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E2s Act Sequentially in the Assembly of Ubiquitin Chains by the Anaphase-Promoting Complex

by

Mónica C. Rodrigo Brenni

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Cellular Biology

in the

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of the

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by

Mónica C. Rodrigo Brenni

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by

Monica C. Rodrigo Brenni

Abstract

Ubiquitination is used as a signaling mechanism by many processes in the cell. The ubiquitin signal can encode changes in intracellular trafficking, binding partners or protein turnover. Ubiquitin is a 76 amino acid protein that is covalently attached to other proteins via its C-terminus. The ubiquitination of proteins involves a cascade of three enzymes whose functions are to activate ubiquitin and attach it to lysine side chains of proteins. The last member of the cascade is an E3, or ubiquitin-protein ligase, that brings together substrates and ubiquitin-charged E2s. E3s allow for the transfer of ubiquitin from a catalytic cysteine on the E2 to a lysine side chain on the substrate. If lysines on ubiquitin itself are used during ubiquitination then polyubiquitin chains are built on the substrates. The Anaphase-Promoting Complex (APC) is an E3 ubiquitin ligase that assembles polyubiquitin chains on substrates important for cell cycle progression. In budding yeast, the APC collaborates with two E2 ubiquitin-conjugating enzymes, Ubc4 and Ubc1. We have demonstrated that Ubc4 and Ubc1 have very different enzymatic behaviors: APC reactions with Ubc4 result in rapid monoubiquitination of multiple nonspecific lysines on the substrate, whereas APC reactions with Ubc1 result in the assembly of polyubiquitin chains

that are linked specifically through lysine 48 (K48) of ubiquitin. We have also found two residues, threonine 84 and glutamine 122, on Ubc1 that are required for K48-linked polyubiquitination. Both of these residues are on flexible loops near the catalytic cysteine, in a position where they could influence catalysis. Threonine 84 is involved in catalyzing K48-dependent ubiquitination, whereas glutamine 122 is involved in reducing the ubiquitination of nonspecific lysines on the substrate. We propose that Ubc1 is able to catalyze the assembly of K48-specific chains at the expense of substrate lysine ubiquitination.

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Chapter 1

Introduction

One of the most spectacular events in every eukaryotic cell is the segregation of their genetic material or chromosomes. In a period that goes from mere seconds to a few minutes, the previously aligned chromosomes are synchronously separated and pulled to opposite poles of the cell. This single event, the splitting of chromosomes, has huge consequences for the survival of the cell. If anything goes wrong, it could mean the death of those cells. How does the cell ensure that this process happens correctly every single time? What are the master regulators of this crucial transition in the life of a cell?

Cell-cycle Regulation: Waves of Protein Phosporylation/Dephosphorylation and Regulated Protein Turnover

Every cell must coordinate chromosomal duplication with segregation in order to prevent errors during cell division. One of the master regulators of the cell cycle is cyclin dependent kinase, or Cdk, an enzyme that phosphorylates a wide variety of substrates, most of which are involved in one way or another with the cell cycle (Morgan, 1997; Morgan, 2007). Cdk, as the name implies, is regulated by a cofactor, cyclin. Cyclins not only recruit substrates but also change the specific activity of Cdk (Loog and Morgan, 2005). There are a number of cyclins and each isoform accumulates and is then degraded once per cell cycle. It is this cyclical activation of Cdk by its partner cyclins that allows the cell cycle to proceed in an orderly fashion. Cyclins interact with various substrates and allow Cdk to phosphorylate them. The phosphorylation, in turn, changes some property of the protein: often its localization, its binding partners

or its stability (Bloom and Cross, 2007). This phosphorylation is reversible, and there are a number of phosphatases, most notably Cdc14, that oppose Cdk phosphorylation and hence bring about a reversal in the overall cellular protein phosphorylation state (Stegmeier and Amon, 2004).

Another key regulator of cell division is the Anaphase-Promoting Complex, or APC. The APC is intimately involved in the segregation of chromosomes since its activity precipitates chromosome separation and their eventual segregation. The APC is a ubiquitin-protein ligase that adds the small protein ubiquitin to key cell cycle regulators, most notably securin, an enzyme that opposes chromosome separation. Ubiquitination by the APC leads to proteasomal targeting and destruction. It is this destruction that allows many processes inside the cell to be unidirectional (Peters, 2006; Thornton and Toczyski, 2006).

Cells closely coordinate Cdk and APC activity. The APC ubiquitinates, and hence marks for destruction, a number of cyclins, including all S-phase and M-phase cyclins, thereby abrogating late mitotic Cdk activity (Sullivan and Morgan, 2007). Cdk, on the other hand, is both a repressor and an activator of the APC. In G1 cells, Cdk phosphorylates one of the activators of the APC, Cdh1. When Cdh1 is phosphorylated it cannot activate the APC (Jaspersen et al., 1999). This prevention of APC activation in G1 allows for the accumulation of both S- and M-phase cyclins. After S-phase, Cdk phosphorylates the APC, allowing another activator, Cdc20, to bind and activate the APC in mitosis (Rudner and Murray, 2000) (Pesin and Orr-Weaver, 2008).

The coupled activities of Cdk and APC give the cell cycle its main properties: order and directionality (Figure 1). Oscillations in Cdk activity cause cyclical changes in the phosphorylation state of many cell-cycle regulators. These cell-cycle regulators in turn initiate cell-cycle events in a determined order. For instance, an increase in Cdk activity at the beginning of mitosis phosphorylates proteins needed for spindle assembly. At the end of mitosis, Cdk activity drops and phosphatases remove phosphates from these same proteins, allowing for spindle disassembly (Sullivan and Morgan, 2007). A crucial point of regulation is the metaphase-to-anaphase transition. At this transition the cell introduces regulated-protein destruction by the APC. The APC targets securin for degradation, which in turns allows for chromosomal separation and segregation (Cohen-Fix et al., 1996). The APC also targets cyclins for degradation at this point. The drop in Cdk activity allows for spindle disassembly and mitotic exit (Woodbury and Morgan, 2007). Thus, the APC, by destroying proteins required to maintain the mitotic state, introduces unidirectionality to the cell cycle.

The APC: a Multisubunit Ubiquitin-Protein Ligase

The APC targets proteins for destruction by attaching a ubiquitin signal to lysine side chains. Proteins that attach ubiquitin to other proteins belong to a group of enzymes called ubiquitin-protein ligases or E3s. In eukaryotes, there are two major families of ubiquitin ligases: the HECT domain and the RING domain family (Pickart and Eddins, 2004). HECT domain ligases contain a

catalytic cysteine that is intimately involved in the ubiquitination cascade, whereas RING domain ligases do not (Kee and Huibregtse, 2007). The APC belongs to this latter category. RING ligases have two protein substrates: the protein to be ubiquitinated and the enzyme that brings the ubiquitin to the complex, or E2. Many RING ligases are quite simple: a protein with a RING domain that interacts with both substrates and the E2 (Deshaies and Joazeiro, 2009).

The APC is much more complex, with at least 13 subunits in the budding yeast *Saccharomyces cerevisiae* (Peters, 2006; Thornton and Toczyski, 2006). Nine of these subunits are essential, underscoring their importance for APC activity, but very little is known about their exact function. Besides the RING subunit, Apc11, the APC contains a cullin subunit, Apc2, making it a member of the Cullin/RING ligases. The APC also has two activators, Cdc20 and Cdh1, which recruit substrates to the APC (Pesin and Orr-Weaver, 2008). APC substrates have small sequences, termed D-boxes and KEN boxes, which are recognized by Cdc20 and Cdh1 (King et al., 1996; Pfleger and Kirschner, 2000). There is also evidence for a substrate-binding site on the APC core (Yamano et al., 2004). Analysis of yeast APC interactions found two sub-complexes. One sub-complex contains Apc11, Apc2 and Doc1, and it is believed to interact with E2s. Another sub-complex contains Cdc27, Cdc16 and Cdc23 (Thornton et al., 2006). These three proteins contain a large number of TPR motifs, a protein interaction domain. Although previous work had implicated Cdc27 in activator binding, APC lacking Cdc27 is still able to ubiquitinate substrates, indicating that

there are other activator- binding sites on the APC (Thornton et al., 2006). Mutational analysis on the TPR subunits of Cdc27 and Cdc23 uncovered a role for these subunits in activator binding. The mutations created increase the rate of activator dissociation. More importantly, they did not affect substrate ubiquitination. From these studies a bivalent binding mechanism was proposed. In this model, the APC, the activator and the substrate will form a trimolecular complex with all proteins interacting with one another (Matyskiela and Morgan, 2009).

The Ubiquitination Cascade

Ubiquitination involves at a minimum three enzymes: an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme and an E3 ubiquitin-protein ligase (Figure 2) (Pickart and Eddins, 2004). In budding yeast there is a single E1 for ubiquitin (McGrath et al., 1991). E1s have three domains, the Ub adenylation domain, the Cys domain and the ubiquitin-fold domain (UFD) (Pickart, 2001). The ubiquitination reaction begins by the ordered binding of Mg-ATP and then ubiquitin. In fact, E1 has very low affinity towards ubiquitin in the absence of ATP (Haas and Rose, 1982). Ubiquitin is then adenylated, and thus activated and then transferred to the catalytic cysteine in the Cys domain. At this point another molecule of ATP and ubiquitin bind the adenylation domain so that E1 always carries two activated ubiquitins. The UFD domain recruits E2 enzymes allowing the thiol-linked ubiquitin to be transferred to the E2 (Capili and Lima, 2007).

The thiol-linked E2 interacts with the next enzyme in the cascade, an E3 ubiquitin-protein ligase. The *S. cerevisiae* genome encodes 13 E2 genes, or UBC genes. Of these, *UBC12* is specific towards Nedd8 conjugation, and *UBC9* is the E2 for SUMO. Both Nedd8 and SUMO are ubiquitin-like proteins and they have their own E1 and E3 enzymes (Kerscher et al., 2006). The remaining UBC genes have all been shown to have ubiquitin-conjugating activity. The human genome contains at least 40 E2s (Michelle et al., 2009). All E2 enzymes share a catalytic core domain, or UBC domain, of about 150 amino acids containing a catalytic cysteine (Michelle et al., 2009). E2s containing only this domain are called class I E2 enzymes. Class II E2s contain an addition C-terminal extension, whereas class III E2s contain a N-terminal extension (Jentsch et al., 1990). The catalytic core of the E2 contains residues that mediate the interaction with E1 and E3 enzymes in a mutually exclusive manner (Eletr et al., 2005). Hence the E2 conjugated to ubiquitin must first disengage from E1 in order to interact with an E3 enzyme. The reverse is also true; the uncharged E2 must dissociate from the E3 in order to be charged again. This implies that the E3 enzyme is able to discern between a charged and uncharged E2 enzyme, and this appears to be the case at least for HsUbc2b and E3α and Ubc4 with $SCF^{\beta\text{-}TRCP}$ (Siepmann et al., 2003). Since substrate modification by ubiquitin usually requires multiple rounds of additions, the mutually exclusive interactions could impinge on how fast a substrate can be modified.

The final step in the cascade is the transfer of ubiquitin from the E2 catalytic cysteine to the ε amine of a lysine side chain in the substrate. If a HECT

domain ligase is involved, then the transfer will happen from the catalytic cysteine on the E3 to the lysine on the substrate (Kee and Huibregtse, 2007). RING ligases bring together a ubiquitin- charged E2 and a substrate to be ubiquitinated. This interaction can be direct, meaning mediated solely by the RING subunit, or it can require a number of adaptor proteins (Petroski and Deshaies, 2005). In the case of Cullin/RING ligases, the RING subunit usually mediates the interaction with the charged E2 enzyme and the Cullin subunit interacts with adaptors that bring the substrate to be ubiquitinated (Deshaies and Joazeiro, 2009). The genome of *S. cerevisiae* encodes more than 40 E3 ligases alone, without taking into account different substrate-recruitment factors. In higher eukaryotes the number of proteins with recognizable RING domains approaches 300. Unfortunately bioinformatics falls short because there are many RING fold ligases that are not related by sequence and are harder to predict. Instead, if we look at different substrate-recruitment factors, an estimate of over 600 RING ligases could potentially exist in the human proteome (Li et al., 2008). This is comparable to the approximately 500 genes encoding kinase domains.

Mechanism of Ubiquitin Transfer

How exactly is the thiol-linked ubiquitin on the E2 transferred to the ε amine of a lysine side chain thus forming an isopeptide? This is a question that remains to be fully answered. From a chemical point of view, the attack of the thiolester by an amine should depend on groups to stabilize the negative charge

on the carbonyl group of ubiquitin, an oxyanion hole, and a general base might be needed to deprotonate the attacking group (Passmore and Barford, 2004) (Pickart and Eddins, 2004). No such stabilizing residue or general base stands out in either the E2 or the E3. In fact, both E3s and E2s are devoid of any strong candidates to aid in catalysis. It has been proposed that the E3 is merely a platform that brings together the ubiquitin-conjugated E2 and the substrate and that its "catalytic role" is merely due to an increase in local concentration (Zheng et al., 2002; Hao et al., 2007). Although this is the case in most systems studied, it is also clear that the ability of the charged E2 to be attacked by a lysine is increased in the presence of a ligase (Saha and Deshaies, 2008). This observation brought about the model of allosteric activation, in which binding of E3 to charged E2 would cause small changes in a number of amino acids that are connected via a network that culminates at the catalytic cysteine, making it more reactive (Ozkan et al., 2005). Allosteric activation and increase in local concentration provide insights on how E3 ligases might be acting upon the E2 enzymes, but these models do not bring us closer to understanding the chemistry behind ubiquitination.

While looking for residues that might be important for catalysis, Wu and coworkers were intrigued by the absolute conservation of an asparagine near the catalytic cysteine of all E2s (Wu et al., 2003). Mutating this residue in Ubc13 caused a specific defect: isopeptide bond formation was compromised if the asparagine was mutated. This was also true for the E2 enzyme E2-25K and for the SUMO-specific E2, Ubc9. The authors proposed a role for the asparagine in

stabilizing the oxyanion intermediate and brought us the first catalytic residue, besides the cysteine, involved in ubiquitination (Wu et al., 2003). Although this asparagine faces in the opposite direction in the known tertiary structures of E2s, it could be reposition upon E3 binding.

The finding of this catalytic residue brings us closer to what is needed for ubiquitination, but there is still a lack of a general base to deprotonate the ε amino group of the attacking lysine. The ε amino group of lysine has a pK close to 10 in most proteins, making its deprotonation at physiological pH almost impossible in the absence of a general base. This was addressed in the SUMO pathway (Yunus and Lima, 2006). SUMO is a ubiquitin-like molecule that uses analogous chemistry to be covalently attach to proteins. It has its own E1 and E2, Ubc9. A unique aspect of sumoylation is that some substrates can interact directly with Ubc9 in the absence of an E3 ligase. While looking for residues in Ubc9 that affect sumoylation, a microenvironment near the catalytic cysteine was found that effectively lowers the pK of the substrate lysine from around 10 to around 8.5 and orders the attacking lysine in the active site. This microenvironment will take the role of the general based proposed to be needed for catalysis. These experiments were done with a mixture of structural and enzymatic studies. The residues involved in this microenvironment, Y87, D127 and N85 of Ubc9 directly contact the substrate's lysine, but rather than being involved in binding, they are involved in catalysis. N85 is the asparagine that is conserved across E2s and besides stabilizing the oxyanion hole, it aids catalysis by positioning the loop containing D127. D127 in turn interacts with the attacking

lysine and orders it in the active site. D127 is highly conserved as well and when it is not an aspartate it is a serine. At least in one case the serine is under phosphoregulation: when phosphorylated, the E2 activate site would mimic an aspartate at that position, when not phosphorylated it would lack a crucial member of the microenvironment (Sarcevic et al., 2002). Y87 provides a hydrophobic platform to also position the lysine in the active site. This residue is not as highly conserved; in fact in most E2s is either an asparagine or an aspartate. When residue 87 is not a tyrosine there is a leucine that comes in contact with the substrate's lysine. This leucine, although it approaches the lysine from the opposite side, provides hydrophobic chain that occupies the same space as Y87 would. All these residues are also involved in suppressing the lysine pK by replacing hydrogen bonds between the lysine and the solvent with interactions between the lysine and the E2 side chains (Figure 3)(Yunus and Lima, 2006).

The example above provides a great working model for the catalysis of ubiquitination, but it was discovered utilizing the SUMO pathway. How is this relevant for ubiquitination? The only other structure of a charged E2 in complex with the attacking lysine is in the Ubc13/Mms2 system (Eddins et al., 2006). This system does carry out ubiquitination, but specifically at residue K63 of ubiquitin (this will be discussed later). In the crystal structure it was seen that the attacking lysine approached the active site in a similar manner as in the SUMO pathway. Many of the residues implicated in lysine ordering and pK suppression, N85 and D127, are conserved in Ubc13 as N79 and D119. Although Y87 is not

conserved (it is an aspartate) the position is still important because mutating it to alanine in Ubc13 causes a reduction in K63-linked ubiquitination. And, as Yunus and Lima proposed, there is a leucine, L121, which fills the space that Y87 would occupy otherwise. These observations will be greatly aided by the same kinetic characterization done in the SUMO pathway. As of now we know these residues are positioned in a similar manner as in the Ubc9 structure, but we do not know if they also help in the pK suppression, which seems a critical idea of the model.

Not every lysine was created equal

Although it is in principle true that ubiquitination can happen at any lysine, there seems to be a preference, most noticeable seen in the case of polyubiquitination, where a ubiquitin chain is built on a substrate. In fact, ubiquitination can be divided into two general reactions: the attachment of ubiquitin to a substrate's lysine, or monoubiquitination, and the attachment of ubiquitin to lysines on ubiquitin itself, or polyubiquitination. A special case of monoubiquitination, multi-monoubiquitination, occurs when multiple lysines on the substrate are ubiquitinated. What determines the pattern of ubiquitination depends on the specific reaction being followed, but often the substrate/E3 interaction plays a role, as well as the nature of the E2. Let us first considered monoubiquitination and how the first ubiquitin is attached.

As mentioned earlier, E3 ligases bring substrates in close proximity to a charged E2. The mere interaction of E3 ligases and substrates precludes some lysines from attacking the charged E2 because they will be in the binding

interface. This will also imply that some lysines are more prone to be ubiquitinated due to the optimal distance from their E3 recruitment/interaction region and the site of ubiquitination (Pickart, 2001). Data from the Morgan lab favors the idea that ubiquitination happens at an optimal distance from the substrate's recruitment region and that in most proteins this is restricted to the N-terminus of the protein. Moreover, in many cases the N-terminus contains an unstructured region with many lysines. The presence of multiple lysines could make it easier for the substrate to be ubiquitinated because it does not have to depend on the correct position of the lysines. Also, ubiquitination sometimes happens in the context of complexes, where one subunit gets ubiquitinated and degraded but others do not. The protein-protein interaction in the complex will also preclude some lysines (Petroski and Deshaies, 2003).

The context of ubiquitination has been explored more extensively in a different E3 system. The SCF (Skp1-Cullin-Fbox) is a RING ligase also important for cell-cycle progression. One of its substrate recruitment factors, the Fbox protein Cdc4, interacts with substrates that have been phosphorylated by Cdk. SCF provides an excellent system to study protein ubiquitination since, although it has multiple subunits, it is not as complex as the APC. One of SCF's substrates is Sic1, whose turnover is important for cells to enter the cell cycle (Petroski and Deshaies, 2003).

This substrate is ubiquitinated while still bound to its binding partner, Cdk. Using an *in vitro* system, it was discovered that Sic1 can be ubiquitinated at any of its lysines, but some of them are physically blocked by its binding to Cdk.

Interestingly, when ubiquitination is forced to happen at lysines usually not available, Sic1 is degraded very slowly by the proteasome. Of the 6 N-terminal lysines that are not blocked by Cdk binding, all were able to sustain ubiquitination and proteasomal targeting of Sic1, albeit to different degrees, underscoring the importance of where ubiquitination occurs. Another insight from this work was their demonstration that a single ubiquitin chain is both necessary and sufficient for proteasomal targeting and degradation *in vivo*. Up to this point all experiments showing the requirement of a ubiquitin chain for proteasomal targeting had been done *in vitro* and with model substrates (Petroski and Deshaies, 2003).

Mechanisms of Polyubiquitination

Some proteins get polyubiquitinated, where a chain of ubiquitin is built on a substrate's lysine (Hochstrasser, 2006). In this reaction, the site of ubiquitination is predetermined. How can the same enzyme carry out both types of ubiquitination: a nonspecific substrate ubiquitination and a specific ubiquitin attachment? The answer comes from studies of E2 enzymes and how they determine linkage- specificity of RING ligases.

At a minimum, in order for polyubiquitination to happen the charged E2 must be able to interact with ubiquitin, the attacking moiety. Thus far there are four mechanisms in the literature explaining how a chain can be built. The first one involves Cdc34, the E2 for the SCF ligase. Cdc34 builds lysine 48-linked ubiquitin chains on substrates as well as free in solution. Interestingly the two

activities of Cdc34, the attachment of ubiquitin to substrate's lysines and to K48 of ubiquitin occur at markedly different rates, with the attachment of the first ubiquitin to the substrate being rate limiting. Moreover, these two activities can be separated by specific mutations (Petroski and Deshaies, 2005).

Cdc34 contains an acidic loop near its catalytic cysteine that when mutated abrogates K48-linked chains but does not affect substrate ubiquitination. Kinetic studies also demonstrated that charged Cdc34 interacts noncovalently with ubiquitin with an apparent affinity of 600 μM. Although this might be a low affinity interaction given the intracellular concentration of ubiquitin (10 μM) (Riley et al., 1988), it allows for the SCF-bound substrate that is already ubiquitinated to be the predominant attacking species because it will be in the millimolar range. Further studies showed that the acidic loop is not involved in ubiquitin binding but in catalysis because mutating it does not affect K_M but rather decreases maximal activity towards ubiquitin. When reactions were done in the presence of SCF, a Vmax effect was also observed, but the K_M for ubiquitin was unchanged. This demonstrated that Cdc34 contains a binding site for ubiquitin that can be allosterically stimulated by SCF.

The same study looked at residues important in ubiquitin for K48-linked ubiquitination. The hydrophobic patch, a region centered on isoleucine 44 of ubiquitin, mediates most known binding interactions with ubiquitin (French et al., 2005). Ubiquitin carrying mutations in the hydrophobic patch was unable to attack charged Cdc34. Unfortunately we do not know what the defect in these mutations is because they did not have any measurable activity under the

conditions tested. What we do know is that the acidic loop is restricted to Cdc34 and its orthologs, so it is unclear how relevant this mechanism will be for E2s that do not have it. What is evident is that the same E2 can carry out both nonspecific substrate ubiquitination and highly specific polyubiquitination on the "priming" ubiquitin. It appears that there is a trade off because the first step is slow in comparison to the second step. This is circumvented by the E3 ligase that both increases the local concentration of the substrate and allosterically activates the E2 (Saha and Deshaies, 2008).

The second mechanism involves UbcH5, a human E2 enzyme extensively used *in vitro* because it appears to work with almost every ligase. When UbcH5 is paired with the E3 ligase BRCA1 it results in the autoubiquitination of BRCA1 and the formation of high molecular weight products. When the solution structure of UbcH5 was solved in the presence of ubiquitin, a noncovalent interaction between the two molecules was observed (Brzovic et al., 2006). This interaction occurs on the "backside" of UbcH5, far from its catalytic cysteine and the surfaces proposed to interact with both E1 and E3. Moreover, this noncovalent interaction allowed UbcH5 to self-assemble into high molecular weight complexes. When a critical residue in the interface, S22, was mutated, UbcH5's self-assembly was abolished as well as its ability to form highly ubiquitinated products. This interaction appears to be important for the processive ubiquitination of substrates and not necessarily for a specific lysine to be used. In fact, UbcH5 family members are known to build every type of ubiquitin-ubiquitin linkage possible depending on the system being studied. Although this

model is very attractive it has yet to be demonstrated in other family members, or in an *in vivo* relevant system.

The third mechanism involves an E2 already discussed, Ubc13. Ubc13, in combination with Mms2, builds K63-linked ubiquitin chains; both free in solution and attached to substrates. Mms2 is an E2 variant – it has the same fold as an E2 and most residues are conserved but it lacks the catalytic cysteine. When the tertiary structure of charged Ubc13/Mms2 was solved, a binding interaction between heterodimers was observed (Eddins et al., 2006). In the heterodimer, K63 of ubiquitin of one heterodimer was poised to attack the ubiquitin attached to Ubc13 of a second heterodimer. The structure clearly shows the role of Mms2 in positioning the attacking ubiquitin in the active site. The interaction is mediated by isoleucine 44 of ubiquitin and isoleucine 57 of Mms2, two residues that had been shown previously to be important for chain formation. Other residues involved are S27 and T44 of Mms2. All these residues are far away from the heterodimer interface as expected. A competition assay was used to determine the defect with the mutants in Mms2. Inert polyubiquitin chains inhibit Ubc13/Mms2's chain-building activity because they occupy the acceptor ubiquitin site on Mms2. Mutants in Mms2 that prevented chain assembly were inhibited to a lesser extent by these chains, indicating a binding defect rather than a catalytic defect. The relevance of this mechanism for other E2/E3 systems is unclear, since it relies on an E2-variant to position the attacking ubiquitin.

The final mechanism involves the preassembly of a K48-linked chain and its transfer en bloc to a substrate's lysine. Gp78 is a human RING ligase that

works with the E2 Ube2g2 at the endoplasmic reticulum and it is essential for the ubiquitination of misfolded ER proteins. While studying this system, it was discovered that K48-linked chains were being built on the catalytic cysteine of Ube2g2 in a RING-dependent manner (Li et al., 2007). Moreover, this assembly required residues on an acidic loop on Ube2g2, akin to Cdc34, but the acidic loop of Ube2g2 is interacting with the donor ubiquitin (the one thiol-linked to the E2) rather than with the acceptor (attacking) ubiquitin. The chain was built by aminolysis-based transfer between two Ube2g2 molecules. Two charged Ube2g2s interact such that K48 of ubiquitin of one Ube2g2 attacks the thiol-linked ubiquitin of another. In this model, the most recently added ubiquitin is at the bottom of the chain, still attached to the E2 via the catalytic cysteine, and there is a sew-saw mechanism at play. One prediction of this model is that the E2 may form dimmers. A small population of the E2 preparation appeared to be in higher ordered structures. More intriguingly, gp48 forms homodimers and it may promote the formation of E2 dimers. In fact, recently two groups have shown that gp48 contains a region distinct from the RING domain that interacts with Ube2g2, promotes its binding to gp48 and polyubiquitination at the catalytic cysteine (Das et al., 2009; Li et al., 2009). This is clearly different from most E2/E3 interactions, which are transient in nature. One group proposes that this enhanced affinity, in the nanomolar range, would promote processive ubiquitination: the addition of multiple ubiquitins in a single substrate binding event, even when the substrates have low affinity for the E3 (Li et al., 2009). This would be especially useful in the ER-associated degradation (ERAD)

pathway because a small number of ligases have to deal with potentially hundreds of different misfolded proteins (Li et al., 2009).

This preassembly mechanism has been seen in another E2 enzyme, budding yeast Ubc7. Ubc7 is also an ER-associated E2, it also contains an acidic loop, but the only time that a chain has been observed is when Ubc7 is overproduced and mislocalized. The chain is built upon Ubc7 catalytic cysteine and can be transferred to a lysine on a substrate, lending more evidence to the preassembly-transfer mechanism (Ravid and Hochstrasser, 2007).

All the above examples show different ways by which a ubiquitin chain can be built. This is very interesting in light of how highly homologous the E2s are across species. It would indicate that small changes could lead to completely new activities on the E2s. As in the Cdc34 example, there is also a trade off between substrate ubiquitination and polyubiquitination. A comprehensive study involving the E3 BRCA1 and different E2s found that some E2s only do monoubiquitination, whereas others only do polyubiquitination if the substrate already has a ubiquitin, and a third group can do both (Christensen et al., 2007). One common feature of the ubiquitin-specific E2s was their ability to bind ubiquitin noncovalently, albeit by very different methods. The enzyme that can do both, UbcH5, might represent an ancestral version of the E2 enzymes, since although it can do polyubiquitination it does so in a nonspecific manner. As the E2s gained the ability to carry out polyubiquitination, they lost the ability to ubiquitinate substrate's lysines. In this evolutionary trajectory, we are bound to

find enzymes at all stages of the route, with different degrees of specificity for the two reactions.

Polyubiquitination and the APC: a Tale of Multiple E2s

All APC substrates studied to date are short-lived proteins that are degraded by the 26S proteasome. *In vitro* studies demonstrated that highly ubiquitinated proteins are degraded more efficiently by the proteasome. The nature of these high molecular weight species was not understood until chemical mapping was done on a model substrate, b-galactosidase. It was shown that there was a chain of ubiquitins attached to a single lysine on b-galactosidase. Not only was ubiquitin being ubiquitinated, but also it was specifically ubiquitinated on lysine 48 (Chau et al., 1989; Gregori et al., 1990). The importance of K48-linked ubiquitination is highlighted by the cell- cycle arrest associated with mutants lacking K48. Yeast expressing K48R ubiquitin as the only copy of ubiquitin had a marked inhibition in the degradation of most short-lived proteins and they arrested in both the G1 and G2/M phases of the cell cycle (Finley et al., 1994). These arrests points coincide with the time that SCF and APC are active. Although ubiquitin is highly conserved across all species, alanine scanning of surface residues found few residues important for yeast survival under normal laboratory conditions (Sloper-Mould et al., 2001).

The ability to create chains of defined lengths further expanded our understanding of proteasomal targeting. Using these chains, it was found that a chain of four ubiquitins linked via K48 was the minimal signal for efficient

proteasomal degradation (Piotrowski et al., 1997). Why would such a specific signal be needed? There are a number of benefits of having the targeting signal be a ubiquitin chain. For instance, a long chain could persist longer on a substrate and allow for efficient targeting even in the presence of competing deubiquitinating activities. Conversely, since a chain must be built, the substrate must remain bound to the ligase long enough to acquire such chain. This would increase the fidelity of degradation. Also, the ubiquitin chain might provide a unique signal that is recognized by receptors on the proteasome (Pickart, 1997). This last point has some structural evidence, since ubiquitin chains built via different linkages form different structures in solution (Varadan et al., 2002; Varadan et al., 2004; Eddins et al., 2007). Recent evidence does suggest that other linkages, and even short-clustered chains do allow for proteasomal targeting although has only been demonstrated *in vitro* (Kirkpatrick et al., 2006).

The most compelling study using *in vivo* data showed that yeast cells build every possible linkage on ubiquitin and that K11-linked chains constitute a large proportion of linkages, almost as common as K48 (Xu et al., 2009). This study also showed that K11 linkages are important for ERAD and specifically Ubc6 function. How to reconcile this data with the importance of K48 of ubiquitin? One idea is that K48 is used by enzymes whose substrates, or rather the degradation of those substrates, is important for cell-cycle progression. On the other hand, K11 would only be required upon ER stress and it would not be essential for viability of yeast cells under normal laboratory growth conditions.

In light of the above, it was expected that APC should build ubiquitin chains, quite possibly via K48. When the APC was first discovered, two E2s were found to work with it and allow for ubiquitination of cyclin B, UbcH5 (also known as Ubc4) and UbcH10 (UbcX and E2-C in some species) (Hershko et al., 1994; Aristarkhov et al., 1996; Mathe et al., 2004). Initial characterizations showed that UbcH10 converted more substrate to a ubiquitinated species than UbcH5, but the conjugates were lower in molecular weight (Yu et al., 1996). A more careful mass spectroscopy study showed that both enzymes modify multiple lysines on cyclin B with short polyubiquitin chains. These chains displayed very little linkage specificity although UbcH5 tended to favor the formation of K48-linked chains and UbcH10 favored K11-linked chains (Kirkpatrick et al., 2006). This was an early indication that the ubiquitination pattern is dictated by the nature of the E2. Cyclin B ubiquitinated by these two enzymes was recognized by the proteasome and degraded, albeit slowly. It has since been demonstrated that UbcH10 builds K11-linked chains in other APC substrates and that these types of chains are important for the degradation of substrates in lysates (Jin et al., 2008).

