RESEARCH PAPER

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Centrosomal AKAP350 modulates the G_1/S transition

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AKAP350 (AKAP450/AKAP9/CG-NAP) is an A-kinase anchoring protein, which recruits multiple signaling proteins to the Golgi apparatus and the centrosomes. Several proteins recruited to the centrosomes by this scaffold participate in the regulation of the cell cycle. Previous studies indicated that AKAP350 participates in centrosome duplication. In the present study we specifically assessed the role of AKAP350 in the progression of the cell cycle. Our results showed that interference with AKAP350 expression inhibits G_1/S transition, decreasing the initiation of both DNA synthesis and centrosome duplication. We identified an AKAP350 carboxyl-terminal domain (AKAP350CTD), which contained the centrosomal targeting domain of AKAP350 and induced the initiation of DNA synthesis. Nevertheless, AKAP350CTD expression did not induce centrosomal duplication. AKAP350CTD partially delocalized endogenous AKAP350 from the centrosomes, but increased the centrosomal levels of the cyclin-dependent kinase 2 (Cdk2). Accordingly, the expression of this AKAP350 domain increased the endogenous phosphorylation of nucleophosmin by Cdk2, which occurs at the G_1/S transition and is a marker of the centrosomal activity of the cyclin E-Cdk2 complex. Cdk2 recruitment to the centrosomes is a necessary event for the development of the G_1/S transition. Altogether, our results indicate that AKAP350 facilitates the initiation of DNA synthesis by scaffolding Cdk2 to the centrosomes, and enabling its specific activity at this organelle. Although this mechanism could also be involved in AKAP350-dependent modulation of centrosomal duplication, it is not sufficient to account for this process.

Introduction

AKAP350 (AKAP450/CG-NAP/AKAP9) is a multiple spliced A-kinase anchoring protein (AKAP), which assembles protein complexes at the Golgi apparatus and the centrosomes.[1-3] Largely known as the main microtubule nucleating organelle, centrosomes have garnered interest during the last decade because of their central role in various signaling events, including those involved in the establishment of cell polarity,[4] and the regulation of the cell cycle progression.[5] In this regard, AKAP350 recruits to the pericentriolar matrix several signaling proteins implicated in the regulation of these processes, including PKA, Cdk2,[6] the Ran small GTPase,[7] and the γ-TURC associated proteins GCP2/3.[8] Although AKAP350 participation in the establishment of cell polarity has been well established,[9,10] the relevance of this scaffold protein in the regulation of the cell cycle has not been characterized to date.

AKAP350 localization at the centrosomes is mediated by a carboxyl-terminal 92 aminoacid region (AKAP450 3699–3790), the PACT domain, which also interacts with calmodulin and is highly conserved in functionally related centrosomal proteins.[11] Previous studies demonstrated that expression of the PACT domain induces a decrease in AKAP350 localization at the centrosomes, and inhibition of centrosomal duplication.[12] A different study suggested that AKAP350-dependent recruitment of cyclin E-Cdk2 to the centrosomes could induce centrosomal amplification.[6] Even though both studies pointed to a facilitative role of AKAP350 in centrosomal duplication, their data are not convergent, nor they make focus, on the understanding of how AKAP350 participates in the cell cycle progression.

Centrosomal duplication is coordinated with DNA replication, both events initiated at the G_1/S transition.[13] A key enzyme in the coordination of these events is Cdk2. At late G_1, there is an increase in cyclin E levels, which leads to activation of Cdk2. The cyclin E–Cdk2 complex phosphorylates specific substrates involved in the activation of both DNA synthesis and centrosomal duplication.[14] Interestingly, the initiation of DNA synthesis is dependent, not only on cyclin E–Cdk2 activity, but also on the proper location of this complex at the centrosomes.[15]

