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DISSERTATION

Characterization of the c11orf51 subunit of the Anaphase-Promoting Complex / Cyclosome

Verfasserin

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angestrebter akademischer Grad

Doktorin der Naturwissenschaften (Dr.rer.nat.)

Wien, 2011

Studienkennzahl: A 091490

Dissertationsgebiet: Molekulare Biologie (A 091490)

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Zusammenfassung

Das Durchlaufen des Zellzyklus hängt vom Abbau bestimmter regulatorischer Proteine ab, zu denen Securin und mitotische Cycline zählen. Diese Abbaureaktionen werden von der E3 Ubiquitin-Ligase Anaphase-Promoting Complex / Cyclosom (APC/C) eingeleitet, welche selbst zellzyklisch reguliert wird. Der APC/C ist ein 1.5 MDa großer Proteinkomplex, welcher bei Vertebraten aus mindestens 13 Untereinheiten aufgebaut ist, und er fügt Ubiquitin-Ketten an Substratproteine an. Proteine, die mehrerer solcher Ubiquitin-Moleküle besitzen, werden vom 26S proteasome erkannt und proteolytisch abgebaut. Dieser unwiederrufliche Vorgang versichert, dass der Zellzyklus unidirektional verläuft und dass das nächste Stadium im Zellzyklus gesichert stattfinden kann. Da dieser Prozess ein „alles-oder-nichts"-Vorgang ist, muss diese Ubiquitylierungsreaktion sehr gut reguliert werden und sie muss zellzyklus-abhängig stattfinden. Das wiederrum bedeutet, dass die Aktivität des APC/C´s stark kontrolliert werden muss. Dies wird durch mehrere Faktoren gewährleistet. Dazu gehören Phosphorylierungs- und Dephosphorylierungsvorgänge, das transiente Binden der APC/C-spezifischen aktivatorischen Proteine Cdc20 und Cdh1, sowie die Interaktion mit inhibitorischen Proteinen wie etwa dem mitotic checkpoint complex (MCC). Obwohl der APC/C schon seit mehr als einem Jahrzehnt intensiv erforscht wird , wissen wir immer noch wenig über die Regulation dieses Komplexes während des Zellzyklus und für viele seiner Untereinheiten sind die biochemischen Funktionen und Eigenschaften weitestgehend unbekannt.

Erst kürzlich wurde gezeigt, der Genabschnitt C11ORF51 (chromosome 11 open reading frame 51) für ein Protein kodiert, welches für den Zellzyklusprozess von Bedeutung ist. Zudem fand man heraus, dass es mit humanem APC/C assoziert. Dies wurde in Proteinaufreingungsexperimenten gezeigt, bei denen APC/C Untereinheiten als „bait" benutzt wurden. Wir beschlossen, das c11orf51 Protein näher zu charakterisieren und seine biologische Funktion zu analysieren. Dabei haben wir eine Kombination aus biochemischen und (quantitativ-) massenspektrometrischer Methoden angewandt. Das Protein c11orf51 wurde in unseren Untersuchungen als eine neue Untereinheit des humanen APC/C´s identifizieren und seine biochemischen Eigenschaften wurden bestimmt.

In biochemischen Experimenten, teilweise in Kombination mit Massespektrometrie, konnte ich zeigen, dass c11orf51 während des gesamten Zellzyklus mit dem APC/C interagiert. Des Weiteren ist zelluläres c11orf51 zum größten Teil an den Komplex gebunden und die mit c11orf51-assozierte Form des APC/C´s ist aktiv gegenüber dem Substratprotein cyclin B1, wie *in vitro* Ubiquitylierungsassays zeigen. Mit Hilfe der Antikörper-Markierungsmethode, bei welchem wir c11orf51-spezifische Peptidantikörper verwendeten, konnten wir in negative staining-elektronenmikroskopischen Studien die Bildung von APC/C-Dimeren beobachten. Dies weist darauf hin, dass c11orf51 ein Protein ist das fest mit dem APC/C assoziert. Deletion von c11orf51 durch RNA-Interferenz ergab einen Phänotyp in sich teilenden humanen Zellen in Kultur. Dabei war die Zeit, welche die Zellen in der Metaphase verbrachten, im Vergleich zu kontroll-transfizierten Zellen signifikant verlängert. Um weiter zu bestätigen, dass c11orf51 eine konstitutive APC/C-Untereinheit ist, wurde eine quantitative Proteomanalyse angewandt. Dabei arretierte ich humane HeLa Zellen in verschiedenen Zellzyklus-Stadien und reinigte den APC/C auf, welcher weiteres mit dem iTRAQ Reagenz chemisch markiert und anschließend mit quantitativer Massenspektrometrie analysiert wurde. Zusätzlich wurden noch weitere APC/C-Bindungsproteins quantifiziert, wie etwa die Co-Aktivatoren. Diese Methode kann zukünftig dazu dienen, eine zusammenfassende Studie über die Zusammensetzung des APC/C´s während des Zellzykluses zu erhalten.

Abstract

Cell cycle progression depends on the degradation of specific regulatory proteins, such as securin and mitotic cyclins. Degradation of these proteins is initiated by the cell cycle-regulated activity of the E3 ubiquitin ligase anaphase-promoting complex / cyclosome (APC/C). The APC/C is a 1.5 MDa protein complex, composed of 13 individual subunits, which assembles ubiquitin chains on substrate proteins. Polyubiquitylation targets these proteins for recognition by the 26S proteasome, resulting in their subsequent degradation. This pathway ensures unidirectionality of the cell cycle process and forces full commitment to progress to the next stage. Because this process is an "all-or-nothing"-event, substrate ubiquitylation has to be highly regulated in a cell cycle-specific manner. Hence, APC/C activity has to be tightly controlled. This is ensured by different events, such as phosphorylation and dephosphorylation, transient binding of its co-activatory subunits Cdc20 and Cdh1 as well as on association of inhibitory proteins, like the mitotic checkpoint complex (MCC). Even though the APC/C has been subjected to intensive studies for more than a decade now, we still know little about APC/C regulation during the cell cycle and what the function of its many subunits are. Moreover, despite of APC/C´s large size it seems as if there are more associating proteins waiting to be discovered.

Only recently, a protein encoded by C11ORF51 (chromosome 11 open reading frame 51) was shown to be required for correct cell cycle progression. Moreover, it was found to associate with human APC/C after tandem affinity purification using tagged APC/C subunits as bait. We aimed to characterize the c11orf51 protein further and to analyze its biological function. Therefore, we applied a combinatorial approach of biochemical and quantitative mass spectrometric analysis which allowed us to comprehensively look at human APC/C composition during the cell cycle. We could identify the c11orf51 protein as a constitutive APC/C subunit and characterized its biochemical properties.

In different biochemical assays, partially combined with mass spectrometry, I could show that c11orf51 protein associates with the APC/C during the entire cell cycle, which identifies the c11orf51 protein as a novel and constitutive subunit of human APC/C. *In vitro* ubiquitylation assays revealed that the APC/C containing c11orf51 protein is active towards its mitotic

substrate cyclin B1. Antibody-labelling using c11orf51 peptide specific antibodies resulted in APC/C-Dimer formation in negative staining electronmicroscopy studies, which confirms that c11orf51 must tightly bind to the complex. Depletion of the c11orf51 protein by RNA interference (RNAi) resulted in a metaphase arrest phenotype in proliferating human cultured cells as analyzed by immunofluorescence microscopy on fixed cells. Furthermore, live cell imaging experiments indicated that the time from nuclear envelope breakdown (NEBD) to anaphase onset was significantly prolonged, as compared to mock-transfected cells. To further verify that c11orf51 protein is a constitutive APC/C subunit, we applied a quantitative proteomics approach. To this end, immunopurified APC/C from human cultured cells that had been arrested at different cell cycle stages were used for iTRAQ labeling and quantitative mass spectrometry. This approach allowed us to resolve the composition of human APC/C during the cell cycle. We could show that the protein levels of APC/C-associated c11orf51 did not significantly fluctuate during the cell cycle, which confirms that c11orf51p is a *bona fide* subunit of human APC/C.

1 Introduction

1.1 The cell cycle

The cell cycle is an ordered series of events, in which one cell divides into two daughter cells, each containing a copy of the chromosomes derived from the parental cell. The cell cycle is fundamental to the development and function of all life. The different stages of a eukaryotic cell cycle can be defined based on morphological changes of the chromosomes. Duplication of the parental chromosomes occurs only once per cell cycle, which is during the synthetic or S phase, with each of the derived daughter chromosomes then distributed to the two daughter cells in mitosis, or M phase. Both phases are separated by two gap phases, G1 and G2. In these "interphases" the cell synthesizes proteins, membranes and other cell organelles and it prepares for the subsequent cell division. The mitotic cell division in most eukaryotic cells (except for fungi) starts with the disassembly of the nucleus and chromosome condensation in prophase. The bipolar mitotic spindle begins to form, which captures the chromosomes in prometaphase. In metaphase, all chromosomes are aligned to form the so-called metaphase plate. Sister chromatids that are held together by a mechanism called cohesion are now being captured by microtubules that come from opposite poles of the spindle. Upon loss of cohesion at anaphase onset, sister chromatids are pulled towards the opposite poles of the spindle. Telophase marks chromosome decondensation and formation of the nucleus. After nuclear division, the two daughter cells are finally separated by cell membrane ingression in a process called cytokinesis (Morgan, 2007). Replication and segregation of chromosomes during the cell cycle must be precisely controlled and reliable over countless generations. Therefore, cell cycle events must be processed in the right order and with high fidelity. Proper cell cycle regulation is essential in all living organisms, and aberrant division can lead to cell death or hyperproliferation, as it is found in cancer.

1.2 Cell cycle control

Cell cycle events are highly regulated through oscillating waves of cyclin-dependent kinase (Cdk) activity. As the cell progresses through the cell cycle, abrupt changes in the enzymatic activities of these kinases lead to changes in the phosphorylation state of their target proteins, which are major player for cell cycle progression. Cdk-mediated phosphorylation of target proteins may alter their activity or their binding properties (Morgan, 2007). Cdks can only fulfill their function in association with their regulatory subunit, the cyclins, leading to biochemical oscillation waves. While the Cdk levels stay constant and are in large excess over the cyclin levels throughout the cell cycle, cyclin concentrations are dramatically changing as the cell progresses through different cell cycle stages (Murray, 2004). Cdk activity is high during DNA synthesis and early mitosis and low during cytokinesis and G1. Budding and fission yeast only have one Cdk, which associates with nine different cyclins, whereas higher organisms have several Cdks, which form different cyclin-Cdk complexes that are active at different time points in the cell cycle. Concentrations of cyclin A and B rise during interphase and fall during mitosis, whereas cyclin E remains constant. The activity of Cdk1, which binds cyclin A and B, accumulates slowly during S and G2 and reaches maximal activation in mitosis, whereas Cdk2, which binds cyclin A and E, reaches its peak of activity earlier in S phase (Guardavaccaro and Pagano, 2006; Murray, 2004). Hence, in animal cells, Cdk4 and Cdk6- paired with D-type cyclins, are active in G1; Cdk2 associated with A-type and E-type cyclins initiates DNA replication and centrosome duplication, and Cdk1 together with B-type cyclins promotes mitotic entry (Murray, 2004; Pagano and Jackson, 2004).

Furthermore, cyclin-Cdk specificity is determined by their specific localization and expression levels, rather than by their chemical properties. In addition, the activity of cyclin-Cdk complexes is fine-tuned by another control mechanism, involving phosphorylation/dephosphorylation as well as binding of inhibitors, of which levels also fluctuate. Abolished Cdk activity is for example necessary to exit from mitosis and for loading of pre-replicative complexes (pre-RCs) at the origins of replication in G1. The origins of replications only start firing in S phase, when E- and A-type cyclins accumulate and activate Cdk again at the end of G1 (reviewed by Diffley, 2004). Oscillations of both cyclins

and Cdk inhibitors are partially controlled on the transcriptional level, but largely accomplished by the action of the ubiquitin proteasome system, leading to irreversible substrate degradation (Morgan, 2007). This mechanism ensures that the cell cycle processes in a unidirectional fashion with irreversible cell cycle transitions.

1.3 Ubiquitin-dependent proteolysis

Ubiquitin-mediated degradation of regulatory proteins plays important roles in the control of many different processes that require rapid alterations in protein levels, including cell cycle progression, signal transduction, transcriptional regulation, receptor down-regulation, and endocytosis (Amerik and Hochstrasser, 2004; Hershko and Ciechanover, 1998). Proteolysis is particularly critical at the metaphase-to-anaphase transition, where sister-chromatid separation and mitotic exit are triggered by the irreversible destruction of mitotic cyclins and proteins that control sister-chromatid cohesion. Protein degradation is accomplished by the attachment of multiple copies of the small protein ubiquitin. Ubiquitin is a highly conserved 76 amino acid protein found in all eukaryotes. The attachment of ubiquitin to target proteins, a process known as ubiquitilation, ultimately leads to their proteosomal degradation (Morgan, 2007). The minimal targeting signal on a protein for its proteasomal degradation is a tetraubiquitin chain, whereas the attachment of only one ubiquitin acts as a distinct type of signal, for example in endocytosis (Thrower et al., 2000). The ubiquitilation process is carried out in a series of reactions by the action of three enzymes: an ubiquitin activating enzyme (E1), an ubiquitin conjugating enzyme (E2) and an ubiquitin ligase (E3). In the first ATP hydrolysis dependent step, the E1 activates ubiquitin by first adenylating ubiquitin and then creating a thioester bond between its catalytic site cysteine and the C-terminal glycine residue of ubiquitin. The E1-ubiquitin conjugate is then transferred to an active site cysteine residue of an E2 in a transesterification reaction. The final step is most tightly regulated and is conducted by the E3 that contributes the substrate specificity to the reaction process. The E3 recognizes the target protein and catalyzes the formation of an isopeptide bond between the C-terminus of ubiquitin and a substrate´s lysine side chain. The attachment of ubiquitin to lysine residues within ubiquitin itself results in formation of long polyubiquitin chains on the

target protein. These are recognized by receptors on the proteasome, leading to their proteolytic degradation (reviwed by Pickart, 2001). (**Figure 1-1).**

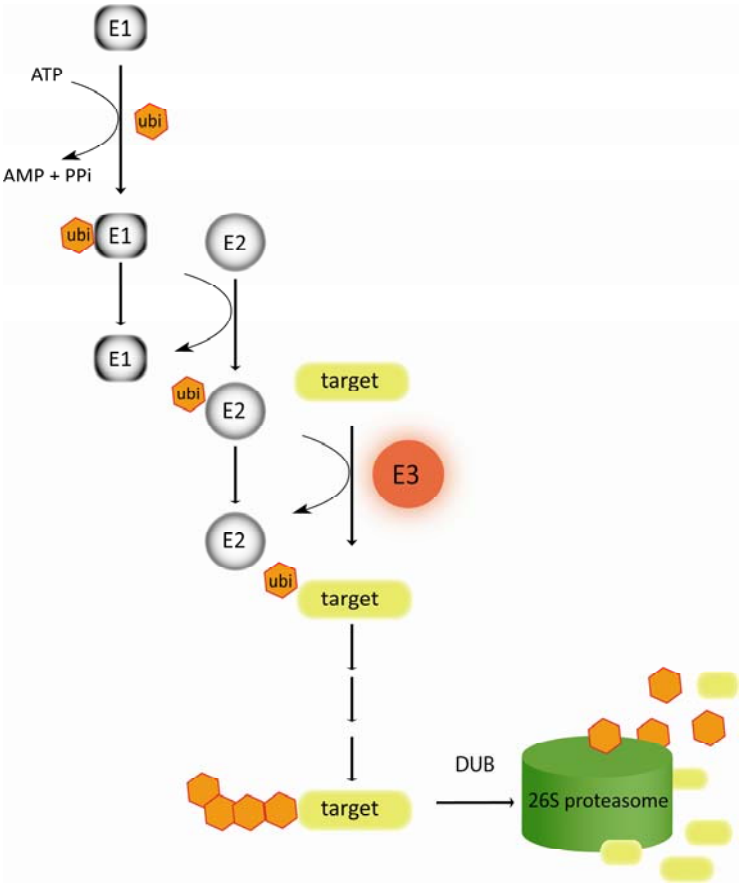


Figure 1-1: The ubiquitin-proteasome pathway. Two enzymes and one ubiquitin ligase act in a cascade to attach ubiquitin moieties to target proteins. Other steps include ubiquitin precursor processing as well as the ATP-dependent processes of ubiquitin activation and translocation of substrates to the proteasome, which are not depicted here. Target proteins can become monoubiquitilated, multiubiquitilated (i.e. several distinct substrate lysines are ubiquitilated) or polyubiquitilated (as shown). Deubiquitilating enzymes (DUB) can additionally act at other steps in the pathway. See text for details.

Ubiquitin-protein conjugates are highly dynamic structures. While an array of enzymes directs the attachment of ubiquitin to substrates, there are also many deubiquitilating enzymes (DUBs) that can reverse the process. DUBs are required to maintain a sufficient pool of free ubiquitin within the cell and for generating conjugation-competent ubiquitin from precursors (Amerik and Hochstrasser, 2004). Furthermore, the disassembly of ubiquitin from the substrate protein might serve as a final safeguard against the proteolysis of poorly, and

perhaps erroneously, ubiquitinated proteins (Lam et al., 1997). It has also been shown that some DUBs act in a target-specific manner (Li et al., 2002).

Importantly, substrate specificity in the ubiquitilation process is only conferred by the Ubiquitin ligases (E3s) (Wickliffe et al., 2009). Two groups of proteins belong to this class of E3s: HECT (Homologous to E6AP C-Terminus) and RING (Really Interesting New Gene). HECT type E3s can directly bind to ubiquitin via their active-site cysteine forming a thioester linkage, before the molecule is passed over to the substrate protein (Passmore and Barford, 2004). In contrast, RING-finger E3s do not form a thioester bond with ubiquitin and therefore they do not directly take part in the transfer of ubiquitin from E2 to the substrate. RING-finger E3s directly bind E2-ubiquitin conjugates and the substrate proteins, thereby bringing them into close reaction proximity (reviewed by Pickart, 2001).

1.4 The E3 ubiquitin ligases SCF and APC/C: unequal twins

The two main ubiquitin ligases catalyzing cell cycle transitions are the SCF (Skp1-Cul1-F-box) and the APC/C (Anaphase Promoting Complex / Cyclosome). Both are distantly related multi-subunit complexes which belong to the family of RING ubiquitin ligases (reviewed by Passmore and Barford, 2004). While various SCF complexes are active at many cell cycle stages as well as beyond the cell cycle, the APC/C controls mitotic progression and remains active until the end of G1 phase (Nakayama and Nakayama, 2006; Petroski and Deshaies, 2005; Vodermaier, 2004). Despite the different timing of their activity during the cell cycle, both RING-type protein complexes share structural and biochemical similarities. In both cases, the catalytic subunit that recruits the E2-ubiquitin conjugate is represented by a small zinc-binding RING-finger subunit (Roc1/Rbx1/Hrt1 in SCF and Apc11 in APC/C). In both cases, the RING-finger protein is anchored to the C-termini of proteins containing cullin domains (Cul1 in SCF and Apc2 in APC/C). Both complexes function through their adaptor proteins that recruit the substrate to the catalytic core. These adaptor proteins (different F-box proteins for the SCF, Cdc20/Fzz and Cdh1/Fzr for the APC/C) confer the substrate specificity and allow those complexes to bind to their numerous substrates via recognition motifs that are implicated to mediate protein-protein interactions (Petroski and Deshaies, 2005; reviewed by

Vodermaier, 2004). The co-activator proteins of the APC/C will be described in more detail in chapter 1.7.3.

Both E3 ligases share a similar structural backbone and display similarities in their core domains. Therefore, it has been proposed that they might also have similar modes of mediating substrate ubiquitilation (Ohi et al., 2007). A crucial difference, however, is how substrate recognition is mediated and how their activity is regulated. The SCF is a constitutively active complex and phosphorylation of substrate proteins is the driving force for their recruitment to the SCF via the F-box adaptor proteins (Petroski and Deshaies, 2005). The APC/C in contrast becomes phosphorylated itself, allowing activator proteins to bind and activate the complex (reviewed by Vodermaier, 2004). Thus, whereas for SCF substrate availability is restrained, it is the complex itself which is regulated in case of the APC/C. Correct regulation of the APC/C is ensured on multiple levels, such as phosphorylation and dephosphorylation events of many proteins, including the APC/C itself, association with activator proteins Cdc20 or Cdh1, as well as binding of inhibitory proteins (reviewed by Peters, 2006) (described in more detail in chapter 1.7.3).

1.5 Roles of the APC/C in the cell cycle

The APC/C is the largest and most complex E3 RING-ligase known to date. It is endowed with elaborate regulatory, catalytic and specificity properties. While it only takes three to five subunits to build a functional SCF complex, the APC/C is assembled from at least 13 individual subunits and for many of them the exact biological role is not well understood.

The discovery of the APC/C reaches back to the year 1995, where studies on clam oocytes, *Xenopus* egg extracts as well as budding yeast mutants identified a cell cycle-regulated E3 ubiquitin ligase that was responsible for cyclin B degradation in mitosis (Irniger et al., 1995; King et al., 1995; Sudakin et al., 1995). At that time, cyclin had already been identified as the activating component of the maturation-promoting factor (MPF), which drives the cell into mitosis. It was also evident that cyclin proteolysis, which is dependent on the ubiquitin-proteasomal pathway, is required for completion of mitosis (Glotzer et al., 1991; Murray and

Kirschner, 1989). Later, yeast genetic studies as well as biochemical fractionation experiments in cell-free systems led to the discovery of the APC/C as the ubiquitin ligase responsible for both anaphase onset and mitotic exit (reviewed by Hershko, 2010; Irniger et al., 1995).

As mentioned earlier, the cell cycle is driven by periodic fluctuations in Cdk1 activity. While high Cdk activity promotes many mitotic events, Cdk1 inactivation is important for correct mitotic exit. This is accomplished by degradation of its activating subunits cyclin A or cyclin B. Both cyclins are targeted by the APC/C. Upon loss of its regulatory cyclin subunit, Cdk1 undergoes a conformational change. This prevents both ATP hydrolysis and access of protein substrates to the active site of the kinase, resulting in complete inactivation of Cdk1 (Jeffrey et al., 1995). This process is initiated once all sister chromatids have been attached to the mitotic spindle in a bipolar manner. Complete cyclin B degradation mediated by the APC/C establishes and maintains a period of low Cdk activity that is necessary for finishing mitosis (Amon et al., 1994; Morgan, 1999; Sullivan and Morgan, 2007). It allows dephosphorylation of Cdk substrates which is essential for disassembly of the mitotic spindle, reformation of the nuclear envelope, chromosome decondensation and cytokinesis. After mitotic exit, the APC/C remains active until the end of G1, maintaining low Cdk1 activity. This allows formation of pre-replicative complexes on origins of replication on the DNA strand, ensuring correct S phase entry. Initiation of DNA synthesis by DNA polymerases is dependent on high Cdk activity, thus the APC/C prevents premature S phase entry and restricts DNA replication to only once per cell cycle (reviewed by Diffley, 2004)

Apart from its function to ensure correct mitotic exit, the APC/C fulfills another important role which led the APC/C to its name, namely to initiate anaphase onset by targeting securin for ubiquitylation. Securin is an inhibitor of the protease separase. Separase can cleave the Scc1 subunit of a protein complex named cohesin. Cohesin is a ring-shaped multisubunit complex which embraces sister chromatids and thereby holds them together. Proteasomal degradation of securin leads to activation of separase, which releases cohesin from chromatids, thereby initiating anaphase onset (reviewed by Nasmyth, 2001). In vertebrates, the role of APC/C in separase activation may not be restricted to degradation of securin. Moreover, it requires proteolysis of cyclin B, because Cdk1-dependent phosphorylation of

separase also inactivates the protease and thereby prevents sister chromatid separation. Therefore, low Cdk1 activity accomplished by the APC/C might fully promote sister chromatid separation (Gorr et al., 2005; Stemmann et al., 2001).

In addition to the essential processes mentioned above, it has become clear that the APC/C fulfills additional tasks within the cell cycle by targeting other protein substrates. These include mitotic kinases (NIMA-related kinases, Plk1, Aurora A and B), proteins involved in DNA replication (geminin, Cdc6 in mammals and Dbf4 in yeast), and proteins involved in spindle function (Ase1, Kip1, Cin8 in yeast and Xkid in frogs) as well as the APC/C co-activator protein Cdc20 itself. For some of the mentioned proteins additional forms of regulation apart from proteolysis exist. Plk1 for example can be inactivated at the end of mitosis by either proteolysis or dephosphorylation (Lindon and Pines, 2004). Moreover, studies in budding yeast have shown that the only essential APC/C targets, at least in this organism, are securin and mitotic cyclins (Thornton et al., 2006). Such findings and phylogenetic analysis of Cdk evolution (Krylov et al., 2003) support the hypothesis that the cyclin degradation machinery and the Cdk-based-oscillator co-evolved during evolution and took control over mitotic processes that had originally been regulated by other ancestral enzymatic activities (reviewed by Murray, 2004).

1.6 Topology of individual APC/C subunits

The APC/C is composed of at least thirteen individual subunits. To better understand the process of APC/C-mediated ubiquitilation and how the different subunits orchestrate their functions to build an active APC/C, it is necessary to take a closer look at what is known about the structure and the organization of this multi-subunit complex.

Knowledge about the topology of individual APC/C subunits could first be gained by dissociating the complex into smaller subcomplexes, either biochemically (Vodermaier et al., 2003) or after mutation or deletion of individual subunits in yeast (Schwickart et al., 2004; Thornton et al., 2004; Thornton et al., 2006). Thornton and Toczisky could draw a detailed subunit assembly map of the APC/C by creating a budding yeast strain that allowed the

deletion of normally essential APC/C subunits (Thornton et al., 2004) (**Figure 1-2**). Hereby, the subunit composition of the APC/C was analyzed by sequential deletion of single subunits, followed by purification and biochemical analysis of the remainder of the APC/C (Thornton et al., 2006). The molecular characterization of those subunits shed some light on the mechanism of APC/C catalysis. Their data suggests that the largest subunit, Apc1 together with Apc4 and Apc5 provides a structural scaffold that associates independently with two other subcomplexes. One of those subcomplexes contains the catalytic core proteins Apc2, Apc11 and Doc1/Apc10, and is therefore referred to as the "catalytic subcomplex". The other "TPR subcomplex" comprises the TPR subunits Cdc27/Apc3, Cdc16/Apc6 and Cdc23/Apc8, with Cdc27/Apc3 being the most peripheral and Cdc23/Apc8 the most internal protein (**Figure 1-2**). According to earlier findings, the non-essential subunits Apc9, Swm1/Apc13 and Cdc26 most likely also belong to the TPR subcomplex (Passmore et al., 2003; Schwickart et al., 2004; Zachariae and Nasmyth, 1996). Those three subunits are important for structural integrity of yeast APC/C. Mutations or loss of any of these proteins leads to dissociation of other APC/C subunits or the destabilization of subcomplexes (Passmore et al., 2003; Schwickart et al., 2004; Zachariae et al., 1998b). If Apc9 is absent, levels of Cdc27/Apc3 are significantly reduced (Passmore et al., 2003; Zachariae et al., 1998b). Furthermore, Swm1 is required for integration of the subunits Cdc16/Apc6, Cdc27/Apc3, Apc9 and Cdc26 into the APC/C as well as for sporulation during meiosis (Schwickart et al., 2004; Ufano et al., 1999). However, the function of the three subunits providing the structural scaffold is not fully understood.

The catalytic subunits Apc2 and Apc11 were shown to be essential for the assembly of polyubiquitin chains from ubiquitin donated E2 enzyme (Vodermaier et al., 2003). High-salt washes not only led to dissociation of Apc2 and Apc11 from APC/C but also to a reduction of Doc1/Apc10 protein levels (Thornton et al., 2006). The situation appears to be different in human APC/C, where only partial loss of Doc1/Apc10 is observed in an APC/C complex that is lacking Apc2 and Apc11. This might be due to the observation that Doc1/Apc10 associates with human APC/C through binding to TPR subunits (Buschhorn et al., 2010; Da Fonseca, 2011; Vodermaier et al., 2003).

Vertebrate APC/C contains four tetratricopeptide repeat (TPR) proteins, called Cdc27/Apc3, Cdc16/Apc6, Apc7, and Cdc23/Apc8, whereas yeast APC/C contains only three; Apc7 is exclusively found in higher eukaryotes (Pal et al., 2007a; Yu et al., 1998). The TPR domain containing proteins were first discovered as a macromolecular complex in yeast with essential mitotic function (Irniger et al., 1995; Lamb et al., 1994) and they represent the largest group of structurally related proteins within the APC/C. The TPR motif is a ~34-residue helix-turn-helix structure and multiple TPRs can pack into into a superhelical domain that is believed to mediate protein-protein interactions (Das et al., 1998). Moreover, TPR domains can serve as receptors for C-terminal peptide motifs (Gatto et al., 2000). Consistent with this, it has been shown that TPR subunits of the APC/C can bind to peptides that correspond the C-terminal part of Cdh1, Cdc20 (Vodermaier et al., 2003), and Doc1/Apc10 (Wendt et al., 2001). The APC/C contains multiple copies of several TPR subunits per complex (Dube et al., 2005; Huang and Raff, 2002; Ohi et al., 2007; Passmore et al., 2005b). They might function as versatile acceptor sites for interactions with a variety of regulatory proteins (Vodermaier et al., 2003) and possibly substrates (Hayes et al., 2006) (see chapter 1.7). This could partially explain some of APC/C´s complexity.

Apc1 shows sequence homologies to the Rpn1 and Rpn2 subunits of the proteasome (Lupas et al., 1997), suggesting that Apc1 might have a proteasome-related function such as delivery of polyubiquitinated substrates to the proteasome or unfolding of protein substrates prior to proteolysis (Kajava, 2002). To date, no experimental data could support this hypothesis. Apc4 on the other hand is predicted to contain a WD40 domain, which can fold into a propeller-like structure (Herzog et al., 2009; reviewed by Peters, 2006). Apc1, Apc4, Apc5 and Cdc23/Apc8 associate interdependently, such that loss of any of them greatly reduces binding of the remaining three proteins (Thornton et al., 2006).

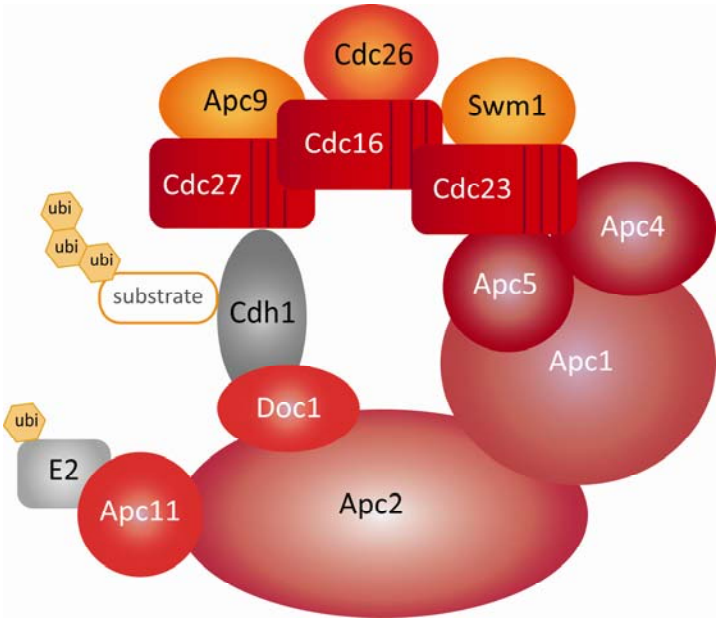


Figure 1-2: APC/C subunit interaction map and substrate ubiquitilation modified after (Peters, 2006; Thornton et al., 2006). The "catalytic subcomplex" contains the cullin protein Apc2, the RING-finger protein Apc11 and Doc1/Apc10. The ubiquitin-charged E2 is recruited to the RING-finger protein Apc11. In analogy to the Cul1 subunit of the SCF complex, Apc2 serves as a scaffold to bring the E2 in close vicinity to the substrate receptor site containing the co-activator and Doc1/Apc10. The substrate is recruited to the APC/C through binding to an APC/C co-activator (in this case Cdh1) via its recognition element (here: a D-box) and with the help of the processivity factor subunit Doc1/Apc10. APC/C then catalyzes poly-ubiquitilation of its substrate proteins. The "TPR-subcomplex" conists of Cdc27/Apc3, Cdc16/Apc6, and Cdc23. Vertical stripes indicate TPR-containing subunits. The Cdc26 subunit is a chaperone of Cdc16/Apc6. Metazoan APC/C has the additional TPR-subunit Apc7, which is not depicted here and Apc9 has so far only been found in yeast. Swm1/Apc13, Cdc26 and Apc9 play a role in stabilizing the Cdc27/Apc3 and Cdc16/Apc6 interaction with the rest of the APC/C. Of the core subunits, Apc1, Apc2, Cdc27/Apc3, Apc4, Apc5, Cdc16/Apc6, Cdc23, and Apc11 are essential in yeast, wheras Apc9, Doc1, Cdc26 and Swm1 are not (**Table 1-1**). See text for details.

1.7 Composition of the APC/C and mechanistic implications

The APC/C is an unusually complex multi-subunit E3 ubiquitin ligase (**Table 1-1**) (reviewed by Peters, 2006). The complexity of the APC/C is somewhat surprising, since other relative Cullin-RING ligases (such as the SCF, see chapter 1.4) only consist of a single or few subunits, indicating that the ubiquitin ligase reaction *per se* does not require complex multi-subunit enzymes (Passmore and Barford, 2004). Although the APC/C has been studied in

great detail for more than a decade now, it is still not entirely clear why the APC/C is so complex and what the functions of its different subunits are.

Recent cryo electron microscopy studies on human and yeast APC/C led to a more precise definition of the APC/C and provided first implications of how the ubiquitylation reaction might be catalyzed by this complex (Buschhorn et al., 2010; Da Fonseca, 2011). Moreover, a combinational approach of using electron microscopy, mass spectrometry and docking of crystallographic and homology-derived coordinates has very recently provided a detailed picture of the organization and structure of all essential APC/C subunits, and resulted in a pseudo-atomic model covering at least 70% of the APC/C (Schreiber et al., 2011).

As mentioned in chapter 1.4, the APC/C is a family member of ubiquitin ligases that contain a Zinc-coordinated RING-finger domain and a cullin domain (reviewed by Thornton and Toczyski, 2006). The cullin-RING finger ubiquitin ligase Cul1-Rbx1-Skp1-F box^{Skp2}-SCF complex is structurally related to the APC/C (Zheng et al., 2002). Although it is by far not as complex as the APC/C, its structure might help to understand the underlying mechanism for APC/C-mediated ubiquitilation. The Cul1-subunit is an elongated protein that serves as a rigid scaffold, providing binding interfaces for the RING-finger protein Rbx1 at its C-terminus and for the Skp1-F box^{Skp2} at its N-terminus. The structure suggests that Cul1 may contribute to catalysis through positioning of the substrate adaptor Skp2, and the ubiquitin conjugation enzyme E2. Cul1 and Apc2 are paralogs which share no detectable sequence homology beyond the cullin homology domain. However, the superimposed crystal structure of non-homologue yeast Apc2 peptide provided a first hint that despite the low sequence homology, Apc2 and Cul1 may adopt similar structures (Zheng et al., 2002).

