Mammalian Sterile 20-like Kinase 1 (Mst1) Enhances the Stability of Forkhead Box P3 (Foxp3) and the Function of Regulatory T Cells by Modulating Foxp3 Acetylation*

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Regulatory T cells (Tregs) play crucial roles in maintaining immune tolerance. The transcription factor Foxp3 is a critical regulator of Treg development and function, and its expression is regulated at both transcriptional and post-translational levels. Acetylation by lysine acetyl transferases/lysine deacetylases is one of the main post-translational modifications of Foxp3, which regulate Foxp3’s stability and transcriptional activity. However, the mechanism(s) by which the activities of these lysine acetyl transferases/lysine deacetylases are regulated to preserve proper Foxp3 acetylation during Treg development and maintenance of Treg function remains to be determined. Here we report that Mst1 can enhance Foxp3 stability, its transcriptional activity, and Treg function by modulating the Foxp3 protein at the post-translational level. We discovered that Mst1 could increase the acetylation of Foxp3 by inhibiting Sirt1 activity, which requires the Mst1 kinase activity. We also found that Mst1 could attenuate Sirt1-mediated deacetylation of Foxp3 through directly interacting with Foxp3 to prevent or interfere with the interaction between Sirt1 and Foxp3. Therefore, Mst1 can regulate Foxp3 stability in kinase-dependent and kinase-independent manners. Finally, we showed that treatment of Mst1−/− Tregs with Ex-527, a Sirt1-specific inhibitor, partially restored the suppressive function of Mst1−/− Tregs. Our studies reveal a novel mechanism by which Mst1 enhances Foxp3 expression and Treg function at the post-translational level.

Tregs3 play very important roles in maintaining immune tolerance and homeostasis. Genetic abnormalities or environmental factors that affect development or functions of Tregs can cause or predispose to autoimmunity (1). Foxp3, a member of the Forkhead transcription factor family, is critical for the development and suppressive function of Tregs. Foxp3 initiates and maintains the expression of the Treg signature genes to guarantee the normal development and function of Tregs (2–4). Loss-of-function mutations in Foxp3 result in Treg deficiency and severe autoimmune diseases in both mouse and human (2, 4–6). Ectopic expression of Foxp3 in naive T cells leads to their conversion to Tregs (3). Although the molecular pathways that control the expression of Foxp3 gene at the transcription level have been studied extensively, the molecular mechanism(s) underlying regulation of Foxp3 at the post-translational level is not fully understood.

Post-translational modifications such as acetylation, phosphorylation, and ubiquitination have been demonstrated to regulate protein stability and activity. Acetylation is important for maintaining Foxp3 protein expression levels and transcriptional activity, and consequently is also vital for Treg cell-mediated suppression (7, 8). Tip60 and P300 acetylases can acetylate Foxp3, whereas Sirt1, an NAD+-dependent lysine deacetylase, deacetylates Foxp3 (7–12). Mice treated with broad spectrum lysine deacetylase inhibitors such as trichostatin A- or SIRT-specific inhibitor nicotinamide all demonstrated reduced proinflammatory immune responses in colitis, arthritis, and cardiac transplantation models, resulting in lower disease scores (9, 13–15). However, how the activities of those lysine acetyl transferases or lysine deacetylases are regulated in vivo and their importance under homeostatic conditions remain to be determined.

Mst1 encoding a serine/threonine kinase is a core component of the Hippo pathway and is involved in multiple cellular processes such as morphogenesis, cell proliferation and differentiation (16–20), stress response (21), apoptosis (22–24), and T cell egress and homing (25–28). Recently, it has been shown that Mst1 deficiency results in autoimmune diseases in both humans and mice (25, 29–31) due to impaired development and function of Tregs (30), demonstrating that Mst1 plays crucial roles in preventing autoimmunity. Although phosphoryl-
tion of Foxo1/3 at the Forkhead domains by Mst1 augments their nuclear entry in 293T cells and granule neurons (21), we have recently shown that Mst1 promotes Foxp3 expression at the transcription level through regulating the protein stability of Foxo1/3 by phosphorylating Foxo1/3 in T cells (30). Foxp3 is also a Forkhead protein. Therefore, it remains unknown whether Mst1 can affect Foxp3 protein expression at the post-translational level to regulate Treg development and function.