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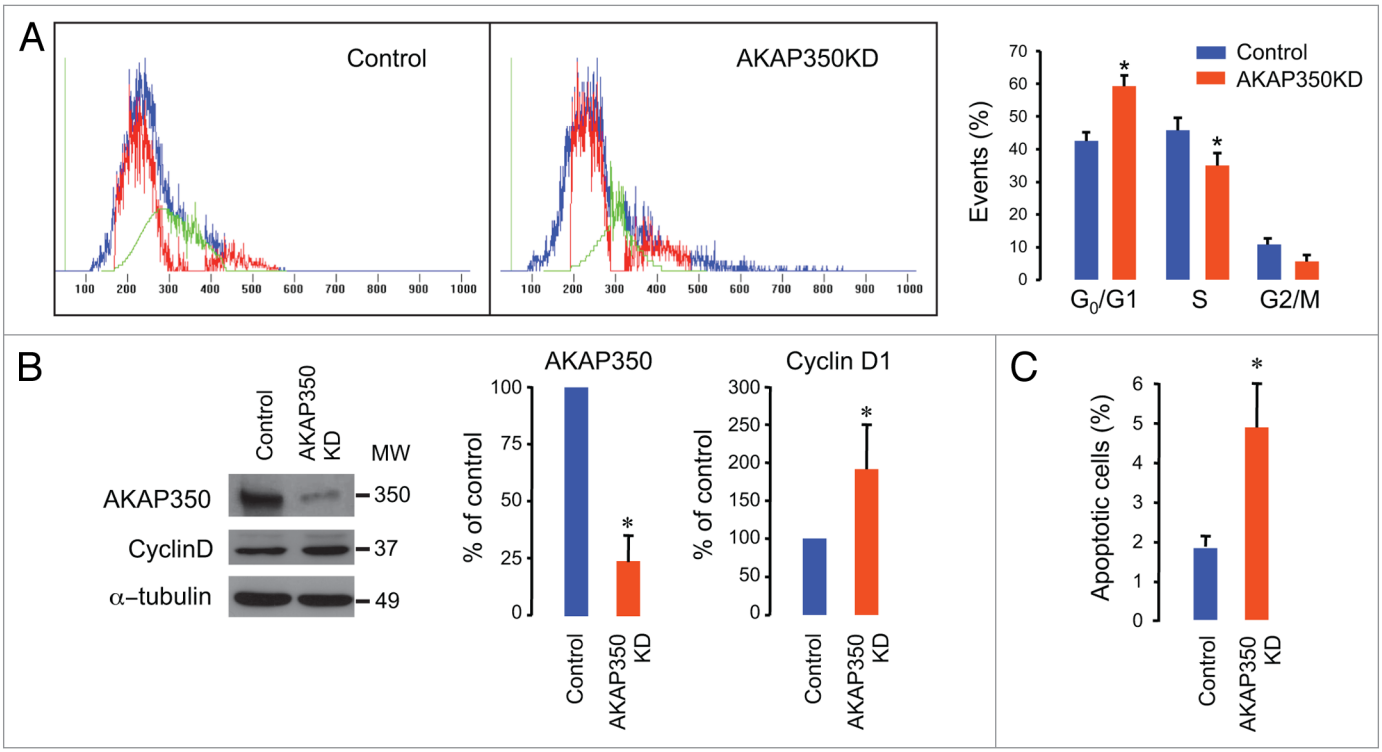

Figure 1. Effect of AKAP350 knock down on the cell cycle. HepG2 cells were transfected with siRNA1 or siRNA2 (AKAP350KD), specifically targeted to AKAP350 mRNA, or with a scrambled control (control), and allowed to grow for 48 h. (**A**) Control and siRNA1 transfected cells were fixed with cold 70% ethanol, marked with propidium iodide and analyzed by flow cytometry. The figure shows typical outputs of the cell population distribution of control and AKAP350 KD cells (left) and the histogram representing the percentage of cells in the different phases of the cell cycle. Similar results were obtained with siRNA2 transfected cells. (**B**) AKAP350 and Cyclin D1 expression were analyzed by western blot in control and siRNA1 transfected cells. α-Tubulin was used as loading control. These results were confirmed in siRNA2 transfected cells. (**C**) Control and AKAP350KD HepG2 cells were stained with Annexin V-FITC and propidium iodide (PI) and the percentages of apoptotic (Annexin$^+$) and dead (PI$^+$) cells were determined by flow cytometry analysis. Bars represent the percentages of cells undergoing early apoptosis (Annexin$^+$/PI$^-$). Results are means ± s.e.m. of four (**A and B**) or three (**C**) independent experiments. *$P < 0.05$

The aim of our work was to analyze AKAP350 participation in cell cycle progression by taking advantage of specific loss-of-function studies in proliferating cells and to obtain insights into the mechanisms involved.

Results

AKAP350 participates in G_1/S transition

In order to evaluate the participation of AKAP350 in cell cycle progression, we studied the effect of specifically decreasing AKAP350 expression on DNA content in HepG2 cells. Cells were transfected with a non-specific (control) and two AKAP350-specific (AKAP350KD) siRNAs. We found that the decrease in AKAP350 expression induced a 40% increase in the population of 2N-containing cells (**Fig. 1A**). To discriminate if this increased population corresponded to cells in G_0 (quiescent) or G_1 phase, we analyzed cyclin D1 levels. AKAP350KD cells showed a significant increase in cyclin D1 (**Fig. 1B**), thus indicating that the accumulation of 2N-containing cells was due to an increase in cells that had entered the cell cycle. We further characterized AKAP350KD cells regarding their viability. We did not find any change in the total number of viable cells, as measured by MTT assay, or in the number of dead cells, assessed by propidium iodide incorporation of non-fixed cells (data not shown), but we detected an increase in early apoptosis in AKAP350KD cells (**Fig. 1C**). Altogether, our results indicate that the decrease in AKAP350 expression inhibits G_1/S transition, inducing G_1 arrest.

AKAP350CTD induces the initiation of DNA synthesis

AKAP350 recruits to the centrosomes several proteins involved in G_1/S transition, including PKA and Cdk2. To investigate the mechanism involved in AKAP350 participation in G_1/S transition, we prepared stable cell lines expressing the AKAP350(3330–3595) domain, equivalent to AKAP450(3643–3908), fused to GFP (AKAP350CTD) or GFP (control). AKAP350CTD harbors the centrosomal targeting domain of AKAP350, the PACT domain, and the 50 amino acids located upstream the PACT domain. We first characterized two different clones (AKAP350CTD 1 and 4) for the localization of the fusion protein, and that of the endogenous AKAP350. Our results showed that AKAP350CTD localized exclusively at the centrosomes in both clones (**Fig. 2A**), inducing a partial displacement of the endogenous AKAP350 (**Fig. 2B**).

