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14. ABSTRACT LncRNAs CRNDE is one of the most significantly elevated-expressed genes in HCC. CRNDE levels are reversely correlated with HCC patients' prognosis. Based on our previous data, we assumed that HCC-derived CRNDE acts as inhibitory messenger molecular to impair host immunoreaction in the tumor microenvironment, and thus causing immune evasion and promoting HCC progression. During the 1 st year of the performance, we evaluated the impact of CRNDE on tumor growth <i>in vivo</i> using a novel mouse model of HCC induced by HDTV (Hydrodynamic tail vein) injection of transposase (sleeping beauty)-based plasmids expressing Yap ^{S127A} and β -catenin ^{ΔN90} . we observed that co-injecting CRNDE-expressing plasmids with Yap ^{S127A} / β -catenin ^{ΔN90} promotes HCC growth; while co-injecting shCRNDE plasmids with Yap ^{S127A} / β -catenin ^{ΔN90} attenuate Yap/ β -catenin-induced HCC growth. Further RNA-seq analyses of these mouse HCC tissues indicate that immune system activation level was greatly elevated in CRNDE-depleted HCC when compared with control HCC, demonstrating as higher expression of immune-response related genes; more infiltrating CD8 T-cells, CD4 memory T-cells and NK cells in tumor; and higher level of CD8 T-cells activation in tumor tissues. Next, in order to determine the effect of CRNDE on TILs functions, we carried out granzyme B ELISPOT assays using mouse TILs, which were isolated from mouse HCCs induced by HDTV injection of Yap ^{S127A} / β -catenin ^{ΔN90} in combination with CRNDE over-expression or shCRNDE plasmids and their respective control plasmids. Our data demonstrated that HCCs with CRNDE over-expression/depletion have much less/more granzyme B-secreting TILs than their corresponding control HCCs respectively. These results further support our initial assumption that the immune responses of TILs are suppressed by CRNDE in HCC.		

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1. Introduction

Tumor-infiltrating lymphocytes (TILs) in HCC tissues are functionally compromised, impeding anti-tumor immunity. While inhibitory receptor-ligand pathways partly attribute to compromised TIL response in HCC, additional mechanisms likely also exist in the HCC microenvironment. Our preliminary results indicate that long non-coding RNA CRNDE can be transported from HCC cell into TIL, and the transferred HCC-derived CRNDE acts as a crucial factor to inhibit TIL anti-tumor functions in the HCC microenvironment. The objective of this project is to assess the potential of CRNDE as a novel therapeutic target for effective anti-HCC immunotherapy. In this project, we plan to explore the functional impact of CRNDE on hepatic carcinogenesis through regulating TILs activation; we also plan to determine the mechanism by which HCC-derived CRNDE inhibits TIL functions; as well as to evaluate the potential therapeutic effects of CRNDE specific gapmer-ASO (next-generation anti-sense oligonucleotides) in HCC.

2. Keywords

Hepatocellular carcinoma (HCC)
Long non-coding RNA (lncRNA)
Colorectal Neoplasia Differentially Expressed (CRNDE)
Immunosuppression
Tumor Infiltrating Lymphocyte (TIL)
Exosome
Hydrodynamic injection

3. Accomplishments

- Major goals of the project (anticipated date of completion, actual completion date)

First year (9/15/2019 to 9/15/2020):

- To obtain ACURO approval (12/15/2019, 1/16/2020)
- To determine the effect of CRNDE on TIL functions (2/15/2020, 2/10/2020)
- To characterize extracellular vesicles which transport HCC-derived CRNDE to TILs (3/30/2020, 3/15/2020)
- To delineate the molecular mechanism of CRNDE-CypB interaction in TILs (8/15/2020, 7/10/2020)

Second year (9/16/2020 to 9/15/2021):

- To assess CRNDE effects on hepatocarcinogenesis (12/30/2020, 50% completion)
- To determine the downstream molecular targets of CRNDE-CypB complex (1/31/2021, 10% completion)
- To assess therapeutic efficacy of CRNDE specific ASOs, alone or in combination with checkpoint antibodies nivolumab/ipilimumab. (8/31/2021, 10% completion)

- Accomplishments under major goals

- To obtain ACURO approval.

The animal protocol of this projects was approved by Tulane IACUC on 12/30/2019 and by ACURO on 1/16/2020.

- To determine the effect of CRNDE on TIL functions.

