

Molecular Characterization of the Doc1 Subunit of the Anaphase-Promoting Complex

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Zusammenfassung

Damit eine Zelle den Zellzyklus durchlaufen kann, müssen bestimmte regulatorische Proteine, zu denen Securin und mitotische Cykline gehören, abgebaut werden. Diese Abbaureaktionen werden vom Anaphase-promoting Complex/Cyclosome (APC) initiiert, einem 1.5 MDa grossen Ubiquitinligase-Komplex, der aus mindestens einem Dutzend Untereinheiten besteht. Die Substrat-Ubiquitinketten werden vom APC auf eine prozessive Art und Weise generiert. Für diese Prozessivität wird die APC-Untereinheit Doc1 benötigt. Wie allerdings Doc1 diese Prozessivität vermittelt ist nicht bekannt.

Um Proteine zu identifizieren, die mit Doc1 interagieren, habe ich eine Photocrosslinking-Methode etabliert. Dazu habe ich APC aus der Bäckerhefe sowie rekombinantes Doc1, welches einen spezifischen Photocrosslinker enthält, verwendet. Diesen Crosslinker habe ich an verschiedenen Stellen innerhalb von Doc1 eingebaut, von denen gezeigt wurde, dass sie entweder für Doc1s Funktion als Prozessivitätsfaktor oder für die Bindung von Doc1 an den APC wichtig sind. Außerdem habe ich den Crosslinker an verschiedenen Stellen auf der Oberfläche des Doc1 Moleküls eingebaut und damit die Interaktionen von 10 % der Aminosäurereste von Doc1 untersucht. Ich habe herausgefunden, dass Doc1 innerhalb des APC an die „tetra-trico peptide repeat“ (TPR) Proteine Cdc16 und Cdc27 bindet. Dazu verwendet Doc1 seine C-terminale Region beziehungsweise seinen C-terminalen „IR tail“. Desweiteren konnte ich Aminosäurereste in Doc1 identifizieren, die direkt die größte Untereinheit des APC, Apc1, kontaktieren. Ich habe diese Technik auch verwendet, um nach Bindungspartnern der N-terminalen Loop-Region von Doc1 zu suchen. Der Grund dafür war, dass postuliert wurde, dass Doc1 über diese Region die Prozessivität in Ubiquitinierungsreaktionen vermittelt. Allerdings haben diese Experimente nicht zu der Identifikation eines möglichen Liganden geführt.

Um die Rolle von Doc1 innerhalb des APC zu verstehen und um einen Einblick in die dreidimensionale (3D) Organisation der 13 APC Untereinheiten zu bekommen, haben wir damit begonnen, den APC aus der Bäckerhefe mittels Elektronenmikroskopie zu analysieren. Wir haben ein 3D Modell für den Wildtyp-APC erstellt, der eine asymmetrische und insgesamt trianguläre Form aufweist. Um Doc1 innerhalb des APC zu lokalisieren, haben wir ein Modell für eine APC-Mutante, der Doc1 fehlt, erstellt. Indem wir diese Struktur mit der des Wildtyp-APC verglichen haben, konnten wir eine zusätzliche Masse im Wildtyp-APC

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ausfindig machen, bei der es sich wahrscheinlich um Doc1 handelt. Um weitere APC Untereinheiten zu lokalisieren, haben wir einen Satz von Hefestämmen hergestellt, in denen ein 50 kDa großer Tag mit dem C-terminus von jeweils einer der APC Untereinheiten fusioniert wurde. Diese Tags sind groß genug, um sie mittels Negativfärbung und Elektronenmikroskopie sichtbar zu machen. Wir haben APC aus diesen Hefestämmen gereinigt und dabei herausgefunden, dass für neun der getaggten APC Untereinheiten die Komposition des APC nicht beeinflusst wurde. Bisher wurden Strukturen für zwei der neu generierten APC Versionen ermittelt; in einem der Komplexe war Cdc27 getaggt und in dem anderen Apc5. Der Vergleich dieser Strukturen mit der des Wildtyp-APC hat uns ermöglicht, die C-termini von Cdc27 und Apc5 präzise zu lokalisieren. Während Apc5 ein Teil der "Plattform" des APC zu sein scheint, befindet sich der C-terminus von Cdc27 in unmittelbarer Nähe der Masse, bei der es sich wahrscheinlich um Doc1 handelt. Die Interaktion zwischen Doc1 und Cdc27, die ich mittels Photocrosslinking ermittelt habe, ist damit konsistent mit unserem 3D Modell des APC. Doc1 könnte die katalytische Aktivität des APC beeinflussen, indem es mit einem TPR Protein Subkomplex assoziiert, welcher Cdc27 und Cdc16 beinhaltet. Durch gleichzeitige Bindung an eine zweite Region innerhalb des APC, welche möglicherweise Apc1 enthält, könnte Doc1 strukturelle Veränderungen innerhalb des APC verursachen, welche die prozessive Substratubiquitinierung ermöglichen.

Abstract

Progression through mitosis depends on the degradation of certain regulatory proteins, such as securin and mitotic cyclins. These degradation reactions are initiated by the anaphase-promoting complex/cyclosome (APC), a 1.5 MDa ubiquitin ligase complex that is composed of at least a dozen subunits. APC assembles ubiquitin chains on substrates in a processive manner. This processivity depends on the APC subunit Doc1, but how Doc1 confers processivity to the APC is unknown.

To identify the binding partners of Doc1, I have established a photocrosslinking approach using budding yeast APC and recombinant Doc1 mutants containing a site-specific crosslinker. I have inserted this photocrosslinker at different sites which were shown to be important for Doc1's function as a processivity factor and for its binding to the APC. In addition, I have inserted the crosslinker at various sites on the surface of the Doc1 structure and thereby mapped interactions of about 10 % of Doc1's amino acid residues. I have found that within the APC, Doc1 binds to the tetratricopeptide repeat (TPR) proteins Cdc16 and Cdc27 via Doc1's C-terminal region and the C-terminal IR tail, respectively. In addition, I could identify sites which directly contact APC's largest subunit, Apc1. I also used this technique to search for interaction partners which might bind to an N-terminal loop region in Doc1, because this region has been proposed to mediate processivity in ubiquitination reactions. However, these experiments did not result in the identification of a potential ligand.

To understand Doc1's role within APC and to get insight into the three-dimensional (3D) organisation of all 13 APC subunits, we have started to analyze budding yeast APC by electron microscopy (EM). We have generated a 3D model for *wild type* APC which displays an asymmetric and overall triangular shape. To localize Doc1 within APC, we have obtained a 3D model of APC lacking Doc1. Comparing this structure with that of *wild type* APC has revealed one additional density in the *wild type* structure; this mass is likely to represent Doc1. In order to analyze the localization of further APC subunits we have created a set of yeast strains which each carry a 50 kDa-tag fused to the C-terminus of one of the APC subunits. These tags are large enough to be visualized by negative staining EM. We have purified APC from these strains and have found that for nine tagged APC subunits, APC composition remained unaffected. Structures have so far been generated for two APC versions; in one

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complex Cdc27 was tagged and in the other one Apc5. Comparison of those structures with that of *wild type* APC has allowed the precise localization of the C-termini of Cdc27 and Apc5. Whereas Apc5 appears to be part of the “platform” of APC, the C-terminus of Cdc27 localizes close to the mass which likely represents Doc1. The Doc1-Cdc27 interaction detected by photocrosslinking is therefore in good agreement with our 3D model of APC. Doc1 might affect the catalytic activity of APC by associating with a TPR protein-containing subcomplex including Cdc27 and Cdc16; through simultaneous binding of a second region within APC, which might include Apc1, Doc1 might cause structural rearrangements within APC which allow processive substrate ubiquitination.

1 Introduction

1.1 The cell cycle

The cell cycle is an ordered series of events which lead to cell division and the production of two daughter cells, each containing exact copies of the parental cells' chromosomes. In the case of a unicellular organism, cell division leads to proliferation, whereas in a multicellular organism, cell division results in growth. Duplication of the parental chromosomes occurs during the S phase of the cycle, and one of the resulting daughter chromosomes is distributed to each daughter cell during mitosis. These two cell cycle phases are separated by two gap phases, G1 and G2 phases, which allow cells to prepare for the subsequent duplication and division stages by synthesizing RNA and protein, and by growing in size. During mitosis, the cell undergoes major morphological changes. In most eukaryotic cells except for fungi, the nucleus disassembles in prophase and chromosomes start to condense. Sister chromatids are held together by a mechanism called cohesion. The formation of a bipolar mitotic spindle begins, onto which chromosomes start to align in prometaphase. In metaphase all chromosomes are aligned to form a so-called metaphase plate. Cohesion is lost at the onset of anaphase and sister chromatids move to opposite spindle poles. During telophase, the chromosomes decondense and the nucleus reforms. Finally, the two daughter cells become separated by cell membrane ingression in a process called cytokinesis (Morgan, 2007). Precise temporal control of the cell cycle events ensures that both chromosome replication and chromosome segregation to daughter cells occur in the proper order and with high fidelity. Regulation of the cell cycle is critical for the normal development of all organisms, and loss of controlled division can ultimately lead to cell death or hyperproliferation as it is found in cancer.

1.2 Cell cycle control

The cell cycle is driven onwards by a system of highly regulated oscillating waves of cyclin-dependent kinase (Cdk) activity. Cdk activity is high during DNA synthesis and early mitosis and low during cytokinesis and G1. High Cdk activity results from association of the catalytic subunit Cdk with its regulatory cyclin subunit (Murray, 2004). Budding and fission

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yeasts only use a single Cdk, whereas higher organisms have several Cdks. Budding yeast Cdk1 (initially termed Cdc28), can associate with nine different cyclins to induce major cell cycle events including the commitment to cell division (with G1 phase cyclins Cln1 to 3, (Nasmyth, 1993)), DNA replication (with S phase cyclins Clb5 and 6, (Murray, 2004)) and entry into mitosis (with M phase cyclins Clb1 to 4, (Andrews and Measday, 1998)). In animal cells, Cdk4 and Cdk6, paired with D-type cyclins, are active in G1; Cdk2 associated with A-type and E-type cyclins initiates DNA replication, and Cdk1 together with B-type cyclins promotes mitotic entry (Pagano and Jackson, 2004).

Whereas Cdks are constantly present during the cell cycle, levels of their activating cyclins are periodically fluctuating (Murray, 2004). Moreover, Cdk activities are controlled by Cdk inhibitors, whose levels also fluctuate. Oscillations of both cyclins and Cdk inhibitors are partially controlled on the transcriptional level, but are largely accomplished by the action of the ubiquitin proteasome system (Morgan, 2007). Proteolysis therefore ensures irreversible cell cycle transitions and moves the cell cycle forward in a unidirectional fashion.

1.3 Ubiquitin dependent proteolysis

Cyclins and many other cell cycle regulators are targeted to the proteasome (Voges *et al.*, 1999) by the addition of ubiquitin chains. Ubiquitin is a highly conserved 76 amino acid protein found in all eukaryotes. The C-terminus of ubiquitin is conjugated to lysine residues of target proteins by the action of three enzymes: a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2) and a ubiquitin ligase (E3). In a first step, the E1 activates ubiquitin by first forming a ubiquitin-adenylate which requires ATP hydrolysis and then creating a thioester bond between its catalytic cysteine and the C-terminal glycine residue of ubiquitin. The activated ubiquitin molecule is then transferred to the catalytic cysteine of an E2 in a transesterification reaction. In the final and most tightly regulated step, an E3 recognizes the target protein and catalyzes the formation of an isopeptide bond between the C-terminus of ubiquitin and a substrate's lysine side chain. When ubiquitins are conjugated to lysine residues within ubiquitin itself in subsequent reactions, polyubiquitin chains are formed on the target protein ((Pickart, 2001); Figure 1–1).

By targeting proteins for degradation, ubiquitin converts many proteasomal substrates into very short-lived proteins. Ubiquitin itself, however, escapes degradation and is removed

from its conjugates by deubiquitinating enzymes (DUBs), allowing recycling of ubiquitin and maintenance of a pool of free ubiquitin in the cell (Amerik and Hochstrasser, 2004). Furthermore, DUBs are required for generating conjugation-competent ubiquitin from precursors (Amerik and Hochstrasser, 2004). It has been proposed that DUBs might serve a proofreading function in that poorly ubiquitinated or slowly degraded proteins are diconjugated from their ubiquitin chains and therefore rescued from degradation before proteasomal processing could take place (Lam *et al.*, 1997). Apart from their general role in ubiquitin processing and recycling, DUBs have been implicated in specific biological processes (Amerik and Hochstrasser, 2004). Furthermore, it has been shown that some DUBs are target-specific (Cohen *et al.*, 2003; Li *et al.*, 2002). DUBs might therefore be involved in “fine-tuning” of ubiquitin dependent proteolysis by contributing to balancing the levels between ubiquitination and deubiquitination.

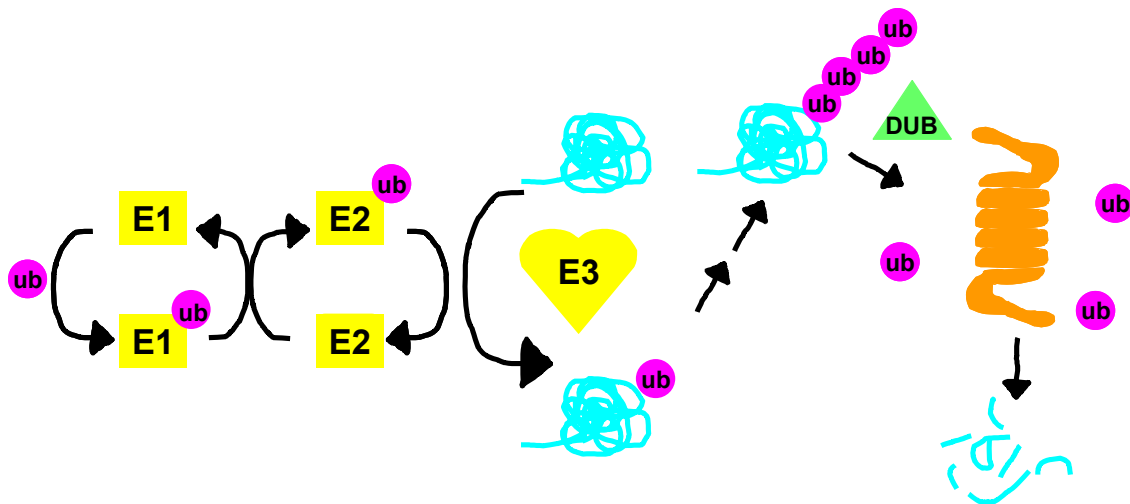


Figure 1-1: The ubiquitin proteasome pathway. Three enzymes act in a cascade to attach ubiquitin moieties to target proteins. Note that steps including ubiquitin precursor processing and two ATP dependent processes, ubiquitin activation and translocation of substrates into the proteasome, were omitted for simplicity. Attachment of ubiquitin can result in monoubiquitination, multiubiquitination (i.e. several distinct substrate lysines are ubiquitinated) and polyubiquitination (as shown). Deubiquitinating enzymes (DUB) can also act at other steps in the pathway. See text for details.

The first implication that ubiquitin dependent proteolysis might underlie cell cycle regulation came from the observation that the temperature sensitive mouse cell line ts85, which carries a point mutation in the E1 enzyme, arrests the cell cycle in G2 (Finley *et al.*, 1984). This hypothesis was proven to be correct when mitotic cyclins were shown to be ubiquitinated

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and degraded by the proteasome (Glotzer *et al.*, 1991; Hershko *et al.*, 1991). The two key enzymes which are involved in major cell cycle transitions by mediating the conjugation of ubiquitin to cell cycle regulators were identified as the anaphase promoting complex/cyclosome (APC) (Irniger *et al.*, 1995; King *et al.*, 1995; Sudakin *et al.*, 1995; Tugendreich *et al.*, 1995) and the Skp1/Cullin/F box complex (SCF) (Feldman *et al.*, 1997; Skowyra *et al.*, 1997).

1.4 Roles of the APC

The cell cycle is driven by periodic fluctuations in the activity of Cdk1. Whereas activation of Cdk1 is a prerequisite for the initiation of many mitotic events including entry into mitosis, nuclear envelope breakdown, spindle assembly and chromosome condensation, inactivation of Cdk1 must occur before a cell can exit mitosis. Inactivation of Cdk1 is accomplished by degradation of its activating subunits cyclin A and cyclin B. Before the ubiquitin ligase responsible had been discovered, it became evident that the same activity is also responsible for degrading an inhibitor of anaphase onset (Holloway *et al.*, 1993). Yeast genetic studies as well as biochemical fractionation experiments then identified APC as the ubiquitin ligase responsible for both anaphase onset and mitotic exit in yeast, clam and frog egg extracts (Hershko *et al.*, 1991; Irniger *et al.*, 1995; King *et al.*, 1995). At the metaphase to anaphase transition, when all chromosomes have been attached to both poles of the mitotic spindle, APC mediates the ubiquitination of securin, which is an inhibitor of separase. Once separase is active, it cleaves the cohesin complex, allowing anaphase onset (Nasmyth, 2002). Cohesin is a ring-shaped complex which embraces sister chromatids and thereby holds them together until cleavage of cohesin's Scc1 releases cohesin from chromatids (Nasmyth, 2002). APC ubiquitination of cyclin B establishes and maintains a state of low Cdk activity that is necessary for mitotic exit (Amon *et al.*, 1994; Morgan, 1999; Sullivan and Morgan, 2007). Dephosphorylation of Cdk1 substrates is essential for disassembly of the mitotic spindle, reformation of the nuclear envelope, decondensation of chromosomes and cytokinesis. In vertebrates, APC might not only activate separase by mediating securin destruction, but also by ubiquitinating cyclin B. Phosphorylation of separase and association with Cdk1-cyclin B inactivates the protease; the low Cdk state created by APC might therefore fully activate separase (Gorr *et al.*, 2005; Stemmann *et al.*, 2001). Persisting APC activity during G1 and

therefore low Cdk1 activity allows formation of prereplicative complexes on origins of replication. Initiation of DNA synthesis by DNA polymerases is dependent on high Cdk activity. APC activity therefore prevents premature S phase entry (Diffley, 2004; Zachariae and Nasmyth, 1999).

Apart from cyclins and securin, a growing number of additional cell cycle substrates have been identified as APC targets including proteins involved in DNA replication (geminin and Cdc6 in mammals, Dbf4 in yeast), mitotic kinases (yeast and human polo kinases), the APC cofactor Cdc20 and proteins involved in spindle function in yeast (Ase1, Kip1, Cin8) or frogs (Xkid). For some of those, additional forms of regulation apart from proteolysis exist. One example is Plk1, which can be inactivated at the end of mitosis by either degradation or dephosphorylation (Lindon and Pines, 2004). Moreover, studies in budding yeast have shown that the only essential APC targets, at least in this organism, are securin and mitotic cyclins (Thornton *et al.*, 2006).

1.5 APC and SCF

The main two ubiquitin ligases catalyzing cell cycle transitions are APC and SCF. Whereas APC is mainly active during mitosis and G1 phase (see above), various SCF complexes are acting at many cell cycle stages as well as beyond the cell cycle (Petroski and Deshaies, 2005; Vodermaier, 2004). APC and SCF are distantly related multi-subunit complexes which belong to the family of really interesting new gene (RING) ubiquitin ligases (Passmore and Barford, 2004). These ligases use a small zinc-binding RING finger protein (Apc11 in APC and Roc1/Rbx1/Hrt1 in SCF) to recruit E2 enzymes. There is no evidence that RING proteins directly take part in transfer of ubiquitin from E2 to a substrate. This is in contrast to a second family of ubiquitin ligases which uses homology to E6-AP C-terminus (HECT) domain proteins to form a thioester linkage with ubiquitin (Passmore and Barford, 2004). In both APC and SCF the RING subunits are associated with C-termini of proteins containing cullin domains (Apc2 in APC, Cdc53 in SCF). Substrate binding in SCF complexes is accomplished by substrate adaptors. These typically contain an N-terminal domain which contacts the cullin subunit and a C-terminal domain which binds to substrates (Petroski and Deshaies, 2005). One of the two cullin binding domains known so far comprises an approximately 40 amino acid F box motif. F box proteins contain substrate-binding domains

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such as WD40 and leucine-rich repeats to bind to their numerous substrates (Petroski and Deshaies, 2005). WD40 repeats are also found in APC coactivator proteins which play an important role in APC substrate recognition and binding (see below). Structural information available for SCF (Schulman *et al.*, 2000; Zheng *et al.*, 2002) allows insight into the function of the SCF ubiquitin ligase. The structures suggest that the main function of SCF is to form a scaffold (built by the rigid cullin subunit) which correctly positions ubiquitin-charged E2 (bound via cullin and the RING finger) and substrate (bound by the F box protein) to facilitate ubiquitination. Despite the fact that SCF contains three to five, and APC at least twelve subunits, it was proposed that both complexes might share a similar structural backbone and therefore similar modes of mediating substrate ubiquitination (Ohi *et al.*, 2007).

Although SCF and APC display basic similarities in their core domains, there is a fundamental difference in their regulation during the cell cycle. SCF is a constitutively active complex and the regulation takes place at the substrate level. In most cases, phosphorylation targets SCF substrates to the ligase, but other signals including oligosaccharides have also been observed (Petroski and Deshaies, 2005; Yoshida, 2007). APC in contrast becomes phosphorylated itself, which allows activator proteins to bind and activate the complex (Vodermaier, 2004). Thus, whereas for SCF substrate availability is restrained, it is the enzyme itself which is regulated in case of APC (see below). However, it has become evident that substrate modification provides an additional layer of regulation to control APC activity. Substrates including yeast Pds1 (Wang *et al.*, 2001), aurora A (Littlepage and Ruderman, 2002), Cdc6 (Mailand and Diffley, 2005) and Skp2 (Rodier *et al.*, 2008) are protected from APC-mediated destruction by phosphorylation.

1.6 APC regulation in mitosis

Since inappropriate APC-mediated protein degradation could have fatal consequences for a cell or an organism, APC activity is restrained at multiple levels by several cellular mechanisms (Peters, 2006). To become active, APC has to associate with one of its coactivator proteins, which in mitosis are Cdc20 and Cdh1. APC-Cdc20 is activated early in mitosis, whereas APC-Cdh1 takes over in late mitosis and remains active until late G1 phase (Peters, 2006). An important basis for the strictly regulated time frames in which these two

forms of APC are active is the opposing effect that phosphorylation has on APC-Cdc20 and APC-Cdh1: Whereas Cdc20 can only bind to and activate phosphorylated APC, Cdh1 is kept in an inactive state by phosphorylation (Peters, 2006). Levels of Cdc20 oscillate in a cell cycle dependent manner with transcription and protein expression profiles being similar to those of cyclins promoting mitosis (Shirayama *et al.*, 1998). Cdc20 accumulates during late S phase and is present until late mitosis. Phosphorylation of APC by Plk1 and Cdk paired with mitotic cyclins allows activation of APC-Cdc20 (Kraft *et al.*, 2003; Kramer *et al.*, 1998; Rudner and Murray, 2000; Shteinberg *et al.*, 1999), which then mediates the destruction of mitotic B-type cyclins, thereby creating a “low Cdk activity state”. Phosphatases such as Cdc14 in yeast are activated which remove inhibitory phosphates on Cdh1. APC-Cdh1 becomes active (Shou *et al.*, 1999; Visintin *et al.*, 1998; Zachariae *et al.*, 1998a) and promotes Cdc20 destruction. Thus, Cdc20 not only mediates the degradation of mitotic cyclins, but indirectly also its own (Prinz *et al.*, 1998; Shirayama *et al.*, 1998). In contrast to Cdc20, Cdh1 is present throughout the cell cycle. APC-Cdh1 remains active during G1, and this prevents reaccumulation of mitotic cyclins. At the G1/S phase transition, however, APC has to be inactivated to allow reaccumulation of APC substrates required for DNA replication and mitotic entry. This is, in part, accomplished by Cdk1-mediated phosphorylation which promotes Cdh1 dissociation from APC (Jaspersen *et al.*, 1999; Kramer *et al.*, 1998; Zachariae *et al.*, 1998a). Phosphorylation of Cdh1 might also target it for degradation by SCF in S phase (Benmaamar and Pagano, 2005; Kramer *et al.*, 1998; Yamaguchi *et al.*, 2000).

A model for autonomous APC regulation has been proposed based on the observation that APC-Cdh1 promotes its own inactivation by mediating destruction of UbcH10 (Rape and Kirschner, 2004), an E2 enzyme that appears to collaborate exclusively with APC (Yu *et al.*, 1996). Cyclin A degradation is critically dependent on levels of UbcH10 (Rape and Kirschner, 2004). During mitosis, APC-Cdh1 together with UbcH10 is “busy” degrading APC substrates. At the end of mitosis, however, when most of APC substrates have been degraded, APC-Cdh1 promotes UbcH10 autoubiquitination and degradation. Rising levels of cyclin A result in inactivation of APC-Cdh1 by cyclin A-Cdk2-mediated phosphorylation (Rape and Kirschner, 2004). In addition, APC-Cdh1 is directly inhibited by a protein called early mitotic inhibitor-1 (Emi1) in vertebrate cells and regulator of cyclin A (Rca1) in *Drosophila* during S and G2 phases (Dong *et al.*, 1997; Grosskortenhaus and Sprenger, 2002; Hsu *et al.*, 2002), allowing accumulation of cyclin A and other APC substrates. Emi1 was

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reported to also prevent APC-Cdc20 activation prior to and in early mitosis, when Cdk1 activity might already activate APC and promote Cdc20 binding (Guardavaccaro *et al.*, 2003; Margottin-Goguet *et al.*, 2003). Emi1 can directly bind to APC coactivators (Hsu *et al.*, 2002; Reimann *et al.*, 2001; Reimann and Jackson, 2002) and core APC (Miller *et al.*, 2006) and might thereby inhibit substrate recruitment to the APC. A more recent study has shown that Emi1 destruction is not required for mitotic entry, which implies that Emi1 might not regulate APC-Cdc20; however, it is important for inhibiting APC-Cdh1 during interphase to prevent rereplication (Di Fiore and Pines, 2007). Emi1 degradation is initiated by Plk1-mediated phosphorylation which targets Emi1 for SCF-mediated degradation at prometaphase onset (Hansen *et al.*, 2004; Moshe *et al.*, 2004).

1.7 APC regulation by the spindle assembly checkpoint

A surveillance mechanism known as the spindle assembly checkpoint (SAC) ensures proper chromosome segregation in mitosis (Musacchio and Hardwick, 2002). Checkpoints are regulated transition points at which progression to the next cell cycle state can be arrested if certain conditions arise. In case of the SAC, a single unattached kinetochore can lead to a prolonged prometaphase. The SAC targets APC-Cdc20 and delays its ubiquitination activity towards securin and cyclin B, whose degradation is required for anaphase onset and mitotic exit, respectively. Remarkably, the SAC inhibits APC in a substrate-specific manner: While degradation of securin and cyclin B is prevented, cyclin A and Nek2A degradation remains unaffected (den Elzen and Pines, 2001; Geley *et al.*, 2001; Hames *et al.*, 2001; Hayes *et al.*, 2006). SAC activation is thought to generate a diffusible signal, which delays mitotic progression until all chromosomes have become attached to both poles of the mitotic spindle.

Several proteins have been implicated in the function of the spindle assembly checkpoint, including Mad1, Mad2, BubR1, Bub1 and Bub3 (Musacchio and Hardwick, 2002; Musacchio and Salmon, 2007). Direct binding of Mad2 to Cdc20 is essential for SAC function (Hwang *et al.*, 1998; Kim *et al.*, 1998). Mad2 and BubR1 have been shown to directly bind to APC *in vivo* and to impair its ubiquitination activity *in vitro* (Fang *et al.*, 1998; Sudakin *et al.*, 2001; Tang *et al.*, 2001a). An elegant so-called “template model” has been proposed to explain how a weak signal caused by only one unattached kinetochore could be rapidly amplified to affect

the entire cell: Upon SAC activation, Mad2 is recruited to unattached kinetochores by Mad1 (Sironi *et al.*, 2002). Mad2 of Mad1-Mad2 complexes forms an asymmetric dimer with diffusible Mad2 (Mapelli *et al.*, 2007) and as part of this complex, Mad2 may function as a template for the assembly of Mad2-Cdc20 complexes (De Antoni *et al.*, 2005; Luo *et al.*, 2002). Newly assembled Mad2-Cdc20 complexes may themselves function as templates for additional Mad2-Cdc20 complexes, thereby rapidly multiplying their amounts. A mitotic checkpoint complex consisting of Mad2, BubR1/Mad3, Bub3 and Cdc20 (Fraschini *et al.*, 2001) might be an effector of the SAC; MCC efficiently inhibits APC upon SAC activation (Morrow *et al.*, 2005; Sudakin *et al.*, 2001). The mechanism of APC inhibition remains to be understood. Recent data suggest that SAC activation and MCC association prevent substrate binding to APC-Cdc20 and effect APC's catalytic activity (Herzog *et al.*, manuscript in preparation). Once all requirements of the checkpoint are satisfied, Cdc20 has to be rapidly liberated from its inhibition. It has been proposed that APC itself might relieve Cdc20 by ubiquitination, leading to dissociation of BubR1 and Mad2 from Cdc20 (Reddy *et al.*, 2007). In this setting, the deubiquitinating enzyme Usp44 antagonizes APC activity towards Cdc20 by disassembling ubiquitin chains, thereby stabilizing BubR1-Mad2-Cdc20 complexes required for SAC function (Stegmeier *et al.*, 2007).

1.8 What is known about APC subunits

APC is an unusually complex ubiquitin ligase (Table 1-1). So far, 13 subunits have been detected in budding and fission yeasts and twelve in humans (Peters, 2002; Peters, 2006). The complexity of the APC is somewhat surprising because many other RING ubiquitin ligases consist of a single or few subunits, indicating that the ubiquitin ligase reaction *per se* does not require complex multi-subunit enzymes (Passmore and Barford, 2004). The reason for the complexity of the APC is still a mystery and the molecular function of many APC subunits unknown. The cullin-containing subunit Apc2 and the RING finger Apc11 are required for APC ubiquitination activity (Section 1.5). Apc11 and the E2 enzyme UbcH5 alone were reported to assemble ubiquitin chains on substrates *in vitro* (Gmachl *et al.*, 2000; Levenson *et al.*, 2000; Tang *et al.*, 2001b) whereas the cullin domain of Apc2 is required in addition when UbcH10 is used instead of UbcH5 (Tang *et al.*, 2001b). These reactions display low substrate specificity (Gmachl *et al.*, 2000; Levenson *et al.*, 2000; Tang *et al.*, 2001b).

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One might therefore speculate that Apc11 alone or together with Apc2 is responsible for APC's ubiquitin ligase activity and the remaining subunits are required to regulate APC activity and to confer substrate specificity.

Table 1-1: APC subunits and coactivators identified to date in human cells, *Saccharomyces cerevisiae* (*S.c.*) and *Schizosaccharomyces pombe* (*S.p.*). Modified from (Peters, 2006; Thornton and Toczyski, 2006). anaphase promoting complex/cyclosome (APC); regulatory particle non-ATPase (Rpn); ubiquitin conjugating enzyme (E2); tetratricopeptide repeats (TPR); really interesting new gene (RING); tryptophane aspartate (WD40); degradation of cyclin B protein-1 (Doc1); temperature sensitive (ts); spore wall maturation protein-1 (Swm1); meiotic nuclear division protein-2 (Mnd2).

human	<i>S. c.</i>	<i>S. p.</i>	essential?	known motifs or functions
core APC subunits				
Apc1	Apc1	Cut4	yes	homology to Rpn1, Rpn2 (proteasomal subunits)
Apc2	Apc2	Apc2	yes	cullin domain; catalytic activity, E2 binding
Apc3	Cdc27	Nuc2	yes	TPRs; coactivator binding
Apc4	Apc4	Lid1	yes	WD40 repeats; bridges Apc1 and TPR subunits
Apc5	Apc5	Apc5	yes	bridges Apc1 and TPR subunits
Apc6	Cdc16	Cut9	yes	TPRs; required for Cdc27 association with APC
Apc7	-	-	?	TPRs
Apc8	Cdc23	Cut23	yes	TPRs; required for Cdc16 and Cdc27 association
-	Apc9	-	no	promotes association of Cdc27
Apc10	Doc1	Apc10	essential in <i>S.p.</i> deletion <i>ts</i> in <i>S.c.</i>	DOC domain, IR tail; processivity, substrate binding
Apc11	Apc11	Apc11	yes	RING finger; catalytic activity, E2 binding
Cdc26	Cdc26	Hcn1	deletion <i>ts</i> in <i>S.c.</i>	upregulated at higher temperature
Apc13	Swm1	Apc13	essential in <i>S.p.</i> deletion <i>ts</i> in <i>S.c.</i>	promotes association of Cdc16,Cdc27,Cdc26, Apc9, required for sporulation
		Apc14	no	
	Mnd2	Apc15	no	Inhibition of Ama1 in meiosis
APC coactivators				
Cdc20	Cdc20	Slp1	yes	WD40 repeats, C box, IR tail; substrate recruitment
Cdh1	Cdh1	Ste9	no	WD40 repeats, C box, IR tail; substrate recruitment
Ama1	-	-	no	WD40 repeats, C box, IR tail; substrate recruitment

APC activity depends on the association of one of several coactivator proteins, the best studied of which are the mitotic APC coactivators Cdc20 and Cdh1. Additional meiosis-

specific coactivators have so far only been identified in budding yeast and *Drosophila* (Table 1-1, (Peters, 2006)). APC coactivators have several sequence motifs in common: They contain a WD40 domain which is predicted to fold into a seven-bladed propeller (Orlicky *et al.*, 2003; Wu *et al.*, 2003). This domain is required for the direct interaction of Cdh1 with a prominent substrate recognition element, the D box (Kraft *et al.*, 2005). Two sequence elements mediate coactivator binding to APC. The C-terminal IR tail associates with the TPR subunit Cdc27 (see below), whereas the internal C box (Schwab *et al.*, 2001) mediates binding to APC's catalytic subunits (Thornton *et al.*, 2006).

The small protein Doc1 most likely represents the best-studied APC subunit. *DOC1* mutation or deletion results in temperature sensitive budding yeast strains (Hwang and Murray, 1997). In fission yeast and *Drosophila*, Doc1 is essential (Kominami *et al.*, 1998; Pal *et al.*, 2007b). Moreover, disruption of *Apc10/Doc1* alleles was found to be responsible for a radiation-induced phenotype in mice that is associated with oligosyndactylism (Pravtcheva and Wise, 2001; Wise and Pravtcheva, 2004). Doc1 almost entirely consists of the conserved so-called DOC domain (Grossberger *et al.*, 1999) which is also found in Doc1 orthologs and in several predicted multidomain proteins (Grossberger *et al.*, 1999; Kominami *et al.*, 1998). All these proteins have in common that they possess additional domains, such as cullin homology regions, RING fingers or HECT domains, which imply a role for them in ubiquitination reactions. It was therefore speculated, that DOC domain-containing proteins might play a general role in ubiquitination reactions (Grossberger *et al.*, 1999; Kominami *et al.*, 1998). In the meantime, some of these proteins have indeed been found to be ubiquitin ligases or to function in ubiquitin-dependent processes (DiAntonio *et al.*, 2001; Dias *et al.*, 2002; Nikolaev *et al.*, 2003). Furthermore, based on structural, biochemical and yeast genetic data, Doc1 has been proposed to be involved in APC substrate recognition and ubiquitination, possibly by directly binding to APC substrates (Section 1.12).

The non-essential APC subunits Apc13/Swm1, Cdc26 and Apc9 play a role in the structural integrity of yeast APC. Mutations or loss of any one of these proteins leads to the dissociation of other APC subunits or the destabilization of subcomplexes (Passmore *et al.*, 2003; Schwickart *et al.*, 2004; Zachariae *et al.*, 1998b). In absence of Apc9, levels of Cdc27 are significantly reduced (Passmore *et al.*, 2003; Zachariae *et al.*, 1998b). Furthermore, Swm1 is required for the association of Cdc16, Cdc27, Apc9 and Cdc26 with APC (Schwickart *et al.*, 2004). Cdc26 fulfills a similar function as Swm1 with the exception that Swm1 incorporation

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into APC does not require Cdc26. Both Cdc26 and Swm1 are only required at temperatures above 37°C, whereas at 25°C either subunit is sufficient to promote the formation of active APC (Schwickart *et al.*, 2004). In addition, Swm1 is required for sporulation during meiosis (Schwickart *et al.*, 2004; Ufano *et al.*, 1999). Another APC subunit with meiosis-specific function is Mnd2 (Rabitsch *et al.*, 2001; Yoon *et al.*, 2002). Mnd2 APC bound to the coactivator Ama1 during meiotic S phase and prophase I. This ensures the timely destruction of Pds1 and prevents premature sister chromatid separation (Oelschlaegel *et al.*, 2005; Penkner *et al.*, 2005).

1.9 Analysis of APC subcomplexes

Insight into APC subunit organization has been gained by dissociating the complex into smaller subcomplexes, either biochemically (Vodermaier *et al.*, 2003) or after mutation or deletion of individual subunits (Schwickart *et al.*, 2004; Thornton *et al.*, 2006). Apc2 and Apc11 form a stable subcomplex with Apc1, Apc4 and Apc5 (Vodermaier *et al.*, 2003). When dissociating Apc2 and Apc11 from APC by high-salt washes, levels of Doc1 are also reduced, indicating that Doc1 interacts with Apc2 or Apc11, but also with (an)other APC subunit(s) (Vodermaier *et al.*, 2003). Vertebrate APC contains four tetratricopeptide repeat (TPR) proteins, whereas yeast APC has only three of them; Apc7 is exclusively found in higher eukaryotes (Pal *et al.*, 2007a; Yu *et al.*, 1998). TPR domains were discovered in the first identified APC subunits (Irniger *et al.*, 1995; Lamb *et al.*, 1994) and represent the largest group of structurally related proteins within APC. TPR domains were proposed to serve as receptors for C-terminal peptide motifs (Gatto *et al.*, 2000). Consistent with this, TPR subunits can bind to peptides corresponding to the C-termini of Cdh1, Cdc20 (Vodermaier *et al.*, 2003), and Doc1 (Wendt *et al.*, 2001). Several TPR subunits which are each present more than once per complex (Dube *et al.*, 2005; Huang and Raff, 2002; Ohi *et al.*, 2007; Passmore *et al.*, 2005b) might therefore function as versatile acceptor sites for interactions with a variety of regulatory proteins (Vodermaier *et al.*, 2003) and possibly substrates ((Hayes *et al.*, 2006), Section 1.11).

Thornton and Toczisky have identified conditions under which APC becomes nonessential in yeast, allowing the deletion of otherwise essential APC subunits (Thornton *et al.*, 2006). By systematic deletion of subunits, followed by purification and subunit analysis of the

remainder of APC, the authors have been able to draw a detailed subunit assembly map ((Thornton *et al.*, 2006), Figure 1-2). According to their map, the largest subunit Apc1 builds a structural scaffold together with Apc4 and Apc5 and associates independently with two separable subcomplexes. The functions of these three subunits are not understood. Based on sequence homologies to the Rpn1 and Rpn2 subunits of the proteasome (Lupas *et al.*, 1997) it has been proposed that Apc1 might have a proteasome-related function such as delivery of polyubiquitinated substrates to the proteasome or unfolding of substrates (Kajava, 2002; Lupas *et al.*, 1997), but there are no experimental data supporting this hypothesis. Furthermore, Apc4 was predicted to fold into a WD40 propeller (Peters, 2006).

