

Deregulation of cyclin E in human cells interferes with prereplication complex assembly

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Deregulation of cyclin E expression has been associated with a broad spectrum of human malignancies. Analysis of DNA replication in cells constitutively expressing cyclin E at levels similar to those observed in a subset of tumor-derived cell lines indicates that initiation of replication and possibly fork movement are severely impaired. Such cells show a specific defect in loading of initiator proteins Mcm4, Mcm7, and to a lesser degree, Mcm2 onto chromatin during telophase and early G1 when Mcm2–7 are normally recruited to license origins of replication.

Because minichromosome maintenance complex proteins are thought to function as a heterohexamer, loading of Mcm2-, Mcm4-, and Mcm7-depleted complexes is likely to underlie the S phase defects observed in cyclin E–deregulated cells, consistent with a role for minichromosome maintenance complex proteins in initiation of replication and fork movement. Cyclin E–mediated impairment of DNA replication provides a potential mechanism for chromosome instability observed as a consequence of cyclin E deregulation.

Introduction

Cyclin E, a positive regulatory subunit of Cdk2, normally accumulates periodically at the G1/S transition, where it promotes entry into S phase and other DNA replication–associated functions (Sauer and Lehner, 1995; Ekholm and Reed, 2000). In somatic mammalian cells, cyclin E levels specifically decline during S phase, reaching low or undetectable levels by the time replication is complete (Ekholm et al., 2001). However, in many types of human cancer cyclin E is overexpressed, and in some cases its expression becomes deregulated relative to the cell cycle (Keyomarsi et al., 1995; Sandhu and Slingerland, 2000; Erlanson and Landberg, 2001; Erlandsson et al., 2003; Schraml et al., 2003; Reed et al., 2004). That cyclin E deregulation is directly implicated in the etiology of cancer is supported by at least two lines of evidence. First, mice carrying a transgene programmed to express cyclin E at an elevated level and without cell cycle regulation in the mammary epithelium during pregnancy

and lactation develop mammary adenocarcinomas (Bortner and Rosenberg, 1997). Second, the gene encoding hCdc4, a protein required for turnover of cyclin E during S phase, is found to be mutated and to have undergone allelic loss in several types of cancer, leading to cyclin E deregulation (Moberg et al., 2001; Strohmaier et al., 2001; Spruck et al., 2002; Rajagopalan et al., 2004). In the latter case, *bCDC4* mutation and concomitant cyclin E deregulation correlate with higher tumor grade, more advanced stage, and metastasis, compared with tumors without cyclin E deregulation (Spruck et al., 2002). Together, these observations suggest that deregulation of cyclin E is a functionally significant factor in the development and progression of malignant disease.

Although it is not yet known how cyclin E deregulation promotes tumorigenesis, one possible mechanism may be through the generation of aneuploidy (Duesberg and Li, 2003; Fabarius et al., 2003). Deregulation of cyclin E expression in nontransformed rodent fibroblasts and human mammary epithelial cells caused elevated frequencies of chromosome losses and gains, as well as polyploidy (Spruck et al., 1999; Loeb and Loeb, 2000). Therefore, cyclin E–mediated

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Abbreviations used in this paper: c-Ad, control adenovirus; E-Ad, cyclin E recombinant adenovirus; MCM, minichromosome maintenance complex; ORC, origin recognition complex; PCNA, proliferating cell nuclear antigen; preRC, prereplication complex; siRNA, small interfering RNA.

genomic instability may constitute a functional link to malignancy, although this remains to be demonstrated in an *in vivo* model.

The generation of aneuploid cells can come about through a variety of mechanisms ranging from DNA damage that is not correctly repaired to defects in chromosome segregation during mitosis (Loeb and Loeb, 2000; Jallepalli and Lengauer, 2001; Masuda and Takahashi, 2002). Of potential significance is the paradoxical observation that deregulated cyclin E expression accelerates the G1/S transition (Ohtsubo and Roberts, 1993; Resnitzky et al., 1994; Wimmel et al., 1994), yet leads to a slowing of S phase (Ohtsubo and Roberts, 1993; Resnitzky et al., 1994; Spruck et al., 1999). In principle, impairment of DNA replication could elevate the frequency of cells with incompletely replicated chromosomes undergoing mitosis. The inevitable result of such regulatory accidents would most likely be chromatid nondisjunction and subsequent aneuploidy.

The apparent paradox of cyclin E deregulation on the one hand accelerating the rate of entry of cells into S phase, but on the other causing inefficient progression through S phase can be resolved if one considers the role(s) of Cdks in regulating DNA replication. Cdk activity is clearly required for initiating DNA replication (Lei and Tye, 2001; Nishitani and Lygerou, 2002; Woo and Poon, 2003), and it is likely that cyclin E–Cdk2 has a role in this context, consistent with deregulated expression of cyclin E accelerating the G1/S transition. At the same time, investigation of the requirements for assembly of prereplication complexes (preRCs) in yeast and *Xenopus* egg-based *in vitro* DNA replication systems has indicated that Cdk activities must be reduced to low levels or eliminated for this process to occur (Lei and Tye, 2001; Nishitani and Lygerou, 2002; Woo and Poon, 2003). PreRCs are formed by the six-subunit origin recognition complex (ORC) as well as initiation factors Cdc6, Cdt1, Mcm2–7, and possibly other proteins (Lei and Tye, 2001; Nishitani and Lygerou, 2002). Therefore, the negative effect of cyclin E deregulation on DNA replication could be a consequence of inappropriate Cdk activity at the time when preRC complexes are normally assembled—the end of mitosis and the beginning of G1. To clearly define the link between cyclin E deregulation and replication impairment, an analysis of preRC assembly was performed in human cells ectopically expressing high levels of cyclin E via adenoviral transduction. In this paper, we show that deregulation of cyclin E expression does indeed interfere with preRC assembly, leading to defects in replication initiation and possibly in fork movement.

Results

Deregulation of cyclin E expression accelerates S phase entry

To study the effect of constitutive cyclin E expression on DNA replication in mammalian cells, KB cells were transduced with a recombinant adenovirus containing a cDNA encoding human cyclin E (E-Ad; see Materials and methods). At an multiplicity of infection of 100 almost all cells were shown to be positive for cyclin E immunofluorescence

staining, whereas in cells transduced with a control adenovirus (c-Ad) only 50% of the cells were found to be positive for cyclin E immunofluorescence staining, a level also found in nontransduced cells (unpublished data).

It has been shown previously that premature expression of cyclin E results in shortening of the G1 phase and accelerated S phase entry (Ohtsubo and Roberts, 1993; Resnitzky et al., 1994; Wimmel et al., 1994). To confirm that acute cyclin E expression obtained by adenoviral transduction affects the duration of G1 phase and timing of entry into S phase, KB cells, chosen for their high efficiency of adenoviral transduction, were transduced with E-Ad or c-Ad. Transduced cells were then synchronized by mitotic shake-off, plated onto glass slides in the presence of BrdU, and analyzed by immunofluorescence microscopy for BrdU staining at different times after mitosis. As can be seen in Fig. 1 a, cells transduced with E-Ad entered S phase synchronously between 4 and 10 h after exiting mitosis, with 50% of the cells scoring positive for BrdU 5 h after mitosis. In control cells, entry into S phase began later with more heterogeneous kinetics. Not until 8 h after mitosis did 50% of the population score positive for BrdU. These results are in agreement with previous reports showing that constitutive expression of cyclin E accelerates S phase entry (Ohtsubo and Roberts, 1993; Resnitzky et al., 1994; Wimmel et al., 1994) and that cells enter S phase at a relatively fixed interval after the accumulation of cyclin E (Ekholm et al., 2001), accounting for the more synchronous entry into S phase of the E-Ad–transduced population.

Deregulation of cyclin E impairs DNA replication

Deregulated expression of cyclin E has also been inferred to result in slowing of S phase progression (Ohtsubo and Roberts, 1993; Resnitzky et al., 1994; Spruck et al., 1999), although no direct analysis of DNA synthetic rate or of dose responsiveness to cyclin E levels was reported. Therefore, two-dimensional flow cytometric analysis of cells transduced with E-Ad and c-Ad and then subjected to a short pulse of BrdU incorporation was performed to investigate the effect of deregulated cyclin E expression on DNA replication. As shown in Fig. 1 b, 24 h after transduction 48% of cells transduced with E-Ad were in S phase, whereas only 35% of cells transduced with c-Ad were in S phase. Furthermore, this effect was shown to be dose dependent, as the percentage of cells in S phase was found to increase with increasing multiplicity of infection (Fig. 1 b).

