Functional analysis of the cell cycle regulator Rca1

in *Drosophila melanogaster*

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Abstract

Tight regulation of APC/C activity is essential for cell cycle progression. An important class of negative APC/C regulators are the Rca1/Emi1 family proteins. All members of the Rca1/Emi1 family share a conserved zinc binding region (ZBR) which is essential for their inhibitory activity. The Rca1/Emi1 proteins belong to the class of F-box proteins that are known to act as substrate recognition subunits in SCF-E3-ligase complexes. Emi1 and Rca1 interact *in vitro* with members of the Skp family via the F-box. However, no F-box dependent function has been ascribed to these proteins. In *Drosophila*, Rca1 is required in G2 to prevent premature activation of the APC/C by Fzr. Loss of Rca1 results in an arrest during G2 of the 16th embryonic cell cycle due to premature cyclin degradation. In order to map the essential domains for Rca1 function, a series of deletion constructs was tested for their ability to inhibit APC/C-Fzr activity *in vivo*. A C-terminal Rca1 fragment including the ZBR was sufficient to restore mitosis 16 in *rca1* mutant embryos. This observation confirms that the ZBR is the only protein motif essential for APC/C-Fzr inhibition by Emi1/Rca1. Moreover, this result indicates that the F-box is dispensable for APC/C-Fzr inhibition during embryogenesis. However, analysis of Rca1 function during larval development revealed that Rca1 has a secondary role as an F-box protein. Using the MARCM technique, wing disc cells were generated in which endogenous Rca1 was replaced by an Rca1 construct lacking the F-box. These cells displayed a reduced proliferation rate and prolonged G1-phase. Conversely, overexpression of Rca1 accelerates the G1-S transition in imaginal discs in an F-box dependent manner. Hence, it is likely that Rca1 regulates S-phase entry as part of a yet uncharacterized SCF-complex. In addition, the effect of Rca1 on endoreplication was analyzed. Overexpression of Rca1 during salivary gland development leads to a reduction of polyploidization. This phenotype also depends on a functional F-box. Endoreplication cycles are driven by oscillating waves of Cyclin E/Cdk2 activity, whereas Cdk1 and the mitotic cyclins are transcriptionally downregulated. Furthermore, APC/C-Fzr activity seems not to be required once the endoreplication program has been initiated. Cells overexpressing Rca1 displayed elevated levels of Cyclin E, although Cyclin E is not a target of the APC/C-Fzr complex. It has been shown that continuous expression of Cyclin E interferes with DNA-licensing. Thus, the reduced DNA content in Rca1 overexpressing cells might be due to elevated Cyclin E levels. Additionally, Rca1 overexpressing cells displayed markers for mitotic cells such as Cdk1 and nuclear Cyclin A. The accumulation of Cdk1, Cyclin A and Cyclin E cannot simply be explained by APC/C inhibition. It rather appears that Rca1

activates the transcription of these genes by an unknown mechanism. Nevertheless it cannot be excluded that the APC/C-Fzr complex indirectly contributes to this process. Altogether, Rca1 might act as an F-box protein in an SCF complex that is involved in maintaining diploidy.

1. Introduction

All living organisms are comprised of cells that reproduce by the interplay of cell growth and cell division. Division of already existing cells is the only possibility to generate novel cells and consequently the only way to inherit the genome of the progenitor cell. Thus, the capability of cell division is a fundamental prerequisite for the continuance of life. In unicellular organisms each cell division results in the production of an entire new organism. In multicellular organisms, however, it requires numerous divisions to create a novel organism from a fertilized oocyte. Since cell division and cell differentiation cannot occur simultaneously, cell division has to be tightly coordinated with the developmental program. In multicellular organisms, cell division occurs not only during development but is rather required throughout the whole lifespan. Continuous replacement of dead and degenerated cells is a fundamental process to maintain the health of an organism. Hence, impaired cell division can result in severe defects during development as well as in adult organisms.

In principle, cell division requires two different steps that have to be orchestrated. In the first step, cells duplicate their DNA, which is equally distributed between both daughter cells during the actual division step. Since both events occur in an ordered fashion and cannot be separated from each other, the reproduction process of a eukaryotic cell is generally referred as cell cycle (for a general review see Morgan, 2006). The eukaryotic standard cell cycle is divided into four distinct phases, which are named G1, S, G2 and M-phase. The actual division process takes places during M-phase, which is subdivided into mitosis and cytokinisis. The period between two subsequent divisions is termed interphase and is comprised of the three remaining phases. Interphase begins in G1, where the cells are highly metabolic active and increase their cell mass. After completion of this gap phase, cells undergo S-phase to duplicate their DNA. DNA-replication results in the generation of two sister chromatids that will be evenly distributed during mitosis. Before initiation of mitosis, cells enter the G2-phase, in which they undergo further growth. Mitosis is also referred as nuclear division since it only results in the formation of two new nuclei. In the following cytokinisis, these daughter nuclei are then distributed into separate cells.

Based on morphological criteria, mitosis is subdivided into five sections: The first part of mitosis, where the DNA begins to condensate, is named prophase. It is followed by prometaphase and correlates with the initiation of nuclear envelope breakdown. During

metaphase, sister chromatids are attached to the mitotic spindle and form the metaphase plate in the middle of the cell. In the following anaphase, sisterchromatids separate and are subsequently pulled towards the spindle poles. The formation of new nuclei marks the terminal phase of mitosis and is termed telophase. At this time the DNA starts to decondensate, a process that persists till onset of interphase.

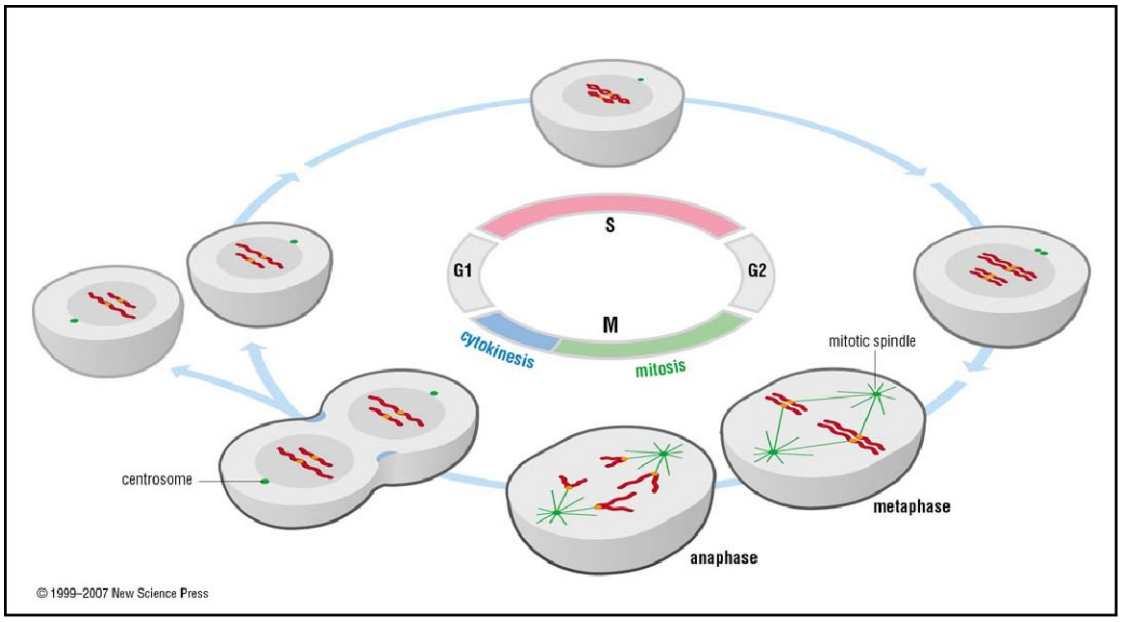

Figure 1 The eukaryotic standard cell cycle (adapted from Morgan, 2006). The standard cell cycle is comprised of four phases. In S-phase the DNA of the cell becomes duplicated. During M-phase, the replicated DNA is equally distributed between both daughter cells. S and M-phase are separated by two Gap-phases (G1 and G2) in which the cell increases their mass by growth. The M-phase is dived into two sections, mitosis and cytokinisis. During mitosis sisterchromatids are distributed into two daughter nuclei which become, in the following cytokinisis separated, by a new cell wand. Mitosis is subdivided into five phases, whereby metaphase and anaphase are of particular interest. During metaphase, sister chromatids are held in the middle of the cell by the mitotic spindle. In the following anaphase, sister chromatids fall apart and are drawn to opposite poles of the cell.

1.1. Cell cycle regulation during *Drosophila* development

The eukaryotic standard cell cycle is not the only of cell cycle mode that is applied. The cells of multicellular organisms rather display different types of cell cycle regulation. This plasticity is necessary to adapt the cell cycle to the different demands of a certain tissue or a particular developmental process. The fruit fly, *Drosophila melanogaster* is an outstanding model organism to study the mechanisms coordinating cell proliferation with the developmental program (for review see Edgar and Lehner, 1996; Lee and Orr-Weaver, 2003; Swanhart et al., 2005). During the course of *Drosophila* development, cells exhibit a variety of different cell cycle types. Even during the short period of embryogenesis, the cells have to undergo three different modes of cell cycle regulation (Figure 2). After completion of

embryogenesis a feeding larvae arises from the embryo. The larvae grows dramatically, but instead of increasing cell numbers, most cells of the larval tissues undergo a specialized cell cycle called endocycle. Most of the adult structures derive from imaginal discs which divide mitotically during the larval period. Of particular interest are the imaginal discs that comprise of the prospective eye (Figure 3) and wing (Figure 4), since they display a cell cycle mode that resembles the standard cell cycle. Finally, nurse and follicle cells of the *Drosophila* ovary have been proofed as very useful for the investigation of DNA replication and endoreplication, respectively.

1.1.1. Adaptation of cell cycle regulation during *Drosophila* embryogenesis

The first ten cell cycles during *Drosophila* embryogenesis are nuclear divisions that occur in a common cytoplasm and give rise to a syncytium. These syncytial cell cycles are very rapid, since they consist only of S and M-phases without intervening Gap-phases. At the onset of embryogenesis, all nuclei are located in the centre of the syncytium. However, at the end of the seventh division cycle three-quarters of the nuclei start to migrate to the surface. The remaining nuclei, by contrast, develop into yolk nuclei that exit the cell cycle after completion of the tenth cycle and then initiate endoreplication. During endoreplication, mitosis is bypassed resulting in an increased DNA content (for review see Edgar and Orr-Weaver, 2001; Lilly and Duronio, 2005). In the course of the ninth division cycle, the first migrating nuclei arrive at the posterior pole of the zygote. During the following cell cycle, cellularization becomes initiated and thereby these nuclei lose their synchrony with the remainder of the nuclei. These early forming posterior cells are termed as pole cells and give rise to the germ cells. The remaining nuclei reach the surface at the beginning of the tenth cell cycle. These nuclei undergo four additional syncytial divisions until they initiate cellularization. These divisions originate at the poles and spread then wavelike to the middle of the embryo. Similar to the first ten divisions, these cell cycles (10-13) lack any intervening Gap-phases, but are a bit slower.

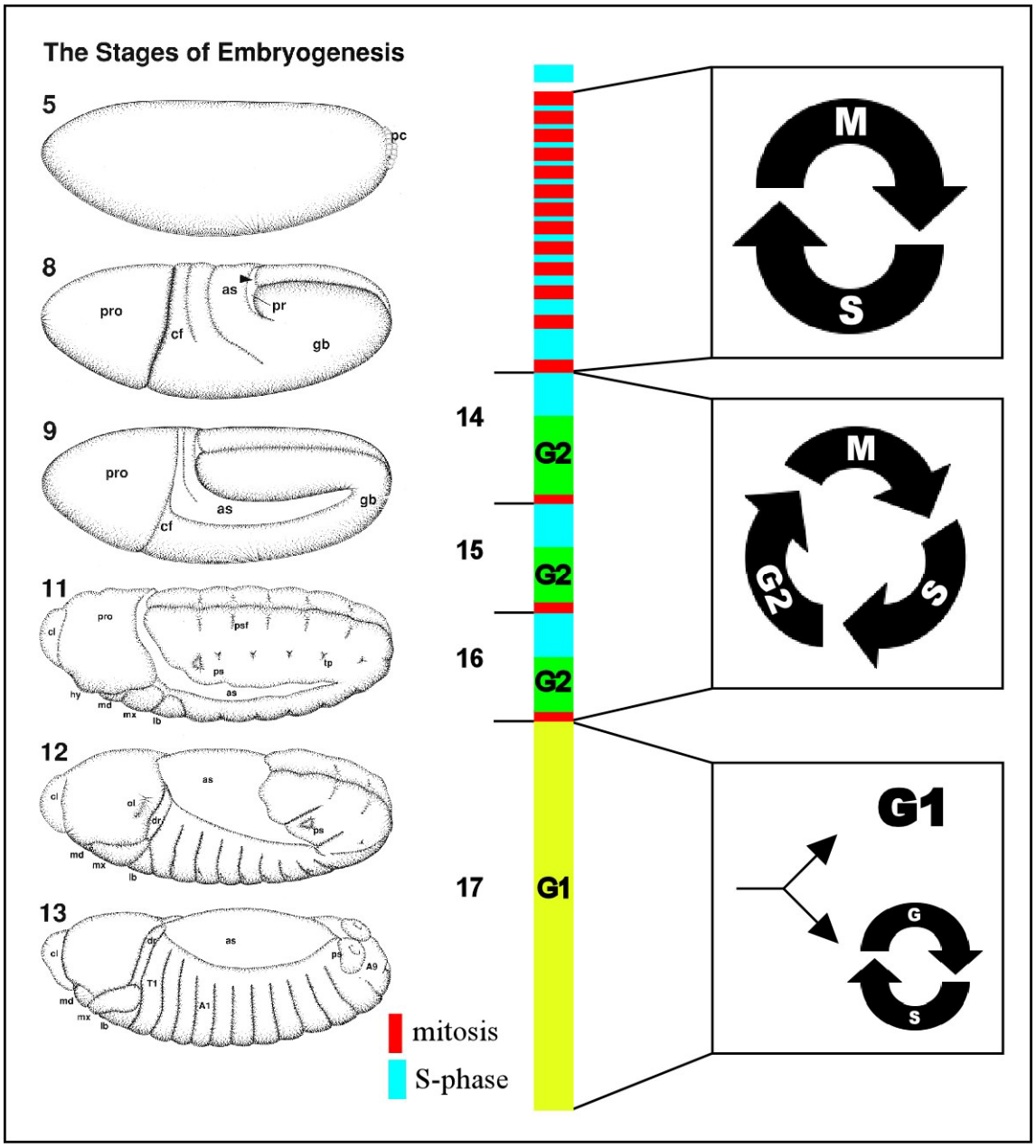

Figure 2 Cell cycle regulation during *Drosophila* embryogenesis. The first thirteen cell cycles are very rapid and only consist of S- and M-phases. At the stage of cellularization, the midblastula transition occurs and thereby cells change from maternal to zygotic transcription. The following three divisions are termed postblastoderm cell cycles. During these stages, cells undergo a distinct G2-phase, but enter S-phase without an intervening G1-phase. Most of the epidermal cells persist in this terminal G1-phase until the end of embryonic development, while the cells of certain internal tissues such as the gut or the salivary glands enter endocycles. An exception are the cells of the developing nervous system which remain mitotic. The drawings of embryonic stages on the right side are adapted from Hartenstein (1993).

During the 14th embryonic cell cycle the remaining nuclei initiate cellularization, a stage that is called cellular blastula. With the onset of cell cycle 14, the nuclei lose their synchrony and the divisions occur in an invariant spatiotemporal pattern of 25 mitotic domains (Foe, 1989). The first 13 divisions are driven by maternal stockpiles. At the cellular blastula stage these maternal transcripts are exhausted and zygotic gene expression becomes essential (Merrill et

al., 1988; Wieschaus and Sweeton, 1988). The initiation of zygotic transition is called midblastula transcription and results in the introduction of the first G2-phase (Edgar and O'Farrell, 1989; Edgar and O'Farrell, 1990). The first G1-phase is established after completion of mitosis16 (Edgar and O'Farrell, 1990). Most of the epidermal cells reside in this terminal G1 phase until the end of embryogenesis. Groups of 10-50 imaginal cells that develop into adult structures are separated from the remainder of cells and re-initiate proliferation only upon onset of larval development. By contrast, cells that give rise to the larva proper (e.g. gut, fat body and salivary glands) initiate endoreplication cycles and become polyploid (Smith and Orr-Weaver, 1991). The cells of the developing nervous system are an exception, because they continue to proliferate mitotically during late embryogenesis.

1.1.2. Third instar eye imaginal disc display a linear arrangement of cell cycle stages

The imaginal discs of *Drosophila* are monolayered epithelial sacs that undergo extensive proliferation during the larval stages. The adult eyes as well as some structures of the head originate from the posterior part of the eye-antenna disc. Eye imaginal discs are an excellent system to study cell cycle control during organ development. A major advantage of the eye imaginal disc is that alterations of the cell cycle often results in aberrant eye morphology (de Nooij and Hariharan, 1995). Eye phenotypes are easy recognizable and therefore facilitate the identification of defects in the cell cycle program. During the first two larval stages, eye imaginal disc cells proliferate in a unpatterned manner that resembles the standard cell cycle. However, during the third instar stage the differentiation of eye disc cells is initiated. The differentiation into photoreceptor cells is coordinated by the movement of the morphogenetic furrow. The morphogenetic furrow sweeps from anterior to posterior and thereby creates a linear arrangement of cell cycle stages (Figure 3). Undifferentiated cells anterior to the morphogenetic furrow divide asynchronously, whereas cells in the posterior part initiate differentiation. The cells within the morphogenetic furrow are synchronized in G1. A subset of these cells, termed as preclusters, exits the cell cycle and differentiates into the photoreceptor cells R8, R2, R5, R3 and R4 (Ready et al., 1976; Wolff and Ready, 1991a). The remainder of cells enter a terminal cell cycle called second mitotic wave. The cells of this second mitotic wave give rise to the photoreceptor cells R1, R6, R7, the cone cells, the pigment cells as well as the precursors of the mechanosensory bristles (Ready et al., 1976; Wolff and Ready, 1991a). The remaining undifferentiated cells undergo apoptosis (Wolff and Ready, 1991b).

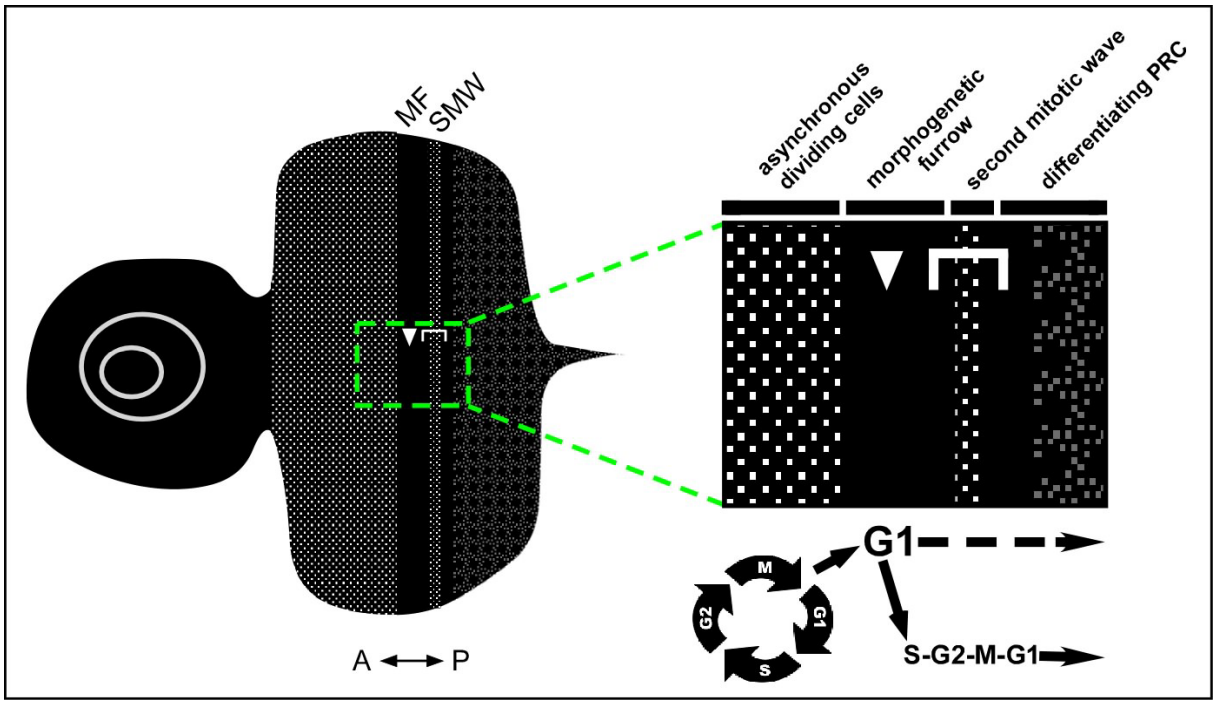

Figure 3 Eye imaginal disc cells exhibit morphologically distinguishable G1-S transition. The eye imaginal disc represents the posterior part of the eye-antenna disc. During the third instar stage the morphogenetic furrow (MF) moves from posterior to anterior and thereby initiates the differentiation into photoreceptor cells (PRC). Cells in the posterior part of the disc divide asynchronously until they become synchronized in G1 by the anterior sweeping furrow. The cells within the morphogenetic furrow subsequently separate into two subpopulations. One fraction terminates proliferation and initiates differentiation immediately, while the remaining cells enter a terminal cell cycle called second mitotic wave (SMW).

1.1.3. Cell cycle regulation in wing imaginal discs

Wing imaginal discs gives rise to two different adult structures. The ventral part of the disc develops into the wing, whereas the dorsal part differentiates into the notum. Wing imaginal disc cells exhibit a mode of cell cycle regulation that resembles the proliferation behaviour of vertebrate cells. Cells in wing imaginal disc undergo a standard cell cycle with four distinct phases. Moreover, unlike embryonic divisions, these cell cycles are accompanied with cell growth ensuring that these cells maintain a constant size during proliferation. Wing imaginal disc cells undergo apoptosis only occasionally, therefore it is thought that most of the disc growth results from proliferation (James and Bryant, 1981). In contrast to the cells of the developing eye, cell division in wing imaginal discs occurs in a largely unpatterned fashion (for review see Milan, 1998). Wing disc cells rather divide in clusters of synchronized cells that are randomly distributed through out the disc. However at the end of larval development, a stripe of cells at the dorso-ventral boundary enter a developmentally programmed cell cycle arrest (O'Brochta and Bryant, 1985) and therefore this region of the disc was named “zone of none proliferating cells” (ZNC; Figure 4). Cells of the ZNC cease proliferation around 30

hours earlier than remaining cells, but re-initiate the cell cycle for a couple of division during the pupal stage (Hartenstein and Posakony, 1989; O'Brochta and Bryant, 1985). The zone of none proliferating cells is subdivided into four domains (Figure 4; Johnston and Edgar, 1998). Cells in the centre of the anterior part as well as the whole posterior part of the ZNC undergo a G1 arrest, whereas the two outer cell rows of the anterior part stay in the G2-phase.

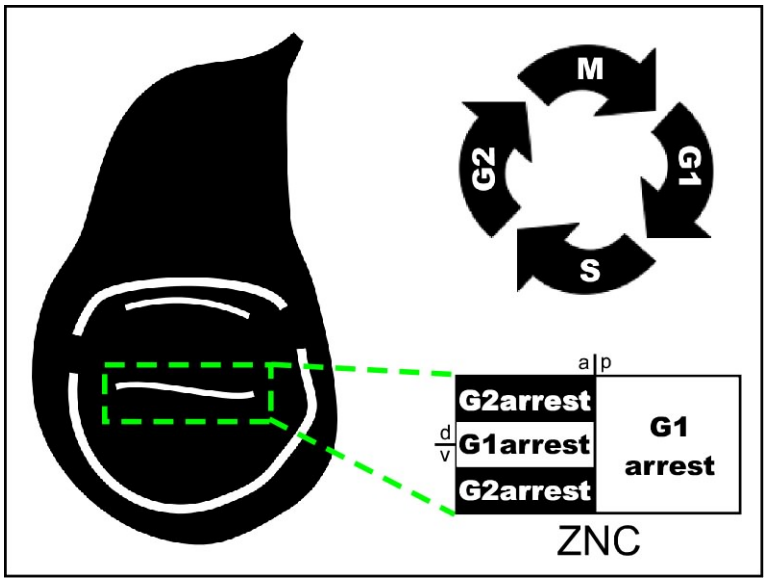

Figure 4 Cell division during wing development. Cells in wing imaginal discs proliferate extensively during the larval period. Thereby, these cells undergo a standard cell cycle with four distinct phases. Cell division occurs in an asynchronous and unpatterned manner. Only at the end of larval development cells of the ZNC undergo a developmentally programmed cycle arrest. The cells in the middle of the anterior part of the ZNC arrest in G1, while the adjacent cell rows undergo a G2 arrest. Cells in the posterior domain of the ZNC uniformly arrest in G1.

1.1.4. Endoreplication cycles

The endoreplication cycle or endocycle is a cell cycle variant that is employed by various tissues in *Drosophila* (for review see Edgar and Orr-Weaver, 2001; Lilly and Duronio, 2005). During endoreplication, cells undergo repeated rounds of DNA replication without intervening mitosis which result in increased DNA contents. Endocycling cells exhibit a distinct Gap-phase, in which no DNA-replication occurs. Endoreplication is an effective strategy of cell growth and is therefore frequently found in cells that give rise to tissues with high metabolic activity (for review see Edgar and Nijhout, 2004). Cells of several internal organs such as the gut, fat body, malipighian tubules and salivary glands, initiate endoreplication cycles during late embryogenesis and maintain endoreplication cycles during the larval period (Smith and Orr-Weaver, 1991). In addition, several adult tissues like the gut and the ovary harbor endoreplicating cells (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Endoreplication results in multiple copies of the genome that can be organized in different chromosomal arrangements. Generally, it is distinguished between polyploidy and polyteny. Polyploid cells contain multiple copies of their chromosomes that

are clearly distinguishable from each other. In polytene cells, by contrast, sisterchromatids remain closely associated. A well known example of polyteny, are the giant chromosomes of the larval salivary gland that exhibit a DNA content of 1024-2048C. The border between polyploidy and polyteny is uncertain and numerous intermediate configurations can be observed. An interesting example for the plasticity of the chromosome arrangement is found in the nurse cells of the *Drosophila* ovary, which switch from polyteny to polyploidy. In the first five endoreplication cycles during nurse cell development the chromosomes remain aligned to each other (Dej and Spradling, 1999). After S-phase of the fifth cycle, however, the chromosomes condense and separate from each other. From this point on the chromosomes of the nurse cells maintain the polyploid configuration and continue endoreplication until they reach a DNA content of 1024 C. Besides the nurse cells, the *Drosophila* ovary harbors another cell type that becomes polyploid. During oogenesis, the somatic follicle cells first undergo five mitotic divisions and then initiate endoreplication. Follicle cells execute five endocycles that give rise to DNA content of 16C. However, DNA replication stops completely at this stage. Several loci maintain DNA replication and become amplified. Among these amplified loci are genes required for the formation the chorion of the eggshell. Therefore, this process was termed chorion gene amplification (Calvi et al., 1998).

1.2. The cell cycle control system

During cell division the cell is confronted with numerous problems challenging the correct inheritance of the genetic information of the progenitor cell. Cells have to ensure that the genome is only duplicated once per cell cycle. Then, the chromosomes must be distributed evenly between both daughter cells and thereby each cell must receive a full copy of the genome. I most cases, the cell cycle must be coordinated with cell growth to maintain a constant cell size. In order to prevent the inheritance of severe chromosomal defects, safeguard mechanisms have to interrupt cell cycle progression after genomic damage. In multicellular organisms proliferation must be coordinated with the demands of the developmental program and the housekeeping mechanisms, respectively. Finally, it must be ensured that these events occur in a ordered manner and that the cell cycle proceeds only in one direction. To achieve all these tasks eukaryotic cells have evolved a tightly regulated cell cycle control system that is basically conserved throughout the animal kingdom (for review see Morgan, 2006; Murray, 2004). The heart of the cell cycle control system are the Cyclin dependent kinases (Cdk) and their regulators, the cyclins. At certain stages of the cell cycle,

Cdk's become activated by interaction with a particular Cyclin. Cdk levels remain constant throughout the cell cycle, whereas cyclin levels oscillate. Thus, it is achieved that Cyclin/Cdk activity peaks at specific points of the cell cycle and thereby initiates the next series of cell cycle event. Generally, it is distinguished between two types of cyclins. Cyclins involved in mitosis are referred as mitotic cyclins, whereas cyclins implicated in the initiation of DNA replication are termed G1 cyclins. In *Drosophila*, mitotic cyclins A, B and B3 as well as the G1 cyclins D and E have been identified. Moreover, orthologues of Cdk1, Cdk2 and Cdk2/4 are know in *Drosophila* (for review see Edgar and Lehner, 1996; Lee and Orr-Weaver, 2003; Swanhart et al., 2005).

1.2.1. Initiation and execution of mitosis

Mitosis is initiated in late G2 by the activation of Cyclin/Cdk1 complexes (Minshull et al., 1989; Murray and Kirschner, 1989). Cdk1 only forms complexes with a subset of cyclins, which were generally referred as mitotic cyclins. In *Drosophila,* entry into mitosis is regulated by Cdk1 and the mitotic cyclins A, B and B3 (Jacobs et al., 1998; Knoblich and Lehner, 1993; Lehner and O'Farrell, 1989; Lehner and O'Farrell, 1990a; Lehner and O'Farrell, 1990b). It is thought that the mitotic cyclins partly overlap in their functions, since only loss of cyclin A results in embryonic lethality (Jacobs et al., 1998; Knoblich and Lehner, 1993; Lehner and O'Farrell, 1989; Lehner and O'Farrell, 1990b). Transcription of mitotic cyclins is initiated during late S-phase and results in the accumulation of cyclin/Cdk1 complexes during G2. To prevent premature entry into mitosis, Cdk1 activity is restrained by inhibitory phosphorylation at threonine residue 14 and tyrosine residue 15. These phosphorylations are mediated by kinases of the conserved Wee1/Myt1 family (Morgan, 1995). To initiate mitotic entry, these inhibitory phosphorylations are removed by a Cdc25 phosphatase (Russell and Nurse, 1986). The *Drosophila* genome bears two different isoforms of Cdc25, named *string* and *twine*. String activity becomes essential for the first time after midblastula transition and is then required for all mitotic divisions throughout development (Edgar et al., 1994a; Edgar and O'Farrell, 1990). By contrast, the activity of Twine is restricted to meiosis (Edgar and Datar, 1996)

After mitotic entry, the Cyclin/Cdk1 complex must be inactivated to allow proper progression through mitosis (Murray et al., 1989). The downregulation of Cdk1 activity is achieved by degradation of the Cyclin subunit (Glotzer et al., 1991). In *Drosophila*, mitotic cyclins are

sequentially degraded (Figure 5; Sigrist et al., 1995). Cyclin A degradation is initiated in metaphase just before chromosome separation. Cyclin B degradation occurs in early anaphase when the chromosomes are separated. Cyclin B3 gets degraded in late anaphase after chromosome segregation. The degradation of B-type cyclins depends on a conserved protein motif named destruction box (Glotzer et al., 1991; King et al., 1996). Deletion of this destruction box (D-box) results in a stable Cyclin B protein. Overexpression of this stable Cyclin B in *Drosophila* embryos leads to an arrest in early anaphase (Sigrist et al., 1995). Moreover, overexpression of a stable version of Cyclin B3 specifically arrest the cell cycle in late anaphase (Sigrist et al., 1995). So far no particular motif that mediates Cyclin A destruction has been identified. However, a N-terminal truncated version of Cyclin A is refractory to degradation and overexpression of this fragment results in an metaphase arrest (Sigrist et al., 1995). Based on these observations, it has been proposed that the successive steps required for the completion of mitosis are ordered by the sequential degradation of mitotic cyclins (Sigrist et al., 1995).

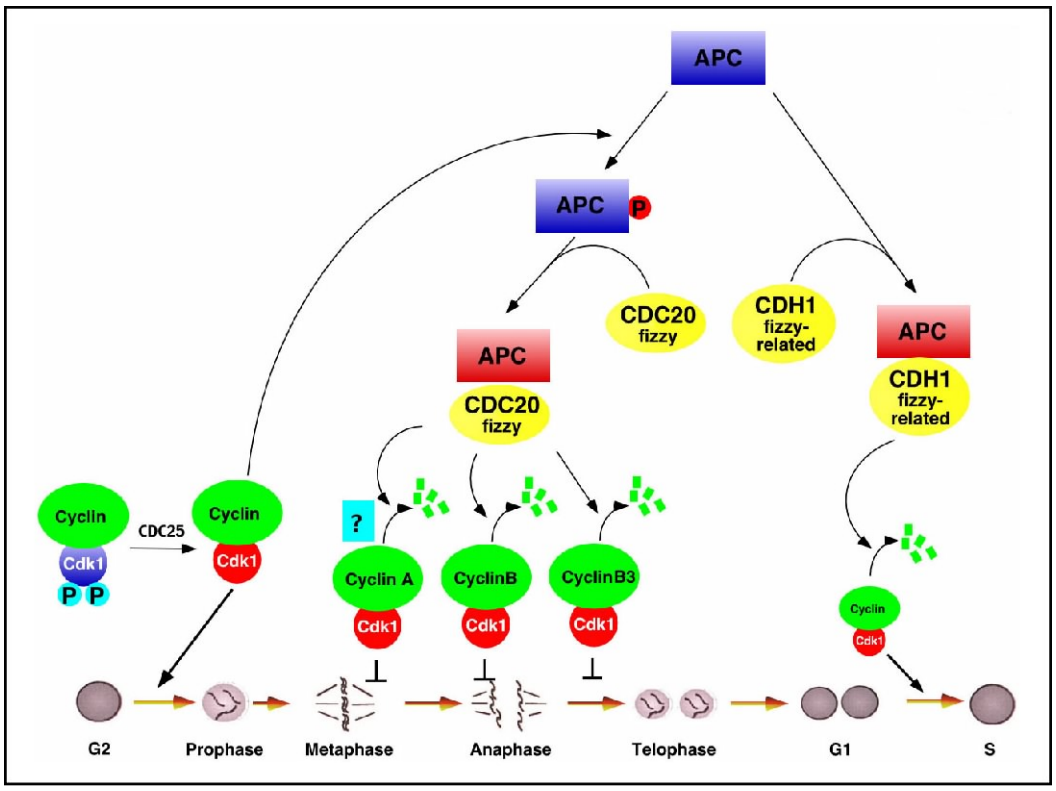

Figure 5 Regulation of the anaphase promoting complex/cyclosome (APC/C). To initiate mitosis, Cyclin/Cdk1 is activated by the phosphatase String/Cdc25. The APC/C gets subsequently phosphorylated and thereby activated through Cdc20/Fzy binding. The activated APC/C-Cdc20 complex initiates in turn the sequential degradation of mitotic cyclins and thus the downregulation of Cdk1 activity. After depletion of Cdk1 activity the APC/C forms a complex with another activator protein, Cdh1/Fzr. The activity of APC/C-Cdh1complex is crucial for the initiation of the following G1-phase.

The degradation of mitotic cyclins and numerous other proteins is mediated by the 26S proteasome, a multi-subunit protease specific for multi-ubiquitinated substrates (for review see Baumeister et al., 1998; Coux et al., 1996; Hochstrasser, 1996). In order to mark proteins for proteasomal degradation, multi-ubiquitin chains are transferred in a three-step reaction to the substrate. In the first step, ubiquitin is activated by forming a high-energy thioester between a cysteine of its active site and the C-terminus of ubiquitin. The activated ubiquitin is subsequently transferred to one of several ubiquitin-conjugating enzymes that are also named E2-enzymes. Finally, the ubiquitin is covalently attached to the substrate protein by an ubiquitin-protein ligase or E3-enzyme, respectively. The ubiquitin ligase mediating the proteasomal degradation of mitotic cyclins is called anaphase promoting complex or cyclosome (for review see Peters, 2006; Pines, 2006; Zachariae and Nasmyth, 1999). The anaphase promoting complex (APC/C) is a high molecular weight complex that consists of at least eleven subunits (Gieffers et al., 2001; Passmore et al., 2005). The activity of the APC/C depends of its phosphorylation state and the abundance of two WD40 activator proteins, Cdc20 and Cdh1 (Schwab et al., 1997; Visintin et al., 1997). The APC/C-Cdc20 complex gets only activated during mitosis to mediate the proteasomal degradation of mitotic cyclins and other cell cycle regulators. The activation of the APC/C by Cdc20 depends on the phosphorylation state of the APC/C (Kraft et al., 2003; Peters et al., 1996). Cdc20 can only bind to the APC/C once several APC/C subunits have been phosphorylated (Kramer et al., 2000) (Kramer et al., 1998). In vertebrates, APC/C phosphorylation is achieved by mitotic kinases such as Cyclin/Cdk1 and polo like kinase 1 (Descombes and Nigg, 1998; Patra and Dunphy, 1998). Hence, APC/C-Cdc20 activity is restricted to early mitosis. By contrast, Cdh1 can only activate the APC/C at stages with low Cdk activity (Kramer et al., 2000; Zachariae et al., 1998). It is thought that Cdk1 and Cdk2 phosphorylate Cdh1 thereby preventing APC/C activation (Kramer et al., 2000; Sorensen et al., 2000; Zachariae et al., 1998). Thus, the APC/C-Cdh1 complex is only active during late mitosis and G1, when Cyclin/Cdk activity is dampened. In addition to a destruction box, substrates of the APC/C-Fzr complex frequently contain a KEN-box (Burton and Solomon, 2001; Hilioti et al., 2001; Pfleger et al., 2001). The *Drosophila* orthologues of Cdc20 and Cdh1 are encoded by the genes *fizzy (fzy)* and *fizzy-related (fzr)*, respectively. Cells in *fizzy* mutant embryos fail to downregulate mitotic cyclins and subsequently arrest in metaphase (Dawson et al., 1993; Dawson et al., 1995; Sigrist et al., 1995). Epidermal cells of embryos lacking *fizzy-related* cannot establish the terminal G1-phase and undergo an additional seventeenth mitosis (Sigrist and Lehner, 1997). Closer inspection of *fizzy-related* mutants revealed that completion of this

additional mitosis (including cyclin degradation) does not require *fizzy-related* (Jacobs et al., 2002). Moreover, cells in eye discs derived from hypomorphic *fizzy-related* mutants fail to become synchronized in G1 and enter ectopic S-phases (Pimentel and Venkatesh, 2005). Therefore APC/C-Fzr activity is thought to be only required for establishment and maintenance of the G1 state (Figure 5).

1.2.2. Regulation of the G1-S transition

In vertebrates, the transition from G1 to S-phase is regulated by three different kinases, Cdk2, Cdk4 and Cdk6. In response to external growth signals Cyclin D expression is stimulated (Matsushime et al., 1994; Sherr, 1993). Cyclin D activates Cdk4/6 and members of retinoblastoma (Rb) tumour suppressor family are subsequently inhibited. The inhibition of Rb leads to the release of a transcription factor of the E2F family (Attwooll et al., 2004; Blais and Dynlacht, 2004; Kato et al., 1993). E2F stimulates the transcription of Cyclin E and Cyclin A (DeGregori et al., 1995; Pagano et al., 1992). Besides these cyclins, E2F activates the transcription of numerous other genes required for DNA replication such as ribonucleotide reductase (RNR) and the DNA polymerase δ accessory subunit, PCNA (DeGregori et al., 1995). Cyclin E and Cyclin A activate Cdk2 and initiate in turn DNA replication (Dutta and Stillman, 1992; Pagano et al., 1992). Moreover, Cdk2 also phosphorylates Rb and enhances thereby its own activation by increased Cyclin E and A transcription. In *Drosophila*, single genes for Cdk4 and Cyclin D have been identified (Datar et al., 2000; Meyer et al., 2000; Sauer et al., 1996). *Drosophila* Cyclin D/Cdk4 is not directly implicated in the transition from G1 to S-phase, although it can phosphorylate Rb (Datar et al., 2000; Meyer et al., 2000; Xin et al., 2002). It rather appears that the Cyclin D/Cdk4 complex is involved in growth regulation, but it remains to be clarified how this function is achieved (Datar et al., 2000; Meyer et al., 2000). Recently it has been demonstrated that this pathway requires mitochondrial activity and Hph, a hydroxylase implicated in the cellular response to low oxygen (Frei and Edgar, 2004; Frei et al., 2005). In *Drosophila,* S-phase induction mainly relies on Cyclin E/Cdk2 activity (Knoblich et al., 1994; Richardson et al., 1995). Cyclin E/Cdk2 phosphorylates the sole Rb ortholog in *Drosophila* (Rbf) and stimulates thereby transcription of S-phase genes via E2F1 (Du et al., 1996; Duronio and O'Farrell, 1994; Duronio and O'Farrell, 1995; Duronio et al., 1995). In addition, S-phase can be induced by Cyclin A overexpression or by loss of the Cyclin A/Cdk1 inhibitor Roughex, respectively (Foley et al., 1999; Sprenger et al., 1997; Thomas et al., 1997). Exit from S-

phase is facilitated by downregulation of Cyclin E dependent kinase activity. After autophosphorylation, Cyclin E is therefore targeted for degradation by an SCF ubiquitin ligase complex (Koepp et al., 2001; Moberg et al., 2001; Schwab and Tyers, 2001). In *Drosophila*, Cyclin E degradation is mediated by the F-box protein Archipelago that was initially identified in a screen for mutants causing overproliferation (Moberg et al., 2001).

The SCF ubiquitin ligases are named by their core subunits Skp, Cullin, and F-box protein (for review see Ang and Wade Harper, 2005; Jackson et al., 2000; Vodermaier, 2004). Apart from the three core subunits, SCF complexes contain a RING finger protein as well as an E2-enzyme (Jackson et al., 2000). SCF complexes are implicated in a plethora of processes such as cell cycle regulation, signalling pathways, circadian rhythms and apoptosis (Grima et al., 2002; Koepp et al., 1999; Maniatis, 1999; Nateri et al., 2004). SCF complexes are only distinguishable by their F-box proteins that confer substrate specificity (Skowyra et al., 1997). F-box proteins are characterized by a conserved motif that was first identified in Cyclin F and thus named F-box (Bai et al., 1996). In addition F-box proteins frequently contain protein motifs involved in protein-protein interaction (Jin et al., 2004; Winston et al., 1999). It is thought that these domains are required for substrate binding. Substrate recognition by many F-box proteins depends on phosphorylation of the substrate, thereby allowing temporal control of degradation (Orlicky et al., 2003; Skowyra et al., 1997). The F-box protein is attached to the cullin scaffold by an Skp protein that recognizes the F-box (Schulman et al., 2000; Zheng et al., 2002). Mammals and yeast have only a single Skp gene (Skp1), while six Skp proteins (SkpA-F) have been identified in *Drosophila* (Nayak et al., 2002; Yamanaka et al., 2002). So far only SkpA has been characterized in greater detail (Murphy, 2003) and it remains to be elucidated whether the other homologues are implicated in SCF complexes.