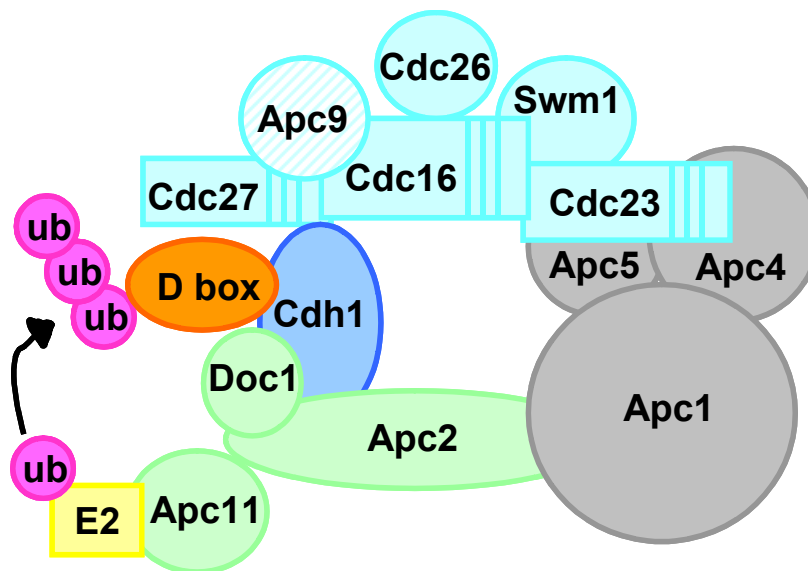


Figure 1-2: Model depicting APC subunit interactions and substrate ubiquitination. Modified after (Peters, 2006; Thornton *et al.*, 2006). Interactions are described in the text in detail. Subunits belonging to the “catalytic subcomplex” are light green and those of the “TPR subcomplex” are turquoise. The ubiquitin-loaded E2 enzyme is recruited by the RING finger Apc11. The cullin Apc2 might, in analogy to SCF complexes, serve as a scaffold that positions substrate receptor and the E2 with the help of Apc11. The substrate is recruited via its recognition element (here: a D box) by the coactivator and probably by a core APC subunit, which might be Doc1. APC then assembles ubiquitin chains onto substrate proteins. Doc1 functions as a processivity factor for APC ubiquitination and aids in substrate binding. Vertical stripes indicate TPR subunits. Metazoan APC contains an additional TPR subunit, Apc7, which is not depicted here. Apc9, which has so far only been found in yeast, is hatched. Of the core subunits, Apc1, Apc2, Cdc27, Cdc16, Apc4, Apc5, Cdc16, Cdc23, and Apc11 are essential in yeast, whereas Apc9, Doc1, Cdc26 and Swm1 are not (see Table 1-1).

One of the subcomplexes identified by Thornton *et al.* is composed of Apc2, Apc11 and Doc1, therefore referred to as the “catalytic subcomplex” (Thornton *et al.*, 2006). The “TPR

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subcomplex” contains the TPR subunits and, according to earlier findings most likely Apc9, Swm1 and Cdc26 (Passmore *et al.*, 2003; Schwickart *et al.*, 2004; Zachariae *et al.*, 1996). Among the TPR subunits, Cdc27 is the most peripheral and Cdc23 the most internal component (Figure 1-2). Apc1, Apc4, Apc5 and Cdc23 associate interdependently, such that loss of any of them greatly reduces binding of the remaining three proteins (Thornton *et al.*, 2006). Upon deletion of Apc2, Apc11 and Doc1 are lost (Thornton *et al.*, 2006). The situation appears to be different in human APC, where only partial loss of Doc1 is observed in an APC version lacking Apc2 and Apc11 (Vodermaier *et al.*, 2003).

1.10 Electron microscopic analysis of APC structures

An idea how APC’s many subunits might assemble into a three-dimensional (3D) structure comes from recent cryo-electron microscopy (EM) data. Structures obtained for APC purified from frog egg extracts and human cells (Dube *et al.*, 2005; Gieffers *et al.*, 2001), as well from budding (Passmore *et al.*, 2005b) and fission yeasts (Ohi *et al.*, 2007) have revealed that the APC is an asymmetric triangular complex with an internal cavity. The structures differ from each other significantly with respect to the size of the cavity, which might be too small to accommodate all factors required for substrate ubiquitination (substrate, E2, ubiquitin) in the mammalian, frog and budding yeast APCs; the recent structure of fission yeast APC in contrast includes a prominent cavity in which the ubiquitination reaction was proposed to take place (Ohi *et al.*, 2007). Labeling experiments performed with human APC contradict the existence of an inner reaction chamber since Cdh1 and the cullin domain of Apc2 are located on the outside of the complex (Dube *et al.*, 2005). Since most of the subunits and many functions are conserved among APC molecules from different species, it is plausible that the overall structural organization and the reaction mechanism should be similar as well. Further experimental and structure determination approaches will have to clarify this issue. Vertebrate APC contains two domains referred to as “platform” and “arc lamp” which exhibit a large degree of flexibility relative to each other. A movement of the domains resulting in a diminished angle between “platform” and “arc lamp” can be observed upon Cdh1 binding to APC which implies that coactivator binding might induce conformational changes within APC (Dube *et al.*, 2005). Two comprehensive antibody labeling data sets are largely consistent with the outlined APC subunit map (Schwickart *et al.*,

2004; Thornton *et al.*, 2006; Vodermaier *et al.*, 2003); Figure 1-2). Ohi *et al.* have presented an almost-complete set of localized fission yeast APC subunits, the interpretation of which is however somewhat limited due to the fact that subunit positions are only available in two dimensions (2D) (Ohi *et al.*, 2007). Subunit localization of eight human APC subunits, performed similar as described in an earlier study (Dube *et al.*, 2005), has yielded an impressive and largely “annotated” structure of human APC (Herzog *et al.*, manuscript in preparation).

1.11 APC substrate recognition

Most APC substrates contain either one or both of two *cis* elements called the destruction box (D box, consensus sequence R-X-X-L-X-X-X-X-N, (Glutzer *et al.*, 1991)) or the KEN box (K-E-N-X-X-X-E/D/N, (Pfleger and Kirschner, 2000)) that target them for ubiquitination by APC. Other less well characterized degrons include the A box (Littlepage and Ruderman, 2002), the GxEN motif (Castro *et al.*, 2003) and the O box (Araki *et al.*, 2005). Several studies have shown a direct association between coactivators and various APC substrates *in vitro* (Vodermaier, 2001), indicating that the coactivators might function similarly to the WD40 repeat-containing F box proteins in SCF complexes. Not all reports, however, were consistent with respect to the questions which regions within the coactivators are responsible for substrate binding and whether or not the coactivator-substrate interaction is D/KEN box dependent (Peters, 2006; Vodermaier, 2001). A direct interaction between the WD40 domain of Cdh1 and a D box-containing substrate peptide has recently been reported (Kraft *et al.*, 2005). Importantly, residues within the WD40 domain required for the D box interaction were identified; mutations in these residues result in a Cdh1 version which is not able to efficiently ubiquitinate substrates anymore. This implies that the WD40 domain is a functionally important D box receptor (Kraft *et al.*, 2005). The first evidence that core APC might directly interact with substrates can be found in a study by Yamano *et al.* who used a tandem D box affinity matrix to isolate APC from frog egg extracts (Yamano *et al.*, 2004). Cdc20 was depleted and since Cdh1 is believed to be absent from these extracts, they did not contain any coactivators; yet APC was able to bind (Yamano *et al.*, 2004). Some support for Yamano’s finding comes from a study on Emi1, which proposed that Emi1 inhibit APC by acting as a pseudosubstrate inhibitor (Miller *et al.*, 2006). The Emi1-APC

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binding depends on Emi1's D box and was also observed in frog egg extracts from which Cdc20 was depleted; lending support to Yamano's findings that holo-APC contains a D box receptor (Miller *et al.*, 2006). Substrates have recently been shown to be targeted to APC via a C-terminal MR motif (Hayes *et al.*, 2006). This interaction occurs independently of Cdc20 and is reminiscent of the IR tail-dependent binding of coactivators and Doc1 to APC core subunits (Vodermaier *et al.*, 2003; Wendt *et al.*, 2001).

Elegant experiments have put forward the idea of a stoichiometric APC-coactivator-substrate complex. Passmore and Barford have reported that APC, coactivator and substrate form a ternary complex, and that the amount of APC-substrate binding directly correlates with the amount of APC-coactivator association (Passmore and Barford, 2005). "Isotope trapping" experiments have shown that a complex between APC and Cdc20 binds substrate with higher affinity than APC or Cdc20 alone (Eytan *et al.*, 2006). Importantly, substrate binding to APC in the absence of coactivators occurs with highly reduced selectivity and affinity (Eytan *et al.*, 2006), which again underlines the important role of coactivators in this process. Some studies have implicated specific APC subunits as APC substrate receptors. Whereas a yeast two-hybrid screen identified an interaction between the D box of the B-type cyclin Clb2 and Cdc23 (Meyn *et al.*, 2002), several studies proposed Doc1 as an APC substrate receptor (Carroll *et al.*, 2005; Nourry *et al.*, 2004; Passmore *et al.*, 2003).

1.12 ...and the proposed role of Doc1

Substrates have been reported to bind to APC in the absence of coactivators (Eytan *et al.*, 2006; Hayes *et al.*, 2006; Yamano *et al.*, 2004) and core APC has been shown to contribute to efficient substrate recognition together with coactivators (Burton *et al.*, 2005; Eytan *et al.*, 2006; Kraft *et al.*, 2005; Passmore and Barford, 2005) – yet the identity of the putative APC substrate receptor is still unknown. The best candidate so far is Doc1. APC lacking Doc1 can no longer bind substrates but is still able to associate with Cdc20 and Cdh1 (Passmore *et al.*, 2003). This indicates that Doc1 contributes to substrate binding either directly or together with other APC subunits, but not via Cdc20 or Cdh1 (Passmore *et al.*, 2003). Kinetic analysis has revealed a defect of APC^{doc1Δ} in processivity (Carroll and Morgan, 2002). In a processive ubiquitination reaction, the substrate remains bound to APC while repeated cycles of ubiquitination occur; in absence of Doc1 only short substrate-ubiquitin conjugates

are formed (Carroll and Morgan, 2002). Residual APC^{doc1Δ} activity is not further reduced if the D box in substrates is mutated, indicating that Doc1 might contribute to D box recognition (Carroll *et al.*, 2005).

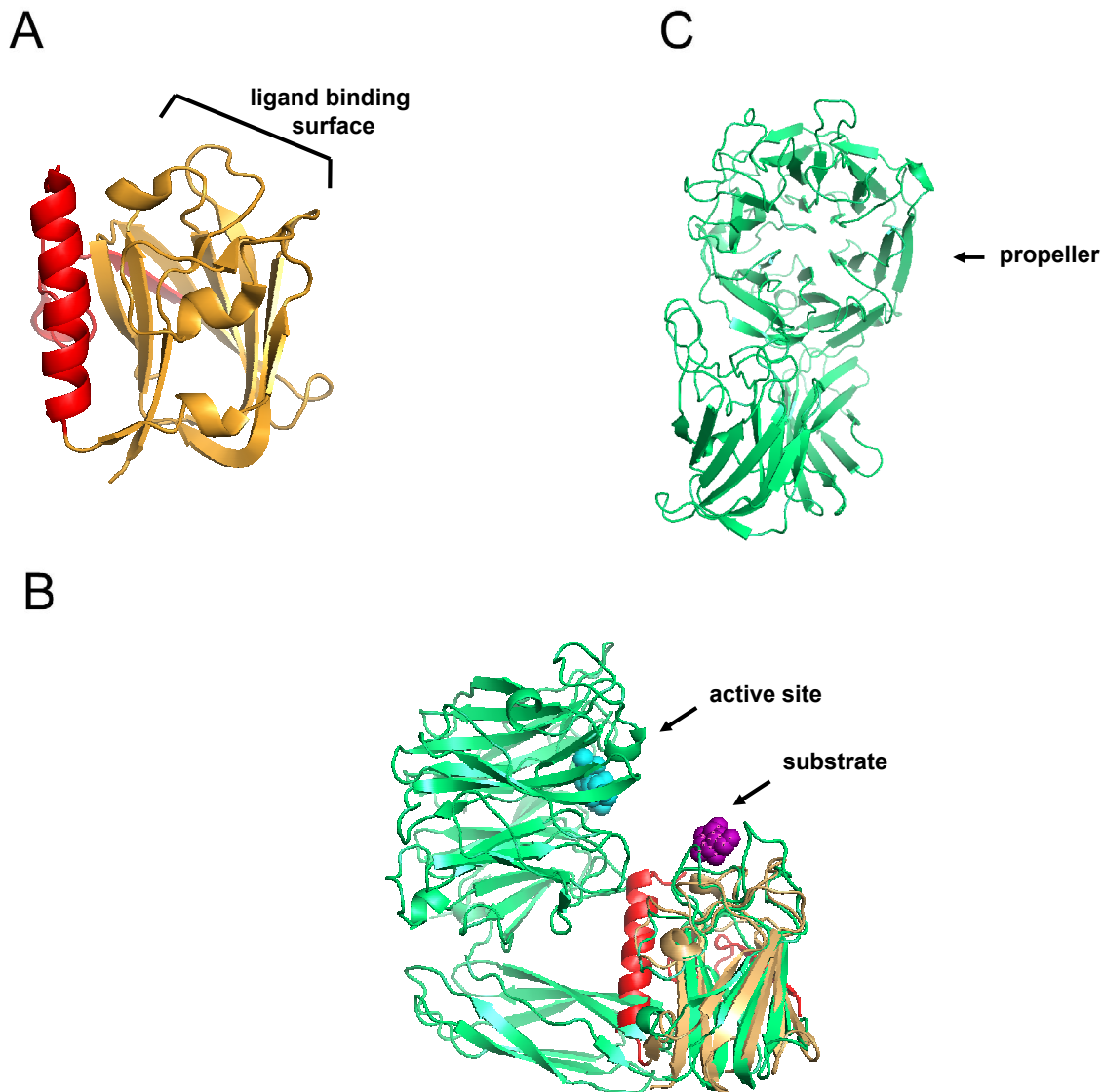


Figure 1-3: Crystal structures of yeast Doc1 and a bacterial sialidase; structure overlay. A) Crystal structure of yeast Doc1. The Doc domain is shown in yellow and the putative ligand binding region is depicted. B) Three-domain structure of bacterial sialidase. The galactose binding domain which folds into a jelly-roll is overlaid with the similar Doc1 structure. Sialidase consists of a 41 kDa propeller domain which was both crystallized separately bound to an inhibitor (shown in turquoise) and together with the IgG linker domain and the galactose binding domain bound to galactose (shown in purple). C) The sialidase structure is rotated by 90° to show the propeller fold of the 41 kDa domain.

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The crystal structures of both human and budding yeast Apc10/Doc1 have been solved (Au *et al.*, 2002; Wendt *et al.*, 2001). Apc10/Doc1 exhibits a jelly-roll fold (Figure 1-3A) which is also found in other proteins; all these proteins have in common that they use the same surface to bind to their diverse ligands (Au *et al.*, 2002; Wendt *et al.*, 2001). Interestingly, residues conserved among Doc1 orthologs are found in a region of Doc1 that corresponds to this ligand-binding surface (Figure 1-3A). Mutational analysis has shown that Doc1 can no longer function as a processivity factor if four residues within this ligand binding surface are changed to alanines (Carroll *et al.*, 2005). Doc1 might therefore use this surface to bind to a putative ligand, which could be an APC substrate or any other factor important in the ubiquitination reaction. A ternary APC-coactivator-substrate complex might therefore be formed when a substrate (via its D box) interacts with the WD40 propeller domain of a coactivator and with an APC subunit such as Doc1, either in a simultaneous or a sequential fashion (Figure 1-3B). Such a Doc1-coactivator arrangement would be strikingly similar to the domain structure of sialidase. The crystal structure of this sugar-hydrolyzing enzyme has revealed a propeller domain (Figure 1-3C) and a jelly-roll fold domain, connected by a linker domain, which collaborate in substrate binding and catalysis (Figure 1-3B; (Gaskell *et al.*, 1995; Peters, 2006)).

1.13 Aim of this study

The mechanisms of substrate recognition and ubiquitination by APC are only poorly understood. The APC subunit Doc1 has been shown to be a processivity factor for APC ubiquitination. The molecular basis for this function is not known. Doc1 might mediate processive ubiquitination reactions by directly binding to an APC substrate or any other factor important in the ubiquitination reaction. Alternatively Doc1 could have an indirect effect on APC activity by causing structural rearrangements within APC once a substrate is bound to the complex. I therefore set out to study the role of Doc1 in APC-mediated substrate ubiquitination by identifying the interaction partners of this subunit, both within APC and among factors involved in APC-catalyzed ubiquitination reactions. Because interactions involved in APC substrate recognition are thought to be very transient, I have chosen a site-specific photo crosslinking approach which allows “freezing” of transient interactions between Doc1 and its possible binding partners.

Understanding how APC subunits, including Doc1, cooperate to recognize and ubiquitinate substrates will ultimately require information about their 3D organization within APC. We have decided to extend our EM studies to budding yeast APC, to be able to generate and analyze mutant and engineered APC versions. The first aim was to generate a 3D model of yeast APC. We then wanted to analyze mutant complexes such as APC lacking Doc1. Comparing 3D models of *wild type* APC and APC lacking Doc1 should reveal the localization of Doc1. Furthermore, we set out to analyze the localizations of additional APC subunits by fusing big globular tags to these proteins, which would not interfere with APC subunit assembly and function. Comparing an APC structure which contains a tagged protein with the *wild type* structure should reveal an additional mass which indicates the localization of the epitope tag. By using this strategy for many APC subunits we aimed at generating an APC subunit topology map which is more precise than those presented before.

2 Results

2.1 Site-specific photocrosslinking to identify interaction partners of Doc1

2.1.1 Yeast Doc1 comprises 250 and not 283 amino acids

DOC1 was first identified as a gene involved in cyclin proteolysis in a genetic screen for mitotic arrest mutants (Hwang and Murray, 1997). Hwang and Murray had isolated a Doc1 mutant, *doc1-1*, whose phenotype was rescued with a minimal sequence containing an open reading frame (ORF) which encoded a protein of 283 amino acids (Hwang and Murray, 1997). The *Saccharomyces* genome database (SGD, <http://www.yeastgenome.org/>) entry for Doc1, however, comprises only 250 amino acids. Most Doc1 publications, including the work by Carroll *et al.* that I will be referring to often, used the 283 amino acid version (Carroll *et al.*, 2005).

In order to find out which version of Doc1 exists in the cell, I generated Doc1 from DNA templates encoding the “long” (Dl) and the “short” (Ds) version by *in vitro* translation (IVT). These DNA templates were generated by polymerase chain reaction (PCR)-amplification from genomic yeast DNA. For this I used two different forward primers which annealed upstream of either of the two proposed start codons and contained a T7 promotor and a Kozak sequence; the reverse primer used for both reactions annealed downstream of the stop codon. The “long” DNA template therefore contained two translation initiation sites. I separated the proteins obtained from the *in vitro* translation reactions by SDS-PAGE along with yeast cell extracts and purified APC. Doc1 was visualized by immunoblotting using a Doc1 antibody and by phosphorimaging (Figure 2–1). The phosphorimage shows that when using DNA encoding the “long” Doc1 for the translation reaction, two versions of Doc1 were generated, presumably because the template contained both translation start sites (Figure 2–1, lane 3). In contrast, only one Doc1 version was obtained in the reactions using the “short” Doc1 template had the same size as the faster migrating band in the “long” Doc1 sample (Figure 2–1, lane 2). The endogenous Doc1 protein that was present in the cell extract (Figure 2–1, lane 5) and in the purified APC sample (Figure 2–1, lane 4) clearly

comigrated with the short version of Doc1 (Figure 2–1, lane 2). I therefore decided to use the 250 amino acid long version of Doc1 in my *in vitro* assays.

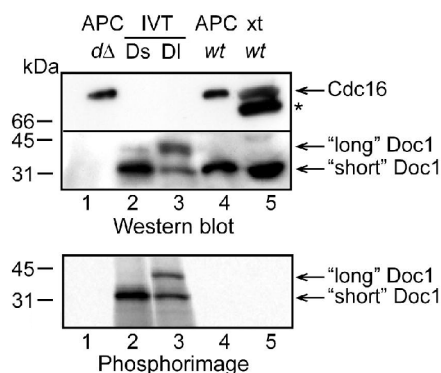


Figure 2-1: Doc1 contained in yeast cell extracts comigrates with the 250, and not the 283 amino acid long version. The product of an *in vitro* translation (IVT) using the short *DOC1* ORF version as a template (lane 2) was loaded next to an IVT that had been generated from a template that also comprised the earlier translation start site. Lanes 1 and 4 contain TAP-purified APC from *doc1Δ* (*dΔ*) and *wild type* (*wt*) strains and lane 5 the extract (xt) of a *wild type* strain for size comparison. Samples were analyzed by immunoblotting using Cdc16 and Doc1 antibodies and by phosphorimaging of the same membrane. The asterisk marks an unspecific signal recognized by the Cdc16 antibody on cell extracts. On the phosphorimage, *in vitro* translated proteins labeled with [³⁵S]methionine are visualized.

2.1.2 APC can be reconstituted from APC^{doc1Δ} and recombinant Doc1

To identify interaction partners of Doc1 I wanted to replace endogenous Doc1 with a form of Doc1 that carries a photocrosslinker at a defined amino acid site. It has been shown before that adding back recombinant Doc1 to mutant APC^{doc1Δ} fully restores APC's ubiquitin ligase activity (Passmore *et al.*, 2003). APC was purified to high homogeneity from a yeast strain carrying a TAP-tag on Apc4 via the TAP-method ((Figure 2–2A), Passmore *et al.*, 2005a; Puig *et al.*, 2001). *DOC1* was deleted from this strain (which renders it temperature sensitive) to allow complete replacement with a recombinant crosslinker-containing version, and the deletion was analyzed by silver staining (Figure 2–2A) and by immunoblotting (Figure 2–2B). APC lacking Doc1 is stable and was reported to contain all other core subunits at levels similar to wild type APC (Carroll and Morgan, 2002; Passmore *et al.*, 2003; Schwickart *et al.*, 2004). In my preparations, all subunits seemed to be unaffected with the exception of Apc11, whose levels were slightly reduced (Figure 2–2A, auto contrasted panel).

RESULTS

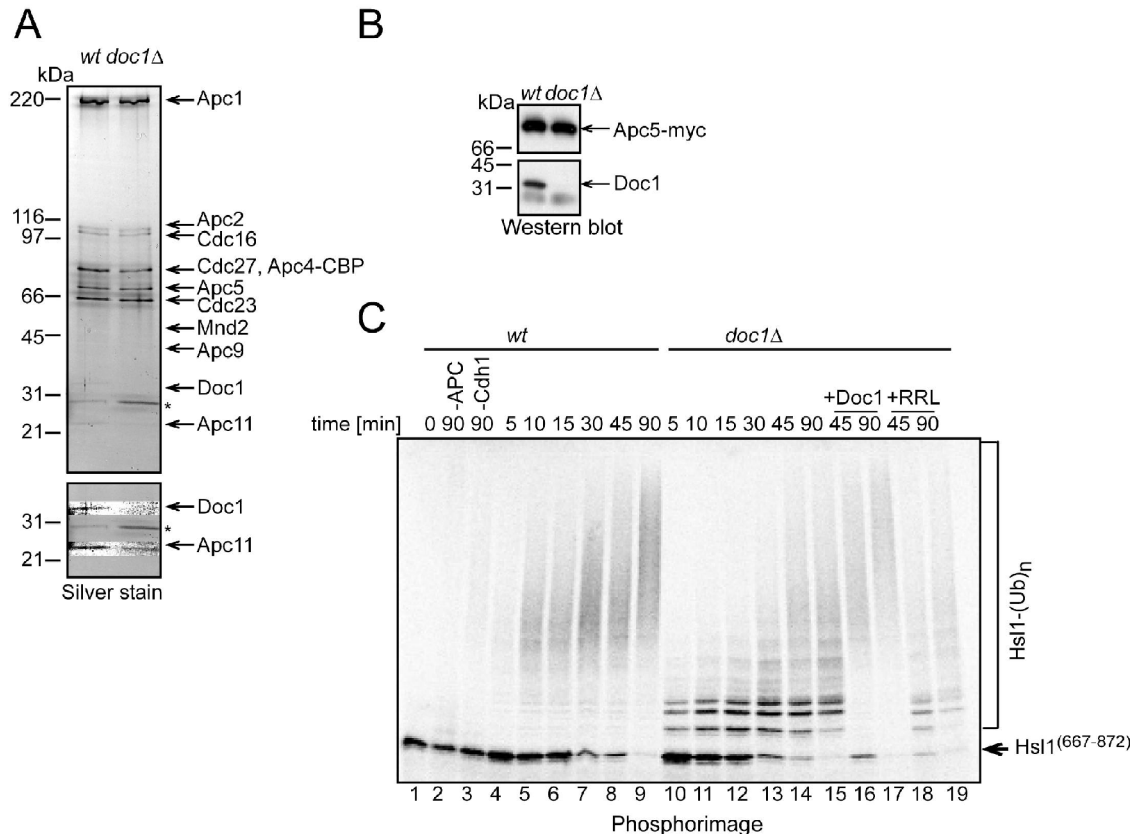


Figure 2-2: Addition of *in vitro* translated Doc1 to APC lacking Doc1 restores *wild type* activity. A) APC from a *wild type* strain (*wt*) and a strain lacking Doc1 (*doc1Δ*) was purified by the TAP-method and analyzed by SDS-PAGE and silver staining. APC subunits were identified by their electrophoretic mobility and by mass spectrometry. The asterix marks a contaminating band which does not contain APC subunits. Preparations were from strains J319 (*wt*) and J323 (*doc1Δ*). B) The Doc1 protein is absent from yeast strains after *DOC1* deletion. APC purified from *wild type* (*wt*) and *doc1Δ* yeast strains was analyzed by immunoblotting using myc and Doc1 antibodies. Both yeast strains (J42, J187) carried a myc-tag on Apc5. C) APC lacking Doc1 shows impaired ubiquitination activity, and activity of the *wild type* can be restored by adding recombinant Doc1. *In vitro* ubiquitination assay using an [³⁵S]methionine-labeled Hsl1-fragment (amino acids 667 to 872) as model substrate. Substrate and ubiquitin conjugates are visualized on a phosphorimage.

I carried out *in vitro* ubiquitination assays in which recombinant *in vitro* translated Cdh1 was used to activate APC, to measure APC activity. *Wild type* APC assembled polyubiquitin chains on the yeast Hsl1⁽⁶⁶⁷⁻⁸⁷²⁾ model substrate (Figure 2–2C, lanes 1 to 9). In contrast, substrates processed by APC^{doc1Δ} were modified with short conjugates only (Figure 2–2C, lanes 10 to 15). These results are consistent with previous data (Carroll *et al.*, 2005; Passmore *et al.*, 2003) and are in line with the notion that Doc1 acts as a processivity factor for APC ubiquitination (Carroll *et al.*, 2005). The activity of the *wild type* enzyme can be largely restored by adding back Doc1 to APC^{doc1Δ} ((Carroll *et al.*, 2005; Passmore *et al.*, 2003), Figure 2–2B,

lanes 16 and 17). Despite the complication that the reticulocyte lysate contributes to enzymatic activity (Figure 2–2C, lanes 18 and 19), I used Doc1 generated by IVT for restoring APC activity, because I was using this system also for incorporation of the photocrosslinker (see below).

2.1.3 Identification of Doc 1 interaction partners within the APC

Photocrosslinking techniques have recently been adopted to study APC-substrate interactions (Kraft *et al.*, 2005). We decided to use the crosslinker compound L-4'-(3-[trifluoromethyl]-3H-diazirine-3-yl) phenylalanine ((tmd)phe, Figure 2–3A), which can be introduced into a protein in an *in vitro* transcription/translation system using a suppressor tRNA approach (Brunner, 1993). (Tmd)phe is a structural analogue of phenylalanine which upon photolysis generates a very reactive carbene that is capable of forming covalent interactions with a broad range of organic bonds ((Brunner, 1993), Figure 2–3A). Because of its reasonably small size, (tmd)phe may be incorporated into proteins without seriously affecting protein structure or the protein's interactions with adjacent components (High *et al.*, 1993). The incorporation of the modified amino acid is directed by the presence of an “amber” stop codon (TAG) which is introduced into the coding sequence of the cDNA to be expressed. In an *in vitro* transcription/translation reaction, the use of an *amber* suppresser tRNA coupled to (tmd)phe ((tmd)phe-tRNA) allows translation beyond the stop codon, resulting in a protein carrying a photoactivatable amino acid at one specific site.

Figure 2–3B shows an example of an *in vitro* translation product of a Doc1 *amber* mutant in the presence or absence of (tmd)phe-tRNA; the position of the *amber* mutations is described below. As predicted, a truncated version of the protein was generated in the absence of (tmd)phe-tRNA (Figure 2–3C, lane 1). After addition of the compound, most of the translation product represents full-length protein with incorporated crosslinker (Figure 2–3C, lane 2) which had the same size as a translation product obtained from *wild type* cDNA (Figure 2–3C, lane 3). The incorporation efficiency was about 75 % on average for all Doc1 mutants generated (not shown). An efficient suppression required the presence of magnesium acetate in the reaction, which in turn decreased the total translation efficiency about three to four-fold (compare Figure 2–3C, lanes 2 and 3; quantification not shown).

RESULTS

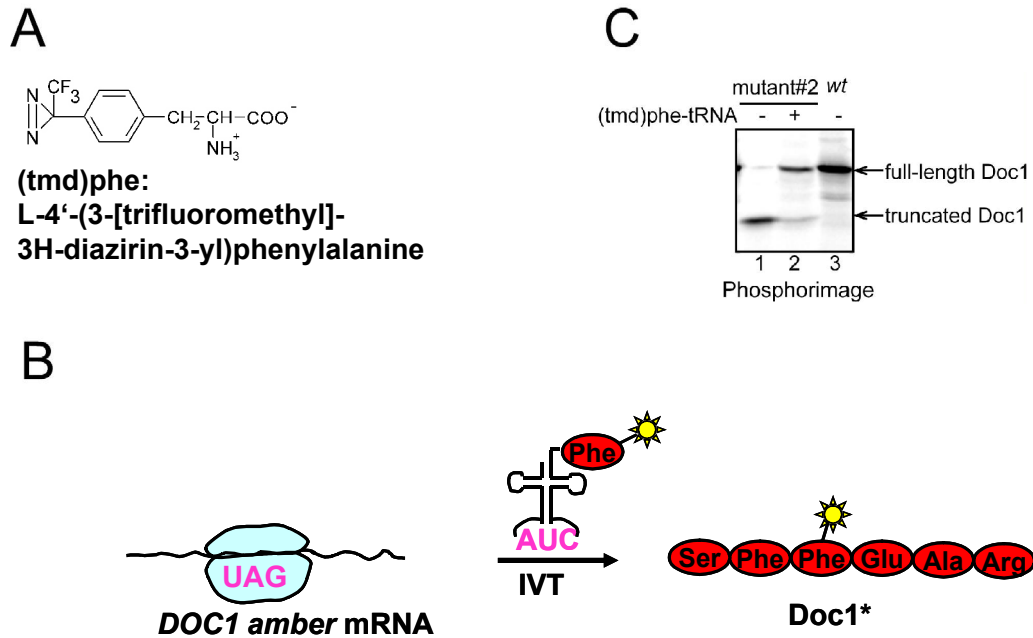


Figure 2-3: Introduction of (tmd)phe into Doc1 by *in vitro* translation. A) Structure of L-4'-(3-[trifluoromethyl]-3H-diazirin-3-yl) phenylalanine ((tmd)phe). B) An *amber* stop codon is introduced into the cDNA encoding the protein of interest. The protein is *in vitro* translated in the presence of a non-natural suppressor tRNA coupled to a (tmd)phe and [35 S]methionine. C) Every full-length protein contains the photoreactive amino acid. Phosphorimage showing the *in vitro* translation of Doc1 *wild type* (*wt*) and mutant#2 in absence and presence of (tmd)phe-tRNA.

To set up the conditions for photocrosslinking experiments with Doc1, I first introduced the modified amino acid within a region which was known to be important for Doc1 interaction with the APC. A mutational analysis had shown that two amino acids close to the C-terminus of Doc1 (depicted in Figure 2-4A), which are highly conserved among Doc1 orthologs, are important for Doc1 binding to the APC (Carroll *et al.*, 2005). Carroll *et al.* had found that mutation of these two sites, K129 and R130, to alanine and simultaneous deletion of the C-terminus, greatly reduced the binding of Doc1 to APC (Carroll *et al.*, 2005). I therefore decided to introduce the photocrosslinker at three different sites within this “C-terminal region” (at K129, R130 and an adjacent site, S128, Figure 2-4A). These three mutants were used in photocrosslinking experiments. The experimental setup is outlined in Figure 2-4B. I incubated APC^{doc1Δ} that was immobilized on IgG sepharose beads via a TAP-tag on Apc4 with the Doc1 crosslinker versions, washed off the reticulocyte lysate translation mixture and activated the photocrosslinker with UV-light. SDS-PAGE and phosphorimage analysis revealed two major crosslink products for mutants S128*amber* and

K129*amber* at sizes of ~120 kDa and ~200 kDa (Figure 2–4C, lanes 2 and 3). No crosslink products were obtained for mutant R130 (not shown). These results indicate that Doc1's interaction partners can be identified with this approach.

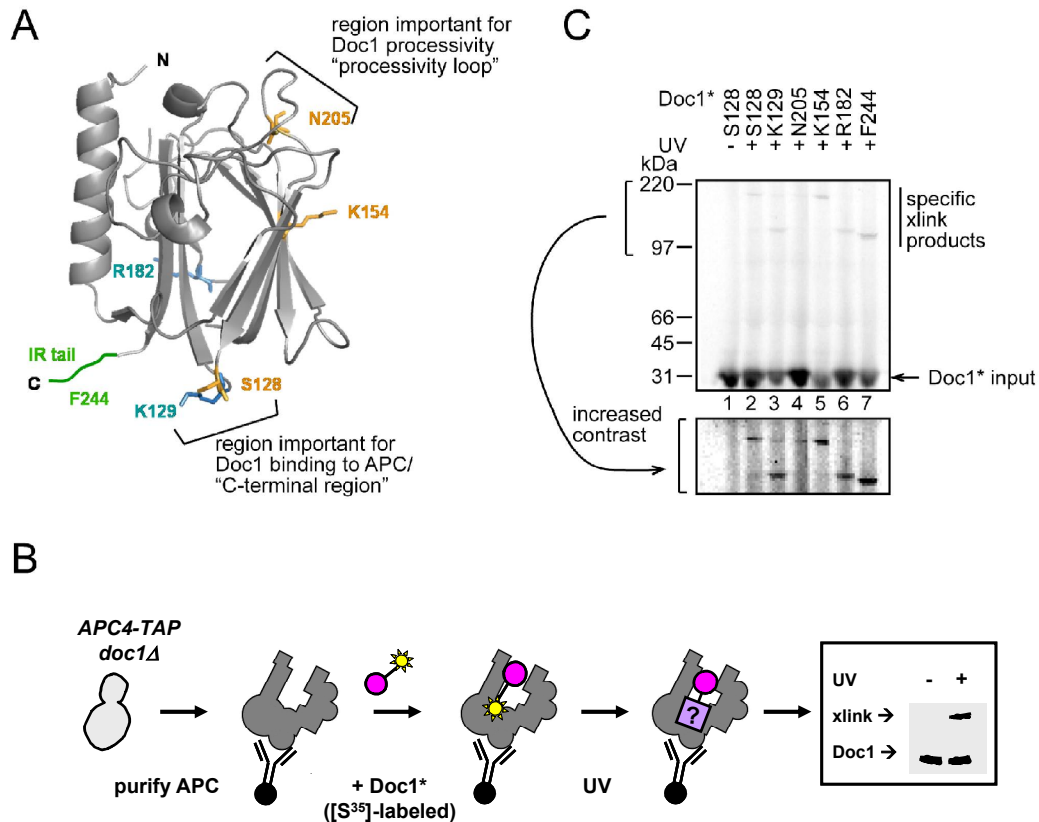


Figure 2-4: Insertion of a photocrosslinker at single sites within several regions of Doc1 results, upon photolysis, in specific crosslink products with different electrophoretic mobilities. A) Structure of *S. cerevisiae* Doc1 generated with pymol (DeLano, W.L. The PyMOL Molecular Graphics System (2002), <http://www.pymol.org>). Two regions which were proven to be important for Doc1 function (Carroll *et al.*, 2005) are depicted. When a photoreactive amino acid was inserted at the sites shown in the structure, specific crosslink (xlink) products were obtained. B) Experimental outline. APC from strains lacking Doc1 was bound to beads via a TAP-tag on Apc4. Radiolabeled Doc1 containing a photocrosslinker is bound to beads and the photocrosslinker is activated by UV-light. Samples were analyzed by SDS-PAGE and phosphorimaging for mobility shifts occurring upon UV-irradiation. C) Overview of crosslink products obtained with Doc1*amber* mutants carrying the photocrosslinker at the positions indicated in A). APC was bound to beads and incubated with Doc1 versions carrying photocrosslinker at the given positions. After UV-irradiation, bead-bound proteins were eluted by SDS sample buffer and samples were analyzed by phosphorimaging. The contrast settings of the phosphorimage were changed to visualize the crosslink products, which ran at three different sizes.

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I then went on to test further 21 Doc1 *amber* mutants under the above conditions. Subsets of these mutants contained the photocrosslinker within regions or at sites that were described as being important for Doc1 function before (Carroll *et al.*, 2005), such as the putative ligand

Table 2-1: List of all sites in Doc1 which were exchanged for the photoreactive amino acid. For easier comparison with studies such as the one from (Carroll *et al.*, 2005), the corresponding positions in the “long” Doc1 versions are also given. Regions within Doc1 were roughly classified based on the view shown in Figure 2-4A. Abbreviations are: back side (BS), N-terminal helix (NH), front side (FS), C-terminal region (CR), processivity loop (PL), IR tail (IR).

site of incorporation	site in “long” Doc1	location of site in structure
R37	R70	BS
H67	H100	NH
Q71	Q104	NH
K91	K124	FS
K96	K129	FS
L97	L130	FS
F103	F136	FS
D110	D143	FS
D116	D149	FS
S128	S161	CR
K129	K162	CR
R130	R163	CR
E146	E179	BS
K154	K187	FS
R182	R215	BS
R199	R232	FS
N205	N238	PL
H206	H239	PL
E207	E240	PL
N208	N241	PL
K210	K243	PL
D211	D244	PL
E239	E272	IR
F244	F277	IR

binding region (Figure 2–4A, Section 2.1.4). In addition, I selected sites for crosslinker incorporation in all areas of the surface of Doc1. In total I exchanged 24 residues for

(tmd)phe, corresponding to 10 % of all Doc1 amino acid residues. Table 2–1 lists all mutants that I generated and tested.

I obtained crosslink products with six mutants, as shown in Figure 2–4C. Mutants K154*amber* and N205*amber* gave a similar 200 kDa crosslink product as mutant S128*amber* (in Figure 2–4C, lanes 2, 4, 5). Using R182*amber* resulted in a crosslink product similar in size to the one observed with K129*amber* (in Figure 2–4C, lanes 2 and 6). Finally, I obtained a product which ran at a size of about 120 kDa with mutant F244*amber* (in Figure 2–4C, lane 7) and with mutant E239*amber* (not shown).

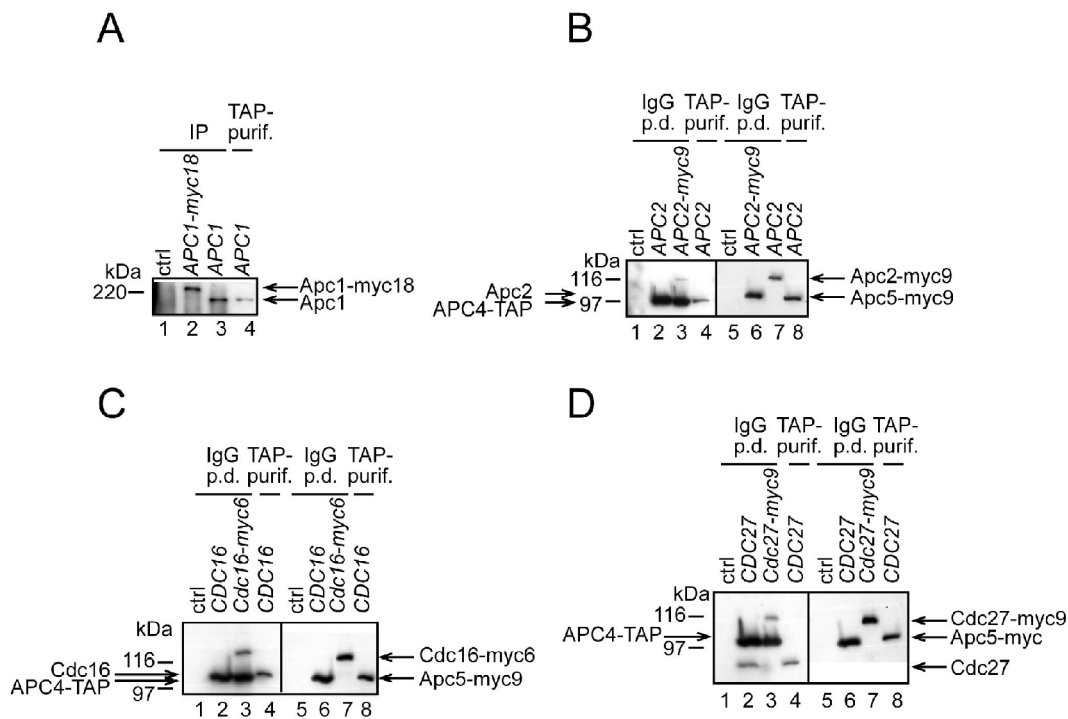


Figure 2-5: Myc-tagging of APC subunits results in significant mobility shifts. APC was purified from tagged and untagged strains by myc-immunoprecipitation (IP) in (A) or by myc-IP and IgG pull-down (IgG p.d., B to D). APC subunits were visualized by immunoblotting using antibodies against Apc1 (A), Apc2 (B), Cdc16 (C) and Cdc27 (D) and the myc-epitope (B to D). All strains used also carried a myc-tag on Apc5. Visualizing the shift of Apc5 upon tagging with the myc9-epitope could not be demonstrated in a similar way since an antibody against yeast Apc5 was not available. The following strains were used: strains J61 and J134 for (A); J189 and J187 for (B); J235 and J187 for (C); J266 and J187 for (D); as a control strain (ctrl), J202 was used; the TAP-purified (TAP-purif.) samples were from J180.