To determine whether deregulated expression of cyclin E affects the efficiency of DNA replication, individual pulse-labeled cells with an early S phase BrdU staining pattern (Nakamura et al., 1986; Nakayasu and Berezney, 1989) were compared from the E-Ad– and c-Ad–transduced populations after analysis by immunofluorescence deconvolution microscopy. Compared with control cells, cells with deregulated cyclin E expression contained a reduced number of BrdU foci, and these foci exhibited a significantly lower fluorescence intensity (Fig. 1 c). Although the exact nature of such BrdU foci is not known, presumably they correspond to clusters of newly replicated DNA strands. Therefore, these data are consistent with the notion that deregulation of cyclin E results in a reduced rate of DNA synthesis.

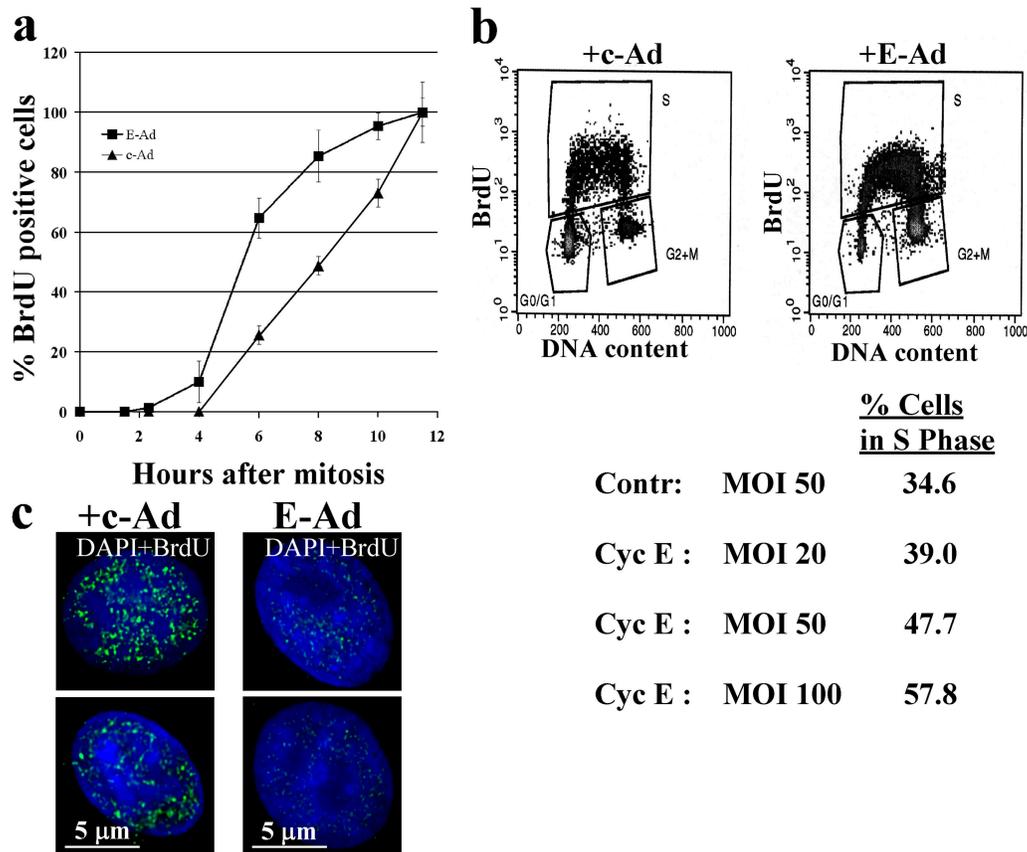


Figure 1. Cell cycle effects on deregulation of cyclin E expression. (a) Time course of S phase entry of cyclin E-deregulated and control cells. Cells transduced with cyclin E recombinant adenovirus (E-Ad) and control adenovirus (c-Ad) were synchronized by mitotic shake-off and replated into medium containing BrdU. S phase entry was scored by immunofluorescence microscopy using anti-BrdU antibodies. The experiment was performed in triplicate and error bars represent 1 SD. (b) Flow cytometric cell cycle analysis of cyclin E-deregulated and control cells. Asynchronous cells transduced with c-Ad or E-Ad were subjected to a 15-min BrdU pulse before fixation and preparation for flow cytometric analysis. Abscissa indicates nuclear DNA content based on propidium iodide staining; ordinate indicates BrdU incorporation based on immunofluorescence. (c) Replication foci in early S phase cells with deregulated cyclin E. E-Ad- and c-Ad-transduced cells, respectively, were subjected to a 15-min BrdU pulse and labeled DNA was analyzed by immunofluorescence. Two representative early S phase cells from each population are shown. DAPI-stained DNA is shown in blue; incorporated BrdU is shown in green.

This provides a mechanistic basis for the accumulation of S phase cells observed by flow cytometric analysis (Fig. 1 b).

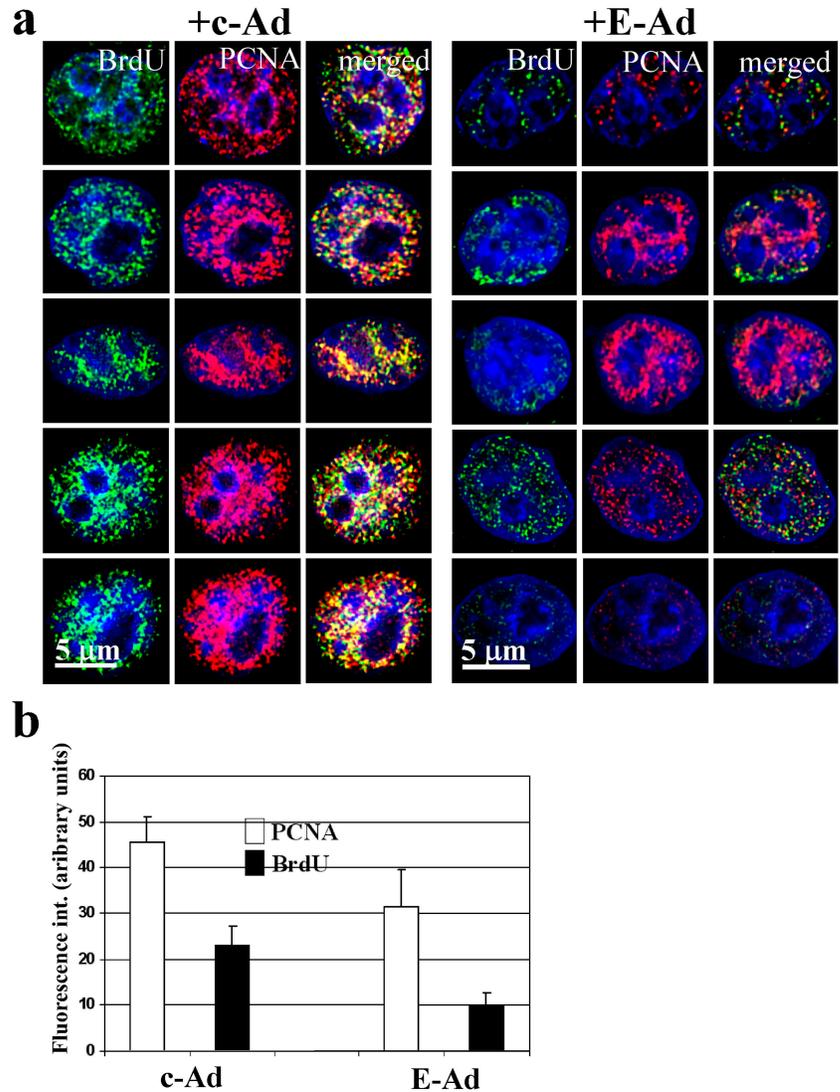
Initiation or elongation?

