Control of G₁ in the developing *Drosophila* eye: *rca1* regulates Cyclin A

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In the developing eye of *Drosophila melanogaster*, cells become synchronized in the G₁ phase of the cell cycle just prior to the onset of cellular differentiation and morphogenesis. In *roughex* (*rux*) mutants, cells enter S phase precociously because of ectopic activation of a Cyclin A/Cdk complex in early G₁. This leads to defects in cell fate and pattern formation, and results in abnormalities in the morphology of the adult eye. A screen for dominant suppressors of the *rux* eye phenotype led to the identification of mutations in *cyclin A*, *string* (*cdc25*), and new cell cycle genes. One of these genes, *regulator of cyclin A* (*rca1*), encodes a novel protein required for both mitotic and meiotic cell cycle progression. *rca1* mutants arrest in G₂ of embryonic cell cycle 16 with a phenotype very similar to *cyclin A* loss of function mutants. Expression of *rca1* transgenes in G₁ or in postmitotic neurons promotes Cyclin A protein accumulation and drives cells into S phase in a Cyclin A-dependent fashion.

[Key Words: *cyclin A*; *rca1*; *roughex*; G₁-S phase; cell cycle; *Drosophila*]

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During the G₁ phase of the cell cycle, cells receive and interpret information from their environment and either commit to a complete round of cell division or withdraw from the cell cycle to become quiescent or to terminally differentiate (Pardee 1989). Specific cyclin-dependent kinase complexes (Cyclin/Cdks) have been shown to play crucial roles in regulating the passage of cells through G₁ and into S phase in yeast, mammalian cells, and *Drosophila* (Knoblich et al. 1994; Sherr 1994). For normal progression through G₁, not only is the accumulation and activation of each specific Cyclin/Cdk required at the appropriate time, Cyclin/Cdk complexes that function in S and G₂ must be inhibited (Amon et al. 1994; Schwob et al. 1994).

G₁ progression in *Drosophila* and mammalian systems is similar in many respects. In mammalian cells, sequential activation of Cyclin/Cdks occurs during G₁ with Cyclin D/Cdk4 activity peaking first, followed in late G₁ by Cyclin E/Cdk2, and then Cyclin A/Cdk2 (Sherr 1993). Cyclin D/Cdk4 plays a crucial role in regulating G₁ by phosphorylating the Retinoblastoma (Rb) protein (Matsushime et al. 1994; Meyerson and Harlow 1994). In its hypophosphorylated form, Rb binds to and inhibits

the activity of the transcription factor E2F. Upon hyperphosphorylation of Rb, E2F is released, activating transcription of genes required for DNA replication (Nevins 1992). In *Drosophila*, Cyclin E and E2F have been shown to play essential roles in regulating the G₁/S transition in the embryonic cell cycles (Knoblich et al. 1994; Duronio and O'Farrell 1995; Duronio et al. 1995). Although *Drosophila* homologs of Rb and Cyclin D have also been identified, mutations in them have not yet been reported (Du et al. 1996; Finley et al. 1996).

Several features of cell cycle regulation in the developing *Drosophila* eye are favorable for analyzing the control of G₁ progression. First, cells become synchronized in G₁ within a specific spatial domain that coincides with the onset of pattern formation and differentiation (Thomas et al. 1994). Eye development has been described in considerable detail (Ready et al. 1976). In brief, the eye forms from a columnar epithelium called the eye imaginal disc. Toward the end of larval development a wave of morphogenesis initiated at the posterior edge of the eye disc sweeps anteriorly. The front of this wave is marked by a depression in the disc epithelium referred to as the morphogenetic furrow (MF). Anterior to the MF, cells are unpatterned and undifferentiated and progression through the cell cycle occurs in an asynchronous fashion. Within the MF all cells are synchronized in G₁ (Thomas et al. 1994). These cells will segregate into two populations, those entering S phase and those becoming postmitotic and differentiating into neurons. Second, the spatial order of Cyclin accumulation within the synchro-

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nized G_1 domain is highly stereotyped and parallels the order of Cyclin/Cdk activation during the mammalian cell cycle (Thomas et al. 1994; Richardson et al. 1995; Finley et al. 1996). The length of time a cell has been in G_1 can be determined by its location along the anterior/posterior axis in the MF region. Finally, the eye is well suited to the isolation of dominant suppressors and enhancers of weak loss-of-function and dominant gain-of-function phenotypes. Indeed, such screens have led to the identification of genes, essential for viability, that participate in various signal transduction pathways, most notably components downstream from receptor tyrosine kinases (Simon et al. 1991; Dickson et al. 1996; Karim et al. 1996).

The analysis of the *roughex* (*rux*) gene has provided insight into the mechanism synchronizing cells in G_1 (Thomas et al. 1994). Cell cycle synchronization occurs in a domain of cells just anterior to the MF (see Fig. 2A). Cells within the anterior part of this domain are either in G_1 or G_2 . A burst of *string* (*stg*, encoding a cdc25-type phosphatase) (Edgar and O'Farrell 1989; Kumagai and Dunphy 1991) expression within this domain is induced by developmental patterning genes (Heberlein et al. 1995). This drives those cells in G_2 through mitosis and into G_1 . All cells now in G_1 are prevented from entering S phase through the action of the *rux* locus; in *rux* mutants cells become synchronized but enter S phase precociously (Thomas et al. 1994). Although the biochemical function of Rux is not known, several lines of evidence support a model in which Rux prevents precocious activation of Cyclin A/Cdk complex in early G_1 (Gönczy et al. 1994; B.J. Thomas and S.L. Zipursky, in prep.) (see Discussion). Whereas *cyclin A* has been shown in the *Drosophila* embryo to be essential for G_2 /M (Lehner and O'Farrell 1989), ectopic expression of Cyclin A in the MF drives G_1 cells into S phase. Together these findings are consistent with recent studies in yeast demonstrating negative regulation of G_2 cyclins in G_1 to be essential for normal G_1 progression (e.g., Amon et al. 1994).

