Analysis of Rca1 function at the G1-S transition in *Drosophila melanogaster*

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Abstract

Tight control of APC/C-Cdh1^{Fzr} activity is essential for progression through mitosis and establishment of the G1 phase. Rca1 is a nuclear protein that inhibits the APC/C-Cdh1^{Fzr} complex during G2 to allow cyclin accumulation and subsequent entry into mitosis. In this thesis, a localisation study of Rca1 was performed revealing that a nuclear localisation sequence (NLS) and other domains in the protein mediate efficient nuclear accumulation.

Besides its function in G2, Rca1 expression can promote S phase entry. Rca1 belongs to the family of F-box proteins that mediate substrate specificity in SCF (Skp-Cul1-F-box) ubiquitin ligases. Functional studies demonstrated that the F-box is crucial for S phase induction by Rca1, suggesting that Rca1 has a secondary function in an SCF complex.

A major part of this thesis was devoted to characterise the putative SCF/Rca1 complex and its target proteins. In a yeast two-hybrid screen, the SCF subunit SkpA was identified as a binding partner of Rca1. Coimmunoprecipitation studies confirmed this interaction and indicated moreover that SkpA binding relies on the F-box in Rca1. Furthermore, endogenous Cul1 coprecipitated with Rca1 and this also in an F-box dependent manner. Altogether, these experiments demonstrated that Rca1 forms a complex with the SCF core subunits SkpA and Cul1. The SCF/Rca1 complex could promote S phase entry by degrading a negative regulator of the G1-S transition. Cyclin A/Cdk1 is a potent S phase inducer, but its activity is normally dampened by the CKI Rux, inhibitory phosphorylation by Wee and APC/C-Cdh1^{Fzr} dependent degradation of Cyclin A. Protein levels of these S phase inhibitors were not altered by coexpression of Rca1 suggesting that these proteins are not targets of the SCF/Rca1 complex.

Overexpression of Rca1 in larval salivary glands results in impaired endoreplication and accumulation of Cyclin A, Cyclin E and Cdk1. Expression profiling revealed that mitotic genes (e.g. Cyclin A/B, Cdk1) are normally downregulated in salivary glands, but ectopic Rca1 expression promotes transcription of these genes. In addition, qRT-PCR analysis showed elevated transcript levels of the E2F1 targets Rnr2 and PCNA, suggesting that Rca1 overexpression leads to increased E2F1 activity. Cyclin E is also a known E2F1 target and hence, this might explain the marked increase in Cyclin E transcript levels. Finally, the gene expression analysis indicated that components of the APC/C and its target Geminin were present in larval salivary glands, thus supporting the idea that rereplication in endoreplicating cells is controlled by Geminin and the APC/C-Cdh1^{Fzr} complex.

1. Introduction

1.1 The cell cycle

Cell proliferation is a fundamental process for the development of all organisms and function and maintenance of life. Cells arise by division of already existing cells and the resulting daughter cells contain the same genetic information as their progenitors. In unicellular organisms, cell division results in an entire new organism. However, multicellular organisms develop by countless divisions of a founder cell giving rise to a vast number of cells that make up the tissues and organs. Furthermore, cell proliferation enables self renewal of various tissues throughout the whole lifetime of an adult organism including the haematopoetic system or skin and intestinal epithelium. Cell reproduction occurs by a well defined series of events, a process that is also known as the cell cycle. All cell cycle events have to take place in correct order and timing to ensure precision and reliability over generations. These requirements are achieved by a complex regulatory network, the cell cycle control system. Different extrinsic and intrinsic factors like nutritional status, growth factors, cell density and developmental state promote or restrain cell division, cell cycle exit or apoptosis. Defects in the system can lead to abnormal, uncontrolled cell growth and cell proliferation during development or adulthood and eventually promote tumorigenisis.

The cell cycle can roughly be divided into two broad periods: First, cells increase their cell mass and DNA is duplicated, whereas in the following step the cell splits itself into two daughter cells consisting of the same DNA content. In a standard cell cycle both processes are tightly coupled and consist of four distinct phases: G1 (Gap1) phase, S (Synthesis) phase, G2 (Gap2) phase and M (Mitotic) phase (Figure 1.1). The first three stages G1, S, G2 are collectively known as interphase. Usually these phases are morphologically indistinguishable, but each stage is determined by specific processes that prepare the cell for initiation of cell division. During G1, cells show a high rate of biosynthetic activities resulting in the synthesis of various proteins that are required in S phase. This phase is marked by DNA replication which leads to duplication of all chromosomes. After S phase completion, cells enter the G2 phase, in which additional proteins are synthesised for the following M phase. The M phase consists of two major events: the nuclear division

(mitosis) and the cytoplasmic division (cytokinesis). During mitosis, the duplicated DNA is divided equally into two daughter nuclei, a process which can be subdivided into different phases. First, the chromatin begins to condensate into highly ordered chromosome structures during prophase and this is followed by nuclear envelope break down in prometaphase. During metaphase, chromosomes are attached to spindle microtubules and align at the metaphase plate or equatorial plane. Chromosome segregation occurs in anaphase resulting in separated sister chromosomes that are pulled to opposite spindle poles. Mitosis ends with telophase when both chromosome sets are surrounded by a new nuclear envelope and DNA unfolds into chromatin. Cell division is completed after cytokinesis, a process in which the mother cell is eventually divided into two daughter cells both harbouring a complete copy of the parental genome. Some cells stop dividing at a certain developmental stage and remain in a quiescent G_0 state for long periods of time, which is common for fully differentiated cells.

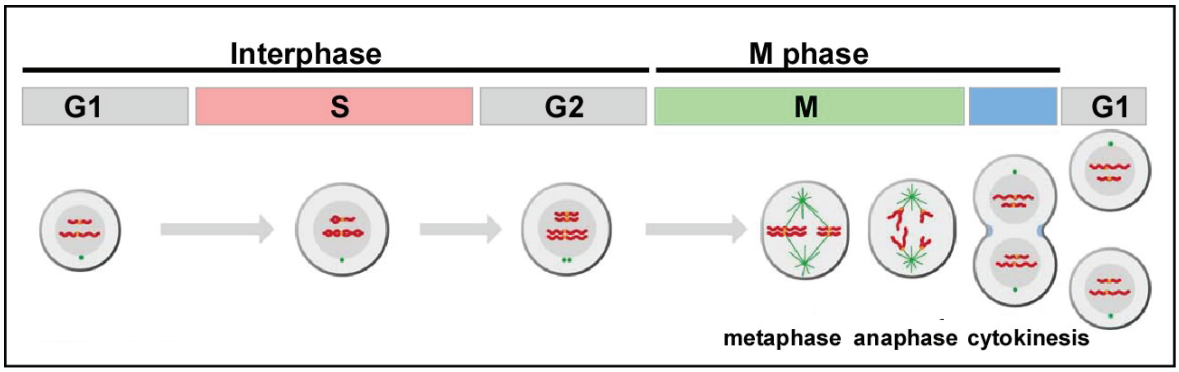


Figure 1.1 The eukaryotic standard cell cycle (adapted from Morgan, 2006).
The standard cell cycle consists of four phases: G1, S, G2 and M phase. During interphase, the DNA is replicated in S phase resulting in duplicated chromosomes. The Gap phases G1 and G2 provide time for cell growth and serve as important regulatory transitions for cell cycle progression. M phase is composed of two major events: mitosis and cytokinesis. During mitosis, chromosomes align at the metaphase plate and in anaphase sister chromatides are separated and distributed to opposite poles. In the following cytokinesis the cell divides into two new daughter cells that both exhibit a complete copy of the parental genome.

1.2 Cell cycle modes in *Drosophila melanogaster*

The fruit fly *Drosophila melanogaster* serves as a model organism to study cell proliferation and its coordination with cell growth and differentiation (for review see Edgar and Lehner, 1996). The standard cell cycle consisting of G1-S-G2-M is not the only cell cycle mode that is applied in *Drosophila*. The embryonic development of *Drosophila* exhibits three cell cycle variants as an adaption to specific developmental circumstances (Lee and Orr-Weaver, 2003; Swanhart et al., 2005). The first 13 embryonic cell cycles are

driven by maternal components and consist only of S-M phases. These rapid nuclear divisions occur in a shared cytoplasm that is named syncytium. During division 10-13, cellularisation of the embryo takes place resulting in the formation of a cell layer beneath the embryo's surface which is called the cellular blastoderm. Gastrulation starts at cell cycle 14 transforming the simple blastoderm into a multilayered embryo, a process that is accompanied by cell movement and differentiation. The cell cycle lengthens and acquires for the first time a G2 phase. The postblastoderm divisions 14-16 are composed of S-G2-M phases and are driven by zygotic gene products since maternal supplies are almost depleted (Merrill et al., 1988; Wieschaus and Sweeton, 1988). Furthermore, nuclei start dividing asynchronously, but in programmed spatial and temporal patterns, the so-called mitotic domains (Foe, 1989). After mitosis 16, the first G1 phase is introduced in cycle 17 (Edgar and O'Farrell, 1990). Some epidermal cells exit the cycle after mitosis 16 and arrest in the terminal G1 phase of cell cycle 17 (Edgar and O'Farrell, 1990). These G1-arrested epithelial cells are programmed to become imaginal disc cells and reinitiate the standard cell cycle upon the onset of larval development. During *Drosophila* development, the imaginal discs give rise to adult structures like wing, notum and eyes. Cells of the gut, the fat body or the salivary glands enter endoreplication by the end of embryogenesis and continue throughout larval stages (Smith and Orr-Weaver, 1991). In addition, cells of the gut and the ovary undergo endoreplication during adulthood (Dej and Spradling, 1999; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Endoreplication or the endocyle is a cell cycle mode in which cells bypass mitosis resulting in G-S cycles (for review see Edgar and Orr-Weaver 2001; Lilly and Duronio, 2005). Cells undergo multiple rounds of initiation of DNA replication resulting in increased DNA content and polyploidy due to complete replication of the genome or amplification of specific chromosome regions (Calvi et al., 1998). This strategy of endoreplication helps to support the rapid growth after the larva hatches and enables an increased production of numerous gene products.

1.3 Regulation of the cell cycle

The eukaryotic cell cycle is a highly regulated series of events leading to cell reproduction. These molecular events occur in a sequential fashion and are irreversible. In response to growth factors, the cell cycle is initiated first leading to duplication of cell organelles and increased protein synthesis. After this phase of massive cell growth, cells enter S phase and

duplicate their chromosomes. This process must be tightly regulated to ensure complete replication of the genome and only once per cycle. During M phase duplicated chromosomes and cell components must be distributed equally into individual daughter cells. Both processes must be achieved with extreme precision and in the correct order to guarantee accurate inheritance of the genetic information. Eucaryotic cells contain a complex regulatory network, the so-called cell cycle control system that regulates timing and coordination of each cell cycle event (for review see Morgan, 2006; Murray, 2004). This system drives progression through cell cycle transitions called checkpoints and interrupts the cell cycle upon DNA damage or failures in mitosis, thereby preventing uncontrolled or defective cell division. Components of this regulatory network are highly conserved throughout all organisms providing a very robust and reliable control system. The central components of the cell cycle control system are the cyclin-dependent kinases (Cdks) and their regulatory subunits called cyclins. Enzymatic activity of these kinases depends on changes in their phosphorylation state and tight binding of the cyclin which stimulates the catalytic activity. Cdk protein levels remain constant throughout the cell cycle, so that oscillations in Cdk activity depend primarily on fluctuations in the protein level of the corresponding cyclin. During the cell cycle, different types of cyclins and Cdks are synthesised resulting in the formation and activation of several cyclin-Cdk complexes at different time points. These cyclin-Cdk complexes control the progression through the three major cell cycle checkpoints: G1-S, G2-M and metaphase-anaphase transition. In mammalian cells, four different Cdk proteins (Cdk1, Cdk2, Cdk4 and Cdk6) are involved directly in cell cycle controls. In *Drosophila*, orthologues of Cdk1, Cdk2 and Cdk4 have been identified (for review see Edgar and Lehner, 1996; Lee and Orr-Weaver, 2003; Swanhart et al., 2005), whereas in budding and fission yeast just one main Cdk, known as Cdc28 or Cdc2 has been identified (Beach et al., 1982). The cyclins can be divided into two main groups that oscillate during different cell cycle phases. Mitotic cyclins are responsible for the G2-M transition and mitosis, whereas the G1 cyclins drive progression from G1 into S phase and induce DNA replication. In *Drosophila*, the mitotic cyclins A, B and B3 and the G1 cyclins D and E are present.

1.3.1 Cyclin-Cdk activity initiates mitosis

Entry into mitosis is driven by the activity of cyclin-Cdk complexes. In *Drosophila*, mitotic cyclins A, B and B3 are expressed during S phase and accumulate in G2 (Sigrist et al., 1995). All mitotic cyclins can form a complex with Cdk1 and the activity of each cyclin-Cdk1 complex triggers a certain event during mitosis (Jacobs et al., 1998; Knoblich and Lehner, 1993; Lehner and O'Farrell, 1989; Lehner and O'Farrell, 1990b). Cyclin A is essential for chromatin condensation and nuclear envelop breackdown during prophase whereas Cyclin B is needed in metaphase and Cyclin B3 for events during anaphase (Ramachandran et al., 2007). Altough all three mitotic cyclins perform a certain task during mitosis, Cyclin A seems to be the most important one since Cyclin A mutants are embryonic lethal due to a cell cycle arrest in G2 of mitosis 16 (Jacobs et al., 1998; Knoblich and Lehner, 1993; Lehner and O'Farrell, 1989). Cdk activity depends on phosphorylation by the Cdk-activating kinase (CAK) at threonine residue 161 (Morgan, 2006). In *Drosophila*, the CAK is a trimeric complex consisting of a Cdk-related protein Cdk7, Cyclin H and Mat1. Furthermore, Cdk activity is negatively regulated by the Wee1/Myt1 kinase that phosphorylates Cdk at threonine residue 14 and tyrosine residue 15 (Campbell et al., 1995; Morgan, 1997). The phosphatase Cdc25 removes these inhibitory phosphates and thereby allows entry into mitosis (Russell and Nurse, 1986). In *Drosophila*, s*tring* and *twine* are the Cdc25 homologues. At cell cycle 14, maternal *string* transcript supplies are exhausted which advances midblastula transition (Edgar and O'Farrell, 1989). The embryo initiates zygotic *string* expression that regulates all mitotic divisions throughout development (Edgar and O'Farrell, 1990; Edgar et al., 1994a). In contrast, Twine activity is restricted to meiosis (Edgar and Datar, 1996).

1.3.2 Cyclin degradation promotes mitotic exit

Cell cycle progression must occur unidirectional and irreversible. These requirements can be achieved by proteolytic destructions of the regulatory proteins. Cdk activity is essential for mitotic entry, but for further progression and exit from mitosis Cdk activity must be restrained. Since Cdk levels remain stable throughout the cell cycle, Cdk activity is mainly regulated by oscillating cyclin levels. Cyclins are expressed before entry into mitosis and get degraded during mitosis resulting in decreased Cdk activity (Murray and

Kirschner, 1989). Destruction of the mitotic cyclins A, B and B3 occurs in a sequential fashion during mitosis (Sigrist et al., 1995). Expression of stable versions of Cyclin A, B or B3 results in a mitotic arrest in metaphase, early or late anaphase respectively, demonstrating that Cyclin A degradation is essential for sisterchromatide segregation, Cyclin B degradation for stable kinetochor-spindle attachments during anaphase and Cyclin B3 destruction for late mitotic events (Parry and O'Farrell, 2001; Ramachandran et al., 2007). Mitotic cyclins and other cell cycle regulators are targeted for ubiquitin-dependent degradation. Therefore, multiple ubiquitin molecules are attached to substrates resulting in polyubiquitinated proteins that are recognised and destroyed by the 26S proteasome (for review see Murray, 2004). Ubiquitination is a process carried out in a series of reactions including ubiquitin activation, conjugation and ligation to the substrate. First, ubiquitin is covalently attached through its carboxyl terminus to the sulfhydryl group of a cysteine in the active site of the ubiquitin-activating enzyme (E1), a reaction that requires ATP hydrolysis. Subsequently, the activated ubiquitin is transferred to the ubiquitin-conjugating enzyme (E2). In the final step, the ubiquitin is transferred from the E2-ubiquitin conjugate to the amino group of a lysine residue in the target protein. This reaction is catalysed by the ubiquitin-protein ligase (E3) and occurs in a successive transfer of several ubiquitin molecules. Finally, the polyubiquitinated proteins are recognised and destroyed by the proteasome (Hochstrasser, 1996). The anaphase promoting complex/cyclosome (APC/C) is a large, multisubunit ubiquitin-protein ligase that mediates proteasomal destruction of mitotic cyclins and thereby progression through mitosis (for review see Peters, 2006; Pines, 2006; Yu, 2007; Zachariae and Nasmyth, 1999). The APC/C is a complex of 11-13 subunits including a cullin (APC2) and a RING subunit (APC11) that binds the E2-ubiquitin conjugate (Harper et al., 2002). APC/C activity depends on its phosphorylation state and association with the two WD40 activator proteins named Cdc20 and Cdh1 in yeast and mammals (Kramer et al., 2000; Visintin et al., 1997). In *Drosophila*, the genes *fizzy* (*fzy*) and *fizzy-related* (*fzr*) encode for Cdc20 and Cdh1 (Dawson et al., 1993; Dawson et al., 1995; Sigrist et al., 1995). Binding and activation of the APC/C by $Cdc20^{Fzy}$ or $Cdh1^{Fzr}$ occurs at different phases of the cell cycle (Figure 1.2). The APC/C-$Cdc20^{Fzy}$ complex is only active during early mitosis when Cdk activity is high resulting in phosphorylation of several APC/C subunits, a prerequisite for $Cdc20^{Fzy}$ binding to the APC/C (Kramer et al., 2000). It has been proposed that the early mitotic inhibitior 1 (Emi1) inhibts APC/C-$Cdc20^{Fzy}$ activity and that degradation of Emi1 upon

phosphorylation by Polo-like kinase (Plk1) is essential for APC/C-Cdc20^{Fzy} activation (Reimann et al., 2001a; Hsu et al., 2002). However, a more recent study demonstrated that Emi1 degradation is not needed for APC/C-Cdc20^{Fzy} activation during early mitosis (Di Fiore and Pines, 2007). Moreover, APC/C-Cdc20^{Fzy} activity is restrained by the spindle assembly checkpoint until metaphase. The spindle checkpoint proteins Bub1, BubR1 and Mad2 sense unattached kinetochors and prevent APC/C-Cdc20^{Fzy} activity by binding and subsequent withdrawal of Cdc20 (Yu, 2006). As soon as all sister chromatides are attached to the spindle microtubules, the APC/C-Cdc20^{Fzy} complex is fully active and triggers the metaphase/anaphase transition by degradation of mitotic cyclins. By the end of mitosis, Cdk activity drops resulting in dissociation and inactivation of APC/C-Cdc20^{Fzy} until the cells enter the next mitosis. In addition to mitotic cyclins, the Separase inhibitor Securin is a major target of the APC/C-Cdc20^{Fzy} (Hagting et al., 2002). Upon Securin destruction, Separase is released and cleaves the cohesin complex holding sister chromatides together and thereby enables chromosome segregation (Nasmyth, 2001). In *Drosophila*, the Securin homologue Pimples forms a complex with Three rows and Separase which is required for sister chromatide separation (Leismann et al., 2000). Embryos mutant for *fizzy* arrest in metaphase of mitosis 16 when maternal supplies of Fizzy are exhausted resulting in APC/C-Cdc20^{Fzy} inactivation and subsequent accumulation of mitotic cyclins (Dawson et al., 1993; Dawson et al., 1995; Sigrist et al., 1995). Cdh1^{Fzr} can only bind the APC/C during late mitosis and G1 phase, when Cdk activity is low and Cdh1^{Fzr} is unphosphorylated (Zachariae et al., 1998; Kramer et al., 2000). Thereby, cyclin degradation is maintained throughout late mitosis and the G1 phase. In addition, APC/C-Cdh1^{Fzr} mediates destruction of Cdc20^{Fzy} as well as Geminin which is a DNA licensing inhibitor. At the G1-S transition, APC/C-Cdh1^{Fzr} gets inhibited by vertebrate Emi1 or *Drosophila* Rca1, respectively (Hsu et al., 2002, Zielke et al., 2006). In the *Drosophila* embryo, epidermal cells mutant for *fizzy-related* fail to establish the terminal G1 and undergo an additional seventeenth mitosis (Sigrist and Lehner, 1997).

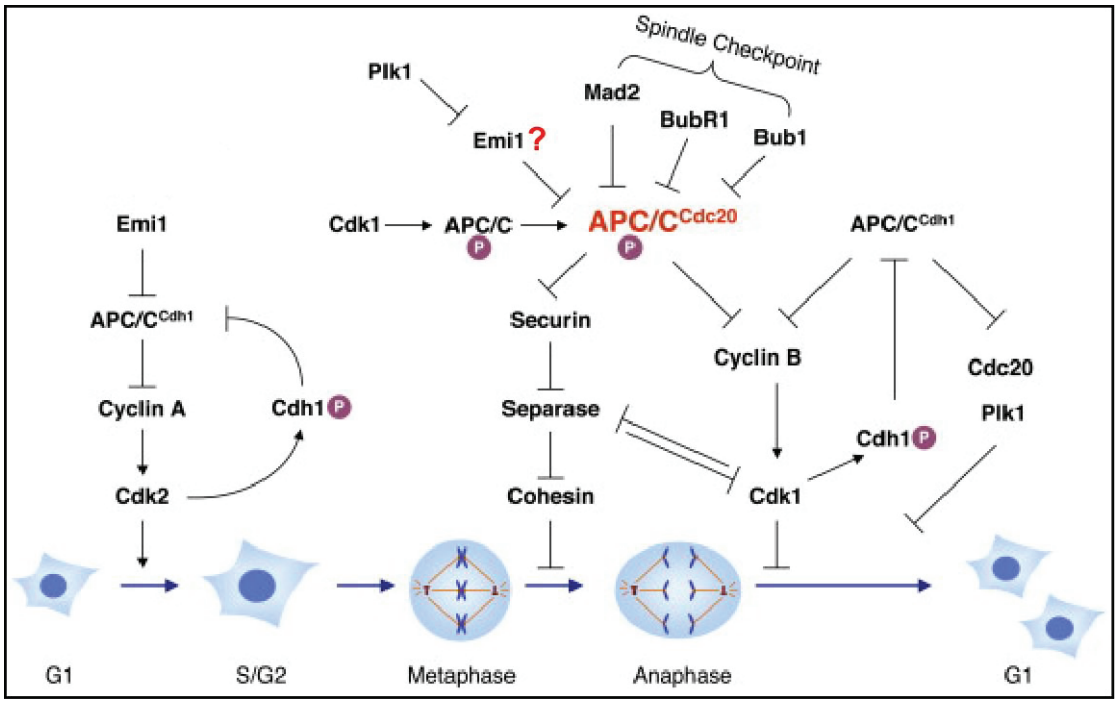


Figure 1.2 Regulation of the APC/C during the standard cell cycle (adapted from Yu, 2007).
The APC/C-Cdh1 mediates degradation of mitotic cyclins during late mitosis and G1. At the G1/S transition, the APC/C-Cdh1 is inhibited and subsequently Cyclin A is stabilised which activates Cdk2 and triggers S phase events. Active Cdk2 phosphorylates Cdh1 and further inhibits APC/C-Cdh1. In early mitosis, Cdk activity is high and APC/C subunits get phosphorylated which promotes Cdc20 binding. APC/C-Cdc20 activity is restrained by the spindle checkpoint. When all kinetochors are attached to spindle microtubules, inactivation of the spindle checkpoint results in APC/C-Cdc20 activity and Cyclin B and securin degradation. In turn, separase is activated which cleaves the cohesin complexes and thereby enables sister chromatide separation and metaphase-anaphase transition. Degradation of mitotic cyclins reduces Cdk activity resulting in dephosphorylation of APC/C subunits and Cdh1. Cdh1 activates the APC/C during late mitosis and the G1 phase, when Cdk activity is low. At late G1, APC/C-Cdh1 activity gets inhibited by Emi1.

APC/C target proteins contain a conserved amino acid sequence that is important for substrate recognition. The most widespread motif is the destruction box (D-box) mediating degradation of B-type cyclins and securin (Glotzer et al., 1991; King et al., 1996). The D-box is recognised by both complexes, APC/C-Cdc20^{Fzy} and APC/C-Cdh1^{Fzr}. Mutations in the D-box result in stable Cyclin B or Cyclin B3 proteins that lead to an early or late anaphase arrest when overexpressed in the *Drosophila* embryo (Sigrist et al., 1995). Degradation of Cyclin A is more complex and depends on other destruction elements in addition to the D-box (Ramachandran et al., 2007). Another important substrate recognition motif is the KEN-box which is mainly present in APC/C-Cdh1^{Fzr} substrates (Burton and Solomon, 2001; Pfleger et al., 2001; Pfleger and Kirschner, 2000). Most APC/C substrates contain either or both destruction elements for their efficient ubiquitination. Both activator proteins Cdc20^{Fzy} and Cdh1^{Fzr} function as substrate receptors which bind to the D-box or KEN-box (Kraft et al., 2005; Vodermaier, 2001). In yeast, the

APC core subunit Doc1 (APC10) contributes to D-box binding and is required for ubiquitination processivity in vitro (Carroll et al., 2005). Furthermore, it was demonstrated that the APC/C-Cdc20^{Fzy} complex binds substrates with a higher affinity than the APC/C core does alone (Passmore et al., 2005).

1.3.3 Establishment and maintenance of the G1 phase

The first G1 phase is established after mitosis 16 during late *Drosophila* embryogenesis. Most epidermal cells reside in the terminal G1 until they reinitiate the standard cell cycle upon larval hatching. During G1, reactivation of cyclin-Cdk complexes is prevented to allow controlled entry into the next cycle upon cell growth or other regulatory factors. Cdk inhibition in G1 is achieved by several mechanisms. Expression of mitotic cyclins is decreased and protein levels remain low due to ongoing cyclin degradation by the APC/C-Cdh1^{Fzr}. In addition, Cdk activity is supressed by Cdk inhibitor proteins (CKIs) that bind and inactivate cyclin-Cdk complexes and thereby promote a G1 arrest (Peter and Herskowitz, 1994; Sherr and Roberts, 1999). In *Drosophila*, Dacapo (Dap) inhibits increasing Cyclin E/Cdk2 activity and enables a stable G1 state (de Nooij et al., 1996; Lane et al., 1996). Furthermore, Roughex (Rux) is a specific inhibitor of Cdk1 and is also involved in mitotic exit (Foley et al., 1999; Foley und Sprenger, 2001; Thomas et al., 1994; Thomas et al., 1997). Embryos mutant for *rux* exhibit a delay in the metaphase-anaphase transition resulting from prolonged Cyclin A/Cdk1 activity. In addition, the inhibitory effect of Rux is also essential for a stable G1. Loss of Rux leads to premature cyclin-Cdk1 activation which promotes S phase entry and results in a rough eye phenotype (Thomas et al., 1994; Thomas et al., 1997).

1.3.4 Regulation of the G1-S transition

In mammalian cells, Cyclin E/Cdk2 and Cyclin D/Cdk4/6 drive the G1-S transition (Matsushime et al., 1994; Sherr, 1993). Cyclin D expression is induced by growth factors thereby linking extrinsic growth signals and cell proliferation. Cyclin D/Cdk4/6 complexes phosphorylate and inactivate members of the Retinoblastoma (Rb) tumor supressor family. Rb forms a heterodimer with the transcription factor E2F and represses E2F activity. E2F complexes are heterodimers consisting of two subunits, E2F subunit and one of the

Dp family. Phosphorylation of the Rb protein leads to dissociation from E2F (Attwooll et al., 2004; Blais and Dynlacht, 2004; Kato et al., 1993). The released E2F factor initiates the transcription of Cyclin E and Cyclin A (DeGregori et al., 1995; Pagano et al., 1992) and other genes involved in DNA replication such as ribonucleotide reductase (RNR), the DNA polymerase δ accessory subunit PCNA (DeGregori et al., 1995) and the vertebrate APC/C inhibitor Emi1 (Hsu et al., 2002). During late G1, the APC/C-Cdh1^{Fzr} complex is inhibited by vertebrate Emi or Rca1 in *Drososphila* resulting in Cyclin A accumulation. Simultaneously, CKIs normally restraining Cdk activity are degraded by the SCF (Skp-Cullin-F-box protein) complex (Cardozo and Pagano, 2004; Nakayama and Nakayama, 2006). As a result, Cyclin/Cdk activity increases thereby driving the G1-S transition and initiating DNA replication. Furthermore, Cyclin E/Cdk2 complexes enhance the transcription of Cyclin E by phosphorylating Rb and thereby promoting its own activity in a positive feedback loop. In *Drosophila*, orthologues of Cyclin D and Cdk4 have been identified, but Cyclin D/Cdk4 complexes are rather involved in regulating cell growth than in G1-S control (Datar et al., 2000; Meyer et al., 2000). S-phase induction mainly relies on Cyclin E/Cdk2 activity (Knoblich et al., 1994; Richardson et al., 1995). It is assumed that Cyclin E transcripts accumulate prior to the S phase in endocycling and mitotically dividing cells (Edgar and Nijhout, 2004). Cyclin E/Cdk2 phosphorylates the Rb-related proteins (Rbf1/Rbf2) and stimulates the transcription of E2F target genes like *cyclinE*, *rnr* and *PCNA* (Du et al., 1996; Duronio and O'Farrell, 1994; Duronio and O'Farrell, 1995; Duronio et al., 1995). In *Drosophila*, a single DP and two E2F subunits (E2F1 and E2F2) do exist: E2F1 acts as a transcriptional activator and E2F2 as a repressor on the same promoters (Frolov et al., 2001). Thus, in mitotically dividing cells Cyclin E/Cdk2 activity is regulated on the transcriptional level by E2F1. In addition, the CKI Dacapo inhibits Cyclin E/Cdk2 complexes (de Nooij et al., 1996; Lane et al., 1996). In addition to Cyclin E/Cdk2 activity, overexpression of Cyclin A or loss of the Cyclin A/Cdk1 inhibitor Rux can induce S phases (Foley et al., 1999; Sprenger et al., 1997; Thomas et al., 1997). Moreover, SCF complexes are also involved in G1-S regulation since several vertebrate CKIs are targeted for ubiquitin-dependent degradation by the proteasome (for review see Nakayama and Nakayama, 2006).

1.3.5 Protein degradation by SCF complexes

SCF complexes belong to a superfamily of Cullin-RING ubiquitin ligases (CRLS) that can be found throughout all eukaryotes. All CRLs share the catalytic core of Cullin-RING, but despite of that they show a great diversity in terms of composition and function, including cell cycle regulation, signalling pathways, circadian rhythms and apoptosis (Grima et al., 2002; Koepp et al., 1999; Maniatis, 1999; Nateri et al., 2004). Different CRLS are characterised by the Cullin isoform which is assembled into the complex. In humans, seven different Cullin proteins have been identified and each can nucleate into a multisubunit ubiquitin ligase. The archetypical CRL is the SCF ubiquitin ligase which is named by its core subunits Skp1, Cullin1 (Cul1) and F-box protein (for review see Nakayama and Nakayama, 2006; Petroski and Deshaies, 2005; Vodermaier, 2004). Cul1 acts as a scaffold protein binding to Skp1 and the RING-domain known as Roc1/Rbx1/Hrt1. The Roc1 subunit recruits a ubiquitin-conjugating enzyme (E2), while Skp1 acts as an adapter through binding the F-box motif of F-box proteins (Bai et al., 1996; Zheng et al., 2002). Besides Cul1-Skp1 based SCF complexes, the family of CRLS also includes the classes of Cul2/5-Elongin B/C, Cul3-BTB and Cul4-DBB1 based ubiquitin ligases. Specificity of the SCF complex is determined by the F-box protein which binds the target via protein-protein interaction motifs such as WD40 or leucine-rich repeats (Kipreos and Pagano, 2000; Skowyra et al., 1997). Many different F-box proteins exist, but not all of them are directly involved in SCF mediated protein degradation (Hermand, 2006). Phosphorylation of the target is a prerequisite for substrate recognition. In yeast, the most prominent F-box protein Cdc4 regulates the stability of Sic1, Far1, Cdc6 and the Cyclins Cln1/2 (Tyers and Jorgensen, 2000). In *Drosophila*, the F-box protein Slimb mediates destruction of the Dorsal /NFκB inhibitor Cactus/INFκB and of the transcription factors Cubitus interruptus (Ci) and Armadillo (Arm) which are involved in Hedgehog and Wingless signalling (Maniatis, 1999; Jiang and Struhl, 1998; Spencer et al., 1999). Furthermore, Cyclin E degradation depends on the SCF/Archipelago (Ago) and is essential for downregulation of Cyclin E/Cdk2 activity and exit from S phase (Koepp et al., 2001; Moberg et al., 2001; Schwab and Tyers, 2001). In addition, Myc protein is also degraded by the SCF/Ago complex (Moberg et al., 2004). The *Drosophila* genome harbours six different Cullin (Cul1-6) isoforms and several Skp proteins (A-F), whereas in mammals and yeast just a single Skp1 protein exists (Nayak et al., 2002; Yamanaka et al., 2002). Furthermore, three

different Roc proteins (Roc1a, Roc1b and Roc2) have been identified in *Drosophila*, but so far there is only evidence for SkpA and Roc1a to be part of an SCF complex that regulates cell cycle progression (Murphy, 2003; Noureddine et al., 2002). Which members of the Cullin, Roc and Skp family preferentially associate and form an active SCF complex *in vivo* still remains unclear.

1.3.6 Regulation of DNA replication

During S phase, DNA replication takes place resulting in duplication of all chromosomes. This process must be tightly regulated to prevent uncontrolled DNA synthesis and to ensure genome duplication once per cycle. Several factors are involved in initiation of replication and DNA synthesis (for review see Bell and Dutta, 2002; Machida et al., 2005; Sivaprasad et al., 2007). High Cyclin E/Cdk2 activity drives initiation of DNA replication at discrete regions on the chromosomes called origins of replication. These origins are organised in clusters that are distributed throughout the chromosomes and are activated simultaneously in S phase. During late mitosis and G1, the pre-replicative complex (pre-RC) assembles at origins, a process also known as DNA licensing in which origions are prepared for activation by preloading replication factors and the DNA helicase. Pre-RC formation starts with a six-subunit complex (ORC1-6) called origin recognition complex (ORC) that is constantly bound to origins. The proteins Cdt1 and Cdc6 bind to the ORC complex followed by recruitment and loading of the minichromosome maintenance complex (MCM) onto the DNA. This hexameric MCM complex consists of the proteins MCM 2-7 and acts as a replicative helicase that unwinds the DNA helix using energy by ATP hydrolysis. Thereby, it enables other downstream factors to gain access to the DNA in order to initiate replication (Fletcher et al., 2003; Pape et al., 2003). After binding of the MCM complex, DNA licensing is completed and the pre-RC, now consisting of ORC 1-6, Cdt1, Cdc6 and MCM 2-7 is activated upon S phase entry. The components of the pre-RC are conserved throughout many organisms including *Drosophila* with the Cdt1 orthologue *double-parked* (*dup*) (Crevel et al., 2005; Thomer et al., 2004; Whittaker et al., 2000). Origin firing requires Cdk activity resulting in phosphorylation of pre-RC components and recruitment of essential replication factors including Cdc45, Mcm10, RPA, proliferating cell nuclear antigen (PCNA) and DNA polymerases α and δ. After initiation of DNA replication, the MCM helicase moves away from the origin with the replication forks. The

other pre-RC proteins remain at the origin and are destroyed or inhibited to ensure origin firing only once in the same cycle (for review see Bell and Dutta, 2002; Blow and Dutta, 2005). Thereby, cells proceed into mitosis after DNA replication. The pre-RC remains inactive until late mitosis and prevents MCM loading until formation of a novel pre-RC. In multicellular organisms, pre-RC assembly in G1 is promoted by the activity of the ubiquitin ligase APC/C-Cdh1^{Fzr} which mediates the destruction of Cyclin A and Geminin. Geminin inhibits pre-RC formation by association and inactivation of Cdt1 (Wohlschlegel and Dwyer, 2000; Tada, 2007). Vertebrate Emi1 has been proposed to link DNA replication and mitosis and to prevent rereplication (Di Fiore and Pines, 2007; Machida and Dutta, 2007). At late G1, the APC/C-Cdh1^{Fzr} is inhibited by vertbrate Emi1 or *Drosophila* Rca1 resulting in Geminin and Cyclin A accumulation upon S phase entry (Machida and Dutta, 2007; N. Zielke, personal communication). Geminin binds and inhibits Cdt1 from S phase until late mitosis and prevents MCM loading after origin firing (McGarry and Kirschner, 1998; Tada, 2007; Wohlschlegel and Dwyer, 2000). Depletion of Geminin or overexpression of Cdt1 is sufficient to cause rereplication in mammals (Melixetian et al., 2004; Takeda et al., 2005; Zhu et al., 2004). In *Drosophila*, an orthologue for Geminin has been identified and also here depletion of Geminin or overexpression of Cdt1/Dup has been shown to induce rereplication (Mihaylov et al., 2002; Quinn et al., 2001; Thomer et al., 2004). In yeast, rereplication is prevented by phosphorylation of Cdc6 and MCM proteins followed by nuclear export. In addition, phosphorylated Cdc6 is degraded by the SCF^{Cdc4} complex (Perkins, et al., 2001). Besides Geminin, Cyclin/Cdk activity has been reported to prevent rereplication (Machida and Dutta, 2007; Mihaylov et al., 2002). While in yeast constitutive inactivation of Cdk is sufficient to cause rereplication (for review see Machida et al., 2005), the effect of cyclin/Cdk complexes on pre-RC formation are poorly understood in metazoa. In *Drosophila,* Geminin depletion or inactivation of Cyclin A/Cdk results in rereplication demonstrating that both mechanisms are nonredundant (Mihaylov et al., 2002). Cdk activity prevents pre-RC formation by phosphorylation of Cdc6 and Cdt1. While Cdc6 is exported from the nucleus, phosphorylated Cdt1 is a target of ubiquitin-dependent degradation. In vertebrates, the Cul4-DDB1^{CDT2} ubiquitin ligase and the SCF^{Skp2} complex have been reported to mediate Cdt1 degradation (Li et al., 2003; Liu et al., 2004; Nishitani et al., 2001; Sugimoto et al., 2004; Zhong et al., 2003). In *Drosophila*, the Cdt1 orthologue

Dup is also degraded throughout the cell cycle, but a potential SCF complex has not been identified so far (Thomer et al., 2004).

