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ARTICLES

ATM is Activated by Mitotic Stress and Suppresses Centrosome Amplification in Primary but not in Tumor Cells

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Abstract Centrosome amplification has been proposed to contribute to the development of aneuploidy and genome instability. Here, we show that Ataxia-Telangiectasia Mutated (ATM) is localized to the centrosome and co-purified with γ-tubulin. The importance of ATM in centrosome duplication is demonstrated in *Atm*-deficient primary mouse embryonic fibroblasts that display centrosome amplification. Interestingly, centrosome amplification was not observed in tumor cell lines derived from *Atm* and *p21* double deficient mouse. Our results also indicate that both p53 and p21 operate in the same pathway as ATM in regulating centrosome biogenesis. Finally, a potential role of ATM in spindle checkpoint regulation is demonstrated by which ATM protein is activated by mitotic stress. These results suggest a role of ATM in spindle checkpoint regulation and indicate that ATM suppresses genome instability and cellular transformation by regulating centrosome biogenesis. J. Cell. Biochem. 99: 1267–1274, 2006.

Key words: Ataxia-Telangiectasia; ATM; centrosomes; micronuclei; mitotic spindle checkpoint; mitotic stress; p53

The metazoan centrosome, also known as the microtubule-organizing centers, consists of two centrioles and surrounding amorphous peri-centriolar materials. Centrosome duplication occurs at the G1/S transition and is coordinated with the onset of DNA synthesis. The centrosome duplication cycle has several distinct phases that include centriolar splitting, centrosome duplication, maturation, and separation. Centrosome duplication completes prior to the onset of mitosis and centrosomes function as spindle poles that direct the formation of bipolar mitotic spindles during mitosis [Doxsey, 2001; Hinchcliffe and Sluder, 2001]. The coordination of centrosome duplication with onset of DNA synthesis can be partly explained by the dual requirement of cyclin dependent kinase 2 (CDK2) in these processes [Hinchcliffe and Sluder, 2002].

Since centrosomes are required for G1/S progression, proper orientation of the mitotic spindle, and cytokinesis, centrosome irregularity may contribute to deregulation of cell cycle. Indeed, centrosome abnormalities are common features of tumor samples [Pihan et al., 1998; Nigg, 2002]. Some of these abnormalities include numerical (usually increased numbers of centrosomes, or amplification) and structural (i.e., centrosome size variation and acentriolar bodies). In particular, centrosome amplification is detected in ductal carcinomas in situ (DCIS) of the breast, suggesting that centrosome abnormalities are early events in tumorigenesis. In addition, centrosome amplification has been observed in over 80% of invasive breast

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carcinomas and is correlated with aneuploidy and chromosomal instability [Lingle et al., 2002]. These results collectively argue that centrosome abnormalities precede chromosomal instability, and centrosome amplification is the driving force in genome instability frequently observed in cancer cells [Pihan et al., 1998; Nigg, 2002].

Recent evidence indicates that centrosome duplication not only couples to the cell cycle progression but also is linked to DNA damage response and repair pathways [Sibon et al., 2000; Su and Vidwans, 2000; Dodson et al., 2004]. For example, deficiency of a number of genes involved in DNA repair and damage responses, such as *Brca1, Brca2, Rad51, p53, Parp-1*, has been linked to centrosome abnormalities [Fukasawa et al., 1996; Tutt et al., 1999; Deng, 2002; Kanai et al., 2003; Dodson et al., 2004]. Ataxia-Telangiectasia Mutated (ATM) is a protein kinase of the PIKK family that is activated by DNA damage and oncogene overexpression. The protein kinase ATM is essential in sensing DNA damage and activates cell cycle checkpoints through regulation of downstream target proteins. Some of these ATM targets, such as Brca1, p53, MDM2, and CHK2/hCDS1 have been causatively linked to S-phase checkpoint regulation and cancer development [Shiloh, 2003]. Furthermore, ATM signaling pathway is activated during the early stages of tumor progression and ATM activation has been suggested as the early host response to suppress cellular transformation [Bartkova et al., 2005; Gorgoulis et al., 2005]. Mutations in ATM gene cause Ataxia-Telangiectasia (A-T), which is characterized by progressive cerebellar degeneration, immune deficiencies, premature aging, and predisposition to cancer [Shiloh, 2003].

