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# Centrosome amplification induced by hydroxyurea leads to aneuploidy in pRB deficient human and mouse fibroblasts

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**Abstract**

Alterations in the number and/or morphology of centrosomes are frequently observed in human tumours. However, it is still debated if a direct link between supernumerary centrosomes and tumorigenesis exists and if centrosome amplification could directly cause aneuploidy. Here, we report that hydroxyurea treatment induced centrosome amplification in both human fibroblasts expressing the HPV16 -E6-E7 oncoproteins, which act principally by targeting p53 and pRB, respectively, and in conditional pRB deficient mouse fibroblasts. Following hydroxyurea removal both normal and p53 deficient human fibroblasts arrested. On the contrary pRB deficient fibroblasts entered the cell cycle generating aneuploid cells. Also the majority of conditional Rb deficient MEFs showed supernumerary centrosomes and aneuploid cells which increased over time. Finally, our results suggest that pRB dysfunction both in human and murine fibroblasts transiently arrested in G1/S by hydroxyurea allows centrosomes amplification, in the absence of DNA synthesis, that in turn could drive aneuploidy.


*Keywords:* pRB; Centrosome amplification; CIN; Aneuploidy

## 1. Introduction

Centrosomes are the microtubule-organizing centre of animal cells, that helps assembling of the mitotic spindle necessary for the correct segregation of chromosomes. Generally, the number of centrosomes determines the number of mitotic spindle poles so that the two centrosomes normally present in a cell organize a bipolar spindle. Conversely, supernumerary centrosomes could result in the formation of a multipolar spindle leading to chromosome missegregation. Centrosome amplification is observed in the majority of solid tumors analyzed so far and it is believed an early feature of tumor initiation/progression. A growing number of reports indicate that chromosome instability (CIN) parallels the degree of centrosome alterations in tumors of different origins as well as in derived cell lines [1]. Although CIN might be caused by mutations in genes involved in the mitotic checkpoint [2,3,4], our previous results [5] suggested that dysfunction of the Retinoblastoma protein (pRB) could play an important role in this process. In fact, by allowing DNA re-replication and centrosome

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re-duplication, pRB dysfunction enabled aneuploid cells to proliferate despite the presence of supernumerary centrosomes. CIN could be a phenomenon consisting in a two stages process, where centrosome amplification would lead to aberrant spindles and catastrophic variations in chromosome number. Most of the progeny of cells with dysfunctional spindles would die, leaving an occasional progenitor with just the right combination of chromosomes to transform into a tumour cell [6]. Even though many reports suggested the existence of a relationship between reduplicated centrosomes and aneuploidy, is still debated if centrosome defects could lead directly to chromosome instability. To this aim we analyzed the effects of the presence of multiple centrosomes in human fibroblasts both wild type and expressing the HPV16-E6-E7 oncoproteins as well as in conditional pRB deficient mouse fibroblasts. Here, we show that deregulation of the centrosome duplication cycle by hydroxyurea (HU) in pRB deficient primary human fibroblasts as well as in pRb null MEFs resulted in centrosome amplification that in turn drove aneuploidy.

## 2. Materials and methods

### 2.1. Cells and cell culture

Normal human fibroblasts IMR90 (diploid human embryonic lung fibroblasts, A.T.C.C. CCL-186), stably expressing a neomycin resistance gene (IMR90), or neomycin resistance and the gene encoding the HPV16-E7 protein (IMR90E7) or the HPV16-E6 protein (IMR90E6) were generated by retroviral gene transduction. Cells were at passage 8 when infected and they were used within 4–6 passages from the infection. Mouse Embryonic Fibroblasts with conditional Rb alleles ($Rb^{LoxP/LoxP}$ MEFs) were infected (MOI: 300, that ensures 90–95% of successful infected fibroblasts) with the empty vector J-pCA13 or with adenoviruses (Ad-Cre) expressing the Cre recombinase under control of the human cytomegalovirus (hCMV) immediate-early promoter to generate MEFs devoid of pRB ($Rb^{-/-}$MEFs). All adenoviruses used in this study were replication-deficient (E1 region deleted). Cells were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin and 0.1 mg/ml streptomycin (Euroclone Ltd, UK).

