MST2 Phosphorylation at Serine 385 in Mitosis Inhibits its Tumor Suppressing Activity

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Abstract

Mammalian sterile 20-like kinase 1/2 (MST1/2) are core tumor suppressors in the Hippo signaling pathway. MST1/2 have been shown to regulate mitotic progression. Here, we report a novel mechanism for phospho-regulation of MST2 in mitosis and its biological significance in cancer. We found that the mitotic kinase cyclin-dependent kinase 1 (CDK1) phosphorylates MST2 *in vitro* and *in vivo* at serine 385 during antimitotic drug-induced G2/M phase arrest. This phosphorylation occurs transiently during unperturbed mitosis. Mitotic phosphorylation of MST2 does not affect its kinase activity or Hippo-YAP signaling. We further showed that mitotic phosphorylation-deficient mutant MST2-S385A possesses higher activity in suppressing cell proliferation and anchorage-independent growth *in vitro* and tumorigenesis *in vivo*. Together, our findings reveal a novel layer of regulation for MST2 in mitosis and its role in tumorigenesis.

Key words: MST2; mitotic phosphorylation; CDK1; tumor suppressor

Abbreviations: CDK1: cyclin-dependent kinase 1; LATS1/2: large tumor suppressor 1/2; MST1/2: mammalian sterile 20-like kinase 1/2; PLK1: polo-like kinase 1; TAZ: transcriptional co-activator with PDZ binding domain; YAP: yes-associate protein.

1. Introduction

Mammalian sterile 20-like kinase 1/2 (MST1/2) are protein kinases that belong to the serine/threonine kinase family (MST1 and MST2 are also called STK4 and STK3, respectively). MST1/2 are the core components of the Hippo pathway and transduce their kinase activity mainly through directly phosphorylating large tumor suppressor 1/2 (LATS1/2) [1,2]. Once phosphorylated and activated, LATS1/2 subsequently phosphorylate and inhibit the downstream effectors Yes-associated protein (YAP) and transcriptional co-activator with PDZ binding domain (TAZ) [1-4]. Neither MST1 nor MST2 alone is required for embryonic development, but double knock out of MST1/2 mice exhibit early embryonic lethality, suggesting a redundant and overlapping function between MST1 and MST2 [5]. Recent studies using conditional MST1/2 knockout animal models demonstrated that MST1/2 function as tumor suppressors [5-8]. In addition to their role as tumor suppressors in the Hippo signaling pathway, MST1/2 also phosphorylate several other proteins to exercise their functions in various cellular processes, mainly in cell proliferation and apoptosis [9].

Mitotic aberration-induced genomic or chromosome instability is a characteristic of human malignancy [10,11]. Several recent studies have shown that MST1/2 are important regulators for the mitotic machinery. MST1 phosphorylates and inhibits Aurora B kinase activity and is required for accurate kinetochore-microtubule attachment [12]. PLK1 (Polo-like kinase 1) directly phosphorylates MST2 (possibly MST1 as well) in mitosis and this phosphorylation allows Nek2A kinase activity to promote centrosome disjunction [13]. These studies suggest that MST1/2 function as tumor suppressors through dysregulation of mitosis.

We have recently shown that several upstream regulators (KIBRA and Ajuba) [14-16] and downstream effectors (YAP and TAZ) [17,18] of the Hippo pathway are phosphorylated during mitosis. During these previous studies, we found that the Hippo core kinase MST2 is also phosphorylated during antimitotic drug-induced G2/M phase arrest. In this report, we further characterized the phospho-regulation of MST2 in mitosis and examined the functional significance of the phosphorylation. Our data showed that mitotic phosphorylation inhibits MST2 tumor suppressing activity.

