The *Drosophila* Cdc6/18 protein has functions in both early and late S phase in S2 cells

Gilles Crevel[1], Endre Mathe[2] and Sue Cotterill[1,*]

[1]Department Basic Medical Sciences, St Georges Hospital Medical School, London, SW17 0RE, UK
[2]Genetics and Molecular Biology Research Group, Institute of Biology and Environmental Sciences, University College of Nyiregyhaza, Sostoi ut 31/B, Nyiregyhaza 4400, Hungary
*Author for correspondence (e-mail: s.cotterill@sghms.ac.uk)

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Summary

The Cdc6/18 protein has been mainly characterised for its role in the initiation of DNA replication. Several studies exist, however, which suggest that it may also have a role in controlling the G2/M transition. Here we present studies on the *Drosophila* Cdc6 (DmCdc6) protein that support this dual function for the protein.

First we show that its location is consistent with a cellular role post replication initiation as it remains nuclear throughout G1, S and G2 phases. In addition, we have been able to reduce the level of DmCdc6 protein to nondetectable levels in S2 cells using RNAi. This causes DNA fragmentation and cell cycle abnormalities which have some similarities with phenotypes previously observed in yeasts and are consistent with the cells entering mitosis with incompletely replicated DNA. Finally, we have stably overexpressed the DmCdc6 protein to a high level in S2 cells. Despite a large excess of protein the effects on the S2 cells were minimal. We did, however, detect a slight stalling of the cells in the late S phase of the cell cycle, which further supports the proposal that DmCdc6 has a role in controlling the transition from the S to M phases of the cycle.

Key words: Cdc6/18, *Drosophila*, DNA replication, Cell cycle

Introduction

The Cdc6/cdc18 protein is thought to have two important roles during the progression of the cell cycle. One is in the initiation of DNA replication, and the other is in checkpoint processes controlling the passage of the cell through the later stages of the cell cycle.

Of the two, its role in DNA replication has been more extensively studied (reviewed by Bell and Dutta, 2002). In this case Cdc6 is one of the earliest proteins required and is involved in the formation of the prereplicative complex. Together with cdt1, Cdc6 binds after the hetero-hexameric origin recognition complex (ORC), but its binding precedes and is needed for the loading of the six-membered minichromosome maintenance protein (MCM) complex. In the absence of Cdc6 no prereplicative complex can be formed. The prereplicative complex then provides the platform from which bulk DNA synthesis is launched. Although this sequence of events has been well studied, the exact biochemical activities catalysed by these proteins remains to be clarified, with the exception of the MCM complex which is thought to be the replicative helicase (reviewed by Bell and Dutta, 2002).

The checkpoint role of Cdc6/18 is less well understood. In *Saccharomyces cerevisiae Cdc6* has been suggested to play a direct role in mitotic exit by inactivating cdc28 kinase complexes (Bueno and Russell, 1992). In *Schizosaccharomyces pombe* it is thought to be needed for the replication arrest checkpoint acting through cds1 (chk2) (Murakami et al., 2002). A replication checkpoint-related role has also been reported in *Xenopus*, although in this case it acts through chk1 (Oehlmann et al., 2004). In *Xenopus* Chk1 also seems to be involved in the observed role of Cdc6 to couple the S and M phases of the cell cycle (Clay-Farrace et al., 2003).

Species-specific differences have been reported in the behaviour of the Cdc6/18 during the cell cycle (reviewed by Kearsey and Cotterill, 2003). *Cdc6*/cdc18 in *S. cerevisiae*/*S. pombe* is an unstable protein that is present only in a narrow window of the cell cycle (Baum et al., 1998; Piatti et al., 1995), whereas in higher eukaryotes the protein seems to be more stable but has been reported to show cell cycle changes in localisation (Saha et al., 1998; Petersen et al., 1999). Several reports have suggested that the protein is nuclear and probably chromatin bound in G1, and then moves to the cytoplasm in S. Although it is worth noting that many of the observations providing localisation data were carried out using overexpressed protein, a recent paper has suggested that endogenous Cdc6 in mammalian cells may remain nuclear and chromatin associated throughout the whole cycle (Alexandrow and Hamlin, 2004).

Differences in Cdc6 behaviour between species might be responsible for the species-specific variations that are observed when Cdc6/18 is overexpressed. Overexpression of cdc18 in *S. pombe* causes overreplication of the DNA, particularly in combination with cdt1 (Yanow et al., 2001). In *S. cerevisiae* a delay in the initiation of M phase is seen, although the overall growth rate is not affected (Elsasser et al., 1996). In higher eukaryotes the effect of increased expression of Cdc6 is dependent on the phase of the cell cycle in which it is carried out. In nonsynchronised cells retrovirally overexpressed

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protein shows no measurable effects (Petersen et al., 2000). However, if high levels of protein are microinjected into cells in the G2 phase of the cell cycle the cells are prevented from entering mitosis (Clay-Farrace et al., 2003). Finally, adenoviral directed expression in quiescent mammalian cells (from which it is normally absent) causes MCM loading and, if coupled with serum stimulation of the cells, advancement of S phase entry (Cook et al., 2002).

The effect of knocking out or mutating the gene also varies depending on the species under study. In *S. pombe cdc18* null mutants go through mitosis without having first replicated their DNA, producing a cut phenotype that is lethal (Kelly et al., 1993). Reduction division and lethality is also observed for null mutants in *S. cerevisiae* (Piatti et al., 1995). In higher eukaryotes in vivo knockout experiments for *Cdc6* have not been reported; however, depletion of the protein in *Xenopus* extracts causes a block before S phase (Coleman et al., 1996). Dominant negative mutants for mammalian cells (Walker A and B box mutants) cause a stop during the S phase of the cell cycle (Herbig et al., 1999), a result that is consistent with what is observed for analogous mutants in *S. cerevisiae*.

In the studies reported in this paper we have looked at DmCdc6 in the three areas where behavioural differences have been reported in other species. In terms of cell cycle localisation we found that both endogenous and overexpressed DmCdc6 remain nuclear during S phase. We have also looked at the effect of knocking out DmCdc6 in vivo by exploiting the amenability of *Drosophila* S2 cells to RNAi. We show that reduction of DmCdc6 protein to nondetectable levels causes DNA fragmentation and cell cycle abnormalities that have some similarities with phenotypes previously observed in yeasts. Knockout experiments have not previously been reported for other higher eukaryotic systems. Finally, we have shown that, consistent with observations in most systems, with the exception of *S. pombe*, even high-level stable overexpression of DmCdc6 in nonsynchronised S2 cells has only minimal effects on cell functioning.

