Titel der Dissertation
Substrate binding on the anaphase promoting complex/cyclosome

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1. Abstract

Cell division is a complex process that occurs in a series of events called the cell cycle and results in the generation of two genetically identical daughter cells. To ensure the correct order of events, the cell produces regulatory proteins that delay cell cycle progression until all necessary requirements to continue are satisfied. Once all prerequisites to progress are fulfilled, polyubiquitin chains are linked to these regulatory proteins which label them to be disposed in the cellular waste container.

The anaphase promoting complex/cyclosome (APC/C) is a multisubunit ubiquitin-protein ligase which is essential for cell division. In metaphase, when all chromosomes are bioriented on the mitotic spindle, APC/C becomes active and polyubiquitinates proteins that inhibit cell cycle progression, which targets them for proteasomal degradation. Removal of Securin and Cyclin B1 initiates sister chromatid separation in anaphase and mitotic exit. Ubiquitination of substrate proteins by the APC/C depends on either of two co-activator proteins, called Cdc20 and Cdh1. These proteins have been implicated in substrate recruitment and allosteric activation of the APC/C. However, the exact mechanism of how APC/C and the co-activator proteins recruit substrates is incompletely understood.

To obtain insight into this process electron microscopy is used to determine how Cdh1 and substrate proteins interact with APC/C. A protocol is established to isolate human APC/C which is bound to stoichiometric amounts of CDH1 and substrate proteins. Single particle electron microscopy and angular reconstruction suggest the existence of two CDH1 binding sites that mediate binding of two substrate molecules, which is confirmed by biochemical analysis. One substrate molecule is intercalated between CDH1 and
DOC1 in the central cavity of APC/C. The second CDH1/substrate binding site is located in the platform region, presumably between CDC23 and APC1. The identification of a second co-activator/substrate binding site helps unmasking the mystery why APC/C requires at least 13 different protein subunits to catalyze a relatively simple chemical reaction. Furthermore, conformational changes in the catalytic subunits APC2 and/or APC11 could be observed which upon substrate binding contact the proximal co-activator protein. This conformational rearrangement could reflect a co-activator induced stimulation of APC/C’s E3 activity.

To study how co-activator proteins interact with substrates at atomic resolution, CDH1 purification protocols are optimized to allow enrichment of full-length CDH1 in a milligram range. A truncated version of CDH1 in complex with a substrate fragment led to crystal formation. However, the obtained crystals require further optimization to determine a high-resolution structure of the CDH1-substrate complex.
2. Zusammenfassung

Die Teilung einer Zelle (Zellzyklus) folgt einem präzisen Ablauf von Ereignissen und resultiert in zwei genetisch identischen Tochterzellen. Die Zelle produziert regulatorische Proteine, die an bestimmten Kontrollpunkten (Checkpoints) das Fortschreiten des Zellzyklus verhindern, solange dafür nicht alle notwendigen Voraussetzungen erfüllt sind. Sobald der Zellzyklus fortgesetzt werden kann, werden Ubiquitinketten an diese regulatorischen Zellzyklusproteine gehaftet, wodurch diese zu zellulärem Müll deklariert werden, was zu deren Abbau führt.

Der „Anaphase einleitende Komplex“ oder „Zyklosom“ (APC/C) ist eine molekulare Nanomaschine, die die Anheftung von Ubiquitinketten an Substratproteine katalysiert (Ubiquitin-Ligase). Wenn alle Chromosomen in korrekter Weise (biorientiert) mit dem mitotischen Spindelapparat verbunden sind (Metaphase) wird der APC/C aktiviert und kann somit die inhibitorischen Proteine Securin und Zyklin-B1 ubiquitinieren. Die Degradation dieser zwei Proteine ermöglicht letztendlich, dass die genetisch identischen Schwesterchromatiden von den Spindelfasern an die entgegengesetzten Zellpole gezogen werden können (Anaphase), welches eine Voraussetzung für die anschließende Zellteilung (Zytokinese) darstellt. Der APC/C kann nur aktiviert werden, wenn er mit einem der zwei Koaktivatorproteinen, Cdh1 oder Cdc20, interagiert. Die Koaktivatorproteine helfen Substratproteine an den APC/C zu binden und haben vermutlich auch eine katalytische Funktion. Der genaue Mechanismus, wie die Substrate durch Koaktivatorproteine zum APC/C rekrutiert werden und was genau dessen katalytische Aktivität stimuliert, ist nicht vollständig verstanden.

3. Introduction

3.1. The Cell Cycle

Cell reproduction occurs by a complex series of events called the cell cycle, which regulate the duplication of all cellular components to allow the generation of two functional daughter cells by cell division (Morgan 2007). Fundamental to cell division is the precise duplication and equal distribution of the genome, which carries the genetic information. In multicellular organisms, missegregation of the duplicated genome in the germline causes developmental defects (trisomies) and can lead to cancer when happening during the division of somatic cells.

To ensure the generation of genetically identical daughter cells, genome duplication and segregation occur in distinct cell cycle phases that are usually separated by gap phases (Fig.1a). In S phase (synthesis phase), the genome is duplicated by a process called DNA replication. During M phase, the duplicated genome is split and segregated into two identical sets of genetic information (mitosis) prior to division of the entire cell (cytokinesis). The gap phases (G1 and G2) between S and M phase provide additional time for cell growth and ensure that genome duplication is completed before an attempt is made to split and segregate the genetic information (Morgan 2007).

The cell-division cycle employs a complex regulatory network (cell-cycle control system) that ensures the correct order and timing of cell-cycle events. Central components of the cell-cycle control system are the cyclin-dependent kinases (Cdks), whose kinase activity is stimulated by their association with the regulatory subunits known as cyclins. Presence of mitogens triggers cyclin gene expression which initiates early cell cycle
events by switching on Cdk activity at late G1. During mitosis, at the metaphase-to-anaphase transition, cyclins are proteolytically degraded which inactivates cyclin-dependent kinases.

![Cell Cycle Diagram]

**Figure 1** The eukaryotic cell cycle. (a) Cell cycle phases. DNA (blue) replication occurs in the nucleus during S phase. During pro- and prometaphase, the nuclear envelope disintegrates and the mitotic spindle forms. In anaphase, the replicated DNA is segregated and the entire cell divides during cytokinesis. G1, S and G2 phase are summarized as interphase. Start indicates cell cycle entry site. Prophase (P), prometaphase (PM), metaphase (M), anaphase (A), telophase (T). (b) The mitotic chromosome consists of two genetically identical DNA strands called the sister chromatids. Cohesin connects the sister chromatids at the centromer forming a centromeric constriction. Kinetochores connect sister chromatid DNA to the mitotic spindle.

### 3.2. The eukaryotic genome

One important advance in evolution was the transition from single-cell (pro- and eukaryotes) to multicellular organisms (only eukaryotes) which was accompanied by
increased complexity of the organism’s genetic information. The human genome constitutes the largest set of genetic information which is encoded in about 3 billion base pairs. Due to their increased size eukaryotic genomes were fragmented into several pieces of linear DNA double-strands (chromosomes; a human cell contains 46 chromosomes), whose replication and subsequent segregation must be synchronized in order to generate genetically identical daughter cells. The duplicated DNA double-strands (sister chromatids) are physically connected during DNA replication in S phase to ensure that the sister chromatids can be identified, split and segregated during mitosis (Fig.1a, b). The physical connection (cohesion) is mediated by a molecule called cohesin, which is believed to entrap the duplicated DNA double-stands in a ring-like structure (Peters et al. 2008).

### 3.3. Mitosis

![Figure 2](image-url)

*Figure 2* Key events in mitosis. In prometaphase, unattached kinetochores produce an ‘anaphase-wait’ signal that inhibits APC/C. In metaphase, all chromosomes are bioriented on the mitotic spindle which activates APC/C. Ubiquitylation and subsequent proteasomal degradation of securin and cyclin B activates separase which proteolytically cleaves cohesin to allow sister chromatid separation in anaphase.
During mitosis the sister chromatids of every single chromosome are split and segregated which requires substantial morphological changes of the cell interior allowing classification of different mitotic stages, namely prophase, prometaphase, metaphase, anaphase and telophase. In early mitosis (pro- and prometaphase), the nuclear envelope breaks down and the replicated DNA condenses into compact structures called mitotic chromosomes. The bipolar mitotic spindle aligns and starts contacting the chromosomes on their kinetochores (Morgan 2007). Once every chromosome is attached to spindle fibers coming from opposing poles (metaphase), a configuration which is called biorientation, the anaphase promoting complex/cyclosome (APC/C) becomes active (Fig.2). APC/C activity leads to proteolytic cleavage of cohesin which now enables the sister chromatids to be pulled to opposite cell poles by the mitotic spindle (anaphase). Premature initiation of sister chromatid separation can lead to chromosome missegregation and cancer and therefore must be prevented. This is ensured by a surveillance mechanism called the spindle assembly checkpoint (SAC) which monitors chromosomal attachment to the mitotic spindle and generates a diffusible signal (mitotic checkpoint complex; MCC) that binds and inhibits APC/C until the last chromosome is bioriented (Musacchio and Salmon 2007; Herzog et al. 2009).

3.4. The anaphase promoting complex/cyclosome (APC/C)

3.4.1. APC/C targets cell cycle regulators for proteasomal degradation

The anaphase promoting complex/cyclosome is an 1.5MDa protein complex that is conserved in all eukaryotic species and has essential functions in sister chromatid separation and mitotic exit (Peters 2006). APC/C initiates these events by assembling polyubiquitin chains on substrate proteins (Fig.3) which targets them for degradation by the 26S proteasome. Prominent APC/C substrates are securin (Cohen-Fix et al. 1996;
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Ciosk et al. (1998), A- and B-type cyclins (King et al. 1995; Geley et al. 2001), Aurora kinases (Honda et al. 2000), Polo-like kinase-1 (Sorensen et al. 2000), geminin (McGarry and Kirschner 1998), sororin (Rankin et al. 2005) and the protein kinase Hsl1 (Burton and Solomon 2000). Proteasomal degradation of these substrates regulates important cell cycle events: (i) removal of securin activates separase, a protease that cleaves cohesin to allow sister chromatid separation in anaphase (Ciosk et al. 1998); (ii) degradation of A- and B-type cyclins inactivates cyclin-dependent kinases (Cdks) which is important for the disassembly of the mitotic spindle, chromosome decondensation, regeneration of the nuclear envelope and formation of the cytokinetic cleavage furrow at the end of mitosis (Peters 2006); (iii) destruction of geminin enables incorporation of the MCM complex into pre-replication complexes (pre-RCs) which permits DNA replication during S phase of the succeeding cell cycle (McGarry and Kirschner 1998).

Figure 3 Ubiquitin transfer reaction. (a) Enzymatic cascade leads to activation and transfer of ubiquitin to substrate proteins catalyzed by the enzymes E1, E2 and E3. (b) E3 brings E2~Ub and substrate in close proximity. An E2 active site asparagine is believed to stabilize the oxyanion transition state of the E2~Ub thioester bond which facilitates the nucleophilic attack of the substrate lysine. RING E3s might promote adjustment of the asparagine residue.
Polyubiquitination of substrates requires an enzymatic cascade (Fig.3a) containing an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and an ubiquitin ligase (E3) (Deshaiies and Joazeiro 2009). The APC/C is a RING domain E3 ubiquitin ligase that binds a substrate and Ubch10 (E2) thioesterified with ubiquitin (E2~Ub) which brings them in close proximity so that the ubiquitin can be transferred to substrate lysines (Fig.3b) (Summers et al. 2008). Apart from bringing together a substrate and E2~Ub, RING domain E3s might also play a more active role in catalysis by inducing conformational changes in E2~Ub that could enhance ubiquitin discharge (Fig.3b) (Deshaiies and Joazeiro 2009; Ye and Rape 2009).

### 3.4.2. APC/C activity is regulated by multiple mechanisms

In addition to E2 enzymes, APC/C activity is strictly dependent on the co-activator proteins Cdc20 and Cdh1. The co-activator proteins assist APC/C by facilitating substrate recruitment (Visintin et al. 1997; Burton and Solomon 2001; Hilioti et al. 2001; Schwab et al. 2001) and presumably by activating the ubiquitin transfer reaction (Vodermaier et al. 2003; Kimata et al. 2008).

The co-activator proteins contain conserved sequence elements known as the C-box (Schwab et al. 2001) and the IR-tail (Vodermaier et al. 2003) that are required for binding to APC/C (Fig.4). The C-box might have additional functions in activating APC/C’s E3 activity (Kimata et al. 2008). Substrate binding to APC/C is believed to be mediated by the co-activator’s C-terminal WD40 domain (Kraft et al. 2005) by interacting with specific recognition motifs of APC/C substrates, called the D-box (consensus sequence: RxxLxxxxN/D/E) (Glotzer et al. 1991) and the KEN-box (consensus sequence: KENxxxN/D/E) (Pfleger and Kirschner 2000).
Association of APC/C with its co-activator proteins is tightly regulated and primarily determines when during the cell cycle APC/C becomes active (Fig.5). Cyclin-dependent kinase activity phosphorylates APC/C subunits in early mitosis which promotes APC/C-Cdc20 assembly (Kraft et al. 2003), whereas phosphorylation of Cdh1 prevents association with APC/C (Kramer et al. 2000). At the metaphase-to-anaphase transition, when cyclin-dependent kinase activity is downregulated by cyclin degradation, protein phosphatases dephosphorylate APC/C and Cdh1 (Visintin et al. 1998) which disassembles APC/C-Cdc20 and favors association of APC/C-Cdh1. Cdc20 itself is a substrate of APC/C-Cdh1 which strengthens inactivation of APC/C-Cdc20 during mitotic exit (Prinz et al. 1998). Cdh1 keeps APC/C active throughout G1 and its inactivation in late G1 is believed to occur by APC/C-dependent ubiquitylation and subsequent degradation of its cognate E2 enzyme Ubch10 (Rape and Kirschner 2004), which helps the cell reentering a new cell division cycle by enabling upregulation of cyclin-dependent kinase activity (Peters 2006).
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Figure 5 Regulation of APC/C activity throughout the cell cycle by phosphorylation, inhibitors, co-activators and APC/C-dependent degradation of UbcH10. Cdk activity is shown in the center. Lines indicate when during the cell cycle the regulatory proteins or complexes are expressed or assembled, respectively. APC/C becomes active at the metaphase-to-anaphase transition. In late G1, APC/C activity declines due to autonomous degradation of UbcH10. Emi1 might also be involved in APC/C inactivation during late G1.

Besides the regulation of APC/C activity by phosphorylation and association with co-activator proteins, inhibitors have been identified that block APC/C’s ubiquitylation activity even though co-activator proteins are bound (Peters 2006) (Fig.5). The mitotic checkpoint complex (MCC) and Emi1 (early mitotic inhibitor 1) inhibit APC/C activity by employing similar recognition motifs to those found in APC/C substrates (D-box and KEN-box) which enables them to outcompete substrate binding on APC/C (Miller et al. 2006; Herzog et al. 2009). This led to the hypothesis that APC/C inactivation is achieved
by a mechanism called pseudosubstrate inhibition (Lara-Gonzalez et al.; Miller et al. 2006; Burton and Solomon 2007). However, it is unknown why substrates and not inhibitors become ubiquitylated upon APC/C binding, even though similar recognition motifs are employed. It is possible that the inhibitors block APC/C’s ability to mediate the ubiquitin transfer reaction which could be achieved by multiple mechanisms (Miller et al. 2006; Herzog et al. 2009).

3.4.3. APC/C architecture and subunit assembly

The anaphase promoting complex/cyclosome is a 1.5MDa ubiquitin ligase and is composed of at least 13 core subunits (Thornton et al. 2006). In its molecular dimensions APC/C is comparable to the ribosome which makes it the largest enzyme that is known to catalyze ubiquitylation of substrates. It remains a big mystery why APC/C is built up by so many subunits while the SCF complex (Skp, cullin, F-box containing complex), another E3 ubiquitin ligase involved in cell cycle regulation, requires only 4 protein subunits to be functional (Ang and Wade Harper 2005; Peters 2006).

Structural analysis of APC/C from different organisms using electron microscopy and single particle analysis revealed that APC/C adopts an asymmetric triangular structure that is composed of two large domains called the ‘arc lamp’ and the ‘platform’ that together enclose a central cavity (Dube et al. 2005; Ohi et al. 2007; Herzog et al. 2009) (Fig.6a). Genetic, biochemical and structural analysis provided the first insights into APC/C subunit interactions, subunit topology within the 3D model of APC/C and binding surfaces of regulatory proteins (Wendt et al. 2001; Vodermaier et al. 2003; Dube et al. 2005; Thornton et al. 2006; Ohi et al. 2007; Herzog et al. 2009). The ‘arc lamp’ is build up by the tetratricopeptide repeat (TPR) proteins Cdc27/Apc3, Cdc16/Apc6, Cdc23/Apc8
and Apc7 in vertebrate APC/C whereas the ‘platform’ is composed of Apc1, Apc4 and Apc5 (Thornton et al. 2006; Herzog et al. 2009).

Apc2, a member of the cullin domain family is located in APC/C’s central cavity between the ‘arc lamp’ and the ‘platform’ domain (Herzog et al. 2009). Apc2 associates with the RING-finger domain Apc11 which in turn mediates interaction with the E2 ubiquitin-conjugating enzyme (Gmachl et al. 2000; Leverson et al. 2000; Tang et al. 2001). Also, Apc2 has been shown to interact with Doc1/Apc10, an APC/C subunit that is required for processive substrate ubiquitylation (Carroll and Morgan 2002; Thornton et al. 2006). Very recently Doc1 has been localized in the structures of yeast and human APC/C (Fig. 6b) which was still unpublished when the project was initiated that is presented in this study. Co-activator proteins have been shown to interact with Cdc27/Apc3 (Wendt et al. 2001; Kraft et al. 2005) and bind to the ‘arc lamp’ domain opposite of Apc2 and Doc1, leading to a configuration in which Apc2, Doc1 and the co-activator face APC/C’s central cavity (Herzog et al. 2009). Since co-activators are believed to facilitate substrate
binding and Apc2/11 recruit E2^Ub, it is likely that substrate ubiquitylation occurs in the central cavity.

### 3.4.4. Substrate recognition by the APC/C

The co-activator proteins Cdc20 and Cdh1 have a essential role in APC/C activation (Visintin et al. 1997). The co-activators contain a C-terminal WD40 propeller which is a protein domain that serves as a protein-protein interaction platform that can be found in substrate adaptor proteins of other E3 ubiquitin ligases such as SCF (Kraft et al. 2005). Therefore, since their discovery the essential role of APC/C co-activators has been suspected to be the recruitment of substrates to the APC/C (Peters 2006).

Cdc20 and Cdh1 bind specific recognition motifs (destruction motifs) that can be found in APC/C substrates, called the D-box (destruction box) and the KEN-box (Burton and Solomon 2001; Hilioti et al. 2001; Schwab et al. 2001). The D-box has been shown to directly contact the WD40 domain of Cdh1 and this interaction is required for APC/C-dependent substrate ubiquitylation (Kraft et al. 2005). Previous studies showed that apart from the co-activator proteins also APC/C core subunits seem to be involved in substrate recognition (Passmore et al. 2003; Yamano et al. 2004; Burton et al. 2005; Carroll et al. 2005; Passmore and Barford 2005). Deletion of the APC/C core subunit Doc1 interferes with substrate recruitment to the APC/C even though Doc1 is dispensable for co-activator binding (Passmore et al. 2003). Doc1 crystal structures revealed a jellyroll fold that is highly similar to ligand binding domains of several bacterial and eukaryotic proteins (Wendt et al. 2001; Au et al. 2002). Single residue mutations in Doc1’s putative ligand binding domain caused processivity defects in the ubiquitylation reaction of APC/C substrates (Carroll et al. 2005). Therefore, the ability of the co-activator proteins to recruit substrates to the APC/C is required but not sufficient to promote APC/C-dependent substrate ubiquitylation. Interestingly, Doc1’s
contribution in mediating processive substrate ubiquitylation depends on the presence of the D-box destruction motif (Carroll et al. 2005) which suggests a direct or indirect role of Doc1 in D-box recognition. It is therefore possible that substrates interact with both the co-activator and Doc1 via their D-box, either simultaneously or sequentially, to facilitate the formation of a ternary APC/C–co-activator–substrate complex (Burton et al. 2005; Passmore and Barford 2005).

Two model substrates have been shown to be particularly suitable for studying APC/C substrate binding in vitro, namely Hsl1 667-872 and N70-2x. The Hsl1 fragment 667-872 contains a D-box and a KEN-box and has been shown to act as an APC/C substrate in multiple assays (Burton and Solomon 2000; Burton and Solomon 2001; Burton et al. 2005). N70-2x is an artificial fusion protein made of two copies of the D-box containing 70 N-terminal amino acids originated from fission yeast cyclinB which has been used in several studies (Yamano et al. 2004; Eytan et al. 2006).

3.4.5. APC/C mediated substrate degradation

Early in mitosis, phosphorylation of core subunits activates APC/C which initiates the degradation of various APC/C substrates in a sequential order while the cell progresses through mitosis (den Elzen and Pines 2001; Lindon and Pines 2004). CyclinA1 and Nek2A are degraded in prometaphase (‘early substrates’) (den Elzen and Pines 2001; Hames et al. 2001) while destruction of other substrates (securin, B-type cyclins) is inhibited by the spindle assembly checkpoint and only starts at the metaphase-to-anaphase transition (‘late substrates’) (Geley et al. 2001; Hagting et al. 2002). The sequential degradation has been attributed to kinetic differences in the ubiquitylation reaction of these substrates (Rape et al. 2006). However, despite the fact that Cdc20 and Cdh1 are believed to mediate some degree of substrate specificity (Schwab et al. 1997; Visintin et al. 1997; Wan and Kirschner 2001) the exact mechanism of how such kinetic differences
might be achieved is vaguely understood and currently under debate (Di Fiore and Pines; van Zon et al.; Rape et al. 2006; Stegmeier et al. 2007; Wolthuis et al. 2008).

Once a substrate is bound to APC/C, ubiquitin chain assembly is catalyzed by two different ubiquitin-conjugating enzymes. UbcH10 (Ubc4 in yeast) catalyzes monoubiquitination of substrate lysines which are elongated to ubiquitin chains by Ube2S (Ubc1 in yeast) (Wu et al.; Rodrigo-Brenni and Morgan 2007; Garnett et al. 2009; Williamson et al. 2009b). Human APC/C catalyzes noncanonical K11-linked polyubiquitin chains that act as a functional degradation signal in the proteasome pathway (Matsumoto et al.; Jin et al. 2008).
4. **Aim of the study**

The anaphase promoting complex/cyclosome (APC/C) regulates key cell cycle events by catalyzing ubiquitin chain assembly on regulatory proteins which targets them for proteasomal degradation. APC/C serves as a huge platform that specifically recognizes adapter proteins (co-activators) and substrates and brings them in close spatial proximity to an ubiquitin-conjugating enzyme.

