μM TAME or control compounds for 22 °C for 30 min. Pre-incubation was performed in the presence of 100μg/ml cycloheximide to prevent re-incorporation of free labeled amino acid and 1/20th volume of energy mix (150 mM creatine phosphate, 20 mM ATP, 2 mM EGTA and 20 mM MgCl₂, pH 7.7). 90 μl of extract was then added to 15 μl of labeled cyclin B1-ubiquitin conjugates and incubated at 22°C for the indicated amount of time. Reactions were stopped by the addition of an equal volume (105μl) of chilled 2% perchloric acid. The mixture was incubated on ice for 30 min and centrifuged at 14,000 rpm for 10 min at 4 °C. 168 μl of supernatant was mixed with 20 μl 2 M Tris base and 6 ml ultima gold scintillation fluid (Perkin Elmer).
Samples were mixed well and counted with a scintillation counter.

**Covalect coupling of Cdc27 antibody to protein A beads**

Protein A affiprop beads were coupled with Cdc27 antibody as described above. After coupling, the beads were washed with TBST for 10 min followed by two additional quick washes with TBST. Dimethyl pimelimidate (DMP, PIERCE, 21666) was freshly dissolved in 100 mM sodium tetraborate decahydrate, pH 9.0 at 20 mM. The beads were mixed with ten beads volume of DMP solution and incubated on a rotating wheel for 45 min in the dark at room temperature. The beads were then washed twice quickly with 200 mM Tris, pH 8.0 twice, followed by a final 1 h wash. Beads were then washed twice in TBST and twice in XB prior to APC immunoprecipitation.

**IR peptide immobilization on iodoacetyl resin**

A 20-aa Cdh1 C-terminal peptide with one cysteine residue added at the N-terminus was synthesized along with a control peptide lacking the C-terminal IR residues. The lyophilized peptide was re-dissolved at 400 μM in 100 mM HEPES, 5 mM EDTA, pH 7.9. To reduce the disulfide bonds, TCEP (Sigma C4706) was dissolved at 10 mM in 100 mM HEPES, 5 mM EDTA, pH 7.9 and added to the peptide solution at a final concentration of 200 μM (stoichiometric amount to reduce disulfide bonds). The peptide was reduced at room temperature for 15 min before mixing with Ultralink iodoacetyl resin (Pierce 53155) that was pre-equilibrated with 100 mM HEPES, 5 mM EDTA, pH
7.9. A ratio of 35 μl resin volume per 110 μl reduced peptide was used. For the negative control, freshly prepared 50 mM cysteine in 100 mM HEPES, 5 mM EDTA, pH 7.9 was used instead of the peptide. The coupling reaction was carried out at room temperature on a rotating wheel for 1 h and unreacted sites on the resin were blocked by further incubation with 50 mM cysteine for 30 min. The resin was then washed with 1 M NaCl followed by two washes with XB and stored at 4 °C before APC pull down.

**APC pull down by IR peptide resin**

10 μl of resin coupled with IR peptide, ΔIR peptide or cysteine as described above was mixed with 100 μl interphase Xenopus egg extract and incubated on a rotating wheel for 30 min at 4 °C. The resin was then washed twice with XB high salt and once with PBS. The resin was boiled with 10 μl sample buffer and the amount of Cdc27 was analyzed by immunoblot.

**Conjugation of IR peptide with photoinactive crosslinker**

The IR and ΔIR peptides were reduced as described above. The photoinactive crosslinker, Profound Mits-AIF-Biotin label transfer reagent (Pierce 33093), was dissolved at 40 mg/ml in DMSO. The crosslinker was added to the reduced peptide at a 1:1 molar excess and the reaction was left at room temperature in the dark for 1 h. The reaction mixture was centrifuged at 12,000 rpm for 1 min and the supernatant was loaded onto HPLC for purification. The purified conjugated peptide showed >99% purity on HPLC. The identity
of the conjugated peptide was confirmed by mass spectrometry.

Crosslinking assay

Purified conjugated IR or ΔR peptide was diluted in XB to a final concentration of approximately 2 μM. The following additives were included when necessary: 10 μM of unconjugated IR peptide with the cysteine modified with N-ethyl maleimide to show competition with the conjugated peptide, 20 μM or 200 μM TAME to show inhibition of crosslinking, and 200 μM AAME as a negative control. APC was immunoprecipitated with protein A affigel beads covalently crosslinked with Cdc27 antibody as described above. After washing, 5 μl aliquot of beads were mixed with 50 μl conjugated peptide and transferred to a 96-well polypropylene clear conical bottom plate. The plate was illuminated at a distance of 10 cm from a 300 watt long wavelength UV lamp for 3 min. The beads were then transferred back to 0.5 ml tubes and mixed with 10 μl sample buffer and boiled for 5 min. APC subunits that were crosslinked were analyzed by streptavidin-HRP blot. To confirm the nature of the crosslinked subunits, APC was immunoprecipitated from interphase extract as described above and run on the same gel of the crosslinked sample and coomassie stained. The bands were subjected to mass spectrometry analysis.

3H-TAME binding assay in interphase extract

3H-TAME (15 Ci/mmol) was added to interphase extract (100 μl) to a final concentration
of 200 nM, and subject to immunoprecipitation (4 °C for 1.5 h) using 5 µl protein A
affiprep beads coupled with Cdc27 antibody as described above. Protein A beads without
Cdc27 antibody were used as a negative control to measure background level of binding
(mock IP). The beads were washed quickly twice with XB high salt and twice with XB.
The beads were then transferred to scintillation vials, mixed with scintillation fluid, and
counted in a scintillation counter. Alternatively, the extract was subjected to one or two
rounds of immunoprecipitation before the addition of ³H-TAME. For competition assays,
different concentrations of unlabeled TAME or other derivatives were added along with
200 nM ³H-TAME into the extract. Specific binding under each condition was obtained
by subtracting the value of mock IP.

