Hepatology

ORIGINAL ARTICLE

CHK2 overexpression and mislocalisation within mitotic structures enhances chromosomal instability and hepatocellular carcinoma progression

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ABSTRACT

Objective Chromosomal instability (CIN) is the most common form of genomic instability, which promotes hepatocellular carcinoma (HCC) progression by enhancing tumour heterogeneity, drug resistance and immunity escape. CIN per se is an important factor of DNA damage, sustaining structural chromosome abnormalities but the underlying mechanisms are unknown.

Design DNA damage response protein checkpoint kinase 2 (Chk2) expression was evaluated in an animal model of diethylnitrosamine-induced HCC characterised by DNA damage and elevated mitotic errors. Chk2 was also determined in two discrete cohorts of human HCC specimens. To assess the functional role of Chk2, gain on and loss-of-function, mutagenesis, karyotyping and immunofluorescence/live imaging were performed by using HCT116, Huh7 and human hepatocytes immortalised with hTERT gene (HuS).

Results We demonstrate that mitotic errors during HCC tumorigenesis cause lagging chromosomes/DNA damage and activation of Chk2. Overexpression/phosphorylation and mislocalisation within the mitotic spindle of Chk2 contributes to induce lagging chromosomes. Lagging chromosomes and mitotic activity are reversed by knockdown of Chk2. Furthermore, upregulated Chk2 maintains mitotic activity interacting with Aurora B kinase for chromosome condensation and cytokinesis. The forkhead-associated domain of Chk2 is required for Chk2 mislocalisation to mitotic structures. In addition, retinoblastoma protein phosphorylation contributes to defective mitoses. A cohort and independent validation cohort show a strong cytoplasm to nuclear Chk2 translocation in a subset of patients with HCC.

Conclusions The study reveals a new mechanistic insight in the coinvolvement of Chk2 in HCC progression. These findings propose Chk2 as a putative biomarker to detect CIN in HCC providing a valuable support for clinical/therapeutical management of patients.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the primary malignancy of hepatocytes and the most frequent solid tumour of the liver. Half a million cases occur annually and the WHO has recently ranked HCC as the second most lethal cancer worldwide.[1] Different aetiological factors such as hepatitis B

Significance of this study

What is already known on this subject?
► Chromosomal instability (CIN) is a major hallmark of hepatic tumorigenesis and hampers prognosis and therapy. The underlying mechanisms are not well characterised.
► CIN is regarded as an ongoing elevated frequency of mitotic errors that leads to numerical and structural chromosome aberrations in a significant proportion of cell divisions leading to intratumoural heterogeneity and drug resistance.
► DNA damage caused by genotoxic agents activates DNA damage response checkpoint kinase 2 (Chk2) protein expression.
► Mislocalisation of proteins, including oncoproteins, tumour suppressors and other cancer-related proteins, can interfere with normal cellular function and cooperatively drive tumour development.

What are the new findings?
► Mitotic errors represented by lagging chromosomes cause DNA damage and overexpress Chk2 throughout hepatic tumorigenesis.
► Chk2 and the activation/phosphorylated forms of Chk2 mislocalise within the mitotic structures in the presence of DNA damage contributing to increased numbers of cells with lagging chromosomes. In contrast, knockdown of Chk2 reduces the number of cells with lagging chromosomes and the mitotic index.
► The Chk2 forkhead-associated domain is responsible for the mislocalisation of the protein at the mitotic structures.
► Chk2 is expressed at the nuclear level in a subset of human hepatocellular carcinoma (HCC) and this aspect correlates with HCC progression. Moreover, CHK2 expression is significantly higher in patients with HCC with mutated TP53, a hallmark of genomic instability, than in those with mutated CTNNB1/β-catenin.

Significance of this study

How might it impact on clinical practice in the foreseeable future?

▶ The study reveals a new mechanistic insight in the coinvolvement of Chk2 in CIN, that is, Chk2 mislocalisation, hence indicating the possibility to use Chk2 as a new drug target in HCC.

▶ The findings of this study suggest Chk2 as a putative biomarker that can be used to detect DNA damage and chromosomal instability in HCC providing a valuable support, for prognosis, and for identifying patients who are likely to have a response to therapy.

and C virus, alcohol and diabetes cause liver injury followed by inflammation, necrosis and hepatocytes proliferation.[2] Continuous cycles of this destructive–regenerative process culminates in liver cirrhosis which is characterised by regenerating nodules that progress to dysplastic nodules and ultimately HCC.[3 4] More than 90% of HCC, including dysplastic nodules in cirrhotic liver, possess chromosomal aberrations suggesting that chromosomal defects occur at early stages of tumour development and several attempts have been made to connect different chromosomal aberration patterns as specific subsets of HCC, although with limited success.[5–7] A common feature of human HCC and dysplastic nodules is represented by chromosomal instability (CIN). Various types of chromosomal aberrations, including numerical (aneuploidy) and structural (eg, translocations, deletions), are found in HCC and are linked to tumorigenesis.[8 9] An important aspect in studying HCC tumorigenesis is to distinguish between the state of the karyotype and the rate of karyotypic change. In fact, aneuploidy is not synonymous with CIN, because aneuploid tumours are stable with uniform karyotype and phenotype.[10–12] Conversely, there is an increased rate of CIN generating diverse karyotypes within the same tumour. This yields a heterogeneous tumour cell population that has the ability to undergo selective evolution in term of drug resistance and immune system escape mechanisms.[13–15] CIN is regarded as an ongoing elevated frequency of mitotic errors that leads to additional chromosome gains or losses in a significant proportion of cell divisions. Our hypothesis is that CIN per se is also an important mechanism of DNA damage inducing structural chromosome aberrations in HCC. To verify this hypothesis, we used checkpoint kinase 2 (Chk2) as a central effector of the cell response to DNA damage. In fact, Chk2 has been described to detect a variety of DNA lesions caused by exogenous and endogenous genotoxic agents. Our aim is to investigate the expression of this kinase in hepatic carcinogenesis and its correlation with the genesis of structural chromosomal aberrations. Here, we provide evidence that defective chromosome segregation, that is, lagging chromosomes cause DNA damage and determine an overexpression/phosphorylation of Chk2. Furthermore, the mislocalisation of Chk2 with components of the mitotic spindle contributes to chromosome missegregations. These data are corroborated with the observation of an increased Chk2 and nuclear translocation in two independent cohorts of human HCC specimens.

MATERIALS AND METHODS

Please refer to the online supplementary materials and methods for more detailed descriptions.

Tissue samples and immunohistochemistry assays

Florence cohort, University Hospital of Florence: formalin-fixed, paraffin-embedded tissues were obtained from an archive of liver biopsies of patients diagnosed with HCC. H&E-stained tissue sections of 49 patients were reviewed, and areas of cirrhotic and HCC were identified. Chk2 antigen retrieval was performed by heating in citrate buffer pH 6.0 at 98°C for 30 min. Non-specific signal was eliminated by peroxidase block for 10 min at room temperature. Primary antibody was incubated at room temperature overnight in a humidified chamber followed by Horseradish peroxidase (HRP)-conjugated secondary antibody incubation for 30 min at room temperature. Antibody binding was revealed by 3,3′-Diaminobenzidine and reaction was stopped by immersion of tissue sections in distilled water once brown colour appeared. Tissue sections were counterstained by haematoxylin, dehydrated in graded ethanols and mounted. All reagents for immunohistochemistry were from Dako. Appropriate positive and negative controls were included for each run of immunohistochemistry. Chk2 expression was determined according to H-score method. For H-score assessment, 10 fields were chosen at random at ×400 magnification and the staining intensity in the malignant cell nuclei was scored as 0, 1, 2 and 3 corresponding to the presence of negative, weak, intermediate and strong brown staining, respectively. The total number of cells in each field and the number of cells stained at each intensity were counted. The average percentage positive was calculated, and a H-score between 0 and 300 was obtained where 300 was equal to 100% of tumour cells stained brown strongly. The positive cut-off was considered ≥100 (see online supplementary figure S1). Chk2 expression in biopsy sections was scored by a pathologist and two researchers independently.

London cohort, Royal Free Hospital: formalin-fixed, paraffin-embedded surgical specimens from consecutive patients diagnosed with HCC were retrieved from Royal Free Hospital archives. All samples were stained with H&E and the sections were reviewed and suitable areas of cirrhosis and HCC were identified in 20 samples. Antigen retrieval was performed by microwaving (720 W) in pH 6.0 sodium citrate buffer for 30 min. Endogenous peroxidase and non-specific binding of primary antibody was blocked. The primary Chk2 antibody was incubated overnight at 5°C. Primary antibody detection was carried out using the Novolink polymer detection system (Novocastra). Tissue sections were counterstained with Mayer's haematoxylin.