1.2.3. Initiation and regulation of DNA replication

At the transition from G1 to S-phase, DNA replication is initiated by increasing Cdk activity. DNA synthesis occurs at specific sites of the chromosomes which are named origins of replication. During late mitosis and early G1 the pre-replicative complex (pre-RC) gets recruited to the replication origins. This process is also known as DNA licensing. Upon S-phase entry the pre-RC gets activated and subsequently triggers DNA replication. The pre-RC contains the helicase that unwinds the DNA and promotes assembly of the actual replication machinery. The formation of the pre-RC relies on the origin recognition complex (ORC). The

ORC complex consists of six subunits and is constantly bound to the replication origins. The ORC can only promote pre-RC formation during late G1 (Figure 6; Bell and Dutta, 2002; Chesnokov et al., 1999; Gossen et al., 1995). In late mitosis and early G1, the actual licensing process begins with binding of Cdt1 and Cdc6 to the ORC complex (Figure 6). After recruitment of Cdt1 and Cdc6 to the ORC, minichromosome maintenance (MCM) proteins are loaded onto the DNA and remain associated with the DNA until S-phase (Figure 6). The MCM2-7 proteins are arranged in a bilobed hexameric structure that surrounds the DNA (Fletcher et al., 2003; Pape et al., 2003). Once the MCM2-7 complex is associated with the DNA the licensing process is completed and the pre-RC can be activated. The components of the pre-RC, namely ORC1-6, Cdt1, Cdc6 and MCM2-7, are conserved among all eukaryotes, including *Drosophila* (Bell and Dutta, 2002; Chesnokov et al., 1999; Gossen et al., 1995; Su et al., 1996). The *Drosophila* ortholog of Cdt1 is also known as *double-parked* (*dup*) (Thomer et al., 2004; Whittaker et al., 2000).

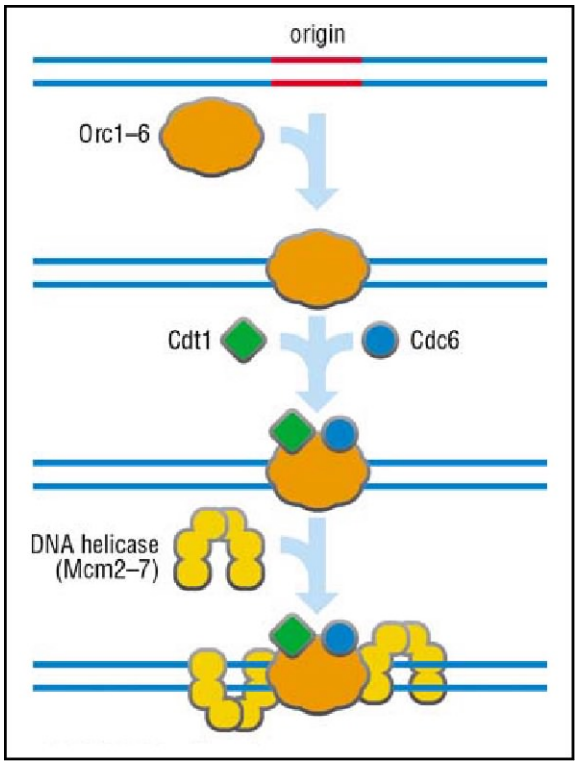

Figure 6 Assembly of the pre-replicative complex (adapted from Morgan, 2006). DNA replication begins at specific sites on the chromosomes named origins. The ORC1-6 complex binds constantly to the replication origins and serves as scaffold for the formation of the pre-replicative complex (pre-RC). In late mitosis and early G1, Cdt1 and Cdc6 are recruited to the ORC complex. Upon binding of Cdt1 and Cdc6, the MCM2-7 helicases are loaded to the DNA.

The precise duplication of the genome is crucial for the survival of an organism. In multicellular organisms any genome instability potentially gives rise to cancer and thus compromises the life of the whole organism. Therefore, several safeguard mechanisms have been evolved ensuring that the DNA is only replicated once per cell cycle (for review see Bell and Dutta, 2002; Blow and Dutta, 2005). After initiation of DNA replication, the MCM2-7

complexes are release from the replication origin and move with the replication fork along the DNA. The release of the MCM2-7 complexes results in inactivation of the pre-RC. Thus, new MCM2-7 complexes cannot be recruited until formation of a novel pre-RC. The assembly of pre-RCs is restricted to late mitosis and early G1, thereby ensuring that mitosis proceeds DNA-replication. In multicellular organisms this is largely achieved by two mechanisms. In late G1, Geminin binds to Cdt1 and prevents the formation of pre-RCs (McGarry and Kirschner, 1998). Geminin is a target of the APC/C which mediates its proteasomal degradation during late mitosis and G1 (McGarry and Kirschner, 1998). At the end of the G1 phase, the APC/C is inactivated, resulting in accumulation of Geminin and subsequent inhibition of Cdt1. A *Drosophila* orthologue of Geminin has been identified, but it remains to be clarified whether *Drosophila* Geminin levels oscillate throughout the cell cycle (Quinn et al., 2001). Since APC/C activity depends on Cdk phosphorylation, the assembly of pre-RCs is indirectly coupled to Cyclin/Cdk activity. However, Cyclin dependent kinase activity contributes also directly to the formation pre-RC. It is assumed that APC/C activity is dispensable for the endoreplication cycles in *Drosophila* (Edgar and Orr-Weaver, 2001; Lilly and Duronio, 2005). However, overexpression of Cyclin E promotes MCM2-7 loading and prevents thereby endoreplication (Follette et al., 1998; Su and O'Farrell, 1998; Weiss et al., 1998). Although phosphorylation of pre-RC subunits appears to be important to inhibit pre-RC assembly, the mechanisms are only poorly understood. In higher eukaryotes (including *Drosophila*), Cdt1 protein levels fluctuate throughout the cell cycle (Nishitani et al., 2001; Thomer et al., 2004). Evidence from several organisms suggest that two different ubiquitin-ligase complexes contribute to the proteasomal degradation of Cdt1. In human cells, a Skp2 containing SCF-complex interacts with Cdt1, whereby Cdt1 binding requires phosphorylation by Cdk2 or Cdk4 (Li et al., 2003; Liu et al., 2004; Nishitani et al., 2001; Sugimoto et al., 2004). In *C. elegans*, an SCF-like complex based on Cullin 4 has been identified that is required to downregulate Cdt1 at the end of G1 (Zhong et al., 2003). In *Drosophila* Dup/Cdt1 degradation seems to be Cyclin E/Cdk1 dependend, but it remains to be clarified whether SCF-E3 ligases are implicated in this process (Thomer et al., 2004).

1.3. The cell cycle regulator Rca1

Tight regulation of APC/C activity is crucial to ensure normal cell cycle progression (for review see Peters, 2006; Pines, 2006; Zachariae and Nasmyth, 1999). The activity of the APC/C depends on its phosphorylation state and the presence of the WD40 activator proteins Fzy/Cdc20 and Fzr/Cdh1 (Schwab et al., 1997; Visintin et al., 1997). During mitosis, phosphorylation of APC/C subunits is prerequisite for its interaction with Fzy, whereas activation of APC/C by Fzr is prevented via phosphorylation (Kramer et al., 2000; Sorensen et al., 2000; Zachariae et al., 1998). Therefore, APC/C-Fzr activity is restricted to later mitotic stages and G1 when Cdk activity is low. Several additional molecules have been identified that regulate APC/C activity by other mechanisms (for review see Peters, 2006; Pines, 2006). Prominent members among these regulators are the vertebrate Emi proteins that restrict APC/C activity at different cell cycle stages (for review see Schmidt et al., 2006). The *Drosophila rca1* gene encodes an APC/C-Fzr inhibitor that is related to the Emi1 proteins (Grosskortenhaus and Sprenger, 2002). Embryos homozygous mutant for *rca1*, fail to execute the 16th mitosis of *Drosophila* embryogenesis. Due to this G2 arrest, *rca1* mutants display a reduced number of epidermal cells compared to wild-type (Dong et al., 1997). Since this phenotype resembles mutants for Cyclin A (Lehner and O'Farrell, 1989), the gene was named *regulator of Cyclin A* 1 (*rca1*). The G2 arrest in *rca1* mutants is caused by premature degradation of the mitotic cyclins A and B (Grosskortenhaus and Sprenger, 2002). In *Drosophila,* degradation of mitotic cyclins is mediated by the APC/C and the two activator proteins, Fizzy (Fzy) and Fizzy-related (Fzr) (Dawson et al., 1995; Sigrist et al., 1995; Sigrist and Lehner, 1997). Mutants for *fzr* fail to establish the terminal G1-phase and execute an extra cell cycle (Sigrist and Lehner, 1997). Since overexpression of Cyclin A abolishes the terminal G1 arrest (Sprenger et al., 1997), this additional mitosis 17 is probably due to accumulation of Cyclin A. By contrast, overexpression of Fzr prevents accumulation of mitotic cyclin and entry into mitosis 16 (Sigrist and Lehner, 1997). Double mutants for *rca1* and *fzr* display epidermal cell numbers similar to wild-type, indicating that mitosis 16 occurs normally in these embryos (Grosskortenhaus and Sprenger, 2002). Moreover, simultaneous overexpression of Fzr and Rca1 allows normal cyclin accumulation and execution of mitosis 16 (Grosskortenhaus and Sprenger, 2002). Hence, these experiments demonstrated that Rca1 has a negative effect on Fzr. In addition, co-immunoprecipitation experiments revealed that Rca1 and Fzr also interact physically (Grosskortenhaus and Sprenger, 2002). Altogether, these data give rise to the model that Rca1 restrains APC/C-Fzr activity during G2 of cell cycle 16 to allow cyclin accumulation and subsequent entry into terminal mitosis (Figure 7).

Moreover, overexpression of Rca1 can overcome the G2 arrest in Cyclin A mutant embryos (Dienemann and Sprenger, 2004). This suggests that Cyclin A/Cdk1 activity also contributes to APC/C-Fzr inhibition in G2 of cell cycle 16 (Figure 7).

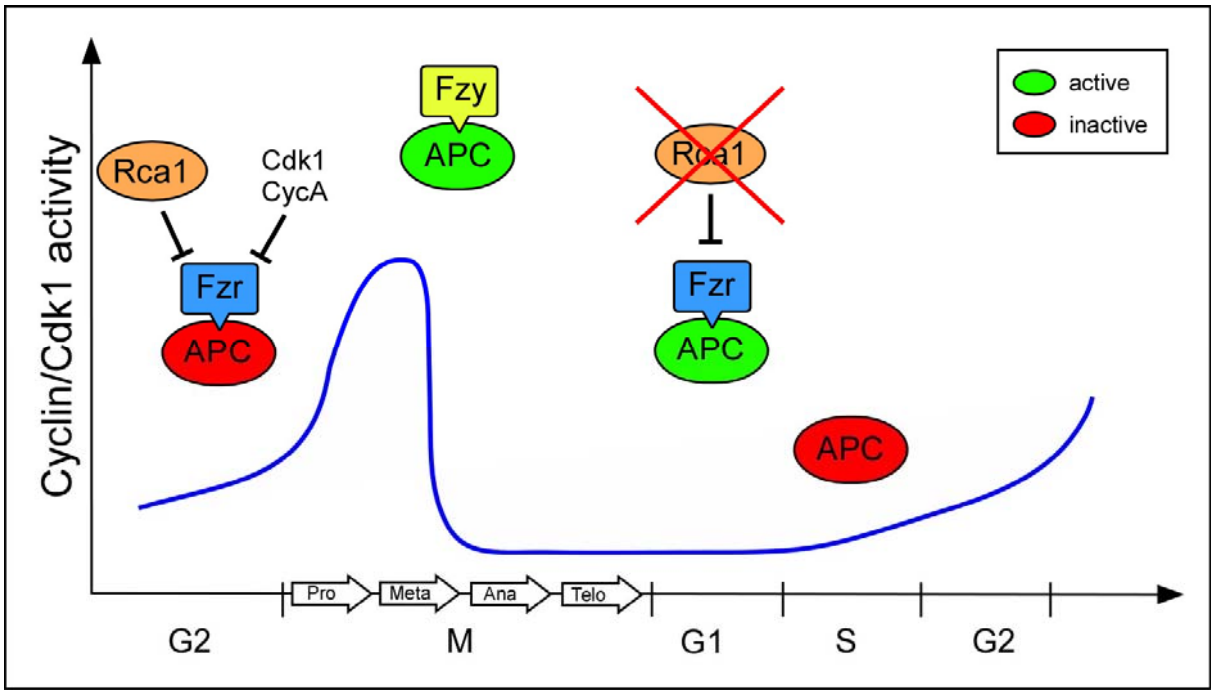

Figure 7 Rca1 prevents untimely activation of APC/C-Fzr complex. APC/C activity is crucial for execution of mitosis and the establishment of the G1-phase**.** At the metaphase-anaphase transition the APC/C is activated by Fzy and mediates the degradation of mitotic regulators such as cyclins**.** Since APC/C activation by Fzy requires high kinase activity, APC/C-Fzy activity is restricted to mitosis. By contrast, Fzr can only bind to the APC/C at stages with low kinase activity as found in late mitosis, G1 and G2. During G2, APC/C-Fzr activity is dampened by Rca1 to allow the accumulation of Cyclin/Cdk1 activity and entry into mitosis. In addition, recent evidences suggest that Cyclin/Cdk1 activity also contributes to APC/C-Fzr inhibition in G2. In G1, Rca1 activity has to be restricted to allow APC/C-Fzr activity.

Rca1 was initially identified in a screen for suppressors of the *roughex* eye phenotype (Dong et al., 1997). Roughex is an inhibitor of Cyclin A depend kinase activity (Foley et al., 1999). Flies carrying weak alleles of *roughex* display a rough eye phenotype (Thomas et al., 1994). In *roughex* mutants, cells of eye imaginal discs enter S-phase prematurely because they fail to downregulate Cyclin A/Cdk1 activity in G1 (Thomas et al., 1997). Moreover, eye imaginal disc cells in hypomorphic *fzr* mutants fail to undergo the G1 arrest in the morphogenetic furrow and display elevated levels of mitotic cyclins (Pimentel and Venkatesh, 2005). Hence, demonstrating that two different mechanisms contribute to the inhibition of Cyclin A/Cdk1 activity in G1. On the one hand, Cdk1 activity is restricted by the action of the Cdk1 inhibitor Roughex (Sprenger et al., 1997; Thomas et al., 1997). On the other hand, S-phase entry is prevented by APC/C-Fzr complex which mediates the destruction of Cyclin A. Mitotic cyclins accumulate upon entry into S-phase, suggesting that APC/C-Fzr activity is

downregulated after progression through G1. It has been demonstrated that Cyclin A/Cdk1 acts negatively on APC/C-Fzr activity (Dienemann and Sprenger, 2004). However, if APC/C-Fzr inactivation is required for Cyclin A accumulation, this rises the question how Cyclin A/Cdk1 activity can accumulate and inactivate Fzr. In human cell culture, it has been shown that Emi1, the vertebrate ortholog of Rca1, promotes S-phase entry by inhibiting the APC/C-Cdh1 complex (Hsu et al., 2002). In late G1-phase, E2F stimulates the transcription of Emi1 to allow Cyclin A accumulation and subsequent entry into S-phase (Hsu et al., 2002). Depletion of Emi1 levels by RNAi prevents S-phase entry while cells overexpressing Emi1 progress faster through G1 (Hsu et al., 2002). Overexpression of Rca1 also drives cells into ectopic S-phases (Dong et al., 1997). Furthermore, the premature entry into S-phase in *roughex* mutants can be suppressed by reduced activity of Rca1 (Dong et al., 1997). Therefore, it seems conceivable that Rca1 might have a similar function at the G1-S transition as Emi1. Moreover, a recent study proposed that Emi1 might have a secondary function at the G1-S transition beyond APC/C inhibition and this could be possible for Rca1 (Rape and Kirschner, 2004).

1.3.1. The Rca1/Emi1 family

Rca1 shares limited homology (18% identity) to the vertebrate Emi1 proteins (Reimann et al., 2001a). Emi1 was initially identified as an APC/C inhibitor specific for early mitotic stages, but further work revealed that Emi1 is also implicated in the transition from G1 to S-phase (Hsu et al., 2002; Reimann et al., 2001a). In contrast to Rca1, the Emi1 proteins are able to inhibit Cdc20 and Cdh1 dependent APC/C activity (Hsu et al., 2002; Reimann et al., 2001a; Reimann et al., 2001b). Despite their low identity, Rca1 and Emi1 display an intriguingly similar arrangement of their functional domains (Figure 8). All Rca1/Emi1 proteins contain an F-box in their central region followed by a zinc binding region (ZBR) in the C-terminal part. The ZBR is assumed to be involved in protein-protein interaction. Additionally, they harbor different putative nuclear localization signals as well as several sequence motifs that might be involved in degradation. Finally, they contain several potential Cdk1 phosphorylation sites distributed throughout the protein. Two recent reports described the identification of a novel meiosis specific homologue of Emi1 called Emi2/XErp1 (Schmidt et al., 2005; Tung et al., 2005). Emi2/XErp1 is an inhibitor of the APC/C-Fzy complex that seems to be required for maintenance of the cyctostatic 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 display a ZBR as well as an F-box domain (Schmidt et al., 2005; Tung et al., 2005).

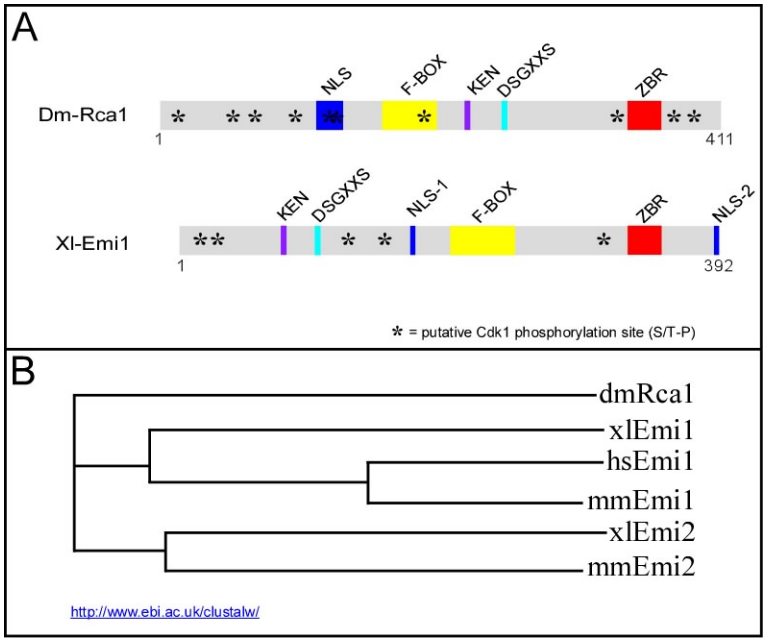

Figure 8 Rca1 is related to APC/C inhibitors of the Emi1/Emi2 family. (A) Overview of the conserved motifs in Rca1 and *Xenopus* Emi1. Both proteins contain an F-Box and a C-terminal zinc binding region (ZBR). The N-terminal part of the xlEmi1 harbours a conserved DSGxxS that is crucial for Emi1 degradation. Rca1 displays a similar motif and a KEN-box in its central region. Finally, both proteins contain various numbers of putative Cdk phosphorylation sites marked by an asterisk. xlEmi1 was used as showcase and the features described for xlEmi1 apply basically to all Emi1/Emi2 proteins. **(B)** Rca1 is a distant relative of the Emi1/Emi2 family. The phylogenetic tree was generated by the ClustalW multiple sequence alignment tool.

All Rca1/Emi family members belong to the class of F-box proteins. F-box proteins are part of SCF (Skp-Cullin-F-box) ubiquitin ligases that are involved in targeting of numerous substrates for degradation (Ang and Wade Harper, 2005; Kipreos and Pagano, 2000; Maniatis, 1999). Emi1 was identified in a screen for Skp1 interaction partners and deletion of the F-box prevents Skp1 binding *in vitro* (Reimann et al., 2001a). In addition, yeast two-hybrid data indicated that Emi2/XErp1 interacts also with Skp1 in a F-box dependent manner (Schmidt et al., 2005). A genome wide yeast two-hybrid analysis demonstrated that Rca1 interacts with *Drosophila* SkpA and B (Giot et al., 2003). These observations indicate that the Rca1/Emi proteins contain functional F-box domains. However, several studies demonstrated that the F-box of the Emi1 proteins is dispensable for its inhibitory effect on the APC/C (Reimann et al., 2001a; Schmidt et al., 2005). Thus, the *in vivo* function of the F-box remains unclear. It has to be elucidated whether Rca1/Emi proteins act as classical F-box proteins in an SCF complex that targets proteins for proteasomal degradation.

1.3.2. Regulation of Rca1/Emi1 activity

Since APC/C-Fzr activity is crucial for the establishment of the G1-phase (Jacobs et al., 2002; Pimentel and Venkatesh, 2005), Rca1 activity has to be eliminated at this stage. During embryogenesis, Rca1 is degraded specifically at the stage when the epidermal cell enter the

terminal G1-phase (Grosskortenhaus and Sprenger, 2002). At the moment it is unclear which pathways mediates Rca1 degradation in G1 and how this is regulated. However, much more is known about the regulation of Emi1 and these mechanisms could also apply to Rca1 (Figure 9).

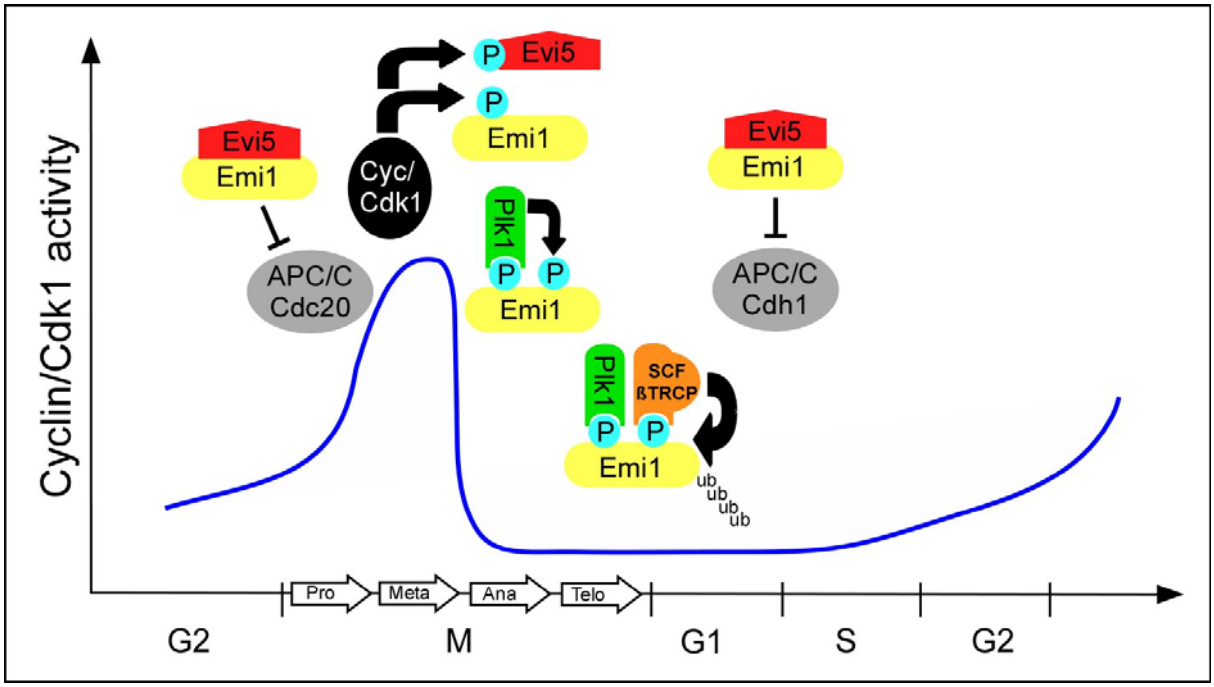

Figure 9 Emi1 degradation is regulated at multiple levels. During early mitosis, Emi1 inhibits APC/C-Cdc20 activity and enables thereby the accumulation of mitotic cyclins. In addition, Emi1 promotes S-phase entry by APC/C-Cdh1 inhibition. The stabilizing factor Evi5 accumulates in late G1 and maintains Emi1 levels during S/G2. During prophase Evi5 is phosphorylated by polo like kinase 1 (plk1) and becomes subsequently degraded. After Cdk1 phosphorylation, Emi1 is then also phosphorylated by Plk1 1. Phosphorylated Emi1 is recognized by the SCF/ßTRPC complex, which subsequently targets Emi1 for proteasomal degradation.

Emi1 activity is necessary for normal progression through mitosis and promotes the transition from G1 to S-phase (Hsu et al., 2002; Reimann et al., 2001a). In early mitosis Emi1 prevents premature APC/C-Cdc20 activation to facilitate the increase of Cyclin/ Cdk1 activity (Reimann et al., 2001a). To allow normal cyclin destruction in mitosis, Emi1 is degraded during prophase (Reimann et al., 2001a). The proteasomal degradation of Emi1 is mediated by the SCF/ßTRCP complex and persists until G1 (Guardavaccaro et al., 2003; Margottin-Goguet et al., 2003). The F-box protein ßTRCP specifically recognizes a DSGxxS consensus site in Emi1. ßTRCP can only bind to the DSGxxS degron when both serines have been phosphorylated by polo like kinase 1 (Plk1) (Hansen et al., 2004; Moshe et al., 2004). Plk1 activity originates in G2 and persists until early G1. Therefore, premature activation of Emi1 degradation must be prevented in early mitosis. The initiation of Emi1 degradation by Plk1 requires the previous phosphorylation by Cyclin/Cdk1 and is thereby directed to later mitotic

stages (Margottin-Goguet et al., 2003; Reimann et al., 2001a). In addition, the Evi5 oncogene has been identified as a stabilizing factor for Emi1 (Eldridge et al., 2006). Evi5 accumulates in early G1 and shields Emi1 from Plk1 phosphorylation by binding to a site adjacent to the DSGxxS degron. After progression through early mitosis, Evi5 degradation is triggered by Plk1 and Emi1 is then accessible for Plk1. The basic components of this pathway such as ßTRCP and Plk1 are conserved in *Drosophila* (Barr et al., 2004; Jiang and Struhl, 1998). Moreover, Rca1 also contains a putative DSGxxS degron in its central region (Figure 8). Therefore it is conceivable that Rca1 degradation is achieved in a similar manner.

2. Aim

APC/C activity is crucial for normal progression through mitosis and establishment of the G1-phase. During mitosis, APC/C activity depends on the WD40 proteins Cdc20/Fzy, while in G1 the APC/C is activated by Fzr. Previous work has shown that Rca1 is an inhibitor of the APC/C-Fzr complex. During G2 of the terminal cell cycle of *Drosophila* embryogenesis, Rca1 prevents the untimely activation of the APC/C-Fzr complex . At this stage APC/C-Fzr activity is also antagonized by Cyclin A/Cdk activity. Using different genetic approaches, it should be addressed whether this also applies to earlier cell cycles. Moreover, it should be elucidated whether other Cyclin dependent kinases contribute to APC/C-Fzr inhibition. Overexpression of Rca1 during eye development promotes S-phase entry, suggesting that Rca1 might be implicated in the transition from G1 to S-phase. A major goal of this study is to determine whether Rca1 has a second function at the G1-S transition and whether this function relies on its inhibitory effect on the APC/C-Fzr complex. Furthermore, Rca1 contains several conserved protein motifs. The role of these domains for Rca1 function and regulation should be elucidated in a structure/function analysis. Since Rca1 contains a conserved F-Box with so far unknown function, this analysis should be particularly focused on this F-box motif.

3. Results

3.1. Rca1 regulates mitotic entry in postblastoderm embryos in concert with Cyclin/Cdk complexes

The last three embryonic cell cycles (14-16) which take place just before most of the epidermal cells become quiescent are generally referred as postblastoderm cell cycles. During this stage cell division occurs in an invariant spatiotemporal pattern that is tightly linked to the developmental program (Edgar et al., 1994a; Edgar et al., 1994b; Foe, 1989; Hartenstein and Campos-Ortega, 1986). These divisions are the first cell cycles that rely on zygotic gene expression. The postblastoderm cell cycles are G2-M controlled and lack a distinct G1-phase, since the mediators of S-phase entry are still expressed constitutively (Edgar and O'Farrell, 1990; Sauer et al., 1995). The decision to enter mitosis at the G2-M transition is mainly regulated by Cdk1/cyclin complexes (Edgar et al., 1994a; Minshull et al., 1989; Murray and Kirschner, 1989). The spatiotemporal pattern of mitosis originates from the differential expression of the phosphatase String/Cdc25 which is crucial for Cdk1 activation (Edgar et al., 1994a; Edgar et al., 1994b; Edgar and O'Farrell, 1989). Due to large maternal stocks, the amount of Cdk1 and mitotic cyclins is not limiting for mitotic entry until interphase 16 (Knoblich and Lehner, 1993; Lehner and O'Farrell, 1989). Following mitosis 16, the developmental program introduces a major transition of the cell cycle program by preparing cells to enter a terminal G1-phase (Edgar and O'Farrell, 1990). This G1 arrest is achieved by the developmentally programmed downregulation of Cyclin E dependent kinase activity (Knoblich et al., 1994).

3.1.1. Cyclin E dependent kinase activity contributes to downregulation APC/C-Fzr activity during G2

In most multicellular organisms mitosis is controlled generally by two classes of cyclins, which are named A- and B-type cyclins. In addition, certain species display also a third class of mitotic cyclins, named Cyclin B3-type that is less important for progression through mitosis (Humbert et al., 2004). In *Drosophila* and several other species, loss of Cyclin B and Cyclin B3 respectively does not affect viability, whereas mutants for Cyclin A are embryonic lethal (Humbert et al., 2004; Jacobs et al., 1998; Knoblich and Lehner, 1993; Lehner and O'Farrell, 1989; Lehner and O'Farrell, 1990b). Epidermal cells of Cyclin A mutant embryos fail to execute the 16^{th} mitotic division resulting in reduced numbers of epidermal cells. A

phenotype that is also found in embryos mutant for the *rca1* (regulator of Cyclin A 1) gene (Dong et al., 1997; Grosskortenhaus and Sprenger, 2002). Rca1 is an inhibitor of the APC/C-Fzr-complex in G2 and mitotic cyclins become prematurely degraded in *rca1* mutants (Dong et al., 1997; Grosskortenhaus and Sprenger, 2002). Interestingly, Cyclin B disappears in Cyclin A mutants just before entry into mitosis 16 (Dienemann and Sprenger, 2004; Lehner and O'Farrell, 1990b). This observation raised the idea that in *cycA* mutants APC/C-Fzr activity might be upregulated as well. The premature disappearance of Cyclin B in *cycA* mutants can be prevented by Rca1 overexpression (Dienemann and Sprenger, 2004). Therefore it has been proposed that APC/C-Fzr activity is also restricted by inhibitory phosphorylation mediated by the Cdk1/Cyclin A complex (Dienemann and Sprenger, 2004). This function can apparently not be fulfilled by Cdk1/Cyclin B, providing a reasonable explanation why Cyclin A is the only mitotic cyclin with a lethal phenotype.

Previous studies in human cell culture suggested that in G2 APC/C-Fzr activity is inhibited by Cdk2 mediated phosphorylation (Kramer et al., 2000; Lukas et al., 1999; Sorensen et al., 2001). In *Drosophila*, Cdk2 only forms complexes with Cyclin E (Knoblich et al., 1994), whereas in vertebrates it was shown that Cdk2 inhibits APC/C-Fzr activity only in conjunction with Cyclin A and not with Cyclin E (Lukas et al., 1999). However, overexpression of Cyclin E suppresses the $rca1^{2}$ mutant phenotype (Grosskortenhaus and Sprenger, 2002), suggesting that APC/C-Fzr activity is also negatively regulated by Cyclin E/Cdk2. To test this hypothesis, Cyclin E was overexpressed in $cycA^{C8LR1}$ mutant embryos utilizing the *paired-Gal4* driver-line. Quantification of epidermal cell numbers indicated that Cyclin E overexpression in $cycA^{C8LR1}$ mutants results in an increase of epidermal cell numbers (Figure 10A-D). Furthermore, inspection of cyclin B levels in these embryos revealed that Cyclin E overexpression prevents premature degradation of Cyclin B (Figure 10E&F). Hence, these results demonstrate that overexpression of Cyclin E restores mitosis 16 in $cycA^{C8LR1}$ mutants. The stabilization of Cyclin B suggests moreover that the APC/C-Fzr complex is also negatively regulated by Cyclin E dependent kinase activity.

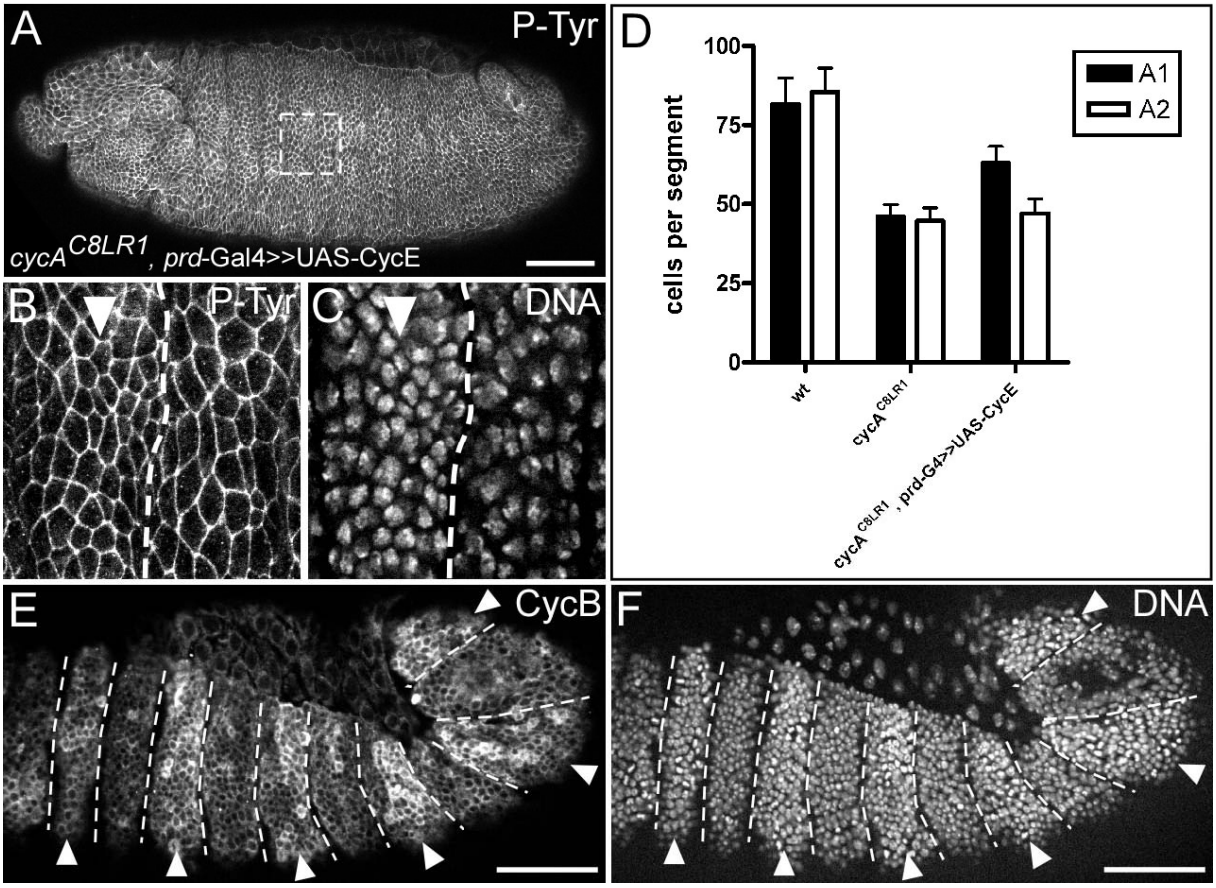

Figure 10 Cyclin E overexpression restores mitosis 16 in *cycA*^{C8LR1} mutants. UAS-Cyclin E was overexpressed in the *cycA*^{C8LR1} mutant background using the *paired*-Gal4 driver-line. Scale bar: 50µm
(A-D) Cyclin E overexpressing segments display increased epidermal cell numbers, demonstrating that excess Cyclin E rescues entry into mitosis 16 in *cycA*^{C8LR1} mutant embryos. Cells were visualized with antibodies against p-Tyr as well as DNA staining. The diagram on the right side shows a comparison of epidermal cell numbers determined in the indicated genotypes. Epidermal cell numbers were quantified in the first two abdominal segments as described in Figure 11.
(E&F) Overexpression of Cyclin E stabilizes Cyclin B levels in *cycA*^{C8LR1} mutants, suggesting that Cyclin E/Cdk2 prevents upregulation of APC/C-Fzr activity.

3.1.2. The Cyclin A phenotype is enhanced by downregulation of Cyclin E/Cdk2 activity

The observations that entry into mitosis 16 in *cycA* mutant embryos can be rescued by overexpression of Cyclin E, suggests that APC/C-Fzr activity is also inhibited by Cyclin E/Cdk2. However, at this stage Cyclin E/Cdk2 activity decays due to upregulation of the CKI Dacapo and downregulation of Cyclin E transcription (de Nooij et al., 1996; Knoblich et al., 1994; Lane et al., 1996), raising the question whether this is a general mechanism or an artefact due to overexpression. APC/C-Fzr activity is thought to be required for cell cycle exit, since Fzr expression culminates at the end of cell cycle 16 (Sigrist and Lehner, 1997). However, examination of Fzr levels in embryo extracts revealed that Fzr is also abundant at earlier stages (Raff et al., 2002). Furthermore, *rca1*^{2}*; cycA*^{C8LR1} double mutants displayed a

dramatically reduced number of epidermal cells, even compared to single mutants for *rca1*^{2} and *cycA*^{C8LR1}, respectively (Figure 11; Grosskortenhaus and Sprenger, 2002). It is therefore assumed that APC/C-Fzr activity is also present during cell cycle 15.

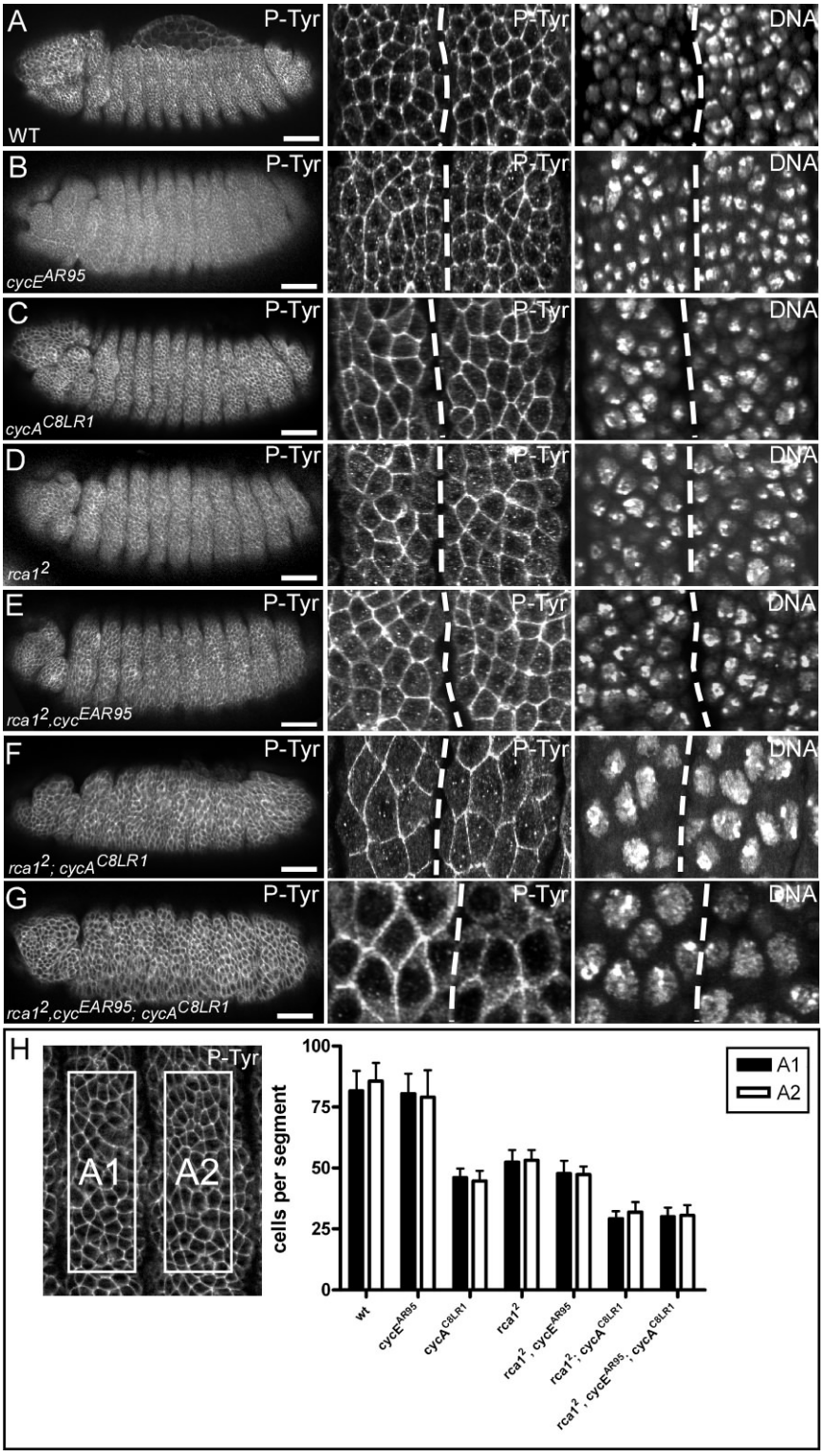

Figure 11 APC/C-Fzr is active during cell cycle 15 of *Drosophila* embryogenesis.
To compare cell cycle progression in the indicated genotypes epidermal cell numbers were quantified (H). The number of epidermal cells were determined in defined regions of the first two abdominal segments in stage 13 embryos (n≥10). Cells were visualized with antibodies against p-Tyr as well as DNA staining. An example of each genotype is depicted in the upper part of the Figure. Scale bar : 50µm
(A-H) Due to the G2 arrest in cell cycle 16, *rca1*^{2} mutant embryos (D) contain a reduced number of epidermal cells compared to wild-type (E) embryos of the same stage. *cycA*^{C8LR1} embryos (C) display slightly reduced numbers of epidermal cells compared to *rca1*^{2} mutants, but it appears that they also arrest during the 16th cell cycle. In *rca1*^{2}*; cycA*^{C8LR1} mutants (F) the epidermal cell number is remarkably reduced compared to *rca1*^{2} mutant embryos (D), indicating that the epidermal cells of this embryos arrest already during the 15th cell cycle. Genetic elimination of Cyclin E in *rca1*^{2} and *rca1*^{2}*; cycA*^{C8LR1} mutants has no significant effect on epidermal cell numbers (E&G), suggesting that either Cyclin E/Cdk2 activity is not required at this stage or that Cyclin E/Cdk2 is no completely removed in *cycE*^{AR95} mutants.

