From the Department of
Pathology-Oncology, Cancer
Center Karolinska, Karolinska
Institutet

THE ROLE OF CYCLIN E IN CELL CYCLE REGULATION AND GENOMIC INSTABILITY

Susanna Ekholm-Reed

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To Jan and Kajsa,

ABSTRACT

Cyclin E, a positive regulatory subunit of Cdk2, normally accumulates periodically at the G_1/S transition, where it promotes entry into S phase and other DNA replication-associated functions. However, in many types of human cancer, cyclin E is overexpressed and in some cases its expression becomes deregulated, a phenomenon generally associated with poor prognosis. Although it is not yet known how cyclin E deregulation promotes tumorigenesis, one possible mechanism may be through the generation of genomic instability, a common characteristic of tumor cells.

The restriction point (R) is a checkpoint in mid G_1 phase, defined as the point after which cells can complete a division cycle independently of mitogenic signals. Regulation at this checkpoint is often lost in cancer cells. Passage through R has been shown to depend on the accumulation of a labile protein, suggested to be cyclin E. We carried out single cell analysis of cyclin E expression to determine the exact timing of cyclin E protein accumulation in relation to previous mitosis, passage through the restriction point (R) and subsequent S phase. By time-lapse video microscopy and immunofluorescence staining, we showed however, that passage through R is independent of the accumulation of cyclin E and does not require Cdk2 activity. These results rule out the hypothesis that passage through R is dependent on the accumulation of cyclin E but suggest that passage through R is a prerequisite for cyclin E accumulation.

Deregulation of cyclin E with respect to the cell cycle has been linked to a lengthening of S phase. We therefore investigated the effect of deregulated cyclin E on pre-replication complex (pre-RC) assembly in telophase cells. By immunofluorescence and biochemical analysis of chromatin bound pre-RC components in telophase cells, we show that loading of Mcm2, Mcm4 and Mcm7, believed to function in a complex with other Mcm proteins as a replicative DNA helicase, is severely impaired in these cells. We also show that DNA replication is impaired in cells constitutively expressing cyclin E. These results suggest that cyclin E interferes with proper pre-RC assembly, which may explain the observed replication defects that these cells exhibit. Cyclin E-mediated impairment of DNA replication constitutes a potential mechanism for the chromosome instability that has been observed in cells constitutively expressing cyclin E and might thereby represent the link between deregulation of cyclin E and tumorigenesis.

Recent studies demonstrate a link between cancer and mutations in *hCdc4*, the gene encoding the F-box protein that facilitates SCF-mediated degradation of cyclin E. However, in many of these tumors cyclin E is not overexpressed. We investigated whether deregulation of cyclin E expression rather than simple overexpression might be a more critical factor in tumorigenesis. We found a strong correlation between *hCDC4* mutation and deregulation of cyclin E expression, suggesting that *hCDC4* function is necessary for proper regulation of cyclin E expression. Thus, deregulation of cyclin E-Cdk2 activity and as a result, specific phosphorylation events, may be the critical factor in cyclin-E-mediated pathology.

LIST OF ABBREVIATIONS

BrdU	Bromodeoxyuridine
c-Ad	Control adenovirus
Cdk	Cyclin dependent kinase
CKI	Cyclin dependent kinase inhibitor
DAPI	4', 6-diamidino-2-phenylindole
E-Ad	Cyclin E adenovirus
FITC	Fluorescein isothiocyanate
HDAC	Histone deacetylase
HDF	Human diploid fibroblasts
G_1-pm	G_1-post mitosis
G_1-ps	G_1-pre-DNA –synthesis
LOH	Loss of heterozygosity
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
Pre-RC	Pre-replication complex
PI	Propidium Iodide
R	Restriction point
RNAi	RNA interference
siRNA	Small interfering RNA
TLC	Time-lapse cinematography
TLV	Time-lapse videomicroscopy

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

I. **Ekholm, S.V., Zickert, P., Reed, S.I. & Zetterberg, A.** Accumulation of cyclin E is not a prerequisite for passage through the restriction point. *Mol Cell Biol* **21**, 3256-65 (2001).

II. **Ekholm-Reed, S*., Méndez, J*, Zetterberg, A., Stillman, B., Tedesco, D. and S. I. Reed.** Deregulation of Cyclin E in Human Cells Interferes with Pre-replication Complex Assembly. Accepted by JCB for publication 2004.

III. **Ekholm-Reed, S.*, Spruck, C.H.*, Sangfelt, O., van Drogen, F., Mueller-Holzner, E., Widschwendter, M., Zetterberg, A and S. I. Reed.** Mutation of *hCDC4* leads to Cell Cycle Deregulation of Cyclin E in Cancer. *Cancer Research* **64**, 795-800 (2004).

* Authors contributed equally to this work

Additional papers:

- **Ekholm S.V, Reed SI.** Regulation of G_1 cyclin-dependent kinases in the mammalian cell cycle. *Curr Opin Cell Biol*., 2000,(6): 676-84.
- **Erlandsson F, Linnman C, Ekholm S, Bengtsson E, Zetterberg A.** (2000) A detailed analysis of cyclin A accumulation at the G_1/S border in normal and transformed cells. *Exp Cell Res*. 2000; 259(1): 86-95.
- **Erlandsson F, Wahlby C, Ekholm-Reed S, Hellstrom AC, Bengtsson E, Zetterberg A.** Abnormal expression pattern of cyclin E in tumor cells. *Int J Cancer*, 2003; 104(3): 369-75.

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TABLE OF CONTENTS

1. INTRODUCTION

Cyclin E is a regulatory subunit of Cdk2, an important positive regulator of the G_1/S transition. Cyclin E normally exhibits a periodic expression pattern with protein levels accumulating in late G_1, peaking at the G_1/S transition and being downregulated during S phase. This strict regulation of cyclin E levels suggests that persistent expression of cyclin E might be deleterious. Consistent with this, cyclin E overexpression and/or deregulation has been observed in many human cancers, correlating with poor prognosis and more aggressive disease. Furthermore, hCdc4, a protein responsible for targeting cyclin E for degradation, is mutated both in primary tumors and in tumor cell lines. However, the mechanism by which improper expression of cyclin E contributes to tumorigenesis remains unclear. The work presented in this thesis was conducted to increase understanding of the role of cyclin E in normal cell cycle regulation and in tumorigenesis.

1.1 THE CELL CYCLE

The cell cycle is the cyclic succession of events that allow a cell to grow, double in size, duplicate its DNA and finally divide into two identical daughter cells. The cell cycle is comprised of four different phases: G_1 phase, S (DNA synthesis) phase, G_2 phase and M (mitosis) phase (Fig. 1). When cells are not cycling because of lack of nutrients, differentiation, anti-mitotic factors or contact inhibition, they enter a resting state, called quiescence or G_0. During S phase, DNA is synthesized to make two identical copies of the genome, which segregate to generate two daughter cells during M phase. Separating M phase and S phase are the two gap phases; G_1 phase, separates M and S and G_2 phase, separates S and M. During the G_1 and G_2 phases, cells prepare for the following phase by synthesizing proteins and growing in size but it is also during the G_1 and G_2 phases that most of cell cycle regulation occurs. This thesis is mainly focused on G_1 phase and S phase.

1.2 CELL CYCLE REGULATION

Cell cycle progression occurs in sequential steps. Only when all events associated with a cell cycle phase have been completed, will transition to the following phase occur. Regulation of cell cycle progression is executed by evolutionary conserved serine/threonine protein kinases, called cyclin-dependent kinases (Cdks).

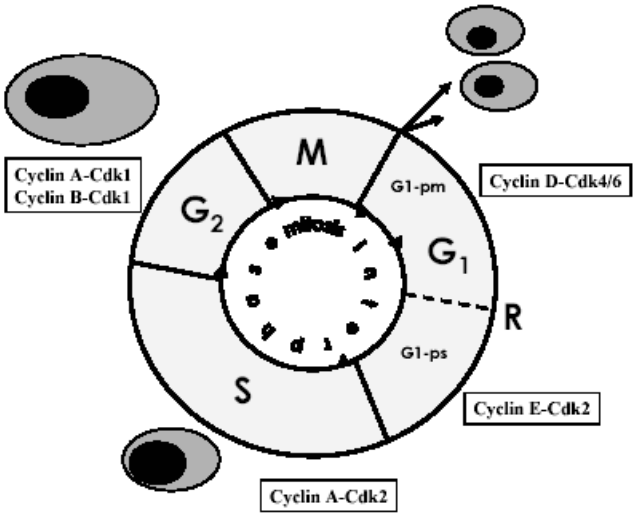


Figure 1. The eukaryotic cell cycle. In G_1, the newly divided cell grows in size and starts synthesizing enzymes and other proteins required for DNA replication. Progression through the first part of G_1 is regulated by cyclin D-Cdk4/6 and the G1/S transition is regulated by cyclin E-Cdk2. During S (DNA synthesis) phase, the cell duplicates its genome. At the onset of S phase, cyclin A starts accumulating and regulates Cdk2 activity throughout S phase. During G_2, cyclin A and cyclin B become associated with Cdk1and regulate the G_2/M transition. During M phase, the DNA, which now contains two genomes and the cytoplasm, which has doubled in size, segregates perfectly into two identical daughter cells.

The catalytically inactive Cdk subunit is associated with a regulatory subunit, a cyclin, which also confers substrate specificity to the complex. Cyclin-Cdk complexes control events that drive the transitions between cell cycle phases by phosphorylating phase-specific substrates.

Cdks were initially discovered by genetic analysis of the cell cycle in yeast and through the analysis of M phase inductive activities in frog and marine invertebrate eggs and early embryos. However, it soon became evident that Cdks are a universal hallmark of the eukaryotic cell cycle and that Cdks, directly or indirectly, control the major cell-cycle transitions and phases in all eukaryotic organisms (Hunt, 1991; Morgan, 1995; Nigg, 1995; Norbury and Nurse, 1992; Pines, 1995; Reed, 1992; Reed, 1997; Sherr, 1994; Solomon, 1993).

Cyclins were first identified in clam and sea urchin embryos where they were found to accumulate during interphase, and be degraded during mitosis (Evans et al., 1983). Based on homology to invertebrate and frog embryonic cyclins, cyclin A and cyclin B were the first human cyclins to be identified (Pines and Hunter, 1989; Wang et al., 1990). The human G_1 cyclins, the D-type cyclins and cyclin E, were identified by screening of human cDNA libraries for sequences that could complement G_1 cyclin mutations in *Saccharomyces cerevisiae* (Koff et al., 1991; Lew et al., 1991; Xiong et al., 1991).

In yeast, there is only one cell-cycle associated Cdk (Cdk1), which binds distinct cyclins as cells progress through the cell cycle, forming complexes with different substrate specificities. In mammalian cells, a number of different Cdks and cyclins form phase specific complexes (Fig.1)(Hunt, 1991; Norbury and Nurse, 1992; Pines, 1995; Reed, 1992). Even though different Cdk-cyclin complexes have been shown to have unique substrate specificity there has also been shown to exist a great degree of redundancy (Ortega et al., 2002) (Diehl, 2004; Murray, 2004). In the remaining part of this thesis, I will mainly discuss findings related to regulation of cyclin E and cyclin E-Cdk2 activity.

1.3 REGULATION OF CDK2 ACTIVITY

Because of their central regulatory roles in cell proliferation, Cdks themselves are subject to many modes and levels of regulation in response to both intracellular and extracellular signals (reviewed by (Ekholm and Reed, 2000). The first level of regulation is the binding of the Cdk subunit to the cyclin subunit, which is regulated by cyclin availability. The Cdk-cyclin complex is further regulated by both activating and inhibitory phosphorylation and by binding of Cdk inhibitors (CKIs) (Fig2).

1.3.1 Regulation of cyclin E levels

Cyclin abundance oscillates during the cell cycle as a result of periodic, programmed synthesis and degradation, thereby restricting Cdk activation to a limited window. Cyclin E protein starts accumulating in late G_1, peaks at the G_1/S transition (Dulic et al., 1992; Ekholm and Reed, 2000; Keyomarsi and Herliczek, 1997; Reed, 1997) and is degraded during S phase, via the ubiquitin/proteasome pathway (Clurman et al., 1996; Strohmaier et al., 2001; Won and Reed, 1996). Accumulation of cyclin E is believed to be a result of transcriptional activation when the cyclin E promoter is derepressed by partial phosphorylation of the tumor suppressor Retinoblastoma protein (pRb) (see below, "Regulation of the G_1 phase: The RB pathway")(reviewed by Ekholm and Reed, 2000). Cyclin E is degraded during S phase by ubiquitin-mediated proteolysis by the 26S proteasome (Clurman et al., 1996; Strohmaier et al., 2001; Won and Reed, 1996). Degradation of Cdk2-bound cyclin E is dependent on phosphorylation on threonine 380 (T380) (Won and Reed, 1996) and T62, as well as other residues (Welcker et al., 2003). Some of these phosphorylation events are performed by cyclin E-Cdk2 itself and are believed to constitute a self-regulatory feedback loop, linking cyclin E-Cdk2 activation to rapid degradation of cyclin E (Won and Reed, 1996). In a recent study of the requirements for cyclin E turnover *in vivo*, it was shown that phosphorylation of T380 is not mediated by autophosphorylation but by GSK3 kinase and that Cdk2 activity is

instead required for phosphorylation of T62 and S372 (Welcker et al., 2003).

