p53 Displacement from Centrosomes and p53-mediated G1 Arrest following Transient Inhibition of the Mitotic Spindle*

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Growing evidence indicates a central role for p53 in mediating cell cycle arrest in response to mitotic spindle defects so as to prevent reeplication in cells in which the mitotic division has failed. Here we report that a transient inhibition of spindle assembly induced by nocodazole, a tubulin-depolymerizing drug, triggers a stable activation of p53, which can transduce a cell cycle inhibitory signal even when the spindle-damaging agent is removed and the spindle is allowed to reassemble. Cells transiently exposed to nocodazole continue to express high levels of p53 and p21 in the cell cycle that follows the transient exposure to nocodazole and become arrested in G1, regardless of whether they carry a diploid or polyploid genome after mitotic exit. We also show that p53 normally associates with centrosomes in mitotic cells, whereas nocodazole disrupts this association. Together these results suggest that the induction of spindle damage, albeit transient, interferes with the subcellular localization of p53 at specific mitotic locations, which in turn dictates cell cycle arrest in the offspring of such defective mitoses.

Polyploidization is regarded as a crucial step leading to aneuploidy in tumor cells (1, 2), which in turn is associated with high malignancy and poor prognosis. A complex network of structural and regulatory factors ensures that chromosomes segregate evenly in daughter cells and prevents resumption of DNA synthesis before mitosis has been completed. This process is subject to control mechanisms collectively referred to as the post-mitotic checkpoint (3).

A large body of data clearly documents the key role of the p53 tumor suppressor gene in the G1/S checkpoint in response to DNA damage, whereby DNA replication is prevented until repair has occurred, or apoptosis is induced when repair is ineffective (reviewed in Ref. 4). Both p53 and its major regulatory target, the cyclin-dependent kinase inhibitor p21, also act at later cell cycle stages and are implicated in the G2 checkpoint, which prevents mitotic entry following post-replicative DNA damage (5, 6). These p53-de‐pendent regulatory pathways rest upon the ability of p53 to act as a “sensor” of DNA damage and are now well clarified. A more intricate aspect of cell cycle checkpoints is being unraveled in a growing number of studies that indicate that p53 also contributes to a checkpoint triggered in response to mitotic spindle defects (3, 7, 8; reviewed in Ref. 9).

Nocodazole (Noc) is a well known tubulin-depolymerizing agent that inhibits spindle assembly and triggers a response known as the mitotic spindle checkpoint, following which mitotic progression is arrested (10–12 and references therein). In previous work (13) we sought to investigate the requirement of p53 activity in response to failure of the mitotic spindle. Two human isogenic cell lines, i.e. the K562 (p53-negative) erythroleukemia cell line and a parvovirus-resistant derivative clone named KS, which reexpresses p53, were continuously exposed to Noc. Human lymphoblastoid AHH1 cells were also examined in parallel experiments. We found that the activation of the proper spindle checkpoint, revealed by a delay in mitotic progression in the presence of spindle damage, was independent of p53. However, mitotic arrest was transient, and, after a delay of variable length, cells achieved mitotic exit even in the absence of spindle (mitotic slippage). At that stage, the cell cycle patterns of cell lines differed dramatically depending on their p53 status; only p53-deficient K562 cells progressed through the cell cycle in the presence of Noc, resuming DNA synthesis and hence entering a polyplloid cell cycle. On the contrary, p53-expressing KS and AHH1 cells stopped cell cycle progression after the first mitotic round, degraded cyclin B, and arrested in the following G2 phase with a 4C DNA content. These results converge with data obtained by other groups (reviewed in Ref. 9) indicating that G1 arrest in tetraploid cells (post‐mitotic checkpoint) is p53-dependent.

In a further step to understand the mechanism of action of p53 in the post-mitotic checkpoint, we examined the response of cells to transient exposure to Noc, i.e. over the duration of one cell cycle only. We report here that transient exposure to Noc generates a prolonged signal for p53 activation, which persists during the first cell cycle after Noc removal, i.e. when a functional spindle is in fact allowed to reassemble. In addition, Noc alters the subcellular localization of p53 during mitosis. Indeed, p53 associates with centrosomes in normal mitotic cells; in contrast, this association is disrupted by Noc and is not reestablished in cells that achieve mitotic exit after transient exposure to Noc. Thus, the inhibition of spindle assembly by Noc is reflected by the failure of p53 to associate with structures of the mitotic apparatus; in turn, delocalization and

