Protein phosphatase 4 plays dual roles during cell proliferation

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Abstract

Objectives: Protein phosphatase 4 (PP4) has been reported to be indispensable for cell proliferation and survival. Deletion of PP4 has been shown to induce abnormal and even lethal events in growth and development both in lower eukaryotes and in mammals. However, until now, effects of PP4 up-regulation have remained unclear.

Materials and methods: To test effects of PP4 on cell proliferation, cell cycle and morphology in HepG2 cells, it was down-regulated using PP4 siRNA or its activity was inhibited using PP4RL (a PP4 phosphatase-dead mutant) adenoviruses. Alternatively, PP4 was up-regulated using PP4 adenoviruses. Next, we used a functional proteomic approach to identify proteins that may interact with PP4. Furthermore, we performed rescue experiments to verify the possible mechanisms.

Results: To our surprise, we found that both up-regulation and inhibition of PP4 inhibited cell proliferation. Unlike PP4 inhibition, PP4 up-regulation induced prominent arrest at the prometaphase/metaphase transition by causing defects in chromosome alignment and spindle assembly. Moreover, we identified scaffold attachment factor A (SAF-A) (an important protein required for kinetochore-microtubule attachment that participates in the prometaphase/metaphase transition), to be a novel protein that interacts with PP4, using a proteomic approach. Thus, mutual regulatory mechanisms exist between PP4 and SAF-A. Interactions between PP4 and SAF-A played a role in prometaphase/metaphase transition.

Conclusions: Our data demonstrate a novel regulatory mechanism involving PP4 in cell proliferation.

Abbreviations

PP4: protein phosphatase 4

PP4-RL: PP4 phosphatase-dead mutant

SAF-A: scaffold attachment factor A

AD: adenovirus

GFP: Green fluorescent protein

Introduction

Mitosis involves complex processes in which reversible phosphorylation of proteins plays crucial roles. In the human genome, there are 40 potential serine/threonine phosphatases that counter the activity of 428 kinases known or predicted to phosphorylate serine/threonine residues. The elaborate interplay between kinases and phosphatases results in changes in the phosphorylation of substrates that ensures the completion of mitosis. In the past few decades, multiple kinases and phosphatases, including Cdk1, Aurora-A, Cdc25C, protein phosphatase 1 (PP1) and protein phosphatase 4 (PP4), have been identified as key regulators in cell division (1,2).

PP4 is an evolutionarily conserved protein serine/threonine phosphatase that belongs to the PP2A/PP4/PP6 family (3,4). This phosphatase has been shown to participate in multiple diverse cellular processes including the DNA damage response, spliceosomal assembly, glucose metabolism and multiple signalling pathways, including mTOR, Jun-terminal protein kinase and NF- κ B (5–11) signalling. PP4 can dephosphorylate KAP1 and is involved in the non-homologous end-joining (NHEJ) pathway, which is essential for the response to DNA damage. PP4 has been shown to dephosphorylate HDAC3, which regulates its activity. PP4 is also involved in the regulation of hepatic glucose metabolism through dephosphorylation of CRT2 (5–12).

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During proliferation, PP4 is considered to be indispensable for growth, development and proliferation in organisms ranging from the lower eukaryotes, including *C. elegans* and *Drosophila*, to mammals. In *C. elegans*, depletion of PP4 by RNAi causes a semi-lethal phenotype with aberrations in spindle formation that were similar in mitosis and in sperm meiosis (13). Disruption of the PP4 gene in *Drosophila* also produces a semi-lethal phenotype (14). In a vertebrate, zebrafish, PP4 functions in dorsoventral patterning of the early embryos (15). Similarly, genetic ablation of PP4 resulted in embryonic lethality of mice before E9.5. Conditionally knocking out PP4 in mouse T cells or B cells inhibited the development of the T cells or B cells (16,17). Additionally, *in vitro* experiments showed a delay in G2 before entry into prophase in mouse embryonic fibroblast (MEF) cells isolated from mice in which PP4 had been disrupted mice by Cre-loxP recombination (18). Depletion of PP4 by lentivirus-delivered stable gene silencing in HEK293 cells led to a delay in prophase (19). Zhuang *et al.* reported that knock-down of PP4 by siRNAs in HEK293 cells caused an increase in the S-phase cell population (2). These data indicate that depletion of PP4 induces abnormal and even lethal events in growth development.

Recently, Martin Voss *et al.* have reported that in contrast to other phosphatases, PP4 is not continuously activated through the entire cell cycle. The activity of PP4 undergoes dynamic changes during the cell cycle with a peak in the G1 phase and a decrease during the S phase, reaching the lowest value in the G2/M phase (12). These results indicate that PP4 is not continuously activated through the entire cell cycle, and up-regulation of PP4 may also effect the cell cycle process. However, the role of PP4 in the cell cycle remained largely unclear.