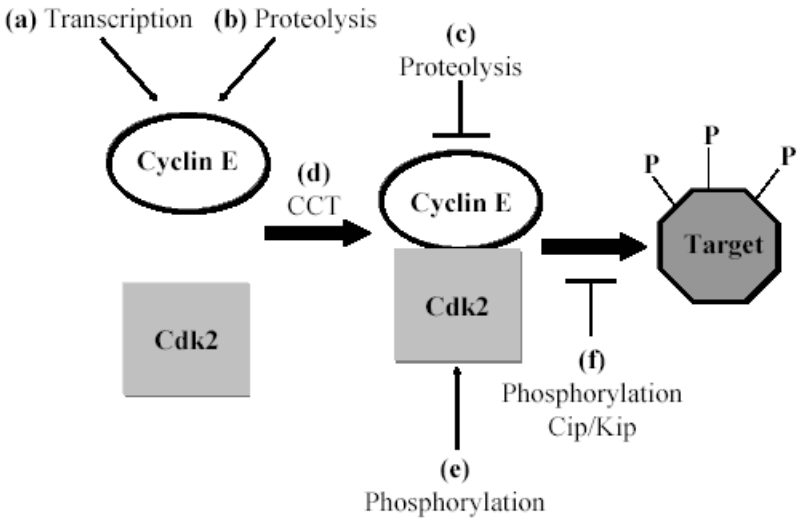


Figure 2. Regulation of cyclin E–Cdk2. Cyclin E accumulation has been shown to be regulated at the level of **(a)** transcription and degradation. Degradation pathways differentially target **(b)** free cyclin E and **(c)** Cdk2-bound cyclin E (hCdc4). Maturation and binding of cyclin E to Cdk2 also requires the activity of the **(d)** CCT chaperonin complex. Cyclin E-bound Cdk2 must be activated by **(e)** T-loop phosphorylation and cyclin E-Cdk2 complexes can be regulated by **(f)** inhibitory phosphorylation and Cip/Kip inhibitors.

Phosphorylation-dependent ubiquitylation of proteins and subsequent degradation by the 26S proteasome relies on the sequential action of a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a protein-ubiquitin ligase (E3) (Fig. 3)(reviewed by (Hershko and Ciechanover, 1992). E1 is a ubiquitin-activating enzyme, which initiates the reaction by activating ubiquitin by adenylation (Haas et al., 1982). Activated ubiquitin is then transferred to a E2 ubiquitin-conjugating enzyme, which in theory can attach ubiquitin residues directly to a lysine residue on a substrate but for most ubiquitylation reactions, an E3 ubiquitin-ligase is required. The E3 ligases are a large family of protein complexes involved in substrate recognition (reviewed by (Deshaies, 1999; Joazeiro and Weissman, 2000).

The SCF (Skp1–Cullin1–F-box) family of ubiquitin ligases is involved in the phosphorylation-dependent ubiquitylation of G_1 cyclins in both yeast (Deshaies et al., 1995; Skowyra et al., 1997; Willems et al., 1996) and mammalian cells (Strohmaier et al., 2001). SCF complexes contain a substrate specificity factor known as an F-box protein (Fig. 3).

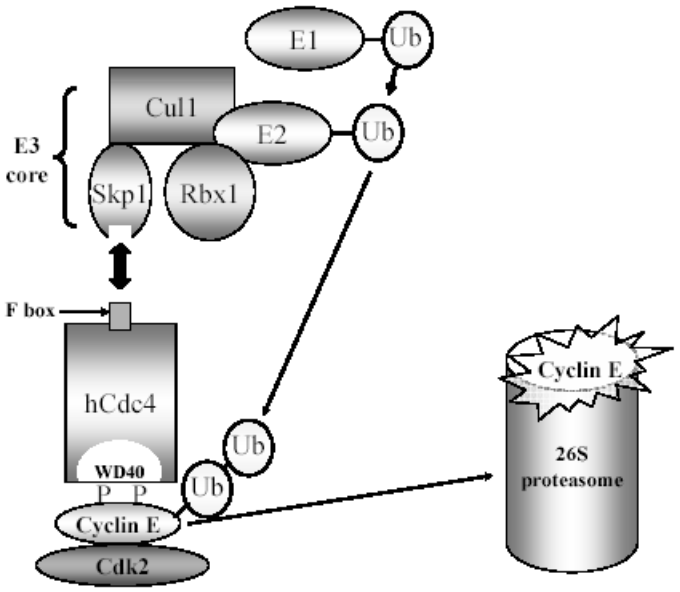


Figure 3. SCF-hCdc4-ubiquitin ligase complex-mediated ubiquitination of phosphorylated cyclin E. The basic components of this E3 complex include Skp1, Cul1 and the F-box protein, hCdc4 recognizes and links the phosphorylated cyclin E to the complex, allowing the ubiquitylation of cyclin E by ubiquitin-conjugating enzyme E2, following the activation of ubiquitin by ubiquitin- activating enzyme E1. The ubiquitylated cyclin E is recognized by the 26S proteasome, unfolded and drawn into the central cylinder where it is degraded.

hCdc4 (Fbw7/Ago), was recently identified as the F-box protein that targets phosphorylated cyclin E to the SCF complex (Koepp et al., 2001; Moberg et al., 2001; Strohmaier et al., 2001). Another SCF associated F-box protein, Skp2, has been suggested to be involved in the ubiquitin-dependent degradation of cyclin E. Free, non-Cdk bound cyclin E and the Cdk inhibitor p27 were shown to accumulate to elevated levels in Skp2$^{-/-}$ cells (Nakayama et al., 2000). However, it is not clear that elevated cyclin E in Skp2 nullizygous cells is a direct effect of loss of Skp2 or an indirect effect mediated by stabilizing p27 (Nakayama et al., 2000). Also supporting an SCF-dependent mechanism for degradation of cyclin E is the observation that embryonic cells from Cullin1$^{-/-}$ mice contain high levels of cyclin E (Dealy et al., 1999; Wang et al., 1999). Degradation of free, Cdk2 unbound, cyclin E has been suggested to be mediated through a different mechanism, involving Cul3 (Singer et al., 1999). Cul3 is non-SCF relative of Cul1, which was shown to interact with cyclin E in a phosphorylation independent fashion. However, in Cul3$^{-/-}$ embryos, cyclin E accumulates in only a few cell types, leaving the role of the Cul3 pathway in cyclin E degradation unclear (Singer et al., 1999).

1.3.2 Cdk Inhibitors

CDK inhibitors (CKIs) accumulate in response to a cell's need to cease dividing, because of entry into a post-mitotic terminally

differentiated state or into a resting state or because of checkpoint activation. This mode of Cdk inhibition has also been proposed as a mechanism for creating a sharp transition between G_1 and S phase (Reed, 2003).

There are three classes of CKIs, based on structure homology and Cdk affinity: the INK4 family, the Cip/Kip family and the pocket proteins (reviewed by (Sherr and Roberts, 1999). The INK4 family includes: p15, p16, p18 and p19 and the Cip/Kip family includes: $p21^{WAF1/CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$ (Sherr and Roberts, 1999; Vidal and Koff, 2000). The INK4 proteins bind specifically to Cdk4 and Cdk6 complexes, while the Cip/Kip proteins can bind and inhibit both cyclin D-Cdk4, 6 complexes and cyclin E/A-Cdk2 complexes. In addition, the pocket proteins p107, p130, and the retinoblastoma protein, comprise a third family of Cdk inhibitors that target Cdk2 (Garriga et al., 1998; Grana et al., 1998; Lipinski and Jacks, 1999; Stiegler et al., 1998).

The mechanism by which binding of the Cip/Kip family of CKIs inhibits Cdk2 activity was demonstrated by the recently determined three-dimensional structure of a ternary complex containing the Cdk-inhibitory domain of p27, Cdk2 and the carboxy-terminal (Cdk-activating) half of cyclin A (Russo et al., 1996). It revealed that p27 binding dramatically rearranges the structure of the Cdk active site, thereby inactivating it (Russo et al., 1996). Structure homology in key regions of p21 and p57, strongly suggests an equivalent mechanism of inhibition.

p21 is transcriptionally activated by p53 in response to DNA damage (Harper et al., 1993; Harper et al., 1995). This model is confirmed in cells from mice lacking p21, which are defective in the p53-induced DNA damage checkpoint (Brugarolas et al., 1995; Deng et al., 1995). Expression of p21 also occurs in a p53 independent fashion throughout embryogenesis, in various tissues and is associated with cell cycle-exit due to terminal differentiation (Macleod et al., 1995; Parker et al., 1995). Expression of p57 is also tissue specific during development, suggesting a similar role for p57 in cell cycle-exit during terminal differentiation.

p27 mRNA levels remain fairly constant throughout the cell cycle but p27 protein levels and rate of synthesis increase dramatically in fibroblasts as they exit the cell cycle as a result of serum withdrawal or contact inhibition (Hengst and Reed, 1996; Zhang and Lin, 1997). p27 also has a role at the G_1/S transition, in creating an abrupt, switch-like transition. In G_0 and throughout G_1 phase, cyclin E-Cdk2 complexes are kept inactive in a ternary complex with p27. Entry into S phase occurs when p27 is phosphorylated by cyclin E-Cdk2 activity and thereby targeted to SCF-mediated degradation (Carrano et al., 1999; Montagnoli et al., 1999; Nguyen et al., 1999; Shirane et al., 1999; Tsvetkov et al., 1999). The problem of how p27 is phosphorylated by the

inhibited substrate has been proposed to involve titration of cyclin E-Cdk2 complexes against a fixed pool of p27. At the G_1/S transition, as cyclin E levels increase, cyclinE-Cdk2 complexes will at some point outnumber the fixed pool of p27 molecules, resulting in activation of cyclin E-Cdk2. Phosphorylation and degradation of p27, which activates more cyclin E-Cdk2 complexes, is then believed to function as a positive feedback loop to rapidly facilitate S phase entry. p27 phosphorylated by cyclin E-Cdk2 is targeted for degradation via the SCF complex in association with the F-box protein Skp2, which is synthesized periodically at the G_1/S phase boundary (Lisztwan et al., 1998).

1.3.3 CKI exchange

Accumulation of D-type cyclins and binding to Cdk4, 6 in G_1 promotes the G_1/S transition in two ways: by phosphorylating pRb and other pocket proteins and by sequestering Cip/Kip inhibitors away from cyclin E–Cdk2 complexes, thereby activating cyclin E-Cdk2. Conversely, accumulation of INK4 inhibitors results in the disruption of cyclin D-Cdk complexes and liberated Cip/Kip inhibitor molecules are then allowed to associate with cyclin E–Cdk2 complexes, thereby inhibiting cyclin E-Cdk2 (Reynisdottir et al., 1995).

CKI exchange is believed to be the primary mechanism for activation of cyclin E–Cdk2 by c-Myc. Expression of c-Myc, in response to mitogenic stimulation, leads to transactivation of the cyclin D1 and D2 genes. Increased levels of cyclin D result in the redistribution of p27 from cyclin E-Cdk2 complexes to cyclin D-Cdk complexes and thereby activation of cyclin E–Cdk2 (Bouchard et al., 1999; Coller et al., 2000; Perez-Roger et al., 1999).

1.3.4 Regulation of Cdk activity by phosphorylation

In addition to binding of Cip/Kip inhibitors, inhibitory phosphorylation of Cdk2 on threonine 14 and tyrosine 15 maintains cyclin E–Cdk2 complexes in an inactive state before the G_1/S phase transition (Blomberg and Hoffmann, 1999; Vigo et al., 1999). Phosphatase treatment *in vitro* leads to hyperactivation of Cdk2 kinase activity (Heichman and Roberts, 1994). Mammalian cells contain three specialized phosphatases, CDC25A, B and C that reverse inhibitory Cdk phosphorylation (Donzelli and Draetta, 2003). CDC25A is activated late in G_1 and is assumed to be the phosphatase responsible for Cdk2 activation (Vigo et al., 1999). Consistent with this, ectopic expression of CDC25A accelerates the G_1/S transition by premature dephosphorylation of cyclin E–Cdk2 complexes (Blomberg and Hoffmann, 1999). CDC25A has an essential role in the G_1 response to DNA damage. In mammalian cells, UV or ionizing radiation results in

rapid ubiquitin/proteasome-mediated degradation of CDC25A and cell cycle arrest (Mailand et al., 2000). This response precedes the p53-induced accumulation of p21 and is essential for DNA repair and survival. Cdks are also subjected to activating phosphorylation. Cdk2 needs to be phosphorylated by CAK (Cdk-activating kinase) to be activated (Fisher and Morgan, 1994).

Finally, accumulation of cyclin E has also been shown to depend on the cytosolic chaperonin CCT, which mediates folding of newly translated cyclin E into a mature form that can associate with Cdk2 (Won et al., 1998).

1.4 CHECKPOINTS

In addition to regulating normal cell cycle progression, Cdks are part of intricate regulatory networks called checkpoints, which monitor the intracellular and extracellular environment and respond to inappropriate conditions. Without these control systems, serious genetic alterations would occur, leading to cell death or malignant cell growth. For example, if cells have damaged or unreplicated DNA, the cell cycle will arrest at checkpoints to allow for completion of replication and repair before the cell can enter mitosis. If the damage is too severe to be repaired, the checkpoint will induce programmed cell death, known as apoptosis. Another checkpoint, the Restriction point, operates in early G_1 to halt cell cycle progression when extracellular conditions are unfavorable, for example if the cell is starved of essential growth factors or if protein synthesis is inhibited. This checkpoint will be discussed in detail below.

Numerous studies have provided evidence, linking impairment of checkpoint mechanisms to tumorigenesis. Proteins involved in checkpoint control and cell cycle regulation are often mutated in tumors (reviewed by (Sherr, 2004). Consistent with this, most tumors are impaired in Restriction point control and cannot respond to serum withdrawal by exiting the cell cycle. Cyclin E has been suggested to have a role in the control of this checkpoint (Dou et al., 1993; Reed et al., 1992; Zetterberg et al., 1995).

1.4.1 The Restriction Point

When normal cells progress through G_1, they require the continuous presence of growth factors and a high rate of protein synthesis. If cells are deprived of growth factors or allowed to grow to confluency, cell cycle progression arrests at a point in mid- to late G_1 and cells exit from the cell cycle and enter a quiescent state (G_0) (Dulbecco and Elkington, 1973; Pardee, 1989; Todaro et al., 1965; Zetterberg and Auer, 1970; Zetterberg and Larsson, 1985). This is a reversible arrest and cell cycle progression will continue when growth factors are restored. Cells that have already passed the Restriction

point are not affected but will progress through the remainder of the cell cycle and only arrest after mitosis. This phenomenon was observed as early as 1971 by Temin (Temin, 1971), who demonstrated that the division of chicken fibroblasts become independent of external mitogenic growth factors during G_1 several hours before entry into S phase. Pardee introduced the term Restriction point (R), to define this point in G_1 after which cells can complete a division cycle independently of mitogenic signals (Fig.1) (Pardee, 1974).