Structural analysis will be applied to obtain insight into how co-activator proteins and substrates interact with APC/C. To achieve this goal, a protocol needs to be established that allows isolating sufficient amounts of APC/C that interact with co-activators and substrates in a manner that is functionally relevant. Sample preparation techniques have to be improved to allow studying structures of APC/C at subnanometer resolution using cryo-electron microscopy and single particle analysis.

Furthermore, crystallographic analysis could potentially lead to a better understanding of how co-activator proteins interact with destruction motifs that mark proteins as APC/C substrates.
5. Results

5.1. Substrate binding on the anaphase promoting complex/cyclosome (APC/C)

5.1.1. *In vitro* reconstitution of substrate bound APC/C complexes

During cell cycle progression the anaphase promoting complex/cyclosome (APC/C) is activated at the metaphase-to-anaphase transition and stays active until late G1 phase. APC/C serves as a platform to bring substrate lysines in close proximity of the E2~ubiquitin active site cysteine to allow processive ubiquitin transfer. The co-activator proteins Cdc20 and Cdh1 are believed to mediate binding between APC/C and its substrate molecules by recognizing destruction motif sequences that lie in the primary sequence of the substrates. Therefore, presence of co-activator proteins and wild-type destruction motifs within the substrates can be used to test specific substrate binding to APC/C *in vitro*.

To *in vitro* reconstitute substrate bound APC/C complexes, endogenous human APC/C was purified from logarithmically grown HeLa cells (log-phase HeLa) using anti-CDC27-antibody beads. APC/C bound to anti-CDC27-antibody beads was incubated with recombinantly expressed and purified co-activator and/or substrate proteins which allowed removal of unbound material during subsequent washing steps (*Fig.7a*). The complexes were peptide eluted and analyzed via silver stain and western blot. The majority of log-phase HeLa cells are in G1 phase of the cell cycle which explains why
Figure 7 In vitro reconstitution of substrate bound APC/C complexes. (a) Schematic representation of the in vitro reconstitution protocol. (b) Binding of 3Myc-His-CDC20 and 3Myc-His-CDH1 to log-phase APC/C purified. (c) APC/C incubated with wild-type (wt) or D-box, KEN-box mutant (m) form of His-Flag-td2-Hsl1$^{667-872}$ in the absence or presence of CDH1. (d) APC/C incubated with wild-type (wt) or double D-box mutant (m) form of N70-2x-td2-Flag-His in the absence or presence of CDH1. (e) APC/C incubated with wild-type (wt) or D-box, KEN-box mutant (m) form of Securin-Flag-His in the absence or presence of CDH1. (f) APC/C incubated with wild-type (wt) or KEN-box mutant (m) form of Sororin-td2-Flag-His in the absence or presence of CDH1. Asterisk indicates position of Sororin-td2-Flag-His in the silver gel.
CDH1, not CDC20, preferentially binds APC/C purified from these cells (Fig.7b). Therefore, in this study CDH1 was used to facilitate substrate binding to APC/C.

Using the above described in vitro reconstitution protocol, the four tested substrates (Hsl1^{667-872}, N70-2x, Securin and Sororin) showed specific APC/C binding, meaning that substrate binding was dependent on the co-activator protein CDH1 and wild-type destruction motifs in the substrate molecules (Fig.7c-f).

5.1.2. Model substrates Hsl1^{667-872} and N70-2x bind particularly stably to APC/C

To visualize a substrate molecule bound to APC/C using electron microscopy (EM) and single particle analysis, the complexes need to fulfill three prerequisites. First, the complexes should be relatively abundant (10-100 pmol). Second, the complexes must survive long hours of biochemical purification procedures, before they can be looked at under the microscope. Third, sample homogeneity is of fundamental importance to accelerate subsequent steps calculating a 3D model of the complex of interest. The model substrates Hsl1^{667-872} (Burton and Solomon 2001) and N70-2x (Yamano et al. 2004) seem to satisfy the abundance threshold, since in both cases corresponding substrate bands could clearly be visualized in silver staining experiments (Fig.7c, d).

To test the stability of substrate binding on APC/C, APC/C-CDH1-substrate complexes were subjected to off-rate experiments, in which dissociation rates of the different substrates were examined. Substrate molecules were bound to APC/C as described above. To allow substrates to dissociate off APC/C, beads were exposed to buffer (unbound) for 2, 4 and 6 hours before the complexes were peptide eluted (APC/C bound) and analyzed by western blot (Fig.8). Securin and Sororin tend to dissociate over time (Fig.8c, d), whereas more than ~90% of Hsl1^{667-872} and N70-2x remain APC/C bound after six hours (Fig.8a, b). Furthermore, the presence of a substrate molecule seems to further stabilize the interaction between CDH1 and APC/C (Fig.8a), confirming previous
Results (Matyskiela and Morgan 2009). Therefore, Hsl1\textsuperscript{667-872} and N70-2x seem to be suitable substrate candidates to pursue electron microscopy experiments.

\textbf{Figure 8} Off-rate experiments. (a) Off-rate determination of CDH1 and His-Flag-td2-Hsl\textsubscript{667-872} in absence or presence of Hsl1. (b) Off-rate determination of N70-2x-td2-Flag-His. (c) Off-rate determination of Securin-Flag-His. (d) Off-rate determination of Sororin-td2-Flag-His.

5.1.3. Re-IP protocol to homogenously purify APC/C-CDH1-substrate complexes

\textit{In vitro} reconstitution experiments of APC/C-CDH1-substrate indicated that substrates were bound in substoichiometric amounts (\textbf{Fig.7d, f} and data not shown). Therefore, the possibility remained that APC/C complexes not bound to substrates (apo-APC/C and APC/C-CDH1) were still present, causing heterogeneity of the sample. To homogenously purify substrate bound APC/C-CDH1, a second immunoprecipitation step (re-IP) was introduced pulling on the Flag-tag of the substrate molecules (\textbf{Fig.9a}). Anti-FLAG-M2-antibody beads were tested for their ability to unspecifically recognize APC/C-CDH1, a sample that did not contain any Flag-tag sequences. The Flag peptide eluate only contained traces of APC/C-CDH1, demonstrating its high specificity towards the Flag-tag (\textbf{Fig.9b}).
Next, Securin, N70-2x and Hsl1 were tested for their ability to re-IP purify stoichiometric APC/C-CDH1-substrate complexes, a procedure that takes between 3 and
4 hours. Consistent with substrate off-rate experiments shown in **Figure 8**, highest yields of re-IP purified complexes were obtained only in case of the stably binding model substrates N70-2x and Hsl1\(^{667-872}\) (**Fig.9c-e**). Comparing silver staining intensity of substrate bands with those of APC/C subunits, substrate bound APC/C complexes appear to be more stoichiometric after re-IP purification (**Fig.9d**). Direct comparison of lanes ‘APC/C bound’ and ‘depleted’ allows estimating relative amounts of APC/C complexes that were bound to a substrate molecule. Re-IP purification of APC/C-CDH1-Hsl1\(^{667-872}\) leaves only very little APC/C in the depleted supernatant. Compared to Securin and N70-2x, a much higher portion of APC/C complexes seems to be bound to Hsl1\(^{667-872}\) (**Fig.9e**).

Since the model substrate Hsl1\(^{667-872}\) stably binds a relatively high portion of APC/C molecules purified from log-phase HeLa cells, Hsl1\(^{667-872}\) has been chosen for subsequent electron microscopy experiments.

### 5.1.4. **In vitro** reconstituted APC/C-CDH1-Hsl1 complexes show ubiquitination activity

Efficient binding of Hsl1\(^{667-872}\) to APC/C was strictly dependent on wild-type destruction motifs in Hsl1 and only occurred in the presence of CDH1 (**Fig.7c**), indicating that Hsl1 was bound as a functional substrate. Interestingly, Hsl1 was found to stably bind APC/C for hours (**Fig.8a**). Substrate binding stability of such kind cannot exist *in vivo* since this would contradict the idea of having rapid substrate turnover on APC/C. Therefore, the possibility remains that *in vitro* Hsl1\(^{667-872}\) binds in a manner that is non-physiological.

**APC/C-CDH1-substrate in vitro** reconstitutions as well as off-rate experiments were carried out at 4°C, which might increase stability of substrate binding. Therefore, APC/C-CDH1-Hsl1 complexes were subjected to dissociation experiments carried out at 4°C, 22°C and 37°C to study the influence of the temperature on Hsl1\(^{667-872}\) binding. These experiments showed that at 37°C, Hsl1\(^{667-872}\) dissociates off APC/C-CDH1 much faster.
compared to lower temperatures (Fig.10a). Thus, the in vitro conditions further stabilize Hsl1\textsuperscript{667-872} binding on APC/C.

![Image](image.jpg)

**Figure 10** Biochemical characterization of APC/C-CDH1-Hsl1 complexes. (a) Hsl1 dissociation experiments at different temperatures after 1 hour incubation. (b) APC/C-CDH1-Hsl1 shows substrate ubiquitination activity after incubation with E1, ATP, UBCH10 and ubiquitin (ubi-mix) for 1 hour. (c) CDH1 and Hsl1 off-rate experiments in the absence and presence of the ubi-mix.

To provide further evidence that Hsl1\textsuperscript{667-872} binds as a bona fide substrate in vitro, APC/C-CDH1-Hsl1 complexes were incubated with E1, ATP, UBCH10 and ubiquitin (ubi-mix) at 4°C, which resulted in high molecular weight products (ubiquitin conjugates) that could be detected using an anti-Hsl1-antibody (Fig.10b). Furthermore, Hsl1\textsuperscript{667-872} and CDH1 dissociation rates were drastically increased in the presence of the ubi-mix (Fig.10c), providing the interesting possibility that substrate ubiquitination might also have a role in disassembling APC/C-CDH1-substrate complexes in vivo.

Taken together, in vitro Hsl1\textsuperscript{667-872} binding does not contradict ideas of processive substrate turnover but seems to reflect a particularly stable intermediate of the substrate ubiquitination pathway that can exist only in isolation (in the absence of
accessory proteins required during later steps of the substrate ubiquitination reaction) and only at low temperatures.

5.1.5. D-box and KEN-box of Hsl1 have different binding properties

Figure 11a shows the domain architecture of the Hsl1\textsuperscript{667-872} construct as it was used in binding experiments. Hsl1\textsuperscript{667-872} is fused to the C terminus of tdimer2 (fusion protein of two RFP molecules), which carries a hexahistidine-tag followed by a Flag-tag on its N terminus. The His6-Flag-tdimer2-tag allowed keeping Hsl1\textsuperscript{667-872} soluble during His-tag purification. The Hsl1\textsuperscript{667-872} fragment contains a destruction box (D-box) and a KEN-box, two destruction motifs that are required for co-activator mediated binding to APC/C (Burton and Solomon 2001).

To dissect the contribution of either destruction motif to APC/C binding, mutants were generated in which either the D-box (dbm), the KEN-box (kbm) or both (dkm) were mutated to allow studying their individual binding properties. When APC/C was incubated with CDH1 and equal concentrations of the Hsl1\textsuperscript{667-872} mutants, Hsl1-wt and Hsl1-kbm (only D-box present) were bound in comparable amounts (Fig.11b), much less was bound in the reaction that contained Hsl1-dbm (only KEN-box present), whereas no binding could be detected for the Hsl1-dkm double mutant. Incubation of these complexes with ubi-mix shows a gradual reduction of ubiquitin molecules that are conjugated to Hsl1 (i.e. processivity of the substrates decreases) from Hsl1-wt over Hsl1-kbm to Hsl1-dbm (Fig.11c). Decreased processivity of the ubiquitin transfer reaction was related to decreased binding affinity of substrates to APC/C (Carroll and Morgan 2002; Rape et al. 2006), suggesting that the D-box in Hsl1 has a higher binding affinity than the KEN-box. Therefore, off-rate experiments were performed to study the individual contribution of the two different destruction motifs to the stability of Hsl1\textsuperscript{667-872} binding on APC/C (Fig.11d). These experiments reproducibly showed that in vitro the D-box of
Hsl1 binds APC/C more stably than the KEN-box, however the D-box alone was not sufficient to restore wild-type Hsl1^{667-872} binding (Fig.11e). These observations suggest that D-box and KEN-box have additive or cooperative effects on APC/C binding when present in one substrate molecule which could imply that D-box and KEN-box...
destruction motifs bind different sites on APC/C-CDH1 (Burton et al. 2005; Carroll et al. 2005).

5.1.6. **Substrate binding on APC/C occurs between the co-activator protein CDH1 and the processivity factor DOC1**

Since stable APC/C-CDH1-Hsl1 complexes could be purified in sufficient amounts, it seemed feasible to study these complexes by electron microscopy and single particle analysis. EM experiments were carried out in collaboration with Prof. Dr. Holger Stark at the Max-Planck Institute for Biophysical Chemistry in Goettingen. APC/C-CDH1-Hsl1 complexes were re-IP purified and subjected to gradient fixation (GraFix) (Kastner et al. 2008) to further stabilize the complexes. Particles in the peak fractions were adsorbed to electron microscopy grids that were coated with a thin carbon layer, negative stained using 2% (w/v) uranyl formate and snap-frozen in liquid nitrogen (**Fig.12a**). About 14,000 2D projections of individual particles were classified and averaged to increase the signal-to-noise ratio (**Fig.12b**). Angular reconstruction of the averaged 2D projections (class averages) produced a 3D model of APC/C-CDH1-Hsl1 which revealed two additional densities compared to the APC/C-CDH1 reference structure (**Fig.12c**). One extra density was intercalated between the co-activator protein CDH1 and the APC/C core subunit DOC1 (Destruction of Cyclin B1). This density does not disappear by increasing surface rendering thresholds, meaning that nearly 100% of the particles contained an extra mass in this position. Therefore, the additional mass between CDH1 and DOC1 is referred to as the major density. The second additional density could be resolved in the platform region and partially disappeared by increasing surface rendering thresholds. Since the second extra mass seemed to be less abundant (present only in a subpopulation of APC/C molecules contained in the dataset), it is referred to as the minor density (**Fig.12c**).
The Hsl1^{667-872} fragment has been shown to bind co-activator proteins via both D-box and KEN-box recognition motifs (Burton et al. 2005), suggesting that both motifs bind in close proximity of CDH1 in the 3D model of APC/C-CDH1-Hsl1. The APC/C subunit DOC1 has been implicated in substrate recognition (Passmore et al. 2003; Carroll et al. 2005) and processive substrate ubiquitination (Carroll and Morgan 2002), which led to the hypothesis that on APC/C, apart from the co-activator proteins, also DOC1 has a direct role in stabilizing substrate binding. Since the 3D model of APC/C-CDH1-Hsl1 revealed a
prominent density intercalated between CDH1 and DOC1, this density most likely corresponds to the Hsl1$^{667-872}$ fragment (Fig.13a, b).

Furthermore, it could be observed that substrate binding seems to induce structural rearrangements in the catalytic module consisting of the subunits APC2 and APC11. Normally APC2/11 form a contact to an unknown subunit in the platform domain which is absent in APC/C-CDH1-Hsl1. Instead, in the substrate bound form APC2/11 contact CDH1 in close proximity to where the substrate molecule binds (Fig.13b). These results
Results suggest that a substrate molecule forms several contacts on APC/C and induces conformational changes in the catalytic subunits APC2 and APC11. These findings were published in “Buschhorn and Petzold et. al., Substrate binding on the APC/C occurs between the coactivator Cdh1 and the processivity factor Doc1, Nat.Struct.Mol.Biol., 18 (6-12), 2011.” which can be found in the appendix of this thesis.

Even though the major density could be inferred to be the Hsl1^{667-872} fragment, the source that was causing the minor density was still unknown (Fig.12c). Three different hypotheses could potentially explain the nature of the minor density (Fig.13c). First, the Hsl1^{667-872} fragment contains two different destructions motifs, D-box and KEN-box, which showed different binding properties in dissociation experiments and might have different binding sites on APC/C (Burton et al. 2005; Carroll et al. 2005). Therefore, it could be possible that major and minor density correspond to D-box and KEN-box regions in Hsl1^{667-872}, respectively. This assumption is supported by the fact that the volume of the major density is too small to accommodate a 206 amino acid fragment (Hsl1^{667-872}), suggesting that parts are either unstructured or bound elsewhere. Second, since no physical connection could be resolved between major and minor densities, it could even be possible that two individual substrate molecules were bound to APC/C. Third, since tdimer2-Hsl1 has been used for EM experiments, it could be that the globular tdimer2-tag by chance was located in the platform region causing a less abundant extra density (due to flexibility) in this position.

5.1.7. D-box and KEN-box simultaneously bind APC/C-CDH1

EM experiments of APC/C-CDH1-Hsl1 revealed two additional densities in the structure of the APC/C. The major density could be inferred to parts of Hsl1^{667-872}, whereas the source of the minor density could not be identified yet.
To follow the idea that D-box and KEN-box motifs bind different sites on APC/C-CDH1, in principle it should be possible to bind Hsl1-kbm and Hsl1-dbM to APC/C-CDH1 simultaneously. To discriminate two substrate molecules being bound to APC/C using re-IP experiments, the two substrate constructs must differ in at least two parameters. Therefore, maltose binding protein tagged Hsl1 (MBP-Hsl1) was created that first differs in size compared to Flag-tdimer2-Hsl1 (Flag-td2-Hsl1) and second does not contain a Flag-tag. After incubation of APC/C-CDH1 with both substrate constructs, one can ask whether anti-Flag immunoprecipitation (pulling on Flag-td2-Hsl1) can co-precipitate the non-Flag-tagged MBP-Hsl1 (Fig.14a). Presence of both substrate constructs in the Flag-peptide eluate can be visualized on a protein gel due to size differences of Flag-td2-Hsl1 and MBP-Hsl1.

In order to test whether independent binding sites for D-box and KEN-box destruction motifs exist, APC/C-CDH1 was incubated with either Flag-td2-Hsl1-kbm, MBP-Hsl1-dbM or an equimolar mixture of both constructs. After CDC27-peptide elution (Fig.14b “APC/C bound”), the eluate was incubated with anti-FLAG-M2-antibody beads to re-IP purify all APC/C complexes that were bound to Flag-td2-Hsl1-kbm (Fig.14b “Flag”), leaving remaining complexes in the depleted supernatant (Fig.14b “depleted”). Lanes 1-6 in Figure 14b show that anti-FLAG-M2-antibody beads are specific towards the Flag-tag sequence in Flag-td2-Hsl1, since APC/C complexes bound to MBP-Hsl1 could not be re-IP purified (compare lane 3 and 6). Interestingly, when APC/C was incubated with an equimolar mixture of both constructs, MBP-Hsl1-dbM could be found in the Flag-peptide eluate (Fig.14b lane 9), indicating that MBP-Hsl1-dbM was bound to APC/C-CDH1-[Flag-td2-Hsl1-kbm]. Importantly, in control experiments containing the Hsl1 constructs and CDH1 but no APC/C, MBP-Hsl1 cannot be co-purified in such amounts (Fig.14c, see also Fig.15b, compare lane 4 and 9). However, a faint band representing MBP-Hsl1-dbM could still be co-purified in control experiments containing CDH1 and Flag-td2-Hsl1-kbm which was dependent on the KEN-box motif (Fig.14c, lane 4 and 5).
This demonstrates that in vitro CDH1 does not bind D-box and KEN-box sequences in a 1:1 stoichiometry. The possibility remains that the in vitro conditions lock CDH1 in a certain conformational state that does not allow simultaneous binding of the two destruction motifs. However, it could also be possible that binding of CDH1 to APC/C is
required to allow direct interaction of both destruction motifs with the co-activator protein.

Taken together, D-box and KEN-box sequences in Hsl1 can be bound to APC/C-CDH1 at the same time. However, it is unclear whether both D-box and KEN-box destruction motifs were bound to CDH1 in the major position, or whether one of the two motifs would cause the minor density in the platform region.

5.1.8. Identification of a second bona fide substrate binding site on APC/C-CDH1

D-box and KEN-box recognition sequences in Hsl1 simultaneously bind APC/C-CDH1. However, it is unclear whether this could explain the existence of a second extra density in the platform region of the APC/C-CDH1-Hsl1 structure (Fig.12c). Since no physical connection could be resolved between major and minor density, the possibility remained that the two densities correspond to two individual Hsl1 molecules that were bound in either position.

To test this possibility, in vitro binding of the two substrates was repeated, but this time using Hsl1\(^{667-872}\) sequences that both contained wild-type destruction motifs. Unexpectedly, incubation of APC/C-CDH1 with equimolar concentrations of Flag-td2-Hsl1-wt and MBP-Hsl1-wt considerably increased the portion of non-Flag-tagged Hsl1 that could be co-purified via APC/C-CDH1-[Flag-td2-Hsl1] complexes (Fig.15a). Co-purification of MBP-Hsl1 was clearly dependent on the presence of APC/C (Fig.15a, compare lanes 9 – 11), which clearly demonstrates the existence of a second substrate binding site on APC/C.

Repetition of these experiments using different combinations of destruction motifs in either substrate construct showed that, apart from the presence of APC/C, the ability to co-purify a second substrate molecule was strictly dependent on the existence of at least one destruction motif in the non-Flag-tagged Hsl1 molecule (Fig.15b). However, no
second substrate molecule could be co-purified when using two KEN-box recognition sequences in both constructs (Fig. 15b, lane 5), which could be due to the fact that a D-box domain is necessary to stimulate association between a CDH1-substrate complex and the APC/C (Burton et al. 2005).

Figure 15 Identification of two substrate binding sites on APC/C-CDH1. (a) Two wild-type Hsl1 molecules can be bound to APC/C-CDH1. Asterisks indicate position of His-MBP-Hsl1-wt in the Flag eluate after re-IP purification. (b) Different combinations of Hsl1 wild-type (wt), KEN-box mutant (kbm), D-box mutant (dbm) and KEN-box, D-box mutant (dkm) re-IP purified in the presence of APC/C-CDH1, only CDH1 or in the absence of both.
Co-purification experiments of two substrate molecules bound to APC/C produced best results when two wild-type Hsl1 molecules were used. Therefore, it seems that the second Hsl1 binding site represents a so far unidentified second *bona fide* substrate binding site on APC/C, to which substrates bind in a manner dependent on both D-box and KEN-box destruction motifs.

