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Substrate Binding by the Anaphase-Promoting Complex

by

Mary E. Matyskiela

DISSERTATION

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By

Mary E. Matyskiela

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Substrate Binding by the Anaphase-Promoting Complex

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Mary E. Matyskiela

Abstract

The anaphase-promoting complex or cyclosome (APC/C) is a ubiquitin ligase essential for the completion of mitosis in all eukaryotic cells. Substrates are recruited to the APC/C by activator proteins (Cdc20 or Cdh1), but it is not known where substrates are bound during catalysis. We explored this problem by analyzing mutations in the tetratricopeptide repeat (TPR)-containing APC/C subunits. We identified residues in Cdc23 and Cdc27 that are required for APC/C binding to Cdc20 and Cdh1 and for APC/C function *in vivo*. Mutation of these sites increased the rate of activator dissociation from the APC/C but did not affect reaction processivity, suggesting that the mutations have little effect on substrate dissociation from the active site. Further studies revealed that activator dissociation from the APC/C is inhibited by substrate, and that substrates are not bound solely to activator during catalysis but interact bivalently with an additional binding site on the APC/C core.

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Chapter One

Introduction

Ubiquitin ligases in the cell

The function of a ubiquitin-protein ligase, as its name implies, is to catalyze the ligation of the small protein ubiquitin to other proteins. Ubiquitin attachment occurs primarily at lysine residues, and in many cases the reaction is repeated at several lysines on the same protein and on ubiquitin itself, resulting in decoration of the target protein with multiple ubiquitin polymers, which send the protein to the proteasome for destruction or alter other aspects of protein function. Protein ubiquitination is a versatile and effective mechanism for controlling protein behavior, and has been adapted for diverse purposes by many regulatory systems in the eukaryotic cell.

There is enormous complexity in the mechanisms that allow ubiquitin-protein ligases to recognize and repeatedly modify specific substrate proteins. We have been interested in understanding these mechanisms as they apply an unusually large and complex ubiquitin-protein ligase called the anaphase-promoting complex or cyclosome (APC), which promotes the proteasomal destruction of key mitotic regulators in all eukaryotic cells (Peters, 2006; Thornton and Toczyski, 2006). The APC, like other ubiquitin-protein ligases or E3s, promotes the transfer of ubiquitin to a target protein from another protein called a ubiquitin-conjugating enzyme or E2 (Figure 1a). The APC can therefore be viewed as a platform with specific binding sites for two substrates: the E2-ubiquitin conjugate and the target protein, which are positioned by the APC to allow the ϵ -amino

group of a target lysine to attack the thioester bond that links the C-terminus of ubiquitin to the active-site cysteine of the E2 (Figure 1b).

The APC must bind substrate proteins with high specificity to ensure that only the appropriate targets are destroyed. In addition, the APC and/or its substrates must be flexible enough to allow the transfer of ubiquitin to multiple lysines on the target protein and to specific lysines on ubiquitin itself. We are interested in the basis for this balance of specificity and flexibility in APC mechanism and have focused on two key issues: substrate binding and the catalysis of ubiquitin transfer.

Key components of the APC reaction

Protein ubiquitination by the APC requires the cooperation of four protein components: the APC core, activator subunit, E2, and substrate (Figure 2). Activators, E2s, and substrates all bind reversibly to the APC core with varying affinities, and interact with each other as well. To understand the contribution of each of these components to the ubiquitination reaction, it is helpful to first summarize their basic features.

APC core. The APC is a ~1 MDa, tightly-associated complex of 11-13 subunits that are generally well-conserved in eukaryotes (Table 1). The APC is a cullin-RING-type ubiquitin-protein ligase (Deshaies and Joazeiro, 2009), in which the

Apc2 and Apc11 subunits contain the cullin and RING domains, respectively. As in other cullin-RING ligases, the RING domain of Apc11 interacts directly with the E2, and the cullin domain of Apc2 binds Apc11 and likely provides an extended scaffold that connects these two subunits to the rest of the enzyme.

The analysis of APC purified from yeast strains lacking individual subunits has led to the identification of APC subcomplexes (Figure 3) (Thornton et al., 2006). One contains Apc2 and Apc11, as well as a third subunit, Doc1. Doc1 contains a -barrel structure known as a Doc domain, which in other proteins is involved in binding to small ligands, and this subunit may contribute to substrate binding, as discussed later. The other APC subcomplex contains three large subunits (Cdc27, Cdc16, and Cdc23 in yeast) that carry ten or more copies of a 34-residue sequence motif called a tetratricopeptide repeat (TPR). These subunits seem to associate sequentially with the APC, such that the association of Cdc27 depends on Cdc16, and the association of Cdc16 depends on Cdc23 (Thornton et al., 2006). Stoichiometry calculations suggest that the TPR subunits are present in two copies on the APC (Dube et al., 2005; Ohi et al., 2007). TPRs generally form protein-binding grooves, and thus the multiple TPR subunits are likely to provide a large array of interaction surfaces on the APC core.

The two APC subcomplexes are held together by the largest APC subunit, Apc1 (Figure 3). Apc4 and Apc5 help connect the base of the TPR subcomplex, Cdc23, to Apc1. The non-essential subunits Cdc26 and Swm1 (not shown in the

figure) help stabilize the association of the TPR subunits with the rest of the APC (Schwickart et al., 2004; Zachariae et al., 1998b). The functions of other APC subunits remain unclear.

Several electron microscopic (EM) analyses have provided a glimpse of the size and shape of the APC (Dube et al., 2005; Gieffers et al., 2001; Herzog et al., 2009; Ohi et al., 2007; Passmore et al., 2005). At a resolution of ~ 30 Å, the yeast APC seems to form a triangular particle, and the localization of individual subunits by antibody labeling is roughly consistent with the architecture determined from subcomplex studies (Herzog et al., 2009; Ohi et al., 2007). In the highest resolution EM structure, the TPR subunits are localized to an “arc lamp” structure, and Apc2 is found in an adjacent “platform” region where E2s are likely to bind (Herzog et al., 2009).

Activator. Despite its large size, the APC core has little activity in the absence of one of its activator proteins, Cdc20 and Cdh1 (a third activator, Ama1, is expressed solely in meiosis and will not be discussed here; (Oelschlaegel et al., 2005)). Cdc20 associates with the APC in early mitosis, leading to the destruction of targets that control the onset of anaphase. Cdc20 binding to the APC is promoted by phosphorylation of multiple APC subunits (Golan et al., 2002; Kraft et al., 2003; Rudner et al., 2000; Rudner and Murray, 2000; Shteinberg et al., 1999). Later in mitosis, Cdc20 is replaced by Cdh1, which maintains activity through the following G1. The association of Cdh1 with the APC depends on

Cdh1 dephosphorylation (Jaspersen et al., 1999; Kramer et al., 2000; Zachariae et al., 1998a).

Activator proteins participate in substrate recognition by the APC. The C-terminal regions of Cdc20 and Cdh1 contain a WD40 domain that is thought to form a propeller-like binding platform that binds APC substrates (Burton and Solomon, 2001; Hilioti et al., 2001; Kraft et al., 2005; Schwab et al., 2001). It is likely that sequence variations in the WD40 domains of Cdc20 and Cdh1 result in different substrate specificities: while Cdc20 targets a small number of substrates for destruction at metaphase, Cdh1 possesses a broader specificity, targeting these proteins and many more in late mitosis and G1 (Schwab et al., 1997; Visintin et al., 1997).

Activators also contain at least two sequence motifs, the IR and C-box, that are required for activator binding to the APC core. The IR motif consists of the two residues at the C-terminus of the activator, and the C-box is an 8-residue motif near the N-terminus (Passmore et al., 2003; Schwab et al., 2001; Vodermaier et al., 2003). Activator binding to the APC is at least partly mediated by the TPR subunits (Figure 3): Cdh1 binds directly to Cdc27 *in vitro* (Kraft et al., 2005; Vodermaier et al., 2003), and specific residues in the protein-interaction grooves formed by the TPRs in Cdc27 and Cdc23 are required for the binding of both Cdh1 and Cdc20 (Matyskiela and Morgan, 2009). The activator IR motif binds to the TPRs of Cdc27, and an additional unidentified activator region seems to bind

the TPRs of Cdc23 (Matyskiela and Morgan, 2009). The C-box binding site remains unknown, but one possibility is the Apc2 subunit, as removal of Apc2 from the APC reduces activator binding (Thornton et al., 2006). Together, these multiple interactions generate very high-affinity binding of activator to the APC core, and it is likely that the activator remains bound during multiple substrate-binding events (Matyskiela and Morgan, 2009). Recent EM analyses suggest that the activator is found between the TPR “arc lamp” and Apc2, in an ideal position to present substrates to attack incoming E2-ubiquitin conjugates (Herzog et al., 2009).

Substrates. While both the E2 and the target protein are chemically altered during ubiquitination, for clarity we will use the term ‘substrate’ to refer to the ubiquitinated target and not the E2. The two essential substrates of the APC are securin and the mitotic cyclins (Thornton and Toczyski, 2003). The degradation of securin triggers sister-chromatid separation, and the degradation of the mitotic cyclins is required for the completion of mitosis. The APC, particularly when bound to Cdh1, also ubiquitinates numerous other proteins involved in various aspects of mitotic exit (Sullivan and Morgan, 2007b).

Substrates bind specifically to the APC-activator complex through degradation sequences, the best understood of which are the D-box (RXXLXXXN) and KEN-box (KEN) (Glotzer et al., 1991; Pfleger and Kirschner, 2000). Although D- and KEN-box sequences are required for the ubiquitination of many substrates by the

APC, they are often not sufficient, suggesting that substrates contain additional unidentified degradation sequences (Glotzer et al., 1991; Pflieger and Kirschner, 2000). Numerous APC substrates contain non-canonical degradation sequences that lack any clear sequence similarities (Araki et al., 2003; Benanti et al., 2009; Castro et al., 2003; Enquist-Newman et al., 2008; Hildebrandt and Hoyt, 2001; Ko et al., 2007; Littlepage and Ruderman, 2002; Sullivan and Morgan, 2007a). It is likely that most, if not all, APC substrates contain multiple degradation sequences and might therefore be capable of multivalent interactions with the APC-activator complex.

Degradation sequences and ubiquitinated lysines are often found in substrate regions that are likely to be disordered. For example, the globular Cdk-binding domain of cyclins is generally preceded by a disordered ~100-residue N-terminal region that contains the critical D- and KEN-box sequences, along with numerous lysines. Securin is also likely to possess a disordered N-terminal APC-recognition region adjacent to a C-terminal functional domain. The separation of degradation and functional domains might prevent the degradation signal from interfering with the normal function of the protein, and thus might facilitate the evolution of regulatory degradation. As unfolded sequences at the N- or C-termini of proteins are required for their efficient unfolding and translocation into the proteasome pore for degradation (Prakash et al., 2009; Prakash et al., 2004), these unfolded regions may be a hallmark of all degradation targets.

E2. E2s share a conserved core domain of approximately 150 amino acids, including the central cysteine residue at which ubiquitin is attached; some E2s also contain N- or C-terminal extensions that lend specificity to their functions. E2s are charged with ubiquitin by the ubiquitin-activating enzyme E1 (Figure 1a). Since E2s use the same binding interface to interact with both the E1 and E3, E2s must dissociate from the E3 to be recharged with ubiquitin (Eletr et al., 2005). The rate of E2 turnover is very fast: during *in vitro* ubiquitination experiments, the APC can add as many as ten ubiquitins to a substrate within seconds (M.R.-B. and M.E.M., unpublished results).

Ubiquitin-protein ligases like the APC catalyze two distinct reactions: the ligation of ubiquitins to different substrate lysines (termed multiple monoubiquitination) and the transfer of ubiquitins to specific lysines on previously attached ubiquitins, leading to the formation of ubiquitin chains (termed polyubiquitination). Lysine specificity is determined primarily by the E2. In yeast, for example, Ubc4 promotes addition of ubiquitins to substrate lysines, while Ubc1 catalyzes ubiquitination of lysine 48 (K48) of a previously attached ubiquitin, leading to K48-linked chains that are recognized by the proteasome (Rodrigo-Brenni and Morgan, 2007). The different preferences of these E2s allow them to collaborate *in vivo*, such that Ubc4 attaches the initial ubiquitins to substrate lysines and Ubc1 extends these ubiquitins into K48-linked chains. In vertebrates, UbcH5, like

its yeast ortholog Ubc4, tends to generate non-specific linkages to substrate lysines (Jin et al., 2008; Kirkpatrick et al., 2006), while E2-25K, like yeast Ubc1, generates K48-linked chains (Chen and Pickart, 1990; Rodrigo-Brenni and Morgan, 2007). UbcH10 allows the APC to make K11-linked chains (Jin et al., 2008).

The APC reaction

I will now examine how the components of the APC reaction work together to catalyze the varied ubiquitination activities of the APC.

Substrate binding. Activators clearly contribute to the recognition of specific substrates for ubiquitination by the APC, but there is evidence that substrates also bind to the APC core: a peptide containing repeated D-boxes binds directly to the APC in the absence of activators (Yamano et al., 2004). The location of the D-box receptor is not known, but one possibility is the APC subunit Doc1. Doc1 enhances substrate binding to the APC-activator complex in native gel assays (Passmore et al., 2003), thereby increasing substrate residence time and the processivity of ubiquitination (Carroll and Morgan, 2002). The enhanced substrate affinity provided by Doc1 is dependent on the substrate D-box (Carroll et al., 2005).

Notably, Doc1 does not promote substrate binding by stimulating binding of the activator subunit. The concentration of activator required for half-maximal APC stimulation is unaffected by the removal of Doc1 (Carroll and Morgan, 2002). Additionally, steady-state activator binding and the rate of activator dissociation from the APC are unaffected by mutations in Doc1 that are known to reduce substrate binding (Matyskiela and Morgan, 2009). Thus, Doc1 seems to promote activator-independent substrate binding to the core APC.

Several models of substrate recognition by the APC have been proposed to take into account both activator and APC core binding sites (Yu, 2007). One possibility is a multivalent mechanism, where substrates are shared between substrate receptors on both the activator and APC. Multivalent substrate binding is supported by recent evidence that substrate enhances the affinity of activator for the APC, through a mechanism that depends on Doc1 (Burton et al., 2005; Matyskiela and Morgan, 2009). The simplest explanation for these results is that a multivalent substrate binds simultaneously to both activator and APC, although it is not possible to rule out more complex mechanisms such as substrate-induced conformational changes in the activator. A multivalent substrate-binding model is attractive for several reasons. Multiple low-affinity interactions would explain why individual interactions between substrates and the APC have been so difficult to detect, and allow high-affinity substrate binding to the APC-activator complex despite low-affinity individual interactions. Multivalency might also allow

greater specificity in substrate recognition by requiring two or more separate binding interactions for efficient substrate recognition.

Catalysis. After substrate binding, ubiquitin transfer requires the nucleophilic attack of the E2-ubiquitin thioester bond by the ϵ -amino group of a substrate lysine, leading to the formation of an isopeptide bond between the C-terminus of ubiquitin and the substrate lysine by way of an oxyanion intermediate (Figure 1b). The formation of this isopeptide bond likely requires the deprotonation of the attacking amide group, as well as stabilization of the negatively-charged oxyanion intermediate (Passmore and Barford, 2004; Pickart and Eddins, 2004; Wu et al., 2003).

The catalytic residues that promote ubiquitin transfer reside in the active site of the E2 itself. Biochemical studies of Ubc9, which conjugates the ubiquitin-like molecule SUMO, have identified residues conserved in most E2s that enhance deprotonation of the substrate lysine – not by abstracting a proton from the lysine but by creating a local microenvironment of reduced lysine pK (Yunus and Lima, 2006). Additionally, a conserved asparagine in the E2 may provide a positive charge that stabilizes the oxyanion intermediate (Wu et al., 2003). While this residue is positioned far from the E2 active site in structures of unconjugated E2s, it rotates towards the active-site cysteine upon ubiquitin or SUMO conjugation, or E3 binding (Reverter and Lima, 2005). It therefore appears that

the ubiquitin-protein ligase does not catalyze the chemical step directly, but instead facilitates ubiquitination by positioning the substrate and E2 in a productive orientation.

Because the catalytic residues for ubiquitin transfer are located within the E2, high concentrations of substrate lysines drive a low rate of ubiquitin transfer from an E2-ubiquitin conjugate, even in the absence of an E3 (Petroski and Deshaies, 2005; Saha and Deshaies, 2008), (M.R.-B. and D.O.M., unpublished results). This activity can be detected with a diubiquitin synthesis assay, which measures transfer of ubiquitin from the E2 to monomeric ubiquitin in solution. At least in some cases (including the E3s SCF and APC), the rate of ubiquitin transfer by E2s is stimulated by interaction with a RING E3 (Gmachl et al., 2000; Ozkan et al., 2005; Petroski and Deshaies, 2005). Because the ubiquitin substrate in these experiments is free in solution and is therefore not positioned by the E3, this stimulation may be due to an activating conformational change induced in the E2 by the RING domain.

