



# NEOPLASIA

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**Proteomics Reveals an Association of Cytokeratins with Survival in Lung Cancer**

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**7q32-q33 Linked to Prostate Cancer Aggressiveness**

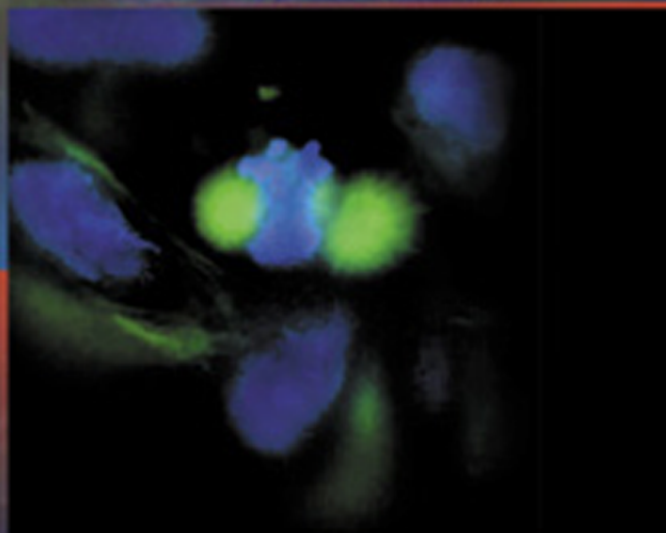
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**Pharmacokinetic Studies of BFA-Directed Cancer Therapy**

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**pRB: A Regulator of Cellular Ploidy**

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# Functional Inactivation of pRB Results in Aneuploid Mammalian Cells After Release From a Mitotic Block<sup>1</sup>

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## Abstract

The widespread chromosome instability observed in tumors and in early stage carcinomas suggests that aneuploidy could be a prerequisite for cellular transformation and tumor initiation. Defects in tumor suppressors and genes that are part of mitotic checkpoints are likely candidates for the aneuploid phenotype. By using flow cytometric, cytogenetic, and immunocytochemistry techniques we investigated whether pRB deficiency could drive perpetual aneuploidy in normal human and mouse fibroblasts after mitotic checkpoint challenge by microtubule-destabilizing drugs. Both mouse and human pRB-deficient primary fibroblasts resulted, upon release from a mitotic block, in proliferating aneuploid cells possessing supernumerary centrosomes. Aneuploid pRB-deficient cells show an elevated variation in chromosome numbers among cells of the same clone. In addition, these cells acquired the capability to grow in an anchorage-independent way at the same extent as tumor cells did suggesting aneuploidy as an initial mutational step in cell transformation. Normal Mouse Embryonic Fibroblasts (MEFs) harboring *LoxP* sites flanking exon 19 of the *Rb* gene arrested in G2/M with duplicated centrosomes after colcemid treatment. However, these cells escaped the arrest and became aneuploid upon pRB ablation by CRE recombinase, suggesting pRB as a major component of a checkpoint that controls cellular ploidy.

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**Keywords:** pRB, cell cycle control, CIN, centrosomes, aneuploidy.

## Introduction

Cancer cells are characterized not only by mutations in specific oncogenes and tumor-suppressor genes, but they also display gain and loss of chromosomes as well as alterations in chromosome number (aneuploidy) [1–4]. Chromosomal instability (CIN) [2] could account for the heterogeneity observed in individual tumors and could underlie the clonal differences seen in the same solid tumor. Recently, it has been suggested that CIN in cancer cells could be associated with mutations in mitotic checkpoint genes [5]. Near diploid tumor cells without the *hSecurin* gene, a component of the complex of proteins responsible for chromatid cohesion, became aneuploid over several ge-

nerations [6]. However, to date inactivation of *hSecurin* has not been observed in human cancers suggesting that altered expression of other genes must be responsible for the aneuploidy observed in cancer. Also, it has been shown that mammalian cells with reduced expression of MAD2 show CIN even though additional mutations are likely necessary for cellular transformation to occur readily [7,8].

An alternative view is that CIN is not generated by gene mutation/s; instead it could result from an initial abnormal chromosome complement within the cell [9]. This hypothesis comes from studies that showed that treatments with non-genotoxic drugs induce a near-complete aneuploidy [10].

Previously we showed that normal human fibroblasts lacking functional pRB re-replicate their DNA when challenged with antimicrotubule drugs [11]. These cells probably do not go through a “point of no return”; instead they decondense their chromosomes and return to a G2 phase [12,13] from which they can re-replicate their DNA. Here we show that transient polyploidization, by uncoupling DNA replication and cytokinesis, can trigger CIN both in human and murine pRB-deficient primary fibroblasts and that cells derived following colcemid treatment are capable of unanchored growth in soft agar to the same extent as tumor cells. By using mouse embryonic fibroblasts (MEFs) harboring *LoxP* sites flanking exon 19 of the *Rb* gene [14], we demonstrate a direct relationship between pRB ablation, centrosome duplication, and generation of aneuploid cells.

