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Centrosomal Localization of DNA Damage Checkpoint Proteins

Suisheng Zhang, Peter Hemmerich, and Frank Grosse*

Leibniz Institute for Age Research, POB 100813, D-07708 Jena, Germany

Abstract During mitosis, the phosphatidylinositol-3 (PI-3) family-related DNA damage checkpoint kinases ATM and ATR were found on the centrosomes of human cells. ATRIP, an interaction partner of ATR, as well as Chk1 and Chk2, the downstream targets of ATR or ATM, were also localized to the centrosomes. Surprisingly, the DNA-PK inhibitor vanillin enhanced the level of ATM on centrosomes. Accordingly, DNA-PKcs, the catalytic subunit of DNA-PK, was also found on the centrosomes. Vanillin altered the phosphorylation of Chk2 in the centrosomes and in whole cell extracts. Nucleoplasmic ATM co-immunoprecipitated with Ku70/86, the DNA binding subunits of DNA-PK, while vanillin diminished this association. Vanillin did not affect microtubule polymerization at the centrosomes but, surprisingly, caused a transient enhancement of α-tubulin foci in the nucleus. Interestingly, γ-tubulin was also present in the nucleus and co-immunoprecipitated with ATR or BRCA1. DNA damage led to a reduction of the mentioned checkpoint proteins on the centrosomes but increased the level of γ-tubulin at this organelle. Taken together, these results indicate that DNA damage checkpoint proteins may control the formation of γ-tubulin and/or the kinetics of microtubule formation at the centrosomes, and thereby couple them to the DNA damage response. J. Cell. Biochem. 101: 451–465, 2007.

Key words: ATM; ATR; centrosomal organization; checkpoint control; chromosomal stability; DNA-PK; genomic stability; Ku70; Ku80

ATM (ataxia telangiectasia mutated), ATR (ATM and Rad3 related), and DNA-PK are three protein kinases involved in the DNA damage checkpoint and signal transduction to its downstream targets Chk1, Chk2, and p53. The latter either promote DNA repair or lead to cell-cycle arrest or apoptosis [Smith and Jackson, 1999; Abraham, 2001]. Although all above protein kinases belong to the family of phosphatidylinositol-3 (PI-3) kinases, they participate in different damage response pathways depending on the primary DNA lesion. For example, a DNA double-strand break (DSB) prefers to activate ATM, while single-stranded DNA (ssDNA) from a collapsed replication fork is specifically recognized by ATR [Kurz and Lees-Miller, 2004; Lisby and Rothstein, 2004]. A recent study unveiled the presence of a common structural motif in Nbs1, ATRIP, and Ku86, which are the physical and functional interaction partners of ATM, ATR, and DNA-PK, respectively [Falck et al., 2005]. Indeed, complementary functions of ATM and DNA-PK have been recognized in the DNA damage response to DSBs [Stiff et al., 2004] or in DNA repair by non-homologous end joining (NHEJ) [Riballo et al., 2004]. ATM and ATR also display a redundant role in the maintenance of telomeres [Bi et al., 2005]. It seems that after recognition and processing of a DNA damage site, for example, a DSB that may be initially targeted by ATM or the MRN complex (Mre11-Rad50-Nbs1), the ATM response is initiated. The Mre11 nuclease of the MRN complex subsequently generates ssDNA, which in turn can activate ATR [Jazayeri et al., 2006; Myers and Cortez, 2006]. Moreover, ATM and DNA-PK are both involved in NF-κB-mediated signaling pathways that determine the cell fate during the DNA damage response [Panta et al., 2004; Wu et al., 2006].

Here, we examined the localization of ATM and ATR in different phases of the cell cycle. We found that these two kinases were apparently

*Correspondence to: Frank Grosse, Biochemistry Group, Leibniz Institute for Age Research (Fritz Lipmann Institute), Beutenbergstrasse 11, D-07745 Jena, Germany.
E-mail: fgrosse@fli-leibniz.de

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localized to the centrosomes during mitosis. The centrosomes are well-known microtubule organization centers (MTOC) that organize spindle pole formation, which is necessary for the segregation of the chromosomes in mitosis [Urbani and Stearns, 1999]. Centrosome duplication, separation, and maturation are tightly coordinated with cell division and regulated by many tumor suppressor proteins with well-known roles in maintaining genome stability [Fisk et al., 2002; Lange, 2002; Nigg, 2002; Doxsey et al., 2005; Tsou and Stearns, 2006]. The presence of ATR or ATM in centrosomes led us to think about a direct involvement of these checkpoint kinases in the regulation of centrosomal activities. We found that related DNA damage checkpoint proteins, such as ATRIP, Chk1, or Chk2, were also present in the centrosomes of mitotic cells. Since the DNA-PK-specific inhibitor vanillin [Durant and Karran, 2003] provoked an enhancement of ATM in centrosomes, we looked at and found that DNA-PK was also localized to this organelle. It seems that ATR, ATM, and DNA-PK can complement each other on centrosomes, which may be necessary for the phosphorylation of the downstream targets Chk1, Chk2, or BRCA1. Based on these findings we propose that the PI3-like kinases may play a regulatory role in centrosomal cycling and/or the synthesis of microtubules.

MATERIALS AND METHODS

Antibodies

Rabbit polyclonal antibodies against ATM (H248), DNA-PKcs (H-163), Chk1 (FL-476), and BRCA1 (C-20) as well as a goat polyclonal antibody against ATR (N-19) and mouse monoclonal antibodies against DNA-PKcs (G4), Ku86 (B-1), and Ku70 (A-9) were from Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit polyclonal antibody against Chk2/hCds1 (Ab-1) was from Oncogene (San Diego, CA). Rabbit antibodies against phospho-Chk2 at Thr26/Ser28, Thr68, or Thr432 were obtained from Cell Signaling Technology. A rabbit polyclonal antibody against ATRIP was purchased from Upstate Biotechnology (Charlottesville, VA). Mouse monoclonal antibodies against γ-tubulin (clone GTU-88) and α-tubulin (clone DM1A) and a rabbit polyclonal antibody against γ-tubulin were from Sigma.

Cell Cultures

Human HeLa or MCF-7 cells were maintained in DMEM or RPMI supplemented with 10% fetal bovine serum (Gibco-BRL, Karlsruhe, Germany) and incubated at 37°C in the presence of 10% CO_2. For the treatment of cells, vanillin was added to the medium to 1 mM, followed by incubation for 1 h before subsequent experiments. DNA damage was induced by treating the cells with 4NQO (4-nitroquinoline-1-oxide) at different concentrations (see Fig. 10) for 1 h.

Immunofluorescence

Immunofluorescence measurements were as described before [Zhang et al., 2004a]. Cells after washing in PBS were fixed in 4% paraformaldehyde for 15 min and then permeabilized by 0.5% Triton X-100 in PBS for another 15 min. Alternatively, cells were fixed in cold methanol without or with a pre-permeabilization step for 30 s by 0.5% Triton-X 100 in a solution of 10 mM PIPES, pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, and 1 mM EGTA. Reactive centers were then blocked with 5% bovine serum albumin (BSA) in PBS for 30 min. Primary antibodies were added at a dilution of 1 to 1,000 for the mouse antibodies against γ-tubulin (GTU-88) and α-tubulin (DM1A) or the rabbit antibodies against γ-tubulin and ATRIP. The dilutions were 1 to 200 for the rabbit antibodies against ATM (H-248), BRCA1 (C-20), Chk1 (FL-476), Chk2/Cds1 (Ab-1), phospho-Chk2, and the goat antibody against ATR (N-19); and 1 to 20 for the mouse monoclonal antibody against DNA-PKcs (G4). Secondary antibodies were a FITC-conjugated anti-mouse IgG or a Cy-3-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories), which were used at a dilution of 1 to 100. The cells were finally washed with PBS, stained by Topro3 at a 1 to 500 dilution for visualizing DNA, and then placed over an aliquot of antifade mounting medium on the supporting glass slide (ProLong Gold, Invitrogen).