1.7.1 The catalytic core

Both, the RING-finger protein Apc11 and the cullin containing Apc2 are required for ubiquitilation activity of the APC/C. However, substrate specificity is reduced if the co-activator is absent (Gmachl et al., 2000; Leverson et al., 2000). Apc11 directly interacts with the E2 enzyme UbcH5 and both alone can promote association of ubiquitin chains on

substrates *in vitro* (Gmachl et al., 2000; Leverson et al., 2000; Tang et al., 2001). Apc11 can also interact with the cullin homology domain in Apc2, which is required when UbcH10 is used as the E2 instead of UbcH5 (Tang et al., 2001; Vodermaier et al., 2003). UbcH10 is an E2 enzyme more specific for ubiquitilation reactions catalyzed by the APC/C (Yu et al., 1996). Another E2 enzyme, Ube2S is further required in this catalytic module to elongate the ubiquitin chains on the substrate (Garnett et al., 2009). It is therefore believed that Apc11 together with Apc2 orchestrate the ubiquitin ligase reaction of the APC/C and that remaining subunits are necessary to regulate its activity and to confer substrate specificity to this complex. Some subunits have been implicated to be important for structural integrity of the APC/C (Hall et al., 2003) (see chapter 1.6) and it is conceivable that some of the subunits are necessary to spatially control the access of substrates to the catalytically important subunits within the APC/C (Gieffers et al., 2001). Biochemical experiments revealed that binding of the co-activator and substrate adaptor protein Cdh1 is reduced upon Apc2 deletion, indicating that the Cdh1 binding site to the APC/C is close to its catalytic core (Thornton and Toczyski, 2006). This finding could be confirmed in recent structural studies (Da Fonseca, 2011; Herzog et al., 2009; Schreiber et al., 2011).

1.7.2 The processivity factor Doc1/Apc10

The small subunit Doc1/Apc10 is among the known APC/C subunits most likely best studied. Besides crystal structures of a C-terminal fragment of yeast Apc2, and parts of the tetratricopeptide repeat (TPR) subunits Cdc27/Apc3, Cdc16/Apc6 and Apc7 (Han et al., 2009; Wang et al., 2009; Zhang et al., 2010; Zheng et al., 2002), Doc1/Apc10 is the only APC/C subunit where a crystal structure had been solved (Au et al., 2002; Wendt et al., 2001). Mutation or deletion of *DOC1* results in temperature sensitive budding yeast strains (Hwang and Murray, 1997). In fission yeast and *D. melanogaster*, Doc1 is essential (Kominami et al., 1998; Pal et al., 2007b). It is a small one-domain protein and characterized by the presence of a conserved DOC domain, which is also found in several other putative E3 ubiquitin ligases. Therefore it might fulfill a function common to this type of E3 protein complexes (Grossberger et al., 1999; Kominami et al., 1998). In fact, many of these multidomain proteins

have been identified to be ubiquitin ligases or to be linked to ubiquitin-dependent processes (DiAntonio et al., 2001; Dias et al., 2002; Nikolaev et al., 2003). Structural studies revealed that the DOC domain can fold into a "jellyroll" structure which is virtually identical to domains found in various other prokaryotic and eukaryotic enzymes that can bind ligands such as sugars, nucleotides and polypetides (Au et al., 2002; Wendt et al., 2001). Hence, this first raised the possibility that Doc1/Apc10 might participate in APC/C catalysis by stabilizing the interaction with substrate proteins (Grossberger et al., 1999; Passmore et al., 2003). Moreover, deletion of Doc1 abolishes APC/C´s ability to ubiquitylate its substrates in a processive manner (Carroll and Morgan, 2002). The important role of Doc1/Apc10 in substrate recognition and processive ubiquitylation could later be verified in structural studies (Buschhorn et al., 2010; Da Fonseca, 2011) (see chapter 1.8). In addition to the DOC domain, Doc1 contains a C-terminal IR-tail which allows association with the tetratricopeptide repeat (TPR) domain proteins Cdc27/Apc3 and Cdc16/Apc6 *in vitro* (Buschhorn et al., 2010; Wendt et al., 2001). Cross-linking experiments indicated that Doc1 also interacts with Apc1 and EM data on yeast APC/C revealed that Doc1 is located next to Apc2 and Apc11 (Buschhorn et al., 2010). Moreover, the substrate protein was observed to be located directly in between Doc1 and Cdh1, which strongly implies that Doc1 directly interacts with the substrate, similarly to the co-activator (Buschhorn et al., 2010). The TPR containing subunits as well as Apc1, Apc4 and Apc5 are essential APC/C subunits and mutations of these proteins cause a profound metaphase arrest due to the inability to induce sister chromatid separation and cyclin B1 degradation (Kramer et al., 1998; Lamb et al., 1994; Zachariae and Nasmyth, 1996; Zachariae et al., 1998b).

1.7.3 The co-activators

This already complex composition is still not sufficient to constitute an active APC/C. The APC/C still requires additional co-activator proteins in order to promote substrate ubiquitylation in a specific fashion. The two main co-activator proteins are Cdc20 and Cdh1. They can directly associate with the APC/C in a cell cycle-regulated and substoichiometric manner and are essential for cell cycle progression (Schwab et al., 1997; Visintin et al., 1997).

Both proteins activate the APC/C at different time points during mitosis (described in more detail in chapter 1.7.3). CDC20 and CDH1 are encoded in all known eukaryotic genomes. Both proteins contain specific sequence elements, known as C-box and IR-tail. The internal C-box mediates binding to the catalytic subunits of the APC/C (Schwab et al., 2001; Thornton et al., 2006), whereas the C-terminal IR-tail is required for binding to the TPR subunit Cdc27/Apc3 (Kraft et al., 2005; Passmore et al., 2003; Vodermaier et al., 2003). In addition they contain a WD40 domain which is predicted to fold into a seven-bladed propeller like structure (Orlicky et al., 2003; Wu et al., 2003), mediating binding to specific recognition elements in substrate proteins, known as the D-box or the KEN-box (Burton and Solomon, 2001; Burton et al., 2005; Kraft et al., 2005).

However, additional meiosis-specific APC/C co-activator proteins were identified in yeast and *D. melanogaster* (Cooper et al., 2000; reviewed by Peters, 2006). Ama1 in budding yeast (Cooper et al., 1997) and Fzr1/Mfr1 in fission yeast (Asakawa et al., 2001) are essential for exit from the second meiotic division and thereby required for spore formation (Penkner et al., 2005). Ama1 (activator of meiotic APC/C) is a developmentally regulated member of the Cdc20 family of APC/C activators that controls the first meiotic division. AMA1 gene transcription only occurs in meiotic cells where APC/C^{Ama1} function is necessary for spore wall assembly and expression of late meiotic genes (Cooper et al., 2000). The subunit Mnd2 in budding yeast is a meiosis-specific inhibitor of APC/C^{Ama1}. Mnd2 is required during S- and prophase I to prevent premature sister chromatid separation on sequences around centromeres and chromosomal arms by inhibiting the APC/C^{Ama1}-dependent proteolysis of Pds1. Pds1 is the orthologue to securin in mammalian cells and required for cohesin cleavage. (Oelschlaegel et al., 2005; Penkner et al., 2005). Mnd2 mediated Pds1 stabilization therefore maintains cohesion established on chromosome arms after segregation of homologues in meiosis I and retains cohesin on centromeres until the second division in meiosis II, where sister chromatids are finally pulled apart (Oelschlaegel et al., 2005). Mnd2 had been shown to be essential for meiosis but not for normal mitotic cell division (Rabitsch et al., 2001).

human	*S. c.*	*S. p.*	essential	Known motifs or functions
Core APC/C subunits				
Apc1	Apc1	Cut4	yes	Homologue to Rpn1, Rpn2 (proteosomal subunits)
Apc2	Apc2	Apc2	yes	Cullin domain; catalytic activity, E2 binding
Apc3	Cdc27	Nuc2	yes	TPRs; co-activator binding, APC/C function *in vivo*
Apc4	Apc4	Lid1	yes	WD40 repeats; bridges Apc1 and the TPR containing subunits
Apc5	Apc5	Apc5	yes	Bridges Apc1 and TPR containing subunits
Apc6	Cdc16	Cut9	yes	TPRs; required for association of Cdc27 to APC/C
Apc7	-	-	?	TPRs
Apc8	Cdc23	Cut23	yes	TPRs; required for Cdc16 and Cdc27 association
-	Apc9	-	no	Promotes association of Cdc27
Apc10	Doc1	Apc10	Deletion *ts* in *S.c.* Essential in *S.p.*	DOC domain; IR tail; processivity; substrate binding
Apc11	Apc11	Apc11	yes	RING finger, catalytic activity, E2 binding
Cdc26	Cdc26	Hcn1	Deletion *ts* in *S.c.* Essential in *S.p* .	Upregulated at higher temperatures; promotes association of Cdc16, Cdc27, Cdc26, Apc9
Apc13	Swm1	Apc13	Deletion *ts* in *S.c.*	Required for sporulation
-	-	Apc14	no	
-	Mnd2	Apc15	no	Inhibition of APC/C^{Ama1} in meiosis
Apc16	-	-	yes	APC/C activity in mitosis
APC/C co-activators				
Cdc20	Cdc20	Slp1	yes	WD40 repeats, C-box, IR-tail; substrate recruitment
Cdh1	Hct1	Ste9	no	WD40 repeats, C-box, IR-tail; substrate recruitment
Ama1	-	-	no	WD40 repeats, C-box, IR-tail; substrate recruitment

Table 1-1: APC/C subunits and co-activators identified to date in human cells, *Saccharomyces cerevisiae (S.c.)* and *Schizosaccharomyces pombe (S.p.)* (reviewed by Peters, 2006; Thornton and Toczyski, 2006). Anaphase promoting complex / cyclosome (APC/C); regulatory particle non-ATPase (Rpn); ubiquitin conjugating enzyme (E2); tetratrico peptide repeats (TPR); really interesting new gene (RING); tryptophane aspartate (WD40); degradation of cyclin B protein 1 (Doc1); temperature sensitive (ts); spore wall maturation protein-1 (Swm1); meiotic nuclear division protein-2 (Mnd2); activator of meiotic APC/C protein-1 (Ama1).

Soon after the APC/C had been discovered, it became evident that its composition is much more complex compared to other Cullin-RING finger ubiquitin ligases. APC/C-mediated reactions are known to be only regulated on the level of the APC/C, whereas SCF-depenend reactions are controlled on the level of substrates. Hence, it seems likely that the large composition of the APC/C is necessary to allow regulation of ubiquitin reactions at the level of the ubiquitin ligase (Gieffers et al., 2001). However, still today, fifteen years after its

discovery, we still know very little about the biological functions of different APC/C subunits and we still have no satisfying explanation why this protein complex is so large in size.

Only recently, Apc16 had been identified as a novel constitutive subunit of human APC/C (Hutchins et al., 2010; Kops et al., 2010), increasing the number of known subunits in vertebrate APC/C to thirteen. Apc16 is required for APC/C activity towards mitotic substrates, and is therefore important for the transition from metaphase to anaphase (Hutchins et al., 2010; Kops et al., 2010). However, unlike some other APC/C subunits, Apc16 is dispensable for structural integrity of the APC/C holocomplex and for its assembly (Kops et al., 2010). Antibody labeling using an Apc16-specific antibody in electron microscopic analysis of the APC/C revealed that the small protein is located at the top of a region called the "arc lamp" of the complex in close confirmation to Cdc27/Apc3 (Hutchins et al., 2010). The protein is conserved among higher metazoans but has no homology in *fungi* (Kops et al., 2010). Apc16 is encoded by the gene region C10ORF104 (chromosome 10 open reading frame 104). Due to its small size of only 11.7 kDa, it might have previously escaped standard detection in protein gels or in mass spectrometric analysis.

1.8 Analysis of APC/C structures by Electron microscopy

To better understand how APC/C components organize to form a tightly regulated, multi-protein E3 ubiquitin ligase, it is necessary to determine the structure formed when they come together. Recent cryo-electron microscopy studies (EM) shed light into the assembly of those multiple subunits, and provide a three-dimensional (3D) structure of the APC/C. Together with the already existing biochemical data; those structural studies are valuable for understanding the mechanism of APC/C activity. The first structures were obtained for APC/C purified from frog egg extracts and human cells (Dube et al., 2005; Gieffers et al., 2001), as well as from budding (Passmore et al., 2005b) and fission yeast (Ohi et al., 2007) and have revealed that the APC/C is an asymmetric triangular complex, 180 to 200 Å in size, with an internal cavity enclosed by an outer wall and a "head"-like structure at one end. Vertebrate APC/C consists of two domains referred to as "platform" and "arc lamp" which exhibit a large degree of flexibility relative to each other (Dube et al., 2005). The dimensions

of the platform domain are similar in yeast, *Xenopus* and human, but the arc lamp domain of yeast APC/C is shorter than the corresponding domain in vertebrate APC/C (Dube et al., 2005). The arc lamp is mainly composed of TPR subunits, raising the possibility that the density only found in vertebrate APC/C is due to Apc7, a TPR subunit thus far identified only in higher eukaryotes. Otherwise, the structures of yeast and vertebrate APC/C are similar in shape and size, indicating the APC/C´s structure has been largely conserved during evolution (Buschhorn et al., 2010).

Recent higher resolution structures have led to a better understanding of APC/C organization and composition. Antibody-labeling and cryo-EM experiments (Herzog et al., 2009) could show that Apc5 is located at the bottom of the platform domain, in close vicinity to the interface of the platform and the arc lamp. The Apc4 subunit sits at the front and Apc1 at the right-hand side. Part of Apc4 has a ring-shaped structure, confiming the bioinformatic prediction that Apc4 contains an N-terminal propeller-shaped WD40 domain. Cdc27/Apc3 is located at the headlike protrusion at the top end of the arc lamp domain and Apc2 is located in the central cavity on the front side of APC/C, residing in close vicinity to Apc1 and Cdc27/Apc3. Antibody labeling of Cdc16/Apc6 and Apc7 revealed that those subunits bind to multiple locations on the arc lamp domain, presumably because they are present in substoichometric amounts. Moreover, this structure shows that the arc lamp domain is bent and has an unusual repetitive regularity, features that are characteristic of TPR domains that are present in Cdc16/Apc6 and Apc7 and also in Cdc27/Apc3 and Cdc23/Apc8, indicating that a major part of the arc lamp domain consists of TPR subunits (Herzog et al., 2009). With the exception of Apc2 and Apc4 topology, this 3D model of subunit topology is consistent with the 2D positions of subunits determined in fission yeast APC/C (Ohi et al., 2007).

Studies on *Xenopus* APC/C could show a reduction in the angle between the platform and the arc domain upon binding of Cdh1 to the complex, indicating that co-activator binding might induce conformational changes within the APC/C, that are of importance for the function and regulation of this ligase (Dube et al., 2005). In contrast to this, studies on budding yeast APC/C did not reveal any significant structural difference between APC/C and APC/C^{Cdh1}, except for the additional density coming from the co-activator (Da Fonseca, 2011).

Interestingely, however, engagement of the substrate protein Hsl1 with APC/C^{Cdh1} did result in a profound structural change involving Cdh1 and Doc1/Apc10, implicating that substrate binding might promote the formation of new connections between the co-activator and Doc1/Apc10 (Da Fonseca, 2011). This finding is consistent with direct co-activator-substrate interactions and emphasizes the function of Doc1/Apc10 in mediating substrate binding (Da Fonseca, 2011). Furthermore, studies on human APC/C showed that Doc1/Apc10 is binding in close vicinity to the cullin-RING module Apc2-Apc11 and that the substrate binding site is located in the inner cavity, between Doc1 and Cdh1, which again implies that Cdh1 and Doc1 might form a bipartite substrate receptor on the APC/C, thereby contributing to processive substrate ubiquitylation (Buschhorn et al., 2010; Da Fonseca, 2011). However, Doc1 and Cdh1 are conformationally not interdependent (Da Fonseca, 2011). Binding of Doc1 to the APC/C is mediated through interaction with the TPR subunits Cdc27/Apc3, Cdc16/Apc6 as well as the scaffold protein Apc1, as shown in EM-studies on budding yeast and human APC/C (Buschhorn et al., 2010). Higher resolution structures of budding yeast could also show that the co-activator interacts with Cdc27/Apc3 through its C-terminal IR-tail, while its N-terminal C-box contacts Apc2 (Da Fonseca, 2011; Schreiber et al., 2011). Yeast Swm1/Apc13 is located in the vicinity of Cdc16/Apc6 and Cdc27/Apc3, consistent with the observation that Swm1/Apc13 stabilizes interactions between these two subunits (Buschhorn et al., 2010; Schwickart et al., 2004). Two other non-essential yeast subunits, Cdc26 and Apc9 also seem to have a role in stabilizing the Cdc27/Apc4 and Cdc16/Apc6 association with the rest of the complex (Thornton and Toczyski, 2006; Wang et al., 2009; Zachariae and Nasmyth, 1996; Zachariae et al., 1998a). Despite some differences of yeast and vertebrate APC/C, all comprehensive EM data sets are largely consistent with the outlined APC/C subunit map (Thornton et al., 2006) (**Figure 1-2**) confirming biochemical mapping experiments.

1.9 Substrate recognition by the APC/C and substrate ordering

Most APC/C substrates contain at least one of specific amino acid sequence motifs, so called degrons that are usually located in low complexity regions of the protein and are required for

their ubiquitilation. The most widespread motifs are the “destruction-box” (D-box, consensus sequence RxxLxxxxN/D/E) (Glotzer et al., 1991; King et al., 1996) and the “KEN-box” (consensus sequence KENxxxN/D) (Pfleger and Kirschner, 2000). Other less well characterized degrons include the “A-box” in Aurora A (Littlepage and Ruderman, 2002), the “GxEN” motif in XKid (Castro et al., 2003) and the “O-box” (Araki et al., 2005). Mutations in these recognition sites abolish substrate ubiquitylation and therefore stabilize the protein, mostly because they serve as a binding motif for the APC/C co-activator proteins Cdc20 and Cdh1. Those sequences lack conserved lysine residues that are capable of accepting ubiquitin, therefore these degrons are not sufficient for substrate degradation (King et al., 1996). Moreover, it seems likely that the context in which the degron is placed is important. It has also been shown that the KEN-box is more specific to be targeted by the form of APC/C associated with Cdh1 (Zur and Brandeis, 2002). The degrons are portable and chimeric proteins containing for example the N-terminal D-box of cyclin B are degraded as if they were cyclins (Amon et al., 1994; Glotzer et al., 1991; Yamano et al., 1996). Moreover, amino acid sequences that lie outside the recognition motifs seem to influence the substrate ubiquitylation efficiency. For example, it is sufficient to fuse a D-box-baring fragment of *Xenopus* cyclin B1 (amino acids 13-66) to protein A to render it degradable in a cell cycle-dependent manner, whereas a fragment of 13-53 amino acids is not (Glotzer et al., 1991). However, there seem to be differences among the D-boxes of different cyclins. In contrast to the D-box-containing fragment of cyclin B1, the degron fragment of *Xenopus* cyclin A1 on protein A stabilized the fusion protein (Klotzbuecher et al., 1996). In addition, studies of chimeric proteins have shown that fusing the D-box-containing N-terminus of *Xenopus* cyclin A1 to the C-terminus of cyclin B1 rendered the fusion proteins stable, which was not the case the other way round (King et al., 1996; Klotzbuecher et al., 1996). It therefore seems likely, that the position of the degrons in the context of the overall protein sequence as well as degron-intrinsic properties partly account for differences in substrate recognition by the APC/C.

1.9.1 Substrate ordering

Substrate ordering refers to the right order and specificity of substrate degradation by APC/C-mediated ubiquitylation, and it is crucial for the correct sequence of events in mitosis and G1. The degradation of different APC/C substrates is sequential and occurs in temporally distinct events, which defines the different cell cycle stages (Hunt et al., 1992; Sigrist et al., 1995; Whitfield et al., 1990). Therefore, proteolysis of APC/C substrates cannot simply be regulated by activation or inactivation of the APC/C but must also include a layer of selectivity on the substrate level. Selective substrate degradation is mainly ensured by cell cycle-regulated sequential association of the APC/C co-activator proteins Cdc20 and Cdh1 to the complex, which both confer different substrate specificity to the ligase. Cdc20 specific substrates are early mitotic regulatory proteins that are targeted by the APC/C from metaphase till the end of anaphase, whereas Cdh1 recognizes specific substrates until the end of mitosis and throughout G1. Substrate ordering through sequential recruitment to the APC/C can nicely be explained by the budding yeast Pds1, Clb2 and Ase1 proteins (Juang et al., 1997; Schwab et al., 1997; Shirayama et al., 1999; Visintin et al., 1997). Cdc20 is required for degradation of Pds1 in early mitotic stages but not for Clb2 and Ase1. Pds1 destruction leads to sister chromatid separation, which promotes transition from metaphase to anaphase. In contrast, APC/C^{Cdh1} drives mitotic exit and events in G1, because Cdh1 is more specific towards Ase1 and Clb2 later in mitosis but not towards Pds1. The Ase1 protein associates with the mitotic spindle and its destruction is required for disassembly of the spindle. Together with Clb2 destruction, its proteolysis is essential for exiting the mitotic stage (Visintin et al., 1997). Both co-activator proteins together ensure that different APC/C substrates are degraded at the right time during mitosis. Pds1 destruction, along with sister chromatid separation, also triggers Cdc14 phosphatase activation, leading to a decline in Clb2-mediated phosphorylation of other proteins, including Cdh1 (Visintin et al., 1997). Dephosporylation of Cdh1 in turn promotes its association with the APC/C, and therefore changes APC/C´s substrate specificity (reviewed byBaker et al., 2007; Peters, 2006) . Hence, degradation of the later Cdh1-specific substrates Ase1 and Clb2, which ensures proper cytokinesis and correct mitotic exit, can only occur after the sister chromatids had been separated. Correct sister chromatid separation is a highly regulated process in itself and will be described in chapter 1.10.4.

Another mechanism regulating substrate ordering is substrate-intrinsic and depends on the ubiquitylation processivity of different substrates by the APC/C (Rape et al., 2006; Stegmeier et al., 2007). In this model, substrate ordering is based on relative differences in the processivity of multiubiquitilation of the various substrates. These differences can originate from the catalytic rate of multiubiquitilation as well as from the rate of dissociation from the APC/C. Thus, processive substrates obtain their multiubiquitin chain in a single binding event, while more distributive substrates continuously shuttle on and off the APC/C; hence it takes longer time until they obtain multiubiquitin chains necessary for their degradation. Processivity in substrate ubiquitylation strongly correlates with the relative timing of their degradation during the cell cycle. The more processive the multiubiquitilation of a substrate is, the earlier it is degraded relative to other substrates. Thus, only degradation of the more processive substrates will allow for efficient multiubiquitilation of the distributive ones. Moreover, multiubiquitilation of distributive substrates require multiple rounds of APC/C binding, hence it renders them sensitive to lower APC/C concentrations, competition by processive substrates, and deubiquitilation events. This additional layer of processivity differences in APC/C mediated ubiquitilation allow ordered substrate degradation without prior substrate modification. This in turn allows for signal amplification mechanisms and fine-tuning and it implies that substrate ordering by the APC/C is self-organizing. Biochemical and structural studies have shown that processivity of multiubiquitilation and thus substrate ordering is strongly influenced by the D-box within the substrate (Buschhorn et al., 2010; Da Fonseca, 2011; Rape et al., 2006) (see chapter 1.9.1). For example, the distributive nature of cyclin A as an APC/C substrate is thought to be determined by its D-box sequence, which differs from the D-boxes of other substrates (Rape, 2010) (see chapter 1.9.2). The more distributive the multiubiquitilation of a substrate is, the more it is likely that it will be converted into the basal state by the activity of deubiquitilating enzymes (DUBs). DUBs can therefore amplify small differences in ubiquitilation processivity and are likely to have profound effects on the timing of substrate degradation (Rape et al., 2006; Stegmeier et al., 2007). It is therefore believed that substrates are initially recognized and delivered to the APC/C by Cdc20 and Cdh1. The processivity of the ubiquitilation reaction, however, will in part depend on the stability of this interaction, which is in turn is dependent on the D-box of the substrate protein (Da Fonseca, 2011; Rape et al., 2006). Substrate degradation can also be

influenced by their Cdk-mediated phosphorylation status. It has been shown that phosphorylation of residues in proximity to APC/C-recognition motifs can selectively inhibit the degradation of specific APC/C substrates by blocking their recognition through the co-activators (Song and Rape, 2011). For example, the APC/C-dependent degradation of Aurora A was found to be inhibited by cyclin B1/Cdk1-mediated phosphorylation of a specific serine residue within its recognition motif, the A-box in early mitosis (Crane et al., 2004; Littlepage and Ruderman, 2002). In order to be efficiently degraded during mitotic exit, Aurora A needs to be dephosphorylated by the protein phosphatase PP2A (Horn et al., 2007). Other post-translational modifications might serve similar roles in regulation of APC/C-dependent substrate proteolysis. Acetylation of BubR1 or cyclin A for example has been reported to modulate their stability during mitosis, potentially by interfering with APC/C-dependent ubiquitilation (Choi et al., 2009; Mateo et al., 2009).

1.9.2 Co-activator independent substrate recognition by the APC/C

The protein kinase Nek2A (NIMA-related kinase 2A) and cyclin A are substrate proteins that are already degraded in prometaphase, soon after the nuclear envelope breakdown (NEBD). This occurs before other substrates such as B-type cyclins or Pds1/securin are targeted by the APC/C (see chapter 1.9.2). Therefore, both proteins are believed to be recognized by the APC/C somewhat differently than other substrates. Nek2A recruitment to the APC/C has been reported to be Cdc20-independent, although its ubiquitylation requires the N-terminal C-box domain of the co-activator (Kimata et al., 2008). Nek2A can directly bind to the APC/C via its C-terminal methionine-arginine (MR) tail. This motif resembles the isoleucine-arginine dipeptide (IR-tail) found at the C terminus of the co-activator proteins Cdc20 and Cdh1 and in the small subunit Doc1, which promotes binding of these proteins to the APC/C (Hayes et al., 2006). Cyclin A1 degradation in early mitosis is dependent on its binding to Cdk, as a mutation in cyclin A that abolishes Cdk binding delays its degradation until anaphase (den Elzen, 2001; Geley et al., 2001; Sorensen et al., 2001; Stewart et al., 1994; Wolthuis et al., 2008). However, Cdk binding to cyclin A is not sufficient for its ubiquitylation because deletion of the D-box sequence of cyclin A but an intact Cdk1 binding domain stabilizes the

protein (Geley et al., 2001; Kobayashi et al., 1992). The difference in timing of cyclin A1 degradation compared to cyclin B1 degradation might be explained by the D-box sequence of cyclin A, which is 10-20 residues longer than that of cyclin B. This feature is conserved among cyclin A1 and A2 molecules across species and it has been proposed that A type cyclins are targeted for ubiquitylation by an "extended D-box" sequence. This sequence might in addition contain the Cdk binding site, thereby promoting its recognition by APC/C^{Cdc20} (Geley et al., 2001). Alternatively, association with its Cdk might induce a structural change in cyclin A, which makes its N-terminal D-box motif more accessible (Wolthuis et al., 2008). Moreover, it has been shown that unlike cyclin B, cyclin A binds to Cdc20 in G2 phase, which is before APC/C becomes active in mitosis. This reasoned that the co-activator might be a liminting factor for cyclin A degradation, also because Cdc20-depleted cells showed high levels of cyclin A, but not of cyclin B (Wolthuis et al., 2008). Cyclin A forms a trimeric complex with Cdk and a Cks protein. Cks proteins bind to Cdks *in vitro* and are though to facilitate binding to previously phosphorylated Cdk consensus sites (Bourne et al., 1996). The Cks subunit of the cyclin A-Cdk-complex has been shown to strongly bind to Cdk-phosphorylated APC/C (Fry and Yamano, 2006). Since the timely destruction of cyclin A is depend on its binding to Cdk and coincides with an increase in APC/C phosphorylation, it is possible that cyclin A gets recruited to phospho-APC/C through its Cks subunit, where it can then be targeted for ubiquitylation (Wolthuis et al., 2008). This would explain why cyclin A gets degraded before other mitotic substrates and why this can happen independently of the spindle assembly checkpoint (Fry and Yamano, 2006; Wolthuis et al., 2008) (see chapter 1.10.4). Such multivalent binding might positively affect the processivity of ubiquitin chain formation, which is a major determinant of the timing of APC/C substrate degradation (Rape et al., 2006). Therefore, binding partners such as the Cks subunit of cyclin A-Cdk-complex might provide additional ways to APC/C recruitment and are effective of increasing the processivity of the reaction, thereby accelerating substrate degradation during the cell cycle (Song and Rape, 2011). It is also conceivable that subtle intrinsic differences in cyclins might contribute to the tight regulation of cell cycle transitions. However, unknown features might in addition determine substrate ordering events.

1.10 Regulation of APC/C activity

Irreversible proteolysis of key regulatory proteins by the APC/C initiates irreversible cell cycle progression, thereby confering unidirectionality to this process. It ensures that the cell can divide the genome equally to daughter cells which must occur only once per cell cycle and that mitotic exit is performed in an ordered fashion. All of these important events depend on APC/C activity. Since this ligase is required for accurate execution of the mitotic program, the APC/C has to be tightly controlled. This is in part achieved by reversible phosphorylation events, sequential and cell cycle-regulated association of its co-activators, and association of inhibitory proteins, such as the mitotic checkpoint complex (MCC), the effector of the spindle assembly checkpoint (SAC).

1.10.1 Regulation of APC/C activity by phosphorylation

Protein phosphorylation is a fundamental regulatory mechanism in biology, with essential functions in signaling, metabolism, and cell cycle control. Clusters of multiple phosphate groups can significantly change the surface charge distribution of a molecule. Hence, phosphorylation can induce structural changes within a molecule by altering protein interactions or it can change the affinity for other molecules. The TPR-containing subunits and Apc1 are hyperphosphorylated in mitosis (King et al., 1995; Peters et al., 1996; Yamada et al., 1997). It has been shown that phosphorylation of APC/C is functionally important, as dephosphorylation of mitotic APC/C abolishes its ubiquitin-ligase activity (King et al., 1995; Lahav-Baratz et al., 1995; Shteinberg et al., 1999). Although Cdc20 protein levels rise during S and G2 phase, Cdc20 can only associate with the APC/C early in mitosis, which is dependent on phosphorylation of the TPR-subunits by mitotic kinases (Kraft et al., 2003; Kramer et al., 2000; Rudner and Murray, 2000; Shteinberg et al., 1999). Immunofluorescence microscopy using phospho-specific antibodies revealed that APC/C-phosphorylation is initiated in prophase, when cyclin B1 starts to enter the nucleus. In prometaphase phospho-APC/C accumulates on centromeres where ubiquitylation of cyclin B is initated. Later, it

appears throughout the cyctosol and disappears during mitotic exit. Hence, activation of the APC/C is already initiated in the nuclei of late prophase cells (Kraft et al., 2003).

Cdc20 also gets phophorylated in mitosis but this does not seem to affect its mitotic activity (Kramer et al., 2000). Rather it was proposed that Cdc20 phosphorylation plays a role in the function of the spindle assembly checkpoint (Chung and Chen, 2003; Tang et al., 2004). In contrast, phosphorylation of the other co-activator Cdh1 prevents its binding to the APC/C (Jaspersen et al., 1999; Kramer et al., 2000). In vertebrates, mitotic activity of the APC/C through phosphorylation is mainly stimulated by the two kinases Cdk1/cyclin B and Plk1, but Cdk1 seems to have a more important role than Plk1 (Descombes and Nigg, 1998; Patra and Dunphy, 1998). *In vitro*, the APC/C is directly phosporylated to the largest extent, when both kinases are combined (Golan et al., 2002; Kraft et al., 2003). Once Cdc20 has bound to the complex, it mediates cyclin B proteolysis in metaphase, which reduces Cdk1 activity. This allows protein phosphatases such as Cdc14 in yeast to dephosphorylate Cdh1 (Jaspersen et al., 1999; reviewed by Stegmeier and Amon, 2004; Visintin et al., 1998) which enables Cdh1 to associate and activate APC/C. The human genome encodes two Cdc14 homologues. The roles of these two phosphatases are poorly understood, but it is plausible that they are involved in mitotic exit and cytokinesis (Kaiser et al., 2002). In yeast, additional dephosphoylation of the Cdk1 inhibitor Sic1 leads to its activation and therefore to inhibition of Cdk1 activity. This in turn allows dephosphorylation of Cdh1 and promotes its binding to the APC/C (Donovan et al., 1994; Verma et al., 1997). Cdc20 contains the D-box motif necessary for APC/C-mediated ubiquitylation and is therefore itself a target of APC/C. Dephosphorylation of APC/C subunits and of Cdh1 thus leads to dissociation of Cdc20 from the complex and to activation of APC/C by Cdh1, which now targets Cdc20 for degradation at the end of mitosis (Fang et al., 1998b; Prinz et al., 1998; Shirayama et al., 1998). In contrast to Cdh1 levels, which are constant throughout the cell cycle, Cdc20 levels fluctuate. Cdc20 protein is absent during S phase, whereas its level peaks in mitosis and declines as cells enter G1 phase (Prinz et al., 1998). Therefore, APC/C^{Cdc20} activity is prevented during S phase and in early mitosis. Unlike Cdc20 degradation in G1, Cdc20 proteolysis in S phase and early mitosis is mediated by a process which does not seem to depend on Cdc20´s D-box or on Cdh1; moreover it requires direct binding of Cdc20 to components of the mitotic checkpoint complex and functional APC/C (Pan and Chen, 2004; Prinz et al., 1998; Thornton and Toczyski, 2006) (see

chapter 1.10.5). Hence, mitotic kinases promote phosphorylation of the APC/C core complex and of both co-activator proteins, importantly of Cdh1, which allow for the switch from APC/C^{Cdc20} to APC/C^{Cdh1} activity, thereby keeping Cdk1 activity low until late G1 phase. This ensures that mitotic exit only occurs after successful anaphase and it is a prerequisite for initiation of DNA replication. The kinases that promote APC/C activity in mitosis are the cyclin-Cdk/Polo/Polo-like kinases, whereas Protein kinase A has been shown to inhibit APC/C activity (Descombes and Nigg, 1998; Kotani et al., 1998; Yamashita et al., 1996).

