The long non-coding RNA rhabdomyosarcoma 2-associated transcript exerts anti-tumor effects on lung adenocarcinoma via ubiquitination of *SOX9*

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Background: Long non-coding RNAs (lncRNAs) play an important role in the post-translational modification of proteins, but the importance of lncRNAs in protein ubiquitination remains unclear. This study investigated to role of the lncRNA rhabdomyosarcoma 2-associated transcript (*RMST*) in lung adenocarcinoma (LUAD).

Methods: The expression of *RMST* was analyzed in LUAD samples and normal lung tissues using data from The Cancer Genome Atlas (TCGA) and The Genotype-Tissue Expression (GTEx) public databases. Colony formation and transwell assays were used to determine the anti-tumor effects of *RMST* in human LUAD progression. RNA pull-down assays, RNA immunoprecipitation assays, and mass spectrometry were used to determine the mechanisms by which *RMST* induces the ubiquitination of SRY-box transcription factor 9 (*SOX9*). Furthermore, animal models were used to determine the effects of *RMST* on LUAD tumorigenicity *in vivo*.

Results: Compared with normal tissues, *RMST* expression was significantly downregulated in LUAD samples. This abnormal expression of *RMST* led to significant changes in the proliferation and migration of LUAD cells both *in vitro* and *in vivo*. The experiments demonstrated that *RMST* binds directly to the *SOX9* protein, resulting in the ubiquitination of *SOX9* and this was mediated by F-box and WD repeat domain-containing 7 (*FBW7*). Clinically, *RMST* expression was shown to be positively correlated with the overall survival of LUAD patients.

Conclusions: These findings revealed that *RMST* suppressed the *SOX9* signaling pathway to inhibit LUAD growth and metastasis. The *RMST*-induced ubiquitination of *SOX9* via *FBW7* may be a potential therapeutic target for the treatment of patients with LUAD.

Keywords: Long non-coding RNA (lncRNA); lung adenocarcinoma (LUAD); rhabdomyosarcoma 2-associated transcript (*RMST*); SRY-box transcription factor 9 (*SOX9*); ubiquitin

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Introduction

Lung adenocarcinoma (LUAD) is the most common cancer worldwide. Despite advances in surgical treatment and chemotherapy, the prognosis of LUAD patients is still poor due to metastasis and recurrence. Furthermore, the mechanisms involved in the growth and metastasis of LUAD remain unclear. Understanding the molecular mechanisms underlying the growth and metastasis of LUAD will facilitate the development of improved prognostic indicators and therapeutic targets.

Long noncoding RNAs (lncRNAs) are RNA transcripts with a length of more than 200 nucleotides, that lack important protein coding ability (1). In the past two decades, the mechanisms of microRNAs (miRNAs) and mRNAs have been widely studied. However, the role of lncRNAs remains unclear. LncRNAs can act on tumor stem cells, affecting tumor initiation, development, and metastasis (2). Recently, lncRNAs have been described as ubiquitin-mediated protein hydrolysis inducers and identification of their interacting partners may further our understanding regarding the molecular mechanisms of lncRNAs (3).

Rhabdomyosarcoma 2-associated transcript (*RMST*) is a lncRNA located in chromosome band 12q23.1.24. Recent studies have reported an association between *RMST* and glioblastoma multiforme and breast cancer (4,5). Indeed, *RMST* plays an anti-tumor role in triple negative breast cancer by inhibiting cell proliferation, invasion, and migration. Previous studies have demonstrated that *RMST* can act as a scaffold to bind different proteins or different protein complexes together (6). For example, *RMST* interacts with sex determining region Y-box 2 (*SOX2*) to regulate neurogenesis (6). Peng *et al.* reported that *RMST* can enhance DNA methyltransferase 3 (*DNMT3*) expression through interaction with human antigen R (*HuR*) (7). However, to date, the expression and function of *RMST* in LUAD has not been reported.

This study revealed that the expression of *RMST* was significantly downregulated in LUAD samples compared to healthy controls. Furthermore, *RMST* suppressed the proliferation and migration of LUAD cells *in vitro* and *in vivo*. Notably, *RMST* was found to directly bind *SOX9* to induce its degradation via F-box and WD repeat domain-containing 7 (*FBW7*). These results suggested that the combination of *RMST* and *SOX9* may be a valuable prognostic factor for LUAD patients.

We present the following article in accordance with the ARRIVE reporting checklist (available at https://dx.doi.org/10.21037/atm-21-6052).

Methods

Tissues and database

Data from The Cancer Genome Atlas (TCGA) and The Genotype-Tissue Expression (GTEx) public databases were used for this study. Furthermore, tissue samples were collected from LUAD patients who underwent surgery in the Zhejiang Provincial People's Hospital. A total of 90 pairs of tumor and adjacent non-tumor tissues were assessed. Written informed consent was obtained from all patients. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The present study was approved by the Ethics Committee of Zhejiang Provincial People's Hospital.

Cell culture

The human LUAD cell lines A549 and H1650 were obtained from the American Type Culture Collection (ATCC) and cultured as previously described (8). Cell lines were cultured in RPMI-1640 medium containing 10% fetal bovine serum with 5% CO_2 at 37 ℃.