Experimental hepatocarcinogenesis

Twenty-five female Wistar rats (210–225 g) were given diethylnitrosamine 50 mg/L ad libitum in their drinking water for a period of 10 weeks as previously described.[16] Another group of five animals were maintained as controls. All animals were administered a standard laboratory diet and were weighed each week. Five animals for each time point were sacrificed on week 4, 6, 8, 10 and 14. Tumours and liver tissues were removed and fixed in 10% neutral buffered formalin. Liver sections (5 μm) were cut from paraffin-embedded blocks and stained Chk2 (Abcam, #ab47433), phospho-Histone H2A.X (Cell Signaling, #9718) and H&E for histological examination and expression were determined according to H-score method.

CHK2 expression in HCC samples from The Cancer Genome Atlas database

The Cancer Genome Atlas (TCGA)-Liver Hepatocellular Carcinoma (LIHC) cohort, containing 377 cases, was used to validate CHK2 expression levels in distinct subtypes of HCC.

The TCGA database (https://gdc-portal.nci.nih.gov) was accessed on 16 November 2016 through the cGbioportal website (http://www.cbioportal.org/) to retrieve patients with either TP53 or CTNNB1 mutation. The TCGA_participant_barcode was used to download CHK2 expression from RNA-seq data using the firebrowse portal (http://firebrowse.org). Patients with co-occurrent mutations of TP53 and CTNNB1 were excluded from the analysis. CHK2 expression levels were retrieved for 101 patients with TP53 mutation and 85 patients with CTNNB1 mutation. Statistical analysis was performed using the Mann-Whitney test with GraphPad 6 software.

RESULTS

Chk2 is overexpressed in a subset of human HCC

To test our hypothesis that hepatic tumorigenesis per se could be a cause of DNA damage and structural chromosome aberrations, an animal model of diethylnitrosamine-induced HCC was employed which induced macroscopical visible tumours after 14 weeks (figure 1A). This model has previously been established to induce elevated mitotic errors, DNA damage and chromosome aberrations.[17] Using this model, we can compare the expression levels of DNA damage response proteins induced by the genotoxic activity of diethylnitrosamine with a later phase represented exclusively by tumorigenesis. Therefore, Chk2, a DNA damage response checkpoint kinase, and phospho-Histone H2A.X (Ser139), a component of chromatin which becomes phosphorylated following DNA damage (eg, DNA double-stranded breaks), were investigated.[18] As shown in figure 1B, several nuclei of cancer cells exhibited an increased expression of Chk2 and phospho-Histone H2A.X revealing the presence of DNA damage. A timeline analysis of liver sections demonstrated a twofold increase in Chk2 basal levels at 4 weeks followed by decreased values after 8 weeks (figure 1C). In contrast, by 14 weeks tumours were macroscopically visible and histologically evident in 80% of animals. Immunostaining of HCC sections showed a significant increased expression of Chk2 and phospho-Histone H2A.X (figure 1C, D) when compared with liver sections without evidence of tumours (8 weeks). Taken together, these data showed that the increased expression of Chk2 reflected a response triggered by DNA damage during tumorigenesis.

To extend these findings to humans, the expression of Chk2 in patients with cirrhotic-related HCC and normal liver was examined in liver biopsies from hospital archives (Florence cohort, see online supplementary table S1). A nuclear staining of Chk2 was detectable in distinctive histotypes of HCC (see online supplementary figures S2 and S3). More specifically, the expression level of Chk2 in different grades of HCC was evaluated. Remarkably, within histological grades 2 and 3, significant quantitative differences between positive and negative Chk2 patients were found (p<0.001). Overall, these data demonstrated that Chk2 nuclear positivity was found in 63.2% (31 of 49) of HCCs analysed (see online supplementary table S2). A separate validation cohort of patients was examined at Royal Free Hospital, London (see online supplementary table S3). Normal liver tissue showed a moderate and diffuse cytoplasmic staining, whereas grade 1 specimens were characterised with weak focal cytoplasmic Chk2 staining. In grade 2, a Chk2 perinuclear and nuclear accumulation was observed. Specimens with grade 3 showed exclusively a clear and strong nuclear staining,

Figure 1 Immunohistochemical detection of checkpoint kinase 2 (Chk2) and phospho-Histone H2A.X (Ser139) in diethylnitrosamine-induced hepatocellular carcinoma (HCC). (A) Representative image of livers of female Wistar rats used as control (left panel) and rats HCC (right panel) which were given diethylnitrosamine 50 mg/L ad libitum in their drinking water for a period of 10 weeks. Scale bar, 10 mm. (B) Liver sections (HCC 14 weeks) were analysed for Chk2 and phospho-Histone H2A.X(Ser139) expression (see nuclear staining in the right panels; scale bar, 200 μm.). (C and D) Five animals for each time point were sacrificed on weeks 4, 6, 8, 10 and 14. Chk2 and phospho-Histone H2A.X(Ser139) expression were determined according to H-score method. The average positive percentage was calculated, and a H-score between 0 and 300 was obtained where 300 was equal to 100% of tumour cells stained brown strongly. The positive cut-off was considered ≥100. The levels of Chk2 and phospho-Histone H2A.X(Ser139) expression are indicated as fold increase of cut-off values.*p<0.001 versus 8 weeks. Chk2 expression was evaluated in the nucleus of the HCC tissue as indicated by the brown staining.
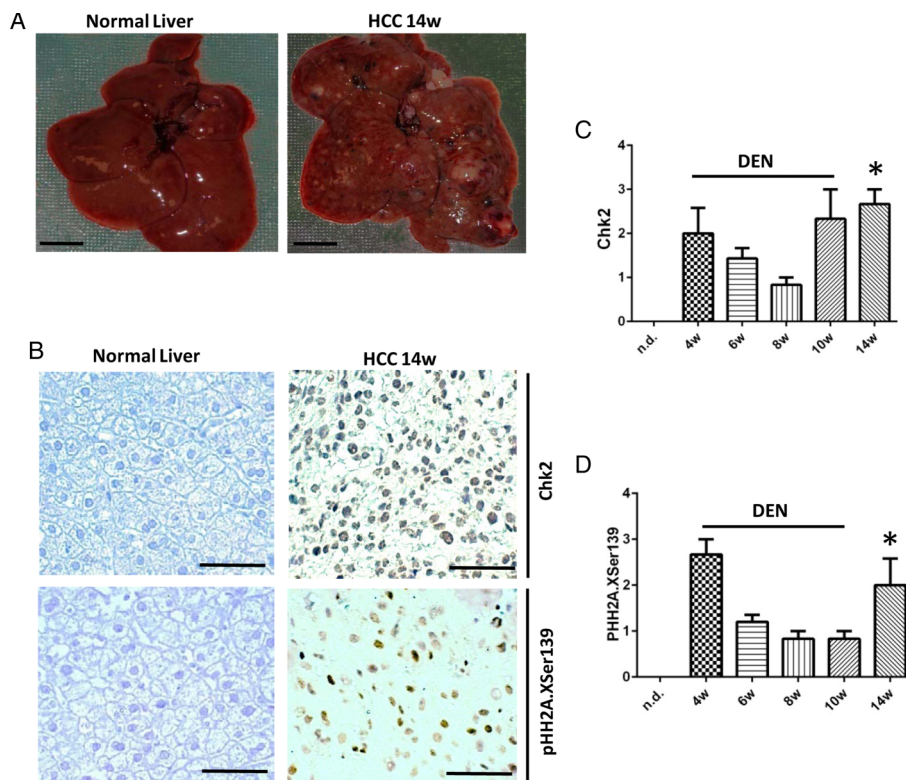
without cytoplasmic and perinuclear Chk2 staining (figure 2A and see online supplementary table S4). Furthermore, the expression levels of CHK2 were analysed in a public available cohort of HCC from the TCGA database. We found a highly significant increase of CHK2 expression in HCC with mutated TP53, a hallmark of genomic instability, with respect to those with CTNNB1/β-catenin mutation (figure 2B).