### 2.2. Cell cycle analysis

Asynchronously growing cells, $10^6$cells/100 mm dish, were treated with 2 mM hydroxyurea (HU) for 48 h and then released into complete medium without HU. To monitor cells actively engaged in DNA synthesis they were labelled with BromodeoxyUridine (BrdU, 10 μM for 2 h), then harvested and stained with an anti-BrdU antibody. DNA content was determined by Propidium Iodide (PI) staining. Analysis of BrdU labelled cells was conducted as described previously [5] and samples were analyzed on a Beckman Coulter Epics-XL. Experiments were repeated at least twice with similar results. For each sample 10,000 events were analyzed by EXPO32 software and representative experiments are shown.

### 2.3. Immunoblot analysis

For immunoblot analyses cells 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 50 mg of protein (as determined by the Bradford assay) was loaded per lane on a SDS PAGE gel. After gel electrophoresis proteins were electrotransferred onto Immobilon-PVDF membrane (Millipore) blocked in 5% (w/v) no-fat milk in TBST buffer (10 mM Tris pH8.0,150 mM NaCl, 0.1% Tween 20) at room temperature and incubated overnight at 4 °C with the primary antibody. After three washes with TBST buffer the blot was incubated in horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, diluted 1:2000) for 1 h RT. To detect amount of E7 oncoprotein, p53 and pRb proteins in human fibroblasts blots were probed with mouse monoclonal antibodies HPV16-E7 (sc-6981, Santa Cruz), p53 (sc-126, Santa Cruz) and pRB (554136, Becton Dickinson), respectively. MEFs' western blot was probed with a mouse monoclonal anti pRB antibody (554136, Becton Dickinson). Equal loading of proteins was evaluated by probing the blot with a mouse monoclonal antibody β-actin (A53-16 Sigma).

Blots were developed with chemiluminescent reagent (SuperSignalWest Pico, Pierce Rockford, IL) and exposed to CL-Xposure film (Pierce Rockford, IL) for 1–5 min.

### 2.4. Determination of ploidy

Asynchronous cells were treated with 0.2 mg/ml colcemid (Demecolcine, Sigma, St Louis, MO) for 4 h. 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 min. Chromosome numbers were evaluated using a Zeiss Axioskop microscope under a 100× objective. At least 50 metaphases were analyzed at each time point.

### 2.5. Immunofluorescence microscopy

To detect centrosomes cells ($4\times10^4$) left untreated or treated with HU for 48 h were grown on glass coverslips, fixed in methanol/acetone at −20 °C, permeabilized with 0.1% Triton X (Sigma, St Louis, MO) and blocked with 0.1% BSA both at room temperature. Then, coverslips were incubated with a mouse monoclonal antibody against γ-tubulin (Sigma, diluted 1:250 in PBS) overnight at 4 °C, washed in PBS and incubated with a FITC-conjugated goat anti-mouse IgG secondary antibody (Sigma, diluted 1:100 in PBS) for 1 h at 37 °C. Nuclei were visualized with 4′,6-Diamidino-2-phenylindole (DAPI) and examined on a Zeiss Axioskop microscope equipped for fluorescence, images were captured with a CCD digital camera (Axiocam, Zeiss) and then transferred to Adobe PhotoShop for printing.

## 3. Results

### 3.1. Centrosome reduplication occurs and drives aneuploidy in pRB deficient human fibroblasts

In the attempt to discriminate between centrosome amplification and ploidy alteration (transient tetraploidy) as the trigger for CIN, we investigated whether after treatment with hydroxyurea (HU), an inhibitor of ribonucleotide reductase activity that accumulate cells at the G1/S border, centrosomes amplification occurs both in human and murine fibroblasts as observed in rodent and human tumor cell lines [7,8]. To this aim we used both normal and p53- and pRB-compromised human fibroblasts, because of the presence of the Human Papilloma Virus type 16 (HPV16)-E6 and -E7 oncoproteins, that are potent viral oncoproteins extensively used to study defects in the pRb and p53 pathways. Centrosome numbers were detected by γ-tubulin staining in normal and p53-, pRB- deficient human fibroblasts, both untreated and HU treated, growing onto coverslips. Immunocytochemistry and fluorescence microscopy revealed that untreated cells showed a normal number of centrosomes (Fig. 1a,c). Our finding that in proliferating human fibroblasts lack of pRB, caused by HPV16-E7 oncoprotein expression, did not affect centrosome number differently of what previously observed in human keratinocytes [9] at a first glance appearing unusual, could be related to cell type specific differences. In fact, primary fibroblasts could respond differently to the presence of the HPV oncoproteins in comparison to keratinocytes, which are the natural target of HPV lesions. Even though fibroblasts stably expressing the HPV16-E7 oncoprotein could increase S phase entry this could not cause centrosome amplification as long as the two processes, DNA and centrosome duplication, remain coupled. Then, fibroblasts stably expressing the HPV oncoproteins could be more refractory to re-duplicate centrosomes, though they could be able under some circumstances to accumulate supernumerary centrosomes. Following HU treatment (Fig. 1a,c) control cells (IMR90) accumulated with duplicated centrosomes and did not show cells with more than two centrosomes. On the contrary, supernumerary centrosomes were present in both E7 (IMR90E7) and E6 (IMR90E6) expressing cells (Fig. 1a,c). Both IMR90E6 and IMR90E7 fibroblasts showed (Fig. 1c) a marked reduction of cells with one centrosome (23 and 28%, respectively) and an increase in cells harbouring two (60 and 43%, respectively) or more than two centrosomes (17 and 29%, respectively) suggesting that these cells are lacking of a putative surveillance system monitoring the occurrence of centrosome amplification. Conversely, the fact that IMR90 cells showed only two