The observed reduction of both the number and staining intensity of BrdU foci in cells with constitutive cyclin E expression (Fig. 1 c) indicates a reduced rate of replication, but doesn't distinguish between impairment of initiation or impairment of fork progression. Therefore, we analyzed the localization patterns of BrdU and proliferating cell nuclear antigen (PCNA) in individual early S phase cells with or without constitutive cyclin E expression. As a processivity factor for DNA polymerase δ during replication (Prelich et al., 1987; Krishna et al., 1994; Fukuda et al., 1995; Kelman, 1997), PCNA has been shown to be localized at the replication fork and to colocalize with newly synthesized DNA labeled with BrdU (Hozak et al., 1993; Takanari et al., 1994; Somanathan et al., 2001). Therefore, we used PCNA as a quantitative marker for replication forks. Presumably, the greater the intensity of PCNA staining, the greater the number of replication forks assembled. In early S phase, the number of replication forks should be roughly proportional

to the number of origins used. As can be seen in Fig. 2, early S phase cells transduced with E-Ad exhibit reduced BrdU staining as well as reduced PCNA staining. For PCNA, there was a reduction in the number of foci and of staining intensity of individual foci (Fig. 2), as observed for BrdU (Fig. 1 c and Fig. 2). These results suggest that deregulation of cyclin E expression results in a reduction in the number of origins used in early S phase, consistent with a defect in replication initiation. Interestingly, however, in the E-Ad-transduced population many nuclei were observed in which PCNA foci did not correspond to BrdU foci, compared with nuclei in the c-Ad-transduced population. Such a pattern is consistent with inactive or stalled replication forks. Thus, deregulation of cyclin E expression may also impair replication fork movement.

Before proceeding further in this investigation, we confirmed that cyclin E levels under viral transduction conditions were comparable to those in tumors with deregulated cyclin E. Therefore, we compared both cyclin E protein levels and associated Cdk2 kinase activity in E-Ad-transduced KB cells to those in selected breast cancer-derived cell lines and found them to fall within an equivalent range (Fig. 3 a).

Figure 2. Titration of replication forks by PCNA staining in early S phase nuclei with deregulated cyclin E. c-Ad- and E-Ad-transduced cells were subjected to a 15-min BrdU pulse and were analyzed simultaneously for BrdU incorporation and chromatin-bound PCNA by immunofluorescence deconvolution microscopy. (a) Images of representative early S phase cells are shown. Red, PCNA; green, BrdU; blue, DNA (DAPI). (b) Histograms representing average values of integrated intensities (in arbitrary units) of nuclear staining shown in panel a. Error bars are equivalent to 1 SD.



A potentially trivial explanation for impairment of DNA replication in E-Ad-transduced cells is competition with cyclin A for a limiting pool of the catalytic subunit Cdk2. Cyclin A-Cdk2 has been shown to be required for progression through S phase. Therefore, Cdk2 was immunoprecipitated from extracts prepared from E-Ad- and c-Ad-transduced S phase cells and was analyzed for cyclin A binding by immunoblotting. Although there is significantly more cyclin E in S phase extracts prepared from E-Ad-transduced cells, the amount of cyclin A bound to Cdk2 is comparable in the two extracts (Fig. 3 b). Therefore, S phase effects of cyclin E deregulation under the conditions used in this paper cannot be attributed to competition with cyclin A.

Altered chromatin loading of MCM proteins in cells constitutively expressing cyclin E

Previous reports in yeast and *Xenopus* egg extracts have shown that Cdk activity must be reduced to low levels to allow assembly of preRCs, a criterion that is normally met during late mitosis and early G1 phase in mammalian somatic cells (Yan and Newport, 1995; Coverley et al., 1996; Wuarin and Nurse, 1996; Hua et al., 1997; Nishitani and Lygerou,

2002). To determine whether constitutive cyclin E expression in late mitosis and early G1 impairs preRC assembly, we studied chromatin loading of various preRC components in telophase cells, with or without constitutive cyclin E expression. First it was confirmed that in E-Ad-transduced cells, cyclin E was expressed at the time when preRCs are normally assembled. After adenovirus transduction, cells were synchronized by mitotic shake-off and were analyzed for cyclin E expression by immunofluorescence deconvolution microscopy at ~ 1 h after mitosis. In the control population cyclin E was not detected in any telophase cells. However, for the E-Ad-transduced population, most telophase cells were cyclin E positive (Fig. 3 c). For comparison, telophase SUM149PT cells processed in parallel are shown (Fig. 3 c). SUM149PT is a breast cancer-derived cell line mutated for *bCDC4/FBW7* encoding a critical specificity factor required for cyclin E turnover (Strohmaier et al., 2001). As can be seen, the typical level of cyclin E in telophase E-Ad-transduced cells is comparable to that in a nontransduced tumor-derived cell line deregulated for cyclin E expression as a result of mutation.

The association of preRC components with chromatin during telophase was first determined by immunofluores-

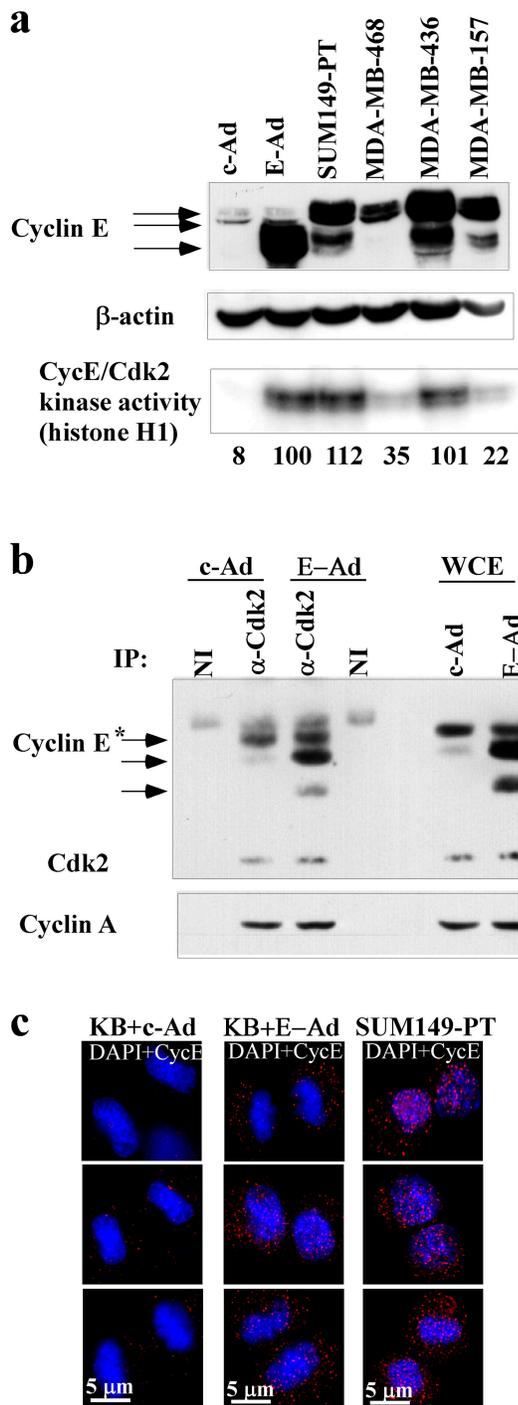


Figure 3. Status of cyclin E and cyclin A in adenovirus-transduced cells. (a) Levels of cyclin E and cyclin E–Cdk2 kinase activity in cancer-derived cell lines. Protein extracts were prepared from c-Ad– and E-Ad–transduced KB cells as well as from four breast cancer–derived cell lines (SUM149-PT, MDA-MB-468, MDA-MB-436, and MDA-MB-157). The same extracts were used for SDS-PAGE followed by immunoblotting (top panels) and immunoprecipitation for histone H1 kinase assay (bottom panel). Quantitation of cyclin E–Cdk2 kinase activity (32 P incorporation) is shown. (b) Cyclin E and cyclin A association with Cdk2 in S phase cyclin E–transduced cells. c-Ad– and E-Ad–transduced cells were synchronized in S phase by treatment with thymidine. Cell lysates were subjected to immunoprecipitation using anti-Cdk2 antibody followed by SDS-PAGE and immunoblotting, or were analyzed directly by SDS-PAGE and immunoblotting. NI, nonimmune serum; WCE, whole-cell

cence microscopy of cells that were subjected to a short detergent extraction to remove proteins not tightly bound to chromatin (see Materials and methods). Constitutive cyclin E expression was found to dramatically reduce chromatin loading of Mcm4 in telophase cells compared with control cells (Fig. 4 b). However, the total level of Mcm4 protein was not altered because nondetergent-extracted cells showed no difference in the Mcm4 staining pattern (Fig. 4 a). For Mcm3 and Mcm7 there was a lesser but significant decrease in chromatin loading in telophase cells with deregulated cyclin E expression (Fig. 5). For Mcm2 and Mcm6 no significant decrease was observed (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200404092/DC1>).

