REPORT

Depletion of JMJD5 sensitizes tumor cells to microtubule-destabilizing agents by altering microtubule stability

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ABSTRACT

Microtubules play essential roles in mitosis, cell migration, and intracellular trafficking. Drugs that target microtubules have demonstrated great clinical success in cancer treatment due to their capacity to impair microtubule dynamics in both mitotic and interphase stages. In a previous report, we demonstrated that JMJD5 associated with mitotic spindle and was required for proper mitosis. However, it remains elusive whether JMJD5 could regulate the stability of cytoskeletal microtubules and whether it affects the efficacy of microtubule-targeting agents. In this study, we find that JMJD5 localizes not only to the nucleus, a fraction of it also localizes to the cytoplasm. JMJD5 depletion decreases the acetylation and detyrosination of α -tubulin, both of which are markers of microtubule stability. In addition, microtubules in JMJD5-depleted cells are more sensitive to nocodazole-induced depolymerization, whereas JMJD5 overexpression increases α -tubulin detyrosination and enhances the resistance of microtubules to nocodazole. Mechanistic studies revealed that JMJD5 regulates MAP1B protein levels and that MAP1B overexpression rescued the microtubule destabilization induced by JMJD5 depletion. Furthermore, JMJD5 depletion significantly promoted apoptosis in cancer cells treated with the microtubule-targeting anti-cancer drugs vinblastine or colchicine. Together, these findings suggest that JMJD5 is required to regulate the stability of cytoskeletal microtubules and that JMJD5 depletion increases the susceptibility of cancer cells to microtubule-destabilizing agents.

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Introduction

Microtubules, which are core components of the cytoskeleton, are composed of heterodimers of α - and β -tubulin subunits.¹ The dynamics and stability of microtubules play pivotal roles in a variety of cellular activities, including cell migration, cell division, and intracellular trafficking.² The stability of microtubules is reported to be tightly regulated by a variety of microtubule-associated proteins (MAPs).^{3,4} The α - and β -tubulin subunits undergo various post-translational modifications,^{5,6} and post-translational acetylation and detyrosination are commonly used as markers of microtubule stabilization.^{7–10}

Increased levels of microtubule acetylation and detyrosination have been observed in multiple types of cancer cells,^{11–15} and both microtubule-stabilizing and microtubule-destabilizing agents have been widely used in cancer treatment.^{16–18} However, the clinical applications of these agents have shown the emergence of drug-resistant tumor cells, due to the overexpression of different beta-tubulin isoforms,^{19,20} or tubulin mutations.²¹

JMJD5 is a member of the JmjC domain-containing protein family, which has been shown to obtain H3K36me2 histone demethylase and hydroxylase activities.^{22,23} JMJD5 was reported to function in multiple biological processes, including embryonic

development, stem cell differentiation, osteoclastogenesis, circadian rhythm regulation, hepatitis B virus (HBV) replication, cell metabolism and cancer progression.^{23–32} In addition, we previously reported that JMJD5 associated with the mitotic spindle and regulated mitotic spindle stability during mitosis.³³ However, it remains unclear about the functional role of JMJD5 in regulating cytoskeletal microtubule stability and its molecular mechanism.

In this study, we reveal that JMJD5 localizes not only to the nucleus but also to the cytoplasm. JMJD5 significantly affects the acetylation and detyrosination of α -tubulin. In addition, JMJD5 modulates microtubule stability by regulating MAP1B protein levels. Furthermore, we provide evidence that JMJD5 depletion markedly increases the sensitivity of cancer cells to microtubule-destabilizing agents.

