Title
The Role of E2 Affinity in Ubiquitination by the Anaphase-Promoting Complex

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The Role of E2 Affinity in Ubiquitination by the Anaphase-Promoting Complex

by

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DISSERTATION

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The text of Chapter 2 is a reprint of the material in revision for publication at *The Journal of Biological Chemistry* at the time this dissertation was presented (citation below). The coauthor David O. Morgan listed in this publication directed and supervised the research in this chapter. The second author Jeanette L. Tenthorey performed the experiments found in Figures 1A & B of this chapter.

Girard, J. R., Tenthorey, J. L., and Morgan, D. O. (2015) An E2 Accessory Domain Increases Affinity for the Anaphase-Promoting Complex and Ensures E2 Competition. *J Biol Chem. In Revision.*

Abstract

The anaphase-promoting complex/cyclosome (APC/C) is a large, multi-subunit E3 ubiquitin ligase that governs key mitotic events in eukaryotes. The APC/C catalyzes the transfer of ubiquitin from an E2 ubiquitin-conjugating enzyme to a protein substrate, building polyubiquitin signals that mark substrates for destruction by the proteasome. In yeast, the APC/C collaborates with two E2s, Ubc4 and Ubc1: APC/C^{Ubc4} catalyzes the attachment of the initial ubiquitin to the substrate, while APC/C^{Ubc1} elongates ubiquitin chains. Both E2s seem to interact with the same site on the APC/C, and it is not clear how their competing activities collaborate to generate a polyubiquitin chain that is sufficient for proteosomal recognition. We hypothesized that E2 synergy requires a finely tuned balance of the affinities of the two E2 proteins for the APC/C, allowing E2s to alternate on the APC/C. In this work, we uncovered new insights into this problem by studying the role of a C-terminal ubiquitin-associated (UBA) domain in Ubc1. Deletion of the UBA domain decreased the length of polyubiquitin chains and increased the concentration of Ubc1 required for half-maximal APC/C activity *in vitro*. Surprisingly, the stimulatory effect of the UBA domain does not depend on previous initiation of a ubiquitin chain on the substrate, suggesting that the UBA domain does not promote polyubiquitination by interacting with ubiquitin on a substrate. Instead, deletion of the UBA domain reduced Ubc1 binding to the APC/C. Finally, deletion of the UBA domain from Ubc1 decreased its ability to compete with Ubc4 and reduced polyubiquitin chain length, while attachment of the UBA domain to Ubc4 increased its ability to compete with Ubc1 and reduced polyubiquitin chain length. Thus, the extra affinity provided by the UBA domain of Ubc1 ensures efficient polyubiquitination of substrate by balancing Ubc1 affinity with that of Ubc4, resulting in an efficient collaboration between the two E2s.

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Chapter 1

Introduction

The Cell Cycle and Mitosis

All living organisms are composed of cells and these cells depend on the cell cycle to reproduce (1). The cell cycle is a highly ordered and regulated process composed of distinct stages, during which cells duplicate their contents and then divide them equally into two daughter cells (1). One key component of the cell that must be copied and divided without error is the genetic information encoded in the cell's chromosomes (1-3). Errors in chromosome segregation can cause many problems for single-celled and multicellular organisms such as cell death, cancer, and genetic disorders (1-3).

Duplicated chromosomes are segregated during the mitosis stage of the cell cycle (1-3). Chromosome segregation is controlled by an important and highly conserved cell cycle-regulatory enzyme called the anaphase-promoting complex/cyclosome (APC/C) (1-3). APC/C activity is essential for progression through mitosis, and its activation is highly regulated to prevent premature segregation of chromosomes (1-3).

The Anaphase-Promoting Complex/Cyclosome (APC/C) in Mitosis

The APC/C governs passage through the metaphase-to-anaphase transition during mitosis (1-3). In metaphase duplicated chromosomes attach to the mitotic spindle, the molecular machinery that pulls chromosomes apart. However, despite being attached to the mitotic spindle, chromosomes do not separate until anaphase because they are held together by protein rings that resist the pulling forces of the spindle (1-3). The APC/C promotes anaphase by initiating events that lead to cleavage of the protein rings that hold chromosomes together as well as elongation of the mitotic spindle that segregates the chromosomes (1-3). The APC/C does this by ubiquitinating key protein substrates, thus marking these substrates for destruction (proteolysis) (1-4).

Protein Ubiquitination

Ubiquitin is a small, 76-residue protein important for many cellular processes, including signaling and proteolysis. Ubiquitin gets attached to a protein substrate via a three-enzyme cascade involving E1, E2, and E3 enzymes (Fig. 1A) (1,4,5).

First, an E1 ubiquitin-activating enzyme uses the energy of ATP to covalently link the C-terminus of ubiquitin to the E1's catalytic cysteine residue, creating a highly labile thioester bond (1,4,5). An E2 ubiquitin-activating enzyme then binds to the E1, allowing the transfer of the ubiquitin from the catalytic cysteine of the E1 to the catalytic cysteine of the E2, once again creating a labile thioester (1,4,5). The E2 then binds to an E3 ubiquitin ligase, which also binds to a substrate protein, and the ubiquitin is transferred from the E2 to a lysine residue on the substrate, creating a stable isopeptide bond (Fig. 1A) (1,4,5).

There are two major classes of E3 ubiquitin ligases, RING domain ligases which promote transfer of the ubiquitin directly from the E2 to the substrate, and HECT domain ligases that transfer ubiquitin indirectly (4,5). The APC/C is a RING domain E3 ubiquitin ligase and therefore promotes direct transfer of ubiquitin from the E2 to the substrate (Fig. 1B) (1-5).

Distinct Steps of Protein Ubiquitination

The first step of protein ubiquitination is the attachment of ubiquitin to a lysine residue on a substrate protein; this process is called monoubiquitination or ubiquitin chain-initiation (Fig 1A,B) (1,4-6). After ubiquitin is attached to a substrate lysine, ubiquitin can be attached to one of seven lysine residues on ubiquitin itself (1,4-6). This process is called polyubiquitination or ubiquitin chain elongation, since several of these transfers result in the formation of a polyubiquitin chain on a substrate (Fig. 1A) (1,4-6).

Polyubiquitin chains can be linked through specific lysine residues, and the type of linkage is important for determining what happens downstream of ubiquitination (1,4-6). For example, polyubiquitin chains linked through lysine 48 (K48) promote proteolysis of substrates

(1,4,5,7-9). The proteasome recognizes these polyubiquitin signals and destroys the proteins they are attached to (1,4,5,7-9). Detailed study of the proteasome *in vitro* demonstrated that it efficiently destroys substrates with a K48-linked polyubiquitin chain containing 4 or more ubiquitins (9). Thus, the length of the polyubiquitin chain is also important for determining whether a protein gets degraded.

The specificity of the ubiquitination reaction in terms of the products formed (i.e. whether a substrate is monoubiquitinated or polyubiquitinated through a specific lysine) is generally encoded by the E2 active site, not the E3 (1,5-7,10-16). Therefore, the outcome of the ubiquitination reaction is determined in part by the specificity of the E2s that work with a given E3.

In the budding yeast, *Saccharomyces cerevisiae* the APC/C works with two different E2s, Ubc4 and Ubc1, to carry out the different steps of ubiquitination (6). The APC/C collaborates with Ubc4 to initiate ubiquitin chains on substrate lysines, and it collaborates with Ubc1 to elongate ubiquitin chains specifically through K48 of ubiquitin (Fig. 2) (6,12).

Both of these E2s are important for APC/C function *in vivo* (6). However, as one might predict, Ubc1 is more important for promoting proteolysis of APC/C substrates because it generates K48-linked polyubiquitin signals that are efficiently recognized by the proteasome (6). Therefore, it is not surprising that Ubc1 is essential for progression through anaphase while Ubc4 is dispensable (6). In the absence of Ubc4, Ubc1 can initiate ubiquitin chains albeit with lower efficiency, resulting in a delay in the timing of APC/C substrate degradation, but ultimately normal progression through anaphase (6).

E2 Binding to the APC/C

The APC/C is a large (>1 MDa), complex enzyme composed of many subunits (1-4). In the budding yeast *Saccharomyces cerevisiae,* the APC/C has 13 core subunits, most of which are essential for progression through mitosis (1,2,4). The APC/C also requires the association of an

activator subunit that acts as a substrate adaptor and causes a conformational change in the APC/C that enhances E2 binding (2,3,17).

Until about five years ago, structural information about the APC/C, such as subunit locations, structures of individual subunits, and how the subunits associate with one another, was limited. However, recent advances in high-resolution electron microscopy have given us a wealth of structural information about both the yeast and human APC/C, which are very similar structurally (2,18-25). Structures of the APC/C bound to an activator and substrate have been determined (19,22-25), but structure of the APC/C bound to an E2 are technically challenging due to low affinity interactions between E2s and E3s.

Nevertheless, some structures of the human APC/C bound to its cognate E2s have been published in the last few months (18,20). While this research has illuminated some aspects of how E2s bind to the APC/C, several questions remain about E2-APC/C collaboration. One such important question is whether or not the two E2s that carry out the two different steps of ubiquitination bind to the same site on the APC/C.

The human APC/C uses the E2s UbcH10 and Ube2s to initiate and elongate ubiquitin chains, respectively (15,16). While UbcH10 binds to the APC/C using a well-characterized interface (18,20) that has been demonstrated for several other E2-RING E3 pairs (5,26-28) (See structure in Fig. 3), Ube2s appears to depend more heavily on non-canonical binding (10,15,19). This introduces the possibility that the two E2s bind to different sites on the APC/C. If this is the case, do these E2s bind simultaneously or sequentially?

Biochemical studies using yeast APC/C suggest that the E2s Ubc4 and Ubc1 do not bind simultaneously, but instead directly compete for APC/C binding (6,17). This raises the question of how the competing E2 activities balance to generate a polyubiquitin signal sufficient for proteasomal recognition?

In this study, we hypothesized that E2s Ubc4 and Ubc1 finely balance their affinities for the APC/C. We addressed the question of what structural elements contribute to the balance of E2

affinities by studying the role of an accessory domain of Ubc1. Both Ubc4 and Ubc1 have characteristic E2 ubiquitin-conjugating domains (UBCs), which contain residues important for catalysis and reaction specificity, but Ubc1 also contains an extra domain at its C-terminus (29) (Fig. 3). The domain is a type of ubiquitin binding domain called a ubiquitin associated (UBA) domain (29,30). In this study we addressed the role of the UBA domain in balancing Ubc1's affinity with that of Ubc4. We found that deleting the UBA domain decreases Ubc1 affinity for the APC/C and decreases competition between Ubc1 and Ubc4.

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Figure 1. Ubiquitination occurs by a three-enzyme cascade. (A) Ubiquitin (Ub, yellow) is first activated by an E1, or ubiquitin-activating protein (purple square), which couples ATP hydrolysis to 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, magenta). In the case of the APC/C and many other E3s, this final step is repeated several times with the same substrate, resulting in ubiquitination of multiple lysines. In addition, specific lysines on ubiquitin itself can be modified, resulting in the assembly of polyubiquitin chains. (B) The APC/C is a member of the RING-domain family of E3s. These proteins facilitate the final step in ubiquitination by positioning the E2-ubiquitin conjugate next to the substrate, allowing the ε-amino group of a lysine on the substrate to nucleophilically attack the E2-ubiquitin thioester bond, resulting in direct transfer of ubiquitin as shown here. Figure and legend reproduced from Matyskiela and Morgan 2009 (4) with permission.

