Published in final edited form as:
*Cell Cycle*. 2006 December ; 5(24): 2899–2902.

# p21$^{Waf1/Cip1}$ deficiency stimulates centriole overduplication

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## Abstract

Inactivation of the cyclin-dependent kinase (CDK) inhibitor p21$^{Waf1/Cip1}$ (CDKN1; hereafter p21) has previously been implicated in the induction of numerical centrosome alterations. It is unclear, however, whether p21 deficiency deregulates the centrosome duplication cycle itself or causes an accumulation of centrosomes due to cell division failure and/or polyploidization. Using a novel marker for maternal centrioles, Cep170, we show here that knock-down of p21 protein expression in murine myeloblasts can stimulate excessive centriole numbers in the presence of only one or two mature centrioles. These results indicate that p21 deficiency can trigger a *bona fide* overduplication of centrioles and that aberrant centrosome numbers cannot solely be explained by polyploidization as suggested by previous studies. Our findings underscore that impaired p21 expression may function as a driving force for chromosomal instability and highlight the importance of markers for maternal centrioles such as Cep170 to elucidate the pathogenesis of numerical centriole aberrations in tumor cells.

### Keywords

p21; centrosome; CDK2; genomic instability; cancer

Aberrant centrosome numbers are detected in virtually all cancers where they can increase the risk for cell division errors and chromosomal instability[1–3]. Centrosomes function as major microtubule organizing centers during interphase and mitosis in most animal and human cells[4]. Each centrosome consists of two centrioles, short barrel-shaped microtubule cylinders, embedded in a pericentriolar protein matrix. In order to ensure bipolar mitotic spindle formation, the single centrosome of a cell duplicates precisely once prior to mitosis[5]. During this process, the two pre-existing centrioles disengage followed by a de novo synthesis of a single daughter centriole adjacent to each of the older centrioles during S phase[6]. The restriction to a single round of centriole duplication per cell cycle and the formation of only one daughter per maternal centriole prevents multipolar mitotic spindles and their potentially deleterious consequences. Aberrant centrosome numbers in tumor cells can in principle arise through two different mechanisms: a genuine overduplication i.e., excessive formation of daughter centrioles, or, centriole accumulation after failed mitosis and/or polyploidization[1,7]. This distinction is critical since a persistent failure of a cell to complete mitosis and to produce

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viable progeny does not propagate genomic instability in a tumor. Conversely, cells that acquire supernumerary centrioles through overduplication during S phase are likely to have an increased propensity to generate abnormal mitotic spindles in a subsequent mitosis[1, 7].

Among the first molecules that have been implicated in the control of centrosome numbers was the CDK inhibitor p21[8, 9]. p21 mainly inhibits cyclin/CDK2 complexes thereby arresting the cell cycle and also binds to PCNA to block DNA synthesis[10, 11]. p21 has not only antiproliferative activities but is also involved in the assembly of D-cyclin-containing CDK complexes[12]. p21 is a transcriptional target of p53 and an important mediator of a stress-induced cell cycle arrest[13]. Mutations of p21 are rare but genetic and/or epigenetic alterations of p21 regulators including p53 or MYC are common findings in malignant tumors.

In one of the first studies related to the role of p21 in centrosome homeostasis, cells deficient of p21 and exposed to ionizing radiation were found to accumulate abnormal numbers of centrosomes following abortive mitoses[14]. In keeping with these findings, depletion of p21 in human hematopoietic cells was found to cause abnormal centrosome numbers together with a deformed nuclear architecture and polyploidy[15]. Additional support for a role of p21 in centrosome duplication came from studies showing that p21 overexpression can inhibit centrosome overduplication associated with a prolonged treatment of cells with hydroxyurea[16]. Similar results were obtained when the related CDK inhibitor $p27^{Kip1}$ was overexpressed[17, 18]. Conversely, cells lacking p21 were prone to centrosome amplification following a prolonged S phase arrest[19]. Inhibitory effects of Cip/Kip type CDK inhibitors on repeated centrosome reproduction were reported in frog embryos[18] and Xenopus egg extracts[20]. Furthermore, the human papillomavirus type 16 E7 (HPV-16 E7) oncoprotein, which inactivates p21 [21, 22] and several other key regulators of the G1/S cell cycle checkpoint, was found to stimulate centrosome overduplication[23]. Cells deficient of p53 frequently show abnormal centrosome numbers[24] and the failure to upregulate p21 following genotoxic stress in such cells may contribute importantly to this phenotype[25].

Based on these results, no straightforward explanation for the development of abnormal centrosome numbers in p21-deficient cells exists and it is possible that these alterations develop as a secondary phenomenon of cell division failure and/or polyploidization[14, 15]. This notion is further supported by findings implicating p21 in the prevention of polyploidization[26–28].