We have recently obtained evidence indicating that *Atm*-deficient cells exhibit both numerical chromosomal instability as well as structural chromosomal instability [Shen et al., 2005]. We found that p21 specifically suppressed further numerical chromosomal instability in an *Atm*-deficient background. Furthermore, we observed that Atm null cells are defective in chromosomal segregation during metaphase–anaphase transition with increased anaphase bridges being observed in Atm-deficient cells. Given that centrosome amplification and ATM activation have been proposed as early events in cellular transformation and tumor progression, the role of ATM in centrosome biogenesis was examined in this study. Here, we show that ATM is detected in purified centrosomes. *Atm*-deficiency in primary cells derived from mouse embryos contributes to defective centrosome biogenesis exemplified by centrosome amplification. Interestingly, centrosome amplification was not a general feature in tumor cell lines derived from *Atm* and *p21* deficient animals. Finally, we show that ATM is activated by mitotic stress, suggesting a role of ATM in mitotic progression. Collectively, our data suggest that ATM suppresses genome instability and cellular transformation by regulating centrosome biogenesis.

MATERIALS AND METHODS

Cell Culture

Primary MEFs culture for wild-type, $p21^{-/-}$, $Atm^{-/-}$, and $Atm^{-/-}p21^{-/-}$ were carried out as described previously [Wang et al., 1997]. $Atm^{-/-}p53^{-/-}$ MEFs were kindly provided by C. Westphal and P. Leder and cultured as described [Westphal et al., 1997]. Atm-deficient tumor cell lines were generated from primary tumors derived from Atm and p21 double knock out mice. Tumors were minced with razor followed by trypsin digestion for 30 min at 37°C. Tumor cell lines were established from these primary cultures without extensive passage in culture. T7211, T1063, and T1066 cell lines were derived from large embryonic-type tumor, small-cell mammary adenocarcinoma, and osteosarcoma of the $Atm^{-/-}p21^{-/-}$ mice, respectively. HeLa cells was obtained from ATCC. AT22IJE-T cell line and ATM-complemented AT22IJE-T cells were provided by Drs. M. Kastan and Y. Shiloh. All primary cells as well as cell lines were cultured in DMEM containing 10% fetal bovine serum and antibiotics.

Micronuclei Analysis

Primary MEFs at passage 1 or 2 or tumor cell lines were plated onto 2-well Nunc slides overnight in growth media. MEFs or tumor cell lines were stained with 5 μg/ml propidium iodide and examined under the fluorescence microscope. At least 200 cells for each cell type were counted from three experiments.

Production of Monoclonal Antibody

N-terminal sequence from amino acid 1 to 93 of ATM was PCR amplified with a pair of AT26

and AT27 primers (AT26: 5′-gatggatccgccaccatgagtctagtacttaatgatctgc-3′; AT27: gatgaattccttttctgcctggaggcttgtg-3′) using full length ATM cDNA as template (kindly provided by M. Kastan). The PCR product was digested with BamHI and EcoRI and subcloned into pGEX6p-1. The bacterial expression plasmid was transformed into BL21(DE3)pLysS cells and protein production was induced by IPTG and affinity purified. Purified GST-ATM N-terminal fusion protein was used to immunize mice for monoclonal antibody production according to the standard protocol [Harlow and Lane, 1988]. Eleven monoclonal hybridoma cell lines were produced, two of these clones (8F5 and 7B2) were further characterized by immunoblot, immunoprecipitation, and immunokinase assay. Both monoclonal antibodies were isotyped to be IgG2a and could be used for immunoblot, immunoprecipitation, and ATM kinase assay. Both monoclonal antibodies were further verified for cross reactivity to other commercial ATM antibodies (unpublished observation). 7B2 monoclonal antibody was used in this study.

Purification of Centrosomes

Centrosome purification from HeLa cells was performed as described [Hsu and White, 1998] with slight modification in which cytochalasin D and nocodazole treatment prior to cell harvesting were omitted since this alteration of procedure does not affect centrosome purification and nocodazole activates ATM (see Results).