Figure 1

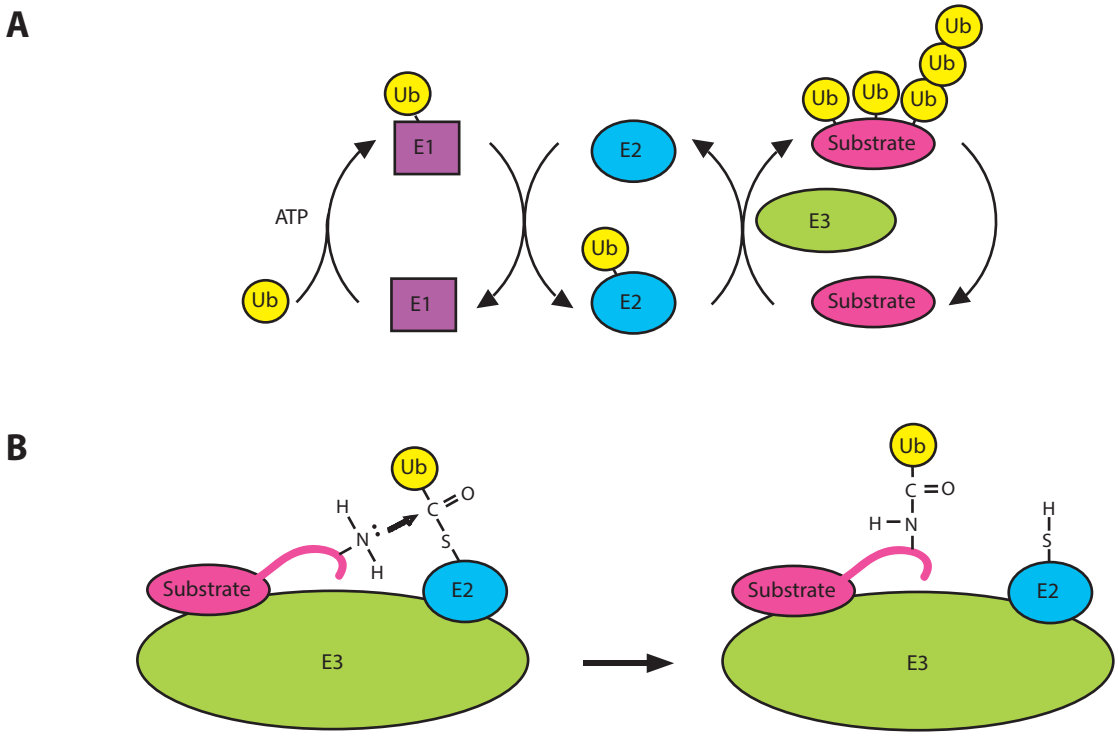
A
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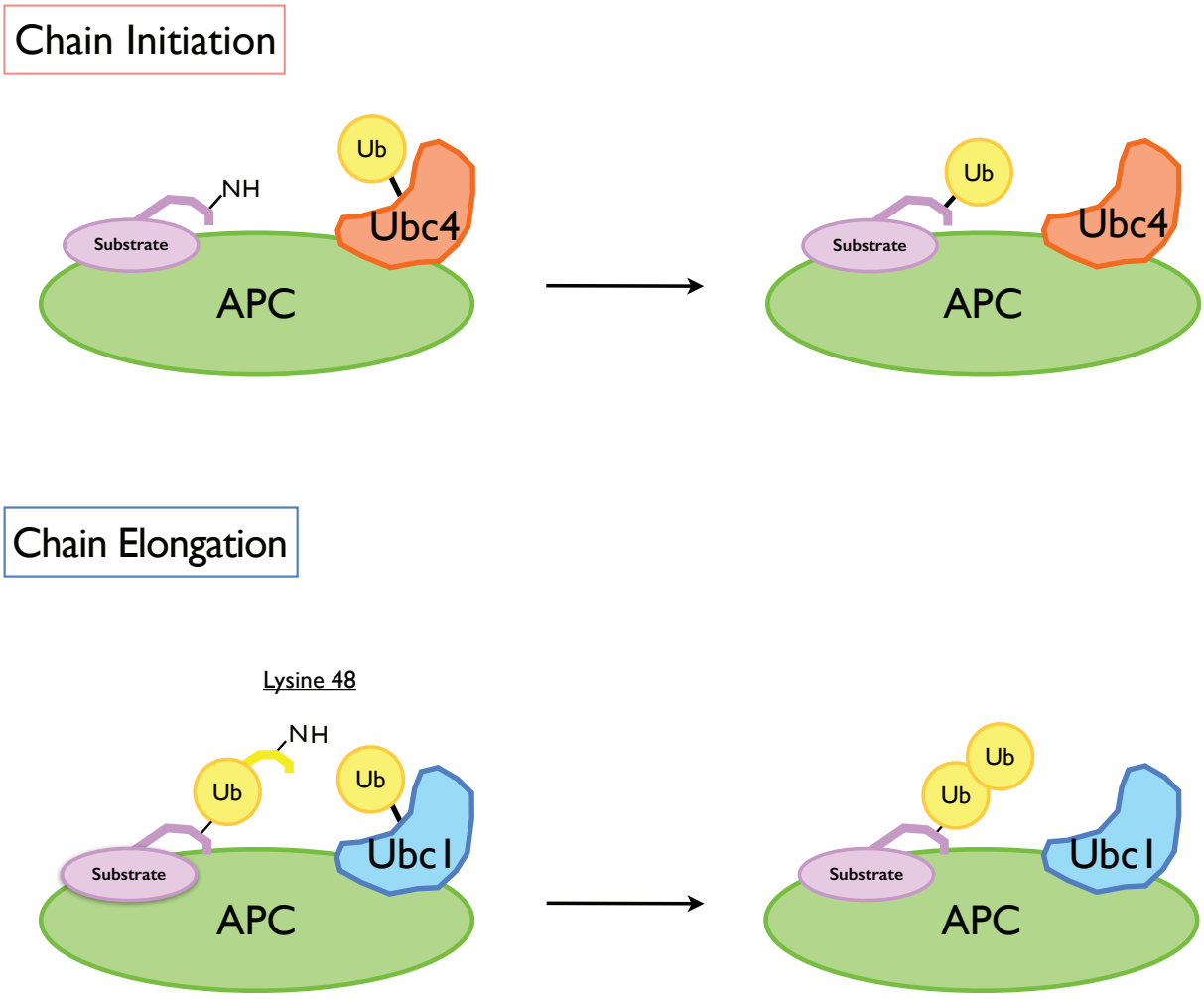
Figure 2. The APC/C uses two E2 ubiquitin-conjugating enzymes, Ubc4 and Ubc1, to initiate and elongate polyubiquitin chains. APC/C (green) associates with the E2 Ubc4 (orange) in order to transfer ubiquitin (Ub, yellow) from the E2 to a lysine on the substrate (magenta). This process is called monoubiquitination or ubiquitin chain initiation (highlighted in orange). Subsequently, APC/C associates with the E2 Ubc1 (blue) in order to transfer ubiquitin from the E2 to lysine 48 of a pre-attached ubiquitin on the substrate. This process is called polyubiquitination or ubiquitin chain elongation (highlighted in blue).

Figure 2


Chain Initiation
Ub
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Substrate
Ubc4
APC
Ub
Substrate
Ubc4
APC
Chain Elongation
Lysine 48
NH
Ub
Ub
Substrate
Ubc1
APC
Ub
Ub
Substrate
Ubc1
APC

Figure 3. The structure of the human APC/C bound to the E2 Ubch10. Three-dimensional structure of the human APC/C determined recently by high-resolution cryo-electron microscopy with close-up of the active site (with subunit names). The individual subunits of the APC/C all appear in different colors. In the active site, a fragment of a substrate (orange line) is sandwiched between the APC/C activator Cdh1 and the APC/C subunit Apc10. The E2 Ubch10 has a covalently linked ubiquitin (Ub, in green which is modeled into this structure in the closed conformation) and binds to the catalytic RING subunit Apc11 (magenta) in the vicinity of the substrate it will modify. The APC/C subunits Apc1 and Apc2 are also close to the E2 binding site. This figure is reprinted by permission from Macmillan Publishers Ltd: (Chang *et al,* Nature, copyright 2015) (20).

Figure 3

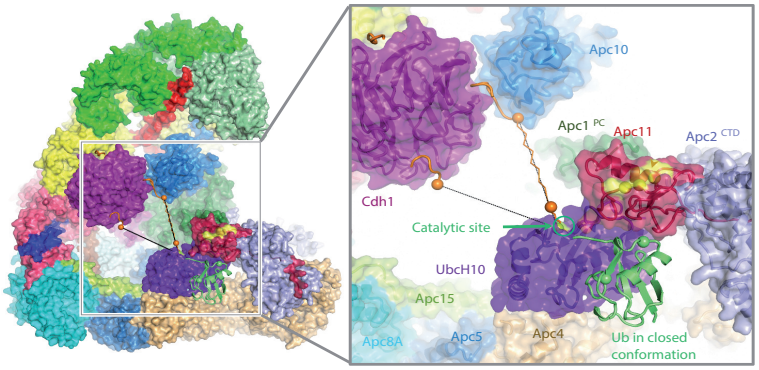

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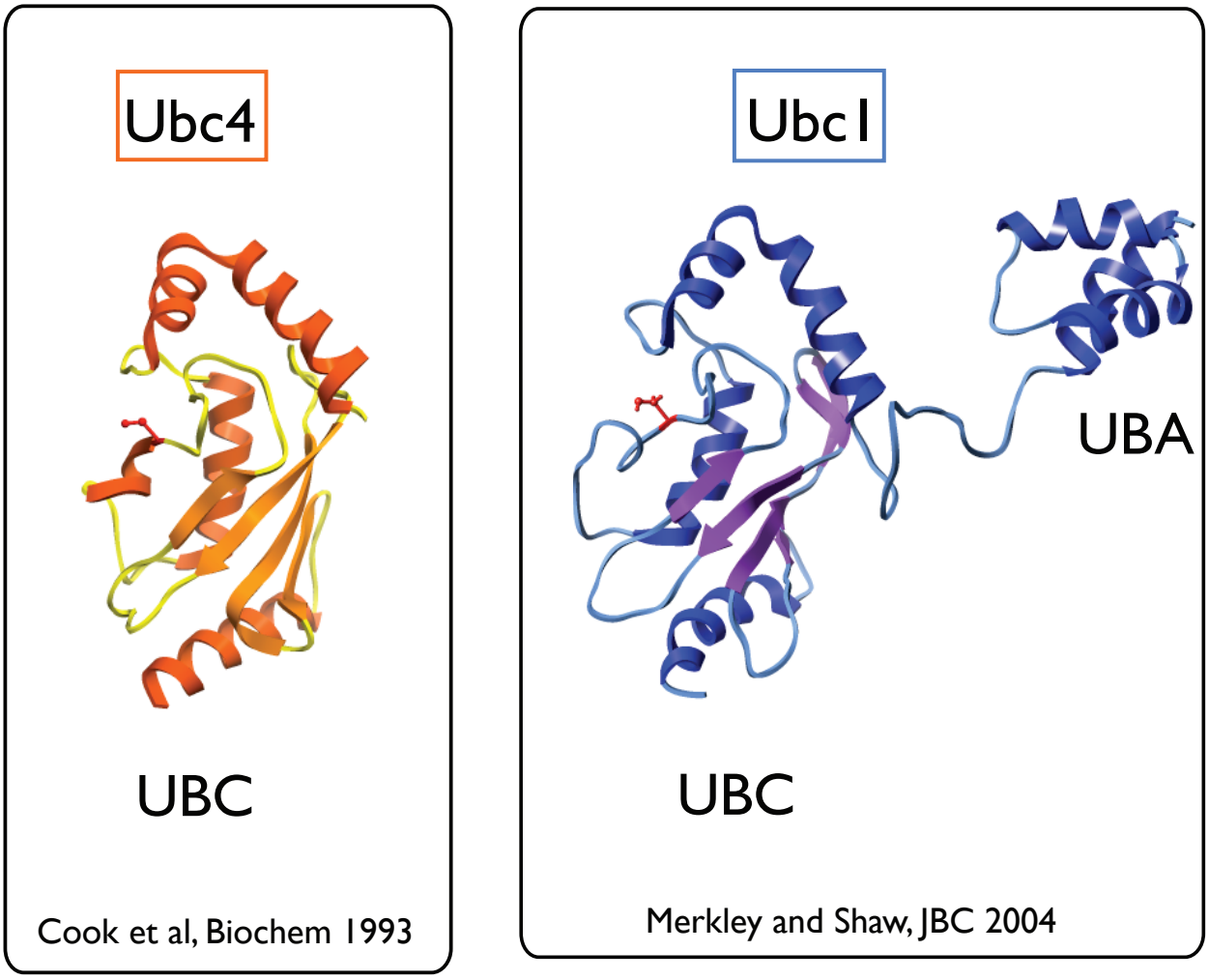
Figure 4. The structures of the E2s Ubc4 and Ubc1. Three-dimensional structures of Ubc4 (orange) and Ubc1 (blue) as determined by x-ray crystallography and nuclear magnetic resonance, respectively. Both E2s contain a UBC domain, a well-characterized structural fold that all E2 ubiquitin-conjugating enzymes possess. For both E2s, the residues important for catalysis and reaction specificity are contained in the UBC domain. In addition to its UBC domain Ubc1 contains an accessory domain at its C-terminus. This domain is an ubiquitin-associated (UBA) domain, characterized by a three-helix bindle. The UBA domain is connected to the UBC domain of Ubc1 via a 22-residue flexible tether. Structures correspond to PDB files IQCQ (Ubc4) and 1TTE (Ubc1) and these structures were determined in the following studies: Cook *et al*, Biochemistry 1993 (31) (Ubc4) and Merkley and Shaw, Journal of Biological Chemistry 2004 (29) (Ubc1).

Figure 4


Ubc4
UBC
Cook et al, Biochem 1993
Ubc1
UBA
UBC
Merkley and Shaw, JBC 2004

Chapter 2

An E2 Accessory Domain Increases Affinity for the Anaphase-Promoting Complex and Ensures E2 Competition

Abstract

The anaphase-promoting complex (APC/C) is a member of the RING family of E3 ubiquitin ligases, which promote ubiquitin transfer from an E2 ubiquitin-conjugating enzyme to a substrate. In budding yeast, the APC/C collaborates with two E2s, Ubc4 and Ubc1, to promote the initiation and elongation, respectively, of polyubiquitin chains on the substrate. Ubc4 and Ubc1 are thought to compete for the same site on the APC/C, but it is not clear how their affinities are balanced. Here, we demonstrate that a C-terminal ubiquitin-associated (UBA) domain enhances the affinity of Ubc1 for the APC/C. Deletion of the UBA domain reduces apparent APC/C affinity for Ubc1 and decreases polyubiquitin chain length. Surprisingly, the positive effect of the UBA domain is not due to an interaction with the acceptor ubiquitin attached to the APC/C substrate or the donor ubiquitin attached to Ubc1 itself. Also, the UBA domain does not bind to the APC/C activator. Instead, our evidence suggests that the UBA domain binds to a site on the APC/C core, thereby increasing Ubc1 affinity and enhancing its ability to compete with Ubc4. The UBA domain is required for normal Ubc1 function and E2 competition *in vivo*. Thus, the UBA domain of Ubc1 ensures efficient polyubiquitination of substrate by balancing Ubc1 affinity with that of Ubc4.

