The dynamic localisation of the *Drosophila* APC/C: evidence for the existence of multiple complexes that perform distinct functions and are differentially localised

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Summary

In *Drosophila* cells, the destruction of cyclin B is spatially regulated. In cellularised embryos, cyclin B is initially degraded on the mitotic spindle and is then degraded in the cytoplasm. In syncytial embryos, only the spindle-associated cyclin B is degraded at the end of mitosis. The anaphase promoting complex/cyclosome (APC/C) targets cyclin B for destruction, but its subcellular localisation remains controversial. We constructed GFP fusions of two core APC/C subunits, Cdc16 and Cdc27. These fusion proteins were incorporated into the endogenous APC/C and were largely localised in the cytoplasm during interphase in living syncytial embryos. Both fusion proteins rapidly accumulated in the nucleus prior to nuclear envelope breakdown but only weakly associated with mitotic spindles throughout mitosis. Thus, the global activation of a spatially restricted APC/C cannot explain the spatially regulated destruction of cyclin B. Instead, different subpopulations of the APC/C must be activated at different times to degrade cyclin B. Surprisingly, we noticed that GFP-Cdc27 associated with mitotic chromosomes, whereas GFP-Cdc16 did not. Moreover, reducing the levels of Cdc16 or Cdc27 by >90% in tissue culture cells led to a transient mitotic arrest that was both biochemically and morphologically distinct. Taken together, our results raise the intriguing possibility that there could be multiple forms of the APC/C that are differentially localised and perform distinct functions.

Key words: Anaphase promoting complex, APC, APC/C, Mitosis, Cell cycle

Introduction

The anaphase promoting complex or cyclosome (APC/C) is a large multi-subunit complex that promotes the ubiquitination and subsequent proteosome-mediated destruction of several proteins during mitosis (Cerutti and Simanis, 2000; King et al., 1996; Page and Hieter, 1999; Peters, 1999; Zachariae and Nasmyth, 1999). The activity of this complex is tightly regulated, and it is widely believed that the sequential activation of the APC/C towards different substrates at different times is essential for coordinating the exit from mitosis (Morgan, 1999; Townsley and Ruderman, 1998).

How the APC/C targets different proteins for destruction at different times remains a mystery. Part of the answer to this problem appears to lie in the requirement for the APC/C to bind to either Fizzy (Fzy/Cdc20) or Fizzy-related (Fzr/Cdh1) to recognise its substrates. In budding yeast, for example, the APC/C-Fzy complex is required to initiate the destruction of the securin Pds1 and the mitotic cyclin clb2 early in the exit from mitosis. The APC/C-Fzr complex catalyses the destruction of clb2 and several other proteins later in the exit from mitosis and G1 (Baumer et al., 2000; Kramer et al., 2000; Shirayama et al., 1999; Visintin et al., 1997; Yeong et al., 2000). This requirement for Fzy and Fzr, however, cannot be the only way in which the activity of the APC/C towards different substrates is temporally regulated. In higher eukaryotes, for example, cyclin A always appears to be degraded before cyclin B (den Elzen and Pines, 2001; Geley et al., 2001; Lehner and O'Farrell, 1990; Minshull et al., 1990; Whitfield et al., 1990), yet the destruction of both proteins is Fzy dependent (Dawson et al., 1995; Sigrist and Lehner, 1997).

Recent studies have shown that in addition to being temporally regulated, the destruction of cyclin B is also spatially regulated (Clute and Pines, 1999; Huang and Raff, 1999; Yanagida et al., 1999). In *Drosophila*, the destruction of cyclin B appears to be initiated at centrosomes and then spreads towards the spindle equator (Huang and Raff, 1999; Wakefield et al., 2000). Once the spindle-associated cyclin B has been degraded, any remaining cytoplasmic cyclin B is then degraded. These two phases of destruction appear to be separable, as in early syncytial embryos only the spindle-associated cyclin B is degraded at the end of mitosis (Huang and Raff, 1999; Wakefield et al., 2000). This appears to explain why cyclin B is only partially degraded at the end of mitosis in *Drosophila* syncytial embryos (Edgar et al., 1994).

It is unclear how the destruction of cyclin B is spatially regulated. An attractive possibility is that the APC/C is globally activated to ubiquitinate cyclin B but is itself spatially restricted. Thus the APC/C might initially be concentrated at centrosomes, then 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 been shown to be concentrated on centrosomes and spindles in mammalian cells (Tugendreich et al., 1995). The localisation of the APC/C, however, is controversial. In *Drosophila* embryos, Cdc16 and Cdc27 appear to be present throughout the embryo and only weakly associate with mitotic spindles (Huang and Raff, 1999); in *A. nidulans*, the Cdc27 homologue BimA is concentrated on the spindle pole bodies (Mirabito and Morris, 1993); whereas in vertebrate cells the APC2/Tsg24 subunit of the APC/C appears to be concentrated at centromeres, although Cdc16 and Cdc27 were not detected at centromeres (Jorgensen et al., 1998). These data suggest that the localisation of APC/C may vary between species and may even vary depending on which subunit of the APC/C is being studied. All of these studies, however, were performed using indirect immunofluorescence methods on fixed tissues and so could be prone to fixation artefacts.

To investigate the localisation of the APC/C in *Drosophila* embryos without fixation, we have fused GFP onto Cdc16 and Cdc27. We show that these fusion proteins are not highly overexpressed and are incorporated into the endogenous APC/C. Both proteins are mainly cytoplasmic in interphase but also accumulate in the nuclear envelope region. As embryos enter mitosis, both proteins rapidly accumulate in the nuclear region but then only weakly bind to the spindle throughout mitosis. Thus, the APC/C cannot be globally activated to degrade cyclin B at the end of mitosis in *Drosophila* embryos.

Surprisingly, we noticed that GFP-Cdc27 binds to mitotic chromatin, whereas GFP-Cdc16 does not. This observation prompted us to test whether Cdc16 and Cdc27 might perform different functions. We used double-stranded RNA-mediated interference (RNAi) to lower the level of both proteins in S2 tissue culture cells. Although this treatment lowered the levels of both proteins by >90%, the mitotic arrest produced by reducing the levels of either protein was both morphologically and biochemically distinct. Taken together, these findings raise the intriguing possibility that there may be multiple forms of the APC/C that are differentially localised and perform different functions.

Materials and Methods

Generation of GFP-Cdc16 and GFP-Cdc27 transformed lines

The N-terminus of full-length Cdc16 and Cdc27 cDNAs were modified by PCR to include an appropriate restriction enzyme site just after the initiating ATGs. mGFP6 (Schuldt et al., 1998) was then subcloned, in frame, into this site, and the modified GFP-Cdc16 or GFP-Cdc27 cDNAs was then subcloned into the pWR-Pubq transformation vector (Nick Brown, Wellcome/Cancer Research UK Institute, Cambridge, personal communication; full cloning details available on request), which placed these cDNAs under the influence of the polyubiquitin promoter. These constructs were used to generate several stably transformed lines using standard P-element-mediated transformation methods (Roberts, 1986). Similar methods were used to create transformed flies expressing the C-terminally fused Cdc27-GFP fusion protein (full cloning details available on request).

Gel filtration chromatography

High-speed extracts were made from 0-4 hour-old wild-type embryos or from embryos expressing either the GFP-Cdc16 or GFP-Cdc27 fusion proteins as described previously (Huang and Raff, 1999). The extracts were then passed over a Superose 6 gel filtration column (Pharmacia) using a Bio-Logic HR workstation (Bio-Rad). 1 ml fractions were collected, precipitated with Trichloroacetic acid and resuspended in protein sample buffer (Laemmli, 1970). After neutralisation with ammonium chloride, the samples were run on 8% or 10% SDS-polyacrylamide gels and blotted to nitrocellulose. These blots were then probed with appropriate antibodies. Protein standards from the MW-GF-1000 kit (Sigma) were used to calibrate the Superose 6 column.

Immunoprecipitation

Immunoprecipitations were performed as described previously (Huang and Raff, 1999) using high-speed extracts made from embryos expressing either the GFP-Cdc16 or GFP-Cdc27 fusion proteins.

Rescue of the *cdc27* mutation

The *cdc27*^{L7123} mutant stock was obtained from the Bloomington stock centre. This mutation is on the third chromosome, and standard genetic methods were used to generate a stock containing this mutation plus a copy of the Pubq-GFP-Cdc27 transgene on the second chromosome.

Time-lapse confocal microscopy

Embryos of the appropriate genotype were observed using time-lapse confocal microscopy as described previously (Huang and Raff, 1999). Images were transported into Adobe Photoshop, and all images were adjusted to use the full range of pixel intensities. Movies of embryos were then compiled in Adobe Premier.