Isolation of Centrosomes

HeLa cells were pretreated with nocodazole at 0.2 μM and cytochalasin D at 1 μg/ml for 1 h and then collected for the isolation of centrosomes as described [Bornens and Moudjou, 1999]. After ultracentrifugation of centrosomes through sucrose steps of 40%, 50%, and 70%,

fractions were collected and precipitated by 10% (v/v) trichloroacetic acid. The pellets were dissolved in SDS–PAGE loading buffer for Western blotting. The purity of the purified centrosomes was examined by phase contrast under a light microscope. Alternatively, centrosomes were sedimented into a 60% sucrose cushion for comparing the associated proteins under different conditions, for example, after treatment with increasing concentrations of 4NQO.

Microtubule Polymerization Assay

HeLa cells were either untreated or treated with vanillin as described above before depolymerization of the microtubules by nocodazole (1 μg/ml) for 1 h on ice. The cells were then returned to a normal medium or media containing the same concentrations of vanillin to allow microtubules regrowth at 37°C. Cells were withdrawn at different times for immunofluorescence by methanol fixation following prepermeabilization with 5% Triton X-100 (see above). Microtubules were visualized using the mouse monoclonal antibody against α-tubulin (DM1A) and the FITC-conjugated secondary antibody.

Immunoprecipitation and Chromatin Fractionation

Immunoprecipitations were performed from nucleoplasmic extracts of HeLa cells as described [Mischo et al., 2005]. The rabbit polyclonal antibody against ATM (H248) or the mouse monoclonal antibody against γ-tubulin (GTU88) were used for obtaining the ATM- or γ-tubulin-containing immunoprecipitates. Equal amounts of rabbit or mouse IgG (Santa Cruz) were used as control antibodies.

Chromatin fractionations from HeLa cells, untreated or treated by vanillin, were performed as described before [Zou et al., 2002]. Supernatants and pellets of centrifugations after micrococcal nuclease (MNase) digestion and subsequent EDTA extractions were suspended in an equal volume of SDS–PAGE loading buffer. The samples were heated for 5 min at 95°C and analyzed by Western blotting (see below).

In Vitro Phosphorylation by DNA-PK

The assay of DNA-PK was as described before [Zhang et al., 2004b]. Purified DNA-PK was obtained from Promega. Chk2 as a GST-fusion protein was obtained from Upstate. To inhibit DNA-PK, 1 mM vanillin or 10 μM wortmannin were given to the assay mixtures. Phosphorylated Chk2 was identified by Western blotting.

Western Blot

Whole cell lysates for Western blotting were obtained by immediately dissolving the cells on culture dishes with SDS–PAGE loading buffer. After heating to 95°C samples were electrophoresed through an SDS–polyacrylamide gel and then transferred to a Hybond C extra nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK) using a semi-dry blotter (Sigma). A dilution of 1 to 200 was used for the goat antibody against ATR (N-19) and the rabbit antibodies against BRCA1 (C-20), DNA-PKcs (H-163), and ATM (H-248), and a dilution of 1 to 1,000 for the rabbit antibodies against phospho-Chk1 and Chk2 or the mouse antibodies against γ-tubulin (GTU-88), Ku86 (B-1), and Ku70 (A-9). Secondary anti-rabbit or anti-mouse IgGs coupled with avidin as well as a streptavidin-horseradish peroxidase complex was diluted at 1 to 5,000 for immunodetection by enhanced luminescence (Amersham Biosciences).

RESULTS

Localization of DNA Damage Checkpoint Protein Kinases ATR and ATM to the Centrosomes

Using immunofluorescence of paraformaldehyde-fixed HeLa cells, the intranuclear localization of ATR and ATM was identified at the centrosomes of mitotic cells. ATR was initially recognized under the light microscope at the centrosomes (see Fig. 1A,B and A′,B′ at two panels), where we observed an apparent accumulation of ATR near to and flanking the metaphase chromosomes. These positions turned out to be centrosomes by comparing the localization of ATR with γ-tubulin on the confocal microscope (see Fig. 1C–E and C′–E′ at two panels), although in interphase cells there was hardly a co-localization of ATR and γ-tubulin (Fig. 1F–H). Interestingly, ATM was also found at the centrosomes of metaphase cells as shown by confocal microscopy (Fig. 1I–K), although again in interphase cells there was barely any ATM at centrosomes compared to its obvious presence there in mitosis (Fig. 1L–N).

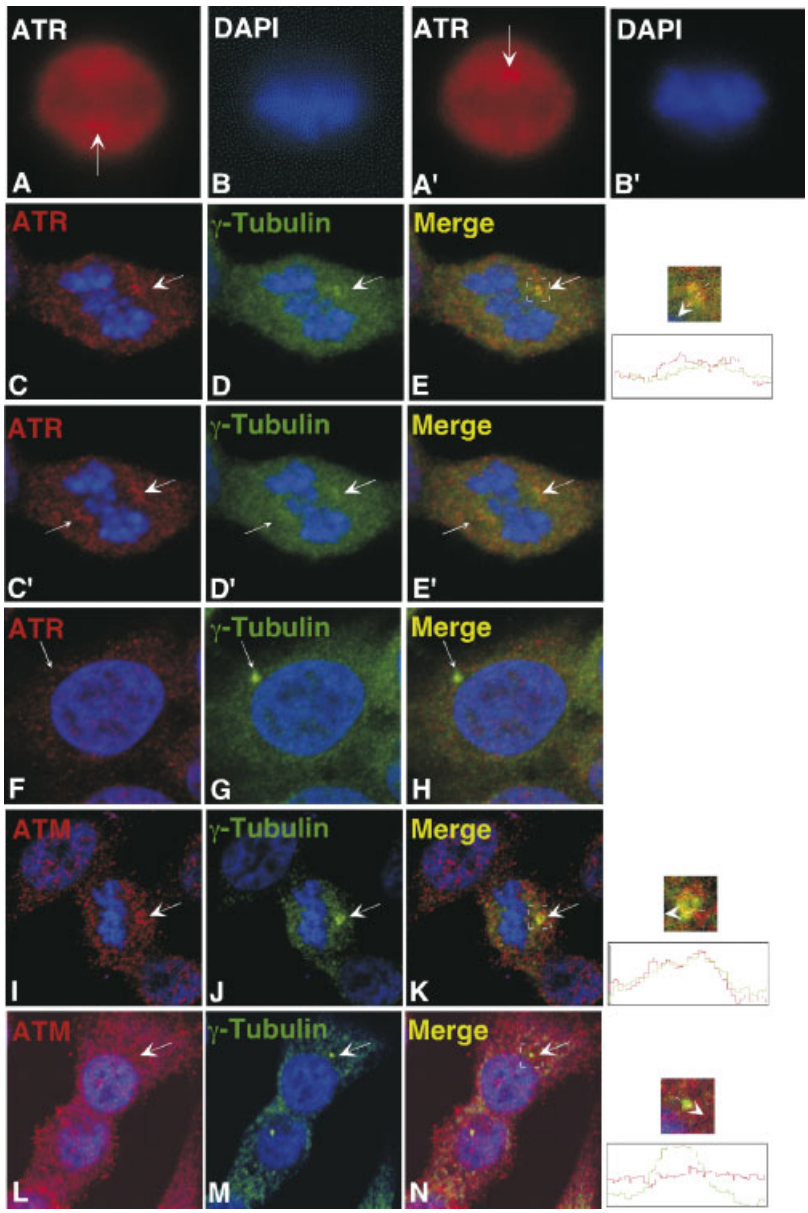

Fig. 1. ATR and ATM are present on centrosomes of mitotic cells. HeLa cells were fixed by 4% paraformaldehyde and examined for ATR by immunofluorescence under the light microscope (two typical examples in **A**–**B** and **A′**–**B′**). Double-immunofluorescence of ATR and γ-tubulin was performed by confocal microscopy in mitotis (**C**–**E** and **C′**–**E′**) or interphase (**F**–**H**). Double-immunofluorescence images of ATM and γ-tubulin in mitosis (**I**–**K**) or interphase (**L**–**N**) are also shown. Solid arrows indicate the centrosomes. A magnified area framed in (E), (K), and (N) is presented together with a fluorescence scan along the dashed arrow.