### 5.1.9. Identification of a second co-activator binding site on APC/C

Substrate binding on APC/C is dependent on co-activator proteins (Fig.7). *In vitro* substrate binding experiments revealed the existence of two *bona fide* substrate binding sites on human APC/C-CDH1 which raises the possibility that binding of the second substrate molecule is mediated by a second CDH1 molecule. To test the existence of a second CDH1 binding site on APC/C, two differently labeled CDH1 molecules were used that differed in size due to the presence of a 3Myc-tag followed by a hexahistidine-tag (3MH6-tag) on the N terminus of one of the co-activator constructs.

CDH1 is a delicate protein that easily precipitates in non-optimal buffer conditions. Therefore, when 3Myc-CDH1 and untagged CDH1 were mixed in a buffer containing 150mM NaCl, 3Myc-CDH1 seemed to co-purify the untagged form of CDH1 (Fig.16a), which actually reflects CDH1 aggregation, not dimerization (see section 5.2.2. Optimization of the 3MH6-CDH1 purification protocol). Different buffer conditions were screened for their ability to abolish CDH1 aggregation (Fig.16b). Only 180mM ammonium sulfate completely stopped CDH1 aggregation in this assay which is consistent with recent buffer optimizations used for the purification of recombinant human CDH1 (see section 5.2.2. Optimization of the 3MH6-CDH1 purification protocol). However, due to the fact that high-salt conditions cause APC/C disassembly (Vodermaier et al. 2003) (Fig.16c, d), 250mM KCl seemed to be the best compromise between CDH1 stability and APC/C integrity, even though ubiquitination activity was lost.
due to loss of the small subunit APC11 which recruits E2-Ub to APC/C (Leverson et al. 2000). Nevertheless, loss of APC11 (and partial loss of APC2) did not markedly change CDH1 and Hsl1 binding to APC/C (Fig. 16c, d lanes 1-3) which indicates that APC11 is dispensable for APC/C co-activator interaction (Vodermaier et al. 2003; Thornton et al. 2006).

Figure 16 Optimization of buffer conditions for APC/C-CDH1 biochemistry. (a) Myc-IP purification of 3Myc-His-CDH1, untagged CDH1 or a mixture of both in the absence of APC/C. (b) Myc-IP purification of a mixture of 3Myc-His-CDH1 and untagged CDH1 in different buffer conditions in the absence of APC/C. (c) In vitro reconstitution of APC/C-CDH1-Hsl1 in different buffer conditions and peptide eluted with antigenic CDC27 peptide. (d) Peptide eluted APC/C-CDH1-Hsl1 reconstituted in different buffer conditions incubated with ubi-mix for 1 hour at 4°C.
Figure 17 Identification of two CDH1 binding sites on APC/C. (a) Schematic representation of the protocol used to distinguish two co-activator binding sites on APC/C. (b) Myc-IP purification of 3Myc-His-CDH1, untagged CDH1 or a mixture of both in the presence and absence of APC/C using a 250mM KCl buffer. (c) Myc-IP purification of 3Myc-His-CDH1, untagged CDH1 or a mixture of both in the presence of His-Flag-td2-Hsl1-kbm in the presence and absence of APC/C.
To explore the possibility that APC/C has two co-activator binding sites, APC/C was first incubated with either 3Myc-CDH1, untagged CDH1 or an equimolar mixture of both proteins in the absence of substrate proteins (Fig. 17a). After CDC27-peptide elution (“APC/C bound”), the eluate was incubated with anti-Myc-antibody beads to re-IP purify all APC/C complexes that were bound to 3Myc-CDH1 (“Myc”), leaving remaining complexes in the depleted supernatant (“depleted”). Lanes 1 – 6 in Fig. 17b show that anti-Myc-antibody beads are specific towards the Myc-tag in 3Myc-CDH1, since APC/C complexes bound to untagged CDH1 could not be re-IP purified (compare lane 3 and 6). Unexpectedly, when APC/C was incubated with an equimolar mixture of both constructs, untagged CDH1 could be co-purified with APC/C bound to 3Myc-CDH1 which was strictly dependent on the presence of APC/C (Fig. 17b, compare lane 9 and 10).

The same experiment was repeated, this time including Hsl1\textsuperscript{667-872-kbm} (only D-box present). Presence of a substrate molecule seemed to increase the portion of co-purified untagged CDH1 (Fig. 17c, lane 9), which might be explained by the finding that co-activator binding on APC/C is stabilized in the presence of a substrate molecule (Matyskiela and Morgan 2009) (Fig. 8a).

Taken together, co-purification experiments using two differently labeled CDH1 constructs revealed the presence of a second CDH1 binding site on APC/C. CDH1 binding to this second site can occur in the absence of substrates and is at most stabilized (but not induced) upon substrate binding. Nevertheless, the second CDH1 molecule most likely mediates binding of the second substrate molecule. Since two additional densities were observed in the structure of APC/C-CDH1-Hsl1, it is tempting to speculate that on human APC/C two CDH1-substrate binding modules exist, one in the major co-activator binding region and one in the platform region (Fig. 18).
5.2. Purification and crystallization of human CDH1

5.2.1. Human co-activator proteins are stabilized by an N-terminal 3Myc-His6-tag

The co-activator proteins Cdh1 and Cdc20 are required to mediate cell cycle regulated proteolysis of APC/C substrates (Visintin et al. 1997). Co-activator proteins contain a C-terminal WD40 domain that binds APC/C substrates (Kraft et al. 2005) by recognizing so-called destruction motifs that are short in sequence and poorly conserved. Due to their poor conservation (D-box: RxxLxxxN/Q/E; KEN-box: KENxxxN/D/E) multiple destruction motifs can be found in the primary sequence of APC/C substrates, but only a subset of these sequences act as actual degradation signals (Goh et al. 2000; Pfleger and Kirschner 2000). Structural information will be required to provide a rationale of how co-activator proteins interact with cognate destruction motifs. Folding of APC/C co-activators has been reported to depend on the CCT chaperonin complex (Camasses et al. 2003) which is the eukaryotic counterpart of the bacterial
GroEL/GroES protein folding machinery. So far, purified amounts of co-activator proteins were very small despite the fact that eukaryotic expression systems have been utilized (Kraft et al. 2006).

**Figure 19** 3Myc-His6-tagged human CDH1 (3MH6-CDH1) yields high amounts of functional protein. (a) 3MH6-CDH1 compared to an older CDH1 construct previously used for protein expression and purification. Similar amounts of the insoluble fraction after cell lysis and centrifugation and Ni-affinity purified protein form the soluble fraction were run on SDS-PAGE for comparison. (b) 3MH6-CDH1 binds APC/C and can be used for re-IP purification of APC/C-CDH1 via the Myc-tag. 3MH6-CDH1 comigrates with with the APC/C subunit CDC23. (c) 3MH6-CDH1 mediates binding of Securin to APC/C in a D-box, KEN-box dependent manner similar to previously used CDH1 constructs. Asterisks indicate endogenous CDH1.

New CDH1 constructs were designed with the aim of doing APC/C re-IP purifications pulling on the co-activator protein CDH1. When CDH1 was tagged with an N-terminal 3Myc-His6-tag (3MH6-CDH1), Ni-affinity purification from similar amounts of Baculovirus-infected Sf9 cells led to ~15-fold higher protein yields compared to other
CDH1 constructs (**Fig.19a**). Using N-terminally tagged 3MH6-CDH1, less protein was found in the insoluble fraction after cell lysis (**Fig.19a**). Similar stabilizing effects could be observed for 3MH6-CDC20 (data not shown), suggesting a general mechanism of how the 3Myc-His6-tag stabilizes human co-activator proteins. 3MH6-CDH1 binds and re-IP purifies APC/C (**Fig.19b**) and facilitates substrate binding in a D-box/KEN-box dependent manner that is similar to previously used CDH1 constructs (**Fig.19c**). 3MH6-tagged co-activator proteins triggered APC/C-dependent substrate ubiquitination and destruction in a variety of assays (data not shown).

Thus, the 3Myc-His6-tag largely stabilizes human co-activator proteins without affecting their functional properties.

### 5.2.2. Optimization of the 3MH6-CDH1 purification protocol

Even though the N-terminal 3Myc-His6-sequence seemed to stabilize CDH1 during cell lysis and Ni-affinity purification, a large portion of the protein eluted in the void volume after size exclusion chromatography (SEC), suggesting that CDH1 aggregated over time using regular buffer compositions (Kraft et al. 2006) (**Fig.20**). Therefore, buffer conditions had to be optimized to stabilize CDH1 during the entire purification procedure. The Hofmeister series classifies ions with respect to their ability to stabilize proteins (Zhang and Cremer 2006). According to Hofmeister’s series ammonium sulfate is a better kosmotrope (kosmotropes stabilize proteins by strengthening hydrophobic interactions) than potassium chloride, so ammonium sulfate was tested for its ability to stabilize CDH1. Using ammonium sulfate, yields after Ni-affinity purification did not change; however, during size exclusion chromatography 3MH6-CDH1 elutes in a single peak and is absent in the void volume (**Fig.21**). Usage of ammonium sulfate did not markedly change the elution volume of the 3MH6-CDH1 peak, suggesting that no major differences are introduced into the folded state of CDH1 using different salt conditions.
Size exclusion chromatography of 3MH6-CDH1 revealed an apparent molecular weight of \( \sim 154 \text{kDa} \) which could correspond to a CDH1 dimeric form. However, 3D models of APC/C bound to CDH1 and CDC20 only showed a bound monomeric form (Herzog et al.)
2009) (Fig.13a, b), and in vitro experiments confirmed that in ammonium sulfate conditions, 3MH6-CDH1 did not co-purify untagged CDH1 (Fig.16c). Therefore, the unusually large hydrodynamic radius during size exclusion chromatography either reflects an elongated structure of full-length CDH1, presence of disordered regions (e.g. 3Myc-His6-tag, N terminus of CDH1), or both.

**5.2.3. PreScission protease cleavage of the 3MH6-tag does not destabilize CDH1**

The N-terminal 3Myc-His6-tag has a huge impact on CDH1 stability/solubility during cell lysis and Ni-affinity purification. However, size exclusion chromatography is consistent with disordered regions being present in 3MH6-CDH1, which could hinder ordered protein crystallization.

![Figure 22](image)

Figure 22 PreScission cleavable version 3Myc-His6-tagged human CDH1. (a) Schematic representation of 3MH6-CDH1 and the PreScission cleavable form 3MH6-PS-CDH1. (b) PreScission protease treatment of purified 3MH6-PS-CDH1 over time at 4°C.

Since the 3Myc-His6-tag was suspected to be unstructured, a PreScission cleavage site was inserted (3MH6-PS-CDH1) to study the influence of the 3Myc-His6-tag on CDH1
stability and its hydrodynamic radius during size exclusion chromatography (Fig. 22a).

Incubation of Ni-affinity purified 3MH6-PS-CDH1 with excess amounts of PreScission protease resulted in almost complete cleavage after one hour incubation at 4°C (Fig. 22b). Interestingly, cleaved CDH1 did not seem to precipitate during PreScission protease treatment.

Figure 23 Purification of PreScission cleavable 3MH6-PS-CDH1. (a) SDS-PAGE of Ni-affinity purified 3MH6-PS-CDH1. Dialysis and PreScission protease cleavage was carried out at 4°C for 2 hours. Ni-affinity purification of the PreScission protease treated 3MH6-PS-CDH1 efficiently removed uncleaved 3MH6-PS-CDH1 and contaminating proteins. Cleaved CDH1 could be found in the flow through after the Ni-affinity column. Asterisks indicate uncleaved 3MH6-PS-CDH1. (b) Elution profile of 3MH6-PS-CDH1 (dashed blue) and cleaved CDH1 (yellow) after size exclusion chromatography using a S200 16/60 column. Asterisks indicate fractions of cleaved CDH1 used for SDS-PAGE. (c) SDS-PAGE of cleaved CDH1 before and after size exclusion chromatography. Peak fractions of cleaved CDH1 were pooled and concentrated up to 3mg/ml final protein concentration.
To remove the released 3Myc-His6-tag, uncleaved 3MH6-PS-CDH1 and His6-PreScission protease, a second Ni-affinity purification was introduced after PreScission protease treatment and imidazole removal via dialysis. The flow through of the second Ni-affinity purification showed efficient removal of uncleaved 3MH6-PS-CDH1 and contaminating proteins, leaving almost pure cleaved CDH1 (Fig.23a). When this sample was concentrated and applied to size exclusion chromatography, cleaved CDH1 eluted in a single peak and was absent in fractions of the void volume (Fig.23b, c). Cleaved CDH1 (Mr = 55kDa) eluted with an apparent molecular weight of ~126kDa; protein concentration via centrifugal filter devices before and after size exclusion chromatography was very slow and cleaved CDH1 could not be concentrated above 3mg/ml (Fig.23c).

Therefore, removal of the 3Myc-His6-tag after Ni-affinity purification does not destabilize cleaved CDH1. However, behavior during protein concentration suggested the construct to be suboptimal for protein crystallography. Protein crystals could never be grown using cleaved full-length CDH1.

5.2.4. The N terminus of CDH1 is predicted to be disordered

Full-length 3Myc-His6-tagged and cleaved CDH1 could not be concentrated to 10mg/ml or higher, suggesting that the full-length protein is not suitable for protein crystallography. Therefore, bioinformatic analysis was performed to define a deletion construct of CDH1 that would be suitable for crystallization experiments. CDH1 primary sequence was analyzed using PONDR-FIT (Xue et al.) to identify intrinsically disordered regions, which predicted the first 200 amino acids to be unstructured (Fig.24a). Secondary structure prediction found 6 WD40-repeats ranging from amino acids Gln218 to Asn471. However, in CDC20 only the first 120 amino acids were predicted to be disordered and 7 WD40-repeats were found by the Single Modular
Architecture Research Tool (SMART) (Letunic et al.) (Fig.24b). Multiple sequence alignment of Cdh1 and Cdc20 protein sequences from different species revealed high conservation of the central and C-terminal region, starting from residue Ser163 in human CDH1 and Ser159 in human CDC20 (Fig.24c).

Figure 24 Bioinformatic analysis of human CDH1 and CDC20. (a) Analysis of intrinsically disordered regions in human CDH1 using PONDR-FIT. A disorder disposition above 0.5 illustrates regions that are predicted to be disordered. Secondary structure prediction using SMART predicts 6 WD40-repeats in the C-terminal region of human CDH1 ranging from amino acid 218 to 471. (b) Analysis of intrinsically disordered regions in human CDC20 using PONDR-FIT as shown in a. Secondary structure prediction using SMART predicts 7 WD40-repeats in the C-terminal region of human CDC20 ranging from amino acid 169 to 471. (c) Multiple protein sequence alignment of full-length CDH1 and CDC20 proteins from Homo sapiens, Mus musculus, Bos taurus, Danio rerio and Xenopus tropicalis using Jalview. Physical properties of conserved residues are ClustalX color coded. Asterisks indicate positions of residues that were implicated in D-box binding (Kraft et al. 2005).
This region has been implicated in mediating substrate binding (Hilioti et al. 2001). Considering the high degree of sequence conservation between Cdh1 and Cdc20 and given that both recognize APC/C substrates by binding destruction motifs (Glotzer et al. 1991; Pfleger and Kirschner 2000; Burton and Solomon 2001; Burton et al. 2005), it is likely that CDH1 residues Ser163 to Trp212/Ser213 contain a WD40-repeat that could not be predicted.

Furthermore, existence of an additional N-terminal WD40-repeat in CDH1 is supported by the fact that CDH1 residues Leu179 and Pro182 were implicated in D-box binding (Kraft et al. 2005) (Fig.24c).

5.2.5. Limited proteolysis removes the N terminus of CDH1

Bioinformatic analysis suggested that CDH1 N-terminal regions are disordered. To determine boundaries of the WD40 domain by biochemical means (Fig.25a), CDH1 was treated with 1μg chymotrypsin to allow proteolytic cleavage of disordered regions. Two stable fragments with a size of ~34kDa appeared after 30 minutes that could not be detected using antibodies directed against N-terminal regions (Fig.25b). At later time points also the antibody directed against the very C terminus of CDH1 failed to detect these stable fragments. Similar results were obtained using 1μg trypsin. EDMAN sequencing revealed identical N-terminal sequences for both fragments starting at residue Ser149 (Fig.25c), suggesting differences in the C terminus. Importantly, chymotrypsin failed to cleave at position Trp176, Trp188 and Tyr189, which were predicted to be high-specificity cleavage sites using the ExPASy PeptideCutter tool (Wilkins et al. 1999), indicating that the WD40 domain of CDH1 starts around residue Ser163 (Fig.24c).
Figure 25 Limited proteolysis of cleaved CDH1. (a) Schematic representation of cleaved human CDH1. N- and C-terminal regions recognized by peptide antibodies used in b. (b) Limited proteolysis time course of cleaved CDH1 at 4°C. Peptide antibodies were used that recognize sequences in N- and C-terminal regions as shown in a. (c) Primary sequence of human CDH1 from Asp141 to Leu190. Sequence derived from EDMAN sequencing of the two degradation products is shown in yellow. Asterisks indicate high-probability chymotrypsin cleavage sites.

5.2.6. 3MH6-CDH1 deletion constructs bind Hsl1

Based on bioinformatic and biochemical analysis and with great help of Marc Jarvis different combinations of N- and C-terminal deletions (Fig.26) were tested in Ni-affinity purifications and size exclusion chromatography. Solubility of the deletion constructs was still dependent on the presence of an N-terminal 3Myc-His6-tag. Constructs lacking the last 21 amino acids at the C terminus (dC21) were insoluble. N-terminal deletions dN148 and dN162 behaved similarly during size exclusion chromatography, indicating
that deleting the first 162 amino acids did not destabilize the fold of the WD40 domain. Therefore, 3MH6-CDH1 dN162 dC14 (162-14) was selected for further experiments. During size exclusion chromatography 162-14 eluted in a single peak with an apparent molecular weight of 36kDa (Fig.27a), suggesting a compact fold of the WD40 domain (Fig.27c).

**Figure 26** Multiple sequence alignment of full-length CDH1 from *Homo sapiens, Mus musculus, Bos taurus, Danio rerio, Gallus gallus, Xenopus tropicalis, Saccoglossus kowalevskii, Salmo salar, Drosophila melanogaster* and *Shizosaccharomyces pombe* (from top to bottom). (a) Schematic representation of the aligned regions shown in b and c. (b) Sequence alignment showing the N-terminal boundary of the WD40 domain. Asterisks indicate two possible ends of the first WD40 repeat in CDH1 (end with WS). (c) Sequence alignment of CDH1 at its C terminus. Asterisk indicate end of the seventh WD40 repeat (ends with WN).
Importantly, after co-expression of 162-14 and untagged Hsl1\textsuperscript{667-872}, 162-14 could co-purify Hsl1\textsuperscript{667-872} (162-14 + Hsl1 complex), meaning that the CDH1 WD40 domain was still interacting with the substrate protein Hsl1\textsuperscript{667-872}. During size exclusion chromatography the 162-14 + Hsl1 complex eluted in one peak with an apparent molecular weight of 108kDa (Fig.27a, b) which differed from the predicted molecular weight by an extra mass of 44kDa (Fig.27c).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure27.png}
\caption{Purification of 3MH6-CDH1 dN162 dC14 (162-14) in absence and presence of Hsl1\textsuperscript{667-872}. (a) Elution profiles of 162-14 (dashed blue) and 162-14 + Hsl1\textsuperscript{667-872} (yellow) after size exclusion chromatography using a S200 16/60 column. (b) Peak fractions of 162-14 + Hsl1\textsuperscript{667-872} after size exclusion chromatography. (c) Predicted molecular weights of 162-14 +/- Hsl1\textsuperscript{667-872} compared to apparent molecular judged from the hydrodynamic radius after size exclusion chromatography. (d) Limited proteolysis of 162-14 +/- Hsl1\textsuperscript{667-872}. (e) N-terminal primary sequence of 3MH6-CDH1 dN162. Asterisks indicate high-probability trypsin cleavage sites.}
\end{figure}
Therefore, either large portions of the 206 amino acids of Hsl1<sup>667-872</sup> were unstructured or two CDH1 WD40 domains were interacting with one Hsl1<sup>667-872</sup> molecule. Only minor cleavage products of 3MH6-CDH1 dN162 dC14 were observed in limited proteolysis experiments using 1μg trypsin (Fig.27d) which presumably cleaved in the 3Myc-His6-tag (Fig.27e), confirming compactness of 162-14. Interestingly, when the 162-14 + Hsl1 complex was subjected to limited proteolysis Hsl1<sup>667-872</sup> rapidly disappeared which supports the hypothesis that large portions of Hsl1<sup>667-872</sup> are unstructured (Fig.27d). 162-14 and 162-14 + Hsl1 could be concentrated to 10mg/ml and higher (Fig.27b) making those suitable candidates for protein crystallography. However, no crystals could be grown using either construct.

### 5.2.7. 3MH6-CDH1 dN162 dC14 interacts with minimal version of Hsl1

Since major parts of Hsl1<sup>667-872</sup> seemed to be disordered when bound to 3MH6-CDH1 162-14, Hsl1 was trimmed to a minimal version that still contained D-box and KEN-box recognition sequences (Hsl1<sup>768-842</sup>; Hsl1-Mini). Hsl1-Mini could still bind 162-14 and eluted in one peak during size exclusion chromatography (Fig.28a-c). 3MH6-CDH1 162-14 + Hsl1-Mini could be crystallized in the presence of 100mM sodium cacodylate pH6.5, 300mM ammonium sulfate and 30% (w/v) PEG8000 (Fig.28d). Unfortunately, these crystals produced only faint and streaky X-ray diffraction patterns at the ESRF synchrotron in Grenoble (Fig.28e) suggesting serious crystal defects (high mosaicity).

### 5.2.8. 1MH6-CDH1 dN162 dC14 crystallizes in the presence of a D-box peptide

The 162-14 + Hsl1-Mini crystals seemed to be highly disordered (Fig.28e). In vitro experiments showed that full-length CDH1 could bind only one destruction motif at a time (Fig.14c). Furthermore, full-length CDH1 seems to have a preference towards the
D-box recognition motif since only little CDH1 could be co-purified with Flag-Hsl1-dbm (only KEN-box present; Fig.29a, b).

Figure 28 Purification of 162-14 in complex with Hsl1-Mini. (a) Elution profile of 162-14 + Hsl1-Mini after size exclusion chromatography using a S75 16/60 column. Asterisks indicate 162-14 + Hsl1-Mini peak fractions used for SDS-PAGE. (b) SDS-PAGE of 162-14 + Hsl1-Mini peak fractions after size exclusion chromatography. (c) Predicted molecular weights of 162-14 +/- Hsl1-Mini compared to apparent molecular judged from the hydrodynamic radius after size exclusion chromatography. (d) 162-14 + Hsl1-Mini crystals. (e) X-ray diffraction of a 162-14 + Hsl1-Mini crystal. Resolution at the upper left corner equals 4Å.