To test whether Rca1, Cyclin A/Cdk1 and Cyclin E/Cdk2 act in concert to restrain Fzr-activity at earlier stages, *rca1*^{2}, *cycE*^{AR95} double mutants as well as *rca1*^{2}, *cycE*^{AR95}; *cycA*^{C8LR1} triple mutants were generated. In *cycE*^{AR95} mutant embryos, the amount of Cyclin E is remarkable reduced, but the maternal supply is sufficient to drive the cells through mitosis 16 (Knoblich et al., 1994; Vidwans et al., 2002). Consistently, the epidermal cells in Cyclin E deficient embryos proliferate normally, resulting in equal epidermal cell numbers as in wild-type embryos (Figure 11A&B). The slight reduction of Cyclin E activity does neither enhance the *rca1*^{2} mutant nor the *rca1*^{2}; *cycA*^{C8LR1} double mutant phenotype, indicated by of epidermal cell numbers comparable to the origin stocks (Figure 11). Thus, it remains unclear whether Cyclin E/Cdk2 activity contributes to the downregulation of APC/C-Fzr activity in postblastoderm embryos.

The investigation whether Cyclin E/Cdk2 restrains APC/C-Fzr activity during cell cycle 15, was mainly impeded by the presence of large amounts maternally derived Cyclin E protein. Therefore, an alternative approach had to be found to avoid this problem. In order to introduce a G1 state, Cyclin E/Cdk2 activity becomes inhibited by the CKI Dacapo (de Nooij et al., 1996; Lane et al., 1996). Overexpression of Dacapo in otherwise wild-type embryos using *paired*-Gal4, prevents entry into mitosis, evidenced by reduced numbers of epidermal cells in the overexpressing segments (Figure 12; Lane et al., 1996). Dacapo overexpression has no influence on progression through mitosis 15 and execution of the subsequent S-phase, although the protein is readily detectable (Lane et al., 1996). This approach can therefore be used to reduce Cyclin E/Cdk2 activity during cell cycle 15, without interfering with entry into S-phase 16. Overexpression of Dacapo in *cycA*^{C8LR1} embryos using *paired*-Gal4 results in a significant reduction of epidermal cell numbers in the overexpressing segments (Figure 12), suggesting that these cells arrest at an earlier point. Furthermore, examination of Cyclin B levels in these embryos, revealed premature disappearance of Cyclin B in Dacapo overexpressing segments. Hence, these experiments demonstrate that Cyclin E dependent kinase activity is necessary to prevent premature activation of the APC/C-Fzr complex during cell cycle 15. Furthermore, these results indicate that Rca1, Cyclin A/Cdk1 and Cyclin E/Cdk2 cooperate to restrain APC/C-Fzr activity in postblastoderm embryos.

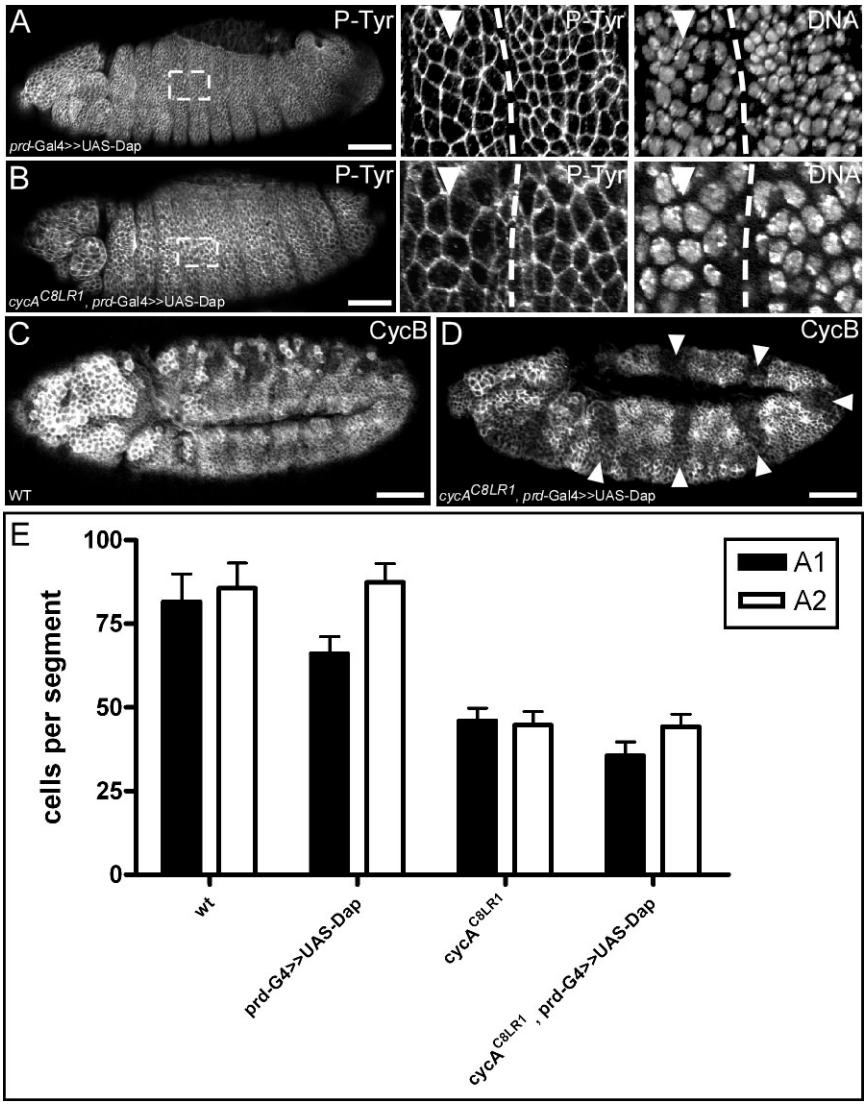

Figure 12 Overexpression of Dacapo prevents execution of cell cycle 15. UAS-Dacapo was overexpressed in wild-type or in the *cycA*^{C8LR1} mutant background using the *paired*-Gal4 driver-line. Cells were visualized with antibodies against p-Tyr as well as DNA staining. The diagram in (E) shows a comparison of epidermal cell numbers determined in the indicated genotypes. Epidermal cell numbers were quantified in the first two abdominal segments as described in Figure 11. Scale bar: 50µm.
(A&E) Dacapo overexpressing segments display a reduced number of epidermal cells compared to the neighbouring wild-type segments. Comparison of epidermal cell numbers reveals that these cells arrest at the same stage as *cycA*^{C8LR1} mutants (E), indicating that progression through cell cycle 15 is not affected by Dacapo overexpression.
(B&E) Overexpression of Dacapo in *cycA*^{C8LR1} mutants results in a further reduction of epidermal cells in the overexpressing segments, demonstrating that these cells arrest at an even earlier point than the adjacent *cycA*^{C8LR1} cells. The number of epidermal cells in these segments is comparable to cell numbers found in *rca1*^{2}*; cycA*^{C8LR1} double mutants, suggesting that reduction of Cyclin E/Cdk2 activity in *cycA*^{C8LR1} mutants prevents completion of cell cycle 15.
(C) Cyclin B expression in wild-type embryos. (D) Cyclin B disappears prematurely in Dacapo overexpressing segments, suggesting that downregulation of Cyclin E dependent kinase activity results in upregulation of APC/C-Fzr activity.

3.1.3. Downregulation of Dacapo activity is not sufficient to restore mitosis 16 in Cyclin A mutants

To establish the first G1-pase, Cyclin E dependent kinase activity becomes downregulated during cell cycle 16 by two different mechanisms (Knoblich et al., 1994). At one hand Cyclin E transcription becomes terminated and at the other hand Cyclin E/Cdk2 is inhibited by upregulation of Dacapo (de Nooij et al., 1996; Lane et al., 1996). Epidermal cells in *dap*^{4} mutants divide normally until the end of mitosis 16, but fail then to arrest in G1 and enter an additional S-phase (de Nooij et al., 1996; Lane et al., 1996). The additional S-phase observed in *dap*^{4} mutants is followed by an additional mitosis, indicated by mitotic figures at a stage

when epidermal cell have normally ceased proliferation (de Nooij et al., 1996; Lane et al., 1996). Since Cyclin E/Cdk2 activity contributes to APC/C-Fzr inhibition during cell cycle 15, the question emerges whether loss of Cyclin A/Cdk1 activity can be compensated by extended Cyclin E/Cdk2 activity. To address this question, the phase of Cyclin E/Cdk2 activity was prolonged until mitosis 16, by generating *dap*^{4}*; cycA*^{C8LR1} double mutants. Unexpectedly, mitosis 16 was not restored in *dap*^{4}*; cycA*^{C8LR1} double mutants as evidenced by epidermal cell numbers similar to *cycA*^{C8LR1} single mutants (Figure 13), suggesting that downregulation of Cyclin E/Cdk2 activity by Dacapo cannot occur before entry into mitosis 16. Furthermore, this observation implies that termination of Cyclin E transcription is of greater importance for establishment of G1 state than downregulation of Cyclin E dependent kinase activity by Dacapo.

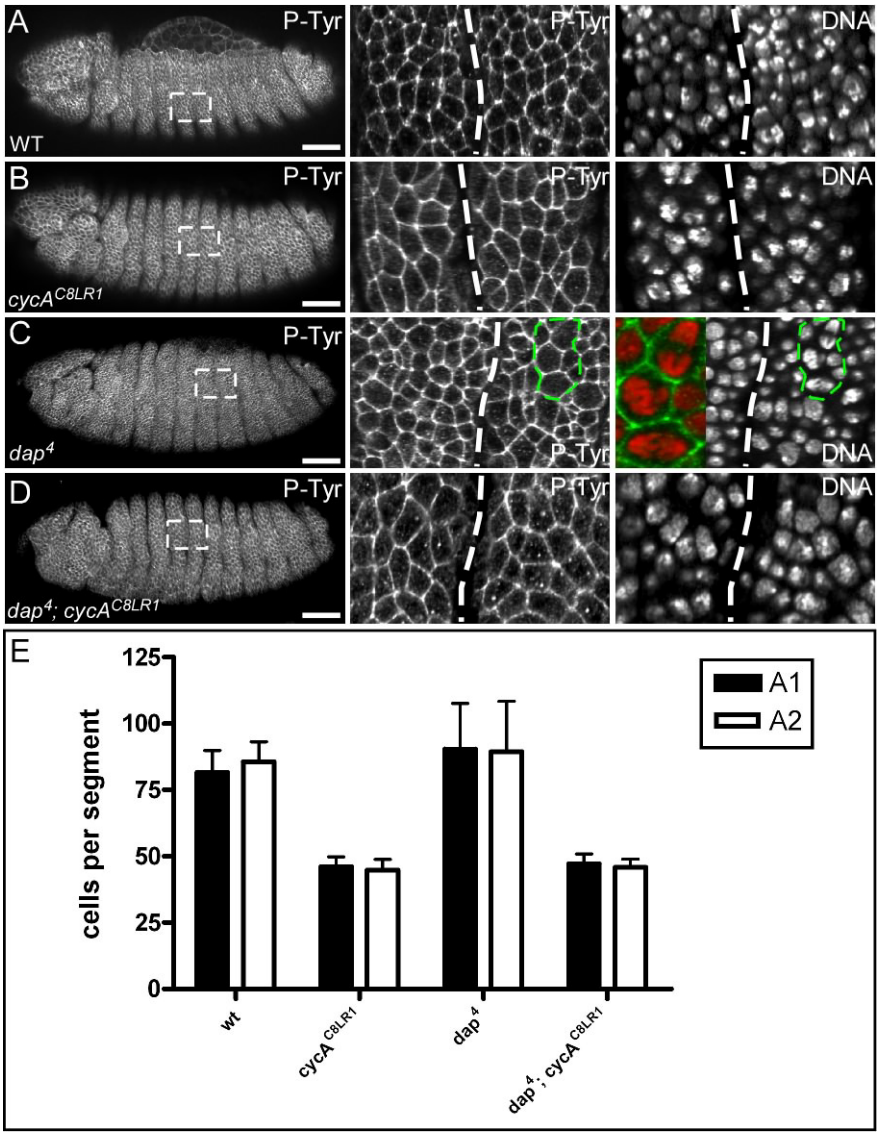

Figure 13 Dacapo; Cyclin A double mutants fail to execute mitosis 16. Cells were visualized with antibodies against p-Tyr as well as DNA staining. The diagram in (E) shows a comparison of epidermal cell numbers determined in the indicated genotypes. Epidermal cell numbers were quantified in the first two abdominal segments as described in Figure 11. Scale bar: 50µm.
(A&B) *CycA*^{C8LR1} mutants fail to enter mitosis 16 as evidenced by reduced number of epidermal cells compared to wild-type embryos.
(C) *Dap4* single mutants progress normal through mitosis 16, but fail to establish the subsequent G1 phase. Cells in *dap4* mutants undergo an additional cell cycle, evidenced by mitotic figures (insert) in embryos in that epidermal cells are normally quiescent. The encircled region indicates the depicted in the insert. P-Tyr is shown in green and DNA in red.
(D) *Dap4; cycA*^{C8LR1} double mutants display epithelial cell numbers comparable to *cycA*^{C8LR1} single mutants, demonstrating that these cells fail to enter mitosis 16. Thus, premature APC/C-Fzr activation in *cycA*^{C8LR1} mutants cannot be compensated by extended Cyclin E/Cdk2 activity.

In summary, these experiments demonstrate that Rca1, Cyclin A/Cdk1 and Cyclin E/Cdk2 cooperate to prevent premature APC/C-Fzr activation during postblastoderm cell cycles. After downregulation of Cyclin E/Cdk2-activity, APC/C-Fzr activity is restricted only by Rca1 and Cyclin A/Cdk1. Loss of one of these factors results in premature APC/C-Fzr activation and a subsequent failure to execute mitosis 16. Thus, these experiments explain why *rca1*^{2} mutants arrest specifically in G2 of cell cycle 16 and not directly after switching to G2-M control during cell cycle 14.

3.2. Functional analysis of the Rca1 protein

The Rca1 protein contains several conserved motifs (Figure 14A). The N-terminal half of Rca1 contains a bipartite nuclear localization signal (NLS), whereas the central part of Rca1 harbors three conserved sequence elements: the F-box, a KEN-box and a DSGxxS motif that has been implicated in Emi1 destruction (Guardavaccaro et al., 2003; Margottin-Goguet et al., 2003). The C-terminus contains the ZBR, a sequence motif with a characteristic cysteine-spacing that is conserved among all Rca1/Emi members (Reimann et al., 2001a). Additionally, ten putative Cdk1 phosphorylation sites (S/T-P) are distributed throughout the protein. In order to analyze the function of these protein motifs *in vivo,* we have generated a series of transgenic flies expressing different deletion constructs under control of a UAS-promoter (Rorth, 1998). All constructs were equipped with an N-terminal hemagglutinin (HA)-tag to facilitate their detection.

3.2.1. Rca1 inhibits APC/C-Fzr activity in G2 by an F-box independent mechanism

The *rca1*^{2} phenotype is characterized by premature activation of APC/C-Fzr complex during G2. During embryogenesis *rca1*^{2} mutants fail to execute the 16th mitosis resulting in fewer cells compared to wild-type embryos (Figure 14B). In order to determine the structural requirements for APC/C-Fzr inhibition, different Rca1 constructs were tested for their ability to replace endogenous Rca1 during embryogenesis. The Rca1 constructs shown in Figure 14A were expressed in *rca1* mutant embryos using the *paired*-Gal4 driver line. The resulting expression in alternating segments allows direct comparison of *rca1* mutant cells (A2 segment) and those expressing a certain Rca1 deletion construct (A1 segment). Expression of HA-Rca1 restores cell numbers to wild-type levels in *rca1* mutants (Figure 14B). Each construct has been tested in this assay and the results are summarized in Figure 14B. N-terminal truncations up to residue 203 were able to restore mitosis 16 in *rca1* mutant segments. The deleted region includes the NLS and the F-box as well as 7 potential Cdk phosphorylation sites indicating that these motifs are dispensable for inhibition of the APC/C-Fzr complex in G2. A further deletion up to residue 255 abolished the activity of the Rca1 protein. In addition, mutation of a conserved cysteine residue (C346S) in the ZBR eliminated Rca1 activity. Internal deletions removing only one protein domain did not impair the functionality during cell cycle 16. Finally, Rca1 activity is not affected by simultaneous mutation of all ten putative Cdk1 phosphorylation sites to alanine. Thus, a C-terminal Rca1 fragment including an intact ZBR is sufficient to restrict of APC/C-Fzr activity. To verify the

observation that the F-box is dispensable for APC/C-Fzr inhibition, both constructs lacking the F-box (HA-Rca1Δ203 and HA-Rca1ΔF-box) were tested for their ability to prevent premature degradation of Cyclin A in *rca1*^{2} mutants. Similar to the full-length protein (Grosskortenhaus and Sprenger, 2002), both constructs were able to stabilize Cyclin A in *rca1*^{2} embryos (Figure 14C&D). In summary, these experiments indicate that Rca1 inhibits APC/C-Fzr activity in G2 by an F-box independent mechanism.

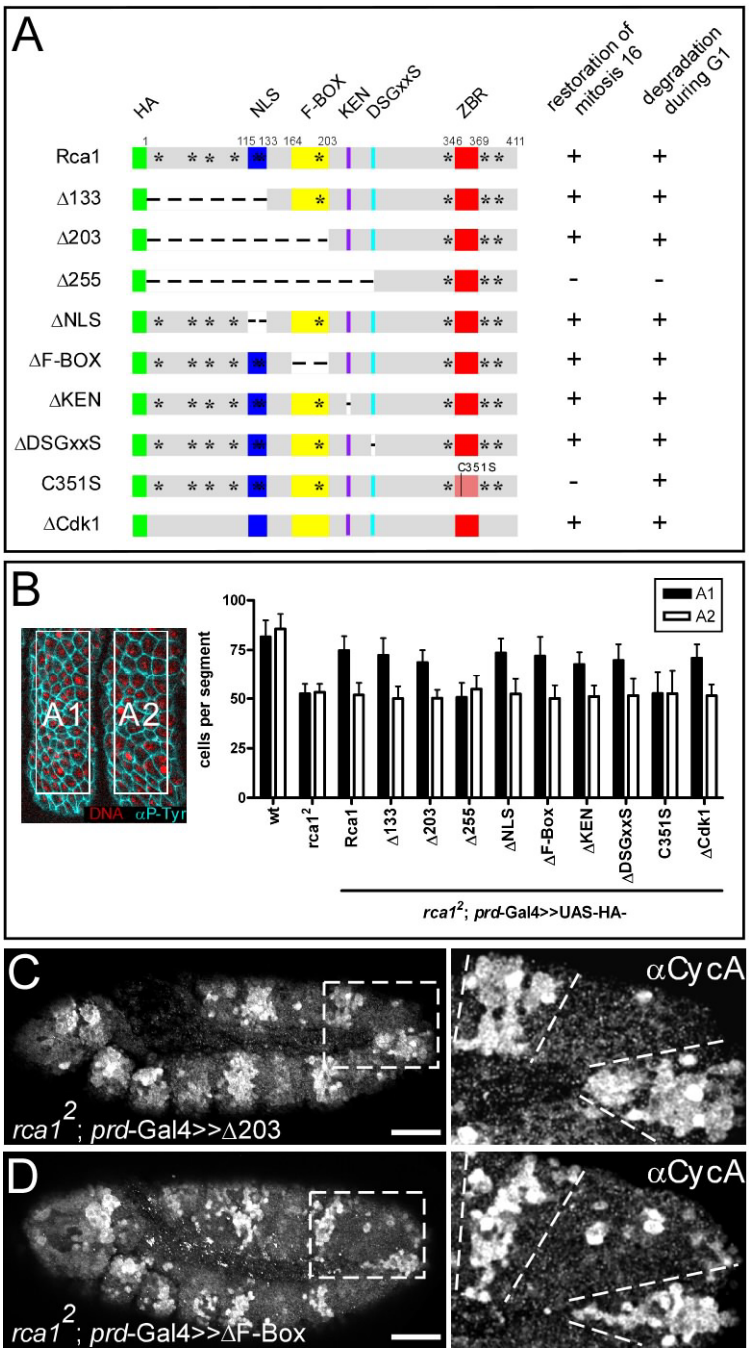

Figure 14 Functional analysis of the Rca1 protein.
(A) Schematic representation of the analyzed Rca1 constructs. All constructs were tested for their ability to restore mitosis 16. In addition, the stability of a certain construct during G1 was determined. The asterisks indicate putative Cdk1 phosphorylation sites (S/T-P).
(B) A functional assay for Rca1 activity during embryogenesis. Epidermal cells were counted in a given region (marked by the white box) of stage 13 *rca1*^{2} embryos. For quantification cells were visualized with P-Tyr antibodies and DNA staining. In the A1 segment, the expression of different constructs was induced by the *paired*-Gal4 driver line. Cell numbers in segments A1 and A2 were quantified after expression of the indicated Rca1 constructs and compared to wt and *rca1*^{2} mutants (n≥10). *rca1*^{2} mutants display fewer epidermal cells compared to wild-type embryos. Expression of several Rca1 constructs results in the restoration of epidermal cell number in the A1 segment of *rca1*^{2} mutants. In summary, these experiments reveal that a C-terminal fragment of Rca1 is sufficient for APC/C-Fzr inhibition during embryogenesis.
(C&D) The F-box of Rca1 is not essential for stabilization of Cyclin A in *rca1*^{2} embryos. HA-Rca1Δ203 (C) and HA-Rca1ΔF-box (D) were expressed in *rca1*^{2} mutants using *paired*-Gal4. In both cases, Cyclin A degradation, that is apparent in the *rca1*^{2} mutant segments, is suppressed in the *paired* expressing regions. Scale bar: 50µm.

3.2.2. Rca1 gets degraded by different mechanisms than Emi1

During *Drosophila* development, the first G1 phase occurs after mitosis 16 and most epidermal cells persist in that G1 phase until the end of embryogenesis. Previously, it was shown that Rca1 becomes degraded during this G1 phase (Grosskortenhaus and Sprenger, 2002). In order to identify elements that regulate Rca1 degradation, different Rca1 deletion constructs (Figure 14) were expressed in wild-type embryos using *paired*-Gal4. At stage 11 of *Drosophila* embryogenesis, most of the epidermal cells are in interphase of cell cycle 16 and contain mitotic cyclins, like Cyclin A. At this stage, full-length HA-Rca1 is able to stably accumulate (Figure 15A). In embryos at developmental stage 13, most of the epidermal cells reside in G1 as indicated by the absence of Cyclin A (Figure 15B). Confocal sections reveal that HA-Rca1 is not detectable in these epidermal cells (Figure 15B). However, HA-Rca1 is visible in trace amounts in the proliferating cells of the developing nervous system (Figure 15B). Like the full-length protein, HA-Rca1Δ203 which lacks the whole N-terminal region gets degraded in G1 (Figure 15C) demonstrating that the N-terminus is dispensable for Rca1 degradation. In contrast, further deletion up to amino acid 255 results in a stable protein, which is detectable even in epidermal cells that reside in G1 (Figure 15D). These observations suggest that the region between amino acid 203 and 255 is involved in Rca1 destruction. This part of Rca1 contains two putative destruction elements: the KEN-box, a destruction element recognized by Fzr (Pfleger and Kirschner, 2000) and the DSGxxS motif, which is implicated in the turnover of Emi1. During prometaphase degradation of Emi1 is initiated by polo like kinase 1 (Plk1) that phosphorylates the DSGxxS motif (Hansen et al., 2004; Moshe et al., 2004). The SCF/ß-TRCP ubiquitin ligase subsequently recognizes the phosphorylated DSGxxS motif and targets Emi1 for proteasomal degradation (Guardavaccaro et al., 2003; Margottin-Goguet et al., 2003). In order to test whether the DSGxxS degron is also involved in Rca1 destruction the serine residues in the DSGxxS motif were mutated (S253A, S256A S257A). In contrast to Emi1, this triple mutant gets degraded like full length Rca1 (Figure 15E). In addition, deletion of the KEN-box does not increase the halftime of the Rca1 protein (Figure 15F). These observations indicate that the region between amino acid 203 and 255 might harbor a so far unknown degradation element. For Emi1 it was furthermore shown that Cdk1 phosphorylation is required to initiate Emi1 destruction (Margottin-Goguet et al., 2003; Reimann et al., 2001a). In Rca1, simultaneous mutation of all putative Cdk1 phosphorylation sites does not stabilize the protein (Figure 15G). Altogether, these observations show that Rca1 degradation is achieved by other mechanisms than those described for Emi1.

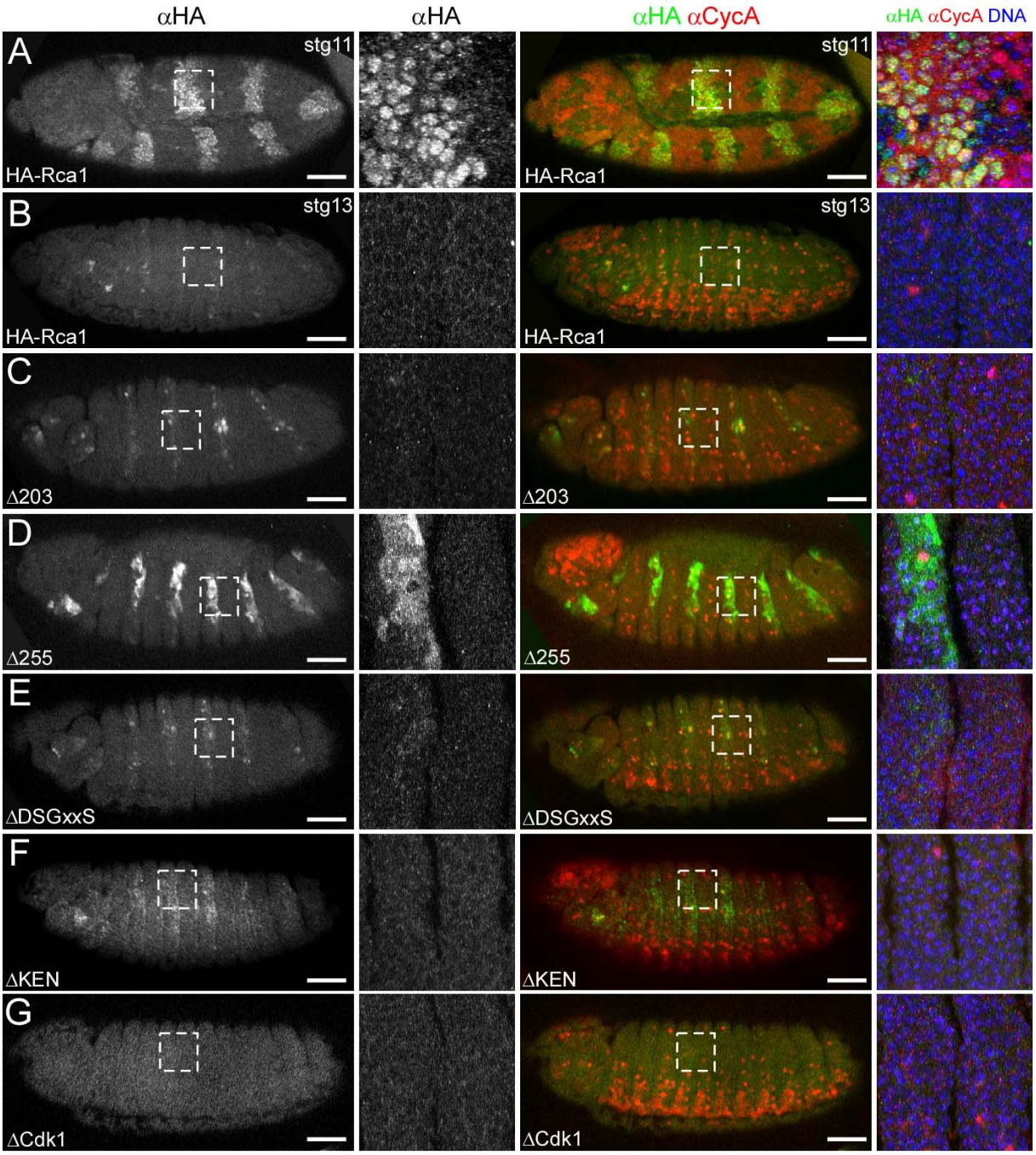

Figure 15 Stability of Rca1 in embryonic G1 cells. Certain HA-tagged Rca1 constructs (Figure 14A) were expressed in stripes in otherwise wild-type embryos using *prd-Gal4* and stained for HA, Cyclin A and DNA. The box marks the region of the embryo magnified to the right. Scale bar: 50µm.
(A) In stage 10 embryos most cells are in G2 indicated by high levels of Cyclin A and can accumulate HA-Rca1.
(B) During stage 13, most epidermal cells are in G1 and lack Cyclin A. Here HA-Rca1 is unstable and fails to accumulate. The Cyclin A staining seen in lower magnifications results from cells of the nervous system that reside in deeper levels of the embryo.
(C&D) HA-Rca1Δ203 which lacks the complete N-terminal region gets degraded during G1 like full-length Rca1 (C). In contrast, HA-Rca1Δ255 is refractory to degradation in G1 (D), suggesting that the region between amino acid 203 and 255 is essential for Rca1 turnover.
(E&F) Neither, deletion of the DSGxxS motif (E) nor mutation of the KEN-box (F) stabilizes Rca1 in G1. Although, these putative destructions elements are located in the region between amino acid 203 and 255, these results demonstrate that both motifs are not essential for Rca1 degradation.
(G) Mutation of the ten putative Cdk1 phosphorylation site does not affect the stability of Rca1.

3.3. Rca1 is implicated in the G1-S transition during imaginal disc development

Previous studies revealed that Emi1, the vertebrate ortholog of Rca1 is implicated in the transition from G1 to S-phase (Hsu et al., 2002; Rape and Kirschner, 2004) raising the idea that Rca1 might have a similar function beside its requirement during G2. Up to this point all experiments were done in postblastoderm embryos that never undergo a G1-S transition. To elucidate whether Rca1 has an additional function at the G1-S transition, it was therefore necessary to switch to a cell type that displays a cell cycle mode with all four phases. Imaginal disc cells are an ideal model system to address this question since they proliferate continuously during larval development and undergo a standard cell cycle with a distinct G1-S transition.

3.3.1. Rca1 and Fzr are expressed in complementary domains during eye imaginal disc development

Eye imaginal discs display a morphological defined G1-S transition (Figure 3), so that they are an appropriate model to study S-phase entry. During the third instar stage the morphogenetic furrow sweeps from posterior to anterior. Cells in front of the morphogenetic furrow are undifferentiated and proliferate asynchronously, whereas cells within the morphogenetic furrow are synchronized in G1. Cells posterior to the furrow separate into two subpopulations. One group stays in G1 and differentiates into photoreceptor cells, whereas the remaining cells enter a terminal cell cycle called second mitotic wave (Thomas et al., 1994). Previous work revealed that Emi1 gets upregulated in late G1 by E2F dependent transcription (Hsu et al., 2002). A well characterized E2F1 target in *Drosophila* is PCNA, a gene essential for DNA replication (Thacker et al., 2003). The expression pattern of PCNA, that can be visualized by a GFP-reporter construct (Figure 16A-C), therefore reflects the spatial distribution of E2F1 activity. *In situ* hybridization experiments were performed to test whether Rca1 transcription gets upregulated in an E2F1 dependent manner prior to S-phase. However, these experiments revealed no match with the PCNA reporter construct. Rca1 is rather uniformly expressed in the proliferating cells in the anterior half of the eye disc (Figure 16 A-C), suggesting that its transcription does not depend on E2F1 activity. Interestingly, these *in situ* hybridization experiments indicated that Rca1 is expressed in a pattern complementary to that of Fzr. *In situ* hybridization experiments as well as antibody staining showed that Fzr is only detectable in the morphogenetic furrow as well as in the postmitotic photoreceptor cells in the posterior part of the disc (Figure 16D-F; Pimentel and Venkatesh,

2005). Fzr activity is thought to be required for cell cycle exit (Sigrist and Lehner, 1997). It seems therefore reasonable that the expression of its antagonist Rca1 is terminated in the quiescent photoreceptor cells.

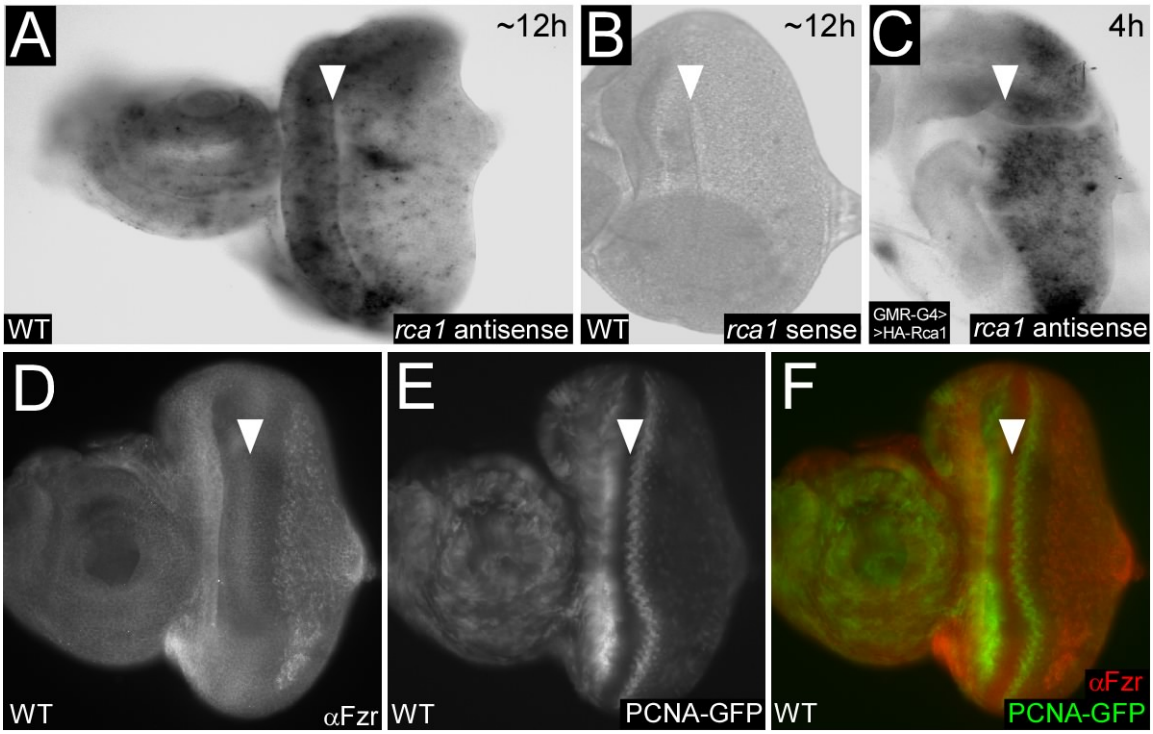

Figure 16 Rca1 and Fzr are expressed in complementary patterns during eye development.
(A-C) Expression of Rca1 visualized in third instar eye imaginal discs by *in situ* hybridization. (A) Rca1 is ubiquitously expressed in the proliferating part of third instar eye discs anterior to the morphogenetic furrow. Control *in situs* with a sense probe (B) and (C) with the antisense probe after overexpression of *HA-Rca1* using GMR-Gal4.
(D-F) Fzr expression was determined by antibody staining of eye imaginal disc expressing the S-phase marker PCNA-GFP. Fzr is detectable at low levels within the morphogenetic furrow, whereas it is absent in the remaining proliferating cells. Consistent with is implication in cell cycle exit, Fzr is readily detectable in the differentiating photoreceptor cells in the posterior part of the disc.

3.3.2. Overexpression of Rca1 promotes S-phase entry in eye imaginal discs

A previous study showed that expression of Rca1 during eye development causes ectopic S-phases and a rough eye phenotype (Dong et al., 1997). In contrast, a more recent study failed to show ectopic BrdU labeling after expression of Rca1 (Araki et al., 2003). In order to verify this observations, HA-Rca1 was overexpressed in all cells posterior to the morphogenetic furrow using GMR-Gal4 driver line. In agreement with the first study, overexpression of HA-Rca1 resulted in a rough eye phenotype (Figure 17B). Moreover, BrdU labeling (Figure 17A) and FACS analysis (Figure 18D) revealed that misexpression of Rca1 drives cells posterior to the second mitotic wave into ectopic S-phases. Finally, phosphohistone 3 staining revealed mitotic cells posterior to the second mitotic wave (Figure 18D). This suggests that excess

Rca1 activity not only speeds up the cell cycle, it rather drives the cells in at least one extra cell cycle.

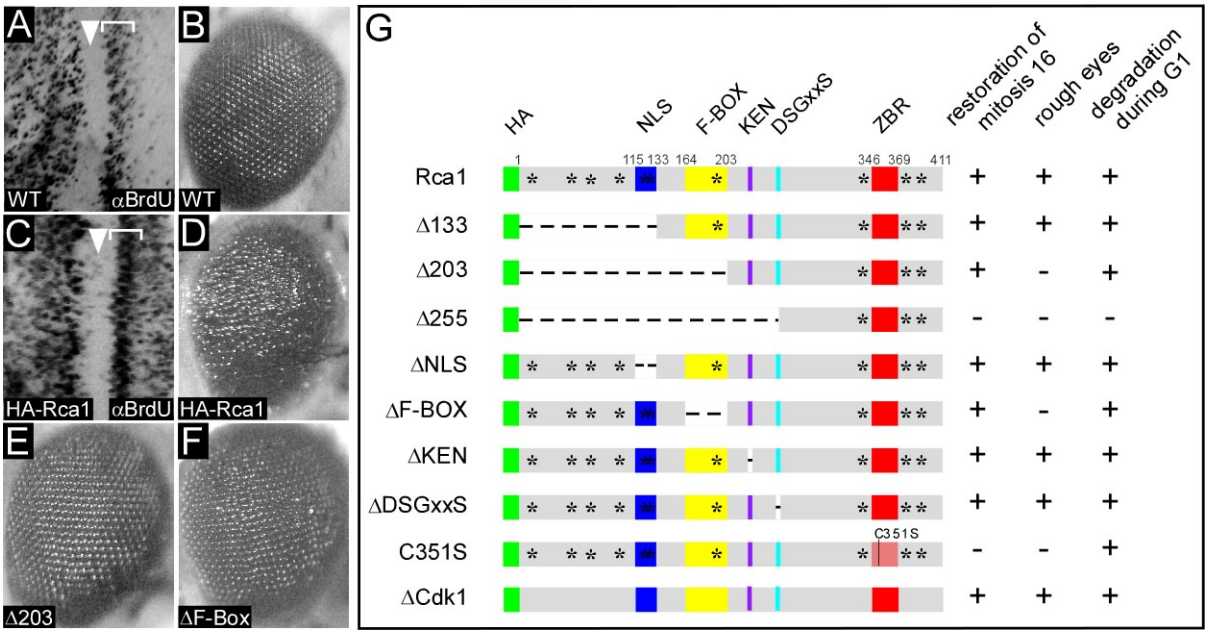

Figure 17 Overexpression of HA-Rca1 results in rough eyes due to ectopic S-phases.
(A&B) BrdU pattern and eye morphology in wild-type. The brackets indicate the second mitotic wave and the arrowhead the morphogenetic furrow.
(D&E) Overexpression of HA-Rca1 using GMR-Gal4 induces ectopic S-phases indicated by BrdU incorporation in the posterior part of the eye disc and a rough eye phenotype.
(E&F) Overexpression of HA-Rca1Δ203 or HA-Rca1ΔF-box using GMR-Gal4 does not affect eye morphology.
(G) Structural requirements for S-phase induction by Rca1. All constructs tested in the embryo were examined for their ability to induce rough eyes after overexpression with GMR-Gal4. The induction of the rough eye phenotype requires basically the structural elements that were necessary for APC/C-Fzr inhibition during embryogenesis. However, HA-Rca1Δ203 or HA-Rca1ΔF-box which were able to rescue mitosis 16 in *rca1*^{2} mutant embryos failed to induce rough eyes.

3.3.3. The F-box is essential to drive cells prematurely into S-phase

Previous work demonstrated that APC/C-Fzr activity is required to maintain the G1 state. Hypomorphic *fzr* mutants display aberrant BrdU incorporation in the eye disc and a rough eye phenotype in the adult. This raises the idea that the ectopic S-phases as well as the rough eye phenotype induced by Rca1 could be due to APC/C-Fzr inhibition. In this case, the induction of the rough eye phenotype would rely on the same parts of the Rca1 protein that are required for APC/C-Fzr inhibition during embryogenesis. All constructs tested for functionality in the embryo, were therefore examined also for their ability to induce rough eyes after overexpression with GMR-Gal4. In most of the cases, the ability to promote S-phase entry, correlated with the capability to restrict APC/C-Fzr activity in G2. For instance, the NLS is dispensable for restriction of APC/C-Fzr activity as well as for induction of the rough eye phenotype. Furthermore, HA-Rca1Δ255 and HA-Rca1C351S that were unable to inhibit the

APC/C-Fzr complex during embryogenesis, failed also to induce rough eyes. Interestingly, both constructs lacking the F-box (HA-Rca1Δ203 and HA-Rca1ΔF-box) had no effect on eye morphology after overexpression with GMR-Gal4. Moreover, both constructs failed to promote S-phase entry as indicated by FACS analysis (Figure 18J&M), suggesting that S-phase induction by Rca1 relies on an F-box dependent mechanism.

