The Doc1 subunit is a processivity factor for the anaphase-promoting complex

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Ubiquitin-mediated proteolysis of securin and mitotic cyclins is essential for exit from mitosis. The final step in ubiquitination of these and other proteins is catalysed by the anaphase-promoting complex (APC), a multi-subunit ubiquitin-protein ligase (E3). Little is known about the molecular reaction resulting in APC-dependent substrate ubiquitination or the role of individual APC subunits in the reaction. Using a well-defined *in vitro* system, we show that highly purified APC from *Saccharomyces cerevisiae* ubiquitinates a model cyclin substrate in a processive manner. Analysis of mutant APC lacking the Doc1/Apc10 subunit ($APC^{doc1\Delta}$) indicates that Doc1 is required for processivity. The specific molecular defect in $APC^{doc1\Delta}$ is identified by a large increase in apparent K_M for the cyclin substrate relative to the wild-type enzyme. This suggests that Doc1 stimulates processivity by limiting substrate dissociation. Addition of recombinant Doc1 to $APC^{doc1\Delta}$ fully restores enzyme function. Doc1-related domains are found in mechanistically distinct ubiquitin-ligase enzymes and may generally stimulate ubiquitination by contributing to substrate–enzyme affinity.

The covalent attachment of ubiquitin to proteins is a common regulatory mechanism in a number of cellular processes. Ubiquitination of a substrate requires the activity of a cascade of three enzymes, beginning with the ubiquitin-activating enzyme (E1), which forms a covalent thioester bond with ubiquitin on an active-site cysteine[1,2]. In the second step, an ubiquitin-conjugating enzyme (E2) transiently receives the activated ubiquitin from E1, again on a conserved cysteine residue. Finally, a ubiquitin-protein ligase (E3) transfers ubiquitin from E2 to a lysine side-chain on the target protein. In most cases, repeated cycles of the ubiquitination reaction result in modification of several lysines in the target protein. In addition, lysines within ubiquitin itself can be ubiquitinated, resulting in the assembly of poly-ubiquitin chains.

The substrate specificity and regulation of ubiquitination is usually conferred by the E3 ubiquitin-protein ligase, which binds directly to the target protein and is typically the rate-limiting component in the ubiquitination cascade[2]. All known ubiquitin-protein ligases belong to one of two families: the HECT-domain family and the RING-H2-domain family. The mechanism by which HECT-domain E3 enzymes catalyse substrate ubiquitination is well understood and requires the formation of a covalent E3–ubiquitin intermediate on an active-site cysteine[3]. In contrast, it is not clear how RING-H2-domain enzymes catalyse substrate ubiquitination, although their reaction mechanism probably involves the direct transfer of ubiquitin from an E2 to the protein substrate. Therefore, to catalyse the ubiquitination reaction, RING-H2 enzymes must bind simultaneously to both an E2–ubiquitin conjugate and a target substrate. It is not known if RING-H2 E3s function simply as scaffolds to position E2 next to the substrate, or whether they are involved more directly in catalysing substrate ubiquitination.

In late mitosis and throughout G1, several proteins, including securin (an inhibitor of sister chromatid separation) and mitotic cyclins, undergo poly-ubiquitination, a process that targets them to the proteasome for destruction[4,5]. The ubiquitination of these and other mitotic proteins is controlled by the APC or cyclosome, a RING-H2-domain E3 ligase. The APC is a multi-subunit enzyme consisting of at least 12 proteins that are conserved across eukaryotic species.

The activity and substrate specificity of APC are controlled by sub-stoichiometric activator proteins called Cdc20 and Cdh1/Hct1 (refs 4, 5). Activation of the APC in mitosis is triggered by association with Cdc20, whereas the subsequent binding of Cdh1 maintains APC in an active state throughout G1. Cdc20 and Cdh1 also contribute to the substrate specificity of the APC. Both proteins bind directly to target substrates and are thought to recruit them to the APC for ubiquitination[6–9]. Target-proteins contain short amino-acid sequence motifs, called the destruction box and KEN box, that are required for their interaction with Cdc20 and Cdh1.

Two subunits of the core APC, Apc11 and Apc2, are thought to form the catalytic centre of the enzyme[4,5]. Apc11 is a small subunit that contains the RING-H2 domain. In common with many proteins with RING-H2 domains, recombinant Apc11 alone is sufficient to bind E2 and catalyse substrate ubiquitination, but this process is inefficient and lacks the specificity of the APC holoenzyme[10–12]. A second APC subunit, Apc2, belongs to the cullin family of proteins, whose members are also found in other ubiquitin ligases. The cullin domain of Apc2 interacts with Apc11 and may also contribute to E2 binding in some cases[12]. RING-H2–cullin heterodimers are also found in the APC-related E3 enzymes VBC and SCF, suggesting that they possess a similar molecular architecture.

The Doc1 subunit of the APC is likely to be important in the ubiquitination reaction. Cells containing temperature-sensitive mutations in *DOC1* arrest in mitosis and are unable to target substrates for destruction at the restrictive temperature[13,14]. Doc1 binds directly to Apc11, placing it in close proximity to the catalytic centre of the enzyme[12]. Furthermore, sequence analysis has identified domains related to the Doc1 protein (called 'Doc domains') in a number of other putative ubiquitin-ligase enzymes[15]. Interestingly, a subset of the putative E3 enzymes containing Doc domains belong to the HECT-domain family of ubiquitin-ligases, suggesting that Doc domains are involved generally in protein ubiquitination reactions.