³H-TAME binding assay in HeLa cell lysate

Protein A affiprep beads coupled with Cdc27 antibody were prepared as described above.
HeLa cells were harvested in lysis buffer (10mM potassium phosphate pH 7.5, 0.1mM
EDTA, 0.5mM EGTA, 50mM β-glycerophosphate, 1mM sodium vanadate, 1mM DTT,
0.5% Triton X-100 and leupeptin, chymostatin and pepstatin each at 10 µg/ml). For
4,000,000 cells, 100 µl lysis buffer was used. The cell lysate was centrifuged at 10,000
rpm for 10 min to remove cell debris. ³H-TAME (15 Ci/mmol) was added to cell lysate to
a final concentration of 200 nM. For each aliquot of 5 µl beads, 100 µl lysate was used
for APC immunoprecipitation at 4 °C for 1 h. Protein A beads without Cdc27 antibody
was used as a negative control to measure background level of binding (mock IP). The
beads were washed quickly with lysis buffer high salt (500 mM sodium chloride in addition to above components) twice and lysis buffer twice. The beads were then transferred to scintillation vials, mixed with scintillation fluid, and radioactivity measured by scintillation counting. Alternatively, the lysate was subjected to one or two rounds of immunoprecipitation before the addition of $^{3}$H TAME. For competition assays, 10 μM unlabeled TAME or AAME was added along with 200 nM $^{3}$H TAME into the lysate. Specific binding under each condition was obtained by subtracting the value of mock IP.

**Synthesis of proTAME (14) and proAAME (15)**

![Chemical Structures](image)

$N^2$-[4-(methylphenyl)sulfonyl]-$N^2$-[phenylnmethoxy]carbonyl]-L-ornithine

$1,2$-dimethylethyl ester (1): A mixture of $N^2$-[4-(phenylnmethoxy)carbonyl]-L-ornithine $1,2$-dimethylethyl ester HCl (718 mg, 2 mmol), acetone (15 mL) and sat. aq. NaHCO$_3$ (15 mL) was treated with $p$-toluenesulfonyl chloride (420 mg, 2.2 mmole) in acetone (15 mL) at 0 °C and then stirred at room temperature for 16 h. The mixture was diluted with EtOAc, washed with brine, dried over anhydrous sodium sulfate, filtered, concentrated in vacuo to afford an oil, which was purified by silica gel column chromatography using 40% EtOAc in hexane to give compound 1 (910 mg, 95%): $^1$H NMR (500 MHz, CDCl$_3$): δ1.23 (s, 9H), 1.56 – 1.65 (m, 3H), 1.73 – 1.77 (m, 1H), 2.39 (s, 3H), 3.21 (q, J = 6.0, 2H), 3.72 –
$N^2$-acetyl-$N^2$-[(phenylmethoxy)carbonyl]-L-ornithine 1,2-dimethylethyl ester (2) was prepared in a manner similar as 1, with acetyl chloride used in place of p-toluenesulfonyl chloride. $^1$H NMR (500 MHz, CDCl$_3$): δ1.46 (s, 9H), 1.49 – 1.57 (m, 2H), 1.63 – 1.69 (m, 1H), 1.81 – 1.88 (m, 1H), 2.01 (s, 3H), 3.22 (q, J = 5.5, 2H), 4.07 – 4.15 (m, 1H), 4.94 (br, 1H), 5.09 (s, 2H), 6.11 (br d, J = 7.5, 1H), 7.30 – 7.36 (m, 5H).

$N^2$-[(4-methylphenyl)sulfonyl]-L-ornithine 1,2-dimethylethyl ester (3): A mixture of 1 (500 mg, 1.05 mmole), methanol (1.5 mL), ethanol (15 mL) and 10% Pd-C (200 mg) was stirred under a hydrogen atmosphere at room temperature for 3 h. The mixture was filtered through a pad of Celite, concentrated in vacuo to give crude compound 3 (399mg, 99%). This material was stored in the freezer and then used without further purification.

$N^2$-acetyl-L-ornithine 1,2-dimethylethyl ester (4) was prepared in a manner similar as 3.
O-Chloromethyl S-(phenylmethyl) carbothioate (5): was prepared following a literature procedure (Folkmann, 1990).

[[phenylmethylthio]carboxyl][oxy]methyl benzenecacetate (6): A mixture of 5 (6.327 g, 29.2 mmol), phenylacetic acid (3.68 g, 27 mmole), K₂CO₃ (3.74 g, 27 mmole), and cat. KI in acetone (100 mL) was refluxed for 16 h. The mixture was diluted with EtOAc, washed with brine, dried over anhydrous sodium sulfate, filtered, concentrated \textit{in vacuo} to afford an oil, which was purified by silica gel column chromatography with 5% EtOAc in hexane to give
1 (5.80 g, 92%): \(^1\)H NMR (500 MHz, CDCl\(_3\)): 8 3.68 (s, 4H), 4.12 (s, 4H), 5.82 (s, 4H), 7.26 – 7.33 (m, 10H).

\((\text{chlorocarbonyl})\text{oxy}\)methyl benzoate (7): was prepared following a literature procedure (Folkanara, 1990).

3-(methylthio)-5,9-dioxo-10-phenyl-\((\text{phenylacetyl})\text{oxy}\)methyl
6,8-dioxa-2,4-diazadec-2-enoate (8): was prepared following a literature procedure (Saulnier, 1994). \(^1\)H NMR (500 MHz, CDCl\(_3\)): 8 2.44 (s, 3H), 3.70 (s, 4H), 5.84 (s, 4H), 7.26 – 7.35 (m, 10H).