1.3.7 APC/C and endoreplication

In a standard cell cycle, pre-RC formation and subsequent DNA replication once per cell cycle depends on oscillating APC/C activity. During late mitosis and G1, the APC/C-Cdh1^{Fzr} mediates degradation of Cyclin A and Geminin, thereby allowing pre-RC formation. At late G1, vertebrate Emi1 or *Drosophila* Rca1 inhibit the APC/C-Cdh1^{Fzr} resulting in Geminin and Cyclin A accumulation that both prevent rereplication (Machida and Dutta, 2007; N. Zielke, personal communication). Emi1 or Rca1 depletion results in cells with giant nuclei which is due to rereplication (Grosskortenhaus and Sprenger, 2002; Machida and Dutta, 2007). If the APC/C also regulates pre-RC formation during endoreplication still remains unclear. In *Drososphila*, APC/C-Cdh1^{Fzr} activity is essential for downregulation of cyclin/Cdk1 activity and entry into the endocycle (Edgar and Orr-Weaver, 2001; Lilly and Duronio, 2005; Sigrist and Lehner, 1997). In epidermal cells of the *Drosophila* embryo, deletion of *fzr* triggers rereplication, but in endoreplicating cells it prevents entry into the endocycle program suggesting a cell type specific role of Fzr. Mitotic cyclins and other mitotic regulators (e.g. Cdk1 and Cdc25/String) are assumed to be transcriptionally downregulated when cells switch from mitotic cycles to endocycles (Klebes et al., 2002; Smith and Orr-Weaver, 1991; Sauer et al., 1995; Schaeffer et al., 2004). So far, it has been assumed that APC/C activity is dispensable once cells have entered the endocycle (Edgar and Orr-Weaver, 2001; Lilly and Duronio, 2005). Also in mammals, a direct role of the APC/C during endoreplication has not yet been confirmed since trophoblast cells of Emi -/- embryos endoreplicate normally while other cells fail to proliferate (Lee et al., 2006). However, there are similarities in the regulation of G-S cycles in mitotic and endocycling cells (Edgar and Orr-Weaver 2001; Lee and Orr-Weaver 2003). Endocycles are driven by oscillating waves of Cyclin E/Cdk2 activity (Lilly and Spradling 1996; Royzman et al., 1997; Weng et al., 2003) requiring regulated accumulation and destruction of Cyclin E. Continuous expression of Cyclin E interferes with the DNA licensing program and results in an endocycle block (Follette et al., 1998; Su and O'Farrell 1998; Weiss et al., 1998). It is proposed that Cyclin E/Cdk2 activity is low during the Gap phase in order to allow relicensing of origins and increases upon S phase entry. DNA

licensing in endoreplicating cells includes the same pre-RC components. Several mechanisms are assumed to influence the periodic expression of Cyclin E, but a final model of the Cyclin E oscillator has not been defined so far. It has been suggested that the SCF/Archipelago complex contributes to Cyclin E fluctuations by targeting phosphorylated Cyclin E for degradation (Koepp et al., 2001; Moberg et al., 2001; Schwab and Tyers, 2001). In addition, Cyclin E levels are regulated by E2F1 which activates transcription of S phase genes such as *cyclin E*, *PCNA* and *Rnr2* (Duronio et al., 1995; Royzman et al., 1997). The Cyclin E/Cdk2 specific inhibitor Dacapo (Dap) is also assumed to influence G-S kinetics during endoreplication by oscillating out of phase with Cyclin E. Dacapo binds and inhibits Cyclin E/Cdk2 and thereby promotes entry into the Gap phase (de Nooij et al., 1996; Lane et al., 1996). A recent study has given evidence for Dacapo being essential during endocycles of *Drosophila* oocytes (Hong et al., 2007). Geminin prevents rereplication in mitotic cells, but to what extent Geminin contributes to endocycle regulation remains elusive.

1.4 The cell cycle regulator Rca1

The APC/C promotes cell cycle progression by triggering the periodic degradation of mitotic and other cell cycle substrates (for review see Nakayama and Nakayama, 2006; Peters, 2006; Pines, 2006; Zachariae and Nasmyth, 1999). Activation of the APC/C requires association with the activator proteins $Cdc20^{Fzy}$ and $Cdh1^{Fzr}$ and depends on its phosphorylation state (see section 1.3.2). While the APC/C-$Cdc20^{Fzy}$ complex is active during early mitosis, degradation of cell cycle substrates is maintained during late mitosis and G1 by APC/C-$Cdh1^{Fzr}$ activity (Figure 1.3). APC/C activity can be restrained by different factors including components of the spindle checkpoint and members of the Emi family (for review see Schmidt et al., 2006). In *Drosophila*, the gene *rca1* encodes for an APC/C inhibitor that belongs to the family of Emi proteins (Grosskortenhaus and Sprenger, 2002). Initially, Rca1has been identified in a screen for dominant suppressors of the *roughex* (*rux*) eye phenotype (Dong et al., 1997). The CKI Rux specifically inhibits Cyclin A/Cdk1 activity and thereby contributes to maintain a stable G1 state. Loss of *rux* results in a rough eye phenotype due to unrestrained Cyclin A/Cdk1 activity and premature S phase entry (Thomas et al., 1997). Overexpression of Rca1 or Cyclin A in the developing eye resembles the rough eye phenotype. Embryos mutant for *rca1* arrest in G2 of cell cycle

16 resulting in a reduced number of epidermal cells compared to wild-type embryos (Dong et al., 1997). The *rca1* mutant phenotype is similar to *cyclin A* mutants (Lehner and O'Farrell, 1989) indicating that Rca1 controls Cyclin A/Cdk1 activity and naming the gene *regulator of cyclin A* (*rca1*). The G2 arrest in *rca1* mutant cells is due to premature cyclin degradation by the APC/C-Cdh1^{Fzr} complex (Grosskortenhaus and Sprenger, 2002). Rca1 has a negative effect on Fzr. Embryos mutant for *fzr* fail to establish the terminal G1 and undergo an additional cell cycle 17 (Sigrist and Lehner, 1997). Since overexpression of Cyclin A results in the same phenotype, premature reaccumulation of Cyclin A seems to be responsible for mitosis 17 (Sigrist and Lehner, 1997). However, overexpression of Fzr prevents entry into mitosis 16 similar to *rca1* mutants (Sigrist and Lehner, 1997). Simultaneous overexpression of Rca1 and Fzr or double mutants for *rca1* and *fzr* both show normal cyclin accumulation and mitosis 16 (Grosskortenhaus and Sprenger, 2002). In addition, Rca1 and Fzr have been shown to interact physically (Grosskortenhaus and Sprenger, 2002). Rca1 is a nuclear protein, but Fzr and mitotic cyclins have been shown to localise in the cytoplasm during interphase (Dienemann and Sprenger, 2004; Grosskortenhaus and Sprenger, 2002). It remains unclear in which compartment of the cell the active APC/C-Cdh1^{Fzr} complex resides and how Rca1 can inhibit Fzr in G2 despite their spatial separation.

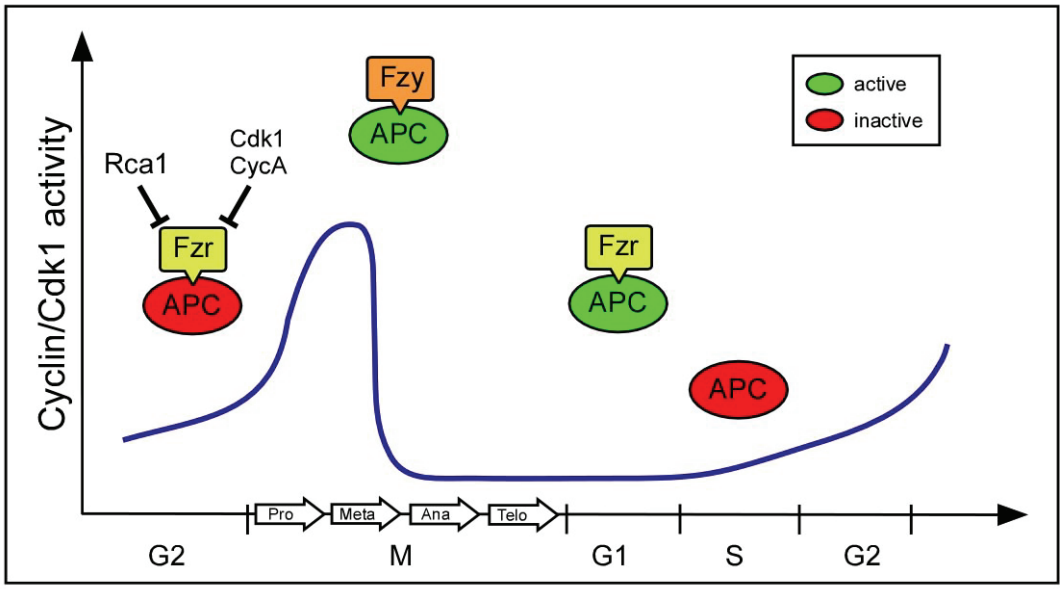


Figure 1.3 Rca1 restrains the APC/C-Cdh1^{Fzr} activity in G2. APC/C promotes cell cycle progression by degradation of different substrates like mitotic cyclins. The activity of APC/C-Cdc20^{Fzy} depends on high cyclin/Cdk activity and triggers the metaphase-anaphase transition. Cyclin/Cdk activity drops during late mitosis allowing Cdh1^{Fzr} to bind and activate the APC/C. Cyclin degradation is maintained during late mitosis and G1 by APC/C-Cdh1^{Fzr} activity. In G2, APC/C-Cdh1^{Fzr} activity is inhibited by Rca1 to allow cyclin accumulation and entry into mitosis. In addition, Cyclin A/Cdk1 activity contributes to APC/C-Cdh1^{fzr} inhibition.

Hence, these data show that Rca1 regulates Cyclin A/Cdk1 activity by inhibiting the APC/C-Cdh1^{Fzr} during G2 of *Drosophila* development. Subsequently, APC/C-Cdh1^{Fzr} mediated protein degradation is restrained resulting in the accumulation of mitotic cyclins and entry into mitosis. In addition, Cyclin A/Cdk1 and Cyclin E/Cdk2 activity contributes to restrain APC/C-Cdh1^{Fzr} activity by phosphorylation of Cdh1^{fzr} (Dienemann and Sprenger, 2004; Zielke, 2007). During G1, Cyclin A/Cdk1 activity is limited by the CKI Rux and the APC/C-Cdh1^{Fzr} which targets Cyclin A for ubiquitin dependent degradation by the proteasome.

1.4.1 The Rca1/Emi1 family

The Emi protein family consists of several members including *Drosophila* Rca1 and vertebrate Emi1 and Emi2/XErp1 (Reimann et al., 2001a; Schmidt et al., 2005; Tung et al., 2005). Rca1 and Emi1 are both inhibitors of the APC/C-Cdh1^{Fzr} complex and have been reported to be involved in the G1-S transition (Dong et al., 1997; Hsu et al., 2002; Reimann et al., 2001a; Zielke et al., 2006; Zielke, 2007).

Sequence analysis of Rca1 and Emi1 revealed a limited sequence homology of 18%, but both proteins share a set of conserved functional domains (Figure 1.4). The carboxy-terminus contains a conserved zinc binding region (ZBR) which is known to mediate protein-protein interactions. A C-terminal fragment of Emi1 and Rca1 has been shown to be sufficient for its function to restrict APC/C-Cdh1^{Fzr} activity in G2 (Reimann et al., 2001a; Zielke et al., 2006). Furthermore, a point mutation in the ZBR (C351S) abolishes the inhibitory effect of Rca1 and Emi1 (Reimann et al., 2001a; Reimann et al., 2001b; Zielke et al., 2006). Emi1 harbours a D-box (RxxL) that has been shown to bind the APC/C core (Miller et al., 2006). While the D-box in Emi1 enables binding to the APC/C-Cdh1^{Fzr} complex, the ZBR is assumed to prevent substrate access (Miller et al., 2006). Mutation of the ZBR converts Emi1 to a D-box substrate suggesting that Emi acts as a pseudosubstrate inhibitor (Miller et al., 2006). Rca1 also contains a minimal D-box between amino acid 203 and 255. Although this region seems to harbour elements essential for Rca1 function (Radermacher, 2007; Zielke et al., 2006), there is so far no evidence for Rca1 acting as a pseudosubstrate inhibitor of the APC/C-Cdh1^{Fzr}. In addition, Rca1 and Emi1 contain different putative localisation signals (NLS) and an F-box motif in the central region classifying both proteins to the family of F-box proteins (see section 1.3.5).

Finally, several putative Cdk1 phosphorylation sites, a DSGxxS degron and a KEN-box can be found in both proteins.

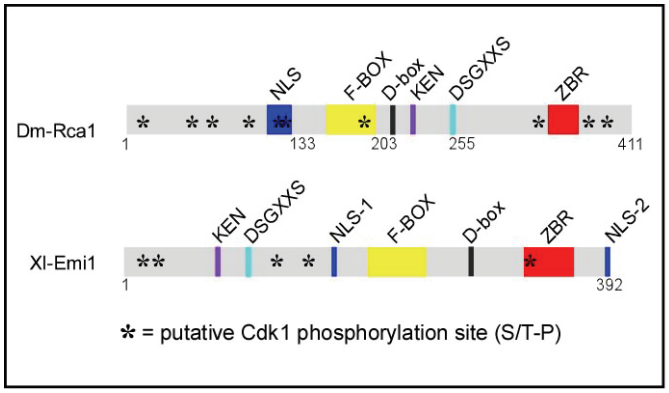


Figure 1.4 Schematic overview of Rca1 and *Xenopus* Emi1 protein structure. The C-terminal part of both proteins harbours a ZBR which is essential for APC/C inhibition. Furthermore, an F-box is located in the middle part as well as a KEN-box and a DSGxxS degron that is crucial for Emi1 degradation. Several putative Cdk1 phosphorylation sites (marked by an asteriks) and nuclear localisation sequences (NLS) are present in both proteins. All conserved domains described for xlEmi1 apply basically to all Emi1/Emi2 proteins.

In vertebrates, Emi1 degradation is initiated by Cdk1 phosphorylation that facilitates Plk1 binding and phosphorylation of the DSGxxS motif. The $SCF^{\beta TRCP}$ ubiquitin ligase recognises the phosphorylated degron and targets Emi1 for proteasomal degradation (Guardavaccaro et al., 2003; Hansen et al., 2004; Margottin-Goguet et al., 2003; Moshe et al., 2004). The proteins Evi5 and Pin1 have been reported to stabilise Emi1 levels during G2 by preventing Plk1 mediated phosphorylation and subsequent degradation (Bernis et al., 2007; Eldridge et al., 2006). Mutation of the DSGxxS degron or all Cdk1 phosphorylation sites results in a stable Emi protein (Margottin-Goguet et al., 2003). Rca1 has been shown to be degraded during the terminal G1 to allow APC/C-$Cdh1^{Fzr}$ dependent cyclin degradation and a stable G1 state (Grosskortenhaus and Sprenger, 2002; Jacobs et al., 2002; Pimentel and Venkatesh, 2005). Surprisingly, Rca1 turnover neither relies on Cdk1 phosphorylation nor on phosphorylation of the DSGxxS degron suggesting that Rca1 degradation occurs in a different way than Emi1 degradation (Radermacher 2007; Zielke et al., 2006). Furthermore, deletion of the KEN-box does not affect Rca1 protein levels demonstrating that Rca1 is not a target of the APC/C-$Cdh1^{Fzr}$ (Zielke et al., 2006). Which pathways eventually mediate Rca1 degradation still remains unclear. The Emi2/XErp1 proteins inhibit the APC/C-$Cdh1^{Fzr}$ during meiosis and are required for maintenance of the cytostatic factor (CSF) arrest during *Xenopus* oocyte maturation (Rauh et al., 2005; Schmidt et al., 2005; Tung et al., 2005). The C-terminal part of Emi2/XErp1 shows significant homology to Emi1 and displays a ZBR and F-box domain (Schmidt et al., 2005; Tung et al., 2005).

1.4.2 F-box dependent function of Rca1 at the G1-S

Eye imaginal disc cells mutant for *fzr* fail to downregulate Cyclin A/Cdk1 activity in G1 resulting in ectopic S phases and a rough eye phenotype (Pimentel and Venkatesh, 2005; Thomas et al., 1994). At the G1-S transition, the APC/C-Cdh1^{Fzr} is inhibited by Rca1/Emi proteins to allow cyclin accumulation upon S phase entry. Overexpression of Rca1 during eye development also causes ectopic S phases and a rough eye phenotype (Dong et al., 1997; Zielke et al., 2006). This indicates that Rca1 has a secondary function at the G1-S transition besides APC/C inhibition in G2. Furthermore, wing imaginal disc cells overexpressing Rca1 progress faster through G1 also demonstrating that excess Rca1 protein accelerates the G1-S transition (Zielke et al., 2006). Moreover, S phase induction by excess Rca1 depends on the F-box motif (Zielke et al., 2006). Since Rca1 lacking the F-box does not affect APC/C-Cdh1^{Fzr} inhibition during G2 (Zielke et al., 2006), S phase induction by Rca1 must occur independently from its function as an APC/C-Cdh1^{Fzr} inhibitor. Rca1/Emi1 proteins belong to the family of F-box proteins which are part of SCF ubiquitin ligases (see section 1.3.5). Hence, it has been proposed that Rca1 might act as an F-box protein in an SCF complex that regulates the G1-S transition (Zielke et al., 2006).
Furthermore, the role of Rca1 for G-S cycles of endoreplicating cells has been analysed in *Drosophila* salivary glands. Endocycles are driven by oscillating waves of Cyclin E/Cdk2 activity, whereas mitotic regulators including Cdk1, Cdc25/string and the mitotic cyclins are assumed to be transcriptionally downregulated (see section 1.3.6). Furthermore, APC/C-Cdh1^{Fzr} activity is essential for endocycle entry, but once cells have entered the endoreplication program, APC/C-Cdh1^{Fzr} activity is assumed to be dispensable. Rca1 has been shown to be dispensable for endocycle progression (Zielke, 2007). Overexpression of Rca1 during salivary gland development blocks the endocycle resulting in reduced polyploidy (Zielke, 2007). Interestingly, this phenotype also depends on a functional F-box. In addition, Rca1 overexpressing cells show elevated Cyclin E levels as well as mitotic proteins Cdk1 and Cyclin A. Since continuous expression of Cyclin E interferes with DNA-licensing, the endoreplication block caused by Rca1 might be due to elevated Cyclin E levels. The accumulation of Cdk1, Cyclin A and Cyclin E cannot simply be explained by APC/C-Cdh1^{Fzr} inhibition, because Cyclin E is not a target of the APC/C-Cdh1^{Fzr} and Cyclin B levels are not affected (Zielke, 2007). In endoreplicating cells, Rca1

seems to activate the transcription of these genes by an unknown mechanism and it could be possible that the APC/C-Cdh1^{Fzr} complex also contributes to this process.

2. Aim

The APC/C ubiquitin ligase mediates degradation of mitotic cyclins by the proteasome and thereby regulates cell cycle progression. APC/C activity depends on the proteins Cdc20^{Fzy} and Cdh1^{Fzr}, Cdk phosphorylation and the inhibitory Rca1/Emi1 proteins. Rca1 restrains APC/C-Cdh1^{Fzr} activity during the G2 phase of *Drosophila* embryogenesis, thereby allowing cyclin accumulation and entry into mitosis. Furthermore, Rca1 has a second function at the G1-S transition that depends on the conserved F-box domain. F-box proteins are part of the SCF (Skp-Cul1-F-box protein) complexes, another ubiquitin ligase that marks different cell cycle substrates for degradation. It has been proposed that Rca1 might act as an F-box protein in a so far uncharacterised SCF complex which is involved in regulating the G1-S transition (Zielke et al., 2006).

A major goal of my thesis was to give evidence for the physical existence of the proposed SCF/Rca1 complex by performing different interaction studies in *Drosophila* S2 cells. The idea that Rca1 might be part of an SCF ubiquitin ligase raises the question which substrates could be targeted for degradation by the putative SCF/Rca1 complex. Potential targets should be identified using different biochemical approaches and by performing a yeast two-hybrid screen for Rca1 interacting proteins. In addition, the F-box motif and other protein domains were tested for their relevance in Rca1 localisation. Previous studies have shown that Rca1 has also an F-box dependent effect when overexpressed in endoreplicating *Drosophila* salivary glands. Overexpression of Rca1 results in an endoreplication block and accumulation of Cyclin A, Cyclin E and Cdk1 protein. Mitotic genes such as Cyclin A and Cdk1 are assumed to be transcriptionally downregulated in endoreplicating cells (Klebes et al., 2002). This assumption has to be verified by performing a gene expression analysis of endoreplicating salivary glands. Furthermore, it has to be elucidated if Rca1 overexpression activates the transcription of Cyclin A, Cyclin E and Cdk1 since accumulation of these proteins cannot solely be explained by Rca1 mediated APC/C-Cdh1^{Fzr} inhibition.

3. Results

3.1 Subcellular localisation studies of Rca1

Rca1 is an inhibitor of APC/C-Cdh1^{Fzr} activity during G2 of *Drosophila* embryogenesis resulting in cyclin accumulation and subsequent entry into mitosis (Grosskortenhaus and Sprenger, 2002). In addition, overexpression of Rca1 in the developing eye can induce ectopic S phases that will result in a rough eye phenotype (Dong et al., 1997). Moreover, Rca1 can accelerate the G1-S transition in other tissues like the wing imaginal discs (Zielke et al., 2006).

Rca1 is a nuclear protein, but the APC/C activiator Cdh1^{Fzr} and the APC/C substrates, mitotic cyclins, accumulate in the cytoplasm during interphase (Grosskortenhaus and Sprenger, 2002). How Rca1 can act on Cdh1^{Fzr} despite their spatial separation still remains unclear. Moreover, it has to be elucidated if nuclear accumulation is a prerequisite to inhibit APC/C-Cdh1^{Fzr} activity during G2 or to promote the G1-S transition. A first step to address these questions is the identification of the protein domains that are responsible for targeting Rca1 to the nucleus. The Rca1 protein contains several motifs that are highly conserved throughout the Rca1/Emi1 family (Figure 3.1). A structure/function analysis of Rca1 revealed the relevance of each protein motif for its functions: First, as an APC/C-Cdh1^{Fzr} inhibitor during G2 of *Drososphila* embryogenesis and second, as a G1-S regulator that can induce ectopic S phases resulting in a rough eye (results are summarised in Figure 3.1; Radermacher, 2007; Zielke et al., 2006; Zielke, 2007). The C-terminus harbours a zinc-binding region (ZBR) which is essential for both functions during G1 and G2. Furthermore, a N-terminal truncations up to amino acid 255 result in a nonfunctional protein (Zielke et al., 2006; Zielke, 2007). The central part of Rca1 harbours a minimal D-box, a KEN-box and the DSGxxS degron which mediates Emi1, but not Rca1 degradation (Guardavaccaro et al., 2003; Margottin-Goguet et al., 2003; Zielke, 2007). Each of these protein motifs and all Cdk1 phosphorylation sites have been shown to be dispensable for Rca1 function (Zielke et al., 2006; Zielke, 2007). Deletion of amino acid 203-255 resulted in a protein which was not able to restrain APC/C-Cdh1^{Fzr} activity during G2 of embryogenesis. However, it was still capable to induce S phases (Radermacher, 2007). Deletion of the F-box alone or N-terminal truncations including the F-box cannot induce S

phases and a rough eye phenotype. Thus, the F-box motif is required for S phase induction, but is dispenasble for APC/C-Cdh1^{Fzr} inhibition since Rca1 lacking the F-box can still restrain APC/C-Cdh1^{Fzr} activity (Zielke et al., 2006; Zielke, 2007). In addition, the N-terminus harbours a bipartite nuclear localisation signal (NLS).

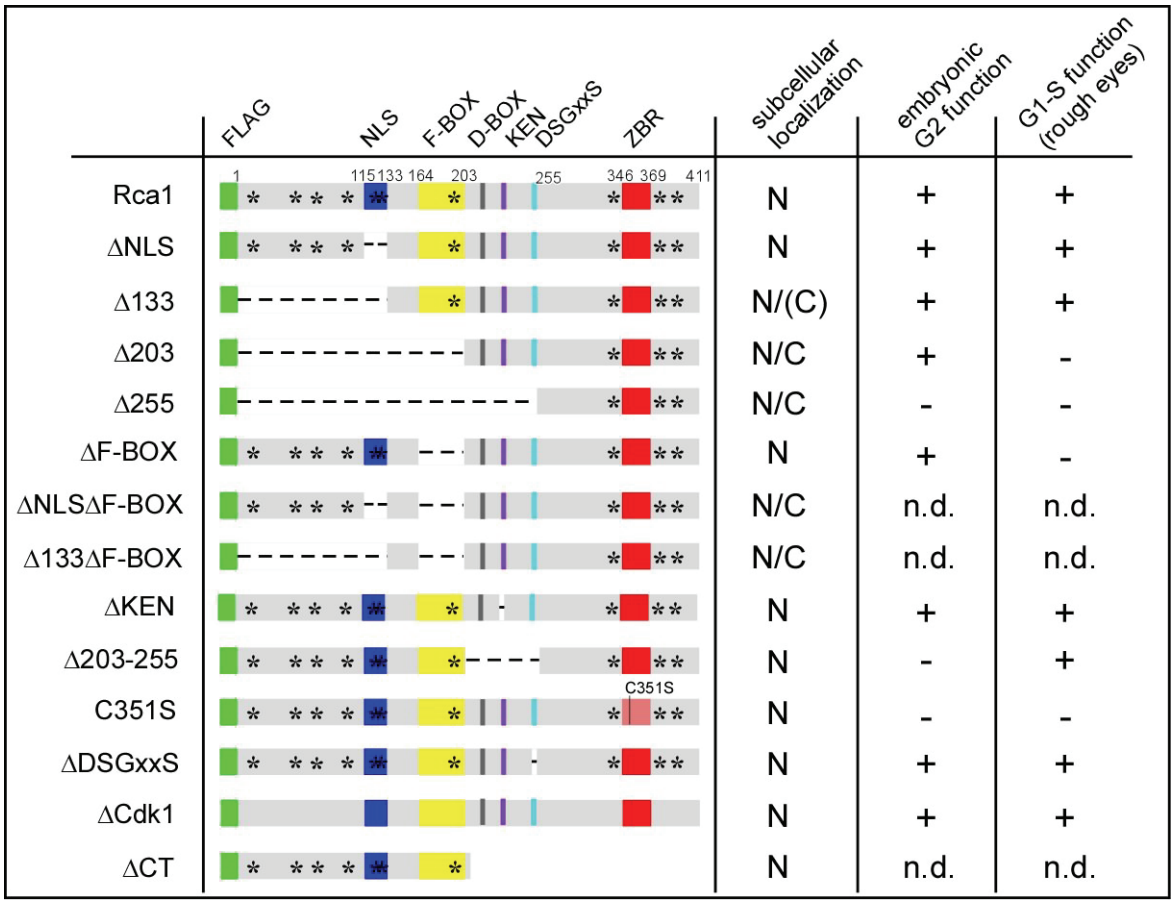
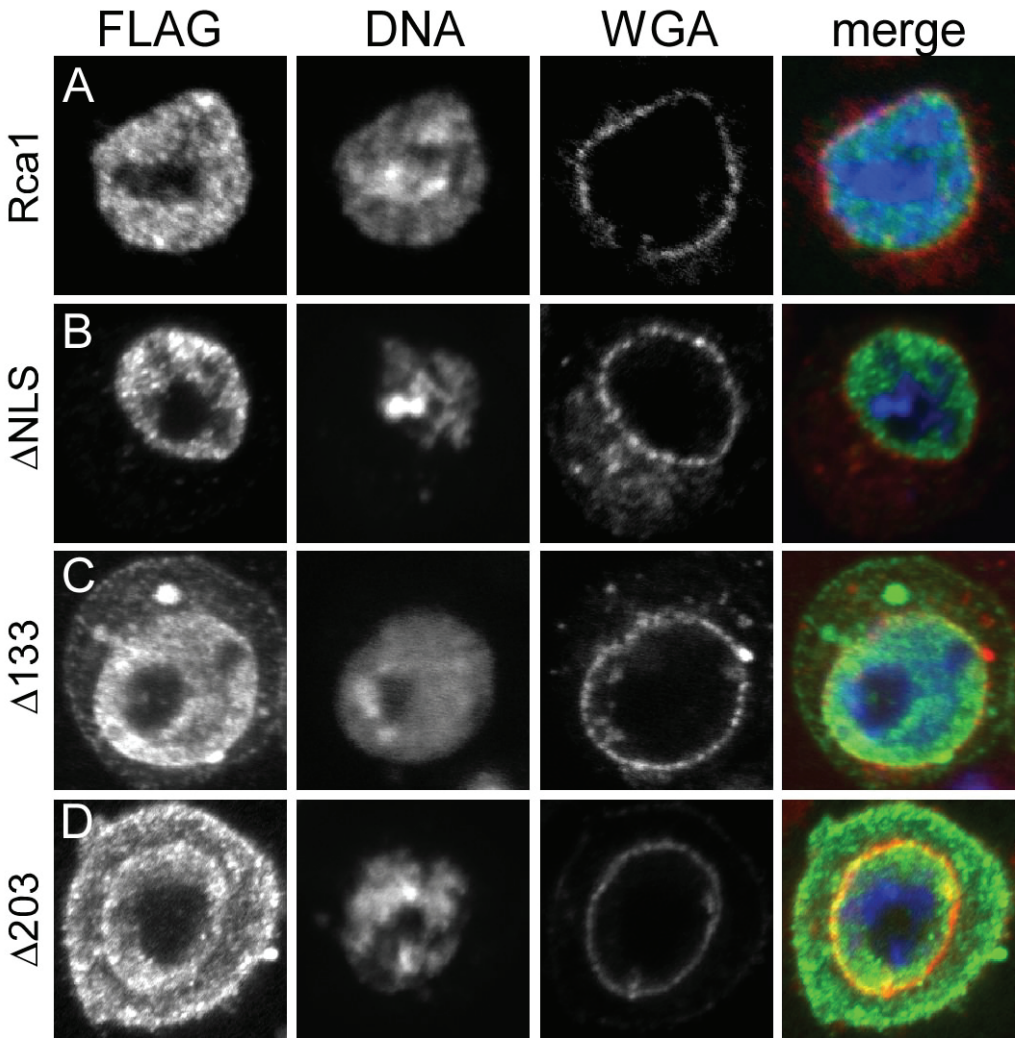
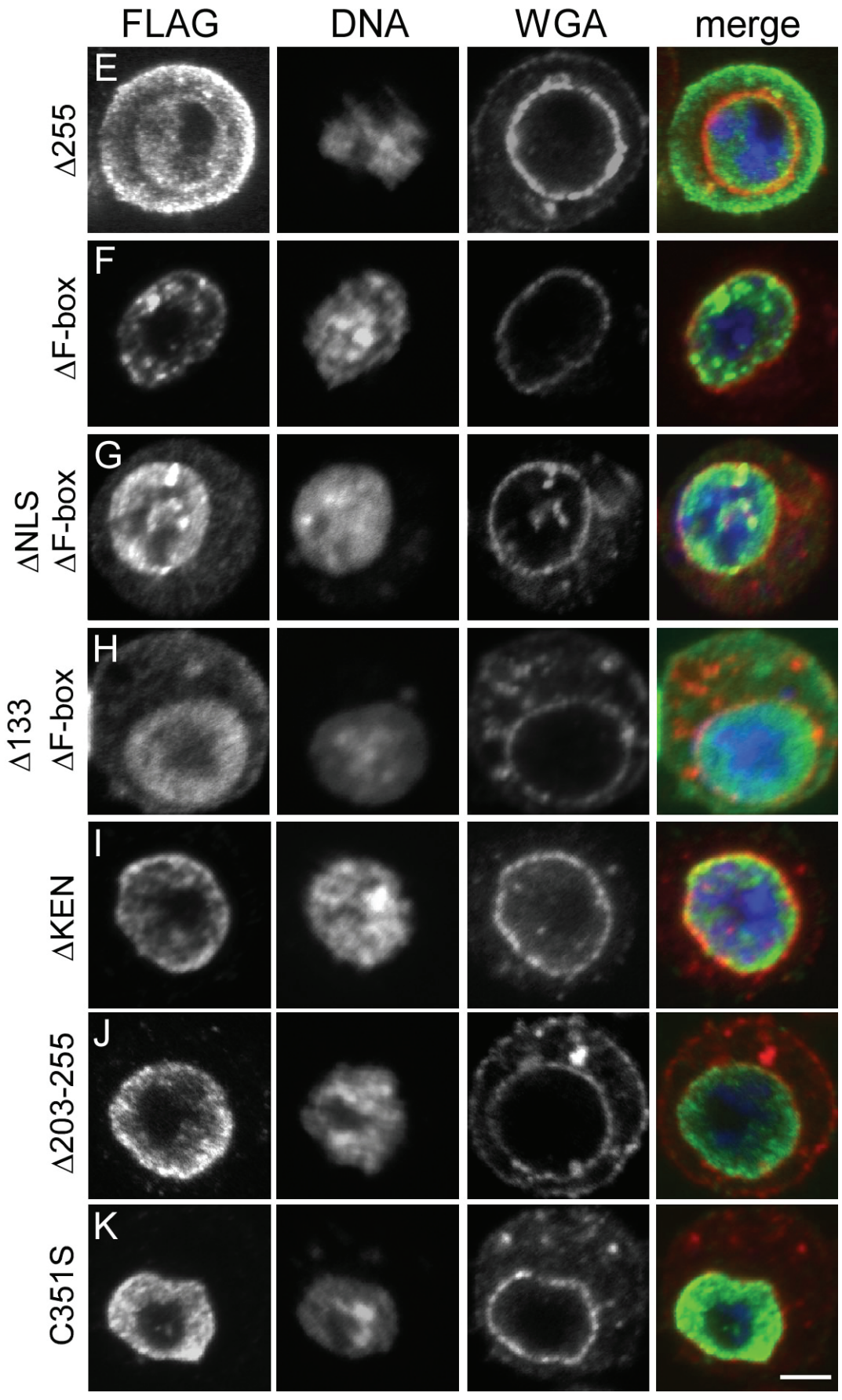


Figure 3.1 Schematic overview of Rca1 deletion proteins tested for subcellular localisation in *Drosophila* S2 cells and functionality in the fly.
Schematic representation of FLAG-tagged full length Rca1 and different Rca1 deletion constructs. All constructs were overexpressed in S2 cells. After immunostaining, nuclear (N) or cytoplasmic (C) localisation of Rca1 deletion proteins was determined. In addition, the relevance of each protein motif for Rca function during G2 of embryogenesis or for induction of the rough eye phenotype are also shown in the table (Radermacher, 2007; Zielke et al., 2006; Zielke, 2007). The asteriks indicate putative Cdk1 phosphorylation sites (S/T-P). The function of some Rca1 deletion constructs has not been determined (n.d.) so far.
C351S, point mutation of the cysteine residue at position 346 in the ZBR; CT, C-terminus; Δ, deletion; D-box, destruction box which is an APC/C substrate recognition motif; DSGxxS, GSK3 phosphorylation site; F-box, conserved protein motif in F-box proteins; FLAG, FLAG tag (DYKDDDDK); KEN-box, KEN-box (recognition motif of APC-Cdh1^{Fzr} substrates); NLS, nuclear localisation signal; Rca1, regulator of Cyclin A; ZBR, zinc binding region.

