p53 Localization at Centrosomes during Mitosis and Postmitotic Checkpoint Are ATM-dependent and Require Serine 15 Phosphorylation

A. Tritarelli,* E. Oricchio,* M. Ciciarello,* R. Mangiacasale,* A. Palena,* P. Lavia,* S. Soddu,[†] and E. Cundari*[‡]

*Istituto di Biologia e Patologia Molecolari Consiglio Nazionale delle Ricerche, 00185 Rome, Italy; and

[†]Dipartimento di Oncologia Sperimentale, Laboratorio di Oncogenesi Molecolare, Istituto Regina Elena, 00158 Rome, Italy

Submitted December 17, 2003; Accepted May 25, 2004

Monitoring Editor: Mark Solomon

We recently demonstrated that the p53 oncosuppressor associates to centrosomes in mitosis and this association is disrupted by treatments with microtubule-depolymerizing agents. Here, we show that ATM, an upstream activator of p53 after DNA damage, is essential for p53 centrosomal localization and is required for the activation of the postmitotic checkpoint after spindle disruption. In mitosis, p53 failed to associate with centrosomes in two ATM-deficient, ataxia-telangiectasia–derived cell lines. Wild-type ATM gene transfer reestablished the centrosomal localization of p53 in these cells. Furthermore, wild-type p53 protein, but not the p53-S15A mutant, not phosphorylatable by ATM, localized at centrosomes when expressed in p53-null K562 cells. Finally, Ser15 phosphorylation of endogenous p53 was detected at centrosomes upon treatment with phosphatase inhibitors, suggesting that a p53 dephosphorylation step at centrosome contributes to sustain the cell cycle program in cells with normal mitotic spindles. When dissociated from centrosomes by treatments with spindle inhibitors, p53 remained phosphorylated at Ser15. AT cells, which are unable to phosphorylate p53, did not undergo postmitotic proliferation arrest after nocodazole block and release. These data demonstrate that ATM is required for p53 localization at centrosome and support the existence of a surveillance mechanism for inhibiting DNA reduplication downstream of the spindle assembly checkpoint

INTRODUCTION

We have recently shown that, at the onset of mitosis, after nuclear envelope breakdown, p53 localizes at centrosomes. However, microtubule-depolymerizing agents disrupt the p53 centrosomal association, provoking p53 stabilization and arrest in the G1 phase of the following cell cycle (Ciciarello *et al.*, 2001). Although the latter finding strongly supports a previously identified role for p53 in the so-called postmitotic checkpoint (for details, see Minn *et al.*, 1996; Lanni and Jacks, 1998; Casenghi *et al.*, 1999), the biological significance of p53 localization at the centrosomes in mitosis still remains to be elucidated. It may be speculated that microtubule-driven transport to centrosomes prevents p53 from being activated “by default” in mitosis and that the failure of p53 association with centrosomes constitutes the trigger for postmitotic checkpoint activation.

In mitotic cells, where nucleus/cytoplasm compartmentalization is transiently disrupted, centrosomes are thought to provide a specialized environment in which the coordination of complex molecular interactions takes place (Palazzo *et al.*, 2000). Besides their activity as microtubule-organizing centers, centrosomes concentrate a number of

enzymes, e.g., protein phosphatases, kinases, and substrates that are not necessarily related to microtubule-organizing center function (Andreassen *et al.*, 1998; Palazzo *et al.*, 2000). These lines of evidence suggest a view of centrosomes as the “terminal hub” for the microtubule-mediated transport system in the cell (Rieder *et al.*, 2001). This system seems to be critical in p53 regulation because, after DNA damage, p53 is transported to the nucleus in a microtubule-mediated, dynein-dependent manner in interphase (Giannakakou *et al.*, 2000). The question arises as to which factor(s) contribute(s) to localize p53 at centrosomes and/or activate(s) p53 when this association is disrupted.