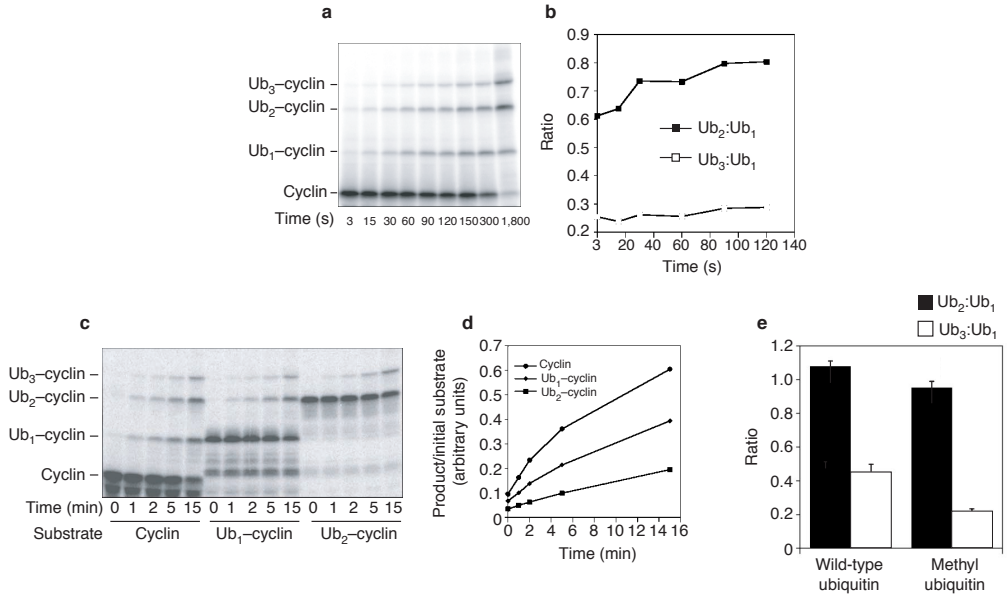

Figure 1 **The APC is processive *in vitro*. a**, Purified APC (~0.5 nM) was incubated for the indicated times in a complete reaction mixture (see Methods) with an ^{125}I-labelled fragment of sea urchin cyclin B (~100 nM). Ub_1-, ub_2- and ub_3-cyclin fragments represent the mono-, di- and tri-ubiquitinated forms of cyclin, respectively. This data is representative of results from three independent experiments. **b**, The data from **a** was quantified and the ratios of di- to mono- (ub_2:ub_1) and tri- to mono- (ub_3:ub_1) ubiquitinated cyclins is plotted as a function of time. The ratios of ub_2:ub_1 and ub_3:ub_1 at 1,800 s were 2.27 and 1.58, respectively. **c**, Individual reaction products were extracted from gels as described[24]. Gel-extracted unmodified, mono (ub_1)-, or di (ub_2)-ubiquitinated cyclins were then re-used as substrates in APC reactions, as in **a**. The concentration of substrate in these reactions was ~0.25 nM. This data is representative of results from three independent experiments. **d**, The data from **c** was quantified and the ratio of product to initial substrate is plotted as a function of time. **e**, APC reactions were performed as in **a**, using either wild-type or methylated ubiquitin. Reactions were stopped after 2 min, analysed by SDS–PAGE and quantified. The ratios are the average of three experiments; error bars represent the standard error of the mean.

Little is known about the mechanisms underlying the ubiquitination of substrates by the APC. It is not clear, for example, how the APC and E2 enzymes are coordinated to promote multi-ubiquitination of a single protein target. One simple possibility is that the reaction is distributive, whereby the addition of each new ubiquitin involves a separate interaction between the substrate and the APC. Alternatively, the reaction could be processive, such that repeated cycles of ubiquitination occur while the substrate remains bound to the APC. In this work, we examined these and other issues by studying the substrate ubiquitination reaction with highly purified budding yeast APC and the activator Cdh1. We show that the APC can processively ubiquitinate a model cyclin substrate in a reaction that requires multiple E2 molecules. The Doc1 subunit enhances the processivity of the APC, probably by increasing the affinity of the substrate–enzyme complex. We conclude that Doc domains in other ubiquitin ligases may have similar functions.

Results

The APC is a processive enzyme *in vitro*. Previously, we reconstituted the ubiquitination of a model cyclin substrate *in vitro* using APC purified from *S. cerevisiae*[16]. In addition to APC, ubiquitination of the cyclin substrate required E1, E2, ubiquitin, ATP and the activator Cdh1. Efficient ubiquitination also required that the cyclin substrate have an intact destruction box. The requirements for cyclin ubiquitination *in vitro* were therefore similar to the requirements for cyclin ubiquitination *in vivo*, suggesting that this is a valid system for studying APC-dependent protein ubiquitination.

In this study, we explored the APC-dependent reaction mechanism in more detail. We isolated highly purified APC from a budding yeast strain in which the APC subunit Cdc16 is modified with a carboxy-terminal tandem-affinity purification (TAP) tag. The TAP tag allows for the rapid and efficient purification of protein complexes using two sequential affinity steps (see Fig. 3 and Methods)[17]. TAP-tagged Cdc16 is expressed under the control of its endogenous promoter and is fully functional *in vivo* (data not shown). APC purified from asynchronous cultures by this method is highly active (see below) and produces the same reaction products as APC immunoprecipitates.

We measured the ability of purified APC to add ubiquitin to a radiolabelled cyclin substrate. In a complete reaction, purified APC generated at least three distinct products, representing mono (Ub_1)-, di (Ub_2)- and tri (Ub_3)-ubiquitinated cyclin substrates (Fig. 1a). All three products were detectable at the earliest times in the reaction and the ratio of these products was constant over time (Fig. 1b). After long reaction times (over 30 min), unmodified substrate was gradually depleted and ubiquitinated substrates were re-used, generating increased ratios and higher molecular-weight ubiquitin conjugates. The presence of multi-ubiquitinated products, despite the high relative concentration of unmodified substrate at early time points, strongly suggests that cyclin multi-ubiquitination occurs processively (that is, during a single cyclin–APC binding event). Consistent with this, product distributions are also constant over a wide range of substrate and Cdh1 concentrations (data not shown, but see Fig. 6).