3-chloro-5,9-dioxo-10-phenyl-\((\text{phenylacetyl})\text{oxy}\)methyl
6,8-dioxa-2,4-diazadec-2-enoate (9): was prepared following a literature procedure (Saulnier, 1994).

1,2-dimethylethyl

(S)-2-\((4\text{-methylphenyl})\text{sulfonyl}\)amino-4-\((\text{bis}[\text{(phenylacetyl)oxy}])\text{methoxy}\)carbonyl\([\text{amino}][\text{methylene}]\)amino\)[pentanate (10) and 1,2-dimethylethyl

(S)-2-\((\text{acetyl})\text{amino}-4-\((\text{bis}[\text{(phenylacetyl)oxy}])\text{methoxy}\)carbonyl\([\text{amino}][\text{methylene}]\)amino\)[pentanate (11): were prepared following a literature procedure (Saulnier, 1994).
10: $^1$H NMR (500 MHz, CDCl3): $\delta$ 1.23 (s, 9H), 1.57 – 1.62 (m, 1H), 1.71 – 1.77 (m, 3H), 2.39 (s, 3H), 3.45 (q, J = 6.5, 2H), 3.68 (s, 2H), 3.71 (s, 2H), 3.77 – 3.79 (m, 1H), 5.23 (d, J = 8.5 Hz, 1H), 5.80 (s, 2H), 5.82 (s, 2H), 7.24 – 7.36 (m, 12H), 7.71 – 7.73 (m, 2H), 8.29 (br t, J = 5.3 Hz, 1H), 11.61 (br s, 1H).

11: $^1$H NMR (500 MHz, CDCl3): $\delta$ 1.46 (s, 9H), 1.56 – 1.71 (m, 3H), 1.86 – 1.90 (m, 1H), 2.22 (s, 3H), 3.42 – 3.48 (m, 2H), 3.67 (s, 2H), 3.71 (s, 2H), 4.51 – 4.54 (m, 1H), 5.80 (s, 2H), 5.82 (s, 2H), 6.19 (d, J = 7.5 Hz, 1H), 7.26 – 7.36 (m, 10H), 8.31 (br t, J = 5.5 Hz, 1H), 11.61 (br s, 1H).

(S)-2-[[4-methylphenyl)sulfonyl]amino-4-[[bis[[phenylacetyl]oxy]methoxy]carbon yl]amino)methylene][amino]pentanoic acid (12) and

(S)-2-(acetyl)amino-4-[[bis[[phenylacetyl]oxy]methoxy]carbon yl]amino)methylene]pentanoic acid (13) : were prepared following a literature procedure (Bryan, 1977). Briefly, each ester (10 and 11) was deprotected with 30% TFA in DCM at room temperature for 2 h and then purified by silica gel column chromatography with 2.5% MeOH in dichloromethane to give each acid 12 and 13 in 30-40% yield.

12: $^1$H NMR (500 MHz, CDCl3): $\delta$ 1.66 – 1.75 (m, 3H), 1.83 – 1.89 (m, 1H), 2.40 (s, 3H), 3.36 – 3.46 (m, 2H), 3.68 (s, 2H), 3.71 (s, 2H), 4.01 – 4.05 (m, 1H), 5.48 (d, J = 8.5 Hz, 1H), 5.78 (s, 2H), 5.82 (s, 2H), 7.24 – 7.35 (m, 12H), 7.72 – 7.74 (m, 2H), 8.32 (br t, J = 5.3 Hz, 1H), 11.55 (br, 1H).
13: $^1$H NMR (500 MHz, CDCl$_3$): δ 1.67 – 1.77 (m, 3H), 1.93 – 1.97 (m, 1H), 2.04 (s, 3H), 3.36 – 3.40 (m, 1H), 3.51 – 3.55 (m, 1H), 3.67 (s, 2H), 3.70 (s, 2H), 4.54 – 4.58 (m, 1H), 5.78 (s, 2H), 5.81 (s, 2H), 6.93 (d, J = 7.5 Hz, 1H), 7.26 – 7.35 (m, 10H), 8.41 (br s, J = 5.5 Hz, 1H), 11.63 (br, 1H).

Methyl[(S)-2-[(4-methylphenyl)sulfonyl]amino-4-[(bis)phenylacetyl]oxy]methoxy|carbonyl]amino|methylene|amino|pentanoate (14) and methyl (S)-2-(acetyl)amino-4-[(bis)phenylacetyl]oxy|methoxy|carbonyl]amino|methylene|amino|pentanoate (15): were prepared following a literature procedure (Tangirala, 2006). Briefly, each acid (12 and 13) was methylated with TMSCHN$_2$ (2 M solution in hexane) in dry benzene at room temperature and then purified by silica gel column chromatography with 40% EtOAc in hexane for 14, 80% EtOAc in hexane for 15 to give each ester 14 and 15 in 40 to 45% yield, respectively.