Introduction

The anaphase-promoting complex or cyclosome (APC/C) is a large, multi-subunit E3 ubiquitin ligase that governs key mitotic events in eukaryotes (1,2). Like other members of the RING family of ubiquitin ligases, the APC/C catalyzes the transfer of ubiquitin directly from an E2 ubiquitin-conjugating enzyme to a lysine residue on a protein substrate. Subsequent ubiquitin attachment to lysines on ubiquitin itself then leads to the assembly of polyubiquitin signals that mark substrates for destruction by the proteasome.

Polyubiquitin chain assembly by the APC/C depends on the sequential actions of two distinct E2s. In the budding yeast *Sacharomyces cerevisiae*, the APC/C collaborates with the E2s Ubc4 and Ubc1: first,

APC/C interacts with Ubc4 to catalyze attachment of the initial ubiquitin to a lysine residue on the substrate, after which it interacts with Ubc1 to catalyze ubiquitin attachment to lysine 48 (K48) of a pre-attached ubiquitin, thereby promoting K48-linked polyubiquitin chain assembly (3,4). In human cells, the APC/C collaborates with the E2s UbcH10 and Ube2S to initiate and elongate K11-linked polyubiquitin chains, respectively (5-12).

Members of the RING family of ubiquitin ligases are generally thought to bind the E2 via a canonical interface between the RING subunit of the E3 and the conserved ubiquitin-conjugating (UBC) domain of the E2 (13). In many cases, this RING-E2 interaction enhances catalysis, primarily by promoting a productive 'closed' orientation of the ubiquitin linked to the E2 active-site cysteine (7,14-19).

Interestingly, some E2s appear to interact with non-canonical sites on the E3. In the case of the human APC/C, the initiating E2, UbcH10, participates in canonical RING binding as well as binding the winged-helix bundle of the cullin subunit Apc2, using the backside of the UBC domain (9). Both of these interaction surfaces are critical for APC/C^{UbcH10} activity (9).

The second E2 that operates with the human APC/C, Ube2S, appears to depend almost entirely on non-canonical interactions with the E3. Ube2S has a disordered C-terminal extension that binds a site on the Apc2 subunit (5,10-12,20). Deletion of this C-terminal extension greatly reduces the binding of Ube2S to the APC/C *in vitro* (5,8,10). In contrast, mutations in the canonical E2-binding site of the RING subunit Apc11 do not cause a defect in APC/C^{Ube2S} activity (10). In addition, a distinct face of the RING subunit seems to interact with the ubiquitin that attacks the Ube2S-ubiquitin conjugate, suggesting that Ube2S is not activated by the canonical mechanism (10). Finally, there is recent evidence that the binding of the activator subunit Cdh1 to the APC/C causes a conformational change that exposes the canonical E2-binding site of the RING subunit, thereby enhancing UbcH10 binding but having little effect on Ube2S binding (8,10). These results suggest that UbcH10 and Ube2S bind to different sites on the human APC/C, raising the possibility that they can bind simultaneously to promote polyubiquitin chain assembly.

Unlike the E2s that operate with the human APC/C, the yeast E2s, Ubc4 and Ubc1, both seem to interact with the canonical RING binding site. For example, Ubc1 inhibits the rapid substrate turnover catalyzed by Ubc4 in APC/C reactions *in vitro*, suggesting that the two E2s compete for the same binding site (4). Furthermore, addition of the activator Cdh1 to the APC/C promotes the binding of both Ubc4 and Ubc1, suggesting that both E2s employ the canonical binding site on the RING subunit (21).

Although yeast Ubc1 seems to depend on a canonical RING interaction for its function, it also carries an additional feature that may modulate its interactions with the APC/C. The C-terminus of Ubc1 is linked by a 22-residue flexible tether to a ubiquitin-associated (UBA) domain, a type of ubiquitin-binding domain characterized by a three-helix bundle of approximately 50 residues (22). The UBA domain of Ubc1 has been shown to bind mono-ubiquitin with low affinity (K_D ~230 μM) (23) but has a ~6-fold higher affinity (K_D ~37 nM) for K48-linked tetra-ubiquitin (24).

Deletion of the UBA domain and flexible tether (Ubc1ΔUBA) results in a correctly folded and catalytically active UBC domain (25) that is charged normally with ubiquitin by E1 ubiquitin-activating enzyme and retains its catalytic specificity for K48 of ubiquitin (4). Ubc1ΔUBA and wild-type Ubc1 exhibit similar APC/C-independent catalytic rates with ubiquitin as substrate (3), further suggesting that the UBA domain is not required for catalytic activity.

However, studies of the APC/C reaction *in vitro* indicate that deletion of the UBA domain reduces the length of K48-linked polyubiquitin chains on APC/C substrates. In addition, the concentration of Ubc1ΔUBA required for half-maximal APC/C activity is increased 10-fold relative to wild-type (4), suggesting that the UBA domain promotes binding to some site on the APC/C-substrate complex. However, previous studies indicate that the UBA domain does not bind the ubiquitin covalently linked to the UBC domain (the donor ubiquitin) (26) or the ubiquitin attacking the E2-ubiquitin conjugate (the acceptor ubiquitin) (3). In addition, UBA domains in other proteins have been shown to bind non-ubiquitin folds (27-29). It therefore remains unclear how a putative ubiquitin-binding domain promotes a productive interaction between Ubc1 and the APC/C.

Here, we set out to understand the mechanism by which the UBA domain exerts its effects on APC/C activity *in vitro* and *in vivo*. Our evidence suggests that the UBA domain binds not to ubiquitin but directly to the APC/C core, thereby boosting Ubc1 affinity and allowing it to compete effectively with Ubc4.

Results

The UBA domain does not contribute to RING-mediated stimulation of E2 catalytic activity

Previous evidence suggests that the UBA domain does not contribute to the APC/C-independent E2 catalytic rate or affinity for the acceptor ubiquitin (3). Here, we tested whether the UBA domain has an impact on these parameters when Ubc1 is bound to its RING E3 partner. In other systems, binding of an E2 to the RING orients the donor ubiquitin in a 'closed' conformation that greatly enhances E2 catalytic function (7,14-19). Moreover, the RING subunit of human APC/C promotes Ube2S activity through a unique interaction between the acceptor ubiquitin and a specific surface of the RING domain (10). It was therefore conceivable that the UBA domain could contribute to Ubc1 catalytic rate or acceptor ubiquitin affinity only in the presence of the RING, or that the UBA domain could bind directly to the RING. To explore these possibilities, we first set out to examine what role, if any, the RING subunit of APC/C plays in catalytic activation of Ubc1 or binding the acceptor ubiquitin.

We addressed this question with a diubiquitin synthesis assay, in which Ubc1 is charged with radiolabeled K48R-ubiquitin (donor), after which unlabeled wild-type ubiquitin (acceptor) is added at increasing concentrations, leading to the formation of radiolabeled K48-linked diubiquitin, which cannot be elongated further. Ubc1 cannot be recharged by E1 in this assay due to inactivation of E1 and free E2, and thus the assay measures a single turnover of E2, allowing estimates of Ubc1 catalytic rate and affinity for acceptor ubiquitin (3,18).

We carried out these studies with purified Apc11, the RING domain-containing subunit of APC/C. We found that Apc11 could be expressed recombinantly in *E. coli* after deletion of the N-terminal 34

residues, which contain the cullin-binding region and a flexible linker. The truncated Apc11 protein was expressed and purified as an N-terminal GST fusion (GST-Apc11ΔN) and added to diubiquitin synthesis assays.

GST-Apc11ΔN caused a massive stimulation of diubiquitin synthesis by Ubc1 (Fig. 1A), to the extent that reactions containing GST-Apc11ΔN were carried out on ice for 5 seconds to prevent depletion of the charged E2. Quantification of results from three separate experiments indicated that GST-Apc11ΔN stimulated the maximal catalytic rate of Ubc1 about 700-fold, from 0.0002 s^{-1} to 0.14 s^{-1} (Fig. 1A). GST-Apc11ΔN caused a minor ~2-fold reduction in K_M for the acceptor ubiquitin, from ~840 μM to ~435 μM (Fig. 1A), suggesting that Apc11 does not stimulate Ubc1 by interacting with the acceptor ubiquitin but rather by allosterically activating the E2 or by orienting the E2-donor ubiquitin conjugate for successful attack. This mechanism is distinct from the mechanism by which human APC/C stimulates the E2 Ube2S, which is primarily through decreasing the K_M for acceptor ubiquitin by ~40-fold via Apc11 binding directly to the acceptor ubiquitin (10).

If Ubc1 and Ubc4 bind to the same site on Apc11, then one might expect that Apc11 can also stimulate Ubc4 catalytic activity. Because Ubc4 does not readily form diubiquitin in this assay, we used a modified assay in which unlabeled sea urchin cyclin B N-terminal fragment ($CycB^N$) was used as an acceptor substrate instead of ubiquitin. Due to the low affinity of $CycB^N$ for Ubc4, it is difficult to saturate with substrate. Thus, instead of measuring activity across a range of substrate concentrations, we added increasing amounts of GST-Apc11ΔN to Ubc4 and Ubc1 assays with sub-saturating substrate. The catalytic rate of both E2s increased significantly with the concentration of GST-Apc11ΔN (Fig. 1B). The concentration of GST-Apc11ΔN required for half-maximal activity was ~4-fold lower for Ubc1 (35 μM) than it was for Ubc4 (135 μM), suggesting that Ubc1 might have a slightly higher affinity for the RING.

To measure the contribution of the UBA domain to stimulation by GST-Apc11ΔN, we compared Ubc1 and Ubc1ΔUBA in diubiquitin synthesis assays. We used a sub-saturating concentration of GST-Apc11ΔN to prevent depletion of the charged E2 and reduce experimental variability; since GST-

Apc11ΔN greatly stimulates Ubc1, RING-stimulated Ubc1 activity represents the majority of the activity in this assay. We found that deletion of the UBA domain had a minor effect on activation by Apc11; in multiple experiments, Ubc1ΔUBA exhibited a slightly lower catalytic rate than wild-type Ubc1 (0.0016 s^{-1} and 0.0040 s^{-1}, respectively), and a slightly higher K_M for the acceptor ubiquitin (425 μM and 145 μM, respectively) (Fig. 1C). In a GST-Apc11ΔN dose response, the UBA domain had no impact on the half-maximal concentration of GST-Apc11ΔN (25 μM for Ubc1ΔUBA and 30 μM for Ubc1), indicating that the UBA domain does not affect E2 affinity for Apc11 (Fig. 1D). Deletion of the UBA domain caused a minor decrease in maximal catalytic activity (0.03 s^{-1} for Ubc1 and 0.02 s^{-1} for Ubc1ΔUBA) (Fig. 1D). Our results suggest that the UBA domain does not contribute significantly to E2 affinity for the acceptor or donor ubiquitin, E2 affinity for the RING subunit, or RING-dependent Ubc1 activation.

The UBA domain does not bind ubiquitin attached to APC/C substrate

Our results suggest that the UBA domain does not bind to the acceptor ubiquitin in assays where Ubc1 is bound to the RING subunit of the APC/C. It remained possible, however, that some other component of the APC/C orients the acceptor ubiquitin on an APC/C substrate, allowing the ubiquitin to bind the UBA domain. To rule out this possibility, we determined the effects of the UBA domain in APC/C reactions with a substrate that lacks pre-attached ubiquitin.

We used ^{125}I-labeled single-lysine $CycB^N$ as substrate, and carried out APC/C reactions with K48R ubiquitin, ensuring that the substrate could be modified only once with a single ubiquitin. We found that the half-maximal concentration of Ubc1ΔUBA with this substrate was 10-fold higher than the half-maximal concentration of wild-type Ubc1 (0.6 μM for Ubc1 and 6 μM for Ubc1ΔUBA; Fig. 2). This is the same difference in apparent Ubc1 affinity that is seen with wild-type ubiquitin in reactions with conventional substrates (4), suggesting that Ubc1ΔUBA is defective in both initiation of a ubiquitin chain and subsequent polyubiquitination. Thus, the UBA domain promotes ubiquitination even when there is no

ubiquitin on the substrate, further arguing that the effects of the UBA domain do not depend on its interaction with ubiquitin.

The UBA domain does not bind to APC/C activator

Since the UBA domain does not interact with ubiquitin attached to APC/C substrate, we asked if the UBA domain binds other components of the APC/C. We first tested the possibility that the UBA domain interacts with the activator subunit, by analyzing the effect of the UBA domain in reactions where activator subunit is not present.