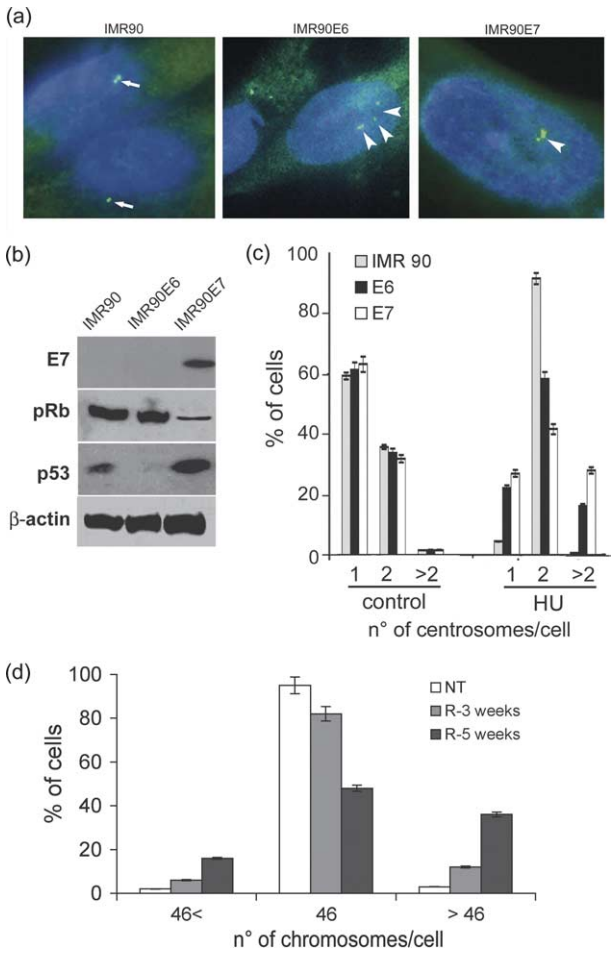


Fig. 1. Centrosome amplification and aneuploidy in human fibroblasts after HU treatment. (a) Centrosomes were revealed by γ-tubulin detection (green) and nuclei were stained with DAPI (blue). After HU treatment the majority of IMR90 fibroblasts showed two centrosomes (arrows), on the contrary both IMR90E6 and IMR90E7 fibroblasts showed the presence of supernumerary centrosomes (arrowheads). (b) Western-blot analysis showing decreased levels of p53 and pRB in IMR90E6 and IMR90E7 cells (expressing HPV16-E6 and -E7, respectively) and detection of HPV-16 E7 protein in IMR90 cells stably expressing this protein. (c) Histograms summarizing the percentage of cells with one, two or more than two centrosomes. An average of 100 cells were analyzed. Each bar indicates mean + SE of at least three independent experiments. (d) Histograms showing percentage of cells with diploid (46 chromosomes), hypodiploid (<46 chromosomes) and hyperdiploid (>46 chromosomes) metaphases. An average of 50 metaphases were analyzed for each time point, each bar represents mean + SE of at least two experiments.

