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Regulation of the Anaphase-Promoting Complex in Late Mitosis

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Regulation of the Anaphase-Promoting Complex in Late Mitosis

by

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Till Johan, utan någon konkurrens det bästa resultatet under mina år som doktorand

To Johan, without any competition the best result during my years in graduate school

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Regulation of the Anaphase-Promoting Complex in Late Mitosis

By

Maria Enquist-Newman

Abstract

Polyubiquitination is an important mechanism by which proteins in all cellular processes are targeted for destruction. To understand this process we wanted to identify proteins in budding yeast that perform it, using an *in vitro* system to follow activities that add ubiquitin chains to a substrate that had already been modified by monoubiquitination. We detected a strong polyubiquitinating activity in yeast lysates. Initially we attempted to fractionate the lysates, but ultimately we identified the protein responsible for this strong polyubiquitinating activity using a candidate approach. It is the HECT-domain containing protein-ubiquitin ligase Tom1 that causes the large modification to the substrate in our *in vitro* assays. Tom1 appears to be a general polyubiquitinating activity in the cell, and as it is conserved all the way up to humans, it may be a general activity in all eukaryotic systems.

The Anaphase-Promoting Complex (APC) is a protein-ubiquitin ligase that targets cell cycle proteins for destruction. It is a tightly regulated enzyme which needs to be associated with one of two activating subunits (Cdc20 or Cdh1) to be active. The ability of Cdh1 to bind and therefore activate the APC is regulated through phosphorylation by Cyclin-Dependent Kinase (CDK). An additional level

of regulation is through binding of the protein inhibitor Acm1. Our studies show that on a molecular level, Acm1 is a specific inhibitor of APC^{Cdh1}, and has no affect on the activity of APC^{Cdc20}. Further, we showed that the inhibitory activity is dependent on a central region of Acm1 that contains motifs similar to the recognition motifs in APC-substrates. We also showed that Acm1 binds very tightly to Cdh1 independently of the inhibition region, with a K_d likely in the nano-molar range. Next we addressed the regulation of Acm1. The protein levels fluctuate through the cell cycle, and we showed that the destruction of Acm1 is mediated by APC^{Cdc20}. Moreover, we showed that Acm1 is phosphorylated by CDK and that this phosphorylation affects the localization and stability of Acm1.

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Chapter 1

Introduction

General overview

The process of cell division is critical for the survival and evolution of all living organisms. From single-celled organisms, where cell division results in a new organism, to humans, where a single fertilized egg divides to make the whole human body consisting of 100 trillion cells, cell division has to occur accurately every time. In the case of a single-celled organism, a mistake in DNA segregation may lead to death or a major growth disadvantage. In a multicellular organism, mistakes in DNA segregation can give rise to cancer or developmental defects.

To ensure the fidelity of cell division, the processes leading up to the creation of two cells are tightly controlled. The most fundamental regulator of the cell cycle is the Cyclin-Dependent Kinase (CDK) and its associated cyclin, which is required for CDK activity (Morgan, 1997). Oscillations in the levels of the cyclin proteins, and thus the activity of CDK, control the molecular and chemical landscape inside the cell, allowing different processes to occur at distinct times. For example, it is critical that chromosome replication occurs before segregation, or massive missegregation of the DNA would take place.

Another key player in cell cycle regulation is the Anaphase-Promoting Complex (APC). The APC is a ubiquitin-protein ligase that promotes polyubiquitination of key cell cycle components and thereby targets them for destruction (Peters, 2006). The removal of components at one stage of the cell

cycle allows for entry into the next stage, and also ensures that the cycle will not go backwards.

The Anaphase-Promoting Complex (APC)

The APC is a large, multisubunit ubiquitin-protein ligase that is present in all eukaryotes. The APC has two major functions: by targeting the protein securin for degradation in metaphase, it promotes the onset of anaphase (hence the name), and it targets the mitotic cyclins for degradation, allowing the exit from mitosis and entry into the next cell cycle (Shirayama *et al.*, 1999) (Thornton and Toczyski, 2003).

Though most of them are essential, very little is known about most of the APC subunits (Peters, 2006; Thornton and Toczyski, 2006). To date it has 13 identified subunits, of which 9 are essential, and it also needs to be associated with one of two different mitotic activating subunits, Cdc20 and Cdh1 (there is a third activator, Ama1, present in meiosis). The catalytic core consists of Apc11, a RING protein, and Apc2, a Cullin-domain containing protein. There are 3 proteins containing tetratricopeptide repeats (TPR), Cdc16, Cdc23, and Cdc27, that are known to be phosphorylated and are believed to be where the activating subunits bind. Apc1 is the largest subunit and is believed to form a scaffold. Nothing is known about Apc4, Apc5, or Apc9. As for the non-essential subunits, Doc1 is the best understood. It has been shown to have a role in the ability of the APC to add multiple ubiquitins in a single binding event (processivity) and possibly in

substrate recognition. Mnd2 is known to act as an inhibitor of the APC specific activator Ama1. Cdc26 is induced upon heat shock, and might act as a chaperone, while Swm1 is important for spore wall maturation.

Regulation of the APC

The core APC is present throughout the cell cycle, but its activity is tightly regulated through association of the two different activating subunits, without which the APC is inactive. The activating subunits are called Cdc20 and Cdh1. Cdc20 is the activator that initially activates the APC in metaphase. Cdc20 transcription is regulated and peaks in G2/M (Spellman *et al.*, 1998), and its association with the APC is promoted by phosphorylation of the core APC by CDK in early mitosis (Shteinberg *et al.*, 1999) (Rudner and Murray, 2000).

Cdh1 -dependent APC activity rises later in anaphase and remains high through G1. Cdh1's association with the APC is regulated through CDK phosphorylation of Cdh1. When phosphorylated, Cdh1 cannot bind and activate the APC (Zachariae *et al.*, 1998) (Jaspersen *et al.*, 1999). As CDK activity remains high through metaphase, Cdh1 is phosphorylated and therefore inactive. When the cell enters anaphase and CDK levels have been lowered by Cdc20-dependent cyclin destruction, the phosphatase Cdc14 is activated and Cdh1 is dephosphorylated. Cdh1 then activates the APC and targets the rest of the mitotic cyclins for destruction. The Cdh1-APC activity remains high through G1 and does not drop until APC-resistant G1 cyclins accumulate and phosphorylate

Cdh1 in late G1, thereby tuning off the APC and allowing the mitotic cyclins to accumulate again.

APC inhibitors

A number of APC inhibitor proteins have been identified through the years. The best understood involves the spindle-assembly checkpoint. This checkpoint mechanism blocks Cdc20-APC activation until all chromosomes have attached to the mitotic spindle in a bipolar fashion. The checkpoint acts on Cdc20, which is kept from activating the APC by a group of proteins that together keeps the spindle-assembly checkpoint functional. The key player is Mad2, which interacts directly with Cdc20. Mad2 does not appear to interfere with the ability of Cdc20 to interact with the APC; instead, a model has been proposed where it may interfere with substrate release (Yu, 2006).

Another APC inhibitor is Emi1, which appears to inhibit both Cdh1- and Cdc20-activated APC in *X. laevis* egg extracts (Reimann *et al.*, 2001). However, the *D. melanogaster* orthologue RCA1 appears to inhibit only Cdh1-APC (Grosskortenhaus and Sprenger, 2002). Emi1 is targeted for destruction in early mitosis. Initial studies suggested that Emi1 binds directly to APC activators and prevents their association with substrates. However, since then it has been shown that Emi1 binds tightly to the core APC, and that it appears to act as a pseudosubstrate inhibitor that competes with substrates for D-box binding sites in the core APC (Miller *et al.*, 2006).

Additional APC inhibitors have been identified in the meiotic cell cycle. In budding yeast the APC subunit Mnd2 prevents the meiosis specific APC activator Ama1 from activating the APC until anaphase I onset (Oelschlaegel *et al.*, 2005). How it functions to inhibit the APC and how it gets inactivated in anaphase I are both unknown. In meiosis in fission yeast, an APC inhibitor named Mes1 has been shown to bind to the WD40 region of Cdc20, implying it may be a competitive inhibitor for substrate binding (Izawa *et al.*, 2005). A few other APC inhibitors have also been described, but little is known about the mechanism of inhibition of any of these inhibitors.

Substrate recognition

Besides the temporal difference in the Cdc20 and the Cdh1 activation of the APC, it also appears that the activators contribute different substrate specificity to the APC. The activators bind directly to the substrate and bring them to the APC. The activators contain a WD40 propeller motif that is thought to bind the substrates. The first recognition motif on an APC substrate that was identified was in Cyclin B, and the minimal motif was termed the destruction box (D-box), which consists of the sequence RxxLxxxxN/D/E (Glotzer *et al.*, 1991). D-box containing substrates appear to be recognized by both Cdc20- and Cdh1-activated APC. A second APC recognition motif was identified in human Cdc20, and this motif was termed the KEN-box, as the minimal motif here is KENxxxD/N (Pfleger and Kirschner, 2000). The KEN-box is believed to be recognized only by

Cdh1. Though these motifs are definitely necessary, they are not sufficient for a protein to be recognized by the APC. Even in the initial study with Cyclin B destruction, the D-box needed to be surrounded by 24 other residues, and even in that case, the protein had a half life more than 4-fold longer than that of a 79-residue fragment (King *et al.*, 1996). It is not known if this depends on size constraints and lysine availability or on additional determinants for APC recognition that cannot be identified in a primary sequence (which might depend on secondary or tertiary structures). In addition, numerous other motifs have been recognized in APC substrates, such as the A-box (Littlepage and Ruderman, 2002) and the O-box (Araki *et al.*, 2005). From this it is clear that the activator-substrate interaction is poorly defined, and that we are far from being able to identify new APC substrates by simply looking at the primary sequence of proteins.

The Ubiquitin Pathway

Ubiquitin is a small protein that can be added to other proteins as a post-translational modification. This addition occurs in a multistep process (Kerscher *et al.*, 2006) (Pickart and Eddins, 2004). First, ubiquitin is activated by a ubiquitin-activating enzyme (E1) that covalently links the C-terminus of ubiquitin to an active-site cysteine through a thioester linkage in a step requiring ATP. Next, the ubiquitin is transferred to an active-site cysteine in a ubiquitin-conjugating enzyme (E2). Lastly, the ubiquitin is transferred to a lysine residue in a substrate

protein in a step that is dependent on a ubiquitin-protein ligase (E3). This occurs either by the E3 simultaneously binding to the charged E2 and the substrate, and the ubiquitin getting transferred straight from the E2, as is the case with RING-domain E3s, or, as is the case with HECT-domain E3s, the ubiquitin is first transferred to an active-site cysteine in the E3 before being transferred to the substrate protein.

Polyubiquitination

In the case of some protein substrates, a single ubiquitin is all that is transferred, and this can act as a post-translational modification similar to phosphorylation or methylation. However, in many cases the substrate is modified with mulitple ubiquitins (Hochstrasser, 2006) (Pickart and Fushman, 2004). This can occur either by the addition of single ubiquitins to multiple lysine residue in the target protein, or by the addition of ubiquitin to a lysine residue in an already attached ubiquitin. When the latter happens repeatedly, this gives rise to ubiquitin chains, also referred to as polyubiquitination. When polyubiquitinating, an E3 tends to have a preference for a specific lysine in the attached ubiquitin. The resulting chains are named on the basis of this specifictiy (e.g. K48 chains refer to chains in which ubiquitins are added to lysine 48 in the previous ubiquitin molecule; K63 chains are those in which the linkage occurs through lysine 63). K48 chains that consist of 4 ubiquitins or more are recognized by the proteasome, and the target protein will be degraded promptly (Thrower *et*

al., 2000). K63 chains are not believed to be recognized by the proteasome, but rather appear to have a role in signaling (Chan and Hill, 2001).

The APC and polyubiquitination

The APC adds multiple ubiquitins to its substrates, and does so in a processive manner (Carroll and Morgan, 2002). That is, it adds multiple ubiquitins to the target protein in a single binding event. When associated with the E2 enzyme Ubc4, this occurs primarily by addition of ubiquitins to separate lysines within the substrate protein. This in vitro result was confusing since APC substrates do get degraded in vivo, and therefore must get ubiquitin chains added. Light was recently shed upon this mystery when it was shown that the APC can work with a different E2 enzyme, Ubc1, and in this context the APC adds long K48-linked chains to its substrates (Rodrigo-Brenni and Morgan, 2007). It was also clear that Ubc1 and Ubc4 are working together in the cell to help target APC substrate for degradation.

Chapter 2

Identification of a polyubiquitinating activity in budding yeast

Introduction

Ubiquitin is a 76 amino acid protein that is added to substrate proteins as a posttranslational modification (Kerscher *et al.*, 2006) (Pickart and Eddins, 2004). Protein ubiquitination occurs in a series of steps, beginning with ubiquitin activation by a ubiquitin-activating enzyme, or E1, that uses the energy of ATP hydrolysis to form a thioester linkage between a cysteine residue in the E1 and the C-terminus of ubiquitin. The ubiquitin is then transferred to an E2, or ubiquitin-conjugating enzyme, with a similar thioesterbond. An E3, or ubiquitin-protein ligase, then transfers the ubiquitin from the E2 to the protein substrate. In the case of RING-domain ubiquitin ligases, the E3 is a scaffold that simultaneously binds to the E2-ubiquitin conjugate and the substrate and brings them into close proximity, allowing direct transfer of the ubiquitin from the E2 to the substrate. A second class of E3s is called HECT-domain ubiquitin ligases, and here the ubiquitin is transferred from the E2 to the E3 before being transferred to the substrate lysines.

Ubiquitins can be added either as single modifications, or alternatively multiple ubiquitins can be transferred to multiple lysines on a single substrate. In addition, ubiquitins can be added to a lysine residue on an already attached ubiquitin, forming polyubiquitin chains. The proteasome efficiently recognizes chains of 4 or more ubiquitins, and this leads to substrate degradation (Thrower *et al.*, 2000). Polyubiquitination is therefore a key regulatory process in the cell. It is also believed that the proteasome only recognizes chains where the ubiquitins

are linked to lysine 48 in the previous ubiquitin molecule (K48 chains). However, it is known that there are other chains formed *in vitro* and *in vivo* (e.g. K63 chains), but the function of of these chains is less clear (Chan and Hill, 2001). Overall, the process of poly-ubiquitination is a poorly understood yet critical process.

The Anaphase-Promoting Complex (APC) is a large multisubunit RING-domain ubiquitin ligase that ubiquitinates cell cycle components and thereby targets them for destruction by the proteasome. *In vitro*, yeast APC, with the yeast Ubc4 E2 enzyme, adds ubiquitins at multiple lysines in the target protein but has only a limited ability to catalyze polyubiquitination (Carroll and Morgan, 2002). This raises a very important question: if APC/Ubc4 cannot add chains to its substrates, how are these substrates recognized and destroyed by the proteasome? One possibility is that the APC used in the assays has lost an activity or a subunit during purification; however, we have no evidence for this. Another possibility is that a separate enzymatic activity steps in after the APC has “primed” its substrate with mono-ubiquitins. This type of separate “extending activity” further modifies the substrates by building with ubiquitin chains.

While generating novel substrates for our APC assays, we started *in vitro* transcribing and translating different yeast substrates using a reticulocyte lysate system in the presence of ^{35}S-Methionine, thereby radiolabeling the substrates. When doing this, we noticed an activity present in these lysates that had the ability to extend ubiquitin chains on the substrates after the APC had done the

initial multi-ubiquitination. Through further investigation we identified and characterized this activity in yeast lysates. The enzyme responsible for the extending activity in yeast lysates was identified to be Tom1, a HECT-domain ubiquitin-ligase. Though Tom1 has no obvious *in vivo* connection with the APC, the presence of a potent polyubiquitinating activity in reticulocyte lysates and in yeast raises the possibility that this conserved ubiquitin ligase is a general eukaryotic ubiquitin chain extender.

Results

APC-Ubc4 acts primarily to promote monoubiquitination at multiple target lysines

It had previously been observed in our lab that the APC adds multiple ubiquitins to a substrate in a single binding event (Carroll and Morgan, 2002). To investigate whether these ubiquitins are added to separate lysines or added as a chain (polyubiquitination), we took advantage of methylated ubiquitin. Methylated ubiquitin has had all its lysines blocked with methyl groups, and is therefore unable to support chain formation. In figure 2-1, APC-Ubc4 reactions were performed with either unmodified ubiquitin (wt) or with methylated ubiquitin (Me), using Pds1 as the substrate. If the APC normally acted by forming chains, one would expect a large difference in the kinetics of these reactions, since in the case of the methylated ubiquitin there are significantly fewer lysines available for the APC to modify. Instead, we observed that the kinetics are similar in the two cases, supporting the idea that the APC acts mainly through ubiquitination of multiple substreate lysines.

Reticulocyte lysates contain a ubiquitination activity that affects APC substrates

The possibility of a separate activity that extends ubiquitin chains on substrates looked very attractive after we observed APC reactions performed in

the presence of reticulocyte lysates. If the APC reaction was performed with unpurified substrate (in the presence of reticulocyte lysate), the substrate was modified in a very dramatic way, creating a smear of products in the entire lane, all the way up to the top of the gel (figure 2-2, lanes 1 and 2). However, if the substrate was first purified from the reticulocyte lysates by immunoprecipitation, the high smear was no longer observed, suggesting that the reticulocyte lysate contained an activity capable of extending ubiquitin chains on APC-modified substrates. In a pure system (our regular APC assays), addition of reticulocyte lysates also drove formation of the high smear (figure 2-3, lane 3).

To address whether these gel-retarding modifications on APC substrates were due to ubiquitination, initial APC reactions were performed either with unmodified or methylated ubiquitin. Reticulocyte lysates and additional E1/E2 mix (all with wt ubiquitin) were then added. Only in the case where the original reaction was performed with wt ubiquitin could the activity in the lysate significantly modify the substrate further, indicating that the modifications made by the lysate are mainly by addition to the ubiquitins originally put there by the APC (figure 2-3).

Extending activity in reticulocyte lysates appears to be independent of APC activity

We attempted to address if the activity present in reticulocyte lysates is from the APC or a separate enzymatic activity. Substrate was modified by the APC while kept bound to IgG beads, and then washed to remove any APC, after which it was cleaved off the beads. After the wash and cleavage, the modified substrate was split in 5 reactions to examine the high molecular weight products formed. The first sample was boiled in sample buffer to examine the APC-dependent modifications (lane 3, figure 2-4): no chain extensions were seen after the initial APC reaction. The second sample was put through a second APC reaction to see how the APC modified it further. Better depletion of unmodified substrate occurred (presumably free substrate is a better substrate for the APC than substrate bound to beads), but no higher products were observed (lane 4). To the third sample reticulocyte lysate was added and this led to a collapse of the modifications, due to de-ubiquitinating activities present in the reticulocyte lysate (lane 5). To the 4^{th} sample, reticulocyte lysate was added in addition to E1/E2 mix (containing E1, E2 (Ubc4), ATP, and ubiquitin). In this case, the high smear appeared on the gel (lane 6). To the last sample, APC was also added in addition to reticulocyte lysate and E1/E2 mix. No further modifications were observed in this case (lane 7). From these experiments we conclude that the APC is not necessary for the ubiquitin extending activity present in reticulocyte lysates.