In this study, we demonstrated that PP4 played dual roles during cell proliferation. Both up-regulation and inhibition of PP4 inhibited cell proliferation. Up-regulation of PP4 blocked the prometaphase/metaphase transition by inducing defects in chromosome alignment and spindle assembly. Moreover, we demonstrated that scaffold attachment factor A (SAF-A) was an important target of PP4 and was involved in the PP4-induced prometaphase arrest. Our data provide a novel regulatory mechanism involving PP4 in cell proliferation.

Materials and methods

Plasmids, siRNA and adenoviral vectors

The PP4 and SAF-A genes were amplified by PCR and cloned into the PCMV6 vector. A PP4RL (a PP4 phosphatase-dead mutant) was obtained using the Quick-

Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) to substitute leucine for arginine at amino acid 235 in the phosphatase domain of PP4 construct, and this was subcloned into the pEGFP-C1 vector. A non-specific siRNA duplex was used for control transfection. Two siRNA duplexes (siRNA1: 5'-GGUUA CAAGUGGCACUUCATT-3' and siRNA2: 5'-GGA CGAGCAUCUCCAGAAATT-3') that target human PP4 were used to deplete PP4 (Genepharma, Shanghai, China). The previously reported siRNA duplexes were used to deplete SAF-A (20). The adenoviral vectors containing pAdxsi-PP4 or pAdxsi-PP4RL and the corresponding control adenoviral vectors were obtained from the Chinese National Human Genome Center.

Antibodies

The antibodies against PP4 (ab16475), SAF-A (ab10297), Bcl-2, Bcl-xl and GFP were purchased from Abcam (Cambridge, MA, USA). The antibodies against cyclin B and cyclin E were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). The antibodies to PP4 (Sc-6118), p53, GAPDH and actin were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). The antibodies to SAF-A (16365-1) and BubR1 were obtained from Proteintech (Chicago, IL, USA). The antibodies against α -tubulin and γ -tubulin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

HepG2 cells were maintained at 37 °C in minimum Eagle's medium (Invitrogen Life Technologies, St. Louis, MO, USA) supplemented with 10% foetal bovine serum (Hyclone, Waltham, MA, USA), 100 units/ml penicillin and 0.1 mg/ml streptomycin (Invitrogen Life Technologies). The cell viability was determined using the MTT assay (Wuhan Boster Biotech, Wuhan, Hubei, China) or the CCK8 assay (Wuhan Boster Biotech, Wuhan, Hubei, China).

Immunoprecipitation

HepG2 cells were lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 1% NP-40, 120 mM NaCl, 1 mM EDTA, 6 mM EGTA, 1 mM dithiothreitol, 50 μ M PMSF and 2 μ g/ml aprotinin. PP4 was immunoprecipitated with an anti-PP4 antibody (Sc-6118), whereas SAF-A was immunoprecipitated with an anti-SAF-A antibody 16365-1. The immunoprecipitates were washed three times with buffer containing 50 mM HEPES (pH 7.4), 0.1% Triton X-100 and 500 mM NaCl.

Phosphatase assays

Phosphatase assays were performed using the Ser/Thr phosphatase assay kit 1 (Upstate Biotechnology, New York, NY, USA), according to the manufacturer's protocol.

Identification of proteins by mass spectrometry

Discrepant bands were excised from the immunoprecipitates, destained and subjected to in-gel trypsin digestion according to the manufacturer's instructions. Briefly, an aliquot (1 μ l) of the peptide extract was mixed with 1 μ l of a saturated solution of α -cyano-4-hydroxycinnamic acid matrix (10 mg/ml) prepared in TFA (0.1% trifluoroacetic acid and 50% acetonitrile) and submitted to MALDI-QTOF mass spectrometry using Qstar Pulsar I Quadrupole TOF-MS (Applied Biosystems/MDS Sciex, Toronto, ON, Canada). The following calibrants were used for external calibration of the equipment: Bradykinin fragments 1–7 (monoisotopic peak (M+H) is 757.3997) and ACTH fragments 18–39 (human) (monoisotopic peak (M+H) is 2461.1989). The mass accuracy was less than 5 ppm. Known contaminant peaks of keratin and trypsin self-digestion ions were excluded. The molecular masses of the tryptic peptide profiles were used to search the National Center for Biotechnology Information (NCBI) database using ProFound software (ProteoMe-trix) using peptide fingerprinting.

Live cell imaging

The HepG2 cells were transfected with the PP4 or control adenoviruses and used for live cell imaging for 32 h by placing the culture dishes onto a heated sample stage within a heated chamber (37 °C). Live imaging was performed using a live cell imaging system equipped with an Observer Z1 inverted microscope (Zeiss, Göttingen, Germany). Images were captured with 10-ms exposure times in 15-min intervals by an MRm CCD camera, and different Z sections were then projected using the Softworx suite.