Results

A fraction of JMJD5 localizes in the cytoplasm

First, we investigated the subcellular localization of JMJD5. Cells were transfected with Flag-JMJD5 and analyzed using immunofluorescence staining. As shown in Fig. 1A, although

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Figure 1. A fraction of JMJD5 localizes to the cytoplasm. (A) The cellular distribution of Flag-tagged JMJD5 in HeLa cells. HeLa cells transfected with Flag-JMJD5 were stained with anti-Flag (green), anti- α -tubulin (red) and DAPI (blue). Scale bars, 5 μ m. (B) Western blot analysis of the cytosolic and nuclear fractions of Flag-tagged JMJD5. Histone H1 and α -tubulin were used as the nuclear and cytosolic loading controls, respectively. (C) Immunofluorescence staining of HeLa cells using anti-JMJD5 (red), anti- α -tubulin (green) and DAPI (blue). Scale bars, 5 μ m. (D) Western blot analysis of the cytosolic and nuclear fractions of endogenous JMJD5.

Flag-JMJD5 primarily localized to the nucleus, some of the Flag staining was also detected in the cytoplasm. Next, we isolated the cytoplasmic and nuclear fractions of protein extracts and examined the distribution of Flag-JMJD5 using western blot. A subset of total cellular Flag-JMJD5 was observed in the cytoplasmic fraction (Fig. 1B). To verify the result, the subcellular localization of endogenous JMJD5 was investigated using immunofluorescence staining and western blot. As shown in Fig. 1C and D, a small fraction of total endogenous JMJD5 localized to the cytoplasm. The cytoplasmic localization of JMJD5 suggests that it plays a role in the cytoplasm.

JMJD5 depletion significantly reduces α -tubulin acetylation and detyrosination and destabilizes cytoskeletal microtubules in HeLa cells

In a previous study, we demonstrated that JMJD5 regulates the stability of the mitotic spindle.³³ To determine if JMJD5 regulates the stability of cytoplasmic microtubules, we evaluated cells transfected with 2 distinct siRNAs targeting JMJD5. As shown in Fig. 2A and B, siRNA-mediated knockdown of JMJD5 expression significantly reduced tubulin acetylation. Additionally, tubulin detyrosination, another marker of microtubule stability, also decreased in JMJD5-depleted cells (Fig. 2A and C). However, JMJD5 depletion or overexpression has no effects on the protein level of α -tubulin (Fig. S1). Further, cells were synchronized into different stages by double thymidine block, we found that JMJD5 depletion decreases the acetylation and detyrosination of α -tubulin in both interphase and mitotic cells (Fig. S2). To further examine the effect of JMJD5 on tubulin modifications in interphase cells, we evaluated JMJD5-depleted cells using immunofluorescence staining. The results showed that siRNAs targeting JMJD5 can

efficiently knockdown the levels of JMJD5 in both cytoplasm and nuclear (Fig. S3), and JMJD5 depletion markedly decreased the levels of acetylated and detyrosinated cytoskeletal microtubules (Fig. 2D to G).

Both acetylation and detyrosination of tubulin are markers of microtubule stability.⁷⁻¹⁰ To gain further insight into the role of JMJD5 in microtubule stability, cells were transfected with siRNAs against JMJD5 and then incubated with nocodazole for 0, 20, 40 or 60 min to depolymerize microtubules. Cell extracts that contained polymeric or soluble dimeric tubulin were prepared as described in Materials and Methods and analyzed using western blot. As shown in Fig. 3A and B, nocodazole induced microtubule depolymerization in a time-dependent manner, and the levels of polymerized tubulin decreased in JMJD5-depleted cells compared with control cells. To verify this result, we evaluated nocodazole-treated cells using immunofluorescence staining (Fig. 3C). Consistent with the results observed in western blot assays, fewer polymerized microtubules were observed after nocodazole treatment in JMJD5-depleted cells compared with control cells (Fig. 3D). These results suggest that JMJD5 depletion reduces the stability of microtubules.

