Published June 24, 2002

JCB Article

The roles of Fzy/Cdc20 and Fzr/Cdh1 in regulating the destruction of cyclin B in space and time

Jordan W. Raff, Kim Jeffers, and Jun-yong Huang

Department of Genetics, Wellcome/Cancer Research UK Institute, Cambridge CB2 1QR, UK

In *Drosophila* cells cyclin B is normally degraded in two phases: (a) destruction of the spindle-associated cyclin B initiates at centrosomes and spreads to the spindle equator; and (b) any remaining cytoplasmic cyclin B is degraded slightly later in mitosis. We show that the APC/C regulators Fizzy (Fzy)/Cdc20 and Fzy-related (Fzr)/Cdh1 bind to microtubules in vitro and associate with spindles in vivo. Fzy/Cdc20 is concentrated at kinetochores and centrosomes early in mitosis, whereas Fzr/Cdh1 is concentrated at centrosomes throughout the cell cycle. In syncytial embryos, only Fzy/Cdc20 is present, and only the spindle-associated cyclin B is degraded at the end of mitosis. A destruction box–mutated form of cyclin B (cyclin B triple-point mutant [CBTPM]–GFP) that cannot be targeted for destruction by Fzy/Cdc20, is no longer degraded on spindles in syncytial embryos. However, CBTPM–GFP can be targeted for destruction by Fzr/Cdh1. In cellularized embryos, which normally express Fzr/Cdh1, CBTPM–GFP is degraded throughout the cell but with slowed kinetics. These findings suggest that Fzy/Cdc20 is responsible for catalyzing the first phase of cyclin B destruction that occurs on the mitotic spindle, whereas Fzr/Cdh1 is responsible for catalyzing the second phase of cyclin B destruction that occurs throughout the cell. These observations have important implications for the mechanisms of the spindle checkpoint.

Introduction

The sequential activation and inactivation of cyclin-dependent protein kinases (cdks)* ensures the proper timing and order of cell cycle events. The complex of cdc2 and cyclin B is a major regulator of the entry into mitosis, and multiple factors control the timing of its activation (Harper and Elledge, 1996; Lew and Kornbluth, 1996). The inactivation of cdc2/cyclin B is essential for the proper exit from mitosis, and this is usually achieved by degrading cyclin B via the polyubiquitination/26S proteosome pathway (Glotzer et al., 1991). The destruction of cyclin B is initiated by the anaphase-promoting-complex, or cyclosome (APC/C), a large multisubunit complex that specifically targets cyclin B for ubiquitination at the metaphase/anaphase transition (Hershko et al., 1994; Irniger et al., 1995; King et al., 1995).

As well as being temporally regulated, the destruction of cyclin B also appears to be spatially regulated. For example, in fused vertebrate cells that contain two spindles, the spindles can exit mitosis independently of each other, suggesting that cyclin B is not being degraded everywhere in the cell at the same time (Rieder et al., 1997). In syncytial *Drosophila* embryos, the destruction of cyclin B is essential for the exit from mitosis (Su et al., 1998), but Western blotting experiments revealed that cyclin B is only partially degraded at the end of mitosis (Edgar et al., 1994). This suggests that only a specific sub-population of cyclin B is degraded at the end of mitosis in these embryos. More recently, the destruction of cyclin B–green fluorescent protein (GFP) fusion proteins has directly been observed to be spatially regulated in *Drosophila* (Huang and Raff, 1999), human (Clute and Pines, 1999), and yeast (Yanagida et al., 1999; Decottignies et al., 2001) cells.

In *Drosophila*-cellularized embryos, the destruction of cyclin B–GFP appears to initiate at centrosomes and spreads to the equator of the spindle. The degradation of the cytoplasmic cyclin B is then initiated slightly later in mitosis, and continues into the next cell cycle. Unfortunately, the question of how cyclin B is only partially degraded in syncytial embryos could not be directly addressed, as the destruction of cyclin B–GFP could not be followed in syncytial embryos. This is probably because GFP has to undergo an intramolecular rearrangement before it becomes fluorescent, and, in flies,

The online version of this paper contains supplemental material.

Address correspondence to Jordan W. Raff, Department of Genetics, Wellcome/Cancer Research UK Institute, Cambridge CB2 1QR, UK. Tel.: 44-122-333-4114. Fax: 44-122-333-4089. E-mail: j.raff@welc.cam.ac.uk

Kim Jeffers's present address is Virology Research and Development, Commonwealth Serum Laboratories, 45 Poplar Rd., Parkville 3052, Australia.

*Abbreviations used in this paper: aa, amino acid(s); APC/C, anaphase-promoting complex/cyclosome; CBTPM, cyclin B triple-point mutant; cdk, cyclin-dependent protein kinase; D-box, destruction box; Fzy, Fizzy; Fzr, Fizzy-related; GFP, green fluorescent protein; SPB, spindle pole body; TLCM, time-lapse confocal microscopy; WT, wild-type.

Key words: Cdc20; Cdh1; anaphase-promoting complex; cyclin B; mitosis

Supplemental Material can be found at:
http://jcb.rupress.org/content/suppl/2002/07/12/jcb.200203035.DC1.html

Published June 24, 2002

this takes ~1 h (Hazelrigg et al., 1998). Thus, in syncytial embryos (where cyclin B is continually synthesized and then partially degraded approximately every 10 min [Edgar et al., 1994]), many cyclin B–GFP molecules may not survive long enough to become fluorescent, so the signal is very weak. Nevertheless, when the behavior of the endogenous cyclin B was analyzed in fixed embryos, cyclin B was clearly degraded on the spindle, but it did not appear to be degraded in the cytoplasm (Huang and Raff, 1999). This suggests that in *Drosophila* there are two phases of cyclin B destruction that are temporally and spatially separable: the first phase destroys the spindle-associated cyclin B, whereas the second phase destroys the cytoplasmic cyclin B. In syncytial embryos, only the first phase of destruction seems to be initiated.

An attractive explanation for this spatially regulated destruction of cyclin B is that the APC/C is globally activated to degrade cyclin B, but is itself spatially restricted. Thus, the APC/C might initially be concentrated at centrosomes, move into the spindle, and finally be released into the cytoplasm. In support of this possibility, two core APC/C components, Cdc16 and Cdc27, have previously been shown to be concentrated on centrosomes and spindles in mammalian cells (Tugendreich et al., 1995). However, we have shown that only a small fraction of the APC/C associates with spindles in *Drosophila* embryos (Huang and Raff, 1999, 2002), suggesting that the APC/C cannot be globally activated to degrade cyclin B.

Two proteins, Fizzy (Fzy)/Cdc20 and Fzy-related (Fzr)/Cdh1, bind to the APC/C and are thought to target the APC/C to its various substrates (Pfleger et al., 2001). Experiments in yeasts and flies have led to the suggestion that Cdc20–APC/C complexes target proteins for destruction early in the exit from mitosis, whereas Cdh1–APC/C complexes target proteins for destruction later in the exit from mitosis and into G1 (Sigrist and Lehner, 1997; Visintin et al., 1997; Kramer et al., 1998, 2000). Therefore, we proposed that the subpopulation of the APC/C that associates with Fzy/Cdc20 might be responsible for the first phase of cyclin B destruction (that is restricted to the spindle), whereas a different subpopulation of the APC/C that associates with Fzr/Cdh1 might be responsible for the second phase of cyclin B destruction (that occurs in the cytoplasm) (Huang and Raff, 1999, 2002). In this paper we set out to test the respective roles of Fzy/Cdc20 and Fzr/Cdh1 in regulating the destruction of cyclin B in space and time.

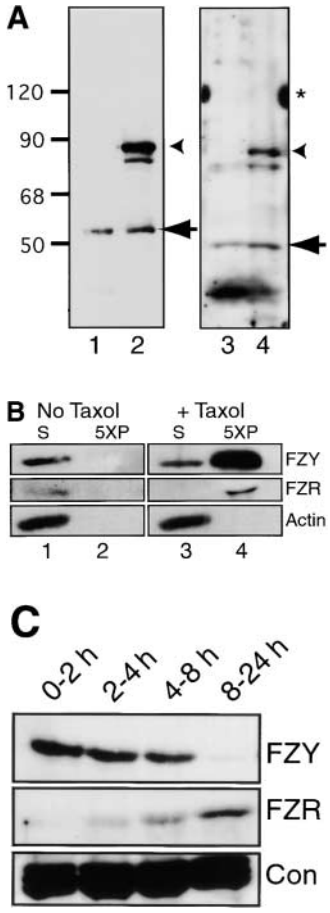

Figure 1. **The behavior of Fzy/Cdc20 and Fzr/Cdh1 in Western blotting experiments.** (A) WT embryos (lanes 1 and 3) or embryos expressing either GFP-Fzy (lane 2) or GFP-Fzr (lane 4) were probed with affinity-purified anti-Fzy (lanes 1 and 2) or anti-Fzr (lanes 3 and 4) antibodies. Arrows highlight the position of Fzy or Fzr; arrowheads highlight the position of GFP-Fzy or GFP-Fzr. The anti-Fzr antibodies also recognize several other proteins in the embryo extract, as well as the 120-kD marker protein (asterisk). The position of marker proteins is indicated at the left of the figure. (B) A microtubule spindown experiment probed with anti-Fzy (top), anti-Fzr (middle), or anti-actin (bottom) antibodies. In control experiments, where no taxol is added, all three proteins remain in the extract supernatant (S, lane 1) and are not present in the pellet (5 × P, lane 2; the 5× indicates that 5× more of the pellet was loaded on the gel relative to the supernatant). If taxol is added to the embryo extracts, a significant fraction (~50–70%) of Fzy/Cdc20 and Fzr/Cdh1 copellet with the microtubules, whereas actin does not (lane 4). (C) The developmental expression of Fzy (top) and Fzr (bottom) proteins. Equal numbers of 0–2-, 2–4-, 4–8-, or 8–24-h-old embryos were loaded in each lane. The bottom panel shows the levels of a nonspecific band recognized by the Fzr antibodies, shown here as a loading control.