Immunofluorescence microscopy

The cells on coverslips were briefly washed in PBS and fixed in cold methanol for 10 min at –20 °C or 4% paraformaldehyde in PBS for 3 min at room temperature. The fixed cells were permeabilized with 0.2% Triton X-100/PBS for 10 min at room temperature followed by blocking with 1% BSA/0.2% Tween 20/PBS. The cells were then incubated with primary antibodies (diluted in PBS containing 1% BSA) at 4 °C overnight. The cells were then washed three times with PBS and incubated with secondary antibodies for 1 h at

room temperature. For Hoechst staining, the cells were incubated with 10 mM Hoechst 33342 to stain the DNA. The cells were mounted in 90% glycerol/PBS.

For TUNEL staining, the fixed cells were incubated with reagents from a commercial apoptosis detection kit (Roche Diagnostics, Indianapolis, IN, USA) combined with Hoechst 33342. Between 500 and 700 cells in 10 randomly chosen fields from each coverslip were counted to determine the percentage of apoptotic nuclei. Each data point indicates the results from 1600 to 2000 cells from four independent experiments.

For image analysis, the cells were analysed with standard FITC, TRITC, rhodamine and DAPI filter sets using a ZEISS LSM700 immunofluorescence microscope. The images were captured with CCD and Axio-Vision image software.

Western blot analysis

The cell lysates (15–30 μ g of protein) were separated using 10% SDS-PAGE, transferred to PVDF membranes (Millipore Corporation, Billerica, MA, USA), blocked with 5% non-fat dry milk, and probed with antibodies at 4 °C overnight. The blots were incubated with HRP-conjugated anti-IgG followed by detection with ECL (Vilber Lourmat, Marne-la-Vallée, France).

Flow cytometry analysis

The cells that had been transduced with up-regulation-inducing or siRNA-expressing adenoviruses were incubated with trypsin–EDTA at 37 °C for 5 min, and the aspirated medium was used to resuspend the cells. For propidium iodide (PI) staining, the cells were incubated with 0.1% sodium citrate hypotonic solution containing 0.25 mg/ml RNaseA and 50 g/ml PI for 30 min at 4 °C. Finally, the cells were subjected to FACS using a Vantage SE instrument (BD).

Phosphoprotein detection with phos-tag

The HepG2 cells transfected with adenovirus-PP4 and adenovirus-PP4RL or control adenovirus vectors were lysed, and the lysates were subjected to SDS-PAGE on an 8% polyacrylamide gel containing 40 μ M phos-tag AAL-107 (Wako Pure Chemical Industries, Chuo-ku, Japan), followed by immunoblotting with indicated antibodies.

siRNA and plasmid transfection

HiPerfect transfection reagent (Qiagen) was used for the transfection with PP4 and SAF-A siRNA transfection, and Effectene transfection reagent (Qiagen) was used for

SAF-A plasmid transfection. At 48 h after transfection, the expression of the target proteins was detected by Western blotting.

Statistical analysis

All values are presented as the means $\pm$ SD of the indicated number of measurements. A one-way analysis of variance test was used to determine significance, requiring $P < 0.05$ for statistical significance.

Results

Both up-regulation and inhibition of PP4 inhibit cell proliferation

To test the effect of PP4 on the proliferation of HepG2 cells, PP4 was down-regulated by transfection of the PP4 siRNA- or PP4RL-expressing adenoviruses, or up-regulated using PP4-expressing adenoviruses. In accordance with a previous study, reduced proliferation

Figure 1. Both up-regulation and inhibition of PP4 inhibit cell proliferation. (a) The expression of PP4 was suppressed following siRNA transfection. (b) HepG2 cell proliferation was strongly inhibited following PP4 inhibition as detected using the MTT assay. (c) The expression of PP4 was elevated, the phosphatase activity of PP4 was suppressed (d), and cell proliferation was strongly inhibited (e) in a dose-dependent manner following AD-PP4RL transfection. (f) The expression of PP4 was elevated and cell proliferation was strongly inhibited (g) in a dose-dependent manner following AD-PP4 transfection. (h) The growth curve indicated that up-regulation of PP4 suppressed proliferation. (i) Screenshots from time-lapse microscopy show a large number of rounded cells following PP4 up-regulation. * $P < 0.05$ versus control, ** $P < 0.01$ versus control, *** $P < 0.001$ versus control, ### $P < 0.001$ versus control AD.

occurred (Fig. 1b) following PP4 down-regulation (Fig. 1a). PP4RL, in which arginine 236 is replaced by leucine, specifically inhibits endogenous PP4 activity by competitive inhibition with endogenous PP4 (Fig. 1c,d) as previously described (6,8,21). As expected, the proliferation of HepG2 cells transduced with the PP4RL-expressing adenovirus was strongly inhibited in a dose-dependent manner (Fig. 1e).