## Materials and Methods

### Cell Culture

Normal human fibroblasts IMR90 (diploid human embryonic lung fibroblasts, ATCC CCL-186), stably expressing a neomycin resistance gene (*IMR90neo*), or neomycin resistance and the gene encoding the human papillomavirus type

Abbreviations: MEFs, mouse embryonic fibroblasts; HPV16, human papillomavirus type 16; PI, propidium iodide; BrdU, bromodeoxyuridine; FISH, fluorescence *in situ* hybridization. Address all correspondence to: Dr. Aldo Di Leonardo, Department of Cell and Developmental Biology “A. Monroy,” University of Palermo, viale delle Scienze 90128, Palermo, Italy. E-mail: adileon@unipa.it

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16 (HPV16) E7 protein (IMR90E7) were obtained by retroviral gene transduction. Cells expressing E7 have nonfunctional RB protein. MEFs with conditional *Rb* alleles (*Rb*<sup>LoxP/LoxP</sup> MEFs) were kindly provided by Dr. Marco Crescenzi (Istituto Superiore di Sanità, Rome). *Rb*<sup>LoxP/LoxP</sup> MEFs were infected (MOI: 300, which ensures 90% to 95% of successful infected fibroblasts) with the empty vector J-pCA13 or with adenoviruses (AdCre) expressing the Cre recombinase under control of the human cytomegalovirus (hCMV) immediate-early promoter to generate MEFs devoid of pRB (*Rb*<sup>-/-</sup> MEFs). All adenoviruses used in this study were replication-deficient (E1 region deleted). Cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Euroclone, Milan, Italy).

#### Cell Cycle Analysis

Asynchronously growing cells were treated with 0.2  $\mu\text{g}/\text{ml}$  colcemid (Demecolcine, Sigma) for 72 hours and then released into complete medium without colcemid. DNA content was determined using PI staining alone by treating the cells with PBS containing 4  $\mu\text{g}/\text{ml}$  of PI and 40  $\mu\text{g}/\text{ml}$  RNase. BrdU incorporation (10  $\mu\text{M}$  for 4 hours) was used to determine DNA synthesis of the cells released in medium without colcemid. Analysis of BrdU-labeled cells was conducted as described previously [15] and samples were analyzed on a Becton Dickinson (NJ, USA) FACScan. Experiments were repeated twice; 5000 events were analyzed for each sample, and representative experiments are shown.

#### Fluorescence In Situ Hybridization

Centromeric probes specific for chromosome 2 and 6 (kindly provided by Dr. Mariano Rocchi, University of Bari, Italy) were used for interphase and metaphase fluorescence *in situ* hybridization (FISH). FISH was done following published procedures [15] with minor modifications. Briefly, after RNase and proteinase K treatment and DNA denaturation in 70% formamide/2 $\times$  SSC, cells were dehydrated in an ethanol series. Following overnight incubation at 37°C in a hybridization mixture consisting of 50% deionized formamide, 5% dextrane sulfate, 2 $\times$  SSC, 1% Tween 20, 100  $\mu\text{g}/\text{ml}$  salmon sperm DNA, and 100 ng biotinylated and digoxigenated pX2 (chr. 2) and pEDZ6 (chr. 6) probes, the slides were washed and blocked in 4 $\times$  SSC/0.5% nonfat dry milk. Slides were layered with FITC-conjugated avidin or antidigoxigenin rhodamine (Roche Molecular, Mannheim, Germany) in 4 $\times$  SSC/0.5% blocking reagent. Slides were stained with Hoechst 33258 (0.05  $\mu\text{g}/\text{ml}$ ), examined on a Zeiss (Gottingen, Germany) Axioskop microscope equipped for fluorescence, and the observed nuclei and metaphases were captured on a CCD camera (Zeiss). Images were transferred to Adobe PhotoShop for printing.

#### Determination of Ploidy

Asynchronous cells were treated with 0.2  $\mu\text{g}/\text{ml}$  colcemid (Demecolcine, Sigma) for 4 hours. Cells were harvested by trypsinization, swollen in 75 mM KCl at 37°C, fixed with 3:1

methanol:acetic acid (v:v), and dropped onto clean, ice-cold glass microscope slides. The slides were air dried and stained with 3% Giemsa in phosphate-buffered saline for 10 minutes. Chromosome numbers were evaluated using a Zeiss Axioskop microscope under a 100 $\times$  objective.

#### Immunofluorescence Microscopy

To visualize multipolar spindles, cells grown on rounded glass coverslips were treated with Taxol (Paclitaxel, Sigma) 1  $\mu\text{g}/\text{ml}$  for 2 hours and then fixed with 3.7% formaldehyde for 10 minutes at 37°C, permeabilized with 0.1% Triton X (Sigma) in PBS for 15 minutes, and blocked with 0.1% BSA for 30 minutes, both at room temperature. Coverslips were incubated with a mouse monoclonal antibody against  $\beta$ -tubulin (Sigma; diluted 1:200 in PBS) overnight at 4°C, followed by a goat anti-mouse IgG-FITC conjugated secondary antibody (Sigma; diluted 1:100 in PBS) for 1 hour at 37°C. For immunostaining of centrosomes of asynchronously grown cells, we used a mouse monoclonal antibody against  $\gamma$ -tubulin (Sigma; diluted 1:4000 in PBS) incubated overnight at 4°C. Cells were washed in PBS buffer and exposed to a FITC-conjugated goat anti-mouse IgG secondary antibody (Sigma; diluted 1:100 in PBS) for 1 hour at 37°C. Nuclei were visualized with Hoechst 33258 (0.05  $\mu\text{g}/\text{ml}$ ) and examined on a Zeiss Axioskop microscope equipped for fluorescence and images were captured with a CCD digital camera (Zeiss). Images were transferred to Adobe PhotoShop for printing.

