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Report

DNA Damage-Induced Accumulation of Centrosomal Chk1 Contributes to its Checkpoint Function

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ABSTRACT

The checkpoint kinase Chk1 is an established transducer of ATR- and ATM-dependent signalling in response to DNA damage. In addition to its nuclear localization, Chk1 localizes to interphase centrosomes and thereby negatively regulates entry into mitosis by preventing premature activation of cyclin B-Cdk1 during unperturbed cell cycles. Here, we demonstrate that DNA damage caused by ultraviolet irradiation or hydroxyurea treatment leads to centrosomal accumulation of endogenous Chk1 in normal human BJ fibroblasts and in ATR- or ATM-deficient fibroblasts. Chemical inhibition of ATR/ATM by caffeine led to enhanced centrosomal Chk1 deposition associated with nuclear Chk1 depletion. In contrast to normal or ATM-deficient fibroblasts, genetically ATR-deficient Seckel-fibroblasts showed detectable constitutive centrosomal accumulation of Chk1 even in the absence of exogenous insults. After DNA damage, the centrosomal fraction of Chk1 was found to be phosphorylated at ATR/ATM phosphorylation sites. Forced immobilization of kinase-inactive but not wild-type Chk1 to centrosomes resulted in a G_2/M checkpoint defect. Finally, both DNA damage and forced centrosomal expression of Chk1 in the absence of genotoxic treatments induced centrosome amplification in a subset of cells, a phenomenon which could be suppressed by inhibition of ATM/ATR-mediated signaling. Taken together, our results suggest that accumulation of phosphorylated Chk1 at centrosomes constitutes an additional element in the DNA damage response. Centrosomal Chk1 induces G_2/M cell cycle arrest and may evoke centrosome amplification, the latter possibly providing a backup mechanism for elimination of cells with impaired DNA damage checkpoints operating earlier during the cell cycle.

ABBREVIATIONS

AKAP, protein kinase A-anchoring protein; AT, ataxia teleangiectasia; ATM, ataxia teleangiectasia-mutated; ATR, ATM- and Rad3-related; Cdc25, cell division cycle 25; Cdk1, cyclin-dependent kinase 1; Chk1, checkpoint kinase 1; DAPI, 4',6-diamidino-2-phenylindol; GFP, green fluorescent protein; HU, hydroxyurea; PACT, pericentrin-AKAP450 centrosomal targeting domain; UV, ultraviolet radiation

INTRODUCTION

Cancer is a disease commonly characterized by genomic instability.[1] To protect their genome, eukaryotic cells have evolved signalling pathways that arrest cell cycle progression in response to DNA damage and facilitate DNA repair or, in case of too severe damage, eliminate potentially hazardous cells through apoptosis.[2-7] Recent findings indicate that the DNA damage response is commonly activated in premalignant lesions and early cancer, while evolution to advanced cancer is associated with abrogation of this response, further arguing for an essential role of the DNA damage response pathway in protecting the organism against malignant disease.[8-11]

A central mediator of the DNA damage response is the checkpoint kinase Chk1. Triggered by a variety of genotoxic insults such as ultraviolet (UV) radiation or hydroxyurea (HU) treatment, Chk1 is phosphorylated at Ser317 and Ser345 by the ATR kinase, after which activated Chk1 phosphorylates and inactivates Cdc25 phosphatases, finally leading to inhibition of cyclin-dependent kinases (Cdks) and cell cycle arrest.[7,12-17]

Additional functions of Chk1 during unperturbed cell cycles include suppression of potentially harmful supraphysiological Cdc25A levels, protection of cells against

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aberrantly increased initiation of DNA replication and DNA breakage, and suppression of supraphysiological Cdc25B levels that would otherwise impede coordination between mitotic chromatin condensation and spindle assembly.[17-19]

Under physiological conditions, the major pool of Chk1 shows nuclear localization.[20,21] In addition, Chk1 localizes to interphase centrosomes with the centrosomal localization being critical for prevention of premature activation of cyclin B-Cdk1, as this activation is initiated at centrosomes.[22,23] Accordingly, interference with centrosomal Chk1 activity leads to premature entry into mitosis.[22]

In addition to their function as organizers of interphase cytoskeleton and bipolar mitotic spindle, centrosomes serve as spatiotemporal organizers of a variety of cell cycle regulatory events. Also, there is growing evidence that centrosomes are involved in the cellular response to DNA damage.[24] In syncytial Drosophila embryo cells, DNA damage leads to increased DmChk2 localization to centrosomes followed by centrosome inactivation and subsequent anaphase chromosome segregation failure.[25,26] Hence, a centrosome inactivation checkpoint has been proposed to induce mitotic catastrophe and apoptosis in order to ensure elimination of damaged cells. In mammalian cells, amplification or fragmentation of centrosomes after DNA damage has been described.[27-29] However, only little is known about the causes and consequences of DNA damage-induced centrosome aberrations.