Materials and Methods

Cloning of DmCdc6

DmCdc6 was identified by searching databases with *S. cerevisiae*, *S. pombe* and human Cdc6 protein sequences. The Flybase gene code is CG5971, with a Flybase accession number of FBgn0035918. The full-length clone was amplified from a *Drosophila* early embryo cDNA library and sequenced to ensure that there were no alterations from the sequence on Flybase.

Antibody production

The C-terminus (AA 343 to AA 662) of DmCdc6 was amplified by PCR and cloned in frame with the 6×His tag of pRSETa.

The plasmid was transformed into BL21 cells and the production of protein was induced by IPTG for 3 hours at 37°C. The 6Xhis-tagged protein was purified using Talon resin (BD Bioscences) following the manufacturer's instructions for the purification of proteins under denaturing conditions in urea. The purified protein was used to immunise two rabbits (Neosystem, Strasbourg).

Cell fractionation

Cell fractionation was carried out as described previously (Crevel et al., 2001; Mathe et al., 2000). Briefly, cells were spun down and lysed by homogenisation in PBS. The nuclear pellet was separated from the cytoplasmic fraction by centrifugation at 5000 ***g*** in an eppendorf bench centrifuge. The pellet was then resuspended in PBS containing 0.5% Triton, and centrifuged at 5000 ***g***. The supernatant after centrifugation was the nucleoplasmic fraction, and the remaining pellet was resuspended in SDS loading buffer and constituted the chromatin fraction. Samples were analysed by PAGE and western blotting.

RNAi

Two nonoverlapping regions of DmCdc6 were chosen as the targets for the RNA interference experiment (nucleotides 457-1026 and 1048-1589 of the mRNA; Fig. 3A).

Sequence-specific oligos were designed for each of these regions and a 5′ T7 RNA polymerase binding site was added on each primer.

The double-stranded RNA was obtained using the MEGAscript T7 kit (Ambion) as described by the manufacturers, with the following modifications: the reaction was carried out in 40 μl overnight at 37°C. To denature secondary structures the RNA was heated at 65°C for 30 minutes and left to slowly cool down to room temperature. The double-stranded RNA was precipitated with isopropanol, washed with ethanol 70% and resuspended in 50 μl H_2O. Typically this yielded 8-9 μg/μl of RNA.

The RNAi experiment was carried out on S2 cells in exponential growth phase exactly as described by Maiato et al. (Maiato et al., 2003). Fifteen micrograms of RNA were added per well (10^6 cells) and the cells were monitored by cell count, FACS analysis, western blotting, RT PCR and immunofluorescence over a period of 7 days. Control RNAi experiments used a similar size piece of RNA directed against a nonrelated human protein. In each case the experiment was carried out multiple times and the results presented represent the combined data of several independent experiments.

Drug treatment of S2 cells

Aphidicolin treatment: Cells were grown to a density of 5×10^6 and then split into two parts. One part remained untreated and to the other part was added aphidicolin to a final concentration of 4 μg/ml. The incubation was then continued for 2 days, with a sample of the cells being taken on each day. Analysis was by FACS analysis and western blotting.

Ecdysone treatment: Cells were grown to a density of 5×10^6 and then split into two parts. One part remained untreated and the other part was treated with ecdyosone exactly as described by MacAlpine et al. (MacAlpine et al., 2004).

RTPCR

CDNA was made using the cell to cDNA kit from Ambion as described in the manufacturer's instructions. 500,000 cells were lysed and 50,000 of these were then used to make cDNA. The equivalent of 5000 cells was then amplified using appropriate primers as described in the text and Fig. 3A using Megamix blue (Microzone).

FACS analysis

Cells at appropriate stages were harvested and fixed using ethanol. Immediately before use they were treated with 10 μg/ml RNase (in the presence of EDTA) and the DNA was stained with propidium iodide. The flow cytometry analysis was carried out on a Epics XL (Coulter Beckman) supplied with Expo32 ADC software.

Immunofluorescence

At each time point an aliquot of the cells was deposited on polylysine treated coverslips. The cells were fixed using 4% paraformaldehyde

diluted in a buffer containing: 1.1 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 137 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 5 mM Pipes, 5.5 mM glucose, pH 6.1 (Maiato et al., 2003). After permeabilisation in PBS, 1% BSA and 0.1% triton X100, the coverslips were processed for immunofluorescence using primary antibodies for: mouse anti-alpha tubulin (Sigma, clone DM 1A), mouse anti-lamin (gift from D. Glover lab) and rabbit anti-phospho H3 (Upstate). The secondary antibodies were from Molecular Probes: Alexa fluor488 anti-mouse and Alexa fluor 594 anti-rabbit. The DNA was counterstained with TOTO3 iodide (Molecular Probes). The coverslips were mounted in mounting medium Vectashield (Vector), and analysed by confocal microscopy (Biorad).

Overexpression of DmCdc6 in S2 cells

Full-length DmCdc6 was cloned into the *Not*1 and *Xho*1 sites of the pMT/V5 hisA vector (Invitrogen) in such a way that it was His- and SV5-tagged at the C terminus and under the control of the inducible metallothionein promoter. The construct was introduced into S2 cells along with the pCoBlast vector using the calcium phosphate procedure, and cells stably transfected with the *cdc6* gene were selected using blasticidin according to the manufacturer's instructions.

Results

Identification of the DmCdc6 protein

The *DmCdc6* gene (CG5971) was identified by searching the annotated Flybase gene collection using the sequences of *cdc6* genes from several other species. A comparison of the protein sequence with Cdc6/18 homologues from other species (Fig. 1) shows a high degree of homology; however, the *Drosophila* protein is 662 amino acids, which is about 100 amino acids longer than the protein from other species. This includes three regions of 20-24 amino acids inserted in the first 124 amino acids of DmCdc6 and one of 17 amino acids inserted at position 222. By database searching none of these regions seems to contain any particular motif; however, all of these regions are highly charged, either positive or negative depending on the region.

The C-terminal portion of the protein was produced as a His-tagged fusion protein, and the overproduced protein was used to produce two rabbit polyclonal antibodies. Both antibodies reacted with a band of around 75 kDa on western blots of crude *Drosophila* extracts from both cells and embryos (Fig. 2A). This is consistent with the predicted size of the protein from the amino acid composition. An additional band of approximately 60 kDa was also seen on most gels. This band did not, however, react with antibody that had been affinity purified against the overproduced DmCdc6 protein; we therefore do not believe that this band is related to DmCdc6. Occasionally, some degradation products could be seen much lower down the gel, which was not unexpected because of the lability of the DmCdc6 protein. Care was therefore taken in all western blotting experiments to minimise the appearance of such bands; however, where they were present their behaviour was the same as that for the full-length protein.