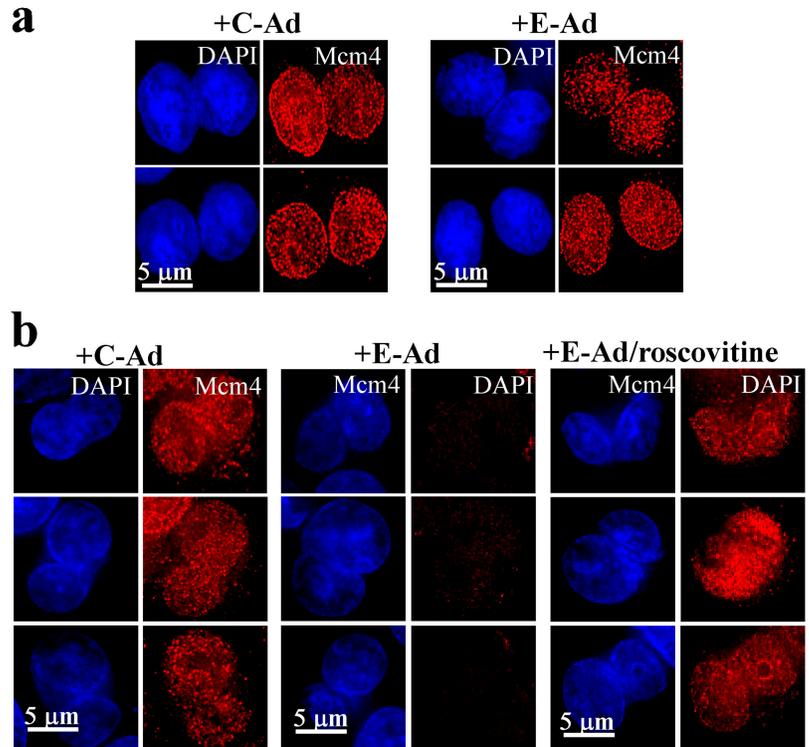
The presence of initiator proteins on chromatin was also analyzed in cells transduced with c-Ad or E-Ad by immunoblotting after biochemical fractionation (Fig. 6 a; Méndez and Stillman, 2000). The chromatin localization of components of the human ORC (Orc1, Orc2) or initiator protein Cdc6 was not affected by constitutive expression of cyclin E (Fig. 6 b). However, a strong impairment of chromatin loading was noticed for both Mcm4 and Mcm7, with much more modest effects observed for the other four minichromosome maintenance complex (MCM) proteins (Fig. 6 b).

Kinetics of MCM protein loading after mitotic exit

Experiments described above document a cyclin E–mediated impairment of Mcm4 and Mcm7 loading, and to a lesser degree, loading of other MCM subunits onto chromatin during telophase. To determine whether a deficiency of Mcm4 and Mcm7 (as well as other subunits) persists into G1 and early S phase, two experiments were performed. First, c-Ad– or E-Ad–transduced cells were subjected to mitotic synchronization and shake-off. In this experiment, cells were collected at prometaphase by treatment with nocodazole after release from a double-thymidine block in order increase the yield of mitotic cells. After shake-off in the presence of nocodazole, cells were replated in nocodazole-free medium and harvested at intervals after mitosis, and MCM protein binding to chromatin was determined by biochemical fractionation and immunoblotting. In control, c-Ad–transduced cells, a significant fraction of Mcm4 was detected on chromatin as early as 1 h after mitosis, as was seen in previous experiments without nocodazole (Fig. 4 b and Fig. 6 b). In contrast, very little Mcm4 was detected in the chromatin fraction in E-Ad–transduced cells (Fig. 7 a). At subsequent time points progressively more Mcm4 was loaded, although the amount never reached parity with the control population (Fig. 7 a). Loading of other MCM proteins is shown numerically after quantitation of immunoblots and normalization to the Orc2 signal, which is presumed to be constant (Fig. 7 b). This experiment shows a significant impairment of Mcm7 loading over the time course similar to

extract. Asterisk indicates principal isoform of endogenous cyclin E. (c) c-Ad– and E-Ad–transduced cells, as well as the SUM149PT breast cancer–derived cell line, were synchronized by thymidine block–release; telophase cells were analyzed in parallel for cyclin E level by immunofluorescence deconvolution microscopy. Images of representative telophase cells are shown. Red, cyclin E; blue, DNA (DAPI).

Figure 4. Analysis of chromatin-bound Mcm4 in telophase cells with deregulated cyclin E. c-Ad- and E-Ad-transduced cells were synchronized by mitotic shake-off. At 1 h after mitosis, cells were either (a) directly fixed and analyzed for Mcm4 by immunofluorescence deconvolution microscopy or (b) subjected to mild detergent extraction before fixation and then analyzed for Mcm4 by immunofluorescence deconvolution microscopy. In parallel, E-Ad-transduced cells were incubated with the Cdk2 inhibitor roscovitine subsequent to mitotic shake-off. Images of representative telophase cells are shown for each population. Mcm4 is shown in red; DAPI staining of DNA is shown in blue.



that of Mcm4 loading. In addition, there is an obvious impairment of Mcm2 loading, but only at time points subsequent to 1 h after mitosis. Therefore, impairment of loading of Mcm2, Mcm4, and Mcm7 are likely to contribute to the S phase phenotypes associated with cyclin E deregulation.

To visualize the chromatin-bound Mcm4 levels during early S phase, asynchronous cells were subjected to a short BrdU pulse, and after detergent extraction were analyzed for BrdU and Mcm4 by immunofluorescence deconvolution microscopy. Early S phase cells selected based on the pattern of BrdU foci were quantitated for Mcm4 signal (Fig. 7 c). A comparison of the E-Ad-transduced and control populations indicated that even in early S phase, cells experiencing deregulated cyclin E expression had a deficiency in Mcm4 bound to chromatin, although this deficiency was not quantitatively as great as in telophase (Fig. 7 c). To confirm that a partial reduction in Mcm4 chromatin loading could have a significant impact on DNA replication, RNA interference was used to reduce the total intracellular level of Mcm4, and asynchronous cells were given a 15-min pulse of BrdU to mark S phase cells. Microscopic analysis of detergent-extracted early S phase cells and biochemical analysis of chromatin indicated that partial reduction of chromatin-bound Mcm4 correlated with a significant reduction in BrdU incorporation (Fig. 8). Thus, Mcm4 loading is rate-limiting for DNA replication.

Altered chromatin loading of Mcm4 is dependent on Cdk2 activity

MCM proteins are normally phosphorylated and gradually dissociate from chromatin as DNA replication proceeds (Kubota et al., 1995; Todorov et al., 1995; Coue et al., 1996; Fujita et al., 1996; Krude et al., 1996; Lei et al., 1996;

Holthoff et al., 1998; Méndez and Stillman, 2000). The kinase activities responsible for this phosphorylation are believed to be Dbf4/cdc7 and cyclin A-Cdk2 (for review see Lei and Tye, 2001). To investigate the possibility that deregulated cyclin E-Cdk2 activity might phosphorylate Mcm4 and thereby prevent its binding to chromatin during telophase, a Cdk2 inhibitor, roscovitine, was added to the medium when cells were replated after mitotic shake-off. As can be seen in Fig. 4 b, Mcm4 chromatin loading in telophase was restored when cells with constitutive cyclin E levels were treated with roscovitine as they proceeded through mitosis, confirming that the observed reduction of Mcm4 chromatin binding when cyclin E is deregulated is dependent on Cdk2 kinase activity. However, this experiment does not distinguish between a direct effect mediated by phosphorylation of Mcm4 or an indirect effect mediated by phosphorylation of other proteins. It is noteworthy that under appropriate SDS-PAGE separation conditions, hyperphosphorylated Mcm4 could be detected in the nonchromatin-bound fraction of telophase cells experiencing deregulated cyclin E expression (Fig. 9 a). Treatment of the soluble nuclear fractions with alkaline phosphatase eliminated the slowest migrating isoform (Fig. 9 b, lanes 2 and 6). Interestingly, the chromatin-bound fraction of Mcm4 was not hyperphosphorylated (Fig. 9 a, c-Ad P3; Fig. 9 b, lane 4). However, this experiment does not prove that hyperphosphorylation of Mcm4 under these conditions is mediated by cyclin E-Cdk2.