So far, in mammalian cells, experimental proof for a link between the function of Chk1 as a mediator of DNA damage response and its centrosomal localization is lacking. In the present study, we demonstrate that after DNA damage, human Chk1 accumulates at centrosomes in its phosphorylated form. Moreover, we provide evidence that implicates centrosomal Chk1 in the induction of G_2/M cell cycle arrest and centrosome amplification.

MATERIALS AND METHODS

Cell culture and cell lines. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (U2OS) or 15% (fibroblasts) fetal calf serum (Gibco) and antibiotics. U2OS cells conditionally expressing tetracycline-inducible wild-type GFP-Chk1-PACT(AKAP) or kinase-dead GFP-Chk1(D130A)-PACT(AKAP), respectively, have been described.[22] U2OS cells stably expressing GFP-AKAP were generated using standard technologies.

Immunofluorescence. Cells grown on glass coverslips were fixed in -20°C methanol/acetone (1:1) for 7 min. Indirect immunofluorescence staining was done using mouse monoclonal antibodies to γ-tubulin (GTU-88; Sigma), Chk1 (DCS-310),[17,22] Chk2 (DCS-270 and DCS-273),[30] and a rabbit polyclonal antibody to γ-tubulin (T 5192; Sigma). Secondary antibodies were from Molecular Probes (Alexa Fluor 488, highly cross-adsorbed) or from Jackson ImmunoResearch (Cy3-conjugated goat anti-mouse IgG). Cells were mounted in Vectashield with DAPI (H 1200; Vector Laboratories) diluted (1:3) with Vectashield (H 1000; Vector Laboratories). Fluorescence images were captured and processed using a Zeiss Axiovert 200 M microscope and Zeiss AxioVision software.

Centrosome extraction and immunoblotting. Centrosomes were isolated from U2OS cells as described.[31] A mouse monoclonal antibody to Chk1 (DCS-310),[17,22] and rabbit polyclonal antibodies to phospho-Ser317-Chk1 (Cell Signaling #2344), phospho-Ser345-Chk1 (Cell Signaling #2341), and centrin (C 7736; Sigma) were used for immunoblotting.

Flow cytometry. Cells were trypsinized, fixed with ice-cold 70% ethanol for 1 h, washed with PBS supplemented with 1% fetal calf serum (Gibco), treated with 0.25% Triton X-100 (Sigma)/PBS for 15 min on ice and consecutively washed and incubated with a rabbit polyclonal antibody to phospho-Ser10-histone H3 (Upstate #06-570), a secondary antibody from Molecular Probes (Alexa Fluor 488, highly cross-adsorbed), and PBS containing 10 μg/ml propidium iodide (Molecular Probes) and 0.25 mg/ml RNase A. Using a FACScan flow cytometer, the percentage of mitotic cells was assessed by gating in cells with 4N DNA and bright phospho-Ser10-histone H3 and gating out doublets by their propidium iodide staining pattern.

Statistical analysis. The significance of quantitative differences was calculated using the heteroscedastic, two-tailed Student's t-test.

RESULTS

Chk1 accumulates at centrosomes after DNA damage. It has been previously shown that endogenous Chk1 localizes to interphase centrosomes, from where it is released at the onset of mitosis.[22] To test whether this centrosomal localization is responsive to DNA damage, different human fibroblast cell lines were treated with 20 J/m^2 UV or 2 mM HU. Both types of DNA-damaging treatments led to centrosomal accumulation of endogenous Chk1 in all tested cell lines (Figs. 1A and B). Centrosomal Chk1 accumulation was evident 3 h after UV treatment, or 3 h after addition of HU to cells, and after 48 h, this accumulation was even more pronounced for both forms of damage. Centrosomal Chk1 accumulation was found in normal BJ fibroblasts as well as in ATR- or ATM-deficient fibroblasts (Fig. 1 and data not shown). The most pronounced effect was seen in ATR-Seckel (F02-98) fibroblasts, which contain a hypomorphic ATR mutation[32] (Fig. 1A and B).

Interestingly, the constitutive level of Chk1 centrosomal localization was enhanced in ATR-deficient Seckel cells, compared with either normal BJ or ATM-deficient AT fibroblasts (Fig. 2, mock), possibly reflecting the constitutive endogenous replication stress reported for Seckel cells.[32] Centrosomal Chk1 accumulation after 20 J/m^2 UV was observed in BJ, AT or ATR-Seckel cells, and it was not suppressed by the ATM/ATR inhibitor caffeine (10 mM) in any of the cell types examined (Fig. 2). Instead, caffeine alone led to an increase in centrosomal Chk1 accumulation even in the absence of DNA damage, which was associated with a decrease in the amount of nuclear Chk1 (Figs. 2 and 3A). This caffeine-induced loss of nuclear Chk1, associated with increased centrosomal Chk1 loading, was verified in U2OS cells, which possess a higher pool of nuclear Chk1 than fibroblasts (Fig. 3A). Furthermore, the nuclear pool of Chk1 was low in ATR-Seckel fibroblasts, while Chk2 was strongly localized to the nucleus in these cells (Fig. 3B). Both damaged and undamaged BJ fibroblasts showed a stronger nuclear Chk1 signal as compared to ATR-Seckel cells under identical conditions (Fig. 1A). We conclude that while DNA damage leads to accumulation of Chk1 at interphase centrosomes in non-transformed human fibroblasts, in the absence of DNA damage ATR (and possibly ATM) limit centrosomal Chk1 accumulation, which explains both the remarkably high centrosomal Chk1 load in ATR-deficient fibroblasts and the induction of centrosomal Chk1 accumulation by caffeine. Whether this reflects a direct role of ATR in enhancing nuclear retention of Chk1 under normal conditions, or indirect effects due to endogenous DNA damage in Seckel cells, remains to be elucidated.