To test if the observed crosslink products represent Doc1 bound to APC subunits, and to determine the identity of these subunits, we generated a new set of yeast strains. These

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strains, in addition to the *doc1* deletion and the TAP-tag on *APC4*, each carried a myc-tag on one of the candidate subunits. Since the observed crosslink products were in the range of 100 to 200 kDa, the molecular weight of Doc1's possible interaction partners was predicted to be 80 kDa or more. We therefore created yeast strains a tag on Apc1, Apc2, Cdc27, Cdc16 or Apc5; we used myc6-, myc9- or myc18-tags. Figure 2–5 shows that these tags were big enough to clearly alter the electrophoretic mobility of the tagged APC subunits.

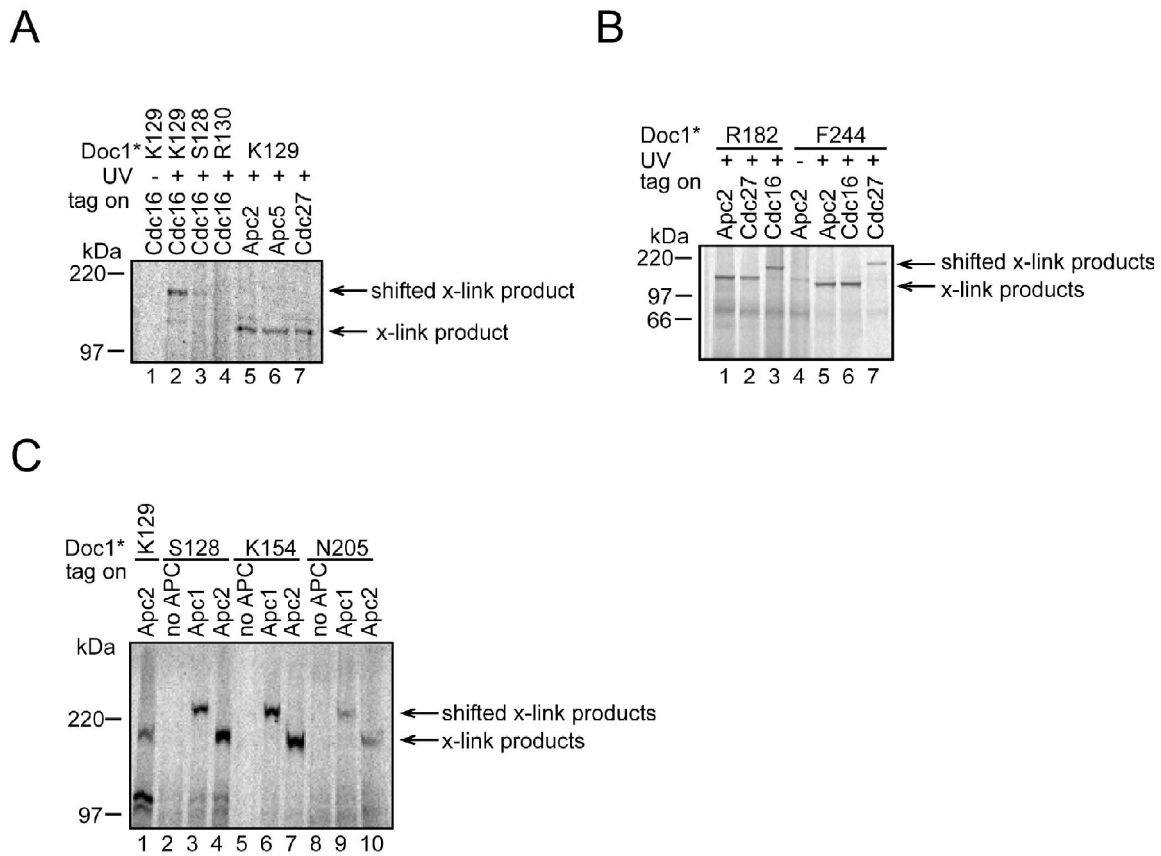


Figure 2-6: Identification of Doc1's interaction partners within APC. Doc1 directly interacts with Cdc16 (A and B), Cdc27 (B) and Apc1 (C). Crosslinker-containing Doc1 versions were incubated with APC immobilized on beads. Photolysis was induced by UV-irradiation and samples were analyzed by SDS-PAGE and phosphorimaging. A) Doc1 binds to Cdc16 via K129. To identify the subunit, strains carrying tags on Cdc16, Apc2, Apc5 and Cdc27 (J235, J189, J187, J266) were used. B) Doc1 interacts with Cdc16 via K182 and with Cdc27 via F244. The experiment was carried out using strains with myc-tagged Apc2, Cdc16 and Cdc27 (J189, J235, J266). C) Doc1 contacts Apc1 via S128, K154 and N205. Strains carrying myc-tags on Apc1 and Apc2 were used (J182, J189).

Using these strains in crosslinking experiments should therefore result in an additional mobility shift compared to the initial crosslink once the “right” strain was used for APC

isolation and Doc1 was crosslinked to a myc-tagged subunit. Following this strategy led to the identification of Cdc16 as a binding partner of Doc1's residue K129 in the C-terminal region (Figure 2–6A, compare lane 2 with lanes 5 to 7). The same interaction was also found with the mutant R182*amber* (Figure 2–6B, compare lane 3 with lanes 1 and 2). R182 is in proximity to K129 and emanates from the lower back of the molecule (Figure 2–4A).

Residues in Doc1's C-terminal protrusion, the IR tail, directly interacted with Cdc27 (Figure 2–6B, compare lane 7 with lanes 5 and 6, and data not shown). Two mutants crosslinked strongly to Apc1; one of them carries the crosslinker in the C-terminal region (S128, Figure 2–6C, compare lanes 3 and 4) and in the other one it extends the lower right from a β -sheet (K154, Figure 2–6C, compare lanes 6 and 7). In addition, N205*amber*, in which the crosslinker is located in the putative ligand binding region, crosslinked weakly to Apc1 (Figure 2–6C, compare lanes 9 and 10). With mutant K129*amber*, which strongly crosslinked to Cdc16, I also obtained a weak crosslink to Apc1 (not shown). Table 2–2 lists successful crosslinker mutants and the identified interaction partners.

Table 2-2: List of all Doc1 interactions identified by photocrosslinking. +++ marks a strong and + a crosslink.

site of incorporation	site in “long” Doc1	interaction partner
S128	S161	Apc1 (+++) Cdc16 (+)
K129	K162	Cdc16 (+++) Apc1 (+)
K154	K187	Apc1 (+++)
R182	R215	Cdc16 (+++)
N205	N238	Apc1 (+)
F244	F277	Cdc27 (+++)

In the budding yeast APC subunit map by Thornton et al., Cdc16 is required for the association of Cdc27 with APC (Thornton et al., 2006). The same authors have reported that APC isolated from a *cdc27* Δ strain retains residual ubiquitination activity, whereas *cdc16* Δ APC is inactive (Thornton et al., 2006). This suggests that Cdc16 might have an additional function beyond mediating Cdc27 binding to APC. We hypothesized that the interaction of Doc1 with Cdc16 might be important for the residual ubiquitination activity of *cdc27* Δ

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APC. One prerequisite for this would be that the Doc1-Cdc16 interaction can take place also in the absence of Cdc27. I therefore decided to analyze the effect of Doc1's IR tail, which directly binds to Cdc27, on the interaction with Cdc16. For this, I first had to find conditions, under which wild type Doc1 and a version lacking the C-terminal 16 amino acids (Doc1 Δ IR) were binding to APC^{doc1 Δ} at similar levels. TAP-tagged APC^{doc1 Δ} bound to IgG sepharose was incubated with the two *in vitro* translated Doc1 versions and subsequently eluted by TEV protease cleavage at different time points. At early time points, more *wild type* Doc1 was bound to APC than Doc1 Δ IR, but the effect of the IR tail deletion decreased over time with no difference left after 60 min (Figure 2–7A, lanes 15 and 16). Comparable levels

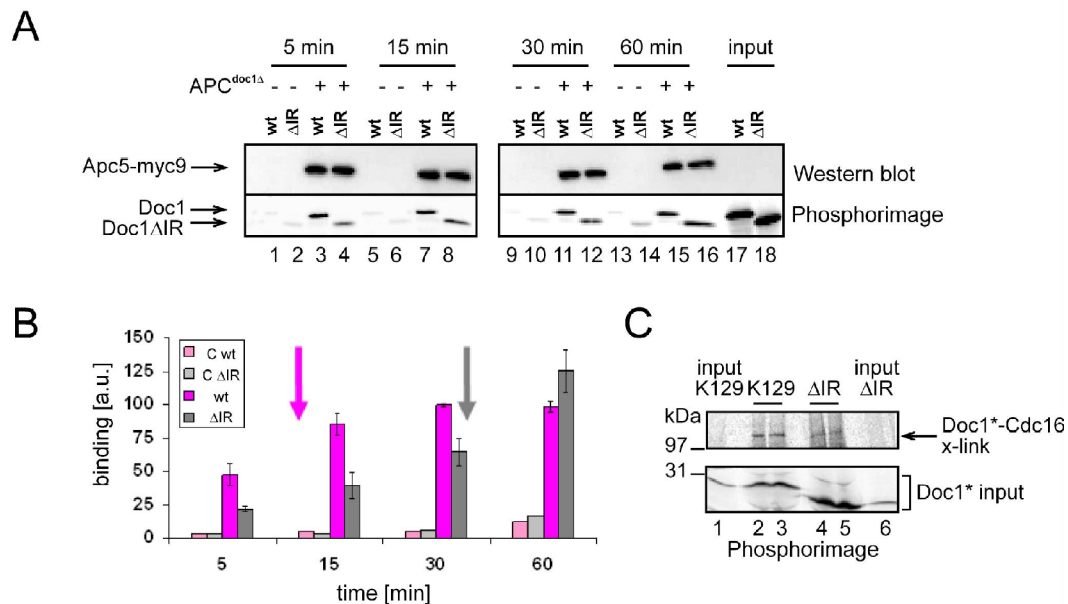


Figure 2-7: Doc1's C-terminal IR tail enhances binding of Doc1 to APC. A) The effect of a C-terminal deletion of Doc1 decreases over time. TAP-tagged APC was bound to IgG sepharose, incubated with *in vitro* translated [³⁵S]-labeled Doc1 *wild type* (wt) and a truncated version (Δ IR). Bound material was eluted by TEV cleavage and analyzed by SDS-PAGE and immunoblotting as well as phosphorimaging. 10 % of the input was also analyzed. Yeast strains used in this experiment contained myc-tagged Apc5, which was stained with 9E10 antibody. B) Quantification of the experiment shown in (A) and three independent similar experiments using ImageQuant. C) The crosslink of Doc1 to Cdc16 via Doc1's K129 is four-fold reduced after IR tail deletion. Doc1 and Doc1 Δ IR both carrying a crosslinker at K129 were bound to APC-beads for the times indicated with arrows in (B) to achieve equal amounts being bound of both versions. After photolysis, samples were analyzed by SDS-PAGE and phosphorimaging. Samples were produced in duplicates.

of both Doc1 versions were bound when allowing *wild type* Doc1 to bind for 10 and Doc1 Δ IR for 30 min. (Figure 2-7B, arrows).

I then used this information for a crosslinking experiment, assuming that the presence of the photocrosslinker would have no or the same effect on the binding of both Doc1 versions. The photocrosslinkers were inserted into *wild type* Doc1 and Doc1 Δ IR at K129, proteins were bound to APC^{doc1 Δ} -beads, and after UV-irradiation and SDS-PAGE analysis I compared the crosslink intensities by quantifying the phosphorimage (Figure 2-7). The crosslink obtained with *wild type* Doc1 is about four times stronger than the one with Doc1 Δ IR (Figure 2-7, compare lanes 2 and 3 with lanes 4 and 5). The binding of Cdc27 to Doc1's IR tail therefore significantly enhances the Doc1-Cdc16 interaction.

2.1.4 Searching for a putative ligand of Doc1's "processivity loop"

The results above demonstrated that the photocrosslinking approach allows the identification of Doc1 interacting proteins. We therefore wanted to use this technique to exploit the mechanism underlying the role of Doc1 in processive APC-mediated ubiquitination reactions (Carroll and Morgan, 2002; Passmore *et al.*, 2003). Doc1's "processivity loop" (Figure 2-4A) has been proposed to bind to a putative ligand (Au *et al.*, 2002; Wendt *et al.*, 2001), and biochemical and yeast genetic studies implied that this ligand might be an APC substrate (Carroll *et al.*, 2005; Passmore *et al.*, 2003). Importantly, a mutational analysis has revealed four amino acids located in the putative ligand binding region, which are essential for Doc1's function in processivity (Carroll *et al.*, 2005).

We therefore speculated that Doc1 might use this "processivity loop" to bind to a putative ligand, which could be the substrate or any other component present in the ubiquitination reaction. Since in an *in vitro* ubiquitination assay a severe difference in the presence and absence of Doc1 can be observed ((Carroll and Morgan, 2002; Passmore *et al.*, 2003), Figure 2-2), I performed crosslinking in this experimental set up (Figure 2-8A). Figure 2-8B shows that purified APC was able to ubiquitinate the substrate used in this set of experiments, GST-Hsl1⁽⁶⁶⁷⁻⁸⁷²⁾. I had decided to use GST-Hsl1⁽⁶⁶⁷⁻⁸⁷²⁾ in this set of experiments, because the Hsl1 fragment was shown to be an APC substrate (Burton and Solomon, 2000; Burton and Solomon, 2001) which is processively ubiquitinated by APC (Carroll *et al.*, 2005).

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Furthermore, the fusion with GST rendered it a 55 kDa protein. Possible crosslinks with Doc1 (33 kDa) would therefore be bigger than 70 kDa; at 70 kDa I often observed unspecific crosslinks, possibly due to Doc1 molecules reaction with each other (see below). APC lacking Doc1 was immobilized on beads and incubated with Doc1 crosslinker versions in which one of the “processivity loop” residues had been exchanged for the photoactivatable amino acid. Before UV-irradiation I added the components of an *in vitro* ubiquitination assay mix (termed ubiquitination mix), namely E1 and E2 enzymes, Cdh1, substrate, ubiquitin, ATP and 0.12 μ M substrate (lanes 1, 4 and 7 in Figure 2-8B, C and D). In an additional sample set, I added 100-fold more substrate (12 μ M) to facilitate the formation of a possible crosslink to a substrate (lanes 3, 6 and 9 in Figure 2-8B, C and D). A control set was incubated with ubiquitination buffer instead of the ubiquitination mix (lanes 2, 5 and 8 in Figure 2-8B, C and D). Phosphorimages after SDS-PAGE were then specifically analyzed for crosslink products which were dependent on the presence of the ubiquitination mix in the crosslinking experiment. Because the substrate is the most prominent candidate for a Doc1 interactor (Carroll *et al.*, 2005; Passmore *et al.*, 2003), I also tested another model substrate, human cyclin B (amino acids 1 to 87) which is also well ubiquitinated by yeast APC in similar assays (not shown). All conditions were tested at least twice and radioactive gels were exposed to screens for up to eight weeks in order to enhance possible weak bands. Nevertheless, no specific bands could be detected when comparing samples that were UV-irradiated in absence and presence of the ubiquitination mix. Furthermore, I tried leaving out individual components of the ubiquitination mix when performing the crosslinking, but this also did not lead to the loss of any of the obtained bands (not shown). The mobility shift pattern obtained due to the interactions of Doc1 with Cdc16, Cdc27 and Apc1 was not affected by the addition of ubiquitination mix during the UV-exposure (lanes 1 to 3 in Figure 2-8B, C and D). The intensity of the crosslinks, however, was decreased in some cases (Figure 2-8B, compare lanes 1 and 3 with lane 2, and data not shown) when the samples were incubated with the ubiquitination mix before UV-irradiation.

As Figure 2-8B to C shows, the phosphorimages obtained from experiments employing the Doc1 loop *amber* mutants were not empty, but indeed contained several bands, the most prominent of which were running at 45 kDa, 70 kDa, and between 120 to 140 kDa. Two reasons, however, made us think that these bands do not represent crosslinks of Doc1 to its

putative ligand. First of all, these bands, and in particular those at 45 kDa and 70 kDa size were observed frequently and with several mutants which carried the crosslinker at different positions on Doc1. According to the manufacturer's manual provided with our reticulocyte lysate translation system (Promega), labeling of a 42 kDa protein is frequently obtained when using rabbit reticulocyte lysate with [S^{35}]methionine of a quality below "cell labeling grade"

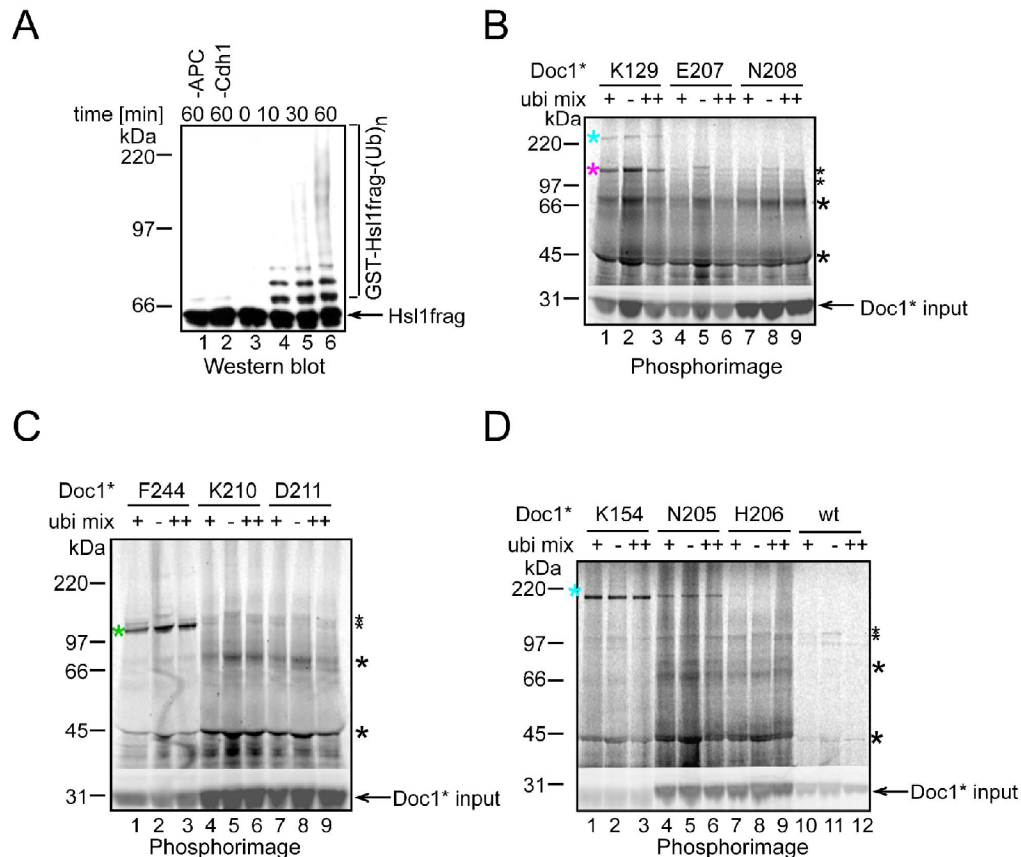


Figure 2-8: Crosslinking experiments employing Doc1 mutants containing a photocrosslinker in the putative ligand binding region. A) The GST-Hsl1 fragment (amino acids 667 to 872, GST-Hsl1⁽⁶⁶⁷⁻⁸⁷²⁾) is ubiquitinated by purified APC. TAP-purified APC was incubated with E1, E2, Cdh1, ubiquitin, ATP and GST-Hsl1⁽⁶⁶⁷⁻⁸⁷²⁾ for the times indicated and samples were analyzed by SDS-PAGE and immunoblotting using a Hsl1 antibody (1740). B) Crosslinking experiment testing Doc1 mutants K129, E207 and N208. Each mutant was tested in presence of ubiquitination mix containing 0.12 μ M GST-Hsl1⁽⁶⁶⁷⁻⁸⁷²⁾ (+), 12 μ M GST-Hsl1⁽⁶⁶⁷⁻⁸⁷²⁾ (++) and in absence of the ubiquitination mix (-). K129 served as a positive control, it crosslinks to Cdc16 independent of the ubiquitination mix. After 10 min of UV-exposure, samples were analyzed by SDS-PAGE and phosphorimaging. C) Same experimental set-up as in (B) but this time using mutants F244 (positive control, crosslinks to Cdc27), K210 and D211. D) Same as in (B) but testing mutants N205 and H206 with K154 (crosslinks to Apc1) as a positive control. For the gel region above the Doc1 input contrast settings were changed to facilitate visualization of weak bands. The black asterices mark unspecific crosslink products and the colored asterices crosslinks to APC subunits (see text).

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(Jackson and Hunt, 1983). I could, despite the use of different labels including the recommended company and grade, often observe a band in the 45 kDa region in translation reactions generating Doc1 versions or other proteins (not shown). One explanation for the existence of the putatively unspecific 42 kDa bands could therefore be that the labeled rabbit reticulocyte lysate 42 kDa protein was not washed off before the UV-exposure. This is supported by the observation that the 42 kDa band was also present in control reactions that had not been exposed to UV-light (not shown). The band at 70 kDa might contain Doc1 dimers resulting from two Doc1 molecules bound to each other. Formation of this band is not dependent on APC, because this band was also observed in control reactions for which IgG beads were incubated with extracts of yeast strains that did not contain a TAP-tag (not shown). Crosslinker containing Doc1 versions might therefore bind non-specifically to IgG beads and crosslink to each other in an unspecific manner. Accordingly, weaker unspecific bands observed at a size of 120 to 140 kDa could be due to formation of Doc1 trimers and tetramers. It is important to note, however, that the crosslinks to Cdc16 can clearly be distinguished from the unspecific bands. The specific crosslinks not only ran at a slightly lower size when proteins on the gel were well-separated (Figure 2-6A lanes 2 and 5), but importantly the mobility of the unspecific bands was not changed upon use of myc-tagged APC strains (Figure 2-6A).

2.1.5 Functionality of photocrosslinker containing Doc1 versions

For those mutants with which I could identify specific crosslink products (Table 2-2), I also tested if these mutants can restore APC activity in ubiquitination assays. I made use of the fact that APC lacking Doc1 (APC^{doc1Δ}) shows impaired ubiquitination activity (Carroll and Morgan, 2002; Passmore *et al.*, 2003) and that *wild type* ubiquitination activity can be restored by adding back recombinant Doc1 (Figure 2-2; (Carroll *et al.*, 2005; Passmore *et al.*, 2003)). To assess whether the crosslinker-containing Doc1 versions were also able to restore *wild type* ubiquitination activity to APC^{doc1Δ}, I carried out *in vitro* ubiquitination assays (Figure 2-9A). Mutant APC was pre-incubated with Doc1 mutants and then assayed for its ability to assemble ubiquitin chains onto the Hsl1 fragment after addition of E1 and E2 enzymes, Cdh1, ubiquitin and ATP. Since Doc1, Cdh1 and Hsl1⁽⁶⁶⁷⁻⁸⁷²⁾ were produced by *in vitro* translation in reticulocyte lysate, strong background activity in the absence of Doc1 was

observed (Figure 2-9A, lanes 2 and 3). Mutants that carry the crosslinker in the C-terminus showed more activity in this assay than those with the mutated amino acid inserted within the processivity loop (Figure 2-9A, lanes 10 and 11, data not shown). In particular, Doc1 versions with crosslinkers inserted at K129, R182, and F244 largely restored *wild type* ubiquitination activity (Figure 2-9A, lanes 8, 9, 14 to 19), indicating that the crosslinks to Cdc16 and Cdc27 were obtained with functional mutants. Mutants carrying the photocrosslinker at K154 and N205, which crosslinked to Apc1, clearly retained only little (Figure 2-9A, lanes 10 to 13), but notably more than background activity. S128, which also crosslinked predominantly to Apc1, showed intermediate activity (Figure 2-9A, lanes 6 and 7).

Next, we performed growth test experiments to examine whether the temperature sensitivity of a *DOC1* deleted strain could be rescued by Doc1 mutants which carry single phenylalanine mutations under the endogenous *DOC1* promoter. Strains lacking Doc1 are temperature sensitive and can not grow at 37°C (Figure 2-9C). This phenotype can be partially rescued by introducing *wild type DOC1* on an integrative plasmid under the endogenous *DOC1* promoter (Figure 2-9C). We then mutated all sites which by photocrosslinking had been shown to interact with an APC subunit (Table 2-2) to phenylalanine. Since the photoactivatable amino acid (tmd)phe is a phenylalanine analog (Figure 2-3A), we reasoned that with this amino acid exchange we would mimick the “real” situation best and might be able to estimate the effect of (tmd)phe on Doc1 function “*in vivo*”. Importantly, no difference could be observed with our phenylalanine mutants in this assay (Figure 2-9C), indicating that the overall function of these Doc1 versions might not be impaired by insertion of a bulky amino acid. In particular, if sites that interacted with Cdc16, Cdc27 or Apc1 were exchanged for a phenylalanine, Doc1 was still able to exert its overall cellular function. These observations further support the notion that the crosslinks were obtained with at least partially functional Doc1 versions.

Doc1 versions carrying a photocrosslinker in the processivity loop were greatly impaired in their ability to restore activity to mutant APC, as measured in *in vitro* ubiquitination assays using TAP-purified APC (Figure 2-9A and data not shown). Nevertheless, the measured activities were above background levels (Figure 2-9A and data not shown). In order to explore directly whether ubiquitination could be stimulated by Doc1 under conditions of a

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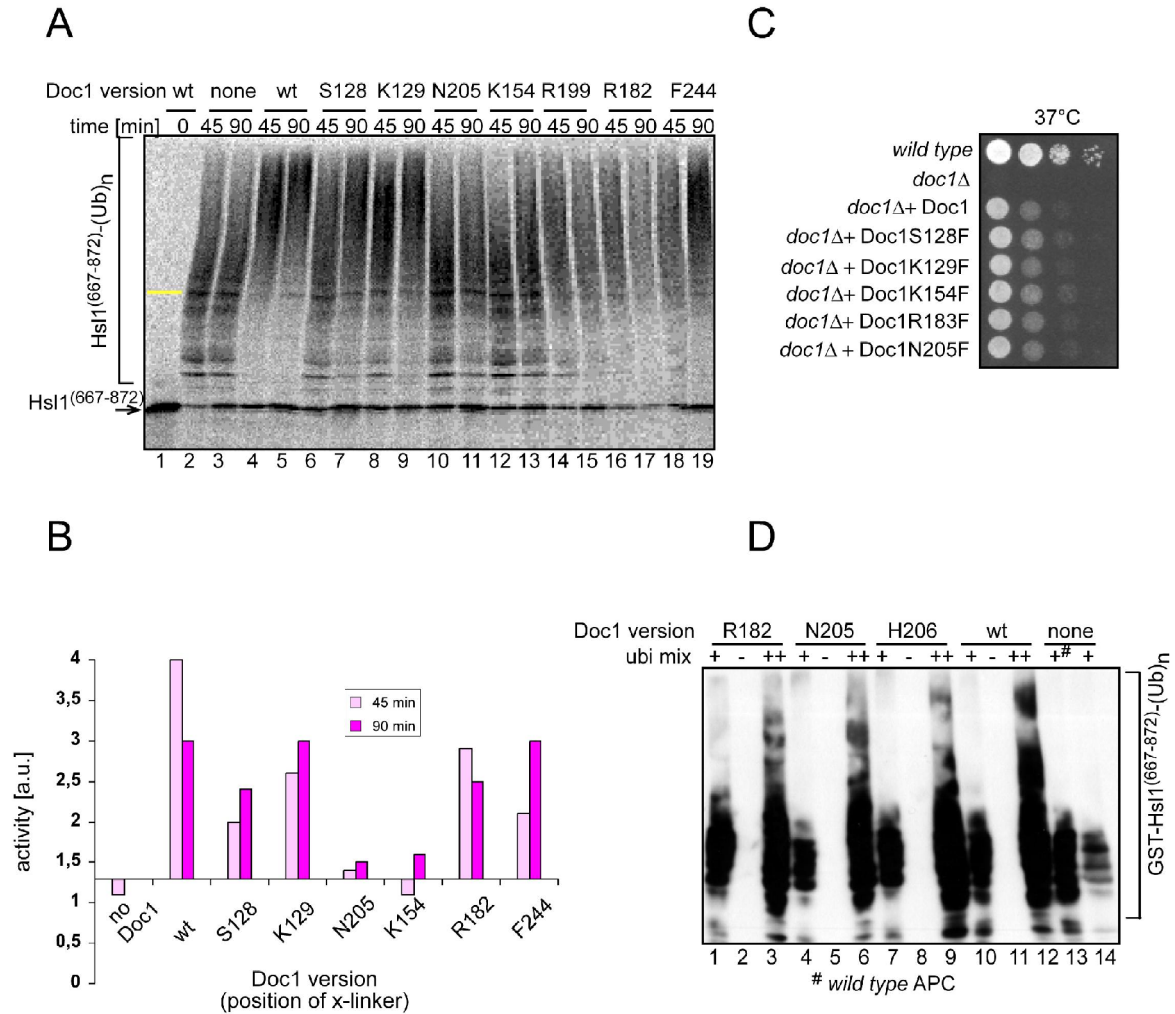


Figure 2-9: Analyzing functionality of Doc1 versions containing a photocrosslinker.

A) *In vitro* ubiquitination assay of APC reconstituted from mutant APC lacking Doc1 and from crosslinker-containing Doc1 versions. TAP-purified mutant APC was pre-incubated with the indicated Doc1 versions that had been generated by *in vitro* translation. As a control, reticulocyte lysate was used. E1, E2, Cdh1, ubiquitin, ATP and a [35 S]-labeled Hsl1-fragment (Hsl1⁽⁶⁶⁷⁻⁸⁷²⁾) were added and reactions were stopped after 45 and 90 minutes. Samples were analyzed by SDS-PAGE and phosphorimaging. B) Quantification of the experiment shown in (A) using ImageQuant. Intensity of ubiquitin conjugates above the yellow mark was divided by the intensity below the mark to obtain an arbitrary activity value. The axis intersection was set to the value obtained with the reticulocyte lysate control after 90 min incubation (lane 3 in (A)). Crosslinks obtained initially with mutant R199 were not reproducible and are not shown in this study. Therefore, R199 was not included in the quantification. C) Doc1 growth assay. The temperature sensitivity of *doc1Δ* strains transformed with Doc1 *wild type*, Doc1 Phe-mutants (see text) or empty plasmid as a control was analyzed by spotting assays and incubation at 37°C. In the mutant which crosslinked to Cdc27 (F244), a phenylalanine had been exchanged for (tmd)phe. D) Test of ubiquitination activity of Doc1 mutants carrying the crosslinker in the processivity loop during the crosslinking experiment. Supernatants from the experiment shown in Figure 2-8D were analyzed by immunoblotting using an antibody against ubiquitin. Lanes 13 and 14 contain equally treated samples to control ubiquitination activity. No Doc1 was added to mutant APC in lane 14 and *wild type* APC was analyzed in lane 13 (ubiquitination mix (ubi mix), see text).

crosslinking experiment, I analyzed the formation of substrate-ubiquitin conjugates during the actual crosslinking experiment. I took advantage of the fact that substrates can be largely separated from bead-bound APC by centrifugation. APC^{doc1Δ} on beads, bound Doc1 and potentially crosslinked ubiquitination mix components were analyzed by phosphorimaging (Section 2.1.4). At the same time the supernatants could be separated by SDS-PAGE and analyzed by immunoblotting with antibodies specific for the substrate or ubiquitin. Figure 2-9A shows such an analysis with supernatant samples taken from the experiment in Figure 2-8D. All samples were therefore generated by incubating APC lacking Doc1 bound to beads with Doc1 crosslinker mutants, before adding an ubiquitination mix containing GST-Hsl1⁽⁶⁶⁷⁻⁸⁷²⁾ as a substrate for 15 min. After UV-exposure bead-bound material was removed. A sample to which no Doc1 had been added (Figure 2-9D, lane 14) and a sample in which *wild type* APC was used (Figure 2-9D, lane 13) were analyzed in parallel. The experiment showed that Doc1 loop mutants were indeed able to stimulate APC activity (Figure 2-9D, compare lanes 1, 4 and 7 with lanes 10 and 13 for *wild type* activity and with lane 14 for “no Doc1” activity). Although this was not a quantitative immunoblot and the assay did not seem to be very sensitive (since only little difference between *wild type* Doc1 and mutant Doc1 could be observed, compare lanes 1, 4 and 7 with lane 10 in Figure 2-9D), the result clearly showed that Doc1-mediated ubiquitination was occurring during the actual crosslinking experiments. I therefore carried out my search for a putative Doc1 ligand under conditions which allowed at least partially functional Doc1 versions to stimulate substrate ubiquitination.

2.2 Role of Doc1 in potential APC dimerization

In order to determine the size of yeast APC, yeast extracts have been sedimented through glycerol gradients. Yeast APC sediments as a 36S particle (Zachariae *et al.*, 1996), whereas *Xenopus* and HeLa APC have been reported to sediment as 20S and 22S particles, respectively (Gieffers *et al.*, 2001; King *et al.*, 1995). When running purified APC on non-denaturing gels, a doublet can be observed with the slower migrating form being about twice as large as the faster migrating one, which in turn matches estimates of the size of yeast APC (Passmore and Barford, 2005). Monomeric and dimeric form can be separated from each other. When used in activity assays, the dimeric form seems to process its substrate with an

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increased processivity compared to the monomeric version (Passmore *et al.*, 2005b). This raises the interesting question whether Doc1 contributes to the processivity of APC-catalyzed ubiquitination reactions by mediating APC dimerization.

Because Apc1 is the only APC subunit that has never been proposed to be present more than once per complex, I used the ability of Apc1 to self-associate (Zachariae *et al.*, 1996) as a second readout for APC dimerization. Similar to Zachariae *et al.* (Zachariae *et al.*, 1996), I used a yeast strain which contained two different epitope-tagged versions of Apc1. I performed co-immunoprecipitation experiments to determine whether one epitope-tagged component could co-immunoprecipitate the other epitope-tagged version of itself, and *vice versa*. In a diploid strain expressing Apc1-myc6 and Apc1-HA3, the myc antibody immunoprecipitated both Apc1-myc6 and Apc1-HA3 (Figure 2-10, lanes 8 and 9). Apc1-HA3 was not pulled down from a strain producing only Apc1-myc6 (Figure 2-10, lane 8), indicating the specificity of the antibody. In the reciprocal experiment, anti-HA immunoprecipitates obtained from the diploid *APC1-myc6 APC1-HA3* strain, but not the single myc-tagged, strain contained Apc1-myc6 in addition to Apc1-HA3. (Figure 2-10, lane 3). We then constructed a similar set of epitope-tagged strains containing homozygous deletions of *DOC1* and carried out the same experiments again (Figure 2-10, lanes 4 to 6 and 10 to 12). The absence of Doc1 did not lead to a significant decrease in Apc1-self-association. Doc1 therefore, at least in this assay, does not contribute to APC dimerization.

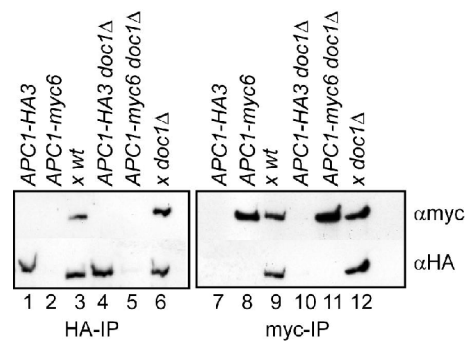


Figure 2-10: Self association of Apc1 is not impaired in the absence of Doc1. Apc1 was immunoprecipitated from extracts of yeast strains expressing the indicated epitope-tagged Apc1 versions in a *wild type* (*wt*) or *doc1Δ* background. Antibodies to myc and HA epitopes were used for immunoprecipitation. Bound proteins were eluted with glycine and analyzed by immunoblotting with myc and HA antibodies. Strains J109, J110, J325, J326, J327, J328 were used in this experiment.

2.3 Electron microscopic analysis of yeast APC

2.3.1 Obtaining a 3D model of budding yeast APC

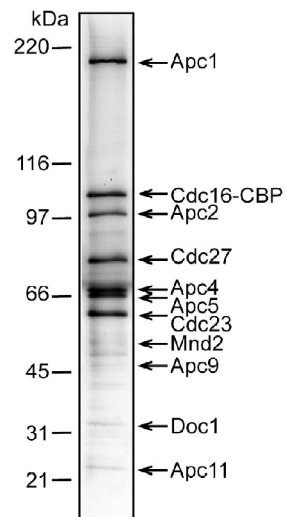
Substrate ubiquitination mediated by APC requires the concerted action of more than a dozen subunits (Passmore and Barford, 2004; Peters, 2006). A mechanistic understanding of how APC catalyzes these reactions and how this is regulated during the cell cycle requires information about the overall APC structure and the 3D organization of its many subunits. We have decided to extend our structural studies from human and frog APC (Dube *et al.*, 2005; Gieffers *et al.*, 2001) to the budding yeast complex. The reasons for this are manifold. Comparing complexes from different organisms and thereby discovering similarities and differences might provide valuable hints for preserved mechanisms, distinct regulations, etc. In addition, the EM structure published of budding yeast APC (Passmore *et al.*, 2005b) surprisingly different from the structures of vertebrate APC. We therefore decided to compare the structures of yeast and human APC under identical imaging conditions. Furthermore, establishing a novel strategy for localizing individual subunits within the 3D APC model, we will in the future create a more detailed 3D map of APC subunit localizations.

To this end, I purified budding yeast APC by the TAP-method from strains that were deleted of the protease Pep4 and contained a TAP-tag on either Cdc16 or Apc4. The purified complex was then enriched and fixed by dual glycerol-glutaraldehyde density gradient centrifugation (Kastner *et al.*, 2008) and fractions containing APC were analyzed by EM of double carbon foil negative stain preparations (Figure 2-11B). Most of the APC particles on the grids were monomeric, but we could also observe dimeric particles among them, in agreement with the study by Passmore *et al.* ((Passmore *et al.*, 2005b), Figure 2-11B). In some cases it was difficult to judge whether a particle was dimeric or whether two monomers were just very close to each other. The purity of the preparation was analyzed by SDS-PAGE and silver staining (Figure 2-11A).