Although budding yeast has homologous enzymes for UbcH5 and UbcH10, Ubc4 and Ubc11 respectively, only Ubc4 was known to work with yeast APC (Townsley and Ruderman, 1998). *In vitro* APC reactions with Ubc4 lead to ubiquitination of the model substrate sea urchin cyclin B on multiple lysines but does not promote the formation of chains (Carroll and Morgan, 2002; Passmore et al., 2005). This is also true when APC-Ubc4 ubiquitinates the *in vivo* relevant

substrate, yeast securin. Although Ubc4 works with many ligases *in vitro*, the activity of Ubc4 with the APC is not an *in vitro* artifact because deletion of Ubc4 is synthetically lethal with mutations that diminish APC function (Irniger et al., 1995). The mere fact that, unlike the APC, Ubc4 is not essential suggests that other E2s can work with the APC.

During the course of my studies I discovered a novel yeast APC-dependent E2 enzyme, Ubc1. Ubc1 was known to be an E2 enzyme and to be able to catalyze the formation of unanchored ubiquitin chains, although it does this very slowly (Hodgins et al., 1996). Most importantly, there were not any ligases known to work with Ubc1. Chapter two of my dissertation deals with the discovery of Ubc1 as an APC-dependent E2 enzyme. Not only does Ubc1 work with the APC, but also it facilitates the formation of K48-linked polyubiquitin chains on APC substrates. I also demonstrate that Ubc4 and Ubc1 have complementing activities: Ubc4 leads to the rapid monoubiquitination of substrates and Ubc1 uses these pre-ubiquitinated species to rapidly build a K48-linked chain. *In vivo* the pair of enzymes is essential for APC function and cell cycle progression (Rodrigo-Brenni and Morgan, 2007). This was the first work that showed two E2 enzymes working synergistically with the same E3 to polyubiquitinate substrates (Christensen et al., 2007; Windheim et al., 2008).

The third chapter of my dissertation deals with the K48 specificity of APC-Ubc1 reactions. In the course of these studies I discovered important residues in Ubc1 required for both K48-linked ubiquitination and restriction of multi-monoubiquitination. Both residues mapped to an area near the catalytic cysteine

of Ubc1. Interestingly these residues seem to be involved in catalysis and not in ubiquitin recognition. In trying to decipher the catalytic mechanism of Ubc1 ubiquitination I found a small but reproducible suppression of the pK of the attacking lysine similar to that seen in the SUMO field (Yunus and Lima, 2006). It will be interesting to see if pK suppression is a general mechanism used to promote specific lysine ubiquitination.

I also looked at residues in ubiquitin important for its interaction with Ubc1. I found that tyrosine 59 was important for K48-linked chain formation. This residue was also found to be important for polyubiquitin formation by E2-25K, a human ortholog of Ubc1 that also carries out K48-linked ubiquitination (Pickart et al., 1992). At this point it is unclear whether Y59 is involved in catalysis or binding, but a general defect on ubiquitin conformation or K48 position can be discounted because this mutant does not affect Cdc34-dependent reactions, which also rely on K48 of ubiquitin (Pickart et al., 1992).

During the course of my studies, we have gained a better understanding of the importance of E2 enzymes in the ubiquitination of substrates. Until recently, E2 enzymes were thought to be just carriers of ubiquitin and that the important enzyme was the E3. E3s bring diversity by recognizing different substrates, but ultimately the E2 enzyme determines the fate of those substrates because it is the factor that establishes the ubiquitination pattern. Much is left to learn about these enzymes, but this work has shed some light on their amazing activities.

Figure 1. A simplified view of the cell-cycle control system

Levels of the three major cyclin types oscillate during the cell cycle (top), providing the basis for oscillations in the cyclin–Cdk complexes that drive cell-cycle events (bottom). Reproduced with permission from The Cell Cycle, Principles of Control (Morgan, 2007).

Figure 1

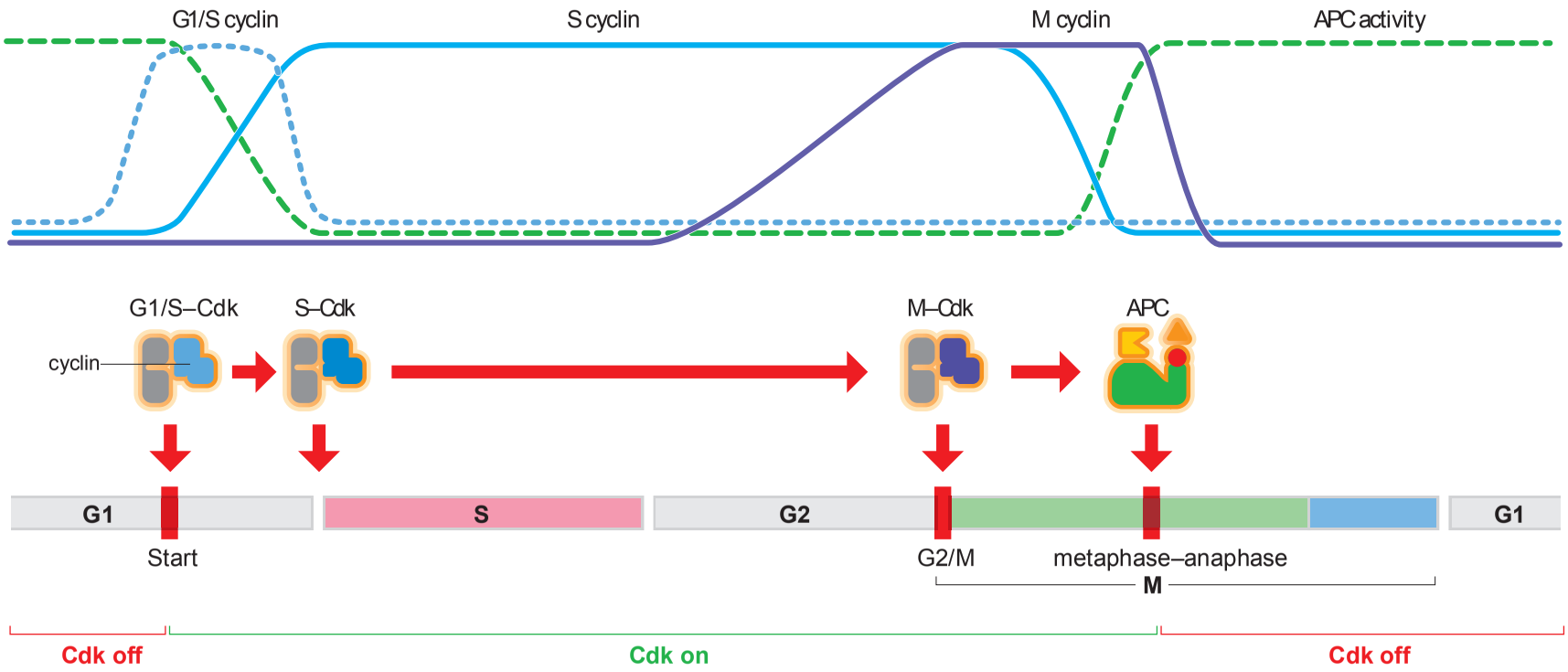

G1/S cyclin
S cyclin
M cyclin
APC activity
G1/S–Cdk
S–Cdk
M–Cdk
APC
cyclin
G1
S
G2
G1
Start
G2/M
metaphase–anaphase
M
Cdk off
Cdk on
Cdk off

Figure 2. The Ubiquitination Cascade

Ubiquitination begins by the activation of ubiquitin and its attachment to a catalytic cysteine of an E1 enzyme. E1 interacts with a number of E2 enzymes and the ubiquitin is transferred from the catalytic cysteine of E1 to a catalytic cysteine of E2. E2s interact with E3 ligases. E3s bring protein targets and ubiquitin-charged E2 together. A lysine on the protein target attacks the E2-bound ubiquitin. The E3-protein target complex is more stable than the E3-E2 complex; hence multiple rounds of ubiquitination occur in a single protein target-binding event.

Figure 2

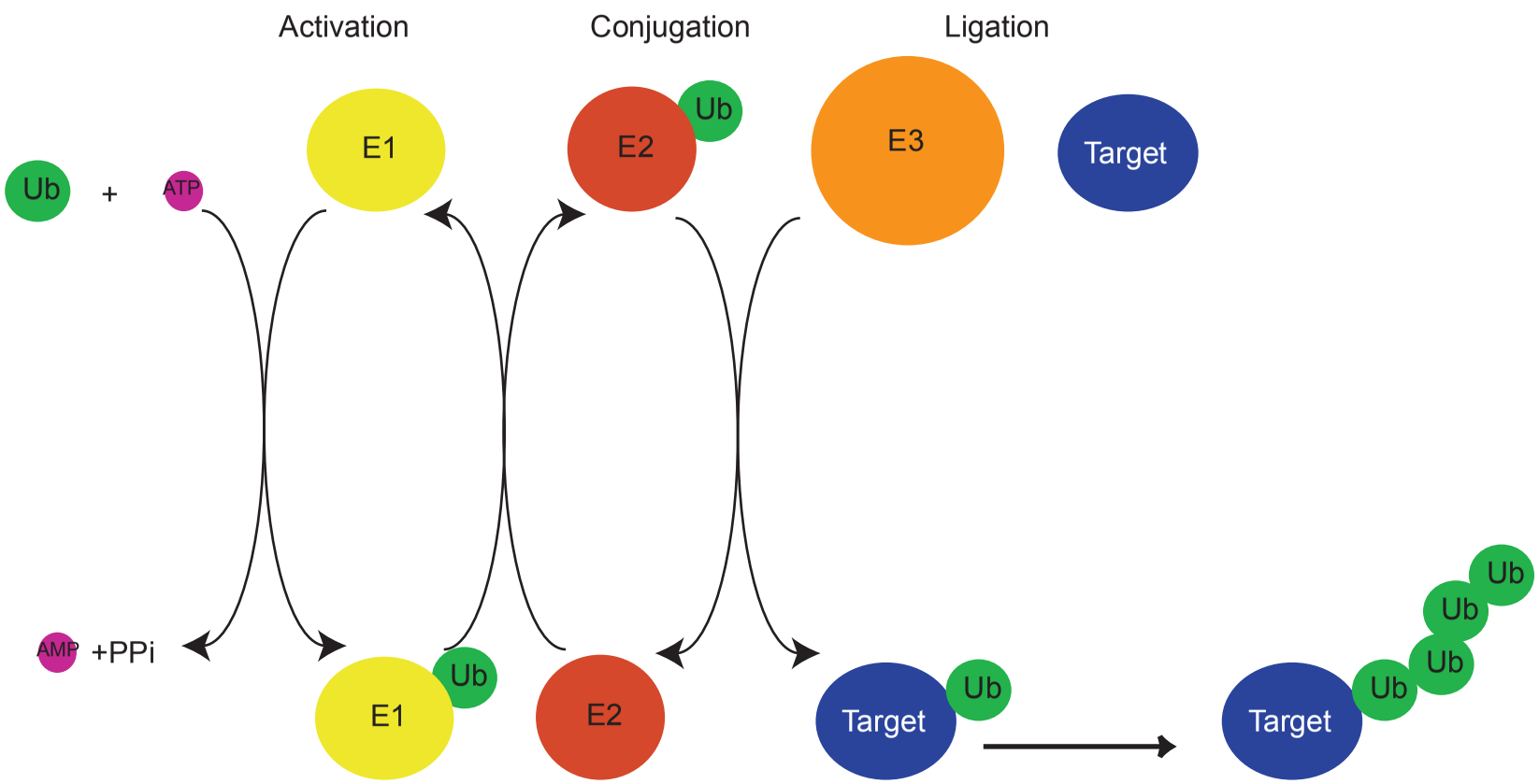

Activation
Conjugation
Ligation
Ub
+
ATP
E1
E2
Ub
E3
Target
AMP
+PPi
E1
Ub
E2
Target
Ub
Target
Ub
Ub
Ub
Ub

Figure 3. Propose Mechanism of Ubiquitination

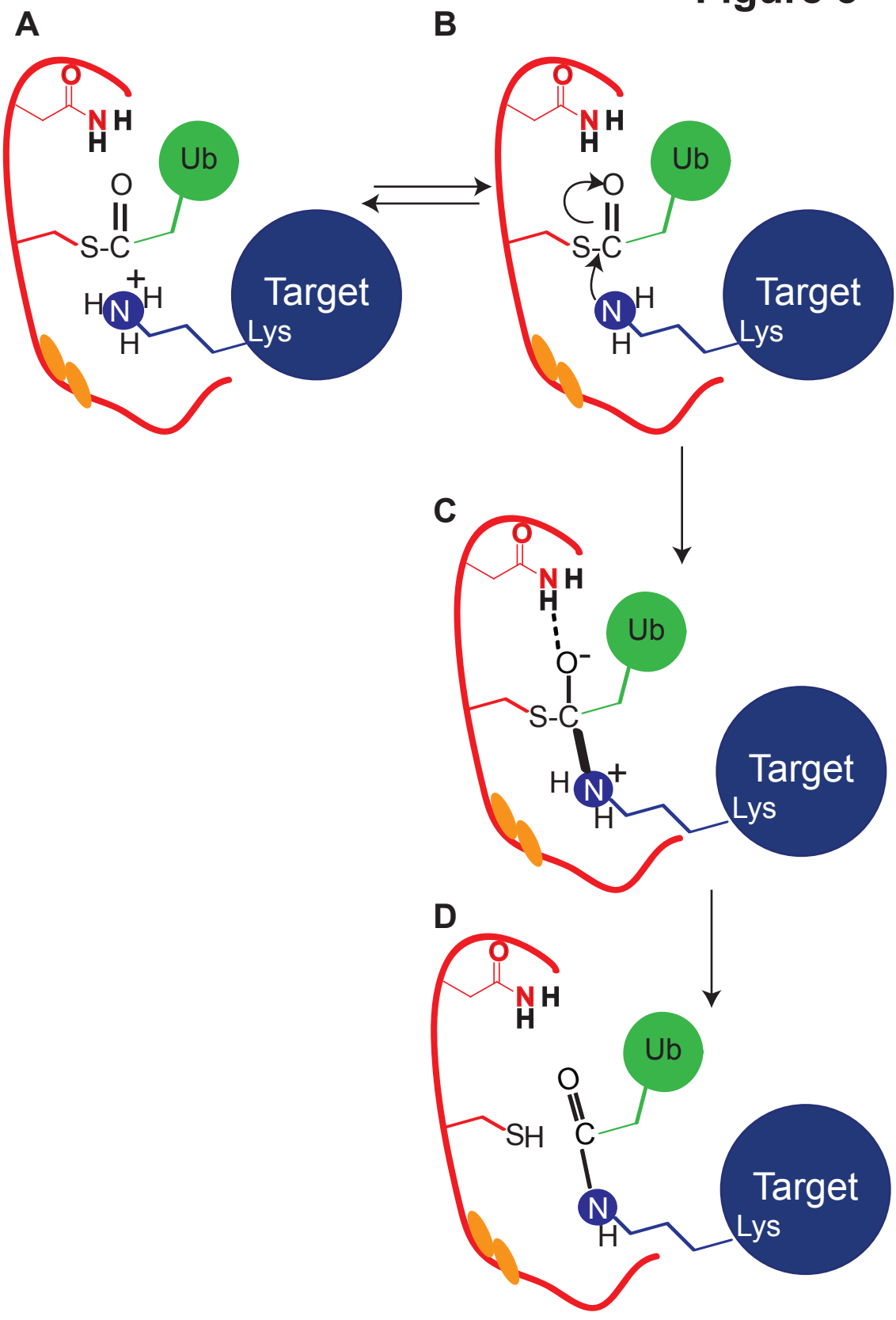
The E2 active site is shown in red, the rest of the protein has been omitted for simplicity. The E3 ligase does not appear to have a catalytic role and it has been omitted as well, but both the E2 and the protein target are brought together by the E3. Also, although a protein target is shown, the lysine can belong to another ubiquitin species in a polyubiquitination reaction.

(A and B) Equilibrium between protonated and deprotonated lysine side chain. The catalytic cysteine is shown bound to the C-terminus of ubiquitin (green). A lysine on the protein target (blue) is in the active site. The ammonium cation will be unable to attack the carbonyl carbon unless it is deprotonated. The E2 lacks a general base to extract the proton, but rather it has been proposed that it contains residues (in orange) that promote deprotonation by replacing optimal interactions between the lysine and the solvent with suboptimal interactions between the E2 side chains and the lysine. Once deprotonated the lysine will be able to attack the carbonyl carbon, creating a negative charge on the oxygen. A conserved asparagine has been proposed to be part of an oxyanion hole and stabilize the oxyanion intermediate.

(C) Transition state, showing the zwitterionic intermediate. The conserved asparagine is shown H-bonding to the oxyanion species, thereby stabilizing it.

(D) Final step in the reaction. The bond between the E2 cysteine and the c-terminus of ubiquitin is broken, releasing the ubiquitinated lysine on the substrate.

Figure 3



Chapter 2

Sequential E2s drive polyubiquitin chain assembly on APC targets

Monica C. Rodrigo-Brenni and David O. Morgan

Departments of Physiology and Biochemistry & Biophysics, University of California, San Francisco, CA.

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Abstract

The anaphase-promoting complex (APC), or cyclosome, is an E3 ubiquitin-protein ligase that collaborates with E2 ubiquitin-conjugating enzymes to assemble polyubiquitin chains on proteins important for cell-cycle progression. It remains unclear how the APC – or many other E3s – promotes the multiple distinct reactions necessary for chain assembly. We addressed this problem by analyzing APC interactions with different E2s. We screened all budding yeast E2s as APC co-enzymes in vitro and found that two, Ubc4 and Ubc1, are the key E2 partners for the APC. These proteins display strikingly different but complementary enzymatic behaviors: Ubc4 supports the rapid monoubiquitination of multiple lysines on APC targets, while Ubc1 catalyzes K48-linked polyubiquitin chain assembly on pre-attached ubiquitins. Mitotic APC function is lost in yeast strains lacking both Ubc1 and Ubc4. E2-25K, a human homolog of Ubc1, also promotes APC-dependent chain extension on pre-attached ubiquitins. We propose that sequential E2 proteins catalyze K48-linked polyubiquitination and thus proteasomal destruction of APC targets.

INTRODUCTION

Protein ubiquitination is a critical regulatory modification, particularly in the control of protein degradation, whereby the addition of multiple ubiquitins to a protein triggers recognition and destruction by the proteasome (Pickart and Eddins, 2004; Kerscher et al., 2006). This process is catalyzed by an enzymatic cascade involving a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-protein ligase (E3). E1 employs ATP hydrolysis to catalyze formation of a thioester bond between the C-terminus of ubiquitin and an active-site cysteine on E1. The E1-ubiquitin conjugate binds to an E2, resulting in transfer of the ubiquitin C-terminus from E1 to a cysteine in the E2. An E3 then promotes transfer of the ubiquitin from the E2-ubiquitin conjugate to a lysine side chain on the target protein. Repeated cycles of this reaction can lead to the attachment of ubiquitins to several lysines on the target and to specific lysines on ubiquitin itself, resulting in polyubiquitin chains.

E3s contain specific binding sites for two substrates: an E2-ubiquitin conjugate and a protein target. In the case of the large RING-domain family of E3s, the ε-amino group on a lysine side chain in the target protein attacks the E2-ubiquitin thioester linkage, resulting in the formation of an isopeptide bond between the C-terminus of ubiquitin and the target lysine. Catalysis depends on residues provided by the E2 (Wu et al., 2003; Passmore and Barford, 2004; Pickart and Eddins, 2004). RING-domain ligases facilitate the reaction by bringing the two substrates in close proximity and possibly by activating the catalytic function of the E2 (Ozkan et al., 2005).

Polyubiquitin chain formation results from a linkage between the C-terminus of one ubiquitin and a lysine side chain in another (Pickart and Eddins, 2004; Hochstrasser, 2006). Ubiquitin contains several lysines that can be used as ubiquitination sites (Peng et al., 2003), but most chains are linked through lysine 48 (K48) or lysine 63 (K63). A K48-linked chain of four or more ubiquitins provides an important recognition signal for destruction in the proteasome (Thrower et al., 2000). In most cases, polyubiquitin chain assembly is thought to occur by the sequential addition of ubiquitin to a lysine on a ubiquitin already attached to the target (Hochstrasser, 2006).

Polyubiquitin chain assembly by sequential addition involves two reactions with different lysine specificity. The monoubiquitination reaction – direct attachment of ubiquitin to the protein target – often occurs at multiple lysines in a somewhat random pattern that does not seem to depend on the amino-acid sequence context (King et al., 1996; Petroski and Deshaies, 2003). In contrast, the polyubiquitination reaction typically requires modification of a single specific lysine, such as K48, in ubiquitin. Many E3s are capable of promoting both of these reactions – raising the intriguing question of how a single E3 can carry out two reactions of such distinct specificity. The RING-domain ligase SCF solves this problem by collaborating with a single E2, Cdc34, that catalyzes both the slow attachment of ubiquitin to a lysine in the substrate as well as the rapid extension of a K48-linked polyubiquitin chain (Petroski and Deshaies, 2005). In another case, the two steps of chain assembly involve entirely different enzymes: the ubiquitination of PCNA begins with one E3-E2 pair that places the initial

ubiquitin on the target, after which a different E3-E2 complex extends a K63-linked ubiquitin chain (Hoege et al., 2002; Eddins et al., 2006).

The anaphase-promoting complex (APC), or cyclosome, is a large, multisubunit RING-domain E3 required for the completion of mitosis in all eukaryotes (Peters, 2006; Thornton and Toczyski, 2006; Morgan, 2007). The APC ubiquitinates securin, allowing chromosome segregation, and mitotic cyclins, promoting mitotic exit and the establishment of G1. APC activity is regulated by its association with activator subunits, Cdc20 and Cdh1, which help mediate the interaction between the APC and specific destruction sequences on its substrates. During mitosis, the APC is activated first by association with Cdc20, which targets securin and some cyclins at the metaphase-to-anaphase transition. Cdh1 is activated later in mitosis, resulting in further cyclin destruction and the maintenance of APC activity in G1.

A major unsolved problem in APC biology is the identity of the E2s that collaborate with the APC in vivo. In higher eukaryotes, APC function is thought to depend on at least two E2 proteins, whose names in human cells are UbcH5 (also known as Ubc4) and UbcH10 (also named UbcX and E2-C in some species) (Hershko et al., 1994; King et al., 1995; Aristarkhov et al., 1996; Yu et al., 1996; Townsley et al., 1997; Mathe et al., 2004; Kirkpatrick et al., 2006). Homologs of these two proteins are both essential for timely mitotic progression and cyclin destruction in fission yeast (Seino et al., 2003). A recent analysis suggests that vertebrate UbcH5 and UbcH10 promote monoubiquitination of APC targets, as well as the assembly of short polyubiquitin chains that display little

lysine specificity (Kirkpatrick et al., 2006). K48-specific polyubiquitination by purified APC has not been observed, suggesting either that it does not occur in vivo or depends on E2s that remain to be identified.

In the budding yeast, *Saccharomyces cerevisiae*, the relevant E2s are not clear. The yeast genome encodes proteins that are related to both UbcH5 and UbcH10 (Ubc4 and Ubc11, respectively), but cells depleted of both enzymes progress normally through the cell cycle (Townsley and Ruderman, 1998). Ubc4 has a close homolog in yeast, Ubc5, but cells lacking both Ubc4 and Ubc5 are viable, although their growth is impaired (Seufert and Jentsch, 1990). The same is true of cells depleted of both Ubc5 and Ubc11. Thus, there is no single or multiple E2 defect known to result in a complete loss of APC function, suggesting that we have not yet found all the E2s that collaborate with the yeast APC.

Budding yeast Ubc4 is a well-established APC-dependent E2 that has been used in all previous studies of yeast APC enzymology (Carroll and Morgan, 2005; Passmore et al., 2005). Deletion of *UBC4* is lethal in strains with diminished APC activity (Irniger et al., 1995). However, Ubc4-dependent reactions with the APC or other E3s do not result in efficient formation of polyubiquitin (Carroll and Morgan, 2002; Wu et al., 2003), indicating that other E2s may be required to promote chain formation by the APC.

To clarify the mechanisms by which the APC modifies its targets, we identified and characterized the E2s that work with the budding yeast APC. Two E2s were found to be critical: Ubc4 and Ubc1. A detailed comparison of their activities revealed that they act in sequence to promote the two steps in

polyubiquitin chain assembly: Ubc4 monoubiquitinates APC targets at multiple lysines, providing a substrate upon which Ubc1 assembles K48-linked polyubiquitin chains.

RESULTS

Identification of APC-dependent E2s

The yeast genome encodes 13 ubiquitin-conjugating enzyme family members (Ubc1-Ubc13). We purified each protein and tested its activity with the APC in vitro. E2s were purified from a library of yeast strains carrying TAP-tagged proteins under the control of their endogenous promoters (Ghaemmaghami et al., 2003). Seven of the thirteen E2 proteins were readily prepared from extracts of proliferating cells (Figure 1A). Of the remaining six E2s, Ubc6 and Ubc7 were present in denaturing lysates but could not be recovered from native lysates, presumably because these two enzymes participate in the ERAD pathway and are connected to the ER membrane (Sommer and Jentsch, 1993; Biederer et al., 1997). The remaining four enzymes (Ubc5, Ubc10, Ubc11 and Ubc12) were not detected in denatured whole lysates and thus appear to be poorly expressed in cycling cells.

The seven Ubc enzymes present in native lysates from log-phase cells were purified and added to reactions containing E1, ATP, ubiquitin, APC, Cdh1 and radiolabeled securin to monitor ubiquitination. As expected, TAP-tagged Ubc4 purified from yeast promoted APC activity toward securin. Of the remaining six E2s tested, only Ubc1 also supported robust APC-dependent securin ubiquitination (Figure 1A).

We also tested the E2s that we could not detect in proliferating cells. Ubc11 and Ubc12 were produced by overexpression in yeast. Ubc12, as

expected for a Nedd1-dependent enzyme, was not able to conjugate ubiquitin (data not shown) (Liakopoulos et al., 1998). Ubc11 promoted a low level of APC-dependent ubiquitination of securin and sea urchin cyclin B (Supplemental Figure 1A and data not shown). We also tested Ubc5 produced in bacteria. As expected from its high degree of homology to Ubc4, Ubc5 also promoted APC-dependent ubiquitination of both substrates (Supplemental Figure 1B and data not shown). Like Ubc4, both Ubc11 and Ubc5 catalyzed the addition of relatively few ubiquitins per substrate. Although Ubc11 and Ubc5 are able to support some APC activity in vitro, their undetectable expression in proliferating cells, their increased expression in meiosis, and the minimal effects of their gene deletions, all suggested to us that they are not critical APC partners in mitosis (Seufert and Jentsch, 1990; Chu et al., 1998; Townsley and Ruderman, 1998). We therefore focused our efforts on Ubc1 and Ubc4.

Ubc1 and Ubc4 generate different reaction products

We purified 6-histidine-tagged Ubc1 and Ubc4 from *E. coli* by metal affinity chromatography. Both enzymes accepted ubiquitin from E1 (see Figure 2A below) and supported APC-dependent ubiquitination of securin in vitro in a purified system (Figure 1B). Ubc4 was able to completely deplete the initial substrate under these conditions, while Ubc1 used only a small fraction of the initial substrate. Ubc4 therefore displays greater activity toward unmodified substrate. However, Ubc1 promoted the formation of higher molecular weight products than Ubc4, indicating that more ubiquitins were added per substrate

molecule. Ubc1 also generated higher molecular weight products in reactions with other substrates (Figure 1C).

The activating subunit in these experiments was Cdh1, which is more readily prepared in active form than Cdc20. Similar results were obtained using Cdc20 that was produced by translation in vitro (Supplemental Figure 2), indicating that the distinct behaviors of Ubc1 and Ubc4 are seen with both activating subunits.

Ubc1 promotes the formation of K48-linked polyubiquitin chains

To test whether the highly ubiquitinated products generated by Ubc1 were polyubiquitin chains, we carried out reactions with methylated ubiquitin, in which all 7 lysines in ubiquitin are chemically blocked and thus not available for chain formation. Incubation of methyl-ubiquitin with Ubc4 or Ubc1 resulted in the formation of E2-Ub conjugates that could be detected on a non-reducing polyacrylamide gel, confirming that both E2s are able to accept the different ubiquitin species equivalently (Figure 2A).

We first used the N-terminus of sea urchin cyclin B as a substrate. With Ubc4 as the E2 in the reaction, we mainly observed mono-, di- and tri-ubiquitinated species in reactions with wild-type ubiquitin (Figure 2B). As seen in our previous work, when methyl-ubiquitin was used, tri-ubiquitinated species were slightly reduced but the mono- and di-ubiquitinated species were unaffected (Carroll and Morgan, 2002). Thus, Ubc4 primarily promotes monoubiquitination of multiple lysines in cyclin B but is not effective in promoting chain formation. The

slight reduction in the tri-ubiquitinated species, as well as the loss of the heterogeneous bands above the tri-ubiquitinated species, suggests that limited chain formation did occur.

We carried out the same analysis with Ubc1. Reaction products with wild-type ubiquitin included at least eight ubiquitinated species, present in roughly equal amounts. Only a single band was observed in reactions with methyl-ubiquitin, indicating that the higher species seen with unmodified ubiquitin are polyubiquitinated. Thus, Ubc1 catalyzes polyubiquitin chain formation.

The same result was obtained using yeast securin as substrate, although in the case of Ubc4 with ubiquitin or methyl-ubiquitin the pattern was different, indicating that some polyubiquitin chains were present (Figure 2C).

We next carried out reactions with three different ubiquitin point mutants in which single lysines were mutated (K29R, K48R and K63R). All mutants were conjugated efficiently to both Ubc1 and Ubc4 (Figure 2A). In Ubc4-dependent reactions with these point mutants, the pattern of ubiquitination was the same as that with wild-type ubiquitin, indicating that the few chain linkages in these reactions were not occurring at specific lysines in ubiquitin (Figure 2B, C). In reactions with Ubc1, however, the K48R mutant specifically lacked polyubiquitinated species, indicating that Ubc1-dependent chains are linked at lysine 48.

Ubc1 catalyzes processive modification of pre-ubiquitinated substrate

Our studies suggested that Ubc1 catalyzes the formation of polyubiquitin chains, but turnover of unmodified substrate into ubiquitinated species appeared slower than that with Ubc4 (Figure 1B). We characterized these differences further by analyzing substrate ubiquitination at various concentrations of Ubc4 and Ubc1 (Figure 3A). Half-maximal stimulation of cyclin ubiquitination occurred at roughly equal concentrations of the two E2s (1.19 +/- 0.12 μM Ubc1 and 2.80 +/- 0.32 μM Ubc4; Figure 3B). Most importantly, at saturating E2 concentrations the rate of cyclin turnover (that is, depletion of unmodified substrate) was about ten-fold higher with Ubc4 than with Ubc1, consistent with the earlier indication that Ubc4 was able to convert more original substrate into ubiquitinated species (Figure 1B).

To assess processivity in these reactions, we also calculated the amounts of ubiquitin added per molecule of modified cyclin B (Figure 3C) (Carroll and Morgan, 2005). As anticipated, Ubc1 was 2-3-fold more processive than Ubc4 by this measure.