Here we show that Mst1 can stabilize the Foxp3 protein and increase its transcriptional activity and Treg function by modulating the protein at the post-translational level. We demonstrate that Mst1 enhances Foxp3 acetylation by inhibiting Sirt1 activity in a kinase-dependent manner. We also found that Mst1 or Mst1K59R (a kinase-inactive mutant functioning as a dominant negative form) could attenuate Sirt1-mediated Foxp3 deacetylation by preventing or interfering with the interaction between Sirt1 and Foxp3 in a kinase-independent manner. Finally, we uncovered that the suppressive function of Mst1−/− Tregs was partially restored when they were treated with Ex-527, a Sirt1-specific inhibitor. Our results reveal a novel regulatory mechanism by which Mst1 enhances Foxp3 expression and Treg function through post-translational modification.

Experimental Procedures

Mice—Mst1−/− mice are described in previous work (28). All mice were maintained on a 129/Sv genetic background and raised in a specific pathogen-free facility. Experiments were conducted with consent from the Animal Care and Use Committee of the Institute of Developmental Biology and Molecular Medicine at Fudan University, Shanghai, China.

Cell Culture, Plasmids, and Antibodies—Murine embryonic fibroblasts (MEFs) were isolated from Mst1+/+ and Mst1−/− mice. Human embryonic kidney 293T cells were cultured in DMEM with 10% FBS. CD4+/CD25+ Treg cells and Jurkat cells were cultured in RPMI 1640 with 10% FBS. Human Mst1K59R gene was derived from a human Mst1 cDNA by site-directed mutagenesis. A HA-tagged human Mst1 and Mst1K59R genes were then cloned into expression vector pCDNA3.1. The Mst1 RNA interference (shMst1) plasmid is described previously (32). The IL-2 luciferase reporter plasmid was derived via inserting a PCR-amplified IL-2 promoter fragment (−305 to +39) from Jurkat cells into a luciferase reporter vector, pGL4.20 (Promega). The plasmids expressing Foxp3, or shSirt1, or Sirt1, or Epo300 were gifts from Drs. Bin Li (Institut Pasteur of Shanghai, Chinese Academy of Sciences (CAS), Shanghai, China), Qiwei Zhai (Institute for Nutritional Sciences, CAS, Shanghai, China), and Shimin Zhao (Fudan University, Shanghai, China). Antibodies against the following proteins were used for our studies: CD4, CD25, Foxp3, IL-2, and IFN-γ (eBioscience); CD3 and CD28 (BD Biosciences); phospho-Ser (Millipore); acetylated lysine, ubiquitin, and Sirt1 (Cell Signaling); HA and FLAG (Sigma); Myc (SC-40), GFP, and actin (Santa Cruz Biotechnology); and GAPDH (Beyotime).

Cell Purification and Flow Cytometry Analysis—CD4+ T cells and CD4+CD25− Treg cells were purified using the mouse CD4 kit or CD4+CD25+ Treg cells kit (11461D and 11463D; Invitrogen). CD4+CD25− cells were purified by depleting CD25+ cells with an anti-CD25/anti-rat IgG kit (11035; Invitrogen), followed by further purification using a mouse CD4 kit. Intracellular staining for Foxp3, IL-2, and IFN-γ was performed using a kit (00-5523; eBioscience) or as described elsewhere (33). 7-Aminoactinomycin D (Sigma-Aldrich) was used to label dead cells. All samples were analyzed with a FACS Calibur or CyAn ADP analyzer and FlowJo software (TreeStar).

Quantitative PCR—Total RNA was extracted from purified peripheral CD4+CD25+ Treg cells with TRIzol (Invitrogen). RT-PCR was carried out with Takara RNA PCR kit (AMV) according to the manufacturer’s instructions. Quantitative PCR reactions were performed on Mx3000P (Stratagene) with the Fast SYBR Green QPCR Master Mix (Stratagene), and data were analyzed with the MxPro software. Expression of Gapdh was used as internal control for real-time PCR. The primer sequences are as follows: Nrp1-F, 5′-TCTGCGATTCTGTTACGCTGTG-3′; Nrp1-R, 5′-ACTCCTAGACACATCCTGTC-3′.
GGG-3'; Pde3b-R, 5'-GCACTCTATGATGCCTGGG-3'; Pde3b-F, 5'-CCTGCTTCTTCTTTTGTGG-3'; Gapdh-L, 5'-TGTTCCATTCCCCAATGTGTCC-3'; and Gapdh-R, 5'-GGAAGGTCGTTGAAGTGCA-3'.