We used a recently devised APC/C assay in which it is possible to measure activity in the absence of activator (21). Although the activator subunit is normally required for substrate recruitment, this requirement can be bypassed by using a radiolabeled substrate (the N-terminal region of securin) directly fused to the Apc10 subunit of the APC/C ($Securin^{N}$-Apc10). Some ubiquitination of this substrate occurs in the absence of activator, but addition of activator enhances activity and E2 affinity due to an activator-induced conformational change (8,21).

Deletion of the UBA domain increased the half-maximal E2 concentration in the presence of the APC/C activator Cdh1 or Cdc20 (Fig. 3A), as seen in previous studies with soluble substrate (4). Most importantly, deletion of the UBA domain also caused an ~18-fold increase in the half-maximal E2 concentration in the absence of added activator, from 0.8 μM to 14 μM (Fig. 3B). Thus, the enhanced binding provided by the UBA domain does not require activator or depend on the conformational change caused by activator. Notably, the maximal catalytic rate of Ubc1ΔUBA was comparable to that of wild-type Ubc1 (Fig. 3A, at maximal E2 concentrations), providing evidence that deletion of the UBA domain does not affect catalysis in the presence of the APC/C.

To explore further whether the UBA domain interacts with the activator, we measured the concentration of Cdh1 needed for half-maximal APC/C activity, using soluble ^{35}S-labeled $Securin^{N}$ substrate and saturating amounts of either Ubc1 or Ubc1ΔUBA. Half-maximal Cdh1 concentrations were

similar for mutant and wild-type Ubc1 (Fig. 3C), further suggesting that the UBA domain functions independently of activator.

The APC/C activator Cdc20 autoubiquitinates at multiple lysines during the course of the cell cycle (30). To test whether the UBA domain of Ubc1 binds to ubiquitin conjugated to Cdc20, we carried out APC/C^{Ubc1} reactions with a mutant form of Cdc20, Cdc20-5K, that is poorly ubiquitinated because most of its ubiquitinated lysines are mutated to arginine (30). Although the activity of the Cdc20-5K mutant was low relative to wild-type Cdc20, the average chain length was the same, suggesting that there is no defect in Ubc1 binding (Fig. 3D). Similar results were obtained with APC/C$^{Ubc1\Delta UBA}$, further indicating that the UBA domain does not bind ubiquitin conjugated to activator (Fig. 3D).

The UBA domain acts independently to promote APC/C binding

We next hypothesized that the UBA domain binds to the APC/C core. To explore this possibility, we tested if adding the UBA domain alone to an APC/C^{Ubc1} reaction inhibited processivity *in trans*. Recombinant UBA domain was prepared in *E. coli*, and high concentrations of the protein reduced chain length in APC/C^{Ubc1} reactions to the length seen in reactions with Ubc1ΔUBA (Fig. 4A). UBA domain did not significantly affect APC/C$^{Ubc1\Delta UBA}$ reactions (Fig. 4A). The IC_{50} of the UBA domain in the wild-type Ubc1 reactions was ~25 μM (Fig. 4A). These results suggest that the UBA domain can reduce reaction processivity independently of the UBC domain.

We further explored the modularity of the UBA domain by creating a chimeric E2, Ubc4-UBA, in which the UBA domain and flexible tether of Ubc1 are fused to the C-terminus of Ubc4. Adding the UBA domain to Ubc4 lowered its half-maximal concentration in a conventional APC/C reaction (Fig. 4B), suggesting that the UBA domain boosts Ubc4 affinity for the APC/C. The increase in apparent affinity was 10-fold, from 2 μM to 0.2 μM (Fig 4B), identical to the loss of Ubc1 affinity upon deletion of the UBA domain.

Fusion of the UBA domain to Ubc4 did not significantly affect its maximal catalytic activity (Fig. 4B) or its specificity for ubiquitin chain initiation (see Fig. 5A, B below, first two lanes). Thus, the UBA domain can confer a boost in APC/C affinity to a distinct E2 with different lysine specificity, providing more evidence that the UBA domain does not interact with the UBC domain or K48-linked polyubiquitin, but is binding some site that is common to the functions of both E2s, such as the APC/C core.

The core subunit Apc10/Doc1 is a short distance from the E2-binding site of the APC/C. We tested its role in UBA domain binding by measuring Ubc1-dependent activity with APC/C lacking the Apc10 subunit. This subunit is involved in substrate binding, and deletion of Apc10, or mutation of key substrate-binding residues (the apc10-4A mutant), greatly reduces the processivity of ubiquitination (31,32). For these experiments, we used a fragment of the APC/C substrate Hsl1, $Hsl1^{F}$ (residues 667-882), which binds extremely tightly to the APC/C and is modified with very high processivity. We found that *apc10Δ* APC/C and *apc10-4A* APC/C showed similar defects in polyubiquitin chain length with Ubc1, and deletion of the UBA domain from Ubc1 caused a major decrease in processivity regardless of the presence or absence of Apc10 (Fig. 4C). It is therefore unlikely that the UBA domain binds to the Apc10 subunit. Since our earlier work ruled out Apc11 as a binding site (Fig. 1B), it seems likely that the UBA domain binds some site on the nearby Apc1 or Apc2 subunits. We were unable to test this possibility because deletion of either of these subunits abolishes APC/C activity, and neither subunit can be expressed stably as a recombinant protein for binding experiments.

The UBA domain ensures E2 competition

Ubc1 and Ubc4 likely compete for the same canonical binding site on the RING subunit Apc11. Our evidence suggests that the UBA domain of Ubc1 provides an extra affinity boost, and we hypothesized that deleting the UBA domain should decrease the ability of Ubc1 to compete with Ubc4. We assessed E2 competition by analyzing the products of APC/C reactions with each E2 alone or in combination. As seen in previous work (4), Ubc4 alone rapidly modified the substrate at multiple lysines to generate short monoubiquitinated products, whereas Ubc1 generated long polyubiquitin chains but turned over less

substrate because it is less efficient than Ubc4 in attachment of the initial ubiquitin (Fig. 5A, B). When the two E2s were mixed at equal concentrations, the high initiating activity of Ubc4 was reduced by competition with Ubc1, but the total amount of polyubiquitin chains increased slightly due to the increased number of initial ubiquitins relative to Ubc1 alone (see quantification of activity in Fig. 5A, B). In addition, deletion of the UBA domain from Ubc1 decreased its ability to compete with Ubc4, resulting in higher substrate turnover, lower average polyubiquitin chain length, and a pattern of modification similar to that with Ubc4 alone (Fig. 5A). Similar results were obtained in reactions with a single lysine substrate, Securin^{N} 1K, which exhibits decreased substrate turnover (since Ubc4 can only modify the substrate with 1 or 2 ubiquitins) and very few ubiquitin chains (Fig. 5B).

Since attaching the UBA domain to Ubc4 increased its apparent affinity for APC/C, we hypothesized that Ubc4-UBA should compete more effectively with Ubc1 as compared to Ubc4. Indeed, addition of Ubc4-UBA to a Ubc1 reaction reduced average polyubiquitin chain length, resulting in a pattern of reaction products more closely resembling that seen in a reaction with Ubc4 alone (Fig. 5A). Also, APC/C activity was higher in the Ubc1 + Ubc4-UBA reaction relative to an Ubc1 + Ubc4 reaction, indicating that Ubc1 cannot compete as effectively with Ubc4-UBA as it can with Ubc4. The Ubc1 + Ubc4-UBA reaction exhibited a more heterogeneous banding pattern than the Ubc1 + Ubc4 reaction (Fig. 5A), likely because Ubc1 extended chains on substrates that had been monoubiquitinated at multiple lysines by Ubc4-UBA. The average ubiquitin chain length was also slightly shorter in a Ubc1 + Ubc4-UBA reaction with a single lysine substrate (Fig. 5B). Thus, we propose that the extra affinity provided by the UBA domain of Ubc1 ensures efficient polyubiquitination of substrate by balancing Ubc1 affinity with that of Ubc4, resulting in the optimal modification of APC/C substrates for proteasomal recognition.

The UBA domain is important for APC/C activity *in vivo*

We assessed the importance of the UBA domain for Ubc1 function *in vivo* in *S. cerevisiae*. First, we deleted the UBA domain at the endogenous *UBC1* locus and also introduced a C-terminal 1XFlag6XHis tag. As a control, we introduced the same tag at the wild-type locus. Ubc1ΔUBA was expressed at a

slightly lower level than Ubc1 (data not shown). Deletion of the UBA domain did not appear to have any effect on growth or the timing of destruction of the APC/C substrate securin (Fig. 6A).

Tetrad analysis revealed that when we sensitized the system by deleting *UBC4*, the UBA domain became essential for yeast survival (data not shown). To further explore the phenotype of *ubc4Δ ubc1ΔUBA* strains, we created a conditional system in which we placed the endogenous copy of *UBC1* under the control of the *GAL* promoter (with an N-terminal 3XHA tag) and introduced a second copy of *UBC1* (either wild-type, *ubc1ΔUBA*, or an empty vector) under the control of the endogenous promoter (tagged with 9XMYC) at the *LEU2* locus. In this system, we could shut off expression of *pGAL-UBC1* and observe the effects of the *ubc1ΔUBA* mutation in strains with either *UBC4* or *ubc4Δ*. As in our earlier experiments, the *ubc1ΔUBA* mutant displayed no colony growth defect, but the *ubc4Δ ubc1ΔUBA* double mutant did not proliferate at all (Fig. 6B). Importantly, all the strains grew similarly when *pGAL-UBC1* was expressed (Fig. 6B). The expression of Ubc1 and Ubc1ΔUBA were comparable in these strains (data not shown).

To determine if *ubc4Δ ubc1ΔUBA* double mutants have a defect in cell cycle progression due to a loss of APC/C activity, we released these strains from a G1 arrest after shutting off *pGAL-UBC1*. *ubc4Δ ubc1ΔUBA* cells arrested with high levels of the APC/C substrate securin and large buds (Fig. 6B), consistent with a pre-anaphase arrest like that seen in *apc* mutants. We conclude that the UBA domain is particularly important for Ubc1 function in the absence of Ubc4. These results are consistent with our biochemical evidence that deletion of the UBA domain does not simply cause a defect in ubiquitin chain elongation by Ubc1 but also causes a defect in chain initiation (Fig. 2, 5), and cell survival requires that Ubc1 must carry out this task efficiently in the absence of Ubc4.

We used a similar system to test the effect of attaching the Ubc1 UBA domain to Ubc4. We found that *UBC4-UBA* cells displayed wild-type viability (Fig. 6C). However, deletion of the UBA domain from Ubc1 together with attachment of the UBA domain to Ubc4 resulted in synthetic lethality (Fig. 6C). This effect was not due to low Ubc4-UBA expression (data not shown) or a lack of Ubc4-UBA activity, since our earlier results (Fig. 4B) indicate that this E2 is fully active *in vitro*. Instead, we believe that fusing the

UBA domain to Ubc4 and deleting the UBA domain of Ubc1 increases the affinity of Ubc4 for the APC/C while decreasing the affinity of Ubc1. This imbalance in E2 affinities cannot support proper APC/C activity, likely because Ubc1 cannot perform its essential function in chain elongation (Fig 5A, last lane).

Discussion

We report that the UBA domain enhances Ubc1 affinity for the APC/C, ensuring that Ubc1 binds with sufficient affinity in the presence of the competing E2 Ubc4. The UBA domain is linked to the UBC domain of Ubc1 by a 22-residue flexible tether. In theory, a disordered linker of this size could reach up to ~75 Å away from Ubc1, allowing it to interact with numerous sites on the 150 Å-wide APC/C (8,12). However, assuming that the linker is not entirely unstructured, the UBA domain is most likely to interact with a site near the primary E2-binding site on the RING subunit Apc11. We ruled out an interaction with the Apc11 and Apc10 subunits, and so nearby regions of Apc2 or Apc1 represent the likeliest candidates.

It is unlikely that the flexible linker of Ubc1 contributes directly to APC/C binding, as the recombinant UBA domain alone (containing only the last 3 residues of the linker) inhibited APC/C^{Ubc1} processivity *in trans* (Fig. 4A). Nevertheless, the length and flexibility of the tether are likely to be important for allowing the UBA domain to reach its binding site on the APC/C.

In the human APC/C, the chain-elongating E2, Ube2S, also uses a C-terminal extension to bind the APC/C at a site distinct from the canonical RING site (5,10-12,20). The C-terminal extension of Ube2S is required for its interaction with the APC/C (5,8,10), and deletion of the C-terminal extension decreases Ube2S processivity *in vitro* (6,10). Thus, it appears that in both yeast and humans, chain-elongating E2s have independently evolved extensions to enhance interactions with the APC/C, perhaps suggesting that this is a common feature of E3s that use sequential E2s for chain initiation and elongation.

In the case of the human APC/C, this strategy may allow both E2s to bind the APC/C simultaneously, perhaps enabling more efficient chain assembly. In the case of the yeast APC/C, however, the UBC

domains of the two E2s are likely to interact with the same canonical binding site, resulting in competition—and therefore requiring finely balanced affinities to allow the two E2s to alternate. The yeast strategy may be relevant to other E2-E3 modules, such as the human SCF complex, which uses the E2s UbcH5c and Cdc34 to initiate and elongate K48-linked ubiquitin chains, respectively (18,33,34). These E2s are thought to bind the canonical RING binding site (33,34). Here again, the chain-elongating E2, Cdc34, has a C-terminal extension that binds to the cullin subunit of the SCF (35). This extension may tune Cdc34 affinity for SCF in the face of competition from UbcH5c.

