The cyclin-dependent kinase inhibitor Roughex is involved in mitotic exit in *Drosophila*

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Background: Exit from mitosis is a tightly regulated event. This process has been studied in greatest detail in budding yeast, where several activities have been identified that cooperate to downregulate activity of the cyclin-dependent kinase (CDK) Cdc28 and force an exit from mitosis. Cdc28 is inactivated through proteolysis of B-type cyclins by the multisubunit ubiquitin ligase termed the anaphase promoting complex/cyclosome (APC/C) and inhibition by the cyclin-dependent kinase inhibitor (CKI) Sic1. In contrast, the only mechanism known to be essential for CDK inactivation during mitosis in higher eukaryotes is cyclin destruction.

Results: We now present evidence that the *Drosophila* CKI Roughex (Rux) contributes to exit from mitosis. Observations of fixed and living embryos show that metaphase is significantly longer in *rux* mutants than in wild-type embryos. In addition, Rux overexpression is sufficient to drive cells experimentally arrested in metaphase into interphase. Furthermore, *rux* mutant embryos are impaired in their ability to overcome a transient metaphase arrest induced by expression of a stable cyclin A. Rux has numerous functional similarities with Sic1. While these proteins share no sequence similarity, we show that Sic1 inhibits mitotic Cdk1–cyclin complexes from *Drosophila* in vitro and in vivo.

Conclusions: Rux inhibits Cdk1–cyclin A kinase activity during metaphase, thereby contributing to exit from mitosis. To our knowledge, this is the first mitotic function ascribed to a CKI in a multicellular organism and indicates the existence of a novel regulatory mechanism for the metaphase to anaphase transition during development.

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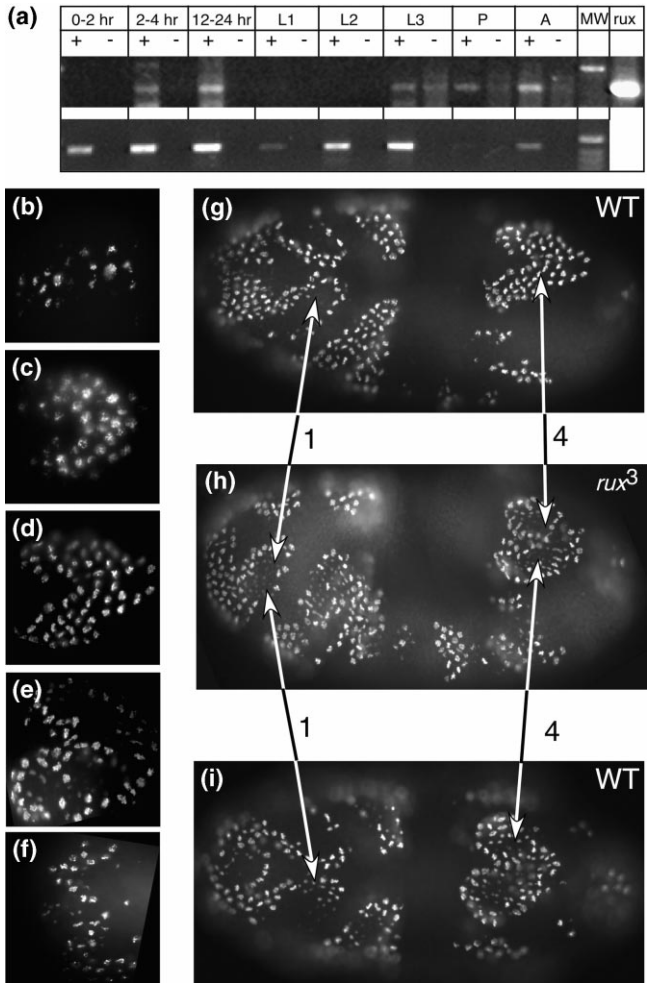
Background

Cell cycle progression is governed by the activity of a family of kinases, the cyclin-dependent kinases (CDKs) [1]. High levels of CDK activity are required for mitosis and DNA replication. Low levels are required for exit from mitosis and licensing of replication origins. Orderly progression through the cell cycle is essential for the development and survival of all metazoans. Faithful segregation of sister chromatids to two cells during mitosis is a crucial event in this process and aberrant mitoses are hallmarks of lethal conditions such as cancer [2]. Exit from mitosis is best understood in budding yeast [3]. Several distinct cellular activities act in a coordinated manner to downregulate the activity of the CDK–cyclin B complex Cdc28–Clb at the correct stage and induce an exit from mitosis. These activities include irreversible proteolytic destruction of cyclins by the ubiquitin ligase termed the anaphase promoting complex/cyclosome (APC/C) and inhibition of Cdc28–Clb activity by the cyclin-dependent kinase inhibitor (CKI) Sic1 [4]. During mitosis the phosphatase Cdc14 is released from the nucleolus in a Tem1-dependent manner and dephosphorylates Hct1, Swi5 and Sic1 [5, 6]. Unphosphorylated Hct1 activates the APC/C as an E3 ligase for Clb2 [7, 8]. Unphosphorylated Swi5 accumulates in the nucleus and transcriptionally activates Sic1 [9, 10]. Unphosphorylated Sic1 is considerably more stable than its phosphorylated isoform [11]. In concert with APC/C-mediated proteolysis of CLBs, Sic1 downregulates Cdc28 kinase activity during mitosis.

Studies in higher eukaryotes have demonstrated that irreversible proteolysis of mitotic cyclins has been conserved as a mechanism of downregulating Cdk1 activity during mitosis [12]. Proteolysis is also essential for exit from mitosis because expression of indestructible forms of cyclins arrests cells at distinct points of mitosis [13]. However, it has not been demonstrated that CKI activity also contributes to downregulation of Cdk1 during mitosis in higher eukaryotes.

