

ORIGINAL ARTICLE

Cyclin E and chromosome instability in colorectal cancer cell lines

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Aims/Background: The development of colorectal cancer depends on at least two distinct pathways involving genetic instability, namely: chromosome instability (CIN) and microsatellite instability. Cyclin E is involved in aneuploidy and several cancer types show an abnormal number of chromosomes.

Methods: Cyclin E protein and mRNA values were analysed in human fetal skin fibroblasts and five colorectal cancer cell lines.

Results: Cells with an aberrant number of chromosomes had higher cyclin E mRNA values and a significant increase in protein concentrations.

Conclusions: These data suggest that cyclin E regulation is altered in aneuploid cells and is an important factor in the CIN pathway.

The cell cycle is a tightly regulated process that makes cell division possible through the activity of cyclin/cyclin dependent kinase (cdk) complexes. The enzymatic activity of the cdk is controlled by the regulatory subunits of the complexes (the cyclins, which are synthesised and then destroyed), whereas cdk protein concentrations remain constant.¹ Cyclin/cdk complexes catalyse the phosphorylation of substrates essential for the commitment of the cell to pass through checkpoint barriers.¹ The precisely timed degradation of cyclins is one mechanism that regulates cdk activity, ensuring that the cells move through the cell cycle.¹

During the G1 phase of the cell cycle, cyclin E gene transcription is induced by several signals, such as E2F transcription factors, and cyclin E protein concentrations rise, reaching a maximum at the G1–S transition.¹ Cyclin E binds to and activates cdk2, allowing the cell to enter into S phase. Conversely, impaired activity of the cyclin E/cdk2 complex arrests the cell before new DNA is synthesised.¹ Cyclin E is degraded by ubiquitin dependent proteolysis.^{2,3} Phosphorylation of cyclin triggers its ubiquitination, which is a common theme in regulated protein turnover.^{2,3} Often, phosphorylation of a protein is the end result of a signal transduction pathway and represents a mechanism that allows ubiquitin conjugating enzymes to recognise the proteins. Cyclin E protein is directly phosphorylated by its associated kinase^{2,3}; thus, cyclin E/cdk2 activity is inherently self limited, because cdk2 activity initiates the pathway leading to the destruction of cyclin E.^{2,3}

In many human tumours the cyclin E gene is overexpressed and protein concentrations and related kinase activity are often altered.^{4–6} Cyclin E regulation is crucial for several cellular processes that influence the fidelity of chromosome transmission, including the licensing of the origins of DNA replication and centrosome duplication.⁹

“The development of colorectal cancer depends upon two distinct pathways, both characterised by genetic instability: microsatellite instability and chromosome instability”

Recently, Spruck and co-workers⁹ demonstrated that induction of the cyclin E transgene in rat embryo fibroblasts and human breast epithelial cells affects chromosome stability, thus causing aneuploidy, whereas the overexpression of cyclin

A and cyclin D1 does not induce changes in chromosome number.⁹ These data seem to indicate that aberrant regulation of cyclin E could be responsible for chromosome instability (CIN).

CIN is one of the pathways that lead to tumorigenesis—cancer cells frequently show an abnormal chromosome number.¹⁰ Several recent studies link cyclin E and cancer aneuploidy, showing that cyclin E overexpression is a common finding in cancer cells and correlates with poor prognosis, whereas the overexpression of other cyclins is not related to prognosis.^{11–16}

The development of colorectal cancer depends upon two distinct pathways, both characterised by genetic instability: MIN (microsatellite instability) and CIN. The MIN phenotype is a recessive trait and results from mismatch repair (MMR) deficiency.¹⁰ CIN, as has been demonstrated by cell fusion experiments with MIN cell lines, acts in a dominant fashion.¹⁰ The cause of CIN remains to be established.¹⁰ These data suggest that during early colorectal tumorigenesis one or more genes affecting chromosome segregation are mutated in a dominant fashion, and that the resulting instability drives the tumorigenic process in the same way as MIN does in MMR deficient tumours.¹⁰

Based on recent findings,⁹ the cyclin E gene seems to be one of the candidates responsible for the CIN phenotype. Cyclin E gene amplification has been seen in a very small proportion of both primary colorectal tumours and colon cancer cell lines.^{17,18} In addition, consistent with a high rate of cell proliferation, higher cyclin E protein concentrations have been found in cancerous tissue compared with non-neoplastic mucosa.^{19,20}

In this report, we analyse cyclin E protein and mRNA values in human fetal skin fibroblasts (hFSF) and in five human colorectal cancer cell lines. Four of the five cancer cell lines showed the CIN phenotype. Our data support the idea that cyclin E overexpression correlates with the CIN pathway, probably as a consequence of an altered balance of transcription, translation, and post-translational regulation.

