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# Cell cycle-dependent localization of CHK2 at centrosomes during mitosis

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## Abstract

**Background:** Centrosomes function primarily as microtubule-organizing centres and play a crucial role during mitosis by organizing the bipolar spindle. In addition to this function, centrosomes act as reaction centers where numerous key regulators meet to control cell cycle progression. One of these factors involved in genome stability, the checkpoint kinase CHK2, was shown to localize at centrosomes throughout the cell cycle.

**Results:** Here, we show that CHK2 only localizes to centrosomes during mitosis. Using wild-type and CHK2<sup>-/-</sup> HCT116 human colon cancer cells and human osteosarcoma U2OS cells depleted for CHK2 with small hairpin RNAs we show that several CHK2 antibodies are non-specific and cross-react with an unknown centrosomal protein(s) by immunofluorescence. To characterize the localization of CHK2, we generated cells expressing inducible GFP-CHK2 and Flag-CHK2 fusion proteins. We show that CHK2 localizes to the nucleus in interphase cells but that a fraction of CHK2 associates with the centrosomes in a Polo-like kinase 1-dependent manner during mitosis, from early mitotic stages until cytokinesis.

**Conclusion:** Our findings demonstrate that a subpopulation of CHK2 localizes at the centrosomes in mitotic cells but not in interphase. These results are consistent with previous reports supporting a role for CHK2 in the bipolar spindle formation and the timely progression of mitosis.

**Keywords:** CHK2, CHK1, Centrosome, Mitosis, Cell cycle

## Background

The serine/threonine checkpoint kinases CHK1 and CHK2 are core components of the cell response to genotoxic stresses [1-3]. In response to DNA damage, CHK1 and CHK2 are activated by Rad3-related (ATR) and ataxia-telangiectasia mutated (ATM) protein kinases respectively, targeting many downstream substrates that coordinate cell cycle checkpoint activation, DNA repair and apoptosis [4,5]. Interestingly, recent studies have highlighted roles for CHK1 and CHK2 during normal cell cycle regulation in the absence of DNA damage. CHK1 has been reported to play a role in unperturbed S phase and in delaying the entry of cells into mitosis [6-10]. CHK1 has also been shown to be required for mitosis progression and for spindle assembly checkpoint

function [11-14]. Recently, a role for CHK2 in the assembly of the bipolar mitotic spindle and normal mitosis progression has been reported [15]. This DNA-damage-independent function of CHK2 is mediated by the phosphorylation of the tumor suppressor BRCA1 on Ser 988, and is required to maintain chromosomal stability in the HCT116 human colorectal tumor cell line [15].

Centrosomes function primarily as microtubule-organizing centres and play a crucial role in the formation of bipolar spindles and chromosomal segregation during mitosis [16,17]. In addition, increasing evidence suggests that centrosomes also play roles in regulating various cell signalling pathways including cell cycle regulation and the DNA damage response [17-19]. Numerous cell cycle regulatory molecules have been identified at the centrosomes which are thought to function as integration sites of positive and negative pathways to regulate cell cycle progression [17,18,20-25]. A growing number of components of the DNA damage response network, including p53, ATM, ATR, CHK1, CHK2, Rad 51, BRCA1 and BRCA2 have also been localized at the

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centrosomes supporting a role for centrosomes in the DNA damage response [8,9,26-35]. At the G2/M boundary, the molecular events that initiate entry into mitosis occur at the centrosomes where the activation of the mitotic kinase CDK1-cyclinB is initiated [36-39]. Interestingly, several studies report that a subpopulation of CHK1 localizes to the interphase centrosomes to regulate the entry of cell into mitosis by inhibiting centrosomal CDK1-cyclin B activation [8-10].

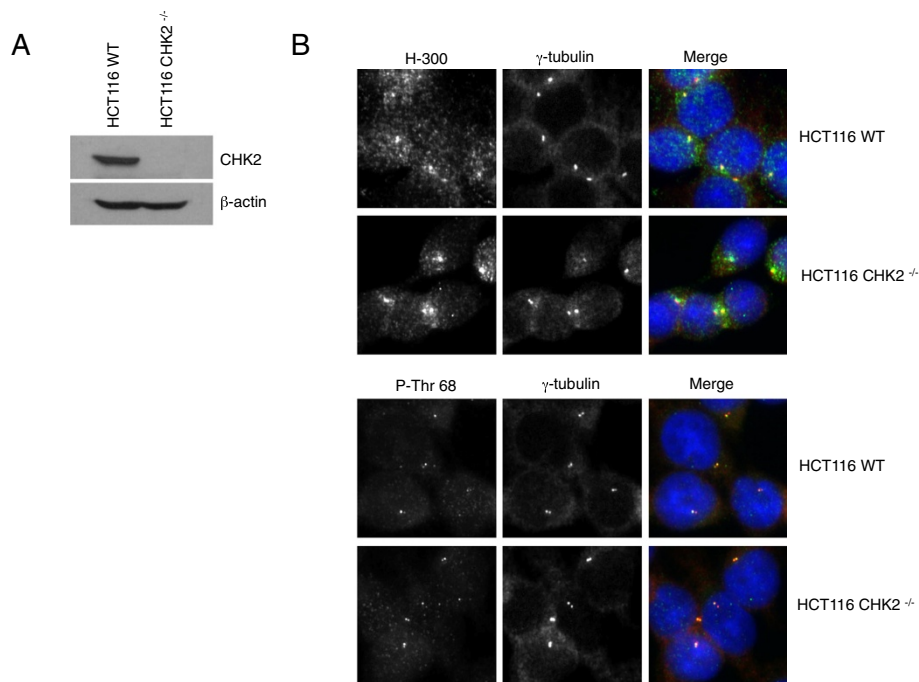
Although CHK2 is mainly nuclear in somatic cells, several reports document the presence of a subpopulation of CHK2 at centrosomes. Strikingly, in embryonic stem (ES) cells, CHK2 has been shown to localize exclusively at the centrosomes and studies performed in *Drosophila melanogaster* embryos suggest that DmCHK2 localizes at the centrosomes to disrupt spindle assembly and chromosome segregation in response to DNA damage [29,31,40]. Using antibodies against CHK2-phospho-Thr68 in immunofluorescence experiments (IF), Tsvetkov and colleagues reported that a subpopulation of CHK2 localizes at centrosomes in interphase and mitotic cells in the absence of induced DNA damage [30]. In the same study, a centrosomal localization for a subset of HA-tagged CHK2 protein under specific IF conditions with pre-extraction of cells was reported [30]. The Thr383/387-phosphorylated form of CHK2 has also been localized at centrosomes in response to DNA damage, and CHK2 was found to co-purify with centrosomes in gradient-purified centrosome preparations [8,41]. Despite these observations, the centrosomal localization of CHK2 remains controversial and there are doubts regarding the specificity of the antibodies used to demonstrate expression of CHK2 at the centrosomes by immunofluorescence. In the present study we show that anti-CHK2 antibodies used to stain CHK2 at centrosomes cross-react non-specifically with an unknown centrosomal protein(s). By using cell lines expressing Flag or GFP-tagged CHK2 proteins, we demonstrate that CHK2 localizes exclusively in the nucleus of cells in interphase. However, we observed that a small portion of CHK2 localizes to centrosomes in mitotic cells, from late prophase until cytokinesis supporting a role for CHK2 during mitosis [15].