**Treg Suppression Assay**—A total of $6 \times 10^4$ splenic CD4$^+$CD25$^-$ T cells labeled with CFSE were cultured with CFSE were cultured with mitomycin C-treated T cell-depleted splenocytes and concanavalin A (2 mg/ml) in the presence of $3 \times 10^4$ Tregs (spleen CD4$^+$CD25$^-$ cells) for 72 h and then analyzed by FACS. The suppressive capacity of Treg cells was calculated using the following formula: ($100 \times (1 - \%$ of CFSE$^{low}$CD4$^+$CD25$^-$ T cells in co-culture/% of CFSE$^{low}$CD4$^+$CD25$^-$ T cells alone)), as described previously (34). Relative Treg suppression was calculated using the following formula: ($\frac{\%$ of CFSE$^{low}$CD4$^+$CD25$^-$ T cells in co-culture}{\%$ of CFSE$^{low}$CD4$^+$CD25$^-$ T cells alone}) $\times 100\%$, respectively, in DMSO or Ex-527.

**Luciferase Reporter Assay**—The luciferase reporter driven by the NFAT2 promoter was co-transfected with NFAT2, Foxp3, Sirt1, and Mst1 expression plasmids as indicated (see Fig. 5A) into 293T cells using Lipofectamine 2000. Cells were harvested 40 h after transfection. Jurkat T cells were transfected with the same luciferase reporter only, or co-transfected with plasmids expressing Mst1, shMst1, Foxp3, or shMst1/Foxp3, respectively by electroporation (Pulsar II, Bio-Rad). 40 h after electroporation, the cells were stimulated with PMA and ionomycin for another 6 h. Luciferase activity was analyzed as described previously (35) and normalized to renilla luciferase activity.

**GST Pulldown Assay**—The GST-Foxp3 fusion protein (GST-Foxp3) and the His$_6$-tagged Mst1 protein (His-Mst1) were expressed in Escherichia coli. The GST-Foxp3 and His-Mst1 were purified by glutathione-Sepharose 4B (GE Healthcare) or Ni-NTA Agarose (Clontech), respectively. The GST pulldown assay was performed as described previously (36). GST and the GST-Foxp3 protein were visualized by GST antibody, and His-Mst1 was detected by His antibody on Western blots.

**Immunoblotting and Immunoprecipitation**—Immunoblotting was performed as described previously (28). For immunoprecipitation, the transfected 293T cells or T cells freshly isolated from 8-week-old WT and Mst1$^{-/-}$ mice were lysed in Nonidet P-40 lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM PMSF, 1X protease inhibitors (Roche Diagnostics)). Then, Mst1 or Foxp3 proteins were immunoprecipitated from the supernatants with anti-HA, FLAG, or Myc-agarose beads or the appropriate antibodies with protein A-agarose beads and analyzed by immunoblotting. Images were collected with a Tanon-5200 or by x-ray film exposure. For quantification of protein levels, the appropriate film exposures were scanned, and the density of