DmCdc6 is a nuclear protein

Analysis of the cellular location of the endogenous DmCdc6 by immunofluoresence was not possible as in S2 cells neither of our DmCdc6 antibodies gave staining above background. We therefore looked at the location of this protein by fractionating S2 cells into cytoplasmic, nuclear and nucleoplasmic compartments. Using this protocol efficient fractionation was achieved because the histones were confined solely to the nuclear compartment. For an example of the pattern obtained in the fractionation, see Fig. 3D.

These cell fractionation studies suggested that the protein

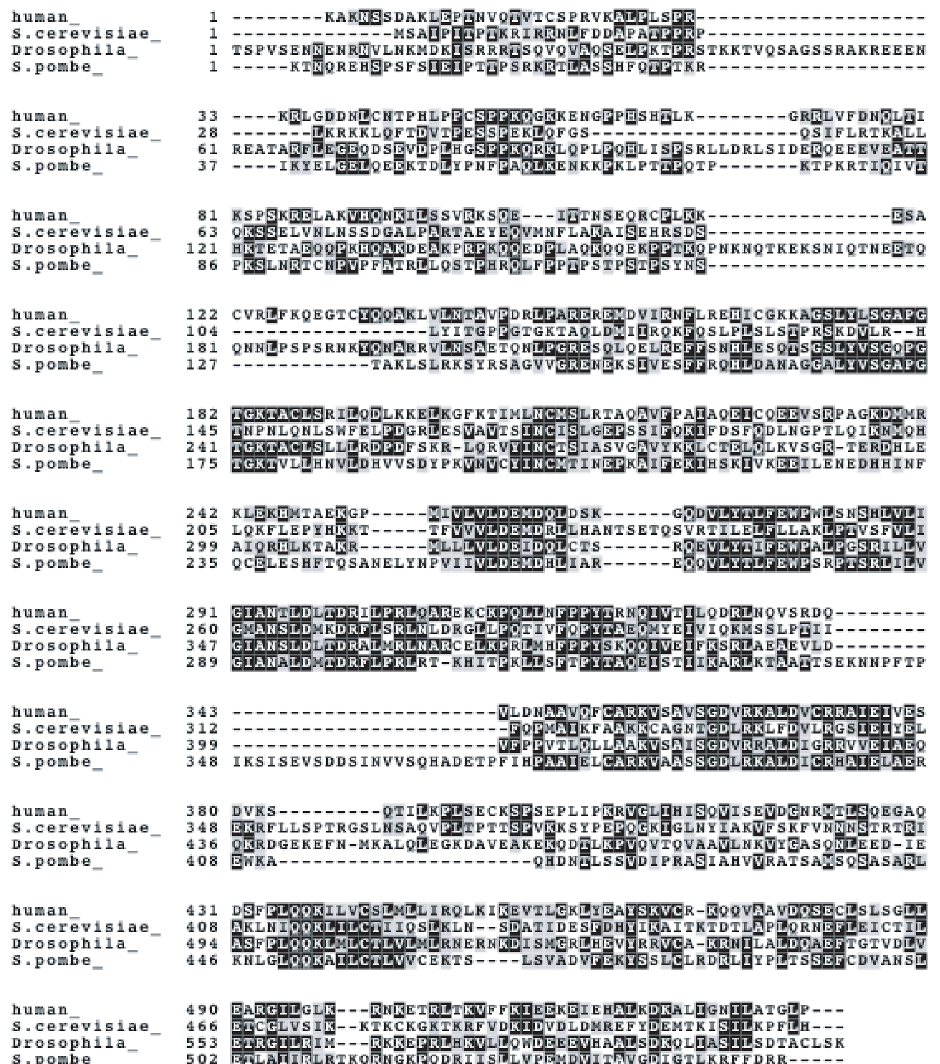

Fig. 1. Sequence comparison of the cdc6 proteins from *Drosophila*, human, *S. cerevisiae* and *S. pombe*. Black shading represents identity and grey shading represents similarity.

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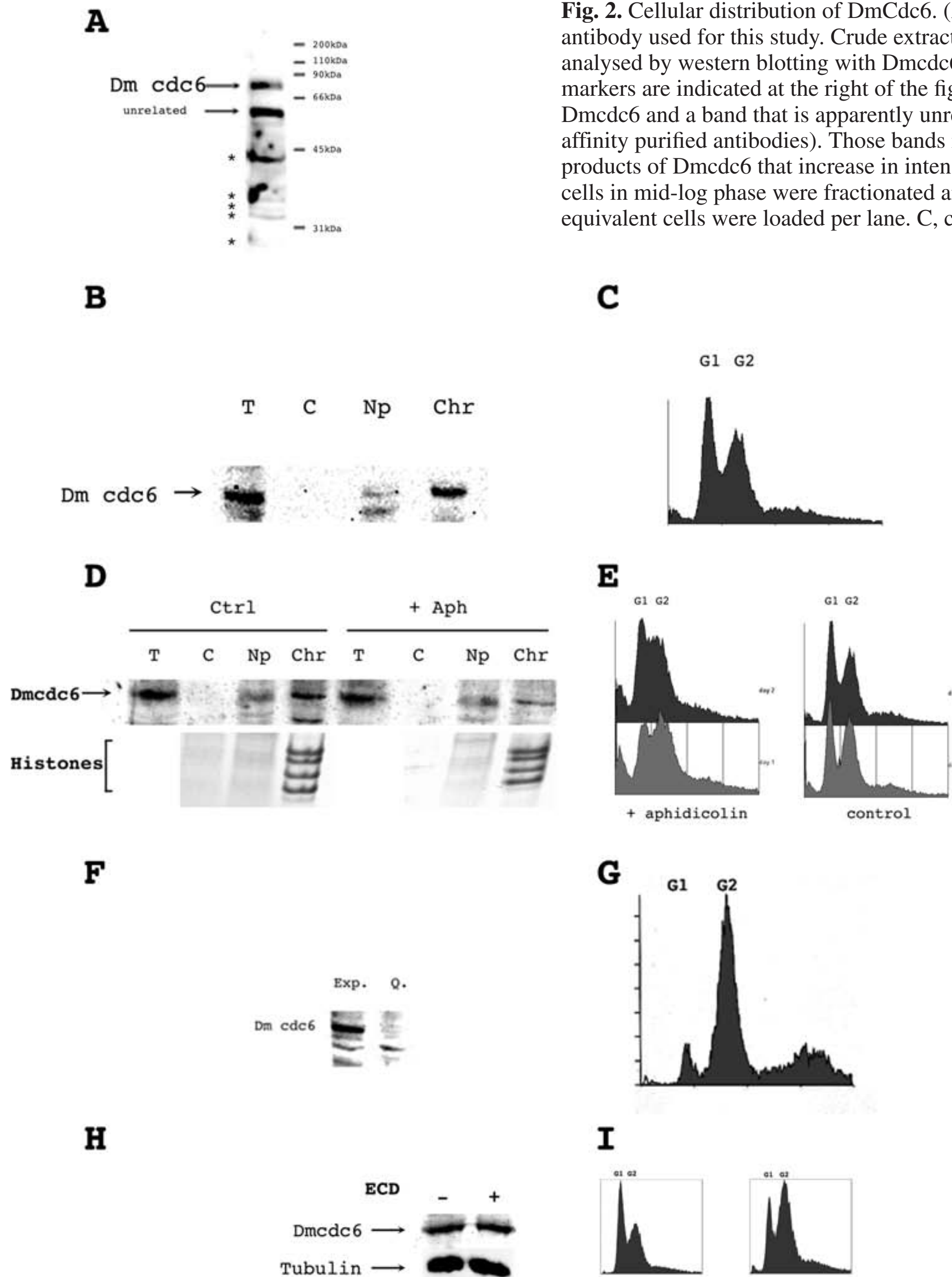