JMJD5 overexpression promotes tubulin detyrosination and microtubule stability

To further investigate the role of JMJD5 in cytoplasmic microtubule stability, we examined cells transfected with Flag or Flag-JMJD5. JMJD5 overexpression had little effect on tubulin acetylation levels (Fig. 4A and B). However, tubulin detyrosination levels increased in JMJD5-overexpressing cells (Fig. 4A and C). Consistent with this finding, immunofluorescence staining also showed that JMJD5-

Figure 2. JMJD5 depletion reduces tubulin acetylation and detyrosination. (A–C) HeLa cells were transfected with the control or JMJD5 siRNAs. JMJD5, acetylated α -tubulin, detyrosinated α -tubulin and α -tubulin levels were examined using western blot analysis with the indicated antibodies (A). The relative ratios of acetylated α -tubulin (acetyl-tubulin) to α -tubulin (B) and detyrosinated α -tubulin (detyr-tubulin) to α -tubulin (C) were measured by densitometric analysis of the western blot data. Values are presented as the mean $\pm$ s.e.m. of 3 independent experiments. (D–G) HeLa cells were transfected with the indicated siRNAs and then stained with anti-acetylated-tubulin (D) or anti-detyrosinated-tubulin (F) (red) and anti- α -tubulin (green). DNA was stained with DAPI (blue). Scale bars, 10 μ m. The fluorescence intensities of acetyl-tubulin, detyr-tubulin and α -tubulin were quantified using NIS-Elements software. The relative ratios of acetyl-tubulin to α -tubulin (E) and detyr-tubulin to α -tubulin (G) were measured. The error bars indicate $\pm$ s.e.m., * p < 0.05 (Student's t -test).

overexpressing cells contained more detyrosinated microtubules compared with control cells (Fig. 4D). Together, these results indicate that JMJD5 overexpression promotes α -tubulin detyrosination.

Next, we used western blot analysis to examine the distribution of tubulin in the polymeric and the soluble dimeric fractions in cells transfected with Flag or Flag-JMJD5 and incubated with nocodazole for 0, 20, 40 or 60 min. Compared

Figure 3. JMJD5 depletion destabilizes microtubules in HeLa cells. (A and B) HeLa cells transfected with the indicated siRNAs were treated with nocodazole for 0, 20, 40 or 60 min. The polymeric and soluble dimeric fractions of tubulin were isolated and examined using western blot (A). The ratio of polymeric tubulin to total tubulin was analyzed (B). Error bars indicate $\pm$ s.e.m. * $p < 0.05$; n.s., not significant (Student's *t*-test). (C and D) The cells transfected with the indicated siRNAs were treated with nocodazole for 20 or 40 min. After pre-treatment with PHEM buffer, the cells were fixed with cold methanol and stained with anti- α -tubulin (red) and DAPI (blue) (C). The fluorescence intensity of the remaining microtubules was measured (D). The values in the graph represent the mean $\pm$ s.e.m. of data from 3 independent experiments, * $p < 0.05$; n.s., not significant (Student's *t*-test).

with the control cells, the polymeric fraction of tubulin was greater in JMJD5-overexpressing cells (Fig. 4E and F), indicating that JMJD5 promoted microtubule resistance to nocodazole. These results suggest that JMJD5 overexpression promotes microtubule stability.

JMJD5 modulates the stability of microtubules by regulating MAP1B protein levels

Microtubule dynamics and stability are highly regulated by microtubule-associated proteins (MAPs).³ To gain further

insight into the functional role of JMJD5 in microtubule stability, we analyzed the effects of JMJD5 on the expression of MAPs. As shown in Fig. 5A, JMJD5 depletion reduced MAP1B protein levels, whereas little effect on MAP1S protein levels was observed. Consistent with this finding, JMJD5 overexpression increased MAP1B levels but exerted little effect on MAP1S expression (Fig. 5B).