To allow the re-accumulation of cyclins and other APC/C substrates that are needed for subsequent S-phase and for a new round of mitotic cell division, APC/C^{Cdh1} has to be inactivated at the G1-S transition. This in part is mediated through phosphorylation of Cdh1 by S-phase specific cyclin-Cdk complexes, which has been shown to promote dissociation and inactivation of APC/C^{Cdh1} (Huang et al., 2001; Jaspersen et al., 1999; Kramer et al., 2000; Lukas et al., 1999; Zachariae et al., 1998a). However, the mechanism of how the cyclin-Cdk complex levels rise to a sufficient threshold in late G1 seems to vary in different organisms. In *D. melanogaster*, Cdh1 re-phosphorylation and thereby APC/C inactivation has been implicated to be due to cyclin E-Cdk1 activity (Knoblich et al., 1994). Another model suggests that APC/C^{Cdh1} promotes its own inactivation by catalyzing the autoubiquitilation and subsequent degradation of the ubiquitin-conjugating enzyme (E2) UbcH10. This leads to accumulation of cyclin A and therefore to increasing cyclin A-Cdk2 activity, which in turn further inactivates APC/C^{Cdh1}. The other E2 UbcH5 is stable and remains associated with the APC/C, but it does not target cyclin A efficiently. Autoubiquitilation of UbcH10 can only be initiated, once other APC/C substrates have been degraded in G1 (Rape and Kirschner, 2004; reviewed by Thornton and Toczyski, 2006). This mechanism ensures that APC/C is active during mitotic progression but inactive before S-phase entry and it suggests that the metazoan cell cycle is built around a self-perpetuating but highly regulated oscillator (Rape and Kirschner, 2004). In *D. melanogaster* and in vertebrate cells, APC/C^{Cdh1} activity is additionally repressed by an APC/C inhibitor known as Rca1 (regulator of cyclin A1) or Emi1 (early mitotic inhibitor 1), respectively. Association of inhibitory proteins to the complex allows accumulation of cyclin A and of other APC/C substrates in S and G2 (Dong et al., 1997; Grosskortenhaus and Sprenger, 2002) and will be discussed in chapter 1.10.3.

1.10.2 Regulation of APC/C activity by co-activator proteins

The APC/C is only fully activated upon binding of one of its co-activatory proteins. Cdc20 (Fizzy in *D.melanogaster*, Slp1 in *S. pombe*) transiently associates with the APC/C in mitosis until anaphase, whereas Cdh1 (Fizzy-related in *D. melanogaster*, Srw1/Ste9 in *S.pombe*, Hct1 in *S.cerevisae*, Fzr1 in *Homo sapiens*) keeps the APC/C active until the end of G1 phase (reviewed by Yu, 2007). Other activators include the meiosis-specific Ama1 protein in budding yeast (Penkner et al., 2005) (see chapter 1.10.3) and Cort in *D.melanogaster* (Swan and Schupbach, 2007). Substrate specificity of the ubiquitylation reaction is largely conferred by the co-activator proteins, where Cdc20 preferentially targets D-box containing substrates, while Cdh1 can additionally recognize the KEN-box motif (Schwab et al., 1997; Schwab et al., 2001; Visintin et al., 1997; Wan and Kirschner, 2001; Zur and Brandeis, 2002). Cdc20 and Cdh1 are both highly conserved in all eukaryotes. In yeast, most genes that encode apo-APC/C-subunits as well as Cdc20 are essential for viability (Zachariae et al., 1998a) and mutations of Fizzy in *D.melanogaster* cause a metaphase arrest phenotype (Dawson et al., 1993). Regulation of Cdc20 protein levels have been shown to be critical for cell cycle progression and cell viability, since ectopic expression of CDC20 is lethal (Visintin et al., 1997) and overexpression of Cdc20 is sufficient to cause a bypass of the DNA damage and mitotic spindle assembly checkpoint arrest (Hwang et al., 1998). Thus, tight regulation of Cdc20 protein levels is not only critical for proper cell cycle progression but also during cell cycle arrest induced by DNA damage or by mitotic spindle defects (Prinz et al., 1998). In contrast, RNA interference (RNAi) of Cdh1 in human cells does not seem to significantly perturb the cell cycle (Qi and Yu, 2007) and Cdh1-deficient mice are viable (Garcia-Higuera et al., 2008). Moreover, Cdh1 protein levels are constant throughout the cell cycle, and Cdh1 is inhibited by cyclin/Cdk-mediated phosphorylation (Kraft et al., 2003). Cdc20 was originally identified as a *cdc* gene required for APC/C-dependent proteolysis of Pds1, whereas Cdh1 (Cdc20 homolog 1) was implicated to be important for degradation of Clb2 and Ase1 (Schwab et al., 1997; Visintin et al., 1997). Later it was shown that Cdc20 and Cdh1 directly bind to the APC/C to activate its ligase activity towards cyclin B *in vitro* (Fang et al., 1998).

Cdc20 and Cdh1 contain a WD40 repeat domain in their C-terminal region. This motif can fold into a rigid propeller-like structure that has been found in a variety of proteins with different functions. Moreover, this motif has been proposed to mediate protein-protein interactions (reviewed by Smith et al., 1999; Visintin et al., 1997). The folding of the WD40 propeller in Cdc20 and Cdh1 requires the CCT chaperonin complex that has been shown to associate with the activator proteins during the cell cycle (Camasses et al., 2003). The WD40 domain of Cdc20 and Cdh1 is believed to be the adaptor sequence for binding to the recognition motifs in APC/C-substrates. Importantly, mutations in residues within the WD40 domain of Cdh1 significantly abolish the substrate ubiquitylation efficiency (Kraft et al., 2005). This is analogous to the function of WD40-domain containing F-box substrate adaptor proteins of the SCF, which also recruit substrates via their propeller (reviewed by Nakayama and Nakayama, 2005; Zheng et al., 2002). Recent electron microscopy studies confirmed binding of the substrate´s degron motifs to the co-activator proteins and their recruitment to the APC/C (Da Fonseca, 2011). In budding yeast, the binding site for the substrate´s D-box is shared between the WD40 domain of the co-activator and the β-sandwich of Doc1 (Da Fonseca, 2011). In the same study, *In vitro* binding experiments using D-box and KEN-box peptides also revealed an intrinsic difference in both motifs in that only D-box substrates could promote a physical interconnection between Cdh1 and Doc1 (Da Fonseca, 2011).

In addition to the WD40 domain, other short sequences within the co-activator proteins were shown to be conserved and of particular function. The IR tail at the very C-terminus is required for binding to the TPR-subunit Cdc27/Apc3 of human and yeast APC/C (Matyskiela and Morgan, 2009; Thornton et al., 2006; Vodermaier et al., 2003), although the IR tail of Cdc20 does not seem to be important for viability in yeast (Thornton et al., 2006). Activity of the co-activatory proteins seems to depend on their C-box, a seven amino acid sequence at the N-terminus. This motif promotes binding of the protein to the APC/C and stimulates its ligase activity in ubiquitylation reactions (Kimata et al., 2008; Schwab et al., 2001; Thornton et al., 2006; Vodermaier et al., 2003). Structural studies suggest that the C-terminal Ile-Arg (IR) tail of Cdh1 contacts the APC/C subunit Cdc27/Apc3, while its N-terminal C-box is positioned to contact Apc2 (Buschhorn et al., 2010; da Fonseca et al., 2011). Although there is clear evidence that Cdc20 and Cdh1 activate the APC/C in a cell cycle-regulated manner and

confer substrate specificity to the ligase (Burton and Solomon, 2001; Burton et al., 2005; Hilioti et al., 2001; Kraft et al., 2005; Pfleger et al., 2001b; Schwab et al., 2001), some degree of substrate-binding has also been attributed to apo-APC/C. First evidence came from a study, where a tandem D-box affinity matrix could isolate the APC/C from *Xenopus* egg extracts where Cdc20 was depleted and which naturally does not contain Cdh1 (Yamano et al., 2004). It has also been reported that substrates like Nek2A are targeted to the APC/C via a C-terminal MR motif (Hayes et al., 2006), an interaction that is independent of Cdc20 and reminiscent of the IR-tail-dependent binding of co-activators and Doc1 to the APC/C core (Vodermaier et al., 2003; Wendt et al., 2001). Doc1/Apc10 is a good candidate for being a substrate-receptor at the APC/C core, since yeast Doc1 is required for D-box-dependent and processive substrate degradation in an *in vitro* ubiquitylation reaction whereas APC/C lacking Doc1 can no longer bind substrate but is still able to bind the co-activator proteins (Carroll et al., 2005; Carroll and Morgan, 2002; Passmore et al., 2003). Electron microscopy studies on yeast and human APC/C now confirmed that Doc1 directly binds to the TPR subunits Cdc27/Apc3, Cdc16/Apc6, Apc1 and the Apc2 catalytical subunit. This interaction helps to mediate optimal substrate binding by forming a bipartite substrate receptor with the co-activator protein, engaging Doc1´s ligand binding region and the WD40 domain of the co-activator proteins (Buschhorn et al., 2010; Da Fonseca, 2011). This cooperatively substrate binding mechanism between the co-activator proteins and the APC/C is further strengthend by the observation that although substrates can directly associate with the APC/C, their binding selectivity and affinity is reduced in absence of co-activators (Eytan et al., 2006); (Matyskiela and Morgan, 2009; Passmore and Barford, 2005; reviewed by Yu, 2007).

1.10.3 Regulation of APC/C activity by inhibitory proteins

Another principle for controlling the activity of this complex ligase is by association with inhibitory proteins. As described in the previous chapters, binding of Cdc20 and Cdh1 is in part regulated through phosphorylation events on both, the activators and the APC/C core. Cdc20 can only bind to the phosphorylated form of the APC/C, whereas phosphorylation of Cdh1 prevents its binding to the complex. Activation of APC/C^{Cdh1} is hence only induced

after APC/C^{Cdc20}-mediated degradation of mitotic cyclins which leads to reduced Cdk activity in early mitosis and in G1. Later in the cell cycle, APC/C^{Cdh1} is inactivated at the G1/S boundary due to the increasing Cdk activity and Cdh1 phosphorylation, leading to its dissociation from the APC/C (Kraft et al., 2003). However, Cdk activity is also low in G2 phase. Moreover, overexpression of the co-activators *in vivo* can activate the APC/C at any cell cycle stage, indicating that mitotic APC/C phosphorylation is not sufficient to explain the timing of APC/C activity (Schwab et al., 1997; Visintin et al., 1997). Several studies suggest that other mechanisms regulate APC/C^{Cdh1} activity in interphase. In budding yeast, for example, the protein Acm1 (APC/C^{Cdh1} modulator 1) in complex with Bmh1/Bmh2 was found to associate with Cdh1 from late G1 until late M phase (at cell cycle stages when APC/C^{Cdh1} activity is absent) inhibiting APC/C^{Cdh1}-dependent proteolysis of mitotic cyclins (Martinez et al., 2006). Acm1´s inhibitory activity is dependent on pseudosubstrate regions within its sequence, including minimal D-box and KEN-box binding sites that function by competitively inhibiting binding to other APC/C targets (Enquist-Newman et al., 2008). Acm1 is targeted for ubiquitylation by APC/C^{Cdc20} in anaphase. Therefore, Cdc20 not only promotes Cdh1 activation through the destruction of mitotic cyclins and less directly through activation of the phosphatase Cdc14, resulting in Cdh1 dephosphorylation and activation, but also through destruction of the Cdh1 inhibitor Acm1, making the activation process more robust (Enquist-Newman et al., 2008). Moreover, Cdh1 dephosphorylation triggers APC^{Cdh1} activation that is sufficient for Acm1 ubiquitilation and destruction. Therefore, APC/C^{Cdh1} can promote the destruction of its own inhibitor (Enquist-Newman et al., 2008). The regulatory mechanism of pseudosubstrate inhibiton of APC/C is reminiscent of other proteins, such as the fission yeast Mes1, a meiosis-specific APC/C inhibitor which itself is a substrate and competes for ubiquitylation with other APC/C targets (Izawa et al., 2005).

In vertebrate cells, the E2F-dependent expression of cyclin A and its assembly with Cdk2 prevents activation of APC/C^{Cdh1} through inhibitory phosphorylation of Cdh1, thus allowing accumulation of APC/C targets such as cyclin B1, leading to S phase entry and progression (Lukas et al., 1999). Since cyclin A itself is a target of APC/C^{Cdh1}, the question arises how cyclin A can accumulate to inactivate Cdh1. This was shown to be mediated by the pseudosubstrate inhibitor Emi1 (early mitotic inhibitor 1) which was found to inactivate APC/C^{Cdh1} at the G1-S transition in vertebrates, allowing accumulation of cyclin A (Hsu et

al., 2002). In *Xenopus* egg extracts, Emi1 has been reported to prevent substrate binding to both APC/C^{Cdc20} and APC/C^{Cdh1} by directly associating with the co-activators (reviewed by Baker et al., 2007; Reimann et al., 2001a; Reimann et al., 2001b). Inhibition of APC/C^{Cdc20} by Emi1 happens prior to mitosis, allowing accumulation of cyclin B, driving the cell into mitosis (Reimann et al., 2001a). Emi1 has also been reported to inhibit APC/C^{Cdc20} activity in prophase, allowing activation of Cdk1 by cyclin A leading to phosphorylation of the APC/C and thus to association with Cdc20, prior to cyclin A ubiquitylation in prometaphase. Thus, Emi1 has to be degraded in early mitosis to ensure transition from early to late prophase and to allow activation of APC/C^{Cdc20} (reviewed by Baker et al., 2007; Guardavaccaro et al., 2003; Margottin-Goguet et al., 2003). Like cyclin A, Emi1 is transcriptionally induced by the E2F transcription factor at the G1-S transition (Hsu et al., 2002) and phsophorylation of Emi1 by Plk1 in prophase targets it for $SCF^{\beta TrCP1}$-dependent ubiquitylation and subsequent degradation (Hansen et al., 2004; Moshe et al., 2004), which is required for the destruction of cyclin A and cyclin B (Guardavaccaro et al., 2003; Margottin-Goguet et al., 2003). The C-terminal region of Emi1 exhibits a D-box motif, which mediates binding to both Cdh1 and the APC/C core, thereby competitively preventing substrate binding to the APC/C *in vitro*. In addition, Emi1 contains a conserved zinc-binding region (ZBR) which antagonizes APC/C E3 ligase activity independently of tight APC/C binding through sterical hindrance of substrate binding to the APC/C. Mutation of this ZBR renders Emi1 into a D-box-dependent APC/C^{Cdh1} substrate (Miller et al., 2006). The highly conserved meiosis-specific homolog of Emi1, Erp1/Emi2, which also contains the D-box and the ZBR motif, has been shown to inhibit APC/C activity in Meiosis II to prevent activation of cyclin destruction in unfertilized eggs (Schmidt et al., 2006; Tung et al., 2005). In *D. melanogaster*, the Emi1 homolog Rca1 (regulator of cyclin A1) specifically inhibits APC/C^{Cdh1} activity allowing cyclin A accumulation in G2 (Grosskortenhaus and Sprenger, 2002). Emi1 provides a good example of how E3 substrates evolved to become pseudosubstrate inhibitors by combining conserved degron sites with a catalysis-inhibitory function (Miller et al., 2006).

In early mitosis, the messenger RNA export factor Rae1 interacts with the nucleoporin Nup98 to form a complex that specifically binds to Cdh1 and inhibits APC/C^{Cdh1}-mediated ubiquitylaton of securin but not cyclin B (Jeganathan et al., 2005). Release of the Rae1/Nup98 complex from APC/C^{Cdh1} coincides with the release of the mitotic checkpoint complex

protein BubR1 from APC/C^{Cdc20} at the metaphase to anaphase transition. APC/C^{Cdc20} specifically targets cyclin B for ubiquitylation and since Rae1/Nup98 specifically inhibits securin degradation, it is tempting to speculate that the release of this complex from APC/C^{Cdh1} contributes to the right timing of anaphase initiation (reviewed by Baker et al., 2007; Jeganathan et al., 2005). However, the mechanism of the synchronized release with the MCC component BubR1 is not clear.

A further mechanism of APC/C^{Cdh1} inhibition already briefly discussed is that of the E2 UbcH10, which promotes its autoubiquitylation and degradation at the end of G1. This in turn allows cyclin A to accumulate and phosphorylate Cdh1, leading to APC/C^{Cdh1} inhibition (Rape and Kirschner, 2004). Also, the Mad2-like protein Mad2B was identified as an APC/C^{Cdh1} inhibitor *in vitro and in vivo* (Chen and Fang, 2001; Pfleger et al., 2001a). It has been reported that Mad2/Mad2B inhibit APC/C activation by Cdc20/Cdh1 *in vitro*, but neither can inhibit preactivated APC/C complexes, although they are capable of forming a ternary complex with activator and APC/C (Chen and Fang, 2001). However, Emi1 can inhibit APC/C that has already been activated by Cdc20 or Cdh1, and it has been suggested that it fulfills its inhibitory function by preventing substrate binding to the co-activator and not through interfering with the enzymatic core components Apc2/Apc11 (Reimann et al., 2001b).

As mentioned earlier, meiotic APC/C in budding yeast is not only regulated by Cdc20 and Cdh1 but also by the protein Ama1 (Blanco et al., 2001; Chu et al., 1998). Activity of APC/C^{Ama1} is selectively inhibited in early meiosis by the constitutive APC/C subunit Mnd2 (Oelschlaegel et al., 2005; Penkner et al., 2005). Meiosis is a specialized process, which gives rise to haploid cells that originate from a diploid progenitor. The two main differences in meiosis that differ from the mitotic cell division are the establishment of chiasmata between homologous chromosomes followed by two consecutive nuclear divisions, separating first homologous chromosomes and then sister chromatids (Penkner, 2005). Therefore, some cohesin has to be protected at kinetochores from cleavage in metaphase I (MI) (reductional segregation) to allow another chromosome alignment in metaphase II (MII) (equational segregation) that separates each sister chromatid, resulting in a haploid cell (Morgan, 2007; Penkner et al., 2005). Persistence of sister chromatid cohesion after MI is especially

important, since the first nuclear division is preceded by an extended prophase, which can last for several decades in the case of human oocytes (Morgan, 2007; Oelschlaegel et al., 2005). The protease separase (Esp1 in budding yeast) cleaves cohesin once all chromosomes are correctly aligned on the metaphase spindle, resulting in metaphase to anaphase transition. Separase activity depends on APC/C-mediated destruction of its inhibitory binding partner securin (Pds1) (Cohen-Fix et al., 1996; Funabiki et al., 1996; reviewed by Harper et al., 2002; Uhlmann et al., 1999). In meiosis, cohesion that gets established during pre-meiotic S phase, mediates two rounds of chromosome segregation (Oelschlaegel et al., 2005). Another protein, shugoshin (Sgo1) was identified to protect cohesin at kinetochores in meiosis I and disappears from centrosomes at the onset of anaphase II (Katis et al., 2004; Kitajima et al., 2004; Marston et al., 2004; Rabitsch et al., 2004). The budding yeast APC/C subunit Mnd2 has been shown to prevent APC/C^{Ama1}-dependent Pds1 and Sgo1 degradation in meiotic prophase (Penkner et al., 2005). Ama1 and Mnd2 are both upregulated in meiosis (Cooper et al., 2000; Rabitsch et al., 2001) and Mnd2 is stably associated with APC/C subunits in both, mitotic and meiotic cells (Hall et al., 2003; Penkner et al., 2005; Yoon et al., 2002). Similar to the APC/C-subunits Cdc16, Cdc27, and Cdc23, Mnd2 becomes strongly phosphorylated in mitosis (Torres and Borchers, 2007). This modification does not seem to affect mitotic progression and it is not important for binding to the APC/C, but it has been shown to be important for the APC/C^{Ama1}-inhibitory function of Mnd2 in meiosis (Torres and Borchers, 2007). Mnd2-mediated APC/C^{Ama1} inhibtition is partly regulated by degradation of the protein that occurs late in meiosis after anaphase II, a process which may require Mnd2 phosphorylation (Oelschlaegel et al., 2005; Penkner et al., 2005). Although Mnd2 is present in equal abundance throughout the cell cycle, there is no evidence for an inhibitory function of Mnd2 towards the co-activators Cdc20 and Cdh1 (Penkner et al., 2005). Budding yeast Mnd2 may also play a role in mitosis since anaphase entry is delayed in a Mnd2 deletion strain as shown by accumulation of G2 and M phase cells. However, in contrast to its function in meiosis, the Mnd2 protein does not seem to be essential for mitotic progression (Hall et al.).

1.10.4 Inhibition of APC/C activity by the spindle assembly checkpoint

Checkpoint controls ensure correct cell cycle transitions dependent on the completion of earlier events. In early mitosis, APC/C^{Cdc20} activity is regulated by the spindle assembly checkpoint (SAC), a ubiquitous surveillance mechanism that links APC/C-mediated degradation of mitotic regulators to the chromosome cycle.

The SAC functions in prometaphase to ensure correct anaphase onset by inhibiting APC/C^{Cdc20}-mediated degradation of securin and cyclin B, until all sister chromatids are aligned at the metaphase plate and captured by microtubules in a bipolar manner. The SAC remarkably senses one single unattached kinetochore, and produces a diffusible "anaphase wait" signal to prevent precocious sister chromatid separation and hence aneuploidy (reviewed by Musacchio and Hardwick, 2002). It is believed that the SAC senses the lack of microtubule attachment at kinetochores and the lack of tension between sister centromeres caused by incorrect attachments (reviewed by Musacchio and Salmon, 2007; Pinsky and Biggins, 2005; Stern and Murray, 2001). The inhibitory effector of the SAC is the mitotic checkpoint complex (MCC), comprising the proteins Mad2, BubR1, Bub3 and Cdc20. All checkpoint proteins are highly conserved during evolution, which emphasizes their important function in the cell cycle (Musacchio and Hardwick, 2002). In addition to the MCC proteins, the SAC includes many other proteins, such as kinases, motor proteins or structural components. The MCC proteins are localized to unattached kinetochores during mitosis in all organisms that have been examined and are removed once faithful chromosome segregation can occur (reviewed by Musacchio and Salmon, 2007). Microtubules are highly dynamic structures and their attachment to kinetochores results in intermediate attachment states. Checkpoint signaling is activated by non-bipolar attachments which are sensed by lack of stretching within the kinetochore (Maresca and Salmon, 2009; Uchida et al., 2009). If this microtubule-kinetochore interface lacks tension, the attachment gets actively destabilized by Aurora B kinase activity, which recruits the SAC proteins (Ditchfield et al., 2003). Unattached kinetochores then enter a new round of microtubule capture until bipolar attachment is achieved (Lampson et al., 2004; Tanaka et al., 2002). Aurora B is thought to

exert its destabilizing function by phosphorylating Ndc80/Hec1 and Dam1 complexes, proteins that are located at the kinetochore to capture microtubules that emanate from the spindle poles. Phosphorylation of these proteins diminishes their microtubule-binding capacity (Cheeseman et al., 2002; Cheeseman et al., 2006; DeLuca et al., 2006). Aurora B is located at the inner kinetochore and phosphorylation of kinetochore proteins was shown to depend on their distance from this site as these proteins become separated from the kinase upon bipolar attachment (Liu et al., 2009). In cultured cells, the SAC can be activated by spindle poison drugs that effect microtubule dynamics. Agents such as nocodazole interfere with microtubule polymerization and can destabilize microtubules, whereas the drug taxol stabilizes microtubule structures. Both events result in checkpoint signaling by the SAC. However, the effects caused by taxol treatment can be overridden with Aurora B-inhibiting agents, such as hesparadin (Ditchfield et al., 2003; Hauf et al., 2003), which silences the SAC.

It is believed that the effecter complex of the SAC, the MCC, exerts its inhibitory function through direct binding to the APC/C-activator Cdc20, thereby preventing Cdc20 binding to the APC/C (Sudakin et al., 2001). In yeast, Mad2 (mitotic arrest deficient 2) was found to bind to Cdc20 and Mad2-binding deficient Cdc20 mutants become insensitive to the SAC (Hwang et al., 1998; Kim et al., 1998). Mad2 gets recruited to unattached kinetochores by its binding partner Mad1, whereas kinetochores that are fully captured by microtubules do not contain detectable Mad2 (Chen et al., 1996; Shah et al., 2004; Sironi et al., 2001). However, the complete MCC is believed to form in a two-step process, where Mad2-Cdc20 complex formation promotes BubR1-Bub3-Cdc20 interaction (Burton and Solomon, 2007; Davenport et al., 2006; Fang, 2002; Fraschini et al., 2001; Kulukian et al., 2009). Thereby, Mad2 may serve as a template for the assembly of Mad2-Cdc20 complexes, in that Mad2 first has to stably bind to Mad1 at unattached kinetochores (DeAntoni et al., 2005; Luo et al., 2002). This "template model" provides a reasonable explanation of how the inhibitory signal emanating from one single unattached kinetochore can be amplified and how it diffuses to effectively silence the APC/C. Crystal structures of Mad2 bound to a Cdc20-mimicking peptide showed that Mad2 can exist in two structural conformations (Mapelli et al., 2007; Sironi et al., 2001). It can adopt an open form (O-Mad2) or a closed form (C-Mad2). Soluble Mad2 exist predominantly in its open conformational state, but Cdc20 binding is facilitated by the closed form (Mapelli et al., 2007). The implicated mechanism of Mad2 activation towards Cdc20

implies that a C-Mad2 confomer that is stably bound to Mad1 at unattached kinetochores binds an O-Mad2 conformer from the cytosol, which promotes conversion into C-Mad2. This closed conformer can then capture Cdc20 (DeAntoni et al., 2005; Luo et al., 2004; Mapelli et al., 2007; reviewed by Musacchio and Salmon, 2007). Fluorescence recovery after photobleaching (FRAP) experiments could show rapid exchange of kinetochore-bound Mad2, BubR1 and Cdc20 with their cytosolic pools (Howell et al., 2000; Shah et al., 2004).

Like Mad2, the kinase protein BubR1 (budding uninhibited by benomyl related-1) has been shown to directly inhibit APC/C activity by blocking the formation of APC/C^{Cdc20} (Tang et al., 2001a). *In vitro*, BubR1 has been shown to be a more potent inhibitor of APC/C^{Cdc20} than Mad2 (Tang et al., 2001a), but the highest inhibitory potency is achieved through simultoaneous binding of both proteins (Fang, 2002; Fang et al., 1998; Li and Benezra, 1996; Sudakin et al., 2001; Tang et al., 2001a). In vertebrates, Mad2 and BubR1 are believed to act synergistically at physiological concentrations to inhibit APC/C *in vivo* by directly binding to Cdc20 (Sudakin et al., 2001). BubR1 localizes to unattached kinetochores, where it is sensitive for Aurora B activity, but it can also be found on microtubule-occupied kinetochores that lack tension (Ditchfield et al., 2003; Skoufias et al., 2001). Furthermore, the protein directly binds to the kinesin-like motor protein CENP-E, which is located at the kinetochore to promote chromosomal alignment in metaphase (Mao et al., 2003). Direct association of CENP-E with BubR1 activates BubR1 kinase activity, which is necessary for mitotic checkpoint signaling, but dispensible for Cdc20 binding (Mao et al., 2003; Sudakin et al., 2001). Kinetochore enrichment of BubR1 in checkpoint activated cells is dependent on the SAC components Bub1 and Bub3 (Millband and Hardwick, 2002)

Although it is known that the MCC inhibits APC/C^{Cdc20} activity in early mitosis, the underlying mechanism is still not clear. The MCC subcomplexes Mad2-Cdc20 and BubR1-Bub3-Cdc20 could be identified *in vivo* (Kulukian et al., 2009), but the presence of a ternary complex has also been reportet (Sudakin et al., 2001). *In vitro* studies have shown that recombinant Mad2 or BubR1 prevent association of APC/C with its co-activator by binding to Cdc20 (Reimann et al., 2001b; Tang et al., 2001a). However, Mad2 and BubR1 were also found in complex with the APC/C bound to Cdc20, and direct binding of MCC proteins to the APC/C has also been observed (Braunstein et al., 2007; Kallio et al., 1998; Morrow et al.,

2005). Although both Mad2 and BubR1 can directly interact with Cdc20 to inhibit APC/C^{Cdc20} activity *in vitro*, there is increasing evidence that the Mad2-Cdc20 interaction is required for activation of the SAC (Davenport et al., 2006; Hwang et al., 1998; Kulukian et al., 2009). However, this does not rule out the possibility that the BubR1-Bub3 complex may sequester different pools of Cdc20 and thereby may act in a parallel pathway to inhibit APC/C^{Cdc20} (Fang, 2002; Tang et al., 2001a). Budding yeast cells lacking Mad2 or Mad3/BubR1 exhibit differences in their response to microtubule toxins, and their ability to align chromosomes, supporting the notion that Mad3/BubR1 may function differently than Mad2 (Ditchfield et al., 2003; Skoufias et al., 2001). There is increasing evidence that the rate-limiting Mad2-Cdc20 complex promotes formation of the final MCC by recruiting Cdc20 to BubR1, where Mad2 binding is believed to induce a conformational change in Cdc20, thereby facilitating association of Cdc20 to BubR1 (Davenport et al., 2006; Kulukian et al., 2009). Consistent with this, *S. cerevisiae,* Mad2 is required for Cdc20 binding to BubR1 (Hardwick et al., 2000) and Mad2 depletion reduced the amount of Cdc20 bound to BubR1 in SAC-arrested and MG132 treated HeLa cells, but not *vice versa* (Nilsson et al., 2008).

Cryo-EM studies on human APC/C revealed that the MCC binds in vicinity of the subunits Apc2, Apc4, and Apc5 (Herzog et al., 2009). Importantly, the MCC binding site partially overlaps with the Cdc20 binding site on APC/C, which raises the possibility that association of MCC might induce repositioning of Cdc20. Moreover, MCC binding induces structural changes within the APC/C and locks the otherwise flexible complex in a “closed” state, which prevents binding and ubiquitylation of a wide range of substrates (Herzog et al., 2009).

1.10.5 Silencing of the spindle assembly checkpoint

SAC silencing upon correct bipolar attachment of sister chromatids restores APC/C activity. This process has to be fast to ensure that chromosome segregation directly follows SAC inactivation. While progress has been made towards understanding the mechanism by which the spindle checkpoint inhibits APC/C in response to spindle defects, the mechanism underlying checkpoint silencing is still not fully understood. SAC inactivation requires that the respective proteins are removed from the kinetochore, which has been shown to depend on

dynein motility along microtubules (Howell et al., 2001; reviewed by Musacchio and Salmon, 2007). Checkpoint components like Mad2 and BubR1 are localized at unattached kinetochores, hence it is possible that microtubule attachment and increasing kinetochore tension leads to dissociation of Mad2, which would also decrease the efficiency of Mad2-Cdc20 complex formation (Howell et al., 2000). However, recent studies suggest that MCC dissociation and therefore SAC silencing is assisted by an active mechanism. Structural studies on the Mad1-Mad2 and Mad2-Cdc20 complexes have revealed that the dissociation of these complexes require the partial unfolding of the C-terminal region of Mad2 (referred to as the "safety belt" mechanism), which imposes a significant energetic barrier on these processes (Luo et al., 2002; Sironi et al., 2002), suggesting the existence of an active mechanism. Intriguingly, this implies that other factors might exist that facilitate the disassembly of Mad2-Cdc20 containing complexes upon checkpoint silencing.

The human Mad2-binding protein $p31^{comet}$ (formerly known as Cmt2; Caught by MAD Two) might be part of one such active mechanism for checkpoint silencing (Habu et al., 2002; Xia et al., 2004). $P31^{comet}$ selectively binds to the closed Mad2 conformer and it can effectively compete with O-Mad2 for binding C-Mad2, since this interaction is stronger than that of O-Mad2. Thereby, $p31^{comet}$ prevents the dimerization of C-Mad2 with O-Mad2 (Mapelli et al., 2006; Vink et al., 2006), which is the catalyzing step in MCC formation based on the "template model". $P31^{comet}$ can also interact with C-Mad2 bound to Cdc20 and association of endogenous $p31^{comet}$ to Mad2 coincides with Mad2-Cdc20 dissociation (Habu et al., 2002). It has been proposed that during checkpoint inactivation in HeLa cells, $p31^{comet}$, Mad2 and Cdc20 transiently form a ternary complex *in vitro* and *in vivo*, which is believed to stimulate APC/C activity and to promote mitotic exit (Xia et al., 2004). Thereby, $p31^{comet}$ seems to be required for efficient checkpoint silencing established by extensive spindle damage by counteracting the APC/C inhibitory activity of Mad2 (Vink et al., 2006; Xia et al., 2004). However, $p31^{comet}$ is not sufficient to break up the Mad2-Cdc20 interaction. Therefore, it is possible that it is not directly involved in the disassembly of the Mad2-Cdc20 inhibitory complex. This suggests that $p31^{comet}$ might collarborate with other factors to promote activation of APC/C^{Cdc20} (Xia et al., 2004). It is also noteworthy that despite the high conservation of MCC components from yeast to humans, $p31^{comet}$ homologues have not yet

been found in *S. cerevisiae* (Habu et al., 2002). This indicates that checkpoint regulation could be rather different in different organisms (Mapelli et al., 2006).

Another study in HeLa cells further proposed that dissociation of Mad2 and BubR1 from Cdc20 is dependent on APC/C-dependent multi-ubiquitilation (Reddy et al., 2007). In nocodazole arrested cells, Cdc20 is multi-ubiquitilated by the APC/C. This process requires the catalytic activity of the APC/C-specific ubiquitin-conjugating (E2) enzyme UbcH10 and is Cdh1-independent (Reddy et al., 2007). Cdc20 ubiquitilation is counteracted by the de-ubiquitilating enzyme USP44 (Stegmeier et al., 2007). Moreover, UbcH10 together with $p31^{comet}$ had a synergistic effect and accelerated the rate of substrate degradation by mitotic APC/C (Reddy et al., 2007). The activity of these proteins may allow mitotic APC/C to promote dissociation of checkpoint proteins, which indcates that the APC/C itself may drive the process of checkpoint silencing (Reddy et al., 2007). Moreover, these observations imply that Cdc20 ubiquitilation by mitotic APC/C is an early mitotic event which may contribute to disossiation of checkpoint components from the APC/C. In particular, Cdc20 ubiquitylation mediated by UbcH10 and $p31^{comet}$ might contribute to actively disrupt the Mad2-Cdc20 inhibitory complex, since C-Mad2 not only dissociated from ubiquitylated Cdc20, but it was also unable to re-bind to the modified co-activator. Dissociation of Mad2-Cdc20 may thereby liberate the APC/C, which can then trigger inactivation of additional Mad2-Cdc20 complexes by ubiquitylating Cdc20, leading to a switch-like metaphase to anaphase transition (Reddy et al., 2007).

However, it is unclear if the activities of UbcH10 and $p31^{comet}$ as well as USP44 in ubiquitylating / deubiquitylating Cdc20 are directly regulated by the SAC.