The exact position of R in G_1 and its relationship to the previous mitosis and following S phase was determined previously by time-lapse cinematography (TLC) analysis of mouse and human cells (Fig. 3) (Larsson et al., 1989; Zetterberg and Larsson, 1985; Zetterberg and Larsson, 1991; Zetterberg et al., 1995). Passage through R was found to occur between 3 to 4 hours after the end of mitosis, in all cells studied so far. This analysis also revealed that the G_1 phase in cycling cells consists of two functionally different intervals. During the first 3 to 4 hours after mitosis, the G_1-pm (post mitosis) period, cell cycle progression is dependent on the continuous presence of serum growth factors and on a high rate of protein synthesis. If growth factors are withdrawn from the medium or if protein synthesis is inhibited during this interval, cells will rapidly (within 30 to 60 min) leave the cell cycle and enter G_0. The part of G_1 that follows R, known as the G_1-ps (pre-DNA-synthetic) period, is highly variable in duration. Some G_1-ps cells enter S phase immediately after passage through R, while others may spend up to 20 h in G_1-ps before initiating DNA replication. This variability in the duration of G_1-ps, suggests that even though passage through R is necessary for further progression through the cell cycle, other regulatory events must be completed during this interval in order for cells to enter S phase.

Early work on the regulation of R showed that passage through this checkpoint depends on the accumulation of a labile protein (Pardee, 1974; Rossow et al., 1979), suggested to be cyclin E (Dou et al., 1993; Keyomarsi and Herliczek, 1997; Reed et al., 1992; Zetterberg et al., 1995). However, the molecular basis for this switch, from growth factor-dependent to growth factor-independent progression, remains unclear but most likely does not involve cyclin E.

1.5 REGULATION OF THE G_1 PHASE: THE RB PATHWAY

Progression through the G_1 phase, at the molecular level, is controlled by phosphorylation of the tumor suppressor Retinoblastoma protein (pRb) at multiple sites (Ezhevsky et al., 1997). These phosphorylation events are performed by two cdk complexes: Cdk4, 6 in association with the D-type cyclins (cyclin D1-3) and Cdk2 in association with E-type cyclins (cyclin E and cyclin E2). The *RB* gene is sporadically mutated in a wide range of cancers. In addition to direct

mutation of the *RB* gene, the RB protein (pRb), is functionally inactivated in many tumor cells by binding of viral proteins or through mutations of proteins involved in a regulatory pathway that controls the activity of pRB, like p16, cyclin D1, and Cdk4 (Bartkova et al., 1996). These regulators, together with pRb, make up the so-called "RB pathway," a regulatory cascade that controls progression through the G_1 phase of the cell cycle (Fig. 4). Current data indicate that nearly all tumor cells harbor mutations or gene silencing that result in inactivation of pRb (Sherr, 1996).

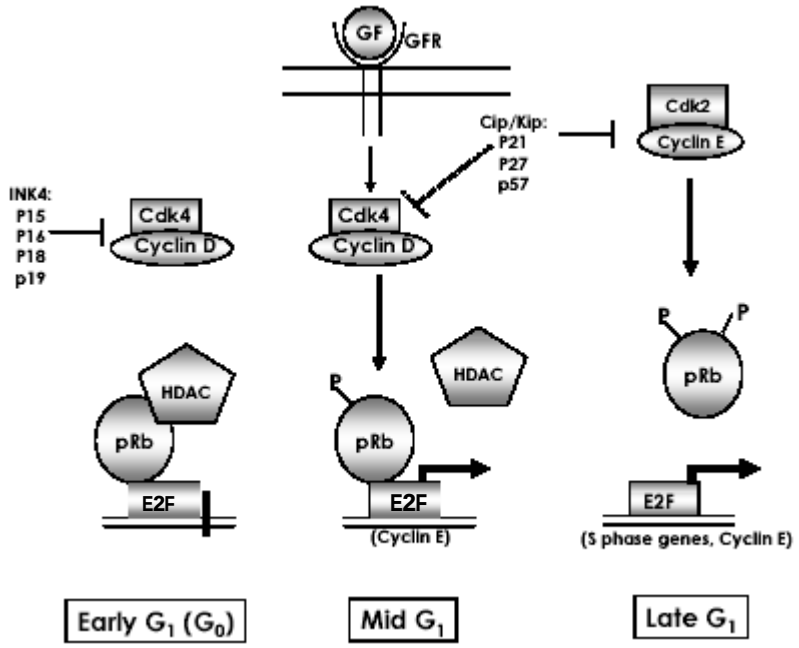


Figure 4. Regulation of the G_1 phase: The RB-pathway. Progression through G_1 is controlled by phosphorylation of pRb by cyclin D-Cdk4, 6 and cyclin E-Cdk2. In early G_1, pRb is hypophosphorylated and represses transcription of E2F regulated genes by binding transcription factors of the E2F family and chromatin remodeling enzymes like SWI/SNFs or histone deacetylases (HDACs). Cyclin D starts accumulating in response to mitogenic signals. Cyclin D-Cdk4, 6 phosphorylation of pRb results in release of HDAC and transcriptional activation of cyclin E in late G_1. Subsequent hyper-phosphorylation of pRb by cyclin E-Cdk2 results in inactivation of pRb and transcriptional activation of S phase genes.

During early G_1, pRb is present in its active, hypophosphorylated form (Ezhevsky et al., 1997) (Fig. 4). As cells enter G_1 from G_0, D-type cyclins (cyclin D1-3) start accumulating in response to mitogenic signals. Cyclin D associates with Cdk4 and/or Cdk6 and regulates progression through the first part of G_1 phase (Kato et al., 1993; Zhang et al., 2000) by phosphorylating pRb. pRb is phosphorylated by Cdk activity at multiple sites during progression through G_1. In its hypophosphorylated state, pRb represses transcription of genes required for progression through G_1 and entry into S phase through two different mechanisms. Firstly, pRb can bind to transcription factors of the E2F-family that are present on the promoters of cell cycle regulated genes and thereby directly block E2F activity (Geng et al., 1996). Secondly, chromatin remodeling enzymes like SWI/SNFs or histone deacetylases (HDACs) can be recruited by pRb tethered to

promoters by E2F and thereby efficiently repress transcription. Phosphorylation of Rb by cyclin-D–Cdk4/6 has been shown to disrupt binding of pRb to HDAC, allowing transcriptional activation of cyclin E in mid-late G_1 (Le Cam et al., 1999; Zhang et al., 2000) (Fig. 4). It has been proposed that cyclin E then associates with Cdk2, which also phosphorylates pRb, but at different sites than Cdk4, 6, resulting in hyper-phosphorylation and inactivation of pRb and transcriptional activation of proteins required for DNA synthesis (Weinberg, 1995). However, recent data argue against this model (see below). Overexpression of D-type cyclins or cyclin E during early G_1 leads to premature S phase entry (Ohtsubo et al., 1995; Resnitzky et al., 1994), suggesting that the G_1 cyclins are at least partially rate limiting for S phase entry and confirming that regulation of Cdk activity by cyclin accumulation has biological consequences.

It has been suggested that passage through R, at the molecular level, might represent inactivation of pRb and subsequent accumulation of cyclin E to a critical level, which allows progression through the remainder of the cell cycle independently of extracellular mitogenic signals.

1.6 DEREGULATION OF CYCLIN E AND CANCER

A link between cyclin E and malignant transformation has been established by several independent studies. Cyclin E is often overexpressed and/or deregulated relative to the cell cycle in many types of human cancer (Erlandsson et al., 2003; Erlanson and Landberg, 2001; Keyomarsi et al., 1995; Sandhu and Slingerland, 2000; Schraml et al., 2003). High cyclin E protein levels are associated with a more aggressive tumor phenotype and were shown to be a prognostic marker for poor outcome in breast cancer (Foulkes et al., 2004; Porter et al., 1997), as well as in several other types of cancer, particularly in combination with low p27 levels (Erlanson et al., 1998; Hayashi et al., 2001). That cyclin E has a direct role in tumorigenesis is supported by at least two lines of evidence. Firstly, mice carrying a transgene programmed to constitutively overexpress cyclin E in the mammary epithelium during pregnancy and lactation, were demonstrated to develop mammary adenocarcinomas (Bortner and Rosenberg, 1997). Secondly, the *hCDC4 gene* (Fbw7/*Ago*), which codes for the F-box protein that targets phosphorylated cyclin E to SCF-mediated ubiquitylation and degradation, was recently demonstrated to be mutated in primary tumors, in particular in endometrial tumors (16%), ovarian tumors (3/10) and in a few cancer-derived cell lines (Koepp et al., 2001; Moberg et al., 2001; Rajagopalan et al., 2004; Spruck et al., 2002; Strohmaier et al., 2001). Overexpression of cyclin E levels can also be based on gene amplification, which has been observed in about 20% of ovarian carcinomas (Marone et al., 1998) and in about 1% of breast carcinomas (Courjal et al., 1996).

The observed correlation between deregulated cyclin E levels and cancer, suggests that the presence of cyclin E outside of the very limited window when it is normally expressed might result in impairment of normal regulatory functions, possibly ultimately leading to malignancy. It is still unclear how cyclin E contributes to tumorigenesis. However, it was recently shown that constitutive overexpression of cyclin E induced chromosome instability in both immortalized rat embryo fibroblasts and human breast epithelial cells (Spruck et al., 1999), suggesting that the mechanism of cyclin E-mediated carcinogenesis might be based on accelerated loss of heterozygosity (LOH) at tumor suppressor loci.

1.7 THE ROLE OF CYCLIN E FOR CELL CYCLE REGULATION

The normal cyclin E expression pattern is consistent with a role in initiation of DNA replication, demonstrated by several studies (Jackson et al., 1995; Krude et al., 1997; Ohtsubo et al., 1995). A few cyclin E-Cdk2 targets involved in S phase related processes, like activation of histone transcription, centrosome duplication and initiation of DNA replication have been identified. These three S phase events are initiated simultaneously at the onset of S phase. Recent evidence indicates that failure to properly coordinate these events causes stalling of replication forks and contributes to DNA damage.

Deregulated cyclin E expression has been shown to have two distinct effects on cell cycle regulation. At the same time that deregulated cyclin E accelerates the G_1/S transition (Resnitzky et al., 1994; Resnitzky and Reed, 1995; Wimmel et al., 1994), it also causes inefficient progression through S phase (Ohtsubo et al., 1995; Resnitzky et al., 1994; Spruck et al., 1999). In fact, cyclin E-Cdk2 has been shown to have a dual role in the regulation of DNA replication. Cdk activity is required at the G_1/S transition to initiate DNA replication and it is additionally involved in the process that prevents re-replication. Cdk activity regulates the temporal order of S phase and M phase by regulating the formation of pre-replication complexes (pre-RCs) (Nasmyth, 1996; Nurse, 1994). Studies of the requirements for pre-RC assembly in yeast and Xenopus egg-based *in vitro* DNA replication systems, has suggested that Cdk activities must be reduced to very low levels in early G_1 phase for this process to occur (Kelly and Brown, 2000; Lei and Tye, 2001; Nishitani and Lygerou, 2002; Woo and Poon, 2003a).

1.7.1 Initiation of DNA replication

At the G_1/S transition, the pre-RC is converted into an active replication fork. This process depends on Cdk2 and Cdc7-Dbf4 activity

for structural reorganization of the pre-RC, which involves release of Cdc6 and recruitment of Cdc45, followed by origin unwinding, RPA binding, and recruitment of DNA polymerases (Aparicio et al., 1999; Aparicio et al., 1997; Chou et al., 2002; Woo and Poon, 2003b). It is however still unclear what the specific role of cyclin E-Cdk2 activity is in this process.

1.7.2 Regulation of histone transcription

During S phase, DNA synthesis is tightly coordinated with both histone synthesis and assembly of newly synthesized DNA and histones into chromatin. Histones are the building blocks of nucleosomes and are essential for packaging of newly synthesized DNA into chromatin. In a screen for cyclin E-Cdk2 substrates, a protein associated with histone promoters, $p220^{NPAT}$, was identified (Zhang et al., 1998). p220 is localized in nuclear organelles called Cajal bodies (Zhao et al., 2000). Cajal bodies associate with histone gene clusters and these sites also co-localize with cyclin E (Ma et al., 2000; Wei et al., 2003). p220 was shown to bind to cyclin E and to be phosphorylated by cyclin E-Cdk2, both *in vitro* and *in vivo* and mutation of Cdk2 phosphorylation sites of the p220 gene prevents activation of the histone H2B promoter by p220 (Ma et al., 2000; Wei et al., 2003). These findings are all consistent with a role for cyclin E-Cdk2 in p220 activation and histone transcription.

1.7.3 Regulation of centrosome duplication

Centrosome duplication is another S phase associated event that is regulated by cyclin E. In interphase, cells contain a single centrosome, which duplicates at the onset of S phase to generate two daughter centrosomes. During mitosis, daughter centrosomes organize a bipolar spindle, thus ensuring correct chromosome segregation. In Xenopus egg extracts, repeated centrosome duplication depends on cyclin E-Cdk2 activity (Hinchcliffe et al., 1999; Lacey et al., 1999). In mammalian cells, centrosome duplication was found to be initiated by cyclin E-Cdk2 phosphorylation of a centrosome associated protein, nucleophosmin (NPM/B23). This phosphorylation event activates centrosome duplication *in vivo* (Okuda, 2002; Okuda et al., 2000). Conversely, centrosome duplication is blocked by the expression of a mutated, non-phosphorylatable NPM/B23, or when nuclei are preincubated with a NPM/B23 antibody that prevents phosphorylation of NPM/B23 by cyclin E-Cdk2 (Tarapore et al., 2002). Duplicated centrosomes remain free of NPM/B23 until mitosis, when the nuclear membrane breaks down and NPM/B23 re-localizes to centrosomes and each daughter receives one centrosome bound by NPM/B23. Thus, NPM/B23 could represent a licensing system for centrosome duplication, regulated by periodic cyclin E-Cdk2 activity to ensure that centrosomes are duplicated only once per cell cycle. Abnormal

centrosome numbers have been observed in many human tumors and have been suggested to be a consequence of cyclin E deregulation. However, no increase in the numbers of cells exhibiting abnormal centrosome numbers was found in cells constitutively overexpressing cyclin E and exhibiting chromosome instability (Spruck et al., 1999). Also, centrosome duplication occurs normally in fibroblasts nullizygous for cyclin E (Geng et al., 2003).