Defective chromosome segregations cause DNA damage

The previous observations prompted us to investigate the functional role of Chk2. For this reason, three different cell lines were employed. The colon carcinoma cell line HCT116 was used, that is, a near-diploid cell line that faithfully segregates chromosomes to maintain a stable karyotype and is commonly employed in CIN studies.[19 20] Second, the HCC cell line Huh7 was used which is a stable hyperdiploid karyotype with altered chromosomal structures, and finally HuS cells were used marked with an unstable karyotype.[21] HuS cells are human hepatocytes immortalised with human telomerase reverse transcriptase (hTERT), a gene described commonly activated in human HCC.[8 9] HuS cells just established in culture (0 generation) and hereafter referred to as HuS0gen showed a karyotype
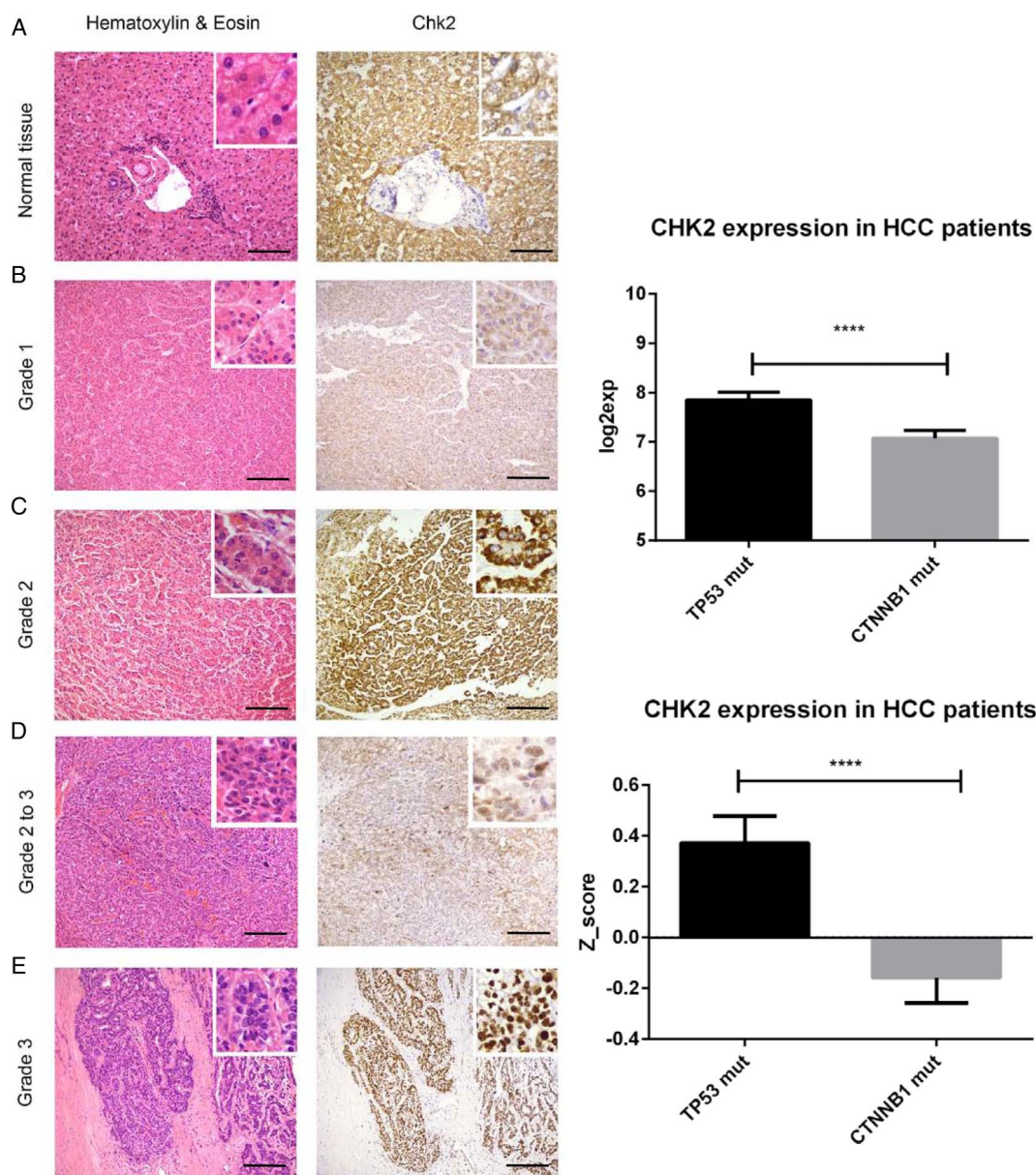

Figure 2 (A) Detection of checkpoint kinase 2 (Chk2) protein in human hepatocellular carcinoma (HCC) by immunohistochemistry. Paraffin-embedded tissue sections were stained with a specific antibody for Chk2 and H&E. (A) Normal liver with a moderate, diffuse cytoplasmic Chk2 staining. (B) Chk2 immunoreactivity in cancer tissue from a tumour classified grade 1 characterised by a weak focal cytoplasmic Chk2 staining. (C and D) Chk2 immunoreactivity in cancer tissue from a tumour classified grade 2 with a strong Chk2 perinuclear and moderate nuclear accumulation. (E) Chk2 immunoreactivity in cancer tissue from a tumour classified grade 3 with a clear and strong nuclear staining, without cytoplasmic and perinuclear Chk2 staining. Scale bar, 100 μm. (B) CHK2 expression in HCC subtypes from The Cancer Genome Atlas database. CHK2 expression from RNA-seq data was retrieved for 101 patients with TP53 mutations and 85 patients with CTNNB1/β-catenin mutations, using the Liver Hepatocellular Carcinoma (LIHC) cohort of 377 cases (accessed on 16 November 2016). Patients with both TP53 and CTNNB1 mutations were excluded from the analysis. Expression levels are expressed as log2exp (absolute levels) or z-score (expression levels in HCC samples normalised to the expression levels in the reference population). Graph shows mean±SEM, *p<0.0001.

Figure 3 Defective chromosome segregation causes checkpoint kinase 2 (Chk2) overexpression in hTERT immortalised human hepatocytes. (A) Examples of chromosome numbers from metaphase spreads of HCT116, Huh7 and HuS0gen cells (upper panels). In the bottom panels, individual chromosome numbers percentage from metaphase spreads of HCT116, Huh7 and HuS0gen cells are represented (n=20). (B) Representative immunoblot out of three independent experiments demonstrating the protein levels of Chk2 in indefinite cultured HCT116 and Huh7 cells, in HuS0gen cells and HuS0gen cells after 30 generations in culture (HuS30gen). (C) Example of chromosomes from metaphase spreads of HuS30gen exhibiting numerical and structural (red arrow) alterations; the histogram shows individual chromosome numbers percentage in HuS30gen (n=20). (D) Representative immunoblot out of three independent experiments demonstrating the protein levels of phospho-Histone H2A.X (Ser139), a hallmark of DNA damage, in indefinite cultured HCT116, Huh7, HuS0gen and HuS30gen cells.
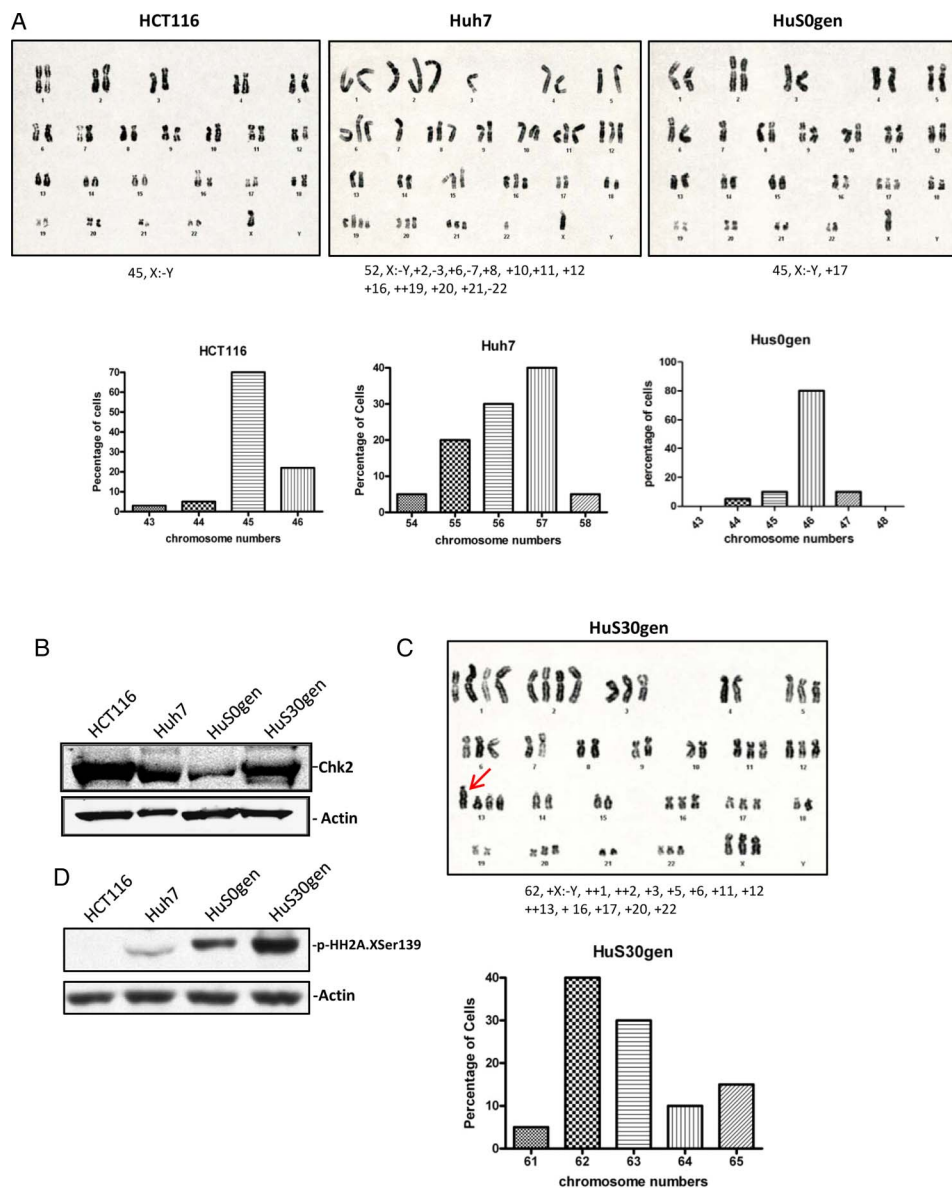

with a distribution of 46 chromosomes (figure 3A) and expressed low levels of Chk2 protein in comparison to HCT116 and Huh7 (figure 3B). The HuS0gen cells were maintained in culture and after 30 consecutive generations (HuS30gen) were re-examined to verify the karyotype status. The cells showed a distribution near triploid, with many structural chromosomal defects (figure 3C and see online supplementary figure S4A), and importantly this aspect was associated with increased expression of Chk2 protein (figure 3B). Likewise with the murine model, we evaluated the expression levels of phospho-Histone H2A.X (Ser139) in all the cell lines studied. As shown in figure 3D, both HuS0gen and HuS30gen cells exhibited high levels of phospho-Histone H2A. X, although with different levels of expression. These findings indicate the presence of DNA double-strand breaks, a hallmark of DNA damage, in HuS cells.