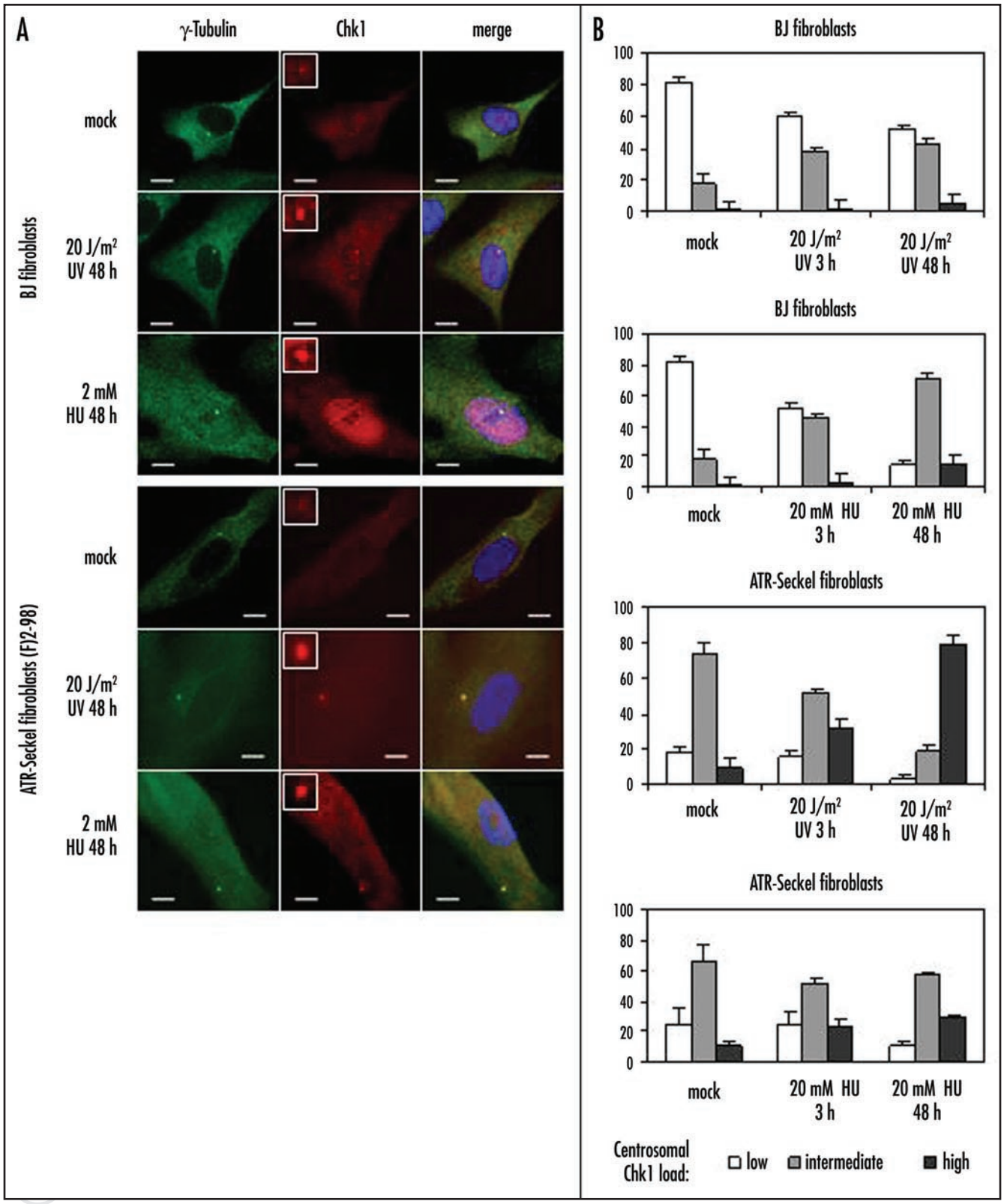

Figure 1. Chk1 accumulates at the centrosome in response to both UV and HU treatment. (A) BJ, ATR-Seckel (F02-98) or AT fibroblasts were harvested 3 h or 48 h after irradiation with 20 J/m^2 UV or after addition of HU to a final concentration of 2 mM. Cells were immunostained with antibodies to γ-Tubulin (green) and Chk1 (red). DNA was counterstained with DAPI (blue). Scale bars represent 10 μm. Insets show higher magnifications of the centrosomal Chk1 load. (B) Quantification of the centrosomal Chk1 load as shown in (A). At least three times 100 cells were counted per data bar. Results are given as mean ± standard deviation.