1.7.4 Licensing

Pre-RCs are formed by the sequential loading of Cdc6, Cdt1 and the six mini-chromosome maintenance proteins, Mcm2-7, onto chromatin where the six-subunit origin recognition complex (ORC) is already bound (Fig. 5) (Lei and Tye, 2001; Nishitani and Lygerou, 2002). This process, also called licensing, renders the DNA competent for replication and is believed to be part of the mechanism allowing DNA to be replicated only once per cell cycle. Licensing takes place during late mitosis and early G_1 when Cdk activity is low in the cell (Fig. 6A). At the G_1/S transition, as Cdk activity increases, pre-RCs are activated by phosphorylation and origins are fired (Fig. 6A). Two distinct kinases, cyclin E-Cdk2 and Cdc7-Dbf4, cooperate for activation of pre-RCs and initiation of DNA replication (Aparicio et al., 1999; Aparicio et al., 1997; Chou et al., 2002; Woo and Poon, 2003a).

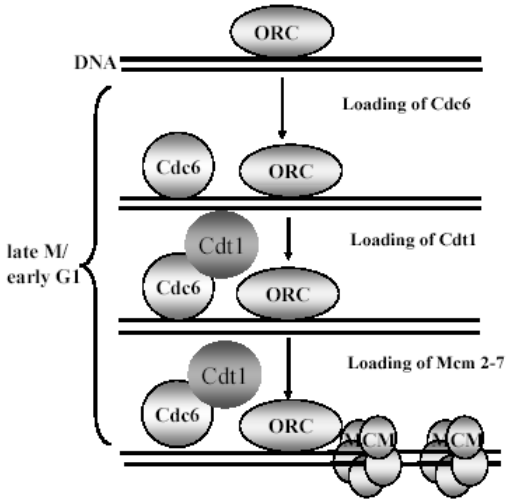


Figure 5. Assembly of the pre-Replication complex (pre-RC). Pre-RCs assembly onto chromatin, at origins of replication, during telophase and early G_1 phase. Cdc6, Cdt1 associate with origins, where ORC is already bound and serve as loading factors for the MCM proteins, Mcm2-7.

During S phase and M phase, high Cdk activity prevents binding of Cdc6 and MCM proteins, blocking re-licensing and re-replication until after mitosis, when Cdk activity is downregulated (Fig. 6A). The negative effect of cyclin E deregulation on DNA replication, could therefore be a consequence of inappropriate Cdk activity at the time

when pre-RCs are normally assembled, the end of mitosis and the beginning of G_1 (Fig. 6B).

All of the pre-RC components are potential targets for cyclin E-Cdk2 activity. Cdc6 is phosphorylated right before entry into S phase and is displaced from the DNA and transported out of the nucleus (Coverley et al., 2000; Fujita et al., 1999; Jiang et al., 1999; Pelizon et al., 2000; Petersen et al., 1999; Takei et al., 1999). MCM proteins are also phosphorylated and dissociate from the chromatin but with slower kinetics, as S phase proceeds (Coue et al., 1996; Fujita et al., 1996; Holthoff et al., 1998; Krude et al., 1996; Kubota et al., 1995; Lei et al., 1996; Mendez and Stillman, 2000; Todorov et al., 1995).

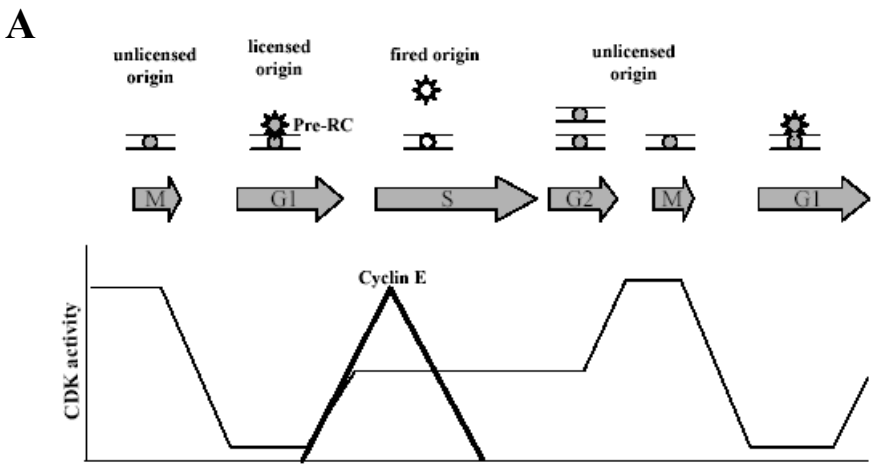
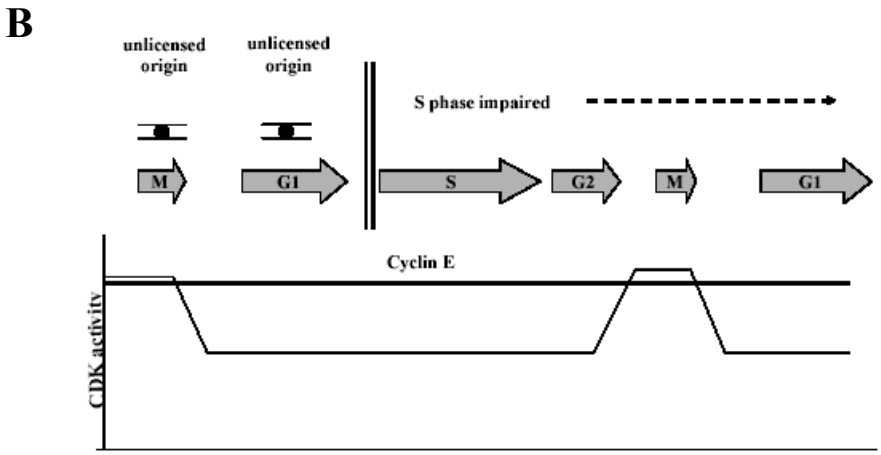


Figure 6. Cdk activity and origin function. (A) Origins of replication are licensed during late M and early G_1 phase when Cdk activity is low in the cell. As Cdk activity increases at the G_1/S transition, origins are fired, pre-RC components are phosphorylated and dissociate from the chromatin, some immediately at the G_1/S transition (Cdc6) and others as S phase proceeds (MCM proteins). High Cdk activity ensures that origins are kept unlicensed until late in mitosis, when Cdk activity is once again downregulated. **(B)** When cyclin E is deregulated, Cdk activity persists during late M and early G_1, which might interfere with pre-RC assembly and ultimately with DNA replication.



How the MCM proteins are physically organized is not clear. Different MCM protein complexes and subcomplexes have been detected in various organisms. The six subunits have been shown to interact and form a ring-shaped hexameric complex containing one of each of the subunits (Ishimi, 1997; Lee and Hurwitz, 2000; Madine et al., 1995; Prokhorova and Blow, 2000), while subcomplexes have also been isolated, suggesting that different subunits might be differentially loaded and regulated (Ishimi, 1997; Kearsey and Labib, 1998; Prokhorova and Blow, 2000). A subcomplex consisting of Mcm 4,6 and 7 was shown to have weak helicase activity (Ishimi, 1997). Binding of a dimer consisting of Mcm3 and Mcm5 or binding of Mcm2 was shown to inhibit the helicase activity (Lee and Hurwitz, 2000).

MCMs are a family of proteins related to ATP-dependent helicases that have a role in both initiation and elongation of DNA replication, presumably by unwinding of the DNA helix ahead of the replication fork (Ishimi, 1997; Labib et al., 2000; Lei and Tye, 2001; You et al., 2002). Depletion of Mcm proteins during G_1 blocks initiation of DNA replication and depletion of any of the MCM proteins after initiation, using conditional degron mutants, results in an irreversible DNA replication block (Labib et al., 2000). MCM proteins also interact with transcription factors and with RNA polymerase II, suggesting that they might have a role in transcription as well (Yankulov et al., 1999; Zhang et al., 1998).

1.8 THE ROLE OF *hCDC4* IN REGULATION OF CYCLIN E

The link between deregulation of cyclin E and tumorigenesis suggests that proper degradation of cyclin E may be important to prevent malignant transformation. Consistent with this view, mutations in the gene encoding hCdc4, the F-box protein required for targeting phosphorylated cyclin E for ubiquitylation, have been linked to cancer (Koepp et al., 2001; Moberg et al., 2001; Rajagopalan et al., 2004; Spruck et al., 2002; Strohmaier et al., 2001). In a study of endometrial tumors, 16% of the tumors analyzed were found to harbor loss of function mutations in the *hCDC4* gene(Spruck et al., 2002). In most cases, *hCDC4* mutations could be shown to be accompanied with loss of heterozygosity (LOH), consistent with Knudson's "two-hit" hypothesis of tumor suppression. Based on Knudson's observations of sporadic and inherited forms of retinoblastoma, he concluded that tumorigenesis associated with mutations in a tumor suppressor gene generally requires loss or mutation of both alleles (Fig. 7)(Knudson, 1979). Also supporting a tumor suppressor role for hCdc4 is that the *hCDC4* gene is located to 4q32, a known tumor suppressor locus, reported to be deleted in 31% of all cancers and in 17% of endometrial tumors (Knuutila et al., 1999).

The *hCDC4* gene locus consists of ten common 3'exons that are alternatively spliced to three different 5'exons: α, β and γ, generating three different mRNAs and three distinct proteins (Spruck et al., 2002). The α-isoform appears to be expressed in all tissues and is also the predominantly expressed isoform in cancer-derived cell lines. The β and γ-isoforms are expressed at very low levels except in skeletal muscle, brain and heart (Spruck et al., 2002). Interestingly, one tumor with an α-isoform specific *hCDC4* mutation and two with β-isoform specific mutations were identified, suggesting that the function of these two alternatively spliced variants are not redundant (Spruck et al., 2002).

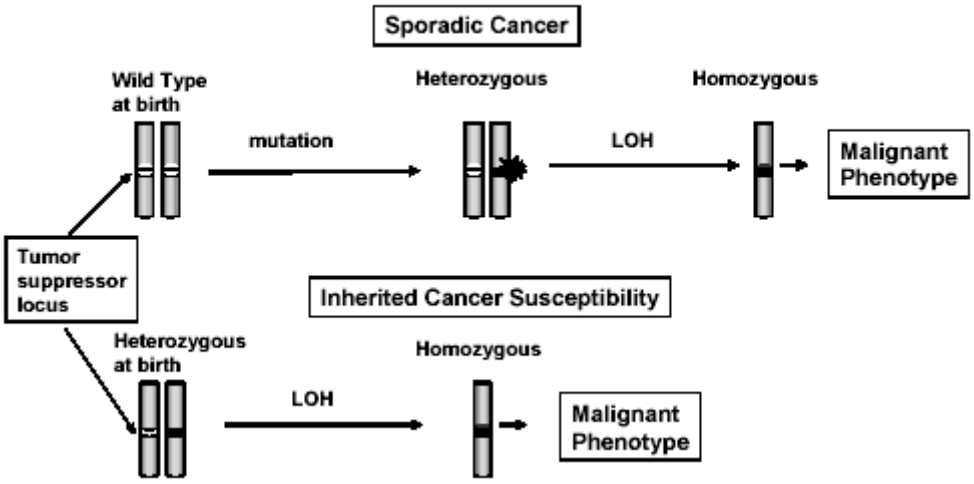


Figure 7. Knudson's two-hit hypothesis for tumorigenesis. In sporadic cancer involving mutation of tumor suppressor genes, both mutations ("hits") are somatic. Separate mutations of the two alleles are required to inactivate the tumor suppressor gene in a single cell and to initiate tumor formation. Since the chance of two mutations occurring in an individual cell is relatively small, sporadic cancer involving tumor suppressor inactivation is generally rare. In hereditary forms, a first mutation, or "hit", is present in a germ cell, which gives a predisposition to the development of malignancy. The "second hit", refers to a second mutation that is somatically acquired. Since somatic mutations are relatively frequent, individuals with germline mutations of tumor suppressor genes often develop multiple tumors.

hCdc4 has been demonstrated to play a central role in the ubiquitin-mediated degradation of cyclin E (Koepp et al., 2001; Moberg et al., 2001; Strohmaier et al., 2001). However, in endometrial tumors, no strong link between loss of hCdc4 function and high cyclin E levels could be established (Spruck et al., 2002). Most tumors with mutated *hCDC4* were shown to contain hyperphosphorylated cyclin E but not necessarily high total levels of cyclin E, suggesting that other pathways for degradation of cyclin E, maybe the Cul3 pathway, might be upregulated in these tumors. SSCP analysis revealed that the mutations were predominantly located in the WD40 substrate-binding domain, consistent with the observation that cyclin E accumulates in its hyperphosphorylated form in these tumors, presumably because of an inability of cyclin E to bind to and be targeted by mutated hCdc4.

These findings suggest that temporal deregulation of cyclin E and Cdk2 activity, relative to the cell cycle, might be the critical link between *hCDC4* mutation and tumorigenesis, rather than simple overexpression.

2. AIMS OF THE STUDY

The general aim of this study was to investigate the role of cyclin E in normal cell cycle regulation and the mechanism by which deregulated cyclin E contributes to genomic instability and tumorigenesis.

The specific aims were:

1. To determine whether cyclin E is the R point protein.

2. To investigate whether deregulated cyclin E expression interferes with pre-replication complex assembly during telophase and G_1 phase.

3. To investigate the role of *hCDC4* for cell cycle regulation of cyclin E.

3. MATERIAL AND METHODS

The methods used in this study are described in detail in Paper I-III and will here only be listed and briefly commented upon.