The failure of HA-Rca1Δ203 and HA-Rca1ΔF-box to promote S-phase entry might be rather due to a dose effect than to a requirement of the F-box. In order to exclude this possibility, expression levels of these deletion constructs were compared to those of full-length Rca1. Antibody staining against the HA-tag demonstrates that HA-Rca1Δ203 and full-length HA-Rca1 accumulate to comparable levels (Figure 18E&H), whereas HA-Rca1ΔF-box displays a reduced expression level (Figure 18K). To rule out that the failure of HA-Rca1ΔF-box to induce S-phase results from its reduced expression level, two copies of HA-Rca1ΔF-box were crossed together. Although, simultaneously overexpression of two copies of HA-Rca1ΔF-box results in expression levels comparable to HA-Rca1 (Figure 18P), these flies display normal eye morphology (Figure 18O). Thus, these experiments lead to the conclusion that S-phase induction by Rca1 requires a functional F-box.

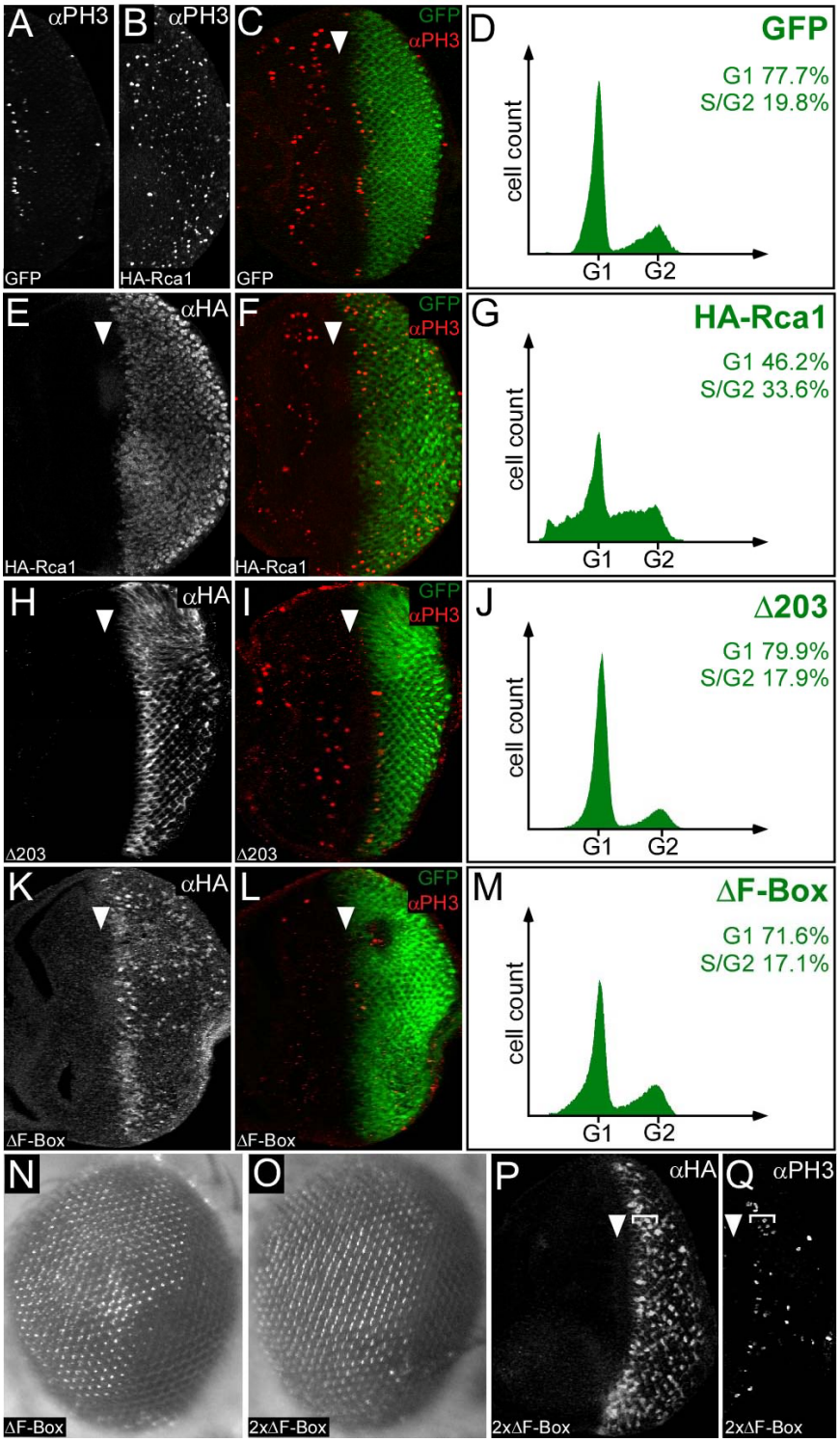

Figure 18 Rca1 overexpression in eye imaginal discs promotes S-phases entry in an F-box dependent manner. All constructs were coexpressed with GFP posterior to the morphogenetic furrow using GMR-Gal4. To monitor the effect of Rca1 overexpression, the cell cycle profile of the GFP positive cells was analyzed by flow cytometry. In addition, mitotic cells were visualized by PH3 staining. In order to compare expression levels, discs were stained against HA. The brackets indicate the second mitotic wave and the arrowhead the morphogenetic furrow.
(**A, C&D)** Cell cycle profile and PH3 staining in control (GFP only) eye imaginal discs.
(B, E-H) Overexpression of HA-Rca1 results in a remarkable increase of cells in S-phase, whereas the G1 population declines (H).
(G-N) Overexpression of both constructs lacking the F-box (HA-Rca1Δ203 and HA-Rca1ΔF-box) does not affect cell cycle progression in eye imaginal discs indicated by cell cycle profiles and PH3 patterns similar to controls. HA staining reveals that HA-Rca1Δ203 accumulates to similar amounts as full length Rca1 (E&H), whereas HA-Rca1ΔF-box displays a reduced expression level (K).
(**O-Q)** To exclude that the failure of HA-Rca1ΔF-box to induce S-phase is due to its reduced expression levels, we have overexpressed two copies of HA-Rca1ΔF-box. However, even with two copies of HA-Rca1ΔF-box the flies displayed normal eye morphology (N&O) and a PH3 pattern similar to wild-type (Q). HA staining indicates that HA-Rca1ΔF-box now reaches the level of full length Rca1 (P), arguing against a dose effect. In addition, HA staining reveals a reduced number of HA positive cells compared to discs overexpressing HA-Rca1. This observation reflects the failure of HA-Rca1ΔF-box to induce S-phase, since both proteins are unstable in G1. In conclusion, these experiments demonstrate that Rca1 requires the F-box to accelerate S-phase entry.

3.3.4. Excess Rca1 activity accelerates G1-S transition in wing imaginal discs

To determine if Rca1 can induce G1-S transitions in other imaginal discs as well, clones that continuously express GFP and HA-Rca1 were generated in wing imaginal discs by the use of the „flpout“ technique (Ito et al., 1997). Flow cytometric analysis of late third instar wing discs (Neufeld et al., 1998) revealed a significant decrease of G1 cells after Rca1 overexpression, whereas the amount of cells in S/G2 simultaneously increased (Figure 19A). The decline of G1 cells indicates that the Rca1 overexpressing cells progress faster through G1. The concomitant increase in G2 cells is likely due to a recently discovered compensatory mechanism that keeps the overall cell cycle duration constant (Reis and Edgar, 2004). In agreement with this, forward scattering revealed no significant change in cell size in Rca1 overexpressing cells (Figure 19A). These results demonstrate that excess Rca1 protein can change the cell cycle profile also in wing imaginal discs by accelerating the transition from G1 into S-phase, suggesting that this a general effect of Rca1 overexpression.

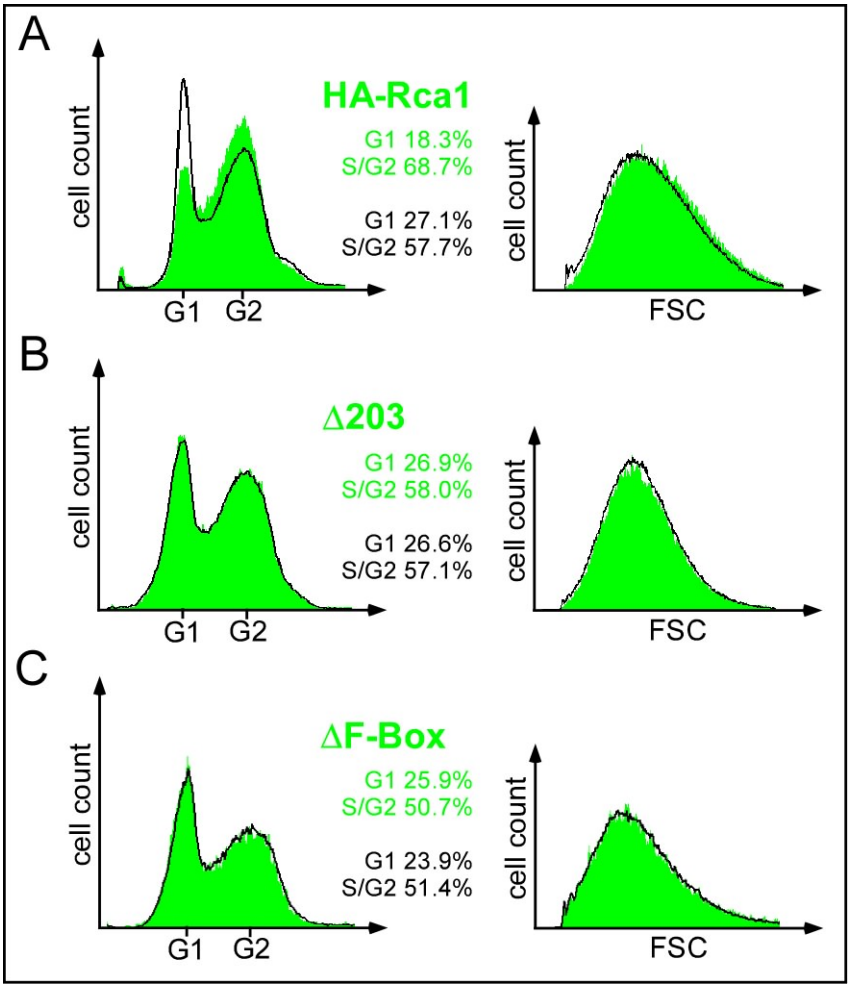

Figure 19 Wing disc cells overexpressing Rca1 progress faster through G1. To confirm the results obtained in eye disc, HA-Rca1 and both constructs lacking the F-box were overexpressed in wing imaginal disc using the “flpout” technique and analyzed by flow cytometry. The green curve represents the overexpressing cells whereas wild-type cells are shown in black.
(A) Overexpression of HA-Rca1 leads to a decrease of cells residing in G1, indicating that these cells progress faster through G1. Simultaneously, the fraction of G2 cells increases suggesting that these cells undergo cell cycle length compensation. Forward scattering (FCS) reveals only slight size differences between GFP positive and negative cells.
(B&C) Overexpression of HA-Rca1Δ203 or HA-Rca1ΔF-box does not affect the cell cycle profile, confirming the observation that a functional F-box is necessary to drive cells ectopically into S-phase.

Furthermore, both constructs lacking the F-box were tested in the same manner. Consistent to the results obtained in the eye, neither HA-Rca1Δ203 (Figure 19B) nor HA-Rca1ΔF-box (Figure 19C) affected the cell cycle profile in wing discs, further supporting the idea that S-phase induction by Rca1 depends on a functional F-box. In both cases forward scattering revealed no size difference between wild-type and overexpressing cells (Figure 19B&C), suggesting that the marginal size shift observed in the HA-Rca1 overexpressing cells (Figure 19A) is most likely due to the increased number of G2 cells, which are slightly bigger.

3.3.5. Rca1 overexpression stabilizes mitotic cyclins in G1

The expression pattern of mitotic cyclins reflects the different cell cycle modes within third instar eye imaginal discs. Many of the asynchronously dividing cells ahead of the morphogenetic furrow and the cells of the second mitotic wave display high levels of mitotic cyclins. Within the morphogenetic furrow, cells are synchronized in G1 and mitotic cyclins are downregulated (Figure 20B&G). Fzr expression is upregulated within the morphogenetic furrow (Figure 16D) and hypomorphic *fzr* mutants display elevated levels of Cyclin B protein (Pimentel and Venkatesh, 2005), suggesting that mitotic cyclins become degraded within the morphogenetic furrow by the APC/C-Fzr complex. Moreover, previous studies demonstrated that premature activation of Cyclin A/Cdk1 in G1 results in ectopic S-phases (Dong et al., 1997; Sprenger et al., 1997; Thomas et al., 1994; Thomas et al., 1997). Hence, accumulation of Cyclin A due to APC/C-Fzr inhibition could be an explanation for the ectopic S-phases observed in Rca1 overexpressing cells. To determine whether overexpression of Rca1 leads to stabilization of mitotic cyclins, “flpout“ clones were generated within the morphogenetic furrow. S-phase cells were visualized by a GFP-reporter construct that reflects PCNA expression (Figure 20A), a gene essential for DNA replication (Thacker et al., 2003). Overexpression of Rca1 results in ectopic activation of this reporter, confirming that excess Rca1 activity promotes S-phase entry (Figure 20E). Furthermore, these cells showed high levels of Cyclin A (Figure 20E) and Cyclin B (Figure 20E). Since both cyclins are targets of the APC/C-Fzr complex, these results suggest that APC/C activity gets downregulated in Rca1 overexpressing cells. This inactivation of the APC/C could be the direct cause of the inhibitory activity of Rca1. Alternatively, it could be a secondary consequence of the induced S-phase in which the APC/C is normally inactive.

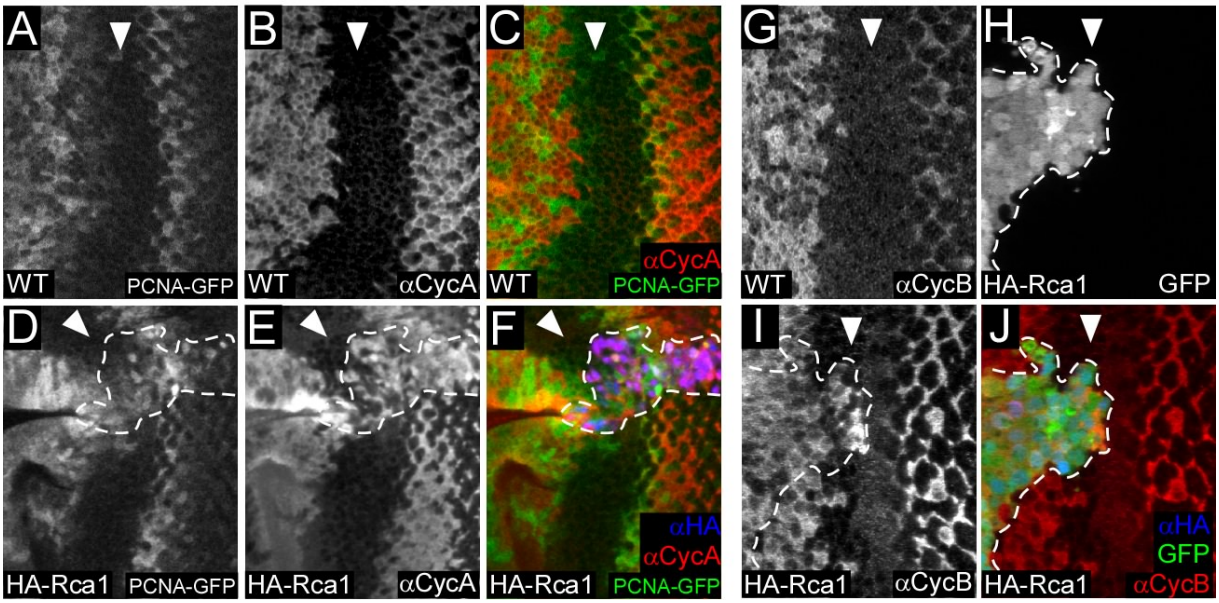

Figure 20 Overexpression of Rca1 stabilizes mitotic cyclins within the morphogenetic furrow. HA-Rca1 was overexpressed under control of the actin promoter using the "flpout" method. Clones were visualized by GFP expression or anti-HA staining. The arrowheads indicate the morphogenetic furrow.
(A-C&G) Expression pattern of PCNA-GFP, Cyclin A and Cyclin B in wild-type eye discs highlight the downregulation of mitotic cyclins in the morphogenetic furrow and their subsequent accumulation in cells that undergo S-phase.
(D-F) HA-Rca1 overexpression results in ectopic PCNA-staining and accumulation of Cyclin A within the MF.
(H-J) Cyclin B can also accumulate in clones overexpressing HA-Rca1.

3.3.6. Cyclin A accumulation accompanied with Rca1 overexpression relies on a functional F-box

In order to determine whether stabilization of Cyclin A within the morphogenetic furrow requires a functional F-box, Cyclin A levels were analyzed in cell clones overexpressing HA-Rca1Δ203. Although, HA-Rca1Δ203 is an effective inhibitor of APC/C-Fzr activity (Figure 14C), the cells of the morphogenetic furrow displayed no ectopic Cyclin A staining (Figure 21E-H). This result shows that these cells remain in G1 and explains why HA-Rca1Δ203 fails to induce the rough eye phenotype. Furthermore, this experiment demonstrates that the ectopic S-phase induced by Rca1 cannot be simply explained by its inhibitory effect on the APC/C-Fzr complex. F-box proteins are part of SCF (Skp-Cullin-F-box)-ubiquitin-ligases that mediate degradation of numerous substrates. In such an SCF complex, F-box proteins are attached to the Cullin scaffold by a Skp protein and act as substrate recognition subunits (Vodermaier, 2004). A genome wide yeast two-hybrid screen demonstrated that Rca1 interacts with *Drosophila* SkpA and B (Giot et al., 2003) suggesting that Rca1 might be part of an SCF complex. Hence, a reasonable explanation for the requirement of the F-box in G1, could be that Rca1 gets incorporated in a SCF-complex which inhibits APC/C-Fzr, resulting in accumulation of mitotic Cyclin A and subsequent entry into S-phase. Alternatively,

SCF/Rca1 could promote S-phase entry by a yet unknown mechanism and thereby inactivate APC/C-Fzr indirectly.

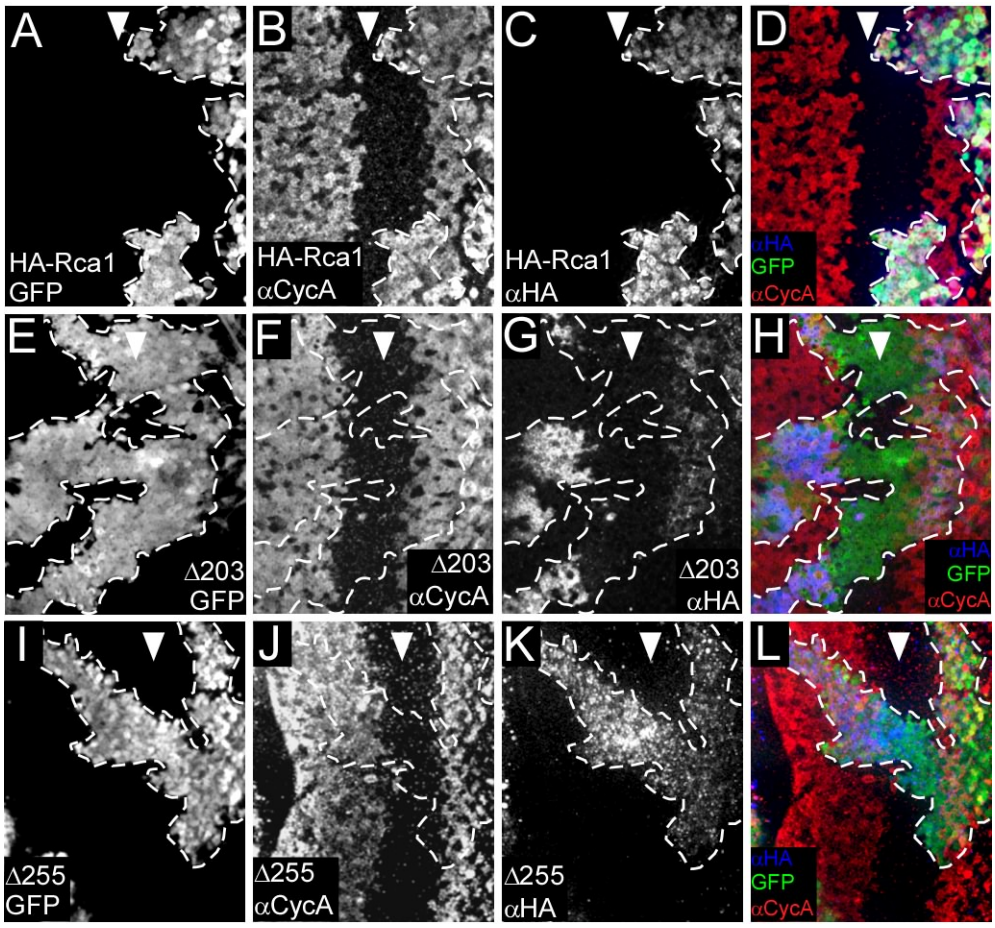

Figure 21 The F-box of Rca1 is required for Cyclin A stabilization in the morphogenetic furrow. Certain Rca1 constructs were overexpressed under control of the actin promoter using the "flpout" method. Clones were positively marked by GFP. The arrowheads indicate the morphogenetic furrow.
(A-D) Overexpression of HA-Rca1 results in ectopic Cyclin A staining within the morphogenetic furrow. HA-Rca1 is detectable throughout the clone.
(E-H) Overexpression of HA-Rca1Δ203 does not stabilize Cyclin A within the morphogenetic furrow. HA staining reveals that HA-Rca1Δ203 is instable within the morphogenetic furrow.
(I-L) HA-Rca1Δ255 is stable within the MF, but has no effect on Cyclin A.

3.3.7. Rca1 gets degraded within the morphogenetic furrow

In situ hybridization experiments showed that Rca1 mRNA is expressed ubiquitously in the anterior part of eye imaginal discs (Figure 16A). Moreover, it was previously demonstrated that Fzr accumulates within the morphogenetic furrow and that APC/C-Fzr activity is required to maintain the G1 state (Pimentel and Venkatesh, 2005). These experiments revealed that both expression patterns overlap within the morphogenetic furrow, raising the problem how Fzr can activate the APC/C in presence of Rca1. To examine whether Rca1 gets

posttranscriptional inactivated to allow APC/C-Fzr activity, certain constructs were overexpressed in clones spanning the morphogenetic furrow. HA-Rca1Δ203 gets degraded in the G1 cells of the morphogenetic furrow and accumulates again once cells enter S-phase (Figure 21E-H). Furthermore, HA-Rca1Δ255, which is stable in the embryo, is also refractory to degradation within the MF (Figure 21I-L). These observations imply that Rca1 gets degraded in the morphogenetic furrow by the same mechanisms that also mediate Rca1 destruction in G1 cells of postblastoderm embryos. Interestingly, full-length HA-Rca1, which is subject to degradation in G1, can accumulate in cell clones within the morphogenetic furrow (Figure 21A-B). However, these cells have been shifted from G1 into S-phase where Rca1 degradation is switched off, explaining why HA-Rca1 appears stable. Hence, G1 degradation within the morphogenetic furrow can only be observed for constructs lacking the F-box since they cannot promote S-phase entry.

3.3.8. Rca1 lacking the F-box fails to restore the proliferation disadvantage of *rca1* mutant clones

To test whether the F-box dependent function of Rca1 is required for proliferation in general, endogenous Rca1 was replaced by Rca1 constructs lacking the F-box. Therefore, $rca1^2$ mutant clones expressing appropriate transgenes were generated in wing imaginal discs and examined for their proliferation potential. As reported previously, cells in $rca1^2$ clones display a severe proliferation disadvantage compared to wild-type cells indicated by a dramatically reduced average clone size (Figure 22A-D, K and Grosskortenhaus and Sprenger, 2002). Overexpression of HA-Rca1 in $rca1^2$ clones using the MARCM-System (Lee and Luo, 1999) rescues this proliferation disadvantage evidenced by an average clone size similar to wild-type (Figure 22E&F, K). Remarkably, neither HA-Rca1Δ203 nor HA-Rca1ΔF-box were able to completely restore the proliferation potential of $rca1^2$ mutant cells (Figure 22G-J), although they perfectly restored mitosis 16 in $rca1^2$ mutant embryos (Figure 14). In both cases the average clone size was only a third of wild-type levels (Figure 22K). This indicates that these cells can only undergo limited number of divisions, demonstrating that the F-box function of Rca1 is required for normal proliferation of wing disc cells.

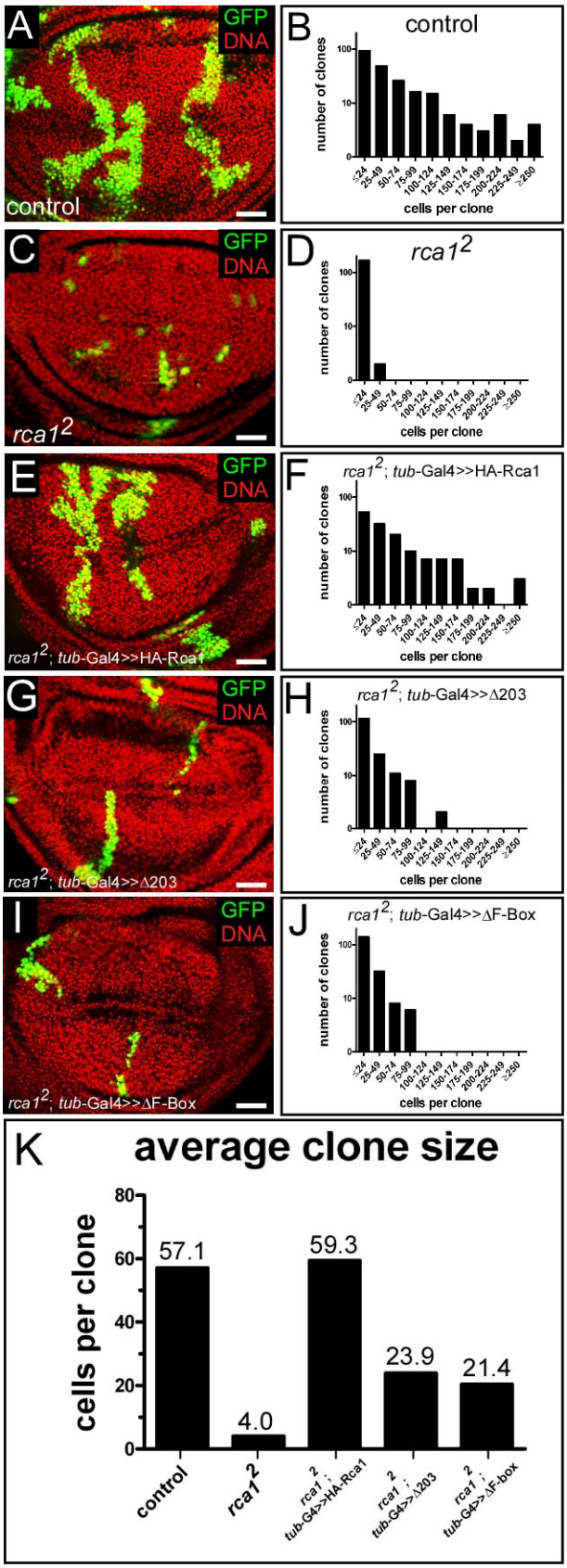

Figure 22 Rca1 lacking the F-box fails to restore the proliferation disadvantage of *rca1²* mutant cells. *rca1²* mutant clones positively marked with GFP were generated by the MARCM-technique which allows the simultaneous overexpression of UAS-constructs under control of the *tubulin*-Gal4 driver line. The proliferation rate was determined by counting the number of cells per clone in the ventral part of wing imaginal discs (n=25). An example for each genotype is shown on the left side. The right side shows the distribution of the number of cells per clone. The diagram in (K) shows the average clone size for each genotype. Scale bar: 100µm.
(A&B) Control (GFP only) clones exhibit a broad range of clone sizes varying from small clones that contain only few cells up to large clones with more than 250 cells per clone. In average, these clones contained 57.1 cells per clone.
(C&D) *rca1²* mutant clones barely reach sizes of more than 25 cells (4.0 cells in average) indicating that loss of *rca1²* results in a severe proliferation disadvantage.
(E&F) Expression of HA-Rca1 in *rca1²* mutant clones using *tubulin*-Gal4 overcomes the proliferation disadvantage, indicated by a size distribution and average clone size (59.3) similar to controls. The proliferation rate does not exceed the wild-type level since wing disc cells overexpressing HA-Rca1 can compensate the accelerated G1-S transition by a prolonged G2 phase (Figure 19A).
(G-J) Expression of constructs lacking the F-box (HA-Rca1Δ203 and HA-Rca1ΔF-box) stimulates proliferation of *rca1²* mutant cells, indicated by the appearance of clones with more than 50 cells. However, these cells cease proliferation after a certain time , thus cells with more than 150 cells per clone were never found. The limited proliferation potential of these cells results in average clone sizes (Δ203: 23.9, ΔF-box: 21.4) between control (57.1) and *rca1²* mutant cells (4.0).

In agreement with its function as an inhibitor of Fzr dependent APC/C activity, *rca1*^{2} clones display reduced amounts of Cyclin A protein. Additionally, these cells displayed increased DNA levels indicating that they underwent DNA endoreplication (Figure 23A-C; Grosskortenhaus and Sprenger, 2002). Examination of *rca1*^{2} clones rescued by expression of HA-Rca1 revealed cells with normal DNA content and wild-type levels of Cyclin A. (Figure 23D-F). In contrast, *rca1*^{2} mutant clones that overexpress HA-Rca1Δ203 exhibited significantly reduced levels of Cyclin A and normal DNA content (Figure 23G-I). In rare cases, cells with higher DNA content were observed within these clones (Figure 23J-L). In addition, HA-Rca1Δ203 was absent in large regions of the clones (Figure 23I&L). Considering that HA-Rca1Δ203 and Cyclin A are unstable in G1 (Figure 20B&G), this suggests that these cells persist longer in G1. In summary, these findings demonstrate that HA-Rca1Δ203 cannot completely replace endogenous Rca1 in proliferating wing disc cells. This result is surprising since HA-Rca1Δ203 can inhibit APC/C-Fzr activity during the G2 of the 16th embryonic cell cycle. The major difference between both cell cycle types is the presence of a G1 state in imaginal disc cells, suggesting that the F-box function of Rca1 is necessary for an effective G1-S-transition.

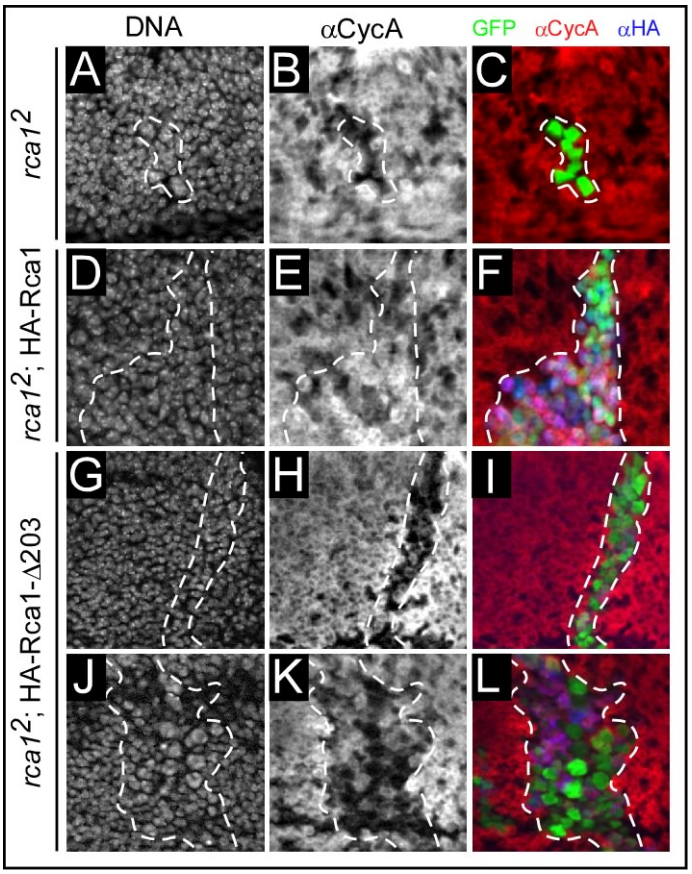

Figure 23 Cells lacking the F-box function of Rca1 reside longer in G1. Areas of wing imaginal discs containing MARCM-clones (marked with GFP) of the indicated genotypes. Discs were stained for Cyclin A, DNA and HA.
(A-C) *rca1*^{2} mutant clones fail to maintain Cyclin A levels and undergo endocycles, suggested by the increased nuclear size.
(D-F) Expression of HA-Rca1 in *rca1*^{2} mutant clones prevents premature degradation of Cyclin A and allows normal proliferation. HA-Rca1 is readily detectable in all cells of the clone.
(G-L) Most *rca1*^{2} mutant clones that express HA-Rca1Δ203 display reduced amounts of Cyclin A. Moreover, HA-Rca1Δ203 itself is unstable in these cells (I&L). Since both proteins are unstable in G1 cells, this suggests that these cells persist longer in a G1-state. Occasionally these clones exhibit cells with increased DNA level (J).

3.3.9. Rca1 promotes S-phase by a mechanism independent of Cyclin E/Cdk2

The G1-S transition in *Drosophila* eye discs is normally mediated by Cyclin E/Cdk2 (Knoblich et al., 1994; Richardson et al., 1995). To discriminate whether Rca1 requires Cyclin E/Cdk2 activity or whether Rca1 can drive cells into S-phase independently from Cyclin E, HA-Rca1 was coexpressed with the Cyclin E inhibitor Dacapo (dap). As showed above, the overexpression of HA-Rca1 results alone in ectopic BrdU labeling within the morphogenetic furrow (Figure 24B&C). Overexpression of *dap* should completely inhibit Cyclin E dependent kinase activity regardless of other upstream inputs (de Nooij et al., 1996; Reis and Edgar, 2004). According to this model, Dap overexpression prevents BrdU incorporation in cells of the second mitotic wave, indicating that these cells cannot enter S-phase when Cdk2 activity is suppressed (Figure 24D&E). Interestingly, clones that coexpress Dap and HA-Rca1 show ectopic BrdU incorporation ahead of the second mitotic wave (Figure 24F&G; de Nooij et al., 1996). Thus, overexpression of DAP cannot suppress S-phase induction by HA-Rca1, suggesting that Rca1 promotes S-phase entry by an alternative pathway.

F-box proteins are part of SCF complexes and mediate the degradation of target proteins. The F-box dependent S-phase induction by Rca1 suggests that SCF/Rca1 might mediate degradation of a negative regulator of S-phase entry. Hence, Dap might be a good candidate for a target of the putative SCF/Rca1 complex. Excess SCF/Rca1 activity could force Dap turnover, explaining why Dap fails to prevent S-phase entry after coexpression with HA-Rca1. In wild-type eye imaginal discs, endogenous Dap protein can be detected at low levels in the posterior part of the morphogenetic furrow (de Nooij et al., 2000; de Nooij et al., 1996). However, Dap levels were not altered in Rca1 overexpressing clones spanning the morphogenetic furrow (Figure 24H-K), thus demonstrating that Dap stability is not regulated by SCF/Rca1. In summary, these experiments demonstrate that ectopic Rca1 expression accelerates the G1-S transition independently from Cyclin E/Cdk2 and that Dap protein becomes not targeted for degradation by the SCF/Rca1 complex.

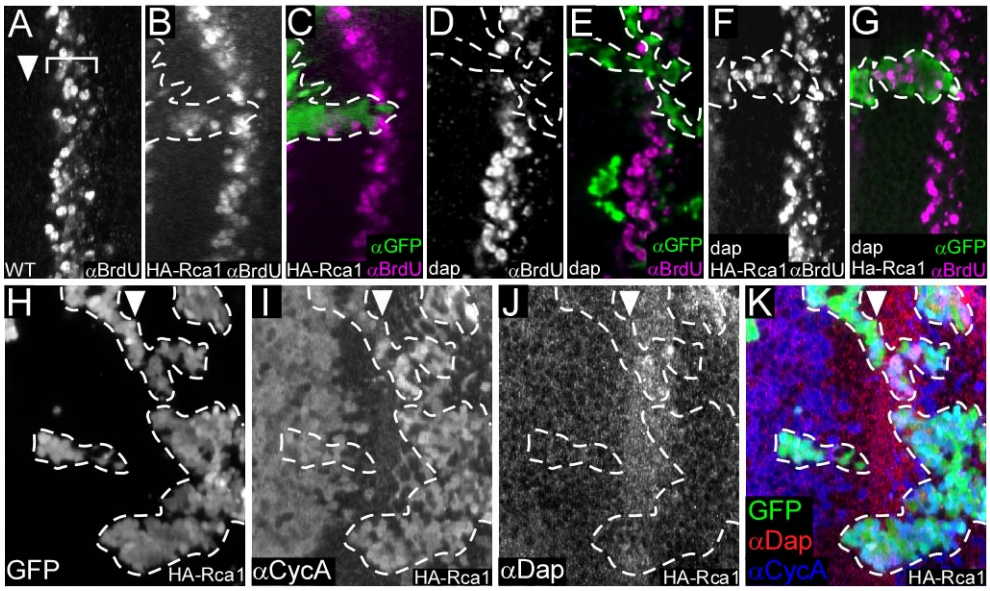

Figure 24 Rca1 overexpression can induce S-phase independent from Cyclin E/Cdk2. HA-Rca1 and Dacapo (dap) were overexpressed under control of the actin promoter using the "flpout" method. Clones were positively marked by GFP. The arrowheads indicate the morphogenetic furrow and the brackets mark the second mitotic wave.
(A) BrdU expression in wild-type eye discs. The brackets indicate the second mitotic wave and the arrowheads the morphogenetic furrow.
(B&C) HA-Rca1 overexpression results in ectopic S-phases within the morphogenetic furrow.
(D&E) Overexpression of Dap blocks S-phase entry in the second mitotic wave indicated by the absence of BrdU-incorporation in the clone area.
(F&G) Coexpression of Dap and HA-Rca1 does not affect Rca1's ability to induce S-phases.
(H-K) Endogenous Dap levels were not influenced by overexpression of Rca1, demonstrating that Dap is not a target of the putative SCF/Rca1 complex.

3.3.10. Fzr is not a target of the SCF/Rca1 complex

The experiments described above showed that Rca1 promotes S-phase entry independently from the main S-phase inducer Cyclin E/Cdk2, suggesting that Rca1 acts through an alternative pathway. Previous work revealed that Cyclin A/Cdk1 can trigger S-phase in *Drosophila*. However, the S-phase inducing activity of CyclinA/Cdk1 is normally diminished by at least three mechanisms (Pimentel and Venkatesh, 2005). On the one hand, S-phase entry is prevented by activation of the APC/C-Fzr complex which mediates the destruction of Cyclin A. On the other hand, Cdk1 activity becomes restricted by the action of the CKI *roughex* as well as inhibitory phosphorylation (Sprenger et al., 1997; Thomas et al., 1997). Since Rca1 interacts physically with Fzr and the APC/C it was assumed that Rca1 inhibits APC/C-Fzr activity in a competitive manner (Grosskortenhaus and Sprenger, 2002), a model that is further supported by recent work on Emi1 (Miller et al., 2006). However, ectopic S-phases induced by Rca1 cannot simply be explained by competitive APC/C-Fzr inhibition, since cyclin accumulation and S-phase entry induced by Rca1 requires a functional F-box although this motif is dispensable for APC/C-Fzr inhibition. Therefore, a reasonable model

how Rca1 mediates S-phase entry could be that Rca1 rather mediates the degradation of Fzr in G1 cells. To test this hypothesis, HA-Fzr and Rca1 were coexpressed in eye imaginal discs. BrdU-labelling reveals that coexpression of HA-Fzr blocks the ability of Rca1 to induce ectopic S-phases (Figure 25), whereas HA-Fzr overexpressed alone had no effect on S-phase entry in the second mitotic wave. However, HA-Fzr levels were not changed upon coexpression with Rca1 in eye imaginal discs (Figure 25L-O), indicating that Rca1 does not target Fzr for degradation. Therefore, the absence of ectopic S-phases after coexpression of Rca1 and HA-Fzr is likely caused by a competitive effect of Fzr.

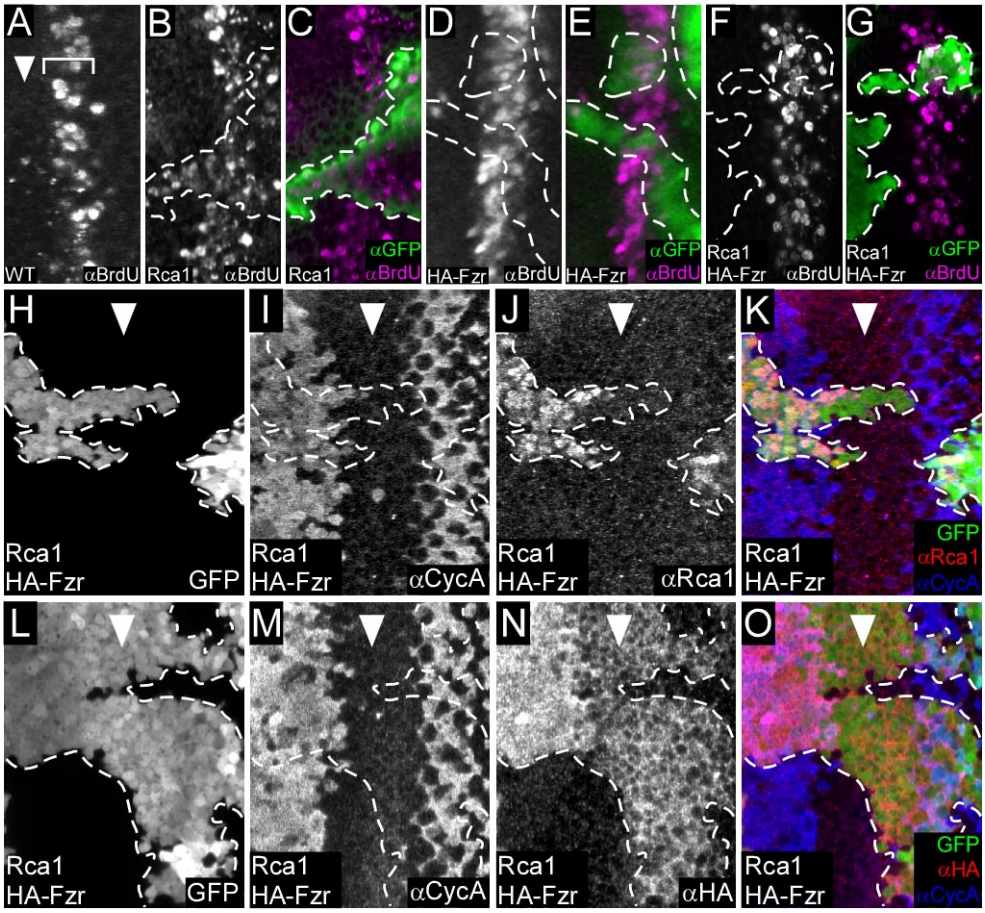

Figure 25 Fzr is not a target of the SCF/Rca1-complex. Rca1 and HA-Fzr were overexpressed under control of the actin promoter using the "flpout" method. The brackets indicate the second mitotic wave and the arrowheads the morphogenetic furrow.
(A) BrdU incorporation in wild-type eye discs.
(B&C) Rca1 overexpression promotes S-phase entry shown by ectopic BrdU-labelling.
(D&E) HA-Fzr overexpression does not affect S-phase entry in the second mitotic wave.
(F&G) Coexpression of Rca1 and HA-Fzr blocks Rca1 ability to accelerate S-phase entry.
(H-K) Clones expressing both, Rca1 and HA-Fzr fail to accumulate Cyclin A demonstrating that HA-Fzr overexpression can neutralize the effects of Rca1 overexpression.
(L-O) HA-Fzr is readily detectable in clones coexpressing Rca1 and HA-Fzr suggesting that Fzr is not a target of the putative SCF/Rca1-complex.