A series of Rca1 deletion constructs was cloned in a vector suitable for expression in *Drosophila* Schneider (S2) cells (Figure 3.1). Each construct was equipped with an N-terminal FLAG tag to facilitate their detection. Protein expression occured under control of an inducable metallothionein promotor (Bunch et al., 1988). After transient transfection and induction of protein expression, cells were fixed and incubated with FLAG-antibody to visualise the localisation of the Rca1 protein inside the cell (Figure 3.2). In addition, cells were stained for DNA and the nuclear envelope was visualised by wheat germ agglutinin (WGA) staining (Figure 3.2).




FLAG
DNA
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Δ255
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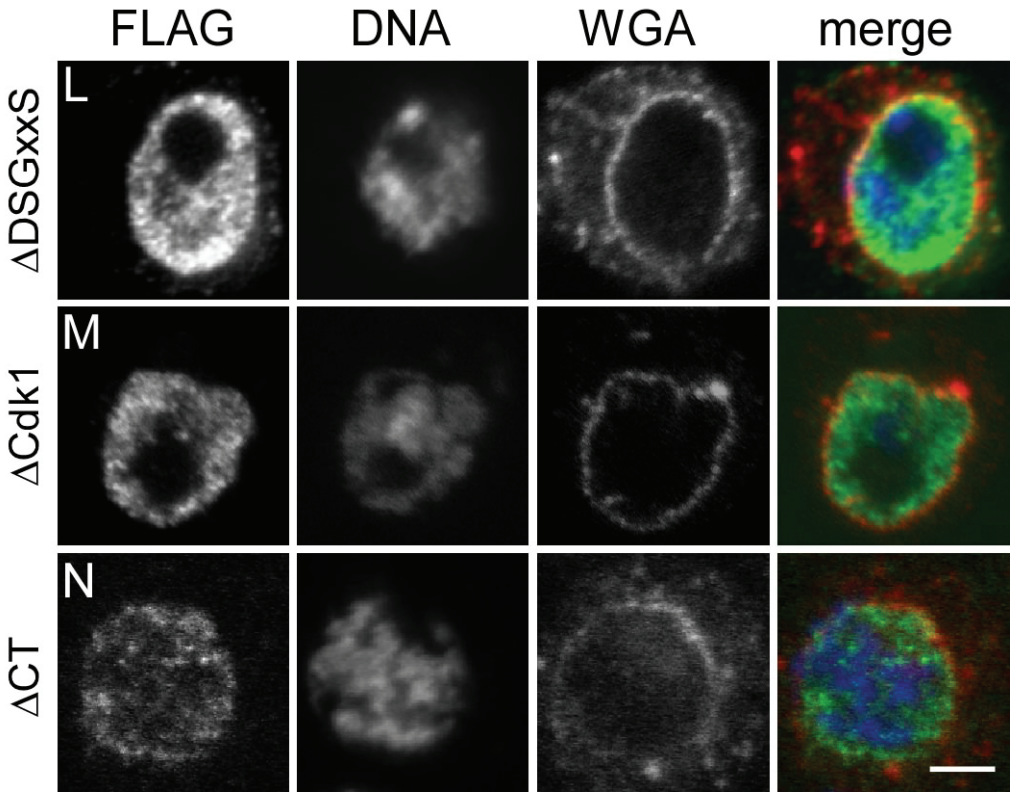


Figure 3.2 Subcellular localisation studies of Rca1 in *Drosophila* S2 cells.
Different FLAG-tagged Rca1 deletion constructs were expressed in S2 cells. Cells were stained with FLAG antibody to detect localisation of the Rca1 proteins in the cell. In addition, the nuclear membrane (WGA) and DNA were visualised. Scale bar: 1µm.
(A) Full length Rca1 protein accumulates in the nucleus.
(B) Deletion of the NLS does not affect nuclear localisation of FLAG-Rca1ΔNLS.
(C-E) Truncation of the first 133, 203 or 255 amino acids of the N-terminus results in proteins distributed throughout the nucleus and cytoplasm suggesting that N-terminal regions contribute to nuclear localisation.
(F-H) Besides the NLS, the F-box is a prominent element in the N-terminal region that might be involved in targeting Rca1 to the nucleus. However, FLAG-Rca1 ΔF-box localises only in the nucleus, but FLAG-Rca1ΔNLSΔF-box and FLAG-Rca1Δ133ΔF-box translocate to the cytoplasm.
(I-N) FLAG-Rca1 proteins lacking the KEN-box or the region Δ203-255 accumulate in the nucleus. The same phenotype has been observed for FLAG-Rca1C351S, FLAG-Rca1ΔDSGxxG, FLAG-Rca1ΔCdk1 and FLAG-Rca1ΔCT.

In *Drosophila* S2 cells, full length Rca1 (FLAG-Rca1) accumulates in the nucleus (Figure 3.2A). This recapitulates the localisation in embryonic cells (Grosskortenhaus and Sprenger, 2002). Since Rca1 habours a nuclear localisation signal (NLS) in the N-terminus, this protein motif is the most likely element responsible for mediating nuclear localisation of Rca1. However, deletion of NLS (FLAG-Rca1ΔNLS) does not change the prominent nuclear localisation of Rca1 suggesting that the NLS is not the only motif that is essential for nuclear targeting of Rca1 (Figure 3.2B). Deletion of the first 133 amino acids of the N-terminus including the NLS results in a predominantly nuclear protein, but slight amounts of FLAG-Rca1Δ133 can be detected in the cytoplasm. These trace amounts of

FLAG-Rca1Δ133 in the cytoplasm indicate that the region upstream of the NLS contains further unknown motifs which are involved in the transport into the nucleus (Figure 3.2C). Further shortening of the N-terminus up to residue 203 leads to a protein that is evenly distributed throughout the cell (Figure 3.2D). A similar protein distribution has been observed for Rca1 lacking the first 255 amino acids (Figure 3.2E). Since high amounts of FLAG-Rca1Δ203 accumulate in the cyctoplasm, the region between 133 and 203 seems to be important for Rca1 localisation. The F-box is the most prominent element in this region suggesting that it mediates localisation of Rca1. F-box proteins are known to bind the SCF component Skp via the F-box. In *Drosophila*, SkpA protein localises throughout the cytoplasm and nucleus in diploid tissues such as the CNS and during embryogenesis (Murphy, 2003). However, it is slightly more concentrated in the nucleus of some diploid cells suggesting that SkpA gets located to the nucleus by interaction with nuclear transport proteins (Murphy, 2003). Therefore, nuclear localisation of Rca1 might depend on SkpA binding and indirect interaction with nuclear proteins that mediate nuclear import. However, Rca1 lacking the F-box (FLAG-Rca1ΔF-box) still localises to the nucleus indicating that Rca1 localisation does not solely rely on the NLS or F-box. Moreover, a combination of different motifs might mediate nuclear transport of Rca1. Therefore, different putative localisation motifs were deleted simultaneously and tested *in vivo*. Rca1 constructs lacking the F-box and the NLS or the first 133 residues (FLAG-Rca1ΔNLSΔF-box or FLAG-Rca1Δ133ΔF-box) were localised partly in the cytoplasm, but high levels still accumulated in the nucleus (Figure 3.2G and H). Thus, nuclear accumulation could not be abolished by deletion of the NLS and F-box. Furthermore, no significant increase in the cytoplasmic fraction was observed when the F-box was mutated in the background of the 133 amino acid deletion. Finally, several constructs with altered activity profile were tested for their subcellular distribution (Figure 3.2I-N). In all of these constructs an intact NLS was present and all these proteins localised to the nucleus. Altogether these data suggest that nuclear localisation of Rca1 apparently relies on several, partly redundant domains within the Rca1 protein. Some additional elements that contribute to nuclear targeting of Rca1 are located in the N-terminal part, but their precise nature was not identified in this study.

3.2 Yeast two-hybrid screen

Rca1 restrains APC/C-Cdh1^{Fzr} activity during G2 and this inhibitory function requires association with components of the APC/C complex (Grosskortenhaus and Sprenger, 2002). Moreover, Rca1 was proposed to be part of an SCF complex that accelerates the G1-S transition by mediating degradation of unknown substrates (Zielke et al., 2006).
A yeast two-hybrid screen has been performed to identify binding partners of Rca1. This approach should reveal possible subunits of the APC/C and SCF complex that interact with Rca1. Moreover, the screen should result in the identification of possible substrates of the SCF/Rca1 complex. For screening, a bait vector was constructed in which the complete coding region of Rca1 was cloned in frame to the Gal4-DNA binding domain into the pB27 vector (Figure 5.1E). The screen was carried out by the Y2H service Hybrigenics (Paris) using *Drosophila* whole embryo RP2 (0-12+12-24) libary as prey. About 133.92 million diploids were tested resulting in the identification of five putative interaction partners of Rca1 (Table 3.1).

Table 3.1 Yeast two-hybrid screen results for *Drososphila* Rca1.

Gene	Name	Number of clones	Frame	Interaction score
CG16983	SkpA	71	IF	A
CG2508	Cdc23 (APC8)	1	IF	D
CG11494	BtbVII	4	IF	E
CG6716	Paired	1	IF	E
CG13651	Distal antenna-related	1	IF	E

Interaction score:
A: very high confidence in the interaction
B: high confidence in the interaction
C: Good confidence in the interaction
D Moderate confidence in the interaction (hardly detectable interactions because of folding, toxicity in yeast, or low representation of the mRNA in the libary)
E Potential false positives
IF CDS fragments cloned in frame to the Gal4AD

Drosophila SkpA showed the highest interaction score of all candidates that were identified as interacting partners of Rca1. This is consistent with a genome wide yeast two-hybrid screen (Giot et al., 2003), in which *Drosophila* SkpA was also shown to bind Rca1.

The second best interaction score was seen for Cdc23, an APC/C subunit that is also named APC8. The other three candidates BtbVII, Distal Antenna-related and Paired showed a very low score of interaction and were thus classified as potential false positives. Therefore, the validation of the yeast two-hybrid data was prioritised for SkpA and Cdc23.

3.2.1 Interaction of Rca1 and Cdc23

Based on the yeast two-hybrid data, Cdc23 is a moderate interacting partner of Rca1. Cdc23, also known as APC8, is one subunit of the large APC/C ubiquitin ligase that targets mitotic cyclins and other substrates for proteasomal degradation. Since Rca1 restrains APC/C-Cdh1^{Fzr} activity during G2, the inhibitory effect of Rca1 has been proposed to rely on interaction between Rca1 and components of the APC/C. A physical interaction between Rca1 and the APC/C-Cdh1^{Fzr} complex has already been demonstrated. Precipitation of Rca1 from embryonic extracts and subsequent Western blot analysis using specific antibodies revealed coprecipitation of Cdh1^{Fzr} and the APC/C subunit Ccd27 (Grosskortenhaus and Sprenger, 2002). The protein(s) that mediate direct interaction between Rca1 and the APC/C subunits remain to be identified. Cdc23 might be a good candidate for interacting directly with Rca1 and promoting complex formation between Rca1, Cdh1^{Fzr}, Cdc27 and the remaining APC/C subunits. Interaction studies on Emi, the vertebrate orthologue of Rca1, have also claimed interaction between Emi and Cdh1^{Fzr} (Reimann et al., 2001a) and several APC/C subunits including Cdc23 (APC8) (Miller et al., 2006).

To verify the interaction between Rca1 and Cdc23, immunoprecipitaion experiments were performed in *Drosophila* S2 cells. Cdc23 was equipped with a FLAG tag and Rca1 with an HA epitope tag to allow detection of these proteins with the corresponding antibodies. FLAG-Cdc23 and HA-Rca1 were coexpressed in S2 cells and either of these proteins was immunoprecipitated from cell lysates using HA or FLAG antibodies. Cell lysates and immunoprecipitates were analysed by SDS-PAGE and Western blots were probed with HA or FLAG antibodies to detetct precipitated proteins (Figure 3.3A and B).

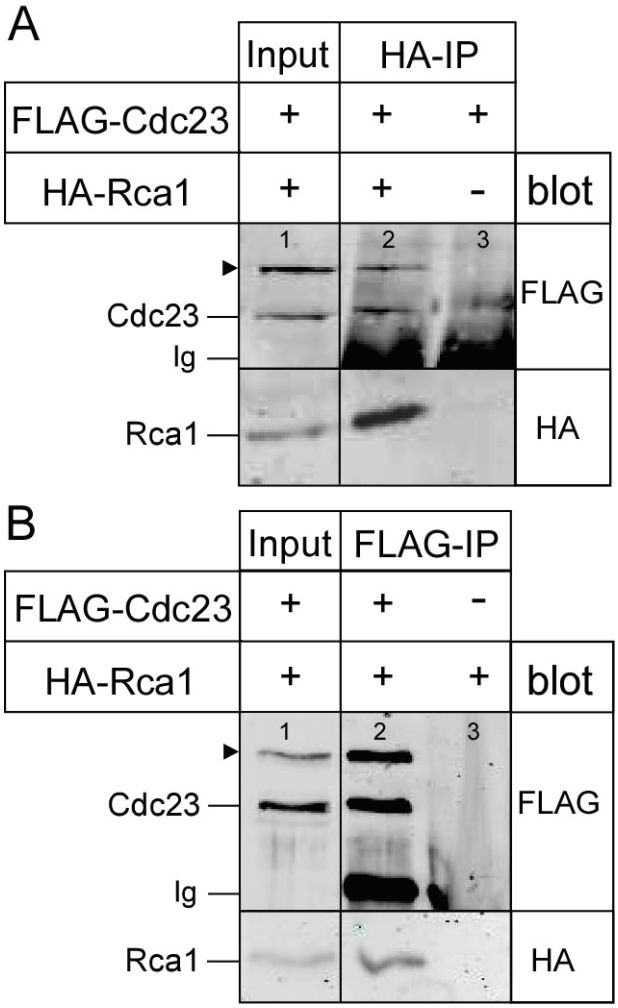


Figure 3.3 Interaction studies between Rca1 and Cdc23.
FLAG-tagged Cdc23 and HA-tagged Rca1 were transiently transected into S2 cells. After induction of protein expression, cells were lysed for immunoprecipitation (IP) experiments using either HA or FLAG antibodies. FLAG-Cdc23 and HA-Rca1 lysates (Input) and immunoprecipitates were analysed by SDS-PAGE and Western blotting using HA and FLAG antibodies to detect tagged proteins. The arrowhead marks an additional band for Cdc23 at approximately 80 kDa presumably due to hyperphosphorylation of Cdc23 (FLAG blots, lane 1 and 2).
(A) HA-Rca1 was precipitated from S2 lysates resulting in a 47 kDa protein band (HA Blot; lane1 and 2). FLAG-Cdc23 was detected at the expected protein size of 68 kDa and coprecipitated with HA-Rca1 (FLAG blot, lane 1 and 2).
(B) FLAG antibody was used to precipitate FLAG-Cdc23 from S2 lysates (FLAG blot; lane 1 and 2). HA-Rca1 coprecipitated with Cdc23 (HA blot; lane 2) giving evidence for a physical interaction between Rca1 and the APC/C subunit Cdc23.

Expression of HA-Rca1 was monitored by Western blot analysis in which HA-Rca1 migrates as an approximately 47 kDa protein (Figure 3.3A and B; HA blots, lane1 and 2). The FLAG antibody recognised specifically two bands after expression of FLAG-Cdc23. The faster migrating protein reveals Cdc23 at the expected size of 68 kDa (Figure 3.3A and B; FLAG-blots; lane 1 and 2). Furthermore, an additional protein band was detected at approximatlly 80 kDa (Figure 3.3A and B; FLAG blots, arrowheads). Since APC/C subunits are known to be phosphorylated upon Cdk activity (Kraft et al., 2005), this additional band could correspond to a hyperphosphorylated form of Cdc23. However, there is no direct evidence that this band is a result of Cdc23 phosphorylation. HA-Rca1 or FLAG-Cdc23 was efficiently precipitated from S2 cell lysates (Figure 3.3A and B; lane 3). In both immunoprecipitation assays, the other protein was coprecipitated, respectively. Moreover, it was shown that coprecipitation of either protein depends on the presence of

the other one (Figure 3.3 A and B, lane 3) demonstrating that the interaction between Cdc23 and Rca1 is specific. Thus, immunoprecipitation studies in *Drosophila* S2 cells confirmed the yeast two-hybrid interaction of Rca1 and the APC/C subunit Cdc23.

3.2.2 Interaction of Rca1 and SkpA depends on the F-box motif

The yeast two-hybrid screen identified SkpA as a binding partner of Rca1 (Table 3.1). This interaction was already found in a genome wide yeast two-hybrid screen (Giot et al., 2003). Moreover, vertebrate Emi1 and Emi2/XErp1 were shown to interact with Skp1 in an F-box dependent manner (Reimann et al., 2001a; Schmidt et al., 2005). Skp is a subunit of the SCF (Skp-Cullin-F-box protein) ubiquitin ligase which mediates degradation of several cell cycle substrates (Ang and Wade Harper, 2005; Kipreos and Pagano, 2000; Maniatis, 1999). In an SCF complex, Skp binds the F-box protein via the F-box motif and mediates interaction with the scaffold protein Cullin (Vodermaier, 2004). F-box proteins are responsible for substrate recognition and specificity of the SCF complex (Skowyra et al., 1997). Rca1/Emi proteins harbour an F-box motif and therefore belong to the family of F-box proteins. Recently, an F-box dependent function of Rca1 at the G1-S transition has been identified (Zielke et al., 2006). Thereby, Rca1 overexpression promotes S phase entry and this function relies on the F-box motif. This implicates that Rca1 might function as an F-box protein in an SCF complex that is involved in G1-S regulation. The observed interaction of Rca1 and SkpA in the yeast two-hybrid system suggests that Rca1 is incorporated into an SCF complex by the F-box motif. However, this idea has not yet been verified experimentally. Therefore, it was necessary first to give evidence for an interaction between Rca1 and SkpA in a different system and second, to verify that this interaction relies on the F-box motif in Rca1. FLAG-tagged variants of Rca1, Rca1 lacking the F-box (FLAG-Rca1ΔFbox) or Rca1 harbouring a point mutation in the ZBR (FLAG-Rca1C351S) were coexpressed with HA-SkpA in *Drosophila* S2 cells. Proteins were precipitated from cell lysates using HA or FLAG antibodies (Figure 3.4A and B). Cell lysates and immunoprecipitates were analysed by SDS-PAGE and Western blotting using antibodies that specifically detect the tagged proteins.

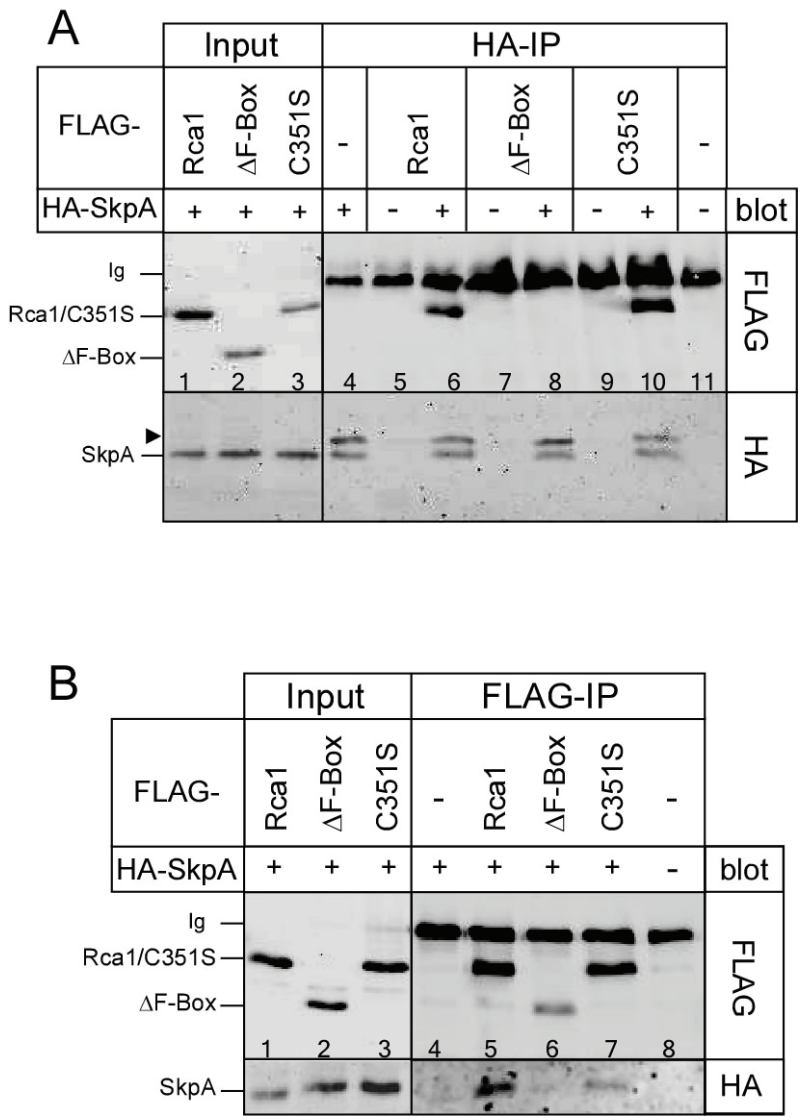


Figure 3.4 Rca1 and SkpA interact in an F-box dependent manner. FLAG-tagged full length Rca1 (FLAG-Rca1), Rca1 lacking the F-box motif (FLAG-Rca1ΔF-box) or harbouring a point mutation in the ZBR (FLAG-Rca1C351S) were coexpressed with HA-tagged SkpA in S2 cells. Cell lysates (Input) and immunoprecipitates (IP) were analysed by SDS-PAGE. Proteins were detected on Western blots via their epitope tag using FLAG or HA antibodies. Immunglobulin (Ig) heavy chain was visible in FLAG blots. **(A)** HA-SkpA was efficiently precipitated from cell lysates with HA antibodies (HA-IP/HA-blot). An additional, unspecific band was detected in HA-SkpA precipitates (arrowhead). While FLAG-Rca1 or FLAG-Rca1C351S coprecipitated with HA-SkpA (FLAG-blot, lane 6 and 10), Rca1 lacking the F-box could not bind SkpA (FLAG-blot, lane 8). Thus, the F-box motif within Rca1 specifically mediates interaction with SkpA. **(B)** Immunoprecipitations were performed similarly, but using FLAG antibodies to precipitate FLAG-Rca1 proteins (FLAG-blost, lane 5, 6, 7). HA-SkpA coprecipitated with FLAG-Rca1/C351S, but not with FLAG-Rca1ΔF-box (HA blot, lane 5, 6, 7) demonstrating that the F-box is essential for Rca1 and SkpA interaction.

Expression of HA-SkpA resulted in a 23 kDa protein (Figure 3.4A and B; HA blots, lane 1-3). HA-SkpA was efficiently precipitated from S2 cell lysates using HA antibody (Figure 3.4A; HA blot, lane 4, 6, 8, 10). FLAG-Rca1 or FLAG-Rca1C351S protein was detected as a 47 kDa protein band, whereas FLAG-Rca1ΔFbox resulted in a slightly smaller protein of approximatly 43 kDa (Figure 3.4A and B; FLAG blots, lane 1-3). FLAG-Rca1 and FLAG-Rca1C351S were both able to coprecipitate with HA-SkpA (Figure 3.4A; FLAG blot, lane 6 and 10). Although disruption of the ZBR by a point mutation (C351S) results in a protein unable to inhibit the APC/C-Cdh1^{Fzr} complex (Zielke et al., 2006), this mutation did not perturb SkpA binding (Figure 3.4A; FLAG blot, lane 10). Importantly, Rca1 lacking the F-box failed to interact with SkpA demonstrating that the F-box motif is essential for SkpA

binding (Figure 3.4A; FLAG blot, lane 8). Interaction of Rca1 and SkpA is specific since all FLAG-Rca1 proteins cannot be precipitated in the absence of SkpA (Figure 3.4A; FLAG blot, lane 5, 7, 9). To verify this interaction, immunoprecipitations were performed *vice versa* using FLAG antibody to pull down FLAG-Rca1, FLAG-Rca1ΔF-box or FLAG-Rca1C351S proteins from S2 cell lysates (Figure 3.4B, FLAG blot, lane 5, 6, 7). HA-SkpA coprecipitates with full length Rca1 or Rca1C351S (Figure 3.4B; HA blot, lane 5 and 7). However, SkpA failed to interact with Rca1 lacking the F-box (Figure 3.4B; HA blot, lane 6). Altogether these experiments verified the yeast two-hybrid interaction between Rca1 and SkpA suggesting that Rca1 could incorporate in an SCF complex via F-box dependent binding to SkpA.

The F-box and the ZBR motif of Rca1 are necessary for the ectopic S phase induction by Rca1 (Figure 3.1; Zielke et al., 2006). APC/C-Cdh1^{Fzr} inhibition by Rca1 relies on the ZBR, but the F-box is dispensable for this function (Figure 3.1; Zielke et al., 2006). Interaction of Rca1 and the SCF subunit SkpA depends on the F-box suggesting that a potential SCF/Rca1 complex is responsible for S phase induction, but not for the G2 function during embryogenesis. Rca1 harbouring a point mutation in the ZBR (C351S) cannot induce ectopic S phases, but is capable for SkpA binding (Figure 3.4A and B) demonstrating that SCF/Rca1 complex formation does not rely on the ZBR. Besides the ZBR, the amino acid region 203-255 has been shown to play a role for the inhibitory effect on the APC/C-Cdh1^{Fzr} complex (Figure 3.1; Radermacher, 2007; Zielke et al., 2006). However, overexpression of Rca1Δ203-255 can still induce ectopic S phases (Figure 3.1; Radermacher, 2007). Since this region is not essential for Rca1 function at the G1-S transition, it should not affect interaction between Rca1 and SkpA. To test this, FLAG-Rca1 or FLAG-Rca1Δ203-255 was coexpressed with HA-SkpA in S2 cells. For immunoprecipitation studies, FLAG-tagged proteins were precipitated from cell lysates using FLAG antibody. Precipitates were analysed by SDS-PAGE and Western blots probed with FLAG or HA antibodies to detect proteins via their tags.

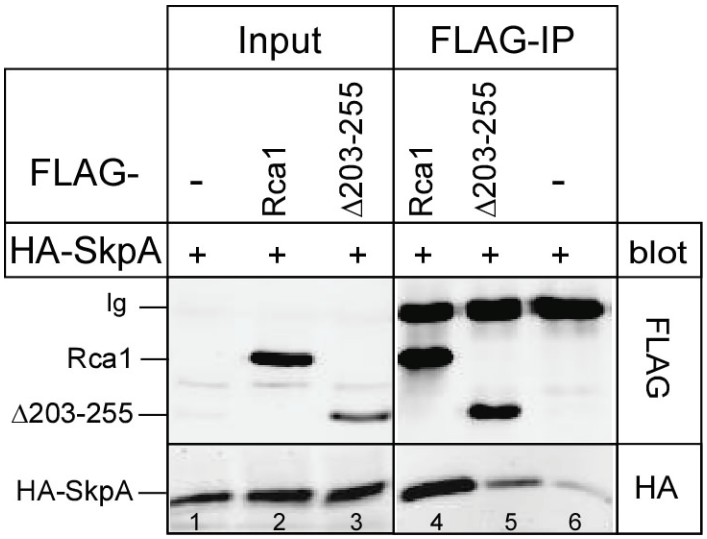


Figure 3.5 Rca1Δ203-255 can still interact with SkpA.
Rca1 lacking the region 203-255 was tested for its ability to bind SkpA. FLAG-Rca1 or FLAG-Rca1Δ203-255 was co-expressed with HA-SkpA in S2 cells. Rca1 proteins were precipitated from cell lysates using FLAG antibody. Cell lysates (Input) and immunoprecipitations (FLAG-IP) were analysed by SDS-PAGE and Western blots probed with FLAG and HA antibodies. HA-SkpA coprecipitated with FLAG-Rca1 or FLAG-Rca1Δ203-255 (FLAG/HA blots, lane 4 and 5). HA-SkpA was still able to coprecipitate with FLAG-Rca1Δ203-255 (HA blot, lane 5, but the amount of SkpA pulled down was reduced to 40%.

FLAG-Rca1 or FLAG-Rca1Δ203-255 was efficiently precipitated from S2 cell lysates and resulted in protein bands detectable at 47 kDa or 41 kDa (Figure 3.5; FLAG blot, lane 4 and 5). HA-SkpA was coprecipitated with full length Rca1 as well as Rca1 lacking the region 203-255 (Figure 3.5; HA blot, lane 4 and 5). However, the binding of HA-SkpA to FLAG-Rca1Δ203-255 was less efficient and the amount of coprecipitated HA-SkpA was about 40% reduced (Figure 3.5; HA blot, lane 5).

3.3 Rca1 interacts with SCF subunits

SCF complexes consist of the core subunits Skp, Cullin1 (Cul1) and an F-box protein (for review see Nakayama and Nakayama, 2006; Petroski and Deshaies, 2005; Vodermaier, 2004). In an SCF complex, the F-box protein is attached to the Cul1 scaffold protein via the Skp subunit that recognises the F-box (Schulman et al., 2000; Zheng et al., 2002). Cul1 associates with a member of the Roc or RING finger protein family which recruits the ubiquitin-conjugating enzyme (E2). In *Drosophila*, six Skp proteins (SkpA-F) and three different Roc proteins (Roc1a, Roc1b and Roc2) have been identified (Donaldson et al., 2004; Nayak et al., 2002; Noureddine et al., 2002; Yamanaka et al., 2002). There is evidence for SkpA primarily acting as part of multiple SCF complexes: First, SkpA is highly similar to human and yeast Skp1 which forms different SCF's in *vitro* and in *vivo* (Deshaies, 1999). Secondly, SkpA forms a complex with *Drosophila* Cul1, Roc1a and the

F-box protein Slimb or Ppa (Bocca et al., 2001; Raj et al., 2000) indicating that SkpA forms at least two different SCF complexes in *Drosophila*. Furthermore, SkpA and Cul1 function as core subunits of the SCF/Ago complex which mediates ubiquitination and degradation of Cyclin E (Moberg et al., 2001; Murphy, 2003). A function of any other Skp protein acting in an SCF complex in *Drosophila* has not been ascribed so far. Roc1a is the only member of the *Drosophila* Roc family which has been investigated in more detail. Roc1a can interact with Cul1 and SkpA and has been ascribed to mediate Cubitus interruptus (Ci) degradation in an SCF complex with the F-box protein Slimb (Bocca et al., 2001; Donaldson et al., 2004; Jiang and Struhl, 1998; Maniatis, 1999). Roc1b and Roc2 have been shown to interact with Cul3 or Cul5 proteins presumably acting in other Cullin-RING ubiquitin ligases than the SCF complex (Donaldson et al., 2004).

3.3.1 Endogenous Cul1 coprecipitates with Rca1 or SkpA

Rca1 was shown to bind SkpA via the F-box (Figure 3.4A and B). However, it remains to be elucidated if Rca1 and SkpA form a complex with other SCF subunits. If this notion holds true, the third SCF core subunit Cul1 should interact via the adaptor SkpA with the F-box protein Rca1. Therefore, FLAG-tagged Rca1 or HA-tagged SkpA were overexpressed in S2 cells. Proteins were precipitated from cell lysates using FLAG or HA antibodies. Cell lysates and immunoprecipitation samples were separated by SDS-PAGE. For Western blot analysis, FLAG or HA antibodies were used to detect Rca1 and SkpA protein in cell lysates and immunoprecipitates (Figure 3.6A and B). In addition, Western blots were probed with Cul1 antibody which recognises endogenous Cul1 protein.

FLAG-Rca1 or HA-SkpA were efficiently precipitated from S2 cell lysates (Figure 3.6A and B; FLAG and HA blot, lane 1 and 2). Western blots were incubated with Cul1 antibody to detect endogenous Cul1 protein with a protein size of approximatley 83 kDa in cell lysates and immunoprecipitates (Figure 3.6A and B; Cul1 blot, lane 1 and 2). Endogenous Cul1 protein specifically coprecipitated with immunoprecipitated FLAG-Rca1 or HA-SkpA (Figure 3.6A and B; Cul1 blot, lane 2). Thus, the third SCF subunit specifically binds to SkpA as well as to the F-box protein Rca1 resulting in a complete SCF core complex. Since Cul1 is known to interact with the F-box protein via the adaptor SkpA, coprecipitation of endogenous Cul1 and FLAG-Rca1 occurs indirectly via endogenous SkpA protein.

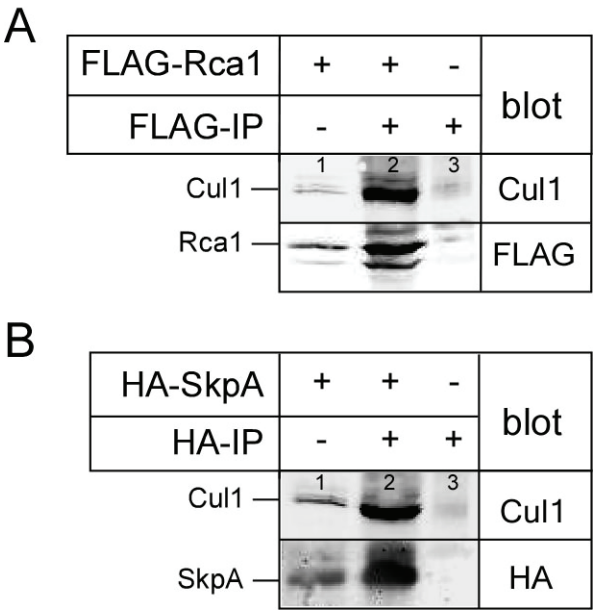


Figure 3.6 Endogenous Cul1 protein coprecipitates with FLAG-Rca1 or HA-SkpA. FLAG-Rca1 or HA-SkpA were expressed in S2 cells and proteins were precipitated from cell lysates using FLAG or HA antibodies. Cell lysates (lane1) and immunoprecipitates (FLAG/HA-IP) were analysed by SDS-PAGE. Western blots were incubated with HA, FLAG or Cul1 antibodies to detect epitope tagged proteins or endogenous Cul1. **(A)** Immunoprecipitated FLAG-Rca1 (FLAG-IP) was detected on FLAG blots as a 47 kDa protein band (lane 2). Endogenous Cul1 resulted in a 83 kDa protein band and coprecipitated with FLAG-Rca1 (Cul1 blot, lane 2), presumably via binding to endogenous SkpA. **(B)** HA-SkpA was precipitated with HA antibodies resulting in a 23 kDa protein band (HA blot, lane 2). Endogenous Cul1 coprecipitated with HA-SkpA (Cul1 blot, lane 2) indicating that Cul1 binds to SkpA and forms a complex.

3.3.2 Rca1 forms a complex with Cul1 and SkpA

The finding that endogenous Cul1 coprecipitates with FLAG-Rca1 or HA-SkpA from S2 cell lysates (Figure 3.6A and B) supports the idea that Rca1 is part of an SCF complex. To verify this assumption, all three SCF core proteins were overexpressed in S2 cells and cell lysates were used for immunoprecipitation studies. All constructs were equipped with different tags to allow precipitation and detection of every single protein with the corresponding antibodies. FLAG-Rca1, HA-SkpA and Myc-Cul1 were simultaneously expressed in S2 cells and Rca1 or SkpA were precipitated from cell lysates using FLAG or HA antibodies. Cell lysates and immunopreciptates were analysed by SDS-PAGE and Western Blotting using HA, FLAG or Myc antibodies (Figure 3.7A and B).

FLAG-Rca1 or HA-SkpA were efficiently precipitated from cell lysates using FLAG or HA antibodies (Figure 3.7A and B; FLAG or HA blot, lane 4) and resulted in the coprecipitation of Myc-Cul1 and HA-SkpA/FLAG-Rca1 (Figure 3.7A and B; lane 4). Furthermore, coprecipitation of all three proteins was shown to be specific (Figure 3.7A and B; lane 5 and 6). Thus, the SCF core subunits SkpA and Cul1 physically interact with the F-box protein Rca1 providing further evidence for the proposed SkpA-Cul1-Rca1 complex.