Results

Fzy/Cdc20 is concentrated at centrosomes and kinetochores, whereas Fzr/Cdh1 is concentrated at centrosomes in *Drosophila* embryos

To investigate the role of Fzy/Cdc20 and Fzr/Cdh1 in regulating the destruction of cyclin B, we raised antibodies against both proteins and expressed GFP fusions of both proteins in *Drosophila* embryos (see Materials and methods). In Western blots, affinity-purified anti-Fzy antibodies recognized a prominent protein of ~55 kD (Fig. 1 A, lane 1), and they also recognized an extra band of the expected size in embryos expressing the GFP–Fzy fusion protein (Fig. 1, lane 2). The affinity-purified anti-Fzr antibodies recognized a protein of ~50 kD (Fig. 1, lane 3), and they also recognized an extra band of the expected size in embryos expressing the GFP–Fzr fusion protein (Fig. 1, lane 4).

To observe the dynamic localization of Fzy/Cdc20 and Fzr/Cdh1, we followed the behavior of GFP-Fzy and GFP-Fzr in living syncytial embryos using time-lapse confocal microscopy (TLCM) (Fig. 2). In interphase, GFP-Fzy was concentrated at centrosomes and was slightly excluded from nuclei (which appeared slightly darker than the surrounding cytoplasm; Fig. 2 A, 0:0). As soon as the nuclear envelope

Published June 24, 2002

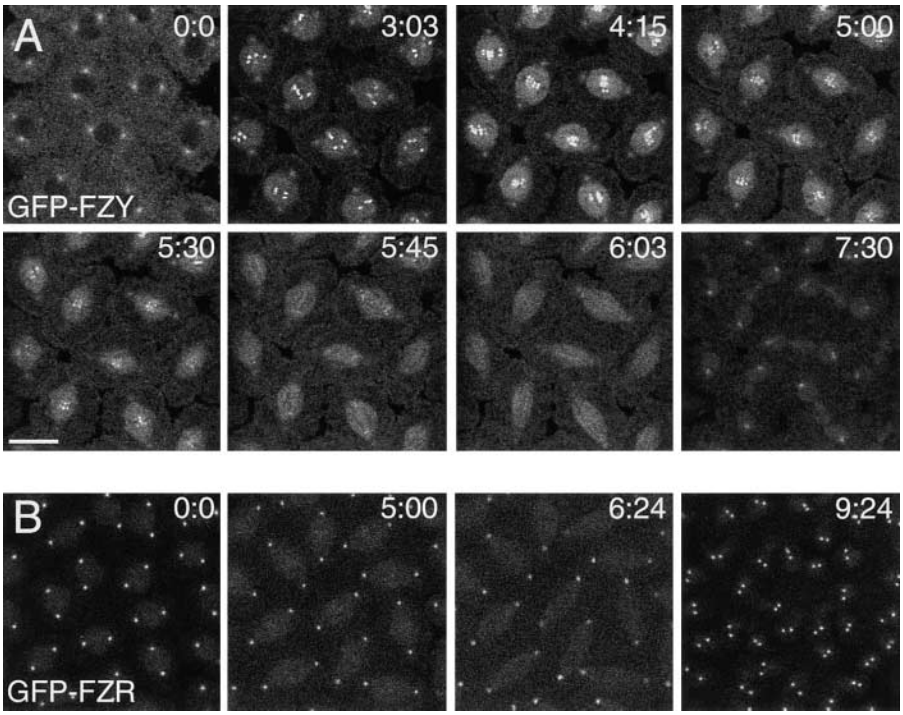

Figure 2. **The behavior of GFP-Fzy and GFP-Fzr in living syncytial embryos.** The GFP-Fzy (A)– and GFP-Fzr (B)–expressing embryos shown here were followed from interphase to telophase (see text for details). Time in minutes is shown at the top right of each panel. Bar, 20 μm.

started to break down, GFP-Fzy rapidly accumulated in the nuclear region where it associated with a small number of bright dots that appeared to be the kinetochores, as they rapidly lined up in pairs at the metaphase plate (Fig. 2 A, 3:03–4:15). Throughout this time, GFP-Fzy remained concentrated to a lesser extent on centrosomes and on the mitotic spindle (Fig. 2 A, 3:03–4:15). Once chromosomes aligned at the metaphase plate, GFP-Fzy started to disappear from the kinetochores, centrosomes, and spindle, although it was still detectable on the kinetochores as they moved toward the poles in anaphase (Fig. 2 A, 5:30–5:45). In late telophase, the protein was no longer detectable at kinetochores (Fig. 2 A, 7:30). A very similar localization pattern was observed in cellularized embryos (unpublished data).

In interphase, GFP-Fzr was slightly concentrated in the nuclei, and it was strongly concentrated at centrosomes throughout the cell cycle (Fig. 2 B). In mitosis, the protein was also detectable on the spindle, and it very weakly associated with regions of the mitotic chromatin late in mitosis; these regions are probably the kinetochores (see below). A very similar localization pattern was observed in cellularized embryos (unpublished data).

Fzy/Cdc20 and Fzr/Cdh1 interact with microtubules, but only Fzy/Cdc20 requires microtubules for its centrosomal localization during mitosis

As both GFP-Fzy and GFP-Fzr associate with centrosomes and spindles in mitosis, we tested whether the endogenous proteins physically interacted with microtubules. We added taxol to 0–24-h embryo extracts to polymerize the microtubules, and then pelleted the microtubules (together with any associated proteins) through a sucrose cushion. In control extracts, where no taxol was added, both Fzy/Cdc20 and Fzr/Cdh1 remained in the soluble fraction (Fig. 1 B, lane 1). In the presence of taxol, a significant fraction of both proteins (~50–70%) was detectable in the microtubule pellet (Fig. 1 B, lane 4). Thus, both proteins can interact with microtubules, although it is not clear whether this interaction is direct.

As both proteins interacted with microtubules, we tested whether either protein required microtubules for their localization at kinetochores and/or centrosomes. We injected living GFP-Fzy or GFP-Fzr embryos with colcemid during interphase of nuclear cycle 10–12, and observed the embryos on the confocal microscope. GFP-Fzy remained concentrated at interphase centrosomes after colcemid injection, although the localization was more diffuse than normal (Fig. 3 A, 0:0). As the embryos entered mitosis, GFP-Fzy levels decreased at centrosomes and increased at kinetochores (Fig. 3 A, 1:00–2:00). Injected embryos then appeared to terminally arrest in this mitotic state, with elevated levels of GFP-Fzy on the kinetochores (Fig. 3 A, 6:40). In some of these embryos we inactivated the colcemid with a short pulse of UV light (at 7:00 min for the embryo shown in Fig. 3 A). GFP-Fzy levels increased on the centrosomes and microtubules, and decreased on the kinetochores as the chromosomes became aligned on the reforming spindles (Fig. 3 A, 8:40–12:00). Thus, the localization of Fzy/Cdc20 to centrosomes during mitosis appears to be microtubule dependent, whereas its localization at kinetochores appears to be microtubule independent.

When colcemid was injected into GFP-Fzr–expressing embryos, it remained concentrated at centrosomes as the embryos entered, and then arrested in, mitosis (Fig. 3 B). In addition, GFP-Fzr eventually accumulated on a small number of chromatin-associated dots (Fig.3 B, 12:40, arrow). We suspect that these dots are the kinetochores, and that it is these structures that are normally weakly labeled by GFP-Fzr during mitosis in untreated embryos. Thus, Fzr/Cdh1 does not appear to require microtubules to concentrate at centrosomes.