Colony formation assay, cell counting kit-8 (CCK8) assays and transwell assay

Colony formation assays, CCK8 assays and transwell assays were performed as previously described (8). In transwell assay, 700 μL RPMI-1640 containing 10% fetal bovine serum was added to the lower chamber, at the same time, 200 μL serum-free medium containing 10^5 cells was added to the upper chamber. After 24 hours, chambers were fixed in 4% paraformaldehyde and stained with 0.2% crystal violet.

RNA extraction and quantitative polymerase chain reaction (qPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) and reverse transcribed using the reverse transcription kit from Promega (Madison, USA). The SYBR Green Mix (ABI, CA, USA) was used for qPCR reactions. All primers used in the present study are listed in Table S1.

Western blot analysis

Western blots were performed as previously described (9). The protein was extracted from cell lysate, denatured, transferred to PVDF membrane by electrophoresis, and then incubated with antibody. Antibodies used in this study are listed in Table S2.

Co-immunoprecipitation (Co-IP) assay

Co-IP assays were performed as previously described (10). Cell lysates were incubated with antibodies and magnetic beads before washing and electrophoresis. Antibodies used in this study are listed in Table S2.

Constructs

The sequences of the small interfering RNAs (siRNAs) si*RMST*, si*FBW7*, and the negative control (siNC) are listed in Table S3. The *RMST* overexpression plasmid pcDNA3.1-*RMST* was purchased from Integrated Biotech Solutions (Shanghai, China).

RNA pull-down assay, mass spectrometry, and RNA immunoprecipitation (RIP) assay

The RiboMAX Large Scale RNA Production Systems (Promega, USA) and the Biotin RNA Labeling Mix (Roche, USA) were used to synthesize full-length *RMST in vitro* for the RNA pull-down assays. The biotin-labelled RNA was incubated with the cell lysate and the streptavidin-coated beads in accordance with the manufacturer's instructions. The lysate was then washed three times and examined via Western blot analysis. The protein bands were identified by mass spectrometry.

RIP assays were performed using the RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) according to the manufacturer's instructions. Briefly, cell lysates were incubated with anti-*SOX9* and anti-IgG antibodies. The precipitated RNA was reverse transcribed into cDNA, followed by amplification with *RMST* primers.

Fluorescence in situ hybridization (FISH)

The FISH Tag RNA Green Kit (Invitrogen, USA) was used according to the manufacturer's protocol. *RMST* detection probes were synthesized using linearized plasmids containing T7 RNA polymerase. Hybridization and washing were performed as per the manufacturer's instructions.

Animal model

Six-week-old male BALB/c nude mice were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and housed in specific pathogen-free (SPF) conditions. Mice were randomly assigned to the experimental group and the control group. The LUAD cell lines A549 and H1650 were transfected with plasmids overexpressing *RMST* or silencing *RMST* (si*RMST*), and their respective plasmid negative controls (NC) with Lipofectamine 2000. The transfected cells were subcutaneously injected into mice at a concentration of 2×10^6 cells/100 μL per mouse. All mice were euthanized at the end of the experiment. The tumor grafts from the mice were harvested and fixed. Tumors were measured and recorded every 5 days, and the tumor volumes were calculated as previously described (11). Animal experiments were performed under a project license [No. KS(Y)21220] granted by ethics board of Zhejiang Provincial People's Hospital, in compliance with the Guide for the Care and Use of Laboratory Animals of Zhejiang Provincial People's Hospital. A protocol was prepared before the study without registration.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 (La Jolla, CA, USA) and SPSS software version 19.0 (IBM Corporation, Armonk, NY). The experimental data was analyzed using *t*-tests. A P value <0.05 was considered statistically significant.

Results

Down-regulation of RMST is correlated with progression of lung adenocarcinoma

Sequencing data of LUAD from the TCGA and GTEx databases revealed that *RMST* expression was downregulated in LUAD samples compared to normal tissues (*Figure 1A,1B*). In addition, *RMST* expression was downregulated at all stages of LUAD compared with adjacent healthy tissues (*Figure 1C*).

The downregulation of *RMST* in LUAD was validated using clinical samples obtained from patients admitted to our hospital with LUAD (*Figure 1D*). Kaplan-Meier analysis