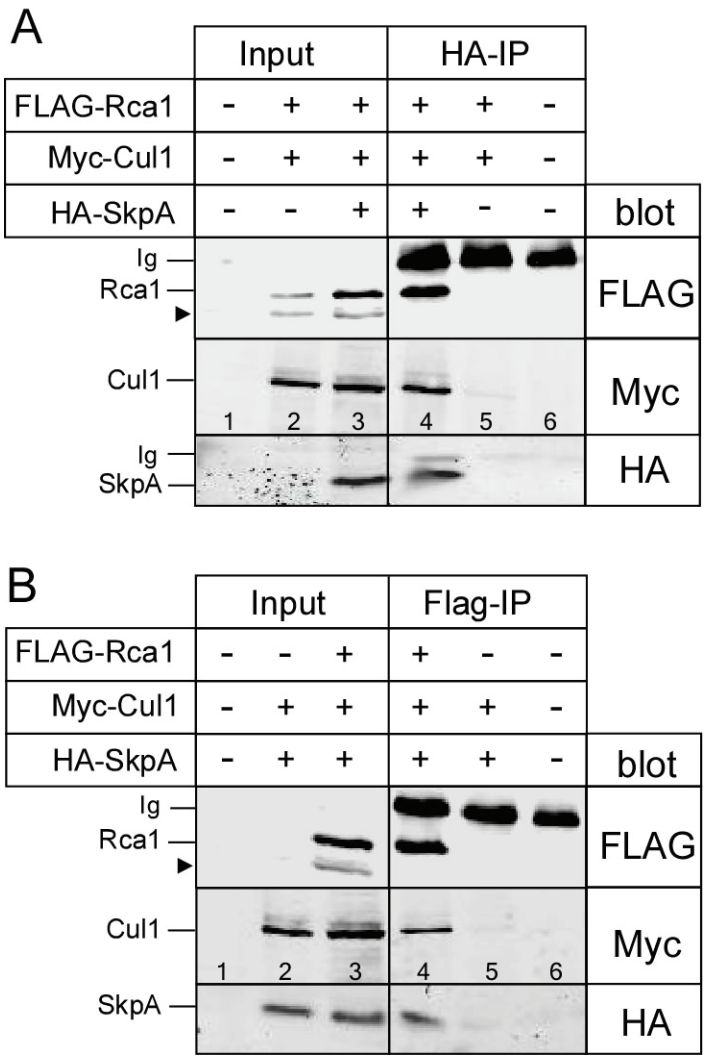


Figure 3.7 Rca1, SkpA and Cul1 can physically interact and form a complex. FLAG-Rca1, HA-SkpA and Myc-Cul1 were coexpressed in S2 cells. Rca1 or SkpA were precipitated from cell lysates using FLAG or HA antibodies. Cell lysates (Input) and immunoprecipitates (FLAG/HA-IP) were separated by SDS-Page. Western blots were probed with FLAG, HA and Myc anibodies to detect epitope tagged proteins. Heavy or light chains of immunglobulins (Ig) as well as an unspecific protein band (arrowhead) in the FLAG-blots (lane 3) were visible.
(A) HA-SkpA is precipitated from cell lysates using HA antibodies and could be detected as a 23 kDa protein band (HA blot, lane 4). Myc-Cul1 (83 kDa) and FLAG-Rca1 (47 kDa) were specifically coprecipitated with HA-SkpA (FLAG/Myc blot, lane 4) confirming an interaction between the three SCF proteins.
(B) FLAG-Rca1 was efficiently precipitated from cell lysates with FLAG antibodies (FLAG blot; lane 4). Western blots were probed with Myc and HA antibodies to detect coprecipitated Myc-Cul1 and HA-SkpA (Myc/HA blot; lane 4). Immunoprecipitation of all three proteins together is specific since Cul1 and SkpA fail to coprecipitate in the absence of FLAG-Rca1 (lane 5). Thus, Rca1, Cul1 and SkpA can form a core SCF complex.

3.3.3 Complex formation of Rca1-SkpA-Cul1 depends on the F-box

Rca1 was shown to form a complex with the SCF subunits SkpA and Cul1. In this complex, interaction of all three proteins occurs via the adaptor SkpA that binds to both proteins, the F-box protein Rca1 and the scaffold protein Cul1. Rca1 lacking the F-box fails to interact with SkpA (Figure 3.4A and B) and presumably cannot form a complex with Cul1. To test this, full length Rca1 (FLAG-Rca1) or Rca1 lacking the F-box (FLAG-Rca1ΔF-box) was coexpressed with Myc-Cul1 in S2 cells. FLAG-tagged Rca1 proteins were precipitated from cell lysates using FLAG antibody. Cell lysates and

immunoprecipitation samples were analysed by SDS-PAGE and Western blotting using FLAG or Myc antibodies to detect the proteins via their tags.

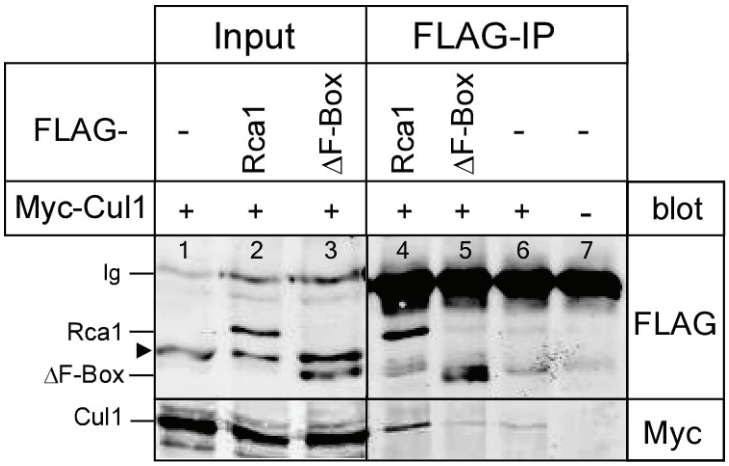


Figure 3.8 Interaction of Rca1 and Cul1 depends on the F-box.
FLAG-Rca1 or FLAG-Rca1ΔF-box was coexpressed with Myc-Cul1 in S2 cells. FLAG-tagged proteins were precipitated from cell lysates using FLAG antibody. Cell lysates (Input) and precipitates (FLAG-IP) were analysed by SDS-PAGE and Western blotting with FLAG and Myc antibodies. Myc-Cul1 coprecipitated specifically with FLAG-Rca1, but not with FLAG-Rca1ΔF-box (Myc blot, lane 4 and 5) indicating that this interaction relies on the F-box motif within Rca1. An unspecific protein band was detected in the FLAG blot (arrowhead, lane 1-3).

FLAG-Rca1 or FLAG-Rca1ΔF-box were readily detectable in the FLAG blot indicating that both proteins were efficiently precipitated and resulted in a 47 kDa or 43 kDa protein band (Figure 3.8; FLAG blot, lane 4 and 5). Myc-Cul1 was detetced on Myc blots with a protein size of approximately 83 kDa (Figure 3.8; Myc blot). Myc-Cul1 coprecipitated with full length Rca1 (Figure 3.8; Myc blot; lane 4), but failed to interact with Rca1 lacking the F-box (Figure 3.8; Myc blot, lane 5). Thus, the interaction between Rca1 and Cul1 also depends on the F-box motif similar to Rca1 and SkpA binding (Figure 3.4A and B). In an SCF complex, F-box proteins bind the Cul1 subunit indirectly via the adaptor protein SkpA. This indicates that binding of FLAG-Rca1 and Myc-Cul1 seems to occur indirectly via endogenous SkpA protein. Thus, in a putative SCF/Rca1 complex SkpA binds to Cul1 and also to the F-box motif within Rca1 and thereby mediates interaction of all three SCF components.

3.4 What are the targets of the putative SCF/Rca1 complex?

Rca1 promotes S phase entry in proliferating cells by an F-box dependent mechanism (Zielke et al., 2006). Furthermore, Rca1 binds the SCF components SkpA and Cul1 (Figure 3.4-3.8). This interaction depends on the F-box motif suggesting that Rca1 acts as an F-box protein in an SCF complex. F-box proteins are known to mediate substrate recognition and thereby initiate degradation of target proteins by the proteasome. Thus, the F-box dependent S phase induction by Rca1 indicates that the SCF/Rca1 complex might mediate degradation of a negative regulator of the G1-S transition. In *Drosophila*, Cyclin E/Cdk2 activity drives the G1-S transition (Knoblich et al., 1994; Richardson et al., 1995). Several factors negatively regulate Cyclin E/Cdk2 activity: The Retinoblastoma protein (Rb) influences Cyclin E transcript levels by inhibiting E2F1 (Du et al., 1996; Du and Dyson, 1999; Frolov et al., 2001), whereas the SCF/Ago complex mediates degradation of Cyclin E protein (Koepp et al., 2001; Moberg et al., 2001; Schwab and Tyers, 2001). Moreover, Cyclin E/Cdk2 activity is restrained by the CKI Dacapo (de Nooij et al., 1996; Lane et al., 1996). Cyclin A/Cdk1 activity can also trigger S phase entry, but in general its S phase potential is prevented by different mechanisms: First, by APC/C-Cdh1^{Fzr} dependent Cyclin A degradation (Pimentel and Venkatesh, 2005), second, by restriction of Cdk1 activity due to Wee dependent inhibitory phosphorylation (Campbell et al., 1995; Sprenger et al., 1997) and third, by the action of the CKI Rux (Sprenger et al., 1997; Thomas et al., 1997). Loss of any of these inhibitory mechanisms results in premature Cyclin/Cdk1 activity and ectopic S phases.

3.4.1 Fzr, Rux or Wee are not targets of the SCF/Rca1

Fzr is a negative regulator of the G1-S transition since the APC/C-Cdh1^{Fzr} complex restrains Cyclin A/Cdk activity by mediating degradation of Cyclin A. S phase induction by Rca1 could either be achieved by competitive inhibition of the APC/C-Cdh1^{Fzr} complex or by SCF/Rca1 dependent degradation of Fzr. In addition, Wee and Rux could also be targets of the SCF/Rca1 complex since both proteins are inhibitors of Cyclin A/Cdk complexes. To test this, Fzr, Rux or Wee were equipped with an HA tag and coexpressed with FLAG-tagged Rca1 in S2 cells. Protein expression of Fzr, Rux or Wee was monitored by immunostainings and Western blot analysis in the abscence or presence of FLAG-Rca1.

S2 cells were transfected with HA-tagged Fzr, Rux or Wee constructs and protein expression was visualised by HA antibody staining. In addition, cells were stained for DNA and the nuclear envelope was visualised by wheat germ agglutinin (WGA) staining (Figure 3.9).

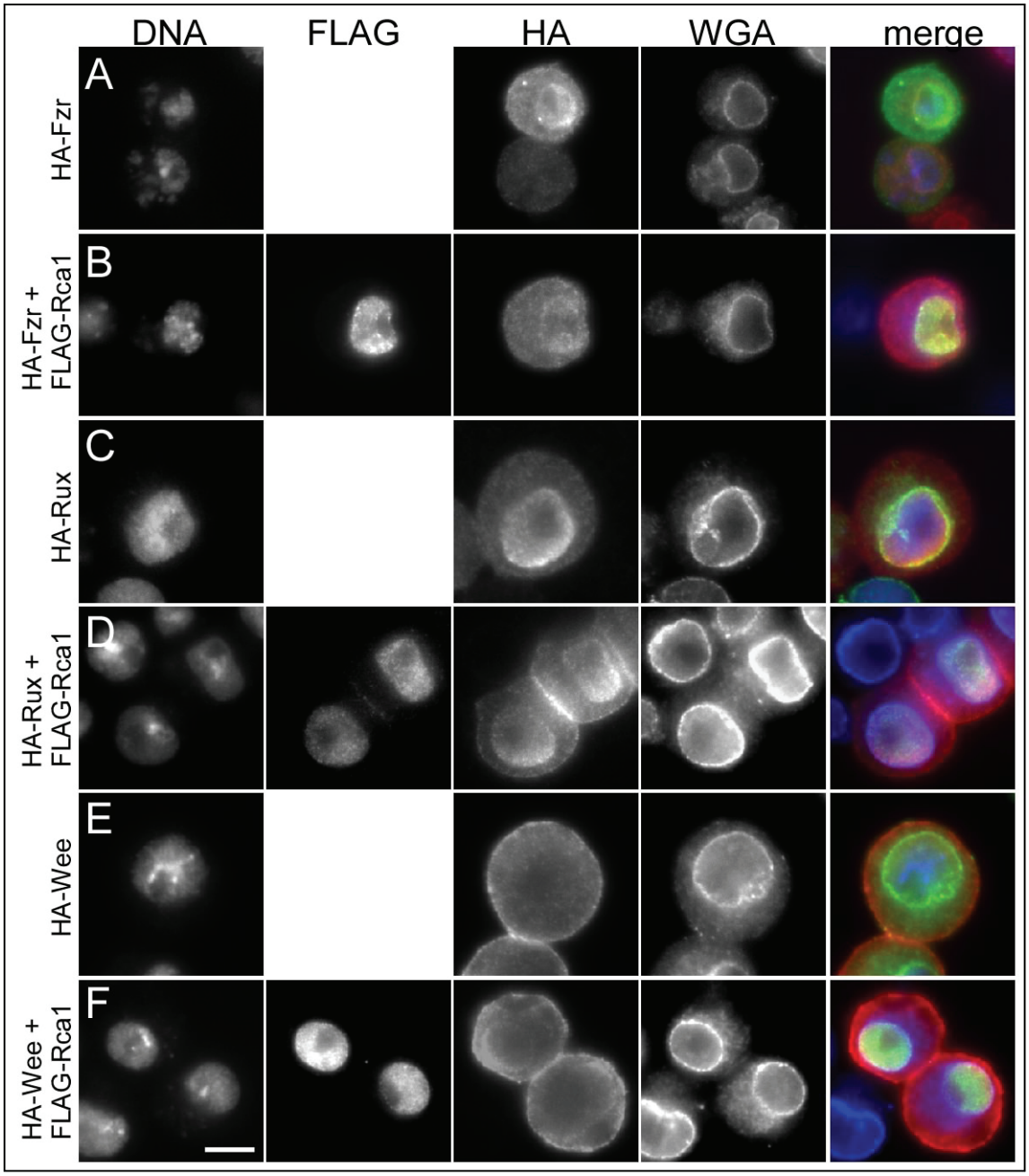


Figure 3.9 Protein levels of Fzr, Rux and Wee were not affected by coexpression of Rca1.
HA-tagged Fzr, Rux or Wee were expressed in S2 cells with or without FLAG-Rca1. Cells were fixed and stained with HA antibody to detect Fzr, Rux or Wee protein. FLAG-Rca1 was visualised using FLAG antibody. In addition, the nuclear membrane (WGA) and DNA were stained. Scale bar: 2µm.
(A, C, E) Fzr, Rux or Wee can be detected throughout the cytoplasm and nucleus with Rux showing the highest nuclear accumulation.
(B, D, F) FLAG-Rca1 is a nuclear protein. Coexpression of Rca1 did not affect Fzr, Rux or Wee protein levels within the cell demonstrating that these proteins are not targets of the putative SCF/Rca1 complex.

Fzr, Rux or Wee protein could be detected throughout the cytoplasm and nucleus, but Fzr and Rux seemed to accumulate in the nucleus (Figure 3.9A and C and E). Cells coexpressing FLAG-Rca1 were stained with FLAG antibody to monitor nuclear localisation of Rca1 (Figure 3.9B and D and F). However, protein levels of Fzr, Rux or Wee did not change upon Rca1 coexpression indicating that these proteins are not targets of the SCF/Rca1 complex. In addition, these observations were confirmed by Western blot analysis. Lysates of cells expressing HA-tagged Fzr, Rux or Wee alone or with FLAG-Rca1 were separated by SDS-PAGE. Western blots were probed with HA and FLAG antibodies to detect expressed proteins via their tag (Figure 3.10). To normalise protein levels, endogenous actin was detected using actin antibody. Protein levels of Fzr, Rux or Wee were not affected upon Rca1 coexpression (Figure 3.10; HA blots, second lane) demonstrating that these proteins are not degraded by the putative SCF/Rca1 complex.

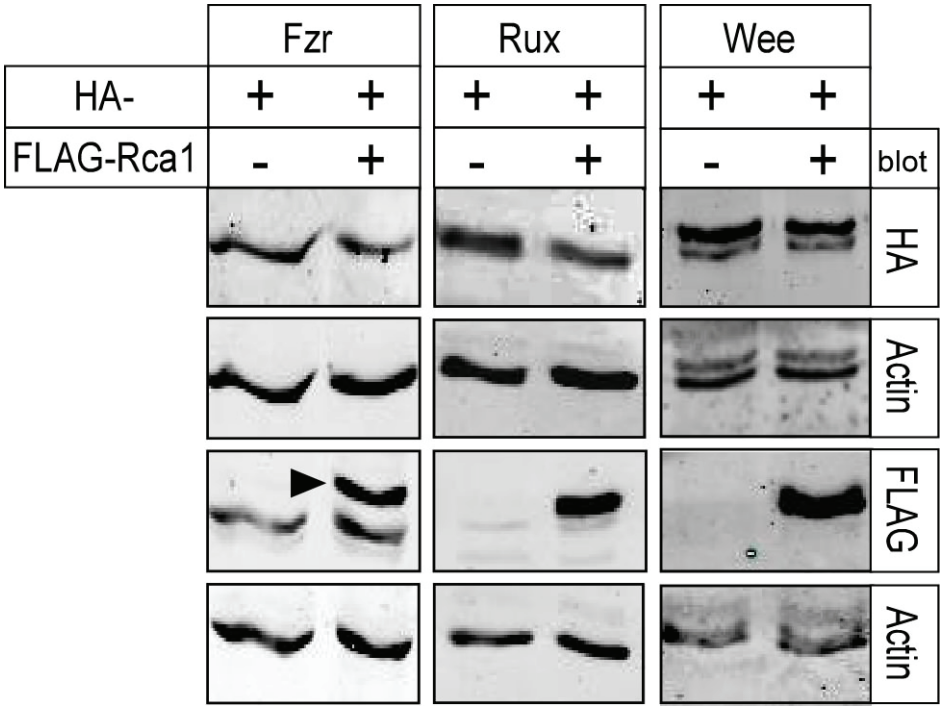


Figure 3.10 Fzr, Rux or Wee are not a target of the putative SCF/Rca1 complex.
HA-tagged Fzr, Rux or Wee were expressed alone or with FLAG-Rca1 in S2 cells (HA blots first or second lane). Protein lysates were analysed by SDS-PAGE and Western blots were probed with HA and FLAG antibodies. To normalise protein levels, endogenous actin was detected using actin antibody (Actin blots). The arrowhead marks FLAG-Rca1 protein. Protein levels of HA-Fzr, HA-Rux or HA-Wee did not change upon FLAG-Rca1 coexpression (HA blots, second lane). This indicates that Fzr, Rux or Wee protein are not degraded by the putative SCF/Rca1 complex.

3.4.2 Peptide Mass Fingerprinting analysis (PMF)

Rca1 seems to act in two different complexes during the cell cycle. First, it restrains APC/C-Cdh1^{Fzr} activity in G2 and therefore associates with APC/C components (Figure 3.3; Grosskortenhaus and Sprenger, 2002). Secondly, Rca1 drives the G1-S transition in an F-box dependent manner (Zielke et al., 2006). Since the F-box is essential for interaction with the SCF subunits SkpA and Cul1 (Figure 3.4 and 3.8), a putative SCF/Rca1 complex could induce S phase entry by targeting different G1-S inhibitors for degradation. Potential substrates of the SCF/Rca1 complex should be identified by immunoprecipitation experiments combined with Peptide Mass Fingerprinting (PMF) analysis. Furthermore, Rca1 binding partners like APC/C or SCF subunits should also be found by this method. Therefore, FLAG-Rca1 was overexpressed in S2 cells and immunoprecipitated from cell lysates with ANTI-FLAG-M2 affinity gel (Sigma). FLAG-Rca1 precipitates was separated by SDS-PAGE and analysed by Western blotting or Colloidal Coomassie staining. Western blots were probed with FLAG and Cul1 antibodies to detetct efficient pull-down of FLAG-Rca1 and coprecipitation of endogenous Cul1. Protein bands were excised from Coomassie stained gels and proteins were identified by PMF analysis. FLAG-Dof (1-552) protein was precipitated as a control to observe unspecific protein binding on Coomassie stained gels.
FLAG-Rca1 and FLAG-Dof protein were efficiently precipitated from S2 cell lysates resulting in 47 kDa and 65 kDa protein band (Figure 3.11A; FLAG blot, arrowheads). Endogenous Cul1 coprecipitated only with FLAG-Rca1 indicating that this interaction of Rca1 and Cul1 is specific (Figure 3.11A; Cul1 blot, lane 3). Coomassie stained protein gels revealed several bands of coprecipitated proteins in the FLAG-Rca1 or FLAG-Dof precipitates (Figure 3.11B). Some protein bands were observed in both immunoprecipitation samples indicating that these proteins unspecifically bind to the affinity gel. However, six specific protein bands were detected in FLAG-Rca1 immunoprecipitates which were exciced and identified by PMF analysis (results are summarised in Table 3.2).

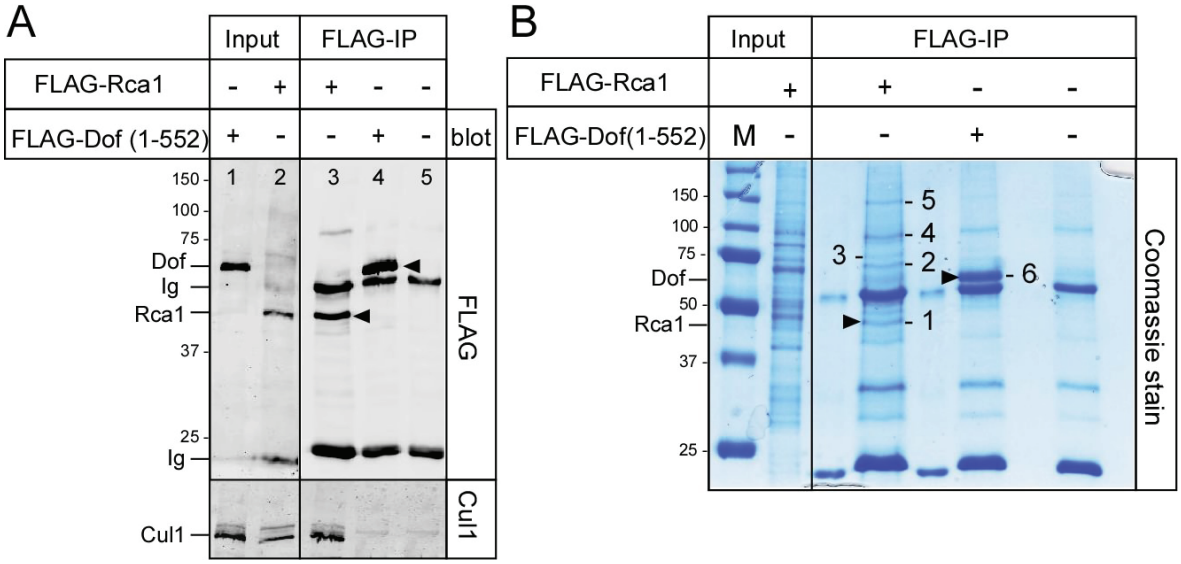


Figure 3.11 Peptide Mass Fingerprinting (PMF) analysis of FLAG-Rca1 coprecipitated proteins.
FLAG-Rca1 or FLAG-Dof (1-552) was precipitated from cell lysates with ANTI-FLAG affinity gel. Cell lysates (Inputs) and precipitates (IP) were separated by SDS-PAGE for Western Blotting or Coomassie staining.
(A) Precipitated FLAG-Rca1 or FLAG-Dof protein was detected by FLAG antibody (lane2 and 3; arrowheads). Endogenous Cul1 protein coprecipitated with FLAG-Rca1 (Cul1 blot; lane 3), but not with FLAG-Dof indicating that the interaction between Rca1 and Cul1 is specific. Moreover, immunglobulin heavy and light chains are visible (Ig).
(B) Colloidal Coomassie staining of FLAG-Rca1 or FLAG-Dof precipitates resulted in detection of several protein bands. Specific protein bands 1-6 were excised and analysed by PMF (arrowheads indicate FLAG-Rca1 or FLAG-Dof protein).

Table 3.2 PMF results of proteins precipitated with FLAG-Rca1.
Scores were defined as -10*Log (P); P is the probability that the observed match is a random event. Protein scores greater than 60 were set as significant ($p<0.05$).

Nr.	Identified Proteins	Protein Score
1	Regulator of Cyclin A1 (Rca1)	198
2	Heat shock protein cognate 4	290
3	Heat shock protein cognate 3 Heat shock protein cognate 72	80 80
4	Structure specific recognition protein CG4817-PA	84
5	Dre4 CG1828-PB, isoform A/B	184
6	Dof	210

PMF analysis identified the protein bands 1 and 6 as precipitated FLAG-Rca1 and FLAG-Dof protein (Figure 3.11B; Table 3.2; protein band 1 and 6). The remainder bands of coprecipitated proteins were identified as a mixture of several proteins. Proteins with a significant score of more than 60 are listed in Table 3.2. Different heat shock protein cognates, a structure specific recognition protein and the transcriptional regulator gene Dre4 were identified to coprecipitate with Rca1 (Figure 3.11B; Table 3.2; protein band 2, 3, 4 and 5). Immunoprecipitation of FLAG-Rca1, Coomassie staining and PMF analysis were repeated several times. In addition, HA-Rca1 was precipitated with HA antibody from S2 cell lysates followed by identification studies of coprecipitated proteins (data not shown). Protein bands at the same height were excised in all experimental set-ups and analysed by PMF. However, each PMF analysis resulted in the identification of different proteins. Significant protein identification was only observed for precipitated Rca1 or Dof protein. Effective coprecipitation of endogenous Cul1 was observed on Western blots (Figure 3.11A; Cul1 blot, lane 3) demonstrating once again a specific interaction between Rca1 and Cul1. However, Cul1 was not identified by PMF analysis although a protein band of the expected size (83 kDa) was excised from Coomassie stained gels and analysed by PMF. Rca1 pull-down experiments followed by Coomassie staining and PMF analysis were repeated several times using a variety of different immunoprecipitation assays. Neither Cul1 nor the SCF subunit SkpA, which was also shown to interact specifically with Rca1 (Figure 3.4A and B), was ever identified by Peptide Mass Fingerprinting analysis suggesting that this method is not suitable for the identification of potential SCF/Rca1 targets.

3.5 Rca1 function during endoreplication

Endoreplication is a specialised cell cycle variant in which cells undergo repeated rounds of DNA replication that are separated by distinct Gap phases (for review see Edgar and Orr-Weaver, 2001; Lilly and Duronio, 2005). Mitosis is bypassed in endoreplicating cells resulting in cells with increased DNA content and polyploidy/polyteny. (Calvi et al., 1998; Lilly and Spradling, 1996). During *Drosophila* development, various tissues like the gut, the fat body or the salivary glands undergo endoreplication (Smith and Orr-Weaver, 1991). In addition, endoreplication also occurs during adulthood in cells of the gut and the ovary

(Dej and Spradling, 1999; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Several proteins that are known to regulate the G1-S transition in mitotic cells are also proposed to be essential for endoreplication control (Edgar and Orr-Weaver, 2001; Lilly and Duronio, 2005; Sauer et al., 1995). By contrast, the regulatory network that governs progression through mitosis is inactivated in endoreplicating cells.

3.5.1 Gene expression analysis of endoreplicating cells

Since mitosis is bypassed in endoreplicating cells, mitotic genes like Cyclin A and B, Cdc25/String and Cdk1 get transcriptionally downregulated (Klebes et al., 2002). APC/C-Cdh1^{Fzr} activity is crucial for downregulation of cyclin/Cdk1 activity and entry into the endocycle (Edgar and Orr-Weaver, 2001; Lilly and Duronio, 2005; Sigrist and Lehner, 1997). It is assumed that APC/C-Cdh1^{Fzr} activity is not required for endocycle progression since its main targets, the mitotic cyclins, are not expressed and the APC/C-Cdh1^{Fzr} inhibitor Rca1 is not essential for endoreplication (Zielke, 2007). However, Rca1 overexpression in salivary glands results in an endocycle block and elevated Cyclin A and Cdk1 levels (Zielke, 2007). This indicates that Rca1 somehow promotes the expression of Cyclin A and Cdk1 in endoreplicating cells or that recent data about the transcriptional downregulation of mitotic genes is questionable. In order to refine the expression data for endoreplicating cells, a gene expression assay of *Drosophila* salivary glands was performed. Therefore, RNA of mitotically dividing cells from *Drosophila* embryos (4-8h after egg deposition) as well as RNA of endoreplicating cells from larval salivary glands was isolated. After reverse transcription, cDNA was analysed by quantitative real-time PCR. Several genes involved in different cell cycle processes were tested in this qRT-PCR study and subdivided into the following groups: genes regulating the G1-S transition, genes of the G2-M network and genes encoding for APC/C subunits. Transcript levels of these genes were determined relatively to expression of the endogenous control GAPDH. The relative gene expression levels in the embryo were set as reference/calibrator to monitor expression changes of genes in endoreplicating cells of larval salivary glands.

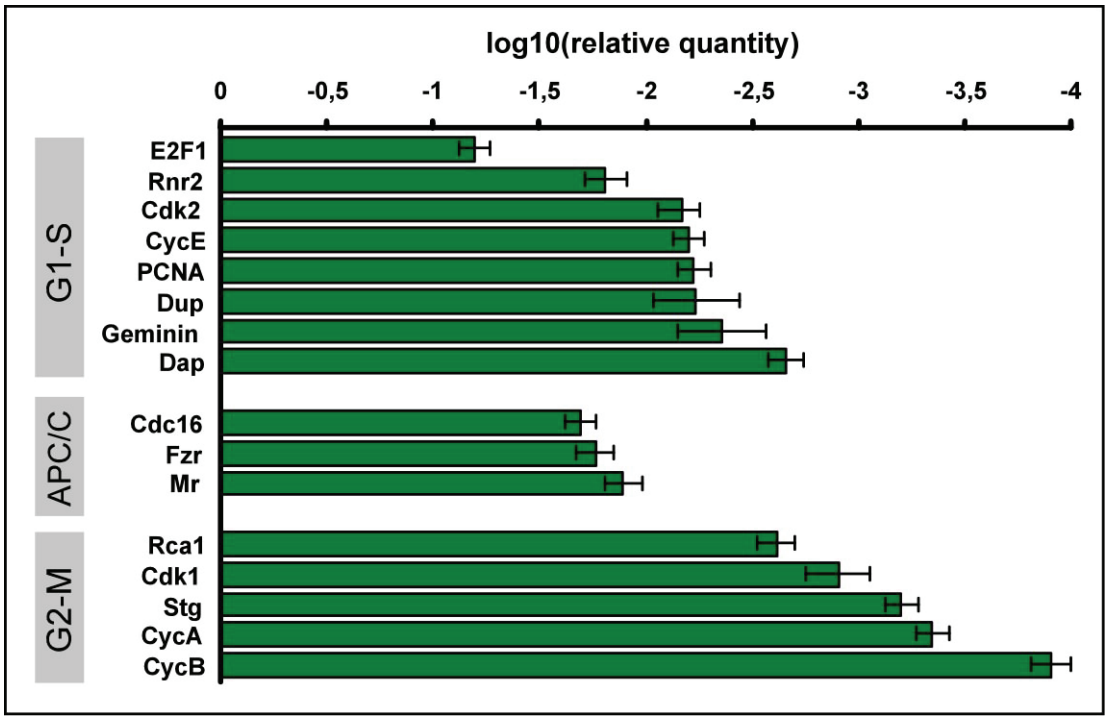


Figure 3.12 Gene expression analysis of *Drosophila* salivary glands by qRT-PCR.
Genes regulating the transition from G1-S, G2-M and genes encoding for APC/C subunits were analysed for endoreplicating (third instar feeding larvae salivary glands) and mitotically dividing cells (embryos 4-8h after egg deposition) by quantitative real-time PCR. The relative expression quantity of the indicated genes was determined by the ΔΔCT method. GAPDH was used as endogenous control to normalise gene expression. The relative expression levels in embryos were set as reference to monitor relative fold differences in gene expression of salivary glands as the log10 (relative quantity). Transcript levels of all all tested genes were reduced in endoreplicating cells. Mitotic gene expression (CycA/B or Cdc25/Stg) was dramatically decreased compared to G1-S genes (e.g. CycE, Cdk2) that are known to be essential for endoreplication. Interestingly, the APC/C components Cdc16 and Morula as well as the activator protein Fzr are expressed at levels comparabel to those of G1-S genes.

Relative fold differences in gene expression of endoreplicating and mitotically dividing cells were determined by the ΔΔCT method. The resulting relative expression quantity (RQ) for each gene in endoreplicating cells was shown as log10 (RQ) in a gene expression diagram (Figure 3.12). In endoreplicating cells of *Drosophila* salivary glands, transcript levels of all genes analysed in this assay were generally reduced. However, the degree of reduction varied significantly. In salivary glands, genes involved in regulation of the G1-S transition and DNA replication (such as E2F1, CycE, Cdk2, Dup/Cdt1, PCNA, Rnr2 and Geminin) are expressed about 10-100 times lower than in embryonic cells (Figure 3.12). However, Dacapo (Dap) expression is significantly lower than the remaining G1-S genes. Most genes of the G2-M network (Rca1, Cdk1, String/Cdc25, Cyclin A and Cyclin B) display a 400-8000 fold lower expression in salivary glands. Consistent with previous data (Klebes et al., 2002), mitotic genes like Cyclin A, Cyclin B and Cdk1 are dramatically

downregulated in endoreplicating cells. Interestingly, the APC/C subunits *morula*/APC2 (*mr*) and *dmCdc16*/APC6 (Chen et al., 2007; Kashevsky et al., 2002; Reed and Orr-Weaver, 1997;) as well as the APC/C activator Fzr are expressed at the same level as the G1-S regulators which are known to be involved in endoreplication (Figure 3.12). APC/C-Cdh1^{Fzr} activity is assumed to be dispensable for the endocycle, since its main targets, the mitotic cyclins, are transcriptionally downregulated. The APC/C-Cdh1^{Fzr} substrate Geminin, however, is expressed at levels comparable to the G1-S genes. Since Cdc16, Morula and Fzr are expressed at the same level as genes involved in the G1-S transition, the APC/C-Cdh1^{Fzr} complex might have a function during endoreplication. This idea is supported by the finding that Fzr protein is present in larval salivary glands (C. Lehner, personal communication).

3.5.2 HA-Rca1 overexpression increases Cyclin E, Rnr2 and PCNA transcript levels in endoreplicating cells

Overexpression of Rca1 in *Drosophila* salivary glands perturbed endocycle progression in an F-box dependent manner (Zielke, 2007). Furthermore, these cells show elevated protein levels of Cyclin E and mitotic Cyclin A and Cdk1 (Zielke, 2007). Continous expression of Cyclin E also results in an endocycle block accompanied by increased Cyclin A and Cdk1 protein levels (Follette et al., 1998; Weiss et al., 1998; Zielke, 2007). Increased Cyclin E/Cdk2 activity interferes with the DNA licensing machinery and has been shown to downregulate APC/C-Cdh1^{Fzr} activity during postblastoderm cell cycles (Grosskortenhaus and Sprenger, 2002; Reber et al., 2006; Zielke, 2007). Since Cyclin E levels are elevated upon Rca1 overexpression, the endocycle breakdown could be due to increased Cyclin E/Cdk2 activity. Thus, Cyclin A accumulates as a result of APC/C-Cdh1^{Fzr} inhibition by either Rca1 and/or increased Cyclin E/Cdk2 activity. However, Cyclin E and Cdk1 are not targets of the APC/C-Cdh1^{Fzr} suggesting that Rca1 influences the expression of these genes independently from restraining APC/C-Cdh1^{Fzr} activity. A possible explanation could be that Rca1 overexpression stimulates transcription of Cyclin A/E and Cdk1. To test this, a gene expression analysis of HA-Rca1 as well as Cyclin E overexpressing salivary glands was performed. Quantitative real-time PCR was carried out on RNA isolated from both tissues. In this assay the same set of genes was tested as in section 3.7.1. Expression

changes upon HA-Rca1 or Cyclin E overexpression were determined relatively to transcript levels of wild-type salivary glands. ß-Actin was used as the endogenous control to normalise gene expression.