Overexpressed Chk2 mislocalises with mitotic structures in HuS30gen cells but not in HCT116 and Huh7

The finding of overexpressed Chk2 in both karyotyped stable HCT116 and Huh7 and in unstable karyotyped HuS30gen did not demonstrate a specific role for Chk2 in defective chromosome segregation. Therefore, we analysed the cell lines HCT116, Huh7, HuS0gen and HuS30gen by immunofluorescence to examine a possible altered localisation of Chk2 with the mitotic structures. Chk2 showed a localisation to the spindle poles of metaphase mitotic structures in HCT116, Huh7 and HuS0gen (figure 4A). Very importantly, HuS30gen cells showed a robust localisation of Chk2 with all metaphase mitotic structures indicating a clear mislocalisation of Chk2 (figure 4A and see online supplementary figure S4B). These results suggest that in HuS0gen cells, expressing low levels of DNA damage, Chk2 weakly colocalised with the mitotic spindle components, whereas Chk2 is increased and strongly associated with all mitotic structures in HuS30gen cells that are expressing high levels of DNA damage. To further address these findings, we stably transfected HuS0gen with hemagglutinin (HA)-tagged Chk2, HuS0gen overexpressing Chk2 exhibited Chk2 localisation with mitotic structures as shown in figure 4B. Next, we measured the number of lagging chromosomes (figure 4C) in HuS0gen, HuS30gen and in HuS0gen stably transfected with HA-tagged Chk2 to mimic overexpression of Chk2. As shown
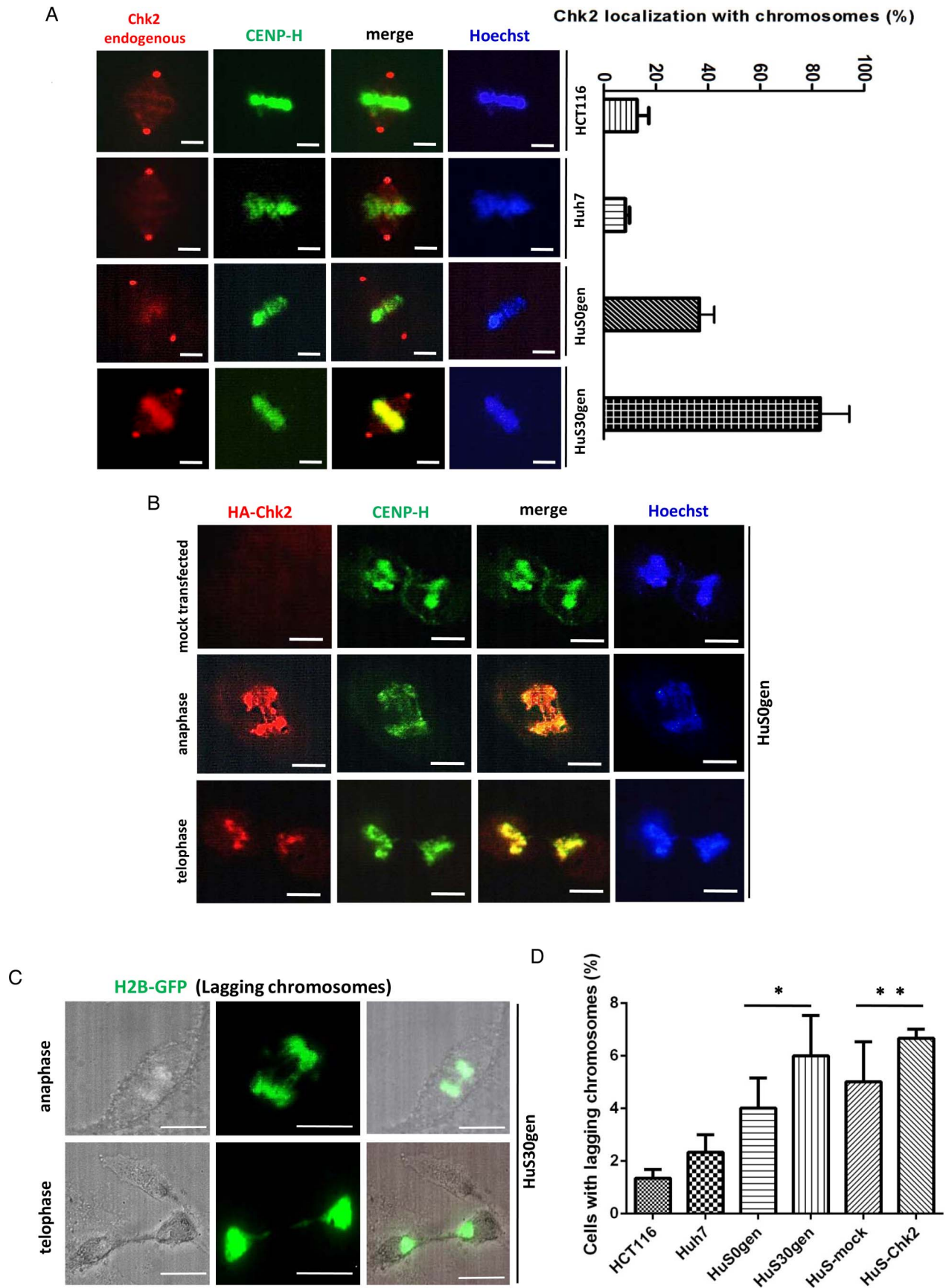
in figure 4D, overexpression of Chk2 significantly correlated with high number of mitoses with lagging chromosomes in HuS30gen, and in HuS0gen overexpressing HA-tagged Chk2. These data clearly indicate that indeed overexpression and mislocalisation of Chk2 itself contributes to defective chromosome segregations. Furthermore, to assess that the increased expression of Chk2 was specifically related to those mitoses with defective chromosome segregations and not a random deregulation of proteins, the expression of Nek2 (NIMA-related kinase 2) protein was evaluated. Nek2 is a protein located at the centrosome/spindle poles and responsible for chromosomal defective segregations.[22] HCT116 and Huh7 cells showed a strong Nek2 expression, whereas both HuS0gen and HuS30gen cells expressed low levels of Nek2 (see online supplementary figure S5) suggesting that the previously observed overexpression of Chk2 in Hus30gen was a specific event that coincided with structural chromosomal defects and DNA damage.

Chk2 overexpression is correlated with increased chromosomal missegregations

The findings that HuS30gen cells express high levels of Chk2 protein correlating with DNA damage (figure 3B,D) prompted us to investigate the effects of knocking down expression of CHK2 in HuS30gen cells by retroviral-mediated short hairpin RNA (shRNA) transduction. A pool of four shRNA targeting Chk2 resulted in effective decrease of the protein (figure 5A). It is important to note that silencing CHK2 coincided with a significant reduction of the number of HuS30gen cells with lagging chromosomes and HuS30gen mitotic index. To address any off-target effects of the hairpins, we rescued the mitotic index and the number of cell with lagging chromosomes of the knockdown HuS30gen by expressing a myc-tagged version of Chk2 that circumvents the shRNA directed against human Chk2 (figure 5B,C). These data demonstrate that Chk2 overexpression is correlated with increased chromosomal missegregation and mitotic index.

Defective mitoses-related DNA damage promotes Chk2 phosphorylation and mislocalisation within mitotic structures

Human CHK2 is a 543-amino-acid protein that consists of a N-terminal SQ/TQ cluster domain (SCD), a central forkhead-associated (FHA) domain and a C-terminal serine/threonine kinase domain.[23] The SCD consists of multiple SQ/TQ (Ser-Gln/Thr-Gln) motifs with Thr68 being the primary site to be phosphorylated in response to DNA damage followed by phosphorylation of Thr387 in the activation loop of the kinase domain.[24 25] Therefore, the protein expression of phospho-Thr68-Chk2 and phospho-Thr387-Chk2 in HuS30gen and HuS0gen stably transfected with HA-tagged Chk2 were assessed. As shown in figure 5D, phospho-Thr68-Chk2 and phospho-Thr387-Chk2 were highly expressed in HuS30gen and

in HuS0gen stably transfected with HA-Chk2, but not in HuS0gen as expected. Furthermore, phospho-Thr68-Chk2 revealed a robust localisation with components of the mitotic spindle as previously shown for Chk2. In particular, a prominent association of phospho-Thr68-Chk2 was observed with mitotic structures such as the spindle poles, kinetochores and midbody in Hus30gen cells (figure 5E, upper panels). In contrast, Huh7 cells expressing low levels of DNA damage (see figure 3D) did not show colocalisation of phospho-Thr68-Chk2 with mitotic structures (figure 5E, lower panels). Taken together, these results reveal an overexpression/mislocalisation of phosphorylated Chk2, which is correlated with its activation status, and manifestations of lagging chromosomes-induced DNA damage.