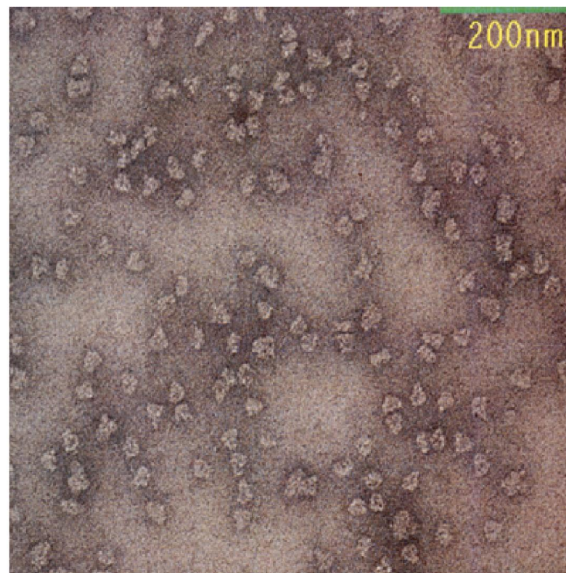
The 3D model was determined by angular reconstitution from cryo-negative stain images. Figure 2-11C shows our 3D model in comparison with that published by the Barford lab (Passmore *et al.*, 2005b). Our structure appears slimmer and strikingly more asymmetric. When looking at both models in several views, similarities become apparent, such as the overall triangular shape with one vertex being more pronounced and the other two being

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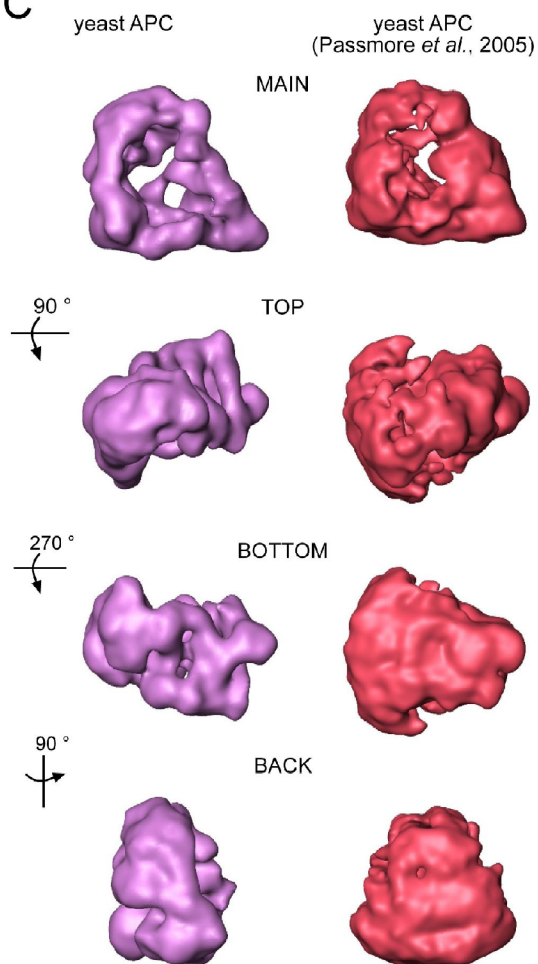
A



B



C



D

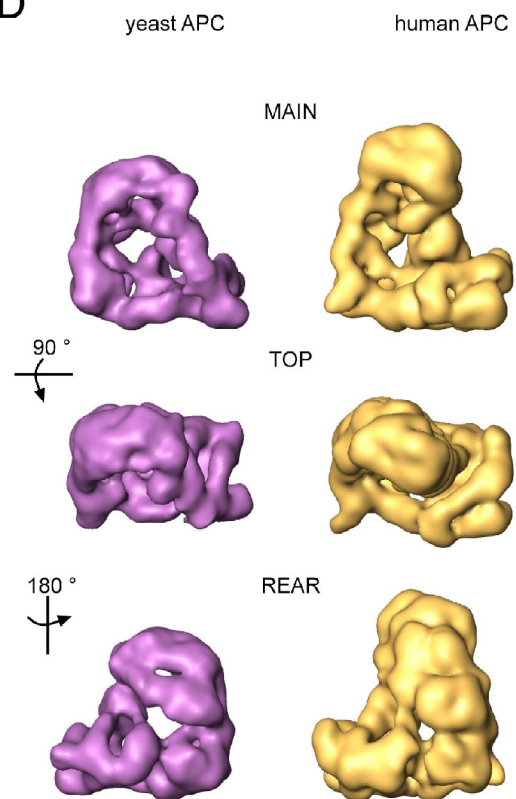


Figure 2-11: Purification and 3D reconstruction of budding yeast APC after negative staining EM. A) APC was purified from strain J315 (*CDC16-TAP pep4Δ*) via the TAP-method and analyzed by SDS-PAGE and silver staining. The sample was taken before the preparation was further purified and fixed by glycerol-glutaraldehyde centrifugation. Subunits were identified by their electrophoretic mobility and by mass spectrometry. B) Typical EM raw image of yeast APC prepared by the double carbon foil sandwich technique. C) Our yeast APC structure (purple) in comparison with a previously published budding yeast structure shown in red (Passmore 2005), which was obtained by cryo-EM and angular reconstitution. Both structures are shown in several comparable orientations. Arrows and angles are in reference to the top structures. D) Surface views of yeast APC (purple) and human APC (yellow) structures obtained by angular reconstitution. Both structures are shown in several orientations. Arrows and angles are in reference to the top structures.

more “rounded” (“main” view in Figure 2-11C). The yeast APC model resembles that of human APC (which was obtained in a similar way, Herzog *et al.*, manuscript in preparation) in many details but also displays some striking differences. Similar to human APC, yeast APC has an asymmetric shape with a convexly curved “back” side and a concavely curved “front” side (Figure 2-11D). Both complexes contain a central cavity and prominent ridges at the back side. Human APC has a pronounced head-like structure at one end ((Dube *et al.*, 2005), Figure 2-11D) which is significantly smaller in yeast APC. The overall structure of yeast APC appears somehow compressed in comparison to human APC. Moreover, yeast APC contains a prominent extra mass at the right side of the complex (Figure 2-11D, “front” view). The “rear” side views of both APC models implicate that yeast APC lacks one of the ridges (Figure 2-11D) which, together with the smaller head region, might create the more compressed appearance.

2.3.2 Localization of Doc1 within APC

Using site-specific photocrosslinking, I have identified the interaction partners of Doc1 within APC (see 2.1). Localization of Doc1 within the 3D structure of APC could, combined with the possible localization of further subunits (see below) or with available subunit maps and interaction data ((Dube *et al.*, 2005; Kraft *et al.*, 2005; Ohi *et al.*, 2007; Passmore *et al.*, 2005b; Thornton *et al.*, 2006; Vodermaier *et al.*, 2003), Herzog *et al.*, manuscript in preparation) help to verify the observed interactions and might lead to a better understanding of Doc1’s role within APC. Deletion of Doc1 does not affect the overall subunit composition of APC (Carroll and Morgan, 2002; Passmore *et al.*, 2003). Determining a 3D map of APC purified from a strain lacking Doc1 and comparison with that of *wild type*

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APC cells should therefore allow the localization of Doc1. APC was purified as before from yeast cells lacking Doc1 and the purified sample was processed and analyzed as before (Section 2.3.1, Figure 2-12).

The 3D models of APC and APC^{doc1Δ} are indeed similar in their overall shapes (Figure 2-12B). APC is slightly more flexible in the absence of Doc1 and the 3D model for APC^{doc1Δ} is therefore not as well defined as the *wild type* structure. APC^{doc1Δ} reveals small differences in few areas compared to *wild type* APC. There is, however, only one single location where APC^{doc1Δ} lacks a prominent density compared to APC (Figure 2-12B). This extra density in *wild type* APC is located in the center of the complex (front view) beneath the head region and to the right-hand side of the central cavity (Figure 2-12B). The volume and the round shape of this “mass” are consistent with a globular protein of 35 kDa size. To confirm the localization of Doc1, we are planning to reconstitute APC from purified APC^{doc1Δ} and from recombinant Doc1 purified from *E. coli*, and to analyze this complex by negative staining EM. I obtained very pure His-Doc1 from *E. coli* by Ni-NTA batch purification followed by gel filtration (Figure 2-12C). Doc1 from this preparation can restore *wild type* ubiquitination activity when added to APC^{doc1Δ} ((Passmore *et al.*, 2003) and data not shown) indicating that Doc1 binds to its physiological site on APC. EM analysis of a reconstituted APC^{doc1Δ}-Doc1 sample might therefore be suitable to confirm the Doc1 localization.

2.3.3 Localization of further APC subunits within the 3D model

To gain inside into the organization of APC subunits within the complex, antibody labeling experiments have been carried out. This approach has led to the localization of several subunits of yeast and human APC within the corresponding 3D models ((Dube *et al.*, 2005; Ohi *et al.*, 2007; Passmore *et al.*, 2005b), Herzog *et al.*, manuscript in preparation). Combining these data with those obtained from interaction studies of vertebrate and yeast APC subunits (Kraft *et al.*, 2005; Thornton *et al.*, 2006; Vodermaier *et al.*, 2003) has already contributed to a better understanding of the 3D organization of APC (Peters, 2006). Precise subunit localizations have not been feasible so far due to technical limitations. For human APC, for example, it has not been possible to generate a 3D structure of APC in complex with an antibody because of the inherent flexibility of the antibody (Franz Herzog and Holger Stark,

personal communication). Subunits have therefore been localized by computational analysis of 2D images and projection onto a 3D structure ((Dube *et al.*, 2005) Herzog *et al.*, manuscript in preparation). Alternatively, subunits have been localized in 2D only (Ohi *et al.*, 2007).

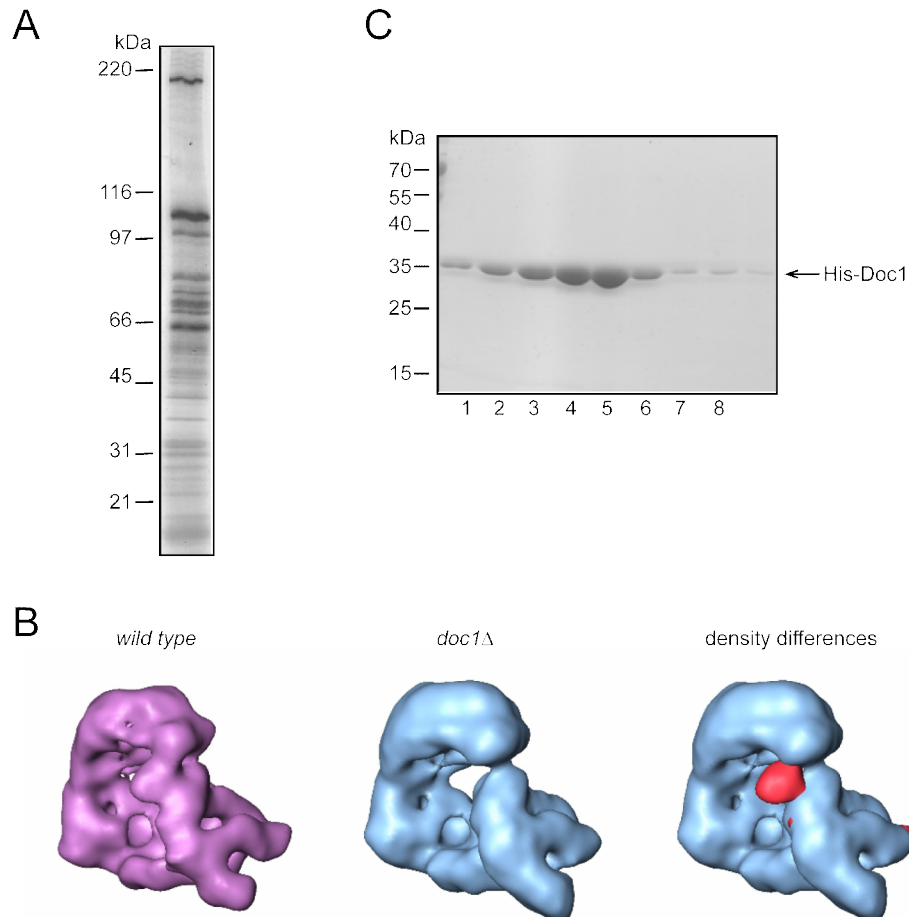


Figure 2-12: Localization of Doc1 on APC. A) Purification of APC^{doc1Δ} from yeast strain J320 (*CDC16-TAP doc1Δ pep4Δ*). The sample was taken before fixation by glycerol-glutaraldehyde centrifugation and analysis by negative staining EM. B) The 3D model obtained for APC purified from a *wild type* strain is shown in purple (left), and that for APC purified from a *doc1Δ* strain in blue (middle). In the model to the right, the differences in density found between the two structures are shown in red. C) Purified Doc1 which will be used to confirm the Doc1 localization. His-tagged Doc1 was purified from *E. coli* by Ni-NTA chromatography (batch mode) and gel filtration. Samples of Doc1 containing fractions obtained after gel filtration were separated by SDS-PAGE and stained with Coomassie.

To be able to localize subunits in 3D, we decided to fuse relatively large globular tags to the C-termini of individual APC subunits. We wanted the tags to be large enough to be visible

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on electron micrographs but ideally they should not interfere with APC assembly and function. Purification of APC carrying such a tag and generating a 3D model by negative staining EM should, when this 3D model is compared to that of untagged APC, lead to the identification of an additional mass. This mass should then indicate the localization of the C-terminus of the tagged subunit. Tdimer2 (td2) is a tandem dimer of a dimeric DsRed variant and has a size of about 50 kDa (Campbell *et al.*, 2002). This protein has been successfully used for the localization of three subunits of a yeast splicosomal subcomplex by a “tagging approach” similar to the one outlined before (personal communication from Irina Haecker

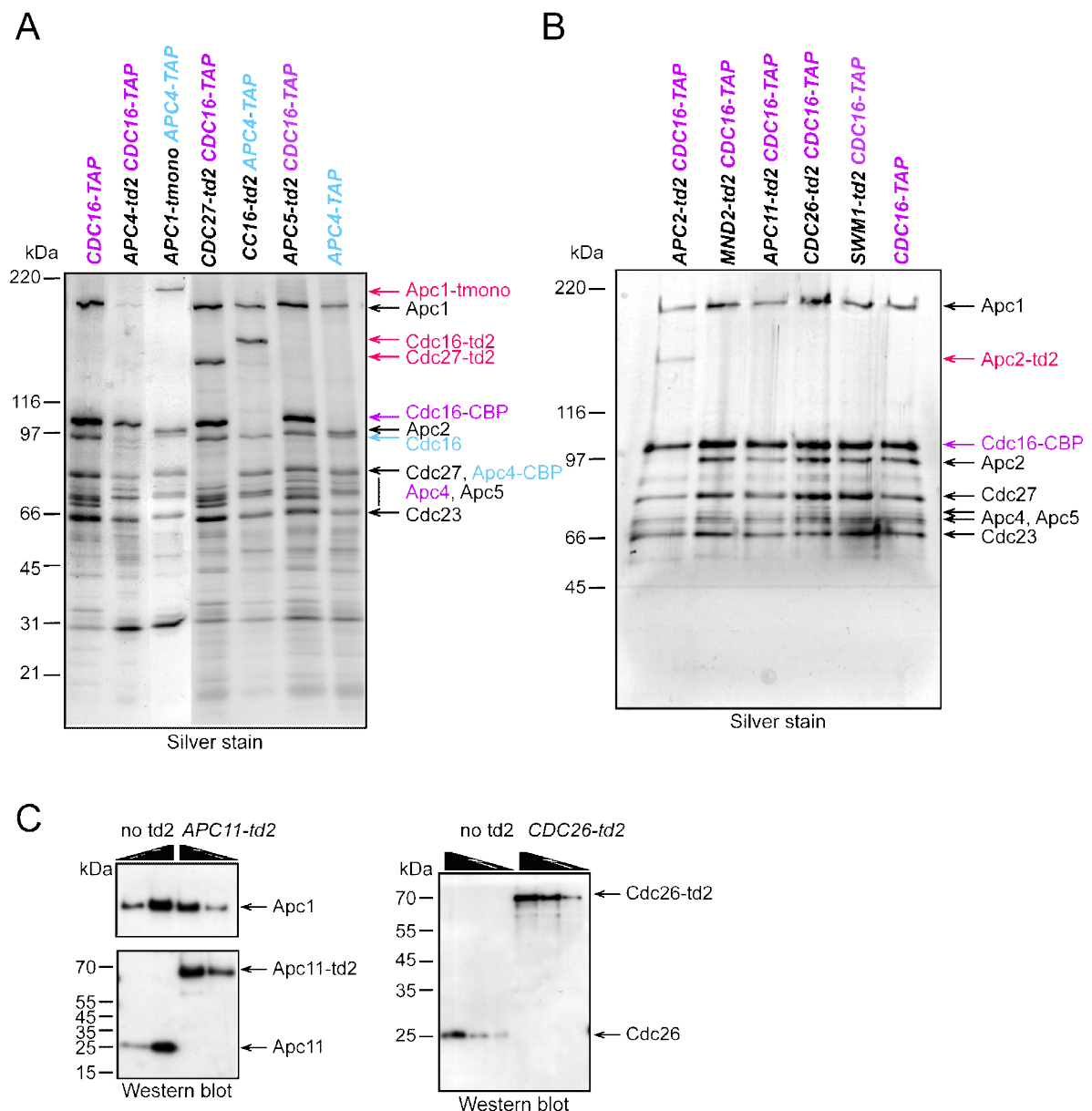


Figure 2-13: Subunit composition of APC purified from various yeast strains each carrying a td2-tag on a different APC subunit. A) Preparations from strains carrying a td2-tag on Apc4 (J347), Cdc27 (J376), Cdc16 (J378) or Apc5 (J380), and from a strain carrying a tmono-tag (see text) on Apc1 (J329). APC was purified using the TAP-method via a TAP-tag on Apc4 or Cdc16 and analyzed by SDS-PAGE and silver staining. Preparations from strains without td2- or tmono-tags (*CDC16-TAP*, J315 and *APC4-TAP*, J319) are shown for reference. All strains in addition were *pep4Δ*. Subunits were identified by analysis of the electrophoretic mobility pattern. The contrast settings were changed within the left half of the gel to visualize weak bands. B) Preparations from strains carrying a td2-tag on Apc2 (J408), Mnd2 (J407), Apc11 (J422), Cdc26 (J406) or Swm1 (J428), and from a strain carrying no td2-tag (J315). All strains carried TAP-tags on APC 4 and were deleted of *Pep4*. APC was purified using the TAP-method and analyzed by SDS-PAGE and silver staining. Subunits were identified by their electrophoretic mobility. C) Immunoblot analysis demonstrating the successful tagging of Apc11 (left) and Cdc26 (right panel) and the resulting mobility shifts. Different amounts of samples from the *APC11-td2 CDC16-TAP* and *CDC26-td2 CDC16-TAP* preparations shown in (B) were loaded together with control samples (*CDC16-TAP*) and analyzed by immunoblotting using antibodies against Apc1, Apc11 and Cdc26.

and Reinhard Lührmann). We therefore set out to generate a set of yeast strains which each carried a C-terminal td2-tag on one of the APC subunits. We succeeded in tagging the C-termini of *APC2*, *CDC27*, *CDC16*, *APC4*, *APC5*, *APC11*, *CDC26*, *SWM1* and *MND2* with td2 (Table 2-3) using available one-step tagging cassettes (Sheff and Thorn, 2004). Tagging of *APC1* was only possible with a modified version of the tag which consisted of a DsRed monomer (tmono). We did not obtain any viable spores when we tried to tag *CDC23* or *APC9* with td2 or tmono (not shown). Strains were then crossed into *APC4-TAP pep4Δ* and *CDC16-TAP pep4Δ* backgrounds and APC was purified from these strains using the TAP-method. The subunit composition was analyzed by SDS-PAGE and silver staining. APC purified from all but one strain carrying a td2-tag appeared to have a normal APC subunit composition (Figure 2-13A and B). APC purified from *APC4-td2* cells was the only complex which seemed to have lost some subunits, such as Apc1, or contain them at substoichiometric levels (Figure 2-13A). Mass spectrometry analysis after in solution digest, however, revealed the presence of all 13 APC subunits in this preparation (not shown). We therefore decided to subject APC purified from all of these newly generated td2- or tmono-tagged strains for negative staining EM analysis. So far, electron micrographs have been prepared for APC purified from strains carrying tags on Apc1, Cdc27, Apc4, Apc5 and Cdc16 (Table 2-3). To this end, APC was purified by the TAP-method as before, followed by glycerol glutaraldehyde centrifugation. For APC carrying td2-tags on Apc1, Cdc27, Apc5 and Cdc16, negatively stained electron micrographs revealed asymmetric particles which

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looked similar to those of *wild type* APC (not shown). No intact particles could be found for the Apc4-td2sample, the silver gel analysis of which had already indicated unusual subunit stoichiometry (Figure 2-13A). Tagging of Apc4 might have therefore led to the structural destabilization of APC. APC purified from the remaining five yeast strains will be purified and analyzed by EM after submission of this thesis (see Table 2-3).

Table 2-3: Generated yeast strains containing td2-tagged APC subunits and current status of their EM analysis. Doc1 was not tagged since C-terminal tagging avoids association of Doc1 with APC (Carroll *et al.*, 2005).

APC subunit	strain	subunit composition?	EM analysis?	3D model obtained?	tag visible?
Apc1	J329	ok	in process	no	
Apc2	J408	ok	to be done		
Cdc27	J376	ok	in process	yes	yes
Apc4	J347	not ok	impossible		
Apc5	J380	ok	in process	yes	yes
Cdc16	J378	ok	in process	yes	yes
Cdc23	not viable				
Apc9	not viable				
Apc11	J422	ok	to be done		
Cdc26	J406	ok	to be done		
Swm1	J428	ok	to be done		
Mnd2	J407	ok	to be done		

Figure 2-14 shows the 3D model obtained for Cdc27-td2-tagged APC after EM negative staining and angular reconstitution. After building class averages from EM raw images, the tag is clearly visible as a “horn-like” protrusion which is not present in corresponding views of untagged APC (Figure 2-14). According to this, the 3D model contains one additional blob which is missing in the *wild type* structure, indicating the localization of Cdc27’s C-terminus (Figure 2-14). It is located directly next to the mass that might represent Doc1 (Figure 2-12B), close to the head region and at the edge of the prominent back-side ridges. Passmore *et al.* have analyzed the stoichiometry of budding yeast APC by iodine-labeling and found two to three copies of Cdc27 per complex (Passmore *et al.*, 2005b). We only found one major additional mass. This does not, however, exclude the possibility that further additional masses are buried within the complex.

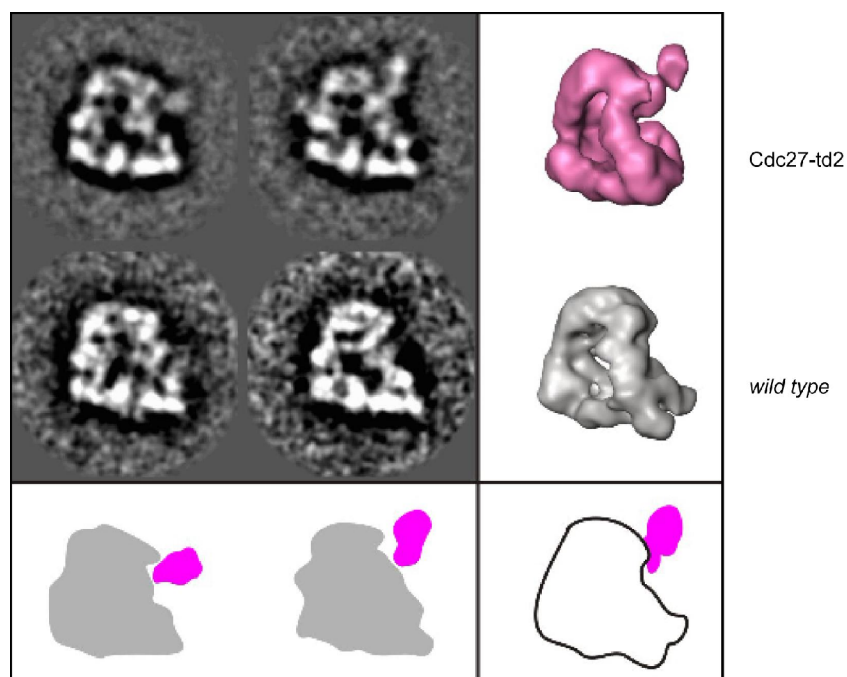


Figure 2-14: EM of APC carrying a td2-tag on Cdc27 and of untagged APC. Two class averages are shown each for td2-tagged and untagged APC in corresponding projection directions. Contour lines of the class averages are below the particles to outline the similar projection directions. The pink shape outlines the additional mass which is only present in the upper row images. Front views of the resulting 3D models of tagged (pink) and untagged (grey) APC are shown.

We also obtained a precise localization of Apc5 after analysis of APC purified from the *APC5-td2* cells. Figure 2-15 shows the resulting 3D model in comparison with that of *wild type* APC, yielding the localization Apc5 within the “platform” of the complex. By analyzing the remaining complexes in a similar fashion we will create a detailed APC subunit map which is more precise than the ones which have been obtained previously.

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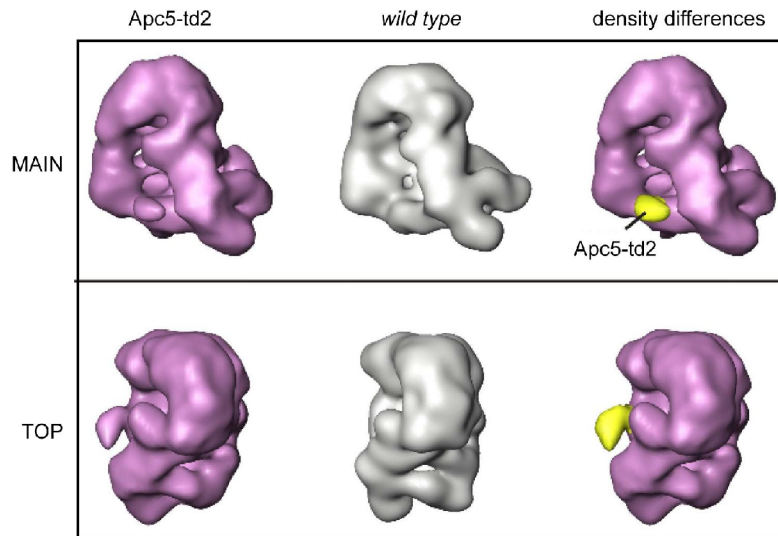


Figure 2-15: A 3D model of APC carrying a td2-tag on Apc5 in comparison to that of untagged APC. The model obtained for APC purified from a yeast strain carrying a td2-tag on Apc5 is shown to the left and that for APC purified from a *wild type* strain in the middle. To the right the main density difference which can be found between the two structures is shown in yellow. The bottom panel shows the structures in the “top” view. Class averages of EM raw images are not shown because the additional mass was not clearly visible on them.

2.4 Conditions for photocrosslinking with the artificial substrate N70_{2x}-GST

The mechanism of substrate recognition by APC and the role of APC core subunits largely remain a mystery. Our Doc1 photocrosslinking approach has not enabled us to identify the interaction partner of Doc1’s putative ligand binding loop (Section 2.1.4). Several pieces of evidence have implied that Doc1 might use this loop to bind to APC substrates and that way function as a processivity factor for APC ubiquitination (Carroll *et al.*, 2005; Passmore *et al.*, 2003; Wendt *et al.*, 2001); likewise Doc1 might, via this loop, interact with other factors important in the ubiquitination reaction. We have decided to undertake a more unbiased approach to continue our search for the APC substrate receptor. In this section, I am describing the conditions which I established for a photocrosslinking approach employing substrate constructs which contain a photoactivatable amino acid at various sites. This approach might allow us to identify the APC substrate receptor, which would be an important step towards a better understanding of APC substrate recognition.

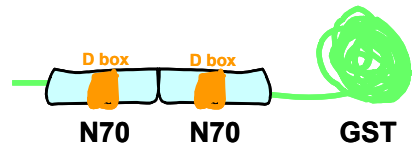
APC coactivators clearly play an important role in this process, as they were shown to directly interact with the substrates' D and KEN boxes and are believed to thereby recruit substrates to the APC (Burton *et al.*, 2005; Kraft *et al.*, 2005). There is evidence that also core APC subunits contribute to APC substrate recognition (Carroll *et al.*, 2005; Eytan *et al.*, 2006; Passmore *et al.*, 2003; Yamano *et al.*, 2004) and strikingly, *Xenopus* and human APC was found to bind to D box sequences even in the absence of coactivators (Eytan *et al.*, 2006; Yamano *et al.*, 2004). The latter two studies both used the same artificial substrate construct (first introduced by Yamano *et al.*, (Yamano *et al.*, 1996; Yamano *et al.*, 1998)), expressed in and purified from *E. coli*, which consists of a double fragment of the N-terminal 70 amino acids of fission yeast cyclin B fused to a C-terminal GST-tag (termed N70_{2x}-GST). Importantly, Ivana Primorac in our lab could show that binding of this construct to APC is not only dependent on its D boxes, but is also largely diminished if APC is inhibited by MCC (Herzog *et al.*, manuscript in preparation). This indicated that binding of N70_{2x}-GST to APC might be highly specific and that the construct might indeed resemble a physiological APC substrate.

However, the identity of the APC subunits which mediate substrate interactions remains unknown. We therefore decided to use N70_{2x}-GST in site-specific photocrosslinking experiments. Similar as described in section 2.1, I decided to use an *in vitro* translation system and non-natural *amber* suppressor tRNA coupled to a photocrosslinkable amino acid for the crosslinker incorporation. Setting up the experimental conditions therefore required the specific binding of N70_{2x}-GST generated by *in vitro* translation to APC. To test if *in vitro* translated N70_{2x}-GST binds to APC specifically the following experiment was performed: APC isolated from HeLa cells which had been arrested in mitosis with an inactive spindle assembly checkpoint (SAC) was incubated with *in vitro* translated N70_{2x}-GST versions. Bound proteins were eluted by addition of SDS sample buffer and analyzed by immunoblotting and phosphorimaging. As shown in Figure 2-16B, the *wild type* construct strongly bound to APC (lanes 9 and 11) whereas a version in which both D boxes were mutated bound only very poorly (lanes 10 and 12). The same result was obtained when using purified proteins (Figure 2-16B, lanes 5 and 6). When analyzing phosphorimages (Figure 2-16B, lower panel) and long exposures of immunoblots (not shown), I observed strong ubiquitination of *in vitro* translated N70_{2x}-GST, which was dependent on the reticulocyte lysate translation mix and largely on addition of purified APC (Figure 2-16B, compare lane 3,

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input substrate incubated at room temperature with lanes 9 and 11, substrate after incubation with APC; data not shown).

A



B

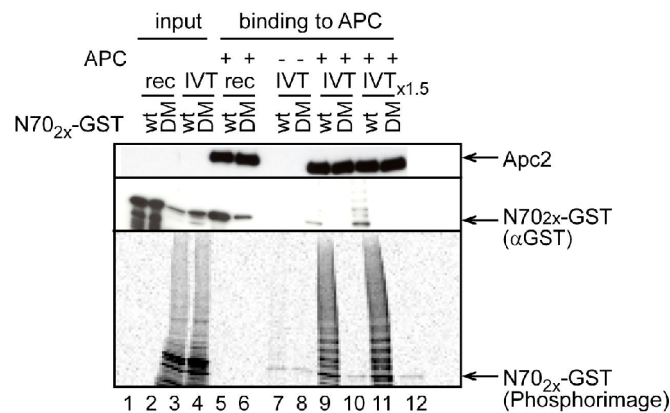


Figure 2-16: N70_{2x}-GST produced by *in vitro* translation binds to mitotic APC. A) Schematic drawing depicting the substrate construct used for these experiments. A tandem fragment comprising the N-terminal 70 amino acids of fission yeast cyclin B (Cdc13) is fused to GST. In a D box mutant, the R-x-x-L sequence is changed to A-x-x-A. B) APC was immunoprecipitated from HeLa arrested in mitosis with a Cdc27 antibody and subsequently incubated with N70_{2x}-GST protein purified from *E. coli* (rec, lanes 5 and 6) or generated by *in vitro* translation (IVT, lanes 7 to 12). *Wild type* (wt) and a version with a mutated D box (DM) were analyzed in parallel. In lanes 11 and 12, a 1.5-fold amount of protein was added. Bound protein was eluted with glycine and analyzed by SDS-PAGE and immunoblotting with Apc2 and GST antibodies. A phosphorimage of the same membrane that was used for immunoblotting shows the radiolabeled *in vitro* translated proteins.

These circumstances were problematic for my photocrosslinking experiments for two reasons. First, low levels of unmodified substrate would decrease the chances of detecting a crosslink signal, since usually only a small fraction of the input shifts to a higher molecular weight upon photolysis ((Kraft *et al.*, 2005) and Figure 2-4C). Second, the presence of ubiquitin chains covalently attached to radiolabeled substrate in the samples might cover a weak crosslink product on phosphorimages after SDS-PAGE and would therefore hinder the analysis. I therefore set out to avoid ubiquitination or revert it by four different means.

N-ethylmaleimide (NEM) is commonly used for blocking sulfhydryl groups and should not chemically attack the photocrosslinker's diazirin group (personal communication from Josef Brunner). UbcH10^{C114S} (Townsend *et al.*, 1997) is a dominant negative version of the E2 enzyme which is predominantly used by APC and should, when added at an excess, “counteract” the activity of E2 enzymes present in the lysate (similarly to its effect after addition to HeLa lysates, (Rape *et al.*, 2006; Reddy *et al.*, 2007)). N70_{2x}-GST generated by *in vitro* translation was therefore treated with NEM before incubation with APC (Figure 2-17A). Alternatively, UbcH10^{C114S} was added to the APC-substrate binding reaction (Figure 2-17B). In both cases, the ubiquitination of N70_{2x}-GST was reduced with increasing concentrations of the chemical or biological inhibitor. Ubiquitination can be reverted by deubiquitinating enzymes (Amerik and Hochstrasser, 2004). When seeking for a deubiquitination enzyme that would also cleave APC generated ubiquitin chains *in vitro*, I chose Usp2-cc (Ryu *et al.*, 2006), the catalytic core of the deubiquitinating enzyme Usp2. This enzyme does not contain a domain conferring substrate specificity and was reported to cleave all ubiquitin fusions, including linear or branched mono- or multiubiquitin chains after the ubiquitin's C-terminal glycine (Baker *et al.*, 2005; Catanzariti *et al.*, 2004; Ryu *et al.*, 2006). Addition of Usp2-cc during the APC-substrate binding reaction similar as in Figure 2-17B completely prevented the formation of N70_{2x}-GST-ubiquitin conjugates at a concentration of 4 μ M (not shown). Furthermore, Usp2-cc added to substrate-APC beads after the APC-substrate binding step and removal of the reticulocyte lysate by several washes also completely removed ubiquitin chains (Figure 2-17C, lanes 2 to 6). Prior UV-treatment of samples slightly reduced the deubiquitinating effect of Usp2-cc and lead to a weak “smear” being left after Usp2-cc treatment (Figure 2-17C lanes 7 to 11). Ubiquitination was still greatly reverted, but two bands at about 120 kDa and 200 kDa size could not be removed (Figure 2-17C lanes 7 to 11). These bands could represent unspecific crosslink products which were sometimes generated even when using proteins that carried no crosslinker (e.g. in Figure 2-8D, lane 12 and not shown). Alternatively, UV-exposure might have denatured long polyubiquitin chains resulting in ubiquitin moieties that could not be recognized and cleaved by Usp2-cc anymore. In a fourth attempt to interfere with N70_{2x}-GST ubiquitination by APC and cofactors present in the reticulocyte lysate, I sought after a translation system which would allow efficient production of N70_{2x}-GST. Bacterial S30 extracts (Promega) do not contain an ubiquitin proteasome system. Translating the N70_{2x}-GST *wild type* construct

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resulted in protein levels 5 to 10 times higher than those obtained with the reticulocyte lysate system (Figure 2-17D, lane 1; data not shown). I have, however, not tested yet whether the S30-translated N70_{2x}-GST binds to APC with similar specificity as the reticulocyte lysate system-produced protein

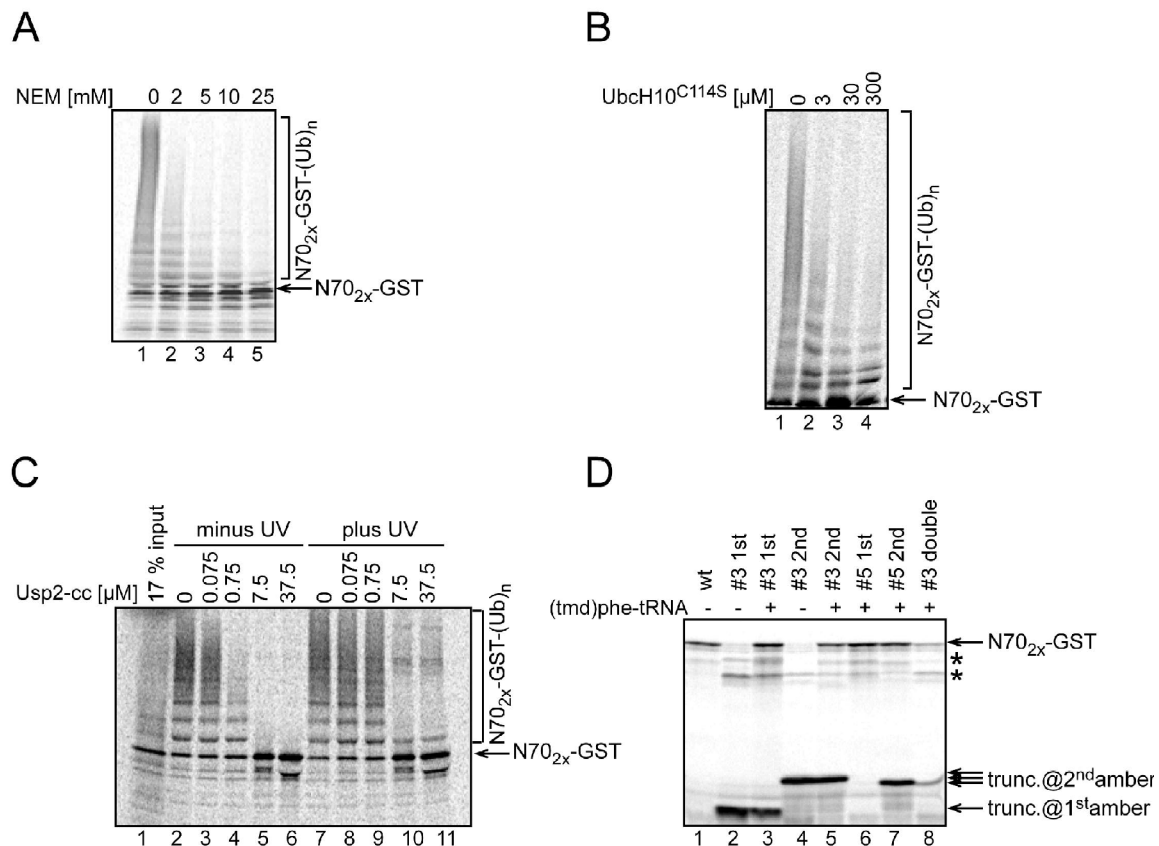


Figure 2-17: Four different ways of avoiding or reverting ubiquitination of N70_{2x}-GST when it is bound to mitotic APC. A) Reticulocyte lysate extracts containing translated N70_{2x}-GST were treated with the indicated amounts of N-ethylmaleimide (NEM) and then incubated with bead-bound APC. Bound proteins were washed and eluted from beads by SDS sample buffer. Samples were analyzed by SDS-PAGE and phosphorimaging. B) N70_{2x}-GST produced by *in vitro* translation in reticulocyte lysate was bound to bead-bound APC. During this binding reaction, the indicated amounts of a dominant negative version of UbcH10 (UbcH10C114S) were added. Samples were analyzed as in (A). C) N70_{2x}-GST produced by *in vitro* translation in reticulocyte lysate was bound to bead-bound APC and reticulocyte lysate was washed off. Samples were then incubated for 10 min at 8°C while they were exposed to UV-light (lanes 7 to 11). Control reactions were not exposed (lanes 2 to 6). Samples were then incubated with the indicated amounts of purified Usp2-cc. Samples were analyzed as in (A). D) *In vitro* translation of N70_{2x}-GST in bacterial S30 extracts. *Wild type* N70_{2x}-GST and different constructs were translated in the presence or absence of (tmd)phe-tRNA. Full-length protein is only obtained in reactions which contained (tmd)phe-tRNA. The translation efficiency in this extract system is only about 35 %; therefore, a high portion of truncated versions (trunc.@1st/2nd amber) was generated. The asterisks mark unspecific translation products.

