Centrosomes have a role in regulating the destruction of cyclin B in early *Drosophila* embryos

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We reported previously that the disappearance of cyclin B at the end of mitosis in early *Drosophila* embryos starts at centrosomes and spreads into the spindle [1]. Here, we used a novel mutation, *centrosome fall off* (*cfo*), to investigate whether centrosomes are required to initiate the disappearance of cyclin B from the spindle. In embryos laid by homozygous *cfo* mutant mothers, the centrosomes co-ordinately detached from the mitotic spindle during mitosis, and the centrosomeless spindles arrested at anaphase. Cyclin B levels decreased on the detached centrosomes, but not on the arrested centrosomeless spindles, presumably explaining why the spindles arrest in anaphase in these embryos. We found that the expression of a non-degradable cyclin B in embryos also caused an anaphase arrest, but most centrosomes remained attached to the arrested spindles, and non-degradable cyclin B levels remained high on both the centrosomes and spindles. These findings suggest that the disappearance of cyclin B from centrosomes and spindles is closely linked to its destruction, and that a connection between centrosomes and spindles is required for the proper destruction of the spindle-associated cyclin B in early *Drosophila* embryos. These results may have important implications for the mechanism of the spindle-assembly checkpoint, as they suggest that unattached kinetochores may arrest cells in mitosis, at least in part, by signalling to centrosomes to block the initiation of cyclin B destruction.

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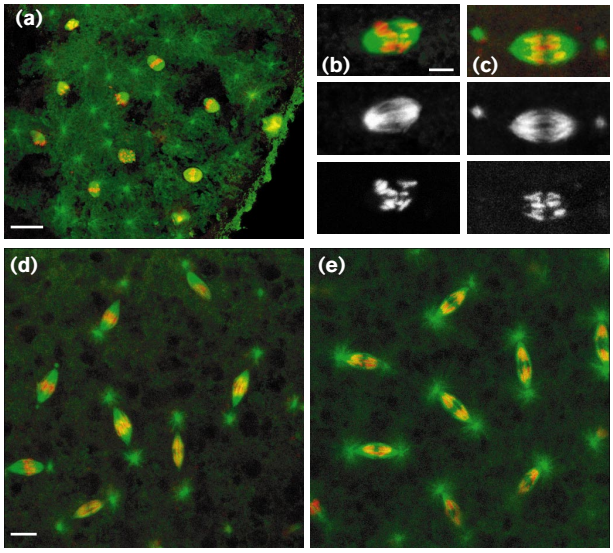
Figure 1



Centrosomes detach from spindles at anaphase in *cfo* mutant embryos. **(a)** Typical *cfo* mutant embryo stained to reveal the distribution of DNA (red) and microtubules (green). The images shown in this panel and in (d,e) are projections of several different focal planes, and some of the spindles are not entirely located within the focal planes shown. **(b,c)** Close-up view of (b) a centrosomeless spindle and (c) an anaphase spindle in which the centrosomes are apparently just detaching from the spindle. In (b,c), the middle panels show the tubulin signal (green), and the bottom panels the DNA signal (red); the merged images are shown in the upper panels. **(d)** Lower-magnification view of a *cfo* embryo in which the majority of the centrosomes appear to be coordinately detaching from their spindles. **(e)** Wild-type embryo at anaphase of the same nuclear cycle as that in (d). Embryos were fixed with methanol and stained with the DM1a anti-α-tubulin antibody (Sigma) and propidium iodide (to visualise the DNA), as described previously [1]. The scale bar represents 20 μm in (a), 5 μm in (b,c) and 10 μm in (d,e).

Results and discussion

In embryos laid by females homozygous for *cfo*, fewer than 10% of embryos developed normally to the blastoderm stage. The majority (> 80%) of the pre-blastoderm embryos were arrested at nuclear cycles 1–7 (as judged by the number of spindles in the embryo) in a mitotic-like state, with large barrel-shaped spindles surrounding clusters of mitotic chromatin (Figure 1a). Almost all of these spindles were devoid of centrosomes, and free centrosomes were usually randomly distributed throughout the cytoplasm. Most of the chromosomes on these spindles were in an anaphase-like state, with the sister chromatids separated to some extent (Figure 1b). This was confirmed by staining *cfo* embryos with antibodies against either the *proliferation disruptor* (*prod*) gene product or the GAGA transcription factor, which label centromeric heterochromatin [2,3] (data