CDH1-KEN-box interaction does not seem to be outcompeted by adding a D-box containing substrate (Fig.29b compare lane 1 and 4), suggesting that both motifs bind to different sites on CDH1. Assuming that 162-14 exhibits similar D-box/KEN-box binding properties mosaicity of the 162-14 + Hsl1-Mini crystals might either be caused by
Results

complexes that are heterogeneous in their KEN-box bound state or simply by flexibility of ~60 amino acids in Hsl1-Mini due to an unbound KEN-box (Fig.29c). Therefore, it was decided that in future experiments D-box peptides are going to be used to eliminate the possibility that flexible substrate sequences cause crystal disorder.

In addition, 162-14 constructs with N-terminal 3Myc-, 2Myc- and 1Myc-His6-tag were tested for solubility during Ni-affinity purification and size exclusion chromatography. 1MH6-CDH1 162-14 could be purified in usual amounts and was as stable as the other two constructs (Fig.30a, b), providing an even more compact version of 162-14.

![Figure 29](image)

**Figure 29** *In vitro* binding of D-box and KEN-box destruction motifs to full-length CDH1. (a) Flag eluates after re-IP purification of His-Flag-tet2-Hsl1-kbm and/or His-MBP-Hsl1-dbm in the presence or absence of CDH1. (b) Flag eluates after re-IP purification of His-Flag-tet2-Hsl1-dbm and/or His-MBP-Hsl1-kbm in the presence or absence of CDH1. Blots shown in a and b were run on one gel and blots are derived from the same exposure. Asterisks indicate C-terminal degradation products of His-Flag-tet2-Hsl1<sup>667-872</sup>.

(c) Possible scenarios that potentially cause high mosaicity of the 162-14 + Hsl1-Mini crystals.

With the help of Marc Jarvis, purified 1MH6-CDH1 162-14 mixed with equimolar amounts of an Hsl1 D-box peptide (EQKPKRAALSĐTNSFNKM) led to the formation of
small crystals in the presence of 100mM sodium cacodylate pH6.5 and 1.0M sodium citrate. Intriguingly, sodium cacodylate was already found to help crystallization of 162-14 + Hsl1-Mini (Fig.28d) which prompted us to repeat commercially available crystal screens adding 100mM sodium cacodylate to each condition. By doing so, crystal growth could be observed in a variety of different conditions.

Figure 30 3Myc-, 2Myc- and 1Myc-His6-CDH1 dN162 dC14. (a) Ni-affinity purification of 3Myc-, 2Myc- and 1Myc-His6-CDH1 dN162 dC14. (b) Elution profile of 3Myc-, 2Myc- and 1Myc-His6-CDH1 dN162 dC14 after size exclusion chromatography using a S200 10/30 column. In all cases, 162-14 did not elute in the void volume. (c) X-ray diffraction of a 1MH6-162-14 + D-box peptide crystal bundle.
Crystals grown in 150mM sodium cacodylate, 2.0M ammonium sulfate and 10mM magnesium sulfate showed major improvements judged from their X-ray diffraction (Fig.30c) that was recorded with the help of Brenda A. Schulman and Nicholas G. Brown. However, since 162-14 + Hsl1 D-box peptide crystals grew in small bundles containing multiple crystals, diffraction patterns were caused by multiple orientations which so far impede structure determination.

5.3. APC/C sample preparation for high resolution cryo-EM

5.3.1. Prerequisites for high resolution cryo-electron microscopy

The anaphase promoting comlex/cyclosome (APC/C) is a 1.5MDa multiprotein complex which in its dimensions is comparable to the eukaryotic ribosome. Structural elucidation of APC/C is hampered by the following limitations: (i) inherent conformational flexibility of the complex (Dube et al. 2005) and (ii) limited amounts of endogenous APC/C that can be purified from eukaryotic cells (Herzog and Peters 2005). Subnanometer resolution (<10Å) of the holoenzyme will be required to obtain detailed mechanistic insight into the molecular functioning of this unusually large E3 ubiquitin-ligase (Peters 2006).

Electron microscopy has been shown to be suitable for determining high resolution structures (Zhang et al.) which at the same time requires markedly less material (about 20μl of ~50μg/ml APC/C; can be used for 2-3 EM grids) compared to x-ray crystallography (milligram range). Sample homogeneity is a necessary prerequisite to allow determination of 3D models at subnanometer resolution. However, some degree of heterogeneity can be compensated by computational sorting using sensitive
algorithms recognizing small conformational differences (Fischer et al.). Chemical fixation of protein complexes during glycerol gradient centrifugation (GraFix) prior to electron microscopy has been shown to considerably improve sample quality and can be used for unstained cryo-EM (Kastner et al. 2008).

The following improvements need to be made to allow the determination of an APC/C structure at subnanometer resolution: (i) purify APC/C at higher concentrations, (ii) establish protocols for gentle glycerol removal subsequent to gradient centrifugation (without affecting particles) which is required for unstained cryo-EM and (iii) find conditions that conformationally stabilize APC/C complexes to accelerate high-resolution structure determination.

5.3.2. Two step cell lysis protocol to increase the concentration of purified APC/C

Inherent conformational flexibility of the APC/C complex complicates the calculation of a 3D model at subnanometer resolution. However, structural heterogeneity can be compensated by recording millions of single particles that afterwards will be sorted into subpopulations that are conformationally identical. The expensive process of image acquisition can be accelerated by increasing the number of recorded particles per image, which in essence requires a higher abundance of APC/C complexes on the EM grid compared to previous experiments (Dube et al. 2005; Herzog et al. 2009). However, conventional protein concentration procedures such as centrifugal filter devices are believed to affect the shape of the complexes and thus cannot be applied. Therefore, mild procedures are desired to purify APC/C at higher concentrations.

Endogenous human APC/C is affinity purified from HeLa cell extracts using anti-CDC27-antibodies beads recognizing the C-terminal 20 amino acids of the APC/C subunit CDC27. Antibodies bind their antigen with a certain affinity constant $K_a$, which is the inverted dissociation constant ($K_d = 1/K_a$). The dissociation constant $K_d$ relates
equilibrium binding to molar concentrations of free antibody [Ab], free antigen [Ag] and the antibody-antigen complex [AbAg] contained in the reaction as follows:

$$K_d = \frac{[Ab][Ag]}{[AbAg]}; \text{ converted to the antibody-antigen complex: } [AbAg] = \frac{[Ab]}{K_d} [Ag].$$

This equation shows that the concentration of the antibody-antigen complex [AbAg] will be increased by using elevated concentrations of the antigen [Ag] at a constant antibody concentration [Ab].

**Figure 31** Two step cell lysis protocol. (a) Schematic representation of the two step cell lysis protocol. (b) Negative stain of apo-APC/C purified from a cell extract derived from a single cell lysis step. (c) Negative stain of apo-APC/C purified from a cell extract derived from the two step cell lysis procedure. Adsorption time to the EM grid was comparable. Black bars equal 50nm.
Therefore, a two step cell lysis protocol was established to increase the concentration of the soluble cell extract and thus the concentration of the contained APC/C molecules. Adherently grown HeLa cells were resuspended in APC/C extract buffer, lysed using a dounce homogenizer and cell debris was removed by centrifugation. To increase the protein concentration in the extract, a second cell lysis step was introduced but this time HeLa cells were resuspended in the soluble cell extract derived from the first cell lysis step (Fig.31a). APC/C complexes bound to anti-CDC27-antibody beads are recovered by elution with antigenic peptides. To increase APC/C recovery after peptide elution the concentration of the competing CDC27 peptide was doubled compared to previous experiments (Herzog et al. 2009).

Using a combination of both concentrated cell extracts for binding and increased peptide concentration for elution, APC/C complexes could be purified at 2-3 times higher concentrations compared to previous experiments (Herzog et al. 2009) (Fig.31b, c). Importantly, no differences in the appearance of APC/C particles could be detected indicating that the two step cell lysis protocol provides a gentle alternative to conventional protein concentration procedures. However, only adherently grown HeLa cells could be used for the two step cell lysis procedure (extracts from adherently grown HeLa cells are of extraordinary clarity compared to those derived from HeLa cells grown in suspension).

5.3.3. Glycerol removal using gravity flow desalting columns

With the aim of studying APC/C at subnanometer resolution sample preparation techniques that fully maintain the hydrated state of the molecule are required. Samples embedded in vitreous ice (“frozen hydrated”) lead to the best preservation of the macromolecule with all its atomic features which has been demonstrated by comparing structures at atomic resolution derived from either x-ray crystallography or single-
particle cryo-EM (Zhang et al.; Ludtke et al. 2004; Frank 2006). Glycerol negatively affects the already low contrast of protein samples embedded in vitreous ice. Therefore, glycerol originated from gradient fixation (GraFix) has to be removed prior to specimen preparation (Grassucci et al. 2007).

**Figure 32** Glycerol removal using a gravity flow PD Minitrap G-25 column. APC/C was purified from cell extract derived from a single cell lysis step. Dashed circles show aggregated APC/C complexes appearing after the desalting column. Black bars equal 50 nm.

Gravity flow desalting columns were used to transfer the complexes into a non-glycerol buffer. Only in the presence of the detergent octyl-β-D-glucopyranoside (OGP) APC/C molecules could pass the column and appeared to be intact (Fig. 32). However, larger
protein aggregates were observed after buffer exchange (dashed circles) indicating that APC/C complexes fell apart during the procedure. APC/C immunoprecipitation is done in Tris which is known for its stabilizing effects towards protein structure and function. Unfortunately, Tris is incompatible with the amine-reactive homobifunctional crosslinker glutaraldehyde used during gradient fixation (Kastner et al. 2008). Therefore, Hepes was used for gradient fixation (Herzog et al. 2009) and buffer exchange experiments.

During experiments in which APC/C had to withstand as harsh treatments as centrifugal filtration (Fig. 33a), when a Tris buffer was used APC/C remained stable during entire procedure (Fig. 33b), kept ubiquitination activity and subunit integrity (Fig. 33c).

Figure 33 Robustness test of APC/C integrity in different buffers. (a) Schematic representation of the robustness test. (b) SDS-PAGE of apo-APC/C before and after centrifugal filtration using a Tris buffer. (c) APC/C-CDH1-Hsl1 before and after centrifugal filtration. APC/C-CDH1-Hsl1 could be re-IP purified via the Flag-tag on Hsl1 after the complexes were treated with centrifugal filtration. Each sample was incubated with ubi-mix for 1 hour at 4°C. (d) SDS-PAGE of apo-APC/C before and after centrifugal filtration using a Hepes buffer.
However, repeating the same procedure using Hepes, APC/C did not survive centrifugal filtration (Fig.33d). This suggested that Hepes is not suitable to protect APC/C towards shear forces which might apply also to glutaraldehyde fixed complexes (Fig.32). Based on these results, BisTris was used during gradient fixation and subsequent buffer exchange which led to less frequent protein aggregation in the peak fraction after the desalting column (Fig.34).

Figure 34 Apo-APC/C purified from a cell extract derived from the two step cell lysis procedure before and after glycerol removal using a BisTris buffer. Dashed circles show aggregated APC/C complexes appearing after the desalting column. Black bars equal 50nm.

5.3.4. Cryo-EM 3D model of apo-APC/C at a resolution of approximately 15Å

Sample homogeneity is a parameter that can markedly accelerate the calculation of an APC/C 3D model at subnanometer resolution. Therefore, APC/C populations used for high-resolution single particle electron microscopy should not only be homogenous in terms of subunit composition but also regarding their conformational state. Unfortunately, human apo-APC/C has been found to possess a large degree of conformational flexibility between the ‘arc lamp’ and the ‘platform’ domain (Dube et al.
2005). To compensate for such large conformational heterogeneity, the particles need to be sorted into classes of similar conformation which requires a multitude of recorded particles in the dataset. For apo-APC/C, approximately 1-2 million particles will need to be recorded, picked and sorted to give rise to a structure at subnanometer resolution. Since automated particle picking is still hampered by APC/C aggregation during glycerol removal, so far only semi-automated particle picking procedures could be employed. About 140,000 particles of apo-APC/C were picked, sorted and used for angular reconstruction leading to an initial 3D model at a resolution of approximately 15Å which so far is the highest resolution ever obtained for human APC/C (Fig.35).

Figure 35 Comparison of human apo-APC/C 3D models derived from either cryo-negative staining EM or unstained cryo-EM. Different views are shown. The resolution of the apo-APC/C 3D model derived from cryo-negative staining is ~25Å. The structure was calculated from about 50,000 particles and was taken from Herzog et. al., Science, 2009. The resolution of apo-APC/C derived from cryo-EM is approximately 15Å. Rotations refer to the front view orientation shown on the left side.
6. Discussion

The anaphase promoting complex/cyclosome (APC/C) is an unusually large ubiquitin ligase that is essential for cell division. APC/C serves as a platform that brings together substrates and E2~Ub to allow the ubiquitin being transferred to substrate lysines. APC/C mediated polyubiquitylation targets key regulatory proteins for proteasomal degradation.

APC/C requires the assistance of co-activator proteins and E2~Ub to fulfill its function. Co-activator proteins are required to bind substrates to the APC/C. However, substrate binding to APC/C is not sufficient to enable substrate polyubiquitylation. Two more factors seem to be involved. First, APC/C depleted for its core subunit Doc1 can ubiquitylate bound substrates but displays severe processivity defects in the ubiquitylation reaction (Carroll and Morgan 2002). Second, co-activator proteins contain an N-terminal sequence element called the C-box, which seems to be required for APC/C binding and to activate its E3 function (Vodermaier et al. 2003; Kimata et al. 2008). The function of the Apc11 core subunit is to recruit E2~Ub to APC/C, but does not seem to be involved in binding co-activators (Thornton et al. 2006) and substrates (Fig.16d).

In the present study, biochemical and EM experiments were used to obtain a better understanding of how co-activators and APC/C interact with substrate proteins. Analysis of in vitro reconstituted human APC/C complexes bound to stoichiometric amounts of CDH1 (co-activator) and Hsl1 (substrate) revealed the existence of two co-activator binding sites as well as two substrate binding sites (Fig.15-17) which also could be resolved in the EM model of APC/C-CDH1-Hsl1 (Fig.12 and Fig.13). One substrate
molecule is intercalated between one CDH1 molecule and the APC/C core subunit DOC1. This finding strongly implies that DOC1 directly contacts the substrate, as was previously shown to be the case for CDH1 (Kraft et al. 2005) and is supported by the fact that binding of Hsl1 to APC/C further stabilizes CDH1 binding (Matyskiela and Morgan 2009) (Fig. 8a). We therefore believe that CDH1 and DOC1 form a bipartite substrate receptor (Buschhorn et al. 2011; da Fonseca et al. 2011) composed of the WD40 domain of CDH1 (Kraft et al. 2005) and DOC1’s ligand binding region (Wendt et al. 2001; Au et al. 2002; Carroll et al. 2005) (Fig. 13). This hypothesis is further supported by the observation that mutation of the D-box in APC/C substrates and deletion of Doc1 reduce substrate ubiquitylation in similar and functionally redundant manners (Carroll et al. 2005). The density intercalated between CDH1 and DOC1 did not disappear by increasing the surface rendering threshold which indicates that nearly all APC/C-CDH1-Hsl1 complexes were bound to a substrate molecule in this position (Fig. 12). This observation is consistent with the hypothesis that Doc1 contributes to processive substrate ubiquitylation by decreasing substrate dissociation (Carroll and Morgan 2002). The second co-activator/substrate binding module is believed to be located in the platform region of APC/C (Fig. 18).

The localization of APC/C subunits in the structures of yeast and human APC/C defined exact positions and boundaries of almost all APC/C subunits (Buschhorn et al. 2011; Schreiber et al. 2011; Herzog et al. 2009) which is consistent with previous genetic analyses from budding yeast (Thornton et al. 2006). The TPR subunits Cdc23, Cdc16, Cdc27 and Apc7 (in vertebrates) exist as dimers and are the major components that build up the ‘arc lamp’ domain (Schreiber et al. 2011; Zhang et al.; Zhang et al.; Han et al. 2009) (Fig. 36). Two of these TPR subunits (Cdc27 and Cdc23) have been implicated in co-activator recruitment to the APC/C, however, it was assumed that one co-activator protein interacts with both subunits at the same time (Matyskiela and Morgan 2009).
Interestingly, the two co-activator/substrate binding modules observed in our APC/C-CDH1-Hsl1 3D model overlap with the predicted positions of CDC27 and CDC23 in the complex of human APC/C (Buschhorn et al. 2011; Schreiber et al. 2011; Herzog et al. 2009) (Fig. 37).

Figure 36 APC/C architecture and subunit topology. (a) 3D model of human APC/C. ‘Arc lamp’ and ‘platform’ domain enclose the central cavity. (b) Schematic model of how the major subunits build up the APC/C complex based on Herzog et. al., Science, 2009; Buschhorn and Petzold et. al., NSMB, 2011 and Schreiber et. al., Nature, 2011.

Figure 37 The binding of two co-activator proteins could be mediated by the APC/C subunits Cdc27 and Cdc23. (a) 3D model of human APC/C-CDH1-Hsl1 at low surface rendering thresholds. (b) Predicted positions of the two co-activator/substrate binding modules. Schematic model of how human APC/C mediates binding of two co-activators and two substrates (white boxes).
Therefore, it is tempting to speculate that the two co-activators bind either TPR subunit, Cdc27 or Cdc23, in an IR-tail dependent manner and that each co-activator protein mediates binding of one substrate molecule. This assumption is supported by the fact, that in vitro one co-activator molecule could not bind two substrate molecules at the same time (Fig.15) and that in the structure of APC/C-CDH1-Hsl1 no physical connection could have been resolved between the co-activator/substrate modules in the major and minor position. This model also implies that the second co-activator/substrate module contacts APC1 (Buschhorn et al. 2011; Schreiber et al. 2011), possibly via the substrate molecule (Fig.37) and that this substrate molecule seems to dissociate more easily (Fig.13).

In the structure of APC/C-CDH1, the catalytic subunits APC2 and/or APC11 form a contact to a subunit in the platform region. Upon substrate binding, this connection is resolved and instead APC2 and/or APC11 contact the proximal co-activator protein (major position), close to where the substrate binds (Fig.13 and Fig.38a). Yeast APC/C depleted for the catalytic subunit Apc2 showed a reduction in Cdh1 binding (Thornton et al. 2006), even though this might be explained by severe structural aberrations in the complex. The C-box of Cdh1 binds APC/C differently than the IR-tail (Matyskiela and Morgan 2009) and furthermore seems to be required to stimulate its ubiquitylation activity. Taken together, one could speculate that once a substrate molecule is bound the C-box interacts with the catalytic core (e.g. Apc2) to eventually stimulate APC/C activity.

In early mitosis, the co-activator protein Cdc20 is part of the mitotic checkpoint complex (MCC) which inserts into APC/C’s central cavity and inhibits its ability to recruit and ubiquitylate substrates (Herzog et al. 2009) (Fig.38b). By binding to APC/C, the mitotic checkpoint complex occupies the entire minor position between Cdc23 and Apc1 which strongly suggests that binding of a co-activator/substrate module is prevented by sterical means. In the major position, Cdc20 seems to be pulled apart from its canonical
binding site (Herzog et al. 2009) and these drastic conformational changes might interfere with substrate binding in the major position. Importantly, in APC/C-MCC no connection can be observed between the Cdc20 density and APC2 and/or APC11 (Fig.38b). If the connection between co-activators and Apc2 and/or Apc11 is required to stimulate APC/C activity, then the logical implication would be that APC/C-MCC is not only inhibited towards substrate binding but also towards its ability to become catalytically activated by the co-activator protein per se.

The existence of two co-activator/substrate binding sites helps revealing why APC/C, compared to other E3 ubiquitin ligases, requires much more protein subunits to catalyze the ubiquitin transfer reaction. However, even though this finding provides one explanation for APC/C’s unusual dimensions, it also raises important biological questions. In vitro APC/C ubiquitylates different substrates with different kinetics which led to the hypothesis that substrate ordering in vivo depends on the relative processivity of substrate polyubiquitylation by the APC/C (Rape et al. 2006; Sullivan and Morgan 2007). Processive substrates would obtain a polyubiquitinchain during one binding event, whereas distributive substrates require several binding events to become
polyubiquitylated (Rape et al. 2006; Stegmeier et al. 2007). Therefore, if the model is correct, processive substrates would be degraded earlier in mitosis, whereas distributive substrates would be rather late substrates. The identification of two substrate binding sites on APC/C could provide an important extension to this model. Our EM experiments showed that the abundance of the densities that we believe correspond to the substrates differ between the major (high abundance) and the minor (low abundance) position (Fig.39) which could imply that the two sites exhibit different substrate binding characteristics. Although highly speculative, processive or early substrates might bind in the major position because the substrates in this position were bound particularly stably whereas distributive or late substrates might bind in the minor position.

Several experimental approaches will be required to test the outlined hypotheses. First it will be very important to repeat binding of two co-activator proteins using CDC20 and mitotic APC/C to find out whether binding two co-activators is a general feature of human APC/C or unique to CDH1. Recombinant human APC/C could be used to introduce mutations that potentially disrupt the co-activator binding site on CDC27 and/or CDC23. These mutant APC/C complexes could be used to identify residues important for co-activator binding in both TPR subunits. If one co-activator binding site can be mutated, it will be important to ask whether this also interferes with the ability
to recruit a second substrate molecule. Once the two co-activator binding sites have been identified, one could use BAC TransgeneOmics and RNAi to replace endogenous CDC27 and/or CDC23 with the mutant versions and study the *in vivo* effects during cell cycle progression (Poser et al. 2008) providing that these mutant versions are incorporated into the complex and do not interfere with MCC function on APC/C. Using time-lapse light microscopy, one could follow the kinetics of substrate degradation *in vivo* and study the influence of the two co-activator/substrate binding sites on the degradation rate of early and late APC/C substrates.

Importantly, the structure of APC/C is conserved from yeast to human (Buschhorn et al. 2011) which would imply that also the ability to bind two co-activators and two substrates might have been conserved. Biochemical studies in yeast clearly showed that residues in Cdc27 and Cdc23 contribute to co-activator binding (Matyskiela and Morgan 2009). However, recent structural analysis of yeast APC/C bound to Cdh1 and different substrate sequences could neither resolve a second co-activator/substrate binding module in the platform region nor provide an explanation for how Cdc23 might interact with Cdh1 (da Fonseca et al. 2011; Schreiber et al. 2011). Important differences during sample preparation and/or image processing could provide an explanation for the existing discrepancy (Buschhorn et al. 2011; da Fonseca et al. 2011; Sander et al.; Kastner et al. 2008). However, the ability of yeast APC/C to dimerize does not seem to be conserved from yeast to human and could provide an important piece to this complex puzzle. Therefore, to resolve this inconsistency, it will be essential to investigate whether also yeast APC/C mediates binding of two co-activator and two substrate molecules in its monomeric form.