Having found conditions under which *wild type* N70_{2x}-GST (translated in reticulocyte lysate) binds specifically to APC and which allow detecting possible crosslinks, I generated crosslinker containing mutants similarly as described for Doc1 (see Section 2.1). To this end I chose six sites within a single “N70” fragment which I decided to exchange for the photocrosslinker. I generated constructs which carried the modified amino acid in the first, the second, in both or in a single N70 fragment of the construct (Figure 2-18). Incorporation of (tmd)phe worked with 40 to 70 % efficiency when reticulocyte lysate was used as a translation system. Crosslinker containing versions of N70_{2x}-GST were still able to bind to APC beads (not shown), but binding was reduced though in constructs carrying the photocrosslinker within the D box (observed e.g. for mutant “#4 1st amber”, not shown). Tmd(phe) incorporation was also possible when translating the substrate construct in S30 extracts. The incorporation efficiency, however, did not exceed 35 % in this system (Figure 2-17D, lanes 2 to 8; quantification not shown). Because the translation efficiency is a lot higher in S30 extracts, the amount of full length crosslinker containing proteins generated in this system was still up to 25 times higher compared to those translated in the reticulocyte lysate system (not shown). As for the *wild type* construct, I have not tested whether S30-produced crosslinker containing versions of N70_{2x}-GST can bind to APC beads. Likewise we

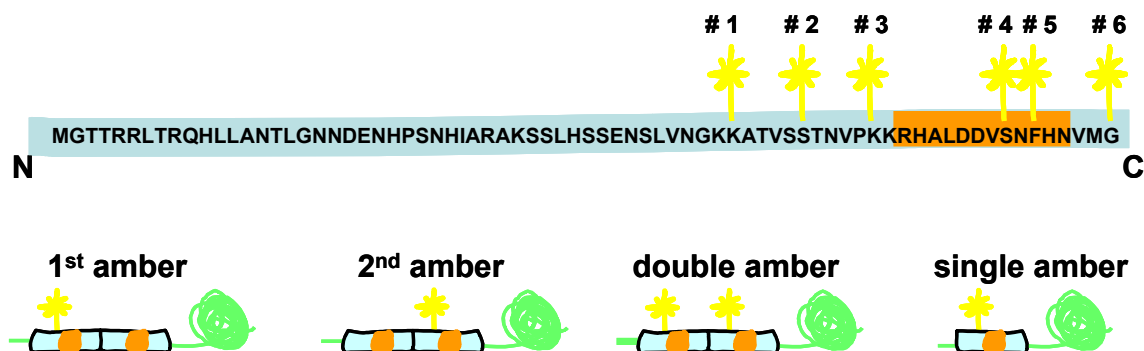


Figure 2-18: Schematic drawing of N70_{2x}-GST constructs which were generated for substrate photocrosslinking experiments. Colors and symbols are similar as in Figure 2-16. The sequence of N70_{2x}-GST is shown. The D box sequence is highlighted in orange and sites of photocrosslinker incorporation are indicated with a yellow mark. For each indicated residue four different constructs were generated. In “1st amber” the crosslinker was inserted within the first N70 fragment; “2nd amber” carried the crosslinker in the second fragment, “double amber” in both fragments. “Single amber” only consists of one “N70” fused to GST.

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do not know if the higher amount of incomplete translation products might interfere with binding. These and the actual crosslinking experiments will be carried out after submission of this thesis. In the future I might use both, N70_{2x}-GST generated in the reticulocyte lysate system and bacterial S30 lysate-produced N70_{2x}-GST in photocrosslinking experiments with mitotic human APC. Crosslinking experiments performed with reticulocyte lysate system-generated N70_{2x}-GST will be followed by incubation with Usp2-cc for removal of ubiquitin chains. In case I will obtain specific crosslink products, I will analyze their identity by denaturation and re-immunoprecipitation using APC subunit specific antibodies, similar as in Kraft *et al.* (Kraft *et al.*, 2005).

2.5 The protein encoded by *c10ORF104* is a novel subunit of vertebrate APC

As described in Section 2.4, we are carrying out substrate photocrosslinking experiments to identify the D box receptor on APC. We are hoping to detect specific crosslink products in these experiments which might result from interactions between the substrate and APC. In order to then be able to identify the substrate-interacting APC subunit we need to know all subunits of human APC. APC composition has been analyzed intensively in the last decade and twelve human subunits have been identified so far (Table 1-1). We were therefore surprised that Martina Sykora and Jim Hutchins as part of their MitoCheck work (www.mitocheck.org) were able to detect a protein whose association with APC had not been described before. They had analyzed APC purified by the localization and affinity purification (LAP) method from HeLa cells expressing LAP-tagged Apc1, Apc5, Apc6/Cdc16, Apc8/Cdc23, Cdc26, Apc13 or Cdc20. In all these preparations, the gene product of *c10orf104* was found by mass spectrometry after in solution digest. Importantly, it was never found in purifications of non-APC subunits or interactors (Martina Sykora and Jim Hutchins, personal communication). Furthermore, the *c10orf104* gene product was associated with APC in immunoprecipitations using the Cdc27 antibody followed by peptide elution (Franz Herzog, personal communication).

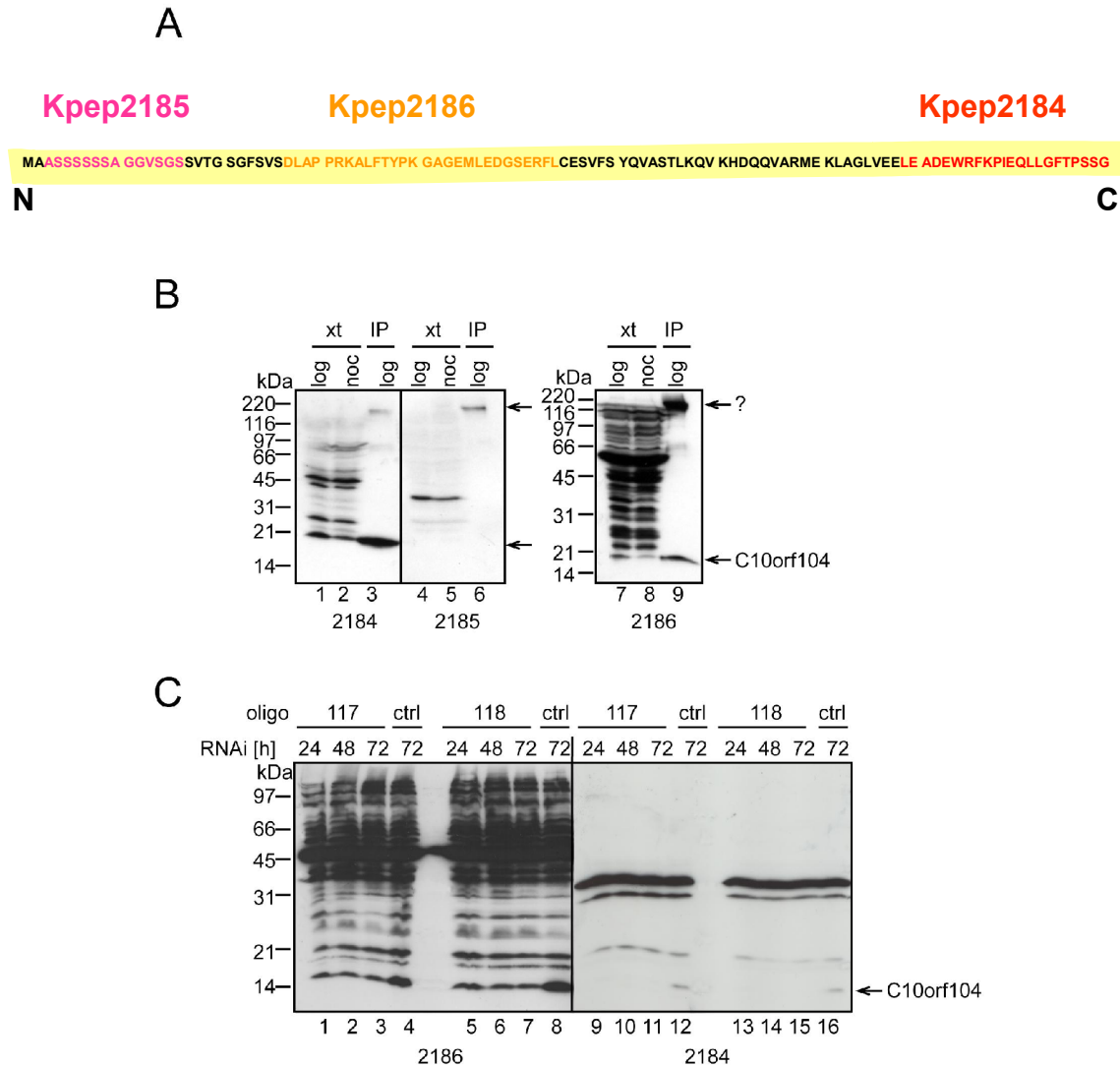


Figure 2-19: Antibodies raised against three regions of the C10orf104 protein specifically recognize the protein on Western blots. C10orf104 can be depleted by two different siRNAs. A) Protein sequence of C10orf104 depicting the peptides used for antibody generation. B) Antibodies recognize a band at 15 kDa size on protein extracts (xt) and on Cdc27-immunoprecipitates (IP). HeLa extracts prepared from logarithmically grown cells (log) and from cells that had been arrested in mitosis with nocodazole (noc) were loaded next to a glycine-eluate of a Cdc27-immunoprecipitation from log-cells. Immunoblots were analyzed with the glycine elutions of purified 2184, 2185 and 2186 antibodies at 2 µg/ml. C) The band specifically recognized by 2186 and 2184 is reduced after siRNA treatment. Cells were treated with siRNAs 117 or 118 or with water as a control for the indicated times. Protein extracts were prepared and the soluble fraction was analyzed by SDS-PAGE and immunoblotting using glycine elutions of purified antibodies as in (B).

We decided to analyze this protein and its putative interaction with APC further. *C10orf104* encodes a hypothetical protein comprising 110 amino acids that is remarkably conserved among higher eukaryotes but could not be found in yeasts, worms or flies (Maria

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Novatchkova, personal communication, Section 5.3). We generated antibodies against the *c10orf104* gene product by immunization of rabbits with peptides which were derived from three regions of the *c10orf104* protein sequence (Figure 2-19A). In immunoblot experiments, a 15 kDa band was recognized by these antibodies in APC purifications, which was also detected in crude extracts from interphase or mitotic HeLa cells (Figure 2-19B).

These antibodies also stained a band at about 200 kDa size in APC immunoprecipitates (Figure 2-19B, lanes 3, 6, 9 and not shown), the identity of which is presently unknown. We then performed RNAi experiments using two siRNAs directed against the *c10orf104* sequence. After transfecting cells with siRNA specific for *c10orf104* the 15 kDa band was strongly reduced (Figure 2-19C). These observations indicate that the 15 kDa band represents C10orf104.

In immunoprecipitates obtained with one of the C10orf104 antibodies (2186) from extracts of logarithmically growing HeLa cells, the C10orf104 protein and Apc2 were detected by immunoblotting at similar levels as in Cdc27 immunoprecipitates (Figure 2-19, lanes 7, 9 and 15). This supports the hypothesis that the C10orf104 protein is an APC interactor.

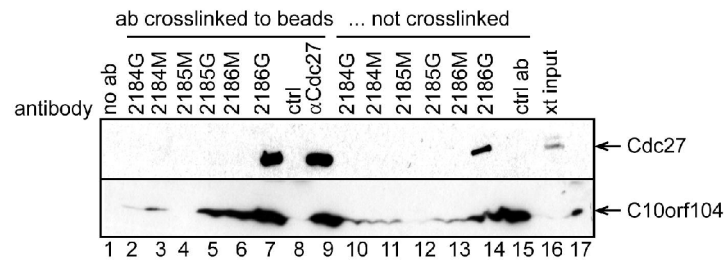


Figure 2-20: Antibodies raised against C10orf104 immunoprecipitate APC. Glycin (G) and magnesium (M) elutions of purified antibodies (ab) raised against C10orf104, bound to protein A beads and crosslinked if indicated, were incubated with cleared extracts of logarithmically grown HeLa cells. Crosslinked Cdc27 antibodies were used as a control. Bound protein was eluted with glycine and analyzed by SDS-PAGE and immunoblotting with Cdc27 and C10orf104 (2184) antibodies.

To address which fraction of the C10orf104 protein is associated with APC in cell extracts, sucrose density centrifugation experiments were performed. Cdc16, Apc10/Doc1 and the C10orf104 protein sedimented in the same fractions (Figure 2-21). However, not all of C10orf104 is stably associated with APC since a subpopulation could also be detected in fractions 7 to 9 (Figure 2-21). This also explains why the C10orf104 band recognized in the

input lanes was stronger than the Cdc16 input band, but in the APC peak fractions, the Cdc16 staining was more intense than the C10orf104 staining (Figure 2-21).

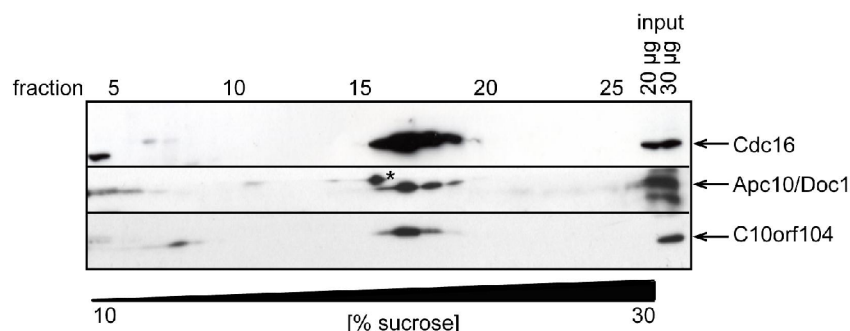


Figure 2-21: C10orf104 co-sediments with APC. Sucrose density gradient centrifugation of extracts prepared from logarithmically grown HeLa cells. Extracts were sedimented through a 10 to 30 % sucrose gradient for 18 h and fractionated into 28 fractions per gradient. APC sedimentation was analyzed by immunoblotting with Cdc16, Doc1 and C10orf104 (2186) antibodies. The asterix marks an unspecific band recognized by the Apc10/Doc1 antibody.

Immunoblotting on samples taken every 90 minutes within 18 hours after release from a double thymidine arrest showed that – although immunoblot signals were slightly decreased at time points 13.5, 15 and 16.5 – the C10orf104 protein is expressed continuously throughout the cell cycle (Figure 2-22). Further analysis will be required to test whether levels are constant or whether the apparent decrease of C10orf104 protein levels (Figure 2-22, lanes 10 to 12) is a reproducible and meaningful phenomenon.

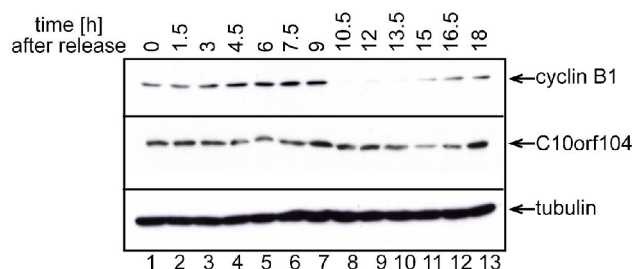


Figure 2-22: C10orf104 is expressed throughout the cell cycle. Samples were taken after cells had been synchronized by a double thymidine arrest release protocol. At the time of the second release, the first time point was taken (lane 1); cells therefore were in early S phase. Cells then accumulate cyclin B while proceeding through G1 (lanes 4 to 7). Cyclin B (lane 8) degradation marks mitosis. Time points were taken until cells again were in early S phase. Equal numbers of cells were lysed by adding SDS sample buffer and by sonication. Immunoblotting was performed using antibodies against cyclin B, C10orf104 (2184) and tubulin.

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APC is bound to and its activity inhibited by the MCC during early mitosis until all chromosomes are properly attached to the mitotic spindle (Musacchio and Salmon, 2007). The association of the C10orf104 protein with APC could therefore either be direct or could be mediated by the MCC. The observation that the C10orf104 antibody immunoprecipitated Apc2 from interphase cells, where APC is not associated with the MCC (Sudakin *et al.*, 2001), already indicated that C10orf104 is part of APC and not MCC. To confirm this notion, we performed immunoprecipitation experiments with C10orf104 (2186) and Cdc27 antibodies from extracts of interphase, mitotic and S phase HeLa cells. All samples showed the typical “APC pattern” when analyzed by SDS-PAGE and silver staining (Figure 2-23A). This indicates that C10orf104 is bound to APC throughout the cell cycle, in contrast to MCC. To test whether the C10orf104 antibody precipitates active APC, we carried out ubiquitination assays. APC purified from interphase extracts using the C10orf104 antibody was similarly active in ubiquitination assays as Cdc27 immunoprecipitates (Figure 2-23B).

We then wanted to get insight into the function of C10orf104 as an APC subunit. To this end, we depleted C10orf104 by siRNA transfection for 48 hours and tested whether cells accumulated in mitosis – as one might predict given APC’s essential function at the metaphase to anaphase transition (Peters, 2006) by counting the mitotic index of fixed cells and by analyzing cyclin B1 levels. Accumulation of cells in mitosis leads to an increased mitotic index and to higher levels of cyclin B1. However, we found the contrary. Whereas in the control sample we determined a mitotic index of 3.2, we found only 1.6 % of the cells to be mitotic in the siRNA treated samples (Figure 2-24A). Immunoblot analysis of cyclin B levels in these cells demonstrated that the amount of cyclin B1 was reduced upon C10orf104 depletion (Figure 2-24A). To further investigate whether this observation was due to a reduced number of mitotic cells we depleted C10orf104 by RNAi and arrested cells with thymidine to block them in early S phase. Six hours after release from thymidine we added taxol for three hours to activate the spindle checkpoint and let cells accumulate in mitosis. Analysis of the cells before and after taxol treatment by immunofluorescence microscopy using cyclin B1 and BubR1 antibodies showed that under control conditions about 90 % of cells were in S or G2 phase six hours after thymidine release. These cells were positive for BubR1 and cyclin B1 in the cytosol; the DAPI staining showed that the chromatin was in a decondensed state. After three hours of taxol treatment 50 % of control cells were in a

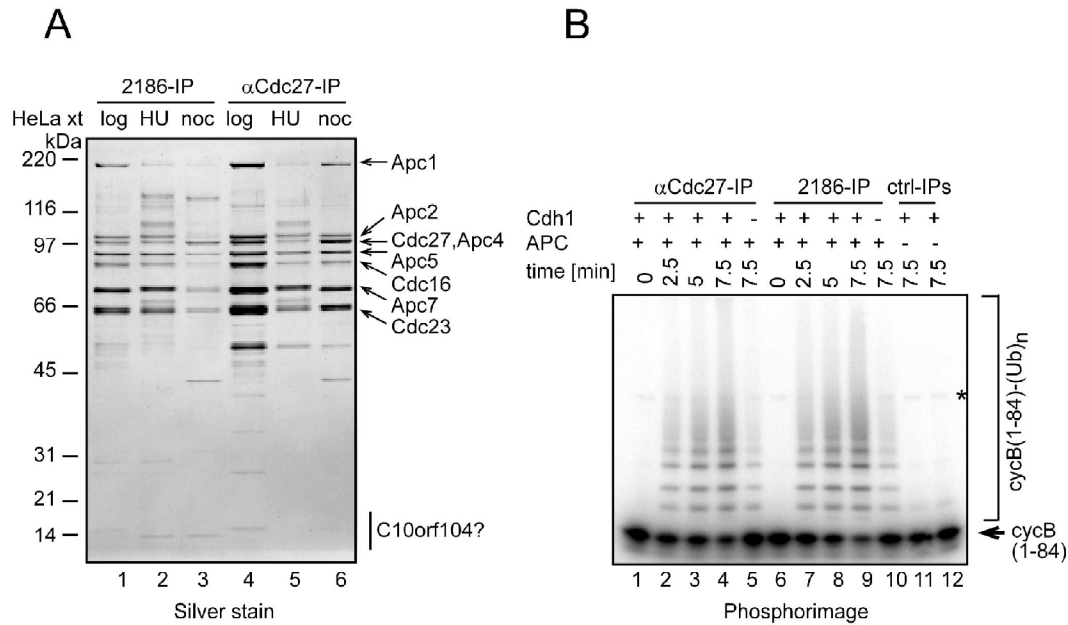


Figure 2-23: C10orf104 is a subunit of human APC. A) APC was purified by immunoprecipitation (IP) using C10orf104 (2186) and Cdc27 antibodies. Cell extracts used for this experiment were prepared from logarithmically grown HeLa cells (log), from HeLa cells that had been arrested in S phase by addition of hydroxyurea (HU) or in mitosis by nocodazole treatment (noc). Bound proteins were eluted with glycine and analyzed by SDS-PAGE and silver staining. APC subunits were identified by their electrophoretic mobility. C10orf104 could not be detected by silver staining. The candidate bands at around 15 kDa were excised and analyzed by mass spectrometry. C10orf104 was not detected. B) C10orf104 antibody pulls down active APC. *In vitro* ubiquitination assay using a [¹²⁵I]-labeled human cyclin B fragment (amino acids 1 to 84, cycB (1-84)) as a model substrate. Immunoprecipitates obtained with Cdc27 and C10orf104 antibodies were incubated with E1 and E2 enzymes, recombinant Cdh1, substrate, ubiquitin and ATP for the times indicated and analyzed by SDS-PAGE and phosphorimaging. As controls, antibodies specific for the condensin complex (lane 12) and empty protein A beads (lane 11) were used. The asterisk marks a contaminating band present in the substrate preparation.

prometaphase state with BubR1 staining the kinetochores, condensed chromatin and cytoplasmic staining of cyclin B1 (Figure 2-24B). In contrast, cells depleted of C10orf104 did not accumulate in mitosis. They retained cytosolic BubR1 and cyclin B1 staining, which is indicative of a G2 or S phase state (Figure 2-24B). There were no cells that had passed metaphase which would have been cyclin B1 negative. This indicates that cells lacking C10orf104 might be delayed in their progression through S and G2 or in mitotic entry. Although its function is not clear yet, the above findings strongly indicate that the *c10orf104* gene product is a novel subunit of human APC and we therefore propose to rename it to “Apc16”.

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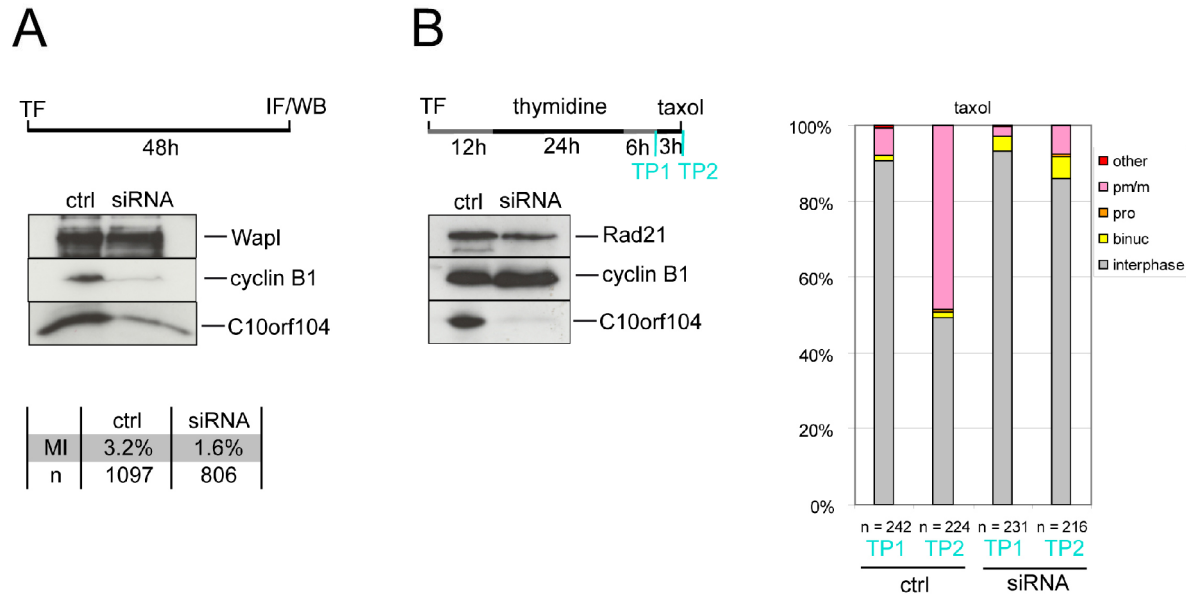


Figure 2-24: Depletion of C10orf104 leads to a reduction of cyclin B1 levels in asynchronously growing HeLa cells and decreases the number of mitotically arrested cells after taxol treatment. A) Cells were transfected with *c10orf104* siRNA for 4 h and afterwards split onto coverslips and tissue culture plates. Cells on coverslips were PFA-fixed and stained with DAPI and antibodies against H3S10ph to count the mitotic index 48 h after transfection. Remaining cells were harvested, extracted and analyzed by SDS-PAGE and immunoblotting. Membranes were probed with cyclin B1, C10orf104 (2184G) and WAPL antibodies. B) Cells were transfected with *c10orf104* siRNA for 12 h and then split onto coverslips and tissue culture plates. Afterwards, cells were treated with 2 mM thymidine for 24 h, then released into fresh medium and fixed 6 (time point 1, TP1) or 9 (TP2) h later after a 3 h-treatment with 10 μ M taxol. Cells were fixed with PFA and stained with BubR1 and cyclin B1 antibodies as well as DAPI. More than 200 cells were counted at each time point and scored as interphase (BubR1/cyclin B1 negative nuclei), prophase (cyclin B1-positive nuclei before nuclear envelope breakdown) or prometa- and metaphase cells (BubR1-positive kinetochores and condensed chromosome morphology). For the immunoblot control cells were harvested at time point 2 and processed as in (A). An antibody against Rad21 was used as a loading control. In both experiments, control cells (ctrl) were transfected with water.

2.6 Contributions

Marta Galova created most of the yeast strains which contain myc- or td2-tagged APC subunits and carried out TAP-purifications of some of the created td2-tagged APC strains to analyze APC composition. She also created the pRS316-Doc1 plasmid and all mutations in this plasmid. The yeast growth test (Figure 2-9C) was performed by Claudine Kraft (ETH Zurich, Switzerland). Electron microscopy experiments were carried out in collaboration with Prakash Dube and Holger Stark at the Max Planck Institute for Biophysical Chemistry

in Göttingen, Germany. The C10orf104 experiments were performed together with Björn Hegemann. Maria Novatchkova performed bioinformatical analyses on C10orf104

3 Discussion

3.1 Site-specific crosslinking identifies three Doc1 binding partners within APC

The APC subunit Doc1 has been shown to be required for processive APC-mediated ubiquitination reactions (Carroll and Morgan, 2002; Passmore *et al.*, 2003; Carroll and Morgan, 2002) and is a prominent candidate for being involved in substrate recognition (Carroll *et al.*, 2005; Passmore *et al.*, 2003; Wendt *et al.*, 2001). The molecular basis of this processivity function of Doc1 is unknown. Doc1 might directly bind to the substrate or any other factor being important in the ubiquitination reaction. For example, Doc1 might bind a substrate and thereby keep it in place while ubiquitin moieties are being attached to it. Doc1's "DOC domain" also exists in a few unrelated proteins, some of which have been predicted or demonstrated to function as ubiquitin ligases (DiAntonio *et al.*, 2001; Dias *et al.*, 2002; Nikolaev *et al.*, 2003). Therefore, one could also imagine Doc1 assisting in efficient ubiquitination by binding a general component of ubiquitination reaction, such as ubiquitin or an E2 enzyme. Alternatively, Doc1 might exert its processivity function indirectly by causing structural rearrangements within APC once a substrate is bound to the complex. Understanding Doc1's role in APC processivity therefore requires information about Doc1's direct interaction partners. To identify these, I have set up a photocrosslinking approach using budding yeast APC and recombinant Doc1 mutants containing a site specific photocrosslinker. I have inserted this photocrosslinker within different regions of Doc1, some of which have been shown to be important for Doc1's function as a processivity factor or for its binding to the APC (Carroll *et al.*, 2005); other sites have been chosen randomly. I have found that within the APC, Doc1 binds to the TPR proteins Cdc16 and Cdc27 via its C-terminal region and the IR tail, respectively. In addition, I could identify sites which directly contact APC's biggest subunit, Apc1. However, this technique did not identify a possible interaction partner of the Doc1 loop region, which has been shown to be important for APC processivity (Carroll *et al.*, 2005).

3.1.1 Doc1 interacts with the TPR subunits Cdc16 and Cdc27

It has been suggested that within APC, TPR proteins might serve as versatile adaptors for IR tail containing proteins (Hayes *et al.*, 2006; Vodermaier *et al.*, 2003; Wendt *et al.*, 2001). More than half of the total mass of APC consists of tandem arrays of TPR motifs which in budding yeast APC are present in the three subunits, Cdc16, Cdc23 and Cdc27 (Lamb *et al.*, 1994; Zachariae and Nasmyth, 1996). Each subunit contains eight to ten copies of the TPR motif in tandem arrays. A TPR motif contains 34 amino acids; the structural consensus sequence is composed of eight residues which are conserved in terms of size, hydrophobicity and spacing rather than in sequence (Blatch and Lassar, 1999; Lamb *et al.*, 1995). Structural analysis of other TPR proteins has shown that this motif forms into a pair of antiparallel α -helices, and clusters of tandem TPR motifs fold into a right-handed superhelical structure (Passmore, 2004). Within APC, the TPR subunits form a stable subcomplex together with Cdc26, Apc9 and Swm1 (Schwickart *et al.*, 2004; Thornton *et al.*, 2006; Vodermaier *et al.*, 2003; Zachariae *et al.*, 1996). TPR subunits might form a scaffold-like structure onto which other APC subunits, regulators and substrates might assemble (Vodermaier *et al.*, 2003).

Recruitment of the coactivators Cdc20 and Cdh1 to APC requires IR tails at the C-termini of coactivators and TPR proteins within APC (Passmore *et al.*, 2003; Vodermaier *et al.*, 2003). Both Apc3/Cdc27 and Apc7 can bind IR-containing peptides (Vodermaier *et al.*, 2003). Crosslinking studies have identified Apc3/Cdc27 as the APC subunit to which Cdh1 binds (Kraft *et al.*, 2005). Binding of Cdc20 and Cdh1 is reduced in mutants lacking their IR tails (Kraft *et al.*, 2005; Thornton *et al.*, 2006). Doc1 is the only core APC subunit which contains an IR tail – in fact, it is an “ILR tail” in budding yeast – and its interaction with the TPR subcomplex has been proposed based on *in vitro* binding assays (Wendt *et al.*, 2001). In these experiments, full-length Doc1, C-terminally truncated versions or peptides corresponding to the C-terminus of human Apc10/Doc1 have been coupled to a matrix and incubated with lysate of insect cells which expressed different human APC subunits (Wendt *et al.*, 2001). Cdc27 was found to interact with the peptide corresponding to Doc1’s C-terminus as well as with the full-length Apc10/Doc1; the binding was abolished when the last 14 or 23 amino acids of Doc1 were missing (Wendt *et al.*, 2001). In our crosslinking experiments we have identified one crosslinker mutant, which carries the photoreactive amino acid close to the C-terminus (three residues distance to the IR tail) and interacts with Cdc27 (Section 2.1.3).

DISCUSSION

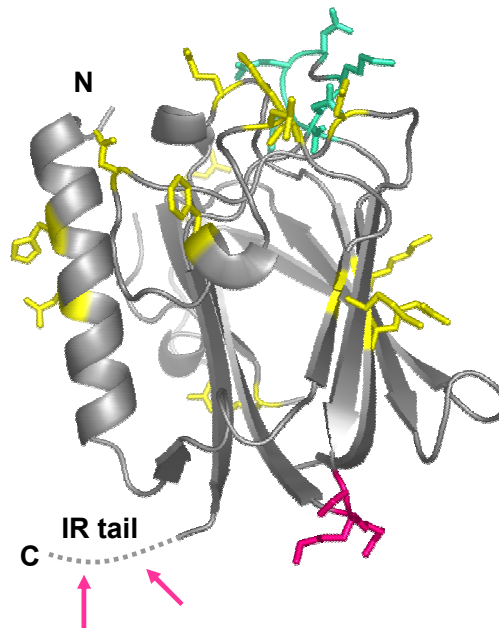


Figure 3-1: Sites on Doc1 which have been exchanged for the photocrosslinker. A total of 24 Doc1 versions have been created, each carrying the photoreactive amino acid at one site. Sites have been picked based on earlier data (indicated in pink or turquoise) or arbitrarily (yellow). Sites within an N-terminal loop region have been shown to be important for Doc1's ability to stimulate the processivity of APC-mediated ubiquitination reactions (Carroll *et al.*, 2005). Therefore, all six sites within this loop – one at a time – were exchanged for the photocrosslinker. These sites are indicated in turquoise. A Doc1 mutant that has two residues in the C-terminal region exchanged for alanines and in addition lacks 23 residues at the C-terminus including the so-called IR tail is impaired in binding to APC (Carroll *et al.*, 2005). The photocrosslinker was therefore inserted at three sites within the C-terminal region and at two sites within the IR tail, indicated in pink. In addition, 13 residues at various sites spread all over Doc1 were exchanged for the photocrosslinker. These sites are shown in yellow. The C-termini of both human and yeast Apc10/Doc1 have not been included in the crystal structures. This region is therefore represented as a grey dotted line.

Using a second Doc1 version with the crosslinker in the C-terminus (eight residues distance to the IR tail) resulted in a crosslinking product of the same size. Since the size of this product is clearly different from the size of the Doc1-Cdc16 crosslink products, we assume that it is result of a Doc1-Cdc27 interaction (not shown). Our data therefore fully support the previously reported interaction between Doc1's C-terminal region and Cdc27. Moreover, they provide the first evidence of a direct interaction between an IR tail and a TPR subunit, observed with full-length proteins and in the context of the intact APC.

We have found a second TPR subunit which directly interacts with Doc1. Doc1 crosslinks to Cdc16 via two residues which are both located in its “C-terminal region”. One site has been

found in the study of Carroll *et al.* to be important for Doc1 binding to APC (Carroll *et al.*, 2005) and was therefore replaced with a crosslinker amino acid residue; the other site, emanating from the lower back side of the molecule, has successfully been exchanged as one of the “randomly” picked residues. After deletion of the C-terminal IR tail the interaction between Doc1 and Cdc16 was four-fold reduced (Section 2.1.3). The IR tail-dependent interaction of Doc1 with Cdc27 therefore stimulates the interaction of Doc1 with Cdc16. In the study mentioned above, Wendt *et al.* have also tested binding of insect cell-expressed Cdc16 to the Doc1 matrices. Cdc16 bound to full length Apc10/Doc1, but this association was weaker compared to that of Cdc27. Importantly, Cdc16 and not Cdc27 was able to weakly associate with the Doc1 version lacking the C-terminal 23 amino acids (Wendt *et al.*, 2001). This is consistent with a Doc1-Cdc16 interaction being promoted by, but not dependent on the Doc1 IR tail. Peculiarly, in these experiments both Cdc16 and Cdc27 did not bind to the Doc1 version lacking the C-terminal 14 amino acids (Doc1 Δ C14). It has to be noted though, that this Doc1 version was not, as the other Doc1 versions used in this experiment, a GST-fusion protein bound to a glutathione matrix; instead, the untagged Doc1 Δ C14 was coupled to a matrix via cyanogen bromide activation (Wendt *et al.*, 2001) which might have caused damage to the protein.

3.1.2 Does Doc1 interact with Apc1 or Apc2?

Budding yeast APC lacking Cdc27 (Kraft *et al.*, 2005; Thornton *et al.*, 2006) or Cdc16 (Thornton *et al.*, 2006) contains normal levels of Doc1, indicating that Doc1 might be able to interact with the APC also via different subunits. A C-terminally truncated Doc1 mutant which in addition has two conserved residues within the C-terminal region changed to alanines is impaired in binding to APC *in vitro*; this defect can, however, be overcome by adding higher levels of Doc1 (Carroll *et al.*, 2005). This indicates that Doc1 must bind to additional APC subunits via sites distinct from the C-terminal region and the IR tail.

Which subunit apart from Cdc16 and Cdc27 does Doc1 bind to? A subcomplex of human APC which is lacking Apc2 and Apc11 shows reduced levels of Doc1 (Vodermaier *et al.*, 2003). This suggests Apc2 or Apc11 as candidate binding partners for Doc1 but clearly indicates that binding of Doc1 to APC also occurs via subunits except Apc2 and Apc11. In contrast, Thornton *et al.* observed complete loss of Doc1 from the APC in strains lacking

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Apc2 (Thornton *et al.*, 2006). Likewise, Apc11 was absent from these preparations. Moreover, APC purified from an *apc11Δ* strain contains reduced levels of Doc1 and Apc2, whereas Doc1 deletion does not affect any other known subunits (Thornton *et al.*, 2006). This study therefore implicates Apc2 as a direct interaction partner of Doc1. In our crosslinking experiments, we have not detected an interaction between Doc1 and Apc2 or Apc11, although I have “scanned” 10 % of Doc1’s amino acids and thereby tried to cover all surfaces (Section 2.1; Figure 3-1). This does, however, not rule out the possibility that Doc1 directly binds to Apc2. I could have missed the Apc2-interacting surface when choosing the sites of crosslinker-incorporation. Alternatively, the structures of some of the mutants could have been distorted due to crosslinker insertion; these mutants, possibly including those carrying the crosslinker at putative Apc2-interacting sites, might have therefore not been functional in crosslinking experiments.

Instead, however, I have obtained crosslinks to Apc1 with four different Doc1 crosslinker mutants (Section 2.1.3). Two mutants crosslink strongly to Apc1; in one of these mutants the crosslinker is located in the C-terminal region, right next to the site which strongly interacts with Cdc16; in the other mutant the crosslinker is emerging from a β -sheet to the lower right side. Weak crosslinks have been obtained with a mutant, in which the crosslinker is located in the putative ligand binding region and with a mutant which strongly crosslinked to Cdc16. Conversely, the Doc1 version which has a crosslinker in the C-terminus and interacts strongly with Apc1 also interacts weakly with Cdc16 (for overview see Table 2-2). These observations raise several questions. Is it conceivable that a single crosslinker mutant can interact with two different proteins? We believe that if two sites located directly next to each other can strongly interact with two different proteins, then it might be plausible that they can also weakly contact the respective neighbor protein, since there should be some flexibility in the crosslinker itself. Another puzzling question becomes evident when looking at Figure 3-2B. The sites on Doc1 which crosslink to Apc1 cover an extensive surface area; Apc1 would have to be “wrapped” around Doc1 if the two proteins were simultaneously making contacts via these residues. Although unusual, there is some precedence for interactions in which one protein embraces its binding partner. One example is the ubiquitin ligase SCF^{Skp2} which requires the accessory protein Cks1 to recognize and ubiquitinate the substrate p27^{Kip1} (Ganoth *et al.*, 2001; Spruck *et al.*, 2001). Structural data available for this complex reveal that Skp2 “embraces” Cks1 by making contacts with residues which are

(within structure and sequence) distant from each other (Hao *et al.*, 2005). Remarkably, within the above complex, the 9 kDa protein Cks1 directly interacts with three different proteins and Cks1 residues which make contacts to different proteins lie directly next to each other (Hao *et al.*, 2005). It is therefore conceivable that Doc1 would be embraced by Apc1 in a similar fashion.