Upregulated Chk2 cooperates with Aurora B for chromosome condensation and cytokinesis

As previously shown, the typical localisation of activated Chk2 in HuS30gen cells was observed in kinetochores and specifically the midbody, suggesting a possible interaction with Aurora B kinase. This protein is known to be involved in chromosome condensation, the assembly of the spindle midzone in anaphase and the midbody during telophase. Furthermore, Aurora B kinase has been identified as an effective predictor of aggressive HCC recurrence, in relation to genomic instability.[26] Therefore, HuS0gen cells were transfected with HA-tagged Chk2 and immunofluorescence analyses revealed a colocalisation of Chk2 with Aurora B throughout all mitotic phases, and this colocalisation was also present in cells marked with lagging chromosomes (figure 6A, B). Aurora B is responsible for the phosphorylation of histone H3 (pHH3), and pHH3 serine residue (Ser-10) is considered to be a crucial event for the onset of mitosis. The utility of pHH3 to identify mitoses has been demonstrated in several cancer studies.[27–29] As the colocalisation of Aurora B with Chk2 was demonstrated, we further explored the colocalisation of overexpressed HA-Chk2 with pHH3. As shown in figure 6C, Chk2 exhibited a striking colocalisation with pHH3. Overall, the data so far demonstrate that in unstable HuS cells during mitosis, lagging chromosomes cause DNA damage, overexpression of Chk2 which localises to all mitotic structures. This overexpressed and mislocalised Chk2 protein contributes to a constant mitotic activity and more important Aurora B kinase and pHH3 interact with Chk2. This favours a possible scenario where an overexpressed and mislocated Chk2 overrules the signalling specificity at distinct mitotic checkpoints inducing itself chromosomal aberrations, hence CIN.

The FHA domain determines Chk2 mislocalisation to mitotic structures and is essential for DNA damage response

To understand how Chk2 is recruited to the mitotic structures in such a specific manner, we investigated Chk2 specific domain(s) that are required for Chk2 localisation. A series of red

Figure 4 Checkpoint kinase 2 (Chk2) overexpression is linked to lagging chromosomes. (A) Chk2 colocalises with chromosomal structures only in HuS30gen cells. Asynchronously growing HCT116, Huh7, HuS0gen and HuS30gen cells were synchronised in metaphase by releasing cells from thymidine treatment into MG132. Metaphase mitotic spindles were monitored by immunofluorescence and typical examples are given, endogenous Chk2 (red); kinetochores, CENP-H (green); chromosomes are stained with Hoechst 33342 (blue). Chk2 colocalisation with chromosome structures was quantified in metaphases (mean±SD; n≥ 300 metaphases). (B) HuS0gen cells that express low levels of protein Chk2 were stably transfected with HA-tagged Chk2 and asynchronously growing cells were evaluated for lagging chromosomes; typical examples of lagging chromosomes containing anaphase and telophase are given (HA-Chk2, red; CENP-H, green; chromosomes, Hoechst 33342, blue). (C) Asynchronously growing HuS30gen cells expressing H2B-green fluorescent protein (GFP) (to visualise in vivo lagging chromosomes) were analysed by live cell microscopy. Left, phase-contrast images; middle, fluorescence images; right, merge. Scale bar, 10 µm. (D) Asynchronously growing HCT116, Huh7, HuS0gen, HuS30gen and HuS0gen cells stably transfected with HA-tagged Chk2 (HuS-Chk2) were stained with CENP-H (constitutive component of kinetochores) and Hoechst 33342 (chromosomes). Anaphase and telophase lagging chromosomes were counted by immunofluorescence (mean±SD; n=500 cells). *p<0.01 versus HuS0gen. **p<0.05 versus HuS-mock. CENP-H, centromere protein; HA, hemagglutinin.

