Biochemical and Biophysical Research Communications 450 (2014) 1690–1695

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Overexpression of Mps1 in colon cancer cells attenuates the spindle assembly checkpoint and increases aneuploidy

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ARTICLE INFO

Article history:
Received 9 July 2014
Available online 22 July 2014

Keywords:
Mps1
Overexpression
Spindle assembly checkpoint
Tumorigenesis
Aneuploidy

ABSTRACT

The spindle assembly checkpoint kinase Mps1 is highly expressed in several types of cancers, but its cellular involvement in tumorigenesis is less defined. Herein, we confirm that Mps1 is overexpressed in colon cancer tissues. Further, we find that forced expression of Mps1 in the colon cancer cell line SW480 enables cells to become resistant to both Mps1 inhibition-induced checkpoint depletion and cell death. Overexpression of Mps1 also increases genome instability in tumor cells owing to a weakened spindle assembly checkpoint. Collectively, our findings suggest that high levels of Mps1 contribute to tumorigenesis by attenuating the spindle assembly checkpoint.

1. Introduction

Lack of fidelity in chromosome segregation during mitosis causes aneuploidy, a hallmark of cancers associated with high risk for tumorigenesis [1]. The spindle assembly checkpoint (SAC), also termed the mitotic checkpoint, prevents mis-segregation of chromosomes by retaining cells in metaphase until all the chromosomes are properly aligned. The SAC can be triggered by many factors such as DNA damage affecting centromere integrity, disturbed spindle assembly and unaligned chromosomes [2]. Chromosomes unaligned in the metaphase plate generate an "anaphase wait" signal by promoting the formation of a mitotic checkpoint complex (MCC), containing Mad2, BubR1, Bub3 and Cdc20 [2–4]. The MCC binds and inactivates the E3-ubiquitin ligase APC/C (Anaphase Promoting Complex/Cyclosome), protecting securin and cyclin B from destruction [3,4]. Degradation of these two proteins is required to activate the protease separase; activated separase cleaves the cohesion complex and allows sister chromatids to separate, satisfying the SAC. The SAC will remain until the signal for the checkpoint is extinguished. However, if the SAC components are dysregulated or the SAC persists for a long period, its maintenance will fail and induce aberrant exit of the cell from mitosis. SAC maintenance failure, also termed mitotic slippage, causes polyploidy and predisposes cells to be malignantly transformed [5]. Mitotic slippage occurs in the presence of an active SAC and requires the destruction of cyclin B [6]. The generation of the SAC signal is a complicated process, regulated by many other proteins besides the MCC component, including Mps1, Aurora B, PLK1, Bub1 and others [7]. The mechanism by which the SAC is maintained differs from that by which it is generated; the latter involves many other proteins such as Aurora A, histone deacetylase and BubR1 [5,8,9].

Mps1 kinase is an essential component of the SAC. It resides predominantly in the cytoplasm during interphase and relocates to the nucleus late in G2 phase and then associates with the kinetochore from prophase to metaphase [10–12]. The kinase activity of Mps1 is required for both SAC generation and maintenance [10]. In response to the SAC signal, Mps1 binds to the unoccupied kinetochore via association with Tpr (*T*ranslocated *p*romoter *r*egion), a human nuclear pore complex protein; depletion of Tpr affects both the generation and maintenance of the SAC [13]. Once associated with kinetochore, Mps1 further recruits Mad2 and directs formation of the MCC by transforming Mad2 from open-form to closed-form [14]. Mps1 phosphorylates BubR1 and contributes to MCC formation [15]. Kinetochore-associated Mps1 contributes to the hyperphosphorylation of BubR1, which can be

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http://dx.doi.org/10.1016/j.bbrc.2014.07.071
0006-291X/

jointly regulated by Aurora B and Polo kinase [16]. Besides SAC regulation, Mps1 is also involved in centrosome duplication, chromosome alignment, cytokinesis, DNA damage response and organ development [17].