Fig. 2. Cellular distribution of DmCdc6. (A) Characteristics of the Dmcdc6 antibody used for this study. Crude extracts of *Drosophila* S2 cells were loaded and analysed by western blotting with Dmcdc6 antibody. The sizes of molecular weight markers are indicated at the right of the figure. Text indicates the position of the Dmcdc6 and a band that is apparently unrelated (as determined by the use of affinity purified antibodies). Those bands marked with an asterisk are degradation products of Dmcdc6 that increase in intensity as S2 cell fractions are aged. (B) S2 cells in mid-log phase were fractionated and analysed by western blotting. 500,000 equivalent cells were loaded per lane. C, cytoplasmic extract; Chr, chromatin-bound extract; Np, nucleoplasmic extract; T, total cell extract. (C) FACS analysis of exponential phase S2 cells. (D) Proliferating S2 cells were treated with aphidicolin (as described in Materials and Methods) and fractionated as in Fig. 2A. The top panel shows the distribution of DmCdc6 in cellular fractions of untreated cells (Ctrl) and cells treated with aphidicolin (Aph). The bottom panel shows a Coomassie Blue-stained gel of the region of gel between 14 and 21 kDa to show that the distribution of histones is as would be expected for an efficient fractionation. (E) FACS profile of control and aphidicolin-treated cells on days 1 and 2 of the experiment. The fractionation above corresponds to the day 1 sample, although no significant difference was seen between day 1 and day 2. (F) Total cell extract from 500,000 cells exponentially growing (Exp.) or quiescent (Q.) were analysed for the presence of Cdc6 by western blot. (G) FACS profile of nondividing S2 cells prepared as described in Materials and Methods. (H) Proliferating S2 cells were treated with ecdysone as described in Materials and Methods, and the total amount of DmCdc6 in untreated (–) and treated (+) was analysed by western blotting. (I) FACS profile of untreated cells (left) compared with cells that had been treated with ecdysone as described in Materials and Methods (right).

was largely nuclear. In cells growing in mid-exponential phase most of the protein appeared to be quite tightly bound into the nucleus (Fig. 2B). When an analysis of the cell cycle stage of the cells used for the fractionation by FACS analysis was carried out the cell populations were shown to consist of roughly equal populations of cells in the G1 and G2 phases of the cell cycle (Fig. 2C). This would suggest that the DmCdc6 does not become cytoplasmic at either of these stages of the cell cycle.

In other systems it has been suggested that Cdc6 moves out of the nucleus during the S phase. To test whether this was the case in *Drosophila* cells the cell fractionation was performed with cells that had been stalled in the S phase of the cell cycle using aphidicolin (Fig. 2D,E). In this case most of the DmCdc6 protein was still seen to be nuclear, indicating that in the *Drosophila* system DmCdc6 remains nuclear throughout S phase. Although the protein remained nuclear, aphidicolin-blocked cells did consistently show an increased proportion of the DmCdc6 in the nucleoplasm rather than being tightly bound into the nucleus.

Finally, we looked at the situation in nondividing cells. In metazoans when cells enter a quiescent state the levels of Cdc6 can be seen to decrease dramatically. Nondividing S2 cells were produced by allowing the cells to grow to a high density (10 days) – at this point no further increase in cell numbers was detected and the cells were largely in G2 (Fig. 2G). Fig. 2F shows the results that were obtained when equal numbers of dividing and nondividing cells were examined for the