Roughex (Rux) is a *Drosophila* CKI that inhibits the activity of mitotic CDK–cyclin complexes in vitro and in vivo [14]. *rux* mutants are male sterile and have rough eyes [15, 16]. Analysis of *rux* mutants showed that Rux is a *Drosophila* cell cycle regulator required to establish and maintain a G1 state during development [17, 18]. However, strong mutations in the gene are semi-lethal indicat-

Figure 1



Mitosis 14 is disturbed in *rux* mutants. **(a)** Developmental expression profile of *rux* using RT-PCR (upper panel). As a control, the expression profiles for the ribosomal gene *rp49* were analyzed (lower panel). *rux* transcript is absent in 0–2 hr embryos, indicating the absence of maternally provided transcript. *rux* is detected in older embryos (2–4 and 12–24 hr), larvae (L1, L2 and L3), pupae (P) and adult (A) flies (lanes marked '+'). As a control the same mRNA was amplified in PCR reactions without a preceding reverse transcription reaction (lane marked '–'). MW indicates molecular weight marker and in the lane 'rux' a PCR reaction was loaded in which the same primers were used with *rux* plasmid DNA to indicate the expected product length. **(b–i)** Mitosis 14 occurs in 25 different domains in a strict spatiotemporal pattern. Mitosis is visualized with an antibody that recognizes a phosphorylated form of histone H3 (PH3). (b–f) Progression through mitosis 14 for domain 4. The central cells are the first to enter mitosis (b), followed by the peripheral cells (c). All cells are in metaphase for a brief period (d), before the central cells exit mitosis, giving the domain a hollow appearance (e) followed by the peripheral cells (f). (g–i) The relative timing of mitosis for specific domains was compared for a wild-type and a mutant embryo. A wild-type (g) and rux^3 mutant embryo (h) were set such that domain 1 was hollowed out for both embryos (arrows '1'). In the wild-type embryos domain 4 is in a uniform metaphase, whereas many mutant cells have exited mitosis (arrows '4'). In a second comparison, a wild-type domain 4 (i) was set at the identical stage as a mutant domain 4 (h) (arrows '4'). In this case mitosis is further advanced in domain 1 of the wild-type than the mutant embryo (arrows '1'), indicating that the spatiotemporal pattern of mitotic domains is disturbed in *rux* mutants.

ing that the gene product has functions in addition to its roles in eye development and meiosis. Rux is a nuclear protein that interacts with mitotic cyclins [14]. Rux physically interacts with both mitotic cyclins A (CycA) and B (CycB) and overexpression of Rux causes an aberrant nuclear accumulation of these cyclins during interphase [14]. Rux inhibits the in vitro H1 kinase activity of Cdk1–CycA or Cdk1–CycB complexes [14]. Overexpression of Rux inhibits mitosis in embryos and induces cycles of endoreplication in proliferating imaginal discs [14, 17]. Rux does not inhibit Cdk2–CycE H1 kinase activity in vitro and overexpressing *rux* does not inhibit CycE-dependent progression through S phase, indicating that Rux is a CKI specific for mitotic cyclins [14, 17].

We now present evidence that, similar to Sic1 in yeast, Rux contributes to downregulation of Cdk1 activity during mitosis. Observations of fixed and living embryos revealed that metaphase length was doubled in *rux* mutants. Furthermore, overexpression of Rux was sufficient to induce an exit from mitosis in cells arrested in metaphase. *rux* mutants also had a considerably reduced ability to overcome a transient metaphase arrest induced by expression of an indestructible form of CycA in *Drosophila* embryos. We also demonstrate that Sic1 mimics Rux overexpression in embryos and inhibits *Drosophila* Cdk1, but not Cdk2. These data indicate that Rux and Sic1 are functionally related and raise the possibility that mechanisms of downregulating CDK activity during mitosis have been conserved during evolution and may exist in other metazoans.

Results

Progression through mitosis is disturbed in *rux* mutants

Endogenous *rux* is expressed at low levels and cannot be detected by *in situ* hybridization, so we used RT-PCR to determine the stages of *rux* expression. *rux* is zygotically expressed through most of *Drosophila* development (Figure 1a). Interestingly, *rux* is already expressed in 2–4 hr embryos, which are in the 14th and 15th cell cycles [19]. These are the first two cellular divisions during embryogenesis and occur without any G1 phase. S phases in cycles 14 and 15 immediately follow the preceding mitoses and a prolonged interphase is established during a G2 state [20]. Transition from G2 to mitosis is determined by the temporally controlled transcription of $cdc25^{stg}$, the *Drosophila* homologue of Cdc25. We analyzed *rux* mutants for cell cycle alterations to determine whether *rux* is required during the 14th cell cycle. Initially, we focused on rux^3 mutants. The wild-type Rux protein has 335 amino

acids and the *rux*^{3} allele carries a frameshift mutation that encodes a protein with 21 out of frame amino acids after amino acid 320 (data not shown). Although the *rux*^{3} mutants have rough eyes, they are not male sterile and can be maintained as a homozygous stock. We compared the pattern of mitosis 14 in *rux*^{3} mutant embryos and wild-type embryos. Mitosis 14 is the first zygotically controlled division and occurs in a spatiotemporal pattern of domains [21]. Additionally, cells in individual domains proceed through mitosis in a stereotyped sequence. Figure 1b–f shows this for domain 4. This domain starts as a thin wisp of cells, expands laterally and assumes defined contours. The central cells are the first to exit mitosis, followed by the peripheral cells.

Germband extension is a morphological process which occurs independent of cell cycle progression [22]. We therefore used the extent of germband extension as a marker for developmental stage. We did not find any differences in the timing of entry into mitosis between wild-type files and *rux*^{3} mutants for individual domains (data not shown). However, we noticed changes in the overall pattern of mitotic domains. To study this difference we compared the rate of progression through mitosis 14 for wild-type and *rux* mutant embryos by selecting embryos in which one domain appeared identical and compared the progression of the other domains (Figure 1g–i). When we chose embryos in which domain 4 was set at the same stage for wild-type (Figure 1i) and mutant embryos (Figure 1h) we observed that nearly all cells of domain 1 in wild-type embryos had completed mitosis and only a few telophase cells of the peripheral region of this domain were present. In contrast, domain 1 in *rux* mutants lagged behind with most central cells in telophase and all peripheral cells of this domain in metaphase. Similarly, when we compared embryos in which domain 1 appeared identical in wild-type and *rux* mutant embryos, we observed that mitosis lagged behind in mutant embryos when we compared other domains, such as domain 4 (Figure 1g,h). Thus, patterns of mitotic domains co-exist in *rux* mutants that are temporally separated in wild-type embryos, suggesting that individual mitotic domains persist longer in mutant embryos.

The effect described above is not specific to the *rux*^{3} allele and is not the result of a background mutation in the *rux*^{3} genotype. The same observations described in Figure 1 were made for *rux*^{2} mutants and a heteroallelic combination of *rux*^{8} and *rux*^{3}: *rux*^{8} is a null allele [16] and *rux*^{2} bears a mutation in the *rux* promoter region (data not shown). At least 10 embryos of the individual genotypes were compared with wild-type embryos of the same stage. Each mutant genotype showed a similar deviation from the wild-type pattern as that observed for *rux*^{3} (data not shown).