High levels of Mps1 kinase are found in several tumor cell lines including U937, HeLa, HEY, OCC1, Bewo, T987, and a set of breast cancer lines. Genetic and pharmaceutical blockades of Mps1 kinase induce tumor cell death while leaving the transformed normal cell unaffected [18–20], making it a potential target for tumor therapy. However, it is not clear whether high levels of Mps1 contribute to tumorigenesis because tumors are heterogeneous. In this paper, we developed a model in which FTHMps1 is expressed ectopically in SW480 colon cancer cells. In contrast to the previous finding that overexpression of SAC components hyperactivates the checkpoint, we found for the first time that overexpressed Mps1 did not promote but actually attenuated the SAC. As a result, the SW480 with FTHMps1 had a higher rate of misalignment and chromosomal aneuploidy. These findings indicate that high levels of Mps1 can promote tumorigenesis by down-regulating the SAC.

2. Materials and methods

2.1. Generation of stable cell lines

Flag-tag fused Mps1 was inserted into the retroviral expression vector pRex as described previously [21]. The fusion gene was confirmed by DNA sequencing. The procedure for generating SW480 with stable FTHMps1 expression was as described [21].

2.2. Cell synchronization

Cells were synchronized at G1/S phase by double thymidine treatment as described by Zhang et al. [21]. To obtain mitotic cells, cells arrested at G1/S were released into medium containing 100 ng/ml nocodazole for 12 h and collected by shaking off.

2.3. Western blotting

Cells were washed twice with cold D-PBS and lyzed in buffer (20 mM Tris–Cl (pH 8.0), 0.2 M NaCl, 0.5% NP40, 1 mM EDTA, 1 mM PMSF, 20 mM NaF, 0.1 mM Na_3VO_4, 1X Protease inhibitors (Roche)) for 5 min prior to scraping. Cell extracts were clarified by spinning at 13,000 rpm for 10 min. The protein concentrations were determined by a BSA assay. Equal amounts of total proteins were resolved on 8% or 10% SDS–PAGE gels and transferred to nitrocellulose membranes. Primary and secondary antibodies were applied sequentially. The blots were developed in Super Signal WestDura (Pierce) according to the manufacturer's instructions.

2.4. Immunofluorescence

Cells for immunofluorescence were grown on cover glasses. Prior to staining, they were treated with reagents at the indicated durations and doses. They were washed three times with D-PBS and fixed for 10 min in D-PBS plus 1% paraformaldehyde, then stained with primary antibody for 1.5 h at room temperature, followed by Alexa Fluor 488-conjugated goat anti-mouse secondary antibodies (Invitrogen, Eugene, OR). Before the mounting solution was applied, 50 μg/ml propidium plus RNAase A was used to stain the DNA. After staining, the cover glasses were mounted on precleaned microscope slides with D-PBS containing 50% glycerol and sealed with nail oil. Images were acquired on a Zeiss LSM 510 equipped with a 100× objective.

2.5. Immunohistochemistry

Paraffin-embedded colon cancer tissues spotted on a chip (T054a, Alenabio Inc, Xi'an) were immunohistochemically stained with a mouse monoclonal antibody, Anti-Mps1 N1 (Abcam). Tris–ethylenediaminetetraacetic acid buffer (pH 9.0) was used for heat-induced epitope retrieval. The slide was incubated with an antibody diluted 1:100 for 2 h and staining was detected with a DAB IHC Detection System (Invitrogen). Images were acquired with a Leica SCN400 slide scanner and then arranged using Photoshop software.

2.6. Flow cytometry

Reversine-treated SW480 cells were trypsinized, washed three times with D-PBS, fixed with 70% ethanol, and stored at −20 °C. The fixed cells were resuspended in D-PBS containing 10 mg/ml RNase A and 20 mg/ml PI for 30 min at 37 °C. Cellular DNA content was determined by flow cytometry using a FACSCalibur and CellQuest software (Becton Dickinson, Tokyo, Japan). Twenty thousand cells were counted in each sample.

2.7. Chromosome spread

Cells were treated with 100 ng/ml nocodazole for 12 h and mitotic cells were collected by the shaking-off method. The mitotic cells (2×10^5/ml) were incubated in a hypotonic buffer (50 mM Tris (pH 7.4) and 55 mM KCl), fixed with freshly-prepared Carnoy's solution (75% methanol and 25% acetic acid), dropped on to glass slides, and dried at 80 °C. The slides were mounted in D-PBS buffer plus 1.5 μg/ml DAPI and 50% glycerol, sealed with nail oil and processed for fluorescence microscopy. To prepare a representative figure for a chromosome spread, the pictures were inverted and contrast-adjusted using Adobe Photoshop CS2 software.

