

# Terminal mitoses require negative regulation of Fzr/Cdh1 by Cyclin A, preventing premature degradation of mitotic cyclins and String/Cdc25

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*Cyclin A* expression is only required for particular cell divisions during *Drosophila* embryogenesis. In the epidermis, *Cyclin A* is strictly required for progression through mitosis 16 in cells that become post-mitotic after this division. By contrast, *Cyclin A* is not absolutely required in epidermal cells that are developmentally programmed for continuation of cell cycle progression after mitosis 16. Our analyses suggest the following explanation for the special *Cyclin A* requirement during terminal division cycles. *Cyclin E* is known to be downregulated during terminal division cycles to allow a timely cell cycle exit after the final mitosis. Cyclin E is therefore no longer available before terminal mitoses to prevent premature Fizzy-related/Cdh1 activation. As a consequence, Cyclin A, which can also function as a negative regulator of Fizzy-related/Cdh1, becomes essential to provide this inhibition before terminal mitoses. In the absence of Cyclin A, premature Fizzy-related/Cdh1 activity results in the premature degradation of the Cdk1 activators Cyclin B and Cyclin B3, and apparently of String/Cdc25 phosphatase as well. Without these activators, entry into terminal mitoses is not possible. However, entry into terminal mitoses can be restored by the simultaneous expression of versions of Cyclin B and Cyclin B3 without destruction boxes, along with a Cdk1 mutant that escapes inhibitory phosphorylation on T14 and Y15. Moreover, terminal mitoses are also restored in *Cyclin A* mutants by either the elimination of *Fizzy-related/Cdh1* function or *Cyclin E* overexpression.

**KEY WORDS:** Cyclin A, Fzr/Cdh1, String/Cdc25, Mitosis, G1 phase, Cell proliferation arrest

## INTRODUCTION

Mitotic cyclins accumulate during the S and G2 phases of the cell cycle. Their C-terminal cyclin boxes mediate binding to cyclin-dependent kinase 1 (Cdk1). Their rapid degradation during late M and G1 phase depends on the D- and KEN-boxes in their N-terminal domains. These degradation signals are recognized by Fizzy/Cdc20 (Fzy) and Fizzy-related/Cdh1 (Fzr), which recruit the mitotic cyclins to the anaphase-promoting complex/cyclosome (APC/C) during M and G1, respectively. The ubiquitin ligase activity of the APC/C allows cyclin poly-ubiquitination and consequential proteolysis (for reviews, see Murray, 2004; Peters, 2002).

Metazoan species express three different types of mitotic cyclins: A, B and B3. The specific functions of these different cyclins are not understood in detail. The presence of single genes coding for either Cyclin A (*CycA*), Cyclin B (*CycB*) or Cyclin B3 (*CycB3*) has facilitated a genetic dissection of their functional specificity in *Drosophila melanogaster*. In this organism, development to the adult stage requires the zygotic function of *CycA*, but not of *CycB* or *CycB3* (Jacobs et al., 1998; Lehner and O'Farrell, 1989). Initial analysis of the embryonic cell proliferation program in *CycA* mutants revealed that epidermal cells fail to progress through the sixteenth round of mitosis (Lehner and O'Farrell, 1989; Knoblich and Lehner, 1993). Cyclin A is also required for mitosis 16 in the epidermis of *dup/Cdt1* mutant embryos, in which mitosis 16 is no longer dependent upon completion of the preceding S phase (Jacobs et al., 2001). The failure of mitosis 16 in *CycA* mutants therefore does not simply result from the activation of a DNA replication or damage checkpoint – a possibility suggested by evidence obtained

in vertebrate cells in which Cyclin A binds not only to Cdk1 but also to Cdk2, and provides crucial functions during S phase (Coverley et al., 2002; Machida et al., 2005; Pagano et al., 1992).

The accumulation of Cyclin B and Cyclin B3 during cycle 16, which also occurs in *CycA* mutants (Lehner and O'Farrell, 1990) (see below), complicates the explanation of why mitosis 16 in the epidermis requires Cyclin A. In *Xenopus* egg extracts, Cyclin B can trigger entry into mitosis in the absence of Cyclin A (Murray and Kirschner, 1989). Conversely, mitosis is clearly inhibited in cultured human cells after the microinjection of antibodies against cyclin A (Pagano et al., 1992). Cyclin A-Cdk1 complexes are thought to have special properties, important for starting up a positive-feedback loop that confers a switch-like behavior on the Cdk1 activation process (Clarke et al., 1992; Pomeroy et al., 2005). In this feedback loop, Cdk1 activity results in phosphorylation and suppression of the inhibitory Wee1 kinase, as well as in phosphorylation and activation of the String/Cdc25 phosphatase, which removes the inhibitory phosphate modifications from Cdk1. However, our analyses indicate that the Cyclin A requirement in *Drosophila* is not linked to this positive-feedback loop. Rather, it is linked to the fact that the sixteenth round of mitosis during embryogenesis is the last cell division for the great majority of the epidermal cells.

After mitosis 16, most epidermal cells enter a G1 phase and become mitotically quiescent (Edgar and O'Farrell, 1990; Knoblich et al., 1994). By contrast, all the previous embryonic divisions (mitoses 1–15) are followed by an immediate onset of S phase. The G1 phase after mitosis 16 is therefore the first G1 phase during development. Entry into this G1 phase is dependent upon a complete, developmentally controlled inactivation of Cyclin E-Cdk2 and Cyclin A-Cdk1, because both complexes can trigger entry into S phase (Knoblich et al., 1994; Sprenger et al., 1997). Cyclin E-Cdk2 inactivation results from transcriptional *CycE* downregulation and concomitant upregulation of *dacapo*, which encodes the single *Drosophila* CIP/KIP-type inhibitor specific for Cyclin E-Cdk2 (de

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Nooij et al., 1996; Knoblich et al., 1994; Lane et al., 1996). Cyclin A-Cdk1 inactivation is dependent on Fzr (Rap – FlyBase), which is also transcriptionally upregulated. Moreover, Fzr is activated as a consequence of Cyclin E-Cdk2 inactivation (Sigrist and Lehner, 1997). Importantly, this cell cycle exit program is initiated already during G2 of the final division cycle.

Although cycle 16 is the final division cycle for most epidermal cells, some defined regions do not activate the cell cycle exit program during cycle 16 (Knoblich et al., 1994). Instead, they maintain *CycE* expression, enter S phase immediately after mitosis 16 and complete an additional division cycle 17. In these regions, mitosis 16 is not fully inhibited in *CycA* mutants. Cyclin A is therefore especially important for terminal mitoses preceding G1 and cell cycle exit. We show that the downregulation of Cyclin E-Cdk2 before terminal divisions, in preparation for the imminent cell cycle exit, converts Cyclin A from a redundant into an indispensable, negative regulator of Fizzy-related/Cdh1, preventing premature degradation of the mitotic inducers String/Cdc25 and the mitotic cyclins. The significance of the basic cell cycle regulator Cyclin A therefore depends on the developmental context.

## MATERIALS AND METHODS

### Fly stocks

Most of the fly lines have been described previously: *CycA<sup>C8</sup>*, *CycA<sup>C8LRI</sup>*, *CycA<sup>neol14</sup>*, *CycA<sup>183</sup>* (Knoblich and Lehner, 1993; Lehner et al., 1991), *UAS-CycA* (Weiss et al., 1998), *CycE<sup>AR95</sup>* (Knoblich et al., 1994), *dap<sup>f</sup>* and *UAS-CycE* (Lane et al., 1996), *fzr<sup>je28</sup>* and *fzr<sup>G0326</sup>* (Jacobs et al., 2002), *UAS-fzr* (Sigrist and Lehner, 1997), *Hs-CycB*, *Hs-ΔCycB*, *Hs-CycB3* and *Hs-ΔCycB3* (Sigrist et al., 1995), *Hs-stg* (Edgar and O'Farrell, 1990), *Hs-Cdk1* (Stern et al., 1993), *Hs-Cdk1<sup>AF</sup>* (Sprenger et al., 1997) and *prd-GAL4* (Brand and Perrimon, 1993). *UAS-fzr<sup>psm</sup>* lines were generated by standard germ line transformation. The *CycA*, *CycE* and *fzr* alleles were kept over the blue balancer chromosomes *TM3*, *Sb<sup>1</sup> P{35UZ}2*, *CyO*, *P{ftz/lacB}E3* and *FM7i*, *P{ftz/lacC}YH1*, respectively. The characteristic *lacZ* expression patterns driven by the transgenes on these blue balancers allowed embryo genotype identification after anti-β-galactosidase immunolabeling. As all transgenes were established in a *w<sup>1</sup>* background, we used this strain for some control experiments.

### Construction of the *UAS-fzr<sup>psm</sup>* transgene

To generate *UAS-fzr<sup>psm</sup>* lines, we first changed the codons for the serines at positions 3, 15, 60, 121, 136, 148, 211, 307 and 437, and for the threonines at positions 30, 109 and 285 in a *fzr* cDNA into alanine codons using site-directed mutagenesis (QuikChange, Stratagene). Moreover, the codon for serine 349 was changed into a cysteine codon, which is found at the corresponding position in *Drosophila*, *Xenopus* and budding yeast Fzy/Cdc20. The mutated cDNA was subsequently inserted into the *EcoRI* and *BglIII* sites of pUAST (Brand and Perrimon, 1993). All mutations were verified by DNA sequence analysis.