3.4. Investigation of the S-phase promoting activity of Rca1 in endoreplicating cells

In *Drosophila* larvae, cells of many tissues like the gut, the fat body or the salivary glands pass through a specialized cell cycle called endoreplication cycle or endocycle (Edgar and Orr-Weaver, 2001; Lilly and Duronio, 2005). Endoreplicating cells undergo multiple rounds of DNA replication without intervening mitosis. Recent studies have demonstrated that the G1/S regulatory machinery that is present in mitotic cells is also involved in regulation of endoreplication cycles (Edgar and Orr-Weaver, 2001; Lilly and Duronio, 2005; Sauer et al., 1995). Several studies have demonstrated that Cyclin E/ Cdk2 activity is essential for endocycle progression (Knoblich et al., 1994; Lilly and Spradling, 1996). Furthermore, it has been shown that the activities of the Cyclin E/ Cdk2 inhibitor Dacapo as well as the transcription factor E2F1 are particularly important during endoreplication (de Nooij et al., 2000; Duronio and O'Farrell, 1995). Since mitosis is bypassed in endoreplicating cells, transcription of mitotic regulators such as String/Cdc25, Cdk1, as well as mitotic cyclins has been terminated in these tissues (Klebes et al., 2002; Sauer et al., 1995). The endocycle resembles therefore a mitotic cycle in which the S-phase controls are maintained, but the cells are no longer forced to enter mitosis (Lilly and Duronio, 2005). Thus, endocycling cells are a valuable model to study S-phase entry without interfering with mitotic controls, a feature that greatly facilitates further analysis of Rca1 function.

3.4.1. Rca1 is not required for endocycle progression

The experiments described above, suggest that Rca1 is implicated in the transition from G1 to S-phase, at least in imaginal discs. This observation raised the question whether Rca1 activity contributes also to S-phase entry in endocycling tissues. Since mitosis is bypassed and the cells cannot arrest at the G2/M transition, this celltype enables to study S-phase entry in absence of Rca1. To test whether loss of Rca1 activity perturbs endocycle progression, clones mutant for $rca1^2$ were generated in salivary glands by the MARCM technique (Lee and Luo, 1999). Since mitotic recombination does not occur in polytene cells, clones were induced during embryogenesis within the salivary placode. However, at the third instar stage $rca1^2$ mutant cells did not show any abnormalities in their DNA content (Figure 26). This observation indicates that $rca1^2$ mutant cells underwent the same number of endoreplication cycles as the surrounding control cells and demonstrates furthermore that Rca1 activity is not essential for endocycle progression in salivary glands.

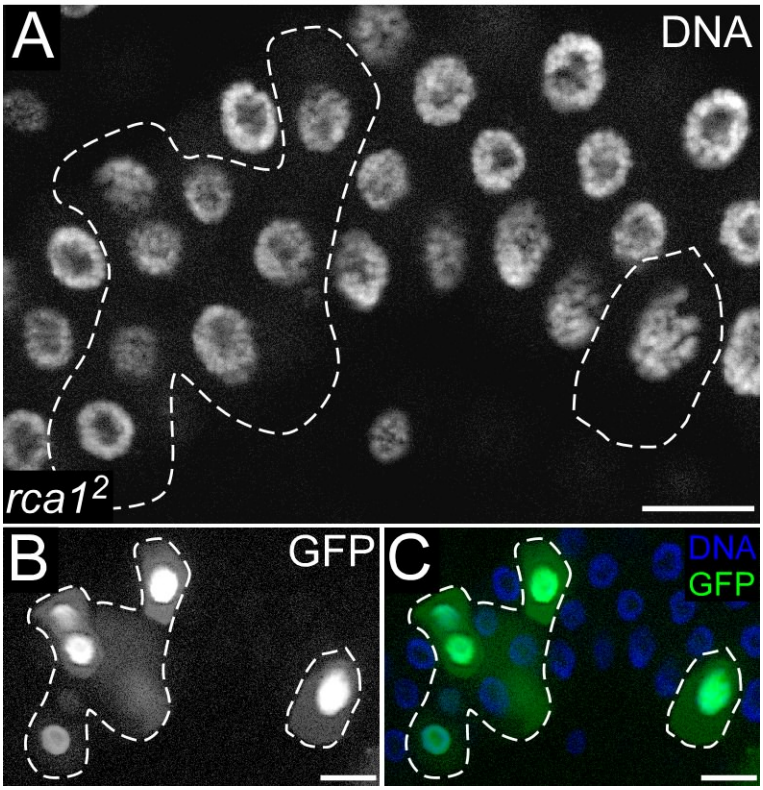

Figure 26 Endocycle progression is not affected in *rca1²* mutant clones. Section of a third instar salivary gland containing cells mutant for *rca1²*: Clones mutant for *rca1²* were generated in the embryonic salivary placode by the MARCM technique. *rca1²* mutant cells are marked by GFP expression. Furthermore, salivary glands were stained for DNA to monitor endocycle progression. Scale bar : 100µm
(A-C) Cells lacking Rca1 display similar DNA content than the surrounding control cells, indicating that Rca1 is not essential for endocycle progression in salivary glands.

3.4.2. Overexpression of Rca1 perturbs endocycle progression in a F-box dependent manner

Rca1 activity is apparently not essential for S-phase entry in endoreplicating tissues. However, this observation does not exclude that Rca1 overexpression affects S-phase entry in endocycling cells in a similar manner than in imaginal discs. To test this hypothesis HA-Rca1 was continuously overexpressed in salivary glands using the "flpout" method (Ito et al., 1997). Previously, it has been shown that APC/C-Fzr activity is essential for the switch from mitotic to endoreplication cycles during embryogenesis (Sigrist and Lehner, 1997), suggesting that Rca1 overexpression might affect endocycle initiation rather than S-phase entry. Therefore, expression of HA-Rca1 was induced during first the instar stage when cells of the salivary gland had already entered the endoreplication program. To monitor the effects of Rca1 misexpression, the DNA content in salivary glands from third instar larvae was determined by fluorescence microscopy. Cells continuously expressing HA-Rca1 display a significant reduction of the DNA content compared to the adjacent wild-type cells (Figure 27A). At first glance, this result appears be contrary to the accelerated S-phase entry observed in imaginal discs after Rca1 overexpression. However, it has previously been shown that continuous expression of the S-phase inducer Cyclin E prevents endocycle progression (Follette et al., 1998; Weiss et al., 1998). Hence, the reduced DNA content found in Rca1 overexpressing cells might be caused by the S-phase promoting function of Rca1.

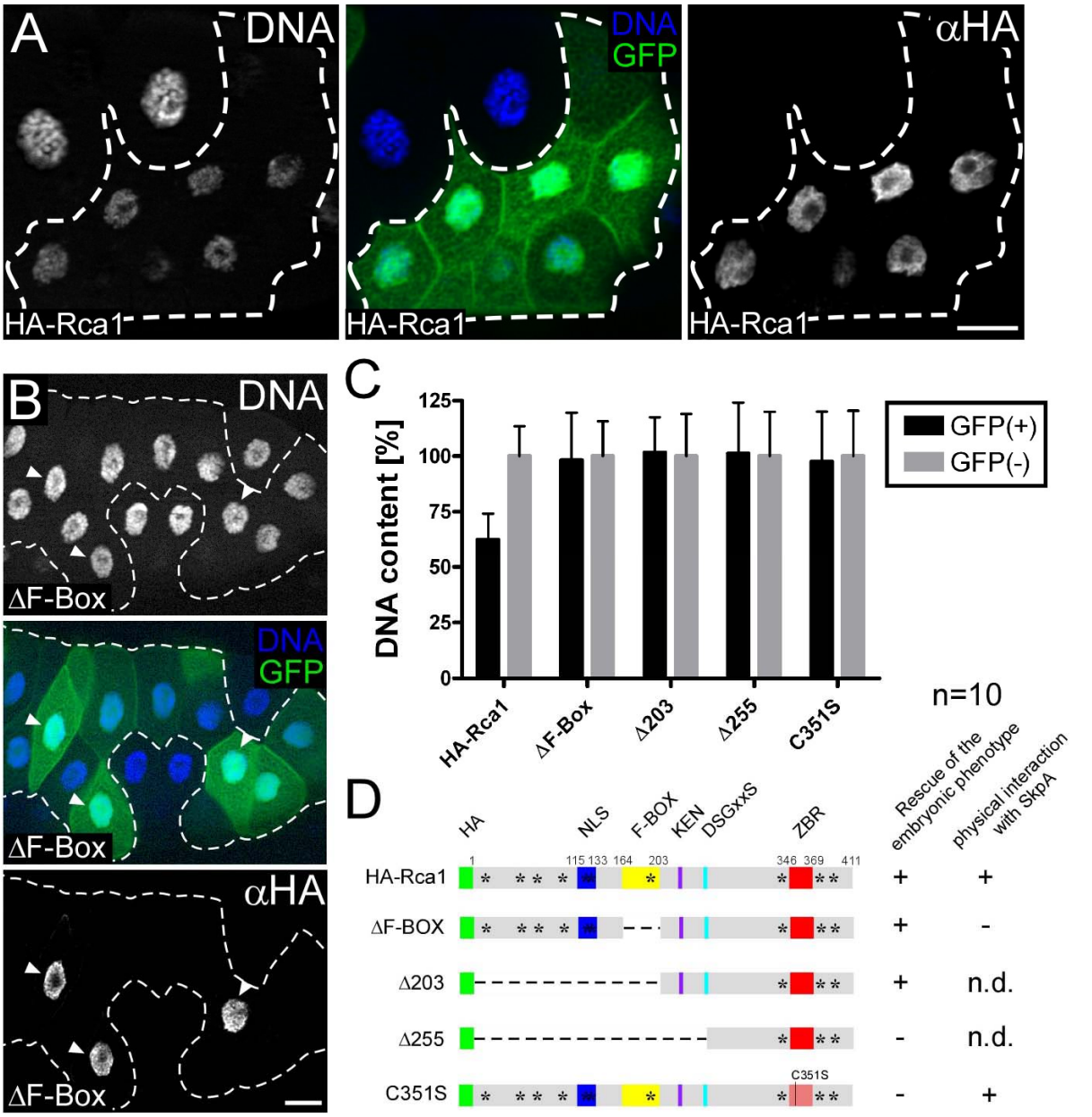

Figure 27 Rca1 overexpression prevents polyploidization in an F-box dependent manner. Certain Rca1 constructs were overexpressed in larval salivary glands under control of the actin promoter using the "flpout" method. Overexpressing cells were visualized by coexpression of GFP and antibody staining against the HA-tag. DNA contents of nuclei from at least 10 different salivary glands were quantified by fluorescence microscopy (C). Scale bar: 100µm.

(A) Salivary gland cells overexpressing HA-Rca1 display reduced DNA levels compared to wild-type cells, demonstrating that excess Rca1 activity blocks endoreplication.

(B-D) Overexpression of Rca1 constructs lacking the F-box (HA-Rca1Δ203 and HA-Rca1ΔF-box) does not perturb endocycle progression indicated by DNA contents similar to wild-type cells (B&C). Both proteins are functional since they are capable to rescue the embryonic phenotype of *rca1* (D). Moreover, HA-Rca1 and HA-Rca1ΔF-box accumulate to similar levels, demonstrating that the failure of HA-Rca1ΔF-box to impair endoreplication is not due to a dose effect (A&B). Hence, these results lead to the conclusion that the F-box is essential for Rca1´s effect on endocycle progression, suggesting that Rca1 is part of a yet uncharacterized SCF-complex. A construct with a point mutation in the ZBR (C351S), that does not prevent SkpA binding, fails to block endoreplication, indicating that the effect of Rca1 overexpression does not result from a dominant negative effect.

The endocycle breakdown upon Rca1 overexpression might be due to the S-phase inducing function of Rca1, thus raising the question whether the structural elements required for S-phase induction in mitotic cells are also essential for endocycle disruption. Therefore, salivary glands overexpressing certain Rca1 constructs in scattered cells were inspected for their DNA content (Figure 27C). Remarkably, these experiments revealed that the block of endoreplication caused by Rca1 overexpression relies like S-phase induction in imaginal discs on a functional F-box. Overexpression of both Rca1 constructs lacking the F-box (HA-Rca1Δ203 and HA-Rca1ΔF-box) did not affect the DNA content in third instar salivary glands (Figure 27B&C), although both proteins are potent inhibitors of the APC/C-Fzr complex in the embryo (Figure 14). Furthermore, expression levels were compared to exclude that the inactivity of HA-Rca1ΔF-box is due to a dose effect. However, staining with antibodies against the HA-tag, revealed that HA-Rca1 and HA-Rca1ΔF-box were expressed in similar amounts (Figure 27B&C), so that the failure of HA-Rca1ΔF-box to block endoreplication cannot be explained by a dose effect. Rca1 activity is normally not required in endoreplicating cells raising the problem that the phenotype observed upon Rca1 overexpression might be due to a dominant negative effect. Rca1 could perturb endocycle progression by sequestering the SkpA subunit of an SCF-complex that is essential for endoreplication. To exclude this possibility, a construct (HA-Rca1C351S) was used that is inactive due to a pointmutation in its ZBR, but still binds SkpA (S. Querings, personal communication). Overexpression of HA-Rca1C351S has no influence on endocycle progression in salivary glands (Figure 27C&D), so that a dominant negative effect can be ruled out. In summary, these findings demonstrate that excess Rca1 activity can promote S-Phase entry also in endoreplicating salivary gland cells and that this occurs most likely by the same F-box dependent mechanism as in imaginal discs.

3.4.3. Continuous expression of Rca1 increases Cyclin E levels in salivary glands

In the recent years, comprehensive research on endoreplication discovered that the re-replication controls that ensure that each DNA sequence is only replicated once per cycle are also present in endocycling cells (Edgar and Orr-Weaver, 2001; Lilly and Duronio, 2005). In this regard the interplay between Cdk´s and the machinery that mediates the licensing of DNA replicating origins was of particular interest. During a replication cycle, the cell is faced with two contrary requirements. DNA licensing or more precisely the formation of pre-replication complexes can only occur at stages with low Cyclin/Cdk activity, whereas the actual initiation

of DNA replication requires high levels of Cyclin/Cdk activity (Bell and Dutta, 2002; Blow and Dutta, 2005). In *Drosophila*, initiation of DNA replication mainly relies on Cyclin E/Cdk2 activity (Knoblich et al., 1994; Lane et al., 2000). Several studies revealed that Cyclin E levels oscillate in endoreplicating tissues (de Nooij et al., 2000; Lilly and Spradling, 1996; Weng et al., 2003). Moreover, continuous expression of Cyclin E resulted in endocycle breakdown due to interference with the DNA licensing machinery (Follette et al., 1998; Su and O'Farrell, 1998; Weiss et al., 1998). The phenotype observed after continuous Rca1 expression (Figure 27A) appeared very similar to that resulting from Cyclin E overexpression (Figure 32), although the impaired DNA replication was not analyzed in detail. This correlation raised the question whether Rca1 misexpression affects Cyclin E oscillation. Therefore, Cyclin E levels were examined in third instar salivary glands that express HA-Rca1 in scattered cells. The amount of Cyclin E protein was significantly increased in cells continuously expressing Rca1 (Figure 28A-D), suggesting that the diminished DNA replication is due to the elevated Cyclin E levels. The abundance of Cyclin E protein does not rely on APC/C activity, therefore this result supports the notion that Rca1 has additional functions besides its role as an inhibitor of the APC/C-Fzr.

In endoreplicating cells, the levels of Cyclin E oscillate not only at the protein level, but also on the level of transcription (Duronio and O'Farrell, 1995; Sauer et al., 1995). To investigate whether the upregulation of Cyclin E levels by Rca1 is due to increased transcription, expression of Cyclin E was monitored by a reporter construct that contains 16.4 kb of the regulatory region of the Cyclin E gene fused to lacZ (Jones et al., 2000; Wu et al., 2003). Inspection of third instar salivary glands that overexpress Rca1 in scattered cells revealed that the Cyclin E reporter construct is highly activated in the Rca1 expressing cells (Figure 28E-H). At the first glance this result suggests that Rca1 activates the transcription of Cyclin E. However, in endoreplicating cells, Cyclin E and the transcription factor E2F1/Dp cooperate in an autoregulatory feedback loop which ensures that the accumulation of Cyclin E peaks prior to S-phase entry (Duronio and O'Farrell, 1995; Sauer et al., 1995). Cyclin E/Cdk2 activates E2F1 by phosphorylation of its inhibitor Rbf. E2F1 in turn promotes transcription of Cyclin E. Since, Cyclin E can indirectly stimulate its own transcription by derepressing E2F1, the enhanced Cyclin E transcription observed after Rca1 overexpression must not necessarily result from a direct effect of Rca1 on Cyclin E transcription. Hence, it remains unclear whether the upregulation of Cyclin E by Rca1 occurs at transcriptional or posttranscriptional level, but it confirms the result obtained by staining with antibodies against Cyclin E.

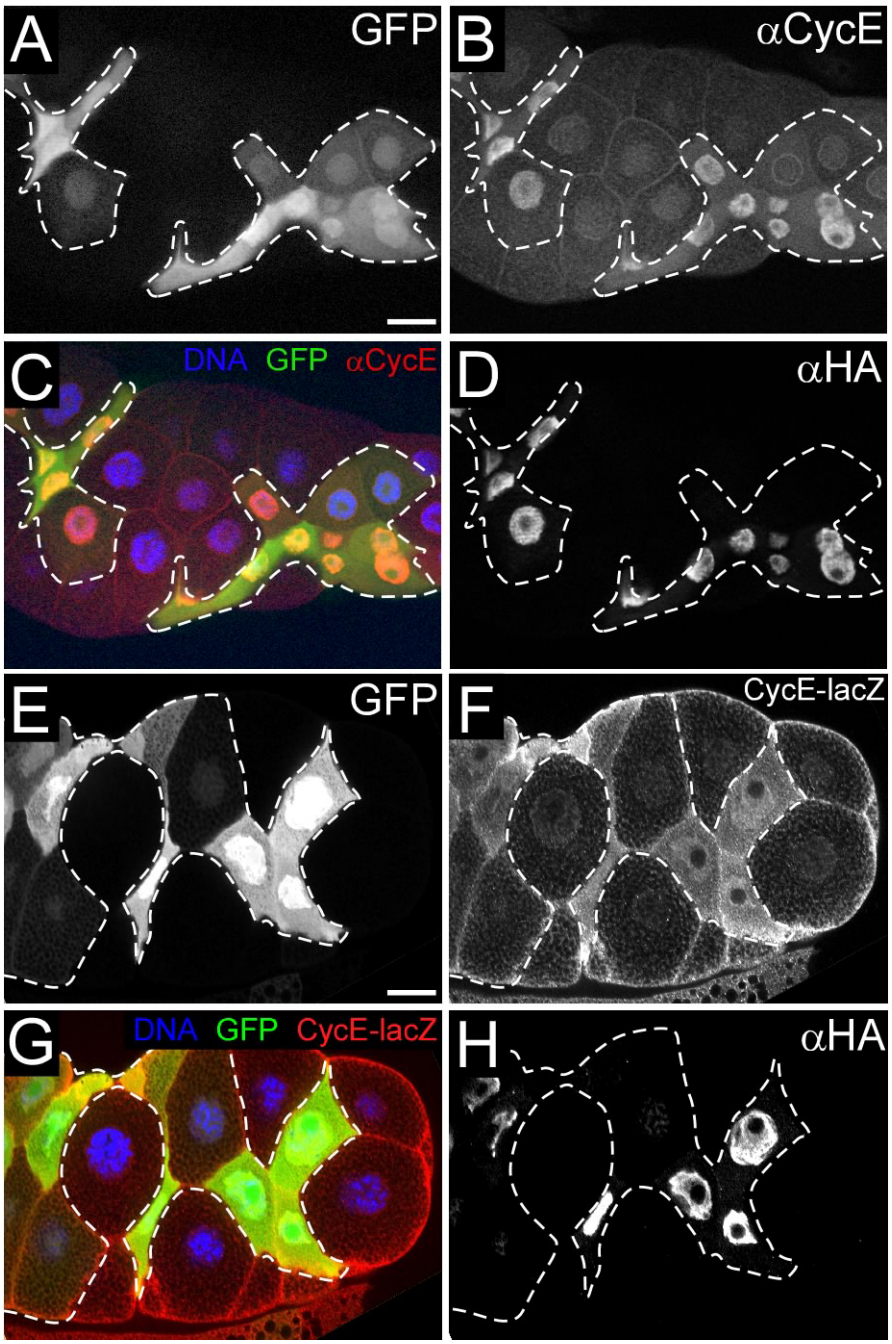

Figure 28 Rca1 overexpression results in increased Cyclin E levels. HA-Rca1 was overexpressed in larval salivary glands under control of the actin promoter using the "flpout" method. Cells overexpressing HA-Rca1 were visualized by coexpression of GFP and antibody staining against the HA-tag. Scale bar : 100µm. **(A-D)** Excess Rca1 leads to stabilization of Cyclin E protein (B). Since continuous Cyclin E activity perturbs endocycle progression, the reduced DNA content (C) observed in Rca1 overexpressing clones is likely due to the elevated levels of Cyclin E. **(E-F)** Overexpression of Rca1 leads to increased transcription of Cyclin E mRNA, visualized by a CycE-lacZ reporter construct. Cyclin E is part of an autoregulatory feedback loop. Therefore, it remains unclear whether Rca1 stimulates Cyclin E transcription directly or indirectly by increasing Cyclin E level posttranscriptional.

3.4.4. Rca1 expression forces endoreplicating cells to re-enter a mitotic state

In *Drosophila*, the commitment of Cyclin A and its kinase partner Cdk1 is crucial for entry into mitosis (Lehner and O'Farrell, 1989; Stern et al., 1993). Cells lacking either of these genes fail to accomplish mitosis and enter endoreplication cycles instead (Grosskortenhaus and Sprenger, 2002; Smith and Orr-Weaver, 1991; Stern et al., 1993; Weigmann et al., 1997). Several studies revealed moreover that entry into the endocycle is accompanied with transcriptional downregulation of several mitotic regulators including Cyclin A, Cyclin B, Cyclin B3, Cdk1 and String/Cdc25 (Klebes et al., 2002; Sauer et al., 1995; Shcherbata et al.,

2004). Concomitantly to the transcriptional downregulation of mitotic regulators, transcription of the APC/C activator fizzy-related (Fzr) gets upregulated (Schaeffer et al., 2004; Sigrist and Lehner, 1997). In *fzr* mutants, embryonic salivary gland cells fail to enter endoreplication, demonstrating that proteasomal degradation of mitotic cyclins by the APC/C-Fzr complex is absolutely crucial for endocycle entry (Sigrist and Lehner, 1997). The activation of the APC/C-Fzr complex results in rapid degradation of mitotic cyclins and thereby creates a sharp drop of Cdk1 activity that drives the cells into the endocycle. Hence, it is thought that activation of the APC/C-Fzr is the actual trigger for endocycle entry. The transcriptional downregulation of mitotic cyclins seems to be rather required to maintain the endocycle by preventing re-entry into mitosis. It is assumed that APC/C-Fzr activity is not required once the endoreplication program has been initiated (Edgar and Orr-Weaver, 2001; Lilly and Duronio, 2005). However, both mechanisms may also cooperate after endocycle entry to sustain low levels of Cdk1 activity. Inhibition of APC/C-Fzr activity and subsequent accumulation of mitotic cyclins might therefore be a reasonable explanation for the endocycle block observed after Rca1 overexpression. In order to test this hypothesis, the levels of Cyclin A and Cyclin B in third instar salivary glands that overexpress HA-Rca1 were analyzed by fluorescence microscopy and Western blotting (Figure 29). Interestingly, only Cyclin A accumulates after Rca1 overexpression (Figure 29B&I), whereas Cyclin B levels are unaffected (Figure 29I). If Rca1 inhibits APC/C-Fzr activity one would expect that both proteins accumulate simultaneously. Moreover, if Cyclin A protein accumulates due to APC/C-Fzr inhibition, this would at least require the abundance basal levels of Cyclin A transcript. However, several studies have demonstrated that Cyclin A transcription is turned off in endoreplicating cells (Klebes et al., 2002; Sauer et al., 1995). Finally, the accumulation of Cyclin E in Rca1 overexpressing cells argues against this model, since Cyclin E is not a target of the APC/C-Fzr complex. Thus, the impaired endoreplication upon Rca1 overexpression cannot be simply explained by downregulation of APC/C-Fzr activity. It seems that Rca1 overexpression rather stimulates Cyclin A transcription.

Fluorescence microscopy indicated furthermore that Cyclin A accumulates in the nucleus upon Rca1 overexpression (Figure 29B&I), whereas Cyclin A expressed from a transgene is predominantly detectable in the cytoplasm (Figure 30B). In mitotic cells Cyclin A enters the nucleus just after entry into mitosis (Dienemann and Sprenger, 2004; Lehner and O'Farrell, 1989), thus this observation strongly supports the idea that Rca1 overexpression promotes re-entry into mitosis. After endocycle entry, transcription of Cdk1 becomes terminated as well,

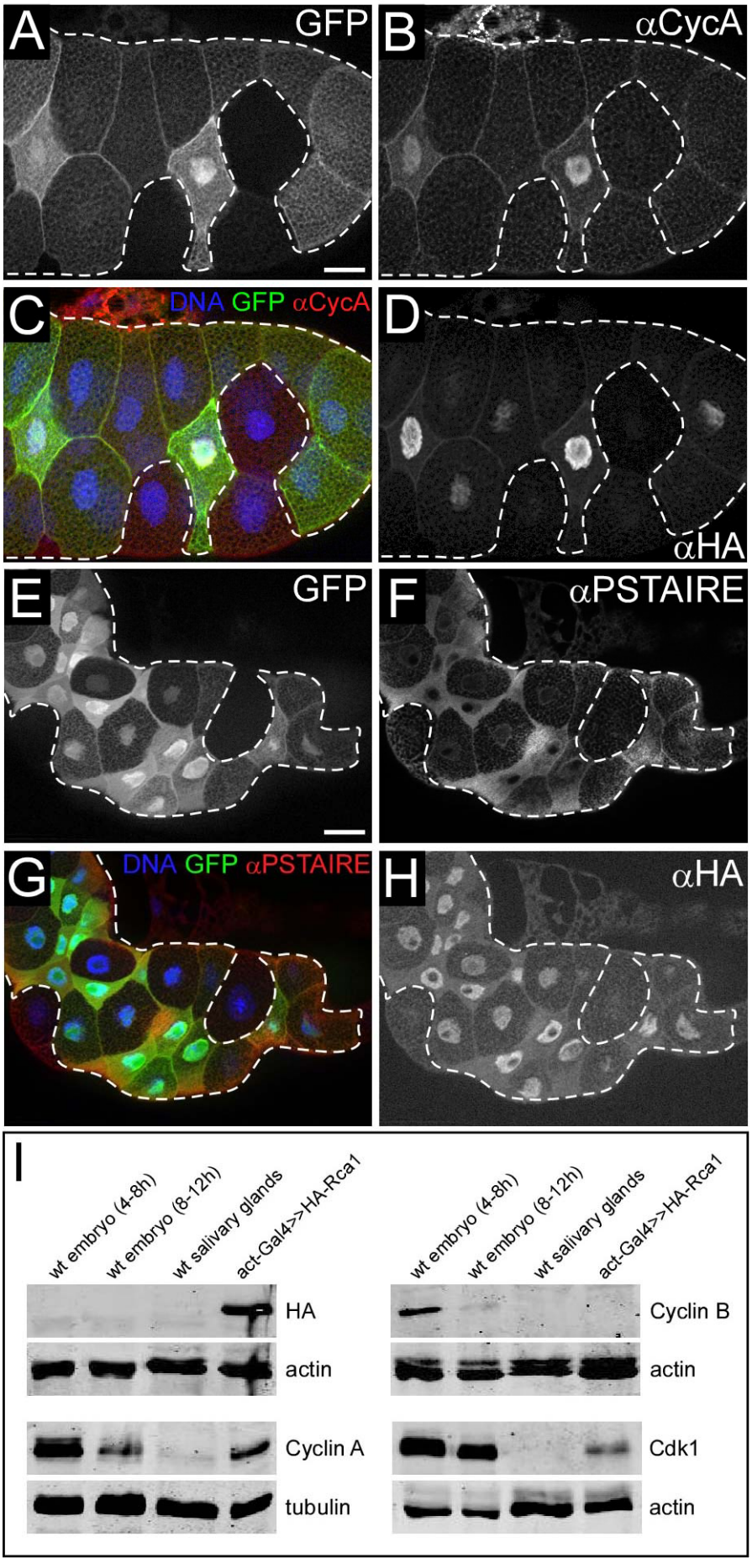

Figure 29 Rca1 overexpression leads to re-accumulation of mitotic proteins. HA-Rca1 was overexpressed in larval salivary glands under control of the actin promoter using the "flpout" method. Cells overexpressing HA-Rca1 were visualized by coexpression of GFP and antibody staining against HA. Scale bar : 100µm.

(A-D) Rca1 overexpression results in accumulation of Cyclin A (B). This result is surprising since Cyclin A expression becomes terminated upon endocycle entry. Interestingly, Cyclin A is accumulates in the nucleus, whereas Cyclin A derived from a transgene is predominantly cytoplasmic (Figure 30). Considering that Cyclin A enters the nucleus at the onset of mitosis (D), this suggests that Rca1 overexpressing cells try to re-enter a mitotic state.

(E-H) Cdk1 protein accumulates in cells overexpressing Rca1 demonstrated by antibody staining against the PSTAIRE motif of Cdk1.

(I) Extracts derived from embryos of the indicated age and larval salivary glands were analyzed by Western blotting. Cyclin A, Cyclin B and Cdk1 are absent in wild-type salivary glands. However, after expression of HA-Rca1 Cyclin A and Cdk1 were readily detectable, whereas Cyclin B was still absent. Since Cyclin A and B are substrates of the APC/C, this observation indicates that the block of endoreplication is not caused by Rca1's inhibitory effect on APC/C-Fzr.

raising the idea that Rca1 overexpression could also stimulate accumulation of Cdk1, which is not subject of APC/C mediated degradation. To investigate this idea, salivary glands overexpressing HA-Rca1 were inspected for Cdk1 protein (Figure 29). Fluorescence microscopy as well as Western blotting demonstrate that Cdk1 accumulates at high levels in salivary glands which overexpress HA-Rca1 (Figure 29). In summary, these findings suggest that Rca1 overexpression forces re-entry in mitosis by activating the transcription of mitotic regulators. However, it remains to be elucidated whether this occurs via downregulation of APC/C-Fzr activity or by employment of an alternative pathway.

3.4.5. Overexpression of Cyclin A cannot impair endoreplication in larval salivary glands

DNA replication in *Drosophila* is normally initiated by Cyclin E/Cdk2 (Knoblich et al., 1994; Lane et al., 2000), but several studies have demonstrated that under certain circumstances Cyclin A/Cdk1 can trigger DNA replication as well (Sprenger et al., 1997; Thomas et al., 1994; Thomas et al., 1997). Upregulation of Cyclin A/Cdk1 activity should therefore block endoreplication in a similar manner than Cyclin E overexpression. To investigate whether Cyclin A overexpression can perturb endocycle progression, continuous expression of HA-CycA was induced during the first instar stage. Inspection of DNA levels in third instar salivary glands revealed that misexpression of HA-CycA does not affect endoreplication (Figure 30D&F). Furthermore, antibody staining indicated that Cyclin E levels are not affected by overexpression of HA-CycA (Figure 30D&F). Thus, this experiment excludes that the accumulation of Cyclin A in Rca1 overexpressing cells enhances Cyclin E protein levels indirectly. During embryogenesis, when residual amounts of Cdk1 protein are still abundant, misexpression of Cyclin A can perturb endoreplication (F. Sprenger, personal communication; Follette et al., 1998). The incapability of Cyclin A to prevent endoreplication in larval salivary glands is therefore most probably due to the lack of its kinase partner Cdk1 at this stage. As mentioned above, HA-CycA is localized predominantly in the cytoplasm (Figure 30D&F), whereas endogenous Cyclin A accumulates in the nucleus after Rca1 overexpression. Since nuclear targeting of Cyclin A requires Cdk1 activity, this observation confirms that Cdk1 protein is not abundant in larval salivary glands. Furthermore, this strongly supports the idea that Cyclin E, Cyclin A and Cdk1 levels are simultaneously increased after Rca1 overexpression.

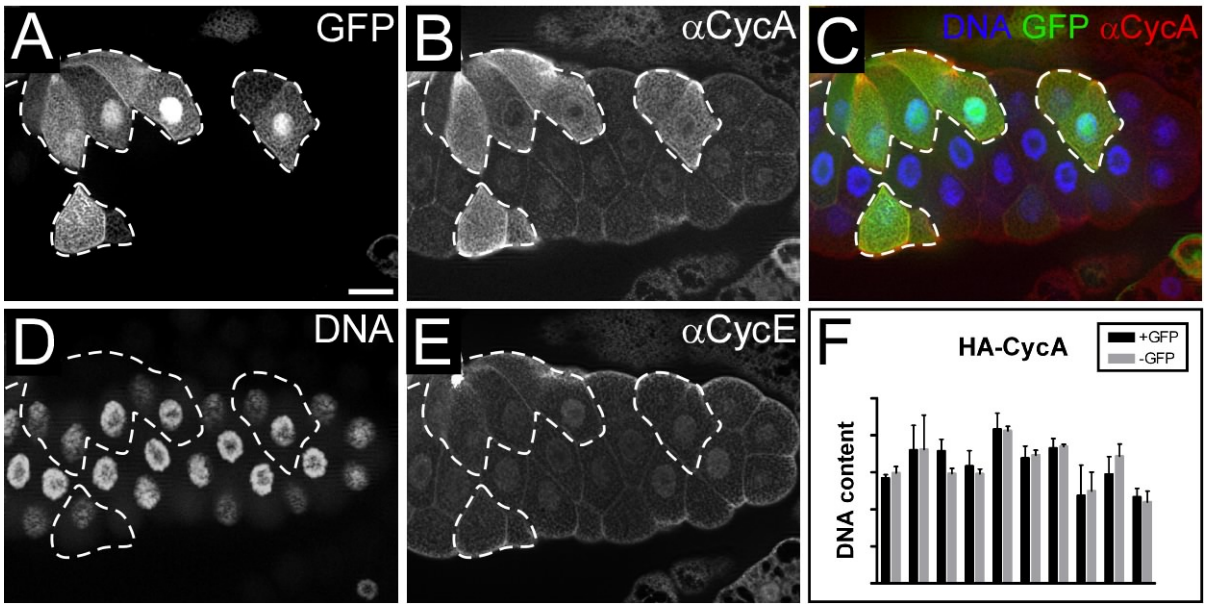

Figure 30 Excess Cyclin A does not affect endocycle progression. HA-Cyclin A was overexpressed in larval salivary glands under control of the actin promoter using the "flpout" method. Cells overexpressing Cyclin E were visualized by coexpression of GFP. Scale bar: 100µm.
(A-E) Overexpression of Cyclin A does not affect the DNA content (B&F), demonstrating that the endocycle program is not influenced by excess Cyclin A. Furthermore, the levels of Cyclin E are not influenced by Cyclin A overexpression (A). The failure of Cyclin A to block endoreplication is likely due to the absence of its kinase partner Cdk1 in salivary glands.
(F) Quantification of DNA contents by fluorescence microscopy. Mean values of several nuclei of individual salivary glands are represented by each set of bars (n=10).

3.4.6. The endocycle breakdown induced by Rca1 overexpression is due to impaired DNA licensing

The simultaneous accumulation of Cyclin E, Cyclin A and Cdk1 upon Rca1 overexpression raises the question whether the endocycle breakdown is caused by the enhanced Cyclin E levels or it is due to the rise of Cyclin A/Cdk1 activity. Continuous Cyclin E expression halts the endocycle by interfering with the DNA licensing machinery (Follette et al., 1998; Su and O'Farrell, 1998; Weiss et al., 1998). It is thought that Cyclin A/Cdk1 can perturb DNA licensing as well (F. Sprenger, personal communication; Follette et al., 1998). However, an alternative explanation might be that Cyclin A/Cdk1 drives the cells into a mitotic state and prevents thereby endoreplication. To discriminate between these possibilities, Rca1 and the Cdk1 inhibitor *roughex* (rux) were coexpressed in larval salivary glands. Although Cyclin A/Cdk1 activity was damped by Rux, HA-Rca1 was capable to disrupt endoreplication (Figure 31C-F), whereas overexpression of Rux alone has no effect on endocycle progression (Figure 31A&B). Hence, these results indicate that the endocycle breakdown observed upon Rca1 overexpression is due to impaired DNA licensing. If Cyclin E/Cdk2 prevents DNA licensing alone or both, Cyclin E/Cdk2 and Cyclin A/Cdk1 contribute to this process remains unclear. The latter explanation however seems to be more likely.

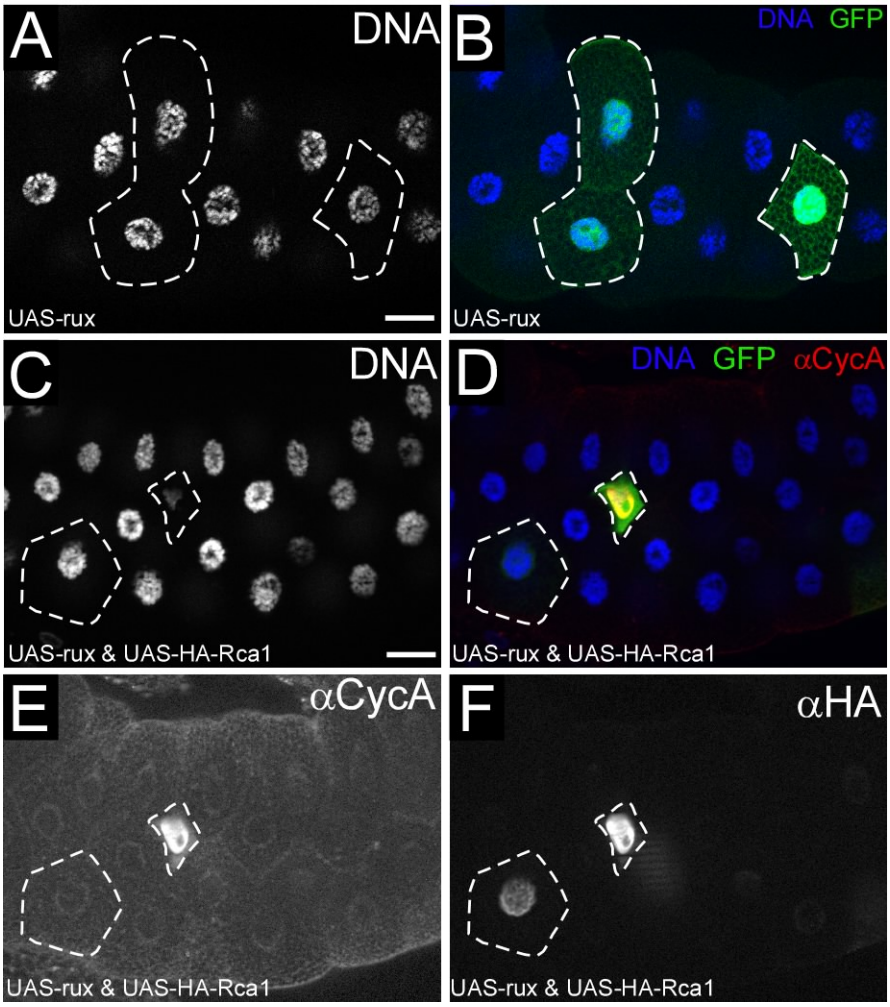

Figure 31 The accumulation of Cyclin A/Cdk1 is not crucial for endocycle breakdown induced by Rca1. HA-Rca1 and Rux were overexpressed in larval salivary glands under control of the actin promoter using the "flpout" method. Overexpressing cells were visualized by coexpression of GFP and antibody staining against the HA-tag. Scale bar : 100µm
(A-B) Overexpression of Rux does not impair endocycle progression evidenced by DNA contents similar to the surrounding wild-type cells.
(C-F) Excess HA-Rca1 blocks endocycle progression, although Cdk1 activity is damped by coexpression of Rux.