Another interesting observation was made when *in vitro* reconstituted APC/C-CDH1-Hsl1 complexes were incubated with the ubi-mix containing E1, ATP, UBCH10 and ubiquitin. Under experimental conditions, APC/C-CDH1-Hsl1 was compositionally stable for several hours (*Fig. 8a*), but upon addition of the ubi-mix Hsl1 and CDH1 dissociated.
within minutes (Fig.10c). This observation might reveal a co-activator/substrate release function of one or several components contained in the ubi-mix, i.e. E2 and/or E2~Ub. In a pathway model, substrate molecules bind APC/C-CDH1 and only upon their modification should be released again. Therefore, coupling substrate polyubiquitylation to APC/C-CDH1-substrate disassembly via E2~Ub could provide an appealing mechanism. In the in vitro experiment, only unmodified CDH1 and Hsl1 dissociated off APC/C (Fig.10c) which might be explained by excess amounts of free E2 (over E2~Ub) that could cause complex disassembly without substrate modification. Therefore, the assay requires further optimization to study the effects of ubi-mix components on APC/C-CDH1-Hsl1 disassembly.

Several substrate binding and co-pull down experiments confirmed that the D-box is required to stimulate association of CDH1-substrate complexes with APC/C (Carroll et al. 2005; Burton et al. 2005) (Fig.15b; compare lanes 1-4 with lane 5). Off-rate experiments showed that the D-box of Hsl1 binds APC/C more stably than the KEN-box, however the D-box alone was not sufficient to restore binding of Hsl1-wt (Fig.11d, e). These observations suggest that D-box and KEN-box have additive or cooperative effects on APC/C binding when present in one substrate molecule. Each of the two co-activator proteins seems to mediate bona fide substrate binding (dependent on D-box and KEN-box motifs) on APC/C (Fig.15b; compare lane 1 to lanes 2-5) suggesting that each co-activator binds both destruction motifs. The D-box of Hsl1 could bind much more CDH1 than the KEN-box (Fig.29). However, the D-box could not outcompete a bound KEN-box (Fig.29b; compare lane 1 and 4) which would be expected if the D-box binds to the same site but with higher affinity, again supporting the idea that both destruction motifs bind different sites on CDH1 (Fig.29). However, in vitro the co-activators were not able to interact with two substrate molecules at the same time (Fig.15b and Fig.29). It is therefore possible, that the ability of CDH1 to interact with both destruction motifs changes upon association with APC/C.
Improved protocols for the purification of APC/C co-activators were established that will be important to answer further detailed molecular questions concerning substrate binding and will potentially provide an understanding of how cognate destruction motifs interact with the WD40 domain of CDH1 at atomic resolution.

Also, sample preparation techniques for single particle electron microscopy have been optimized that in combination with state of the art EM equipment and conformationally stalled APC/C complexes can lead to the determination of a real subnanometer 3D model of endogenous human APC/C. Furthermore, recombinant APC/C will be an essential necessity to obtain a detailed mechanistic understanding of the molecular workings that make the APC/C an exceptional member of RING domain ubiquitin ligase family.
7. Material and Methods

7.1. Protein expression and purification using *E. coli* cells

7.1.1. Constructs used for bacterial expression

Constructs designed in this study used for expression and purification from *E. coli* cells are summarized in Table 1.

<table>
<thead>
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<th>C-terminal tag</th>
<th>Vector</th>
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<td>His-Flag-td2-</td>
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<td>pET21a</td>
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*Table 1* Bacterial expression constructs designed in this study.
7.1.2. Protein expression in *E. coli*

All APC/C substrates used in this study as well as UBCH10 and ubiquitin were expressed in either *E. coli* BL21 (DE3) or *E. coli* Rosetta (DE3) strains using the pET System (Novagen). Chemically competent cells were heat shock transformed using standard protocols and plated onto LB-Agar containing respective antibiotics. After over-night incubation, pre-cultures were grown in 50ml LB-medium containing respective antibiotics for approximately 8 hours using a shaker incubator (200rpm). Pre-culture cells were spun down at 4000rpm at 4°C and resuspended in 10ml LB-medium. 1ml of resuspended pre-culture cells was used to inoculate 1L LB-medium containing respective antibiotics and 1.5% (w/v) D-(-)-lactose monohydrate (Roth) (main-culture) to induce T7 RNA polymerase driven protein expression. Main-cultures were grown in a shaker incubator (Infors) at 200rpm for 14-16 hours at 30°C. Main-culture cells were centrifuged at 4000rpm at 4°C, pellets were resuspended in 40 ml 1xPBS and centrifuged again. Pellets were frozen in liquid nitrogen and stored at -80°C.

7.1.3. Purification of His-tagged proteins expressed in *E. coli*

*E. coli* cells derived from 2L main-culture were resuspended in 40 ml lysis buffer (20mM Tris, pH8.0, 150mM NaCl, 50mM Imidazol, 5% (v/v) glycerol) containing 1 tablet of EDTA-free protease inhibitor cocktail (Roche), supplemented with 1mg/ml Lysozyme (Sigma) and incubated on a rotary shaker for 1 hour at 4°C. Lysed cells were sonicated on ice and centrifuged at 20,000rpm for 30 minutes at 4°C. Soluble cell extracts were incubated with 1ml Ni-NTA beads (Qiagen) on a rotary shaker for 1 hour at 4°C. Ni-NTA beads were washed with at least 20 bead volumes lysis buffer, bound protein was eluted with 2 bead volumes elution buffer (20mM Tris, pH8.0, 150mM NaCl, 500mM Imidazol, 5% (v/v) glycerol) and incubated on a rotary shaker for 15 minutes at 4°C. The
elution step was repeated twice (3 elutions in total), eluted protein was pooled and concentrated using Amicon centrifugal filter units (Millipore). The concentrated protein was diluted in lysis buffer and concentrated again until the predicted concentration of imidazole was below 50mM. Aliquots of the concentrated protein were frozen in liquid nitrogen and stored at -80°C.

The Hsl1<sup>667–872</sup> sequence was derived from <i>S. cerevisiae</i>, Sororin and Securin from <i>H. sapiens</i>.

### 7.2. Protein expression and purification using Sf9 insect cells

#### 7.2.1. Constructs used for insect cell expression

Constructs designed in this study used for expression and purification from insect cells are summarized in **Table 2** and **Table 3**.

<table>
<thead>
<tr>
<th>N-terminal tag</th>
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<th>CDH1 modifications</th>
<th>Vector</th>
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</thead>
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<td>full-length wild-type</td>
<td>pFastBac1</td>
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<td>CDH1</td>
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<td>pFastBac1</td>
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<tr>
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<td>CDH1</td>
<td>dN162 dC21</td>
<td>pFastBac1</td>
</tr>
<tr>
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<td>CDH1</td>
<td>dN162 dC14</td>
<td>pFastBac1</td>
</tr>
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<td>full-length wild-type</td>
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</tr>
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<td>C-box mutant</td>
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<td>pFastBac1</td>
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<tr>
<td>3Myc-His-PreScissionSite-</td>
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<td>dN171 dC14</td>
<td>pFastBac1</td>
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</table>

**Table 2** Insect cell expression constructs designed in this study.
### 7.2.2. Generation of a recombinant bacmids carrying the gene of interest

All CDH1 constructs described in this study were expressed using the Bac-to-Bac Baculovirus Expression System (Invitrogen).

A baculovirus was generated by chemical transformation of *E. coli* DH10Bac or *E. coli* EMBacY (Trowitzsch et al.) (contain baculovirus shuttle vector; bacmid) with a pFastBac vector carrying the gene of interest. Transformed cells were incubated in a shaker incubator for at least 3 hours at 37°C to allow transposition of the pFastBac expression construct into the lacZ gene of the bacmid. Afterwards, cells were plated onto LB-Agar containing 50μg/ml kanamycin, 7μg/ml gentamycin, 10μg/ml tetracycline, 100μg/ml X-gal and 40μg/ml IPTG to enable blue-white selection of the clones containing the gene of interest expression construct (white colonies). White colonies were re-streaked and re-evaluated. Positive clones were amplified in a over-night cultures containing 50μg/ml kanamycin, 1.2μg/ml gentamycin and 10μg/ml tetracycline in 5ml LB-medium using a shaker incubator (200rpm) at 37°C.

To purify recombinant bacmid DNA, a modified version of the Qiagen MIDIprep protocol was used. Cells were resuspended in 300μl Qiagen buffer P1, mixed with 300μl Qiagen buffer P2 and incubated at room temperature for 5 minutes to lyse the cells. Addition of 300μl Qiagen buffer P3 and incubation on ice for 10 minutes precipitates cell debris. Lysate was cleared by centrifugation at 13,000rpm for 10 minutes at 4°C. 700μl cleared

<table>
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<th>Vector</th>
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<tr>
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<td>CDH1 + Hsl1-Mini</td>
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<td>CDH1 + Hsl1-Mini</td>
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<td>CDH1</td>
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</table>

*Table 3* Bicistronic insect cell expression constructs designed in this study.
lysate was added to 800μl ice cold isopropanol and incubated for 10 minutes on ice. Precipitated bacmid DNA was pelleted by centrifugation at 13,000rpm for 15 minutes at room temperature. The bacmid DNA pellet was washed with 70% (v/v) Ethanol and resuspended in 50μl steril ddH₂O under steril conditions (laminar flow cabinet).

7.2.3. Generation of a recombinant baculovirus and protein expression in insect cells

To generate a recombinant baculovirus, the transfection mix containing 5μl resuspended bacmid DNA and 5μl Cellfectin II Reagent (Invitrogen) in 200μl Sf-900 II serum-free medium (Gibco) was incubated for 30 minutes in a laminar flow cabinet. To transfect insect cells, approximately 9x10⁵ adherently grown Sf9 cells per well grown in a 6-well plate (Nunc) were washed with 2ml Sf-900 II serum-free medium. 200μl transfection mix and 800μl Sf-900 II serum-free medium were added to one well and incubated for 4 hours at 27°C. After transfection, insect cells were washed once with Sf-900 II serum-free medium and then incubated in 3ml Sf-900 II serum-free medium for 4-5 days. The baculovirus will start to be released into the medium 3 days post-transfection and transfected cells appear larger in size and tend to detach.

To generate the 1⁰ virus amplification, 1ml baculovirus containing supernatant derived from bacmid transfection was added to semi-confluent Sf9 cells in a T-175 flask containing 50ml Grace’s insect medium supplemented with 0.2mM L-glutamine (Sigma-Aldrich), 1% penicillin-streptomycin solution (Sigma-Aldrich) and 10% heat inactivated fetal bovine serum (Gibco). After 5-6 days incubation at 27°C, baculovirus containing supernatant was filtered using 0.22μm Steriflips (Millipore) and stored at 4°C protected from light. To generate the 2⁰ virus amplification, 1ml of the 1⁰ virus amplification was added to semi-confluent Sf9 cells in a T-175 flask containing 50ml Grace’s insect medium supplemented with 0.2mM L-glutamine (Sigma-Aldrich), 1% penicillin-
streptomycin solution (Sigma-Aldrich) and 10% heat inactivated fetal bovine serum (Gibco). Usually, 3rd virus amplification was used in protein expression experiments. For protein expression, 750ml 0.8-1.0x10^6 Sf9 suspension cells grown in Grace’s insect medium supplemented with 0.2mM L-glutamine (Sigma-Aldrich), 1% penicillin-streptomycin solution (Sigma-Aldrich), 0.1% PLURONIC F-68 and 10% heat inactivated fetal bovine serum (Gibco) were inoculated with 5-10ml 3rd virus amplification and incubated in a shaker incubator (100rpm) at 27°C for 60-72 hours. After protein expression, cells were spun down at 1,500rpm for 30 minutes at 4°C, 1xPBS washed and pelleted again. Insect cell pellets were liquid nitrogen frozen and stored at -80°C.

7.2.4. Purification of 3Myc-His6-tagged full-length CDH1

Frozen insect cells expressing 3Myc-His6-CDH1 (3MH6-CDH1) were resuspended in CDH1 lysis buffer (20mM Hepes, pH 7.0, 100mM (NH₄)₂SO₄, 20mM Imidazol, 1mM DTT, 5% (v/v) glycerol; 20ml per 10g of cells) and lysed on ice using a dounce homogenizer (Wheaton). Lysates were cleared at 35,000rpm for 30 minutes at 4°C using a 45 Ti rotor (Beckman Coulter). Cleared lysates were incubated with Ni-NTA (Qiagen) beads (1ml Ni-NTA beads per 10g of cells) on a rotary shaker at 4°C for 1 hour. Beads were washed twice with at least 20 bead volumes CDH1 wash buffer (20mM Hepes, pH 7.0, 300mM (NH₄)₂SO₄, 20mM Imidazol, 1mM DTT, 2.5% (v/v) glycerol), bound protein was eluted using 2 bead volumes CDH1 elution buffer (20mM Hepes, pH 7.0, 300mM (NH₄)₂SO₄, 250mM Imidazol, 2.5% (v/v) glycerol) and incubated on a rotary shaker for 15 minutes at 4°C. The elution step was repeated twice (3 elutions in total), eluted protein was pooled and concentrated using 50,000 MWCO Amicon centrifugal filter units (Millipore). Concentrated 3MH6-CDH1 was purified using a Superdex S200 16/60 size exclusion column equilibrated in CDH1 SEC buffer (20mM Hepes, pH 7.0, 300mM (NH₄)₂SO₄, 1mM DTT, 2.5% (v/v) glycerol) operated by an ÄKTA purifier 100 (GE Healthcare). 3MH6-CDH1
peak fractions were pooled and concentrated using 50,000 MWCO Amicon centrifugal filter units, aliquots were frozen in liquid nitrogen and stored at -80°C.

7.2.5. Purification of PreScission cleavable 3MH6-PS-CDH1

PreScission cleavable 3MH6-PS-CDH1 (full-length) was Ni-affinity purified as described for 3MH6-CDH1. PreScission protease was added to the pooled eluate (5 units PreScission protease (GE Healthcare) per 1 ml eluate) and was supplemented with 1 mM DTT (final concentration). 3MH6-PS-CDH1 containing PreScission protease was dialysed against CDH1 SEC buffer for 2-3 hours at 4°C to allow cleavage and remove excess imidazole derived from the CDH1 elution buffer. To remove uncleaved 3MH6-PS-CDH1, cleaved 3MH6-PS-tag and His6-tagged PreScission protease a HisTrap FF column (1 ml, GE Healthcare) was equilibrated in CDH1 SEC buffer ((20 mM Hepes, pH 7.0, 300 mM (NH₄)₂SO₄, 1 mM DTT, 2.5% (v/v) glycerol)) and the dialysed protein sample was manually applied using a syringe (approximately 15-20 ml eluate per 1 ml HisTrap FF column) at a flow rate of approximately 1 ml/min. The flow through containing cleaved CDH1 was concentrated using 50,000 MWCO Amicon centrifugal filter units (Millipore). Concentrated cleaved CDH1 was purified using a Superdex S200 16/60 size exclusion column (GE Healthcare) equilibrated in CDH1 SEC buffer. Peak fractions of cleaved CDH1 were pooled and concentrated using 50,000 MWCO Amicon centrifugal filter units, aliquots were frozen in liquid nitrogen and stored at -80°C. Protein concentration of cleaved CDH1 before and after size exclusion chromatography took hours and cleaved CDH1 could not be concentrated above 3 mg/ml.
7.2.6. Purification 3MH6-, 2MH6- and 1MH6-CDH1 deletion constructs and CDH1-Hsl1

CDH1 deletion constructs +/- Hsl1 fragments were purified with 1.5ml Ni-NTA beads (Qiagen) per 10g of insect cells. During gel filtration, 300mM ammonium sulfate could be replaced by 300mM sodium chloride (but was not always done). Apart from that, the purifications were carried out as described for 3MH6-tagged full-length CDH1. Insertion of a PreScission cleavage site between the 3MH6-tag and a CDH1 deletion constructs resulted in most of the protein being insoluble. Therefore, the PreScission cleavage protocol could not be applied to the CDH1 deletion constructs. Yet, it has not been tried to insert a PreScission cleavage site between the 1MH6- or 2MH6-tag and a CDH1 deletion construct.

7.3. CDH1 biochemistry and crystallization

7.3.1. Limited proteolysis of CDH1 constructs

310μl of ~1.0mg/ml cleaved full-length CDH1 or 3MH6-162-14 +/- Hsl1 \(^{667-872}\) were incubated with 1μg of either trypsin or chymotrypsin on ice. Samples were taken at indicated time points and analyzed via coomassie stain and western blot.

7.3.2. Crystallization of CDH1 deletion constructs

Commercial crystal screens (Hampton Research, Emerald Biosystems, Jena Bioscience) and MRC 2 96-well crystallization plates (Swisssci) were used to screen conditions for protein crystallization. Crystal screens were set up using a Mosquito crystallization
robot. The purified protein was concentrated up to 10-15 mg/ml, mixed with the respective reservoir solution in a ratio of 1:1 (100nl protein + 100nl reservoir) and 1:2 (200nl protein + 100nl reservoir) and subjected to 80μl reservoir solution via sitting drop and vapor diffusion methods. Plates were kept at 19°C for several days.

Different forms of purified CDH1 were subjected to crystallization experiments. Full length 3MH6-CDH1 and cleaved full-length CDH1 never gave rise to crystal formation. 3MH6-CDH1 dN162 dC14 only crystallized in complex with Hsl1-Mini (purified in a 300mM (NH₄)₂SO₄ SEC buffer) at protein concentrations between 10 and 15 mg/ml in the presence of 100mM sodium cacodylate pH6.5, 300mM ammonium sulfate and 30% (w/v) PEG8000. Unfortunately, crystal growth was not very reproducible which is why screening different salts and precipitants did not lead to conclusive results.

Crystal trials using 10mg/ml 1MH6-CDH1 dN162 dC14 were set up only in the presence of an equimolar concentration of the Hsl1 D-box peptide (EQKPRAALSDITNSFNM). 1MH6-CDH1 dN162 dC14 was purified in a SEC buffer containing 300mM NaCl, 100mM sodium cacodylate pH6.5 and 1.0M sodium citrate lead to initial protein crystal formation. Since sodium cacodylate was contained in both conditions that led to crystal formation of different CDH1 deletion constructs, sodium cacodylate was used as an additive in subsequent crystallization screens using commercial crystal screens. Addition of 100mM sodium cacodylate caused crystal formation in a variety of different conditions. Crystals grown in 150mM sodium cacodylate, 2.0M ammonium sulfate and 10mM magnesium sulfate and frozen in the presence of 30% glycerol showed major improvements judged from their x-ray diffraction pattern (crystals were grown and frozen by Nicholas G. Brown and Brenda A. Schulman). However, these crystals seemed to be twinned and require further improvement.
7.4. **APC/C purification and biochemistry**

7.4.1. **HeLa cell culture**

HeLa cells were grown in DMEM supplemented with 0.2mM L-glutamine (Sigma), 1% penicillin-streptomycin solution (Sigma) and 10% heat inactivated fetal bovine serum (Gibco) in either adherent or suspension cultures at 37°C and 5% CO₂. Adherent HeLa cells were grown to confluence in 245x245cm tissue culture dish (Nunc), harvested in 1xPBS using a cell scraper and spun down at 1,500rpm for 20 minutes at 4°C, washed once with 1xPBS and pelleted again. One tray usually produced 0.5-0.6g frozen HeLa cell pellet. Adherently grown HeLa cells were used for electron microscopy experiments that required the two step cell lysis protocol described in section 5.3.2.

![Figure 40](image_url)  
*Comparison of different HeLa cell lines grown in either suspension or adherent cultures. These cells were used to perform anti-CDC27-IP to compare purity and quality of purified APC/C analyzed by SDS-PAGE and silver staining.*
HeLa suspension cultures were grown in 3L Wheaton spinner flasks filled with 1.5L DMEM medium using regular HeLa cells, not HeLa S3 or Hela Kyoto. 1.5L DMEM suspension culture was inoculated with trypsinated adherent HeLa cells from 3 confluent 245x245cm trays and grown for 4 days at 65rpm at 37°C and 5% CO₂ (Williamson et al. 2009a). 1.5L HeLa suspension culture usually produced ~7.5g frozen HeLa cell pellet. HeLa suspension cells can be used for all biochemistry experiments which do not require the two step cell lysis protocol. Comparison of different HeLa cell lines showed that regular HeLa cells yielded most pure APC/C purifications from both adherent and suspension cells (Fig.40).

7.4.2. Coupling antibodies to Affi-prep protein A beads

To generate reusable antibody beads, purified antibodies generated in rabbit were covalently bound to affi-prep protein A beads (Bio-Rad). All steps were performed at room temperature.

The required amount of protein A beads was 2 times washed with at least 10 bead volumes TBS-T (20mM Tris pH7.5, 150mM NaCl, 0.05% (v/v) Tween-20) and resuspended in 10 bead volumes TBS-T. 1.3μg antibody was added per 1μl protein A beads and incubate on a rotary shaker for 1h. Unbound protein was removed by washing 3 times with at least 10 bead volumes TBS-T and subsequently with at least 20 bead volumes of 0.2M sodium tetraborate pH9.2. Beads were resuspended in 20 bead volumes 0.2M sodium tetraborate pH9.2 and solid dimethylpimelimidate was added to a final concentration of 20mM. This suspension was incubated on a rotary shaker for 30 minutes to crosslink antibodies via their constant domain (Fc) to protein A. The crosslinking reaction was stopped by washing the beads 2 times with at least 20 bead volumes of stop buffer (200mM Tris pH7.5, 150mM NaCl) for 10 minutes using a rotary shaker. Afterwards, beads were 3 times washed with TBS-T, 2 times with 0.1M glycine.
pH2.2 and again 3 times with at least 20 bead volumes TBS-T to restore pH. Beads were stored at 4°C in TBS-T in the presence of 0.03% NaN₃.