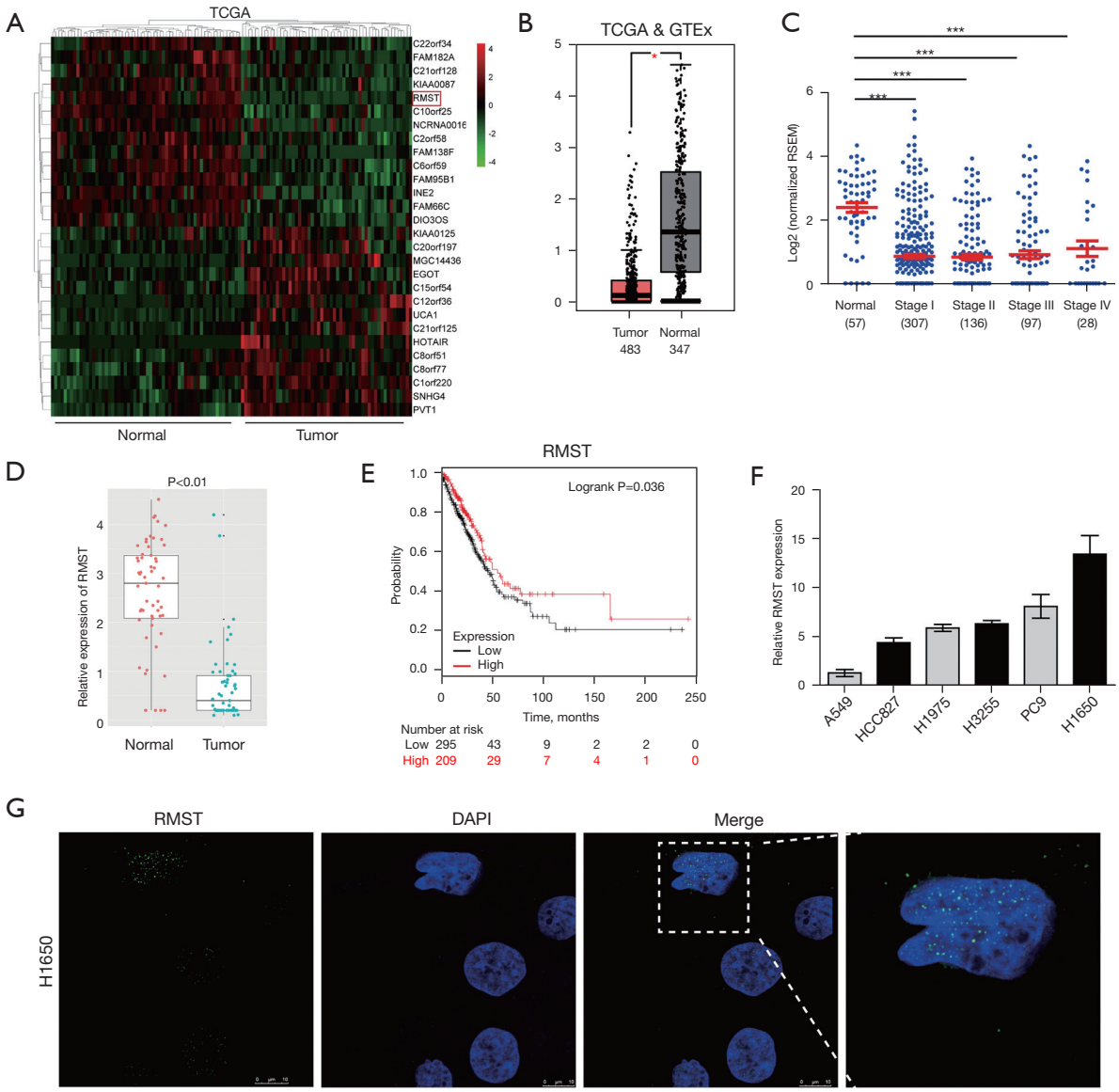

Figure 1 Down-regulation of *RMST* is correlated with LUAD progression. (A) A heatmap showing the differently expressed lncRNAs in the LUAD samples from the TCGA database. (B) Differential expression of *RMST* between tumor samples and normal tissues in the TCGA and GTEx databases. (C) *RMST* expression at different pathological stages of LUAD. (D) *RMST* expression in clinical samples. (E) Kaplan-Meier curve analysis of LUAD patients with high and low expression of *RMST*. (F) *RMST* expression in LUAD cell lines. (G) Fluorescent labeled hybridization probes were used and confocal micrographs showing *RMST* localization. *P<0.05, ***P<0.001. LUAD, lung adenocarcinoma; RMST, rhabdomyosarcoma 2-associated transcript; LUAD, lung adenocarcinoma, lncRNA, long non-coding RNA; OD, optical density; TCGA, The Cancer Genome Atlas; GTEx, The Genotype-Tissue Expression.

revealed that low expression of *RMST* was correlated with poor survival in LUAD patients (*Figure 1E*).

RMST expression was also detected in different LUAD cell lines. As shown in *Figure 1F*, A549 cells had the lowest *RMST* expression, while *RMST* was highly expressed in H1650 cells. FISH demonstrated that *RMST* was mostly

localized to the nucleus (*Figure 1G*).

RMST suppresses cell proliferation and migration in vitro and tumorigenicity in vivo

To evaluate the functional role of *RMST* in LUAD cells, colony formation and cell migration assays were performed. *RMST* was overexpressed in A549 cells (A549/*RMST* cells) and knocked down in H1650 cells (H1650/si*RMST*) and qPCR was used to validate the overexpression and knockdown efficiencies (*Figure 2A*). Colony formation assays, Cell Counting Kit-8 (CCK8) assays, and transwell assays demonstrated that *RMST* overexpression in A549 cells resulted in decreased cell proliferation and migration, while *RMST* knockdown in H1650 accelerated cell proliferation and migration (*Figure 2B-2D*). These results indicated that *RMST* suppressed cell proliferation and migration *in vitro*.