Figure 5 Overexpressed/phosphorylated checkpoint kinase 2 (Chk2) is correlated with increased chromosomal missegregation. (A) HuS30gen cells were stably retrovirally transduced either with a control shRNA (shCTL) or shRNAs targeting CHK2 (shChk2). Chk2-depleted HuS30gen cells were used to reconstitute expression of CHK2 by introducing a shRNA-resistant mutant of CHK2 (myc-Chk2resist). The protein levels were determined by immunoblotting and a representative example is shown. (B) Asynchronously growing HuS30-shCTL, HuS30-shChk2 and Chk2-depleted HuS30gen cells reconstituted with shRNA-resistant mutant of CHK2 were stained with CENP-H (kinetochores) and Hoechst 33342 (chromosomes). Anaphase and telophase lagging chromosomes counted by immunofluorescence (mean ±SD; n=500 cells), *p<0.01 versus controls. (C) The mitotic index of asynchronously growing HuS30-shCTL, HuS30-shChk2 and Chk2-depleted HuS30gen cells reconstituted with shRNA-resistant mutant of CHK2 was measured by counting the mitotic figures (prophase, metaphase, anaphase and telophase) positively stained for phosphorylated histone H3. The mitotic index was calculated as percentage of mitotic cells/total cells as described in the section 'Materials and Methods'. Error bars show the SD from the means of three independent experiments, *p<0.01 versus controls. (D) Chk2 phosphorylation levels on Thr68 and Thr387. Representative immunoblot out of three independent experiments showing asynchronously growing HCT116, HuS0gen, HuS30gen and HuS0gen cells stably transfected with Chk2 (HuS-Chk2). Cell lysates were immunoblotted against p-Thr68-Chk2 and p-Thr387-Chk2. (E) Activated Chk2 localises with mitotic structures in HuS30gen cells. Examples of metaphase, anaphase and telophase from unstable HuS30gen cells and stable hepatocellular carcinoma cell line, Huh7. Activated Chk2 (p-Thr68-Chk2, red) colocalises with CENP-H, constitutive component of kinetochores (green) only in HuS30gen. Arrowheads indicate lagging chromosomes (Hoechst 33342, blue). Scale bar, 10 μm. CENP-H, centromere protein.
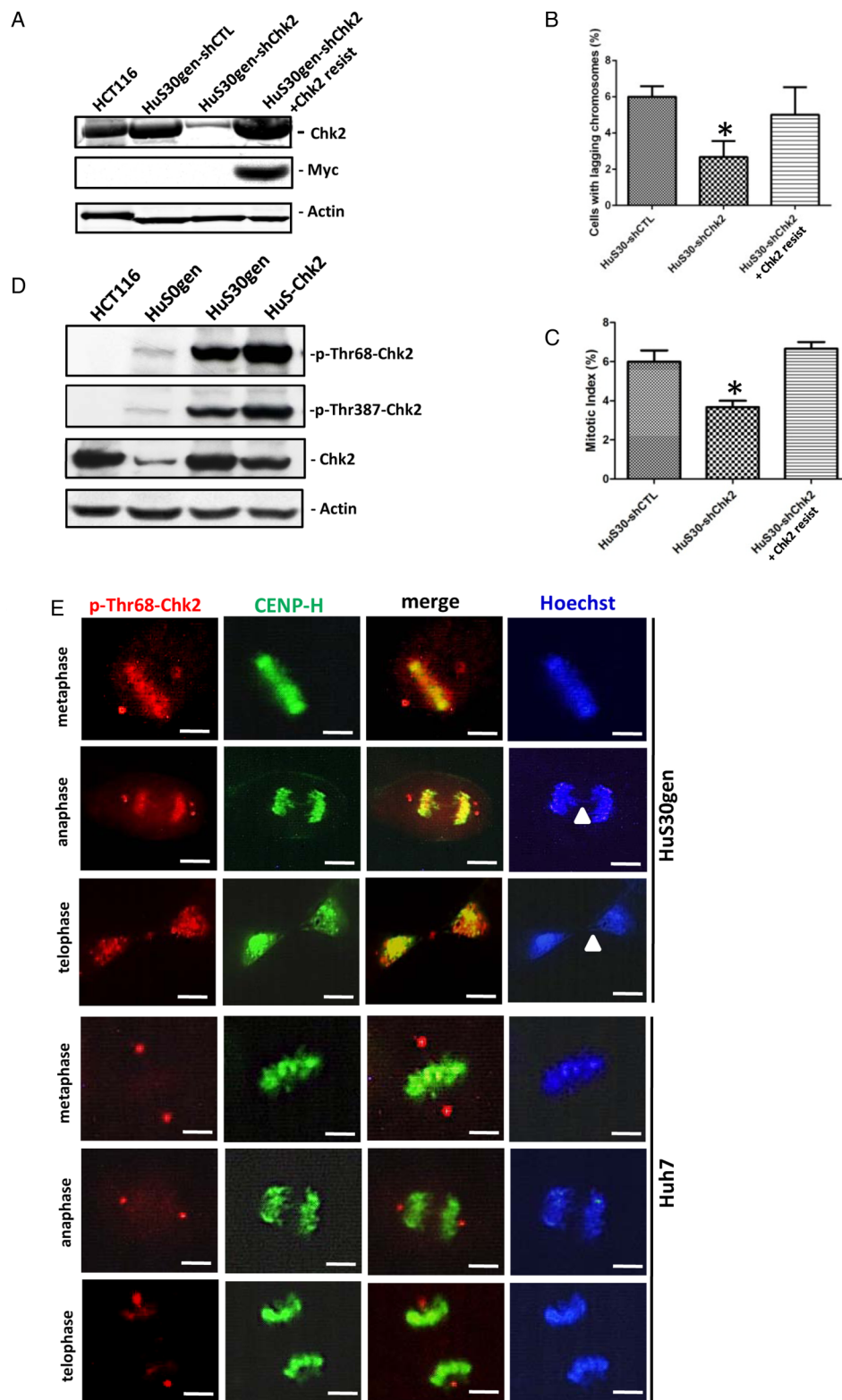
fluorescent protein (RFP)-tagged Chk2 variants were created, each variant carrying a point mutation or a deletion, and were expressed and localised individually in HuS30gen. The RFP-Chk2D347A, a kinase-inactive mutant, localised similarly as shown for RFP-Chk2 (figure 7A). This indicated that the kinase activity is dispensable for Chk2 mislocalisation (figure 7B). The FHA domain of Chk2 has been proposed to mediate localisation of Chk2 to mitotic structures for the reason that FHA domains function as phospho-threonine-binding protein motifs. The deletion of the Chk2 FHA domain disrupted the localisation of the protein to mitotic structures such as the spindle poles and kinetochores indicating that the deletion of
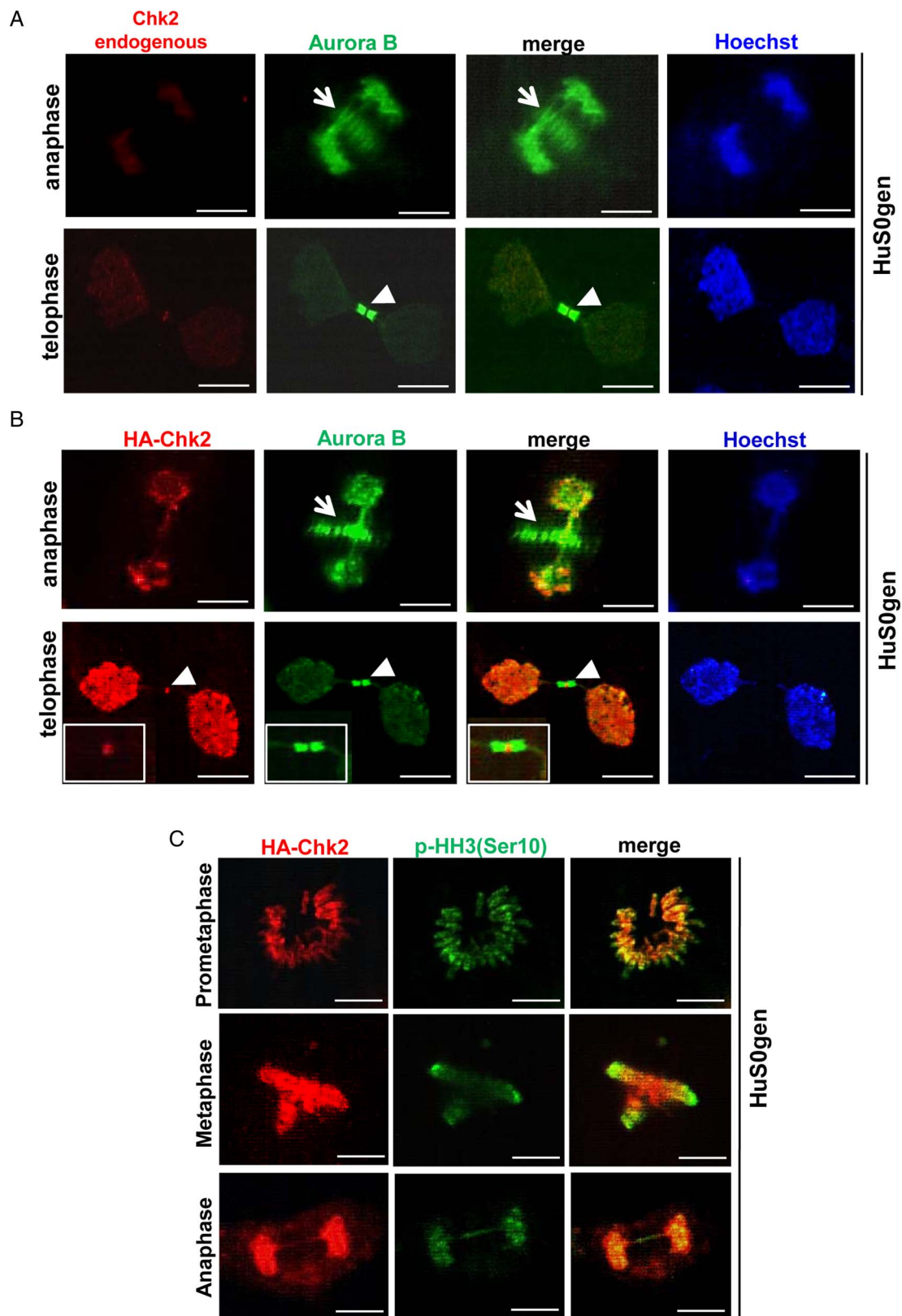