Protein Extraction and Immunoblot Analysis

HeLa cells were treated with nocodazole or taxol for several hours and both floating (mitotic cells) and attached cells were harvested. After washing in cold PBS, the cells were extracted with NP40 buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8, and 1% IGEPAL CA630) containing 1× cocktail of proteinase inhibitors (Roche) and total cell lysates were collected after a centrifugation step at 14 k rpm for 10 min at 4°C. Hundred micrograms of total proteins were fractionated in 10% SDS–PAGE gel and the fractionated proteins were transferred to PVDF membrane (BioRad). After a brief blocking of non-specific binding sites in milk, the PVDF membrane was incubated with the appropriate primary antibody followed by an appropriate HRP-conjugated secondary antibody. Signals were visualized by ECL method (Amersham). Antibodies for ATMpS1981 and Aurora A were purchased from Cell Signaling; MCM2 and PLK1 antibodies were from SantaCruz; γ-tubulin and β-actin antibodies were from Sigma.

Immunofluorescence Analysis

Cells were plated on Nunc slide overnight in growth media and fixed for 10 min at room temperature in 2.5% paraformaldehyde in PBS buffer containing 25 mM $MgCl_2$. Cells were then permeabilized in PBS containing 0.2% Triton X-100 after washing the cells with PBS containing 0.3 M glycin. Incubation with anti-γ-tubulin diluted at 1:1,000 was carried out overnight at 4°C [Fukasawa et al., 1996]. After three washes with PBS following incubation with primary antibody, cells were incubated with FITC conjugated antimouse IgG for 30 min at room temperature, followed by nuclear staining with 4′,6′-diamidino-2-phenylindole (DAPI) for 5 min. Slides were mounted with antifade (Molecular Probes) and examined under a fluorescence microscope.

RESULTS

We have previously observed increased aneuploidy and chromosomal translocations in $Atm^{-/-}$ MEFs [Shen et al., 2005]. To further characterize genome instability observed in $Atm^{-/-}$ cells, we examined micronuclei formation (broken pieces of chromosomes) as an index for measuring genome instability. Early passage cells (passage 1 from 2) were stained with propidium iodide and micronuclei in $Atm^{-/-}$ cells were determined. We found that there were increased micronuclei (μN) in $Atm^{-/-}$ cells compared to wild-type cells (Fig. 1A,B,D). In addition, a slight increase in macronuclei (mN) due to either cell fusion or endoreduplication was also observed in $Atm^{-/-}$ cells while those cells were never seen in wild-type population (Fig. 1C,D). Since we found previously that there is an increased numerical chromosomal instability in $Atm^{-/-}p21^{-/-}$ cells, we wish to examine whether this is correlated with increased micronuclei formation [Morita et al., 1997]. In addition, we also analyzed micronuclei formation in $Atm^{-/-}p53^{-/-}$ cells to ascertain the epistatic interaction of Atm, p53, and, p21 in the suppression of micronuclei formation and genome instability. We found that there is no