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1 The abbreviations used are: Noc, nocodazole; BrdUrd, 5-bromo-2’-deoxyuridine; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorter; DAPI, 4’,6-diamidino-2-phenylindole.
prolonged activation of p53 are associated with maintenance of a cell cycle inhibitory signal in the G1 phase that follows the removal of the spindle-damaging agent.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—Human cell lines AHH1 (non-transformed lymphoblastoid cell line) and K562 (erythroleukemic cell line) were grown in RPMI 1640 medium (Euroclone) supplemented with 10% fetal calf serum in a 5% CO2 atmosphere at 37 °C. Exponentially growing cultures were used for all experiments. Where indicated, AHH1 cells were transfected by electroporation (975 microfarads and 250 V) with a plasmid (pG48)-resistant construct encoding a temperature-sensitive version of the p53 protein (p53V135A135), which at 38 °C acts as a dominant negative factor for wild-type p53. Control cells in these experiments were generated by transfection with empty vector carrying the p53 sequence. Both transfected cell lines were kept under selection by culturing in the presence of 50 μg/ml geneticin (Life Technologies, Inc.) and maintained as polyclonal populations (AHH1p53V135 cells). Cell cultures were exposed to Noc for 20 h, then washed in fresh medium, and cultured in noc- free medium for the times indicated in the text. Noc was dissolved in Me2SO (both from Sigma) at a 1 mg/ml concentration. The required volume of 1 mg/ml Noc solution was directly added to exponentially growing cultures so as to obtain a final concentration of 0.2 μg/ml.

**Protein Extract Preparation and Western Immunoblotting Assays**— Aliquots of 5 × 106 cells were withdrawn from the cultures at the indicated times and centrifuged at low speed at 4 °C. Pelleted cells were washed in ice-cold phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride (Sigma) and lysed as described (14). Cell lysates were centrifuged at 14,000 rpm at 4 °C for 30 min. Protein concentrations were determined using the Bradford assay kit (Bio-Rad). Protein extracts were resuspended in loading buffer (4% SDS, 100 mM dithiothreitol, 0.5 mM EDTA, 20% glycerol, 100 mM Tris- HCl, pH 6.8, 0.1% bromophenol blue), boiled for 8 min, briefly centrifuged, and finally subjected to 10–12% SDS-polyacrylamide gel electrophoresis. Electrophoresed proteins were electrotransferred at 100 mA for 75 min onto a Trans-blot nitrocellulose membrane (Schleicher & Schuell). Membranes were blocked in 5% (w/v) fat milk in TBST buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween 20) at 4 °C overnight and then incubated at room temperature with the following primary antibodies in 5% milk, TBST: anti-cyclin B1 (GNS1, Santa Cruz Biotechnology, sc-245), anti-p53 (DO-7, Dako, M7001), anti-p21 (C-19, Santa Cruz Biotechnology, sc-29), anti-cyclin E (Ab-1, Calbiochem, C056), all primary antibodies were diluted at 0.5 μg/ml. Immunoreactive proteins were detected using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and revealed using the enhanced chemiluminescence system (ECL-plus, Amersham Pharmacia Biotech).

**Flow Cytometry Analysis**—DNA replication was monitored essentially as described (15) after addition of BrdUrd (5-bromo-2-deoxyuridine, 45 μM final concentration) to the culture medium 30 min before harvesting the cells. Cells were fixed in 70% ethanol (30 min, 4 °C), washed twice in 0.5% Tween 20 in phosphate-buffered saline, and incubated in 3 M HCl for 45 min to denature the DNA. Cells were then exposed to anti-BrdUrd monoclonal antibody (Dako clone Bu20a, M0744) and to secondary fluorescein isothiocyanate (FITC)-conjugated antibody (Vector Laboratories, FI2000) and were finally stained with propidium iodide. Samples were analyzed using a FACStar (fluorescence-activated cell sorter) Plus flow cytometer (Becton-Dickinson) and the WinMDI software. 10,000 events were recorded for each sample. The amplification scale was logarithmic for FSC-H and FL1-H parameters and linear for SSC-H, FL2-A, FL2-H, and FL2-W. Photomultiplier tension was set so as to place the peak corresponding to 2C DNA content (G0/G1) at channel 200 in the FL2-H histogram. A similar procedure, except for the HCl step, was adapted to measure the DNA content and the fluorescence associated with anti-cyclin B1 (Santa Cruz Biotechnology), anti-p53 (see above for details), and MPM-2 (16; Dako M3514) antibodies. A negative control was also prepared for each sample by treating the cells with a mouse nonspecific IgG and secondary FITC-conjugated antibody (Santa Cruz Biotechnology). In all types of analyses, all cell aggregates were carefully gated out using FL2-W versus FL2-A bivariant graphs.