### Bromodeoxyuridine pulse labeling and immunolabeling

Pulse labeling of embryos (6.5–7.5 hours after egg deposition) with 5'-bromodeoxyuridine (BrdU) for 20 minutes and immunolabeling with antibodies specific for BrdU (Becton-Dickinson), β-galactosidase (ICN/Cappel and Promega), α-tubulin (Sigma), phospho-histone H3 (Upstate Biotechnology), Cyclin E (Lane et al., 1996), Cyclin B and Cyclin B3 (Jacobs et al., 1998), and with the DNA stain Hoechst 33258, were carried out essentially as described previously (Lehner et al., 1991). For secondary antibodies, we used goat antibodies conjugated with Cy3 (Jackson ImmunoResearch) or Alexa Fluor 488 (Molecular Probes). Images were captured using cooled CCD cameras and processed using PhotoShop (Adobe).

### Temperature shifts

Heat shocks were applied as described previously (Sigrist et al., 1995). Eggs were collected for 1 hour and aged for 5.5 hours at 25°C before the application of a heat shock (30 minutes, 37°C). After a recovery period at 25°C of either 30 minutes (data not shown) or 120 minutes (Fig. 2), embryos were fixed.

### Immunoblotting

For determination of String/Cdc25 phosphatase levels in *fzr* mutant embryos, eggs were collected from fly stocks with either *fzr<sup>je28</sup>* or *fzr<sup>G0326</sup>* over the *FM7i*, *P{ftz/lacC}YH1* blue balancer chromosome for 2 hours and aged for 8 hours at 25°C. Hemi- or homozygous *fzr<sup>je28</sup>* and *fzr<sup>G0326</sup>* embryos were sorted from sibling blue balancer embryos after methanol fixation and anti-β-galactosidase immunolabeling, as described previously (Jacobs et al., 2002). Moreover, for control experiments, eggs were also collected from *w<sup>1</sup>* and *Hs-stg* fly stocks for 2 hours. After ageing for 8 hours at 25°C, collections were divided into two aliquots. One of these was exposed to a heat shock (15 minutes, 37°C) and allowed to recover (15 minutes, 25°C), whereas the other was kept at 25°C. Embryo extract preparation and immunoblotting was done as described (Knoblich et al., 1994). Antibodies against String (Edgar et al., 1994) and α-tubulin (Sigma) were used for detection.

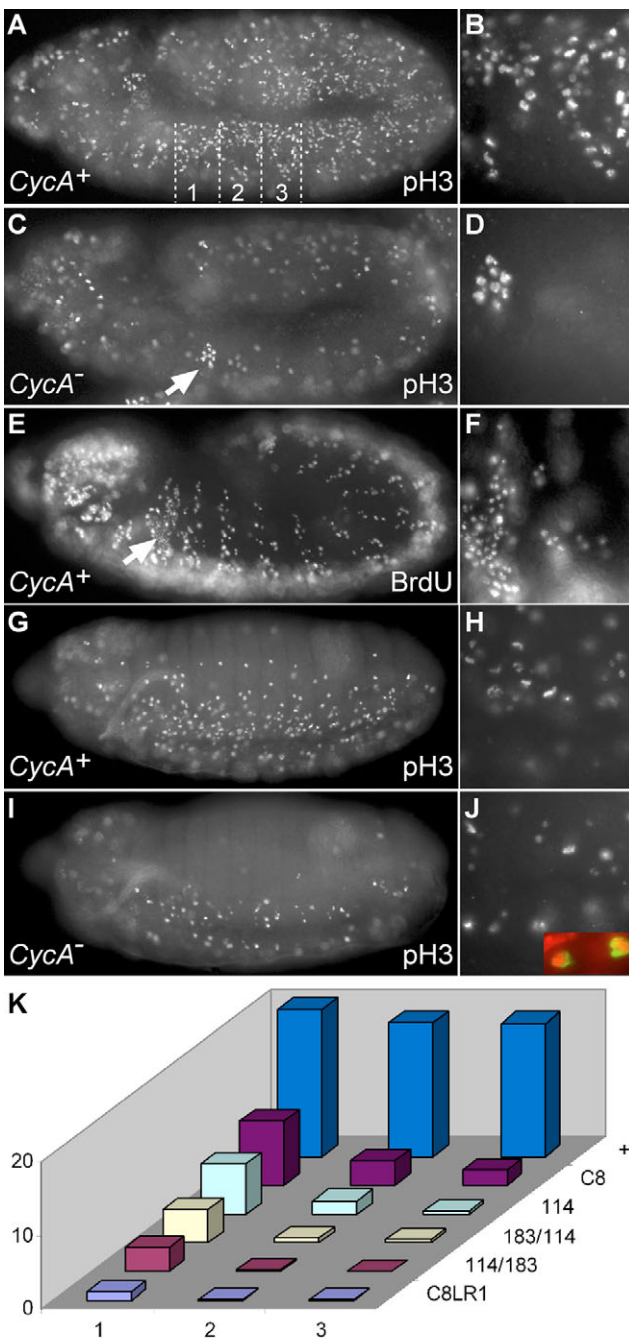
## RESULTS

### Cyclin A is predominantly required for terminal divisions

Our previous analysis of the cell proliferation program in *CycA* mutant embryos had indicated that most epidermal cells fail to undergo mitosis 16. However, a few epidermal cells still progressed into mitosis 16 in these mutants (Fig. 1) (see also Jacobs et al., 2001; Lehner et al., 1991). Interestingly, these mitotic cells were not distributed in a random pattern within the epidermis. Instead, they were found within defined regions where epidermal cells progress through a seventeenth division cycle instead of becoming quiescent after mitosis 16. The largest of these domains corresponds to the prospective anterior spiracle region situated mostly in the first, and to a minor extent in the second, thoracic segment. In wild-type embryos, this epidermal region is efficiently labeled during a BrdU pulse after mitosis 16, in contrast to the adjacent postmitotic epidermal cells (Fig. 1E,F) (see also Knoblich et al., 1994; Sauer et al., 1995). Quantitative analyses of mitotic cells at the stage of mitosis 16 in embryos with various strong or null alleles of *CycA* clearly revealed that 80% of the mitotic cells in the epidermis were confined to the prospective anterior spiracle region in the mutants (Fig. 1C,D,K). By contrast, mitotic cells were almost evenly distributed over the thoracic and abdominal segments, and were not confined to the prospective anterior spiracle region in *CycA<sup>+</sup>* control embryos (Fig. 1A,B,K). The pattern of mitotic cells in *CycA* mutants at the stage of mitosis 16 indicates therefore that zygotic *CycA<sup>+</sup>* expression is primarily required for entry into mitosis in those cells that enter G1/0 and exit from the cell cycle after mitosis 16. By contrast, mitosis is not absolutely dependent on zygotic *CycA<sup>+</sup>* function in cells that continue to proliferate after mitosis 16.

We emphasize that not all of the epidermal cells in the anterior spiracle region progress normally through mitosis 16 in *CycA* mutants. Previous analyses have clearly demonstrated that a fraction of these cells fail to divide and yet continue with an additional round of DNA replication (Sauer et al., 1995). At the stage of mitosis 16, therefore, the requirement for zygotic *CycA* expression is maximal for terminal mitoses and variable for non-terminal mitoses. Some of the latter mitoses appear to be normal, and only restricted aspects of mitosis like the re-licensing of DNA replication are realized in others.

The notion that Cyclin A is especially crucial for terminal divisions is further supported by observations in the nervous system. Lear et al. (Lear et al., 1999) have reported that the divisions of ganglion mother cells in various neuroblast lineages are preferentially inhibited in *CycA* mutants. Ganglion mother cell divisions are terminal mitoses. The resulting daughter cells exit from the cell cycle and differentiate into post-mitotic neurons. By



contrast, the asymmetric neuroblast divisions, which were found to be less inhibited in *CycA* mutants, are not terminal mitoses. The resulting daughter cells, a neuroblast and a ganglion mother cell, progress through additional divisions in the wild type. Consistent with these findings, mitotic cells (including normal anaphase figures) were readily observed in the nervous system of *CycA* mutant embryos during the later stages 12-14 (Fig. 1I,J). BrdU incorporation has previously been observed in the central nervous system of *CycA* mutants during these late stages (Lehner et al., 1991). We conclude, therefore, that at least some cells continue to progress through apparently normal division cycles in *CycA* mutant embryos, even at late stages, long after the depletion of the maternal contribution (Knoblich and Lehner, 1993) and long after the failure of the terminal mitosis 16 in the epidermis.

