The EMBO Journal vol.14 no.19 pp.4827–4838, 1995

Exit from mitosis is regulated by *Drosophila fizzy* and the sequential destruction of cyclins A, B and B3

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While entry into mitosis is triggered by activation of cdc2 kinase, exit from mitosis requires inactivation of this kinase. Inactivation results from proteolytic degradation of the regulatory cyclin subunits during mitosis. At least three different cyclin types, cyclins A, B and B3, associate with cdc2 kinase in higher eukaryotes and are sequentially degraded in mitosis. We show here that mutations in the *Drosophila* gene *fizzy* (*fzy*) block the mitotic degradation of these cyclins. Moreover, expression of mutant cyclins (Δcyclins) lacking the destruction box motif required for mitotic degradation affects mitotic progression at distinct stages. Δcyclin A results in a delay in metaphase, Δcyclin B in an early anaphase arrest and Δcyclin B3 in a late anaphase arrest, suggesting that mitotic progression beyond metaphase is ordered by the sequential degradation of these different cyclins. Co-expression of Δcyclins A, B and B3 allows a delayed separation of sister chromosomes, but interferes with chromosome segregation to the poles. Mutations in *fzy* block both sister chromosome separation and segregation, indicating that *fzy* plays a crucial role in the metaphase/anaphase transition.

Keywords: anaphase/cyclin degradation/*fizzy*/mitosis/sister chromosome separation

Introduction

The characteristic, rapid degradation of A- and B-type cyclins during mitosis results in the inactivation of mitosis-promoting cdc2 kinase activity. Mitotic cyclin destruction involves ubiquitin-dependent proteolysis and is dependent on the presence of a small conserved motif, the destruction box, located in the N-terminal region of the cyclin proteins (Glotzer *et al.*, 1991). Cyclins with N-terminal truncations deleting this destruction box (Δcyclins) are no longer degraded during mitosis. As a consequence, cdc2 kinase is not inactivated and exit from mitosis is blocked (Murray *et al.*, 1989; Ghiara *et al.*, 1991; Luca *et al.*, 1991; Gallant and Nigg, 1992; Holloway *et al.*, 1993; Surana *et al.*, 1993).

Not only the degradation of cyclins but also that of additional hypothetical proteins appears crucial for exit from mitosis according to experiments with inhibitors of the ubiquitin-dependent protein degradation system. Such inhibitors delay mitotic divisions *in vitro* in *Xenopus* egg extracts during metaphase, before chromosome separation, in contrast to sea urchin Δ90cyclin B, which arrests these divisions only after chromosome separation (Holloway *et al.*, 1993; Morin *et al.*, 1994). These observations are consistent with the attractive idea that sister chromosome cohesion, which is known to be required for correct orientation of the chromosomes at the metaphase plate, might be provided by those substrates of the ubiquitin-dependent degradation system that need to be degraded for progression beyond metaphase. According to this idea, therefore, sister chromosome separation and segregation in anaphase results from degradation of hypothetical cohesion proteins and telophase from degradation of cyclins (Holloway *et al.*, 1993).

The ubiquitin-dependent protein degradation system degrades substrates throughout the cell cycle. However, A- and B-type cyclins (and presumably also the unidentified additional mitosis-specific targets) become substrates only during mitosis. The regulatory mechanisms controlling this change in protein stability, and thereby also exit from mitosis, are poorly understood. Specialized ubiquitin-conjugating enzymes appear to be involved (Seufert *et al.*, 1995; Sudakin *et al.*, 1995). In addition, cyclin degradation in Clam or *Xenopus* egg extracts is a consequence of activation of the cyclin B–cdc2 kinase complexes during entry into mitosis (Murray and Kirschner, 1989; Murray *et al.*, 1989; Felix *et al.*, 1990; Luca *et al.*, 1991; Sudakin *et al.*, 1995). However, in most cells cyclin B degradation is not only dependent on cyclin B–cdc2 kinase activity, but also on the presence of functional mitotic spindles, and the presence of kinetochores that are incorrectly oriented or not connected to spindle poles is thought to inhibit cyclin B degradation (Gorbsky and Ricketts, 1993; Minshull *et al.*, 1994; Gorbsky, 1995). Inhibitors of microtubule polymerization arrest mitosis in prometaphase and prevent mitotic degradation of cyclin B (Minshull *et al.*, 1989; Whitfield *et al.*, 1990; Hunt *et al.*, 1992). The inhibitory effect of microtubule inhibitors on cyclin B degradation involves activation of a MAP kinase in *Xenopus* egg extracts (Minshull *et al.*, 1994). Cyclin A degradation, which occurs in normal division before that of cyclin B, is not prevented by microtubule inhibitors, suggesting that degradation of the different cyclins is controlled independently (Whitfield *et al.*, 1990; Hunt *et al.*, 1992; Minshull *et al.*, 1994). Clam Δcyclin A has also been shown to block exit from mitosis in *Xenopus* eggs, but this arrest has not been characterized in detail (Luca *et al.*, 1991).

Mutations in the *Drosophila* gene *fzy* result in a metaphase arrest (Dawson *et al.*, 1993). Here we show that neither cyclin A nor cyclin B are degraded in this arrest. Moreover, cyclin B3, a recently identified, evolutionarily conserved cyclin (Gallant and Nigg, 1994; Kreutzer *et al.*, 1995; H.Jacobs, J.Knoblich and C.F.Lehner, unpublished observations), is also stable in *fzy* arrest. Comparison of

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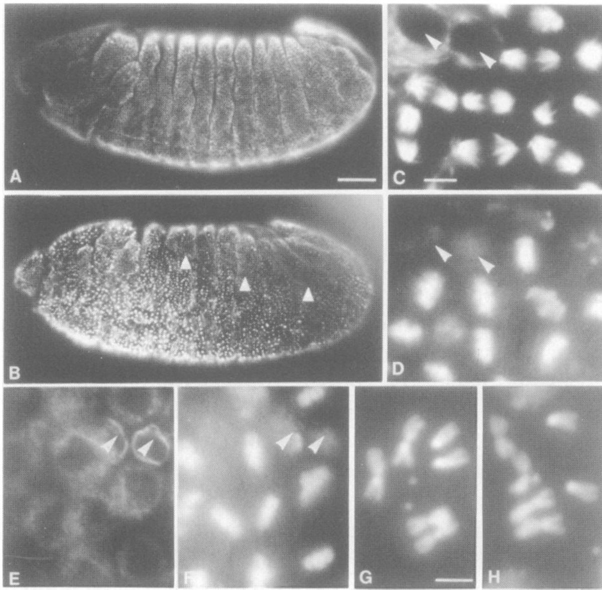


Fig. 1. Mutations in *fzy* result in a metaphase arrest. Wild-type (**A**) or *fzy*[1]/*fzy*[1] embryos (**B–H**) were fixed during stage 12 and used for immunofluorescent labelling with antibodies against β-tubulin (A–C) or against a nuclear envelope antigen (E). Double labelling with the DNA stain Hoechst 33258 corresponding to (C) and (E) is shown in (D) and (F) respectively. While the epidermal cells in wild-type embryos (A) are post-mitotic, essentially all cells in the ventral region of *fzy*[1]/*fzy*[1] embryos (B) are arrested in metaphase, but the dorsal epidermis (see arrowheads) is not affected. (C–F) Regions of the ventro-lateral epidermis at high magnification with arrowheads indicating non-arrested interphase cells. Cytological analyses with *fzy*[1]/*fzy*[1] embryos with (G) or without (H) pre-incubation in colcemid revealed that sister chromosomes in the mitotically arrested cells are still joined in the centromeric region. Bars in (A), (C) and (G) correspond to 50, 3 and 2.5 μm respectively.

the *fzy* mutant phenotype with the phenotypes resulting from expression of Δcyclins A, B or B3 suggests that *fzy* is not only required for mitotic cyclin degradation because *fzy* mutations, but not Δcyclins, block chromosome separation. Interestingly, however, the three Δcyclins cause distinct defects at sequential steps during completion of mitosis.

Results

fzy is required for sister chromosome separation and cyclin degradation

The *fzy* locus was identified in a screen for recessive embryonic lethal mutations which affect the cuticular pattern (Nüsslein-Volhard *et al.*, 1984). A recent phenotypic characterization indicated that all mitotically proliferating cells in embryos homozygous for *fzy* null alleles arrest in mitosis after exhaustion of the maternal *fzy* contribution, which is deposited in the egg during oogenesis (Dawson *et al.*, 1993). To characterize this mitotic arrest, we analysed the mitotic spindles after anti-tubulin labelling, as well as the chromosome cytology (Figure 1). At late stage 12, essentially all of the epidermal cells in wild-type embryos display an interphase microtubule organization, since mitotic proliferation in the epidermis is essentially completed at this stage (Figure 1A). In contrast, mitotic spindles are present in almost all cells of the ventral epidermis in *fzy* mutant embryos at the same stage (Figure 1B and C). In the dorsal epidermis of *fzy* mutants the normal interphase organization is observed (Figure 1B, see arrowheads). Dorsal epidermal cells terminate their mitotic proliferation before ventral epidermal cells in wild-type embryogenesis (C.F.Lehner, unpublished observation). The maternal *fzy* contribution, therefore, appears stable enough to allow completion of mitotic proliferation in the case of the dorsal epidermis, but not in the case of the ventral epidermis (Dawson *et al.*, 1993).