Extending activity is dependent on E2 enzyme and ubiquitin

The extending activity in the reticulocyte lysates depends on the E1/E2 mix. To identify which components of this mix (E1, E2, ATP, ubiquitin) are necessary for the activity, we repeated the previous reactions but added only one component of the E1/E2 mix. Addition of the individual components of the E1/E2 mix (E1, E2, ATP, ubiquitin) alone could not support the extending activity (Figure 2-5 lanes 4-7). However, when E2 enzyme was added in combination with ubiquitin, this was sufficient to support the extending activity (lane 9). Adding ATP and E1 in addition to this did not promote any further activity (lanes 10 and 11). In conclusion, the extending activity present in reticulocyte lysates depends on additional supplied E2 enzyme and ubiquitin.

Reticulocyte lysates contain ubiquitination machinery, and therefore contain E1 enzyme, explaining why additional E1 is not necessary (Wilkinson *et al.*, 1980). Also, to support transcription and translation, reticulocyte lysates contain ATP. It is surprising that additional ubiquitin was needed for the extending activity. However, careful examination of the lane where only E2 enzyme was added reveals a slight upshift in the products formed (lane 12), so clearly there is some ubiquitin in the lysates.

Extending activity is present in yeast lysates

To address whether this extending activity could be a general activity of all eukaryotic systems, we looked for it in yeast lysates. We found that yeast lysates contain an extending activity similar to that found in reticulocyte lysates (figure 2-

6, “lysate”). However, since the activity at times was hard to follow in whole cell lysate due to opposing deubiquitinating activities, we decided to partially purify it using an anion exchange column (a Quarternary Amine column, HiTrap Q, from Pharmacia) to separate it from some of these other activities. The activity bound well and a scheme was optimized to purify the activity. This consisted of washing the bound activity with 300 mM NaCl and then eluting with a 300-600 mM gradient over 20 fractions. The activity reproducibly came off in a peak around fractions 13 and 14. Figure 2-6 shows an example of such elution from a Q column.

The extending activity in yeast lysates is APC-independent

To address whether the extending activity in yeast lysate is dependent on the APC, we took advantage of the APC bypass-suppressed strain from the Toczyski lab (Thornton and Toczyski, 2003). A lysate was made from an *apc11_ apc2_* strain, which contains no APC activity. This lysate was put over a Q column as described above, and the extending activity was present and behaved the same as in the lysate from wild-type yeast (data not shown). However, it was still possible that the activity was helping the APC already present in the reactions. To address the dependence on the APC, the following scheme was followed. As described previously, substrate was modified by purified APC-Ubc4 while still bound to beads, after which the APC and all reaction components were washed away before cleaving the substrate off the beads (lane 1 and 2, figure 2-

7). This modified substrate was then used in 5 sets of duplicate reactions: the first pair was put through a second APC reaction (lanes 3 and 4). To the following reactions (lanes 5-12), one of the peak fractions of extending activity from the *apc11_ apc2_* strain (fraction 12) was added. Addition of the fraction alone caused no change in the modification profile (lanes 11 and 12). However, if the E1/E2 mix was added in addition to the fraction, the extension of substrate ubiquitination chains was seen (lanes 9 and 10). Addition of Cdh1 (lanes 7 and 8) or Cdh1 + APC (lanes 5 and 6) did not add any additional activity to that seen when adding only E1/E2 mix. This result led to the conclusion that the extending activity detected in yeast lysates is completely independent of APC activity.

Fractionation

Our goal was to identify the protein responsible for the extending activity seen in yeast lysates. To do this, multiple approaches were available, including taking advantage of the power of yeast genetics and screening, following a candidate approach, or biochemical fractionation of the yeast lysates. We initially favored this last approach given its unbiased nature. We made lysates from wild-type yeast by bead beating and put these lysates through different separation techniques. The lysate, or the fractions thereof, were then added to an APC reaction that had been performed for 30 min. From the beginning, we understood that we would need multiple good purification steps to be able to reduce the

complexity of the yeast lysate to a level where the individual components could be identified.

The main approach planned for fractionation of the yeast lysate was affinity chromatography. As described above, the activity bound well to an anion exchange column, and this was a good first step in the fractionation scheme. Though this was a very good step to reduce the complexity of the yeast lysate, there were still hundreds of different proteins present in the peak fractions, and more purification steps were necessary. To further purify the activity, we investigated the binding properties of the activity to numerous different columns, including S, Heparin, 6 different dye ligands (Cibacron Blue 3GA, Reactive Blue 4, Reactive Brown 10, Reactive Green 19, Reactive Red 120, Reactive Yellow 86), 6 different hydrophobic columns (Phenyl FF (high sub), Phenyl FF (low sub), Phenyl HP, Butyl, Octyl), Ubiquitin agarose, and E2 coupled to affigel. However, for all of these strategies the activity was in the flow-through, and thus none of these could be used for further purification.

When salting out, the activity came out in one of the fractions with the highest overall protein concentration (between 30% and 50% ammonium sulfate, along with 40% of all proteins). Thus, this strategy was largely ineffective as well.

The extending activity migrates with a size between 1.5 and 8 MDa

Overall, the attempts to purify the activity were unsuccessful as it did not bind to any of the resins tested other than the Q. Another strategy is to separate based on size. In initial tests, the activity was not retarded by Sephacryl-100, 200, or 300 columns, implying that it has a size larger than 1.5 MDa. However, the activity was retarded on an S-400 column, which separates proteins up to 8 MDa. Figure 2-8 shows a typical elution from a large (200 ml) S-400 column, where the activity came off in fractions 45-48 reproducibly. This led us to conclude that the activity has a size of between 1.5 and 8 MDa.

The extending activity in yeast lysates depends on the Tom1 HECT-domain ubiquitin-protein ligase

Fractionation had been unsuccessful in identification of the extending activity present in yeast lysates. An alternative approach was to take a candidate approach. Budding yeast contains 39 candidate E3 or E3-related proteins. Our first approach was to do small scale preparations from a library of TAP-tagged strains, but no activity was detected in any of the pull-downs (data not shown).

Our second approach was to test the non-essential E3 ubiquitin-ligases by using the budding yeast deletion collection to look for a strain lacking the extending activity. 34 of the 39 candidates are non-essential. We prepared lysates from 20 of these 34 strains (14 were excluded since they appeared to be very unlikely candidates due to already assigned activities) and flowed them over a Q column as previously described. The fractions where the peak of the

extending activity comes off (12-15) were assayed for the presence of the activity.

In one of the deletion strains tested the extending activity was absent. This was in the strain deleted for *TOM1* (figure 2-9). All the fractions from the *tom1_* lysates were examined and no trace of the extending activity was detected. Tom1 is a HECT-domain E3 ligase, and explains why the initial candidate approach did not work: the TAP library has C-terminal tags and HECT-domain ligases are inactive when C-terminally tagged. Consistent with this, lysates prepared from Tom1-TAP cells were examined over a Q column as well, and no extending activity was detected (data not shown).

Tom1 shows no *in vivo* relevance in conjunction with the APC but rather appears to be a more general activity

Tom1 is a HECT-domain ubiquitin-ligase. It is conserved in all eukaryotes, from yeast to humans. The yeast protein is very large (the 4th largest protein in the yeast genome at 374 kDa). Although it has been given a name, very little is known about the protein. The published data suggest roles in transcriptional regulation and mRNA transport (Saleh *et al.*, 1998) (Duncan *et al.*, 2000). The name stands for Temperature dependent Organization in Mitotic nucleus (Utsugi *et al.*, 1999). The deletion is viable, but temperature-sensitive at 37°C. The protein has been shown to localize to the nucleolus.

After identifying Tom1 as the enzyme responsible for the extending activity in yeast lysates, we attempted to identify a connection with the APC *in vivo*. None of the attempts showed any connection to the APC or the cell cycle. Crosses were done to multiple APC mutants, cytological studies were done at permissive and non-permissive temperature to look for a cell-cycle phenotype, and the stability of APC substrates was assessed in the *tom1_* strain, but none of these assays revealed any phenotype (data not shown).

The lack of connection to the APC suggests a more general role for Tom1, where it might be a factor in the constant battle between ubiquitinating and de-ubiquitinating activities inside the cell.

Discussion

We have identified a novel APC-independent activity present in reticulocyte lysates that adds long ubiquitin chains to substrates pre-ubiquitinated by the APC *in vitro*. Further, we have demonstrated that a similar extending activity is present in yeast lysates. Attempts to fractionate the activity failed, but a candidate approach, looking in strains deleted for potential E3s in yeast, identified the HECT-domain ubiquitin ligase Tom1 as the enzyme responsible for the extending activity in yeast lysates.

Analysis of the complex size associated with the activity revealed a size of between 1.5 and 8 MDa, while Tom1 has a predicted size of only 374 kDa. This discrepancy may be explained in a number of ways. Tom1 may associate with a complex of proteins (e.g. the proteasome) or multimerize. Because gel filtration actually measures Stoke's radius and not molecular weight, an oddly shaped molecule could also explain the behavior of Tom1.

Whether the activity seen in reticulocyte lysates is due to a homologue of Tom1 is unknown. There is a rabbit homologue of Tom1, and we have shown that the activity present in reticulocyte lysates is sensitive to treatment by N-Ethylmaleimide (NEM, see figure A1-2), indicating the activity is likely due to a HECT-domain ubiquitin ligase (NEM blocks active-site cysteines). Either way, it is clear that there is a similar activity present in both yeast and in rabbits, supporting the idea that this could be a general eukaryotic activity.

Future work with Tom1 will address the role of Tom1 in the cell. Identifying Tom1-associated proteins would be a first step in this direction. If one can get pass the hurdle of purification (both N- and C-terminal tags are non-functional), some very interesting *in vitro* studies could be done. This includes what a Tom1 substrate actually consists of: is mono-ubiquitination of any protein sufficient for recognition? Can any ubiquitin chain be recognized? Is there chain specificity, both in the case of the substrate recognition and in the case of the Tom1 products?

The actual function of Tom1 *in vivo* is unknown. Our data suggests that it is not specific to ubiquitination of APC substrates as we did not see an obvious defect in APC substrate degradation *in vivo*. However, we cannot exclude that Tom1 contributes to APC substrate degradation under certain conditions. Alternatively, Tom1-mediated ubiquitin chain building could be a general activity in the cell that is part of the constant balance between ubiquitin ligases and deubiquitinases. If it does turn out to be associated with the proteasome, which some data have suggested, one could imagine it having a role in quality control, where it might help ensure that proteins tagged for destruction are recognized by the proteasome. For example, there may be cases in which a protein is targeted for destruction by a ubiquitin ligase, but there are not enough ubiquitins added for a tight association with the proteasome. This could lead to this protein continuing to perform its cellular role when this is not longer desired. By having an activity such as Tom1, the destruction of this substrate in a timely manner would be ensured.

Materials and Methods

APC and extension assays

APC reactions were performed as described (Carroll and Morgan, 2005). Briefly, in a typical APC reaction, the E1/E2 mix is first mixed (0.5 _l E1 (purified from yeast), 0.5 _l E2 enzyme (yeast Ubc4 purified from bacteria), 1.5 _l ATP (10 mM stock, Sigma), 1.5 _l ubiquitin (1 mM stock, Boston Biochem)) and incubated for 15 min at room temperature. To this is then added 0.2-1 _l APC (purified using TAP-tag from yeast, as described in (Carroll and Morgan, 2005)), 0.5-1 _l Cdh1 (purified from SF9 cells), 2 _l Pds1 (^{35}S-labeled, purified from reticulocyte lysates), and lastly QAH buffer (20 mM Hepes pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$) up to a total of 15 _l. Reactions were then incubated for 30-60 min at room temperature (or as indicated). If lysates were to be added to the reactions, 1 _M ubiquitin aldehyde was also added to the reaction (to inhibit de-ubiquitinases). When doing the extending assays, a normal APC reaction was performed for 30 min, then lysate was added (usually 2-5 _l to a 15 _l APC reaction) and incubated for an additional 30 min at room temperature.

Substrate purification

Pds1, with a C-terminal TEV protease cleavage site followed by a ZZ-tag, was *in vitro* transcribed and translated (TnT quick coupled transcription/translation system from Promega). Protein was then bound to IgG-

beads; sepharose beads in the case of regular preps (GE Healthcare), and magnetic beads in the case where the substrate was used in APC assays while still bound to the beads (rabbit IgG from Sigma coupled to epoxy beads from Dynal). Binding reactions were done at 4°C for 1-2 hrs (50 _l TnT reaction + 25 _l beads, TAP-core buffer (20 mM Hepes pH 8.0, 150 mM NaCl, 0.1 % NP-40) was added to a total volume of 500 _l). After binding, 3-5 washes with 1 ml TAP-core buffer were performed. Beads were then resuspended in 50 _l TAP-core + 1 mM DTT + 1 _l TEV protease, and left for 2 hours at 16°C. After cleavage, glycerol was added to a final concentration of 20%, and the substrate was snap frozen in liquid nitrogen.

Fractionation

Yeast was lysed into Buffer A (10 mM Tris 7.5, 0.5 mM DTT) + protease inhibitors using bead beating. A high speed spin (1 hr, 50k rpm in ultra centrifuge) and filtration using a 20 _m syringe filter followed. Columns were connected to an FPLC, and used as per manufacturer's instructions. High salt buffer for elutions (Buffer B) consisted of Buffer A + 1 M NaCl.

Figure 1. The APC (with the Ubc4 E2 enzyme) acts mainly through monoubiquitination of multiple substrate lysines

APC assays were performed with either unmodified (wt) or methylated (me) ubiquitin, over a time course of 60 minutes. Substrate used was yeast ^{35}S-labeled securin (Pds1).

Figure 1

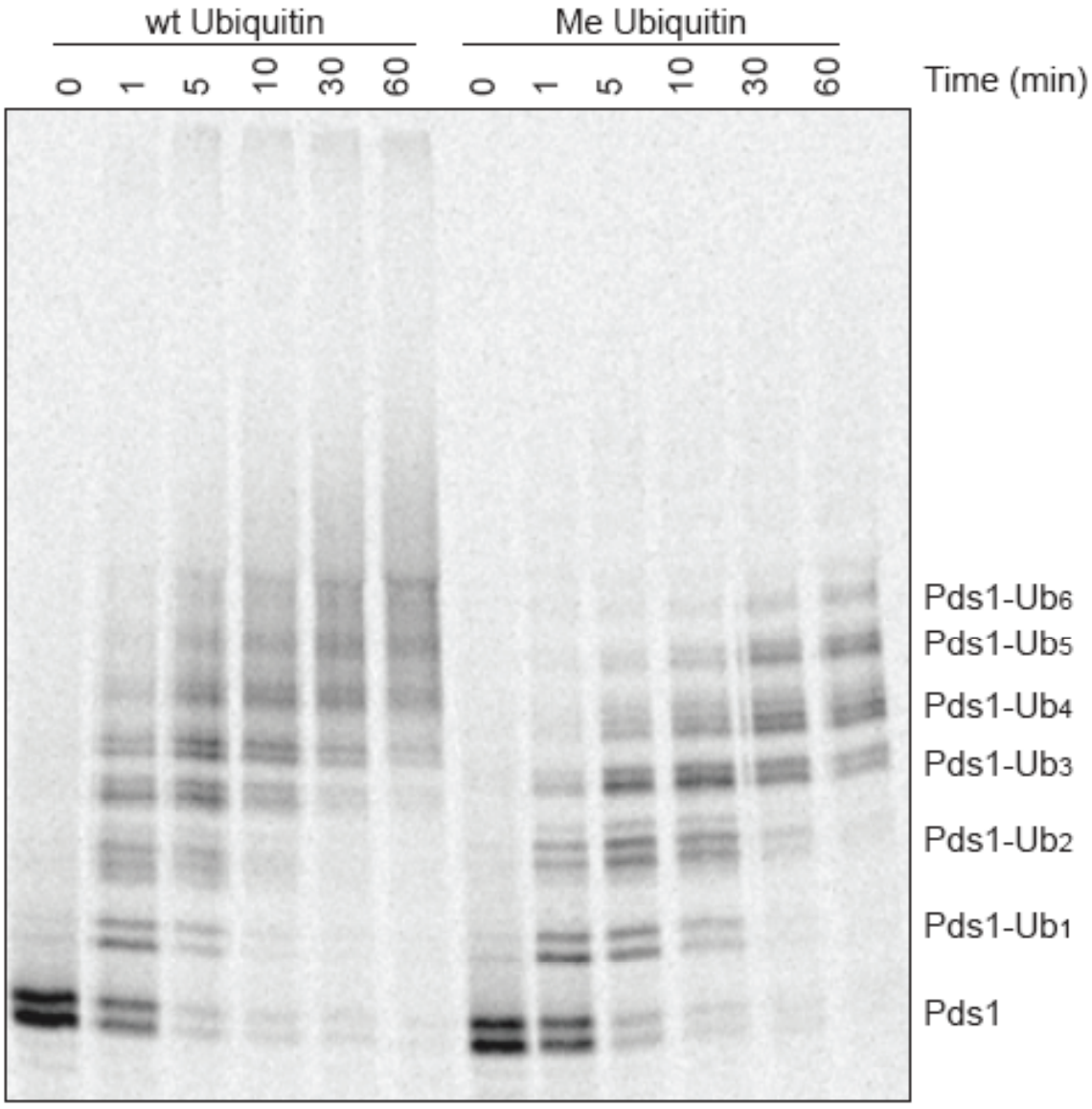

wt Ubiquitin
Me Ubiquitin
0
1
5
10
30
60
0
1
5
10
30
60
Time (min)
Pds1-Ub6
Pds1-Ub5
Pds1-Ub4
Pds1-Ub3
Pds1-Ub2
Pds1-Ub1
Pds1

Figure 2. Reticulocyte lysates contain an extending activity

Substrates (Pds1, ZZ-Pds1, Pds1-ZZ) were *in vitro* transcribed and translated. The tagged versions were bound to IgG-beads and purified. APC assays were performed with these purified proteins as substrates or the untagged Pds1 that was still in reticulocyte lysates.

Figure 2

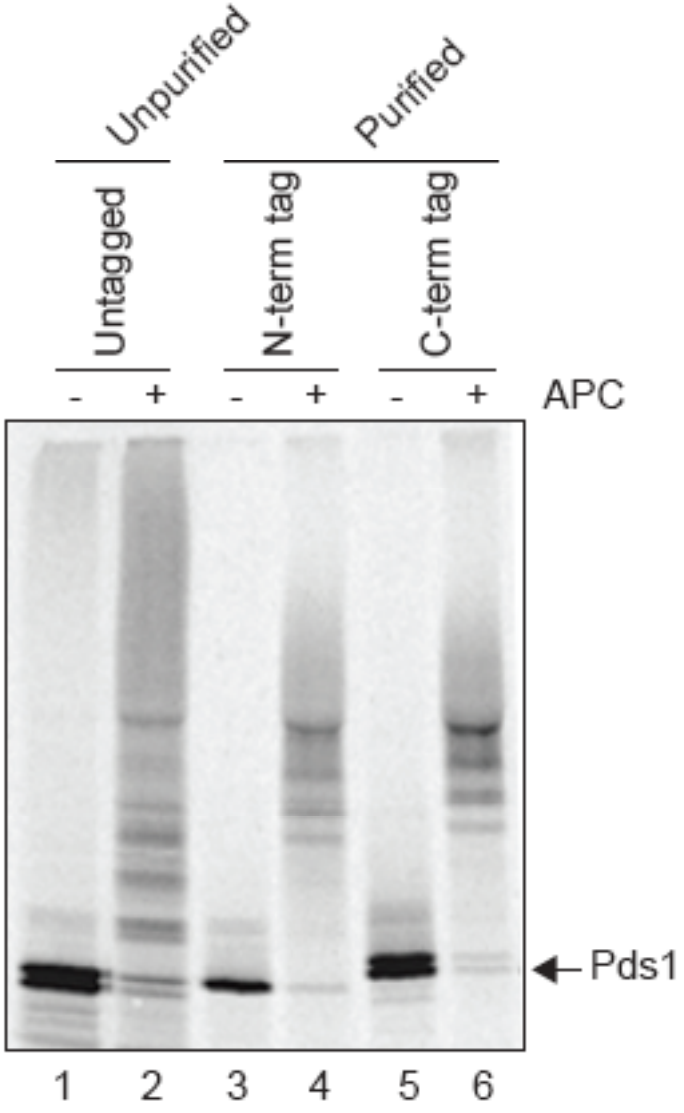

Unpurified
Purified
Untagged
N-term tag
C-term tag
- + - + - +
APC
Pds1
1 2 3 4 5 6

Figure 3. Modifications by reticulocyte lysates are due to formation of ubiquitin chains

APC assays were performed with either unmodified (wt) or methylated (Me) ubiquitin and then incubated with reticulocyte lysate.