**Fig. 1. Cyclin A is preferentially required for terminal mitoses.** (A-J) Mitotic cells were labeled with antibodies against phospho-histone H3 (A-D,G-J; pH3) and  $\beta$ -galactosidase for genotyping (not shown). Mitotic cells are observed throughout the thoracic and abdominal epidermis in sibling control embryos at the stage of mitosis 16 (A,B; *CycA*<sup>+</sup>). By contrast, mitotic cells are restricted mainly to the prospective anterior spiracle region in the *CycA*<sup>C8LR1</sup> mutant epidermis (C,D; *CycA*<sup>-</sup>; white arrow). Cells in this restricted epidermal region are exceptional because they progress through an additional division cycle 17 after mitosis 16 during wild-type embryogenesis, as indicated by BrdU pulse labeling (BrdU) of control embryos at the stage of S phase 17 (E,F; white arrow), while essentially all the other epidermal cells exit from the mitotic cycle after mitosis 16. Mitotic divisions were also observed in the central nervous system of stage 14 *CycA*<sup>C8LR1</sup> embryos (I,J; *CycA*<sup>-</sup>). B,D,F,H,J present high magnification views from the embryos shown in A,C,E,G,I, respectively. Inset in J illustrates the presence of normal anaphase figures in late *CycA*<sup>C8LR1</sup> embryos, with DNA and anti-phospho-histone H3 staining shown in red and green, respectively. (K) Embryos at the stage of mitosis 16 were immunolabeled with anti-tubulin and anti- $\beta$ -galactosidase for genotyping. The number of mitotic cells in thoracic segments 1-3 (see 1, 2 and 3 separated by dashed lines in A) was counted in control (+) and *CycA* mutants carrying various alleles (C8, *CycA*<sup>C8</sup>, 114, *CycA*<sup>neo114</sup>, 183, *CycA*<sup>183</sup>, C8LR1, *CycA*<sup>C8LR1</sup>). Columns represent the average number of mitotic cells ( $n=10$  embryos).

### Premature disappearance of B-type cyclins and String/Cdc25 phosphatase before the terminal division in the epidermis of *CycA* mutant embryos

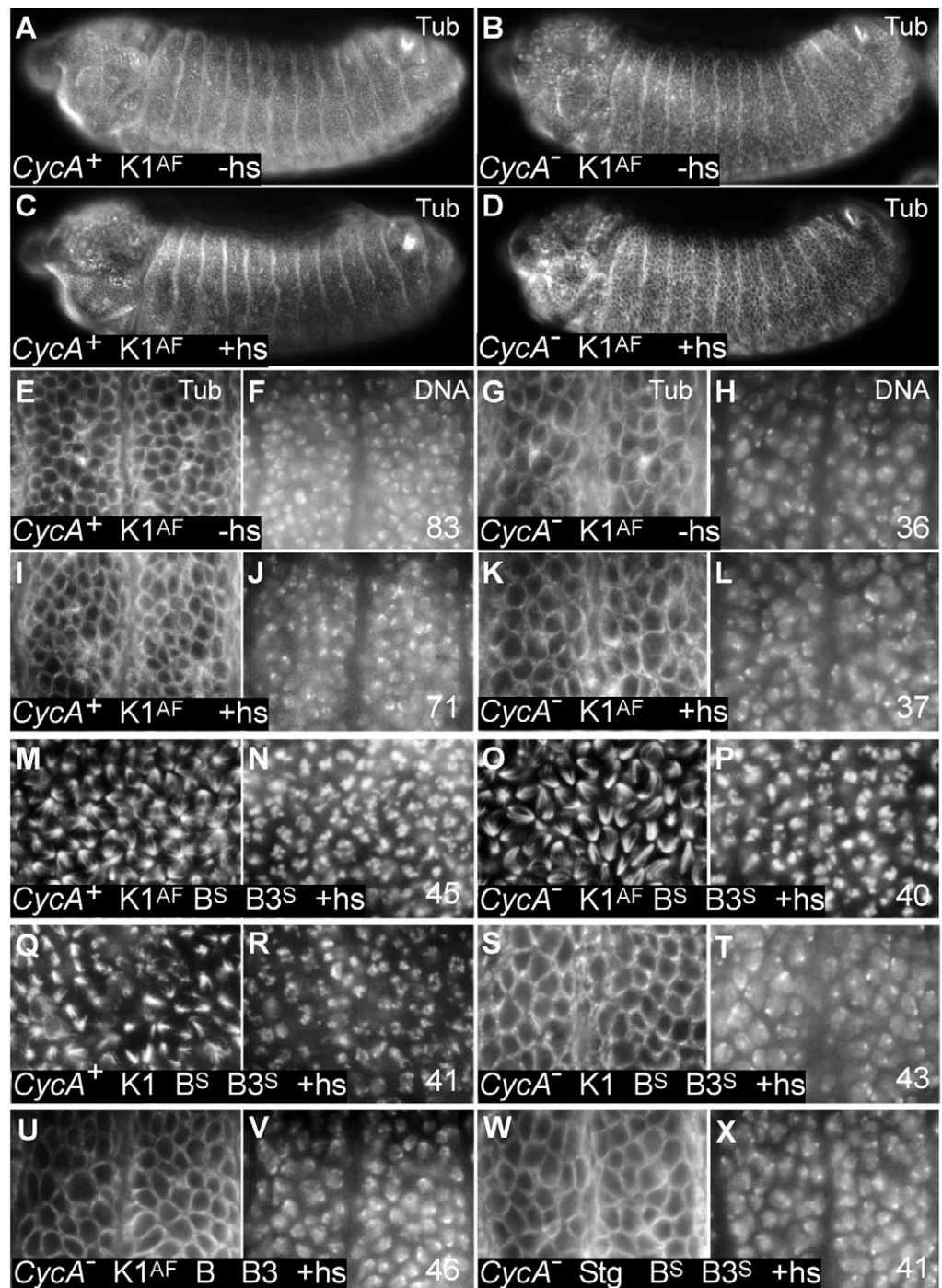
Why does entry into terminal epidermal mitoses depend on Cyclin A? When bound to Cyclin A, Cdk1 is not a substrate of the inhibitory Wee1/Myt1-like kinases in *Xenopus* extracts (Clarke et al., 1992). Cyclin A-Cdk1 activity might therefore start the positive Cdk1-activation loop, involving the inhibition of Wee1 kinase and activation of the Cdk1 activator String/Cdc25 phosphatase. Although Cyclin A-Cdk1 complexes in *Drosophila* embryos are subject to inhibitory phosphorylation (Sprenger et al., 1997), it is conceivable that the responsible kinases in this organism also work less efficiently on Cyclin A-Cdk1 complexes than on Cdk1 complexes containing B-type cyclins. Accordingly, epidermal cells of *CycA* mutant embryos might fail to enter mitosis 16 because of an inability to start up the positive Cdk1-activation loop. In this case, expression of a mutant Cdk1 (Cdk1<sup>AF</sup>) (Sprenger et al., 1997), which cannot be phosphorylated by Wee1/Myt1-like kinases, should restore entry into mitosis 16 in the epidermis of *CycA* mutants. For an experimental test, we crossed a transgene that allows the heat-inducible expression of Cdk1<sup>AF</sup> into *CycA* mutants. However, expression of this transgene did not restore mitosis 16 in the mutant epidermis (Fig. 2A-L). This observation also suggested that absence of String/Cdc25 activity is not the only cause for the failure of mitosis 16 in the mutants and raised the possibility that other Cdk1 activators might be lacking.

Therefore, we carefully re-evaluated the behavior of Cyclin B and Cyclin B3 in *CycA* mutants. Our previous analysis of *CycA* mutants had clearly demonstrated that Cyclin B re-accumulates in the embryonic epidermis after degradation during mitosis 15 and eventually disappeared again late in cycle 16 (Lehner and O'Farrell, 1990). The precise timing of this disappearance was difficult to assess with fixed *CycA* mutants because it occurs during a phase with little morphological change. In addition, mitotic divisions,



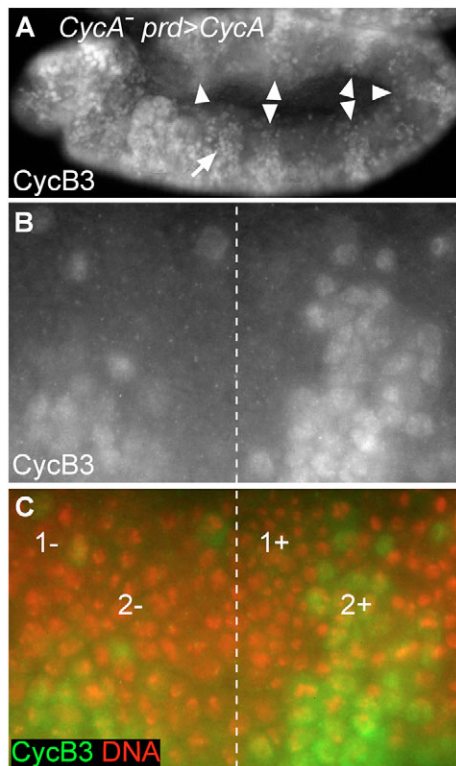
**Fig. 2. Restoration of terminal mitosis 16 in the epidermis of *CycA* mutant embryos requires the simultaneous expression of stabilized B-type cyclins and String/Cdc25-independent Cdk1.**