RNAi treatment and analysis of S2 tissue culture cells

Cdc16 and Cdc27 cDNA templates were amplified by PCR using the primer pairs: for Cdc16, 5′-TAA TAC GAC TCA CTA TAG ATG CCC GGG GAC ACG GAA AAC ACA-3′, and 5′-TAA TAC GAC TCA CTA TAG TGC CAG CGG TAA ATG ATG CAT TAG-3′; for Cdc27, 5′-TAA TAC GAC TCA CTA TAG AGC TGG GTG CAG TCG CTA ATC GGA-3′, AND 5′-TAA TAC GAC TCA CTA TAG TAG GTT CCG TGG TGC TGC GCC TGC-3′; the 5′ end of each primer also contained the T7 RNA polymerase promoter site (5′-TAA TAC GAC TCA CTA TAG-3′). PCR products (~700 bp in length) were purified using the QIA quick Gel Extraction Kit according to the manufacturers instructions. Purified PCR products (final concentration ~100 μg/ml) were used to produce double-stranded RNA (dsRNA) using a Megascript T7 transcription kit (Ambion). The RNA was purified according to the manufacturer's instruction, heated at 65°C for 30 minutes and then placed in a beaker of water at 65°C and left on the bench to cool to room temperature. Each batch of RNA was analysed on an agarose gel to ensure the quality of dsRNA. S2 cells were grown in Schneider's Insect medium (Sigma) supplemented with 10% fetal calf serum (FCS, Gibco) and 50 μg/ml streptomycin and penicillin at 27°C. The RNAi treatment and subsequent FACs and viable cell count analysis of S2 tissue culture cells was performed essentially as described previously (Adams et al., 2001; Clemens et al., 2000; Giet and Glover, 2001).

For immunofluorescence analysis, cells were fixed with cold (–20°C) methanol/3% EGTA and processed as described previously (Gergely et al., 2000). To quantify the total cellular fluorescence of immunostained cells we obtained serial confocal sections through the entire volume of a cell, compiled a 3D projection of the cell and then imported this projection into NIH Image. The area of the cell was defined manually, and the average pixel intensity of the cell was calculated. We found that even if we collected images of cells at the same stage of the cell cycle, on the same coverslip and using the same confocal settings, there was a considerable variation in the average pixel intensity of cells, although there was a clear trend towards lower total cellular levels of cyclin A and B as cells exited mitosis. Thus, we currently cannot reliably estimate what proportion of cyclin A or B has been degraded in any individual cell using these methods. To accurately measure the

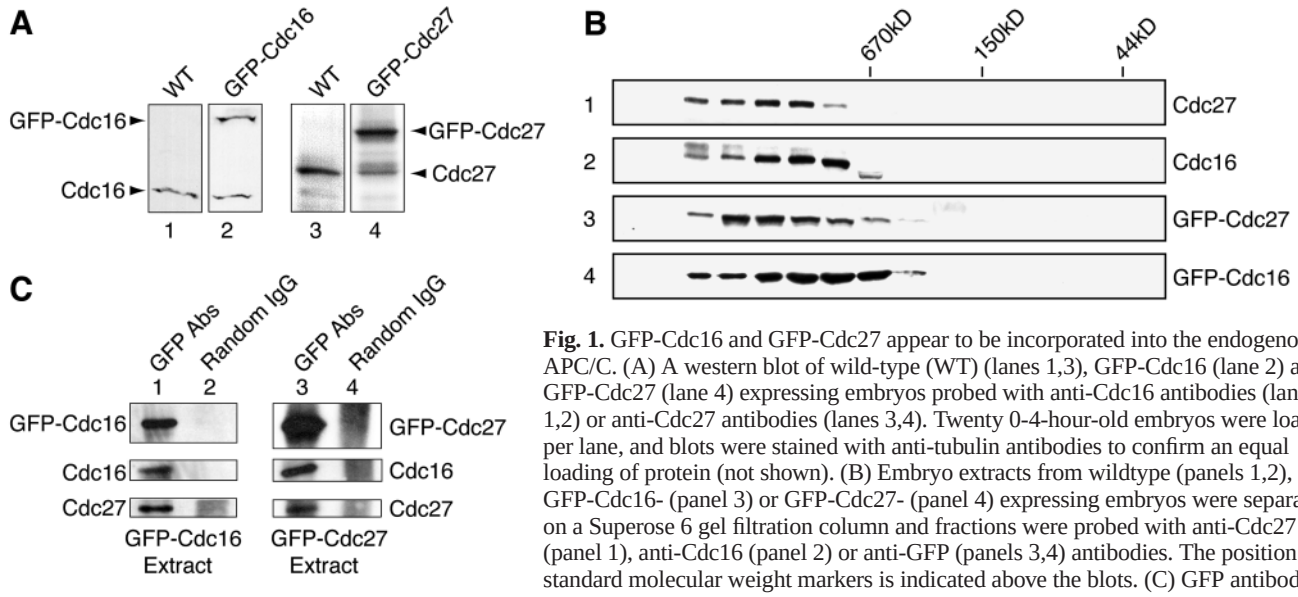


Fig. 1. GFP-Cdc16 and GFP-Cdc27 appear to be incorporated into the endogenous APC/C. (A) A western blot of wild-type (WT) (lanes 1,3), GFP-Cdc16 (lane 2) and GFP-Cdc27 (lane 4) expressing embryos probed with anti-Cdc16 antibodies (lanes 1,2) or anti-Cdc27 antibodies (lanes 3,4). Twenty 0-4-hour-old embryos were loaded per lane, and blots were stained with anti-tubulin antibodies to confirm an equal loading of protein (not shown). (B) Embryo extracts from wildtype (panels 1,2), GFP-Cdc16- (panel 3) or GFP-Cdc27- (panel 4) expressing embryos were separated on a Superose 6 gel filtration column and fractions were probed with anti-Cdc27 (panel 1), anti-Cdc16 (panel 2) or anti-GFP (panels 3,4) antibodies. The position of standard molecular weight markers is indicated above the blots. (C) GFP antibodies (lanes 1,3) or Random rabbit IgG (lanes 2,4) were used to immunoprecipitate proteins from embryos expressing GFP-Cdc16 (lanes 1,2) or GFP-Cdc27 (lanes 3,4). Blots were probed with anti-GFP (top panels), anti-Cdc16 (middle panels) or anti-Cdc27 antibodies (bottom panels).

degradation of cyclin A and B in RNAi-treated cells we will have to follow the behaviour of GFP-tagged versions of these proteins in living cells (Clute and Pines, 1999). Similar 3D reconstructions were made of whole cells to analyse the distribution of the CID protein.

SDS-PAGE and western blotting

SDS-PAGE, western blotting and the quantification of western blots was performed as described previously (Huang and Raff, 1999).

Antibodies

The following antibodies were used in this study: our own affinity-purified rabbit anti-Cdc16, anti-Cdc27, anti-cyclin B, anti-cyclin A and anti-GFP antibodies have been described previously (Huang and Raff, 1999); the mouse monoclonal DM1a (Sigma) was used to detect tubulin; anti-phospho-histone H3 (Upstate Technology) was used to detect phospho-histone H3; our own affinity-purified rabbit anti-fizzy antibodies were raised and purified against an MBP fusion protein containing the N-terminal 194 amino acids of *Drosophila* fizzy as described previously (Huang and Raff, 1999). The anti-CID antibody has been described previously (Blower and Karpen, 2001). All affinity-purified antibodies were used at 1-2 μg/ml in western blotting or immunofluorescence experiments. The DM1a and anti-phospho-histone H3 antibodies were used at a 1:500 dilution in western blotting and immunofluorescence studies.

TUNEL analysis

To assess the percentage of cells that were in an apoptotic state, around 6×10^5 cells were transferred to an 8-well Permanox slide chamber and allowed to settle for 15 minutes. The chamber was centrifuged at 480 ***g*** for 5 minutes, and the chamber was washed three times in PBS. The cells were fixed in freshly prepared 2% paraformaldehyde solution in PBS, pH7.4 for 45 minutes. The cells were washed 3×2 minutes in PBS, then permeabilised with 0.1% Triton X-100, 0.1% sodium citrate in fixation solution for 2 minutes at 4°C. The samples were washed 3×2 minutes in PBS, then the TUNEL assay was performed using the 'In Situ Cell Death Detection Kit, TMR red' (Roche) following the manufacturer's instructions.