A likely explanation for these results is that the depletion of cyclin in these assays is limited by the attachment of the first ubiquitin; that is, the rate of substrate depletion is determined by the rate at which the first ubiquitin is added to an unmodified substrate. Given its preference for K48 of ubiquitin, Ubc1 might have low activity in this first step but have much higher activity in subsequent steps with substrates that are already ubiquitinated. To test this possibility, we isolated pre-ubiquitinated species from the products of a Ubc4-dependent

reaction, which generally produces equal amounts of mono-, di- and tri-ubiquitinated species (for examples, see Figures 2B, 3A). We isolated these species by separating reaction products on a polyacrylamide gel and then extracting radiolabeled proteins from excised gel fragments. Different species were then used in reactions with either Ubc1 or Ubc4. As we showed previously (Carroll and Morgan, 2002), Ubc4 did not display a preference for pre-ubiquitinated substrates (Figure 4A). In contrast, Ubc1 had significantly higher activity with substrates that were already ubiquitinated, using mono-ubiquitinated cyclin B at least ten-fold more rapidly than unmodified cyclin B (Figure 4B, C).

When we compared the rate of ubiquitin incorporation as opposed to cyclin turnover, we found that Ubc1 was twice as fast as Ubc4 when mono-ubiquitinated cyclin was the substrate (Figure 4D). This observation further suggests that Ubc1 prefers to make chains and that the slow, rate-limiting step in Ubc1 reactions with unmodified substrate is addition of the first ubiquitin to a lysine on the substrate. Once the substrate carries a ubiquitin, however, Ubc1 catalyzes more rapid ubiquitin transfer (through a K48 linkage) than Ubc4 (which modifies nonspecific lysines in the substrate or ubiquitin).

These results suggest that Ubc4 and Ubc1 act synergistically in the ubiquitination of APC targets. We tested this hypothesis by measuring APC-dependent ubiquitination of cyclin B in the presence of both E2 enzymes under limiting APC conditions (Figure 4E). When we measured the appearance of polyubiquitinated cyclin carrying more than 3 ubiquitins, we observed that chain formation was far more rapid in the presence of both E2s than it was in the

presence of either E2 alone (Figure 4F). This high polyubiquitination activity was specific to the combination of Ubc4 and Ubc1, as it was not seen in reactions with twice the amount of Ubc4 alone or Ubc1 alone (data not shown). These data suggest that Ubc4 and Ubc1 can act sequentially, with Ubc4 products being extended by Ubc1 as seen in Figure 4B.

Measurements of the total turnover of unmodified cyclin B revealed that Ubc4 alone was better at converting cyclin B to ubiquitinated species than either Ubc1 alone or Ubc1 and Ubc4 together (Figure 4G). Ubc1 therefore inhibits the ability of Ubc4 to modify the substrate under these conditions of limiting APC levels, as might be expected if the two E2s compete for the same or overlapping binding sites on the APC.

The UBA domain of Ubc1 contributes to the processivity of chain assembly

Ubc1 is unique among the yeast E2s in that it contains not only a ubiquitin-conjugating domain (UBC) but also a ubiquitin-associated (UBA) domain at its C-terminus (Merkley and Shaw, 2004). Previous studies have shown that a deletion of the UBA domain on Ubc1 alters the pattern of its autoubiquitination, but its function has not been assessed in an E3-dependent reaction (Hodgins et al., 1996). UBA domains bind ubiquitin (Hicke et al., 2005), and thus a reasonable hypothesis is that the UBA domain of Ubc1 aids in its ability to form polyubiquitin chains. We tested this possibility by creating a version of Ubc1 that lacks this domain, Ubc1-ΔUBA, and produced it in bacteria with a C-terminal 6-histidine tag. This truncated protein was as effective as Ubc1 in the

conjugation of ubiquitin and ubiquitin mutants (data not shown), indicating that the core ubiquitin-conjugating activity of Ubc1 does not depend on the UBA domain.

As seen in Figure 5A, removal of the UBA domain resulted in a significant decrease in the number of ubiquitins incorporated into an APC substrate, although the number of ubiquitins was still greater than that seen with Ubc4 (compare Figures 2B and 5A). The distribution of products was altered: shorter products were more common than longer products in the case of Ubc1-ΔUBA, whereas full length Ubc1 tended to generate equal amounts of the different species. The UBA domain is therefore required for the full processivity of Ubc1.

Like full-length Ubc1, the truncated mutant promotes the formation of polyubiquitin chains, as indicated by the loss of activity in reactions with methyl-ubiquitin (Figure 5A). We also tested the linkage specificity and again observed a complete preference for K48-linked chains in the absence of the UBA domain. We conclude that the UBA domain contributes to polyubiquitin chain assembly, but that the core ubiquitin-conjugating domain determines specificity for the lysine on ubiquitin.

We also compared the rates and products of the ubiquitination reactions at different concentrations of wild type and truncated Ubc1 (Figure 5B, C). Half-maximal stimulation of cyclin turnover with Ubc1-ΔUBA occurred at ten-fold higher concentrations than those for Ubc1 (Figure 5D), suggesting that Ubc1-ΔUBA has a lower affinity for the APC or substrate. Interestingly, maximal activity varied in the opposite manner, with Ubc1-ΔUBA having a ten-fold higher V_{max}

than Ubc1, resulting in identical $k_{cat}/K_{1/2}$ values. In a comparison of processivities (Figure 5E), Ubc1 reached a higher value at a lower E2 concentration than Ubc1-ΔUBA, with the latter never able to achieve the processivity of Ubc1. We therefore suspect that Ubc1-ΔUBA has a defect in binding to the APC-substrate complex (resulting in a processivity defect) but is not defective in catalysis or linkage specificity. The high cyclin-depleting activity of Ubc1-ΔUBA may indicate that the UBA domain reduces activity toward unmodified targets, and in its absence this restriction is relaxed.

Human E2-25K promotes modification of pre-ubiquitinated APC targets

Our studies of yeast Ubc1 raised the possibility that higher eukaryotes might also employ an E2 that is specialized for the second, K48-specific, step in polyubiquitin chain assembly on APC substrates. On the basis of amino acid sequence, the closest human homolog of yeast Ubc1 is E2-25K/Hip2, a well-known E2 that was among the first to be characterized in biochemical detail (Chen and Pickart, 1990; Chen et al., 1991). Like Ubc1, E2-25K contains a C-terminal UBA domain (Haldeman et al., 1997; Merkley and Shaw, 2004) and is known to catalyze the formation of unanchored K48-linked polyubiquitin chains (Chen and Pickart, 1990). Little is known about its biological function or E3 partners; recent studies suggest that E2-25K, like yeast Ubc1, may serve as an E2 for E3s involved in the ER-associated degradation (ERAD) pathway (Flierman et al., 2006). Thus, there are clearly many parallels between yeast Ubc1 and human E2-25K, suggesting that E2-25K may contribute to APC function.

We tested the activity of E2-25K with APC^{Cdh1} immunopurified from extracts of human HeLa cells (Figure 6A) or from frog embryonic cells (data not shown). In both cases, E2-25K displayed low but reproducible activity with unmodified substrates, including cyclin (Figure 6A) and securin (data not shown). More importantly, we noted that mixing E2-25K with human UbcH10 consistently resulted in greater polyubiquitination of substrates than was achieved with UbcH10 alone (Figure 6A), suggesting that E2-25K, like Ubc1, might be specialized for the modification of substrates carrying pre-attached ubiquitins. We therefore measured E2-25K activity toward cyclin that was already modified with one or two ubiquitins, as in our studies of Ubc1. The activity of E2-25K was stimulated about 3-fold by the presence of one ubiquitin on the substrate, and two ubiquitins resulted in a dramatic 15-fold increase in activity (Figure 6B, top). Thus, E2-25K, like Ubc1, displays a clear preference for pre-ubiquitinated targets and is well suited for the second step in polyubiquitin chain formation. Because E2-25K is known to possess E3-independent ubiquitin chain-forming activity (Chen and Pickart, 1990), we also analyzed its activity in the absence of APC (Figure 6B, bottom). A low level of activity was detected, but the addition of APC increased this activity 6- to 8-fold with any of the three substrates. We therefore conclude that E2-25K behaves much like Ubc1 and might therefore serve as an APC-dependent chain-extending E2 in human cells.

Ubc1 and Ubc4 are required for APC function in vivo

A prediction from our studies in vitro is that both Ubc1 and Ubc4 collaborate with the APC in the yeast cell to promote ubiquitination of substrates important for cell-cycle progression. Previous studies have shown that Ubc4 is not required for the efficient degradation of mitotic cyclins (Townsley and Ruderman, 1998), but the contribution of Ubc1 to APC function has not been assessed. We first characterized the effects of deleting the *UBC1* gene and found that Ubc1 is essential in our strain background (W303). We therefore turned to conditional expression of *UBC1* by placing the chromosomal copy of *UBC1* under the control of the P_{GAL1} promoter, with a 3xHA tag at the protein's N-terminus. This strain was viable on media containing galactose, but displayed a severe growth defect when *UBC1* expression was repressed on media containing glucose (Figure 7A). When we streaked for single cells on dextrose plates, we observed multiple rounds of division, resulting in microcolonies containing filamentous clusters of cells lacking a uniform bud-size phenotype (data not shown). We also constructed a P_{GAL1}*UBC1* strain in which *UBC4* was deleted. When plated on repressive glucose media, this double mutant strain was completely inviable (Figure 7A).

Loss of APC function is known to result in a metaphase arrest with high levels of securin and mitotic cyclins. Deletion of all APC-dependent E2s should generate a similar phenotype, unless those E2s have important collaborations with E3s involved in other processes. To further analyze the cell-cycle defects in the P_{GAL1}*UBC1* and P_{GAL1}*UBC1 ubc4*Δ strains, cells were arrested in G1 with

alpha factor, in the presence of galactose. Glucose was added to repress *UBC1* transcription, and after two hours cells were released from the alpha factor arrest into media containing glucose. No Ubc1 was detectable by western blotting of lysates from either strain (Figure 7B, Ubc1 western). We compared cell-cycle events and APC substrate levels in wild type, P_{GAL1}*UBC1*, *ubc4Δ*, and P_{GAL1}*UBC1 ubc4Δ* cells (Figure 7B).

Cells lacking *UBC1* alone displayed several mitotic defects. Analysis of DNA and spindle morphology revealed a delay in the initiation of spindle elongation and chromosome segregation, as well as a delay in spindle disassembly. The final step in cell separation was highly defective, as indicated by the observation that the two daughter cells remained connected to the end of the experiment. This connection could be severed by digesting the cell wall with zymolyase. Analysis of APC substrate levels was consistent with these phenotypes: securin accumulated to abnormally high levels and was destroyed only after a delay, while the destruction of Clb2 and Cdc20 was completely blocked. Ubc1 is therefore required for the normal destruction of APC targets and is particularly critical for that of Clb2 – resulting in various partial defects in late mitotic events.

Cells lacking *UBC4* also displayed defects in late mitotic events, including delays in anaphase onset and spindle disassembly that were accompanied by partial defects in the destruction of securin and Clb2 (Figure 7B). Double mutants lacking both *UBC1* and *UBC4* displayed more severe defects than either single mutant. The metaphase-to-anaphase transition was completely blocked, resulting

in a complete arrest with a single DNA mass over a short pre-anaphase spindle at the bud neck. All APC substrates tested were stabilized in this arrest. Ubc1 and Ubc4 are therefore required for APC activity during mitosis.

We also analyzed cell-cycle progression in P_{GAL1}*UBC1 ubc5*Δ and P_{GAL1}*UBC1 ubc11*Δ cells. When these cells were released from a G1 arrest in the presence of glucose to repress *UBC1* expression, the mitotic defects in double mutants were the same as those in the absence of *UBC1* alone (data not shown). Thus, Ubc5 and Ubc11 do not make significant contributions to APC function in the absence of Ubc1.

Our observation that cells lacking *UBC1* are particularly defective in Clb2 destruction and late mitotic events suggested that the inviability of this strain is due primarily to defects in Cdk1 inactivation in late mitosis. Consistent with this possibility, we found that P_{GAL1}*UBC1* cells are viable on glucose plates when they are engineered to express high levels of the Cdk inhibitor Sic1 (Supplemental Figure 3).

DISCUSSION

The biochemical features of Ubc4 and Ubc1 suggest that they operate in sequence to promote the two steps of polyubiquitination: Ubc4 is best suited for the nonspecific monoubiquitination of multiple lysines in the target, yielding an ideal substrate upon which Ubc1 can assemble K48-linked polyubiquitin chains.

The K48 linkage specificity of Ubc1 is not affected by removal of its C-terminal UBA domain, indicating that specificity is determined by the core ubiquitin-conjugating domain. The K48 linkage specificity of Cdc34 (Ubc3), the primary E2 for the ubiquitin-protein ligase SCF, depends on an acidic loop near its catalytic cysteine (Petroski and Deshaies, 2005). This loop is not present in Ubc1, and so the sequence determinants of Ubc1 specificity remain unclear.

Removal of the UBA domain results in shorter reaction products than those seen with wild-type Ubc1. In addition, the concentration of Ubc1-ΔUBA required for half-maximal activity is far higher than that for the wild-type protein. A likely explanation for these results is that the UBA domain interacts with ubiquitin on a pre-ubiquitinated target, thereby enhancing the affinity of Ubc1 for the APC-substrate complex. It remains unclear if Ubc1 promotes modification of K48 in the UBA-bound ubiquitin itself or in an adjacent ubiquitin. Even if the substrate carries only a single ubiquitin, the interaction of that ubiquitin with Ubc1 might stimulate monoubiquitination of nearby lysines in the target protein, resulting in additional substrates upon which chains can be built.

Ubc1 and Ubc4 collaborate in vivo to promote APC target destruction

Our studies of *ubc1*Δ and *ubc4*Δ cells indicate that the sequential actions of the two E2s are critical for the normal timing of mitotic events: the loss of either E2 results in the partial stabilization of APC substrates and delays in the onset and completion of anaphase and other events. The loss of both Ubc1 and Ubc4 results in a complete arrest in metaphase, clearly arguing that these two proteins alone serve as the E2 partners for the APC in the mitotically proliferating yeast cell.

Repression of *UBC1* expression results in a late mitotic arrest that appears to be due primarily to a defect in Clb2 destruction and thus Cdk1 inactivation, as it is suppressed by overproduction of the Cdk1 inhibitor Sic1. This phenotype appears to be a less severe form of the phenotype that results from expression of a nondegradable Clb2 mutant, which is also rescued by Sic1 overproduction (Wasch and Cross, 2002). Given that Clb2 destruction depends partly on Cdc20, and that Cdh1 is not essential for viability, these results argue that the *ubc1*Δ phenotype is not simply the result of a defect in Cdh1-specific APC activity.

Cells lacking Ubc4 alone display a minor mitotic delay but eventually degrade all major APC targets; thus, the poor monoubiquitination activity of Ubc1 may be sufficient in vivo, or other factors in the cell may boost this activity. Cells lacking Ubc1 alone display a more pronounced defect in APC-dependent protein destruction: these cells eventually degrade securin and Clb5 but not Cdc20 and Clb2. When *UBC4* is also deleted, destruction of all major APC targets is

blocked. Thus, Ubc4 alone appears capable of promoting the destruction of some APC targets despite its poor polyubiquitin chain-forming activity. As suggested recently, an array of short chains produced by Ubc4-APC may be sufficient to target some proteins for destruction (Kirkpatrick et al., 2006). Alternatively, chain-extending activities or E4s (Hoppe, 2005) may assist Ubc4-APC in the polyubiquitination of some targets. These mechanisms may explain why securin is eventually destroyed in the absence of Ubc1, but it remains unclear why Clb2 and Cdc20 remain stable. One possibility is that Clb2 and Cdc20 are relatively poor substrates of APC-Ubc4, so that their rate of ubiquitination is insufficient to oppose the rate of deubiquitination in the cell. The timing of substrate destruction in vivo is likely to depend, at least in part, on the processivity of substrate ubiquitination by the APC (Rape et al., 2006), and the processivity of Clb2 and Cdc20 ubiquitination by APC-Ubc4 may not be sufficient for its destruction in vivo.

E2s other than Ubc4 and Ubc1 do not appear to make significant contributions to mitotic APC activity. We did find, however, that Ubc5 and Ubc11 catalyze APC-dependent monoubiquitination in vitro. Interestingly, expression of the genes encoding these two proteins is induced upon entry into meiosis, whereas *UBC4* expression is repressed (Chu et al., 1998). Perhaps one or both of these E2s fulfills the role of Ubc4 in the meiotic program.

Sequential E2 action may be a conserved mechanism in APC function

Our work is reminiscent of previous studies in fission yeast, where two E2s have been implicated in cyclin destruction and mitotic progression (Seino et al., 2003). Depletion of the Ubc4 homolog, UbcP1, leads to the accumulation of low molecular weight cyclin-ubiquitin species in vivo, while depletion of a UbcH10 homolog, UbcP4, results in the accumulation of nonubiquitinated cyclin. These results are consistent with the possibility that the two E2s generate different products, but detailed biochemical studies of these products have not been performed. The vertebrate homologs of these E2s, UbcH5 and UbcH10, are not believed to generate extensive polyubiquitin chains (Kirkpatrick et al., 2006), and so it remains possible that UbcP1 and UbcP4 are supplemented by a fission yeast Ubc1 homolog or some other chain-extending activity.

In vertebrates, UbcH5 and UbcH10 are thought to be the two major E2s that collaborate with the APC. Both were identified biochemically as the major APC-dependent E2 activities in extracts of frog and clam embryos (King et al., 1995; Aristarkhov et al., 1996; Yu et al., 1996). RNAi-mediated depletion of UbcH10 from human cells, or the homologous protein, Vihar, in *Drosophila*, causes partial mitotic defects accompanied by stabilization of APC targets (Mathe et al., 2004; Rape and Kirschner, 2004). Apart from these experiments, there has been no systematic analysis in higher eukaryotes of the requirement for different E2s in APC function, and there is no evidence to rule out the possibility that E2s other than UbcH5 and UbcH10 are involved.

Our studies suggest that E2-25K also contributes to APC function in vertebrate cells. E2-25K is the closest human homolog of Ubc1 by sequence, and it shares many other features with yeast Ubc1: it catalyzes K48-linked polyubiquitin formation (Chen and Pickart, 1990), it contains a UBA domain at its C-terminus (Haldeman et al., 1997), and it has been implicated in the ERAD pathway (Flierman et al., 2006). We found that E2-25K is an effective catalyst of APC-dependent ubiquitination of substrates carrying pre-attached ubiquitins. It has little activity with unmodified substrates, perhaps explaining why it was never identified as a major APC-dependent E2 activity in previous biochemical studies. We therefore speculate that sequential E2s operate in human cells as in yeast: UbcH5 and UbcH10 may add the first ubiquitins to APC targets, after which E2-25K extends K48-linked chains on those ubiquitins.

Sequential E2 action as a general mechanism in polyubiquitin chain formation

Our work reveals how the APC solves an important general problem in the mechanism of protein polyubiquitination: how a single E3 can efficiently promote both monoubiquitination of nonspecific lysines in a target and highly specific K48 ubiquitination of ubiquitins on that target. The strategy used by the APC to solve this problem – a pair of E2s, each specialized for one of the two steps in chain formation – contrasts with the strategy used by the related E3, SCF, which employs a single E2, Cdc34, to catalyze both steps. What advantage might there be to using two E2s instead of one? One possibility is that two specialized E2s

allow more rapid polyubiquitination than a single E2. In the case of Cdc34, for example, the initial monoubiquitination step is slow and limits the overall rate of polyubiquitination; in the case of the APC, this problem is solved by introducing a second E2, Ubc4, that drives rapid nonspecific monoubiquitination of targets. We speculate that it would be difficult to evolve a single E2 that is efficient at both steps in chain formation: the first step requires a flexible active site environment that allows any lysine to attack, while the second step requires a more selective active site that channels lysine 48 of ubiquitin for attack.

The use of sequential E2s may be a general mechanism in polyubiquitin chain assembly by other E3s. There are numerous examples of ubiquitin-dependent degradation processes that appear to depend on multiple E2s. For example, Ubc7, Ubc6, and Ubc1 all contribute to the degradation of proteins in the yeast ERAD pathway (Meusser et al., 2005), and human ERAD may depend on homologs of Ubc1 and Ubc7 (Fliermann et al., 2006). In these and other cases, different E2s may display distinct biochemical features that together allow more efficient polyubiquitination than is possible with a single E2.

EXPERIMENTAL PROCEDURES

Strains and Plasmids

All yeast strains were derived from W303, except strains from the TAP library. *P_{GAL1}3xHA-UBC1* was constructed by standard PCR-targeting techniques by direct replacement of the *UBC1* promoter with the *GAL1* promoter and 3xHA sequence (Longtine et al., 1998). Construction of Ubc1 and Ubc4 for protein expression was carried out by standard techniques. *UBC1* and *UBC4* were PCR-amplified lacking the stop codon and ligated into the pET23d vector to generate a C-terminally 6xHis tagged protein. *UBC1* was PCR-amplified using an internal 3' oligonucleotide to create Ubc1-ΔUBA (residues 1-150).

Expression and Purification of E2 proteins

Yeast strains expressing C-terminally TAP-tagged *UBC* genes were obtained from the library constructed at UCSF (Ghaemmaghami et al., 2003). One liter cultures were grown to an OD_{600} of 1.0, harvested and lysed by bead-beating into 2.5 volumes of TAP lysis buffer (20 mM HEPES pH 8.0, 150 mM NaCl, 0.1% NP40) supplemented with 50 mM β-glycerophosphate, 50 mM NaF, 1 mM DTT, 1 mM PMSF, and 1 μg/ml each of aprotinin, pepstatin and leupeptin. Lysate was clarified by ultracentrifugation (1 h, 100,000 x g, 4°C) and added to 50 μl of IgG beads pre-equilibrated in TAP lysis buffer. After an overnight incubation at 4°C, beads were washed twice in TAP lysis buffer and once in TEV cleavage buffer (TAP lysis buffer plus 0.5 mM EDTA and 1 mM DTT). Beads were incubated 30 min at room temperature in 50 μl of TEV cleavage buffer and

2 μl of recombinant TEV protease, and cleaved proteins were recovered for ubiquitination assays.

For bacterial expression, plasmids containing C-terminally tagged *UBC1* and *UBC4* were transformed into BL21 cells. A single colony for each gene was incubated overnight in 100 ml LB/Amp media at 37°C, diluted into 1 liter of fresh media and grown at 37°C to an OD_{600} of 0.60, after which IPTG was added to 1 mM for 4 h at 37°C. Cells were harvested by centrifugation, washed in cold water and frozen in liquid nitrogen. Frozen pellets were melted rapidly and dripped into a mortar containing liquid nitrogen, where they were ground with a pestle to a fine powder. The powder was added to 4 volumes of breakage buffer (50 mM HEPES pH 7.4, 300 mM NaCl, 10% Glycerol plus protease inhibitors lacking DTT or any reducing agent) and the mixture was sonicated 5 times for 30 s each, alternating with 45 s breaks. Lysates were centrifuged (1 h, 100,000 x g, 4°C) and supernatants were subjected to purification by metal affinity chromatography on a 1-ml Pharmacia Hi-Trap column charged with Co^{+2}. Fractions containing purified protein were pooled and dialyzed into QAH buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$) plus 10% glycerol and 1 mM β-mercapto-ethanol. The resulting stocks of Ubc1 (5 mg/ml), Ubc4 (2.5 mg/ml) and Ubc1-ΔUBA (5 mg/ml) were stored at -80°C.

Ubiquitination Assays

Preparation of reaction components (E1, ATP, Ubiquitin, APC, Cdh1) was described previously (Carroll and Morgan, 2005), except that the final calmodulin-binding step was omitted from the APC preparation. To prepare

ubiquitin-E2 conjugates, E1 (0.2 μl of 1 mg/ml), ATP (1.0 μl of 16.7 mM) and ubiquitin (1.5 μl of 10 mg/ml) were incubated with E2 proteins for 15 min at room temperature. In separate tubes, APC (1.5 μl of 10 nM), Cdh1 (0.5 μl of 1.5 μM) and radiolabeled substrate were mixed at room temperature. Reactions were initiated by combining the E1/E2 mix with the APC mix in a final volume of 20 μl, except for dose response assays in Figure 3 and Figure 5 (10 μl final volume). Reaction products were analyzed by SDS-PAGE and visualized with a PhosphorImager. Data were quantified as described using ImageQuant and analyzed using the ligand-binding module of SigmaPlot (Carroll and Morgan, 2005). The expression, purification and labeling of the N-terminus of sea urchin cyclin B has been described previously (Carroll and Morgan, 2005). All other substrates were produced by coupled transcription and translation in rabbit reticulocyte extracts. Unless specified, substrates produced by this method were not purified; instead, the TNT mixture was treated with NEM prior to ubiquitination reactions, which we find inactivates chain-extending activities present in the lysate. Purification of pre-ubiquitinated cyclin B species was described previously (Carroll and Morgan, 2002).

Cell Cycle analysis

Strains were grown at room temperature and arrested in G1 by the addition of alpha factor (1 μg/ml) for 3 h in the presence of 2% raffinose and 2% galactose. 2% glucose was added to all strains for another 2 h. Cells were washed free of alpha factor and placed in fresh media containing 2% glucose, and samples were taken every 15 min. Alpha factor (1 μg/ml) was added back

after 90% of the cells had budded. Protein extracts were prepared by bead-beating in urea lysis buffer. 3HA-Ubc1 was detected on western blots with 12CA5 monoclonal antibody (Roche). Securin-13Myc was detected with 9E10 monoclonal antibody. Cdc20 and Clb5 were detected with polyclonal antibodies (Santa Cruz BioTech). Polyclonal anti-Clb2 antibodies were a generous gift of Doug Kellogg (University of California, Santa Cruz). DNA was visualized by DAPI staining, and tubulin was visualized by indirect immunofluorescence with antibody YOL1/34 using spheroplasts. The percentage of budded cells was measured from intact cells fixed in ethanol.

Human APC analysis

HeLa cells were grown and lysates were prepared as previously described (Kraft et al., 2006). Twenty 15-cm dishes of confluent cells were lysed in 5 ml HeLa lysis buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 20 mM β-glycerophosphate, 5 mM $MgCl_2$, 1 mM NaF, 0.2% NP-40, 10% Glycerol) plus protease and phosphatase inhibitors. The lysate was clarified by centrifugation (1h, 100,000 x g, 4^0C); final protein concentration was 18 mg/ml. APC was purified by immunoprecipitation as follows. 50 μl of rabbit anti-Cdc27 antiserum was incubated with 125 μl protein A magnetic beads (Dynal, Invitrogen) for 30 min at room temperature. After one wash in HeLa lysis buffer, 15 mg of lysate was added to the beads and incubated with rocking for 2 h at 4^0C. Beads were washed once with wash buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 0.1% NP-40, 10% Glycerol), twice with wash buffer plus 600 mM NaCl, and once more with wash buffer. Beads were resuspended in 100 μl of wash buffer and

distributed into ten ubiquitination reactions. To prepare ubiquitin-E2 conjugates, mammalian E1 (0.55 mg/ml, BostonBiochem), ATP (2.0 μl of 16.7 mM), and ubiquitin (1.5 μl of 10 mg/ml) were incubated 20 min at room temperature with each E2 protein (purified human E2-25K and UbcH10 were gifts of Vincent Chau and Hongtao Yu, respectively). After confirming by gel electrophoresis that ubiquitin conjugation had occurred, E2-ubiquitin conjugates were mixed with human Cdh1 (1.0 μl, a gift of Hongtao Yu), radiolabeled substrate (2.0 μl), and bead-bound human APC (10 μl). Reactions (23 μl) were incubated 1 h at 37^0C.

ACKNOWLEDGEMENTS

We are grateful to Vincent Chau for a gift of purified E2-25K and Hongtao Yu for *Xenopus* embryonic extracts, human UbcH10, and human Cdh1. We thank M. Sullivan, M. Matyskiela, E. Griffis, and members of the Morgan Lab for technical assistance and thoughtful discussions, R. Deshaies and R. King for insightful comments, S. Nguyen for technical assistance, D. Kellogg for reagents, and M. Sullivan, M. Matyskiela, G. Tully, and T. Matsuguchi for critical reading of the manuscript. This work was supported by funding from the National Institute of General Medical Sciences (GM53270) and a fellowship from the National Science Foundation.

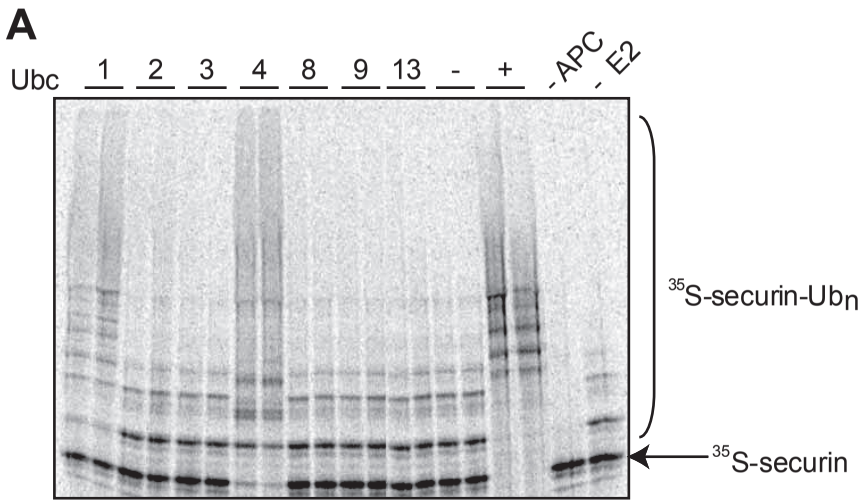
Figure 1. Ubc1 is an APC-dependent E2 enzyme

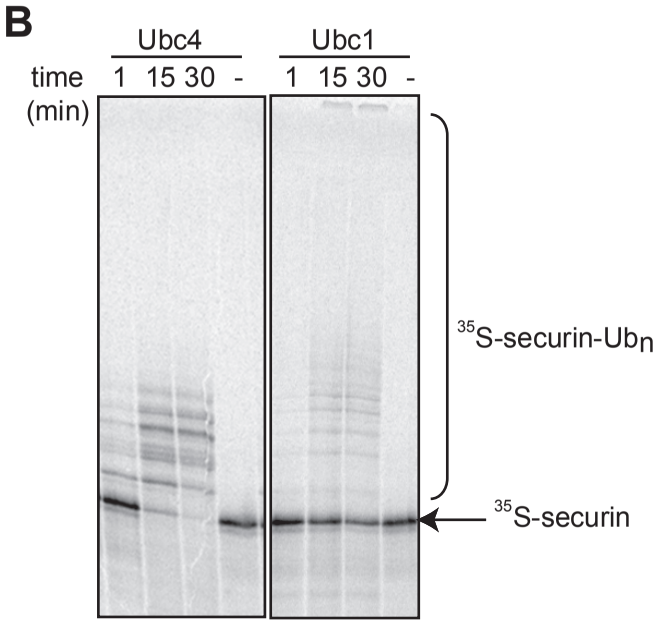
(A) The seven indicated Ubc enzymes (100-200 ng) were purified from yeast and incubated for 1 h at room temperature in duplicate APC^{Cdh1}-dependent ubiquitination assays, using purified ^{35}S-securin as the substrate. Control reactions were performed with a mock-purified lysate from a strain lacking a TAP-tagged E2 (-) or Ubc4 purified from bacteria (+). Final lanes indicate negative control reactions in which substrate was incubated with recombinant Ubc4 alone (-APC) or with APC alone (-E2).

(B) Recombinant Ubc4 (1.25 μg) or Ubc1 (1.0 μg) was incubated for 15 min with E1, ATP and ubiquitin, added to the APC^{Cdh1} mix containing ^{35}S-securin, and incubated for the indicated time. 30-minute control reactions (-) lacked APC.

(C) Recombinant Ubc4 (0.5 μg) or Ubc1 (1.0 μg) was incubated with E1, ATP and ubiquitin for 15 min and then added to APC^{Cdh1} reactions containing the indicated ^{35}S-labeled substrates for 15 min at room temperature. Hsl1 is a truncated protein (residues 667-882), and cyclin B1 is an N-terminal fragment of sea urchin cyclin B1 (residues 11-113).