2. Materials and methods

2.1. Expression constructs, cell culture and transfection

Flag-MST2 has been described [19]. Point mutations were generated by the QuikChange Site-Directed PCR Mutagenesis Kit (Stratagene) and verified by sequencing. HEK293T, HEK293GP, and HeLa cell lines were purchased from American Type Culture Collection (ATCC) and cultured as ATCC instructed. Attractene (Qiagen) was used for transient overexpression of proteins in HEK293T and HEK293GP cells following the manufacturer's instructions. SiRNA oligos were purchased from Dharmacon (the target sequences were: siMST2-1: CCACAAGCACGATGAGTGA; siMST2-2: GCCCATATGTTGTAAAGTA; siMST2-3: GAACTTTGGTCCGATGATT) and transfected with HiPerfect reagent from Qiagen (at the final concentration of 40 nM). Transient transfections were done with Attractene reagent (Qiagen) following the manufacturer's instructions. Nocodazole (100 ng/ml for 16 h) and Taxol (100 nM for 16 h) (Selleck Chemicals) were used to arrest cells in G2/M phase. VX680 (Aurora-A, -B, -C inhibitor), BI2536 (PLK1 inhibitor), Purvalanol A (CDK1/2/5 inhibitor), SB216763 (GSK-3 inhibitor) and MK2206 (Akt inhibitor) were also from Selleck Chemicals. RO3306 (CDK1 inhibitor) was from ENZO Life Sciences. Kinase inhibitors for MEK-ERK (U0126) and p38 (SB203580) were from LC Laboratory. All other chemicals were either from Sigma or Thermo Fisher.

2.2. Tet-On-inducible expression system

The MST2 or MST2-S385A mutated cDNA was cloned into the Tet-All vector [20] to generate Tet-On-inducible overexpression constructs. Retrovirus packaging, infection, and subsequent selection were done as we have described previously [21]. The transduced cells were selected with neomycin (G418) (400 µg/ml) to establish pooled cell lines. Cells were maintained in medium containing Tet system-approved fetal bovine serum (Clontech Laboratories).

2.3. Recombinant protein purification and in vitro kinase assay

GST-tagged MST2 or MST2-S385A (cloned in pGEX-5X-1) was bacterially expressed and purified on GSTrap FF affinity columns (GE Healthcare) following the manufacturer's instructions. GST-MST2 (1 µg) was incubated with 5-10 U recombinant CDK1/cyclin B complex (New England Biolabs) or 50-100 ng CDK1/cyclin B (SignalChem) in kinase buffer (New England Biolabs) in the presence of 5 µCi γ -³²P-ATP (3000 Ci/mmol, PerkinElmer) as we

previously described [15]. Active CDK2, CDK5, p38, JNK1, JNK2, MEK1, ERK1, and PLK1 kinases were also purchased from SignalChem.

2.4. Antibodies

Rabbit polyclonal phospho-specific antibodies against human MST2 S385 were generated and purified by AbMart. The peptide used for immunizing rabbits was KRNAT-pS-PQVQR. The corresponding non-phosphorylated peptide was also synthesized and used for antibody purification. Anti- β -actin (SC-47778) and anti-cyclin B (SC-752) antibodies were from Santa Cruz Biotechnology. Glutathione S-transferase (GST) (A190-122A), Mst1 (A300-465A), Mst2 (A300-467A), and Lats1 (A300-478A) antibodies were from Bethyl Laboratories. MST2 antibodies from Cell Signaling Technology (3952) were also used. Phospho-S10 H3 (3377), phospho-S127 YAP (4911), phospho-S909 Lats1 (9157), phospho-S1079 Lats1 (8654), phospho-T183 MST1/T180 MST2 (3681), and cleaved caspase 3 (9664) antibodies were also from Cell Signaling Technology. Anti-PLK1 antibodies were from Biolegend (667701). Phospho-T210 PLK1 antibodies were purchased from BD Bioscience (558400).