In contrast to these findigs, studies in yeast propose that Cdc20 turnover is important to maintain the SAC upon spindle damage (King et al., 2007; Pan and Chen, 2004). In this scenario, Cdc20 levels are reduced under a certain threshold to prevent premature activation of the APC/C by Cdc20 (Pan and Chen, 2004). Thereby, the SAC may be composed of a dual control mechanism. First, unattached kinetochores stimulate binding of Cdc20 to the MCC, which prevents premature APC/C activation. Second, the SAC mediates Cdc20 degradation to keep the available Cdc20 low until bipolar attachment of microtubules is achieved (Pan and Chen, 2004). Furthermore, these studies showed that yeast Cdc20 is mostly bound to Mad3

(the homologue of BubR1 in yeast) in checkpoint activated cells, with only very little Mad2 (Kulukian et al., 2009; Pan and Chen, 2004) and most of cellular Mad2 was found as a monomer, which is less capable of binding to Cdc20 (Luo et al., 2004). Moreover, fission yeast Mad3 is essential for Mad2 to block cells in mitosis (Millbrand and Hardwick, 2002). This indicates that the more prominent APC/C-inhibitory complex is Mad3-Bub3-Cdc20 and not Mad2-Cdc20. However, binding of Cdc20 to BubR1 still requires previous binding of Cdc20 to Mad2 (Davenport et al., 2006).

Given that the SAC components are highly conserved in various organisms, it is plausible that fundamental mechanisms underlying SAC activity may also be well conserved through evolution. Consistent with the observations in yeast, studies in mammalian cells suggest that the SAC is maintained through BubR1-Bub3 presenting Cdc20 to the APC/C as a substrate, which requires previous Mad2-Cdc20 complex formation. Moreover, a form of Cdc20 that could not be ubiquitylated was sufficient to overcome the SAC arrest (Nilsson et al., 2008), indicating that maintainance of the SAC might require Cdc20 ubiquitylation. Cdc20 degradation was further shown to depend on previous binding to Mad2 and BubR1, which implies that the SAC causes Cdc20 to activate its own ubiquitylation by the APC/C (Nilsson et al., 2008). Why this mechanism is important for SAC maintainance in mammalian cells is not clear because overexpression of wild type Cdc20 does not override the SAC, as it is the case in *S. cerevisae* (Nilsson et al., 2008; Pan and Chen, 2004).

Although it is not clear if Cdc20 degradation is important for SAC maintainance or for its inactivation, it is believed that regulation of the SAC requires Cdc20 turnover. Therefore, the mentioned observations raise the interesting question of how the checkpoint proteins can facilitate regulated Cdc20 turnover upon spindle damage. It may be possible, that this is not entirely mediated by the MCC proteins, but that it requires an additional protein that specifically targets Cdc20 in early mitosis. Alternatively, other mechanisms could exist which ensure that early mitotic APC/C^{Cdc20} is exclusively responsible for Cdc20 degradation and not for targeting its prominent mitotic substrates. It may also be possible that Cdc20 is only recognized by the APC/C as a substrate when it is bound to SAC components, as it has been shown that degradation of Cdc20 in budding yeast is dependent on its binding to Mad2 (Pan and Chen, 2004).

1.11 Can the APC/C get any bigger?

Despite its already large size, more components of the APC/C have recently been identified, such as c10orf104/Apc16 (Hutchins et al., 2010; Kops et al., 2010). Recently, the protein encoded by 11ORF51 (chromosome 11 open reading frame 51) has also been linked to the APC/C. The c11orf51 protein (from now on called c11orf51) was first found in an endoribonuclease-prepared siRNA (esiRNA) screen in human cells that aimed to find genes important for cell cycle progression (Kittler et al., 2004). Later studies then suggested that it associates with human APC/C (Hubner et al., 2010).

The endoribonuclease-prepared siRNA (esiRNA) screen performed by Kittler *et al.* in 2004 intented to discover and study genes involved in cell division (Kittler et al., 2004). Endoribonuclease-prepared short interfering RNA´s are generated from cDNA clones by *in vitro* transcription and digestion of the resulting long dsRNA, using bacterial RNase III enzyme or recombinant Dicer enzyme (Kittler and Buchholz, 2005). This generates a heterogeneous pool of siRNAs that target multiple sites on the same target mRNA, leading to less off-target effects and efficient gene silencing (reviewed by Buchholz et al., 2006). Kittler *et al.* generated a genome-scale library of esiRNAs from a sequence-verified complementary DNA collection that initially represented 15,497 genes. Out of these genes, 5,305 esiRNAs were used to screen for genes that are required for cell division in human HeLa cells. By combining a primary high-throughput cell viability screen followed by a secondary, more stringent high content videomicroscopy assay, they could observe severe cell division phenotypes for 37 genes (Kittler et al., 2004). From these 37 genes, C11ORF51 (chromosome 11 open reading frame 51; DKFZP564M082) was one out of seven previously uncharacterized genes. RNAi-mediated depletion of c11orf51 resulted in aberrant spindle formation and cell cycle progression defects in human HeLa cells (Kittler et al., 2004).

This study was the basis for a later clinical trial. From the 37 genes, Olson *et al.* selected 30 and examined them for association between genetic variation and risk of breast cancer in a clinical study. C11ORF51 contained one SNP in a region that suggests a role in gene function and/or influence the expression levels of this gene (Olson et al., 2010). Carriers of the minor allele of this SNP were at 40% increased risk of breast cancer when compared with non-

carriers. Although only one SNP could be assigned to this small gene, it was significantly associated with breast cancer in their population. Notably, they also found SNPs associated with breast cancer in the APC/C subunits-encoding genes CDC16 and CDC27 (Olson et al., 2010).

The first hint that the c11orf51 protein could be linked to human APC/C came one year later, in 2010. The protein was found by mass spectrometry to specifically bind to the APC/C isolated from asynchronous HeLa cells after tandem affinity purification (TAP) experiments using GFP-CDC23 as bait (Hubner et al., 2010).

Independently, we could also find c11orf51 in our APC/C samples. As part of the MitoCheck work (www.mitocheck.org), Björn Hegemann and Jim Hutchins could detect c11orf51 in both, mitotically arrested and asynchronous HeLa cells after Apc3-immunoprecipitations. They had also analyzed APC/C from CDC16-LAP expressing cells. After in solution-digest using trypsin, chymotrypsin or subtilisin, the gene product of C11ORF51 was found by mass spectrometry (see **Figure 1-3**). Importantly, it was never found in purifications of non-APC/C subunits or interactors (Björn Hegemann and Jim Hutchins, personal communication).

condition	digest	c11orf51 # unique peptides	c11orf51 sequence coverage	c11orf51 MS/MS score
log (interphase)	subtilisin	8	45%	345
Noc + BI4834	trypsin	3	37%	69
Noc (mitosis)	subtilisin	7	32%	325
Noc + BI4834	subtilisin	7	32%	319
log (interphase)	trypsin	2	31%	63
Noc + BI4834	chymotrypsin	4	29%	154
Noc + Hesp/MG132	subtilisin	6	28%	283
Noc + Hesp/MG132	chymotrypsin	3	28%	139
log (interphase)	chymotrypsin	3	24%	113
Noc (mitosis)	trypsin	1	23%	22
Noc (mitosis)	chymotrypsin	2	18%	80
CDC16-LAP	trypsin	1	7%	42

Figure 1-3: c11orf51 was found in mitotically arrested and in interphase (log) HeLa cells after Apc3-immunoprecipitations by mass spectrometric analysis. To obtain prometaphase cells with an active SAC, either nocodazole (Noc) alone was used for 18 hours or in combination with 250 mM BI4834 for the last two hours. Nocodazole treatment (18 hours) in combination with 100 nM of the Aurora B inhibitor Hesperadin and 10 µg/ml of the proteasome inhibitor MG132 for the last two hours arrested cells in metaphase with an inactive SAC. The purified proteins were enzymatically digested for mass spectrometric analysis.

Trypsin cleaves after arginin and lysin residues, chymotrypsin cuts after tryptophan, tyrosine, phenylalanine, leucine and methionine and subtilisin is an unspecific cutter (www.mitocheck.org; Bjoern Hegemann, Jim Hutchins and Otto Hudec).

1.12 Aim of this study

APC/C regulation is tightly linked to its composition; both of which are very complex. Biological functions to particular subunits or subunit assemblies could be assigned over the last decade, such as the catalytic core of the APC/C (described in chapter 1.7). However, other APC/C subunits are poorly characterized and we still have no satisfying explanation why this complex is so large in size. Despite its already large composition, improved biochemical / proteomics approaches and sensitive detection techniques could lately identify novel APC/C-associated proteins. For example, the lately identified APC/C-subunit c10orf104/Apc16, has escaped standard detection techniques in the past, perhaps because of Apc16's small size. The same might be true for the c11orf51 protein. As mentioned in the previous chapter , TAP and mass spectrometry studies could reproducibly find that c11orf51 associated with human APC/C (Hubner et al., 2010). Moreover, depletion of c11orf51 by RNAi caused cell cycle defects and genetic variation of C11ORF51 has been correlated with breast cancer formation (Kittler et al., 2004; Olson et al., 2010). However, otherwise this protein remained uncharacterized and the molecular basis for its function was not known. The aims of this study were to characterize the c11orf51 protein and to elucidate its biological function. If c11orf51 specifically interacts with APC/C, it could be a novel subunit, a regulator, an inhibitor or a substrate. It could function as a constitutive binder which is present throughout the entire cell cycle or its association with the APC/C might be cell cycle-regulated. Moreover, the cell cycle defect upon c11orf51 RNAi could be due to a direct effect on APC/C or because c11orf51 is linked to a mechanism that controls APC/C regulation. To characterize c11orf51, we applied biochemical analysis, partly combined with mass spectrometry and negative staining electron microscopy studies. Loss-of-function experiments in combination with immunfluorescence microscopy were performed for phenotypic characterization of c11orf51. Furthermore, iTRAQ labeling (isobaric tags for relative and absolute quantification) of human APC/C and quantitative mass spectrometry were established to resolve the composition of the APC/C during the cell cycle. Future studies can be directed towards the

composition of other APC/C associating proteins using this technique, which might lead to a better understanding of APC/C regulation.

2 Results

2.1 C11ORF51 is evolutionary conserved among metazoans

Several studies have suggested a role for C11ORF51 during the eukaryotic cell cycle. Interestingly, the encoded protein was found to associate with human APC/C (Hubner et al., 2010; Kittler et al., 2004; Olson et al., 2010). We first asked if homologues of this protein exist in other species. C11ORF51 encodes a hypothetical protein (it will be called c11orf51 throughout this thesis) comprising of 121 amino acids. Bioinformatic analysis revealed that c11orf51 is remarkably conserved among different species, ranging from fungi to humans, although the polypeptides show higher degree of conservation among metazoan species, such as frog, fish, mouse, etc. (in collaboration with Maria Novatchkova, IMP, Vienna) (see **Figure 2-1**). This implies that c11orf51 might play an important role in eukaryotic cells. The alignment was performed by predicting compositional bias (Promponas et al., 2000). Interestingly, it suggests that this protein is a distant homologue of the meiosis-specific APC/C-inhibitor Mnd2 in *S. cerevisiae*. Mnd2 had previously only been identified in budding yeast as an inhibitor of the meiosis-specific APC/C^{Ama1}. However, a homologue of Mnd2 has not been identified to date. C11orf51 is only 14.3 kDa in size and it is predicted to be largely unstructured. Especially its C-terminal part can be considered to be a low complexity region. With a high degree of uniformity, several bioinformatic methods (quick2d) have predicted a helical structure and a small β-sheet at the N-terminus of c11orf51, which are marked in grey above the aligned sequences (**Figure 2-1**). Prediction of other structural motifs is rather uncertain. In addition, using the Meta protein disorder prediction-Server (metaPrDOS; http://prdos.hgc.jp/cgi-bin/meta/top.cgi) (Ishida and Kinoshita, 2007) confirms the bioinformatic analysis that this protein is largely unstructured.

One striking feature of c11orf51 is the acidic stretch at its C-terminus containing several aspartic acid (D) and glutamic acid (E) residues. However, the lengths of these acidic residues differ among different species. Higher metazoans contain a larger array, whereas in plant and fungi it is significantly shorter.

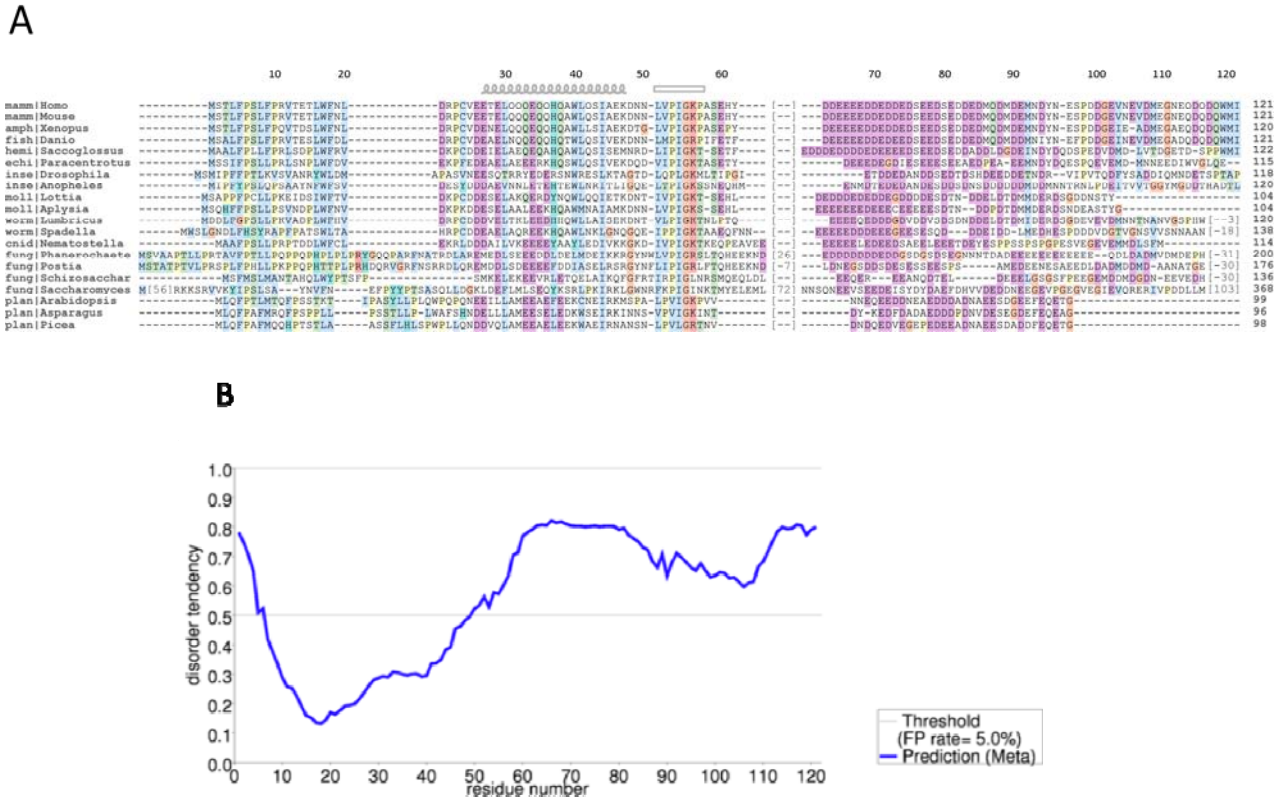


Figure 2-1: Sequence alignment of chromosome 11 open reading frame (C11ORF51). (A) C11ORF51 is evolutionary conserved among metazoans, ranging from fungi to humans. Bioinformatic analysis revealed that c11orf51 is largely unstructured, except for its N-terminal part where it contains an α-helix and a small β-sheet (marked in gray on the top of the alignment). The C-terminus is composed of an array containing aspartic acid and glutamic acid residues. For better comparison with (B), amino acid residue numbers are marked on top of the alignment. (B) Structural disorder prediction analysis of the c11orf51 protein using the metaPrDOS server. Threshold is set at a disorder tendency rate of 0.5.

2.2 The protein encoded by C11ORF51 associates with human APC/C *in vivo*

Hubner *et al.* could identify c11orf51 as a protein associated with human APC/C after immunoprecipitation experiments using CDC23 as bait (Hubner et al., 2010) (see chapter 1.11). We also identified c11orf51 in several immunopurified APC/C samples from both asynchronous and mitotic population of HeLa cells by mass spectrometry (**Figure 1-3** and

chapter 1.11). Theses experiments had been carried out independently of the Hubner *et al.* study by the MitoCheck project, but because only a single c11orf51 peptide was identified in these experiments, c11orf51 was initially not included in the list of APC/C associated polypeptides (Hutchins et al., 2010). In order to confirm our APC/C IP result, we performed reciprocal IP experiments, using C11ORF51 as the bait in a cell line that expresses a LAP tagged form of either the human or mouse c11orf51 protein.

These constructs contain either a LAP-tag at the C-terminus or a FLAP-tag at the N-terminus of the protein. The LAP-tag is depicted in **Figure 2-2**. It is composed of a GFP-tag and S-peptide sequences, which allows performing tandem affinity purification (TAP). To identify c11orf51-associating proteins, I performed tandem affinity purification combined with mass spectrometric analysis (in collaboration with Otto Hudecz and Karl Mechtler, IMP, Vienna). TAP was performed with each of the four different constructs expressing mouse or human c11orf51 (mouse/C-LAP; mouse/N-FLAP; human/C-LAP; human/N-FLAP) from asynchronous human HeLa Kyoto cells. Mass spectrometric analysis of the eluates indicated that the C-terminal LAP-tagged human c11orf51 (hc11orf51-LAP) could co-purify the majority of human APC/C subunits (**Figure 2-2**), whereas no APC/C was detected using the other three constructs. The co-activator Cdc20 was also found. C11orf51 could be found with one peptide hit after tryptic in-solution digestion. The captured peptide sequence is marked in red (**Figure 2-2**). However, the protein amount purified after TAP for MS/MS analysis was below detection limit for silver stain (data not shown). This result indicates that c11orf51 associates with human APC/C *in vivo*. **Figure 2-2** shows that HeLa interphase cells expressing hc11orf51-LAP can be stained with GFP antibodies in immunofluorescence microscopy experiments, whereas HeLa cells not expressing hc11orf51-LAP can not. However, GFP signal could be detected in both the nucleus and the cytoplasm, whereas APC/C is thought to be present predominantly in the nucleus. It is therefore not clear if all hc11orf51-LAP is associated with the APC/C.

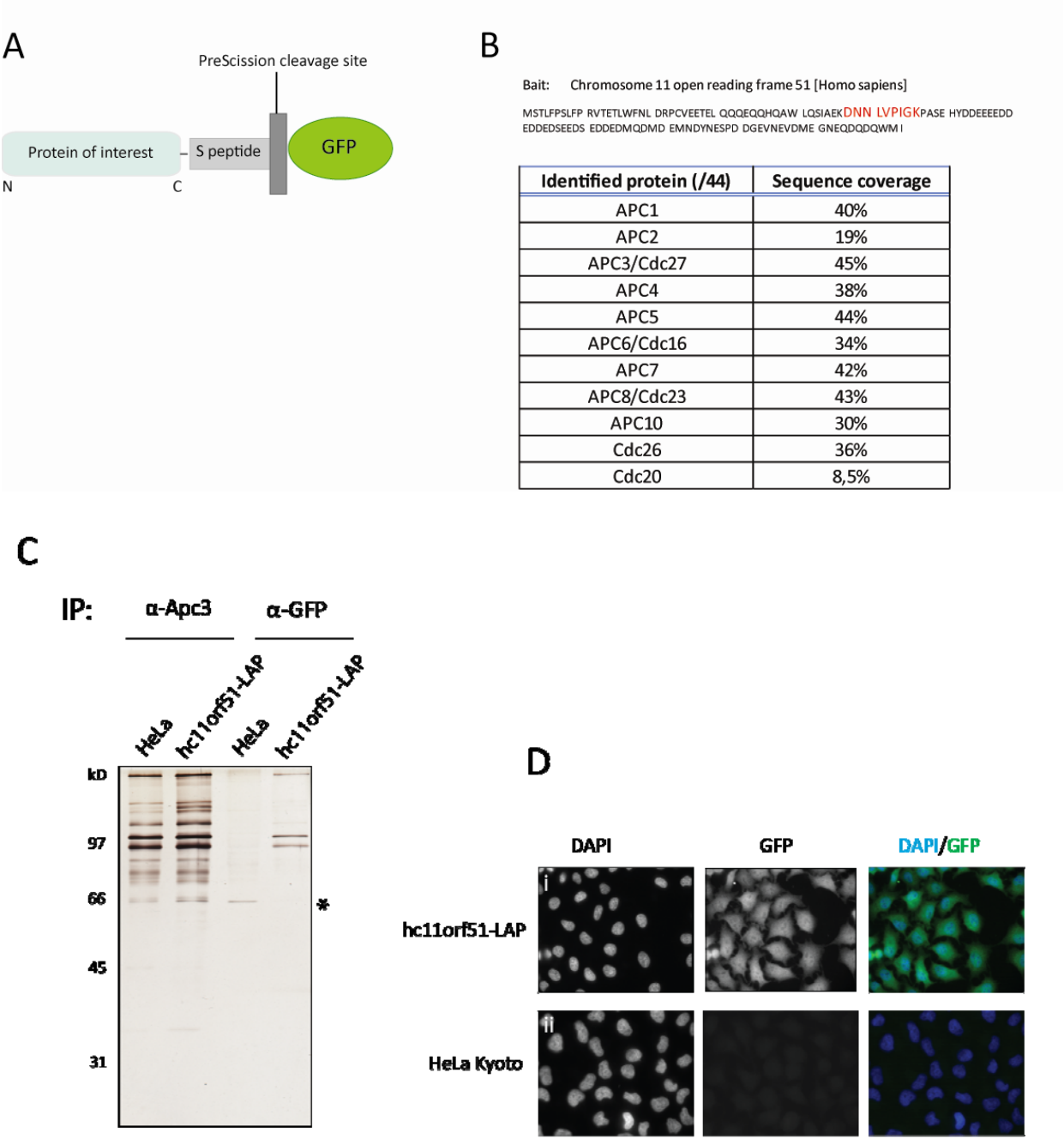


Figure 2-2: c11orf51 protein associates with human APC/C *in vivo*, as confirmed by mass spectrometric (MS/MS) analysis. (A) Schematic picture of the LAP-tag (33.5 kDa) located at the C-terminus of a protein of interest. The FLAP-tag contains an additional TEV cleavage site after the S-peptide, followed by a flag-tag. The FLAP-tag is not depicted here. (B) Identification and peptide sequence coverage of APC/C subunits after tandem affinity purification followed by MS/MS analysis of LAP-tagged c11orf51. The asterix marks a contaminant band. (C) Silver staining of APC/C subunits after Apc3- or GFP-IP experiments from HeLa Kyoto cells or hc11orf51-LAP expressing HeLa cells. (D) Immunofluorescence microscopy using GFP antibodies and DAPI confirms expression of the c11orf51 protein in hc11orf51-LAP cells.

2.3 C11orf51 antibody generation and antibody testing

In order to recognize the endogenous protein, we raised peptide antibodies against c11orf51. Three rabbits were immunized with a peptide corresponding to a sequence located close to the C-terminus of c11orf51. The respective peptide sequence is depicted in **Figure 2-3**. All three antibodies were coupled to protein A beads for IP-experiments to test their specificity in purifying APC/C from asynchronous HeLa cells. Bound proteins were eluted with glycine and the eluates were analyzed by SDS-PAGE and silver staining. Of the three antibodies, the glycine eluate of antibody number 1006 (1006 G) could IP the largest amount of APC/C from extract of asynchronous HeLa cells, as indicated by silver staining (**Figure 2-3**). Antibody number 1007 G could also purify APC/C, but to a lesser extent. Western blotting with Apc4 and Apc16 antibodies confirmed that c11orf51 antibody 1006 G was most efficient in purifying APC/C from HeLa extracts (**Figure 2-3**). Therefore, antibody 1006 G was used for further IP-experiments.

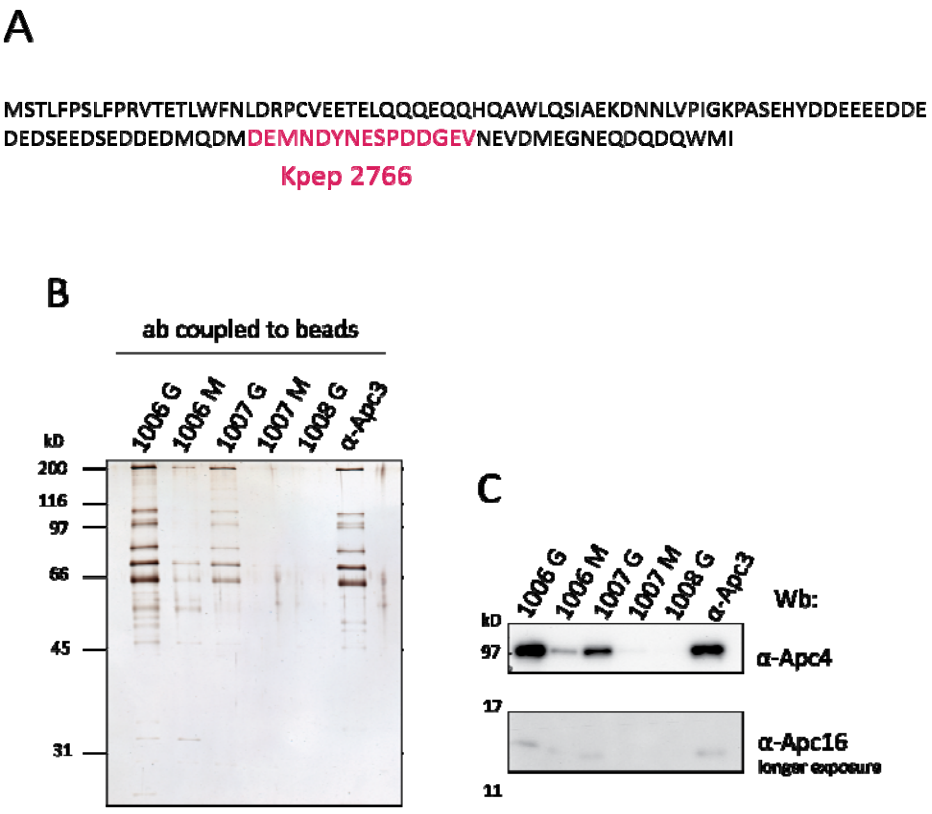


Figure 2-3: Testing c11orf51 antibodies in IP and WB. (A) Protein sequence of human c11orf51 depicting the peptide sequence (marked in red) used to generate the antibody. (B) Silver stain of purified APC/C after immunoprecipitation (IP)-experiments using three different α-c11orf51 antibodies. Glycin (G) and magnesium (M) eluates of purified antibodies (ab) raised against c11orf51 protein. (C) Western blotting analysis of APC/C subunits after c11orf51 IP.

The hypothetical protein encoded by C11ORF51 has a size of 14.3 kDa. To identify the size of c11orf51 protein in immunoblot experiments, I performed Western blots of c11orf51-immunoprecipitated APC/C and from HeLa lysates. The antibodies 1006 and 1007 could both purify APC/C in the previous IP (**Figure 2-3**). Therefore, I also tested their ability to recognize the c11orf51 protein on Western blots. **Figure 2-4** shows that 1006 G and 1007 G could recognize a band at about 20 kDa in the elution after c11orf51-IP and in the HeLa cell lysate.

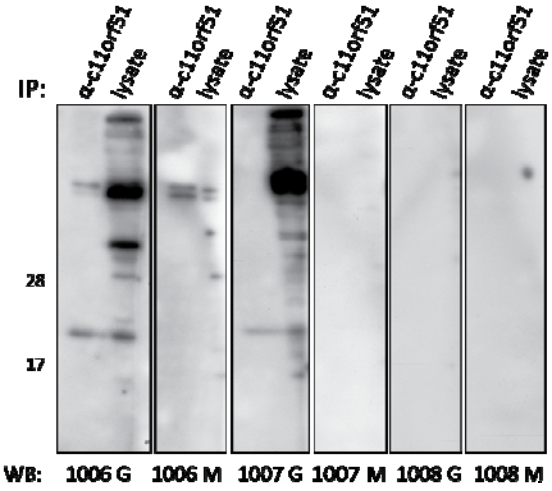


Figure 2-4: Reactivity of different c11orf51 antibodies in Western blot analysis. Antibodies 1006 G and 1007 G could recognize a band at about 20 kDa in eluates after c11orf51-IP and in the lysate. No band could be detected using the 1008 antibody.

To confirm that the 20 kDa band recognized by antibodies 1006 G and 1007 G represents the c11orf51 protein, APC/C was immunopurified using antibodies against c11orf51, Apc3 and GFP from extracts of logarithmically growing HeLa and from c11orf51-LAP-expressing HeLa cells. Western blot analysis of the resultant immunoprecipitates showed that 1006 G could detect both the endogenous and LAP-tagged c11orf51 proteins (**Figure 2-5**, lanes 1, 2, 9 and 10). Consistently, the GFP antibody could detect only the c11orf51-LAP protein (**Figure**

2-5, lanes 4, 8 and 12) and in addition, both 1006 G and α-GFP antibodies detected a product with similar mobility only in the LAP- tagged cell line (**Figure 2-5**, lanes 2, 4, 6, 8, 10 and 12).

In addition, RNAi experiments were performed using esiRNA-mediated decay of c11orf51 transcripts described by Kittler *et al.* (Kittler et al., 2004). HeLa cells were transfected with 1 µg esiRNA for 68 hours and cell lysates were used for immunoblotting. The 20 kDa band detected by c11orf51 antibodies was strongly reduced upon RNAi of c11orf51 (**Figure 2-5**). These observations indicate that the 20 kDa band represents the c11orf51 protein.

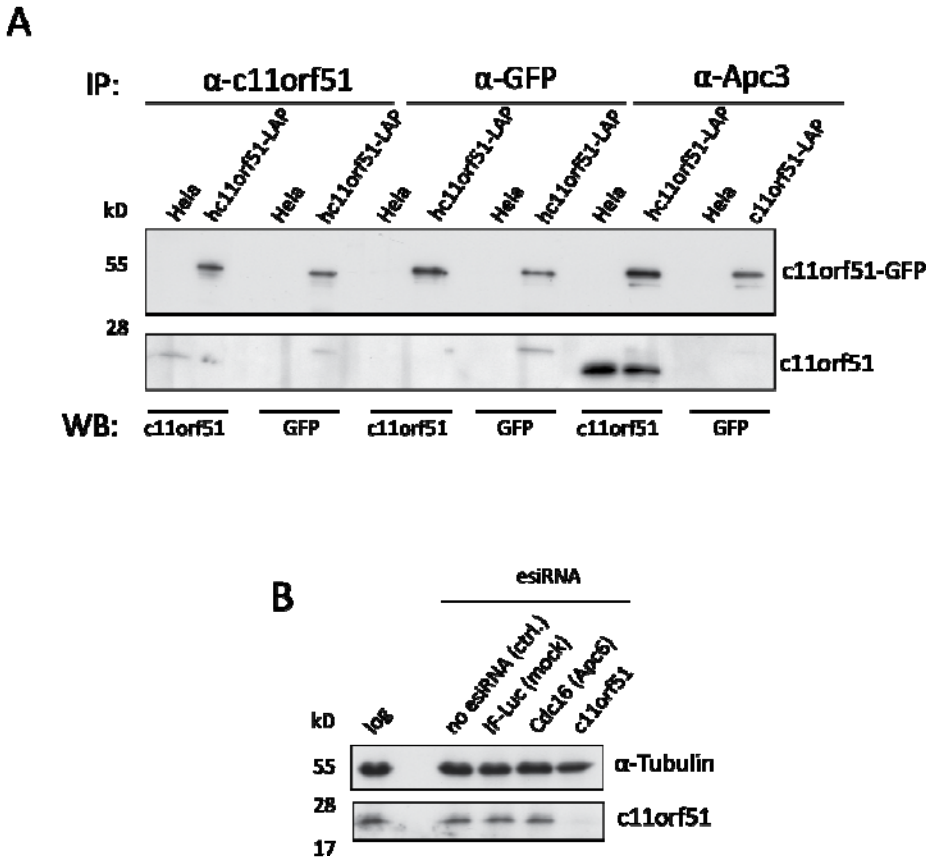


Figure 2-5: Detection of c11orf51 protein product by Western blotting (WB) analysis (A) Western blotting analysis to detect for c11orf51p after APC/C-IP from HeLa and c11orf51-LAP tagged HeLa cells. (B) esiRNA-mediated decay of endogenous c11orf51 transcripts. Controls used were Firefly Luciferase, Apc6 and no esiRNA treatment. α-Tubulin protein levels confirm equal loading.

2.4 The c11orf51 protein associates with human APC/C at all cell cycle stages

To further verify the interaction of c11orf51 with human APC/C, α-c11orf51-beads were used for immunoprecipitation (IP) experiments from extracts of asynchronous (log) HeLa cells. The positive control IP-experiment was performed with Apc3 antibody-coupled beads. Immunoprecipitated proteins were eluted with glycine and analyzed by SDS-PAGE and silver staining.

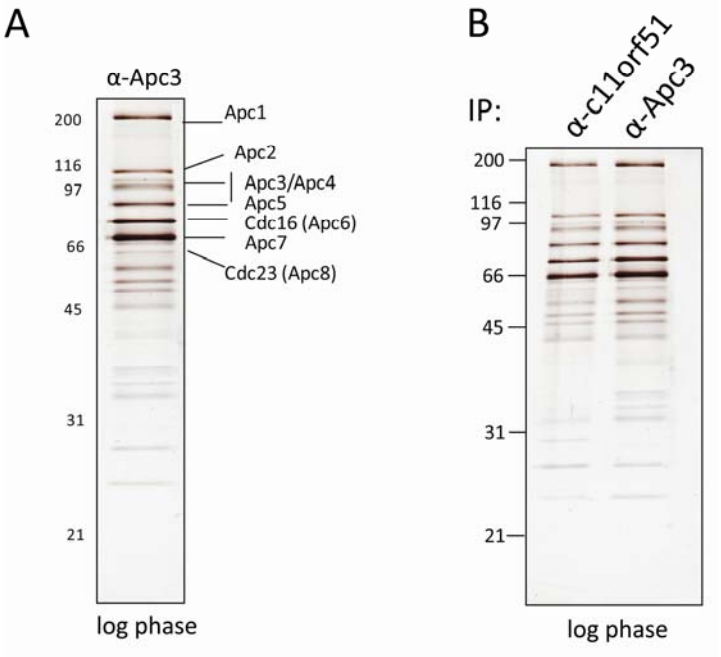


Figure 2-6: C11orf51 antibody coupled protein A beads can immunoprecipitate (IP) APC/C from logarithmically grown (log) HeLa cells. (A) Silver staining pattern characteristic of APC/C subunits after α-Apc3-IP. (B) C11orf51 antibody-coupled beads purify the APC/C in comparable amounts and purity as α-Apc3 antibody, confirming that c11orf51 associates with APC/C *in vivo*.