**Immunofluorescence Assays**—Cells were washed, spread on glass coverslips by centrifugation, and fixed in 3% formaldehyde for 15 min at 4 °C. Cell preparations were permeabilized in 0.2% Triton X-100 in phosphate-buffered saline for 5 min at room temperature and 100% methanol for 10 min at −20 °C. Fixed and permeabilized cells were pre-incubated in 20% goat serum for 30 min at 37 °C in a humidified chamber and then incubated for 2 h with anti-p53 antibody. Mouse monoclonal clone DO-7 (Dako M 7001, 1:100) was mostly used (4 μg/ml); in certain experiments sheep polyclonal clone Ab-7 (Oncogene Science PC35, 1:200) was used. Antibodies to centrosomal components included the following: rabbit anti-centrin antibody, described in Ref. 17 (1:500 dilution), rabbit anti-γ tubulin (Sigma T3559, 1:2000 dilution), and anti-glutamylated tubulin (GT355), as indicated in Ref. 17. Antibodies were diluted in 5% goat serum. After three washes in 0.05% Tween 20 in phosphate-buffered saline, cells were incubated with rhodamine-conjugated secondary antibody against centrin or γ-tubulin antibody (Santa Cruz Biotechnology, sc2091, 1:400) for 30 min at 37 °C. After three more washes cells were incubated with FITC-conjugated secondary antibody (Vector Laboratories FI2000, 1:300) directed against anti-p53 antibody for 30 min. Cell spreads were counterstained with 0.2 μg/ml DAPI for 10 min at room temperature to stain the DNA and mounted on glass slides in Vectashield (Vector Laboratories). Cell preparations were examined under an Olympus AX70 microscope equipped with epifluorescence, and photographs were taken (×100 objective) using a cooled camera device (Photometrics).

**RESULTS**

**p53-expressing Cells Undergo a Prolonged yet Reversible Arrest after Transient Exposure to Noc**—In previous experiments designed to analyze the mechanisms preventing polyploidyization, we examined human non-transformed AHH1 cells that were continuously cultured in the presence of Noc. The cell cycle progressed normally until the first mitotic division; after a transient mitotic delay, cells resumed mitosis and eventually arrested in a G1-like tetraploid state. This pattern was similar to that observed in the p53-proficient KS cell line and differed from that of K562 cells, which functioned p53 and continue to progress into the cell cycle in the presence of Noc (13).2 Thus, Noc activates a similar post-mitotic checkpoint pathway both in AHH1 cells and in KS cells.

To further investigate the cell cycle inhibitory mechanisms triggered by spindle failure, we examined cell cultures that were exposed to Noc for a length of time allowing one cell division and subsequently cultured in the absence of Noc. AHH1 and K562 cells were exposed to Noc for 20 h, then washed, and further incubated in Noc-free growth medium. Cell cycle progression was examined by simultaneous FACs analysis of BrdUrd incorporation into newly replicating DNA and of the genomic content revealed by propidium iodide incorporation. In these experiments both cell lines were effectively arrested in G2/M in the presence of Noc. Upon Noc removal, both cell lines rapidly exited mitosis; 2 h after the block release, about 50% of the cells had reached the G1 phase (Fig. 1A). Thereafter a dramatic difference in cell cycle resumption was observed between cell lines; 20 h after Noc removal, DNA replication was actively resumed in G2/M in the presence of Noc. Upon Noc removal, both cell lines rapidly exited mitosis; 2 h after the block release, about 50% of the cells had reached the G1 phase (Fig. 1A). Thereafter a dramatic difference in cell cycle resumption was observed between cell lines; 20 h after Noc removal, DNA replication was actively resumed in p53-proficient K562 cells, the cell cycle profile of which was similar to that of asynchronously cycling cultures (Fig. 1A, lower row). In contrast, AHH1 cultures remained blocked with a similar cell cycle profile for at least 20 h after release from Noc arrest (Fig. 1A, upper row); after 48 h some resumption of cell cycle progression began; only 72 h after Noc removal, roughly corresponding to the length of three cell cycles in normal conditions, did AHH1 cells eventually recover the typical distribution of asynchronously cycling cultures (Fig. 1A). These data therefore depict a significant difference between p53-proficient and p53-defective cells in the ability to induce a durable arrest after spindle failure; this difference is most evident 20 h after Noc release. By Western immunoblotting assays (Fig. 1B), AHH1 cells accumulated p53 during Noc exposure as expected (13, 18); furthermore, p53 levels peaked at 20 h after Noc removal and gradually declined thereafter (Fig. 1B). The gene encoding p21 is the best charac-