Activator proteins may enhance the rate of ubiquitin transfer by the APC through a mechanism that is distinct from their role in substrate binding. Studies of the unusual APC substrate Nek2A suggest that activators induce an activating conformational change in the APC. Nek2A contains a C-terminal dipeptide motif (MR) that is similar to the IR motif at the C-terminus of activators, and it uses this

motif to bind the APC in the absence of activator. However, bound Nek2A is not ubiquitinated unless activator is also added (Hayes et al., 2006). Ubiquitination of Nek2A is even stimulated by an N-terminal fragment of Cdc20 that lacks the substrate-binding WD40 domain (Kimata et al., 2008). The N-terminal activator region, which includes the C-box, might therefore promote an activating change in APC structure that is independent of the activator's substrate-recruiting function.

Insights into the role of activator-induced conformational changes in the APC might be found in recent studies of the ubiquitin-protein ligase, SCF. Modification of the cullin subunit of SCF with the ubiquitin-like molecule Nedd8 enhances catalytic rate (Saha and Deshaies, 2008), probably as a result of a conformational change that reduces the distance between the RING domain (where the E2 binds) and the substrate-binding site (Duda et al., 2008). While there is no evidence that the APC is neddylated, it is possible that activator binding to the APC results in a similar conformational change in the cullin subunit Apc2, thereby enhancing the rate of ubiquitination.

Ubiquitination by the APC is processive; that is, multiple ubiquitins are added during a single substrate-binding event. Processivity is readily observed in ubiquitination assays with the E2 Ubc4, in which multiubiquitinated substrates appear in the first seconds of a ubiquitination reaction despite the presence of a large excess of unmodified substrate. In these Ubc4-dependent reactions, the

APC does not display higher activity with pre-ubiquitinated substrates, and thus would not preferentially re-bind them. Thus, substrates must remain bound to the APC for a sufficient time to allow multiple E2s to bind, transfer ubiquitin, and dissociate. The residence time of a substrate on the APC is determined by its dissociation rate. This rate varies among different substrates, so that different substrates acquire a different number of ubiquitins in a single binding event. Substrate dissociation rate, and thus the degree of processivity, is influenced by the activator subunit: for example, Cdc20 may have a higher affinity for securin than Cdh1, as this substrate is ubiquitinated with higher processivity by APC-Cdc20 than it is by APC-Cdh1 (Matyskiela and Morgan, 2009).

An increase in the number of ubiquitins added to a substrate may increase the likelihood of substrate recognition by the proteasome in the face of competing deubiquitinating activities in the cell. Thus, substrates that are ubiquitinated more processively by the APC may have an enhanced degradation rate in vivo (Rape et al., 2006). However, this variable degradation efficiency could be achieved by varying substrate affinities in both processive and distributive ubiquitination reactions, and so it is not clear what advantage a processive ubiquitination mechanism might confer.

The lysine to which ubiquitin is attached determines the efficiency of substrate recognition by the proteasome. Polyubiquitin chains linked by K48 of ubiquitin are

generally considered to be the ideal proteasome targeting signal (Pickart and Fushman, 2004). However, multiple monoubiquitinations and small nonspecific chains might also be sufficient for proteasomal targeting in some cases (Kirkpatrick et al., 2006; Rodrigo-Brenni and Morgan, 2007), and recent evidence suggests that linkages at K11 or indeed any lysine other than K63 can be recognized by the proteasome (Jin et al., 2008; Xu et al., 2009). In yeast, the APC (with the E2 Ubc1) generates polyubiquitin chains linked by K48; in human cells, the APC (with UbcH10) produces K11-linked chains (Jin et al., 2008).

As mentioned earlier, E2s have different lysine preferences. In yeast, for example, Ubc4 is specialized for promoting ubiquitin transfer to lysines on a large number of substrates, while Ubc1 promotes the formation of K48-linked polyubiquitin. The molecular basis of this lysine specificity is not yet understood, but presumably depends on residues surrounding the active-site cysteine of the E2, which are expected to position the attacking lysine (Eddins et al., 2006; Petroski and Deshaies, 2005). Although Ubc1 has a C-terminal extension containing a ubiquitin-binding domain, this domain is not required for the K48 specificity of the Ubc1 catalytic domain (Rodrigo-Brenni and Morgan, 2007); instead, this specificity is provided by specific residues near the active-site cysteine in the catalytic domain (M.R.-B. and D.O.M., unpublished data).

The ability to modify many different lysines and accommodate growing ubiquitin chains implies some degree of flexibility in the APC active site. As discussed earlier, many APC substrates are predicted to contain unstructured N-terminal sequences, which would allow variable positioning of attacking lysines and might also allow a growing substrate to loop out of the active site. Multivalent substrate binding might allow the APC to better position these unstructured regions for attack. Furthermore, the APC core may also provide a source of flexibility. EM structural analysis suggests that the angle between the “arc lamp” and the “platform” might vary to accommodate substrates of various sizes or ubiquitin chain length (Herzog et al., 2009). Additionally, neddylation of some cullin-RING ligases results in more flexible tethering of the RING domain to the associated cullin subunit, providing flexibility in the positioning of the RING-E2 complex (Duda et al., 2008). Perhaps similar conformational changes occur in the APC upon activator binding, as mentioned earlier.

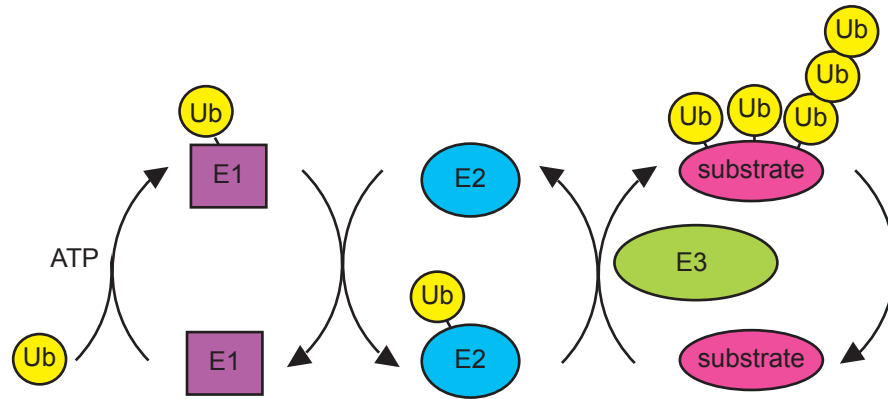
Figure 1. Ubiquitination occurs by a three-enzyme cascade

(a) Ubiquitin (Ub, yellow) is first activated by an E1, or ubiquitin-activating protein (purple), which couples ATP hydrolysis with the formation of a thioester bond between the active-site cysteine of the E1 and the carboxyl-terminus of ubiquitin. The E1 then transfers the activated ubiquitin to the active-site cysteine of an E2, or ubiquitin-conjugating enzyme (blue). Finally, the E3, or ubiquitin-protein ligase (green), facilitates the transfer of the ubiquitin from the E2 to a lysine on the target protein (substrate, pink). In RING-type E3s like the APC, this transfer is direct: ubiquitin is not conjugated to the E3, but is transferred directly from E2 to substrate.

(b) The final step of ubiquitin transfer from E2 to substrate occurs when the ϵ -amino group of a lysine on the substrate nucleophilically attacks the E2-ubiquitin thioester bond. RING-type E3s like the APC facilitate this reaction by positioning the E2 and substrate for efficient attack.

Figure 1

(a)



(b)

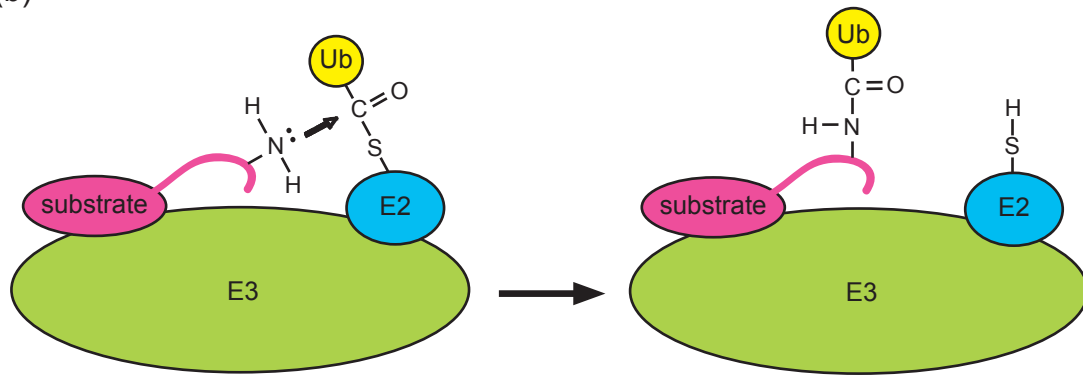


Figure 2. The four major protein components in an APC reaction

Catalysis depends on cooperative interactions among the APC core, activator, substrate, and E2.

Figure 2

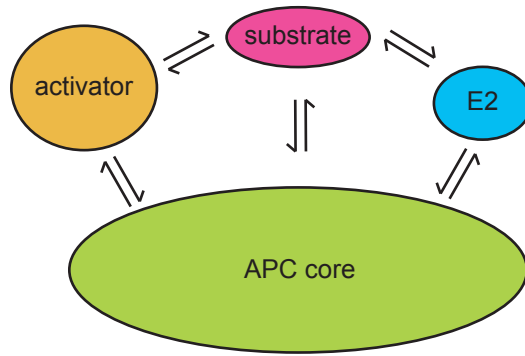


Figure 3. The APC core contains multiple subcomplexes

The budding yeast APC core is a ~1 MDa complex of 13 subunits (Table 1), including the nine key subunits shown here. One subcomplex (shaded in dark green) contains the cullin subunit Apc2 and the RING domain protein Apc11, which recruits E2s. Another subcomplex (shaded in light green) contains the three TPR-containing subunits, Cdc27, Cdc23, and Cdc16, as well as two subunits, Apc4 and Apc5, that help connect them to the rest of the APC via Apc1. The TPR-containing subunits provide binding sites for the activator, which contains at least two APC-interaction motifs, the IR and C-box, as well as a large WD40 repeat sequence that is likely to form a propeller-like binding site for substrate.

Figure 3

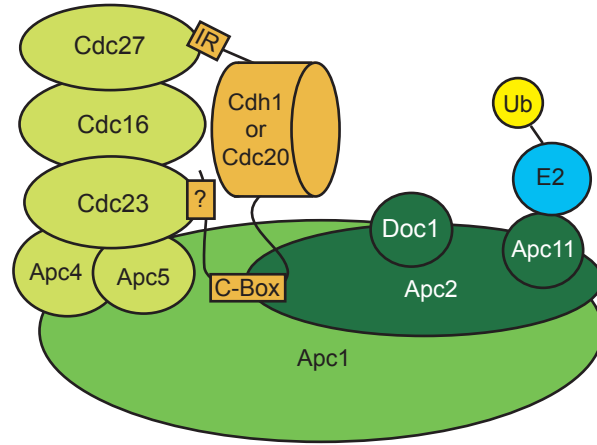


Table 1. APC subunits in different organisms

Dashes indicate that no homologous subunit has been identified in that species.

Most core subunits are not yet identified in *Drosophila melanogaster*. Motifs in APC subunits include tetratricopeptide repeats (TPRs) and WD40 repeats, both of which form domains that are typically involved in protein-protein interactions.

Modified from reference (Morgan, 2007).

Table 1

<i>S. cerevisiae</i>	<i>S. pombe</i>	Mammals	<i>D. melanogaster</i>	Comments
Core subunits:				
Apc1	Cut4	Apc1/Tsg24		
Apc2	Apc2	Apc2		Cullin domain
Cdc27	Nuc2	Cdc27		TPRs
Apc4	Lid1	Apc4		
Apc5	Apc5	Apc5		
Cdc16	Cut9	Apc6		TPRs
-	-	Apc7		TPRs
Cdc23	Cut23	Cdc23		TPRs
Apc9	-	-		
Doc1	Apc10	Apc10		Doc domain
Apc11	Apc11	Apc11		RING finger
Cdc26	Hcn1	Cdc26		
Swm1	Apc13	-		
Mnd2	Apc15	-		
-	Apc14	-		
Activators:				
Cdc20	Slp1	Cdc20/p55 ^{CDC}	Fizzy/FZY	WD40 repeats
Cdh1/Hct1	Srw1/Ste9	Cdh1	Fizzy-related/FZR	WD40 repeats
Ama1	Mfr1			Meiosis-specific

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Chapter Two

**Analysis of activator-binding sites on the APC/C
supports a cooperative substrate-binding
mechanism**

**Analysis of activator-binding sites on the APC/C supports a cooperative
substrate-binding mechanism**

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Summary

The anaphase-promoting complex or cyclosome (APC/C) is a ubiquitin ligase essential for the completion of mitosis in all eukaryotic cells. Substrates are recruited to the APC/C by activator proteins (Cdc20 or Cdh1), but it is not known where substrates are bound during catalysis. We explored this problem by analyzing mutations in the tetratricopeptide repeat (TPR)-containing APC/C subunits. We identified residues in Cdc23 and Cdc27 that are required for APC/C binding to Cdc20 and Cdh1 and for APC/C function *in vivo*. Mutation of these sites increased the rate of activator dissociation from the APC/C but did not affect reaction processivity, suggesting that the mutations have little effect on substrate dissociation from the active site. Further studies revealed that activator dissociation from the APC/C is inhibited by substrate, and that substrates are not bound solely to activator during catalysis but interact bivalently with an additional binding site on the APC/C core.

Introduction

Protein ubiquitination is a critical regulatory modification in the control of numerous cellular processes. The final step in ubiquitination is catalyzed by a ubiquitin ligase or E3, which facilitates the transfer of an activated ubiquitin from a ubiquitin-conjugating enzyme (E2) to a lysine on a specific substrate or on ubiquitin itself, thereby forming ubiquitin chains that target the substrate for degradation by the proteasome. The anaphase-promoting complex/cyclosome (APC/C) is a multisubunit E3 required for the initiation of anaphase and the completion of mitosis in all eukaryotic cells (Peters, 2006; Sullivan and Morgan, 2007b; Thornton and Toczyski, 2006). While the most important substrates of the APC/C are securin and the mitotic cyclins (Thornton and Toczyski, 2003), the APC/C ubiquitinates numerous additional substrates involved in late mitotic events. Most of these substrates contain short amino acid sequences, called destruction (D-) boxes and KEN-boxes, that target them to the APC/C.

Ubiquitination by the APC/C is processive: that is, multiple ubiquitins are added in a single substrate-binding event (Carroll and Morgan, 2002). The processivity of ubiquitination is determined by two factors: how long the substrate remains bound to the APC/C active site, and the catalytic rate of the enzyme during that time. Thus, any factor that increases the substrate dissociation rate will cause a decrease in processivity.

APC/C activation requires association with one of two related activator proteins, Cdc20 or Cdh1. Cdc20 activates the APC/C during the metaphase-to-anaphase transition, after which Cdh1 activates the APC/C during mitotic exit and the following G1. Activators are thought to recruit substrates to the APC/C. Both Cdh1 and Cdc20 contain a C-terminal WD-40 domain that binds substrates (Burton and Solomon, 2001; Hilioti et al., 2001; Kraft et al., 2005; Schwab et al., 2001). Both activators also contain at least two conserved sequences important for binding to the APC/C core: an 8-residue N-terminal motif called the C-box, and an IR motif at the C-terminus (Passmore et al., 2003; Schwab et al., 2001; Vodermaier et al., 2003).

In addition to recruiting substrates, activator proteins may promote changes in APC/C conformation that increase activity toward those substrates. Evidence for this possibility comes primarily from studies of the unusual APC/C substrate Nek2A, which can bind the APC/C in the absence of activator but is not ubiquitinated unless activator is also added (Hayes et al., 2006). Recent studies reveal that ubiquitination of Nek2A can be stimulated by an N-terminal fragment of Cdc20 containing the C-box motif but lacking the WD-40 domain (Kimata et al., 2008). The C-box region might therefore promote an activating change in APC/C conformation that is independent of the activator's substrate-recruiting function. Consistent with this possibility, electron microscopic (EM) analyses suggest that the APC/C adopts a different conformation in the presence of Cdh1 (Dube et al., 2005).

Two lines of evidence suggest that substrate binding to the APC/C is not mediated entirely by the activator protein. First, there is evidence for a direct, low-affinity interaction between substrate and the APC/C in the absence of activator (Eytan et al., 2006; Yamano et al., 2004). Second, the APC/C subunit Doc1 promotes substrate binding through a mechanism that appears to be independent of activator binding (Passmore et al., 2003). Removal of Doc1 increases the rate of substrate dissociation from the active site, resulting in a decrease in reaction processivity (Carroll et al., 2005; Carroll and Morgan, 2002). Substrate binding to the APC/C might therefore involve contributions from a binding site on the activator and a site on the APC/C core, but the importance of each site during ubiquitination is not known. Since substrate binds the APC/C-activator complex with higher affinity than it binds the APC/C core alone (Eytan et al., 2006; Passmore and Barford, 2005), activator-APC/C binding might trigger a conformational change that enhances substrate-binding affinity, or substrates might be bivalent and interact simultaneously with sites on the activator and APC/C core (Eytan et al., 2006; Passmore and Barford, 2005; Yu, 2007).