Published June 24, 2002

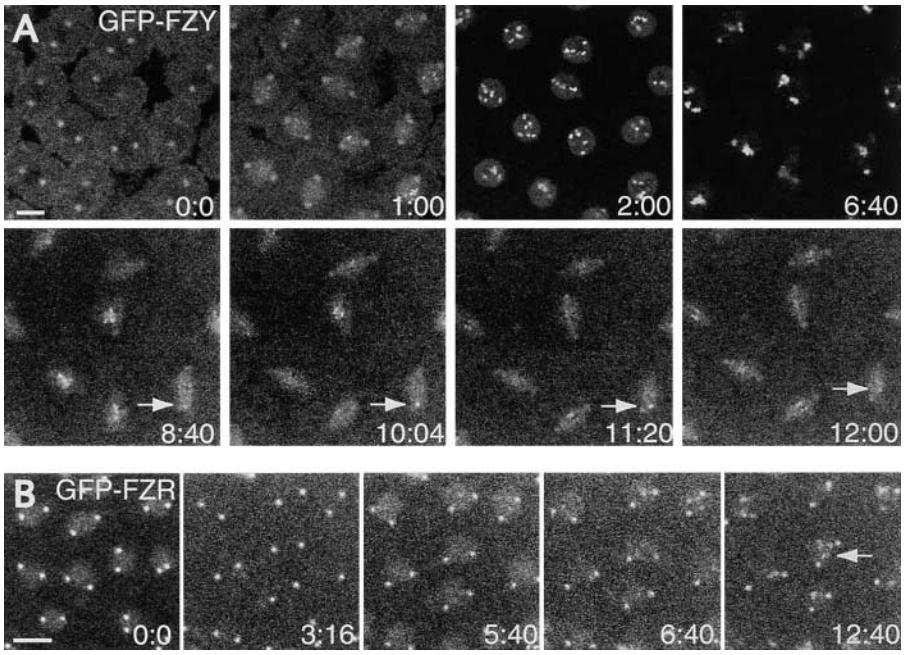

Figure 3. **The behavior of GFP-Fzy and GFP-Fzr in living embryos injected with colcemid.** Embryos were injected in interphase and they then arrested in a mitotic state (see text for details). The GFP-Fzy expressing embryo was then subjected to a single pulse of UV light at 7:00 min to inactivate the colcemid. The arrow in A highlights the position of a kinetochore that is delayed in lining up on the metaphase plate of the reforming spindle, and retains high levels of GFP-Fzy until it does so. The arrow in B highlights the GFP-Fzr dots that appear to associate with the chromosomes as the embryos arrest in mitosis. Time in minutes is shown in the bottom right of each panel. Bars, 20 μm.

Fzy/Cdc20 catalyzes the destruction of the spindle-associated cyclin B in syncytial embryos

In fixed syncytial embryos, cyclin B appears to be degraded only on the spindle (Huang and Raff, 1999). In this previous study, the destruction of cyclin B–GFP in living syncytial embryos could not be followed directly (Introduction). However, by expressing four copies of the cyclin B–GFP transgene in cyclin B–null mutant embryos, we have now followed the behavior of cyclin B–GFP in living syncytial embryos (Fig. 4). In these embryos, the spindle-associated cyclin B is degraded at the end of mitosis, but the levels of cytoplasmic cyclin B remain unchanged, directly confirming that only the spindle-associated cyclin B is degraded in syncytial embryos.

We previously suggested that the two phases of cyclin B destruction might be catalyzed sequentially by Fzy/Cdc20 and Fzr/Cdh1, and that the second phase of cyclin B destruction (that normally degrades cyclin B in the cytoplasm) might not occur in syncytial embryos because Fzr/Cdh1 is either not present or is inactive (Huang and Raff, 1999). Therefore, we probed Western blots of staged embryos to examine the expression profile of both proteins (Fig. 1 C). Fzy/Cdc20 was relatively abundant in 0–2-, 2–4-, and 4–8-h embryos, and was then dramatically downregulated in 8–24-h embryos. In contrast, Fzr/Cdh1 protein was essentially undetectable in 0–2-h embryos, and levels gradually increased as the embryos aged. Thus, only Fzy/Cdc20 is present in syncytial embryos, suggesting that Fzy/Cdc20 alone is responsible for targeting the spindle-associated cyclin B for degradation in these embryos.

To confirm that cyclin B in syncytial embryos was only being targeted for destruction by Fzy/Cdc20 (and that there was no contribution from a small pool of Fzr/Cdh1 that was undetectable by Western blotting), we tested whether a form of cyclin B that can only be degraded by Fzr/Cdh1 could be degraded in syncytial embryos. We noticed that *Drosophila* cyclin B has a KEN box as well as a destruction box (D-box). It has previously shown that Fzy/Cdc20 requires that its targets contain a D-box, whereas Fzr/Cdh1 can also recognize other, less well-defined sequences, such as a KEN box (Pfleger and Kirschner, 2000). Therefore, we reasoned that cyclin B molecules containing a mutated D-box would no longer be targeted for degradation by Fzy/Cdc20, but would be targeted for degradation by Fzr/Cdh1 (see below). We previously constructed transgenic flies containing an inducible form of

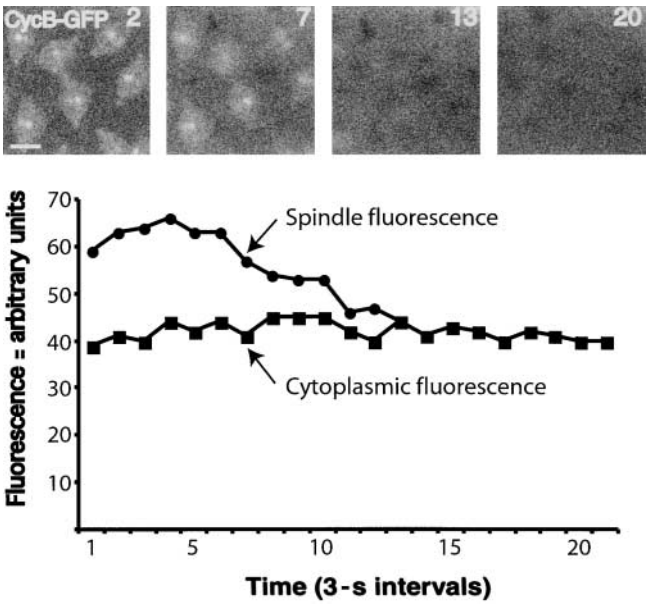

Figure 4. **The destruction of cyclin B–GFP in living syncytial embryos.** Selected images of a syncytial embryo exiting mitosis are shown here. The graph shows the quantitation (Materials and methods) of fluorescence intensity on the spindle (●) and in the cytoplasm (■) measured at 3-s intervals as the embryo exits mitosis. The number in the top right-hand corner of each image corresponds to the number on the time line of the graph. Bar, 20 μm.

Published June 24, 2002

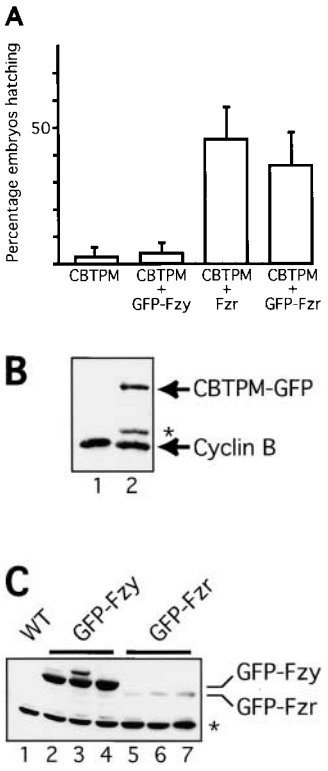

Figure 5. **The expression of Fzr/Cdh1 or GFP-Fzr partially rescues the lethality associated with expressing CBTPM–GFP in early embryos.** (A) A graph showing the percentage of embryos that hatch when CBTPM–GFP alone is expressed in embryos, or when CBTPM–GFP is coexpressed with GFP-Fzy, GFP-Fzr, or Fzr alone (as indicated below each bar). Error bars represent the standard deviation. (B) A Western blot showing the relative expression level of CBTPM–GFP in syncytial embryos compared with the endogenous cyclin B. Equal numbers of WT (lane 1) or CBTPM–GFP-expressing (lane 2) embryos were loaded in each lane. Arrows show the position of the endogenous cyclin B and of the CBTPM–GFP. The asterisk shows the position of a prominent breakdown product of CBTPM–GFP. (C) A Western blot showing the relative levels of expression of GFP-Fzy and GFP-Fzr. Equal numbers of syncytial embryos from three different lines expressing GFP-Fzy (lanes 2–4), GFP-Fzr (lanes 5–7), or a WT control (lane 1) were probed with anti GFP antibodies. The asterisk marks a crossreacting band that is recognized by the GFP antibodies in embryo extracts, shown here as a loading control. In all lines tested, the GFP-Fzy protein is expressed at ~10-fold higher levels than GFP-Fzr.

cyclin B–GFP that is mutated at all three of the most conserved D-box residues (called cyclin B triple-point mutant [CBTPM]–GFP) (Wakefield et al., 2000). When CBTPM–GFP was expressed in syncytial embryos, the vast majority of embryos failed to develop significantly (Fig. 5 A) and arrested in mitosis during the early syncytial divisions with their spindles in an anaphase-like state (as shown previously [Wakefield et al., 2000]). A small number of CBTPM–GFP-expressing embryos did develop (presumably because CBTPM–GFP was only expressed at relatively low levels in these embryos; Fig. 5 B). When we observed these developing embryos by TLCM, we found that CBTPM–GFP was not detectably degraded, and that it remained concentrated on centrosomes and spindles throughout the exit from mitosis (Fig. 6 A, compare with wild-type [WT] cyclin B–GFP in Fig. 4). Thus, in syncytial embryos, CBTPM–GFP cannot be degraded, strongly suggesting that Fzy/Cdc20 alone is normally responsible for degrading cyclin B in these embryos.