Taken together, these observations indicate that c11orf51 is a specific interaction partner of human APC/C. As described in chapter 1.10, regulation of APC/C activity is mediated through binding of different proteins to the complex (Baker et al., 2007; Peters, 2006). It was therefore conceivable that c11orf51 is a novel APC/C subunit, a regulator, a substrate or an

inhibitor of the APC/C. The APC/C is inhibited by the MCC during early mitosis. The association of c11orf51 with the APC/C could also be either direct or indirectly mediated by the MCC. The observation that the c11orf51 antibody immunoprecipitated APC/C from interphase cells, where MCC is not associated with the complex (Sudakin et al., 2001), already indicated that c11orf51 is not part of the MCC. To confirm this notion and to further characterize c11orf51, we aimed to see if this protein is associated with the APC/C during all cell cycle stages. Therefore, I performed a cell cycle synchronization experiment, by a double thymidine arrest-release protocol (dTAR) to obtain cells arrested in early S-, G1- and in the G2 states (see Materials and Methods). In order to arrest cells in the prometaphase stage in mitosis, HeLa cells were treated with nocodazole. Efficient cell cycle arrest was confirmed by FACS analysis using PI buffer (**Figure 2-7**). In this experiment, only Apc3 antibody-coupled beads were used to isolate APC/C from lysates and the presence of the c11orf51 protein was analyzed by immunoblotting (**Figure 2-7**). α-IgG beads were used as a negative control. Silver staining allowed direct comparison of the "APC/C pattern" of purified APC/C from all cell cycle stages. Moreover, it served to control the protein loading for further Western blots, and electrophoretic mobility shifts of the APC/C subunits Apc1, Apc3 and Apc8 due to phosphorylation indicated efficient mitotic arrest (Kraft et al., 2003). Furthermore, silver staining confirmed that α-IgG beads did not purify any APC/C (**Figure 2-7**). Nocodazole treatment activates the SAC, therefore MCC proteins are associated with the APC/C in prometaphase cells (see chapter 1.10.4). Consistently, BubR1 and Mad2 exclusively co-purified with APC/C in prometaphase arrested cells. Furthermore, very little Cdc20 was found to co-IP with G1, S and G2 APC/C and on the other hand, significantly higher levels of Cdc20 was observed in prometaphase-arrested cells. This is consistent with the fact that Cdc20 is the co-activator of APC/C during early stages in mitosis, in addition to the fact that it remains bound to MCC upon SAC activation. Therefore, the synchronization protocol works efficiently. Apc4 provides us with a good loading control. Importantly, c11orf51 protein could be detected during all cell cycle stages, indicating that it associates with the APC/C during the entire cell cycle. Furthermore, immunoprecipitation analysis of c11orf51 shows that all APC/C subunits co-immunoprecipitate irrespective of the cell cycle stage. This suggests that c11orf51 is a constitutive APC/C subunit rather than an APC/C regulator which associates with the APC/C only during specific times of the cell cycle.

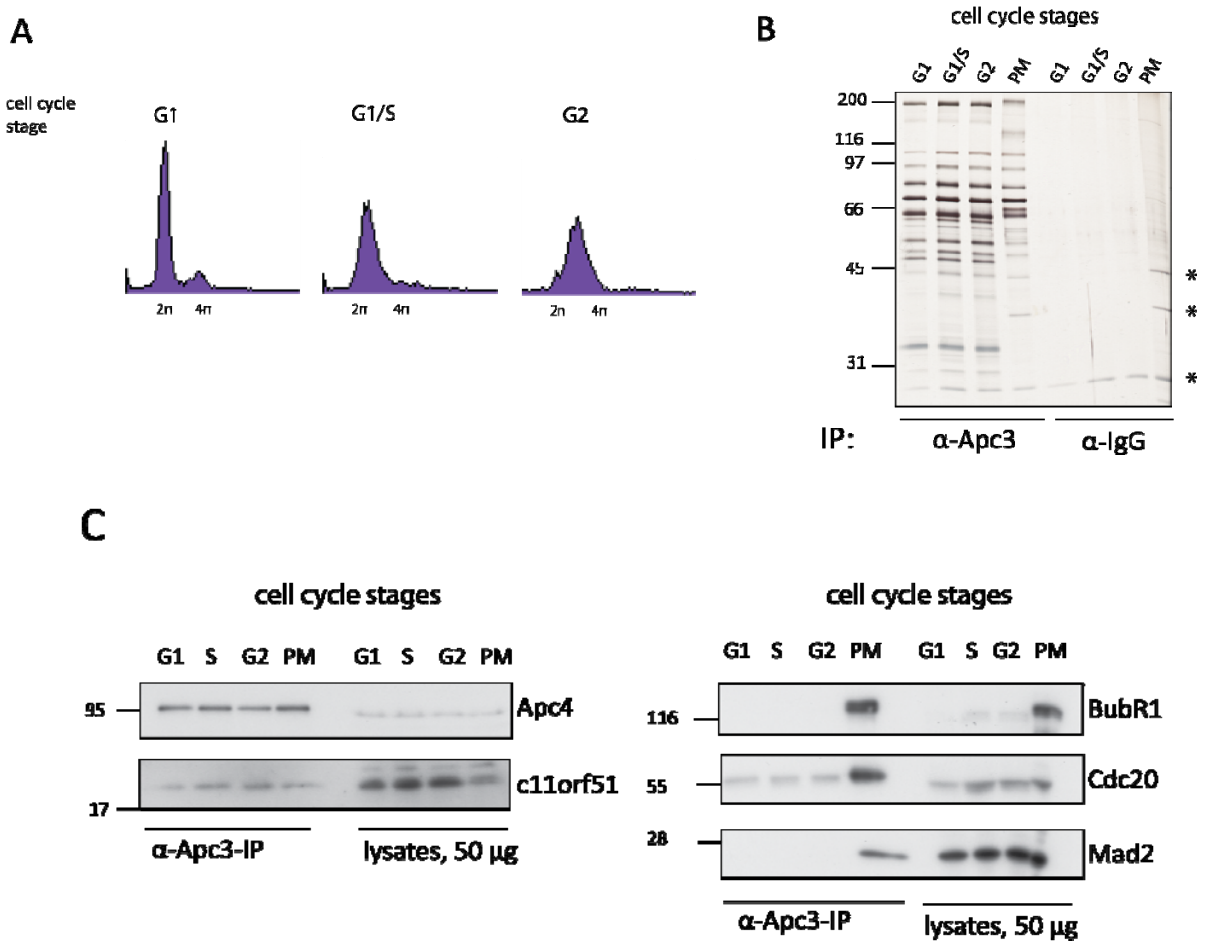


Figure 2-7: C11orf51 protein is associated with the APC/C at all cell cycle stages. HeLa cells were synchronized by double thymdidine arrest release (dTAR) in G1, G1/S, G2 and prometaphase (PM). Immunoprecipitation (IP) experiments were performed using Apc3 antibody-coupled protein A beads to purify APC/C from all cell cycle stages. α-IgG beads served as negative control. (A) The cell cycle arrests in G1, G1/S and G2 were confirmed by FACS analysis using PI buffer (B) Silver stain shows characteristic "APC/C pattern" after Apc3-IP. The asterix mark indicates contaminant proteins. Phosphoshifts for Apc1, Apc3 and Apc8 was observed in mitotic extracts indicating efficient synchronization of HeLa cells. (C) Coimmunoprecipitation of MCC complex - BubRI, Cdc20 and Mad2 in mitotic extracts.

As mentioned in chapter 2.1, c11orf51 protein contains a distinct acidic stretch at its C-terminus. Because this acidic stretch could affect elution by glycine, I compared glycine elution with that of peptide elution after c11orf51-IP. As a positive control, I performed an Apc3-IP, which showed no difference between the two methods of elution. However, for the c11orf51-IP, higher amount of protein was released as a result of peptide elution in

comparison to the glycine elution (data not shown). Therefore, in all subsequent experiments, I performed antigenic peptide elution for eluting protein after immunoprecipitation experiments.

2.5 The c11orf51 protein is associated with the active form of human APC/C

In order to address how much of c11orf51 in HeLa cell extracts is associated with the APC/C, sucrose density gradient centrifugation experiments were performed. Immunoblotting with Apc2, Apc4, Apc6 and c11orf51 antibodies revealed that all proteins co-sedimented in the same fractions (**Figure 2-8**).

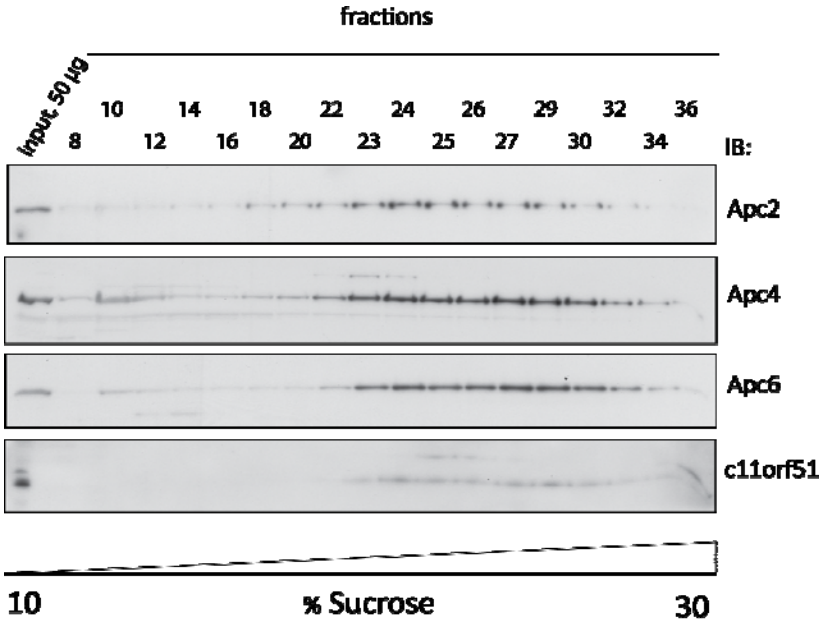


Figure 2-8: C11orf51 co-sediments with the APC/C. Sucrose density gradient centrifugation of extracts prepared from logarithmically grown HeLa cells. Extracts were sedimented through a 10% to 30% sucrose gradient for 18 hours and fractionated into 36 fractions per gradient. APC/C sedimentation was analyzed by immunoblotting (IB) with Apc2, Apc4, Apc6 and c11orf51 antibodies.

Sucrose density gradient centrifugation indicated that the majority of cellular c11orf51 is associated with the APC/C. However, the intensity of the Western blot signal for c11orf51 after density gradient centrifugation was weak compared to my earlier experiments in which cellular extracts were analyzed, perhaps because the concentration of c11orf51 in the sucrose density gradient fraction was too low. Therefore, it could be possible that the c11orf51 protein is distributed all across the gradient. Another possibility is that the antibody is not as sensitive as other anti-APC/C antibodies in recognizing the c11orf51 protein despite all of the protein being associated with the APC/C. To test if all APC/C is associated with c11orf51, I performed a Re-IP experiment for c11orf51-associated APC/C after a first Apc3-IP experiment from logarithmically grown HeLa cells. To ensure that c11orf51 was depleted from the extract, I used a large excess of beads compared to the input (APC/C).

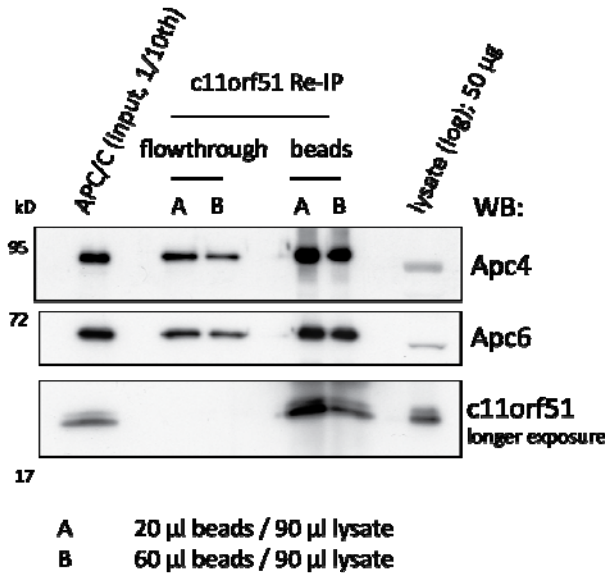


Figure 2-9: Presence of two APC/C populations: c11orf51-bound and -unbound. Native APC/C (input) was purified via Apc3 antibody-coupled beads and was subsequently used for a Re-IP step using c11orf51 antibody-coupled beads. To ensure complete depletion of c11orf51p, beads were provided in excess and the Re-IP step was performed using two different beads to lysate volumes (A and B). SDS-PAGE and Western blotting (WB) was performed on Apc3-immunopurified native APC/C (lane 1), the flowthrough after c11orf51 Re-IP (lane 2 and 3), c11orf51 antibody coupled beads after Re-IP (lane 4 and 5) and lysate from logarithmically (log) grown HeLa cells (lane 6).

Western blotting analysis using c11orf51 antibody confirmed that most c11orf51 bound to α-c11orf51-beads since no c11orf51-specific band could be observed in the unbound fraction

(panel 3, lane 2 and 3). However, some APC/C could be detected in the unbound fraction after c11orf51 Re-IP in Western blot using Apc4 and Apc6 antibodies (panel 1 and 2). This indicates that not all APC/C molecules are associated with c11orf51 and that c11orf51 is limiting in amounts in comparison to other APC/C subunits. Whether or not this has a functional implication towards APC/C activity remains to be seen. It would therefore be interesting to see the differences in activities of c11orf51-bound and c11orf51-free APC/C populations.

Immunoblotting on samples taken every 90 to 120 minutes within 14 hours after release from a double thymidine arrest showed that the c11orf51 protein is present continously during the cell cycle (**Figure 2-10**). However, c11orf51 signal intensities were slightly increased at some time points in Apc3-IP eluates (lane 4 to 6; mitotic and G1 stage). Further analysis will be required to test whether c11orf51 levels are constant or whether the apparent increase of c11orf51 protein levels is a reproducible observation.

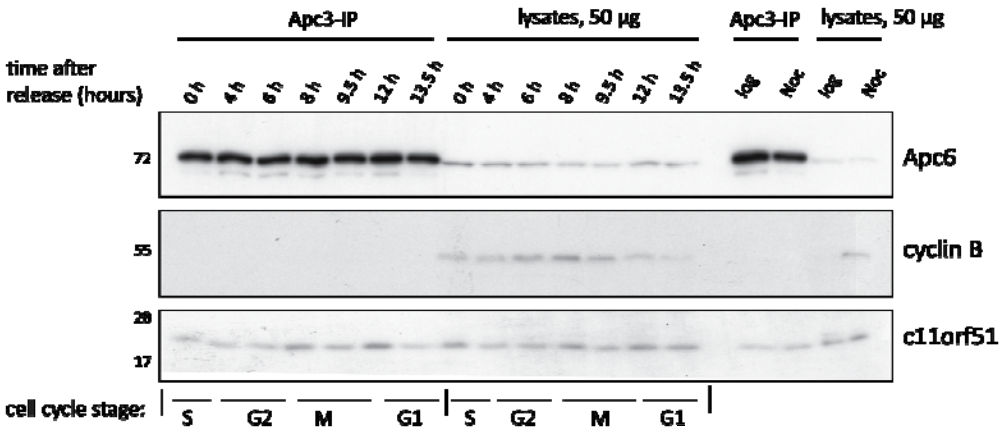
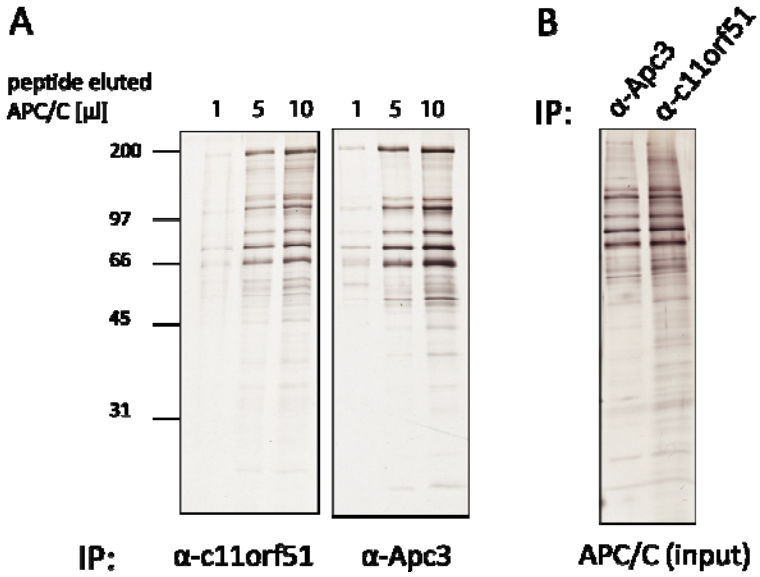


Figure 2-10: Analysis of the c11orf51 protein levels during different cell cycle stages. Cells were synchronized by double thymidine arrest/release protocol and samples were taken at the indicated time points. Apc6 serves as a loading control. Cyclin B levels are low during S (0h to 4h) and G2 (4h to 6h), accumulate as cells reach the mitotic stage (M) at around 8h and decrease again in G1 (12h and 13.5h) due to degradation of cyclin B.

In order to address if c11orf51 is associated with active APC/C, ubiquitilation assays were performed using APC/C that was immunoprecipitated with c11orf51 antibody. APC/C was immunopurified from interphase extracts using c11orf51 antibody (1006 G) or Apc3

antibody-coupled beads. Bound protein was eluted with α-c11orf51 or α-Apc3 antigenic peptide solution to isolate native APC/C. A fraction of the eluate was analyzed by SDS-PAGE and silver staining to adjust for similar amounts of APC/C to be used in the ubiquitilation reactions (**Figure 2-11**). Purified APC/C was used in an *in vitro* ubiquitylation assay, where I^{125}-labeled human cyclin B1 fragment (amino acids 1-87) served as the substrate protein. The phosphorimage depicted in **Figure 2-11** shows that the c11orf51 antibody purifies APC/C from interphase extracts that is similarly active as Apc3-immunoprecipitated complex. Moreover, APC/C activity can be stimulated by addition of the co-activator Cdh1. We conclude that c11orf51 protein associates with the active form of APC/C, which further indicates that c11orf51 protein does not inhibit APC/C ubiquitylation activity towards its mitotic substrates, at least not if APC/C is activated by Cdh1.



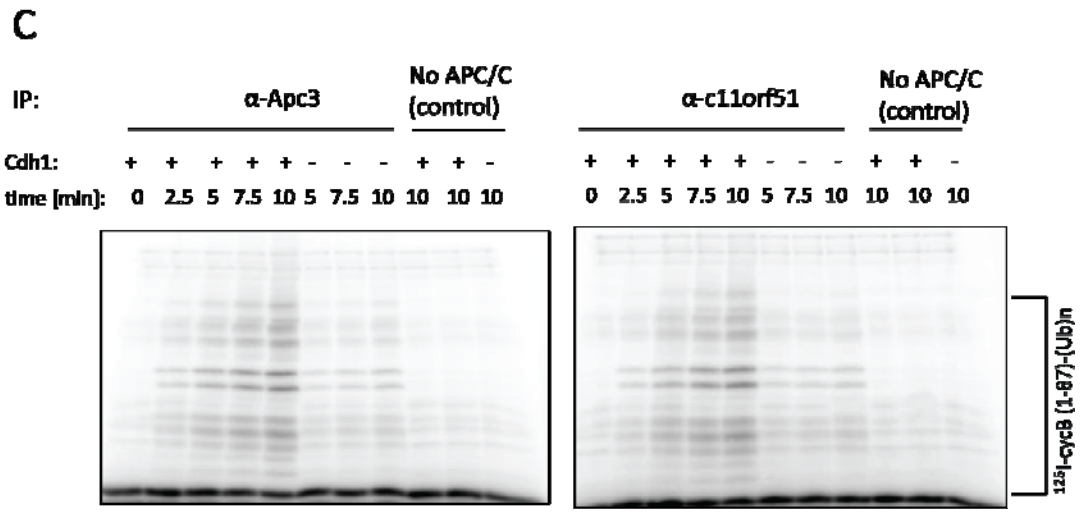


Figure 2-11: Ubquitin-ligase activity of APC/C after immunopurification from interphase extracts using α-APC3 and α-c11orf51 antibodies. (A) Silver staining of the immunopurified APC/C complex. (B) Normalized for APC/C concentration purified using different antibodies before performing the ubiquitilation assays. (C) *In vitro* ubiquitilation assay using [I^{125}]-labeled human cyclin B fragment (amino acids 1 to 87, cycB (1-87)) as model substrate. As controls, the ubiquitilation reaction mix including the antigenic peptide solution without APC/C was used.

2.6 Localization of the c11orf51 protein within the APC/C by electron microscopy

The APC/C performs its enzymatic function through the concerted action of its numerous subunits (Passmore and Barford, 2004). Information about the overall APC/C structure and the 3D organization of its subunits is necessary for understanding the mechanisms of ubiquitylation reactions mediated by this complex. Moreover, localization of c11orf51 protein within the 3D structure of APC/C might help to better understand the role of c11orf51 within APC/C and to interpret interaction studies or experimental observations.

All experiments so far showed that c11orf51 is associated with the APC/C during all cell cycle stages, indicating that it is a constitutive subunit of human APC/C. We used antibody-labeling and negative staining electron microscopy (EM) analysis of human APC/C to address the localization of c11orf51 in the APC/C complex.

Therefore, I isolated human APC/C from asynchronous HeLa cells using Apc3 antibody-coupled beads, followed by peptide elution to obtain functional APC/C. The purified complex was further prepared for EM analysis (in collaboration with Holger Stark, MPI-Göttingen, Germany). To localize APC/C components by antibody labeling, the antibody has to specifically recognize its native epitope. As previously shown in **Figure 2-3**, c11orf51 antibody 1006 G could specifically purify APC/C from HeLa cell lysate, indicating that the 1006 G antibody recognizes its epitope on c11orf51 within the APC/C. Therefore, the antibody 1006 G was used for this experiment. Native APC/C was incubated with decreasing amounts of antibody, starting at a molar IgG to APC/C ratio of 1:2. Theses samples were analyzed by negative staining for the formation of immunocomplexes that contain two APC/C particles (dimers) which are crosslinked via one c11orf51-specific immunoglobulin (**Figure 2-12**). An antibody to APC/C ratio of about 1:5 yielded samples with the highest number of dimerized APC/C. The antibody-mediated APC/C dimer formation confirmed that c11orf51 is a constitutive subunit. Based on the raw image data, we suspect that the c11orf51 protein is located at the platform domain of the APC/C. A more detailed analysis of the localization of c11orf51 within the APC/C by negative-staining EM is currently under investigation (Holger Stark, MPI-Göttingen, Germany).

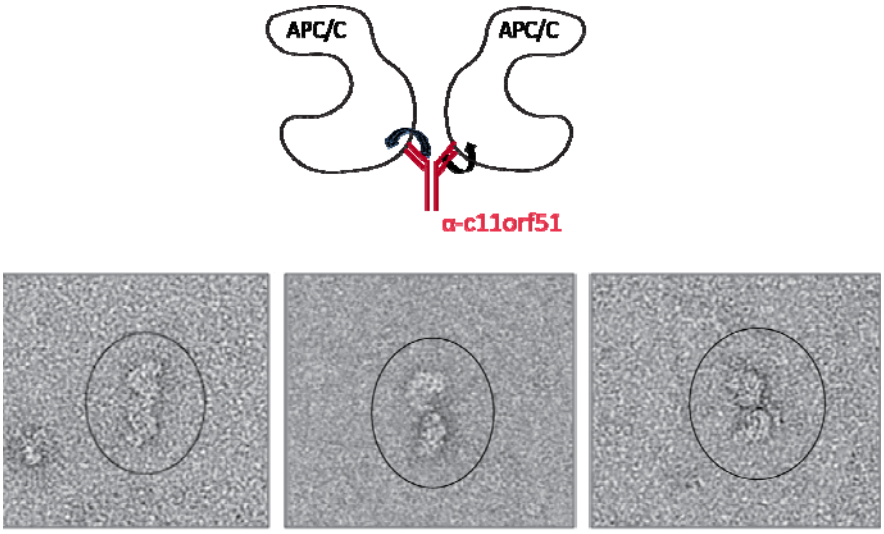


Figure 2-12: c11orf51 antibody labeling and negative staining-electron microscopy of human APC/C. (A) Selected raw images of human APC/C. Native APC/C was incubated with c11orf51 antibody 1006 G at a ratio of IgG to APC/C of about 1:5, resulting in formation of the APC/C-IgG-APC/C complex. Complexes were formed by crosslinking two APC/C

molecules to one c11orf51-specific immunoglobulin. The arrows marked in the drawing on the top represent that the APC/C can rotate at the IgG binding site.

2.7 iTRAQ labeling and quantitative mass spectrometric analysis of human APC/C

The biochemical and EM-structural data so far supports the notion that c11orf51 is a constitutive subunit of human APC/C. However, because it is not possible to obtain quantitative data by Western blotting experiments based on enhanced chemiluminescence, we applied a quantitative proteomics approach to further verify this observation. If c11orf51 is a *bona fide* subunit of human APC/C, protein levels of APC/C-bound c11orf51 should remain constant throughout the cell cycle. In addition, we aimed to establish a protocol allowing quantifying APC/C components and APC/C associating proteins during the cell cycle, which might help in better understanding the regulation of APC/C during the cell cycle.

In this experiment, immunopurified APC/C was chemically labeled using isobaric tags for absolute and relative quantification (iTRAQ) in combination with mass spectrometry (LC-MS/MS) (in collaboration with Thomas Koecher and Karl Mechtler, IMP, Vienna). One major advantage of this method is that it allows quantifying proteins from different samples in one single MS/MS run, thereby reducing experimental errors. Therefore, I immunopurified APC/C from different stages during the cell cycle. Since peptide and glycine elutions are not conducive for quantitative MS/MS approach through iTRAQ labeling, I tested different APC/C elution procedures as will be described later in this section.

iTRAQ is a chemical labeling technique based on stable isotopes that allows multiplexing of up to eight different samples on the peptide level (Pichler et al., 2010). In this study, we applied the four-plex labeling strategy. Purified protein is subjected to reduction and alkylation steps, followed by digestion with trypsin and derivatization of total peptide with the iTRAQ reagents, where primary amines are tagged via N-hydroxysuccinimide (NHS) chemistry (Ross et al., 2004). The iTRAQ labels consist of a charged reporter group, that is unique to each of the four reagents, an amine-reactive group and a neutral balancing group in-between, which is necessary to maintain the total isobaric mass of 145 Da for all labels

(isobaric, by definition, implies that any two or more species have the same mass but different configuration) (Burkhart et al., 2011; Zieske, 2006). The reporter group is a tag with a mass of about 114, 115, 116 or 117 Da, depending on isotopic combinations of $^{12}C/^{13}C$ and $^{16}O/^{18}O$ in each of the four reagents. (Yan and Chen, 2005). Since the label does not change the physiochemical property of the peptide in LC-MS, identical peptides with different labels are selected for fragmentation as a single precursor, which increases sensitivity. Following collision-induced dissociation (CID), the iTRAQ-tagged peptides fragment to release the four reporter group ions which appear as distinct masses between m/z 114.1-117.1, while the remainder of the sequence informative y- and b- ions remain as additive isobaric signals (Zieske, 2006). This enables MS/MS-based relative quantification of the same peptide across the four labeled samples in one single measurement, which minimizes experimental errors. Chemical noise on the MS/MS level is also minimized, which improves accuracy, especially of low-abundant proteins in a complex sample. The iTRAQ labeling strategy is particularly useful for comparing different biological states (e.g. normal, diseased and drug-treated samples) simultaneously or to quantify proteins during a time course study, as it is the case for the cell cycle. We applied this method to study changes in the protein composition of human APC/C during the cell cycle stages G1, S, G2 and prometaphase. Thereby, protein levels of APC/C-subunits should remain constant throughout the cell cycle.

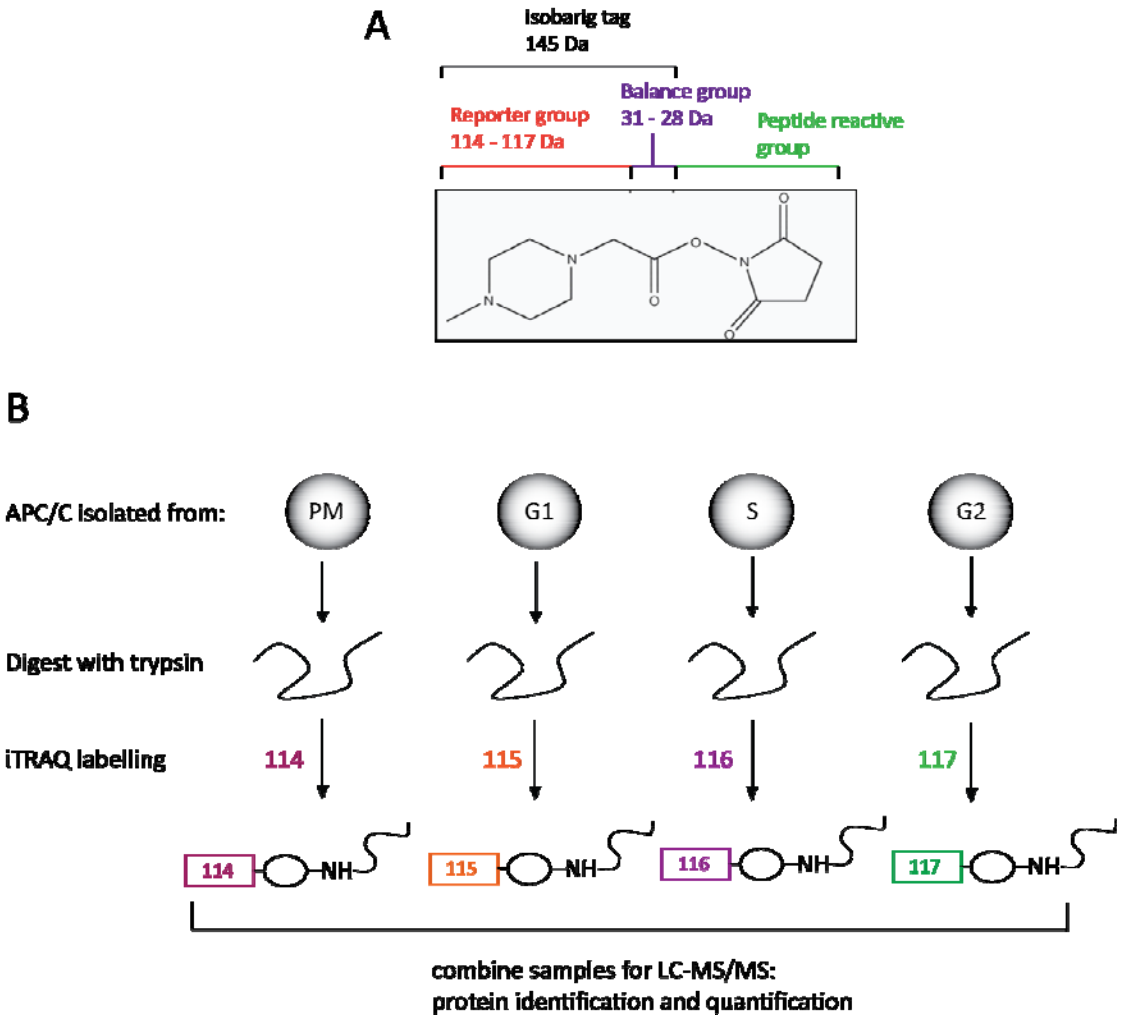


Figure 2-13: Isobaric tags for relative and absolute quantification (iTRAQ). Modified after (Yan and Chen, 2005). (A) Structure of iTRAQ reagents, consisting of a reporter group, a balance group and an amine-specific reactive group. (B) Strategy of iTRAQ labeling. APC/C isolated from four different cell cycle stages is proteolysed by trypsin. The resultant peptides are labelled with individual iTRAQ reagents which differ in the length of the reporter group. The labelled peptides are combined and analyzed by liquid chromatography and tandem mass spectrometry (LC-MS/MS).

Purified proteins have to be compatible for iTRAQ labeling. Because the N-hydroxysuccininimide-reactive group that is used for derivatization reacts rapidly with any primary or secondary amine, buffer or washing solutions that contain an amine group have to be omitted in immunoprecipitation experiments. For this reason, Tris buffer was replaced by 1x PBS for washing steps and TEAB was used to neutralize the protein sample after acidic elution. Moreover, glycine could not be used for elution. Therefore, I first tested iTRAQ-

compatible elution solvents for their capability to elute immunopurified APC/C. The silver stain in **Figure 2-14** shows that hydrochloric acid (HCl) (with and without 30% MeOH), Trifluoroacidic acid (TFA) or 2% formic acid (CH_2O_2) all worked equally well. For the following experiments, I decided to use HCl as the elution solvent.