An intriguing possibility is that the UBC domain of Ubc1 can dissociate transiently while its UBA domain remains bound to the APC/C. This could be relevant for E2 competition: the same surface of the E2 UBC domain binds E3 and E1, and so the UBC domain must dissociate from the E3 to be recharged with ubiquitin by E1. However, if the UBA domain allows Ubc1 to remain bound to the APC/C while recharging, it could perform multiple rounds of ubiquitin transfer in a single E3-binding event.

Ubiquitin chains containing four or more ubiquitins represent the canonical recognition motif for the proteasome (36), and several deubiquitinating enzymes (DUBs) in yeast exhibit preference for mono- and diubiquitinated substrates (37). Thus, the binding of the ubiquitin chain-elongating E2 Ubc1 is particularly important for the ability of the APC/C to effectively target its substrates for destruction, which is further demonstrated by the fact that Ubc1, but not Ubc4, is essential *in vivo* (4). The existence of a second APC/C binding site for the chain-elongating E2 introduces the possibility that this site can be regulated; inhibition of this site, for example, could prevent the elongation of short ubiquitin chains that are spuriously initiated by APC/C^{Ubc4}, allowing their rapid removal by DUBs. A similar sort of regulation has been demonstrated for the human APC/C inhibitor Emi1, which reduces chain elongating APC/C activity by blocking binding of the Ube2S C-terminal tail to APC/C (20).

Given our result that the UBA domain of Ubc1 binds to the APC/C, it is surprising that this domain retains conserved ubiquitin-binding residues and has the ability to bind ubiquitin and tetra-ubiquitin with significant affinity. Previous evidence suggests that the UBA domain does not bind to the donor ubiquitin, and evidence presented here suggests that it does not bind to the acceptor ubiquitin or ubiquitin

conjugated to the APC/C activator. To our knowledge, there is no experimental evidence to suggest that ubiquitin is present in significant quantities on any other APC/C subunit. There are also no obvious ubiquitin or ubiquitin-like sequences encoded by any APC/C subunit.

Nevertheless, it is possible that the UBA domain binds both the APC/C and ubiquitin under certain circumstances. Several previously characterized UBA domains can bind both ubiquitin and another partner, sometimes simultaneously (27-29). Since the affinity of the UBA domain for a single ubiquitin is low (~230 μM) but is considerably higher for a ubiquitin chain (~37 nM for K48-linked tetraubiquitin), it is possible that as the ubiquitin chain on substrates grows longer, it interacts with the UBA domain. It is unclear if the UBA domain of Ubc1 could bind ubiquitin and the APC/C simultaneously, or if it switches from binding APC/C to binding the ubiquitin chain. This mechanism could allow Ubc1 to compete more effectively with Ubc4 when the substrate carries a polyubiquitin chain. Also, it may explain why mixing the two E2s at equal concentrations leads to a banding pattern that is identical to that of Ubc1 alone: Ubc1 binding may become dominant at longer chain lengths, thereby ensuring that the substrate has an adequate signal for recognition by the proteasome.

Experimental Procedures

Cloning, expression, and purification of proteins

To make ^{32}P-labeled K48R mutant ubiquitin for diubiquitin synthesis assays, GST-TEV-PKA-K48R-Ubiquitin (a gift of Ray Deshaies (18)) was expressed in *E. coli*, radioactively labeled, and purified as described (3). E2 constructs, including Ubc1-6XHis, Ubc1ΔUBA-6XHis, Ubc4-6XHis, and Ubc4-UBA-6XHis were expressed in *E. coli* and purified as described (4). The Ubc4-UBA chimera was created by amplifying DNA encoding the flexible linker and UBA domain of Ubc1 (residues 151-215) from the Ubc1-6XHis expression vector by PCR and ligating into the Ubc4-6XHis vector.

The yeast E1 Uba1 was expressed in *E. coli* and purified as described (31). APC/C was purified from yeast cells using tandem affinity purification as described (31). Where indicated, APC/C carrying TAP-

tagged Cdc16 was immunoprecipitated from yeast cells using IgG-coupled Dynabeads (Invitrogen, Carlsbad, CA), and remained on beads for the duration of the experiment. Cdh1 was expressed in insect cells and purified as described (38). Sea urchin Cyclin B N-terminal fragment ($CycB^N$, residues 13-110), either wild-type $CycB^N$ or a version containing a single lysine ($CycB^N$ 1K; K60 (37)), were expressed in *E. coli*, purified, and labeled with ^{125}I (where indicated) as previously described (38). All APC/C substrates labeled with ^{35}S-methionine were expressed and translated in rabbit reticulocyte lysates using the TnT Quick Coupled Transcription/Translation System (Promega, Madison, WI). Unlabeled Cdc20 and Cdh1 were also produced by this method where indicated. Proteins were purified from rabbit reticulocyte lysates by immunoprecipitation with IgG-coupled dynabeads and cleavage from the beads with TEV protease.

Truncated *APC11* (encoding residues 35-165) was amplified from yeast genomic DNA by PCR and ligated into a pGEX-4T1-derived expression vector containing an N-terminal GST. GST-Apc11ΔN was expressed in E. coli and purified with Glutathione Sepharose 4B (GE).

DNA encoding the UBA domain of Ubc1 (residues 167-215) was amplified from the Ubc1-6XHis expression vector by PCR and ligated into a pET28a-derived expression vector containing an N-terminal 6XHis1XGB1 tag followed by a recognition site for TEV protease. The UBA domain construct was expressed in *E. coli* and purified with Ni-NTA Agarose (Qiagen, Venlo, Holland). The 6XHis1XGB1 tag was cleaved by TEV protease during dialysis, and the tag and protease were removed by incubation with Ni-NTA agarose prior to concentration. This leaves the N-terminus of the protein with the sequence GGSGID, in which the last three residues, GID, are the final residues of the Ubc1 flexible linker.

Diubiquitin synthesis assays

All reactions were done in QAH buffer (50 mM Hepes pH 7.4, 100 mM NaCl, 10% glycerol, 1 mM $MgCl_2$) and stopped by addition of 6X non-reducing sample buffer (375 mM Tris-HCl pH 6.8, 6% SDS, 30% glycerol, 0.03% bromophenol blue, 60 mM NEM). Ubc1 was charged with ^{32}P-radiolabeled K48R ubiquitin in the following manner: E1 (300 nM), ATP (1 mg/ml), ^{32}P-labeled K48R ubiquitin (~1 mg/ml),

and Ubc1 (0.5 μM) were incubated at room temperature for 20 min. E1 and uncharged E2 were inactivated by incubation with NEM (10 mM) and EDTA (50 mM) for 15 min at room temperature. Tubes were transferred to 4°C and incubated with GST-Apc11ΔN (0-80 μM) for 3 min. Wild-type ubiquitin (Boston Biochem, Cambridge, MA) or $CycB^N$ (where indicated) was added to the reactions at the concentrations indicated to start the reactions. Proteins were separated by SDS-PAGE and dried gels were exposed to a storage phosphor screen (GE) overnight. Screens were scanned on a Typhoon phosphorimager (GE) and autoradiographs were quantified using ImageQuant software (GE). k_{obs} was calculated by dividing the diubiquitin signal by the charged E2 signal, then dividing by the reaction time in seconds. Data were fit to the Michaelis-Menten equation in Prism software (GraphPad, La Jolla, CA).

APC/C assays

E2s were charged in the following manner: E1 (300 nM), ATP (1 mg/ml), ubiquitin (100 mM), and E2 (0-40 μM) were incubated at room temperature for 20 min. APC/C, activator (either Cdh1 or Cdc20), and radiolabeled substrate were pre-incubated for 10 minutes and reactions were started by mixing the E2 charging mix with the APC/C mix, except where otherwise indicated. All reactions were carried out in QAH buffer pH 7.4 for the amount of time indicated, and reaction products were separated by SDS-PAGE and visualized by autoradiography. APC/C activity was calculated by combining signal from all modified substrate bands and dividing by the reaction time in seconds. APC/C processivity was calculated by quantifying individual ubiquitinated products, multiplying the amount of product by the number of ubiquitins in the product, and dividing by the total amount of modified products.

For the assays in which substrate was fused to the APC/C, APC/C was immunoprecipitated from *cdh1Δ doc1Δ* cells using IgG-coupled Dynabeads (Invitrogen) and remained bead-bound throughout the course of the reactions. ^{35}S-Methionine-labeled N-terminal securin fragment (residues 1-110) fused to the N-terminus of Apc10 (^{35}S-Securin^N-Apc10) was incubated with the APC/C on beads, and unbound ^{35}S-

Securin^{N}-Apc10 was washed away. Ubc1 (either wild-type or ΔUBA) charged with methylated ubiquitin (Boston Biochem) was added in increasing concentrations.

Yeast strains and analysis

All yeast strains were in the W303 background and are listed in Table 1. Strains were generated using standard yeast cloning techniques for transformation, mating, sporulation, and tetrad dissection. For yeast growth assays, strains were grown to mid-log phase at 30°C in the indicated media. Cells were diluted to OD_{600}=0.1 and plated on the indicated media. Plates were scanned and images prepared with Adobe Photoshop. For cell-cycle analysis, asynchronous yeast cultures were grown to OD_{600}=0.2 in the indicated media at 30°C and then arrested in G1 by incubation with α factor (1 μg/ml) for at least 3 h. Cultures were released from G1 arrest by washing away α factor and resuspending in the indicated media (zero timepoint). Cell samples were taken at the indicated times, lysed, and analyzed by western blotting against the indicated proteins. Where shown, parallel samples were taken and a budding index was counted by microscopy. For western blot analysis, Securin-9XMyc and Ubc1-9XMyc were detected by monoclonal 9E10 anti-Myc antibody (Covance, Princeton, NJ, 1:1000), Cdk1 was detected by polyclonal sc-53 anti-Cdk1 antibody (Santa Cruz, Dallas, TX, 1:1000), and Ubc1-1XFlag6XHis was detected by monoclonal M2 Flag antibody (Sigma, 1:5000).

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Figure 1. The UBA domain does not contribute to acceptor ubiquitin binding or RING enhancement of E2 catalysis. (A) Ubc1 (wild-type, 0.5 μM) was charged with ^{32}P-radiolabeled K48R ubiquitin and incubated with either GST-Apc11ΔN (80 μM) or buffer. Wild-type ubiquitin was added at the indicated concentrations, and reactions were carried out at 4°C for 5 s (+GST-Apc11ΔN) or 10 min (buffer alone). Reaction products were analyzed by SDS-PAGE and autoradiography with a PhosphorImager. Right panel displays the quantification of diubiquitin synthesis assays, showing the dependence of catalytic rate (k_{obs}) on ubiquitin concentration. Autoradiographs were quantified using ImageQuant and data were fit to the Michaelis-Menten equation in Prism software. The average of 3 experiments is shown. Error bars represent standard error of the mean (SEM). (B) Ubc1 (wild-type) and Ubc4 (each at 0.5 μM) were charged with ^{32}P-radiolabeled K48R ubiquitin and incubated with buffer or increasing concentrations of GST-Apc11ΔN. Unlabeled $CycB^{N}$ (200 μM, Ubc4 reactions) or ubiquitin (100 μM, Ubc1 reactions) was added, and reactions were carried out at 4°C for 5 s. Reactions were analyzed as in panel A. Results are representative of 3 independent experiments. (C) Ubc1 (wild-type or ΔUBA, at 0.5 μM) reactions were carried out as in panel A, except that a sub-saturating concentration of GST-Apc11ΔN (6.5 μM) was used. Reactions were carried out at room temperature for 3 min (wild-type Ubc1) or 5 min (Ubc1ΔUBA). The average of 3 experiments is shown. Error bars represent SEM. (D) Ubc1 and Ubc1ΔUBA were charged as in panel A and incubated with buffer or increasing concentrations of GST-Apc11ΔN. Unlabeled wild-type ubiquitin (100 μM) was added, and reactions were carried out, visualized, and quantified as in panel A. Results are representative of 3 independent experiments.