CBTPM-GFP appears to be degraded by Fzr/Cdh1 throughout the cell

To confirm that CBTPM–GFP could be degraded by Fzr/Cdh1, we ectopically expressed either Fzr, GFP-Fzr, or GFP-Fzy in syncytial embryos that also expressed CBTPM–GFP. The coexpression of Fzr or GFP-Fzr allowed ~30–50% of the CBTPM–GFP-expressing embryos to develop normally, whereas the overexpression of GFP-Fzy had no rescuing affect (Fig. 5 A), indicating that ectopically expressed Fzr or GFP-Fzr allows CBTPM–GFP to be degraded. The difference in the ability of GFP-Fzy and GFP-Fzr to overcome the mitotic arrest associated with expressing CBTPM–GFP was not due to differences in their expression levels. We observed that GFP-Fzy was always expressed at ~10-fold higher levels than GFP-Fzr in syncytial embryos (Fig. 5 C), even though the expression of both proteins was driven from the same promoter and both transgenic mRNAs contained identical 5′ and 3′ UTR's. Thus, in syncytial embryos, Fzr/Cdh1 appears to be intrinsically less stable than Fzy/Cdc20, perhaps explaining why Fzr/Cdh1 protein levels are so low in syncytial embryos, even though Fzr mRNA levels are relatively high (Sigrist and Lehner, 1997).

Our finding that CBTPM–GFP can be degraded by Fzr/Cdh1, but not by Fzy/Cdc20, allowed us to test how cyclin B might normally be degraded by Fzr/Cdh1 alone. We expressed CBTPM–GFP in otherwise wild-type cellularized embryos (that contain both Fzy/Cdc20 and Fzr/Cdh1). In cellularized embryos the nuclei no longer enter mitosis in synchrony (as occurs during the syncytial divisions). Instead, small domains of cells enter and exit mitosis at approximately the same time (Foe, 1989). We found that expressing CBTPM–GFP in cellularized embryos did not arrest cells in mitosis, and the protein was completely degraded at the end of mitosis (Fig. 6 B, arrows highlight the two domains of cells that have exited mitosis and have low levels of CBTPM–GFP fluorescence). However, on closer inspection it was clear the CBTPM–GFP was not degraded with normal kinetics. In cellularized embryos expressing WT cyclin B–GFP, the fusion protein initially started to disappear from the spindle, whereas protein levels in the cytoplasm remained relatively constant (Fig. 7 A). As the level of spindle fluorescence approached that of the cytoplasm, the cell entered anaphase and cyclin B–GFP started to disappear throughout the cell. In cellularized embryos expressing CBTPM–GFP (Fig. 7 B), the kinetics of destruction were more variable. In most cells, the rate of disappearance of CBTPM–GFP from the spindle was much slower than normal. As a result, when cells entered anaphase, CBTPM–GFP was almost always still detectable on the spindle, and the spindle remnants (the midbody, or central spindle) always contained high levels of CBTPM–GFP long after mitosis had finished (Fig. 6 B, arrowheads). Thus, Fzr/Cdh1 appears capable of catalyzing the destruction of CBTPM–GFP throughout the cell, although with slowed kinetics.

Published June 24, 2002

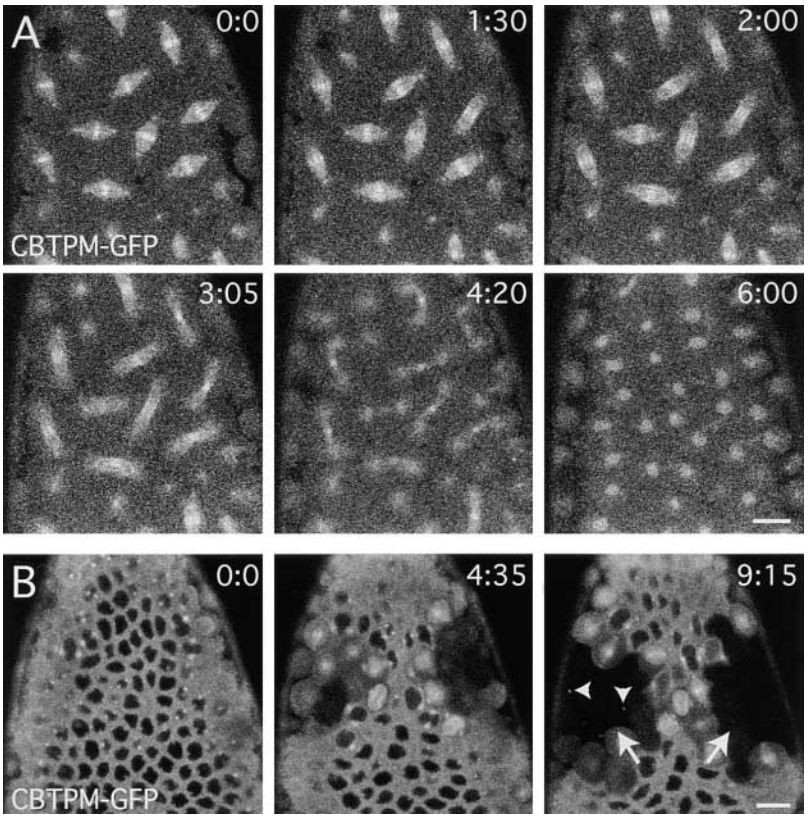

Figure 6. **The behavior of CBTPM–GFP in living syncytial or cellularized embryos.** (A) CBTPM–GFP usually arrests syncytial embryos in mitosis (Wakefield et al., 2000). However, in the syncytial embryo shown here, the spindles exit mitosis but CBTPM–GFP remains concentrated on centrosomes and spindles throughout this time (WT cyclin B–GFP is normally degraded on the spindle at this time; Fig. 4). (B) In a cellularized embryo, CBTPM–GFP is degraded at the end of mitosis (the arrows highlight the position of two mitotic domains that have exited mitosis). However, the kinetics of degradation are not normal, and the spindle remnants still contain CBTPM–GFP even after mitosis is finished (arrowheads). Time in minutes is shown in the top right of each panel. Bars, 10 μm.

GFP-Fzr turns over rapidly at the centrosome

Our finding that Fzr/Cdh1 is concentrated at centrosomes, raised the possibility that it could initially be activated to degrade cyclin B at centrosomes (see Discussion). If Fzr/Cdh1 is activated at centrosomes, how could it catalyze the destruction of cyclin B throughout the cytoplasm? To test whether the centrosomal pool of GFP-Fzr might rapidly exchange with the cytoplasmic pool, we performed a FRAP analysis. As controls, we also tested the behavior of two other GFP fusion proteins that are concentrated at centrosomes: tubulin-GFP (Grieder et al., 2000) and D-TACC-GFP (Gergely et al., 2000). We observed embryos expressing these fusion proteins on the confocal microscope, photobleached a small area of the embryo, and monitored the recovery of fluorescence (Fig. 8). As expected, the centrosomal fluorescence of tubulin-GFP recovered very rapidly, ($T_{1/2}$ ~10 s). The centrosomal fluorescence of GFP-Fzr recovered more slowly ($T_{1/2}$ ~45 s), but the centrosomal fluorescence of D-TACC-GFP recovered even more slowly ($T_{1/2}$ ~2 min). Thus, although GFP-Fzr is concentrated at centrosomes, it seems to exchange with a cytoplasmic pool of GFP-Fzr relatively rapidly. Note that for ease of presentation, the embryos shown here all remained in interphase throughout the time course of the experiment. However, similar results were obtained for all these GFP fusion proteins when embryos were analyzed at any stage of the cell cycle (unpublished data).

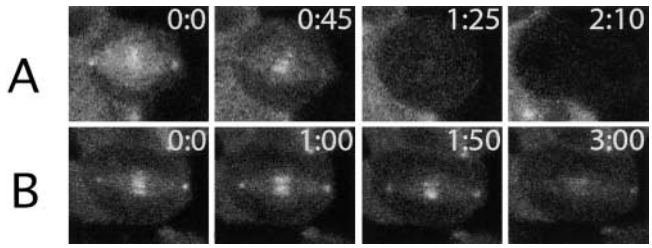

Figure 7. **A comparison of the destruction of cyclin B–GFP and CBTPM–GFP in cellularized embryos.** Whereas cyclin B–GFP (A) is no longer detectable on spindles as they enter anaphase, CBTPM–GFP (B) can still be detected on anaphase spindles. Quantitation of the fluorescence levels in these cells (Materials and methods) revealed that both fusion proteins were eventually completely degraded in both cells, apart from a small amount of protein that remained on the spindle remnants of the CBTPM–GFP-expressing cell (unpublished data; Fig. 6 B). The number in the top right-hand corner corresponds to the time in minutes.