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Figure 6 Upregulated checkpoint kinase 2 (Chk2) cooperates with Aurora B for chromosome condensation and cytokinesis. **(A)** HuS0gen cells showing expression and localisation of endogenous Chk2 (red), Aurora B kinase (green) and chromosomes (Hoechst 33342, blue). (B) Colocalisation of Aurora B kinase (green) with HuS0gen overexpressing HA-tagged Chk2 (red) in mitoses with lagging chromosomes (Hoechst 33342, blue). Cells were fixed and analysed by fluorescence microscopy. Arrows indicate spindle midzone region; arrowheads indicate midbody region. (C) HuS0gen cells overexpressing HA-tagged Chk2 were stained with antibody anti-HA (red) and phospho-histone H3 (green) to show colocalisation of Chk2 with missegregating chromosomes. Scale bar, 10 μm. HA, hemagglutinin; pHH3, phosphorylated histone H3.
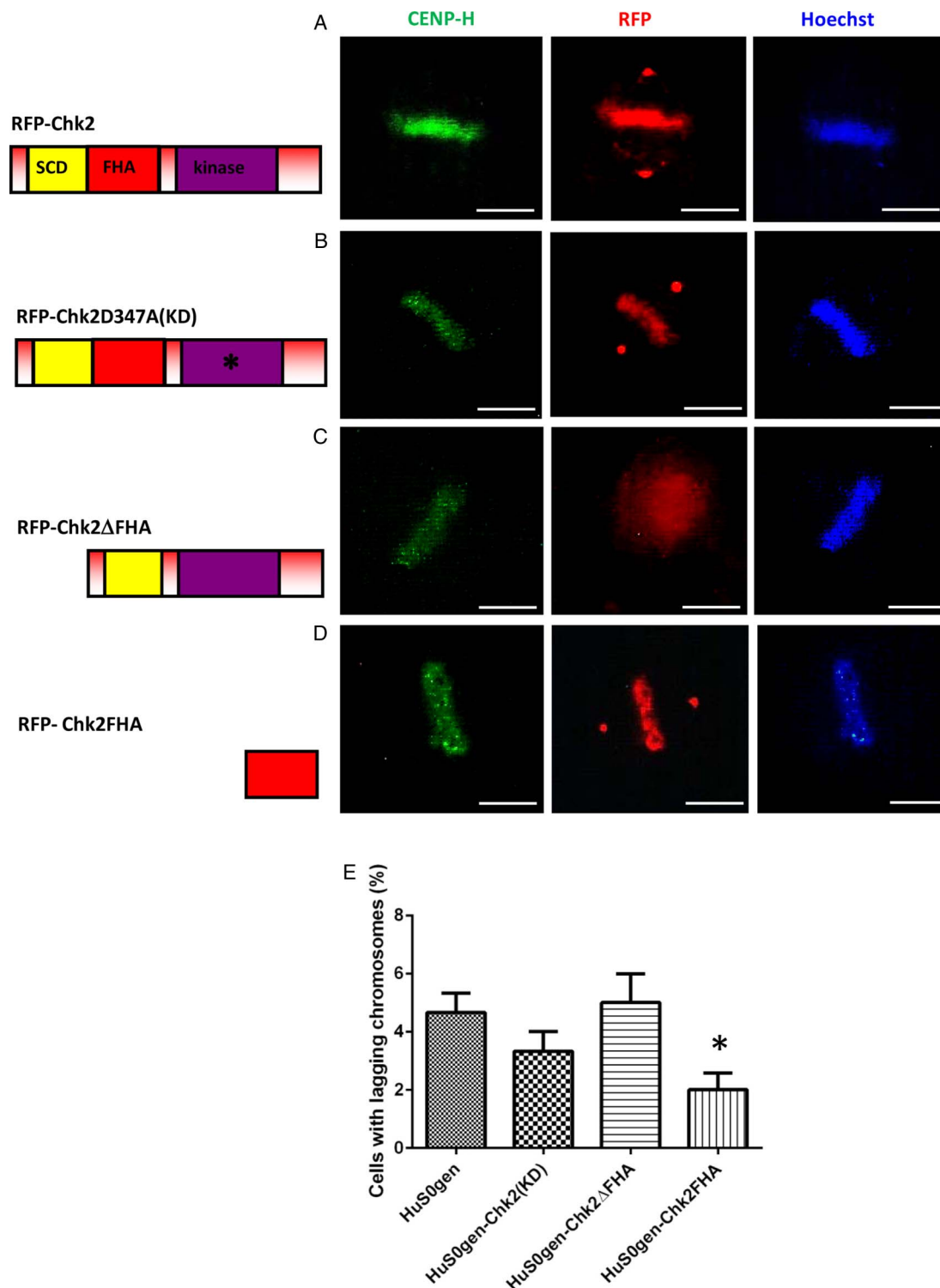
Figure 7 Forkhead-associated (FHA) domain is essential for Chk2 localisation and contributes to sustain lagging chromosomes. Red fluorescent protein (RFP) was conjugated at the N-terminus of each Chk2 mutant. Left, schematic diagrams. RFP was omitted. RFP-Chk2 mutants were transfected in HuS30gen cells, synchronised and blocked in metaphase by treatment with MG132 for 2 hours; the cells were analysed by fluorescence microscopy. (A) Expression and subcellular localisation of RFP-Chk2. RFP-Chk2 decorates the spindle poles (two red dots), and localises with kinetochores (CENP-H, green); chromosomes are stained with Hoechst 33342, blue. (B) RFP-Chk2D347A, kinase dead mutant (KD), localises similarly to RFP-Chk2. (C) RFP-Chk2ΔFHA does not localise to mitotic structures. (D) RFP-FHA localises to spindle poles, kinetochores. FHA domain tagged with RFP is similar to RFP-Chk2. Scale bar, 10 μm. (E) Quantification of lagging chromosomes during anaphases and telophases in HuS0gen cells stably expressing Chk2(KD), Chk2ΔFHA and Chk2FHA mutants. Chk2 FHA mutant significantly reduces the number of lagging chromosomes as a result of the dominant negative effect towards endogenous Chk2. Mean±SD; n=400 anaphases and telophases. *p<0.01 versus control. CENP-H, centromere protein.
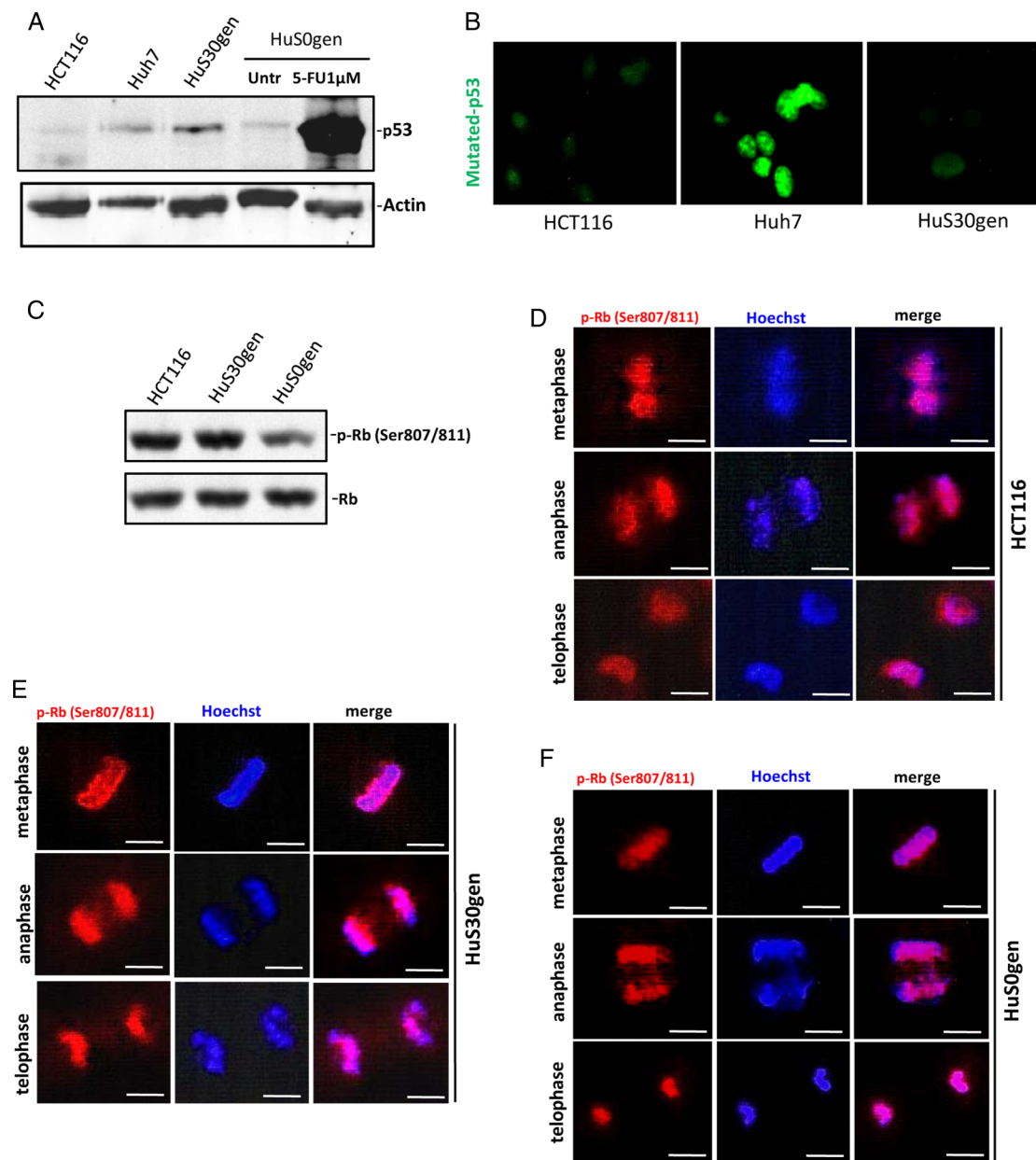
Figure 8 P53 and phosphorylated retinoblastoma (Rb) expression in HCT116, HuS30gen and HuS0gen. (A) Representative immunoblot out of three independent experiments showing the protein levels of p53 in HCT116, Huh7, HuS30gen and HuS0gen cells. In the same blot are represented p53 expression levels of HuS0gen cells after treatment with DNA-damaging agent 5-fluorouracil (5-FU, 1 μM). (B) Representative images of HCT116, Huh7 and HuS30gen cells fixed and stained with an antibody directed against the mutated codon TGT at position 220 of p53 protein. Huh7 cells, previously characterised as p53 mutated,[30] are used as positive control. (C) Representative immunoblot out of three independent experiments showing the protein levels of Rb and phosphorylated Rb, p-Rb (Ser807/811), in HCT116, HuS30gen and HuS0gen cells. (D, E and F) Mitoses of asynchronously growing HCT116, HuS30gen and HuS0gen cells stained with the antibody specific for phosphorylated Rb and analysed by fluorescence microscopy; p-Rb (Ser807/811) red; chromosomes (Hoescht 33342, blue). Scale bar, 10 μm.