A major activator of p53 is the protein kinase ATM, impaired in the human cancer-prone syndrome ataxia telangiectasia (AT) (Savitsky *et al.*, 1995). After DNA damage, possibly due to the ensuing chromatin conformational changes, ATM is activated by autophosphorylation (Bakkenist and Kastan, 2003). This corresponds to a burst of ATM kinase activity that occurs within a few minutes after the induction of even a small number of double strand breaks. Once activated, ATM can phosphorylate p53 and its principal negative regulator MDM2, thus preventing p53 cytoplasmic relocalization and degradation (Shieh *et al.*, 1997; Maya *et al.*, 2001; McGowan, 2002). ATM phosphorylates p53 at serine residue 15 (Ser15) (Shieh *et al.*, 1997), and this modification seems to facilitate other posttranslational modifications, including further phosphorylation at other sites, acetylation, and association to p300 (Sakaguchi *et al.*, 1998; Ashcroft *et al.*, 1999; Dumaz and Meek, 1999). Because ATM is considered to be one of the main activators of p53 and is directly or

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E03-12-0900. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E03-12-0900.

[‡] Corresponding author. E-mail address: enrico.cundari@uniroma1.it.

Abbreviations used: AT, ataxia-telangiectasia; DAPI, 4',6-diamidino-2-phenylindole; PBS, phosphate-buffered saline.

indirectly involved in checkpoint arrest at almost all cell cycle phases (Kastan and Lim, 2000; for review, see Shiloh, 2003), ATM itself might be a good candidate as an activator of p53 when the latter is dissociated from the mitotic apparatus, e.g., after inhibition of mitotic spindle formation by microtubule-depolymerizing agents.

This work analyzes p53 subcellular localization during mitosis in human lymphoblastoid cell lines derived from a healthy individual or two AT patients. We unequivocally show that ATM is involved in p53 localization to the centrosomes in mitosis and phosphorylation at Ser15 is a critical step in this process. We also provide evidence that the ATM-dependent association of p53 to centrosomes is strictly linked to the postmitotic checkpoint response to spindle disruption. This constitutes a novel function for ATM and describes a novel phenotype for AT cells.

MATERIALS AND METHODS

Cell Culture and Transfection

Cells from the human lines AHH1 (normal lymphoblastoid), GM02782 and GM03189 (AT lymphoblastoid), and K562 (erythroleukemia) were grown in RPMI 1640 medium (Euroclone Life Science Division, Perd, Italy) supplemented with 15% fetal calf serum in a 5% CO₂ atmosphere at 37°C. Exponentially proliferating cultures were used for all experiments. When required, caffeine or NaF was directly added to the growth medium at the final concentrations of 5 μ M for 20 h and 20 mM for 1 h, respectively. Where indicated, 3×10^6 cells/sample were transfected with 10 μ g of plasmid DNA in 400 μ l of cold phosphate-buffered saline (PBS) by electroporation (975 μ F and 250 mV) with a Gene Pulser II (Bio-Rad, Hercules, CA). The following plasmids were used: hemagglutinin (HA)-tagged wild-type ATM kinase cDNA (Baskaran *et al.*, 1997), human wt-p53 cDNA, or the p53-S15A mutant cloned in the pCAG3.1 vector.