Most cell cycle genes in *Drosophila* that have been described so far are essential for viability. In contrast, *rux* mutants are viable and have a rough-eye phenotype resulting from precocious activation of a Cyclin A/Cdk complex in G_1 . *rux* thus provides a suitable genetic background to identify regulators and targets of Cyclin A/Cdk complexes that can drive entry into S phase. In this paper, we describe the isolation and characterization of suppressors of *rux* and the detailed phenotypic and molecular characterization of a locus regulating Cyclin A, *regulator of cyclin A* (*rca1*).

Results

Identification of Suppressors of *rux*

An F1 screen to identify dominant suppressors of the adult rough-eye phenotype of a weak *rux* allele, *rux*³, was performed (Fig. 1). Some 71,000 F1 males, including 14,000 from X-ray and 57,000 from ethyl methane sulfonate (EMS) mutagenized fathers, were screened. Sixty-

Figure 1. Screen for dominant suppressors of *roughex*. (A) Scheme used to identify *S(rux)* mutations (indicated by asterisks). (B–E) SEMs of adult eyes. (B) Rough-eye phenotype of *rux*³. (C–E) Suppression of *rux*³ results in the more uniform array of facets called ommatidia. Each ommatidium contains a precise arrangement of neuronal and non-neuronal cells (see Table 1). Genotypes are (C) *rux*³; *rca1*/+; (D) *rux*³; *S(rux)2B*/+; (E) *rux*³; *S(rux)2C*/+. (B,C,E) Anterior is to the left. (D) Anterior is to the right.

three suppressor mutations, designated *S(rux)*, were identified, which fell into 22 lethal and 3 viable complementation groups. Two lethal complementation groups containing multiple alleles correspond to *cyclin A* and *stg*. Previously, we identified these two loci as suppressors during a screen of a large panel of deficiencies for regions of the genome capable of dominantly suppressing *rux* (Gönczy et al. 1994; Thomas et al. 1994). Three other complementation groups containing multiple alleles, *S(rux)2A*, *S(rux)2B*, and *S(rux)2C*, define new loci (Table 1). One of these genes, *S(rux)2A*, has been studied in considerable detail. Based on its phenotype, this locus has been renamed *regulator of cyclin A* (*rca1*) to reflect its function more accurately. Nine of the *S(rux)* loci (excluding *cyclin A* and *stg*) were mapped by meiotic recombination. None correspond to previously identified *Drosophila* cell cycle genes (Table 1; data not shown).

S(rux) mutations restore normal G_1 progression in *rux* mutant eye discs

The G_1 domain within the MF can be visualized in preparations double-labeled for *stg* mRNA and 5-bromo-2-deoxyuridine (BrdU) (marking cells in S phase). The

Table 1. Summary of *S(rux)* complementation groups

Genotype	alleles (no.)	Genetic location	<i>rux</i> ³ Pseudopupil ^a (%)	<i>rux</i> ⁸ Male fertility ^b
+/+	N.A.	N.A.	11	sterile
<i>rca1</i> /+	18	2-21 (27B4-C1)	95	fertile
<i>S(rux)2B</i> /+	3	2-54 (37D)	80	sterile
<i>S(rux)2C</i> /+	3	2-64 (47C)	97	sterile
<i>S(rux)3A</i> /+	4	3-99 (99A)	98	sterile
<i>S(rux)3B</i> /+	14	3-37 (68E)	96	fertile

Suppression of *rux*³ was quantified using pseudopupil analysis, an optical technique for assessing the position and morphology of photoreceptor neurons (R cells). The wild type eye is a reiterated pattern of hexagonally arranged facets called ommatidia. Each ommatidium contains 8 R cells arranged in a precise fashion. In *rux*³, ommatidia contain abnormal numbers of R cells. (N.A.) Not applicable.

^aPseudopupil analysis was performed on 3 day flies raised at 18°C with the genotype *rux*³; *S(rux)*/+. For this analysis, from 4 to 10 eyes were used to score between 500 and 2000 ommatidia per genotype and the results are expressed as a percentage of wild-type ommatidia.

^bMale fertility assays were performed at 18°C on flies (raised at 18°C) of the genotype *rux*⁸; *S(rux)*/+. Male flies were individually crossed to wild-type females and vials were examined 8 days later for the appearance of larvae. *S(rux)3A* and *S(rux)3B* mutations were found to be allelic to *string*, *cyclin A*, respectively.

onset of *stg* expression is some one to three cell diameters anterior to the onset of *cyclin D*, which marks early G₁; the posterior boundaries of *cyclin D* and *stg* are similar (Fig. 2A,B) (Finley et al. 1996). Previously, we have shown that in the strong loss-of-function *rux*⁸ mutant entry into S phase is precocious, resulting in marked overlap between the *stg*-expressing and BrdU-labeled cells (Thomas et al. 1994). In contrast to wild type, where only a subpopulation of cells enter an additional round of cell division (Fig. 2A), in *rux*⁸ all cells reenter the cell cycle. In *rux*³ eye discs the phenotype is less severe. Although all cells enter S phase and do so earlier than in wild type, the overlap between the *stg*-expressing and BrdU-labeled cells is less extensive (Fig. 2C), indicating that some cells establish a G₁ phase, albeit shorter in length. Figure 2D shows that the G₁ gap is restored in *rux*³ eye discs heterozygous for *rca1*. In these discs, a small number of BrdU-labeled cells within the G₁ domain was observed in most preparations (Fig. 2D, arrows) indicating that in a few cells the phenotype is not suppressed. In addition, in *rux*³ many normally quiescent cells in the posterior region of the disc continue to divide. *rca1* also dominantly suppressed these cell divisions (data not shown). *S(rux)2B*, *S(rux)2C*, *stg*, and *cyclin A* mutations also suppress the cell cycle defects in the *rux*³ eye discs (data not shown); other complementation groups were not tested. Hence, the suppression of the *rux* adult eye phenotype results from restoration of a normal G₁ in the MF.