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**FIGURE 2.** Mst1 enhances the protein stability of Foxp3. A–C, the Mst1 protein enhances Foxp3 protein level in transfected cells. Left and right panels, Western blot (left panel) and quantitative (right panel) analyses of the level of exogenous Foxp3 protein in 293T cells when Foxp3 was co-expressed with exogenous WT Mst1 gene (A) or dominant negative Mst1K59R (C), or when endogenous Mst1 expression was knocked down by shMst1 (B). Mst1KD, Mst1K59R mutant. D, the reduced endogenous Foxp3 protein level in Mst1$^{-/-}$ splenic CD4$^+$CD25$^-$ Treg cells freshly isolated from 8-week-old mice. E–G, Mst1 blocks proteasome-mediated Foxp3 degradation. Left and right panels, Western blot (left panel) and quantitative (right panel) analyses of the level of exogenous Foxp3 proteins co-expressed with shMst1 in 293T cells with/without MG132 treatment (E) or co-expressed with WT Mst1 in 293T cells treated with cycloheximide (CHX) for the indicated times (G), or analyses of the level of endogenous Foxp3 in splenic CD4$^+$CD25$^-$ Treg cells freshly isolated from 8-week-old WT and Mst1$^{-/-}$ mice and treated with MG132 or DMSO (F). When the protein level or stability of exogenous Foxp3 was analyzed in 293T cells, exogenous EGFP was co-expressed with Foxp3 and treated with MG132 or DMSO (F). When the protein level or stability of exogenous Foxp3 was analyzed in 293T cells treated with proteasome inhibitor MG132 ($100\mu$M), the reduced endogenous Foxp3 protein level in Mst1$^{-/-}$ splenic CD4$^+$CD25$^-$ Treg cells freshly isolated from 8-week-old mice. E–G, Mst1 blocks proteasome-mediated Foxp3 degradation. Le
bands was determined with ImageJ and normalized with endogenous GAPDH or exogenous EGFP.

In Vitro Kinase Assay—Kinase assay was performed as described by Tao et al. (37). Briefly, HA-tagged Mst1 was immunoprecipitated from the lysate of 293T cells transfected with pcDNA3.1-HA-Mst1 using anti-HA-agarose beads and incubated with 1 μg of GST-Foxp3 purified from E. coli in the presence of [\gamma^{32}P]ATP. The reactions were subjected to SDS-PAGE followed by autoradiography to visualize signals of phosphoproteins.

Statistical Analysis—Statistical analysis was conducted using an unpaired t test by GraphPad Prism. A p value < 0.05 was considered significant.

Results

Mst1 Interacts with the Foxp3 Protein—It has been shown that Mst1 enhances the stability of Foxo1/3 proteins in T cells (30) and promotes nuclear entry of the Foxo1/3 proteins in 293T cells and granule neurons (21) by phosphorylating the proteins at the conserved sites within a Forkhead domain. Mst1 deficiency impairs Treg development and results in autoimmunity in mice by diminishing Foxo1/3-mediated Foxp3 transcription (30). Because Foxo1/3 and Foxp3 proteins share a conserved Forkhead domain, we tested whether the Foxp3 protein could also be a substrate of the Mst1 kinase. Although we failed to detect obvious Mst1-mediated Foxp3 phosphorylation (Fig. 1A), Foxp3 was found to be co-immunoprecipitated with Mst1 when co-transfected in 293T cells (Fig. 1, B and C). This result was also repeated for endogenous Foxp3 and Mst1 proteins in mouse lymphoid T cells (Fig. 1D). To assess the potential consequences of Mst1-Foxp3 interaction, we expressed exogenous GFP-Foxp3 fusion protein in WT and Mst1−/− MEFs. We found that the GFP-Foxp3 fusion protein was located in 100% of nuclei of both WT and Mst1−/− MEFS (Fig. 1E), indicating that Mst1 deficiency does not affect the subcellular localization of the Foxp3 protein.

Mst1 Stabilizes Foxp3 Protein in Vitro and in Vivo—Although Mst1 deficiency has no effect on the Foxp3 nuclear localization, the Foxp3 protein level was higher when Foxp3 and Mst1 were co-expressed in 293T cells when compared with that in the control cells transfected with Foxp3 alone (Fig. 2A). Conversely, the Foxp3 protein level was significantly lower in 293T cells co-transfected with Foxp3 and shMst1 (with a Mst1 knockdown plasmid) (Fig. 2B), or Foxp3 and dominant negative Mst1K59R, than the controls (Fig. 2C). The Foxp3 protein level was also significantly lower in Mst1-deficient Tregs (Fig. 2D). Furthermore, the lower levels of Foxp3 proteins in Mst1 knockdown 293T cells (Fig. 2E) or in Mst1-deficient Tregs (Fig. 2F) could be recovered when the cells were treated with MG132, a proteasome inhibitor. These results suggest that Mst1 enhances Foxp3 stability. To further confirm the effect of Mst1 on Foxp3 stability, we evaluated the degradation of Foxp3 when protein production was blocked by cycloheximide. Our results reveal that the degradation of ectopically expressed Foxp3 protein was significantly slowed in the cells co-transfected with Mst1 when treated by cycloheximide (Fig. 2G).