The one-megadalton, 13-subunit APC/C is a complex protein machine whose parts are not well understood. A recent analysis of the budding yeast APC/C identified two subcomplexes (Thornton et al., 2006). One subcomplex contains Doc1 and the essential catalytic subunits Apc2 and Apc11, which binds the E2. The other subcomplex contains three large subunits, Cdc16, Cdc23, and

Cdc27, each of which contain multiple tetratricopeptide repeats (TPRs). These three subunits associate with the APC/C in series: Cdc23 is the innermost TPR subunit and anchors Cdc16, which anchors Cdc27. A similar arrangement of subunits is supported by EM analysis of APC/C structure (Ohi et al., 2007).

Previous work suggests that the TPR subunit Cdc27 contributes to activator binding to the APC/C: peptides containing the IR motif of activators bind to Cdc27 (Vodermaier et al., 2003), activator can be crosslinked to Cdc27 (Kraft et al., 2005), and removing Cdc27 from the APC/C reduces activator binding affinity (Thornton et al., 2006). However, the APC/C can still be stimulated by activator in the absence of Cdc27, indicating that additional activator-binding sites exist on the APC/C (Thornton et al., 2006).

TPR motifs are thought to mediate protein-protein interactions. A single repeat consists of 34 residues folded into two anti-parallel α -helices, and multiple repeats can pack together to form a protein-binding groove (D'Andrea and Regan, 2003). Each of the TPR subunits of the APC/C has at least nine repeats (an unusually high number (D'Andrea and Regan, 2003)), and stoichiometry calculations suggest that each TPR subunit is present in 2-3 copies on each APC/C molecule (Dube et al., 2005; Ohi et al., 2007; Passmore et al., 2005). Given that the TPR subunits associate with each other, it appears that the APC/C contains a very large array of TPRs on its surface.

To explore the molecular basis of activator and substrate binding by the APC/C, we constructed and analyzed a series of APC/C mutants with single point mutations in the TPR grooves of yeast Cdc16, Cdc23, or Cdc27. We identified residues in Cdc27 and Cdc23 that are required for activator binding. Detailed biochemical characterization of these TPR mutants, together with studies of the effects of substrate on activator binding, revealed that substrates are not bound solely to activators during catalysis but are shared with a second binding site on the APC/C core.

Results

Identification of residues in the TPR grooves that are important for activator binding

We used a site-directed mutagenesis approach to determine whether the TPR repeats of Cdc16, Cdc23, or Cdc27 mediate the binding of activators or substrates to the APC/C. TPRs have a well-defined structure due to their alpha-helical content, and the residues that lie on the surface of the protein interaction groove can be predicted accurately (Cortajarena et al., 2004; Magliery and Regan, 2005). We focused our analysis on the side chains that were predicted to line the TPR grooves and that were conserved between yeast and humans, as those seemed most likely to mediate binding to other conserved domains.

The three TPR subunits of the budding yeast APC/C contain at least 9 or 10 TPRs each (Figure 1A). The consensus TPR sequence defines the interactions between the two helices A and B in the repeat (Main et al., 2003). Consensus residues create an alternating sequence of large hydrophobic and small residues that interlock to form a helix-turn-helix motif (Figure 1B, C; green residues). Adjacent repeats pack in parallel but at a slightly offset angle, leading to a spiral of anti-parallel helices that form a groove. This groove is lined by residues that are not part of the TPR consensus sequence but instead interact with peptide ligands that bind in the groove. In co-crystal structures of peptides bound to TPR grooves, the residues that most often line the surface of TPR grooves are at positions 2, 6, 9, and 13 of the repeats (Figure 1B, C; pink residues) (Cortajarena et al., 2004; Gatto et al., 2000; Magliery and Regan, 2005; Scheufler et al., 2000). We selected well-conserved residues at these positions in Cdc16, Cdc23, and Cdc27 for alanine scanning by site-directed mutagenesis. We also considered residues at these positions that were conserved between the three yeast proteins. In total, we mutated 10, 21, and 11 residues in Cdc27, Cdc16, and Cdc23, respectively.

Since mutations that reduce the function of TPR subunits might be lethal, we integrated each mutant gene under the control of its own promoter into a strain of *Saccharomyces cerevisiae* in which the APC/C is not essential and the chromosomal TPR subunit gene is deleted (Thornton and Toczyski, 2003). Using a TAP tag on the Apc1 subunit, we immunopurified each mutant APC/C and

divided the preparation for analysis by two different assays: an activity assay to identify enzymatic defects and a Cdh1-binding assay to determine whether any activity defect was accompanied by reduced activator binding. In addition, the APC/C preparation was analyzed by Coomassie Blue staining to assess subunit stoichiometry and the amount of enzyme purified.

In multiple experiments, three mutations in Cdc27 generated reproducible activity defects (Figure 1D): N548A in TPR 5, Y575A in TPR 6, and L579A in TPR 6. N548A (referred to hereafter as Cdc27-A1) and L579A (referred to hereafter as Cdc27-A2) had the most significant defects, and a double mutation of these two residues (Cdc27-A1A2) had greatly reduced activity, although not as low as that of Cdc27 APC/C. Two mutations in Cdc23 generated reproducible activity defects (Figure 1D): N405A in TPR 5 and E442A in TPR 6. N405A (referred to hereafter as Cdc23-A) had a more significant defect and was the only Cdc23 mutation pursued further. Interestingly, Cdc23-A is mutated in the same position in TPR 5 as Cdc27-A1.

None of the mutations in Cdc27 or Cdc23 disrupted APC/C subunit composition or the levels of the mutant subunits (Figure 1D; bottom panels). Thus, the mutated residues are not required for interaction of TPR subunits with each other or with other subunits of the APC/C.

Importantly, the Cdc27 and Cdc23 mutants with major activity defects all displayed reduced binding to Cdh1 (Figure 1D; middle panels). Thus, the activity defects of the mutant enzymes were accompanied by a reduced affinity for activator.

We tested 20 single mutations in Cdc16. None produced a significant defect in activity, APC/C integrity, or activator binding (Figure 2). In general, the residues facing into the groove at positions 2, 6, 9, and 13 of the repeats are not as well conserved in Cdc16 as they are in Cdc23 and Cdc27, and so it is possible that Cdc16 function depends on other residues in the TPRs.

We also examined the processivity of ubiquitination. As in our previous work (Carroll and Morgan, 2005; Rodrigo-Brenni and Morgan, 2007), we calculated processivity by determining the ratio of the amount of ubiquitin added to the amount of substrate modified. Interestingly, none of the TPR-mutant APC/Cs displayed a significant defect in the processivity of cyclin B ubiquitination (generally about 1.6 ubiquitins/cyclin B). For comparison, Figure 1D (right panel) illustrates the major processivity defect (1.1 ubiquitins/cyclin B) that is observed when substrate affinity is reduced by a mutation in the Doc1 subunit (the Doc1-4A mutant (Carroll et al., 2005)). These results suggest that none of the TPR mutations have major effects on substrate binding during ubiquitination, despite their Cdh1-binding defects.

These experiments, and many of those that follow, were performed with an ^{125}I -labeled sea urchin cyclin B fragment as substrate, because this substrate allows the most accurate measurement of processivity. We obtained similar results in experiments where the substrate was ^{35}S -labeled yeast securin (Pds1) (see Figure 4 below).

A defect in activator binding to the APC/C should result in a shift in the amount of Cdh1 required for half-maximal APC/C stimulation. We therefore purified each mutant APC/C and examined its ability to respond to increasing amounts of Cdh1 (Figure 3). The concentration of Cdh1 required for half-maximal stimulation of wild-type APC/C was about 50 nM, as seen in previous studies (Carroll and Morgan, 2002; Jaspersen et al., 1999). Consistent with their Cdh1-binding defects, the Cdc27-A1, Cdc27-A2, Cdc27-A1A2, and Cdc23-A mutations all caused at least a five-fold increase in half-maximal Cdh1 concentration. Accurate half-maximal values could not be calculated because we could not achieve saturating concentrations of Cdh1 (Figure 3).

We also quantitated the processivity of ubiquitination at each concentration of Cdh1. Wild-type and mutant APC/Cs all had a processivity of about 1.6 ubiquitins/cyclin B (Figure 3), demonstrating again that the defect in activator binding did not result in an apparent defect in substrate residence time at the active site.

Mutations in Cdc27 and Cdc23 affect both Cdc20- and Cdh1-dependent activity

It is not known whether Cdc20 and Cdh1 bind at the same sites on the APC/C. We found that purified Cdc27-A1, Cdc27-A2 and Cdc23-A APC/Cs all displayed significant defects in Cdc20-dependent activity as well as Cdh1-dependent activity in vitro, using ³⁵S-securin as substrate (Figure 4). These results argue that Cdc20 and Cdh1 interact with the same sites on the TPRs of Cdc27 and Cdc23. We have been unable to develop a Cdc20 binding assay in vitro and so we cannot directly test the effects of these mutations on Cdc20 binding.

Ubiquitination by APC/C^{Cdc20} was generally more processive than ubiquitination by APC/C^{Cdh1} in these assays, and we have observed similar differences in reactions with other substrates (data not shown). Notably, however, longer exposures of these autoradiographs indicate that the Cdc27-A1, Cdc27-A2 and Cdc23-A mutations do not decrease the processivity of securin ubiquitination with Cdc20 or Cdh1 (data not shown).

Mutations in Cdc27 or Cdc23 cause a mild APC/C defect in vivo

To characterize the effects of Cdc27 and Cdc23 mutations in the cell, we used two-step gene replacement to exchange the wild-type genes with mutant alleles at their endogenous loci. None of the mutations had an effect on growth rates in

liquid culture at 30°C or 37°C (Figure 5A). Mutant strains were also indistinguishable from wild type when grown on plates (Figure 6).

We also analyzed the *cdc27-A2* and *cdc23-A* strains through a single cell cycle after release from a G1 arrest (Figure 5B). *cdc27-A2* cells displayed only minor defects in mitotic progression, while *cdc23-A* cells exhibited a significant delay: after 195 minutes, 30% of the *cdc23-A* cells had not divided. Both *cdc27-A2* and *cdc23-A* mutants exhibited a decreased ability to degrade the APC/C substrate Clb2, with the defect being more dramatic in *cdc23-A* cells. While delayed mitotic exit in *cdc23-A* cells seems inconsistent with the absence of a growth defect in an asynchronous population, we have seen previously that yeast with mild APC/C mutations show more significant cell-cycle defects following an alpha-factor release (Carroll et al., 2005). These cells are clearly able to degrade enough securin to separate their sister chromatids, and we presume that excess Clb-Cdk1 activity is inhibited by the Cdk1 inhibitor Sic1. These phenotypes are therefore characteristic of cells with moderate defects in APC/C activity.

We also found that the *cdc27-A2* and *cdc23-A* strains are resistant to the growth defects that result from overexpression of *CDH1* or *CDC20* (Figure 6). Thus, mutations in the TPR grooves of Cdc27 or Cdc23 reduce the ability of the APC/C to bind Cdc20 and Cdh1 both in vitro and in vivo, further demonstrating that both activators bind to these sites on the APC/C.

***cdc27-A2 cdc23-A* double mutant cells arrest in metaphase with high levels of mitotic cyclins**

To analyze the combined effects of mutations in Cdc27 and Cdc23, we mated a *cdc27-A2 MAT#* strain to a *cdc23-A MATa* strain. Tetrad dissection of spores from the resulting diploid strain indicated that the *cdc27-A2* and *cdc23-A* mutations are synthetically lethal. As Cdh1 is not essential for viability, this lethality is likely to be due to defective Cdc20 binding. This result is consistent with our data showing that the mutations in Cdc23 and Cdc27 affect Cdc20-dependent APC/C activity in vitro (Figure 4).

To determine the terminal phenotype of the double *cdc27-A2 cdc23-A* mutant, we used the conditional expression of a wild-type copy of *CDC23* to allow integration of both the *cdc27-A2* and *cdc23-A* mutations into the same strain. We integrated wild-type *CDC23*, *cdc23-A*, or an empty vector at the *ADE2* locus of a strain where the chromosomal copy of *CDC23* had been placed under the control of the *P_{GAL1}* promoter and *cdc27-A2* had replaced *CDC27* at the endogenous locus. These strains were arrested in alpha factor in the presence of galactose. Glucose was added to suppress transcription of the chromosomal *CDC23* for two hours before releasing from the arrest into media containing glucose.

Cells carrying an integrated copy of *CDC23* displayed minimal defects like those seen in the *cdc27-A2* strain (Figure 5B, C). However, cells in which the

cdc23-A gene was integrated (resulting in the *cdc27-A2 cdc23-A* double mutant) arrested with large buds, single DNA masses, and short metaphase spindles. The same phenotype was observed in cells carrying an empty vector (resulting in a *cdc27-A2 cdc23Δ* double mutant). Both the *cdc27-A2 cdc23-A* and *cdc27-A2 cdc23Δ* strains accumulated large amounts of the APC/C substrate Clb2. Thus, the *cdc27-A2 cdc23-A* double mutant phenotype is the same as an APC/C-null phenotype, demonstrating that without at least one of the two TPR subunits to bind activator, the APC/C cannot ubiquitinate substrates efficiently enough to allow progression into anaphase.

The IR motif of Cdh1 interacts with the TPR groove of Cdc27, but the C-box binds elsewhere

To determine whether the well-characterized APC/C-interaction motifs on Cdh1 interact with the TPR grooves of Cdc27 or Cdc23, we tested the ability of Cdh1 lacking either its C-box or IR motifs to activate wild-type and TPR-mutant APC/C. Mutation of either the C-box or IR motifs in Cdh1 causes a defect in APC/C activity. If an activator motif interacts with the TPR residues that are changed in our mutants, then we would expect to see no further activity defect when activator and TPR mutations are paired together.

We mutated the C-terminal IR residues of Cdh1 to AA (IR), and used a previously characterized C-box mutant (I58A P59A, referred to here as the C-box mutant) (Thornton et al., 2006). The IR and C-box mutations each caused

a large reduction in wild-type or Cdc27-A2 APC/C activity (Figure 7A; normalized in Figure 7B for the amount of enzyme added and background activity).

Importantly, Cdh1- IR stimulated wild-type and Cdc27-A2 APC/Cs to a similar extent. Thus, in the absence of the IR motif on Cdh1, the Cdc27-A2 mutation had no further defect, implying an interaction between the two. However, since mutation of the IR motif on Cdh1 still reduced activity of Cdc27-A2 APC/C, there must be other residues on the APC/C that are important for IR binding.

In contrast, the Cdh1- C-box stimulated wild-type APC/C but did not generate detectable activity with Cdc27-A2 APC/C. The C-box is therefore essential in the Cdc27-A2 mutant, suggesting that the C-box binds elsewhere to help generate the remaining APC/C activity in this mutant.

In the Cdc27-A1A2 mutant, all IR-dependent activity was eliminated (Figure 7C, D). It is therefore unlikely that there are other significant IR-binding sites on the APC/C. These results are consistent with previously published experiments demonstrating that the IR interacts with Cdc27 but the C-box does not (Thornton et al., 2006). Additionally, the lack of a growth defect in the Cdc27-A1A2 strain despite the loss of all IR-dependent activity (Figure 5A) is consistent with previous evidence that mutation of the IR motif in Cdc20 has little apparent effect in vivo (Thornton et al., 2006).

Mutation of either the IR motif or C-box greatly reduced the activity of the Cdc23-A mutant (Figure 7E, F), suggesting that these motifs do not interact with the residue mutated in Cdc23-A. We presume that the IR mutations reduced activity in this mutant because the interaction with Cdc27 was lost (Figure 7A and 7B), and the C-box mutation reduced activity because the C-box interacts with another, unidentified site. Therefore, the residue mutated in Cdc23-A is likely to interact with a third, unknown site on Cdh1. When the Cdc23-A mutant is combined with either the C-box or the IR mutation, two out of three interactions are disrupted, reducing the affinity to a level that is too low to allow detectable ubiquitination in our assays.

Detailed analysis of C-box and IR mutants revealed that these mutations have no major effects on the processivity of substrate ubiquitination (Figure 8). These results are consistent with our evidence that processivity is not affected when activator-APC/C affinity is reduced by mutations in Cdc27 or Cdc23.

TPR mutations increase the rate of activator dissociation from the APC/C

To obtain a more detailed understanding of the activator-binding defects of TPR-mutant APC/Cs, we measured the rate at which radiolabeled Cdh1 dissociates from immunopurified APC/C (Figure 9A). Cdh1 dissociated from wild-type APC/C with a half-time of 25 min ($k_{\text{off}} = 4.6 \times 10^{-4} \text{ s}^{-1}$) and dissociated more rapidly from the TPR mutants: the half-time was 10 min ($k_{\text{off}} = 1.1 \times 10^{-3} \text{ s}^{-1}$) with Cdc27-A2

APC/C and 3 min ($k_{\text{off}} = 3.8 \times 10^{-3} \text{ s}^{-1}$) with Cdc23-A APC/C. These results support two conclusions: first, that activator binds the APC/C with very high affinity; and second, that decreased activator affinity in the TPR mutants is due, at least in part, to an increased dissociation rate.