Abbreviations: cdk, cyclin dependent kinase; CIN, chromosome instability; hFSF, human fetal skin fibroblasts; MIN, microsatellite instability

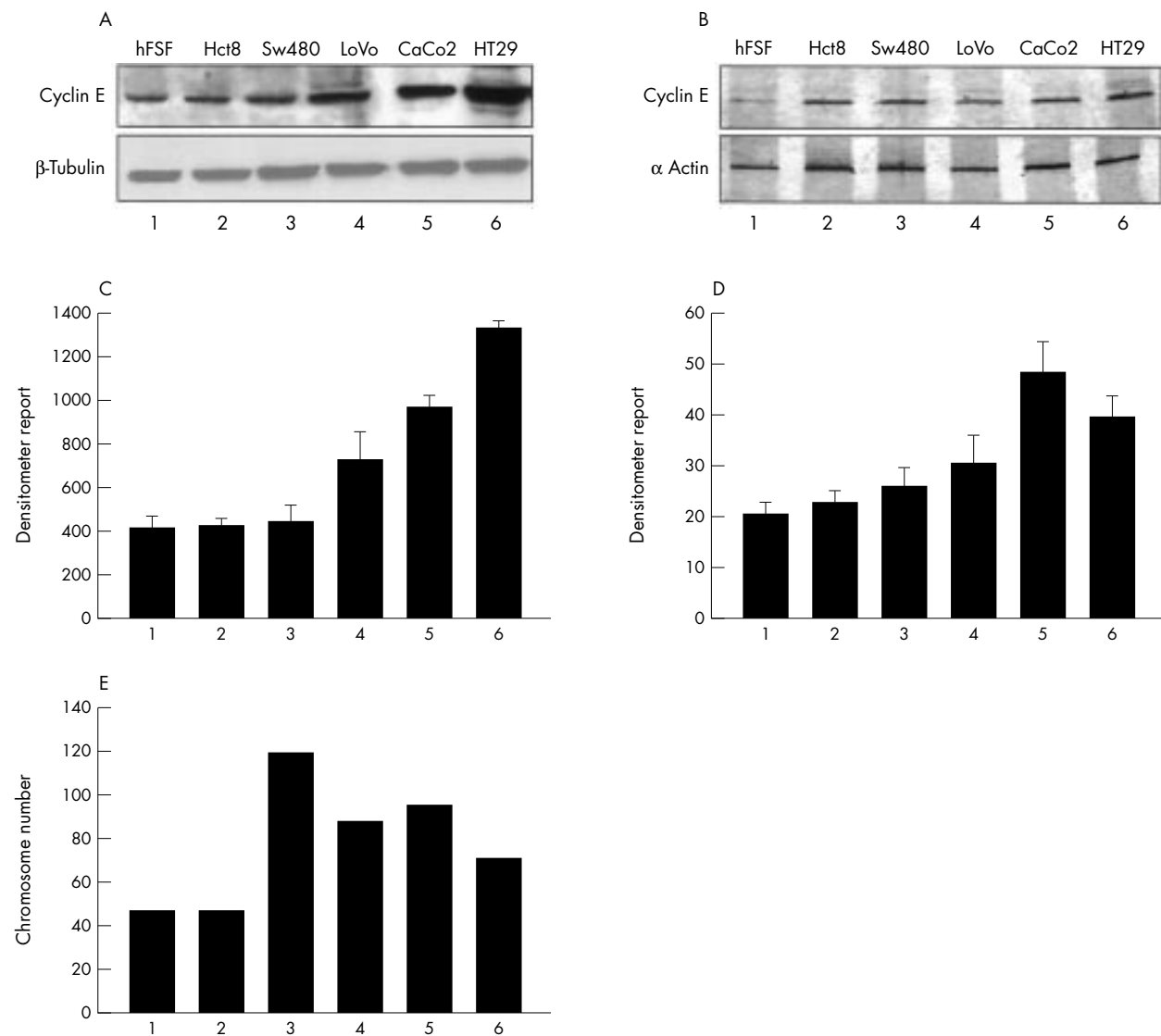


Figure 1 Overexpression of the cyclin E protein in aneuploid colon cancer cells depends on the increased transcription of cyclin E mRNA. (A,C) Immunoblot assay of 50 µg of total cell lysate from the indicated cell lines probed with anti-cyclin E antibody. The same extracts were probed with anti-β-tubulin and the results were normalised after densitometer analysis. (B,D) The reverse transcription polymerase chain reaction was performed on total RNA extracted from the same cells used for immunoblot analysis. cDNA was obtained with actin and cyclin E specific primers and resolved on a polyacrylamide gel. The data presented were obtained with densitometer analysis and normalisation on actin values. (E) The histogram illustrates the modal number of chromosomes for each cell line.

MATERIALS AND METHODS

Cell lines

hFSF were obtained by standard methods.²¹ The LoVo, Hct8, SW480, CaCo2, and HT29 cell lines were obtained from the ATCC. The chromosomal content of each cell line was determined by conventional cytogenetic karyotyping and was in accordance with data presented in the literature.¹⁰ All cell lines were grown in DMEM + 10% fetal bovine serum, avoiding confluency at any time.

Protein analysis