To our surprise, PP4 up-regulation (Fig. 1f) also strongly inhibited cell proliferation in a dose-depen-

dent manner (Fig. 1g). The effect of PP4 up-regulation on cell proliferation was confirmed by monitoring cell growth up to 5 days after adenoviral transfection with PP4 (Fig. 1h). Moreover, the effect of PP4 up-regulation was monitored by time-lapse microscopy for up to 32 h following adenoviral transfection with PP4. As shown in Fig. 1i, compared to the control cells that exhibited normal proliferation, the proliferation was strongly inhibited following PP4 up-regulation.

Figure 1. (continued)

Up-regulation of PP4 produced cell cycle-arrested phenotypes and nuclear morphology different from PP4 inhibition

We found that a large number of rounded cells were present following PP4 up-regulation (Fig. 1i). All of the rounded cells displayed condensed chromosomes as indicated by Hoechst staining in Fig. 2a. Similar data were also obtained in HeLa cells treated with the PP4 adenovirus at 50 MOI for 24 h (Fig. 2b) as well as MCF-7 cells (data not shown), indicating that this effect of PP4 was not cell-type specific.

Because cells undergoing apoptosis show proliferation inhibition accompanied by condensed chromosomes, we investigated whether these rounded cells were apoptotic. No obvious apoptosis had occurred in the cells with condensed chromosomes as indicated by a lack of TUNEL staining (Fig. 2c). Then, we assessed whether these cells were arrested in mitosis. As shown in Fig. 2d, the cells with condensed chromosomes showed enhanced cyclin B staining, which is a marker of the entry into mitosis. Similar results were obtained from the Western blot data: no obvious changes occurred in the apoptosis-related proteins, Bcl-2 and Bcl-xl, but changes in the cell cycle-related proteins, including an elevated expression of cyclin B and reduced expression of cyclin E, were observed (Fig. 2e,f). These data strongly suggested that the PP4-induced inhibition of cell proliferation was due to mitotic arrest but not apoptosis. Moreover, compared to the control cells, an increased G2/M-phase cell population was detected by flow cytometry in the PP4-up-regulated cells in a dose-dependent manner (Fig. 2g), which further confirmed that up-regulation of PP4 led to the accumulation of mitotic cells.

In contrast, cells in which PP4 activity had been inhibited by AD-PP4RL transfection demonstrated no obvious G2/M phase accumulation. However, an increase in the G1- and S-phase populations and a reduction in the G2/M cell population occurred (Fig. 2h). Moreover, the percentage of cells with condensed chromosomes was not increased following PP4RL overexpression (Fig. 2i). In addition, as expected, the expression of cyclin B was decreased in PP4RL-overexpressing cells (Fig. 2j). Similar data were

obtained in cells in which PP4 had been down-regulated by siRNA transfection (Fig. 2k–m).

Up-regulation of PP4 produced cells arrested at prometaphase with disrupted spindles and misaligned chromosomes

To better understand the details of the PP4-induced mitotic arrest, we assessed distribution among the mitotic phases in AD-PP4-transfected cells using immunofluorescence analyses for α -tubulin and nuclear staining (Fig. 3a). First, we found that the mitotic index increased dramatically from $8.38 \pm 0.42\%$ in the control AD-transfected cells to $48.60 \pm 2.01\%$ in the AD-PP4 transfected cells (Fig. 3b). More importantly, the distribution among the mitotic phases changed dramatically. As shown in Fig. 3c, the control AD-transfected cells showed normal prometaphase, metaphase, anaphase and telophase processes with only 13.5% in prometaphase, whereas in the AD-PP4 transfected cells, 89.6% of mitotic cells were arrested in prometaphase.

Next, we analysed the mitotic spindles and chromosomal alignment in the AD-PP4 transfected cells. As shown in Fig. 3d,e, the percentages of cells with aberrant spindles and misaligned chromosomes increased dramatically in the AD-PP4-transfected cells compared with the control AD-transfected cells. Specifically, among the 49% of mitotic cells in the AD-PP4-transfected cells, 43% of cells showed disorganized spindles and 45.5% of cells showed misaligned chromosomes, whereas only 0.2% and 0.6% of cells showed aberrant spindles and misaligned chromosomes, respectively, among the 8% mitotic cells in the control AD-transfected cells.

As predicted, the spindle checkpoint remained active in the cells with disrupted spindles and misaligned chromosomes. As indicated in Fig. 3f, clear staining of spindle checkpoint protein, BubR1, was observed at the kinetochores of the non-aligned chromosomes. This result demonstrated that the mitotic cell population in the AD-PP4-transfected cells were arrested before metaphase entry.

Together, these data suggested that PP4 activation induced cell accumulation at prometaphase with disrupted spindles and misaligned chromosomes.