We analyzed the effect of AKAP350CTD expression on the DNA content of HepG2 cells. The expression of this AKAP350 domain induced a decrease in the population of 2N-containing cells, with a concomitant increase in 2N < x < 4N cells, both

in AKAP350CTD1 and AKAP350CTD4 cell lines (**Fig. 3A**). These results indicated an increase in the S phase population and a decrement of G_0/G_1 cells. In agreement with these results, we found that AKAP350CTD cells expressed higher levels of cyclin A (+130%, $P < 0.01$) (**Fig. 3B**). These findings indicated that AKAP350CTD expression activated the G_1/S transition. We studied if this effect was accompanied by changes in cell proliferation. Our results showed that AKAP350CTD expressing clones did not differ in the total number of viable or dead cells (**Fig. 3C and D**), which indicated that AKAP350CTD expression did not affect cell proliferation or death in these conditions.

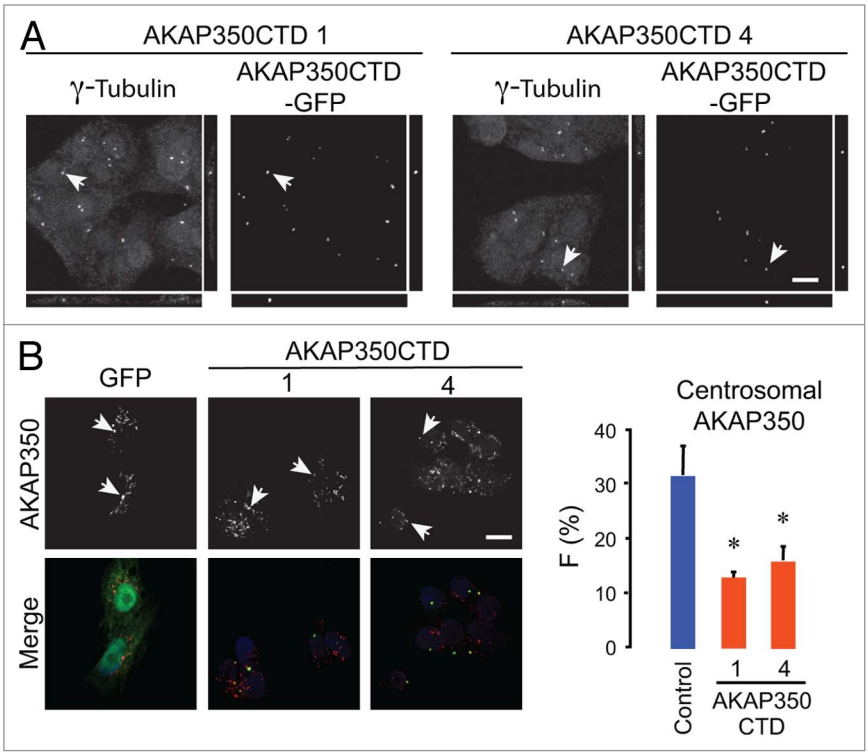

Figure 2. Characterization of AKAP350CTD cells. AKAP350CTD1 and AKAP350CTD4 cells were cultured for 24 h, fixed with methanol and stained for γ-tubulin (**A**) or fixed with paraformaldehyde and stained for AKAP350 (**B**). Immunofluorescence images were obtained by confocal microscopy. (**A**) Images show γ-tubulin staining and AKAP350CTD-GFP fluorescence. Arrows indicate the centrosomes with orthogonal view. (**B**) Images show the z-projection of AKAP350 staining. Arrows indicate centrosomal localization of AKAP350. Bars represent the mean ± s.e.m. of the % of AKAP350 fluorescence intensity associated with centrosomes for three independent experiments. Bar: 5 μm.

AKAP350 participates in centrosomal duplication

Previous studies indicated that the expression of AKAP450 (3699–3796) inhibited the initiation of centrosomal duplication,[12] while expression of AKAP450 (2895–3908) led to centrosomal amplification.[6] We analyzed the effect of the specific decrease in AKAP350 expression on the number of centrosomes per cell (**Fig. 4A**). Our results showed that AKAP350KD cells have an increased population of cells with one centrosome with a concomitant decrease in cells with two centrosomes, which indicates that the loss of AKAP350 expression leads to the inhibition of centrosomal duplication. Considering that the initiation of DNA synthesis was also inhibited in AKAP350KD cells, and that AKAP350CTD expression was enough to promote this process, we further studied the effect of AKAP350CTD expression on centrosomal duplication. We analyzed the number of centrosomes per cell, and their extent of separation in AKAP350CTD cells. We discriminated cells containing one centrosome from those containing two attached and two separated centrosomes, which would correspond to cells in G_0/G_1, S, and G_2/mitosis phases, respectively. No significant difference in the number of cells from each population was observed (**Fig. 4B**). We also did not observe centrosomal amplification in AKAP350CTD cells. Thus, the expression of AKAP350CTD was capable of inducing the initiation of DNA synthesis, but not centrosomal duplication. These results indicated that, while AKAP350 participation in DNA synthesis lies in its carboxyl-terminal domain, there are other fragments of the molecule involved in the modulation of centrosomal duplication.