2.5. Phos-tag and Western blot analysis

Phos-tagTM was obtained from Wako Pure Chemical Industries, Ltd. (304-93521) and used at 20 μ M (with 100 μ M MnCl₂) in 8% SDS-polyacrylamide gels as we previously described [16]. Western blotting, immunoprecipitation, and lambda phosphatase treatment assays were done as previously described [15,22].

2.6. Cell proliferation and colony formation assays

For cell proliferation assays, cells (50,000/well) were seeded in a 6-well plate in triplicate. Cells were counted by a hemacytometer. Colony formation assays in soft agar were performed as described [19]. Cells (5,000/well) were seeded in a 6-well plate and colonies were counted by ImageJ online.

2.7. Animal studies

For *in vivo* xenograft studies, 2.0×10^6 HeLa cells expressing Tet-All-MST2 or Tet-All-MST2-S385A (non-phosphorylatable mutant) were subcutaneously injected into flanks (both left and right) of 6-week-old male athymic Ncr-nu/nu nude mice (Harlan). Five animals were used per group. Tumor sizes were measured every four days using an electronic caliper starting at 10 days after injection. Tumor volume (V) was calculated by the formula: $V = 0.5 \times \text{length} \times \text{width}^2$ [19]. The animals were housed in pathogen-free facilities. All animal experiments were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.

2.8. Statistical analysis

Statistical significance was analyzed using a two-tailed, unpaired Student's *t*-test. Pearson Chi-Square analysis was used to determine the statistical significance in Figure 6C.

3. Results and Discussion

3.1. MST2 is phosphorylated during antimitotic drug-induced G2/M arrest

Using a Phos-tag SDS-polyacrylamide gel system, we recently examined the phosphorylation status of the Hippo pathway proteins during G2/M arrest induced by Taxol or Nocodazole. During these experiments, we found that MST2, but not MST1, was upshifted on a SDS-polyacrylamide gel during G2/M arrest (Fig. 1A, B) [16]. Lambda phosphatase treatment largely abolished the mobility upshift of MST2, suggesting that MST2 is phosphorylated during G2/M arrest (Fig. 1A). The phosphorylation on T183-MST1 (T180-MST2) in the activation loop was not altered under these conditions (Fig. 1B).

3.2. Identification of the corresponding kinase for MST2 phosphorylation

We used various kinase inhibitors to identify the candidate kinase for MST2 phosphorylation. Inhibition of p38 kinase (with SB203580), JNK1/2 (with SP600125), MEK-ERK (with U0126), Akt (with MK-2206), PLK1 (with BI2536), Aurora-A, -B, -C (with VX680) or GSK-3 (with SB216763) failed to alter the mobility/phosphorylation of MST2 during G2/M arrest (Fig. 1C, lanes 5-11). These inhibitors are effective under the conditions used [17,23]. Interestingly, treatments with RO3306 (CDK1 inhibitor) or Purvalanol A (CDK1/2/5 inhibitor) almost completely reverted the mobility shift/phosphorylation (Fig. 1C, lanes 3-4). These data suggest that CDK1 is likely the corresponding kinase for MST2 phosphorylation induced by Taxol or Nocodazole treatment.

3.3. CDK1 phosphorylates MST2 *in vitro*

Next, we performed *in vitro* kinase assays with bacterially purified MST2 proteins as substrates to determine which kinase can directly phosphorylate MST2. Figure 2A shows that purified CDK1/cyclin B kinase complex robustly phosphorylated GST-MST2 proteins *in vitro* (Fig. 2A). No or very mild phosphorylation was detected when CDK2, CDK5, p38, JNK1, JNK2, MEK1, or ERK1 kinase was used in these assays, though these kinases recognize the same consensus sequence as CDK1 kinase. These results indicate that CDK1 specifically and directly phosphorylates MST2 *in vitro*.