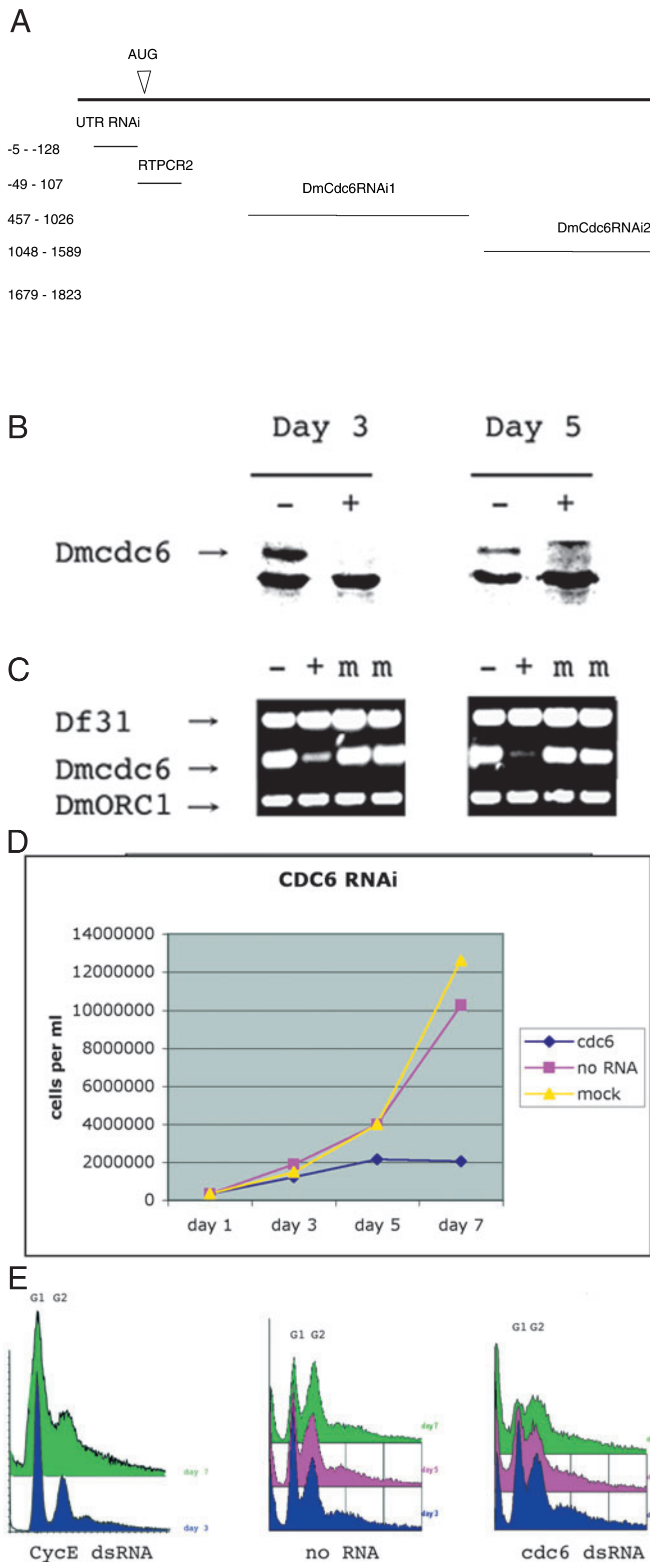

Fig. 3. *DmCdc6* RNA interference. (A) The regions of the *DmCdc6* gene used for RNAi and RT-PCR in all experiments presented in the paper. *DmCdc6* RNAi 1 and 2 were used in the standard *Dmcdc6* knockouts presented in Fig. 3, and RT-PCR 1 is used in both this Figure and Fig. 7 to check for the presence of bulk *DmCdc6*. UTR, RNAi and RTPCR2 are used to knockout and check for, respectively, the presence of the endogenous DmCdc6 in the experiments discussed in Fig. 7. (B) Western blot analysis of *Cdc6* depletion by RNAi: total cell extracts of 500,000 cells untreated (–) or treated with *DmCdc6* double-stranded RNA (+) at day 3 and day 5 of the experiment were loaded in each well. (C) Depletion of *DmCdc6* mRNA detected by RT-PCR at day 3 and day 5. –, no treatment; +, treated with *Dm Cdc6* double-stranded RNA and m, treated with unrelated double-stranded RNA. The Df31 gene amplification serves as a nonspecific control and the ORC1 gene amplification serves as a specific control to test for cross reaction of the probes with the *DmORC1* gene. (D) Comparison of the growth of untreated S2 cells, cells treated with *DmCdc6* double-stranded RNA and cells treated with an unrelated double-stranded RNA. (E) FACS analysis of S2 cells treated with *DmCdc6* RNAi compared with mock treated cells and cells treated with RNAi against cyclin E.

presence of DmCdc6. (Equal loading was also checked by staining the western blots using anti tubulin antibodies – data not shown.) From these results it is clear that these cells do not contain detectable levels of DmCdc6. We have also noted that for cells approaching a nondividing state a high percentage of the protein is often observed in the nucleoplasmic fraction (data not shown). To check if the lack of DmCdc6 in nondividing cells is due to the cells being in the G2 phase of the cycle, S2 cells were enriched for G2 cells using ecdysone as described previously (MacAlpine et al., 2004) (Fig. 2I). When western blotting was used to compare the amount of DmCdc6 in these cells with the levels in unsynchronised cells no significant differences were observed (Fig. 2H).

DmCdc6 depletion by RNAi results in cells that are slow in the S phase of the cell cycle and show abnormal morphology

Cdc6/18 knockouts in yeasts cause a block to S phase entry. In higher eukaryotes gene knockouts have not been carried out, but adding dominant negative protein mutants (usually mutated in the walker A and B ATPase motifs that are seen in cdc6) led to slow progression through S phase. To determine the effect of performing a knockout of Cdc6 in a higher eukaryotic system we carried out RNAi in S2 cells against the DmCdc6 protein. The experiments were carried out with RNAi against two different regions of the protein (Fig. 3A), and the same results were obtained in each case. Using this technique we were able to significantly reduce the levels of both DmCdc6 protein (Fig. 3B) and RNA (Fig. 3C) in these cells.

S2 cells lacking DmCdc6 were less healthy than mock treated controls. Analysis of cell counts suggested that the doubling time of the depleted cells was severely compromised (Fig. 3C). More detailed examination of these cells by FACS analysis (Fig. 3D) revealed that as early as day 3 after RNAi addition there was a significant increase in the numbers of the treated cells that were in the S phase of the cell cycle, suggesting that they were having problems in passing through this stage. In addition, we also saw a significant number of cells that had sub-G1 DNA content. The numbers of cells in both of these categories increased at longer time points. Abnormalities in the cells were also observed when they were studied by indirect immunofluorescence. At day 3 in the experiment

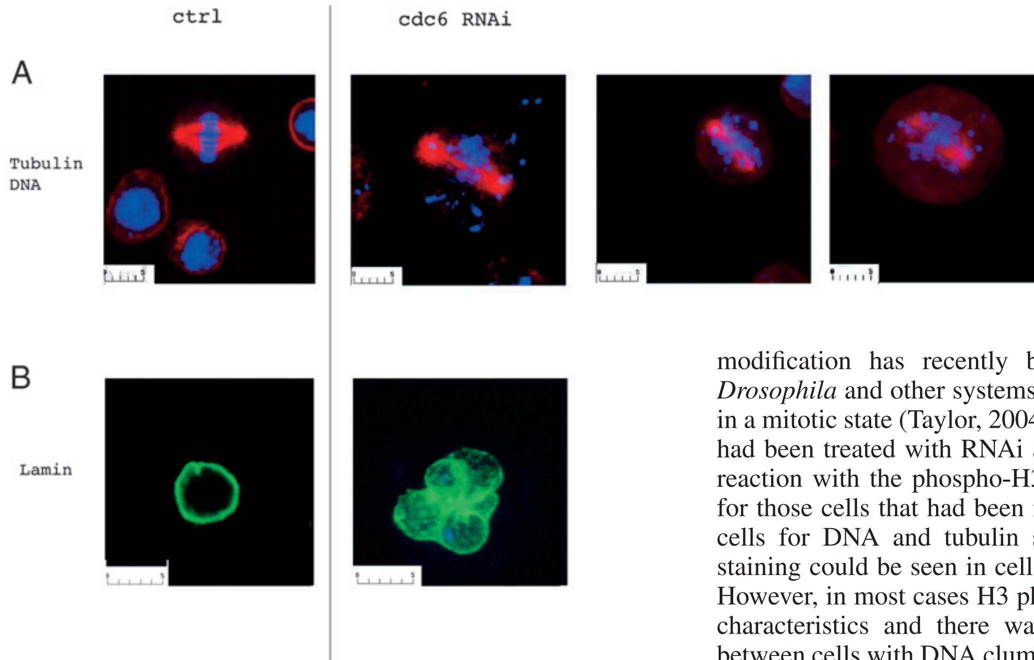

Fig. 4. S2 cells treated with *DmCdc6* double-stranded RNA show abnormalities. Cells treated with *DmCdc6* double-stranded RNA (Cdc6 RNAi) or untreated (Ctrl) were stained for tubulin (red) and DNA (blue) (A), or for lamin (green) and DNA (blue) (B).