Figure 1

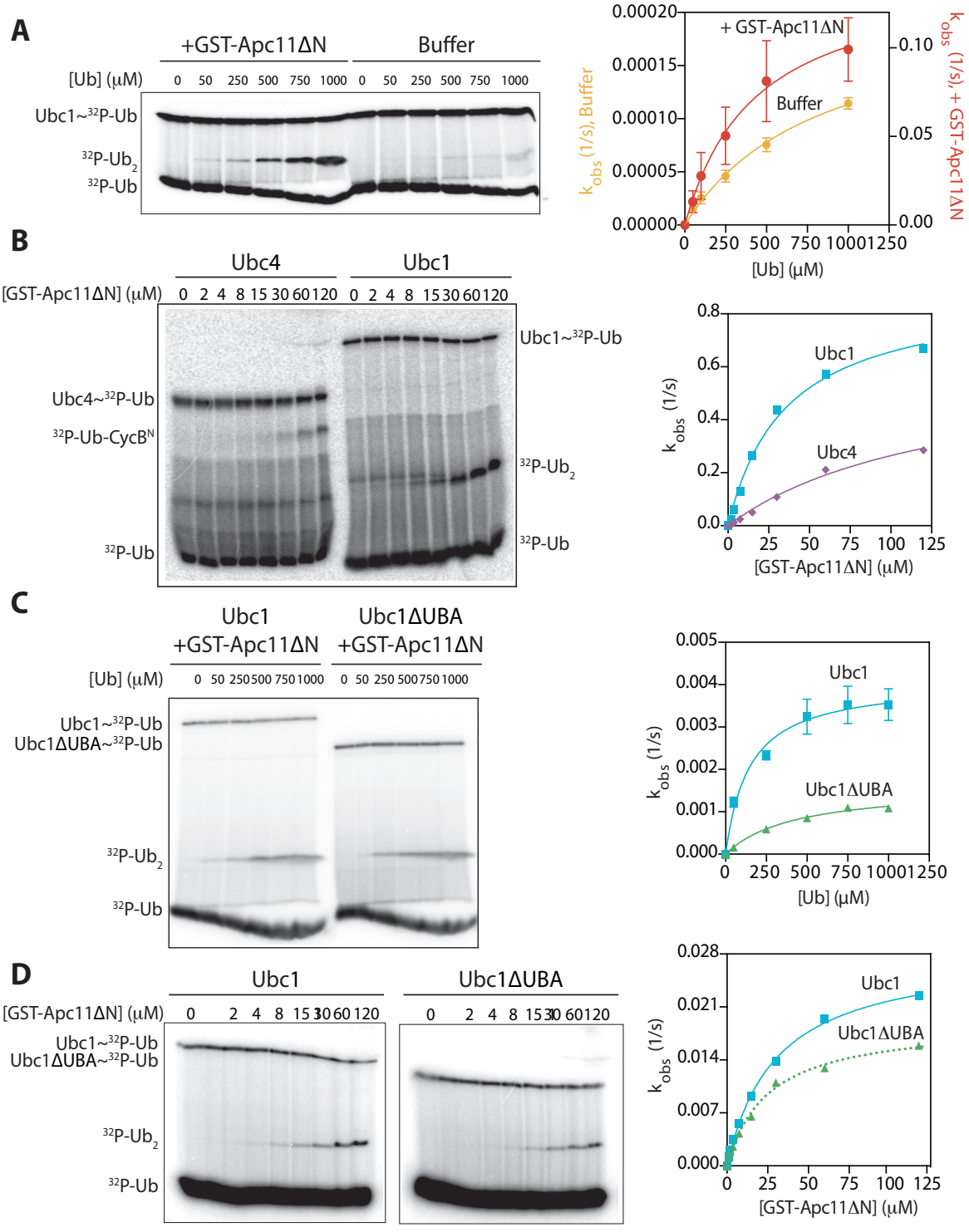
A
+GST-Apc11ΔN
Buffer
[Ub] (μM)
0 50 250 500 750 1000 0 50 250 500 750 1000
Ubc1~32P-Ub
32P-Ub2
32P-Ub
+ GST-Apc11ΔN
Buffer
kobs (1/s), Buffer
kobs (1/s), + GST-Apc11ΔN
[Ub] (μM)
B
Ubc4
Ubc1
[GST-Apc11ΔN] (μM)
0 2 4 8 15 30 60 120 0 2 4 8 15 30 60 120
Ubc1~32P-Ub
Ubc4~32P-Ub
32P-Ub-CycBN
32P-Ub2
32P-Ub
Ubc1
Ubc4
kobs (1/s)
[GST-Apc11ΔN] (μM)
C
Ubc1
+GST-Apc11ΔN
Ubc1ΔUBA
+GST-Apc11ΔN
[Ub] (μM)
0 50 250 500 750 1000 0 50 250 500 750 1000
Ubc1~32P-Ub
Ubc1ΔUBA~32P-Ub
32P-Ub2
32P-Ub
Ubc1
Ubc1ΔUBA
kobs (1/s)
[Ub] (μM)
D
Ubc1
Ubc1ΔUBA
[GST-Apc11ΔN] (μM)
0 2 4 8 15 30 60 120 0 2 4 8 15 30 60 120
Ubc1~32P-Ub
Ubc1ΔUBA~32P-Ub
32P-Ub2
32P-Ub
Ubc1
Ubc1ΔUBA
kobs (1/s)
[GST-Apc11ΔN] (μM)

Figure 2. The UBA domain is independent of ubiquitin on substrate. APC/C purified from yeast cells was mixed with Cdh1 and ^{125}I-CycB 1K. Ubc1 (either wild-type or ΔUBA) charged with K48R mutant ubiquitin was added at the indicated concentrations, and reactions were carried out at room temperature for 15 min. Reaction products were analyzed by SDS-PAGE and autoradiography with a PhosphorImager. Bottom panel displays quantification of results, showing the dependence of APC/C activity on concentration of Ubc1 or Ubc1ΔUBA. Autoradiographs were quantified using ImageQuant and data were fit to Michaelis-Menten equation in Prism software. The average of 4 experiments is shown. Error bars represent SEM. PIU/s, PhosphorImager Units per second.

Figure 2

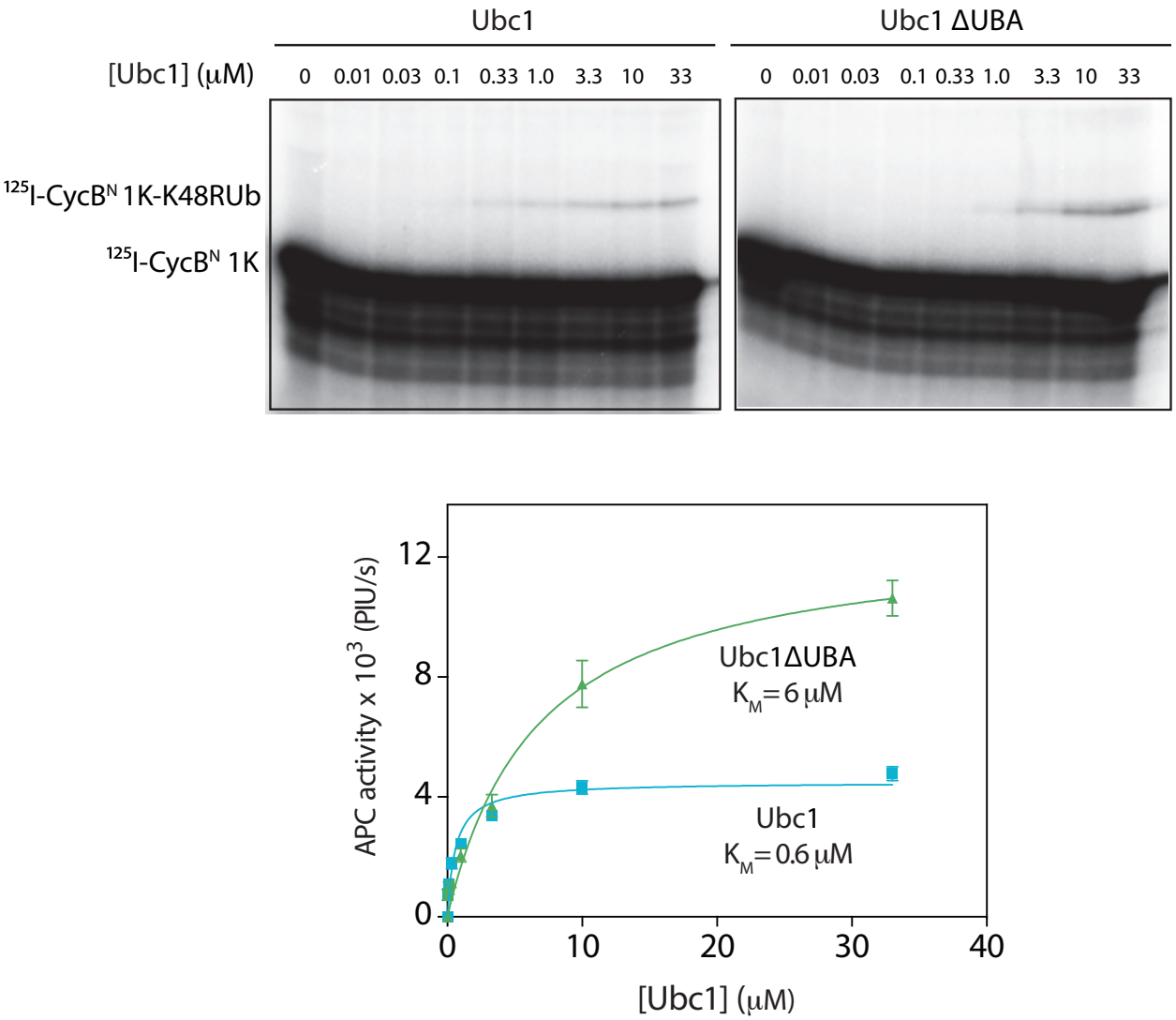
Ubc1
Ubc1 ΔUBA
[Ubc1] (μM)
0 0.01 0.03 0.1 0.33 1.0 3.3 10 33
0 0.01 0.03 0.1 0.33 1.0 3.3 10 33
125I-CycBN 1K-K48RUb
125I-CycBN 1K
APC activity x 10^3 (PIU/s)
12
8
4
0
0
10
20
30
40
[Ubc1] (μM)
Ubc1ΔUBA
KM = 6 μM
Ubc1
KM = 0.6 μM

Figure 3. UBA domain does not bind to APC/C activator. (A) ^{35}S-Securin^{N}-Apc10, Cdh1, and Cdc20 were generated by *in vitro* translation in rabbit reticulocyte lysate. ^{35}S-Securin^{N}-Apc10 was bound to immunoprecipitated APC/C (*apc10*Δ *cdh1*Δ), and unbound substrate was washed away. Cdh1 and Cdc20 were purified from reticulocyte lysate and mixed with fusion substrate-bound APC/C. Ubc1 (either wild-type or ΔUBA) charged with methylated ubiquitin was added in increasing concentrations, and reactions were carried out at room temperature for 15 min. Reaction products were analyzed by SDS-PAGE and autoradiography with a PhosphorImager. Results are representative of 3 independent experiments. (B) APC/C assays were performed as in panel A, except that no activator subunit was added. The graph at right shows mean values (+/- SEM), normalized to maximal Ubc1 activity, of 3 independent experiments. (C) ^{35}S-Securin^{N} and Cdh1 were generated by *in vitro* translation in rabbit reticulocyte lysate and purified. Immunoprecipitated APC/C was mixed with ^{35}S-Securin^{N} and increasing amounts of Cdh1. Ubc1 or Ubc1ΔUBA (10 μM) were charged with wild-type ubiquitin, and reactions were carried out at room temperature for 15 min. Reaction products were analyzed as in panel A. The graph at right shows quantification of APC/C activity as a function of Cdh1 concentration for the experiment at left. Results are representative of 3 independent experiments. PIU/s, PhosphorImager Units per second. (D) ^{35}S-Securin^{N}, Cdc20, and Cdc20-5K were generated as in panel C and mixed with purified APC/C. E2s (each at 5 μM) charged with wild-type ubiquitin were added, and reactions were carried out at room temperature for 30 min. Reaction products were analyzed as in panel A. Results are representative of 3 independent experiments.