Immunofluorescence Analysis

Cells were washed, spread on glass coverslips by centrifugation, and fixed in 3.7% formaldehyde for 15 min at 4°C. Cells were permeabilized in 0.25% Triton X-100 for 5 min at room temperature and 100% methanol for 10 min at -20°C. Fixed and permeabilized cells were preincubated in 20% goat serum for 30 min at 37°C in a humidified chamber, and then incubated for 2 h with mouse monoclonal anti-p53 antibody (1:100 dilution, clone DO-7; DakoCytomation Denmark A/S, Glostrup, Denmark), rabbit anti-phospho-p53 (Ser15) antibody (1:2000 dilution, #9284; Cell Signaling Technology, Beverly, MA), rabbit anti- γ -tubulin antibody (1:2000 dilution, T3559; Sigma-Aldrich, St. Louis, MO), all in 5% goat serum. After three washes in PBS + 0.05% Tween 20, cells were incubated with rhodamine-conjugated anti-rabbit antibody (1:600 dilution, sc2091; Santa Cruz Biotechnology, Santa Cruz, CA), and fluorescein isothiocyanate-conjugated anti-mouse antibody (1:600 dilution, FI2000; Vector Laboratories, Burlingame, CA) in 5% goat serum for 30 min at 37°C. Cell spreads were thoroughly washed in PBS + 0.05% Tween 20, counterstained with 0.1 μ g/ml 4',6-diamidino-2-phenylindole (DAPI), and incubated for 1 min at room temperature to stain the DNA. Spreads were then washed in PBS and finally mounted on glass slides in Vectashield (Vector Laboratories). Cell preparations were examined under an AX70 microscope (Olympus, Tokyo, Japan) equipped with epifluorescence, and photographs were taken using a charge-coupled device camera device (Photometrics, Tucson, AZ).

Protein Extract Preparation and Western Immunoblotting Assays

Aliquots of 5×10^6 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 saline containing 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich) and lysed essentially as described previously (Ciciarello *et al.*, 2001). Cell lysates were centrifuged at 14,000 rpm, 4°C, for 30 min. Protein concentrations were determined using the Bradford assay kit (Bio-Rad, Hercules, CA). 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, Dassel, Germany). Membranes were blocked in 5% (wt/vol) low-fat milk in TBST buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween 20) at 4°C overnight, then incubated for 1 h at room temperature with the following primary antibodies in 5% milk/Tris-buffered saline/Tween 20: anti-cyclin B1 (GNS1; Santa Cruz Biotechnology), anti-p53 (DO-7; DakoCytomation Denmark A/S), anti-p21 (C-19; Santa Cruz

Biotechnology), and rabbit anti-phospho-p53 (Ser15) antibody (#9284; Cell Signaling Technology). All primary antibodies were used at 0.5 μ g/ml. Immunoreactive proteins were detected using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and revealed using the enhanced chemiluminescence system (ECL Plus; Amersham Biosciences, Piscataway, NJ).

Flow Cytometry Analysis

DNA replication was essentially monitored as described previously (Ciciarello *et al.*, 2001), after addition of 5-bromo 2'-deoxyuridine (BrdUrd; 45 μ M 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, and incubated in 3 M HCl for 45 min to denature the DNA. Cells were then exposed to anti-BrdUrd monoclonal antibody (DakoCytomation Denmark A/S), to the secondary fluorescein isothiocyanate-conjugated antibody (Vector Laboratories), and finally stained with propidium iodide (PI). Ten thousand events were acquired for each sample using a FACStar Plus fluorescence-activated cell sorter (BD Biosciences, San Jose, CA) flow cytometer and analyzed by FACS WinMDI software. 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 as to place the peak corresponding to 2C DNA content (G0/G1) at channel 200 in the FL2-H histogram. Cell aggregates were carefully gated out using FL2-W versus FL2-A bivariate graphs.