AKAP350CTD increases centrosomal Cdk2 activity

Among the proteins involved in G_1/S transition and centrosomal duplication, Cdk2 interacts with AKAP450 (2895–3908),[6] while PKA has two different interaction motifs located at positions 1439–1456 and 2551–2565 of the AKAP450 sequence,[1-3] which are excluded from AKAP350CTD. Immunofluorescence analysis indicated that Cdk2 localizes at the centrosomes both in control and AKAP350CTD cells (**Fig. 5A**), but was not suitable for quantitative analysis (see Materials and Methods). Therefore, we investigated if AKAP350CTD affected the centrosomal localization of Cdk2 by analyzing Cdk2 expression in centrosome-enriched subcellular fractions. We found that AKAP350CTD expression did not induce any change in total Cdk2 expression, but increased centrosomal Cdk2 levels (**Fig. 5B**). We further studied AKAP350CTD effects on centrosomal activity of Cdk2 by determining the phosphorylation status of its centrosomal substrate nucleophosmin (NPM). NPM is a well-characterized centrosomal target of cyclin E-Cdk2, whose phosphorylation promotes the initiation of centrosomal duplication.[16] We analyzed NPM phosphorylation at its specific Cdk2 site, and found that the expression of AKAP350CTD induced an increase in NPM phosphorylation by Cdk2 (**Fig. 6**).

Discussion

Previous studies demonstrated the participation of AKAP350 in centrosomal duplication.[6,12] The first of these studies showed that the expression of the PACT domain induced abrogation of cell cycle progression either in G_2 phase or in G_1 phase, in HeLa