Figure 2. Up-regulation of PP4 and PP4 inhibition showed different phenotypes of cell cycle arrest and nuclear morphology. (a) The rounded cells induced by AD-PP4 transfection displayed condensed chromosomes as indicated by white arrows. (b) HeLa cells treated with PP4 adenovirus showed condensed chromosomes as indicated by white arrows. (c) No obvious apoptosis occurred in the cells with condensed chromosomes as shown by the lack of TUNEL staining. (d) Enhanced cyclin B staining was detected in the cells with condensed chromosomes. (e) Western blot analysis showing expression levels of Bcl-2, Bcl-xl, cyclin B and cyclin E. (f) The statistical data for Bcl-2, Bcl-xl, cyclin E and cyclin B. (g) An increased cell population in the G2/M phase occurred in the AD-PP4-transfected cells. (h) A decreased population in G2/M phase, with no obvious condensed chromosomes (i) and decreased expression of cyclin B (j) was detected in AD-PP4RL-transfected cells. (k) A decreased population in G2/M phase, with no obvious condensed chromosomes (i) and decreased expression of cyclin B (j) occurred following PP4 siRNA transfection. Scale bars: 10 μ m. * $P < 0.05$ versus control, *** $P < 0.001$ versus control.

Figure 2. (continued)

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The SAF-A nuclear scaffold protein is a novel interacting protein of PP4

To investigate the possible mechanism of PP4 in the cell cycle, we applied a functional proteomic approach to the HepG2 cell lysates (Fig. 4a). By mass spectrometry, a group of potential PP4-interacting proteins was identified, including PP4R2 (the regulatory subunit of PP4)

and several other spindle-interacting proteins (data not shown). Among the multiple potential PP4-interacting proteins, the SAF-A nuclear scaffold protein was of great interest (Fig. 4b). SAF-A is a spindle regulator that contributes to the attachment of the spindle microtubules to the kinetochores and participates in spindle organization (20). In accordance with previous studies,

Figure 3. Up-regulation of PP4 in cells arrested at prometaphase with disrupted spindles and misaligned chromosomes. (a) Cells in which PP4 was up-regulated displayed an increased mitotic index following α -tubulin and Hoechst staining (b) and an elevated percentage of prometaphase cells (c). (d, e). An increased percentage of abnormal prometaphase phenotypes, including disrupted spindles and chromosomes, was found in AD-PP4-transfected cells compared to control cells. (f) BubR1 was present at the chromosomes in the prometaphase-arrested cells. Scale bars: 10 μ m. The data represent the means $\pm$ SD ($n = 4$ independent experiments), *** $P < 0.001$ versus control AD.

SAF-A displayed an obvious localization with mitotic spindle (Fig. 4c) and underwent dynamic localization during mitosis (data not shown). Furthermore, the siRNA-mediated down-regulation of SAF-A (Fig. 4d)

increased the G2/M cell population (Fig. 4e), and the cells were arrested in prometaphase/metaphase transition with aberrant chromosome alignment (Fig. 4f). More important, a dramatic increase in the number of mitotic

Figure 4. The SAF-A nuclear scaffold protein is a novel protein that interacts with PP4. (a) Isolation of PP4-interacting proteins by immunoprecipitation with an anti-PP4 antibody. (b) A representative tandem mass spectrometry spectrum that identified the 120-kDa band as SAF-A. (c) SAF-A localized to the mitotic spindle in metaphase. (d, e) Suppression of SAF-A by siRNA induced G2/M accumulation and aberrant chromosome alignment (f) as well as an elevated level of prometaphase cells (g). (h) Immunoprecipitation/Western blotting was used to co-immunoprecipitate endogenous SAF-A with PP4 when PP4 was immunoprecipitated with an anti-PP4 antibody and vice versa. Scale bars: 5 μ m. * P < 0.05 versus control, *** P < 0.001 versus control.

cells in prometaphase was observed in the SAF-A-silenced cells (Fig. 4g). To confirm the interaction between PP4 and SAF-A, we performed immunoprecipitation

and Western blotting analysis and found that the endogenous SAF-A was immunoprecipitated with PP4 by an anti-PP4 antibody and vice versa (Fig. 4h). Taken

together, we identified SAF-A as a novel protein that interacted with PP4. It was interesting that PP4 up-regulation and SAF-A inhibition showed similar patterns.