Localization of ATRIP, Chk1, and Chk2 to the Centrosomes

Next we asked whether the interaction partner of ATR, that is, ATRIP [Cortez et al., 2001], was also localized to the centrosomes. Double-immunofluorescence of paraformaldehyde-fixed HeLa cells for ATRIP and γ-tubulin revealed that ATRIP was present at the centrosomes of metaphase cells (Fig. 2A–C and A′–C′ at two panels), whereas in the centrosomes of interphase cells ATRIP was not as apparent as in the mitotic phase (Fig. 2D–F).

In the DNA damage response, ATR or ATM phosphorylates the downstream kinases Chk1 and Chk2, leading to DNA repair, cell-cycle arrest, or apoptosis [Smith and Jackson, 1999; Abraham, 2001]. Since ATM, ATR, and ATRIP

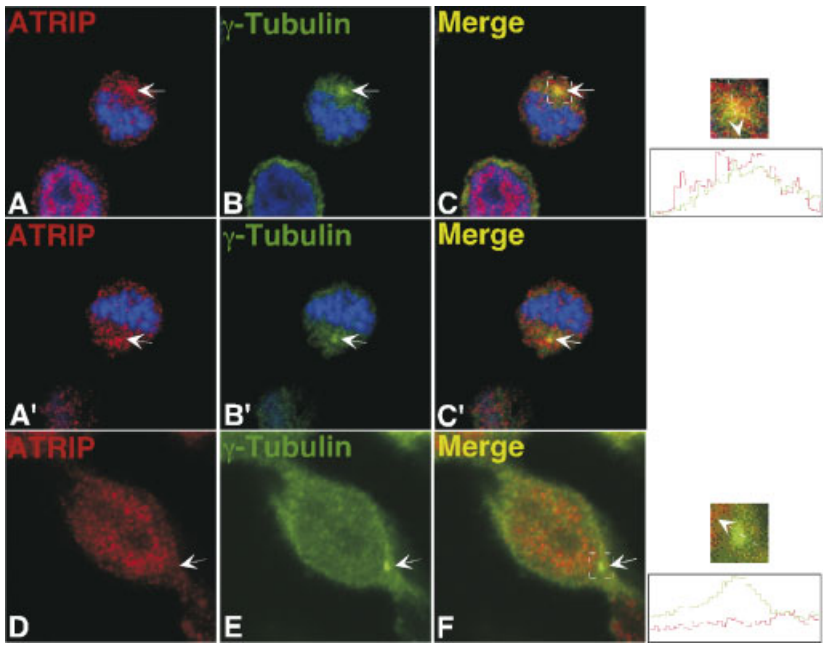

Fig. 2. The ATR interaction partner ATRIP is also present in centrosomes of mitotic cells. Double-immunofluorescence of ATRIP and γ-tubulin was performed with paraformaldehyde-fixed HeLa cells and observed by confocal microscopy of metaphase (**A**–**C** and **A′**–**C′**) or interphase cells (**D**–**F**). Solid arrows indicate the centrosomes. A magnified area framed in (C) or (F) is presented together with a fluorescence scan along the dashed arrow.

were found at the centrosomes, we also performed double-immunofluorescence studies of Chk1 or Chk2 and γ-tubulin. Both Chk1 (Fig. 3A–C and A′–C′) and Chk2 (Fig. 3D–F) were accumulated at the centrosomes of metaphase cells indicating a centrosomal co-localization of Chk1 and Chk2 as well as their upstream kinases ATR or ATM.

The DNA-PK Inhibitor Vanillin Enhanced a Localization of ATM to the Centrosomes

Because of the complementary functions of the three PI3-like checkpoint kinases, we asked whether DNA-PK is also involved in centrosomal processes. Recently, it has been shown that the DNA-PK-specific inhibitor vanillin influences DNA repair by NHEJ [Durant and Karran, 2003]. NHEJ requires DNA-PK, both for its autophosphorylation and for the phosphorylation of other proteins, such as XRCC4/DNA ligase IV, in order to reseal a DSB. However, when HeLa cells were treated with 1 mM vanillin for 1 h before immunofluorescence, centrosomal ATM of metaphase cells became so much enhanced that it was easily detectable over the background by ordinary light microscopy, while the background decreased in the presence of vanillin (Fig. 4A,B vs. C,D). This finding was corroborated by confocal microscopy showing a strong increase of ATM at metaphase centrosomes of vanillin-treated cells (Fig. 4E–G vs. Fig. 1I–K). This effect of vanillin seems to be specific to ATM since we failed to detect a similar enhancement of the ATR signal, either under the light microscope (Fig. 4H,I) or by confocal microscopy (Fig. 4J–L). Moreover, we neither detected an apparent enhancement of Chk1 (compare Fig. 4M–O with Fig. 3A–C) nor of Chk2 (compare Fig. 4P–R with Fig. 3D–F) at centrosomes of vanillin-treated cells. These results point to a closer physical or functional relationship of DNA-PK and ATM compared to ATR, or a better compensation of DNA-PK by ATM (compared to ATR) to maintain the phosphorylation of Chk1 or Chk2 at centrosomes.

Detection of DNA-PK at Centrosomes

Since the DNA-PK inhibitor vanillin enhanced the ATM signal on centrosomes, we asked whether DNA-PK is also present in centrosomes as shown above for ATR and ATM. To identify the catalytic subunit of DNA-PK (DNA-PKcs) at centrosomes by immunofluorescence we had to

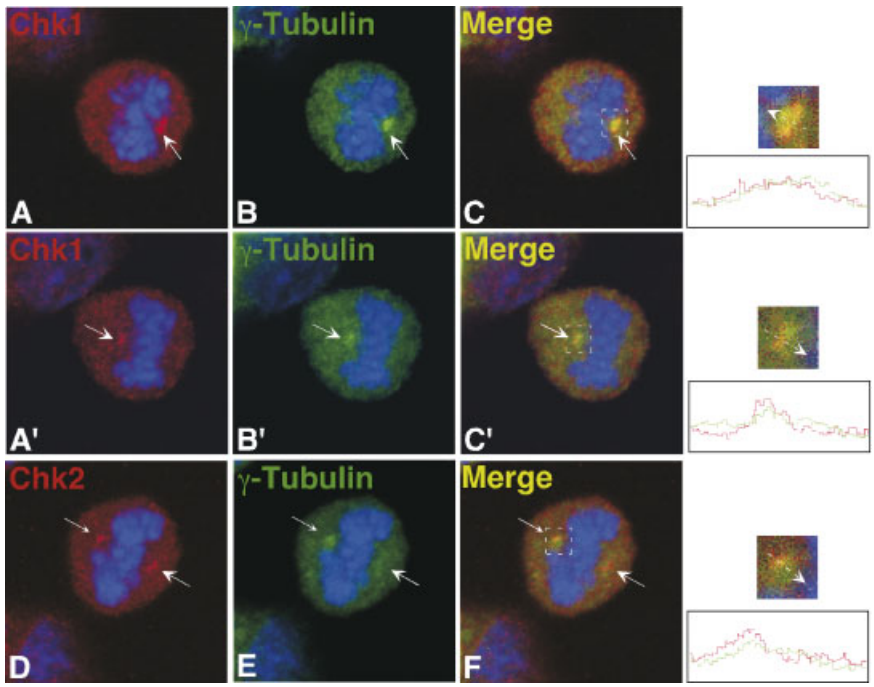

Fig. 3. Presence of Chk1 and Chk2 in centrosomes of mitotic cells. Double-immunofluorescence of Chk1 (**A**–**C** and **A′**–**C′**) and Chk2 (**D**–**F**) with γ-tubulin was performed using paraformaldehyde-fixed HeLa cells. Solid arrows indicate the centrosomes. A magnified area framed in (C), (C′), or (F) is presented together with a fluorescence scan along the dashed arrow.