3. Results

3.1. Mps1 is overexpressed in colon cancer tissues

High levels of Mps1 mRNA are observed in several tumor cell lines. Consistent with these findings, Mps1 mRNA is elevated in the majority of cancer tissues, including breast, colon, esophageal, liver, and lung cancers, as demonstrated by analyzing the annotated microarray data in the Oncomine website (Fig. 1A, Fig. S1A, [22,23]). Specifically, we determined Mps1 mRNA levels in colon cancers by analyzing the microarray database in another publicly-accessible online tool, canEvolve (http://www.canevolve.org/). Consistent with the results from Oncomine, there is a significantly higher level of Mps1 in adenocarcinoma tissues than in normal colonic tissues (Fig. 1B). To determine whether the mRNA level correlates with the protein level, we measured the Mps1 protein levels in seven cultured cancer cell lines. As demonstrated, the Mps1 protein levels in these cancer cell lines, including colon cancer cell lines, are significantly higher than in the immortal breast epidermal cell MCF-10A (Fig. 1C). Finally, we analyzed 48 cases from 12 subjects with colon cancer. Consistent with the Mps1 level in cancer cell lines, all adenocarcinoma cases exhibited stronger Mps1 staining patterns than normal colon tissues (Fig. 1D). Notably, the high levels of Mps1 appear to be limited to certain types of colon cancer tissues, such as adenocarcinoma and papillary adenocarcinoma, but they are not high in all mucinous adenocarcinomas, suggesting a special requirement for the formation of certain types of colon cancer (Fig. S1B).