Figure 3

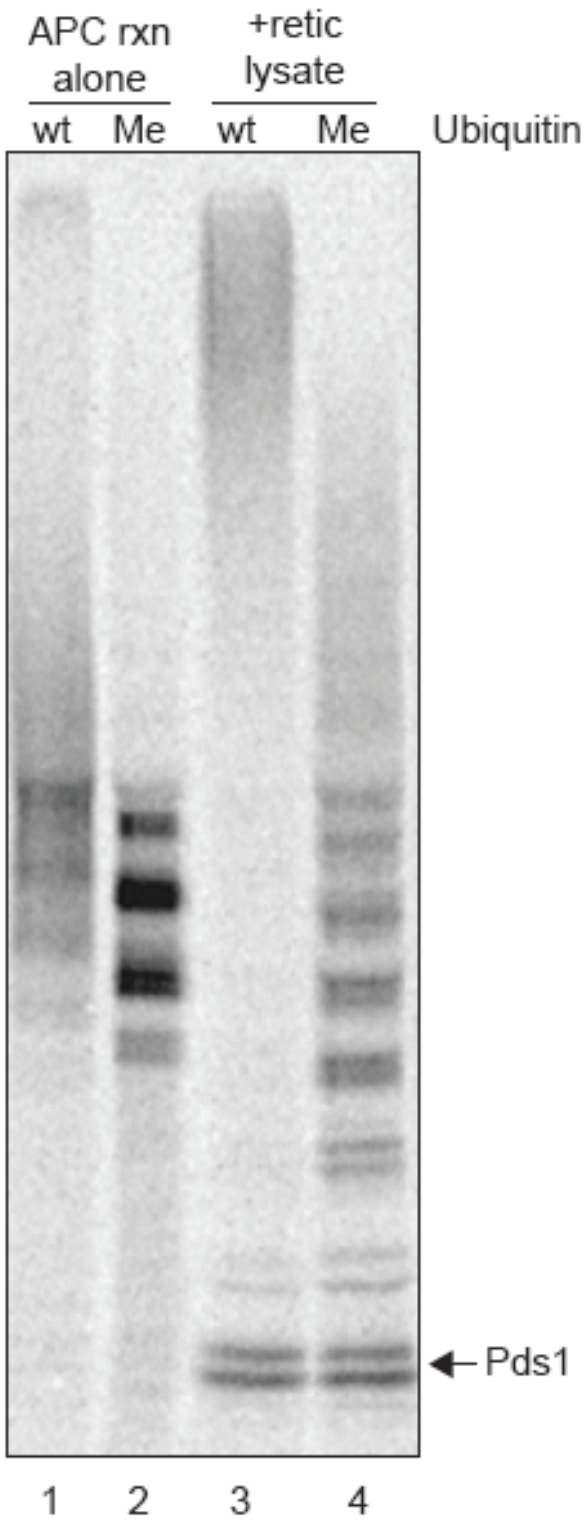


Figure 4. Extending activity in reticulocyte lysates is independent of APC activity

APC assays were performed with purified Pds1 substrate bound to beads. After washing away the APC reaction components, the substrate was cleaved off of the beads. Different components were added to this modified substrate as indicated and ability to polyubiquitinate was monitored. TnT=the originally translated protein, FT=flow through after binding to IgG beads, E1/E2 mix=E1, yeast Ubc4, ATP, ubiquitin.

Figure 4

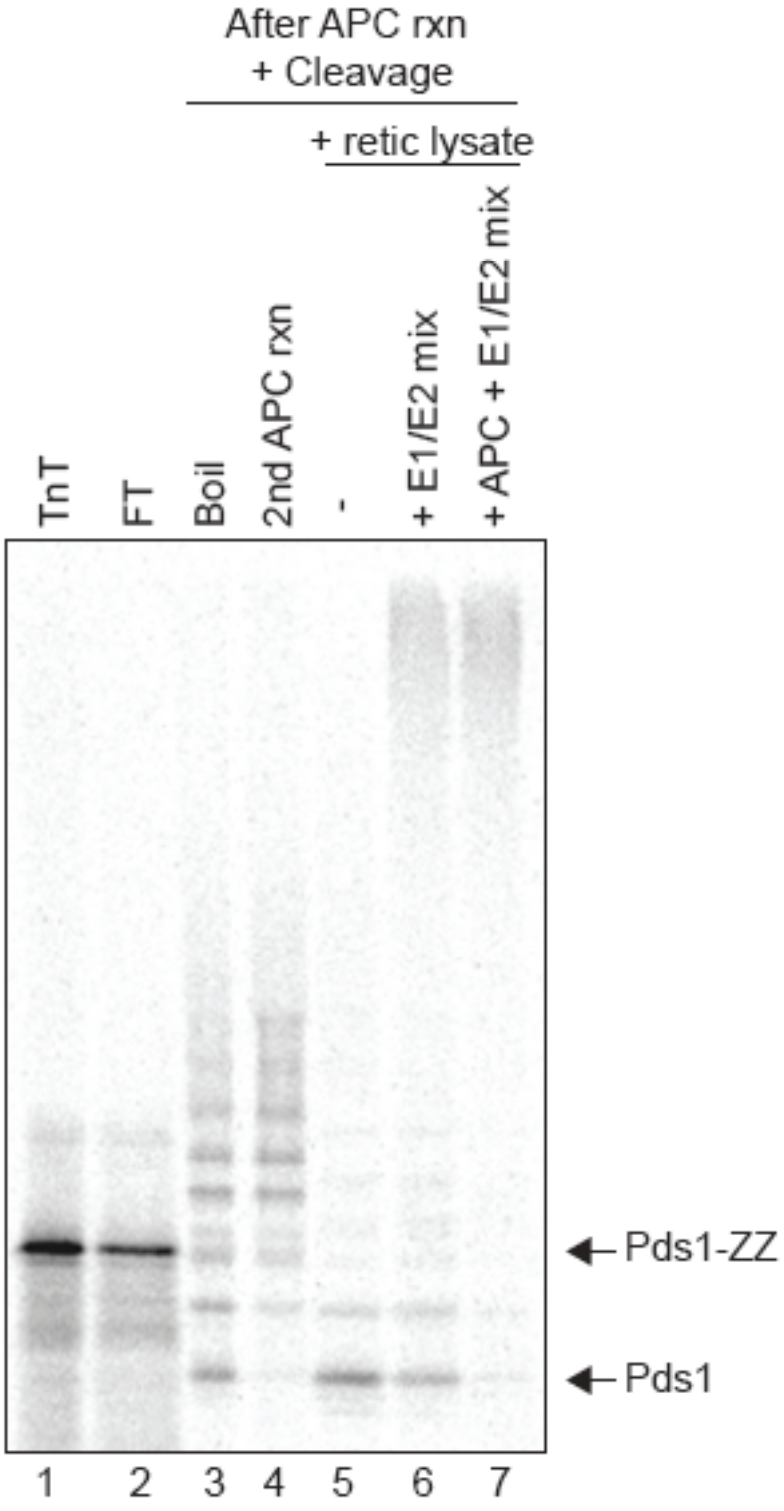

After APC rxn
+ Cleavage
+ retic lysate
TnT
FT
Boil
2nd APC rxn
-
+ E1/E2 mix
+ APC + E1/E2 mix
Pds1-ZZ
Pds1
1
2
3
4
5
6
7

Figure 5. Extending activity in reticulocyte lysates is dependent on the addition of E2 enzyme and ubiquitin

APC assays were performed with purified Pds1 substrate bound to beads. After washing away the APC reaction components, the substrate was cleaved off of the beads. Different components were added to this modified substrate as indicated and ability to polyubiquitinate was monitored. Ub=ubiquitin, TnT=reticulocyte lysates, FT=flow through, E2=yeast Ubc4.

Figure 5

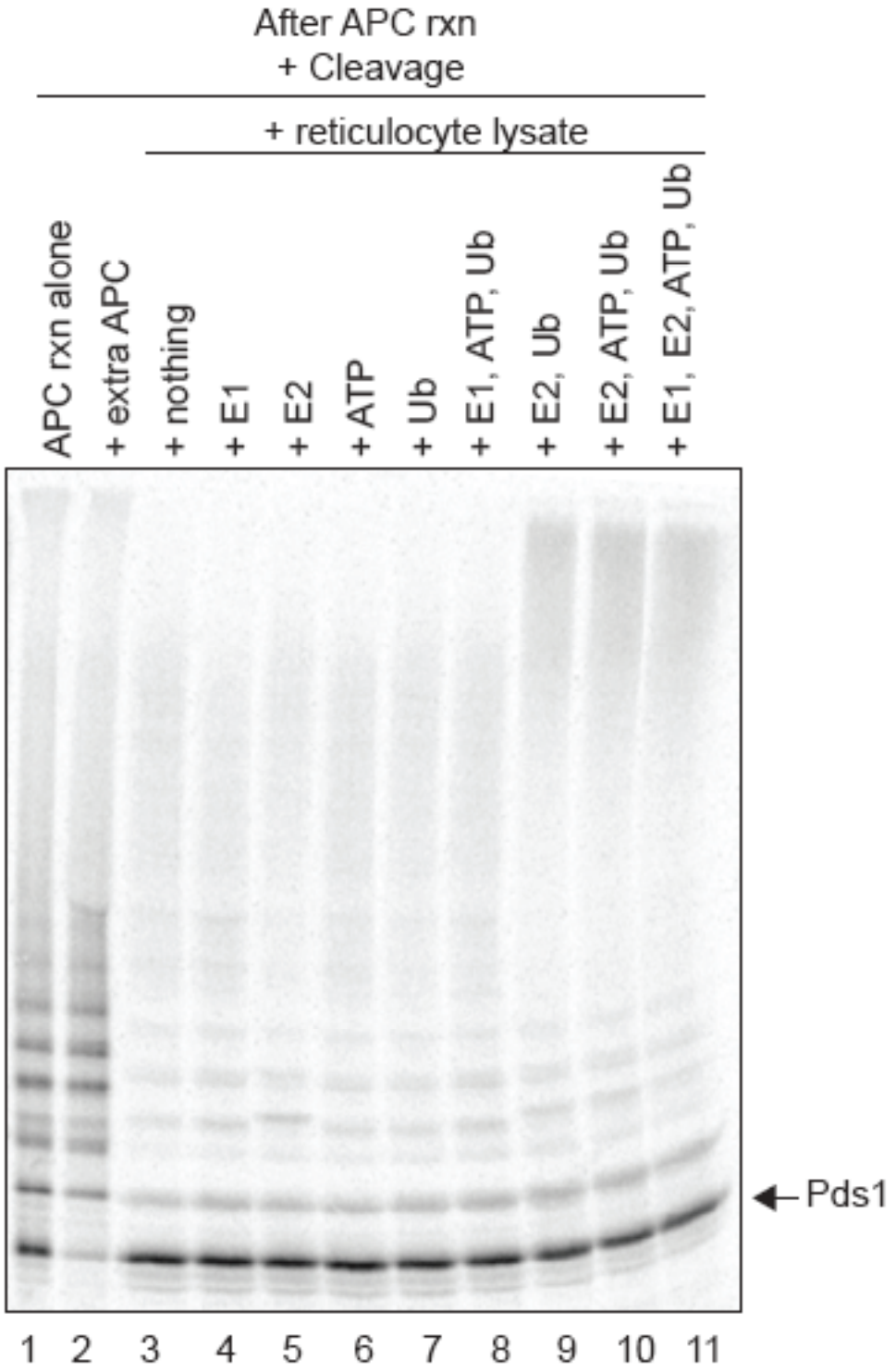

After APC rxn
+ Cleavage
+ reticulocyte lysate
APC rxn alone
+ extra APC
+ nothing
+ E1
+ E2
+ ATP
+ Ub
+ E1, ATP, Ub
+ E2, Ub
+ E2, ATP, Ub
+ E1, E2, ATP, Ub
Pds1
1 2 3 4 5 6 7 8 9 10 11

Figure 6. An extending activity is present in yeast lysates and fractionates on a Q-column

Lysates were made from wild type yeast, and these lysates were loaded onto a HiTrap Q-column. Column was washed with 300 mM NaCl, and eluted with a 300-600 mM NaCl gradient. The presence of the extending activity in the different fractions was assayed by addition of 3 _l (to 10 _l APC reaction) of the fraction to an already performed APC reaction. The behavior of activity peaking in the fractions 12-15 (* marked) was reproducible (in over 10 experiments). Note the strong deubiquitinating activity in fractions 8-11. There is also another polyubiquitinating activity in fractions 7 and 8, though not as strong as the one seen in 12-15. Another possibility is that there is only one polyubiquitinating activity that comes off in fractions 7-15, and that the deubiqitinating activity in 8-11 masks it in these fractions.

Figure 6

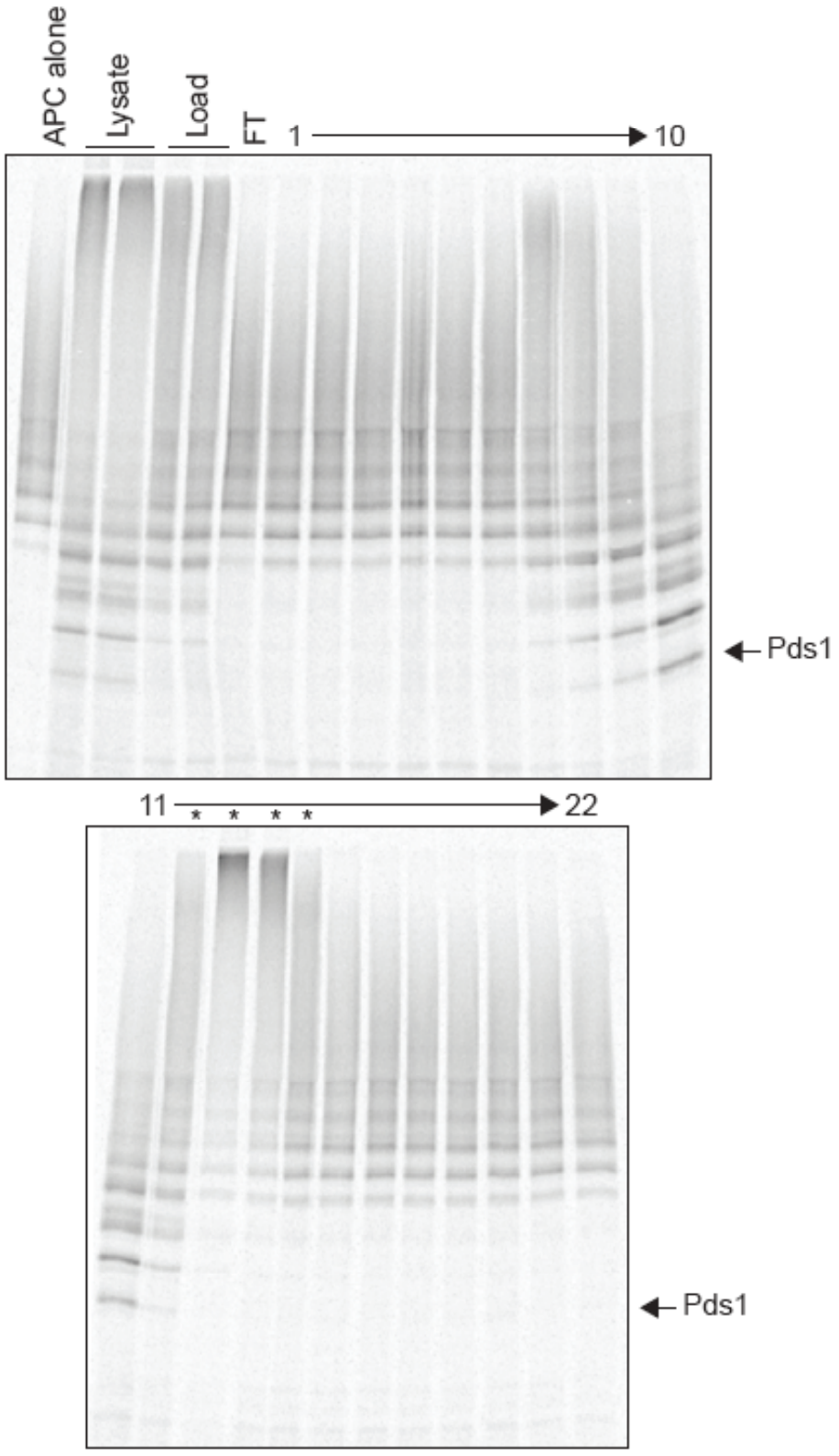


Figure 7. The yeast extending activity is APC-independent

APC assays were performed with purified Pds1 substrate bound to beads. After washing away the APC reaction components, the substrate was cleaved off of the beads (APC #1). Different components were added to this modified substrate as indicated and ability to polyubiquitinate was monitored. F12 is the peak fraction off of a Q column where the fractionation was performed with an *apc11_ apc2_* strain, lacking all APC activity. All reactions were done in duplicates. E1/E2=E1, E2 (Ubc4), ATP, ubiquitin.