Results

GFP-Cdc16 and GFP-Cdc27 are incorporated into the endogenous APC/C

Several transgenic lines that expressed either a GFP-Cdc16 or GFP-Cdc27 fusion protein were generated by standard P-element-mediated transformation (see Materials and Methods). These transgenic lines all behaved in a similar manner, and the results reported here have been pooled from several different lines. In early embryos, the GFP-Cdc16 transformed lines expressed the fusion protein at about the same level as the endogenous Cdc16 protein (Fig. 1A, lanes 1,2), whereas the GFP-Cdc27-transformed lines appeared to express the fusion protein at about two to three times the level of the endogenous Cdc27 protein (Fig. 1A, lanes 3,4). We noticed, however, that the levels of the endogenous Cdc27 consistently appeared to be downregulated in the presence of GFP-Cdc27, suggesting that total Cdc27 levels might be regulated in the embryo (see below).

To test whether the GFP-fusion proteins were incorporated into the APC/C, we made embryo extracts from either wildtype, GFP-Cdc16- or GFP-Cdc27-expressing embryos and separated the extracts on a Superose 6 gel filtration column (Fig. 1B). In WT extracts the endogenous Cdc16 and Cdc27 both migrated as a large complex, although Cdc16 reproducibly migrated at a slightly smaller size than Cdc27 (compare Fig. 1B, panel 1 with panel 2). GFP-Cdc16 and GFP-Cdc27 largely co-migrated with the endogenous Cdc16 and Cdc27 (compare Fig. 1B, panels 1,3 and 2,4), and GFP-Cdc16 reproducibly migrated at a slightly smaller size than GFP-Cdc27 (compare Fig. 1B, panel 3 with panel 4). To confirm that the fusion proteins were incorporated into the APC/C we performed immunoprecipitation experiments with anti-GFP antibodies using extracts from either GFP-Cdc16 or GFP-Cdc27 embryos. Anti-GFP antibodies precipitated both the endogenous Cdc16 and Cdc27 from embryos expressing either of the fusion proteins, whereas random IgG antibodies precipitated neither protein (Fig. 1C). The anti-GFP antibodies did not precipitate any of these proteins from wild-type extracts

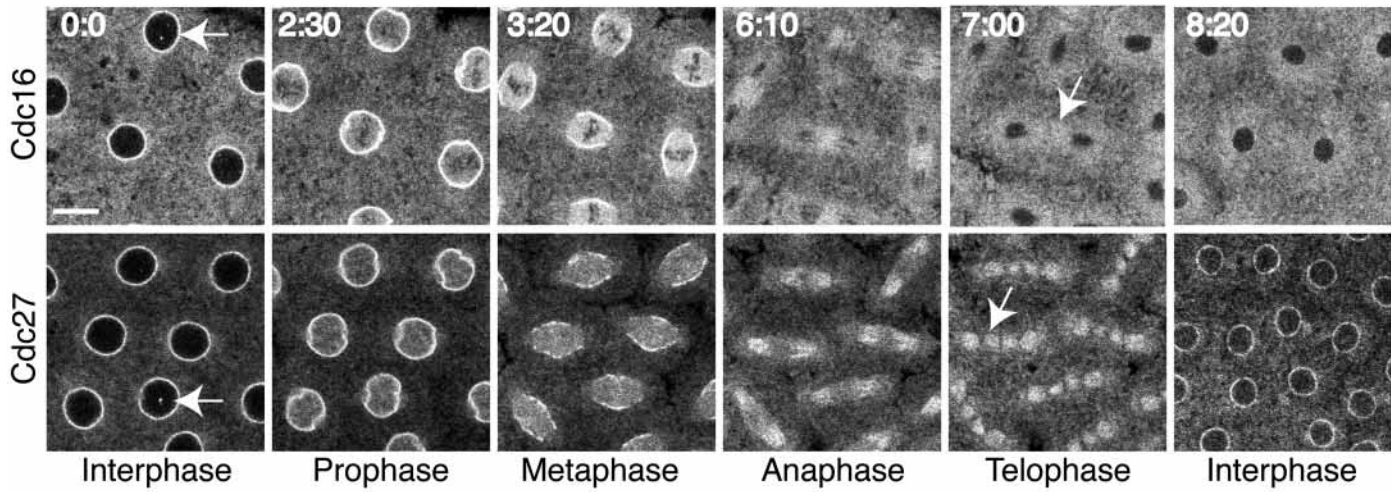


Fig. 2. The behaviour of GFP-Cdc16 (top panels) and GFP-Cdc27 (bottom panels) in living syncytial embryos. The time (in minutes) is shown in the top left-hand corner. Arrows indicate the small dots seen in interphase nuclei (0:0) or the position of the spindle midbody (7:00). Bar, 10 μm.

(data not shown). Thus, both GFP-Cdc16 and GFP-Cdc27 appear to be incorporated into the endogenous APC/C.

GFP-Cdc27 can rescue a mutation in the *cdc27* gene

To test whether the GFP-Cdc27 fusion protein was functional, we assayed its ability to rescue a mutation in the *cdc27* gene (unfortunately, there are no available mutations in the *cdc16* gene). The mutant line *cdc27*^{L7123} contains a P-element insertion 519 bp upstream of the initiating ATG of the *cdc27* gene. This mutation is semi-lethal, and the occasional homozygous fly survived to adulthood. These rare homozygous flies had many eye and bristle defects, were invariably sterile and only lived for a few days. If the GFP-Cdc27 transgene was introduced into this stock, however, homozygous mutant flies were readily recovered. These 'rescued' flies had almost no eye or bristle defects, were fertile and could be maintained as a homozygous laboratory stock. Western blot analysis of embryos laid by these flies confirmed that they expressed the GFP-Cdc27 fusion protein but had severely reduced (<10%) levels of the endogenous Cdc27 protein (data not shown). Thus, the GFP-Cdc27 fusion protein can rescue the phenotypes associated with the *Cdc27*^{L7123} mutation.

The dynamic localisation of the *Drosophila* APC/C

To analyse the localisation of the APC/C, we followed the behaviour of the GFP-Cdc16 and GFP-Cdc27 fusion proteins in living syncytial embryos using time-lapse confocal microscopy (Fig. 2). During interphase, both proteins were abundant in the cytoplasm and were largely excluded from the nuclei, although they were both concentrated in the nuclear envelope region (Fig. 2; 0:0). Both proteins were also concentrated in a small number of dots within the nucleus; these dots moved about rapidly in the nucleus, and only one or two dots were visible at any one time in any confocal section (arrows, Fig. 2, time 0:0).

As the nuclei started to enter mitosis, both fusion proteins entered the nuclear region (Fig. 2, 2:30) and then weakly concentrated on the forming mitotic spindle (Fig. 2, 3:20). Both proteins remained weakly concentrated on the spindle microtubules throughout mitosis, although neither protein was particularly concentrated at centrosomes (Fig. 2, 3:20-7:00). Strikingly, the GFP-Cdc27 protein accumulated on mitotic chromosomes, most obviously during anaphase/telophase (Fig. 2, 2:30-7:00), whereas the GFP-Cdc16 fusion protein appeared to be excluded from the mitotic chromosomes, which could be visualised as dark 'shadows' in the GFP-Cdc16 fluorescence (Fig. 2, 2:30-7:00). At the end of mitosis, GFP-Cdc16 was already excluded from the reforming nuclei (Fig. 2, 8:20), whereas GFP-Cdc27 was only gradually excluded from the reforming nuclei and appeared to accumulate in the nuclear envelope region during this period (Fig. 2, 7:00-8:20).

During mitosis in cellularised embryos, the localisation of GFP-Cdc16 and GFP-Cdc27 was similar to that seen in syncytial embryos (not shown): both proteins were enriched in the nuclear region as the nuclear envelope broke down and then weakly associated with the spindle throughout mitosis. As in the syncytial divisions, GFP-Cdc27 associated with the mitotic chromosomes, whereas GFP-Cdc16 was excluded from mitotic chromosomes. The enrichment of both proteins in the nuclear envelope region, however, was much less pronounced in cellularised embryos (not shown).