## Results

### CHK2 is localized in the nucleus but not at the centrosomes in interphase cells

Several antibodies raised against CHK2 have been reported to stain the centrosomes by immunofluorescence in HEK293T and U2OS cells [8,30]. We confirm that both the rabbit polyclonal affinity-purified antibodies raised against the N-terminal amino acids 1 to 300 of CHK2 (H-300) as well as the CHK2-phospho-Thr68 antibody stain the centrosomes of U2OS cells fixed with 100% methanol. To validate the specificity of

these antibodies in immunofluorescence experiments, we used an isogenic human colorectal cancer HCT116 cell line with a targeted deletion of CHK2 [42]. The expected 62 kDa band corresponding to the molecular weight of CHK2 protein was not detected in the HCT116 CHK2<sup>-/-</sup> cells by Western blotting, but was detected in the wild type (WT) cells (Figure 1A). Although we obtained positive staining at the centrosomes with the CHK2 H-300 and phospho-Thr68 antibodies in IF experiments there was no significant difference in the staining at the centrosomes between the HCT116 WT and CHK2 knockout cells (Figure 1B). To confirm the results obtained with the HCT116 cell lines, we generated human osteosarcoma (U2OS) cell lines stably transduced with small hairpin *CHK2* RNAs targeting different regions of *CHK2* ORF. Lentiviral shRNAs directed against N-terminal sequences of the *CHK2* ORF located either at 3' (shRNA-1) or 5' (shRNA-2) of Thr68 were used to infect U2OS cells. U2OS cell lines transduced with a *CHK2* shRNA targeting the 3'UTR sequence (shRNA-3) or with a combination of shRNA 2 + 3 were also generated. We confirmed the shRNA-mediated silencing of CHK2 in the stably transduced cells by immunoblotting, and noted that the efficiency of CHK2 depletion varied between small hairpins with shRNA-1 providing the best depletion (Figure 2A). We then stained the cell lines generated with the anti-CHK2 H-300 and phospho-Thr68 antibodies. Although the intensity of staining with the H-300 antibody in the U2OS cell lines depleted for CHK2 decreased in the nucleus, the centrosomes remained positively stained in all cell lines. There was no significant decrease of the centrosomal signal in CHK2-depleted cell lines compared to the untransduced U2OS cells or cells transduced with control GFP-shRNAs (Figure 2B-E). To validate the methodology we used to quantify the fluorescence intensity signal at the centrosomes, we generated a stable U2OS cell population expressing a doxycycline-inducible GFP protein fused to the PACT (pericentrin-AKAP450-centrosome targeting) domain of AKAP450 [43]. Confirming previous reports, we found that the GFP-PACT fusion protein localized predominantly at the centrosomes (Figure 2F). We induced the expression of the GFP-PACT fusion protein by adding increasing doses of doxycycline to the culture media. We observed a dose-dependent increase in the expression of the GFP-PACT fusion protein that was proportional to the intensity of the fluorescence measured at the centrosomes (Figure 2G and H). In summary, the staining pattern we obtained with the U2OS cell lines is in agreement with that observed in the HCT116 cells. We confirm that the CHK2 H-300 and CHK2-phospho-Thr68 antibodies bind non-specifically to protein(s) other than CHK2 localized at the centrosomes in interphase cells.