3.4. CDK1/cyclin B complex phosphorylates MST2 at S385 *in vitro*

CDK1 phosphorylates substrates at a minimal proline-directed consensus sequence [24]. MST2 only contains a total of 2 S/TP motifs (S107 and S385) and S107 also exists in MST1. Therefore, S385 was chosen for further investigation. Interestingly, mutating S385 to alanine completely abolished the ³²P incorporation in GST-MST2, suggesting that S385 is the main CDK1 site in MST2 *in vitro* (Fig. 2B). A recent report showed that MST2 is also phosphorylated by the mitotic kinase PLK1 [13]. Consistent with that study, we confirmed that MST2 is also a suitable substrate for PLK1 (Fig. 2C); however, mutating S385 to alanine failed to significantly reduce the phosphorylation of MST2 mediated by PLK1 (Fig. 2C). These observations suggest that PLK1 and CDK1 phosphorylate different sites in MST2 *in vitro*.

We have generated phospho-specific antibodies against S385. *In vitro* kinase assays confirmed that CDK1 readily phosphorylates MST2 at S385 (Fig. 2D). Mutating S385 to alanine abolished the phosphorylation, confirming the specificity of our antibody (Fig. 2D). These data indicate that CDK1 phosphorylates MST2 at S385 *in vitro*.

3.5. CDK1 phosphorylates MST2 at S385 *in cells*

Next, we explored whether this phosphorylation occurs in cells. Taxol treatment significantly increased the phosphorylation of MST2 S385 (Fig. 3A). Addition of RO3306 or Purvalanol A, but not the PLK1 kinase inhibitor BI2536, greatly inhibited MST2 S385 phosphorylation, suggesting that these antibodies specifically recognize phosphorylated MST2 and that phosphorylation of MST2 S385 is CDK1 kinase dependent (Fig. 3A). As expected, the signal of MST2 S385 was significantly reduced in MST2 knockdown cells (Fig. 3B). Using immunoprecipitated samples, we further demonstrated that MST2 is phosphorylated on S385 during Taxol-induced G2/M in a CDK1-dependent manner (Fig. 3C).

3.6. MST2 phosphorylation on S385 occurs during normal mitosis

To determine whether phosphorylation of MST2 S385 occurs during normal mitosis, a double thymidine block and release method was used [25]. Figure 3D shows that the p-MST2 S385 signal was significantly increased in cells after 11 hours of being released from double thymidine block (Fig. 3D). A significant portion of cells is in mitosis, as revealed by increased cyclin B levels (Fig. 3D). These results indicate that the phosphorylation of MST2 S385 occurs dynamically during normal mitosis.

3.7. Mitotic phosphorylation of MST2 does not impact Hippo-YAP activity

MST2 is a core kinase in the Hippo-YAP signaling. We first tested whether this phosphorylation affects its kinase activity. The non-phosphorylatable (MST2-S385A) mutant has similar basal kinase activity revealed by phosphorylation at T180 as wild type MST2 (Fig. 4A), suggesting that S385 phosphorylation of MST2 does not impact its kinase activity. As expected, YAP S127 (a major phosphorylation site mediated by LATS1/2 kinases) phosphorylation was significantly increased upon MST2 overexpression [19,21]. However, ectopic expression of MST2-S385A had similar effects as wild type MST2 on YAP S127 phosphorylation (Fig. 4B). We further established doxycycline-induced MST2 or MST2-S385A in HeLa cells, and in the presence of doxycycline, both wild type MST2 and MST-S385A were modestly induced at a similar level (Fig. 4C). No significant changes were detected in the Hippo-YAP signaling activity under these conditions (Fig. 4C). These observations suggest that phosphorylation of MST2 at S385 does not affect Hippo-YAP activity.