(A–X) Heat-inducible transgenes allowing expression of the String/Cdc25 phosphatase-independent Cdk1<sup>AF</sup> mutant (K1<sup>AF</sup>), wild-type Cdk1 (K1), String/Cdc25 (Stg), Cyclin B (B), Cyclin B3 (B3), or stabilized versions of these B-type cyclins lacking the degradation signals for APC/C-mediated proteolysis (B<sup>S</sup> and B3<sup>S</sup>) were crossed into the *CycA*<sup>CBLR1</sup> mutant background. Embryos before mitosis 16 were either exposed to a heat shock (+hs) or not (–hs). After ageing to stage 13, embryos were labeled with anti-tubulin (Tub), a DNA stain (DNA) and anti-β-galactosidase (not shown) for the identification of *CycA*<sup>CBLR1</sup> mutant embryos (*CycA*<sup>–</sup>) and sibling control embryos (*CycA*<sup>+</sup>). E–L present high magnification views from the embryos shown in A–D. Cell density in *CycA* mutants after failure of the terminal mitosis 16 is only half of that observed in control embryos (compare A,E,F with B,G,H) and is not increased by Cdk1<sup>AF</sup> expression (compare B,G,H with D,K,L). Co-expression of Cdk1<sup>AF</sup> and stabilized B-type cyclins, however, restores entry into the terminal mitosis 16 in *CycA* mutants and leads to an arrest in this mitosis in both control (M,N) and *CycA* mutants (O,P), as evidenced by the presence of spindles and condensed chromosomes. Co-expression of wild-type Cdk1 and stabilized B-type cyclins leads to an arrest during mitosis 16 in control embryos (Q,R), but does not restore mitosis 16 in *CycA* mutants (S,T). Cdk1 activators carrying degradation signals for APC/C-mediated proteolysis also fail to restore mitosis 16 in *CycA* mutants. Neither expression of a combination of Cdk1<sup>AF</sup> with wild-type B-type cyclins (U,V) nor expression of wild-type String/Cdc25 phosphatase with stabilized B-type cyclins (W,X) restores the epidermal cell density in the *CycA* mutants to normal. The numbers in lower right panel corners represent the cell counts in the regions of the right segments shown in E–X.



which normally allow for precise staging because of the dynamic but stereotypic nature of the division program during wild-type embryogenesis, do not accompany this disappearance of Cyclin B in *CycA* mutants. However, by crossing *prd-GAL4* and *UAS-CycA* into *CycA* mutants, embryos with segmentally alternating regions either with or without zygotic *CycA* expression can be generated (Jacobs et al., 2001). The *CycA*-expressing segments in these embryos progress normally through the wild-type division program.

By contrast, the intervening *CycA* mutant segments fail to progress through the terminal mitosis 16 in the epidermis. Immunolabeling of such embryos demonstrated that Cyclin B3 disappeared rapidly from the epidermal regions lacking Cyclin A just before the stage where entry into terminal mitosis 16 normally occurs in the neighboring *CycA*-expressing segments (Fig. 3). Immunolabeling with antibodies against Cyclin B revealed an analogous premature disappearance before terminal mitoses (data not shown) (Dienemann



**Fig. 3. Cyclin A prevents a premature disappearance of Cyclin B3 before mitosis 16.** (A-C) The *prd-Gal4* transgene was used to drive *UAS-CycA* expression in alternating segments of *CycA<sup>CBLR1</sup>* mutant embryos. Embryos were immunolabeled with anti-Cyclin B3 (CycB3, green in C) and a DNA stain (red in C). The *UAS-CycA* expressing segments (white arrowheads in A) contain more anti-CycB3-positive cells than do the segments without *UAS-CycA* expression. Among the segments without *UAS-CycA* expression, however, the one with most of the prospective anterior spiracle region (white arrow) has an exceptionally high number of CycB3-positive cells. The high magnification views in B and C indicate that mitosis 16 has already been completed in the anti-CycB3-negative regions in the segment with *UAS-CycA* expression to the right of the dashed white line. These regions (see for example cells around '1+') are characterized by a higher density of cells with smaller nuclei than in regions with CycB3-positive cells in G2 before mitosis 16 (around '2+'). The anti-CycB3-negative region '1-' in the segment without *UAS-CycA* expression to the left of the dashed line, which corresponds to region '1+', has a low cell density, reflecting the failure of mitosis 16. Importantly, in this segment without *UAS-CycA* expression, Cyclin B3 is not only absent from region '1-' but also from the region around '2-', indicating that in the absence of Cyclin A, Cyclin B3 disappears prematurely before mitosis 16, as the corresponding region '2+' in the segment with *UAS-CycA* expression still consists of anti-CycB3-positive cells in G2 before mitosis 16.

and Sprenger, 2004). Interestingly, premature disappearance was not observed within the prospective anterior spiracle region of *CycA* mutant embryos where mitosis 16 is not the terminal mitosis (Fig. 3A, arrow).

To determine whether the observed premature disappearance of Cyclin B and Cyclin B3 in the *CycA* mutant epidermis before terminal mitoses is responsible for the failure of these divisions, we crossed heat-inducible transgenes into the *CycA* mutant background, allowing the expression of either wild-type Cyclin B and Cyclin B3, or mutant versions lacking functional degradation signals. However, expression of these transgenes did not restore mitosis 16 (data not

shown), suggesting that at least one Cdk1 activator other than the mitotic A- and B-type cyclins might also become eliminated before terminal mitoses in *CycA* mutants.

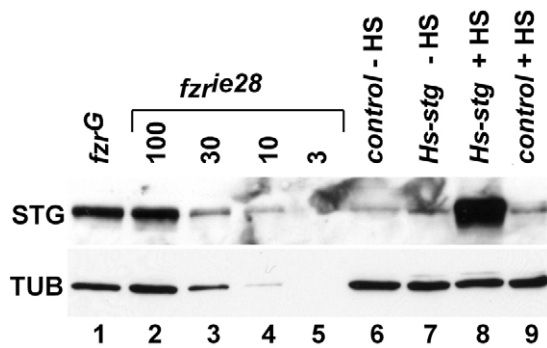
Therefore, we addressed whether String/Cdc25 phosphatase might disappear prematurely in *CycA* mutants before mitosis 16, as described above for Cyclin B and Cyclin B3. Previous observations have strongly suggested that String/Cdc25, which includes a KEN box, is also an APC/C substrate (Edgar and Datar, 1996; Edgar et al., 1994). If absence of Cyclin A was to cause a premature APC/C activation before terminal mitoses, String/Cdc25 might be eliminated along with the mitotic cyclins. Our comparison of String/Cdc25 levels in wild-type and *fzr* mutant embryos by immunoblotting experiments supported the idea that String/Cdc25 is an APC/C substrate (Fig. 4). In *fzr* mutants, String/Cdc25 levels were found to be elevated about tenfold. Unfortunately, the available antibodies against String/Cdc25 did not work in immunolabeling experiments with *CycA* mutant embryos expressing *prd-Gal4* and *UAS-CycA*. However, as an experimental test for the hypothesis that the failure of entry into the terminal mitosis 16 in the epidermis of *CycA* mutant embryos reflects the premature combined degradation of B-type cyclins and String/Cdc25, we simultaneously induced three heat-inducible transgenes in *CycA* mutant embryos, resulting in the expression of a String/Cdc25-independent Cdk1 (Cdk1<sup>AF</sup>) along with N-terminally truncated Cyclins B and B3 lacking destruction boxes. Expression of these three transgenes fully restored entry into mitosis 16 (Fig. 2M-P). After entry into mitosis, epidermal cells were unable to complete this division because of the presence of the non-degradable B-type cyclins, as expected (Sigrist et al., 1995).

Simultaneous overexpression from analogous heat-inducible transgenes of wild-type Cdk1 in combination with the two non-degradable B-type cyclins did not restore mitosis 16 in the epidermis of *CycA* mutants (Fig. 2Q-T). The same result was also obtained with combinations of non-degradable B-type cyclins and wild-type String/Cdc25 (Fig. 2U, V), or wild-type B-type cyclins and Cdk1<sup>AF</sup> (Fig. 2W, X). Finally, the combination of wild-type String/Cdc25 and Cyclin B and Cyclin B3 with destruction boxes did not restore mitosis 16 either (data not shown). These results indicate therefore that the failure of terminal mitosis 16 in *CycA* mutant embryos results from the combined loss of B-type cyclins and String/Cdc25. Moreover, the instability of these mitotic inducers before the terminal mitosis 16 in *CycA* mutant embryos appears to be so extensive that it cannot be overcome by the overexpression of wild-type Cdk1 activator proteins with functional degradation signals.

### Terminal mitosis 16 in *CycA* mutants is restored by the loss of *Fzr* or gain of Cyclin E

The loss of String/Cdc25 activity and B-type cyclins before the terminal mitosis in the epidermis of *CycA* mutant embryos might reflect a premature activation of *Fzr* activity. *Fzr* has been shown to be required for the degradation of mitotic cyclins during the G1 phase, when the epidermal cells exit from the division cycle after mitosis 16 (Sigrist and Lehner, 1997). The level of *fzr* transcripts is upregulated during wild-type embryogenesis when cells exit from the division cycle (Sigrist and Lehner, 1997). Moreover, in parallel with *fzr* upregulation, *CycE*, which acts as a negative regulator of *Fzr*, is downregulated (Knoblich et al., 1994). Because these changes are initiated already before mitosis 16, but only in epidermal cells programmed to exit from the cell cycle after mitosis 16, we investigated the possibility that the onset of this cell cycle exit program might create the higher Cyclin A requirement during terminal mitoses.