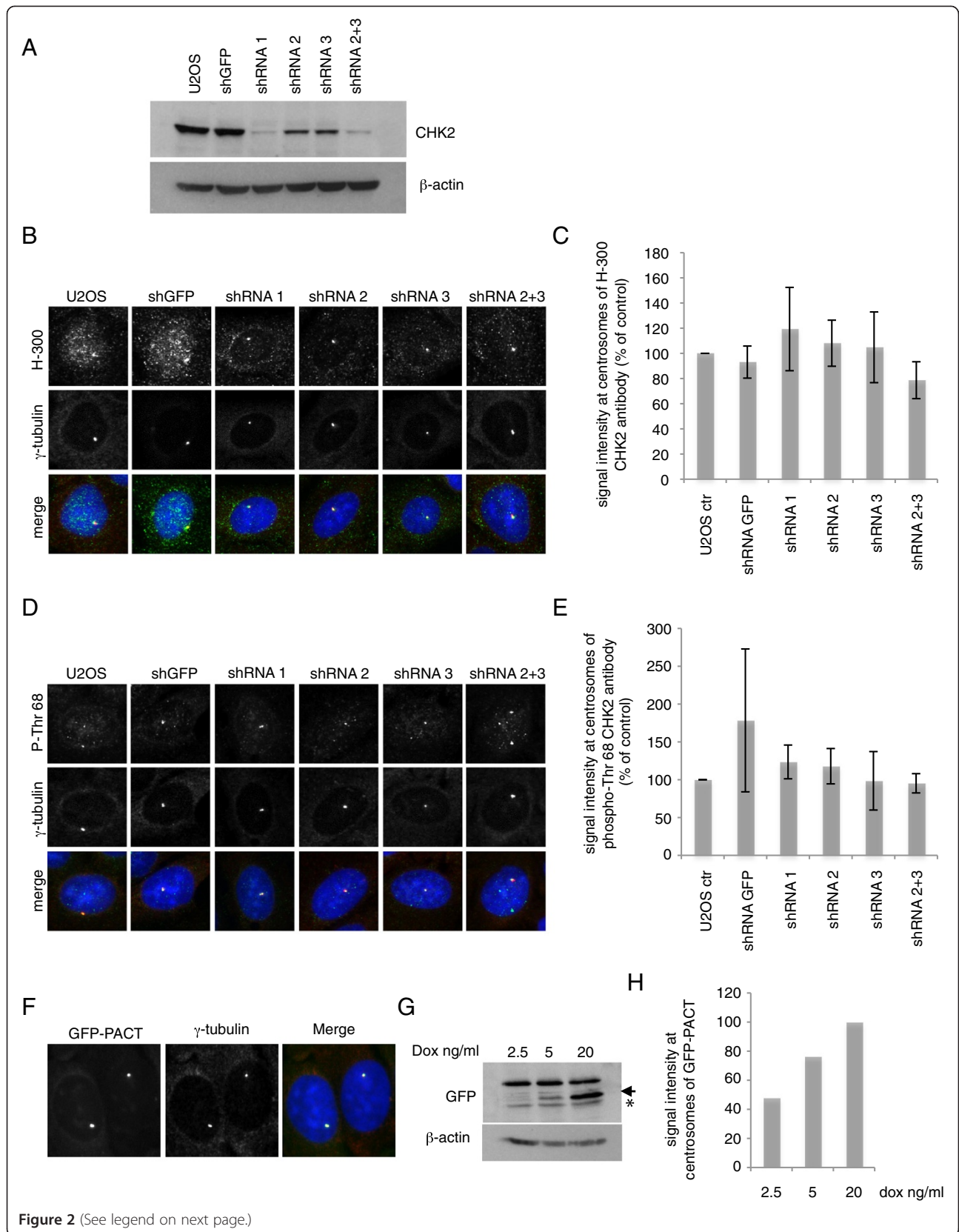
**Figure 1 Centrosomes stain positive for CHK2 in human colon cancer cells deficient in CHK2. (A)** There is no expression of CHK2 in HCT116 CHK2<sup>-/-</sup> cells. HCT116 WT and HCT116 CHK2 knockout cells were subjected to Western blotting with anti-CHK2 antibody. **(B)** We did not detect differences in the staining between WT and CHK2 knockout cells. Exponentially growing HCT116 WT and CHK2<sup>-/-</sup> cells were fixed and immunostained with antibodies against the N-terminal portion of CHK2 (H-300) or the Thr68-phosphorylated form (P-Thr 68) (green). Cells were costained for  $\gamma$ -tubulin (red) and counterstained with DAPI (blue).

### CHK2 localizes at the centrosomes in mitotic cells

To examine more closely the localization of CHK2, we generated stable U2OS cell lines that express the fusion protein GFP-CHK2 or Flag-CHK2 under the control of the doxycyclin-inducible promoter (Figure 3A). We first assessed the functionality of the GFP and Flag-tagged CHK2 proteins by examining their phosphorylation status in response to DNA damage induced by ionizing radiation ( $\gamma$ -IR). In response to gamma rays, the exogenous CHK2 fusion proteins were found phosphorylated on Thr 68, Thr 383/387 and Ser 516 suggesting that the kinases are fully active (see Additional file 1). In addition, we detected an increase in the phosphorylation of the CHK2 substrate CDC25 A (Ser 123) indicative of GFP- and Flag-CHK2 activation in the transduced cell lines relative to U2OS control cells (see Additional file 1). These data suggest that the doxycycline-inducible GFP-CHK2 and Flag-CHK2 proteins are functional kinases [2,3].

In direct immunofluorescence experiments performed 48 h following doxycycline addition to the culture medium, we found GFP-CHK2 to be exclusively localized in the nucleus of interphase cells with no apparent centrosomal staining (Figure 3B). However, we detected GFP-CHK2 at the centrosomes in mitotic cells. We found a small subpopulation of GFP-CHK2 at the centrosomes from the late prophase/early prometaphase

stage, where centrosomes separate and migrate to cell poles, until cytokinesis. We also observed GFP-CHK2 concentrated at the mid-body in late mitosis (Figure 3B). Similar results were obtained in immunostained cells expressing Flag-CHK2 fusion protein (Figure 3C). The centrosomal localization of GFP-CHK2 and Flag-CHK2 in mitotic cells was maintained when microtubules were depolymerized by nocodazole treatment, suggesting that CHK2 localizes to mitotic centrosomes in a microtubule-independent manner (see Additional file 2). To further control the reliability of these results, we also generated U2OS cell lines transduced with the doxycycline-inducible GFP, GFP-CHK1 and Flag-CHK1 constructs. Unlike GFP-CHK2 and Flag-CHK2, the GFP, GFP-CHK1 and Flag-CHK1 proteins, although induced at high levels in the U2OS cell lines, did not localize to the centrosomes in mitotic cells (see Additional file 3). In interphase cells expressing very high levels of GFP protein, some centrosomes appeared slightly stained but most cells did not stain at the centrosomes (see Additional file 3). Although these results suggest that the centrosomal localization revealed for GFP-CHK2 and Flag-CHK2 is specific, we decided to further support our findings by performing live-cell microscopy experiments. In living cells, we observed that a fraction of GFP-CHK2 was associated with the centrosomes during mitosis from early prometaphase



**Figure 2** (See legend on next page.)



(See figure on previous page.)