First, we induce the HCCs in C57BL/6 mice through hydrodynamic tail vein injection of Yap^{S127A}/β-catenin^{ΔN90}/SB plasmids. After tumor fully developed (6-8 weeks later), mouse livers were perfused with PBS and harvested. Live TILs were then isolated with method of mechanical homogenization and Percoll (33% in PBS) density gradient centrifugation.

Next, the isolated mouse TILs (co-activated with 2ug/ml anti-CD3 and 2ug/ml anti-CD28) were incubated with exosomes isolated from culture media of CRNDE knockdown or control Hepa1-6 cells (5×10^6 cells cultured in 10ml serum-free DMEM medium for 24h), then IL-2, TNF α and IFN γ mRNA levels in the TILs were measured by qRT-PCR. The results demonstrated that all three cytokines mRNA levels are significantly increased in activated TILs cells incubated with exosomes from CRNDE depleted Hepa1-6 cells as compared with TILs cells incubated with exosomes from control Hepa1-6 cells. Meanwhile, we also observed that exosome incubation (regardless from which cell strain the exosomes were isolated) dramatically decreased IL-2, TNF α and IFN γ mRNA levels in TILs (Fig1, Left). The CRNDE levels in TILs incubated with different exosomes were also analyzed by qRT-PCR (Fig1, Right). These results indicate that CRNDE could inhibit TIL activation in HCC.

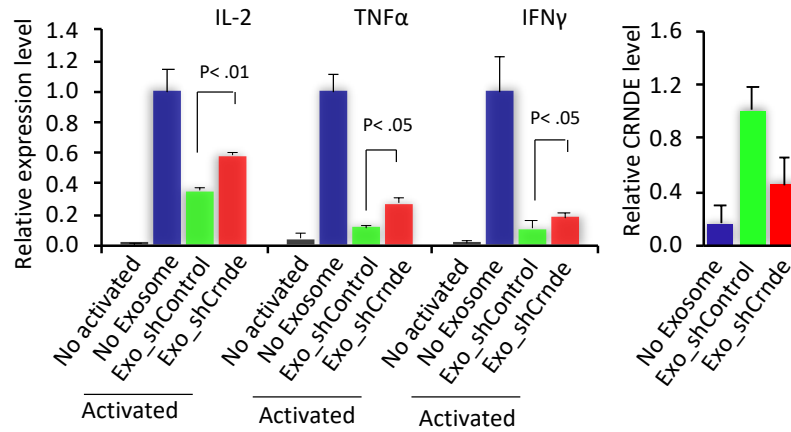


Fig 1. Isolated mouse TILs (co-activated with anti-CD3 and anti-CD28) were incubated with exosomes from CRNDE knock-downed or control Hepa1-6 cells for 24h. IL-2, TNF α and IFN γ mRNA levels in the treated TILs were analyzed by qRT-PCR. The right panes show the CRNDE levels in TILs incubated with exosomes from Hepa1-6 cells with or without CRNDE knockdown.

Then, in addition to the original protocol that HCC is induced by Yap^{S127A}/β-catenin^{ΔN90}, we have modified the protocol by inclusion of CRNDE expression or shRNA plasmids to the mixed solution for HDTV injection. This modification creates a unique opportunity to overexpress or knockdown CRNDE in HCC cells, which will enable us to assess the impact of CRNDE on TILs during the hepatic carcinogenic processes. Using the same methods as aforementioned, TILs were isolated from HCC tissues with CRNDE overexpression or knockdown. Then, these isolated TILs were subjected to Granzyme B ELISPOTS assays. As shown in Fig2, the number of Granzyme B-secreting TILs in CRNDE overexpression HCC tissues decreased by 54% compared with that in vector control HCC tissues (43 vs 94 per 5×10^5 cells); while in CRNDE knockdown tissues, the corresponding number increase by 2.5-fold when compared with that in shControl HCC (234.3 vs 94.3 per 5×10^5 cells). When co-stimulated the TILs with 2ug/ml anti-CD3 and anti-CD28, the differences are even greater: the number of Granzyme B-secreting TILs in CRNDE overexpression HCC tissues decreased by 70% compared with that in vector control HCC tissues (75 vs 256 per 5×10^5 cells); while in CRNDE knockdown tissues, the corresponding number increase by 2.75-fold when compared with that in control HCC tissues (247.6 vs 681.3 per 5×10^5 cells). These results suggest that HCC-derived CRNDE has important effect on the cytotoxic function of TILs.