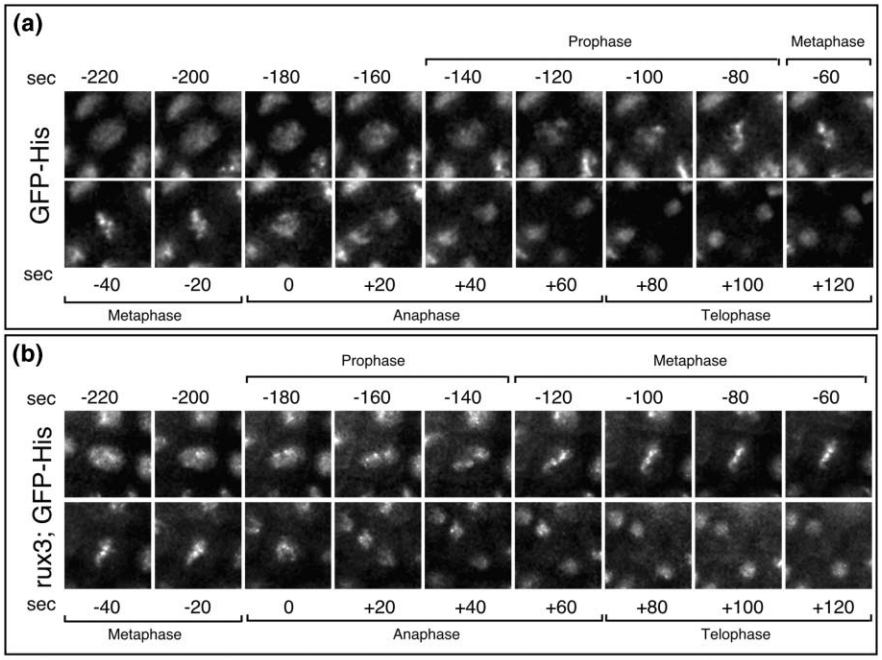
To analyze the basis of this phenotype, we followed mitoses in living wild-type and *rux*^{3} mutant embryos using time-lapse video microscopy where DNA had been marked with a green fluorescent protein (GFP)-tagged histone transgene (His2AvD) [23]. We compared mitosis 14 in single cells of domains 2 and 5 from 10 living wild-type and *rux*^{3} embryos to determine the duration of the individual phases of mitosis. A typical cell for each genotype is shown in Figure 2a,b. Sister chromatid separation was set as time point 0. We defined metaphase as the time of maximal DNA alignment on the metaphase plate. Cells in which chromosomes were observed outside the metaphase plate were still considered to be in prophase. The duration of prophase, metaphase, anaphase and telophase was determined for a representative population of cells for both genotypes (Figure 2c). We found that metaphase was on average 79% longer in *rux*^{3} mutants than wild-type embryos (Figure 2). The other stages of mitosis were not significantly altered in *rux* mutant embryos. An individual cell from domain 5 is depicted for a GFP–His2AvD embryo in Figure 2a and a *rux*^{3}; GFP–His2AvD embryo in Figure 2b. Whereas prophase, anaphase and telophase are the same length in both embryos, metaphase is twice as long in the *rux* mutant embryo (metaphase lasts ~60 s in the wild-type and 120 s in the mutant embryos).

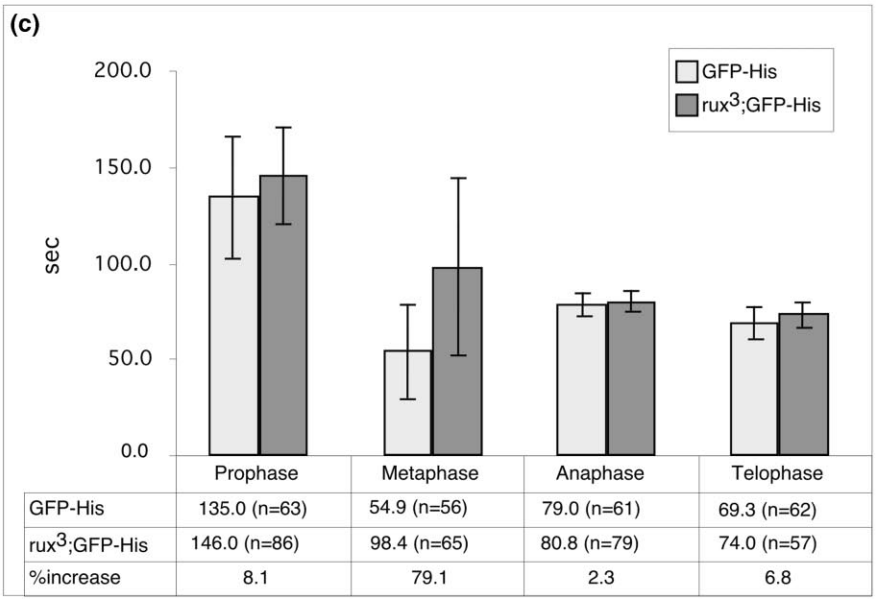
Therefore, *rux* mutant embryos appear to be compromised in their ability to execute the metaphase to anaphase transition. As Rux is a CKI that specifically inhibits Cdk1–CycA and interacts both genetically and physically with CycA, we infer from these data that Rux is required to inhibit Cdk1–CycA kinase activity during metaphase, thereby facilitating the transition to anaphase.