**Figure 2 Centrosomal staining for CHK2 antibodies persists in cells expressing CHK2 targeting shRNAs.** U2OS cells were stably transduced with various shRNAs directed against CHK2 or GFP. **(A)** Western blots showing the expression level of endogenous CHK2 in the transduced cell lines. **(B and D)** Cells were fixed and immunostained with the indicated anti-CHK2 antibodies (green) and costained for centrosomes (red) and DNA (blue). Representative fields of the immunofluorescence stainings obtained with the indicated cell lines are shown. **(C and E)** The fluorescence intensity of the CHK2 antibodies H-300 and P-Thr 68 was quantified and represented as a ratio of the control  $\gamma$ -tubulin signal at centrosomes. This ratio was set at 100% in control untransduced cells. Results are representative of 3 independent experiments where the intensity at the centrosomes was monitored in 200 cells. Error bars represent the standard deviation from the mean of 3 experiments. To validate the method of quantification of the fluorescence intensity signal at centrosomes, stable transduced GFP-PACT U2OS cells were generated. **(F)** A representative field showing the centrosomal localization of the GFP-PACT fusion protein. 48 h after 20 ng/ml doxycycline addition, cells were fixed and stained with anti- $\gamma$ -tubulin antibody (red) and DAPI (blue). GFP-PACT was visualized by direct fluorescence. **(G, H)** GFP-PACT expression was induced with 2.5, 5 or 20 ng/ml doxycycline and cells were stained as in **(F)**. **(G)** The expression of GFP-PACT at each doxycycline concentration was analyzed by Western blotting using anti-GFP antibody. The arrow indicates the bands corresponding to GFP-PACT and the asterisk designates non-specific signal. **(H)** The fluorescence intensity at centrosomes of GFP-PACT was quantified as in **C** and **E** and set at 100% in cells induced with 20 ng/ml doxycycline. Graphs represent the mean of intensity  $\pm$  s.d. of 3 independent experiments.

until anaphase. In metaphase, GFP-CHK2 also localizes at the bipolar mitotic spindle. In contrast to GFP-CHK2, no specific enrichment of GFP at any particular structure was detected in mitotic cells (Figure 4 and Additional movie files 4 and 5). Collectively, our results indicate that a subpopulation of CHK2 localizes at centrosomes specifically in mitotic cells. These data are consistent with the novel function proposed for CHK2 in the mitotic spindle assembly and the progression of mitosis reported recently by others [15].

#### **Polo kinase 1 is required for CHK2 localization at centrosomes**

Polo-like kinase 1 (PLK1) is a key mitotic kinase that regulates mitotic entry and mitosis progression. PLK1 localizes to centrosomes and kinetochores in early mitotic stages and at the central spindle and mid-body in anaphase and cytokinesis [44,45]. Since PLK1 localizes at the centrosomes and regulates CDK1-Cyclin B activation, centrosome maturation and separation as well as spindle assembly, we examined whether it is required for the recruitment of CHK2 at mitotic centrosomes. U2OS cells induced for GFP-CHK2 expression were treated with BI 2536 an inhibitor of PLK1, or monastrol an inhibitor of the Eg5 kinesin [46-48]. We found that both treatments inhibit centrosomes separation resulting in the formation of monopolar spindles and the arrest of cells in prometaphase (Figure 5A). In cells treated with monastrol, GFP-CHK2 was present at the unseparated centrosomes of the monopoles while it was not associated with centrosomes in BI 2536-treated cells, suggesting that PLK1 kinase activity is required for CHK2 recruitment at the centrosome during mitosis. To confirm the role of PLK1 in targeting CHK2 at the centrosome, we depleted the expression of PLK1 using short interfering RNA (siRNA).

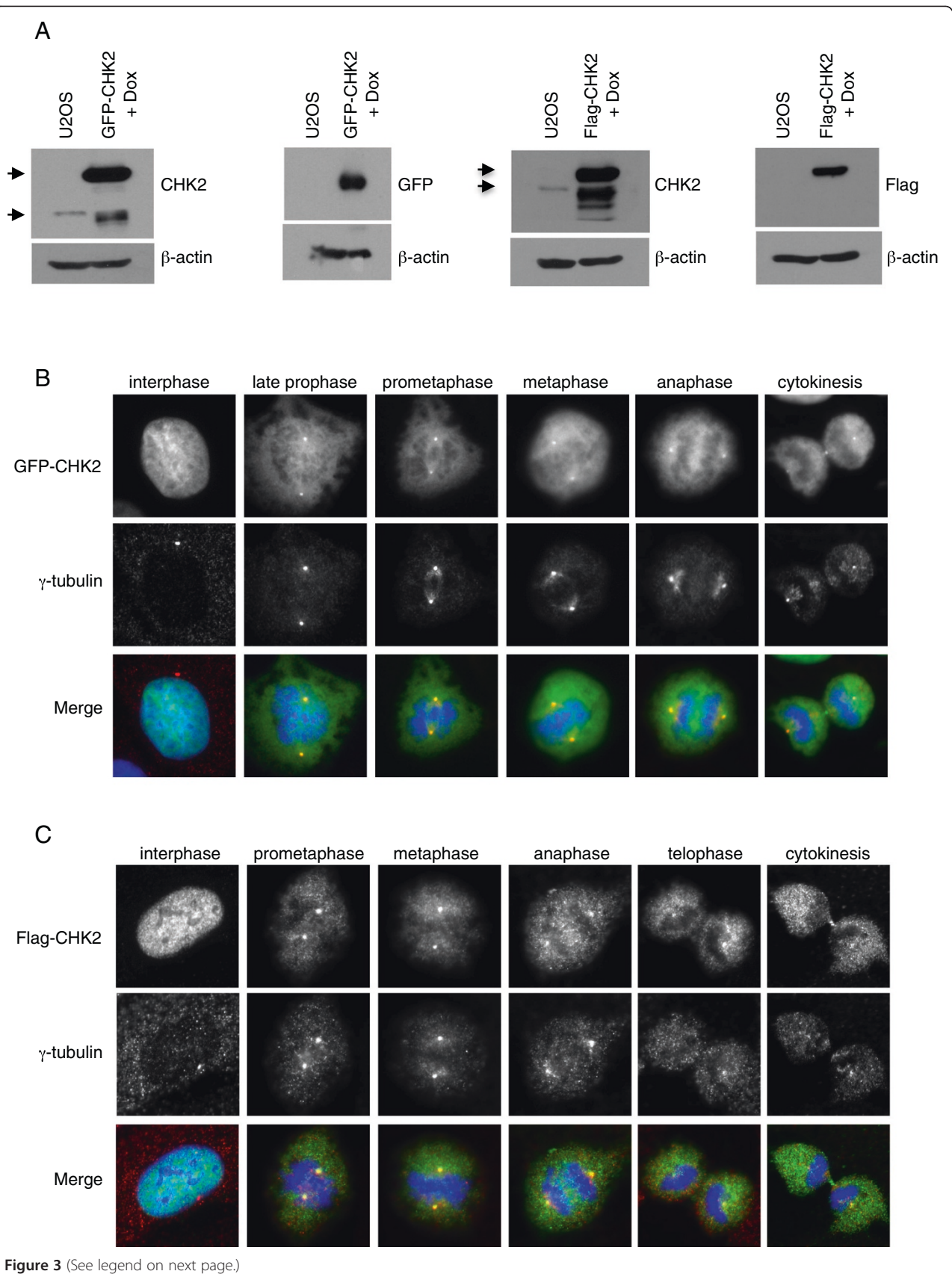
Unlike BI 2536 that strongly inhibits centrosome separation, cells transfected with siRNA against *PLK1* arrested at late prophase/early prometaphase with