We also performed a FRAP analysis with GFP-Fzy and found that the centrosomal GFP-Fzy also turned over rapidly during interphase ($T_{1/2}$ ~45 s, unpublished data). As GFP-Fzy normally starts to disappear from kinetochores, centrosomes, and spindles as the chromosomes align at the metaphase plate, it was impossible to measure a half life of GFP-Fzy on these structures during mitosis. Nevertheless, it was clear that GFP-Fzy also rapidly turned over at kinetochores, centrosomes, and spindles, at least during the early stages of mitosis. This is consistent with the possibility that GFP-Fzy may constantly be loading onto spindles via kinetochores during the early stages of mitosis (see Discussion).

Discussion

We have followed the subcellular localization of Fzy/Cdc20 and Fzr/Cdh1 throughout the cell cycle in living *Drosophila* embryos. We show that GFP-Fzy is concentrated on kineto-

Published June 24, 2002

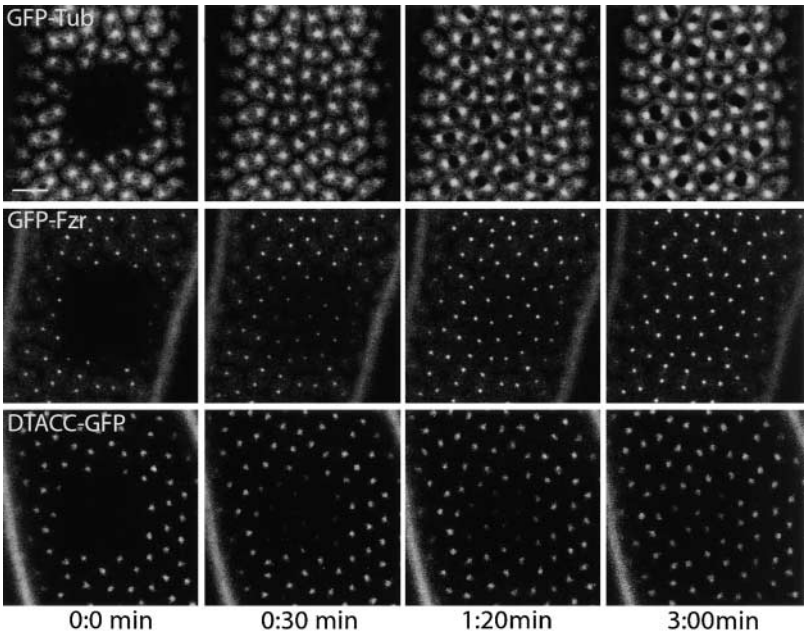

Figure 8. **GFP-Fzr is rapidly turned over at centrosomes.** Living embryos expressing GFP-tubulin (A), GFP-Fzr (B), or D-TACC-GFP (C) were followed on the confocal microscope. During interphase of nuclear cycle 10–12, a small region of the embryo was bleached with 100% laser power, and the recovery of fluorescence was followed. GFP-tubulin recovers quickly ($T_{1/2}$ ~10 s, Materials and methods); GFP-Fzr recovers at an intermediate rate ($T_{1/2}$ ~45 s); D-TACC-GFP recovers more slowly ($T_{1/2}$ ~2 min). Time, in minutes, after photobleaching is indicated at the bottom of the figure. Bar, 20 μm.

chores, centrosomes, and spindles early in mitosis, and starts to disappear from these structures once the chromosomes align at the metaphase plate. This localization is similar to that reported previously for $p55^{cdc20}$ in fixed human cells (Kallio et al., 1998), and it fits in well with the proposed role of Fzy/Cdc20 in linking the spindle assembly checkpoint to the APC/C. In higher eukaryotes, the spindle checkpoint system consists of several proteins, including the Mad and Bub proteins (Wells, 1996; Shah and Cleveland, 2000) as well as CenpE, Mps1, Rod, and ZW10 (Abrieu et al., 2000, 2001; Basto et al., 2000; Chan et al., 2000). As cells enter mitosis, most of these proteins accumulate on unattached kinetochores, and are then lost from the kinetochores once the chromosomes align at the metaphase plate. Several of these checkpoint proteins can bind to Fzy/Cdc20, and this appears to inhibit the ability of Fzy/Cdc20 to activate the APC/C (He et al., 1997; Fang et al., 1998; Hwang et al., 1998; Kallio et al., 1998; Lorca et al., 1998; Sudakin et al., 2001; Tang et al., 2001). Therefore, an unattached kinetochore is thought to continuously generate inhibitory checkpoint protein/Fzy (Cdc20) complexes, thus ensuring that the APC/C is not activated until all of the chromosomes have aligned properly at the metaphase plate.

The checkpoint proteins Mad2, BubR1, CENP-E, Rod, and ZW10 have all been shown to bind to kinetochores and then move along microtubules to the centrosomes in a dynein-dependent manner (Basto et al., 2000; Chan et al., 2000; Howell et al., 2001; Scaerou et al., 2001; Wojcik et al., 2001). We show that during mitosis, the localization of GFP-Fzy to kinetochores is microtubule independent, whereas its localization at centrosomes is microtubule dependent. This is consistent with the possibility that Fzy/Cdc20 may also load onto kinetochores and then move along microtubules to the centrosomes.

In contrast to GFP-Fzy, GFP-Fzr is strongly concentrated at centrosomes throughout the cell cycle, apparently in a microtubule-independent fashion. The concentration of Fzr/Cdh1 at centrosomes was unexpected, as we had previously proposed that Fzr/Cdh1 catalyzed the second phase of cyclin B destruction that occurs in the cytoplasm (Huang and Raff, 1999). However, our FRAP analysis suggests that Fzr/Cdh1 is rapidly turned over at centrosomes. Although the significance of this turnover is unclear, it is possible that Fzr (Cdh1)–APC/C complexes activated at centrosomes could diffuse throughout the cell to catalyze the destruction of cyclin B.

The respective roles of Fzy/Cdc20 and Fzr/Cdh1 in degrading cyclin B

We find that Fzy/Cdc20 protein is abundant in syncytial embryos, whereas Fzr/Cdh1 protein is virtually undetectable. Moreover, a D-box–mutated form of cyclin B (CBTPM–GFP), which cannot be targeted for destruction by Fzy/Cdc20, is not degraded on spindles in syncytial embryos. CBTPM–GFP can be targeted for destruction by Fzr/Cdh1, and, in cellularized embryos, where Fzr/Cdh1 is normally present, CBTPM–GFP is destroyed throughout the cell but with slowed kinetics. Taken together, these findings indicate that Fzy/Cdc20 alone is responsible for catalyzing the destruction of cyclin B on the spindle in syncytial embryos, whereas Fzr/Cdh1 can catalyze the destruction of cyclin B throughout the cell in cellularized embryos.

These results suggest a model of how the destruction of *Drosophila* cyclin B is regulated in space and time (Fig. 9). Early in mitosis (Fig. 9 A), inhibitory checkpoint protein/Fzy (Cdc20) complexes form at unattached kinetochores. We propose that these complexes are restricted to the spindle microtubules, and spread from the kinetochore to the centrosome, and then throughout the spindle. As the kinetochores align at the metaphase plate (Fig. 9 B), inhibitory complexes no longer form, and this leads to the activation of Fzy (Cdc20)–APC/C complexes. Exactly where and how this activation occurs is unclear, but we propose that only the specific pool of Fzy/Cdc20 that has passed through the kinetochore (and so is restricted to the spindle) is activated to degrade cyclin B. The destruction of cyclin B on the spindle then initiates the second phase of cyclin B destruction by ac-

Published June 24, 2002

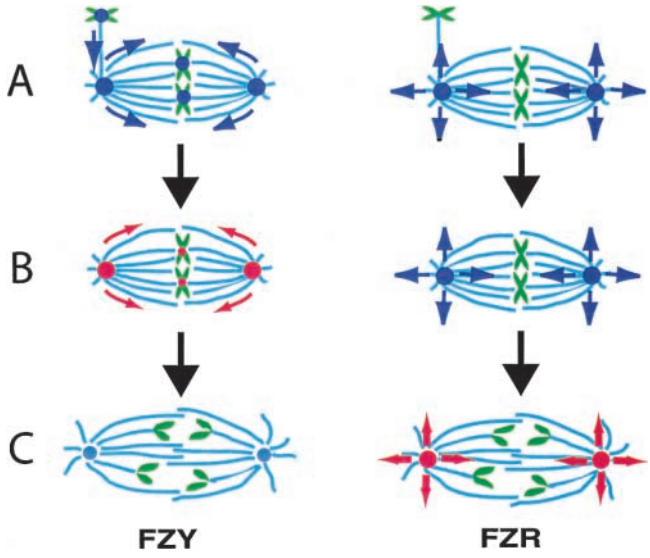

Figure 9. **A model of how the sequential activation of Fzy/Cdc20 and Fzr/Cdh1 regulates the destruction of cyclin B in space and time.** Chromosomes are shown in green and microtubules in light blue. When Fzy/Cdc20 (left) and Fzr/Cdh1 (right) are inactivate they are shown as dark blue, and when activated to degrade cyclin B they are shown as red. The motion of these molecules is depicted with arrows. (A) Early in mitosis, inactive Fzy (Cdc20)/checkpoint protein complexes are formed at unattached kinetochores, transported toward the centrosome, and then spread throughout the spindle. Fzr/Cdh1 constantly turns over at centrosomes, (indicated by the arrows showing the protein leaving the centrosome) but is inactive as cyclin B/cdc2 activity is high. (B) Once all the chromosomes align at the metaphase plate, inhibitory complexes of Fzy (Cdc20)/checkpoint proteins are no longer generated at kinetochores and Fzy/Cdc20 is activated to degrade cyclin B at centrosomes (or perhaps at kinetochores). The activated Fzy/Cdc20 complexes spread along the spindle microtubules degrading cyclin B in a wave that appears to spread from the centrosome to the spindle equator. (C) The destruction of cyclin B lowers cdc2 kinase activity at the centrosome, leading to the activation of the Fzr/Cdh1 at the centrosome. The active Fzr/Cdh1 complexes are not restricted to microtubules, and can degrade cyclin B throughout the cell.