Figure 1


A
Ubc
1
2
3
4
8
9
13
-
+
-APC
-E2
35S-securin-Ubn
35S-securin


B
Ubc4
Ubc1
time
(min)
1
15
30
-
1
15
30
-
35S-securin-Ubn
35S-securin

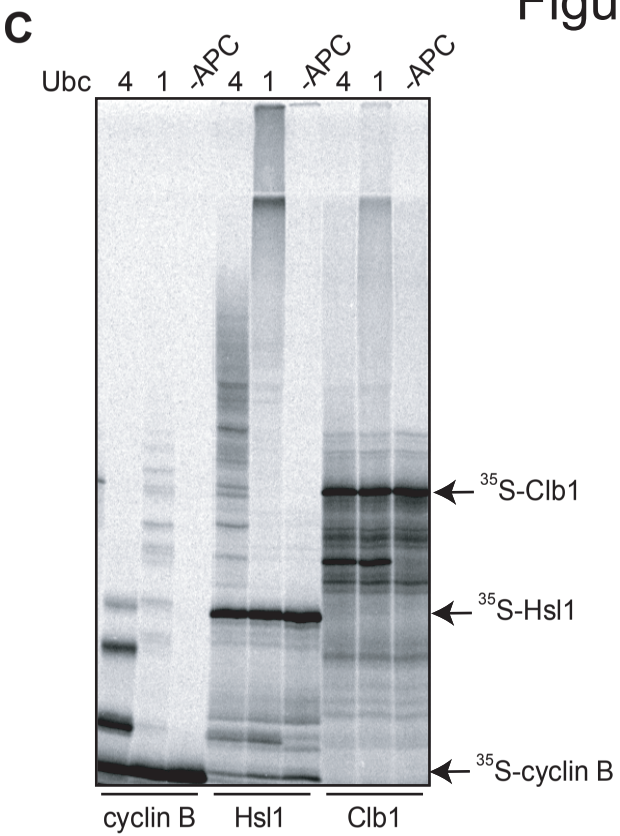

C
Ubc
4
1
-APC
4
1
-APC
4
1
-APC
35S-Clb1
35S-Hsl1
35S-cyclin B
cyclin B
Hsl1
Clb1

Figure 2. Ubc1 promotes the formation of K48-linked ubiquitin chains

(A) To confirm that they display normal ubiquitin-conjugating activity, purified Ubc4 (1.25 μg) or Ubc1 (1.25 μg) was incubated with E1, ATP and 5 μg of the indicated ubiquitin species for 15 min at room temperature. Reactions were stopped in SDS sample buffer lacking DTT and analyzed by non-reducing SDS-PAGE and Coomassie Blue staining. The position of the protein with or without ubiquitin is indicated.

(B) The reactions from panel A were used in ubiquitination reactions. APC, Cdh1 and ^{125}I-cyclin B were added to the E1/E2 mix and incubated for 30 min at room temperature. The reactions were stopped by addition of SDS sample buffer, analyzed by SDS-PAGE and visualized with a PhosphorImager. The number of ubiquitins on cyclin is indicated.

(C) Same reactions as in panel B but using purified ^{35}S-securin as the substrate.

Figure 2

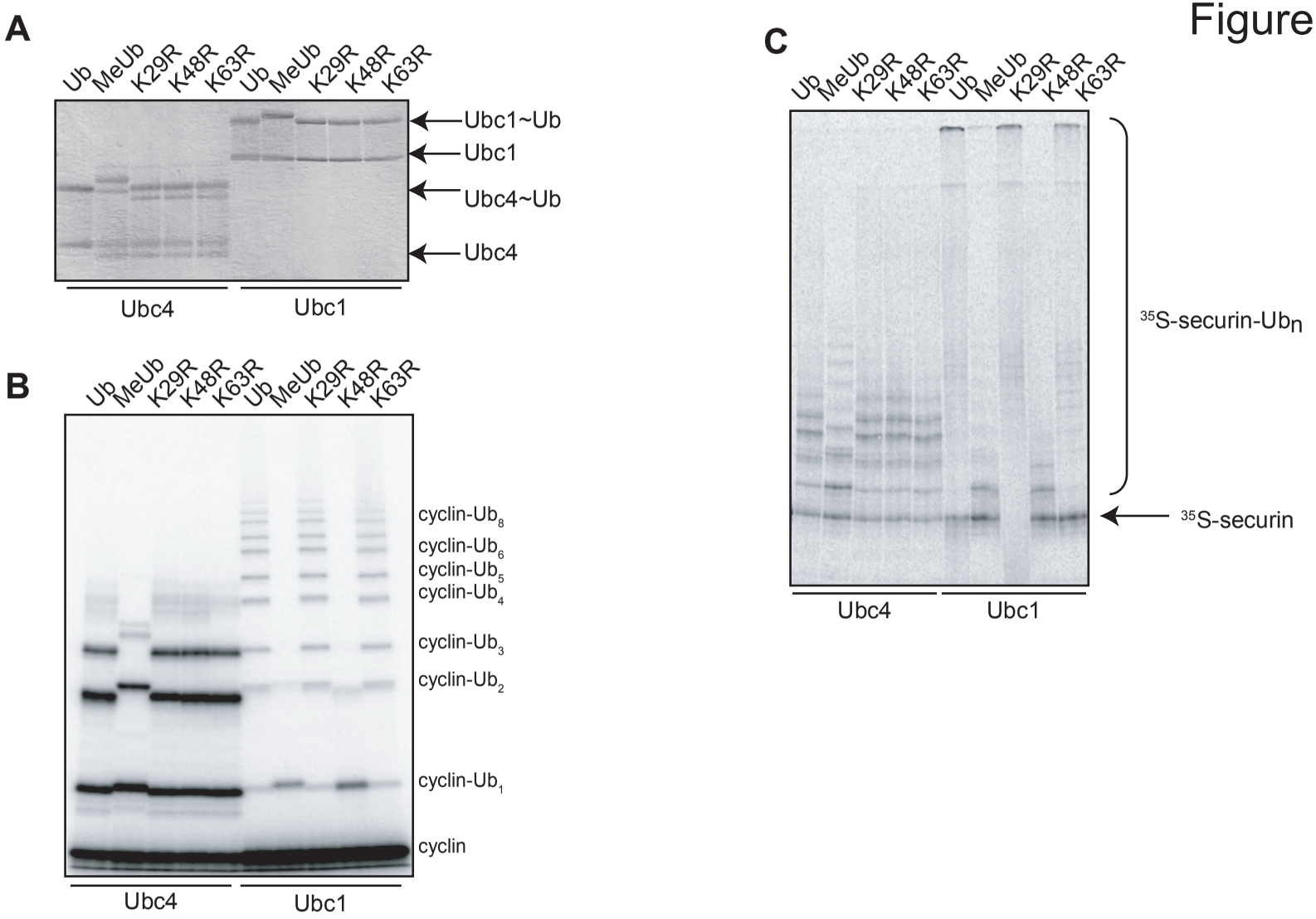

A
Ub
MeUb
K29R
K48R
K63R
Ubc1~Ub
Ubc1
Ubc4~Ub
Ubc4
Ubc4
Ubc1
B
cyclin-Ub8
cyclin-Ub6
cyclin-Ub5
cyclin-Ub4
cyclin-Ub3
cyclin-Ub2
cyclin-Ub1
cyclin
C
35S-securin-Ubn
35S-securin

Figure 3. Ubc1 is more processive than Ubc4

(A) Recombinant Ubc4 (4.8 nM to 48 μM in equal steps) or Ubc1 (6.6 nM to 66 μM in equal steps) was added to E1, ATP and ubiquitin and incubated for 15 min at room temperature. The E1/E2 mix was added to the APC^{Cdh1} mix containing ^{125}I-cyclin B and allowed to react until no more than 2% of the initial substrate was used. Reactions were stopped by addition of SDS sample buffer, analyzed by SDS-PAGE and visualized with a PhosphorImager.

(B) The rate at which cyclin was converted to any ubiquitinated species was determined by quantifying the total amount of ubiquitinated substrate. For Ubc4 reactions, the mono-, di- and tri-ubiquitinated species were quantified, while for Ubc1, the 8 major ubiquitinated species were quantified. Data were fit to a rectangular hyperbola using the ligand-binding module provided with Sigma Plot. This data is representative of three independent experiments. The inset provides a scaled version of the Ubc1 curve.

(C) Processivity was determined by calculating the ratio of ubiquitin to cyclin in the products as described (Carroll and Morgan, 2005). Data were fit to a rectangular hyperbola as in B.

Figure 3

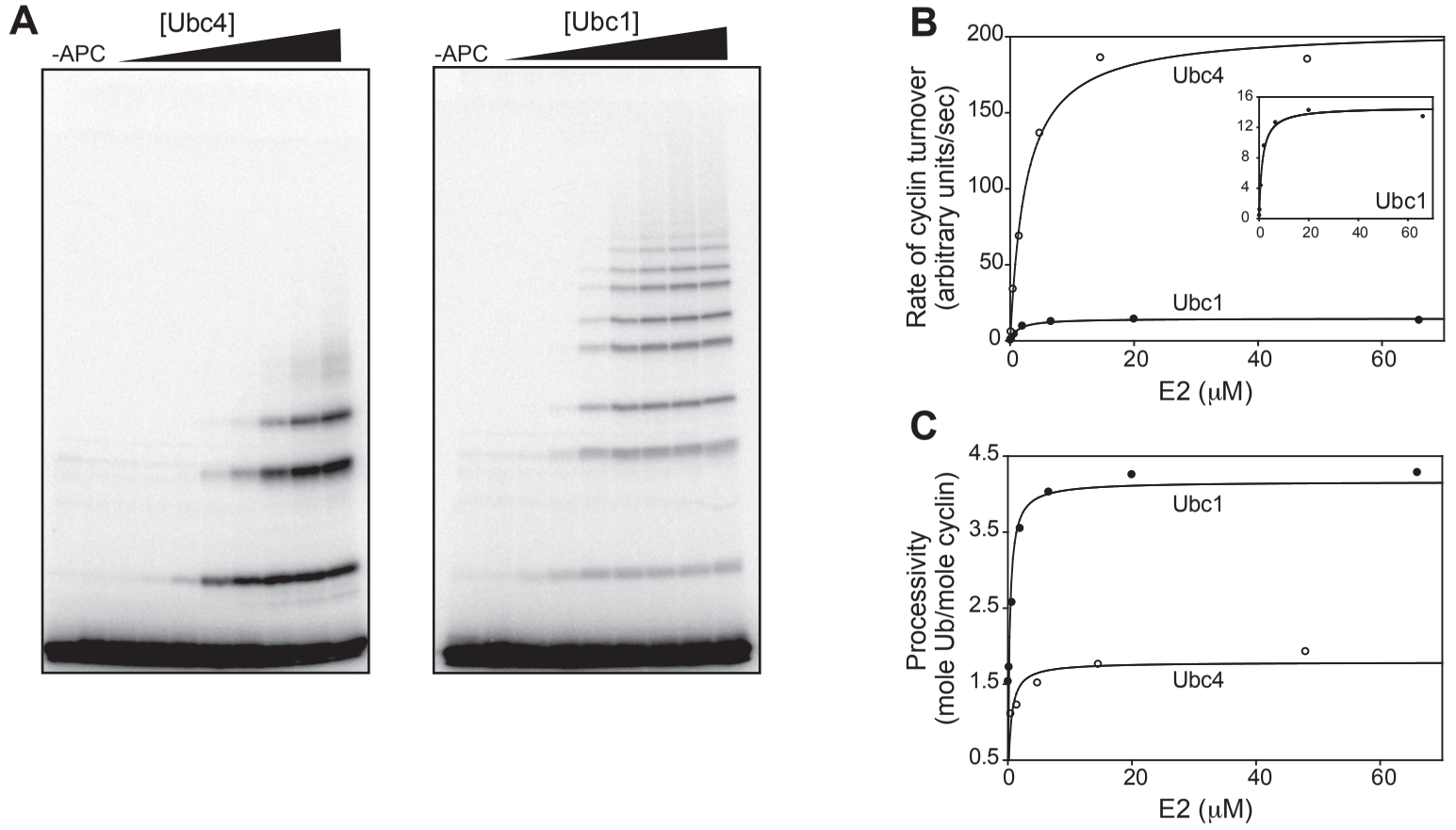

A
[Ubc4]
-APC
[Ubc1]
-APC
B
Rate of cyclin turnover
(arbitrary units/sec)
200
150
100
50
0
Ubc4
Ubc1
0
20
40
60
E2 (μM)
16
12
8
4
0
C
Processivity
(mole Ub/mole cyclin)
4.5
3.5
2.5
1.5
0.5
Ubc1
Ubc4
E2 (μM)

Figure 4. Ubc1 catalyzes rapid ubiquitination of pre-ubiquitinated species

Mono- and di-ubiquitinated ^{125}I-cyclin were prepared by subjecting a large Ubc4-APC^{Cdh1}-dependent reaction to SDS-PAGE and extracting reaction products from excised gel fragments. Recombinant Ubc4 (A, 1.25 μg) or Ubc1 (B, 1.25 μg) was combined with E1, ATP and ubiquitin and added to APC^{Cdh1} mixes containing the indicated substrate. After the indicated times, reactions were stopped by addition of SDS sample buffer, analyzed by SDS-PAGE and visualized with a PhosphorImager.

(C and D) Cyclin turnover and ubiquitin incorporation were measured for reactions using cyclin and mono-ubiquitinated cyclin as starting substrates. Note that in panel D the two Ubc4 curves are superimposed.

(E) APC reactions were carried out in the presence of Ubc4 (0.5 μg), Ubc1 (0.5 μg) or a combination of both enzymes at these concentrations. After the indicated times, reactions were stopped by addition of SDS sample buffer, analyzed by SDS-PAGE and visualized with a PhosphorImager.

(F) The amount of ubiquitinated products above the tri-ubiquitinated species was quantified using ImageQuant.

(G) All ubiquitinated products were quantified, providing a measure of the total amount of cyclin converted to ubiquitinated species.

Figure 4

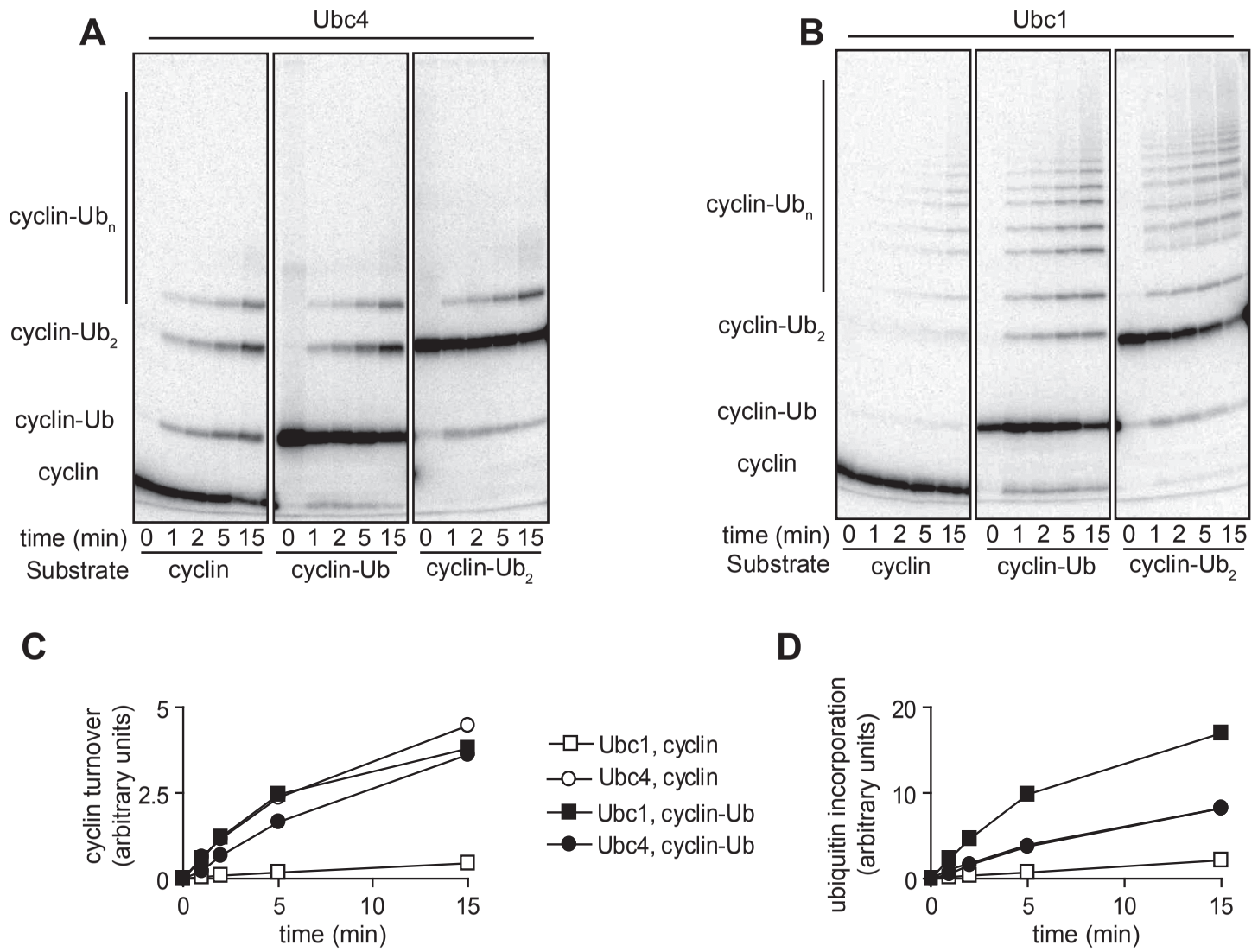

A
Ubc4
cyclin-Ub$_n$
cyclin-Ub$_2$
cyclin-Ub
cyclin
time (min) 0 1 2 5 15 0 1 2 5 15 0 1 2 5 15
Substrate cyclin cyclin-Ub cyclin-Ub$_2$
B
Ubc1
cyclin-Ub$_n$
cyclin-Ub$_2$
cyclin-Ub
cyclin
time (min) 0 1 2 5 15 0 1 2 5 15 0 1 2 5 15
Substrate cyclin cyclin-Ub cyclin-Ub$_2$
C
cyclin turnover (arbitrary units)
5
2.5
0
0 5 10 15
time (min)
Ubc1, cyclin
Ubc4, cyclin
Ubc1, cyclin-Ub
Ubc4, cyclin-Ub
D
ubiquitin incorporation (arbitrary units)
20
10
0
0 5 10 15
time (min)

Figure 4, part 2

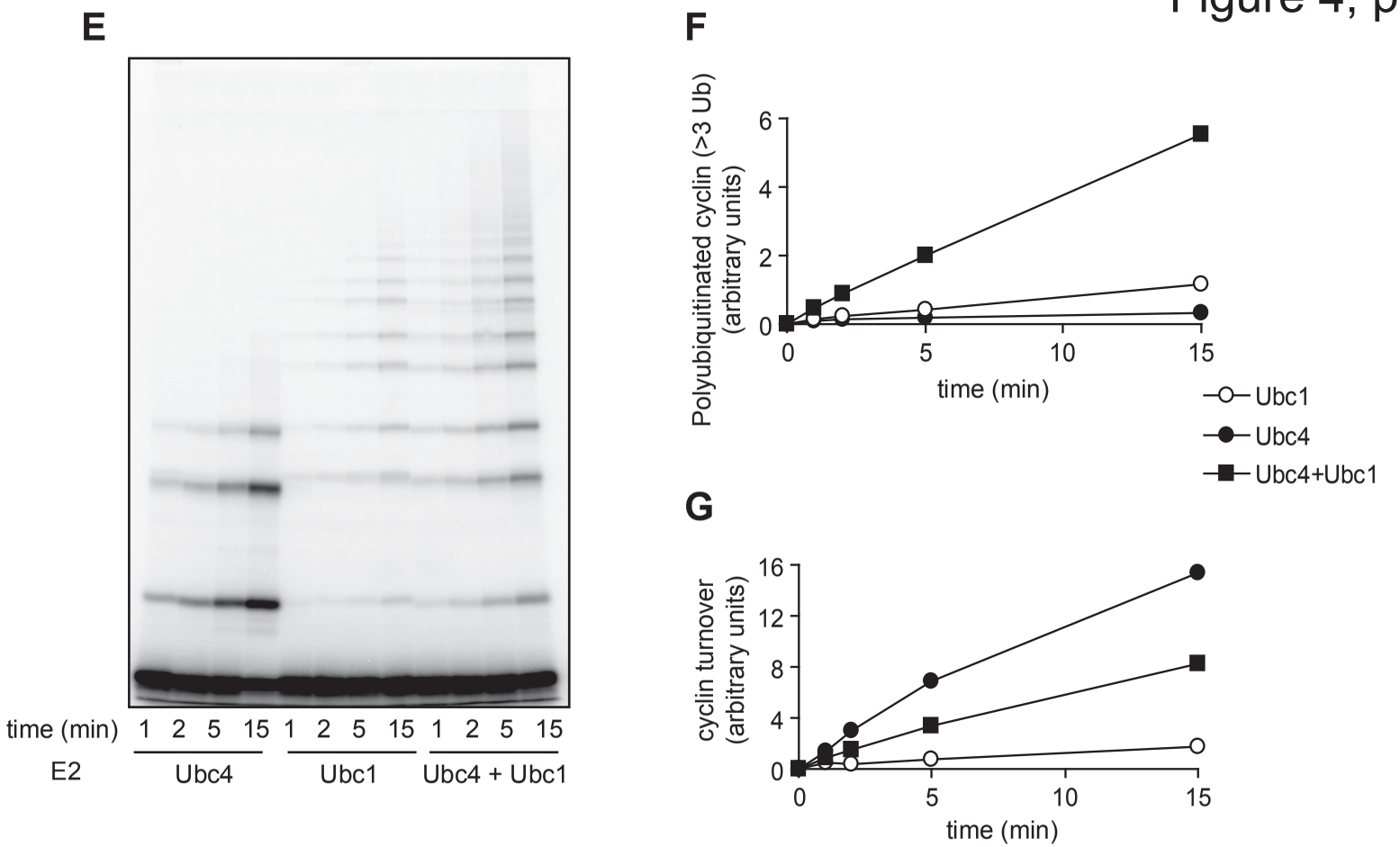


Figure 5. The UBA domain of Ubc1 participates in processivity

(A) Reactions were carried out as in Figure 2A and 2B. Ubc1 (1.25 μg) or Ubc1-ΔUBA (1.25 μg) was incubated with the indicated ubiquitin species in an APC^{Cdh1}-dependent reaction with ^{125}I-cyclin. Reaction products were analyzed by SDS-PAGE and visualized with a PhosphorImager.

(B and C) Recombinant Ubc1 (6.6 nM to 66 μM in equal steps) or Ubc1-ΔUBA (6.6 nM to 66 μM in equal steps) was used in APC^{Cdh1} ubiquitination assays as in Figure 3A.

(D and E) Total cyclin turnover and reaction processivity were measured as described in Figures 3B and 3C.

Figure 5

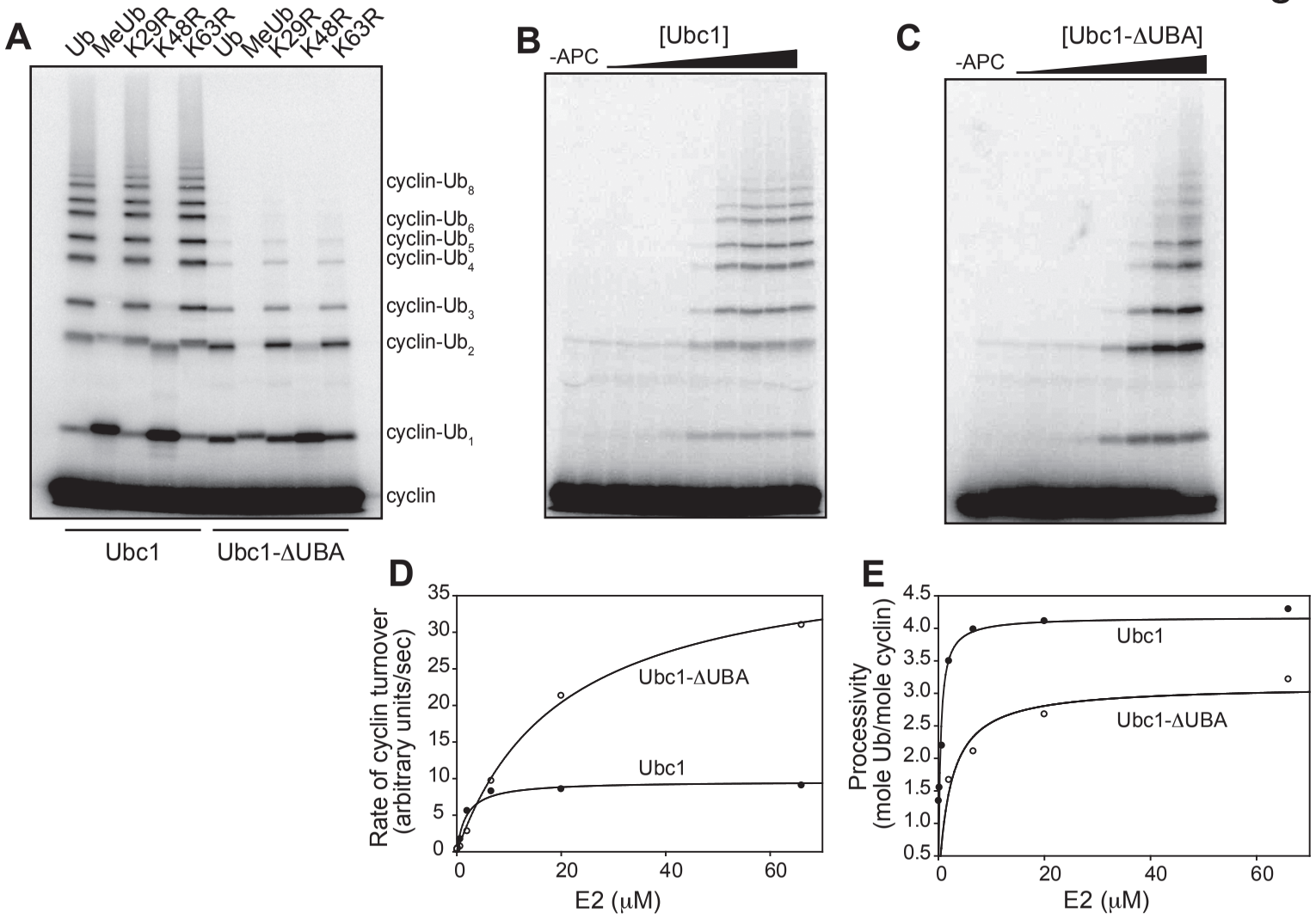

A
Ub
MeUb
K29R
K48R
K63R
Ub
MeUb
K29R
K48R
K63R
cyclin-Ub8
cyclin-Ub6
cyclin-Ub5
cyclin-Ub4
cyclin-Ub3
cyclin-Ub2
cyclin-Ub1
cyclin
Ubc1
Ubc1-ΔUBA
B
-APC
[Ubc1]
C
-APC
[Ubc1-ΔUBA]
D
Rate of cyclin turnover (arbitrary units/sec)
Ubc1-ΔUBA
Ubc1
E2 (μM)
E
Processivity (mole Ub/mole cyclin)
Ubc1
Ubc1-ΔUBA
E2 (μM)

Figure 6. Human E2-25K catalyzes APC-dependent modification of pre-ubiquitinated species

(A) Purified UbcH10 (1 μg) or E2-25K (4 μg, 8 μg) or the indicated combinations were conjugated to ubiquitin by incubation with mammalian E1 (0.55 μg for UbcH10, 1.1 μg for E2-25K and the combination), ATP, and ubiquitin. E2-ubiquitin conjugates were then incubated with immunopurified human APC supplemented with human Cdh1 and ^{125}I-cyclin. Reaction products were analyzed by SDS-PAGE and visualized with a PhosphorImager. Control reactions (first lane) lacked E2 protein.

(B) Mono- and di-ubiquitinated ^{125}I-cyclin were prepared as described in Figure 4. E2-25K (4 μg) was incubated with E1, ATP, and ubiquitin, and added to human APC supplemented with Cdh1 and the indicated substrate (top). Control reactions (bottom) were mixed with buffer lacking APC^{Cdh1}. After the indicated times at 37^0C, reactions were stopped by addition of SDS sample buffer, analyzed by SDS-PAGE and visualized with a PhosphorImager. Note that a de-ubiquitinating activity co-purifies with the APC in these experiments, resulting in some loss of ubiquitin during the reaction.

Figure 6

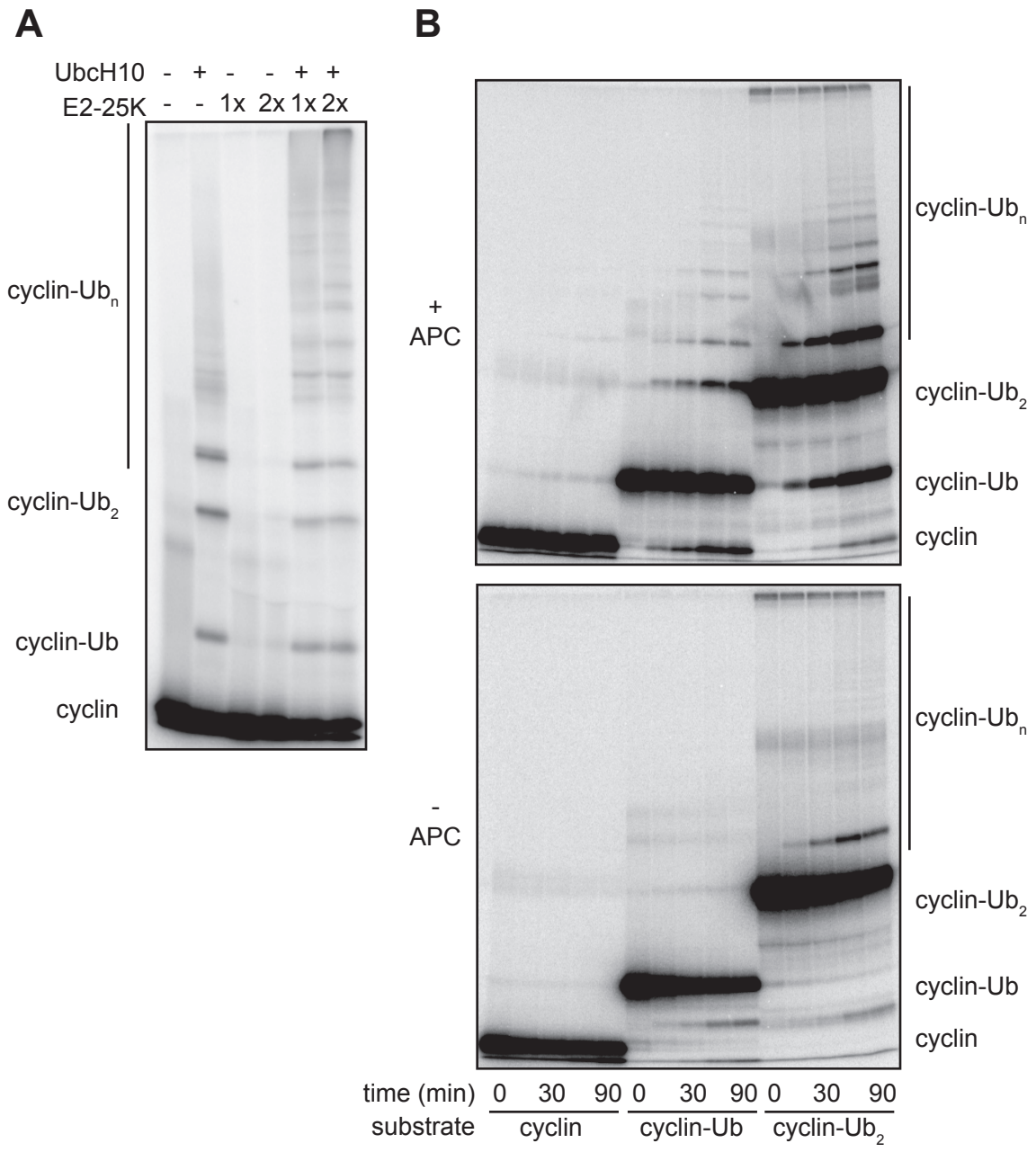

A
UbcH10 - + - - + +
E2-25K - - 1x 2x 1x 2x
cyclin-Ub$_n$
cyclin-Ub$_2$
cyclin-Ub
cyclin
B
+ APC
cyclin-Ub$_n$
cyclin-Ub$_2$
cyclin-Ub
cyclin
- APC
cyclin-Ub$_n$
cyclin-Ub$_2$
cyclin-Ub
cyclin
time (min) 0 30 90 0 30 90 0 30 90
substrate cyclin cyclin-Ub cyclin-Ub$_2$

Figure 7. Ubc4 and Ubc1 are the only E2s required for APC function in vivo

(A) The indicated strains were grown in 2% galactose to mid-log phase. 10^7 cells were centrifuged and serially diluted onto plates containing galactose or glucose and incubated at 30^{o}C for two days.