pre-permeabilize cells for 30 s with 0.5% Triton X-100 and fix them in cold methanol, whereas fixation with paraformaldehyde gave poor results. This change of the immunofluorescence protocol allowed a visualization of DNA-PKcs in centrosomes, in interphase (Fig. 5A–C), and in metaphase (Fig. 5D–F and D′–F′ at two panels). To further confirm this finding, we isolated centrosomes from HeLa cells by sucrose gradient ultracentrifugation and observed co-sedimentation of DNA-PKcs and γ-tubulin by Western blotting (data not shown). Taken together, these results indicate that DNA-PKcs is present in the centrosomes.

Vanillin Altered the Phosphorylation of Chk2 at Centrosomes and in Whole Cell Extracts

We further examined whether vanillin affects the phosphorylation of Chk2 that contains multiple consensus sequences recognized and phosphorylated by ATM, ATR, or DNA-PK [Smith and Jackson, 1999; Abraham, 2001]. Immunofluorescence with phospho-specific antibodies revealed that Chk2 phosphorylated at Ser 28, Thr68, and Thr432 was present at centrosomes of mitotic or interphase cells (Fig. 6A). In mitotic cells these different phosphorylation forms of Chk2 were also observed in the centrosomes, after vanillin treatment, however, phosphorylation at Ser28 and Thr432 was reduced (Fig. 6B). Examination of MCF-7 whole cell extracts revealed that vanillin hardly affected the protein levels of Chk2 but reduced its phosphorylation at Ser28 while phosphorylation at Thr68 or Thr432 increased (Fig. 6C). In vitro, the phosphorylation of Chk2 by DNA-PK, for example, at Thr432, was inhibited by vanillin to a similar extent as inhibition by

Fig. 4. The DNA-PK inhibitor vanillin enhanced the ATM signal on the centrosomes. Immunofluorescence was performed by fixing the HeLa cells with paraformaldehyde as described above. HeLa cells untreated (**A** and **B**) or after incubation with 1 mM vanillin for 1 h (**C** and **D**) were examined by immunofluorescence to observe ATM under the light microscope. Confocal microscopic images of ATM and γ-tubulin in metaphase vanillin-treated cells are shown in **panels E**–**G**. Light microscopic images of ATR (**H** and **I**) and confocal double-immunofluorescence images of ATR (**J**–**L**), Chk1 (**M**–**O**), and Chk2 (**P**–**R**) with γ-tubulin from vanillin-treated cells are also shown. Solid arrows indicate the centrosomes. A magnification of (A), (C), and (H) is presented with a fluorescence scan along the dashed line crossing over two centrosomes at both sides of the metaphase chromosomes. A fluorescence scan along the dashed arrow in (G), (L), (O), and (R) is also presented.

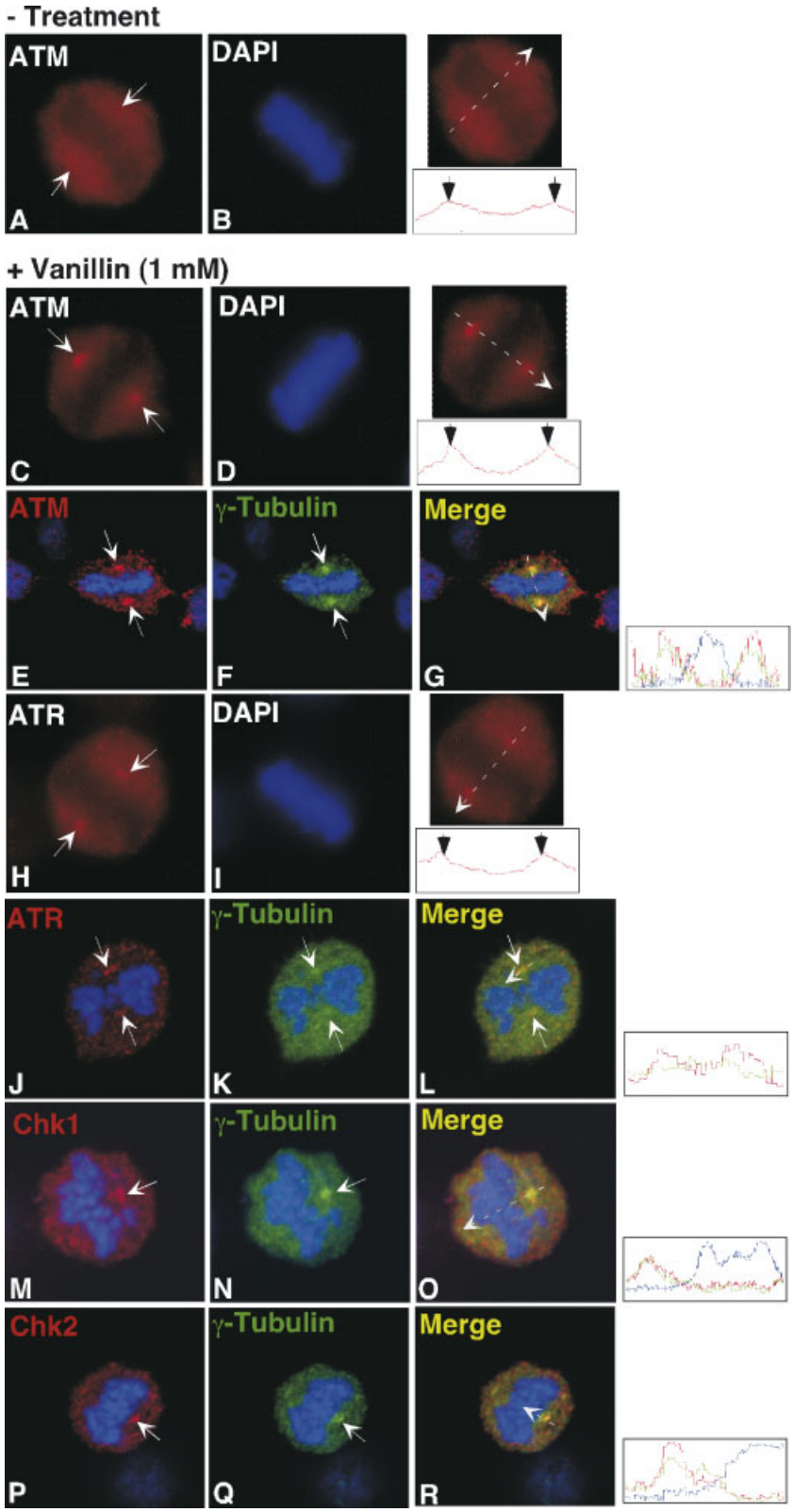

Fig. 4. (*Continued*)