3.1 CELLS AND MEDIA

All cell lines were maintained in a 5% CO_2-95% air mixture in a humidified incubator at 37°C. The different cell lines that were utilized in this study and their specific growth requirements, are listed below:

- **Paper I:** Early-passage human diploid fibroblasts (HDF) from embryonic lungs were cultured in a 1:1 mixture of modified Eagle's medium (MEM) and Ham's F-12 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 U each of penicillin and streptomycin per ml. hTERT-BJ cells were cultured in a 4:1 ratio of Dulbecco's MEM containing Medium 199 with 10% fetal bovine serum, 4 mM L-glutamine, 1 mM sodium pyruvate and 50 U each of penicillin and streptomycin per ml.
- **Paper II:** KB cells, were originally classified as a human nasopharyngeal epidermoid carcinoma cell line but were recently demonstrated to be derived from contaminating HeLa cells. KB cells were cultured as monolayers in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin and L-glutamine. Sum149PT, a breast cancer derived cell line, was grown in DFCI-1 medium.
- **Paper III:** ZR75-1 (breast cancer-derived cell line, ductal), MDA-MB-157, -436 and –468 (breast cancer-derived cell lines), HEK239 (derived from embryonal kidney cells, transformed with adenovirus) and 293 Phoenix cells (derived from 293 cells, retroviral packaging cell line) were grown in DMEM, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin and L-glutamine. hTERT-immortalized human breast epithelial cells (IME) were grown in MCDM 131 media, supplemented with 1% fetal bovine serum, 10 ng/ml EGF, 30 ng/ml bovine pituitary extract, 5μg/ml insulin, 0.5 μg/ml hydrocortisone, 5 μg/ml human holo-transferrin, 100 U/ml penicillin, 0.1 mg/ml streptomycin and L-glutamine.

3.2 ANALYSIS OF PROTEIN LEVELS IN SINGLE CELLS OF KNOWN AGE

To investigate the relationship of cyclin E accumulation to passage through the R point and entry into S phase, we used time-lapse analysis in combination with immunocytochemical analysis.

3.2.1 Time-lapse videomicroscopy (TLV)

Time-lapse recordings of cells in culture enable analysis of individual cells of an unperturbed, asynchronously growing population. This method is a powerful tool for detailed kinetic analysis of transition events in the cell cycle because, unlike synchronization procedures, it addresses the problem of intercellular variability in cell cycle times, particularly G_1 variability. An inverted microscope equipped with a monochrome, cooled CCD camera was placed in an incubator with temperature and CO_2 regulation. Humidity was excluded to protect the instruments. A petri dish with cells growing on a gridded coverslip was placed under the microscope, and a field of 20 to 50 well-separated cells was chosen for recording (Fig. 8). Images were captured every 4 to 10 min by a framegrabber card and stored in a personal computer. After the final photograph was taken, cells were fixed and stained for immunofluorescence or immunoperoxidase as described below (Fig. 8).

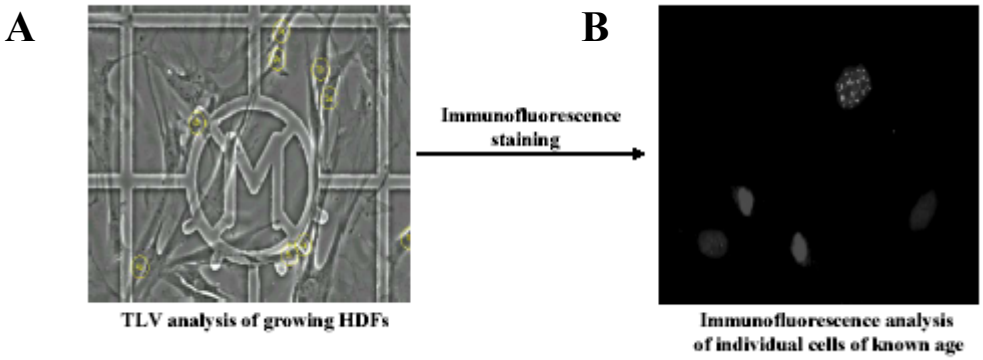


Figure 8. Time-lapse video microscopy (TLV) and subsequent immunofluorescence analysis of protein levels in single cells of known age. (A) A petri dish with HDFs growing on a gridded coverslip was placed under the microscope. Images were captured every 4 to 10 min. After the final photograph was taken, cells were fixed and immunofluorescence stained. **(B)** TLV analyzed cells could easily be identified after immunofluorescence staining. Images were collected and fluorescence intensity was quantified with the Delta Vision system.

3.2.2 Immunocytochemistry

Fixation. Three different methods for fixing cells before immunostaining have been used in this study. (1) 100% methanol fixation, (2) methanol/acetone fixation. Organic solvents such as alcohols and acetone remove lipids and dehydrate the cells, while precipitating the proteins on the cellular structure. This fixation was always used before staining of cyclin E and BrdU. (3) Paraformaldehyde fixation (4%), which is a cross-linking reagent, forms intermolecular bridges, normally through free amino groups, thus creating a network of linked antigens. Cross-linkers preserve cell structure better than organic solvents, but may reduce the antigenicity of some cell components,

and require the addition of a permeabilization step, to allow access of the antibody to the specimen. This type of fixation was used before staining of pre-RC components.

Immunoperoxidase. Fixed cells were permeabilized and endogenous peroxidase was blocked by soaking slides in H_2O_2 before incubation with the primary antibody. Cells were then washed and blocked with serum, before the primary antibody was detected using Dako StreptABComplex/HRP. Diaminobenzidine was used as a chromogen, and nuclei were counterstained with Meyer's hematoxylin. The amount of immunoperoxidase material was determined from light absorption at a wavelength of 480 nm after subtraction of the counterstain intensity of immunoperoxidase-negative cells.

Immunofluorescence. Fixed cells were treated with a buffer containing bovine serum albumin (BSA) and detergent (Tween) to block out unspecific interactions and to permeabilize the cell membranes. Cells were then incubated with primary antibody and washed several times in a detergent-containing buffer. Before detection of the primary antibody, cells were treated with serum from the species of the secondary antibody to prevent non-specific interactions between the cells and the secondary IgG. For detection of the primary antibody, cells were incubated with a fluorochrome- or biotin-conjugated secondary antibody. For detection of the biotin conjugated secondary antibody, cells were incubated with fluorochrome-conjugated streptavidin. Coverslips were mounted with glycerol containing DAPI and antifade reagent. All immunofluorescence analysis was done by deconvolution microscopy.

The major methodological drawback in the use of immunocytochemistry for determination of protein levels is that it is only semiquantitative. Although there is a rough correlation between protein content and immunostaining intensity, epitope availability is often sensitive to variation in fixation and other parameters of sample preparation. However, the fact that cyclin E could be detected during G_1-pm when cyclin E was ectopically expressed makes it unlikely that epitope masking is a factor in this analysis.

3.3 DECONVOLUTION MICROSCOPY (PAPER I-III)

Fluorescence intensity data were collected and analyzed with the Delta Vision system for deconvolution microscopy. Deconvolution is a computational method used to reduce out-of-focus fluorescence in three-dimensional (3D) microscope images. Compared to other methods for 3D microscopy, like confocal microscopy, the advantage of deconvolution microscopy is that data can be collected at very low light levels, thus allowing image acquiring at multiple focal-planes over

long periods of time. A conventional fluorescence microscope (Zeiss), equipped with a movable z-axis stage that permits imaging of the specimen at different focus positions and a CCD camera for quantification of the light emitted by the specimen, was used. Images were captured at intervals of 0.2 μm. All images shown were generated from a single central section of the 3-dimensional image stack (z-stack). Images were processed via a constrained iterative deconvolution algorithm and intensity calculations were carried out using the Delta Vision Softworks package.

3.4 KINETICS OF R POINT PASSAGE (PAPER I)

To investigate whether human diploid fibroblasts (HDFs) can pass through R in the absence of Cdk2 activity, cells were treated with the Cdk2 inhibitor roscovitine. HDFs growing on glass coverslips were serum starved for 60 h and then incubated in medium with 10% FBS with or without 10 μM roscovitine. This medium was replaced at 6, 9, or 15 h post release from G_0 with medium without serum but with BrdU. To control for recovery from roscovitine treatment, duplicate roscovitine-treated samples received BrdU-containing medium with 10% FBS at 6, 9, and 15 h, respectively. Additional controls were continuous incubation in serum-free medium and continuous treatment with roscovitine in the presence of 10% FBS. Cells not treated with roscovitine were fixed at 22 h post release from G_0, when none of the cells in the population had reached M phase, based on visual observation. Cells treated with roscovitine were fixed at 34 h post release from G_0 in order to allow recovery from roscovitine treatment when, similarly, none of the cells in the population had reached M phase. All fixed cells were immunostained for BrdU reactivity. Since there is some time-dependent toxicity associated with roscovitine treatment (Alessi et al., 1998), experimental data (roscovitine-treated cells from which serum was withdrawn) were normalized to control data (roscovitine-treated cells from which serum was not withdrawn) for all time points.

3.5 IMMUNOHISTOCHEMISTRY (PAPER III)

To analyze protein levels in cells from primary endometrial tumors, paraffin-embedded tumor material was sectioned (2μm) and deparaffinized. Antigen retrieval was performed by boiling sections for 10min in antigen unmasking solution. Imunohistochemical staining with anti-cyclin E and anti-cyclin A antibodies was performed using the immunoperoxidase method.

3.6 WESTERN BLOT ANALYSIS (PAPER I-III)

Western blotting is a standard method for immunodetection of proteins. Total proteins from cell lysates are size-separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to

a nylon membrane. Detection of a specific protein is achieved by incubating the membrane with an antibody directed against the protein. To visualize the primary antibody, a horseradish peroxidase (HRP)-conjugated secondary antibody is bound to the primary antibody. The HRP cleaves a substrate, resulting in enhanced chemiluminescence (ECL). The light emitted from this chemical reaction is captured on film. The sensitivity of this method depends on the specificity and quality of the antibody.

3.7 IMMUNOPRECIPITATION PROCEDURES (PAPER III)

hCdc4 protein is expressed at very low levels in cells and cannot be detected by Western Blotting. For immunodetection, hCdc4 protein was first immunoprecipitated with an anti-hCdc4 antibody and then detected by Western blotting.

3.8 RECOMBINANT ADENOVIRUS PROCEDURES (PAPER I AND II)

Transduction of cells with a recombinant adenovirus is an efficient technique for gene transfer, resulting in transient gene expression. In Paper I, a recombinant adenovirus containing the human cyclin E cDNA was used to verify that cyclin E could be detected during G_1-pm. In Paper II, the same adenovirus was used to facilitate the study of the effects of acute overexpression and deregulation of cyclin E on G_1 progression and DNA replication.

3.9 RECOMBINANT RETROVIRUS PROCEDURES (PAPER III)

Recombinant retrovirus transduction can be used to introduce a gene and produce stable cell lines expressing the gene product. In Paper III, Sum149PT cells were transduced with a recombinant retrovirus containing the cDNA for hCDC4α and a stable cell population expressing hCdc4α protein was obtained after Puromycin selection.

3.10 RNAi (PAPER II AND III)

RNAi (RNA interference) refers to the introduction of homologous double stranded RNA to specifically target a gene's product, causing degradation of the complementary mRNA. Two different techniques for RNAi were used in this study. In Paper II, HeLa cells were transfected with a 25-nucleotide long siRNA (small interfering RNA) duplex, corresponding to a sequence of the Mcm4 mRNA. The introduced siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs), unwinding in the process. The siRNA strands subsequently guide the RISCs to complementary RNA molecules, where they cleave and destroy the complementary RNA. Cells were transfected every 24 h with oligofectamine and 24 h after the third transfection, cells were pulse labeled with BrdU, fixed and

processed for immunofluorescence analysis. By transfecting cells with siRNA, the silencing effect lasts for several days and does appear to be transferred to daughter cells but it does eventually diminish.

Recently, expression vectors have been developed which continually express siRNAs in stably transduced mammalian cells. This method was used to silence hCdc4 expression in Paper III. HEK293 cells were transduced with a pSuperRetro virus, expressing siRNA against a region corresponding to in exon3 of the *hCDC4* gene. Stable cell populations were obtained by Puromycin selection. As a control, cells were transduced with a pSuperRetro virus expressing siRNA against green fluorescence protein.

3.11 LASER SCANNING CYTOMETRY (PAPER III)

Laser scanning cytometry is a method that provides equivalent data to a flow cytometer but which is slide-based. It allows light scatter and fluorescence measurements but also records the position of each measurement. Cells of interest may be re-located, visualized, re-stained, re-measured and photographed. In common with some flow cytometers, the LSC measures multicolor fluorescence and light scatter on a single cell basis. A laser scanning cytometer (CompuCyte, Cambridge, MA), equipped with a 20-mW argon-ion air-cooled laser and a 5-mW HeNe laser and a digital camera, was used to measure PI and FITC fluorescence. The user defines a threshold parameter, which is used to delineate the specific fluorescence from the cells from the background fluorescence of the slide. The computer program then draws a threshold contour within which a number of parameters can be measured, eg area (the number of pixels occupied by the cell), the integral value (the sum of the fluorescence values for each pixel in the contour) and the maximum pixel intensity.

3.12 RT-PCR (PAPER III)

RT-PCR (reverse transcription PCR) is a method to amplify a specific sequence of RNA by the polymerase chain reaction (PCR). To determine the efficiency of the siRNA silencing of hCDC4, quantitative RT-PCR was performed on poly-A+ RNA in a 20-cycle PCR reaction.

3.13 CYCLIN E DEREGULATION AND G_1 DURATION (PAPER II)

3.13.1 S-phase entry assay

Cells were synchronized with a triple thymidine block (see Paper II), transduced with a recombinant adenovirus expressing the human cyclin E cDNA (see under Recombinant Adenovirus procedures), collected by mitotic shake-off and replated in the presence of BrdU. At the indicated time points, cells were harvested, fixed in methanol and

processed for BrdU immunofluorescence staining and scored for BrdU positivity by immunofluorescence microscopy.