tivating Fzr/Cdh1–APC/C complexes (Kramer et al., 2000). Unlike the Fzy/Cdc20 complexes, activated Fzr/Cdh1 complexes are not restricted to spindle microtubules, and can target cyclin B for destruction throughout the cell (Fig. 9 C).

As the destruction of cyclin B appears to initiate at centrosomes, we suspect that the Fzy (Cdc20)–APC/C complexes initially become activated to degrade cyclin B at centrosomes. Presumably, the activated complexes then spread along the microtubules toward the spindle equator. This would explain why, in syncytial *Drosophila* embryos where only Fzy/Cdc20 is present, the attachment between centrosomes and spindles appears to be essential for the destruction of the spindle-associated cyclin B (Wakefield et al., 2000). Why Fzy/Cdc20 might initially be activated at centrosomes is unclear. Perhaps the disassembly of the inhibitory checkpoint protein/Fzy (Cdc20) oligomers that form at the unattached kinetochores requires some activity that is concentrated at centrosomes.

We stress that this model applies only to the destruction of cyclin B. For example, cyclin A is also targeted for destruction by Fzy (Cdc20)–APC/C complexes (Dawson et al., 1995), but it is not concentrated on spindles (Pines and Hunter, 1991). It seems unlikely that Fzy/Cdc20 also catalyzes the destruction of cyclin A only on the spindle. Therefore, we speculate that there must be separate pools of Fzy/Cdc20 that are responsible for degrading cyclin A and B. An attractive aspect of our model is that it explains how these different pools are generated. Only the pool of Fzy/Cdc20 that passes through the kinetochore is inhibited from activating the APC/C by the spindle checkpoint system, and only this pool of Fzy/Cdc20 is competent to catalyze the destruction of cyclin B. In this way, the destruction of cyclin B is inhibited by the spindle checkpoint system, whereas the destruction of cyclin A is not (Whitfield et al., 1990; den Elzen and Pines, 2001; Geley et al., 2001).

A general mechanism for regulating the destruction of cyclin B?

Could this mechanism for regulating cyclin B destruction in *Drosophila* embryos apply to other systems? If two vertebrate mitotic cells are fused to form a single cell, the presence of an unattached kinetochore on one spindle (spindle A) does not block the exit from mitosis on the other spindle (spindle B) once the chromosomes on spindle B have aligned (Rieder et al., 1997). Moreover, once spindle B exits mitosis, spindle A exits mitosis soon afterwards, even if some of its kinetochores remain unattached. These observations are consistent with our model. We would predict that the Fzy (Cdc20)/checkpoint-protein complexes generated at the unattached kinetochores of spindle A are restricted to microtubules and so cannot inhibit the exit from mitosis on the neighboring spindle B. Moreover, the activation of Fzy/Cdc20 on spindle B would eventually activate Fzr (Cdh1)–APC/C complexes on spindle B. These complexes can then spread throughout the cell, ultimately degrading cyclin B on spindle A and forcing it to exit mitosis. The degradation of clb2 in *S. cerevisiae* also occurs in two phases that appear to be catalyzed sequentially by Fzy/Cdc20 and Fzr/Cdh1 (Yeong et al., 2000), although the spatial organization of this destruction has not been investigated.

However, our model cannot explain how cyclin B is degraded in early *Xenopus* embryo extracts. Like early *Drosophila* embryos, these extracts contain Fzy/Cdc20, but lack Fzr/Cdh1 (Kramer et al., 2000). Nonetheless, cyclin B is completely degraded at the end of mitosis in these extracts, even if no nuclei or spindles are present. Thus, Fzy/Cdc20 can catalyze the destruction of cyclin B that is not spindle associated in *Xenopus* extracts. The reason for this apparent difference is unclear. However, we note that early *Xenopus* extracts do not have a functional spindle checkpoint (Minshull et al., 1994). As discussed above, the mechanisms that link the destruction of cyclin B to the spindle checkpoint may also be required to restrict Fzy/Cdc20 complexes to the mitotic spindle.

The role of the centrosome in regulating the exit from mitosis

Our finding that Fzy/Cdc20 and Fzr/Cdh1 are concentrated at centrosomes highlights the potential importance of this organelle in regulating the exit from mitosis (Rieder et al.,

Published June 24, 2002

2001). We speculate that the concentration of these proteins at centrosomes might serve two purposes. First, it might enhance the fidelity of their sequential activation. The inactivation of cyclin B/cdc2 triggered by Fzy/Cdc20 seems to start at centrosomes, and cyclin B levels might only have to fall below a certain threshold level at the centrosome (rather than throughout the whole cell) to trigger the activation of the centrosomal Fzr/Cdh1. Second, in budding yeast there is a second, Bub2-dependent checkpoint that monitors the positioning of the spindle between the mother and daughter cell (Bardin et al., 2000; Daum et al., 2000; Pereira et al., 2000). Bub2 is concentrated at the spindle pole body where it is thought to suppress the activation of the mitotic exit network, and so block the activation of Fzr/Cdh1 and the exit from mitosis (Gardner and Burke, 2000). It is not clear if mammalian cells also have a spindle orientation checkpoint, but if they do, the concentration of Fzr/Cdh1 at centrosomes may be important for the function of this checkpoint.

Materials and methods

Production of antibodies

PCR was used to amplify the DNA encoding amino acids (aa) 2–194 of the Fzy protein and aa 2–137 of the Fzr protein. This DNA was subcloned, in frame, into the pMal vector (NEB), and the resulting MBP fusion proteins were purified from bacteria according to the manufacturer's instructions. The purified fusion proteins were used to generate antibodies in rabbits. All injections and bleeds were performed by Eurogentec. The antibodies were affinity purified and stored as described previously (Huang and Raff, 1999). The antibodies were used at 1–2 μg/ml in Western blotting experiments.

Construction of GFP-Fzy– and GFP-Fzr–expressing lines

Full-length cDNAs for Fzy and Fzr were obtained from Research Genetics and from Christian Lehner (University of Bayreuth, Bayreuth, Germany), respectively. The coding sequences were modified by PCR so that mGFP6 (Schuldt et al., 1998) could be cloned, in frame, onto the NH_2 terminus of both proteins. The GFP fusion proteins were then subcloned into the pWR-pUBq *Drosophila* transformation vector, putting their expression under the control of the polyubiquitin promoter that is expressed at relatively high levels throughout *Drosophila* development (Lee et al., 1988). Full details of these cloning procedures are available upon request. These plasmids were then used to generate stable fly lines using standard P-element–mediated transformation (Roberts, 1986).

Transgenic lines expressing CBTPM–GFP

The transgenic lines expressing CBTPM–GFP under the control of the UASp promoter (Rorth, 1998) have been described previously (the conserved D-box sequence RXXLXXXXN has been mutated to GXXAXXXXA, Wakefield et al., 2000). The expression of this protein in cellularized embryos was achieved by crossing males carrying the UAS-CBTPM–GFP transgene to females carrying a transgenic Gal4/VP16 fusion protein whose expression was under the control of the 67C maternal α-tubulin promoter (that drives high levels of expression during oogenesis, Micklem et al., 1997). In this way, the early embryo has high levels of the Gal4/VP16 fusion protein, but it only drives significant transcription of the CBTPM–GFP fusion protein (that comes into the embryo on the male chromosomes) at cellularization, when bulk transcription is initiated. To observe the effect of expressing CBTPM–GFP in syncytial embryos, embryos were collected from the females produced in this first cross. These embryos are derived from females carrying both the UAS-CBTPM–GFP and Gal4/VP16 transgenes, so expression of CBTPM–GFP is driven throughout oogenesis and early embryos express significant amounts of this protein.

Microtubule spindown experiments

Microtubule spindown experiments were performed with extracts made from 0–24-h embryos as described previously (Raff et al., 1993).