RESULTS

The ATM Kinase Is Required for the Mitotic Localization of p53 at Centrosomes

We have previously shown that, during mitosis, the p53 onco-suppressor protein is reorganized in the form of "spots" associated with centrosomes. Furthermore, the induction of mitotic spindle depolymerization is associated with p53 displacement from centrosomes and the activation of the p53-dependent postmitotic checkpoint (Ciciarello *et al.*, 2001). To investigate whether the protein kinase ATM is involved in this process, the subcellular localization of p53 in mitosis was assessed in two human lymphoblastoid cell lines derived from AT patients (GM02782 and GM03189) and compared with the pattern previously characterized in the AHH1 lymphoblasts derived from a healthy donor (Ciciarello *et al.*, 2001). Immunostaining of centrosomes (γ -tubulin) and p53 confirmed the centrosomal localization of p53 during mitosis in normal AHH1 cells (Figure 1A). In contrast, in cells from both AT lines, p53 was still distributed in discrete "spots" that failed to colocalize with centrosomes (Figure 1, B and C), suggesting a causal role for ATM in the centrosomal localization of p53, even independently of induction of mitotic spindle failure (i.e., nocodazole [NOC]-induced activation of postmitotic checkpoint). To test this hypothesis, ATM was inhibited in the normal AHH1 cells by exposing to caffeine for 20 h (Blasina *et al.*, 1999). This treatment provoked a complete dissociation of p53 from centrosomes in mitosis (Figure 1D). Consistent with this, expression of exogenous wild-type ATM protein, obtained by transient transfection of AT lymphoblastoid cells and verified by Western blotting (our unpublished data), corrected the p53 mislocalization. Indeed, >95% of mitotic GM03189 cells transfected with empty vector had dispersed p53 spots in the cytoplasm (our unpublished data), whereas 48% of mitotic cells exhibited centrosome-associated p53 in the ATM-corrected cultures (Figure 1E). Because in our experiments the average efficiency of transfection was $\approx$ 50%, as determined by parallel transfection with a green fluorescent protein (GFP)-expressing vector at the same molarity, we can conclude that almost all of the transfectants were able to relocalize p53 at the centrosome. Together, these data demonstrate that ATM is critical for p53 association to centrosomes during mitosis.

ATM-dependent Phosphorylation of p53 at Ser15 Is Required for Centrosomal Localization

A priori, the function of ATM in targeting p53 to mitotic centrosomes may have been exerted either by directly phos-

Figure 1. Immunostaining of p53 and centrosomes (γ -tubulin) in mitotic lymphoblastoid cells derived from a normal donor (AHH1) or two AT patients (GM02782 and GM03189). In red, γ -tubulin; in green, p53; and in blue, DNA counterstained with DAPI. (A) AHH1 cells. (B) GM02782 (AT). (C) GM03189 (AT). (D) AHH1 cells treated with 5 μ M caffeine. (E) GM03189 transfected with wild-type ATM. Bar, 10 μ m.

phorylating p53 at critical site(s), or by activating the cascade of downstream effectors such as chk1 or chk2 (Shiloh, 2003). Because the only ascertained site of direct phosphorylation by ATM on p53 is Ser15, we used a mutant form of p53, in which the Ser15 was replaced with an alanine (p53-S15A), which cannot be phosphorylated by ATM (Khanna *et al.*, 1998), to transiently transfect the human p53-null K562 cells. Parallel cultures were transfected with constructs expressing wild-type-p53. K562 cells were immunostained for p53 and γ -tubulin, and the proportion of cells showing the p53/centrosome association during mitosis was determined. In our analysis, only those cells in which both centrosomes exhibited the p53 signals were considered positive for the association. Cells in which only one or no centrosomes colocalized with p53 were considered negative. Figure 2 de-

Figure 2. Immunostaining of p53 and centrosomes (γ -tubulin) in mitotic K562 cells. In red, γ -tubulin; in green, p53; and in blue, DNA counterstained with DAPI. (A) K562 cells transfected with wt-p53. (B) K562 cells transfected with p53-S15A. Bar, 10 μ m.

picts examples of K562 mitotic cells transfected with either wt-p53 (Figure 2A) or p53-S15A mutant (Figure 2B). We found that wild-type-p53 protein was detected at both centrosomes in almost all K562 mitotic cells (76%), whereas only a minority of the cells (24%) exhibited either one or no p53 spots at centrosomes. On the contrary, the nonphosphorylatable p53-S15A mutant did not colocalize with centrosomes. The quantitative evaluation of these experiments is reported in Figure 3. Together, these data indicate that the correct localization of p53 at centrosomes during mitosis in human lymphoblastoid cells is ATM dependent, and furthermore, identify phosphorylation at Ser15 as a functional requirement in this process.