7.4.3. Purification of endogenous human APC/C for biochemistry

Cell extracts were prepared by lysis of frozen log-phase HeLa cells in ice cold APC/C extract buffer (20mM Tris-HCl, pH7.5, 150mM NaCl, 2mM EDTA, 10% (v/v) glycerol, 0.05% (v/v) Tween-20) using a dounce homogenizer (Wheaton) followed by centrifugation. Human apo-APC/C was immunoprecipitated (IP) from the soluble fraction by incubation with CDC27 antibodies cross-linked to Affi-prep protein A beads (Bio-Rad) for 1 hour at 4 °C. Beads were washed four times with at least 20 bead volumes of TBS-T (20mM Tris-HCl, pH7.5, 150mM NaCl, 0.05% (v/v) Tween-20) for 3 minutes at 4 °C. Purified APC/C bound to CDC27 antibody beads could be used for in vitro reconstitution experiments or recovered by elution with two bead volumes 1mg/ml antigenic peptides (20mM Tris pH7.5, 150mM NaCl, 2.5% (v/v) glycerol, 0.05% (v/v) Tween-20).

7.4.4. In vitro reconstitution of APC/C bound to CDH1 and substrates

To reconstitute co-activator- and/or substrate-bound APC/C complexes in vitro, APC/C-bound anti-CDC27-antibody beads were resuspended in 2 bead volumes binding buffer (20mM Tris-HCl, pH 7.5, 150mM NaCl, 0.05% (v/v) Tween-20, 4mg/ml BSA (Sigma; 99% pure); for EM experiments Tween-20 was replaced by 0.1% (w/v) Octyl-β-D-glucopyranoside) supplemented with 3.5μM recombinant human CDH1 and/or 150nM recombinant wild-type or mutant (D-box and/or KEN-box mutated) His-Flag-ttd2-Hsl1667–872, wild-type or KEN-box mutant Sororin-ttd2-Flag-His or wild-type or mutant (D-box and KEN-box mutated) Securin-Flag-His. After 1 hour incubation at 4 °C, the excess of
recombinant proteins was removed by washing the beads four times with at least 20 bead volumes of APC/C wash buffer for 3 minutes at 4 °C, and APC/C complexes were recovered by elution with antigenic peptides.

To demonstrate activity of *in vitro* reconstituted APC/C-CDH1–Hsl1 complexes, 50μl eluate was incubated with 5μl reaction mix containing preloaded UBCH10-ubiquitin, E1, ATP and DTT for 1 hour at 4 °C.

APC/C-CDH1-substrate complexes were enriched by re-IP using Anti-FLAG-M2-Agarose (Sigma-Aldrich). APC/C-CDH1 complexes were enriched by re-IP using anti-Myc-antibody beads.

### 7.4.5. CDH1 and substrate off-rate experiments

*In vitro* reconstituted substrate-bound APC/C-CDH1 on 25μl anti-CDC27-antibody beads was resuspended in 50μl wash buffer and incubated on a rotary shaker. At indicated time points, anti-CDC27-antibody beads were spun down, wash buffer was saved (unbound fraction) for subsequent SDS-PAGE analysis and APC/C complexes were recovered by elution with 50μl antigenic peptides. APC/C-bound and unbound fractions were analyzed by western blot.

Quantification of western blot signals was done using ImageJ. To compare and quantify APC/C bound and APC/C unbound Hsl1 (wt, kbm, dbm), samples containing APC/C bound fractions were diluted 1:10 and together with undiluted APC/C unbound samples run on the same SDS-PAGE. This allowed the acquisition low expose blots for both APC/C bound and unbound samples and therefore more accurate measurements of band signal intensities (I). Percent unbound ($P_{\text{unbound}}$) was calculated using the following formula:

$$P_{\text{unbound}} = \frac{I_{\text{unbound}}}{(I_{\text{bound}} \cdot 10 + I_{\text{unbound}})} \cdot 100 \equiv \frac{\text{unbound}}{\text{total}} \cdot 100$$
7.4.6. Binding two substrate molecules to APC/C

All incubation and elution steps were performed on a rotary shaker at 4°C for 1 hour. 200µl anti-CDC27-antibody beads bound to APC/C were resuspended in 2 bead volumes binding buffer (20mM Tris-HCl, pH 7.5, 150mM NaCl, 0.05% (v/v) Tween-20, 4mg/ml BSA) and incubated with 2.5µM recombinant human CDH1 and either 75nM His-Flag-td2-Hsl1 (wt, kbm, dbm), 75nM His-MBP-Hsl1 (wt, kbm, dbm, dkm) or an equimolar mixture of both substrate constructs (Fig.41). Beads were 3 times washed with 75 bead volumes APC/C wash buffer and APC/C complexes were eluted using 2 bead volumes CDC27 antigenic peptide (“APC/C bound”). 300µl of the peptide eluate were incubated with 50µl anti-Flag-antibody beads, the depleted supernatant was kept (“depleted”) and Flag-beads were 4 times washed with at least 10 bead volumes APC/C wash buffer. Bound complexes were eluted using 2 bead volumes Flag antigenic peptide (“Flag”). 7.5µl protein sample were used for SDS-PAGE and western blot.

Figure 41  Equal loading of the different Hsl1 mutant constructs that are tagged with either His-Flag-tdimer2-tag or His-MBP-tag.

Control experiments in the absence of APC/C were performed using 25µl anti-Flag-antibody beads that were incubated with 0.5µM recombinant human CDH1 and 37.5nM substrate to reach comparable signal intensities of His-Flag-td2-Hsl1 during western blot. A better way of doing the controls is to keep the co-activator and substrate
concentrations identical and dilute the control sample to reach comparable His-Flag-ttd2-Hsl1 signals on the western blot which will be done in future experiments.

### 7.4.7. Binding two co-activator proteins to APC/C

All incubation and elution steps were performed on a rotary shaker at 4°C for 1 hour. 200μl anti-CDC27-antibody beads bound to APC/C were resuspended in 2 bead volumes KCl binding buffer (20mM Tris-HCl, pH 7.5, 250mM KCl, 0.05% (v/v) Tween-20, 4mg/ml BSA) and incubated with either 1.25μM 3MH6-CDH1, 1.25μM cleaved CDH1 or a mixture of both CDH1 constructs in the absence or presence of 150nM His-Flag-ttd2-Hsl1-kbm. Beads were 3 times washed with 75 bead volumes KCl wash buffer (20mM Tris-HCl, pH 7.5, 250mM KCl, 0.05% (v/v) Tween-20) and APC/C complexes were eluted using 2 bead volumes CDC27 antigenic peptide in 250mM KCl (“APC/C bound”). 300μl of the peptide eluate were incubated with 50μl anti-Myc-antibody beads, the depleted supernatant was kept (“depleted”) and Myc-beads were 4 times washed with at least 10 bead volumes KCl wash buffer. Bound complexes were eluted using 2 bead volumes Myc antigenic peptide (“Myc”). 7.5μl protein sample was used for SDS-PAGE and western blot.

Control experiments in the absence of APC/C were performed using 50μl anti-Myc-antibody beads that were incubated with identical concentrations of co-activator and substrate constructs compared to experiments containing APC/C. To reach comparable western blot signals of 3MH6-CDH1 in samples derived from control and APC/C containing experiments, control samples had to be diluted 1:20.
7.4.8. **APC/C stability and ubiquitination activity in different salt conditions**

All incubation and elution steps were performed on a rotary shaker at 4°C for 1 hour unless otherwise noted. APC/C was purified in regular APC/C extract and wash buffers using anti-CDC27-antibody beads. Purified APC/C on beads (50μl beads per reaction) was 2 times washed with 30 beads volumes of wash buffer containing either 150mM NaCl, 150, 250, 350 or 450mM KCl and then incubated in the respective buffer conditions for 30 minutes. After this treatment, APC/C bound to beads was incubated with binding buffer containing respective salt concentration, 1.25μM cleaved CDH1 and 150nM His-Flag-td2-Hsl1-wt. Beads were washed 5 times with at least 10 bead volumes wash buffer (containing respective salt concentrations) and eluted using 2 bead volumes antigenic peptides (containing respective salt concentrations). 25μl APC/C-CDH1-Hsl1 eluate was incubated with 2.5μl reaction mix containing preloaded UBCH10-ubiquitin, E1, ATP and DTT for 1 hour at 4°C. Reactions were analysed using silver stain and western blot.

7.4.9. **Robustness test of APC/C in different buffers**

All incubation and elution steps were performed on a rotary shaker at 4°C for 1 hour unless otherwise noted. Apo-APC/C and APC/C-CDH1-Hsl1 were purified as described above. Peptide elution using 1mg/ml antigenic peptides was performed in either Tris or Heps buffer (20mM Tris-HCl or Heps, pH7.5, 150mM NaCl, 2.5% (v/v) glycerol, 0.1% (w/v) Octyl-β-D-glucopyranoside). 1ml of the peptide eluate was transferred into a 50,000 MWCO Amicon centrifugal filter units (do not prewash membrane) and diluted with 15ml of dialysis buffer (20mM Tris-HCl or Heps, pH7.5, 150mM NaCl, 5-10% (v/v) glycerol, 0.1% (w/v) Octyl-β-D-glucopyranoside). Concentration was carried out in 3-4 consecutive steps of centrifugation at 2,000 rpm for 5 minutes and subsequent, gentle
sample mixing. After the sample was concentrated up to 1ml, the sample was again diluted with 15ml dialysis buffer and subjected to concentration.

### 7.5. APC/C purification and electron microscopy

#### 7.5.1. Purification of APC/C complexes for electron microscopy

High-concentrated cell extracts were prepared using the two step cell lysis protocol (section 5.3.2). Adherently grown log-phase HeLa cells were resuspended and lysed in ice cold EM extract buffer (20mM Tris-HCl, pH7.5, 150mM NaCl, 2mM EDTA, 10% (v/v) glycerol, 0.1% (w/v) Octyl-β-D-glucopyranoside) using a dounce homogenizer followed by centrifugation (22,000g, 4°C, 30 minutes). To increase the protein concentration of the cell extract adherently grown log-phase HeLa cells were resuspended and lysed in the soluble cell extract derived from the first cell lysis step using a dounce homogenizer followed by centrifugation (22,000g, 4°C, 30 minutes). Human apo-APC/C was immunoprecipitated (IP) from the soluble fraction by incubation with anti-CDC27-antibodies cross-linked to Affi-prep protein A beads (Bio-Rad) for 1 hour at 4 °C. Beads were washed four times with at least 20 bead volumes of EM wash buffer (20mM Tris-HCl, pH7.5, 150mM NaCl, 10% (v/v) glycerol, 0.1% (w/v) Octyl-β-D-glucopyranoside) for 3 minutes at 4 °C. Apo-APC/C bound to CDC27 antibody beads was either used for in vitro reconstitution experiments or/and recovered by elution with 2mg/ml antigenic peptides (20mM Tris pH7.5, 150mM NaCl, 2.5% (v/v) glycerol, 0.05% (w/v) Octyl- β-D-glucopyranoside) for 1 hour.

25g adherently grown log-phase HeLa cells were used to saturate 1ml anti-CDC27-antibody beads. High-concentrated cell extracts were produced by resuspending 10g
HeLa cells in 10ml EM extract buffer and the remaining 15g of HeLa cells were used in the second cell lysis step. APC/C was eluted in one bead volume of 2mg/ml antigenic peptide. *In vitro* reconstitutions were done as described above. 2ml *in vitro* reconstituted APC/C eluate was used to saturate 500μl anti-Flag- or anti-Myc-antibody beads.

After APC/C purification, specimens were subjected to GraFix to further purify and stabilize the complexes (Kastner et al. 2008). Glycerol density gradients (20mM Bis-Tris pH8.0, 150mM NaCl, 10mM Imidazol, 10-40% (v/v) glycerol, 0.025-0.1% (v/v) glutaraldehyde (Electron Microscopy Sciences)) were prepared by pouring a 1 step gradient (~2.2ml 10% glycerol and 0.025% glutaraldehyde on top; ~2.2ml 40% glycerol and 0.1% glutaraldehyde at the bottom using thinwall polyallomer tubes (Beckman) and short caps (Biocomp)) that was mixed using the Biocomp gradient master. 550μl were removed from the gradient top, 150μl cushion (20mM Bis-Tris pH8.0, 150mM NaCl, 10mM Imidazol, 7.5% glycerol) was overlaid and up to 550μl APC/C eluate was applied on top of one gradient using cut tips. Glycerol density gradient centrifugation was performed using a TH-660 Sorvall rotor at 37,000rpm for 14 hours at 4°C. Gradients were fractionated from bottom to top (~130μl fractions) and glutaraldehyde crosslinking was stopped using 7.5mM L-aspartate pH8.0 (final concentration).

### 7.5.2. Glycerol removal for cryo-EM

To remove glycerol from the APC/C peak fractions, 2μl of 10% Octyl-β-D-glucopyranoside was added to each fraction. APC/C peak fractions from two glycerol gradients were then applied to a PD MiniTrap G-25 column (up to 500μl sample can be applied) that was equilibrated in buffer exchange buffer (20mM Bis-Tris pH8.0, 150mM NaCl, 10mM Imidazol, 0.1% Octyl-β-D-glucopyranoside). APC/C complexes were eluted by applying 1ml buffer exchange buffer to the column and collecting 5 fractions of 5
drops each. After the buffer exchange, fraction 3 contained the majority of APC/C complexes.

7.5.3. Grid preparation, image recording and data analysis

Preparation of EM grids, image recording and data analysis were carried out by Prakash Dube and Holger Stark and are described in (Buschhorn et. al.). Cryo grids were prepared with the FEI Vitrobot. Cryo images were recorded with the FEI Titan Krios.
8. References


9. **Abbreviations**

<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>162-14</td>
<td>CDH1 dN162 dC14</td>
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<tr>
<td>2D</td>
<td>Twodimensional</td>
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<tr>
<td>3D</td>
<td>Threedimensional</td>
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<td>Å</td>
<td>Angstroem</td>
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<td>APC/C</td>
<td>Anaphase promoting complex/cyclosome</td>
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<tr>
<td>ATP</td>
<td>Adenosine-triphosphate</td>
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<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
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<td>Bacmid</td>
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<td>Bovine serum albumin</td>
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<td>Chaperonin-containing TCP1 complex</td>
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<td>Dkm</td>
<td>D-box, KEN-box mutant</td>
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<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>Doc1</td>
<td>Destruction of Cyclin B1</td>
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<tr>
<td>DTT</td>
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<td>tdimer2</td>
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<td>Ubiquitin</td>
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<td>Ubi-mix</td>
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<td>WD40-domain</td>
<td>WD40-repeat containing protein fold; also β-propeller</td>
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WD40-repeat  Stretch of ~40 amino acids usually ending with Trp-Asp
Wt          Wild-type
w/v         Weight per volume

Amino acids were abbreviated according to the standard one or three letter nomenclature.
10. Appendix

The appendix section contains a publication in which some data of the present thesis have already been included.

*equally contributing authors
Substrate binding on the APC/C occurs between the coactivator Cdhl and the processivity factor Doc1

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The anaphase-promoting complex/cyclosome (APC/C) is a 22S ubiquitin ligase complex that initiates chromosome segregation and mitotic exit. We have used biochemical and electron microscopic analyses of Saccharomyces cerevisiae and human APC/C to address how the APC/C subunit Doc1 contributes to recruitment and processive ubiquitylation of APC/C substrates, and to understand how APC/C monomers interact to form a 36S dimeric form. We show that Doc1 interacts with Cdc27, Cdc16 and Apc1 and is located in the vicinity of the cullin–RING module Apc2–Apc11 in the inner cavity of the APC/C. Substrate proteins also bind in the inner cavity, in close proximity to Doc1 and the coactivator Cdhl, and induce conformational changes in Apc2–Apc11. Our results suggest that substrates are recruited to the APC/C by binding to a bipartite substrate receptor composed of a coactivator protein and Doc1.

Sister chromatid separation and exit from mitosis are initiated by a 1.5-MDa ubiquitin ligase (E3) composed of at least 13 subunits and known as the anaphase-promoting complex/cyclosome (APC/C). The APC/C initiates these events by ubiquitylating substrate proteins such as securin and cyclin B, which are subsequently degraded by the 26S proteasome. Recognition of substrates by the APC/C depends on coactivator proteins, called Cdc20 and Cdhl, which promote substrate recruitment by interacting both with the APC/C and with recognition motifs in the substrates known as the destruction box (D box) and KEN box. The subsequent ubiquitylation of substrates is mediated by ubiquitin-conjugating (E2) enzymes that interact with a RING-finger subunit of the APC/C, called Apc11 (ref. 2). Before all chromosomes have been bioriented on the mitotic spindle, the spindle-assembly checkpoint (SAC) inhibits the form of the APC/C that is interacting with Cdc20 (APC/C<sub>Cdc20</sub>) by promoting assembly of a mitotic checkpoint complex (MCC) in which Mad2, BubR1 and Bub3 bind to Cdc20 (APC/C<sub>MCC</sub>).

Structural information will be essential to understanding the mechanism of APC/C-mediated ubiquitylation reactions, but so far, only crystal structures of the 35-kDa APC/C subunit Doc1 (also known as Apc10; refs. 4,5) and of parts of the tetratricopeptide repeat (TPR) subunits Cdc16, Cdc27 and Apc7 have been solved6-8. Therefore, electron microscopy (EM) has been used to analyze the structure of the APC/C. For human9,10, Xenopus laevis9 and fission yeast APC/C11, this has revealed that the APC/C complex has a roughly triangular shape and is largely composed of two domains, called the “platform” and the “arc lamp,” that together enclose a central cavity (see discussion below), whereas a model obtained for budding yeast (S. cerevisiae) APC/C shows a more globular structure12. Biochemical and EM subunit mapping experiments in different species9-11,13-15 have indicated that the platform domain contains Apc1, Apc4 and Apc5, whereas the arc lamp domain consists of the TPR proteins Cdc16, Cdc23, Cdc27 and, in case of the vertebrate APC/C, presumably also Apc7. Apc2, a member of the cullin protein family, is located between the platform and the “head” of the arc lamp domain, whereas Cdc20 and Cdhl bind to the arc lamp opposite Apc2, with Apc2 and coactivators both facing the central cavity.

Because Apc2’s interaction partner Apc11 interacts with E2 enzymes and coactivator proteins help to recruit substrates, it has been speculated that ubiquitylation reactions occur in the inner cavity9,10. However, direct evidence for substrate recruitment to this site is so far lacking. Also unknown is the location of Doc1, a subunit that has been implicated in substrate binding to the APC/C16 and in processive substrate ubiquitylation17. How Doc1 contributes to these processes is unknown, but the protein is structurally similar to ligand-binding domains in bacterial sialidases and some other enzymes4,5, and related “Doc domains” are also found in other ubiquitin ligases18,19. It is therefore possible that Doc1 uses its putative ligand-binding region to interact with APC/C substrates. Consistent with this hypothesis, Doc1’s ligand-binding domain is required for its ability to confer processivity to the APC/C20. However, direct evidence for Doc1-substrate interactions has not been obtained. Here we have used biochemical reconstitution experiments to isolate various forms of budding yeast and human APC/C and have

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analyzed these complexes by using photo-cross-linking, single-particle EM and three-dimensional (3D) reconstruction. Our results show that the structure of the APC/C is conserved from budding yeast to vertebrates and that Doc1 is located in close vicinity to the cullin–RING module Apc2–Apc11. Structural analysis of human APC/CCDH1–substrate complexes revealed that the Doc1 cross-links to Cdc27, Cdc16 and Apc1 in yeast APC/C and DOC1 form a bipartite substrate receptor on the APC/C.

RESULTS
Doc1 cross-links to Cdc27, Cdc16 and Apc1 in yeast APC/C
To understand how Doc1 interacts with the APC/C, we generated radio-labeled forms of Doc1 containing a photoactivatable cross-linker at defined sites, allowed these Doc1 proteins to bind to APC/C lacking endogenous Doc1 and used photo-cross-linking followed by SDS-PAGE and phosphorimaging to identify cross-link products between Doc1 and APC/C subunits. We used APC/C from budding yeast for these experiments because previous work had shown that APC/C can be purified from yeast strains from which the DOCl gene has been deleted and because the processivity defect of the resulting APC/C<sub>ΔDoc1</sub> complexes can be restored in vitro by addition of recombinant Doc1 (refs. 16, 17). Earlier studies used a version of Doc1 containing 283 amino acid residues<sup>20,21</sup>, whereas the <i>Saccharomyces cerevisiae</i> genome database (SGD; http://www.yeastgenome.org/) reports only 250 residues for Doc1 (lacking 38 residues at the N terminus). Because we could identify only the latter form of Doc1 associated with endogenous APC/C (Supplementary Fig. 1a), we used a cDNA that encodes the 250-residue sequence of the Doc1 cDNA. We used the resulting mutated cDNAs to generate forms of Doc1 that contain cross-linkers, we introduced amber (TAG) stop codons into different sites in the coding sequence of the Doc1 cDNA. We used the resulting mutated cDNAs as templates in <i>in vitro</i> transcription-translation reactions that contained [35S]methionine, [35S]cysteine and an amber suppressor tRNA coupled to the artificial amino acid L-4’-[(trifluoromethyl)-3H-diazirine-3-yl]phenylalanine (hereafter called (tmd)Phe<sup>22</sup>). Use of the suppressor tRNA allowed translation beyond the amber stop codon, resulting in [35S]-labeled Doc1 carrying (tmd)Phe at the sites specified by the amber codons (Fig. 1c). We inserted (tmd)Phe at any one of 24 different positions, representing nearly 10% of all residues in Doc1 (Supplementary Table 1). When incubated with APC/C<sub>ΔDoc1</sub>, 7 of the 24 Doc1 mutants reproducibly yielded cross-link products with molecular masses of 120 kDa (Phe224amber, Glu239amber), 140 kDa (Lys129amber, Arg182amber) and 200 kDa (Ser128amber, Lys154amber, Arg182amber, Asn205amber; Fig. 1d and Supplementary Table 2).