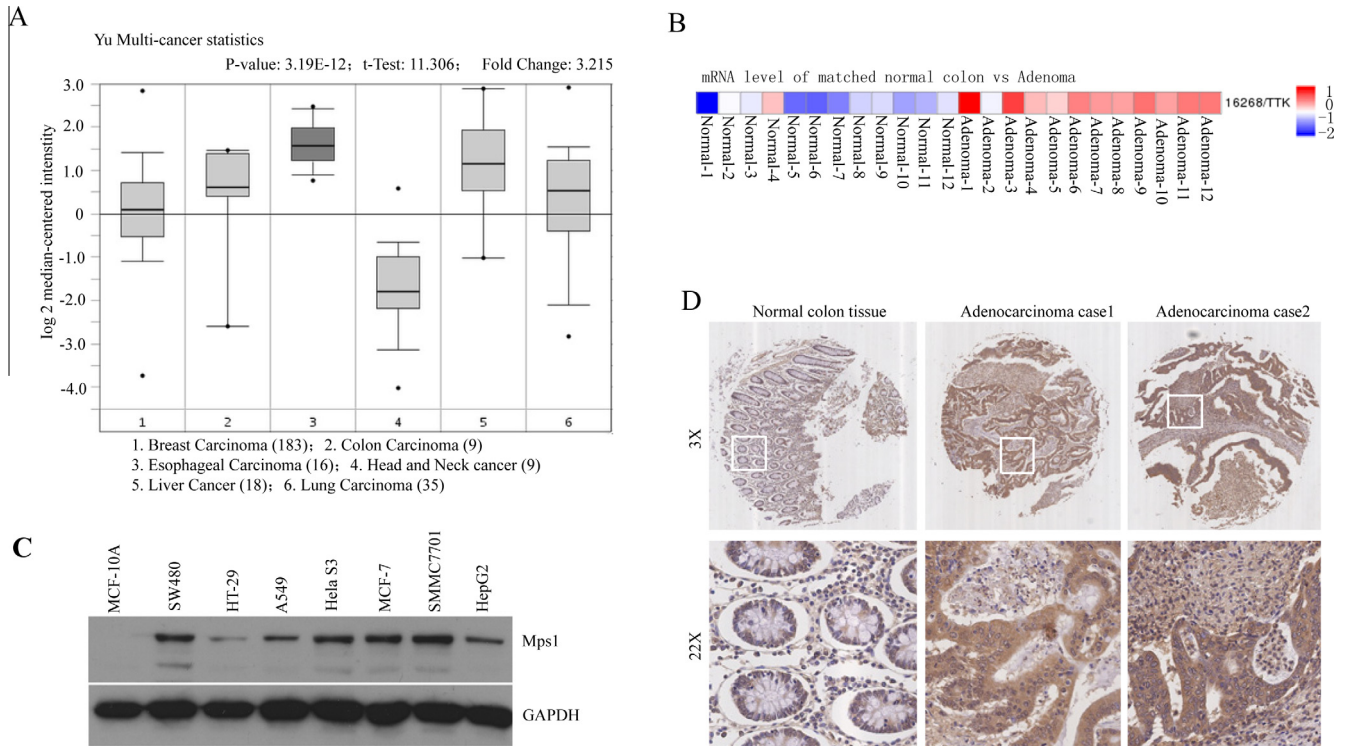

Fig. 1. High levels of Mps1 in a variety of tumor types. (A) Mps1 mRNA levels in breast, colon, esophageal, head and neck, liver, and lung cancers. Data were from the annotated microarray data set and the figure was generated using Oncomine version 4.0 (https://www.oncomine.com/resource/login.html). (B) mRNA level of matched normal colon tissue and adenocarcinoma. Differentiation expression data for the twelve subjects were retrieved from the canEvolve website and presented as a heatmap form. (C) Western blotting of Mps1 protein in the validated tumor cell lines and the immortal breast epidermal cell MCF-10A. GAPDH was set as a loading control. (D) Immunohistochemical staining of Mps1 in normal colonic tissue and colon cancer tissues. Each sample is presented at both 3× and 22× magnification.

3.2. Ectopically expressed Mps1 incorporated functionally into the cell cycle

To examine the effect of overexpression of Mps1 on colon cancers, we generated a stable SW480 cell line constitutively expressing a flag–tagged Mps1 (FTHMps1). The level of FTHMps1 in the SW480-FTHMps1 cells was comparable to the endogenous Mps1 during both interphase and mitosis (Fig. 2A). The kinase activity of Mps1 is required for the spindle assemble checkpoint, and impairment of this checkpoint via Mps1 inhibition causes cell death [18,19,24]. To test whether FTHMps1 acts similarly to endogenous Mps1, we examined the mitotic checkpoint and cell viability after Mps1 inhibition. For mitotic checkpoint depletion, SW480 and SW480-FTHMps1 cells were arrested at prometaphase using a procedure combining single thymidine and nocodazole treatments. Mitotic cells were then collected by shaking off and reseeded on a 12-well plate with fresh medium containing 100 ng/ml nocodazole plus 50 or 100 nM reversine, a specific Mps1 inhibitor [25]. After 2 h, the cells were collected and the mitotic indexes were calculated. As demonstrated, the mitotic indexes of the SW480-FTHMps1 cells upon reversine challenge were significantly higher than those of the parental SW480 (Fig. 2B). For cell viability, SW480 and SW480-FTHMps1 cells were treated with reversine for 3 or 7 days, then followed by flow cytometry or crystal violet staining. Consistent with the role of FTHMps1 in SAC depletion, SW480-FTHMps1 was more highly resistant to Mps1 inhibition than the parental SW480 (Fig. 2C and D). Collectively, these results revealed that FTHMps1 acts as efficiently as the endogenous Mps1, making the SW480-FTHMps1 an ideal model for elucidating the effects of high levels of Mps1.