unseparated centrosomes or at metaphase showing effective centrosome separation (Figure 5B and Additional file 6). Accumulation of these cells in metaphase most likely results from the incomplete silencing of PLK1 by the siRNA, as observed on the Western blots (Figure 5C and Additional file 6B). Alternatively, a potential off-target effect of BI 2536 is also possible [46]. In addition, PLK1 silencing or inhibition has no effect on centrosomes in interphase (Additional file 7). Most importantly, in all mitotic-arrested cells following siRNA-mediated PLK1 depletion or inhibition, the association of GFP-CHK2 with centrosomes was inhibited suggesting that PLK1 activity is required for CHK2 recruitment at the centrosomes during mitosis. However, data presented in Additional files 6 and 7 indicate that CHK2 silencing does not influence centrosome duplication and separation.

#### **Discussion**

Increasing evidence indicates that centrosomes integrate pathways that regulate cell cycle progression in interphase and mitosis. In the past years, key proteins of the DNA damage response have been localized at the centrosomes supporting a role for DNA damage responsive molecules in the control of unperturbed cell cycle progression and mitosis. Several studies revealed a centrosomal localization for CHK2 in interphase and mitotic cells [30,31,41]. We show here that the CHK2 rabbit polyclonal antibodies H-300 and CHK2-phospho-Thr68 stain the centrosomes by cross-reacting with an unknown centrosomal protein(s). By using U2OS cells transfected with Flag and GFP-tagged CHK2 fusion proteins we demonstrate that a subpopulation of CHK2 localizes at the centrosomes specifically in mitotic cells, from the late prophase/early prometaphase stage until cytokinesis. Moreover, we show that the centrosomal localization of CHK2 depends on PLK1 activity.

Our finding is in agreement with a recent work published by Stolz and al., supporting a new function for CHK2 in the mitotic bipolar spindle assembly and the



(See figure on previous page.)

**Figure 3** **CHK2 localizes at centrosomes in mitotic cells but not in interphase.** U2OS cells were transduced with lentiviruses coding for GFP-CHK2 or Flag-CHK2 and stably selected. **(A)** Cells were exposed for 48 h to doxycycline at 5 ng/ml for GFP-CHK2 cells, or 20 ng/ml for Flag-CHK2 cells, and expression of proteins in whole cell extracts was analyzed by Western blotting using the indicated antibodies. The arrows indicate endogenous and exogenous CHK2 proteins. Note that some exogenous CHK2 is expressed without tag.  $\beta$ -actin was used as loading control. **(B and C)** GFP-CHK2 and Flag-CHK2 are exclusively localized in the nucleus of interphase cells whereas a small subpopulation localizes at the centrosomes during mitosis. 48 h following transgene induction cells were fixed in methanol. Centrosomes were stained with anti- $\gamma$ -tubulin antibody (red), and DNA was counterstained with DAPI (blue). The localization of GFP-CHK2 was observed by direct fluorescence and Flag-CHK2 was immunostained with an anti-Flag antibody (green). Cells in interphase and various phases of mitosis were selected.

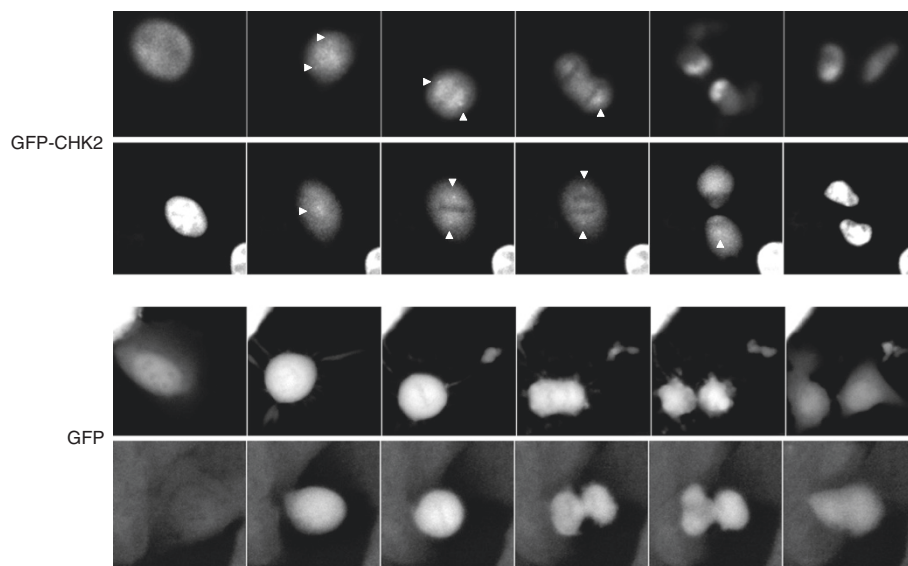
accurate segregation of chromosomes in the human colon cancer cell line HCT116 [15]. In this study, the tumor suppressor BRCA1 has been identified as a mitotic target of CHK2, and alteration of the CHK2-BRCA1 pathway was shown to promote chromosome segregation errors in dividing cells, a feature that is commonly observed in cancer cells and might drive chromosomal instability and cellular transformation [15,49,50]. Whether this new function of CHK2 in mitosis is associated with the centrosomal pool of CHK2 remains to be established. Interestingly, the CHK2 substrate BRCA1 has also been localized at the centrosomes in mitotic cells where it was reported to inhibit microtubule nucleation, an event that initiates entry into mitosis [26,51-54].