3.4.7. Continuous Cyclin E expression results in accumulation of Cyclin A and Cdk1

Overexpression of Cyclin E rescues the G2 arrest in *rca1*^{2} embryos (Grosskortenhaus and Sprenger, 2002), suggesting that Rca1 and Cyclin E have at least partly overlapping functions. Moreover, genetic epitasis experiments indicated that Rca1 and Cyclin E/Cdk2 act in concert to restrict APC/C-Fzr activity during postblastoderm cell cycles (Figure 10; Reber et al., 2006; Sigrist and Lehner, 1997). Although APC/C-Fzr activity is assumed to be not required for endoreplication upon endocycle entry, Western blotting revealed that Fzr is present in larval salivary glands and that the amount of Fzr protein is comparable to that of postblastoderm embryos (C. Lehner, personal communication). The abundance of Fzr in larval salivary glands raises the question whether the APC/C-Fzr complex may have a function in larval salivary glands. If the phenotype observed after Rca1 overexpression is due to downregulation of APC/C-Fzr activity, overexpression of Cyclin E should therefore have similar effects. To address if Cyclin E phenocopies the effect of Rca1 overexpression, third instar salivary glands that overexpress Cyclin E in scattered cells were inspected for Cyclin A

and Cdk1 levels. Both proteins were readily detectable in Cyclin E overexpressing cells (Figure 32), demonstrating that the functions of Cyclin E and Rca1 overlap also in endoreplicating cells. However, it remains unclear whether Cyclin E stimulates transcription of Cyclin A and Cdk1 by downregulation of APC/C-Fzr activity. Nevertheless, a very attractive model might be that after endocycle entry, APC/C-Fzr mediates the degradation of a factor that regulates the transcription of Cyclin A and Cdk1. Hence, inhibition of APC/C-Fzr activity either by Rca1 or by phosphorylation through Cyclin E/Cdk2 would result in subsequent accumulation of Cyclin A and Cdk1.

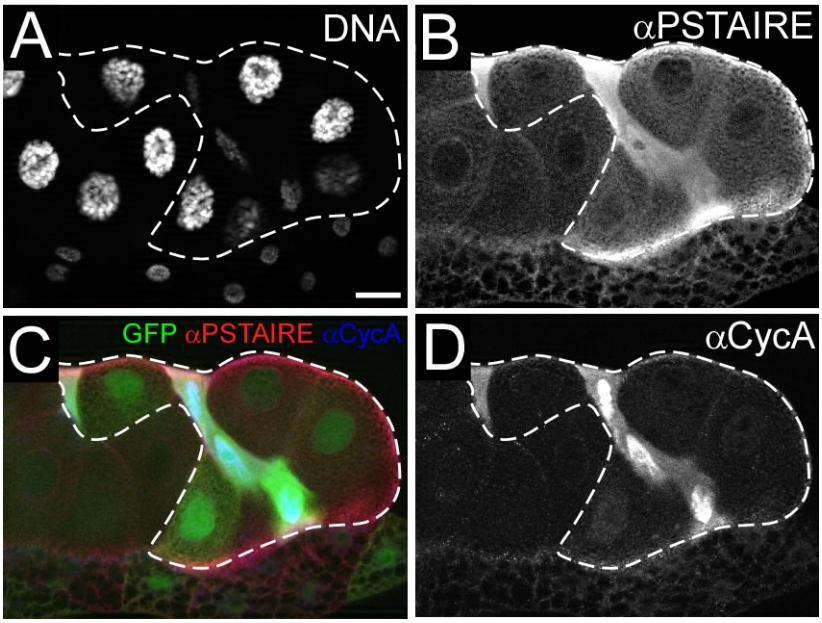

Figure 32 Cyclin A and its kinase partner Cdk1 accumulate after overexpression of Cyclin E. Cyclin E was overexpressed in larval salivary glands under control of the actin promoter using the "flpout" method. Cells overexpressing Cyclin E were visualized by coexpression of GFP. Scale bar : 100µm.
(A) Continuous Cyclin E expression perturbs endocycle progression, evidenced by reduced DNA contents compared to the surrounding wild-type cells.
(B&C) Cyclin A and Cdk1, visualized with an antibody directed against its PSTAIRE helix, accumulate in Rca1 overexpressing cells. The phenotype seems to be dose dependent, since Cdk1 and Cyclin A are only readily detectable in cells with dramatically reduced DNA content. Since, the use of the "flpout" technique in polyploid cells produces a gradient of expression levels, these cells are most likely the cells with the highest Cyclin E levels.

3.4.8. Oscillation of APC/C-Fzr activity is not required for endocycle progression

Since Cyclin E activity is normally present in endoreplication, a general problem emerges challenging the idea that Cyclin E stimulates the transcription of Cyclin A and Cdk1 by inhibiting the APC/C-Fzr activity. If Cyclin E dependent kinase activity is present in endoreplicating cells anyway, how can the APC/C-Fzr complex develop its full activity? A solution for this problem could be that Fzr activity oscillates out of phase with CyclinE/Cdk1 activity. To test this idea HA-Fzr was continuously overexpressed in third instar salivary glands using the "flpout" technique (Ito et al., 1997). Examination of DNA content by fluorescence microscopy indicates that continuous expression of HA-Fzr does not influence endocycle progression (Figure 33). However, Cyclin E /Cdk2 could also inactivate the extra

Fzr protein, so that the APC/C-Fzr complex becomes only hyper activated in phases with low Cyclin E /Cdk1. Furthermore, the amount of APC/C molecules that can be activated could be limiting, thus excess Fzr would not necessarily result in higher APC/C activity. Therefore, one can only exclude from this experiment that Fzr levels fluctuate in endoreplicating tissue since HA-Fzr is readily detectable in all cells that are supposed to express it (Figure 33D).

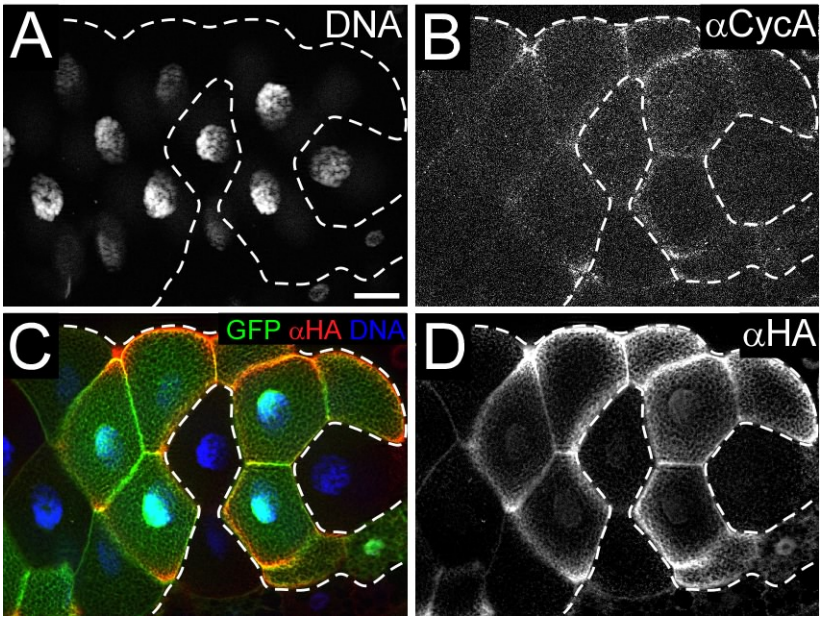

Figure 33 Excess Fzr protein does not prevent endoreplication. HA-Fzr was overexpressed in larval salivary glands under control of the actin promoter using the "flpout" method. Cells overexpressing HA-Rca1 were visualized by coexpression of GFP and antibody staining against the HA-tag. Scale bar **:** 100µm.
(A-D) Continous expression of Fzr has no effect on endocycle progression, since cells overexpressing HA-Fzr display DNA contents simarilar to the adjacent control cells.

4. Discussion

4.1. Rca1 restrains APC/C-Fzr activity in postblastoderm embryos in concert with Cyclin/Cdk complexes

The first thirteen divisions during *Drosophila* embryogenesis are very rapid since they lack intervening G1 and G2-phases. These cell cycles are driven by maternal supplies that persist until the cellular blastoderm stage. At this stage, the cells of the embryo establish the first G2-phase. The following divisions (14-16), which are generally referred as postblastoderm cell cycles, are the first cycles that rely on zygotic transcription (Edgar and O'Farrell, 1989; Edgar and O'Farrell, 1990). Cells in the postblastoderm embryo enter S-phase subsequently after progression through mitosis without an intervening G1-phase. After completion of cell cycle 16, epidermal cells for the first time enter a G1-phase (Edgar and O'Farrell, 1990). Since most epidermal cells persist in this G1-phase until the end of embryogenesis, the 16th cell division is also referred as terminal mitosis. The String/Cdc25 phosphatase controls entry into mitosis during postblastoderm cell cycles by removing inhibitory phosphorylations from Cyclin/Cdk1 complexes (Edgar and O'Farrell, 1989; Edgar and O'Farrell, 1990). Upon activation by String/Cdc25, the Cyclin/Cdk1 complex triggers entry into mitosis (Edgar et al., 1994a; Minshull et al., 1989; Murray and Kirschner, 1989). Mutants that lack either Cyclin A or its kinase partner Cdk1 are not able to enter mitosis (Lehner and O'Farrell, 1989; Stern et al., 1993). Epidermal cells in Cyclin A mutant embryos progress normally through the first two postblastoderm cycles but fail to execute the terminal mitosis 16 (Lehner and O'Farrell, 1989). This is a feature unique to Cyclin A, since mutants for Cyclin B and Cyclin B3 develop normally to adulthood (Jacobs et al., 1998; Knoblich and Lehner, 1993; Lehner and O'Farrell, 1989; Lehner and O'Farrell, 1990b).

In preparation of the terminal G1-phase, APC/C-Fzr activity becomes upregulated (Raff et al., 2002; Sigrist and Lehner, 1997). Premature activation of the APC/C-Fzr complex, however, interferes with execution of the terminal mitosis (Grosskortenhaus and Sprenger, 2002; Sigrist and Lehner, 1997). In embryos lacking the APC/C-Fzr inhibitor Rca1, mitotic cyclins become prematurely degraded resulting in a G2 arrest during cell cycle 16 (Grosskortenhaus and Sprenger, 2002). Furthermore, it has been demonstrated that APC/C-Fzr activity is also restricted by the Cyclin A/Cdk1 complex (Dienemann and Sprenger, 2004). This function appears to be unique to Cyclin A/Cdk1 and explains why only loss of Cyclin A results in embryonic lethality. Thus, Rca1 and Cyclin A have overlapping functions regarding negative

regulation of APC/C-Fzr activity. Double mutants that lack both genes, already arrest during cell cycle 15 (Grosskortenhaus and Sprenger, 2002), supporting the notion that APC/C-Fzr activity emerges during this stage (Raff et al., 2002). This experiment indicates furthermore that Rca1 and Cyclin A cooperate to restrain APC/C-Fzr activity during the last two postblastoderm cell cycles (Figure 34). Large amounts of maternally derived transcripts encoding for both proteins are present in the postblastoderm embryo. These maternal stocks persist till interphase 16, explaining why the preceding division occurs normal in single mutants for *rca1* and *cycA* respectively. However, only loss of either of these genes can be compensated during cell cycle 15. The amount of protein derived from maternal transcripts is apparently not sufficient to replace both genes, thus explaining why *rca1; cycA* double mutants arrest even earlier.

In vertebrates, it has been shown that APC/C-Fzr activity is also restricted by Cyclin A dependent kinase activity (Kramer et al., 2000; Lukas et al., 1999; Sorensen et al., 2001). In this model system, Cyclin A restrains APC/C-Fzr activity in conjunction with Cdk2 (Lukas et al., 1999), a combination that is not found in *Drosophila*. In flies, however, Cdk2 forms only complexes with Cyclin E (Knoblich et al., 1994). This suggests that either Cdk2 dependent kinase activity is not necessary for APC/C-Fzr inhibition in *Drosophila* or that the APC/C-Fzr complex is also regulated by Cyclin E/Cdk2. The latter explanation is supported by the observation that overexpression of Cyclin E in *rca1* mutants prevents premature degradation of mitotic cyclins and thereby rescues entry into terminal mitosis (Grosskortenhaus and Sprenger, 2002). Moreover, Cyclin B levels as well as entry into mitosis 16 can be rescued by overexpression of Cyclin E in *cycA* mutant embryos (Figure 10; Reber et al., 2006). Thus, providing further evidence for the idea that Cyclin E/Cdk2 is a negative regulator of APC/C-Fzr activity besides Rca1 and Cyclin A/Cdk1.

Cyclin E/Cdk2 activity decreases during cell cycle 16 when the epidermal cells prepare the terminal G1-phase (de Nooij et al., 1996; Knoblich et al., 1994; Lane et al., 1996). Hence, Cyclin E/Cdk2 activity cannot be required to restrain APC/C-Fzr activity to allow execution of mitosis 16. However, downregulation of Cyclin E dependent kinase activity in Cyclin A mutants by Dacapo overexpression results in premature Cyclin B degradation and a subsequent arrest during cell cycle 15 (Figure 12). This demonstrates that Cyclin E/Cdk2 activity is necessary to prevent premature APC/C-Fzr activation. Initially, *rca1, cycE* double mutants and *rca1, cycE; cycA* triple mutants were generated, to examine whether Cyclin

E/Cdk2 is involved in APC/C-Fzr inhibition during cell cycle 15. However, even combination with the strongest available Cyclin E allele did not enhance the phenotype of *rca1* single mutants and *rca1; cycA* double mutants, respectively. Although, Cyclin E/Cdk2 activity is crucial for DNA replication epidermal cells in Cyclin E mutants progress normally through postblastoderm cell cycles (Knoblich et al., 1994). It is therefore assumed that loss of zygotic Cyclin E is compensated by maternal stocks (Knoblich et al., 1994; Vidwans and Su, 2001). The absence of an obvious phenotype in *rca1, cycE* double mutants and *rca1, cycE; cycA* triple mutants could be explained by maternally derived Cyclin E. Cells in *rca1, cycE* double mutants as well as cells in *rca1, cycE; cycA* triple mutants displayed increased DNA contents (Figure 11F&G), suggesting that these cells underwent endoreplication. Considering that Cyclin E/Cdk2 activity is essential for initiation of DNA replication (Knoblich et al., 1994; Lane et al., 2000), these findings support the notion that maternally derived Cyclin E protein is abundant in these mutants. This experiment stands in contrast to the observation that *cycE*; *cycA* double mutants arrest during cell cycle 15 (Reber et al., 2006). An explanation for this discrepancy might be that Cyclin A dependent kinase activity is of greater importance for APC/C-Fzr inhibition during cell cycle 15 than Rca1 function. Altogether, these experiments suggest that Rca1, Cyclin A/Cdk1 and Cyclin E/Cdk2 cooperate to restrain APC/C-Fzr activity during cell cycle 15 (Figure 34). However, it remains to be elucidated whether downregulation of Cyclin E/Cdk2 activity enhances the *rca1* phenotype as well. This experiment should confirm the model described in Figure 34 and maybe explains the discrepancies between *rca1, cycE* and *cycE; cycA* double mutants.

In preparation of the terminal G1-pase, the Cdk2 inhibitor Dacapo becomes upregulated during cell cycle 16 (de Nooij et al., 1996; Lane et al., 1996). Beside termination of Cyclin E transcription, upregulation of Dacapo causes a decrease of Cyclin E /Cdk2 activity that is necessary to arrest the epidermal cells in the G1 state (de Nooij et al., 1996; Knoblich et al., 1994; Lane et al., 1996). Considering that Cyclin E/Cdk2 is a potent inhibitor of APC/C-Fzr activity, *dap; cycA* double mutants were generated to test whether loss of Cyclin A/Cdk1 activity can be compensated by extended Cyclin E/Cdk2 activity. These mutants, however, failed to enter the terminal mitosis 16, suggesting that termination of Cyclin E transcription during cell cycle 16 is of higher impact than downregulation of Cyclin E dependent kinase activity by Dacapo.

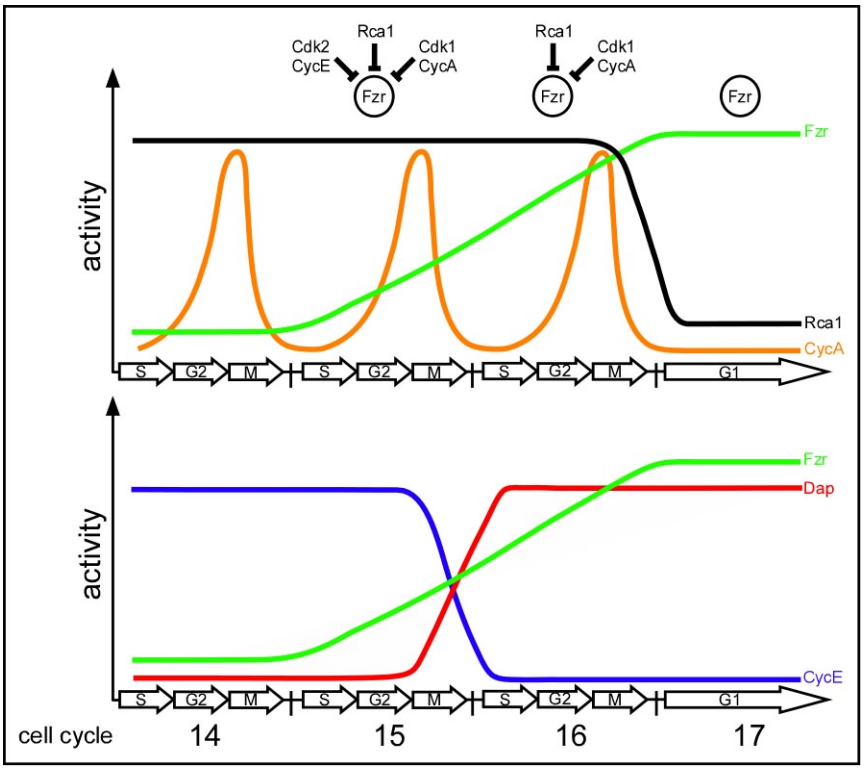

Figure 34 Regulation of late embryonic cell cycles in *Drosophila*. Fzr protein emerges during cell cycle 15 to establish the terminal G1 phase that occurs after execution of mitosis 16. During cell cycle 15 premature activation of the APC by Fzr becomes prevented by Rca1, Cyclin A/Cdk1 and Cyclin E/Cdk2. In the 16th cell cycle, Cyclin E dependent kinase activity decreases due to termination of Cyclin E transcription and upregulation of the Cdk2 inhibitor Dacapo. In G2 of cell cycle 16 APC/C-Fzr activity is therefore only inhibited by Rca1 and Cyclin A/Cdk1. During progression through mitosis 16, Rca1 as well as Cyclin A become inactivated to allow APC/C-Fzr activity, which is necessary to exit the cell cycle as well as for initiation of endoreplication.

In summary, these experiments demonstrate that during postblastoderm cell cycles Rca1, Cyclin A/Cdk1 and Cyclin E/Cdk2 act in concert to restrict APC/C-Fzr activity. In preparation of the terminal G1 state, Cyclin E/Cdk2 activity decreases and APC/C-Fzr inhibition relies only on Rca1 and Cyclin A/Cdk1. Loss of either of these genes causes therefore an arrest during interphase 16 due to premature APC/C-Fzr activation. These results indicate that multiple inputs regulate APC/C-Fzr activity and Rca1 function becomes essential when alternative mechanisms are inactivated by the developmental program. APC/C-Fzr activity is required for the establishment of the first G1 phase in the epidermis as well as for the initiation of endoreplication cycles in internal tissues (Jacobs et al., 2002; Sigrist and Lehner, 1997) In order to enable Fzr dependent APC/C activity, Rca1 itself becomes transcriptionally downregulated and degraded after progression through mitosis 16 (Figure 34 (Dong et al., 1997; Grosskortenhaus and Sprenger, 2002). To understand the function of Rca1 completely it is therefore necessary to understand how Rca1 is regulated at the molecular level.

4.2. Structural requirements for APC/C-Fzr inhibition in G2

Drosophila Rca1 is related to the vertebrate Emi proteins that are inhibitors of Cdc20/Fzy and Cdh1/Fzr dependent APC/C activity (for review see Schmidt et al., 2006). Although their overall identity is not very high, Rca1 and the Emi1 proteins share several structural elements (Figure 8). The N-terminal part of Rca1 contains a putative nuclear localization signal (NLS) and a potential F-box. The C-terminus harbors a so called zinc binding region (ZBR), a motif that is known to mediate protein-protein interactions (van der Reijden et al., 1999). In addition, Rca1 contains a KEN-box and a DSGxxS degron that both are located in the region between the F-box and the ZBR. Finally, the Rca1/Emi1 proteins several potential Cdk1 phosphorylation sites (S/T-P), which are distributed throughout the protein. In order to map the structural elements essential for APC/C-Fzr inhibition, an *in vivo* structure/function analysis was conducted (Figure 14). Mutations in *rca1* result in a failure to execute mitosis 16 due to premature activation of the APC/C-Fzr complex in G2 (Grosskortenhaus and Sprenger, 2002). This G2 arrest as well as the premature degradation of mitotic cyclins can be overcome by Rca1 expression. Thus, rescue of mitosis 16 in *rca1* mutants can be used as an assay to monitor APC/C-Fzr activity *in vivo*. In the course of the structure/function analysis, this assay was utilized to test different Rca1 deletion constructs for APC/C-Fzr inhibition. A C-terminal fragment of Emi1 was shown to be sufficient for APC/C inhibition as revealed by *in vitro* ubiquitinylation experiments (Reimann et al., 2001a). Similar, the C-terminal half of Rca1 (HA-Rca1Δ203) was sufficient for restriction of APC/C-Fzr activity in G2 evidenced by execution of mitosis 16 and normal Cyclin degradation (Figure 14). The deleted region contains the F-box suggesting that Rca1 and Emi1, respectively, do not require this motif for proper APC/C inhibition. In addition, an Rca1 construct just lacking the F-box was capable to rescue the G2 arrest in *rca1* mutants (Figure 14). This supports the notion that Rca1 inhibits the APC/C-Fzr complex by an F-box independent mechanism during embryogenesis.

By contrast, further truncation of the N-terminus (HA-Rca1Δ255) perturbed Rca1 activity suggesting that the region between amino acid 203 and 255 contains elements essential for APC/C-Fzr inhibition (Figure 14). Furthermore, point mutations in the ZBR abolished the inhibitory effect of Rca1 and Emi1, respectively (Figure 14; Reimann et al., 2001a; Reimann et al., 2001b). For Emi1, initially it was shown that the ZBR mediates physical interaction with Fzy/Cdc20 and Fzr/Cdh1, but not with components of the APC/C. Therefore it was postulated that Emi1 inhibits APC/C activity by blocking the substrate binding site in Fzy/Cdc20 (Reimann et al., 2001a; Reimann et al., 2001b). Rca1 co-immunoprecipitates with

Fzr suggesting that Rca1 also inhibits APC/C-Fzr activity by preventing substrate binding to Fzr (Grosskortenhaus and Sprenger, 2002). However, a more recent study indicates that Emi1 interacts with subunits of the APC/C via a minimal destruction box (RxxL) (Miller et al., 2006). The destruction box (D-box) is a conserved degron found in substrates of the APC/C (Glotzer et al., 1991; King et al., 1996). Initially it has been proposed that an adaptor protein (Cdc20/Fzy and Cdh1/Fzr) recognizes the D-box and thereby promotes the APC/C dependent degradation of the target protein (Schwab et al., 1997; Visintin et al., 1997). However, recent studies have revealed that the APC/C core complex also contributes to substrate binding by recognizing the D-box even in the absence of an adaptor protein (Carroll et al., 2005; Yamano et al., 2004). Although Emi1 contains a putative D-box, it has been demonstrated that it is not a substrate of the APC/C (Reimann et al., 2001a). Disruption of the ZBR, however, converts Emi1 into an APC/C substrate (Miller et al., 2006). Hence, it was proposed that Emi1 rather acts as a pseudosubstrate inhibitor instead of preventing substrate binding to the adaptor protein. In this model, the D-box mediates the interaction with the APC/Cdh1 complex, whereas the ZBR seems to be required to prevent substrate access (Miller et al., 2006). In the Emi1/Emi2 proteins, the D-box is located between F-box and ZBR (Figure 35). This particular region also seems to be crucial for APC/C inhibition by Rca1, suggesting that Rca1 might also act as pseudosubstrate inhibitor. The D-box is conserved among all Emi1/Emi2 proteins except *Drosophila* Rca1. However, closer inspection of the region between F-box and ZBR revealed that Rca1 also contains a minimal D-box that is located closer to the F-box (Figure 35). In addition, co-immunoprecipitation experiments revealed that Rca1 interacts also with the APC/C subunit Cdc27 (Grosskortenhaus and Sprenger, 2002). Thus, it remains to be clarified whether Rca1 acts as a pseudosubstrate inhibitor of the APC/C-Fzr complex. In order to elucidate this question it should be tested if deletion of the D-box in Rca1 prevents APC/C-Fzr inhibition during *Drosophila* embryogenesis.

Besides the D-box, the region between F-box and ZBR harbors a KEN-box (Figure 35). The KEN-box is another degron known from APC/C-Fzr substrates (Pfleger and Kirschner, 2000). Most APC/C targets contain one or both of these sequences. The APC/C-Cdc20 complex prefers D-box containing substrates, whereas substrates of the APC/C-Fzr complex frequently contain a D-box as well as a KEN-box (Burton and Solomon, 2001; Hilioti et al., 2001; Pfleger et al., 2001). Emi1 inhibits both, APC/C-Cdc20 and APC/C-Cdh1, while Rca1 only affects APC/C-Fzr activity. Therefore, the presence of D-box and KEN-box could reflect the different substrate specificity of Rca1. However, a construct carrying point mutations in the

KEN-box was still capable to rescue the G2 arrest in *rca1* mutants (Figure 14). This result suggests that APC/C-Fzr inhibition by Rca1 does not solely rely on this motif. It is not known whether the KEN-box is also recognized by the APC/C core complex. Hence, a conceivable explanation could be that the D-box mediates the interaction with the APC/C core complex, whereas the KEN-box is required for Fzr binding. Binding to the APC/C core could be sufficient for APC/C-Fzr inhibition and thus explaining why Rca1 can restrain APC/C activity even in absence of the KEN-box. To test this idea, an Rca1 construct that lacks both, D-box and KEN-box, should be tested for its capability to restrain APC/C-Fzr activity.

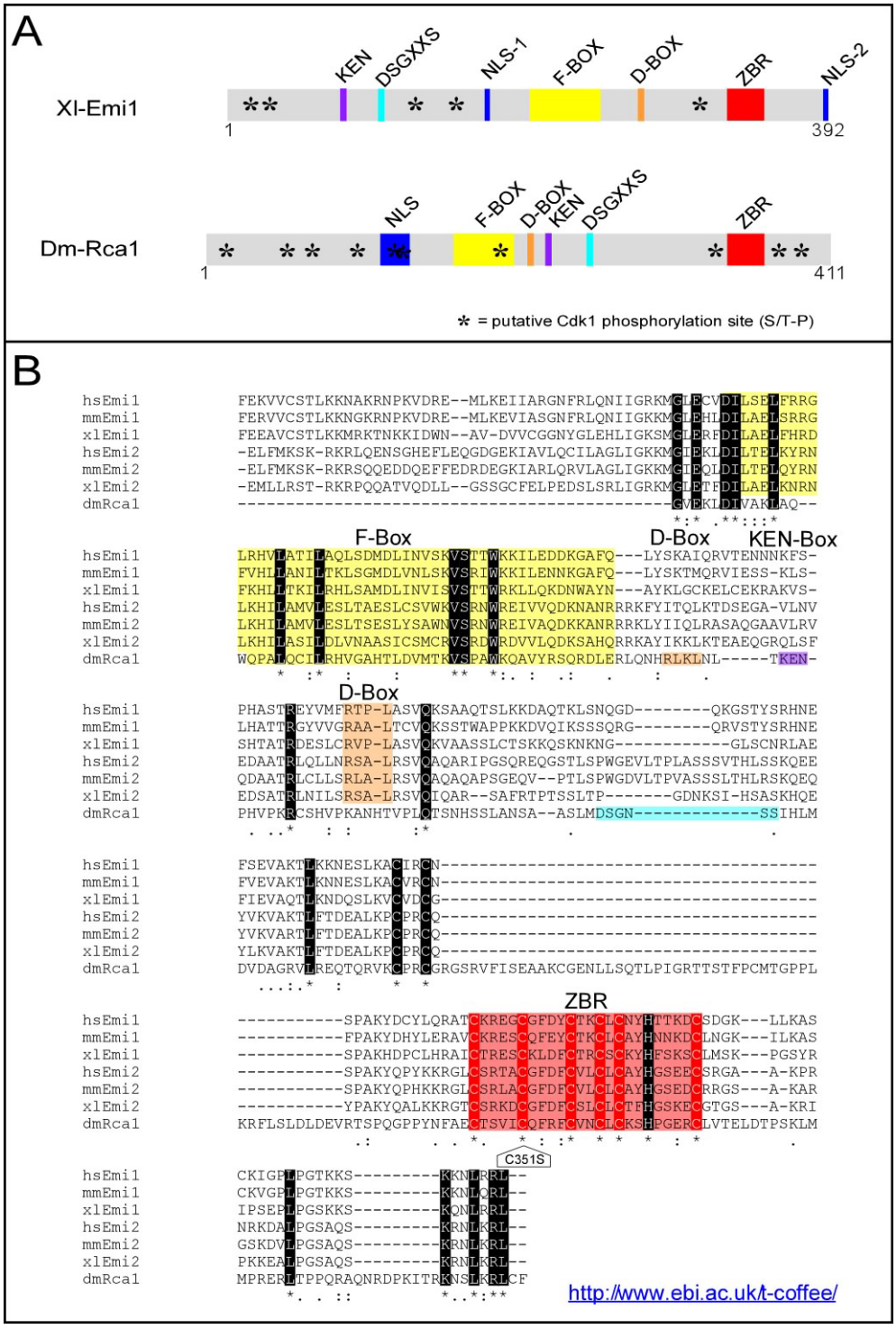

Figure 35 Comparison of the C-terminal region of the Rca1/Emi family. (A) Overview of the domain arrangement of xlEmi1 and dmRca1. The Emi1/Emi2 proteins contain a conserved D-Box (orange) located between F-box (yellow) and ZBR (red). This D-box is not conserved in Rca1. However, Rca1 contains a putative D-box (RxxL) adjacent to the F-Box. In addition, Rca1 contains a KEN-box (purple) close to the D-box. The schematic drawings are derived from the sequence alignment shown in (B) and an earlier alignment that revealed general arrangement of the functional domains (Reimann et al., 2001a). **(B)** Alignment of the Rca1/Emi1 family generated with the T-Coffee multiple sequence alignment tool. Functional domains are visualized in the same colour code as in (A). Residues that are identical in all sequences are indicated by an asterisk "*" and a black bar (red in case of the cysteine residue of the ZBR). Highly similar regions are marked by an ":", whereas semi-conserved regions are visualized by an ".". The triangle indicates the conserved cysteine residue that was substituted by an alanine to disrupt the ZBR in Rca1 and Emi1, respectively.

4.3. Rca1 degradation in G1 is achieved by a so far unknown mechanism

In *Drosophila* and vertebrates, degradation of mitotic cyclins by the APC/C-Fzr complex is crucial for establishment and maintenance of the G1 state (Jacobs et al., 2002; Lukas et al., 1999; Pimentel and Venkatesh, 2005). In order to allow APC/C activity during mitosis and G1, the inhibitors Rca1 and Emi1 have to be eliminated, respectively. Several experiments demonstrated that Rca1 gets degraded when cells enter a G1-state. During embryogenesis, epidermal cells establish the first G1-phase after progression through mitosis 16 and persist in this stage until onset of larval development. It has been shown previously, that Rca1 disappears when the cells enter this terminal G1-phase (Figure 15; Grosskortenhaus and Sprenger, 2002). In third instar eye imaginal disc, cells within the morphogenetic furrow become synchronized in G1. As in the embryonic epidermis, Rca1 gets degraded in eye imaginal discs cells upon entry into G1 (Figure 21). Finally, Rca1 protein levels fluctuate within salivary gland clones (Figure 28), suggesting that the machinery mediating Rca1 degradation is also present in salivary glands, although Rca1 itself is not necessary for endoreplication. In vertebrates, Emi1 degradation is initiated by Cdk1 phosphorylation that facilitates binding of Plk1 and causes subsequent phosphorylation of the DSGxxS motif. The SCF/ßTRCP ubiquitin ligase recognizes this degron and targets Emi1 for proteasomal degradation (Guardavaccaro et al., 2003; Hansen et al., 2004; Margottin-Goguet et al., 2003; Moshe et al., 2004). Emi1 can be stabilized by preventing Cdk1 phosphorylation or by mutating the DSGxxS degron (Margottin-Goguet et al., 2003). In order to map the structural requirements for Rca1 turnover, the stability of certain Rca1 constructs was determined in G1 cells of the embryonic epidermis. This structure/function analysis demonstrated that the region between amino acid 203 and 255 is essential for Rca1 degradation in G1. In particular, a C-terminal fragment of Rca1 (HA-Rca1Δ255) was refractory to degradation in G1 cells of the late embryo (Figure 15) as well as in the morphogenetic furrow of eye imaginal discs (Figure 21). This construct was moreover readily detectable throughout clones generated in salivary glands (data not shown). Inspection of the region between amino acid 203 and 255 indicated that Rca1 contains a DSGxxS motif as well (Figure 8). Surprisingly, Rca1 degradation in embryonic G1 cells could not be prevented by mutating the DSGxxS motif (Figure 15) indicating that this sequence is not crucial for Rca1 turnover. In the Emi1 proteins, the DSGxxS degron is found in the N-terminus, whereas in Rca1 this motif is located in the central part of the protein (Figure 8), suggesting that the actual DSGxxS degron is not conserved to Rca1. In addition, the instability of Rca1 in G1 was not affected by

simultaneous knockout of all putative Cdk1 phosphorylation sites (Figure 15). Hence, Rca1 must be degraded by different mechanisms than Emi1. Beside the DSGxxS sequence, the region between amino acid 203 and 255 harbors a KEN-box (Figure 8). Proteins containing KEN-boxes are recognized by the APC/C-Fzr complex and become subsequently targeted for proteasomal degradation (Pfleger and Kirschner, 2000). In G2 cells, Rca1 is an inhibitor of APC/C-Fzr activity, but it is conceivable that in G1 Rca1 becomes converted into an APC/C-Fzr substrate. Deletion of the KEN-box, however, did not prevent Rca1 degradation in G1 cells, so that a mechanism which relies only on the KEN-box can be excluded. However, a mechanisms that exploits a combination of these motifs cannot be ruled out. Proteasomal degradation is furthermore mediated by SCF-complexes, another type of E3-ligases (Jackson et al., 2000; Vodermaier, 2004). These complexes are named after their three core components Cullin, Skp and F-box protein, whereby the F-box protein acts as substrate recognition subunit. Rca1 harbors an F-box motif in its N-terminus and moreover interacts physically with SCF components (S. Querings, personal communication). In principle, Rca1 could mediate its own degradation by an autocatalytic mechanism. Rca1 might bind to the SCF core complex via its F-box, but instead of sequestering target proteins, Rca1 itself could become ubiquinated and subsequently degraded. However, Rca1 gets degraded normally when the F-box is deleted (data not shown), hence also this autocatalytic mechanism cannot be applied. Altogether, the structure/function analysis so far did not reveal by which mechanism Rca1 could be degraded in G1 cells and it remains to be clarified how it is achieved. Since the region between amino acid 203 and 255 is crucial for Rca1 turnover, further deletion constructs covering this region should be analyzed. The identification of a pathway mediating Rca1 degradation could be facilitated, if a particular degron which is already known from other proteins could be determined. Further insights in Rca1 turnover could also be obtained by testing candidate genes involved in the degradation of other proteins. The SCF/Archipelago complex for instance, mediates the degradation of Cyclin E and dMyc within the morphogenetic furrow (Moberg et al., 2001; Moberg et al., 2004). Since Rca1 gets degraded in G1 cells of the morphogenetic furrow, SFC/Archipelago could be a candidate for mediating Rca1 degradation.

4.4. Rca1 promotes S-phase entry as part of an SCF-complex

APC/C-Fzr activity is required for the establishment and maintenance of G1, but needs to be inactivated at the G1-S transition (Jacobs et al., 2002; Lukas et al., 1999; Pimentel and Venkatesh, 2005). In vertebrates, excess Emi1 activity accelerates S-phase entry, whereas depletion of Emi1 by RNAi results in a delay of the G1-S transition suggesting that APC/C inactivation by Emi1 contributes to S-phase entry (Hsu et al., 2002; Rape and Kirschner, 2004). In order to investigate whether this applies also to *Drosophila*, the effect of Rca1 overexpression on S-phase entry was examined. In eye imaginal discs, Rca1 overexpression results in ectopic S-phases demonstrated by BrdU incorporation in normally quiescent cells posterior to the morphogenetic furrow (Figure 17) as well as altered FACS profiles (Figure 18). Moreover, phosphohistone 3 staining revealed mitotic cells among the differentiating photoreceptor cells (Figure 18), suggesting that these cells undergo an extra cell cycle and do not simply duplicate their DNA for another time. The effect of Rca1 on S-phase is not restricted to eye imaginal discs. Flow cytometric analysis of wing disc clones continuously expressing Rca1 indicated that Rca1 overexpressing cells progress faster through G1 (Figure 19). The Rca1 overexpressing cells exhibited a concomitant increase of the G2/S fraction, suggesting that the accelerated S-phase entry upon Rca1 overexpression is compensated by a postponed G2-M transition (Figure 19). This is most likely due to an recently discovered mechanism which ensures that the overall cell cycle duration stays constant (Reis and Edgar, 2004). By contrast, overexpression of Rca1 during embryogenesis did not promote S-phase entry (Grosskortenhaus and Sprenger, 2002). This result was unexpected because mutants for Fizzy-related enter an additional S-phase 17, followed by an extra mitosis (Sigrist and Lehner, 1997). During the final cell cycle of embryogenesis, Fzr protein accumulates to high levels to introduce the terminal G1 phase (Sigrist and Lehner, 1997). By contrast, in eye imaginal discs Fzr is only barely detectable (Figure 16; Pimentel and Venkatesh, 2005). Hence, the inability of Rca1 to promote S-phase entry in the embryo might be because the amount of Fzr is too high to be inhibited. This is supported by the finding that in eye imaginal discs S-phase entry induced by Rca1 can be prevented by simultaneous overexpression of HA-Fzr (Figure 25). Recent studies have demonstrated that the G1/S regulatory machinery that is present in mitotic cells is also involved in regulation of endoreplication cycles (Edgar and Orr-Weaver, 2001; Lilly and Duronio, 2005; Sauer et al., 1995). Misexpression of Rca1 in endoreplicating salivary cells prevents endocycle progression (Figure 27A). A similar endocycle breakdown was also observed after continuous expression of the S-phase inducer

Cyclin E (Follette et al., 1998; Weiss et al., 1998). This suggests that excess Rca1 activity promotes also S-phase entry in endoreplicating cells, although Rca1 itself is dispensable for endocycle progression (Figure 26).

In *Drosophila*, S-phase is normally induced by the Cyclin E/Cdk2 complex (Knoblich et al., 1994; Richardson et al., 1995). In eye imaginal discs, however, S-phase induction by Rca1 does not rely on Cyclin E dependent kinase activity, because coexpression of the Cdk2 inhibitor Dacapo did not prevent premature S-phase entry (Figure 24). Overexpression of Cyclin A can induce ectopic S-phases. Therefore, it is thought that, besides Cyclin E/Cdk2, also Cyclin A/Cdk1 can trigger S-phase in *Drosophila* (Sprenger et al., 1997; Thomas et al., 1997). Characterization of hypomorphic *fzr* mutants revealed that downregulation of APC/C-Fzr activity results in accumulation of mitotic cyclins and premature entry into S-phase (Pimentel and Venkatesh, 2005). Hence, accumulation of Cyclin A due to APC/C-Fzr inhibition could be a conceivable explanation for the ectopic S-phases observed in Rca1 overexpressing cells. Inspection of mitotic cyclin levels indicated that Cyclin A and Cyclin B accumulate in eye disc clones overexpressing Rca1 (Figure 20). However, APC/C inhibition might not be the only mechanism by which Rca1 promotes S-phase. The C-terminal half of Rca1, which lacks the F-box, is sufficient for APC/C inhibition (Figure 14). Eye imaginal disc clones overexpressing this C-terminal fragment, fail to accumulate mitotic cyclins (Figure 21). Furthermore, Rca1 lacking the F-box cannot induce ectopic S-phases in eye and wing imaginal disc, respectively (Figure 18). Hence in imaginal discs, S-phase induction by Rca1 requires the F-box motif. Furthermore, endocycle breakdown induced by Rca1 overexpression relies on a functional F-box (Figure 27). This effect is probably caused by untimely activation of DNA replication, thus strongly supporting the notion that S-phase entry upon Rca1 overexpression occurs by an F-box dependent mechanism. Conversely, substitution of endogenous Rca1 by a transgene lacking the F-box revealed that the F-box is crucial for endogenous Rca1 function. Rescue experiments have demonstrated that the F-box is dispensable for APC/C-Fzr inhibition during G2 of embryonic cell cycles (Figure 14). By contrast, Rca1 lacking the F-box failed to restore the proliferation disadvantage of *rca1* mutant clones in wing imaginal discs (Figure 22). These cells persist longer in G1 (Figure 23), confirming the notion that Rca1 has a function at the transition from G1 to S-phase. However, these cells did not cease proliferation completely suggesting that Rca1 is not absolutely required for G1-S transition, but apparently makes it more efficient.

F-box proteins are known to act as substrate recognition subunits in SCF-E3-ligases. The Emi1/Emi2 proteins interact with members of the Skp family via the F-box suggesting that they become incorporated into SCF-complexes (Reimann et al., 2001a; Schmidt et al., 2005). Moreover, a genome wide two-hybrid analysis revealed that Rca1 interacts with *Drosophila* SkpA and SkpB (Giot et al., 2003). Co-immunoprecipitation experiments demonstrated that Rca1 forms a complex with SkpA and Cullin 1, whereby both interactions depend on a functional F-box (S. Querings, personal communication). These results strongly support the idea that Rca1 has a second function in an SCF-complex mediating the degradation of a so far unknown target protein. Therefore, a conceivable model how Rca1 accelerates the G1-S transition could be that this putative SCF/Rca1 complex mediates the proteasomal degradation of a negative regulator of S-phase entry. The APC/C-Fzr complex is important for the establishment of G1 state (Pimentel and Venkatesh, 2005; Sigrist and Lehner, 1997). Since Rca1 is an inhibitor of the APC/C-Fzr complex in G2, a potential SCF/Rca1 target could be Fzr itself. However, overexpression of Rca1 did not change Fzr protein levels in eye imaginal disc cells. Nevertheless, SCF/Rca1 could modulate APC/C activity by targeting other APC/C subunits or regulators for degradation (Figure 36). Alternatively, Rca1 could have an additional role beside its function as an APC/C inhibitor. The SCF/Rca1 complex could promote degradation of a yet unidentified negative regulator of S-phase entry (Figure 36). Formally, this putative negative S-phase regulator could either restrain Cyclin A dependent kinase activity or might be directly involved in the transition from G1 to S-phase. Interestingly, such a mechanism was already proposed for Emi1 (Rape and Kirschner, 2004), but so far this APC/C-Cdh1 independent S-phase promoting function of Emi1 has not been identified. A negative effect of Rca1 on Cyclin E/Cdk2 activity, however, can be excluded. Cyclin E is part of an autocatalytic feedback loop (Duronio and O'Farrell, 1995; Sauer et al., 1995), but Cyclin E protein levels as well as Cyclin E transcription were not enhanced in eye disc clones overexpressing Rca1 (data not shown).