Figure 3. Frequency of mitotic cells presenting p53 localized at centrosomes (black bars) or dissociated from centrosomes (hatched bars). At least 200 cells from two independent experiments were counted for each culture condition. Interexperimental variation was always <5%. Bars represent the averages of the two experiments. AHH1, GM02782, and GM03189, untreated cells; AHH1 caff., AHH1 cells treated with 5 μ M caffeine; GM03189 e.v., transfected with empty vector; GM03189 corr., transfected with an ATM expression vector; K562 p53 w.t., transfected with a wt-p53 expression vector; and K562 p53ser/ala15, transfected with the p53-S15A mutant expression vector.

Figure 4. Immunostaining of p53 phosphorylated at Ser15 and centrosomes (γ -tubulin) in AHH1 cells untreated (A), exposed to 20 mM NaF for 1 h (B), exposed to 0.2 μ g/ml NOC for 20 h (C).

Wild-Type p53 Undergoes Rapid Dephosphorylation at Mitotic Centrosomes

Based on the findings reported thus far, it might be expected that p53 is phosphorylated at Ser15 when it is at the centrosome. Surprisingly, when AHH1 cells were immunostained with a phospho-p53ser15-specific antibody (see MATERIALS AND METHODS), no signal was found at the centrosomes of mitotic cells (Figure 4A). However, when the same cells were exposed to the general serine-phosphatase inhibitor NaF before immunostaining with the anti-phospho-p53ser15 antibody, a clear positive signal was detected at the centrosomes of mitotic cells (Figure 4B), suggesting that, when associated to centrosomes, p53 is rapidly dephosphorylated at Ser15. On the contrary, when p53 association to centrosome was prevented by exposure to the microtubule depolymerizing drug NOC (Ciciarello *et al.*, 2001), the p53 spots remained reactive to the anti-phospho-p53ser15 antibody (Figure 4C).

ATM-defective Cells Lack the Postmitotic Checkpoint Response to MT-targeting Drugs

The observation suggests that impairment of the mitotic spindle due to either depolymerization of microtubules or to hampered microtubule dynamics keeps p53 in the phosphorylated form by preventing its localization to the centrosome, where it would otherwise undergo dephosphorylation; the persistence of phosphorylated p53 might therefore constitute the trigger of postmitotic checkpoint. If that hypothesis is correct, then AT cells, which are unable to phosphorylate p53 even when the latter is not associated to centrosomes, should resume proliferation after treatment with NOC. To test this hypothesis, we first verified that AT cells are actually unable to phosphorylate p53 at Ser15 when

treated with NOC. Western blot analysis of phospho-p53ser15 in the normal AHH1 and in two AT cell lines shows that NOC induces a strong signal of p53 phosphorylation only in AHH1 cells but not in AT cell lines (Figure 5A). Cells from all three lines were then exposed to the NOC block-and-release protocol as described previously (Ciciarello *et al.*, 2001), and cell cycle progression was monitored by FACS analysis of BrdU incorporation (Figure 5B). As expected, 20 h after release from the NOC block, AHH1 cells were still arrested and almost no S-phase cell (i.e., positive for BrdU incorporation) was present in the culture. On the contrary, both AT cell lines promptly resumed proliferation, and 20 h after release, the FACS graphs were undistinguishable from those of an asynchronously growing culture. Western blot analysis showed that, in AHH1 cells, cyclin B accumulated during the first 20 h of culture in the presence of NOC, as expected, and was fully degraded within the first 4 h of the block release, indicating that mitosis was resumed and completed. Yet, cyclin B expression was not resumed for as long as 20 h after NOC release. On the contrary, both AT cell lines expressed fluctuating levels of cyclin B as would be expected of an actively proliferating cell culture (Figure 5C).