To further validated these effects *in vivo*, the A549/*RMST* cells and H1650/si*RMST* cells were injected into nude mice subcutaneously. Consistent with the *in vitro* assays, *RMST* overexpression suppressed tumor volume and weight, while *RMST* knockdown showed the opposite effect (*Figure 2E-2G*). The immunohistochemical staining for Ki-67 in tumors harvested form nude mice showed that *RMST* overexpression resulted in a lower Ki-67 index, while *RMST* knockdown increased the Ki-67 index (*Figure 2H*). These results suggested that *RMST* suppressed tumorigenesis of LUAD cells *in vivo*.

RMST interacts with SOX9 and promotes SOX9 degradation via ubiquitination

Since lncRNAs can interact with proteins (12), RNA-pull down assays and mass spectrometry (MS) were performed to identify proteins that interact with *RMST* in LUAD cells. MS analysis revealed that *SOX9* was a potential target for interaction with *RMST* (*Figure 3A*). RNA-pull down assays and RNA immunoprecipitation (RIP) assays validated the interaction between *SOX9* and *RMST* (*Figure 3B*,*3C*). Interestingly, *RMST* overexpression or knockdown did not alter *SOX9* mRNA levels. However, *RMST* overexpression decreased *SOX9* protein levels and *RMST* knockdown elevated *SOX9* protein expression in LUAD cells (*Figure 3D*,*3E*). These results suggested that the *SOX9* protein might be a target for *RMST*.

To investigate the mechanisms by which *RMST* interacts with *SOX9*, LUAD cells were treated with cycloheximide (CHX). As shown in *Figure 3F*, *RMST* overexpression shortened the half-life of the *SOX9* protein compared to control cells. Furthermore, when cells were treated with the proteasome inhibitor MG132, *SOX9* protein levels were elevated compared with control cells (*Figure 3G*). These results suggested that ubiquitination may be involved in the *RMST*-mediated downregulation of *SOX9* protein. Indeed, significantly increased levels of ubiquitinated *SOX9* protein were detected in cells overexpressing *RMST* compared to control cells (*Figure 3H*). Taken together, these results indicated that ubiquitination may be involved in *RMST*-mediated *SOX9* protein degradation.

RMST-FBW7 mediates SOX9 ubiquitination

To further investigate the mechanisms by which *RMST* regulates *SOX9* ubiquitination, the mass spectrometry data was analyzed and ubiquitin ligase *FBW7* was identified. *FBW7* is a ubiquitin ligase component which targets substrates for degradation. As shown in *Figure 4A*, expression of *FBW7* was not altered in cells overexpressing *RMST*. Indeed, co-IP assays demonstrated that the interaction between *SOX9* and *FBW7* was strengthened by *RMST* in A549 cells treated with MG132 (*Figure 4B*,*4C*). These data suggested that *FBW7* might be a mediator for *SOX9* ubiquitination by *RMST*. When *FBW7* expression was silenced in A549 cells, the protein levels of *SOX9* increased (*Figure 4D*). Moreover, *SOX9* ubiquitination was significantly decreased in A549/si*FBW7* cells compared with negative control cells (*Figure 4E*). Collectively, these data indicated that *RMST* may strengthen the interaction between *FBW7* and *SOX9*, and that *FBW7* enhances the ubiquitination and degradation of *SOX9*.

RMST inhibits cell proliferation and migration in a SOX9-dependent manner

Kawai *et al.* reported that *SOX9* can act as a transcriptional factor (TF) (13). To identify the target genes of *SOX9*, the ChIP-seq data in the public database GSE114305 was analyzed (*Figure 5A*). The target genes modulated by *SOX9* include *Smad3*, *Slug*, and *ZEB1*. These genes are important for inducing the endothelial-mesenchymal transition (EMT) process in various types of tumors (14-16). To determine whether *SOX9* modulates *Smad3*, *Slug*, and *ZEB1* in LUAD, ChIP assays were performed. As shown in *Figure 5B*, *SOX9* directly binds to the promoters of *Smad3*, *Slug*, and *ZEB1*. The PCR results showed that *SOX9* knockdown decreased