We also measured the rate at which substrate (^{125}I -cyclin B) dissociates from immunopurified APC/C-Cdh1 complexes. Substrate dissociated from the APC/C with a half-time of ~ 1 min (an average from three experiments, apparent $k_{\text{off}} = 0.01 \text{ s}^{-1}$) (Figure 9B). This is more rapid dissociation than that of Cdh1 from wild-type APC/C, suggesting that activator remains bound to the APC/C for a longer time than substrate. Multiple substrates might therefore be modified during a single activator-binding event. This is not the case with Cdc23-A APC/C, from which substrate and activator dissociate at roughly similar rates.

We also determined the dissociation rate of Cdh1 from wild-type and Cdc23-A APC/C in the presence of E1, E2, ATP and ubiquitin, and found that the presence of these reaction components had no effect on dissociation rate (Figure 10A). Similarly, these components had no significant effect on the rate of substrate dissociation (Figure 10B).

Substrate promotes activator-APC/C binding through a bivalent interaction

We were surprised to observe that APC/C mutants with an increased activator dissociation rate do not display a processivity defect (Figure 3). If substrate is

bound solely to the activator during ubiquitination by these mutants, then that substrate should also have an increased dissociation rate and might therefore be ubiquitinated less processively. The simplest explanation for our results is that substrate is not bound only to activator during the reaction but is passed to and/or shared with a second, activator-independent site on the APC/C. Given that many APC/C substrates contain multiple degradation motifs, an appealing possibility is that a single substrate binds in a bivalent fashion to sites on the activator and the APC/C core. If this were the case, a reduction in activator-APC/C affinity in our TPR mutants would not necessarily have a significant impact on substrate residence time in the active site.

If a substrate binds simultaneously to both activator and APC/C, then it should increase activator-APC/C binding affinity. This possibility has not been tested previously in biochemical detail, but it has been shown that overexpression of the APC/C substrate Hsl1 increases the amount of Cdh1 bound to the APC/C in vivo (Burton et al., 2005). On the other hand, cyclin substrate had no apparent effect on activator-APC/C binding in native-gel binding assays (Passmore and Barford, 2005), and our own results (Figure 9A) show clearly that activator binds very tightly to the APC/C in the absence of substrate. It remained possible, however, that substrate does enhance the already high activator-APC/C affinity but that measurement of this effect requires sensitive analyses of rates of activator dissociation from the APC/C.

We therefore analyzed the effects of substrate on the half-time of Cdh1 dissociation from the APC/C. The addition of 50 μM cyclin prolonged the apparent half-life of activator bound to wild-type APC/C by about 10-fold (Figure 9C). Similarly, substrate caused a significant extension of the half-life of activator bound to the Cdc23-A APC/C, from 3 min (apparent $k_{\text{off}} = 3.8 \times 10^{-3} \text{ s}^{-1}$) in the absence of substrate to 12 min (apparent $k_{\text{off}} = 9.5 \times 10^{-4} \text{ s}^{-1}$) in the presence of 50 μM cyclin. Thus, under these conditions, substrate significantly reduces the apparent rate of activator dissociation, as expected for the bivalent substrate-binding mechanism.

Using our standard activator binding assay rather than half-life measurements, we tested the effects of varying concentrations of the APC/C substrates securin, cyclin, and Hsl1 (Burton and Solomon, 2001) on Cdh1-APC/C binding. The high binding affinity of Cdh1 for wild-type enzyme made the stimulation of activator binding difficult to observe in this assay, but all three substrates clearly promoted Cdh1 binding to Cdc23-A APC/C (Figure 11A). Cyclin and securin stimulated activator binding to the Cdc23-A APC/C at micromolar concentrations, and the effect of cyclin was abolished by mutation of its D-box sequence. Hsl1 was more effective, stimulating activator binding at far lower concentrations (tens of nanomolar). Mutation of APC/C-recognition sequences in Hsl1 prevented its effects. The potency of Hsl1 in this assay is consistent with previous evidence that Hsl1 is the most processively-modified

APC/C substrate known and might therefore possess a particularly high affinity for the APC/C active site (Carroll et al., 2005).

If a bivalent substrate interaction promotes activator binding, then the stimulation of activator binding might be reduced at high substrate concentrations, where the two substrate-binding sites could be occupied by two separate substrate molecules rather than a single bridging molecule. Indeed, the high-affinity substrate Hsl1 displayed this behavior: its stimulatory effects on activator binding were not observed at the highest concentrations tested (Figure 11A).

The bivalent substrate-binding model requires a substrate-binding site on the core APC/C. There is substantial previous evidence that a core binding site exists and depends on the subunit Doc1. Mutation of Doc1 is known to reduce substrate binding and reaction processivity but does not appear to affect activator binding or the concentration of activator required for half-maximal stimulation of APC/C activity (Carroll and Morgan, 2002; Passmore et al., 2003). Using our substrate-dissociation assay, we confirmed that substrate binds very poorly to Doc1-4A APC/C (Figure 12). Thus, if the stimulation of activator-APC/C binding by substrate depends on a bivalent bridge between the activator and the Doc1-dependent core binding site, then it should be abolished by mutation of Doc1.

We therefore analyzed the dissociation rate of activator from Doc1-4A APC/C. Mutation of Doc1 had no effect on activator half-life (Figure 11B), consistent with previous indications that Doc1 does not promote substrate binding through an effect on the activator. Mutation of Doc1 had a striking effect in the presence of substrate: high concentrations of cyclin did not prolong activator binding in the absence of Doc1 function (Figure 11B). These results support the model that substrate promotes activator binding by providing a bivalent bridge between a site on the activator and a Doc1-dependent site on the APC/C core.

Discussion

Our results provide new insights into the mechanism of substrate binding by the APC/C. We identified mutations in the APC/C that increase the rate of activator dissociation but do not significantly affect reaction processivity, suggesting that substrates are not bound solely to the activator during ubiquitination. Further analysis of activator binding by mutant and wild-type APC/Cs then revealed that the affinity of activator for the APC/C is enhanced in the presence of substrate, probably as a result of a bivalent bridging interaction between a substrate-binding site on the activator and another on the APC/C core.

Our results, as well as most previous studies of APC/C substrate-binding mechanisms, are most readily explained by a model diagrammed in Figure 11C

and explored in more detail in Figure 13. According to this scheme, activator, substrate, and the APC/C core form a trimolecular complex in which each of the three components interacts with both of the others. According to established principles of cooperative binding in trimolecular complexes (Goodrich and Kugel, 2007), this model can explain why defects in activator-APC/C affinity have only minor effects on reaction processivity. The model predicts that substrate residence time on the APC/C (and thus reaction processivity) is determined by the rates of dissociation of (1) substrate from the activator-APC/C complex and (2) activator-substrate complex from the APC/C. Based on reasonable estimates of binding affinities (Figure 13), we predict that the first of these dissociation rates is about 200-fold greater than the second. Thus, the first rate is the primary determinant of the overall substrate dissociation rate. In our TPR mutants, where activator-APC/C affinity is reduced, there should be no change in the first rate and perhaps a ten-fold increase in the second, so that the unchanged first rate is still the primary determinant of substrate dissociation rate. In contrast, inactivation of the APC/C-substrate binding site by mutation of Doc1 is predicted to increase both substrate dissociation rates, explaining the major processivity defect of Doc1 mutants. This model can also explain why processivity of securin ubiquitination with Cdc20 is higher than that with Cdh1 (Figure 4): we speculate that Cdc20 has a higher affinity for certain substrates, which would reduce the first substrate dissociation rate and could thus significantly prolong substrate residence time in the active site.

Our model is also consistent with the fact that many, if not all, APC/C targets contain multiple APC/C-recognition motifs. Although the well-established D- and KEN-box sequences are clearly required in many cases for APC/C-dependent ubiquitination, they are often not sufficient, suggesting that many substrates contain additional, as yet unidentified, APC/C-recognition sites. Numerous APC/C substrates contain unconventional targeting motifs (see Sullivan and Morgan, 2007a, and references therein), and so there is a clear potential for substrates to employ multivalent interactions with sites on both the activator and APC/C.

Recent studies suggest that the N-terminal C-box region of the activator induces a conformational change in the APC/C that enhances the rate of substrate ubiquitination (Dube et al., 2005; Kimata et al., 2008). This possibility is not incompatible with our model: the activator might help link the substrate to the APC/C core while also triggering a conformational change that somehow promotes ubiquitin transfer from the E2 to the substrate.

Our studies, as well as previous work (Passmore and Barford, 2005; Thornton et al., 2006), indicate that activator binds the APC/C with high affinity in the absence of substrate; substrate boosts this affinity but is not required for high-affinity activator binding. These results are not consistent with previous speculation that substrate binding to activator is a prerequisite for activator binding to the APC/C (Burton et al., 2005). Instead, our results argue that

activator can associate with the APC/C in the absence of substrate and might even remain bound during multiple rounds of substrate binding and dissociation. On the other hand, we also appreciate that the activator (and substrate) dissociation rates measured in our experiments might not be accurate reflections of these rates inside the cell, where they could be regulated by additional factors.

Mutation of conserved residues predicted to line the TPR grooves of the APC/C revealed that TPR motifs mediate the binding of both Cdc20 and Cdh1 to the APC/C. Our evidence for an IR-binding site on Cdc27 is consistent with previous work showing that the IR motif of Cdh1 interacts with Cdc27 (Kraft et al., 2005; Thornton et al., 2006; Vodermaier et al., 2003). Activator binding to Cdc23, however, has not been reported. Our results do not indicate what part of the activator binds to the TPR motif of Cdc23. TPR grooves tend to bind extended peptides, and so Cdc23 could bind a disordered loop or the N-terminus of the activator. Our results also do not identify the C-box binding site, but previous results suggest that a C-box binding site might exist on Apc2 (Thornton et al., 2006). Additional support for this idea comes from EM structures that locate Apc2 in close proximity to bound Cdh1 (Dube et al., 2005; Ohi et al., 2007).

The role of the TPRs in Cdc16 remains unclear. The removal of Cdc16 from Cdc27 APC/C abolishes the remainder of its activity, even though Cdc23 remains in place (Thornton et al., 2006). Cdc16 might simply stabilize Cdc23 or other essential subunits, or activators may bind to Cdc16 at sites that we did not

mutate. While previous work showed some binding of IR peptides to Cdc16 (Vodermaier et al., 2003), our work shows that the Cdc27-A1A2 mutations are sufficient to eliminate IR binding.

Of the many recently proposed models of APC/C-substrate recognition (reviewed by Yu, 2007), we support ‘multivalency’ models in which substrate is shared by binding sites on the activator and APC/C core, perhaps accompanied by an activator-induced conformational change that promotes activity. In these models, the activator provides additional substrate-binding sites that enhance weak interactions between substrate and the APC/C core. These multivalent mechanisms could also enhance the fidelity of substrate recognition by requiring multiple substrate-binding steps, or provide the APC/C with greater flexibility in substrate recognition by allowing the combinatorial integration of multiple degradation motifs that interact separately with the activator and the APC/C. Thus, these mechanisms might allow the APC/C to recognize more substrates with greater specificity, and might provide an important level of proofreading for an enzyme whose substrates are irreversibly destroyed.

Experimental Procedures

Strains and Plasmids

All yeast strains are in a W303 background. For initial analysis of mutant APC/C behavior (Figure 1), *CDC27* was cloned into pRS305 (*LEU2*) and *CDC16* and

CDC23 were cloned into pRS402 (*ADE2*), and the Quik-change method (Stratagene, La Jolla, CA) was used to generate the indicated point mutations. All mutations were confirmed by sequencing. Wild-type and mutant forms of *CDC27* were expressed from the *CDC27* promoter integrated at the *LEU2* locus in strain MM1, in which *Cdc27* is not essential for viability (Thornton and Toczyski, 2003). Wild-type or mutant *CDC23* was expressed from the *CDC23* promoter integrated at the *ADE2* locus in strain TMN90-1, in which the APC/C is not essential for viability (Thornton and Toczyski, 2003). Wild-type or mutant *CDC16* was expressed from the *CDC16* promoter integrated at the *ADE2* locus in strain TMN89, in which the APC/C is not essential for viability (Thornton and Toczyski, 2003).

To generate the *cdc27-A2* (MM2) and *cdc23-A* (MM4) strains used in Figure 4A and B, mutant genes were cloned into pRS306 (*URA3*) and integrated into wild-type strain DOM90 (*MAT a, bar1*) for pop-in/pop-out two-step gene replacement. To generate the *cdc27-A1A2* strain (MM5) used in Figure 5A, the mutant gene under the control of its own promoter was integrated at the *LEU2* locus of a strain carrying the genomic copy of *CDC27* under the control of the P_{GAL1} promoter, and the yeast were grown in media containing glucose.

For tetrad dissection of spores from *CDC27/cdc27-A2 cdc23-A/CDC23* diploid cells, MM3 (*MAT α cdc27-A2*) was mated to MM4 (*MATa cdc23-A*) and the resulting diploid strain was grown on sporulation media for three days at 30°C.

To determine the terminal phenotype of a *cdc27-A2 cdc23-A* double mutant (Figure 5C), the P_{GAL1} promoter was integrated upstream of the genomic copy of *CDC23* in MM2. A plasmid containing either *CDC23*, *cdc23-A*, or no insert under the control of the *CDC23* promoter was integrated at the *ADE2* locus.

To determine whether the Cdc27 and Cdc23 mutations suppressed the lethality of *CDC20* and *CDH1* overexpression (Figure 6), *CDH1* and *CDC20* were cloned into pRS306 under the control of the P_{GAL1} promoter (replacing the existing *Myc-CDH1* in pDM180, J. Charles). These two vectors, as well as pDM180, were integrated into DOM90, MM2, and MM4, generating strains MM6-MM14.

Ubiquitination and Cdh1 binding assays

Ubiquitination assays contain purified E1 (yeast Uba1), E2 (6xHis-Ubc4 purified from bacteria), ubiquitin, ATP, APC/C, activator, and substrate. Cdh1 was produced either by baculovirus expression in insect Sf9 cells or by coupled transcription and translation in rabbit reticulocyte lysate (Promega, Madison WI). In vitro translated Cdh1 and Cdc20 were purified away from the reticulocyte lysate via an N-terminal ZZ+TEV tag as noted. An amino-terminal fragment of sea urchin cyclin B (residues 13-110) was purified from bacteria and radioiodinated as described (Carroll and Morgan, 2005). ³⁵S-securin with a C-terminal TEV+ZZ tag was purified with IgG-coupled Dynabeads (Invitrogen, Carlsbad CA)

from rabbit reticulocyte lysate after coupled transcription and translation, and cleaved from beads with TEV protease.

For analysis of APC/C activity and binding in immunoprecipitates, cell lysates were prepared from the indicated strains by bead-beating ~300 mg cell pellets in 1.5 ml tubes one min every 5 min for 2.5 h in 750 μ l lysis buffer (60 mM Hepes pH 8, 150 mM NaCl, 0.2% NP-40, 50 mM β -glycerophosphate, 50 mM NaF, 1 mM Na_3VO_4 , 2 mM EDTA, 1 mM DTT, 1 μ g/ml leupeptin, pepstatin, and aprotinin, and 1 mM PMSF). Lysates were incubated with IgG-coupled Dynabeads for 2 h at 4°C, and beads were washed two times in lysis buffer before use in assays.

APC/C was purified using a C-terminal TAP tag on Apc1 as described (Thornton et al., 2006), except for Doc1-4A APC/C, which has a C-terminal TAP tag on Cdc16 (Carroll et al., 2005). Ubiquitination assays contained Uba1, Ubc4, ubiquitin, ATP, APC/C, activator, and substrate, prepared as described (Carroll and Morgan, 2005; Rodrigo-Brenni and Morgan, 2007) and detailed in Supplemental Experimental Procedures. All in vitro translation was performed with the TNT Quick-Coupled Transcription/Translation System (Promega, Madison WI) in rabbit reticulocyte lysate.

For analysis of APC/C activity in immunoprecipitates as in Figure 1, cell lysates were prepared from a ~300 mg cell pellet of the indicated strains by bead-beating

(see Supplemental Experimental Procedures). Lysates were incubated with IgG-coupled Dynabeads for 2 h at 4°C, and beads were washed in lysis buffer before 10% of the preparation was removed, washed with QAH buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM MgCl₂), and resuspended with E1, E2, ATP, ubiquitin, insect cell-derived Cdh1 and ¹²⁵I-cyclin B for 30 minutes. A limiting amount of Cdh1 was used to normalize for the amounts of immunopurified APC/C.

For analysis of Cdh1 binding, 90% of the immunopurified APC/C was incubated with 10 µl in vitro translated ³⁵S-Cdh1 and 50 µl HBST (10 mM HEPES pH 7.4, 150 mM NaCl, 0.1% Triton X-100) for 1 h at room temperature.

Immunoprecipitates were washed three times before separation by SDS-PAGE. The gel was stained with Coomassie Blue before visualization with a PhosphorImager.

For Cdh1 dose responses (Figure 3), wild-type and mutant APC/C were purified in parallel using the full TAP purification protocol (Carroll and Morgan, 2005). Insect cell-derived Cdh1 was added in increasing concentrations.