Discussion

Cyclin E deregulation, aneuploidy, and cancer

Cyclin E is found elevated in many types of tumors, often correlated with aggressive disease and poor prognosis (Key-

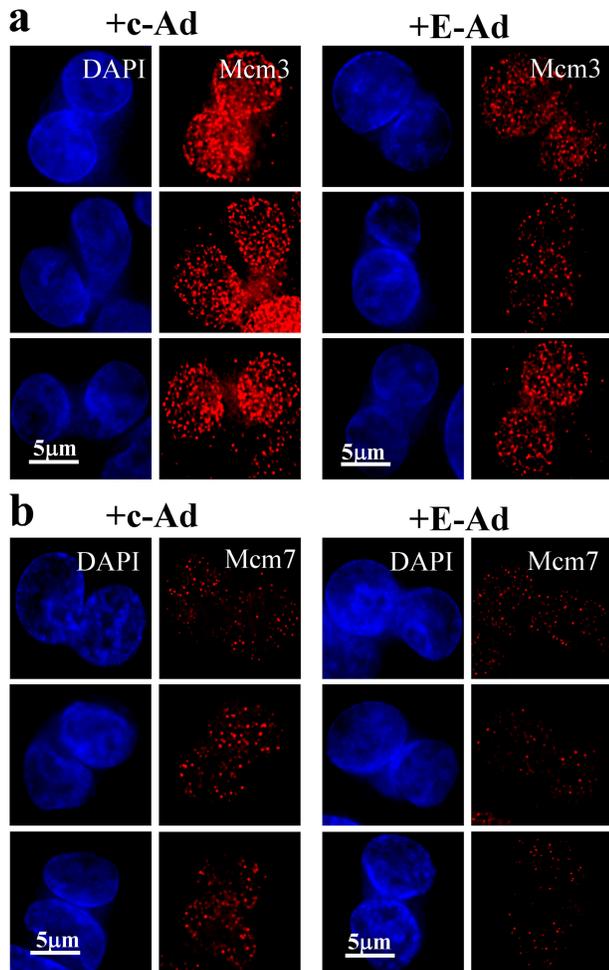


Figure 5. Analysis of chromatin-bound Mcm3 and Mcm7 in telophase cells with deregulated cyclin E. c-Ad- and E-Ad-transduced cells were synchronized by mitotic shake-off. At 1 h after mitosis, cells were subjected to mild detergent extraction, fixed, and analyzed for (a) Mcm3 and (b) Mcm7 by immunofluorescence deconvolution microscopy. Images of representative telophase cells are shown. Red, Mcm3, Mcm7; blue, DNA (DAPI).

marsi et al., 1995; Nielsen et al., 1997, 1998, 1999; Sandhu and Slingerland, 2000; Donnellan et al., 2001; Erlanson and Landberg, 2001; Schraml et al., 2003). However, it is not simple overexpression of cyclin E, but the loss of its cell cycle regulation that might be linked to cancer (Erlandsson et al., 2003; Reed et al., 2004). A recent analysis of endometrial carcinomas revealed that mutation of *hCDC4*, a gene crucial for cell cycle regulation of cyclin E degradation, did not correlate well with elevated levels of cyclin E (Spruck et al., 2002; Reed et al., 2004). However, *hCDC4* mutation correlated extremely well with deregulation of cyclin E relative to the cell cycle.

In an in vitro tissue culture model, deregulation of cyclin E expression was found to promote chromosome instability (Spruck et al., 1999). Accordingly, several hypotheses can be considered to explain how the negative impact of cyclin E deregulation on DNA replication could result in chromosome instability. First, the interference with preRC assembly could lead to a lower number of active replication origins, increasing

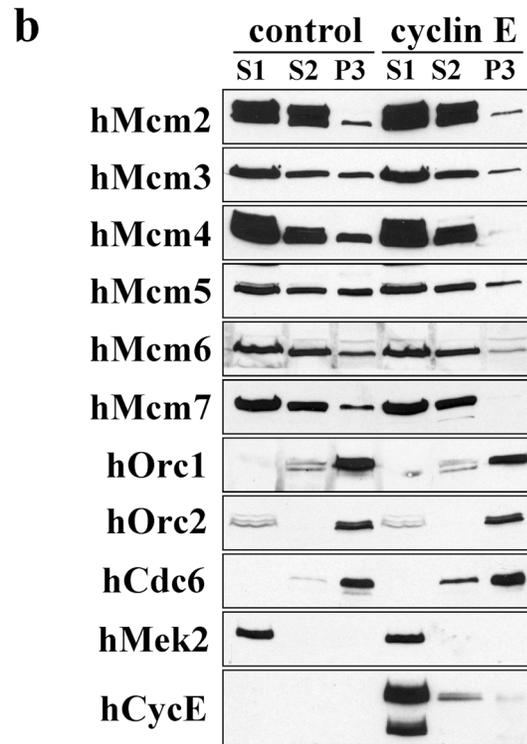
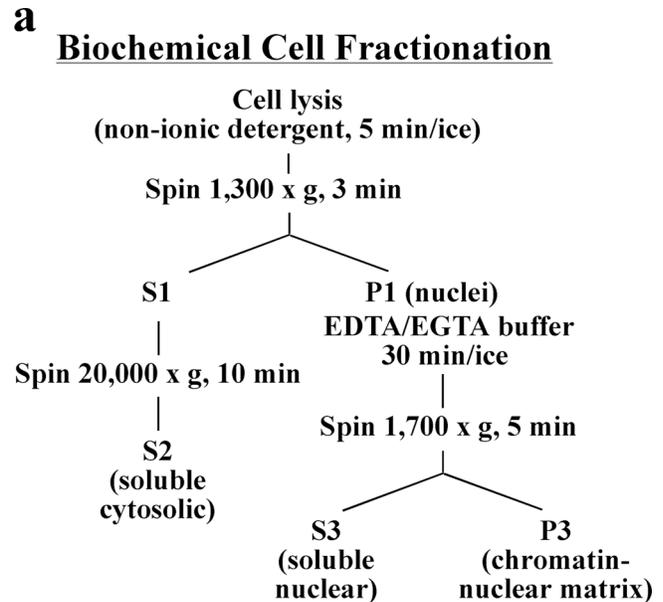


Figure 6. Chromatin loading of proteins associated with preRCs in cells with deregulated cyclin E. (a) c-Ad- and E-Ad-transduced cells were synchronized by mitotic shake-off. At 1 h after mitosis, cells were harvested and fractionated into a cytosolic supernatant (S2), a nuclear supernatant (S3), and a chromatin-enriched pellet (P3). (b) All fractions were separated by SDS-PAGE and were analyzed by Western blotting. Mek2 is used as a control for cytosolic localization, whereas Orc1 and Orc2 are controls for chromatin localization. Expression of cyclin E in the E-Ad-transduced population is also shown.

the average replicon size and resulting in higher frequencies of stalled replication forks and double-stranded DNA breaks. Second, the diminished rate of DNA replication could com-