To determine whether deficiency of p21 can cause a genuine centrosome overduplication, we engineered the murine myeloblast cell line 32Dcl3 (32D) to stably express a hairpin siRNA construct targeting murine p21[29]. A significant reduction of p21 protein expression was detected (Fig. 1A) that was associated with enhanced *in vitro* CDK2 kinase activity towards a C-terminal fragment of the retinoblastoma protein (pRB) that contained serine residues 807 and 811 (Fig. 1B).

Immunofluorescence analysis of p21-deficient and control cells for centrin[30] was performed to visualize individual centrioles. Cells deficient of p21 frequently showed excessive numbers of centrioles (Fig. 1C). Quantification of centriole numbers on a cell-per-cell basis revealed a decrease of cells with normally duplicated centrioles (three or four per cell) from 52% in controls to 35% in p21-deficient populations ($p \leq 0.05$; Fig. 1D). At the same time, an increase of cells with overduplicated centrioles (more than four per cell) was detected. Cells containing five (24%), six (11%) or more than six (22%) centrioles were increased in p21-deficient cells when compared to controls (20%, 7%, and 8%, respectively; Fig. 1D). The increase of the proportion of cells with six or more than six centrioles in p21-depleted populations in comparison to controls was found to be statistically significant ($p \leq 0.05$ and $p \leq 0.005$, respectively).

To analyze whether aberrant centriole numbers were generated through overduplication or accumulation, a double-immunofluorescence analysis for centrin and Cep170 (centrosomal protein of ~170 kD) was performed (Fig. 1E). Cep170 has recently been identified as a novel marker for mature centrioles and can hence be used to distinguish between newly synthesized centrioles (Cep170-negative) and maternal centrioles that have undergone maturation (Cep170-positive)[31]. Cells usually contain one Cep170-positive maternal centriole (Fig. 1E, top panels) until late G2 phase when the second maternal centriole becomes positive (not shown). A genuine centriole overduplication is characterized by the presence of a single mature centriole and more than three immature daughter centrioles [31]. In p21-deficient 32D cells, staining for Cep170 enabled us to distinguish between overduplication of centrioles in the presence of a single Cep170-positive maternal centriole and centriole accumulation leading to multiple Cep170-positive centrioles. The example for centriole overduplication shown in Fig. 1E (middle panels) highlights a cell with a single Cep170-positive mature centriole and five Cep170-negative centrioles whereas centriole accumulation is illustrated by a cell displaying four Cep170-positive centrioles in the presence of the same number of Cep170-negative centrioles (Fig. 1E, bottom panels).

In order to quantify the staining results, we analyzed cells containing more than six centrioles since the difference in the frequency of such cells was most significant between control and p21-deficient populations ($p \leq 0.005$; Fig. 1D). Quantification of the Cep170 staining revealed that 58.8% of control cells with more than six centrioles contained three, four or more than four Cep170-positive centrioles (Fig. 1F, left panel). These results suggest cytokinesis failure and/or polyploidization in a high number of control cells. Centriole overduplication, however, is also detectable in control populations as indicated by cells with more than six centrioles and only one or two Cep170-positive centrioles.

In cells deficient of p21, the steady state level of cells with centriole overduplication had significantly shifted and the majority of cells containing more than six centrioles showed only one or two Cep170-positive maternal centrioles (81.1%; Fig. 1F, right panel). It is noteworthy that the proportion of cells with more than six centrioles and only one Cep170-positive centriole was increased from 15.7% in controls to 43.4% in p21-deficient cells indicating that loss of p21 promotes a genuine centriole overduplication. The presence of cells with three, four or more than four Cep170-positive centrioles in p21-deficient cells furthermore suggests that cytokinesis failure and/or polyploidization contribute to the overall number of cells with more than six centrioles but to a much lesser extent than in controls.

Given that centriole duplication requires CDK2 and that p21-depleted cells showed an increased *in vitro* CDK2 kinase activity (Fig. 1B), we next tested whether chemical CDK inhibitors can abrogate centriole overduplication[32]. The two CDK inhibitors indirubin-3′-oxime (IO) and roscovitine[33, 34] were found to reduce the proportion of p21-deficient cells with excessive centriole numbers to the level detected in control cells (Fig. 1G). In contrast, when we treated cells with 1-methyl-indirubin-3′-oxime (MeIO), no effect on the proportion of cells with centriole overduplication was detected. MeIO is an analogue of IO that lacks the ability to donate a hydrogen bond to the peptidyl carbonyl oxygen of CDK2's amino acid residue glutamine 81 and thus cannot bind the ATP binding pocket and inhibit its kinase activity[33, 35–37]. These results are in line with previous findings showing an abrogation of centriole overduplication in the absence of CDK2 activity[32, 38].