Rux overexpression is sufficient to induce an exit from mitosis

To test whether Rux can downregulate Cdk1–CycA activity during metaphase we expressed an indestructible form of CycA (CycAΔ170) in segmental stripes of the embryonic epidermis by crossing UAS–CycAΔ170 flies with prd–GAL4 flies (prd–GAL4 X UAS–CycAΔ170). This led to an accumulation of cells in metaphase during mitosis 15 in CycAΔ170-expressing stripes of the epidermis (Figure 3a,b). In a parallel experiment, we examined embryos that carried a heat-inducible *rux* transgene in addition to UAS–CycAΔ170 (prd–GAL4 X UAS–CycAΔ170; hs–rux). Overexpression of Rux does not induce a decrease in the levels of CycAΔ170 expressed from a second transgene [18]. Rux expression was induced by a 5 min heat pulse after cells had arrested in metaphase. Administration of a mild heat pulse resulted in *rux* expression throughout the embryo 10 min after induction (Figure 3c,d and E.F. and F.S., unpublished observations). Rux protein was detected 5 min later (data not shown) and after an additional 5 min we observed numerous cells that exited mitosis

Figure 2





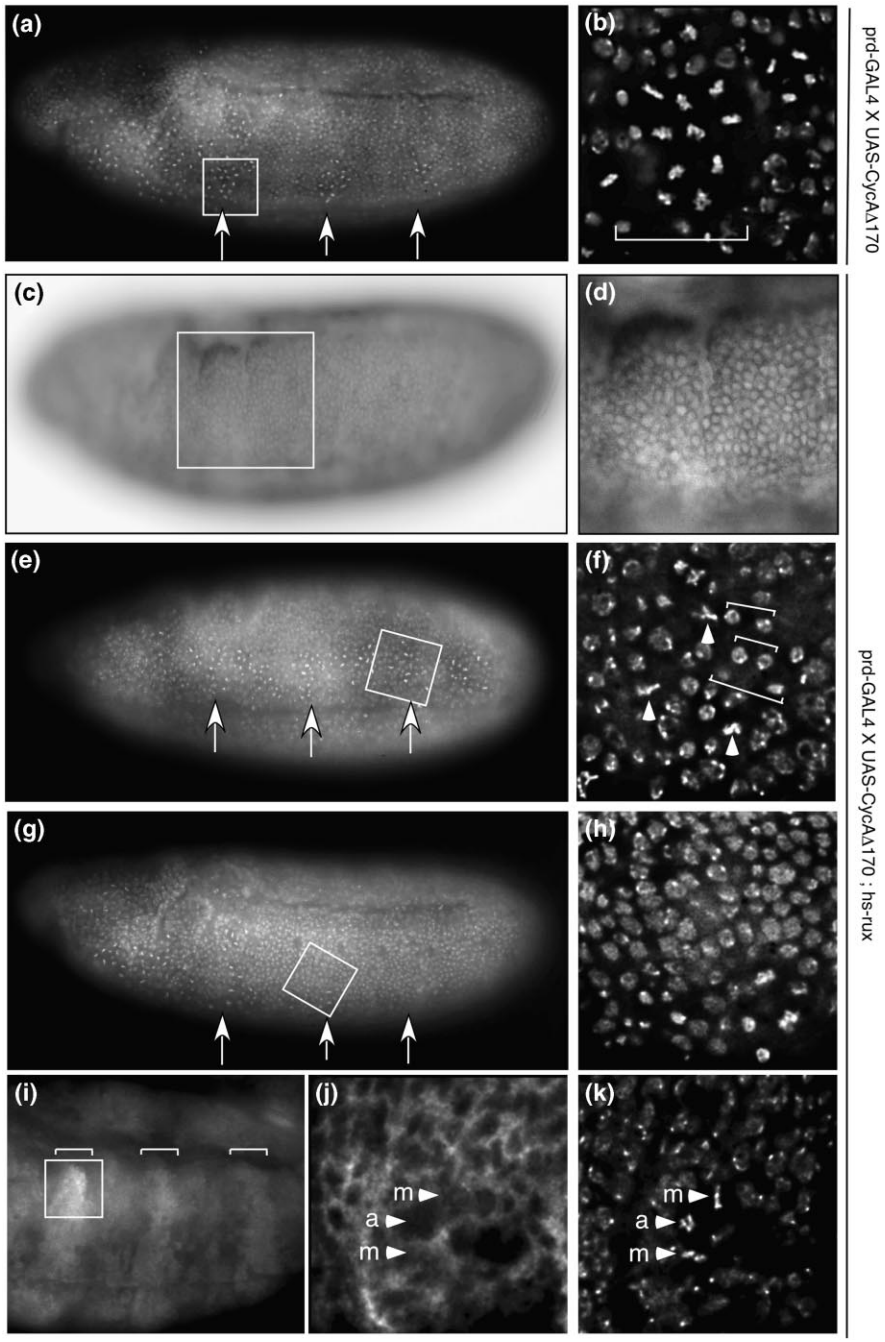
	Prophase	Metaphase	Anaphase	Telophase
GFP-His	135.0 (n=63)	54.9 (n=56)	79.0 (n=61)	69.3 (n=62)
rux3;GFP-His	146.0 (n=86)	98.4 (n=65)	80.8 (n=79)	74.0 (n=57)
%increase	8.1	79.1	2.3	6.8

Metaphase is prolonged in *rux* mutants. **(a,b)** The *rux*^{3} mutation was crossed into a His2AvD (GFP–His) background. Mitosis was followed in a population of living embryos by time-lapse fluorescence microscopy. Images were taken every 10 s. Frames of 20 s intervals from a single cell of domain 5 for the control parental His2AvD strain and the *rux*^{3}; His2AvD strain are presented in (a,b), respectively. The individual stages of mitosis are distinguishable at this magnification. Prophase, anaphase and telophase are of similar lengths in both cells. In contrast, metaphase is significantly longer in the mutant cell. **(c)** The same observations were performed for a representative sample of mutant and wild-type cells from domains 2 and 5. Whereas no significant change in prophase, anaphase and telophase was observed, metaphase was dramatically longer in the mutant strain. On average metaphase is increased by almost 80% in the *rux*^{3} mutant strain in comparison to the wild-type strain. Light gray columns represent the average time in sec for wild-type embryos and dark gray columns represent the average results for mutant embryos. Numbers in parentheses indicate the number of observations made for the individual mitotic stages for both genotypes. Times of the individual stages are given in sec.

in the UAS–CycAΔ170-expressing stripes. Concomitant with the reduction in the number of metaphase cells in these stripes we observed a number of cells in anaphase or telophase (Figure 3e,f). After 1 hr, all cells were in interphase (Figure 3g,h). Thus, Rux expression is sufficient to induce a mitotic exit in CycAΔ170-arrested cells.

Cyclins A, B and B3 are degraded in a sequential manner as cells progress through mitosis [13]. It is not known whether this sequential destruction is a prerequisite for the chromosome movements that accompany progression through mitosis. As we observed anaphase and telophase chromosome structures in prd–GAL4; UAS–CycAΔ170; hs–rux embryos, we examined these embryos for the presence of mitotic cyclins at the point Rux induced an exit from metaphase. Expression of Rux did not affect the levels of CycAΔ170 (Figure 3i), indicating that Rux induced an exit from mitosis independent of CycAΔ170 proteolysis. We also detected CycB protein in cells in which the metaphase to anaphase transition had not yet occurred (Figure 3j, arrowheads), indicating that destruction of endogenous cyclins had not proceeded to completion at the time when we expressed Rux and observed an exit from mitosis.