Altogether, our studies show that Mst1 can up-regulate the expression of Foxp3 protein at the post-translational level, in addition to at the transcriptional level through enhancing the stability of Foxo1/3 proteins (30). Our data also suggest that the Mst1 kinase activity may be required for Mst1-mediated Foxp3 post-translational regulation (Fig. 2C).

Mst1 Enhances Foxp3 Activity—To further investigate the effect of Mst1 protein on the function of Foxp3 protein, we first evaluated Foxp3 transcriptional repressor activity in vitro using a luciferase reporter driven by IL-2 promoter, the activity of which is repressed by Foxp3. The results show that the Foxp3 repressive activity was significantly enhanced when Foxp3 was co-expressed with Mst1 (Fig. 3A, Bar 11), or impaired when Foxp3 was co-transfected with shMst1 to knock down endogenous Mst1 expression (Fig. 3A, Bar 12) in Jurkat T cells stimulated by PMA and ionomycin. Consistent with the above in vitro data, expression of endogenous IL-2, IFN-γ, and Pde3b genes, which were direct targets of Foxp3 and down-regulated by Foxp3, was up-regulated in Mst1−/− Tregs (Fig. 3, B and C). Meanwhile CD25 and Nrp1, which were up-regulated by Foxp3, were down-regulated in Mst1−/− Tregs (Fig. 3, C and D). These data indicate that Mst1 enhances Foxp3 transcription activity.

Mst1 Enhances Foxp3 Acetylation—Post-translational modifications of the Foxp3 protein such as phosphorylation, acetylation, and ubiquitination affect the protein’s stability and activity (7, 8, 10, 38). Because we failed to detect obvious Mst1-mediated Foxp3 phosphorylation (Fig. 1A), we tested whether Mst1 might enhance Foxp3 protein stability by modulating Foxp3 protein acetylation. To analyze the acetylation status of

FIGURE 3. Foxp3 activation by Mst1. A, exogenous Mst1 increases Foxp3 transcriptional activity in T cells cultured in vitro. Shown is quantification of relative Foxp3 transcriptional activity in Jurkat T cells transfected with a luciferase reporter plasmid containing the IL-2 promoter together with the plasmids expressing Foxp3, Mst1, or shMst1 only or both Mst1 and Foxp3, or both shMst1 and Foxp3. 40 h after transfection, the cells were stimulated with PMA and ionomycin for another 6 h. B–D, Mst1 deficiency impairs Foxp3 activity in vivo. B and D, intracellular FACS analyses of the expression of IL-2 and IFN-γ in splenic CD4+ CD25− Tregs freshly isolated from 8-week-old WT and Mst1−/− mice and stimulated with PMA and ionomycin for 6 h (B) or the expression of CD25 in the same cells without any treatment (D). C, the expression of Foxp3 target genes, Nrp1 and Pde3b, was also analyzed at mRNA levels by real-time RT-PCR. The numbers above boxes in B are the percentages of IL-2- or IFN-γ-positive cells. Values in A, C, and D represent the means ± S.E. of three independent experiments. #, p < 0.05; **, p < 0.01, ***, p < 0.001; ns, not significant. % Max, percentage of maximum.
Mst1 Up-regulates Foxp3 Stability and Treg Functions