It is possible, however, that substrate multi-ubiquitination could still be distributive in our experiments if the ubiquitinated species

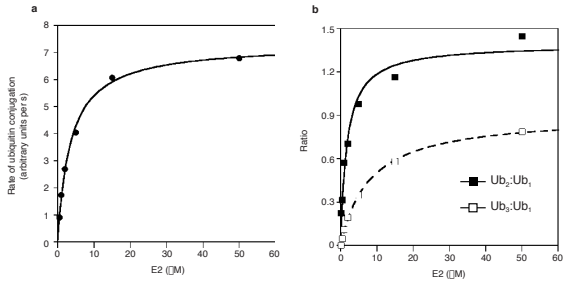

Figure 2 **Substrate multi-ubiquitination requires multiple E2 enzymes.** Recombinant E2 (Ubc4) was added at the indicated concentrations to complete reactions containing ~0.1 nM APC. Reactions were allowed to proceed until approximately 1% of the total cyclin substrate was used, at which point they were stopped and analysed by SDS–PAGE. Total ubiquitin–cyclin ligase activity (**a**) and the ratios of di- to mono- (ub_2:ub_1) and tri- to mono- (ub_3:ub_1) ubiquitinated species (**b**) were determined. Data were fitted to a rectangular hyperbola. This data is representative of results from three independent experiments.

Table 1 Summary of kinetic data

	App K_M E2 (μM)	*1/2 max Cdh1* (nM)	App K_M cyclin (nM)
WT	3.81 ± 0.44	14.11 ± 2.47	63.21 ± 11.05
doc1Δ	3.12 ± 0.77	9.88 ± 2.19	>1500
doc1Δ + rDoc1	N.D.	N.D.	85.00 ± 13.77

The data from Figs 5 and 6 are summarized. All values are the average of three independent experiments with the standard error of the mean. App K_M, apparent K_M; N.D., not determined.

are used much more efficiently than the unmodified species; that is, for example, if $k_{cat}/K_{M(Ub2)} \gg k_{cat}/K_{M(Ub1)} >> k_{cat}/K_{M(Ub0)}$. To address this possibility, we directly compared the rate of ubiquitination of unmodified substrate with that of substrates that had been previously mono- or di-ubiquitinated. APC reactions that generated equal amounts of these products were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and unmodified, mono- or di-ubiquitinated products were individually extracted from gels and re-used as substrates (Fig. 1c). To normalize between samples, we divided the amount of product at each time by the original amount of substrate and plotted the data as a function of time (Fig. 1d). These data demonstrate that the modified cyclin substrates were not used more efficiently than unmodified cyclin, confirming that substrate multi-ubiquitination occurs processively.

Efficient proteasomal targeting requires that a substrate have a poly-ubiquitin chain containing at least four ubiquitin molecules linked through Lys 48 of ubiquitin[18]. To determine if substrate multi-ubiquitination is the result of Lys 48-linked poly-ubiquitin chain assembly, we performed reactions with methylated ubiquitin, a modified form of ubiquitin that cannot form poly-ubiquitin chains (Fig. 1e). Methylated ubiquitin had only minor effects on the distribution of products, demonstrating that the majority of ubiquitin was conjugated directly to cyclin substrate. High-molecular-weight conjugates detected after long reaction times (see Fig. 4a below) are also present in reactions with methylated ubiquitin (data not shown). Similar results were obtained using a mutant form of ubiquitin in which Lys 48 was mutated to alanine (data not shown). We conclude that the APC can processively ubiquitinate a target-protein *in vitro*, but that it does so primarily by ligating multiple ubiquitin molecules to lysine residues in the cyclin substrate. Whether the yeast APC alone has the ability to rapidly assemble Lys 48-linked poly-ubiquitin chains on a substrate remains unclear.

Processive substrate ubiquitination requires multiple E2 enzymes. Our evidence suggests that cyclin substrates generally receive multiple ubiquitins during each interaction with the APC. Given that each E2 enzyme carries only a single ubiquitin, there are two simple models that could explain the E2 dynamics of the reaction. First, a single E2 enzyme may bind the APC with relatively high affinity and receive ubiquitin from E1 within this complex for transfer to the substrate. Re-iteration of this cycle while associated with a single cyclin substrate would result in processive ubiquitination. Alternatively, the APC–E2 interaction could be transient, with multiple APC–E2 interactions occurring per APC–substrate interaction.

These models can be distinguished by examining the relationship between E2 concentration and the distribution of reaction products. If a single E2–APC interaction is sufficient to generate processive multi-ubiquitination of a substrate, then the ratio of those products should be constant over a wide range of E2 concentrations. However, if multiple E2 enzymes are required to processively ubiquitinate a given substrate, the relative fraction of multi-ubiquitinated products should increase with increasing E2 concentration.

We determined the rate of the reaction and the ratio of di-:mono-ubiquitinated and tri-:mono-ubiquitinated species over a wide range of E2 concentrations. As expected, increasing the concentration of E2 enzyme potently stimulated the rate at which cyclin–ubiquitin conjugates were generated (Fig. 2a). The ratio of reaction products was also highly sensitive to the concentration of E2 in these reactions (Fig. 2b). All reactions were performed under conditions where E1, ubiquitin, and ATP concentrations were saturating, suggesting that all the E2 was conjugated with ubiquitin. Thus, we conclude that multiple E2 molecules are required to processively ubiquitinate cyclin substrates. However, we cannot be certain whether there are multiple E2 binding sites per APC or if multiple E2 molecules cycle in and out of a single binding site.

Doc1 is required for APC activity *in vivo*. Having characterized the ubiquitination reaction in detail, we then asked whether this assay could be used to dissect the function of individual subunits within the APC. Of particular interest is analysis of APC lacking the Doc1 subunit. Cells lacking *DOC1* are viable but temperature-sensitive[14]. At the permissive temperature (23 °C), *doc1Δ* cells proliferate slowly, accumulate with large buds and mitotic spindles (Fig. 3a, b), and are defective in the mitosis-specific degradation of the APC substrates securin (Pds1) and the mitotic cyclin Clb2 (data not shown). These data are consistent with a partial defect in APC function that is not confined to a single substrate or specific to the activators Cdc20 or Cdh1.