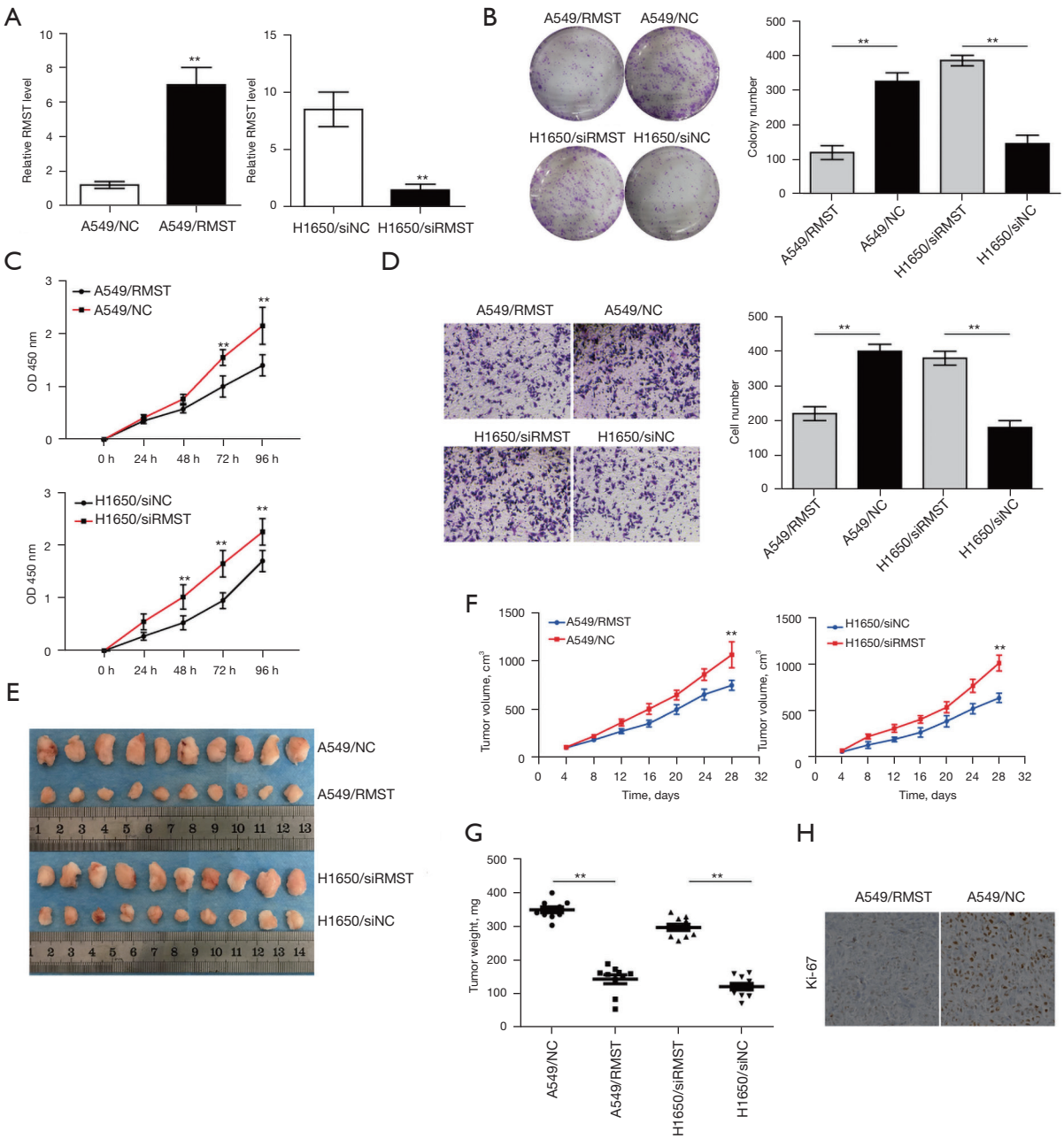

Figure 2 *RMST* suppresses cell proliferation and migration *in vitro* and tumorigenicity *in vivo*. (A) *RMST* overexpression and knockdown efficiency in A549 and H1650 cells. (B) Colony formation ability of cells with *RMST* overexpression or silencing (magnification 400×). (C) Cell proliferation ability in cells with *RMST* overexpression or silencing. (D) Cell migration ability in cells with *RMST* knockdown or overexpression (magnification 400×). (E-G) *RMST* overexpression reduced tumor volume and tumor weight, while *RMST* knockdown induced increased tumor volume and weight. (H) Ki-67 staining of tumor allografts from mice injected with cells overexpression *RMST* and mice injected with negative control cells (magnification 400×). Cells were stained with crystal violet. **P<0.01. *RMST*, rhabdomyosarcoma 2-associated transcript.