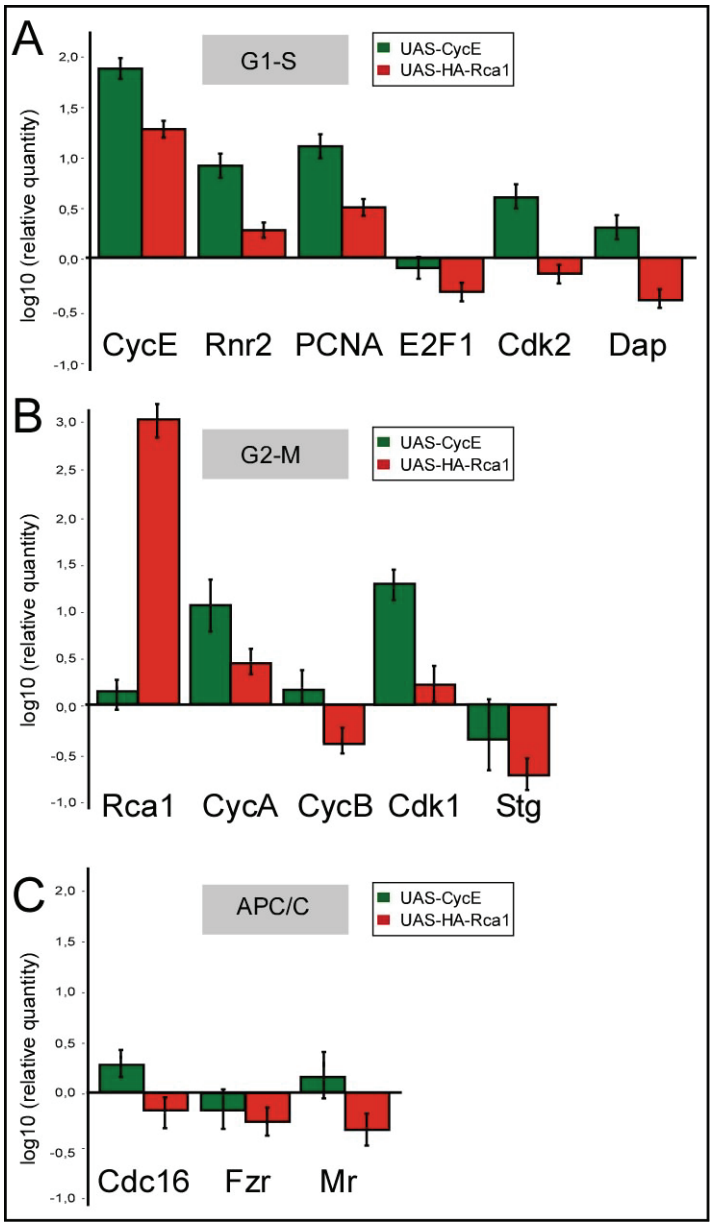


Figure 3.13 Gene expression analysis of HA-Rca1 and CycE overexpressing larval salivary glands.
Genes involved in the transition from G1-S, G2-M or APC/C components were analysed for expression changes upon HA-Rca1 or Cyclin E overexpression in endoreplicating salivary glands. Gene expression in wild-type salivary glands was used as reference. ß-Actin was used as the endogenous control to normalise gene expression. The relative expression quantity (RQ) of the indicated genes was determined by the ΔΔCT method. RQ values of each gene in HA-Rca1 or CycE overexpressing salivary glands were displayed as log10 (RQ) in gene expression diagrams.
(A) CycE transcript levels are elevated upon HA-Rca1 expression. The E2F1 targets Rnr2 and PCNA were upregulated upon Rca1 and Cyclin E overexpression, but E2F1 transcript levels were not increased. Cdk2 and Dap are slightly increased when Cyclin E is overexpressed, but not upon HA-Rca1 expression.
(B) CycA and Cdk1 transcripts were significantly elevated upon Cyclin E expression, but less affected when Rca1 was overexpressed. Cyclin B levels were not significantly increased by ectopic Cyclin E expression and rather reduced after Rca1 expression. Stg levels were reduced in both samples.
(C) Transcripts of the APC/C subunits Cdc16 and Morula as well as APC/C activator Fzr were not dramatically altered upon Rca1 and Cyclin E overexpression.

The ΔΔCT method was used to determine relative fold differences in gene expression. The relative quantity (RQ) of each gene in HA-Rca1 or Cyclin E overexpressing salivary glands was displayed as log10 (RQ) in gene expression diagrams (Figure 3.13A-C). Cyclin E transcript levels were elevated when Rca1 was overexpressed in larval salivary glands confirming the marked increase in Cyclin E protein levels (Zielke, 2007). The E2F1 target

genes Rnr2 and PCNA (Duronio et al., 1995; Thacker et al., 2003) were upregulated upon Rca1 and Cyclin E expression. However, E2F1 transcript levels itself were not affected upon Cyclin E overexpression, but rather reduced after HA-Rca1 expression. The increase in E2F1 target gene expression is therefore not caused by the induction of E2F1 transcription suggesting that E2F1 activity is promoted by other mechanisms. Cdk2 and Dacapo transcripts were slightly increased upon Cyclin E expression, but reduced for HA-Rca1 expression. Cyclin E overexpression in salivary glands increases Cyclin A and Cdk1 levels, but Cyclin B levels do not change significantly. Moreover, String expression was decreased. Cyclin A and Cdk1 expression was only slightly increased after Rca1 overexpression suggesting that the increase in Cyclin A and Cdk1 protein levels is not solely due to APC/C-Cdh1^{Fzr} inhibition (Zielke, 2007). However, Cyclin B levels are decreased upon Rca1 overexpression as well as String transcript levels. Expression levels of the APC/C components Cdc16 and Morula and the APC/C activator protein Fzr do not change upon Cyclin E or HA-Rca1 overexpression in salivary glands. Cdc16 and Morula transcript levels are slightly elevated upon Cyclin expression, but reduced in HA-Rca1 overexpressing salivary glands. Fzr expression is reduced for both Cyclin E and Rca1 overexpressing cells. However, Rca1 or Cyclin E overexpression does not significantly affect the expression of APC/C subunits.

4. Discussion

4.1 Localisation studies of Rca1

Rca1 belongs to the family of Emi1 proteins that are inhibitors of the APC/C-Cdh1^{Fzr} complex (Grosskortenhaus and Sprenger, 2002; Schmidt et al., 2006). During *Drosophila* embryogenesis, Rca1 restrains APC/C-Cdh1^{Fzr} activity in G2 resulting in cyclin accumulation and entry into mitosis 16 (Grosskortenhaus and Sprenger, 2002). At later developmental stages, Rca1 has been shown to accelerate the G1-S transition in imaginal disc cells, presumably as part of a not yet characterised SCF complex (Zielke et al., 2006). During interphase, Rca1 protein accumulates in the nucleus of embryonic cells (Grosskortenhaus and Sprenger, 2002) suggesting that APC/C inhibition by Rca1 occurs mainly in the nucleus. However, its main targets, the mitotic cyclins as well as the activator protein Cdh1^{Fzr} were detected in the cytoplasm during interphase (Dienemann and Sprenger, 2004; Grosskortenhaus and Sprenger, 2002). The spatial separation between Rca1, Cdh1^{Fzr} and APC/C substrates raises the question how nuclear Rca1 protein can negatively regulate APC/C-Cdh1^{Fzr} activity in the cell. If the inhibitory effect of Rca on the APC/C-Cdh1^{Fzr} is restrained to the nucleus, it must be assumed that mitotic cyclins, Fzr and APC/C subunits shuttle between nucleus and cytoplasm and that the APC/C is only active in the nucleus. Drosophila Cyclin A and human Cyclin B1 are cytoplasmic in interphase and accumulate in the nucleus at prophase implying a dynamic shuttling of mitotic cyclins between the cytoplasm and the nucleus (Dienemann and Sprenger, 2004). In contrast, constitutive nuclear accumulation of Rca1 must rely on strong nuclear protein import process. To identify the protein domains in Rca1 that are responsible for its nuclear localisation, the subcellular distribution of different Rca1 constructs with deletions or mutations in certain domains of the protein were analysed in *Drosophila* S2 cells (Table 3.1). Some of these protein motifs are known to be essential for Rca1 function during G2 of embryogenesis and/or S phase induction in imaginal disc cells (Table 3.1; Radermacher, 2007; Zielke et al., 2006; Zielke, 2007). Hence, protein domains which were identified to mediate nuclear localisation of Rca1 should be analysed for their *in vivo* activity. In *Drosophila* S2 cells, Rca1 accumulated exclusively in the nucleus as in embryonic cells (Figure 3.2A). Rca1 harbours a bipartite nuclear localisation signal (NLS) suggesting that

nuclear transport of Rca1 relies mainly on this motif. However, Rca1 lacking the NLS still accumulates in the nucleus indicating that Rca localisation does not solely rely on the NLS (Figure 3.2B). Efficient nuclear targeting rather depends on N-terminal sequences, including the NLS. Cytoplasmic localisation increased when N-terminal residues were incrementally removed. Trace amounts of FLAG-Rca1Δ133 were already detected in the cytoplasm and further N-terminal truncations (FLAG-Rca1Δ203 or FLAG-Rca1Δ255) lead to an even distribution of Rca1 throughout the nucleus and the cytoplasm (Figure 3.2C-E). The dramatic increase in cytoplasmic localisation between Δ133 and Δ203 suggests that this region contributes significantly to nuclear localisation. The F-box is located in the implied region suggesting that this motif mediates nuclear localisation. Rca1 physically interacts with the SCF subunits SkpA and Cul1 via the F-box (Figure 3.4 and 3.8) and was proposed to accelerate the G1-S transition as part of an SCF/Rca1 complex (Zielke et al., 2006). SkpA protein concentrates in the nucleus of some diploid cells, presumably by interaction with nuclear transport proteins (Murphy, 2003). Therefore, it could be possible that Rca1 gets located to nucleus indirectly via SkpA binding. However, deletion of the F-box motif did not reveal any changes in nuclear Rca1 protein (Figure 3F). Since Rca1 lacking the F-box fails to interact with SkpA or Cul1 (Figure 3.4 and 3.8), nuclear accumulation cannot solely rely on SkpA dependent localisation. Simultaneous deletion of the NLS and the F-box (FLAG-Rca1ΔNLSΔF-box) or FLAG-Rca1Δ133ΔF-box still resulted in nuclear accumulation of Rca1, albeit slight amounts of the protein were detected in the cytoplasm (Figure 3G and H). However, cytoplasmic localisation increased in the FLAG-Rca1ΔNLSΔF-box construct compared to the single domain deletions (FLAG-Rca1ΔF-box and FLAG-Rca1ΔNLS), indicating that both elements contribute to efficient nuclear targeting. Altogether, these localisation studies revealed that the NLS and the F-box motif contribute to nuclear import of Rca1. However, the N-terminal region in front of the NLS and the F-box seem to play an important role for nuclear localisation of Rca1. Thus, nuclear import of Rca1 relies on more than one protein motif favouring a model in which a broader N-terminal region, at least up to residue 203 mediates interaction with nucleocytoplasmic shuttling proteins. However, it cannot be excluded that any other protein domains in Rca1 take part in nuclear targeting as well. This could be tested by deletion of each remaining protein motif in a Rca1Δ133ΔF-box or Rca1Δ203 background and subsequent localisation analysis in S2 cells. Since none of the tested deletion proteins resulted in complete cytoplasmic localiation of Rca1, it is more difficult to investigate to

what extent nuclear accumulation contributes to Rca1 function. N-terminal deletions up to residue 203 resulted in a Rca1 protein which is evenly dispersed throughout the cytoplasm and the nucleus (Figure 3.2C and D). FLAG-Rca1Δ133 and FLAG-Rca1Δ203 are both able to inhibit the APC/C-Cdh1^{Fzr} complex during G2 of *Drososphila* embryogenesis (Figure 4.1). However, a further truncation up to amino acid 255 abolished the inhibitory effect of Rca1 on the APC/C-Cdh1^{Fzr} (Table 3.1). Since FLAG-Rca1Δ203 and FLAG-Rca1Δ255 show a similar protein distribution throughout the cell (Figure 3.2D and E), cytoplasmic dislocation of FLAG-Rca1Δ255 cannot be the reason for the functional loss. It is more likely that FLAG-Rca1Δ255 protein structure is severely affected by this large N-terminal deletion which eventually results in a nonfunctional protein. Mutation of the ZBR (C351S) or Rca1 lacking the region 203-255 both lead to proteins which are also not capable to inhibit the APC/C-Cdh1^{Fzr} during G2 (Radermacher, 2007; Zielke et al., 2006). However, functional loss of these proteins is only due to the affected protein domains since both proteins accumulate in the nucleus similar to wild-type Rca1 (Figure 3.2J and K).

S phase induction by Rca1 depends on the F-box motif (Zielke et al., 2006). N-terminal deletions including the F-box motif (ΔF-box, Δ203, Δ255) or mutation of the ZBR (C351S) were not able to promote the G1-S transition resulting in flies with normal eye stucture (Zielke et al., 2006). Since none of these deletions resulted in complete cytoplasmic localisation of the protein in S2 cells, it remains ambiguous how far nuclear accumulation of Rca1 is required for its function at the G1-S transition or during G2. However, a more recent *in vivo* study of different Rca1 deletion proteins harbouring a nuclear export signal (NES) could give evidence that nuclear localisation is essential for Rca1 function as an inhibitor of APC/C-Cdh1^{Fzr} activity during G2 of embryogensis (Radermacher, 2007). These data suggest that the APC/C-Cdh1^{Fzr} complex is active in the nucleus of embryonic cells and this idea is further strengthened by the observation that certain APC/C substrates, e.g. Geminin, are solely nuclear (N. Zielke, personal communication). The requirements of nuclear localisation for S phase induction by Rca1 has not yet been defined (Radermacher, 2007). S phase induction by Rca1 occurs in an F-box dependent manner suggesting that the SCF/Rca1 complex could mediate degradation of a negative G1-S regulator (Zielke et al., 2006). So far, little is known about the localisation of SCF complexes and SCF activity within the cell. The SCF subunit SkpA is distributed throughout the cytoplasm and nucleus in embryonic cells, but is predominantly nuclear in some diploid cells and cells of the fat body and salivary glands (Murphy, 2003).

These data indicate that at least the SCF subunit SkpA shuttles between the nucleus and cytoplasm and therefore could form putative SCF complexes in both parts of the cell. Rca1 and SkpA interact physically in an F-box dependent manner and associate with Cul1 to form the SCF/Rca1 complex (Figure 3.4 and Figure 3.6). Since Rca1 is a nuclear protein (Figure 3.2A), complex formation of Rca1 and SkpA and SCF/Rca1 activity could be restricted to the nucleus. However, it cannot be excluded that after complex formation in the nucleus, the SCF/Rca1 is exported from the nucleus to perform its activity in the cytoplasm. Moreover, SCF complex formation or SCF activity in the nucleus or cytoplasm is cell type specific and requires a dynamic flux of all SCF subunits within the cell.

4.2 Rca1 interacts with the APC/C subunit Cdc23

During G2, APC/C-Cdh1^{Fzr} activity is restrained by Rca1/Emi1 inhibitors which results in accumulation of mitotic cyclins and entry into mitosis (Di Fiore et al., 2007; Grosskortenhaus and Sprenger, 2002; Schmidt et al., 2006). Rca1 has been shown to coprecipitate with APC/C components such as the activator protein Cdh1^{Fzr} or the APC/C subunit Cdc27 (Grosskortenhaus and Sprenger, 2002). In addition, a yeast two-hybrid screen identified the APC/C subunit Cdc23 (APC8) as a binding partner of Rca1 (Table 3.1) and this interaction was confirmed in coimmunoprecipitation experiments using extracts of *Drosophila* S2 cells (Figure 3.3). Interstingly, Cdh1^{Fzr} and Cdc27 were not found in the yeast two-hybrid screen as interacting partners of Rca1. This data indicates that Rca1 might interact with the APC/C-Cdh1^{Fzr} complex via Cdc23 suggesting that Cdc27, Cdh1^{Fzr} or other APC/C subunits associate with Rca1 indirectly. However, it must be considered that expression of *Drosophila* Cdh1^{Fzr} in yeast might result in a nonfunctional protein that cannot bind to Rca1 in the yeast two-hybrid system. To test whether *Drosophila* Cdh1^{Fzr} is functional in yeast, *Drosophila* Cdh1^{Fzr} should be expressed in a Cdh1^{Fzr} mutant yeast strain revealing if *Drosophila* Cdh1^{Fzr} can replace endogenous Cdh1^{Fzr} in yeast.

Rca1 associates with the APC/C subunits presumaby via Cdc23 binding suggesting that this interaction might accomplish its inhibiory function. A conceivable mechansisms could be that Rca1 binding blocks the substrate binding site within the APC/C-Cdh1^{Fzr} complex. A recent study on vertebrate Emi1 showed that several APC/C subunits coprecipitated with

Emi1 and this interaction was in part mediated by a minimal destruction box (RxxL) within Emi1 (Miller et al., 2006). The destruction box (D-box) is a conserved degron that can be found in a variety of the APC/C substrates (Glotzer et al., 1991; King et al., 1996). Emi1 binds to the APC/C core in a D-box dependent manner, but which APC/C subunit could act as a potential D-box receptor remains to be elucidated. Although Emi1 harbours a D-box, it is not a substrate of the APC/C complex (Reimann et al., 2001a). Mutation of the ZBR, however, converts Emi1 from an APC/C inhibitor to a D-box dependent APC/C substrate. Hence, Emi1 was proposed to act as an pseudosubstrate inhibitor whose D-box provides the majority of APC/C binding, while the ZBR retains the ability to block substrate binding (Miller et al., 2006).

The D-box motif is conserved among all Emi proteins except Rca1 (Zielke, 2007). Nevertheless, Rca1 harbours a minimal D-box, which is located in a different region within the protein (Zielke, 2007) suggesting that this motif could mediate Rca1 binding to APC/C-Cdh1^{Fzr} complex. To test this idea, further coimmunoprecipitation experiments should be performed in which the D-box of Rca1 is deleted. These experiments should reveal if Rca1 lacking the D-box is still capable to coprecipitate with Cdc23 and/or Cdc27. Besides the D-box, Rca1 contains a conserved KEN-box which is another substrate recognition motif present in a variety of APC/C targets (Pfleger and Kirschner, 2000). While the APC/C-Cdc20^{Fzy} complex prefers D-box containing substrates, the APC/C-Cdh1^{Fzr} recognises substrates with a D-box and KEN-box (Burton and Solomon, 2001; Hilioti et al., 2001; Pfleger et al., 2001). Cdh1^{Fzr} has been proposed to act as a substrate recognition subunit that binds to the D-box or KEN-box within APC/C targets (Vodermaier, 2001; Kraft et al., 2005). Thus, Rca1 could interact with Cdh1^{Fzr} and/or APC/C subunits (e.g. Cdc23/Cdc27) via the KEN-box. This should also be tested by interaction studies using Rca1 constructs that lack the KEN-box or both, D-box and KEN-box. Moreover, it remains to be elucidated if these interactions occur in a direct manner or are rather due to indirect association of several subunits. Emi1 was proposed to interact first with Cdh1^{Fzr} to be loaded onto the APC/C followed by independent binding to the APC/C receptor via its D-box (Miller et al., 2006). A similar model could also apply to Rca1: First, Rca1 could interact with the activator Cdh1^{Fzr} and then it binds independently to the APC/C core via the KEN-box and/or D-box. However, it cannot be excluded that additional, so far unknown factors contribute to Rca1-APC/C-Cdh1^{Fzr} complex formation or serve as a physical bridge between Rca1 and APC/C core subunits.

4.3 Rca1 is part of an SCF complex

In a yeast two-hybrid screen *Drosophila* SkpA was identified as a binding partner of Rca1 (Table 3.1). Previous yeast two-hybrid data already reported an interaction between Rca1/Emi1 and members of the Skp protein family (Giot et al., 2003; Reimann et al., 2001). In *Drosophila*, six Skp proteins (SkpA-F) have been identified (Nayak et al., 2002; Yamanaka et al., 2002), but so far SkpA is the only member that has been characterised in more detail (Murphy, 2003). SkpA is involved in several cellular processes like centrosome duplication, endoreplication and chromatin condensation (Murphy, 2003). There is evidence that SkpA primarily acts as part of multiple SCF complexes: First, SkpA is highly similar to human and yeast Skp1 which forms different SCF complexes in *vitro* and in *vivo* (Deshaies, 1999). Secondly, SkpA interacts with *Drosophila* Cul1 and the F-box proteins Slimb, Partner of paired and Archipleago (Moberg et al., 2001; Bocca et al., 2001; Raj et al., 2000). Thus, SkpA and Cul1 interact with different F-box proteins resulting in several SCF complexes in *Drosophila* which mediate degradation of many cell cycle substrates (Nakayama and Nakayama, 2006; Kipreos and Pagano, 2000; Maniatis, 1999). In an SCF complex, SkpA acts as an adaptor protein which binds the F-box motif within F-box proteins and associates with the scaffold protein Cul1 (Schulman et al., 2000; Zheng et al., 2002). In addition to SkpA, Cul1 interacts with Roc/RING finger proteins, which are responsible for recruitment of the ubiquitin-conjugating enzyme (E2). Substrate recognition and binding to the SCF complex relies on the different F-box proteins (Skowyra et al., 1997). Rca1 and Emi1 belong to the family of F-box proteins and recently, an F-box dependent function of Rca1 at the G1-S transition has been suggested (Zielke et al., 2006). Overexpression of Rca1 can induce S phase entry in imaginal disc cells and this effect relies on the F-box motif (Zielke et al., 2006). The F-box of Rca1 has been shown to be dispensable for its inhibitory effect on the APC/C-Cdh1^{Fzr} complex, at least during embryogenesis (Zielke et al., 2006). This implicates that Rca1 has a secondary, F-box dependent function at the G1-S transition, presumably as an F-box protein in an SCF complex. The idea that Rca1 is incorporated in an SCF complex was experimentally verified by coimmunoprecipitation studies with *Drosophila* S2 cell lysates. First, the interaction between Rca1 and SkpA has been evaluated successfully. Moreover, it has been shown that this interaction specifically depends on the F-box motif, since Rca1 harbouring a point mutation in the ZBR or an internal deletion between amino acids 203-255 did not

perturb SkpA binding (Figure 3.4 and 3.5). Thus, Rca1 and SkpA interact physically in an F-box dependent manner. Furthermore, the third SCF core subunit Cul1 was shown to bind Rca1 and SkpA. Endogenous Cul1 protein efficiently coprecipitated with Rca1 or SkpA in S2 cell lysates (Figure 3.6). In addition, simultaneous expression of Rca1 and the SCF components SkpA and Cul1 in S2 cells revealed physical interaction between all three proteins (Figure 3.7). Finally, interaction studies demonstrated that coprecipitation of Cul1 depends on the F-box motif within Rca1 (Figure 3.8). Altogether, these experiments support the notion that Rca1 has a secondary function as part of an SCF complex. Complex formation between Rca1 and SCF components was additionally approached using Peptide Mass Fingerprinting (PMF) analysis. By this method, protein bands that specifically coprecipitated with FLAG-Rca1 from S2 cell lysates were characterised by PMF analysis. Efficient precipitation of FLAG-Rca1 from S2 lysates was confirmed by PMF identification (Figure 3.11 and Table 3.2). Moreover, PMF analysis of coprecipitated proteins resulted in the identification of several proteins including different heat shock protein cognates (Table 3.2). However, components of the SCF or APC/C complex that specifically coprecipitate with Rca1 in Western blot analysis failed to be identified by PMF. Importantly, neither SkpA nor Cul1 could be identified by PMF, although both proteins were present in the precipitated fraction judged from Western blot analysis (Figure 3.4-3.8). Apparently, the PMF method has to be improved to detect these proteins and novel interaction partners of Rca1. The identification of heat shock proteins was not followed up since the induction process used in these studies (addition of $CuSO_4$) or the overexpression conditions will induce a stress response resulting in upregulation of heat shock factors. Moreover, it remains to be clarified if the putative SCF/Rca1 complex shows *in vivo* or *in vitro* activity.

Altogether, Rca1 forms a complex with the SCF core subunits SkpA and Cul1, but association with the Roc subunit still has to be tested. In *Drosophila*, three different members of the Roc/Rbx family were identified and named Roc1a, Roc1b and Roc2 (Noureddine et al., 2002). Genetic analysis revealed that these Roc proteins play nonredundant roles during *Drosophila* development (Donaldson et al., 2004). Cul1 interacts most strongly with Roc1a suggesting that the majority of SCF complexes contain Cul1 and Roc1a as core subunits (Donaldson et al., 2004). Roc1b and Roc2 have been shown to associate with Cul3 or Cul5 indicating that these proteins belong to another class of Cullin-RING ubiquitin ligases than the SCF complex (Donaldson et al., 2004). Loss of

Roc1a function results in a cell proliferation block and lethality suggesting that Roc1a containing SCF complexes play a crucial role for cell cycle progression by targeting many substrates for degradation (Noureddine et al., 2002). Roc1a and the F-box protein Slimb have been ascribed to function together in an SCF complex targeting Cubitus interruptus (Ci) for degradation (Jiang and Struhl, 1998, Maniatis, 1999). However, Roc1a is not required for SCF/Slimb mediated degradation of Armadillo (Arm). Therefore, it can be suggested that SCF complexes vary in their subunit composition including different Roc proteins (Donaldson et al., 2004). Moreover, different Roc proteins could be responsible for recruitment of a specific set of E2 enzymes, each acting on different targets and thereby providing an additional level of substrate specificity (Donaldson et al., 2004). Thus, it would be interesting to reveal which particular Roc protein is incorporated into the SkpA-Cul1-Rca1 complex and which E2 enzyme is utilized by the SCF/Rca1 complex.

4.4 Substrates of the putative SCF/Rca1

The putative SCF/Rca1 complex can induce S phases presumably by mediating degradation of so far unknown target proteins (Zielke et al., 2006). In this SCF complex, Rca1 could mediate degradation of so far unknown target proteins. A conceivable model would be that SCF/Rca1 activity leads to proteasomal destruction of a negative regulator of the G1-S transition. Thus, degradation of this inhibitor could cause premature S phase entry upon Rca1 over-expression. In *Drosophila*, S phase entry relies on Cyclin E/Cdk2 activity (Knoblich et al., 1994; Richardson et al., 1995). The CKI Dacapo specifically inhibits Cyclin E/Cdk2 activity during G1 (de Nooij et al., 1996; Lane et al., 1996). To examine whether S phase induction by Rca1 relies on Cyclin E/Cdk2 activity, Rca1 and Dacapo were coexpressed (Zielke et al., 2006). However, Dacapo could not prevent induction of ectopic S phases by Rca1 (Zielke et al., 2006) suggesting that Rca1 either acts independently of Cyclin E/Cdk2 activity or targets the inhibitor Dacapo for SCF/Rca1 mediated degradation. Overexpression of Rca1 did not influence Dacapo protein levels indicating that Dacapo is not a substrate of the SCF/Rca1 complex (Zielke et al., 2006). Moreover, Cyclin E transcript levels were not enhanced in eye disc clones overexpressing Rca1 (N. Zielke, personal communication). Hence, S phase induction by Rca1 does not rely on Cyclin E/Cdk2 activity indicating that negative regulators of Cyclin E such as

Dacapo, the E2F1 repressor Rbf (Du et al., 1996; Du and Dyson, 1999) or SCF/Archipelago (Moberg et al., 2001) are presumably not targets of the SCF/Rca1 complex.

Besides Cyclin E/Cdk2, overexpression of Cyclin A or loss of the Cdk1 inhibitor Rux results in ectopic S phases demonstrating that Cyclin A/Cdk1 activity can also trigger the G1-S transition (Foley et al., 1999; Sprenger et al., 1997; Thomas et al., 1997). Since S phase entry upon Rca1 overexpression occurs independently of Cyclin E, it could rather rely on Cyclin A/Cdk1 activity. The APC/C-Cdh1^{Fzr} complex is essential for establishment of the G1 phase by targeting Cyclin A for proteasomal degradation (Pimentel and Venkatesh, 2005; Sigrist and Lehner, 1997). Hence, ectopic S phases upon Rca1 overexpression could be due to APC/C-Cdh1^{Fzr} inhibition and subsequent Cyclin A accumulation. Since the F-box is essential for S phase induction in eye and wing imaginal disc cells (Zielke et al., 2006), the SCF/Rca1 complex could mediate degradation of Fzr in G1 cells. However, coexpression of Rca1 and Fzr in S2 cells did not result in any changes of Fzr protein levels (Figure 3.9 and Figure 3.10) indicating that Fzr is not a target of the SCF/Rca1 complex. Moreover, it could be possible that the SCF/Rca1 complex targets an APC/C subunit for degradation resulting in APC/C inactivation and subsequent Cyclin A accumulation which in turn drives S phases. Since Rca1 lacking the F-box cannot accelerate the G1-S transition, it must be taken into account that this deletion simply affects the activity of Rca1. However, Rca1 lacking the F-box can still inhibit APC/C-Cdh1^{Fzr} activity, at least during G2 of *Drosophila* embryogenesis (Zielke et al., 2006). Besides APC/C-Cdh1^{Fzr} mediated degradation of Cyclin A, Cyclin A/Cdk1 activity is dampened by the CKI Rux (Sprenger et al., 1997; Thomas et al., 1997) and inhibitory phosphorylation of Cdk1 by Wee kinase (Campbell et al., 1995; Sprenger et al., 1997). Hence, both proteins are negative regulators of the G1-S transition and therefore could be putative targets of the SCF/Rca1 complex. However, coexpression of Rux or Wee with Rca1 in S2 cells did not reveal changes in Rux or Wee protein levels (Figure 3.9 C-F and Figure 3.10) indicating that these G1-S regulators are not targeted for degradation by the SCF/Rca1 complex.

In addition, potential substrates of the SCF/Rca1 complex should be identified by immunoprecipitation of FLAG-Rca1 from S2 lysates followed by Coomassie staining and Peptide Mass Fingerprinting (PMF) analysis of coprecipitated proteins. However, PMF analysis failed to discover a potential SCF/Rca1 target protein. Most proteins identified by

this method did not show a significant interaction with Rca1. Presumably, substrate binding to the SCF/Rca1 complex occurs transiently suggesting that these interactions are rather weak which makes substrate identification more challenging. A dominant negative effect might also explain S phase induction by Rca1 overexpression. In this scenario excess Rca1 protein might titrate out the Skp subunit from other SCF complexes such as SCF/Archipelago (Moberg et al., 2001). However, a structure/function analysis of Rca1 revealed that mutation of the ZBR (C351S) was unable to induce ectopic S phases, but was still capable to bind SkpA via its F-box (Figure 3.4).

Since biochemical methods were apparently not sensitive enough, a genetic approach might be more suitable for the identification of SCF/Rca1 targets. Overexpression of Rca1 during eye development leads to a rough eye phenotype. Defects in eye morphology are easily evaluable and therefore an excellent system to screen for modifiers of the rough eye phenotype induced by Rca1 (for review see St Johnston, 2002). Since the rough eye phenotype relies on the F-box, this strategy might lead to the identification of SCF/Rca1 substrates or other proteins that impinge on Rca1 activity.

4.5 Gene expression in endoreplicating cells

During late *Drosophila* embryogenesis, cells of virtually all internal tissues such as the salivary glands and the gut initiate endoreplication (Edgar and O'Farrell, 1989). Cells undergo multiple rounds of DNA replication without intervening mitosis resulting in increased DNA content and polyploidy/polyteny (Smith and Orr-Weaver, 1991). The regulatory network which controls G-S cycles and DNA licensing in endoreplicating cells has been studied extensively over the last years. A gene expression analysis of wild-type salivary glands revealed that transcript levels of all genes tested in this assay were generally reduced compared to corresponding gene expression levels in the embryo (Figure 3.12). However, the degree of reduction varied significantly. It has been demonstrated that proteins controlling the G1-S transition in mitotic cells are also involved in endoreplication control (for review see Edgar and Orr-Weaver, 2001; Lilly and Duronio, 2005). Consistently with these data, G1-S regulatory proteins including E2F1, Cyclin E, Cdk2, Dup/Cdt1, Rnr2, PCNA and Geminin were expressed in endoreplicating salivary glands (Figure 3.12A). However, transcript levels of all G1-S genes in salivary glands were 10-

100 times reduced when compared to gene expression levels in the embryo. Cyclin E and its kinase partner Cdk2 are known to be crucial for endoreplication and both genes showed comparable expression levels (Figure 3.12A). Cyclin E/Cdk2 activity is required for initiation of DNA replication (Knoblich et al., 1994; Lane et al., 2000). Continuous expression of Cyclin E interferes with the DNA licensing machinery and results in an endocycle block (Follette et al., 1998; Weiss et al., 1998). Therefore, it has been proposed that Cyclin E/Cdk2 activity is low during the Gap phase to allow relicensing of origins and rises prior to S phase entry. Since Cdk2 protein levels remain stable throughout the cell cycle (Stern et al., 1993), Cyclin E/Cdk2 activity changes rely on variations in Cyclin E levels. Several studies revealed that Cyclin E oscillates in endoreplicating tissues (Lilly and Spradling, 1996; de Nooij et al., 2000), presumably due to several regulators including the transcription factor E2F1 or the CKI Dacapo (de Nooij et al., 2000; Duronio and O'Farrell, 1995; Lilly and Spradling, 1996). E2F1 is the most abundant mRNA among all studied transcripts in endoreplicating salivary gland cells (Figure 3.12A). E2F1/Dp activity depends on Cyclin E/Cdk2 which inactivates the inhibitory Retinoblastoma (Rb) protein by phosphorylation and thereby promotes transcription of E2F1 target genes such as *rnr, PCNA* and *cyclinE* itself (Du et al., 1996; Duronio and O'Farrell, 1994; Duronio and O'Farrell, 1995; Duronio et al., 1995). Thus, Cyclin E and E2F1/Dp act in an autoregulatory feedback loop in mitotic and endoreplicating cells resulting in Cyclin E accumulation prior to S phase (Duronio and O'Farrell, 1995; N. Zielke, personal communication; Sauer et al., 1995).

The CKI Dacapo specifically inhibits Cyclin E/Cdk2 activity in G1 of mitotically dividing cells (de Nooij et al., 1996; Lane et al., 1996). Recently, Dacapo has been implicated in the regulation of endocycles and meiosis in *Drosophila* ovarian cysts (Hong et al., 2007). Dacapo expression in *Drosophila* salivary glands is significantly reduced in comparison to to other G1-S genes (e.g. Cyclin E and Cdk2) which are known to be essential for endocycle regulation. Thus, in *Drosophila* salivary glands Cyclin E/Cdk2 activity seems to be restrained by mechanisms independent of Dacapo. However, it plays a role in other endoreplicating tissues like the ovary suggesting a cell type specific function of Dacapo. Since endoreplicating cells do not enter mitosis, it has been assumed that genes usually regulating the G2-M transition in mitotic cells are not required for endocycle control. This idea could be confirmed by qRT-PCR analysis of different mitotic genes that are involved in regulation of the G2-M transition. In *Drosophila* salivary glands, components of the

G2-M regulatory network such as Cdk1, String/Cdc25, Cyclin A and Cyclin B were expressed 400-8000 times lower than in mitotically dividing cells of the embryo (Figure 3.12). Comparison of G1-S and G2-M gene expression revealed that transcript levels of mitotic genes were dramatically reduced suggesting that these genes are presumably not involved in endocycle regulation. These observations are consistent with previous studies in which mitotic cyclins, Cdk1 and String/Cdc25 were reported to get transcriptionally downregulated, when cells switch from mitotic into endocycles (Klebes et al., 2002; Sauer et al., 1995; Shcherbata et al., 2004). Previously, it has been shown that *rca1* mutant cells of salivary glands did not show any abnormalities in their DNA content (Zielke, 2007). In agreement with these data, Rca1 is transcriptionally downregulated in *Drosophila* salivary glands (Figure 3.12) indicating that Rca1 activity is dispensable in endoreplicating cells. APC/C-Cdh1^{Fzr} activity is known to be essential for degradation of mitotic cyclins and entry into endoreplication cycles (Sigrist and Lehner, 1997). So far, it was assumed that APC/C-Cdh1^{Fzr} activity is not required once cells have entered the endocycle, because its main targets, the mitotic cyclins, are transcriptionally downregulated (Edgar and Orr-Weaver, 2001; Klebes et al., 2002; Lilly and Duronio, 2005). Interestingly, gene expression analysis of endoreplicating salivary glands demonstrated that the APC/C subunits *morula*/APC2 (*mr*) and *dmCdc16*/APC6 (Kashevsky et al., 2002; Pal et al., 2007; Reed and Orr-Weaver, 1997) as well as the APC/C activator Fzr were highly expressed (Figure 3.12). The expression levels of APC/C components and Fzr were higher than for most G1-S genes (e.g. Cyclin E, Cdk2) which are definitely essential during endoreplication. Furthermore, Western blot analysis revealed that Fzr protein is present in larval salivary glands (C. Lehner, personal communication). All together these data suggest that the APC/C-Cdh1^{Fzr} complex could play a role during endoreplication. This notion has been confirmed by functional studies. Supression of APC/C-Cdh1^{Fzr} activity by Cdc16 depletion or Rca1 overexpression in *Drosophila* salivary glands resulted in impaired endoreplication (N. Zielke et al., manuscript in preparation). The APC/C is a ubiquitin ligase that targets several substrates for degradation (for review see Peters, 2006; Pines, 2006) raising the question, which target causes the block of endoreplication. The mitotic cyclins cannot be responsible for the endocycle block since these genes are transcriptionally downregulated in salivary glands (Figure 3.12). However, Geminin, another target of the APC/C-Cdh1^{Fzr} complex, is expressed at levels comparable to G1-S genes (Figure 3.12; McGarry and Kirschner, 1998). Geminin has been shown to

accumulate upon APC/C inactivation in *Drosophila* salivary glands (N. Zielke, personal communication). These data indicate that Geminin might play a role in endocycle regulation. In mitotically dividing cells, APC/C-Cdh1^{Fzr} activity mediates degradation of mitotic cyclins and Geminin and thereby allows pre-RC formation during G1 (McGarry and Kirschner, 1998). When DNA replication is initiated, Geminin inhibits Dup/Cdt1 to prevent relicensing of replication origins during S phase (Wohlschlegel and Dwyer, 2000; Tada, 2007). Overexpression of Geminin in the embryo has been shown to inhibit DNA replication (Quinn et al., 2001) suggesting that increased Geminin protein levels observed upon APC/C inactivation cause the endocycle block in endoreplicating salivary glands.
In summary, gene expression analysis of *Drosophila* salivary glands revealed that G1-S genes are expressed, but mitotic genes of the G2-M network are transcriptionally downregulated since mitosis is bypassed in endoreplicating cells. Intriguingly, the APC/C-Cdh1^{Fzr} complex and its target Geminin are expressed in salivary glands. Hence, these results strongly support recent findings that the APC/C-Cdh1^{Fzr} complex mediates Geminin degradation and thereby regulates DNA licensing in endoreplicating cells (N. Zielke, personal communication).