#### Immunoblot Analyses

For immunoblot analyses, MEFs were lysed in SDS/PAGE sample buffer, protein extracts were resuspended in loading buffer (0.125 M Tris-HCl, 4% SDS, 20% v/v glycerol, 0.2 M dithiothreitol, 0.02% bromophenol blue, pH 6.8), and 60  $\mu\text{g}$  of protein (as determined by the Bradford assay) was loaded per lane on a 6% SDS PAGE gel. After gel electrophoresis proteins were electrotransferred onto a Hybond C membrane (Amersham Life Science, Milan, Italy). Electrobotted gel was stained with Coomassie blue and the membrane was reversibly stained with Ponceau S to verify equal loading and transfer. The membrane was blocked in 5% (w/v) nonfat milk in TBST buffer (10 mM Tris pH 8.0, 150 mM NaCl, 0.1% Tween 20) at room temperature and incubated overnight at 4°C with the anti-pRB primary antibody (C-15, Santa Cruz Biotechnology, Santa Cruz, CA) 1  $\mu\text{g}/\text{ml}$  in blocking solution. After three washes with TBST buffer, the blot was incubated in horseradish peroxidase-conjugated anti-goat IgG (Santa Cruz Biotechnology, diluted 1:5000) for 1 hour at room temperature and washed again three times with TBST buffer. Blot was developed with chemiluminescent reagents (SuperSignalWest Pico, Pierce Rockford, IL) and exposed to CL-Xposure film (Pierce, Rockford, IL) for 1 to 5 minutes.

#### Focus Assay

We have used a base layer (2 ml) composed of DMEM, 20% FBS, and 1% Noble agar. Noble agar was allowed to sit in six-well multidish plates of 35-mm diameter (Iwaki, Bibby

Sterlin Staffordshire, England, UK). This substrate was overlaid with 2 ml of a second layer of agar media (0.3% DMEM, 20% FBS) containing a suspension of 10,000 cells/dish and incubated at 37°C in a 5% CO<sub>2</sub> atmosphere. Foci were visible within 10 to 14 days.

## Results

### *pRB-Deficient Human Fibroblasts Become Aneuploid upon Release from Colcemid and Show Potential for Transformation*

Previously, we showed that pRB deficiency in human and mouse fibroblasts leads to increased ploidy in the presence of spindle inhibitors due to re-replication of most, if not all, of the genome without inducing DNA damage [11]. This initial abnormal chromosome complement could be the basis for generating CIN in human cells when associated with malfunction of a checkpoint controlling the correct ploidy or reduplicated centrosomes. To further examine this hypothesis, both normal (IMR90neo) and pRB-deficient (IMR90E7) human fibroblasts were treated with the anti-microtubule drug colcemid for 72 hours to induce transient polyploidization. The DNA content and distribution in E7-expressing cells showed, by FACScan analyses, that the majority of the cells accumulated with a 4 N (70%) and 8 N DNA content (12%) (Figure 1A). In contrast, there were no IMR90neo cells with an 8 N DNA content, and 36% of cells accumulated with a 4 N DNA content. Bivariate FACScan analyses (BrdU vs propidium iodide [PI] incorporation) of IMR90E7 cells colcemid treated for 72 hours and then released for 4 hours in complete medium without colcemid, showed that additional 12% of IMR90E7 cells (Figure 1B) were able to enter S phase with respect to those analyzed at 72 hours. The cell cycle profiles showed that these additional cells in S-phase (BrdU positive) originated from the cells with a 4 N DNA content that re-replicated their DNA (data not shown). On the contrary, IMR90neo cells treated and released in the same way did not show any BrdU incorporation at the indicated times.