Reducing the levels of Cdc16 and Cdc27 in tissue culture produces morphologically distinct affects

These results suggested that Cdc16 and Cdc27 might not always colocalise in embryos. To test whether Cdc16 and Cdc27 could perform different functions in cells, we used RNAi to reduce the levels of each protein in S2 tissue culture cells (Adams et al., 2001; Clemens et al., 2000; Giet and Glover, 2001). Fig. 3 shows a western blot analysis, a FACS analysis, a TUNEL analysis and a count of the total number of viable cells during the four day time course of a typical RNAi experiment. The levels of both Cdc16 and Cdc27 fell during the time course of the experiment, and by 72-96 hours, both proteins were reproducibly depleted by >90% (Fig. 3A). Surprisingly, however, depleting Cdc27was always more deleterious to cells as judged by the FACs analysis (Fig. 3B), the count of total cell numbers (Fig. 3C), the levels of

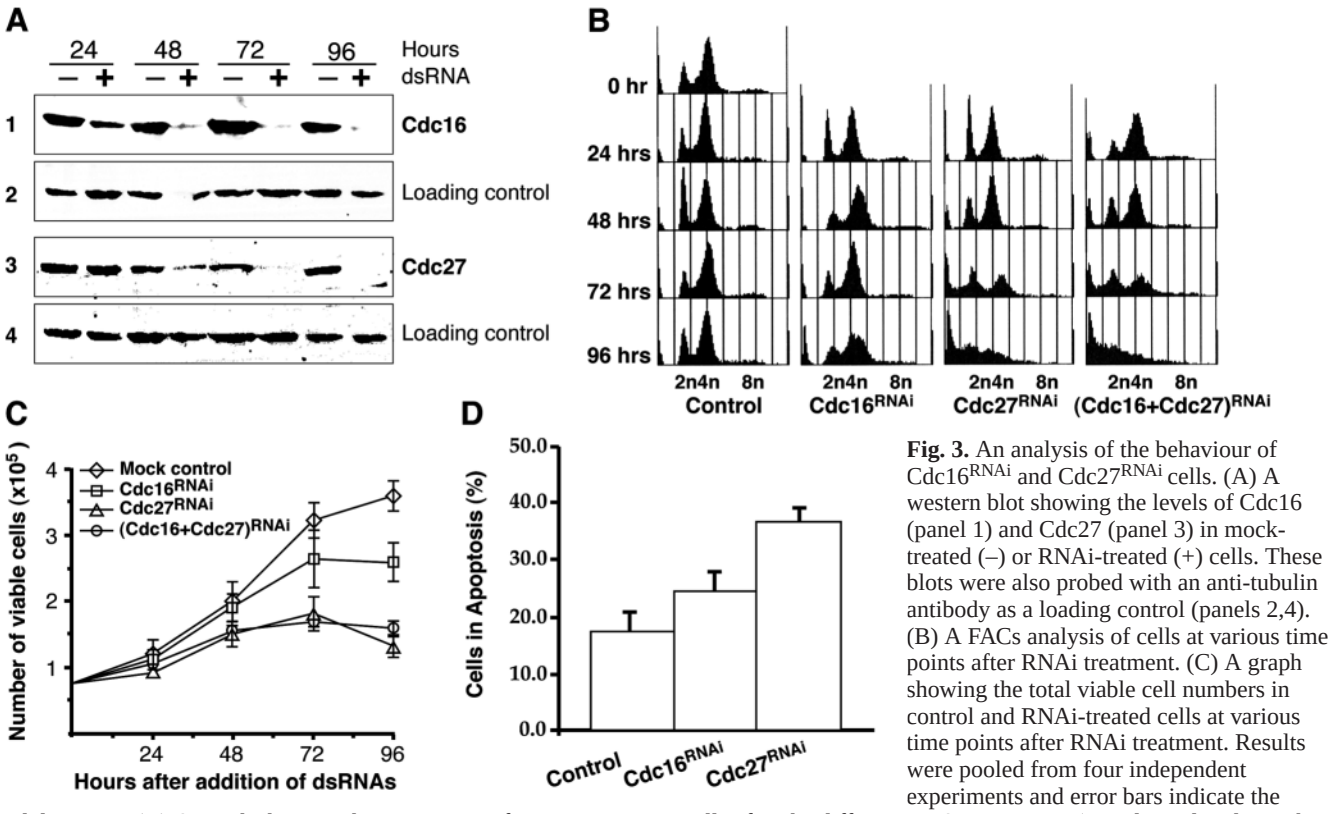


Fig. 3. An analysis of the behaviour of $Cdc16^{RNAi}$ and $Cdc27^{RNAi}$ cells. (A) A western blot showing the levels of Cdc16 (panel 1) and Cdc27 (panel 3) in mock-treated (–) or RNAi-treated (+) cells. These blots were also probed with an anti-tubulin antibody as a loading control (panels 2,4). (B) A FACs analysis of cells at various time points after RNAi treatment. (C) A graph showing the total viable cell numbers in control and RNAi-treated cells at various time points after RNAi treatment. Results were pooled from four independent experiments and error bars indicate the standard deviation. (D) A graph showing the percentage of TUNEL-positive cells after the different RNAi treatments (as indicated under each bar). Only data from the 72 hour time point is shown. Error bars represent the standard deviation.

apoptosis induced by each treatment (Fig. 3D) and the morphology of the treated cells (Fig. 4).

To investigate the affects of depleting Cdc16 and Cdc27 on cells in more detail, we fixed treated cells at the 72 or 96 hour time point and stained them to look at the distribution of microtubules and DNA. Results from either time point were similar, and the results presented here are pooled from both time points. In both $Cdc16^{RNAi}$ and $Cdc27^{RNAi}$ cells there was a marked increase in the mitotic index, although this was always higher in $Cdc27^{RNAi}$ cells (Fig. 4A). Although the mitotic index was increased in treated cells, the majority of cells were not in mitosis, suggesting that cells can ultimately exit mitosis even when levels of the APC/C are greatly reduced. Indeed, we observed some treated cells in a relatively normal telophase configuration (Fig. 4C, panel 4).

Examination of the mitotic cells revealed a clear difference between the $Cdc16^{RNAi}$- and $Cdc27^{RNAi}$-treated cells (Fig. 4B). The majority of $Cdc16^{RNAi}$ mitotic cells (~60%) were well organised, with the majority of chromosomes aligned at the equator of a typical metaphase-like spindle (Fig. 4C, panel 1). By contrast, the majority of $Cdc27^{RNAi}$ mitotic cells (~70%) were in a much more disorganised state, with condensed chromosomes spread throughout an elongated spindle area (Fig. 4C, panel 2). A small proportion of $Cdc16^{RNAi}$- and $Cdc27^{RNAi}$-treated cells appeared to contain a spindle with mitotic chromosomes and also a significant mass of non-condensed (and non-phospho-histone H3 positive) chromatin (Fig. 4C, panel 3). When both Cdc16 and Cdc27 protein levels were reduced at the same time, the proportion of mitotic cells in these two configurations was similar (~45% each), suggesting that reducing the levels of both proteins produces an intermediate phenotype (Fig. 4C).

To more accurately determine the state of the chromosomes in these cells, we looked at the distribution of the CID protein that binds the centromeric regions of chromosomes (Blower and Karpen, 2001). In control cells, the centromeres were closely paired in metaphase and were well separated in early anaphase (Fig. 5A,B). In RNAi-treated cells where the spindles were well formed and the chromosomes appeared to be largely aligned on a metaphase plate (~60% of $Cdc16^{RNAi}$ cells and ~20% of $Cdc27^{RNAi}$ cells; Fig. 4C), the anti-CID staining revealed a striking difference between the two RNAi treatments. In the majority of such $Cdc16^{RNAi}$ cells, all the centromeres were tightly clustered in a metaphase-like alignment (68%; Fig. 5C). In the remaining cells, the sister centromeres appeared to have separated to some degree (4%; Fig. 5D) or were no longer tightly aligned on the metaphase plate, even though the bulk of the chromatin appeared roughly aligned on a well organised spindle (28%; Fig. 5E). In this subset of $Cdc27^{RNAi}$ cells, the proportion of cells in these states was reversed: only 11% of cells had their centromeres tightly aligned on the metaphase plate, 36% of cells showed some degree of centromere separation and 54% of cells had centromeres spread throughout a larger area of the spindle. Thus, in these relatively well organised mitotic cells, the sister chromatids are usually not separated in $Cdc16^{RNAi}$ cells but are usually at least partially separated in $Cdc27^{RNAi}$ cells.

In RNAi-treated cells where the spindle was elongated and the chromosomes were not aligned near the spindle equator (~20% of mitotic $Cdc16^{RNAi}$-treated cells and ~70% of mitotic

Cdc27^{RNAi}-treated cells; Fig. 4C), the centromeres were invariably spread throughout the elongated spindle area (Fig. 5F,G). These cells were usually so disorganised that it was difficult to be sure whether sister chromatids had separated, but in some cells the spindle seemed to be excerpting a force on the centromeres, separating the sister chromatids to some extent (arrows, Fig. 5F). Thus, although the spindles in these cells have elongated, it is not clear whether the sister chromatids have separated.