Figure 1 Incorporation of a photo-cross-linker into Doc1 results in cross-link products between Doc1 and APC/C subunits. (a) Composition of wild-type APC/C (WT) and APC/C lacking Doc1 (ΔDoc1) affinity-purified via a TAP tag on Apc4. Apc4–CBP indicates calmodulin-binding protein that remains on Apc4 after TEV cleavage. Asterisk marks contaminating band. (b) APC/C ubiquitylation of [35S]methionine-labeled Hsl1<sub>667–782</sub> substrate is impaired when Doc1 is absent as compared to when the wild-type form of APC/C is present. (c) Cross-linker incorporation into <i>in vitro</i>-translated Doc1. Full-length Doc1 contains the photoactivatable amino acid. (d) Overview of cross-link products obtained with [35S]-labeled Doc1 amber mutants carrying the photo-cross-linker at six different sites. The two consecutive sites Ser128 and Lys129 each created two cross-link products of different sizes. (e) Doc1 crystal structure of <i>S. cerevisiae</i>. Cross-linker incorporation sites are indicated. Predominant cross-links of APC/C subunits to respective Doc1 sites are color coded (green, Cdc27; blue, Cdc16, orange, Apc1).
and Doc1-Lys129amber, which are located in the C-terminal region of Doc1, and Doc1-Arg182amber, located on the back side of Doc1, formed cross-link products with Cdc16. Two Doc1 mutants, Doc1-Ser128amber and Doc1-Lys154amber, predominantly formed cross-link products with Apc1, but less abundant Apc1 cross-link products were also obtained with Doc1-Lys129amber and Doc1-Asn205amber (note that Doc1-Ser128amber predominantly formed cross-link products with Apc1 but also did so less frequently with Cdc16, whereas Doc1-Lys129amber showed the opposite behavior, forming cross-link products preferentially with Cdc16 but also to a lesser degree with Apc1; for possible interpretations of this result, see Discussion). Doc1-Glu239amber was not analyzed because Doc1-Glu239amber and Doc1-Phe244amber yielded indistinguishable cross-link products, suggesting that they both interact with the same APC/C subunit (Cdc27). These results suggest that Doc1 interacts with the APC/C by contacting at least three other subunits, Cdc27, Cdc16 and Apc1.

**Localization of Doc1 in budding yeast and human APC/C**

To better understand Doc1’s interactions with other APC/C subunits, we identified the position of Doc1 within the structure of APC/C. Because the previously reported 3D model of budding yeast APC/C differed substantially from the structures obtained for human and *Xenopus* APC/C, we first determined a 3D structure of yeast APC/C (Fig. 3a) at ~25-Å resolution by cryo-negative-staining EM (Fig. 3b,c and Supplementary Table 3). This structure revealed that yeast APC/C, like vertebrate APC/C, is composed of a platform and an arc lamp domain that together enclose a central cavity. The dimensions of the platform domain are similar in yeast, *Xenopus* and human APC/C (135 × 130 Å in yeast APC/C), but the arc lamp domain of yeast APC/C is shorter than the corresponding domain in vertebrate APC/C (195 Å high in yeast versus 230 Å in human APC/C). Otherwise, the structures of yeast and vertebrate APC/C are similar in shape and size, indicating that APC/C’s structure has been largely conserved during evolution.

We identified the location of Doc1 within the APC/C by using three different approaches (Fig. 4). First, comparison of the 3D structure of wild-type yeast APC/C with a structure obtained for APC/C<sup>ΔDoc1</sup> revealed that a small density located on top of the inner cavity and under the head of the arc lamp domain was absent in APC/C<sup>ΔDoc1</sup> (Fig. 4a). Second, we generated a strain in which the N terminus of Doc1 was fused to a 56-kDa tag called tdimer2 (td2), which is composed of two copies of the red fluorescent protein DsRed<sup>24,25</sup> (Supplementary Fig. 1c). When APC/C containing only this version of Doc1 was analyzed by EM and 3D reconstruction, an extra density was observed next to the density that was absent in APC/C<sup>ΔDoc1</sup> (Fig. 4a,b). Third, we used antibodies to human DOC1 to map the location of this subunit in human APC/C. We incubated APC/C purified from HeLa cells with DOC1 antibodies at an APC/C-to-antibody ratio that led to the formation of trimers in which two APC/C particles were bound by one IgG molecule, enriched these complexes by glycerol density-gradient...
centrifugation and used negative staining EM to determine the region on APC/C to which the DOC1 antibodies had been bound (Supplementary Fig. 1d,e). These experiments indicated that in the structure of human APC/C, as in that of yeast, DOC1 is located above the inner cavity and below the head of the arc lamp domain.

Localization of yeast APC/C subunits

To determine whether the position of Doc1 within the APC/C structure is consistent with the observation that Doc1 can form cross-link products with Cdc27, Cdc16 and Apc1, we mapped the location of these subunits in the 3D structure of yeast APC/C. We tagged Cdc27 and Cdc16 at their C termini with td2, and Apc1 with a tag that contained a single copy of DsRed (called “tm” for monomer; viable strains expressing Apc1-tm could not be obtained). We purified APC/C containing Cdc27-td2, Cdc16-td2 and Apc1-tm and obtained 3D structures for these complexes (Fig. 5 and Supplementary Fig. 2). These experiments revealed that Cdc27 is located in the “head” of the arc lamp domain and Cdc16 in a central region of the arc lamp domain, next to the putative location of Cdc27 (Fig. 5b,c); in other words, Cdc27 and Cdc16 are located above and behind Doc1. The tag fused to the C terminus of Apc1 protruded from the platform domain into the inner cavity of APC/C; that is, it was located below Doc1 (Supplementary Fig. 2d). Although it cannot be inferred from these data that Doc1 directly contacts Cdc27, Cdc16 and Apc1, the EM data are consistent with this possibility.

We also used the techniques developed for localization of Doc1 to identify the position of other APC/C subunits. In case of Swm1 (called Apc13), one of the few APC/C subunits that are not essential for viability in yeast14,16,26,27, we purified APC/C from a strain from which the SWM1 gene had been deleted. In the APC/C<sup>ΔSWM1</sup> structure, a small density was missing on the front side of the head of the arc lamp domain, next to Cdc16 and Cdc27 (Figs. 4c and 5e). A 3D model of APC/C in which Swm1 had been tagged with td2 revealed an extra globular density right next to the putative Swm1 density identified by difference mapping (Fig. 4c). Swm1 is therefore located in the vicinity of Cdc16 and Cdc27, consistent with the observation that Swm1 stabilizes interactions between these two subunits14.

We attempted to use td2 tagging for the remaining APC/C subunits as well, but we were able to obtain viable yeast strains and APC/C samples suitable for EM only for Apc11 and Apc5. The resulting 3D structures revealed an additional globular domain in the central cavity, close to Doc1, when td2 had been fused to the C terminus of Apc11 (Fig. 5c and Supplementary Fig. 2c,d), consistent with the previously identified location of APC11’s binding partner, Apc2, in human APC/C<sup>9,10</sup>. C-terminal tagging of Apc5 with td2 revealed an extra density on the left side of the platform domain (Fig. 5c and Supplementary Fig. 2b,d), also consistent with the previous localization of APC5 in human APC/C<sup>10</sup>.

EM analysis of dimeric forms of yeast APC/C

The majority of APC/C observed in our EM specimens was monomeric, but dimeric forms of APC/C were also present (Supplementary Fig. 3a), confirming previous reports12,16. As shown before23, we found that APC/C sedimented corresponding to a sedimentation coefficient of 36S when yeast extracts were fractionated in the presence of low salt concentrations (50 mM KCl). However, when the same yeast extracts were analyzed in the presence of 400 mM KCl, most APC/C sedimented as a 22S particle (Supplementary Fig. 3b,c),
Figure 6. Analysis of APC/C dimers. (a) Class averages of dimeric APC/C with different orientations. Size bar, 10 nm. (b) 3D model of dimeric APC/C. The purple APC/C monomer is shown in a side view orientation with the TPR-rich arc lamp domain in the front. The gray APC/C monomer is shown in a bottom view orientation with the bottom of the platform domain in the front. Asterisks mark the face of APC/C’s central cavity. The middle ellipse marks a unique contact lying on the c2 symmetry axis, whereas the other ellipses show contact points that each exist as an asymmetric pair. (c) APC/C dimerization causes conformational rearrangements in the platform domain as well as the head domain compared to monomeric APC/C (yellow). Black lines and asterisk indicate conformational changes between monomeric and dimeric APC/C.

as does purified monomeric yeast APC/C. These observations indicate that many APC/C particles in yeast extracts exist as dimers that dissociate into monomers in the presence of buffers with high ionic strength. Because these findings are consistent with the possibility that yeast APC/C can also exist as a dimer in vivo, we also generated a 3D structure of dimeric APC/C at a resolution of ~35 Å. Images of these dimers could be sorted into class averages of similar orientations (Fig. 6a), indicating that these dimers represent a homogeneous population of particles in which two APC/C monomers adopt a defined orientation.

The resulting 3D structure revealed that APC/C monomers within the dimer interact via five discrete contact points (three of which can be seen in Fig. 6b, marked by ellipses), mostly on the back side of the arc lamp and the platform domains (Fig. 6bc). One of these points lies on a c2 symmetry axis and is generated by a contact between the same regions of the arc lamp domain where Cdc27 is located and thus possibly through a homotypic interaction between two Cdc27 molecules. The other contacts are not located on the c2 axis and thus occur as asymmetrical pairs of interactions (subunit A of monomer 1 interacts with subunit B of monomer 2 and vice versa). The first pair of contacts is generated by interactions between Apc1 in the platform domain and an unidentified subunit located on the back side of the arc lamp domain, possibly Cdc27, Cdc16 or Cdc26. The second pair of contacts is formed between Cdc16 and an unidentified subunit on the back side of the platform, possibly Apc1 or Apc2.

During 3D reconstruction, we applied two-fold symmetry, which prevents the detection of differences in conformation between APC/C monomers within the dimer. However, the conformation of APC/C in the dimer is clearly different from the conformation of monomeric APC/C. Whereas Apc1 has a characteristic bent shape in monomeric APC/C, it adopts a straighter conformation in the dimer, pointing upward into the direction of Doc1 (the conformational change in Apc1 is indicated by long black lines in Fig. 6c). Smaller conformational changes could also be observed for densities that correspond to Apc4, Cdc27 and Doc1. In the monomer, the density of Apc4 is better defined and the contact between Cdc27 and Doc1 (indicated by short black lines in Fig. 6c) is more pronounced than in the dimer.

Because the dimeric form of yeast APC/C has been reported to ubiquitylate substrates with higher processivity than monomeric APC/C, we tested whether the role of Doc1 in APC/C processivity could be explained by a requirement for Doc1 in APC/C dimerization. When we analyzed proteins in whole cell extract from yeast Δdoc1 strains, however, we observed that Cdc16 sedimented as a 36S particle, and in EM specimens of APC/CΔDoc1, dimers could still be detected (data not shown), indicating that APC/CΔDoc1 also exists in a dimeric form (see Supplementary Fig. 3d). Doc1 therefore is not required for APC/C dimerization and must contribute to APC/C processivity through other mechanisms.

Substrate binding to the APC/C occurs between CDH1 and DOC1

To understand how substrates are recruited to the APC/C and whether DOC1 has a direct role in this process, we mapped the location of a substrate protein on the APC/C by EM and 3D reconstruction. We used human APC/C for this analysis because we were able to generate sufficient amounts of human CDH1 (His6-CDH1), but not of yeast coactivator proteins, for these experiments. To evaluate which substrate binds to APC/C stably enough to allow EM mapping experiments, we reconstituted and purified APC/C bound to either budding yeast Hsl1667–872, human sororin or human securin and measured, by immunoblotting over a time course of 6 h, how much substrate remained bound to APC/C. Many sororin and securin molecules dissociated from APC/C during the 6-h time course, whereas the majority of Hsl1 remained associated (Fig. 7ab), consistent with the previous observation that Hsl1 binds to APC/C particularly tightly. These experiments also confirmed that the interaction between APC/C and CDH1 is stabilized by the presence of Hsl1 (refs. 29,30).

We therefore performed all subsequent experiments with the Hsl1667–872 fragment. We used a td2-tagged version of Hsl1 (His6-Flagtd2-Hsl1667–872) for these experiments, hoping that the td2 domain might help in the subsequent visualization of the substrate in the 3D structure. To ascertain that this protein binds as a bona fide substrate, we isolated APC/C on CDC27 antibody-conjugated Sepharose beads and incubated these with either wild-type Hsl1 or a mutant in which the D box and the KEN box were mutated in either the absence or the presence of CDH1. Unbound CDH1 and Hsl1 were removed by washing the beads, and APC/C was subsequently eluted from the antibody beads by an excess of antigenic CDC27 peptide. SDS-PAGE and silver staining confirmed that only the wild-type fragment of Hsl1 could efficiently bind to the APC/C, and that this interaction was greatly increased by the presence of CDH1 (Fig. 7c). Furthermore, incubation of APC/C–CDH1 complexes with E1, UBCH10 and ubiquitin in the ubiquitylation of Hsl1, further indicating that Hsl1 associated with APC/C–CDH1 as a functional substrate (Fig. 7d).

Because substrates have to be turned over rapidly in vivo, it was surprising to find that substrates bind to the APC/C relatively stably. This observation raises the interesting possibility that, in vivo, additional factors might promote substrate release from the APC/C.

To obtain complexes composed of APC/C, CDH1 and substrate in a 1:1:1 stoichiometry, we further re-isolated proteins bound to Hsl1 by immunoprecipitation using antibodies to Flag, again followed by peptide elution (Fig. 7c, last lane). The resulting APC/C–CDH1–Hsl1 complexes were further purified by glycerol density-gradient centrifugation using the GraFix method and analyzed by cryo-negative
CDH1 and DOC1, and that substrate binding induces conformational changes in APC2 and/or APC11.

**DISCUSSION**

Even though the APC/C is essential for cell division, it remains poorly understood how this complex recognizes and ubiquitylates specific substrate proteins, how these processes are controlled in time, and why, in contrast to other ubiquitin ligases, the APC/C comprises at least 13 different subunits. Because structural information will be important for answering these questions, we have used biochemical and EM approaches to identify how the processivity factor Doc1 and a substrate protein interact with the APC/C. Our results also show that the structure of the APC/C is largely conserved between budding yeast and vertebrates, as well as how two APC/C particles interact to form a 36S dimer.

Although our structural analysis of yeast APC/C revealed many similarities with the structure of human APC/C, there are also important differences. The most notable one is the lack of a density in the arc lamp domain, which results in a reduced height (by 15%) for this domain in yeast APC/C (Fig. 8 and Supplementary Fig. 3e). Because the arc lamp domain is predominantly composed of TPR subunits, it is possible that the density only found in vertebrate APC/C is formed by a pre-formed UBCH10–ubiquitin complexes.

A second peculiarity of budding yeast APC/C is its ability to form dimers, which are the predominant form in yeast whole-cell extracts and which therefore might exist in vivo. This notion is further supported by the fact that we were able to obtain a defined 3D structure of APC/C dimers, which would not be possible if the dimers were formed by nonspecific aggregation of monomers. Our 3D structure reveals that two APC/C monomers interact via multiple discrete contacts involving Cdc27, Cdc16, Apc1 and possibly Apc2. Because these contacts are located on the back side of the arc lamp and the platform domains, the substrate-binding sites within the dimer face.

We also noticed that a contact that normally exists between APC2 and a subunit in the platform domain (indicated by a short arrow in Fig. 7e) is absent in APC/C–Hsl1 complexes (Fig. 7e,f and Supplementary Fig. 5). Instead, in APC/C–Hsl1, APC2 appears to contact CDH1 directly, in close proximity to where CDH1 contacts the substrate density. These data are consistent with the possibility that the substrate protein forms several contacts on the APC/C, possibly with
opposite directions. This observation suggests that each monomer within the APC/C dimer can mediate ubiquitylation reactions independent of its dimerization partner. Understanding the functional relevance of APC/C dimerization, and whether such dimers also exist in vivo, will therefore require further investigation.

By combining EM structure determination with the deletion or tagging of different subunits, we have identified the position of seven subunits in yeast APC/C. For four of these, we have also mapped the position of their orthologs in human or Xenopus APC/C\(^9,10\) (Supplementary Table 4). Notably, for all four of these subunits we found that their positions corresponded to each other in the yeast and vertebrate 3D structures. This observation, and the finding that the structures of yeast and human APC/C are similar, makes it possible for the first time to draw an almost complete 3D map of the locations of APC/C subunits (Fig. 8). This topographic map is largely consistent with previous biochemical and genetic data\(^13\)-\(^15\) and indicates that the platform domain is composed of Apc1, Apc4 and Apc5, and the arc lamp domain of Cdc27, Apc11, Swm1, Cdc16, Cdc26, Cdc23 and (in vertebrates) Apc7. Notably, a third, small domain, composed of Apc2, Apc11 and Doc1, is located between the platform and the arc lamp. Because Apc11 is known to recruit E2 enzymes, which transfer ubiquitin residues to substrates\(^1\), we propose to call this domain the "catalytic core."

It is well established that Doc1 is required for efficient substrate recruitment to the APC/C and for processive substrate ubiquitylation\(^16,17,20\), but how Doc1 performs these functions and how Doc1 itself interacts with the APC/C are poorly understood. In cross-linking experiments, we identified Cdc27, Cdc16 and Apc1 as binding partners of Doc1. These results are consistent with our previous observation that Doc1 binds to Cdc27 in vitro\(^9\) and with our EM data, which place Doc1 above Apc1, to the right of Cdc16 and below Cdc27 (Fig. 8). Furthermore, it has been previously shown that the Doc1 residues Lys129 and Arg130 are required for efficient binding of Doc1 to the APC/C (ref. 20; note that in this study these residues were referred to as Lys162 and Arg163 because the 283-residue version of Doc1 was used). This observation, combined with our finding that Lys129 predominantly cross-links to Cdc16 and the neighboring residue Ser128 predominantly to Apc1, indicate that the interaction of Doc1 with Cdc16 and Apc1 is required for efficient binding of Doc1 to the APC/C. Because genetic experiments had shown that Cdc2 is required for recruitment of Doc1 to yeast APC/C\(^15\), it was unexpected that no Doc1–Apc2 cross-link products were obtained, but our EM data reveal that Doc1 is indeed located next to Apc2 and Apc1. It is possible that we were unable to detect Doc1–Apc2 interactions because we did not insert tmd(Phe) into Doc1 residues that contact Apc2. Notably, we observed that two Doc1 residues (Ser128 and Lys129) could each cross-link to two APC/C subunits, Cdc16 and Apc1, which might represent a subunit specific for vertebrate APC/C, such as Apc7.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS
H.S. and J.-M.P. planned and supervised the project. B.A.B., G.P., C.K. and F.H. designed the experiments. B.A.B. performed most of the photo-cross-linking and biochemical experiments on yeast APC/C. G.P. performed the experiments on substrate-bound APC/C. M.G. and C.K. generated yeast strains and performed growth assays and yeast APC/C purifications. F.H. performed antibody labeling on human APC/C. P.D. performed EM. H.S. calculated and analyzed the 3D EM structures. B.A.B., G.P. and J.-M.P. wrote the paper.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Strains and plasmids. Yeast genetic manipulations were carried out using standard protocols. For untagged Doc1 constructs in pME vectors, pME34 (ref. 20) was used and the original STOP codon of the open reading frame (ORF) was kept.

Strains and plasmids.

APC/C purification. Yeast cells were grown in YPD rich medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose). At OD600, cells were harvested by centrifugation and washed with EM lysis buffer (20 mM HEPES-KOH, pH 8.0, 200 mM KCl, 10% (v/v) glycerol, 1.5 mM MgCl2). Yeast extracts were prepared by bead beating for cross-linking experiments. For EM experiments, frozen cells were ground in a mortar grinder (RM100, Retsch), whereas a freezer mill (SPEX Freezer/Mill 6770, 6780) was used for all other applications. APC/C isolation by tandem affinity purification was carried out as described16,36. For each EM experiment, cells from 40 liters of yeast culture were lysed in the presence of 0.03% (w/v) octyl-β-d-glucopyranoside, and APC/C was eluted with 3 mM EGTA. 500 μl APC/C eluate was loaded onto a 10–40% (v/v) glycerol density gradient (20 mM HEPES-KOH, pH 7.9, 150 mM NaCl, 0.05% (v/v) octyl-β-d-glucopyranoside) containing 0.05–0.2% (v/v) glutaraldehyde32 and centrifuged at 37,000 r.p.m. (140,601 g) for 60–90 min at 4 °C in a Beckman SW60Ti rotor. APC/C peak fractions were used for subsequent SDS-PAGE experiments.

Photo-cross-linking. TNT rabbit reticulocyte lysates were used to incorporate photoactivatable cross-linkers into the Doc1 protein. Reactions were supplemented with 1 mM magnesium acetate and 1.6 μl (tmd)PrsA-tRNA (see below) per 50 μl reaction. Photo-cross-linking experiments were carried out with freshly prepared yeast cell extracts (50 mM Tris-HCL, pH 8, 150 mM NaCl, 10% (v/v) glycerol, 0.1% (v/v) NP40). Samples containing photo-cross-linkers were light-protected in all steps. APC/C was bound to IgG-Sepharose beads by incubating extract and Sepharose (Bio-Rad) (1–2 mg protein per 5 μl beads) for 60–90 min at 4 °C. Beads were washed with buffer used for cell lysis before addition of TNT rabbit reticulocyte lysate containing in vitro–translated Doc1 versions carrying a photo-cross-linker at distinct sites. Samples were incubated at room temperature for 20 min and subsequently washed 3–4 times with lysis buffer. For photo-cross-linking, samples with lids opened were kept in a Thermomixer (Eppendorf) (1,800 r.p.m., 9 °C) and exposed to a black-ray long-UV lamp (B–100AP, 100 W, UV) with a wavelength of 360 nm at a distance of 10 cm for 5–8 min. APC/C was eluted by the addition of SDS sample buffer. 

In vitro reconstitution of APC/C bound to human CDH1 and substrates. Cell extracts were prepared by lysis of frozen log-phase HeLa cells in extract buffer (20 mM Tris–HCL, pH 7.5, 150 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol, 0.05% (v/v) Tween–20) using a Dounce homogenizer followed by centrifugation. APC/C was immunoprecipitated (IP) from the soluble fraction by incubation with CDC27 antibodies57 cross-linked to Affi–prep protein A beads (Bio-Rad) for 1 h at 4 °C. Beads were washed four times with at least 20 bead volumes of wash buffer (20 mM Tris–HCL, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 0.05% (v/v) Tween–20) for 3 min at 4 °C. To reconstitute coactivator- and/or substrate-bound APC/C complexes in vitro, APC/C-bound CDC27 beads were resuspended in binding buffer (20 mM Tris–HCL, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween–20, 4 mg ml−1 BSA) supplemented with 3.5 μM recombinant human CDH1 and/or 150 nM recombinant wild-type or mutant (D box and KEN box mutated) His-Flag-td2-Hsl1667–872, sororin-td2-Flag-His or securin-Flag-His. After a 1-h incubation at 4 °C, the excess of recombinant proteins was removed by washing the beads four times with at least 20 bead volumes of wash buffer for 3 min at 4 °C, and APC/C complexes were recovered by elution with elution buffer. APC/C(CDH1–Hsl1) complexes were enriched by re-immunoprecipitation (re-IP) experiments using ANTI-FLAG M2 Agarose (Sigma) and recovered by elution with elution buffer. For EM experiments, APC/C complexes were subjected to GraFix32 to further purify and stabilize the complexes. Human recombinant CDH1 was expressed in baculovirus-infected Sf9 insect cells. Recombinant His-Flag-Hsl1667–872, sororin-td2-Flag-His and securin-Flag-His substrate proteins were expressed and purified from Escherichia coli BL21(DE3) or Rosetta(DE3). The His1667–872 sequence was derived from S. cerevisiae and sororin and securin from Homo sapiens. To demonstrate activity of in vitro–reconstituted APC/C(CDH1–Hsl1) complexes, 50 μl eluate was incubated with 5 μl reaction mix containing preloaded UBCH10–monoubiquitin, E1, ATP and DTT for 1 h at 4 °C. 