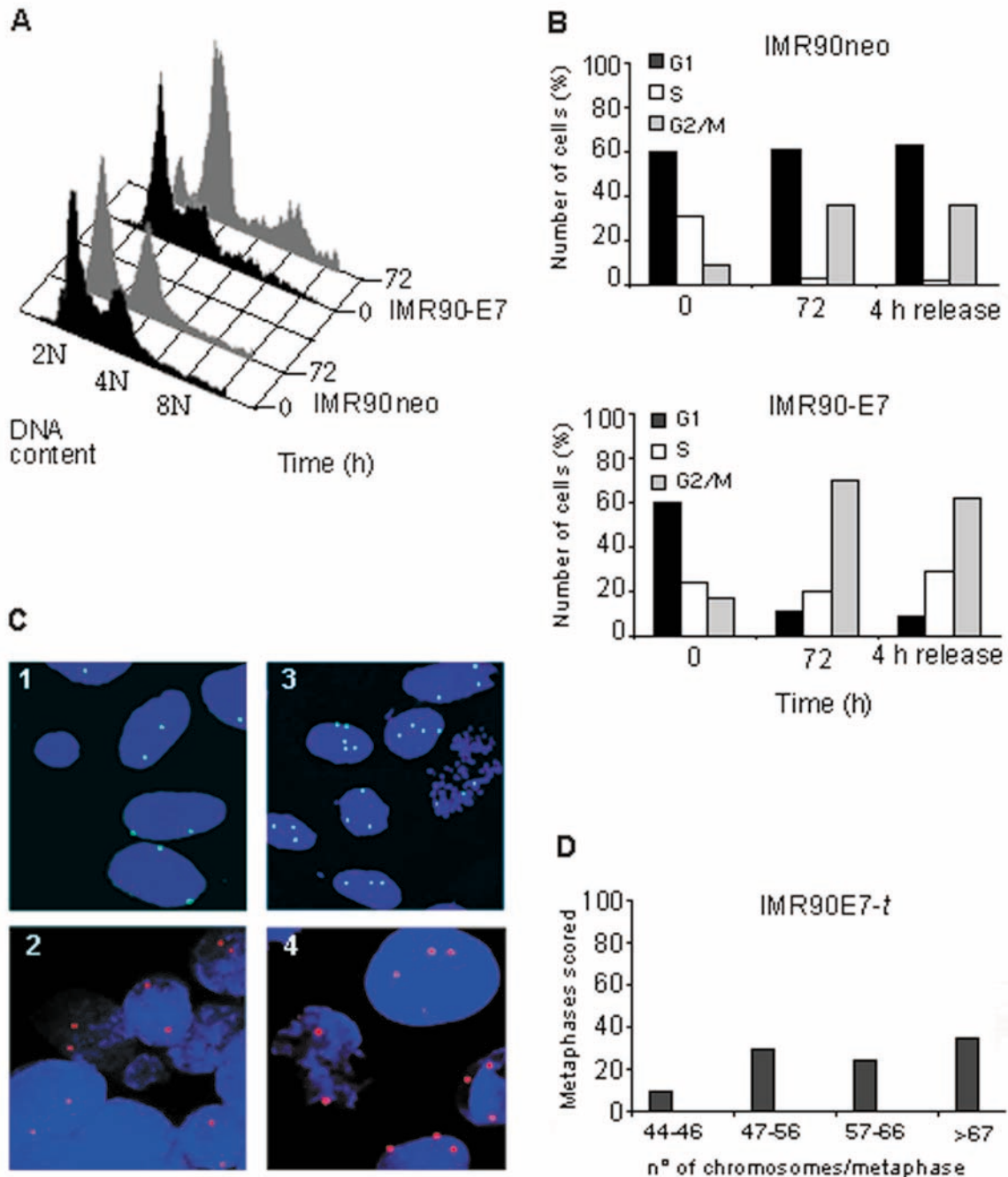
We next asked whether these treated cells (henceforth designated IMR90neo-*t* and IMR90E7-*t*) were capable of further growth when released into complete medium without the drug. During the first week after the release, we estimated that about 40% of IMR90E7-*t* cells died. However, in the second week of culture the remaining cells proliferated at the same rate as IMR90E7 cells (parental) when cultured in the absence of the mitotic inhibitor. In contrast, IMR90neo-*t* cells proliferated very slowly, becoming completely arrested within 2 weeks following the release. We did chromosome counts on 100 metaphase spreads of these IMR90E7-*t* cells to ascertain the ploidy status. Cytogenetic analysis showed more than 90% of the cells were aneuploid (Figure 1D) and contained different numbers of chromosomes per metaphase (range of chromosomes per metaphase 47 to 80). In contrast, all IMR90E7 and IMR90neo cells examined were diploid. Fluorescence *in situ* hybridization (FISH) with chromosome-specific anti-centromeric probes, both on

metaphases and nuclei, confirmed differences in chromosome numbers between untreated and treated E7-expressing cells (Figure 1C). We isolated several single-cell clones from the population of colcemid-released IMR90E7-*t* cells from two independent experiments, to determine ploidy in pRB-deficient cells in which DNA replication is uncoupled from cytokinesis. Clones were allowed to expand, and after 1 week of growth FACScan analyses showed that the DNA content for all of them was greater than that of the parental cells, indicating that these cells had acquired a perpetual aneuploidy (Figure 2A). Alteration of ploidy in these cells was confirmed by determining chromosome numbers (Figure 2B). Cytogenetic analyses of pRB-deficient cells released from the colcemid block and analyses of single-cell clones showed high variability in chromosome numbers from cell to cell suggesting random occurrence of chromosomal gain and loss. These observations were consistently confirmed during at least 3 months of cell culture. The observed heterogeneity in chromosome numbers in these pRB-deficient cells suggests that the initial trigger (transient polyploidy) results in chromosomal instability that allows aneuploidy cells to proliferate and perpetuate.

Aneuploidy in tumors has been associated with the presence of multipolar mitotic spindles strictly correlated with alterations in centrosome numbers. We examined the spindle organization in these growing aneuploid cells by  $\beta$ -tubulin staining (Figure 2C) and found multipolar spindles in all of the cells scored. Chromosomal instability (CIN) is a hallmark of cancer and accounts for the heterogeneity observed in tumor cells. However, the possibility that the acquired aneuploidy is an initial event in the transformation process is still debated. Therefore, we characterized the aneuploid IMR90E7-*t* population and the independently derived aneuploid cell clones for their potential for transformation. Both the IMR90E7-*t* population and cell clones grew in soft agar as discrete colonies indicating the capability of these cells to grow in an anchorage-independent manner. Estimation of the number of foci (Figure 2D) revealed that these cells have a potential for transformation comparable to that displayed from the tumor cells DLD1 used as a positive control. In contrast, wild-type IMR90neo and IMR90E7 cells failed to grow in soft agar (Figure 2D).

### *pRB Ablation Allows Growth of MEFs Arrested with Duplicated Centrosomes*

It has been shown that centrosome duplication requires both E2F and CDK2/cyclin A in Chinese hamster ovary cells [16], and that stable expression of HPV16-E7 oncoprotein in keratinocytes, but not in fibroblasts [17], causes induction of numerical centrosome abnormalities that could represent an early event during neoplastic progression potentially driving genomic destabilization [18]. However, HPV16-E7 oncoprotein causes inactivation of other cellular targets such as p107 and p130 pRB's family protein, p21<sup>WAF1-Cip1</sup> [19] and insulin-like growth factor binding protein 3 (IGFBP-3) [20]. Then, we determined whether the lack of pRB only is sufficient for allowing perpetual aneuploidy associated with centrosome reduplication in mammalian cells.