3.13.2 Flow cytometry analysis

Cells were grown asynchronously in tissue culture flasks, transduced with recombinant adenovirus and harvested for flow cytometry analysis 24 hrs later. Before harvesting, cells were pulse labeled with BrdU and fixed in 70% ethanol. For detection of incorporated BrdU, cells were treated with 2N HCl to denature the DNA and then with $Na_2B_4O_7$ (Borax) to neutralize. Cells were processed for BrdU immunofluorescence, resuspended in propidium iodide and analyzed using a BD FACScan and Cell Quest software.

3.14 ANALYSIS OF CHROMATIN BOUND PROTEINS IN TELOPHASE

3.14.1 Synchronization by mitotic shake-off

For synchronization of cells in late M/early G_1 phase by mitotic shake-off, cells were first synchronized in early S phase (Fig. 9). Three thymidine arrests were required to achieve a synchronized early S phase population, which after release progressed through S and G_2 until collected by mitotic shake-off 10-12 hours later (Fig. 9). Just prior to the shake-off, the volume of the medium was reduced from 15 to 5 ml to increase the cell concentration for immunofluorescence. Cells were detached by banging flasks against the bench 10 times and collected by pipetting off the medium and then transferred to either a dish containing a hematocytometer glass cover slip for immunofluorescence analysis, or to tissue culture flasks for cell fractionation/immunoblotting analysis. Cells were subsequently grown for the indicated periods of time, to allow re-attachment and progression to telophase. Cells were fixed for immunofluorescence or harvested for fractionation/immunoblotting when 50% of the cells were divided but still touching, forming an 8-shape.

3.14.2 Immunofluorescence staining

For detection of pre-RC proteins (MCM, Cdc6, Orc) in telophase cells, cells synchronized and collected by mitotic shake-off were replated onto hematocytometer glass coverslips and then allowed to grow for re-attachment and progression to telophase. Before fixation in 4% fresh paraformaldehyde, cells were detergent extracted (0.5% Triton X-100 in PBS) to remove proteins not tightly bound to chromatin (Todorov et al., 1995). After permeabilization with blocking buffer, cells

were incubated with the indicated primary and secondary antibodies, and further processed for immunofluorescence analysis as described under Immunofluorescence.

(A)For Mcm4/BrdU double staining, cells were grown asynchronously on coverslips, pulse labeled with BrdU and extracted before fixation in paraformaldehyde. After completion of Mcm4 immunofluorescence, Mcm4-stained cells were fixed again for 5 min at room temperature in 4% paraformaldehyde, to fix the secondary antibody. The DNA was then denatured by incubation in 2N HCl and processed for BrdU immunofluorescence.

(B) For PCNA/BrdU double staining, cells were grow asynchronously on glass coverslips and pulse labeled with BrdU as indicated above and fixed first in 2% paraformaldehyde and then in methanol. Cells were subsequently processed for PCNA immunofluorescence. To visualize BrdU, cells were processed as described above.

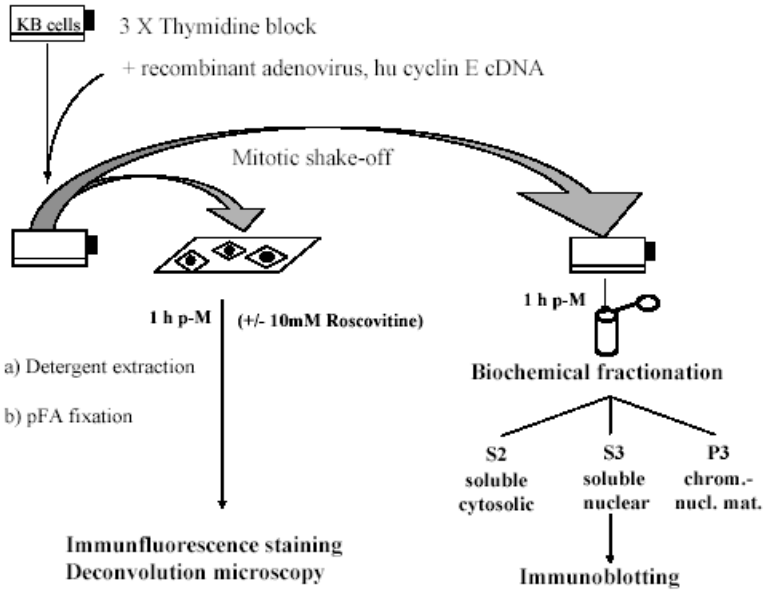


Figure 9. Mitotic shake-off for analysis of chromatin bound protein during telophase. KB cells are synchronized in early S phase by three thymidine blocks. During the last incubation in thymidine, cells are transduced with a recombinant adenovirus expressing human cyclin E. 10-12 hours after release from thymidine, cells are collected by mitotic shake-off and replated (in the presence or absence of roscovitine) for immunofluorescence and deconvolution microscopy or for biochemical fractionation and immunoblotting.

3.14.3 Biochemical fractionation

Cells transduced with c-Ad or E-Ad and collected by mitotic shake-off as described above, were subjected to the biochemical fractionation protocol originally described by Méndez and Stillman

(Mendez and Stillman, 2000) and schematized in Figure 10. This was done to facilitate analysis of chromatin bound proteins. Equal volumes of soluble cytosolic (S2), soluble nuclear (S3) and chromatin/nuclear matrix (P3) extracts were subjected to SDS-PAGE and transferred to nitrocellulose filters. The presence of initiator proteins in each of the fractions was tested by immunoblotting with the indicated antibodies. To test the phosphorylation status of Mcm4 protein, aliquots of the soluble nuclear (S3) extracts from c-Ad or E-Ad transduced cells were incubated with calf intestinal alkaline phosphatase, either in the absence or in the presence of Na_2VO_4 as inhibitor. Immunoblots were quantitated in a FluorChem 8000 digital imaging system. hOrc2p, which is stably associated with the chromatin during this window of the cell cycle, served as a loading control for normalization.

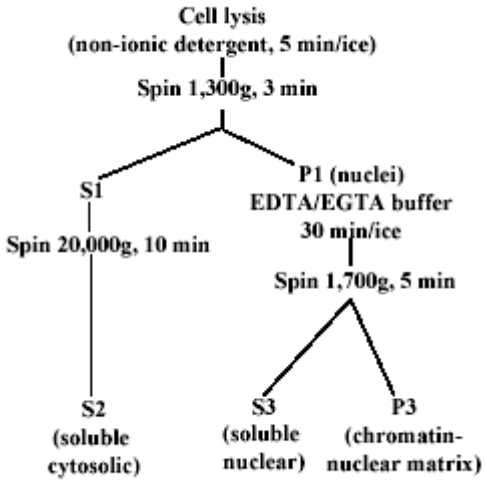


Figure 10. Biochemical fractionation. KB cells, transduced with E-Ad or c-Ad and collected by mitotic shake-off, were transferred to tissue culture flasks. Cells were allowed to grow for re-attachment and to reach telophase before they were harvested and lysed in non-ionic detergent. The illustrated protocol was used to fractionate the cell lysates into a soluble cytosolic (S2), a soluble nuclear (S3) and a chromatin bound (S3) fraction. The different fractions were subjected to SDS-PAGE, transferred to nitrocellulose filters and analyzed for the presence of pre-RC components by western blotting.

3.15 IMUNOPRECIPITATION AND KINASE ASSAY (PAPER III)

Cell lysates from KB cells transduced with E-Ad or c-Ad, SUM149PT, MDA-MB-157, -436 and –468, were subjected to immunoprecipitation followed by histone H1 kinase assay. Briefly, lysates were immunoprecipitated with the anti-cyclin E antibody HE172, for 1 hour on ice. The precipitates were then bound to G-Sepharose beads. After washing the precipitates with a lysis buffer, precipitates were resuspended in reaction buffer, ATP, histone H1 and ^{32}P-labelled ATP and incubated for 30 minutes at 38^{o}C. Reaction products were separated by SDS-PAGE. The gel was fixed in 40% methanol-10% acetic acid, dried and exposed to X-ray film. Signals were quantified with a

phospho imager (Molecular Dynamics STORM 840, Amersham Biosciences, Sweden, Sockholm).

4. RESULTS AND DISCUSSION

4.1 PAPER I

4.1.1 Cyclin E and the Restriction point

Cyclin E accumulation and concomitant activation of Cdk2 have been proposed to constitute the molecular basis for the R point phenomenon (Dou et al., 1993; Reed et al., 1992; Zetterberg et al., 1995). The periodic accumulation of cyclin E, starting in late G_1 phase, was first observed by Dulic et al. (Dulic et al., 1992) and has since been verified by a number of other investigators (Ekholm and Reed, 2000; Keyomarsi and Herliczek, 1997; Reed, 1997). However, the methods used in these studies rely on analysis of populations, which only allow comparison of population averages. Information potentially useful for establishing kinetic and functional relationships could be lost in this averaging process. Thus, it was not possible to draw strong inferences from these studies concerning the relationship of cyclin E accumulation to passage through the R point or entry into S phase. In Paper I, we used time-lapse analysis in combination with immunocytochemical analysis to estimate cyclin E levels in individual unperturbed cells of an asynchronously growing population. This strategy, by focusing on individual cells, allowed us to determine the variability of cyclin E accumulation behavior within the population. In addition, it allowed us to correlate the kinetics of cyclin E accumulation and downregulation with passage through the R point and entry into S phase.

Asynchronously growing HDFs were video recorded and immunostained for cyclin E. The immunostaining intensity was measured and correlated to cell age (time elapsed after the last mitosis). We found that G_1-pm (post-mitosis) cells, cells younger than 3.5 hours after last mitosis, were all negative for cyclin E. Cyclin E was found to start accumulating after passage through R, at different times during the G_1-ps (pre-DNA-synthesis) interval. This finding argues against the hypothesis that passage through the R point is dependent on the accumulation of cyclin E but suggests that passage through the R point is a prerequisite for accumulation of cyclin E. The fact that cells are cyclin E negative when they pass through R indicates that cyclin E is not the labile R point protein defined by Pardee (Dou et al., 1993; Rossow et al., 1979). In support of this conclusion, we also found that direct inhibition of cyclin E-Cdk2 activity with the potent Cdk2 inhibitor roscovitine does not prevent cells from passing R, although it completely blocked entry into S phase, consistent with a replication initiation role for cyclin E.

The mechanism by which cell cycle progression becomes growth factor independent during G_1 remains elusive. Other G_1 events that have been suggested to be the molecular basis for passage through R

are accumulation of cyclin D or inactivation of the cell cycle inhibitor pRb (Blagosklonny and Pardee, 2002). Cyclin D is a labile protein and its transcriptional induction is regulated by growth factors but its accumulation pattern does not correlate temporally with passage through R. Neither does pRb inactivation, which occurs concomitant with cyclin E accumulation. Another G_1 event that might have a role in regulation of R is the assembly of pre-replication complexes (pre-RCs) during early G_1 phase. This process is growth factor dependent and is required for the G_1/S transition. However, in cycling cells, pre-RC assembly occurs in late mitosis and early G_1, presumably well before passage through R. Interestingly, we observed that HDFs that were detached by mitotic shake-off and replated in the absence of serum had lost R point regulation and entered S phase with normal kinetics. This suggests that R point regulation is related to attachment of cells to their substrate and therefore possibly mediated by integrin signaling.

4.1.2 Cyclin E expression in non-transformed cells

Accumulation. TLV (timelapse videomicroscopy) analysis revealed that the timing of cyclin E accumulation after passage through R is highly variable. Previous studies have shown that the duration of G_1-ps, i.e., the time period from R to entry into S phase is highly variable among the cells in a population, ranging from less than 1 h to more than 20 h. In fact, most of the variation in cell cycle length, observed when comparing cells in a population, is believed to be a reflection of G_1-ps variability (Zetterberg and Larsson, 1985). The basis for G_1-ps variability remains to be determined but it has been suggested that adjustment of cell size or cellular protein content could be a factor (Killander and Zetterberg, 1965; Zetterberg, 1966; Zetterberg and Larsson, 1991). Our observations suggest that cyclin E accumulation is likely to be closely linked to the mechanism that sets the length of G_1-ps and triggers the G_1/S phase transition. However, this remains to be shown.

The rapid induction of cyclin E protein in late G_1 has been suggested to be the result of a positive transcriptional feedback loop (Won and Reed, 1996). According to this model, the cyclin E gene is under E2F transcriptional control (Geng et al., 1996). Thus, cyclin E-dependent Cdk2 activity would stimulate induction of additional cyclin E transcription by phosphorylation of pRb and the concomitant derepression of E2F activity. Consistent with this, Rb $^{-/-}$ mouse embryo fibroblasts prematurely induce cyclin E mRNA (Herrera et al., 1996). However, cells with constitutively inactive pRb, such as HeLa cells, exhibit periodic transcription of cyclin E mRNA, suggesting alternative transcriptional regulation (Herrera et al., 1996). It was shown by Oda et al. (Oda et al., 1995) that cyclin E protein levels are induced as a result of mRNA stabilization at the G_1/S transition. Possibly, cyclin E is regulated by two mechanisms, both by pRb and other factors at the

level of transcription and by an additional mechanism that mediates mRNA stabilization at the G_1/S transition.

To study cyclin E accumulation in relation to entry into S phase, TLV-analyzed cells were stained for cyclins E and A and visualized by immunofluorescence microscopy. Cyclin A positivity can be used as a marker for S phase entrance, based on recent data showing a very high correlation between cyclin A immunostaining and BrdU incorporation (Erlandsson et al., 2000). By presenting the immunostaining data as the cumulative number of cyclin E-positive cells or cyclin A-positive cells, as a function of cell age, we could determine the temporal relationship between cyclin E accumulation in G_1 and entry into S phase. These experiments showed that cells become cyclin E positive approximately 2 to 5 h before entry into S phase. Thus, for cells destined to enter S phase, cyclin E accumulates at a variable interval after passage through R but at a relatively fixed interval prior to entry into S phase. However, a substantial proportion of cells were found to accumulate cyclin E without entering S phase or entering S phase after very long delays. This subpopulation of cyclin E-positive of old G_1 cells most likely represents cells that are entering into senescence or have become senescent. This finding is in agreement with previous studies showing that senescent cells are cyclin E positive (Dulic et al., 2000; Stein and Dulic, 1998).