A central feature of the postmitotic checkpoint in normal cells, such as AHH1, is that the levels of expression of both p53 and its transcriptional target p21 increase significantly and constantly, even after release from NOC-induced arrest (Figure 6, top; Ciciarello *et al.*, 2001). In contrast, up-regulation of p53 was largely defective in both AT cell lines; more specifically, no change in p53 expression was recorded in the GM02782 line, whereas in GM03189 cells, the level of p53 increased slightly during exposure to NOC but remained constant after release (Figure 6). This was paralleled by the pattern of p21 expression. In the presence of NOC, both AT cell lines underwent transient up-regulation of p21, similar to AHH1 cells. However, differences became apparent after NOC removal; whereas AHH1 maintained high levels of p21, concomitant with the induction of durable cell cycle arrest (Ciciarello *et al.*, 2001; this study, Figure 5), p21 levels decreased abruptly in both AT cell lines, similar to the pattern observed in K562 cells (Figure 6, bottom row), which do not express p53 and lack the postmitotic checkpoint (Casenghi *et al.*, 1999).

After NOC removal, cells rapidly exited mitosis, and p53 was found to relocalize in the reforming interphase nuclei, both in normal AHH1 cells and in the two AT cell lines (Figure 7). However, postmitotic AHH1 cell nuclei contained very high levels of p53 as revealed by the extremely bright signals recorded by immunofluorescence analysis (Figure 7A). In contrast, AT cell nuclei, analyzed under identical image settings, revealed a very weak immunostaining for p53 (Figure 7, B and C).

DISCUSSION

The present work was undertaken to investigate the role of ATM in the postmitotic checkpoint, and more specifically, in p53 localization to the centrosomes in mitosis. In summary, when ATM is not functional, i.e., in AT cells or after inhibition by caffeine in normal cells, p53 fails to associate to centrosomes in mitosis. The same phenotype can be reproduced in an ATM-proficient cell context by preventing p53 phosphorylation at the ATM-specific target site. These results indicate that ATM relocalizes p53 to mitotic centrosomes, and furthermore that phosphorylation at Ser15 is a critical step in this process. To our knowledge, the present results provide the first evidence for a novel ATM function that, interestingly, is not related to DNA damage signaling.

Figure 5. (A) Western blot analysis of phospho-ser15-p53 (for details, see MATERIALS AND METHODS) in untreated cultures or after exposure to 0.2 μ g/ml NOC for 20 h. (B) Cell-cycle distribution as measured by FACS analysis of DNA content versus BrdU incorporation of asynchronously growing untreated cultures (CTR), after exposure to 0.2 μ g/ml NOC for 20 h (NOC) or at different times of release (rel.) from NOC-induced arrest. (C) Western blot analysis of cyclin B expression in the same experimental conditions as in B.

We previously showed that when cells enter mitosis and nuclear envelope breakdown occurs, p53 is relocated at centrosomes; however, when mitotic spindle assembly is inhibited by microtubule-depolymerizing drugs, p53/centrosome association is prevented. Under these conditions, cells can still achieve the mitotic division, but then arrest in the following G1 (Casenghi *et al.*, 1999; Ciciarello *et al.*, 2001). Interestingly, a similar phenotype has been described after

physical centrosome ablation in S or G2 phases (Hinchcliffe *et al.*, 2001; Khodjakov and Rieder, 2001), or inactivation of centrosomal components by RNA interference (Gromley *et al.*, 2003). In these instances, mitosis progression is not affected per se, but cells cannot undergo further replication and are arrested in the following G1 phase. In other words, centrosomes must be present and functional to permit post-mitotic cells to proliferate. Our data strongly suggest that

Figure 6. Western blot analysis of p53 and p21 expression in cell cultures exposed to NOC block and release as described in Figure 5, B and C.

p53 is phosphorylated by ATM at Ser15 at each mitotic onset and promptly dephosphorylated at the centrosomes.

When the centrosomal localization of p53 is prevented by inhibiting polymerization of the spindle microtubules, p53 remains phosphorylated and cells arrest in G1 after exit from mitosis (Ciciarello *et al.*, 2001; this study). On the contrary, when ATM-dependent p53 phosphorylation is prevented, as in the case of AT cells, even though p53 does not associate to centrosomes, cells resume proliferation after removal of the mitotic-spindle-damaging agent, implying that a signal emanating from the mitotic spindle was defective.