3.8. The non-phosphorylatable mutant MST2 possesses stronger inhibitory activity in cell proliferation and anchorage-independent growth

Next, we compared the effects from doxycycline-induced MST2- or MST2-S385A-expressing HeLa cells to determine the biological significance of S385 phosphorylation of MST2. Interestingly, overexpression of the MST2-S385A mutant significantly reduced cell proliferation when compared to MST2-expressing cells (Fig. 5A). Furthermore, MST2-S385A-expressing cells formed a significantly lower number of colonies in soft agar when compared with MST2-expressing cells (Fig. 5B, C). These data suggest that mitotic phosphorylation inhibits MST2 activity in suppressing cell proliferation and anchorage-independent growth.

3.9. The non-phosphorylatable MST2 mutant inhibits tumorigenesis in vivo

We further evaluated the influence of S385 phosphorylation on tumor growth in animals. An equal number of HeLa cells expressing MST2 or MST2-S385A were subcutaneously inoculated into immunodeficient mice and tumor size was monitored in the presence of doxycycline. Interestingly, in line with the results in Figure 5, tumors from mice bearing MST2-S385A-

expressing cells were significantly smaller when compared with those from mice injected with wild type MST2-expressing cells (Fig. 6A, B). Western blotting analysis showed that MST2 (wild type or S385A) expression levels were similar in most of these tumors (Fig. 6C). Interestingly, expression of MST2-S385A induced stronger apoptosis (detected by cleaved caspase 3) when compared with wild type MST2 (Fig. 6C, Pearson Chi-Square test, $p < 0.1$). These results suggest that phosphorylation of MST2 at S385 inhibits its tumor suppressing activity *in vivo*.

Future studies are needed to further determine whether S385 phosphorylation of MST2 contributes to the fidelity of mitosis and how this phosphorylation links subsequent tumorigenesis. Our results showed that mitotic phosphorylation of MST2 S385 does not impact the LATS and YAP activity and thus, it will be interesting to see what the downstream effector of S385 phosphorylation is.

4. Conclusions

In the current study, we identified a novel phosphorylation site (S385) on MST2 that is dynamically/transiently phosphorylated by CDK1 during mitosis. The mitotic phosphorylation of MST2 inhibits its tumor suppressing activity without affecting its own kinase activity and the Hippo-YAP signaling. Together, we provided a novel layer of regulation of MST2 activity in cancer cells.

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Figure legends

Fig. 1. CDK1-dependent phosphorylation of MST2 during G2/M arrest.

(A) HeLa cells were treated with Taxol as indicated and cell lysates were further treated with (+) or without (-) λ phosphatase (ppase). Total cell lysates were probed with anti-MST2 antibody on Phos-tag SDS-polyacrylamide gels.

(B) HeLa cells were treated with DMSO, Taxol or Nocodazole (Noco). Total cell lysates were probed with the indicated antibodies on Phos-tag or regular SDS-polyacrylamide gels.

(C) HeLa cells were treated with Taxol together with or without various kinase inhibitors as indicated. RO3306 (CDK1 inhibitor, 5 μ M), Purvalanol A (CDK1/2/5 inhibitor, 10 μ M), SB203580 (p38 inhibitor, 10 μ M), SP600125 (JNK1/2 inhibitor, 20 μ M), U0126 (MEK-ERK inhibitor, 20 μ M), MK2206 (AKT inhibitor, 10 μ M), BI2536 (PLK1 inhibitor, 100 nM), VX680 (Aurora-A, B, C inhibitor, 2 μ M), and SB216763 (GSK3 inhibitor, 10 μ M) were used. Inhibitors were added 1-2 h before harvesting the cells (with MG132 to prevent cyclin B from degradation and cells from exiting from mitosis). Total cell lysates were subjected to Western blotting with the indicated antibodies. SE: short exposure; LE: long exposure.

Fig. 2. CDK1 phosphorylates MST2 *in vitro*.

(A) *In vitro* kinase assays with kinases as indicated.

(B) *In vitro* kinase assays with CDK1/cyclin B complex using GST-MST2 or GST-MSTS385A proteins as substrates. RO3306 (5 μ M) was used to inhibit CDK1 kinase activity.