4.6 Overexpression of HA-Rca1 increases Cyclin E and E2F1 target gene expression in endoreplicating salivary glands

Rca1 activity was shown to be dispensable for S phase entry in endoreplicating tissues (Zielke, 2007). Consistent with these results, Rca1 is transcriptionally downregulated in *Drosophila* salivary glands (Figure 3.12). However, overexpression of Rca1 perturbed endocycle progression in an F-box dependent manner (Zielke, 2007). In addition, Cyclin E and the mitotic proteins Cyclin A and Cdk1 accumulated in Rca1 overexpressing cells (Zielke, 2007). Overexpression of Cyclin E results in a similar phenotype, because continuous CyclinE/Cdk2 activity interferes with the DNA licensing machinery resulting in an endocycle block (Follette et al., 1998; Weiss et al., 1998). Cyclin E overexpressing cells also displayed elevated Cyclin A and Cdk1 protein levels (Zielke, 2007). Rca1 and Cyclin E/Cdk2 complexes are known inhibitors of APC/C-Cdh1^{Fzr} activity (Grosskortenhaus and Sprenger, 2002; Reber et al., 2006; Zielke, 2007). The APC/C-Cdh1^{Fzr} complex is expressed endoreplicating cells (Figure 3.12) and is essential for

mediating Geminin oscillations during the endocycle (N. Zielke, personal communication). Since mitotic cyclins are well known targets of the APC/C, Cyclin A protein could accumulate as a result of APC/C-Cdh1^{Fzr} inhibition by either Rca1 and/or Cyclin E/Cdk2 activity. Interestingly, only Cyclin A accumulates after Rca1 overexpression, whereas Cyclin B levels were unaffected (Zielke, 2007). If Rca1 inhibits the APC/C-Cdh1^{Fzr} complex, this should result in simultaneous accumulation of Cyclin A and B. A prerequisite for protein accummulation upon APC/C inhibition is that at least basal transcript levels of these genes are present in endoreplicating cells. In *Drosophila* salivary glands, expression of both genes is very low, but Cyclin B transcript levels are even more decreased than Cyclin A levels (Figure 3.12). These expression differences between Cyclin A and B might be the reason why Cyclin A accumulates upon APC/C inhibition, but not Cyclin B. However, elevated Cdk1 and Cyclin E protein levels upon Rca1 overexpression cannot be simply explained by APC/C inhibition since both proteins are not targets of the APC/C-Cdh1^{Fzr} complex. This raises the idea that Cyclin E, Cyclin A and Cdk1 are rather transcriptionally upregulated in Rca1 overexpressing salivary glands (Figure 3.12). To evaluate this idea, a gene expression study of Rca1 and Cyclin E overexpressing salivary glands was performed (Figure 3.13). Quantitative real-time PCR analysis confirmed the marked increase of Cyclin E transcript in Rca1 overexpressing salivary glands (Figure 3.13A). Furthermore, Cyclin A and Cdk1 expression levels were slightly upregulated indicating that Rca1 stimulates Cyclin A and Cdk1 transcription (Figure 3.13B). In Cyclin E overexpressing salivary glands, Cyclin A and Cdk1 expression was also increased (Figure 3.13B). However, Cyclin A and Cdk1 transcripts levels showed more than double increase when compared to those of Rca1 overexpressing cells (Figure 3.13B). This indicates that transcriptional upregulation of Cyclin A and Cdk1 after Rca1 expression could be a indirect effect due to elevated Cyclin E levels (Figure 3.13B). So far, it is not well defined how Rca1 or Cyclin E overexpression can influence transcription of Cyclin A and Cdk1 and which transcriptional regulators could be involved.

In addition, we found that Rnr2 and PCNA (Duronio et al., 1995; Thacker et al., 2003) were upregulated upon overexpression of either Rca1 or Cyclin E in larval salivary glands (Figure 3.12A). This phenotype was again much stronger for Cyclin E overexpression suggesting that Rca1 might stimulate Rnr2 and PCNA transcription indirectly via Cyclin E. Since Cyclin E, Rnr2 and PCNA are target genes of E2F1 (Duronio et al., 1995; Thacker et al., 2003), E2F1 activity seems to be increased in Rca1/Cyclin E overexpressing cells.

Interestingly, E2F1 transcript levels were not induced after Cyclin E expression or rather reduced after expression of HA-Rca1. Thus, increased E2F1 target gene expression is not a result of induced E2F1 transcription suggesting that Rca1 or Cyclin E influence E2F1 protein levels or the activity of the E2F1 inhibitor Rb (Kato et al., 1993). Recently, it has been reported that HA-Rca1 or Cyclin E overexpression stabilises E2F1 protein levels by preventing its S phase dependent degradation (N. Zielke, personal communication). Rca1 and Cyclin E/Cdk2 are both inhibitors of the APC/C-Cdh1^{Fzr} complex (Grosskortenhaus and Sprenger, 2002; Reber et al., 2006). Overexpression of HA-Rca1 or Cyclin E in endoreplicating salivary glands inactivates the APC/C-Cdh1^{Fzr} complex resulting in Geminin accumulation (N. Zielke, personal communication). High levels of Geminin in turn prevent DNA replication by inhibiting Dup/Cdt1 (Wohlschlegel and Dwyer, 2000; Tada, 2007). Impaired DNA replication upon Geminin overexpression has been reported to result in E2F1 protein stabilisation and increased transcription of E2F1 target genes. Thus, APC/C inhibition by Rca1 or Cyclin E overexpression leads to elevated Geminin and E2F1 protein levels which in turn activates Cyclin E, Rnr2 and PCNA transcription. Geminin and Dup/Cdt1 expression is under control of E2F1 and Cyclin E in mitotically dividing cells (N. Zielke, personal communication; Whittaker et al., 2000) suggesting that Rca1 or Cyclin E overexpression could also increase transcript levels of these genes in endoreplicating cells. The balance between Geminin and Dup/Cdt1 protein levels is a prerequisite to maintain genomic stability (Saxena and Dutta, 2005). It has been reported that elevated Cdt1 levels are accompanied with increased Geminin expression (May et al., 2005). Therefore, Dup/Cdt1 expression could be upregulated in Rca1 or Cyclin E overexpressing salivary glands as a consequence of elevated Geminin and E2F1 protein. To approach this issue, Geminin and Dup/Cdt1 expression should be examined by further qRT-PCR analysis to monitor transcription changes upon Rca1 or Cyclin E overexpression.

Since Cyclin E, Rnr2 and PCNA transcription is increased upon APC/C inactivation by Rca1 or Cyclin E, elevated transcript levels of Cyclin A and Cdk1 could also be a result of stabilised E2F1 protein. In mammalian cells, Cyclin A and Cdk1 gene expression is controlled by E2F (DeGregori et al., 1995; Pagano et al., 1992). However, there is so far no evidence for Cyclin A or Cdk1 being a direct E2F1 target in *Drosophila* suggesting that other transcriptional activators might be stimulated in Rca1 or Cyclin E overexpressing cells. Transcription of Rca1, Cyclin B or Dacapo were not significantly affected by either

Cyclin E or Rca1 expression (Figure 3.13A and B). Dacapo has been shown to be a E2F1 target in *Drosophila* (de Nooij et al., 2000; Dimova et al., 2003). However, Dacapo expression is not affected upon Rca1 or Cyclin E expression although transcript levels of other E2F1 target genes were increased in endoreplicating cells (Figure 3.13A). A possible explanation could be that the *dacapo* gene locus is inaccessible to the transcriptional machinery as a result of gene silencing during salivary gland development. Interestingly, the E2F1 targets, Cdk2 and String/Cdc25 were both downregulated upon Rca1 overexpression. In Cyclin E expressing salivary glands, expression of Cdk2 was upregulated, whereas String/Cdc25 transcript levels were decreased. Since Rca1 and Cyclin E both restrain APC/C-Cdh1^{Fzr} activity resulting in increased E2F1 target gene expression, Cdk2 and String/Cdc25 transcript levels were expected to increase/decrease simultaneously. However, expression changes of Cdk2 and String/Cdc25 differed upon Rca1 or Cyclin E overexpression and so far there is no plausible explanation for these observations.

Gene expression analysis revealed that APC/C subunits (Cdc16 and Morula) and the activator protein Fzr are expressed in endoreplicating salivary glands (Figure 3.12). Moreover, APC/C-Cdh1^{Fzr} activity has been shown to be essential for regulation of Geminin protein levels in endoreplicating cells (N. Zielke, personal communication). When Rca1 or Cyclin E was overexpressed in salivary glands, transcript levels of Cdc16, Morula or Fzr were not influenced (Figure 3.13C). Thus, expression of these genes is not regulated by E2F1 since E2F1 activity is known to be increased in Rca1 or Cyclin E overexpressing cells.

5. Materials and Methods

5.1 Materials

5.1.1 Chemicals

Standard chemicals were purchased at the following companies: Amersham-Pharmacia, Applied Biosystems, Biomol, Biozym, Fluka, Riedel-de-Häen, Serva, Gibco-Invitrogen, Merck, Roche, Roth and Sigma-Aldrich.

5.1.2 Special chemicals and kits

Big Dye Terminator V.3.1	Applied Biosystems
Blocking Buffer for Fluorescent Western Blotting	Rockland
BSA	Invitrogen
Cellfectin Reagent	Gibco Invitrogen
Calf intestinal phosphatase (CIP)	NEB
DNA Gel purification Kit	GE-Healthcare
DNA molecular weight marker	Invitrogen
dNTPs/NTPs	Amersham-Pharmacia
Drosophila Schneider's media	Gibco Invitrogen
Ethidiumbromide	AppliChem
Fetal Bovine Serum (FBS)	Gibco Invitrogen
FLAG Immunoprecipitation Kit	Sigma-Aldrich
Fugene HD	Roche
Glycogene	Invitrogen
Hoechst 33342	Sigma-Aldrich
Klenow fragment	Roche
Lithiume Chloride Solution (8 M)	Sigma-Aldrich
µMACS mRNA Purification Kit	Miltenyi
Nucleobond AX-100 (Midi Prep Kit)	Machery and Nagel
Nucleospin DNA Purification Kit	Machery and Nagel
PCR Purification Kit	GE-Healthcare
Platinum *pfx* DNA Polymerase	Invitrogen
Poly-L-Lysine 0.01% Solution	Sigma-Aldrich
Power SYBR Green PCR Master Mix	Applied Biosystems
Precision Plus Protein – molecular weight marker	BioRad
Protease Inhibitor Cocktail	Sigma-Aldrich
QuantiFast SYBR Green PCR Kit	Qiagen
QuantiTect Primer Assays	Qiagen

SuperSkript III First-Strand cDNA Synthesis Kit	Invitrogen
Red Taq DNA Polymerase	Sigma-Aldrich
Restriction Enzymes	NEB/Roche
T4 DNA Ligase	NEB
TEMED	Sigma-Aldrich
Vectashield	Vector Laboratories

5.1.3 Electronic equipment, computer and software

This thesis was prepared on IBM compatible PC and Apple Macintosh computers using Vector NTI (Invitrogen), Adobe PhotoShop (Adobe Systems), Canvas (Deneba Systems), Axiovision (Zeiss), Microsoft Word and Excel (Microsoft Corp.) and SDS (Applied Biosystems).

Fluorescent images were aquired with a Zeiss AxioCam HRm CCD camera on a Zeiss Axioplan Imaging2e microscope or on a Leica TCS-SP2 confocal microscope.

Western blots were analyzed with the Odyssey infrared imaging system (Li-Cor Biosciences).

DNA sequences were analyzed on an ABI 3730 sequencer (Applied Biosystems).

Quantitative real-time PCR's were performed on an ABI 7900 Real-Time PCR System (Applied Biosystems).

5.1.4 Media, solutions and buffers

Ampicillin stock solution	50 mg/ml in 50% ethanol, dilute to 50 µg/ml final concentration
APS	10% APS in H_2O
BSA blocking solution (5%)	50 mg BSA is resolved in 1 ml Lysisbuffer
DNA loading buffer	0.25% Bromophenol blue 0.25% Xylene cyanol 30% glycerol in H_2O
Colloidal Coomassie staining solution	0.08% Coomassie Brilliant Blue G250 1.6% ortho-phosphoric acid 8% ammonium sulfate Store stock solution at RT and add 20% of methanol before use

Coomassie destaining solution	40% methanol in H_2O
$CuSO_4$ (0.1 M)	1 M $CuSO_4$ in H_2O is diluted to 0.1 M with H_2O and sterile filtrated
Hoechst 33342	0.5 mg/ml in H_2O
Laemmli buffer (4 x)	8% SDS 400 mM DTT 240 mM Tris-HCl pH 6.8 0.004% bromophenol blue 40% glycerol
LB-Medium (1 l)	10 g bactotrypton 5 g bacto yeast extract 10 g NaCl adjust pH to7.2 with NaOH and add H_2O to 1 l final volume (for casting plates add 15 g agar)
Lithiume chloride solution (4 M)	8 M lithiume chloride solution is diluted with DEPC-treated H_2O to a final concentration of 4 M
Lysisbuffer	10% glycerol 50 mM HEPES, pH 7.5 150 mM NaCl 0.5% Triton X-100 1.5 mM $MgCl_2$ 1 mM EGTA store at 4°C and add 4 µl/ml Protease Inhibitor Cocktail before use
Mini-Prep buffer	<u>Resuspension buffer</u> 50 mM glucose 25 mM Tris-HCl, pH 8.0 10 mM EDTA, pH 8.0 100 µg/ml RNAse 5 mg/ml Lysozyme <u>Lysis buffer</u> 200 mM NaOH 1% SDS <u>Neutralization buffer</u> 3 M potassium acetate, pH 5.5
Formaldehyde (4%, 50 ml)	<u>4% Formaldehyde in PBS</u> 5 ml formaldehyde (37%) add PBS to 50 ml final volume

PBS

130 mM NaCl
2.7 mM KCl
7 mM Na_2HPO_4
3 mM KH_2PO_4
adjust pH to 7.4

PBS-0.1% Tween (500 ml)

0.1% Tween-20 in PBS
50 ml 10 X PBS
2.5 ml 20% Tween (in 50% ethanol)
add H_2O to 500 ml final volume

PBS-0.2% Tween (500 ml)

0.2% Tween-20 in PBS
50 ml 10 X PBS
5 ml 20% Tween-20 (in 50% ethanol)
add H_2O to 500 ml final volume

Ponceau S (10 x, 100 ml)

2 g Ponceau S
30 g trichloracetic acid
30 g sulfosalicyclic acid
add H_2O to 100 ml final volume

Powdered milk (5%)

5% milk powder is resolved in PBS-0.2% Tween

RNAse stock solution

10 mg/ml in H_2O
boil for 10 min to remove DNase

Running buffer (10 x, 1 l)

30 g Tris base (0.25 M)
144 g glycine (1.9 M)
100 ml SDS (10%)
add H_2O to 1 l final volume

Separating gel buffer (4 x, 1 l)

181.7g Tris base (1.5 M)
4 ml SDS (10%)
adjust pH to 8.8 with HCl, add H_2O to 1 l final volume

Separating gel mix for SDS-PAGE (100 ml)

	7.5%	11%	12.5%	15%
H_2O (ml)	50.25	39	33.75	25.5
30% acrylamide (ml)	24.75	36	41.25	49.5
4 X Separating gel buffer (ml)	25	25	25	25

Store gel mix at 4°C. For 2 mini gels take 10 ml of the mix and add 200 µl APS and 20 µl TEMED.

Stacking gel buffer (4 x, 500 ml)

30.3 g Tris base (0.5 M)
20 ml 10% SDS
adjust pH to 6.8 and volume to 500 ml with H_2O

Stacking gel mix for SDS-PAGE

	50 ml	100 ml
H_2O (ml)	28.65	57.3
30% acrylamide (ml)	8.6	17.2
4X Stacking gel buffer (ml)	12.75	25.5

Store gel mix at 4°C. For 2 mini gels take 10 ml of the mix and add 200 µl APS and 20 µl TEMED.

TAE (50 x, 1 l)

242 g Tris base (2 M)
57.1 ml glacial acetic acid
100 ml EDTA (0.5 M, pH 8.0)
add H_2O to 1 l final volume

TE

10 mM Tris-HCl pH 8.0
1 mM EDTA pH 8.0

Terrific broth

Solution A
12 g bactotrypton
24 g bacto-yeast-extract
4 ml glycerol
adjust to 900 ml with H_2O

Solution B
2.31 g KH_2PO_4
12.54 g K_2HPO_4
adjust to 90 ml with H_2O

Tetracycline stock solution

10 mg/ml in 50% ethanol, dilute to 20 µg/ml final concentration

Transfer buffer for western-blotting (1 l)

5.82 g Tris base (48 mM)
2.93 g glycine (39 mM)
3.75 ml 10% SDS
200 ml methanol

5.1.5 Bacterial strains

DH5α — *sup*E44 Δ*lac*U169 (ϕ80*lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1

5.1.6 Oligonucleotides

Oligonucleotides were purchased from Roth and Invitrogen.

Table 5.1 Oligonucleotides used for cloning and sequencing.

Name	Sequence	Application
CO-166$^{rca1.1}$	GATGAACGAGTCTGGCTACACATC	sequencing of Rca1
CO-167$^{rca1.2}$	CCAAGCGACGCAAGAAACACTTTC	sequencing of Rca1
CO-168$^{rca1.3}$	CTAATGGACTCGGGCAACTCGAGCATC	sequencing of Rca1
CO-169$^{rca1.4}$	CTAACCAAAGAGAATCCTCACCTGCC	sequencing of Rca1
CO-170$^{rca1.5}$	CCTATTGGACGTACAACCAGCACATTC	sequencing of Rca1
CO-265	GGATAACAATTTCACACAG	sequencing from SP6 promotor
CO-401	GTAATACGACTCACTATAGGGCG	sequencing from T7 promotor
CO-402	CAATTAACCCTCACTAAAGGG	sequencing from T3 promotor
CO-417	AACTTTGTGTTCGGCCAGTC	Tubulin PCR/cDNA test
CO-418	TGTCGATGCAGTAGGTCTCG	Tubulin PCR/cDNA test
CO-453	ATATGGATCCACTAGTCATGTCCGCCTATTATCGGCGC	cloning of SQ30 (Y2H bait vector)
CO-454	ATATGAATTCTTAATTAAACTAAAAACAGAGCCGCTTGAGCG	cloning of SQ30 (Y2H bait vector)
CO-510	GCAGAAGCTCAGAATAAACG	sequencing from 5' globin leader
CO-547	GTGAATCATCTCAGTGCAACTAAAGGG	sequencing from metallothionein promotor
CO-549	ATATGGATCCATGGCGATGCAGAAGTTCTTCAG	cloning of SQ50.2
CO-550	ATATTCTAGACTAATCGATGGATACGCTGGATATTTCC	cloning of SQ50.2
CO-557	CCGCCCGGAGACGTGTTG	sequencing of SQ50.2
CO-558	GGGATTCAGTTTCAGGGCGCG	sequencing of SQ50.2
CO-580	TTGTCCAATTATGTCACACCA	sequencing from 3'globin-poly-A

Oligonucleotides for quantitative real-time PCR using the QuantiFast SYBR Green PCR Kit were purchased from Qiagen.

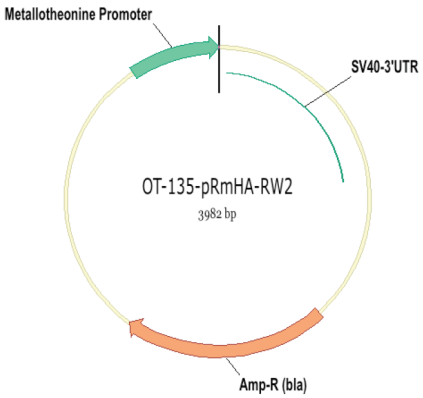
Table 5.2 QuantiTect Primer Assays used for quantitative real-time PCR with SYBR Green dye.

Gene	QuantiTect Primer Assay	Transcript name (Transcript/Amplicon length)
act 87E (Actin 87E)	QT00503503	act87E-RA (1564 bp/61 bp)
gapdh1 (Gapdh1)	QT00941465	gapdh1-RA (1483 bp/96 bp)
geminin (Geminin)	QT00940541	geminin-RA(997 bp/76 bp)
cdc16 (Cdc16)	QT00980644	cdc16-RA (2583 bp/75 bp)
cdc2 (Cdk1)	QT00933961	cdc2-RA (1083 bp/134 bp)
cdc2c (Cdk2)	QT00978782	cdc2c-RB (1220 bp/78bp)
cycA (CyclinA)	QT00962843	cycA-RA (2440 bp/107 bp)
cycB (CyclinB)	QT00952749	cycB-RA (2669 bp/108 bp)
cycE (CyclinE)	QT01087940	cycE-RA (3658 bp/93 bp)
dap (Dacapo)	QT00943096	dap-RA (2535 bp/78 bp)
dup (double parked)	QT00502845	dup-RA (3115/98bp)
e2f1 (E2F1)	QT00979391	e2f1-RA (4926bp/83bp)
mr (Morula)	QT00502425	mr-RA (2557bp/99bp)
mus209 (PCNA)	QT00950769	mus209-RA (1044bp/94bp)
rca1 (Rca1)	QT00931315	rca1-RA (1553bp/76bp)
rnr2 (Rnr2)	QT0094444706	rnrs-RA (1647bp/103bp)
stg (String)	QT00985026	stg-RA (3052bp/112bp)

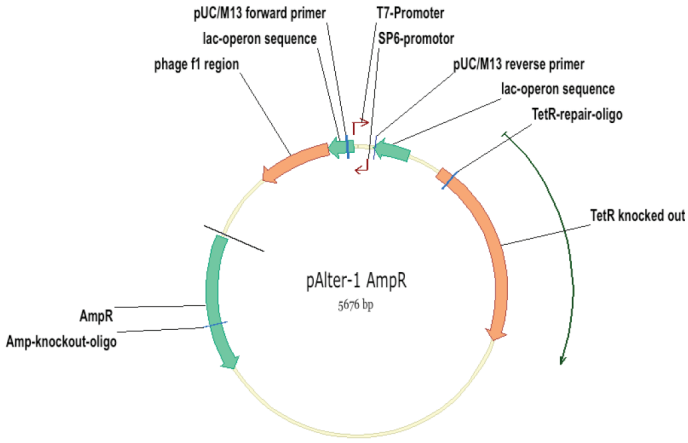
5.1.7 Plasmids

For molecular cloning the following vectors have been used as a matrix (Figure 5.1): **(A)** pOT135-pRmHA-RW2 (Bunch et al., 1988), **(B)** pAlter1 (Promega), **(C)** pBluescript II SK(-) (Stratagene), **(D)** pSP64 and **(E)** pB27 (Hybrigenics Y2H bait cloning vector).

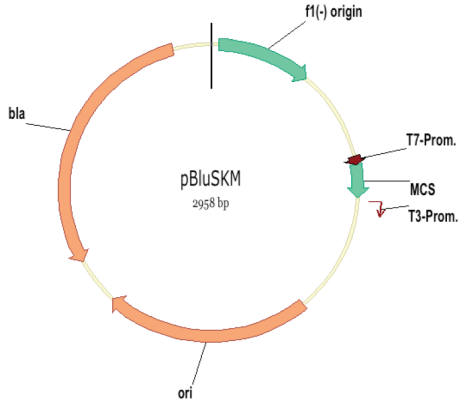
A



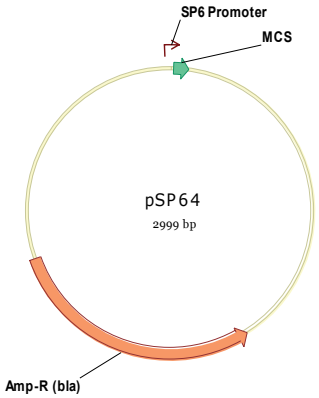
B



C



D



E

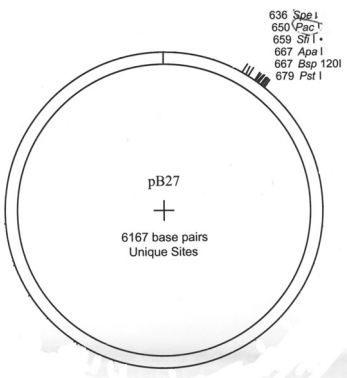


Table 5.3 Plasmids that have been generated or used for cloning or transfection of S2 cells.

NR	Insert	Vector Matrix	Purpose
pAD188	HA-Cyclin A	pBluescript SK(-)	cloning
pHT12	HA-Fzr	pOT135	transfection of S2 cells
pHT13	HA-SkpA	pOT135	transfection of S2 cells
pHT14	FLAG-Rca1	pOT135	transfection of S2 cells
pHT19	HA-Dwee	pOT135	transfection of S2 cells
pHT20	HA-Rux	pOT135	transfection of S2 cells
pHT30	FLAG-Rca1	pOT135	cloning
pHT32	HA-Rca1	pOT135	cloning
pHT37	HA-Rca1	pOT135	cloning
pNZ01	HA-Rca1	pAlter1	cloning
pNZ04	HA-Rca1C351S	pSP64	cloning
pNZ14	HA-Rca1 $\Delta CT^{(370-411)}$	pSP64	cloning
pNZ23	FLAG-Rca1	pSP64	cloning
pNZ26	HA-Rca1$\Delta KEN^{E215A, N216A}$	pAlter1	cloning
pNZ30	FLAG-Rca1$\Delta 255^{(1-255)}$	pSP64	cloning
pNZ31	FLAG-Rca1$\Delta 133^{(1-133)}$	pSP64	cloning
pNZ32	FLAG-Rca1ΔF-box$^{(164-203)}$	pSP64	cloning
pNZ49	HA-Rca1ΔDSGxxS$^{S253A, S256A S257A}$	pAlter1	cloning
pNZ51	HA-Rca1ΔCdk1$^{T14A, T44A, T71A, T104A, S123A, S127A, S187A, S335A, T377A, T388A}$	pAlter1	cloning
pRG26	HA-Rca1	pSP64	cloning
pRG30.3	HA-Rca1 $\Delta NLS^{(117-134)}$	pBluescript SK(-)	cloning
pRG43	Rca1	pSP64	cloning
pRG54	HA-Rca1$\Delta 203^{(1-203)}$	pSP64	cloning
pRW663	FLAG-DofΔ1-552	pOT135	transfection of S2 cells
pOT146	GFP	pOT135	transfection of S2 cells
pOT156	Myc-Cul1	pBluescript SK(-)	cloning
pOT172	Cdc23 cDNA	pBluescript SK(-)	cloning
pSQ01	FLAG-Rca1$\Delta 255^{(1-255)}$	pOT135	transfection of S2 cells
pSQ02	FLAG-Rca1$\Delta 203^{(1-203)}$	pSP64	cloning
pSQ08	FLAG-Rca1$\Delta 133^{(1-133)}$	pOT135	transfection of S2 cells
pSQ09	FLAG-Rca1ΔF-box$^{(164-203)}$	pOT135	transfection of S2 cells
pSQ10	FLAG-Rca1$\Delta NLS^{(117-134)}$	pSP64	cloning
pSQ11	FLAG-Rca1$\Delta NLS^{(117-134)}$	pOT135	transfection of S2 cells
pSQ12	FLAG-Rca1C351S	pSP64	cloning
pSQ13	FLAG-Rca1C351S	pOT135	transfection of S2 cells

NR	Insert	Vector Matrix	Purpose
pSQ14	FLAG-Rca1ΔDSGxxS$^{S253A,\ S256A\ S257A}$	pSP64	cloning
pSQ15	FLAG-Rca1ΔDSGxxS$^{S253A,\ S256A\ S257A}$	pOT135	transfection of S2 cells
pSQ16	FLAG-Rca1ΔCdk1$^{T14A,\ T44A,\ T71A,\ T104A,\ S123A,\ S127A,\ S187A,\ S335A,\ T377A,\ T388A}$	pSP64	cloning
pSQ17	FLAG-Rca1ΔCdk1$^{T14A,\ T44A,\ T71A,\ T104A,\ S123A,\ S127A,\ S187A,\ S335A,\ T377A,\ T388A}$	pOT135	transfection of S2 cells
pSQ20	FLAG-Rca1ΔKEN$^{E215A,\ N216A}$	pSP64	cloning
pSQ21	FLAG-Rca1ΔKEN$^{E215A,\ N216A}$	pOT135	transfection of S2 cells
pSQ29	Rca1	pBluescript SK(-)	cloning
pSQ30	Rca1	pB27	yeast two-hybrid bait vector
pSQ31	FLAG-Rca1 ΔCT$^{(370-411)}$	pSP64	cloning
pSQ32	FLAG-Rca1 Δ133$^{(1-133)}$ΔF-box$^{(164-203)}$	pSP64	cloning
pSQ33	FLAG-Rca1 Δ133$^{(1-133)}$ΔF-box$^{(164-203)}$	pOT135	transfection of S2 cells
pSQ34	FLAG-Rca1ΔNLS$^{(117-134)}$ΔNLS$^{(117-134)}$	pSP64	cloning
pSQ35	FLAG-Rca1ΔNLS$^{(117-134)}$ΔNLS$^{(117-134)}$	pOT135	transfection of S2 cells
pSQ36	FLAG-Rca1 ΔCT$^{(370-411)}$	pBluescript SK(-)	cloning
pSQ37	FLAG-Rca1 ΔCT$^{(370-411)}$	pOT135	transfection of S2 cells
pSQ39	HA-Rca1-SmaI/XmaI	pSP64	cloning
pSQ40	FLAG-Rca1 Δ203-255$^{(203-255)}$	pSP64	cloning
pSQ41	FLAG-Rca1 Δ203-255$^{(203-255)}$	pOT135	transfection of S2 cells
pSQ48	Myc-Cul1	pOT135	transfection of S2 cells
pSQ50.2	FLAG-Cdc23	pOT135	transfection of S2 cells

5.1.8 Fly stocks

The fly stock w^{1118} was used as a wild-type control.

Table 5.4 Fly stocks.

NR	Genotype	Distributor
TF450	w; If/CyO(wg-lacZ); UAS-HA-Rca1/TM6B	Ruth Grosskortenhaus
TF408	w; If/CyO(wg-lacZ); UAS-CycE/TM6B	Ruth Grosskortenhaus
TNZ113	w, hs-Flp$^{1.22}$; AyGal4(25), UAS-GFP/SM6-TM6	Thomas Klein

5.1.9 Antibodies

Table 5.5 Primary antibodies that have been used for immunofluorescence and Western blotting.

NR	Antigen	Source	Flourochrome	Dilution	Distributor
282	HA	Rat	1:1000	1:2000	Roche
294	GFP	Rabbit	1:500	1:2500	Torrey Pines Biolabs
302	FLAG	Mouse	1:2000	1:1500	Sigma-Aldrich
354	Myc	Rabbit	not tested	1:1000	Santa Cruz Biotechnology
347	Cul1	Rabbit	not tested	1:250	Santa Cruz Biotechnology
318	Actin	Rabbit	not tested	1:10000	Sigma-Aldrich

Table 5.6 Secondary antibodies that have been used for immunofluorescence.

NR	Antigen	Source	Flourochrome	Dilution	Distributor
227	Mouse	Goat	Alexa-568	1:500	Invitrogen -Molecular Probes
286	Mouse	Goat	Alexa-488	1:500	Invitrogen -Molecular Probes
298	Mouse	Goat	Rhd-Red-X	1:500	Dianova
182	Rabbit	Goat	Cy5	1:500	Dianova
184	Rat	Goat	Alexa-488	1:500	MoBiTec
198	Rat	Goat	Alexa-568	1:500	MoBiTec
198	Rat	Goat	Alexa-568	1:500	MoBiTec

Table 5.7 Secondary antibodies for western blot analysis using the Odyssey system.

NR	Antigen	Source	Flourochrome	Dilution	Distributor
307	Mouse	Goat	Alexa-680	1:3000	Invitrogen -Molecular Probes
312	Rat	Goat	IRdye-700	1:3000	Rockland Immunochemicals
316	Rabbit	Donkey	IRdye-800	1:3000	Rockland Immunochemicals
312	Rat	Goat	IRdye-700	1:3000	Rockland Immunochemicals

5.2 Methods for molecular cloning

5.2.1 Electrocompetent bacteria cells

DH5a or JM101 cells were grown on LB plates overnight at 37°C. A single colony was picked and grown in a 50 ml LB culture overnight at 37°C on a shaker.
The following day cells were diluted 1:100 with TB and grown again at 37°C until the culture reached an OD_{600} value of 0.5. The bacterial culture was stored on ice for 30 min and then centrifuged at 4000 rpm in a Sorvall GS3 rotor for 10 min at 4°C. The supernatant was discarded while the bacterial pellet was resuspended on ice with 500 ml Millipore water. The cell suspension was spinned down at 8000 rpm in a Sorvall GS3 rotor for 20 min at 4°C and the supernatant was discarded. Cells were resuspended on ice with precooled 10% (w/v) glycerol and centrifuged in a Heraeus bench top centrifuge at 4000 rpm for 7 min at 4°C. Pellets were resuspended in 1 ml ice cold 10% glycerol and splitted into 50 µl aliquots that were frozen in liquid nitrogen and stored at -70°C.

5.2.2 Transformation of electrocompetent bacterial cells

Electrocompetent cells were thawed on ice and 0.5-1 µl of plasmid DNA was added and mixed gently. Electroporation was performed in *E.coli* pulser cuvettes using the Gene Pulser (BIO-RAD). Immediately after the electroporation, cells were resuspended in 1 ml LB media and then grown for 1 h at 37°C on a shaker. Afterwards, cells were plated on LB agarplates containing appropriate antibiotics and grown overnight at 37°C.

5.2.3 Transformation of chemical competent bacteria cells

Chemical competent DH5α were purchased from Invitrogen and stored according to manufacturers' instructions at -80°C. Aliquots of chemical competent cells (50 µl) were thawed on ice. 1-5 µl of plasmid DNA were added, gently mixed and chilled on ice for about 30 min. Transformation by heat shock occured in a 37°C water bath for 20 s. Cells were transferred immediately on ice for 2 min and resuspended in 1ml LB. Cells were

grown for 1 h at 37°C on a shaker and then spread on LB agarplates containing antibiotics. Plates were incubated overnight at 37°C to get single bacterial colonies.

5.2.4 Restriction analysis

Restriction analysis of plasmid DNA or PCR products were performed according instructions provided by suppliers (NEB, Boehringer-Roche) using appropriate buffers, BSA and temperature. Small amounts of DNA (0.5-1 µg) were digested for 1-2 h whereas preparative digests (5-10 µg) for molecular cloning were incubated overnight.

5.2.5 Dephosphorylation of DNA ends

For molecular cloning, overnight DNA digests were treated with 1 µl calf intestinal phosphatase (CIP) and incubated at 37°C for another 30 min. The CIP enzyme removes the 5' phophate from DNA fragments and prevents self-ligation of cloning vectors. The enzyme was not heat-inactivated because digests were directly separated on an agarose gel.