Downregulation. Cyclin E has previously been shown to be downregulated during S phase, but it has not been possible to determine precisely when in S phase this occurs using biochemical approaches. In Paper I, we used three independent methods to analyze the kinetics of downregulation of cyclin E protein levels: (i) calculation of the fraction of cyclin A-positive cells that are also positive for cyclin E, (ii) correlation of cyclin E protein levels with DNA content, and (iii) correlation of cyclin A and E positivity and exclusively cyclin A positivity with cell age. Data obtained by all three approaches, suggested that most of cyclin E protein is degraded very rapidly during early S phase, 1 to 2 h after entry into S phase, although a residual level persists in the nucleus for a significantly longer time. This is in principle consistent with previous studies but provides a surprisingly narrow window for cyclin E persistence during S phase. These observations suggest that cyclin E downregulation is activated upon entry into S phase and that strict regulation of cyclin E levels might be crucial for cell integrity or survival. We have incorporated this view of cyclin E accumulation and downregulation into a schematic model of the cell cycle in Figure 11.

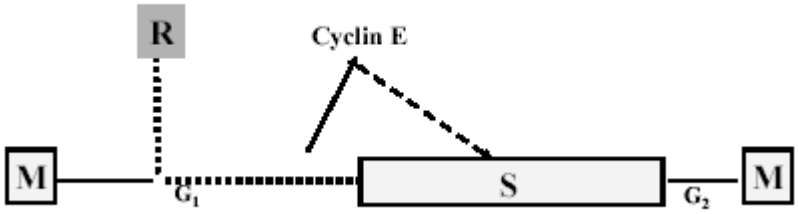


Figure 11. Schematic representation of cyclin E expression in relation to previous mitosis, passage through the Restriction point and entry into S phase. TLV analysis revealed that cyclin E accumulates at different times during the G_1-ps interval, at a relatively fixed interval prior to entry into S phase. Cyclin E levels peak at the G_1/S transition and are rapidly downregulated during early S phase, 1 to 2 h after entry into S phase. However, a residual level persists in the nucleus for a significantly longer time.

At the time when the study presented in Paper I was carried out, the components involved in the degradation of Cdk2-bound cyclin E had not yet been identified. Since then, it has been shown that cyclin E is targeted for ubiquitylation by the SCF-associated F-box protein hCdc4 (Fbw7/Ago). The targeting of cyclin E by hCdc4 for ubiquitylation is regulated by phosphorylation of cyclin E on T380 and T62 as well as on S372 (Won and Reed, 1996) (Welcker et al., 2003). At least some of these modifications correspond to autophosphorylation events (Won and Reed, 1996), which ensures that degradation of cyclin E only occurs after activation of Cdk2 complexes. We observed an inverse relationship between cyclin E and A immunostaining intensity in individual cells (data not shown), which suggests that cyclin A accumulation and cyclin A-controlled Cdk2 activity might have a role in the degradation of cyclin E, possibly by phosphorylation of cyclin E. Additionally, we have observed that cyclins E and A are colocalized during a short period of S phase, implying that the two protein complexes interact physically (data not shown). Thus, cyclin E might be targeted for degradation by both autophosphorylation and cyclin A-dependent phosphorylation.

4.2 PAPER II

4.2.1 Deregulation of cyclin E and DNA replication

Cyclin E is often overexpressed and/or deregulated in many types of human cancer (Erlandsson et al., 2003; Erlanson and Landberg, 2001; Keyomarsi et al., 1995; Sandhu and Slingerland, 2000; Schraml et al., 2003) and appears to have a direct role in tumorigenesis (Bortner and Rosenberg, 1997; Koepp et al., 2001; Moberg et al., 2001; Strohmaier et al., 2001). The mechanism by which deregulated cyclin E promotes tumorigenesis is however still unclear. Previous studies have demonstrated that deregulation of cyclin E accelerates the G_1/S transition (Ohtsubo and Roberts, 1993; Resnitzky et al., 1994; Wimmel et al., 1994) but on the other hand delays progression through S phase

(Ohtsubo and Roberts, 1993; Resnitzky et al., 1994; Spruck et al., 1999). However, these studies utilized either chronic overexpression of cyclin E achieved by retroviral transduction or regulated expression of cyclin E using a tetracycline-repressible transcriptional cassette. In Paper II, we analyzed whether acute, constitutive cyclin E expression obtained by adenoviral transduction affects the duration of G_1 phase and timing of entry into S phase. KB cells, chosen for their high efficiency of adenoviral transduction, were transduced with a recombinant adenovirus containing the cDNA for human E-Ad or with a control adenovirus, c-Ad, carrying a part of the β-globin cDNA (see Material and Methods).

Cells transduced with E-Ad were found to enter S phase with significantly more rapid and synchronous kinetics than control cells, supporting previous studies showing that overexpression of cyclin E results in an acceleration of the G_1/S phase transition (Resnitzky et al., 1994). Deregulated expression of cyclin E has also been shown to result in slowing of S phase progression (Spruck et al., 1999), although no direct analysis of DNA replication rate or of dose responsiveness to cyclin E levels was reported. To investigate the effect of deregulated cyclin E expression on DNA replication, 2-dimensional flow cytometric analysis of E-Ad and c-Ad transduced cells subjected to a short pulse of BrdU incorporation was carried out. In E-Ad transduced cells, an increased percentage of cells were found to be in S phase 24 hours after transduction, compared to control cells. This effect was additionally found to be dose-dependent: the percentage of cells in S phase increased with increasing multiplicity of infection, i.e. increasing cyclin E levels. Immunofluorescence staining of cyclin E and BrdU of transduced cells demonstrated that cells constitutively expressing cyclin E contained a reduced number and fluorescence intensity of BrdU foci, consistent with the flow cytometric analysis and further supporting the notion that deregulation of cyclin E results in a reduced rate of DNA synthesis.

In an attempt to address whether the observed reduction of both the number and staining intensity of BrdU foci is the result of an impairment of DNA replication initiation or an impairment of fork progression (elongation), we analyzed the localization patterns of BrdU and PCNA in individual early S phase cells with or without constitutive cyclin E expression. PCNA forms a sliding clamp that encircles the DNA helix and promotes processivity of DNA synthesis by polymerase δ during DNA replication (Fukuda et al., 1995; Kelman, 1997; Krishna et al., 1994; Prelich et al., 1987). PCNA has also been shown to be localized at the replication fork and to co-localize with newly synthesized DNA labeled with BrdU (Hozak et al., 1993; Somanathan et al., 2001; Takanari et al., 1994) and was therefore used as a quantitative marker for replication forks. Analysis of early S phase cells, identified based on the characteristic early S phase replication pattern, showed that deregulated expression of cyclin E results in a

reduction of both numbers and intensity of both PCNA and BrdU foci. In addition, many PCNA foci were not associated with BrdU foci. The reduction in the number and intensity of PCNA foci suggests that fewer origins are fired, consistent with a defect in replication initiation, while the lack of correlation between PCNA foci and BrdU foci suggests that replication forks might be stalled. Thus, deregulation of cyclin E expression appears to impair both replication initiation and fork movement.

Two recent studies in yeast support the connection between Cdk deregulation and inefficient DNA replication (Lengronne and Schwob, 2002; Tanaka and Diffley, 2002). Deletion of the gene encoding a Cdk1 inhibitor, Sic1, which results in inability to completely down-regulate Cdk activity in G_1, dramatically reduced density of replication origins (Lengronne and Schwob, 2002). In another study, overexpression of yeast G_1 cyclins, known as Clns, also impaired replication, based on elevated rates of plasmid loss (Tanaka and Diffley, 2002). In both cases, deregulation/overexpression of Cdk activity was shown to result in chromosome instability with rearrangements that could be scored genetically.

4.2.2 Cyclin E-Cdk2 interferes with pre-RC assembly

Previous studies in yeast and Xenopus egg extracts have shown that Cdk activity must be reduced to low levels to allow assembly of pre-RCs, a criterion that is normally met during late mitosis and early G_1 phase in mammalian somatic cells (Coverley et al., 1996; Hua et al., 1997; Nishitani and Lygerou, 2002; Wuarin and Nurse, 1996; Yan and Newport, 1995) (Fig. 6A). However, if cyclin E is deregulated and constitutively expressed throughout the cell cycle, Cdk activity will persist during late mitosis and early G_1 phase. This might prevent or interfere with pre-RC assembly, which could result in impairment of DNA replication (Fig. 6B). To investigate whether the negative effects of cyclin E deregulation on DNA replication are a consequence of inappropriate Cdk activity at the end of mitosis and the beginning of G_1, we studied chromatin loading of various pre-RC components in telophase cells, with or without constitutive cyclin E expression.

When telophase cells, transduced with E-Ad or c-Ad, were analyzed for chromatin association of pre-RC components (see Material and Methods, Fig. 9 and 10), we found that chromatin loading of Mcm4 was dramatically reduced in cells constitutively expressing cyclin E, compared to control cells. The total cellular level of Mcm4 however, was not reduced. The observed impairment in Mcm4 chromatin loading was found to be completely restored when cells were treated with the Cdk2 inhibitor roscovitine as they proceeded through mitosis, which confirms that the observed reduction of Mcm4 chromatin binding in cells constitutively expressing cyclin E is dependent on Cdk2 kinase activity. These experiments do not

distinguish between a direct effect of cyclin E-Cdk2 phosphorylation of Mcm4 or an indirect effect mediated by phosphorylation of other proteins. However, Mcm4 present in the non-chromatin-bound fraction of telophase cells, was found to be hyperphosphorylated in cells with deregulated cyclin E expression, while the chromatin-bound fraction of Mcm4 was not hyperphosphorylated. Immunoblot analysis showed that chromatin loading of Mcm3 and Mcm7 were slightly reduced by constitutive cyclin E expression, although this effect was modest compared to the effect on Mcm4 chromatin loading. The telophase chromatin localization of other MCM proteins (Mcm2 and Mcm5) and components of the human ORC (Orc1, Orc2) or initiator protein Cdc6 was not affected by constitutive expression of cyclin E.

In yeast, Cdk activity has been shown to prevent nuclear import of MCM proteins (Tanaka and Diffley, 2002; Wuarin and Nurse, 1996; Zachariae and Nasmyth, 1999) and to promote ubiquitin-mediated proteolysis of Cdc6(Drury et al., 1997; Drury et al., 2000; Sanchez et al., 1999). As both MCM proteins and Cdc6 are required for pre-replication complex assembly, these observations explain how Cdk activity can interfere with this process in yeast. In mammalian cells, MCM proteins are not regulated at the level of nuclear entry but at the level of chromatin loading (Kimura et al., 1994; Krude et al., 1996; Mendez and Stillman, 2000). In addition, Cdc6 is not degraded at the G_1-S boundary (Mendez and Stillman, 2000; Yan et al., 1998). Our data are consistent with this in that deregulation of cyclin E does not affect the nuclear localization of MCM proteins but merely their ability to load onto chromatin. Furthermore, the level and loading efficiency of Cdc6 is not affected.

Based on this and previous work, it is clear that Cdk activity has an ambivalent relationship to DNA replication. On the one hand, it is activation of Cdks in late G_1 that triggers the initiation of S phase. On the other, Cdk activity is antagonistic to pre-RC assembly (Kelly and Brown, 2000; Lei and Tye, 2001; Nishitani and Lygerou, 2002; Woo and Poon, 2003a). These two opposing roles of cyclin E-Cdk2 activity are apparent in the contradictory effects of deregulated cyclin E on the cell cycle. The G_1/S transition is accelerated, consistent with cyclin E's positive role in initiation. However, subsequent DNA replication proceeds at a much slower rate as a consequence of cyclin E-Cdk2 impairment of pre-RC assembly, since all components of the pre-RC are likely required for initiation of DNA replication. Normally, tight temporal regulation of cyclin accumulation eliminates Cdk activity from the critical M phase-G_1 interval. Deregulation of cyclin E apparently interferes with this highly conserved mode of cell cycle regulation, possibly inducing genomic instability and ultimately malignancy.

The well-established view that cyclin E-Cdk2 activity is essential for initiation of DNA replication was recently challenged by a study

demonstrating that cyclin E is dispensable for mouse development (Geng et al., 2003). Mice lacking both cyclin E1 and E2 (cyclin E1 and E2 are highly homologous and are believed to have overlapping functions, (Lauper et al., 1998), die *in utero* at around day 10 *post coitus*, not as a result of developmental deficiencies in the embryo, but because of placental failure (Geng et al., 2003). The trophoblast giant cells, which are enlarged because of endoreplication and serve a critical and central function in placental physiology, were found to be underdeveloped due to a lack of endoreplication. However, by tetraploid complementation the investigators were able to rescue this lethality and concluded that cyclin E is not required for initiation of DNA replication during embryonic development but only for endoreplication events (in both trophoblasts and megakaryocytes). When the investigators further analyzed fibroblasts from embryos (MEFs) lacking both cyclin E genes, they discovered that cells devoid of cyclin E expression were unable to re-enter the cell cycle from G_0 due to an inability in loading of MCM proteins onto chromatin during early G_1 (Geng et al., 2003). In yeast, re-entry into the cell cycle from G_0 has also been shown to depend on G_1 cyclins for loading of MCM proteins (Tanaka and Diffley, 2002). Thus, interestingly, re-entry into the cell cycle from G_0 requires cyclin E activity, while in cycling cells, this interval needs to be free of cyclin E to allow proper MCM loading.