Cyclin A behaves differently in Cdc16^{RNAi} and Cdc27^{RNAi} cells

To further probe the mitotic state of these cells, we stained them with anti-cyclin A and anti-cyclin B antibodies (Fig. 6). Surprisingly, we observed a reproducible difference in the behaviour of cyclin A in Cdc16^{RNAi} and Cdc27^{RNAi} cells. In mock-treated cells, cyclin A antibodies stained the condensing chromosomes during prophase (Fig. 6A, panel 1), but cyclin A was rarely detectable on chromosomes (which were usually visible as a 'shadow') by metaphase (Fig. 6A, panel 2; shadow detectable in 51 out of 54 (~94%) metaphase cells examined). This chromosome shadow was also detectable in most Cdc16^{RNAi} metaphase cells (Fig. 6A, panel 3; shadow detectable in 44 out of 52 (~85%) metaphase cells examined) but not in most Cdc27^{RNAi} metaphase cells (Fig. 6A, panel 4,5; shadow detectable in 11 out of 50 (~22%) metaphase cells examined). Thus, the chromosome-associated fraction of cyclin A appears to be degraded inefficiently in the Cdc27^{RNAi} cells.

We saw no difference in the behaviour of cyclin B between Cdc16^{RNAi} and Cdc27^{RNAi} cells. In mock-treated cells, cyclin B antibodies stained centrosomes in prophase cells (Fig. 6B, panel 1). Cyclin B also stained centrosomes and spindles in some metaphase cells (Fig. 6B, panel 2) but not others (Fig. 6B, panel 3), presumably because the degradation of the spindle-associated cyclin B is initiated in metaphase cells (Clute and Pines, 1999; Huang and Raff, 1999). Cyclin B was not detectable on centrosomes or spindles in mock-treated anaphase cells (Fig. 6B, panel 4). As in control cells, cyclin B staining of the centrosomes and spindles was variable in both Cdc16^{RNAi} and Cdc27^{RNAi} cells in metaphase (not shown), suggesting that cyclin B can be degraded in at least some of these cells. In a very few cells with elongated spindles (<5%), however, cyclin B was still detectable on centrosomes and spindles (Fig. 6C, panel 5), suggesting that at least some of these cells are exiting mitosis without degrading cyclin B properly.

The behaviour of cyclin A is biochemically distinct in Cdc16^{RNAi} and Cdc27^{RNAi} cells

To test whether there was a biochemically detectable difference between Cdc16^{RNAi} and Cdc27^{RNAi} cells, we analysed the behaviour of several cell cycle proteins in these cells by western blotting (Fig. 7). In Cdc16^{RNAi} cells, Cdc16 was reduced by >90%, and the levels of Cdc27 were also reproducibly reduced by ~50-75%. By contrast, Cdc27 was reduced by >90% in Cdc27^{RNAi} cells, but there was no decrease in the levels of Cdc16. This suggests that the stability of at least a fraction of the cellular Cdc27 requires the presence of Cdc16,

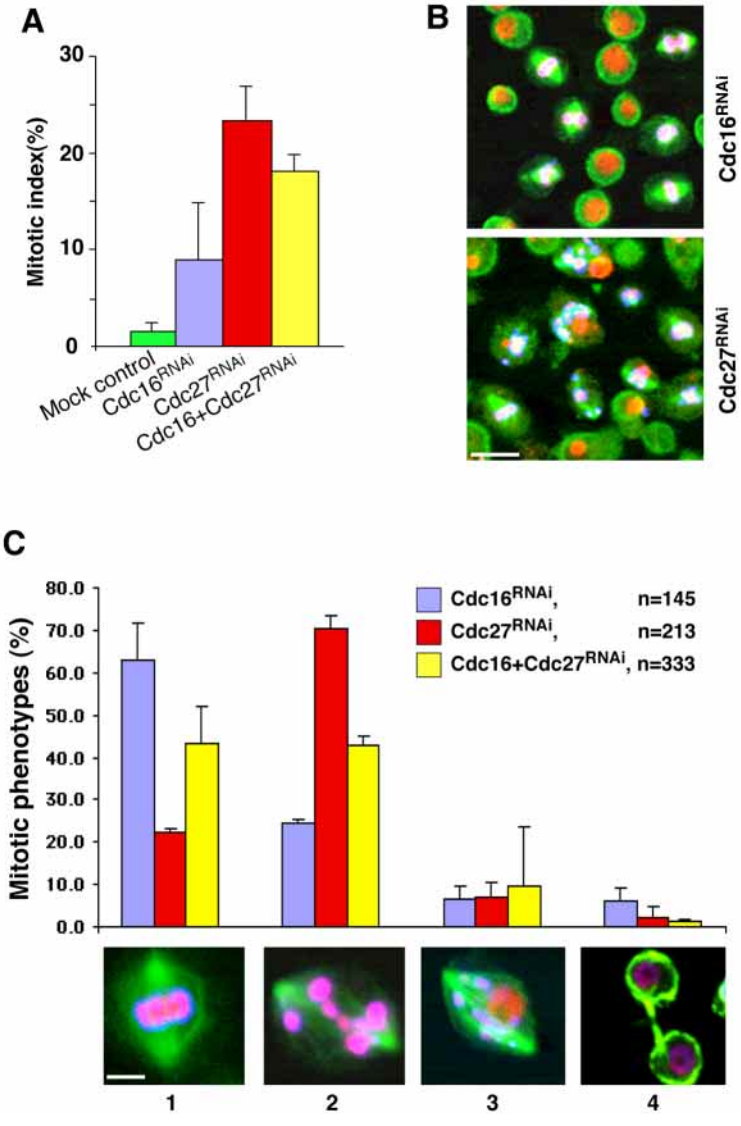
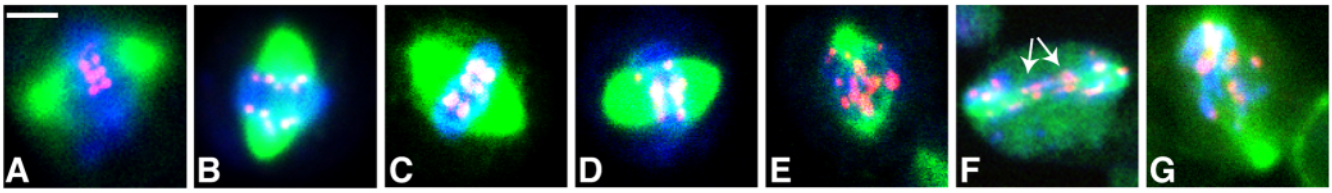


Fig. 4. The mitotic arrest induced in Cdc16^{RNAi} and Cdc27^{RNAi} cells is morphologically distinct. (A) A graph showing the mitotic index of mock-, Cdc16^{RNAi}-, Cdc27^{RNAi}- or Cdc16^{RNAi}- + Cdc27^{RNAi}-treated S2 cells. This was calculated by counting the percentage of cells in mitosis as judged by phospho-histone H3 staining. Error bars represent the standard deviation. Results were pooled from four different experiments. (B) A low magnification view of Cdc16^{RNAi}- (top panel) or Cdc27^{RNAi}- (bottom panel) treated cells stained to reveal the distribution of DNA (red), phosphohistone H3 (blue) and microtubules (green). Note how most of the mitotic Cdc16^{RNAi} cells have their chromosomes roughly aligned at the equator of a metaphase-like spindle, whereas most of the mitotic Cdc27^{RNAi} cells have elongated spindles that are in a much more disorganised state. Bar, 10 μm. (C) A graph showing the proportions of mitotic cells in different states of mitosis. Typical examples of each mitotic state are shown underneath each graph; the colours are the same as in B: chromosomes aligned on a metaphase-like spindle (panel 1); chromosomes spread throughout an elongated spindle (panel 2); condensed and decondensed chromatin (which appears red, as it is not stained by the anti-phosphohistone H3 antibody) in the same cell (panel 3); a telophase-like cell (panel 4). Error bars represent the standard deviation. Bar, 5 μm.