By contrast, Cyclin E accumulates to high levels in salivary gland cells overexpressing HA-Rca1 (Figure 28). Cyclin E dependent kinase activity is absolutely crucial for endoreplication, since it is essential for initiation of DNA replication (Knoblich et al., 1994; Lane et al., 2000; Lilly and Spradling, 1996). Continuous Cyclin E expression, however, impairs endocycle progression by interfering with licensing of replication origins (Follette et al., 1998; Su and O'Farrell, 1998; Weiss et al., 1998). Hence, it seems likely that the endocycle breakdown induced by Rca1 is due to elevated Cyclin E levels. Although, for technical reasons it has not

been tested whether Rca1 overexpression results in aberrant localization of MCM proteins as does continuous Cyclin E expression (Su and O'Farrell, 1998). Since Cyclin E is not an APC/C target, this finding strongly supports the notion that Rca1 has additional functions beside inhibition of the APC/C-Fzr complex. The observation that Cyclin E accumulates only in salivary glands also suggests that Rca1 is a general inducer of S-phase but has different downstream targets. However, an alternative explanation might be that Rca1 exploits different mechanisms to promote S-phase entry in imaginal discs and salivary glands, respectively. Generally, Cyclin E could accumulate either due to increased transcription or by employment of posttranscriptional mechanisms. However, at the moment there is any clue how Rca1 overexpression causes upregulation of Cyclin E.

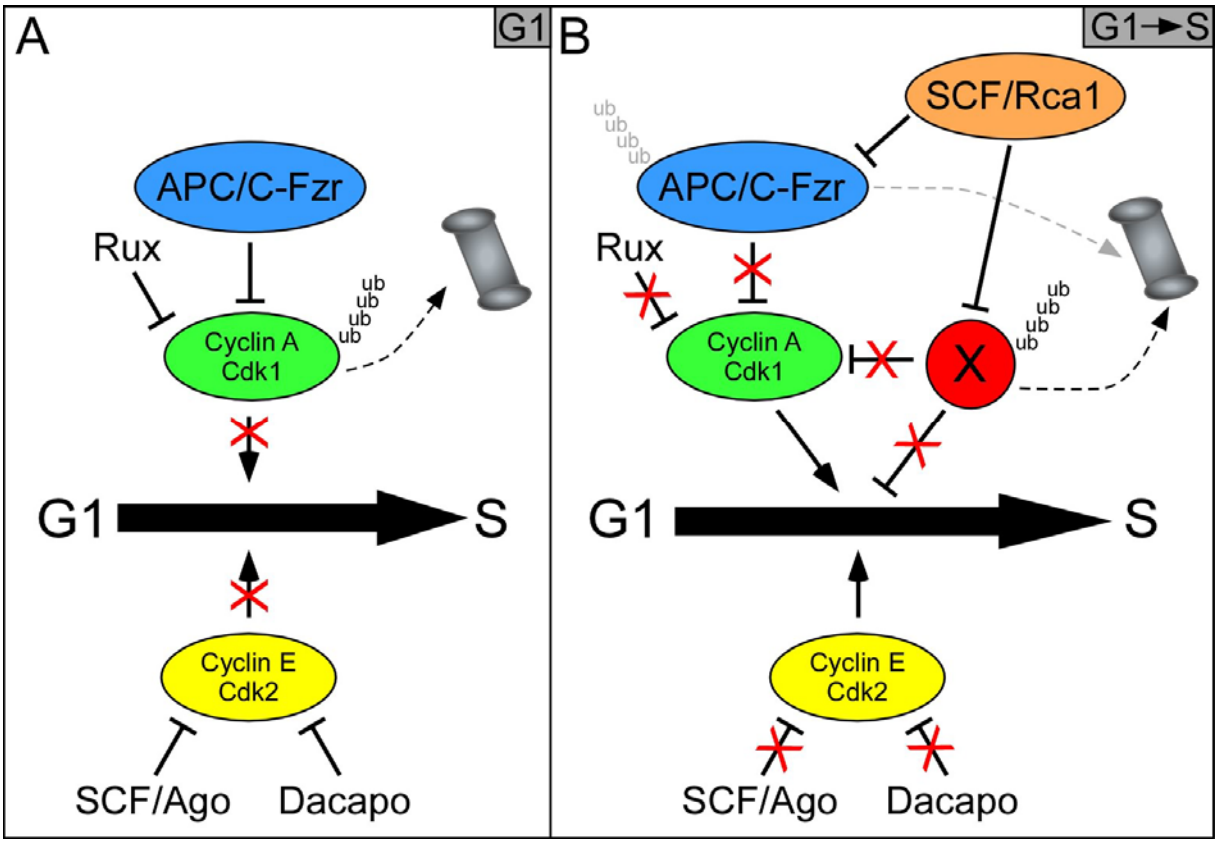

Figure 36 Regulation of S-phase entry by the SCF/Rca1 complex. (A) In *Drosophila,* the G1-S transition is generally mediated by Cyclin E dependent kinase activity, but DNA replication can also be triggered by Cyclin A/Cdk1. In order to establish the G1-state, Cyclin E/Cdk2 activity is restrained by the CKI Dacapo and the SCF/Archipelago (Ago) complex. Furthermore, Cyclin A dependent kinase activity is dampened by the CKI Roughex (Rux) and the APC/C-Fzr complex, which targets Cyclin A for proteasomal degradation. **(B)** When the cell proceeds from G1 to S-phase the SCF/Rca1 complex as well as Cyclin E dependent kinase activity which initiates DNA replication becomes upregulated. Due to upregulation of the SCF/Rca1 complex, Cyclin A/Cdk1 complex also becomes released to assist the Cyclin E/Cdk2. The SCF/Rca1 might prevent APC/C-Fzr activity and subsequently activate Cyclin A/Cdk1 by targeting an APC/C core subunit for degradation by the proteasome. Alternatively or in parallel to its role as a competitive inhibitor of the APC/C-Fzr complex Rca1, could mediate the proteasomal degradation of a so far unknown negative regulator of S-phase (X). This inhibitor could either restrain Cyclin A/Cdk1 activity or affect S-phase entry directly.

In principle, the accelerated G1-S transition observed after Rca1 overexpression could be caused by a dominant negative effect. Excess Rca1 protein might titrate out the Skp subunit from other SCF complexes such as the SCF/Archipelago. Clones mutant for the F-box protein Archipelago fail to downregulate Cyclin E protein resulting in premature entry into S-phase (Moberg et al., 2001). Archipelago is furthermore required for endocycle progression in follicle cells (Shcherbata et al., 2004). Therefore, at least the impaired endoreplication observed in salivary glands upon Rca1 overexpression could be explained by inactivation of SCF/Archipelago and subsequent accumulation of Cyclin E. An Rca1 construct with a single point mutation in the ZBR (HA-Rca1C351S) was unable to induce the rough eye phenotype (Figure 17), although it was capable to bind SkpA in vitro (S. Querings, communication). Furthermore, this construct was not capable to perturb endoreplication after overexpression in salivary glands (Figure 27), thus a dominant negative effect can be excluded. Altogether, an F-box dependent Rca1 function seems to be required for the transition from G1 to S-phase, but the targets of the SCF/Rca1 complex have not been identified yet.

4.5. Rca1 might be required to maintain the mitotic state

Endoreplicating cells duplicate their DNA, but are not obliged to enter mitosis resulting in polyploidy. In *Drosophila,* DNA replication is initiated by Cyclin E dependent kinase activity (Knoblich et al., 1994; Lilly and Spradling, 1996). Therefore, endoreplication relies on Cyclin E/Cdk2 and its regulatory network, which ensures that Cyclin E/Cdk2 activity peaks prior to S-phase (for review see Edgar and Orr-Weaver, 2001; Lilly and Duronio, 2005). By contrast, the machinery required for mitosis is downregulated upon endocycle entry (Klebes et al., 2002; Sauer et al., 1995). During mid embryogenesis, epidermal cells cease mitotic proliferation and enter a terminal G1 state (Edgar and O'Farrell, 1989), whereas the cells of certain internal tissues such as the salivary glands and the gut initiate endoreplication (Smith and Orr-Weaver, 1991). Endocycle entry is mediated by the APC/C-Fzr complex that targets mitotic cyclins and probably also String/Cdc25 for proteasomal degradation (Reber et al., 2006; Schaeffer et al., 2004; Sigrist and Lehner, 1997). Consistently, mitotic cells can be forced to enter endocycles by downregulation of mitotic cyclins or its kinase partner Cdk1 (Hayashi, 1996; Weigmann et al., 1997). The initiation of endoreplication is furthermore accompanied with transcriptional downregulation of mitotic cyclins, Cdk1 and String/Cdc25 (Klebes et al., 2002; Sauer et al., 1995; Shcherbata et al., 2004). It is assumed that APC/C-Fzr activity is not required for endocycle maintenance once the endoreplication program has been

initiated (Edgar and Orr-Weaver, 2001; Lilly and Duronio, 2005). However, Fzr protein is readily detectable in extracts derived from larval salivary glands (C. Lehner, personal communication), raising the idea that Rca1 could be required to prevent Fzr dependent APC/C activation upon endocycle entry. Examination of *rca1* mutant clones generated in the diploid cells of the salivary placode revealed that Rca1 is dispensable for endocycle progression (Figure 26). Moreover, Rca1 transcription becomes terminated during mid embryogenesis when Fzr protein accumulates and the internal tissues subsequently enter endoreplication (Dong et al., 1997; Sigrist and Lehner, 1997). Similar, Rca1 transcription is restricted to the proliferating part of eye imaginal discs (Figure 16), whereas Fzr is upregulated in the differentiating photoreceptor cells that have exited the cell cycle program (Pimentel and Venkatesh, 2005). Thus, Rca1 activity seems to be mainly required in mitotic cells, whereas Fzr becomes upregulated in cells that exit the cell cycle and subsequently differentiate. Furthermore, wing disc cells mutant for *rca1* display ectopic endoreplication (Grosskortenhaus and Sprenger, 2002), while overexpression of Fzr forces imaginal disc cells to enter endocycles (Sigrist and Lehner, 1997). Hence, another function of Fzr beside its requirement during G1 could be initiation of differentiation, particularly if endoreplication is considered as a process of differentiation. Conversely, Rca1 could be regarded as a factor required for maintenance of proliferation, since it counteracts APC/C-Fzr activity.

Although Rca1 itself is not crucial for endocycle progression, endoreplication was perturbed upon overexpression of HA-Rca1 (Figure 27). These cells exhibit elevated levels of cyclin E protein (Figure 28). Endocycles are driven by oscillating waves of Cyclin E dependent kinase activity, while constant cyclin E expression perturbs endoreplication (Follette et al., 1998; Su and O'Farrell, 1998; Weiss et al., 1998). Therefore, the endocycle breakdown observed upon Rca1 overexpression is likely due to Cyclin E accumulation. Since APC/C-Fzr activity is essential for endocycle initiation (Schaeffer et al., 2004; Sigrist and Lehner, 1997), Rca1 expression was induced during the first instar stage when the cells of the salivary gland have already entered the endocycle program. Nevertheless, Cyclin A and Cdk1 can accumulate in Rca1 overexpressing cells, while Cyclin B protein was not detectable (Figure 29). At first glance these observations imply that Rca1 overexpression results in ectopic APC/C-Fzr inhibition and subsequent accumulation of APC/C targets. However, if Cyclin A accumulates due to APC/C-Fzr inhibition, Cyclin B should increase simultaneously. Moreover, Cdk1 is not a substrate of the APC/C-Fzr complex, hence the abundance of these proteins cannot simply be explained by ectopic APC/C-Fzr inhibition. The APC/C-Fzr complex is an ubiquitin-ligase

that can only regulate levels of proteins like mitotic cyclins and other regulators, respectively. Therefore, a prerequisite for protein accumulation upon APC/C-Fzr inhibition is that at least basal levels of the protein are present. Transcription of Cyclin A and Cdk1 is terminated upon initiation of endoreplication (Klebes et al., 2002; Sauer et al., 1995), thus Cyclin A and Cdk1 proteins cannot arise even upon inhibition of the APC/C-Fzr complex.

Interestingly, upon Rca1 overexpression Cyclin A accumulates only in the nucleus (Figure 29), whereas Cyclin A expressed from a transgene is predominantly localized in the cytoplasm (Figure 30). Cyclin A enters the nucleus just after onset of mitosis (Dienemann and Sprenger, 2004; Lehner and O'Farrell, 1989), suggesting that Rca1 expression cells gain some mitotic activity. Furthermore, it is assumed that nuclear targeting of Cyclin A requires Cdk1 activity which is normally absent in endoreplicating tissues. The nuclear accumulation of Cyclin A is therefore a further hint supporting the notion that Rca1 overexpression stimulates also Cdk1 transcription. However, the idea that Rca1 overexpression leads accumulation of Cyclin A/Cdk1 should be confirmed by *in vitro* kinase assays of salivary gland extracts (Foley et al., 1999). In mitotic interphase cells, Cdk1 activity is normally dampened by the inhibitory kinase Wee1/Myt1 (Campbell et al., 1995; Morgan, 1995; Price et al., 2002). At the transition from G2 to mitosis this inhibitory phosphorylation is antagonized by the phosphatase String/Cdc25 (Edgar et al., 1994a; Minshull et al., 1989; Murray and Kirschner, 1989). If Wee1/Myt1 activity is present in endoreplicating tissue, this would imply that String/Cdc25 levels rises concomitantly with Cyclin A and Cdk1. However, due to lack of appropriate antibodies this hypothesis could not be tested so far. Overexpression of Cyclin A cannot perturb endocycle progression in larval salivary glands, most likely due the absence of its kinase partner Cdk1 (Figure 30). In the late embryo, where residual amounts of Cdk1 protein are abundant, however, Cyclin A overexpression can impair endoreplication (F. Sprenger, personal communication; Follette et al., 1998). Cyclin A and Cdk1 accumulate simultaneously after Rca1 overexpression, suggesting that Cyclin A/Cdk1 might cause the perturbation of endoreplication. However, coexpression of the Cyclin A/Cdk1 inhibitor Roughex (Foley et al., 1999) did not prevent the endocycle breakdown induced by Rca1 (Figure 31). Hence, impaired endoreplication due to Rca1 overexpression is rather caused by enhanced Cyclin E/Cdk2 activity than by re-accumulation of Cyclin A/Cdk1 activity.

Generally, two different mechanisms are conceivable how Rca1 overexpression stimulates transcription: The first model implies that APC/C-Fzr activity is not only required to initiate endoreplication program but is also involved in the maintenance of the endocycle (Figure 37A). It is well established that APC/C-Fzr activity is essential for the transition from mitotic division to endoreplication (Schaeffer et al., 2004; Sigrist and Lehner, 1997). In addition, APC/C-Fzr activity could be required to prevent re-entry into mitosis and thereby maintain the endocycle program. This idea is supported by the finding that Fzr protein is abundant in larval salivary glands (C. Lehner, personal communication). Upon endocycle initiation, transcription of mitotic regulators is terminated (Klebes et al., 2002; Sauer et al., 1995). This transcriptional downregulation could also be achieved by APC/C-Fzr activity. APC/C-Fzr could mediate the proteasomal degradation of an unknown transcription factor that stimulates the transcription of Cyclin A, Cdk1 and maybe String/Cdc25. Upon endocycle initiation, APC/C-Fzr dependent degradation of this transcription factor has to be maintained to prevent rise of Cyclin A dependent kinase activity. In addition, Cyclin A protein derived from basal transcription would be targeted for proteasomal degradation by the APC/C-Fzr complex and thus contribute to endocycle maintenance. Overexpression of Rca1 could inhibit the APC/C-Fzr and thereby indirectly stimulate transcription of Cyclin A and Cdk1. Endocycle breakdown induced by Rca1 relies on the presence of a functional F-box, therefore, it is conceivable that Rca1 prevents APC/C-Fzr activity as part of an SCF-complex. This is no absolute necessity, since the F-box is dispensable for APC/C-Fzr inhibition and it is assumed that the endocycle breakdown is rather caused by Cyclin E accumulation. Interestingly, cells overexpressing Cyclin E also displayed elevated Cyclin A and Cdk1 levels. It is thought that Cyclin E dependent kinase activity contributes to APC/C-Fzr inhibition during embryogenesis (Figure 10; Reber et al., 2006; Sigrist and Lehner, 1997). Hence, this observation supports the model that during larval stages APC/C-Fzr is required to maintain the endocycle (Figure 37A). In analogy to the model proposed for Rca1, continuous cyclin E expression could result in ectopic APC/C-Fzr inhibition and thereby induce the accumulation of a transcriptional activator. This transcription factor in turn stimulates Cyclin A and Cdk1 transcription. The idea that APC/C-Fzr might be required to prevent re-entry into mitosis is further supported by the phenotype of the *morula* gene, the *Drosophila* ortholog of the APC/C subunit Apc2 (Kashevsky et al., 2002; Reed and Orr-Weaver, 1997). During oogenesis, ovarian nurse cells first generate polytene chromosomes but later separate their sister chromatids and thereby become polyploid. In *morula* mutants nurse cells undergo several endocycles, but instead of becoming polyploid they accumulate Cyclin B and subsequently arrest in a mitosis like state

(Kashevsky et al., 2002; Reed and Orr-Weaver, 1997). Thus, these observations imply that at least in some endocycles APC/C activity is required to prevent re-entry into mitosis. However, an alternative explanation for the accumulation of Cyclin A and Cdk1 might be that the putative SCF/Rca1-complex mediates the degradation of a transcriptional repressor (Figure 37A). As in the first model, transcription of Cyclin A and Cdk1 again depends on an unknown transcriptional activator. At the transition from mitosis to endocycles, transcription of Cdk1 and Cyclin A ceases due to upregulation of a transcriptional repressor. In mitotic cells, however, the SCF/Rca1 complex could mediate the proteasomal degradation of this transcriptional repressor and thereby maintain the mitotic state. Therefore, overexpression of Rca1 in larval salivary glands would result in aberrant degradation of the repressor protein and subsequently stimulate the transcription of Cyclin A and Cdk1, respectively. Interestingly, wing disc cells enter endocycles when endogenous Rca1 was substituted by a transgene lacking the F-box. This supports the model that SCF/Rca1 is required to maintain the mitotic state.

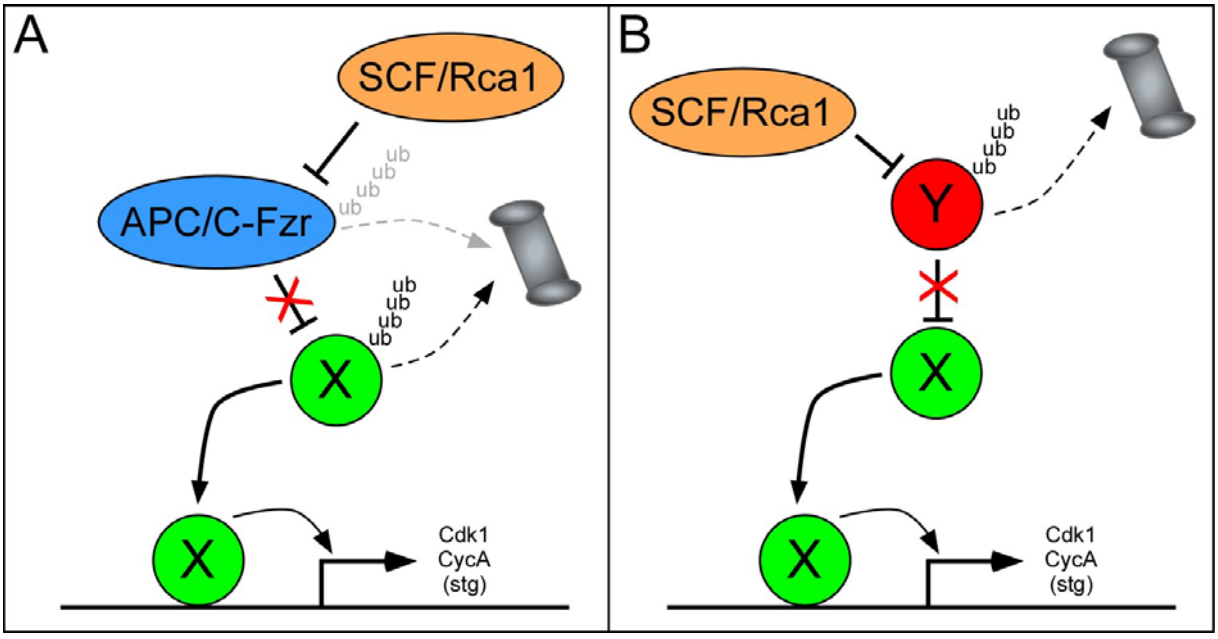

Figure 37 Rca1 overexpression could stimulate Cyclin A and Cdk1 expression by two different mechanisms. (A) Upon initiation of endoreplication, APC/C-Fzr activity might be required to suppress a transcriptional activator (X) which stimulates the transcription of Cyclin A and Cdk1 in mitotic cells. Overexpression of Rca1 prevents ubiquitination of this transcription factor and thereby promotes accumulation of Cyclin A, Cdk1 and maybe String/Cdc25 (stg). **(B)** Alternatively, the SCF/Rca1 complex could maintain diploidy in mitotic cells by mediating the proteasomal degradation of a transcriptional repressor (Y). In mitotic cells, expression of Cyclin A and Cdk1 depends on a transcriptional activator (X), while in endoreplicating tissues the activity of factor X is dampened by the repressor protein (Y). Therefore, overexpression of Rca1 would result in aberrant degradation of the repressor protein and subsequent accumulation of Cyclin A and Cdk1.

At the moment, both mechanisms are highly speculative and there is no direct evidence for any of these models. Moreover, it is not clear how transcription of Cyclin A and Cdk1 is controlled and if both genes are co-regulated. However, a good candidate could be the transcription factor Escargot, which is required to maintain diploidy in imaginal tissues (Hayashi, 1996). Overexpression of Escargot blocks endoreplication in larval salivary glands as indicated by reduced DNA content and impaired BrdU incorporation (Fuse et al., 1994). Conversely, in larvae lacking *escargot* the abdominal histoblasts enter ectopic endocycles and become polyploid (Hayashi et al., 1993). A similar phenotype was also observed in histoblasts mutant for Cdk1 (Hayashi, 1996) suggesting that premature endocycle entry in escargot mutants is due to reduced Cdk1 activity. Moreover, these cells failed to maintain high level of Cyclin A expression, suggesting that Escargot regulates Cyclin A levels and thereby maintains diploidy (Hayashi, 1996). However, it remains to be elucidated whether Escargot is implicated in the transcriptional upregulation of Cyclin A and Cdk1 upon Rca1 overexpression.

4.6. Outlook

The structure/function analysis of the Rca1 protein indicated that the region between amino acid 203 and 255 is crucial for Rca1 turnover and its inhibitory effect on the APC/C-Fzr complex. To identity a particular degron implicated in Rca1 turnover, additional deletion constructs covering this region should be generated and tested for their stability in G1. These deletion constructs should also provide more insights into the mechanism how Rca1 inhibits the APC/C-Fzr complex in G2. Since a putative D-box is located in this region (Figure 35), these experiments should shed light on the question whether Rca1 acts as a pseudosubstrate inhibitor as proposed for Emi1 (Miller et al., 2006). The structure/function analysis was basically initiated to examine the structural requirements for APC/C-Fzr inhibition in embryonic G2 cells. However, extension of this analysis to larval stages revealed that Rca1 has a second function at the transition from G1 to S-phase. Overexpression of Rca1 promotes S-phase entry by an F-box dependent mechanism, suggesting that Rca1 becomes incorporated into an SCF-complex. At the moment it is absolutely unclear which protein is targeted by this Rca1 containing SCF-complex. A putative target might be Double-parked (Dup), the *Drosophila* ortholog of Cdt1 (Whittaker et al., 2000). Dup is degraded during late G1 and it is yet not known how this is achieved (Thomer et al., 2004; Whittaker et al., 2000). Overexpression of Dup in imaginal discs results in polyploidy (Thomer et al., 2004).

Moreover, the Dup overexpressing cells resemble the polyploid cells found in clones lacking the F-Box function of Rca1 (Figure 23). Therefore, it should be tested, by using antibodies, whether Dup accumulates in *rca1* mutant clones overexpressing HA-Rca1Δ203 and HA-Rca1ΔF-box, respectively. Since, the accelerated G1-S transition induced by Rca1 overexpression is accompanied with a rough eye phenotype, a second site modifier screen should be conducted (for review see St Johnston, 2002). This screen could facilitate the identification of proteins targeted by the SCF/Rca1-complex and moreover provide further insights into the mechanism how Rca1 promotes S-phase entry. Considering that Rca1 becomes also degraded during eye development, the screen for modifiers of the rough eye phonotype could also identify genes involved in Rca1 degradation.

Co-immunoprecipitation experiments revealed that Rca1 interacts physically with SkpA and Cullin 1 (S. Querings, personal communication). Beside this two core subunits, the SCF-complex also contains a RING finger protein of the Roc/Rbx family (for review see Jackson et al., 2000). The *Drosophila* genome bears three different Roc/Rbx family members named Roc1a, Roc1b and Roc2 (Donaldson et al., 2004; Noureddine et al., 2002). Genetic analysis revealed that these Roc proteins are not functionally equivalent (Donaldson et al., 2004). Cullin 1 interacts most strongly with Roc1a, suggesting that the majority of SCF-complexes utilize Roc1a (Donaldson et al., 2004). However, it cannot be excluded that Roc1b and Roc2 function in SCF complexes, because both interact weakly with Cullin 1 (Donaldson et al., 2004). Furthermore, Roc1b can suppress the accumulation of SCF/Slimb targets in Roc1a mutant cells (Donaldson et al., 2004). Specific interactions between E2-ubiquitin-activating enzymes and RING domains have been observed, suggesting that the Roc protein can influence which E2 becomes recruited to the complex. It is conceivable that the different Roc proteins recruit a unique set of E2-enzymes that each act on a different set of targets, thereby providing an additional level of substrate specificity (Donaldson et al., 2004). Therefore, it could be interesting to reveal which particular Roc1 protein is incorporated in the SCF/Rca1 complex and which E2-enzyme is utilized for SCF/Rca1 dependent ubiquitination.

Rca1 overexpression prevents endocycle progression in an F-box dependent manner, suggesting that Rca1 overexpression in larval salivary glands leads to ectopic activation of the SCF/Rca1-complex. To elucidate whether Rca1 overexpression perturbs endoreplication in general, it should be tested if Rca1 overexpression has the same effect in the polyploid follicle cells of the *Drosophila* ovary. The Rca1 overexpressing salivary gland cells exhibit elevated

levels of Cyclin E protein, suggesting that the endocycle breakdown is due to enhanced Cyclin E/Cdk2 activity, which is thought to interfere with DNA licensing. However, concomitantly to Cyclin E stabilization, the levels of Cyclin A and Cdk1 protein raise in Rca1 overexpression cells. Since transcription of these proteins gets normally terminated upon endocycle initiation, accumulation of these proteins in larval salivary glands must be due to re-activation of transcription. So far this model is only based on the rise of protein levels and there is no direct evidence that misexpression of Rca1 stimulates the transcription of Cyclin A and Cdk1, respectively. Therefore, RT-PCR as well as microarray experiments should be conducted to confirm that Rca1 overexpression in salivary glands results in transcriptional upregulation of mitotic regulators, such as Cyclin A, Cdk1 and maybe String/Cdc25. Furthermore, Cdk1 activity should be measured to provide further evidence for the notion that Cyclin A and Cdk1 accumulate simultaneously upon Rca1 overexpression. Overexpression of Cyclin A cannot perturb endocycle progression probably because of the absence of its kinase partner Cdk1. Moreover, Cyclin A expressed from a transgene is predominantly found in the cytoplasm, while endogenous Cyclin A accumulates in the nucleus upon Rca1 overexpression. It is thought that nuclear targeting of Cyclin A requires Cdk1 activity, therefore the cyctoplasmatic localization of exogenous Cyclin A is a further hint for the absence of Cdk1 protein in larval salivary glands. To confirm the hypothesis that Cyclin A fails to block endoreplication due to lack of Cdk1, Cyclin A dependent kinase activity could be reconstituted by coexpression of Cdk1. In case that Cdk1 is subject of inhibitory phosphorylation, this could be done by simultaneous coexpression of Cdk1 and String/Cdc25 or a constitutive active form of Cdk1 ($cdc2^{AF}$), respectively (Sprenger et al., 1997). Thereby, Cdk1 activity could be assayed by using endocycle progression as well as nuclear targeting of Cyclin A as read out. It is assumed that APC/C-Fzr activity is not required for endocycle progression once the endoreplication program has been initiated. However, considerable amounts of Fzr protein are abundant in larval salivary glands. Therefore, it cannot be excluded that APC/C-Fzr activity is present at that stage and that the effect of Rca1 overexpression is due to ectopic APC/C-Fzr inhibition. Hence, it would be very helpful to clarify whether APC/C-Fzr activity is present in larval salivary glands. After overexpression of HA-CycB in scattered cells, APC/C-Fzr activity could be monitored by examining whether Cyclin B can accumulate in all cells that are supposed to express it. Furthermore, a Fzr-RNAi construct should be generated to knock down Fzr protein in larval salivary glands. If Fzr depletion leads to the same phenotype as Rca1 overexpression, this would strongly suggest that the endocycle breakdown induced by Rca1 is due to downregulation of APC/C-Fzr. Finally, the

transcription factor Escargot might be implicated in the transcriptional upregulation of mitotic regulators upon Rca1 overexpression. In order to test this idea, the expression levels of Cyclin A and Cdk1 should be determined in Escargot overexpressing cells.

5. Material and Methods

5.1. Material

5.1.1. Chemicals

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

5.1.2. Special chemicals and kits

Altered Sites 2 in vitro Mutagenesis system	Promega
Big Dye Terminator V.3.1	Applied Biosystems
Calf intestinal phosphatase (CIP)	NEB
DNA molecular weight marker	Invitrogen
DIG RNA Labeling Mix	Roche
EasyPure DNA Purification Kit	Biozym
Expand High Fidelity PCR-System	Roche
Hoechst 33258	Sigma
Hoechst 33342	Sigma
Klenow fragment	Roche
Levamisole	Sigma
Normal Goat Serum (NGS)	Dianova
Nucleobond AX-100 (Midi Prep Kit)	Machery & Nagel
Platinum *pfx* DNA Polymerase	Invitrogen
Paraformaldehyde	Electron Microscopy Sciences
Precision Plus Protein - molecular weight marker	BioRad
Propidium Iodide	Sigma
Proteinase K Solution	Quiagen
Restriction enzymes	NEB, Roche
Trypsin-EDTA (10X)	Sigma

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), ImageJ (NIH), Cell Quest software (Becton Dickinson), Microsoft Word and Excel (Microsoft Corp.).

Fluorescent images were captured on a Leica TCS-SP2 confocal microscope or on a Zeiss Axioplan Imaging2e microscope equipped with an Apotome slider module as well as a Zeiss AxioCam HRm CCD camera. Bright-field images were acquired on a Zeiss Axioplan fitted with a Zeiss AxioCam MR5c CCD camera. Pictures of fly eyes were taken on a Zeiss Stemi DRC stereomicroscope equipped with a ProgRes 3008 CCD camera (Kontron Elektronik).

Flowcytometric analyses were performed on a FACS-Vantage (Becton Dickinson) and processed using Cell Quest software (Becton Dickinson).

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

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

5.1.4. Media, solutions and buffers

Ampicillin stock solution	50mg/ml in 50% Ethanol, dilute to 50µg/ml final concentration
APS	10% in H_2O
BCIP stock solution	10mg/ml 5-bromo-4-chloro-indolyl phosphate in Dimethylformamide
BrdU stock solution	10mg/ml in 40% ethanol, store at -20°C
Carbonate buffer (2X)	120mM Na_2CO_3 80mM $NaHCO_3$ pH 10.2, store at -20°C
Chloramphenicol stock solution	34mg/ml in Ethanol, dilute to 30µg/ml final concentration
DNA loading buffer	0.25% Bromophenol blue 0.25% Xylene cyanol 30% Glycerol in H_2O
Hoechst 33342	0,5mg/ml in H_2O
in situ staining solution	100mM NaCl 25mM $MgCl_2$ 100mM Tris-HCl pH 9.5 1mM Levamisole 0.1% Tween-20 in H_2O
in situ hybridization mix	50% Formamide 25% 20X SSC 100µg/ml tRNA 100µg/ml ssDNA 50µg/ml Heparin (in 4X SSC) 0.001% Tween-20 in H_2O
Kanamycin stock solution	10mg/ml in H_2O, dilute to 30µg/ml final concentration

Laemmli buffer (4X)	8% SDS 400mM DTT 240mM Tris-HCl pH6.8 0.004% Bromophenol blue 40% Glycerol
LB-Medium (1L)	10g Bactotrypton 5g Bacto-yeast-extract 10g NaCl (for casting plates add 15g agar)
Mini-Prep buffer	<u>Resuspension buffer</u> 50mM Glucose 25mM Tris-HCl, pH 8,0 10mM EDTA, pH 8,0 100µg/ml RNAse 5mg/ml Lysozyme <u>Lysis buffer</u> 200mM NaOH 1%SDS <u>Neutralization buffer</u> 3M K-acetate, pH 5.5
NBT stock solution	10mg/ml nitroblue-tetrazolium in 70% Dimethylformamide
Paraformaldehyde (4%, 100ml)	<u>4% Paraformaldehyde in PBS</u> 4g Paraformaldehyde 80ml H_2O 8ml 10X PBS dissolve PFA on a stirrer/hotplate add 3 drops of 1M NaOH to help clear cool down to RT adjust pH to 7.2 with NaOH add 1x PBS to make 100 ml store at -20°C
PBS	130mM NaCl 2.7mM KCl 7mM Na_2HPO_4 3mM KH_2PO_4 adjust pH to pH 7.4
PBT (500ml)	<u>0.2% Tween-20 in PBS</u> 50ml 10x PBS 5ml 20% Tween-20 (in 50% Ethanol) add H_2O to make 500ml

PBTX (500ml)

0.3% Triton-X100 in PBS
50 ml 10x PBS
7.5 ml 20% Triton-X100 (in 50% Ethanol)
add H_2O to make 500ml

Ponceau S (10X)

2g Ponceau S
30g Trichloracetic acid
30g Sulfosalicyclic acid

Propidium iodide

10mg/ml in PBS

RNAse stock solution

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

Running buffer for SDS-PAGE (10X, 10L)

300g Tris base (0.25M)
1440g Glycine (1.9M)
1L 10% SDS

Separating gel buffer (4X, 1L)

181.7g Tris base (1.5M)
4ml 10% SDS
adjust pH to 8.8 with HCl

Separating gels for SDS-PAGE

	8.5%	10%	12.5%	15%
H_2O (ml)	4.7	4.2	3.4	2.4
30% Acrylamide (ml)	2.7	3.2	4	5
4X Separating gel buffer (ml)	2.6	2.6	2.6	2.6
APS (µl)	150	150	150	150
TEMED (µl)	7	7	7	7

the amount is sufficient for 2 mini gels.

SSC (20X)

3M NaCl
0.3M Na-citrate

Stacking gel buffer (4X, 500ml)

30.3g Tris base (0,5M)
20ml 10% SDS
adjust pH to 6.8 with HCl

Stacking gels for SDS-PAGE (4%)

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

for 2 mini gels take 10 ml of the prepared stacking gel mix and add 200 µl APS and 10 µl TEMED.

Stop solution (RNA hydrolysis)	0.2M Na-acetate adjust pH to 6.0 with acetic acid
TAE	242g Tris base (2M) 57.1ml glacial acetic acid 500mM EDTA
TE	10mM Tris-HCl pH 8.0 1mM EDTA pH 8.0
Terrific broth	Solution A 12g Bactotrypton 24g Bacto-yeast-extract 4ml Glycerol adjust to 900ml with H_2O Solution B 2.31g KH_2PO_4 12.54g K_2HPO_4 adjust to 90ml with H_2O
Tetracycline stock solution	10mg/ml in 50% Ethanol, dilute to 20µg/ml final concentration
Transfer buffer for western-blotting (1L)	5.82g Tris base (48mM) 2.93g Glycine (39mM) 3.75 ml 10% SDS 200ml 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

ES1301 mutS

*lacZ*53 *mut*S201::Tn5 *thy*A36 *rha*-5 *met*B1 *deo*C IN(*rrn*D-*rrn*E)

JM109

*end*A1 *rec*A1 *gyr*A96 *thi hsd*R17($r_\kappa^- m_\kappa^+$) *rel*A1 *sup*E44 λ^- Δ(*lac-pro*AB) [F′ *tra*D36*pro*A^+B^+ *lac*I^qZΔM15

5.1.6. Oligonucleotides

Oligonucleotides were purchased from the following manufacturers: Eurogentec, Sigma, Roth, and Invitrogen.

Table 1 Oligonucleotides that have been utilized during this thesis.

Name	Sequence	Application
CO-125^{rca1B}	TCGGGATCCATGAGCGCCTATTATCGG	sequencing of Rca1
CO-126^{rca1X}	ATGTCTAGACTAAAAACAGAGCCGCTTGAG	sequencing of Rca1
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-228	GTGTCATCTCCCAGTTTC GG	site-directed mutagenesis C35S1
CO-232	ATTACCCGGGCCATTGCGCCAGCTTGGCCAC	site-directed mutagenesis Q164P, P165G
CO-233	ATTACCGGGCGCCTACAGAACCACCGACTC	site-directed mutagenesis R202P, L203G
CO-265	GGATAACAATTTCACACAG	sequencing from SP6 promotor
CO-266	TTGAATCTAACCAAAGCGGCTCCTCACGTGCCCA AGCGG	site-directed mutagenesis E215A, N216A
CO-267	GAGGTCAGGACTGCACCGCAAGGACCGCC	site-directed mutagenesis S335A
CO-268	CCGAACTGGACGCACCATCCAAATTGATG	site-directed mutagenesis T376A
CO-269	GGGAGCGACTGGCGCCGCCACAACGTGCCCAG	site-directed mutagenesis T388A
CO-278	CGCTGGACGTGATGACCAAGGTATCGCCGG	sequencing of HA-Rca1ΔKEN
CO-319	GTGATGACCAAGGTAGCGCCGGCCTGGAAGCAG G	site-directed mutagenesis S187A
CO-326	CAATTCCACCGCGGAGGCGCCATTTTTATTGG	site-directed mutagenesis T44A
CO-327	GCAAGAAACACTTTCAAGCGCCACACAGTGCCCC CAAGAAGTCC	site-directed mutagenesis S123A, S127A
CO-328	GGCGTTGCGGAAGAAGGCCCCAAGCCGAGGG	site-directed mutagenesis S14A
CO-329	CTTTCGGGGGCTGAACGCGCCCAGTGGCCACC	site-directed mutagenesis T71A
CO-330	GCCTTTCTCTATGGCTCCGCGTCTGCAGG	site-directed mutagenesis T104A
CO-357	GCCTCGCTAATGGACGCGGGCAACGCGGCCATCC ACCTGATGG	site-directed mutagenesis S253A, S256A, S257A
CO-401	GTAATACGACTCACTATAGGGCG	sequencing from T7 promotor
CO-402	CAATTAACCCTCACTAAAGGG	sequencing from T3 promotor
Amp^{R}	GTTGCCATTGCTGCAGGCATCGTGGTG	Altered Sites 2
Tet^{KO}	GCCGGGCCTCTTGCGGGCGTCCATTCC	Altered Sites 2
Amp^{KO}	GTTGCCATTGCGGCATCGTGG TGTCAC	Altered Sites 2
Tet^{R}	GCCGGGCCTCTTGCGGGATATCGTCCA	Altered Sites 2

5.1.7. Plasmids

The following vectors (Figure 38) were utilized as matrix for molecular cloning: pAlter1 (Promega), pBluescript II SK(-) (Stratagene), pSP64 and pUASp (Rorth, 1998).

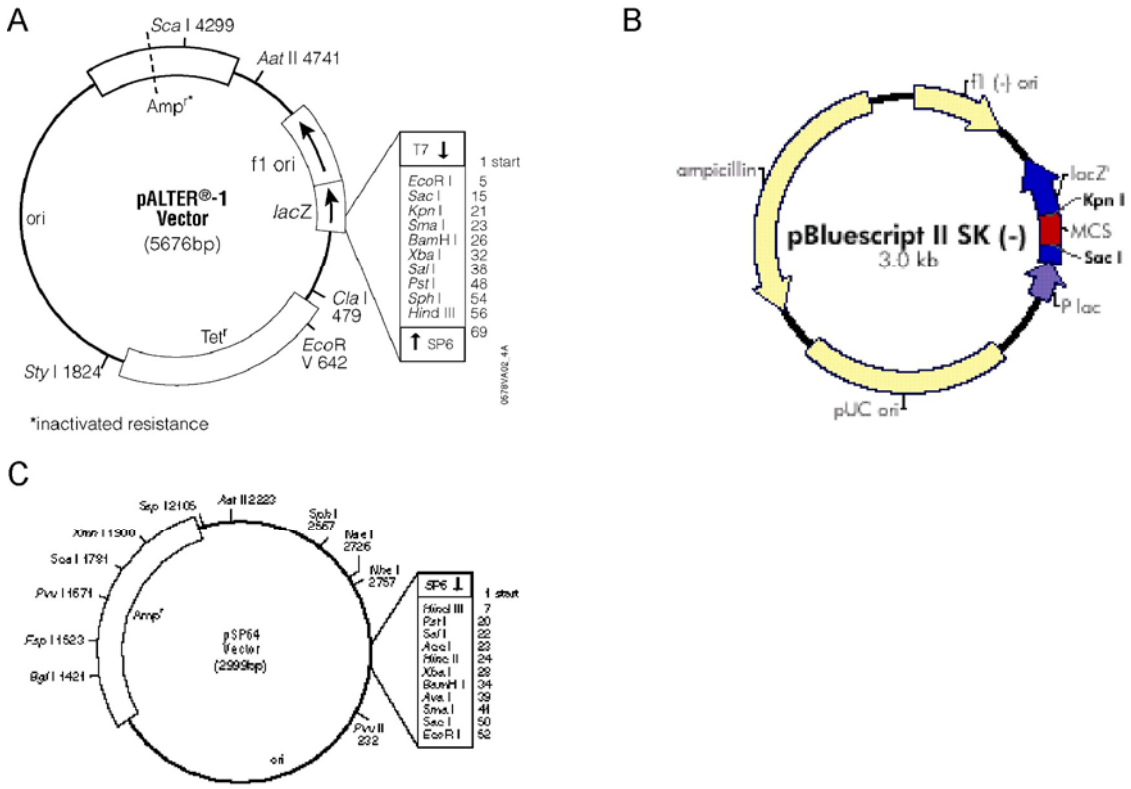

Figure 38 Schematic representation of the vectors used in this study. **(A)** pAlter1 **(B)** pBluescript II SK(-) **(C)** pSP64

Table 2 Plasmids that have been used during this thesis.