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2 M. Ciciarello, R. Mangiacasale, and E. Cundari, unpublished data.
terized transcriptional target of p53, and its expression is triggered by several cell cycle-disrupting agents. Our experiments show that p21 protein levels also peak 20 h after release from Noc arrest and substantially parallel the p53 pattern of expression. Thus, the persistence of G1 arrest in AHH1 cultures in the first cell cycle after removal of Noc coincides with the highest induction of the p53/p21 pathway.

**G1 Arrest after Transient Exposure to Noc Occurs in Diploid and Tetraploid Cell Populations**—The expression of cell cycle markers was examined to determine the molecular features associated with the induction of prolonged arrest in AHH1 cell cultures. In these experiments we focused on the interval of time during which the checkpoint was effective, i.e. the first cell cycle after Noc removal. A time course analysis of AHH1 cell extracts by Western immunoblotting (Fig. 2A) shows that during the first 20 h of culture in the presence of Noc, cyclin B accumulates in the cell cultures, as expected; subsequently, cyclin B becomes fully degraded within the first 4 h of release and fails to be newly synthesized for as long as 20 h after Noc release. Concomitantly, cells gradually accumulate cyclin E during the first 4 h after Noc removal, indicating that mitotic exit has occurred; cyclin E continues to be highly expressed thereafter. Thus, only
a minor fraction, if any, of the AHH1 cells that had been seen to be arrested with a 4C DNA content after 20 h from Noc removal (Fig. 1A, upper row) are capable of reexpressing cyclin B, whereas most of the cell population maintains high levels of cyclin E expression (Fig. 2A). These results suggest that the 4C-containing subpopulation is in fact constituted by tetraploid cells produced by mitotic slippage and arrested in a G1-like state. To further confirm this interpretation, cyclin B expression was directly investigated in AHH1 cell populations separated for their DNA content by biparametric FACS analysis (Fig. 2B). We also investigated the expression of a subset of mitotic markers independent of cyclins, i.e. the G2/M-specific phosphoepitopes reactive to the MPM2 antibody (16). FACS analysis unambiguously indicates that AHH1 cells exit mitosis after Noc removal, as indicated by the disappearance both of cyclin B and of MPM2 antigens in the entire cell population, regardless of the genome content. Thus, exposure of AHH1 cells to Noc during one cell cycle induces a prolonged arrest in the G1 phase of the following cell cycle, in cell populations with a diploid or tetraploid content.

Functional p53 Is Required for Prolonged G1 Arrest in Cells Released from Transient Exposure to Noc—We and others previously showed that the post-mitotic checkpoint activated in the presence of Noc is p53-dependent (3, 5, 7, 8, 13). To assess whether p53 activity was also implicated after release from Noc arrest, we preliminarily used biparametric FACS analysis to determine p53 immunoreactivity in AHH1 cell populations of different genome size; we detected an increased p53 signal in the presence of Noc, i.e. when the cell population is essentially G2/M-arrested; after Noc removal p53 levels continued to increase, consistent with the data in Fig. 1B, and 20 h after removal most AHH1 cells in both the 2C and 4C subpopulations express p53 (data not shown). These data strongly suggest that p53 is functionally implicated in sustaining the prolonged arrest observed in response to transient Noc exposure.