The mitotic spindles present in *fzy* mutants (Figure 1C) have the characteristic features of metaphase spindles and the nuclear lamina appeared disassembled in arrested cells (Figure 1E). DNA staining revealed metaphase plates of normal appearance (Figure 1D and F). Anaphase figures were never observed in late *fzy* mutants. *fzy* is, therefore, clearly required for the segregation of sister chromosomes to the spindle poles. Based on these observations, however, it was not clear whether *fzy* is also required for sister chromosome separation. Sister chromosome cohesion could either be maintained in the *fzy* arrest or lost, but remains undetected because of a failure to segregate the chromosomes to the poles. To investigate the state of sister chromosome cohesion in the *fzy* arrest we developed a protocol for cytological analysis of mitotic chromosomes in embryonic cells (see Materials and methods). At stage 12 the frequency of mitotic figures was >5-fold higher in preparations derived from heterozygous parents (*fzy*/*CyO*) compared with wild-type embryos, where most cells were already post-mitotic at this stage. In 93% of the mitotic figures observed in the *fzy*/*CyO* progeny, the sister chromosomes were still joined in the centromeric region (Figure 1G). Colcemid treatment, which depolymerizes the mitotic spindle, had no effect on the mitotic chromosomes in *fzy* mutants (Figure 1H). Separated sister chromosomes, which are only held together by the static microtubules of a defective spindle, are expected to become distributed after colcemid treatment and mitotic spread preparation. However, since sister chromosome separation in *fzy* mutants was not even observed after colcemid treatment, we conclude that *fzy* is required for sister chromosome separation.

We analysed whether cyclins A, B and B3 are stable in *fzy* arrest by immunofluorescence. Double labelling with antibodies against cyclins A and B resulted in specific signals in the arrested cells of the *fzy* mutants (Figure 2B and C). Post-mitotic, non-arrested cells in the same embryos were not stained, as expected (Lehner and O'Farrell, 1990) and provided an internal control for background levels (Figure 2B and C, arrowhead). The same result (not shown) was also obtained with an antibody against the recently identified *Drosophila* cyclin B3 (H.Jacobs, J.Knoblich and C.F.Lehner, unpublished observation). Immunoblotting confirmed that cyclins A, B and B3 are stable in *fzy* arrest (Figure 3). Increased levels of these cyclins were detected in *fzy* mutant embryos when compared with non-mutant siblings. Moreover, the cyclin forms and also the form of cdc2 kinase, which were found to be enriched in *fzy* mutants (Figure 3, arrowheads), were characteristic of the active complexes observed during pro- and metaphase of wild-type mitosis (Edgar *et al.*, 1994; data not shown).

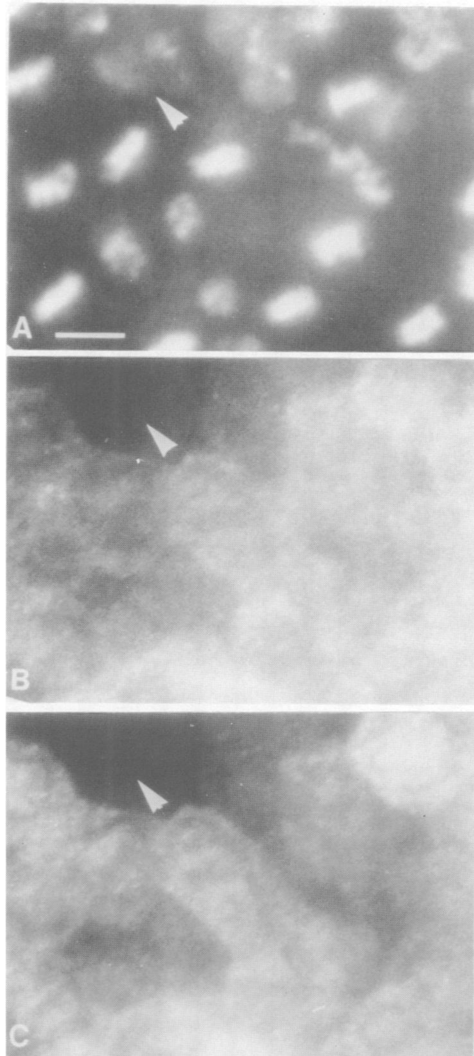


Fig. 2. Cyclins A and B are not degraded in the mitotically arrested cells of *fzy* mutants. *fzy*[1]/*fzy*[1] embryos were fixed at stage 12 and double labelled with the DNA stain Hoechst 33258 (**A**) and with rabbit antibodies against cyclin A (**B**) and a mouse monoclonal antibody against cyclin B (**C**). A region from the ventro-lateral epidermis is shown at high magnification. Cyclin A and B labelling is present in mitotically arrested cells of *fzy* mutants, but absent from non-arrested, post-mitotic cells (arrowhead). Bar in (A) corresponds to 5 μm.

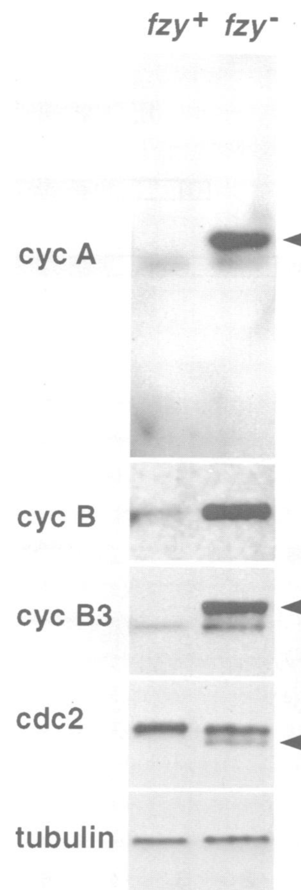


Fig. 3. Mitosis-specific forms of cdc2 kinase and cyclins A, B and B3 accumulate in *fzy* mutants. Embryos from heterozygous parents (*fzy*[1]/*CyO*) were fixed at stage 12 and labelled with the DNA stain Hoechst 33258. *fzy*[1]/*fzy*[1] embryos (*fzy*⁻), characterized by the presence of metaphase plates in the ventral epidermis, were sorted from *fzy*⁺ siblings with the help of an inverted microscope and analysed in immunoblotting experiments with antibodies against cyclin A (cyc A), cyclin B (cyc B), cyclin B3 (cyc B3), cdc2 kinase (cdc2) and β-tubulin. The arrowheads indicate mitosis-specific isoforms which are enriched in *fzy* mutants.

Mitotic defects resulting from expression of Δcyclins A, B and B3

To address whether the *fzy* mutant phenotype results solely from failure to degrade cyclins A, B and B3, we analysed the phenotypic consequences of Δcyclin expression. By P element-mediated germline transformation we established lines carrying transgenes in which a heat shock promoter controls the expression of either Δcyclin A, B or B3 (Figure 4A). Embryos carrying these transgenes were incubated for 30 min at 37°C to induce expression of the Δcyclins. After different times of recovery at 25°C, we analysed the level of the Δcyclins in total embryo extracts by immunoblotting. Δcyclins reached maximal levels within 30–45 min of recovery (Figure 4B) and their levels decreased <2-fold within 2 h (data not shown). After 5 min of recovery we estimated the Δcyclin levels to be 2- to 3-fold higher than the level of normal endogenous cyclins (Figure 4B; data not shown).

To analyse the phenotypic consequences of Δcyclin expression we expressed the transgenes at the stage before the epidermal cells progress through mitosis 15 in the ventral region and through mitosis 16 in the dorsal region. After recovery from the heat treatment, we labelled the embryos with anti-tubulin antibodies. After 30 min of recovery following expression of Δcyclin B, we observed regions with mitotic spindles in both the ventral and dorsal epidermis, as well as apparently normal interphase cells (Figure 5A). With longer recovery periods the regions with mitotic spindles increased. The mitotic spindles accumulated in a pattern reflecting the normal cell division programme. After 135 min of recovery following expression of Δcyclin B we found mitotic spindles in almost all cells of the embryo (Figure 5B). In non-transgenic control embryos exposed to the same heat shock and recovery period, the number of mitotic cells was much lower (Figure 5C) and comparable with the number of cells observed in embryos never exposed to heat (not shown). These observations indicate that Δcyclin B expression has