approximately 10% of cells were seen to have the phenotype that is shown in Fig. 4A. In these cells the DNA staining can be seen to localise in several clumps in the nuclear region. Co-staining of the cells with tubulin suggested that although some DNA clumps were seen in cells with interphase-like tubulin staining, most were seen in cells that had mitotic tubulin staining. At later days (5, 7) up to 30-40% of the cells were seen to be in this state. The number of DNA clumps visible in the cells varied between 5 and 20, so there was no direct correlation between the number of clumps and the number of chromosomes in the cells. These sorts of figures are never observed in mock treated or untreated cells.

One explanation for what we observed is that the DNA is fragmented due to the cells having undergone apoptosis. Apoptosis usually results in the blebbing of the nucleus and the production of fragmented DNA within a single nucleus. However, in this case staining of the cells with DNA and anti-lamin antibodies (Fig. 4B) suggests that the clumps of DNA are enclosed inside several nuclear lamina – each lamina containing significantly less DNA than would be expected for a single intact nucleus. Additional evidence that the phenotypes were not caused by apoptosis was obtained using the apoptosis inhibitors MG132, proteosome inhibitor 1 and lactocystin, which either alone or in combination with each other inhibit the apoptosis induced by cycloheximide treatment of S2 cells (data not shown) (Fraser et al., 1997). If cells that had been treated with RNAi against Dmcdc6 were treated with these inhibitors from the start of the experiment it did not alter the phenotype that was observed (data not shown). It therefore does not seem likely that the cells have undergone apoptosis. A second explanation for the DNA clumps is that the cells have entered mitosis with incompletely replicated DNA. This would be expected to lead to premature chromosome condensation and/or DNA fragmentation. To determine whether this might be the case we stained the cells with antibody that recognises the mitosis-specific phosphorylation of H3 on serine 10. This modification has recently been used extensively in both *Drosophila* and other systems to indicate that the chromatin is in a mitotic state (Taylor, 2004). In all, 40-50% of the cells that had been treated with RNAi against DmCdc6 gave a positive reaction with the phospho-H3 antibody compared with 2.5% for those cells that had been mock treated. Co-staining of the cells for DNA and tubulin showed that some H3 phospho staining could be seen in cells with interphase characteristics. However, in most cases H3 phospho-positive cells had mitotic characteristics and there was a high degree of correlation between cells with DNA clumps and those that stained positive for H3 phospho (Fig. 5). This suggests that the chromatin in these cells is in a mitotic state, even though from FACS analysis substantial amounts of the chromatin did not seem to have been fully replicated. Notably, although most of the chromatin clumps in the *Dmcdc6* RNAi-treated cells stained with the anti-H3 phospho antibody, in some cells it was possible to see clumps of DNA that are negative for this staining.

We do not think that the phenotype we observed with DmCdc6 RNAi was due only to a halt in cell cycle progression because RNAi vs cyclin E, although it caused an arrest at the G1 stage of the cell cycle, did not lead to a reduction in the amount of DNA (Fig. 3D) or give any of the DNA clump phenotypes that were observed with immunofluorescence. In addition, cells blocked with aphidicolin over the same period also did not show these characteristics (data not shown).

Owing to the high degree of homology between cdc6 and DmORC1 it was formally possible that any effects that were observed on *Dmcdc6* RNAi treatment of S2 cells might have been caused by a decrease in the amount of ORC1 in the cell. To control for this, RT-PCR reactions were also carried out to look at the effect of the *DmCdc6* RNAi experiment on DmORC1 levels. As can be seen from Fig. 3B, the RNAi treatment that we carried out showed no effect on the level of DmORC1 in these cells.

Overexpression of DmCdc6 in S2 cells causes a slight S/G2 delay

With the exception of *S. pombe*, overexpression of wild-type Cdc6/18 proteins has been seen to have only subtle effects on nonsynchronised cells. Overexpression studies in higher eukaryotes have mainly been done, however, by injecting protein into cells or transient protein expression. We were therefore interested to determine whether stable overexpression of high levels of DmCdc6 in S2 cells might have a more dramatic effect. We therefore constructed *Drosophila* cell lines containing additional copies of DmCdc6 using the Invitrogen

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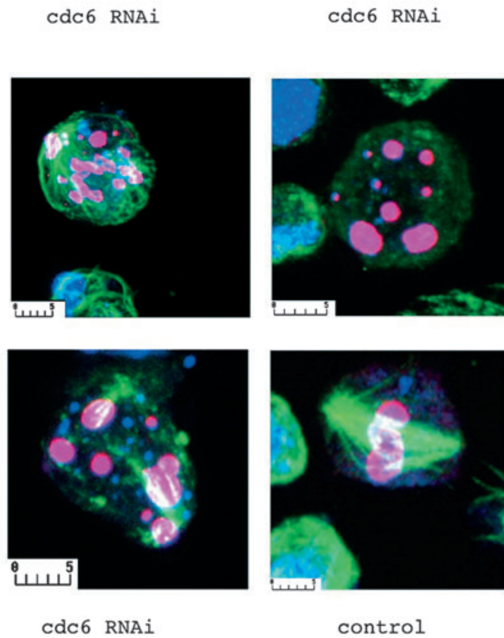

Fig. 5. Treatment of S2 cells *DmCdc6* double-stranded RNA causes a large number of cells to stain with antiphospho H3 antibody. Cells either treated with *Cdc6* double-stranded RNA (Cdc6 RNAi) or untreated (Ctrl)were stained for tubulin (green) phospho H3 (red) and DNA (blue). The DNA and phospho H3 costaining appears purple.