A subset of S(rux) mutations function in mitosis and meiosis

In addition to its function during mitosis, *rux* plays an important role as a negative regulator of Cyclin A during male meiosis (Gönczy et al. 1994) (see Discussion). In strong loss-of-function *rux* mutants (e.g., *rux*⁸), germ cells progress through meiosis I and II normally but then undergo an ectopic round of nuclear division, leading to

male sterility. It was shown previously that *cyclin A* and the germ-line homolog of *string/cdc25* (*twine/cdc25*) (Alphey et al. 1992) dominantly suppress this phenotype (Gönczy et al. 1994; Thomas et al. 1994). Whereas the inappropriate activation of Cyclin A/Cdk complexes in G₁ leads to entry into S phase, failure to inhibit Cyclin A/Cdk activity after meiosis II leads to another round of cell division. This abnormal division is not preceded by an S phase; the extra nuclear division gives rise to cells containing multiple aneuploid nuclei (Gönczy et al. 1994). Hence, suppressors of *rux* are likely to fall into two classes. Mutations suppressing both the meiotic and mitotic phenotypes would be candidates for encoding components regulating Cyclin A/Cdk activity, whereas suppressors rescuing only the eye phenotype may encode components limiting for entry into S phase. All suppressors of the rough-eye phenotype of *rux*³ were tested for their effects on the male sterility of *rux*⁸; 11 of the 25 complementation groups suppressed this phenotype (Table 1; data not shown). We next considered loss-of-function cell cycle phenotypes of *S(rux)* mutants. To ensure that we were assessing the loss-of-function phenotypes, we restricted our analysis to complementation groups containing multiple alleles (Table 1).

S(rux)2B and S(rux)2C are limiting for S-phase entry posterior to the MF

Mutations that only suppress the G₁ phenotype in the eye and not the meiotic defect [e.g., *S(rux)2B*, *S(rux)2C*, and *stg*] may be genes for which germ line-specific isoforms exist (e.g., the germ-line homolog of *stg* is *twine*) or, alternatively, these genes may encode components limiting only for S-phase entry. We therefore examined the loss-of-function phenotypes of *S(rux)2B* and *S(rux)2C*. *S(rux)2B* and *S(rux)2C* mutants die as late larvae or early pupae and show marked defects in cellular proliferation. Larvae homozygous for *S(rux)2C* or *S(rux)2B* show a marked decrease in the size of the eye–

Figure 2. *rca1* restores G_1 in *rux* mutant eye discs. (A) Schematic representation of cell cycle progression and initiation of pattern formation in the MF of the eye imaginal disc. In the anterior region (right side), cells are unpatterned, undifferentiated, and dividing asynchronously as revealed by scattered S-phase cells (brown nuclei). Mitotic figures and patchy *cyclin A* and *cyclin B* staining (not shown) indicates cells in M and G_2 . Cells in G_1 (e.g., *cyclin D* expressing, not shown) are also present in this domain. Just anterior to the MF, cell cycle synchronization is initiated by a burst of *stg* expression (blue); G_2 cells within this domain are driven through mitosis. Within the *stg* domain, all cells become synchronized in G_1 and express *cyclin D*. All cells are in G_1 in the MF and segregate into two populations that either (1) withdraw from the cell cycle, segregate into clusters, and begin to differentiate as photoreceptor neurons [these cells express neural specific antigens in a stereotyped order (yellow)] or (2) emerge from the posterior edge of the MF, express *cyclin E*, enter S phase synchronously, and then divide. Cells derived from this division differentiate into both neuronal and non-neuronal cells, completing ommatidial clusters. (B–D) Eye discs double-labeled for *stg* mRNA (blue) and BrdU (brown) to detect cells in S phase. (B) In wild type (wt), G_1 extends from about the middle of the *stg* domain to just anterior to the BrdU-labeled cells. (C) In *rux*³ many cells enter S phase precociously (arrows indicate the onset of S phase in the *stg* domain). (D) In *rux*³; *rca1*^{+/+} discs, G_1 is largely restored. A few cells (arrows) enter S phase prematurely. Arrowheads indicate the MF. Anterior is to the right. Bar, 10 μ m.

brain complex and a reduction in BrdU incorporation (Fig. 3D,E; data not shown). In larvae carrying a less severe combination of *S(rux)2B* alleles [*S(rux)2B*¹/*S(rux)2B*²], eye discs and optic lobes appear to be of normal size (Fig. 3A,B). However, labeling with BrdU showed a striking decrease in the number of synchronous S-phase cells posterior to the MF (Fig. 3B). In contrast, the onset of neuronal differentiation appears unaffected (data not shown). Hence, cells within the MF cannot proceed from G_1 to S, but can exit the cell cycle and differentiate into neurons.

Interestingly, flies heterozygous for both *S(rux)2B* and *S(rux)2C* [i.e., *S(rux)2B*, +/+; *S(rux)2C*] show an adult rough-eye phenotype and reduced numbers of S-phase cells posterior to the MF (Fig. 3C). This phenotype is similar to that seen with *S(rux)2B*¹/*S(rux)2B*² as well as a viable weak loss-of-function allele of *cyclin E* (H. Richardson, pers. comm.). This is particularly intriguing because *cyclin E* is necessary for entry into S phase in the embryonic cell cycles (Knoblich et al. 1994). Taken together, these data suggest that *S(rux)2B* and *S(rux)2C* are required for entry into S phase. Further characterization of *S(rux)2B* and *S(rux)2C* will be published elsewhere.

rca1 and cyclin A mutants have similar embryonic cell cycle arrest phenotypes

Cell cycle progression in the embryo has been studied in considerable detail (Foe 1989; Edgar and O'Farrell 1990; Foe et al. 1993; Edgar et al. 1994). In wild type, the first 13 cell cycles occur in a cytoplasmic syncytium and oscillate rapidly between S and M phase. After mitosis of cell cycle 13, cellularization occurs and the cell cycle becomes modified by the acquisition of a G_2 phase; cohorts of cells form reproducible mitotic domains differing in the length of G_2 . The first G_1 phases occur during embryonic cell cycle 17. The homozygous phenotype of *stg* and *cyclin A* mutants have been described previously (Lehner and O'Farrell 1989; Edgar and O'Farrell 1990; Lehner et al. 1991). Maternal stores supply Stg and Cyclin A message required for the early embryonic cell divisions (cell cycles 1–13). During the first three zygotic divisions (cell cycles 14–16), cells become dependent upon zygotic transcription of these genes as maternal stores become depleted. Homozygous *stg* mutants arrest in G_2 of cell cycle 14 (Edgar and O'Farrell 1990). In *cyclin A* mutant embryos, S phase of cell cycle 16 occurs as in wild type (Lehner et al. 1991), but cells do not proceed through mitosis 16. Failure of mitosis 16 was evidenced by the absence of mitotic spindles and chromosome figures in mutant cells and a twofold reduction in epidermal cell number at later stages compared with wild type, indicating that mitosis 16 is not simply delayed (Lehner and O'Farrell 1989; Lehner et al. 1991). The *cyclin A* mutant phenotype was therefore described as arrest in G_2 of cell cycle 16. Interestingly, however, cells of the thoracic epidermis, which normally proceed through a 17th mitotic cycle during wild-type embryogenesis, enter S phase 17 on schedule in *cyclin A* mutants, despite the absence of the preceding mitosis (Sauer et al. 1995).