(B) Asynchronous cultures of the indicated cells (-5 h time point) were treated with alpha factor for 3 h (-2 h time point), after which glucose was added for 2 hours. Alpha factor was then washed out (zero time point), and at the indicated times cells were analyzed directly for budding index (black squares). Parallel samples were fixed and treated with zymolyase for analysis of DNA masses with DAPI staining and spindle structure with anti-tubulin antibodies, allowing measurement of chromosome segregation (binucleate cells, white circles) and elongated anaphase spindles (black triangles). Additional samples were prepared for western blotting analysis of the indicated proteins. Note that budding index is generally higher than the percentage of binucleate cells because chromosome masses, but not budding, were counted after treatment with zymolyase, which separates cells connected only by the cell wall.

Figure 7

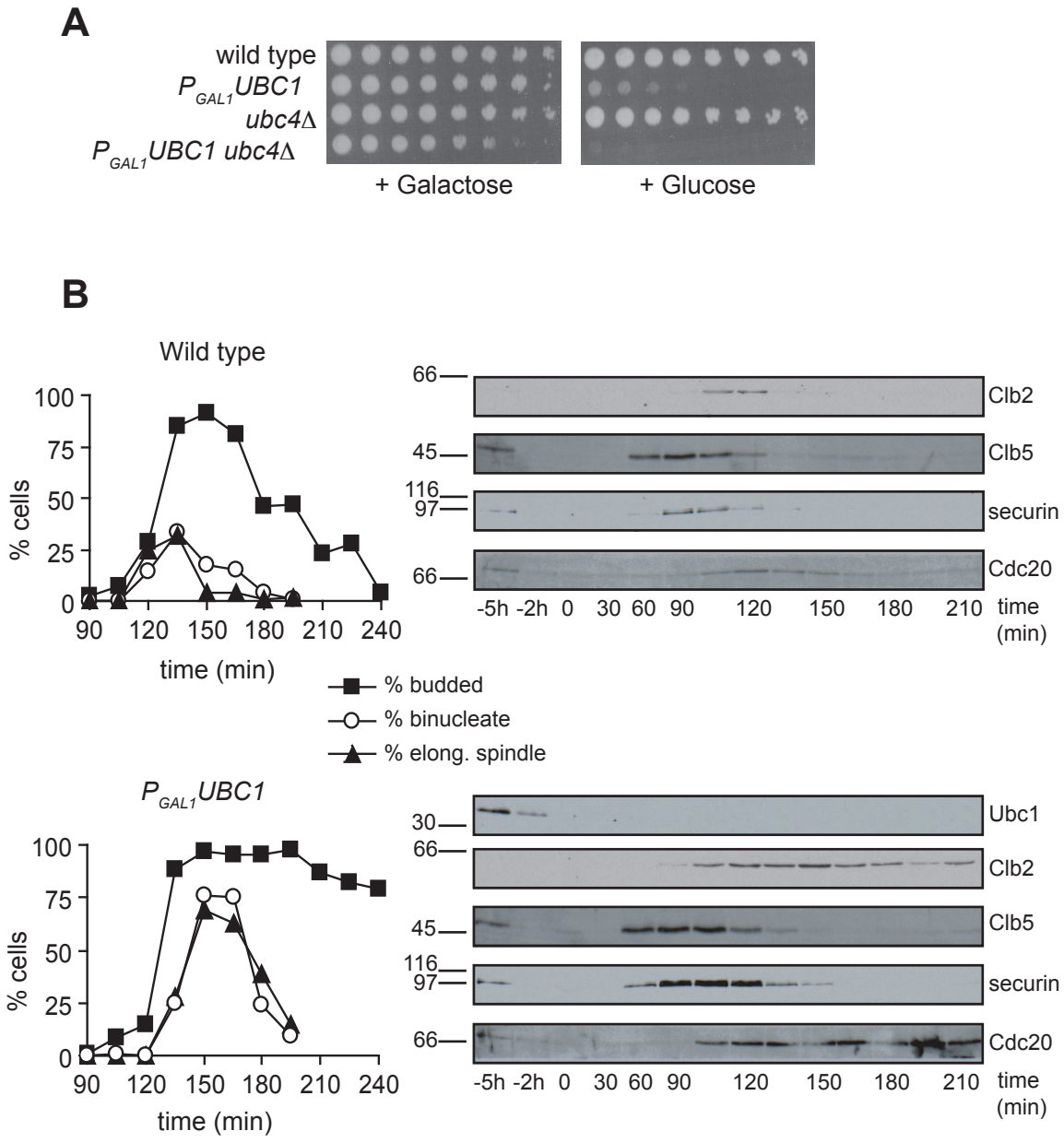
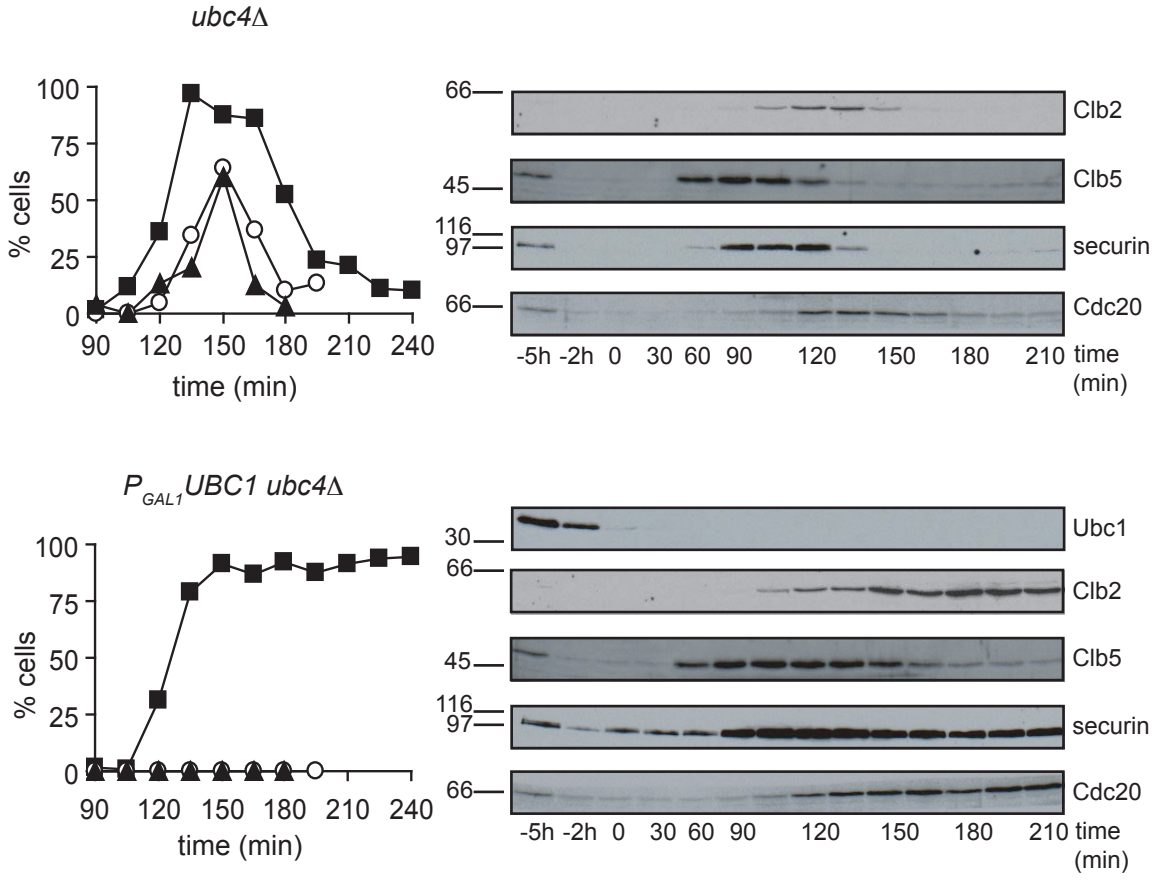

A
wild type
P_{GAL1}UBC1
ubc4Δ
P_{GAL1}UBC1 ubc4Δ
+ Galactose
+ Glucose
B
Wild type
% cells
time (min)
% budded
% binucleate
% elong. spindle
Clb2
Clb5
securin
Cdc20
-5h -2h 0 30 60 90 120 150 180 210
time (min)
P_{GAL1}UBC1
Ubc1

Figure 7 Part 2

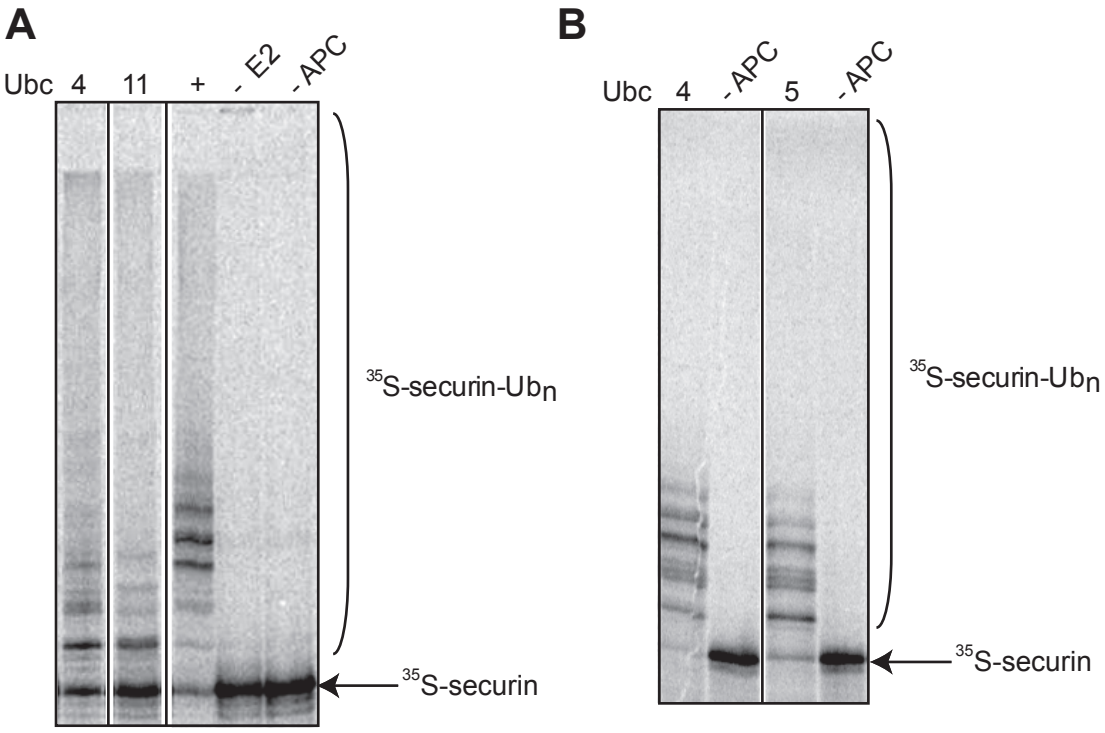


Supplemental Figure 1: Ubc11 and Ubc5 are APC-dependent E2s

(A) Ubc4 or Ubc11 (100-200 ng) was purified from yeast and incubated for 1 h at room temperature in APC^{Cdh1}-dependent ubiquitination assays, using purified ^{35}S-securin as the substrate. Control reactions were performed with Ubc4 purified from bacteria (+). Final lanes indicate negative control reactions in which substrate was incubated with recombinant Ubc4 alone (-APC) or with APC alone (-E2).

(B) Recombinant Ubc4 (1.25 mg) or Ubc5 (1.25 mg) was incubated for 15 min with E1, ATP and ubiquitin, added to the APC^{Cdh1} mix containing ^{35}S-securin, and incubated for 30 min. Control reactions were incubated with Ubc4 or Ubc5 alone (-APC).

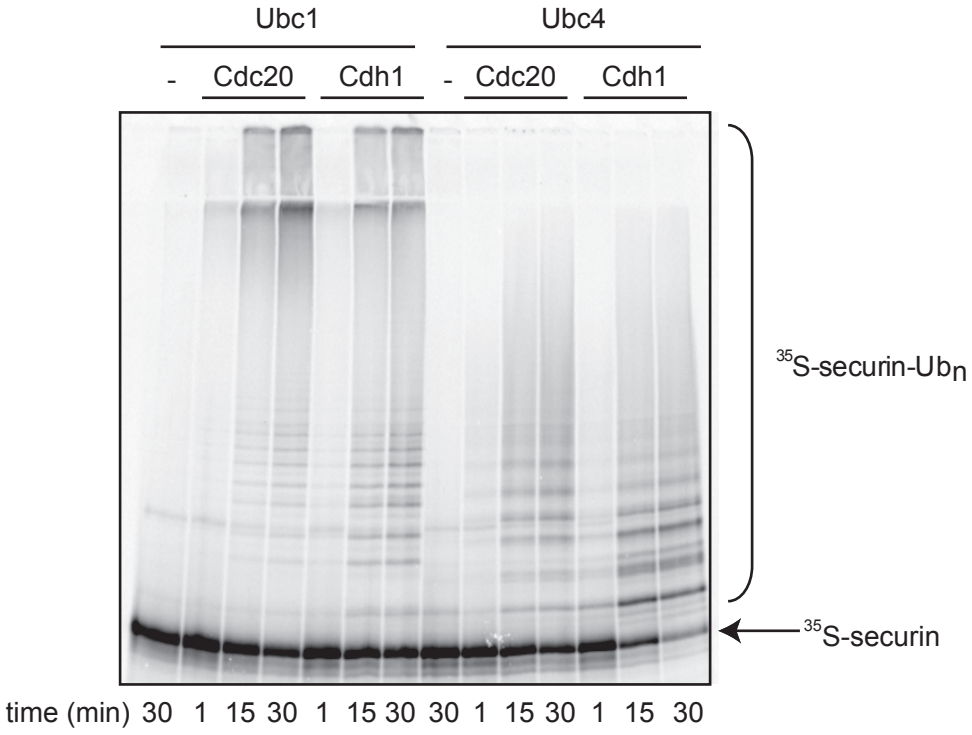
Supplemental Figure 1



Supplemental Figure 2: Activities of Ubc1 and Ubc4 in APC reactions with Cdc20 or Cdh1 as activator subunits

Cdc20 and Cdh1 were produced by coupled transcription and translation in rabbit reticulocyte lysates; each protein was fused to an IgG-binding domain and TEV protease cleavage site at its N-terminus. Translation mixtures were incubated with IgG-coupled magnetic beads for 1 hr at 4^{o}C, washed and then incubated with TEV protease for 30 min at room temperature. Parallel reactions containing 35S-methionine indicated that both proteins were produced and purified in similar amounts (not shown). Recombinant Ubc4 (1.25 mg) or Ubc1 (1.25 mg) was incubated with E1, ATP and ubiquitin and then added to purified yeast APC, purified ^{35}S-securin, and the indicated activator. At the indicated times, reactions were stopped by addition of SDS sample buffer, analyzed by SDS-PAGE and visualized with a PhosphorImager. Control reactions (lanes 1, 8) lacked activator and APC.

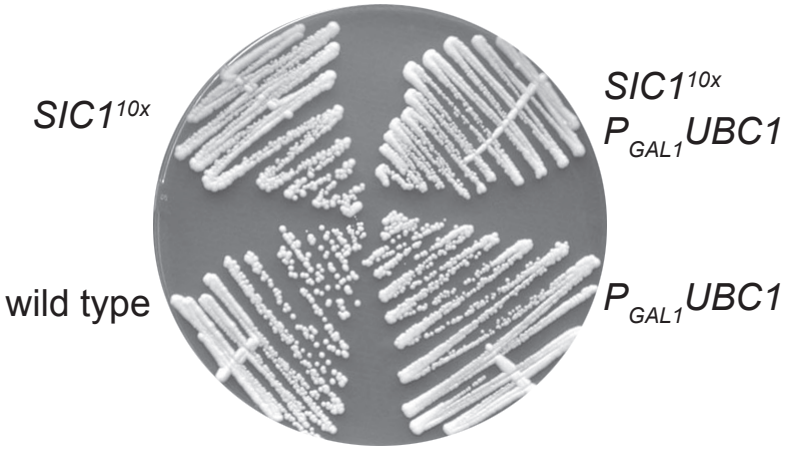
Supplemental Figure 2

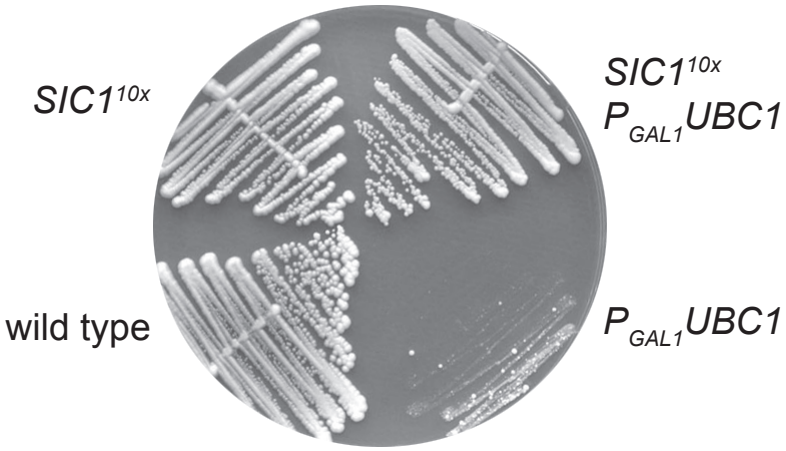


Supplemental Figure 3: The lethality of *UBC1*-repressed cells is rescued by overexpression of *SIC1*.

The indicated strains were streaked onto plates containing 2% galactose or 2% dextrose and incubated at 30^{o}C for two days. The *SIC110X* strain was a gift of David Toczyski.

Supplemental Figure 3





Chapter 3

Ubc1 builds K48-linked chains at the expense of substrate lysine ubiquitination

INTRODUCTION

The covalent attachment of ubiquitin to proteins is used as a signaling mechanism to control a wide range of processes in the eukaryotic cell. Ubiquitination often leads to a change in a protein's properties, whether it is localization, change in binding partners or turnover. Different forms of ubiquitination encode different fates for their protein targets. For instance, the attachment of a single ubiquitin can alter a protein's intracellular trafficking, whereas the attachment of ubiquitin chains often leads to a protein's degradation in the proteasome (Hicke, 1999; Hicke, 2001; Kim and Rao, 2006).

The ubiquitination cascade involves three proteins: an E1 or ubiquitin-activating enzyme, an E2 or ubiquitin-conjugating enzyme, and an E3 or ubiquitin-protein ligase. E1s use ATP hydrolysis to activate the C-terminus of ubiquitin. The activated ubiquitin is then transferred to a cysteine residue on the E1. Ubiquitin-charged E1 interacts with different E2 enzymes and the ubiquitin is transferred to a cysteine on the E2. E3s interact with E2s and promote the transfer of ubiquitin to a lysine side chain on a protein target (Pickart and Eddins, 2004). E3 ligases are divided into two major families, depending on their structure and enzymatic mechanism. The HECT ligases are characterized by their ability to form a covalent intermediate with ubiquitin from an E2, after which the ubiquitin is transferred to the target protein in a separate reaction (Kee and Huibregtse, 2007). RING ligases are thought to bring ubiquitin-charged E2s and protein targets in close proximity and catalyze the transfer of ubiquitin directly

from the E2 to the target protein. Catalysis has been proposed to occur via an increase in local concentration or allosteric activation of the E2 active site (Zheng et al., 2002; Ozkan et al., 2005; Deshaies and Joazeiro, 2009).

While the enzymes involved in ubiquitination has been studied for years, and we have a fairly good understanding of the interactions between the different enzymes, we still do not have a complete understanding of how different ubiquitination signals are achieved. A protein can be ubiquitinated at a single lysine, resulting in monoubiquitination or at multiple lysines (multi-monoubiquitination). The best-studied role of monoubiquitination is as an internalization signal (Hicke, 2001). If instead a ubiquitin chain is built, via the sequential attachment of the C-terminus of ubiquitin to a lysine side chain of a previous ubiquitin, the result is polyubiquitination (Pickart and Fushman, 2004). The best-studied role for polyubiquitin chains is in protein turnover. Turnover involves polyubiquitin chains built on lysine 48 (K48) of ubiquitin (Thrower et al., 2000) . All possible ubiquitin linkages have been observed in the budding yeast *Saccharomyces cerevisiae*, with the predominant species being K48 and K11-linked polyubiquitin chains (Xu et al., 2009).

Polyubiquitination, or the building of a ubiquitin chain, by RING ligases has at least two reaction steps. First, a lysine on the protein target is ubiquitinated, and then a lysine on ubiquitin itself is ubiquitinated. At a minimum either the E3 or the E2 must be able to interact with ubiquitin and position it in a productive manner on the E2 active site.

The synthesis of polyubiquitin chains requires an interaction between a specific lysine in the attacking ubiquitin and the active site of the E2 carrying the donor ubiquitin. These interactions are best understood in the case of K63-linked chain assembly. The formation of K63-linked chains requires Ubc13, an E2, and its binding partner Mms2, an E2-variant. Mms2 has the same overall structure as an E2 but lacks the crucial catalytic cysteine. Ubc13/Mms2 are an obligate heterodimer and act together to build a K63-linked chain. Beautiful structural work showed that Mms2 interacts with a surface region on ubiquitin called the hydrophobic patch, positioning it such that the side chain of K63 is oriented close to the catalytic cysteine of Ubc13, where the C-terminus of ubiquitin is located (Eddins et al., 2006).

In the case of K48-linked chains, the E2 Cdc34 and its partner E3 ligase SCF are the best-studied case. In this system, formation of a ubiquitin chain is a two step mechanism: rate-limiting ubiquitination of a lysine on the protein target, followed by rapid K48-linked chain formation. Through isolation of the second step, ubiquitin-ubiquitin bond formation, mutations were identified that only affected this step and not protein target ubiquitination. These mutations were all located in an insertion near the catalytic cysteine of Cdc34, termed the acidic loop due to its amino acid composition (Petroski and Deshaies, 2005).

Both of these mechanisms clearly indicate that the nature of the E2 enzyme determines chain linkage specificity. This is also the case for the Anaphase-promoting complex/cyclosome (APC), a multisubunit RING ligase that targets many proteins important for cell-cycle progression to the proteasome via

polyubiquitination (Peters, 2006; Thornton and Toczyski, 2006). Previous work from our lab has demonstrated that APC is able to build K48-linked chains with the aid of two E2s, Ubc4 and Ubc1. We established a sequential mechanism that begins when APC-Ubc4 ubiquitinates lysines on the protein target. This priming ubiquitination is rapidly followed by K48-linked polyubiquitination by APC-Ubc1. The K48-specificity of the second step lies in the catalytic core of Ubc1: mutating its known ubiquitin binding domain (UBA) did not result in loss of chains (Rodrigo-Brenni and Morgan, 2007).

To gain a better understanding of the mechanisms underlying K48-specific polyubiquitin chain assembly, we set out to identify the residues in Ubc1 that are required for its K48-specific activity. We found two residues, threonine 84 and glutamine 122, that are required for this function. Both of these residues are on flexible loops near the catalytic cysteine, in a position where they could influence catalysis. Threonine 84 is involved in catalyzing K48-dependent ubiquitination, whereas glutamine 122 is involved in reducing the ubiquitination of nonspecific lysines on the protein target. We propose that Ubc1 is able to catalyze the assembly of K48-specific chains at the expense of substrate lysine ubiquitination.

RESULTS

The catalytic core of Ubc1 binds ubiquitin

We showed previously that Ubc1 assembles K48-linked ubiquitin chains on substrates of the APC (Rodrigo-Brenni and Morgan, 2007). To study this reaction in more detail, we developed diubiquitin synthesis assays that allow careful dissection of the K48-specific polyubiquitination reaction in isolation (Petroski and Deshaies, 2005). Radiolabeled ubiquitin conjugated to Ubc1 is the donor in these assays, and successful attack of this donor by ubiquitin in solution results in the formation of radiolabeled diubiquitin.

We first charged Ubc1 with a radiolabeled ubiquitin moiety that has K48 mutated to arginine. Thus, any unconjugated ubiquitin will not be able to act as the attacking moiety because it lacks K48, a residue previously shown to be required for chain formation. The initial conjugating reaction is stopped by addition of EDTA and NEM, which inactivates all unoccupied active site cysteines on E1 and E2 components in the reaction. These two chemicals therefore allow only a single round of attack by free ubiquitin. Saturating amounts of unlabeled wild-type ubiquitin are then added and aliquots are taken over time. In order to monitor both the appearance of the diubiquitin species and the disappearance of the conjugated species, the reactions are run under nonreducing conditions (Figure 1A). Diubiquitin products can be seen as early as 1 minute into the reaction, and the reaction remains linear with time up to 5 minutes, after which it levels off due to depletion of the conjugated species (Figure 1B).

This single discharge assay allowed us to measure initial rates for the appearance of a diubiquitin species (Figure 1C) over a range of unlabeled ubiquitin concentrations. We plotted the fraction of diubiquitin formed (diubiquitin/(conjugated ubiquitin remaining plus diubiquitin)), which allows us to compare multiple reactions and different Ubc1 species. The plot of initial rates vs. attacking ubiquitin concentration (Figure 1E) reveals a hyperbolic function with a K_M of 350 μM and a maximal velocity of 0.0015 per second. This is equivalent to a maximal velocity of 0.15 pmols ubiquitin linked per second (data not shown).

Ubc1 contains a C-terminal UBA domain. These domains in other proteins have been shown to bind ubiquitin and various diubiquitin species in multiple systems (Raasi et al., 2005). Although the affinities of these interactions are not high, they are similar to the K_M we observed in our diubiquitin assay. We showed previously that the UBA domain is not involved in K48 specificity, but it remained possible that it contributes in some way to recognition of the attacking ubiquitin. We therefore analyzed diubiquitin synthesis by a version of Ubc1 that lacks the UBA domain, Ubc1-ΔUBA. As can be seen in Figure 1D and quantified in Figure 1E, the UBA has only a minimal effect on the initial rate of diubiquitin formation (K_M = 450 μM, Vmax= 0.0013 per second). We conclude that the catalytic core of Ubc1 is the primary site of interaction for the attacking ubiquitin.

Residues near the catalytic cysteine of Ubc1 are involved in chain assembly

We reasoned that residues near the catalytic cysteine (C88) of Ubc1 would be good candidates for binding ubiquitin and/or guiding the side chain of K48 for attack. Since most E2s are highly related, we compared sequences of E2 enzymes that are known to build K48-linked chains (Ubc1 and human E2-25K) with sequences of UbcH5 family members, which don't appear to possess chain specificity (Chen and Pickart, 1990; Carroll and Morgan, 2002). We also used the tertiary structures of Ubc1 and Ubc4 to determine which residues near the catalytic cysteine were likely to be solvent-exposed and thus able to participate in protein-protein interactions (Cook et al., 1993; Hamilton et al., 2001). The primary sequences of Ubc1 and Ubc4 are about 56% identical. We focused on residues that were different between Ubc1 and Ubc4 but identical between E2-25K and Ubc1. We further narrowed our search by looking at residues near the catalytic cysteine that were solvent-exposed. Even without this limitation, most residues that are identical in E2-25K and Ubc1 are found in loops near the catalytic cysteine (Figure S1).

We focused initially on a group of three residues, V83, T84 and A86, which passed all of our criteria. We first mutated all three residues to their sequence in Ubc4, creating the Ubc1-cluster I mutant (V83N, T84Δ, A86N). To rule out gross conformational defects, we monitored E2 conjugation to ubiquitin by E1. The Ubc1-cluster I mutant was charged to equivalent levels as wild-type

Ubc1 (data not shown), indicating that it could interact normally with E1 and that the catalytic cysteine was not greatly affected by the mutations.

We next tested the activity of the Ubc1 mutant in an APC-dependent reaction, with the goal of determining if our mutations affect K48-specific chain assembly (Figure 2A). As in our previous work, wild-type Ubc1 catalyzed the assembly of K48-linked chains on the radiolabeled cyclin substrate. When chain formation was blocked with methyl-ubiquitin or a K48R ubiquitin mutant, Ubc1 catalyzed the attachment of a single ubiquitin to a lysine on the substrate. In contrast, the Ubc1-cluster I mutant had no measurable ability to catalyze assembly of a K48-linked chain. Moreover, this mutant did not catalyze increased multiubiquitination of lysines on cyclin B, as seen with Ubc4 (see Figure 8B for an example of Ubc4 reactions). In fact, the rate of methyl-ubiquitin incorporation, which limits the reactions to the first step of protein target ubiquitination, is indistinguishable between Ubc1 and Ubc1-cluster I (Figure 2B). This mutant therefore displays normal (low) activity in the attachment of the initial ubiquitin to the cyclin substrate, but has no activity in the attachment of ubiquitins to K48 of preattached ubiquitins. We conclude that one or more of the three residues of cluster I are required for K48 specificity.

Threonine 84 in Ubc1 is involved in ubiquitin chain formation

To identify the residues in cluster I that are responsible for K48 specificity, we next tested the activity of single point mutants, Ubc1-V83N, Ubc1-T84Δ and Ubc1-A86N. All mutants were able to conjugate ubiquitin, and hence interact

with E1, to the same degree as wild-type Ubc1 (data not shown). In APC-dependent reactions, both Ubc1-V83N and Ubc1-A86N showed only mild reduction in K48-linked chain assembly, while Ubc1-T84Δ recapitulated the complete loss of K48-specific chain assembly seen with the Ubc1-cluster I mutant (Figure 2C). Again, the first step of protein target ubiquitination was not affected in any of these mutants. When the rate of methyl ubiquitin incorporation was measured, Ubc1-T84Δ showed only a mild defect (Figure 2D).

We also mutated T84 to valine or glycine. Both mutants displayed severe defects like those seen with Ubc1-T84Δ (Figure 3A), but the rate of methyl ubiquitin incorporation was unaffected (Figure 2D). Since the defect seemed to be caused by removal of the hydroxyl group on T84 we also changed T84 to a serine, which contains a hydroxyl group but it is one methylene group shorter. This mutant showed wild-type levels of ubiquitin conjugation, and more importantly, Ubc1-T84S was able to build K48-linked chains at a rate only slightly less than that of wild-type Ubc1 (Figure 3B). We observed the same effects of the T84 mutations in APC reactions with an N-terminal fragment of yeast securin (amino acids 1-110) (Figure 3C). Ubc1-T84Δ was unable to build chains on securin, but Ubc1-T84S restored this activity. We conclude that the hydroxyl group of threonine 84 on Ubc1 is involved in K48-specific chain formation.