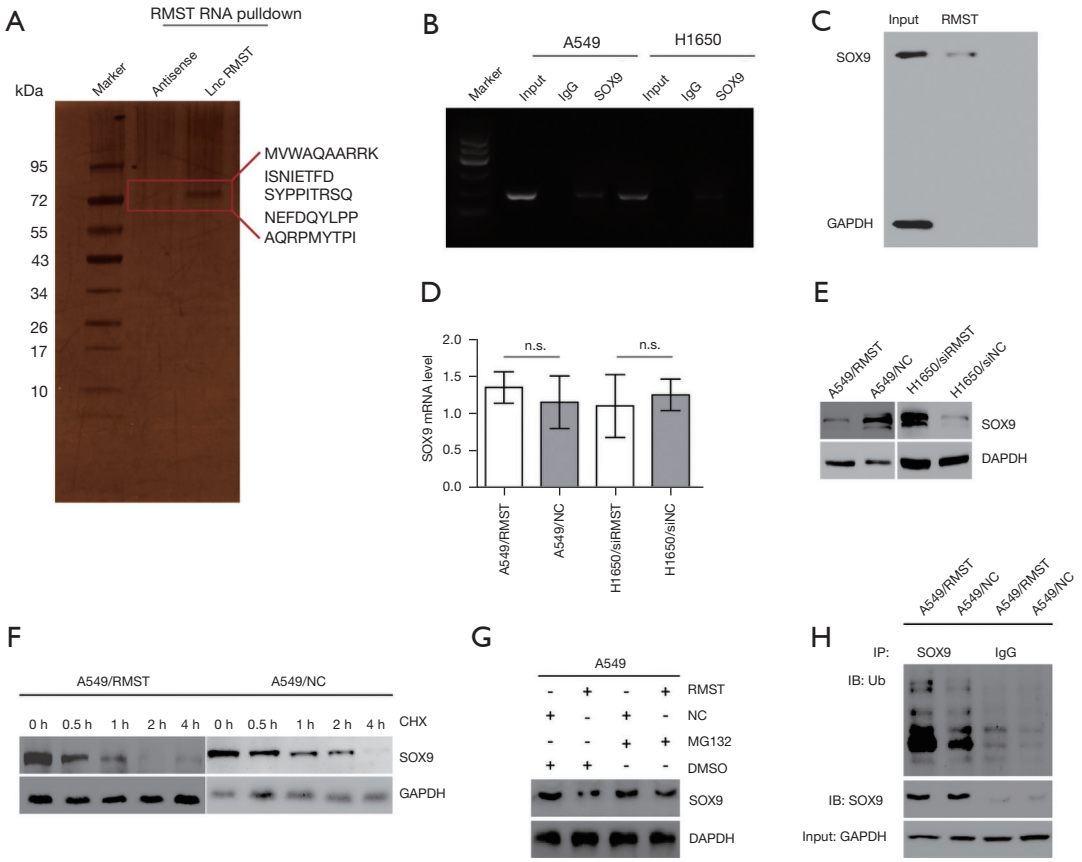

Figure 3 *RMST* interacts with *SOX9* and promotes *SOX9* degradation via ubiquitination. (A) Identification of *SOX9* by silver staining and mass spectrometry. (B) RNA immunoprecipitation assay showing the interaction between *RMST* and *SOX9*. (C) RNA pull-down assay showing the interaction between *RMST* and *SOX9*. (D) *SOX9* mRNA levels in cells with *RMST* overexpression, *RMST* knockdown, and control cells. (E) *SOX9* protein levels in cells with *RMST* overexpression, *RMST* knockdown, and control cells. (F) *SOX9* protein levels in cells overexpressing *RMST* after treatment with CHX. (G) *SOX9* protein levels in cells overexpression *RMST* after treatment with MG132. (H) Ubiquitination levels of *SOX9* in cells overexpressing *RMST* following treatment with MG132. *RMST*, rhabdomyosarcoma 2-associated transcript; *SOX9*, sex determining region Y-box 2; CHX, cycloheximide; +, add the reagent; –, not use the reagent; n.s., not statistically significant.

the expression of *Smad3*, *Slug*, and *ZEB1* (*Figure 5C*). The protein expression of *Smad3*, *Slug*, *ZEB1*, and other EMT markers were also reduced after silencing of *SOX9* (*Figure 5D*). To evaluate whether *RMST* functions in LUAD cells in a *SOX9*-dependent manner, colony-formation and transwell assays were performed. Indeed, *RMST* silencing increased cell proliferation and migration, and this was impaired by the simultaneous silencing of *SOX9* (*Figure 5E*). Conversely, *RMST* overexpression inhibited cell proliferation and migration, and this was reversed by the simultaneous overexpression of *SOX9* (*Figure 5F*).

Discussion

Accumulating research has been attracted to lncRNA because of its crucial role in LUAD. Liu *et al.* reported the relationship between LncRNA and redox (2). Lu *et al.* reported LncRNA induced ubiquitination of EZH2 (17). An increasing number of studies have focused on the post-translational modification of proteins by lncRNA, such as ubiquitination. Evidence shows that ubiquitination modification plays an important role in tumorigenesis. As an important post-translational modification of proteins,