(C) *In vitro* kinase assays with PLK1 kinase using GST-MST2 or GST-MSTS385A proteins as substrates.

(D) *In vitro* kinase assays were done as in B except anti-p-S385 MST2 antibody was used.

Fig. 3. CDK1 mediates the phosphorylation of MST2 S385 in cells.

(A) HeLa cells were treated with Taxol together with or without various kinase inhibitors as indicated. Inhibitors were added 1.5 h before harvesting the cells (with MG132 to prevent cyclin B from degradation and cells from exiting from mitosis). Total cell lysates were subjected to Western blotting with the indicated antibodies.

(B) HeLa cells were transfected with scrambled siRNA (control) or siRNA against MST2 for 48 h and were further treated with (+) or without (-) Taxol for 14 h. The total cell lysates were subjected to Western blotting with the indicated antibodies.

(C) MST2 proteins in HeLa cells were immunoprecipitated and the samples were probed with phospho-S385 MST2 and subsequent MST2 antibodies. Total lysates before immunoprecipitation were also probed with the indicated antibodies. CDK1 inhibitors RO3306

(5 μ M) or Purvalanol A (10 μ M) together with MG132 (25 μ M) were added 1.5 h before the cells were lysed. * marks the IgG heavy chain.

(D) A double thymidine block and release was performed in HeLa cells and samples were collected at the indicated time points. The total cell lysates were probed with the indicated antibodies.

Fig. 4. Mitotic phosphorylation of MST2 does not affect the Hippo-YAP activity.

(A) HEK293T cells were transfected with Flag-MST2 or Flag-MST2-S385A as indicated. The immunoprecipitates (with Flag antibodies) were probed with the indicated antibodies. * marks the IgG heavy chain. WT: wild type.

(B) GFP-YAP was co-transfected with Flag-MST2-WT or Flag-MST2-S385A with or without Flag-LATS2. The cells were harvested at 48 h post-transfection and the total cell lysates were analyzed by Western blotting with the indicated antibodies.

(C) Establishment of Tet-On-inducible HeLa cell lines expressing vector, MST2-WT, or MST2-S385A. Total cell lysates were harvested from these cell lines in the presence of doxycycline (1 μ g/ml for 2 days) and were subjected to Western blotting analysis.

Fig. 5. MST2-S385A suppresses cell proliferation and anchorage-independent growth.

(A) Cell proliferation assays with HeLa cells-expressing Tet-MST2-WT or Tet-MST2-S385A. Cells were kept on Tet-approved FBS and doxycycline was added (1 μ g/ml) to the cells 2 days prior to the experiments. Data were expressed as the mean $\pm$ s.d. of three independent experiments. **: $p < 0.01$; *: $p < 0.05$ (t-test).

(B, C) Colony assays in soft agar to assess anchorage-independent growth of HeLa cells expressing Tet-MST2-WT or Tet-MST2-S385A in the presence of doxycycline. Data were expressed as the mean $\pm$ s.d. of three repeats (B) and representative images were shown (C). **: $p < 0.01$ (t-test). WT: wild type.

Fig. 6. MST2-S385A suppresses tumorigenesis in mice.

(A) Tumor growth curve. HeLa cells expressing Tet-MST2-WT or Tet-MST2-S385A were subcutaneously inoculated into athymic nude mice (n=5, on both left and right flanks) and the mice were kept on doxycycline (0.5 mg/ml)-containing water throughout the experiments. One inoculation (left flank) in the wild type group did not form visible tumor and was excluded from the analysis. Therefore, the tumor volume at each point was the average of 9 (MST2-WT) or 10 (MST2-S385A) tumors. **: $p < 0.01$; *: $p < 0.05$ (t-test).

(B) The tumors in each group were excised and photographed at the endpoint.

(C) Western blotting analysis with tumor samples from **B**. Pearson Chi-Square test showed that it was marginally significant between two groups ($p < 0.1$).

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6