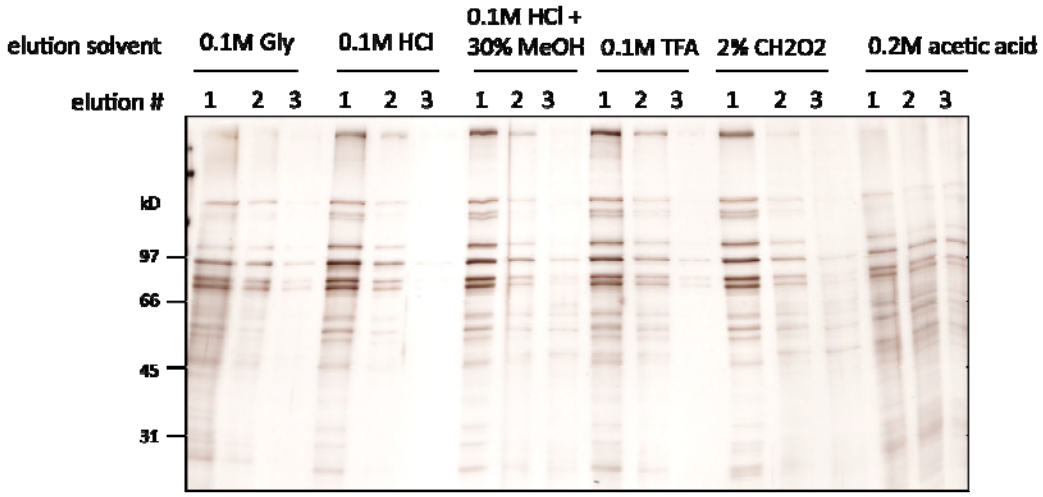
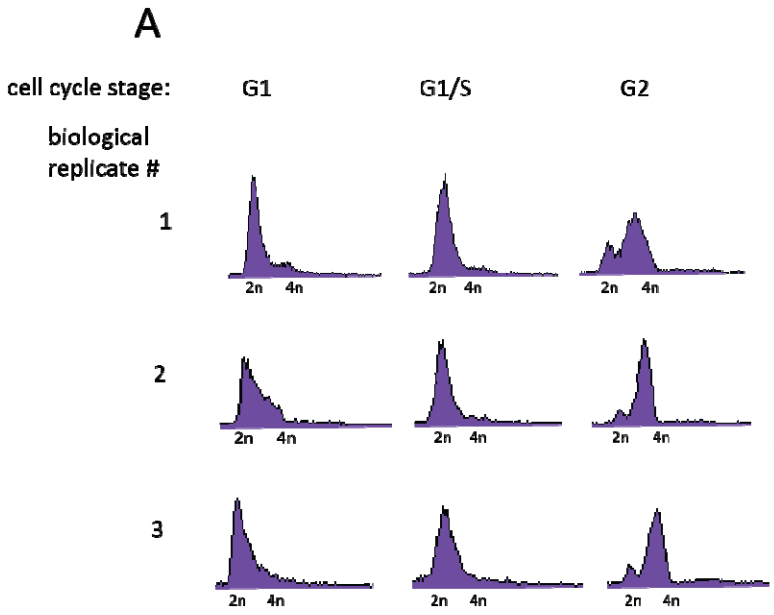
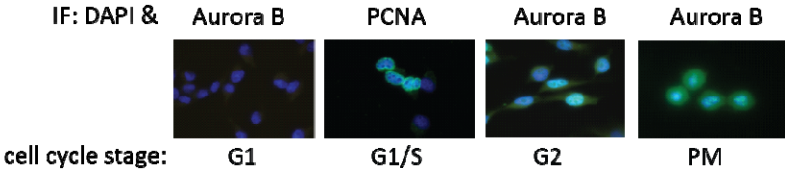


Figure 2-14: Testing different eluants for their ability to release proteins bound to antibody beads.

For iTRAQ labeling experiments, HeLa cells were arrested in the cell cycle stages G1, early S, and G2 by double thymidine arrest release. To obtain prometaphase cells with an active SAC, cells were treated with nocodazole for 16 hours. Cell cycle synchronization efficiency was monitored by FACS analysis using PI buffer and by immunofluorescence microscopy using antibodies against Aurora B kinase for G1, G2 and PM or proliferating cell nuclear antigen (PCNA) for S phase (**Figure 2-15**). Aurora B kinase is present in G2 phase but absent in G1 phase due to its APC/C-dependent proteolytic degradation (Stewart and Fang, 2005). In prometaphase, Aurora B localizes to the centrosome. PCNA is present on the chromatin of cells during DNA synthesis. APC/C was isolated via Apc3 antibody-coupled beads. To ensure that similar amounts of sample were subjected to iTRAQ labeling and quantitative measurements, protein levels were controlled by silver staining. **Figure 2-15** shows the silver stain of the three biological replicates that have been used for quantification of APC/C subunits and MCC proteins. Each replicate was measured three times. Therefore, the final data (**Figure 2-16**) represents the mean values of nine experiments.


A
cell cycle stage:
G1
G1/S
G2
biological replicate #
1
2
3
2n
4n


B
IF: DAPI &
Aurora B
PCNA
Aurora B
Aurora B
cell cycle stage:
G1
G1/S
G2
PM

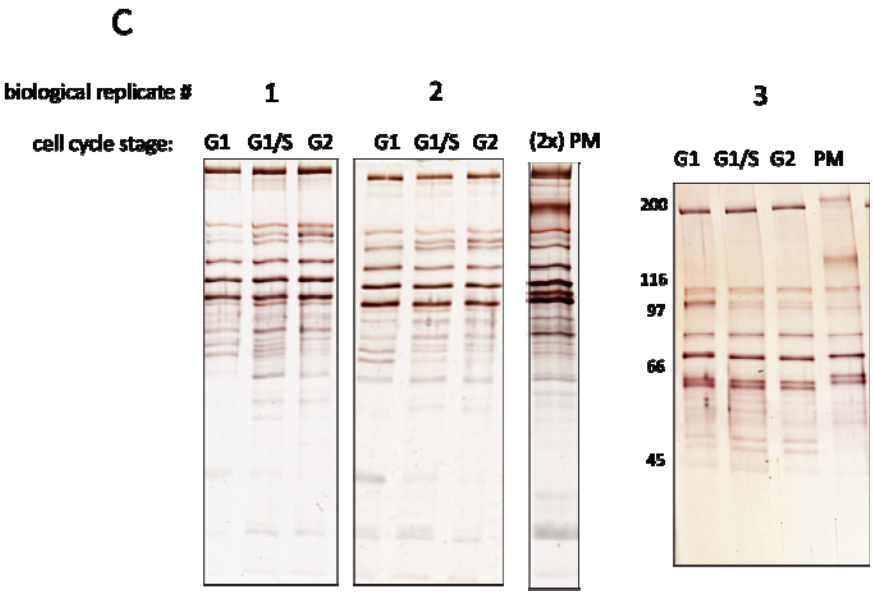
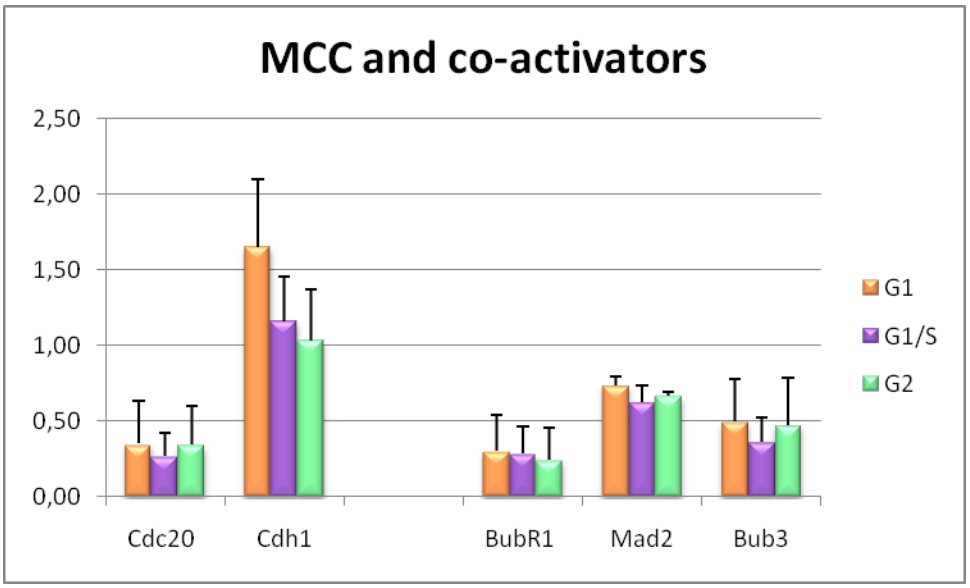

C
biological replicate #
1
2
3
cell cycle stage:
G1
G1/S
G2
G1
G1/S
G2
(2x) PM
G1
G1/S
G2
PM
200
116
97
66
45

Figure 2-15: iTRAQ labeling and quantitative mass spectrometry was performed from three biological replicates. (A) FACS profile using PI buffer confirms that cells have been arrested in the respective cell cycle stage. (B) Immunofluorescence staining of one biological replicate sample using Aurora B and PCNA antibodies. (C) Silver stain of Apc3-immunopurified APC/C which was used for iTRAQ labeling and quantitative MS/MS analysis.

For quantitative mass spectrometry, purified APC/C was alkylated, and digested with trypsin followed by incubation with iTRAQ reagents for derivatisation. Samples from one biological replicate, containing G1, G1/S, G2 and prometaphase were combined and the labeled peptides were separated by liquid chromatography-MS (LC-MS). Collision induced dissociation (CID) resulted in fragmentation of the peptides into reporter ions, which were analyzed by tandem mass spectrometry (MS/MS). To quantify the amount of one protein in one cell cycle stage relative to another cell cycle stage, one state has to be used as reference. We decided to use the prometaphase state as a reference state and normalized the reporter ions of the other iTRAQ channels intensities accordingly. Therefore, fluctuations in protein levels were analyzed relative to the prometaphase state, whose measured value was set to one. We chose prometaphase as the reference stage because it is known that MCC proteins are bound to the APC/C in early mitosis. Consistent with this, we found that levels of BubR1, Mad2, Bub3 and Cdc20 were high in prometaphase and significantly reduced in G1, S and G2. In contrast, Cdh1 protein levels were high in G1 and decreased in the other cell cycle stages. This is consistent with the fact that Cdh1 activates the APC/C later in mitosis and remains associated until the end of G1. Moreover, all APC/C subunits could be quantified, including the recently identified Apc16 subunit. Their protein levels remain largely constant throughout the cell cycle. However, some APC/C subunits seem to be more abundant during some cell cycle stages. The iTRAQ data will be discussed in chapter 3.2.1. Importantly, c11orf51 was also found. Levels of c11orf51 remain largely constant throughout the cell cycle, which confirms the semi-quantitative Western blot results and further supports the notion that this protein is a constitutive subunit of the APC/C. MCC proteins and APC/C co-activator proteins could be accurately quantified, which validates this technique as a sensitive tool to resolve APC/C composition during time. Therefore, future studies can be directed towards the composition of other APC/C associating proteins using this technique, which might lead to a better understanding of APC/C regulation during the cell cycle.

A



B

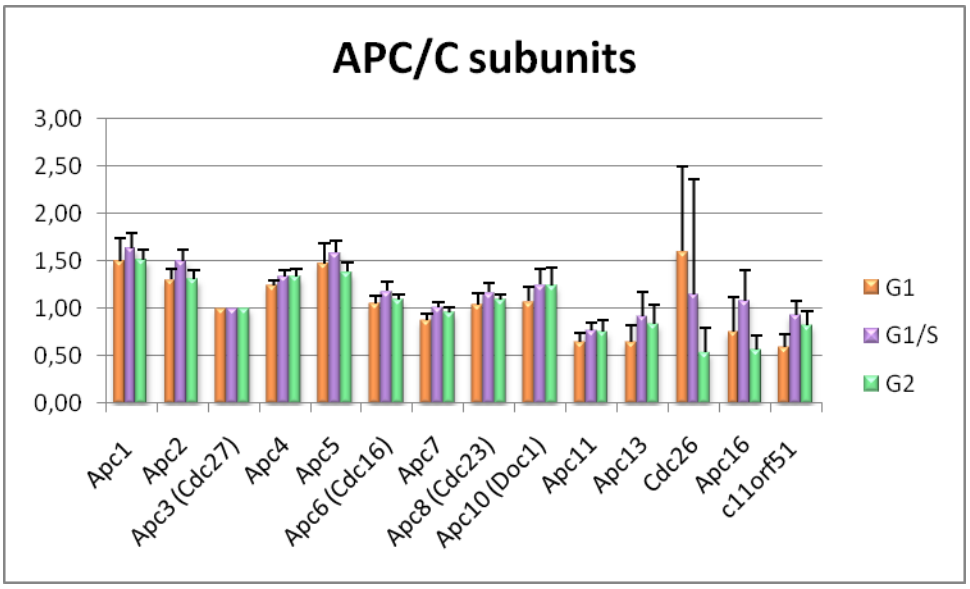


Figure 2-16: combined result of iTRAQ labeling and quantitative MS/MS analysis of three biological replicates. Three replicates were measured three times and the mean value of each experiment was used for these graphs. Prometaphase was used as the reference state and the value measured on this sample was set to one. For simplificity, this is not depicted in the graphs. (A) MCC proteins (Cdc20, Mad2, BubR1, and Bub3) are associated with the APC/C in mitosis, but not in other cell cycle stages. Cdc20 is not only a MCC component but also an

APC/C activator in mitosis, but not in G1. Cdh1 is associated with APC/C in G1, but not in mitosis. (B) Quantification of APC/C subunits from the cell cycle stages G1, S, G2 and PM. All APC/C subunits remain relatively constant throughout the cell cycle. However, levels of Apc1, Apc2, Apc5, and Cdc26 differ significantly, which will be discussed in chapter 3.2.1. The c11orf51 protein could be detected in all cell cycle stages.

2.8 What is the biological function of c11orf51?

The results so far confirmed that c11orf51 is a newly identified subunit of human APC/C, but its function is still largely unknown. Previous studies have shown that depletion of c11orf51 by RNAi causes aberrant cell cycle progression in human cells and that the gene is misregulated in breast cancer cells (Kittler et al., 2004; Olson et al., 2010), indicating that c11orf51 has a function in mitosis, which is consistent with c11orf51 being a subunit of the APC/C. However, cell cycle defects that are caused by APC/C misregulation can have various reasons. APC/C malfunction can occur when the complex is directly perturbed and therefore unable to exhibit its function. Alternatively, defects in other regulatory mechanisms that act on the complex could result in misregulation of the APC/C. To gain better knowledge about the biological role of c11orf51, loss-of-function studies in combination with immunofluorescence microscopy and biochemical analysis were performed.

2.8.1 Phenotypic characterization by RNAi and immunofluorescence microscopy

Loss-of-function studies were combined with microscopic analysis for phenotypic characterization of c11orf51. To this end, the protein was depleted from HeLa Kyoto cells using siRNA (Dharmacon; see materials and methods) and esiRNA (Kittler et al., 2004) (see materials and methods). RNAi in human c11orf51-LAP cells (hc11orf51-LAP) was exclusively performed with esiRNA's. Cell lysates were prepared and knock-down efficiency was controlled by Western blot analysis using c11orf51 antibodies. Untransfected cells which were only treated with RNAi-reaction mix without siRNA or esiRNA, were used as negative controls (**Figure 2-17**). Immunoblotting of cell lysates using c11orf51 antibody confirmed that c11orf51 can be efficiently depleted in both cell lines 48 hours after transfection.

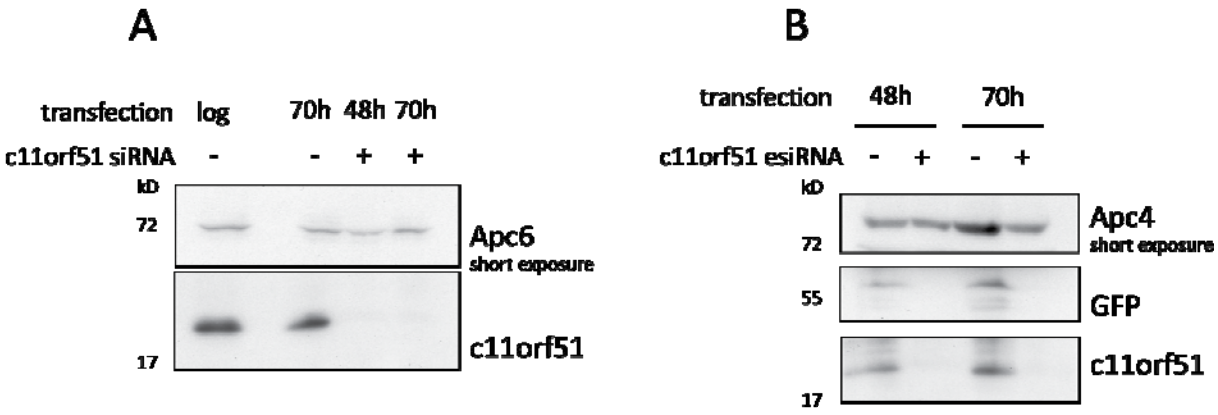
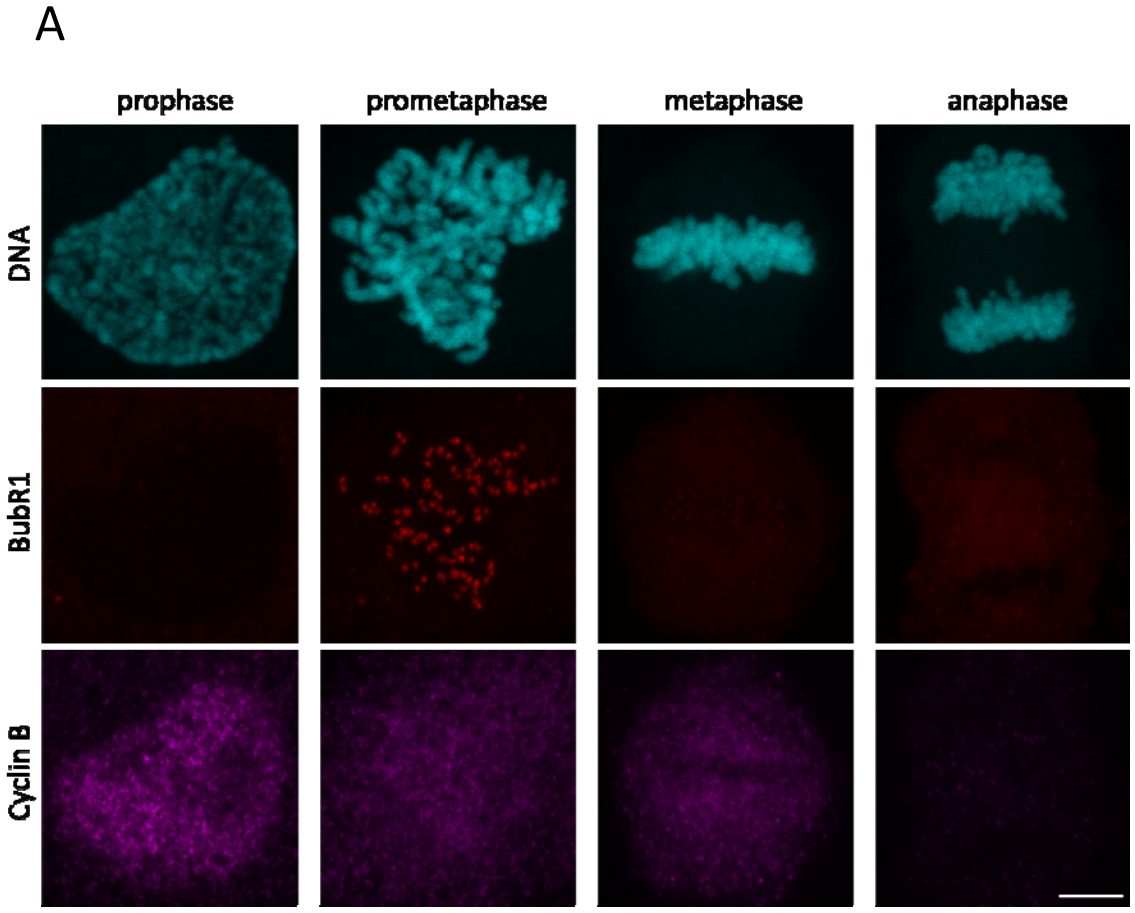


Figure 2-17: c11orf51 can be efficiently depleted in HeLa Kyoto cells and in hc11orf51-LAP expressing cell pool using esiRNA and siRNA. (A) C11orf51 RNAi in Kyoto cells using siRNAs. Western blot using Apc6 antibody confirmed equal protein loading. (B) Depletion of c11orf51p in hc11orf51-LAP expressing cells using esiRNAs. Western blot using α-GFP and α-c11orf51 antibodies confirmed that depletion of both GFP-c11orf51 and endogenous c11orf51 was efficient. In both experiments, protein levels of c11orf51 were significantly reduced 48 hours after transfection.

The c11orf51 protein has been implicated in mitotic progression since depletion of c11orf51 leads to mitotic defects (Kittler et al., 2004). To confirm these results we analyzed the mitotic index after c11orf51 RNAi, by using immunofluorescence staining and microscopy on fixed cells. Cells expressing hc11orf51-GFP (hc11orf51-LAP cell pool) were treated with esiRNA for 70 hours. Cells were fixed with 4% PFA and stained for cyclin B1 and BubR1. DAPI was used to look at chromosome morphology (**Figure 2-18**). We tried to control for depletion efficiency in cells expressing hc11orf51-LAP by immunofluorescence microscopy using GFP antibodies. However, the GFP signal intensity was generally weak and therefore not informative (data not shown). Anaphase onset is characterized by degradation of cyclin B; therefore cyclin B staining was used as a marker for metaphase / anaphase cells. BubR1 is located at the kinetochore in prometaphase cells. The signal intensity of BubR1 decreases as cells enter metaphase and it becomes absent in late anaphase / telophase due to proteasomal degradation of BubR1 (Choi et al., 2009). Therefore, BubR1 was used as an indicator to differentiate the prometaphase and the metaphase state. Although Western blot analysis could confirm efficient c11orf51 depletion in hc11orf51-LAP cells (**Figure 2-17**), we could not observe a significant increase in mitotic index after c11orf51 RNAi compared to control transfected cells (F-Luc). However, when we analyzed mitotic cells only, we observed

differences in the abundance of different mitotic stages. Compared to control cells, depletion of c11orf51 resulted in a twofold higher metaphase arrest (**Figure 2-18**). This is consistent with c11orf51 being required for proper mitotic progression, as previously observed (Kittler et al., 2004). However, the mitotic arrest phenotype as analyzed in this experiment was not profound and conclusive enough. Therefore, we aimed to monitor cell cycle progression in real time by performing live cell imaging after c11orf51 RNAi.



B

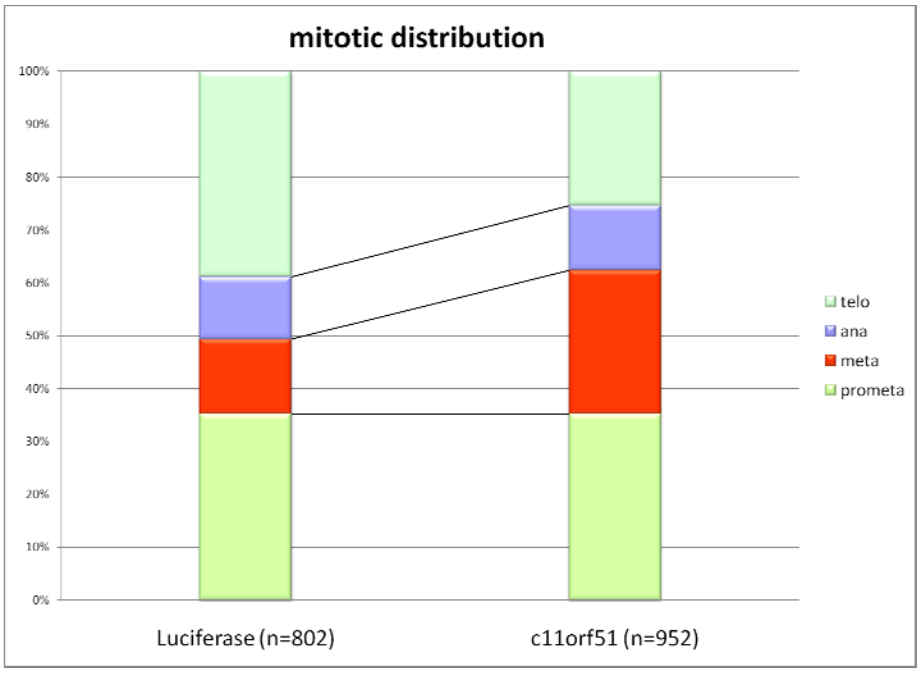


Figure 2-18: Analysis of mitotic stages after c11orf51 RNAi. (A) Immunofluorescence staining on fixed hc11orf51-LAP cells was performed, where c11orf51 was depleted by RNAi. DAPI was used to stain the chromosomes, BubR1 was used as a marker to distinguish prometaphase and metaphase cells, and degradation of cyclin B marks anaphase onset. (B) Distribution of cells during various stages of mitosis in c11orf51 RNAi or control (Luciferase) RNAi cells.

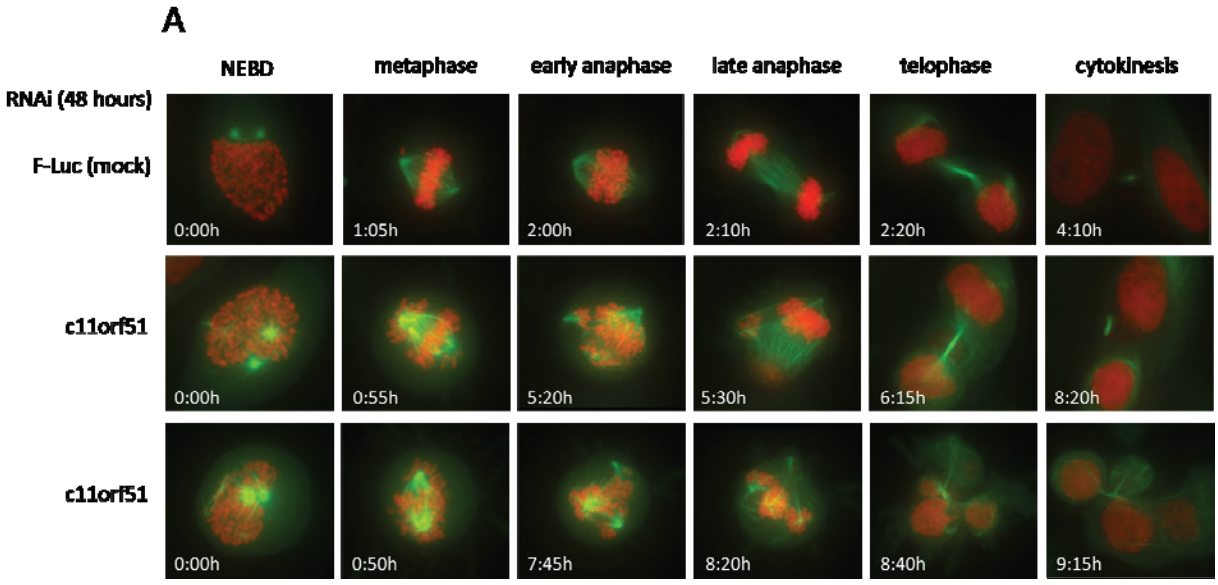
2.8.2 Depletion of c11orf51 causes a mitotic progression defect

The metaphase accumulation phenotype observed in Immunofluorescence microscopy on fixed cells after c11orf51 RNAi was not very severe. This could have several reasons. C11orf51 depletion may have been incomplete. Alternatively, c11orf51 depletion might cause a delay in mitotic progression, rather than an arrest. In this case, it would be difficult to obtain a significant population of cells arrested in mitosis for statistical IF-analysis. We therefore decided to monitor cell cycle progression by live cell imaging in control and c11orf51 RNAi cells (in collaboration with Yusuke Toyoda and Anthony Hyman, MPI Dresden, Germany).

The live cell imaging technique does not only provide a better ability to visualize abnormalities in cell cycle progression but also provides a means by which changes in cell morphology or aberrant cell division kinetics processes can be monitored. For this

experiment, HeLa cells were used that stably express histone H2B-mCherry (red channel) and mouse TUBB-LAP (green channel). H2B-mCherry TUBB-LAP cells were treated with esiRNA´s targeting Apc6, c11orf51, and Eg5. Eg5 (Kiff11) is a kinesin like motor protein required for centrosome separation. Therefore, Eg5 is important for establishment of a bipolar spindle (Harborth et al., 2001). Depletion of Eg5 results in a profound mitotic arrest phenotype with cells that round up and eventually undergo apoptosis. Eg5 was therefore used to control for transfection efficiency. Firefly-Luciferase esiRNA served as negative control. The cells were filmed 24 hours and 48 hours after esiRNA transfection. The filming was performed in 5 min intervals, for 20 hours in total. All cells in the movies were manually annotated for their cell cycle stage (see **Appendix**, chapter 5). Mitosis was defined by the time of nuclear envelope breakdown (NEBD) until anaphase onset. Exit from mitosis was set as the time from anaphase until loss of microtubule bridges of daughter cells (cytokinesis).

The quantification of the movies is depicted in **Figure 2-19**. A mitotic defect was observed 24 hours after Apc6 esiRNA transfection, when compared to Firefly-Luciferase (F-Luc) transfected control cells. This mitotic defect was significant as indicated by a p-value of <0.05. However, fewer Apc6-depleted cells could be counted after 48 hours, which might be the reason for the high standard deviation depicted in **Figure 2-19**. In contrast, c11orf51 depletion showed a significant phenotype 48 hours after transfection compared to control transfected cells, but not after 24 hours. C11orf51 RNAi resulted in an arrest in mitosis (NEBD to anaphase onset); whereas the time those cells took to exit mitosis (anaphase till cytokinesis) was not largely unaffected. Knock-down efficiency of the RNAi target genes were confirmed by immunoblotting using GFP antibody from whole cell lysate of RNAi treated Apc6-LAP and hc11orf51-LAP cells (**Figure 2-19**). The Western blot also shows that maximal c11orf51 depletion was achieved after 48 hours, whereas Apc6 protein levels were already reduced 24 hours post-transfection, which correlates with the appearance of mitotic phenotypes. The median time that cells spent in mitosis upon esiRNA transfections reveals that mitosis lasted on average four times longer in c11orf51 depleted cells, compared to Luciferase-transfected cells (48 hours post-transfection) (**Figure 2-19**). The c11orf51 RNAi-phenotype observed in live cell imaging microscopy showed aberrant mitotic spindle formation and cells that are unable to align their chromosomes and to divide. Moreover, a significant portion of cells formed polylobed nuclei after division (**Figure 2-19**).


A
NEBD
metaphase
early anaphase
late anaphase
telophase
cytokinesis
RNAi (48 hours)
F-Luc (mock)
0:00h
1:05h
2:00h
2:10h
2:20h
4:10h
c11orf51
0:00h
0:55h
5:20h
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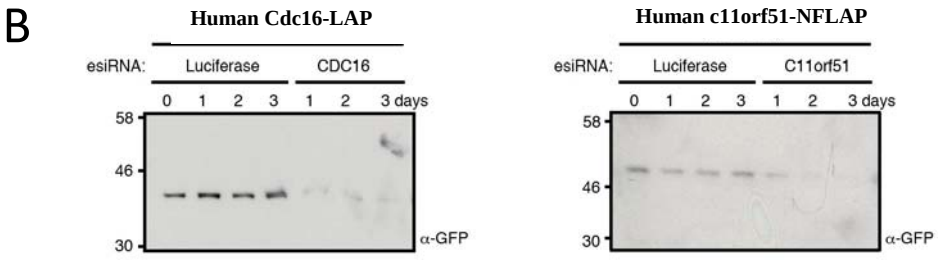
0:50h
7:45h
8:20h
8:40h
9:15h


B
Human Cdc16-LAP
esiRNA:
Luciferase
CDC16
0 1 2 3 1 2 3 days
58
46
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α-GFP
Human c11orf51-NFLAP
esiRNA:
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C11orf51
0 1 2 3 1 2 3 days
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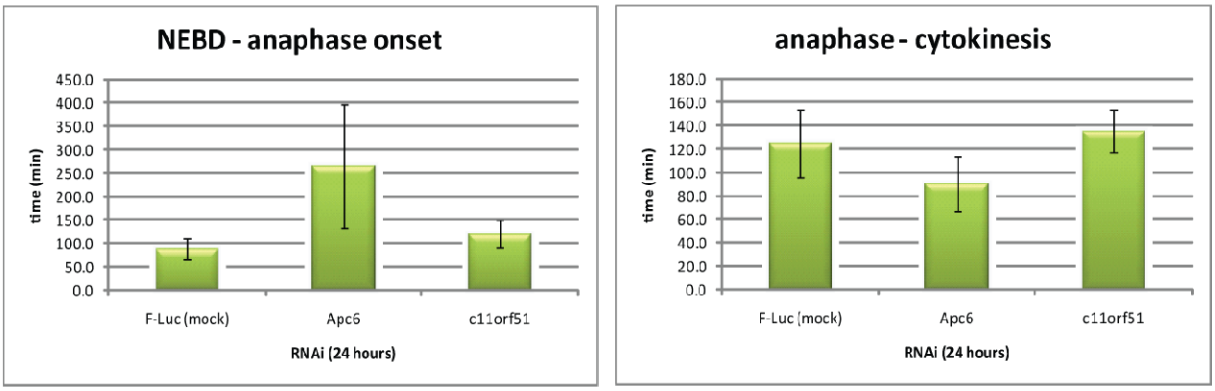
α-GFP

C



D

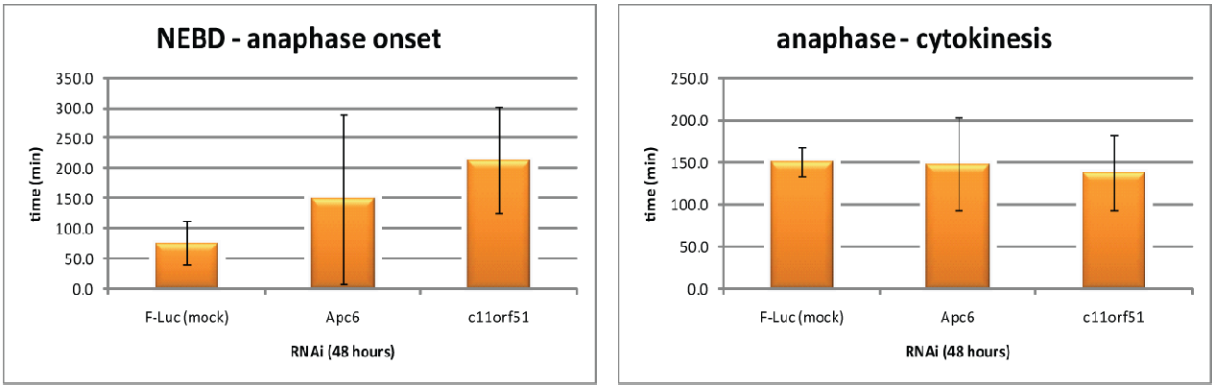


Figure 2-19: c11orf51 RNAi causes defects in mitotic progression. (A) Time lapse microscopy of HeLa cells which stably express histone H2B-mCherry (red) and mouse TUBB-LAP (green) in c11orf51 depleted cells compared to Firefly-Luciferase (mock) transfected cells. (B) Western blots confirming that Apc6 and c11orf51 could efficiently be depleted after one day or two days, respectively. (C) Graph showing the manual annotation of the time in minutes that cells spent in mitosis (prometaphase and metaphase) or that cells needed to exit mitosis (anaphase till cytokinesis) after c11orf51, Cdc16 and F-Luc RNAi for 24 hours. (D) Graph showing the movie result of the 48 hours-RNAi experiment. Apc6 transfected cells were largely apoptotic by that time. Therefore, fewer cells could be counted after Apc6 RNAi.

RNAi and time lapse microscopy experiments confirmed that c11orf51 has an important function in mitosis. However, this experiment still does not answer the question how c11orf51 contributes to correct cell division, particularly in respect to its role as an APC/C subunit. To further analyze the biological function of c11orf51, I combined siRNA-mediated knock-down studies with biochemical analysis.

2.8.3 Depletion of c11orf51 seems to retain MCC proteins bound to the APC/C

As mentioned earlier, there are several reasons for misregulation of the APC/C in the absence of c11orf51. Depletion of c11orf51 could directly decrease the ability of the APC/C to ubiquitylate substrates, or it could indirectly keep the APC/C inhibited by interfering with disassembly of APC/C^{MCC}. To distinguish between these possibilities, we analyzed how much MCC is bound to the APC/C in mitotic cells depleted of c11orf51.