To directly assess that hypothesis, we constructed AHH1 cell lines defective for p53 function. AHH1 cell cultures were stably transfected with a neomycin-resistant expression vector directing the synthesis of a dominant negative allele of p53 (dnp53) (19). The dnp53 protein acts in these cells (henceforth designated AHH1\textsuperscript{dnp53}) as a dominant negative mutant, because it retains the ability to interact with the endogenous, wild-type p53 protein and sequesters it into a functionally inactive complex. AHH1\textsuperscript{dnp53} cells express significantly higher steady-state levels of protein compared with both AHH1 and AHH1\textsuperscript{neo} cells, reflecting the exogenous expression of mutant p53 from the transfected plasmid. FACS analysis was employed to monitor cell cycle progression in AHH1 cell cultures subjected to Noc block and release in the presence or absence of functional p53. AHH1 cells transfected with vector alone (indicated as

**Fig. 3. Functional p53 is required for induction of the post-mitotic checkpoint after transient exposure to Noc.** Upper row, stably transfected AHH1\textsuperscript{neo} (neomycin-resistant vector) cells; lower row, stably transfected AHH1\textsuperscript{dnp53} (neomycin-resistant construct expressing the dominant negative Val-135 mutant of p53) cells; both cell lines were exposed to Noc or harvested at the indicated times after Noc release and then subjected to biparametric FACS analysis of BrdUrd incorporation (y axis) and of the DNA content (x axis). Panels are representative of three independent experiments.

**Fig. 4. Induction of the post-mitotic checkpoint in response to transient exposure to Noc implicates p21 up-regulation.** A, Western blot analysis of p53 (left panels) and p21 (right panels) in parental (AHH1), vector-transfected (AHH1\textsuperscript{neo}), and p53 dominant negative (AHH1\textsuperscript{dnp53}) cell lines during Noc arrest and release. B, quantification of the p21 signals in immunoblots from AHH1\textsuperscript{neo} (plain histograms) and AHH1\textsuperscript{dnp53} (empty histograms) cell extracts. After exposure, autoradiographs of ECL-processed p21 immunoblots were scanned by microdensitometry. Values obtained in each time point after Noc exposure and release were normalized relative to the p21 signal measured in asyn-chronously cycling cells and hence indicate the extent of p21 induction by Noc (represented by histograms). Asy, asynchronous.

AHH1\textsuperscript{neo} cells) responded to transient Noc exposure with a durable cell cycle arrest, revealed by the lack of BrdUrd incorporation, that was indistinguishable from that of the parental AHH1 cell line (Fig. 3, upper row). On the contrary, the AHH1\textsuperscript{dnp53} cell culture, in which the function of endogenous p53 was impaired, failed to induce a post-mitotic arrest (Fig. 3, lower row), similar to what we had previously observed with the p53-defective K562 cell line (see Fig. 1A). Together, these data indicate that the G1 arrest that follows the transient inhibition of the mitotic spindle is dependent on p53 function.

By Western immunoblotting (Fig. 4A, left panel) both the parental (AHH1) and vector-transfected (AHH1\textsuperscript{neo}) cell cultures showed a progressive increase in p53 levels during the first 20 h from Noc release. In contrast, the AHH1\textsuperscript{dnp53} cell line failed to up-regulate p53 levels after exposure to Noc.

In reverse transcriptase-polymerase chain reaction experiments p21 mRNA transcription was significantly up-regulated
Subcellular localization of p53 in normal mitotic cells. A, interphase; B, prometaphase; C, anaphase from asynchronously cycling AHH1 cultures after staining with DAPI to visualize chromosomes (pseudocolored in blue, left column) and processing for indirect immunofluorescence (middle column) to localize centrin (Cen) (rhodamine-conjugated secondary antibody, pseudocolored in red) and p53 (FITC-conjugated secondary antibody, pseudocolored in green); superimposed signals appear in yellow. Merged pictures are shown in the right column.

Fig. 5. Subcellular localization of p53 in normal mitotic cells. A, interphase; B, prometaphase; C, anaphase from asynchronously cycling AHH1 cultures after staining with DAPI to visualize chromosomes (pseudocolored in blue, left column) and processing for indirect immunofluorescence (middle column) to localize centrin (Cen) (rhodamine-conjugated secondary antibody, pseudocolored in red) and p53 (FITC-conjugated secondary antibody, pseudocolored in green); superimposed signals appear in yellow. Merged pictures are shown in the right column.

in AHH1 cells during Noc block and release, but not in p53-deficient K562 cells under similar conditions (data not shown). We directly examined the abundance of the p21 protein in vector-transfected and mutant p53-transfected AHH1 cells by Western blotting assays (Fig. 4A, right panel). In AHH1 and AHH1neo cells, p21 levels increased in the presence of Noc and continued to further increase during the first 20 h of release, substantially paralleling those of p53. In contrast, AHH1dnp53 cells failed to express comparably high levels of p21 when exposed to Noc and after Noc removal. Actually, a low level of p21 induction was observed at late release times (20 h after Noc removal), possibly reflecting the activity of residual wild-type p53 that had evidently not been completely inactivated by the dominant negative mutant. Microdensitometric analysis of the signal intensity (Fig. 4B) indicated, however, that p21 was induced only 2-fold and 3-fold after 8 and 20 h, respectively, of Noc release in AHH1neo cells, compared with 15- and 17-fold, respectively, in AHH1dnp53 cells. Together these results indicate that p53-dependent G1 arrest after spindle damage is associated with up-regulation of p21 protein levels.