Figure 3

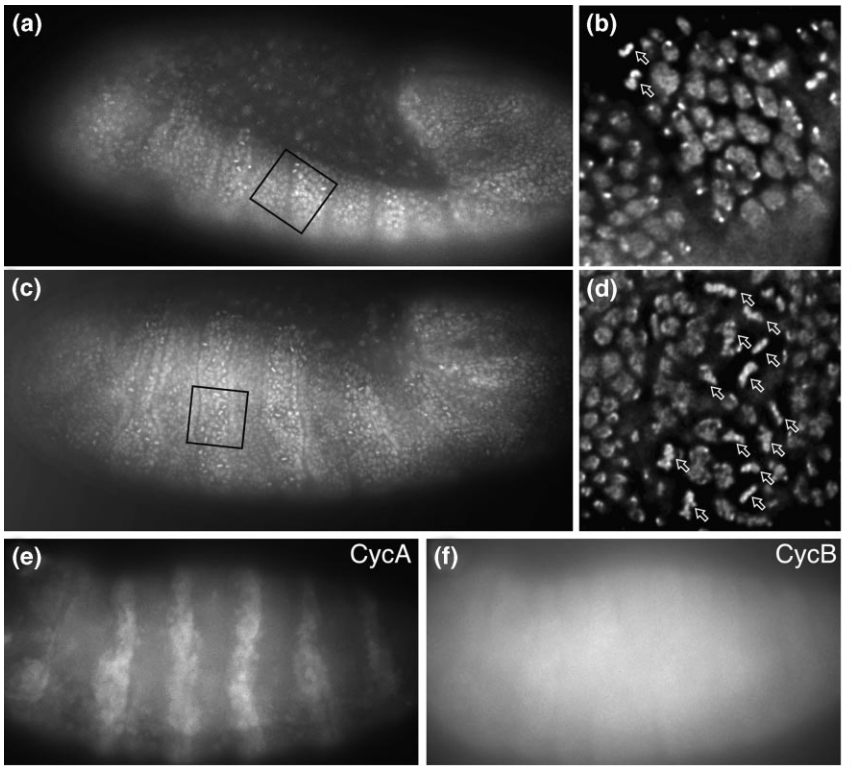


Rux expression is sufficient to induce an exit from mitosis. **(a,b)** A stabilized form of CycA (CycAΔ170) expressed from a UAS transgene (UAS–CycAΔ170) by the paired–GAL4 (prd–GAL4) driver line induces an accumulation of metaphase cells in stripes of the embryonic epidermis (a, arrowheads) during mitosis 15. The region boxed in (a) is magnified in (b). Embryos were exposed to a 5 min heat pulse and examined 20 min later (a,b), confirming that the heat pulse does not abrogate the mitotic arrest. DNA is visualized by staining with bisbenzidine. **(c,d)** A 5 min heat pulse is sufficient to induce expression from a hs–rux transgene throughout the embryo. prd–GAL4 X UAS–CycAΔ170; hs–rux embryos were fixed 10 min after a 5 min heat pulse and examined for *rux* expression by *in situ* hybridization. (c) *rux* transcript is present in the entire embryo including prd–GAL4-expressing cells. (d) A magnification of the boxed region of the embryo depicted in (c) is shown to visualize the expression levels in a prd–GAL4 stripe compared to an interstripe. **(e,f)** Cells exit mitosis 20 min after Rux induction (e). A magnification of a prd-expressing stripe (f) shows a greatly reduced number of metaphase cells (compare arrowheads in (f) with bracketed region in (b)). Furthermore, a number of cells in anaphase/telophase are distinguishable (f, brackets). **(g,h)** Nuclear density is equal throughout the embryo 1 hr after Rux induction (g) and almost all cells have decondensed interphase DNA (h). **(i–k)** Endogenous cyclins are not entirely degraded at the time Rux induces a metaphase exit. CycA is visualized in a prd–GAL4 X UAS-CycAΔ170; hs–rux embryo 20 min after Rux induction (i). A striped pattern of CycA expression is visible (i, brackets), confirming that the prd–GAL4-induced CycAΔ170 is not degraded in response to Rux. The anti-CycA antibody recognizes endogenous CycA 10 times more efficiently than CycAΔ170. The boxed region in (i) is magnified in (j,k). CycB is visualized with an anti-CycB antiserum (j) and DNA with bisbenzidine (k). Two metaphase and one early anaphase cells are indicated with 'm' and 'a' respectively. Endogenous CycB is still visible in the metaphase cells and is declining in the anaphase cells.

rux mutants show impaired ability to overcome a transient metaphase arrest induced by stable CycA

One of the initial pieces of evidence that demonstrated that Sic1 is involved in exit from mitosis in *Saccharomyces cerevisiae* was that low-level expression of a stable cyclin in yeast did not induce a permanent metaphase arrest [24]. The arrest was transitory as Sic1 eventually inhibited Cdc28 to an extent that cells exited mitosis. We examined prd–GAL4 X UAS–CycAΔ170 embryos at later developmental stages and observed that the metaphase arrest described in Figure 3 induced by expression of CycAΔ170 was transitory. The embryo depicted in Figure 4a is approx. 3.5 hr older (stage 12) than the embryo in Figure 3a (late stage 10). The DNA of most cells in prd–GAL4 X UAS–CycAΔ170 embryos of stage 12 was in a decondensed state (Figure 4a,b). The nuclear density of CycAΔ170-expressing stripes was half of that in interstripes, suggesting that cells expressing CycAΔ170 did not segregate

Figure 4



rux mutants are impaired in their ability to overcome a transient metaphase arrest induced by stable CycA. CycA was expressed in segmental stripes of the embryonic epidermis from a UAS transgene by the prd-GAL4 driver line. **(a,b)** The mitotic arrest observed in prd–GAL4 X UAS–CycAΔ170 embryos is only transitory. By developmental stage 12 most cells in prd–GAL4-expressing stripes have exited mitosis (a). The region boxed in (a) is magnified in (b). The DNA is decondensed and the nuclear density is reduced compared with the non-expressing cells. Very few cells are still in metaphase (arrows). **(c,d)** Rux contributes to exit from mitosis in CycAΔ170-expressing cells. *rux*^{3}; prd–GAL4 X *rux*^{3}; UAS–CycAΔ170 embryos were selected at a similar developmental stage (retracting germband) to the embryo in (a). In a *rux* mutant embryo of this stage (c) almost a half of all CycAΔ170-expressing cells remain arrested in metaphase (d, arrows), indicating that Rux contributed to exit from mitosis in prd–GAL4 X UAS–CycAΔ170 embryo (a). **(e,f)** CycB is absent in stage 12 embryos expressing CycAΔ170. A late stage 12 embryo expressing CycAΔ170 was stained for CycA (e) and CycB (f). At this stage endogenous CycB is degraded by Fzr, whose expression is developmentally regulated. Only background levels of CycB are detected (f), while CycAΔ170 is impervious to this degradation (e).