3.3. High levels of Mps1 increase aneuploidy

Genetic evidence has demonstrated that overexpression of the mitotic checkpoint proteins Mad2, Hec1 and Bub3 in mice causes genome instability, increasing the risk for tumorigenesis [26]. Given that Mps1 works as a downstream regulator of Hec1 and the upstream regulator of Mad2 during kinetochore assembly, we speculate that modulating the Mps1 protein level or kinase activity affects genome stability. Consistently with the previous studies, Mps1 inhibition by reversine indeed caused massive polyploidy in SW480 cells, and the change of DNA content was easily identified by flow cytometry or microscopy (Fig. 3A and B). Similarly, overexpression of Mps1 affected genome stability, as there was a significantly higher percentage of cells with chromosome misalignment in SW480-FTHMps1 than the parental SW480 cells (Fig. 3C). Further, the chromosome spread assay demonstrated that the percentage of SW480-FTHMps1 with 50–60 chromosomes was significantly lower than in the parental SW480 ($p < 0.01$); as a result, the number of cells with fewer than 50 or more than 60 chromosomes was proportionally greater in SW480-FTHMps1 (Fig. 3D), suggesting that overexpression of FTHMps1 allows cells with higher aneuploidy to survive. These observations suggest that the overexpressed fraction of Mps1 promotes tumorigenesis by increasing genome instability.

3.4. High levels of Mps1 attenuate the spindle assembly checkpoint

Mps1 is an essential component of the SAC, required for both checkpoint activation and maintenance [10,17,27]. We speculate that increasing Mps1 promotes the threshold of mitotic checkpoint generation or maintenance. To this end, we determined the mitotic

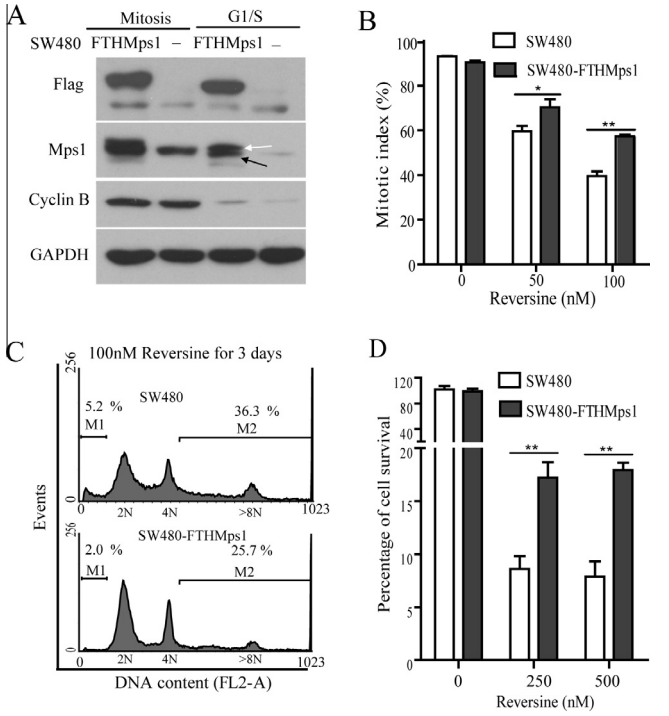

Fig. 2. FTHMps1 incorporated functionally into the cell cycle. (A) Western blotting of Mps1 protein in the SW480 and SW480-FTHMps1 arrested at G1/S and mitosis. Cyclin B is a cell cycle marker. GAPDH was used as a loading control. The white arrow refers to FTHMps1; the black arrow labels the endogenous Mps1. (B) Mitotic index of SW480 and SW480-FTHMps1 cells. The spindle assembly checkpoint was turned on by treating cells with 100 ng/ml nocodazole and then depleted by reversine at a concentration of 50 or 100 nM. At least 300 cells were counted in each independent experiment. (C) Flow cytometry assay for dead cells upon Mps1 inhibition. SW480 and SW480-FTHMps1 were treated with reversine at the indicated concentration for 3 days. (D) Crystal violet assay for living cells upon Mps1 inhibition. SW480 and SW480-FTHMps1 were treated with reversine at the indicated concentration for 7 days. The living cells were fixed and stained with crystal violet. The crystal violet in the fixed cells was then dissolved in methanol and the signals were read using an ELISA reading machine. All error bars this figure represent one standard deviation, $n = 2$ or 3, $^{*}p < 0.05$, $^{**}p < 0.01$.