PMTV/HisA system. In this system the protein is under the control of the metallothionein promoter so that the protein is not constitutive but can be induced by the addition of copper. The expressed protein is tagged with SV5 and hexahis at the C terminus; this makes its apparent size about 3 kDa larger on PAGE. It is therefore distinguishable from the endogenous protein on high-resolution gels. Several independent cell lines were obtained and we chose to characterise two of these, expressing DmCdc6 at different levels (10× or 30-40× endogenous protein levels) (Fig. 6). Although this system is inducible, in our hands the expression of the protein appeared to be somewhat leaky (see Fig. 6), and therefore we were not able to get a true uninduced control with these lines. However, the characterisation of two independent lines should rule out the possibility that the results that we got were due to the insertion sites of the stable transformant.

In terms of the growth characteristics of the cells, neither the low- nor high-expressing cell line was significantly different from what was seen for the control S2 line (data not shown), although some reduction in growth in all cases was caused by the addition of the inducing copper. FACS analysis on both of the overexpressing lines, however, showed that they differed from the control cells in the same way – both of them showed a slightly increased tendency to delay in the late S/G2 phase of the cycle (Fig. 7A). Although the effect observed was very subtle, it was completely reproducible in several independent experiments. Immunofluorescent staining using reagents to visualise DNA, tubulin and lamin did not show any significant differences between the stably transfected and the control S2 cells (data not shown). One possible explanation for the observed lack of effect of overexpressed DmCdc6 was that even though we had observed the overexpressed protein in cell

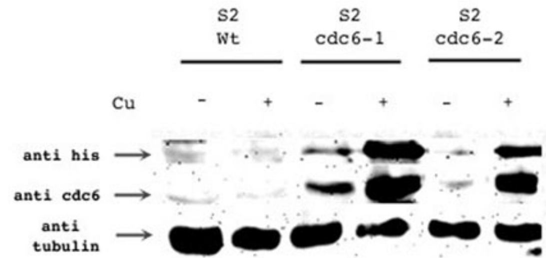

Fig. 6. Overexpression of DmCdc6 in S2 cells. Two independent transformed S2 cell lines (S2 Cdc6-1 and S2 Cdc6-2) were analysed for expression of DmCdc6 either with or without induction by copper. The protein levels were visualised by western blotting with antibody against either the DmCdc6 protein itself or the His tag present in the overexpressed protein. In each case total cell extract from 500,000 cells was loaded. The western was also developed with anti tubulin antibody as a loading control.

extracts it was not localising properly to the nucleus. However, Fig. 7B shows that this is not the case as both of the overexpressed proteins could be seen in the chromatin-associated fraction of the cell. This was further confirmed by the immunostaining of the cells (Fig. 7C). Despite the correct localisation of the overexpressed DmCdc6 proteins it was still possible that some aspect of the construction had inactivated these proteins. Therefore, to show that this was not the case we knocked out the expression of the endogenous DmCdc6 protein in these overexpressing cell lines by targeting RNAi to the upstream untranslated region of the *DmCdc6* mRNA (see Fig. 3A). This caused a reduction in the level of the endogenous RNA to undetectable levels while not measurably affecting the amount of *DmCdc6* mRNA produced from the overexpressed proteins (Fig. 7D). Overexpressing cells in which the endogenous DmCdc6 had been removed were not noticeably affected by the removal of the endogenous DmCdc6 as they continued to grow as well as the cells in which no RNAi had been performed (Fig. 7E). In addition, analysis of the FACS profiles for these cells also failed to show a significant difference between the cells with and without RNAi (data not shown). We therefore conclude that the reason that the overproduced DmCdc6 did not have an effect is not related to the lack of activity of the overexpressed protein, and therefore that constitutive expression of high levels of DmCdc6 – in agreement with what has previously been seen for transient expression – has very little effect on the behaviour of higher eukaryotic cells.

Discussion

Subcellular localisation of DmCdc6

Our results suggest that in *Drosophila* cells DmCdc6 is a nuclear protein in G1, S and G2 phases of the cell cycle. Similar results were recently obtained for the endogenous protein in CHO cells (Alexandrow and Hamlin, 2002). These data are somewhat at odds with earlier data suggesting that Cdc6 in higher eukaryotes leaves the nucleus during the S phase, although as pointed out by Alexandrow the earlier data was largely obtained from the study of overexpressed protein, which seems to behave differently to endogenous protein in mammalian systems. Interestingly, in the *Drosophila* system