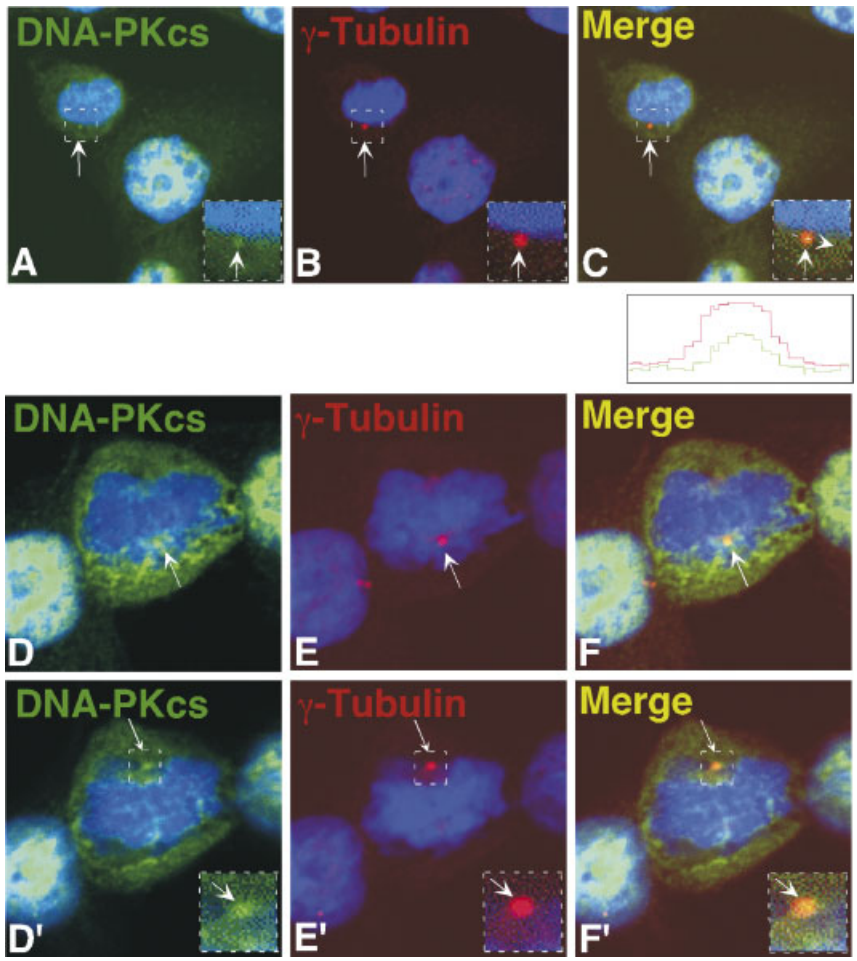

Fig. 5. Presence of DNA-PKcs on centrosomes. HeLa cells were pre-permeabilized with 0.5% Triton X-100 for 30 s and then fixed in methanol as described in Materials and Methods. Immunofluorescence was performed with a mouse monoclonal antibody against DNA-PKcs (G4) and a rabbit antibody against γ-tubulin. The presence of DNA-PKcs in or on the centrosomes is shown for cells in interphase (**A**–**C**) or metaphase (**D**–**F** and **D′**–**F′**). Solid arrows indicate the centrosomes. Magnification from a framed area in (A–C) and (D′–F′) is presented at the lower right corner of the panels. A fluorescence scan is presented for (C) along the dashed arrow in the magnified area.

wortmannin (Fig. 6D). Thus there is a direct inhibition of DNA-PK by vanillin, although in vivo the missing phosphorylation by DNA-PK may be compensated by other protein kinases, for example, ATM. This, however, may lead to an altered phosphorylation pattern, as observed here for Chk2.

Interestingly, ATM co-immunoprecipitated with the Ku86/70 subunits of the DNA-PK holoenzyme from the nucleoplasm of HeLa cells and this association of ATM with DNA-PK was apparently diminished by vanillin (Fig. 7A). Chromatin fractionation from untreated cells showed that Ku86 was nearly completely released from chromatin by MNase digestion and subsequent extraction with EDTA, while Ku86 from vanillin-treated cells displayed an apparent resistance to this extraction procedure (Fig. 7B). This probably indicates an increased condensation of chromatin due to the inhibition of DNA-PK. On the other hand, there was hardly any difference in the chromatin association of ATM under either condition.

Effect of the DNA-PK Inhibitor Vanillin on Microtubule Formation From Centrosomes

Next we asked whether the localization of DNA damage checkpoint kinases with centrosomes might affect microtubule synthesis. To this end HeLa cells were treated with nocodazole to allow a depolymerization of the microtubules and, after withdrawal of nocodazole,

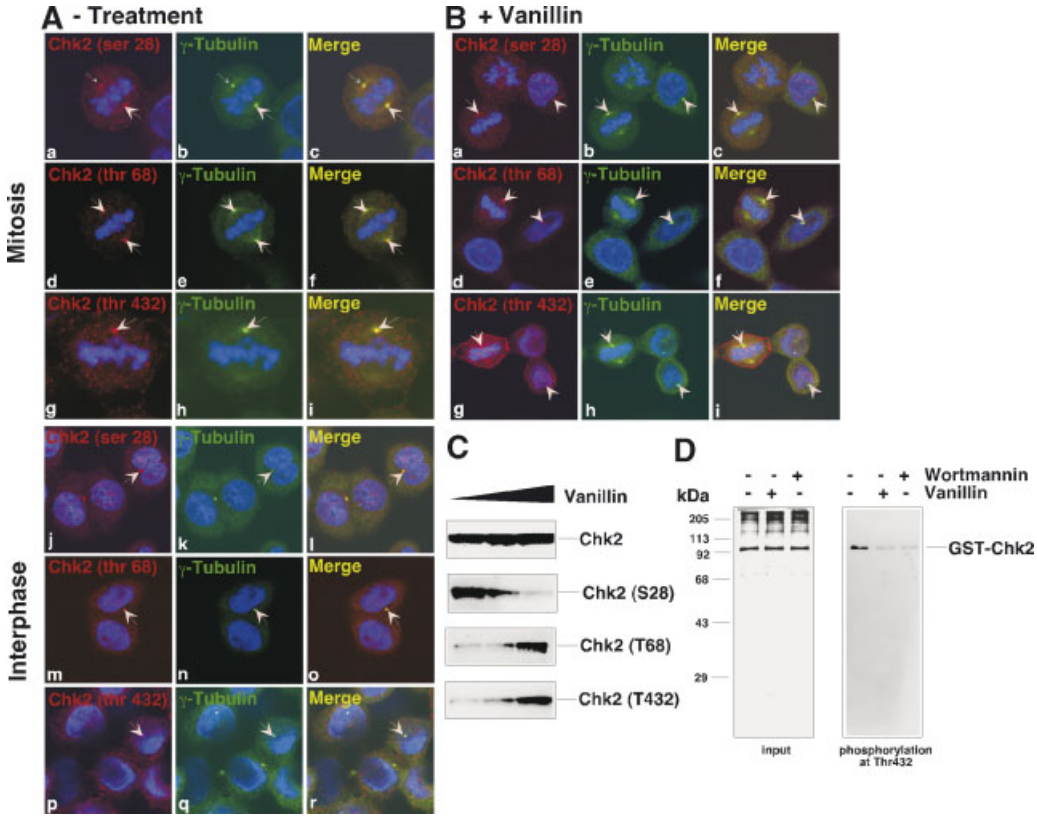

Fig. 6. Vanillin altered the phosphorylation of Chk2 at centrosomes and in whole cell extracts. A co-localization of Chk2 phosphorylated at Ser28, Thr 68, or Thr432 is shown for untreated (**A**) or vanillin-treated HeLa cells (**B**). Immunofluorescence was performed following fixation of HeLa cells with methanol (without pre-permeabilization) and immunostaining with the rabbit antibodies against phospho-Chk2 and the mouse antibody against γ-tubulin (GTU-88). **C**: Chk2 from whole cell lysates of MCF-7 was examined by Western blotting with a rabbit antibody (Ab-1) or with phospho-epitope-specific antibodies as indicated. **D**: In vitro phosphorylation of Chk2 by DNA-PKcs and its inhibition by vanillin or wortmannin are shown for the input by silver staining after SDS–PAGE or Western blot with the rabbit antibody against Chk2 phosphorylated at Thr432.

to allow resynthesis. Cells maintained under normal conditions, that is, without vanillin, rapidly recovered centrosomal microtubules synthesis, since α-tubulin was quickly incorporated into the reforming microtubules after withdrawal of nocodazole (Fig. 8A). In contrast, in vanillin-treated cells, we witnessed an apparent inclusion of α-tubulin into the nucleus 5 min after the withdrawal of nocodazole (see arrow-indicated cell in Fig. 8B, c and c′ for its magnification). These intranuclear α-tubulin signals distributed as dots or even some short rails, which is highly suggestive of microtubule nucleation in the nucleus. These vanillin-induced intranuclear α-tubulin signals vanished 15 min after the induced regrowth of microtubules (see arrow-indicated cell in Fig. 8B, e) suggesting that inclusion of α-tubulin into the nucleus may be affected by microtubules polymerization at the centrosomes.