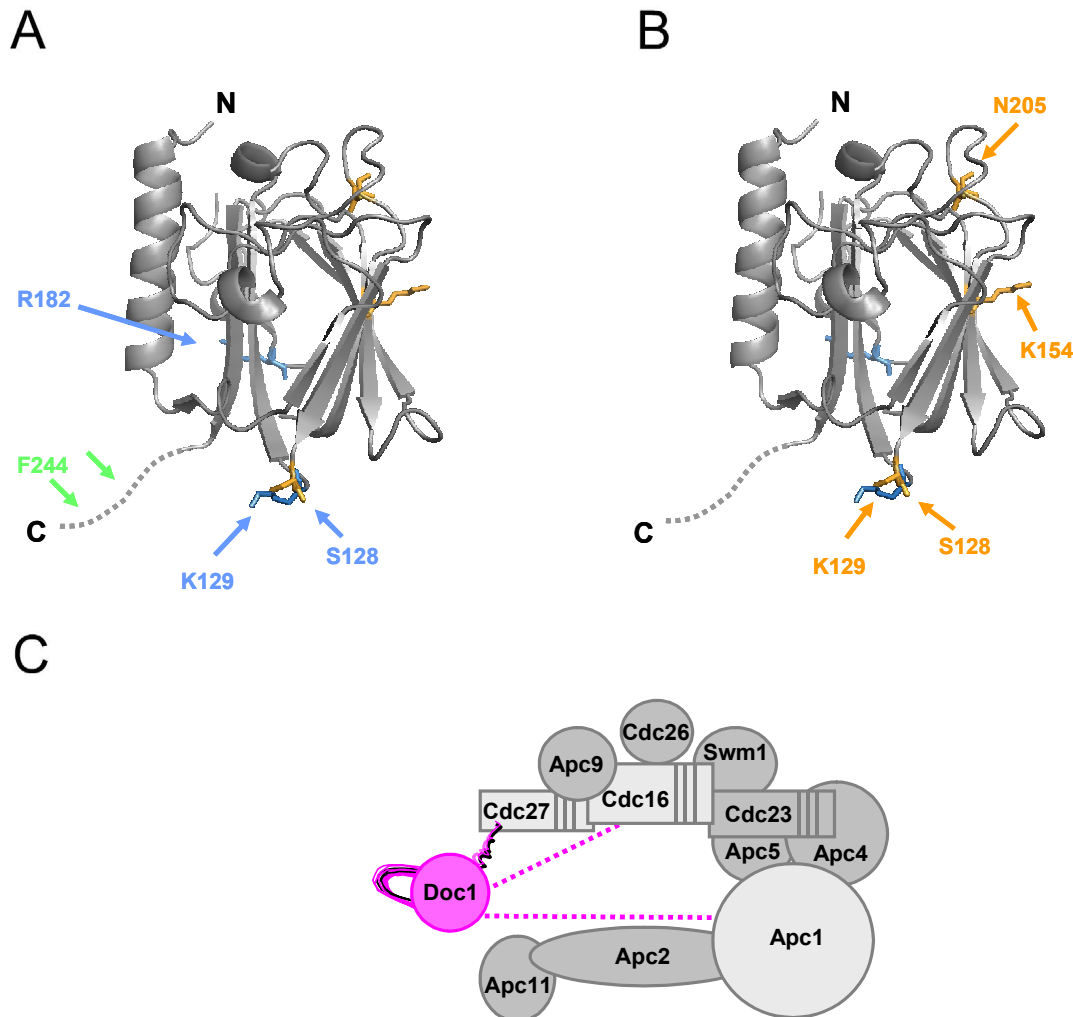


Figure 3-2: Sites within Doc1 which interact with Cdc16, Cdc27 and Apc1; schematic drawing of Doc1 interactions within APC. A) The position of sites which interacted with Cdc16 (blue) or Cdc27 (green) are shown on the crystal structure of budding yeast Doc1. Because the C-terminus was not included in the structure, it is shown as a schematic drawing. B) Same as (A), but here sites which crosslinked to Apc1 are shown in orange. C) Model depicting Doc1 interactions within APC. Modified from (Thornton *et al.*, 2006). Two scribbles on Doc1 indicate Doc1's C-terminus, which directly contacts Cdc27, and the "processivity loop", the putative interaction partner of which has not been identified. Pink dotted lines denote further observed interactions.

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The most important questions which emerge from our interaction data are: Is the identified interaction between Apc1 and Doc1 physiological? And, could Doc1 then bind to Apc1 “instead” of Apc2? Similar to the crosslinks to Cdc16 and Cdc27, we have only observed a crosslink to Apc1 with few (that is four) out of 24 crosslinker mutants; this indicates that the observed interactions might be specific. In contrast to the Doc1 versions which crosslinked to the TPR subcomplex, however, some of the crosslinker mutants which bind to Apc1 are greatly impaired in their ability to stimulate processive ubiquitination. Insertion of the crosslinker might therefore render some Doc1 versions inactive; hence they might bind “inappropriately”. Alternatively, insertion of the crosslinker at sites which are important for a protein’s function (such as those within the “processivity loop”) might inactivate the protein but yet allow it to bind to its native binding partner. We are not able to distinguish between these two options at the moment and therefore have to await results from EM analyses (see below).

How could removal of Apc2 lead to loss of Doc1 from APC (Thornton *et al.*, 2006), although Cdc16, Cdc27 and Apc1 are still present in the complex? Once Apc2 is removed from APC, either by high salt washes in case of human APC (Vodermaier *et al.*, 2003) or upon Apc2 deletion in budding yeast APC (Thornton *et al.*, 2006), Apc1 might adopt a conformation which abolishes the interaction with Doc1. The interaction of Doc1 with Apc1/Apc2 would have to be the predominant one such that Doc1 interactions with the TPR subcomplex only takes place upon binding of Doc1 to Apc1/Apc2. Alternatively, the interactions between Doc1 and Cdc16/Cdc27 may not be strong enough for stable binding of Doc1 to the APC. This might explain why in strains lacking Apc2, Doc1 is lost from APC. Alternatively and more likely, insertion of the crosslinker at certain sites within Doc1 interferes with proper protein folding or function. Doc1 might preferably aggregate with the biggest APC subunit, Apc1. Within these Doc1-Apc1 aggregates, few sites might crosslink to Apc1. A preferable albeit possibly non-physiological association of Cdh1 with human APC was observed in EM experiments. Cdh1 appears to have an additional binding site to which the coactivator binds when added in superstoichiometric amounts (Herzog *et al.*, manuscript in preparation). We will in the future try to explore the physiological relevance of the observed Apc1-Doc1 interaction by EM analysis. We are currently analyzing the localization of the C-termini of Apc1 and Apc2 within our 3D model (Table 2-3). In addition, we are planning to construct a yeast strain carrying an N-terminal td2-tag on Apc1. The localization

of Apc1 within the human complex has been determined by antibody labeling. According to this, the C-terminus of Apc1 is rather distant from Doc1 (Herzog *et al.*, manuscript in preparation). Apc1 is, however, thought to form APC's scaffold in a very "stretched-out" conformation. (Franz Herzog and Holger Stark, personal communication). We therefore consider it worthwhile to identify the position of Apc1's N-terminus as well.

3.2 Functions of the TPR subcomplex

Doc1 interacts with Cdc27 via residues in its C-terminus. We have not addressed whether this interaction is dependent on TPR motifs within Cdc27. Based on earlier findings (Gatto *et al.*, 2000; Vodermaier *et al.*, 2003), however, including those addressing the region responsible for the interaction between human Apc7 and Cdh1 (Vodermaier *et al.*, 2003), we propose that this is likely to be the case. Apc7 is a homolog of Apc3 and is therefore also closely related to yeast Cdc27. *In vitro* interaction studies have shown that recombinant Apc3 and Apc7 can bind to peptides corresponding to the C-termini of Apc10/Doc1, Cdc20 and Cdh1 ((Vodermaier *et al.*, 2003); Christian Gieffers and Hartmut Vodermaier, unpublished observation). The binding of Cdc20 and Cdh1 to yeast APC, which does not contain Apc7, is reduced in strains lacking Cdc27 (Kraft *et al.*, 2005; Thornton *et al.*, 2006). Several pieces of evidence therefore imply that TPR motifs within Cdc27 and possibly its homologue Apc7 are docking sites for C-terminal IR tails present in APC coactivators and Apc10/Doc1. The evidence that one of the other TPR subunits, Apc6/Cdc16 or Apc8/Cdc23, interacts with IR tails, is less convincing. Vodermaier *et al.* only observed weak binding of Cdc16 to peptides derived from the C-termini of Apc10/Doc1, Cdc20 or Cdh1 (Vodermaier *et al.*, 2003). Moreover, the binding of Cdc16 to Apc10/Doc1 appears to occur via a region outside of the IR tail (see 3.1.1). Cdc23 did not bind at all to C-terminal peptides corresponding to the C-termini of Cdc20 and Cdh1 (Vodermaier *et al.*, 2003).

Human APC appears to contain only one copy of Cdc27 and two copies of Apc7 per complex (Dube *et al.*, 2005); the complex might therefore contain several docking sites for IR tails. Fission and budding yeasts do not have Apc7. Fission yeast might "compensate" for this by containing two copies of Cdc27 (Ohi *et al.*, 2007). The Cdc27 copy number in budding yeast, however, is less clear. Whereas Passmore *et al.* have reported two to three copies to be present per complex (Passmore *et al.*, 2005b), we have found evidence that APC

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might contain only one Cdc27 molecule (Sections 2.3.3 and 3.5). In budding yeast the number of Cdc27 versions might therefore be smaller than the number of IR tail-containing binding candidates. This raises the question if Cdc27 can harbor more than one IR tail type at the same time or whether different IR tails bind in a mutually exclusive manner. Some studies implicate Doc1 as a non-constitutive APC subunit (Hwang and Murray, 1997; Kominami *et al.*, 1998); nevertheless it seems unlikely that Cdh1 and Doc1 bind to APC in a mutually exclusive manner, given for example the effect of Doc1 on APC-Cdh1-mediated ubiquitination ((Carroll and Morgan, 2002; Carroll and Morgan, 2005; Passmore *et al.*, 2003); Section 2.1). Rather one could imagine that rearrangements within the TPR-IR interaction module might somehow contribute to regulation of APC activity or substrate recognition, while the proteins remain associated with APC.

Noticeably, Cdc20, Cdh1 and Doc1 bind to APC via additional sites; these interactions seem to be independent of the IR and TPR modules and even functionally predominant (Peters, 2006; Thornton and Toczyski, 2006). Cdh1 retains function independent of the interaction between Cdh1's IR tail and Cdh1 (Thornton *et al.*, 2006). This function is dependent on a sequence called the "C box", which might confer binding of Cdh1 to Apc2. This binding is either direct or indirect via Apc11 or Doc1. Moreover, the interaction between Cdc20's IR tail and APC seems to be dispensable for viability (Thornton *et al.*, 2006). Doc1's binding to APC is dependent on a subunit outside of the TPR subcomplex, possibly Apc2 (Thornton *et al.*, 2006; Vodermaier *et al.*, 2003); Thornton *et al.* reported that in yeast Doc1 does not require TPR subunits for binding to APC (Thornton *et al.*, 2006).

What is the function of TPR subunits then? By providing additional binding sites for IR tail-containing proteins they might be responsible for the correct positioning of proteins within APC. Simultaneous binding of IR tail-containing proteins to subunits of the catalytic and TPR subcomplexes might cause structural rearrangements (see below). Importantly, the IR tail-containing proteins Cdc20, Cdh1 and Doc1 are all required for efficient APC ubiquitination. Even small rearrangements within the IR-TPR binding interface could possibly affect APC activity. When performing crosslinking experiments with a Doc1 version carrying the crosslinker in the IR tail I have observed the following: The Cdc27-Doc1 crosslink was reduced in samples which had been incubated with a ubiquitination mix prior to UV-irradiation, compared to the samples which had been incubated with buffer instead. Importantly, all samples had similar amounts of Doc1 bound to APC (Section 2.1.4).

This ubiquitination mix contained E1 and E2 enzymes, ubiquitin, GST-Hsl1⁽⁶⁶⁷⁻⁸⁷²⁾, ATP and recombinant purified Cdh1. This could observation could mean that, upon Cdh1 binding, the IR tail of Doc1 binds less stably to Cdc27 or is “rearranged” while Doc1 remains bound to APC. We have not followed up on this observation yet and it would be important to first test whether the reduced crosslink is caused by the presence of Cdh1 or any other particular component in the ubiquitination mix.

The many TPR subunits in APC might allow the complex to interact with several proteins at the same time. While Cdc27 might be “specialized” for the interaction with Doc1, Cdh1 and possibly Cdc20 (Section 2.1.3; (Kraft *et al.*, 2005; Vodermaier *et al.*, 2003; Wendt *et al.*, 2001)), functions of other TPR proteins remain to be identified. Likewise, other APC-related proteins contain IR tail-like C-termini, but the APC subunit to which they bind has not been identified yet: Recently it has been reported that the APC substrate Nek2A associates with APC dependent on Nek2A’s C-terminal MR tail (Hayes *et al.*, 2006). This substrate is degraded in a Cdc20 dependent manner and therefore might require simultaneous binding of substrate and coactivator to (two different?) TPR subunits. Finally, apart from providing docking sites for IR tail-containing proteins, Cdc27 has been proposed to have an additional function (Thornton *et al.*, 2006). This hypothesis is based on the observation that a *cdh1Δ cdc20-ΔIR* yeast strain can grow whereas a *cdc27Δ* strain can not (Thornton *et al.*, 2006). If Cdc27’s sole function was to be the IR tail receptor for Cdh1, one would expect the *cdh1Δ cdc20-ΔIR* and *cdc27Δ* strains to show the same growth phenotypes. Cdc27 might therefore have an additional role; the authors suggested that this might be in APC catalysis, especially since *CDC27* deletion does not affect APC subunit composition (Thornton *et al.*, 2006).

3.3 How does Doc1 confer processivity to APC ubiquitination?

Our crosslinking approach has not led to the identification of a Doc1 interaction partner which could directly explain Doc1’s function as a processivity factor. Despite testing many Doc1 versions carrying the photocrosslinker under several conditions (Section 2.1.4), I could have yet “missed” the “right” site or conditions, or both. Alternatively, Doc1 might interact with a non-proteinaceous ligand, and with our experimental set up we would be unable to detect such type of ligand. I have assessed the functionality of my Doc1 versions carrying a

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photocrosslinker in the “processivity loop” which is believed to interact with Doc1’s putative ligand (Au *et al.*, 2002; Carroll *et al.*, 2005; Wendt *et al.*, 2001). The presence of the photocrosslinker has only mildly affected Doc1 function in my experimental set up (Section 2.1.5); it should have therefore been possible to “catch” a ligand with this approach. Importantly, the fact that we have not been successful does not argue against the simplest explanation for Doc1’s effect on processive APC ubiquitination, namely that it directly interacts with the substrate (as suggested by (Carroll *et al.*, 2005; Carroll and Morgan, 2002; Passmore *et al.*, 2003)) or the growing ubiquitin chain on the substrate. To further test these possibilities, I have more recently established the conditions for a photocrosslinking approach employing a photocrosslinker-containing APC substrate which can stably bind to mitotic human APC (Section 2.4). Should this approach be successful we might get insight into the molecular basis of Doc1’s processivity function.

How, if not by directly contacting a substrate or any other component of the ubiquitination reaction, could Doc1 confer processivity to APC ubiquitination? Recently, budding yeast APC has been reported to ubiquitinate substrates in a more processive fashion when it is in a dimeric form, compared to APC monomers (Passmore *et al.*, 2005b). Both monomeric and dimeric APC are able to bind and multiubiquitinate substrates (Passmore *et al.*, 2005b). Since Doc1 has been shown to be a processivity factor for APC ubiquitination it is tempting to speculate that Doc1 might mediate this processivity by contributing to APC dimerization. Passmore and Barford have observed a doublet of bands when running purified APC on non-denaturing gels with the slower one having the estimated size of an APC dimer (Passmore and Barford, 2005). The authors also observed an enrichment of monomeric and dimeric APC versions when separating purified APC by sucrose and glycerol density centrifugations, respectively. When analyzing their APC preparations – which had been obtained by TAP-purification and contained 5 % glycerol – by electron microscopy, they observed monomeric and dimeric particles at a ratio of 3:1. In cryo-EM experiments, which do not allow glycerol to be present in samples, the same authors only observed monomeric APC (Passmore *et al.*, 2005b), indicating that glycerol stabilizes APC dimers. Although a similar TAP-purification protocol including glycerol has been used by Hartmut Vodermaier in our lab, he found that the bulk of APC co-sediments with human APC in sucrose density gradient centrifugations (Kraft *et al.*, 2005); human APC is believed to be monomeric and has an S value of 22 (Gieffers *et al.*, 2001).

The S value of budding yeast APC has been determined to 36 (Zachariae *et al.*, 1996) in glycerol gradient density centrifugation experiments in which total yeasts extracts were analyzed. Similar to Zachariae *et al.* (Zachariae *et al.*, 1996) we have found APC to peak at an S value larger than that for the proteasome and smaller than that of yeast fatty acid synthase when we analyzed whole cell extracts by sucrose density gradient centrifugation (not shown), indicating that in the absence of glycerol APC dimers exist. Similar to Passmore *et al.*, we have also observed APC dimers when analyzing TAP-purified samples by negative staining EM (Section 2.3.1 and not shown). Importantly, our samples are prepared by a fixation step on a gradient containing up to 40 % glycerol prior to grid preparation. Depending on the fraction of the gradient that is being used for grid preparation, we can see a lower or higher frequency of dimers. These dimers seem to have a specific orientation such that APC monomers are oriented to each other in a certain way within a dimer. (not shown). This indicates that APC dimerization might not simply be an artifact due to the presence of glycerol. We are currently analyzing these samples further and are hoping to identify the subunits involved in APC dimerization by combining these data with our subunit localizations obtained for yeast (Sections 2.3.2, 2.3.3) and human (Herzog *et al.*, manuscript in preparation) APC. Furthermore, we will analyze our EM grids of APC^{doc1Δ} samples for the presence of APC dimers and compare *wild type* and *doc1Δ* yeast extracts in glycerol and sucrose density centrifugation experiments; the parallel analysis of TAP-purified APC samples might serve as monomeric control. This might provide insight into whether Doc1 might be involved in the formation of APC dimers.

How could Doc1 mediate this process? Doc1 is a stoichiometric subunit of human and fission yeast APC (Dube *et al.*, 2005; Ohi *et al.*, 2007) and has been reported to be present twice in budding yeast APC (Passmore *et al.*, 2005b). In order to link two complexes, Doc1 would have to be bound stably to one or more subunits within one complex; in addition, it would have to make additional less stable contacts to (a) further subunit(s) within the second complex. For the latter contacts, Doc1 would have to dimerize itself, bind to the same set of subunits again or associate with different subunits. It has been speculated that Doc1 might be stably bound to Apc2 within one complex and by binding to Cdc27 from a second complex, might mediate APC dimerization (Thornton and Toczyski, 2006). This would imply that APC dimers are not “symmetric” in a way that different surfaces would be facing each other.

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Our preliminary biochemical data speak against an involvement of Doc1 in formation of APC dimers (Section 2.2). In co-immunoprecipitation experiments, the ability of Apc1 to self-associate has not been significantly reduced in the absence of Doc1 (Section 2.2). We would like to repeat these experiments under different buffer- and salt-conditions. So far, however, Doc1 may be part of a dimeric interface and somehow act in that setting; but its contributions to the actual formation of an APC dimer seem to be minor. Since APC subunit composition and functions are largely conserved among different species, we would also expect its ubiquitination mechanism to be similar. Therefore it seems unlikely that dimerization is a key mechanism in budding yeast because no APC dimers have been observed in other organisms; the exception might be an observation by Gieffers *et al.* who reported a slower band in native gel electrophoreses (Gieffers *et al.*, 2001). When analyzing human APC the same way as described for budding yeast APC (Kastner *et al.*, 2008) we have so far not observed any dimers (Franz Herzog and Holger Stark, personal communication). Nevertheless, further side-by-side analysis of APC from different species in density gradient centrifugations, negative staining EM and native gels might be worthwhile. It would for example be interesting to see the sedimentation behavior of fission yeast APC when whole cell extracts are separated instead of purified samples. Notably, dimerization has been reported to play an important role for a yeast SCF complex (Tang *et al.*, 2007). This dimerization is mediated by the WD40 domain containing F box proteins β TrCP and Cdc4 and it increases the catalytic efficiency of the ubiquitin ligase. Importantly, however, these adaptor proteins homo-dimerize via a so-called D domain which is missing from the WD40 domain containing APC coactivators (Tang *et al.*, 2007).

Instead of bringing two APCs together or act in such a setting, Doc1 might function by inducing conformational changes within a single complex. Similar to Cdh1, Doc1 seems to interact with both the TPR subcomplex (Vodermaier *et al.*, 2003; Wendt *et al.*, 2001) and with a member of the catalytic subcomplex, probably Apc2 (Thornton *et al.*, 2006; Vodermaier *et al.*, 2003). This might allow APC to close around a substrate and thereby prevent its dissociation during repeated cycles of ubiquitination. According to the budding yeast APC interaction map by Thornton *et al.*, the TPR subunits form a subcomplex with Cdc23 being the innermost member which interacts with Apc4 and Apc5; Cdc27 is the middle and Cdc16 the outermost subunit among the TPR subunits (Thornton *et al.*, 2006). APC isolated from a *cdc27* Δ strain retains residual ubiquitination activity; *cdc16* Δ APC in contrast is inactive

(Thornton *et al.*, 2006). This suggests that Cdc16 might have an additional function beyond mediating Cdc27 binding to APC. Maybe, upon substrate binding to APC via a coactivator, APC needs the interaction between Cdc16/Cdc27 and Doc1 to fully enclose a substrate. The crosslinking data presented in this study would be consistent with this possibility. They suggest that both subunits, Cdc16 and Cdc27, are directly interacting with Doc1's C-terminal region (Section 2.1.3). In a *cdc16Δ* strain, both Cdc16 and Cdc27 are lost from APC; hence, the Doc1 interaction with the TPR subcomplex is abolished.

Both the “dimerization” and the “conformational change” hypothesis do not provide an explanation for a possible involvement of Doc1's putative ligand binding loop in processive APC ubiquitination. Although we have not been able to detect Doc1 binding to Apc2 in our crosslinking experiments and therefore do not know what region of Doc1 it binds to, it seems unlikely that the processivity loop mediates this interaction. Carroll *et al.* have purified APC from a yeast strain which carried four alanine mutations in the processivity loop. These mutations result in a processivity defect; yet is Doc1 associated with APC at normal levels (Carroll *et al.*, 2005). Since in budding yeast Doc1 seems to associate with APC predominantly via Apc2, the processivity loop can not be required for this interaction. It therefore seems more plausible, that Doc1 – possibly upon APC dimerization or induction of conformational changes – employs its processivity loop to act as a processivity factor by binding to a still unidentified ligand.

3.4 How does APC recognize substrates?

Several pieces of evidence suggest that both core APC and an APC coactivator contribute to substrate recognition (Burton *et al.*, 2005; Carroll *et al.*, 2005; Eytan *et al.*, 2006; Hayes *et al.*, 2006; Kraft *et al.*, 2005; Passmore and Barford, 2005; Passmore *et al.*, 2003; Yamano *et al.*, 2004). The D box of an APC substrate has been shown to bind directly to Cdh1 (Kraft *et al.*, 2005). In contrast, the identity of the APC subunit(s) which is (are) binding to substrates is unknown. Our approach employing photocrosslinker-containing Doc1 versions has not revealed whether Doc1 is the APC subunit which interacts with APC substrates (Section 2.1). I have therefore established the conditions for a crosslinking approach which uses the artificial substrate N70_{2x}-GST containing a photoactivatable amino acid. N70_{2x}-GST is a tandem fragment of fission yeast cyclin B/Cdc13 that is C-terminally fused to GST;

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therefore it contains two D boxes as APC substrate degrons (Yamano *et al.*, 1996; Yamano *et al.*, 1998). Substrate photocrosslinking experiments have previously been carried out by Claudine Kraft in our lab. Whereas Claudine has demonstrated that substrate D boxes bind to the WD40 domain of Cdh1 (Kraft *et al.*, 2005), she has not been able to identify a putative APC core substrate receptor ((Kraft *et al.*, 2005); personal communication from Claudine Kraft). Claudine has used short peptides representing parts of the APC substrates cyclin B and securin, in which a photoactivatable amino acid was incorporated in proximity to the D box (Kraft *et al.*, 2005). These peptides were specifically recognized and ubiquitinated by APC; binding to APC, however, could only be detected with the help of the photocrosslinking method (Kraft *et al.*, 2005). We would now like to undertake a new attempt and thereby make use of the substrate construct N70_{2x}-GST, which has been shown to bind to mitotic APC very well in conventional binding assays. Importantly, this binding seems to be specific since it depends on the D boxes within this substrate (Eytan *et al.*, 2006; Yamano *et al.*, 2004). Furthermore, the construct can not bind to APC when APC is associated with the mitotic checkpoint complex. The same was also observed with Hsl1-his, which is a physiological substrate of the APC (Herzog *et al.*, manuscript in preparation). These findings support the notion that its behavior resembles that of a “natural” substrate.

Xenopus APC isolated from frog egg extracts which have been depleted from coactivators is able to bind to N70_{2x}-GST (Yamano *et al.*, 2004). This study by Yamano *et al.* for the first time demonstrated the existence of a D box receptor on APC. Substrate binding is, however, greatly enforced by the presence of Cdc20 (Yamano *et al.*, 2004). Moreover, it has been shown that human APC binds N70_{2x}-GST with relaxed specificity and selectivity upon Cdc20 depletion (Eytan *et al.*, 2006). Eytan *et al.* have reported no difference in the affinity of APC to *wild type* and D box mutant substrates in the absence of Cdc20 (Eytan *et al.*, 2006). In presence of Cdc20, they have found little binding of the D box mutant to APC. This binding was not further reduced after Cdc20 depletion (Eytan *et al.*, 2006). In contrast, Yamano *et al.* have observed reduced binding of an N70_{2x}-GST D box mutant compared to *wild type* N70_{2x}-GST after Cdc20 depletion (Yamano *et al.*, 2004). These experiments indicate that Cdc20 plays an important role in substrate specificity and affinity to APC. It is not clear, however, whether APC can bind to N70_{2x}-GST specifically in absence of Cdc20.

We therefore believe that it is important to carry out photocrosslinking experiments with APC and N70_{2x}-GST in the presence of a coactivator. Adding Cdc20 or Cdh1 to mitotic

APC increased the levels of N70_{2x}-GST that were bound to APC ((Eytan *et al.*, 2006), personal communication from Ivana Primorac). For our crosslinking experiments, we are therefore considering to add recombinant Cdc20, purified from insect cells, to stimulate substrate binding to APC. Since crosslinks between APC substrates and APC coactivators have already been detected in two studies ((Kraft *et al.*, 2005); personal communication from Yuu Kimata and Hiro Yamano), it is not unlikely that we might detect a similar interaction as well, especially when adding extra Cdc20. The position of the crosslinker might, however, determine whether a crosslink product is formed between substrate and Cdc20 or between substrate and APC subunit. Both the crosslinker position relative to the D box and, in case of our tandem fragment, whether it is in the first or second D box, might be important. By employing many N70_{2x}-GST versions, each of which carries the photoactivatable amino acid at a different position, we are hoping to identify a specific crosslink to a core APC subunit (Section 2.4). One might also use APC from mitotic HeLa extracts which have been depleted of Cdc20 for crosslinking experiments. Because this reduces substrate binding and might increase unspecific binding (see above), however, this attempt is not our first choice.

The hypothesis that the photocrosslinker position determines whether the substrate might crosslink to Cdc20 or to an APC subunit is based on APC substrate recognition models proposing “simultaneous” or “multivalent” interactions between APC, Cdc20 and a substrate ((Eytan *et al.*, 2006; Passmore and Barford, 2005; Yu, 2007); Figure 3-3). In a simultaneous binding model, both APC and Cdc20 build a composite binding site for substrates and the substrate’s destruction motif binds at the interface between APC and Cdc20. APC substrates often contain more than one degron. In a multivalency model, APC and Cdc20 each have weak binding sites for D or KEN boxes. An APC-Cdc20 complex might therefore produce high affinity binding due to the synergistic effect of multiple weak interactions. Moreover, substrates might bind sequentially to APC-Cdc20 such that the substrate is first bound by Cdc20 and then transferred to an APC subunit. An allosteric model includes a conformational changes being induced in APC upon coactivator binding. These might expose binding sites on core APC which had been masked before. None of the models can satisfy all the observations that have been made in the past with respect to APC substrate recognition. A valid model would therefore rather contain aspects of several models. It would have to account for the finding that both APC core and coactivators have substrate binding sites; yet the substrate binding is only highly selective and efficient in the

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presence of both entities (Burton *et al.*, 2005; Eytan *et al.*, 2006; Kraft *et al.*, 2005; Passmore and Barford, 2005). The observation that substrate recognition by APC-Cdc20 is more selective than in absence of Cdc20 might support a sequential binding model in which the degrons on APC substrates would be tested for “correctness” at two binding sites before a substrate gets degraded (Eytan *et al.*, 2006). The increased substrate affinity which has been reported for reactions containing APC and coactivator (Eytan *et al.*, 2006; Passmore and Barford, 2005; Yamano *et al.*, 2004) matches a setting in which Cdc20 binding to APC might induce a conformational change within APC. This might be reflected in the observation that Cdh1 binding to APC induces a conformational change in APC (Dube *et al.*, 2005). In line with this, Hershko and coworkers have reported that increasing amounts of Cdc20 increase the catalytic rate of substrate ubiquitination (Eytan *et al.*, 2006). The fact that several APC substrates contain multiple degrons and the observation that elimination of a single degron often does not abolish APC substrate recognition completely (Fang *et al.*, 1998; Zur and Brandeis, 2001) might be explained with a multivalency model. Coactivator and one or more APC subunit(s) might contain substrate receptor sites and several weaker interactions might sum up to a binding of high affinity. Maybe the N70_{2x}-GST construct, which contains two D boxes, binds mitotic APC so well (Eytan *et al.*, 2006; Yamano *et al.*, 2004) because its two degrons can simultaneously interact with two substrate receptors, one of which is Cdc20. Also Hsl1⁽⁶⁶⁷⁻⁸⁷²⁾, which binds human APC with similar specificity (Herzog *et al.*, manuscript in preparation; personal communication from Ivana Primorac), contains a D and a KEN box. It is not clear whether in such a setting contacts between certain degrons and certain substrate receptors would be “predetermined”, or whether interactions happen at random. Likewise, binding of the substrate to its multiple receptors might be highly dynamic. Analysis of APC constructs such as N70_{2x}-GST or Hsl1, which have been mutated by swapping or eliminating degradation motifs or by insertion of linkers, in binding assays might tell us why these proteins are such good APC binders. This could provide valuable information about the mechanism of substrate recognition. Ultimately, however, we will need structural data at atomic resolution to understand how substrate recognition by APC works in detail.

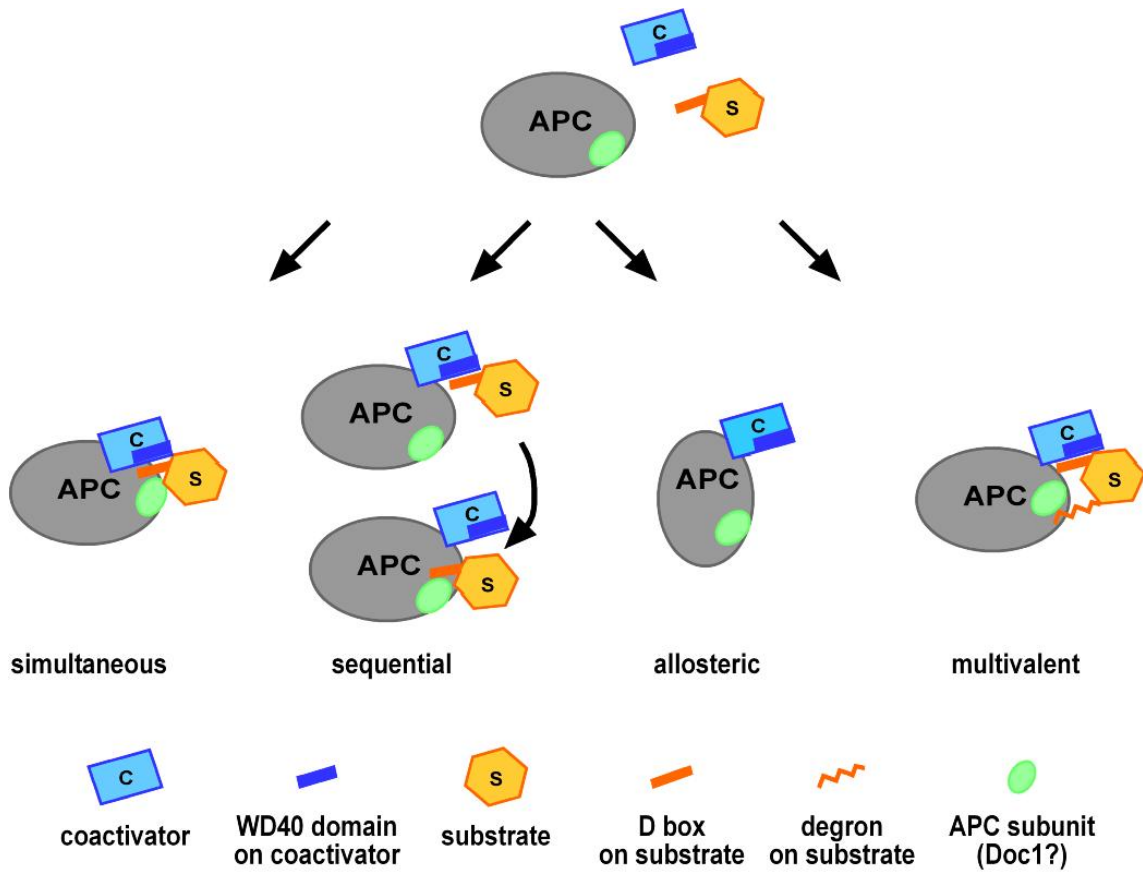


Figure 3-3: Four models for APC substrate recognition involving an APC coactivator and an APC core subunit. Modified from (Buschhorn and Peters, 2006; Yu, 2007). The WD40 domains of Cdc20 and Cdh1 functionally interact with a substrate's destruction box (D box). In addition, a core subunit of APC contributes to substrate recognition. Both coactivator and APC might interact with the D box simultaneously. In the "sequential" setting, the D box might first be recognized by a coactivator and then be transferred to core APC. According to an allosteric model, binding of a coactivator might induce a conformational change in APC which might expose a substrate binding site on core APC. With respect to substrate binding, the allosteric model has to be combined with either of the three other models. Finally, multiple degrons (such as a D box and a KEN box) within substrates might be recognized by substrate binding sites present in both core APC and coactivator. Multivalent weak interactions might produce a binding of higher affinity. The four models are non-mutually exclusive. In fact, APC substrate recognition is likely to feature aspects of several models.

3.5 EM analyses to study APC assembly and function

APC is an essential regulator of cell cycle events and has been intensively studied for the last decade. Yet there are many unresolved questions concerning its regulation and its catalytic mechanism. Answering these questions requires insights into the overall structure of APC and into the 3D topology of its subunits. APC versions from different species contain

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roughly the same number of subunits (see Table 1-1) and the domains which could be identified within these subunits are conserved at the primary amino acid sequence level. Thus, one would expect the structures of APC molecules from different species to be similar. However, structures published so far for APC purified from human cells (Dube *et al.*, 2005; Gieffers *et al.*, 2001), frog egg extracts (Dube *et al.*, 2005), fission yeast (Ohi *et al.*, 2007) and budding yeast (Passmore *et al.*, 2005b) display some striking differences. The budding yeast structure (Passmore *et al.*, 2005b) is particularly different from the structures of vertebrate APC obtained recently in our lab ((Dube *et al.*, 2005), Herzog *et al.*, manuscript in preparation). We therefore decided to generate a 3D model of budding yeast APC under sample preparation and imaging conditions that are comparable to the conditions under which 3D models of vertebrate APC were obtained (Figure 2-11). At first glance, our structure of budding yeast APC shares similarities with the structures of fission yeast (Ohi *et al.*, 2007) and human APC (Figure 2-11C), which is a refined version of the model published in ((Dube *et al.*, 2005), Herzog *et al.*, manuscript in preparation). All these structures are characterized by an asymmetric shape and an inner cavity, though the latter is of different size in the different models. Human and frog APC ((Dube *et al.*, 2005), Herzog *et al.*, manuscript in preparation) have a pronounced “head” domain. In our new structure and in the fission yeast structure, no such prominent “head” domains can be found. It is important to note that Ohi *et al.* did not determine the handedness of their structure; in fact, random conical tilt analyses have shown that the handedness of budding yeast APC is different from the one that was reported by Ohi *et al.* ((Ohi *et al.*, 2007), Holger Stark, personal communication). It is therefore difficult to align and compare our budding yeast structure with the structure of fission yeast APC. It is also surprising in view of the wrong handedness of the fission yeast model that the authors were able to place the atomic structure of the SCF complex into their model (Ohi *et al.*, 2007). All APC 3D models contain prominent ridges at the back of the complex (see below). This includes the published budding yeast structure (Passmore *et al.*, 2005b); although in this model the ridges are less pronounced. However, it is possible that these differences are caused by differences in the image analysis procedures that were used. When having a closer look at Figure 2-11D similar features between the two budding yeast structures become evident. Our structure appears asymmetric, slimmer and more defined. It could be that structural details in the published structure are “smeared out” and the structure might be broadened as a result of a pseudo-symmetry that was not

removed (see also discussion in (Dube *et al.*, 2005)). Maybe, some of the striking differences between the available 3D models of APC stem from difficulties with image processing and structure determination. Removing these will be essential – yet difficult due to the lack of parameters describing structure validity – since only faithful models can contribute to the understanding of APC function.

At the resolutions achieved so far (~ 20 Å for human APC (Herzog *et al.*, manuscript in preparation) 27 Å for fission yeast APC (Ohi *et al.*, 2007) and ~ 20 Å for budding yeast (Passmore *et al.*, 2005b)), the identification of tertiary structure elements and their assignment to individual APC subunits is impossible. Getting insight into the molecular organization of subunits within APC therefore – until higher resolution EM and crystallography approaches are successful – requires approaches such as antibody labeling or mutant analysis to determine subunit localizations. These approaches have already led to an overall idea of APC subunit assembly. Recently, an almost full set of antibody labeling data has been published for fission yeast APC subunits (Ohi *et al.*, 2007). The authors have analyzed the binding sites of antibodies on single APC molecules. Because they could only localize the antibody binding sites on a 2D image, implications from this subunit map are limited (Ohi *et al.*, 2007). Franz Herzog and Holger Stark have generated an impressive 3D model of human APC, onto which they have mapped the localization of 8 APC subunits and the coactivator Cdh1 ((Dube *et al.*, 2005); Herzog *et al.*, manuscript in preparation}. They have analyzed dimers of two APC molecules crosslinked via a single APC subunit-specific immunoglobulin by negative staining EM. By computational analysis of the relative orientations of the two APC molecules to each other they obtained the subunit localizations on the surface of an APC 3D model (Dube *et al.*, 2005, Herzog *et al.*, manuscript in preparation). Due to the flexibility and relatively big size of the antibodies and the fact that a 2D image is mapped onto a 3D structure, these analyses rather lead to assignment of a region to an APC subunit than to a precise localization. Importantly, the area which is assigned to an individual subunit does not represent the entire subunit but marks the antibody binding site.

We have compared the 3D models of APC and APC^{doc1Δ} to localize Doc1 on our budding yeast APC model (Section 2.3.2). This has allowed the assignment of a well defined region to Doc1 and thereby improves the previous localization of Doc1 which had been obtained by antibody labeling (Herzog *et al.*, manuscript in preparation). The overall structure of APC

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does not change upon removal of Doc1. However, whether or not Doc1 association with APC induces small conformational changes – which might be sufficient to affect the processivity of APC ubiquitination – can not be determined at this resolution.

We have started to analyze the 3D APC subunit assembly by fusing big globular tags to individual subunits (Section 2.3.3). The success of this method requires a) the yeast strain to be viable, b) the tag to not interfere with APC assembly or with the integration of the tagged subunit into APC and c) the tag to be exposed to the surface of the molecule so that it is visible. While the data acquisition is elaborate since it requires a 3D model to be generated for each tagged subunit, the data obtained might allow the most precise subunit localization achieved for core APC subunits (except Doc1, see above) to date. We have so far obtained data for the TPR subunit Cdc27 and for Apc5 (Section 2.3.3). According to these, Apc5 localizes to the “basis” or “platform” of APC. A similar localization has been observed for Apc5 in human APC, as determined by antibody labeling experiments (Herzog *et al.*, manuscript in preparation). The tag on Cdc27 localizes close to the head region and at the edge of the prominent back-side ridges. Based on antibody labeling experiments (Herzog *et al.*, manuscript in preparation) and computational analysis (Passmore *et al.*, 2005b) multiple TPR repeats have been proposed to form these ridges, and our result lends support to this. Our subunit localizations indicate that Cdc27 and Doc1 are direct neighbors within APC. This supports previous findings (Vodermaier *et al.*, 2003; Wendt *et al.*, 2001) and supports our crosslinking data (Section 2.1, discussed in Section 3.1). Strikingly, we have only observed one additional mass in the Cdc27-td2-tagged APC model indicating that there might be only one copy of Cdc27 present in budding yeast APC. This would be in disagreement with the studies by Passmore *et al.* and Ohi *et al.* who have proposed a relative Cdc27 copy number of 1.65 and 2, respectively (Ohi *et al.*, 2007; Passmore *et al.*, 2005b). We can not exclude the possibility that the tag of a second Cdc27 protein might be buried within APC. Our observed single mass is consistent with the stoichiometry reported for human APC, where Cdc27 is the only TPR subunit which is present only once per complex (Dube *et al.*, 2005). However, higher eukaryotes contain the TPR subunit Apc7 which is closely related to Cdc27 (Pal *et al.*, 2007a; Yu *et al.*, 1998) and has been reported to be present twice as well (Dube *et al.*, 2005). When having a close look at the human and yeast structures from the “back” view (Figure 2-11C), the yeast model appears to contain fewer ridge-like domains.