NR	Insert	Vector Matrix	Purpose
pRG26	HA-Rca1	pSP64	intermediate construct
pRG28	HA-Rca1	pBluescript SK(-)	intermediate construct
pRG32	HA-Rca1ΔNLS$^{(117-134)}$	pBluescript SK(-)	intermediate construct
pRG36	Rca1	pBluescript SK(-)	RNA probe for in situ hybridization
pRG54	HA-Rca1Δ203$^{(1-203)}$	pSP64	intermediate construct
pRG57	HA-Rca1Δ203$^{(1-203)}$	pBluescript SK(-)	intermediate construct
pRG58	HA-Rca1Δ203$^{(1-203)}$	pUASp	transformation of *Drosophila*
pNZ01	HA-Rca1	pAlter1	site-directed mutagenesis
pNZ02	HA-Rca1	pSP64	intermediate construct
pNZ03	HA-Rca1C351S	pAlter1	intermediate construct
pNZ05	HA-Rca1C351S	pUASp	transformation of *Drosophila*
pNZ07	HA-Rca1Δ255$^{(1-255)}$	pSP64	intermediate construct
pNZ08	HA-Rca1Δ133$^{(1-133)}$	pSP64	intermediate construct

NR	Insert	Vector Matrix	Purpose
pNZ12	HA-Rca1Δ255$^{(1-255)}$	pBluescript SK(-)	intermediate construct
pNZ13	HA-Rca1Δ133$^{(1-133)}$	pBluescript SK(-)	intermediate construct
pNZ17	HA-Rca1Δ255$^{(1-255)}$	pUASp	transformation of *Drosophila*
pNZ18	HA-Rca1Δ133$^{(1-133)}$	pUASp	transformation of *Drosophila*
pNZ21	HA-Rca1$^{(Q164P, P165G, R202P, L203G)}$	pAlter1	intermediate construct
pNZ22	HA-Rca1ΔF-box$^{(164-203)}$	pAlter1	intermediate construct
pNZ25	HA-Rca1 ΔF-box$^{(164-203)}$	pUASp	transformation of *Drosophila*
pNZ26	HA-Rca1ΔKEN$^{E215A, N216A}$	pAlter1	intermediate construct
pNZ27	HA-Rca1ΔNLS$^{(117-134)}$	pUASp	transformation of *Drosophila*
pNZ40	HA-Rca1ΔKEN$^{E215A, N216A}$	pUASp	transformation of *Drosophila*
pNZ41	HA-Rca1$^{S335A, T377A}$	pAlter1	site-directed mutagenesis
pNZ44	HA-Rca1 $^{S187A, S335A, T377A, T388A}$	pAlter1	site-directed mutagenesis
pNZ46	HA-Rca1ΔCdk1$^{T44A, S123A, S127A, S187A, S335A, T377A, T388A}$	pAlter1	site-directed mutagenesis
pNZ49	HA-Rca1ΔDSGxxS$^{S253A, S256A S257A}$	pAlter1	intermediate construct
pNZ51	HA-Rca1ΔCdk1$^{T14A, T44A, T71A, T104A, S123A, S127A, S187A, S335A, T377A, T388A}$	pAlter1	intermediate construct
pNZ52	HA-Rca1ΔCdk1$^{T14A, T44A, T71A, T104A, S123A, S127A, S187A, S335A, T377A, T388A}$	pUASp	transformation of *Drosophila*
pNZ56	HA-Rca1ΔDSGxxS$^{S253A, S256A S257A}$	pUASp	transformation of *Drosophila*

5.1.8. Fly Stocks

Either OregonR or w^{1118} were used as wild-type controls. All used marker genes and balancer chromosomes are described in (Lindsley and Zimm, 1992).

Table 3 Fly Stocks that have been used during this thesis.

NR	Genotype	Distributor
F149	*cycE*^{AR95}/CyO(wg-lacZ)	Christian Lehner
TAD018	*cycA*^{C8LR1}/TM3(ubx-lacZ)	Axel Dienemann
TAD021	w; If/CyO(wg-lacZ); *cycA*^{C8LR1}/TM3(ubx-lacZ)	Axel Dienemann
TF387	*rca1*^{2}/CyO(wg-lacZ)	Ruth Grosskortenhaus
TF389	*rca1*^{2}/CyO(wg-lacZ); *cycA*^{C8LR1}/TM3(ubx-lacZ)	Ruth Grosskortenhaus
TNZ080	*rca1*^{2}, *cycE*^{AR95}/CyO(wg-lacZ)	Ruth Grosskortenhaus
TNZ114	w; *rca1*^{2}, *cycE*^{AR95}/CyO(wg-lacZ); MKRS/TM3(ubx-lacZ)	own production
TNZ194	w; *rca1*^{2}, *cycE*^{AR95}/CyO(wg-lacZ); *cycA*^{C8LR1}/TM3(ubx-lacZ)	own production
TNZ227	*dap*^{4}/CyO	Christian Lehner
TAD19.3	*cycA*^{C8LR1}, *prd*-Gal4 /TM3(ubx-lacZ)	Axel Dienemann
TNZ259	UAS-dap$^{\#660}$/CyO(wg-lacZ); *cycA*^{C8LR1}/TM3(ubx-lacZ)	own production
TF102	UAS-CycE on 2nd	Helena Richardson
TF196	w; UAS-CycE/CyO(wg-lacZ); MKRS/TM6B	own production

NR	Genotype	Distributor
TNZ224	w; UAS-CycE /CyO(wg-lacZ); *cycA*^{C8LR1} /TM3(ubx-lacZ)	own production
TF383	w; UAS-HA-Rca1/CyO(wg-lacZ); MKRS/TM6B	Ruth Grosskortenhaus
TF450	w; If/CyO(wg-lacZ); UAS-HA-Rca1/TM6B	Ruth Grosskortenhaus
TF483	w; UAS-Rca1/CyO(wg-lacZ); MKRS/TM6B	Ruth Grosskortenhaus
TNZ118	w; If/CyO(wg-lacZ); UAS-HA-Rca1Δ133/TM6B	own production
TNZ120	w; UAS-HA-Rca1Δ203/CyO(wg-lacZ); MKRS/TM6B	own production
TNZ121	w; If/CyO(wg-lacZ); UAS-HA-Rca1Δ203/TM6B	own production
TNZ124	w; If/CyO(wg-lacZ); UAS-HA-Rca1Δ255/TM6B	own production
TNZ134	w; If/CyO(wg-lacZ); UAS-HA-Rca1ΔNLS^{TF134}/TM6B	own production
TNZ059	w; UAS-HA-Rca1ΔF-box /CyO(wg-lacZ); MKRS/TM6B	own production
TNZ063	w; If/CyO(wg-lacZ); UAS-HA-Rca1ΔF-box/TM6B	own production
TNZ140	w; If/CyO(wg-lacZ); UAS-HA-Rca1ΔKEN/TM6B	own production
TNZ207	w; If/CyO(wg-lacZ); UAS-HA-Rca1ΔDSGxxS/TM6B	own production
TNZ001	w; If/CyO(wg-lacZ); UAS-HA-Rca1C351S/TM6B	own production
TNZ147	w; UAS-HA-Rca1ΔCdk1/CyO(wg-lacZ); MKRS/TM6B	own production
TNZ251	w; If/CyO(wg-lacZ); UAS-HA-Rca1ΔCdk1/TM6B	own production
TA224.4	w; If/CyO(wg-lacZ); UAS-HA-CycA/TM6B	Axel Dienemann
TF408	w; If/CyO(wg-lacZ); UAS-CycE/TM6B	Ruth Grosskortenhaus
TF470	w; UAS-HA-Fzr/CyO(wg-lacZ); MKRS/TM6B	Ruth Grosskortenhaus
TF473	w; If/CyO(wg-lacZ); UAS-HA-Fzr/TM6B	Ruth Grosskortenhaus
TNZ047	w; UAS-Rca1^{TF483};UAS-HA-Fzr^{TF473}/SM6-TM6	own production
TF313	*prd*-Gal4/TM3(ftz-lacZ)	Christian Lehner
TF374	w; *rca1*^{2}/CyO(wg-lacZ); MKRS/TM6B,	Ruth Grosskortenhaus
TF378	w; *rca1*^{2}/CyO(wg-lacZ); *prd*-Gal4/TM6B	Ruth Grosskortenhaus
TF379	w; *rca1*^{2}/CyO(wg-lacZ); UAS-HA-Rca1^{TF450}/TM6B	Ruth Grosskortenhaus
TNZ005	w; *rca1*^{2}/CyO(wg-lacZ); UAS-HA-Rca1Δ133^{TNZ118}/TM6B	own production
TNZ006	w; *rca1*^{2}/CyO(wg-lacZ); UAS-HA-Rca1Δ203^{TNZ121}/TM6B	own production
TNZ007	w; *rca1*^{2}/CyO(wg-lacZ); UAS-HA-Rca1Δ255^{TNZ124}/TM6B	own production
TNZ147	w; *rca1*^{2}/CyO(wg-lacZ); UAS-HA-Rca1ΔNLS^{TF134}/TM6B	own production
TNZ069	w; *rca1*^{2}/CyO(wg-lacZ); UAS-HA-Rca1ΔF-box^{TNZ063}/TM6B	own production
TNZ148	w; *rca1*^{2}/CyO(wg-lacZ); UAS-HA-Rca1ΔKEN^{TNZ140}/TM6B	own production
TNZ223	w; *rca1*^{2}/CyO(wg-lacZ); UAS-HA-Rca1ΔDSGxxS^{TNZ207}/TM6B	own production
TNZ004	w; *rca1*^{2}/CyO(wg-lacZ); UAS-HA-Rca1C351S^{TNZ001}/TM6B	own production
TNZ254	w; *rca1*^{2}/CyO(wg-lacZ); UAS-HA-Rca1ΔCdk1^{TNZ251}/TM6B	own production
TNZ225	UAS-dap$^{\#660}$ on 2nd	Christian Lehner
TNZ228	w; UAS-dap$^{\#660}$/CyO(wg-lacZ); MKRS/TM6B	own production
	w; UAS-dap$^{\#660}$/CyO(wg-lacZ); UAS-HA-Rca1^{TF450}/TM6B	own production
TF292	GMR-Gal4 on 2nd	Ilan Davis
TK282	w; UAS-GFP/CyO(wg-lacZ); MKRS/TM6B	Thomas Klein
TK351	w; If/CyO(wg-lacZ); UAS-GFP/TM6B	Thomas Klein

NR	Genotype	Distributor
F232	w; If/CyO(wg-lacZ); MKRS/TM6B	Thomas Klein
	W; If/SM6-TM6	Thomas Klein
TNZ087	w; UAS-HA-Rca1^{TF383}; UAS-GFP/SM6-TM6	own production
	w; UAS-HA-Rca1Δ203^{TNZ120}; UAS-GFP/SM6-TM6	own production
	w; UAS-GFP; UAS-HA-ΔF-box^{TNZ063}/SM6-TM6	own production
	w; UAS-HA-Rca1ΔF-box^{TNZ059}; UAS-HA-ΔF-box^{TNZ063}/SM6-TM6	own production
TNZ113	w, hs-Flp$^{1.22}$; AyGal4(25), UAS-GFP/SM6-TM6	Thomas Klein
	w, hs-Flp$^{1.22}$; AyGal4(25), UAS-lacZ/CyO	Antonio Garcia-Belido
TNZ031	PCNA-GFP on 3rd	Robert Duronio
TNZ064	w; If/CyO(wg-lacZ); PCNA-GFP/TM6B	own production
	w; UAS-HA-Rca1^{TF383}; PCNA-GFP/SM6-TM6	own production
TNZ155	CycE$^{16.4kb}$-lacZ on 3rd	Thomas Klein
	w; If/CyO(wg-lacZ); CycE$^{16.4kb}$-lacZ/TM6B	own production
	w; UAS-HA-Rca1^{TF383}; CycE$^{16.4kb}$-lacZ/SM6-TM6	own production
TF179	UAS-rux^{B1} on 2nd	Barbara Thomas
	w; UAS-rux^{B1}/CyO(wg-lacZ); MKRS/TM6B	own production
	w; UAS-rux^{B1}/CyO(wg-lacZ); UAS-HA-Rca1^{TF450}/TM6B	own production
TNZ156	w, hs-Flp$^{1.22}$, tub-Gal4, UAS-GFP/FM7; Frt40A, tub-Gal80/CyO(wg-lacZ)	Thomas Klein
TNZ180	FRT40A on 2nd	Thomas Klein
TF413	w; FRT40A, *rca1^{2}*/CyO(wg-lacZ)	Ruth Grosskortenhaus
TNZ158	w; FRT40A, *rca1^{2}*; UAS-HA-Rca1^{TF450}/SM6-TM6	own production
TNZ195	w; FRT40A, *rca1^{2}*; UAS-HA-Rca1Δ203^{TNZ121}/SM6-TM6	own production
TNZ159	w; FRT40A, *rca1^{2}*; UAS-HA-Rca1ΔF-box^{TNZ063}/SM6-TM6	own production

5.1.9. Antibodies

Table 4 Primary antibodies that have been for immunohistology and western blot analysis.

NR	Antigen	Source	Immuno staining	Western Blot	Distributor
280	HA	Rabbit	1:200	not working	Santa Cruz Biotechnology
282	HA	Rat	1:100	1:3000	Roche
284	Cyclin A	Rabbit	1:250	1:3000	Frank Sprenger
310	Cyclin B	Mouse	1:40	1:2500	Developmental Studies Hybridoma Bank
168	Cyclin B	Rabbit	1:500	not tested	Jordan Raff
278	Cyclin E	Guinea pig	1:800	not working	Terry Orr-Weaver
138	PSTAIRE	Mouse	1:2500	1:50000	Sigma
246	Rca1	Rat	1:1000	1:1000	Ruth Grosskortenhaus
247	Fzr	Rat	1:100	not tested	Ruth Grosskortenhaus
-	Dap		1:4	not tested	Iswar Hariharan

NR	Antigen	Source	Immuno staining	Western Blot	Distributor
318	actin	Rabbit	not tested	1:10000	Sigma
041	tubulin	Mouse	not tested	1:10000	Amersham Life Science
293	p-Histone 3	Rabbit	1:1000	not tested	Upstate-Cell Signaling Solutions
343	p-Histone 3	Mouse	1:2500	not tested	Cell Signaling Technology
102	p-Tyr	Mouse	1:10	not tested	Deborah Morrison
327	BrdU	Mouse	1:20	not tested	Becton Dickinson
059	ß-gal/lacZ	Rabbit	1:500	not tested	Cappel
281	ß-gal/lacZ	Mouse	1:500	not tested	Sigma
294	GFP	Rabbit	1:500	1:1000	Torrey Pines Biolabs
323	DIG-AP		1:2000	not tested	Roche

Table 5 Secondary antibodies that have been used for immunohistology.

NR	Antigen	Source	Flourochrome	Dilution	Distributor
286	Mouse	Goat	Alexa-488	1:500	Invitrogen -Molecular Probes
298	Mouse	Goat	Rhd-Red-X	1:500	Dianova
227	Mouse	Goat	Alexa-568	1:500	Invitrogen -Molecular Probes
297	Mouse	Goat	Alexa-647	1:500-1:250	MoBiTec
184	Rat	Goat	Alexa-488	1:500	MoBiTec
198	Rat	Goat	Alexa-568	1:500	MoBiTec
169	Rat	Goat	Cy5	1:500-1:250	Dianova
290	Rat	Goat	Alexa-647	1:500-1:250	Invitrogen -Molecular Probes
267	Rabbit	Goat	Alexa-488	1:500	MoBiTec
301	Rabbit	Goat	Alexa-568	1:500	Invitrogen -Molecular Probes
182	Rabbit	Goat	Cy5	1:500-1:250	Dianova
205	Guinea pig	Donkey	Texas-Red	1:500	Dianova

Table 6 Secondary antibodies that have been used in combination with the Odyssey system to detect proteins on western blots.

NR	Antigen	Source	Flourochrome	Dilution	Distributor
307	Mouse	Goat	Alexa-680	1:3000	Invitrogen -Molecular Probes
315	Mouse	Sheep	IRdye-800	1:3000	Rockland Immunochemicals
308	Rat	Goat	Alexa-680	1:3000	Invitrogen -Molecular Probes
312	Rat	Goat	IRdye-700	1:3000	Rockland Immunochemicals
317	Rat	Donkey	IRdye-800	1:3000	Rockland Immunochemicals
306	Rabbit	Goat	Alexa-680	1:3000	Invitrogen -Molecular Probes
313	Rabbit	Goat	IRdye-700	1:3000	Rockland Immunochemicals
316	Rabbit	Donkey	IRdye-800	1:3000	Rockland Immunochemicals
319	Guinea pig	Goat	IRdye-700	1:3000	Rockland Immunochemicals

5.2. Molecular cloning

5.2.1. Restriction digests of DNA

Restriction digests were performed in buffers and at temperatures recommended by enzyme manufacturers for at least 2h and up to a maximum of 16h.

5.2.2. Dephosphorylation of DNA ends

After restriction digestion, vector ends were dephosphorylated to prevent self ligation. Therefore, 1µl CIP (calf intestinal phosphatase) was added to a restriction mix and incubated at 37°C for 30min.

5.2.3. Klenow fill in of DNA ends

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

5.2.4. Isolation of DNA fragments

Restriction digests were separated on agarose gels. The fragments of interest were visualized on a UV lamp and excised using a sterile scalpel. DNA was then purified from gel pieces using the EasyPure DNA Purification Kit.

5.2.5. Agarose gel electrophoresis

Agarose gel electrophoresis was performed as described (Sambrook et al., 1989). DNA fragments were separated on a 1% agarose gels, with 10µl 10mg/ml Ethidiumbromide in 100ml TAE buffer.

5.2.6. DNA ligation

Ligation reactions were carried out with a molar ratio of insert to vector of 5:1. The reaction was conducted in a volume of 15µl, whereby 1µl T4 DNA Ligase was added to each reaction. The ligation reactions were incubated overnight at 18°C.

5.2.7. Preparation of electro-competent cells

DH5a, JM101 and ES1301 cells were plated out onto LB plates and grown overnight at 37°C. A colony was picked from this plate and grown overnight in 50ml LB. The following day these cells were diluted 1:100 with TB and grown until an OD600 of 0.5. Next, cells were cooled on ice for 30 min and then centrifuged at 4000rpm in a Sorvall GS3 rotor for 10min at 4°C. The cells were resuspended on ice for 30min in 500ml Millipore water and then centrifuged at 8000rpm in a Sorvall GS3 rotor for 20min at 4°C. Cell were resuspended in ice cold 10% (w/v) glycerol and centrifuged in a Heraeus bench top centrifuge at 4000rpm for 7min at 4°C. Cells were then resuspended in 1ml ice cold 10% glycerol. Finally resuspended cells were divided into 50µl aliquots, frozen in liquid nitrogen and stored at -70°C.

5.2.8. Transformation of electro-competent E. coli

0.5-1µl of plasmid DNA was mixed with a 40µl aliquot of electro-competent E. coli cells. Immediately after transformation cells were resuspended in 1ml LB. After incubation for 1h at 37°C, 20-300µl were plated on LB-Agar plates containing appropriate antibiotics. Plates were incubated overnight at 37°C and individual colonies were picked.

5.2.9. Transformation of chemically-competent E. coli

Chemically-competent DH5α were purchased from Invitrogen and stored according to manufacturers instructions. 1-5µl of plasmid DNA was mixed with a 50µl aliquot of chemically-competent cells and chilled for 30min on ice. Cells were then heat-shocked for 20sec at 37°C and transferred immediately on ice. After 2min recovery, cells were resuspended in 1ml LB and incubated for 1h at 37°C. From this point it was proceeded as described for electro-competent cells.

5.2.10. Isolation of plasmid DNA

To isolate small amounts of plasmid DNA applicable for restriction analysis and sequencing, the following protocol was used: 1.5ml of an overnight culture was centrifuged for 10min at 4000 rpm. The pellet was then resuspended in 100µl resuspension buffer. Afterwards, 200µl lysis buffer were added to the resuspended bacteria, shaken and incubated for 5min at RT. Next, 150µl neutralization buffer were added, shaken and incubated for further 5min at RT. The mix was then centrifuged for 30min at 14000rpm in a bench top centrifuge. After

completion, the supernatant was transferred into a new sample tube, supplemented with 1ml 100% ethanol and centrifuged again for 20min at 14000rpm. The supernatant was discarded, whereas the pellet was supplemented with 1ml 70% ethanol and centrifuged for 5min at 14000rpm. Finally, the pellet was dried and resuspended in 20-50µl H_2O.

To obtain larger quantities of plasmid DNA, the Nucleobond AX-100 Midi Prep kit was used according to the manufacturers information.

5.2.11. Amplification of DNA by PCR (Polymerase Chain Reaction)

For standard PCR either the Expand High Fidelity PCR-System or Platinum *pfx* DNA polymerase were used. The reaction mix was composed according to manufacturers instructions, whereby the reaction was performed in a total volume of 50 µl.

Standard PCR program:

```
start:      5min at 96°C

start:      30sec at 96°C
annealing:  30sec at 50°C          25 cycles
extension:  1min/kb at 68/72°C

end:        10min at 68/72°C
```

5.2.12. Site directed mutagenesis

Site directed mutagenesis was performed according to the Altered Sites 2 manual from Promega.

5.2.13. DNA sequencing

DNA sequencing was performed in the DNA sequencing facility at the University of Cologne. Details about the composition of the sequencing reactions are available at:
http://www.uni-koeln.de/math-nat-fak/genetik/facilities/sequencer/index.html

5.2.14. Preparation of RNA probes for in situ hybridization

For preparation of RNA probes the DIG-RNA labelling kit was used. 200ng linearized plasmid DNA were used. The reaction mix was composed according to manufacturers instructions with the exception that only 200ng linearized plasmid DNA were used for a

reaction. The reaction mix was then incubated for 2h at 37°C. After completion, 2µl reaction mix were tested on an agarose gel, whereas the remaining reaction mix was hydrolysed. Therefore, 30µl H_2O and 50µl 2X carbonate buffer were added to the reaction. After incubation for 40min at 70°C, the reaction was stopped by addition of 100µl stop solution supplemented with 1µl tRNA (100mg/ml). Next, the RNA was precipitated. Therefore, the sample was supplemented with 45µl LiCl (4M) and 1ml Ethanol. The sample was then chilled for 10min at -20° and subsequently centrifuged at 14000 rpm for 15 min in a bench top centrifuge cooled to 4°C. While the supernatant was removed, 1 ml 70% ethanol was added to the pellet. The sample was then centrifuged again for 15 min at 14000 rpm in a bench top centrifuge cooled to 4°C. Finally, the pellet was resuspended in 50µl hybridization mix.

5.3. *Drosophila* techniques

5.3.1. Maintenance of flies

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

5.3.2. Generation of transgenic flies

For the production of transgenic flies the P-element-insertion-method after Rubin and Spradling was used (Rubin and Spradling, 1982). Transgenic flies were made by inserting the DNA sequence of interest into a transposon called P-element. P-elements are mobile genetic elements that occur naturally in *Drosophila*. The integration of the P-element into the genome is mediated by an enzyme called transposase. For the generation of transgenic flies, two modified P-elements, called carrier P-element and helper P-element were utilized. Both P-elements were injected into the forming germ cells of w- embryos. The carrier P element contains the DNA sequence of interest and a mini w+ gene, which serves as marker. Since uncontrolled hopping of P-elements can cause genomic instability, the transposase of the carrier P-element is inactivated. The helper P element provides the transposase, but cannot be integrated into the genome. For transformation of Rca1-constructs, the pUASp vector was used as carrier P-element, which is adapted to the UAS/Gal4-System (Rorth, 1998). The plasmid Δ2.3 was used as helper P element (Rio and Rubin, 1985).

For injection, embryos were collected for 30min on apple juice agar plates and subsequently dechorionated in a 1:1 mixture of water and bleach. About 80 embryos were arranged on an agar block with anterior pole directed outwards. The arranged embryos were subsequently transferred on a coverslip prepared with heptane glue. The glued embryos were then dried for 8-12min in a dessicator. During this drying step, the injection needle was loaded with 1µl injection mix and broken in a 45° angle. The injection mix contained the pUASp vector in a concentration of 400ng/µl and the helper plasmid Δ2.3 in a concentration of 100ng/µl. After drying, the coverslip was installed on a slide and covered with 10S Voltalef oil. The DNA was injected into the posterior pole just before the formation of pole cells. The coverslip with the injected embryos was then transferred to a Petri dish and covered with 3S Voltalef oil. The Petri dish was then placed humid chamber and incubated at 25°C for approximately 24 hours. The newly hatched larvae were transferred into a new vial containing standard fly food supplemented with baker yeast. After approximately 8-9 days, the newly hatched flies were crossed against the double-balancer stock F-232. Positive transformants were identified due to their red eye colour. To map the P-element insertion to a particular chromosome, the red eyed flies were then crossed again with the double-balancer stock F-232.

5.3.3. Collection and fixation of embryos

Embryos were collected at 18°C from overnight clutches. Flies were kept in laying cages fitted with apple juice agar plates. Embryos were dechorionated in a 1:1 mixture of water and bleach for 1-2min, poured through a sieve and washed several times with tap water. The embryos were then transferred into a 2ml sample tube containing 1ml heptane as well as 1ml 4% Formaldehyde in PBS and shaken for 20min at 37°C. Afterwards the lower phase was removed and replaced by 1ml methanol. The embryos were devitellinized by shaking thoroughly for 30s. Finally, embryos were washed three times with methanol and stored at -20°C.

5.3.4. Antibody staining of embryos

For antibody staining, embryos were rehydrated by washing several times in PBS. Rehydrated embryos were then blocked by rotating for 1hr at RT in blocking mix (4% NGS in PBT). Next, the embryos were incubated overnight at 4°C with the desired primary antibody (diluted in 4% NGS in PBT). On the following day, the primary antibody was removed and the embryos were washed three times for 10min in PBT. After washing, an appropriate secondary

antibody (diluted in 4%NGS in PBT) was added to the embryos and incubated for 2h at RT. Afterwards, the embryos were washed three times for 10min in PBT and stained either with Hoechst or propidium iodide (0.2 µg/µl RNAse have to be added to the secondary antibody) for 4min RT. Finally, the embryos were transferred to a slide and mounted in Vecatshield.

5.3.5. Induction of clones

For clonal analysis two different systems were applied, that both rely on the Gal4/UAS system (Brand and Perrimon, 1993). To generate cell clones that overexpress a particular transgene, the "flpout" technique was utilized (Ito et al., 1997).In addition, the MARCM technique was applied to generate overexpressing clones with a certain mutant background (Lee and Luo, 1999). For clonal analysis flies were harvested in big fly vials filled with fly food. Clones were induced 40-48h after egg deposition by heat shock at 37°C. In the case of "flpout" experiments the heat shock was done for 10min, while in the case of MARCM experiments the heat shock had duration of 1h. Imaginal discs as well as salivary glands were analyzed 72h after clone induction. To generate MARCM clones in the salivary placode, embryos were collected for 8h at 25°C and subsequently heat shocked for 1h at 37°C (Hennig et al., 2006).

5.3.6. Dissection of imaginal discs and salivary glands

Imaginal discs and salivary glands were obtained from wandering larvae. Dissection of larval tissues was carried out under a stereomicroscope in a dissecting dish filled with cooled PBS. For dissecting wing imaginal discs, larvae were torn in half with a pair of tweezers. The anterior half of the larvae was then inverted, so that wing discs remained anchored to the body wall and cannot be lost during the staining procedure. For the same reason, eye imaginal discs and salivary glands were kept attached to the mouth hook. To dissect mouth hooks, larvae were hold in the middle with one set of forceps. With a second pair of forceps the mouth hook was then grasped and pulled out of the larvae. In each case, the dissected tissue was then transferred into a multi-well plate equipped with small sieves and immediately used for antibody staining.

5.3.7. Antibody staining of imaginal discs and salivary glands

For antibody staining samples were fixed for 30min at RT in 4% paraformaldehyde. The samples were then washed several times in PBTX and blocked with gentle agitation for 1hr at RT in blocking mix (4% NGS in PBTX). Next, the desired primary antibody (diluted in 4% NGS in PBTX) was added to the samples and incubated overnight at 4°C. On the following day, the primary antibody was removed and the samples were washed three times for 10min in PBTX. After washing, an appropriate secondary antibody (diluted in 4% NGS in PBTX) was added and incubated for 2h at RT. Afterwards, samples were washed three times for 10min in PBTX and stained either with Hoechst for 4min at RT. After completion, samples were transferred to a dissection dish, to separate imaginal discs/salivary glands from the remaining tissue. The dissected imaginal discs/salivary glands were then transferred to a slide and mounted in Vectashield.

5.3.8. BrdU-labelling

BrdU-labelling of eye imaginal discs was carried out according to standard protocols (Baker and Yu, 2001; de Nooij and Hariharan, 1995). The protocol described below enables the simultaneous detection of clones marked with GFP. If this is not necessary, the GFP staining and the Methanol fixation can be omitted. Third instar larvae were feed for 2.5h with yeast supplemented with BrdU (1mg/ml, Sigma). Afterwards, mouth hooks with attached eye imaginal discs were dissected as described above and subsequently fixed for 30min at RT in 4% paraformaldehyde. After completion, the samples were washed several times in PBTX and blocked with gentle agitation for 1hr at RT in blocking mix (4% NGS in PBTX). Next, the samples were incubated overnight at 4°C with the rabbit-anti-GFP antibody (diluted 1:500 in 4% NGS in PBTX). On the following day, the primary antibody was removed and the samples were washed three times for 10min in PBTX. After washing, the secondary antibody (goat-anti-rabbit-Alexa488, diluted 1:500 in 4% NGS in PBTX) was incubated for 2h at RT. After three washing steps (10min in PBTX), samples were fixed for 4min at -20°C in Methanol. The fixed samples were then washed several times in PBTX and denaturated for 1h in 2N HCl. After completion, samples were thoroughly washed and blocked again for 1h in blocking mix (4% NGS in PBTX). Next, the mouse-anti-BrdU antibody (diluted 1:20 in 4% NGS in PBTX) was added to the samples and incubated overnight at 4°C. After completion the samples were washed three times for 10min in PBTX and then supplemented with a further secondary antibody (goat-anti-mouse-Alexa568, diluted 1:500 in 4% NGS in PBTX).

This antibody was incubated for 2h at RT. Finally, samples were washed three times for 10min in PBTX and transferred to a dissection dish, where imaginal discs were separated from the remaining tissue. The dissected imaginal discs were then transferred to a slide and mounted in Vectashield.

5.3.9. Flow cytometry

Flow cytometry was performed according (Neufeld et al., 1998; Reis and Edgar, 2004). Imaginal discs from wandering larvae were dissected as described above. Clones were induced 40-48h after egg deposition by heat shocking for 30min at 37°C. Without fixation, dissected discs were immediately transferred to a 6ml FACS tube filled with 500µl 10X Trypsin-EDTA supplemented with 0.5µg/ml Hoechst 33342. The discs were then dissociated for 2-4h at RT with gentle agitation. Occasionally, samples were shaken by hand to break up clones. To visualize dead cells, samples were supplemented with 10µl propidium iodide and subsequently analyzed on a FACS Vantage sorter.

5.3.10. In situ hybridization of eye imaginal discs

RNA *in situ* hybridizations were preformed according to (Sturtevant et al., 1996). Small batches of mouth hooks with attached eye imaginal discs were dissected from third instar larvae as described above. The dissected tissue was then transferred to a new 1.5ml sample tube and subsequently fixed for 20min at RT in 4% Formaldehyde. After four washing steps with Methanol several batches were pooled in a glass scinti vial, rinsed five times with Ethanol and stored at -20°C.

For *in situ* hybridization, about 25 mouth hooks were transferred to a fresh sample tube and washed three times in Ethanol followed by a washing step in a 1:1 mixture of Ethanol and Xylene. Afterwards, samples were rinsed five times with Ethanol followed by two washing steps in Methanol. Now, samples were fixed again for 20min at RT in 8% Formaldehyde in PBS supplemented with 0.1% Triton-X100. After completion, samples were washed three times for 5min in PBT and digested for 1min at RT with Proteinase K (diluted 1:3000 in PBS). To inactivate the protease, samples were washed twice in glycine (2mg/ml in PBT) followed by two washing steps for 5min in PBT. The samples were then fixed once again in 8% Formaldehyde in PBS and washed then five times for 5min in PBT. Next, the samples were rinsed once with a 1:1 mixture of hybridization mix and PBT and undiluted hybridization mix respectively. Afterwards, the samples were prehybridized for 1h at 55°C in

hybridization mix. During this time, 3µl of the hydrolyzed RNA probe was diluted in 300µl hybridization mix. The diluted probe was then denaturated by heating for 10min at 88°C and placed on ice till use. After prehybridization, most of the hybridization mix was discarded and replaced by the denaturated RNA probe. Hybridization was conducted overnight at 55°C.

On the following day, the probe was discarded and samples were washed for 20min at 55°C in hybridization mix. The samples were then washed for 20min at 55°C with a 1:1 mixture of hybridization mix and PBT. After five washing steps in PBT (5min at RT), samples were incubated for 1h at RT with a Sheep-anti-DIG-AP antibody (diluted 1:2000 in PBT). Samples were then washed three times for 10 min in PBT. After rinsing with staining solution the samples were transferred to an dissection dish containing 500µl staining solution supplemented with 5µl NBT and 5µl BCIP. For staining, samples were kept in the dark for 4-16h at 37°C. After completion, the reaction was stopped by washing in PBT. The samples were then successively rinsed with 30%, 50% and 80% Glycerol and transferred to a slide. Finally, the discs were freed from the remaining tissue and covered with a cover slip.

5.3.11. Production of embryo extracts

For western blot analysis 4-8 or 8-12h wild-type embryos were used. Embryos were collected at 25°C on apple juice agar plates. Embryos were dechorionated in a 1:1 mixture of water and bleach for 1-2min, poured through a sieve and washed several times with tap water. The embryos were then transferred into a 2ml sample tube containing 1ml heptane as well as 1ml Methanol and shaken for 20min at RT. Afterwards, the embryos were washed three times with methanol and stored at –20°C. About 100 embryos were then sorted on an agar block and transferred into a new sample tube filled with 50µl 4x Laemmli buffer. The samples (2 embryos/µl) were subsequently boiled for 10min at 96°C and stored at –80°C until analysis by Western blotting.

5.3.12. Production of salivary gland extracts

Salivary glands from wandering larvae were dissected as described above. “Flpout” clones were induced 40-48h after egg deposition by heat shocking for 30min at 37°C. About 50 salivary glands were dissected in cooled PBS and freed of much fat body as possible. The dissected salivary glands were suspended in 25 µl 4x Laemmli buffer and heated for 10 min at 96°C. Till analysis by Western blotting, samples were stored at –80°C.

5.3.13. SDS-Page and western blot analysis

Samples were separated on 1mm thick polyacrylamide gels using the using the Mini Protean 3 System (BioRad). Gels were run at constant current with a starting voltage of approximately 100V. Gels were blotted onto Amersham Hybond Nitrocellulose membranes for ECL analysis using a dry blotting system (BioRad). Gels were blotted for 30 min at constant current with a starting voltage of approximately 10V. Blotted membranes were then stained for 5min 1X Ponceau S and subsequently destained in tap water. Next, the membrane was washed several times in PBT and blocked for 1h at RT in "Blocking Buffer for Fluorescent Western Blotting" (Rockland). The membrane was then incubated with the desired primary antibody (preabsorbed for 1hr at RT in 5% powdered milk in PBT) and shaken overnight at 4°C. After washing 3 times in PBT, an appropriate secondary antibody (diluted in 5% powdered milk in PBT) was added to the membrane and incubated for 2h at RT. Finally, the membrane was washed three times in PBT and proteins were detected using the Odyssey Infrared Imaging system.

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Note added in proof

Near completion of this work, certain experiments described in section 2.1. were published independently (Reber et al., 2006). In particular, this publication included the observation that the cell cycle arrest in Cyclin A mutants can be rescued by Cyclin E overexpression (2.1.1.) as well as the characterisation of the Dacapo; Cyclin A double mutant (2.1.3.).

Abbreviations

APC/C	Anaphase promoting complex / Cyclosome
APS	Ammoniumperoxidisulfate
ATP	Adenosine Triphosphate
Cdk	Cyclin dependent kinase
Cyc	Cyclin
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethelyne Diamine Tetracetic Acid
Fzr	Fizzy-related
g	gram
h	Hour
hs	Homo sapiens
l	liter
M	mol per litre
m	milli
MF	Morphogenetic furrow
mm	Mus musculus
µ	micro
min	minute
mRNA	messenger RNA
NGS	Normal goat serum
NLS	Nuclear localization signal
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PRC	Photoreceptor cell
Rca1	Regulator of Cyclin A 1
RNA	Ribonucleic acid
rpm	rounds per minute
RT	Room Temperature
SDS	Sodium dodecyl sulfate

SMW	Second mitotic wave
TEMED	N,N,N',N'-Tetramethylendiamin
U	Unit
xl	Xenopus laevis
ZBR	Zinc binding region
ZNC	Zone of none proliferating cells

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 Verlauf der Mitose ist ein strikte Kontrolle des APC/C notwendig. Eine wichtige Klasse von negativen APC/C Regulatoren sind die Proteine der Rca1/Emi1 Familie. Alle Mitglieder der Rca1/Emi1 Familie besitzen eine konservierte zink-bindende Region, welche für ihre Aktivität essentiell ist. Die Rca1/Emi1 Familie gehört zur Gruppe der F-Box Proteine, die als Substrat-Erkennungs Module der SCF-E3-Ligase fungieren. Emi1 und Rca1 binden *in vitro* mit Hilfe ihrer F-Box an Mitglieder der Skp Familie. Bisher wurde jedoch kein Kontext gefunden, in dem die F-Box benötigt wird. In der G2-Phase von Zellzyklus 16 von *Drosophila,* verhindert Rca1 die frühzeitige Aktivierung des APC/C-Fzr Komplex. Der Verlust des *rca1* Gens führt zu einem Arrest in der G2-Phase von Zellzyklus 16 und einem vorzeitigen Abbau der mitotischen Cycline. Rca1 enthält mehrere konservierte Proteinmotive deren Funktion mit Hilfe einer Deletionsanalyse aufgeklärt werden sollte. Diese Analyse zeigte, dass ein C-terminales Rca1 Fragment für die Rettung des Phänotyps von *rca1* Mutanten ausreichend ist. Diese Beobachtung bestätigt, dass die ZBR das einzige essentielle Proteinmotiv für die Inhibition des APC/C durch Mitglieder der Rca1/Emi1 familie ist. Außerdem wird deutlich, dass während der Embryogenese die F-Box nicht für die Inhibition des APC/C benötig wird. Weiterführende Untersuchungen zeigten jedoch, dass Rca1 eine zusätzliche Funktion hat und dass für diese die F-Box benötigt wird. Durch Verwendung der MARCM Technik konnte in Flügel-Imaginalscheibenzellen das endogene Rca1 Protein durch ein Konstrukt ohne F-Box ersetzt werden. Diese Zellen haben eine reduzierte Teilungsaktivität und benötigten länger für das Durchschreiten der G1-Phase. Umgekehrt führte die Überexpression von Rca1 zu einem vorzeitigen Eintritt in die S-Phase. Daher ist es wahrscheinlich das Rca1 Teil eines SCF-Komplexes ist, der den Übergang in die S-Phase reguliert. Zusäztlich dazu wurde der Effekt von Rca1 auf Endoreplikationszyklen untersucht. Die Überexpression von Rca1 im Verlauf der Speicheldrüsenentwicklung führte die zu einer verringerten Polyploidität, wobei dieser Phänotyp ebenfalls eine funktionale F-Box benötigt. Oszillierende Cyclin E/Cdk2 Aktivität ist essentiell für den korrekten Ablauf von Endoreplikationszyklen. In Endoreplikationszyklen wird außerdem die Transkription von Cdk1 und der mitotischen Cyclin abgeschaltet. Weiterhin wurde postuliert dass die Aktivität des APC/C-Fzr Komplex nicht mehr erforderlich ist, sobald das Endoreplikations-Programm initiert wurde. Obwohl Cyclin E kein Substrat des APC/C-Fzr Komplex ist, zeigten Rca1 überexprimierende Zellen erhöhte Mengen an Cyclin E. Es konnte in früheren Experimenten gezeigt werden, dass die kontinuierliche Expression von Cyclin E mit der Initiation der DNA

Replikation interferiert. Daher ist der reduzierte DNA-Gehalt in Rca1 überexprimierenden Zellen wahrscheinlich auf die erhöhte Menge an Cyclin E zurückzuführen. Die Rca1 überexprimierenden Zellen zeigten außerdem Indikatoren für mitotische Zellen, wie Cdk1 und nukleares Cyclin A. Die Akkumulation von Cyclin E, Cyclin A und Cdk1 kann nicht durch Inhibition des APC/C-Fzr Komplexes erklärt werden. Rca1 scheint vielmehr die Transkription dieser Gene zu aktivieren, wobei jedoch nicht ausgeschlossen werden kann, dass der APC/C-Fzr Komplex indirekt an diesem Vorgang beteiligt ist. Zusammengefasst deuten diese Resultate daraufhin, dass Rca1 Teil eines SCF-Komplexes ist, der für die Aufrechterhaltung des diploiden Zustands notwendig ist.

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, November 2006

Norman Zielke

Teilpublikationen

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