centrosomes after HU exposure suggests that this putative control is fully working in normal human fibroblasts. Evaluation of centrosome amplification in IMR90E7 cells at 3 weeks from the HU treatment showed a similar number of cells (30%) harbouring supernumerary centrosomes. This finding suggests that lack of pRB made fibroblasts more permissive to the presence of centrosome abnormalities. As expected western-blot analysis (Fig. 1b) showed in IMR90E7 cells a large reduction of the amount of pRB correlated with the presence of the HPV-E7 oncoprotein that did not affect p53 expression, and the absence of p53 in IMR90E6 cells. Even though HU treated IMR90E7 and IMR90E6 human fibroblasts (Fig. 1c) harboured multiple centrosomes indicating that both oncoproteins are able to disrupt centrosome homeostasis [9], only pRB deficient human fibroblasts were able to proliferate despite of the presence of an

excess of centrosomes. In particular, normal fibroblasts underwent a senescent—like arrest as suggested by their flattened morphology, while p53 deficient partially re-entered the cycle and then arrested within 1 week from the release. Frequently, multiple centrosomes are observed in the majority of solid tumours analyzed so far and they are believed to lead to aneuploidy in tumour cells. To investigate whether the presence of multiple centrosomes in IMR90E7 cells was associated with aneuploidy we analyzed mitoses 3 and 5 weeks after HU treatment. Cytogenetics analyses revealed that near 20% of these cells became aneuploid (Fig. 1d), hypodiploid (range 32–44 chromosomes) as well as hyperdiploid (range 48–88 chromosomes), as soon as 3 weeks from the release in normal medium and the percentage of aneuploid cells increased to more than 50% as shown by 5 weeks after HU treatment (Fig. 1d).

### 3.2. Cell cycle distribution of hydroxyurea treated IMR90, IMR90E7 and IMR90E6 cells

It was showed [10] that wt-REF52 cells as well as their derivatives expressing the L-Tag of SV40 arrested in G1/S after HU or Aphidicolin exposure, suggesting that neither p53 nor pRB are directly involved in the induced arrest. Aphidicolin induced G1/S arrest depended on inhibition of DNA polymerase $\alpha$ and it was linked to loss of MCM proteins from the chromatin. MCM proteins are cell cycle regulated in that they are put in place in G1 and are displaced from chromatin in G2 to inhibit DNA re-replication. Also correct centrosome duplication is important for the right coordination of cell cycle progression. So it is conceivable that already duplicated centrosomes (typical of G2 cells) in cells blocked at the G1/S border might be a signal that does not permit S-phase entry so prolonging the G1 arrest. To assess the cell cycle effects of the presence of supernumerary centrosomes after HU treatment flow cytometry analyses were done in IMR90, IMR90E7 and IMR90E6 cells. Flow cytometry showed that after exposure to HU all cell types accumulated in G1/S (Fig. 2). After the release from HU the majority of both wild-type and E6 expressing cells remained arrested in the G1 phase of the cell cycle and were unable to proliferate further. On the contrary, a higher amount of pRB deficient (E7 expressing) than wild-type and E6 expressing cells (20 versus 6 and 9%, respectively) were able to enter the cell cycle and to proliferate further upon release from the block induced by HU.

### 3.3. Centrosome amplification occurs and drives aneuploidy in pRB deficient murine fibroblasts

It is well-known that HPV16-E7 oncoprotein binds and destabilize pRB. However, it could interfere also with the pRB related pocket proteins p107 and p130 as well as $p21^{WAF1}$ and $p27^{KIP1}$.To assess that the results we obtained in human fibroblasts (amplified centrosomes and aneuploidy) are dependent mainly on pRB inactivation, we shifted to a mouse system. We used Mouse Embryonic Fibroblasts (MEFs) carrying conditional Rb alleles ($Rb^{LoxP/LoxP}$) in which two LoxP sites are inserted into the introns surrounding exon 19 of the *Rb* gene [11] We infected these MEFs with adenoviruses expressing the Cre recombinase (Ad-Cre) in order to generate cells with truncated pRB ($Rb^{-/-}$), by excision of exon 19, that are functionally equivalent to *Rb null* cells. As a control we infected $Rb^{LoxP/LoxP}$ MEFs with adenoviruses that did not express the Cre transgene (pRB proficient, indicated as $Ad\text{-}Rb^{LoxP/LoxP}$ MEFs). Western-blot experiments (Fig. 3a) showed that $Rb^{LoxP/LoxP}$ MEFs infected with Ad-Cre were completely devoid of pRB when compared to uninfected