Figure 3. *S(rux)2B* and *S(rux)2C* are limiting for S-phase entry. (A–E) Cells in S phase were visualized by BrdU incorporation. (B) Eye discs from *S(rux)2B¹/S(rux)2B²* mutant larvae are similar in size to wild-type discs (A); however, the synchronized band of S-phase cells posterior to the MF is missing. (C) Eye discs heterozygous for both *S(rux)2B* and *S(rux)2C* [*S(rux)2B², +/+*, *S(rux)2C³*] also show a marked decrease in BrdU-positive cells posterior to the MF. (E) Eye disc-brain complexes homozygous for *S(rux)2C* [*S(rux)2C¹/S(rux)2C²*] are markedly reduced in size. A corresponding decrease in BrdU incorporation is seen, compared with the wild type (D). (OL) Optic lobe; (ED) eye imaginal disc; (IPC) inner proliferation center; (LPC) lamina precursor cells; (OPC) outer proliferation center. Anterior is to the right. Arrowheads indicate the MF. Bar, 25 μ m.

In *cyclin A* mutants, BrdU incorporation is also seen in cells of the peripheral and central nervous systems (PNS, CNS) that during normal development proceed through several rounds of division beyond mitosis 16 (Lehner et al. 1991). These results indicate that the loss of *cyclin A* can result in the conversion of a mitotic cycle to an endoreduplication cycle and that Cyclin A, in addition to being required for mitosis, also functions during G_2 to prevent re-replication (Sauer et al. 1995). Moreover, Cyclin A function does not appear to be required for S phase in *Drosophila* (Lehner and O'Farrell 1989; see Discussion).

The *rca1* mutant phenotype is strikingly similar to that of *cyclin A* mutants. In *rca1* mutant embryos, mitosis 15 and S phase 16 occur normally (Fig. 4A,B; data not shown), but cells do not proceed through mitosis 16. In wild-type embryos, the mitotic spindles of dorsal and ventral epidermal cells in mitosis 16 were visualized easily, using antitubulin staining (Fig. 4C,E). In contrast, mitotic figures were never seen in *rca1* mutant embryos at this stage (Fig. 4D,F). In the dorsolateral region of each segment of the control embryos, cell division 16 begins around the tracheal pit and spreads outward (Fig. 4C,E). Although the tracheal pit invaginations take place normally in the mutant embryos, no dividing cells were found in these regions (Fig. 4D,F). An examination of *rca1* mutant embryos at later developmental stages also revealed a twofold reduction in epidermal cell number and an increase in cell size compared with identically staged wild-type embryos (Fig. 4G,H). These observations are consistent with the absence rather than a delay of mitosis 16. In addition, pulse-labeling with BrdU of stage-12 *rca1* mutant embryos revealed that, as in *cyclin A* mutants, DNA replication can continue in thoracic

epidermal cells and in the CNS, despite the loss of the previous M phase (data not shown). Thus, *rca1* mutant embryos proceed through the first 15 rounds of mitotic division normally, presumably due to maternally derived *rca1* mRNA (see below). Subsequently, cells in the epidermis of *rca1* embryos arrest in G_2 prior to mitosis 16; however, DNA synthesis can reinitiate on schedule in some of these cells.

The similarity of the *rca1* and *cyclin A* mutant phenotypes, failure to enter mitosis 16 and the conversion in some cells of mitotic cycles to endoreduplication cycles, and the up-regulation of Cyclin A protein in response to ectopic Rca1 expression (see below) prompted us to examine the Cyclin A expression pattern in homozygous *rca1* embryos. Cyclin A protein initially accumulates in the cytoplasm of S/G_2 cells. During cell cycles 14–16, Cyclin A protein accumulates and is degraded in a dynamic pattern throughout the embryo (Lehner and O'Farrell 1989). Cyclin A protein is initially found in the cytoplasm of S/G_2 cells, translocates to the nucleus during prophase, and is rapidly degraded during metaphase (Lehner and O'Farrell 1989; Whitfield et al. 1990). During these cycles, we observed no abnormalities in the pattern of Cyclin A protein expression in *rca1* mutant embryos (data not shown).

rca1 encodes a novel protein

Several lines of evidence suggested that *rca1* and *cyclin A* function in a common pathway: (1) the embryonic cell cycle phenotypes of *cyclin A* and *rca1* are similar; (2) *rca1* and *cyclin A* suppress both the meiotic and mitotic *rux* phenotypes; and (3) *rca1* is a dominant enhancer of a viable allele of *cyclin A*, *cyclin A^{har}* (Ueda et al. 1992;

Figure 4. *rca1* mutants arrest in embryonic cell cycle 16. (A–F) Embryos were subjected to immunofluorescent labeling with anti- β -tubulin antibodies to visualize mitotic figures. (A,B) Ventral view of embryos during cycle 15 (at stage 10). (A) In wild-type embryos, mitotic spindles were seen in ventral (arrowheads) and dorsal (not shown) epidermal cells. (B) Mitosis 15 in *rca1* [i.e., *rca1*²/*rca1*³] mutant embryos proceeds normally. (C,D) Lateral view of embryos during cycle 16 (at stage 11). (C) Many dorsolateral epidermal cells go through mitosis 16 in wild-type embryos. Dividing cells (arrowheads) were observed surrounding the tracheal pits (arrows). (D) No mitotic spindles were observed in *rca1* embryos. Tracheal pit regions are indicated by arrows. (E,F) High-magnification view of tracheal pit regions outlined by broken boxes in panels C and D, respectively. (G,H) Lateral view of stage-13 embryos labeled with anti- β -tubulin antibodies to outline cell profiles of nondividing epidermal cells arrested in G₁ of cell cycle 17. The twofold reduction in cell number and increased cell size in the *rca1* embryo (H) compared with the wild type (G) demonstrates that mitosis 16 did not occur in the mutant. Broken boxes delimit an area of 400 μm^2 containing 81 cells in the wild type and 35 cells in the *rca1* mutant. [Average number of cells per 400- μm^2 box: (wt) 66 $\pm$ 9; (*rca1*) 31 $\pm$ 3; *n*=10.] (A–D) Bar, 20 μm ; (E–H) Bar, 5 μm .