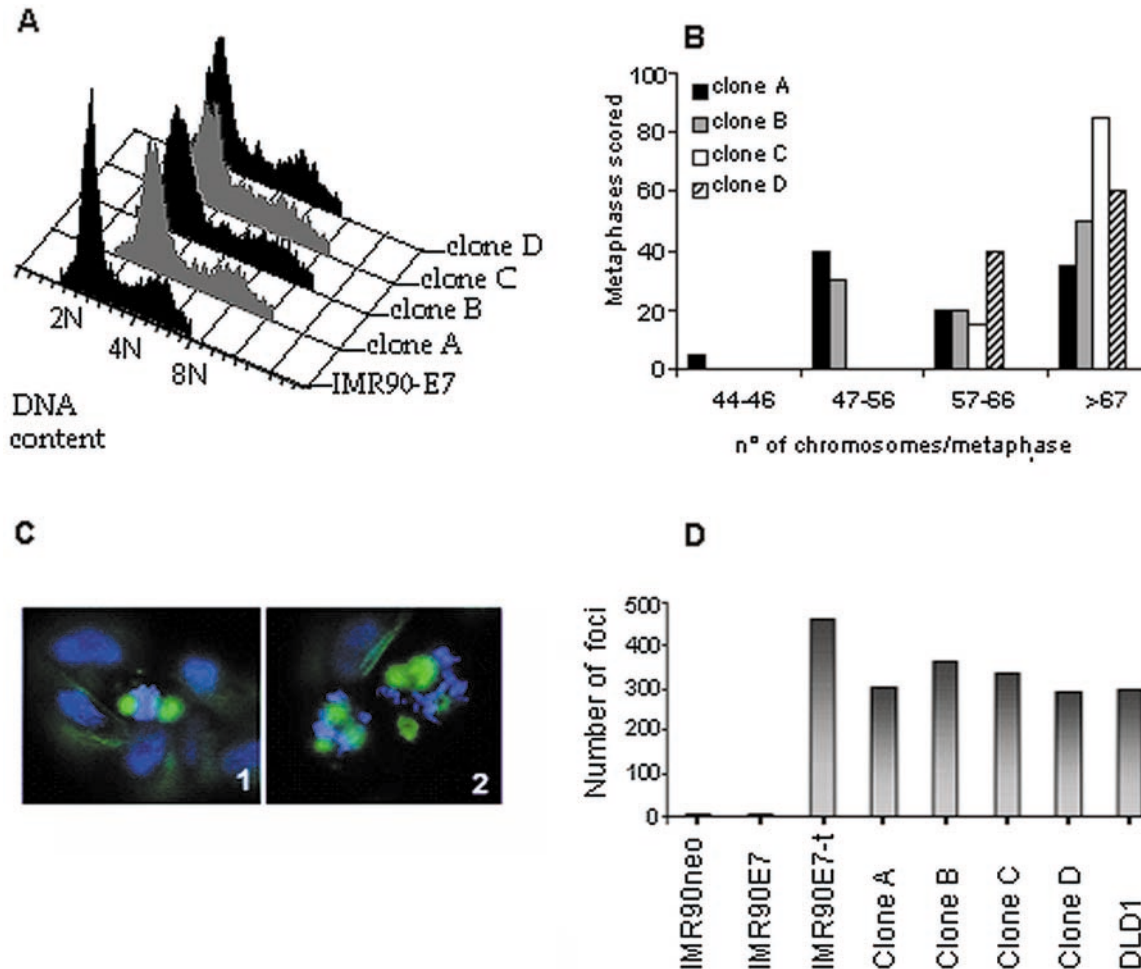


**Figure 1.** Effects of mitotic arrest on DNA replication and ploidy in IMR90neo and IMR90E7 human cells. (A) FACS profiles of PI-stained IMR90neo and IMR90E7 cells untreated or treated with colcemid (0.2  $\mu$ g/ml). (B) Histograms summarizing cell cycle analyses of asynchronous IMR90neo and IMR90E7 colcemid treated and released into medium without colcemid. (C) Detection of aneuploidy in IMR90E7 cells using centromeric probes to human chromosome 2 (green) and 6 (red). 1 and 2: IMR90E7; 3 and 4: IMR90E7 treated for 72 hours with colcemid and released into medium without colcemid. (D) Chromosome/metaphase numbers in IMR90E7 cells treated for 72 hours with colcemid and released for 2 weeks.