Threonine 84 of Ubc1 is involved in catalysis of diubiquitin and not in binding of ubiquitin

To better understand the biochemical defect associated with mutation of threonine at position 84, we attempted to use our diubiquitin synthesis assay (Figure 1) to measure initial rates of diubiquitin formation as a function of ubiquitin concentration using Ubc1-T84G as the E2. However, Ubc1-T84G, or any mutant that lacked chain-building activity, had negligible activity under our normal reaction conditions (data not shown). To determine whether there were better reaction conditions for our diubiquitin assay, we explored its pH dependency. These experiments were motivated in part by previous studies of the pH dependency of E2 enzymes that work with the ubiquitin-related protein SUMO (Yunus and Lima, 2006). These studies suggested that hydrophobic residues near the catalytic cysteine suppress the local pK of the attacking lysine and thereby enhance the rate of sumoylation at physiological pH; activity increased dramatically above pH 8. We therefore determined the pH dependency of our reactions, not simply to find more productive reaction conditions but to assess whether Ubc1, like the SUMO E2, suppresses the pK of the attacking lysine. The rate of diubiquitin formation at saturating amounts of ubiquitin (1 mM) was determined over a range of pH (6.83-10.25). The sigmoidal shape of the plot of activity at each pH (Figure 4A) suggests that activity depends on a single titratable base whose deprotonation is important for catalysis. The active site of Ubc1 is devoid of any titratable groups, so the most plausible base is the deprotonated form of the attacking lysine itself, as in the case of the SUMO

E2. The predicted pK of K48 in ubiquitin is 10.4. Thus the pK of the attacking lysine was suppressed from about 10.4 to 9.4 (Figure 4A). These data clearly reveal that our standard diubiquitin synthesis reactions are carried out at a pH (7.4) that is not ideal for the study of weak mutants like Ubc1-T84G.

We measured initial rates of diubiquitin formation with increasing amounts of ubiquitin for both Ubc1 and Ubc1-T84G at pH 10.26, the highest pH tested in our pH dependency studies. As can be seen in Figure 4B and quantified in Figure 4C, the K_M of both Ubc1 and Ubc1-T84G are similar under these conditions (388 μM for Ubc1 vs 300 μM for Ubc1-T84G), and very similar to values obtained at pH 7.4 (see Figure 1C). Vmax values tell a different story, however. First, the Vmax for wild-type Ubc1 at pH 10.26 is 0.07 per second, almost 50-fold higher than at pH 7.4. More importantly, Ubc1-T84G is only 0.0006 per second, almost 100-fold less than wild-type Ubc1 at the same pH. The fact that Ubc1-T84G affects Vmax but not K_M for ubiquitin suggests that this residue is involved in catalysis and not simply binding of ubiquitin during the attack by K48.

Glutamate 122 in Ubc1 restricts substrate monoubiquitination

We next analyzed another residue, Q122 that is not conserved between Ubc1 and Ubc4 but is identical in Ubc1 and E2-25K and is solvent-exposed near the catalytic cysteine. We constructed a mutant in which Q122 is changed to the residue found in Ubc4 (Ubc1-Q122L). This mutant appeared capable of catalyzing assembly of K48-linked chains (Figure 5A), but the chains were not as

discrete as those seen with wild-type Ubc1. We also observed that this mutant generated more abundant lower products, even in reactions with methyl ubiquitin or K48R-ubiquitin (Figure 5A). A likely explanation for these results is that Ubc1-Q122L is able to ubiquitinate lysines on the protein target more readily than wild-type Ubc1. We confirmed this possibility by measuring the rate of methyl ubiquitin incorporation with Ubc1, Ubc1-Q122L and Ubc4. Ubc1-Q122L was indeed able to more rapidly ubiquitinate lysines on cyclin B (Figure 5B, quantified in Figure 5C). As indicated in our previous work, Ubc4 was particularly active in this assay, readily attaching at least 2 ubiquitins in the first few minutes of the reaction (Figure 5B).

Q122L is a gain-of-function mutation

We next attempted to determine which functional group on Q122 is responsible for the biochemical defect observed in the Q122L mutant. The most conservative mutation is to an asparagine, since it has the same functional groups but is one methylene group shorter. When Q122 was changed to asparagine, we again saw little defect in ubiquitin conjugation, suggesting that the interaction with E1 was intact. When tested in an APC-dependent reaction, Ubc1-Q122N was unable to create long ubiquitinated products (Figure 6A), although short K48-linked products were observed. This reduction in highly ubiquitinated products was not accompanied by an increase in low molecular weight products, indicating that this mutant, unlike Q122L, is not more readily attacked by a protein target lysine. In fact, any mutation we made at Q122, with

the exception of the original mutant, Q122L, resulted in a loss of high molecular weight products (Figure 6A, Ubc1-Q122E or Ubc1-Q122A panels). These results indicate that leucine at position 122 of Ubc1 is a gain of function mutation: it allows Ubc1 to be more readily attacked by nonspecific lysines on the protein target, without losing its K48 specificity in chain formation.

Leucine at position 122 increases the rate of non-K48 lysine ubiquitination

To more rigorously test the idea that Ubc1-Q122L increases the rate of non-K48 dependent ubiquitination, we used the diubiquitin synthesis assay to measure initial velocity of diubiquitin formation as a function of ubiquitin concentration. As shown in Figure 7A and quantified in Figure 7B, Ubc1-Q122L was able to interact with ubiquitin to the same degree as Ubc1, with a K_M of 370 μM (relative to 350 μM for wild-type Ubc1). Although ubiquitin binding was not affected, Vmax was decreased by a factor of 2.5 (from 0.0015 to 0.0006 per second). This lowering of Vmax could indicate a catalytic defect under the conditions tested.

Our results suggest that Ubc1-Q122L has a slight defect in specific catalytic interactions with K48 of ubiquitin, while displaying enhanced interactions with nonspecific lysines on another substrate. This would allow Ubc1-Q122L to be more readily attacked by lysines on cyclin B (see Figure 6). We pursued this idea further by modifying our diubiquitin assay: instead of using unlabeled ubiquitin as the attacking substrate, we used unlabeled sea urchin cyclin B, the same substrate we use in our APC assays. When the rate of ubiquitin

incorporation into cyclin B was measured in the absence of APC, Ubc1 displayed very low activity (0.0027 pmols/min), whereas Ubc1-Q122L had a 30-fold higher rate (0.089 pmols/min) (Figure 7C, quantified in Figure 7D). These results support a catalytic role for leucine at this position, in that it allows non-K48 lysines to attack the catalytic cysteine more readily. Leucine is found at this position in Ubc4, consistent with the high activity of this E2 with substrate lysines.

Mutations at threonine 84 and glutamine 122 are not sufficient for chain assembly

We also characterized a T84Δ Q122L Ubc1 double mutant, reasoning that this mutant might display behavior similar to that of Ubc4, which has high activity toward substrate lysines (like Ubc1 Q122L) and poor K48-specific chain formation activity (like T84Δ). Indeed, we found that the double Ubc1 mutant generated ubiquitinated cyclin B products similar to those seen with Ubc4 (Figure 8A). Although the pattern was similar, however, it did not lead to the same level of substrate ubiquitination: the double mutant had a 3-fold higher activity than Ubc1 in methyl ubiquitin incorporation, whereas Ubc1-Q122L had 5-fold higher activity. Ubc4 reactions incorporate over 16-fold more methyl ubiquitins than Ubc1 (data not shown).

We also attempted to determine if the important side chains we identified in Ubc1 are sufficient to allow K48-specific chain assembly when transferred to Ubc4. This mutant, Ubc4-double cluster (N82V, Δ83T, N84A, L120Q, P122A; see supplemental Figure 1) was able to ubiquitinate multiple lysines on cyclin B

in the presence of APC (Figure 8B), but at a rate slightly lower than that with wild-type Ubc4. K48-specific chains were not observed. Thus, although threonine 84 is required for chain assembly by Ubc1, it is not sufficient, even in the context of multiple mutations near the catalytic cysteine.

Linear fusion between ubiquitin and cyclin B behaves as a monoubiquitinated substrate

We next sought to identify residues in the attacking ubiquitin that are important for K48-specific chain formation by Ubc1 in the context of an E3-dependent reaction. To develop an approach for testing ubiquitin mutations that affected E3-dependent polyubiquitin chain formation, we constructed a linear fusion between the C-terminus of ubiquitin and the N-terminus of our model substrate, cyclin B. This monoubiquitinated substrate allowed us to bypass the first step in substrate ubiquitination and focus on the formation of the K48-specific diubiquitin bond while still recruit the protein target to the complex via its interaction with the APC. We analyzed ubiquitination of the Ub-cyclin fusion protein in APC-dependent and -independent assays with Ubc1 or Ubc4 (Figure 9A). In reactions with Ubc1, a low amount of monoubiquitinated Ub-cyclin was observed in the absence of APC, and the amount of this species was increased upon addition of APC lacking Cdh1, its substrate recruitment factor (compare lane 3 vs 4, Ubc1 panel). In reactions lacking Cdh1, the only product was monoubiquitinated Ub-cyclin. Upon addition of Cdh1, however, large amounts of higher-molecular weight products were observed. When K48R-cyclin was used

as the substrate, these larger products were greatly depleted, leaving a background level of nonspecifically ubiquitinated products that are similar to those seen under the same reaction conditions with cyclin B. In contrast, when Ubc4 was used as the E2, the reaction products were similar with wild-type or K48R (Figure 9A, compare lane 13 and 16). Furthermore, Ubc4 reactions only gave robust ubiquitination in the presence of both APC and Cdh1, in contrast to Ubc1 reactions (Figure 9A, compare lane 4 and 12).

Tyrosine 59 is important for K48-linked chain assembly

Having established that our Ub-cyclin fusion protein behaved as a monoubiquitinated substrate, we mutated residues around K48 of ubiquitin and tested them in our APC-dependent reactions. We charged Ubc1 with unlabeled wild-type ubiquitin and added an APC cocktail with radiolabeled Ub-cyclin fusions. The only reaction being monitored is the formation of ubiquitinated Ub-cyclin species. Our mutations included the hydrophobic patch mutation, I44A, which is known to disrupt K48-specific chain formation by the E2, Cdc34 (Petroski and Deshaies, 2005). Surprisingly, however, this mutation did not affect the assembly of ubiquitin chains by Ubc1 (Figure 10A). In fact, of 8 mutations tested, only the Y59A mutation resulted in a major decrease in the production of ubiquitin chains, similar to the defect seen with the K48R mutation. As expected, the same mutations had little effect in reactions with Ubc4, which does not build chains under the conditions tested.

To further test the role of tyrosine 59 in chain assembly, we constructed ubiquitin species containing either the Y59A or Y59F mutation. In these experiments, we used the mutant allele for both the charging and subsequent attack. We found that tyrosine 59 is not involved in the first step of the reaction, E1 activation, or the second step, transfer of ubiquitin to E2, since monoubiquitination of cyclin B was unaffected (Figure 10B, cyclin-Ub_1 band). However, Y59A-ubiquitin displayed a dramatic defect in chain formation. Interestingly, when tyrosine 59 was mutated to a phenylalanine it was able to support polyubiquitination, indicating that the hydrophobic phenyl ring and not the hydroxyl moiety of Y59 is important for ubiquitin-ubiquitin bond formation. As before, Y59 mutation had little effect in reactions with Ubc4. Unfortunately, because Ubc1 activity with Y59A is so low (even at high pH), we could not distinguish between a binding or catalytic defect.

DISCUSSION

Polyubiquitination is a two-step reaction. First, a lysine on a protein target attacks the E2-ubiquitin thioester, removing ubiquitin from the E2 catalytic cysteine. Second, a specific lysine in ubiquitin (K48 in our case) attacks the E2-ubiquitin thioester to initiate assembly of a polyubiquitin chain. The results presented here shed light on both of these steps.

We demonstrated that Ubc1 is able to make a K48-linked ubiquitin chain at the expense of ubiquitinating lysines on the protein target. These two activities, protein target ubiquitination and K48 ubiquitination, can be separated by specific mutations near the catalytic cysteine of Ubc1. Threonine 84 is involved in K48 ubiquitination, whereas glutamine 122 restricts protein target ubiquitination. Neither mutation appears to be involved in ubiquitin binding, and interestingly, neither is the UBA domain tethered to the C-terminus of Ubc1. Thus, we have not produced mutations that clearly disrupt K48-specific ubiquitin binding, perhaps indicating that the interacting surface depends on multiple low affinity interactions and that mutating single amino acids will not disrupt binding.

Ubc1 reactions are characterized by the slow appearance of K48-linked chains and the small amount of protein target turnover. The rate-limiting step in these reactions is the addition of the first ubiquitin; with rapid chain extension occurring after this priming ubiquitin is present (Rodrigo-Brenni and Morgan, 2007). This is due to Ubc1's ability to bind ubiquitin and guide K48 towards its active site. Previous NMR studies measured an interaction between Ubc1 and

ubiquitin that was in the 400 μM range and was restricted to the UBA domain (Hamilton et al., 2001). Interestingly, these studies did not reveal an interaction between Ubc1 and ubiquitin in the absence of the UBA domain. In our experiments, however, the ability of ubiquitin-charged Ubc1-ΔUBA to bind ubiquitin was assessed. We would argue that our diubiquitin assay is a better index of the physiological interaction between a charged-E2 complex and ubiquitin bound to a protein target. This interaction, in the 300 μM range, would be expected to result in very low levels of unanchored chain formation, as the concentration of ubiquitin inside a cell is in the 10 μM range (Riley et al., 1988).

While initially looking for mutations defective in ubiquitin binding we discovered two residues in the catalytic core of Ubc1 that are involved in ubiquitination. The first residue we characterized, threonine 84, is involved in K48-dependent catalysis rather than binding of ubiquitin. We arrived at this conclusion based on multiple lines of evidence. First, mutating this residue does not inhibit protein target ubiquitination, and therefore lysines are able to attack the catalytic cysteine. Second, even though this mutant does not make chains, it does not lead to more monoubiquitination, suggesting that it can still interact with ubiquitin but in a nonproductive manner. Third, when assayed at a high pH, this mutant displayed an apparent affinity for ubiquitin that is similar to that for wild-type Ubc1. Fourth, even when the mutant is able to interact with ubiquitin it does not lead to efficient diubiquitin formation. Although not rigorously tested, this mutant does not appear to be involved in pK suppression of the attacking lysine.

We are left with a role for threonine 84 in positioning either K48 itself or residues in Ubc1 that are important for K48 positioning.

The second residue we characterized was glutamate 122, which we initially mutated to leucine, uncovering a gain of function mutant. Wild-type Ubc1, when presented with a protein target with multiple lysines, will add one ubiquitin very slowly and then build a K48-linked chain very rapidly. This difference in reaction rate is due in part to Ubc1 binding to ubiquitin. Mutant Q122L revealed another potential mechanism for limited Ubc1 activity in the first step: its catalytic cysteine is generally unreactive towards non-K48 lysines. This was most evident when an APC protein target attacked charged Ubc1 in the absence of the APC. Wild-type Ubc1 ubiquitinated cyclin B very poorly, whereas Ubc1-Q122L ubiquitinated cyclin B 50-fold more rapidly. This mutant was still able to bind ubiquitin and catalyze diubiquitin synthesis, but it had a mild catalytic defect. Preliminary pH dependency studies seem to indicate that Q122L does not change the pK suppression seen with Ubc1 when using K48 as the attacking lysine. We would argue that Q122L allows for more protein target monoubiquitination by suppressing the pK of a non-K48 lysine. This notion is supported by previous studies of the SUMO E2, Ubc9, in which the pK of the attacking lysine is suppressed by creating a hydrophobic microenvironment near the catalytic cysteine. Tyrosine 87 on Ubc9 was crucial for this environment. The same study noted that in Es2 that lack this tyrosine, hydrophobicity may be provided by a leucine at a position equivalent to 122 in Ubc1 (Yunus and Lima, 2006). Interestingly, Ubc1 is unique amongst E2s in that it lacks both tyrosine at

position 87 and leucine at position 122. By creating the Q122L mutant, we may have partly restored the proposed microenvironment and potentially suppressed the pK of a protein target lysine. Interestingly, however, this mutation only affects non-K48 lysines, suggesting that proton removal from the K48 side chain might involve other mechanisms.

We also explored the residues in ubiquitin itself that are important for K48-linked chain formation. To find these residues we created a monoubiquitinated substrate, in which we mutated residues near K48. Under normal reaction conditions, the ubiquitin involved in chain formation is the same that was activated by the E1 and passed to the E2. On the E2, the thioester is attacked by the E3-bound protein target. The linear monoubiquitinated substrate allows us to bypass all earlier steps and focus only on diubiquitin formation. Using this approach we found that tyrosine 59 on ubiquitin is critical for chain formation. Interestingly, isoleucine 44, the classical residue in the hydrophobic patch, was not involved. This is noteworthy since most ubiquitin interactions studied to date have revolved around this hydrophobic patch (French et al., 2005). For example, the hydrophobic patch is important for K48-linked chain formation by the E2, Cdc34, in reactions with the E3, SCF (Petroski and Deshaies, 2005). It is also intriguing to note that iodination of tyrosine 59 was shown many years ago to prevent chain formation by the human Ubc1 ortholog, E2-25K, but not by the human homolog of Cdc34 (Pickart et al., 1992). It appears that there are at least two ways to build a K48 polyubiquitin chain: one (employed by Ubc1) depends on tyrosine 59; the other (employed by Cdc34) depends on isoleucine 44.

Given that it is immediately adjacent to K48, Y59 of ubiquitin might contribute to the hydrophobic environment needed to suppress the pK of the attacking lysine. In this case it would only be able to suppress the pK of K48 of ubiquitin. As mentioned earlier, Ubc1 lacks the residues shown to be important for this suppression by the SUMO E2, Ubc9. One way to make the reaction specific for K48 would be to place residues important for catalysis on the two protein partners such that only when they come together the reaction can proceed at physiological pH. Unfortunately, our efforts to test this idea have proven inconclusive because testing the pH dependency requires amounts of ubiquitin-Y59A that we are unable to produce.

In conclusion, we are left with a clearer picture of the two steps involved in efficient polyubiquitination: priming ubiquitination and chain extension. We would argue that it would be difficult to evolve an E2 enzyme capable of doing both steps equally well. We believe that on one hand there are Ubc4 family members, which have an intact hydrophobic microenvironment and are able to suppress the pK of lysines in many contexts - and therefore display little apparent lysine specificity. On the other hand there is Ubc1, in which the hydrophobic environment is not provided by its own side chains but is restored upon ubiquitin binding in the correct orientation, making it highly specific for K48. Between these extremes there lies Cdc34, which appears to be able to carry out both steps at a reasonable rate – perhaps because it has conserved its hydrophobic environment for pK suppression, while also acquiring an acidic loop to aid in K48-specific catalysis.

EXPERIMENTAL PROCEDURES

Plasmids, Expression and Purification of recombinant proteins

Construction of Ubc1, Ubc4 and Ubc1-ΔUBA for protein expression was previously described (Rodrigo-Brenni and Morgan, 2007). Ubc1 and Ubc4 mutations were created via site-directed mutagenesis on the wild-type plasmids. The expression and purification of the different E2s was previously described and the various mutants were done identically (Rodrigo-Brenni and Morgan, 2007). The final concentration of the different E2 species is Ubc1, Ubc1-ΔUBA and Ubc1-T84Δ, Q122L (5 mg/ml), Ubc1-T84Δ and Ubc1-T84S (10 mg/ml), Ubc1-cluster I, Ubc1-V83N, Ubc1-T84G, Ubc1-T84V, Ubc1-A86N, Ubc4 and Ubc4-double cluster (2.5 mg/ml), and Ubc1-Q122L (1 mg/ml).

The construction of GST-K48R ubiquitin was previously described (Petroski and Deshaies, 2005). The plasmid was transformed into BL21 cells. A single colony was incubated overnight in 100 ml LB/Amp media at 37^{o}C, diluted into 1 liter of fresh media and grown at 37^{o}C to an OD_{600} of 0.60, after which IPTG was added to 1 mM for 4 h at 37^{o}C. Cells were harvested by centrifugation, washed in cold water and frozen in liquid nitrogen. The frozen pellet was melted rapidly and incubated with 4 volumes of breakage buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1mM EDTA, 10% glycerol, protease inhibitor cocktail, 1mM DTT, 1.25 mg/ml lysozyme and 500 U DNase) at room temperature for 30 minutes. The lysate was centrifuged (1 h, 100,000 g, 4^{o}C) and the supernatant was purified on glutathione (GSH) Sepharose (GE Healthcare). The bound material was washed

four times in 10 volumes of wash buffer (50 mM Tris pH 7.4, 500 mM NaCl, 10% glycerol, 1mM EDTA) for 15 minutes. The GST fusion protein was eluted twice by incubation with 3 ml of elution buffer (same as breaking buffer but containing 10 mM reduced glutathione) with agitation for 15 minutes and the fractions were combined. All binding, washing and elution steps were done at room temperature. The purified protein (1 mg/ml) was stored at -80^{o}C.

The liner fusion between ubiquitin and cyclin B was created via standard cloning techniques. Briefly, a PCR product of cyclin B was clone at the 3' end of the C-terminus of GST-ubiquitin and GST-K48R ubiquitin. A 6xHIS tagged was introduced via PCR at the C-terminus of the fusion. The various ubiquitin mutants were created by site-directed mutagenesis.

The fusion protein was purified as described for GST-K48R ubiquitin, except that the GSH purification step was followed by affinity metal chromatography. Briefly, purified GST-fusions were dialyzed overnight into a buffer lacking glutathione (50 mM HEPES 7.4, 100 mM NaCl, 1 mM $MgCl_2$ and 10 % glycerol) and incubated with sepharose beads charged with Co^{+2} for one hour. The bound material was washed with dialysis buffer and eluted twice with 3 ml dialysis buffer containing 500 mM imidazole. The fractions were pooled, dialyzed and the purified proteins (1 mg/ml) stored at -80^{o}C.

All GST encoding plasmids contain a TEV site after the end of the coding sequence of GST. The TEV site is followed by a cAMP-dependent protein kinase A (PKA) phosphorylation site at the N-terminus of ubiquitin and its derivatives. 50 μL of K48R ubiquitin or each ubiquitin-cyclin B fusion was

incubated with 10 μCi γ^{32}P-ATP, 1.67 nmol cold ATP and 1 μL PKA (New England Biolabs) for 2 hour at 30°C. After removal of unincorporated ATP by gel filtration, the eluted proteins were diluted to 250 μL (50 mM HEPES 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 0.5 mM EDTA and 1mM DTT) and incubated with 5 μL 6xHIS-TEV (5 mg/ml) for 1 hour at 30°C. The cleaved material was incubated at 65°C for 15 minutes, followed by 5 minutes on ice. The precipitated material was removed by centrifugation. K48R ubiquitin used in the pH dependency studies was prepared identically, except that the labeled protein was diluted in a buffer containing 50 mM Citrate/Bis-Tris-Propane pH 8.0.

Ubiquitination Assays

Preparation of reaction components (E1, ATP, Ubiquitin, APC, Cdh1 and ubiqutin-E2 conjugates) was described previously (Carroll and Morgan, 2005; Rodrigo-Brenni and Morgan, 2007). 2.5 mg of Ubc1, Ubc4 and all the different mutations were added to each reaction unless otherwise noted. APC (1.5 μl of 10 nM), Cdh1 (0.5 μl of 1.5 μM) and radiolabeled substrate (N-terminus of sea urchin cyclin B, N-terminus of yeast securin, or ubiquitin-cyclin B fusions) were mixed at room temperature. Reactions were initiated by combining the E1/E2 mix with the APC mix in a final volume of 20 μl. Reaction products were analyzed by SDS-PAGE and visualized with a PhosphorImager. The expression, purification and labeling of the N-terminus of sea urchin cyclin B has been described previously (Carroll and Morgan, 2005). The expression, purification and labeling of the N-terminus of yeast securin will be described elsewhere.

Diubiquitin Assays

Ubc1 (0.05 μL of 5 mg/ml) was incubated in the presence of E1 (0.2 μL of 1 mg/ml) ^{32}P-labeled K48R ubiquitin (5 μL of 250 μL labeling reaction) and ATP (1 μL of 10 mg/ml) in 50 mM HEPES 7.4, 100 mM NaCl, 1 mM $MgCl_2$ and 10% glycerol unless otherwise noted for 15 minutes at room temperature. The charging reaction was treated with 10 mM N-ethylmaleimide (NEM) and 50 mM EDTA fro 15 minutes at room temperature. The treated reaction (7 μL final volume) was incubated with either saturating amounts of ubiquitin (1mM) or a range of ubiquitin concentrations (see figure legends) in a final volume of 20 μL. The reactions were stopped by addition of nonreducing sample buffer containing 10 mM NEM, analyzed by SDS-PAGE and visualized by PhosphorImager. The appearance of diubiquitin and the remaining E2~Ub conjugates were quantified with ImageQuant by drawing rectangles around the appropriate bands and converting counts detected to pmol of ^{32}P-K48R ubiquitin incorporated.

pH Dependency Studies

Single turnover assays were carried out at a range of pH (6.86-10.26) in an identical manner to that described above but with a few exceptions. The labeling of ^{32}P-K48R ubiquitin was carried out in 50 mM Citrate/Bis-Tris-Propane pH 8.0, 100 mM NaCl, 1 mM MgCl2 and 10% glycerol. The charging reactions were stopped by treatment with NEM and EDTA. The treated reactions were added to reactions containing 1 mM ubiquitin at a range of pH values. The pH of

the 50 mM Citrate/Bis-Tris-Propane was adjusted by adding different dilutions of HCl and NaOH (added as a 1/20 the volume of the final reaction). The final pH was measured at room temperature. Samples were removed at various times, denatured in nonreducing sample buffer containing 10 mM NEM, analyzed by SDS-PAGE and visualized with a PhosphorImager. The rate of diubiquitin formation at different pH values was calculated from linear curve fitting of the plots of pmol diubiquitin formed as a function of time. The rates were plotted against pH and the pK was estimated by nonlinear curve fitting using the sigmoidal function module of SigmaPlot. The shape of the curve indicates that only the basic form of a general base is involved in catalysis (Fersht, 1999).

Figure 1. The catalytic core of Ubc1 binds ubiquitin

(A) Purified Ubc1 was incubated with ^{32}P-labeled K48R ubiquitin in the presence of E1 and ATP for 15 minutes and treated with NEM and EDTA to prevent recharging of Ubc1. 1mM unlabeled ubiquitin was added to the E1/E2 mix and aliquots were taken at the indicated times. The aliquots were added to nonreducing sample buffer followed by SDS-PAGE and visualization with a PhosphorImager. Free ubiquitin (Ub), diubiquitin (diUb) and charged Ubc1 (Ubc1~Ub) are indicated. The asterisk denotes a nonspecific protein.

(B) The experiment show in A was quantified for the loss of the ubiquitin-charged Ubc1 (white rectangles) or the appearance of diubiquitin (black circles).

(C) Same as A, but the E1/E2 mix was added to reactions with increasing amounts of ubiquitin (50 μM to750 μM). Reactions were allowed to react for two minutes. Reactions were stopped by addition of nonreducing sample buffer, analyzed by SDS-PAGE and visualized with a PhosphorImager.

(D) Same as C but Ubc1-ΔUBA was used instead of Ubc1.

(E) The rates of diubiquitin formation as a function of ubiquitin concentration for reactions shown in C (n=5, black circles) and D (n=3, white circles) were determined. The fraction of diubiquitin formed (total diubiquitin / (diubiquitin plus conjugated ubiquitin remaining) was quantified using ImageQuant and plotted against ubiquitin concentration. Data were fitted to a rectangular hyperbola using the ligand-binding module of SigmaPlot. Error bars represent the standard error.

Figure 1

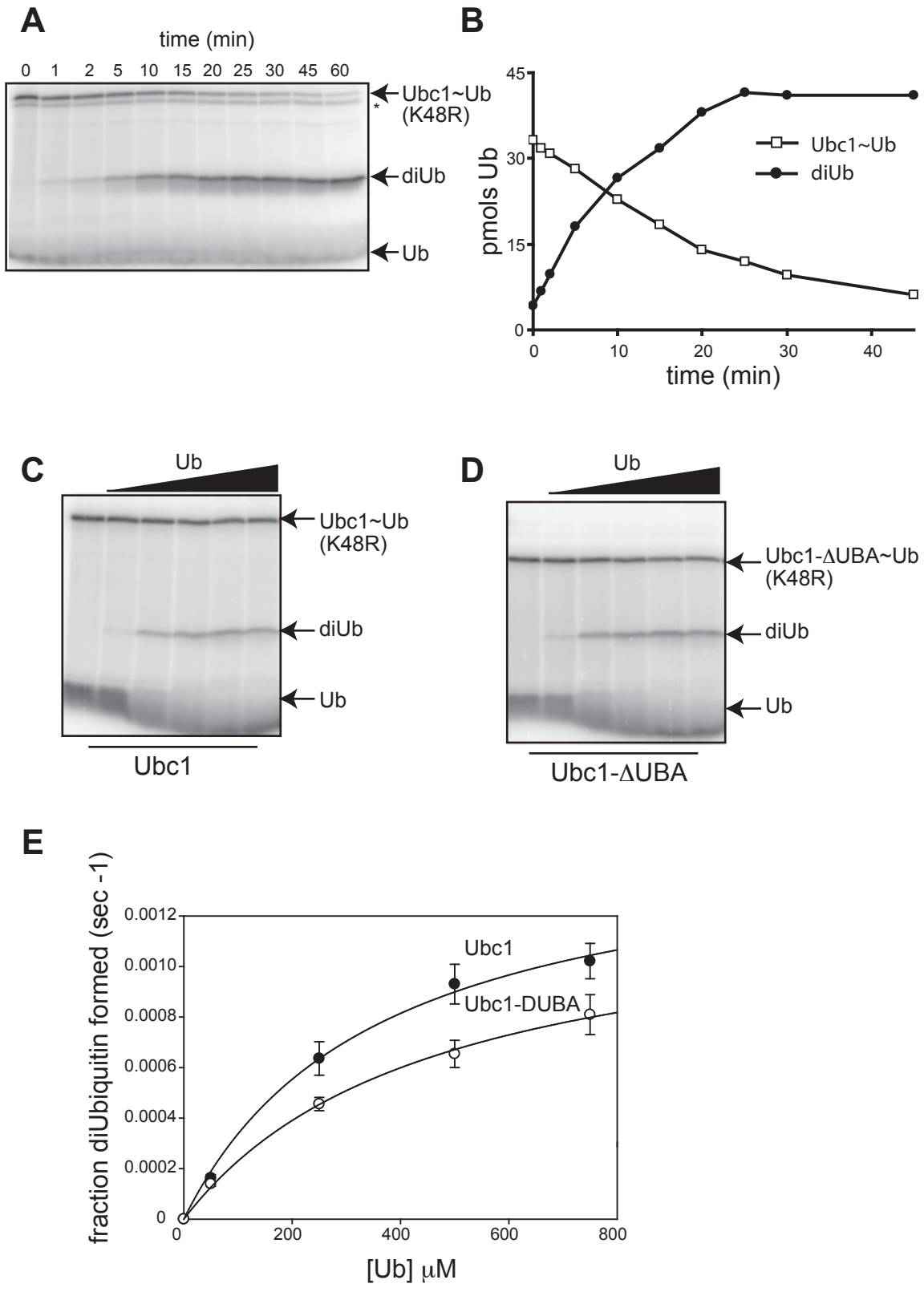

A
time (min)
0 1 2 5 10 15 20 25 30 45 60
Ubc1~Ub
(K48R)
*
diUb
Ub
B
pmols Ub
45
30
15
0
0 10 20 30 40
time (min)
Ubc1~Ub
diUb
C
Ub
Ubc1~Ub
(K48R)
diUb
Ub
Ubc1
D
Ub
Ubc1-ΔUBA~Ub
(K48R)
diUb
Ub
Ubc1-ΔUBA
E
fraction diUbiquitin formed (sec -1)
0.0012
0.0010
0.0008
0.0006
0.0004
0.0002
0
0 200 400 600 800
[Ub] μM
Ubc1
Ubc1-DUBA

Figure 2. Ubc1-cluster I mutant lacks K48-linked polyubiquitination activity

(A) Purified Ubc1 or Ubc1-cluster I was incubated for 15 minutes with E1, ATP and the indicated ubiquitin species. E1/E2 mixes were added to APC, Cdh1 and ^{125}I-cyclin B and incubated for 45 minutes at room temperature. The reactions were stopped by addition of sample buffer, analyzed by SDS-PAGE, and visualized with a PhosphorImager.