JMJD5 has been reported to suppress gene transcription through its histone H3K36me2 demethylase activity,^{22,25} or positively promote gene transcription through binding to gene promoters.³² To investigate if JMJD5 regulates the transcription

Figure 4. JMJD5 overexpression promotes tubulin detyrosination and microtubule stability. (A–C) HeLa cells were transfected with Flag or Flag-JMJD5 plasmids. Flag-JMJD5, acetylated α -tubulin, detyrosinated α -tubulin and α -tubulin levels were then examined using western blot with the indicated antibodies (A). The relative ratios of acetyl-tubulin to α -tubulin (B) and detyr-tubulin to α -tubulin (C) were measured by densitometric analysis of the western blot data. The values represent the mean $\pm$ s.e. m. of 3 independent experiments, $*p < 0.05$ (Student's *t*-test). (D) HeLa cells were transfected with Flag-JMJD5 and then stained with anti-Flag (green), anti-detyrosinated-tubulin (red) and DAPI (blue). Scale bars, 10 μ m. (E and F) HeLa cells transfected with the Flag or Flag-JMJD5 plasmids were treated with nocodazole for 0, 20, 40 or 60 min. The polymeric and soluble dimer fractions of tubulin were isolated and examined using western blot (E). The ratio of polymeric tubulin to total tubulin was analyzed (F). Error bars indicate $\pm$ s.e.m. $*p < 0.05$; n.s., not significant (Student's *t*-test).

of MAP1B, we evaluated MAP1B expression in JMJD5-depleted cells, JMJD5-overexpressing cells, and control cells using RT-real time PCR. As shown in Fig. 5C and D, no significant differences in MAP1B transcription levels were observed in JMJD5 depleted cells or JMJD5-overexpressing cells, suggesting that JMJD5 does not regulate MAP1B at the transcriptional level. To investigate whether JMJD5 regulates MAP1B at the protein level by regulating its degradation, we treated

siRNA-transfected cells with MG132 to block the proteasome-dependent degradation of MAP1B. We found that MG132 treatment reduced the difference of MAP1B expression between JMJD5-depleted and control cells (Fig. 5E). Further, cells were treated with cycloheximide (CHX) to block the newly synthesized proteins. Time-course experiments showed that the protein level of MAP1B gradually decreased after the treatment of CHX and this decrease occurred much faster when JMJD5

Figure 5. JMJD5 modulates microtubule stability by regulating MAP1B protein levels. (A) HeLa cells were transfected with control or JMJD5 siRNAs. The levels of JMJD5, MAP1B, MAP1S and α -tubulin were then examined using western blot with the indicated antibodies (left panel). Relative MAP1B levels were then measured by densitometric analysis of the western blot data (right panel). The values are presented as the mean $\pm$ s.e.m. of 3 independent experiments, $*p < 0.05$ (Student's *t*-test). (B) HeLa cells were transfected with Flag or Flag-JMJD5 plasmids. Flag-JMJD5, MAP1B, MAP1S and α -tubulin levels were then examined using western blot with the indicated antibodies (left panel). Relative MAP1B levels were then measured by densitometric analysis of the western blot data (right panel). The values presented are the mean $\pm$ s.e.m. of 3 independent experiments, $*p < 0.05$ (Student's *t*-test). (C and D) JMJD5 does not regulate MAP1B at the transcriptional level. JMJD5 were depleted (C) or overexpressed (D) in HeLa cells. Relative mRNA levels of JMJD5 and MAP1B were measured using quantitative real-time PCR. The results represent the mean values of 3 independent experiments. (E) HeLa cells transfected with the indicated siRNAs were treated with 20 μ M MG132 for 12 h. JMJD5, MAP1B and α -tubulin levels were then examined using western blot. (F) HeLa cells transfected with siRNAs were treated with 100 μ g/ml CHX for the indicated hours. JMJD5, MAP1B and α -tubulin levels were then examined using western blot. (G) MAP1B partially rescued the reductions of tubulin acetylation and detyrosination induced by JMJD5 depletion. JMJD5-depleted HeLa cells were transfected with GFP or GFP-MAP1B. JMJD5, GFP, acetylated α -tubulin, detyrosinated α -tubulin and α -tubulin levels were then examined using western blot with the indicated antibodies (left panel). The relative ratios of acetylated α -tubulin to α -tubulin (middle panel) and detyrosinated α -tubulin to α -tubulin (right panel) were measured by densitometric analysis of the western blot data. Values are presented as the mean $\pm$ s.e.m. of 3 independent experiments.