14: $^1$H NMR (500 MHz, CDCl$_3$): δ 0.65 – 1.72 (m, 2H), 1.76 – 1.81 (m, 1H), 2.41 (s, 3H), 3.34 (q, J = 6.5, 2H), 3.48 (s, 3H), 3.68 (s, 2H), 3.71 (s, 2H), 3.93 – 3.97 (m, 1H), 5.28 (d, J = 9.0 Hz, 1H), 5.80 (s, 2H), 5.83 (s, 2H), 7.24 – 7.36 (m, 12H), 7.71 – 7.72 (m, 2H), 8.28 (br t, J = 5.8 Hz, 1H), 11.61 (s, 1H). $^{13}$C NMR (125 MHz,CDCl$_3$): 21.5, 24.8, 30.2, 40.4, 40.8, 41.0, 52.6, 55.3, 80.5, 81.2, 127.2, 127.3, 127.5, 128.6, 128.7, 129.3, 129.4, 129.7, 132.7, 133.3, 136.5, 143.8, 152.4, 156.3, 162.1, 169.9, 170.3, 171.8. HRMS calcd for C$_{32}$H$_{29}$N$_2$O$_7$S (M + H)$^+$ 727.2285; found 727.2280.
H5: H NMR (125 MHz, CDCl3): δ 1.59 – 1.73 (m, 3H), 1.88 – 1.92 (m, 1H), 2.03 (s, 3H), 3.40 – 3.45 (m, 1H), 3.45 – 3.52 (m, 1H), 3.68 (s, 2H), 3.71 (s, 2H), 3.75 (s, 3H), 4.63 – 4.67 (m, 1H), 5.80 (s, 2H), 5.82 (s, 2H), 6.28 (d, J = 8.0 Hz, 1H), 7.25 – 7.36 (m, 10H), 8.32 (br t, J = 5.5 Hz, 1H), 11.61 (br s, 1H). 13C NMR (500 MHz, CDCl3): δ 23.4, 25.4, 29.6, 40.6, 41.0, 41.2, 52.1, 52.8, 80.8, 81.4, 127.5, 127.8, 128.8, 129.0, 129.6, 129.7, 132.9, 133.5, 152.7, 156.5, 162.9, 170.2, 170.6, 172.9. HRMS calc for C29H38N8O14 (M + H)⁺: 615.2302; found 615.2297.

ProTAME activation analysis

ProTAME was added to interphase *Xenopus* extract at 50 μM or cell growth media at 20 μM. For interphase extract, 800 μl of sample was collected at 0 min, 10 min, 20 min and 30 min after addition of proTAME and diluted to 8 ml with XB. For cell culture, approximately 800,000 cells were collected at 1 h, 2 h and 3 h after addition of proTAME and lysed in 400 μl lysis buffer as described above and subsequently diluted to 4 ml with lysis buffer. The diluted extract or cell lysate was extracted with 1.5 volume of ethyl acetate. The extracts were dried in vacuo and the dry extracts were resuspended in 200 μl of methanol for LC/MS analysis. LC/MS data were obtained using an Agilent series 1200 LC / 6130 MS system with a reversed-phase C18 column (Phenomenex Luna C18(2), 4.6 mm × 100 mm, 5 μm) and a CH3CN/H2O gradient solvent system beginning with 10% aqueous CH3CN and ending at 100% CH3CN at 20 min. 10 μl of each sample was injected for each analysis. The collected LC/MS profiles were further analyzed by
extracting specific ions such as 343 (TAME) and 727 (proTAME) in the positive ion MS mode.

**Odyssey scanner for western signal quantification**

Secondary antibodies coupled to fluorophores (anti-mouse Alexa-Fluor 750 and anti-rabbit Alexa-Fluor680, Invitrogen) were used to detect and quantify signals from rabbit anti-Cdc20 (Santa-Cruz, sc-8358) and mouse anti-GAPDH (AbCam, ab8245) antibodies on the same membrane using an Odyssey (Li-Cor Biosciences) scanner. Quantifications are reported as CDC20/GAPDH signal ratio, normalized to control treatment.

**Cdc20/Cdh1 binding assay in HeLa cells**

HeLa cells in DMEM 10% FBS were plated in T25 flasks at 20% confluence one day prior to the experiment. They were then synchronized by a double thymidine block (18 h for the first block, 8 h release and another 18 h for the second block, thymidine concentration: 2 mM). For analysis of Cdh1 binding, cells were released into 300 nM from the second thymidine block for 13 h and then washed into fresh medium. Six h later, cells were treated with 12 μM proTAME or proAAME or 0.06% DMSO for 2 h and then collected by trypsin digestion. For analysis of Cdc20 binding, cells were transfected with indicated siRNAs during the first release from thymidine block after two washes with DPBS (CellGro 21-034-CV) and addition of 6.3 ml OptiMEM. A volume (79 μl) of 20 μl
Control siRNA#2 or a 1:1 mix of 20 μl MAD2 siRNA and BubR1 siRNA (Dharmacon D-004101-01, 5’-GGAAGAAGAGAUCUAGAGUAGUAU-3’) was mixed in 1381 μl OptiMEM in a tube, 23.7 μl OligoFectamine were mixed with 94.1 μl OptiMEM in a second tube. After 5 min incubation at RT, the tubes contents were mixed and siRNA-reagent complexes were allowed to form for 20 min at RT. The transfection mixes were added to cells in OptiMEM and FBS was added to 10% after 5 h transfection.

Cyclin B1-Δ107 expressing adenovirus (1:100) was added at the start of the second thymidine block and kept in the medium for all subsequent steps. Cells were treated at 10 h after release with 100 nM okadaic acid, 25 μg/ml cycloheximide and 12 μM proTAME as indicated. After 2 h treatment, cells were collected by mitotic shake-off. Cell pellets were washed twice with DPBS and flash-frozen with liquid nitrogen and stored at -80°C until use. Cell lysis and APC immunoprecipitation were performed as described above.

**Live cell imaging**

The imaging plate was mounted onto a motorized stage (Prior ProScan II) on a Nikon TE2000E PFS inverted microscope fitted with an incubation chamber maintained at 37°C and supplied with 5% CO₂. A 20X Plan Apo 0.75 NA or 40X Plan Fluor 0.75 NA objective lens was used as indicated and images were collected with 2x2 binning. DIC or GFP fluorescent images were taken every 12 min (unless otherwise specified) for 36 h with a Hamamatsu ORCA cooled CCD camera and Nikon Elements Software. TIF files of each image were exported from Elements and used to build stacks and QuickeTime
movies with Metamorph imaging software (Molecular Devices). For manual analysis, mitotic duration is counted as the time between the first frame of chromosome condensation and the frame of chromosome segregation (anaphase) or decondensation (mitotic exit in the presence of nocodazole) or cell death (chromosomes shrinking to a small bright dot).