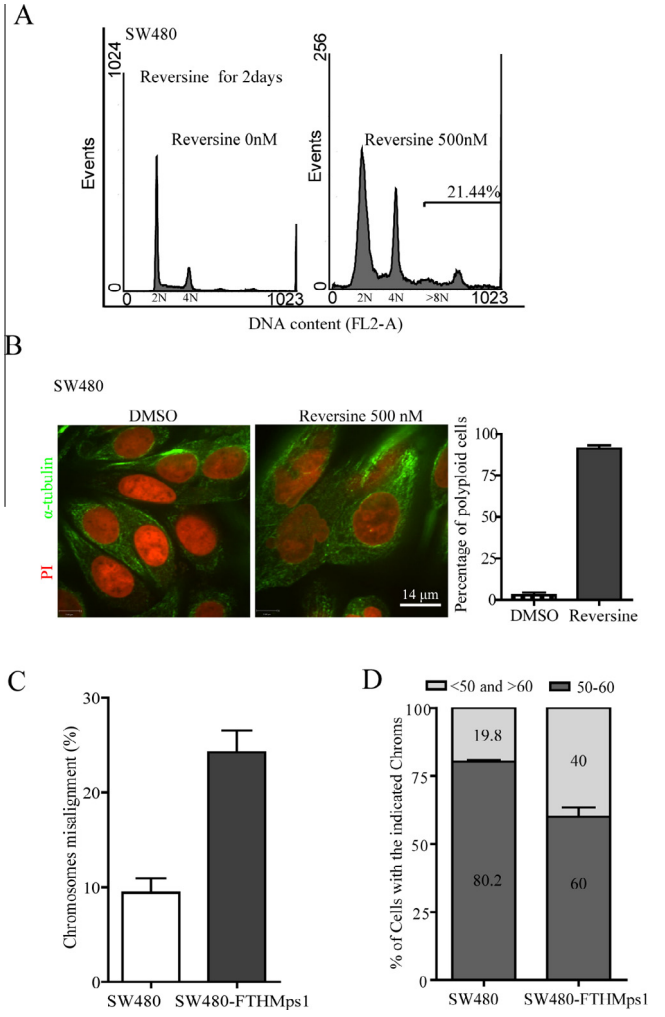

Fig. 3. Overexpression of Mps1 promotes genome aneuploidy. (A) Flow cytometry of polyploid cells upon Mps1 inhibition. SW480 cells were treated with reversine at a concentration of 500 nM. (B) Mps1 inhibition by reversine caused polyploidy. The left panel is a representative picture of the polyploid cells. The right panel is the statistical result of the ratio of polyploidy in SW480 cells induced by reversine. At least 300 cells were counted in each independent experiment. (C) Ectopic expression of Mps1 increased misalignment of chromosomes. Nocodazole-treated SW480 and SW480-FTHMps1 were released into fresh DMEM with the proteasome inhibitor MG132 (10 μM) for 3 h. The ratio of cells with misaligned chromosomes was then counted after immunofluorescence staining. At least 230 cells were counted in each independent experiment. (D) Karyotype analysis of aneuploid cells in SW480 and SW480-FTHMps1. Mitotic cells from SW480 and the derived SW480-FTHMps1 were collected and subjected to chromosome spread analysis. According to the distribution of chromosome numbers, those cells were divided into two subpopulations: cells with fewer than 50 and more than 60 chromosomes, cells with between 50 and 60 chromosomes. At least 120 cells were counted in each independent experiment. All error bars in Fig. 3 represent one standard deviation, $n = 2$ or 3.