68%	4%	28%	Cdc16^{RNAi} Cells
11%	36%	54%	Cdc27^{RNAi} Cells

Fig. 5. The distribution of the anti-centromere antibody CID in Cdc16^{RNAi} and Cdc27^{RNAi} cells. Control (A,B) or Cdc16^{RNAi} or Cdc27^{RNAi} (C-G) cells were stained to reveal the distribution of CID (red), DNA (blue) and microtubules (green). All the images shown are projections of several serial sections that were taken through the entire cell. In control cells during metaphase (A), the centromeres were aligned in closely spaced pairs on the metaphase plate; these pairs were clearly separated by early anaphase (B). In Cdc16^{RNAi} or Cdc27^{RNAi} cells in which the majority of the chromosomes appeared to be roughly aligned on a metaphase plate (C-E), the behaviour of the centromeres fell into three classes (the percentage of cells in each class for each RNAi treatment are shown beneath; C-E). Centromeres were either aligned in closely spaced pairs (C) or were aligned as pairs that appeared to have separated to some extent (D) or were more randomly distributed on the spindle (E). In Cdc16^{RNAi} or Cdc27^{RNAi} cells in which the spindles were elongated (F,G), the centromeres were spread throughout the spindle region. The arrows in F shows a region where it appears that sister chromatids are being pulled apart by tension on the spindle. Bar, 5 μm.

perhaps because Cdc27 is unstable if it is not incorporated into the APC/C. This could also explain why expressing GFP-Cdc27 appears to downregulate the levels of the endogenous Cdc27 (Fig. 1A).

There was very little difference in the overall levels of cyclin A between mock-treated and either Cdc16^{RNAi} or Cdc27^{RNAi} cells, but cyclin B levels were reproducibly slightly elevated in both RNAi-treated cells. Strikingly, however, there was a slower migrating form of cyclin A that was always present at elevated levels in Cdc27^{RNAi} cells but not in Cdc16^{RNAi} cells (arrowhead, Fig. 7). This form of cyclin A was also present in cells that had reduced levels of both Cdc16 and Cdc27. In addition, the Fzy protein, which is degraded at the end of mitosis in many cell types (Fang et al., 1998; Goh et al., 2000; Kramer et al., 1998; Prinz et al., 1998; Shirayama et al., 1998), was always present at significantly elevated levels in both the Cdc16^{RNAi} and Cdc27^{RNAi} cells, suggesting that depleting either protein interferes with the normal degradation of Fzy to a similar extent.

Discussion

We have previously shown that the destruction of cyclin B in *Drosophila* cells is spatially regulated and occurs in two phases. The destruction of cyclin B initiates at the centrosomes and then spreads to the spindle equator. Once the cyclin B on the spindle has been degraded, the remaining cytoplasmic cyclin B is then degraded. These phases appear to be separable, as in syncytial embryos only the spindle-associated cyclin B is degraded at the end of mitosis. The localisation of GFP-Cdc16 and GFP-Cdc27 in living syncytial embryos suggests that only a small fraction of the APC/C is associated with mitotic spindles. Thus, the APC/C cannot be globally activated to degrade cyclin B at the end of mitosis. Instead, subpopulations of the APC/C must be activated at different times and at different places in order to explain the spatially regulated destruction of cyclin B. We speculate that the APC/C is present in excess relative to two of its key regulators, Fzy/Cdc20 and Fzr/Cdh1. The targeting of Fzy and Fzr to different locations in the cell may explain how the destruction of cyclin B is regulated in space and time (Raff et al., 2002).

Surprisingly, we noticed that GFP-Cdc27 associated with mitotic chromatin whereas GFP-Cdc16 did not, suggesting that these two core APC/C components are not always associated with one another in *Drosophila* embryos. This prompted us to test whether these proteins might perform distinct functions. We found that depleting either protein by >90% from cells in culture produced a mitotic arrest that was both morphologically and biochemically distinct. As we discuss below, these data raise the intriguing possibility that the APC/C may exist as several related complexes that could perform different functions.

The localisation of the APC/C

A crucial question in interpreting our data is whether the localisation of GFP-Cdc16 and GFP-Cdc27 accurately reflects the localisation of the endogenous proteins. We think this is likely for several reasons. First, both fusion proteins are expressed at levels roughly comparable to the endogenous proteins, and we cannot detect a fraction of either protein that does not behave as though it is part of a large complex that largely co-migrates with the endogenous Cdc16 and Cdc27 on a gel filtration column. Anti-GFP antibodies can precipitate the endogenous Cdc16 and Cdc27 from extracts expressing either fusion protein, demonstrating that these complexes also contain endogenous APC/C components. Second, the GFP-Cdc27 fusion protein can rescue a *cdc27* mutation, suggesting that it is functional. Third, although the distribution of GFP-Cdc16 and GFP-Cdc27 are not identical, they are very similar, and no other GFP fusion proteins that we are aware of have this localisation pattern. It seems unlikely that both fusion proteins would be artefactually mislocalised in such a similar way.

It is possible, however, that the localisation of both fusion proteins is largely correct, but the differences we observe in the localisation of the two fusion proteins are artefactual. Perhaps, for example, a fraction of GFP-Cdc27 is not incorporated into the APC/C and can bind non-specifically to mitotic chromatin.

We think this unlikely for two reasons. First, we cannot detect any pool of monomeric GFP-Cdc27 on gel filtration columns. Second, we have expressed a C-terminal fusion of GFP with Cdc27 (Cdc27-GFP). This fusion protein is non-functional: it is not incorporated into the endogenous APC/C, it does not rescue a *cdc27* mutation and it does not bind to mitotic chromatin but is instead localised throughout the cytoplasm (J.-Y.H. and J.W.R., unpublished). Thus, even if there were a small pool of monomeric GFP-Cdc27, it seems unlikely that it would bind to mitotic chromatin. Alternatively, perhaps GFP-Cdc16 is incorporated into the APC/C, but the GFP moiety specifically prevents the complex interacting with chromatin. Although this would be surprising, as the presence of even multiple copies of the GFP-Cdc16 transgene does not appear to have any deleterious affects on flies (J.-Y.H. and J.W.R., unpublished), we cannot at present rule this possibility out. We note, however, that a previous study has shown that Cdc27 biochemically co-purifies with mitotic chromatin whereas Cdc16 does not (Jorgensen et al., 1998). Thus, in both *Drosophila* and mammalian cells there is evidence that Cdc27 associates with mitotic chromatin whereas Cdc16 does not.

Depleting Cdc16 or Cdc27 produces distinct phenotypes

To test whether Cdc16 and Cdc27 could perform distinct functions, we reduced the levels of each protein in *Drosophila* tissue culture cells using RNAi. Although this procedure depletes both proteins by >90%, the affect of depleting Cdc27 was always much more deleterious to cells than depleting Cdc16. Moreover, cyclin A is normally undetectable on metaphase chromosomes, and this was true in Cdc16^{RNAi} cells but not in Cdc27^{RNAi} cells. This suggests that a chromosome-associated fraction of cyclin A can be degraded when Cdc16 is depleted but not when Cdc27 is depleted, correlating with our observation that Cdc27 associates with mitotic chromatin whereas Cdc16 does not. Intriguingly, a slower migrating form of cyclin A was also reproducibly detectable in western blots of Cdc27^{RNAi} cells but not Cdc16^{RNAi} cells. Perhaps this slower migrating form of cyclin A represents a chromatin-bound form of cyclin A that is not degraded properly when Cdc27 is depleted.

It is possible, however, that the different phenotypes induced by depleting Cdc16 and Cdc27 could be explained if depleting Cdc27 simply inactivated the APC/C more efficiently than