Several studies have suggested that abnormal centrosome numbers in p21-deficient cells arise from centrosome accumulation[14, 15]. Here, we provide evidence that p21-deficiency can trigger a bona fide centriole overduplication. Our results do not preclude that cell division failure and/or polyploidization followed by centrosome accumulation contribute to aberrant centrosome numbers in cells with reduced p21 expression. Nonetheless, the fact that p21-

deficiency represents an oncogenic stimulus that can provoke centriole overduplication may help to explain previous findings reporting that p21-deficient cells become aneuploid[39]. A recent report has shown that loss of the CDK inhibitor $p16^{INK4A}$ can promote chromosomal instability through the induction of supernumerary centrosomes[40]. Despite probable differences in the underlying mechanisms that lead to numerical centrosome abnormalities, these findings, together with results presented here, lend support to the notion that impaired function of CDK inhibitors can trigger centrosome-mediated chromosomal instability. Centriole overduplication results from disruption of the centriole duplication cycle itself and our results suggest that p21 helps to prevent centriole overduplication, most likely by regulating CDK2 activity[16–20, 25]. Further studies are warranted to explore the precise mechanism through which disruption of the p21/cyclin/CDK2/pRB axis leads to centriole overduplication.

**Acknowledgements**

We are grateful to Erich A. Nigg (Max Planck Institute for Biochemistry, Martinsried, Germany), Giulia Guarguaglini (University of Rome “La Sapienza”, Rome, Italy), Jeffrey L. Salisbury (Mayo Clinic, Rochester, MN, USA) and Laurent Meijer (CNRS, Roscoff, France) for sharing important reagents. We would like to thank Giulia Guarguaglini for critical reading of the manuscript and helpful comments. Supported by NIH/NCI grant R01 CA112598 and the Susan G. Komen Breast Cancer Foundation.

## Abbreviations

**CDK2**
: cyclin-dependent kinase 2

**DAPI**
: 4′,6′-diamidino-2-phenylindole

**HPV-16**
: human papillomavirus type 16

**pRB**
: retinoblastoma protein

**shRNA**
: short hairpin RNA

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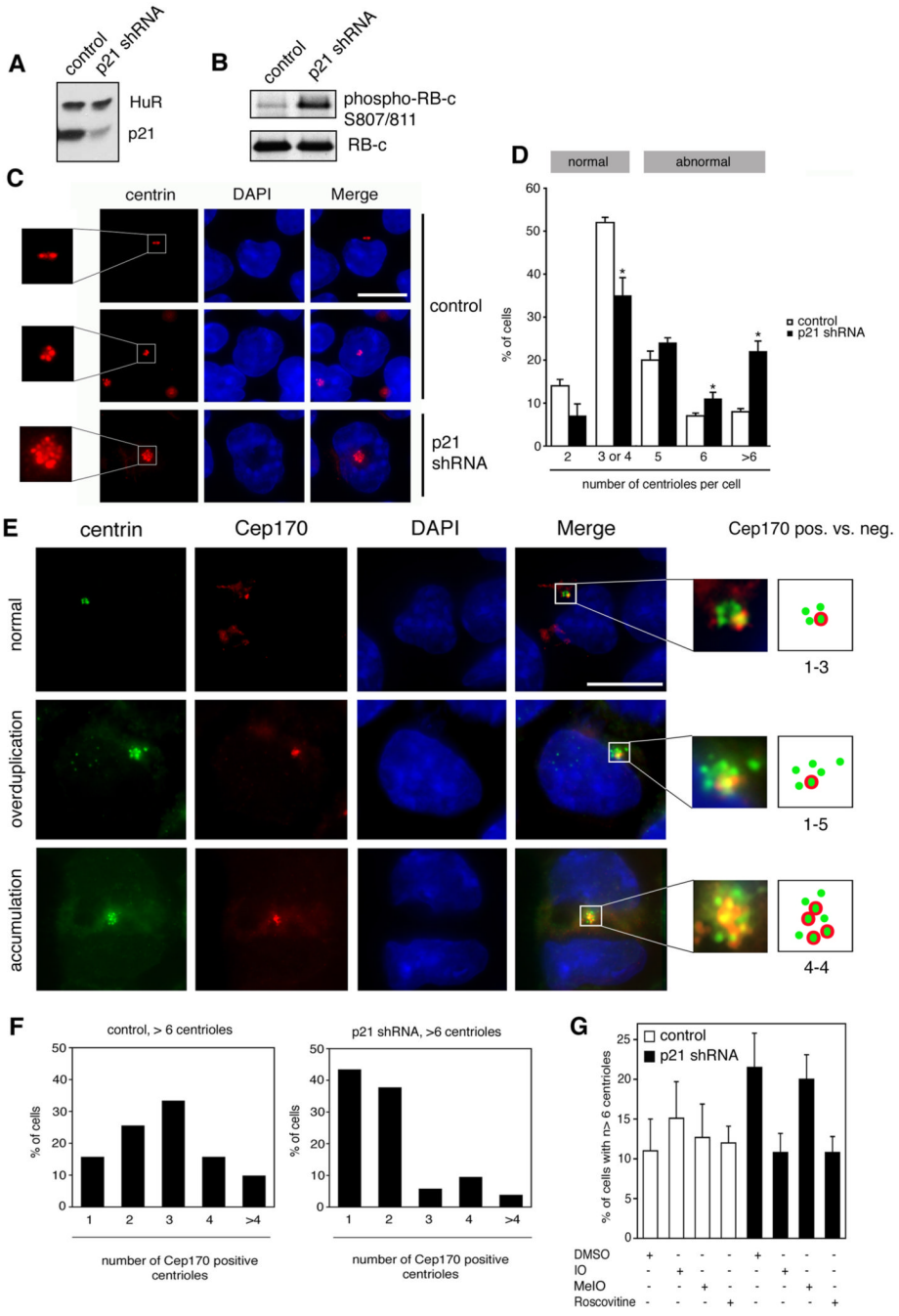