The kinase CHK2 is a checkpoint kinase known for its functions in the DNA damage response and is detected in the nucleus of interphase cells. In response to DNA damage CHK2 phosphorylates several targets *in vivo* including the phosphatases CDC25 A and C, the tumor suppressor BRCA1, the promyelocytic leukemia protein (PML), as well as the transcription factors p53, E2F1 and FOXM1 which

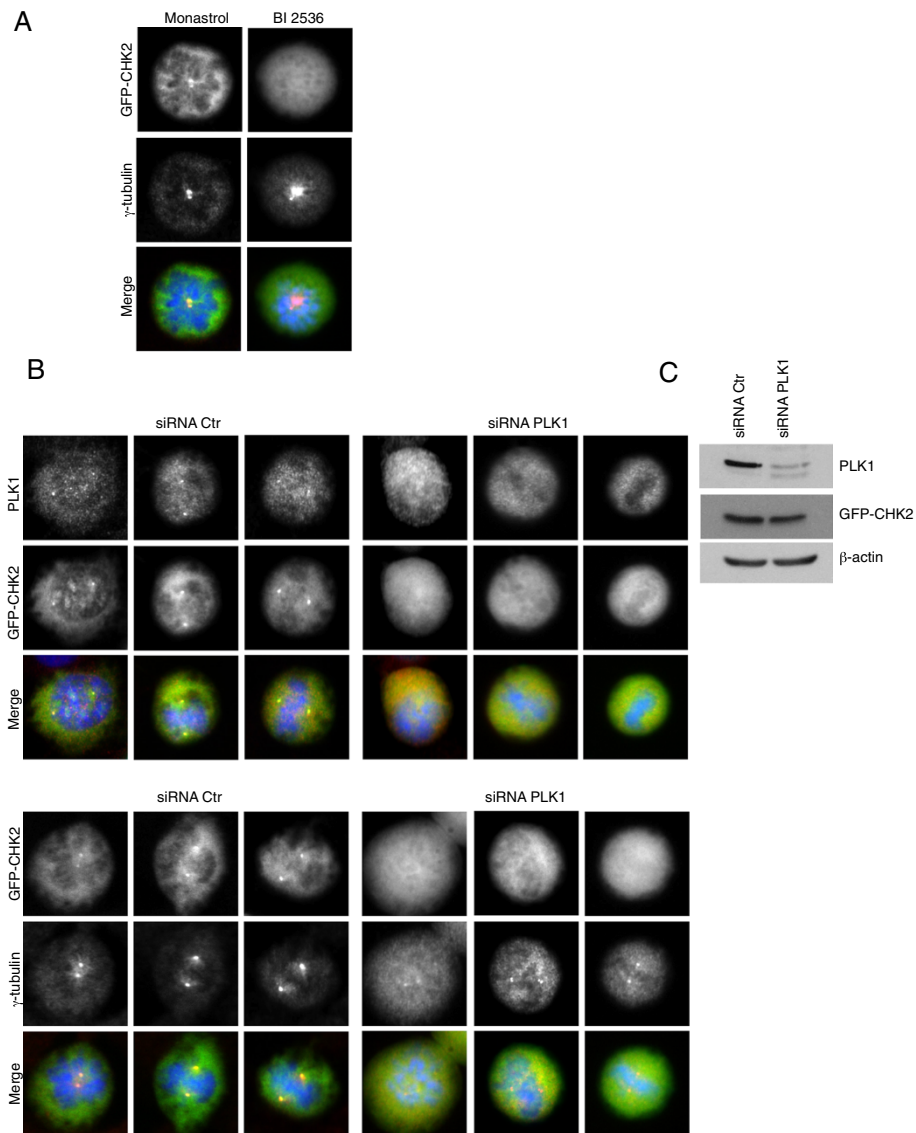
are all involved in cell cycle checkpoints, programmed cell death and DNA repair [55-60]. More recently, CHK2 has been demonstrated to be required for the proper progression of mitosis in HCT116 cells [15]. Interestingly, recent findings indicate that several other key proteins of the DNA damage response, including CHK1, ATM, DNA-PK, MDC1 and BRCA2 also play roles in regulating the proper progression of mitosis and/or the mitotic spindle assembly checkpoint (SAC), supporting the existence of a functional crosstalk between proteins of the DNA damage response and the mitotic regulatory network [8-13,22,61-66].

### Conclusion

Despite several recent reports documenting the presence of CHK2 at centrosomes there was some doubt about the specificity of the antibodies that stained positively for CHK2 at the centrosomes by immunofluorescence. In this study we conclusively show that CHK2 does not localize at the centrosomes in interphase cells, but rather, that a sub-population of CHK2



**Figure 4** **GFP-CHK2 localizes at centrosomes and spindle in mitotic living cells.** Representative images of the live cell videos showing mitotic U2OS cells expressing GFP-CHK2 or GFP proteins. Arrowheads indicate centrosomes.



**Figure 5 Polo kinase 1 is required for CHK2 localization at centrosomes.** (A) Inhibition of PLK1 activity with BI 2536 inhibits GFP-CHK2 association with centrosomes. U2OS GFP-CHK2 cells were incubated with doxycycline at 5 ng/ml for 32 h and treated for an additional 16 h with BI 2536 (100 nM) or monastrol (100  $\mu$ M) in doxycycline-containing medium. Cells were then fixed and stained for centrosomes (red) and DNA (blue) and GFP-CHK2 was visualized by direct fluorescence (green). (B) Inhibition of PLK1 expression prevents GFP-CHK2 association with centrosomes during mitosis. U2OS GFP-CHK2 cells were transfected with a siRNA directed against *PLK1* or control siRNA. 48 h post-transfection cells were fixed to examine GFP-CHK2 localization by fluorescence microscopy as in (A). (C) Western blot analysis of PLK1 expression shows a significant decrease in the expression of PLK1. Cell lysate from PLK1 siRNA-transfected U2OS cells was prepared from arrested mitotic cells collected by shake-off 48 h post-transfection. As a control, U2OS cells transfected with control siRNA were synchronized in mitosis by a 16 hours nocodazole block and protein extracts were prepared from the mitotic cells collected by shake-off.

associates with centrosomes in a PLK1-dependent manner during mitosis.