Figure 3

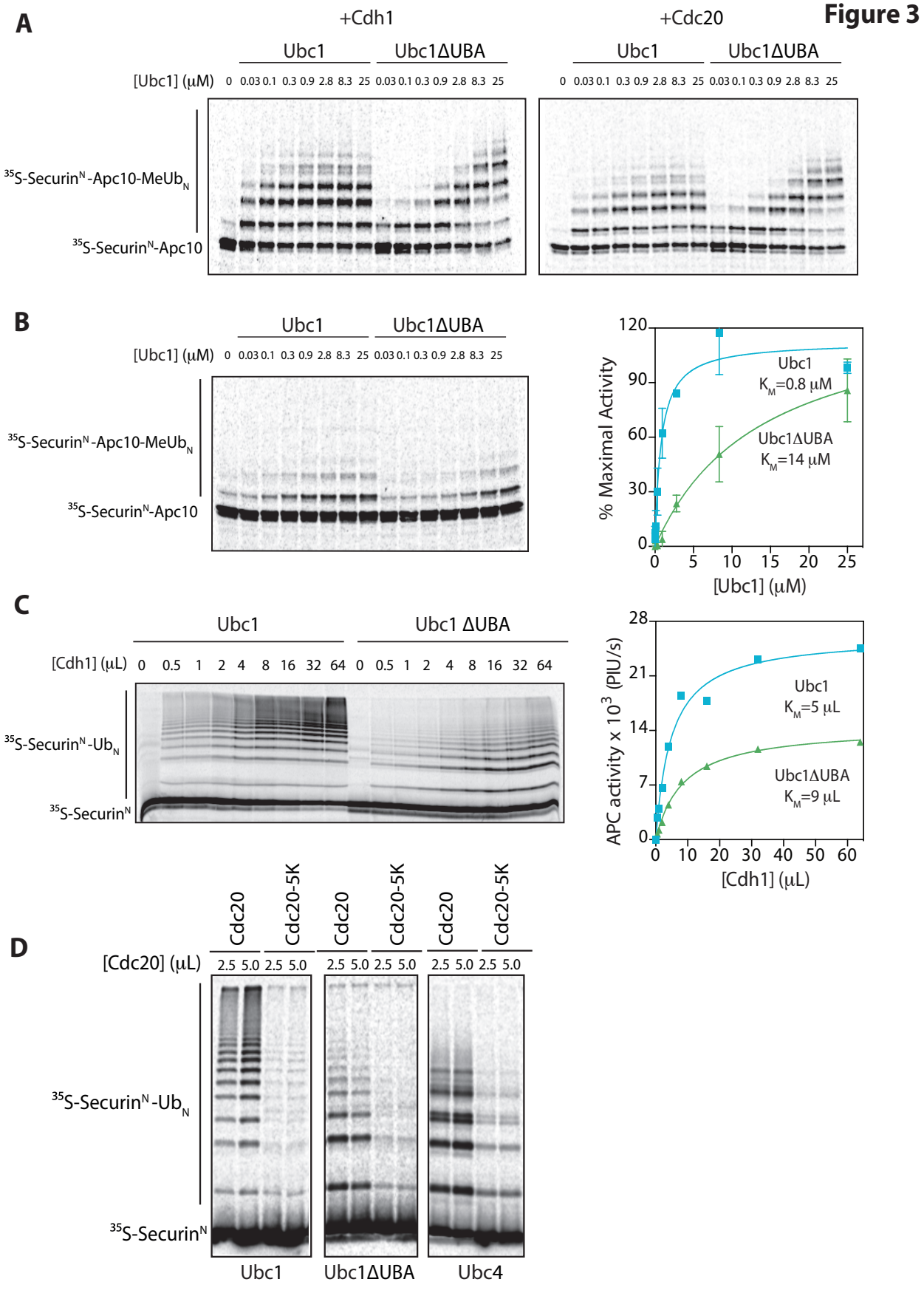
A
+Cdh1
+Cdc20
Ubc1
Ubc1ΔUBA
[Ubc1] (μM) 0 0.03 0.1 0.3 0.9 2.8 8.3 25 0.03 0.1 0.3 0.9 2.8 8.3 25
35S-SecurinN-Apc10-MeUbN
35S-SecurinN-Apc10
B
Ubc1
Ubc1ΔUBA
[Ubc1] (μM) 0 0.03 0.1 0.3 0.9 2.8 8.3 25 0.03 0.1 0.3 0.9 2.8 8.3 25
35S-SecurinN-Apc10-MeUbN
35S-SecurinN-Apc10
% Maximal Activity
Ubc1
KM=0.8 μM
Ubc1ΔUBA
KM=14 μM
[Ubc1] (μM)
C
Ubc1
Ubc1 ΔUBA
[Cdh1] (μL) 0 0.5 1 2 4 8 16 32 64 0 0.5 1 2 4 8 16 32 64
35S-SecurinN-UbN
35S-SecurinN
APC activity x 10^3 (PIU/s)
Ubc1
KM=5 μL
Ubc1ΔUBA
KM=9 μL
[Cdh1] (μL)
D
Cdc20
Cdc20-5K
[Cdc20] (μL) 2.5 5.0 2.5 5.0 2.5 5.0 2.5 5.0 2.5 5.0 2.5 5.0
35S-SecurinN-UbN
35S-SecurinN
Ubc1
Ubc1ΔUBA
Ubc4

Figure 4. The UBA domain promotes APC/C binding. (A) ^{35}S-Securin^{N} was translated *in vitro* in rabbit reticulocyte lysate, purified, and mixed with purified APC/C, Cdh1, and varying concentrations of recombinant UBA domain. Ubc1 (either wild-type or ΔUBA, 10 μM) charged with wild-type ubiquitin was added, and reactions were carried out at room temperature for 15 min. Reaction products were analyzed by SDS-PAGE and autoradiography with a PhosphorImager. Autoradiographs were quantified using ImageQuant. The right panel displays quantification of processivity of APC/C^{Ubc1} and APC/C$^{Ubc1\Delta UBA}$ as a function of the concentration of free UBA domain. Processivity was calculated by quantifying individual ubiquitinated products, multiplying the amount of product by the number of ubiquitins in the product, and dividing by the total amount of modified products. Data were fit to the log(inhibitor) vs. response equation in Prism software. The average of 3 experiments is shown. Error bars represent SEM. (B) Purified ^{35}S-Securin^{N}, APC/C, and Cdh1 were combined as in panel A. Ubc4 or Ubc4-UBA charged with wild-type ubiquitin was added at the indicated concentrations, and reactions were carried out at room temperature for 15 min. Reaction products were analyzed as in panel A. The right panel displays quantification of the dependence of APC/C activity on concentration of Ubc4 or Ubc4-UBA. Data were fit to Michaelis-Menten equation in Prism software. The average of 3 experiments is shown. Error bars represent SEM. The inset shows a close-up of the graph at lower E2 concentrations. PIU/s, PhosphorImager Units per second. (C) ^{35}S-Hsl1^{F} was translated *in vitro* in rabbit reticulocyte lysate, purified, and mixed with Cdh1 and APC/C immunoprecipitated from wild-type, *apc10Δ* mutant, or *apc10-4A* mutant yeast. E2s (all at 5 μM final concentration) were charged with wild-type ubiquitin and added, and reactions were carried out at room temperature for 15 min. Reaction products were analyzed as in panel A.

Figure 4

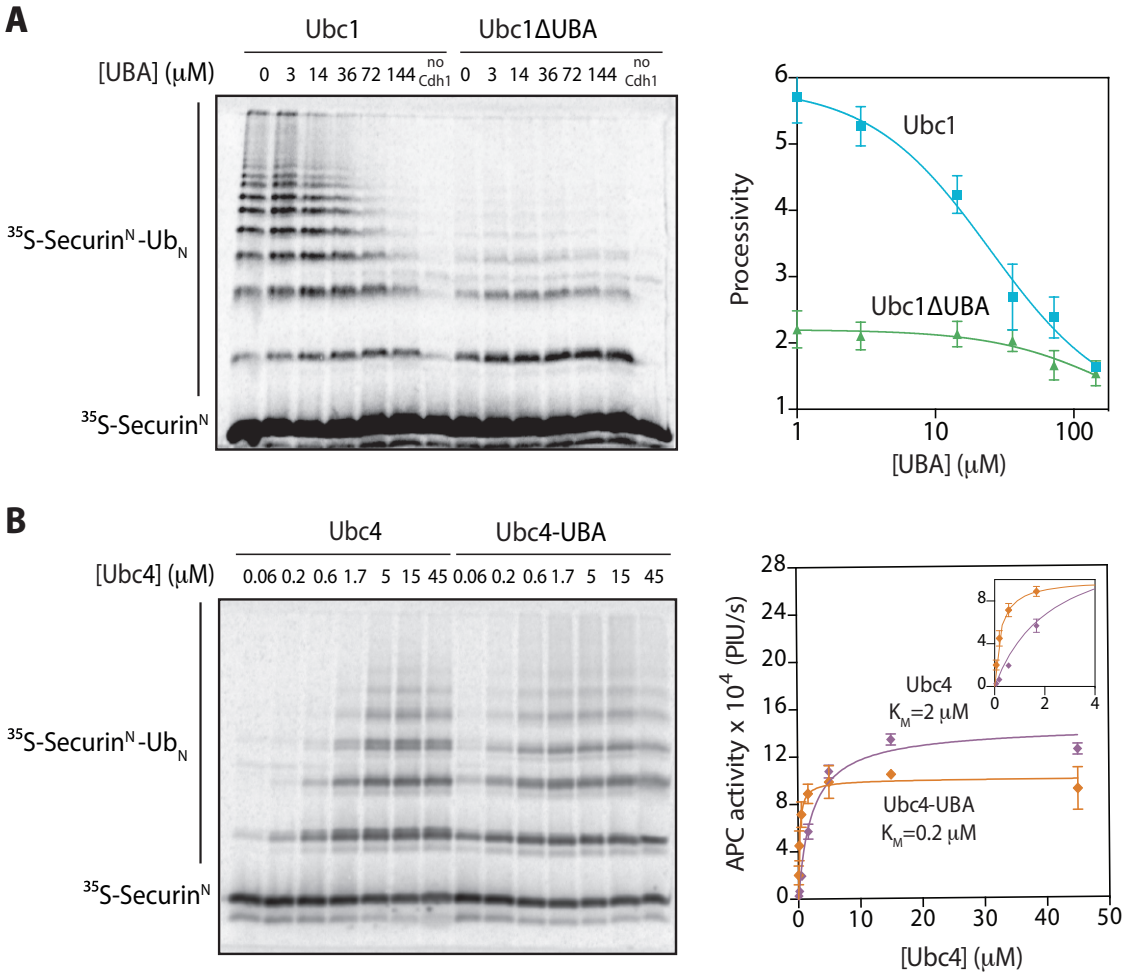
A
Ubc1
Ubc1ΔUBA
[UBA] (μM)
0 3 14 36 72 144 no Cdh1 0 3 14 36 72 144 no Cdh1
35S-SecurinN-UbN
35S-SecurinN
Processivity
Ubc1
Ubc1ΔUBA
[UBA] (μM)
B
Ubc4
Ubc4-UBA
[Ubc4] (μM)
0.06 0.2 0.6 1.7 5 15 45 0.06 0.2 0.6 1.7 5 15 45
35S-SecurinN-UbN
35S-SecurinN
APC activity x 10^4 (PIU/s)
Ubc4
KM=2 μM
Ubc4-UBA
KM=0.2 μM
[Ubc4] (μM)
C
APC:
WT
apc10-4A
apc10Δ
WT
Ubc1:
WT
ΔUBA
WT
ΔUBA
WT
ΔUBA
–
35S-Hsl1F-UbN
35S-Hsl1F

Figure 5. The UBA domain is important for E2 competition. (A) ^{35}S-Securin^{N} was translated *in vitro* in rabbit reticulocyte lysate, purified, and mixed with purified APC/C and Cdh1. The indicated E2s (each at 3 μM final concentration) were charged with wild-type ubiquitin and added. Reactions were carried out at room temperature for 20 min. Reaction products were analyzed by SDS-PAGE and autoradiography with a PhosphorImager. The numbers below show quantification of APC/C activity (i.e. total modified substrate) in each lane, with Ubc4 activity normalized to 100. Results are representative of 3 independent experiments. (B) APC/C assays were performed as in panel A, except that ^{35}S-Securin^{N} 1K was used as the substrate. Results are representative of 3 independent experiments.

Figure 5

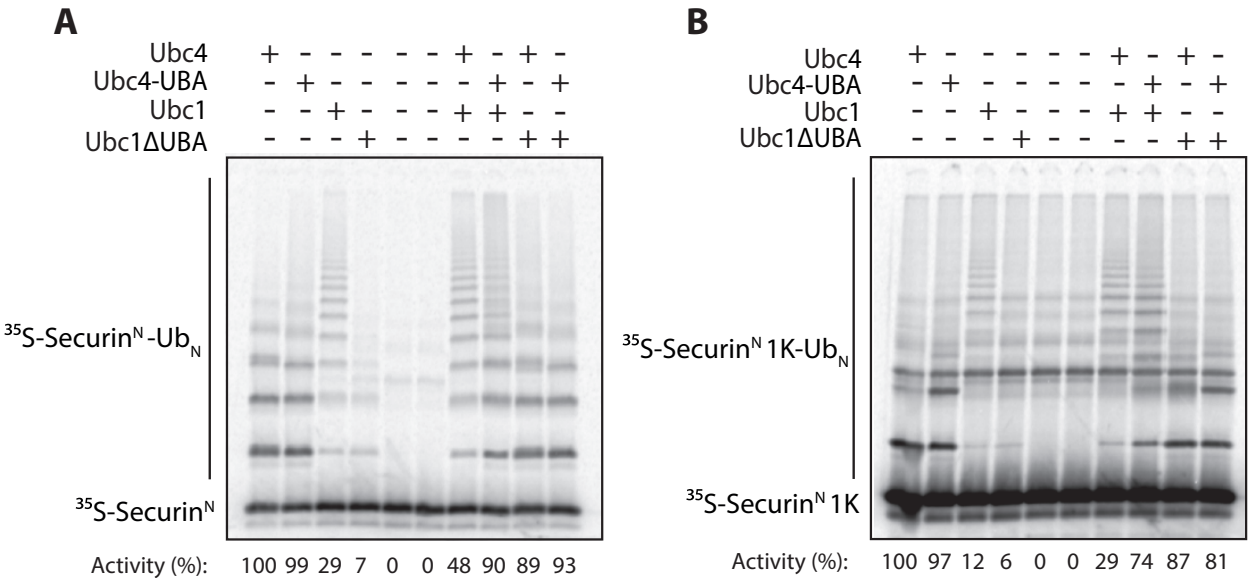
A
Ubc4 + - - - - - + - + -
Ubc4-UBA - + - - - - - + - +
Ubc1 - - + - - - + + - -
Ubc1ΔUBA - - - + - - - - + +
35S-SecurinN-UbN
35S-SecurinN
Activity (%): 100 99 29 7 0 0 48 90 89 93
B
Ubc4 + - - - - - + - + -
Ubc4-UBA - + - - - - - + - +
Ubc1 - - + - - - + + - -
Ubc1ΔUBA - - - + - - - - + +
35S-SecurinN 1K-UbN
35S-SecurinN 1K
Activity (%): 100 97 12 6 0 0 29 74 87 81

Figure 6. The UBA domain is important for Ubc1 function *in vivo*. (A) (Left) Strains were grown to mid-log phase at 30°C in media containing 2% glucose, diluted to OD_{600}=0.1, plated as serial dilutions on 2% glucose, and grown for 2 days at 30°C. (Right) Asynchronous cultures (OD_{600}=0.2) were arrested in G1 with α factor (1 μg/ml) for 3 h, and released from G1 arrest by washing away α factor (zero timepoint). Cell samples were taken at the indicated times, lysed, and analyzed by western blotting against the indicated proteins. Results are representative of 3 independent experiments. (B) (Top) Strains were grown to mid-log phase at 30°C in media containing 2% galactose and raffinose, diluted to OD_{600}=0.1 and plated as serial dilutions on 2% gal/raff or 2% glucose and grown for 2 days at 30°C. Results are representative of 3 independent experiments. (Bottom) Asynchronous cultures were arrested in G1 with α factor (1 μg/ml) for 5 h. During the last 2 hours of α factor treatment, cultures were incubated with 2% glucose. Cells were released from G1 by washing away α factor, and resuspended in media containing 2% glucose (zero timepoint). Cell samples were taken at the indicated times, lysed, and analyzed by western blotting against the indicated proteins. Parallel samples were taken and a budding index was counted by microscopy. Results are representative of 3 independent experiments. (C) The indicated strains were grown and plated as in panel B.