**Em1 knockout and proTAME rescue**

HeLa H2B-GFP cells were plated in glass-bottom 24-well plates at 20% confluence one day prior to the experiment. Cells were transfected with a pool of Em1 siRNA (Dharmacon M-012434-01, 5'-GAAAGGCCUGUCAUGUUAUG-3'; 5'-CAACAGACACUUAUAGUA-3'; 5'-CGAAGUGUCUGUAAUAUA-3'; 5'-GUACGAGUGUCUGUAAGA-3') or Control3 siRNA (described above) at 18.5 nM with DharmaFect3. After 24 h, cells were treated with 0.06% DMSO or 12 μM proTAME. Live cell imaging was set up as described above.

**ProTAME dose-response**

HeLa H2B-GFP cells were plated in a 24-well plate in DMEM 10% FBS at 20% confluence one day prior to experiment and synchronized by double thymidine block as described above. ProTAME was added to final concentrations of 780 nM, 3 μM or 12 μM and proAAME was added to a final concentration of 12 μM at 8 h after release from the second thymidine block. 0.06% DMSO was used as the negative control. Live cell
Imaging was set up as described above.

**Exogenous cyclin-GFP expression, live-cell imaging, and quantitation**

HeLa H2B-RFP cells were transduced with cyclin B1-GFP or cyclin A2-GFP adenovirus for 40 h. Phenol Red-Free DMEM (Mediatech) supplemented with 10% FBS and 1:100 Penicillin-Streptomycin-Glutamine (Mediatech) was used as imaging medium. 20 μM proAAME, 20 μM proTAME or 150 nM nocodazole was added 45 min prior to the start of imaging. Live cell imaging was set up as described above except that the cells were plated in a 8-well chambered coverglasses (NUNC Lab-tek 155411). Four positions per treatment group were imaged with DIC transmitted light, red fluorescence, and green fluorescence (Semrock GFP/HeRd “Pinkel” filter set) at 12 min intervals for 24 h.

Stacks of red and green fluorescence were merged, saved as AVI video files and analyzed using ImageJ. For quantitation, the first GFP-positive cells that undergo mitosis in three separate movies were chosen, giving at least 30 cells quantitated for each treatment group. Mean intensity values for the green channel were collected for a cytoplasmic region of a cell upon mitotic entry, mitotic exit, or after 1 h of mitotic arrest. At the same time points, background mean green intensity was determined and individually subtracted from the cytoplasmic mean intensity. This background corrected mean intensity value was then used to determine the percentage of the original GFP signal remaining at the completion of division (for the control cells) or after 1 h of mitotic arrest (for the nocodazole and proTAME treated cells). The average values for all quantitated cells were plotted with the
error bars representing standard error of the mean.

**Immunofluorescence**

HeLa cells grown in DMEM + 10% FBS were plated on 25 mm glass coverslips in a 6-well dish at a density of 130,000/ml. 3 ml 48 h prior to treatment. They were then treated with 0.06% DMSO, 12 μM proTAME, 300 nM nocodazole or 300nM taxol for 2 h. The cells were washed twice with PBS and fixed with 3% paraformaldehyde for 15 min. The cells were then washed with PBS and permeabilized with PBS plus 0.5% Triton X-100 for 2 min. The cells were then washed with PBS and blocked with PBS plus 5% FBS for 1 h. CREST antiserum diluted 1:50 into PBS was added to the cells and incubated at room temperature for 1 h. The cells were washed with PBS and incubated with 1:1000 anti-human-Alexa 568 (Invitrogen, A21090) and 1:100 anti-a-tubulin FITC for 1 h. The cells were then washed with PBS and the nuclei were stained with 1 μg/ml Hoechst 33342. The cover slips were mounted in 0.1M N-propylgalllate in 9:1 glycerol:PBS.

Z-series images were taken on a Nikon TE2000 microscope with PerkinElmer spinning disk confocal device. Maximal Z-projection images of individual cells were made by Image J. To measure interkinetochore distances, a straight line was drawn across a kinetochore pair in the same confocal plane and pixel intensities along the line were plotted so that each kinetochore would be represented by a peak on the line. The interkinetochore distance was calculated as the distance between the peaks. Fifty-five kinetochore pairs from 5 cells treated with DMSO or proTAME were measured and the
p-value was calculated with a paired student test.

**Mad2 knockdown and time point analysis**

HeLa H2B-GFP cells were plated in a 24-well plate in DMEM + 10% FBS at 20% confluence one day prior to the experiment and synchronized by double thymidine block as described above. The cells were released from the first thymidine block into 200 µl OptiMem without FBS. Transfection was performed immediately after the first thymidine release. To prepare the transfection mixture for one well, 40 µl OptiMem was mixed with 2.5 µl of 20 µM Mad2 siRNA stock (GGAAACACUGAAAGAUGGdTdT, synthesized by DHARMACON) or control (D-001210-01-20, DHARMACON), and 6.5 µl of OptiMem was mixed with 1 µl of Oligofectamine (Invitrogen, 12252-011). The two mixtures were left at room temperature for 5 min before being mixed together and incubated for additional 20 min and then added to the cells to a final volume of 250 µl. 4 h after transfection, 250 µl of DMEM + 20% FBS were added to cells. 8 h after the release, 500 µl of 4 mM thymidine in DMEM + 10% FBS was added to each well to make the final concentration of 2 mM and the cells were incubated for another 18 h before being released into growth medium. At 8 h after release, cells were treated 0.06% DMSO, 12 µM proTAME, 300 nM nocodazole or 12 µM proTAME plus 300 nM nocodazole in growth medium. Cell samples were collected at 4 h, 8 h, 10 h, 12 h, 14 h, 16 h and 20 h post-release and protein levels were analyzed by Western blot.
Cdc20 knockdown sensitization to proTAME treatment

HeLa H2B-GFP cells were plated in glass-bottom 24-well plates (Greiner Bio-One 662892) at 20% confluence one day prior to the experiment. Cells were transfected with DharmaFect3, following the manufacturer’s protocol at a final concentration of 18.5 nM control siRNA#3 or a mix of 1.85 nM Cdc20 siRNA completed to 18.5 nM with control siRNA#3. After 24 h transfection, cells were treated with DMSO or 4 μM proTAME and live cell imaging was set up immediately as described above.