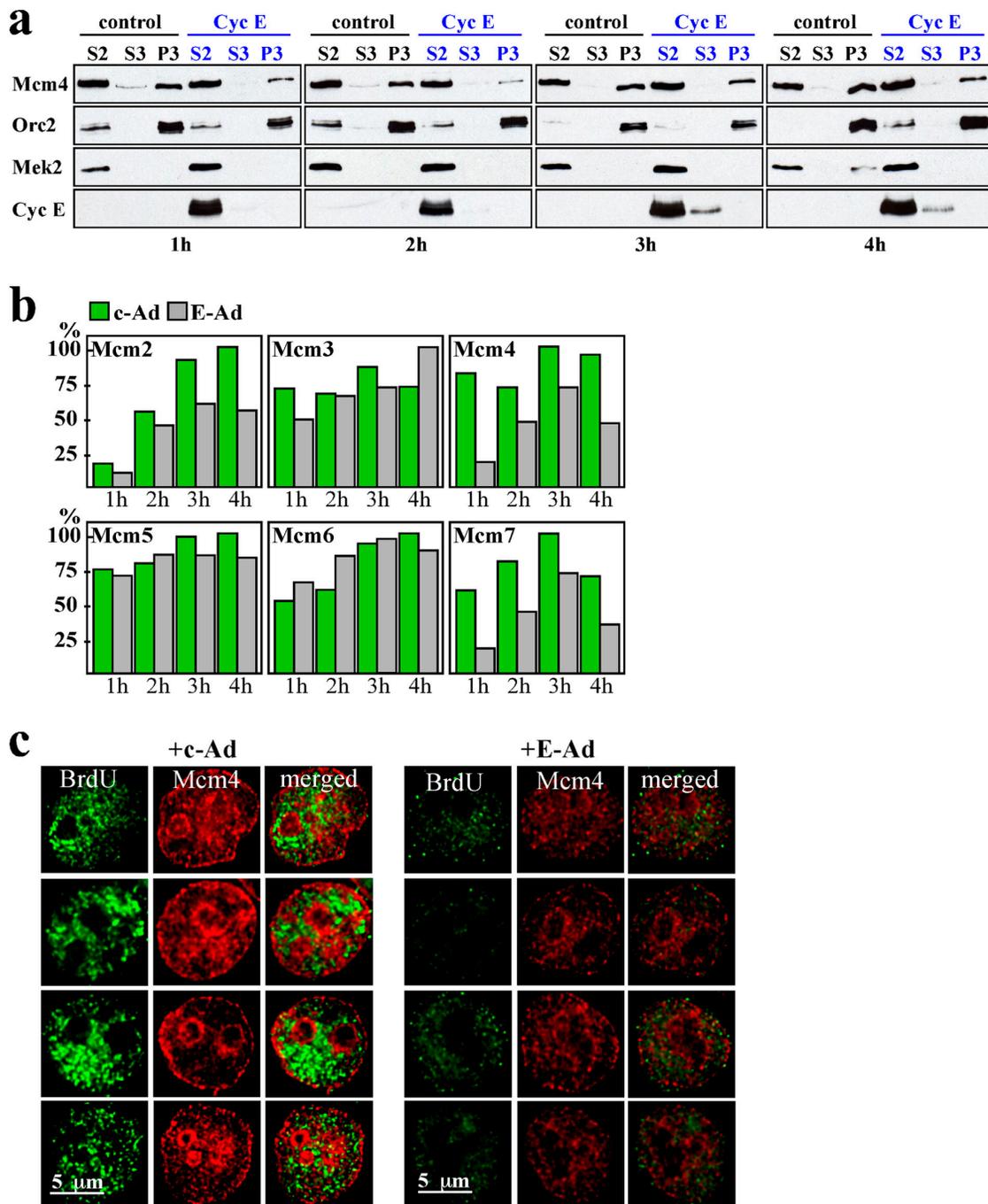


Figure 7. Effects of deregulated cyclin E on chromatin association of MCM proteins as cells progress through G1 and in early S phase. c-Ad- and E-Ad-transduced cells were synchronized by nocodazole block and mitotic shake-off. At hourly intervals after mitosis, cells were harvested and fractionated as in Fig. 6. Western blotting of fractions separated by SDS-PAGE was performed with antibodies specific for Mcm2, Mcm3, Mcm4, Mcm5, Mcm6, Mcm7, Orc2, Mek2, and cyclin E. (a) Immunoblot of time course showing analysis of Mcm4, Orc2, Mek2, and cyclin E. (b) Quantitation of immunoblots of chromatin-bound fractions for MCM proteins relative to Orc2. The data are expressed as percentage of the maximal ratio observed during the time course. (c) c-Ad- and E-Ad-transduced cells were subjected to a 15-min BrdU pulse, and then were detergent extracted, fixed, and analyzed for BrdU incorporation and chromatin-bound Mcm4 by immunofluorescence microscopy. Typical early S phase cells were chosen based on BrdU focus pattern. Red, Mcm4; green, BrdU.

promise the processes of chromosome condensation and sister chromatid cohesion because both these processes are coupled to DNA synthesis at the replication fork. Third, slow DNA replication could lead to incompletely replicated chromosomes at the time when cells would normally enter mitosis, leading to nondisjunction events and ultimately karyotypic

anomalies. Eukaryotic cells possess checkpoint mechanisms to prevent such catastrophes, but these presumably fail at a low but finite frequency. Finally, deregulation of cyclin E may directly compromise the normal intra-S phase checkpoint mechanisms and consequently allow propagation of DNA damage. These various scenarios are not mutually exclusive.

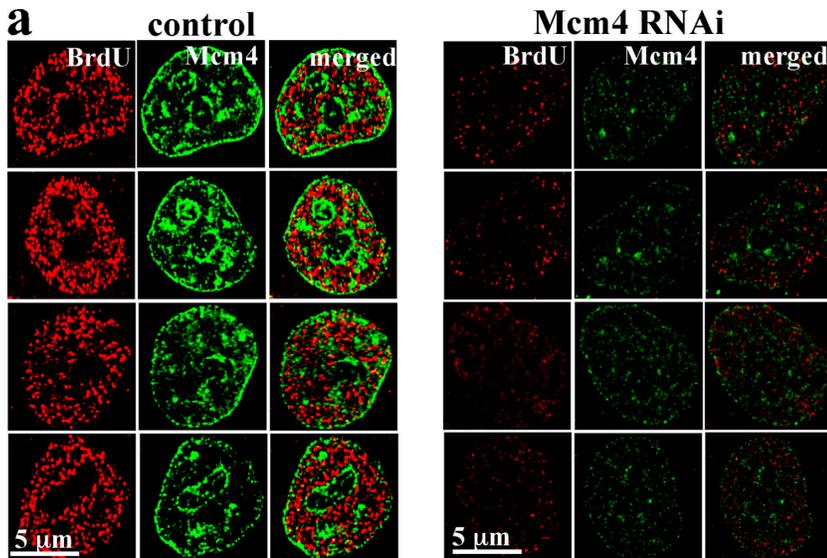
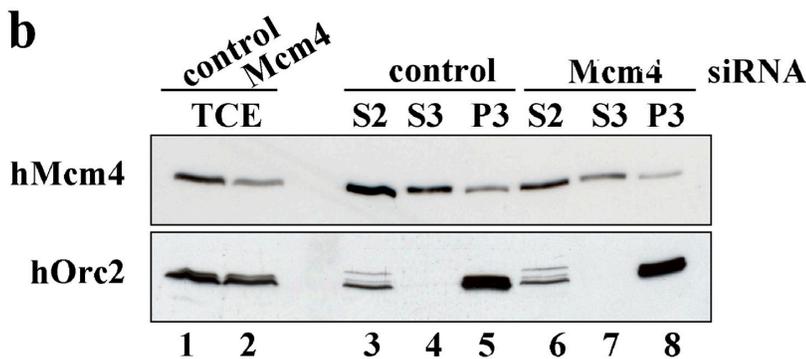


Figure 8. Partial reduction of Mcm4 expression by RNA interference results in impairment of DNA replication. (a) HeLa cells were transfected with firefly luciferase siRNA (left) or with Mcm4 siRNA (right), pulse labeled with BrdU, and analyzed by immunofluorescence microscopy after mild detergent extraction. Images of representative early S phase cell are shown. Green, Mcm4; red, BrdU. (b) HeLa cells transfected with firefly luciferase siRNA and Mcm4 siRNA as in panel a were fractionated into soluble and chromatin-enriched fractions as described in Fig. 6. Total cell extracts (TCE) as well as subcellular fractions were separated by SDS-PAGE and blots were probed with Mcm4 and Orc2 antibodies, respectively. Orc2 serves as a loading control for the chromatin-enriched fraction.