**Fig. 4. String/Cdc25 phosphatase levels are regulated by the APC/C activator Fizzy-related/Cdh1.** The levels of String/Cdc25 phosphatase in embryo extracts after the stage of mitosis 16 were analyzed by immunoblotting with antibodies against String (STG) and tubulin (TUB) to control for loading. Extract from 60 embryos was loaded in each of the lanes except for lanes 3-5, which contain a threefold dilution series of the extract loaded in lane 2. The embryos loaded in lanes 8 and 9 were exposed to a heat shock (+HS) before extract preparation. The following genotypes were analyzed: *fzr<sup>G0326</sup>* (lane 1, *fzr<sup>G</sup>*), *fzr<sup>ie28</sup>* (lanes 2-5, *fzr<sup>ie28</sup>*), *w<sup>1</sup>* (lane 6 and 9, control) and *Hs-stg* (lane 7 and 8, *Hs-stg*). Comparison of anti-STG signal intensities indicates that the levels of String/Cdc25 phosphatase are at least 10-fold higher in *fzr* mutant embryos.

To evaluate whether the premature loss of String/Cdc25 activity and B-type cyclins that is responsible for the failure of the terminal mitosis in the epidermis of *CycA* mutants is brought about by Fzr, we analyzed *fzr CycA* double mutant embryos. In these embryos, mitosis 16 was fully restored in the normal pattern (Fig. 5A-D) (Sigrist and Lehner, 1997) (and data not shown), and premature B-type cyclin degradation before mitosis 16 no longer occurred. Moreover, the epidermal cells in these double mutant embryos even progressed through an additional seventeenth division cycle accompanied by B-type cyclin accumulation and degradation during interphase and mitosis, respectively (Fig. 5G), as was also observed in *fzr* single mutant embryos (Fig. 5F) (Jacobs et al., 2002; Sigrist and Lehner, 1997). Although not all of the late mitotic figures in *fzr CycA* double mutant embryos were normal, most epidermal cells appeared to progress successfully through mitosis 17, as was also observed in the *fzr* single mutants (Fig. 5H,I). These results indicate that the Cyclin A requirement for mitosis is minimal in the absence of Fzr. Moreover, they suggest that a premature Fzr activation is responsible for the loss of String/Cdc25 and B-type cyclins that prevents entry into the terminal mitosis 16 in the epidermis of *CycA* mutants.

To evaluate whether developmentally programmed downregulation of endogenous *CycE* expression contributes to the failure of the terminal mitosis, we expressed *UAS-CycE* in *CycA* mutants using *prd-GAL4*. The *UAS-CycE* expression driven by *prd-GAL4* extends beyond the stage where the cell cycle exit program is initiated and endogenous *CycE* expression is downregulated (Knoblich et al., 1994). This extended *UAS-CycE* expression fully restored mitosis 16 in *CycA* mutant embryos (Fig. 6), suggesting that the downregulation of endogenous *CycE* expression indeed contributes to the arrest before the terminal mitosis observed in the epidermis of *CycA* mutants. As expected, we also no longer observed premature Cyclin B3 degradation in the *UAS-CycE*-expressing segments of the *CycA* mutants (Fig. 6A,B). *prd-GAL4*-driven *UAS-CycE* expression not only restored progression through

mitosis 16 in *CycA* mutants, but also promoted progression through an extra S phase 17 (data not shown), as was observed in wild type (Knoblich et al., 1994).

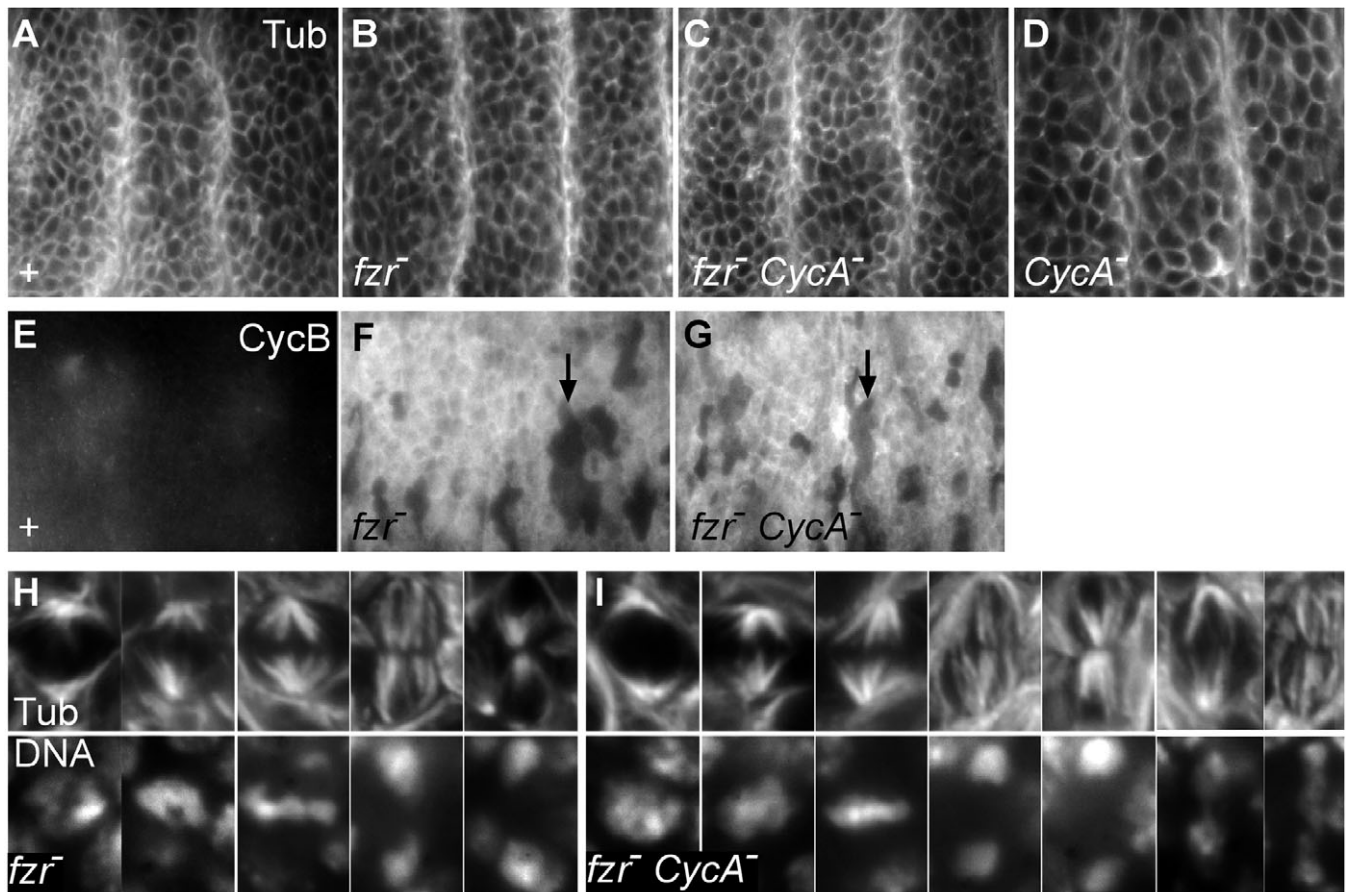
### Cyclin E and Cyclin A are both negative regulators of Fzr

Although the elimination of *fzr* function in *CycA* mutants restored the terminal mitosis 16, *fzr* overexpression is known to phenocopy *CycA* mutants. In segments with *prd-GAL4*-driven *UAS-fzr* expression, mitotic cyclins disappear prematurely just before the stage of mitosis 16, and this terminal division is inhibited (Fig. 7C,D) (Sigrist and Lehner, 1997). This premature *fzr* activation resulting from *UAS-fzr* overexpression is suppressed by *UAS-CycE* co-expression (Sigrist and Lehner, 1997). The premature *fzr* activation apparent in *CycA* mutants suggested that Cyclin A might also function as a negative Fzr regulator, like Cyclin E. In addition, a redundant ability of Cyclin A and E to suppress mitotic cyclin degradation during G2 has also been suggested by previous studies (Sprengrer et al., 1997; Widwans et al., 2002). Accordingly, co-expression of *UAS-CycA* should also suppress the *UAS-fzr* overexpression phenotype, as previously described for *UAS-CycE*. This was found to be the case (Fig. 7E,F).

In addition, an analysis of *CycA CycE* double mutant embryos also provided evidence consistent with the notion that Cyclin A and E might co-operate as negative Fzr regulators. In these double mutant embryos, cells were observed to arrest before mitosis 15 (Fig. 8), whereas this mitosis is not affected in single mutants. BrdU pulse labeling did not reveal abnormalities during the preceding S phase 15 in the double mutants (data not shown), arguing against the possibility that the arrest before mitosis 15 reflects the activation of a DNA replication check point. However, immunolabeling with antibodies against Cyclin B (data not shown) or Cyclin B3 (Fig. 8B) indicated that these mitotic cyclins had disappeared already around the stage of mitosis 15 in *CycA CycE* double mutants and thus even earlier than in *CycA* single mutants. Low levels of Fzr, mostly maternally derived, which are present during cycle 15 (Raff et al., 2002), therefore appear to become activated efficiently in *CycA CycE* double mutants. All of our observations therefore support the notion that both Cyclin A and Cyclin E act as negative Fzr regulators.