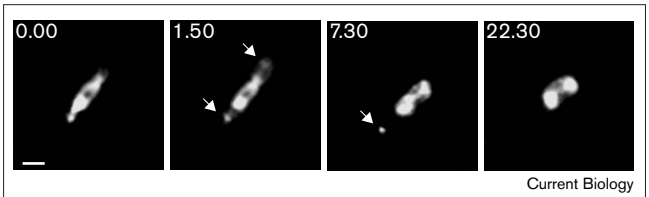
not shown). As the majority of the embryos were in this anaphase-like state, we assume that it represents a terminal-arrest stage. The same phenotype was seen in embryos transheterozygous for *cfo* and *Df(3L)Ac1*, a deficiency that removes the *cfo* gene (data not shown).

Failure of *cfo* embryos to maintain a connection between centrosomes and spindles at anaphase

Although most fixed *cfo* embryos were arrested in this anaphase-like state, a small number of pre-blastoderm embryos in interphase or early mitosis looked normal (data not shown). Some pre-blastoderm embryos in anaphase also looked normal, but in many of these anaphase embryos the centrosomes appeared to be co-ordinately detaching from the mitotic spindle (Figure 1c,d). As there were no other detectable defects in these embryos, it seems likely that the primary defect is a failure to maintain the connection between centrosomes and spindles during anaphase.

To observe the *cfo* defect more directly, we expressed a fusion between the tau protein and the green fluorescent protein (tau–GFP) in *cfo* embryos and followed the behaviour of microtubules in living embryos using time-lapse video microscopy [4]. In 13 of the 15 embryos we followed, the centrosomes were already separated from the spindles by the time the spindles were close enough to the cortex to be seen clearly. In the other two embryos, however, we observed several normal looking metaphase spindles near the cortex. As these spindles were in different focal planes and in different orientations, we could follow in detail only one at a time. The spindle shown in Figure 2 was oriented at a slight angle, and so the top spindle pole is slightly out of the plane of focus. This spindle was observed in an apparently normal metaphase-like state for about 90 seconds before both centrosomes simultaneously started to detach from the spindle (arrows, 1.50 minutes). After several minutes, one centrosome (7.30 minutes), and then the other (22.30 minutes) moved out of the plane of focus. The remaining centrosomeless spindle looked very similar to those seen in fixed embryos, and it stayed arrested in this state for a further 10 minutes, before we stopped following it. We followed several other spindles that had already lost their centrosomes by the time we began observing them, and in all cases they remained arrested in a mitotic-like state throughout the 10–30 minute period of observation (mitosis normally lasts for only about 3 minutes in these early syncytial divisions [5]). These observations suggest that the detachment of the centrosomes from the spindles at anaphase is the primary defect in *cfo* embryos. We next investigated why centrosome detachment might arrest spindles in mitosis.

Figure 2

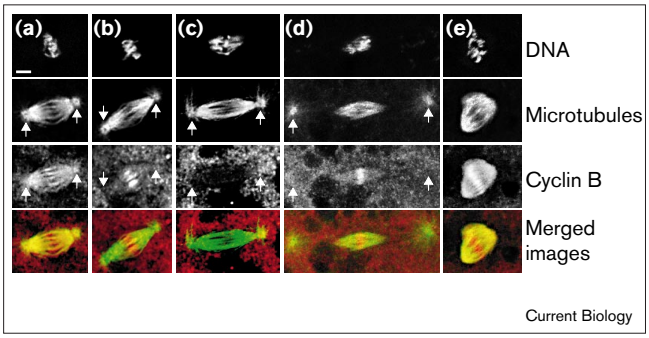


The centrosome-detachment phenotype in living *cfo* embryos. The microtubules in living *cfo* mutant embryos were visualised with a tau–GFP fusion protein, as described previously [4]. Time (in min) is shown at the top left of each panel. Arrows highlight the positions of the centrosomes as they detach from the spindle. Note that the top of the spindle is slightly out of the focal plane of the rest of the spindle and so the centrosome appears fainter as it detaches from the spindle. The scale bar represents 5 μm.