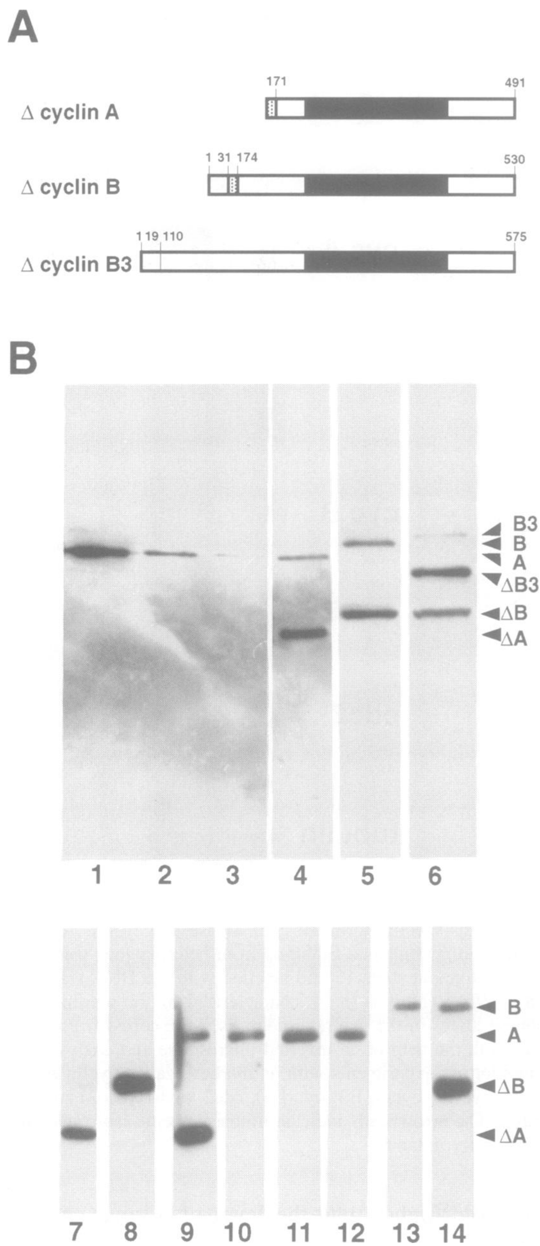


Fig. 4. Heat-inducible expression of Δcyclins. (**A**) Transgenic lines were constructed allowing the expression of Δcyclins, which lack the region with the destruction box motif required for mitotic degradation, under the control of a heat shock promoter. The structure of the Δcyclin proteins is illustrated schematically. Numbers designate amino acid positions. The black box represents the cyclin box, which is required for association with cdc2 kinase. The stippled box represents an epitope tag (HA-tag). (**B**) Early stage 11 embryos carrying either the *Hs-Δcyclin A* (lanes 4, 7, 9, 11 and 13), the *Hs-Δcyclin B* (lanes 5, 8, 10, 12 and 14) or the *Hs-Δcyclin B3* (lane 6) transgene were incubated for 30 min at 37°C followed by 30 min recovery at 25°C before immunoblot analysis. Lanes 1–3, 3, 1 and 0.5 ng bacterially produced cyclin A were resolved respectively. Lanes 1–4, rabbit anti-cyclin A; lane 5, mouse anti-cyclin B monoclonal antibody (Mab) F2; lane 6, rabbit anti-cyclin B3 antibodies; lanes 7 and 8, anti HA-tag Mab; lanes 9 and 10, anti-cyclin A Mab A2; lanes 11 and 12, anti-cyclin A Mab A19; lanes 13 and 14, anti-cyclin B Mab F2. The position of cyclins A (A), B (B) and B3 (B3) and Δcyclins A (ΔA), B (ΔB) and B3 (ΔB3) are indicated by arrowheads on the right side. The band at the position of Δcyclin B in lane 6 represents a cross-reaction of the anti-cyclin B3 antibodies with an unknown protein (data not shown).

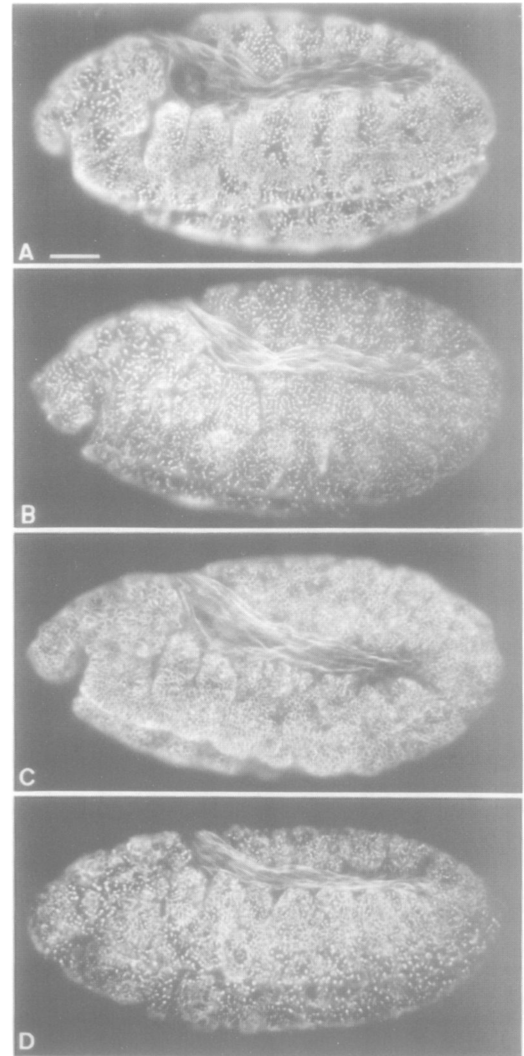


Fig. 5. Expression of Δcyclins blocks cell cycle progression after entry into mitosis. Early stage 11 *Hs-Δcyclin B* embryos (**A** and **B**), control embryos (**C**) and *Hs-Δcyclin A* embryos (**D**) were heat treated (30 min, 37°C) and allowed to recover at 25°C for 30 min (A) or 135 min (B–D) before immunolabelling with antibodies against β-tubulin. The mitotic spindle asters in the mitotically arrested cells appear as bright dots at this magnification. For higher magnifications see Figure 6. Bar in (A) corresponds to 50 μm.

no apparent consequences during interphase, but prevents exit from mitosis, as expected (Murray *et al.*, 1989; Luca *et al.*, 1991; Gallant and Nigg, 1992; Surana *et al.*, 1993).

The phenotypic consequences of Δcyclin A expression appeared similar (Figure 5D). However, double labelling with a DNA stain revealed clear differences, as illustrated in Figure 6, showing the same regions of the ventral epidermis from control embryos (Figure 6A and B), *Hs-Δcyclin A* (Figure 6C and D) and *Hs-Δcyclin B* embryos (Figure 6E and F) at high magnification. Expression of Δcyclin A resulted in a significant enrichment of cells with apparently normal metaphase spindles and metaphase plates (Figure 6C, D, M and N). Apart from the enrichment of metaphase figures, we also always detected anaphase and telophase figures in the mitotically active regions (Figure 6C and D, arrowheads; data not shown). The presence of these late mitotic figures (which were often characterized by abnormal spindle structures) indicated

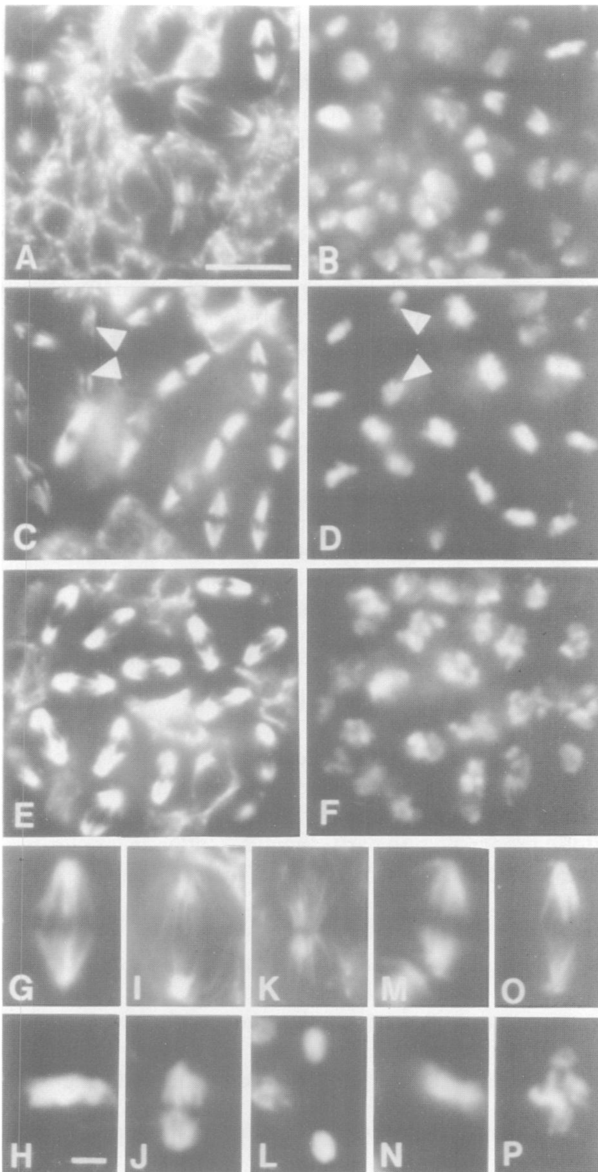


Fig. 6. Δcyclin A and Δcyclin B block mitosis at different stages. Early stage 11 control (**A**, **B** and **G–L**), *Hs-Δcyclin A* (**C**, **D**, **M** and **N**) and *Hs-Δcyclin B* embryos (**E**, **F**, **O** and **P**) were heat treated (30 min, 37°C) and allowed to recover (60 min, 25°C) before double labelling with antibodies against β-tubulin (A, C, E, G, I, K, M and O) and the DNA stain Hoechst 33258 (B, D, F, H, J, L, N and P). High magnification views from the same region of the ventral epidermis are shown in (A–F) and representative cells in (G–P). Arrowheads in (C) and (D) indicate a cell in anaphase. While all mitotic stages are observed in the control embryos, predominantly metaphase figures are observed in *Hs-Δcyclin A* embryos and early anaphase figures in *Hs-Δcyclin B* embryos. Bars in (A) and (H) correspond to 10 and 2 μm respectively.

that Δcyclin A expression does not result in a tight block of mitosis. This interpretation is consistent with the observation that the number of mitotic figures did not increase with longer recovery periods. Consequently, the number of mitotic cells after 135 min of recovery was significantly lower than in the case of Δcyclin B (compare Figure 5B and D). Therefore, our observations indicate that Δcyclin A expression results in a significant mitotic delay in metaphase, but not in a tight mitotic block.