The *Drosophila* CIP/KIP-type Cdk inhibitor Dacapo specifically inhibits Cyclin E-Cdk2 (de Nooij et al., 1996; Lane et al., 1996). *dapcapo* (*dap*) expression is known to be upregulated during the final division cycle in the embryonic epidermis (de Nooij et al., 1996; Lane et al., 1996). Therefore, we analyzed whether genetic elimination of *dap* function restores progression through the final epidermal mitosis in *CycA* mutants. However, mitosis 16 was not restored in *dap, CycA* double mutants (Fig. 8N, data not shown). Interestingly, the epidermal cells in *dap, CycA* double mutants entered another S phase after the stage of mitosis 16 (Fig. 8N). It appears therefore that the inhibition of Cyclin E-Cdk2 activity after *dap* upregulation is not effective before mitosis 16. However, Dap is required thereafter to prevent Cyclin E-Cdk2 from inducing entry into S phase after origin re-licensing during exit from mitosis 16 in wild-type embryos (Fig. 8M) (de Nooij et al., 1996; Lane et al., 1996), or resulting from premature Fzr activation in *CycA* mutants (Fig. 8N).

In both budding yeast and human cells, phosphorylation of Fzr/Cdh1 by Cdks has an inhibitory effect (Kramer et al., 2000; Lukas et al., 1999; Sorensen et al., 2001; Zachariae et al., 1998). In these organisms, Cdh1 proteins with mutations in Cdk consensus phosphorylation sites were shown to be hyperactive and resistant



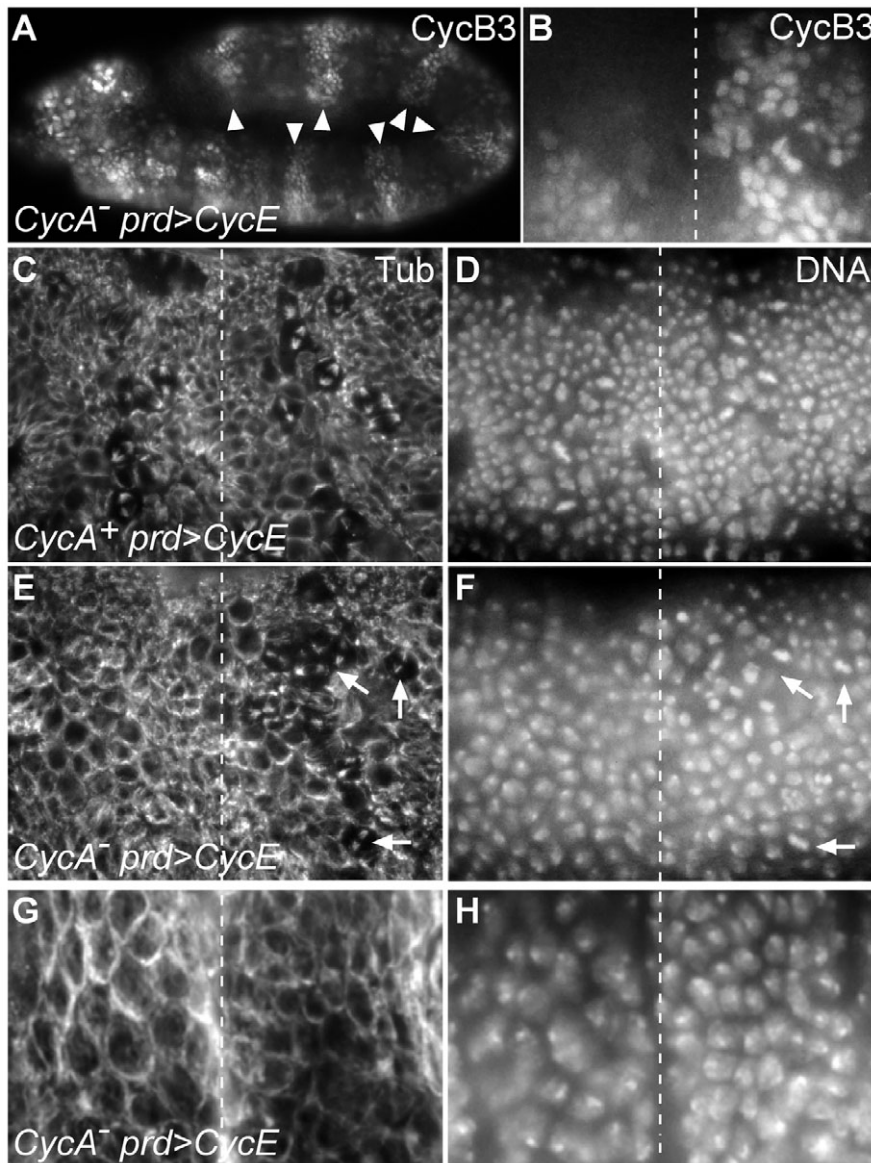
**Fig. 5. Elimination of zygotic *fizzy*-related expression restores progression through mitosis 16 and induces an extra division cycle in the epidermis of *CycA* mutants.** (A-D) High magnification views of the embryonic epidermis after anti-tubulin labeling (Tub) at stage 12 when epidermal cells are already post-mitotic in wild-type embryogenesis. The reduced cell density resulting from the failure of the terminal mitosis 16 in *CycA<sup>CBLR1</sup>* mutants (D, *CycA*<sup>-</sup>) is not present in *fzr<sup>ie28</sup>*; *CycA<sup>CBLR1</sup>* double mutants (C, *fzr<sup>ie28</sup> CycA*<sup>-</sup>), which have the same cell density as sibling control embryos (A, +) and *fzr<sup>ie28</sup>* single mutant embryos (B, *fzr*<sup>-</sup>). (E-G) High magnification views of the embryonic epidermis after anti-Cyclin B labeling (CycB) at stage 13. Although Cyclin B does not accumulate after mitosis 16 in the post-mitotic epidermal cells of sibling control embryos (E, +), it re-accumulates during an extra division cycle 17 not only in *fzr<sup>ie28</sup>* single (F, *fzr*<sup>-</sup>) mutants (Sigrist and Lehner, 1997), but also in *fzr<sup>ie28</sup>*; *CycA<sup>CBLR1</sup>* double mutant embryos (G, *fzr<sup>ie28</sup> CycA*<sup>-</sup>). Cyclin B is also again degraded during the additional mitosis 17 (for examples see arrows in F,G). (H,I) Mitotic figures observed during the additional cell division 17 in *fzr<sup>ie28</sup>* single (H, *fzr*<sup>-</sup>) and in *fzr<sup>ie28</sup>*; *CycA<sup>CBLR1</sup>* double (I, *fzr<sup>ie28</sup> CycA*<sup>-</sup>) mutant embryos after anti-tubulin (Tub) and DNA labeling (DNA). Normal prophase, prometaphase, metaphase, anaphase and telophase figures are shown from left to right in H and I. In addition, abnormal anaphase and telophase figures with chromatin bridges were also present in the *fzr<sup>ie28</sup>*; *CycA<sup>CBLR1</sup>* double mutant embryos, as illustrated by the two right most panels in I.

against inhibition by Cdk activity (Lukas et al., 1999; Sorensen et al., 2001; Zachariae et al., 1998). Interestingly, however, Cdk2 has been shown to inhibit Cdh1 in human cells only in conjunction with Cyclin A, and not with Cyclin E (Lukas et al., 1999). In an attempt to address the mechanisms whereby Cyclin A and Cyclin E inhibit Fzr in *Drosophila* embryos, we also mutated all of the thirteen Cdk consensus phosphorylation sites in Fzr (all positions with S or T followed by P) and analyzed its function. *prd-GAL4*-mediated expression of *UAS-fzr<sup>psm</sup>* (psm: phosphorylation sites mutated) resulted in the same phenotype that had been previously observed with *UAS-fzr* wild type, indicating that the Fzr<sup>psm</sup> protein is still functional (compare Fig. 7C,D with 7G,H). Unexpectedly, however, this *UAS-fzr<sup>psm</sup>* phenotype was still suppressed by co-expression of either *UAS-CycE* (Fig. 7I,J) or *UAS-CycA* (Fig. 7K,L), in contrast to the findings in human cells. It is possible, therefore, that Cyclin E and Cyclin A inactivate Fzr by phosphorylation of non-consensus sites or that these cyclins might act on additional targets to inhibit Fzr-APC/C-mediated degradation.