(B) Methyl-ubiquitin incorporation was measured for APC-Ubc1 or APC-Ubc1-cluster I reactions. Briefly, Ubc1 or Ubc1-cluster I was incubated for 15 minutes with E1, ATP and methyl-ubiquitin. E1/E2 mix was added to an APC^{Cdh1} mix containing ^{125}I-cyclin B, samples were taken at the indicated times and added to sample buffer. The reactions were analyzed by SDS-PAGE, visualized with a PhosphorImager and quantified with ImageQuant.

(C) The ability of various Ubc1 mutants to support APC-dependent ubiquitination was assessed under the same reactions conditions as A.

(D) The ability of various Ubc1 mutants to support APC-dependent methyl-ubiquitin incorporation was assessed under the same reactions conditions as B.

Figure 2

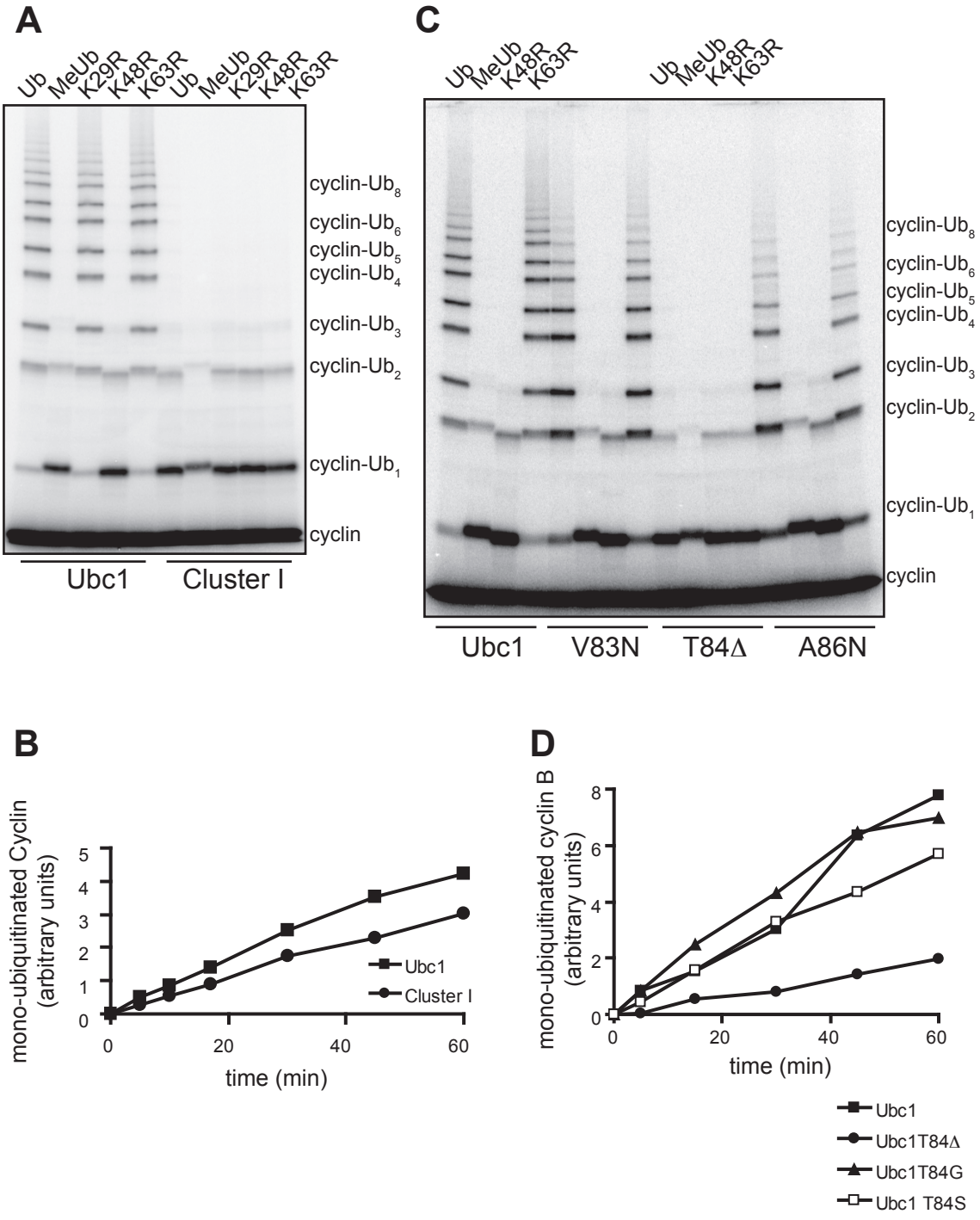

A
Ub
MeUb
K29R
K48R
K63R
Ub
MeUb
K29R
K48R
K63R
cyclin-Ub8
cyclin-Ub6
cyclin-Ub5
cyclin-Ub4
cyclin-Ub3
cyclin-Ub2
cyclin-Ub1
cyclin
Ubc1
Cluster I
C
Ub
MeUb
K48R
K63R
Ub
MeUb
K48R
K63R
cyclin-Ub8
cyclin-Ub6
cyclin-Ub5
cyclin-Ub4
cyclin-Ub3
cyclin-Ub2
cyclin-Ub1
cyclin
Ubc1
V83N
T84Δ
A86N
B
mono-ubiquitinated Cyclin
(arbitrary units)
0
1
2
3
4
5
0
20
40
60
time (min)
Ubc1
Cluster I
D
mono-ubiquitinated cyclin B
(arbitrary units)
0
2
4
6
8
0
20
40
60
time (min)
Ubc1
Ubc1T84Δ
Ubc1T84G
Ubc1 T84S

Figure 3. The hydroxyl group of threonine 84 is critical for K48-linked polyubiquitination

(A) Ubc1, Ubc1-T84V or Ubc1-T84G was incubated with E1, ATP and the indicated ubiquitins species for 15 minutes. E1/E2 mix was added to an APC^{Cdh1} mix containing ^{125}I-cyclin B and incubated for 45 minutes at room temperature. The reactions were stopped by addition of sample buffer, analyzed by SDS-PAGE and visualized by PhosphorImager.

(B) Same as A but using Ubc1 or Ubc1-T84S in the E1/E2 mix.

(C) Same as A, but E1/E2 mix was incubated with an APC^{Cdh1} mix containing ^{35}S-securin.

Figure 3

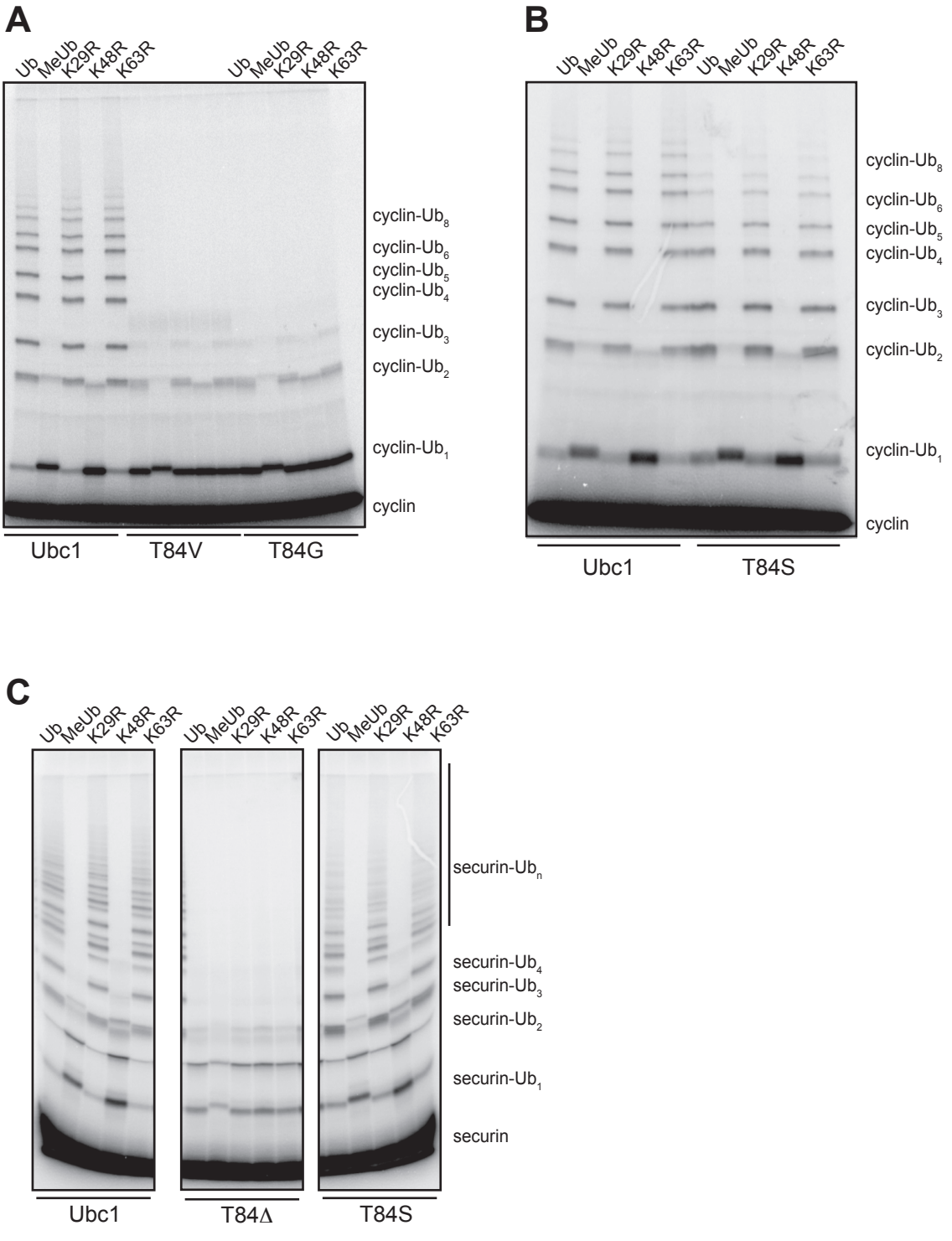

A
Ub
MeUb
K29R
K48R
K63R
cyclin-Ub8
cyclin-Ub6
cyclin-Ub5
cyclin-Ub4
cyclin-Ub3
cyclin-Ub2
cyclin-Ub1
cyclin
Ubc1
T84V
T84G
B
cyclin-Ub8
cyclin-Ub6
cyclin-Ub5
cyclin-Ub4
cyclin-Ub3
cyclin-Ub2
cyclin-Ub1
cyclin
Ubc1
T84S
C
securin-Ubn
securin-Ub4
securin-Ub3
securin-Ub2
securin-Ub1
securin
Ubc1
T84Δ
T84S

Figure 4. Threonine 84 in Ubc1 is involved in K48-dependent catalysis and not ubiquitins binding

(A) Reactions as those shown in Figure 1A were carried out at a range of pH values (6.86-10.26). Briefly, purified Ubc1 was charged with ^{32}P-labeled K48R ubiquitin (50mM Citrate/Bis-Tris-Propane buffered) in the presence of E1 and ATP, and treated with NEM and EDTA to prevent recharging of Ubc1. 1mM unlabeled ubiquitin at a range of pH (50mM Citrate/Bis-Tris-Propane buffer pH 8.0, adjusted with either HCl or NaOH dilutions, 100mM NaCl, 1mM MgCl2 and 10% glycerol) was added to the E1/E2 mix and aliquots were taken at different times. The appearance of diubiquitin was plotted as a function of time and fitted to a linear function using Excel. The rate of diubiquitin formation (the slope of the linear function) was plotted as a function of pH and fitted to a sigmoidal function using SigmaPlot. Experiments were done in triplicate and the error bars represent standard error.

(B) Same as A, but the E1/E2 mix was added to reactions with increasing amounts of ubiquitin (50 μM to750 μM) at pH 10.26. Ubc1 reactions were allowed to react for 10 seconds and Ubc1-T84G reactions were allowed to react for 2 minutes. Reactions were stopped by addition of nonreducing sample buffer, analyzed by SDS-PAGE and visualized with a PhosphorImager.

(C). The results of three experiments as those shown on B were quantified using ImageQuant and plotted. The plots were fitted to a rectangular hyperbola using SigmaPlot. Error bars represent standard error.

Figure 4

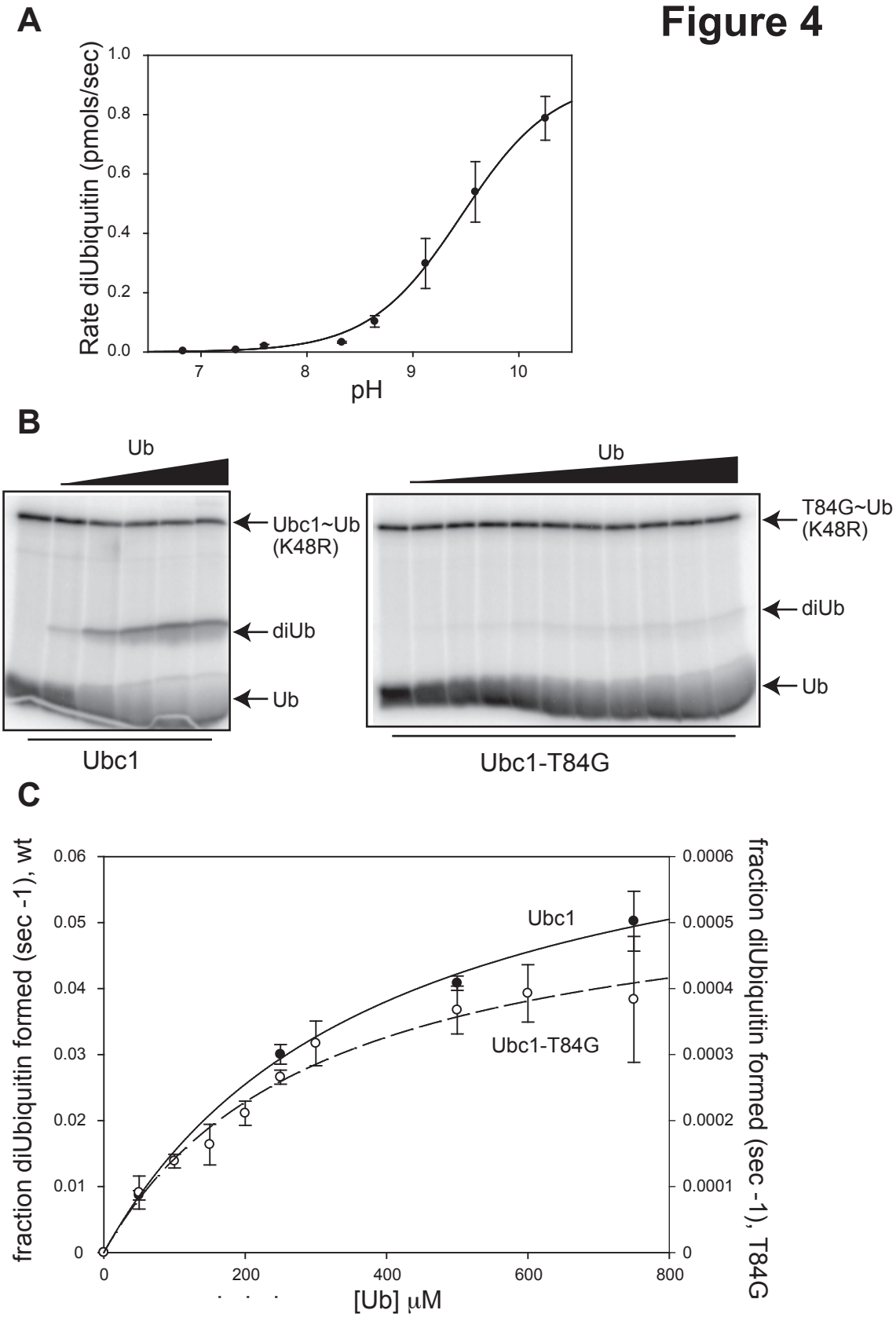

A
Rate diUbiquitin (pmols/sec)
1.0
0.8
0.6
0.4
0.2
0.0
7
8
9
10
pH
B
Ub
Ubc1~Ub
(K48R)
diUb
Ub
Ubc1
Ub
T84G~Ub
(K48R)
diUb
Ub
Ubc1-T84G
C
fraction diUbiquitin formed (sec -1), wt
0.06
0.05
0.04
0.03
0.02
0.01
0
Ubc1
Ubc1-T84G
0
200
400
600
800
[Ub] μM
fraction diUbiquitin formed (sec -1), T84G
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0

Figure 5. Ubc1-Q122L ubiquitinates more substrate's lysines.

(A) Ubc1 or Ubc1-Q122L was incubated with E1, ATP and the indicated ubiquitin species for 15 minutes. E1/E2 mix was added to an APC^{Cdh1} mix containing ^{125}I-cyclin B and incubated for 45 minutes at room temperature. The reactions were stopped by addition of sample buffer, analyzed by SDS-PAGE and visualized by PhosphorImager.

(B and C) Methyl-ubiquitin incorporation was measured for APC-Ubc1 and APC-Ubc1-Q122L reactions. Briefly, Ubc1 or Ubc1-Q122L was incubated for 15 minutes with E1, ATP and methyl-ubiquitin. E1/E2 mix was added to an APC^{Cdh1} mix containing ^{125}I-cyclin B and samples were taken at the indicated times and added to sample buffer. The reactions were analyzed by SDS-PAGE, visualized with a PhosphorImager and quantified with ImageQuant.

Figure 5

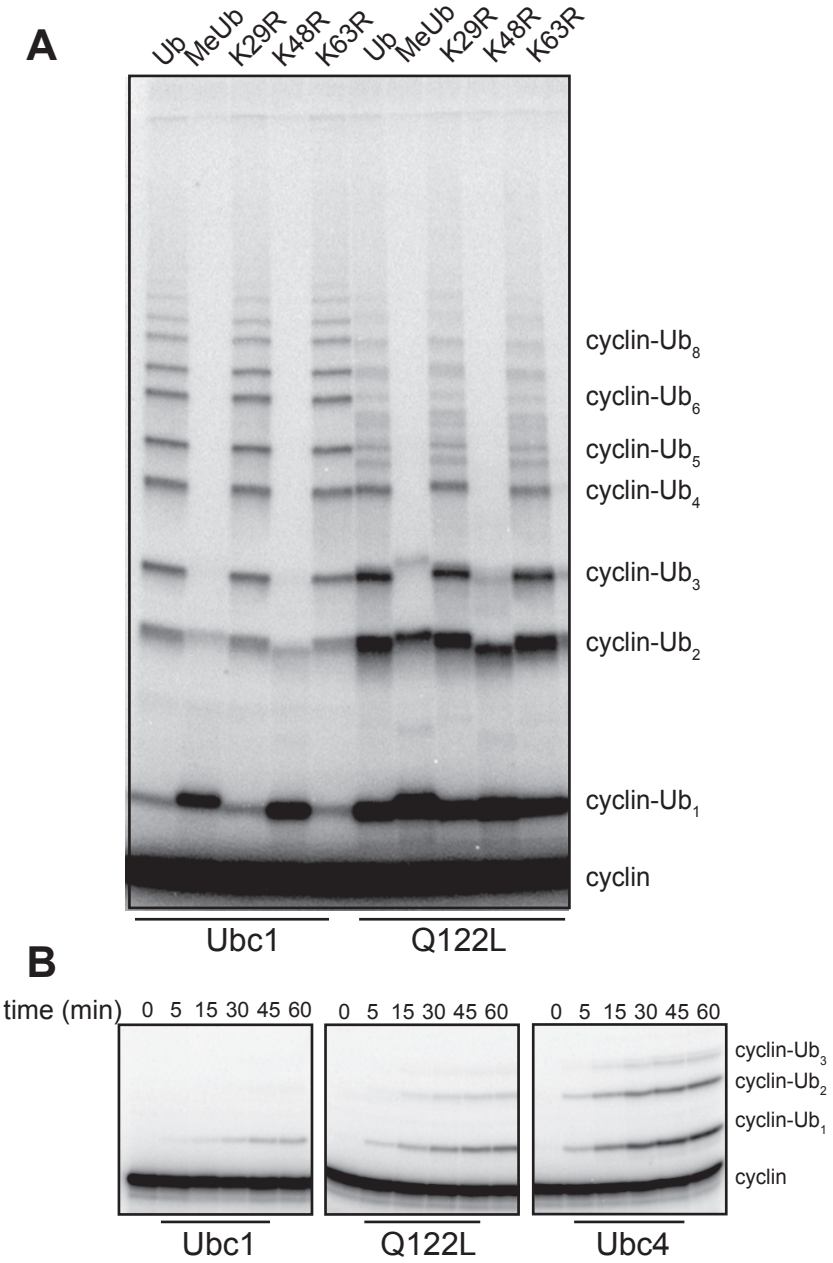

A
Ub
MeUb
K29R
K48R
K63R
Ub
MeUb
K29R
K48R
K63R
cyclin-Ub8
cyclin-Ub6
cyclin-Ub5
cyclin-Ub4
cyclin-Ub3
cyclin-Ub2
cyclin-Ub1
cyclin
Ubc1
Q122L
B
time (min) 0 5 15 30 45 60 0 5 15 30 45 60 0 5 15 30 45 60
cyclin-Ub3
cyclin-Ub2
cyclin-Ub1
cyclin
Ubc1
Q122L
Ubc4
C
Ubiquitinated cyclin B (arbitrary units)
80
60
40
20
0
0
15
30
45
60
time (min)
Ubc1
Ubc1 Q122L
Ubc4

Figure 6. Only reactions with Ubc1-Q122L result in more substrate ubiquitination

(A) Purified Ubc1 or Ubc1 mutated at glutamine 122 was added to E1, ATP and the indicated ubiquitin species. E1/E2 mix was added to an APC^{Cdh1} mix containing ^{125}I-cyclin B and incubated for 45 minutes at room temperature. Reactions were stopped by addition of sample buffer, analyzed by SDS-PAGE and visualized with a PhosphorImager.

Figure 6

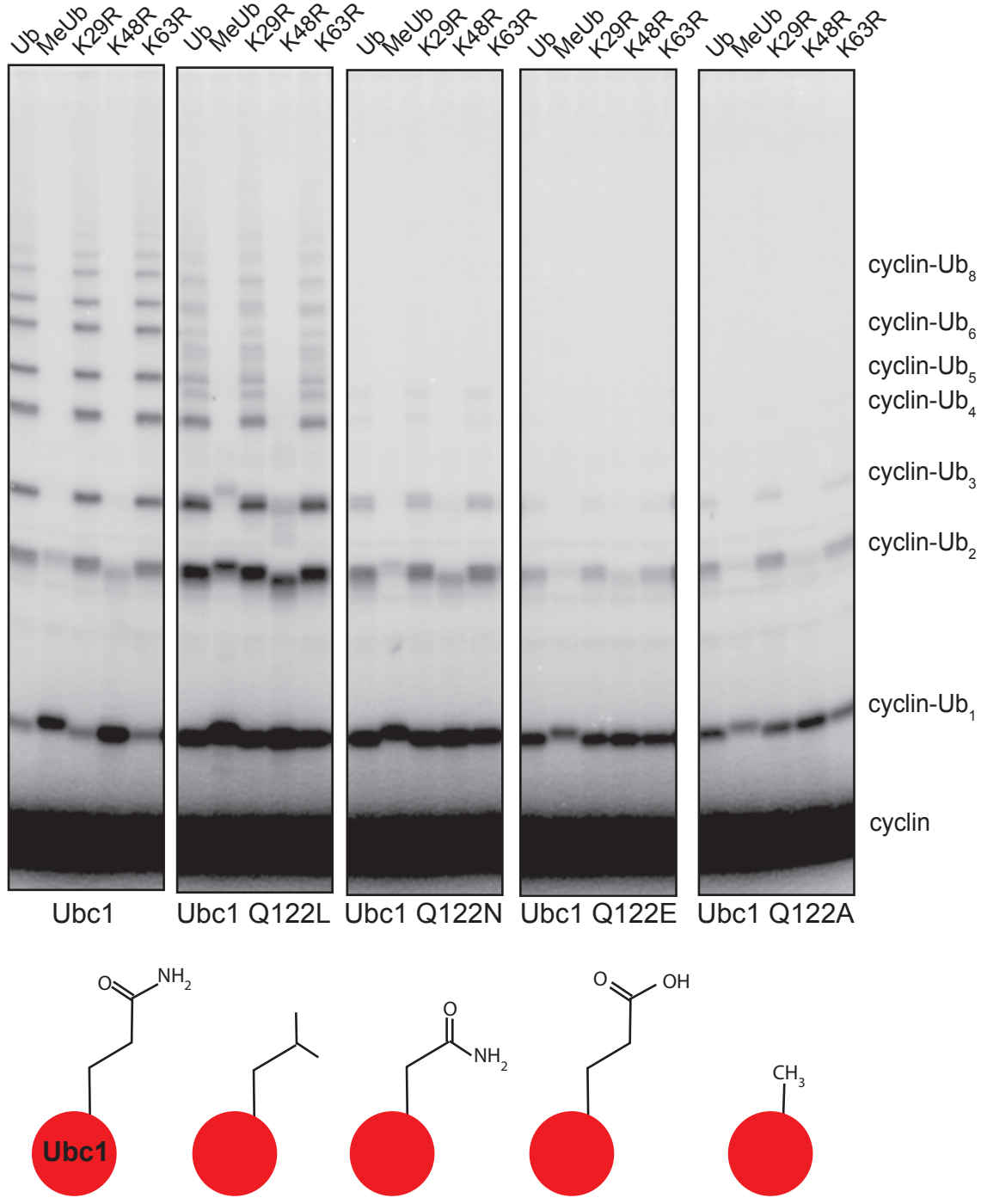

Ub
MeUb
K29R
K48R
K63R
cyclin-Ub8
cyclin-Ub6
cyclin-Ub5
cyclin-Ub4
cyclin-Ub3
cyclin-Ub2
cyclin-Ub1
cyclin
Ubc1
Ubc1 Q122L
Ubc1 Q122N
Ubc1 Q122E
Ubc1 Q122A
O
NH2
OH
CH3
Ubc1

Figure 7. Ubc1-Q122L has a wild-type apparent affinity towards ubiquitin but has higher activity towards non-K48 lysines.

(A) Purified Ubc1-Q122L was incubated with E1, ATP and ^{32}P-ubiquitin for 15 minutes. NEM and EDTA were added to the E1/E2 mix to stop further charging of E1 and E2. E1/E2 mix was added to reactions with increasing amounts of ubiquitin (50 μm to 750 μM) and incubated for 2 minutes at room temperature. Reactions were stopped by addition of nonreducing sample buffer, analyzed by SDS-PAGE and visualized with a PhosphorImager.

(B) The results of experiments (n=3 for Ubc1-Q122L) were quantified, plotted as a function of ubiquitin concentration and fitted to a rectangular hyperbola using SigmaPlot. The Ubc1 plot from Figure 1E is reproduced for comparison.

(C) Purified Ubc1 or Ubc1-Q122L was incubated with E1, ATP and ^{32}P-ubiquitin for 15 minutes. NEM and EDTA were added to the E1/E2 mix. E1/E2 was added to reactions containing 12 mg of purified sea urchin cyclin B. Samples were taken at the indicated times, added to nonreducing sample buffer, analyzed by SDS-PAGE and visualized with a PhosphorImager.

Figure 7

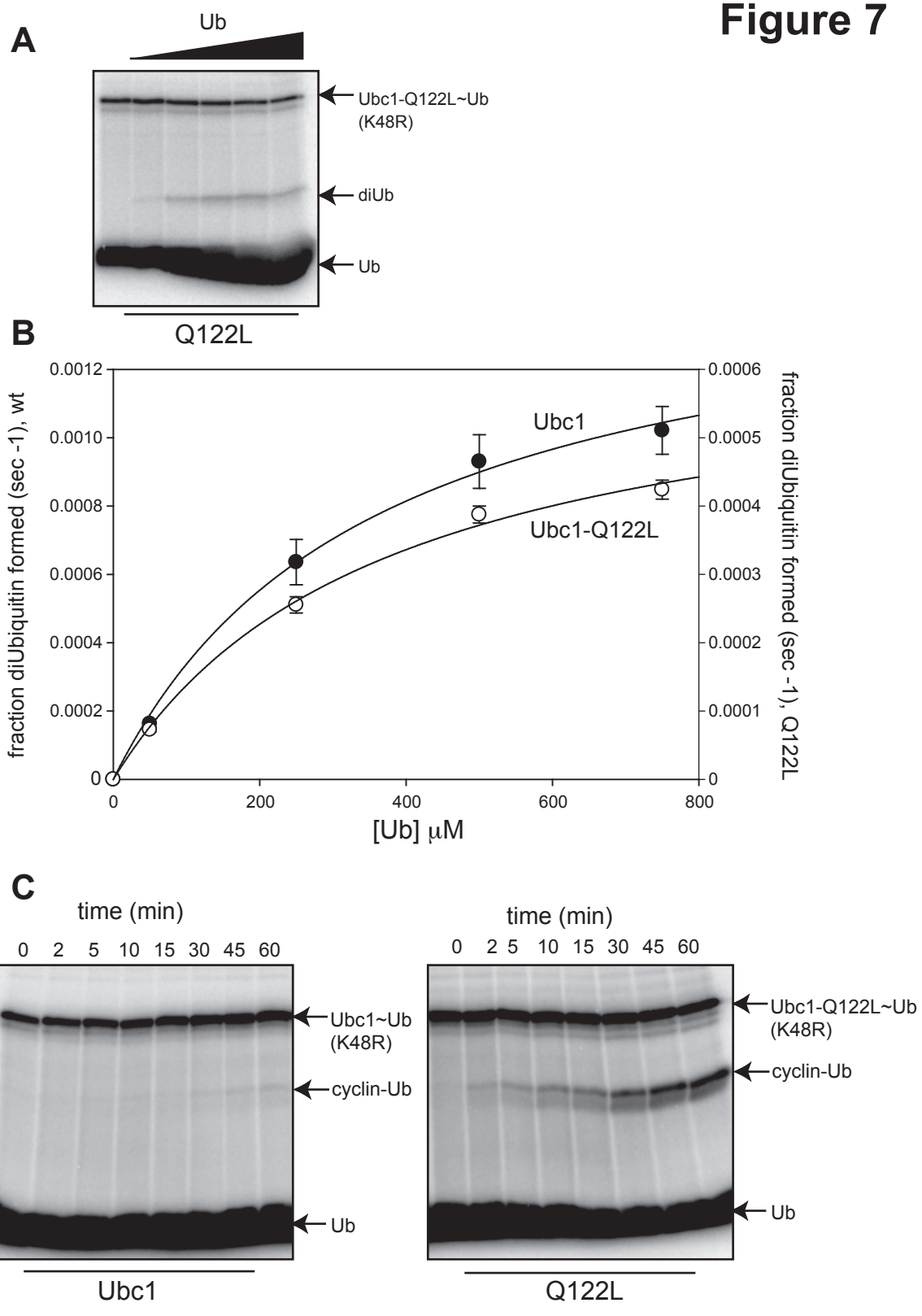
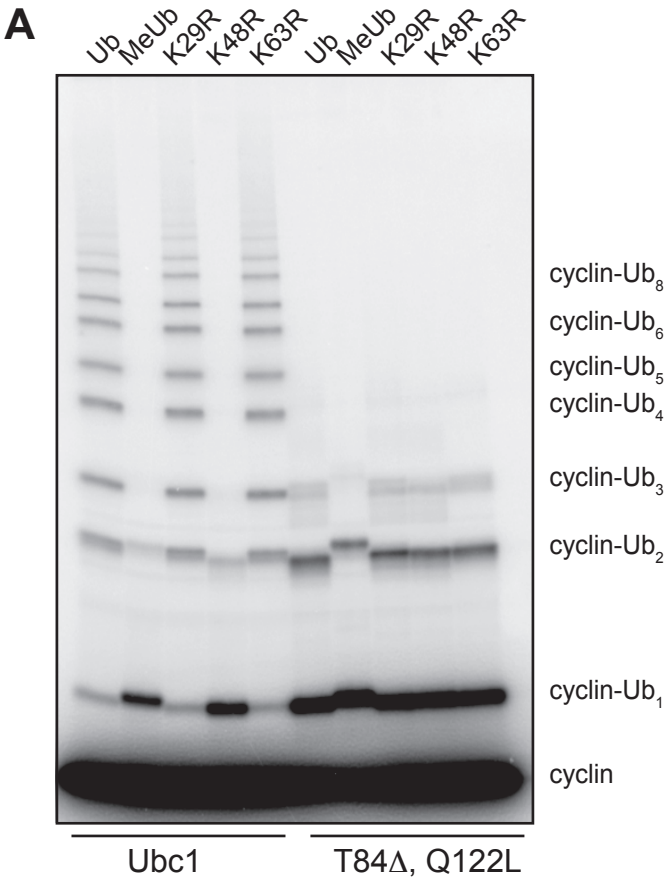

A
Ub
Ubc1-Q122L~Ub
(K48R)
diUb
Ub
Q122L
B
fraction diUbiquitin formed (sec -1), wt
fraction diUbiquitin formed (sec -1), Q122L
Ubc1
Ubc1-Q122L
[Ub] μM
C
time (min)
0 2 5 10 15 30 45 60
Ubc1~Ub
(K48R)
cyclin-Ub
Ub
Ubc1
Ubc1-Q122L~Ub
(K48R)
Q122L

Figure 8. Mutations at threonine 84 and glutamine 122 are not sufficient for K48-dependent polyubiquitination.