UbcH10 and Cdc27 knockdown and hesperadin treatment

HeLa H2B-GFP cells were plated in glass-bottom 24-well plates at 20% confluence one day prior to the experiment and synchronized by double thymidine block as described above. Cells were transfected with UbcH10 siRNA (Dharmacon D-004693-15, 5'-UAAAUUAAGCCUCGGUUGA-3'), Cdc27 siRNA (Dharmacon J-003229-11, 5'-GGAAUUGCCAGAUGGAUAA-3') or Control#3 siRNA (described above) at 18.5 nM with DharmaFect3, during the release from the first thymidine block. Cells were treated with 100 nM Hesperadin or DMSO 8 h after release from the second thymidine block and live cell imaging was set up as described above.

Measuring cycloheximide-sensitivity of drug-induced arrest

HeLa H2B-GFP cells were plated in a 24-well plate in DMEM + 10% FBS at 20% confluence one day prior to experiment and synchronized by double thymidine block as
synchronized above. At 8 h after release from the second block, cells were treated with 12 μM proTAME, 300 nM nocodazole or 150 nM taxol and 4 h later, cells were left untreated or treated with an addition of 25 μg/ml cycloheximide. Live cell imaging was set up as described above.

**Measuring hesperadin-sensitivity of drug-induced arrest**

HeLa H2B-GFP cells were plated in a 24-well plate in DMEM + 10% FBS at 20% confluence one day prior to experiment and synchronized by double thymidine block as described above. At 8 h after release from the second block, 100 nM hesperadin, 12 μM proTAME with or without 100 nM hesperadin, 300 nM nocodazole with or without 100 nM hesperadin or 150 nM taxol with or without 100 nM hesperadin were added to cells. Untreated cells were used as the control. Live cell imaging was set up as described above. For experiments with MG132, at 10 h after release from the second block, 3 μM MG132, or 3 μM MG132 plus 100 nM hesperadin, or 3 μM MG132 plus 100 nM hesperadin and 12 μM proTAME were added to cells. Alternatively, at 10 h after release from the second block, 10 μM MG132 with or without 25 μg/ml cycloheximide was added to the cells. 30 min after, cells were left untreated or treated with 100 nM hesperadin or 100 nM hesperadin and 12 μM proTAME. Live cell imaging was set up as described above.

**Measuring Mad2-dependence of MG132-induced arrest**

HeLa H2B-GFP cells were plated in a 24-well plate in DMEM + 10% FBS at 20%
confluence one day prior to experiment and synchronized by double thymidine block as described above. Mad2 siRNA transfection was performed as described above. At 10 h after release from the second block, 10 µM MG132 with or without 25 µg/ml cycloheximide was added to the cells. Live cell imaging was set up as described above. Manual analysis was focused only on cells that entered mitosis after MG132 addition.

**Chromosome congression analysis**

HeLa H2B-GFP cells were plated in 35mm glass-bottom dishes (MatTek) in DMEM + 10% FBS at 20% confluence one day prior to the experiment and synchronized by double thymidine block as described above. Drugs were added as follows to a final volume of 3 ml from 2X concentrated preparation in culture medium. DMSO (0.06%), proTAME (3 and 12µM) or 10µM Nocodazole were added 8 h after release from the second block, while MG132 (10µM) was added at 10 h. H2B-GFP was imaged for 4 hrs every 3 min at 40X magnification as described above.

**Click-IT chemistry labeling of de novo-translated proteins**

HeLa H2B-GFP cells (600,000) were plated in 3ml DMEM + 10% FBS in 6-well plates 24 h prior to synchronization. Cells were arrested in interphase by 2mM Thymidine treatment for 24 h. To label proteins translated in S/G2 phase, three hours after release from thymidine-block, the cells were washed once with warm DPBS with Mg²⁺/Ca²⁺ and switched to filter-sterilized labeling medium (Methionine-free medium from Sigma,
catalog #D0422, supplemented with 10mL FBS pre-dialyzed against 1L DPBS, 2mM Glutamine and 568 µM L-Cysteine). After 30 min pre-incubation to deplete the remaining intracellular pool of Methionine, the methionine analog L-azidohomocysteine (AHA) was added at 250 µM (Invitrogen, catalog #C10102) and the cells were incubated for 3 h in the presence or absence of 25 µg/mL cycloheximide. To label proteins translated in mitosis, cells were treated with 300 nM nocodazole or 12 µM proTAME at 5 h after release from thymidine block and allowed to enter mitosis. Mitotic cells were collected by mitotic shake off, washed once in warm DPBS with Mg²⁺/Ca²⁺ and switched to labeling medium. After 30 min pre-incubation, 250 µM AHA was added. Labeling was allowed to occur for 12 h in the presence or absence of 25 µg/mL cycloheximide. After labeling, the cells were collected by trypsinization (interphase cells) or mitotic shake-off (mitotic cells), washed twice with DPBS with Mg²⁺/Ca²⁺ and lysed in 50 µL lysis buffer (Tris-HCl 50 mM pH 8.0, SDS 1% supplemented with 250 U/mL Benzonase, VWR, catalog #80108-806 and EDTA-free protease inhibitors, Roche). After 15 min on ice, cells were vortexed and centrifuged at 15,000g at 4°C for 5 min. Supernatants were collected and protein concentrations were determined using the BCA assay (Pierce). Proteins (200 µg) were labeled with biotin-azide following the manufacturer's protocol (Invitrogen, catalog #B10184) and the protein reaction buffer kit (catalog #C10276). Labeled proteins were desalted with desalting columns (Thermo Scientific, catalog #89889) pre-washed with incubation buffer (NP-40 1%, SDS 0.1% in DPBS with Ca²⁺/Mg²⁺, with protease inhibitors). Ten percent of proteins were kept aside as total
protein control for western blots of specific proteins, and another 10% were conserved to run Streptavidin-HRP western blots to detect all labeled proteins. The remaining sample was incubated at room temperature with Neutraavidin agarose resin pre-washed with incubation buffer (Thermo scientific, catalog #29200) to purify biotin-labeled proteins. The resin was washed once with incubation buffer and three times with wash buffer (NP-40 1% in DPBS with Ca²⁺/Mg²⁺, with protease inhibitors). Purified proteins were boiled in SDS-PAGE loading buffer and tested by western blotting.