For Cdc20-dependent APC/C assays (Figure 4), Cdc20 was purified from rabbit reticulocyte lysate (see Supplemental Experimental Procedures) and added to APC/C reactions containing purified ³⁵S-securin and wild-type or mutant APC/C

that had been purified in parallel by the full TAP purification procedure (Carroll and Morgan, 2005).

For C-box and IR mutant Cdh1 assays (Figures 7 and 8), wild-type or mutant activator was translated in vitro and added directly to ubiquitination assays in the translation mix, as these mutants have such weak activity that they often do not survive purification. Equivalent amounts of reticulocyte lysate without in vitro translated Cdh1 were added to control reactions.

For analysis of the stimulation of Cdh1 binding to APC/C by substrate (Figure 11A), Cdc23-A APC/C was immunopurified using IgG-coupled Dynabeads and incubated with 10 μ l in vitro translated ³⁵S-Cdh1 and 50 μ l HBST containing varying concentrations of APC/C substrates for 1 h at room temperature.

Immunoprecipitates were washed two times for 30 s with HBST and samples were separated by SDS-PAGE. Gels were stained with Coomassie Blue to confirm that equivalent amounts of APC/C were immunopurified in each lane.

The cyclin substrate used in these experiments is the sea urchin cyclin fragment described above and its D-box mutant (N50A). The securin substrate is a fragment of yeast Pds1 (residues 1-110), containing both a KEN- and D-box, that was expressed in bacteria. The Hsl1 substrate is a bacterially-expressed MBP fusion of an Hsl1 fragment (residues 667-872) (Burton and Solomon, 2001). We also tested the D/KEN-box double mutant (RAALSDITN starting at residue 828

changed to AAAASDITA, and KEN starting at residue 775 changed to AAA)
(Burton and Solomon, 2001).

Cell cycle analysis

For Figure 5B, strains were grown at 30°C in 2% glucose and arrested in G1 by the addition of # factor (1 µg/ml) for 2.5 h. Cells were washed free of # factor, placed in fresh medium containing 2% dextrose, and samples were taken every 15 min. # factor was added back after 90% of the cells had budded. For Figure 5C, strains were grown at 30°C in the presence of 2% galactose and 2% raffinose and arrested in G1 by the addition of # factor (1 µg/ml) for 3 h. 2% dextrose was then added to all strains for an additional 2 h. Cells were washed free of # factor, placed in fresh medium containing 2% dextrose, and samples were taken every 15 min. # factor was added back after 90% of the cells had budded. Protein extracts were prepared by bead-beating in urea lysis buffer. Polyclonal anti-Clb2 antibodies were a generous gift of Doug Kellogg (University of California, Santa Cruz). Anti-Cdk1 and anti-Cdc20 antibodies were from Santa Cruz Biotechnology, Inc. DNA was visualized by DAPI staining and tubulin was visualized by indirect immunofluorescence with antibody YOL1/34 using spheroplasts.

Dissociation Rates

To measure activator dissociation rates, wild-type, Cdc27-A2, Cdc23-A, or Doc1-4A APC/C was immunopurified using IgG-coupled Dynabeads from lysate made

by bead-beating a ~1.3 g cell pellet, and incubated with 60 μ l of reticulocyte lysate containing in vitro translated ^{35}S -Cdh1 and 400 μ l HBST (either with or without 50 μ M cyclin) for 1 h at room temperature. After two rapid washes, 1.2 ml reticulocyte lysate containing a 20-fold excess of unlabeled in vitro translated Cdh1 (either with or without 50 μ M cyclin) was added as a competitor, so that any ^{35}S -Cdh1 that dissociated would not re-bind. Samples were taken at various times to monitor the amount of bound ^{35}S -Cdh1, and visualized using SDS-PAGE and PhosphorImager. A “pre-mix” control lane, in which labeled and unlabeled Cdh1 are added to the APC/C at the same time, was used to demonstrate that the amount of unlabeled Cdh1 is in excess of the amount of APC/C present. Control experiments also showed that bound ^{35}S -Cdh1 did not decrease when incubated with reticulocyte lysate lacking translated Cdh1 over three hours. Data points were analyzed using the graphing program Prism and fit to an exponential decay to determine dissociation rate (k_{off}) and $t_{1/2}$ ($t_{1/2} = 0.69/k_{\text{off}}$).

To measure substrate dissociation rate, wild-type or Doc1-4A APC/C was immunopurified using IgG-coupled Dynabeads from lysate made by bead-beating a ~2.4 g cell pellet, and incubated with 400 μ l in vitro translated Cdh1 and 400 μ l HBST for 1 h at room temperature. Unbound activator was removed and the remaining APC/C^{Cdh1} was incubated with 70 μ l of ~10 μ M ^{125}I -cyclin B for 1 h at room temperature. Unbound ^{125}I -cyclin B was removed and reticulocyte lysate containing an excess of in vitro translated Acm1 (100 μ l) was added, so that any ^{125}I -cyclin B that dissociated would not re-bind. Samples were taken at various

times to monitor the amount of ^{125}I -cyclin B that remained bound to the APC/C^{Cdh1} on beads.

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Figure 1. TPR groove mutations in Cdc27 and Cdc23 cause Cdh1 binding defects

(A) The TPRs of Cdc27, Cdc23 and Cdc16 are shown in numbered boxes.

Residues that we show are important for activator binding are labeled in TPR 5 and TPR6 of Cdc27 and TPR5 of Cdc23.

(B) A sequence alignment of TPR 6 from Cdc27 homologs illustrates the TPR consensus residues (green) that mediate the interactions between Helix A and Helix B and are distinct from those predicted to form the surface of the TPR groove (pink).

(C) Structure of TPR 3 from the TPR2A domain of Hop (Scheufler et al., 2000), with TPR consensus residues in green and groove-forming residues in pink. This image was generated with MacPyMOL (DeLano, 2007).

(D) Cdc27 and Cdc23 mutants have reduced activator binding. APC/C was immunoprecipitated from strains lacking the wild-type subunit and expressing the indicated Cdc27 or Cdc23 mutant subunit. 10% of the preparation was used to measure ubiquitination activity with ¹²⁵I-cyclin B (top panels). A limiting amount of Cdh1 was used to normalize for the amounts of immunopurified APC/C. 90% of the immunopurified APC/C was used for measurement of ³⁵S-Cdh1 binding (middle panels). The gel was stained with Coomassie Blue (bottom panels; starred band indicates background IgG from beads) to assess integrity of the APC/C. Three different amounts of wild-type APC/C were tested for comparison (WT lanes). This experiment is representative of three separate experiments, except that the lower amounts of APC/C protein obtained with the Cdc23-K466A

and G470A mutants in this experiment were not reproducible. Mutants pursued further are marked with asterisks and labeled. Doc1-4A APC/C was immunopurified using a TAP-tag on Cdc16 rather than Apc1, resulting in the shifted mobility of subunits on the Coomassie Blue-stained gel.

Figure 1

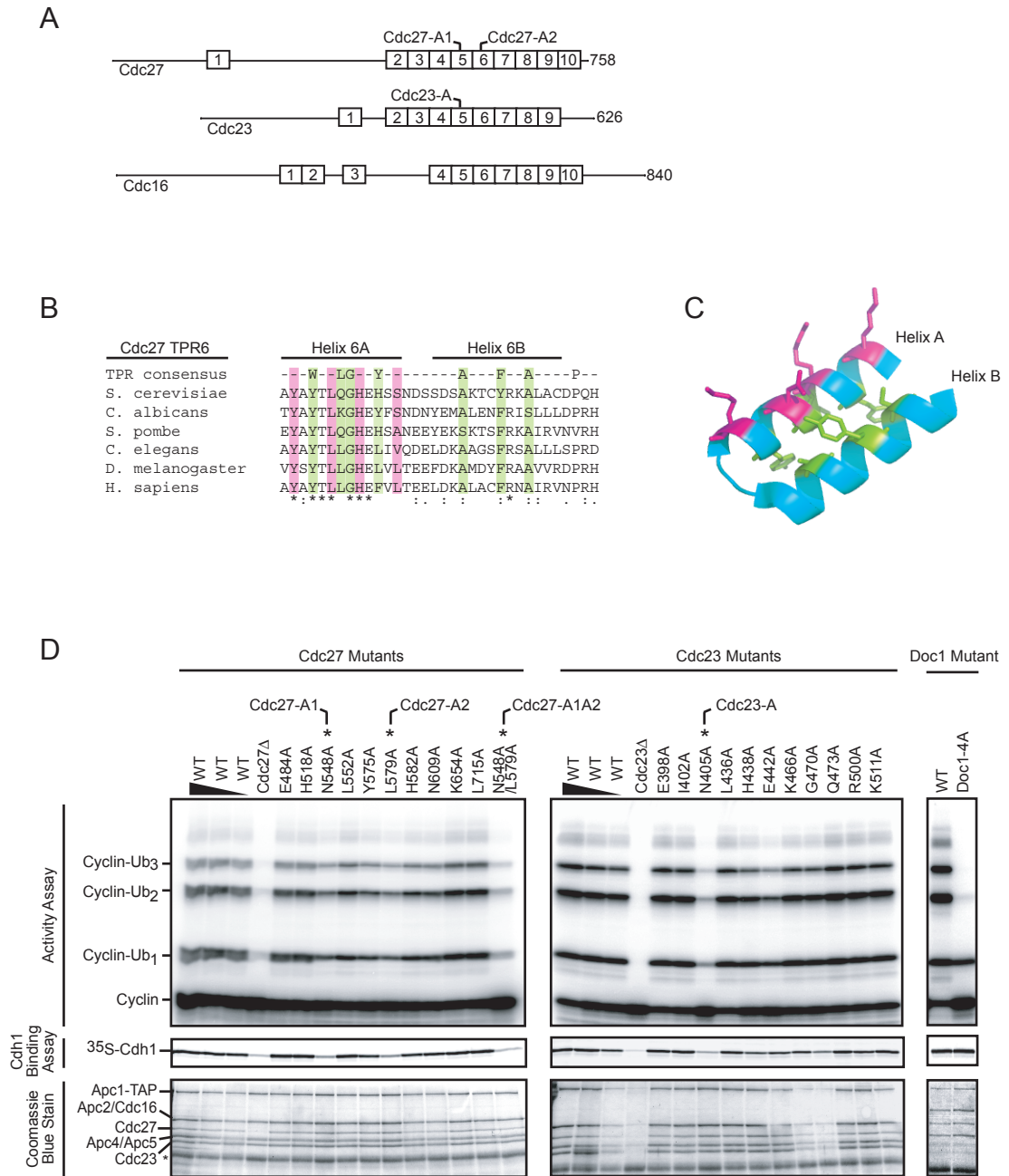


Figure 2. Cdc16 mutants do not have reduced activity or activator binding

APC/C was immunopurified as in Figure 1D from a *cdc16!* strain expressing wild-type *CDC16* or various mutants from the *CDC16* promoter integrated at the *ADE2* locus. APC/C was split into two parts and analyzed as in Figure 1D, except that APC/C amounts were assessed by silver staining (only Apc1 is shown). The lower activity of the V641A mutant in this experiment was not reproducible in other experiments.

Figure 2

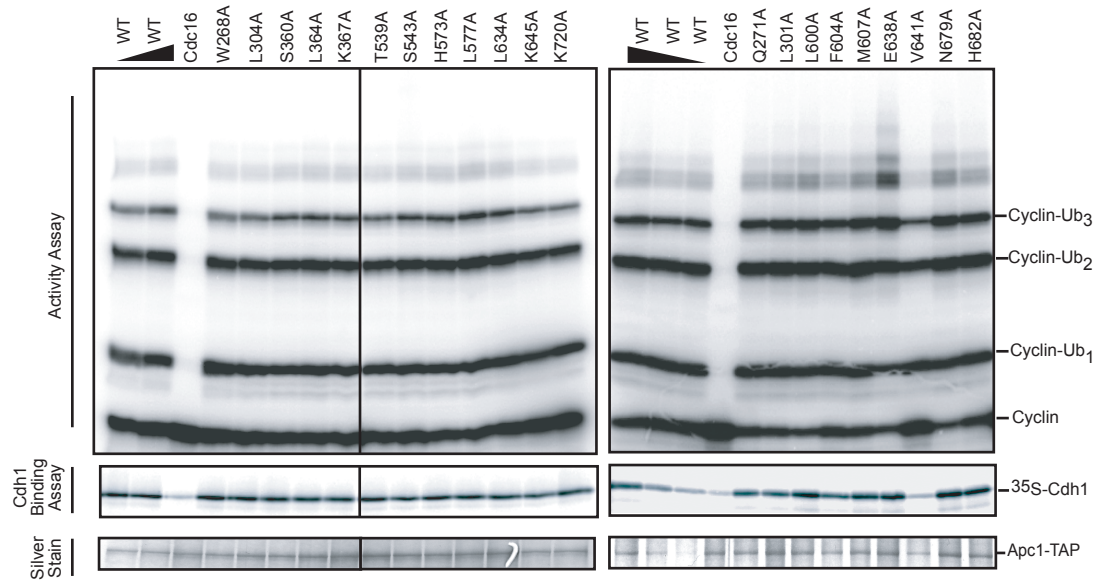


Figure 3. Mutations in Cdc27 and Cdc23 reduce Cdh1 response and activity but not processivity

Recombinant 6xHis-Cdh1, purified from baculovirus-infected insect cells, was titrated into ubiquitination reactions containing E1, E2, ATP, ubiquitin, ¹²⁵I-cyclin B and either wild-type or mutant TAP-purified APC/C. Cdh1 concentrations were 0, 3, 10, 30, 100, 300, 600, and 1000 nM. After 30 min at room temperature, reaction products were analyzed by SDS-PAGE and PhosphorImager (A, C, E, G). These results are representative of three separate experiments. The amounts of mono- (cyclin B-Ub₁), di- (cyclin B-Ub₂) and tri- (cyclin B-Ub₃) ubiquitinated species were quantified and combined to provide the total amount of ubiquitinated cyclin B. The processivity of ubiquitination was determined by calculating the ratio of ubiquitins to cyclin (B, D, F, H). To observe significant activity with the Cdc27-A1A2 mutant, we used five-fold greater amounts of APC/C, and the quantification reflects the normalized activity per mole of enzyme.

Figure 3

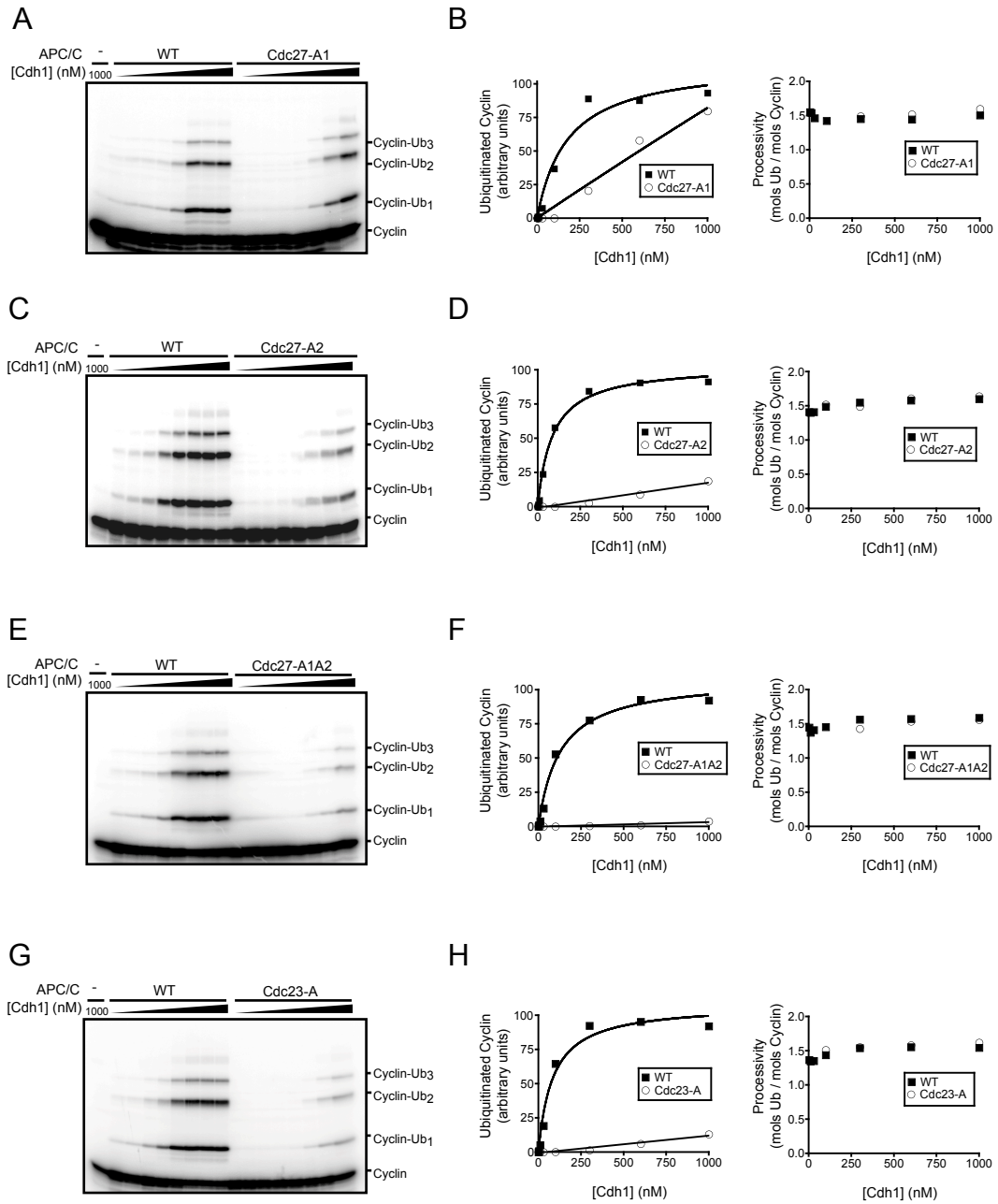


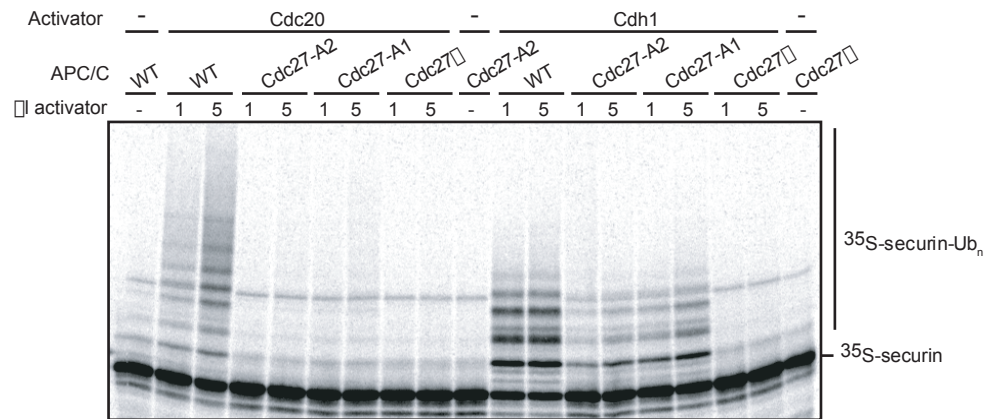
Figure 4. Cdc20-dependent APC/C activity is defective in Cdc27 and Cdc23 mutants in vitro