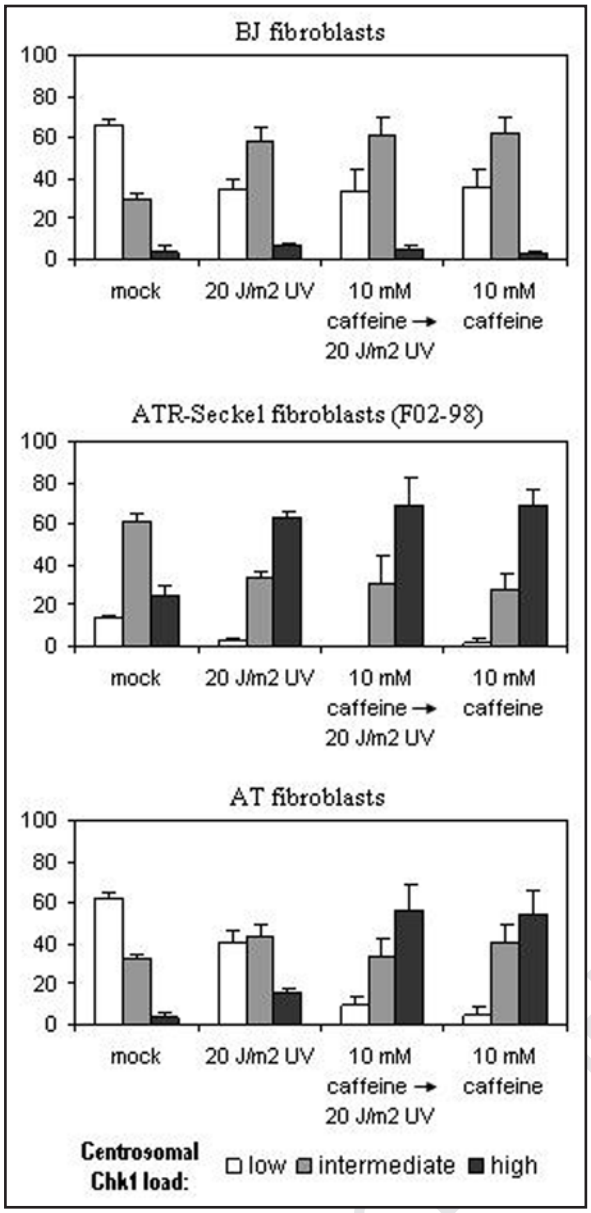

Figure 2. Centrosomal Chk1 accumulation is increased by caffeine. BJ, ATR-Seckel (F02-98) or AT fibroblasts were harvested 48 h after irradiation with 20 J/m^2 UV or mock treatment. When indicated, caffeine was added to a final concentration of 10 mM 1 h before UV or mock treatment. Cells were immunostained with antibodies to γ-Tubulin for identification of centrosomes and Chk1. The centrosomal Chk1 load was quantified by counting at least three times 100 cells per data bar. Results are given as mean ± standard deviation.