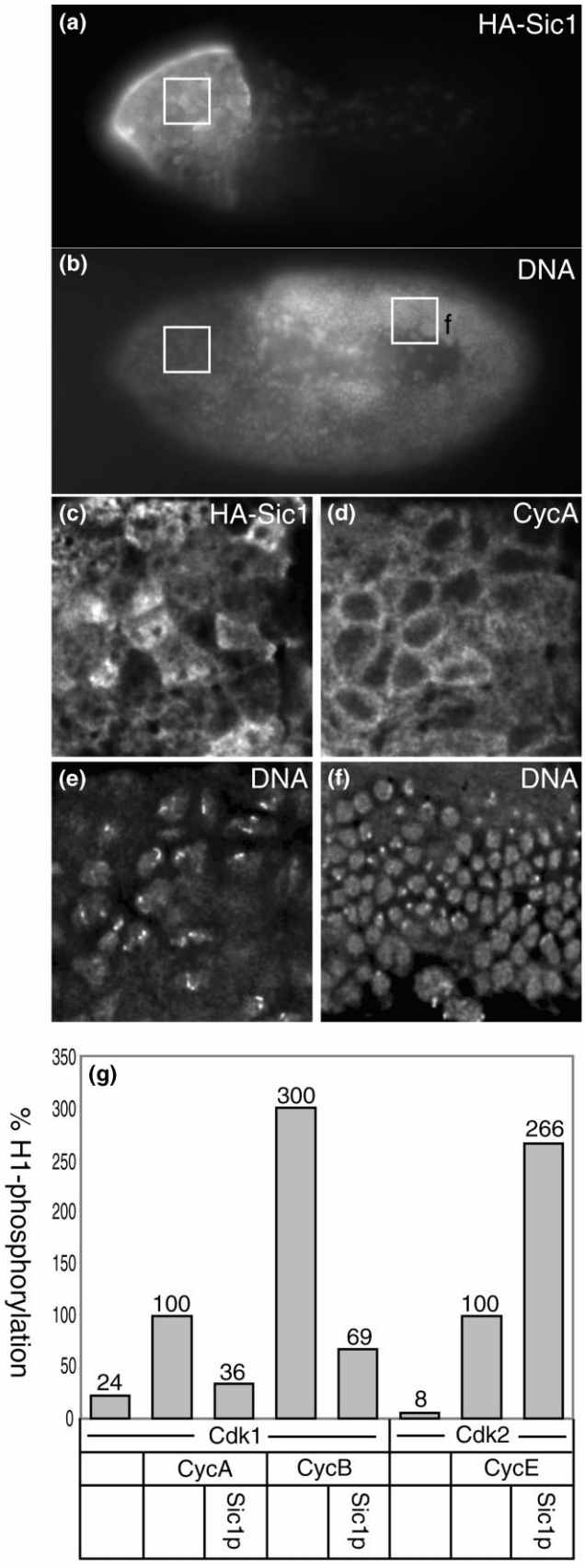
sister chromatids before entering interphase. When we examined *rux*^{3}; prd–GAL4 X *rux*^{3}; UAS–CycAΔ170 embryos of the same developmental stage (Figure 4c), we noticed that numerous cells expressing stable CycA in a *rux* mutant embryo remained trapped in metaphase (Figure 4d). Whereas almost all cells in the prd–GAL4 X UAS–CycAΔ170 embryo depicted in Figure 4b are in interphase, approximately half the cells in a *rux* mutant are still in metaphase (Figure 4c,d). Therefore, Rux is required to downregulate Cdk1–CycAΔ170 activity and allow a metaphase exit in these embryos.

In contrast to the Rux-induced metaphase exit described in Figure 3, the CycAΔ170 expressing cells in Figure 4 exited mitosis without segregation of sister chromatids. These cells exited mitosis at a much later stage than those described in Figure 3. We therefore considered the possibility that all endogenous CycB had been destroyed prior to metaphase exit in the embryos in Figure 4 and that the absence of CycB at the time of metaphase exit prevents anaphase movements. To address this question we examined CycB protein levels in stage 12 embryos expressing CycAΔ170. Whereas the CycAΔ170 protein persisted in metaphase-arrested cells of embryos of stage 12 (Figure 4e), we did not detect any CycB protein above background levels (Figure 4f). Endogenous CycB is degraded in embryos of this stage as a result of the developmentally controlled transcription of the APC/C component *fizzy-related* (*fzr*) [25]. In the absence of CycB protein cells apparently do not execute anaphase and instead decondense their chromosomes without segregation of sister chromatids.

Sic1 phenocopies Rux in *Drosophila* embryos

The data presented above suggest a role for Rux in mitotic exit. Progression through mitosis, as determined by the pattern of domains in mitosis 14 is prolonged in all *rux* mutants. Live observations revealed that the length of metaphase is almost doubled in *rux* mutants. In addition, *rux* expression induces an exit from a metaphase imposed by CycAΔ170 and *rux* mutants expressing CycAΔ170 are compromised in their ability to exit metaphase. Thus, Rux appears to perform a similar function in *Drosophila* to Sic1 in *S. cerevisiae*. There is no obvious sequence homology between Rux and Sic1. However, Sic1 and the CKI rum1 from *Schizosaccharomyces pombe* can functionally replace each other, even though the sequence similarity between the two proteins is minimal [26]. This raises the possibility that the sequence requirements for CKIs specific for mitotic cyclins are not very stringent, making it difficult to identify them on the basis of the primary amino acid sequence.