The interplay between PP4 and SAF-A plays a role in prometaphase/metaphase transition

We further explored the regulatory mechanisms between PP4 and SAF-A. First, we investigated whether PP4 regulated SAF-A expression. As shown in Fig. 5a, no obvious changes in the SAF-A expression were detected in the cells that had been transfected with PP4 siRNA. Moreover, we investigated the effect of the PP4-associated phosphatase activity on SAF-A. As shown in Fig. 5b,c, neither up-regulation of PP4 by AD-PP4 nor inhibition of PP4 activity by AD-PP4RL had any obvious impact on SAF-A expression. Considering that PP4 is a phosphatase, we investigated whether PP4 dephosphorylated SAF-A in HepG2 cells. As indicated in Fig. 5d, multiple serine/threonine phosphorylation sites were identified in SAF-A by Phosphosite Plus. As expected, PP4 up-regulation induced high levels of dephosphorylated SAF-A, while PP4 inhibition led to hyperphosphorylation of SAF-A as indicated by phos-tag AAL-107 (Fig. 5e). Next, we investigated whether SAF-A regulated the expression of PP4. As shown in Fig. 5f, knock-down of SAF-A by siRNA led to increased PP4 expression, whereas up-regulation of SAF-A by transfection of a SAF-A plasmid reduced PP4 expression (Fig. 5g). Taken together, these data suggested that mutual regulatory mechanisms exist between PP4 and SAF-A. Specifically, PP4 regulates the phosphorylation of SAF-A, whereas SAF-A may be involved in the regulation of PP4 expression.

Then, we wondered whether the dysfunction of SAF-A accounted for the prometaphase arrest induced by AD-PP4 transfection. As shown in Fig. 5h, up-regulation of SAF-A partly reversed the accumulation of cells in G2/M induced by AD-PP4 transfection as indicated by flow cytometry, and it also decreased the ratio of aberrant chromosome condensation induced by AD-PP4 transfection (Fig. 5i). Furthermore, SAF-A up-regu-

lation decreased the mitotic index dramatically from 42.8% to 31.6% in the AD-PP4-transfected cells. More importantly, the ratio of prometaphase-arrested cells was decreased (Fig. 5j). Western blot analysis also showed that up-regulation of SAF-A partially rescued the increased expression of cyclin B induced by AD-PP4 transfection (Fig. 5k). Then, we investigated whether PP4 participated in the prometaphase arrest induced by SAF-A inhibition. We found that inhibition of PP4 by AD-PP4RL transfection also partially reversed the prometaphase arrest induced by SAF-A siRNA transfection. As indicated in Fig. 5l–o, inhibition of PP4 partly reversed the SAF-A inhibition-induced accumulation of cells in G2/M, as indicated by flow cytometry, and also decreased the ratio of aberrant chromosome condensation induced by SAF-A inhibition, as indicated by Hoechst staining. Finally, PP4 inhibition dramatically decreased the mitotic index as well as the expression of cyclin B.

Taken together, the data indicate that the interactions between PP4 and SAF-A play a role in prometaphase/metaphase transition.

Discussion

In the present study, we demonstrated that PP4 played dual roles during cell proliferation. Although both up-regulation and suppression of PP4 inhibited proliferation of cells, the phenotypes of the cell cycle-arrested cells for the two conditions differed. Inhibition of PP4 arrested the cells in interphase, while up-regulation of PP4 arrested the cells at prometaphase/metaphase transition. We also identified SAF-A as one of the important targets of PP4 in the prometaphase/metaphase transition, and showed that the interactions between PP4 and SAF-A played a role in prometaphase/metaphase transition.

Experiments in which PP4 was inhibited by various means including deletion of PP4 by Cre-loxP recombination in MEF cells, lentivirus-delivered stable gene silencing in HEK293 and knock-down of PP4 by siRNAs in HEK293 have shown that PP4 is indispensable to the cell cycle process. Data from experiments in

Figure 5. The interplay between PP4 and SAF-A plays a role in prometaphase/metaphase transition. (a) No obvious change in SAF-A was detected in cells in which PP4 was inhibited using siRNA. (b, c) Neither AD-PP4 nor AD-PP4RL transfection had obvious effects on SAF-A expression. (d) The diagram shows that multiple serine/threonine phosphorylation sites were present in SAF-A. (e) AD-PP4 and AD-PP4RL transfection had strong effects on SAF-A phosphorylation. (f) Suppression of SAF-A using siRNA up-regulated PP4 expression. (g) Up-regulation of SAF-A decreased the expression of PP4. (h) Up-regulation of SAF-A partly reversed the accumulation of cells in G2/M induced by PP4 up-regulation, decreased the ratio of aberrant chromosome condensation (i), and partially decreased the expression of cyclin B (k) as well as the mitotic index and the ratio of prometaphase-arrested cells (j). (l) Inhibition of PP4 activity reversed the accumulation of cells in G2/M, decreased the ratio of aberrant chromosome condensation (m), and decreased the expression of cyclin B (o) as well as the mitotic index and the ratio of prometaphase-arrested cells (n). White arrows indicate misaligned chromosomes. Red arrows indicate normally aligned chromosomes. Scale bars: 10 μ m. The data represent the means $\pm$ SD ($n = 4$ independent experiments). * $P < 0.05$ versus control AD or as indicated by line. ** $P < 0.01$, *** $P < 0.001$ versus control.

which PP4 was depleted by Cre-loxP recombination in MEF cells showed that PP4 activity is required for multiple events in interphase, especially in the G2 phase.