Nucleoplasmic ATR Was Associated With γ-Tubulin

To examine a possible nucleation of microtubules in the nucleus, we reexamined the intracellular distribution of γ-tubulin by immunofluorescence using a rabbit polyclonal antibody. This led to the finding that γ-tubulin was also localized to the nucleus in addition to its presence in centrosomes (Fig. 9A). We further performed immunoprecipitations of γ-tubulin from nucleoplasmic extracts and found that nuclear γ-tubulin was associated with ATR (Fig. 9B). Nucleoplasmic γ-tubulin also co-immunoprecipitated with BRCA1 (Fig. 9B), a tumor suppressor protein that was present in the centrosomes of untreated (Fig. 9C, a–c) and vanillin-treated HeLa cells (Fig. 9C, d–f). The presence of ATR and BRCA1 in centrosomes and their association with intranuclear

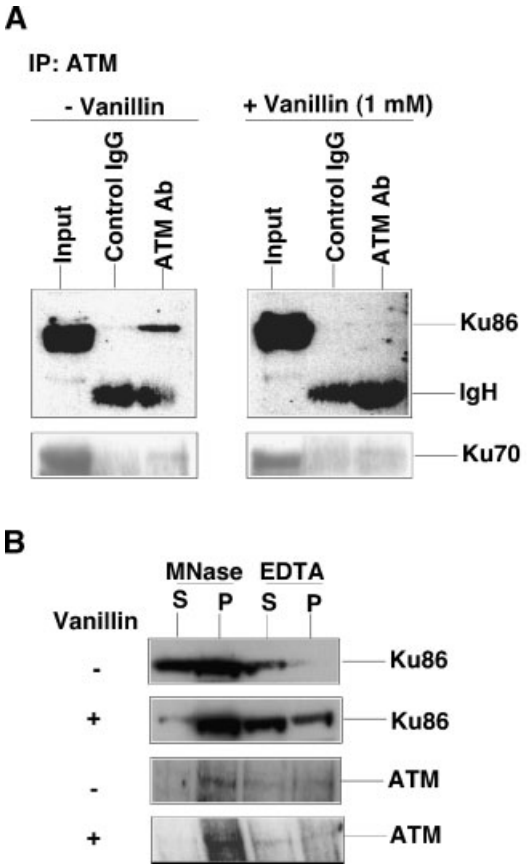

Fig. 7. Vanillin diminished the association of ATM with Ku86 in the nucleus. **A**: Co-immunoprecipitation of ATM with Ku86 from the nucleoplasm of HeLa cells. HeLa cells were untreated or treated with vanillin for 1 h, followed by preparing the nucleoplasmic extracts and immunoprecipitation with the rabbit antibody against ATM (H-248) or equal amounts of a rabbit IgG control. The presence of Ku86 or Ku70 in the inputs and the ATM immunoprecipitates was examined using the mouse monoclonal antibody against Ku86 (B-1) or Ku70 (A-9). **B**: Association of Ku86 and ATM with chromatins in untreated or vanillin-treated cells. Chromatin fractionation was performed as described before [Zou et al., 2002]. Western blotting was performed for examining the presence of Ku86 or ATM in the supernatants or the pellets after centrifugation.

γ-tubulin support the view that these proteins may be involved in the regulation of microtubule nucleation by γ-tubulin, both at the centrosomes and at non-centrosomal sites of the nucleus.

DNA Damage Led to a Reduction of Centrosomal DNA Damage Checkpoint Proteins and to an Enhancement of Centrosomal γ-Tubulin

Because all the examined proteins are involved in the DNA damage response with different effects on DNA repair, cell-cycle progression, or apoptosis, we asked how DNA damage might affect the centrosomal composition. To this end, we examined the presence of DNA damage checkpoint proteins in centrosomes after treating unsynchronized cells with the DNA damage reagent 4NQO for 1 h before immunofluorescence. As a result, BRCA1 (Fig. 10A, a–c), ATR (Fig. 10A, d–f), ATRIP (Fig. 10A, g–i), ATM (Fig. 10A, j–l), Chk1 (Fig. 10A, m–o), and Chk2 (Fig. 10A, p–r) all significantly disappeared from the centrosomes of metaphase cells. Moreover, isolated centrosomes (Fig. 10B, a) from HeLa cells treated with different concentrations of 4NQO lower the amount of DNA-PKcs, possibly due to protein degradation (see degradation products in Fig. 10B, b). Nevertheless, there was an increased level of γ-tubulin in isolated centrosomes when cells were treated with increasing amounts of 4NQO. Taken together, these results suggest that DNA damage checkpoint proteins may negatively regulate the turnover of γ-tubulin at centrosomes, while induction of the DNA damage response may abrogate this effect.

DISCUSSION

So far there is some evidence that the DNA damage checkpoint proteins ATM [Oricchio et al., 2006], Chk2 [Tsvetkov et al., 2003], or Chk1 [Kramer et al., 2004] are localized to the centrosomes of human cells. According to these results, ATM was found in the centrosomes of mitotic cells [Oricchio et al., 2006]; Chk2 was present in the centrosomes of interphase and mitotic cells [Tsvetkov et al., 2003]; while the presence of Chk1 in centrosomes seemed to be confined to the interphase [Kramer et al., 2004]. Although our results are consistent with a centrosomal localization of these DNA damage checkpoint proteins, we found that Chk1 also localized to centrosomes in mitotic cells. It could be that the (slightly) different observations are due to the use of different cell lines, antibodies or fixation protocols.

In addition, ATR and its interaction partner ATRIP were also localized to the centrosomes in mitosis. These findings are novel and have not been reported before. Previous results showed that a complete knockout of ATR led to chromosomal fragmentation and early embryonic death, which is probably due to a defective replication checkpoint [Brown and Baltimore, 2000]. Similar phenotypes were also observed in animals with a deletion of both alleles of Chk1