Figure 7

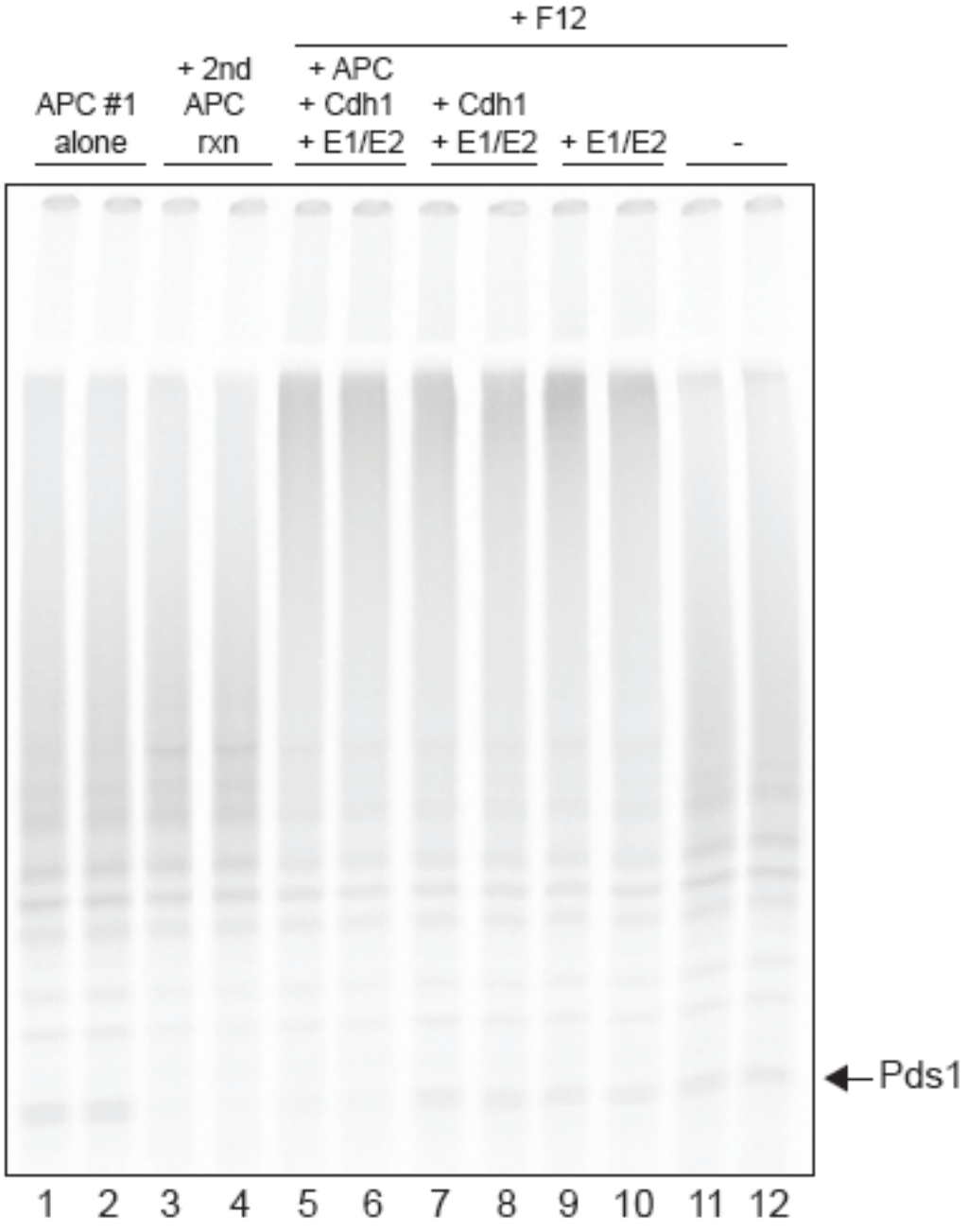

+ F12
APC #1 alone
+ 2nd APC rxn
+ APC + Cdh1 + E1/E2
+ Cdh1 + E1/E2
+ E1/E2
-
Pds1
1 2 3 4 5 6 7 8 9 10 11 12

Figure 8. Extending activity from yeast over a S-400 gel filtration column

Peak fractions of the extending activity off of a Q column were pooled and loaded onto a gel filtration column. Fractions were assayed for the presence of the extending activity as in Fig 2-5. The extending activity migrates with a size larger than 1.5 MDa (it is in the void off of an S-300 column, data not shown), but smaller than 8 MDa since it does separate on an S-400 column. The peak at fractions 45-48 (* marked) behaves reproducibly on an S-400 column (in over 5 experiments).

Figure 8

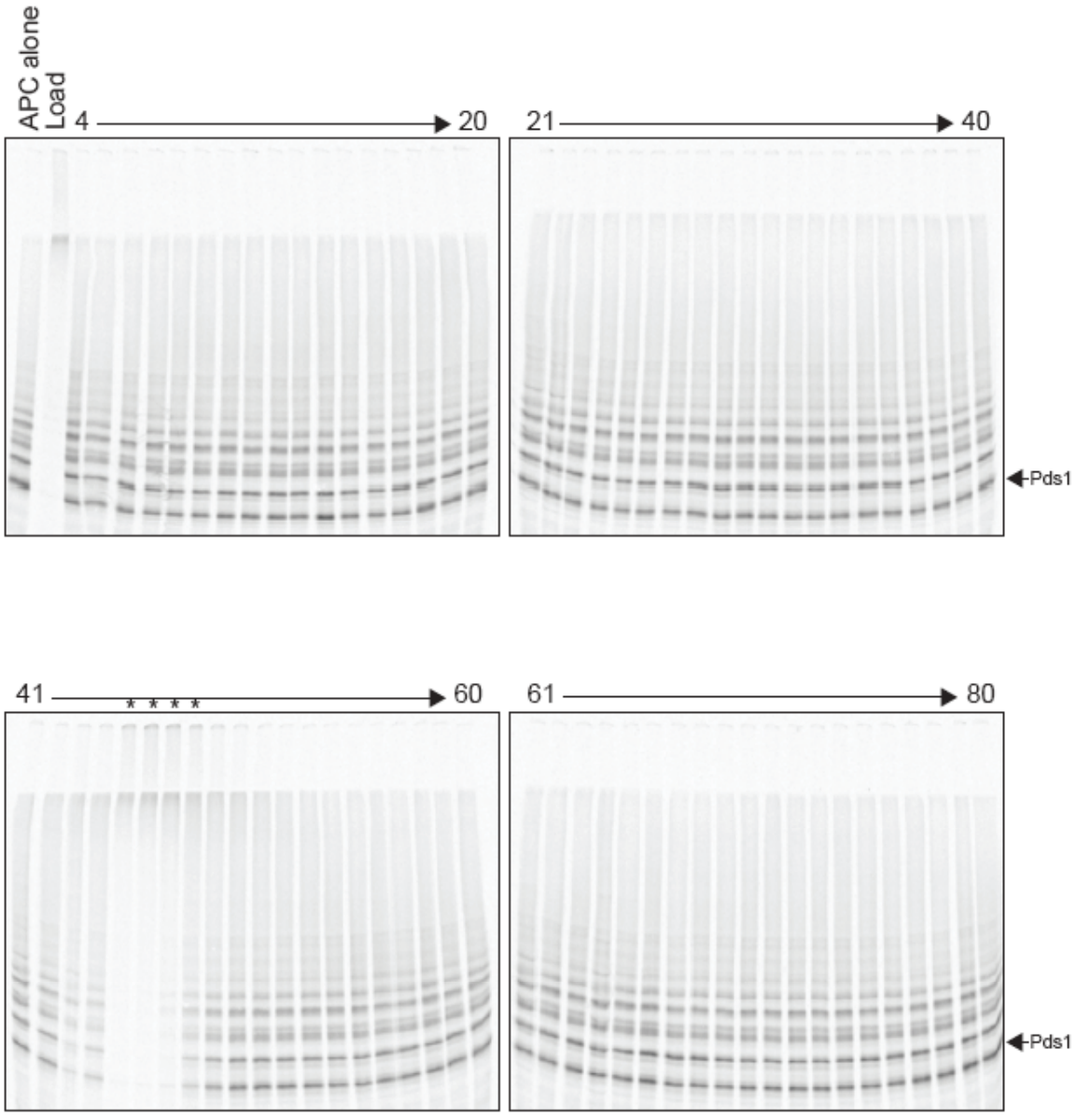
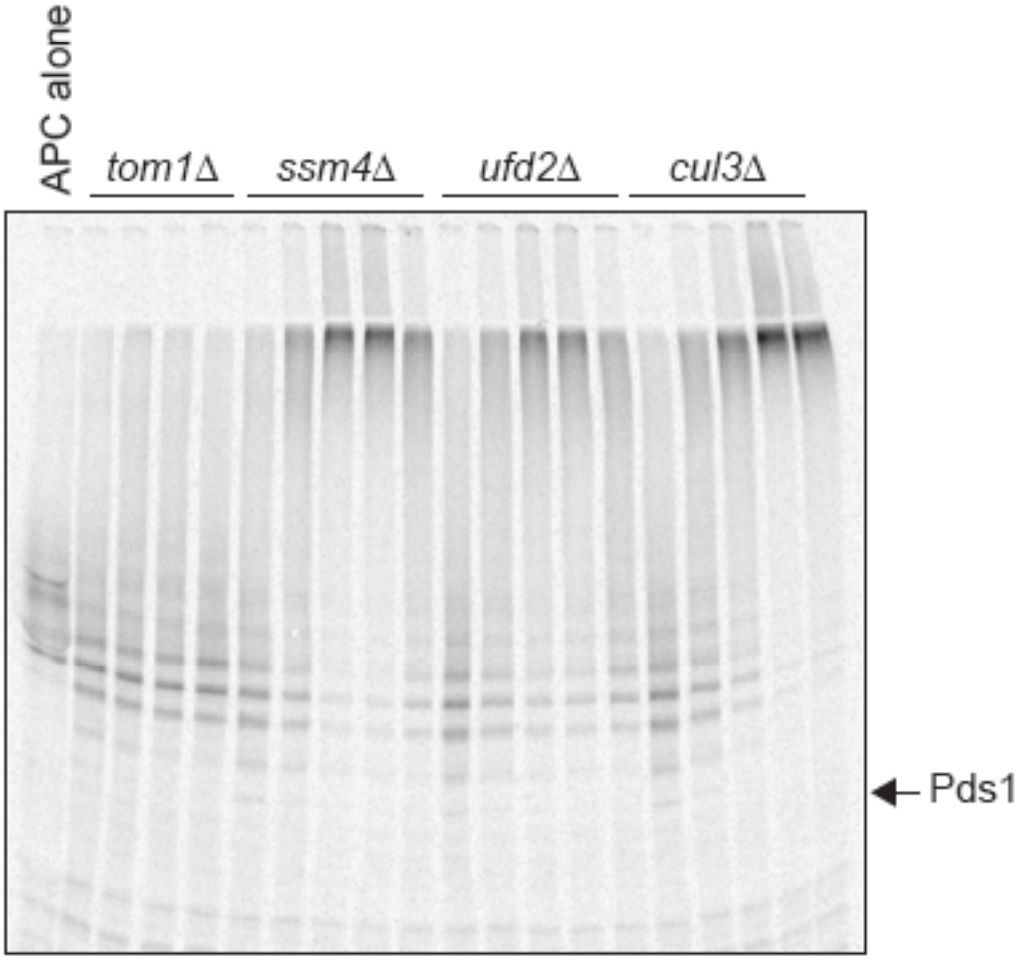


Figure 9. The yeast extending activity is due to Tom1

Yeast lysates were made from 20 different strains from the yeast deletion collection and fractionated over a Q-column. The peak activity fractions (11-15, 12-15 for *tom1_*) were assayed for the presence of the extending activity, as in figure 2-5. Specifically the strain where the *TOM1* gene was deleted showed a loss of extending activity.

Figure 9



Chapter 3

Inhibition of the Anaphase-Promoting Complex in Late Mitosis

Introduction

The anaphase-promoting complex (APC) is a key E3 ubiquitin-protein ligase that polyubiquitinates important cell-cycle components and thereby targets them for destruction by the proteasome. The primary targets of the APC are securin, the destruction of which induces the metaphase-to-anaphase transition, and the mitotic cyclins, the destruction of which allows the cell to exit mitosis and start the next cycle (Shirayama *et al.*, 1999) (Thornton and Toczyski, 2003).

The activity of the APC is regulated through association with activator proteins called Cdc20 and Cdh1. The APC is first activated by Cdc20. Later in mitosis and throughout G1, Cdh1 binds and activates the APC. Cdc20 and Cdh1 also contribute to the substrate specificity of the APC as they bind directly to APC substrates and recruit them to the APC core (Kraft *et al.*, 2005). Thus multiple activator proteins provide a mechanism for the APC to destroy two distinct pools of substrates at different times in mitosis. Substrates contain short amino-acid motifs, called the destruction box (D-box) and the KEN box, that are required for their association with Cdc20 and Cdh1 (Glotzer *et al.*, 1991) (Pfleger and Kirschner, 2000).

The consequences of failing to adequately regulate APC activity would be disastrous. For example, cyclins are necessary for mitosis, and it is critical that they be protected from APC-mediated degradation until anaphase. Previous work has shown that one critical regulatory mechanism is CDK phosphorylation on Cdh1 itself (Zachariae *et al.*, 1998) (Jaspersen *et al.*, 1999). When Cdh1 is

phosphorylated, it is incapable of binding to the core APC and therefore lacks the ability to activate it. Upon activation of the protein phosphatase Cdc14 in anaphase, Cdh1 is dephosphorylated and binds and activates the APC. APC^{Cdh1} activity remains high until CDK activity rises again at the end of the subsequent G1.

Regulation of Cdh1 activity may be more complex than just Cdk-mediated inhibition. Recent work has identified an inhibitor of Cdh1 activity named Acm1 (for APC-Cdh1 Modulator 1) (Martinez *et al.*, 2006) (Dial *et al.*, 2007). Acm1 is a small protein that normally associates with Cdh1 *in vivo*, and can suppress the lethality associated with excess Cdh1 activity. However, a number of key questions about the function of Acm1 remain: for example, is Acm1 specific to APC^{Cdh1} or can it inhibit all APC-activator complexes? What is the mechanism of Acm1 inhibition? And how is Acm1 activity itself regulated?

In this work we examine the molecular mechanism of inhibition by Acm1 on the APC. We find that Acm1 is a specific inhibitor of APC^{Cdh1} and has no impact on APC^{Cdc20} activation, and inhibition correlates with a specific, strong binding between Acm1 and Cdh1. Further, we find that a specific region of Acm1, which contains sequences resembling APC destruction motifs, is completely required for Cdh1 inhibitory activity. However, this region is not strictly required for binding to Cdh1, which seems to depend primarily on adjacent regions of the protein. Our data suggest a model in which Acm1 functions as a pseudo-substrate.

We also examine the regulation of Acm1 and demonstrate that degradation of Acm1 by APC^{Cdc20} is a key event that down-regulates it, and thus promotes the timely liberation of Cdh1 in anaphase. This provides another mechanism by which the activity of APC^{Cdc20} promotes the later activity of APC^{Cdh1}. We also find that Acm1 is heavily regulated by Cdk-dependent phosphorylation. When unphosphorylated, Acm1 is located in the nucleus and Cdk phosphorylation drives it into the cytoplasm. This spatial regulation of Cdh1 by Acm1 may keep Cdh1 apart from the APC core and help maintain adequate levels of APC^{Cdh1} substrates through the cell cycle.

Results

Acm1 is a specific inhibitor of APC^{Cdh1}

Acm1 can inhibit APC^{Cdh1} *in vitro* and *in vivo* (Dial *et al.*, 2007) (Martinez *et al.*, 2006), but it has not been determined if Acm1 inhibits APC^{Cdh1} specifically or is a general inhibitor of all APC/activator complexes. To address this we compared the ability of Acm1 to inhibit APC^{Cdh1} and APC^{Cdc20} in ubiquitination of budding yeast securin, Pds1. APC assays were performed with either Cdc20 or Cdh1 as activator, and *in vitro* translated Acm1 or reticulocyte lysate alone (negative control) was added. At the indicated time points, samples were removed and analyzed by gel electrophoresis (Figure 1a). Addition of Acm1 strongly inhibited Pds1 ubiquitination by APC^{Cdh1}. In contrast, no inhibition of APC^{Cdc20} was seen by addition of Acm1. We conclude that Acm1 is a Cdh1-specific APC inhibitor.

To investigate how Acm1 inhibits APC^{Cdh1} but not APC^{Cdc20}, we analyzed Acm1-activator binding. Initially, Cdh1 and Cdc20 were translated *in vitro* with a ZZ-tag and labeled with ^{35}S-methionine, and their binding to IgG-beads was monitored. From this control it was clear that ZZ-Cdh1 and ZZ-Cdc20 bound to the IgG-beads with similar affinity (Figure 1b). Next, Acm1 was translated with ^{35}S-methionine and mixed with control lysate, ZZ-Cdh1 or ZZ-Cdc20 (both translated with unlabelled methionine). After binding to IgG beads and washing, analysis of the immunoprecipitates revealed that Acm1 showed significant binding to ZZ-Cdh1. In contrast, there was no difference in binding of Acm1

between the control lysate and ZZ-Cdc20. From this we conclude that Acm1 specifically binds to Cdh1 independently of the APC. Further, this result suggests that binding to Cdh1 may be an important part of the mechanism of Acm1 inhibition of APC^{Cdh1}.

A specific region of Acm1 is necessary for its inhibitory activity

To understand how Acm1 inhibits APC^{Cdh1} we examined in more detail the domain structure of Acm1. Our attention was drawn to a specific region of Acm1, amino acids 98-122, for two reasons. First, this region contains sequences that resemble APC-substrate recognition motifs, suggesting that this region could be an inhibitory domain. Second, sequence alignment of Acm1 homologs from multiple yeast species showed conservation of this region. We therefore created a version of Acm1 lacking this region (Δ98-122) and tested its ability to inhibit APC^{Cdh1}. As seen in Figure 2a, addition of wild type Acm1 strongly inhibited ubiquitination of Pds1 by APC^{Cdh1}. In contrast, addition of comparable amounts of Acm1-Δ98-122 did not inhibit Pds1 ubiquitination, suggesting that the central region of Acm1 (98-122) is critical for inhibition of APC^{Cdh1}.

The central region of Acm1 (98-122) contains three sequences that resemble APC destruction motifs: one KEN-box and two D-boxes. Sequence alignment of yeast Acm1 homologs showed conservation of the KEN box and the second D-box (119-122) but no conservation of the first D-box (111-114). To

investigate the importance of these regions we mutated them. An Acm1 mutant KEN→AAA lost all APC^{Cdh1} inhibitory activity (Figure 2a). Single mutations of any of the K, E or N residues were sufficient to abolish inhibitory activity (data not shown). This effect was specific to the KEN amino acids, as mutation of either the two upstream or two downstream residues did not affect the ability of Acm1 to inhibit APC^{Cdh1} (data not shown). Mutation of the second D-box by either deletion or alanine mutation (Figure 2a and data not shown) also reduced Acm1 inhibitory activity. In contrast, mutation of the first D-box motif had no effect (data not shown). Thus, in keeping with the sequence conservation predictions, the second but not first D-box of Acm1 is critical for inhibition.

To confirm if loss of binding inhibition activity *in vitro* corresponded to loss of *in vivo* function, we tested the above Acm1 mutants' ability to inhibit APC^{Cdh1} in yeast cells. Overexpression of a variant of Cdh1 that contains mutations in all of the CDK phosphorylation sites (*CDH1-m11)* is lethal. This can be suppressed by co-overexpression of Acm1 as shown in Figure 2b(Martinez *et al.*, 2006). However, overexpression of Acm1 proteins carrying mutations in the 98-122 region in either the KEN box or D-box did not suppress the lethality of Cdh1-m11. Thus, loss of *in vitro* inhibition activity of Acm1 correlates with loss of *in vivo* functionality, and confirms the critical role of the amino-acid region 98-122 in inhibition of APC^{Cdh1}.