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Exposure to Noc Disrupts the Subcellular Localization of p53 in Mitotic Cells—The results thus far indicate that p53 is central in mediating a prolonged cell cycle arrest that persists for several hours after removal of the spindle-damaging agent, when spindle assembly can actually be recovered. We therefore decided to investigate the intracellular distribution of p53 before and after induction of spindle damage by indirect immunofluorescence.

In normal interphase cells, p53 shows a weak and diffuse pattern throughout nuclei (Fig. 5A). During mitosis, p53 is redistributed in a typical organization consisting of discrete spots. Because the number and arrangement of p53 spots in mitotic cells resembled that of centrosomes, double immunofluorescence experiments were performed using anti-p53 antibody in combination with antibodies against centrosomal markers, i.e. centrin, a major constituent of centrioles (20–21), g-tubulin, forming the nucleating material (reviewed in Refs. 22–24), and glutamylated tubulin, a specifically modified form of tubulin associated with centriolar microtubules (17). Fig. 5, B and C, shows results obtained with anti-centrin antibody in normal mitotic cells; p53 spots are associated with or in close proximity of centriole pairs visualized by centrin staining. Similar results were obtained using two anti-p53 antibodies of different clonal origin, in combination with antibodies against independent centrosomal markers (see below).

We then examined cells exposed to Noc, i.e. typically blocked in prometaphase and completing mitosis during the release from Noc arrest (Fig. 6). In Noc-exposed prometaphase-arrested cultures the intensity of the p53 signal increased compared with mitotic cells from asynchronous cultures; most importantly, p53-positive spots failed to associate with centrosomes (Fig. 6A) and were organized in variable numbers. Furthermore, p53 was neither down-regulated in abundance nor redistributed in its normal pattern after Noc removal, despite the rapid resumption of mitotic progression; indeed, within 80 min from Noc removal cells had progressed to anaphase (Fig. 6, B and C), with a substantial recovery of their chromosome segregation capability at each pole, and yet p53 spots continued to be intense in fluorescence and variable in number and did not reassociate with centrosomes. The abundance of p53 continued to increase even after cells had completed mitosis and reached the following G1, when p53 massively reentered the nucleus (Fig. 6D). To fully assess the significance of these findings, we had to rule out the possibility that the effect in Fig. 6 reflected merely an increase in centriole splitting following Noc exposure. We first sought to quantify the actual occurrence of centriole splitting in NOC-arrested prometaphase cells by analyzing anti-centrin reactive spots. No
evidence of splitting was seen in 95 of 100 examined mitoses, three cells showed more than two centriole pairs, and only in two cells were centrioles abnormally separated; these figures are not significantly different from mitotic figures from asynchron-ous cultures. Immunofluorescence experiments were also repeated in cells processed with anti-p53 and anti-g-tubulin antibodies (Fig. 7); these experiments confirmed that in control mitotic cells p53 spots colocalize with g-tubulin, as indicated by the yellow pseudocoloration of centrosomes (Fig. 7, A and B). In contrast, in Noc-arrested and released cultures, the association of p53 with centrosomes was again found to be inhibited (Fig. 7, C and D). Similar patterns were observed in cultures processed using an anti-glutamylated tubulin antibody (data not shown).

Data are quantified in Table I. In mitotic figures from Noc-exposed and released cultures p53 spots were never found to be associated with centrosomes. This contrasts with the typical pattern observed in asynchronous control cultures, in which 80% of mitotic cells showed centrosome-associated spots. In conclusion, therefore, these results indicate that the stabilization and activation of p53 occurring during exposure to Noc is accompanied by the lack of association of p53 with mitotic centrosomes, which persists during mitotic exit after Noc removal.