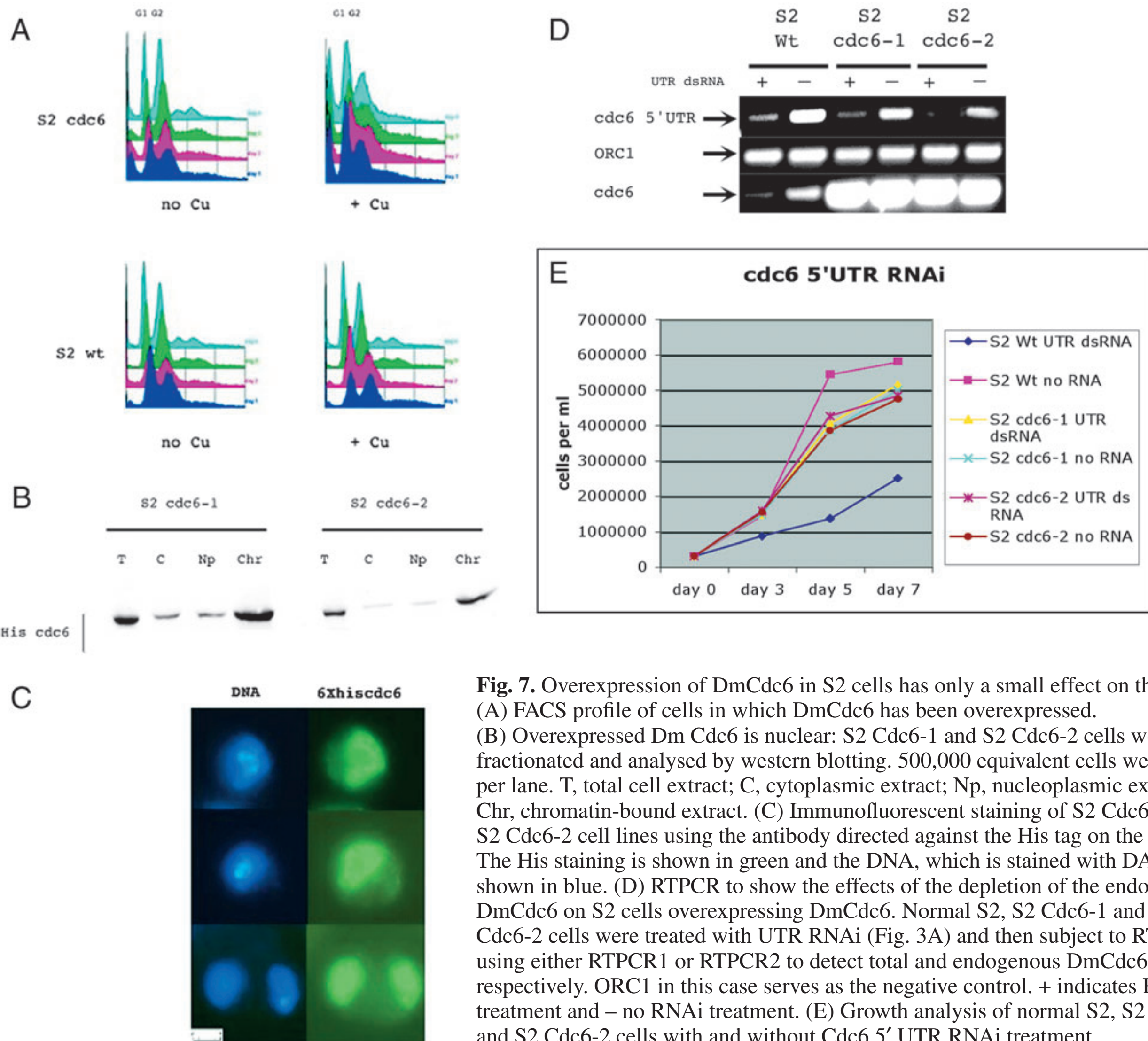

Fig. 7. Overexpression of DmCdc6 in S2 cells has only a small effect on the cells. (A) FACS profile of cells in which DmCdc6 has been overexpressed. (B) Overexpressed Dm Cdc6 is nuclear: S2 Cdc6-1 and S2 Cdc6-2 cells were fractionated and analysed by western blotting. 500,000 equivalent cells were loaded per lane. T, total cell extract; C, cytoplasmic extract; Np, nucleoplasmic extract; Chr, chromatin-bound extract. (C) Immunofluorescent staining of S2 Cdc6-1 and S2 Cdc6-2 cell lines using the antibody directed against the His tag on the protein. The His staining is shown in green and the DNA, which is stained with DAPI, is shown in blue. (D) RTPCR to show the effects of the depletion of the endogenous DmCdc6 on S2 cells overexpressing DmCdc6. Normal S2, S2 Cdc6-1 and S2 Cdc6-2 cells were treated with UTR RNAi (Fig. 3A) and then subject to RTPCR using either RTPCR1 or RTPCR2 to detect total and endogenous DmCdc6, respectively. ORC1 in this case serves as the negative control. + indicates RNAi treatment and – no RNAi treatment. (E) Growth analysis of normal S2, S2 Cdc6-1 and S2 Cdc6-2 cells with and without Cdc6 5′ UTR RNAi treatment.

even heavily overproduced protein is still largely nuclear, although whether this is related to differential control of the protein in different species or is because of the use of different methodology is not clear. In *Drosophila* a high percentage of the DmCdc6 at G1, S and G2 phases of the cycle seems to be tightly associated with chromatin. This prolonged association of DmCdc6 with chromatin is consistent with DmCdc6 having a role in the cell after the initiation of DNA replication as suggested by others (Oehlmann et al., 2004; Clay-Farrace et al., 2003). However, in aphidicolin-blocked cells we see a reproducible increase in the amount of DmCdc6 in the nucleoplasm (i.e. detergent soluble). This might reflect a change in the nature of the DmCdc6 complex with chromatin related to this second function, although we cannot rule out that this is a specific effect caused by the perturbation of the cell cycle due to drug treatment.

Overexpression of DmCdc6

Most of the previous studies carried out to look at the effects of overexpression of Cdc6 in higher eukaryotes have involved protein microinjection or transient expression. These studies suggest that the effects of this overexpression in nonsynchronised cells are very limited. Using *Drosophila* S2 cells it is possible to make stable cell lines that express DmCdc6 under an inducible promoter to high levels. The levels and duration of expression in this type of system are more likely to reveal overexpression effects of the protein. Furthermore, the tight chromatin association of the bulk of the DmCdc6 overexpressed in this way, and the ability of the overexpressed protein to substitute when the endogenous cellular DmCdc6 has been removed by RNAi suggests functionality of the protein. Despite this, in agreement with earlier studies in other systems, we see that even the highest level of overexpression of the protein does not have a drastic effect on the cells – in particular, we see no evidence of over replication of chromatin. We do, however, see a small but reproducible lag in the G2/M transition of these cells. In human cells injection of Cdc6 into synchronised G2 cells has been reported to block entry into mitosis, which has been attributed to a role for Cdc6 in linking the S and M phases of the cell cycle. Our data are suggestive of a similar mechanism operating in *Drosophila* cells.

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Underexpression of DmCdc6

The most penetrative event that is observed when the DmCdc6 protein is removed from *Drosophila* cells is the inability of the cells to progress through the S phase of the cell cycle (as observed by FACS analysis). This suggests, unsurprisingly, that as for other organisms the DmCdc6 protein plays a vital role in DNA replication. Further analysis of the cells by immunofluorescence does not, however, show cells that are arrested with S phase morphology. A high percentage of the cells – although they do not apparently have fully replicated DNA – have passed into mitosis, as measured by the presence of the phospho-H3 antigen on the chromatin. A few of these cells have a morphology that looks like premature chromatin condensation (PCC). PCC has often been observed during the analysis of larvae carrying mutations in *Drosophila* replication proteins (Krause et al., 2001; Loupart et al., 2000; Pflumm and Botchan, 2001). In addition, depletion of other replication proteins in S2 cells (cdc45, MCM2, MCM5 and MCM10) has also been reported to show PCC (Christensen and Tye, 2003). For many of the DmCdc6-depleted cells, however, the phenotype looks more severe as the DNA appears to be heavily fragmented, spindles are often present and in some cases subgenome quantities of DNA appear to be surrounded by individual nuclear laminae. This phenotype has some similarities with what has been observed for *Drosophila cdt1* RNAi in S2 cells (Mihaylov et al., 2002) and also with the cut phenotype obtained from knocking out the *S. pombe* Cdc6 homologue cdc18 (Kelly et al., 1993). The observed state of these DmCdc6-depleted S2 cells suggests that, in addition to their replication defect, they are also missing a checkpoint to prevent mitotic entry with unreplicated DNA. The observation of spindles and nuclear laminar reformation around subgenomic DNA masses further suggest that several aspects of the cell cycle, not just the chromosome cycle, are also affected. This, therefore, provides further evidence that in *Drosophila* as well as in other higher eukaryotes the Cdc6 protein may have a second role in the cell cycle concerned with the co-ordination of the S and M phases.

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