(A) APC/C reactions were performed with either wild-type, Cdc27-A2, Cdc27-A1, or Cdc27⁻ APC/C using ³⁵S-securin as substrate and the indicated amount of in vitro translated, ZZ-tagged Cdh1 or Cdc20 purified from rabbit reticulocyte lysates. Control lanes (-) contained mock activator purifications from 5 μ l rabbit reticulocyte lysate without activator. Due to the different levels of activity with Cdc20 and Cdh1, it is not clear whether the mutations had a greater effect on Cdc20-dependent activity than Cdh1-dependent activity.

(B) APC/C reactions were performed with either wild-type, Cdc23-A or Cdc23⁻ APC/C as in (A). Note that in the reaction with wild-type APC/C and 5 μ l Cdh1, apparent processivity is increased due to substrate depletion.

Figure 4

A



B

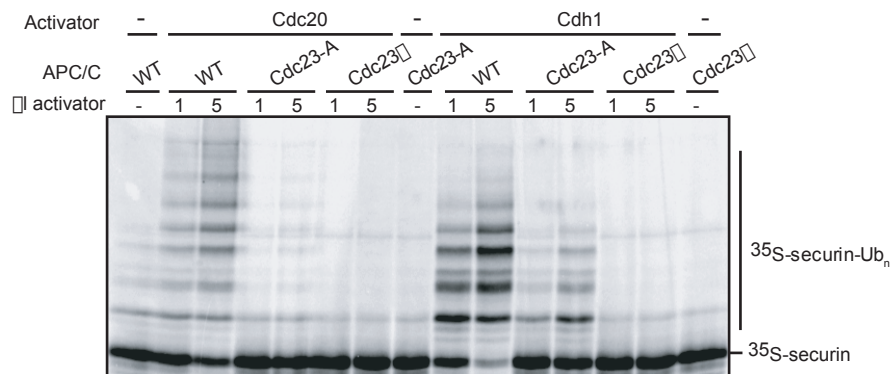


Figure 5. Cdc27 and Cdc23 mutants have mild individual defects in vivo and together are synthetically lethal

(A) Log-phase growth rates, at 30°C and 37°C, of strains in which the indicated mutant gene replaced the endogenous gene. Results are representative of three individual experiments.

(B) Asynchronous cultures of the indicated cells (AS timepoint) were treated with # factor (1 µg/ml) for 2.5 h. # factor was then washed out (zero time point) and cells were harvested at the indicated times. # factor was added back after 90% of the cells were budded. Parallel samples were analyzed directly for budding index (black squares) and fixed, treated with zymolyase, and stained with DAPI to measure chromosome segregation, and with anti-tubulin antibodies to measure spindle elongation. We counted binucleate cells that had elongated spindles (open circles). Additional samples were prepared for western blotting with anti-Clb2 antibodies and anti-Cdk1 (as a loading control).

(C) Asynchronous cultures of the indicated P_{GAL1} -*CDC23* strains in galactose-containing media (AS timepoint) were treated with # factor for 3 h (# time point), after which dextrose was added for 2 h to turn off expression of wild-type *CDC23*. # factor was washed out (zero time point) and cells released into dextrose-containing media. Samples were taken at the indicated times for analysis of budding index, chromosome segregation, spindle formation, and Clb2 levels as in (B).

Figure 5

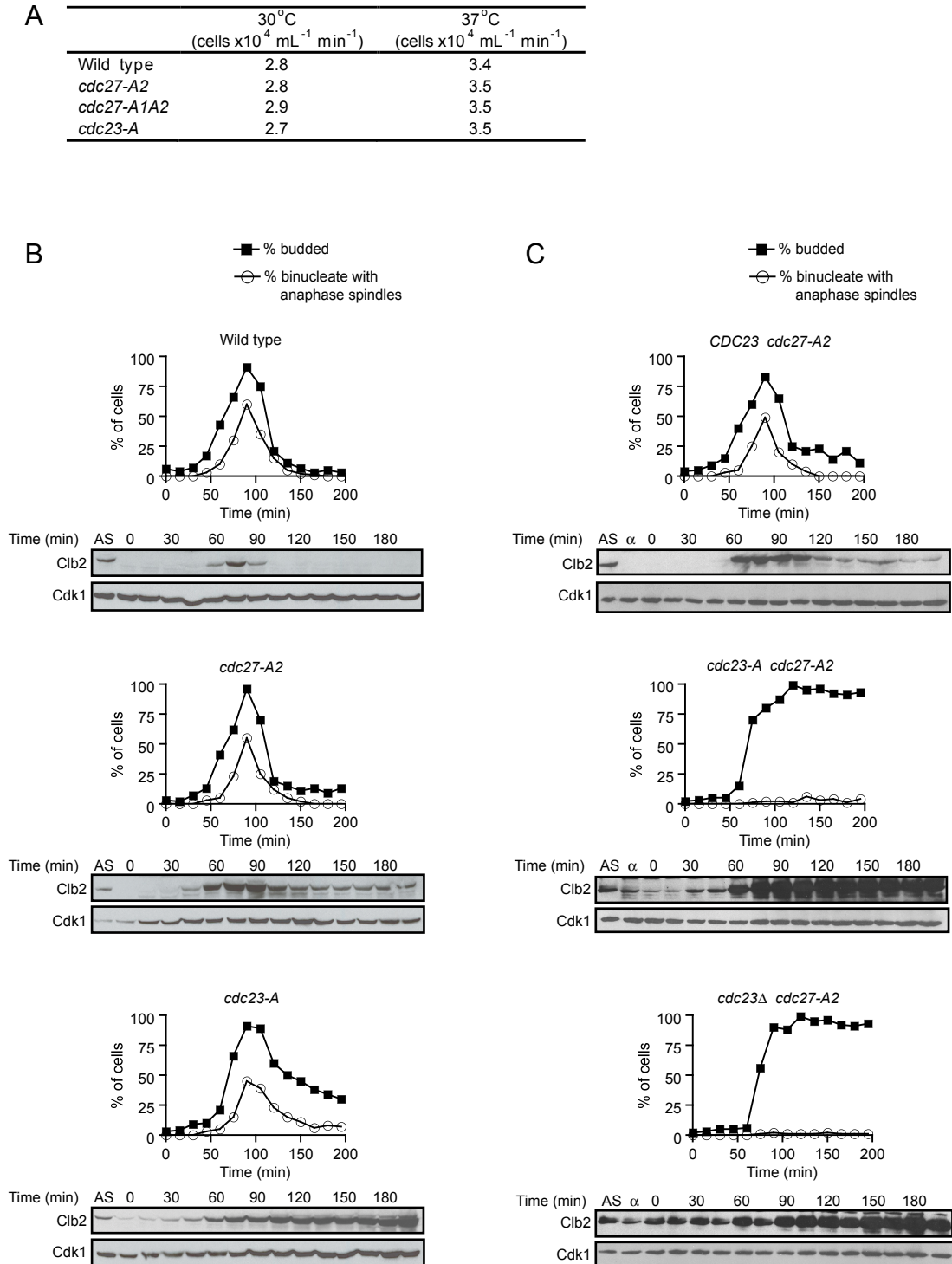


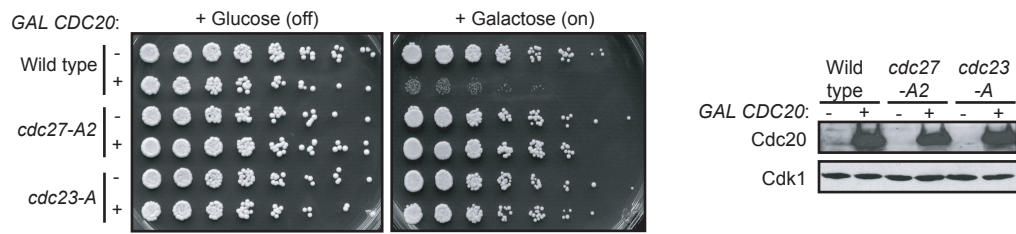
Figure 6. Cdc27 and Cdc23 mutations reduce activator function in vivo

(A) Indicated strains carrying either a vector containing P_{GAL1} -*CDC20* (+) or an empty vector (-) integrated at the *URA3* locus were grown to mid-log phase in raffinose-containing media. 10^6 cells were serially diluted 3-fold onto plates containing galactose or glucose and incubated at 23°C for five days. To analyze Cdc20 levels in these strains, P_{GAL1} -*CDC20*-containing strains were grown to mid-log phase in raffinose-containing media, washed, and grown for 2 h in galactose-containing media. Lysates of these cells were then analyzed by western blotting with anti-Cdc20 antibody (right panel).

(B) Indicated strains carrying either a vector containing P_{GAL1} -*Myc-CDH1* (+) or an empty vector (-) integrated at the *URA3* locus were grown to mid-log phase in glucose-containing media. 10^6 cells were serially diluted 3-fold onto plates containing galactose or glucose and incubated at 23°C for five days. To analyze Cdh1 levels in these strains, P_{GAL1} -*Myc-CDH1*-containing strains were grown to mid-log phase in glucose-containing media, washed, and grown for 2 h in galactose-containing media. Lysates of these cells were then analyzed by western blotting with anti-Myc antibody (right panel). The *cdc27-A* and *cdc23-A* mutations also suppressed the growth defect caused by *CDH1* overexpression (data not shown). The N-terminal Myc tag on Cdh1 had no effect on viability.

Figure 6

A



B

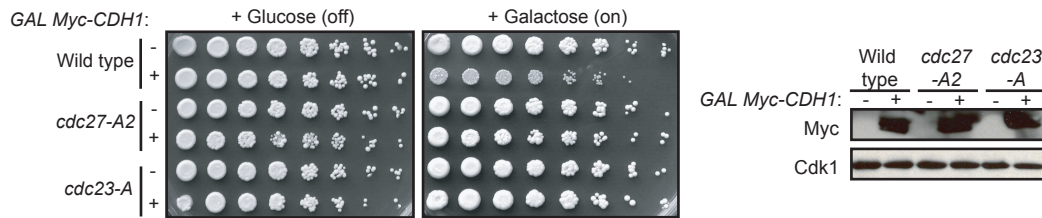


Figure 7. Analysis of activator C-box and IR motif interactions with Cdc27 and Cdc23 mutants

(A, C, E) Reactions containing ^{125}I -cyclin B and either wild-type or Cdc27-A2 APC/C (A), Cdc27-A1A2 APC/C (C), or Cdc23-A APC/C (E) were performed with in vitro translated wild-type, IR (IR to AA), or C-box (IP to AA) Cdh1 in rabbit reticulocyte lysate. An equivalent amount of lysate without activator was added to control reactions (-).

(B, D, F) Quantification of reactions shown in left-hand panels, normalized for background activity (i.e. activity in equivalent (-) lanes was subtracted) and the amount of enzyme added.

Figure 7

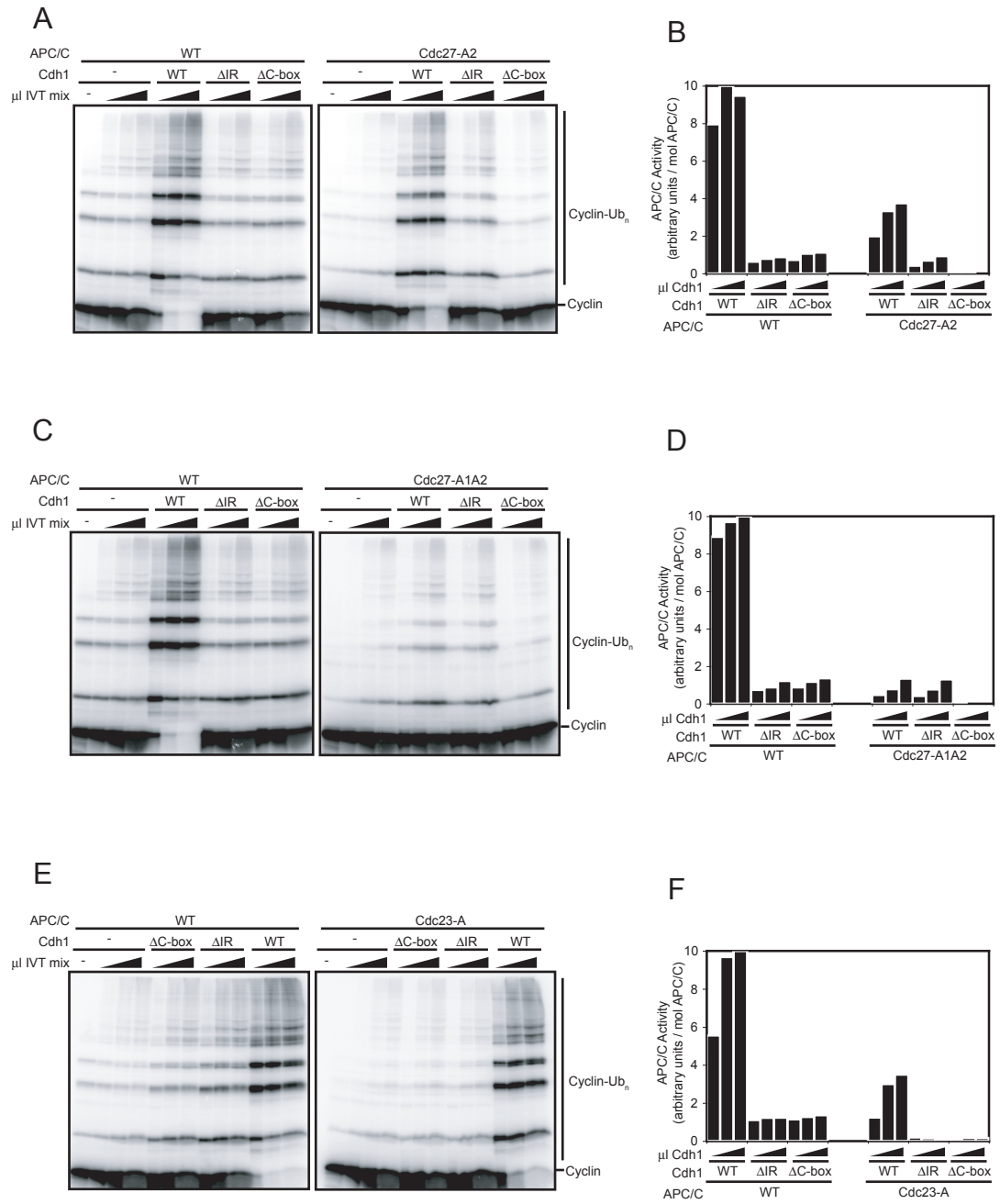


Figure 8. Decreased activator affinity does not affect processivity of ubiquitination by the APC/C

APC/C reactions were performed with wild-type APC/C using ¹²⁵I-cyclin B and the indicated amount of in vitro translated wild-type, IR or C-box Cdh1 in rabbit reticulocyte lysates. Control lanes (-) contained reticulocyte lysates without Cdh1. The processivity of the reactions was determined by calculating the number of ubiquitins per cyclin as in Figure 2. To allow more accurate measurements of processivity, reticulocyte lysates were treated with 10 mM N-ethyl maleimide, followed by 20 mM DTT, to inactivate ubiquitin chain-extending activities.