Figure 5



To test whether Sic1 can mimic Rux function in *Drosophila*, we transiently expressed a haemagglutinin (HA)-tagged *SIC1* construct in embryos by injecting mRNA into embryos immediately before cellularization. Embryos of this stage have completed S phase of cycle 14 and are in a prolonged G2 phase. Embryos were injected with HA–*SIC1* mRNA into the anterior end of the embryo and fixed 2 hr later. During this time, most cells normally complete the 14th cell cycle and are in interphase 15. As a control for the injection procedure, we injected an HA-tagged CycA construct (see Supplementary material, Figure S1). Cell cycle progression and development is not disturbed in the control injected embryos. The embryos injected with HA–*SIC1* were immunostained for HA-tagged *SIC1* and endogenous CycA (Figure 5d) and were also stained for DNA with bisbenzidine. *SIC1* expression is seen in the anterior part of the embryo (Figure 5a,c). Based on the extent of germband extension, anterior cells of this embryo would normally be in the 15th cell cycle (see Supplementary material, Figure S1). However, the nuclear density in the region of the embryo expressing *SIC1* is about half of that for the remainder of the embryo (Figure 5b,e,f). This finding is identical to the previously described HA–Rux mRNA injected embryos [14] and indicates that *SIC1*-expressing cells failed to progress through mitosis 14. The DNA in the anterior region is in a

Sic1 has functional similarities to Rux. **(a–f)** Sic1 inhibits cell cycle progression in *Drosophila*. HA–*SIC1* mRNA was injected into the anterior of pre-blastoderm embryos and the protein visualized 2 hr later by indirect immunofluorescence with anti-HA antibodies (a). DNA was visualized by staining with bisbenzidine (b). Higher magnification of the boxed anterior region in (b) confirms that Sic1 is present throughout the cell and in some cells slightly enhanced in the nucleus (c). CycA is mainly cytoplasmic in the same cells (d). The nuclear density in the HA–*SIC1*-expressing part of the embryo, visualized by DNA staining in (e, boxed anterior region in b), is greatly reduced compared with a region without HA–*SIC1* expression (f, boxed posterior region in b). In addition, DNA is decondensed in all *SIC1*-expressing cells, indicating that *SIC1* expression induces a G2 arrest in *Drosophila* embryos. **(g)** Sic1 inhibits the in vitro kinase activity of *Drosophila* Cdk1. HA–Cdk1, HA–Cdk2, CycA, CycB, CycE and Sic1 were translated and co-incubated with embryonic extract as a source of CAK. Cdk1–cyclin complexes were immunoprecipitated with anti-HA antibodies and assayed for their ability to phosphorylate histone H1. Cdk1 alone is a relatively inert kinase (g, column 1) that is activated by association with CycA or CycB (g, columns 2 and 4 respectively). Sic1 reduces the kinase activity of Cdk1/CycA and Cdk1/cycB by ~66% (g, columns 3 and 5 respectively). In contrast, Sic1 does not inhibit Cdk2–CycE. Cdk2–CycE is greater than 2.5 times more active after incubation with Sic1 than Cdk2–CycE alone (columns 8 and 7 respectively). The results presented in (g) are averages of three separate experiments. The kinase activity of Cdk1–CycA was set to 100%. Numbers above columns indicate kinase activity in percentage. Column 1: 5 μl Cdk1 alone. Column 2: 5 μl Cdk1 + 20 μl CycA. Column 3: 5 μl Cdk1 + 20 μl CycA + 30 μl Sic1. Column 4: 5 μl Cdk1 + 20 μl CycB. Column 5: 5 μl Cdk1 + 20 μl CycB + 30 μl Sic1. Column 6: 5 μl Cdk2. Column 7: 5 μl Cdk2 + 20 μl CycE. Column 8: 5 μl Cdk2 + 20 μl CycE + 30 μl Sic1. Each reaction was made up to a final volume of 55 μl with mock-translated reticulocyte lysate where necessary.

decondensed, interphase state and since *SIC1* expression initiated after completion of S-phase of cycle 14, these cells were in G2. In summary, these data indicate that the overexpression of Sic1, like Rux, induces a G2 arrest.

We also followed the effects of Sic1 on the kinase activity of *Drosophila* Cdk1–CycA and Cdk1–CycB complexes. ^{35}S-Met labeled, in vitro translated Sic1 was incubated with ^{35}S-Met labeled HA–Cdk1, CycA or CycB, and a crude 0–1 hr embryonic extract as a source of CDK activating kinase (CAK). Cdk1–cyclin complexes were immunoprecipitated with anti-HA antibodies and assayed for their ability to incorporate radiolabeled phosphate into the in vitro substrate histone H1. The same experiments were performed with HA–Cdk2 and CycE. We have previously demonstrated using this in vitro assay that Rux inhibits the kinase activity of Cdk1–CycA and Cdk1–CycB complexes, but not of Cdk2–CycE [14]. Figure 5g shows that Sic1 acts as an inhibitor of *Drosophila* Cdk1 kinase activity. The kinase activities of Cdk1–CycA or Cdk1–CycB were reduced by ~66% upon co-incubation with Sic1 (Figure 5g). In contrast, Cdk2–CycE was not inhibited but activated by Sic1 (Figure 5g). We have no explanation for the activation of Cdk2–CycE at present. However, this experiment demonstrates that, like Rux, Sic1 specifically inhibits mitotic cyclins and does not inhibit Cdk2–CycE.

Discussion

Our understanding of mitotic exit in yeast has advanced rapidly in the last decade and a picture of multiple intrinsic cellular activities controlling the process has emerged. The only aspect known so far to be conserved in multicellular organisms is proteolytic destruction of cyclins. We believe that the more complex nature of metazoans necessitates an equal if not more rigorous regulation of exit from mitosis. We propose that Rux performs functions similar to Sic1 from *S. cerevisiae* and that Rux cooperates with other mechanisms to trigger exit from mitosis. Removal of Rux function does not abrogate the ability of a cell to leave mitosis; the process is delayed, however. This delay is specific to metaphase, although Rux can inhibit both Cdk1–CycA and Cdk1–CycB, at least in vitro. Apparently, Rux functions during mitosis mainly as a negative regulator of Cdk1–CycA, which must be downregulated to exit metaphase. It appears that Rux-dependent inhibition of CycB is not limiting for anaphase.

Rux is not transcribed during the first 2 hr of embryogenesis which corresponds to the period of nuclear divisions. These cell cycles are extremely rapid and aberrant divisions at this stage are not repaired; instead, the resulting nuclei are destroyed [27]. During the cellular cycles this option is no longer available, as cellular loss at such a critical developmental period is potentially deleterious to the entire organism. In a situation where a metaphase plate has correctly formed, it is advantageous to the cell to complete metaphase promptly and thereby ensure a faithful segregation of chromatids to two sister cells. Rux is transcribed during the cellular cycles and we propose that Rux functions during these cycles by contributing to Cdk1–CycA kinase inactivation.