Cell pellets were split into two. One part was used for immunoblot assays and the other for mRNA detection. The extracts were obtained by lysing the cells in lysis buffer (50mM Tris, 5mM EDTA, 250mM NaCl, 50mM NaF, 0.1% Triton, 0.1mM Na₃VO₄, and 10 µg/ml aprotinin, leupeptin, and phenylmethylsulphonyl fluoride). The protein concentration was determined by means of the Bradford assay (Biorad, California, USA), following the manufacturer's instructions, and

using bovine serum albumin as a standard. The samples (50 µg of total cell lysate) were resolved on a 10% sodium dodecyl sulfate/polyacrylamide gel and transferred to a PVDF filter (Millipore Corporation, Bedford, Massachusetts, USA) at 4°C and 70 V for two hours. To ensure equal transference, 0.5% Ponceau red was used. The blots were blocked with Tris buffered saline (×1)—Tween 20 (0.1%) (TBST) containing 5% non-fat dry milk. Anti-cyclin E (1/500 dilution; Rockland, Pennsylvania, USA) polyclonal antibody and anti-β-tubulin (1/100 dilution; T-4026) monoclonal antibody were used in TBST containing 5% non-fat dry milk, according to the Western blot conditions suggested by Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, California, USA). Peroxidase conjugated antirabbit and antimouse antibodies (1/20000 dilution; Amersham, Arlington Heights, Illinois, USA) and the ECL detection system (NEN™; Du Pont, Mississippi, USA) were used for detection. The results were quantified by scoring the intensity of the cyclin E band with a densitometer (Ultrascan XL; LKB-Pharmacia) and normalised with the intensity of the β-tubulin band.