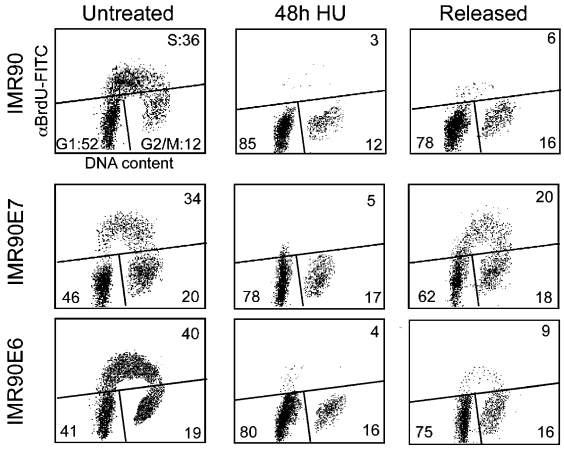


Fig. 2. Cell cycle distribution after HU treatment in primary human fibroblasts. Representative FACS profiles of IMR90, IMR90E7 and IMR90E6 fibroblasts untreated, HU treated or released for 24 h are shown.

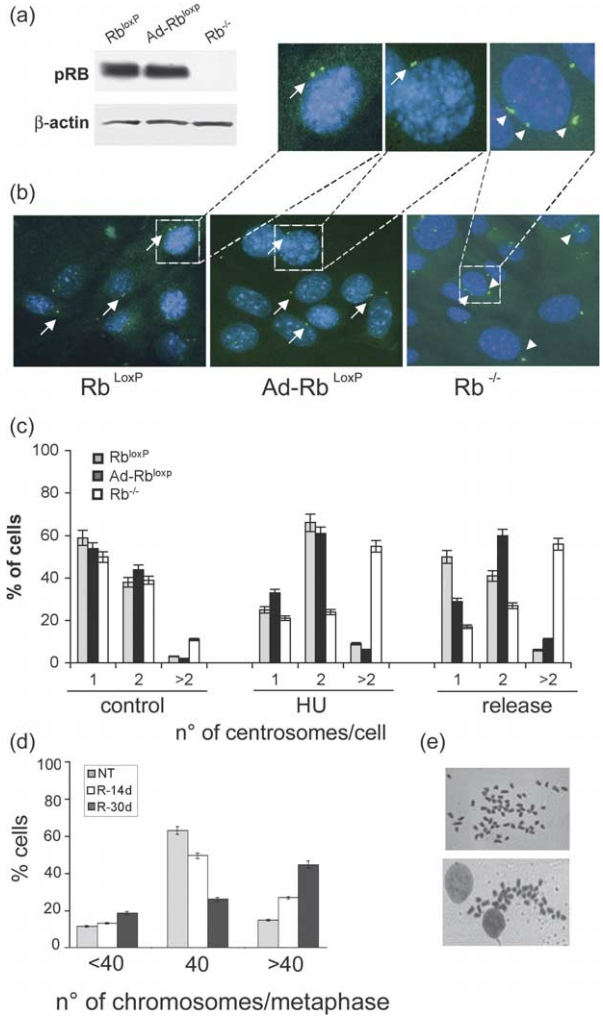


Fig. 3. Centrosome amplification and aneuploidy in pRB deficient MEFs following HU treatment. (a) Western blot analysis showing the level of pRB expression after 96 h from the adenoviral infection: $pRb^{loxP/loxP}$ MEF uninfected (lane 1), infected with the empty adenoviral vector (lane 2) and with the Ad-Cre (lane 3). (b) Centrosomes were revealed by γ-tubulin detection (green) and nuclei were stained with DAPI (blue). The majority of $pRb^{loxP/loxP}$ MEF uninfected or infected with the empty vector showed normal number of centrosomes (arrows). Arrowheads indicate abnormal centrosomes in $Rb^{-/-}$ MEFs, insets illustrate centrosomes at higher magnification. (c) Histograms indicating the percentage of centrosomes numbers in untreated, HU treated (48 h) and released (72 h) MEFs. (d) Histograms showing percentage of cells with normal (40 chromosomes), hypodiploid (<40 chromosomes) and hyperdiploid (>40 chromosomes) metaphases after 14 and 30 days following HU treatment. Each bar indicates mean + SE of at least three independent experiments. (e) Karyotypic analysis of metaphase spreads showing the presence of aneuploid metaphases by using a Zeiss Axioskop microscope under a 63× objective.