Inhibition domain mutants of Acm1 still bind to Cdh1

We next analyzed the effects of Acm1 mutations on binding to Cdh1, using methods like those described in Figure 1b. ZZ-Cdh1 was bound to IgG beads, and multiple long washes were then performed to investigate the rate of protein-bead dissociation. Before washing, all of the Acm1 variants bound with approximately similar amounts, with the exception of a 70-211 mutant, which showed less initial binding (Figure 3). Analysis of the rate of dissociation of the Acm1 mutants from Cdh1 revealed that mutants in the KEN or second D-box had slightly enhanced dissociation rates as compared to wild type Acm1. The Acm1 mutant lacking the entire central region (98-122) has a higher rate of dissociation relative to either wild type Acm1 or the KEN and D-box mutants. However, binding between Cdh1 and the _98-122 mutant is still significant even after 120 minutes, indicating a very strong association. These results demonstrate that the 98-122 region makes some contribution to binding of Acm1 to Cdh1, but the majority of binding comes from elsewhere on the Acm1 protein. One possibility is that this region of Acm1 acts as a pseudo-substrate inhibitory domain that blocks substrate recognition by occupying the KEN and D-box receptors in Cdh1. A separate strong binding domain elsewhere on Acm1 ensures that the local concentration of this inhibitory region of Acm1 around Cdh1 is extremely high.

Acm1 levels fluctuate in the cell cycle

APC^{Cdh1} is activated in late mitosis and persists through G1. As Acm1 can specifically inhibit Cdh1 function, then how is Acm1 function blocked at these

parts of the cell cycle? Two common mechanisms for controlling activity of proteins are regulated degradation and post-translational modification, most commonly by phosphorylation. To investigate the regulation of Acm1, we began our studies by following the levels of Acm1 through a yeast cell cycle. Cells carrying Acm1 C-terminally tagged with myc epitopes were arrested in G1 phase by alpha-factor treatment, released into fresh media, and samples were collected every fifteen minutes. Alpha-factor was re-added after 100 minutes to arrest the cells in the following G1 phase. Western blot analysis revealed that Acm1 levels were low in G1, accumulated as cells progressed into S phase, and then disappeared in mitosis (Figure 4b). Single-cell analysis revealed that cells containing short metaphase spindles had high Acm1 levels (Figure 4a/c). In contrast, cells containing elongating anaphase spindles had undetectable amounts of Acm1. Together, these results show that the levels of Acm1 dramatically fluctuate through the cell cycle. The key transition seems to occur at the metaphase-to-anaphase transition, when Acm1 levels drop precipitously.

Acm1 is a substrate of APC^{Cdc20}

Acm1 levels decline at anaphase onset, which is the same time as Cdc20 activity rises, suggesting that Acm1 could be a substrate of APC^{Cdc20}. To investigate this, we performed an *in vitro* APC^{Cdc20} assay with Acm1 as the substrate. As can be seen in Figure 4d, APC^{Cdc20} strongly promoted the ubiquitination of Acm1, suggesting that it is indeed a substrate. Cdc20

recognizes its substrates through D-box sequence motifs on the substrates. Previously, we had identified a potential D-box on Acm1 necessary for inhibition of Cdh1 at amino acids 119-122. However, there was no loss of *in vitro* ubiquitination of a mutant lacking this region or indeed of a mutant lacking the entire central inhibitory domain region (data not shown), strongly suggesting that Cdc20 recognizes Acm1 through another, unidentified D-box. Sequence analysis of Acm1 revealed another potential D-box close to the N-terminus, a typical place for APC destruction motifs. Encouragingly, this D-box appeared conserved across a range of Acm1 homologs from various yeast species (data not shown). To investigate the importance of this region we constructed an Acm1 mutant lacking it (Δ8-11) and tested its ability to be ubiquitinated by APC^{Cdc20}. As shown in Figure 4d, ubiquitination of an Acm1 mutant lacking amino acids 8-11 (hereafter called Acm1-dbm) by APC^{Cdc20} was severely reduced relative to ubiquitination of wild type Acm1. This result demonstrates that APC^{Cdc20} recognizes Acm1 through an N-terminal D-box. Acm1 lacking this region was still fully capable of inhibition of APC^{Cdh1} both *in vitro* and *in vivo* (data not shown).

Acm1-dbm persists in anaphase when Cdc20 activity is high

The *in vitro* and *in vivo* results suggest that Acm1 is a substrate of APC^{Cdc20} and thereby targeted for destruction at the onset of anaphase. Analysis of the sequence motifs on Acm1 revealed that an N-terminal D-box is required to mediate APC^{Cdc20} dependent ubiquitination *in vitro*. Removal of this N-terminal D-

box from Acm1 *in vivo* should promote the anaphase stabilization of Acm1. To test this, we analyzed the behavior of an *ACM1-dbm* mutant tagged with C-terminal Myc epitopes. Cells were arrested in alpha factor and released through the cell cycle as for wild type Acm1 (Figure 4a). Analysis of Acm1 levels by Western blot revealed that the Acm1-dbm mutant persisted for approximately 20 minutes longer than wild type Acm1 (Figure 4b). Single cell analysis revealed that all cells with an anaphase spindle contained appreciable levels of Acm1, similar to the amount seen in a metaphase cell (Figure 4c). This result further demonstrates that APC^{Cdc20} degrades Acm1 at anaphase onset and that this is due to an N-terminal D-box. Notably, the levels of Acm1-dbm did drop later, suggesting that another pathway other than APC^{Cdc20} has the potential to contribute to Acm1 degradation (see below). Also, analysis of the migration pattern of Acm1-dbm revealed a shift in late mitosis, precisely the time point where Acm1 levels normally drop (Figure 4b). This suggested that Acm1 might be subject to cell cycle regulated phosphorylation (see below).

Acm1-dbm degradation depends upon APC^{Cdh1}

In a normal mitosis, Cdc20 activity promotes the degradation of Acm1 at anaphase onset, and an Acm1 mutant resistant to APC^{Cdc20}-dependent degradation persists into late mitosis. However, the Acm1-dbm protein is not fully stabilized as its levels do decline significantly later in mitosis. One possibility was that the degradation of Acm1-dbm was due to APC^{Cdh1} activity. To investigate

this, we monitored the levels of Acm1-dbm in synchronized *Δcdh1* cells. As Cdh1 is necessary for alpha-factor mediated arrest, we took advantage of a reversible temperature-sensitive mutant in the Cdc15 kinase that is necessary for mitotic exit. *CDH1* and *Δcdh1* cells, carrying both *ACM1-dbm* and *cdc15-2*, were arrested at 37°C for 2.5 hours to synchronize the cells in anaphase. Cells were shifted to 23°C to release the mitotic arrest and samples taken every 15 minutes. Western blot analysis revealed that the levels of Acm1-dbm dropped significantly after 45 minutes in the *CDH1* strain but remained high in the *Δcdh1* cells (Figure 4e). Single cell analysis confirmed this result and also demonstrated that spindle breakdown (a marker of mitotic exit) occurred equivalently between the strains (Figure 4f). Thus the instability of Acm1-dbm depends upon APC^{Cdh1}, suggesting that Cdh1 has some ability to target its inhibitor for degradation. However, in a normal, unperturbed mitosis Acm1 would be destroyed before APC^{Cdh1} activation by APC^{Cdc20}.

Delayed degradation of Clb2 and Cdc5 in *ACM1-dbm* cells

In anaphase, once liberated from inhibition by both phosphorylation and Acm1, Cdh1 associates with the APC and mediates the destruction of its substrates. APC^{Cdc20} is thus the master regulator that drives activation of Cdh1 as it promotes the destruction of both the mitotic cyclins and Acm1. Failure to destroy the major mitotic cyclin Clb2 prevents mitotic exit, but it is unknown what impact failure to destroy Acm1 has on Cdh1 activity. To investigate this, we

compared the degradation of a number of Cdh1 substrates in cells carrying either *ACM1* or *ACM1-dbm*. Cells were synchronized by alpha factor treatment and followed through a single cell cycle as described previously. Western blot analysis of the degradation of Clb2 and Cdc5 revealed a small but reproducible stabilization in late mitosis (Figure 4g). This delay in Clb2 and Cdc5 degradation is consistent with the increased longevity of the Acm1-dbm protein over Acm1 (Figure 4b). We also examined the degradation of two other APC^{Cdh1} substrates, Cin8 and Kip1, but found no discernible difference in their degradation kinetics (data not shown). Notably, these substrates were degraded later than both Clb2 and Cdc5, and thus a minor delay in APC^{Cdh1} activation may not significantly impact their degradation.

Acm1 is regulated by phosphorylation

As described above, Acm1-dbm in anaphase migrated faster than that in metaphase (Figure 4b) suggesting that a change in the post-translational modification of Acm1 occurs in mitosis. The most common modification in cells is protein phosphorylation. Interestingly, Acm1 contains 5 consensus Cdk sites (R/K-x-S/T-P), and our previous work has suggested that Acm1 is an extremely good substrate of the cyclin-dependent kinase (Cdk1) *in vitro*(Ubersax *et al.*, 2003). To investigate if the migration change in mitosis depends upon Cdk phosphorylation, we assessed the impact of induced expression of the anti-Cdk phosphatase Cdc14 on Acm1 mobility. Cells expressing either Acm1-myc or

Acm1-dbm-myc were arrested in metaphase by nocodazole treatment, after which Cdc14 expression was induced by addition of galactose to the culture media. Western blot analysis revealed that both Acm1 and Acm1-dbm migrated faster upon Cdc14 induction (Figure 5a). The migration change of Acm1-dbm here is similar to that seen in Acm1-dbm between metaphase and anaphase (Figure 4b). This result suggests that in metaphase Acm1 is phosphorylated by Cdk-dependent phosphorylation.

What is the purpose of phosphorylation of Acm1? We noticed a striking correlation between Acm1 localization and phosphorylationstate. In early S-phase, Acm1 was localized to the nucleus. Later in G2 and metaphase, Acm1 relocated to the cytoplasm at a time that correlates with rising levels of Cdk activity. Examination of Acm1-dbm showed a dramatic relocalization into the nucleus at anaphase onset (Figure 4c), consistent with the idea that Acm1 dephosphorylation, triggered by a Cdc20-dependent decrease of Cdk activity and increase in Cdc14 activity, drives Acm1 into the nucleus. Further evidence comes from analysis of the effect of Cdc14 on Acm1 localization. Initially Acm1 is localized to the cytoplasm, but upon induction of Cdc14 it relocates to the nucleus (Figure 5b). Together these results suggest that one of the functions of phosphorylation is to regulate the localization of Acm1 between the nucleus and cytoplasm. When Cdk1 activity is low, Acm1 is nuclear, and when Cdk activity is high, Acm1 is cytoplasmic.

Cdc14 activity contributes to nuclear localization of Acm1-dbm in anaphase

Cdc20-dependent APC activity drives Acm1 into the nucleus in anaphase (Figure 4c). Cdc20 contributes to the reversal of Cdk activity by direct destruction of mitotic cyclins (particularly Clb5) and by activation of the Cdc14 phosphatase. Cdc20 completely destroys the mitotic cyclin Clb5 at anaphase onset and this helps reverse Clb5-Cdk substrate phosphorylation. However, our previous work indicates that phosphorylation of Acm1 by Cdk is not Clb5 specific, and thus significant Cdk activity remains after APC^{Cdc20} activation. This suggests that a key role of Cdc20 in dephosphorylation, and thus permitting nuclear entry of Acm1, may be through Cdc14 activation. To examine this directly, we monitored the nuclear localization of Acm1 in cells arrested in late mitosis by use of a temperature-sensitive mutation in the Cdc14 protein. Cells carrying either *ACM1-myc* or *ACM1-dbm-myc* and the *cdc14-1* allele, were arrested at 37°C for 2.5 hours or in nocodazole at permissive temperature. Analysis of the Acm1 protein in the *cdc14-1* arrest revealed that wild type Acm1 was absent while Acm1-dbm was present at high levels (Figure 5c). Examination of the localization of Acm1-dbm in the *cdc14-1* arrest revealed that that it was distributed throughout the nucleus and cytoplasm, in contrast to the wholly nuclear localization seen in wild type anaphase cells (Figure 5d). We believe that this localization defect is directly due to Cdc14 activity and not the late mitotic arrest, as cells arrested in a late mitosis with a *cdc15-2* temperature sensitive mutant had nuclear Acm1-dbm (cells lacking Cdc15 activity still activate Cdc14 at anaphase onset). Thus these

results indicate that Acm1 is a substrate of Cdc14 in anaphase, and dephosphorylation drives Acm1 into the nucleus.

Acm1-5A never leaves the nucleus

To directly prove that Cdk1-dependent phosphorylation of Acm1 regulates Acm1 localization, we created an Acm1 mutant that cannot be phosphorylated by Cdk1. Acm1 contains 5 consensus Cdk sites, and all 5 sites are conserved through multiple yeast homologs (data not shown). We mutated all 5 of the serine/threonine residues to alanine to create an Acm1-5A protein resistant to Cdk1 regulation. We took *ACM1-myc* and *ACM1-5A-myc* cells and arrested them in mitosis by nocodazole treatment. As seen previously, wild-type Acm1 was localized throughout the nucleus and cytoplasm. In contrast, Acm1-5A was located exclusively in the nucleus (Figure 6a). Therefore, phosphorylation by Cdk1 promotes cytoplasmic localization of Acm1. To further characterize the impact of phosphorylation we examined the status of Acm1-5A in a synchronous cell cycle. Cells, carrying either *ACM1-myc* or *ACM1-5A-myc*, were arrested in G1 by alpha factor treatment and released through the cell cycle. While wild type Acm1 started in the nucleus at G1/S and then moved to the cytoplasm before anaphase destruction, Acm1-5A was found exclusively in the nucleus throughout the time course (Figure 6b). Thus phosphorylation of Acm1 by Cdk promotes nuclear export in S phase. There may also be a secondary contribution of Cdk phosphorylation on regulation of Acm1 as we also noticed that Acm1-5A is less

stable than wild type Acm1 (Figure 6b). This suggests that phosphorylation by Cdk also promotes Acm1 accumulation but the mechanism of this remains unclear.

What is the function of anaphase dephosphorylation of Acm1 in wild type cells? One possibility is that dephosphorylation contributes to efficient anaphase degradation of Acm1. APC^{Cdc20} is believed to be present in the nucleus and thus Acm1 dephosphorylation would bring the substrate and activator into the same spatial compartment. Additionally, or alternatively, dephosphorylation of Acm1 may assist the anaphase relocalization of Cdh1 from the cytoplasm to the nucleus and thus bring Cdh1 to the APC core. However, we were not able to directly assay the impact of Acm1 phosphorylation on the localization of Cdh1 due to technical difficulties of visualizing Cdh1 probably arising from its low cellular abundance.

Discussion

We had shown that Acm1 is an inhibitor specific to the Cdh1-dependent APC. We showed that this inhibition is dependent on a central region of Acm1 that contains motifs similar to those found in APC substrates. Moreover, we showed that Acm1 binds very strongly to Cdh1. The dissociation we observed between Acm1 and Cdh1 is similar to the dissociation observed between the ZZ-tag (Protein A) of Cdh1 and the IgG linked to the magnetic beads. This would imply that the Acm1-Cdh1 interaction is of a similar magnitude as the Protein A-IgG interaction, which is known to be in the nanomolar range, extremely tight for a protein-protein interaction. The fact that there is no detectable binding between Acm1 and Cdc20 suggests a mechanism for the specificity of Acm1. Strong binding appears to be a prerequisite for inhibition, since Acm1 mutants that had lost this tight interaction with Cdh1 also showed a loss of inhibition (data not shown). It could also be that the pseudosubstrate region in Acm1 is better at mimicking a Cdh1-specific substrate, and that it is not well recognized by Cdc20.

Future experiments will include a closer examination of how Acm1 acts as an inhibitor. For technical reasons we have not been able to purify soluble recombinant Acm1, but if this problem is solved, investigating Acm1 as an inhibitor *in vitro* would give key information. What type of inhibitor does it behave as? What is its K_i? What is the K_d of the Cdh1-Acm1 interaction? Another important area is to better understand the specificity of the Acm1-Cdh1 interaction. Cdh1 and Cdc20 are closely related proteins, and by identifying what

region of Cdh1 is important for the Acm1 interaction, one can compare these regions in Cdh1 and Cdc20 and better understand how these two APC activators can function differently.

Our investigation of the regulation of Acm1 suggests that it is degraded through a Cdc20-dependent mechanism at anaphase onset. This suggests a novel, direct role for APC^{Cdc20} to be able to flip the switch between the APC^{Cdc20} and APC^{Cdh1}. Moreover, this adds another protein to the short list of Cdc20-specific APC-substrates. One area of great interest for future experiments is substrate ordering: the exact timing of degradation of wild type Acm1 is not only dependent on Cdc20 activation, but also on dephosphorylation by Cdc14 and the localization of Acm1. The understanding of the exact roles these contributing factors play in the destruction of Acm1 will be important to fully understand the regulation of this novel APC inhibitor. Another area to be investigated is a possible role of Acm1 in meiosis.

We also showed that Acm1 is regulated through CDK phosphorylation. It is clear that the phosphorylation state of Acm1 affects its localization, where phosphorylation drives Acm1 out of the nucleus, while dephosphorylated Acm1 is exclusively nuclear. The behavior of the Acm1-5A mutant that is resistant to all CDK phosphorylation suggests two possible roles of the phosphorylation. Acm1-5A is constitutively nuclear, and it is also less stable than wild-type Acm1. One possibility is that it is the premature localization to the nucleus that leads to premature degradation of Acm1, in which case the CDK

phosphorylation is important to protect Acm1 from premature degradation. The other possibility is that the destabilization of Acm1-5A is due to a more direct role of the phosphorylation. Maybe one or more of the phospho-groups normally acts to protect Acm1 from degradation by the APC. Another possibility is that the phosphorylation is necessary for full inhibition of Cdh1, although we know that the nonphosphorylated Acm1 produced by translation *in vitro* is a potent inhibitor. Mutants of Acm1 that no longer inhibit Cdh1 become very good APC^{Cdh1} targets *in vitro* (data not shown). In this case, it is possible that dephosphorylated Acm1 does not inhibit Cdh1 as well, and when Cdh1 is dephosphorylated by Cdc14 and therefore can bind to the APC it might be able to start ubiquitinating Acm1 and thereby target it for destruction at an earlier time.

In this work we have identified a novel way for APC^{Cdc20} to directly activate APC^{Cdh1} at the beginning of anaphase by targeting an APC^{Cdh1} inhibitor for destruction. Moreover, we have shown on a molecular level how a protein can inhibit the APC by being a tight binder with a separate pseudosubstrate region. Overall, these results greatly add to our understanding of the regulation of the Anaphase-Promoting Complex.

Materials and Methods

APC assays and inhibition assays

APC assays were performed as described (Carroll and Morgan, 2005). Activators were *in vitro* transcribed and translated using TnT Quick Coupled Transcription/Translation Systems (Promega). Substrates used were *in vitro* transcribed and translated with ^{35}S-Methionine. Pds1 was transcribed from a plasmid and Acm1 from PCR products (as described in Tully & Morgan, in preparation). Inhibitor was pre-incubated with the activator for 10 minutes before added to the APC assay.

Binding assays

ZZ-tagged activator was *in vitro* transcribed and translated from plasmids. Acm1 was *in vitro* transcribed and translated from PCR products in the presence of ^{35}S-Methionine. Translated products were incubated with IgG-magnetic beads (IgG (Sigma) coupled to Epoxy Magnetic Beads (Dynabeads)) for 2 hours at 4°C. 3 washes were then performed with binding buffer (20mM Hepes pH 8.0, 150mM NaCl, 0.1% NP-40). Beads were then boiled in SDS Sample Buffer. For the time course an additional wash was performed at each timepoint and the long washes were performed at room temperature.

Immuno fluorescence and growth conditions

Methods were used as previously described {Sullivan, 2001 #1420}. Protein extracts were prepared using urea buffer as described {Ubersax, 2003 #1637}.