Figure 6. JMJD5 depletion sensitizes HeLa cells to microtubule-destabilizing agents. (A and B) HeLa cells were transfected with the indicated siRNAs and treated with various concentrations of vinblastine (A) or colchicine (B) for 48 h. Cell viability was measured using a CCK-8 kit. (C and D) HeLa cells were transfected with the indicated siRNAs and treated with 1 nM vinblastine (C) or 100 nM colchicine (D) for different periods of time. Cell viability was measured using a CCK-8 kit. Error bars indicate $\pm$ s.e.m. * $p < 0.05$, ** $p < 0.01$ (Student's *t*-test).

was depleted (Fig. 5F). These results suggest that JMJD5 potentially regulates MAP1B stability.

To investigate whether MAP1B is responsible for the JMJD5-associated regulation of microtubule stability, we over-expressed MAP1B in JMJD5-depleted cells. As shown in Fig. 5G, the decrease of tubulin acetylation and detyrosination observed in JMJD5 knockdown cells was rescued by MAP1B overexpression. Together, these findings suggest that JMJD5 can regulate microtubule stability, potentially by regulating MAP1B protein levels.

JMJD5 depletion increases the sensitivity of cancer cells to microtubule-destabilizing agents

As JMJD5 regulates microtubule stability, we hypothesized that it may also affect the sensitivity of cancer cells to microtubule-destabilizing agents. To address this hypothesis, we treated JMJD5-depleted and control HeLa cells with increasing concentrations of vinblastine or colchicine and analyzed cell viability using the Cell Counting Kit-8 (CCK-8, Beyotime) assay. As shown in Fig. 6A and B, JMJD5-depleted cells were more susceptible to the cytotoxic effects of vinblastine and colchicine at low concentrations. In addition, we evaluated the viability of cells treated with vinblastine (1 nM) or colchicine (100 nM) for 0 to 60 hours. The viability of JMJD5-depleted cells decreased at a greater rate than that of control cells after treated with microtubule-destabilizing agents; this effect was particularly pronounced in cells treated with vinblastine (Fig. 6C and D). Together, these results indicate that JMJD5 depletion significantly increases the sensitivity of cancer cells to microtubule-destabilizing agents.

To determine if this effect was associated with cell apoptosis, we evaluated apoptosis in HeLa cells transfected with JMJD5 siRNA or control siRNA. The cells were treated with 2 different

concentrations of vinblastine or colchicine, and cell apoptosis was analyzed by FACS after Annexin V-FITC and propidium iodide (PI) staining. Consistent with the cell viability assay, the proportion of apoptotic cells increased in the JMJD5-depleted cells treated with vinblastine and colchicine compared with the control cells (Fig. 7A and B).

We further confirmed the increase in apoptosis by examining the levels of cleaved poly ADP ribose polymerase (PARP), a marker of apoptosis.³⁴ As shown in Fig. 7C and D, the cleaved PARP levels increased in cells treated with vinblastine or colchicine in a dose-dependent manner, and the levels of cleaved PARP appeared greater in JMJD5-depleted cells than in control cells. These findings indicated that apoptosis was enhanced in JMJD5-depleted cells. It has been reported that the anti-apoptotic activity of Bcl-2 is inhibited by phosphorylation in response to microtubule-targeting drugs.³⁵⁻³⁷ We observed an increase in the levels of phosphorylated Bcl-2 in JMJD5-depleted cells compared with control cells after treated with vinblastine or colchicine (Fig. 7C and D). Together, these results suggest that JMJD5 depletion may enhance the sensitivity of cancer cells to microtubule-destabilizing agents.