cells and cells infected with adenoviruses that did not express the Cre recombinase. Then parental and infected cells were treated with HU, 2 weeks after infection with Ad-Cre, to detect any centrosome amplification by γ-tubulin staining (Fig. 3b). All of the untreated MEFs showed a similar percentage of cells with 1 or 2 centrosomes (Fig. 3c). After HU treatment both $Rb^{LoxP/LoxP}$ and $Ad\text{-}Rb^{LoxP/LoxP}$ MEFs showed (Fig. 3c) similar number of cells with one or two centrosomes (25–30 and 65–60%, respectively). On the contrary, we scored a high increase in cells harbouring more than two centrosomes (55–60%) in

the $Rb^{-/-}$ MEFs, when compared to the untreated cells (8%). $Rb^{-/-}$ MEFs showing amplified centrosomes were still present at 72 h of release in drug free medium (Fig. 3c). These results suggest that pRB ablation allowed additional rounds of centrosome duplication in absence of DNA synthesis. Cytogenetic analyses performed 2 and 4 weeks after the release from the block imposed by HU showed that the majority of $Rb^{-/-}$ MEFs became aneuploid (Fig. 3d,e), hypodiploid as well as hyperdiploid, suggesting also that pRB absence allows aneuploid cells to proliferate.

## 4. Discussion

Centrosome amplification and aneuploidy occur in virtually all of solid tumors analyzed so far and could be considered early features in tumor initiation and progression [1]. Centrosome alterations were reported in high grade prostate cancer, pancreatic carcinoma and cervix tumors, and were correlated with the presence of multipolar mitoses and chromosomal instability [12,13,14]. Our results show that human fibroblasts expressing HPV16-E6 and -E7 proteins, that target p53 and pRB, respectively, as well as conditional pRB deficient MEFs underwent centrosome amplification after hydroxyurea treatment, whereas wild-type cells did not. Because centrosome duplication normally occurs only once per cell cycle these cells have lost the synchrony between the DNA and centrosome duplication likely by undergoing a prolonged G1/S arrest. Recently, it was reported that centrosome duplication was unaffected by HU treatment in p53 competent MCF7 cells but not in MCF7 cells with altered p53 [8]. In addition, our results are consistent with previous reports showing that dysfunction of p53 [15] and pRB [5] results in deregulation of centrosome duplication [16].

Interestingly, after hydroxyurea removal normal human fibroblasts accumulated with two centrosomes and underwent a senescent—like arrest, while p53 deficient fibroblasts partially re-entered the cycle and then arrested within one week from the release. This finding was similar to that previously noted for p53 deficient (E6 expressing) HeLa cells [10] which underwent a partial replication on release from HU, though this was followed by death likely an alternative outcome to arrest in G1/S in these tumor cells.

On the contrary, pRB deficient (E7 expressing) cells re-entered the cell cycle and proliferated despite the presence of amplified centrosomes. More interestingly, pRB deficient human fibroblasts, analyzed 3 and 5 weeks after hydroxyurea treatment, showed an increase in the number of aneuploid cells. These findings suggest that pRB not only could have a role in centrosome homeostasis, but it might be part of pathway that act downstream by arresting cells that have extra centrosomes. This putative checkpoint could be activated in pRB competent cells by the presence of an incorrect number of centrosomes (for the G1 cell cycle phase) or their related effects (i.e. chromosomal instability) halting further cell cycle progression. We cannot rule out that a certain degree of DNA damage after HU could elicit the G1 arrest we observed in IMR90 cells. Activation of the G1/S checkpoint triggered by DNA damage will block centrosome re-duplication as an additional safeguard mechanism for the integrity of the cell. However, it was reported that antimetabolites such as HU arrested cells at the G1/S border in the absence of any detectable DNA damage [18].

Recently, it was reported that expression of HPV-16 E7 oncoprotein could induce abnormal centrosome duplication independently of its ability to inactivate pRB, p107, and p130 pocket proteins [17]. However, centrosome amplification was less pronounced in pRB negative than in pRB positive cells, indicating that degradation of this protein by E7 contributes to centrosome duplication errors. Our findings that HU treated pRB deficient MEFs behaved similar to human fibroblasts expressing the HPV16-E7 oncoprotein, strongly indicate that lack of pRB could allow generation of multiple centrosomes in a single cell cycle as well as their maintenance. Finally, our results in pRB deficient fibroblasts support the model proposed by Nigg [19] to explain the occurrence of centrosome amplification in one cell cycle and his effects on ploidy, suggesting that multiple centrosome could be a prerequisite in order to generate aneuploidy in cells that lack important functions such as those under pRB control.

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