Cyclin B is not degraded normally on *cfo* spindles

It has previously been shown in several systems that mitotic spindles arrest in an anaphase-like state if the normal degradation of cyclin B is prevented [6–8]. This arrest looks similar to that seen in *cfo* embryos, except that no centrosome-detachment phenotype has been reported. To test whether cyclin B is degraded normally in *cfo* embryos, we stained the embryos with antibodies against cyclin B. We showed previously that cyclin B is concentrated on mitotic spindles and is particularly enriched at the equator of the spindle, where the interpolar microtubules overlap [1] (Figure 3a). Late in metaphase,

Figure 3



Cyclin B is not degraded normally in *cfo* embryos. The distribution of DNA, microtubules (green) and cyclin B (red) is shown in **(a–c)** fixed wild-type spindles in (a) early metaphase, (b) late metaphase and (c) anaphase, and in **(d,e)** *cfo* spindles at anaphase. (a) Note how cyclin B is concentrated on the wild-type spindle in early metaphase (the spindle is yellow in the merged image, and orange in the middle of the spindle where cyclin B levels are highest). (b) In late metaphase, cyclin B at the poles had decreased to background levels but remained high in the middle of the wild-type spindle. (c) By anaphase, cyclin B was at background levels throughout the wild-type spindle. (d) In *cfo* spindles in which the centrosomes were just detaching, cyclin B on the centrosomes had decreased to background levels but remained high on the spindle, even though the chromosomes were in anaphase. (e) Levels of cyclin B on *cfo* spindles that were terminally arrested in anaphase were very high; the gain on the cyclin B channel in this figure has been lowered so that the spindle staining can be seen in detail. Arrows highlight the positions of the centrosomes. Embryos were fixed with methanol and stained with affinity-purified anti-cyclin B antibodies, the DM1a anti-α-tubulin antibody, and propidium iodide, as described previously [1]. The scale bar represents 5 μm.

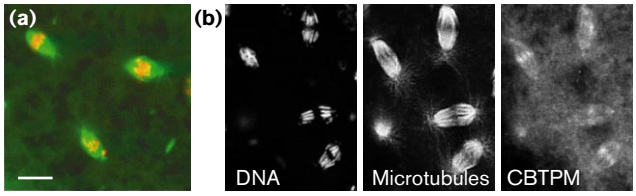
cyclin B starts to decrease at the spindle poles but remains high in the middle of the spindle (Figure 3b). By the time the spindles enter anaphase, however, cyclin B throughout the spindle has fallen to essentially background levels ([1]; Figure 3c). (In methanol-fixed embryos, there was substantial non-spindle cyclin B staining that was unevenly distributed in the cytoplasm (Figure 3; see also Figure 6 in [1])). This can make the normal disappearance of cyclin B from centrosomes and spindles difficult to appreciate. The spatially regulated disappearance of cyclin B at the end of mitosis is most easily seen in movies of living embryos (see Supplementary material).

In *cfo* embryos where the chromosomes were in anaphase and the centrosomes were just detaching from the spindle, cyclin B was at background levels on the detached centrosomes but was still detectable on the spindles (compare Figure 3d with Figure 3c). This contrasts with the situation in wild-type embryos in anaphase, and this failure to properly degrade the spindle-associated cyclin B presumably explains why these spindles arrest in mitosis. In terminally arrested *cfo* embryos, cyclin B reached abnormally high levels on the centrosomeless spindles (Figure 3e), and it also reaccumulated on the detached centrosomes (these centrosomes are not in the same focal plane as the arrested spindle shown in Figure 3e). Thus, cyclin B appears to be stable in the terminally arrested *cfo* embryos, and it can accumulate on the arrested spindles and centrosomes.

The stability of cyclin B on *cfo* spindles does not appear to explain the centrosome-fall-off phenotype

In principle, the failure to properly degrade cyclin B on the spindle could be the cause of the centrosome-fall-off phenotype. To test this possibility, we expressed a non-degradable form of cyclin B in early embryos using the Gal4 system [9,10]. Western blotting showed that this protein was expressed in embryos at similar levels to the endogenous cyclin B protein (data not shown). In 0–2 hour collections, most embryos (> 80%) were arrested in an anaphase-like state during nuclear cycles 1–7. In contrast to the situation in *cfo* embryos, most of the centrosomes appeared to remain attached to the arrested spindles (Figure 4a). In 2–4 hour collections, many embryos appeared to have been arrested in mitosis for some time (as judged by the overall disorganisation of the embryo). In these embryos, some of the centrosomes had detached from the arrested spindles (data not shown). Importantly, however, we failed to observe any cyclin-arrested embryos where the majority of centrosomes were co-ordinately detaching from the spindles, as seen in *cfo* embryos. Thus, it seems unlikely that the centrosome-fall-off phenotype is simply a consequence of a failure to degrade cyclin B. As noted above, no centrosome-fall-off phenotype has been reported in any of the other systems where mitosis has been arrested by blocking the destruction of cyclin B.