To begin to explore the molecular function of Doc1, we purified the APC from wild type and *doc1Δ* cells. Analysis of complexes from cells growing at the permissive temperature revealed that, with the exception of the Doc1 subunit, the overall levels and subunit stoichiometry of the APC were identical in wild type and *doc1Δ* cells, as judged by silver staining (Fig. 3c) and western blotting (Fig. 3d). The Apc11 and Cdc26 subunits stain poorly and are difficult to visualize by silver stain; however, both are present in equal quantities in the two complexes (data not shown and Fig. 3d).

When cells were grown at the restrictive temperature, the yield of $APC^{doc1\Delta}$ was reduced at least tenfold when compared with wild-type APC from cells at 37 °C or to $APC^{doc1\Delta}$ from cells at the permissive temperature (data not shown). Therefore, Doc1 seems to affect multiple features of APC function *in vivo*. At 37 °C, it is required for the maintenance of normal APC levels. However, at 23 °C, Doc1 is not required for the assembly or stability of the APC, suggesting that some other aspect of APC function must be defective.

Doc1 is required for efficient substrate ubiquitination *in vitro*. To understand the biochemical basis of the APC defect in *doc1Δ* cells

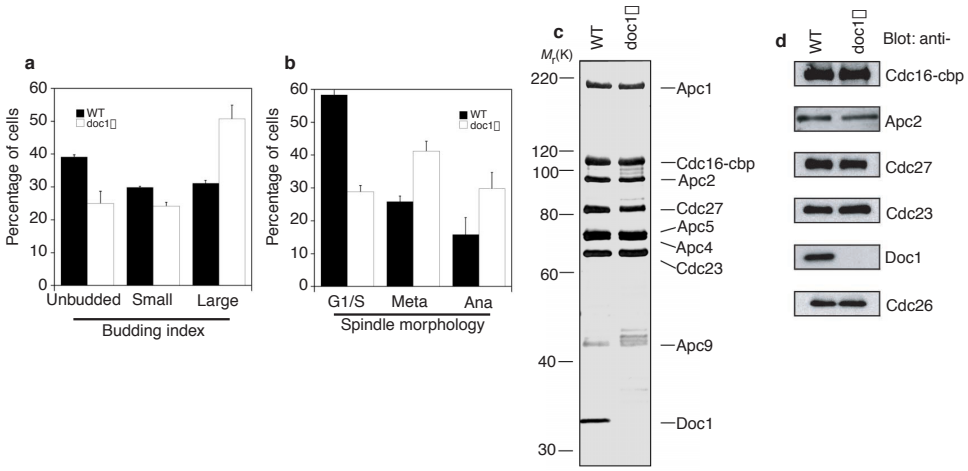

Figure 3 **Doc1 is required for APC activity but not assembly.** Asynchronous wild-type and *doc1Δ* cells were grown to log phase at 23 °C and fixed in 3.7% formaldehyde. For each sample, we measured the percent of unbudded, small budded or large budded cells (**a**) and the percentage of G1/S, metaphase (meta) or anaphase (ana) spindles (**b**). Spindles were visualized by indirect immunofluorescence with anti-tubulin antibodies as described[25]. The data is the average of three independent experiments; error bars represent the standard error of the mean. 200 cells were counted for each sample. **c**, APC was purified from approximately 40 g (wet weight) of wild-type or *doc1Δ* yeast cells using the TAP-tag purification strategy (see Methods). Equal amounts (20 μl) of the peak fractions from each purification were separated on 7.5–15% gradient gels and visualized by silver staining. Background bands present in the *doc1Δ* preparation (above Apc9, for example) are present, but less abundant, in the wild-type preparation. **d**, Equal amounts (1 μl) of the peak fractions of wild-type and *doc1Δ* APCs were separated by SDS–PAGE and transferred to nitrocellulose for western blotting with the specified antibodies.

growing at the permissive temperature, we compared the activities of wild-type and *doc1Δ* APCs *in vitro* (Fig. 4a). As described earlier, the wild-type enzyme initially produced multiple products that are eventually re-used at later times to generate high-molecular-weight cyclin–ubiquitin conjugates. In contrast, the ubiquitin–cyclin ligase activity of APC$^{doc1\Delta}$ was severely compromised. Importantly, addition of purified recombinant Doc1 (rDoc1) to the reaction restored activity. This result indicates that loss of Doc1 function is the primary defect in the mutant enzyme, and also suggests that the Doc1 binding site on the APC is accessible to the solvent. Addition of rDoc1 to the wild-type enzyme had no effect, indicating that Doc1 is probably not a sub-stoichiometric component of the APC.

Two major problems are apparent in ubiquitination reactions catalysed by APC$^{doc1\Delta}$. First, the rate at which the enzyme uses substrate is markedly reduced (compare the amount of total products at the 30-min time point for APC$^{doc1\Delta}$ with the 1-min time point for wild-type APC in Fig. 4a). Second, products generated by APC$^{doc1\Delta}$ are almost exclusively mono-ubiquitinated, suggesting a defect in processivity. Both these defects are abolished by addition of recombinant Doc1.

Dose-response curves indicate that half-maximal stimulation of APC$^{doc1\Delta}$ occurred at ~3 nM rDoc1 (Fig. 4b), suggesting that Doc1 binds the APC with high affinity. Typically, the addition of saturating amounts of rDoc1 to APC$^{doc1\Delta}$ resulted in a reaction rate that was approximately fourfold lower than that of the wild-type enzyme (data not shown, but see Fig. 6a). Most importantly, analysis of the product distribution demonstrated that rDoc1 fully restored processivity to APC$^{doc1\Delta}$, as shown by the ratio of di-:mono- and tri-:mono-ubiquitinated species (Fig. 4c).