**FIGURE 4. Enhanced acetylation and stability of the Foxp3 protein by Mst1.** A, Mst1 enhances Foxp3 acetylation. Left and right panels, IP-Western blot (left panel) and quantitative (right panel) of the acetylation level of exogenous Foxp3 analyses in 293T cells transfected with FLAG-tagged Foxp3 alone, or with HA-tagged Mst1. The acetylation of immunoprecipitated Foxp3 was visualized using pan-acetylated lysine antibody. B, Mst1 inhibits Foxp3 ubiquitination. Myc-tagged-Foxp3 was expressed alone or with HA-tagged Mst1 in 293T cells, and 24 h later, the transfected cells were treated with MG132 for 2 h. Then, the Foxp3 protein was immunoprecipitated followed by Western blot with anti-ubiquitin antibodies. C and D, Mst1 rescued Sirt1-mediated reduction of protein level and acetylation of Foxp3. E and F, Western blot (C) and IP-Western blot (D) were used for analyzing protein levels (C) and acetylation levels of Foxp3. G, respectively, of exogenous Foxp3 in 293T cells transfected with the different set of genes as indicated. E-G, inhibition of Sirt1 blocked Mst1-mediated effects on the Foxp3 protein. The acetylation (E) and protein level (F and G) of exogenous Foxp3 were analyzed by IP-Western blot (E) or Western blot (F and G), respectively, in 293T cells co-transfected with Myc-Foxp3 and HA-Mst1 (E), or Myc-Foxp3 and shMst1 (F) separately, followed by 50 µM Ex-527 treatment for 20 h, or co-transfected with Myc-Foxp3 shMst1 and shSirt1 together (G). Co-transfected EGFP was used to monitor transfection efficiency and a loading control in C and D. G. Foxp3 acetylation in A, D, and E was detected using pan-acetylated lysine antibodies. Immunoprecipitated Foxp3 in A, B, D, and E served as a loading control accordingly. Blots shown are representative of three experiments with similar results. Right panels in A and C-G are quantitative analyses of the corresponding blots in A and C-G. Values represent the means ± S.E. of three separate experiments. Ack-Foxp3, acetylated Foxp3. *p < 0.05; **, p < 0.01; *** p < 0.001; ns, not significant.

Foxp3, Foxp3 and Mst1 were co-transfected into 293T cells and the Foxp3 protein was immunoprecipitated from cell lysate, followed by blotting with an anti-acetyl lysine antibody. The results show that the acetylation level of Foxp3 was increased when Mst1 was co-transfected (Fig. 4A), indicating that Mst1 may increase Foxp3 protein stability by enhancing Foxp3 acetylation. Acetylation of Foxp3 results in increased protein levels by preventing polyubiquitination (7, 10). Consistent with these previous studies, we also found that expression of exogenous Mst1 reduced Foxp3 ubiquitination (Fig. 4B), probably due to Mst1-mediated increase of Foxp3 acetylation.

**Mst1 Enhances Foxp3 Acetylation and Stability by Inhibiting Sirt1 Activity**—The transcription activity and stability of Foxp3 can be dynamically regulated by reversible acetylation (7, 8, 10, 38). Sirt1 is one of the deacetylases that deacetylates Foxp3 (10), and Sirt1’s activity can be inhibited by Mst1-mediated phosphorylation (32). Thus, we hypothesized that Mst1 enhanced Foxp3 stability by inhibiting Sirt1 activity. To test this possibility, we first examined whether Mst1 could affect Sirt1-mediated Foxp3 degradation. The results show that expression of exogenous Mst1 suppressed Sirt1-mediated Foxp3 degradation (Fig. 4C). Then, we assessed the acetylation of Foxp3 in 293T cells expressing exogenous Ep300, Foxp3, and Sirt1 with or without Mst1 by IP-Western blot. Our study demonstrates that Mst1 could inhibit Sirt1-mediated deacetylation of the Foxp3 protein (Fig. 4D). We also show that Mst1 was unable to further enhance acetylation of Foxp3 when Sirt1 activity was inhibited by Ex-527, a specific inhibitor for Sirt1 (Fig. 4E). Furthermore, treatment of Ex-527 or knockdown endogenous Sirt1 was able to prevent Mst1 knockdown-mediated degradation of the Foxp3 proteins (Fig. 4, F and G). All these data demonstrate that Mst1 enhanced Foxp3 stability by inhibiting Sirt1 activity.