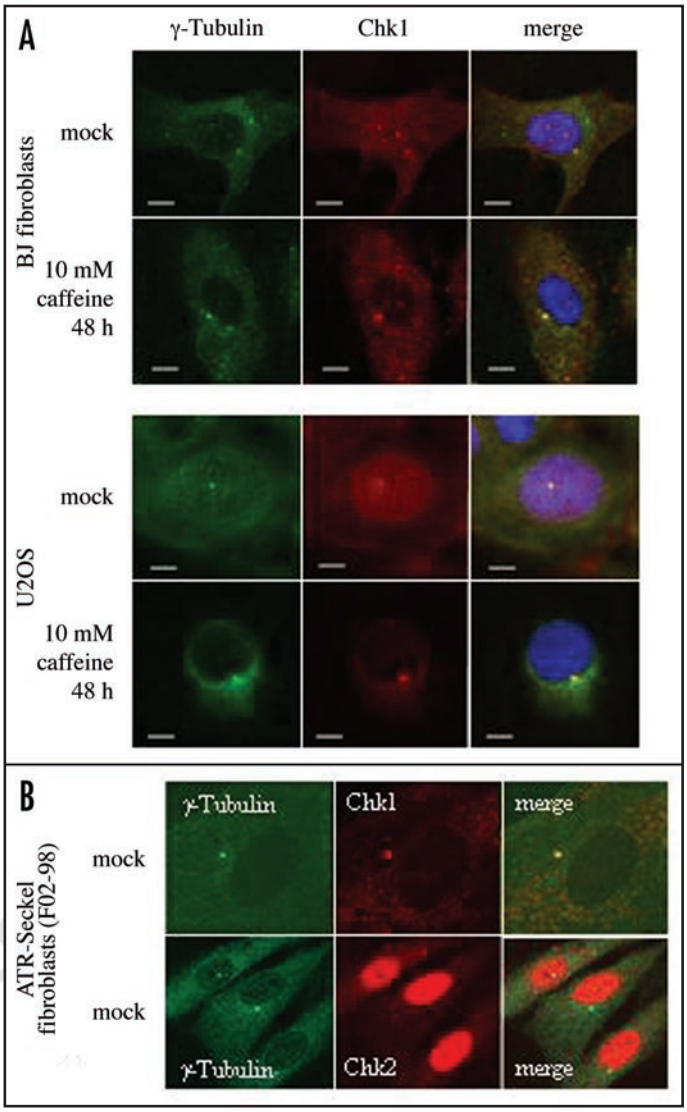

Figure 3. The nuclear Chk1 load correlates with ATR activity. (A) BJ or U2OS cells were harvested 48 h after mock treatment or addition of caffeine to a final concentration of 10 mM. Cells were immunostained with antibodies to γ-Tubulin (green) and Chk1 (red). DNA was counterstained with DAPI (blue). Scale bars represent 10 μm. (B) ATR-Seckel fibroblasts (F02-98) were immunostained with antibodies to γ-Tubulin (green) and Chk1 or Chk2, respectively (red).

To test whether centrosomal Chk1 is activated by ATR/ATM in response to DNA damage, we used centrosome extracts from U2OS cells, which are ATR-, ATM- and G_2/M checkpoint-proficient. Enrichment of centrosomal components in these extracts was demonstrated by immunoblotting with an antibody to centrin (Fig. 4). 3 h after 20 J/m^2 UV, phosphorylation of centrosomal Chk1 at the ATR/ATM phosphorylation sites Ser317 and Ser345 was strongly enhanced in comparison to mock-treated centrosome extracts (Fig. 4). Moreover, the phosphorylated form of Chk1 was preferentially loaded at centrosomes as whole cell lysates showed a weaker enhancement of Chk1 phosphorylation at Ser317 and Ser345 under identical conditions (Fig. 4). We conclude that DNA damage leads to accumulation of ATR/ATM-phosphorylated Chk1 at centrosomes.

G_2/M checkpoint activation by centrosomal Chk1. To further strengthen the link between centrosomal Chk1 and the DNA damage response, we used U2OS cells conditionally expressing wild-type or kinase-dead versions of GFP-Chk1-PACT(AKAP). In these cells, Chk1 is targeted to the centrosome by fusion of GFP-Chk1 to the PACT (pericentrin-AKAP450 centrosomal targeting) domain of AKAP450, an immobile protein of the pericentriolar matrix.[22,33] The PACT(AKAP) motif was previously verified to target Chk1 selectively to centrosomes (apparently not affecting the nuclear pool of Chk1), and neither PACT(AKAP) nor GFP tagging interfered with Chk1 function.[22]

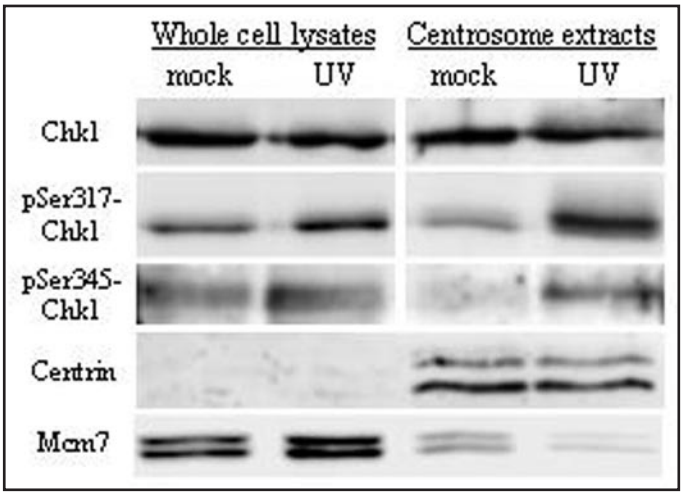

Figure 4. Chk1 phosphorylated at Ser317 and Ser345 is enriched at centrosomes after UV. Isolated centrosome preparations or whole cell lysates from U2OS cells harvested 3 h after 20 J/m^2 UV or mock treatment were subjected to immunoblotting for the indicated proteins.