The E2–APC and Cdh1–APC interactions are similar in wild-type and doc1Δ enzymes. In principle, the processivity of the ubiquitination reaction is most probably determined by the balance between the apparent k_{cat} of the APC and the substrate off-rate (that is, how many times the enzyme turns over before the substrate dissociates). Perturbations that decrease the apparent k_{cat} of the APC or increase the substrate off-rate will result in a less processive enzyme. For example, when E2 concentrations are reduced to limiting levels, there is a decreased probability that an APC will interact with an E2 while associated with a cyclin substrate (Fig. 2). Under these conditions, the apparent k_{cat} is reduced and the enzyme is less processive. Therefore, the defect in APC$^{doc1\Delta}$ could reflect a reduced apparent k_{cat}, resulting from poor binding of the E2 or a decrease in the intrinsic catalytic efficiency of the enzyme. Alternatively, the defect in APC$^{doc1\Delta}$ could be caused by an elevated substrate off-rate, resulting from a reduced affinity for the substrate itself or the activator Cdh1.

To identify the specific molecular defect in APC$^{doc1\Delta}$, we first examined the interaction between the APC and E2 or Cdh1. For practical reasons, these assays were performed at a relatively low cyclin substrate concentration (100 nM), where the maximum velocity of APC$^{doc1\Delta}$ was significantly lower than that of the wild-type enzyme (Fig. 5a, b; see insets). Nevertheless, dose-response curves indicate that the apparent K_M for E2 and the concentration of Cdh1 required for half-maximal activity are identical in the wild-type and doc1Δ enzymes (Fig. 5a, b and Table 1). The half-maximal Cdh1 concentration determined in these experiments is in good agreement with previously determined values[19]. We conclude that interactions between the APC and E2 or Cdh1 are not compromised in the absence of Doc1 and cannot explain the observed defects in cyclin ubiquitination.

Doc1 stimulates cyclin ubiquitination by modulating the affinity of the APC–substrate complex. Next, we determined the apparent K_M of the cyclin substrate for APC purified from wild-type and *doc1Δ* cells (Fig. 6a, b and Table 1). Wild-type APC had a low apparent K_M (63.2 ± 11.1 nM) for cyclin and the reaction was inhibited slightly at cyclin concentrations in excess of 1 μM. In marked contrast, the rate of the reaction for APC$^{doc1\Delta}$ was essentially linear for all cyclin concentrations tested, making it impossible to determine an accurate K_M. We were unable to test cyclin concentrations above 4 μM.

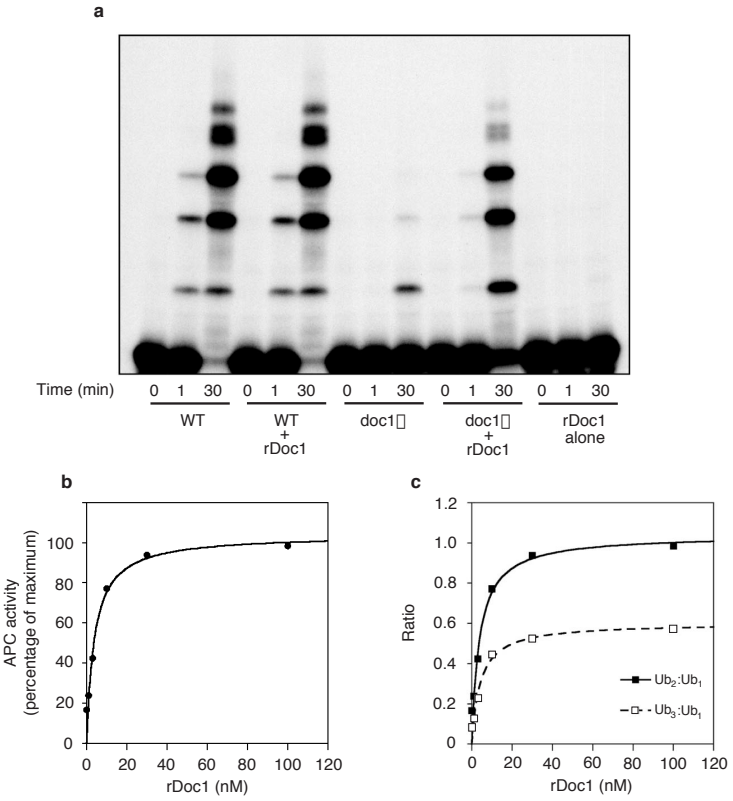

Figure 4 **The Doc1 subunit is required for processive substrate ubiquitination. a**, The activities of wild-type (wt) and doc1Δ APCs (~1.2 nM) were compared in complete ubiquitination reactions in the presence or absence of saturating concentrations of rDoc1 (~300 nM) .**b**, **c**, rDoc1 was titrated into complete reactions containing ~1.2 nM APC$^{doc1\Delta}$ and ~100 nM cyclin substrate. Reactions were stopped after ~1% of the cyclin substrate was converted to products. Total cyclin–ubiquitin ligase activity (**b**), and the ratios of di- to mono (ub2:ub1)-, and tri- to mono (ub3:ub1)-ubiquitinated species (**c**), were quantified. Total ubiquitin–cyclin ligase activity of wild-type APC is typically fourfold greater than that of APC$^{doc1\Delta}$ + rDoc1. The ub2:ub1 and ub3:ub1 ratios at the highest rDoc1 concentration (100 nM) are 1.06 and 0.56, respectively, and are similar to the ratios determined for the wild-type enzyme (ub2:ub1 = 1.11 ± 0.04; ub3:ub1 = 0.44 ± 0.04). Data were fitted to a rectangular hyperbola. This data is representative of results from three independent experiments.

Importantly, addition of saturating amounts of rDoc1 to APC$^{doc1\Delta}$ restored the apparent K_M of cyclin back to a value comparable with that of the wild-type enzyme (85.0 ± 13.8 nM; see Table 1). Therefore, the primary defect in APC lacking the Doc1 subunit is demonstrated by a >20-fold increase in K_M for the cyclin substrate.