To this aim we used MEFs carrying conditional *Rb* alleles (*Rb*<sup>LoxP/LoxP</sup> MEFs) in which two LoxP sites are inserted into the introns surrounding exon 19 of the *Rb* gene [14]. We infected *Rb*<sup>LoxP/LoxP</sup> MEFs with adenoviruses expressing the Cre recombinase (AdCre) to excise the exon 19 and to generate cells with truncated pRB (*Rb*<sup>-/-</sup> MEFs), which are functionally equivalent to *Rb* null cells. *Rb*<sup>LoxP/LoxP</sup> MEFs were also infected with

adenoviruses that did not express the Cre transgene to generate isogenic murine cells (pRB proficient, indicated as *Rb*<sup>LoxP/LoxP</sup>) to be used as a control. Western blot experiments (Figure 3A) showed that as early as 96 hours *Rb*<sup>LoxP/LoxP</sup> MEFs infected with AdCre were completely devoid of pRB when compared to the same cells infected with adenoviruses that did not express the Cre transgene. Infected cells were allowed to expand and 2 weeks after





**Figure 2.** Cell cycle analyses and features of IMR90E7-t derived clones. (A) FACS profiles of asynchronous PI-stained IMR90E7 and IMR90E7-t derived clones showing the presence of hyperdiploidy in all the clones isolated. (B) Chromosome/metaphase numbers in IMR90E7-t derived clones displaying the presence of aneuploid metaphases. (C) Presence of multipolar spindles in the clone A. The spindle was visualized by immunofluorescence staining for  $\beta$ -tubulin, and nuclei were visualized by Hoechst 33258. (1) Control metaphase (IMR90E7); (2) metaphases of clone A displaying asymmetrical distribution of the condensed chromosomes. (D) Numbers of foci scored after 2 weeks growth in 0.3% agar supplemented DMEM for IMR90E7-t cells and the derived clones: A-B-C-D; DLD1: colon cancer cells used as a positive control.

they were colcemid treated. We determined by FACScan analysis the DNA content of both  $Rb^{LoxP/LoxP}$  and  $Rb^{-/-}$  MEFs after exposure to colcemid. Figure 3B shows typical flow cytometry results at 0 and 72 hours. Colcemid-treated (time 72 hours)  $Rb^{LoxP/LoxP}$  MEFs showed increase in 4 N cells but not in 8 N cells when compared to untreated (time 0 hours)  $Rb^{LoxP/LoxP}$  MEFs that showed the majority of the cells with a 2 N DNA content. As expected PI analyses revealed that untreated  $Rb^{-/-}$  MEFs were predominantly

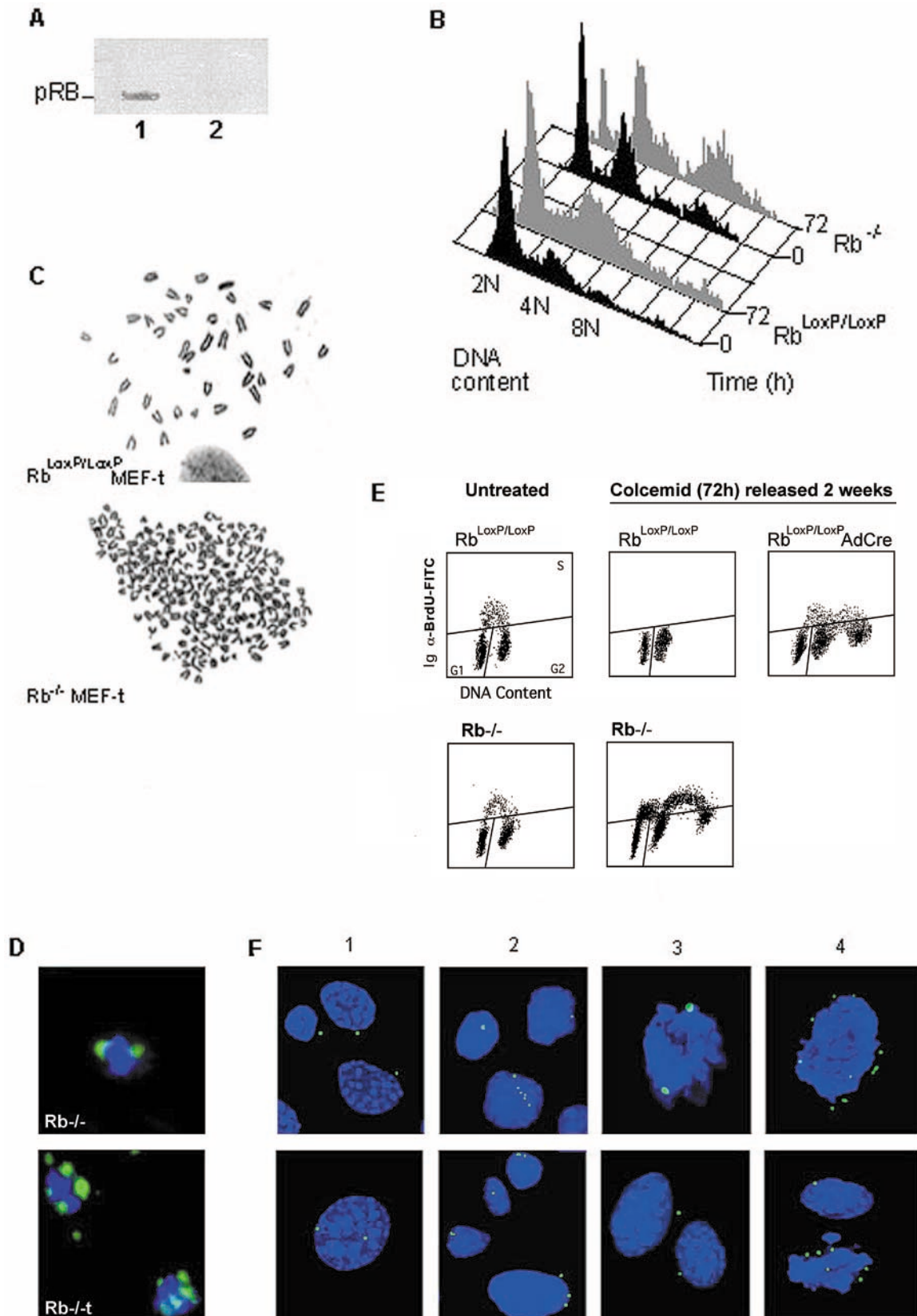
2 N or 4 N with very few >4 N cells. In contrast the majority of  $Rb^{-/-}$  MEFs treated with colcemid accumulated with a 4 N and >4 N of DNA content. Additional cytogenetic analyses showed that more than 50% of these colcemid-treated  $Rb^{-/-}$  MEFs were polyploid (Figure 3C). Evaluation of centrosome numbers, by  $\gamma$ -tubulin detection, in these colcemid-treated  $Rb^{-/-}$  MEFs revealed that 42% of cells had more than two centrosomes, 46% had two centrosomes, and only 12% had one centrosome. This indicates that DNA