Discussion

In addition to inhibiting mitosis, a growing body of evidence indicates that disrupting microtubule dynamics during the interphase stage is another critical mechanism mediating the therapeutic efficacy of microtubule-targeting agents.¹⁷ In a previous study, we demonstrated that JMJD5 regulated the stability of the mitotic spindle and played an important role in mitosis.³³ In the present study, we evaluated the function of cytoplasmic JMJD5. We found that JMJD5 modulated the stability of cytoskeletal microtubules by regulating MAP1B

Figure 7. JMJD5 depletion increases cell apoptosis induced by microtubule-destabilizing agents. (A and B) siRNA-transfected cells were treated with the indicated concentrations of vinblastine (A) or colchicine (B) for 36 h. Cells stained with PI/Annexin V-FITC were analyzed using FACS, and the proportions of cells in early apoptosis and late apoptosis were measured in 3 independent assays. Error bars indicate $\pm$ s.e.m. * $p < 0.05$, ** $p < 0.01$; n.s., not significant (Student's *t*-test). (C and D) JMJD5 depletion enhances PARP1 cleavage and Bcl2 phosphorylation induced by microtubule-destabilizing agents. JMJD5 siRNA-transfected cells and control cells were treated with the indicated concentrations of vinblastine (C) and colchicine (D) for 36 h, and the cell extracts were analyzed using western blot with the indicated antibodies.

protein levels and that JMJD5 depletion significantly increased the sensitivity of cancer cells to microtubule-destabilizing agents.

JMJD5 localization and function are reported primarily restricted to the nucleus.^{22,23} Huang and colleagues reported that a functional nuclear localization signal (NLS) and a nuclear export signal (NES) reside in the N-terminal domain of JMJD5.³⁸ JMJD5 localization is tightly regulated by Importin α/β - and transportin-1-mediated nuclear import and CRC1-dependent nuclear export, suggesting that JMJD5 translocation in and out of the nucleus may be associated with a novel function. In this study, we found that a fraction of JMJD5 localized to the cytoplasm and that it regulated the stability of cytoskeletal microtubules. Consistent with our finding that JMJD5 plays

a role in the cytoplasm, a recent study reported that JMJD5 facilitated HBV replication in the cytoplasm.²⁸ It will be interesting to explore if the effects of JMJD5 on microtubule stability also mediate its function in HBV replication. Furthermore, we predict that additional cytoplasmic functions of JMJD5 will be identified in future studies.

In this study, we found that JMJD5 regulated MAP1B protein levels in cancer cells. MAP1B is predominantly expressed in the central nervous system, and MAP1B expression gradually decreases during neuronal development.³⁹ However, RT-PCR analysis revealed that MAP1B is transcribed in a variety of other tissues.⁴⁰ MAP1B expression is higher in the brain, testis, kidney and lung than in the heart, muscle, liver, and spleen.⁴⁰

Also, MAP1B is reportedly expressed in certain types of cancer cells, such as breast cancer, ovarian cancer, neuroblastoma and colorectal cancer.⁴¹⁻⁴⁴ Exogenous MAP1B protects microtubules against nocodazole-induced depolymerization,⁴⁵ similar to the effect of JMJD5. Our data reveal that JMJD5 depletion reduces the levels of acetylated and detyrosinated tubulin and that MAP1B overexpression can reverse these effects, suggesting that JMJD5 regulates microtubule stability via MAP1B. JMJD5 regulates MAP1B protein levels but has little effect on MAP1B transcription. Thus, JMJD5 regulates MAP1B at the post-transcriptional level. Interestingly, JMJD5 has been reported to regulate NFATc1 protein levels and the stability of HBV capsids at the post-transcriptional steps.^{23,28} Further studies are needed to investigate the precise mechanisms underlying JMJD5-mediated regulation of MAP1B.