(A) Purified Ubc1 or Ubc1-T84D, Q122L was incubated with E1, ATP and the indicated ubiquitin species for 15 minutes. E1/E2 mix was added to an APC^{Cdh1} mix containing ^{125}I-cyclin B and incubated for 45 minutes at room temperature. Reactions were stopped by addition of sample buffer, analyzed by SDS-PAGE and visualized with a PhosphorImager.

(B) Purified Ubc4 or Ubc4-double cluster was assayed as in A.

Figure 8


A
Ub
MeUb
K29R
K48R
K63R
Ub
MeUb
K29R
K48R
K63R
cyclin-Ub8
cyclin-Ub6
cyclin-Ub5
cyclin-Ub4
cyclin-Ub3
cyclin-Ub2
cyclin-Ub1
cyclin
Ubc1
T84Δ, Q122L

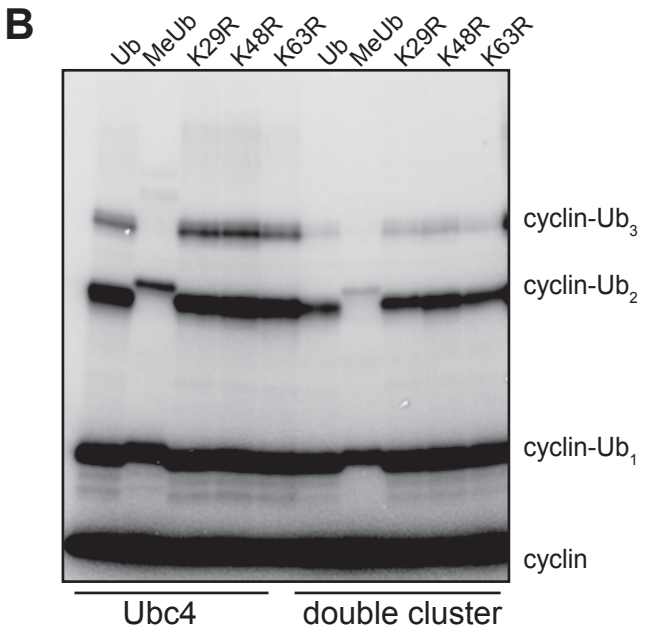

B
Ub
MeUb
K29R
K48R
K63R
Ub
MeUb
K29R
K48R
K63R
cyclin-Ub3
cyclin-Ub2
cyclin-Ub1
cyclin
Ubc4
double cluster

Figure 9. A linear fusion between ubiquitin and cyclin B behaves as a monoubiquitinated substrate.

Purified Ubc1 or Ubc4 was incubated with E1, ATP and ubiquitin for 15 minutes. The ability of ubiquitin-cyclin B fusion and K48R-ubiquitin-cyclin B fusion to be substrate was assayed. E1/E2 mix was added to mixes containing substrate alone (lanes 3, 6, 11 and 14), substrate and APC (lanes 4, 7, 12 and 15) or substrate, APC and Cdh1 (lanes 5, 8, 13 and 16). Reactions were allowed to react for 15 minutes and were stopped by addition of sample buffer, analyzed by SDS-PAGE and visualized by PhosphorImager.

Figure 9

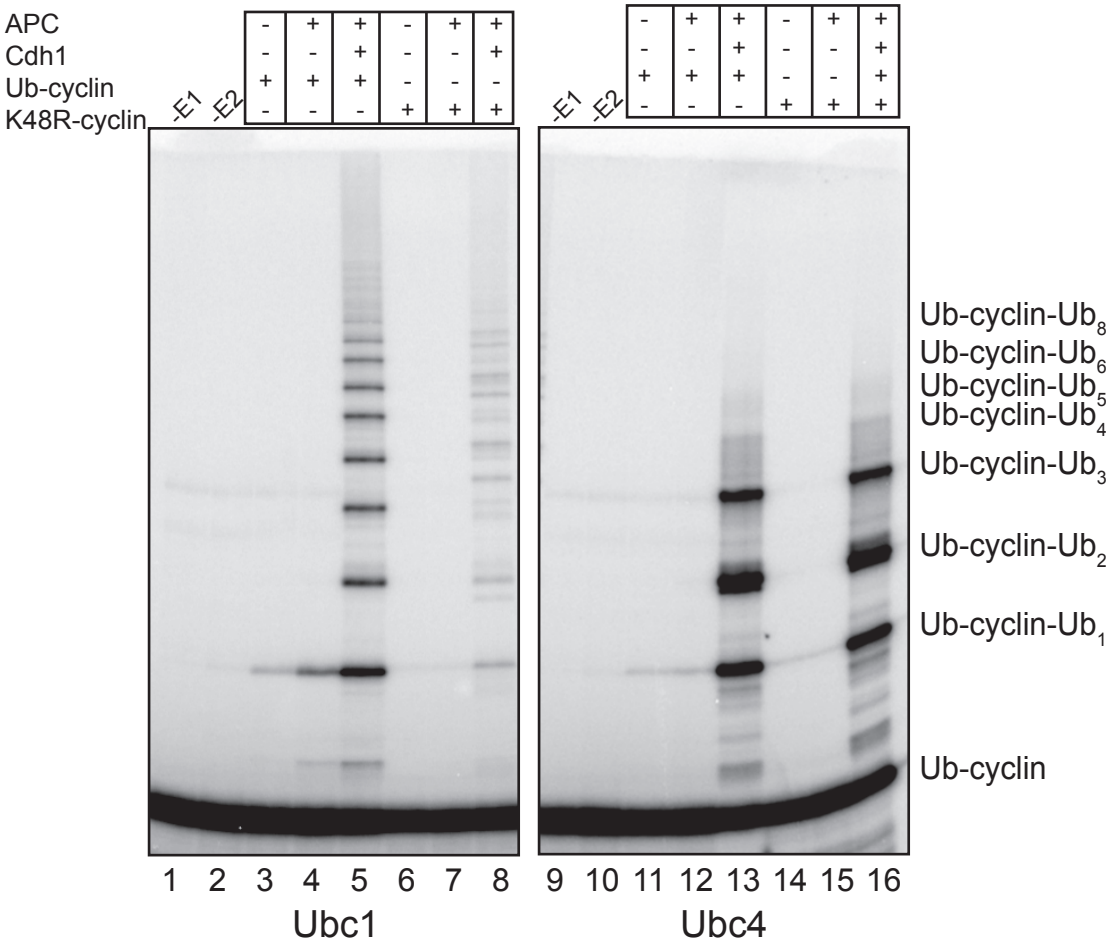
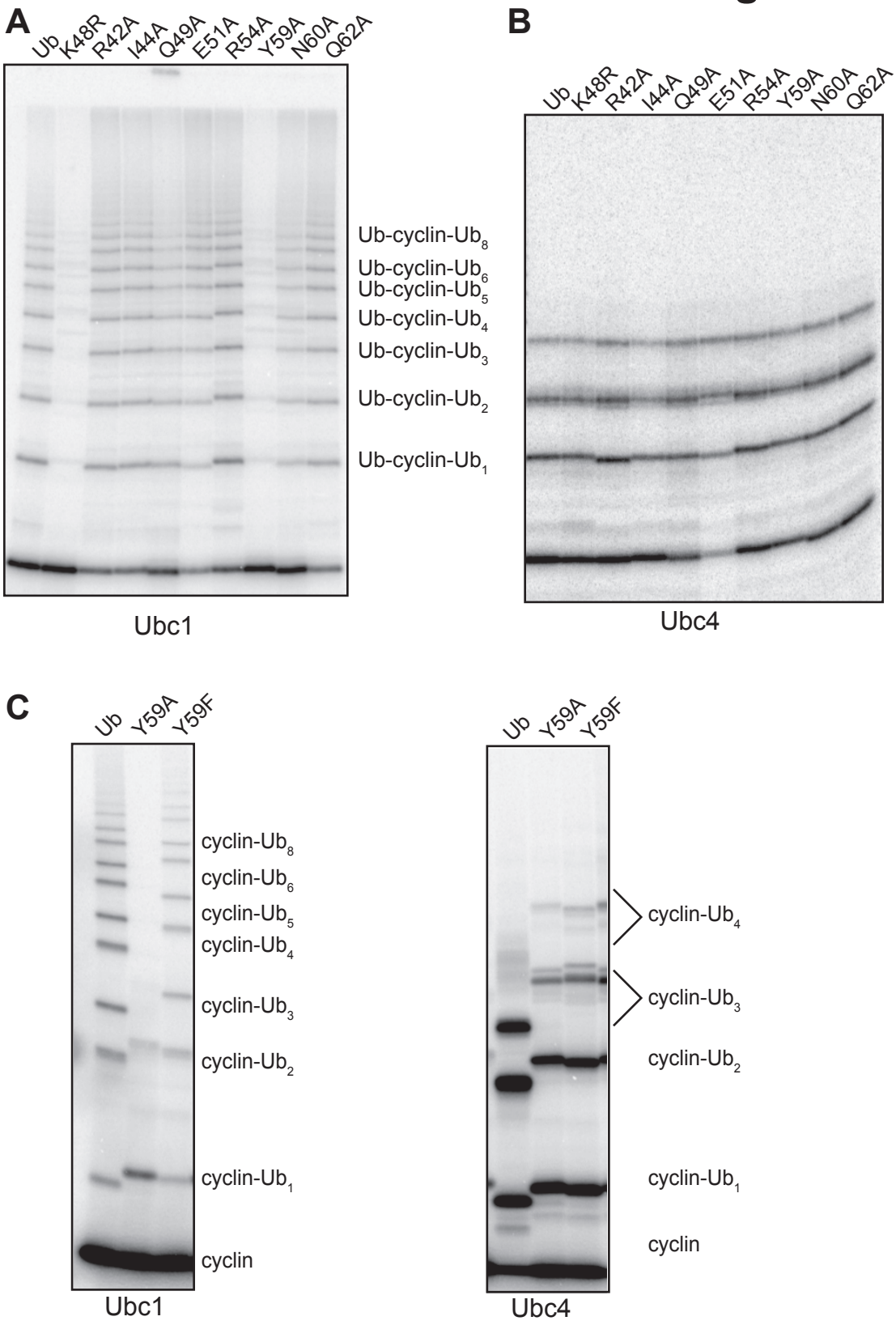

APC
Cdh1
Ub-cyclin
K48R-cyclin
-E1
-E2
Ub-cyclin-Ub8
Ub-cyclin-Ub6
Ub-cyclin-Ub5
Ub-cyclin-Ub4
Ub-cyclin-Ub3
Ub-cyclin-Ub2
Ub-cyclin-Ub1
Ub-cyclin
1 2 3 4 5 6 7 8
Ubc1
9 10 11 12 13 14 15 16
Ubc4

Figure 10. Tyrosine 59 of ubiquitin is critical for K48-dependent polyubiquitination

Purified Ubc1 (A) or purified Ubc4 (B) was incubated with E1, ATP and ubiquitin for 15 minutes. E1/E2 mix was added to APCCdh1 mix containing the indicated 32P-labeled ubiquitin-cyclin b fusions. The reactions were stopped after 15 minutes by addition of sample buffer, analyzed by SDS-PAGE and visualized with a PhosphorImager.

(C). Purified Ubc1 or Ubc4 was incubated with E1, ATP and the indicated ubiquitin species for 15 minutes. E1/E2 mix was added to APCCdh1 containing125I-cyclin B and incubated for 45 minutes at room temperature. The reactions were stopped by addition of sample buffer, analyzed by SDS-PAGE and visualized with a PhosphorImager.

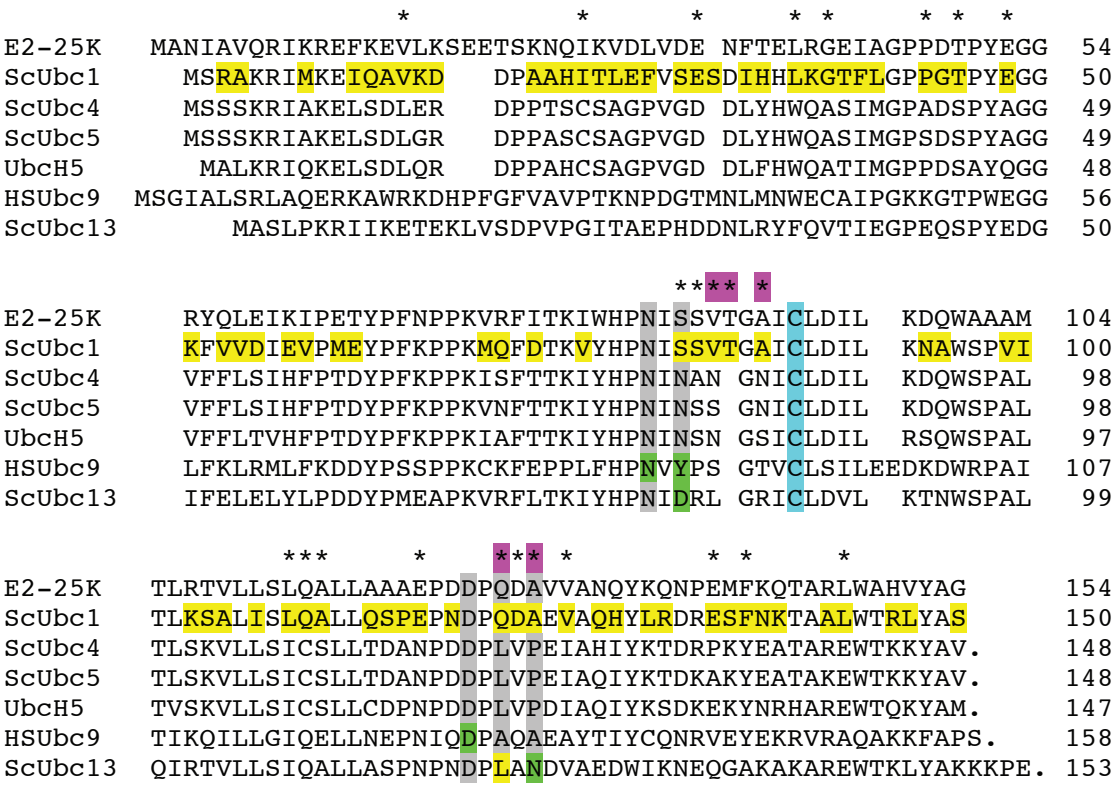
Figure 10


A
Ub
K48R
R42A
I44A
Q49A
E51A
R54A
Y59A
N60A
Q62A
Ub-cyclin-Ub8
Ub-cyclin-Ub6
Ub-cyclin-Ub5
Ub-cyclin-Ub4
Ub-cyclin-Ub3
Ub-cyclin-Ub2
Ub-cyclin-Ub1
Ubc1
B
Ub
K48R
R42A
I44A
Q49A
E51A
R54A
Y59A
N60A
Q62A
Ubc4
C
Ub
Y59A
Y59F
cyclin-Ub8
cyclin-Ub6
cyclin-Ub5
cyclin-Ub4
cyclin-Ub3
cyclin-Ub2
cyclin-Ub1
cyclin
Ubc1
Ub
Y59A
Y59F
cyclin-Ub4
cyclin-Ub3
cyclin-Ub2
cyclin-Ub1
cyclin
Ubc4

Supplemental Figure 1. Alignment of different E2 enzymes

E2-25K and *Saccharomyces cerevisiae* (sc) Ubc1 are E2s known to build K48-linked chains. ScUbc4, ScUbc5 and UbcH5 have not been observed to have linkage specificity. *Homo sapiens* (HS) Ubc9 is the E2 for the SUMO pathway. It is present here to show the residues important for lysine ordering and suppression of its pK. ScUbc13 builds K63-linked chains and residues important for this activity are shown.

Figure S1



1. Blue indicates catalytic cysteine.
2. Yellow indicates residues in Ubc1 that are different from Ubc4.
3. Asterisks indicate Ubc1 residues that are different from Ubc4 but the same in E2-25K.
4. Magenta asterisks indicate Ubc1/E2-25K-specific side chains exposed to solvent near catalytic cysteine.
5. First gray 'N' is asparagine that helps catalyze Ub transfer (Wu et al. [2003] EMBO J. 22:5241).
6. Green indicates three residues in human Ubc9 (N85, Y87, D127) that help catalyze SUMO transfer by reducing pK of lysine (Yunus and Lima [2006] NSMB 13:491).
7. Green indicates N123 in yeast Ubc13, which contributes to orientation of Lys63 in crystal structure (Eddins et al. [2006] NSMB 13:915). Green also highlights D81 in Ubc13, which is analogous to Y87 of Ubc9 and whose mutation in Ubc13 greatly reduces activity (VanDemark et al. [2001] Cell 105:711)
8. Yellow 'L' in Ubc13 is site of important hydrophobic side chain that Yunus and Lima 2006 suggest compensates for lack of Y at position 87: in their Fig 5a, Y at 87 tends to be correlated with A at 129 (as in HsUbc9), while D (or N) at 87 tends to be correlated with an L at 129 (as in ScUbc13): i.e. the L serves as the hydrophobic platform when the Y is absent. Ubc1 is an unusual E2 because it has an S at the 87 position and a Q at 129. See also Gazdoiu et al. [2007] MCB 27:7041 for overview of Cdc34 mutagenesis at some of these sites

Chapter 4

Conclusions

Ubiquitination is used as a signaling mechanism to control a variety of cellular processes. The nature of the ubiquitination signal, whether it is monoubiquitination, multi-monoubiquitination or polyubiquitination, ultimately determines the fate of the protein (Hicke, 2001; Li and Ye, 2008). The knowledge that we have about the enzymes involved in the ubiquitination cascade is quite detailed, in direct contrast to what we understand about the mechanism of ubiquitination itself. The ubiquitination field has come to a bifurcation in the road to discovery. We can pour our energy into finding new substrates for ligases and even novel ligases, or we can try to study mechanistically the ubiquitination process. Recent advances in mass spectroscopy techniques have greatly advance the discovery of novel substrates, but many of them still need to be validated (Peng and Cheng, 2005). I believe that at this point we have enough information to move into a more mechanistic realm and that the most exciting era of the ubiquitination field is yet to come. Only once we have mechanistic understanding of ubiquitination, down to the atomic level, we will be able to really appreciate the work that these enzymes carry out.

This dissertation deals with a very complex E3 ligase, the APC, and its E2 enzymes. During the course of these studies Ubc1 was found to be an APC-dependent E2 enzyme. Moreover, a novel sequential mechanism of action by complementing E2 enzymes is proposed. In this model, APC-Ubc4 is able to rapidly ubiquitinated substrate's lysines. The priming ubiquitination is followed by

rapid polyubiquitination carried out by APC-Ubc1 (Rodrigo-Brenni and Morgan, 2007). I will expand on this model and its implications for protein ubiquitination.

Sequential action of E2 enzymes: the best of both worlds

The attachment of ubiquitin chains to substrates requires two activities: ubiquitination of a substrate lysine and ubiquitination of a specific lysine on ubiquitin itself. Although chemically speaking these reactions should be the same, in reality there seem to be specific activities associated with each. In the present study I found that APC is able to interact with two E2 enzymes with complementary activities. Ubc4 reactions primarily result in rapid ubiquitination of substrate's lysines (Carroll and Morgan, 2002; Rodrigo-Brenni and Morgan, 2007). Ubc1 reactions, on the other hand, are very slow in this step. Instead, Ubc1 prefers an already ubiquitinated substrate and builds a K48-linked chain onto the first ubiquitin (Rodrigo-Brenni and Morgan, 2007). How did this mode of action come to be? What advantages does it have over a single E2 enzyme carrying out both steps?

Ubc4, and UbcH5 in humans, are used extensively *in vitro*. Unfortunately these enzymes have little linkage specificity. In fact they have been observed to form every kind of linkage possible (Brzovic and Klevit, 2006). In light of the exquisite level of regulation that different types of linkages encode, how can we reconcile this fact with the activity seen for the biggest family of E2s? One possibility is that the polyubiquitination seen with Ubc4 is mostly multiubiquitination with very short ubiquitin chains. This appears to be the case

in the APC field, where both UbcH5 and Ubc4 have been shown to mostly multiubiquitinate substrates. The fact that short little chains are seen can reflect the unrestricted reactivity of the Ubc4 active site. I would argue that Ubc4 family members are able to ubiquitinated non-ubiquitin lysines most efficiently. This is due to the presence of unstructured regions in substrates, the presence of multiple lysines on such regions, and the presence of residues near the catalytic cysteine that might aid in suppressing the normally high pK of the attacking lysine. Polyubiquitination, on the other hand, would only occur under conditions of saturating monoubiquitinated substrates. It would be impossible to approach this level of monoubiquitinated substrates inside the cell. How then can a chain be built?

In the case of APC, having two E2s with different activities work together solves this problem. The combined action of Ubc4 and Ubc1 leads to efficient polyubiquitination of APC targets *in vitro* and efficient degradation *in vivo*. Yeast cells are able to survive with Ubc1 as the sole APC-E2, but the metaphase-to-anaphase transition is much longer and APC substrates are degraded slowly. Although the population of cells survives, it would be interesting to see if they have any defects in chromosomal segregation at the single-cell level. Interestingly, in the absence of Ubc1, Ubc4 is sufficient to carry out ubiquitination and degradation of yeast securin, but not yeast cyclin B (Rodrigo-Brenni and Morgan, 2007). We do not understand this difference at this point, but some avenues to explore are differences in affinities between the two substrates,

differences in ubiquitination pattern, and whether yeast securin is more efficiently recognized by the proteasome, regardless of its ubiquitination signal.

Ubc1 is an interesting E2 enzyme because, besides the catalytic core common to all E2s, it contains a ubiquitin-binding domain (UBA) tethered to its C-terminus by a long flexible linker (Hamilton et al., 2001). UBA domains in isolation have been shown to bind ubiquitin and various diubiquitin species in solution. In fact, the UBA domain of Ubc1 was shown to bind K63-linked diubiquitin the best (Raasi et al., 2005). It has been proposed that a UBA domain can have two seemingly opposite roles: binding of ubiquitin to the UBA domain would inhibit further chain elongation by the E2 catalytic domain: alternatively, because the UBA domain binds polyubiquitin chains much better than monomeric ubiquitin, it could aid in the transfer of polyubiquitinated substrates to the proteasome. We have data to support a role for the UBA domain in E3-dependent ubiquitination. Ubc1-ΔUBA is able to carry out polyubiquitination of APC substrates, albeit the products are not as long. Chain linkage is not affected in this mutant, but the amount of protein needed to achieve half maximal activity with the APC is 10 fold higher than with wild-type Ubc1. One scenario is that the UBA domain interacts with the APC core and allows more time for the E3-E2 interaction. This increase in interaction time might be needed to efficiently build long polyubiquitin chains because the most distant ubiquitin must find its way back to the E2 active site. Another possibility is that the UBA domain is involved in ubiquitin binding. I tested the ability of Ubc1-ΔUBA to catalyze diubiquitin formation and I saw wild-type apparent affinities, indicating that the core contains

a ubiquitin binding site. This though does not refute that possibility that the UBA is involved in binding species with more ubiquitins. A role for the UBA could be to bind diubiquitin on the long ubiquitin chains and bringing back the most distant ubiquitin towards the E2 active site. More studies are needed to address this possibility.

Another aspect that distinguishes Ubc1 from Ubc4 is its preference for a pre-ubiquitinated substrate. This is due to its interaction with ubiquitin, even in the absence of the APC. The known ubiquitin-binding domain in Ubc1, its UBA, is not involved in this interaction and thus far I have not been able to disrupt it. One possibility is that the interaction is mediated by a number of low affinity sites, such that mutating single amino acids on Ubc1 would not disturbed it. In this case, multiple mutations might be needed. NMR has been used in the past to probe protein-protein interactions and I believe it is the best-suited technique to use due to the transient nature of the E2-ubiquitin interaction. Previous studies did not find an interaction between the catalytic core of Ubc1 and saturating amounts of ubiquitin. These studies were done with an uncharged Ubc1, which normally does not have to bind ubiquitin. It is possible that small conformational changes upon ubiquitin conjugation can open a ubiquitin-interaction site. One way to determine if conjugation is a prerequisite is to use a charged species in the NMR studies. Unfortunately, the thioester-linked ubiquitin is short-lived, and it will react with the attacking ubiquitin, making it their interaction fleeting and hard to measure. Instead, mutating the catalytic cysteine to a serine will allow for the formation of an oxyester, which is longer-lived and resistant to attack by

lysines. This will allow the interaction between Ubc1 and ubiquitin to be characterized using *in vivo* relevant complexes.

While looking for residues important in K48-linked polyubiquitin chain formation I expected to find mutations that disrupted ubiquitin binding. This was not the case. Instead I found mutations that gave us insight into Ubc1's unique activities: K48-linked ubiquitination with reduced substrate's lysines ubiquitination. I looked for mutations near the catalytic cysteine that could affect chain extension on APC targets. I found such a mutation; threonine 84 on Ubc1 was specifically defective in extending chains. Interestingly mutating T84 did not result in more substrate ubiquitination, even though there are more lysines available in the substrate. If T84 were involved in ubiquitin binding, then I would have expected to see a loss of chains with an increase in monoubiquitination. The fact that we see the same amount of substrate turnover indicates that mutations in T84 do not affect binding of ubiquitin, but rather catalysis of diubiquitin formation. This is interesting because catalysis of substrate-ubiquitin was not affected. The difference between the two reactions is the lysine itself. Diubiquitin formation relies on K48 of ubiquitin, whereas substrate-ubiquitin formation relies on a non-K48 lysine. Why is T84 important for K48 ubiquitination and not for substrate ubiquitination? Unfortunately I do not know at this time. Preliminary pH dependency studies do not show a difference in the pK of the attacking lysine when T84 is mutated. This leads me to believe that T84 is involved in ordering K48 on the active site, or positioning another residue in Ubc1 involved in this ordering. This activity is near impossible to decipher

biochemically and a structure of charged Ubc1 with ubiquitin poised to attack the thioester might be the only way to really understand catalysis.

The other mutation I found gave us a very interesting defect. Glutamate 122 was mutated to a leucine since that is residue found in Ubc4. When this mutant is used in APC reactions, it leads to an increase in multiubiquitination. This mutant still catalyzes the formation of K48-linked chains, but each substrate has more than one chain built on it. Again, this mutant did not affect ubiquitin binding, but rather increase the rate of substrate ubiquitination, even in the absence of APC. This is remarkable since Ubc1 does not interact with APC substrates. I believe this mutation makes lysines on substrates more reactive, possibly by suppressing their pK. Ubc1-Q122L is a gain-of-function mutation because mutating Q122 to any other residue resulted in loss of highly ubiquitinated products. At this point the idea that Q122L is involved in suppressing the pK of substrate's lysine is just that, an idea. Unfortunately pH dependency studies require large amounts of concentrated proteins and I do not have them. One possibility is to use a lysine mimic, hydroxylamine, and monitor the loss of the E2-ubiquitin conjugate. This could tell us whether the active site of Ubc1-Q122L is generally more accessible and reactive. Again, structural studies might be the only way to really address the role of Q122 in catalysis.

What is the role of APC in polyubiquitination? Unfortunately yeast APC is not easily produced in large quantities. The closest to a concentrated APC is to immunoprecipitate it using magnetic beads and assay its activity while bound to the beads. Although I tried to use immunoprecipitated APC in the diubiquitin

assay, the results were not very convincing. Every time I saw about a 3-fold increase in diubiquitin formation, but it saturated only after about 50% of the immunoprecipitated material was used. Although this might be a true result, it is hard to believe that I have some much APC that the reaction is saturated. Also, 3-fold stimulation over diubiquitin formation in the absence of APC seems very low if we compare it to the SCF system where 40-fold stimulation was seen. There are a couple of possibilities and lines of experiments that might be able to bring light to this issue. First the stimulation has to be done with known amounts of the APC and really see what its effects are. Second, Ubc1 has a higher level of E3-independent activity than Cdc34 and the low stimulation seen could reflect the level of stimulation that is needed *in vivo*. Third, Ubc1 has very low activity towards APC substrates in the absence of the APC. APC stimulation might be more important for these substrates rather than ubiquitin. Initial velocity measurements in the presence of saturating substrate with and without APC would greatly aid our understanding of ligase stimulation. One roadblock is our inability to make substrates in large quantities, but this can be overcome. We already have hints that part of Ubc1's specificity comes from its inability to react with substrate's lysines (the Q122L mutant uncovered this). The fact that Ubc1 works at all with the APC substrates could be due to a substrate-specific stimulation.

The APC uses two E2 enzymes extremely well suited to accomplish its goal: rapid polyubiquitination via K48 of ubiquitin. APC-Ubc4 carries out monoubiquitination extremely fast and processively: multiple ubiquitins are added

in one round of substrate interaction. Since multiple E2s are needed for this processivity, Ubc1 can come in and build a ubiquitin chain on the substrate. The UBA domain seems to allow longs chains to be built. Long chains made very rapidly will increase the likelihood that the substrate will be degraded by the proteasome instead of being deubiquitinated by DUBs. There does not seem to be a trade off when using two different E2 enzymes since the E2 needs to disengage from the E3 to bring another ubiquitin. The APC has come up with a clever solution: because the E2 needs to be exchange, why not exchange it for an E2 better suited for the reaction at hand? At this point I do not know whether both E2s bind the APC on the same site. I do know that when both enzymes are present, the lower products characteristic of APC-Ubc4 reactions are reduced and less overall substrate is converted to a ubiquitinated species. This indicates that either both enzymes interact with the APC on the same site, or they bind at different sites but the APC can only stimulate one E2 at a time. This last possibility is quite enticing because it could mean that both Ubc1 and Ubc4 are bound to the APC at the same time and as soon as Ubc4 puts the first ubiquitin, Ubc1 can put the second. Also, since Ubc1 has E3-independent activity, it might not need to be stimulated by the APC but rather it uses the APC to recruit its substrates. Many more experiments will be needed to study this possibility.

In conclusion, this dissertation and the work published from it have increased the knowledge of both the APC and the ubiquitination field. Much is left to do, but the tools, expertise and drive are there to push both fields forward. I am looking forward to the next chapter in APC enzymology.

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