Targeted disruption of PP4 leads to abnormal activation of CDK1 and increased T219-mediated phosphorylation of NDEL1 during interphase, which causes a delay in

Figure 5. (continued)

the G2 phase. MEF cells in which PP4 was depleted rarely display chromosome condensation and phosphorylation of histone H3, which indicates that the arresting phase occurs before entry into M phase (18). Depletion of PP4 by lentivirus-delivered stable gene silencing in HEK293 cells causes a delay in the cell cycle. Under conditions of 85% depletion of PP4, the cells progress slowly through G2 or are delayed in G2. These PP4-depleted cells exhibit nuclei with aberrant morphology, and the cells cannot normally proceed through mitosis. Moreover, an 85% depletion of PP4 is lethal, at least in part, as a consequence of apoptosis induction by caspase 3/7 activation. However, 70% depletion of PP4 induces a considerable decrease in cell number but no significant apoptosis or autophagy (19). Moreover, Zhuang *et al.* reported that knock-down of PP4 by siRNAs in HEK293 cells causes an increase in the S-phase cell population (2). The discrepancies among the various reports may be associated with the use of different cell types and inhibition techniques. Cristina Martin-Grados *et al.* provided evidence that the PP4-regulated processes might be differentially affected by different levels of PP4 depletion. Similar data were obtained in our study, in which inhibition of the expression or activity of PP4 by siRNA transfection or AD-PP4RL transfection, respectively, suppressed the proliferation of cells and arrested cells at G1 and S phase (Figs. 1 and 2). Overall, the data indicated that PP4 was required for interphase and participated in multiple events in interphase, on the basis that inhibition of PP4 further arrested cells in a phase without increasing the mitotic index.

Reversible phosphorylation of proteins plays crucial roles in proliferation, especially during mitosis. The phosphatase activities of multiple phosphatases including PP1, PP2A-B55 and PP2A-B56 are depressed at the time of mitotic commitment (22) and are important for reorganization of multiple cellular architectural structures. Recently, Martin Voss *et al.* reported that, in contrast to other phosphatases, PP4 is not continuously activated through the entire cell cycle. The activity of PP4 undergoes dynamic changes during the cell cycle, with a peak in the G1 phase and decreases during the S phase, with the lowest activity occurring during the G2/M phase (12). This indicates that PP4 is not continuously activated through the entire cell cycle, and up-regulation of PP4 may also effect the cell cycle process. Whether PP4 is needed at all times during the cell cycle is unclear. Here, we found that both up-regulation and inhibition of PP4 suppressed proliferation in HepG2 cells (Fig. 1). These results strongly suggested a dual role of PP4 in the cell cycle. Moreover, inactivation of PP4 was necessary for the prometaphase/metaphase transition

on the basis that forced up-regulation of PP4 induced a dramatic prometaphase arrest with defects in chromosome alignment and spindle assembly (Figs. 2 and 3). Similar phenotypes were obtained in HeLa cells and MCF-7 cells, which indicates that this effect of PP4 was not cell-type specific. Our data further confirmed Martin Voss's results that PP4 is not continuously activated through the entire cell cycle and that it is necessary for PP4 to be inactivated in G2/M phase, especially prometaphase. Moreover, we also found obvious hepatoma formation inhibition effect of PP4 up-regulation in nude mice (data not shown), indicating that PP4 overexpression could strongly suppressed cell proliferation *in vivo*.

Accurate capture of the kinetochores by microtubules and proper alignment of chromosomes at the spindle were essential for the prometaphase/metaphase transition. The mechanisms underlying the PP4 activation-induced prometaphase/metaphase arrest were unknown. Using a proteomic approach, we identified SAF-A as a novel protein that interacts with PP4 (Fig. 4a,b). Previous studies had shown that SAF-A was one of the proteins identified in the proteome analysis of human mitotic spindles (23,24). It participates in multiple processes in mitosis including kinetochore-microtubule attachment, chromosome condensation, and stabilization of the kinetochore fibres as well as cytokinesis. Depletion of SAF-A by RNAi induces mitotic delay, reduces the tension between the kinetochores of non-aligned chromosomes, and decreases the stability of kinetochore-microtubules. SAF-A also forms a complex with the peripheral chromosomal protein, nucleolin and two spindle regulators, Aurora-A and TPX2. The elimination of TPX2 or Aurora-A from the cells abolishes the association of SAF-A with the mitotic spindle. Moreover, SAF-A also contributes to the targeting of Aurora-A to mitotic spindle microtubules. In summary, SAF-A is a spindle regulator and contributes to the attachment of the spindle microtubules to the kinetochores and regulates the spindle organization (20). Similar data were obtained in our study (Fig. 4). We were interested to find that cells in which PP4 was up-regulated (Fig. 3) showed a similar cell cycle distribution and morphology to those in which SAF-A was inhibited (Fig. 4). We therefore explored the regulatory interactions between PP4 and SAF-A. First, no obvious changes in SAF-A expression were detected in cells in which PP4 was inhibited or up-regulated or in which PP4 activity was inhibited (Fig. 5a–c). Next, we determined whether PP4 regulated the phosphorylation of SAF-A. We showed that SAF-A contained multiple serine/threonine phosphorylation sites (Fig. 5d), which suggested that the different phosphorylation sites of SAF-A might be associated with the different functions