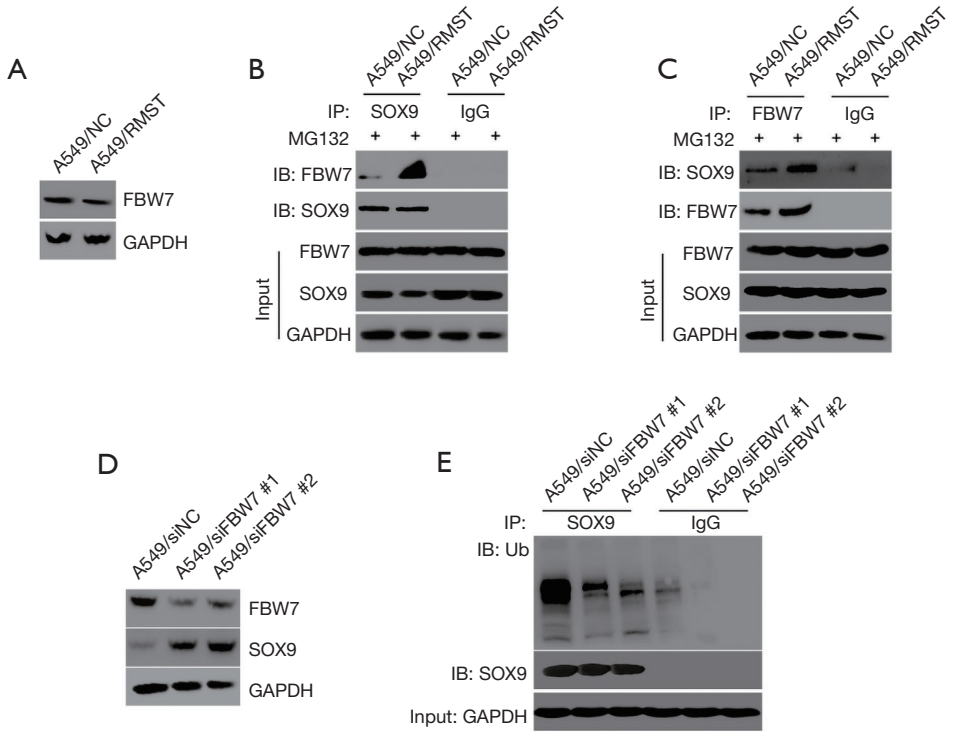

Figure 4 *RMST-FBW7* mediates *SOX9* ubiquitination. (A) *FBW7* protein levels in A549/*RMST* cells. (B,C) Cell lysates of A549/*RMST* cells and A549/NC cells were immunoprecipitated with anti-*SOX9* and anti-*FBW7* antibodies and analyzed by Western blot. (D) *FBW7* and *SOX9* protein levels in A549/si*FBW7* cells and A549/siNC cells. (E) Cell lysates of A549/siFBW7 cells and A549/siNC cells were immunoprecipitated with anti-*SOX9* antibodies and analyzed by Western blot. *RMST*, rhabdomyosarcoma 2-associated transcript; *FBW7*, F-box and WD repeat domain-containing 7; *SOX9*, sex determining region Y-box 2; si, small interfering RNA; NC, negative control; +, add the reagent.

ubiquitination has always been the focus and hotspot of tumor biology research. Ubiquitination modification is a reversible enzyme cascade reaction, which is precisely regulated by ubiquitin ligases. This current study demonstrated that the lncRNA *RMST* could suppress the growth and metastasis of LUAD cells through ubiquitination and degradation of *SOX9*, and this process was mediated by *FBW7*. These findings revealed the important role of *RMST* in the growth and metastasis of LUAD and further revealed a novel function of *RMST* as a mediator for the ubiquitination of *SOX9* in LUAD cells.

While *RMST* has been reported to act as a tumor suppressor in various cancers such as thyroid cancer and head and neck squamous cell carcinomas (18,19), other studies have demonstrated that *RMST* plays a role as a tumor activator (18). In this current study, *RMST* expression was downregulated in LUAD tumor tissues compared to normal healthy tissues and high expression of *RMST* was associated with improved survival in LUAD patients. This seemingly contradictory role of *RMST* in cancer cells suggests that *RMST* may exhibit cellular- and tissue-specific functions in a context-dependent manner.

SOX9, which acts as a tumor activator, belongs to the sex determining region Y-related HMG-box (*SOX*) transcription factor family which includes *SOX2*, *SOX4*, *SOX6*, and *SOX9* (20). Many studies have shown that *SOX9* is aberrantly expressed in various malignancies and is associated with tumor progression in lung adenocarcinoma, breast cancer, and colorectal cancer (21-23). Ma *et al.* reported that *SOX9* accelerated tumor growth and metastasis, and activated the EMT process in breast cancer (21). This was consistent with our observations in LUAD cells. While Ng *et al.* previously reported an interaction between *RMST* and *SOX2* (6), the present study documented a direct binding between *RMST* and *SOX9* in LUAD cells. It is unclear whether *SOX2* and