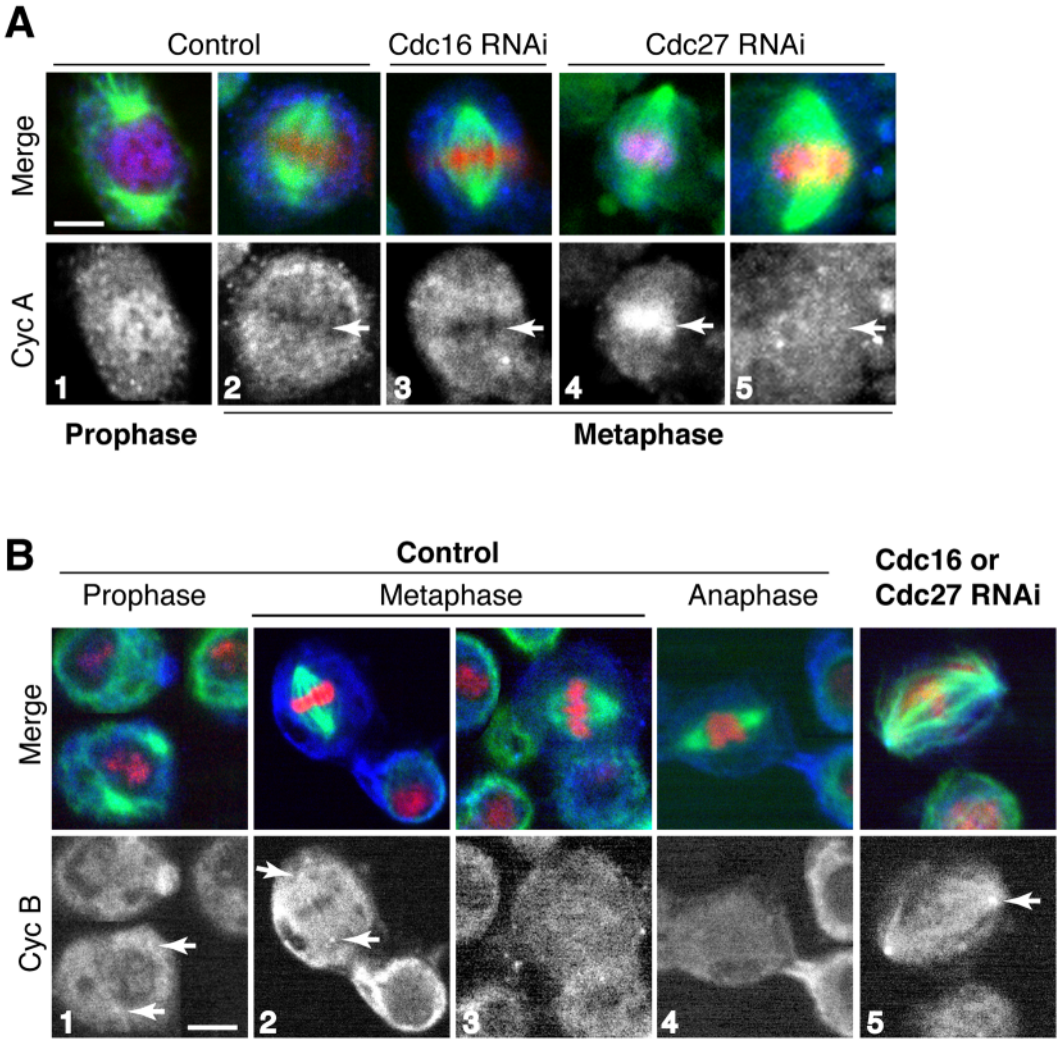


Fig. 6. The distribution of Cyclin A and Cyclin B in Cdc16^{RNAi} and Cdc27^{RNAi} cells. (A) The distribution of Cyclin A (blue in merged image; shown alone in black and white), DNA (red) and microtubules (green). In mock-treated cells, Cyclin A stains the condensing mitotic chromatin in prophase cells (panel 1) but is not detectable on the chromosomes by metaphase when the chromosomes are usually visible as a 'shadowed' region in the cyclin A channel; arrow, panel 2). This is also true of Cdc16^{RNAi} cells (panel 3) but is not true of Cdc27^{RNAi} cells where Cyclin A sometimes strongly stains metaphase chromosomes (panel 4), and the DNA 'shadow' normally seen in the cyclin A channel is usually not detectable (panel 5). (B) The distribution of Cyclin B (blue in merged image or shown alone in black and white), DNA and microtubules (colours as in A). In mock-treated cells, Cyclin B stains centrosomes in prophase (arrows, panel 1) and centrosomes and spindles in some metaphase cells (panel 2) but not others (panel 3). We failed to detect Cyclin B on centrosomes or spindles in any mock-treated cells in anaphase (panel 4). In Cdc16^{RNAi} or Cdc27^{RNAi} cells, Cyclin B behaved in a similar manner to mock-treated cells, but it was very occasionally detectable on centrosomes and spindles in anaphase-like cells, particularly in Cdc27^{RNAi}-treated cells (panel 5), suggesting that cyclin B is not being degraded properly in at least some of these cells. Bar, 5 μm.

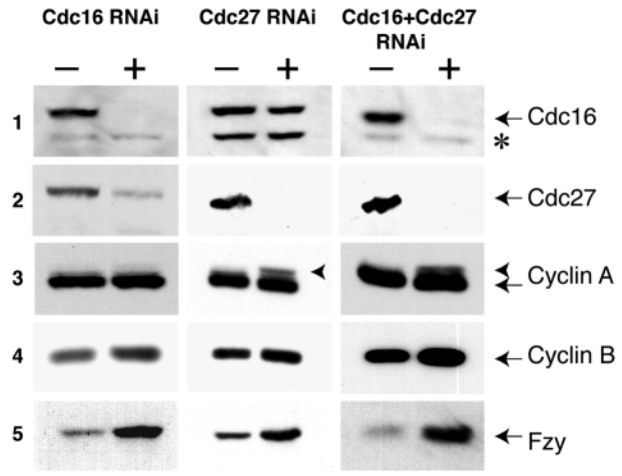


Fig. 7. An analysis of the levels of various cell cycle proteins in $Cdc16^{RNAi}$ and $Cdc27^{RNAi}$ cells. The western blots show the levels of Cdc16 (panel 1), Cdc27 (panel 2), Cyclin A (panel 3), Cyclin B (panel 4) and Fzy (panel 5) in mock-treated (–) or RNAi-treated (+) cells. All blots were also probed with anti-tubulin antibodies to confirm an equal loading (data not shown), and we show the levels of a non-specific band that is variably recognised by the anti-Cdc16 antibodies (asterisk, panel 1). A slower migrating form of Cyclin A is highlighted with an arrowhead in the $Cdc27^{RNAi}$- and $Cdc16^{RNAi}$- and $Cdc27^{RNAi}$-treated cells.

depleting Cdc16. This would be surprising, as previous studies have suggested that both proteins are 'core' components of the APC/C that are present in roughly stoichiometric amounts. And, perturbing the function of either protein by mutation or antibody injection causes the same phenotype - a strong metaphase arrest (Hirano et al., 1988; Irniger et al., 1995; Lamb et al., 1994; Mirabito and Morris, 1993; Tugendreich et al., 1995). Thus, one would not predict that depleting either protein by >90% would produce such different affects on total APC/C activity. In addition, two lines of evidence suggest that in our experiments depleting Cdc27 is not simply inducing a stronger version of the same phenotype induced by depleting Cdc16. First, depleting either protein weakly stabilises cyclin B and strongly stabilises Fzy/Cdc20 to about the same extent, suggesting that at least some aspects of APC/C function are equally inhibited by the depletion of either protein. Second, cells in which both proteins are simultaneously depleted by >90% appear to have an intermediate chromosome/spindle morphology phenotype, arguing that the $Cdc27^{RNAi}$ phenotype is not simply a more extreme version of the $Cdc16^{RNAi}$ phenotype.

The interpretation of this RNAi data is complicated, however, as we are analysing the behaviour of a population of cells that appear to only transiently arrest in mitosis as they run out of Cdc16 or Cdc27. How these cells eventually exit mitosis is unknown, but we note that *Drosophila* tissue culture cells are notoriously difficult to arrest in mitosis, even with microtubule destabilising agents (Mirkovitch et al., 1988) (M. Heck, personal communication). This 'mitotic slippage' mechanism probably explains why we only ever observe a maximum of ~25% of RNAi treated cells arrested in mitosis. A similar failure to completely arrest cells in mitosis has been made in *Drosophila* larval neuroblasts mutant in the ida/APC5 subunit of the APC/C (Bentley, 2002). We therefore remain cautious in our interpretation of these experiments. Nevertheless, these data are at least consistent with the possibility that Cdc16 and Cdc27 could exist in multiple complexes that perform at least partially non-overlapping functions.

Are there multiple APC/C complexes?

The APC/C has been purified from several systems, and in all cases it has been found to contain homologues of Cdc16 and Cdc27 (Page and Hieter, 1999; Peters, 1999). In human cells, APC/C complexes are homogeneous enough that a structure has been derived from cryo-electron microscopy and angular reconstitution studies (Gieffers et al., 2001). Moreover, previous studies in several systems have shown that perturbing APC/C activity always produces a similar phenotype - a strong mitotic arrest (Hirano et al., 1988; Irniger et al., 1995; Lamb et al., 1994; Mirabito and Morris, 1993; Tugendreich et al., 1995). How can these findings be reconciled with our suggestion that the APC/C could exist in several complexes?