The transition from metaphase to anaphase is tightly regulated: DNA must align properly on the metaphase plate and CycA-dependent kinase activity must be downregulated. DNA damage or incorrectly oriented spindles induce metaphase arrest [28, 29]. We did not detect misaligned chromosomes, a delay of entry into mitosis, abnormal spindles or lagging chromosomes in *rux* mutants (data not shown), suggesting that the metaphase delay is not caused by activation of a checkpoint. An additional requirement for the metaphase to anaphase transition is inactivation of Cdk1–CycA. In *Drosophila*, CycA function is required for metaphase execution and expression of an indestructible form of CycA prevents the metaphase to anaphase transition [13, 30]. Rux interacts genetically and physically with CycA and inhibits the in vitro kinase activity of Cdk1–CycA [14, 16]. Therefore, we believe that the most likely explanation of the results above is that Rux contributes to the inactivation of Cdk1–CycA during metaphase. In the absence of Rux function, CycA–Cdk1 activity is only downregulated by cyclin proteolysis and this leads to an extension of metaphase.

Rux is expressed at levels that are insufficient to completely inactivate Cdk1–CycA at the beginning of mitosis. However, CycA levels drop rapidly during metaphase after the initiation of cyclin proteolysis and even low levels of Rux become significant for the inhibition of the residual CycA–Cdk1 complexes and for the transition into anaphase. When we express higher levels of Rux, Cdk1 can be can be quickly inhibited in a manner independent of cyclin proteolysis and cells exit mitosis in a normal fashion (see Figure 3).

Overexpression of Rux in cells that had been arrested in metaphase by a stable form of CycA was sufficient to induce an exit from mitosis. Cells exited mitosis by proceeding though anaphase and telophase and segregating sister chromatids into two distinct cells. *rux* mutants expressing stable CycA were impaired in their ability to exit mitosis. When these cells eventually exited mitosis they did so without a separation of sister chromatids. We believe that the presence or absence of endogenous cyclins is the cause of the two different forms of mitotic exit. Expression of an indestructible cyclin does not inhibit destruction of endogenous cyclins [13]. However, Rux was induced in cells at a time when endogenous CycB was still present in the case of prd–GAL4; UAS–CycAΔ170; hs–rux embryos. We believe that this endogenous CycB then contributed to execution of anaphase. In the case of *rux*$^{-/-}$; prd–GAL4; UAS–CycAΔ170 mutants, cells exit

mitosis at a much later stage; ~3.5 hr later. At this point in development the APC/C component Fzr is active and all endogenous CycB is destroyed. In the absence of CycB cells exit metaphase without separation of sister chromatids. These observations also support a model in which sequential destruction of mitotic cyclins is a prerequisite for the chromosome movements that occur during mitosis.

The only other CKI known to perform a mitotic function is Sic1. Rux and Sic1 have many similarities. Both specifically inhibit mitotic cyclin–Cdk1 complexes. Sic1 interacts with cyclin molecules via a classic RXL motif ([31]; in single letter amino-acid code where X is any amino acid) and we have observed that Rux–CycA interactions also rely on an RXL motifs in Rux (E.F., unpublished observations and [32]). Both are non-essential because they cooperate with other mechanisms such as cyclin proteolysis. However, while Sic1 acts as a late step during mitosis, Rux is involved in the metaphase–anaphase transition. Both genes are then also required to establish a G1 phase. Sic1 is stable during G1 and is destroyed at the G1–S transition by the proteasome [11]. We have observed that Rux is also stable during G1 and is destroyed at the G1–S transition by the proteasome (E.F. and F.S., unpublished observations and [17]). The analogy between Sic1 and Rux is also strengthened by our data that Sic1 is able to inhibit mitotic cyclin–Cdk1 complexes from *Drosophila*, both in vivo and in vitro. As the *SIC1* and *rux* genes have evolved separately there appears to be an evolutionarily conserved advantage behind such gene products, raising the possibility that such CKIs remain to be discovered in other eukaryotes.

Conclusions

We have demonstrated that the *Drosophila* CKI Roughex plays a role in exit from mitosis. Inhibition by Rux cooperates with destruction of CycA to downregulate Cdk1–CycA activity during metaphase, thereby ensuring a rapid transition to anaphase. This is a novel mechanism of Cdk1 inactivation during mitosis in metazoans. We propose that this process is advantageous to organism survival, because prolonging metaphases in undamaged cells increases the possibility of disturbing segregation of sister chromatids. In this context it is noteworthy that *rux* mutants have a greatly reduced viability.

Materials and methods

Molecular biological techniques

Histone H1 kinase assays and the RNA injection protocol have been described previously [14]. For the RNA injection, embryos were aged until the beginning of cellularization became apparent. The RNA was then injected and deposited close to the periphery of the embryo to facilitate uptake of the RNA into the forming cells. Embryos were fixed 2 hr after injection, divitellinized by hand and immunostained with primary and fluorescently labeled secondary antibodies. DNA was visualized using bisbenzidine. For the RT-PCR total RNA was isolated from the different developmental stages using phenol as a grinding material. Polyadenylated mRNA was isolated using the QuickPrep Purification Kit from (Amersham).

Antibodies and microscopy

The primary antibodies against Rux, CycA, phosphorylated histone H3 (PH3) and HA and the secondary antibodies have been described previously [14,18,33]. The anti-CycA antibody recognizes endogenous CycA ten times more efficiently than CycAΔ170. Immunofluorescent pictures were taken on a Zeiss Axiovert 10 using a CCD-Camera (Photometrics). For high magnification pictures, z-stacks were obtained using a piezoelectric mover and deconvolved using the AutoDeBlur program (Autoquant). Images were assembled using Adobe Photoshop and Canvas (Deneba). For time-lapse video microscopy, images were taken every 10 s on the Zeiss Axiovert 10 microscope.

Drosophila techniques

The UAS–rux, hs–rux and prd–GAL4 stocks have been described previously [17,18] Heat shocks were performed by floating the embryos on thin apple juice agar plates on a 37°C water bath for 5 min.

Supplementary material

A figure showing that cell cycle progression is normal in control injected embryos is available with the electronic version of this article at http://current-biology.com/supmatin.htm.

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