Finally, total RNAs were isolated from mouse HCC induced by Yap^{S127A}/β-catenin^{ΔN90} in combination with shCRNDE or shControl and subjected to RNA sequencing and bioinformatic analyses. The proportions of major leukocyte subsets in HCC were estimated by CIBERSORT software using a default 22-immune cell types matrix (LM22). The results show that the proportions of CD8 T-cell, CD4 memory T-cell and NK cell are higher in CRNDE depleted HCC tissues than in control HCC tissues. GO Analyses show that the difference expressed genes between CRNDE

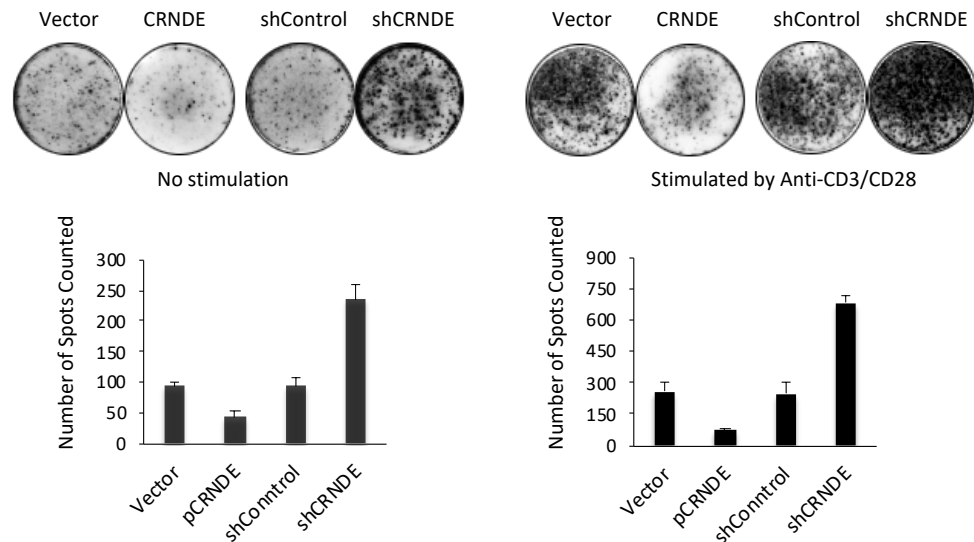


Fig2 TILs were isolated from YapS127A/ β -catenin Δ N90-induced mouse HCC tissues with CRNDE over-expression or depletion and their respective control HCC tissues. The isolated TILs (5×10^5 cells) were cultured in RPMI-1640 medium with indicated stimulation for 24 hours and then subjected to ELISpot assays for Granzyme B.

knockdown and control HCC are significantly enriched ($p < 0.01$) in multiple immune response related GO terms. GSEA analyses demonstrate that CRNDE knockdown in mouse HCC tissues led to enhanced expression of a group of genes related to CD8 T-cell survival, proliferation and cytotoxicity (Fig3). These data provide further evidences that HCC-derived CRNDE could inhibit the tumor-killing effects of TILs in HCC.