data not shown). To gain insight into the function of *rca1*, we characterized it in more detail.

rca1 was mapped by meiotic recombination and by deficiencies and duplications to 27B–27C on the second chromosome. A P-element induced allele of *rca1* was identified from a collection of P-element lethal mutations in the region (Karpen and Spradling 1992). A 5.8-kb DNA fragment flanking the insertion site was isolated by plasmid rescue and was used to clone ~17 kb of genomic DNA. A 9.2-kb genomic DNA fragment covering the insertion site was used to probe a 0- to 24-hr embryonic cDNA library. Sixteen cDNAs corresponding to a single transcription unit were isolated. The longest cDNA was 1.6 kb in length and spanned the P-element insertion site. Representative cDNA clones were sequenced.

The cDNAs encode a 412 amino acid protein with no homology to any other proteins in the data base (Fig. 5A,B). The P-element insertion site was mapped 42 bp upstream of the start codon. We examined 18 EMS alleles of *rca1* by polymerase chain reaction (PCR) to detect deletions in coding sequence. One allele, *rca1*¹, contained a 282-bp deletion in the *rca1* coding sequence. This gives rise to an in-frame deletion predicted to result

in a polypeptide of 318 amino acids (i.e., deletion of residues 285–378). Consistent with these findings, a 10-kb genomic DNA fragment containing the *rca1* transcript rescues the lethality of the *rca1* mutants. These data indicate that we have identified the cDNA corresponding to *rca1* and that it encodes a novel protein required for normal cell cycle progression in *Drosophila*.

The *rca1* expression pattern during embryogenesis was determined by in situ hybridization with *rca1* RNA probes. Antisense but not sense probes revealed a dynamic pattern of expression during development (Fig. 5C–F). Early embryos prior to cellularization stain densely and uniformly for *rca1* mRNA, reflecting maternal *rca1* message deposited in the egg. The pattern of *rca1* staining becomes dynamic following cellularization of the blastoderm. As germ-band elongation proceeds during stages 8 and 9, the *rca1* transcript is predominantly found in the mesoderm and within the anterior and posterior midgut primordia. During stages 10 and 11, *rca1* is expressed at high levels throughout the embryo, with the exception of the amnioserosa. Expression decreases as germ-band retraction proceeds and, by stage 13, *rca1* staining is restricted to the proliferating cells of the CNS. In summary, *rca1* is expressed in a dynamic

Figure 5. Predicted amino acid sequence and embryonic expression pattern of the *rca1* cDNA. (A) The *rca1* cDNA isolated from a 0- to 24-hr embryonic cDNA library encodes a single long open reading frame of 412 amino acids (heavy line). The cDNA also contains a 25-bp poly(A) tail (not shown). The P-element insertion site of the *rca1*^{P1} allele, 42 bp upstream of the initiating ATG, is indicated by an arrow. The position of the 282-bp in-frame deletion of the *rca1*¹ allele is indicated. (B) Predicted amino acid sequence encoded by the *rca1* cDNA. The *rca1*¹ allele results in a deletion of residues 285–378. Three consensus Cdc2 phosphorylation sites (S/T-P-x-K/R) (Marin et al. 1992) are underlined. (C–F) Embryos were stained with antisense *rca1* RNA probes to visualize the *rca1* expression pattern during embryogenesis. (C) Stage-2 embryo (1 hr after egg deposition) stains densely and uniformly. (D) In a stage-9 embryo, transcript is found primarily in the mesoderm (ms) and the anterior (am) and posterior (pm) midgut primordia. (E) In a stage-11 embryo, *rca1* RNA is found throughout the embryo, except within the amnioserosa (a). (F) By stage 13, *rca1* expression is restricted primarily to the proliferating cells of the CNS. Weaker staining is also apparent in the anterior (am) and posterior (pm) midgut whose cells are beginning to endoreplicate at this time. Anterior is to the right. Bar, 30 μ m.

pattern that corresponds to the pattern of mitotically proliferating cells. We have been unable to detect an *rca1* expression pattern in third instar imaginal discs with these antisense RNA probes. This may reflect a very low level of expression in this tissue.

Ectopic expression of *rca1* drives cells into S phase

The ability of *rca1* mutations to suppress entry into S phase in a *ruv* mutant background raised the possibility that overexpression of *rca1* would be sufficient to drive cells from G₁ into S phase. To assess this, *rca1* was expressed ectopically under the control of the heat-inducible *hsp-70* promoter (*hs-rca1*). Flies carrying *hs-rca1* developed normally when raised at room temperature. Heat-induced expression of *rca1* during the third larval instar resulted in a rough-eye adult phenotype. This was correlated with induction of many cells into S-phase in eye discs as assessed by BrdU-labeling 60 min after heat shock treatment. Labeled cells were seen throughout the eye disc, with the most dramatic increase in BrdU incorporation occurring just anterior to and within the MF (Fig. 6B). The location of a cell within the MF along the anterior/posterior axis reflects the length of time a cell has been in G₁. In preparations expressing heat-induced *rca1*, all cells regardless of the length of time spent in G₁ are driven into S phase. The pattern of S phases induced by *hs-rca1* was indistinguishable from that seen in response to *hs-cyclin A* (Fig. 6C). Incorporation of BrdU by cells driven from early G₁ into S was most robust. This may reflect the increased activ-

ity of Cyclin A/Cdk complexes attributable to the high levels of Stg within this domain. Indeed, mammalian Cyclin A/Cdk2 complexes have been shown to be activated in vitro by *Drosophila* Stg protein (Gu et al. 1992). In addition to cells in the MF, cells in more posterior regions of the disc that are normally quiescent were also BrdU-labeled.