The precise functions and the corresponding mechanisms mediated by JMJD5 in oncogenesis are rather complex. JMJD5 was initially identified as a putative tumor suppressor that regulates genome integrity in Blm-deficient mice,⁴⁶ and JMJD5 overexpression inhibits cell growth and tumorigenicity in hepatocellular carcinoma cells.³² However, in lung, colon and breast cancers, JMJD5 functions as a putative oncogene that promotes cancer cell proliferation, invasion and cellular metabolism.^{29-31,47} In the present study, we found that JMJD5 depletion increases the susceptibility of cancer cells to microtubule-destabilizing agents by modulating microtubule stability. A recent study revealed that JMJD5 promotes cancer cell proliferation primarily by inhibiting the nuclear accumulation of p53.⁴⁷ In addition, p53 has been reported to localize to microtubules and to undergo nuclear transport via dynein in response to DNA damage.⁴⁸ Treatment with low concentrations of microtubule-destabilizing agents, such as nocodazole, vincristine and colchicine, enhanced p53 nuclear translocation and p53-mediated activation of downstream target genes.⁴⁹ Thus, JMJD5 may inhibit the nuclear translocation of p53 by modulating microtubule stability.

Microtubule-targeting agents, such as vinblastine, vincristine and paclitaxel, have been widely used as chemotherapeutic agents for various types of cancer.¹⁶ However, as with most chemotherapies, their efficacy is limited by dose-related toxicity and drug sensitivity and resistance.¹⁶ Here, we demonstrated that JMJD5 modulates the stability of microtubules and that JMJD5 depletion increases cancer cell sensitivity to the cytotoxic effects of microtubule-destabilizing agents. Therefore, JMJD5 may represent a potential biomarker that can predict the sensitivity of cancer patients to microtubule-targeting agents. Further work is required to determine if the pharmacological inhibition of JMJD5 would have an enhanced chemotherapeutic response in combination with microtubule-targeting agents.

Materials and methods

Reagents and antibodies

Nocodazole, taxol, vinblastine, colchicine, 4',6-diamidino-2-phenylindole (DAPI), PI and the mouse monoclonal antibody against α -tubulin were purchased from Sigma-Aldrich. MG132 and CHX were obtained from Selleckchem, and the antibody

against acetyl- α -tubulin was obtained from Cell Signaling Technology. Antibodies against Flag, GFP, β -tubulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Abmart. The rabbit anti-JMJD5 antibody was generated by Abmart.³³ The antibody against detyrosinated- α -tubulin was obtained from Millipore. Antibodies against MAP1B, MAP1S, PARP and Bcl-2 were purchased from Proteintech, and the antibody against H1 was obtained from Bioworld. FITC- and CY3-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories.

Plasmids and siRNAs

Human JMJD5 cDNA was cloned into the pEF-Neo-Flag vector as previously described.³³ The GFP-MAP1B plasmid was a gift from Phillip Gordon-Weeks (Addgene plasmid # 44396).⁵⁰ The control siRNA (5'-UUCUCCGAACGUGUCACGUTT-3') and siJMJD5-2 (5'-CCAGAUGUGAAGUUAGAAATT-3') were synthesized by GenePharma (Shanghai, China), and siJMJD5-1 (sc-75359) was purchased from Santa Cruz Biotechnology.

Cell culture and transfection

HeLa cells were purchased from the National Platform of Experimental Cell Resources for Sci-Tech, China. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% streptomycin/penicillin at 37°C in a humidified atmosphere with 5% CO₂. siRNAs were transfected using Lipofectamine RNAiMAX (Life technologies), and the plasmids were transfected using Lipofectamine 3000 transfection reagent (Life Technologies) according to the manufacturer's instructions in medium without antibiotics.

Quantitative real-time RT-PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription of RNA to cDNA was performed using a cDNA Synthesis SuperMix kit (TransGen Biotech). Quantitative real-time PCR was performed using UltraSYBR Mixture (CWbio-tech), according to the manufacturer's instructions.