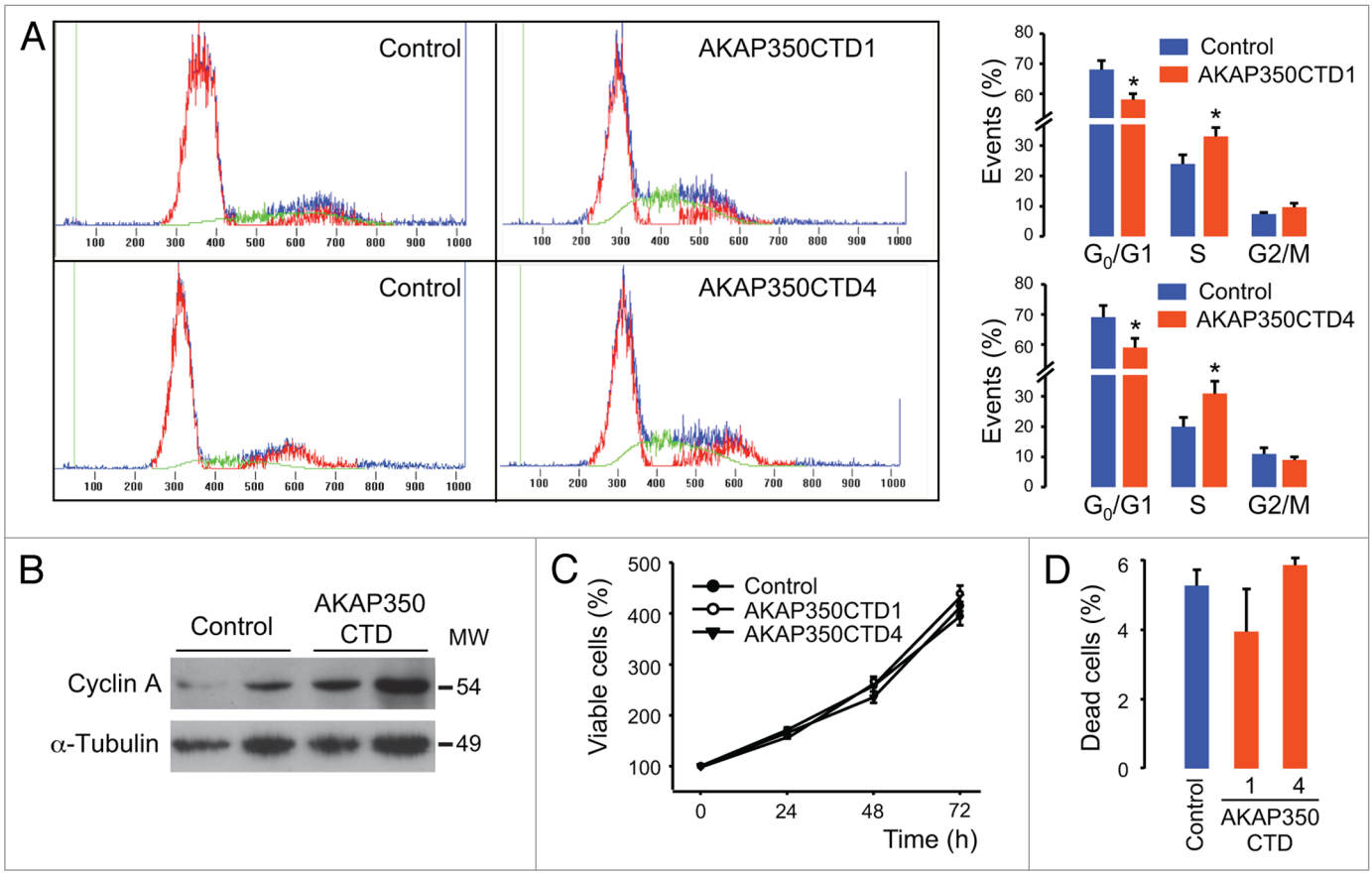

Figure 3. Effect of AKAP350CTD expression on the cell cycle in HepG2 cells. (**A**) GFP (Control), AKAP350CTD1, and AKAP350CTD4 cells were fixed, marked with propidium iodide, and analyzed by flow cytometry. The figure shows typical outputs of control, AKAP350CTD1, and AKAP350CTD4 cells and the histogram representing the percentage of cells in the different phases of the cell cycle. (**B**) Cyclin A expression was analyzed by immunoblot, using α-tubulin as loading control. The image is representative of three independent experiments. (**C**) AKAP350CTD1 and AKAP350CTD4 cells attached to microplates were cultured for 0, 24, 48, and 72 h. MTT assay was performed as described in Materials and Methods. Results are presented as the percentage of the absorbance of the cells cultured for 0 h. (**D**) Control and AKAP350CTD cells were cultured for 72 h and stained with Annexin V-FITC and propidium iodide (PI) and the percentages of apoptotic (Annexin$^+$) and dead (PI$^+$) cells were determined by flow cytometry analysis. Bars represent the percentages of PI$^+$ cells. Results are means ± s.e.m. of three independent experiments.*$P < 0.01$.

or RPE1 cells, respectively.[12] On the other hand, the second study found that the expression of AKAP45O(2895–3908) did not induced any change in the cell cycle distribution of CHO cells.[6] The differences between these studies may lay in two different facts: First, the AKAP350 domains used affect differentially the localization of the endogenous protein. Second, both constructs interact with different cell cycle proteins.[6,11,12] Therefore, although these studies supported the concept that AKAP350 participates in the modulation of cell cycle progression, it was not clear which effects were secondary to endogenous AKAP350 delocalization, and which to the presence of its derived fusion protein at the centrosomes.

We first studied the effect of specifically decreasing AKAP350 expression by RNA interference on the cell cycle distribution of HepG2 cells. We found that the decrease in AKAP350 levels increases the G_0/G_1 population. We have previously demonstrated that the reduction in AKAP350 expression inhibits HepG2 cells differentiation,[10] thus suggesting that the increase in 2N-containing cells was due to an increase in G_1, and not in G_0 cells. The entrance into the cell cycle, from G_0 to G_1, is accompanied by an increase in cyclin D1 levels. We found that cyclin D1 levels were increased in AKAP350KD cells, thus confirming that these cells entered the cell cycle, but could not progress to S phase.

Previous data indicated that disruption of centrosomes by decreasing different centrosomal proteins leads to G_1 arrest in cells with wild-type p53.[17] Our own experiments performed in HepG2 cells, which express functional p53,[18] demonstrated that the increase in G_1 events in AKAP350KD cells was accompanied by an activation of apoptosis, which is consistent with induction of G_1 arrest. We investigated if the effect of AKAP350 knock down on the cell cycle was a general effect, due to a non-specific effect on centrosomal integrity, or if AKAP350 specifically participated in G_1/S transition. We prepared HepG2 cell lines with stable centrosomal expression of an AKAP350 carboxyl-terminal domain, which induced a partial displacement of the endogenous protein. Instead of mimicking AKAP350 knock down effect, the expression of this fragment of the protein lead to an increase in cells with DNA content corresponding to S phase, accompanied by a rise in cyclin A levels. Therefore, AKAP350CTD expression

was enough to induce the G_1/S transition, revealing a specific role of AKAP350 in this process.

The Cdk2 recruitment to centrosomes is a critical event during G_1/S transition.[15] Previous studies demonstrate that expression of the AKAP450(2895–3908) domain increases the centrosomal levels of this mitotic kinase. Thus, we further evaluated if AKAP350CTD, equivalent to AKAP450(3643–3908), participates in the centrosomal recruitment of Cdk2. AKAP350CTD expression induced an increase in Cdk2 protein levels at the centrosome. Although Cdk2 activity is primarily modulated by its interaction with inhibitors, such as p21 or p17, or activators, such as cyclin E and cyclin A, other signaling proteins can further modulate this kinase. In fact, AKAP350 interacts with two proteins which can regulate the G_1/S transition by modulating Cdk2 activity: calmodulin,[11] which promotes this transition by activating cyclin E-Cdk2,[19] and cyclin G_2,[20] which can induce G_1 arrest by inhibiting Cdk2 activity.[21] We speculated that, even though the centrosomal recruitment of Cdk2 by AKAP350 may be necessary to promote the G_1/S transition, it might not be sufficient to ensure the increase of its centrosomal activity. Thus, we analyzed Cdk2 centrosomal activity in AKAP350CTD cells. NPM is a mainly nucleolar protein, whose centrosomal localization is cell cycle dependent. At late G_1 phase, NPM is phosphorylated by cyclin E-Cdk2, phosphorylation which induces NPM release from the centrosomes and the initiation of centrosomal duplication.[16] We found that AKAP350CTD expression induced an increase in Cdk2 specific phosphorylation of NPM, indicating that AKAP350 enables Cdk2 centrosomal activity at the G_1/S transition.