## DISCUSSION

The phenotypical characterization of mutations in the *Drosophila melanogaster* genes encoding the A-, B- and B3-type cyclins have indicated that Cyclin A is the most crucial of these co-expressed mitotic cyclins. Although zygotic *CycB* or *CycB3* function is not essential for cell proliferation and development to the adult stage (Jacobs et al., 1998), null mutations in *CycA* result in embryonic lethality (Lehner and O'Farrell, 1989). Here, we have clarified the molecular basis of the distinct importance of Cyclin A. Our results indicate that the crucial role of Cyclin A is linked to its ability to inhibit Fzr-APC/C-mediated degradation. Moreover, we show that this Cyclin A-dependent negative regulation of the Fzr-APC/C-degradation pathway is of particular importance for progression through the very last mitotic division preceding cell cycle exit and the proliferative quiescence of epidermal cells during embryogenesis. This particular Cyclin A requirement during terminal divisions is caused by a cell cycle exit program that is initiated already before the terminal mitosis. The cell cycle exit





**Fig. 6. Prolonged *CycE* expression during the terminal division cycles restores mitosis 16 in *CycA* mutants.** (A-H) The *prd-Gal4* transgene was used to drive *UAS-CycE* expression in alternating segments of *CycA<sup>CBLR1</sup>* (*CycA<sup>-</sup> prd>CycE*) or sibling control (*CycA<sup>+</sup> prd>CycE*) embryos. Embryos were immunolabeled with anti-Cyclin B3 (A,B; *CycB3*), or with anti-tubulin (C,E,G; *Tub*) and a DNA stain (D,F,H; *DNA*). Premature Cyclin B3 degradation before mitosis 16 is prevented in the *UAS-CycE*-expressing segments of *CycA<sup>CBLR1</sup>* mutants (white arrowheads in A, right side in B). Mitosis 16 is restored in the *UAS-CycE*-expressing segments of *CycA<sup>CBLR1</sup>* mutants (white arrows in E,F), resulting in a twofold higher cell density in the *UAS-CycE*-expressing segments of *CycA<sup>CBLR1</sup>* mutants after the stage of mitosis 16 (compare left and right side in G,H).

program includes downregulation of Cyclin E-Cdk2, which has a comparable ability to inhibit the Fzr-APC/C-degradation pathway to Cyclin A. The downregulation of Cyclin E-Cdk2 by the cell cycle exit program turns Cyclin A into an indispensable inhibitor of the premature degradation of mitotic cyclins and String/Cdc25 via Fzr-APC/C before the terminal mitosis. Accordingly, we have shown here that the terminal mitosis in the epidermis of *CycA* mutants can be restored by overexpression of Cyclin E, by genetic elimination of Fzr, or by simultaneous expression of the String/Cdc25-independent Cdk1<sup>AF</sup> mutant and B-type cyclin versions that are no longer Fzr-APC/C substrates.

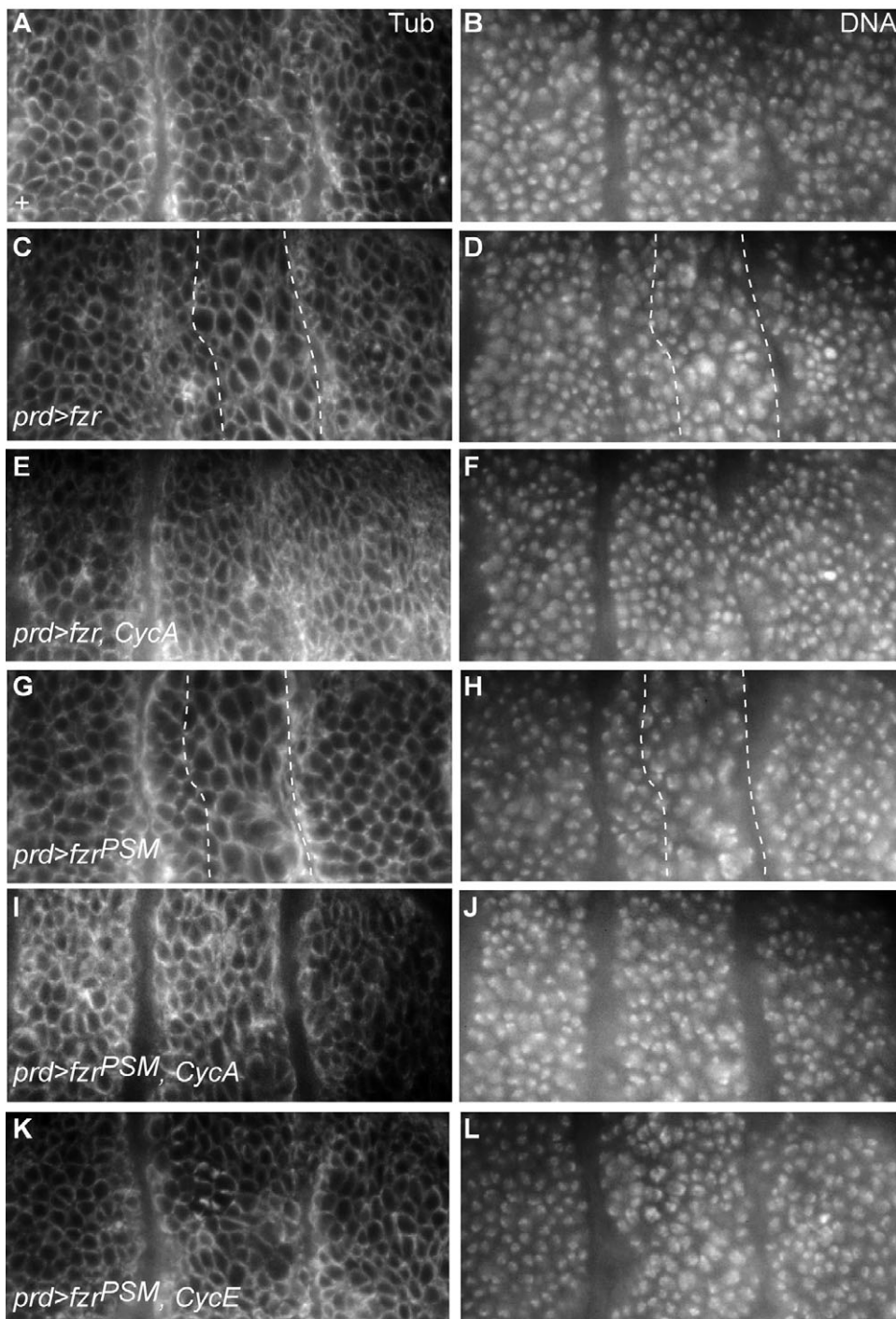
The fact that Cyclin A is also a substrate of Fzr-APC/C-mediated degradation complicates the interpretation of our results (Rape et al., 2006; Sigrist and Lehner, 1997). Two findings, however, strongly suggest that Cyclin A functions not just downstream of Fzr, but also upstream as a negative regulator. The observed premature loss of B-type cyclins in *CycA* mutants is readily explained by a negative effect of Cyclin A on Fzr-APC/C activity and is difficult to explain if Cyclin A was only a Fzr-APC/C substrate. Moreover, the suppression of the *UAS-fzr* overexpression phenotype by co-

expression of *UAS-CycA*, which we describe here, includes the re-accumulation of B-type cyclins and not just the restoration of terminal mitosis 16.

#### Indirect regulation of Fzr by Cyclin A and Cyclin E

Work in mammalian cells has clearly established that Cyclin A functions as a negative regulator of Fzr/Cdh1 (Lukas et al., 1999; Sorensen et al., 2001). Human Cyclin A can bind directly to Cdh1 (Lukas et al., 1999; Sorensen et al., 2001). Moreover, Cyclin A-dependent Cdk activity phosphorylates Cdh1, resulting in the dissociation of Cdh1 from APC/C (Lukas et al., 1999; Sorensen et al., 2001). Conversely, mutations in Cdk consensus phosphorylation sites of human CDH1 were reported to abolish inhibition by Cyclin A (Lukas et al., 1999). Our findings point to alternative modes of Fzr-APC/C-inhibition by Cyclin A. Our Fzr<sup>psm</sup> variant no longer contains canonical Cdk consensus phosphorylation sites (S/T P) and yet its activity is still suppressed by *CycA* overexpression. Fzr inhibition by CyclinA-dependent phosphorylation of non-consensus sites remains a possibility in *Drosophila*. However, we point out that, apart from a potential