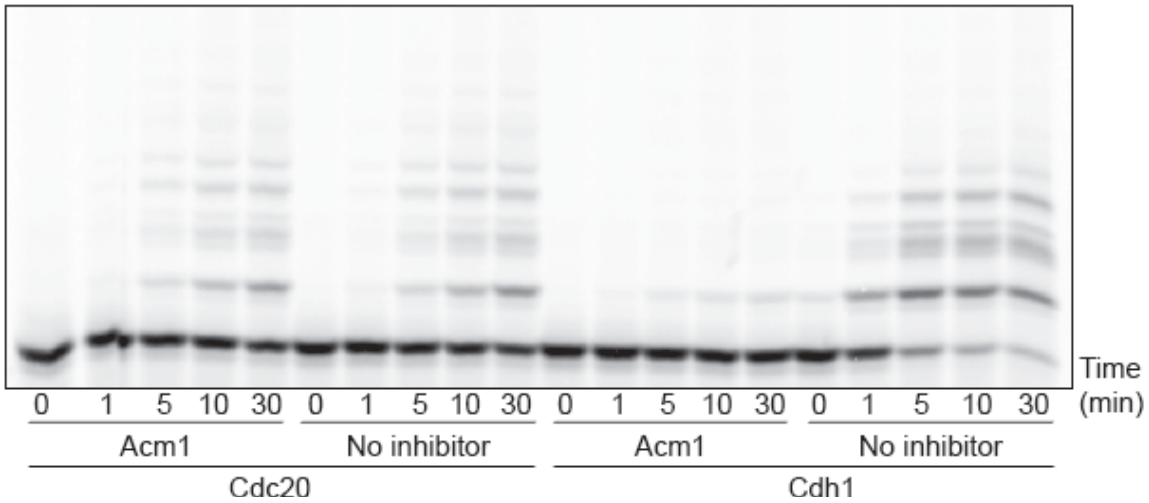
Figure 1: Acm1 is a Cdh1-specific APC inhibitor

(A) An autoradiograph showing APC assays performed with Cdh1 and Cdc20, either with Acm1 added or not. The substrate used in the APC assays is yeast securin, Pds1, which has been *in vitro* translated with ^{35}S-Methionine.

(B) Autoradiographs showing the ability of Acm1 to bind to Cdh1 but not to Cdc20. The top panel shows the activators alone binding to the IgG-magnetic beads, while the bottom panel shows whether Acm1 comes down when either Cdh1 or Cdc20 is pulled down with the IgG beads.

Figure 1

A.


0 1 5 10 30 0 1 5 10 30 0 1 5 10 30 0 1 5 10 30
Time (min)
Acm1
No inhibitor
Acm1
No inhibitor
Cdc20
Cdh1

B.

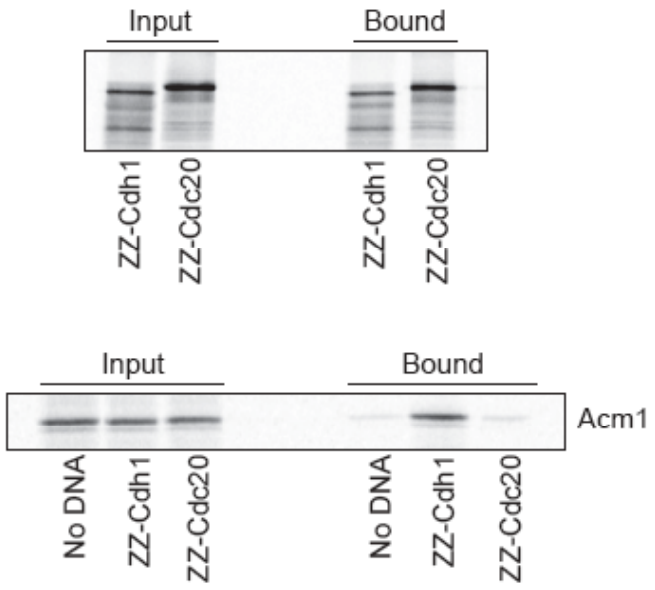

Input
Bound
ZZ-Cdh1
ZZ-Cdc20
ZZ-Cdh1
ZZ-Cdc20
Input
Bound
Acm1
No DNA
ZZ-Cdh1
ZZ-Cdc20
No DNA
ZZ-Cdh1
ZZ-Cdc20

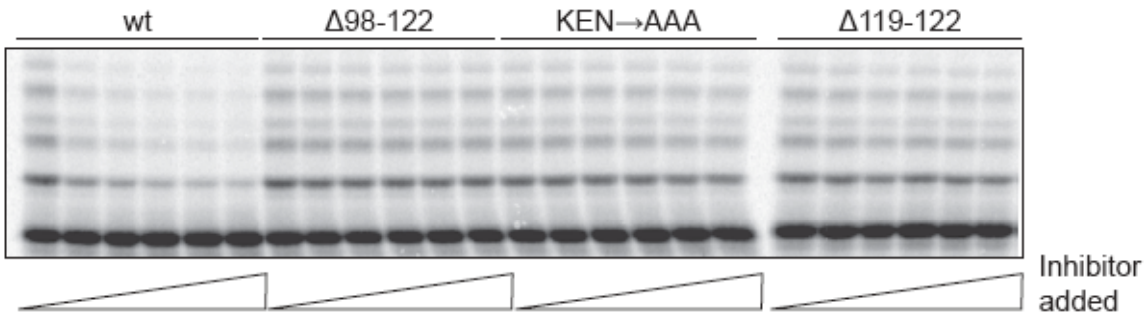
Figure 2: Inhibition by Acm1 is dependent on a pseudosubstrate domain

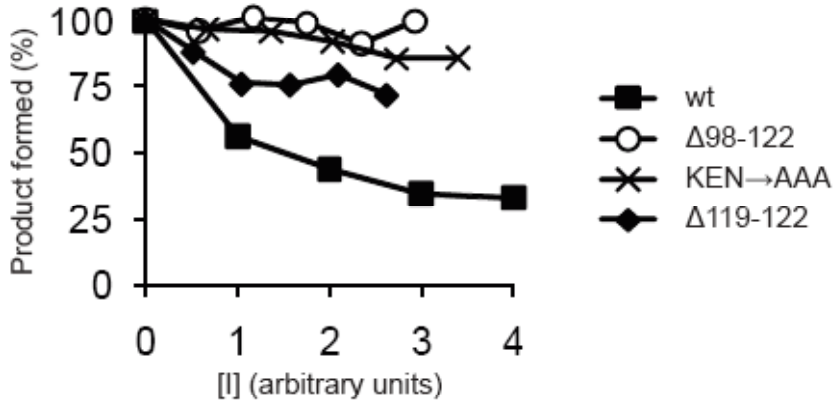
(A) The top panel shows an autoradiograph of APC assays performed with different mutants of Acm1. The bottom panel shows a quantification of the inhibition experiment. KEN->AAA refers to mutations of residues 98-100 to alanines.

(B) A plate showing the effects of the inhibition domain mutants *in vivo*. Con refers to no Acm1 on the plasmid. Wt refers to wildtype Acm1 on the plasmid. KEN to a mutant where residues 98-100 have been mutated to alanines. K refers to K98A, E to E99A, and N to N100A. RL(1) refers to a _114-117 mutant, RL(2) to _119-122, RL(1/2) to _114-117 and _119-122.

Figure 2

A.


wt
Δ98-122
KEN→AAA
Δ119-122
Inhibitor added


Product formed (%)
100
75
50
25
0
0
1
2
3
4
[I] (arbitrary units)
wt
Δ98-122
KEN→AAA
Δ119-122

B.

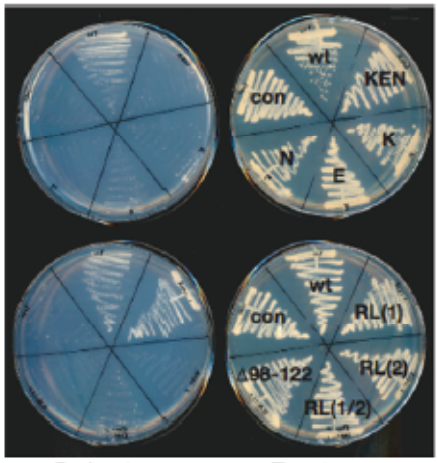
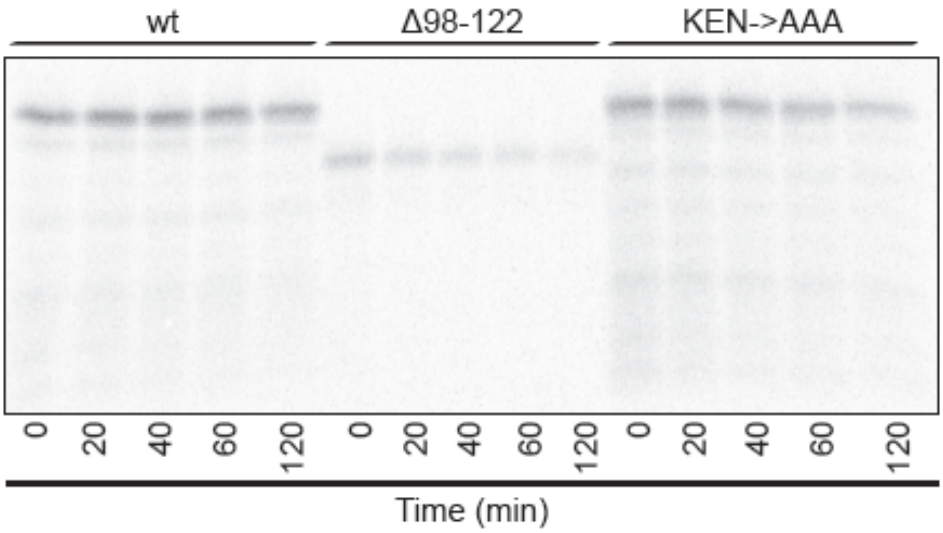

wt
con
KEN
K
N
E
wt
con
RL(1)
Δ98-122
RL(2)
RL(1/2)
Galactose
Dextrose

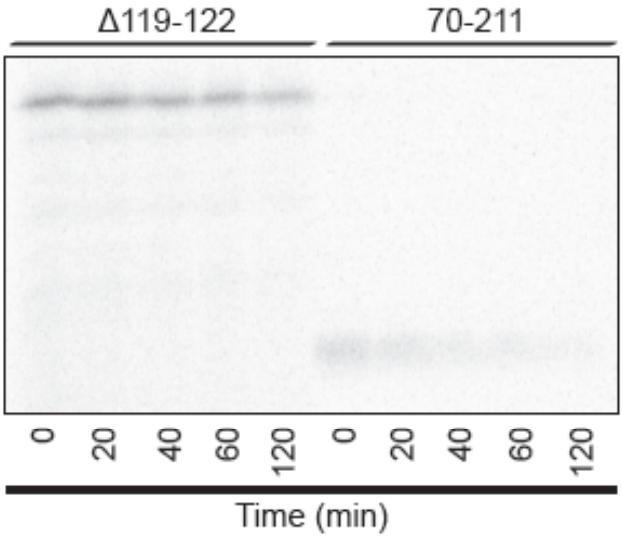
Figure 3: Acm1-Cdh1 binding is independent of the inhibition domain in Acm1

An autoradiograph of different Acm1 mutants binding to ZZ-Cdh1 linked to IgG magnetic beads during a series of washes at room temperature up to a total of 120 minutes. At each time point, another wash was performed. KEN->AAA refers to a mutant where residues 98-100 have been mutated to alanines. 70-211 refers to a mutant where the first 69 amino-acid residues have been deleted.

The bottom panel shows a quantification of the experiment, where the 0 time point is set at 100% for each mutant.

Figure 3


wt
Δ98-122
KEN->AAA
0
20
40
60
120
Time (min)


Δ119-122
70-211
0
20
40
60
120
Time (min)

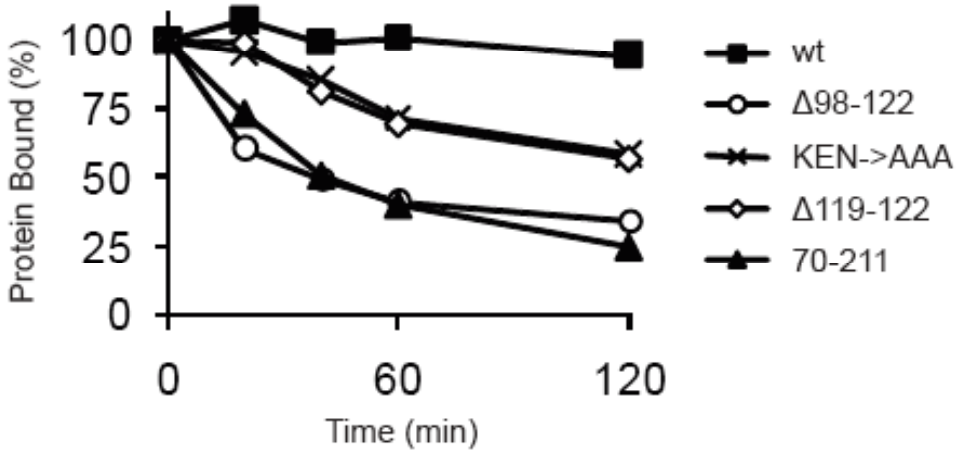

Protein Bound (%)
100
75
50
25
0
0
60
120
Time (min)
wt
Δ98-122
KEN->AAA
Δ119-122
70-211

Figure 4: Acm1 is an APC^{Cdc20} substrate

(A) Synchronous time course of *ACM1* and *acm1-dbm* cells released from alpha factor mediated arrest. Samples were analyzed for budding, chromosome segregation and the level of Acm1 protein.

(B) Western blot of the time course from 4(A) with an anti-myc antibody to follow the levels of Acm1-myc and Acm1-dbm-myc.

(C) Immunofluorescence images from 4(A) showing Acm1, chromosomes and microtubules.

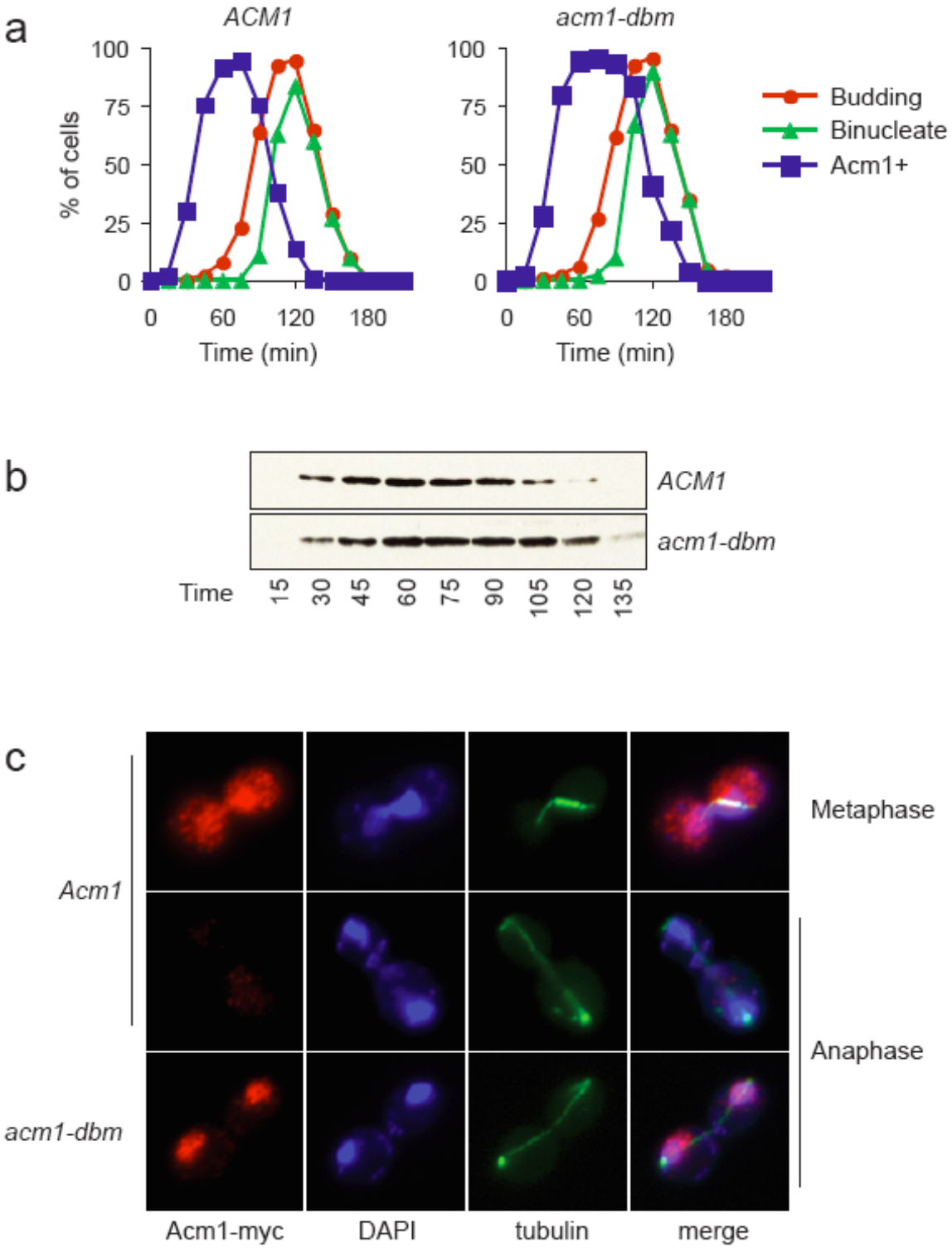
(D) An autoradiograph of APC assays using Cdc20 as the activator and either wt Acm1 or Acm1-dbm as the substrate.

(E) A Western blot showing the stability of Acm1-dbm in either *wt* or *_cdh1* background in cells released synchronously from a *cdc15-2* arrest.

(F) Samples from 4(E) were analyzed for Acm1 protein level and microtubules by immunofluorescence.

(G) Synchronous time course was performed as in 4(A). Western blots were performed against the Cdh1 substrates Clb2 and Cdc5-myc using an anti-Clb2 and an anti-myc antibody respectively.