DISCUSSION

It is now well established that mitotic spindle damage can uncouple mitotic execution from DNA synthesis (rereplication), leading cells to polyploidy and hence to an increased risk of neoplastic transformation. Several works indicate that functional p53 is implicated in regulatory networks that act to prevent polyploidization during cell exposure to spindle-damaging agents (3, 7, 8, 13, 25–26). These studies reported a durable, if not irreversable, p53-dependent inhibition of rereplication. However, in all tested experimental protocols, cells were continuously exposed to mitotic spindle poisons (3, 8, 13). Under those conditions, p21 and pRb can be viewed as downstream effectors of p53 acting to prevent polyploidization in cells in which spindle assembly is continuously inhibited (27–29).

In contrast, the role of p53 in the control of cell cycle progression after removal of spindle-inhibiting drugs has never been examined before. Here we have analyzed the response of cells transiently exposed to Noc and have followed up the subsequent release in Noc-free medium.

The present results indicate that transient exposure to Noc triggers the p53 pathway, which continues to operate in the following cell cycle, at least in human non-tumorigenic hematopoietic cells, by blocking the G1/S transition and preventing the following round of DNA replication. Thus, mitotic impair-
ment by spindle inhibition generates a prolonged signal that is maintained throughout cell division in the following cell cycle. The prolonged G1 arrest implicates p21 up-regulation at the gene and protein levels, and hence, presumably, cdk inhibition. By combining cytometry and immunofluorescence analysis, we have found that the p53 signal is first switched on in prometaphase-arrested cells by Noc, i.e. before chromosome segregation is completed, and hence before any genomic imbalance due to missegregation can be detected in daughter cells. Cell cycle arrest remains effective for at least 20 h after Noc release in AHH1 but fails in the p53−/− K562 cell line (Fig. 1A) and in AHH1 cell populations expressing a dominant negative mutant of p53 (Fig. 3), indicating that prolonged cell cycle arrest is clearly p53-dependent. At later times, i.e. 72 h post-release, arrest-proficient AHH1 cells eventually resume cell cycle progression (Fig. 1A); thus, a typical checkpoint (30) is reversibly activated in response to mitotic spindle failure. The prolonged p53 signal and ensuing cell cycle arrest detected in the present study in response to transient spindle inhibition represent novel aspects of the post-mitotic checkpoint and raise two major questions: (i) the nature of the cell cycle arrest-triggering signal and (ii) the pathway through which the arrest signal is maintained in daughter cells after mitotic exit.

Noc acts with a well established mechanism of action as a non-clastogenic, purely microtubule-directed drug (31; see Ref. 32 for a review). In conditions similar to those used in the present study, Noc lacks any DNA-damaging activity in comet assays.3 Thus, there are grounds to rule out the possibility that p53 induction by Noc reflects a DNA-damaging side effect of

Fig. 7. Colocalization of p53 and centrosomes is lost in Noc-exposed cells. A, metaphase; B, anaphase from asynchronously cycling AHH1 cell cultures; C, Noc-arrested prometaphase cell; D, anaphase exiting mitosis 90 min after Noc release. Chromosomes are visualized by DAPI staining (pseudocolored in blue, left column). γ-tubulin and p53 are pseudocolored in red (rhodamine-conjugated secondary antibody) and green (FITC-conjugated secondary antibody), respectively, in the middle column. Merged pictures are shown in the right column. The yellow signal in A and B indicates that p53 colocalizes with centrosomes in normal mitotic cells but not in Noc-exposed or released cells.

Table 1

| p53 localization                  | Asynchronous | Noc arrest | 45-Min release | 90-Min release |
|-----------------------------------|--------------|------------|----------------|----------------|
| Centrosomal                       | 136          | 0          | 2              | 0              |
| Delocalized from centrosomes      | 34           | 101        | 118            | 104            |
| Counted cells                     | 170          | 101        | 120            | 104            |

a Cells were cultured in Noc for 20 h.

b Centrosomes were visualized by indirect immunofluorescence for either centrin or γ-tubulin.
the drug rather than genuine spindle damage. In addition, the recent observation that p53 stabilization in response to noc is mediated by phosphorylation events that are distinct from those induced by DNA damage (18) further suggests that the pathway(s) of p53 induction in response to noc are specific and depend on transcription cascades distinct from those responsive to DNA damage.