Observation of living embryos by TLCM

Living embryos expressing GFP-Fzy, GFP-Fzr, cyclin B–GFP, or CBTPM–GFP were observed using a Bio-Rad Radiance confocal system mounted on a Nikon microscope as described previously (Huang and Raff, 1999). Embryos that were to be injected with colcemid were observed using a Bio-Rad 1024 confocal system mounted on a Nikon inverted microscope. The embryos were observed on the confocal system until they entered interphase of nuclear cycle 10–12. They were then injected with colcemid (100 mM, dissolved in water), using our own manual injection system mounted on the inverted microscope. The embryos were then followed again on the confocal system. In some embryos, the colcemid was subsequently inactivated by opening the shutter on the microscopes UV lamp and exposing the embryo to either a single pulse of UV light (for 30 s) or to several pulses of UV light (20 s pulses at 2-min intervals). To make movies of these embryos, the image stacks were imported into Adobe® Photoshop® and adjusted to use the full range of pixel intensities. Images were made into movies using Adobe® Premier® .

If the images were to be used to quantitate fluorescence levels, only the raw images were used. Sequential images of embryos were imported into NIH Image, and the average pixel intensity in a manually defined area either on the spindle or in the cytoplasm was calculated. In addition, images were taken of several non-GFP–expressing embryos using the same settings on the confocal microscope. The average pixel intensity from these embryos was used to calculate a zero pixel intensity. For example, in Fig. 4, the pixel intensity on the spindle falls during mitosis until it reaches the same pixel intensity of the cytoplasm. However, the pixel intensity in the cytoplasm is not zero, indicating that this cytoplasmic fluorescence is likely due to cyclin B–GFP and not to a background fluorescence in the embryo.

FRAP analysis

Living embryos expressing either GFP-Fzy, GFP-Fzr, GFP-α-tubulin, or D-TACC-GFP were observed on the Bio-Rad Radiance confocal system. Using our own Macro (written by Alex Sossick) an embryo was imaged, and a small area of the embryo was then selected manually on the computer screen. This area was then photobleached by exposing only this area of the embryo to several passes of the scanning laser on 100% power. Photobleaching was monitored visually, and, when complete, the whole embryo was then imaged again on normal laser power (usually 1–5% of full power). The images acquired during the recovery period were imported into NIH Image. To calculate the half life of the fusion proteins at the centrosome, a line was defined manually that passed through the area of interest (i.e., through the middle of two centrosomes, one in the nonbleached area and one in the bleached area) and the pixel intensity along this line was calculated. The time at which the pixel intensity on the bleached centrosome reached half of the pixel intensity on the nonbleached centrosome could then be estimated.

Western blotting

SDS-PAGE and Western blotting were performed as described previously (Towbin et al., 1979). Blots were probed with affinity-purified antibodies at 1–2 μg/ml, or with DM1α anti-tubulin mouse ascites fluid (Sigma-Aldrich) or with JLA20 mouse monoclonal anti-actin antibody (both at 1/1,000 dilution; Developmental Studies Hybridoma Bank, Iowa City, IA), and then with the appropriate peroxidase-conjugated secondary antibody at 1/2,000 dilution (Amersham Pharmacia Biotech). Blots were developed using the SuperSignal enhanced chemiluminescence kit (Pierce Chemical Co.) according to the manufacturer's instructions.

Supplemental Material

Movies of the embryos shown in Figs. 2, 3, 4, and 6 are available online at http://www.jcb.org/cgi/content/full/jcb.200203035/DC1. Video 1 shows GFP-Fzy in a WT embryo (Fig. 2 A). Video 2 shows GFP-Fzr in a WT embryo (Fig. 2 B). Video 3 shows GFP-Fzy in a colcemid-injected embryo (Fig. 3 A). Video 4 shows GFP-Fzr in a colcemid-injected embryo (Fig. 3 B). Video 5 shows cyclin B–GFP in a WT syncytial embryo (Fig. 4). Video 6 shows CBTPM-GFP in a WT syncytial embryo (Fig. 6 A). Video 7 shows CBTPM-GFP in a WT cellularized embryo. Note that due to memory limitations, only parts of some of the movies are included in these files.

We thank Christian Lehner for supplying a full length anti-Fzr cDNA and the cyclin B^2 mutant fly strain. We thank Alex Sossick for writing the FRAP macro for the confocal microscope, and members of the lab for support and helpful discussions throughout the course of this work.

This work was supported by a Wellcome Trust Senior Research Fellowship in Basic Biomedical Sciences (J.W. Raff).

Submitted: 7 March 2002
Revised: 17 May 2002
Accepted: 17 May 2002

References

Abrieu, A., J.A. Kahana, K.W. Wood, and D.W. Cleveland. 2000. CENP-E as an essential component of the mitotic checkpoint in vitro. *Cell.* 102:817–826.

Abrieu, A., L. Magnaghi-Jaulin, J.A. Kahana, M. Peter, A. Castro, S. Vigneron, T. Lorca, D.W. Cleveland, and J.C. Labbe. 2001. Mps1 is a kinetochore-associated kinase essential for the vertebrate mitotic checkpoint. *Cell.* 106:83–93.

Bardin, A.J., R. Visintin, and A. Amon. 2000. A mechanism for coupling exit from mitosis to partitioning of the nucleus. *Cell.* 102:21–31.

Basto, R., R. Gomes, and R.E. Karess. 2000. Rough deal and Zw10 are required for the metaphase checkpoint in *Drosophila*. *Nat. Cell Biol.* 2:939–943.

Chan, G.K., S.A. Jablonski, D.A. Starr, M.L. Goldberg, and T.J. Yen. 2000. Human Zw10 and ROD are mitotic checkpoint proteins that bind to kinetochores. *Nat. Cell Biol.* 2:944–947.

Clute, P., and J. Pines. 1999. Temporal and spatial control of cyclin B1 destruction in metaphase. *Nat.Cell Biol.* 1:82–87.

Daum, J.R., N. Gomez-Ospina, M. Winey, and D.J. Burke. 2000. The spindle checkpoint of *Saccharomyces cerevisiae* responds to separable microtubule-dependent events. *Curr. Biol.* 10:1375–1378.

Dawson, I.A., S. Roth, and S. Artavanis-Tsakonas. 1995. The *Drosophila* cell cycle gene fizzy is required for normal degradation of cyclins A and B during mitosis and has homology to the CDC20 gene of *Saccharomyces cerevisiae*. *J. Cell Biol.* 129:725–737.

Decottignies, A., P. Zarzov, and P. Nurse. 2001. In vivo localisation of fission yeast cyclin-dependent kinase cdc2p and cyclin B cdc13p during mitosis and meiosis. *J. Cell Sci.* 114:2627–2640.

den Elzen, N., and J. Pines. 2001. Cyclin A is destroyed in prometaphase and can delay chromosome alignment and anaphase. *J. Cell Biol.* 153:121–136.

Edgar, B.A., F. Sprenger, R.J. Duronio, P. Leopold, and P.H. O'Farrell. 1994. Distinct molecular mechanisms regulate cell cycle timing at successive stages of *Drosophila* embryogenesis. *Genes Dev.* 8:440–452.

Fang, G., H. Yu, and M.W. Kirschner. 1998. The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. *Genes Dev*. 12:1871–1883.

Foe, V.E. 1989. Mitotic domains reveal early commitment of cells in *Drosophila* embryos. *Development.* 107:1–22.

Gardner, R.D., and D.J. Burke. 2000. The spindle checkpoint: two transitions, two pathways. *Trends Cell Biol.* 10:154–158.

Geley, S., E. Kramer, C. Gieffers, J. Gannon, J.M. Peters, and T. Hunt. 2001. Anaphase-promoting complex/cyclosome-dependent proteolysis of human cyclin A starts at the beginning of mitosis and is not subject to the spindle assembly checkpoint. *J. Cell Biol.* 153:137–148.

Gergely, F., D. Kidd, K. Jeffers, J.G. Wakefield, and J.W. Raff. 2000. D-TACC: a novel centrosomal protein required for normal spindle function in the early *Drosophila* embryo. *EMBO J.* 19:241–252.

Glotzer, M., A.W. Murray, and M.W. Kirschner. 1991. Cyclin is degraded by the ubiquitin pathway. *Nature.* 349:132–138.

Grieder, N.C., M. de Cuevas, and A.C. Spradling. 2000. The fusome organizes the microtubule network during oocyte differentiation in *Drosophila*. *Development.* 127:4253–4264.

Harper, J.W., and S.J. Elledge. 1996. Cdk inhibitors in development and cancer. *Curr. Opin. Genet. Dev.* 6:56–64.

Hazelrigg, T., N. Liu, Y. Hong, and S. Wang. 1998. GFP expression in *Drosophila* tissues: time requirements for formation of a fluorescent product. *Dev. Biol.* 199:245–249.

He, X., T.E. Patterson, and S. Sazer. 1997. The *Schizosaccharomyces pombe* spindle checkpoint protein mad2p blocks anaphase and genetically interacts with the anaphase-promoting complex. *Proc. Natl. Acad. Sci. USA.* 94:7965–7970.