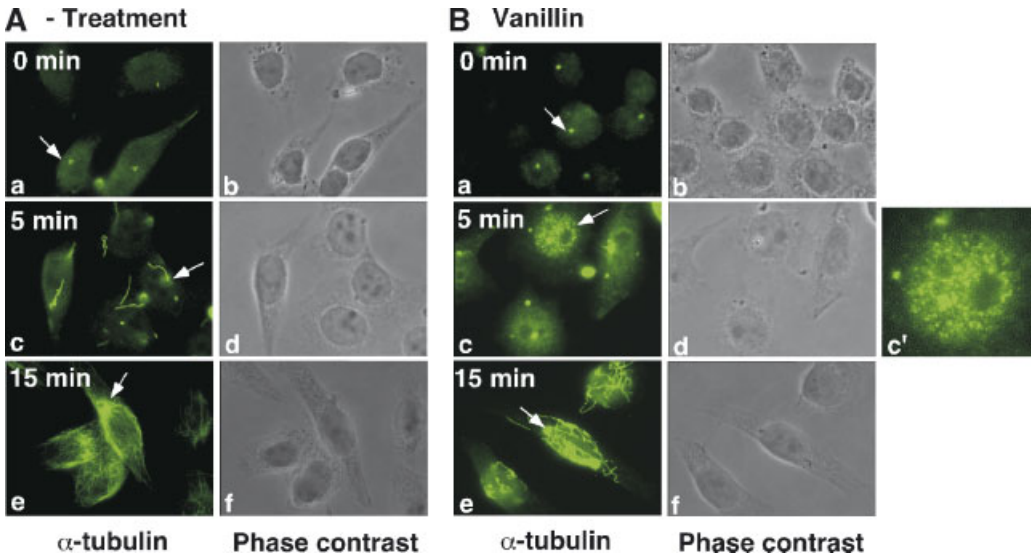

Fig. 8. Effect of vanillin on the assembly of microtubules at centrosomes. The microtubule regrowth assay was performed with untreated HeLa cells (**A**) or HeLa cells after treatment with 1 mM vanillin for 1 h (**B**). The mouse monoclonal antibody against α-tubulin (DM1A) was used for visualizing regrowing microtubules. Arrows indicate centrosomes or intranuclear α-tubulin foci that were seen in vanillin-treated cells (B, (**c**) and its magnification in (**c′**)).

[Takai et al., 2000]. Interestingly, an overexpression of ATR, as found in some tumor cells carrying a duplicated copy of ATR, led to an abnormal amplification of centrosomes and spindles during mitosis [Smith et al., 1998]. Also, a hypomorphic mutation of ATR in the Seckel syndrome is accompanied with an increasing number of centrosomes during mitosis [Alderton et al., 2004]. While the centrosomal abnormality may be due to an insufficient action of ATR during the DNA damage response, which subsequently may affect the centrosomes, the physical presence of ATR and ATRIP in centrosomes suggests a direct involvement of these proteins in the regulation of centrosomal processes.

Most importantly, we recognized an enhancement of ATM signals on centrosomes when cells were treated with the DNA-PK-specific inhibitor vanillin. This led to the finding that DNA-PK is also present in centrosomes, where it may complement the action of ATM. Presently it is unknown why there is an increased recruitment of ATM to centrosomes when DNA-PK is inhibited. It remains possible that these protein kinases target a common component in a manner dependent on the functional state of each other. In the DNA damage response, ATM can directly target damaged DNA, such as a DSB, or ATM follows an initial recognition of DSBs by the MRN complex, where the latter may recruit ATM [Abraham and Tibbetts, 2005]. Similarly, DNA-PK consists of three subunits consisting of the catalytic subunit of DNA-PK and a heterodimer, Ku70/86, responsible for DNA binding [Smith and Jackson, 1999]. Since NBS1 from the MRN complex and Ku86 share a common motif for targeting ATM or DNA-PKcs [Falck et al., 2005], these two protein kinases may exchange their functional partners in a complementary manner [Doherty and Jackson, 2001]. It has been shown before that Ku70 co-immunoprecipitated with Chk1 [Goudelock et al., 2003] or Chk2 [Li and Stern, 2005]. Possibly, Ku70 offers a platform for Chk1 or Chk2 that is phosphorylated at a damaged site. According to our results, ATM co-immunoprecipitated with Ku86/Ku70 from the nucleoplasm while vanillin diminished this association between them. This suggests that ATM also binds to Ku70/86, probably for its sequestration to the chromosomes. A similar situation may also occur at the centrosomes and, when DNA-PK is inhibited by vanillin, ATM may switch to other components of the centrosome and become sequestered. This change may lead to a decreased dissociation rate of ATM from the centrosomes and therefore explains its increased enrichment in this organelle.

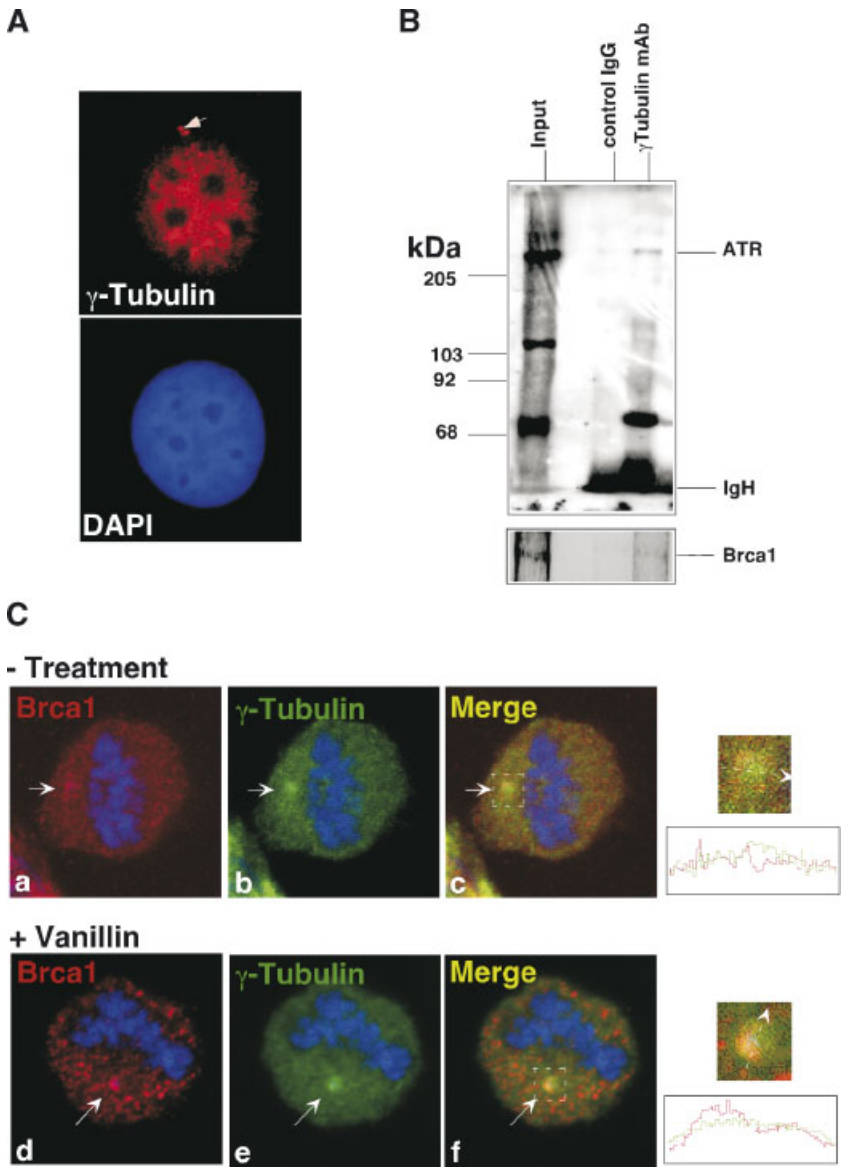

Fig. 9. ATR is associated with γ-tubulin in the nucleus. **A**: The presence of γ-tubulin in the nucleus was unveiled with a rabbit antibody against γ-tubulin and immunofluorescence. **B**: ATR co-immunoprecipitated with γ-tubulin from the nucleoplasm of HeLa cells. Immunoprecipitation was performed with the mouse antibody against γ-tubulin (GTU-88). The immunoprecipitate was examined by Western blotting with the goat antibody against ATR (N-19) or the rabbit antibody against BRCA1 (C-20). **C**: Presence of BRCA1 in the centrosomes. Double-immunofluorescence of BRCA1 and γ-tubulin was performed for HeLa cells untreated (**a**–**c**) or treated by vanillin (**d**–**f**). The immunofluorescence was performed after fixation in paraformaldehyde. Solid arrows indicate the centrosomes. A magnified area framed in (c) or (f) is presented with a fluorescence scan along the dashed arrow.