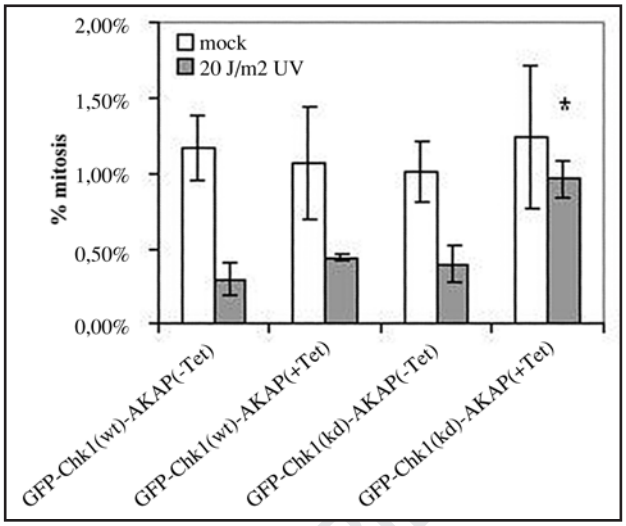

Figure 5. Centrosome-targeted inhibition of Chk1 abrogates G_2/M arrest after DNA damage. U2OS cells conditionally expressing wild-type or kinase-dead GFP-Chk1-PACT(AKAP) were induced to express the transgene by tetracycline for 72 h, irradiated with 20 J/m^2 UV and harvested 1 h after UV. Mock controls were prepared in parallel. The percentage of mitotic cells was assessed by flow cytometry with an antibody to phospho-Ser10-histone H3 as mitosis marker. Results are given as mean ± standard deviation from three independent experiments. The asterisk indicates a significant difference in the percentage of mitotic cells after UV ($p < 0.05$).

In response to DNA damage, G_2/M checkpoint-competent cells rapidly downregulate the proportion of cells in mitosis. This response was proficient in both non-induced cells and cells expressing wild-type Chk1 forced to the centrosome, as shown by flow-cytometric assessment of mitotic cells using phospho-histone H3 staining 1 h after 20 J/m^2 UV treatment (Fig. 5). In contrast, significantly more cells were in mitosis 1 h after 20 J/m^2 UV when kinase-dead centrosome-tagged Chk1 was expressed (Fig. 5). Hence, selective interference with the activity of centrosomal Chk1 leads to a G_2/M checkpoint defect.

Centrosomal Chk1 accumulation leads to centrosome amplification. In response to DNA damage, centrosome amplification or fragmentation has been proposed as a plausible backup checkpoint mechanism to ensure elimination of damaged cells by mitotic catastrophe.[27-29] 48 h after 20 J/m^2 UV, we found amplified centrosomes in a small but significant proportion of ATR-Seckel fibroblasts (Fig. 6A). Under these conditions, centrosome amplification was also found in AT fibroblasts (data not shown) but not in normal BJ fibroblasts (Fig. 6A). Interestingly, the degree of centrosome amplification (the ratio between the number of cells with more than two centrosomes after UV compared to mock-treated cells) correlated with the centrosomal Chk1 load in response to UV in the respective cell lines (Figs. 2 and 6A). Centrosome amplification was suppressed by 10 mM caffeine (Fig. 6A).

These data prompted us to hypothesize that DNA damage-induced centrosome amplification might be regulated by centrosomal Chk1. We tested this hypothesis in U2OS cells conditionally overexpressing the centrosome-localized Chk1. In these cells, upon induction of GFP-Chk1-PACT(AKAP), centrosomes were amplified in a time-dependent manner (Fig. 6B and C). Expression of centrosome-tagged Chk1 led to more pronounced centrosome amplification than 20 J/m^2 UV in non-induced cells, and UV treatment did not further enhance centrosome amplification after induction of wild-type GFP-Chk1-PACT(AKAP) (Fig. 6C), consistent with our prediction that centrosomal Chk1 overexpression and UV treatment may activate the same mechanism of centrosome amplification. Notably, stable expression of a control fusion protein GFP-PACT(AKAP) in U2OS cells was not associated with centrosome amplification (data not shown). Accordingly, centrosome amplification is specifically attributable to expression of centrosome-tagged Chk1. Furthermore, 10 mM caffeine abolished centrosome amplification upon induction of GFP-Chk1-PACT(AKAP) (data not shown). We conclude that both centrosomal localization of Chk1 and its phosphorylation by ATR/ATM are required for centrosome amplification.

DISCUSSION

The principal signalling molecules involved in the DNA damage response are the large serine/threonine kinases ATM and ATR. It is well established that the DNA damage response comprises activation of Chk1 and Chk2 by ATR/ATM, and that interference with these mechanisms leads to disruption of the G_2/M checkpoint, giving rise to frequently aberrant mitoses after genotoxic stress which might affect cell viability.[7,14,16,34-36] In Drosophila embryos, DNA damage-induced centrosome inactivation by centrosomal accumulation of DmChk2 constitutes an additional checkpoint for the elimination of damaged nuclei via induction of mitotic catastrophe.[25,26] In mammalian systems, centrosome aberrations are frequently seen after exposure to DNA damaging agents[27-29] as well as in cells with defective DNA repair and most human malignancies.[37-42]

Our results extend these views by showing that in mammalian cells in response to DNA damage, Chk1 is specifically enriched at centrosomes. This centrosomal recruitment of Chk1 after DNA damage occurred in normal BJ fibroblasts as well as in AT and ATR-Seckel fibroblasts and could not be prevented by inhibition of the upstream kinases ATM and ATR with caffeine. To the contrary,