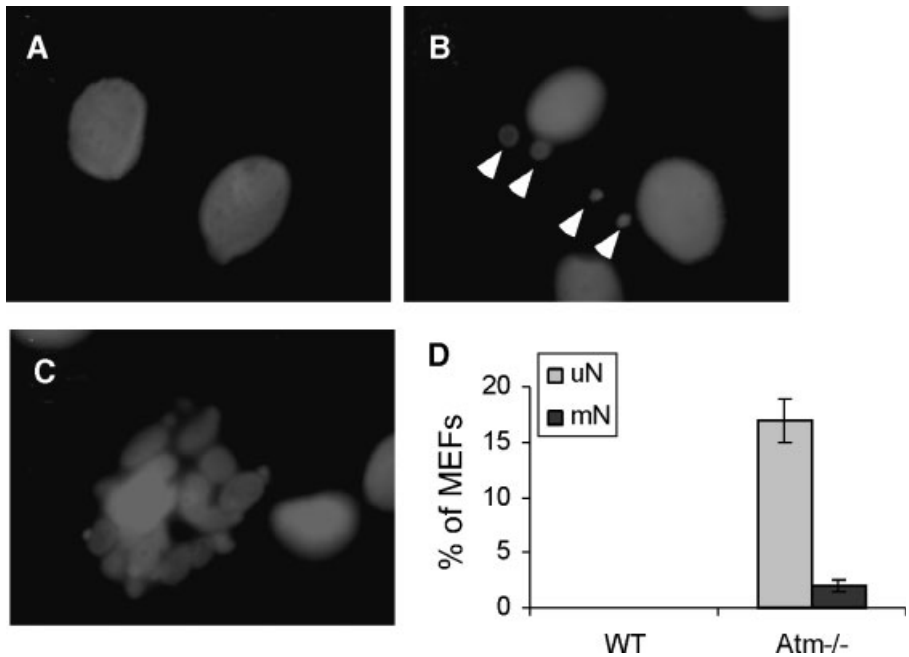

Fig. 1. Micronuclei formation in $Atm^{-/-}$ cells. **A–C**: Propidium iodide staining of mouse embryonic fibroblasts. A: Wild-type MEFs. B: $Atm^{-/-}$ MEFs with micronuclei as indicated by arrows. C: Macronuclei formation due to either cell fusion or failed cytokinesis in $Atm^{-/-}$ MEFs. **D**: Quantitation of micronuclei (μN) and macronuclei (mN) in wild-type and $Atm^{-/-}$ cells. Error bar represents standard deviation from three experiments.

significant increase of micronuclei in either $Atm^{-/-}p21^{-/-}$ cells or $Atm^{-/-}p53^{-/-}$ cells compared to $Atm^{-/-}$ cells (data not shown). Therefore, these results indicate that Atm suppresses micronuclei formation and that p53/p21 act in the same pathway as Atm in suppressing micronuclei formation. These results also imply that increased chromosomal instability observed in $Atm^{-/-}p21^{-/-}$ cells does not correlate with formation of micronuclei.

Centrosome amplification has been proposed as a major driving force of chromosomal instability. To examine the role of ATM in centrosome duplication, we first determined whether ATM is localized to the centrosome. Immunofluorescence analysis using several polyclonal and monoclonal antibodies against ATM in several different cell types did not reveal specific centrosome staining (data not shown). This could be due to the relative low abundance of ATM expression in cells and even lower levels at centrosomes. We, therefore, employed a biochemical approach to determine the expression of ATM on purified centrosomes by immunoblot analysis. As shown in Figure 2, ATM expression was detected in centrosomal fractions of HeLa cells and ATM co-purified with γ-tubulin, a centrosome marker. Furthermore, we demonstrated that ATM expression on purified centrosomes was not due to contamination from either nuclear or cytoplasmic proteins as MCM2 and β-actin, markers for nuclear and cytoplasmic localized proteins, respectively, were not detected in centrosomal fractions (Fig. 2).

It has been shown previously that Brca1 and p53 proteins are localized to centrosomes and cells deficient for either Brca1 or p53 exhibit

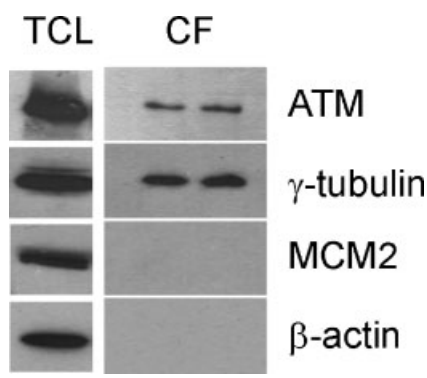

Fig. 2. ATM localization at centrosomes. Presence of ATM in two centrosomal fractions from HeLa cells and ATM co-purifies with γ-tubulin. Nuclear MCM2 as well as cytoplasmic β-actin were not present in the centrosomal fractions. TCL, total cell lysates; CF, centrosomal fractions.

centrosome amplification [Fukasawa et al., 1996; Deng, 2002]. To further characterize the activity of ATM in centrosome biogenesis, we examined the centrosomes in $Atm^{-/-}$ cells through immunofluorescence analysis of γ-tubulin expression. As expected, wild-type MEFs have one or two centrosomes (Fig. 3A). In contrast, an increased number of centrosomes were observed in $Atm^{-/-}$ MEFs (Fig. 3A,B). Therefore, these results suggest that loss of ATM function may contribute to centrosome amplification in primary MEFs. Since increased aneuploidy was observed in $Atm^{-/-}p21^{-/-}$ cells, we also characterized the centrosomes in these cells. We found that loss of both Atm and p21 did not further enhance centrosome amplification. Similarly, loss of both Atm and p53 function did not enhance centrosome amplification significantly over that in $Atm^{-/-}$ cells (Fig. 3B). These results suggest that *Atm*, *p53*, and *p21* are epistatic in regulating centrosome biogenesis.