The enrichment of metaphase cells after Δcyclin A expression is critically dependent on the expression level. Reducing the transgene copy number from two to one or shorter heat treatments reduced the enrichment of mitotic cells significantly (data not shown). Unfortunately, we were unable to increase the level of Δcyclin A expression. More than two Δcyclin A transgene copies could not be introduced into embryos, since all of the six independent insertions isolated were found to be homozygous lethal, presumably because of the toxicity of basal expression from the heat shock promoter. Moreover, increased heat exposure also resulted in a block of entry into mitosis in control embryos lacking Δcyclin transgenes (see also Maldonado-Codina *et al.*, 1993).

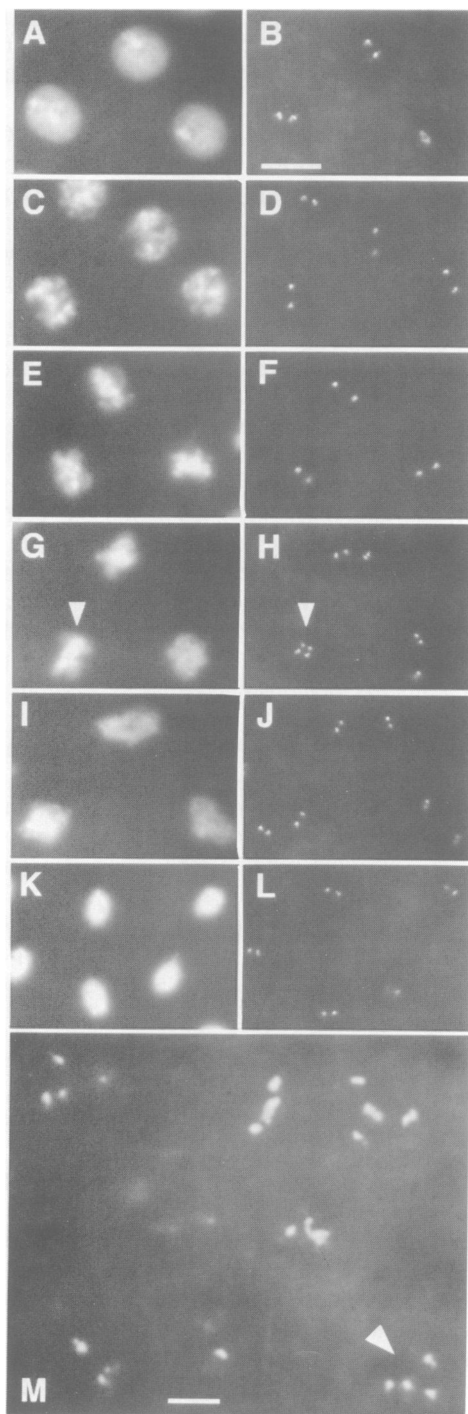
After Δcyclin B expression we never observed anaphase or telophase figures, while prophase and metaphase figures were observed with normal frequency. The vast majority of the cells with mitotic spindles, however, displayed a chromosome distribution (Figure 6E, F, O and P) which is not observed during normal mitosis (Figure 6G–L). While chromosomes always appeared fully condensed in the cells arrested in mitosis by Δcyclin B expression, they were neither arranged at the equatorial plate as in normal metaphase (Figure 6G and H) nor did they show an arrangement characteristic of normal anaphase (Figure 6I and J).

Based on the double labelling of DNA and tubulin, we were unable to determine whether the condensed chromosomes present in the Δcyclin B arrest represented separated sister chromosomes. However, by fluorescence *in situ* hybridization (FISH) we were able to demonstrate that sister chromosomes are separated in cells arrested in mitosis by Δcyclin B (Figure 7). For these experiments, we used a probe hybridizing to the dodeca satellite repeat (Abad *et al.*, 1992). The dodeca satellite repeat has recently been shown to be highly enriched on the right arm of chromosome 3 in a heterochromatic region very close to the centromere (Carmena *et al.*, 1993). Experiments with syncytial embryos demonstrated that separation of the sister chromosomes could be monitored in whole mount preparations with this probe (Figure 7A–L). In interphase two bright dots, reflecting hybridization to the maternal and paternal chromosome 3, were observed in each nucleus (Figure 7A and B). In ~30% of the nuclei the two signals were fused. Two dots (and in rare cases also one dot) were also observed in pro- and metaphase (Figure 7C–F). However, during anaphase and telophase the probe clearly detected four dots (Figure 7G–L), reflecting the separation of sister chromosomes. After expression of Δcyclin B the probe detected four randomly distributed dots in the cells arrested with condensed chromosomes (Figure 7M). We conclude, therefore, that expression of Δcyclin B does not prevent separation of sister chromosomes. However, Δcyclin B expression prevents segregation of the sister chromosomes to the poles.

We also analysed the consequences of Δcyclin B3 expression. Cyclin B3 was originally identified in the chick (Gallant and Nigg, 1994). Our identification of a homologous gene in *Caenorhabditis elegans* (Kreutzer *et al.*, 1995) and *Drosophila* (H.Jacobs, J.Knoblich and C.F.Lehner, unpublished observation) indicates that cyclin B3 is an evolutionarily conserved cyclin type expected to be present in all higher eukaryotes. In contrast to *Drosophila* cyclins A and B, which accumulate predominantly

in the cytoplasm during interphase (Figure 8A; Lehner and O'Farrell, 1990), cyclin B3 accumulates in the nucleus (Figure 8B). Like cyclins A and B, however, cyclin B3 is abruptly degraded during mitosis. While cyclin A is degraded before cyclin B (Lehner and O'Farrell, 1990; Whitfield *et al.*, 1990; Hunt *et al.*, 1992), cyclin B3 disappears after cyclin B, according to double immunolabelling experiments (Figure 8C–E).

Expression of Δcyclin B3 has no phenotypic consequences in interphase cells, according to double labelling with anti-tubulin and a DNA stain. However, in mitotically active regions Δcyclin B3 expression results in a mitotic block in late anaphase (Figure 9A). In addition to late anaphase figures of normal appearance (Figure 9E–G), cells with abnormal features were found, especially if maximal expression of Δcyclin B3 was enforced (Figure 9H–M). In these cells chromosomes were present at the equatorial plate as well as at the poles. In addition, the structure of the mitotic spindle was also clearly abnormal in these cells. The chromosomes at the equatorial plate, which often displayed stretched centromeric regions according to FISH experiments with the dodeca satellite repeat (data not shown), were connected symmetrically to both poles with strong microtubule fibres, as observed during metaphase. In addition, prominent asters extending to the cell cortex, as observed in anaphase, were also present. While we could still detect normal metaphase figures after Δcyclin B3 expression (Figure 9B–D), we never observed telophase figures with decondensing chromosomes after 60 min of recovery. After 120 min of recovery chromosome decondensation could again be observed (not shown). In conclusion, while expression of Δcyclin B3 strongly delays exit from mitosis, it still allows chromosome segregation to the poles. The aberrant mitotic figures observed after high levels of Δcyclin B3 expression might result from attempts to segregate chromosomes prematurely, before sister chromosome separation (see Discussion).



Our results reveal that Δcyclins A, B and B3 have distinct phenotypic consequences. ΔCyclin A causes a mitotic delay in metaphase, Δcyclin B interferes with mitotic progression in early anaphase (after sister chromosome separation) and Δcyclin B3 in late anaphase (after chromosome segregation). Expression of wild-type *Drosophila* cyclins from analogous transgenes did not result in mitotic phenotypes noticeably different from controls, although we cannot exclude minor delays. Interestingly, the apparent temporal order of the phenotypic consequences resulting from either Δcyclin A, B or B3 is correlated with the temporal order of cyclin degradation during wild-type mitosis. These observations therefore suggest that the mitotic processes during exit from mitosis might be ordered by sequential degradation of these cyclins (see Discussion).