Figure 4 A


a
ACM1
acm1-dbm
% of cells
100
75
50
25
0
0
60
120
180
Time (min)
Budding
Binucleate
Acm1+
b
ACM1
acm1-dbm
Time
15
30
45
60
75
90
105
120
135
c
Acm1
acm1-dbm
Metaphase
Anaphase
Acm1-myc
DAPI
tubulin
merge

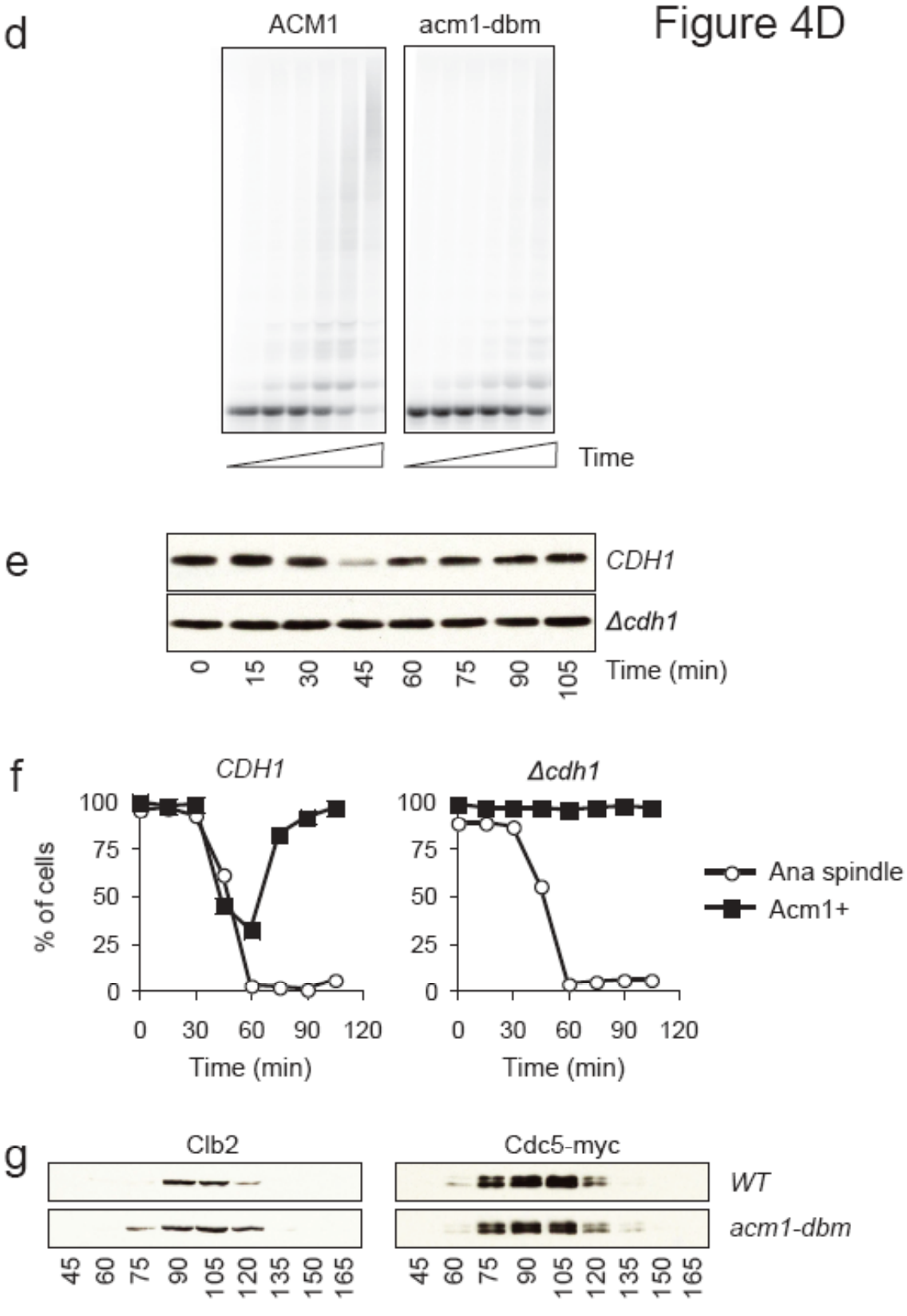

d
ACM1
acm1-dbm
Time
e
CDH1
Δcdh1
0
15
30
45
60
75
90
105
Time (min)
f
CDH1
Δcdh1
% of cells
100
75
50
25
0
0
30
60
90
120
Time (min)
Ana spindle
Acm1+
g
Clb2
Cdc5-myc
WT
acm1-dbm
45
60
75
90
105
120
135
150
165

Figure 4D

Figure 5: Acm1 localization is dependent on its phosphorylation state

(A) *ACM1* and *ACM1-dbm* cells were blocked in mitosis by nocodazole treatment, and Cdc14 with a C-terminal Pk-tag was induced by addition of galactose to the culture media. Western blots were performed to analyze the migration pattern of Acm1 using an anti-myc antibody and to analyze the expression of Cdc14-Pk using an anti-Pk antibody.

(B) Immunofluorescence showing the localization of Acm1-myc in metaphase before and after Cdc14 induction from 5(A).

(C) *ACM1* and *ACM1-dbm* cells carrying either the *cdc15-2* or *cdc14-1* temperature-sensitive mutations were arrested at metaphase by nocodazole treatment or in late mitosis by temperature shift. Western blot was performed to analyze the levels of Acm1 protein.

(D) Immunofluorescence of cells from 5(C) showing the localization of Acm1-dbm in late mitosis.

Figure 5

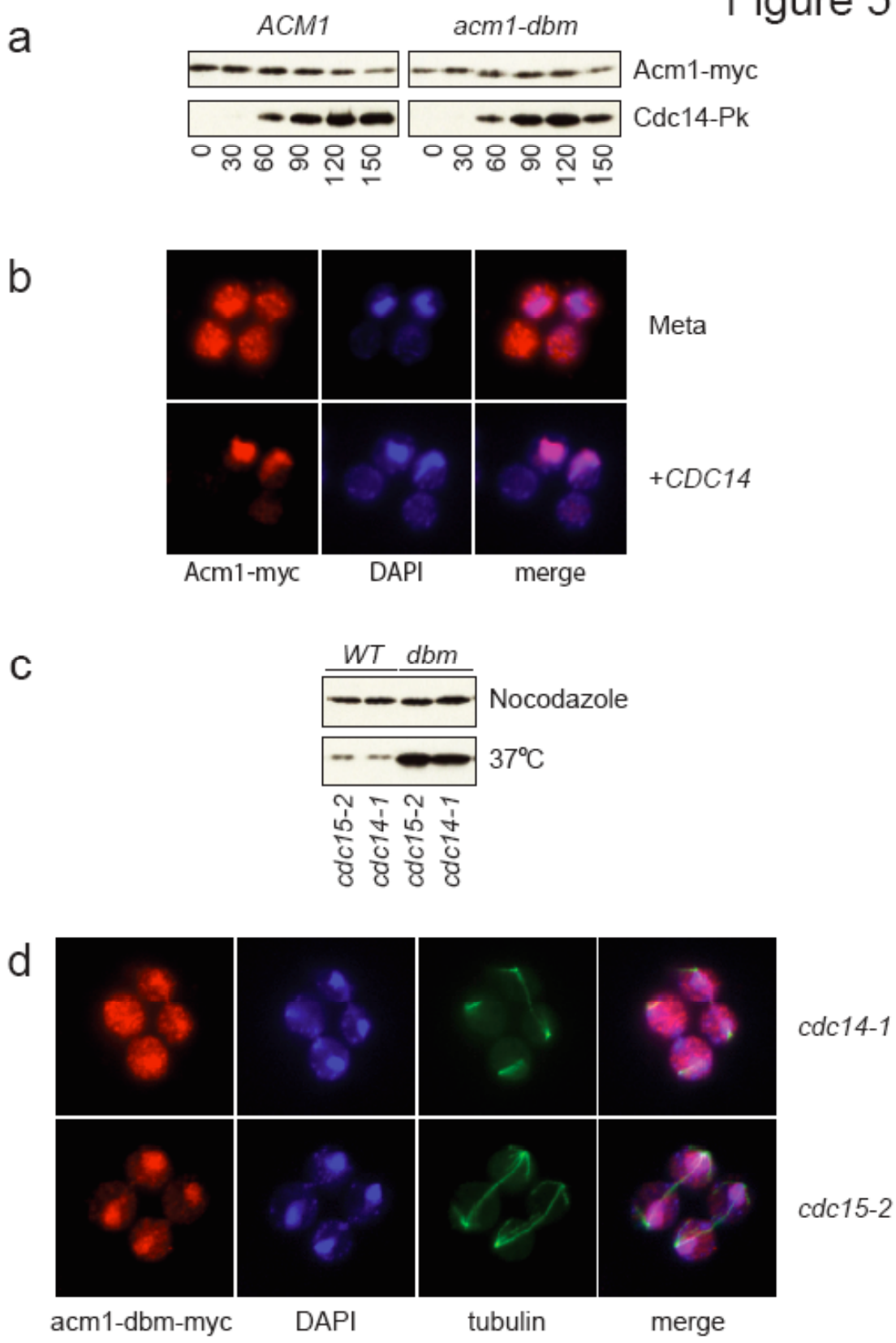
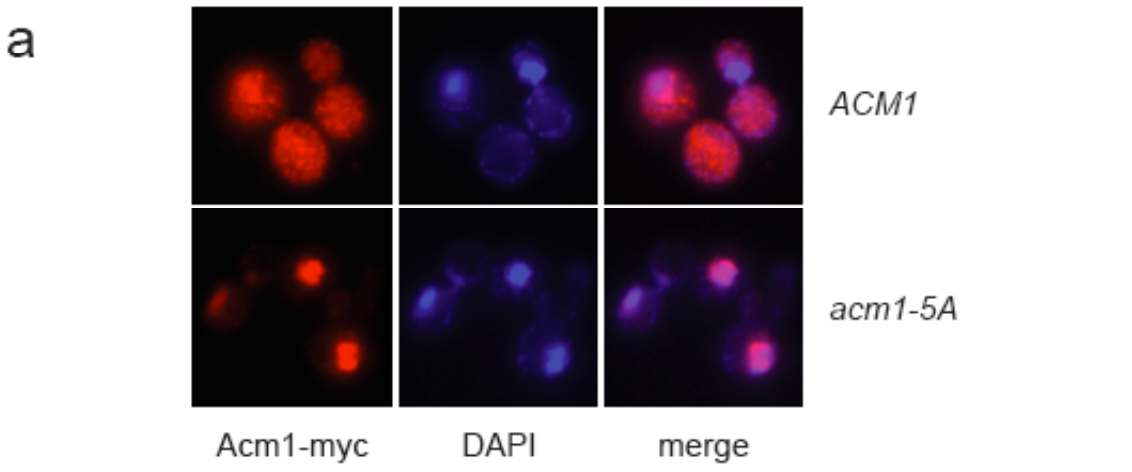

a
ACM1
acm1-dbm
Acm1-myc
Cdc14-Pk
0
30
60
90
120
150
b
Meta
+CDC14
Acm1-myc
DAPI
merge
c
WT
dbm
Nocodazole
37°C
cdc15-2
cdc14-1
cdc15-2
cdc14-1
d
cdc14-1
cdc15-2
acm1-dbm-myc
DAPI
tubulin
merge

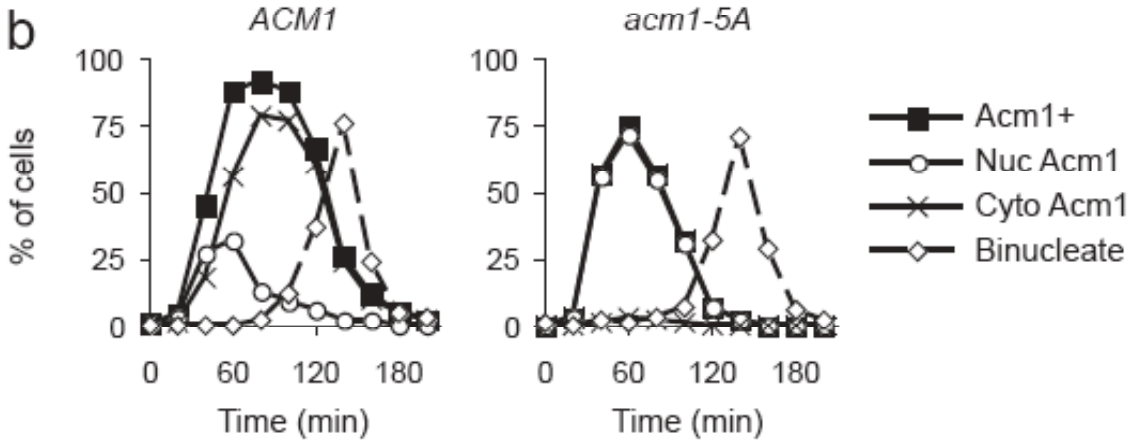
Figure 6: Acm1 localization is dependent on CDK phosphorylation

(A) Cells were arrested in metaphase by nocodazole treatment and immunofluorescence performed to show the localization of Acm1-myc and Acm1-5A-myc.

(B) Synchronous time course of *ACM1* and *ACM1-5A* cells released from alpha factor mediated arrest. Samples were analyzed for chromosome segregation and the level of Acm1 protein. Cells positive for Acm1 staining were characterized as either having exclusively nuclear signal or having cytoplasmic and nuclear staining.

Figure 6


a
ACM1
acm1-5A
Acm1-myc
DAPI
merge


b
ACM1
acm1-5A
% of cells
100
75
50
25
0
0
60
120
180
Time (min)
Acm1+
Nuc Acm1
Cyto Acm1
Binucleate

Chapter 4

Conclusions

Polyubiquitination is a critical mechanism for regulating protein turnover, but we understand little about the enzymology of this process. As we showed by the assays in chapter 2, Tom1 is an excellent polyubiquitinator and could be a useful tool to study how these processes occur. Tom1 is one of only 5 HECT-domain ubiquitin ligases in yeast, and at least 2 of the other 4 (Hul5 and Ufd4) are also known to have the ability to polyubiquitinate(Xie and Varshavsky, 2002; Crosas *et al.*, 2006). Studying these 5 enzymes as a group could be a powerful way of shedding light on the process of polyubiquitination and of HECT-domain ubiquitin-ligase enzymology. Some of the problems to investigate are: substrate recognition, the enzymology of ubiquitin transfer, the requirements for chain formation, and what types of ubiquitin chains that are formed. Other areas to investigate include the roles of these ligases and polyubiquitination *in vivo*. Moreover, it would be interesting to understand the regulation of these enzymes: a strong polyubiquitinator like Tom1 must be kept from attacking everything in the cell, which would cause havoc and premature degradation of random proteins.

An obvious area to further explore Acm1 is how it functions as an inhibitor. A big barrier we encountered was the lack of recombinant protein. If this hurdle can be overcome, it would be interesting to explore the kinetics of the reactions and identify what type of inhibitor it is (most likely competitive or mixed). A K_i could be obtained, and different mutants could be compared. These sorts of studies could be extremely useful for the APC field. Other inhibitors of the APC have been identified, but few studies have been done to characterize these inhibitors on a mechanistic level.

We showed that Acm1 is an inhibitor specific to Cdh1, and understanding this difference between Cdh1 and Cdc20 could help us understand the difference between these two APC activators. Ultimately, a structure of how Acm1 binds to Cdh1 and inserts the pseudo-substrate region would be useful for starting to understand the activator-substrate interaction and ultimately the enzymology of the APC. How can it be that Acm1 does not get ubiquitinated in that context? Does it inhibit some conformational change or does it block some other critical part of Cdh1-APC?

Further understanding the regulation of Acm1 would also be important. What role does phosphorylation play on a molecular level? Does it hide an NLS and thereby affect its localization? Does it have an effect on the interaction with Cdc20, and thereby affect its degradation?

Lastly, the availability of a pure Cdc20-APC assay in conjunction with the already existing Cdh1-APC assay will allow numerous experiments to further explore the mechanisms underlying substrate recognition and processivity of the APC.

Appendix

A functional Cdc20-APC assay and further development of general APC assays

Introduction

The APC is a complex enzyme that ubiquitinates its substrates and thereby targets them for degradation by the proteasome. The APC works in conjunction with 2 activators, Cdc20 and Cdh1, and the difference between the activators is unclear. Previously, we and others have developed APC-Cdh1 *in vitro* assays, but for technical reasons we have not been able to purify active Cdc20. Attempts to purify Cdc20 included purifying it from yeast and baculovirus, all without success. In (Passmore *et al.*, 2003), it was demonstrated that active yeast Cdc20 can be obtained by *in vitro* transcription and translation. Having this functional Cdc20-APC assay allows for comparisons of Cdc20-APC and Cdh1-APC *in vitro*, with regard to many different properties such as kinetics, substrate specificity, and processivity.

Figure 1. Functional Cdc20-APC Assay

As shown in (Passmore *et al.*, 2003), active Cdc20 can be obtained by *in vitro* transcription and translation. *In* vitro translated Cdc20 was added as the activator to APC reactions with securin (Pds1). Time points were taken as indicated, and as can be seen, great stimulation took place when Cdc20 was added as opposed to lysate alone.

Methods: 30 _l of TnT activator was mixed with 20 _l TnT Pds1 (^{35}S-Met labeled), 24 _l E1/E2 mix, 6 _l APC, 2 _l ubiquitin aldehyde (100 _M stock, Boston Biochem). 15 _l were taken out at each timepoint: 0, 1, 2, 5, 10, 30, 50 minutes. Samples were separated by 7.5% SDS-PAGE , fixed, dried and exposed to phosphor screens.

Figure 1

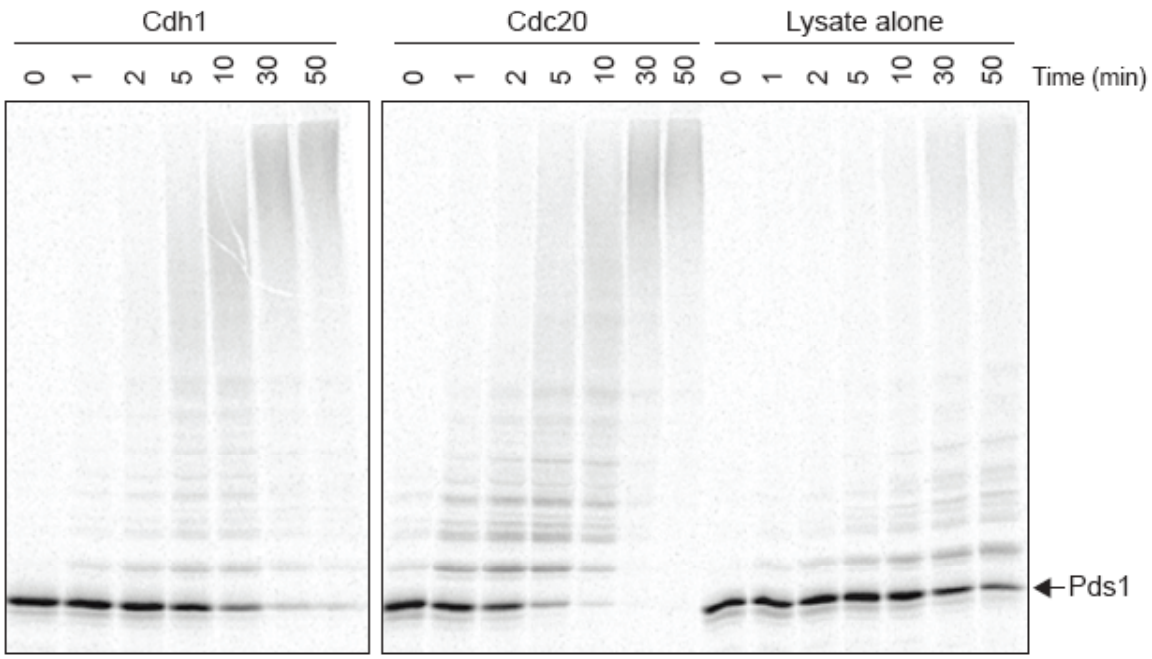

Cdh1
Cdc20
Lysate alone
0 1 2 5 10 30 50
0 1 2 5 10 30 50
0 1 2 5 10 30 50
Time (min)
Pds1

Figure 2. NEM blocks extending activity in reticulocyte lysates and also inactivates Cdc20-APC

We discovered that the extending activity in yeast lysates is due to Tom1, a HECT-domain ubiquitin-protein ligase (see chapter 2). The activity of a HECT-domain ubiquitin-ligase is susceptible to treatment with N-Ethylmaleimide (NEM), which covalently blocks cysteine residues. To see if the extending activity in the rabbit reticulocyte lysate is behaving like a Tom1-homolog, we investigated if its activity was NEM-sensitive.