Figure 4



Non-degradable cyclin B blocks spindles in anaphase, with high levels of cyclin B on the centrosomes and spindles. We have shown previously that a cyclin B–GFP fusion protein has a very similar localisation to the endogenous cyclin B in methanol-fixed *Drosophila* embryos [1]. Here, we constructed a cyclin B–GFP fusion protein containing three point mutations (called CBTPM–GFP) that changed the conserved destruction box sequence *R*AA*L*GDLQ*N* to *AAA*GDLQ*A*. This fusion protein was expressed in early embryos using the Gal4 system [9,10]. The spindles shown in this figure are all projections of several focal planes, and some of the spindles are not entirely located within the focal planes shown. **(a)** Typical embryo arrested in mitosis, showing the distribution of DNA (red) and microtubules (green). Note that most of the centrosomes remain attached to their spindle. **(b)** Distribution of DNA (left panel), microtubules (middle panel) and CBTPM–GFP (right panel, here detected with our own affinity-purified rabbit anti-GFP antibodies), showing that the non-degradable fusion protein was still detectable on the centrosomes and spindles, even though the chromosomes were in anaphase. Embryos were fixed with methanol and stained as described previously [1]. The scale bar represents 10 μm.

The normal disappearance of cyclin B from centrosomes and spindles appears to be closely linked to its destruction

As shown in Figure 4b, cyclin B remained high on both centrosomes and spindles in embryos expressing non-degradable cyclin B. This figure shows an embryo in which the anaphase chromosomes looked relatively normal, suggesting that the spindles were either just arresting in anaphase or were about to complete mitosis. (As mentioned earlier, embryos expressing non-degradable cyclin B usually arrested between nuclear cycles 1–7, suggesting that some embryos can proceed through several rounds of mitosis in the presence of the non-degradable cyclin B, perhaps because the non-degradable cyclin B is not highly overexpressed in these embryos.) Even in such relatively normal looking anaphase spindles, however, the non-degradable cyclin B did not disappear from the spindles or the centrosomes (compare Figure 4b with Figure 3c). This suggests that the normal disappearance of cyclin B from the centrosome and spindle at the end of mitosis is closely linked to its destruction.

Perhaps the simplest interpretation of our findings is that the signal to degrade cyclin B in early *Drosophila* embryos is normally generated at centrosomes. In *cfo* mutant embryos, the link between centrosomes and spindles is broken: although cyclin B degradation may initiate on the centrosomes, the spindle-associated cyclin B is not

degraded properly and, as a result, the spindles arrest in mitosis. One possibility is that the signal to degrade cyclin B normally travels along microtubules from the centrosome to the rest of the spindle and cannot do so when the centrosomes have detached from the spindle. Another possibility is that a specific cell-cycle checkpoint monitors the connection between the centrosomes and spindle, and blocks cyclin B degradation if the connection is lost. In any case, our results suggest that the centrosome may play a key role in controlling the destruction of cyclin B in the early *Drosophila* embryo.

These results could have important implications for the spindle-assembly checkpoint [11,12]. We previously proposed that an unattached kinetochore might block the exit from mitosis, at least in part, by sending a signal to the centrosome to block the initiation of cyclin B degradation [1]. If this putative signal travels along kinetochore microtubules, it could explain why an unattached kinetochore only blocks the exit from mitosis on the spindle to which it is directly attached [13]. Consistent with this hypothesis, several components of the spindle checkpoint are concentrated at centrosomes, as well as on kinetochores [14–16], and it has recently been found that one of these checkpoint components, Mad2, appears to travel along kinetochore microtubules to the centrosomes (Ted Salmon, personal communication). Thus, signalling between kinetochores and centrosomes may play an important role in regulating the exit from mitosis.

Supplementary material

Supplementary material including additional methodological detail and movies showing spatially regulated disappearance of cyclin B at the end of mitosis is available at http://current-biology.com/supmat/supmatin.htm.

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