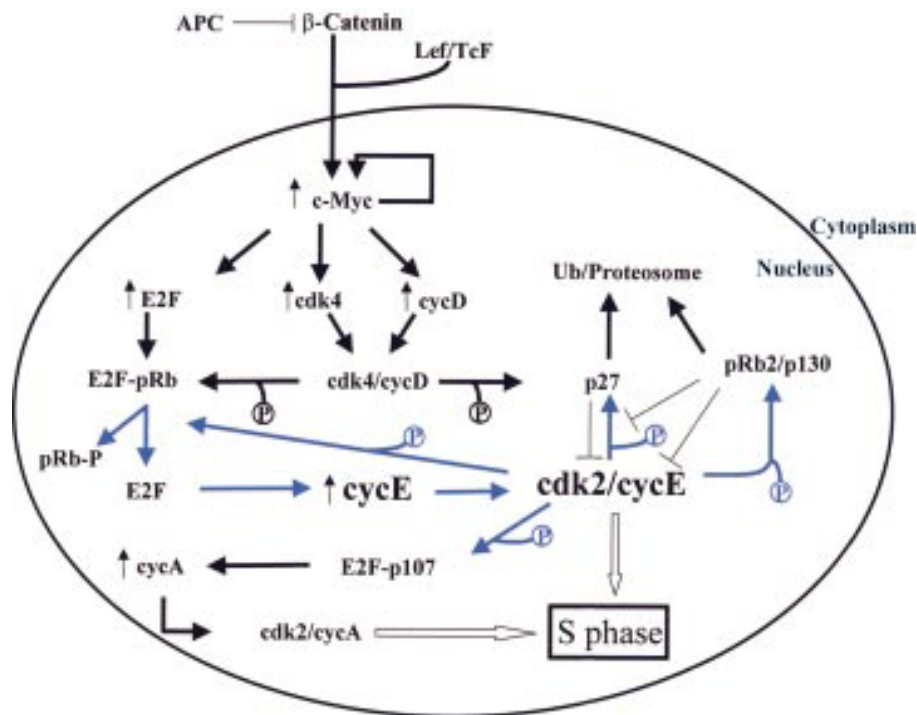


Figure 2 Graphic representation of well characterised pathways that control entry into S phase of the cell cycle. Mutations of the APC gene inhibit its control on β -catenin, which could also be affected by mutation. These carcinogenic events lead to increased Tcf/Lef translocation to the nucleus and the activation of specific gene transcription. One of these genes is c-Myc, which at the end of this process increases cyclin E activity. A detailed explanation is provided in the Discussion section. Cdk, cyclin dependent kinase; cyc, cyclin; P, phosphate; pRb, retinoblastoma protein; Ub, ubiquitin.

RNA extraction and reverse transcription

Total cellular RNA was extracted using Trizol reagent (Gibco Life Technologies). Reverse transcription (RT) was performed using 400 U MMLV-RT point mutation (Promega) in the manufacturer's buffer, 0.5mM dNTPs, and 1 μ g of pd(N)₆ examers.

Polymerase chain reaction (PCR)

The primers for cyclin E were designed to span an intron, hence avoiding confusion between RNA and the genomic DNA derived product, according to the published cDNA sequence (exon 2 CycE forward, 5'-cagcggagcagcccatc-3'; exon 3-4 CycE reverse, 5'-aaaacggtcagcttgccttc-3'). cDNA amplification was performed in 1 \times buffer gold (Perkin Elmer) using 25 pmols of each primer, 0.26mM each dNTP, 2.5 mM MgCl₂, and 1 U Taq polymerase gold in a 50 μ l final volume, with appropriate positive and negative controls. Cycling was carried out on a Hybaid express thermal cycler and the PCR conditions were as follows: variable number of cycles of 94°C denaturation for 30 seconds, annealing at 60°C for 30 seconds, and polymerisation at 72°C for 30 seconds. A final extension step of 72°C for seven minutes was performed to ensure completion of all initiated polymerisation events. The target (cyclin E) and internal standard (β actin) were amplified in the same tube; the ratios of the amount of PCR products generated by target and endogenous standard sequences obtained in repeated experiments using different cancer cell lines were determined and compared. The data from this experiment were obtained before the amplification reaction reached the plateau phase. PCR products were resolved on a 6% non-denaturing polyacrylamide gel. The bands were silver stained. The results of the RT-PCR were semi-quantified by scoring the intensity of the fragment of cyclin E (\approx 130 bp) with a densitometer (Ultrascan XL) and normalised with the intensity of the β actin band.

RESULTS

Figure 1E shows the chromosomal content (expressed as a modal number) of the six cell lines (our unpublished observation).¹⁰

All the data shown in this figure are representative of four independent experiments.

Total lysates obtained from logarithmically growing hFSF, LoVo, Hct8, SW480, CaCo2, and HT29 cancer cell lines were analysed by immunoblot analysis.

From fig 1A,C,E it can be seen that the hFSF and Hct8 cell lines, both euploid (2n = 46), contained comparable amounts of cyclin E. In contrast, the LoVo, CaCo2, and HT29 cell lines, which are clearly aneuploid, showed increased amounts of cyclin E (fig 1A,C, lanes 4-6). It has been shown that the HT29 cell line can maintain the CIN trait after fusion with DLD1, a MIN cell line.¹⁰ Interestingly, Sw480 cells, despite their high chromosome number, showed similar cyclin E protein concentrations to that found in euploid cells.

To understand the nature of cyclin E overexpression, we decided to analyse mRNA values. RT-PCR was performed on total RNA extracted from the same cells used for the immunoblot analysis. In fig 1D, cyclin E mRNA values are shown after normalisation with actin. The increased mRNA values seen can explain the increased cyclin E protein concentrations found in the LoVo, CaCo2, and HT29 cells (fig 1B,D, lanes 4-6). Once again, Sw480 cells showed a distinct feature: their cyclin E mRNA values were comparable to those of the euploid cells.