Off-rate experiments. In vitro–reconstituted substrate-bound APC/C(CDH1) on 25 μl CDC27 antibody–conjugated beads was resuspended in 50 μl wash buffer and incubated on a orbital rotator for 1 min (time point 0), 2, 4 and 6 h. CDC27 antibody–conjugated beads were spun down, wash buffer was saved (unbound fraction) for subsequent SDS-PAGE analysis, and APC/C complexes were recovered by elution with 50 μl antigenic peptides. APC/C-bound and unbound fractions were analyzed by western blotting.

Supplementary Material

Substrate binding on the APC/C occurs between the coactivator Cdh1 and the processivity factor Doc1

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Jan-Michael Peters
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This supplement includes:
Supplementary Methods
Supplementary Figures 1 to 5
Supplementary Tables 1 to 5
Supplementary References
Supplementary Methods

**Doc1 mutagenesis and expression.** To generate “long” and “short” versions of Doc1, PCR products were generated from a *DOC1* cDNA template containing both start codons with primers 5’-ATCGATTAATACGACTCACTATAGGGCTCGAGGCCGCCACCATGGGGCAAAATAAGCGCCGTCTATAT-3’ (forward primer “long”) or 5’-ATCGATTAATACGACTCACTATACTGCTCGAGGCCGCCACCATGGACCCGATTGGAATAAACAAGT-3’ (forward primer “short”) and 5’-GTGCTCTCGAGTTACATCATGCCTCTTAACGTAATATAGCATCCTGGAAAGAATTGTT-3’ (reverse primer for both). PCR products were used as templates in coupled *in vitro* transcription/translation reactions.

**N-terminal tagging of Doc1 with tdimer2.** A *doc1* deletion strain was transformed with a plasmid system (kindly provided by Gwenael Rabut) allowing the exchange of the G418 selection cassette by a tagged gene version of *DOC1*. The *TDIMER2* ORF was subcloned from pGR20 (kind gift of G. Rabut) via PacI/NotI. The *DOC1* promoter, introducing an AgeI site at the 5’ end, and a START codon and a PacI site (ATG TTA ATT AA) at the 3’ end, was amplified from genomic DNA. NotI (plus an extra nucleotide, GCG GCC GCT ATG) and XhoI restriction sites were introduced when amplifying *DOC1* from cDNA and *DOC1* was ligated into pGR51. The resulting construct was cleaved with AgeI and NotI enzymes, and promoter and *TDIMER2* were inserted simultaneously. The resulting plasmid was cut with AgeI and PmeI, releasing the tagged construct, which was transformed into a heterozygous *doc1* deletion strain (Mata/alpha *DOC1/doc1::KanMX*, J464) strain and positive transformants were selected on plates lacking histidine.

**(tmd)Phe-tRNA.** (tmd)Phe was obtained from Botanica GmbH, Sins, Switzerland. Alternatively, it was generated from (tmd)Phe-pdCpA (generous gift from J. Brunner) and tRNA<sup>SUP</sup>(pdCpA) following the protocol by<sup>1</sup>, with two major exceptions. The template for the transcription reaction was generated by PCR amplification from pTHG73 (ref. 2) using primers 5’-GCGGTCCTACTGGGATT-3’ and 5’-AATTGCCTACGACTCCTATAG-3’. The PCR product was extracted with phenol pH 7.9 and chloroform followed by ethanol precipitation.
About 5 μg PCR product were used for a 100 μl transcription reaction. Secondly, N-(4-)
pentenoyl-protected (tmd)Phe was deprotected with 25 mM I₂ in 1:1 tetrahydrofuran-water³.

**Gradient centrifugation and APC/C immunoprecipitation.** CDC16-HA³/CDC16-myc⁶ cells
were lysed in a freezer mill, extracts were cleared by subsequent ultracentrifugation. The soluble
fraction was diluted to a concentration of 4 mg ml⁻¹ in LB50 buffer (20 mM Hepes KOH pH 8.0,
50 mM KCl, 1.5 mM MgCl₂, 0.1% (v/v) NP40, 1 mM DTT) or LB400 buffer (LB50 + 350 mM
KCl), supplemented with 5% (v/v) glycerol. 4 mg of extract were separated through a 10 to 40%
(v/v) glycerol gradient prepared in buffer LB50 or LB400. 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 μl aliquots using an ISCO fractionator.
APC/C was immunoprecipitated in each fraction using 12CA5 (HA) antibody beads. Beads were
washed with LB50 containing 10% (v/v) glycerol. Proteins were eluted with 100 mM glycine pH
2.2 and analyzed by western blotting. Apc1-myc/Apc1-HA coimmunoprecipitations were carried
out as described⁴. Briefly, 12CA5 and 9E10 antibodies were bound to protein A sepharose
(Biorad) and GammaBind G sepharose (GE Healthcare) beads, respectively. Extracts were
prepared in buffer (50 mM Tris pH 7.5, 50 mM NaCl, 10% (v/v) glycerol, 0.2% (v/v) Triton X-
100, 1 mM DTT) by bead beating. 10 μl beads each were incubated with cleared lysates
containing 2 mg protein for 90 min at 4°C. Beads were washed with buffer, and bound proteins
were eluted with 15 μl 100 mM glycine pH 2.2.

**Antibodies.** Antibodies against Apc1, Cdc16, Cdc23, Doc1 and Apc11 were raised by
immunizing rabbits with peptides coupled to Keyhole Limpet Hemocyanin (Apc1:
CDDERSSNGSDisDPTAYLEDKKDIDDDHYG; Cdc16:
CALRKGGHSKTGSNNADDDFDAD; Cdc23: SLaDEsPlRnKQGvPKQMFC; Doc1:
CSNEPHQDTHEWAqTLPETNNV; Apc11: CVDFDEPIRqNDPigRQQV). 9E10 and
12CA5 antibodies were used for immunoprecipitation and immunodetection of the myc- and
HA-epitopes, respectively.

**Cryo-Negative Stain Electron microscopy.** Purified APC/C or APC/C-antibody complexes
were adsorbed to a thin film of carbon and then transferred to an electron microscopic grid
covered with a perforated carbon film. The bound APC/C particles were stained with 2% (w/v) uranyl formate, blotted and air dried for ~1 min at room temperature. For cryo-negative staining the grids were subsequently plunged into liquid nitrogen. Images were recorded at a magnification of 155,000x on a 4k x 4k CCD camera (TVIPS GmbH) using two-fold pixel binning (1.8 Å per pixel) in a Philips CM200 FEG electron microscope (Philips/FEI) operated at 160 kV acceleration voltage. APC/C-antibody complexes were imaged at room temperature using the same magnification.

**Image Processing.** Particle images (200 x 200 pixel) were selected using the semiautomated software boxer as part of the Eman package. Images were coarsened by a factor of two resulting in 100 x 100 pixels per image with a sampling of ~3.8 Å per pixel. After CTF correction\(^5\), images were aligned using an exhaustive multi-reference alignment based on re-sampling to polar coordinates\(^6\). To obtain the initial 3D reconstructions we made use of random-conical-tilt imaging and weighted averaging of 3D volumes\(^7\). Characteristic views were obtained by averaging after multivariate statistical analysis and classification\(^8\). Angular reconstitution\(^9\) was used to determine the relative orientations of the projection images prior to computing the 3D reconstruction. 3D structures were visualized with the software AmiraDev 2.3 (TGS Europe, Merignac Cedex).
Supplementary Figure 1 Doc1 characterization and localization using tdimer2 tagging and antibody labeling. (a) Yeast cell extracts only contained a 30 kDa version of the Doc1 subunit, which can be co-purified with wild type APC/C and is absent in APC/C\textsuperscript{ADoc1}. \textit{In vitro} translated (IVT) products of either the short or the long \textit{DOC1} ORF version serve as reference. The asterisk marks the TAP-tag recognized by the Cdc16 antibody in the cell extract. (b) Doc1 is absent in \textit{doc1} deletions strains. Both wild type and \textit{doc1} deletion strains were used to TAP-tag purify APC/C via the Apc4 subunit. (c) N-terminal tdimer2-labeling of yeast Doc1. Western blot analysis of TAP-tag purified APC/C shows that Doc1 labeling results in a mobility shift from \sim 30 to \sim 80 kDa. (d) Localization of human DOC1 protein by antibody labeling. The orientation of APC/C within the APC/C-antibody complex was evaluated based on the known APC/C structure. Rectangles were modeled to the APC/C 3D structure at the respective binding sites of the antibody and the binding site was determined as the main crossing 3D area of all rectangles\textsuperscript{10}. (e) The antibody epitope is marked on the surface of the human APC/C 3D model and labeling
accuracy is indicated by the size of the area. A structurally similar domain compared to yeast APC/C Doc1 is situated within this area, indicating a conserved localization of the Doc1 subunit in yeast and human APC/C.
Supplementary Figure 2 Yeast APC/C subunit localization using td2-labeling. (a) Subunit labeling of the yeast Apc1 subunit. Apc1 was tagged with a tmmonomer-tag and TAP-tag purified via Apc4. Apc1 undergoes a mobility shift upon tmmonomer labeling in the silver stained SDS-PAGE. Apc4-CBP indicates calmodulin binding protein remaining on Apc4 after TEV cleavage. (b) SDS-PAGE of Apc5-tdimer2 labeled APC/C TAP-tag purified via the Cdc16 subunit. (c) Western blot analysis of tdimer2 labeled Apc11. Yeast APC/C complexes were TAP-tag purified via the Cdc16 subunit. (d) Yeast APC/C 3D models indicating subunit localization of Apc1, Apc5 and Apc11 by colored extra density elements representing the tdimer2 label.
Supplementary Figure 3 Analysis of yeast APC/C dimers. (a) Electron microscopic raw images of cryo-negative stained yeast APC/C monomers and dimers. Continuous circles exemplify monomeric, dashed circles dimeric yeast APC/C. (b) Yeast extracts primarily contain dimeric APC/C, which can be dissociated into monomers applying high salt conditions. Fractions of the
Gradients were analyzed by Western blot. Fatty acid synthase (41 S) and the 26 S proteasome subunit Rpn5 (ref. 11) serve as sedimentation markers. (c) Dimeric APC/C dissociates into monomers under high salt conditions. Extracts from a diploid CDC16-HA2/CDC16-myc6 yeast strain were used for glycerol density gradient centrifugation in the presence of either high or low salt concentrations. After gradient fractionation, APC/C was immunoprecipitated with HA antibodies from each fraction and analyzed by Western blotting. Under both conditions, Cdc16-HA2 co-immunoprecipitated Cdc16-myc6, demonstrating that monomeric yeast APC/C contains at least two copies of the Cdc16 subunit. (d) Self association of Apc1 is not impaired in the absence of Doc1. Wild type or doc1Δ diploid yeast strains carrying indicated epitope-tagged Apc1 versions were used for immunoprecipitation experiments. Apc1 is believed to be present as one copy within monomeric APC/C12,13. Apc1-HA3 could co-immunoprecipitate Apc1-myc9 (and vice versa) when co-expressed in presence and absence of Doc1, which indicates that APC/C dimerization does not depend on Doc1. (e) Yeast APC/C dimer interface involves bulky domains located on the back side of the TPR-rich arc lamp domain, labeled 2 and 3. Due to structural resemblance, similar domains could be identified and allocated in human APC/C. Compared to yeast APC/C the human 3D model carries a significant extra mass inserted within the dimer interface between domain 2 and 3, resulting in a more extended appearance of the arc lamp domain. This insertion might prevent dimerization of human APC/C.
Supplementary Figure 4  APC^{CDH1–Hsl1} and APC^{CDH1} depicted with different surface rendering thresholds. In the 3D model of APC^{CDH1–Hsl1} a second extra density could be resolved near the platform domain. This second density disappears upon increase of the threshold parameter for surface rendering, indicating a structural heterogeneity in the platform region among APC^{CDH1–Hsl1} complexes contained in the dataset. The two extra densities in the APC^{CDH1–Hsl1} 3D model might represent two distinct domains of the bound His-Flag-td2-Hsl1_{667–872} molecule. Importantly, using high threshold parameter settings, the density intercalated between CDH1 and DOC1 remained appreciable compared to APC^{CDH1}. 
Supplementary Figure 5 Comparison of human apo-APC/C, APC/C^{MCC} and APC/C^{CDH1-Hsl1}.
**Supplementary Figure 5** Comparison of human apo-APC/C, APC/C<sup>MCC</sup> and APC/C<sup>CDH1–Hsl1</sup>. The three different complexes are shown in their front, side, back and bottom view orientation. As reported previously<sup>10</sup>, the mitotic checkpoint complex is inserted into the central cavity located at the front side of the platform domain. In the APC/C<sup>MCC</sup> 3D model, the position of the co-activator density (CDC20) is changed compared to the position of CDH1 in the APC/C structure bound to a substrate molecule. These orientational differences might contribute to decreased substrate recognition as shown for APC/C<sup>MCC</sup> (ref. 10) by disrupting the bipartite substrate receptor. In apo-APC/C, the APC2–APC11 module contacts an unknown subunit located in the platform domain, which is also observed in the APC/C<sup>MCC</sup> structure (back view orientation). Interestingly, in the 3D model of APC/C<sup>CDH1–Hsl1</sup> this connection to the platform is absent and instead, APC2–APC11 forms a new contact to the co-activator density (back and bottom view orientation).
**Supplementary Tables**

**Supplementary Table 1** Doc1 sites exchanged with photo-activatable amino acids.

<table>
<thead>
<tr>
<th>Site of incorporation</th>
<th>Site in “long” Doc1</th>
<th>Location of site in structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg37</td>
<td>Arg70</td>
<td>Back side</td>
</tr>
<tr>
<td>His67</td>
<td>His100</td>
<td>N-terminal helix</td>
</tr>
<tr>
<td>Gln71</td>
<td>Gln104</td>
<td>N-terminal helix</td>
</tr>
<tr>
<td>Lys91</td>
<td>Lys124</td>
<td>Front side</td>
</tr>
<tr>
<td>Lys96</td>
<td>Lys129</td>
<td>Front side</td>
</tr>
<tr>
<td>Leu97</td>
<td>Leu130</td>
<td>Front side</td>
</tr>
<tr>
<td>Phe103</td>
<td>Phe136</td>
<td>Front side</td>
</tr>
<tr>
<td>Asp110</td>
<td>Asp143</td>
<td>Front side</td>
</tr>
<tr>
<td>Asp116</td>
<td>Asp149</td>
<td>Front side</td>
</tr>
<tr>
<td>Ser128</td>
<td>Ser161</td>
<td>C-terminal region</td>
</tr>
<tr>
<td>Lys129</td>
<td>Lys162</td>
<td>C-terminal region</td>
</tr>
<tr>
<td>Arg130</td>
<td>Arg163</td>
<td>C-terminal region</td>
</tr>
<tr>
<td>Glu146</td>
<td>Glu179</td>
<td>Back side</td>
</tr>
<tr>
<td>Lys154</td>
<td>Lys187</td>
<td>Front side</td>
</tr>
<tr>
<td>Arg182</td>
<td>Arg215</td>
<td>Back side</td>
</tr>
<tr>
<td>Arg199</td>
<td>Arg232</td>
<td>Front side</td>
</tr>
<tr>
<td>Asn205</td>
<td>Asn238</td>
<td>Processivity loop</td>
</tr>
<tr>
<td>His206</td>
<td>His239</td>
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</tr>
<tr>
<td>Glu207</td>
<td>Glu240</td>
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<tr>
<td>Glu239</td>
<td>Glu272</td>
<td>IR-tail</td>
</tr>
<tr>
<td>Phe244</td>
<td>Phe277</td>
<td>IR-tail</td>
</tr>
</tbody>
</table>

For easier comparison with previous studies, corresponding positions in the “long” Doc1 construct are provided. Regions within Doc1 were classified based on orientation in Figure 1e.
**Supplementary Table 2** List of all Doc1 interactions identified by photo-crosslinking.

<table>
<thead>
<tr>
<th>site of incorporation</th>
<th>site in “long” Doc1</th>
<th>interaction partner</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ser128</td>
<td>Ser161</td>
<td>Apc1 (+++), Cdc16 (+)</td>
</tr>
<tr>
<td>Lys129</td>
<td>Lys162</td>
<td>Cdc16 (+++)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Apc1 (+)</td>
</tr>
<tr>
<td>Lys154</td>
<td>Lys187</td>
<td>Apc1 (+++)</td>
</tr>
<tr>
<td>Arg182</td>
<td>Arg215</td>
<td>Cdc16 (+++)</td>
</tr>
<tr>
<td>Asn205</td>
<td>Asn238</td>
<td>Apc1 (+)</td>
</tr>
<tr>
<td>Phe244</td>
<td>Phe277</td>
<td>Cdc27 (+++)</td>
</tr>
</tbody>
</table>

+++ abundant, + less abundant crosslinks. Listed crosslinks were observed in at least five experiments.
**Supplementary Table 3** Statistics on EM image analysis.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of particles</th>
<th>Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast APC/C monomer</td>
<td>55434</td>
<td>25 Å</td>
</tr>
<tr>
<td>Yeast APC/C dimer</td>
<td>1271</td>
<td>35 Å</td>
</tr>
<tr>
<td>Human APC/C&lt;sup&gt;CDH1-Hs1&lt;/sup&gt;</td>
<td>14813</td>
<td>28 Å</td>
</tr>
<tr>
<td>Human APC/C&lt;sup&gt;CDH1&lt;/sup&gt;</td>
<td>17075</td>
<td>27 Å</td>
</tr>
<tr>
<td>Yeast APC/C&lt;sup&gt;ASwm1&lt;/sup&gt;</td>
<td>6256</td>
<td>33 Å</td>
</tr>
<tr>
<td>Yeast APC/C&lt;sup&gt;ADoc1&lt;/sup&gt;</td>
<td>28383</td>
<td>30 Å</td>
</tr>
<tr>
<td>Yeast APC/C-Apc1-tmonomer</td>
<td>10474</td>
<td>30 Å</td>
</tr>
<tr>
<td>Yeast APC/C-Apc2-tdimer2</td>
<td>12171</td>
<td>32 Å</td>
</tr>
<tr>
<td>Yeast APC/C-Apc3-tdimer2</td>
<td>11767</td>
<td>39 Å</td>
</tr>
<tr>
<td>Yeast APC/C-Apc5-tdimer2</td>
<td>10291</td>
<td>35 Å</td>
</tr>
<tr>
<td>Yeast APC/C-Apc6-tdimer2</td>
<td>14421</td>
<td>38 Å</td>
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<tr>
<td>Yeast APC/C-Apc11-tdimer2</td>
<td>13480</td>
<td>32 Å</td>
</tr>
<tr>
<td>Yeast APC/C-Swm1-tdimer2</td>
<td>6273</td>
<td>34 Å</td>
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<tr>
<td>Yeast APC/C-Doc1-tdimer2</td>
<td>9228</td>
<td>30 Å</td>
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</table>
Supplementary Table 4 Summary of subunit localization experiments. Methods used in this and previous studies for the localization of different subunits and interacting proteins in the 3D structure of the APC/C from different species.

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Subunit deletion</th>
<th>td2 tagging</th>
<th>Antibody labeling</th>
<th>Recombinant protein addition</th>
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<tbody>
<tr>
<td>Species</td>
<td>S. cerevisiae</td>
<td>S. cerevisiae</td>
<td>H. sapiens, X. laevis</td>
<td>H. sapiens, X. laevis</td>
</tr>
<tr>
<td>Apc1</td>
<td>–</td>
<td>1</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>Apc2</td>
<td>–</td>
<td>–</td>
<td>2,3</td>
<td>–</td>
</tr>
<tr>
<td>Cdc27</td>
<td>–</td>
<td>1</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>Apc4</td>
<td>–</td>
<td>–</td>
<td>2</td>
<td>–</td>
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<tr>
<td>Apc5</td>
<td>–</td>
<td>1</td>
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<td>Cdc16</td>
<td>–</td>
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<td>Cdc23</td>
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<td>Apc7</td>
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<td>n.i.</td>
<td>–</td>
<td>–</td>
</tr>
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<td>Apc9</td>
<td>–</td>
<td>–</td>
<td>n.i.</td>
<td>n.i.</td>
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<tr>
<td>Doc1</td>
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<td>–</td>
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<td>Apc11</td>
<td>–</td>
<td>1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Apc16</td>
<td>n.i.</td>
<td>n.i.</td>
<td>4</td>
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<td>–</td>
<td>n.i.</td>
<td>n.i.</td>
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<tr>
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<td>–</td>
<td>–</td>
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<td>–</td>
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Indicated sources are: [1] this study; [2] (ref. 10); [3] (ref. 12); [4] (ref. 15); n.i. not identified.
**Supplementary Table 5** Yeast strains used in this study.

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<th>Strain</th>
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<td>K6201</td>
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<tr>
<td>K6202</td>
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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, GAL psi) and K700 (MATalpha ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1, GAL psi) with the exception of the strains used for spotting experiments which are derivatives of S288C (hi3D1 leu2D0 met15D0 ura3D0 doc1::KanMX). Kl denotes the TRP1 gene from *Klyveromyces lactis*, Sp the HIS5 gene from *Schizosaccharomyces pombe*. 
Supplementary References


11. Acknowledgements

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Still first page, I want to thank Brenda A. Schulman for her determination, support and huge excitement coming from the far side and for investing so much time and effort to push this entire collaboration forward. I especially want to thank her helping us finding solutions to our incapability in some aspects of protein crystallography. I furthermore want to thank her entire lab members for making this collaboration so straightforward and comprehensive.
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Meiner Schwester, Julia Karsubke, möchte ich fuer ihr uneigennütziges Vorhaben danken, mich während meiner Doktorarbeit wöchentlich anzurufen, zu unterstützen und aufzubauen damit sie es diesmal – im Gegensatz zu meiner Diplomarbeit – endlich einmal in die Danksagungen schafft, auch wenn es dann doch nicht jede Woche geklappt hat.


12. Curriculum vitae

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Supervision Dr. Jan-Michael Peters

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Undergraduate assistant scientist in the group of Prof. Dr. Irmgard Sinning  
“Structural characterization of the third SRP GTPase FlhF”

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Publications


* equally contributing authors