Statistical analysis

For each indicated figure and conditions, the data sample size (N), median and average values are reported. Statistical analysis was performed using the software Jmp 8.0 (SAS Institute Inc.). Samples were compared two by two using the Mann-Whitney-Wilcoxon non-parametric statistical test. The p values are reported. The samples were considered statistically significantly different when p was inferior to 0.05. Very small p values were reported as zero by Jmp.

Supplemental References


Figure S1: (A) TAME stabilizes cyclin B1-klotho (cyclB-luc) reporter in mitotic Xenopus extract. Different concentrations of TAME were added to the extract containing the reporter. Samples were collected at 60 min and the remaining reporter level was measured by luciferase activity. Interphase extract was used as a negative control. (B) Structures of TAME derivatives. (C) The derivatives shown in (A) were tested in the luciferase assay at 200 μM. (D) TAME does not inhibit degradation of pre-ubiquitinated cyclin B. Bacterial-expressed and purified [35S]-labeled cyclin B bound to untransfected Cdk1 was first ubiquitinated by APC in an in vitro ubiquitination system and then added into Xenopus extract supplemented with DMSO, 200 μM TAME or 200 μM proteasome inhibitor MG132. At indicated time points, protein was precipitated and the level of radioactivity in supernatant was measured by scintillation counting. (E) TAME does not affect the composition of APC core subunits. Mitotic extract was treated with DMSO or 200 μM TAME, APC was immunoprecipitated and the subunits were resolved by SDS-PAGE and visualized by coomassie stain. Identity of subunits was confirmed by mass spectrometry.
Figure S2: (A) The ability of TAME derivatives to compete with 125I-TAME for binding to the APC correlates with their ability to inhibit cyclin B1-MAP kinase degradation. Two hundred nM 125I-TAME was added into interphase extract with 10 μM of unlabeled TAME derivatized or labeled Cdc27 minus replication, and the amount of bound radioactivity was determined by scintillation counting. Specific binding was obtained by subtracting the value of mock IP with Cdc27 antibody. The relative levels of competition correlate with the trends seen in the cyclin B1-MAP kinase assay as shown in Figure S1C. (B) Coh1 C-terminal peptide crosslinks to the TPR subcomplex in an IR-dependent manner. An IR peptide coupled to a streptavidin matrix is a subset of APC subunits. Left: Coomassie-stained of APC homo-precipitated from interphase Xenopus extract. Right: APC subunits crosslinked by the labeled IR peptide. Identity of APC subunits was confirmed by mass spectrometry. (C) Crosslinking is IR-dependent. The crosslinking assay was performed in the presence of serum, unlabeled IR peptide with the N-terminal cysteine blocked with N-ethylmaleimide (NEM) (lane 2), no UV illumination (lane 3) or with labeled IR peptide (lane 4).
Figure S3: (A) ProTAME is converted to TAME in Xenopus extract. Fifty µM ProTAME was added to interphase Xenopus extract and samples were collected at indicated time points. Enzymatic reaction was performed, and samples were analyzed by liquid chromatography-mass spectrometry (LC/MS). Chromatograms of the abundance of TAME and proTAME are shown. (B) ProTAME is efficiently activated in HeLa cells but not MCF10A cells. HeLa and MCF10A cells were treated with 20 µM ProTAME. Cells were collected after 1 h and lysed. Enzymatic reaction was performed prior to LC/MS analysis. Quantification of the abundance of TAME was normalized to total protein levels is shown. (C) ProTAME does not induce premature accumulation of APO autophagosomes in C12 cells. HeLa cells were synchronized by double thymidine block, released into nocodazole for 12 h and washed out of nocodazole for 6 h. Cells were then treated with DMSO (0.05%), proTAME or proAAME (12 µM). Samples were collected at indicated time points and protein level was measured by immunoblot.
Figure S4: (A) ProTAME induces a mitotic exit delay in HETERT-RPE1 H2B-GFP cells. Asynchronous HETERT-RPE1 H2B-GFP cells were treated with 0.06% DMSO, 6 µM proTAME, or 12 µM proTAME. Cells were imaged at 12 min intervals. Cumulative frequency curves of mitotic duration and cell fate distribution are shown. (B) Cc20 siRNA induces dose-dependent knockdown of Cc20. HeLa H2B-GFP cells were plated in 24-well plates and transfected with indicated siRNAs. Control siRNA was added to final 18.5 nM siRNA concentration in wells with decreasing Cc20 siRNA concentration. Cells were lysed 48 h after transfection and lysates were subjected to western blotting to detect Cc20 and GAPDH. Protein level quantification was performed on a LiCor Odyssey scanner as described in Materials and Methods. (C) Twelve µM but not 3 µM proTAME induces a mild delay in chromosome congression. HeLa H2B-GFP cells were synchronized by double thymidine block and treated with 0.06% DMSO, 3 µM proTAME, 12 µM proTAME, 10 µM INS132, or 10 µM nocodazole at 8 h after release. Cells were imaged at 3 min intervals and 4x magnification. The time between prophase and full metaphase congression was analyzed. Cumulative frequency curves of the congression time were plotted.