5.2.6 Klenow fill-in of DNA ends

To fill 5'overhangs, 1 U Klenow/µg DNA and 33 µM dNTPs were added to a restriction mix and incubated at RT for 15 min. The reaction was stopped by addition of 10 mM EDTA and subsequent heating at 75°C for 10 min.

5.2.7 Agarose gel electrophoresis

DNA fragments were separated by agarose gel electrophoresis following standard methods (Sambrook et al., 1989). In general, agarose gels contained 0.8-1% agarose, 100 ml TAE buffer and 10 µl ethidium bromide solution (10 mg/ml). Resolution of DNA fragments occured at an average voltage of 10 V/cm. After electrophoresis, gels were illuminated with an UV lamp to visualise the DNA bands.

5.2.8 Isolation of DNA fragments

DNA fragments were separated on agarose gels and the DNA bands of interest were excised under UV light using a sterile scalpel. DNA was then purified from gel pieces using the Nucleospin DNA Purification Kit (Machery and Nagel).

5.2.9 DNA ligation

Ligation reactions were carried out in a total volume of 15-20 µl using 1 µl T4-DNA ligase and a molar ratio between vector and insert of 1:5. Reactions were incubated for 2 h at RT or overnight at 18°C.

5.2.10 DNA Mini-Preparation

Small amounts of plasmid DNA were isolated from transformed bacterial cells for restriction or sequencing analysis by alkaline lysis. Therefore, a single colony was cultured in 2 ml LB media with appropriate antibiotics and grown overnight at 37°C. Cells were spinned down in a bench top centrifuge for 5 min at 5000 rpm at RT. Bacterial pellets were first resuspended in 200 µl resuspension buffer, mixed with 200 µl lysis buffer and incubated for 5 min at RT. Afterwards 200 µl neutralization buffer was added and the tube was shaken and incubated for 5 min at RT. Lysates were centrifuged for 30 min at 14.000 rpm at RT. Supernatants were transferred into a new sample tube and plasmid DNA was precipitated by adding 1 ml 100% ethanol. Samples were centrifuged for 20 min at 14.000 rpm. The resulting DNA pellet was washed with 1 ml 70% ethanol followed by centrifugation for 5 min at 14.000 rpm. Finally, the pellet was dried and resuspended in 20-50 µl H_2O.

5.2.11 DNA Midi-Preparation

Larger amounts of plasmid DNA were purified from 50 ml overnight bacterial cultures using the Nucleobond AX-100 Midi Prep Kit (Machery and Nagel). The DNA preparation was performed according to the manufacturers' instructions. DNA pellets were

resuspended in 50-100 µl H_2O and DNA concentrations were determined by photometric measurements at 260 nm.

5.2.12 DNA sequencing

Sequencing analysis of plasmid DNA was performed using either standard (T3, T7 or SP6) or gene specific oligonucleotides and the Big Dye Terminator Sequencing Kit. PCR reactions were performed using a standard PCR (see section 5.2.13) protocol in a 10 µl volume. When the PCR reaction was completed 10 µl water were added to each sample. Samples were analyzed by the CCG sequencing facility at the University of Cologne. Detailed information about the composition of the sequencing reaction and analysis procedure is available at:
http://www.unikoeln.de/mathnatfak/genetik/facilities/sequencer/index.html.

5.2.13 Polymerase chain reaction (PCR)

DNA fragments were amplified from plasmid DNA in a standard PCR reaction using the Expand High Fidelty PCR system (Roche) or the Platinum *pfx* DNA polymerase (Invitrogen). For RT-PCR analysis using agarose gel electrophoresis, 1-2 µl cDNA were amplified in a standard PCR reaction using RedTaq DNA polymerase. PCR reactions were performed in a total volume of 50 µl according to manufacturers' instructions and the following standard PCR program:

1. 95°C	5 min
2. 95°C	30 s (denaturation)
3. 50-60°C	30 s (primer annealing)
4. 68°C/72°C	1 min/1 kb (extension)
5. step 2-4	25-30 cycles
6. 68°C/72°C	10 min (end extension)

For cloning, PCR products were separated by agarose gel electrophoresis, DNA bands of interest were excised under UV light and DNA was purified using Nuclespin DNA Purification Kit (Machery and Nagel).

5.3 *Drosophila* Schneider (S2) cell culture

5.3.1 Maintenance of S2 cells

Drosophila Schneider cells were kindly provided by M. Boutros (DKFZ Heidelberg, Germany) and M. Reth (University of Freiburg, Germany). S2 cells were cultured in 50 ml cell culture flasks at 27°C using *Drosophila* Schneider's media supplemented with 5% fetal bovine serum (FBS). After 2-3 days cells reached a density of about 90% and were splitted in a ratio of 1:2 using fresh media and new flasks. All cell culture operations were performed under sterile conditions in a sterile hood.

5.3.2 Freezing and thawing of S2 cells

Well growing, confluent, young cultures of S2 cells were scraped off the flask bottom and transferred into a 15 ml falcon tube. Cell suspensions were spinned down for 5 min at 1100 rpm in a Haereus centrifuge. The cell pellet was resuspended in freezing media (90% FBS, 10% DMSO) and frozen in cryotubes at -80°C for some days. For long-term storage 1-2 years aliquots were kept in liquid nitrogen.

Cells were recultured by slowly thawing a cryotube of frozen cells on ice. The thawed cell suspension was immediately added to 5 ml *Drosophila* Schneider's media supplemented with 10% FBS and the tube was centrifuged for 5 min at 1100 rpm. The supernatant was discarded and the cell pellet was resupended with fresh *Drosophila* Schneider's media supplemented with 10% FBS. The cell suspension was transferred into a flask and incubated at 27°C for 2-4 days until cells had recovered and started growing. When cells started growing well, the media was changed and slowly adapted to growing media supplemented with 5% FBS.

5.3.3 Transient transfection of S2 cells

Transient transfections of S2 cells were carried out using Cellfectine (Invitrogen) or FuGENE (Roche) as transfection reagents. One day before the transfection experiment, well growing cells were transferred into 6-well plates with 10^6 cells per well in a total

volume of 3 ml media + 5% FBS. For immunostainings cells were grown on Poly-L-Lysine coated coverslips. On the next day the transfection reaction was initially set up as two separate mixes. The first Eppendorf tube contained the DNA mix (100 µl serum free media + 0.5-1 µg plasmid DNA) while the transfection reagent (100 µl serum free media + 10 µl Cellfectine or 5 µl FuGENE) was prepared in the second tube. Both mixes were combined in one tube and the resulting transfection mix was in turn incubated for 30-45 min at RT. In the meantime, the media of cells growing in 6-well plates was replaced by 2 ml serum free media. When the incubation time was completed, the transfection mix was added and cells were incubated at 27°C. After 6 h each well was supplemented with 50 µl of FBS and incubated again at 27°C overnight. The following day, the media was replaced with fresh *Drosophila* Schneider's media supplemented with 5% FBS. The expression of OT135-pRmHA-RW2 derived constructs under the control of a metallothionein promotor was induced by adding 30 µl of $CuSO_4$ (0.1 M). After 24 h cells were either harvested to extract proteins or cells grown on coverslips were immunostained.

5.3.4 Coating coverslips with Poly-L-Lysine

Each glass coverslip was coated with 500 µl Poly-L-Lysine (0.01%) solution under sterile conditions. Coverslips were incubated for 1 h at RT and then washed with sterile water up to ten times. Coverslips were thoroughly dried under the hood and stored in plastic dishes at 4°C.

5.3.5 Immunostaining of S2 cells

S2 cells were grown on Poly-L-Lysine coated coverslips, transiently transfected with different DNA constructs and protein expression was induced by $CuSO_4$ treatment. After 24 h cells were washed once with 2 ml PBS, fixed with 4% formaldehyde for 10 min and permeabilised with PBS-0.5% Tween for 30 s. Following three wash steps with PBS-0.1% Tween for 10 min and blocking with PBS supplemented with 1% BSA for 30 min at RT, cells were incubated with primary antibodies for 1 h at RT. Again, cells were washed three times with PBS-0.1% Tween before secondary antibodies were added for 1 h. After three

washing steps DNA staining with Hoechst 33342 was performed for 5 min at RT. Finally, cells were mounted in Vectashield (Vector Laboratories) and covered with a coverslip.

5.3.6 Immunoprecipitation

24 h after induction of protein expression transfected S2 cells were harvested and spinned down for 5 min at 1100 rpm. The pellet was washed once with 1 ml ice cold PBS and cells were lysed in 500 µl Lysisbuffer supplemented with Protease Inhibitor Cocktail for 45 min on ice. Cell lysates were centrifuged for 20 min at 13.000 rpm at 4°C and the protein containing supernatant transferred to a new Eppendorf tube. For SDS-PAGE, lysates were boiled in 1 x Laemmli buffer and loaded on a polyacrylamide gel.
For immunoprecipitation experiments, 100-500 µl of lysate were incubated with anti-FLAG or anti-HA antibodies for 2 h at 4°C on a shaker. In the meantime, Protein G sepharose beads (20 µl) were blocked in 5% BSA blocking solution at RT, washed two times with Lysisbuffer and added to the lysate samples for another 2 h incubation at 4°C.
Samples were centrifuged for 5 min at 2000 rpm at 4°C and washed three times with 300 µl Lysisbuffer. Finally, the supernatant was removed completely and beads were boiled in 60 µl 2 X Laemmli buffer for 5 min. Samples were fractionated by SDS-PAGE.
For Coomassie staining and subsequent Peptide Mass Fingerprinting (PMF) analysis, coimmunoprecipitations were performed using the FLAG-Immunoprecipitation Kit (Sigma) according to manufacturers' instructions.

5.3.7 SDS-Page, Western blot analysis and Coomassie staining

Protein samples were diluted in Laemmli buffer, boiled at 95°C and separated on 11% polyacrylamide gels (1 mm) using the Mini Protean 3 System (BioRad). Gels were run with constant amperage and a starting voltage of 90 V. For Western blot analysis, proteins were transferred onto Hybond nitrocellulose membranes (Amersham) using a dry blotting system (BioRad) for 30 min with constant amperage and an average voltage of 10 V. Membranes were stained for 5 min with Ponceau S to visualize protein bands and subsequently destained with tap water. After destaining was completed, membranes were blocked for 1 h in "Blocking Buffer for Fluorescent Western Blotting" (Rockland), washed

once with PBS and incubated with primary antibodies diluted in powdered milk (5% milk powder in PBS-0.2% Tween) for 2 h at RT or overnight at 4°C. After washing three times with PBS-0.2% Tween, membranes were incubated with secondary antibodies diluted in powdered milk (5% milk powder in PBS-0.2% Tween) for 1 h at RT. Following three wash steps with PBS-0.2% Tween, the membranes were dried in Whatmann paper and proteins were detected using the Odyssey Infrared Imaging System (Li-Cor Biosciences).
For Coomassie staining, protein gels were incubated in Colloidal Coomassie Stain solution with 20% methanol overnight at RT. On the next day, protein gels were destained with Coomassie Destaining solution for about 15-20 min and washed three times with water. Protein bands of interest were excised and analysed by Peptide Mass Fingerprinting (PMF) in the Bioanalytic facility (ZMMK) of the University of Cologne (http://www.zmmk-bioanalytik.de).

5.4 *Drosophila* techniques and qRT PCR

5.4.1 Maintainance of flies

Flies were maintained under standard conditions (Ashburner, 1989; Wieschaus and Nüsslein-Vollhard, 1986).

5.4.2 Collection of embryos

Wild-type flies were kept in laying cages fitted with apple juice agar plates and incubated at 25°C. After 4 h incubation at 25°C, embryo clutches on the plates were collected and incubated for another 4 h at 25°C. Embryos (4-8 h) were dechorionated in a 1:1 mixture of water and bleach for 1-2 min, poured through a sieve and washed several times with tap water. Finally, embryos were transferred into an Eppendorf tube containing 200 µl of RNA-Lysisbuffer and mRNA was isolated immediately using the µMACS mRNA Purification Kit (Miltenyi).

5.4.3 Dissection of salivary glands overexpressing HA-Rca1 or CycE

For conditional expression of HA-Rca1 and CycE in flies the "flp-out" technique was applied (Ito et al., 1997). Expression of the transgene throughout the salivary glands and the adjacent fatbody was induced 40-48 h after egg deposition by heat shocking the flies at 37°C for 30 min. 60 h after induction of protein expression, salivary glands were dissected from feeding larvae. mRNA from dissected salivary glands was isolated using the µMACS mRNA Purification Kit (Miltenyi).

5.4.4 mRNA isolation and cDNA synthesis

Dechorionised embryos or dissected salivary glands were lysed in 1 ml RNA-Lysisbuffer and pure mRNA was isolated directly from lysates by magnetic labeling with µMACS Oligo (dT) MicroBeads on a magnetic column. RNA preparation using the µMACS mRNA Purification Kit (Miltenyi) was performed according to the manufacturers instructions. After elution from the magnetic column, the mRNA was precipitated by adding 1/10 Vol lithiume chloride solution (4 M), 2.5 Vol ethanol (100%) and 1 µl glycogene followed by overnight incubation at -20°C. On the next day, the precipitate was centrifuged for 20 min at 13.000 rpm at 4°C and the pellet was washed for 5 min with 70% RNase-free ethanol. The air-dried RNA pellet was resuspended in 20 µl Rnase-free water and the RNA concentration was measured with a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies). For reverse transcription of mRNA into single-stranded cDNA the SuperSkript III First-Strand cDNA Synthesis Kit (Invitrogen) was used according to manufacturers' instructions. In a 20 µl synthesis reaction, about 400 ng mRNA was reverse transcribed (10 min 25°C, 50 min 50°C) using random hexamer primers. After synthesis completion, a cDNA aliquot was tested in a standard PCR using Tubulin primers and the remaining cDNA was aliquoted and stored at -20°C.

5.4.5 Quantitative real-time PCR

The mRNA was extracted from wild-type embryos (4-8 h AED), wild-type feeding larvae salivary glands or salivary glands in which HA-Rca1 or CycE was overexpressed. After wards the mRNA samples were reverse transcribed into single-stranded cDNA. cDNA was amplified using SYBR Green PCR Mix (Applied Biosystems/Qiagen) and QuantiTect Primer Assays (Qiagen). Quantitative real-time PCR was performed on an ABI 7900 Real-Time PCR System (Applied Biosystems) supported by SDS 2.1 software (Applied Biosystems). At the beginning, the optimal cDNA concentration had to be determined by generating standard curves of five different cDNA dilutions. Furthermore, the efficiency (90-100%) and specificity of each QuantiTect Primer Assay was verified by a melting curve analysis of each PCR product. Finally, 5 ng cDNA was used for a PCR reaction in a total volume of 10 µl. Real-time PCR was performed in two successive runs using 384-well plates: The actual amplification reaction was followed by a melting curve analysis to verify specificity and identity of the PCR. The following PCR programs and protocol have been used:

PCR program:

1.Run (Relative Quantification Assay):

95°C	5 min
95°C	15 s
60°C	1 min
40 cycles	

2. Run (Absolute Quantification Assay):

95°C	5 min
95°C	15 s
60°C	1 min
2 cycles	
95°C	15 s
60°C	15 s
95°C	15 s

PCR reaction:

5 µl SYBR Green PCR Mix (2 X)

2 µl cDNA (2.5 ng/µl)

1 µl QuantiTect Primer Assay (10 X)

adjust with Rnase-free water to a final volume of 10 µl

ß-actin or glyceraldehyde-3-phosphatase1 (Gapdh1) were used as endogenous controls, because these reference genes show constant, relatively stable expression levels. Each PCR reaction was done in triplicates to ensure statistical significance and included a no template controls (NTC) to detect DNA contamination. All quantification experiments were repeated 3-4 times and each plate included the endogenous control and no template controls for each PCR reaction. SDS software was used to combine and analyse raw data from at least three independent runs in a relative quantification study. Automatic analysis settings for threshold cycle values (CT) and baseline were selected. Wild-type embryos or wild-type salivary glands were used as calibrator, which is the basis for comparative results. CT values were compared and standard deviations (SD) determined for each sample. Outliers (SD $\geq$ 0.3) were identified and ommitted from the study as well as PCR reactions showing DNA contamination (CT (NTC) $\leq$ 8 CT difference to CT (sample)) or more than one clear peak in the melting curve.

Relative fold differences in the expression of candidate genes (gene of interest) to an adequate reference gene (endogenous control) can be determined using the "delta-delta CT" method ($\Delta\Delta$CT method). Therefore, the relative expression quantity (RQ) can be calculated with the following model:

$$\Delta Ct = (Ct_{\text{gene of interest}} - Ct_{\text{endogenous control}})$$

$$\Delta\Delta CT\ (\Delta Ct_{\text{sample}} - \Delta Ct_{\text{calibrator}})$$

$$RQ = 2^{-\Delta\Delta CT}$$

Finally, RQ values for each gene were shown as log10 (RQ) in a gene expression diagram in relation to the calibrator gene (log10 (RQ) = 0).

5.5 Yeast two-hybrid screen

In order to find novel interaction partners of Rca1, a yeast two-hybrid screen was performed by the Hybrigenics Y2H Screening Service (Paris, France). Therefore, the complete coding region of Rca1 was amplified in a standard PCR reaction and cloned into the vector pB27. The pB27 (LexA, C-terminal fusion) vector was provided by the company and contains the DNA binding domain for bait vector cloning. Bait cloning was performed according to manufacturers instructions using the unique restriction sites SpeI/PacI which gave rise to an in frame fusion to the Gal4-DNA binding. Thereby, the Rca1 coding region was fused to the C-terminus of the Gal4-DNA binding domain of pB27 in two cloning steps using gene specific primers with unique restriction sites: Detailed primer sequences are listed in Table 5.1. First, the Rca1 PCR fragment was cloned with *BamHI/XbaI* into pBluescript II SK(-) followed by subcloning of the Rca1 coding region into pB27. The resulting bait vector SQ30 was verified by sequencing and sent to the Hybrigenics Y2H Screening Service. The screen was performed using a prey library of *Drosophila* whole embryo (0-12 + 12-24).

6. References

Ang, X.L. and Wade Harper, J. 2005. SCF-mediated protein degradation and cell cycle control. *Oncogene* **24**(17): 2860-2870.

Attwooll, C., Lazzerini Denchi, E., and Helin, K. 2004. The E2F family: specific functions and overlapping interests. *Embo J* **23**(24): 4709-4716.

Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J.W., and Elledge, S.J. 1996. SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. *Cell* **86**(2): 263-274.

Beach, D., Durkacz, B., and Nurse, P. 1982. Functionally homologous cell cycle control genes in budding and fission yeast. *Nature* **300**(5894): 706-709.

Bell, S.P. and Dutta, A. 2002. DNA replication in eukaryotic cells. *Annu Rev Biochem* **71**: 333-374.

Bernis, C., S. Vigneron, A. Burgess, J. C. Labbe, D. Fesquet, A. Castro and T. Lorca (2007). "Pin1 stabilizes Emi1 during G2 phase by preventing its association with SCF(betatrcp)." *EMBO Rep* **8**(1): 91-8.

Blais, A. and Dynlacht, B.D. 2004. Hitting their targets: an emerging picture of E2F and cell cycle control. *Curr Opin Genet Dev* **14**(5): 527-532.

Blow, J.J. and Dutta, A. 2005. Preventing re-replication of chromosomal DNA. *Nat Rev Mol Cell Biol* **6**(6): 476-486.

Bocca, S.N., Muzzopappa, M., Silberstein, S., and Wappner, P. 2001. Occurrence of a putative SCF ubiquitin ligase complex in Drosophila. *Biochem Biophys Res Commun* **286**(2): 357-364.

Bunch, T. A., Y. Grinblat and L. S. Goldstein (1988). "Characterization and use of the Drosophila metallothionein promoter in cultured Drosophila melanogaster cells." *Nucleic Acids Res* **16**(3): 1043-61.

Burton, J.L. and Solomon, M.J. 2001. D box and KEN box motifs in budding yeast Hsl1p are required for APC-mediated degradation and direct binding to Cdc20p and Cdh1p. *Genes Dev* **15**(18): 2381-2395.

Calvi, B.R., Lilly, M.A., and Spradling, A.C. 1998. Cell cycle control of chorion gene amplification. *Genes Dev* **12**(5): 734-744.

Campbell, S.D., Sprenger, F., Edgar, B.A., and O'Farrell, P.H. 1995. Drosophila Wee1 kinase rescues fission yeast from mitotic catastrophe and phosphorylates Drosophila Cdc2 in vitro. *Mol Biol Cell* **6**(10): 1333-1347.

Cardozo, T. and Pagano, M. 2004. The SCF ubiquitin ligase: insights into a molecular machine. *Nat Rev Mol Cell Biol* **5**(9): 739-751.

Carroll, C.W., Enquist-Newman, M., and Morgan, D.O. 2005. The APC subunit Doc1 promotes recognition of the substrate destruction box. *Curr Biol* **15**(1): 11-18.

Chen, F., Archambault, V., Kar, A., Lio, P., D'Avino, P.P., Sinka, R., Lilley, K., Laue, E.D., Deak, P., Capalbo, L., and Glover, D.M. 2007. Multiple protein phosphatases are required for mitosis in Drosophila. *Curr Biol* **17**(4): 293-303.

Crevel, G., E. Mathe and S. Cotterill (2005). "The Drosophila Cdc6/18 protein has functions in both early and late S phase in S2 cells." *J Cell Sci* **118**(Pt 11): 2451-9.

Datar, S.A., Jacobs, H.W., de la Cruz, A.F., Lehner, C.F., and Edgar, B.A. 2000. The Drosophila Cyclin D-Cdk4 complex promotes cellular growth. *EMBO J* **19**(17): 4543-4554.

Dawson, I., Roth, S., Akam, M., and Artavanis-Tsakonas, S. 1993. Mutations of the fizzy locus cause metaphase arrest in Drosophila melanogaster embryos. *Development* **117**(1): 359-376.

Dawson, I.A., Roth, S., and Artavanis-Tsakonas, S. 1995. The Drosophila cell cycle gene fizzy is required for normal degradation of cyclins A and B during mitosis and has homology to the CDC20 gene of Saccharomyces cerevisiae. *J Cell Biol* **129**(3): 725-737.

de Nooij, J.C., Graber, K.H., and Hariharan, I.K. 2000. Expression of the cyclin-dependent kinase inhibitor Dacapo is regulated by cyclin E. *Mech Dev* **97**(1-2): 73-83.

de Nooij, J.C., Letendre, M.A., and Hariharan, I.K. 1996. A cyclin-dependent kinase inhibitor, Dacapo, is necessary for timely exit from the cell cycle during Drosophila embryogenesis. *Cell* **87**(7): 1237-1247.

DeGregori, J., Kowalik, T., and Nevins, J.R. 1995. Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes. *Mol Cell Biol* **15**(8): 4215-4224.

Dej, K.J. and Spradling, A.C. 1999. The endocycle controls nurse cell polytene chromosome structure during Drosophila oogenesis. *Development* **126**(2): 293-303.

Deshaies, R.J. 1999. SCF and Cullin/Ring H2-based ubiquitin ligases. *Annu Rev Cell Dev Biol* **15**: 435-467.

Di Fiore, B. and Pines, J. 2007. Emi1 is needed to couple DNA replication with mitosis but does not regulate activation of the mitotic APC/C. *J Cell Biol* **177**(3): 425-437.

Dienemann, A. and Sprenger, F. 2004. Requirements of cyclin a for mitosis are independent of its subcellular localization. *Curr Biol* **14**(12): 1117-1123.

Dimova, D.K., Stevaux, O., Frolov, M.V., and Dyson, N.J. 2003. Cell cycle-dependent and cell cycle-independent control of transcription by the Drosophila E2F/RB pathway. *Genes Dev* **17**(18): 2308-2320.

Donaldson, T.D., Noureddine, M.A., Reynolds, P.J., Bradford, W., and Duronio, R.J. 2004. Targeted disruption of Drosophila Roc1b reveals functional differences in the Roc subunit of Cullin-dependent E3 ubiquitin ligases. *Mol Biol Cell* **15**(11): 4892-4903.

Dong, X., Zavitz, K.H., Thomas, B.J., Lin, M., Campbell, S., and Zipursky, S.L. 1997. Control of G1 in the developing Drosophila eye: rca1 regulates Cyclin A. *Genes Dev* **11**(1): 94-105.

Du, W. and Dyson, N. 1999. The role of RBF in the introduction of G1 regulation during Drosophila embryogenesis. *Embo J* **18**(4): 916-925.

Du, W., Vidal, M., Xie, J.E., and Dyson, N. 1996. RBF, a novel RB-related gene that regulates E2F activity and interacts with cyclin E in Drosophila. *Genes Dev* **10**(10): 1206-1218.

Duronio, R.J. and O'Farrell, P.H. 1994. Developmental control of a G1-S transcriptional program in Drosophila. *Development* **120**(6): 1503-1515.

Duronio, R.J. and O'Farrell, P.H. 1995. Developmental control of the G1 to S transition in Drosophila: cyclin Eis a limiting downstream target of E2F. *Genes Dev* **9**(12): 1456-1468.

Duronio, R.J., O'Farrell, P.H., Xie, J.E., Brook, A., and Dyson, N. 1995. The transcription factor E2F is required for S phase during Drosophila embryogenesis. *Genes Dev* **9**(12): 1445-1455.

Edgar, B.A., Sprenger, F., Duronio, R., Leopold, P., and O'Farrell, P. 1994a. Distinct molecular mechanism regulate cell cycle timing at successive stages of Drosophila embryogenesis. *Genes Dev* **8**(4): 440-452.

Edgar, B.A. and Datar, S.A. 1996. Zygotic degradation of two maternal Cdc25 mRNAs terminates Drosophila's early cell cycle program. *Genes Dev* **10**(15): 1966-1977.

Edgar, B.A., Lehman, D.A., and O'Farrell, P.H. 1994b. Transcriptional regulation of string (cdc25): a link between developmental programming and the cell cycle. *Development* **120**(11): 3131-3143.

Edgar, B.A. and Lehner, C.F. 1996. Developmental Control of Cell Cycle Regulators: A Fly's Perspective. *Science* **274**(5293): 1646-1652.

Edgar, B.A. and Nijhout, H.F. 2004. Growth and Cell Cycle Control in *Drosophila*. in *Cell Growth - Control of Cell Size* (ed. M.N. Hall, M. Raff, and G. Thomas), pp. 23-83. CSHL press.

Edgar, B.A. and O'Farrell, P.H. 1989. Genetic control of cell division patterns in the Drosophila embryo. *Cell* **57**(1): 177-187.

Edgar, B.A. and O'Farrell, P.H. 1990. The three postblastoderm cell cycles of Drosophila embryogenesis are regulated in G2 by string. *Cell* **62**(3): 469-480.

Edgar, B.A. and Orr-Weaver, T.L. 2001. Endoreplication cell cycles: more for less. *Cell* **105**(3): 297-306.

Eldridge, A.G., Loktev, A.V., Hansen, D.V., Verschuren, E.W., Reimann, J.D., and Jackson, P.K. 2006. The evi5 oncogene regulates cyclin accumulation by stabilizing the anaphase-promoting complex inhibitor emi1. *Cell* **124**(2): 367-380.

Fletcher, R.J., Bishop, B.E., Leon, R.P., Sclafani, R.A., Ogata, C.M., and Chen, X.S. 2003. The structure and function of MCM from archaeal M. Thermoautotrophicum. *Nat Struct Biol* **10**(3): 160-167.

Foe, V. 1989. Mitotic domains reveal early commitment of cells in Drosophila embryos. *Development* **107**(1): 1-22.

Foley, E., O'Farrell, P.H., and Sprenger, F. 1999. Rux is a cyclin-dependent kinase inhibitor (CKI) specific for mitotic cyclin-Cdk complexes. *Curr Biol* **9**(23): 1392-1402.

Foley, E. and Sprenger, F. 2001. The cyclin-dependent kinase inhibitor Roughex is involved in mitotic exit in Drosophila. *Curr Biol* **11**(3): 151-160.

Follette, P.J., Duronio, R.J., and O'Farrell, P.H. 1998. Fluctuations in cyclin E levels are required for multiple rounds of endocycle S phase in Drosophila. *Curr Biol* **8**(4): 235-238.

Frolov, M.V., Huen, D.S., Stevaux, O., Dimova, D., Balczarek-Strang, K., Elsdon, M., and Dyson, N.J. 2001. Functional antagonism between E2F family members. *Genes Dev* **15**(16): 2146-2160.

Giot, L., Bader, J.S., Brouwer, C., Chaudhuri, A., Kuang, B., Li, Y., Hao, Y.L., Ooi, C.E., Godwin, B., Vitols, E., Vijayadamodar, G., Pochart, P., Machineni, H., Welsh, M., Kong, Y., Zerhusen, B., Malcolm, R., Varrone, Z., Collis, A., Minto, M., Burgess, S., McDaniel, L., Stimpson, E., Spriggs, F., Williams, J., Neurath, K., Ioime, N., Agee, M., Voss, E., Furtak, K., Renzulli, R., Aanensen, N., Carrolla, S., Bickelhaupt, E., Lazovatsky, Y., DaSilva, A., Zhong, J., Stanyon, C.A., Finley, R.L., Jr., White, K.P., Braverman, M., Jarvie, T., Gold, S., Leach, M., Knight, J., Shimkets, R.A., McKenna, M.P., Chant, J., and Rothberg, J.M. 2003. A protein interaction map of Drosophila melanogaster. *Science* **302**(5651): 1727-1736.

Glotzer, M., Murray, A.W., and Kirschner, M.W. 1991. Cyclin is degraded by the ubiquitin pathway. *Nature* **349**(6305): 132-138.

Grima, B., Lamouroux, A., Chelot, E., Papin, C., Limbourg-Bouchon, B., and Rouyer, F. 2002. The F-box protein slimb controls the levels of clock proteins period and timeless. *Nature* **420**(6912): 178-182.

Grosskortenhaus, R. and Sprenger, F. 2002. Rca1 inhibits APC-Cdh1(Fzr) and is required to prevent cyclin degradation in G2. *Dev Cell* **2**(1): 29-40.

Guardavaccaro, D., Kudo, Y., Boulaire, J., Barchi, M., Busino, L., Donzelli, M., Margottin-Goguet, F., Jackson, P.K., Yamasaki, L., and Pagano, M. 2003. Control of meiotic and mitotic progression by the F box protein beta-Trcp1 in vivo. *Dev Cell* **4**(6): 799-812.

Hagting, A., Den Elzen, N., Vodermaier, H.C., Waizenegger, I.C., Peters, J.M., and Pines, J. 2002. Human securin proteolysis is controlled by the spindle checkpoint and reveals when the APC/C switches from activation by Cdc20 to Cdh1. *J Cell Biol* **157**(7): 1125-1137.

Hansen, D.V., Loktev, A.V., Ban, K.H., and Jackson, P.K. 2004. Plk1 Regulates Activation of the Anaphase Promoting Complex by Phosphorylating and Triggering SCF{beta}TrCP-dependent Destruction of the APC Inhibitor Emi1. *Mol Biol Cell* **15**(12): 5623-5634.

Harper, J.W., Burton, J.L., and Solomon, M.J. 2002. The anaphase-promoting complex: it's not just for mitosis any more. *Genes Dev* **16**(17): 2179-2206.

Hermand, D. 2006. F-box proteins: more than baits for the SCF? *Cell Div* **1**: 30.

Hochstrasser, M. 1996. Ubiquitin-dependent protein degradation. *Annu Rev Genet* **30**: 405-439.

Hong, A., Narbonne-Reveau, K., Riesgo-Escovar, J., Fu, H., Aladjem, M.I., and Lilly, M.A. 2007. The cyclin-dependent kinase inhibitor Dacapo promotes replication licensing during Drosophila endocycles. *Embo J* **26**(8): 2071-2082.

Hsu, J.Y., Reimann, J.D., Sorensen, C.S., Lukas, J., and Jackson, P.K. 2002. E2F-dependent accumulation of hEmi1 regulates S phase entry by inhibiting APC(Cdh1). *Nat Cell Biol* **4**(5): 358-366.

Jacobs, H., Richter, D., Venkatesh, T., and Lehner, C. 2002. Completion of Mitosis Requires Neither fzr/rap nor fzr2, a Male Germline-Specific Drosophila Cdh1 Homolog. *Curr Biol* **12**(16): 1435.

Jacobs, H.W., Knoblich, J.A., and Lehner, C.F. 1998. Drosophila Cyclin B3 is required for female fertility and is dispensable for mitosis like Cyclin B. *Genes Dev* **12**(23): 3741-3751.

Jiang, J. and Struhl, G. 1998. Regulation of the Hedgehog and Wingless signalling pathways by the F-box/WD40-repeat protein Slimb. *Nature* **391**(6666): 493-496.

Kashevsky, H., Wallace, J.A., Reed, B.H., Lai, C., Hayashi-Hagihara, A., and Orr-Weaver, T.L. 2002. The anaphase promoting complex/cyclosome is required during development for modified cell cycles. *Proc Natl Acad Sci U S A* **99**(17): 11217-11222.

Kato, J., Matsushime, H., Hiebert, S.W., Ewen, M.E., and Sherr, C.J. 1993. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. *Genes Dev* **7**(3): 331-342.

King, R.W., Glotzer, M., and Kirschner, M.W. 1996. Mutagenic analysis of the destruction signal of mitotic cyclins and structural characterization of ubiquitinated intermediates. *Mol Biol Cell* **7**(9): 1343-1357.

Kipreos, E.T. and Pagano, M. 2000. The F-box protein family. *Genome Biol* **1**(5): REVIEWS3002.

Klebes, A., Biehs, B., Cifuentes, F., and Kornberg, T.B. 2002. Expression profiling of Drosophila imaginal discs. *Genome Biol* **3**(8): RESEARCH0038.

Knoblich, J.A. and Lehner, C.F. 1993. Synergistic action of Drosophila cyclins A and B during the G2-M transition. *Embo J* **12**(1): 65-74.

Knoblich, J.A., Sauer, K., Jones, L., Richardson, H., Saint, R., and Lehner, C.F. 1994. Cyclin E controls S phase progression and its down-regulation during Drosophila embryogenesis is required for the arrest of cell proliferation. *Cell* **77**(1): 107-120.

Koepp, D.M., Harper, J.W., and Elledge, S.J. 1999. How the cyclin became a cyclin: regulated proteolysis in the cell cycle. *Cell* **97**(4): 431-434.

Koepp, D.M., Schaefer, L.K., Ye, X., Keyomarsi, K., Chu, C., Harper, J.W., and Elledge, S.J. 2001. Phosphorylation-dependent ubiquitination of cyclin E by the SCFFbw7 ubiquitin ligase. *Science* **294**(5540): 173-177.

Kraft, C., Vodermaier, H.C., Maurer-Stroh, S., Eisenhaber, F., and Peters, J.M. 2005. The WD40 propeller domain of Cdh1 functions as a destruction box receptor for APC/C substrates. *Mol Cell* **18**(5): 543-553.

Kramer, E.R., Scheuringer, N., Podtelejnikov, A.V., Mann, M., and Peters, J.M. 2000. Mitotic regulation of the APC activator proteins CDC20 and CDH1. *Mol Biol Cell* **11**(5): 1555-1569.

Lane, M.E., Elend, M., Heidmann, D., Herr, A., Marzodko, S., Herzig, A., and Lehner, C.F. 2000. A screen for modifiers of cyclin E function in Drosophila melanogaster identifies Cdk2 mutations, revealing the insignificance of putative phosphorylation sites in Cdk2. *Genetics* **155**(1): 233-244.

Lane, M.E., Sauer, K., Wallace, K., Jan, Y.N., Lehner, C.F., and Vaessin, H. 1996. Dacapo, a cyclin-dependent kinase inhibitor, stops cell proliferation during Drosophila development. *Cell* **87**(7): 1225-1235.

Lee, C., Hong, B., Choi, J.M., Kim, Y., Watanabe, S., Ishimi, Y., Enomoto, T., Tada, S., Kim, Y., and Cho, Y. 2004. Structural basis for inhibition of the replication licensing factor Cdt1 by geminin. *Nature* **430**(7002): 913-917.

Lee, H., Lee, D.J., Oh, S.P., Park, H.D., Nam, H.H., Kim, J.M., and Lim, D.S. 2006. Mouse emi1 has an essential function in mitotic progression during early embryogenesis. *Mol Cell Biol* **26**(14): 5373-5381.

Lee, L.A. and Orr-Weaver, T.L. 2003. Regulation of cell cycles in Drosophila development: intrinsic and extrinsic cues. *Annu Rev Genet* **37**: 545-578.