One might therefore speculate that this “compressed” appearance is due to fewer TPR repeats being present in budding yeast APC.

With more of these tagged APC versions currently being analyzed (Table 2-3) we are hoping to contribute to more precise insights into the 3D topology of APC subunits. In addition, the analysis of APC subcomplexes such as the “TPR subcomplex” and the “catalytic subcomplex” obtained from APC deletion strains (Thornton *et al.*, 2006) might be worthwhile. This might lead to the further identification or confirmation of subunit localizations and possibly to a higher resolution. Creating 3D models of APC purified from different cell cycle stages might tell us whether phosphorylation of APC changes its conformation. Purification of APC from a prometaphase-like stage with the MCC associated with APC has already led to new insights into the mechanism of APC inhibition by the APC (Herzog *et al.*, manuscript in preparation). Likewise, and with highest importance to the focus of this study, localization of an APC substrate might give important hints at the mechanism of substrate recognition, which would then have to be supported and explored further biochemically.

3.6 Apc16 is a novel subunit of human APC

We have identified the gene product of *c10orf104* as a novel component of human APC, which we therefore named Apc16. Apc16 was initially found as a protein that specifically associates with human APC in tandem affinity purifications followed by in solution digest/mass spectrometry analysis (Martina Sykora, Jim Hutchins, personal communication). We have confirmed by reciprocal immunoprecipitation that Apc16 and Cdc27 are part of one complex. Moreover, antibodies specific for Apc16 precipitated the complete APC (as determined by silver staining), which is active in ubiquitination assays upon Cdh1 activation, indicating that Apc16 is a component of active APC. The protein is remarkably conserved among higher eukaryotes with a sequence homology of 99 % (Section 5.3). Therefore, we speculate that Apc16 might also be associated with APC in other species including *Xenopus* and mouse, which we will address in the near future.

In earlier APC preparations obtained with a Cdc27 antibody followed by mass spectrometry analysis, Apc16 was also present, but due to the large number of other copurifying proteins this observation was not followed up (Franz Herzog, personal communication). We have not

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been able yet to detect Apc16 by silver staining. This explains why Apc16 has not been detected earlier despite innumerable SDS-PAGE/silver staining analyses which have been carried out in our lab in the past. The identification of Apc16 therefore underscores the power of tandem affinity purification protocols followed by in-solution mass spectrometry.

When looking at Apc16's protein sequence we were initially reminded of ubiquitin and ubiquitin-like proteins (UBL), because human Apc16 contains a C-terminal glycine. Furthermore, this could explain its high conservation among higher eukaryotes (Section 5.3). However, three sequences – all from fish – do not contain this C-terminal glycine, which contradicts such function, since this glycine is needed for the formation of both a thioester with E1 and E2 enzymes and an isopeptide bond with substrates (Pickart, 2001).

What could then be the function of Apc16? In our preliminary immunofluorescence analysis of Apc16-depleted cells we found a decreased mitotic index. Immunoblot analysis revealed decreased cyclin B1 levels. Furthermore, when cells were synchronized in S phase and then treated with taxol, which activates the SAC, Apc16 depleted cells did not accumulate in mitosis. Instead, they appeared to have problems in either progressing through S and G2 phases or in entering mitosis. Since S and G2 are the phases of the cell cycle where APC is not active, this is a surprising finding. One might therefore speculate that instead of being necessary for APC activity, Apc16 could be involved in keeping APC inactive. APC lacking Apc16 could thus be, at least partially, active and delay the ordered progression through S and G2 phases or the timely entry into mitosis by premature degradation of substrates including cyclin A and cyclin B. Such a role has recently been demonstrated for Emi1 in human cells, where it, apart from preventing rereplication, is required for accumulation of cyclin A and cyclin B (Di Fiore and Pines, 2007). A very speculative hypothesis is that Apc16 is required for Emi1 inhibition of APC in interphase. In further experiments we will first address where exactly cells are delayed by live cell imaging and immunofluorescence microscopy using appropriate S and G2 phase markers such as PCNA and aurora B. We will also carry out ubiquitination assays with Apc16-depleted APC to test whether Apc16 is required for APC activity. Furthermore, understanding the function of this novel APC subunit will also require the thorough parallel analysis of APC after depletion of other APC subunits since only few studies exist in which APC subunits were inactivated (Gimenez-Abian *et al.*, 2005; Wirth *et al.*, 2004).

4 Material and Methods

4.1 cDNA clones

The following cDNA clones were generated and/or used in this study.

- Doc1 in pME (this study)
- Doc1 in pET28 (this study)
- Doc1 plus 500 bp upstream of the translation start site in pRS316 (CEN/ARS, URA3)
- pME-Hsl1 (amino acids 667-882, this study)
- His₆-Cdh1 in pET28 (David Barford)
- His₆-Ubc4 in pET15b (David Barford)
- GST-Hsl1 (amino acids 667-872) in pGEX4T (Ivana Primorac)
- N70_{2x}-GST (*wild type* and D box mutant) in pET15b (Hiro Yamano)
- UbcH10 and UbcH10-His₆^{C114S} in pET28 (Michael Rape)
- His₆-Usp2-cc in pET15b (Ron Kopito)
- cyclin B (amino acids 1-87)-myc-His₆ in pTrcHis2A (Michael Gmachl)

For untagged constructs in pME vectors, pME34 (Carroll *et al.*, 2005) was used and the original STOP codon of the ORF was kept. *DOC1* was cloned into pME using the *EcoRI* and *BamHI* restriction sites and into pET28 using *NheI* and *BamHI*. To introduce *DOC1* including its endogenous promoter into pRS316, the *DOC1* ORF plus 500 bp upstream of the translation start were amplified from yeast genomic DNA and cloned into pRS316 via *EcoRI* sites. The obtained clones were then checked for the correct orientation. The *HSL1* fragment was cloned into pME via *XhoI* and *HindIII*.

Amber (TAG) and *ochre* (TAA) STOP codons as well as alanine and phenylalanine mutations were generated using the Quikchange method (Stratagene). For truncation of Doc1's C-terminus (Doc1ΔIR) the *ochre* STOP codon was introduced at Q235 which resulted in deletion of the last 16 amino acids.

4.2 Peptides and antibodies

For immunoblotting, antibodies were used at 2 µg/ml in 4 % milk-TBS/T unless indicated; glycine elutions were typically preferred over magnesium elutions.

Antibodies against yeast APC subunits: Peptides that were used for rabbit immunization and a basic characterization of the yeast APC and Hsl1 antibodies generated for this study can be found in the Appendix (Section 6). The following antibodies were used: Apc1 (1686), Apc2 (1688), Cdc27 (1518), Cdc16 (1514), Doc1 (1512 and 1513), Apc11 (1742), Hsl1 (1740). Unless indicated, the glycine elutions were used; the concentration was usually 2 µg/ml. The rabbit antibody against yeast Cdc26 (used 1:500) was a gift from David Morgan.

Antibodies against human APC subunits: Antibodies raised against human Apc6/Cdc16 (Grossberger *et al.*, 1999) as well as Apc2 and Apc3/Cdc27 (Gieffers *et al.*, 1999) have been described. The antibody against Apc10/Doc1, raised against recombinant protein, was a gift from Franz Herzog (Herzog *et al.*, manuscript in preparation). C10orf104/Apc16 antibodies raised against peptides 2184 (CLEADEWRFKPIEQLLGFTPSSG), 2185 (ASSSSSSAGGVSGSC) and 2186 (DLAPPRKALFTYPKGAGEMLEDGSEFLC) are described in the result section (Section 2.5).

Other non-commercial antibodies: Monoclonal antibodies against myc (9E10) and HA (12CA5) were made in house. The rabbit anti-BubR1 antibody was a gift from Gregor Kohlmaier.

Commercial antibodies: The goat polyclonal GST antibody was purchased from Amersham (27-4577-01), the mouse monoclonal cyclin B1 antibody from Santa Cruz Biotechnology (GNS1, sc-245), the mouse monoclonal ubiquitin antibody (P4D1) from Santa Cruz, the α tubulin antibody from Sigma (B-512), the mouse Rad21 antibody from Millipore (05-908), and an antibody to Histone H3 phosphorylated on serine 10 from Upstate Biotechnology (05-499). Alexa 488- and Alexa 568-labeled secondary antibodies as well as DAPI were from Molecular Probes (Invitrogen).

4.3 Generation of (tmd)phe-tRNA

4.3.1 Production of tRNA_{CUA}

The template for the transcription reaction was generated by PCR amplification from pTHG73 (Saks and Sampson, 1996) using primers 5'-GCGGTCCTACTGGGATT-3' and 5'-AATTCGTAATACGACTCACTATAG-3'. The PCR product was extracted with phenol pH 7.9 and chloroform followed by ethanol precipitation. Run off transcription reactions were performed using a T7-MEGAscript kit (Ambion). About 5 µg PCR product (dissolved in water) was used for a 100 µl transcription reaction. Transcriptions were typically performed in 500 µl reaction volumes (1 kit) and the total volume was split into 4 x 125 µl aliquots for incubations. Reactions were performed according the manufacturer's protocol. After 2 h incubation (300 rpm, 37°C) 10 µl of the provided DNase was added per tube and incubated for further 15 min. 437 µl nuclease-free water and 63 µl 3 M NaOAc was added per tube, samples were transferred to 2 ml Eppendorf tubes for extraction with phenol, phenol/chloroform and chloroform. (The phenol used had been adjusted to pH 4.5 with 250 mM NaOAc, pH 4.5). RNA transcripts were ethanol precipitated (15 to 20 min at -80°C), centrifuged (13000 rpm, 15 min, 4°C) and washed twice with 70 % ethanol. The pellet was dried and dissolved in 130 µl tRNA loading buffer (50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 50 mM NaCl) per tube. Dissolved transcripts were combined, filtered and the filter washed with 500 µl tRNA loading buffer. Filtrates were combined and loaded onto a 1 ml Resource Q column (GE Healthcare) equilibrated with tRNA loading buffer (flow rate 1 ml/ml). The column was washed with 5 volumes each tRNA loading buffer and tRNA washing buffer (50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 300 mM NaCl) before elution with tRNA elution buffer (50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 1 M NaCl). The collected fractions were subjected to ethanol precipitation. After washing with 70 % ethanol, pellets were dissolved in 100 µl water and the tRNA content was determined by measuring the A₂₆₀ with a NanoDrop. tRNA usually eluted in fractions C6 to C10. tRNA containing fractions were combined, aliquoted into 5 A₂₆₀/tubes, lyophilized and stored at -80°C until ligation. The average yield varied between 5 and 10 A₂₆₀.

4.3.2 Ligation of tRNA_{CUA} with (tmd)phe-pdCpA

The ligation buffer (55 mM Hepes-NaOH pH 8, 15 mM MgCl₂, 3.3 mM DTT, 0.2 µg/µl BSA, 0.8 mM ATP) was prepared freshly before each reaction. In a typical reaction, 10 A₂₆₀ of tmd(phe)-pdCpA were ligated to 10 A₂₆₀ of purified tRNA. 300 µl tRNA (dissolved in water), 100 µl tmd(phe)-pdCpA (in 10 % v/v acetonitril), 100 µl DMSO, 470 µl ligation buffer, 10 µl H₂O and 20 µl RNA ligase (5 U/µl, Epicentre) were mixed, aliquoted to 250 µl per tube and incubated for 90 min at 37°C and 300 rpm. The reaction as boosted with 20 µl enzyme/250 µl reaction after 30 min and 60 min. The ligation product was extracted with phenol (adjusted to pH 4.5 with 250 mM NaOAc, pH 4.5), phenol/chloroform and chloroform. For ethanol precipitation, the extracted product was mixed with 2.5 volumes ice-cold 100 % ethanol and 1/10 volume 3 M NaOAc, incubated for 25 min at -20°C, followed by 15 min centrifugation (13000 rpm, 4°C). The pellets containing ~2.5 A₂₆₀ N-pentenoyl-(tmd)phe-tRNA_{CUA} each were then washed once with ice-cold 70 % ethanol, dried and dissolved in 100 µl ddH₂O. For deprotection, 50 µl of deprotection solution (25 mM iodine in 50 % THF) was added to 100 µl of N-pentenoyl-(tmd)phe-tRNA_{CUA} and incubated at 25°C for 30 min. 150 µl ddH₂O, 30 µl 3 M NaOAc pH 5.2 and 750 µl ice-cold 100 % ethanol were added, incubated for 10 min at -20°C and centrifuged (13000 rpm, 15 min, 4°C). The pellet was washed twice with 1 ml ice-cold 70 % ethanol, dried and resuspended in 25 µl ddH₂O to a concentration of ~0.1 A₂₆₀/µl. (Tmd)phe was aliquoted, snap-frozen in liquid nitrogen and stored at -80°C.

4.4 Recombinant protein expression and purification

4.4.1 Protein production by *in vitro* transcription/translation

Doc1 (*wild type* and Doc1ΔIR) and N70_{2x}-GST (*wild type* and D box mutant) versions as well as Hsl1 (amino acids 667-872) were produced using the TNT T7 coupled reticulocyte lysate system (L4610, Promega). To translate “long” and “short” versions of Doc1 in this system, 8 µl of PCR products generated with primers 5'-ATCGATTAAATACGACTCACTATAGGGCTCGAGGCCGCCACCATGGGGCAAATAAGCGCCGTCTATAT-3' (forward primer “long”) or 5'-ATCGATTAAATACGACTCACTATAGGGCTCGAGGCCGCCACCATGGACCCGATTGGAATAAACAAAGT-3' (forward primer “short”) and 5'-GTGC-

TCTCGAGTTACATCATGGATCCTTAACGTAATATAGCATCCTGGAAGAATTGTT-3' (reverse primer for both) were used directly, without further purification as templates in 25 µl IVT reactions.

For photocrosslinker incorporation into Doc1 or N70_{2x}-GST, the same system was used and reactions were supplemented with 1 mM MgOAc and 1.6 µl (tmd)phe-tRNA per 50 µl reaction. Alternatively, N70_{2x}-GST versions with and without a photocrosslinker were produced with the *E. coli* T7 S30 extract system (L1130, Promega). Yeast Cdh1 was obtained using the TNT Quick coupled reticulocyte lysate system (L1170, Promega). Except when using a PCR generated template as described above, 1 µg of circular plasmid DNA was used as template in a 50 µl reaction. Plasmid DNA was purified using either the QIAGEN plasmid kit or the QIAquick spin mini prep kit followed by ethanol precipitation.

4.4.2 Expression using the baculovirus-insect cell system

The yeast His₆-Cdh1 baculovirus (kindly provided by Topher Carroll and David Morgan) was used for infection of Sf9 cells. One ml virus (4th amplification) was used to infect a 175 cm² filter capped tissue culture flask (Nunc) of 80 % confluent monolayer cells. Forty -48 h after infection, cells were harvested by tapping the culture flask (no scraping!) and washing off remaining cells with a small volume of PBS. Cells were centrifuged in 50 ml Falcon tubes (500 g, 4°C) and pellets were neither washed nor frozen but directly resuspended in 500 µl lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM KCl, 0.1 % Tween 20 and 10 µg/ml each aprotinin, pepstatin, and leupeptin). Cells were lysed on ice by douncing 4 x 5 times with 5 min breaks in between with a glass homogenizer. The soluble protein fraction was recovered by centrifugation (13000 rpm, 15 min, 4°C) in an Eppendorf table top centrifuge. The supernatant was incubated with 20-40 µl Ni²⁺-NTA agarose beads (Qiagen) per harvested flask (pre-equilibrated with water and lysis buffer) for 60-90 min at 4°C on an end-over-end rotator. Sample were washed 5 times with wash buffer containing 50 mM Tris-HCl pH 7.5, 300 mM KCl, 0.1 % Tween 20 (5 min incubation on rotator per washing step) and eluted using 2 times 1 bead volume 50 mM Tris-HCl pH 7.5, 300 mM KCl, 250 mM imidazole. Eluates were dialyzed against 1 liter buffer containing 50 mM Tris-HCl pH 7.5, 100 mM KCl, 10 % glycerol and 1 mM DTT for 3 h at room temperature. Cdh1 purifications only resulted in small amounts of protein; the average yield was about 15-20 µg from ten 175 cm² flasks.

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4.4.3 Expression in *E. coli*

Purified human cyclin B (amino acids 1-87) was a gift from Claudine Kraft, His₆-E1 from Michael Gmachl, *S. cerevisiae* Ubc4 from Hartmut Vodermaier, human Ubc4 from Claudine Kraft and N70_{2x}-GST (*wild type* and D box mutant) from Ivana Primorac.

4.4.3.1 *S. cerevisiae* His₆-Doc1

One liter LB medium supplemented with 25 µg/ml kanamycin was inoculated with 20 ml of a fresh over night culture and grown further until an OD₆₀₀ of 0.6 was reached. Protein expression was induced by addition of 1 mM IPTG and expression was allowed for approximately 20 h at 18°C. Cells were harvested and pellets frozen until further use. Cell pellets were resuspended in 15 ml lysis buffer (50 mM Tris-HCl pH 8, 150 mM KCl, 1 % Triton, 2 mM β-mercaptoethanol) supplemented with 100 µg/ml DNase, ½ tablet Complete EDTA-free (Roche) and 15 mg lysozyme. After 15 min incubation on ice, the cell suspension was sonicated and cell lysate were cleared by centrifugation (18000 rpm, 4°C, SS34 rotor, Sorvall RC 5C PLUS centrifuge, Kendro). The supernatant was added to 500 µl Ni²⁺-NTA agarose beads (Qiagen) that had been prepared by washing with water and lysis buffer. Following an 1 h incubation on an end-over-end rotator at 4°C, bead-bound proteins were washed twice with wash buffer WB1 (50 mM Tris-HCl pH 7.5, 300 mM KCl, 0.5 % Triton, 2 mM β-mercaptoethanol, 20 mM imidazole), twice with WB2 (50 mM Tris-HCl pH 7.5, 150 mM KCl, 0.5 % Triton, 2 mM β-mercaptoethanol) and once with WB3 (50 mM Tris-HCl pH 7.5, 150 mM KCl, 2 mM β-mercaptoethanol). Washing steps were carried out for 5 min at 4°C. For each of the 2 elution steps beads mixed with 500 µl WB3 containing 250 mM imidazol and incubated for 15 min at 4°C. Eluates were dialyzed in slide-a-lyzer dialysis cups (Molecular Weight cut off 10 KDa, Pierce) against buffer containing 50 mM Tris-HCl pH 7.5, 150 mM KCl, 10 % glycerol and 0.5 mM DTT for 12 h including one buffer change after 3 h. Eluates were pooled, filtered using Ultrafree- MC Durapore centrifugal filter units (0.22 µm, Millipore) and subjected to gel filtration chromatography as a second purification step. The eluate was loaded onto a 24 ml Superdex 75 HR 10/30 column (GE Healthcare) at a flow rate of 0.5 ml/min. The injection loop was emptied and fractions of 500 µl each eluted with buffer containing 50 mM Tris-HCl pH 7.5, 150 mM

KCl, 10 % glycerol. Peak fractions were pooled and concentrated using Vivaspin cartridges (Sartorius).

4.4.3.2 GST-Hsl1⁽⁶⁶⁷⁻⁸⁷²⁾

An Hsl1 fragment comprising amino acids 667-872 fused to an N-terminal GST-tag (GST-Hsl1⁽⁶⁶⁷⁻⁸⁷²⁾) was expressed and purified from *E. coli*. One liter *E. coli* BL21(DE3)RP culture inoculated from 20 ml of a fresh overnight culture was grown to an OD₆₀₀ of 0.8. Protein expression was induced by the addition of 0.5 mM IPTG and cells were harvested after 2 h. The cell pellet was resuspended in 15 ml lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM KCl, 0.01 % Triton X-100, 10 % glycerol, 1 mM β -mercaptoethanol) containing ½ tablet Complete EDTA-free (Roche) and 15 mg lysozyme, followed by sonication. The soluble protein fraction was recovered by centrifugation at (18000 rpm, SS34 rotor). The supernatant was added to 500 μ l glutathione sepharose (GE Healthcare) that had been washed with lysis buffer and incubated at 4°C for 1 h on an end-over-end rotator. The beads were washed once with wash buffer 150 (50 mM Tris pH 7.5 150 mM KCl, 0.01 % Tween 20), 3 times with wash buffer 300 (50 mM Tris pH 7.5 300 mM KCl, 0.05 % Tween 20) and once with wash buffer 150 again, each time incubating for 5 minutes on an end-over-end rotator at 4°C. The bound protein was then eluted 3 times with 700 μ l elution buffer (50 mM Tris-HCl pH 8, 150 mM KCl, 0.01 % Tween 20, 0.5 mM β -mercaptoethanol, 50 mM reduced glutathione), each time rotating for 15 min at 4°C. Dialysis was performed against 2 x 1 liter dialysis buffer (20 mM Tris pH 7.5, 150 mM KCl, 10 % glycerole, 0.5 mM β -mercaptoethanol) for 3 h and over night, and eluates were stored in aliquots at -80°C. The yield was about 2.5 mg full-length GST-Hsl1⁽⁶⁶⁷⁻⁸⁷²⁾ from 1 liter culture. It has to be noted that a strong second band was obtained in these preparations which represented GST protein only (as determined by immunoblotting, not shown). About 30-50 % of GST-Hsl1⁽⁶⁶⁷⁻⁸⁷²⁾ was repeatedly degraded in these purifications, also when the expression was performed for 18 h at 18°C.

4.4.3.3 His₆-UbcH10

C-terminally His₆-tagged UbcH10 and UbcH10-His^{C114S} were expressed using the *E. coli* BL21(DE3)RIL strain as recommended in (Rape *et al.*, 2006). One liter of LB-amp medium was inoculated from 20 ml of a fresh overnight culture and grown further to an OD₆₀₀ of 0.8,

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followed by induction of protein expression by adding 0.4 mM IPTG. Cells were harvested after 3 h expression at 37°C and purified essentially as described for UbCH5b in (Kraft *et al.*, 2006) with few changes. The cell pellet was resuspended in 15 ml lysis buffer (50 ml Tris-HCl pH 8, 150 mM KCl, 2 mM β -mercaptoethanol, and 1 % Triton X-100) containing $\frac{1}{2}$ tablet Complete EDTA-free (Roche) and 15 mg lysozyme. Following sonication, lysate were cleared by centrifugation (18000 rpm, 4°C, SS34 rotor) and bound to 800 μ l Ni²⁺-NTA agarose beads (Qiagen) that had been washed with water and lysis buffer beforehand. After 1 h incubation on an end-over-end rotator at 4°C, beads were washed 5 times with WB1 containing 50 ml Tris-HCl pH 8, 500 mM KCl, 2 mM β -mercaptoethanol, and 0.5 % Triton X-100 followed by two washes with WB2 containing 50 ml Tris-HCl pH 8, 150 mM KCl, and 2 mM β -mercaptoethanol. Each washing step included 5-10 min incubation at 4°C. To elute the bound proteins, beads were incubated with 3 x 700 μ l WB 2 containing 250 mM imidazol for 15 min each on an end-over-end rotator. About 30 mg protein was obtained from a 1 liter culture.

4.4.3.4 His₆-Usp2-cc

Usp2-cc comprises amino acids 2-45 of mouse Usp2 (Catanzariti *et al.*, 2004). N-terminally His₆-tagged Usp2-cc was purified following protocol slightly modified from the one described in (Catanzariti *et al.*, 2004). One liter LB-amp medium was inoculated 1:50 with a fresh overnight culture of *E. coli* strain BL21(DE3). At an OD₆₀₀ of 0.8, protein expression was induced by adding 0.4 mM IPTG, with a further 4 h growth. After harvesting cells, pellets were resuspended in 15 ml buffer 2A (50 mM Na₂HPO₄/NaH₂PO₄ pH 7.2, 300 mM NaCl, 20 mM imidazole, 5 mM β -mercaptoethanol, 30 % glycerol) containing 1 mM PMSF, $\frac{1}{2}$ tablet Complete EDTA-free (Roche) and 15 mg lysozyme. Cells were broken by sonication and the lysates cleared by centrifugation (18000 rpm, SS34 rotor, 4°C). 500 μ l of Ni²⁺-NTA agarose beads (Qiagen) were prepared by washing with water and buffer 2A and then incubated with the cleared lysates for 1 h at 4°C on an end-over-end rotator. Bead-bound proteins were washed five times using buffer 2A with 10 min incubation time each. Elution was performed using 3 x 700 μ l elution buffer (buffer 2A containing 250 mM imidazole) by incubating samples for 15 min each time at 4°C on the rotator. Eluates were dialyzed in 2 x 1 liter dialysis buffer (50 mM Na₂HPO₄/NaH₂PO₄ pH 7.2, 150 mM KCl, 10

% glycerol, 1 mM DTT) for 3 h and over night, respectively. This procedure yielded about 16 mg protein.

4.5 Oxidative iodination of proteins

Protein was labeled using the chloramines T procedure according to Parker, as described in (Kraft *et al.*, 2006). In a 21 μ l reaction, 3 μ l of protein (3 μ g/ μ l) was labeled. After dialysis and addition of glycerol, a protein solution of about 60 μ l was obtained, 1 μ l of which was generally used per ubiquitination reaction.

4.6 Yeast methods

The genotypes of all yeast strains used in this study are provided in Table 4–1. Deletion and tagging of genes was performed following the one-step PCR method (Knop *et al.*, 1999; Puig *et al.*, 2001; Rigaut *et al.*, 1999; Wach *et al.*, 1994). For tagging of APC subunits with td2, pKT146 (pFA6a-link-tdimer2-SpHIS5, (Sheff and Thorn, 2004)) was used. Transformations with PCR products derived from this construct were performed using the following protocol (kindly provided by Irina Häcker, MPI-BPC Göttingen). An overnight starter culture was diluted to an OD₆₀₀ of about 0.2 in 50 ml YEP medium containing 2 % glucose. When the OD reached about 0.8 to 1/ml, cells were harvested by centrifugation in 50 ml Falcon tubes (20 min, 2500g, 4°C) and washed with 50 ml 10 mM Tris-HCl pH 7.5. The pellet was resuspended in 25 ml Li-T (10 mM Tris-HCl pH 7.5, 100 mM lithium acetate) containing 10 mM DTT. This suspension was incubated at room temperature for 40 min with gentle shaking, then centrifuged and resuspended in 750 μ l Li-T plus 10 mM DTT.

Transformation reactions containing 50 μ l Li-T, 50 μ l salmon sperm DNA, 20 to 50 μ g PCR fragment, and 100 μ l of cell suspension in Eppendorf tubes were incubated at room temperature for 10 min with gentle shaking. 300 μ l freshly made sterilized PEG solution (2 g PEG 4000 in 2 ml Li-T) and 50 μ l DMSO were added and the mixture was incubated for

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Table 4-1: Yeast strains used in this study. All strains used in this study are derivatives of K699 (*MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1, GALpsi*) and K700 (*MATalpha ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1, GALpsi*) with the exception of the strains used for spotting experiments (Figure 2-9C), which are derivatives of S288C (*hi3D1 leu2D0 met15D0 ura3D0 doc1::KanMX*). *Kl* denotes the *TRP1* gene from *Kluyveromyces lactis*, *Sp* the *HIS5* gene from *Schizosaccharomyces pombe*.

Strain	Relevant genotype	Creator
J9	<i>MATa APC4-TAP::KlTRP1</i>	H. Vodermaier
J42	<i>MATa APC4-TAP::KlTRP1 APC5-myc9::KlTRP1</i>	H. Vodermaier
J61	<i>MATa CDC27-myc9::TRP1</i>	Marta Galova
J109	<i>MATa APC1-ha3::HIS3</i>	(Zachariae <i>et al.</i> , 1996)
J110	<i>MATalpha APC1-myc6::HIS3</i>	(Zachariae <i>et al.</i> , 1996)
J118	<i>MATa APC1-myc18::HIS3</i>	W. Zachariae
J180	<i>MATa APC4-TAP::KlTRP1 doc1::KanMX</i>	this study
J182	<i>MATa APC4-TAP::KlTRP1 APC1-myc18::HIS3 doc1::KanMX</i>	this study
J187	<i>MATa APC4-TAP::KlTRP1 APC5-myc9::KlTRP1 doc1::KanMX</i>	this study
J189	<i>MATa APC4-TAP::KlTRP1 APC2-myc9::TRP1 doc1::KanMX</i>	this study
J203	K699	
J220	<i>MATa doc1::KanMX</i>	this study
J235	<i>MATa APC4-TAP::TRP1 CDC16-myc6::URA3 doc1::KanMX</i>	this study
J266	<i>MATa APC4-TAP::KlTRP1 CDC27-myc9::TRP1 doc1::KanMX</i>	this study
J315	<i>MATalpha CDC16TAP::KlTRP1 pep4::URA3</i>	(Schwickart <i>et al.</i> , 2004)
J319	<i>MATalpha APC4-TAP::KlTRP1 pep4::URA3</i>	this study
J320	<i>MATa CDC16TAP::KlTRP1 doc1::KanMX pep4::URA3</i>	this study
J323	<i>MATa APC4-TAP::KlTRP1 doc1::KanMX pep4::URA3</i>	this study
J325	<i>MATa/alpha APC1-ha3::HIS3/ APC1-myc6::HIS3</i>	this study
J326	<i>MATa APC1-ha3::HIS3 doc1::KanMX</i>	this study
J327	<i>MATalpha APC1-myc6::HIS3 doc1::KanMX</i>	this study
J328	<i>MATa/alpha APC1-ha3::HIS3/ APC1-myc6::HIS3 doc1::KanMX</i>	this study
J329	<i>MATa APC4-TAP::KlTRP1 APC1-tmono::SpHIS5 pep4::URA3</i>	this study
J347	<i>MATalpha CDC16TAP::KlTRP1 pep4::URA3 APC4-tdimer2::SpHIS5</i>	this study
J376	<i>MATalpha CDC16TAP::KlTRP1 CDC27-tdimer2::spHIS5 pep4::URA3</i>	this study
J378	<i>MATa APC4-TAP::KlTRP1 CDC16-tdimer2::spHIS5 pep4::URA3</i>	this study
J380	<i>MATalpha CDC16-TAP::KlTRP1 APC5-tdimer2::SpHIS5 pep4::URA3</i>	this study
J406	<i>MATa CDC16TAP::KlTRP1 pep4::URA3 CDC26-tdimer2::SpHIS5</i>	this study
J407	<i>MATa CDC16TAP::KlTRP1 pep4::URA3 MND2-tdimer2::SpHIS5</i>	this study
J408	<i>MATa CDC16TAP::KlTRP1 pep4::URA3 APC2-tdimer2::SpHIS5</i>	this study
J422	<i>MATa CDC16-TAP::KlTRP1 pep4::URA3 APC11-tdimer2::SpHIS5</i>	this study
J428	<i>MATa CDC16TAP::KlTRP1, pep4::URA3 SWM1-tdimer2::spHIS5</i>	this study

further 10 min with gentle shaking, followed by a 15 min heat shock at 42°C. The cell suspension was briefly centrifuged and the pellet was resuspended in 1 ml YEP-glucose medium. Cells were incubated for 60 min at 30°C and 300 rpm. For plating on selective medium, cells were briefly centrifuged again to remove YEP-glucose, and the cell pellets were resuspended in 100 µl of 10 mM Tris-HCl pH 7.5.

4.7 Yeast cultures and extract preparation

Yeast strains were grown in YEP medium containing 2 % glucose. For APC purifications, an overnight culture was usually diluted to an OD₆₀₀ of 0.2 to 0.3, incubated for further 4 to 5 h until the OD₆₀₀ reached 1-1.5/ml, and then harvested by centrifugation. The cell suspension was aliquoted and cells were stored as 200 µl pellets in Eppendorf tubes or in Falcon tubes. “Large-scale” cultures were grown in a self-made “fermenter” (constructed by the IMP workshop) that is composed of 2 glass beakers of 10 liters each placed in a water-filled basin and a tubing system providing compressed air, which ensured both aeration and agitation (for a description see PhD thesis of Mark Petronczki, IMP). Two or 4 of these 10 liter cultures were harvested using a continuous flow centrifuge (Modell LE, CEPA, 30000 rpm, 4°C), washed 3 times with cold water, aliquoted and stored as pellets in Falcon tubes. When growing cells for electron microscopy purposes, cells were washed twice with water, once in EM lysis buffer (20 mM Hepes-KOH pH 8, 200 mM KCl, 10 % glycerol, 1.5 mM MgCl₂) and then resuspended in one pellet volume of EM lysis buffer supplemented with 0.1 M PMSF and 1 tablet Complete EDTA-free (Roche) per 15 ml buffer. The cell suspension was then dripped directly into liquid nitrogen using a 5 ml glass pipette. This resulted in small yeast beads of about 2 mm diameter, which were stored at -80°C until further use. Harvesting cells from 40 liters of culture at an OD₆₀₀ of around 1.5 usually resulted in about 80 to 100 g of solid wet pellet or six 50 ml Falcons filled with yeast beads.

Frozen yeast pellets were thawed at room temperature, resuspended in 1-1.5 volumes of the appropriate lysis buffer containing protease inhibitors and DNase I (10 µg/ml) and passed through a French Press (Constant Systems LTD, version V4-35-9/96; 2 runs at 2 kpsi). The minimum pellet volume for this procedure was 5 ml. Alternatively, if lysis was to be performed at a smaller scale, acid washed glass-beads (425 µm to 600 µm, Sigma) were added to the cell suspension and cells were broken by vortexing 4 times or 2-3 minutes with 5 min

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on ice in between. Lysates were cleared by centrifugation (19000 rpm, 30 min, 4°C, SS34 rotor or 13000 rpm, 30 min, 4°C, Eppendorf tabletop centrifuge). Yeast beads were ground in a mortar grinder (RM100, Retsch) while constantly adding liquid nitrogen for 12 min. Ground cell powder was thawed in a water bath at 28°C followed by 2 centrifugation steps. Cell extracts were pre-cleared by centrifugation in an SS34 rotor (19000 rpm, 30 min, 4°C), followed by ultra-centrifugation (37000 rpm, 60 min in T865 or 42000 rpm, 50 min in T647.5, both at 4°C).

4.8 Yeast growth assay

For this assay, *doc1Δ* yeast strains were used that had been transformed with pRS316 (*URA3*) plasmids containing *DOC1* versions under the endogenous promoter. As a control, a strain transformed with the empty plasmid was used. Two ml yeast cultures were inoculated from a single colony and grown until late log phase in synthetic medium lacking uracil. Cultures were then diluted to an OD₆₀₀ of 0.02 and grown for another 20 h. After measuring the OD₆₀₀ of the log phase cultures, they were diluted to similar densities. A dilution series was made by diluting cultures 3 times 10-fold. 3.5 µl of each dilution was plated onto solid drop-out medium and incubated at 25°C and 37°C for 1-2 days.

4.9 Cell culture, cell synchronization and RNA interference

Adherent HeLa cells were grown in 145 mm tissue culture dishes (Greiner) at 37°C in the presence of 5 % CO₂ in Dulbecco's Modified Eagle Medium (DMEM, made in house) supplemented with 10 % fetal bovine serum (PAA Laboratories), 0.3 µg/ml L-glutamine (Sigma-Aldrich), 100 U/ml penicillin (Sigma-Aldrich) and 100 µg/ml streptomycin (Sigma-Aldrich). To arrest cells in mitosis by activating the SAC, logarithmically proliferating cells (8x10⁶ cells per plate) were treated with 10 µM taxol (Paclitaxel, Sigma-Aldrich) for 16 h followed by a 40 min-treatment with 10 µM proteasome inhibitor (MG132, Sigma-Aldrich). To then inactivate the SAC, 100 nM hesperadin (Boehringer Ingelheim) was added for 60 min. Cells were harvested by a harsh mitotic shake-off, washed twice with PBS and frozen as pellets. Alternatively, cells were arrested in mitosis by the addition of 100 ng/ml nocodazole for 18 h. S phase arrest was induced by treating cells for 18 h with 2 mM hydroxyurea or by

treating cells for 24 h with 2 mM thymidine. For double thymidine arrest release, HeLa cells were arrested at 50 % confluency by the addition of 2 mM thymidine (Sigma-Aldrich). After 16 h, cells were released by washing once with PBS and addition of fresh medium. Eight h later, the second arrest was induced by the addition of 2 mM thymidine. Cells were released from the second arrest after further 16 h as before and samples were collected at the indicated time points. For RNAi experiments, cells were transfected as previously described (Hirota *et al.*, 2004) using preannealed siRNA oligos targeting c10orf104 from Ambion. The two oligo sequences were 5'-CGCUUAAACAGGUGAAACAtt-3' (117) and 5'-GCUUACAUAAGCCAUCCAGAtt-3' (118).

4.10 Protein purification and fractionation

4.10.1 Yeast APC purification using TAP-method

Table 4-2: Buffers for TAP-purification. The protocol was modified from (Passmore *et al.*, 2005a; Rigaut *et al.*, 1999).

buffer	component
Lysis buffer	50 mM Tris-HCl pH 8.0, 150 mM KCl, 10 % glycerol
TST	50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05 % Tween-20
IgG wash buffer	50 mM Tris-HCl pH 8.0, 150 mM KCl, 10 % glycerol, 0.5 mM EDTA, 0.1 % NP-40
CaM binding buffer	10 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 % glycerol, 10 mM β -mercaptoethanol, 1 mM MgOAc, 2 mM CaCl ₂ , 0.1 % NP-40
CaM elution buffer	10 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 % glycerol, 0.5 mM EDTA, 10 mM β -mercaptoethanol, 1 mM MgOAc, 3 mM EGTA, 0.1 % NP-40
EM lysis buffer	20 mM HEPES-KOH pH 8.0, 200 mM KCl, 10 % glycerol, 1.5 mM MgCl ₂
EM IgG wash buffer	50 mM Tris-HCl pH 8.0, 150 mM KCl, 10 % glycerol, 0.5 mM EDTA, 0.1 % NP-40, 0.03 % octyl- β -D-glucopyranoside (Sigma)
EM CaM binding buffer	10 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 % glycerol, 10 mM β -mercaptoethanol, 1 mM MgOAc, 1 mM imidazole, 2 mM CaCl ₂ , 0.03 % octyl- β -D-glucopyranoside
EM CaM elution buffer	10 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 % glycerol, 10 mM β -mercaptoethanol, 1 mM MgOAc, 1 mM imidazole, 2 mM CaCl ₂ , 3 mM EGTA, 0.03 % octyl- β -D-glucopyranoside

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Yeast extracts were supplemented with 0.5 mM EDTA and 0.1 % NP-40. 100 μ l IgG sepharose 6 Fast Flow (bed volume, Amersham) per 10 g yeast pellet was packed into 0.8 x 4 cm poly prep columns (Biorad) via gravity flow. IgG sepharose was washed with 5 bed volumes of TST, followed by 3 volumes each of 0.5 M acetic acid pH 3.4 (adjusted with ammonium acetate), TST and acetic acid. Beads were then washed with TST until the pH was neutral again, followed by equilibration with 10 ml IgG wash buffer supplemented with 2 mM EGTA. Yeast extract was added and the sealed column was rotated for 90 min at 4°C. The column was then washed by gravity flow with 20 ml IgG wash buffer containing 2 mM EGTA and ½ tablet Complete EDTA-free (Roche), followed by a wash with 15 ml IgG wash buffer containing 1 mM DTT. One ml of the latter buffer and TEV protease (100 units AcTEV, Invitrogen) was added and the sealed column was rotated for 90 to 120 min at room temperature. Eighty μ l calmodulin sepharose 4B (Amersham) was packed into a similar poly prep column and washed with 10 ml water and 20 ml CaM binding buffer. The column was sealed at the bottom, placed underneath the IgG sepharose column and the TEV-eluate was dripped directly into the new column. IgG sepharose beads were washed with 3 ml CaM binding buffer, again directly into the CaM column, and the final CaCl_2 concentration of the eluate was adjusted to 5 mM. The sealed column was rotated for 60 min at 4°C, followed by extensive washes with 40-70 ml CaM binding buffer. APC was eluted by addition of 80 μ l CaM elution buffer. Up to 8 fractions were collected, leaving 3-5 min incubation time in between 2 fractions. Apart from the TEV cleavage, all steps were carried out in a cold room and with chilled buffers.