Figure 8

A

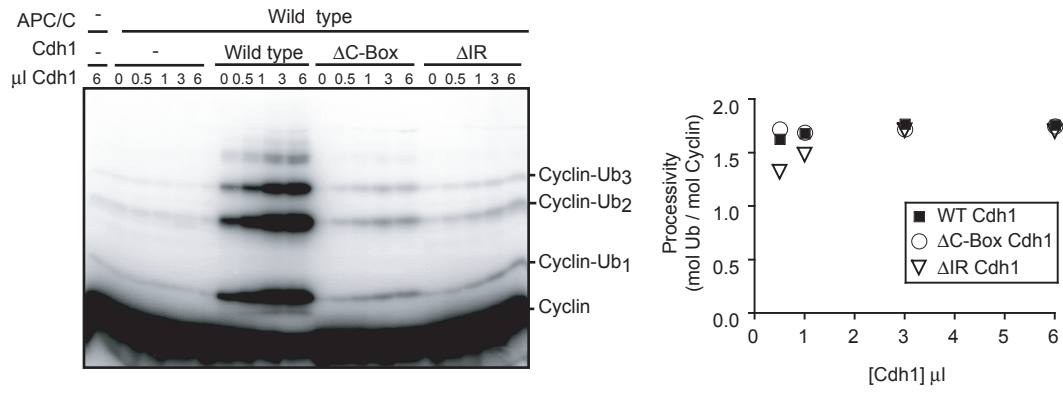


Figure 9. Activator dissociation rate is increased in TPR mutants and decreased in the presence of substrate

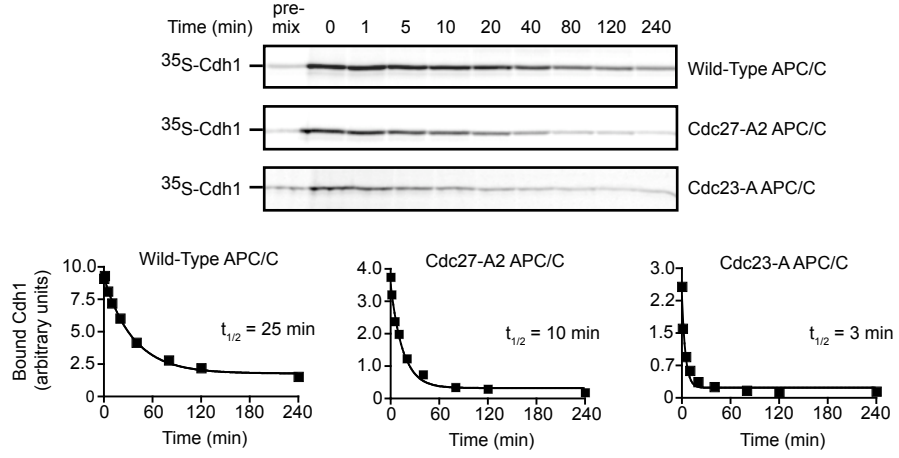
(A) Wild-type, Cdc27-A2, or Cdc23-A APC/C was immunopurified and incubated with in vitro translated ^{35}S -Cdh1 for 1 h at room temperature. After two rapid washes, a 20-fold excess of unlabeled in vitro translated Cdh1 was added as a competitor. Samples were taken at the indicated times to monitor the amount of ^{35}S -Cdh1 that remained bound to the APC/C. When ^{35}S -Cdh1 and unlabeled Cdh1 were added to the APC/C at the same time (pre-mix lane), minimal binding of ^{35}S -Cdh1 was observed. Coomassie staining indicated that the same amount of APC/C was present in each lane.

(B) Wild-type APC/C was immunopurified and incubated with in vitro translated Cdh1 for 1 h at room temperature. Unbound activator was removed and the remaining APC/C^{Cdh1} was incubated with ^{125}I -cyclin B for 1 h at room temperature. Unbound ^{125}I -cyclin B was removed and an excess of in vitro translated Acm1 (Enquist-Newman et al., 2008) was added as substrate competitor. Samples were taken at the indicated times to monitor the amount of ^{125}I -cyclin B that remained bound to APC/C^{Cdh1}. When ^{125}I -cyclin B and Acm1 were added to the APC/C at the same time (pre-mix lane), minimal binding of ^{125}I -cyclin B was observed. Coomassie staining indicated that the same amount of APC/C was present in each lane. A representative experiment is shown on the left, and the graph on the right shows the average of values from three separate experiments (+/- SEM).

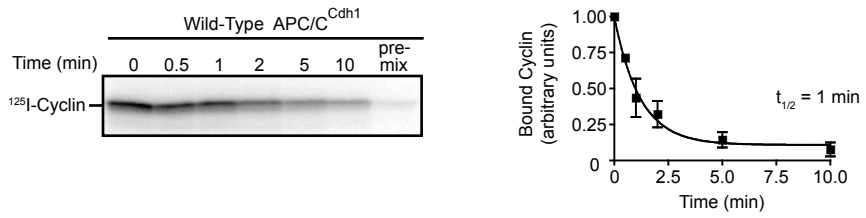
(C) Dissociation of Cdh1 from wild-type or Cdc23-A APC/C was measured as in panel (A), except that 50 μ M cyclin was included as indicated during Cdh1 binding and dissociation. Coomassie staining indicated that the same amount of APC/C was present in each lane.

Figure 9

A



B



C

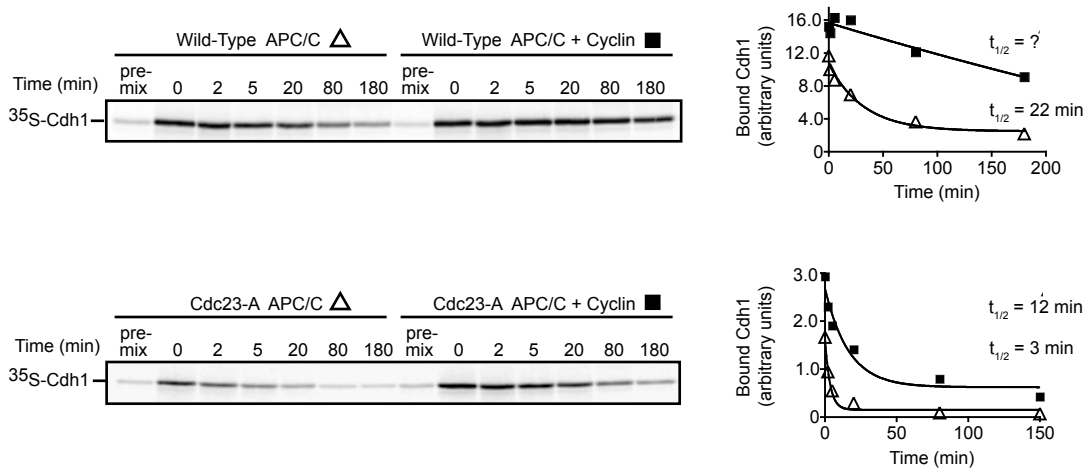


Figure 10. Activator and substrate dissociation rates are unchanged in the presence of ubiquitination reaction components

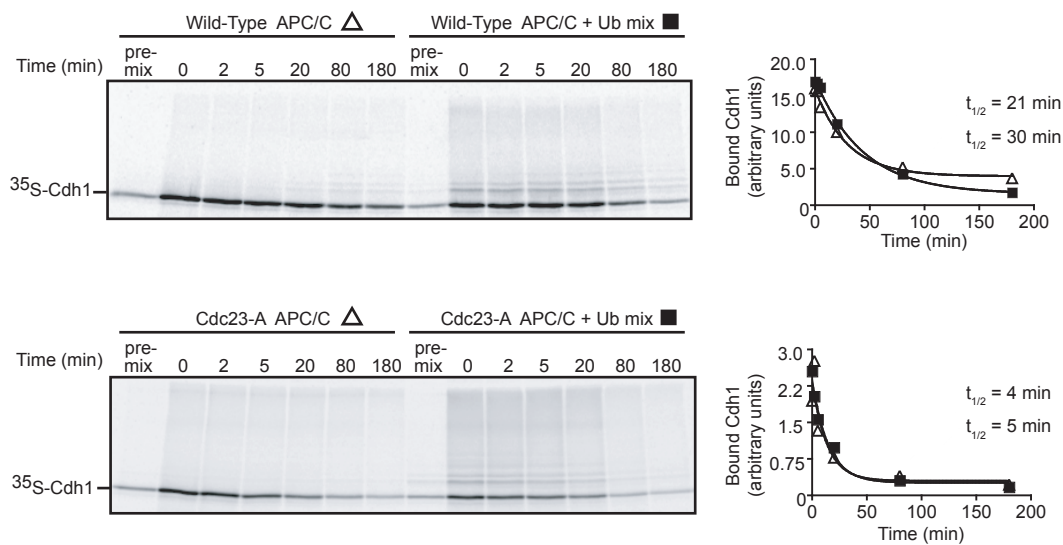
(A) Wild-type or Cdc23-A APC/C was immunopurified and incubated with in vitro translated ^{35}S -Cdh1 and either buffer alone or buffer containing ubiquitination reaction components (Ub mix: 500 nM Uba1, 50 μM Ubc4, 150 μM ubiquitin, and 1 mM ATP, pre-incubated 15 min) for 1 h at room temperature. After two rapid washes, a 20-fold excess of unlabeled in vitro translated Cdh1 was added as a competitor, along with either buffer or buffer containing ubiquitination components. Samples were taken at the indicated times to monitor the amount of ^{35}S -Cdh1 that remained bound to the APC/C. When ^{35}S -Cdh1 and unlabeled Cdh1 (with or without ubiquitination components) were added to the APC/C at the same time (pre-mix lane), minimal binding of ^{35}S -Cdh1 was observed. Coomassie staining indicated that the same amount of APC/C was present in each lane. In the presence of the ubiquitination components, activator is autoubiquitinated at low levels; higher molecular weight conjugates were included in the quantification on the right.

(B) Wild-type APC/C was immunopurified and incubated with in vitro translated Cdh1 for 1 h at room temperature. Unbound activator was removed and the remaining APC/C^{Cdh1} was incubated with ^{125}I -cyclin B for 1 h at room temperature. Unbound ^{125}I -cyclin B was removed and an excess of in vitro translated Acm1 (with or without ubiquitination reaction components) was added as substrate competitor. Samples were taken at the indicated times to monitor

the amount of ^{125}I -cyclin B that remained bound to APC/C^{Cdh1}. When ^{125}I -cyclin B and Acm1 (with or without ubiquitination components) were added to the APC/C at the same time (pre-mix lane), minimal binding of ^{125}I -cyclin B was observed. Coomassie staining indicated that the same amount of APC/C was present in each lane. Quantification of substrate at right includes the high molecular weight ubiquitinated species.

Figure 10

A



B

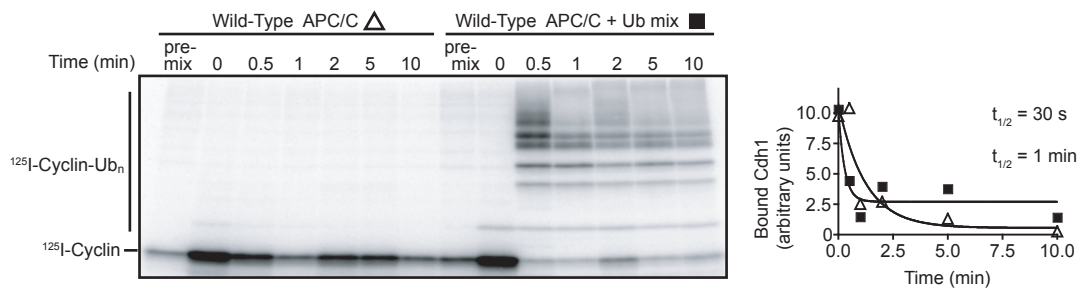


Figure 11. Substrate promotes activator binding through a bivalent bridging interaction

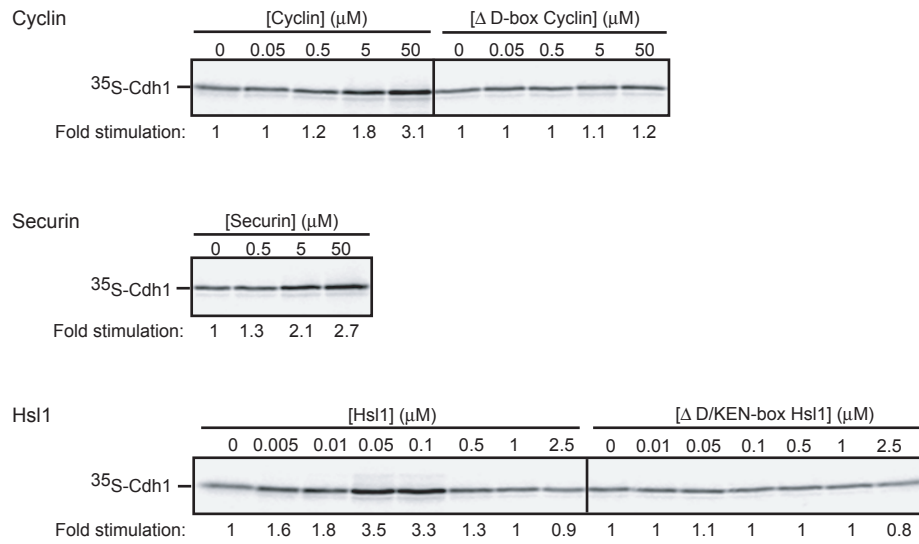
(A) Cdc23-A APC/C was immunopurified and incubated for 1 h at room temperature with in vitro translated ^{35}S -Cdh1 and the indicated concentrations of purified cyclin, securin, or Hsl1 fragments. Immunoprecipitates were washed two times for 30 s each before analysis by SDS-PAGE. D-box mutant cyclin and D/KEN-box mutant Hsl1 (Burton and Solomon, 2001) were also tested. Fold stimulation is the ratio of the amount of ^{35}S -Cdh1 bound in the presence of substrate to the amount bound with no substrate.

(B) Dissociation of Cdh1 from wild-type or Doc1-4A APC/C was measured in the absence and presence of 50 μM cyclin as in Figure 6C. Coomassie staining indicated that the same amount of APC/C was present in each lane. Half-times of Cdh1 dissociation were as follows: wild-type APC/C: 20 min; wild-type APC/C plus cyclin: >200 min; Doc1-4A APC/C: 20 min; Doc1-4A APC/C plus cyclin: 22 min.

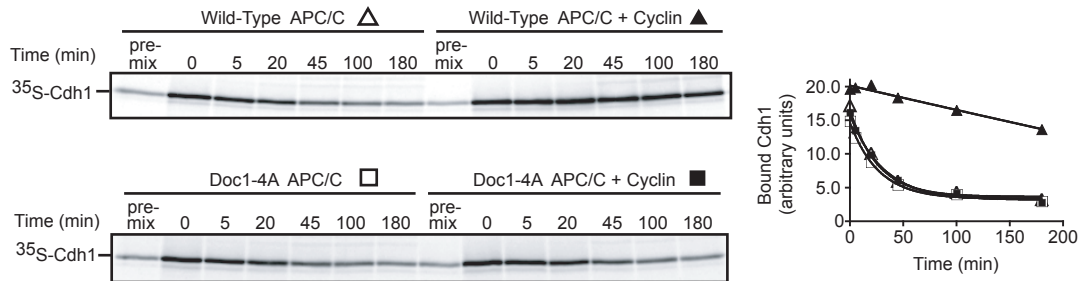
(C) We propose that activator binds the APC/C at sites on Cdc27 and Cdc23 and shares substrates with a site on the APC/C core during catalysis. Our results suggest that Cdc27 interacts with the IR motif of Cdh1 and Cdc23 interacts with an unknown site on the activator. The bivalent substrate-binding model is explored in detail in Figure S10.

Figure 11

A



B



C

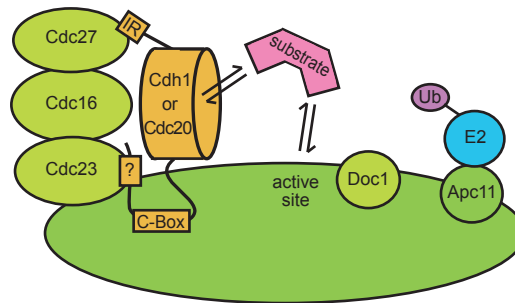


Figure 12. Substrate binding to Doc1-4A APC/C is not detectable

Wild-type or Doc1-4A APC/C was immunopurified and incubated with in vitro translated Cdh1 for 1 h at room temperature. Unbound activator was removed and the remaining APC/C^{Cdh1} was incubated with ¹²⁵I-cyclin B for 1 h at room temperature. Unbound ¹²⁵I-cyclin B was removed and an excess of in vitro translated Acm1 was added. Samples were taken at the indicated times to monitor the amount of ¹²⁵I-cyclin B that remained bound to APC/C^{Cdh1}. When ¹²⁵I-cyclin B and Acm1 were added to the APC/C at the same time (pre-mix lane), minimal binding of ¹²⁵I-cyclin B was observed. Coomassie staining indicated that the same amount of APC/C was present in each lane.