To obtain c11orf51 depleted cells in mitosis, I treated cells with c11orf51 siRNA for 32 hours and added nocodazole for the last 16 hours. C11orf51 depleted interphase cells were obtained by siRNA treatment of asynchronous HeLa cells for 48 hours. Firefly-Luciferase RNAi was performed as control. Apc3-immunopurified APC/C and lysates were analyzed by Western blotting analysis using antibodies against c11orf51, Apc4, Apc6 and the MCC proteins BubR1, Mad2 and Cdc20 (**Figure 2-20**). Western blotting using c11orf51 antibody confirmed that the c11orf51 protein was efficiently depleted, as the c11orf51-specific band was markedly reduced after c11orf51 RNAi (panel 6, lane 1 and 2). Apc4 and Apc6 confirmed equal loading (panel 1 and 2). Notably, the amounts of Apc3-immunopurified BubR1 (panel 3, lane 8), Mad2 (panel 5, lane 8) and Cdc20 (panel 4, lane 8) were increased in c11orf51 was depleted nocodazole arrested cells, compared to control transfected cells. This was despite the fact that the total amount of BubR1 (panel 3, lane 1-4) and Mad2 (panel 5, lane 1-4) in the lysate was similar in all samples. Moreover, cellular Cdc20 protein was increased in c11orf51 depleted cells, upon nocodazole treatment (panel 4, lane 2), compared to Cdc20 levels in control transfected cells.

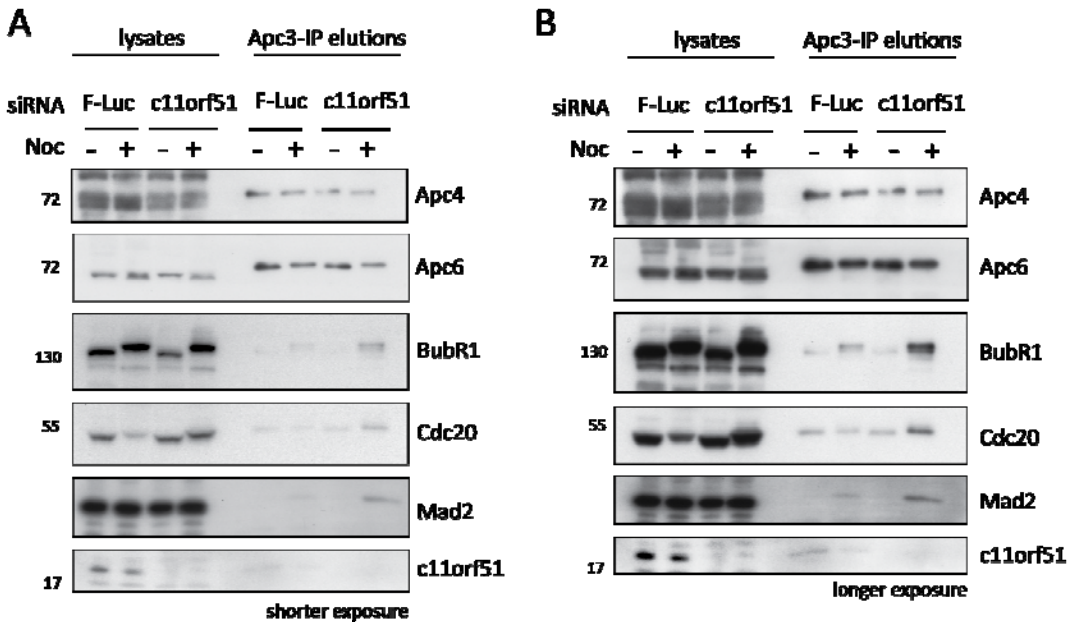


Figure 2-20: Depletion of c11orf51 seems to retain MCC proteins bound to APC/C in cells arrested with an active SAC. Western blotting analysis of lysates and Apc3-eluates of c11orf51 siRNA treated cells with and without nocodazole. Firefly-Luciferase siRNA transfection was used as control. Apc4 and Apc6 confirm equal protein loading. The c11orf51-specific band is markedly reduced after c11orf51 RNAi as it can be seen in the lysate, confirming that the depletion was efficient. (A) Shorter exposure reveals that the cellular protein amount of BubR1 and Mad2 are similar in all samples, whereas Cdc20 levels seem to be reduced in control transfected and nocodazole treated cells. (B) Longer exposure reveals that protein levels of immunopurified BubR1, Mad2 and Cdc20 are higher in c11orf51-depleted cells after activation of the SAC with nocodazole, compared to control transfected cells (panel 3, 4, and 5; lane 6).

It has been shown that Cdc20 is continuously turned over in SAC arrested cells (Nilsson et al., 2008; Pan and Chen, 2004; Prinz et al., 1998; Reddy et al., 2007). Since I see more Cdc20 in c11orf51 depleted cells, the c11orf51 protein might be required for Cdc20 turnover.

However, two opposing theories currently exist regarding Cdc20 turnover in mitosis. Studies in yeast and in human HeLa cells propose that APC/C-mediated Cdc20 ubiquitilation and proteolysis is important to maintain the SAC upon spindle disruption (King et al., 2007; Nilsson et al., 2008; Pan and Chen, 2004). The observation that more Cdc20 can be found in the lysate after depletion of c11orf51 in nocodazole arrested cells, compared to control transfected cells, might indicate that c11orf51 plays a role in regulating Cdc20 turnover in SAC arrested cells. Thereby, loss of c11orf51 would stabilize Cdc20, indicated by higher protein levels of cellular Cdc20.

However, if Cdc20 ubiquitilation and turnover is important to retain the checkpoint proteins on the APC/C, stabilization of Cdc20 should result in dissociation of MCC from the APC/C and should thereby promote premature anaphase onset. We observed the contrary in live cell imaging experiments after c11orf51 RNAi (see chapter2.8.2), where cells were delayed in mitosis, indicative for prolonged SAC activation.

As mentioned in chapter 1.10.5, Cdc20 ubiquitilation has also been proposed to promote disassembly of checkpoint proteins from the APC/C. In this case, Cdc20 ubiquitilation would preceed the release of MCC proteins (Reddy et al., 2007). Structural studies imply that the MCC prevents substrate binding to the APC/C, possibly by repositioning Cdc20 (Herzog et al., 2009). Thereby, ubiquitilation of Cdc20 might be necessary for changing the structural conformation of Cdc20, resulting in MCC disossiaction.

However, this experiment has to be repeated to validate this result. Therefore, the mentioned interpretations are preliminary at this point. Moreover, additional experiments will be necessary to further elucidate the biological role of c11orf51.

3 Discussion

3.1 The c11orf51 protein is evolutionary conserved in metazoans

Bioninformatic sequence alignment of the c11orf51 showed that this protein is evolutionary conserved from yeast to human. This comprises the N-terminal part, which is predicted to contain an α-helix and a small β-sheet, as well as the C-terminal region. Interestingly, the distinct acidic stretch at the C-terminal end of the protein is well conserved in higher metazoans such as humans, mouse, frog, fish and worm. Moreover, the length of this acidic sequence varies among species; it is longer in higher vertebrates and significantly shorter in fungi and plant. Structural prediction using the metaPrDOS-server suggested that this low complexity region is disordered. Natively disordered regions seem to fulfill the primary role of serving as molecular recognition motifs for proteins or DNA and are involved in many biological processes. The flexibility of these regions may be necessary to interact with multiple partners and binding to ligands often leads to disorder-to-order transitions (Dyson and Wright, 2005). In addition, a conserved tail composed of the hydrophobic amino acids tryptophane, methionine and isoleucine (WMI) is exclusively found in the c11orf51 protein of higher vertebrates. This suggests that the c11orf51 protein evolved during evolution and that it might fulfill an important function.

Moreover, the bioinformatic analysis revealed that the c11orf51 protein is a distant homologue of budding yeast Mnd2. Mnd2 is a constitutive subunit of budding yeast APC/C and present in both, mitosis and meiosis (see chapter 1.10.3). This protein has been shown to be important for meiotic progression, whereas in mitosis it is not essential (Penkner et al., 2005). However, Mnd2-depletion in budding yeast caused an accumulation of G2/M cells and it has been shown in *in vitro* transcription / translation experiments that Mnd2 associates with the APC/C subunits Cdc23, Apc5, Apc1 and weakly with Apc2 (Hall et al., 2003). Notably, the mammalian homologue of Mnd2 has not been reported so far. RNAi-mediated depletion of c11orf51 in HeLa cells resulted in a metaphase accumulation phenotype and biochemical experiments indicated an effect on Cdc20 turnover in prometaphase arrested HeLa cells.

However, our observations imply that c11orf51 is not essential in mitosis, but depletion of this protein causes aberrant spindle formation and it delays anaphase onset. However, it does not drive cells into apoptosis, which is the case if other APC/C subunits are absent (see chapter 1.6).

In addition to its function in mitosis, c11orf51 could have an important role in meiosis as it was found to be a distant homologue of budding yeast Mnd2. However, this hypothesis still has to be experimentally confirmed.

To identify binding partners of c11orf51 *in vivo*, cross-linking experiments in HeLa cells could be performed, although this might not be trivial. Alternatively, *in vitro* binding assays could be applied for identifying associating proteins of c11orf51. For example, different Flag-tagged APC/C-subunits and MCC-proteins could be expressed in *in vitro* transcription / translation (IVT) reactions using rabbit reticolysate lysate. Recombinant and e.g. His_6-tagged full length c11orf51 protein and deletion mutants could be added and proteins would be further isolated via α-Flag-antibodies. Immunoblotting using α-His-antibody would reveal presence of bound c11orf51.

3.2 C11orf51 is a constitutive subunit of human APC/C

Tandem affinity purification using the hc11orf51-LAP cell pool could purify the majority of APC/C subunits from asynchronous HeLa cells as analysed by LC-MS/MS. In addition, the APC/C could be immunoprecipitated using c11orf51 antibodies and Apc3-antibodies could co-purify c11orf51, as shown in Western blot experiments. These observations indicate that c11orf51 specifically associates with human APC/C during the entire cell cycle. Sucrose density gradient centrifugation revealed that the majority of cellular c11orf51 is bound to the APC/C as the protein co-fractionated with other APC/C subunits. However, it cannot be ruled out that some trace amounts of free cellular c11orf51 exist, which could not be detected with our α-c11orf51 antibody. Immunoprecipitation experiments using first Apc3- and then c11orf51-antibodies revealed the existence of two APC/C populations in human HeLa cells, with one APC/C fraction containing c11orf51, whereas the other one does not. The question

that arises is if both APC/C populations differ in their activity. Therefore, *in vitro* ubiquitilation assays could be performed. Negative staining electron microscopy showed c11orf51 antibody-mediated formation of APC/C dimers, confirming tight association of c11orf51 with the complex. A more detailed localization study of c11orf51 by negative staining EM, which is currently in progress, will more precisely show the position of c11orf51 within the complex. However, our preliminary EM data suggests that the c11orf51 protein is located at the platform domain of the APC/C.

3.2.1 iTRAQ labeling and quantitative mass spectrometric analysis of human APC/C

To confirm the semi-quantitative Western blot result of c11orf51 being a constitutive subunit of human APC/C, we applied a sensitive and quantitative mass spectrometric approach. ITRAQ labeling (isobaric tags for relative and absolute quantitation) in combination with on-line liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed to analyze c11orf51 protein levels during the cell cycle. Along with this, a protocol was established for arresting cells at all cell cycle stages and for performing iTRAQ-compatible protein purification. In the presented work, only G1, G1/S, G2 and prometaphase arrested cells were used for quantifying APC/C subunits, co-activators and MCC proteins. However, I also performed additional synchronization, immunofluorescence and immunoprecipitation experiments to obtain mitotic APC/C isolated from the cell cycle stages prometaphase, metaphase, anaphase and telophase. The purified proteins from these mitotic stages will be analyzed by the iTRAQ method in near future.

The iTRAQ approach was validated by analyzing protein levels of MCC components and of both co-activators, as cell cycle-regulated association of these proteins with the APC/C had been confirmed in numerous studies (see chapters 1.7.3 and 1.10.4). Therefore, the prometaphase state was used as the reference state and the reporter ions of the other iTRAQ channels intensities were normalized accordingly. In addition, the bait protein Apc3 was used for normalizing the ratios of other APC/C-subunits and interacting proteins in order to compensate for different quantities recovered by the IP. Cdc20 functions in early mitotic

stages; in prometaphase arrested cells, Cdc20 is a component of the MCC, whereas Cdh1 is associated with the APC/C later in mitosis and remains bound to the complex until the end of G1 (see chapter 1.7.3 for details). Consistent with this, the iTRAQ-result shows that Cdc20 protein levels are reduced in G1, G1/S and G2 compared to prometaphase, whereas APC/C-bound Cdh1 is abundant in G1 but not in mitosis. BubR1 and Bub3 protein levels are also highest in prometaphase and significantly reduced in G1, G1/S and G2, as they inhibit APC/C activity in early mitosis by forming the MCC (see chapter 1.10.4 for details). However, Mad2 protein levels seem to be higher in G1, G1/S and G2 when compared to the levels of other MCC components. This might lead to the assumption that Mad2 also associates with interphase APC/C. It has been shown that MCC proteins can bind to interphase APC/C, although only mitotic APC/C can efficiently be inhibited, possibly due to phosphorylation of APC/C subunits (Sudakin et al., 2001). However, it has also been shown that APC/C-bound MCC proteins are present in substoichometric amounts (Sudakin et al., 2001). Therefore, it is unlikely that Mad2 levels are higher in interphase and early S phase as compared to BubR1 and Bub3. Since Mad2 is a small protein (26 kDa) fewer peptides could be identified and quantified by mass spectrometry. The distinct Mad2 profile can be explained by the weaker statistics of the result. However, the general trend of the iTRAQ result on the MCC proteins and co-activators confirms that the iTRAQ approach is sensitive enough to measure fluctuations in protein levels during the cell cycle.

To analyze c11orf51 protein levels during the cell cycle, the APC/C was isolated using Apc3 antibody-coupled beads. As mentioned above, the value measured on the prometaphase sample was used as the reference state and normalized to the intensities of the Apc3 subunit. The other APC/C subunits depicted in **Figure 2-16** can be divided into three classes. The first class comprises the subunits Apc6, Apc7, Apc8, and Apc10, which show the most constant protein levels during the cell cycle. The second class of proteins consists of the subunits Apc1, Apc2, Apc4, and Apc5. These subunits show slightly higher values in G1, early S and G2, as compared to prometaphase. The observed fluctuations of these proteins might be explained by the 16 hours nocodazole treatment, which might have caused biological artifacts. The subunit Cdc26 was found by only one peptide, resulting in a higher standard deviation and fluctuation profile compared to the other proteins. The third class comprises the subunits Apc11, Apc13, Apc16 and c11orf51. All of these proteins are rather small. Therefore,

fluctuations in the protein levels might again be explained by fewer peptides that were used for quantification. Importantly, the iTRAQ-profile of c11orf51 does not significantly differ from the profile of other known APC/C-subunits, such as Apc11 or Apc16, confirming that the c11orf51 protein is a constitutive APC/C subunit.

3.3 What is the biological function of c11orf51?

APC/C associated with c11orf51 was similarly active in ubiquitylating its mitotic substrate cyclin B1 in *in vitro* ubiquitylation assays as Apc3-immunopurified APC/C (**Figure 2-11**). Moreover, its ligase activity could be stimulated when the co-activator Cdh1 was added to the reaction. This indicates that c11orf51 is not inhibiting APC/C^{Cdh1} ubiquitin ligase activity. This is consistent with the fact that RNAi-mediated depletion of c11orf51 resulted in a metaphase accumulating phenotype, which suggests that this protein has more of an active function. To find out if c11orf51 is required for APC/C´s ligase activity, loss-of-function studies and *in vitro* ubiquitylation assays could be performed. It is likely that depletion of c11orf51 does not significantly interfere with the ubiquitylation capacity of the APC/C, at least not in interphase, since *in vitro*-reconstituted APC/C subcomplexes that do not contain c11orf51 show ligase activity (Brenda Schulman, personal communication). Moreover, the catalytic reaction had been assigned to the subunits Apc2, Apc11 and to the processivity factor Apc10 and other subunits are thought to be required for structural integrity of the APC/C or serve as platforms for other proteins to bind to the complex (see chapter 1.7 for details). However, it might be possible, that c11orf51 is specifically required for Cdc20 ubiquitylation in early mitosis (see chapter 1.10.5), as Western blotting revealed increased levels of cellular Cdc20 in c11orf51-depleted and nocodazole arrested HeLa cells (**Figure 2-20**). This could be tested in *in vitro* ubiquitylation assays of APC/C-bound Cdc20 using purified mitotic APC/C which is devoid of c11orf51. Although it is unlikely that depletion of c11orf51 destabilizes the complex, the possibility of structural disruption after c11orf51 RNAi cannot be completely ruled out. Western blotting using Apc4 and Apc6 antibodies confirmed that these subunits are present c11orf51 depleted APC/C isolated after Apc3-IP. Apc4 is a subunit in the "arc lamp" domain; and it connects the catalytic subunit Apc2 to the

TPR-containing subunits via its interaction partner Apc1. The TPR-subunit Apc6 is located between Apc3 and Apc4. The fact that both proteins could be detected after Apc3-IP suggests that at least the head domain and the arc lamp are unperturbed after c11orf51 RNAi. To confirm this notion, Western blot experiments could be performed using more APC/C-subunits or presence of APC/C subunits could be visualized by silver staining.

Depletion of c11orf51 by RNAi in nocodazole-arrested prometaphase cells led to two observations: More MCC proteins remained stably bound to the APC/C, and the amount of cellular Cdc20 was increased, compared to control transfected cells.

Two possibilities might have led to this result:
1. c11orf51 might be needed for MCC disassembly. This would be in agreement with the IF and live cell imaging experiments, where c11orf51-RNAi caused a metaphase accumulation phenotype in HeLa cells, indicative for prolonged APC/C inhibition. Notably, cellular Cdc20 levels were also increased after c11orf51-RNAi.
2. c11orf51 might be needed for Cdc20 turnover. Thereby, c11orf51 would control the amount of cellular Cdc20 in proliferating cells with an active spindle assembly checkpoint.

Cdc20 has been shown to be continuously synthesized and degraded in SAC activated cells (see chapter 1.10.5 and 2.8.3). Furthermore, Cdc20 turnover has been shown to depend on APC/C activity, but not on the co-activator Cdh1 (Pan and Chen, 2004). However, two opposing theories currently exist which address the role of Cdc20 turnover in early mitosis.

Studies in budding yeast and human cells suggest that APC/C-mediated Cdc20 poly-ubiquitylation and degradation is required to maintain the SAC in nocodazole arrested prometaphase cells (King et al., 2007; Nilsson et al., 2008; Pan and Chen, 2004). In contrast to this, another study in human cells could show that Cdc20 poly-ubiquitylation mediates disassembly of the Mad2-Cdc20 complex from the APC/C, which leads to SAC silencing. However, although Cdc20 poly-ubiquitylation was needed for the disassembly of the Mad2-Cdc20 inhibitory complex, Cdc20 proteolysis did not seem to be required for this process. Cdc20 levels are balanced by opposing deubiquitylation events (Reddy et al., 2007; Stegmeier et al., 2007).

We observed more MCC association with the APC/C and more cellular Cdc20 levels in c11orf51 depleted HeLa cells with an active SAC, which favours the hypothesis that Cdc20 turnover is required for MCC disassembly. This is consistent with our IF / live cell imaging data, where c11orf51 depletion delayed anaphase onset, possibly because MCC could not disassemble and the SAC could not be silenced. However, based on our preliminary data, Cdc20 proteolysis seems to be required for disossiaction of MCC proteins from the APC/C. It is believed that the APC/C itself might be capable to some degree of regulating MCC disassembly (see chapter 1.10.5 for details). Hence, it might be possible even though speculative at this point, that c11orf51 would function as an APC/C subunit to promote silencing of the spindle assembly checkpoint by regulating Cdc20 turnover and MCC disassembly.

Live cell imagining experiments after c11orf51 RNAi showed prolonged metaphase arrest in HeLa cells and Western blot analysis revealed increased MCC binding in nocodazole arrested cells. Therefore, we suspect that the mitotic progression defect observed in c11orf51 RNAi experiments is due to prolonged APC/C inhibition mediated by the MCC. To further test this hypothesis, APC/C activity could be measured in *in vitro* ubiquitylation experiments using cyclin B1 as a substrate and APC/C which had been purified from c11orf51 depleted and control transfected cells after release from a nocodazole arrest. In addition, MCC proteins that are bound to the APC/C could be analyzed by Western blotting. C11orf51 RNAi resulted in accumulation of cellular Cdc20 in cells that had been arrested with nocodazole. This further suggests that c11orf51 might be required for Cdc20 turnover which promotes MCC disassembly. Since it has been shown that Cdc20 is continuously synthesized and degraded in early mitosis, the protein synthesis inhibitor cyclohexamide could be used to further confirm this notion. Thereby, Cdc20 protein levels would decrease in siRNA-untransfected and nocodazole arrested cells upon cyclohexamide treatment. If Cdc20 degradation is required for MCC disassembly, these cells should be able to overcome the nocodazole-mediated arrest. However, if c11orf51 promotes Cdc20 proteolysis in early mitosis, c11orf51 depleted cells should arrest longer in prometaphase due to accumulating Cdc20 protein levels and impaired MCC disassembly, which would confirm that c11orf51 is required for Cdc20 turnover in mitosis.

Although c11orf51 RNAi resulted in accumulation of metaphase cells, we could observe that these cells could overcome the arrest after some time. This indicates that c11orf51 is not essential for mitotic progression, although it is important for correct cell division. Aberrant cell division is often caused by premature activation of the APC/C, which promotes cyclin B1 and securin degradation even though the SAC had not been satisfied. Our observation from the live cell imaging experiment suggests that the APC/C could get activated after some time. Besides being a MCC component, Cdc20 is also an APC/C co-activator when not associated with MCC proteins. Thereby, in absence of c11orf51, Cdc20 levels might exceed MCC protein levels after a prolonged metaphase arrest, which could activate the APC/C. At that time, MCC proteins might have captured a large fraction of the APC/C because disassembly of the inhibitory complex is perturbed. Therefore, MCC-unbound APC/C molecules might be too few to target enough securin and cyclin B1 to promote a switch-like metaphase to anaphase transition, resulting in a "mitotic slippage" phenotype. It might be interesting to test how much MCC-unbound APC/C has to be activated before cells can progress into anaphase and how much cellular Cdc20 needs to accumulate to promote this process. This could be addressed by performing (Re-) IP and Western blot experiments using MCC- and Cdc20-antibodies. In addition, accumulation of Cdc20 after c11orf51 RNAi at different mitotic stages could be visualized by immunfluorescence microscopy. However, mitotic exit seems to be largely unaffected, indicating that some mechanism might take over to restore APC/C levels which can be activated by the co-activator Cdh1 later in mitosis.

Notably, all these possibilities and suggestions are very speculative. They are based on only two observations, MCC accumulation on the APC/C and increasing amounts of cellular Cdc20 after c11orf51 RNAi in nocodazole arrested cells. More experiments are necessary to further elucidate the biological function of c11orf51 in mitosis.

4 Material and Methods

4.1 cDNA constructs

Myc-His_6-tagged H.s. cyclin B1 (aa 1-87) in pTrcHis2A was used for cloning of cyclin B1 in *E.coli* as an APC/C substrate for ubiquitylation assays. The construct was generated by Michael Gmachl.

4.2 Antibodies

4.2.1 Antibodies for immunoprecipitation and Western blots

Antibodies for immunoblotting were used at 1-2 µg/mL in 4% milk-TBS-T unless other indicated. The c11orf51 antibodies were raised in rabbits (Gramsch laboratories) against one synthetic peptide covering one region of the protein sequence (see **Figure 2-3** for details). Further antibodies used in immunoprecipitation and Western blots were: rabbit α-Apc3 (Gieffers et al., 1999), , rabbit α-BUB1B (gift from Gregor Kohlmaier), rabbit α-Mad2 (Herzog et al., 2009), goat α-GFP (Poser et al., 2008) and mouse α-GFP (11814460001, Roche), rabbit α-ANAPC16 (Lawo et al., 2009), mouse α-ANAPC2 (Gieffers et al., 1999), and rabbit α-Apc6 (Grossberger et al., 1999).

protein	ID	species	Produced in	Working dilution
Apc2	Apc2-30	H.s.	Mouse (monoclonal)	1:100 (supernatant)
Apc3/Cdc27	3338	H.s.	rabbit	1 µg/mL
Apc4	761	H.s.	rabbit	1 µg/mL
Apc16	2184	H.s.	rabbit	2 µg/mL
BubR1	1676	H.s.	rabbit	1 µg/mL
C11orf51	1006	H.s.	rabbit	2 µg/mL
Cdc20	450/2	H.s.	rabbit	1 µg/mL
Cdh1	452	H.s.	rabbit	1 µg/mL

Commercial antibodies for immunoblotting were used at a concentration of 1 µg/mL and include goat polyclonal GST antibody (Amersham, 27-4577-01), the mouse monoclonal α-Cyclin B1 (GNS1, sc-245 from Santa Cruz Biotechnology), the α-Tubulin antibody (B-512, Sigma), antibody to Aurora B (AIM-1; BD Biosciences) and PCNA (Santa Cruz Biotechnology) as well as an antibody to Histone H3 phosphorylated on serine 10 (05-499, Upstate Biotechnology). Alexa-488 and Alexa-568-labeled secondary antibodies and DAPI for immunofluoresence staining were purchased from Molecular Probes (Invitrogen).

4.2.2 Antibodies for immunofluorescence microscopy

Antibodies were used at the following concentrations: chicken α-EGFP, 1:1000 (kind gift from the laboratory of Dr. Anthony Hyman) (Hutchins et al.); rabbit α-BubR1 (Gramsch Laboratory) kpep 3647, 1:500; mouse α-Cyclin B1 (GNS1, Santa Cruz Biotechnology), 1:1000; rabbit α-Cdc20 (SAT107, Eurogentec) (Gieffers et al., 1999), 1:500; mouse α-Bub1 (MBL International) (Herzog et al., 2009), 1:500. Alexa 488, Alexa 568 and Alexa 633 labeled secondary antibodies as well as DAPI were from Molecular Probes (Invitrogen)

4.3 HeLa cell culture

4.3.1 Cultivation of HeLa-TDS and HeLa Kyoto cells

Adherent HeLa cells were typically grown in 245x245cm tissue culture dishes at 37°C and 5% CO_2 in Dulbecco´s Modified Eagle Medium (DMEM), that is supplemented with 10% (v/v) fetal bovine serum (FBS, from PAA Laboratories GmbH, Pasching, Austria), 0.3 µg/mL L-glutamine (Sigma-Aldrich), 100 untis/mL penicillin (Sigma-Aldrich) and 100 µg/mL streptomycin (Sigma-Aldrich).

4.3.2 Cultivation of HeLa cells expressing human c11orf51-LAP protein

To answer the question if the c11orf51 protein specifically interacts with the APC/C, we made use of a bacterial artificial chromosome (BAC)-transfected cell pool (Poser et al., 2008) expressing tagged c11orf51. Four different cell pools were tested for expression and incorporation into the APC/C. They included the mouse or the human version of the gene, either C-terminally tagged with the LAP-sequence or N-terminally tagged with a FLAP-sequence, which only differs from the LAP-tag in an additional Flag-peptide. The cell pools were kindly provided by the laboratory of Anthony Hyman, MPI Dresden. The LAP-tag is depicted in chapter 2.2, **Figure 2-2**. It is composed of a GFP-tag and S-peptide sequences, which allows performing tandem affinity purification (TAP). The first purification step is via the GFP-moiety, which captures the bait including its associated proteins. Following washing steps, bait-bound proteins are cleaved off the beads by using PreScission protease. The second purification step is via the S-peptide sequence and bound proteins are elution with glycine. The two step purification procedure increases purity and allows for isolating very specific binding partners. The only tagged c11orf51 bait protein that successfully incorporated into the APC/C was the C-terminally tagged human c11orf51 protein (hc11orf51-LAP). The LAP-tag also contains a sequence that encodes for Geneticin resistance. To select for the cells that express the recombinant protein, the growth medium was supplemented with 0.5 mg/mL Geneticin (G418) sulfate (Calbiochem). The hc11orf51-LAP cells were cultivated in 145 x 20 mm (15 cm^2 dishes) round tissue culture dishes at 37°C and 5% CO_2 in Dulbecco´s Modified Eagle Medium (DMEM), supplemented with 10% (v/v) fetal bovine serum (FBS, from PAA Laboratories GmbH, Pasching, Austria), 0.3 µg/mL L-glutamine (Sigma-Aldrich), 100 untis/mL penicillin (Sigma-Aldrich), 100 µg/mL streptomycin (Sigma-Aldrich) and 0.5 mg/mL G418 sulfate. The APC/C was either tandem affinity-purified using the hc11orf51-LAP expressing cell pool or immunoprecipitated from cultured HeLa cells using a c11orf51-peptide specific antibody against the endogenous protein.

4.4 Cell cycle synchronization

4.4.1 Mitotic arrest of HeLa cells

To arrest cells in mitosis with an active spindle assembly checkpoint (SAC-on), one confluent 245x245cm dish of HeLa cells was splitted 1:5 one day before addition of the spindle poison Nocodazole, and treated the next day with 100 ng/mL Nocodazole for 16 – 18 hours. Nocodazole inhibits microtubule polymerization. Hence, drug treated cells cannot form metaphase spindles, leading to a cell cycle arrest in prometaphase. The absence of microtubule attachment to kinetochores activates the spindle assembly checkpoint (see chapter 1.10.4). Detached mitotic cells were collected by shaking the dish ("mitotic shake-off").

4.4.2 Cell cycle synchronization of HeLa cells by double thymidine arrest-release

To arrest cells in the cell cycle stages G1, S and G2, one confluent dish of HeLa cells was split 1:10 into DMEM (containing 10% FBS, 0.3 µg/mL L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin) a few hours before addition of 0.2 mM thymidine (Sigma T1895). Cells were incubated in thymidine under standard conditions for either 24 hours or for 16 hours before they were washed twice with pre-warmed (37°C) PBS and released into fresh DMEM media (10% FBS, 0.3 µg/mL L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin) for 7 hours. Cells were treated a second time with 0.2 mM thymidine for 16 hours and released again into fresh DMEM (10% FBS, 0.3 µg/mL L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin) after two PBS-washes. Cells arrested in early S phase (G1-S) were harvested while they were still in thymidine, G2 phase cells were harvested 4-5 hours after release from the second block, and cells arrested in G1 were harvested 14-15 hours after release.

4.5 FACS analysis

Cells from one 245x245 square plate were resuspended in 20 ml cold PBS and 1/10th to 1/15th of the volume was used for FACS analysis. The cells were centrifuged at 1200 rpm for 4 min in a 15 ml Falcon tube and washed with cold PBS. The pellet was then resuspended in 800 µl PBS and 2.2 mL of cold Methanol (4°C) for fixation. After washing the cells with cold PBS, the pellet was resuspended in 500 µl to 1 mL of PI-buffer (50 µg/mL propidiumiodide, 10 mM Tris-HCl, pH 7.5; 5 mM $MgCl_2$ and 200 µg/mL RNase A) to digest the RNA, and incubated at 37°C for 30 min. The solution can be stored in the dark at 4°C for up to one week. Usually, the sample was analyzed directly using the FACS Calibur system.

4.6 Immunofluorescence microscopy

HeLa cells were grown on 22 mm coverslips and fixed with 4% paraformaldehyde in PBS at RT. Cells that were arrested in G1-S phase of the cell cycle, were fixed in -20°C methanol for 20 min. Cells from the mitotic arrest experiment were prepared on coverslips using the Cytospin centrifuge (Thermo Shandon) at a speed of 1000 rpm, for 5 min. Kyoto cells that were used for loss-of-function studies were grown on 22 mm poly-L-lysine-coated coverslips (Sigma-Aldrich). After fixation in 4% PFA, the cells were washed with PBS, permeabilized with 0.1% Triton-X100 in PBS for 10 min and thereafter blocked for 1hour in 3% BSA-PBS solution containing 0.01% Triton-X100. Coverslips were incubated for 1 hour at RT with primary antibodies in 3% BSA-PBS solution and detected using Alexa 488 and Alexa 568 labeled secondary antibodies (Invitrogen). DNA was counterstained with DAPI and slides were mounted using ProLong Gold (Invitrogen Molecular Probes).