For electron microscopy purposes, extracts prepared by mortar grinding were used. Buffers were slightly modified (see Table 4-2) and the first purification step was carried out in 50 ml Falcon tubes. For a typical purification, cells from 20 liters culture were used with 300 μ l IgG sepharose, 200 μ l calmodulin sepharose, 400 units of TEV protease, and 180 μ l elution buffer per fraction. The first eluted fraction was discarded and fractions 2 to 4 were “blindly” combined and loaded onto glycerol-glutaraldehyde gradients.

4.10.2 APC purification with IgG Sepharose or APC-specific antibodies

Cleared extracts from yeast strains carrying a TAP-tag on Apc4 or Cdc16 were bound to IgG sepharose (Amersham) by incubating for 60-90 min at 4°C. After washing with IgG wash

buffer (50 mM Tris-HCl pH 8, 150 mM KCl, 10 % glycerol, 0.5 mM EDTA, 0.1 % NP40), bead-bound APC was eluted by boiling in SDS sample buffer or by TEV cleavage (10 units AcTEV per 10 μ l beads). Alternatively, bead-bound APC was directly used in activity or crosslinking assays. Immunoprecipitation of APC was carried out with antibodies raised against APC subunits or with myc (9E10) antibodies. Antibodies were used either after pre-incubation with protein A beads (BioRad, in case of APC antibodies) and GammaBind G sepharose (GE Healthcare, for 9E10), or they were crosslinked using dimethylpimelimidate (DMP) as described (Harlow, 1988). About 3 mg yeast cell extract was used per 10 μ l antibody beads and incubated on an end-over-end rotator at 4°C for up to 90 min, followed by washes with TBS/T (TBS containing 0.01 % Tween 20). Elution was carried out using 1-2 bead volumes of 100 mM glycine pH 2.2 or by boiling beads in SDS sample buffer.

4.10.3 Co-immunoprecipitation

12CA5 and 9E10 antibodies were bound to protein A sepharose (Biorad) and GammaBind G sepharose (GE Healthcare) beads, respectively. Extracts of strains J109, J110 and J325 were prepared in buffer (50 mM Tris pH 7.5, 50 mM NaCl, 10 % glycerol, 0.2 % Triton X-100, 1 mM DTT) containing protease inhibitors. 10 μ l beads each were incubated with cleared lysates containing 2 mg protein for 90 min at 4°C on an end-over-end rotator. Beads were washed extensively with buffer, and bound proteins were eluted with 15 μ l 100 mM glycine pH 2.2. Samples were analyzed by immunoblotting using 9E10 and 12CA5 antibodies.

4.10.4 Purification of human APC

HeLa cells were thawed on ice and resuspended in 0.75 to 1 pellet volume lysis buffer (20mM Tris pH7.5, 150mM NaCl, 5 % glycerol, 2 mM EDTA, 0.05 % Tween 20) containing protease inhibitors (0.1 M PMSF, 20 μ g/ml each aprotinin, pepstatin, and leupeptin) and 4 μ g/ml okadaic acid (Alexis). The cell suspension was passed several times through syringe needles (0.8 mm and 0.4 mm in diameter) and the extract was cleared by centrifugation (13000 rpm, 15 minutes, 4°C). APC was isolated with antibodies against Cdc27 or C10orf104/Apc16. Antibodies were coupled to protein A beads (BioRad) and crosslinked (if possible) using DMP as described (Harlow, 1988). About 2-3 mg HeLa cell extract was used

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per 10 μ l antibody beads and incubated on an end-over-end rotator at 4°C for 60-90 min, followed by washes with TBS/T. Elution was carried out using 1-2 bead volumes of 100 mM glycine pH 2.2 or by boiling beads in SDS sample buffer. Alternatively, bead-bound APC was directly used in binding or activity assays.

4.10.5 Sucrose density gradient centrifugation

Sucrose density gradients were prepared in disposable ultra-clear centrifuge tubes (19 x 95 mm, Beckman) by mixing 2 sucrose solutions using a GradientMaster (Biocomp). Cell extracts were pre-cleared in an Eppendorf table-top centrifuge (13000 rpm, 15 min, 4°C) and then centrifuged at 42000 rpm (TLA45 rotor) for 15 min in an Optima MAX ultracentrifuge (Beckman Coulter). Cleared HeLa cell extract (2.5 mg protein) was layered on 10-30 % sucrose gradients in TBS/T. Centrifugation was carried out at 34000 rpm for 18 h at 4°C in a Beckman SW40 rotor in a Beckman Optima MAX ultracentrifuge (Beckman Coulter). Gradients were fractionated into 400 μ l aliquots using an ISCO fractionator at a flow rate of 1 ml/min.

4.10.6 Glycerol glutaraldehyde gradient centrifugation

Gradients were prepared in Beckman polyalomer centrifuge tubes (11 x 60 mm) by mixing a 10 % glycerol solution containing 0.05 % glutaraldehyde (25 %, EM grade, Polysciences) and a 40 % glycerol solution containing 2 % glutaraldehyde using a GradientMaster. Both glycerol solutions were prepared in gradient buffer (20 mM Hepes-KOH pH 7.9, 150 mM NaCl, 2 mM MgCl₂, 0.05 % octyl- β -D-glucopyranoside). 500 μ l from the top of the gradient were replaced by a 10 % glycerol cushion that did not contain glutaraldehyde. 500 μ l TAP-purified APC was carefully layered on the gradient at 4°C and tubes were placed into a Beckman SW60Ti rotor. Centrifugation was performed at 37000 rpm for 14 h at 4°C. The centrifuge was set to “9” for both acceleration and deceleration. Gradients were fractionated in 130 μ l aliquots (5 drops) using a home-made apparatus with a peristaltic pump. APC was detected by online measurement of the absorbance at 280 nm.

4.11 APC assays

4.11.1 Doc1 binding assay

TAP-tagged APC (from strain J187, J220 served as a control) was purified by rotating 20 μ l IgG sepharose beads (Amersham) with yeast lysates containing 5 mg protein for 90 min at 4°C on an end-over-end rotator. After washing with IgG wash buffer (50 mM Tris-HCl pH 8, 150 mM KCl, 10 % glycerol, 0.5 mM EDTA, 0.1 % NP40) supplemented with 1 mM DTT, 30 μ l Doc1 IVT was added and incubated for the indicated times at room temperature. Beads were washed extensively with the same wash buffer and APC was eluted from beads by TEV cleavage (20 units AcTEV (Invitrogen), 90 min, room temperature). Eluates ($\frac{1}{2}$ of the sample) as well as Doc1 input were analyzed by SDS-PAGE followed by phosphorimaging, and by immunoblotting using myc and Doc1 antibodies.

4.11.2 N70_{2x}-GST binding assay

The assay was modified from a protocol described in (Eytan *et al.*, 2006). APC was isolated from extracts of mitotically arrested HeLa cells (“SAC off”) using a Cdc27 antibody bound to protein A beads (Biorad). Bead-bound APC was washed with TBS/TG (TBS containing 0.01 % Tween 20 and 2 % glycerol) and 15 μ l beads were incubated for 60 min at room temperature with 45 μ l of buffer A (50 mM Tris pH 7.5, 10 % glycerol, 1 mM DTT) containing 4 mg/ml BSA and 70 nM purified N70_{2x}-GST- versions, or with 45 μ l or 60 μ l *in vitro* translated N70_{2x}-GST-versions diluted 1:5 in buffer A. Samples were washed 3 times with buffer B (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5 % Tween 20) and bound proteins were eluted with 20 μ l 100 mM glycine pH 2.2. Samples were analyzed by immunoblotting using Apc2 and GST antibodies and by phosphorimaging.

4.11.3 Ubiquitination assay using yeast APC

Ubiquitination assays were performed in 10-14 μ l reaction volumes with 40 mM Tris pH 7.5, 10 mM MgCl₂, 2.7 mM ATP, 7.5 μ g of ubiquitin (Sigma or Affiniti), 0.2 μ g of ubiquitin aldehyde, 4 mM N-acetyl-Leu-Leu-Norleu-aldehyde (LLnL, Sigma), 0.5 μ g of His₆-E1, 1 μ g of His₆-Ubc4, 2 μ l of Cdh1 (recombinant at $\sim$ 0.1 μ g/ μ l, or *in vitro* translated), 1-3 μ l of

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radiolabeled substrate, and 4 µl of purified APC (about 15 ng). Samples were incubated in a thermomixer for up to 90 min at 25°C and 800 rpm. Reactions were stopped with SDS sample buffer, and mixtures were analyzed by SDS-PAGE and autoradiography. Alternatively, when a purified unlabeled protein was used as a substrate, samples were analyzed by immunoblotting.

4.11.4 Ubiquitination assay using human APC

Three -5 µl APC bound to antibody beads were incubated in 7-10 µl XB buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.02 % Tween 20) containing 10 µg ubiquitin, ATP regenerating system (7.5 mM creatine phosphate, 1 mM ATP, 1 mM MgCl₂, 0.1 mM EGTA, 30 U/ml rabbit creatine phosphokinase typeI (Sigma)), 0.25 µg His₆-E1, 1 µg of E2 (His₆-UbcH10 or a mixture of His₆-UbcH10 and His₆-Ubc4), and 0.2 µg purified Cdh1. An iodinated fragment of human cyclin B (amino acids 1-84, 3 µg) was used as a substrate. Reactions were incubated in a thermomixer (1400 rpm, 37°C) for the times indicated and the reaction was stopped by the addition of SDS sample buffer. Samples were analyzed by SDS-PAGE and phosphorimaging.

4.11.5 Photocrosslinking assays

Photocrosslinking experiments were usually carried out with yeast cell extracts prepared freshly by bead beating in lysis buffer (50 mM Tris-HCl pH 8, 150 mM Cl, 10 % glycerol, 0.1 % NP40) containing 1 tablet Complete EDTA-free (Roche) per 15 ml buffer. Samples containing photocrosslinkers were always protected from light in all steps prior to addition of SDS-loading buffer. APC was bound to IgG Sepharose beads (Amersham) by incubating extract and sepharose (1-2 mg protein per 5 µl beads) for 60 to 90 minutes at 4°C on an end-over-end rotator. Beads were washed extensively before addition of reticulocyte lysate containing *in vitro* translated Doc1 versions carrying a photocrosslinker. Samples were rotated at room temperature for 20 minutes followed by 3-4 brief washes with lysis buffer. Lysis buffer containing 1 mM DTT was used for all washing steps apart from the last one before crosslinking and addition of ubiquitination mix, respectively, for which DTT was usually omitted. When crosslinking experiments were carried out in presence of

ubiquitination mix, 28 μ l of ubiquitination buffer containing ubiquitination mix components at concentrations described in Section 4.11.3 were added and the samples were incubated for 5-15 min at 25°C with vigorous shaking. For photocrosslinking samples were incubated with open lids on a shaker (1300 rpm, 9°C) while irradiating them with a black ray long UV-lamp (B-100AP, 100W, UVP) with a wave length of 360 nm at a distance of 10 cm for 5-8 min. In a typical experiment, 15-20 μ l bead-bound APC, 35 μ l reticulocyte lysate containing Doc1 and 28 μ l ubiquitination mix were used.

4.12 Immunofluorescence microscopy

Cells were grown on 18 mm coverslips in 12 well plates and fixed with 4 % PFA. Antibodies were used at a concentration of 2 mg/ml in 3 % BSA, DNA was counterstained with Hoechst 33342 and slides were mounted using Vectashield Mounting Medium (H1000, Vector Laboratories). Image acquisition was performed as described (Waizenegger *et al.*, 2000).

5 Appendix

5.1 Generation of antibodies recognizing yeast APC subunits

For each APC subunit, two peptides from the yeast protein sequences were chosen for rabbit immunization (Table 5–1). The choice of sequence was based on the surface prediction of the respective yeast APC subunits using the software Protean. One peptide per subunit was usually located close to the N- and one peptide close to the C-terminus of the proteins (Table 5–1). All peptides were synthesized by the IMP Protein Chemistry Facility and sent to Gramsch Laboratories in Schwabhausen/Germany for immunization. Antisera obtained from the immunizations were tested for their ability to recognize the antigen on immunoblots. Alternatively, they were immediately purified and the obtained pure antibodies were analyzed for antigen specificity in immunoblotting experiments.

Table 5-1: Peptides derived from APC subunit protein sequences which were used for rabbit immunization.

protein	antigenic peptide	sequence
Doc1	1512	HNKPSVLVLDDRIVDAATKDL
Doc1	1513	CSNEPHQDTHEWAQTLPETNNV
Cdc16	1514	CALRKGGHDSKTGSNNADDDFDAD
Cdc16	1515	THYYKSSKATSNLKSSNRVLC
Cdc23	1517	SLADESPLRNKQGVPKQMFC
Cdc27	1518	CKELTVAMNLDPKGNQVIIDELQK
Cdc27	1519	CIVGRKKDAIKELTVAMNLDPKG
Apc1	1685	KPSTRNDYLPRETHNGEYTGDSPEC
Apc1	1686	CDDERSNGSDISDPTAYLEDKKDIDDHYG
Apc2	1687	SPNDAKSNHQLRPPSLRIC
Apc2	1688	CTLHDSINQDTNITKRDKNKKS
Hsl1	1740	FEKENTLSSSYLEEQKPKRAALSDITC
Apc11	1742	CVDFDEPIRQNTDNPIGRQQV

Affinity purification of antisera was carried out by performing High Performance Liquid Chromatography over a matrix coupled to the immunogenic peptide. Antibodies were eluted from the matrix in two steps: a “magnesium elution” (M) with 1.5 M MgCl₂, 100 mM Na-

acetate, pH 5.2, was followed by a “glycine elution” (G) using 100 mM glycine pH 2.45. Both elutions were usually collected separately and dialyzed in tubings with a Molecular Weight cut off (MWCO) of 3.5KDa against 2 l buffer containing 20 mM Hepes-KOH pH 7.9, 150 mM NaCl and 10 % glycerol for about 8-12 h. The dialysis buffer was changed after 2-4 h. All yeast strains used for the basic characterization of the antibodies are listed in Table 5–2.

Table 5-2: Yeast strains used in antibody tests. All *S.cerevisiae* strains used are derivatives of K699 (*MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1, GALpsi*) and K700 (*MATalpha ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1, GALpsi*).

Yeast strain	Genotype	Creator
J9	<i>MATa APC4-TAP::TRP1</i>	H. Vodermaier
J33	<i>MATa APC4-TAP::K/IRP1 cdc27::KanMX4 myc9-CDH1::LEU2</i> <i>multiple integration of SIC1::HIS3 at SIC1 locus</i>	H. Vodermaier
J42	<i>MATa APC4-TAP::K/IRP1 APC5-myc9::TRP1</i>	H. Vodermaier
J61 (K6197)	<i>MATa CDC27-myc9::TRP1</i>	Marta Galova
J118 (K6329)	<i>MATa APC1-myc18::HIS3</i>	W. Zachariae
J134 (K7898)	<i>MATa APC2-myc9::TRP1</i>	W. Zachariae
J224	<i>MATalpha CDC23-myc9::LEU2</i>	this study
J203	K699	
J224	<i>MATalpha CDC23-myc9::LEU2</i>	this study
J269	<i>MATa APC4-TAP::TRP1 APC11-HA3::TRP1 doc1::kanMX4</i>	this study

5.1.1 Apc1 and Apc2 antibodies

Antisera taken four weeks after immunization were directly tested in immunoblotting for their ability to recognize Apc1 or Apc2 in immunopurified APC preparations and on yeast cell extracts (Figure 5–1A). APC was isolated from yeast strains carrying a myc-tag on Apc1 (J118), Apc2 (J61) or Cdc27 (J134) by immunoprecipitation using an antibody against the myc-epitope (9E10, made in house). As a control, an untagged yeast strain (J203) was used. Purified 9E10 antibody (12.5 µg) and 10 µl GammaBind G sepharose (GE Healthcare) per sample were incubated for 15 min prior to 1.5 h incubation with yeast cell lysate containing 2 mg of total protein. Antibody beads were washed 3 times briefly and 3 times with a 10 min incubation with IP buffer containing 50 mM Tris pH 8, 150 mM KCl, 10 % glycerol and 0.1 % NP-40. All incubation and washing steps were carried out at 4°C on an end-over-end rotator. Elution was carried out by boiling beads in SDS-PAGE sample buffer. 10 µl

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immunopurified APC sample and 75 µg yeast extract per lane were separated by SDS-PAGE and transferred to a PVDF membrane. The membranes were incubated with a 1:250 dilution of the respective antisera in TBS-T containing 4 % milk powder. In order to easily identify an antigen-specific signal, I analyzed APC purifications which contained a tagged version of the antigen next to those containing the untagged antigen. Antigen specific signals were then indicated by a band which appeared at a size corresponding to the size of antigen in immunoprecipitations from strains in which the antigen was untagged and which shifted to a higher molecular weight in preparations from tagged strains. Both Apc1 antisera (1685 and 1686) specifically recognized tagged and untagged Apc1 after immunoprecipitation via the myc-epitope on Apc1 (“*APC1-myc18*”, lanes 3 and 8) and Cdc27 (“*APC1*”, lanes 4, 5, 9, 10), respectively. Similarly, the Apc2 antisera 1688 and 1688 recognized Apc2 versions in APC immunoprecipitates of Apc2-myc9 (“*APC2-myc9*”, lanes 2 and 5) and Cdc27-myc9 (“*APC2*”, lanes 3, 4, 5 and 6). At the conditions tested here, all 4 antisera did not recognize their antigens on yeast cell lysates (Figure 5– 1A, lanes 1 and 6 and data not shown). Purified 1686 antibody, however, stains Apc1 also on extracts (Figure 5– 2, lane 1).

5.1.2 Cdc27 antibodies

To test purified antibodies raised against Cdc27 peptides, I took advantage of a Cdc27 deleted yeast strain (“*cdc27Δ*”, J33) and a corresponding *wild type* strain (“*CDC27*”, J42). Both strains in addition carried a TAP-tag on Apc4. APC was pulled down for 1 h from these strains using IgG Sepharose beads (Amersham, 2 mg total yeast protein per 10 µl beads) and washed extensively with IgG Wash buffer buffer (50 mM Tris pH 8, 150 mM KCl, 10 % glycerol, 0.1 % NP-40 and 0.5 mM EDTA). Bound proteins were eluted using one bead volume SDS-PAGE sample buffer. Sample from 10 µl beads, about 75 ng TAP-purified APC containing Cdc27 (purified from J42) as well as yeast lysates (75 µg total protein) from *CDC27* and *cdc27Δ* strains were separated by SDS-PAGE gels followed by Western blotting. Membranes were developed with the glycine elutions of both antibodies at a dilution of 2 µg/ml. Both 1518 and 1519 antibodies specifically recognized a band corresponding to the size of Cdc27 in purified APC samples. Importantly, this band was not present in APC pull downs from the *cdc27Δ* strain (Figure 5–1C, lanes 3, 4, 7, 8). When using these antibodies on yeast cell extracts, however, several bands were recognized (Figure 5–1C, lanes 1, 2, 5, 6). None of these bands was lost in Cdc27-deleted samples. In addition, none of

the recognized bands had the same size as the band which was recognized in the purified samples, indicating that both Cdc27 antibodies do not recognize their antigen on cell extracts.

5.1.3 Cdc23 antibodies

The immunization program for an N-terminal peptide of Cdc23 (peptide 1516, CYKRSIKASQTVNQNTS) was stopped early because the antiserum did not give a specific signal in immunoblotting experiments. Both 1517 antibody fractions were tested by Western blotting for their ability to recognize tagged and untagged Cdc23 in APC immunoprecipitations, similarly as described in 5.1.1. APC was purified from CDC23-myc9 strains (J224) with the 9E10 antibody (“CDC23-myc9”) using IP buffer and from an Apc4-TAP (J9) strain containing *wild type* Cdc23 allele (“CDC23”) using IgG sepharose beads and IgG wash buffer as in 5.1.1 and 5.1.2. Sample eluted from 10 µl beads per lane were analyzed by SDS-PAGE and Western blotting. The fact that rabbit secondary antibody also stains the Protein A-tag (which is part of the TAP-tag) on Apc4 complicates the interpretation of the result after developing membranes with the 1517 antibody fractions (Figure 5–1D, lanes 1 and 6). Nevertheless, both antibody fractions clearly recognize Cdc23 on purified APC preparations containing either *wild type* or myc9-tagged Cdc23 (Figure 5–1D, lanes 1-6).

5.1.4 Doc1 antibodies

All 4 Doc1 antibody fractions were tested in immunoblotting experiments. Two µl Doc1 *in vitro* translation product, 60 ng APC purified from *DOC1 wild type* and *doc1Δ* yeast strains via the TAP-method and cell extracts from *DOC1* and *doc1Δ* yeast strains (75 µg/lane) were separated by SDS-PAGE and transferred to PVDF membranes. These were incubated with the antibody fractions at a dilution of 2 µg/ml in milk/TBS-T. Both antibodies recognize the *in vitro* translated protein with high specificity. Moreover, the antibodies stain a band corresponding to Doc1’s size (33 kDa) on samples of purified APC, which is not present in

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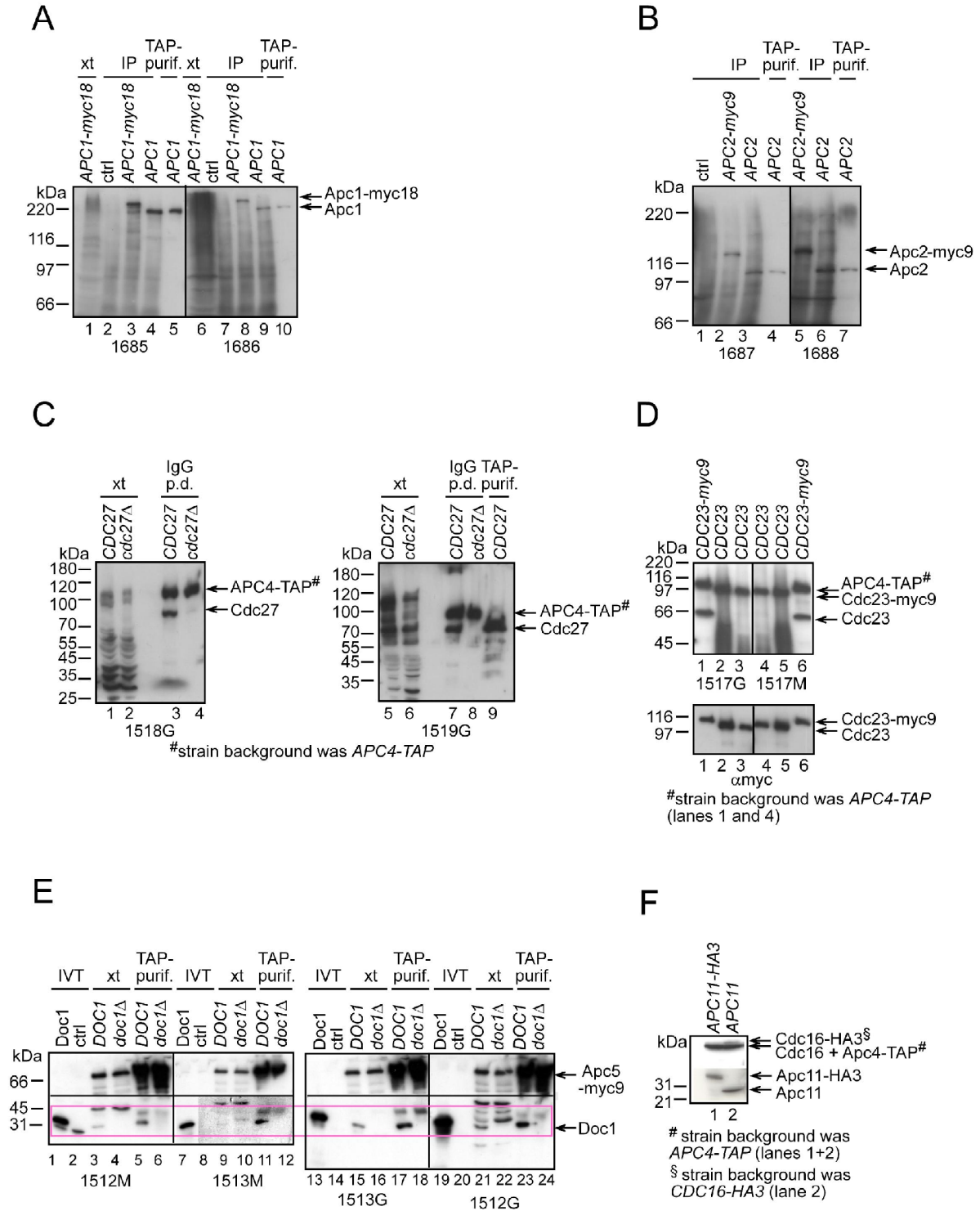


Figure 5-1: Initial specificity tests of peptide antibodies raised against APC subunits.

A – F) Yeast cell lysates or purified samples were separated by SDS-PAGE and analyzed by immunoblotting using the indicated antibodies at 2 µg/ml. Details are described in the text. IgG pull-down (IgG p.d.), TAP-purified APC (TAP-purif.), extract (xt), *in vitro* translation (IVT).

preparations of APC lacking Doc1 (Figure 5–1E). When using these antibodies on yeast cell extracts, only a very faint band (Figure 5–1E, lanes 3 and 15) was recognized or several bands were stained in addition (Figure 5–1E, lane 21). Importantly, however, the 33 kDa band is lost in all samples containing *doc1Δ* extracts, indicating that both 1512 and 1513 antibodies recognize Doc1 on yeast cell extracts.

5.1.5 Apc11 antibodies

The suitability of the 1742 antibody for Western blotting is shown in Figure 5–1F. APC isolated from an *APC11-HA3* (J269) strain was loaded next to a preparation from APC containing untagged Apc11. For both preparations, IgG sepharose pull downs were performed as described in Section 5.1.2 (the yeast strains also contained a TAP-tag on Apc4). After SDS-PAGE, Western blotting and incubation of the membrane with the glycine elution of 1742 (1742G), only one band per lane is stained. This band corresponds to the size of Apc11 in the *APC11* sample (Figure 5–1F, lane 2) and shifts in the *APC11-HA3* sample (Figure 5–1F, lane 1), indicating that the antibody specifically recognizes Apc11 on purified APC.

5.1.6 Yeast APC antibodies in immunoprecipitation experiments

I tested whether the newly generated antibodies are also suitable for purification of the APC under native conditions. Affiprep Protein A beads (Biorad) were washed twice with at least 10 volumes of TBS-T, then resuspended in 10 volumes TBST and 1.5–2 µg purified antibody was added per µl bead volume. Beads and antibodies were incubated for 20 min at room temperature on an end-over-end rotator and were then washed twice with TBST and once with IP buffer containing 50 mM Tris pH 8, 150 mM KCl, 10 % glycerol and 0.1 % NP-40. Yeast extracts containing 2 mg of total protein were added per 10 µl antibody beads. The samples were incubated for 60–90 min at 4°C on an end-over-end rotator and then washed extensively with IP buffer. Elution was carried out by resuspending beads in 1.5 volumes 0.1 M glycine pH 2.2. SDS sample buffer was added to the glycine eluates and the samples were neutralized with 1.5 M Tris pH 9.2 and boiled.

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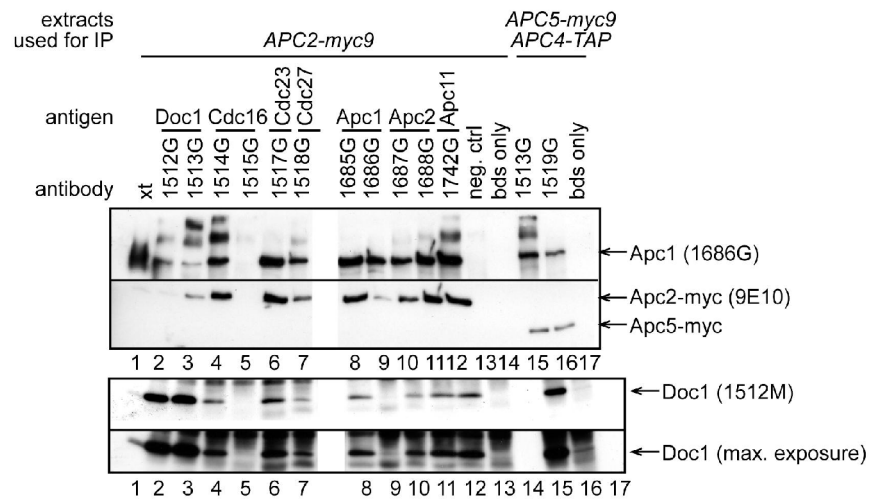


Figure 5-2: Immunoprecipitation of APC with APC-specific antibodies. Details are given in the text.

After SDS-PAGE and transfer to PVDF membranes, the membranes were incubated with antibodies specific for three different APC subunits (Apc1, Apc2-myc9 or Apc5-myc and Doc1) to analyze APC composition. One antibody each generated against Apc1 (1685G), Apc2 (1688G), Cdc27 (1518G), Cdc16 (1514G), Cdc23 (1517G) and Apc11 (1742G) was able to pull down all 3 subunits that were stained for, which implies that they might isolate the complete complex (Figure 5–2, lanes 4, 6, 7, 8, 11, 12). The Doc1 1513 antibody does bring down all three proteins tested for, but a large amount of immunoprecipitated Doc1 is probably not bound to APC (Figure 5–2, lane 3). The 1512, 1515 and 1686 antibodies contain very little or nothing of one or more subunits that were stained for under the conditions tested here. They do immunoprecipitate their respective antigens (Figure 5–2, lanes 2, 5, 9), but they are probably not suitable for purification of the entire APC. Further characterization of these antibodies might be necessary before using them for immunopurifying APC, such as silver staining or blotting for more APC subunits. In summary, I generated peptide antibodies against 7 yeast APC subunits, which specifically recognize their respective antigens in immunoblotting experiments. Some of them also precipitate the entire APC together with the antigen which might make them useful tools for further yeast APC studies.

5.2 Generation of an antibody against Hsl1⁽⁶⁶⁷⁻⁸⁷²⁾

A peptide antibody generated against an sequence within the Hsl1 fragment (which comprises amino acids 667 to 872) was produced as described in Section 5.1. Since I wanted to use the antibody to analyze ubiquitination assays in which Hsl1⁽⁶⁶⁷⁻⁸⁷²⁾ had been used as a substrate, I tested the antibody on purified protein and on a protein mix containing Hsl1⁽⁶⁶⁷⁻⁸⁷²⁾ and the components of a ubiquitination mix (E1 and E2 enzymes, Cdh1, ubiquitin, ATP). No crossreactivities were observed, indicating that this antibody could be suitable as an alternative to the use of radiolabeled substrates Figure (5–3A and B).

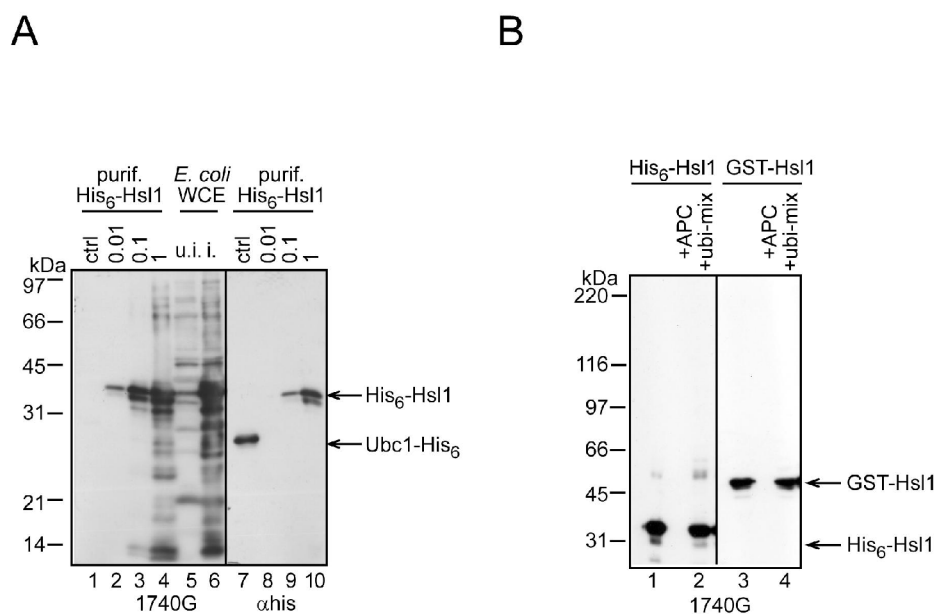


Figure 5-3: Initial specificity test of Hsl1 peptide antibody. A) The indicated amounts (in μg) of purified (purif.) His₆-Hsl1 and 1 μg of Ubc1-His₆ (control, ctrl) as well as lysates (WCE) from uninduced (u.i.) and induced (i.) bacteria that were expressing His₆-Hsl1 were separated by SDS-PAGE and analyzed by immunoblotting using the glycine elution of the Hsl1 antibody (1740G). As a control, a parallel analysis with an anti- His₆ (Penta, Qiagen) antibody was carried out. Both antibodies were used at 2 μg/ml. B) To test the suitability for the 1740G antibody in analysis of ubiquitination assays, 1 μg of purified His₆-Hsl1 or GST-Hsl1 was separated next to a ubiquitination mix (ubi-mix) containing 1 μg of the respective Hsl1 versions as well as E1 and E2 enzymes, TAP-purified APC, purified Cdh1, ubiquitin and ATP. Samples were analyzed by immunoblotting using 1740G at 2 μg/ml.

5.3 Sequence alignment of C10orf104/Apc16 protein sequences

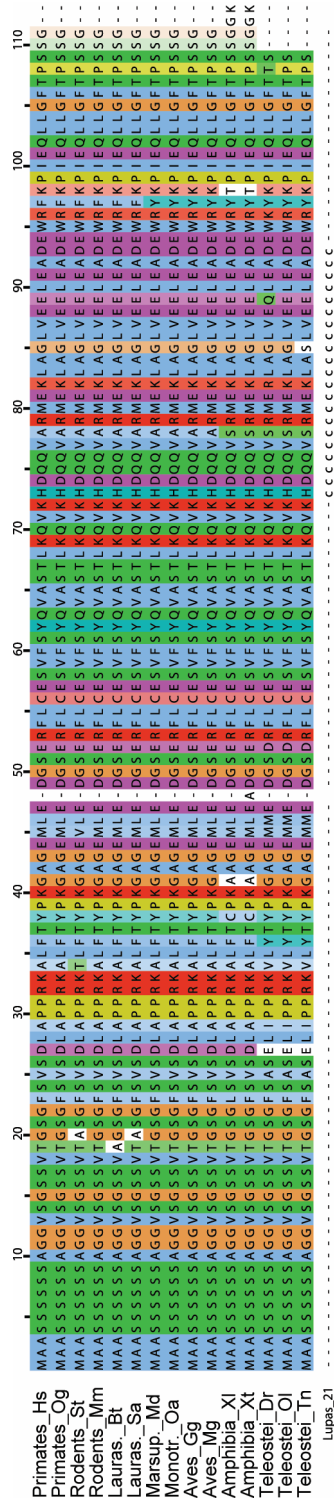


Figure 5-4: Multiple sequence alignment of full-length C10orf104 orthologs from a range of bony vertebrate species. Jalview was used for alignment visualization with ClustalX shading and Lupas coiled-coil prediction (PMID: 14960472). Sequences are derived from NCBI/Entrez and Ensembl databases and are listed in the following order: 1) Primates 1.1) Homo sapiens- NP_775744 1.2) Otolemur garnettii- ENSOGAP00000007350 2) Rodents 2.1) Sperophilus tridecemlineatus ENSSTOP00000000619 2.1) Mus musculus- NP_079790 3) Laurasiatheria 3.1) Bos taurus- NP_001014960 3.2) Sorex araneus- ENSSARP00000001443 4) Marsupials; Monodelphis domestica XP_001362179 5) Monotremes; Ornithorhynchus anatinus XP_001508726 6) Aves 6.1) Gallus gallus- XP_421587 6.2) Meleagris gallopavo- EH290378 7) Amphibia 7.1) Xenopus laevis NP_001085523 7.2) Xenopus tropicalis NP_001004873 8) Teleostei 8.1) Danio rerio- NP_001017878 8.2) Oryzias latipes- ENSORLP000000011498 8.3) Tetraodon nigroviridis- CAG13305. Provided by Maria Novatchkova.

6 Abbreviations

A	alanine
Å	Angström
ab	antibody
Ama1	activator of meiotic APC protein-1
amp	ampicillin
APC	anaphase-promoting complex/cyclosome
ATP	adenosine triphosphate
a.u.	arbitrary unit
A ₂₆₀ , A ₂₈₀	absorption at 260/280 nm
bp	base pairs
BSA	bovine serum albumin
cDNA	complementary DNA
<i>cdc</i>	cell division cycle
Cdk	cyclin dependent kinase
2 D/3 D	two-dimensional/three-dimensional
DAPI	4',6'-diamino-2-phenylindol
DM	D box mutant (R-X-X-L → A-X-X-A)
DMP	dimethylpimelimidate
DNA	deoxyribonucleic acid
DMSO	dimethylsulfoxide
Doc1	degradation of cyclin B protein-1
DTT	dithiothreitol
DUB	deubiquitinating enzyme
E	glutamic acid
E1	ubiquitin-activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin ligase
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediamine tetraacetic acid

ABBREVIATIONS

EGTA	ethylene glycol tetraacetic acid
EM	electron microscopy
F	phenylalanine
G	glycine elution (of purified antibody)
GST	glutathione-S-transferase
H	histidine
h	hour
ha	epitope on influenza virus hemagglutinin (sequence: YPYDVPDYA)
His ₆	His-tag comprising 6 histidines
HECT	homology to E6-AP C-terminus
Hepes	N-2-hydroxyethylpiperazin-N'-2-ethane sulphonic acid
HU	hydroxyurea
ILR	isoleucine-leucine-arginine
IR	isoleucine-arginine
IgG	immunoglobulin G
IPTG	isopropyl β -D-1-thiogalactopyranoside
IVT	<i>in vitro</i> translation
kDa	kilo Dalton
LAP	localization and affinity purification
K	lysine
L	leucine
LB	Luria-Bertani
log	logarithmic
N	asparagine
M	magnesium elution (of purified antibody)
MCC	mitotic checkpoint complex
MDa	mega Dalton
Mnd2	meiotic nuclear division protein-2
MgOAc	magnesium acetate
min	minute
MR	methionine-arginine

ABBREVIATIONS

myc	human c-myc epitope (sequence: EQKLISEEDL)
NaOAc	sodium acetate
NEM	N-ethylmaleimide
Ni-NTA	nickel-nitrilotriacetic acid
noc	nocodazole
OD ₆₀₀	optical density at 600 nm
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pds1	precocious dissociation of sister chromatids
PEG	polyethylenglycole
PFA	paraformaldehyde
PMSF	phenylmethysulphonyl fluoride
PVDF	polyvinylidene fluoride transfer membrane
Q	glutamine
R	arginine
RING	really interesting new gene
RFP	red fluorescent protein
RNA	ribonucleic acid
Rpn	regulatory particle non-ATPase
rpm	rounds per minute
S	Svedberg
S	serine
SAC	spindle assembly checkpoint
<i>S. cerevisiae</i> / <i>S. c.</i>	<i>Saccharomyces cerevisiae</i>
SCF	Skp1/Cul1/F box
SDS	sodium dodecyl sulphate
SGD	<i>Saccharomyces</i> genome database
<i>S. pombe</i> / <i>S. p.</i>	<i>Schizosaccharomyces pombe</i>
Swm1	spore wall maturation protein-1
TAP	tandem affinity purification

ABBREVIATIONS

TBS	tris buffered saline
td2	tdimer2 (tandem dimer of a dimeric DsRed variant)
tmono	“half a tdimer2”; monomeric DsRed
TEV	tobacco etch virus
THF	tetrahydrofuran
(tmd)phe	L-4’-(3-[trifluoromethyl]-3H-diazirine-3yl) phenylalanine
TPR	tetratrico peptide repeat
Tris	tris-(Hydroxymethyl)aminomethane, 2-amino, 2-(hydroxymethyl),1-3-propandiol
tRNA	transfer RNA
UBL	ubiquitin-like
UV	ultraviolet
Usp44	ubiquitin-specific protease-44
v/v	volume per volume
WB	Western blot
WD	tryptophane aspartate
wt	<i>wild type</i>
YPD	yeast extract peptone dextrose

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