The large increase in substrate K_M for APC$^{doc1\Delta}$ relative to the wild-type enzyme could, in principle, reflect a change in the intrinsic catalytic efficiency of the mutant enzyme or a reduced affinity for the substrate. Because we were unable to saturate APC$^{doc1\Delta}$ with substrate, we cannot directly compare the apparent k_{cat} of the mutant enzyme with that of the wild-type APC. However, at near-saturating substrate concentrations (Fig. 6a), addition of rDoc1 had little effect on the rate of cyclin turnover by APC$^{doc1\Delta}$, arguing against a major defect in the catalytic efficiency of APC$^{doc1\Delta}$.

Instead, our data strongly suggest that Doc1 stimulates substrate ubiquitination by limiting substrate dissociation. The precise molecular function of Doc1 is best illustrated by results obtained at cyclin concentrations of 2 μM, where addition of Doc1 to APC$^{doc1\Delta}$ did not have a major impact on the rate of cyclin turnover but greatly stimulated enzyme processivity (Fig. 6c). Under these conditions, the average occupancy of the enzyme active site by cyclin is presumably similar in the presence or absence of Doc1. However, the increased processivity in the presence of Doc1 suggests that Doc1 increases the time that a single cyclin molecule remains in the active site. We conclude that Doc1 is a processivity factor for the APC and probably stimulates the ubiquitination reaction by reducing dissociation of the substrate–enzyme complex.

Discussion

Our results provide several new insights into the APC reaction mechanism (Fig. 7). First, we have shown that APC-dependent multi-ubiquitination of the cyclin substrate occurs processively. Second, processive substrate ubiquitination requires the turnover of multiple E2 enzymes, suggesting that the E2–APC interaction is transient relative to the substrate–APC interaction. In support of this possibility, our data indicate that the apparent K_M of E2 (3.8 μM) is ~50-fold higher than the apparent K_M of cyclin (0.06 μM) in this reaction (Table 1). Third, we have shown that the APC subunit Doc1 stimulates processive substrate ubiquitination, probably by reducing the substrate dissociation rate.

At low substrate concentrations (Figs 4 and 5), APC$^{\Delta doc1}$ is non-processive and ubiquitinates substrates very slowly relative to wild-type APC. At high substrate concentrations (Fig. 6), APC$^{\Delta doc1}$ can

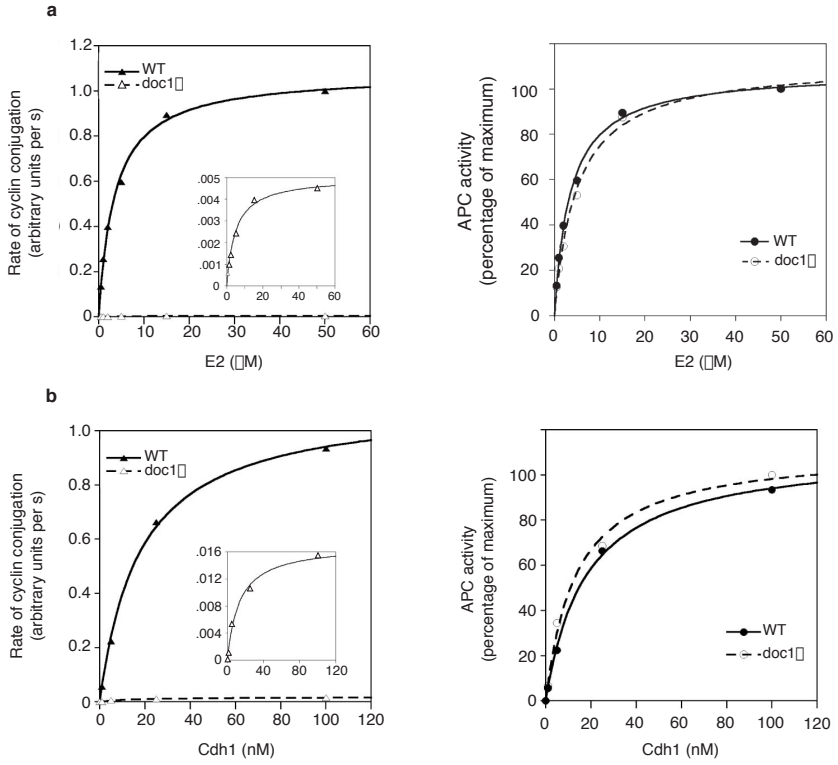

Figure 5 **Doc1 is not required for interaction of APC with E2 or Cdh1. a**, E2 (Ubc4) enzyme was titrated into ubiquitination reactions containing ~0.5 nM of either wild-type (wt) or doc1Δ APC and ~100 nM cyclin substrate. Reactions were stopped after ~1% of the total cyclin substrate was converted to products, and samples were analysed by SDS–PAGE. Total cyclin–ubiquitin ligase activity was measured. Raw data is shown on the left. In the inset, the data from APC$^{doc1\Delta}$ is scaled to demonstrate that the shape of the curve is the same as that for the wild-type enzyme. To emphasize this similarity, the panel on the right shows the same data normalized to the maximal velocity for each enzyme. **b**, APC reactions were performed as in **a**, except that the concentration of Cdh1 was titrated instead of E2. The panel on the left shows the raw data. In the inset, the data from APC$^{doc1\Delta}$ is scaled to demonstrate that the shape of the curve is the same as that for the wild-type enzyme. The right hand panel shows the same data normalized to the maximal velocity of each enzyme. Data in both panels is representative of results from three independent experiments.

turn over substrates at a rate comparable with wild-type APC, but is still non-processive. Doc1 is therefore a processivity factor for the APC. The discrepancy between the rates of cyclin ubiquitination at low and high substrate concentrations is related to the fact that the K_M for the cyclin substrate is higher for APC$^{\Delta doc1}$ than it is for the wild-type APC (Table 1). Because substrate concentration is probably low *in vivo*, we cannot conclude that low processivity *per se* is sufficient to explain the mitotic delay in *doc1Δ* cells. We suspect that it is the combination of poor processivity and the reduced rate of substrate ubiquitination that accounts for the mitotic defects observed in these cells.