Based on observations in other systems (Glotzer *et al.*, 1991; Luca *et al.*, 1991), expression of Δcyclin A, B or B3 is not expected to interfere with degradation of endogenous cyclins. Immunofluorescence experiments with a monoclonal antibody which recognizes only full-

Fig. 7. ΔCyclin B expression does not prevent sister chromosomes separation. Wild-type embryos (**A–L**) or *Hs-Δcyclin B* embryos (**M**) were used for whole mount fluorescence *in situ* hybridization (FISH) with the dodeca satellite repeat, which is located primarily in the centromere-near heterochromatin on chromosome 3. Regions from embryos showing double labelling with a DNA stain (A, C, E, G, I and K) and the hybridization signals (B, D, F, H, J and L) during interphase (A and B), prophase (C and D), metaphase (E and F), early anaphase (G and H), mid-anaphase (I and J) and telophase (K and L) of mitosis stage 12 are shown. Hybridization to two dots per cell are observed before and four dots per cell after sister chromosome separation. The arrowhead in (G) indicates a cell early after sister chromosome separation. (M) Four randomly distributed hybridization dots per cell, indicating that sister chromosome separation has occurred, were observed in cells arrested in mitosis by expression of Δcyclin B using the experimental protocol described in the legend to Figure 6. The arrowhead in (M) indicates a cell where all four dots can be clearly recognized in the same focal plane. Bars in (B) and (M) correspond to 10 and 3 μm respectively.

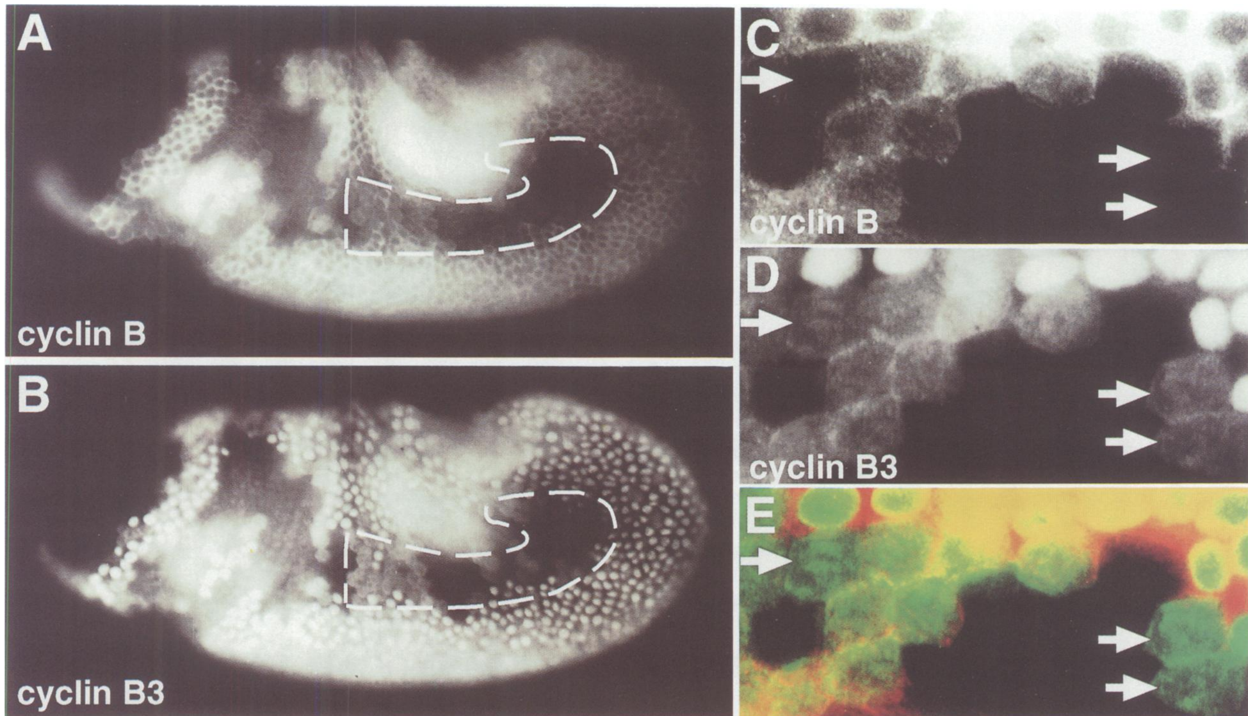


Fig. 8. Cyclin B degradation in mitosis is completed before cyclin B3 degradation. Embryos were fixed at the stage during which the cells in the dorsal epidermis (domain 11 according to Foe, 1989; indicated by the hatched line in A and B) progress through mitosis 14, followed by immunofluorescent double labelling with antibodies against cyclin B (**A** and **C**) and cyclin B3 (**B** and **D**). Labelled cells are either before or during mitosis 14, unlabelled cells have completed mitosis 14. The high magnification views (C–E) reveal cells (white arrows) which are negative for anti-cyclin B labelling (red in E) and positive for anti-cyclin B3 labelling (green in E), indicating that mitotic degradation of cyclin B is completed before that of cyclin B3.

length cyclin A, but not truncated Δcyclin A (Figure 4B, lanes 11 and 12), indicated that cyclin A degradation occured after Δcyclin A expression (data not shown). Similarly, experiments with anti-cyclin B or B3 antibodies indicated that these cyclins were also degraded after Δcyclin A expression. We cannot exclude, however, the possibility that degradation of endogenous cyclins is slightly delayed. The degradation of endogenous cyclins was also not inhibited significantly by Δcyclin B and Δcyclin B3 (data not shown). We must add, however, that we were unable to follow degradation of cyclin B after Δcyclin B expression and degradation of cyclin B3 after Δcyclin B3 expression because we have no antibodies recognizing only cyclin B and not Δcyclin B or only cyclin B3 and not Δcyclin B3.

Since degradation of endogenous cyclins is not inhibited by expression of Δcyclins, it appeared important to analyse the consequences of simultaneous co-expression of all three Δcyclins. Co-expression of Δcyclins A, B and B3 had very similar consequences to expression of Δcyclin B alone (data not shown). Co-expression of these Δcyclins, therefore, interfered with sister chromosome segregation, but not effectively with sister chromosome separation.

Inactivation of cdc2 kinase in the fzy arrest induces a reversion to interphase without completion of mitosis

Degradation of cyclins A, B and B3 in mitosis results in inactivation of the associated cdc2 kinase. The persistence of cyclins A, B and B3 in the *fzy* arrest is, therefore, expected to result in the persistence of cdc2 kinase activity. In fact, the active cdc2 kinase form is clearly enriched in *fzy* mutant embryos (Figure 3). To investigate whether exit from metaphase in *fzy* mutants is blocked solely because of the persistence of cyclins and the consequent persistence of cdc2 kinase activity we analysed the consequences of cdc2 kinase inactivation in the *fzy* arrest. If the persistence of cdc2 kinase activity was the only cause for the mitotic block in *fzy* mutants, inactivation of cdc2 kinase in the *fzy* arrest would be expected to allow normal completion of mitosis. Conversely, if processes other than cyclin degradation and inactivation of cdc2 kinase activity also require *fzy* function, reversion to interphase without completion of mitosis would be expected. For this experiment we crossed the *fzy* mutation into a background with a temperature-sensitive cdc2 kinase (Sigrist *et al.*, 1995). At 18°C, at which this temperature-sensitive cdc2 kinase is active, *fzy* mutant embryos developed and mitotic arrest was observed as expected at stage 11 in the ventral region. At this stage we shifted an aliquot of embryos to the restrictive temperature, while another aliquot was kept at the permissive temperature. Tubulin labelling revealed a drastic reduction in the number of metaphase-arrested cells in embryos shifted to the restrictive temperature (compare Figure 10A and B). Double labelling with a DNA stain revealed decondensed chromatin in the majority of cells of these embryos (Figure 10E and F). An identical temperature shift did not change the number or the morphology of cells arrested in metaphase in *fzy* mutants with a wild-type cdc2 kinase (Figure 10C and D). We conclude, therefore, that inactivation of the temperature-sensitive cdc2 kinase in *fzy* arrest caused a reversion to