This turned out to be true (figure 2). Lane 7 shows a typical APC-Cdh1 reaction that has been treated with reticulocyte lysate, showing the high smear in the lane. In lane 8 the reticulocyte lysate was treated with NEM before addition to the APC-Cdh1 reaction, and no high smear can be seen. This presents a great new tool where substrates can be *in vitro* translated, lysates treated with NEM, and then used straight in assays without purification, and still be analyzed in a quantitative way.

Unpurified Cdh1 in reticulocyte lysates can also be treated with NEM and remain active. This means that quantitative APC assays can be performed using *in vitro* translated Cdh1, which will greatly facilitate studies of Cdh1 mutants.

Unfortunately the same is not true for Cdc20: when unpurified Cdc20 in reticulocyte lysates is treated with NEM it loses its ability to activate the APC

(compare depletion in lanes 3, 4, and 5). Thus Cdc20 is susceptible to NEM treatment and NEM cannot be used to develop a quantitative *in vitro* APC-Cdc20 assay.

Methods: Cdh1 and Cdc20 were *in vitro* transcribed and translated using Promega TnT quick couple transcription/translation kit with unlabeled methionine. The substrate (Pds1) was *in vitro* translated with ^{35}S-labeled methionine. Samples were treated with 10mM NEM where indicated (see figure) for 10 minutes, followed by 20mM DTT where indicated for 10 minutes.

In each reaction, 4 _l E1/E2 mix was mixed with 1 _l APC, 2 _l TnT activator, 2 _l Pds1, 1 _M ubiquitin aldehyde, 5 _l buffer. Reactions were incubated at room temperature for 30 minutes. Samples were separated by 7.5% SDS-PAGE , fixed, dried and exposed to phosphor screens.

Figure 2

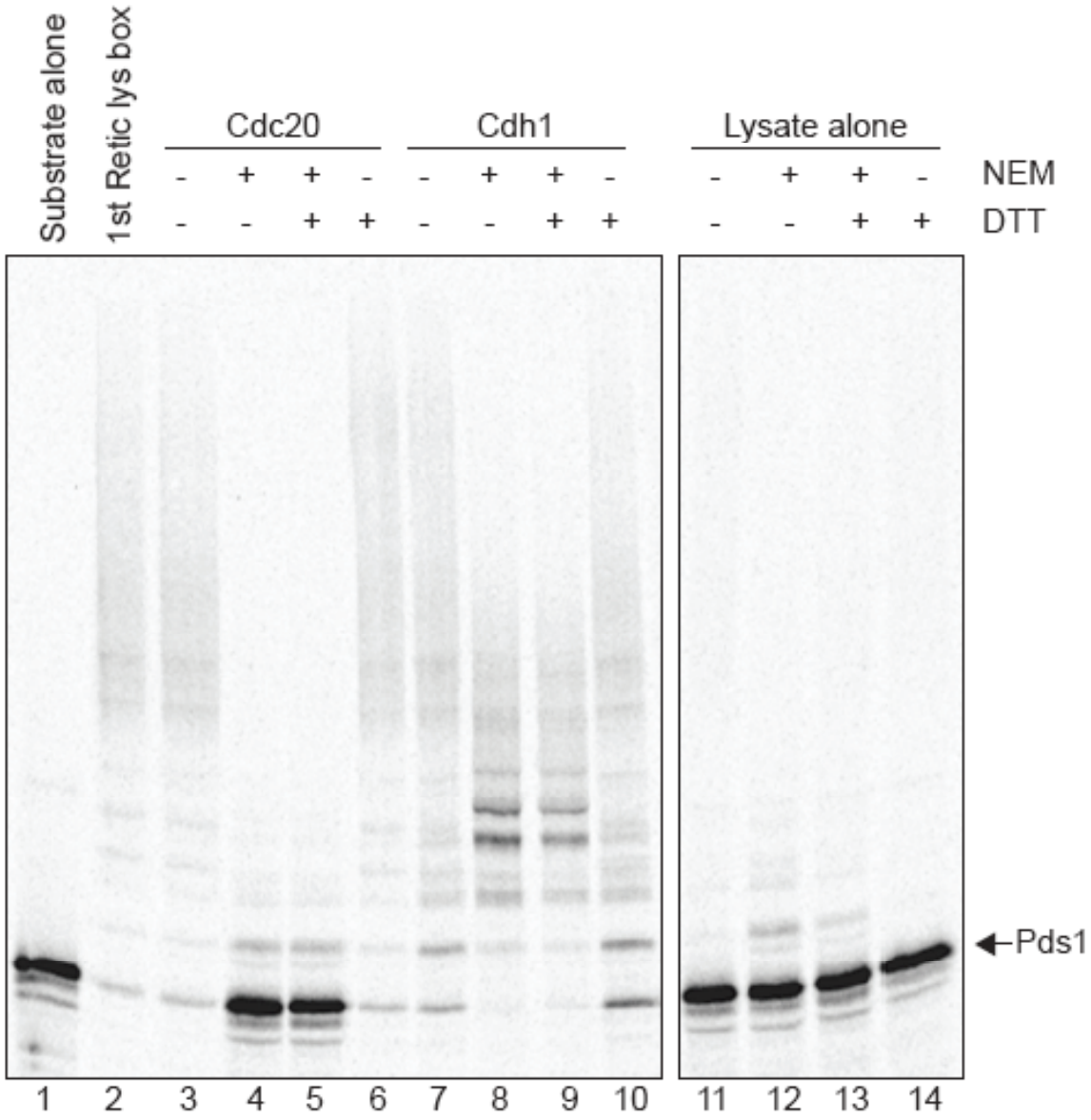

Substrate alone
1st Retic lys box
Cdc20
Cdh1
Lysate alone
NEM
DTT
- + + - - + + - - + + -
- - + + - - + + - - + +
Pds1
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 3. APC assays with purified Cdc20

As seen in Figure 2, Cdc20 activity does not survive NEM treatment. Although we can *in vitro* translate Cdc20 in reticulocyte lysates, the presence of the extending activity prevents quantitative analysis of APC-Cdc20 substrate ubiquitination. To develop a more useful Cdc20-APC assay we attempted to purify Cdc20 away from the reticulocyte lysate after translation. Cdc20 with an N-terminal ZZ-tag followed by a TEV protease cleavage site was *in vitro* transcribed and translated. It was then bound to magnetic IgG beads (either at 4°C or at room temperature) and the reticulocyte lysate was washed away in 3 quick wash steps, after which Cdc20 was cleaved away from the beads with TEV protease. This cleaved product was then added to APC assays as the only source of APC activator. As can be seen in figure A1-3, Cdc20 purified this way can activate the APC. In lane 8, unpurified *in vitro* translated Cdc20 is used in the APC assay and some ubiquitination can be seen. In the following 4 lanes (9-12) different amounts of purified Cdc20 were added to the APC reactions, and one can see significant ubiquitination of the substrate. Also note the depletion of unmodified substrate. Lanes 2-7 show the same experiment but with purified Cdh1 as the activator.

It is very encouraging that this approach to the purification of Cdc20 works, although one can only obtain very small amounts of Cdc20. It clearly has reduced activity. Substrate depletion from APC-Cdc20 requires approximately 10 fold more purified than unpurified material. One likely explanation for the loss of

activity is that Cdc20 requires the CCT chaperone for activity. Immunoprecipitation and washing of Cdc20 may result in a loss of the CCT complex and hence reduced APC-Cdc20 activity. However, even with these flaws it is a big breakthrough to be able to use Cdc20 in a purified *in vitro* system.

Methods: Cdc20 and Cdh1 with an N-terminal ZZ-tag followed by a TEV cleavage site were *in vitro* transcribed and translated (TnT, Promega). 50 _l TnT mix containing the translated activator was added to 50 _l IgG magnetic beads, 400 _l TAP-core buffer (20mM Hepes pH 8.0, 150mM NaCl, 0.1% NP-40) was added, and incubated with rotation, either at 4°C for 1 hour or at room temperature for 20 min as indicated. Beads were washed 3 times with 500 _l TAP-core buffer, then resuspended in 20 _l TAP-core buffer + 1mM DTT and 1 _l TEV protease, and cleaved at room temperature for 20 minutes. Supernatants were collected and used in APC assays.

Each APC assay: 4 _l E1/E2 mix, 0.5 _l APC, 2 _l substrate (Pds1, ^{35}S-labeled and purified away from reticulocyte lysate), activator (2 _l recombinant, or as indicated purified), buffer up to 15 _l. Incubated reactions at room temperature for 30 minutes. Samples were separated by 7.5% SDS-PAGE , fixed, dried and exposed to phosphor screen.

Figure 3

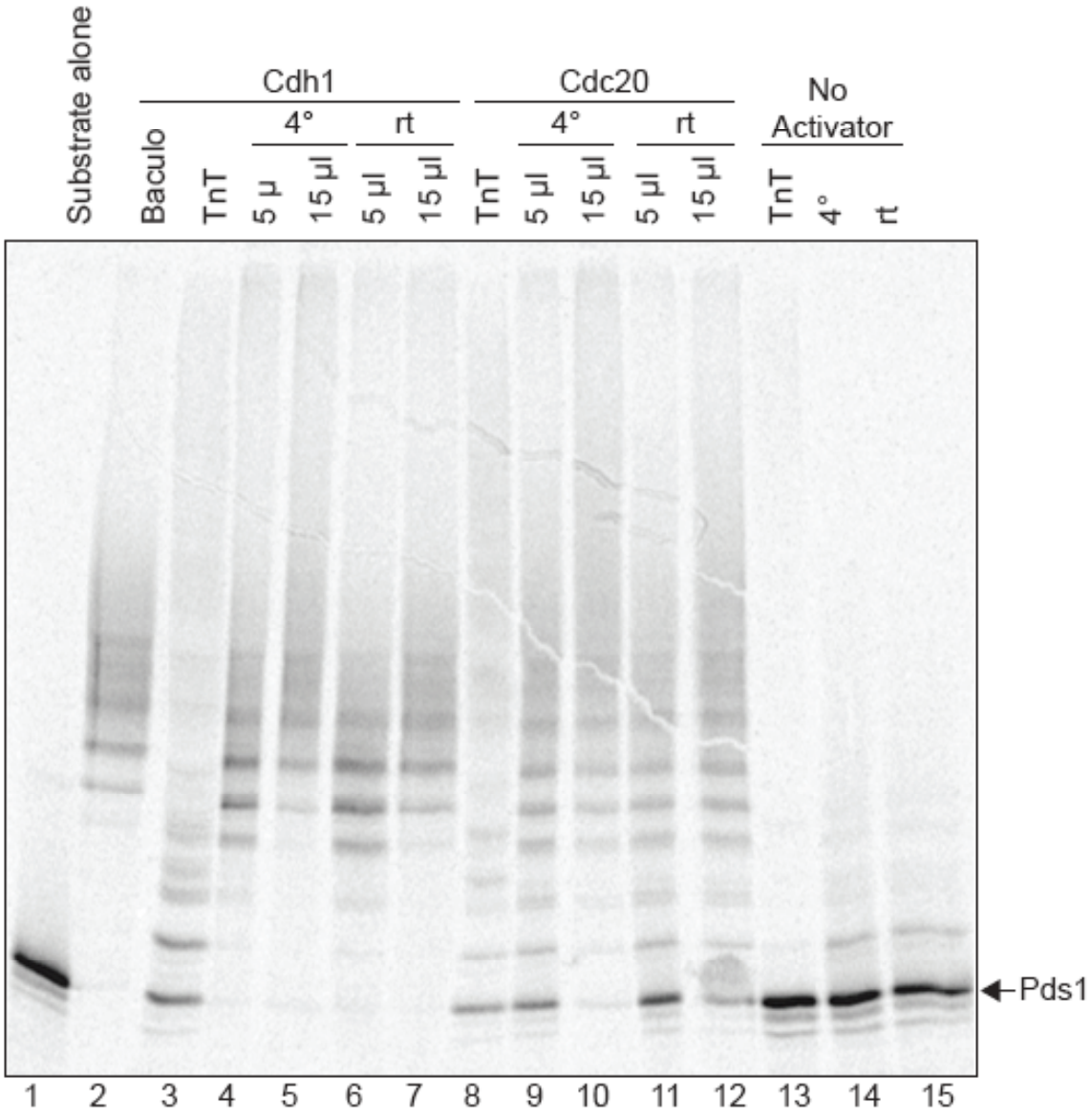

Substrate alone
Cdh1
Cdc20
No Activator
Baculo
TnT
4°
rt
5 µ
15 µl
5 µl
15 µl
TnT
4°
rt
5 µl
15 µl
5 µl
15 µl
TnT
4°
rt
Pds1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 4. Analysis of APC-Cdh1, APC-Cdc20, and APC-Ama1 substrate specificity *in vitro*

One possible difference between different APC-activator complexes is that they target different substrates for degradation. With the developed APC-Cdc20 assay we were able to test this hypothesis by investigating substrate specificity *in vitro*. Additionally we tested the meiotic-specific activator Ama1. All three activators were *in vitro* translated and mixed with APC and a panel of substrates and incubated for 60 minutes.

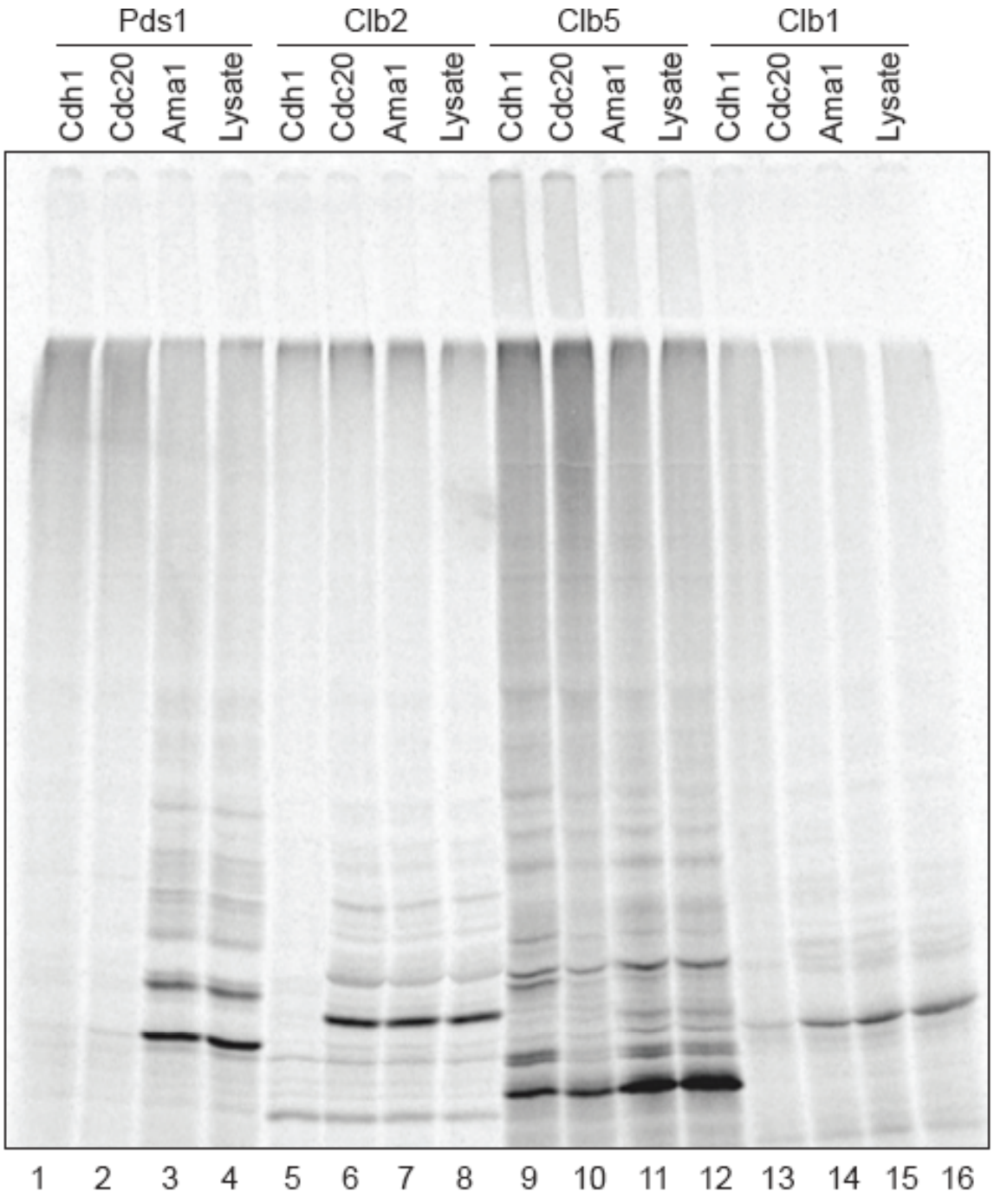
The first thing to note is that Ama1 did not work as an activator of the APC in these assays (figure A1-4, compare Ama1 lanes to lysate alone lanes: lane 3 to 4, 7 to 8, 11 to 12 and 15 to 16). We never managed to get the Ama1 assay to work *in vitro*, even when using APC from an *mnd2_* strain (Mnd2 is a cellular inhibitor of Ama1).

We found that Clb1 and Clb2 behave as Cdh1-specific substrates: they are ubiquitinated by Cdh1-APC (lanes 13 and 5), but not by Cdc20-APC (lanes 14 and 6). Pds1 appears to be a good substrate of both activators (lanes 1 and 2), but relative kinetic differences are likely obscured as the substrate is totally depleted. Lastly, Clb5 appears to be a Cdc20 specific substrate: it is better ubiquitinated by Cdc20-APC (lane 10) than by Cdh1-APC (lane 9). From these results we conclude that different APC-activator complexes display substrate preferences.

Future goals along the lines of these experiments are to test the substrate specificity for all known APC substrates. Mutations of different substrates in their recognition motifs will also be tested, and with these kinds of studies one could develop a much deeper mechanistic understanding of APC substrate recognition.

Methods: Activators (Cdh1, Cdc20 and Ama1) were *in vitro* transcribed and translated. Different substrates (Pds1, Clb2, Clb5, and Clb1) were *in vitro* translated in the presence of ^{35}S-methionine. APC reactions were preformed with 4 _l E1/E2 mix, 1 _l APC, 0.5 _l Ubiquitin aldehyde (100 _M stock), 0.5 _l MG-132 (1mM stock), 5 _l substrate, 5 _l activator. Incubated at room temperature for 1 hour. Samples were separated by SDS-PAGE, fixed, dried and exposed to phosphor screen.

Figure 4


Pds1
Clb2
Clb5
Clb1
Cdh1
Cdc20
Ama1
Lysate
Cdh1
Cdc20
Ama1
Lysate
Cdh1
Cdc20
Ama1
Lysate
Cdh1
Cdc20
Ama1
Lysate
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

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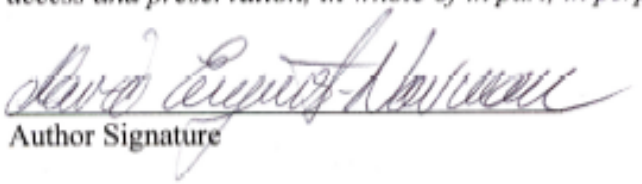

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