Increasing amounts of evidence support the involvement of centrosomes in DNA damage checkpoint signaling [for recent reviews see Fletcher and Muschel, 2006; Loffler et al., 2006]. Inactivation of centrosomes as a result of DNA-replication checkpoint defects has been earlier observed in *Drosophila* embryos that either carry a mutant of grp or of mei-41, which are the homologues of fission yeast Chk1 and human ATM/ATR, respectively [Sibon et al., 2000]. As suggested from these results, a defect in DNA-replication checkpoints may lead to premature mitotic cells that carry incompletely replicated DNA resembling DNA damage. This may induce a centrosomal failure to produce the mitotic spindle for chromosomal segregation

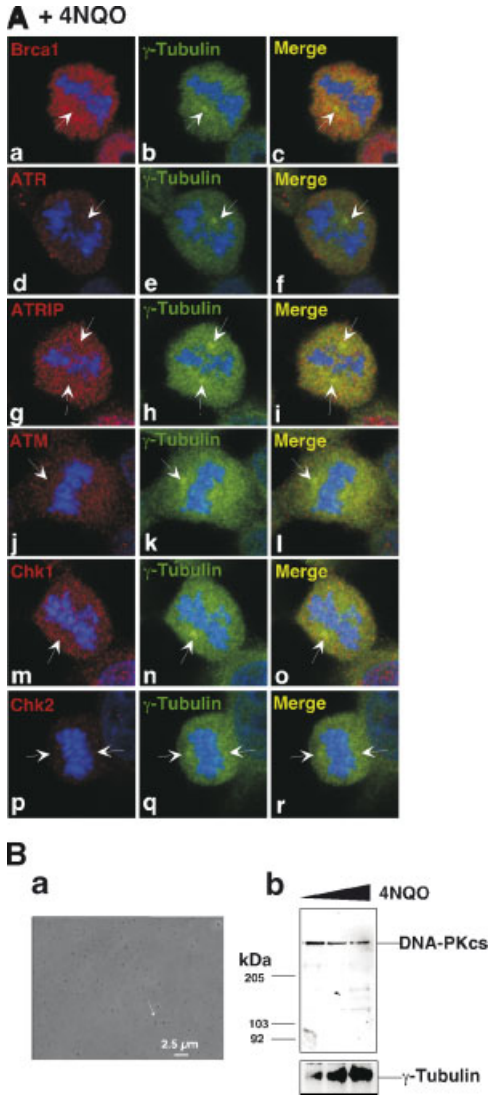

Fig. 10. DNA damage led to a reduction of DNA damage checkpoint proteins at the centrosomes. **A**: Unsynchronized HeLa cells were treated with 1 μM 4NQO for 1 h and then fixed with paraformaldehyde for double-immunofluorescence of BRCA1 (**a**–**c**), ATR (**d**–**f**), ATRIP (**g**–**i**), ATM (**j**–**l**), Chk1 (**m**–**o**), and Chk2 (**p**–**r**) as well as with γ-tubulin. All solid arrows indicate the centrosomes. **B**: Centrosomes were isolated from HeLa cells treated with 4NQO at 0.5, 1, or 3 μM, respectively, for 1 h. The centrosomes were examined by phase contrast with a light microscope (**a**) or after Western blotting to examine the presence of DNA-PKcs and γ-tubulin (**b**).

[Su and Vidwans, 2000]. Later it has been found that after DNA damage Chk2 was recruited to the centrosomes of syncytial *Drosophila* embryos suppressing spindle assembly at the centrosome and chromosomal segregation, which finally eliminated the damaged nuclei from the cortex of the embryo [Sibon, 2003; Takada et al., 2003]. In human cells, centrosomal Chk2 was also phosphorylated by other non-PI-3 kinases involved in cell-cycle regulation, such as the Polo-like kinase 1 (Plk1) [Tsvetkov et al., 2003] or TTK/hMps1 [Wei et al., 2005]. Plk1 exerts a positive regulation on Cdc25C, which promotes the entry into mitosis by activating cyclin B/Cdc2, and regulates the anaphase promoting complex (APC) leading to the degradation of cyclin B for mitotic exit [Glover et al., 1998; Donaldson et al., 2001; van de Weerdt and Medema, 2006]. It was shown that Plk1 was inactivated by a DSB-induced DNA damage checkpoint and this inhibition seemed to be mediated by ATM [Smits et al., 2000]. After DNA damage, Plk1 and its downstream kinase Nek2 mediated an inhibition of centrosome separation that was also dependent on ATM [Zhang et al., 2005]. Moreover, ATM suppressed centrosome amplification in mouse embryonic fibroblasts [Shen et al., 2006] and directed a localization of p53 to the centrosomes in mitosis [Oricchio et al., 2006]. Taken together, the centrosomes may orchestrate multiple signaling networks that involve the DNA-damage checkpoint proteins, either for regulating cell-cycle progression or the DNA damage response in a manner depending on both the differentiation and the functional state of the cell.

Previously it has been shown that wortmannin, an inhibitor of all PI-3 kinases, suppressed the formation of bipolar spindles in mitotic sea urchin eggs [Nadai et al., 1998]. An earlier study identified a physical interaction of PI-3 kinase localized to the centrosomes with α/β tubulin or γ-tubulin in response to insulin [Kapeller et al., 1995]. Interestingly, BRCA1, a gene responsible for a predisposition to breast cancer, was identified as a protein localized to the centrosomes [Hsu and White, 1998] and directly interacting with γ-tubulin in vitro [Hsu et al., 2001]. Recent studies showed that γ-tubulin was ubiquitinated by BRCA1 that associates with its partner BARD1 as an E3 ubiquitin ligase, which may play a role in the regulation of the centrosome number [Starita et al., 2004] or the nucleation of microtubules at the centrosomes [Sankaran et al., 2005]. Such an activity of BRCA1 seems to be dependent on its localization to the centrosomes; an event probably controlled by the DNA damage check-point kinases ATM, ATR, or Chk2 that phosphorylate BRCA1 at several serine residues [Okada and Ouchi, 2003]. A recent study showed that BRIT1/MCPH1 involved in DNA damage

response regulated the ATR-Chk1 pathway and its defect had a similar phenotype as the Seckel syndrome that is caused by a haplodeficiency of ATR [Lin et al., 2005]. Taken together, BRCA1 seems to be a common target of ATR/ATM or Chk1/Chk2 involved in tumor development or growth defects. Interestingly, in our microtubule regrowth assay, we identified an ectopic initiation of microtubules as suggested by α-tubulin foci in the nucleus when vanillin was present. Potentially, a vanillin-induced inhibition of DNA-PK might cause a mislocalization of BRCA1, which in turn might regulate the turnover of γ-tubulin at non-centrosomal sites for microtubule nucleation [Luders et al., 2006]. We recognized that nucleoplasmic ATR was associated with γ-tubulin that was also present in the nucleus and associated with BRCA1. We have so far failed to observe an association of ATM or DNA-PK with γ-tubulin similar as we have seen it for ATR by co-immunoprecipitation (data not shown). Therefore ATR may be directly associated with γ-tubulin, perhaps to regulate its turnover. Alternatively, both ATM and DNA-PK may become dynamically recruited, for example, via intracellular transport, to γ-tubulin and co-operate with ATR to regulate microtubules nucleation. Interestingly, all these DNA damage checkpoint proteins became obviously displaced from the centrosomes after DNA damage. This also holds for some DNA repair proteins present in the centrosomes [Okano et al., 2005]. DNA damage actually led to an increasing level of γ-tubulin at the centrosomes, most likely achieved by a missing recruitment of BRCA1/BARD1 to the centrosome for the ubiquitination and degradation of γ-tubulin. A failure to fulfill this function of BRCA1/BARD1 may lead to a supernumerary of the centrosomes and to genomic instability [Deng, 2006; Sankaran and Parvin, 2006].

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