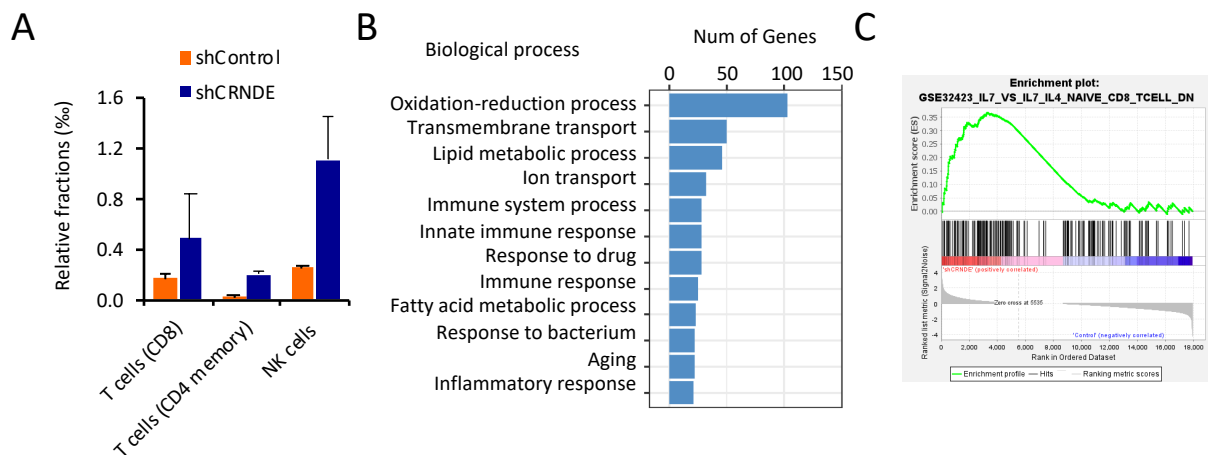


Fig3. RNA sequencing of mouse HCC tissues induced by YapS127A/ β -catenin Δ N90 in combination with shCRNDE or shControl. (A) Estimated proportions of CD8 T-cell, CD4 memory T-cell and NK cell in mouse HCC tissues according to the RNA-seq data. The results were determined by CIBERSORT software using a default 22-immune cell types matrix (LM22) on its webserver. (B) GO Analyses show that the difference expressed genes between mouse HCCs with and without CRNDE knockdown are significantly enriched ($p < 0.01$) in multiple immune response related GO terms. (C) GSEA analyses using RNA-seq data. CRNDE knockdown in mouse HCC tissues led to higher expression of a group of genes related to CD8 T-cell survival, proliferation and cytotoxicity. These gene expressions can also be induced in CD8 T-cells by IL-4 stimulation.

3. To characterize extracellular vesicles which transport HCC-derived CRNDE to TILs.

For this purpose, we transfected Plc/prf/5 and Hep3B cells (both are human HCC cell line) with CRNDE-expression or control plasmids. Then, we isolated exosome RNAs from culture media of

these stable transfected cells and performed RT-PCR using plasmid-CRNDE specific primers. The RT-PCR results demonstrated that the plasmid-derived CRNDEs were able to be detected in exosomes. Next, we incubated Jurkat cells (human T-cell leukemia cell line) with exosomes prepared using the same protocol aforementioned. Twenty-four hours later, RNAs were isolated from Jurkat cells and subjected to RT-PCR analyses using plasmid-CRNDE specific primers. The RT-PCR results clearly demonstrated that the plasmid-derived CRNDEs were able to be detected in Jurkat cells (Fig4). These data indicate that CRNDE can be transferred between cells through exosomes.

To prove the transference of CRNDE from HCC cell to TILs, we prepared TILs from mouse HCC induced by hydrodynamic tail vein injection of Yap^{S127A}/β-catenin^{ΔN90}/SB plasmids, and then incubated these TILs with exosomes isolated from culture medium of Hepa1-6 cells with or without CRNDE knockdown. Twenty-four hours after incubation, the TILs were subjected to RT-PCR assays to determine their CRNDE levels (Fig1). The results showed that CRNDE level in TILs incubated with exosomes from CRNDE-depleted Hepa1-6 cells are 80% lower than that in TILs incubated with exosomes from control Hepa1-6 cells. These experiments indicate that CRNDE could be efficiently transferred from HCC cells to TILs through exosome.

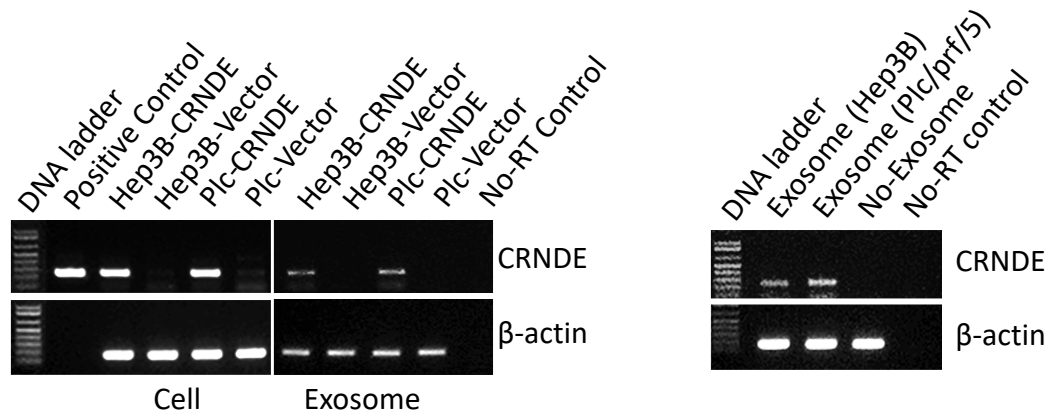


Fig4. left: Plc/prf/5 or Hep3B cells transfected with CRNDE-expression or control plasmids, and then plasmid-derived CRNDEs were detected in cells and exosomes by RT-PCR with plasmid-CRNDE specific primers. Right: Jurkat cells incubated with exosomes isolated from culture media of Plc/prf/5 cells or Hep3B cells transfected with CRNDE-expression plasmids. Twenty-four hours later, plasmid-CRNDEs in Jurkat cells were detected by RT-PCR. (B) Jurkat cells incubated with HCC derived, red-fluorescent labeled exosomes for 24 hours and checked under microscope. Jurkat cells without exosome incubation were used as control.