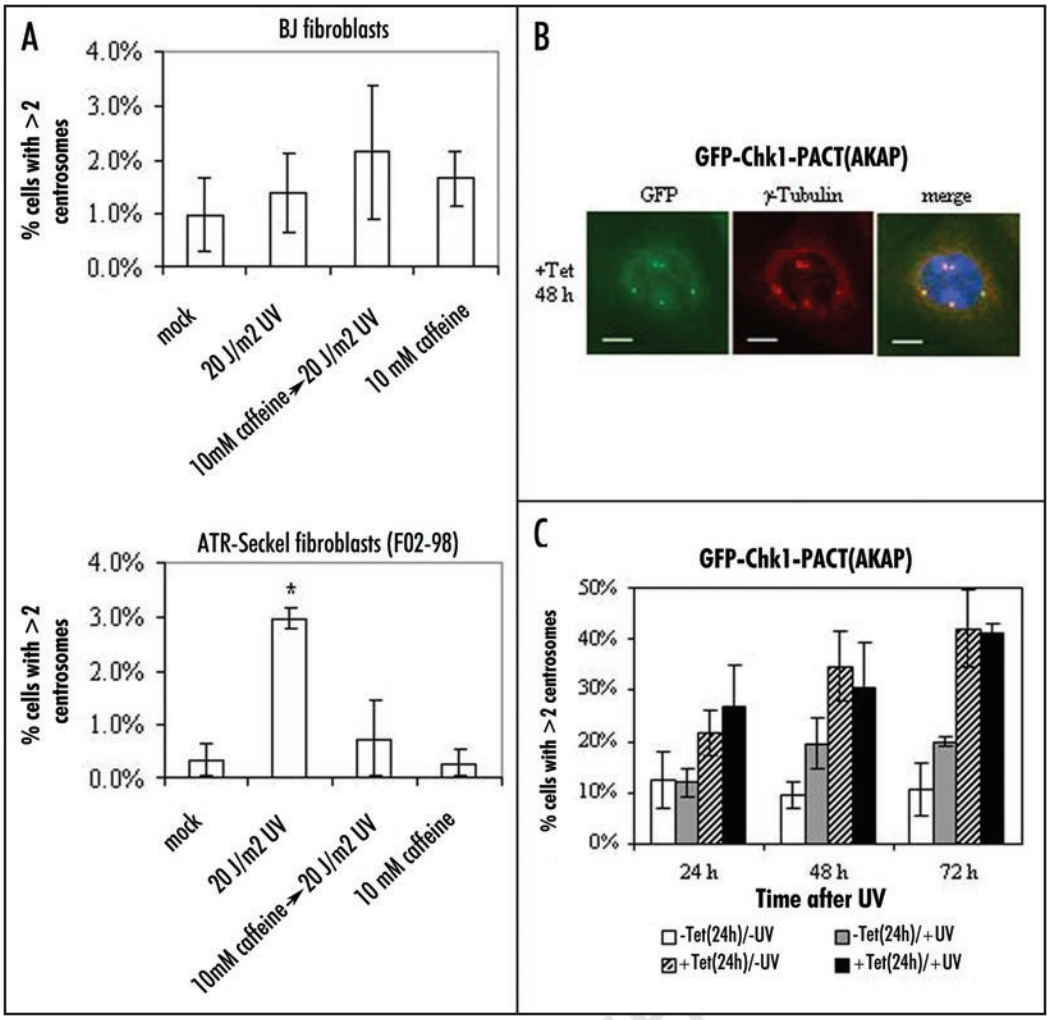

Figure 6. Both ultraviolet irradiation and forced centrosomal expression of Chk1 lead to centrosome amplification. (A) BJ and ATR-Seckel (F02-98) fibroblasts were harvested 48 h after irradiation with 20 J/m^2 UV or mock treatment. When indicated, caffeine was added to a final concentration of 10 mM 1 h before UV or mock treatment. The percentage of cells with more than 2 centrosomes, as identified by γ-Tubulin immunostaining, was evaluated by counting at least three times 200 cells per data bar. Results are given as mean ± standard deviation. The asterisk indicates a significant difference ($p < 0.05$). (B) Centrosome-targeted expression of Chk1 induces centrosome amplification. U2OS cells conditionally expressing wild-type GFP-Chk1-PACT(AKAP) were induced to express the transgene by tetracycline for 48 h. Cells were immunostained with antibodies to γ-tubulin (red). DNA was counterstained with DAPI (blue). Scale bars represent 10 μm. (C) U2OS cells conditionally expressing tetracycline-inducible wild-type GFP-Chk1-PACT(AKAP) were induced to express the transgene by tetracycline for 24 h, irradiated with 20 J/m^2 UV and collected 24 to 72 h after UV. Controls were prepared in parallel. The percentage of cells with more than two centrosomes, as identified by γ-tubulin immunostaining, was evaluated by counting at least three times 100 cells per data bar. Results are given as mean ± standard deviation.