Our results indicate that cyclin E is overexpressed in LoVo, CaCo2, and HT29 colon cancer cell lines and that this overexpression is the consequence of an increased transcription rate and protein translation.

DISCUSSION

Several steps of colorectal cancer progression have now been clarified and the importance of cyclin E altered turnover is

Take home messages

- Those cell lines with an aberrant number of chromosomes (LoVo, CaCo2, and HT29) had higher cyclin E mRNA values and significantly increased protein concentrations
- Cyclin E regulation appears to be altered in aneuploid cells and could be an important factor in the chromosome instability pathway

continually being highlighted. Almost all CIN tumours show mutations at the APC or β -catenin loci (fig 2),²³ and it has been shown that both of these proteins act at the transcriptional level by controlling the Lef and Tcf transcription factors (fig 2).²³ One of the targets of this cascade is the basic helix–loop–helix/leucine zipper transcription factor c-Myc, which has been implicated in at least three distinct genetic pathways controlling progression through the G1 phase of the cell cycle (fig 2).²³ c-Myc controls G1 progression by suppressing the function of p27, inducing the transcriptional activity of the E2F/DP family of transcription factors, and promoting cell growth and increase in cell mass (fig 2).²³ At least two of these three pathways involve cyclin E regulation; in fact, p27 is a cyclin E/cdk2 inhibitor and six E2F sites are present on the promoter of the cyclin E gene (fig 2).^{23,24} Once the cycE/cdk2 complex is activated, it is able to reinforce cyclin E gene transcription through phosphorylation of the retinoblastoma protein (pRb), thereby liberating transcriptionally active E2F. This mechanism involves a positive feedback loop and allows the transition from a mitogen dependent route (via cyclin D) to a mitogen independent route (via cyclin E) for maintaining pRb phosphorylation and progression through the cell cycle (fig 2).²⁵ In addition, this mechanism involves the regulation of the G1–S transition by pRb2/p130, which downregulates cyclin E/cdk2 kinase activity by both physical binding and p27 protein stabilisation. Conversely, cyclin E/cdk2 negatively regulates both p27 and pRb2/p130 protein stability through phosphorylation of specific residues that trigger ubiquitination and degradation via the proteasome pathway (fig 2).²⁶

“Cyclin E overexpression could maintain the altered chromosome number through each cell division and select a population of cells with a high rate of proliferation and altered genotype”

All these data indicate that an abnormal environment exists in colon cancer cells, and that this could affect cyclin E protein regulation. The abnormal cellular environment present in colon cancer cells showing CIN could explain the results seen in the Sw480 cell line. In these cells, characterised by a modal number of 119 chromosomes, cyclin E protein and mRNA values are relatively normal, indicating that the CIN pathway involves many different factors that in turn might be altered to give rise to colon cancer tumorigenesis and that concur to establish the cancer phenotype.

The overexpression of cyclin E could increase cell proliferation through forced entrance into S phase, and the increased proliferation could cause the altered segregation of the chromosomes, thereby promoting aneuploidy. Cyclin E overexpression could maintain the altered chromosome number through each cell division and select a population of cells with a high rate of proliferation and altered genotype.

Further studies aimed at better understanding of the CIN pathway are necessary to identify the correct targets for colon cancer treatment.