Cellular fractionation and Western blot analysis

The cytoplasmic and nuclear fractions were prepared as described previously^{51,52} with some modifications. Briefly, cells were washed with cold PBS, and lysed in hypotonic buffer (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, 1 mM DTT, and protease inhibitors (Roche)), containing 0.1% Triton X-100 for 8 min on ice. Cytoplasmic proteins were separated from nuclei by centrifugation at 2000 × g for 5 min at 4°C. The supernatant was clarified by high-speed centrifugation (13,000 × g, 5 min, 4°C). Nuclei pellets were washed once with hypotonic buffer. Nuclear proteins were extracted in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP40, 1 mM EGTA, 0.5% sodium

deoxycholate, 0.5% SDS, protease and phosphatase inhibitors (Roche)) for 20 min on ice.

For total cell extracts, the cells were washed with cold PBS, and total proteins were extracted using RIPA buffer. Protein extracts were quantified using a bicinchoninic acid (BCA) protein assay kit (Abnova), and 20–40 μ g of protein per lane was separated using SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) and blocked using 5% nonfat milk. The membrane was incubated with the appropriate primary antibody and subsequently incubated with an HRP-conjugated secondary antibody (Abmart). The protein signals were visualized using the eECL Western Blot Kit (CWbiotech), and their intensities were determined using ImageJ software.

Immunofluorescence microscopy

Cells grown on poly-lysine-coated glass coverslips were fixed with pre-chilled methanol for 5 min at -20°C and blocked in PBS-BT buffer [$1\times$ PBS, 3% (m/V) bovine serum albumin (BSA) and 0.1% Triton X-100] at room temperature. The cells were incubated with primary antibodies at 4°C overnight. Then, the cells were incubated with diluted FITC- or CY3- conjugated secondary antibodies at room temperature for 1 h and were subsequently stained with DAPI for 5 min. The coverslips were mounted using Vectashield Mounting Medium (Vector Laboratories). Images were acquired using an A1R MP Multiphoton Confocal Microscope (Nikon) with a $60\times$ or $100\times$ oil objective, and fluorescence intensities were analyzed using NIS-Elements software (Nikon).

Microtubule depolymerization

Cells transfected with the indicated siRNAs or plasmids were treated with 5 μM nocodazole for 0, 20, 40 or 60 min to induce microtubule depolymerization. The residual microtubules were examined using immunofluorescence staining and microscopy.

Detection of polymeric and soluble dimeric tubulin

Cells were treated with nocodazole, and the soluble proteins were extracted using PEMT buffer (100 mM PIPES, 1 mM EGTA, 2 mM MgCl_2 and 0.1% Triton X-100, pH 6.8). The remaining polymeric fraction was dissolved in RIPA buffer. The tubulin levels in the soluble and polymeric fractions were examined using western blotting.

Cell viability assay

Cell viability after drug treatment was measured using a Cell Counting Kit-8 (CCK-8, Beyotime) according to the manufacturer's protocol. Briefly, HeLa cells were transfected with siRNAs and then cultured in 96-well plates. After 24 h in culture, vinblastine was added to final concentrations of 0.1, 0.4, 0.7, 1, 4, 7, 10 and 40 nM, and colchicine was added to final concentrations of 10, 40, 70, 100, 400, 700, 1000 and 4000 nM. DMSO was used as the control treatment. After 36 h of treatment, the CCK-8 solution was added into the wells for 1 h before the optical density (OD) was measured at a wavelength of 450 nm.

Flow cytometry analysis of apoptosis

Cell apoptosis was evaluated using an Annexin V-FITC Apoptosis Detection Kit (Beyotime) according to the manufacturer's protocol. The proportion of apoptotic cells was determined using a BD FACSCalibur flow cytometer (Tsinghua University, Beijing, China), and the data were analyzed using CellQuestTM Pro software (BD).

Disclosure of potential conflicts of interest

The authors declare no competing or financial interests.

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Author contributions

J.W., Z.H., D.-L.W. and F.-L.S. designed research; J.W. and Z.H. performed experiments; J.W., Z.H. and F.-L.S. analyzed data and wrote the paper.

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