ATM/ATR kinase activity actually limited the association of Chk1 with centrosomes, as: (1) genetic ablation of ATR in otherwise unperturbed Seckel fibroblasts increased Chk1 loading onto centrosomes with only little Chk1 left over in the nuclear compartment and (2) chemical inhibition of ATM/ATR by caffeine led to nuclear export and centrosomal accumulation of Chk1 in both BJ fibroblasts and U2OS cells. These findings might be explained by earlier data suggesting that basal activity of ATR is required to keep the chromatin-associated pool of Chk1 within the nucleus in the absence of exogenous DNA damage.[43] Alternatively, an impaired response to deregulated DNA replication might lead to pronounced centrosomal Chk1 accumulation in ATR-Seckel fibroblasts even in the absence of exogenous damage via indirect mechanisms. As a consequence, centrosomal accumulation of Chk1 might prevent cyclin B-cdk1 activation as a backup mechanism in the absence of ATR/ATM activity, a finding that is consistent with the low proliferative potential of ATR-Seckel fibroblasts. Whether and how the compensatory overactivation of ATR described in AT cells[44] affects centrosome-associated Chk1 function, remains to be determined.

To gain insight into how the centrosomal pool of Chk1 might be regulated, we separately analyzed the phosphorylation status of Chk1 at the centrosome. DNA damage led to a predominant phosphorylation of the centrosomal pool of Chk1 as whole cell lysates showed a weaker enhancement of Chk1 phosphorylation at Ser317 and Ser345 under identical conditions. This observation is consistent with a recent report by Niida and coworkers, demonstrating that after DNA damage, phosphorylated Chk1 is predominantly detectable in the cytoplasmic fraction.[45] Therefore, DNA damage seems to trigger both accumulation of Chk1 at centrosomes and ATR/ATM-dependent phosphorylation of Chk1. Whether Chk1 becomes phosphorylated by ATR/ATM directly at the centrosomes, or whether the DNA damage-induced nuclear phosphorylation of Chk1 might lead to an increased nucleocytoplasmic transport and subsequent centrosomal association of Chk1, as suggested earlier,[45] remains to be established. We conclude that the centrosomal pool of Chk1 appears to be, in addition to its role during unperturbed cell cycle progression,[22] involved in the response to DNA damage as well.

Mechanistically, we were able to demonstrate that specific interference with centrosomal Chk1 activity leads to disruption of the DNA damage-induced G_2/M arrest, thereby allocating a specific role for the centrosomal pool of Chk1 during this checkpoint response. This finding is consistent with a recent report demonstrating that, following DNA damage, Chk1 inhibits activation of Cdc25B and Cdk1 at the centrosome with subsequent G_2/M arrest.[47] Also, this emerging concept fits the above mentioned scenario of pronounced centrosomal Chk1 accumulation in ATR-Seckel fibroblasts as a consequence of endogenous DNA damage to prevent mitotic entry as a backup mechanism in the absence of ATR activity.

Recently, it has been described that DNA damage-induced centrosome amplification is dependent on Chk1.[48,49] These observations are corroborated by our finding that centrosomal Chk1 accumulation and centrosome amplification occur concomitantly in response to DNA damage, with the extent of centrosomal accumulation of Chk1 being correlated to the degree of centrosome amplification. Whereas normal BJ fibroblasts show only moderate centrosomal Chk1 enrichment and little centrosome amplification after DNA damage, both effects are more pronounced in AT fibroblasts and most prominent in ATR-Seckel fibroblasts. Noteworthy, despite centrosome amplification was detectable in both ATR- and ATM-deficient cells, it could be suppressed by concomitant inhibition of both ATR and ATM, supporting the conclusion that ATR and ATM might be complementary to each other in this response. Finally and most importantly, expression of centrosome-tethered Chk1 induced centrosome amplification even in the absence of exogenous DNA damage, thereby indicating that centrosomal recruitment of Chk1 is critical in this response.

Taken together, the centrosomal fraction of Chk1 plays an important role for both induction of a G_2/M arrest (the predominant phenotype) and centrosome amplification (in a subset of cells) in response to DNA damage. The principal role of Chk1 during these centrosome-associated processes appears to be to directly relay the DNA damage signal to the centrosome, where it imposes a cell cycle delay by inhibiting Cdk1 activation. In a fraction of cells, centrosomal Chk1 subsequently signals to the centrosome duplication machinery. The mechanistic basis of the decision in terms of which of the cells with centrosome-accumulated Chk1 would undergo centrosome amplification, remains unknown. Given that this phenomenon was more pronounced among ATR/ATM-deficient cells and cancerous U2OS cells compared with normal fibroblasts, we speculate that such DNA damage-induced centrosome amplification might serve as an additional barrier for elimination of cells which have lost or impaired other checkpoint mechanisms and/or reached a threshold of genomic instability beyond repair.

Finally, centrosomal aberrations including numerical amplification are common in a variety of cancers, including premalignant and early lesions,[36,38,39,50,51] where, on the other hand, activation of the DNA damage response pathway does occur frequently.[8,11] Hence, one might speculate that Chk1-dependent centrosome amplification takes place in premalignant lesions and early cancer, where it might serve as a checkpoint mechanism helping to eliminate malignant cells via induction of multipolar mitoses with subsequent mitotic catastrophe. Evolution to advanced cancer has been shown to be associated with abrogation of this checkpoint by clustering of multiple centrosomes into bipolar mitotic spindles.[52-54]

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