Figure 6

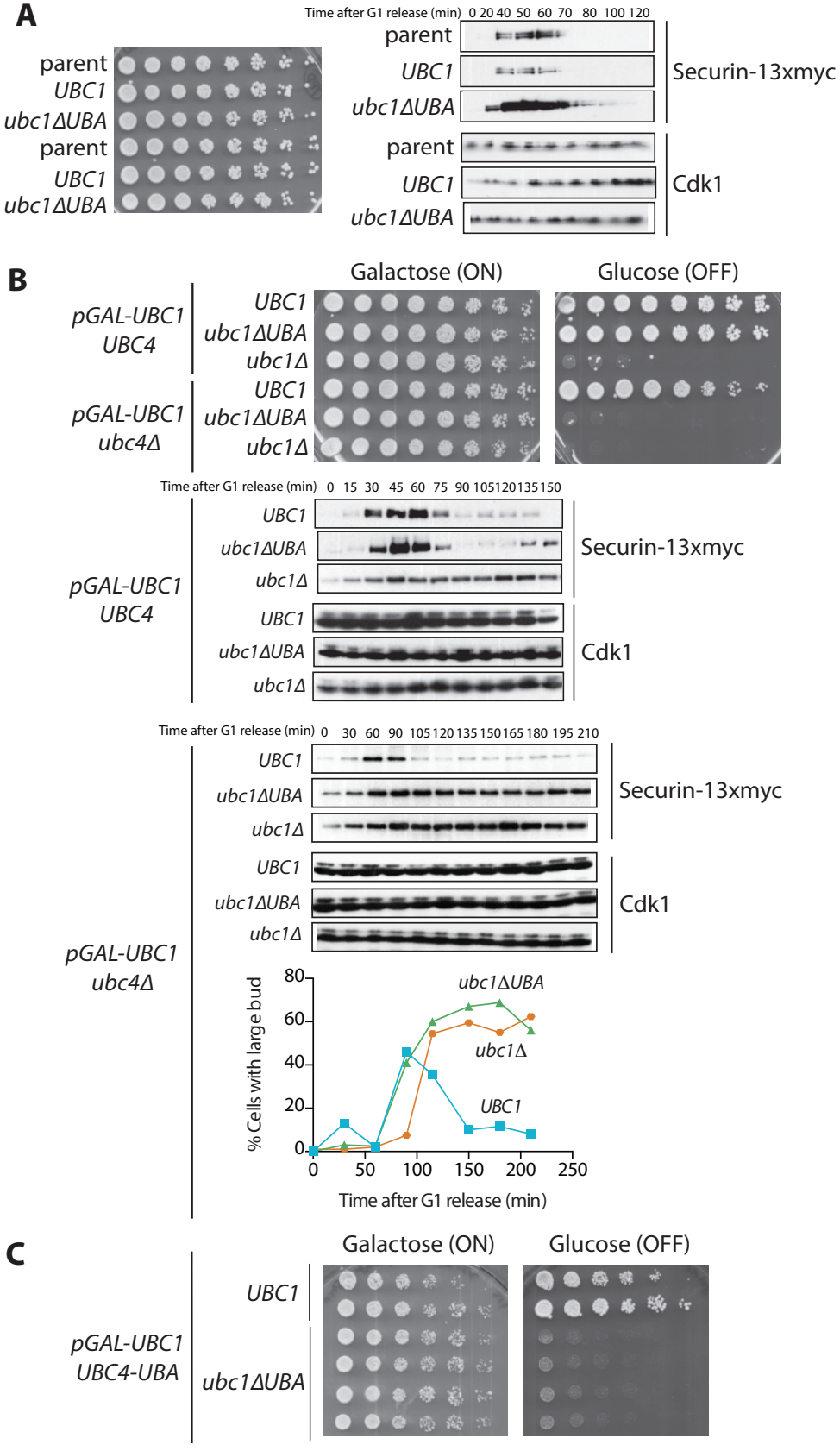
A
parent
UBC1
ubc1ΔUBA
parent
UBC1
ubc1ΔUBA
Time after G1 release (min) 0 20 40 50 60 70 80 100 120
parent
UBC1
ubc1ΔUBA
Securin-13xmyc
parent
UBC1
ubc1ΔUBA
Cdk1
B
Galactose (ON)
Glucose (OFF)
pGAL-UBC1
UBC4
UBC1
ubc1ΔUBA
ubc1Δ
pGAL-UBC1
ubc4Δ
UBC1
ubc1ΔUBA
ubc1Δ
Time after G1 release (min) 0 15 30 45 60 75 90 105 120 135 150
pGAL-UBC1
UBC4
UBC1
ubc1ΔUBA
ubc1Δ
Securin-13xmyc
UBC1
ubc1ΔUBA
ubc1Δ
Cdk1
Time after G1 release (min) 0 30 60 90 105 120 135 150 165 180 195 210
pGAL-UBC1
ubc4Δ
UBC1
ubc1ΔUBA
ubc1Δ
Securin-13xmyc
UBC1
ubc1ΔUBA
ubc1Δ
Cdk1
% Cells with large bud
80
60
40
20
0
0
50
100
150
200
250
Time after G1 release (min)
ubc1ΔUBA
ubc1Δ
UBC1
C
Galactose (ON)
Glucose (OFF)
pGAL-UBC1
UBC4-UBA
UBC1
ubc1ΔUBA

Table 1. List of yeast strains.

All strains listed are W303 and derived from AFS92 (A. Straight).

Strain	Genotype
DOM918	*MAT a; PDS1-13xMyc:HIS3*
yJG2	*MAT a; PDS1-13xMyc:HIS3; pGAL-3xHA-UBC1:TRP1; UBC1-9xMYC::LEU2*
yJG3	*MAT a; PDS1-13xMyc:HIS3; pGAL-3xHA-UBC1:TRP1; ubc1ΔUBA-9xMYC::LEU2*
yJG5	*MAT a; PDS1-13xMyc:HIS3; pGAL-3xHA-UBC1:TRP1; empty vector::LEU2*
yJG10	*MAT a; PDS1-13xMyc:HIS3; UBC1-6xHis1xFlag:URA3(K. lactis)*
yJG11	*MAT a; PDS1-13xMyc:HIS3; ubc1ΔUBA-6xHis1xFlag:URA3(K. lactis)*
yJG14	*MAT a; PDS1-13xMyc:HIS3; pGAL-3xHA-UBC1:TRP1; ubc4Δ:LEU2; UBC1-9xMYC::LEU2*
yJG15	*MAT a; PDS1-13xMyc:HIS3; pGAL-3xHA-UBC1:TRP1; ubc4Δ:LEU2; ubc1ΔUBA-9xMYC::LEU2*
yJG16	*MAT a; PDS1-13xMyc:HIS3; pGAL-3xHA-UBC1:TRP1; ubc4Δ:LEU2; empty vector::LEU2*
yJG81	*MAT α; PDS1-13xMyc:HIS3; UBC4-UBA-9xMyc:LEU2*
yJG82	*MAT a; PDS1-13xMyc:HIS3; UBC4-UBA-9xMyc:LEU2*
yJG85	*MAT a; PDS1-13xMyc:HIS3; pGAL-3xHA-UBC1:TRP1; UBC1-9xMYC::LEU2; UBC4-UBA-9xMyc:LEU2*
yJG86	*MAT α; PDS1-13xMyc:HIS3; pGAL-3xHA-UBC1:TRP1; UBC1-9xMYC ::LEU2; UBC4-UBA-9xMyc:LEU2*
yJG87	*MAT a; PDS1-13xMyc:HIS3; pGAL-3xHA-UBC1:TRP1; ubc1ΔUBA-9xMYC::LEU2; UBC4-UBA-9xMyc:LEU2*
yJG88	*MAT α; PDS1-13xMyc:HIS3; pGAL-3xHA-UBC1:TRP1; ubc1ΔUBA-9xMYC::LEU2; UBC4-UBA-9xMyc:LEU2*

Chapter 3

Conclusion

The anaphase-promoting complex/cyclosome (APC/C) uses two E2 ubiquitin-conjugating enzymes, Ubc4 and Ubc1, to initiate and elongate polyubiquitin chains (1). Prior to this study, some biochemical evidence suggested that these two E2s bind to the same site on the APC and directly compete (1,2). The data presented here supports this model.

We found that the catalytic RING domain-containing subunit of the APC/C greatly enhances the ubiquitination reaction performed by the catalytic UBC domain of the E2s Ubc4 and Ubc1. This suggests that the catalytic domains of both E2s bind to the RING subunit of the APC/C. This is the same well-characterized interaction that has been demonstrated for a number of E2-E3 pairs (3-8).

In addition to its catalytic UBC domain, Ubc1 carries an extra domain, a C-terminal ubiquitin-associated (UBA) domain that affects Ubc1 binding to the APC/C (1,9). In this study we showed that the UBA domain contributes to APC/C binding and enhances competition with Ubc4. Thus, UBA domain of Ubc1 is important for ensuring efficient polyubiquitination of APC/C substrates by balancing Ubc1 affinity with that of Ubc4.

Surprisingly, the UBA domain binding to the APC/C does not seem to depend on the presence of ubiquitin. Instead, the UBA domain is binding directly to the APC/C. We were unable to pinpoint the exact binding site for the UBA domain on the APC/C, but we did rule out several key subunits in the vicinity of the E2 binding site on the catalytic RING subunit of the APC/C. Now only a couple of likely candidate subunits remain. Given this and the recent advances in structural methods (3,10), I believe that we may be able to identify the UBA domain-binding site on the APC/C using cryo-EM (in collaboration with the Barford lab in Cambridge). Alternatively, we could try expressing fragments of the candidate subunits recombinantly in insect cells and directly test binding to the UBA domain.

As with most scientific studies, the findings presented here open up interesting avenues for future study. For example, since Ubc1 contains an extra binding site for the APC/C at a distance from its catalytic domain, it is possible that the catalytic domain of Ubc1 could

transiently dissociate from the APC while the UBA domain remains APC/C-bound. This is an exciting possibility because an E1 enzyme can not normally associate with an E2 while its bound to an E3, since E1 and E3 share the same binding site on E2 (4). However, if the UBA domain binds to the APC/C and allows the catalytic domain of Ubc1 to dissociate from the APC/C, it would make it possible for Ubc1 to bind to an E1 and get recharged with ubiquitin. This would allow multiple round of ubiquitin transfer by Ubc1 in a single APC/C binding event, which could explain how Ubc1 is able to compete so effectively with Ubc4, making more of it's products when the two are mixed *in vitro*.

Another interesting question to explore is whether or not UBA domain binding to the APC/C is regulated in some way. For example, can the association of an inhibitor or a post-translational modification of the APC/C specifically block the UBA domain binding? This would be a way of preventing destruction of APC/C substrates by decreasing the amount of polyubiquitin chains attached to the substrate, a mode of regulation also seen for the human APC/C (3,11,12).

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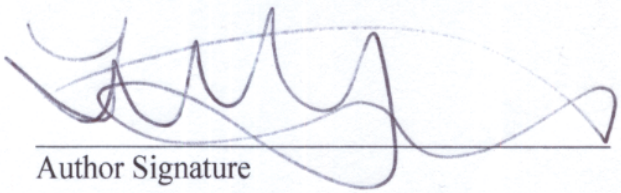

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