Centrosomal duplication is initiated during late G_1 phase by the disengagement of the two centrioles. We found that the initiation of centrosomal duplication was inhibited in AKAP350KD cells, and that AKAP350CTD, in spite of activating DNA synthesis, did not induce the initiation of centrosomal duplication. Thus, we were able to uncouple both events. We concluded that AKAP350 participation in centrosomal duplication does not depend exclusively on the regulation of Cdk2 activity at this organelle. In this regard, PKA participates in the separation of the centrioles by phosphorylating its structural component centrin.[22] It is probable that AKAP350 integrates different events involved in the initiation of centrosomal duplication, such as phosphorylation of centrin by PKA, and phosphorylation of NPM by Cdk2. Further studies will be necessary to understand how these events are coordinated.

Summarizing, our results support a scenario where AKAP350 participates in the G_1/S transition by coordinating the activity of proteins involved in multiple events required for the initiation of both DNA synthesis and centrosomal duplication. On one hand, by means of its carboxyl-terminal domain, AKAP350 enables Cdk2 activity at the centrosomes at this stage of the cell cycle, therefore stimulating the initiation of DNA synthesis. Although the facilitation of Cdk2 activity would also be involved in promoting centrosomal duplication, the recruitment of other signaling proteins by different AKAP350 domains are likewise necessary for this process to be initiated.

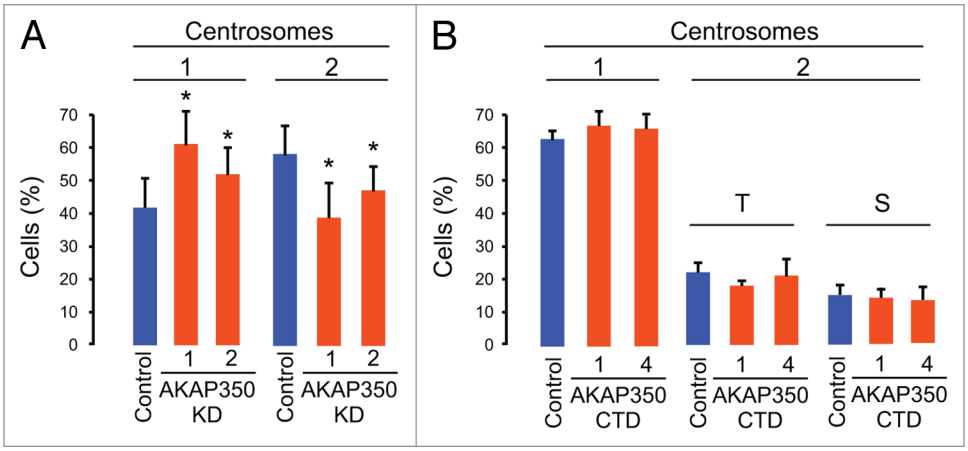

Figure 4. AKAP350KD, AKAP350CTD, and control cells were fixed and stained with γ-tubulin and DAPI. Centrosomes were identified as punctate structures enriched in γ-tubulin. Images were quantified by grouping cells as one-centrosome and two-centrosome cells. (**A**) The bars represent mean values of cells transfected with a control, or two different specific siRNAs (AKAP350KD 1 and 2). (**B**) When analyzing AKAP350CTD cells, two-centrosome cells were further categorized in cells containing the centrosomes together (T) or separated (S), depending whether the distance between them was smaller or greater than 2 μm, respectively. Results are means ± s.e.m. of three independent experiments. *$P < 0.05$.

Materials and Methods

Cell culture

HepG2 cells (ATCC) were grown on plastic dishes in DMEN medium, as previously described.[10]

Reduction of AKAP350 expression by interfering RNA

In order to reduce AKAP350 expression, two specific 21 nucleotide double chain RNA (siRNA), siRNA1 and siRNA2, and a scrambled control were designed as we previously described,[10] and synthesized using an Ambion commercial kit "SilencerTMsiRNA". HepG2 cells were transfected using Dharmafect 4 reagent (Thermo Fisher Scientific), as we previously described.[10] Experiments were performed 48 h after transfection, and the specific decrease in AKAP350 expression was confirmed by immunoblotting.

Generation of stable cell lines (AKAP350CTD1 and AKAP350CTD4)

The AKAP350(3330–3595) domain, equivalent to AKAP450(3643–3908), was cloned into pEGFP-C2 (Clontech) generating a construct coding for GFP fused to the carboxyl-terminal domain of AKAP350 (AKAP350CTD-GFP). We generated populations of HepG2 cells which stably express AKAP350CTD-GFP or GFP (control). HepG2 cells were