indexes of SW480 and SW480-FTHMps1 after the cells were challenged with the microtubule toxin nocodazole. For mitotic checkpoint generation, the cells were arrested at G1/S by a single thymidine treatment for 24 h and then released into fresh medium plus nocodazole for 12 h at the concentration indicated. In contrast to our prediction, overexpression of FTHMps1 did not promote the threshold for checkpoint generation but slightly decreased the mitotic indexes in cells treated with either 50 or 100 ng/ml nocodazole (Fig. 4A). This phenomenon was observed in SW480 cells ectopically expressing a yellow fluorescent protein (YFP) fused-Mps1 (Fig. S2A). For mitotic checkpoint maintenance, SW480 and SW480-FTHMps1 cells were arrested at G1/S by a single thymidine procedure and then released into nocodazole for 12 h. The mitotic cells were collected by shaking off and then reseeded for the durations indicated. The mitotic indexes were calculated as before. As demonstrated, the mitotic indexes for SW480-FTHMps1 cells were significantly lower than for the parental SW480 at 6 h (Fig. 4B), indicating that overexpressed Mps1 attenuates the mitotic checkpoint in cells. Cyclin B is required for mitotic maintenance and its premature degradation causes mitotic slippage [6]. To confirm the negative effect of ectopically expressed FTHMps1 on mitotic maintenance, we also traced the dynamics of the cyclin B level. Consistently, we found that cyclin B was degraded earlier in SW480-FTHMps1 than in the parental SW480 (Fig. 4C and D). As a control, overexpression of GFP-PLK1 yielded cells with relatively robust mitotic checkpoint maintenance (Fig. S2B). Notably, the premature degradation of cyclin B caused by forced expression of FTHMps1 can be attenuated by decreasing the endogenous Mps1 level via a Mps1-specific siRNA (Fig. 4E and F). Taking these findings together, we speculate that high levels of Mps1 are beneficial for tumorigenesis.

4. Discussion

High levels of Mps1 correlate with tumor grades and enable cells with higher aneuploidy to survive. However, the underlying mechanism remains unclear. In this paper, we investigated the