Figure 6D: (A) Same experiment as shown in Figure 6A but with an expanded x-axis to better show the difference between short mitotic durations. (B) proTAME-induced mitotic arrest is Mad2-dependent. HeLa-H2B-GFP cells were transfected with indicated siRNAs between rounds of thymidine treatment. Following release, cells were treated with compounds and samples were collected at indicated time points. Protein levels were measured by immunoblot. (C) Mad2 knockdown efficiently overrides the spindle assembly checkpoint in the presence of nocodazole. Above: Control siRNA-treated cells were treated with 0.05% DMSO. Below: Mad2 knockdown cells were treated with 300 nM nocodazole. Live imaging was done at 40X magnification and 3 min intervals. Scale bar: 10 μm. (D) proTAME inhibits Os9/20 association with human APC if the SAC is activated. Double thymidine synchronized HeLa cells were transfected with Mad2 and BuFR1 siRNA, and infected with cycloheximide for 4 h. After release from thymidine, cells were treated with indicated drugs for 2 h. MB: Mad2/BuFR1 siRNA. Numbers indicate the relative Os9/20 band intensity normalized to β-actin. (E) Hesperadin overcomes a proTAME-induced mitotic arrest. HeLa cells were synchronized with double thymidine block and treated with indicated drugs at 10 h after release for 1 h. Mitotic cells were collected and APC was immunoprecipitated from cell lysates. Protein levels were measured by western blot. (F) Hesperadin induces dephosphorylation of the mitophase plate in proTAME-annealed cells. HeLa H2B-GFP cells were synchronized with double thymidine block and treated with 12 μM proTAME at 6 h after release and with 100 nM hesperadin at 10 h after release. For cells that have entered metaphase prior to hesperadin treatment, changes in the morphology of mitotophase plate were analyzed and one representative cell is shown. The yellow arrow denotes the time of hesperadin addition. A representative cell annealed with proTAME alone is also shown for comparison. Scale bar: 10 μm. (G) Hesperadin overcomes proTAME-induced mitotic arrest of cells released from nocodazole-induced arrest. HeLa-H2B-GFP cells were synchronized with double thymidine block and treated with 300 nM nocodazole at 8 h after release from thymidine block. Cells were washed out of nocodazole at 15 h after release from thymidine block into growth medium, or 12 μM proTAME, or 12 μM proTAME and 100 nM hesperadin. Cells were imaged at 12 h min interval. Cumulative frequency curves of mitotic duration and cell size distribution are shown. Cell samples were collected at different time points as indicated and protein levels were measured by immunoblot. The top diagram shows timing of treatments. N: nocodazole.
Figure S6: (A) Hesperadin overcomes MG132-induced mitotic arrest. HeLa KBG-GFP cells were synchronized with double thymidine block and treated with indicated drugs at 10 h after release. Mitotic duration of each individual cell is plotted against its mitotic entry time point. The red line denotes the time of addition of MG132. (B) Hesperadin reduces the amount of Mad2/BubR1 bound to the APC in cells arrested in mitosis with MG132. HeLa cells were synchronized with double thymidine block and treated with indicated drugs at 10 h after release for 2 h. Mitotic cells were collected and APC was immunoprecipitated from cell lysate. Protein levels were measured by immunoblot. (C) Hesperadin induces deformation of the metaphase plate in MG132-arrested cells. HeLa KBG-GFP cells were synchronized with double thymidine block and treated with 10 µM proTAME at 10 h after release, and with 100 nM hesperadin at 11 h after release. For cells that were arrested in metaphase prior to hesperadin treatment, changes in the morphology of metaphase plate after hesperadin treatment were analyzed and one representative cell is shown. The yellow arrow denotes the time of hesperadin addition. A representative cell arrested with MG132 alone is also shown for comparison. Scale bar: 6 µm.
Figure S7: Proteins required for maintenance of the SAC are synthesized during protracted mitotic arrest. 
HeLa-RED-GFP cells were arrested in interphase by a 24-hour thymidine block (3mM) and released in growth media. To identify newly translated proteins in interphase cells, cells were switched to methionine-free labeling medium 2 hrs after release and de novo translated proteins were labeled by adding the methionine analog L-[35S]-methionine (100uCi) for 3hrs and collected by hypotization. For blockade of proteins newly translated during mitosis, cells were released from thymidine block and nocodazole (500 nM) or nocodazole (500 nM) plus TAME (12uM) was added 4hrs after release. Seven hours later, mitotic cells were collected by aspirated and switched to the labeling medium and incubated with 100uCi AIA for 12 hours. A majority of cells remained in mitosis based on morphology. Following the labeling period, mitotic cells were collected by aspirate off. A labeling reaction including cycloheximide 25ug/ml, as negative control was carried in parallel for each condition. Cells in nocodazole + cycloheximide aged out of mitosis after the 12hrs incubation and were collected by hypotization. Protein lysates were generated for each labeling condition, and newly synthesized proteins were labeled using both azide and purified with nitrocellulose agarose resin as described in the Methods section. Purified proteins from equivalent amounts of total protein were eluted by boiling in SDS sample buffer, separated on SDS-PAGE gels and indicated proteins were detected by western blotting.