Centrosome amplification has been frequently observed in primary tumors and is correlated with genetic instability [Pihan et al., 1998; D'Assoro et al., 2002; Lingle et al., 2002; Nigg, 2002]. We have shown previously that tumor cells from $Atm^{-/-}p21^{-/-}$ mice were extremely heterogeneous with unstable chromosomes as determined by SKY analysis [Shen et al., 2005]. Increased centrosome amplification observed in $Atm^{-/-}$ background is consistent with the notion that centrosome amplification may be the driving force of genomic instability. The extreme heterogeneous nature observed in $Atm^{-/-}p21^{-/-}$ tumor cells suggests that these tumor cells could also have increased centrosome amplification compared to primary cells. Three tumor cell lines derived from $Atm^{-/-}p21^{-/-}$ deficient mice were examined for centrosome amplification. In contrast to expectation, two of the three tumor cell lines exhibit normal centrosome morphology and numbers (Fig. 4A) whereas the third one derived from osteosarcoma (T1066) showed slightly elevated

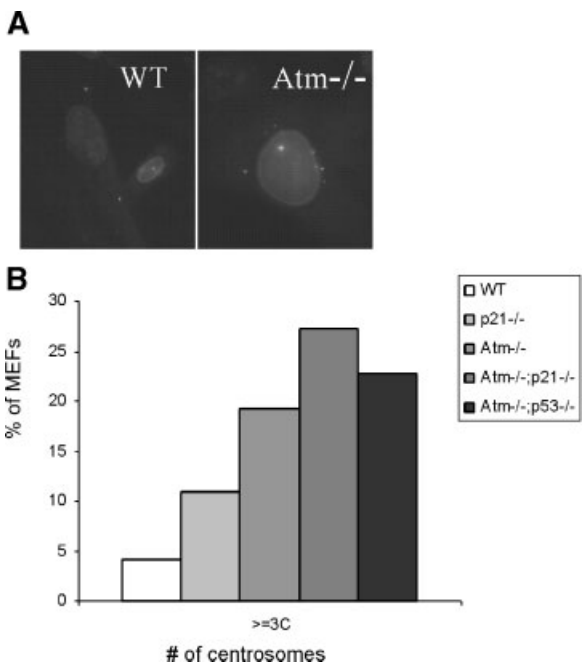

Fig. 3. Centrosome amplification in $Atm^{-/-}$ MEFs. **A**: Immunofluorescence analysis of γ-tubulin in wild-type (WT, left) and Atm null MEFs (right). Numerous centrosomes were seen in $Atm^{-/-}$ MEFs but not in wild-type MEFs. **B**: Increased centrosomes in the $Atm^{-/-}$ background were not affected by the p53 or p21 status. Centrosome numbers that were more than three were presented. Over 200 cells were quantified for each genotype.

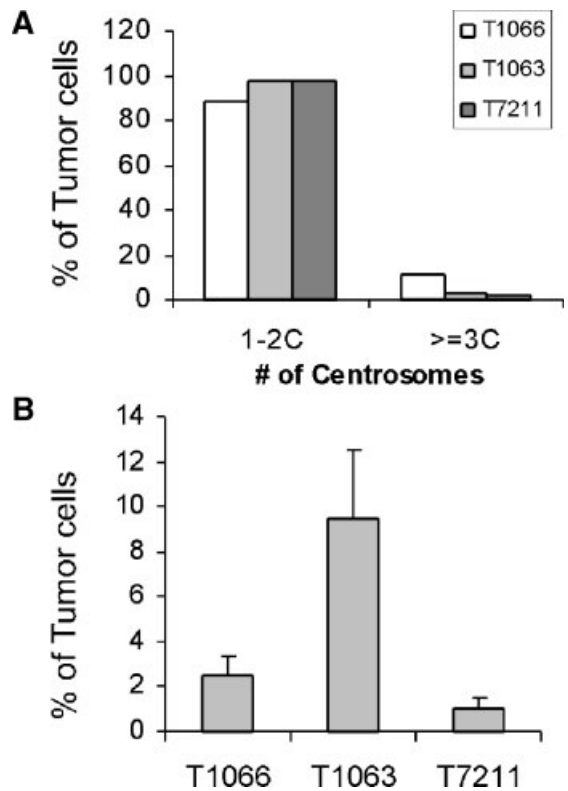

Fig. 4. Centrosome amplification was not detected in $Atm^{-/-}p21^{-/-}$ tumor cell lines. **A**: Centrosome numbers were determined by immunofluorescence analysis of γ-tubulin in these tumor cell lines. None of the tumor cell lines exhibit centrosome amplification. About 200 cells were scored for centrosome amplification. **B**: Analysis of micronuclei in $Atm^{-/-}p21^{-/-}$ tumor cell lines. One of the three cell lines (T1063) analyzed exhibited increased micronuclei, but without accompanying increase in centrosome amplification.