Cdk activity and DNA replication

Based on this and previous work, it is clear that Cdk activity has an ambivalent relationship to DNA replication. On one hand, it is activation of Cdks in late G1 that triggers the initiation of S phase. On the other, Cdk activity is antagonistic to preRC assembly (Kelly and Brown, 2000; Lei and Tye, 2001; Nishitani and Lygerou, 2002; Woo and Poon, 2003). These opposing regulatory modes are apparent in the paradoxical effect of deregulated cyclin E on the cell cycle. Initiation of S phase is advanced, consistent with cyclin E's positive role in initiation. However, because all components of the preRC are likely required for initiation of DNA replication, global replication proceeds much less efficiently as a result of cyclin E–Cdk2 impairment of preRC assembly. The potential tension between Cdk-mediated activation and inhibition of replication is normally averted by imposing tight temporal regulation on cyclin accumulation, thereby excluding cyclin–Cdk activity from the critical M phase/G1 boundary in virtually all cells. Deregulation of cyclin E apparently defeats this highly conserved regulatory safeguard, leading to genomic instability and ultimately malignancy.

Two recent works in yeast support the connection between Cdk deregulation, inefficient DNA replication, and chromosome instability (Lengronne and Schwob, 2002; Tanaka and Diffley, 2002). Deletion of the gene encoding a

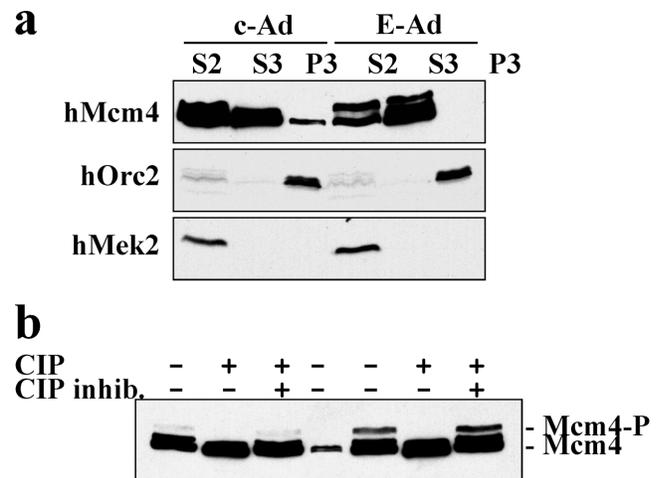


Figure 9. Phosphorylation of Mcm4 in post-mitotic cells with deregulated cyclin E. (a) c-Ad- and E-Ad-transduced cells 1 h after mitosis were analyzed as described in Fig. 6 b, except that SDS-PAGE conditions were used that maximally separate phosphorylated Mcm4 species (10% acrylamide/bisacrylamide instead of 12.5%; longer run time). Orc2, Mek2, and Mcm4 are shown. (b) Aliquots of the S3 fractions shown in panel a were reanalyzed after treatment with alkaline phosphatase, in the absence or in the presence of an inhibitor.

Cdk1 inhibitor, Sic1, which results in inability to completely down-regulate Cdk activity in G1, impaired initiation of DNA replication as was evidenced by high frequency of mini-chromosome loss and an abnormally low density of replication origins (Lengronne and Schwob, 2002). In another paper, gross overexpression of yeast G1 cyclins, known as Clns, also impaired replication, based on elevated rates of plasmid loss (Tanaka and Diffley, 2002). In both cases, there was evidence that the end result of Cdk deregulation and/or overexpression was chromosomal rearrangements that could be scored genetically. Moreover, it has been recently reported that the number of active replication origins influences the frequency of chromosomal rearrangements, increasing it or reducing it in different mutant backgrounds (Huang and Koshland, 2003). However, the relevance of Sic1 loss and Cln overexpression to mammalian cells and cancer must be considered cautiously because the regulation of the G1/S phase transition by Cdks is quite different in yeast compared with mammalian cells, as is the regulation of preRC assembly. In yeast, Cdk activity prevents nuclear import of MCM proteins and promotes degradation of Cdc6, neither of which occurs in mammalian cells (Kimura et al., 1994; Yan and Newport, 1995; Krude et al., 1996; Wuarin and Nurse, 1996; Drury et al., 1997, 2000; Zachariae and Nasmyth, 1999; Méndez and Stillman, 2000; Tanaka and Diffley, 2002). Indeed, our data demonstrate that deregulation of cyclin E does not affect the nuclear localization of Mcm4, but does control its ability to load onto chromatin. Furthermore, the level and loading efficiency of Cdc6 were not affected.

A number of previous papers have analyzed the effects of Cdk activities on association of MCM proteins with chromatin. In *Xenopus* eggs and mammalian cells, high levels of both cyclin A- and cyclin B-associated mitotic kinase activity have been shown to reduce the affinity of MCM complexes for chromatin and to promote release of MCM complexes presumably during late S phase, G2, and mitosis (Fujita et al., 1996; Hendrickson et al., 1996; Findeisen et al., 1999). Analysis of the specific effects of cyclin E on MCM loading onto chromatin has largely been limited to *Xenopus* egg extracts. High cyclin E-Cdk2 activity was found to prevent loading of MCM proteins, but could not dissociate them once loaded (Hua et al., 1997; Findeisen et al., 1999). Interestingly, MCM complexes are not a direct substrate of cyclin E-Cdk2 in the *Xenopus* egg system (Findeisen et al., 1999). However, it is not clear whether Cdk specificities observed for MCM protein phosphorylation in *Xenopus* eggs are also maintained in mammalian somatic cells.

The mechanics of MCM protein loading

In the current work, cyclin E-Cdk2 appears to impair the loading specifically of Mcm2, Mcm4, and Mcm7 at telophase and during G1. This is a surprising result because stable MCM complexes exist both in solution (Schwacha and Bell, 2001) and on chromatin (Ritzi et al., 1998), and because MCM proteins assemble as complexes and subcomplexes (Maiorano et al., 2000) before being loaded onto chromatin. On the other hand, we present data demonstrating that different individual MCM proteins load onto chromatin with distinct kinetic signatures even in the absence of

cyclin E deregulation. In particular, Mcm2 appears to load at a later time than other MCM subunits. This finding may indicate that MCM heteromeric complexes are assembled subsequent to chromatin loading of individual subunits, or that assembled MCM complexes as well as individual MCM subunits can be loaded onto chromatin. If the latter is true, the consequences of the coexistence of alternative loading mechanisms for MCM protein function remain to be explored (Méndez and Stillman, 2003).

Materials and methods

Cell culture and synchronization

KB cells, derived from a human nasopharyngeal epidermoid carcinoma and breast cancer-derived cell lines MDA-MB-157, -436, and -468 were obtained from the American Type Culture Collection (Manassas, VA) and were cultured as monolayers in DME (GIBCO BRL) supplemented with 10% FBS (GIBCO BRL), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and L-glutamine (GIBCO BRL). Sum149PT, a breast cancer-derived cell line, was obtained from the University of Michigan Breast Cell Tissue Bank (Ann Arbor, MI) and was grown in medium recommended by the supplier. All cells were grown at 37°C in 5% CO₂.

For synchronization of cells in late M/early G1 phase by mitotic shake-off, cells were first synchronized in early S phase by a triple-thymidine block according to the following procedure: at ~30% confluence, 2 mM thymidine was added to the medium and cells were incubated for 16 h before the thymidine-containing medium was removed. Cells were washed in PBS and fresh medium was added. 10 h later, 2 mM thymidine was again added to the medium. This procedure was repeated once more. During the third thymidine block, cells were transduced with recombinant adenovirus for 2 h in low volume (3 ml/162 cm² flask). 14 h after the third thymidine block, cells were released into fresh medium and progressed synchronously in the cell cycle until they were collected by mitotic shake-off 10–12 h later. Cells were detached by banging flasks on the bench 10 times, and mitotic cells were collected by pipetting off the medium and were then transferred to (a) 60-mm dishes containing a hemacytometer glass slide for immunofluorescence analysis; or (b) tissue culture flasks for cell fractionation/immunoblotting analysis. In the time-course experiment where 80 ng/ml nocodazole was used to maximize cell yield, it was added after release from a second thymidine block, during which adenoviral transduction was performed. When ~80% of the cells had rounded up, the shake-off procedure described above was used and cells were transferred to tissue culture flasks in medium without nocodazole for the designated incubation times.