4.7 Protein depletion by esiRNA

EsiRNA´s are enzymatically synthesized siRNA´s (Heninger and Buchholz, 2007; Kittler et al., 2007; Yang et al., 2002) which are reported to result in high transfection efficiency with

minimal cross-target effects. HeLa Kyoto cells or hc11orf51-LAP cells were seeded in a 6-well plate (Nunc) at a concentration of $5x10^4$ cells/well in DMEM supplemented with 10% FCS and 0.2 mM L-glutamine 16h before transfection. The media of the hc11orf51-LAP cells contained 100 U/mL penicillin, 100 µg/mL streptomycin, and 500 µg/mL G418 in addition. For transfection, the media of the hc11orf51-LAP cells was changed to media without antibiotics four hours before addition of esiRNA. RNAi depletion was performed using Oligofectamine (Invitrogen) as the transfection reagent and 3500 ng esiRNA of either human Apc6/Cdc16 or human c11orf51. Control transfections included Firefly-Luciferase and Eg5, using a concentration of 1800 ng esiRNA, each. Eg5 is a motor kinesin protein and was used as transfection efficiency control. Cells were harvested 48h or 72h after transfection for Western blot analysis. The esiRNA's were a gift from Dr. Mirko Theis and Dr. Frank Buchholz, MPI Dresden, Germany. The sequences of the long dsRNA's that got digested with RNase III to obtain esiRNA's were the following:

c11orf51 (Ensembl-ID: ENSG00000110200)

CCACTTTGTTCCCCTCACTCTTCCCTCGTGTGACTGAGACTCTGTGGTTTAATCTG
GATCGACCCTGTGTGGAAGAGACAGAGCTGCAGCAGCAGGAACAGCAGCATCAG
GCCTGGCTCCAAAGCATCGCGGAGAAAGACAACAACCTGGTTCCTATTGGCAAG
CCAGCCTCAGAGCACTATGATGACGAGGAAGAAGAGGATGATGAAGATGATGAG
GATAGTGAAGAGGACTCAGAGGATGATGAGGATATGCAGGACATGGACGAGATG
AATGACTACAATGAGTCACCGGATGATGGAGAGGTCAATGAGGTGGACATGGAA
GGCAACGAACAGGATCAGGACCAGTGG

Apc6 (Ensembl-ID: ENSG00000130177)

AACAGGAATTGCTGCGTTTTCTATTTGAGAACAAATTGAAAAAATATAATAAGCC
TAGTGAAACGGTCATCCCTGAATCTGTAGATGGCTTGCAAGAGAATCTGGATGTG
GTAGTGTCTTTAGCTGAGAGACATTATTATAACTGTGATTTTAAAATGTGCTACAA
GCTTACTTCTGTAGTAATGGAGAAAGATCCTTTCCATGCAAGTTGTTTACCTGTAC
ATATAGGGACGCTTGTAGAGCTGAATAAAGCCAATGAACTTTTCTATCTTTCTCAT
AAACTGGTGGATTTATATCCTAGTAATCCTGTGTCTTGGTTTGCAGTGGGATGTTA

CTATCTCATGGTCGGTCATAAAAATGAACATGCCAGAAGATATCTCAGCAAAGCC
ACAACACTTGAGAAAACCTATGGACCTGCATGG

Eg5 (Ensembl-ID: ENSG00000138160)

TCCCCGTAACAAGAGAGGAGTGATAATTAAAGGTTTAGAAGAAATTACAGTACA
CAACAAGGATGAAGTCTATCAAATTTTAGAAAAGGGGGCAGCAAAAAGGACAAC
TGCAGCTACTCTGATGAATGCATACTCTAGTCGTTCCCACTCAGTTTTCTCTGTTA
CAATACATATGAAAGAAACTACGATTGATGGAGAAGAGCTTGTTAAAATCGGAA
AGTTGAACTTGGTTGATCTTGCAGGAAGTGAAAACATTGGCCGTTCTGGAGCTGT
TGATAAGAGAGCTCGGGAAGCTGGAAATATAAATCAATCCCTGTTGACTTTGGGA
AGGGTCATTACTGCCCTTGTAGAAAGAACACCTCATGTTCCTTATCGAGAATCTA
AACTAACTAGAATCCTCCAGGATTCTCTTGGAGGGCGTACA

4.8 Protein depletion by siRNA

HeLa Kyoto cells were seeded in a 6-well plate (Nunc) at a concentration of $5x10^4$ cells/well or on 100 x 20 mm round plates (BD Falcon) at a concentration of about 3.5×10^5 in DMEM supplemented with 10% FCS and 0.2 mM L-glutamine 16h before transfection. For the hc11orf51-LAP cell pool the media additionally contained 100 U/mL penicillin, 100 µg/mL streptomycin, and 500 µg/mL G418. At the day of transfection, the media of the hc11orf51-LAP cells was changed to media without antibiotics. RNAi depletion was performed using a mixture containing four preannealed siRNA oligos targeting different sequences of c11orf51 (Dharmacon;NM_014042):

1.CUACAAUGAGUCACCGGAU

2.UCGCGGAGAAAGACAACAA

3.GGACAUGGAAGGCAACGAA

4.GGAUCGACCCUGUGUGGAA.

Firefly-Luciferase (GL2; Ambion, Cat. No. AM16106) was used as control siRNA and Lipofectamine RNAiMax (Invitrogen) as the transfection reagent. Cells grown in 6-well plates were transfected using 50 nM siRNA; cells grown on 100 x 20 mm plates were treated with 40 nM siRNA. Five hours after transfection, the media was changed to DMEM containing all supplements and the cells were harvested 48h or 72h after transfection.

For Apc3-immunoprecipitation experiments and Western blot analysis, HeLa Kyoto cells were seeded at the respective concentration in 100 x 20 mm plate one day before transfection in growth medium without antibiotics. RNAi depletion was performed using 40 nM of c11orf51 (Dharmacon)- or GL2-siRNA (Ambion). GL2 is Firefly-Luciferase and served as control. The cells were either harvested 48h after transfection or they were treated with 100 ng/µL Nocodazole 32h after transfection. Nocodazole-treated cells were further incubated for 16h and collected by mitotic shake-off 48h after siRNA-transfection.

4.9 Live cell imaging after RNAi

HeLa cells stably expressing histone H2B-mCherry and mouse TUBB-LAP were used for live cell imaging analysis (histone H2B-mCherry plasmid is a gift from Dr. Jan Ellenberg). H2B-mCherry TUBB-LAP HeLa cells were reverse transfected with esiRNA for Cdc16/Apc6, c11orf51, Eg5 (Kiff11), and Firefly Luciferase at 100 nM using oligofectamine reagent (Invitrogen). The cells were cultured in µ-Slide 8 well (ibidi GmbH, Martinsried, Germany). Live cells were filmed after 24 and 48 hours on a DeltaVision sectioning microscope system equipped with an IX71 microscope (Olympus) and a CoolSnap HQ CCD camera (Photometrics). Time-lapse images were taken at 5 min intervals and 13 sections with 1 µm steps along z axis, deconvolved, and maximally projected to generate movies. All cells in the movies were manually annotated for the cell cycle stage to measure the duration of mitosis (from NEBD until anaphase onset) and mitotic exit (from anaphase until the loss of Microtubule Bridge of daughter cells).

To confirm that the knock-down of the RNAi target genes were specific, whole cell lysate of the RNAi treated cells was resolved in SDS-PAGE and transferred onto a nitrocellulose

membrane. The membrane was incubated with primary and secondary antibodies, using α-GFP (mAb, Roche) and α-mouse IgG-HRP conjugate (BioRad, 171-6516), respectively. ECL reagent (GE healthcare) was used for the developing step.

4.10 Sucrose density gradient centrifugation

Sucrose density gradients were prepared in ultra-clear centrifuge tubes (14 x 95 mm, Beckman) by mixing two sucrose solutions using a GradientMaster (Biocomp). Cell extract supernatants were centrifuged at 42 000 rpm (TLA45 rotor) for 15 min in an Optima MAX ultracentrifuge (Beckman Coulter). Supernatant containing 2.4 mg protein was layered on a 10-30% sucrose gradient in TBS-Tween (0.01%). Gradients were centrifuged at 34 000 rpm for 18h 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 at flow rate of 1 mL/min.

4.11 Recombinant protein expression in *E.coli*

To obtain human cyclin B1 as a substrate protein for the ubiquitylation assay, the N-terminal fragment (aa 1-87) of human cyclin B1, fused to a myc-his-tag was expressed in *E.coli*. GST-Hsl1 (aa 667-872) is a fragment of budding yeast protein Hsl1 containing a D-box and a KEN-box motif (Burton and Solomon, 2001), fused *N-terminally* with a GST (glutathione-S-transferase) tag. The constructs were expressed and purified from *E.coli* BL21(DE3) strain. An overnight starter culture was diluted into two liters of LB medium to OD600 = 0.1. The culture was grown at 37°C, until the OD600 reached 0.6. Protein expression was then induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and proteins were expressed for two hours at 37°C and atmospheric air on a shaker.

4.12 Protein purification

4.12.1 Antibody coupling to Protein A beads

The antibodies were first bound and then cross-linked to Affiprep protein A beads (BioRad). One volume protein A beads was washed twice with 10 volumes TBS-T (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20) for equilibration. Beads were resuspended in 10 volumes TBS-T, and affinity purified antibodies were added in a ratio of 1.33 µg antibody per 1 µl beads. The beads were incubated on an end-over-end rotary shaker for one hour at room temperature and subsequently washed three times with TBS-T. After two additional washes with 20 volumes of 0.2 M sodiumborate solution (titrated to pH 9 with HCl), the beads were resuspended in 20 volumes of the same buffer. After the beads had settled down, a small amount of the buffer was used to resuspend solid dimethylpimelimidate (Sigma-Aldrich), which was used at a final concentration of 20 mM to initiate the cross-linking reaction. This solubilized powder was immediately added to the beads and the mixture was rotated at room temperature for 30 minutes. To stop the cross-linking reaction, the beads were incubated twice with 20 volumes of a buffer containing 200 mM Tris-HCl pH 7.5 and 150 mM NaCl with an incubation time of 10 minutes each, on an end-over-end rotatory shaker. The antibody-coupled beads were washed twice with TBS-T and stored at 4°C in TBS-T containing 0.05% NaN_3.

4.12.2 Purification of human APC/C from HeLa cells

HeLa cell pellets grown from the cell cycle stages G1, G1/S, and G2, were thawn on ice and were resuspended in 700 µl / 1g pellet of CytoBuster Protein Extraction Reagent (Novagen). Protease inhibitors (0.1 M PMSF, 20 µg/mL of each aprotinin, pepstatin, and leupeptin) were added to the suspension and the cells were incubated on an end-over-end rotary shaker for 20 min at 4°C to be lysed. Cells arrested in mitosis by means of nocodazole treatment were resuspended in 500 µl – 600 µl / 1g of CytoBuster reagent. In addition, protease inhibitors

(0.1 M PMSF, 20 µg/mL of each aprotinin, pepstatin, and leupeptin) as well as phosphatase inhibitors (4 µg/mL okadaic acid (Alexis), 20 mM NaF, 20 mM beta-glycerophosphate, 10 mM Na-pyrophsophate, 1 mM Na_3VO_4) were added to the suspension. The extract was cleared by centrifugation at 14.500 rpm for 40 min at 4°C. APC/C was isolated with antibodies against Cdc27/Apc3 or c11orf51. Antibodies were coupled to protein A beads (BioRad) and cross-linked using DMP as previously described (Harlow, 1988). The concentration of the HeLa cell extract that was obtained with this method typically ranged from 14 mg/mL to 22 mg/mL. The cleared lysate was further incubated on an end-over-end rotary shaker at 4°C for 60-90 min with 1/10 volume of antibody-coupled beads, followed by washes with TBS-T (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.01% Tween-20 (Sigma-Aldrich), 10% glycerol). The APC/C was eluted off the beads by either using 1.3x the bead volume of 0.1M Glycine-HCl pH 2.2 solution, followed by neutralization of the lysate with $1/10^{th}$ volume 1.5 M Tris pH 9.2, or by two times 30 min incubation with 1.5x–2x the bead volume using 1 mg/mL antigenic peptide buffered solution (20 mM Tris-HCl, pH 7.5; 150 mM NaCl, 5% glycerol, 0.1% Tween-20 (Sigma-Aldrich), 0.5 mM DTT) with pH 7.5. About 20% of the eluate was analyzed by SDS-PAGE and silver staining. The remaining eluate was further used for Western blotting.

The iTRAQ reagent used for quantitative MS/MS analysis of human APC/C reacts with primary and secondary amines. Therefore, Tris buffer was replaced by 1x PBS for washing steps and elution was performed with 0.1M hydrochloric acid (HCl). Eluted protein was neutralized using an equimolar amount (to the elution solvent) of TEAB (triethylammonium bicarbonate).

4.12.3 Tandem Affinity Purification of human APC/C using the hc11orf51-LAP cell pool

LAP-purification was performed using HeLa cells expressing C-terminally LAP-tagged human c11orf51. Cells were extracted in one pellet volume of lysis buffer (50 mM HEPES-KOH, pH 7.5; 5 mM EDTA, 150 mM KCl, 10% glycerol, 1% Triton X-100, 20 mM beta-glycerophosphate, 10 mM NaF, 10 mM Na-pyrophosphate, 0.1 mM PMSF, 1 mM Na_3VO_4, 1

mM DTT and PIM). The extract was cleared by centrifugation at 14.500 rpm for 40 min at 4°C and further incubated using 1/10th of the lysate volume of GFP-beads for 1 hour at 4°C on an end-over-end rotary shaker. After this first purification step, the beads were washed 3x with wash buffer (50 mM HEPES-KOH, pH 7.5; 5 mM EDTA, 150 mM KCl, 10% glycerol, 0.05% NP-40, 20 mM beta-glycerophosphate, 10 mM NaF, 10 mM Na-pyrophosphate, 0.1 mM PMSF, 1 mM Na_3VO_4, 1 mM DTT and PIM) and 2x with cleavage buffer (wash buffer without PIM). For cleavage, the beads were transferred to 1.5 mL low-retention eppendorf tubes and 300 µl cleavage buffer was added per 50 µl beads. After addition of 3 µl PreScission Protease, the beads were incubated for 30 min at 4°C on an end-over-end rotary shaker. After centrifugation at 1000 rpm for 2 min, the supernatant was saved and the beads were washed one additional time with 100 µl cleavage buffer. Both solutions were combined and incubated with 50 µl S-protein beads for 1 hour at 4°C, rotating. The beads were again washed 3x using wash buffer and 3x with 150 mM KCl to remove the detergents for later MS/MS analysis. For elution, the beads were incubated two times for 5 min at 4°C on an end-over-end rotary shaker with 1.3x bead volume of 0.1M Glycine-HCl pH 2.2 solution. Both elutions were combined and the pH was neutralized by addition of 1/10th the elution volume using 1.5 M Tris-HCl pH 9.2. The final pH that was suitable for MS/MS was 8.0. About 20% of the eluate was analyzed by SDS-PAGE and silver staining; the remaining 80% was subjected to in-solution digest using trypsin.

4.13 iTRAQ labeling and protein digestion

iTRAQ (isobaric tag for relative and absolute quantitation) is a quantitative Mass Spectrometric approach to identify and quantify proteins from different sources in one single experiment making use of isotope coded covalent tags (Ross et al., 2004) (see chapter 2.7 for details). For quantitative mass spectrometric analysis of the APC/C during four different cell cycle stages, proteins were tryptically digested and the resultant peptide mixture was labelled using reagents from the iTRAQ reagent kit (Applied Biosystems; Foster City, Ca, USA) as described (Koecher et al., 2009; Ross et al., 2004). Protein eluates were adjusted to pH 8 using 0.5 M triethylammonium bicarbonate (TEAB). Disulfide bonds were reduced in 5 mM

Tris-(2-carboxyethyl)phophine (TCEP) for 1 hour at 60°C, followed by alkylation of the cysteine residues with 10 mM methylmethanethiosulfonate (MMTS) for 30 min at room temperature. Proteins were digested with mass spectrometry-grade modified trypsin (Promega, Madison, WI) at 37°C over night. For labelling, each iTRAQ reagent was dissolved in 70 µl pure ethanol (Merck KGaA, Darmstadt, Germany) and added to the respective peptide mixture. Prior to LC-MS/MS, the labelling reactions were stopped with 0.1% TFA and the four samples were mixed. Ethanol was removed by drying down the solvent to approximately 5 µl in a vacuum centrifuge. The peptide mixtures were again dissolved in 25 µl 0.1% TFA and analyzed by LC-MS/MS.

4.14 HPLC and Mass Spectrometry

All Nano-HPLC-MS/MS analysis were performed on an UltiMate 3000 RSLCnano LC system (Dionex), equipped with an analytical column (Acclaim PepMap C18, 25 cm x 75 µm x 2 µm, 100 Å, Dionex) with the following mobile phases for chromatographic separation: A: 2% acetonitrile, 0.1% formic acid; and B: 80% acetonitrile, 0.08% formic acid and 10% trifluoroethanol. Loading buffer used contains 0.1% trifluoroacetic acid (Pierce).

For analysis of iTRAQ-labeled proteins, a 300 minutes gradient from 100% A to 40% B was used, followed by a short gradient to 90% B (5 min). The HPLC was directly coupled to a nano-electrospray ionization source (Proxeon, Odense, Denmark) mounted on a LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) operating in positive ionization mode. The MS survey scan was performed in the Orbitrap recording a window between 400 and 2000 *m/z*. The resolution was set to 60,000 and the automatic gain control was set to 1,000,000 ions with a maximal acquisition time of 400 ms. Eluting peptides were analyzed in data-depended MS/MS acquisition mode. Minimum MS signal for triggering MS/MS was set to 500 and m/z values were put on an exclusion list for 240 s. In all cases one micro-scan was recorded. The lock mass option was enabled for both MS and MS/MS mode and polydimethylcyclosiloxane ions (protonated $(Si(CH_3)_2O_6)$; m/z 445.120025) were used for internal recalibration of the mass spectra (Olsen et al., 2005). Collision induced dissociation (CID) was performed with a target value of 3,000 in the linear ion trap, maximal acquisition

time 200 ms, collision energy of 35%, Q value of 0.25 and an activation time of 10 ms. HCD (Higher Energy Collision Dissociation) was performed with a target value of 100,000 in the Orbitrap, resolution of 7,500, maximum acquisition time of 250 ms and a collision energy of 35%.peptide.

Mass spectrometric analysis were conducted either on a hybrid linear ion trap/Fourier transform ion cyclotron resonance (FTICR) mass spectrometer with a 7-Tesla superconducting magnet (LTQ-FT Ultra) or on a hybrid linear ion trap/Orbitrap mass spectrometer (both ThermoElectron, Bremen, Germany). The mass spectrometer was equipped with a nano-electrospray ionization source (Proxeon Biosystems, Odense, Denmark). Metal coated nano ESI needles were used (New Objective, Woburn, MA, USA).

For LC separation, samples were loaded onto the trap column at a flow rate of 20 µL/min of loading buffer and were washed for ten minutes. Thereafter, the sample was eluted from the trap column and separated on the separation column with a gradient from 0% to 35% mobile phase B in 85 minutes followed by 35% to 60% in 5 minutes at a flow rate of 300 nL/min.

4.15 Protein identification, data interpretation and protein quantification

Proteins were identified searching the data against the human International Protein Index (IPI) database using Mascot 2.2.0 (Matrix Science, London, UK), against a customised protein sequence database comprising the complete human sequences from Swiss-Prot, TrEMBL, PIR, GenBank, EMBL, DDBJ, RefSeq and Celera (hKBMS), plus the Swiss-Prot entries corresponding to the human ‘bait’ protein c11orf51. In all cases a peptide mass tolerance of 5 ppm was used and fragment ion masses were searched with a 0.5 Da mass window.

For the iTRAQ-labeled proteins, one missed cleavage site for trypsin was allowed. Methylthio-cysteine and iTRAQ reagent labeling at the N-terminus and lysine residues were set as fixed modifications. Variable modifications included oxidation of methionine, phosphorylation of serine, threonine, and tyrosine. Identified proteins were grouped and further analyzed with Protein Center (v2.5.0. Proxeon Biosystems, Odense, Denmark).

Protein grouping was based on 98% homology. Quantitation of iTRAQ labelled peptides was performed with Mascot using the isotopic corrections supplied by the manufacturer (Applied Biosystems; Foster City, Ca, USA).

Data interpretation for iTRAQ: For each selected precursor ion a CID and a HCD spectrum was recorded in order to maximize the number of identified and quantified peptides (Koecher et al., 2009). Both spectra were merged to a combined CID-HCD spectrum in order to obtain quantitative information for all CID spectra. In order to generate CID-HCD data sets, data processing was performed using a Perl script (QuantMerge) (Koecher et al., 2009). In short, intensities from the 4 iTRAQ reporter ions, *m/z* 114.112, 115.1083, 116.116 and 117.1150 were extracted from the mgf-file of each HCD spectrum with a mass tolerance of 10 mDa. The intensities of the reporter ions were normalized to 1 and were combined into the corresponding CID spectrum, deleting at the same time the respective *m/z* region of the original CID spectrum. Details of the procedure can be found in (Koecher et al., 2009).

For MS analysis using c11orf51 as "bait", two missed cleavage sites for trypsin were allowed. Carbamidomethylation on cysteine was set as a fixed modification, oxidation of methionine as a variable modification. Monoisotopic masses were searched within unrestricted protein masses for tryptic peptides. The generation of dta-files for Mascot was performed using the Extract MSn program (version 4.0, Thermo Scientific).

4.16 Electron microscopy

4.16.1 Preparation of APC/C for electron microscopy

HeLa cell lysate of unsynchronized cells was prepared as described in chapter 4.12.2 and the APC/C was isolated using Cdc27/Apc3 antibody-coupled beads. Bound protein was washed four times with 50 bead volumes of TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.5 mM DTT). The APC/C was eluted twice for 15 minutes at 4°C each, using twice the bead volume of a Cdc27 peptide solution in TBS (1 mg/mL stock concentration). Purified APC/C was directly used for antibody labelling.

4.16.2 Localization of c11orf51p on the APC/C by antibody labelling

Peptide eluted human APC/C preparations were incubated with decreasing concentrations of the c11orf51-peptide specific antibody (ID 1006, glycine elution) starting at a molar APC/C to IgG ratio of 2:1. At an antibody concentration that yielded a high percentage of APC/C dimers, the samples were processed using the *GraFix* protocol (Kastner et al., 2008) and analyzed by negative staining EM. To ensure that APC/C-antibody complexes were not formed by accident due to a large number of APC/C molecules, the concentration of particles on the EM grid was chosen to be very low. To determine the antibody binding site in this labeling experiment, 200 – 300 APC/C-antibody complexes were selected. A more detailed localization study of c11orf51 is currently under investigation.

4.17 *In vitro* APC/C activity assay

4.17.1 Oxidative Iodination of proteins

An N-terminal fragment of human cyclin B1 (aa 1-87, myc-his6-tagged) was iodinated using the chloramines T method according to Parker (Parker, 1990). 5 µg of the protein was incubated in a 30 µl reaction containing 250 mM Tris-HCl, pH 8.0; 3 µl Chloramine T (10 mg/mL in H_2O) and 3 µl Na^{125I} (DuPont NEN, NEZ-033A, pH 8-10, 100 µCi/µL) for 3 min at RT. To stop the reaction, 10 µl of 1 M DTT was added, and the solution was dialyzed two times for 2 hours each in a Slide-A-Lyzer Mini Dialysis unit with a molecular weight cut off of 3.5 kDa (Pierce, Rockford, IL) against 2x 250 mL of XB buffer (10 mM HEPES-KOH, pH 7.7; 100 mM KCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 1 mM DTT). The iodinated protein was then mixed in a ratio (v/v) of 1:1 with 87% glycerol and stored at -20°C.

4.17.2 APC/C ubiquitylation assay

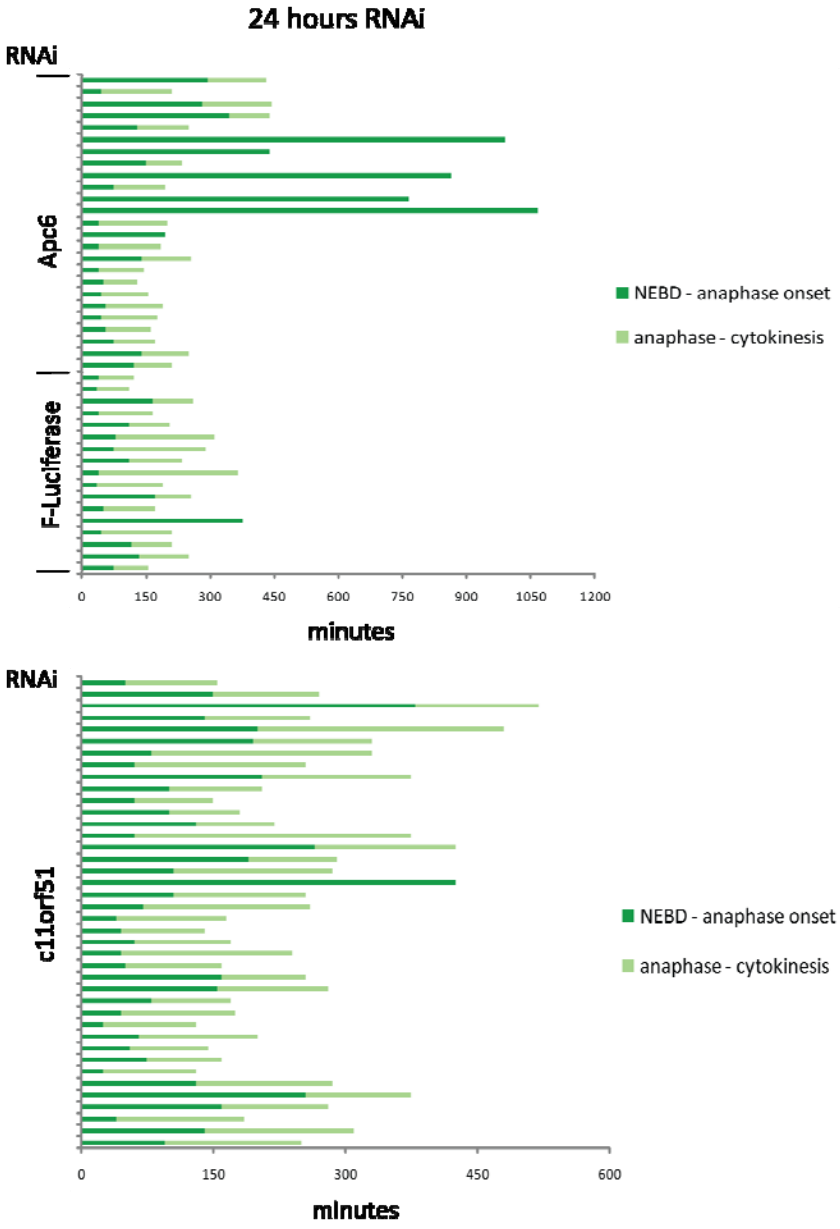
For ubiquitylation assays, 2.5-5 µl of peptide eluted APC/C after either c11orf51 or Cdc27/Apc3 immunoprecipitations was incubated in 10 µl XB buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl, 0.02% Tween-20) containing 10 µg ubiquitin (Sigma), ATP regenerating system (7.5 mM creatine phosphate, 1 mM ATP, 1 mM $MgCl_2$, 0.1 mM EGTA, 30 U/mL rabbit creatine phosphokinase type I (Sigma), 0.25 µg His_6-E1, 1 µg of E2 (His_6-UbcH10 or a mixture of His_6-UbcH10 and His_6-Ubc4) and 0.2 µg purified FZR1/Cdh1 (as indicated). An iodinated fragment of human cyclin B (aa 1-87, myc-his_6-tagged) was used as a substrate. Reactions were incubated in a thermomixer (1500 rpm, 37°C) for the times indicated and the reaction was stopped by addition of 4x SDS-sample buffer (125 mM Tris-HCl, pH 6.8; 4% SDS, 20% glycerol, 200 mM DTT, 0.02% Bromphenolblue). The quantification of the ubiquitylation reaction was analyzed by SDS-PAGE and phosphorimaging.

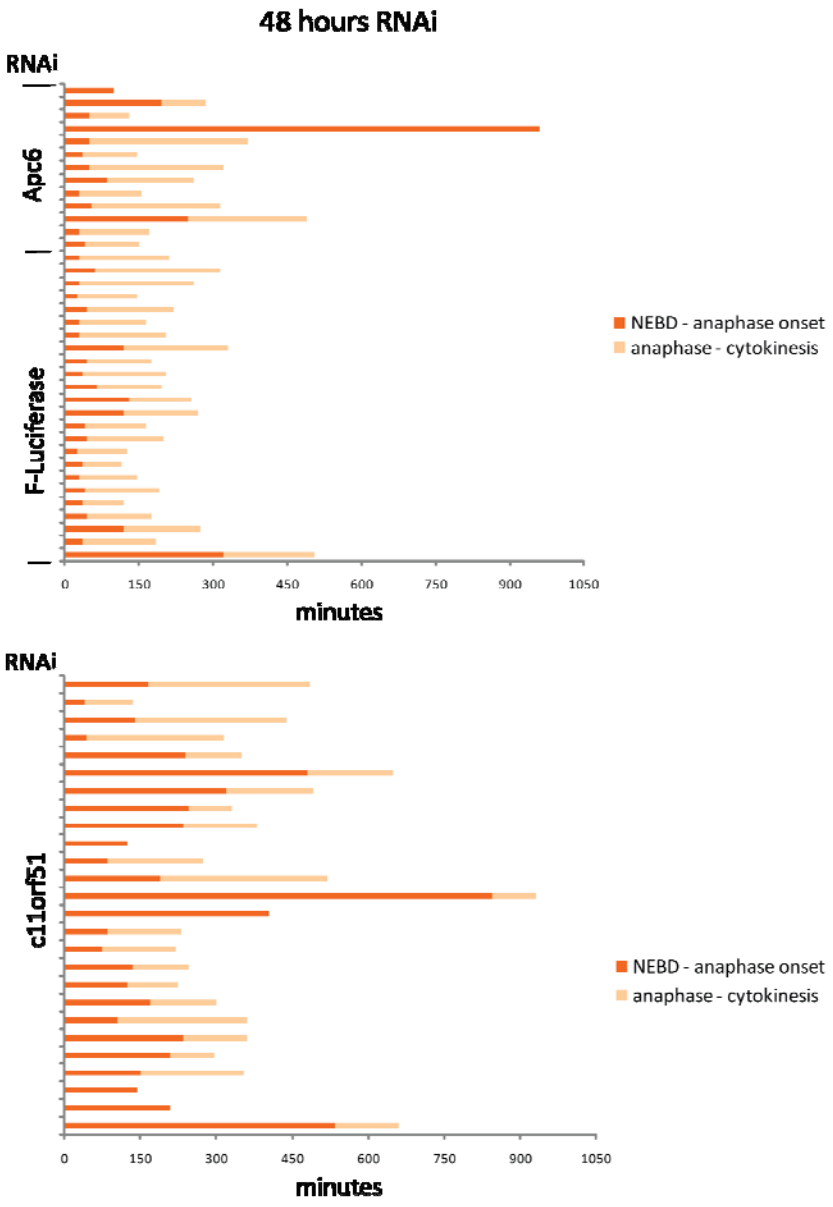
4.17.3 Bioinformatic sequence alignment of c11orf51

Alignment was performed using MAFFT (Katoh, Misawa, Kuma, Miyata 2002 Nucleic Acids Res. 30:3059-3066) and was colored using a clustal-like coloring schema. Conserved secondary structure elements were predicted using JNET and are indicated above the alignment. Structural prediction was performed using the metaPrDOS-Server (http://prdos.hgc.jp/cgi-bin/meta/top.cgi).

5 Appendix

Manual annotation of the time that HeLa cells spent in mitosis (NEBD to anaphase onset) and the time they needed to exit mitosis (anaphase to cytokinesis) after 24h and 48h RNAi (live cell imaging experiment, chapter 2.8.1)




48 hours RNAi
RNAi
Apc6
F-Luciferase
0
150
300
450
600
750
900
1050
minutes
NEBD - anaphase onset
anaphase - cytokinesis
RNAi
c11orf51
0
150
300
450
600
750
900
1050
minutes
NEBD - anaphase onset
anaphase - cytokinesis

6 Abbreviations

Å	Ångström
Ab	antibody
Ama1	activator of meiotic APC protein-1
amp	ampicilin
APC/C	anaphase-promoting complex/ cyclosome
ATP	adenosine triphosphate
a.u.	arbitrary unit
A_{260}, A_{280}	absorption at 260/280 nm
bp	base pairs
BSA	bovine serum albumin
cDNA	complementary DNA
cdc	cell devision cycle
Cdk	cyclin dependent kinase
2D / 3D	two-dimensional / three-dimensional
DAPI	4´,6´-diamino-2-phenylindol
DMEM	Dulbecco´s Modified Eagle Medium
DMP	dimethylpimelimidate
DNA	deoxyribonucleic acid
DMSO	dimethylsulfoxyde
Doc1	degradation of cyclin B protein-1

DTT	dithiothreithol
DUB	deubiquitinating enzyme
E1	ubiquitin-activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin ligase
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
EM	electron microscopy
FACS	Fluorescence activated cell sorting
fl	full length
GST	glutathione-S-transferase
Gly	glycine elution (of purified antibody)
h	hour
HCl	hydrochloric acid
His_6	His-tag comprising 6 histidines
HECT	homology to E6-AP C-terminus
Hepes	N-2-hydroxyethylpiperazin-N´-2-ethane sulphonic acid
H.s.	*homo sapiens*
HU	hydroxyurea
IPTG	isopropyl β-D-1-thiogalactopyranoside
IR	isoleucine-arginine
iTRAQ	isobaric tags for relative and absolute quantification
IVT	*in vitro* translation

kDa	kilo Dalton
LAP	localization and affinity purification
LB	Luria-Bertani
log	logarithmic
MCC	mitotic checkpoint complex
MDa	mega Dalton
Mnd2	meiotic nuclear division protein-2
min	minute
MR	methionine-arginine
Noc	nocodazole
OD_{600}	optical density at 600 nm
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pds1	precocious dissociation of sister chromatids
PFA	paraformaldehyde
PMSF	phenylmethylsulphonyl fluoride
PVDF	polyvinylidene fluoride transfer membrane
RING	really interesting new gene
RNA	ribonucleic acid
Rpn	regulatory particle non-ATPase
rpm	rounds per minute

RT	room temperature
S	Svedberg
SAC	spindle assembly checkpoint
S. cerevisia / S.c.	*Saccharomyces cerevisiae*
SCF	Skp1/Cul1/F-box
SDS	sodium dodecyl sulphate
S. pombe / S.p.	*Schizosaccharomyces pombe*
Swm1	spore wall maturation protein-1
TAP	tandem affinity purification
TBS	tris-buffered saline
TBS-T	tris-buffered saline supplemented with Tween20 detergent
TEAB	triethylammonium bicarbonate
TEV	tobacco etch virus
TPR	tetratrico peptide repeat
Tris	tris-(Hydroxymethyl)aminomethane, 2-amino, 2-(hydroxymethyl), 1-3-propandiol
tRNA	transfer RNA
UV	ultraviolet
v/v	volume per volume
WB	Western blot
WD	tryptophane aspartate
Wt	*wild type*

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Acknowledgements

I would like to thank Jan for his support during my PhD work and for giving me the chance to work on this fascinating complex. His encouragement and his advices have been valuable over the last years. It was inspiring to work in his lab and I learned from him how to analyze and interpret data critically.

I am grateful to the members of my PhD committee, Dr. Stefan Westermann, Dr. Peggy Stolt-Bergner and Dr. Kristina Djinovic-Carugo for their ideas and scientific support in our annual meetings; and to Dr. Geert Kops for kindly agreeing to review this thesis.

I would like to acknowledge our collaborators Dr. Holger Stark for the antibody labeling and cryo-EM structure as well as the following members from the laboratory of Dr. Anthony Hyman (MPI Dresden, Germany): Dr. Yusuke Toyoda for his contribution on the RNAi / live cell imaging experiments, Dr. Mirko Theis for synthesizing the esiRNAs and Dr. Ina Poser for generating and providing the hc11orf51-LAP cell pool. Many thanks to Dr. Thomas Köcher, not only for the iTRAQ labeling and the MS/MS analysis, but also for helpful discussions and for making me laugh when I needed it most.

I would like to thank all the members from the Peters lab, past and present, for creating a very nice working atmosphere and for fun moments in and outside the lab. I am indebted to René for his support on the immunofluorescence microscopy part. I would like to acknowledge Venu for his advices and for always being willing to discuss experiments with me. Thanks go to Georg for performing the antibody labeling in Göttingen and to all other members of the APC/C-group.

Finally, I would like to thank my parents for their constant support and their encouragement throughout my studies. Heartfelt thanks to my friends, especially Cosmas Damian Arnold, Elena Romagnoli, Jesus Fernandez-Rodriguez, Marlene Kelnreiter, and Robert Ananda Lindenthal for their understanding and for unforgettable and joyful moments that were tremendously valuable, especially when times were tough.

Curriculum Vitae

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