centrosome amplification (Fig. 4A). Therefore, these results suggest that centrosome amplification is not essential for the maintenance of the heterogeneous nature of tumors observed in $Atm^{-/-}p21^{-/-}$ background. Although $Atm^{-/-}$ primary cells exhibit increased micronuclei formation and centrosome amplification, these two events may be independent of each other, since cells with increased micronuclei formation do not necessarily exhibit centrosome amplification (Fig. 3A). Consistent with this observation, one of the tumor cell lines (T1063) exhibits increased formation of micronuclei but not centrosome amplification (Fig. 4A,B). Therefore, increased formation of micronuclei and centrosome amplification are independent events that may cooperate to promote chromosomal instability in the early stages of tumor development.

The observations that $Atm^{-/-}$ cells exhibit defective metaphase–anaphase transition [Shen et al., 2005] suggest that ATM is involved in the regulation of mitotic progression. It has been shown previously that ATM is activated through autophosphorylation of serine 1981 [Bakkenist and Kastan, 2003]. To determine whether ATM is activated by mitotic stress, HeLa cells were treated with taxol or nocodazole and examined for ATM activation by immunoblot analysis using anti-ATMpS1981. We found that ATM was gradually phosphorylated at S1981 in mitotic stressed HeLa cells (Fig. 5A). Mitotic stress-induced ATM activation could also be detected in normal fibroblast cell lines but not in ATM deficient cell lines derived from A-T patient (data not shown). These results suggest that ATM is directly involved in mitotic stress response and are consistent with the postulated role of ATM in maintaining chromosome stability. To further explore the function of ATM in spindle assembly checkpoint in response to mitotic stress, we examined the expression of mitotic kinase PLK1 in fibroblast cell line derived from A-T patient (AT22IJE-T) and ATM-complemented AT22IJE-T cells since it has been shown previously that PLK1 expression is regulated in an ATM dependent manner in response to DNA damage [Smits et al., 2000]. Here we found that the expression levels of PLK1 were higher in ATM-deficient AT22IJE-T cells than in ATM-complemented AT22IJE-T cells in response to mitotic stress (Fig. 5B). Since overexpression of Aurora A has been associated with overriding mitotic spindle checkpoint regulation [Meraldi et al., 2004]. We examined the expression of Aurora A and found that it was not altered in either wild-type or A-T cell line, therefore excluding the role of Aurora A as a mechanism for overriding mitotic checkpoint in A-T cells (Fig. 5B). The activation of ATM in mitotic stressed cells is not related to DNA damage response since none of the ATM targets, SMC-1, NBS-1, and CHK-2, were phosphorylated under these conditions (data not shown).

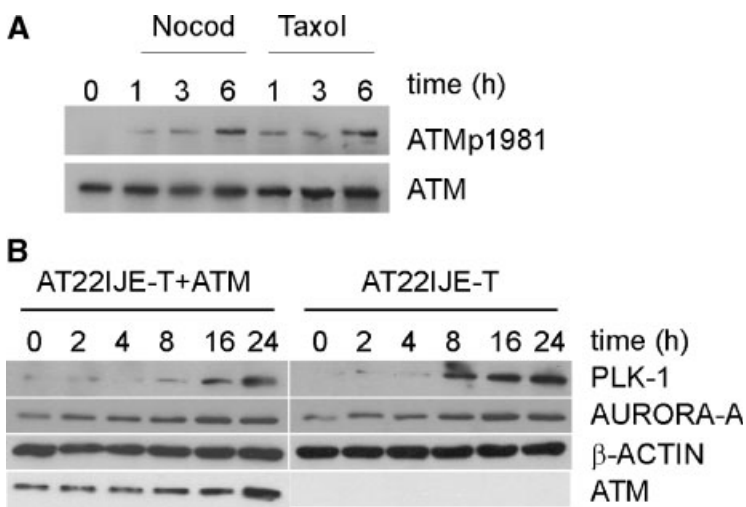

Fig. 5. ATM is activated by mitotic stresses. **A**: ATM activation by taxol and nocodazole in HeLa cells by immunoblot analysis. HeLa cells were treated with 50 nM taxol or 0.5 μg/ml nocodazole for the indicated times and total cell lysates were collected and examined for the expression of ATMp1981 and ATM. **B**: A-T cell line (AT22JE-T) and ATM-complemented AT22JE-T cell line were treated with 0.5 μg/ml nocodazole and the expression of mitotic kinase PLK-1 and Aurora A were determined with immunoblot.