**Figure 3.** Effect of mitotic arrest on DNA replication and ploidy in  $Rb^{loxP/loxP}$  and  $Rb^{-/-}$  MEFs. (A) Western blot analysis showing the level of pRB expression after 96 hours from the adenoviral infection: (lane 1): pRB<sup>loxP/loxP</sup> MEF infected with the empty vector and (lane 2) with the adenovirus expressing the Cre recombinase. Equal loading of proteins on the blot was evaluated by Ponceau S staining, Western blot was probed with a monoclonal anti pRB antibody raised against the carboxy terminus of the RB protein. (B) Cellular ploidy as revealed by FACS profiles of PI-stained  $Rb^{loxP/loxP}$  and  $Rb^{-/-}$  MEFs untreated or treated with colcemid (0.2  $\mu$ g/ml). (C) Metaphase analysis showing the presence of aneuploid metaphase spreads in  $Rb^{-/-}$  MEF-t at 2 weeks from the release. (D) Visualization of the spindle poles by  $\beta$ -tubulin staining in untreated  $Rb^{-/-}$  MEFs and in  $Rb^{-/-}$  MEF-t released for 2 weeks. (E) Bivariate FACS profiles (BrdU vs PI) of  $Rb^{loxP/loxP}$  and  $Rb^{-/-}$  MEFs untreated, treated with colcemid (72 hours) and released for 2 weeks, and of  $Rb^{loxP/loxP}$  MEFs released from colcemid arrest and infected with AdCre. These last cells were analyzed after 144 hours from infection. (F) Centrosome amplification in MEFs released from the colcemid block detected after staining with an antibody directed against  $\gamma$ -tubulin. Top and bottom panels refer to different microscopy fields of the same cell type. Control  $Rb^{loxP/loxP}$  MEFs-t (1) released from colcemid, show one or two centrosomes; the same result was observed in untreated  $Rb^{-/-}$  MEFs (3). Variation in centrosome numbers was observed in  $Rb^{loxP/loxP}$  MEF-t+AdCre (2) and in  $Rb^{-/-}$  MEF-t (4).

re-replication, because of lack of pRB, is accompanied by centrosome reduplication.

Then, we examined the fate of the treated cells by releasing both  $Rb^{LoxP/LoxP}$  and  $Rb^{-/-}$  MEFs (henceforth

indicated as  $Rb^{LoxP/LoxP}$  MEFs-*t* and  $Rb^{-/-}$  MEFs-*t*) from the colcemid block. We observed that  $Rb^{LoxP/LoxP}$  MEFs-*t* remained arrested for at least 2 weeks. In contrast,  $Rb^{-/-}$  MEFs-*t* proliferated as did IMR90E7-*t* cells



**Table 1.** Centrosome Analysis in Mouse Embryonic Fibroblasts.

Cells	% 1 centrosome	% 2 centrosomes	% >2 centrosomes
<i>Rb</i> <sup>LoxP/LoxP</sup> MEF	80	20	–
<i>Rb</i> <sup>LoxP/LoxP</sup> MEF - <i>t</i>	58	37	5
<i>Rb</i> <sup>LoxP/LoxP</sup> MEF - <i>t</i> +AdCre	25	55	20
<i>Rb</i> <sup>-/-</sup> MEF	79	21	–
<i>Rb</i> <sup>-/-</sup> MEF - <i>t</i>	20	64	16

*t*: cells treated with colcemid for 72 hours and then released for 2 weeks.  
*t*+AdCre: cells treated with colcemid for 72 hours released for 2 weeks and then infected with the adenovirus AdCre.