Hershko, A., D. Ganoth, V. Sudakin, A. Dahan, L.H. Cohen, F.C. Luca, J.V. Ruderman, and E. Eytan. 1994. Components of a system that ligates cyclin to ubiquitin and their regulation by the protein kinase cdc2. *J. Biol. Chem.* 269: 4940–4946.

Howell, B.J., B.F. McEwen, J.C. Canman, D.B. Hoffman, E.M. Farrar, C.L. Rieder, and E.D. Salmon. 2001. Cytoplasmic dynein/dynactin drives kinetochore protein transport to the spindle poles and has a role in mitotic spindle checkpoint inactivation. *J. Cell Biol.* 155:1159–1172.

Huang, J., and J.W. Raff. 1999. The disappearance of cyclin B at the end of mitosis is regulated spatially in *Drosophila* cells. *EMBO J.* 18:2184–2195.

Huang, J., and J.W. Raff. 2002. The dynamic localisation of the *Drosophila* APC/C: evidence for the existence of multiple complexes that perform distinct functions and are differentially localised. *J. Cell Biol.* In press.

Hwang, L.H., L.F. Lau, D.L. Smith, C.A. Mistrot, K.G. Hardwick, E.S. Hwang, A. Amon, and A.W. Murray. 1998. Budding yeast Cdc20: a target of the spindle checkpoint. *Science.* 279:1041–1044.

Irniger, S., S. Piatti, C. Michaelis, and K. Nasmyth. 1995. Genes involved in sister chromatid separation are needed for B type cyclin proteolysis in budding yeast. *Cell.* 81:269–278.

Kallio, M., J. Weinstein, J.R. Daum, D.J. Burke, and G.J. Gorbsky. 1998. Mammalian p55CDC mediates association of the spindle checkpoint protein Mad2 with the cyclosome/anaphase-promoting complex, and is involved in regulating anaphase onset and late mitotic events. *J. Cell Biol.* 141:1393–1406.

King, R.W., J.H. Peters, S. Tugendreich, P. Hieter, M. Rolfe, and M.W. Kirschner. 1995. A 20s complex containing cdc27 and cdc16 catalyzes the mitosis specific conjugation of ubiquitin to cyclin B. *Cell.* 81:279–288.

Kramer, E.R., C. Gieffers, G. Holzl, M. Hengstschlager, and J.M. Peters. 1998. Activation of the human anaphase-promoting complex by proteins of the CDC20/Fizzy family. *Curr. Biol.* 8:1207–1210.

Kramer, E.R., N. Scheuringer, A.V. Podtelejnikov, M. Mann, and J.M. Peters. 2000. Mitotic regulation of the APC activator proteins CDC20 and CDH1. *Mol. Biol. Cell.* 11:1555–1569.

Lee, H.S., J.A. Simon, and J.T. Lis. 1988. Structure and expression of ubiquitin genes of *Drosophila* melanogaster. *Mol. Cell. Biol.* 8:4727–4735.

Lew, D.J., and S. Kornbluth. 1996. Regulatory roles of cyclin dependent kinase phosphorylation in cell cycle control. *Curr. Opin. Cell Biol.* 8:795–804.

Lorca, T., A. Castro, A.M. Martinez, S. Vigneron, N. Morin, S. Sigrist, C. Lehner, M. Doree, and J.C. Labbe. 1998. Fizzy is required for activation of the APC/cyclosome in *Xenopus* egg extracts. *EMBO J.* 17:3565–3575.

Micklem, D.R., R. Dasgupta, H. Elliott, F. Gergely, C. Davidson, A. Brand, A. Gonzalez-Reyes, and D. St. Johnston. 1997. The mago nashi gene is required for the polarisation of the oocyte and the formation of perpendicular axes in *Drosophila*. *Curr. Biol.* 7:468–478.

Minshull, J., H. Sun, N.K. Tonks, and A.W. Murray. 1994. A MAP kinase-dependent spindle assembly checkpoint in *Xenopus* egg extracts. *Cell.* 79:475–486.

Pereira, G., T. Hofken, J. Grindlay, C. Manson, and E. Schiebel. 2000. The Bub2p spindle checkpoint links nuclear migration with mitotic exit. *Mol. Cell.* 6:1–10.

Pfleger, C.M., and M.W. Kirschner. 2000. The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1. *Genes Dev.* 14:655–665.

Pfleger, C.M., E. Lee, and M.W. Kirschner. 2001. Substrate recognition by the Cdc20 and Cdh1 components of the anaphase- promoting complex. *Genes Dev.* 15:2396–2407.

Pines, J., and T. Hunter. 1991. Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. *J. Cell Biol.* 115:1–17.

Raff, J.W., D.R. Kellogg, and B.M. Alberts. 1993. *Drosophila* gamma-tubulin is part of a complex containing two previously identified centrosomal MAPs. *J. Cell Biol.* 121:823–835.

Rieder, C.L., A. Khodjakov, L.V. Paliulis, T.M. Fortier, R.W. Cole, and G. Sluder. 1997. Mitosis in vertebrate somatic cells with two spindles: implications for the metaphase/anaphase transition checkpoint and cleavage. *Proc. Natl. Acad. Sci. USA.* 94:5107–5112.

Rieder, C.L., S. Faruki, and A. Khodjakov. 2001. The centrosome in vertebrates: more than a microtubule-organizing center. *Trends Cell Biol.* 11:413–419.

Roberts, D. 1986. *Drosophila*, A Practical Approach. IRL Press, Oxford. 295 pp.

Rorth, P. 1998. Gal4 in the *Drosophila* female germline. *Mech. Dev.* 78:113–118.

Scaerou, F., D.A. Starr, F. Piano, O. Papoulas, R.E. Karess, and M.L. Goldberg. 2001. The ZW10 and rough deal checkpoint proteins function together in a large, evolutionarily conserved complex targeted to the kinetochore. *J. Cell Sci.* 114:3103–3114.

Schuldt, A.J., J.H. Adams, C.M. Davidson, D.R. Micklem, J. Haseloff, D.S. Johnston, and A.H. Brand. 1998. Miranda mediates asymmetric protein and RNA localization in the developing nervous system. *Genes Dev.* 12:1847–1857.

Shah, J.V., and D.W. Cleveland. 2000. Waiting for anaphase: Mad2 and the spindle assembly checkpoint. *Cell.* 103:997–1000.

Sigrist, S.J., and C.F. Lehner. 1997. *Drosophila* fizzy-related down-regulates mitotic cyclins and is required for cell proliferation arrest and entry into endocycles. *Cell.* 90:671–681.

Su, T.T., F. Sprenger, P.J. DiGregorio, S.D. Campbell, and P.H. O'Farrell. 1998. Exit from mitosis in *Drosophila* syncytial embryos requires proteolysis and cyclin degradation, and is associated with localized dephosphorylation. *Genes Dev.* 12:1495–1503.

Sudakin, V., G.K. Chan, and T.J. Yen. 2001. Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2. *J. Cell Biol.* 154:925–936.

Tang, Z., R. Bharadwaj, B. Li, and H. Yu. 2001. Mad2-Independent inhibition of

APCCdc20 by the mitotic checkpoint protein BubR1. *Dev Cell*. 1:227–237.

Towbin, H., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. Natl. Acad. Sci. USA.* 80:4350–4354.

Tugendreich, S., J. Tomkiel, W. Earnshaw, and P. Hieter. 1995. Cdc27hs colocalizes with cdc16hs to the centrosome and mitotic spindle and is essential for the metaphase to anaphase transition. *Cell.* 81:261–268.

Visintin, R., S. Prinz, and A. Amon. 1997. CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis. *Science.* 278:460–463.

Wakefield, J.G., J.Y. Huang, and J.W. Raff. 2000. Centrosomes have a role in regulating the destruction of cyclin B in early *Drosophila* embryos. *Curr. Biol.* 10:1367–1370.

Wells, W.A.E. 1996. The spindle assembly checkpoint: aiming for a perfect mitosis, every time. *Trends Cell Biol.* 6:228–234.

Whitfield, W.G.F., C. Gonzalez, G. Maldonado-Codina, and D.M. Glover. 1990. The A- and B-type cyclins of *Drosophila* are accumulated and destroyed in temporally distinct events that define separable phases of the G2-M transition. *EMBO J.* 9:2563–2572.

Wojcik, E., R. Basto, M. Serr, F. Scaerou, R. Karess, and T. Hays. 2001. Kinetochore dynein: its dynamics and role in the transport of the rough deal checkpoint protein. *Nat. Cell Biol.* 3:1001–1007.

Yanagida, M., Y.M. Yamashita, H. Tatche, K. Ishii, K. Kumada, Y. Nakesako. 1999. Control of metaphase–anaphase progression by protolysis: cyclosome function regulated by the protein kinase A pathway, ubiquitination and localization. *Philos. Trans. R. Soc. Land. B. Biol. Sci*. 354:1559–1569.

Yeong, F.M., H.H. Lim, C.G. Padmashree, and U. Surana. 2000. Exit from mitosis in budding yeast: biphasic inactivation of the Cdc28-Clb2 mitotic kinase and the role of Cdc20. *Mol. Cell.* 5:501–511.