## Methods

### Cells culture and reagents

Human osteosarcoma U2OS cells used in all experiments stably express the tetracycline (Tet) repressor. U2OS T-REx cells were obtained from Dr. Eric Campeau

(University of Massachusetts, USA). HCT116 WT and *CHK2*<sup>-/-</sup> cells were a gift from Dr. Fred Bunz (Johns Hopkins University, Baltimore, USA) to Dr. Francis Rodier. Cells were maintained at 37°C, 5% CO<sub>2</sub> in Dulbecco's modified Eagle's Medium supplemented with 10% fetal calf serum. BI 2536 (Axon Medchem) and monastrol (Enzo Life sciences) were used at 100 nM and 100  $\mu$ M respectively. Nocodazole was purchased from Sigma-Aldrich.



### Generation of U2OS cell lines

To generate inducible U2OS cells lines expressing CHK2 or CHK1 fused to the GFP or FLAG tag, CHK1 and CHK2 cDNAs were cloned into the entry vectors pENTR4-GFP-C3 (Addgene # w393-1) and pENTR4-FLAG (Addgene # w210-2) respectively and the entry vectors were recombined into the inducible destination vector pLenti CMV/TO Puro (Addgene # 670-1). To generate inducible U2OS cells expressing GFP-PACT fusion protein, the PACT domain (amino acids 3643–3808) of AKAP450 was amplified from the GFP-AKAP450 vector provided by Dr. S. Munro (Cambridge, United Kingdom) with the following primers: forward, 5'-AAGCTTGCCAACATTGAAGCCATCATTGCC-3' and reverse, 5'-GAATTCTTATGCACCTTGATTTCAGTCCAAAGC-3'. The amplified sequence was cloned into the entry vector pENTR4-GFP-C3 (Addgene # w393-1) that was recombined into the destination vector pLenti CMV/TO Puro (Addgene # 670-1). Lentiviruses for all the constructs were generated with virus titers to infect 50 to 80% of cells and stably transduced cells were selected with 0.5 microgram /ml puromycin [67].

### RNA interference

RNAi lentiviruses previously shown to effectively reduce CHK2 levels were purchased from Open Biosystems [68]. The short hairpin target sequences used were:

shRNA-1 # TRCN0000039946: 5'-GCCAATCTTGAATGTGTGAAT-3' ;

shRNA-2 #TRCN0000010213: 5'-ACGATGCCAACTCCAGCCAG-3' ;

shRNA-3 #TRCN0000010314: 5'-ACTCCGTGGTTTGAACACGAA-3'.

For PLK1 knockdown, cells were transfected with a siRNA targeting the sequence 5'-AGAUUGGCCUAA GUCUCU-3' [69,70]. Transfection of siRNAs was carried out using DharmaFECT reagent (Thermo Scientific, Dharmacon) according to manufacturer's instructions.

### Immunofluorescence microscopy

Cells were grown on high precision 18 mm/1.5H glass coverslips (Marienfeld) and fixed in -20°C methanol for 15 min and blocked with PBS containing 3% BSA, 2% FCS, 0.3% Triton X-100. Fixed cells were incubated for 1 hour at room temperature with the indicated primary antibodies diluted in blocking buffer, and for 45 min with the Alexa Fluor-conjugated secondary antibodies. DNA was stained for 5 min with DAPI at 100 ng/ml (Sigma). Images were acquired using a Nikon Eclipse 600 fluorescence microscope equipped with a CoolSNAP HQ<sup>2</sup> camera (Photometrics) and processed using NIS-Element AR 3.0 and Photoshop (CS5, Adobe). Cells were immunostained using the following primary antibodies: anti- $\gamma$ -tubulin (GTU-88, Sigma T6557, 1:2000), anti-CHK2 (H300, Santa Cruz,

1:50), anti-phospho-Thr68-CHK2 (Cell signaling 1:50), anti- $\alpha$ -tubulin (DM1A, Sigma 1:500), anti-Flag (M2, Sigma, 1:1000), anti-Flag (F7425, Sigma, 1:250), anti-PLK1 (#06-813, Millipore 1:100) and the secondary antibodies Alexa Fluor 488/TxRed (Molecular Probes, 1: 800). For the quantification of fluorescence signal intensities at the centrosomes, unsaturated images were acquired with the same exposure settings. The areas corresponding to the centrosomes were defined with the  $\gamma$ -tubulin stainings and fluorescence intensities within the determined centrosomal areas were measured using NIS-Element software.

### Western blotting

To prepare protein extracts, cells were lysed in lysis buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 2.5 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF, 5 mM sodium pyrophosphate, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10  $\mu$ M MG132, 20 mM  $\beta$ -glycerophosphate, and a cocktail of proteases inhibitors (Complete™ Roche Applied Science). Proteins were run on 10% SDS-PAGE gels, transferred on nitrocellulose membranes and analyzed by immunoblotting. The antibodies used were anti-GFP (ab290, abcam, 1:1000), anti-Flag (M2, Sigma, 1:1000), anti-CHK2 (B-4, Santa Cruz, 1:500), anti-CHK1 (DCS-310, SantaCruz 1:500), anti-phospho-Thr68-CHK2 (Cell signalling, 1:1000), anti-phospho-Thr387-CHK2 (Assay bioTech, 1:500), anti-phospho-Ser516-CHK2 (Cell signalling, 1:1000), anti-phospho-Ser123-CDC25A (MJS Biolynx 1:250), anti-PLK1 (#06-813, Millipore, 1:500) and anti- $\beta$ -actin (AC-74, Sigma, 1:20 000).

### Time-lapse microscopy

For live cell videomicroscopy, U2OS cells transduced with GFP-CHK2WT or control GFP were grown on CellView™ 35 mm glass bottom dishes (170  $\pm$  10  $\mu$ M bottom thickness) from Greiner-Bio One. Protein expression was induced by a 48 h doxycycline treatment and cells were synchronized at the G1/S transition using a single 24 h thymidine block. 8 h after release from thymidine block they were incubated in FCS-supplemented, phenol red-free Dulbecco's modified Eagle's Medium and placed at 37°C in a heated chamber maintained at 5% CO<sub>2</sub>. Images were collected on a Zeiss Axio Observer Z1 automated microscope every 2.5 minutes for 16 hours using a Plan-Apochromat 20x/0.8NA objective, a Zeiss HRm Axiocam and LED pulsed light illumination setup.