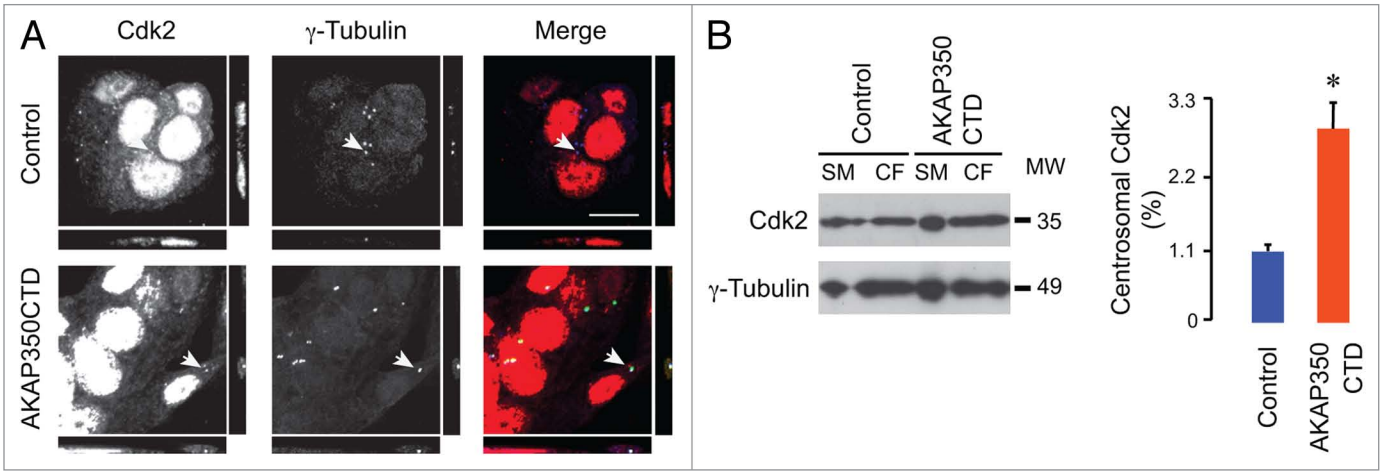

Figure 5. Effect of AKAP350CTD expression on centrosomal levels of Cdk2. (**A**) AKAP350CTD and control cells were cultured for 24 h and fixed with methanol. Fixed cells were double-stained for Cdk2 and γ-tubulin, and analyzed by confocal microscopy. The first and second column show images of the channels corresponding to γ-tubulin and Cdk2, and the third column the overlay view in RGB mode of the channels corresponding to GFP expression (green) and γ-tubulin (blue) and Cdk2 (red) staining. Bar, 10 μm. Arrows indicate centrosomes with orthogonal views. (**B**) Centrosomal enriched fractions (CF) were prepared as described in Materials and Methods, and Cdk2 levels at CF and at the starting material (SM) analyzed by western blot. γ-tubulin was used as loading control. Bars show the percentage of Cdk2 recovered at CF. Results are mean ± s.e.m of 4 independent experiments. *$P < 0.05$.

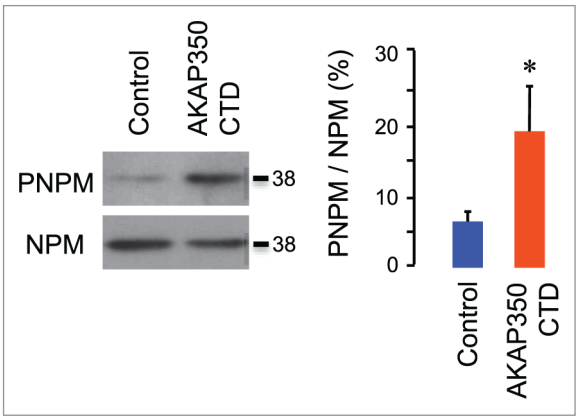

Figure 6. Effect of AKAP350CTD expression on centrosomal activity of Cdk2. NPM and NPM(T199P) (PNPM) levels were analyzed by inmunoblotting in control and AKAP350CTD cells. Bars represent the ratio of the density of the PNPM band, divided by that corresponding to total NPM expression. Results are mean ± s.e.m of three independent experiments. *$P < 0.05$.

transfected by electroporation, as previously described.[10] After 24 h, the antibiotic Geneticin (Invitrogen, 500 μg/ml) was added to the media in order to select the transfected cells. For the maintenance of these cell lines, they were grown in a medium containing Geneticin 200 μg/ml in conditions otherwise similar to parental cells.

Cell viability and proliferation studies

Metabolic activity assessment (MTT assay)

Cells were cultured in 96-well microplates and methylthiazolyldiphenyl-tetrazolium bromide (MTT, Sigma-Aldrich) was added into the culture medium at different time points, as we previously described.[23] After 2 h, cells were lysed by addition of DMSO and absorbance of the metabolite produced from viable cells was detected at 540 nm in a microplate reader (Beckman Coulter LD400). Results were expressed as percentage of absorbance in control cells.

Annexin V/propidium iodide assay

Cells were detached from the petri dishes, gently homogenized in the culture medium/PBS and harvest. Apoptotic externalization of phosphatidylserine and cell death was assessed by staining with Annexin V-FITC and propidium iodide (Sigma Aldrich) or with Annexin V-PE and 7-Animo-Actinomycin (BD Biosciences), for cells expressing GFP associated fluorescence, coupled to flow cytometric analysis (Cell Sorter BD FACSAria II, BD Biosciences), following the manufacturers's instructions.