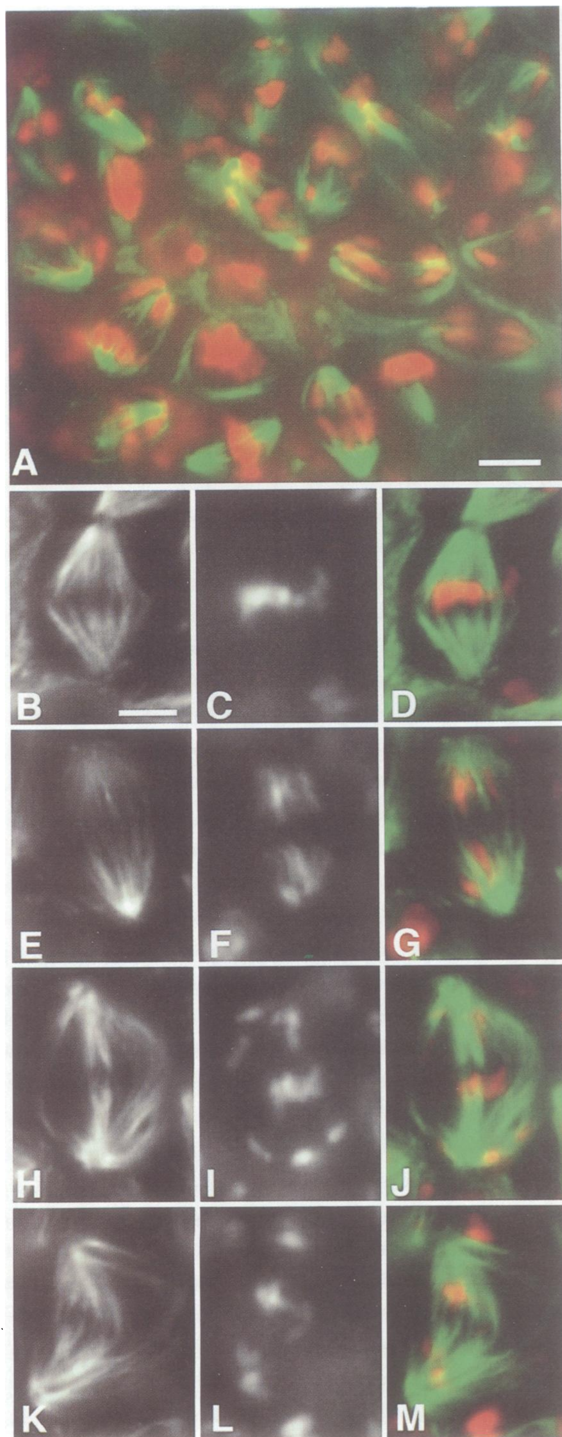


Fig. 9. The mitotic arrest resulting from Δcyclin B3 expression. Early stage 11 *Hs-Δcyclin B3* embryos were heat treated (30 min, 37°C) and allowed to recover (60 min, 25°C) before double labelling with antibodies against β-tubulin (**B**, **E**, **H** and **K**) and the DNA stain Hoechst 33258 (**C**, **F**, **I** and **L**). (**A**, **D**, **G**, **J** and **M**) Superimposition of tubulin (green) and DNA labelling (red). (A) Ventral region corresponding to the regions shown in Figure 6A–F. (B–D) Metaphase of wild-type appearance. (E–G) Anaphase of wild-type appearance. (H–M) Aberrant mitotic figures with both metaphase and anaphase features. Bars in (A) and (B) correspond to 3 and 2 μm respectively.

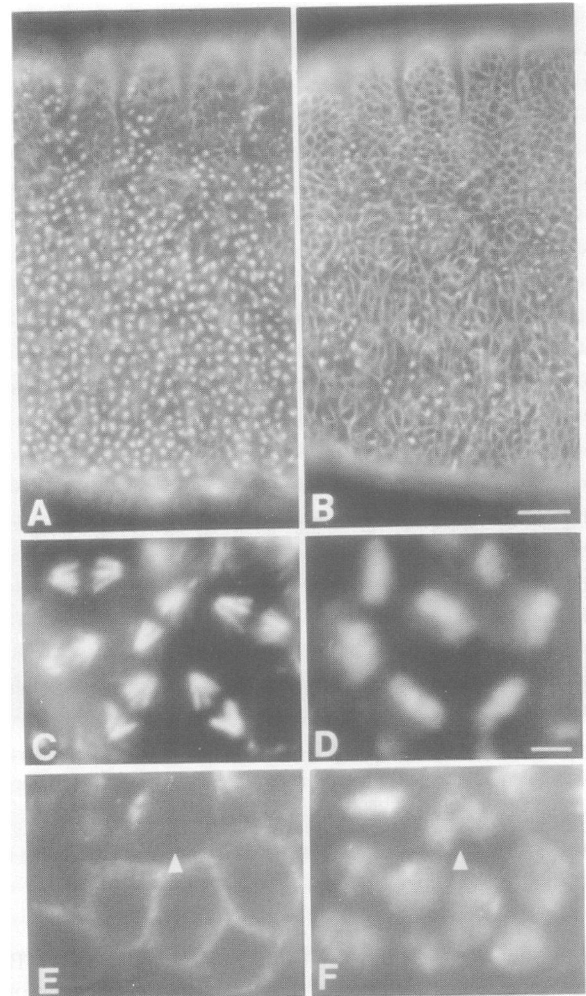


Fig. 10. Inactivation of cdc2 kinase in mitotically arrested cells of *fzy* mutants. *fzy*[1]/*fzy*[1] mutant embryos with either a temperature-sensitive cdc2 kinase (**A**, **B**, **E** and **F**) or a wild-type cdc2 kinase (**C** and **D**) were kept at the permissive temperature (A) or shifted at stage 12 for 1 h to the restrictive temperature (B–F) before fixation and double labelling with antibodies against tubulin (A–C and E) and Hoechst 33258 (D and F). Inactivation of cdc2 kinase in mitotically arrested cells of *fzy* mutants results in exit from mitosis in the absence of anaphase and telophase. A cell during exit from mitosis is indicated by the arrowheads in (E) and (F). Bars in (B) and (D) correspond to 30 and 3 μm respectively.

interphase morphology. This reversion was not accompanied by completion of mitosis. We did not observe an accompanying increase in anaphase cells. Moreover, cell number and cell size (which is reduced with each cell division in *Drosophila* embryogenesis), was also not changed. These observations suggest that *fzy* is needed not only for cyclin degradation and cdc2 kinase inactivation, but also for other aspects required for progression beyond metaphase.

Discussion

Our phenotypic characterizations demonstrate that the *Drosophila fzy* gene is required for separation and segregation of sister chromosomes to the mitotic spindle poles. Moreover, degradation of cyclins A, B and B3 during mitosis also requires *fzy* function. These observations argue that *fzy* plays an important role in triggering the progression beyond metaphase, i.e. exit from mitosis.

In contrast to the regulation of entry into mitosis, we do not understand the regulation of exit from mitosis

mechanistically. The bipolar connection of sister kinetochores, which is established during prophase after activation of the mitosis-promoting cyclin B–cdc2 kinase, results in chromosome congression at the metaphase plate and in tension stabilizing the correct connections. Kinetochores not engaged in a correct bipolar connection are thought to produce an inhibitory signal preventing exit from mitosis (Gorbsky and Ricketts, 1993; Gorbsky, 1995). Accordingly, exit from mitosis is thought to be triggered once all sister kinetochores are correctly connected to opposite spindle poles. The negative control of exit from mitosis by free kinetochores, which appears to involve MAP kinase (Minshull *et al.*, 1994), is not operative during the early cleavage divisions in *Xenopus* embryos, where exit from mitosis appears to follow automatically, with a temporal delay, from activation of cyclin B–cdc2 kinase (Murray and Kirschner, 1989; Murray *et al.*, 1989; Felix *et al.*, 1990; Sudakin *et al.*, 1995). Our understanding of how cyclin B–cdc2 kinase triggers not only entry into mitosis but also exit from mitosis with a temporal delay is very limited. However, it is clear that exit from mitosis requires the degradation of selected proteins. Inhibition of the ubiquitin-dependent degradation system prevents exit from mitosis. Neither chromosome separation nor chromosome segregation to the poles occur if ubiquitin-dependent proteolysis is inhibited in *Xenopus* egg extracts (Holloway *et al.*, 1993; Morin *et al.*, 1994).

Cyclins are among the proteins which need to be degraded for completion of mitosis. Addition of a mutant sea urchin cyclin B lacking the destruction box motif required for ubiquitin-dependent degradation during mitosis prevents exit from mitosis in *Xenopus* egg extracts and results in an arrest in late anaphase, after segregation of sister chromosomes to the poles (Holloway *et al.*, 1993).

Expression of sea urchin Δ90cyclin B, which was used in the experiments with *Xenopus* extracts, had no obvious phenotypic consequences in *Drosophila* embryos. Immunoblotting confirmed expression of sea urchin Δ90cyclin B in these experiments. Phenotypic consequences were observed when a chicken cdc2 kinase was co-expressed with sea urchin Δ90cyclin B (C.F.Lehner, unpublished observations). These observations indicate that sea urchin Δ90cyclin B is active in conjunction with chicken cdc2 kinase, but not with *Drosophila* cdc2 kinase.

Phenotypic consequences vary not only with different heterologous combinations of mutant cyclins and cdc2 kinase, but more importantly also with different types of homologous destruction box-deficient cyclins (Δcyclins). Expression of either *Drosophila* Δcyclin A, B or B3 results in distinct mitotic defects and, interestingly, we find a correlation between the temporal order in which cyclins A, B and B3 are degraded in wild-type mitosis and the temporal order of the mitotic defects caused by Δcyclins A, B and B3. Cyclin A apparently disappears during metaphase, cyclin B during the metaphase/anaphase transition and cyclin B3 during anaphase. Δcyclin A results in a mitotic delay in metaphase, Δcyclin B blocks in early anaphase and Δcyclin B3 in late anaphase. Based on these observations, we propose that the mitotic processes during exit from mitosis are ordered by sequential degradation of the different mitotic cyclins (Figure 11).