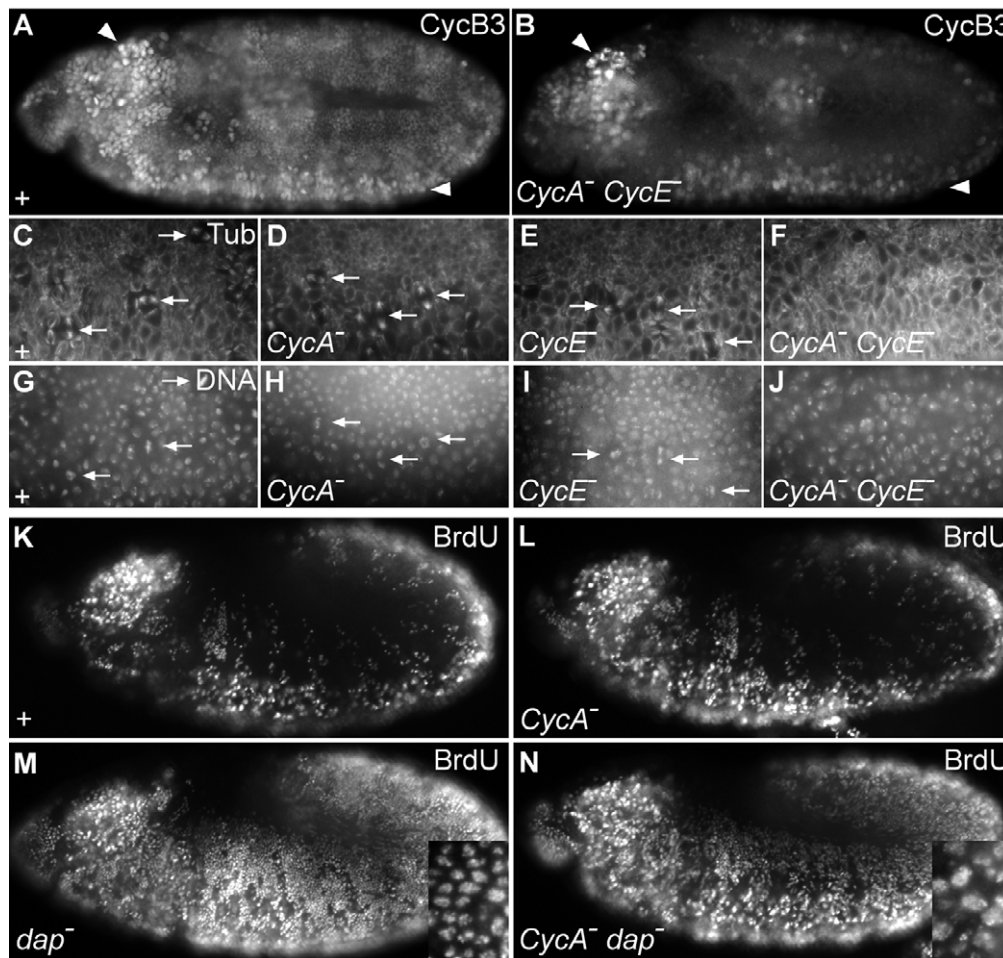


**Fig. 7. Cyclin A and Cyclin E suppress Fizzy-related/Cdh1 lacking all Cdk consensus phosphorylation sites.** (A-L) The *prd-Gal4* transgene was used to drive various *UAS* transgenes in alternating segments. Embryos after the stage of mitosis 16 were immunolabeled with anti-tubulin (Tub) and a DNA stain (DNA) for a comparison of the cell densities in segments with (middle segment) and without *UAS* transgene expression (outer segments). The *UAS* transgenes were *UAS-fzr* (C,D), *UAS-fzr* and *UAS-CycA* (E,F), *UAS-fzr<sup>PSM</sup>* (G,H), *UAS-fzr<sup>PSM</sup>* and *UAS-CycA* (I,J) and *UAS-fzr<sup>PSM</sup>* and *UAS-CycE* (K,L). The *UAS-fzr<sup>PSM</sup>* transgene results in expression of a Fzr version in which all of the Cdk consensus phosphorylation sites (S/T P) are eliminated. A control embryo without an *UAS* transgene is shown in A,B. *UAS-fzr* and *UAS-fzr<sup>PSM</sup>* expression inhibits mitosis 16 and therefore results in a twofold lower cell density. Co-expression of either *UAS-CycA* or *UAS-CycE* suppresses this inhibition of mitosis 16.

control by Cdk phosphorylation, Fzr is inhibited by the Emi1-like *Drosophila* protein Rca1 (Dienemann and Sprenger, 2004). Rca1 overexpression has been shown to prevent premature Cyclin B degradation and restore mitosis 16 in the epidermis of *CycA* mutant embryos (Dienemann and Sprenger, 2004). Based on these observations, the failure of mitosis 16 in *CycA* mutants was proposed to reflect premature Fzr activation (Dienemann and Sprenger, 2004), a suggestion fully confirmed by our work. It is conceivable, therefore, that the Cyclin A-mediated suppression of Fzr<sup>PSM</sup> activity involves Rca1 or other unknown targets. The fact that not only Cyclin A, but also Cyclin E, effectively suppresses *Drosophila* Fzr and Fzr<sup>PSM</sup> provides further support of additional

regulatory complexity. In vertebrate systems, only Cyclin A and not Cyclin E was shown to bind and inhibit Cdh1 (Lukas et al., 1999).

Our findings demonstrate that the Cyclin A requirement in epidermal cells is maximal for progression through the last mitosis of *Drosophila* embryogenesis, which precedes cell cycle exit and proliferative quiescence. A prominent Cyclin A requirement for terminal mitoses appears to exist in neuroblast lineages during development of the embryonic CNS, although definitive proof will require further work. On the basis of our analysis in epidermal cells, a high Cyclin A requirement for entry into mitosis is expected whenever Fzr levels are high and Cyclin E levels low. During the



**Fig. 8. Epidermal cell cycle progression in *CycA<sup>-</sup> CycE<sup>-</sup>* and *CycA<sup>-</sup> dap<sup>-</sup>* double mutants.** (A–J) Embryos at the stage of mitosis 15 were immunolabeled either with anti-Cyclin B3 (A,B; CycB3), or with anti-tubulin (C–F; Tub) and a DNA stain (G–J; DNA). Comparison of the anti-CycB3 signals in *CycE<sup>AR95</sup>; CycA<sup>CBLR1</sup>* double mutants (B; *CycE<sup>-</sup>; CycA<sup>-</sup>*) and sibling control embryos (A; +) reveals a premature Cyclin B3 disappearance in the double mutants. The strong anti-CycB3 signals remaining in the head region and along the ventral midline (arrowheads) of double mutants are from cells programmed to progress through mitosis 14 late. They are still in G2 before mitosis 14 at the stage shown and they therefore also still have residual maternal Cyclin A in the double mutants. Mitotic figures (white arrows) reflecting progression through mitosis 15 are observed in sibling control (C,G; +) and *CycA<sup>CBLR1</sup>* (D,H; *CycA<sup>-</sup>*) and *CycE<sup>AR95</sup>* (E,I; *CycE<sup>-</sup>*) single mutant embryos, but not in *CycE<sup>AR95</sup>; CycA<sup>CBLR1</sup>* double mutant embryos (F,J; *CycA<sup>-</sup> CycE<sup>-</sup>*). (K–N) Embryos were pulse labeled with BrdU. After the stage of mitosis 16, epidermal cells in *dap<sup>4</sup>* single (M; *dap<sup>-</sup>*) and *dap<sup>4</sup>; CycA<sup>CBLR1</sup>* double (N; *CycA<sup>-</sup> dap<sup>-</sup>*) mutants enter S phase, but not in sibling control (K; +) and *CycA<sup>CBLR1</sup>* single mutants (L; *CycA<sup>-</sup>*). Remarkably, epidermal cells in *dap<sup>4</sup>; CycA<sup>CBLR1</sup>* double mutants enter S phase even though they have not progressed through mitosis 16. The failure of mitosis 16 is also evident from a comparison of the nuclear density in the epidermis (insets in M,N).

comparatively slow postembryonic cell cycles of imaginal cells, the periodicity of Cyclin E expression is presumably far more pronounced than during the rapid embryonic cycles in which the persistent presence of maternally contributed Cyclin E eliminates G1 phases. In imaginal cell cycles, which have a G1 phase, Cyclin E expression might therefore be low before each mitosis, and not just before terminal divisions. In combination with Fzr expression, every imaginal mitosis might therefore be strongly dependent upon Cyclin A. By contrast, in the absence of Fzr, progression through mitosis appears to be almost completely independent of Cyclin A, as is evidenced by the observation that the epidermal cells in *fzr CycA* double mutant embryos not only progress successfully through mitosis 16, but also complete an extra division cycle 17. Nevertheless, 10% of the late mitosis 17 figures in these double mutants displayed lagging chromosomes, indicating that cell cycle progression is not entirely normal in the absence of Cyclin A.

The cell cycle exit program, which is activated during the final division cycle in the embryonic epidermis, includes the strong transcriptional upregulation of the CIP/KIP-type Cyclin E-Cdk2 inhibitor Dacapo (de Nooij et al., 1996; Lane et al., 1996), apart from the downregulation of Cyclin E (Knoblich et al., 1994) and the upregulation of Fzr (Sigrist and Lehner, 1997). Accordingly, genetic elimination of *dacapo* function should also restore progression through terminal mitosis 16 in *CycA* mutants. However, mitosis 16 was not observed in the epidermis of *dacapo CycA* double mutants. The contribution of Dacapo to Cyclin E-Cdk2 inhibition appears to be insignificant before mitosis 16. After the stage of mitosis 16, however, the epidermal cells in these double mutants entered an endoreduplication cycle, a behavior that is also displayed by some cells in the prospective anterior spiracle region of *CycA* single mutants (Sauer et al., 1995). This region does not downregulate Cyclin E during cycle 16 in the wild type, it does not upregulate



Dacapo, and it progresses through an additional cycle 17 instead of becoming postmitotic after mitosis 16, in contrast to the great majority of the other epidermal cells. The premature activation of Fzr in *CycA* mutants, therefore, appears to result in DNA replication origin re-licensing, perhaps as a result of B-type cyclin and geminin degradation. Cyclin E-Cdk2 activity might subsequently trigger endoreduplication in cells in which it is not effectively eliminated by both Cyclin E downregulation and Dacapo upregulation. Importantly, not all cells in the anterior spiracle region of *CycA* mutants endoreduplicate, some of the cells still manage to divide. This variability could reflect minor differences in the onset and strength of the zygotic Cyclin E expression. The outcome of insufficient Cyclin A levels appears to be highly dependent on the levels of Cyclin E and Fzr, which, in turn, are subject to developmental regulation, in particular during cell cycle exit. The significance of basic cell cycle regulators in vivo is therefore different in various tissues and developmental stages, and most likely in various cultured mammalian cell types as well.

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