To assess whether expression of *rca1* in late G₁ can drive cells destined to become postmitotic neurons into S phase, *rca1* was expressed under the control of the glass-responsive element, pGMR (*pGMR-rca1*). pGMR drives transgene expression in all cells commencing at the posterior edge of the MF (Ellis et al. 1993; Hay et al. 1994). Cells in this region are either postmitotic and initiating a program of differentiation or are proceeding through the G₁/S transition of the second mitotic wave. *pGMR-rca1* produced an adult rough-eye phenotype. In wild-type eye discs, islands of S-phase cells are regularly spaced between clusters of differentiating R cells just posterior to the MF. This can be seen clearly in preparations pulse-labeled with BrdU and double-stained with the monoclonal antibody directed toward Elav, a widely expressed neuronal nuclear protein (Robinow et al. 1988). BrdU-labeled nuclei are located basally, and the neuronal R cell nuclei apically; no double-labeled cells were detected (Fig. 7A,B). In *pGMR-rca1* eye discs, most cells just posterior to the MF enter S phase, including the normally postmitotic differentiating neurons (Fig. 7C,D). Staining with acridine orange to detect dying cells revealed that ectopic expression by *pGMR-rca1* led to a marked increase in cell death in the most posterior re-

Figure 6. Ectopic expression of *rca1* drives cells into S phase. (A) BrdU-staining pattern of heat shocked wild-type discs. These are indistinguishable from untreated discs. (B) Heat shock *rca1* drives most cells within and just anterior to the MF into S phase. (C) Heat shock *cyclin A* is very similar to heat shock *rca1*. (D) In larvae heterozygous for *cyclin A*, heat shock *rca1* fails to induce ectopic S phases. Third instar larvae were subjected to a 60-min heat shock at 37°C, followed by a 60-min recovery at 25°C. Eye-antennal discs were dissected and pulse-labeled with BrdU for 30 min and stained with anti-BrdU antibody. The MF is indicated by an arrowhead in each panel. Anterior is to the right. Bar, 10 μ m.

gions of the disc (data not shown). Presumably, simultaneous progression through the cell cycle and differentiation is incompatible with cell viability. These data indicate that *rca1* is sufficient to drive postmitotic cells into S phase.

rca1 regulates cyclin A

Both heat shock *rca1* and heat shock *cyclin A* induce G_1 cells in the MF into S phase (Fig. 6B,C). This is consistent with the notion that Rca1 functions with Cyclin A in a common pathway. Indeed, *cyclin A* dominantly suppressed entry into S phase induced by heat shock or pGMR *rca1* (Fig. 6D; data not shown). *rca1*, however, did not dominantly suppress heat shock *cyclin A* (data not shown). These data suggest that *rca1* may function upstream of *cyclin A*.

In support of *rca1* acting upstream of *cyclin A*, *hs-rca1* induced elevated Cyclin A protein levels as revealed by anti-Cyclin A staining of eye discs (Fig. 8). A marked increase was seen in the *stg* domain just anterior to the MF. Elevated levels also were observed within the MF and posterior to it (Fig. 8A,B). Increased levels of Cyclin A protein were observed posterior to the MF in pGMR-*rca1* discs (Fig. 8C) consistent with the pattern of pGMR

expression. An examination of *cyclin A* mRNA levels in a parallel experiments revealed no significant increase in *cyclin A* transcription anterior to and within the MF in response to heat shock *rca1* (Fig. 8D,E). These data support a model in which *rca1* positively regulates Cyclin A by modulating Cyclin A protein levels (see Discussion).

Discussion

We have utilized the developing *Drosophila* eye as a model in which to study cell cycle regulation. We demonstrated that *rux* is a suitable genetic background for identifying new genes required for meiotic and/or mitotic cell cycles. In *rux* mutants, precocious activation of a Cyclin A/Cdk complex during a mitotic cell cycle leads to early entry into S phase (Thomas et al. 1994), whereas failure to inhibit Cyclin A/Cdk during spermatogenesis leads to an additional round of chromosome segregation following meiosis II (Gönczy et al. 1994). Suppressors that rescue only the *rux* eye phenotype include genes encoding proteins that are limiting for S-phase entry; *S(rux)2B* and *S(rux)2C* mutations in a wild-type background inhibit S-phase entry posterior to the MF. Nearly half of the suppressors identified also rescue the male meiotic defect. These genes are likely to suppress the eye phenotype by regulating Cyclin A/Cdk activity. Indeed, as shown in this paper, one of these, *rca1*, is a novel cell cycle gene regulating Cyclin A.

Genetic studies argue that *rux* antagonizes the function of *cyclin A*: (1) *cyclin A* is a dominant suppressor of *rux*; (2) *hs-cyclin A* drives cells in the MF into S phase (this is similar to the *rux* loss-of-function phenotype); (3) *hs-rux* prevents induction of S phase by *hs-cyclin A* (B.J. Thomas and S.L. Zipursky, in prep.); and (4) histological analysis revealed that *rux* regulates Cyclin A protein levels. In *rux* mutants, Cyclin A protein but not mRNA levels are elevated in early G_1 at the anterior edge of the MF (Thomas et al. 1994). Similarly, during male meiosis Cyclin A protein levels are markedly elevated in the cytoplasm of premeiotic G_2 cells in *rux* mutant testis (Gönczy et al. 1994). Conversely, overexpression of Rux in cells posterior to the MF results in a decrease in the number of Cyclin A-positive cells, the abolishment of cytoplasmic Cyclin A, and a transient accumulation of Cyclin A in the nucleus, with no observed effect on Cyclin A mRNA (B.J. Thomas and S.L. Zipursky, in prep.). These data suggest that Rux functions to destabilize Cyclin A protein, perhaps by targeting it for destruction in the nucleus.