the FHA is responsible for the observed mislocalisation (figure 7C). Indeed, the FHA domain tagged with RFP localised to mitotic structures similar to RFP-Chk2 (figure 7D). Along these lines, we can conclude that the FHA domain is required for proper Chk2 localisation to mitotic structures. Furthermore, we analysed the effects of the several Chk2 mutants on lagging chromosomes. Interestingly, transfection of HuS0gen with Chk2 FHA domain significantly reduced the number of lagging chromosomes (figure 7E). This result suggests a dominant negative effect of Chk2 FHA domain towards endogenous Chk2.

Rb phosphorylation contributes to defective mitoses in HuS cells

An important point raised by defective mitoses-related DNA damage as observed in this study is why the cells do not sense signals deployed to curb cell proliferation, as normally the growth of cultured diploid cells is halted by chromosome missegregation. In HCC, p53 deficiency has been described to participate in the development of CIN; however, the relevance of p53 to HCC initiation, progression or both remains an area of active investigation.[2] Therefore, the basal level of p53 in HCT116, Huh7, HuS30gen and HuS0gen was investigated. As shown in
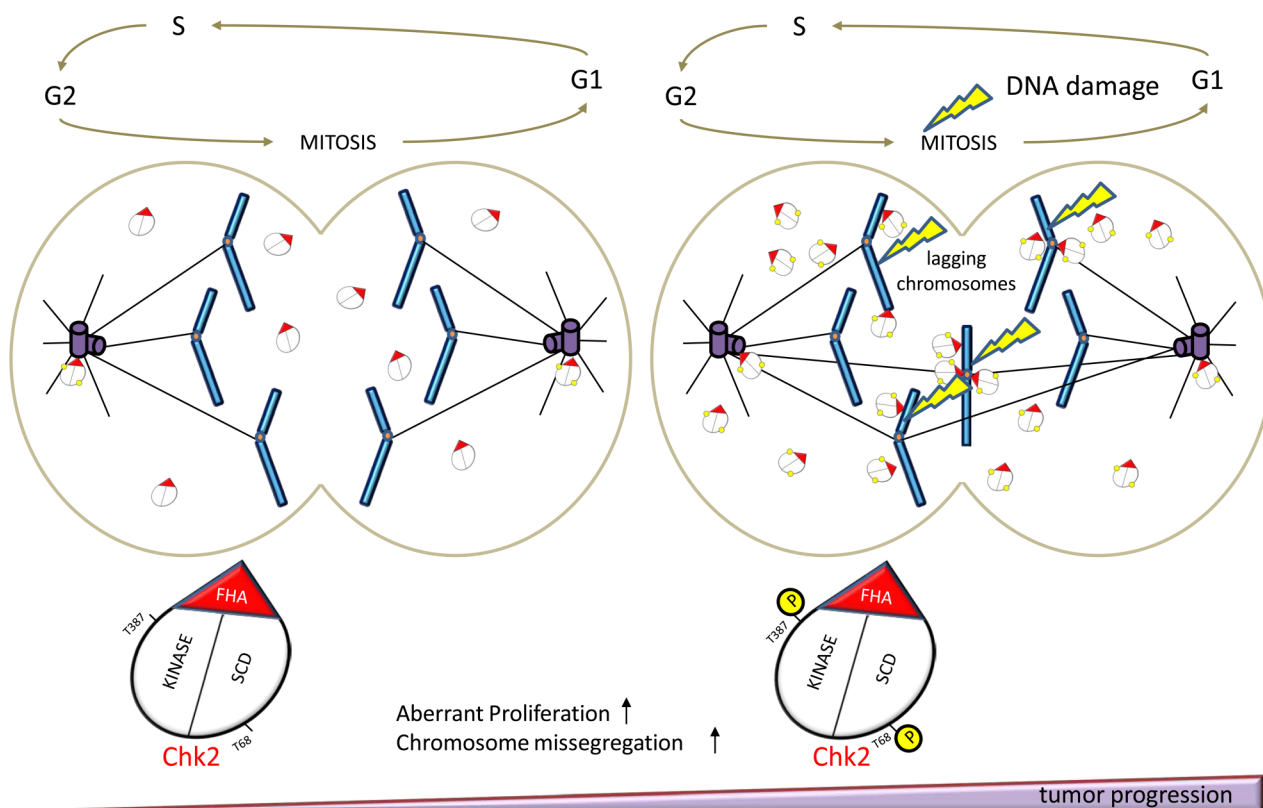
Figure 9 A proposed schema illustrating the mechanism by which checkpoint kinase 2 (Chk2) overexpression and mislocalisation sustains defective mitoses throughout hepatic tumorigenesis. Mitotic errors represented by lagging chromosomes cause DNA damage and overexpress Chk2 in genetically unstable cells. Chk2 and phosphorylated forms of Chk2 mislocalise within the mitotic structures in the presence of DNA damage contributing to increase the numbers of lagging chromosomes. FHA, forkhead associated; SCD, SQ/TQ cluster domain.

figure 8A, p53 was expressed in Huh7 and at low level in HuS30gen cells. HuS0gen cells did not express p53 in basal conditions; however, treatment of the cells with 5-fluorouracil, a DNA-damaging agent, triggered a strong p53 protein expression. Based on these findings, we assume that p53 expression may be a late event in HuS cells, as a result of several defective mitoses.[19] Furthermore, mutated p53 was not present in HCT116 and HuS30gen cells in contrast to Huh7 cells that we used as a positive control (figure 8B).[30]

The possible involvement of the retinoblastoma protein Rb was evaluated since several studies have described Rb as an important regulator of cell division.[31] Specifically, Rb phosphorylation determines Rb inactivation and leads to defective mitoses.[31] Therefore, we evaluated the protein expression levels of phospho-Rb (Ser807/811) in HCT116, HuS0gen cells and HuS30gen. As shown in figure 8C, HuS30gen cells express higher levels of phospho-Rb (Ser807/811) in comparison to HuS0gen cells. Moreover, HCT116 (figure 8D), HuS30gen (figure 8E) and HuS0gen (figure 8F) exhibited a localisation of phospho-Rb (Ser807/811) at the chromosomal level. Taken together, these findings suggest that in HuS30gen Rb phosphorylation is required to sustain mitotic activity which leads to mitotic defects and DNA damage.

DISCUSSION

The neoplastic evolution of HCC proceeds through a multistep histological process that is less well defined than that of other cancer types. Hyperplastic nodules of regenerating hepatocytes have normal karyotype and represent a potential first step

towards HCC. These lesions can progress to dysplastic nodules, which have abnormal chromosome features including numerical and structural alterations. These dysplastic nodules can evolve to HCC marked with recurrent regions of copy number change and allelic imbalances.[3 4] Loss of certain tumour suppressors or gain of specific oncogenes promote cell division as shown in many previous studies.[25 32] We demonstrate that this coincides with defective mitoses which can cause DNA damage. This scenario is conceivable considering that massive deregulation of mitosis is incompatible with cell survival and can even be tumour suppressive.[33] Therefore, subtle rather than massive mitotic defects are expected to underlie CIN in HCC, allowing a tolerable level of chromosome defects. To support this view, we have provided evidence in this study that hepatocarcinogenesis with lagging chromosomes elicits the expression of DNA damage response protein Chk2. Thus, the overexpression of Chk2 and its mislocalisation within structures of the mitotic spindle contribute to sustain cell division and chromosomes missegregation.[34–36] This forms a self-perpetuating mechanism causing DNA damage (figure 9).

We have also found that functional Rb inactivation is necessary but not sufficient to trigger chromosome missegregation. Indeed, phosphorylation of Rb deregulates transcription factor E2F, and well-characterised targets of E2F include multiple genes whose products are required to maintain cell division.[37–39] The integrity of chromosomal DNA is under constant surveillance throughout the cell division. Surprisingly, the fact that a deregulated functional Chk2 itself might cause per se CIN through abnormal spindle localisation is intriguing, as the role

of Chk2 in the maintenance of genetic stability has mainly been ascribed to its function in DNA damage checkpoint signalling.

When DNA damage occurs, Chk2 is first activated in the nucleus during interphase, spreads to the cytoplasm and localises to the various mitotic structures because of the phosphopeptide-binding ability of the FHA domain.[40] During DNA damage, chromosomes accumulate a number of phosphoproteins around DNA damage sites.[41] Chk2 may bind to specific phosphoproteins through the FHA domain forming numerous chromosomal foci and trap Chk2 to the structures. When this FHA domain is deleted, the molecule is excluded from the mitotic chromosomes and therefore does not mislocalise to kinetochores, spindle poles and midbody as was demonstrated in figure 7. Moreover, Chk2 localisation to the kinetochores and midbody region suggests that Chk2 may directly affect spindle assembly function, similar to Aurora B kinase,[42] but only when DNA damage exists. The data obtained in this study fit a model whereby abnormal expression of Chk2 and Aurora B kinase disrupts the sensitive balance of mitotic proteins, which in turn undermines faithful chromosome segregation.[34] If the relative expression levels of the proteins involved in the quality control of the machinery which ensures chromosome segregation fidelity is disrupted by mitotic errors and DNA damage, the resulting imbalance could further compromise chromosome segregation accuracy.

Since Chk2 overexpression can induce oligomerisation and cis/trans phosphorylation of regulatory residues,[43 44] one possibility is that local enrichment of Chk2 at kinetochores and midbody regions facilitates Chk2 oligomerisation and activation. This mechanism may alter regulation of Chk2-dependent processes such as centrosome function, cell cycle progression and chromosome segregation. The discovery of a role for Chk2 in HCC, its association with DNA damage and CIN may have potential diagnostic and prognostic significance. Importantly, grading heterogeneity inside a tumour is frequently observed and may significantly limit diagnosis and choice of treatment. This study suggests that stratification of patients with HCC according to CIN status and/or DNA damage may be useful to minimise the confounding effects of tumour CIN status in clinical trials. Along these lines, we propose that the diagnostic and prognostic value of Chk2 should be further verified in prospective clinical trials.

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CHK2 overexpression and mislocalisation within mitotic structures enhances chromosomal instability and hepatocellular carcinoma progression

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