4.2.3 Kinetics of MCM loading onto chromatin

The finding that deregulation of cyclin E-Cdk2 activity appears to impair the loading specifically of Mcm3, Mcm4 and Mcm7 during telophase is surprising since it was previously demonstrated that stable MCM complexes exist both in solution (Schwacha and Bell, 2001) and on chromatin (Ritzi et al., 1998) and that MCM proteins assemble into complexes and subcomplexes prior to being loaded onto chromatin. However, a recent study of MCM loading kinetics in Xenopus suggested that assembly of the MCM complex onto pre-replicative chromatin might be regulated at the level of distinct subunits (Maiorano et al., 2000). Interference with pre-RC assembly might affect subsequent DNA replication by promoting the formation and chromatin loading of non-functional incomplete MCM complexes or subcomplexes.

When MCM protein binding to chromatin was analyzed at different time points after exit from mitosis throughout G_1 phase, progressively more MCM proteins were found to be loaded in both E-Ad transduced cells and in control cells. However, the amount of Mcm4 and Mcm7 never reached levels comparable to levels in control cells, although the time-dependent loading of Mcm3 was normal. In contrast, loading of Mcm2 was impaired in cells constitutively expressing cyclin E but only at time points subsequent to 1-hour post-mitosis. Therefore, impairment of Mcm7 and Mcm2 loading are also likely to contribute to the S phase phenotypes associated with cyclin E

deregulation. To determine if less Mcm4 was bound to replicating chromatin, Mcm4 was visualized during early S phase by immunofluorescence deconvolution microscopy. Even in early S phase, as determined by BrdU staining pattern, E-Ad transduced cells exhibited a deficiency in Mcm4 bound to chromatin, although the effect was not quantitatively as great as in telophase. To confirm that a partial reduction in Mcm4 chromatin loading could have a significant impact on DNA replication, RNAi was used to reduce the total intracellular level of Mcm4 and asynchronous cells were given a 15-minute pulse of BrdU. Microscopic analysis of detergent-extracted early S phase cells indicated that partial reduction of chromatin-bound Mcm4 correlated with a significant reduction in BrdU incorporation.

Our data suggest that individual MCM proteins load onto chromatin with distinct kinetics 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 both assembled MCM complexes as well as individual MCM subunits can be loaded onto chromatin.

4.2.4 Deregulation of cyclin E and genomic instability

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 non-transformed rodent fibroblasts and human mammary epithelial cells caused elevated frequencies of chromosome losses and gains, as well as polyploidy (Loeb and Loeb, 2000; Spruck et al., 1999). Furthermore, inactivation of *hCDC4*, a gene required for cyclin E turnover, by targeted disruption in karyotypically stable colorectal cancer cells, results in a phenotype associated with micronuclei and chromosomal instability (Rajagopalan et al., 2004). This phenotype was shown to depend on deregulation of cyclin E levels. Additionally, high cyclin E in human breast cancer was shown to correlate with aneuploidy and specific p53 mutations such as insertions and deletions, while p53 in tumors with low cyclin E levels mainly exhibit point mutations, suggesting that gross genomic alterations occur in cells with deregulated cyclin E (Lindahl et al., 2004). Cyclin E-mediated genomic instability, therefore, may constitute a functional link to malignancy but this remains to be demonstrated in an *in vivo* model.

To explain how the negative effect of cyclin E deregulation on DNA replication could result in chromosome instability, several hypotheses might be considered. First, the interference with pre-RC assembly could reduce the number of active replication origins. In this

situation, the average replicon size would increase, resulting in higher frequency of stalled replication forks and double-stranded DNA breaks. Second, the reduced rate of DNA replication could also impair the processes of chromosome condensation and sister chromatid cohesion since 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. If such cells do indeed enter mitosis, incompletely replicated chromosomes will not be able to disjoin at anaphase, and the resulting daughter cells will experience chromosome losses and other chromosomal anomalies. Checkpoint mechanisms prevent such potentially catastrophic events, but the mechanisms underlying the S phase/mitosis checkpoint are poorly understood. For example, the signal(s) preventing mitosis while replication is ongoing have not been identified and the minimum amount of unreplicated DNA or ongoing replication required to trigger this checkpoint is not known. It is possible that a small amount of unreplicated DNA would be sufficient to cause a chromosome non-disjunction event but not create a strong enough signal to trigger a checkpoint response and prevent mitosis. Finally, deregulation of cyclin E may disrupt the normal intra-S-phase checkpoint mechanisms, allowing propagation of DNA damage (Fig.12).

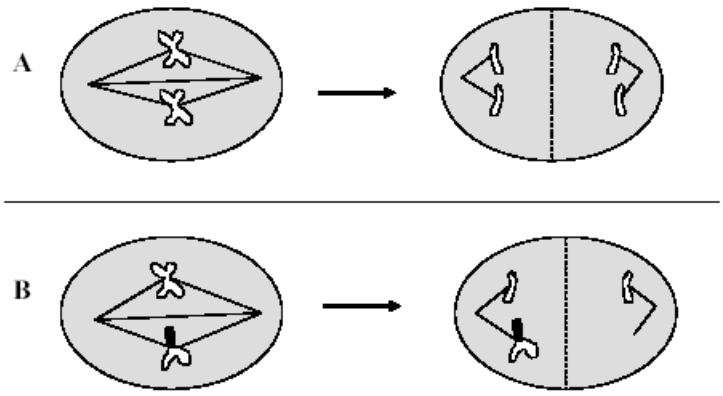


Figure 12. How could impaired DNA replication result in genomic instability? In a normal cell, chromosomes are replicated and sister chromatids are segregated to opposite poles during mitosis. If chromosomes are not completely replicated, sister chromatids will not segregate properly, which could result in non-disjunction events. Checkpoints normally prevent these errors but checkpoints can fail.

4.3 PAPER III

4.3.1 The role of hCDC4 in cell cycle regulation of cyclin E

Loss of *hCDC4* function results in deregulation of cyclin expression in endometrial tumors. Cyclin E levels are downregulated during S phase by SCF-mediated ubiquitylation and subsequent proteolysis by the 26S proteasome (Clurman et al., 1996; Won and Reed, 1996). hCdc4, the F-box protein that targets phosphorylated cyclin E to the SCF complex has been shown to be mutated in both primary tumors and in cancer derived cell lines (Koepp et al., 2001; Moberg et al., 2001; Rajagopalan et al., 2004; Spruck et al., 2002; Strohmaier et al., 2001). Analysis of a set of endometrial tumors indicated that 16% had mutations in *hCDC4* with accompanying loss of heterozygosity (LOH) (Spruck et al., 2002). Yet many of these tumors did not exhibit elevated levels of cyclin E compared to tumors without *hCDC4* mutations. We therefore investigated whether cell cycle regulation of cyclin E might be impaired by functional inactivation of *hCDC4*.

Cyclin E expression was analyzed by immunohistochemistry in sections from 6 tumors harboring mutations that completely eliminated *hCDC4* function and was compared to expression in sections from 7 randomly chosen control tumors. In addition, three tumors with splice variant isoform-specific *hCDC4* mutations were analyzed: two mutated in Exon 1β and one in Exon 1α. This analysis demonstrated that loss of *hCDC4* function is strongly associated with cell cycle deregulation of cyclin E. In control tumors about 30% of the cells stained positive for cyclin E, while 70-100% of the cells of tumors harboring *hCDC4* mutations, stained positive for cyclin E. Neither of the β-isoform-specific mutations showed deregulation of cyclin E, suggesting that this isoform is not involved in cyclin E targeting. However, the α-isoform-specific mutation showed strong deregulation of cyclin E indicating that this isoform is required for cyclin E regulation. These results suggest that mutation of *hCDC4* might promote tumorigenesis via other targets, in addition to cyclin E. In fact, protein levels of the Notch/Lin-12 family of transcriptional activators have also been shown to be regulated by *hCDC4* (Oberg et al., 2001; Tetzlaff et al., 2004a; Tsunematsu et al., 2004) and to be deregulated in human cancer (reviewed by (Allenspach et al., 2002), suggesting that Notch might be targeted by the hCdc4 β-isoform. Interestingly, tumors that had elevated cyclin E levels but were not mutated for *hCDC4* maintained normal cell cycle regulation of cyclin E. Thus, although *hCDC4* mutation in endometrial tumors does not necessarily lead to elevated expression of cyclin E, it always results in loss of cyclin E cell cycle regulation (with the exception of β-isoform-specific mutations). It was previously reported that *hCDC4* mutation correlates with the relatively rare incidence of metastasis in endometrial tumors (Spruck et al., 2002). Cell cycle deregulation of cyclin E, which we show correlates strongly with *hCDC4* mutation ($p<0.01$, Fischer's Exact Test), may

therefore serve as a convenient prognostic marker for individualizing patient treatment.

Loss of *hCDC4* function in the SUM149PT cell line results in deregulation of cyclin E. In order to determine if mutation of *hCDC4* is sufficient to account for loss of cell cycle regulation of cyclin E, we analyzed the relationship between cyclin E expression and hCdc4 function in a cell line that could be manipulated for *hCDC4* expression. SUM149PT is a breast-tumor-derived cell line that is mutated for *hCDC4* and exhibits high cyclin E protein levels (Strohmaier et al., 2001). By immunofluorescence microscopy, SUM149PT cells were shown to express cyclin E in all cells. Transduction of the SUM149PT cell line with a recombinant retrovirus programmed to express the 110 kDa (α) isoform of hCdc4, restored normal cell cycle regulation of cyclin E. The cyclin E and cyclin A expression patterns in SUM149PT+Cdc4 cells were shown to be almost identical to those observed in a randomly chosen mammary carcinoma-derived cell line (ZR75-1) not mutated for *hCDC4* and similar to expression patterns in non-transformed human mammary epithelial (IME) cells. In the case of the IME cells, there is less overlap between cyclin E and cyclin A expressing populations, which is a characteristic of non-transformed cells (Erlandsson et al., 2003). These data collectively confirm that normal cell cycle regulation has been restored.

Inactivation of hCdc4 function in HEK293 cells with RNAi results in deregulation of cyclin E. Knock-down of hCdc4 protein levels using siRNA technology has previously been shown to result in increased levels of cyclin E, but the effects on cell cycle regulation of cyclin E have not been reported (Koepp et al., 2001). HEK293 cells were transduced with a retrovirus programmed to express a small duplex RNA targeted to the region of hCdc4 mRNA corresponding to Exon 3 of the gene, common to all hCdc4 isoforms. Targeting hCdc4 with this siRNA severely reduced both hCdc4 mRNA and protein levels in HEK293 cells, while both the frequency and intensity of cyclin E staining dramatically increased. As in the SUM149PT cell line, knocking down hCdc4 by RNAi in HEK293 cells results in cyclin E being expressed in every cell, even during telophase. Thus, loss of hCdc4 function is sufficient to deregulate cyclin E expression relative to cell cycle progression and causes accumulation of cyclin E at inappropriate times.

Two recent studies describe the generation of mice deficient for *hCDC4* (Fbw7/hAgo)(Tetzlaff et al., 2004b; Tsunematsu et al., 2004) but only in one of these studies was absence of *hCDC4* shown to result in elevated cyclin E levels (Tetzlaff et al., 2004b). Cyclin E cell cycle regulation was however not analyzed. *hCDC4*-null mice die around 10.5 days *post coitus* because of a combination of abnormalities in hematopoietic and vascular development and heart chamber maturation due to elevated levels of the intracellular domain of specific Notch proteins (Tetzlaff et al., 2004b; Tsunematsu et al., 2004).

5. CONCLUSIONS

The three studies included in this thesis were conducted to increase understanding of the role of cyclin E in normal cell cycle regulation and in tumorigenesis. Expression of cyclin E, an important positive regulator of the G_1/S transition, is tightly regulated by transcriptional activation in late G_1 and by phosphorylation-activated degradation during S phase, mediated by the specificity factor hCdc4. In addition, cyclin E is overexpressed and/or deregulated in many human cancers and is believed to be involved in tumorigenesis. Experiments carried out with cells in culture, suggest that deregulation of cyclin E causes genomic instability, possibly explaining the link to tumorigenesis.

In Paper I, we investigated the role of cyclin E and Cdk2 activity for passage through the Restriction point (R). We showed that cyclin E accumulates after passage through R, at different times during the G_1-ps interval and that Cdk2 activity is not required for passage through R. These results strongly suggest that accumulation of cyclin E does not constitute passage through R and that passage through R is a prerequisite for accumulation of cyclin E. The mechanism for R control is still unknown.

Our study of the role of *hCDC4* for regulation of cyclin E levels (Paper III), revealed that loss of *hCDC4* function correlates strongly with deregulation of cyclin E levels relative to the cell cycle. It was previously shown that gene mutations in endometrial tumors were accompanied by loss of heterozygosity and correlated with more aggressive disease. Together, these observations suggest that cyclin E deregulation relative to the cell cycle is likely to be critical for tumorigenesis and might be the specific event inducing genomic instability in these tumors. Thus, loss of *hCDC4* function provides a mechanism by which cyclin E is deregulated.

The correlation between *hCDC4* mutation, metastasis, LOH and deregulated expression of cyclin E, further suggests that deregulation of cyclin E may serve as a convenient prognostic marker for individualizing patient treatment.

In Paper II, we started investigating the mechanism by which cyclin E deregulation might contribute to genomic instability and tumorigenesis. We hypothesized that the impairment of DNA replication, observed in cells expressing cyclin E constitutively, might be the result of cyclin E–Cdk2 activity at inappropriate times, interfering with the formation of pre-replication complexes (pre-RCs) in late mitosis and early G_1 phase. We found that deregulated cyclin E expression indeed interferes with pre-RC assembly, in particular with chromatin

loading of specific MCM proteins. Additional experiments are however necessary to establish whether cyclin E-Cdk2 directly phosphorylates MCM proteins, thereby preventing their association with chromatin or whether this effect is mediated by another protein or other proteins. Furthermore, we showed that deregulated cyclin E expression impairs both initiation and elongation of DNA replication, supporting previous studies showing that MCM proteins are required throughout S phase (Labib et al., 2000). Our observation that some MCM proteins are affected but not all, not even all members of previously identified subcomplexes, suggests that MCM proteins might associate individually with chromatin and assemble into complexes later. It will be important to elucidate the mechanism for MCM loading, complex formation and function to fully understand the effect of deregulated cyclin E levels on pre-RC assembly and subsequent DNA replication.

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