That control of Cyclin A is crucial for G_1 regulation in the MF is underscored by the analysis of *rca1*. Ectopic expression of *rca1* within the MF is sufficient to drive cells into S phase, mimicking the effect of ectopic expression of *cyclin A*. Genetic and molecular epistasis studies argue that *rca1* lies upstream of *cyclin A*; *cyclin A* loss-of-function mutations dominantly suppress ectopic S phases induced by heat shock- or pGMR-driven expression of *rca1*. Loss-of-function *rca1* mutations, however, do not suppress S phases induced by heat shock *cyclin A*. In addition, the embryonic cell cycle pheno-

Figure 7. *rca1* can drive differentiating neurons into S phase. Wild-type and *pGMR-rca1* discs were double-labeled for the neuronal marker Elav (orange) and for BrdU incorporation (black). *pGMR* drives *rca1* in all cells posterior to MF. (A,B) A wild-type disc. (A) In an apical focal plane, nuclei of differentiating clusters of R cells were observed. No double-labeled wild-type nuclei were detected. Arrowhead points to a five-cell precluster. (B) In a more basal focal plane, clusters of BrdU-positive nuclei of cells in S phase were seen. We attribute light BrdU staining seen occasionally in wild-type preclusters (A) to slight overlap in focal planes and to nonspecific background staining. (C,D) *pGMR-rca1* discs. Most cells just posterior to the MF were BrdU-labeled and found both apically (C) and basally (D). (C) Many Elav-positive cells also incorporate BrdU (e.g., five-cell precluster indicated by arrowhead). Arrows indicate the MF. Anterior is to the right. Bar, 5 μ m.

types of *rca1* and *cyclin A* are similar. Ectopic expression of *rca1* in the developing eye disc leads to an increase in Cyclin A protein, but not *cyclin A* mRNA levels. Although *rca1* mutants arrest in embryonic cell cycle 16, no differences in Cyclin A accumulation and degradation

were detected. Hence, the increase in Cyclin A protein levels in the MF in response to increased Rca1 is unlikely to reflect a direct role for Rca1 in regulating Cyclin A protein stability. In summary, these data suggest that Rux and Rca1 function antagonistically to limit Cyclin

Figure 8. Ectopic expression of *rca1* elevates Cyclin A protein levels. (A–C) Eye discs were stained with anti-Cyclin A antibody. (A) In wild-type discs, Cyclin A is expressed anterior to the MF and in cycling cells, posterior to the MF. Very faint, patchy staining is seen within the MF. (B) Heat-shock *rca1* leads to increased Cyclin A protein levels throughout the eye disc. A marked increase is seen at the anterior edge of the MF. (C) In *pGMR-rca1* eye discs, Cyclin A protein levels increased only posterior to the MF, corresponding to the region in which *rca1* is overexpressed. (D,E) In situ hybridization using antisense *cyclin A* RNA as a probe. (D) In wild-type discs, *cyclin A* mRNA signals are weak throughout the entire disc. A slight increase is seen just posterior to the MF. (E) In heat shock *rca1* discs, increased *cyclin A* mRNA levels were not detected anterior to or within the MF. Hence, the marked increase in Cyclin A protein levels in this region is not a result of an increase in *cyclin A* mRNA. (F) In situ hybridization of a wild-type eye disc using a sense *cyclin A* RNA probe as a control. At low magnification there is a marked difference in staining intensity between antisense and sense probes. There are no consistent differences in *cyclin A* mRNA staining between heat shock wild-type and heat shock *rca1* eye discs. Arrows indicate the MF. Anterior is to the right. Bar, 10 μ m.

A activity. Within the MF, a critical balance between these two competing activities and that of String prevents the ectopic activation of a Cyclin A/Cdk complex during G₁.

Taken together our data favor the following model of regulation of the G₂ cyclin, Cyclin A, for normal G₁ progression within the MF. Cyclin A accumulation is suppressed within the MF by Rux. In contrast, *rca1* promotes Cyclin A activity within this region. Cyclin E mRNA and then protein rises in cells destined to reenter the cell cycle toward the posterior edge of the MF. Cyclin E may facilitate the activity and/or the accumulation of Cyclin A in late G₁ by modulating the activities of Rux and/or Rca1. That Cyclin E may inactivate Rux function directly is suggested by the observation that Cyclin E binds to Rux in vitro and in yeast, and that Rux is phosphorylated by *Drosophila* Cyclin E/Cdk complexes (B.J. Thomas and S.L. Zipursky, in prep.). Such features of Cyclin A regulation in the MF are similar to the regulation of G₂ cyclins during G₁ in both budding and fission yeasts (e.g., Amon et al. 1994; Moreno and Nurse 1994; Schwob et al. 1994; Jallepalli and Kelly 1996; Schneider et al. 1996).

Although Cyclin A accumulates at the posterior edge of the MF, whether it is required for entry into S phase in a wild-type eye disc is not known. Several lines of evidence, however, argue for an important role of Cyclin A in controlling the G₁/S phase transition in mammalian somatic cells. Inhibition of Cyclin A, either through antisense technology or microinjection of neutralizing antibodies, prevents entry into and maintenance of S-phase progression (Girard et al. 1991; Pagano et al. 1992). Cyclin A/Cdk2 activity increases toward late G₁ and has been shown to activate DNA replication in extracts prepared from cells in G₁ (D'Urso et al. 1990). Recent studies by Reed and coworkers demonstrated that precocious expression of Cyclin A under the control of a tet-inducible promoter drives Rat-1 cells into S phase earlier than control cells (Resnitzky et al. 1995). However, in the eye disc, precocious entry of cells into S phase, under conditions where Cyclin A levels have been raised (i.e., *rux*, *hs-rca1*, or *hs-cyclin A*), may reflect a dominant gain-of-function wherein Cyclin A assumes the role of another G₁ cyclin such as Cyclin E or indirectly activates an endogenous G₁ Cyclin/Cdk by sequestering inhibitors. Further studies are necessary to determine the role of Cyclin A in regulating entry into S phase in the eye disc.