### Statistical analysis

Statistical analyses were performed using a two-tailed Student t-test, type 3. Difference with P < 0.05 is considered as significant.

## Additional files

**Additional file 1: The exogenous GFP-CHK2 and Flag-CHK2 fusion proteins are functional kinases.** U2OS GFP-CHK2 and U2OS Flag-CHK2 cell lines were incubated with doxycycline for 48 h to induce transgene expression and cells were exposed to  $\gamma$ -IR (10 Gy). (A) 1 h after genotoxic insult whole-cell lysates were prepared and phosphorylation of CHK2 fusion proteins on Thr 68, Thr 383/387 and Ser 516 was assessed by Western blotting using the indicated antibodies. The arrows indicate GFP-CHK2 and Flag-CHK2 proteins phosphorylated on Thr383/387. (B) The phosphorylation of the CHK2 substrate CDC25 A (Ser123) was analyzed by Western blotting of protein extracts prepared 8 h after irradiation. The arrow indicates the band corresponding to P-Ser123-CDC25A. Both GFP-CHK2 and Flag-CHK2 retain full kinase activity.

**Additional file 2: During mitosis the localization of CHK2 at the centrosomes is microtubules independent.** Doxycycline-induced U2OS GFP-CHK2 and U2OS Flag-CHK2 cell lines were incubated for 16 h in nocodazole (0.3  $\mu$ M) to arrest cells in prometaphase. Cells were treated for an additional hour with 10  $\mu$ M nocodazole prior to be fixed and stained with anti- $\gamma$ -tubulin antibody (red) to stain the centrosomes. GFP-CHK2 was visualized by direct fluorescence and Flag-CHK2 was immunostained with an anti-Flag antibody (green). To control microtubules depolymerization cells were also stained for  $\alpha$ -tubulin.

**Additional file 3: GFP, GFP-CHK1 and Flag-CHK1 do not localize to the centrosomes.** U2OS stably transduced with lentiviruses coding for GFP, GFP-CHK1 or Flag-CHK1 were exposed to doxycycline at 5 ng/ml, 10 ng/ml and 20 ng/ml. (A) 48 h following doxycycline addition cells were collected. The expression of exogenous proteins was analyzed by Western blotting using the indicated antibodies. The arrows denote endogenous and exogenous CHK1 proteins.  $\beta$ -actin was used as loading control. (B-D) 48 h post-induction, cells were fixed and immunostained with anti- $\gamma$ -tubulin antibody (red) and costained with DAPI (blue). The localization of GFP and GFP-CHK1 was observed by direct fluorescence and Flag-CHK1 was immunostained with an anti-Flag antibody (green). Cells in interphase and various phases of mitosis were selected.

**Additional file 4: Time-lapse movie showing GFP-CHK2 at centrosomes in mitotic U2OS cells.** U2OS GFP-CHK2 were incubated with doxycycline for 48 h and synchronized by a single 24 h thymidine block. When the synchronized cell population progressed through late G2 phase and mitosis, images were acquired every 2 minutes with a Zeiss Axio Observer Z1 automated microscope.

**Additional file 5: Time-lapse movie showing a mitotic U2OS cell expressing control GFP protein.** Cells were imaged in the same conditions as for Additional file 4.

**Additional file 6: Quantification of centrosome separation in mitotic cells.** (A) Control U2OS cells or cells stably transduced with CHK2 shRNA 1 or CHK2 shRNA 2 + 3 were transfected with a siRNA directed against *PLK1* or incubated with BI 2536 (100 nM). 24 h following transfection or 16 h after treatment with BI 2536, cells were fixed and stained with anti- $\gamma$ -tubulin antibody and DAPI. Representative images of the mitotic-arrested cells are shown. The percentage of each mitotic cellular population was measured. Error bars represent the mean  $\pm$  s.d. of 3 independent experiments, each experiment monitoring 200 mitotic cells (\* $P$  < 0.05; -  $P$  > 0.05). (B) Western blot analysis of *PLK1* expression. Cell lysates from *PLK1* siRNA-transfected U2OS cells were prepared from mitotic cells collected by shake-off 24 h post-transfection. Protein extracts prepared from asynchronous cells or mitotic cells collected by shake-off 24 h following nocodazole treatment serves as control.

**Additional file 7: Quantification of centrosomes duplication/separation in interphase.** (A) Experimental procedure. Control U2OS cells or cells stably transduced with CHK2 shRNA 1 were synchronized at the G1/S boundary by a double thymidine block (DTB). At the indicated times during the cell cycle synchronization protocol, cells were transfected with control or *PLK1* siRNAs, incubated with BI 2536 or left untreated. (B) After release from second thymidine block, cell synchronization was confirmed by FACS analysis at the indicated times. (C) The inhibition of *PLK1* expression was confirmed by Western blotting. Cell lysates from *PLK1* siRNA-transfected cells were prepared from mitotic

cells collected by shake-off 11,5 h after release from DTB. Protein extracts prepared from mitotic cells collected 24 h following nocodazole treatment serves as control. (D) At each time point after release, cells were fixed and stained with anti- $\gamma$ -tubulin antibody and DAPI. The interphase cells with one or two unseparated/separated centrosomes were divided in 4 patterns, as shown in representative images, and cells in each pattern were quantified. Error bars represent the mean  $\pm$  s.d. of 3 independent experiments, each experiment monitoring 200 interphase cells.

### Abbreviations

IF: Immunofluorescence; ORF: Open reading frame;  $\gamma$ -IR: Gamma-irradiation; PLK1: Polo-like kinase 1.

### Competing interests

The authors declare that they have no financial and non-financial competing interests.

### Authors' contributions

GC and IC carried out the experiments. GC developed the method used to quantify centrosomal fluorescence and participated to the design of the experiments. IC and JL established the transduced cell lines. FR generated lentiviruses expressing CHK2 shRNAs and was involved in revisiting the manuscript critically. ES conceived the study, designed the experiments and wrote the manuscript. All authors read and approved the final manuscript.

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