The substantial deletions in the N-terminal regions of cyclins A, B and B3 which we have used in our experiments

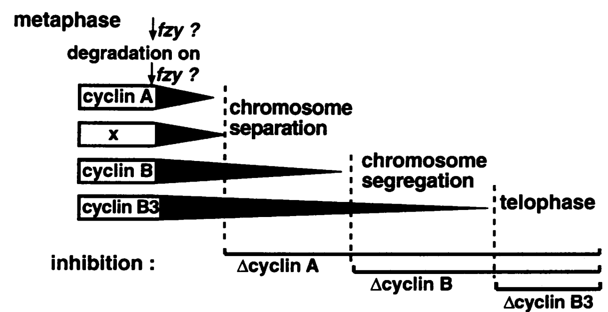


Fig. 11. A hypothetical model illustrating the function of *fzy* and cyclin degradation in the regulation of exit from mitosis. *fzy* is required to activate the ordered mitotic degradation of cyclins, which results in cyclin A disappearing first, cyclin B second and cyclin B3 third. The sequential disappearance of cyclins A, B and B3 is proposed to order successive steps during exit from mitosis (chromosome separation and segregation, telophase), since Δcyclins A, B and B3 (which lack the destruction box motif required for mitotic degradation) arrest mitosis at distinct stages. However, progression beyond metaphase is not only dependent on the degradation of cyclins A, B and B3 and inactivation of cdc2 kinase, suggesting the presence of unidentified regulators (X) which might also be controlled by *fzy* (see Discussion).

might not only interfere with mitotic destruction, but could destroy other functions and result in new activities not displayed by the wild-type cyclin counterparts. While we cannot exclude these possibilities, we emphasize that Δcyclin A and B appear to have retained at least some of their functional specificity, as revealed by complementation experiments in cyclin A mutants. In homozygous cyclin A-deficient embryos epidermal cells no longer enter mitosis after exhaustion of the maternal cyclin A contribution (Lehner and O'Farrell, 1989). As observed with wild-type cyclins, entry into mitosis can be fully restored in these mutants by expression of Δcyclin A, but not of Δcyclin B (Knoblich and Lehner, 1993; S.Sigrist and C.F.Lehner, unpublished observation).

The phenotypes resulting from *Hs-Δcyclin* expression were found to be sensitive to expression levels. Reducing the length of the heat shock or the transgene copy number from two to one resulted in all cases in a lower penetrance of the mitotic defects. However, the characteristic phenotypic differences observed after either Δcyclin A, B or B3 expression were independent of expression level. For instance, a reduction in the level of Δcyclin B expression was accompanied by transformation of the early anaphase arrest into a slow progression through late mitosis (anaphase and telophase), but transformation into the late anaphase arrest characteristic for Δcyclin B3 was not observed. The different phenotypes observed after either Δcyclin A, B or B3 expression, therefore, reflect the specific properties of these proteins and not simply differences in the level of cdc2 kinase activity.

Maximal expression under heat shock conditions still compatible with mitosis in wild-type embryos blocked exit from mitosis completely for >2 h in the case of Δcyclin B, but not in the case of Δcyclins B3 and A. In particular, with Δcyclin A we observed only a transient delay in metaphase. Immunoblotting experiments indicated that Δcyclin levels decreased <2-fold within 2 h, suggesting that exit from mitosis, although with a delay, can occur even in the presence of Δcyclin A. We have been unable to increase Δcyclin A expression levels further

and, therefore, the possibility that higher levels of Δcyclin A result in a complete block at metaphase cannot be excluded. However, we emphasize that Δcyclin A levels were higher than the level of endogenous cyclin A in our experiments. Endogenous wild-type cyclins present in our experiments might actually compete with the Δcyclins in binding to cdc2 kinase. Expression of Δcyclin A in cyclin A-deficient embryos appeared more efficient in delaying progression beyond metaphase. However, aberrant anaphases were also observed in these experiments (S.Sigrist and C.F.Lehner, unpublished observation).

While our experiments indicate that cyclin A activity does at least delay the separation of sister chromosomes, the effects resulting from colchicine treatment argue for the presence of additional unknown regulators controlling sister chromosome separation. As in many other species, colchicine results in *Drosophila* in a mitotic arrest in which cyclin A is degraded, but sister chromosomes are not yet separated (Whitfield *et al.*, 1990; Gonzalez *et al.*, 1991). Cyclin A degradation, therefore, is not sufficient to allow sister chromosome separation.

In contrast to cyclin A, *Drosophila* cyclin B is apparently not required for entry into mitosis. In cyclin B-deficient embryos mitoses occur long after the stage where the maternally contributed cyclin B has become undetectable (Knoblich and Lehner, 1993). These mitoses, however, are abnormal and cytokinesis is initiated before the sister chromosomes have segregated to the poles (Knoblich and Lehner, 1993). In the context of our model chromosome segregation in the absence of cyclin B is predicted to begin at the same time as sister chromosome separation. The simultaneity of separation and segregation might render these processes inefficient and slow. Separation might not be completed, therefore, at the time when cytokinesis starts in cyclin B-deficient embryos.

The effects of Δcyclin B expression in *Drosophila* have been analysed in a recently published independent study (Rimmington *et al.*, 1994). However, in these experiments the level of cyclin B expression and the temporal relation between expression and phenotypic consequences are unknown, making the interpretation of the observed, much more variable phenotypes difficult.

Mutations in cyclin B3 have not yet been isolated. Therefore, we do not know whether entry into mitosis still occurs in the absence of cyclin B3 and whether chromosome segregation and telophase start at the same time as predicted by our model in this case. The consequences of Δcyclin B3 expression at high levels, however, are consistent with our model, which postulates that cyclin B3 activity in the absence of cyclin A and B kinase activity results in chromosome segregation. Δcyclin B3 expression at high levels might result in an excess of cyclin B3-associated kinase activity during prophase and metaphase and, consequently, in premature chromosome segregation during the early mitotic stages. The interpretation that an imbalance of cyclin B3 relative to cyclins A and B is causing the aberrant mitotic arrest is supported by the observation that it is no longer observed after simultaneous expression of Δcyclins A, B and B3.

Simultaneous expression of all three Δcyclins arrests mitosis at the same stage as Δcyclin B alone, i.e. after sister chromosome separation, but before chromosome segregation. Δcyclin B, therefore, might have the highest affinity for cdc2 kinase and might titrate out the other Δcyclins. Alternatively, since Δcyclin A alone causes a metaphase delay, we would expect an enrichment of metaphases after Δcyclin A, B and B3 co-expression if Δcyclin B cannot titrate out all other cyclins and Δcyclin A is also active. Enrichment of metaphases is noticeable in our co-expression experiments, but is difficult to demonstrate unequivocally against the background of the similar chromosome figures characteristically observed after Δcyclin B expression.

Δcyclins A, B and B3 expression did not prevent sister chromosome separation effectively. In contrast, *fzy* function is clearly required for this process. This difference suggests that *fzy* is not only required for degradation of cyclins A, B and B3. Moreover, the idea that *fzy* is exclusively required for cyclin degradation is also disputed by our experiments with the temperature-sensitive cdc2 allele, if we accept the idea that cyclin degradation occurs only for the purpose of cdc2 kinase inactivation. In this case inactivation of cdc2 kinase in the *fzy* arrest would be expected to by-pass the requirement for cyclin degradation and allow completion of mitosis. In fact, inactivation of Cdc28 kinase in budding yeast arrested in mitosis by an undegradable B-type cyclin allows completion of mitosis (Ghiara *et al.*, 1991). In contrast, although the mitotically arrested cells in *fzy* mutants returned to an interphase organization after inactivation of cdc2 kinase, this reversion was not accompanied by anaphase and cytokinesis.

No defects other than the inability to progress beyond metaphase can be detected in *fzy* mutants. Interphase, prophase and metaphase appear normal. Post-mitotic cells are not affected and the cell lethality observed in late *fzy* mutants is dependent on entry into mitosis (Dawson *et al.*, 1993). It is difficult to exclude the possibility that *fzy* is required before the metaphase/anaphase transition and that mitotic arrest results from a checkpoint system recognizing defects during earlier cell cycle stages. Defects in mitotic spindle assembly resulting from colchicine treatment, for example, arrest mitosis before sister chromosome separation (Gonzalez *et al.*, 1991). However, cyclin A is stabilized in the *fzy* arrest, but not in the colchicine arrest (Whitfield *et al.*, 1990), arguing that the *fzy* arrest is not simply the result of a subtle spindle defect. The *fzy* gene has recently been isolated (Dawson *et al.*, 1995; C.F.Lehner, unpublished data) and sequence analysis reveals its similarity to the *CDC20* gene of budding yeast, which is also required for the metaphase/anaphase transition (Sethi *et al.*, 1991).

All our observations, therefore, suggest that *fzy* is an important regulator of exit from mitosis and the events during exit from mitosis (anaphase with sister chromosome separation and segregation, telophase with cytokinesis and reformation of an interphase organization) appear temporally ordered by the sequential degradation of different mitotic cyclins (cyclins A, B and B3).

Materials and methods

Fly strains, egg collections and temperature shifts

The results of the phenotypic analyses shown in Figures 1–3 and 10 were obtained with embryos homozygous for the amorphic allele *fzy*[1], which has been described previously (Nüsslein-Volhard *et al.*, 1984;

Dawson *et al.*, 1993). Essentially identical results were obtained with embryos homozygous for another amorphic allele, fzy^3, or with embryos carrying fzy^1 over the deficiency *Df(2L)RN2*.