Cell cycle analysis by flow cytometry

Cell distribution in the cell cycle was analyzed by determining the cellular DNA content by flow cytometry. 1×10^6 cells were fixed with cold 70% ethanol and then washed with PBS and marked with 50 μg/ml propidium iodide in a buffer containing 0.1% sodium citrate, 0.02 mg/ml RNA, and 0.3% NP-40. Results were analyzed using WinMDi and CellCycle programs.

Immunoblotting

Cells were washed with cold phosphate buffered saline (PBS), scraped and pelleted at 200 *g*, for 5 min at 4 °C. Pelleted HepG2 cells were resuspended in Triton X-100 1%/PBS pH 7.4 with protease and phosphatase inhibitors and subjected to two freeze-thaw cycles. Lysates were centrifuged at 1000 *g* for 5 min and the clear supernatants were conserved. Total protein concentrations were measured according to Lowry et al.[24] Solubilized membranes were heated 10 min at 70 °C in sample buffer (20 mM Tris, pH 8,5, 1% SDS, 400 μM DTT, 10% glycerol). Samples containing equal amounts of proteins were subjected to SDS 4%/10% discontinuous or 12% PAGE. The proteins in the 4% gel were transferred to nitrocellulose membranes (Amersham Pharmacia), while 12% gel proteins were transferred to polyvinyl difluoride

membranes (Perkin Elmer Life Sciences). Blots were blocked with 5% non-fat milk in PBS/0.3% Tween 20. Nitrocellulose membranes were probed with the monoclonal mouse antibody anti-AKAP350 (14G2) (1:500)[1] and polyvinyldifluoride membranes were probed with primary rabbit antibodies: anti-NPM (1:1000, Cell Signaling), anti-Phospho-NPM (1:1000, Cell Signaling), anti-Cdk2 (1:1000, Santa Cruz Biotechnology), goat anti-cyclin A (1:500, Santa Cruz Biotechnology), or mouse anti-β-actin (1:2000, Sigma-Aldrich), anti-α-tubulin (1:2000, Sigma-Aldrich), and anti-Cyclin D1 (1:400, Santa Cruz Biotechnology). The blots were washed and incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies. Bands were detected by chemiluminescence reaction (Amersham Pharmacia), after exposition to Kodak XAR film. Bands were quantified using the Image J program. In preparing the figures, brightness and contrast were adjusted in order to improve visualization.

Immunofluorescence and confocal microscopy

The cells were grown on glass coverslips and, at the end of each experiment, washed with PBS and fixed with 4% paraformaldehyde or in 100% methanol. Fixed cells were permeabilized and blocked with 0.3% Triton X-100/bovine serum albumin 1%/PBS, pH 7.4 for 10 min. For Cdk2 detection, cells were processed as previously described.[6] Then they were incubated with rabbit anti-Cdk2 (1:100) and mouse anti-γ tubulin (1:500) or anti-AKAP350 (1:80) antibodies for 2 h at room temperature. The coverslips were washed, incubated with the secondary Alexa 555 and Cy5 conjugated antibodies and with 4′,6-diamidino-2-phenylindole (DAPI) and mounted with ProLong, as previously described.[10] Fluorescence localization was detected by confocal laser microscopy (Nikon C1SiR with inverted microscope Nikon TE200). Serial optical 0.3 μm thick sections were collected in the *z*-axis. Z-stacks were built, and projections were obtained using Image J tools. In preparing the figures, adjustment in brightness and contrast were equally applied to the entire images using Adobe Photoshop software, in order to improve visualization of fluorescence.

Morphometric analysis of centrosomes

A morphometric analysis was performed on the images obtained by confocal microscopy in order to determine centrosome number and localization. γ-tubulin staining was used to identify centrosomes. Images were quantified by grouping cells in the following categories: one-centrosome cells and two-centrosome cells, considering the centrosomes attached or separated, depending on whether the distance between centrosomeswas smaller/equal or greater than 2 μm. At least 150 cells were analyzed in each group.

Analysis of protein localization in centrosomes

Centrosomal localization of AKAP350 by confocal microscopy was performed as we have previously described.[10] As regards Cdk2, due to the extraction step performed prior to fixation and to its predominant nuclear localization, we found this method inappropriate to estimate its centrosomal localization. Alternatively, we prepared centrosome-enriched fractions by centrifugation in a discontinue sucrose gradient, using a method based on Moudju and Bornens,[25] modified to improve centrosomal proteins resolution.[26] Cells were gently sonicated in cold buffer containing 80 mM HEPES (pH 6.8), 100 mM KCl, 14% sucrose, 1 mM $MgCl_2$, 1 mM EGTA, and protease inhibitors. In order to eliminate unbroken cells and nuclear fractions, cell extracts were centrifuged at 1500 *g* for 15 min. The supernatant was brought to 20% sucrose, 0.1% Triton X-100, and loaded on the top of a sucrose step gradient consisting of 70% and 40% steps. Samples were centrifuged at 100 000 *g* for 20 min and 6 fractions corresponding to the 40–70% sucrose fractions were collected. The best enrichment in centrosomes, assessed by γ-tubulin distribution, was obtained at the fraction corresponding to the 40–70% interface, and the 70% step bottom fraction.

Statistical analysis

Data are expressed as mean ± s.e.m. A paired Student *t* test was used for comparison between groups and non-parametric Mann–Whitney test was used for comparisons within each experiment. $P < 0.05$ was considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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