Because noc primarily or exclusively targets tubulin (33, 34), and yet spindle assembly is rapidly resumed upon noc removal, giving rise to segregation of chromosomes to the poles (see Figs. 6 and 7), two major scenarios may be envisaged for the activation and maintenance of the p53-dependent checkpoint after transient exposure to noc. The checkpoint might be evoked in response to the possible occurrence of aneuploid cells during mitotic exit after noc removal, or the failure of the spindle structure might directly be sensed as a trigger for p53 activation. Although these scenarios are not necessarily mutually exclusive, in our opinion several considerations favor the latter possibility. First, in our experiments both the diploid and tetraploid cell populations, the latter being generated during mitotic slippage in the presence of noc, progress through the mitosis-to-G1 transition within 2 h from noc removal, as judged from the disappearance of mitotic markers (cyclin B- and MPM-reactive antigens) from the entire cell population and the resumption of G1 cyclin synthesis (Fig. 2). This molecular pattern is stabilized during the first 20 h after noc removal (Fig. 2). At this time, the p53 signal reaches its highest level in the cell population (Fig. 1), with a comparable intensity in the diploid and tetraploid cell populations by FACS analysis. These results indicate that G1 arrest takes place both in cells that achieve chromosome segregation and in cells that undergo mitotic slippage. This would suggest that the genome size does not per se constitute the signal for p53 activation. Furthermore, the trigger for p53 stabilization (Figs. 6 and 7) and transcriptional activation of the p21-encoding gene (Fig. 4) is already effective during noc exposure, i.e. before chromosome segregation occurs and before any genomic imbalance can be detected in daughter cells. It remains possible that the occurrence of aneuploid cells contributes to sustain the p53 signal; as a whole, however, the present observations are consistent with the view that mitotic spindle failure, rather than chromosome missegregation, triggers p53-dependent arrest in the cell cycle that follows spindle damage.

Spindle-targeting drugs including taxol, vincristin, and noc have recently been shown to provoke p53 stabilization by inducing specific phosphorylation events (18), implying that the spindle microtubules activate direct and specific pathways culminating with the induction of p53 activity. Consistent with this view, a recent study has highlighted p53 features that may disclose novel p53-dependent signaling mechanism(s). (i) p53 associates with microtubules in vitro and in vivo; (ii) this association is mediated by a specific domain responsible for the association of p53 with a and b tubulin and requires microtubule integrity and dynamics; (iii) p53 is transported along microtubules in a dynein-dependent manner (35). Although these observations are essentially derived from studies of interphase tubulin and microtubules, their implications can be of great relevance to the understanding of the role of p53 in mitosis. Microtubule polymerization inhibitors interfere with the subcellular localization of activities such as NuMA, which after nuclear envelope breakdown relocates from nuclei to the spindle poles via dynein-dependent transport along microtubules (36). Similarly, inhibition of spindle assembly by noc during mitosis may interfere with the localization of p53 to specific structures of the mitotic apparatus. In this work, we found that p53 normally localizes with or in the immediate proximity of the spindle pole component represented by centrosomes in mitotic cells (Fig. 5). This association is disrupted in the presence of noc and is not reestablished in cells that complete mitosis after noc removal (Figs. 6 and 7).

An increasing body of evidence now indicates that centrosomes act as pivotal structures in cell cycle regulatory networks; they actively participate in the cell division regulatory machinery (37 and references therein), and their own biogenesis and duplication are controlled by major cell cycle regulators, including cyclin-dependent kinases (38–42) and p53 itself (43–46). Indeed, impairment of p53 function results in centrosome overduplication (46, 47). Furthermore, in cells transformed with the adenovirus early gene product E1B, p53 is sequestered to cytoplasm, and its association with centrosomes during mitosis is prevented (43), indicating that p53 dysfunction can target the organization or activity of centrosomes at least in certain transformed cell types.

Subcellular localization is crucial to p53 function (35, 43, 48; for reviews see also Refs. 9, 49, and 50). It is tempting to hypothesize that the timely association of p53 with centrosomes constitutes an important regulatory step during the mitotic division. The present results indicate that p53 function, initially triggered by noc, does not operate to arrest mitotic progression itself but becomes effective during the following cell cycle. Because p53 continues to be up-regulated in G1, i.e. after the recovery of the spindle, we would like to suggest that the absence, albeit transient, of a functional spindle generates the signal that triggers the “mitotic” activation of p53. The spindle is the essential structural component that allows mitotic completion. The inhibition, even temporary, of this structure can evidently interfere with signaling processes(ies) mediated by the spindle itself.

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p53 Displacement from Centrosomes and p53-mediated G1 Arrest following Transient Inhibition of the Mitotic Spindle
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