Embryos with a temperature-sensitive cdc2 kinase and homozygous for *fzy* were obtained by crossing females of the genotype $Dmcdc2^{E1-23}$ $fzy^1/Dmcdc2^{E1-23}$; P[w^+, $Dmcdc2^{A171T}$]/P[w^+, $Dmcdc2^{A171T}$] with males of the genotype $Dmcdc2^{E1-23}$ fzy^1/CyO, P[w^+, *ftz-lacZ*]; P[w^+, $Dmcdc2^{A171T}$]/P[w^+, $Dmcdc2^{A171T}$] at 18°C. The amorphic allele $Dmcdc2^{E1-23}$, as well as the transgene encoding a temperature-sensitive allele $Dmcdc2^{A171T}$, have been described previously (Stern *et al.*, 1993; Sigrist *et al.*, 1995). Eggs were collected for 2 h and aged for 18 h at 18°C on standard apple juice–agar plates. Subsequently we incubated the eggs for 1 h at 31°C to inactivate the $Dmcdc2^{A171T}$ kinase before fixation. Controls were kept for an additional 4 h at 18°C until fixation.

Fly strains carrying transgenes allowing heat-inducible expression of cyclins A or B (*Hs-cyclin A*, *Hs-cyclin B*) have been described previously (Knoblich and Lehner, 1993). Additional strains carrying the transgenes *Hs-cyclin B3*, *Hs-Δcyclin A*, *Hs-Δcyclin B*, *Hs-Δcyclin B3* and *Hs-SUΔ90cyclin B* were obtained by standard P element-mediated germline transformation with the CasPeR-hs constructs described below. Since these transgenes were established in the *w* background, we used *w* flies for control experiments. The phenotypes shown are not dependent on the site of insertion of the *Hs-Δcyclin* transgenes, since indistinguishable phenotypes were observed with several independent lines. While the results shown in Figures 5–7 and 9 were from embryos carrying two *Hs-Δcyclin* transgene copies, embryos carrying one copy of *Hs-Δcyclin A*, *B* and *B3* were used for the co-expression experiments. All of the six independent *Hs-Δcyclin A* insertions which were isolated result in lethality when homozygous. *Hs-Δcyclin A* strains were, therefore, kept over balancer chromosomes carrying transgenes which direct the expression of *lacZ*, and progeny with two copies of the *Hs-Δcyclin A* transgene were identified based on an absence of *lacZ* expression.

Severe heat shocks result in various mitotic defects, as described by Maldonado-Codina (1993) in the case of mitosis 14. Since embryos were found to be less sensitive to heat shock at later stages, we applied the heat shocks (30 min, 37°C) at a stage before epidermal cells entered mitosis 15 in the ventral and mitosis 16 in the dorsal regions. Moreover, for the heat shocks we floated the collection plates on a 37°C water bath, resulting in a relatively slow temperature equilibration which also reduces heat shock artefacts. Aliquots of embryos were fixed after incubation for various periods of recovery at 25°C.

Plasmid constructions

For construction of the *Hs-Δcyclin A* transgene we replaced the sequence, including the destruction box, from the *Nco*I site at position 295 to the *Eco*RV site at position 804 in the cyclin A cDNA (Lehner and O'Farrell, 1989) with a double-stranded oligonucleotide coding for an epitope of the influenza virus hemagglutinin protein (HA-tag) (Field *et al.*, 1988).

For construction of the *Hs-Δcyclin B* transgene we replaced the sequence, including the destruction box, from the *Nco*I site at position 338 to the *Eco*RI site at position 770 in the cyclin B cDNA (Lehner and O'Farrell, 1990) with a double-stranded oligonucleotide coding for the HA-tag.

For construction of the *Hs-cyclin B3* transgene we used wild-type cDNA and for the *Hs-Δcyclin B3* transgene we deleted an internal *Pst*I fragment from the cyclin B3 cDNA (H.Jacobs, J.Knoblich and C.F.Lehner, unpublished observation). The SUΔ90cyclin B cDNA (Murray *et al.*, 1989) used for construction of the *Hs-SUΔ90cyclin B* transgene has been described previously and was kindly provided by A.Murray (UCSF, San Francisco, CA).

The cDNAs encoding Δcyclins were introduced into the pCasPeR-hs vector. Further details concerning these constructs are available upon request. The *Drosophila* Δcyclin proteins encoded by the transgenes are schematically illustrated in Figure 4A.

Immunoblotting and immunofluorescence

The antibodies against cyclins A and B, cdc2 kinase and a nuclear envelope protein have been described previously (Lehner and O'Farrell, 1989; Lehner, 1992; Knoblich and Lehner, 1993). Identification of *Drosophila* cyclin B3, as well as production of antibodies against cyclin B3, will be described in another publication. The antibodies against β-tubulin (Amersham), β-galactosidase (Cappel) and HA-tag (Babco) are available commercially.

Immunoblotting was done essentially as described previously. Homozygous fzy^1 embryos were sorted from sibling embryos after fixation and labelling with the DNA stain Hoechst 33258 as described previously (Edgar *et al.*, 1994). The level of endogenous wild-type cyclins and of Δcyclins expressed from the transgenes were quantified by resolving known amounts of bacterially expressed *Drosophila* cyclins in parallel lanes of the gels. In the case of cyclins A and B we used monoclonal antibodies recognizing epitopes present in both wild-type and Δcyclins. In the case of cyclin B3 we used a polyclonal antibody (H.Jacobs, J.Knoblich and C.F.Lehner, unpublished observation). It is possible that this antibody reacts less efficiently with Δcyclin B3 than with cyclin B3 and therefore results in an under-estimation of Δcyclin B3 levels.

Immunofluorescence was done as described previously (Knoblich and Lehner, 1993). In some experiments pictures were acquired with a cooled CCD camera (Photometrics) and superimposed using Adobe Photoshop software.

Cytological analyses

For cytological analysis of mitotic chromosomes we developed a protocol based on methods described for *Drosophila* larval brains (Guest and Hsu, 1973). Eggs were collected and aged on standard apple juice–agar plates. Embryos were dechorionated in 0.5% sodium hypochlorite and washed extensively with 0.7% NaCl, 0.1% Triton X-100. Embryos were permeabilized in a two phase mixture of 750 μl octane and 750 μl Schneider's medium containing 10 μM demecolizin (Sigma) in some experiments. The two phase mixture was removed after 15–30 min. The embryos were washed in 1% sodium citrate containing 10 μM demecolizin in some experiments and gently dissociated and incubated for 5 min in a small volume of the same buffer. Cells were fixed by adding 1 ml of a mixture (1:3) of acetone and methanol (pre-cooled to –20°C) and sedimented by a brief centrifugation at low speed (1000 *g*). After an additional wash in acetone/methanol the cells were resuspended in 100 μl acetone/methanol and dropped onto a slide pre-warmed to 42°C. Slides were air-dried, followed by re-hydration in phosphate-buffered saline (PBS), 0.1% Triton X-100. The preparations were stained in PBS containing 1 μg/ml Hoechst 33258, washed in PBS and mounted in 70% glycerol in PBS containg propyl gallate (10 mg/ml) and *p*-phenylenediamine (0.5 mg/ml).

Fluorescence in situ hybridization

To monitor the separation of sister chromosomes in whole mount preparations of *Drosophila* embryos we followed the protocol of Hiraoka *et al.* (1993), using a dodeca satellite probe (Abad *et al.*, 1992), with the following modifications. The 470 bp *Spe*I insert from pBK6E218 (Abad *et al.*, 1992) was labelled with biotin–16-dUTP by random priming. Embryos were fixed for 20 min at room temperature in a two phase mixture of heptane and 4% formaldehyde in PBS. Embryos were devitellinized by replacing the aqueous phase with methanol. Devitellinized embryos were rinsed three times in methanol, three times in PBS, 0.1% Tween 20 and three times in 2× SSC, 0.1% Tween 20. After 10 min in 4× SSC, 0.1% Tween 20, 20% formamide and 10 min in 4× SSC, 0.1% Tween 20, 50% formamide we incubated the embryos for 1 h at 37°C in 4× SSC, 0.1% Tween 20, 50% formamide. After removal of the pre-hybridization solution we added ~25 ng biotinylated dodeca satellite repeat DNA in 25 μl 4× SSC, 0.1% Tween 20, 50% formamide to an embryo volume of ~15 μl. After gentle mixing chromosomal DNA was denatured by a 15 min incubation at 70°C, followed by hybridization for 18 h at 37°C. After hybridization embryos were washed as described (Hiraoka *et al.*, 1993). The probe was detected by Cy3-coupled avidin (Jackson Immunochemicals) as described (Hiraoka *et al.*, 1993). Signals were recorded using a cooled CCD camera (Photometrics).

Acknowledgements

We thank Eva-Maria Illgen for technical help and the members of the laboratory for discussions and comments on the manuscript.

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Received on April 25, 1995; revised on July 11, 1995