Surprisingly, neither *rux* nor *rca1* shows statistically significant similarities to any open reading frames in the now-complete sequence of the *Saccharomyces cerevisiae* genome. We propose that *rux* and *rca1* represent components of a novel biochemical pathway regulating Cyclin A/Cdk activity. Further genetic, cell biological, and biochemical analyses of additional *S(rux)* genes promise to provide insights into the mechanism of this regulation. Indeed, mutations in several other *S(rux)* complementation groups that suppress both the mitotic and meiotic *rux* defects also interact genetically with *rca1*, suggesting that we have identified multiple mem-

bers of this pathway (K.H. Zavitz and S.L. Zipursky, unpubl.). That higher eukaryotic cells have evolved additional biochemical mechanisms for controlling cell cycle progression is underscored by the central role the Rb protein plays in the mammalian cell cycle and its absence in yeast. Additional layers of regulatory complexity in higher eukaryotic cells may be necessary to coordinate cell cycle progression in diverse cell types during growth and development.

Materials and methods

Drosophila strains and genetics

Fly culture and crosses were performed according to standard procedures. Fly stocks were raised at 18°C or 25°C. Genetic markers and balancer chromosomes are described in Lindsley and Zimm (1992). Male flies (2–4 days old) isogenic for the second and third chromosomes (marked with *cn, bw; ri, e*) were mutagenized by feeding 25 mM ethyl methane sulfonate in a sucrose solution or by x-irradiation (4000 rads) as described (Ashburner 1989) and were mated to homozygous *rux*³ virgin females (*rux*³/*rux*³; +/+; +/+). Approximately 71,000 individual F1 male progeny reared at 25°C (*rux*³/Y; *cn, bw*/+; *ri, e*/+) were screened under the dissecting microscope for suppression of the *rux*³ rough-eye phenotype. Individuals exhibiting a suppressed eye phenotype were recrossed to *rux*³ virgin females, and their male progeny were examined to confirm the suppression. These males were used to map the *S(rux)* mutation to the second or third chromosome and then to balance the mutation. The chromosomes used for segregation analysis and balancing were *SM6A*, *In(2LR)GlaBcElp*, *CyO*, *P[w⁺ftz-lacZ]*, *TM3*, and *TM6B*.

Selected *S(rux)* mutations on the second chromosome were mapped by meiotic recombination with the multiply marked 'a-z' chromosome (*al, dp, b, pr, c, px, and sp*). Females heterozygous for *S(rux)*, *cn, bw/a-z* were crossed to homozygous *a-z* males, and individual male progeny were collected and scored for the *a-z* markers. These males were then crossed to *rux*³ females, and their male progeny were scored for the presence of the *S(rux)* mutation. This analysis yielded approximate map locations that guided further mapping by lethality to deficiency chromosomes. Similar mapping crosses were performed for *S(rux)* mutations on the third chromosome using the markers *th, st, cu, sr, e, c*. A number of P element-induced lethal mutations on the second and third chromosomes (Karpen and Spradling 1992; Torok et al. 1993) used for complementation analysis with the *S(rux)* mutations were provided by the Berkeley *Drosophila* Genome Project (BDGP). The BDGP mutant line *l(2)03300* (mapped to 27B4–27C1 by Todd Laverty, BDGP) corresponds to an allele of *rca1*, designated *rca1*^{P1}. Pseudopupul analysis of the dominantly suppressed *rux*³; *S(rux)*/+ flies was performed as described (Banerjee et al. 1987).

Histology and immunohistochemistry

BrdU pulse-labeling and detection with a mouse monoclonal antibody against BrdU (Becton Dickinson) was performed essentially as described (Truman and Bate 1988). Tissue in situ hybridization was done essentially as described previously (Tautz and Pfeifle 1989) using random prime-labeled digoxigenin DNA probes (Boehringer Mannheim) or RNA probes labeled by in vitro transcription using a digoxigenin RNA labeling kit (Boehringer Mannheim). The anti-Elav monoclonal antibody was a gift from G. Rubin (University of California, Berkeley). The anti-Cyclin A monoclonal antibody was a gift of P. O'Farrell

(University of California, San Francisco). The monoclonal antibody against β -tubulin was from Amersham. Embryos derived from the cross $rca1^{12}/CyO$, $P[w^+,ftz-lacZ] \times rca1^{13}/CyO$, $P[w^+,ftz-lacZ]$ were double-labeled with antitubulin and rabbit polyclonal anti- β -galactosidase antibodies (Cappel). $rca1$ mutant embryos ($rca1^{12}/rca1^{13}$) were identified by the absence of β -galactosidase staining. Secondary antibodies were rhodamine-conjugated goat antimouse IgG and fluorescein-conjugated goat antirabbit IgG (Jackson Labs). Fixation and immunostaining of whole-mount embryos was as described previously [Karr and Alberts 1986].

Molecular biology

Genomic DNA adjacent to the P-element insertion site of $rca1^{P1}$ was isolated by plasmid rescue and subsequently used as a probe to screen a *Drosophila* genomic library in λ EMBL3 [Tamkun et al. 1992]. Five independent clones covering ~17 kb of genomic DNA were isolated. A 9.2-kb genomic DNA fragment spanning the P-element insertion site was used as a probe to screen a 0- to 24-hr embryo cDNA library in λ EXLX(+) [Palazzo et al. 1990]. Approximately 100 positive clones were recovered from screening $\sim 1 \times 10^6$ cDNA recombinant λ phage, 16 of which were subjected to further analysis. All 16 cDNAs corresponded to a single transcription unit. The longest cDNA was 1.6 kb in length. Standard recombinant DNA techniques were as described [Sambrook et al. 1989]. The sequence of the $rca1$ cDNA inserted into pBlueScript KS(-) was determined on both strands primed with synthetic oligonucleotides using Sequenase (United States Biochemical). DNA and protein databases were searched for homologous sequences using the BLAST program [Altschul et al. 1990]. The GenBank accession no. for $rca1$ is U77645.

Transgenic rescue and expression

Germ-line transformation of *Drosophila* was performed using standard methods [Spradling and Rubin 1982]. A 10-kb *Xba*I fragment of genomic DNA surrounding the $rca1$ transcription unit was cloned into the transformation vector pCasper4. Three independent transformant lines were established and crossed to several $rca1$ mutants to assay rescue of lethality. The 1.6-kb $rca1$ cDNA was inserted into the pGMR and Casper-hs transformation vectors. Seventeen independent lines from each construct were generated. The hs-cyclin A line has been described previously [Lehner et al. 1991].

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