Our finding that anti-GFP antibodies can immunoprecipitate Cdc16 from extracts expressing GFP-Cdc16 and can immunoprecipitate Cdc27 from extracts expressing GFP-Cdc27 may give a clue to this apparent paradox. This finding suggests that the APC/C either contains multiple copies of both proteins or that multiple APC/Cs can bind to each other during purification. If the APC/C contains multiple copies of Cdc16 and Cdc27 then different forms of the APC/C could vary in their ratio of Cdc27 to Cdc16. Perhaps a form with a high ratio of Cdc27 to Cdc16 might interact with mitotic chromatin, whereas a form with a low Cdc27 to Cdc16 ratio might not. In our hands, Cdc16 reproducibly migrated at a slightly smaller size on gel filtration columns than Cdc27 (and the same was true of GFP-Cdc16 compared with GFP-Cdc27), supporting the idea that the two proteins may not always exist in identical complexes. Such subtly different complexes, however, might be difficult to detect in purified APC/C preparations. Similarly, if multiple APC/Cs can bind to each other during purification, this might obscure the existence of several related complexes in purified preparations. Interestingly, Cdc16, Cdc27 and another APC/C component, Cdc23, all contain TPR repeats and can bind to themselves and to each other (Lamb et al., 1994). This could explain how the APC/C can contain multiple copies of Cdc16 and Cdc27 or how different APC/C complexes might bind to each other during purification.

In summary, it has widely been assumed that the APC/C exists as a single complex, although there is little direct evidence to support this assumption. Our data raise the possibility that the APC/C may exist as several related complexes that perform at least partially non-overlapping functions. Our observations suggest that there must be subpopulations of the APC/C that are independently activated to degrade cyclin B at different times and at different places. A requirement to regulate overall APC/C activity in a temporally and spatially co-ordinated fashion could explain why the APC/C is so structurally complex.

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References

Adams, R. R., Maiato, H., Earnshaw, W. C. and Carmena, M. (2001). Essential roles of *Drosophila* inner centromere protein (INCENP) and aurora B in histone H3 phosphorylation, metaphase chromosome alignment, kinetochore disjunction, and chromosome segregation. *J. Cell Biol.* **153**, 865-880.

Bentley, A. M., Williams, B. C., Goldberg, M. L. and Andres, A. J. (2002). Phenotypic characterization of Drosophila ida mutants: defining the role of APC5 in cell cycle progression. *J. Cell Sci.* **115**, 949-961.

Baumer, M., Braus, G. H. and Irniger, S. (2000). Two different modes of cyclin clb2 proteolysis during mitosis in *Saccharomyces cerevisiae*. *FEBS Lett.* **468**, 142-148.

Blower, M. D. and Karpen, G. H. (2001). The role of *Drosophila* CID in kinetochore formation, cell-cycle progression and heterochromatin interactions. *Nat. Cell Biol.* **3**, 730-739.

Cerutti, L. and Simanis, V. (2000). Controlling the end of the cell cycle. *Curr. Opin. Genet. Dev.* **10**, 65-69.

Clemens, J. C., Worby, C. A., Simonson-Leff, N., Muda, M., Maehama, T., Hemmings, B. A. and Dixon, J. E. (2000). Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. *Proc. Natl. Acad. Sci. USA* **97**, 6499-6503.

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

Dawson, I. A., Roth, S. and Artavanis-Tsakonas, S. (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.

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

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

Fang, G., Yu, H. and Kirschner, M. W. (1998). Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G1. *Mol. Cell* **2**, 163-171.

Geley, S., Kramer, E., Gieffers, C., Gannon, J., Peters, J. M. and Hunt, T. (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., Karlsson, C., Still, I., Cowell, J., Kilmartin, J. and Raff, J. W. (2000). The TACC domain identifies a family of centrosomal proteins that can interact with microtubules. *Proc. Natl. Acad. Sci. USA* **97**, 14352-14357.

Gieffers, C., Dube, P., Harris, J. R., Stark, H. and Peters, J. M. (2001). Three-dimensional structure of the anaphase-promoting complex. *Mol. Cell* **7**, 907-913.

Giet, R. and Glover, D. M. (2001). *Drosophila* aurora B kinase is required for histone H3 phosphorylation and condensin recruitment during chromosome condensation and to organize the central spindle during cytokinesis. *J. Cell Biol.* **152**, 669-682.

Goh, P. Y., Lim, H. H. and Surana, U. (2000). Cdc20 protein contains a destruction-box but, unlike Clb2, its proteolysisis not acutely dependent on the activity of anaphase- promoting complex. *Eur. J. Biochem.* **267**, 434-449.

Hirano, T., Hiraoka, Y. and Yanagida, M. (1988). A temperature-sensitive mutation of the *Schizosaccharomyces pombe* gene nuc2+ that encodes a nuclear scaffold-like protein blocks spindle elongation in mitotic anaphase. *J. Cell Biol.* **106**, 1171-1183.

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

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

Jorgensen, P. M., Brundell, E., Starborg, M. and Hoog, C. (1998). A subunit of the anaphase-promoting complex is a centromere-associated protein in mammalian cells. *Mol. Cell. Biol.* **18**, 468-476.

King, R. W., Deshaies, R. J., Peters, J. M. and Kirschner, M. W. (1996). How proteolysis drives the cell cycle. *Science* **274**, 1652-1659.

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

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

Laemmli, U. K. (1970). Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lamb, J. R., Michaud, W. A., Sikorski, R. S. and Hieter, P. A. (1994). Cdc16p, Cdc23p and Cdc27p form a complex essential for mitosis. *EMBO J.* **13**, 4321-4328.

Lehner, C. F. and O'Farrell, P. H. (1990). The roles of *Drosophila* cyclins A and B in mitotic control. *Cell* **61**, 535-547.

Minshull, J., Golsteyn, R., Hill, C. S. and Hunt, T. (1990). The A- and B-type cyclin associated cdc2 kinases in *Xenopus* turn on and off at different times in the cell cycle. *EMBO J.* **9**, 2865-2875.

Mirabito, P. M. and Morris, N. R. (1993). BIMA, a TPR-containing protein required for mitosis, localizes to the spindle pole body in *Aspergillus nidulans*. *J. Cell Biol.* **120**, 959-968.

Mirkovitch, J., Gasser, S. M. and Laemmli, U. K. (1988). Scaffold attachment of DNA loops in metaphase chromosomes. *J. Mol. Biol.* **200**, 101-109.

Morgan, D. O. (1999). Regulation of the APC and the exit from mitosis. *Nat. Cell Biol.* **1**, E47-E53.

Page, A. M. and Hieter, P. (1999). The anaphase-promoting complex: new subunits and regulators. *Annu. Rev. Biochem.* **68**, 583-609.

Peters, J. M. (1999). Subunits and substrates of the anaphase-promoting complex. *Exp. Cell Res.* **248**, 339-349.

Prinz, S., Hwang, E. S., Visintin, R. and Amon, A. (1998). The regulation of Cdc20 proteolysis reveals a role for APC components Cdc23 and Cdc27 during S phase and early mitosis. *Curr. Biol.* **8**, 750-760.

Raff, J. W., Jeffers, K. and Huang, J.-Y. (2002). The roles of Fzy/Cdc20 and Fzr/Cdh1 in regulating the destruction of cyclin B in space and time. *J. Cell Biol.* (in press).

Roberts, D. (ed.) (1986). *Drosophila, A Practical Approach.* Oxford: IRL Press.

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

Shirayama, M., Zachariae, W., Ciosk, R. and Nasmyth, K. (1998). The Polo-like kinase Cdc5p and the WD-repeat protein Cdc20p/fizzy are regulators and substrates of the anaphase promoting complex in *Saccharomyces cerevisiae*. *EMBO J.* **17**, 1336-1349.

Shirayama, M., Toth, A., Galova, M. and Nasmyth, K. (1999). APC(Cdc20) promotes exit from mitosis by destroying the anaphase inhibitor Pds1 and cyclin Clb5. *Nature* **402**, 203-207.

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

Townsley, F. M. and Ruderman, J. V. (1998). Proteolytic ratchets that control progression through mitosis. *Trends Cell Biol.* **8**, 238-243.

Tugendreich, S., Tomkiel, J., Earnshaw, W. and Hieter, P. (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., Prinz, S. and Amon, A. (1997). CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis. *Science* **278**, 460-463.

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

Whitfield, W. G. F., Gonzalez, C., Maldonado-Codina, G. and Glover, D. M. (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.

Yanagida, M., Yamashita, Y. M., Tatebe, H., Ishii, K., Kumada, K. and Nakaseko, Y. (1999). Control of metaphase-anaphase progression by proteolysis: cyclosome function regulated by the protein kinase A pathway, ubiquitination and localization. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **354**, 1559-1570.

Yeong, F. M., Lim, H. H., Padmashree, C. G. and Surana, U. (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.

Zachariae, W. and Nasmyth, K. (1999). Whose end is destruction: cell division and the anaphase-promoting complex. *Genes Dev.* **13**, 2039-2058.