Figure 12

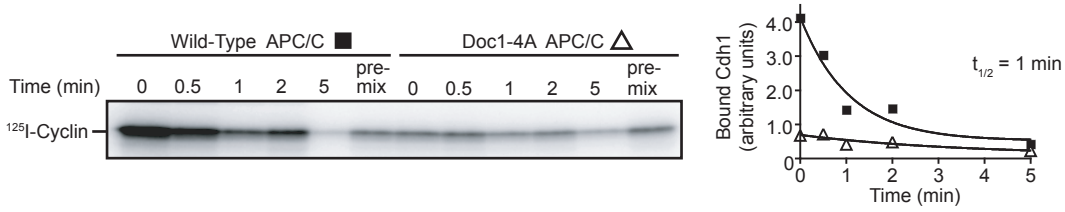


Figure 13. Predicted behaviors in a trimolecular complex of substrate, activator, and APC/C

(A) Our results suggest that the APC/C interacts with activator and substrate to form a trimolecular complex in which each molecule contacts both of the others. Trimolecular binding interactions can be modeled by considering the three bimolecular complexes that can form on the way to the trimolecular complex, as shown here with activator (**A**), substrate (**S**), and APC/C core (**C**). In many cases, one of the three bimolecular complexes has a very low affinity and therefore does not form significantly at physiological concentrations. In these cases, the model can be simplified by considering only the two most commonly formed bimolecular complexes on the path to the trimolecular complex (Goodrich and Kugel, 2007). This is likely the case with the APC/C, as the direct interaction of substrate with the APC/C core is very low affinity. Therefore, this route to trimolecular complex formation is probably negligible, and our model of the formation of the trimolecular complex (**A-C-S**) can be simplified to the four outside interactions, which are labeled with the approximate equilibrium dissociation constants (K_D) we estimate in panel B below. Our results in Figure 6C demonstrate that there is cooperativity in this system: the presence of substrate enhances the affinity of activator for the APC/C. This cooperativity is represented by a cooperativity factor, $\#$, and we can describe the effect of substrate on the K_D of activator for APC/C ($K_{D(AC)}$) as $\#K_{D(AC)}$. A value of $\# < 1$ indicates that there is positive cooperativity, such that substrate enhances the

affinity (i.e. reduces K_D) of activator for APC/C by a factor of $\#$. If there is no cooperativity, $\# = 1$. Importantly, since the overall K_D describing the formation of the trimolecular complex is independent of the order in which the components assemble, $\#$ is a constant in the system. Thus, if substrate affects the affinity of activator for APC/C by a factor of $\#$, then APC/C must affect the affinity of activator for substrate by the same factor.

(B) In this study, we measured three of the four dissociation rates in the equations of the simplified model, allowing us to calculate approximate K_D values for the four interactions as follows. Figure 6A provides k_{off} for the activator-APC/C complex ($5 \times 10^{-4} \text{ s}^{-1}$) and Figure 6C provides an estimate of the reduced k_{off} for this complex in the presence of substrate ($5 \times 10^{-5} \text{ s}^{-1}$). The rate of substrate dissociation from the trimolecular complex is provided in Figure 6B (0.01 s^{-1}). This rate is the sum of two routes of substrate dissociation (illustrated below in panel C): dissociation of substrate monomer from the activator-APC/C complex and dissociation of substrate-activator dimer from the APC/C. We have seen that the latter rate is very slow ($5 \times 10^{-5} \text{ s}^{-1}$), so we can consider it negligible and assume that the substrate dissociation rate we measure primarily reflects substrate dissociation from the activator-APC/C complex. The three measured k_{off} values are inserted in panel B in bold; activator-substrate k_{off} was not measured (n.m.). Assuming a diffusion-limited association rate for globular proteins ($k_{\text{on}} \sim 10^6 \text{ M}^{-1} \text{ s}^{-1}$), we can roughly estimate the K_D values of these interactions ($K_D = k_{\text{off}} / k_{\text{on}}$). Using the values of $K_{D(\text{AC})}$ and $\#K_{D(\text{AC})}$, we find that $\# = \#K_{D(\text{AC})} / K_{D(\text{AC})} = 0.1$ in our system (although our analysis of the Cdc23-A

mutant in Figure 6C might suggest a higher value). This allows us to estimate the value of $K_{D(AS)} = \#K_{D(AS)} / \# = 100$ nM. Clearly, these values are approximations, but they provide a useful illustration of the relative strengths of the various interactions.

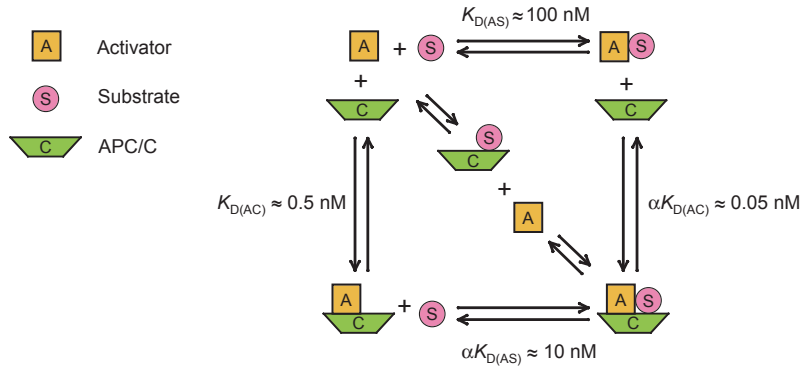
(C) We can use this model to examine how the APC/C mutations analyzed in this study might affect the substrate dissociation rate and thus the processivity of ubiquitination. Substrate dissociation rate from the trimeric **A-C-S** complex is the combination of the two substrate dissociation rates, k_1 and k_2 , labeled on the simplified model. k_1 is the dissociation rate of substrate from the activator-APC/C complex, and k_2 is the dissociation rate of the activator-substrate complex from the APC/C.

(D) In this study, we measured values of k_2 using wild-type, Cdc23-A, and Doc1-4A APC/C (Figures 6C and 7B). We also measured substrate dissociation rate ($\sim k_1$) with wild-type APC/C (Figure 6B) but were unable to measure this rate with Cdc23-A or Doc1-4A APC/C, as steady-state substrate binding to these mutants is not detectable (Figure S9). We were, however, able to estimate these values using the values of $K_{D(AS)}$ and $\#$ that we calculated in panels (A) and (B) above. In Cdc23-A APC/C, $\#K_{D(AS)} = 0.1 \times 100 = 10$ nM (assuming that $\#$ is the same as the value calculated for wild-type, as discussed above). Using an estimated k_{on} of $10^6 \text{ M}^{-1}\text{s}^{-1}$, k_1 for the Cdc23-A mutant is approximately 0.01 s^{-1} . With Doc1-4A APC/C, however, $K_{D(AC)}$ and $\#K_{D(AC)}$ were both ~ 0.5 nM (Figure 7B), and therefore $\# = 1$. With no cooperativity, $\#K_{D(AS)} = 1 \times 100 = 100$ nM, and an estimated k_{on} of $10^6 \text{ M}^{-1}\text{s}^{-1}$ gives a k_1 of 0.1 s^{-1} , significantly greater than wild-type

or Cdc23-A APC/C. These dissociation rates are very approximate estimates, but the relative differences among them provide a clear explanation for the levels of processivity we observe in our activity assays. The total substrate dissociation rate from the enzyme is equal to the sum of k_1 and k_2 . Since k_1 is much larger than k_2 , changes in this rate have a much greater effect on processivity. The key point of the model is that in Cdc23-A APC/C, k_1 is unchanged and k_2 is increased only ten-fold, resulting in only a minor change in the total substrate dissociation rate ($k_1 + k_2$), which we expect would not cause a detectable processivity defect in our assays. In the Doc1 mutant, however, both k_1 and k_2 are increased, resulting in a significantly faster total substrate dissociation rate that corresponds to the major processivity defect we observe in activity assays. A consideration of these dissociation rates might also explain the different processivities of the two activators, as observed in Figure 3. Cdc20 may bind some substrates with increased affinity, resulting in a decreased k_1 that could increase processivity.

Figure 13

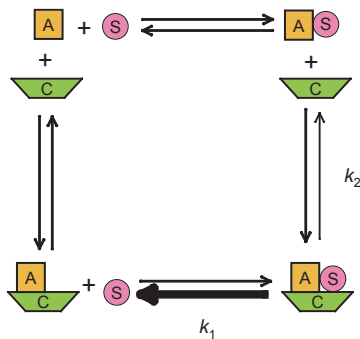
A



B

Interaction	Equation	k_{off} (s^{-1})	Estimated K_D (nM)
Activator - APC/C		5×10^{-4}	$K_{D(AC)} \approx 0.5$
Activator - Substrate in the presence of APC/C		0.01	$\alpha K_{D(AS)} \approx 10$
Activator - APC/C in the presence of substrate		5×10^{-5}	$\alpha K_{D(AC)} \approx 0.05$
Activator - Substrate		n.m.	$K_{D(AS)} \approx 100$

C



D

APC/C	k_1 (s^{-1})	k_2 (s^{-1})	$k_1 + k_2$ (s^{-1})
Wild-Type	0.01	0.00005	0.01005
Cdc23-A	0.01	0.0005	0.0105
Doc1-4A	0.1	0.0005	0.1005

Table 1. Strain List

Strain	Genotype	Source
yBRT138-F1	<i>MATα ade2-1 his3-11 leu2-3,112 trp1-1::(SIC1-TRP1)10x ura3-1 can1-100 cdc27Δ::KanMX pBRT78 (pRS316-CDC27)</i>	D. Toczyski
yMM1	<i>MATα ade2-1 his3-11 leu2-3,112 trp1-1::(SIC1-TRP1)10x ura3-1 can1-100 cdc27Δ::KanMX APC1-TAP::URA3</i>	This study
yTMN89	<i>MATα ade2-1 his3-11 leu2-3,112 trp1-1::(SIC1-TRP1)10x ura3-1 can1-100 clb5! ::HIS3 pds1! ::LEU2 cdc16! ::KanMX APC1-TAP::URA3</i>	D. Toczyski
yTMN90-1	<i>MATα ade2-1 his3-11 leu2-3,112 trp1-1::(SIC1-TRP1)10x ura3-1 can1-100 clb5Δ::HIS3 pds1Δ::LEU2 cdc23! ::KanMX APC1-TAP::URA3</i>	D. Toczyski
DOM90 (AFS92)	<i>MATα ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100 bar1</i>	A. Straight
yMM2	<i>MATα ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100 bar1 CDC27::cdc27-A2</i>	This study
yMM3	<i>MATα ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100 bar1 CDC27::cdc27-A2</i>	This study
yMM4	<i>MATα ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100 bar1 CDC23::cdc23-A</i>	This study
yMM5	<i>MATα ade2-1 his3-11 trp1-1 ura3-1 can1-100 bar1 KanMX-GAL1-CDC27 leu2-3,112::cdc27-A1A2-LEU2</i>	This study
yMM6	<i>MATα ade2-1 his3-11 leu2-3,112 trp1-1 can1-100 bar1 ura3-1::GAL1-CDC20-URA3</i>	This study
yMM7	<i>MATα ade2-1 his3-11 leu2-3,112 trp1-1 can1-100 bar1 CDC27::cdc27-A2 ura3-1::GAL1-CDC20-URA3</i>	This study
yMM8	<i>MATα ade2-1 his3-11 leu2-3,112 trp1-1 can1-100 bar1 CDC23::cdc23-A ura3-1::GAL1-CDC20-URA3</i>	This study
yMM9	<i>MATα ade2-1 his3-11 leu2-3,112 trp1-1 can1-100 bar1 ura3-1::GAL1-CDH1-URA3</i>	This study
yMM10	<i>MATα ade2-1 his3-11 leu2-3,112 trp1-1 can1-100 bar1 CDC27::cdc27-A2 ura3-1::GAL1-CDH1-URA3</i>	This study
yMM11	<i>MATα ade2-1 his3-11 leu2-3,112 trp1-1 can1-100 bar1 CDC23::cdc23-A ura3-1::GAL1-CDH1-URA3</i>	This study
yMM12	<i>MATα ade2-1 his3-11 leu2-3,112 trp1-1 can1-100 bar1 ura3-1::GAL1-MYC-CDH1-URA3</i>	This study
yMM13	<i>MATα ade2-1 his3-11 leu2-3,112 trp1-1 can1-100 bar1 CDC27::cdc27-A2 ura3-1::GAL1-MYC-CDH1-URA3</i>	This study
yMM14	<i>MATα ade2-1 his3-11 leu2-3,112 trp1-1 can1-100 bar1 CDC23::cdc23-A ura3-1::GAL1-MYC-CDH1-URA3</i>	This study

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Chapter Three

Conclusions

A model for ubiquitination by the APC

This leaves us with our current view of protein ubiquitination by the APC (Figure 1). First, an activator binds with very high affinity to the APC core, possibly inducing a conformational change that results in more productive positioning of the binding sites for the E2 and substrate. A substrate then binds to the APC-activator complex using multiple degradation sequences, often found in disordered regions, that interact with sites on both the activator and APC core, possibly via the Doc1 subunit. The E2-ubiquitin conjugate then binds to the APC, positioning the E2 near the substrate and perhaps resulting in a conformational change in the E2 that stimulates its activity. Residues near the E2 active site then promote catalysis. Several E2s may cycle on and off the APC before one is successfully attacked by a substrate lysine; in yeast, the initial successful attack will likely be on a ubiquitin conjugated to Ubc4. After this initial ubiquitin is transferred to the substrate and the E2 dissociates, additional E2s will then bind and be attacked successfully by lysines on the substrate or by specific lysines on ubiquitin itself, as determined by residues near the active site of the E2. Flexibility in the substrate and APC helps accommodate the modification of many different lysines and the formation of long ubiquitin chains. Substrate residence time, and thus the number of ubiquitins added in a single substrate-binding event, is determined by substrate dissociation rate.

Many features of this model remain mysterious. Substrate recognition is still poorly understood: while the roles of some APC subunits are becoming clear, a direct binding site for substrates on the APC core has not been identified, and the amino acid sequences that target substrates to the APC-activator complex are still poorly defined. If substrate binding is multivalent as we propose, it will be interesting to identify which sequences on the substrate are involved in interacting with the activator and which are involved in interacting with the APC core. It is also possible that a single degradation sequence is sandwiched between the activator and APC, such that only one degradation sequence is required to make multiple contacts with the activator and APC core.

Activators clearly play a central role in APC function, but it is not clear how these proteins alter enzymatic activity: they may do more to activate the APC than just participate in substrate binding, and we need to know more about the conformational changes, if any, that activators induce in the APC core. The C-box binding site on the APC core remains to be identified. Locating it will facilitate characterization of the structural changes that may accompany activator binding, providing insight into APC mechanism and regulation. Dare I suggest photoactivatable crosslinking?

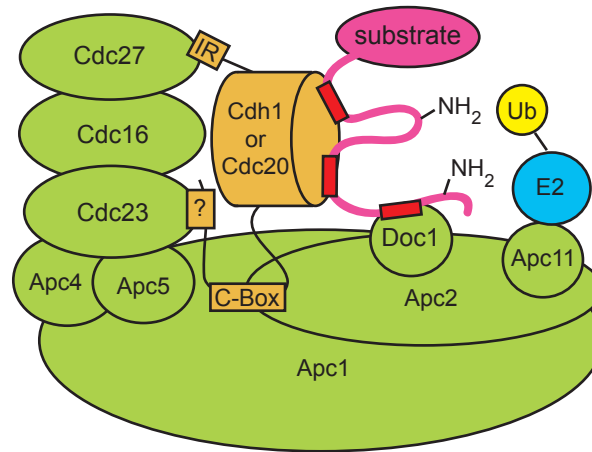
Finally, it is unclear how E2s are stimulated upon binding to the RING domain, and the structural determinants of E2 lysine specificity remain to be identified. It

will be interesting to see how different E2s can position ubiquitin for attack, and what structural aspects determine the preference for unstructured substrates. Clearly, an understanding of these and many other problems will be found in a combination of detailed structural studies and quantitative analyses of protein-protein interactions and enzyme kinetics.

Figure 1. A speculative model of protein ubiquitination by the APC

Substrate is likely to bind multivalently to both activator and the APC using degradation sequences (red rectangles) in disordered substrate regions. The APC positions substrates so that multiple lysine side chains can effectively attack the E2-ubiquitin bond. Residues near the E2 active site promote catalysis, which is stimulated upon binding to the APC. Flexibility in both the substrate and APC may help accommodate the modification of many lysines and the formation of long polyubiquitin chains.

Figure 1

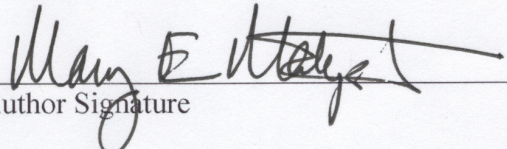


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