of SAF-A. Previous studies had indicated that SAF-A undergoes dynamic phosphorylation during mitosis, and phosphorylation of serines 2, 3, 4, 7, 14, 26, 59, 66, 585 and 674 (25–29) has been reported to be involved in mitosis. As indicated in Fig. 5e, SAF-A showed various mobility shifts as detected by acrylamide-pendant phosphatase. Moreover, dramatic PP4-regulated changes occurred in the phosphorylation status of SAF-A (Fig. 5e). Specifically, PP4 up-regulation induced high levels of dephosphorylated SAF-A, while PP4RL up-regulation led to hyperphosphorylation of SAF-A. However, we did not determine the exact sites of SAF-A that were dephosphorylated by PP4 because antibodies to detect the possible phosphorylation sites were not available. Moreover, it remains unclear how SAF-A maintains the appropriate level of phosphorylation to ensure that cellular mitosis proceeds correctly. The current data indicate that the phosphorylation of SAF-A is elaborately regulated. Several kinases and phosphatases including Aurora-A, polo-like kinase and PP6 have been reported to participate in the regulation of SAF-A regulation. The final phosphorylation status of SAF-A may be determined by multiple kinases and phosphatases, and the interactions among them need further study. Together, the interplay between multiple kinases and phosphatase maintains an appropriate level of SAF-A phosphorylation to ensure the proper cellular mitotic process.

Here, we found that modulating the expression of SAF-A exerted a strong impact on PP4 expression. Knocking down SAF-A using siRNA promoted PP4 expression, and up-regulation of SAF-A induced decreased PP4 expression (Fig. 5f,g). We wondered how SAF-A regulated the expression of PP4. As described previously, SAF-A also acts as a chromatin-associated RNA-binding protein and plays important roles in the regulation of gene expression including transcriptional regulation and RNA metabolism. SAF-A can interact with various transcriptional cofactors such as heterochromatin protein 1 α (30), DNA topoisomerase II β (31) and PCAF (32), and modulate their transcriptional activation. SAF-A also interacts with the SCF β -TrCP-ubiquitin ligase complex to regulate its E3 ligase activity (33,34). Thus, one of these potential mechanisms may account for SAF-A mediated-PP4 expression alteration. However, the precise mechanism for SAF-A to modulate PP4 expression needs further study.

We wondered whether the mutual regulatory mechanisms between PP4 and SAF-A played a role in the prometaphase arrest. First, we investigated whether alteration of SAF-A expression affected the phenotype induced by PP4 up-regulation. As shown in Fig. 5h–k,

up-regulation of SAF-A reversed the accumulation of cells in M phase especially in prometaphase as well as increasing the expression of cyclin B induced by PP4 up-regulation, suggesting that dephosphorylation of SAF-A at some sites could partially account for the phenotype induced by PP4 up-regulation. We also found that inhibition of PP4 also affected the phenotype induced by SAF-A inhibition (Fig. 5l–o). However, SAF-A up-regulation only partially reversed the phenotype induced by PP4 up-regulation, which may due to the poor efficiency of plasmid transfection compared to adenoviral transduction. Moreover, in the human genome, the number of phosphatases is much smaller than the number of kinases, and each protein phosphatase has a large number of substrates and function. SAF-A may not be the only target that is involved in this process. As shown in previous studies, various proteins that interact with PP4 have been identified. Targeted disruption of PP4 leads to abnormal activation of CDK1 in interphase and increased T219-mediated phosphorylation of NDEL1 in interphase, which causes a delay in the G2 phase. PP4 also interacts with γ -tubulin and its associated protein, γ -tubulin complex protein 2, and regulates the microtubule organization at the centrosomes during cell division in response to stress signals including spindle toxins such as paclitaxel and nocodazole (12). Recently, BAF, a highly conserved protein in eukaryotes that is involved in mitotic regulation had been shown to be dephosphorylated by PP4 (2). Although multiple possible target proteins are involved in mitosis, we believe that SAF-A is one of the important targets of PP4 and is involved in the PP4-induced prometaphase arrest.

In conclusion, we showed that PP4 plays dual roles during cell proliferation. We also identified SAF-A as one of the important targets of PP4 in prometaphase/metaphase transition and demonstrated that the interplay between PP4 and SAF-A plays a role in prometaphase/metaphase transition. These findings provide mechanistic insight into the critical role of PP4 in the regulation of the cell cycle.

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Conflict of interest

The authors declare that they have no competing financial interests.

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