Lehner, C.F. and O'Farrell, P.H. 1989. Expression and function of Drosophila cyclin A during embryonic cell cycle progression. *Cell* **56**(6): 957-968.

Lehner, C.F. and O'Farrell, P.H. 1990a. Drosophila cdc2 homologs: a functional homolog is coexpressed with a cognate variant. *Embo J* **9**(11): 3573-3581.

Lehner, C.F. and O'Farrell, P.H. 1990b. The roles of Drosophila cyclins A and B in mitotic control. *Cell* **61**(3): 535-547.

Leismann, O., Herzig, A., Heidmann, S., and Lehner, C.F. 2000. Degradation of Drosophila PIM regulates sister chromatid separation during mitosis. *Genes Dev* **14**(17): 2192-2205.

Li, X., Zhao, Q., Liao, R., Sun, P., and Wu, X. 2003. The SCF(Skp2) ubiquitin ligase complex interacts with the human replication licensing factor Cdt1 and regulates Cdt1 degradation. *J Biol Chem* **278**(33): 30854-30858.

Lilly, M.A. and Duronio, R.J. 2005. New insights into cell cycle control from the Drosophila endocycle. *Oncogene* **24**(17): 2765-2775.

Lilly, M.A. and Spradling, A.C. 1996. The Drosophila endocycle is controlled by Cyclin E and lacks a checkpoint ensuring S-phase completion. *Genes Dev* **10**(19): 2514-2526.

Liu, E., Li, X., Yan, F., Zhao, Q., and Wu, X. 2004. Cyclin-dependent kinases phosphorylate human Cdt1 and induce its degradation. *J Biol Chem* **279**(17): 17283-17288.

Machida, Y.J. and Dutta, A. 2007. The APC/C inhibitor, Emi1, is essential for prevention of rereplication. *Genes Dev* **21**(2): 184-194.

Machida, Y.J., Hamlin, J.L., and Dutta, A. 2005. Right place, right time, and only once: replication initiation in metazoans. *Cell* **123**(1): 13-24.

Maniatis, T. 1999. A ubiquitin ligase complex essential for the NF-kappaB, Wnt/Wingless, and Hedgehog signaling pathways. *Genes Dev* **13**(5): 505-510.

Margottin-Goguet, F., Hsu, J.Y., Loktev, A., Hsieh, H.M., Reimann, J.D., and Jackson, P.K. 2003. Prophase destruction of Emi1 by the SCF(betaTrCP/Slimb) ubiquitin ligase activates the anaphase promoting complex to allow progression beyond prometaphase. *Dev Cell* **4**(6): 813-826.

Matsushime, H., Quelle, D.E., Shurtleff, S.A., Shibuya, M., Sherr, C.J., and Kato, J.Y. 1994. D-type cyclin-dependent kinase activity in mammalian cells. *Mol Cell Biol* **14**(3): 2066-2076.

May, N.R., Thomer, M., Murnen, K.F., and Calvi, B.R. 2005. Levels of the origin-binding protein Double parked and its inhibitor Geminin increase in response to replication stress. *J Cell Sci* **118**(Pt 18): 4207-4217.

McGarry, T.J. and Kirschner, M.W. 1998. Geminin, an inhibitor of DNA replication, is degraded during mitosis. *Cell* **93**(6): 1043-1053.

Melixetian, M., Ballabeni, A., Masiero, L., Gasparini, P., Zamponi, R., Bartek, J., Lukas, J., and Helin, K. 2004. Loss of Geminin induces rereplication in the presence of functional p53. *J Cell Biol* **165**(4): 473-482.

Merrill, P.T., Sweeton, D., and Wieschaus, E. 1988. Requirements for autosomal gene activity during precellular stages of Drosophila melanogaster. *Development* **104**(3): 495-509.

Meyer, C.A., Jacobs, H.W., Datar, S.A., Du, W., Edgar, B.A., and Lehner, C.F. 2000. Drosophila Cdk4 is required for normal growth and is dispensable for cell cycle progression. *Embo J* **19**(17): 4533-4542.

Micchelli, C.A. and Perrimon, N. 2006. Evidence that stem cells reside in the adult Drosophila midgut epithelium. *Nature* **439**(7075): 475-479.

Mihaylov, I.S., Kondo, T., Jones, L., Ryzhikov, S., Tanaka, J., Zheng, J., Higa, L.A., Minamino, N., Cooley, L., and Zhang, H. 2002. Control of DNA replication and chromosome ploidy by geminin and cyclin A. *Mol Cell Biol* **22**(6): 1868-1880.

Miller, J.J., Summers, M.K., Hansen, D.V., Nachury, M.V., Lehman, N.L., Loktev, A., and Jackson, P.K. 2006. Emi1 stably binds and inhibits the anaphase-promoting complex/cyclosome as a pseudosubstrate inhibitor. *Genes Dev* **20**(17): 2410-2420.

Moberg, K.H., Bell, D.W., Wahrer, D.C., Haber, D.A., and Hariharan, I.K. 2001. Archipelago regulates Cyclin E levels in Drosophila and is mutated in human cancer cell lines. *Nature* **413**(6853): 311-316.

Moberg, K.H., Mukherjee, A., Veraksa, A., Artavanis-Tsakonas, S., and Hariharan, I.K. 2004. The Drosophila F box protein archipelago regulates dMyc protein levels in vivo. *Curr Biol* **14**(11): 965-974.

Morgan, D.O. 1997. Cyclin-dependent kinases: engines, clocks, and microprocessors. *Annu Rev Cell Dev Biol* **13**: 261-291.

Morgan, D.O. 2006. *The Cell Cycle - Principles of Control*. New Science Press Ltd.

Moshe, Y., Boulaire, J., Pagano, M., and Hershko, A. 2004. Role of Polo-like kinase in the degradation of early mitotic inhibitor 1, a regulator of the anaphase promoting complex/cyclosome. *Proc Natl Acad Sci U S A* **101**(21): 7937-7942.

Murphy, T.D. 2003. Drosophila skpA, a component of SCF ubiquitin ligases, regulates centrosome duplication independently of cyclin E accumulation. *J Cell Sci* **116**(Pt 11): 2321-2332.

Murray, A.W. 2004. Recycling the cell cycle: cyclins revisited. *Cell* **116**(2): 221-234.

Murray, A.W. and Kirschner, M.W. 1989. Cyclin synthesis drives the early embryonic cell cycle. *Nature* **339**(6222): 275-280.

Nakayama, K.I. and Nakayama, K. 2006. Ubiquitin ligases: cell-cycle control and cancer. *Nat Rev Cancer* **6**(5): 369-381.

Nasmyth, K. 2001. Disseminating the genome: joining, resolving, and separating sister chromatids during mitosis and meiosis. *Annu Rev Genet* **35**: 673-745.

Nateri, A.S., Riera-Sans, L., Da Costa, C., and Behrens, A. 2004. The ubiquitin ligase SCFFbw7 antagonizes apoptotic JNK signaling. *Science* **303**(5662): 1374-1378.

Nayak, S., Santiago, F.E., Jin, H., Lin, D., Schedl, T., and Kipreos, E.T. 2002. The Caenorhabditis elegans Skp1-related gene family: diverse functions in cell proliferation, morphogenesis, and meiosis. *Curr Biol* **12**(4): 277-287.

Nishitani, H., Taraviras, S., Lygerou, Z., and Nishimoto, T. 2001. The human licensing factor for DNA replication Cdt1 accumulates in G1 and is destabilized after initiation of S-phase. *J Biol Chem* **276**(48): 44905-44911.

Noureddine, M.A., Donaldson, T.D., Thacker, S.A., and Duronio, R.J. 2002. Drosophila Roc1a encodes a RING-H2 protein with a unique function in processing the Hh signal transducer Ci by the SCF E3 ubiquitin ligase. *Dev Cell* **2**(6): 757-770.

Ohlstein, B. and Spradling, A. 2006. The adult Drosophila posterior midgut is maintained by pluripotent stem cells. *Nature* **439**(7075): 470-474.

Pagano, M., Draetta, G., and Jansen-Durr, P. 1992. Association of cdk2 kinase with the transcription factor E2F during S phase. *Science* **255**(5048): 1144-1147.

Pape, T., Meka, H., Chen, S., Vicentini, G., van Heel, M., and Onesti, S. 2003. Hexameric ring structure of the full-length archaeal MCM protein complex. *EMBO Rep* **4**(11): 1079-1083.

Parry, D.H. and O'Farrell, P.H. 2001. The schedule of destruction of three mitotic cyclins can dictate the timing of events during exit from mitosis. *Curr Biol* **11**(9): 671-683.

Passmore, L.A., Booth, C.R., Venien-Bryan, C., Ludtke, S.J., Fioretto, C., Johnson, L.N., Chiu, W., and Barford, D. 2005. Structural analysis of the anaphase-promoting complex reveals multiple active sites and insights into polyubiquitylation. *Mol Cell* **20**(6): 855-866.

Perkins, G., Drury, L.S., and Diffley, J.F. 2001. Separate SCF(CDC4) recognition elements target Cdc6 for proteolysis in S phase and mitosis. *Embo J* **20**(17): 4836-4845.

Peter, M. and Herskowitz, I. 1994. Joining the complex: cyclin-dependent kinase inhibitory proteins and the cell cycle. *Cell* **79**(2): 181-184.

Peters, J.M. 2006. The anaphase promoting complex/cyclosome: a machine designed to destroy. *Nat Rev Mol Cell Biol* **7**(9): 644-656.

Petroski, M.D. and Deshaies, R.J. 2005. Function and regulation of cullin-RING ubiquitin ligases. *Nat Rev Mol Cell Biol* **6**(1): 9-20.

Pfleger, C.M. and Kirschner, M.W. 2000. The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1. *Genes Dev* **14**(6): 655-665.

Pfleger, C.M., Salic, A., Lee, E., and Kirschner, M.W. 2001. Inhibition of Cdh1-APC by the MAD2-related protein MAD2L2: a novel mechanism for regulating Cdh1. *Genes Dev* **15**(14): 1759-1764.

Pimentel, A.C. and Venkatesh, T.R. 2005. rap gene encodes Fizzy-related protein (Fzr) and regulates cell proliferation and pattern formation in the developing Drosophila eye-antennal disc. *Dev Biol* **285**(2): 436-446.

Pines, J. 2006. Mitosis: a matter of getting rid of the right protein at the right time. *Trends Cell Biol* **16**(1): 55-63.

Quinn, L.M., Herr, A., McGarry, T.J., and Richardson, H. 2001. The Drosophila Geminin homolog: roles for Geminin in limiting DNA replication, in anaphase and in neurogenesis. *Genes Dev* **15**(20): 2741-2754.

Radermacher, P. 2007. Untersuchung zur Lokalisierung und Stabilität des Zellzyklus-Proteins Rca1 in *Drosophila melanogaster*. In. Universität zu Köln.

Raj, L., Vivekanand, P., Das, T.K., Badam, E., Fernandes, M., Finley, R.L., Brent, R., Appel, L.F., Hanes, S.D., and Weir, M. 2000. Targeted localized degradation of Paired protein in Drosophila development. *Curr Biol* **10**(20): 1265-1272.

Ramachandran, V., Matzkies, M., Dienemann, A., and Sprenger, F. 2007. Cyclin A degradation employs preferentially used lysines and a cyclin box function other than Cdk1 binding. *Cell Cycle* **6**(2): 171-181.

Rauh, N.R., Schmidt, A., Bormann, J., Nigg, E.A., and Mayer, T.U. 2005. Calcium triggers exit from meiosis II by targeting the APC/C inhibitor XErp1 for degradation. *Nature* **437**(7061): 1048-1052.

Reber, A., Lehner, C.F., and Jacobs, H.W. 2006. Terminal mitoses require negative regulation of Fzr/Cdh1 by Cyclin A, preventing premature degradation of mitotic cyclins and String/Cdc25. *Development* **133**(16): 3201-3211.

Reed, B.H. and Orr-Weaver, T.L. 1997. The Drosophila gene morula inhibits mitotic functions in the endo cell cycle and the mitotic cell cycle. *Development* **124**(18): 3543-3553.

Reimann, J.D., Freed, E., Hsu, J.Y., Kramer, E.R., Peters, J.M., and Jackson, P.K. 2001a. Emi1 is a mitotic regulator that interacts with Cdc20 and inhibits the anaphase promoting complex. *Cell* **105**(5): 645-655.

Reimann, J.D., Gardner, B.E., Margottin-Goguet, F., and Jackson, P.K. 2001b. Emi1 regulates the anaphase-promoting complex by a different mechanism than Mad2 proteins. *Genes Dev* **15**(24): 3278-3285.

Richardson, H., O'Keefe, L.V., Marty, T., and Saint, R. 1995. Ectopic cyclin E expression induces premature entry into S phase and disrupts pattern formation in the Drosophila eye imaginal disc. *Development* **121**(10): 3371-3379.

Royzman, I., Whittaker, A.J., and Orr-Weaver, T.L. 1997. Mutations in Drosophila DP and E2F distinguish G1-S progression from an associated transcriptional program. *Genes Dev* **11**(15): 1999-2011.

Russell, P. and Nurse, P. 1986. cdc25+ functions as an inducer in the mitotic control of fission yeast. *Cell* **45**(1): 145-153.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular cloning: a laboratory manual*. Cold Spring Harbour Laboratory Press.

Sauer, K., Knoblich, J.A., Richardson, H., and Lehner, C.F. 1995. Distinct modes of cyclin E/cdc2c kinase regulation and S-phase control in mitotic and endoreduplication cycles of Drosophila embryogenesis. *Genes Dev* **9**(11): 1327-1339.

Saxena, S. and Dutta, A. 2005. Geminin-Cdt1 balance is critical for genetic stability. *Mutat Res.* **6** (1):111-21.

Schaeffer, V., Althauser, C., Shcherbata, H.R., Deng, W.M., and Ruohola-Baker, H. 2004. Notch-dependent Fizzy-related/Hec1/Cdh1 expression is required for the mitotic-to-endocycle transition in Drosophila follicle cells. *Curr Biol* **14**(7): 630-636.

Schmidt, A., Duncan, P.I., Rauh, N.R., Sauer, G., Fry, A.M., Nigg, E.A., and Mayer, T.U. 2005. Xenopus polo-like kinase Plx1 regulates XErp1, a novel inhibitor of APC/C activity. *Genes Dev* **19**(4): 502-513.

Schmidt, A., Rauh, N.R., Nigg, E.A., and Mayer, T.U. 2006. Cytostatic factor: an activity that puts the cell cycle on hold. *J Cell Sci* **119**(Pt 7): 1213-1218.

Schulman, B.A., Carrano, A.C., Jeffrey, P.D., Bowen, Z., Kinnucan, E.R., Finnin, M.S., Elledge, S.J., Harper, J.W., Pagano, M., and Pavletich, N.P. 2000. Insights into SCF ubiquitin ligases from the structure of the Skp1-Skp2 complex. *Nature* **408**(6810): 381-386.

Schwab, M. and Tyers, M. 2001. Cell cycle. Archipelago of destruction. *Nature* **413**(6853): 268-269.

Shcherbata, H.R., Althauser, C., Findley, S.D., and Ruohola-Baker, H. 2004. The mitotic-to-endocycle switch in Drosophila follicle cells is executed by Notch-dependent regulation of G1/S, G2/M and M/G1 cell-cycle transitions. *Development* **131**(13): 3169-3181.

Sherr, C.J. 1993. Mammalian G1 cyclins. *Cell* **73**(6): 1059-1065.

Sherr, C.J. and Roberts, J.M. 1999. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* **13**(12): 1501-1512.

Sigrist, S., Jacobs, H., Stratmann, R., and Lehner, C. 1995. Exit from mitosis is regulated by Drosophila fizzy and the sequential destruction of cyclins A, B and B3. *EMBO J* **14**(19): 4827-4838.

Sigrist, S.J. and Lehner, C.F. 1997. Drosophila fizzy-related down-regulates mitotic cyclins and is required for cell proliferation arrest and entry into endocycles. *Cell* **90**(4): 671-681.

Sivaprasad, U., Machida, Y.J., and Dutta, A. 2007. APC/C - the master controller of origin licensing? *Cell Div* **2**: 8.

Skowyra, D., Craig, K.L., Tyers, M., Elledge, S.J., and Harper, J.W. 1997. F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. *Cell* **91**(2): 209-219.

Smith, A.V. and Orr-Weaver, T.L. 1991. The regulation of the cell cycle during Drosophila embryogenesis: the transition to polyteny. *Development* **112**(4): 997-1008.

Spencer, E., Jiang, J., and Chen, Z.J. 1999. Signal-induced ubiquitination of IkappaBalpha by the F-box protein Slimb/beta-TrCP. *Genes Dev* **13**(3): 284-294.

Sprenger, F., Yakubovich, N., and O'Farrell, P.H. 1997. S-phase function of Drosophila cyclin A and its downregulation in G1 phase. *Curr Biol* **7**(7): 488-499.

St Johnston, D. 2002. The art and design of genetic screens: Drosophila melanogaster. *Nat Rev Genet* **3**(3): 176-188.

Stern, B., Ried, G., Clegg, N.J., Grigliatti, T.A., and Lehner, C.F. 1993. Genetic analysis of the Drosophila cdc2 homolog. *Development* **117**(1): 219-232.

Su, T.T. and O'Farrell, P.H. 1998. Chromosome association of minichromosome maintenance proteins in Drosophila endoreplication cycles. *J Cell Biol* **140**(3): 451-460.

Sugimoto, N., Tatsumi, Y., Tsurumi, T., Matsukage, A., Kiyono, T., Nishitani, H., and Fujita, M. 2004. Cdt1 phosphorylation by cyclin A-dependent kinases negatively regulates its function without affecting geminin binding. *J Biol Chem* **279**(19): 19691-19697.

Swanhart, L., Kupsco, J., and Duronio, R.J. 2005. Developmental Control of Growth and Cell Cycle Progression in *Drosophila*. in *Cell Cycle Control - Mechanisms and Protocols* (ed. T. Humphrey and G. Brooks), pp. 69-94. Humana Press, Totowa, New Jersey.

Tada, S. 2007. Cdt1 and geminin: role during cell cycle progression and DNA damage in higher eukaryotes. *Front Biosci* **12**: 1629-1641.

Takeda, D.Y., Parvin, J.D., and Dutta, A. 2005. Degradation of Cdt1 during S phase is Skp2-independent and is required for efficient progression of mammalian cells through S phase. *J Biol Chem* **280**(24): 23416-23423.

Thacker, S.A., Bonnette, P.C., and Duronio, R.J. 2003. The contribution of E2F-regulated transcription to Drosophila PCNA gene function. *Curr Biol* **13**(1): 53-58.

Thomas, B.J., Gunning, D.A., Cho, J., and Zipursky, L. 1994. Cell cycle progression in the developing Drosophila eye: roughex encodes a novel protein required for the establishment of G1. *Cell* **77**(7): 1003-1014.

Thomas, B.J., Zavitz, K.H., Dong, X., Lane, M.E., Weigmann, K., Finley, R.L., Jr., Brent, R., Lehner, C.F., and Zipursky, S.L. 1997. roughex down-regulates G2 cyclins in G1. *Genes Dev* **11**(10): 1289-1298.

Thomer, M., May, N.R., Aggarwal, B.D., Kwok, G., and Calvi, B.R. 2004. Drosophila double-parked is sufficient to induce re-replication during development and is regulated by cyclin E/CDK2. *Development* **131**(19): 4807-4818.

Tung, J.J., Hansen, D.V., Ban, K.H., Loktev, A.V., Summers, M.K., Adler, J.R., 3rd, and Jackson, P.K. 2005. A role for the anaphase-promoting complex inhibitor Emi2/XErp1, a homolog of early mitotic inhibitor 1, in cytostatic factor arrest of Xenopus eggs. *Proc Natl Acad Sci U S A* **102**(12): 4318-4323.

Tyers, M. and Jorgensen, P. 2000. Proteolysis and the cell cycle: with this RING I do thee destroy. *Curr Opin Genet Dev* **10**(1): 54-64.

Visintin, R., Prinz, S., and Amon, A. 1997. CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis. *Science* **278**(5337): 460-463.

Vodermaier, H.C. 2001. Cell cycle: Waiters serving the Destruction machinery. *Curr Biol* **11**(20): R834-837.

Vodermaier, H.C. 2004. APC/C and SCF: controlling each other and the cell cycle. *Curr Biol* **14**(18): R787-796.

Vodermaier, H.C. and Peters, J.M. 2004. APC activators caught by their tails? *Cell Cycle* **3**(3): 265-266.

Weiss, A., Herzig, A., Jacobs, H., and Lehner, C.F. 1998. Continuous Cyclin E expression inhibits progression through endoreduplication cycles in Drosophila. *Curr Biol* **8**(4): 239-242.

Weng, L., Zhu, C., Xu, J., and Du, W. 2003. Critical role of active repression by E2F and Rb proteins in endoreplication during Drosophila development. *Embo J* **22**(15): 3865-3875.

Whittaker, A.J., Royzman, I., and Orr-Weaver, T.L. 2000. Drosophila double parked: a conserved, essential replication protein that colocalizes with the origin recognition complex and links DNA replication with mitosis and the down-regulation of S phase transcripts. *Genes Dev* **14**(14): 1765-1776.

Wieschaus, E. and Sweeton, D. 1988. Requirements for X-linked zygotic gene activity during cellularisation of early Drosophila embryos. *Development* **104**(3): 483-493.

Wohlschlegel, J.A., Dwyer, B.T., Dhar, S.K., Cvetic, C., Walter, J.C., and Dutta, A. 2000. Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. *Science* **290**(5500): 2309-2312.

Yamanaka, A., Yada, M., Imaki, H., Koga, M., Ohshima, Y., and Nakayama, K. 2002. Multiple Skp1-related proteins in Caenorhabditis elegans: diverse patterns of interaction with Cullins and F-box proteins. *Curr Biol* **12**(4): 267-275.

Yamano, H., Gannon, J., Mahbubani, H., and Hunt, T. 2004. Cell cycle-regulated recognition of the destruction box of cyclin B by the APC/C in Xenopus egg extracts. *Mol Cell* **13**(1): 137-147.

Yu, H. 2006. Structural activation of Mad2 in the mitotic spindle checkpoint: the two-state Mad2 model versus the Mad2 template model. *J Cell Biol* **173**(2): 153-157.

Yu, H. 2007. Cdc20: a WD40 activator for a cell cycle degradation machine. *Mol Cell* **27**(1): 3-16.

Zachariae, W. and Nasmyth, K. 1999. Whose end is destruction: cell division and the anaphase-promoting complex. *Genes Dev* **13**(16): 2039-2058.

Zachariae, W., Schwab, M., Nasmyth, K., and Seufert, W. 1998. Control of cyclin ubiquitination by CDK-regulated binding of Hct1 to the anaphase promoting complex. *Science* **282**(5394): 1721-1724.

Zheng, N., Schulman, B.A., Song, L., Miller, J.J., Jeffrey, P.D., Wang, P., Chu, C., Koepp, D.M., Elledge, S.J., Pagano, M., Conaway, R.C., Conaway, J.W., Harper, J.W., and Pavletich, N.P. 2002. Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex. *Nature* **416**(6882): 703-709.

Zhong, W., Feng, H., Santiago, F.E. and Kipreos, E.T. (2003) CUL-4 ubiquitin ligase maintains genome stability by restraining DNA-replication licensing. *Nature*, 423, 885-889

Zhu, W., Chen, Y., and Dutta, A. 2004. Rereplication by depletion of geminin is seen regardless of p53 status and activates a G2/M checkpoint. *Mol Cell Biol* 24(16): 7140-7150.

Zielke, N. 2007. Functional analysis of the cell cycle regulator Rca1 in *Drosophila melanogaster*. In. Universität Köln.

Zielke, N., Querings, S., Grosskortenhaus, R., Reis, T., and Sprenger, F. 2006. Molecular dissection of the APC/C inhibitor Rca1 shows a novel F-box-dependent function. *EMBO Rep* **7**(12): 1266-1272.

Appendix

Quantitative Realtime PCR (qRT-PCR) gene expression analysis data of *Drosophila* larval salivary glands. Raw data analyses were performed as described in chapter 5.4.5. Data of four independent qRT-PCR were analysed in a Relative Quantification Study using SDS 2.1 software (Applied Biosystems). Relative fold differences in gene expression were determined using the ΔΔ CT method. GAPDH was used as the endogenous control and wild-type embryos (4-8 h AED) as calibrator.

Gene	RQ	log10(RQ)	RQ Min	log10(RQ Min)	RQ Max	log10(RQ Max)	Δ- Log10(RQ)	Δ+Log10(RQ)
CycE	0,0063	-2,2027	0,0053	-2,2786	0,0075	-2,1268	0,0759	0,0759
Cdk2	0,0067	-2,1757	0,0051	-2,2959	0,0080	-2,0963	0,1202	0,0794
Dap	0,0022	-2,6576	0,0018	-2,7417	0,0027	-2,5734	0,0842	0,0842
Geminin	0,0044	-2,3573	0,0027	-2,5619	0,0070	-2,1527	0,2046	0,2046
Dup	0,0058	-2,2382	0,0036	-2,4422	0,0092	-2,0342	0,2040	0,2040
E2F1	0,0637	-1,1958	0,0542	-1,2659	0,0749	-1,1257	0,0701	0,0701
PCNA	0,0059	-2,2276	0,0049	-2,3066	0,0071	-2,1486	0,0790	0,0790
Rnr2	0,0155	-1,8104	0,0123	-1,9112	0,0195	-1,7096	0,1008	0,1008
CycA	0,0004	-3,3491	0,0004	-3,4282	0,0005	-3,2700	0,0791	0,0791
CycB	0,0001	-3,9049	0,0001	-4,0016	0,0002	-3,8081	0,0968	0,0968
Cdk1	0,0012	-2,9067	0,0009	-3,0574	0,0018	-2,7560	0,1507	0,1507
Stg	0,0006	-3,2011	0,0005	-3,2794	0,0008	-3,1229	0,0783	0,0783
Rca1	0,0024	-2,6136	0,0020	-2,6989	0,0030	-2,5283	0,0853	0,0853
Mr	0,0127	-1,8959	0,0104	-1,9819	0,0155	-1,8098	0,0861	0,0861
Cdc16	0,0202	-1,6937	0,0173	-1,7619	0,0237	-1,6254	0,0683	0,0683
Fzr	0,0173	-1,7618	0,0141	-1,8505	0,0212	-1,6731	0,0887	0,0887

Figure A RQ, Relative Expression Quantity value; Log10(RQ), logarithm of RQ value; RQ min, minimum RQ value; RQ max; maximum RQ value; Log10(RQ min), logarithm of RQ min; Log10(RQ max), logarithm of RQ max; Δ-Log10(RQ), Log10(RQ)-Log10(RQ min); Δ+Log10(RQ), Log10(RQ)-Log10(RQ max).

Quantitative Realtime PCR (qRT-PCR) gene expression analysis data of *Drosophila* larval salivary glands overexpressing HA-Rca1. Raw data analyses were performed as described in chapter 5.4.5. Data of four independent qRT-PCR were analysed in a Relative Quantification Study using SDS 2.1 software (Applied Biosystems). Relative fold differences in gene expression were determined using the ΔΔ CT method. Actin was used as the endogenous control and wild-type larval salivary glands as calibrator.

Gene	RQ	log10(RQ)	RQ Min	log10(RQ Min)	RQ Max	log10(RQ Max)	Δ- Log10(RQ)	Δ+Log10(RQ)
CycE	18,9771	1,2782	15,6214	1,1937	23,0537	1,3627	0,0845	0,0845
Cdk2	0,7047	-0,1520	0,5700	-0,2441	0,8696	-0,0607	0,0921	0,0913
Dap	0,4467	-0,3500	0,3795	-0,4208	0,5257	-0,2793	0,0707	0,0707
E2F1	0,4657	-0,3319	0,3905	-0,4084	0,5553	-0,2555	0,0764	0,0764
PCNA	3,1329	0,4960	2,5756	0,4109	3,8109	0,5810	0,0851	0,0851
Rnr2	1,8557	0,2685	1,4925	0,1739	2,3074	0,3631	0,0946	0,0946
CycA	2,7643	0,4416	2,3429	0,3698	3,2616	0,5134	0,0718	0,0718
CycB	0,4730	-0,3251	0,3772	-0,4234	0,5932	-0,2268	0,0983	0,0983
Cdk1	1,4953	0,1747	1,0575	0,0243	2,1143	0,3252	0,1504	0,1504
Stg	0,2229	-0,6519	0,1811	-0,7421	0,2744	-0,5616	0,0903	0,0903
Rca1	1201,0643	3,0796	1035,7117	3,0152	1392,8091	3,1439	0,0643	0,0643
Mr	0,5319	-0,2742	0,4393	-0,3573	0,6440	-0,1911	0,0831	0,0831
Cdc16	0,6999	-0,1550	0,5371	-0,2699	0,9119	-0,0400	0,1149	0,1149
Fzr	0,6055	-0,2179	0,5132	-0,2897	0,7144	-0,1461	0,0718	0,0718

Figure B RQ, Relative Expression Quantity value; Log10(RQ), logarithm of RQ value; RQ min, minimum RQ value; RQ max; maximum RQ value; Log10(RQ min), logarithm of RQ min; Log10(RQ max), logarithm of RQ max; Δ-Log10(RQ), Log10(RQ)-Log10(RQ min); Δ+Log10(RQ), Log10(RQ)-Log10(RQ max).

Quantitative Realtime PCR (qRT-PCR) gene expression analysis data of *Drosophila* larval salivary glands overexpressing Cyclin E. Raw data analyses were performed as described in chapter 5.4.5. Data of four independent qRT-PCR were analysed in a Relative Quantification Study using SDS 2.1 software (Applied Biosystems). Relative fold differences in gene expression were determined using the ΔΔ CT method. Actin was used as the endogenous control and wild-type larval salivary glands as calibrator.

Gene	RQ	log10(RQ)	RQ Min	log10(RQ Min)	RQ Max	log10(RQ Max)	Δ- Log10(RQ)	Δ+Log10(RQ)
CycE	76,7199	1,8849	60,4024	1,7811	97,4455	1,9888	0,1039	0,1039
Cdk2	3,7872	0,5783	2,3717	0,3751	6,0476	0,7816	0,2033	0,2033
Dap	1,8224	0,2607	1,4033	0,1471	2,3668	0,3742	0,1135	0,1135
E2F1	0,8049	-0,0943	0,6252	-0,2040	1,0363	0,0155	0,1097	0,1097
PCNA	12,7635	1,1060	9,6680	0,9853	16,8502	1,2266	0,1206	0,1206
Rnr2	8,1844	0,9130	6,2021	0,7925	10,8002	1,0334	0,1204	0,1204
CycA	11,3651	1,0556	7,7901	0,8915	16,5809	1,2196	0,1640	0,1640
CycB	1,2863	0,1093	0,9871	-0,0056	1,6762	0,2243	0,1150	0,1150
Cdk1	20,7151	1,3163	16,7734	1,2246	25,5830	1,4080	0,0917	0,0917
Stg	0,5163	-0,2871	0,2371	-0,6250	1,1243	0,0509	0,3379	0,3379
Rca1	1,1907	0,0758	0,9385	-0,0276	1,5107	0,1792	0,1034	0,1034
Mr	1,3407	0,1273	0,9183	-0,0370	1,9574	0,2917	0,1644	0,1644
Cdc16	1,7209	0,2358	1,4036	0,1472	2,1099	0,3243	0,0885	0,0885
Fzr	0,7571	-0,1209	0,5506	-0,2592	1,0410	0,0174	0,1383	0,1383

Figure C RQ, Relative Expression Quantity value; Log10(RQ), logarithm of RQ value; RQ min, minimum RQ value; RQ max; maximum RQ value; Log10(RQ min), logarithm of RQ min; Log10(RQ max), logarithm of RQ max; Δ-Log10(RQ), Log10(RQ)-Log10(RQ min); Δ+Log10(RQ), Log10(RQ)-Log10(RQ max).

Abbreviations

APC/C	Anaphase promoting complex/cyclosome
APS	ammoniumperoxidisulfate
ATP	adenosine triphosphate
Cdk	cyclin dependent kinase
Cul1	Cullin1
Cyc	cyclin
Da	dalton
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
Dnase	desoxyribonuclease
DTT	dithiothreitol
EDTA	ethelyne diamine tetracetic acid
FBS	fetal calf serum
Fzy	Fizzy
Fzr	Fizzy-related
g	gram
h	hour
HA	hemagglutinin
hs	homo sapiens
k	kilo
kb	kilo base
l	liter
M	mol per litre
m	milli
µ	micro
min	minute
mol	molar
mRNA	messenger RNA
n	nano
NLS	nuclear localization signal
P	pico
PAGE	polyacrylamide gel electrophoresis

PBS	phosphate buffered saline
PCR	polymerase chain reaction
Rca1	Regulator of Cyclin A 1
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature
s	second
SDS	sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethylendiamin
U	unit
V	voltage
ZBR	zinc binding region

Single and three letter code for amino acids

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartate
E	Glu	Glutamate
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

Zusammenfassung

Für den korrekten Ablauf der Mitose und die Etablierung der G1 Phase ist eine strikte Kontrolle der APC/C-Cdh1^{Fzr} Aktivität notwendig. Rca1 ist ein Kernprotein, das den APC/C-Cdh1^{Fzr} Komplex während der G2 Phase inhibiert und somit eine Akkumulation der Cycline sowie den Eintritt in die Mitose ermöglicht. In der vorliegenden Arbeit wurde eine Studie zur Lokalisation von Rca1 durchgeführt, in der gezeigt werden konnte, dass eine Kernlokalisierungssequenz sowie weitere Domänen im Protein eine effiziente Kernakkumulation vermitteln. Außer der Funktion in der G2 Phase, kann die Expression von Rca1 auch den Eintritt in die S Phase beschleunigen. Rca1 gehört zur Familie der F-box Proteine, welche Substratspezifität in SCF (Skp-Cul1-F-box) Ubiquitinligasen vermitteln. Funktionale Studien konnten zeigen, dass die F-box in Rca1 für die Induktion von S Phasen notwendig ist und führten zur Annahme einer sekundären Funktion von Rca1 in einem SCF Komplex.

Ein großer Teil dieser Arbeit umfasste die Charakterisierung des besagten putativen SCF/Rca1 Komplexes sowie seiner Substrate. Die SCF Untereinheit SkpA wurde in einem Hefe-2-Hybrid System als Bindungspartner von Rca1 identifiziert. Diese Interaktion konnte durch Koimmunopräzipitationsstudien bestätigt werden, welche zudem zeigten, dass die Bindung von SkpA auf der F-box in Rca1 beruht. Des Weiteren kopräzipitierte endogenes Cul1 mit Rca1 und auch dies erfolgte abhängig von der F-box. Diese Experimente zeigten, dass Rca1 einen Komplex mit den SCF Kernuntereinheiten SkpA und Cul1 bilden kann. Dieser SCF/Rca1 Komplex könnte den Eintritt in die S Phase beschleunigen, indem dieser Komplex den Abbau eines negativen Regulators des G1-S Übergangs vermittelt. Cyclin A/Cdk1 kann S Phasen induzieren, aber seine Aktivität ist in der Regel eingeschränkt durch den CKI Rux, inhibitorische Phosphorylierung durch Wee und APC/C-Cdh1^{Fzr} abhängigen Abbau von Cyclin A. Die Koexpression von Rca1 beeinflusste die Proteinmengen dieser S Phase Inhibitoren nicht, so dass diese Proteine vermutlich keine Substrate des SCF/Rca1 Komplexes sind.

Die Überexpression von Rca1 in larvalen Speicheldrüsen beeinträchtigt die Endoreplikation und führt zur Akkumulation von Cyclin A, Cyclin E und Cdk1. Expressionsprofile zeigten, dass mitotische Gene (z.B. Cyclin A/E, Cdk1) in Speicheldrüsen herunter reguliert sind, die Überexpression von Rca1 jedoch deren Transkription verstärkt. Zusätzlich zeigten die qRT-PCR Analysen erhöhte Transkriptlevel der E2F1 Zielgene Rnr2 und PCNA und vermuten,

dass die Überexpression von Rca1 zu einer verstärkten E2F1 Aktivität führt. Da Cyclin E ebenfalls ein E2F1 Zielgen ist, lässt sich somit auch ein Anstieg der Cyclin E Transkripte erklären. Außerdem offenbarten die Genexpressionsanalysen, dass Komponenten des APC/C und dessen Substrat Geminin in Speicheldrüsen vorhanden sind und unterstützen somit die Annahme, dass Geminin und der APC/C-Cdh1^{Fzr} Komplex zur Rereplikationskontrolle in endoreplizierenden Zellen beitragen.

Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist, sowie dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von PD Dr. Frank Sprenger betreut worden.

Köln, Dezember 2007

Silvia Querings

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