It is noteworthy that, when dissociated from centrosomes, p53 is always present under the form of aggregates that are distributed randomly in the mitotic cytoplasm. The formation of these “p53-somes” does not require functional ATM because it occurs in AT mitotic cells as well as in NOC-treated normal cells and is independent of p53 phosphorylation status. It will be of interest to investigate the nature of the p53 spots and the mechanisms underlying their formation.

It is tempting to speculate that the interplay between p53 and centrosomes, mediated by ATM, provides postmitotic cells with a further checkpoint control that can “carry over” a specific arrest signal in response to mitotic spindle impairment, and temporally be placed downstream of the spindle-assembly checkpoint. In this model, ATM would be activated “by default” in mitosis, e.g., by autophosphorylation due to the intervening chromatin modifications, similar to what has been described after DNA damage (Bakkenist and Kastan, 2003). The next step would be p53 phosphorylation at Ser15. In the presence of a functional mitotic spindle, this form is specifically targeted to centrosomes, rapidly dephosphorylated and transmitted to daughter cells in an inactive, proliferation-permissive form. On the contrary, when mitotic spindle is impaired, p53 phosphorylated at Ser15 would transduce a signal to daughter cells, culminating with stabilization and increased levels, which drives G1 arrest with the typical postmitotic checkpoint modality (Ciciarello *et al.*, 2001). ATM would preemptively trigger p53 activation at each mitotic onset so as to render it ready to stop proliferation in case of structural or functional inactivation of the mitotic spindle, or to keep it inactive at the centrosomes when the spindle is correctly in place.

ACKNOWLEDGMENTS

We thank Dr. Barbara Di Fiore for invaluable suggestions and help and Silvia Bonaccorsi for critically revising the manuscript. This work was supported by Consiglio Nazionale delle Ricerche, the Italian Space Agency, and Italian Ministero della Salute. This work is dedicated to the memory of Franco Tatò, a great scientist and a great man.

REFERENCES

- Andreassen, P.R., Lacroix, F.B., Villa-Moruzzi, E., and Margolis, R.L. (1998). Differential subcellular localization of protein phosphatase-1 α , γ 1, and δ isoforms during both interphase and mitosis in mammalian cells. *J. Cell Biol.* 141, 1207–1215.
- Ashcroft, M., Kubbutat, M.H., and Vousden, K.H. (1999). Regulation of p53 function and stability by phosphorylation. *Mol. Cell. Biol.* 19, 1751–1758.
- Bakkenist, C.J., and Kastan, M.B. (2003). DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* 421, 499–506.
- Baskaran, R., *et al.* (1997). Ataxia telangiectasia mutant protein activates c-Abl tyrosine kinase in response to ionizing radiation. *Nature* 387, 516–519.
- Blasina, A., Price, B.D., Turenne, G.A., and McGowan, C.H. (1999). Caffeine inhibits the checkpoint kinase ATM. *Curr. Biol.* 9, 1135–1138.
- Casenghi, M., Mangiacasale, R., Tuynder, M., Caillet-Fauquet, P., Elhajouji, A., Lavia, P., Mousset, S., Kirsch-Volders, M., and Cundari, E. (1999). p53-independent apoptosis and p53-dependent block of DNA rereplication following mitotic spindle inhibition in human cells. *Exp. Cell Res.* 250, 339–350.
- Ciciarello, M., Mangiacasale, R., Casenghi, M., Limongi, M.Z., D'Angelo, M., Soddu, S., Lavia, P., and Cundari, E. (2001). p53 displacement from centro-

Figure 7. Immunostaining of p53 and centrosomes (γ -tubulin) in mitotic lymphoblastoid cells after 20 h of release from NOC-induced arrest. In red, γ -tubulin; in green, p53; and in blue, DNA counterstained with DAPI. (A) AHH1 cells. (B) GM02782 (AT). (C) GM03189 (AT). Bar, 10 μ m.