DISCUSSION

In this report, we presented several lines of evidence suggesting that Atm plays a critical role in spindle checkpoint regulation thereby maintaining genome stability. First, ATM is found in centrosome fractions and co-purified with γ-tubulin. However, we were unable to confirm the presence of ATM at centrosomes directly by immunofluorescence analysis. This could be due to either that ATM is expressed at very low levels in centrosome or that ATM is masked by other proteins. Alternatively, ATM may adopt a novel conformation at centrosomes that was not recognized using the current available antibodies by immunofluorescence analysis but can be detected in the denatured condition via immunoblot analysis. ATM may directly interact and phosphorylate centrosomal components and/or serve as scaffold for the assembly of centrosome components during centrosome duplication. Such interactions might also be required for the appropriate assembly of the mitotic spindle thereby controlling G2/M progression. Second, the localization of ATM on centrosome will also ensure the proper segregation of centrosome to the opposite poles of mitotic cells. Loss of Atm leads to centrosome amplification that is correlated with aneuploidy in these cells. Importantly, we found that loss of Atm causes centrosome amplification in primary cells but not in tumor cell lines, suggesting that centrosome amplification is involved in the development of aneuploidy and chromosomal instability in Atm-deficient cells during early phases of neoplastic progression. This result is reminiscent of the findings that early passages of $p53^{-/-}$ primary cells exhibit centrosome amplification whereas late passage cells show normal and stable centrosomes [Chiba et al., 2000]. Therefore, excess centrosomes may cause mitotic catastrophe and impedes cell proliferation but at the same time also promotes chromosomal instability through disruption of mitotic spindle checkpoint. Third, ATM is autophosphorylated at Ser1981 in response to spindle damage agents that normally activate a mitotic spindle assembly checkpoint. The expression of PLK1, a polo kinase implicated in mitotic progression, is prematurely elevated in ATM-deficient cells in response to mitotic stress. This is consistent with previous studies suggesting that PLK1 is regulated in an ATM-dependent manner in response to DNA damage [Smits et al., 2000]. The increased expression of PLK1 in ATM-deficient cells in response to mitotic stress could be related to the putative role of PLK1 in mitotic checkpoint adaptation since depletion of PLK1 in ATM-deficient cells lead to cell death [Liu and Erikson, 2003].

Our results that $Atm^{-/-}$ primary cells exhibit centrosome amplification appears to contradict with a recent study showing that targeted deletion of Atm in DT-40 cells deficient for either *Rad51* or *Rad54* reduced centrosome amplification [Dodson et al., 2004]. Despite several obvious differences in the model system used, including the difference between primary mouse embryonic fibroblasts used in this study and highly recombinogenic and immortalized chicken DT-40 cells, the most likely explanation for this discrepancy is that in order to generate an Atm deletion clone from a highly genome unstable background in either *Rad51*- or *Rad54*-deficient DT-40 cells, multiple rounds of cell proliferation would have occurred and this process could select against centrosome amplification. Therefore, their results of reduced centrosome amplification are consistent with our findings of the loss of centrosome amplification in Atm-deficient tumor cell lines.

p53 and p21 have been implicated in Atm-mediated cell cycle checkpoint regulation and cooperate with Atm in suppressing tumor development [Shen et al., 2005]. In this study, the role of p53 and p21 in Atm-mediated centrosome biogenesis was further examined. The fact that there is no further increase of centrosome amplification in double knockout cells ($Atm^{-/-}p21^{-/-}$ or $Atm^{-/-}p53^{-/-}$) suggests that p53 and p21 operate in the same pathway as Atm in regulating centrosome biogenesis. This result is consistent with the observation that p53 centrosomal localization is dependent on ATM signaling [Tritarelli et al., 2004]. Collectively, our results suggest a role of ATM in spindle checkpoint regulation and indicate that ATM suppresses genome instability and cellular transformation by regulating centrosome biogenesis.

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