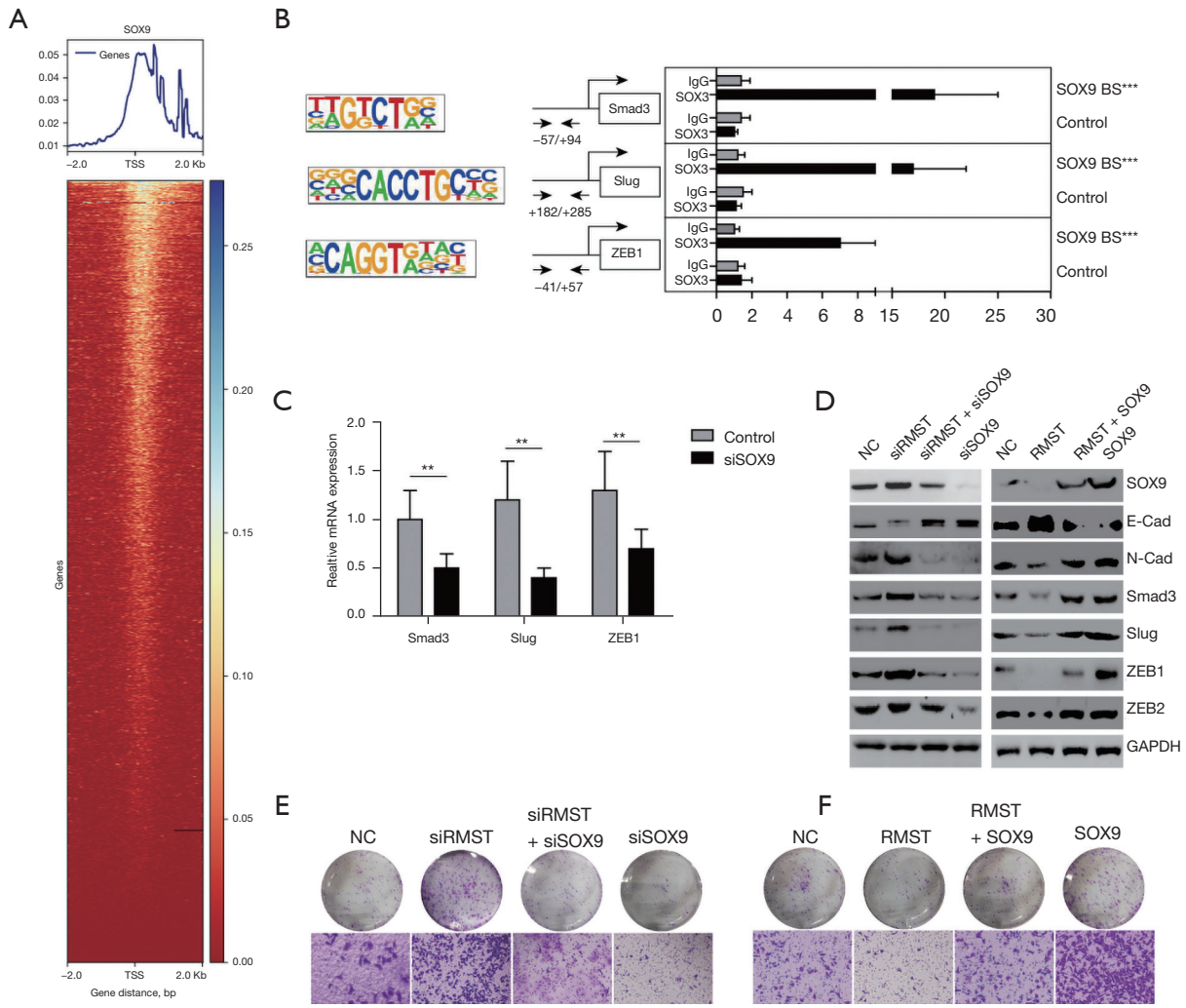

Figure 5 *RMST* inhibits cell proliferation and migration in a *SOX9*-dependent manner. (A) A heatmap of the ChIP-seq data showing that *SOX9* peaks near ±2 kb of the TSS. (B) ChIP assay showing the *SOX9* binding sites which include *Smad3*, *Slug*, and *ZEB1*. (C) Relative mRNA expression of *Smad3*, *Slug*, and *ZEB1* in si*SOX9* cells. (D) *SOX9* regulates *Smad3*, *Slug*, *ZEB1*, and other EMT marker protein levels. (E,F) Colony formation and transwell assays showing that *SOX9* regulates cell proliferation and migration (magnification 400×). Cells were stained with crystal violet. **P<0.01, ***P<0.001. *RMST*, rhabdomyosarcoma 2-associated transcript; *SOX9*, sex determining region Y-box 2; TSS, transcription start sites; EMT, endothelial-mesenchymal transition.

SOX9 have similar structures that enables them to interact with *RMST*. Furthermore, the possible interaction between *RMST* and other members of the *SOX* family warrants further investigation.

The present study revealed that *RMST* could interact with the *SOX9* protein to promote *FBW7*-mediated ubiquitination and degradation of *SOX9*. *FBW7*, an E3 ubiquitin ligase that mediates ubiquitination and degradation of oncoproteins, usually plays a role as a tumor suppressor (24). Many different proteins have been reported as substrates of *FBW7*, including *SOX9* (5,25). This study documented the novel role of *RMST* as a scaffold which interacts with *SOX9* and *FBW7*. These results were verified by RNA pull-down assays, as well as RIP and co-IP assays. We speculate that the interaction between *SOX9* and *RMST* may affect the structure of *SOX9* such that the ubiquitination site of *SOX9* is more readily exposed to *FBW7*. However, the precise mechanisms by which *RMST*

may influence the structure of *SOX9* is unclear and warrants further investigation. In this study, we found that *RMST* inhibited the occurrence and development of lung cancer through *SOX9*. In the next study, we will study how to regulate *RMST* to control lung adenocarcinoma.

Conclusions

In conclusion, this investigation demonstrated a novel lncRNA function whereby *RMST* binds to *SOX9* to mediate the ubiquitination of *SOX9* through ubiquitin E3 ligase *FBW7*. These findings provide new insights into the role of lncRNAs in the progression of LUAD which will contribute to the development of potential strategies for the clinical treatment of LUAD.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://dx.doi.org/10.21037/atm-21-6052

Data Sharing Statement: Available at https://dx.doi.org/10.21037/atm-21-6052

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://dx.doi.org/10.21037/atm-21-6052). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The present study was approved by the Ethics Committee of Zhejiang Provincial People's Hospital and written informed consent was obtained from all patients. Animal experiments were performed under a project license [No. KS(Y)21220] granted by ethics board of Zhejiang Provincial People's Hospital, in compliance with the Guide for the Care and Use of Laboratory Animals of Zhejiang Provincial People's Hospital.

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