- somes and p53-mediated G1 arrest following transient inhibition of the mitotic spindle. *J. Biol. Chem.* 276, 19205–19213.
- Dumaz, N., and Meek, D.W. (1999). Serine15 phosphorylation stimulates p53 transactivation but does not directly influence interaction with HDM2. *EMBO J.* 18, 7002–7010.
- Giannakakou, P., Sackett, D.L., Ward, Y., Webster, K.R., Blagosklonny, M.V., and Fojo, T. (2000). p53 is associated with cellular microtubules and is transported to the nucleus by dynein. *Nat. Cell Biol.* 2, 709–717.
- Gromley, A., Jurczyk, A., Sillibourne, J., Halilovic, E., Mogensen, M., Groisman, I., Blomberg, M., and Doxsey, S. (2003). A novel human protein of the maternal centriole is required for the final stages of cytokinesis and entry into S phase. *J. Cell Biol.* 161, 535–545.
- Hinchcliffe, E.H., Miller, F.J., Cham, M., Khodjakov, A., and Sluder, G. (2001). Requirement of a centrosomal activity for cell cycle progression through G1 into S phase. *Science* 291, 1547–1550.
- Kastan, M.B., and Lim, D.S. (2000). The many substrates and functions of ATM. *Nat. Rev. Mol. Cell. Biol.* 1, 179–186.
- Khanna, K.K., *et al.* (1998). ATM associates with and phosphorylates p 53, mapping the region of interaction. *Nat. Genet.* 20, 398–400.
- Khodjakov, A., and Rieder, C.L. (2001). Centrosomes enhance the fidelity of cytokinesis in vertebrates and are required for cell cycle progression. *J. Cell Biol.* 153, 237–242.
- Lanni, J.S., and Jacks, T. (1998). Characterization of the p53-dependent post-mitotic checkpoint following spindle disruption. *Mol. Cell. Biol.* 18, 1055–1064.
- Maya, R., *et al.* (2001). A.T.M.-dependent phosphorylation of Mdm2 on serine 395, role in p53 activation by D.N.A. damage. *Genes Dev.* 15, 1067–1077.
- McGowan, C.H. (2002). Checking in on Cds1 (Chk2): a checkpoint kinase and tumor suppressor. *Bioessays* 24, 502–511.
- Minn, A.J., Boise, L.H., and Thompson, C.B. (1996). Expression of Bcl-xL and loss of p53 can cooperate to overcome a cell cycle checkpoint induced by mitotic spindle damage. *Genes Dev.* 10, 2621–2631.
- Palazzo, R.E., Vogel, J.M., Schnackenberg, B.J., Hull, D.R., and Wu, X. (2000) Centrosome maturation. *Curr. Top. Dev. Biol.* 49, 449–470.
- Rieder, C.L., Faruki, S., and Khodjakov, A. (2001). The centrosome in vertebrates: more than a microtubule-organizing center. *Trends Cell Biol.* 11, 413–419.
- Sakaguchi, K., Herrera, J.E., Saito, S., Miki, T., Bustin, M., Vassilev, A., Anderson, C.W., and Appella, E. (1998). DNA damage activates p53 through a phosphorylation-acetylation cascade. *Genes Dev.* 12, 2831–2841.
- Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D.A., Smith, S., and Uziel, T. (1995). A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science* 268, 1749–1753.
- Shieh, S.Y., Ikeda, M., Taya, Y., and Prives, C. (1997). DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell* 91, 325–334.
- Shiloh, Y. (2003). ATM and related protein kinases: safeguarding genome integrity. *Nat. Rev. Cancer* 3, 155–168.
- Watters, D., Kedar, P., Spring, K., Bjorkman, J., Chen, P., Gatei, M., Birrell, G., Garrone, B., Srinivasa, P., Crane, D.I., and Lavin, M.F. (1999). Localization of a portion of extranuclear ATM to peroxisomes. *J. Biol. Chem.* 274, 34277–34282.