(Figure 3E). Cytogenetic analyses performed 4 weeks after the release from the block imposed by colcemid showed that the great majority of the *Rb*<sup>-/-</sup> MEFs-*t* were aneuploid (75%) suggesting that lack of pRB allowed aneuploid cells to proliferate. The staining of these cells for  $\beta$ -tubulin revealed the presence of high numbers of tripolar and multipolar spindles (Figure 3D). To examine whether *Rb*<sup>LoxP/LoxP</sup> MEFs-*t* cells, which experienced a prolonged if not permanent arrest upon colcemid treatment, were able to escape the arrest following pRB ablation, they were infected with the adenovirus AdCre to excise by recombination exon 19 of the *Rb* gene and thus inactivate it. These cells (indicated as *Rb*<sup>LoxP/LoxP</sup> MEFs-*t*+AdCre) escaped the arrest and started to proliferate similarly to *Rb*<sup>-/-</sup> MEFs-*t* (Figure 3E). Cytogenetic analyses revealed that more than 50% of these cells become aneuploid. In addition, centrosome analysis (Figure 3F, Table 1), at 1 week after the infection, by  $\gamma$ -tubulin staining of *Rb*<sup>LoxP/LoxP</sup> MEFs-*t*+AdCre cells showed an increase in cells harboring two centrosomes (55%) and more than two centrosomes (20%, range 3 to 8), and only 25% of the cells with one centrosome. On the contrary, *Rb*<sup>LoxP/LoxP</sup> MEFs-*t* cells showed that 58% and 37% of the cells had one or two centrosomes, respectively, as expected whether treated cells are arrested in G1 and G2/M, and only 5% of *Rb*<sup>LoxP/LoxP</sup> MEFs-*t* cells had more than two centrosomes. Both untreated *Rb*<sup>LoxP/LoxP</sup> and *Rb*<sup>-/-</sup> MEFs showed a similar percentage of cells with one or two centrosomes. The increasing number of cells harboring supernumerary centrosomes in *Rb*<sup>LoxP/LoxP</sup> MEFs-*t*+AdCre suggests that pRB ablation allow cells arrested with at least two centrosomes and with altered ploidy to escape the arrest.

## Discussion

A current paradigm in cancer research states that cancer originates from a single founder cell in which mutations in few crucial genes have occurred. However, this paradigm does not seem to be in accordance with either epidemiological data or with multiple mutations and chromosomal aberrations, both structural and numerical, which are found in the majority of tumors. These chromosomal alterations are believed to destabilize the genome and further increase the occurrence of new changes [9]. Aneuploidy observed in the majority of tumors and in early stage carcinomas strongly suggests that it could be considered a prerequisite in

initiation as well as in tumor progression [2,3]. The occurrence of transient polyploidization caused by DNA re-replication [11] could be considered a trigger for generating CIN and perpetual aneuploidy in human cells [10]. Our findings both in human and murine cells suggest that pRB could be a major component of a pathway that controls ploidy when the mitotic checkpoint is activated by antimicrotubule drugs. Our results, showing that pRB-deficient cells are able to tolerate aneuploidy and in turn acquire the ability to grow in an anchorage-independent way, support the hypothesis that aneuploidy may be a driving force in tumorigenesis rather than a side effect of the transformation process driven by mutations in oncogenes and tumor-suppressor genes [21]. Although changes in chromosome numbers might be caused by alterations in specific genes of the mitotic checkpoint [6,8,22], our results suggest that also pRB is playing an important role in this process. In fact, when the mitotic checkpoint is challenged, pRB deficiency predisposes cells to aneuploidy by DNA re-replication [11], and the concomitant centrosome reduplication is necessary to maintain and perpetuate the resulting aneuploid cells. In addition, these findings suggest that lack of pRB is necessary to allow aneuploid cells to proliferate despite the presence of supernumerary centrosomes, which are thought to cause mitotic failure, producing nonviable daughter cells [23]. Mammalian cells that do not have mitotic checkpoints defects, when exposed to spindle inhibitors for a long time, eventually adapt and exit mitosis, escaping the inhibitor-induced arrest. Recently, it has been shown that p53 competent cells exposed to antimicrotubule drugs, after adaptation, arrest in a G1-like cell cycle phase [24]. Surprisingly, p53-deficient human fibroblasts (expressing the HPV-16 E6-oncoprotein) that showed polyploidy after 72 hours of colcemid exposure, arrested with the subsequent passages in culture when released in medium without colcemid (L.L. and A.D.L., unpublished observations). These findings suggest the existence of a pathway dependent on pRB, likely activated in normal diploid fibroblast adapted cells, which is able to monitor alterations in cellular ploidy or centrosome duplication, thereby inducing a prolonged cell cycle arrest.

Many reports have suggested the existence of a relationship between reduplicated centrosomes and the presence of gross aneuploidy. However, it is still debated whether centrosome alterations are a cause of genomic instability or a consequence of alterations in the cell cycle of cancer cells [25]. The ability of both HPV-16 E7 oncoprotein and E2F together with CDK2/cyclin A to induce centrosome duplication in mammalian cells [16,17] suggests that abrogation of pRB functions may have similar effects on centrosome homeostasis that could drive subsequent aneuploidy in tumor cells. Our findings that in proliferating human fibroblasts lack of pRB, caused by HPV16-E7 oncoprotein expression, did not affect centrosome number differently from that previously observed in keratinocytes [17] likely reflects differences in cell types used. This suggests that pRB dysfunction does not have any apparent deleterious effect on centrosomes in asynchronously



growing fibroblasts. However, our results that *Rb<sup>LoxP/LoxP</sup>* MEFs-*t* arrested for 2 weeks with a 4 N DNA content and duplicated centrosomes were able to escape the arrest only after pRB ablation, suggest the existence of a checkpoint, in which pRB plays a central role, which monitors centrosome numbers and when activated arrests cells in a G1-like cell cycle phase.

Finally, our results support the following scenario in mammalian cells. Duplicated centrosomes reduplicate in mitotic arrested cells whether a new round of DNA synthesis occurs because of pRB dysfunction. When the mitotic block is over, the checkpoint is activated by the presence of supernumerary centrosomes and the signal transmitted to pRB, which halts further cell cycle progression. On the contrary pRB-deficient cells could progress in the cell cycle and undergo cytokinesis with more than one centrosome inherited by each daughter cell. Then, some of the aneuploid pRB-deficient cells are capable to proliferate and perpetuate both the CIN phenotype and centrosome hypertrophy.

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