Antibodies

pAbs against hMcm2 (CS732), hMcm6 (CS753), and hMcm7 (CS766) proteins were raised in New Zealand White rabbits against synthetic short peptides (corresponding to aa 131–150 in hMcm2, 9–26 in hMcm6, and 108–127 in hMcm7) conjugated to keyhole limpet hemocyanin. pAbs against hMcm3 (CS738) and hMcm4 (CS739), and mAbs against hCdc6 have been described earlier (Méndez and Stillman, 2000). pAbs against hOrc1 (CS769) and hOrc2 (CS205) have been described before (Gavin et al., 1995; Méndez et al., 2002). mAb PC10 (anti-PCNA) has been described previously (Waseem and Lane, 1990). Other primary antibodies used in this paper are: mouse monoclonal anti-cyclin E (HE12; Dulic et al., 1992), anti-Cdk2 (D12; Santa Cruz Biotechnology, Inc.), sheep polyclonal anti-BrdU (Research Diagnostics, Inc.), and FITC-conjugated mouse monoclonal anti-BrdU (Becton Dickinson). Secondary antibodies Cy3-conjugated donkey anti-rabbit IgG, FITC-conjugated donkey anti-sheep IgG, and Cy3-conjugated donkey anti-mouse IgG were purchased from Jackson Immunoresearch Laboratories. HRP-conjugated anti-rabbit IgG and anti-mouse IgG were obtained from Amersham Biosciences.

Recombinant adenovirus procedures

Recombinant adenovirus carrying the human cyclin E cDNA (E-Ad) was provided by Jeffrey Albrecht (University of Minnesota, Minneapolis, MN). c-Ad carried part of the β -globin gene (John Cogswell and Susan Neill; Glaxo-Wellcome, Research Triangle Park, NC). For transduction, KB cells were incubated in a low volume (3 ml for a 162-cm² tissue culture flask; 500 μ l for a 3-cm dish) with the recombinant adenovirus diluted appropriately in DME and 2% FBS for 2 h. After incubation in fresh medium containing 10% FBS for an additional 24 h, cells were collected by mitotic shake-off for analysis.

S phase entry assay

Cells were synchronized with a triple-thymidine block (see above), transduced with recombinant adenovirus expressing the human cyclin E, collected by mitotic shake-off, and replated in the presence of 10 μ M BrdU. At the indicated times, cells were harvested, fixed in 100% methanol for 1 h at RT, stained with FITC-conjugated anti-BrdU antiserum, and scored for BrdU incorporation by immunofluorescence microscopy. 300 nuclei were scored for BrdU incorporation for each time point.

Flow cytometry analysis

Before harvesting, virally transduced asynchronous cells were pulse labeled for 15 min with 10 μ M BrdU. 10⁶ cells were resuspended in PBS, fixed by adding 5 ml of 70% -20°C ethanol drop-wise while vortexing, and finally processed for propidium iodide and BrdU staining. Cells were collected by centrifugation (for 5 min at 200 g) and treated with 1 ml of 2 N HCl + 0.5% Triton X-100 for 30 min at RT to denature the DNA. The cell suspension was neutralized by addition of 2 ml of 0.1 M Na₂B₄O₇ (Borax). After collecting and washing the cells in PBS, the pellet was resuspended in 50 μ l of 1 \times PBS/1% BSA/0.5% Tween 20 to permeabilize cell membranes and block nonspecific antibody binding. FITC-conjugated anti-BrdU antibody was added to a final concentration of 2.5 μ g/ml and cells were incubated for 1 h at RT. After additional washing in 1 \times PBS/1% BSA/0.5% Tween 20, cells were collected, resuspended in 1 ml of 2 μ g/ml propidium iodide in PBS, and analyzed using a FACScan™ and CellQuest software (Becton Dickinson).

Immunofluorescence and deconvolution microscopy

For immunostaining with anti-hMCM antibodies, cells were replated onto glass coverslips. At the indicated times, cells were washed in PBS, treated with 0.5% Triton X-100 in PBS for 1 min at RT to remove proteins not tightly bound to chromatin (Todorov et al., 1995), and fixed in 4% PFA (wt/vol) in PBS (pH 7.0) for 20 min at RT. After treatment with blocking buffer (1% BSA and 0.5% Triton X-100 in PBS) for 15 min, the cells were incubated with the indicated primary and secondary antibodies and were further processed for immunofluorescence analysis as described previously (Ekholm et al., 2001).

For Mcm4/BrdU double staining, cells were pulse labeled with 10 μ M BrdU for 15 min and processed as indicated above. After completion of Mcm4 staining, cells were fixed again for 5 min at RT in 4% PFA in PBS (pH 7.0). The DNA was denatured by incubation in 2 N HCl at 37°C for 30 min. After three washes in PBS and a 15-min incubation in blocking buffer, cells were incubated with FITC-conjugated anti-BrdU antibody for 1 h at RT.

For PCNA/BrdU double staining, BrdU pulse-labeled cells were first fixed in 2% PFA in PBS for 15 min at RT, and then in 100% methanol for 10 min at RT. Cells were treated with blocking buffer and incubated with PC10 (anti-PCNA antibody) at 4°C overnight. Cy3-conjugated anti-mouse IgG was used as secondary antibody. Cells were washed in TBS + 0.02% Tween 20 after every antibody incubation. To visualize BrdU, cells were processed as described above.

Fluorescence data were collected using a DeltaVision® wide-field optical sectioning microscope system (Applied Precision) based on an inverted epifluorescence microscope (model IX-70; Olympus) as described previously (Reed et al., 2004).

Biochemical fractionation and immunoblot analysis

10⁶ cells, transduced with c-Ad or E-Ad and collected by mitotic shake-off as indicated above, were subjected to the micro-fractionation protocol originally described in Méndez and Stillman (2000) and schematized in Fig. 6 A. To test the phosphorylation status of Mcm4 protein, 8- μ l aliquots of the soluble nuclear (S3) extracts from c-Ad- or E-Ad-transduced cells were incubated in a total volume of 10 μ l with 2 U calf intestinal alkaline phosphatase (New England Biolabs, Inc.), either in the absence or in the presence of 10 mM Na₂VO₄ as an inhibitor, for 30 min at 37°C.

Immunoblots were quantitated in a FluorChem™ 8000 digital imaging system (Alpha Innotech, Inc.). hOrc2p, which is stably associated with the chromatin during this window of the cell cycle, served as a loading control for normalization. Data are presented as the percentage of the maximum amount of each MCM species loaded onto chromatin during the experiment.

Immunoprecipitation and histone H1 kinase assay

Cell lysates were subjected to immunoprecipitation followed by histone H1 kinase assay or Western blotting. In brief, 200 μ g of the lysate was immunoprecipitated with 2 μ l of the anti-cyclin E antibody HE172 or anti-Cdk2 antibody for 1 h on ice. The precipitates were bound to G-Sepharose beads (Boehringer). After washing the precipitates with lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100), cyclin E precipi-

tates were resuspended in 2 \times reaction buffer (40 mM Tris-HCl, pH 7.5, and 15 mM MgCl₂), 20 μ M ATP, 20 μ g histone H1, and 10 μ Ci [³²P]ATP and incubated for 30 min at 38°C. Reaction products were separated by SDS-PAGE and quantified with a phosphorimager (Storm® 840; Molecular Dynamics). Cdk2 immunoprecipitates were resuspended in SDS-PAGE sample buffer and were analyzed on 11% gels.

MCM4 RNA interference

A small interfering RNA (siRNA) duplex corresponding to nucleotides 1595–1615 of MCM4 mRNA was synthesized by Dharmacon Research (Lafayette, CO). HeLa cells growing on coverslips were transfected every 24 h with Oligofectamine™ (Invitrogen) and a 100-nM final concentration of MCM4 siRNA or firefly luciferase siRNA as a control. 48 h after the second transfection, 10 μ M BrdU was added to the medium for 15 min before fixation and immunofluorescence analysis.

Online supplemental material

Analysis of chromatin-bound Mcm2 and Mcm6 is shown in Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200404092/DC1>.

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