**Mst1 Blocks the Access of Sirt1 to Foxp3**—Although Mst1 can enhance Foxp3 acetylation and stability by inhibiting Sirt1
Mst1 Up-regulates Foxp3 Stability and Treg Functions

Transcription factor Foxp3 is essential for the proper development and function of Tregs (39), which are important for maintaining immune homeostasis (40, 41). Because maintenance

Mst1 Promotes Foxp3 Activity and Treg Function by Suppressing Sirt1—Because the above results show that Mst1 could enhance Foxp3 protein stability by inhibiting Sirt1 activity and blocking the access of Sirt1 to Foxp3, we asked whether Mst1 could reverse Sirt1-mediated suppression of Foxp3 transcription repressor activity. To answer this question, we analyzed Sirt1’s inhibition on Foxp3 activity with or without Mst1. Our study shows that when co-transfected with Mst1, the Sirt1-mediated repression of Foxp3 transcription activity was fully eliminated (Fig. 6A). Next, we further asked whether the impaired suppressive function of Mst1+/− Tregs was related to the increase of Sirt1 activity resulting from the loss of Mst1’s inhibition on Sirt1. To address this question, we assessed the suppressive activity of Mst1+/− Tregs with or without Ex-527 treatment. CFSE-labeled WT CD4+CD25− T cells were co-cultured with purified WT or Mst1+/− Tregs (at a 2:1 ratio) in the presence or absence of Ex-527. The results show that impaired suppressive function of Mst1+/− Tregs was partially rescued by Ex-527 treatment (Fig. 6B). Thus, we conclude that Mst1 promotes Foxp3 transcription activity and Treg function by inhibiting Sirt1 activity.

Discussion

Transcription factor Foxp3 is essential for the proper development and function of Tregs (39), which are important for maintaining immune homeostasis (40, 41). Because mainte-
regulates ease due to impaired Tregs and demonstrated that Mst1 up-
shown that expression and Treg development/function by modulating 
protein binding in a kinase-independent manner. Thus, our study 
protein to regulate protein stability through competing for pro-
lylating and inhibiting Sirt1, but also by directly interacting with 
Sirt1-mediated Foxp3 deacetylation not only through phosphor-
lation, and phosphorylation, also plays important roles in the 
control of Foxp3 protein level and Tregs functions (47). In this 
study, we present for the first time evidence that Mst1 enhances 
the stability and transcriptional activity of Foxp3 by inhibiting 
Sirt1-mediated Foxp3 deacetylation not only through phosphor-
ylating and inhibiting Sirt1, but also by directly interacting with 
Foxp3 and physically blocking the binding of Sirt1 to Foxp3. 
Our results indicate that Mst1 can also function as a “scaffold” 
protein to regulate protein stability through competing for pro-
tein binding in a kinase-independent manner. Thus, our study 
reveals a novel mechanism by which Mst1 regulates Foxp3 
expression and Treg development/function by modulating 
Foxp3 protein stability. Because our previous studies have 
shown that Mst1-deficient mice are prone to autoimmune dis-
eease due to impaired Tregs and demonstrated that Mst1 up-
regulates Foxp3 transcription by stabilizing Foxo1/3 proteins 
through direct phosphorylation of Foxo1/3 proteins and indi-
rect inhibition of Akt activity (30), we propose that Mst1 regu-
lates Foxp3 protein expression at both transcriptional and post-
translational levels.

Several deacetylases have recently been shown to regulate 
Foxp3 acetylation and thereby stability and transcriptional 
activity (7–11, 47). However, how the activities of these 
deacetylases are regulated in vivo, and how important they are 
under homeostatic conditions, remains to be determined. The 
results that we show here suggest that upon T cell receptor 
(TCR) stimulation, Mst1 enhances Foxp3 protein stability and 
activity by increasing acetylation of Foxp3 protein through 
inhibiting Sirt1 activity and interfering with Sirt1 binding to 
Foxp3. Therefore, Mst1-mediated post-translational modifica-
tion on Foxp3 may offer a quick way to regulate the cellular 
protein level of Foxp3 in response to extracellular signals.

Mst1 can also regulate P53 stability and activity through 
inhibiting Sirt1 (32). In this study, we have observed that Mst1- 
Sirt1 axis can control Foxp3 stability and activity by modifying 
Foxp3 acetylation. These studies provide strong evidence to 
support that Mst1-Sirt1 axis may be a conserved pathway reg-
ulating multiple biological processes. Therefore, our study 
deepens understanding of the fundamental functions mediated 
or controlled by the Hippo/Mst1 signaling.

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Mst1 Up-regulates Foxp3 Stability and Treg Functions

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