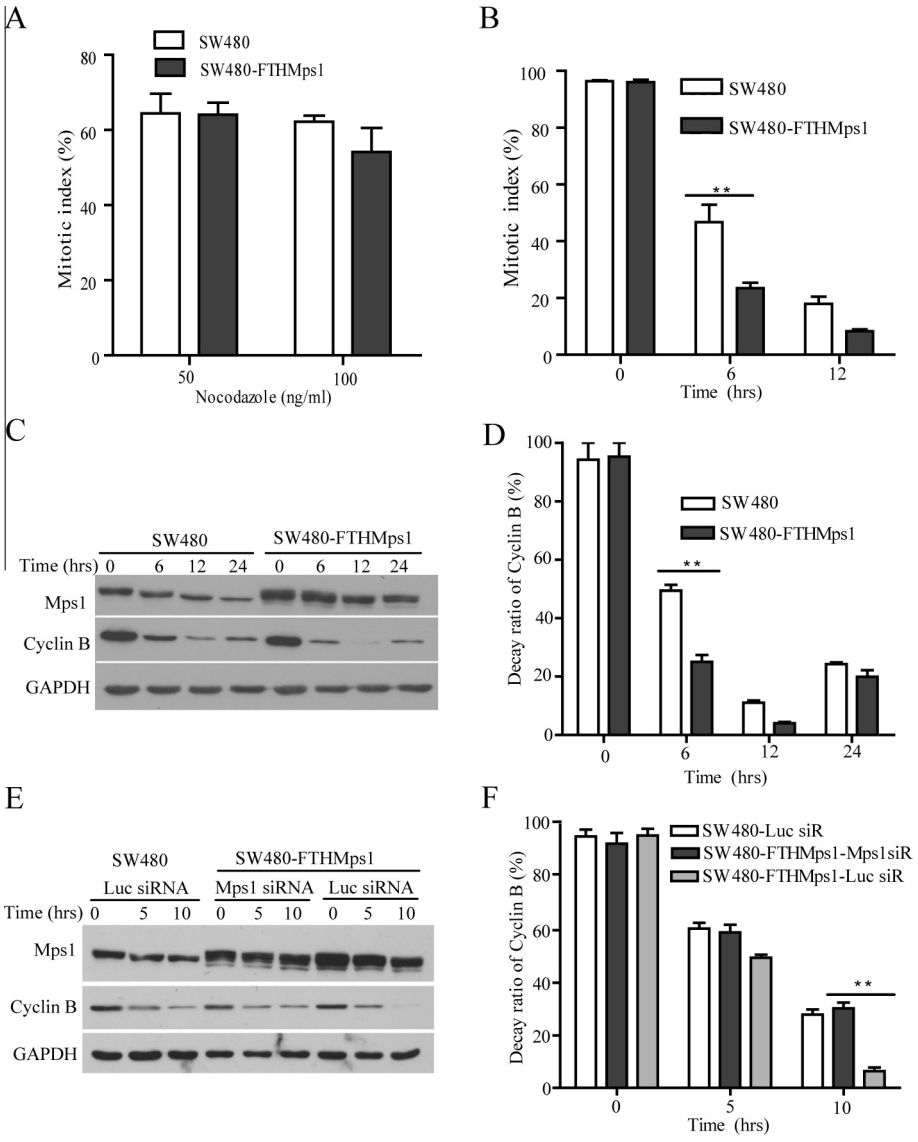

Fig. 4. Overexpression of Mps1 attenuates the spindle assembly checkpoint. (A) Spindle assembly checkpoint generation in SW480 and SW480-FTHMps1 cells. The spindle assembly checkpoint was generated by nocodazole at 50 or 100 ng/ml. At least 300 cells were counted in each independent experiment. (B) Maintenance of SAC in SW480 and SW480-FTHMps1 cells. Arrest of cells in mitosis by 100 ng/ml nocodazole were collected by shaking off and then resuspended in medium with 100 ng/ml nocodazole for a further 6 or 12 h, followed by counting the mitotic indexes. At least 300 cells were counted in each independent experiment. (C and D) Decay of Cyclin B in SW480 and SW480-FTHMps1 cells during mitotic maintenance. Cells were treated as in (B) and lyzed for Western blotting. GAPDH was a loading control. (E and F) Mps1-specific siRNA transfection restored the premature degradation of Cyclin B. Western blotting was conducted to analyze the fluctuation of the Cyclin B level. GAPDH was a loading control. All error bars in this figure represent one standard deviation, $n = 2$ or 3, $^{**}p < 0.01$.

effect of high levels of Mps1 on tumorigenesis by overexpressing FTHMps1 in the colon cancer cell line SW480. FTHMps1 functions physiologically as it predisposes cells to be resistant to mitotic checkpoint depletion and cell death upon Mps1 inhibition. Further, overexpression of Mps1 allows tumor cells with a higher degree of aneuploidy to survive. But in contrast to the reports that overexpressed checkpoint proteins activate SAC, we found that forced expression of FTHMps1 decreases the robustness of the spindle assembly checkpoint, as confirmed by the lower mitotic index and premature degradation of cyclin B during mitotic checkpoint maintenance. Our results primarily suggest that an appropriate level of Mps1 is important for cells to maintain genome stability.

Mps1 is required for both SAC generation and maintenance. In this paper, we found ectopically expressed FTHMps1 to have a minor effect on mitotic checkpoint generation but a significant one on checkpoint maintenance, suggesting that Mps1 regulates these two processes by cooperating with different partners. Consistent with this finding, our previous study demonstrated that mutation of the LXXLL motif in the Mps1 N-terminus attenuates the mitotic checkpoint significantly while affecting checkpoint generation less [11]. The differences between checkpoint generation and maintenance are also apparent in other mitotic checkpoint regulators. The posttranslational modifications of BubR1 affect SAC generation and maintenance differentially. Phosphorylation of BubR1 is required for both SAC generation and maintenance, which can be interdependently regulated by Aurora B, Mps1 and Polo kinase [16]. In contrast, acetylation of BubR1 is required for an intact SAC signal but is dispensable for checkpoint maintenance [28]. A defect in checkpoint maintenance is detrimental to genome stability as mice with loss of BubR1 acetylation spontaneously develop tumors with massive chromosome mis-segregations [28].

Authors' contributions

Y.L. and X.Z. performed the experiments. Q.X., Y.L., X.Z. analyzed the data. Q.X. designed the project and wrote the paper. C.C., H.Z. and R.Z.M. provided the core facilities. P.L., Y.B., B.Z., Z.Z., T.S., C.W., K.G., X.L., Y.Z. shared partial reagents.

Conflict of interest

The authors declared that the financial interests with respect to this manuscript belong to Beijing Institute of Biotechnology.

Acknowledgment

This work was supported by Grants from the National Natural Science Foundation of China (NSFC) to Q.X. (30971444 and 31371371).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.07.071.

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