We have shown that the interaction between the APC and E2 or Cdh1 is not influenced by Doc1. Furthermore, at high substrate concentrations, APC$^{\Delta doc1}$ can turn over cyclin substrate at a comparable level in the presence or absence of Doc1, arguing against a defect in the intrinsic catalytic efficiency of the enzyme. Instead, our data suggest that an increased substrate off-rate is the only satisfactory explanation for the reduced activity of APC$^{\Delta doc1}$.

The mechanism by which Doc1 enhances processivity remains unclear. The prototype for processivity factors is PCNA, a molecular clamp that is required for the processive replication of DNA by DNA polymerase[20]. PCNA exemplifies the two characteristics all processivity factors must have. First, it stabilizes the enzyme–substrate complex, allowing the enzyme to turn over many times before dissociating from a single substrate. Second, it allows for movement between the enzyme active site and substrate, such that the same chemical reaction can be repeated multiple times on a single substrate.

We can only speculate about how Doc1 might limit substrate dissociation and still allow movement of the substrate relative to the active site. The recent determination of the APC structure by cryo-electron microscopy revealed the existence of a large cavity within the enzyme[21]. This cavity may be a reaction chamber within which substrate ubiquitination takes place. The proteasome and chaperonin complex GroEL use reaction chambers to function processively on their respective substrates. In both cases, these enzymes have 'caps' that limit substrate diffusion out of the reaction chamber. It is possible that Doc1 is required for efficient 'capping' of the APC reaction chamber.

A more simple possibility, also completely consistent with our data, is that the effect of Doc1 on substrate affinity is direct. The APC–substrate interface would therefore consist of two distinct interactions. First, the APC would bind substrates through Cdc20 or Cdh1 in a destruction box- or KEN box-dependent manner. Second, Doc1 would recognize a distinct, and as yet unknown, sequence elsewhere in the substrate. Indeed, the recent determina-

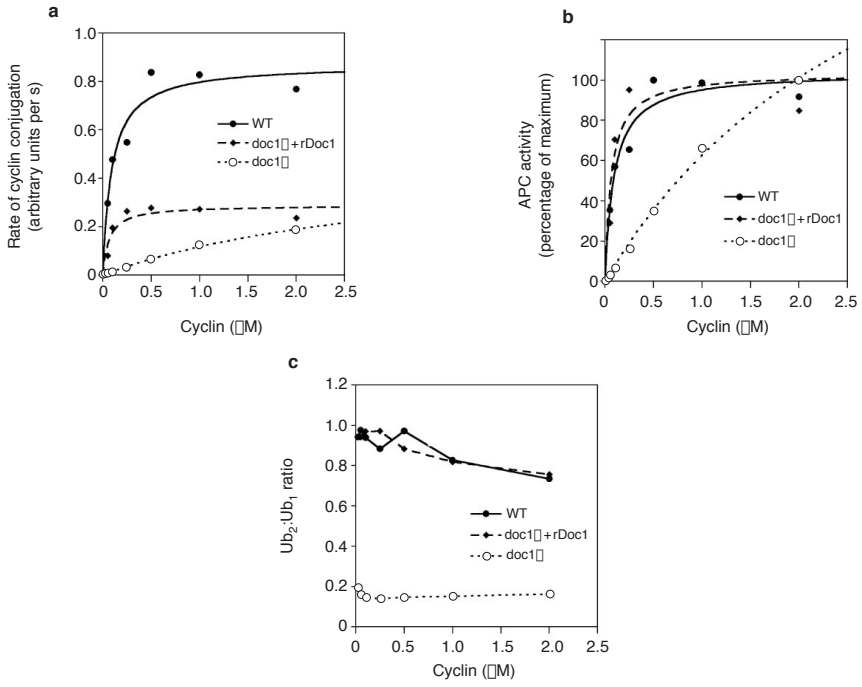

Figure 6 **Doc1 enhances the APC–substrate interaction. a**, Cyclin substrate was titrated into ubiquitination reactions containing ~1 nM of either wild-type (wt) or doc1Δ APC. Where indicated, rDoc1 was added at a concentration of 150 nM. Reactions were stopped after ~1% of the total cyclin substrate had been converted to products. Samples were then analysed by SDS–PAGE and cyclin–ubiquitin ligase activity was measured. **b**, The data from **a** was normalized to the maximum velocity for each condition. **c**, Using the data in **a**, the ratio of di- to mono-ubiquitinated species (ub_2:ub_1) was quantified and plotted as a function of cyclin concentration. The average ratios of tri- to mono-ubiquitinated (ub_3:ub_1) species at all times were 0.32 ± 0.02 and 0.38 ± 0.02 for the wild-type and doc1Δ + rDoc1 APC reactions, respectively. No tri-ubiquitinated species were detectable in the reactions containing only $APC^{doc1\Delta}$.

tion of the crystal structure of Doc1 suggests that the protein contains a conserved ligand-binding domain[15,22]. Furthermore, the recent discovery that Doc1 binds directly to Apc11 places Doc1 in an ideal position to hold substrates in close proximity to the catalytic centre of the enzyme[12]. We were unable to detect a direct interaction between purified Doc1 and cyclin in co-immunoprecipitation experiments (data not shown), but any interaction between these proteins is likely to be low affinity and would require more sensitive methods to detect.

Domains similar to Doc1 have been found in a number of putative ubiquitin-ligase enzymes. To our knowledge, this is the first description of the function of a Doc domain in ubiquitin-protein ligase function. Because of the high conservation of Doc domains in other E3 enzymes, the mechanism by which Doc1 stimulates processive ubiquitination is most probably conserved in other ubiquitin ligases.