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REFERENCES

- 1 **MacLachlan TK**, Sang N, Giordano A. Cyclins, cyclin-dependent kinases and cdk inhibitors: implication in cell cycle control and cancer. *Crit Rev Eukaryot Gene Expr* 1995;**5**:127–56.
- 2 **Clurman BE**, Sheaff RJ, Thress K, et al. Turnover of cyclin E by the ubiquitin–proteasome pathway is regulated by cdk2 binding and cyclin phosphorylation. *Genes Dev* 1996;**11**:1464.
- 3 **Won K**, Reed S. Activation of cyclin E/cdk2 is coupled to site-specific autophosphorylation and ubiquitin-dependent degradation of cyclin E. *EMBO J* 1996;**15**:4182.
- 4 **Keyomarsi K**, Conte D, Toyofuku W, et al. Deregulation of cyclin E in breast cancer. *Oncogene* 1995;**11**:941–50.
- 5 **Keyomarsi K**, Herliczek TW. The role of cyclin E in cell proliferation, development and cancer. *Prog Cell Cycle Res* 1997;**3**:171–91.
- 6 **Dutta A**, Chandra R, Leiter L, et al. Cyclins as markers of tumor proliferation: immunocytochemical studies in breast cancer. *Proc Natl Acad Sci U S A* 1995;**92**:5386–90.
- 7 **Porter PL** et al. Expression of cell cycle regulators p27^{kip1} and cyclin E, alone and in combination, correlate with survival in young breast cancer patients. *Nat Med* 1997;**3**:222–5.
- 8 **Nielsen NH**, Arnerlov C, Cajander S, et al. Cyclin E expression and proliferation in breast cancer. *Anal Cell Pathol* 1998;**17**:177–88.
- 9 **Spruck CH**, Won K-A, Reed S. Deregulated cyclin E induces chromosome instability. *Nature* 1999;**401**:297–300.
- 10 **Lengauer C**, Kinzler KW, Vogelstein B. Genetic instability in colorectal cancers. *Nature* 1997;**386**:623–7.
- 11 **Dou Q-P**, Pardee AB, Keyomarsi K. Cyclin E: a better prognostic marker than cyclin D? *Nat Med* 1996;**2**:254.
- 12 **Erlanson M**, Portin C, Linderholm B, et al. Expression of cyclin E and the cyclin-dependent kinase inhibitor p27 in malignant lymphomas—prognostic implications. *Blood* 1998;**92**:770–7.
- 13 **Sakaguchi T**, Watanabe A, Sawada H, et al. Prognostic value of cyclin E and p53 expression in gastric carcinoma. *Cancer* 1998;**82**:1238–43.
- 14 **Bito T**, Ueda M, Ito A, et al. Less expression of cyclin E in cutaneous squamous cell carcinomas than in benign and premalignant keratinocytic lesions. *J Cutan Pathol* 1997;**24**:305–8.
- 15 **Aoyagi K**, Koufujii K, Yano S, et al. Immunohistochemical study on the expression of cyclin D1 and E in gastric cancer. *Kurume Med J* 2000;**47**:199–203.
- 16 **So JB**, Samarasinghe K, Raju GC, et al. Expression of cell-cycle regulators p27 and cyclin E correlates with survival in gastric carcinoma patients. *J Surg Res* 2000;**94**:56–60.
- 17 **Leach FS**, Elledge SJ, Sherr CJ, et al. Amplification of cyclin genes in colorectal carcinomas. *Cancer Res* 1993;**53**:1986–9.
- 18 **Kitahara K**, Yasui W, Kuniyasu H, et al. Concurrent amplification of cyclin E and CDK2 genes in colorectal carcinomas. *Int J Cancer* 1995;**62**:25–8.
- 19 **Wang A**, Yoshimi N, Suzui M, et al. Different expression patterns of cyclins A, D1 and E in human colorectal cancer. *J Cancer Res Clin Oncol* 1996;**122**:122–6.
- 20 **Sutter T**, Doi S, Carnevale KA, et al. Expression of cyclins D1 and E in human colon adenocarcinomas. *J Med* 1997;**28**:285–309.
- 21 **Rooney DE**, Czepulkowski BH. *Human cytogenetics: a practical approach*. Oxford: IRL Press, 1986.
- 22 **Chung DC**. The genetic basis of colorectal cancer: insights into critical pathways of tumorigenesis. *Gastroenterology* 2000;**119**:854–65.
- 23 **Beier R**, Burgin A, Kiermaier A, et al. Induction of cyclin E-cdk2 kinase activity, E2F-dependent transcription and cell growth by Myc are genetically separable events. *EMBO J* 2000;**19**:5813–23.
- 24 **Geng Y**, Eaton EN, Picon M, et al. Regulation of cyclin E transcription by E2Fs and retinoblastoma protein. *Oncogene* 1996;**12**:1173–80.
- 25 **Hatakeyama M**, Herrera R, Makela T, et al. The cancer cell and the cell cycle clock. *Cold Spring Harbor Symp Quant Biol* 1994;**59**:1.
- 26 **Howard CM**, Claudio PP, De Luca A, et al. Inducible pRb2/p130 expression and growth-suppressive mechanisms: evidence of a pRb2/p130, p27, and cyclin E negative feedback regulatory loop. *Cancer Res* 2000;**60**:2737–44.



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