**Figure 1.**

**(A)** Immunoblot analysis of 32D murine myeloblasts for p21 expression using a polyclonal antibody (C-19; Santa Cruz) following stable expression of p21 shRNA or control shRNA constructs as previously described[29]. Immunoblot for HuR is shown to demonstrate loading of equal amounts of protein.

**(B)** *In vitro* CDK2 kinase assay of control or p21-depleted 32D cells using a recombinant C-terminal fragment of pRB (RB-c; Upstate) as a substrate followed by immunoblot analysis using a phospho-specific pRB antibody (S807/811; Cell Signaling) as previously described[32]. CDK2 complexes were isolated by immunoprecipitation with an anti-CDK2 polyclonal antibody (M-2; Santa Cruz).

**(C)** Immunofluorescence analysis of control or p21-depleted 32D cells for centrin (antibody kindly provided by Jeffrey L. Salisbury, Mayo Clinic, Rochester, MN, USA) as described previously[41]. Examples of cells with unduplicated centrioles (top panels), duplicated centrioles (middle panels) or supernumerary centrioles (bottom panels) are shown. Nuclei stained with DAPI. Scale bar indiates 10 μm.

**(D)** Quantification of the percentage of cells with normal centriole numbers (up to four) or abnormal centriole numbers (five, six or more than six) in control (open bars) or p21-depleted cells (black bars). Each bar represents mean and standard error of at least three different experiments with a minimum of 50 cells counted per experiment. Statistical significance was calculated using Student's t test for independent samples (indicated by asterisks).

**(E)** Co-immunofluorescence analysis of p21-depleted 32D cells for centrin and Cep170[31]. Briefly, cells were stained for centrin as previously described[41] followed by an anti-Cep170 antibody (kindly provided by Erich A. Nigg, Max Planck Institute for Biochemistry, Martinsried, Germany) and a Rhodamine Red-conjugated anti-rabbit secondary antibody (Jackson Immunoresearch). All antibody incubations following the first primary antibody were for 2 h at 37°C. Examples of cells with normal centriole duplication (one Cep170-positive and three Cep170-negative centrioles; top panels), centriole overduplication (one Cep170-positive and five Cep170-negative centrioles; middle panels) or centriole accumulation (four Cep170-positive and four Cep170-negative centrioles; bottom panels) are shown (see also drawings). Nuclei stained with DAPI. Scale bar indicates 10 μm.

**(F)** Quantification of the percentage of control (left panel) or p21-depleted (right panel) cells with more than six centrioles and the indicated numbers of Cep170-positive centrioles. Results of a representative experiment with 50 cells counted per cell population are shown.

**(G)** Quantification of control (open bars) or p21-depleted (black bars) 32D cells with more than six centrioles following 24 h treatment with either 0.1% DMSO used as solvent control or 1 μM indirubin-3′-oxime (IO), 1 μM 1-methyl-indirubin-3′-oxime (MeIO) or 1 μM roscovitine (all reagents kindly provided by Laurent Meijer, CNRS, Roscoff, France). Each bar represents mean and standard error of at least three different experiments with a minimum of 50 cells counted per experiment.