Methods

APC purification

All strains in this work are derivatives of W303. A TAP tag cassette was integrated at the carboxyl terminus of *CDC16* using PCR followed by one-step gene replacement in strains TC7 (*mat*Δ, *bar1*) and TC8 (*mat a, bar1*) to create strains TC79 (*mat*Δ, *bar1, CDC16::CDC16-TAP-HIS3*) and TC80 (*mat a, bar1, CDC16::CDC16-TAP-HIS3*). TC79 was crossed with TC94 (*mat a, BAR1, doc1*Δ*::URA3*) to create strain TC102 (*mat*Δ, *CDC16::CDC16-TAP-HIS3, doc1*Δ*::URA3*). Approximately 20 l of TC79 and TC102 were grown to A_{600} ~1.0 in YPD, and pellets (~40 g wet weight) were frozen down for purification. TAP-tag purification of wild-type and doc1Δ APC were performed essentially as described[23]. For APC complexes purified at high temperatures, both wild-type and *doc1*Δ cultures were arrested in mitosis with 15 μg ml^{-1} nocodazole and 30 μg ml^{-1} benomyl to control for the cell cycle arrest displayed by $APC^{doc1\Delta}$ at 37 °C.

APC Assays

The expression and purification of reaction components has been described elsewhere[16]. Briefly, E1 (Uba1–6His) was expressed in yeast under control of the *CUP1* promoter by addition of 1 mM copper sulphate followed by purification using metal-affinity chromatography on cobalt resin. E2 (Ubc4) was expressed in bacteria and purified by anion-exchange chromatography. 6His–Cdh1 was expressed in Sf9 cells using baculovirus and purified using metal affinity chromatography on cobalt resin[19]. Bovine ubiquitin and methylated ubiquitin were from Sigma (Sigma, St. Louis, MO).

Complete reactions consist of the following: E1 (Uba1, 300 nM), E2 (Ubc4, 50 μM), ubiquitin (150 μM), ATP (1 mM), Cdh1 (150 nM), ^{125}I-labeled cyclin substrate (100 nM) and purified APC (0.1–1 nM) unless indicated otherwise. These concentrations are saturating for all reaction components, with the exception of cyclin substrate. Activity was linear with respect to APC concentration. Typically, E1, E2, ubiquitin and ATP were incubated together at 23 °C for 10 min to charge the E2 before addition of APC, Cdh1 and substrate. For kinetic experiments, reactions were allowed to proceed until ~1% of the total cyclin substrate was modified. Reactions were stopped and then resolved on 15% SDS–PAGE gels before quantification with a Molecular Dynamics PhosphorImager (Amersham Biosciences, Piscataway, NJ). Because the concentration of radiolabelled substrate is low, appropriate amounts of unlabeled cyclin substrate were included in reactions containing 25 nM labelled cyclin substrate to reach the total substrate concentrations indicated in Fig. 6. All reactions were carried out in QA buffer (50 mM Tris-HCl at pH 7.4, 100 mM sodium chloride and 1 mM magnesium chloride).

The extraction of substrates from gels in Fig. 1c was done as described[24]. Briefly, reactions that generated equal amounts of various ubiquitinated products were performed and separated by SDS–PAGE. Slices were cut out of the gel and ground with a small pestle in a 1.5-μl microfuge tube for approximately 10 min. Products were extracted in 1 ml of buffer (Tris-HCl at pH 8.0, 300 mM sodium chloride, 1 mM dithiothreitol (DTT), 0.1% SDS and 0.1 mM EDTA) for 12 h at room temperature followed by acetone precipitation. Precipitated substrate was resuspended in 50 μl QA buffer.

Purification of rDoc1

To express and purify rDoc1, the *DOC1* gene lacking a stop codon was amplified by PCR and integrated by homologous recombination into a linearized 2-μm plasmid containing the *GAL1* promoter and a C-terminal TAP-tag (derived from pRS426). Two litres of cells were grown to log phase at 23 °C in ΔURA raffinose media, followed by induction of Doc1–TAP by addition of galactose to a final concentration of 2% for 4 h. Doc1–TAP was purified as described[23]. Doc1–TAP does not associate with the APC unless the protein-A domain is removed, and therefore does not bring down associated APC activity (data not shown). Although all experiments shown were performed with Doc1 purified from yeast, similar results

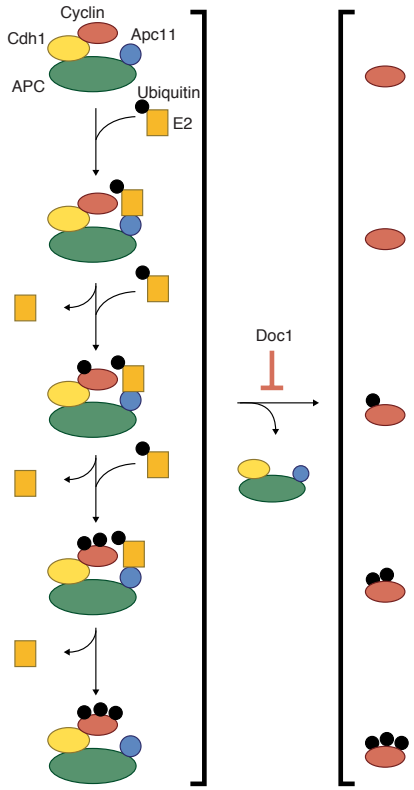

Figure 7 **A model for cyclin ubiquitination by the APC.** The three major principles of this model are as follows: first, substrate multi-ubiquitination occurs processively; second, processive substrate ubiquitination requires multiple E2 enzymes; third, the APC subunit Doc1 stimulates processivity by reducing substrate dissociation.

were obtained with a Doc1–GST fusion protein expressed and purified in bacteria.

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COMPETING FINANCIAL INTERESTS
The authors declare that they have no competing financial interests.