4. To delineate the molecular mechanism of CRNDE-CypB interaction in TILs.

Using RNA pull-down in combination with Mass spectrum assays, we identified that CRNDE binds to CypB in Plc/prf/5 cells (Fig 5A). This conclusion has been verified by Western blot, RNA immunoprecipitation and quantitative RT-PCR in Plc/prf/5 and Jurkat cells (Fig 5B-5D).

5. To assess CRNDE effects on hepatocarcinogenesis.

For this purpose, we modified the HCC hydrodynamic protocol by inclusion of CRNDE expression or shRNA plasmids into the Yap^{S127A}/β-catenin^{ΔN90}/SB plasmid mixture solution for HDTV injection. Eight weeks (for CRNDE over-expression and Vector control groups) or twelve weeks (for shCRNDE and shControl groups) after hydrodynamic tail vein injection, mouse livers were recovered and the liver/body weight ratio for each group was measured. The results demonstrated that the average liver/body weight ratio of shCRNDE group is decreased by 50% when compared with that of shControl group (Fig6); and the average liver/body weight ratio of CRNDE over-expression group is increased by 40% compared with that of vector control group (data not shown). These results suggest that CRNDE could significantly enhance hepatocarcinoma growth through inhibiting anti-tumor activities of TILs.

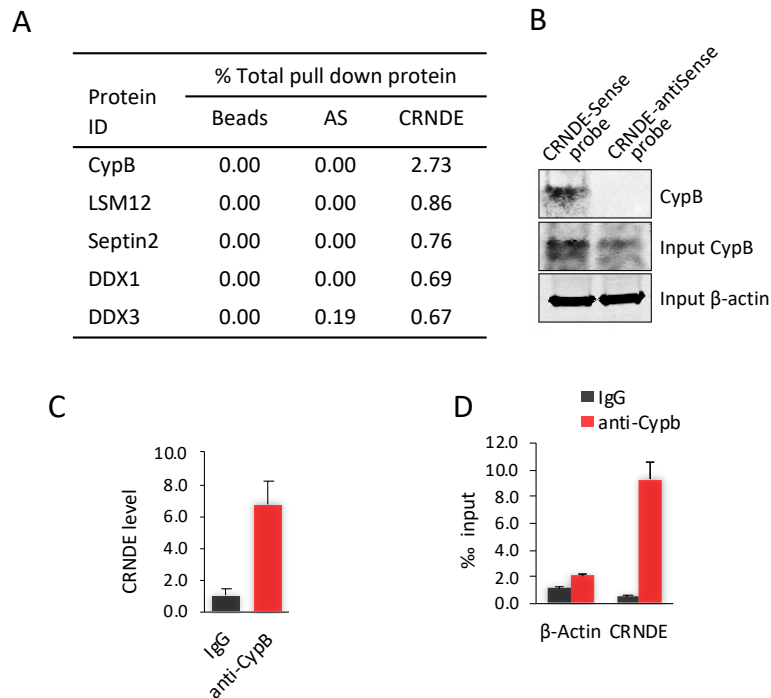


Fig5. CRNDE interact with CypB. (A) The top five proteins with differential-abundance in complexes pull-down by CRNDE specific probe from Plc/prf/5 cell lysate, identified by Mass Spectrum assay. Beads: beads-only control; AS: anti-sense probe; CRNDE: CRNDE sense probe. (B) RNA pull-down with Plc/prf/5 cell lysates using CRNDE sense or anti-sense probes, the pull-down complexes were assessed by Western blot with anti-CypB. (C) RNA immunoprecipitation with whole cell lysate of Plc/prf/5 cells using anti-CypB or IgG, the RNAs in precipitated complexes were extracted and CRNDE was quantified by qRT-PCR. (D) RNA immunoprecipitation with whole cell lysate of Jurkat cells using anti-CypB or IgG.

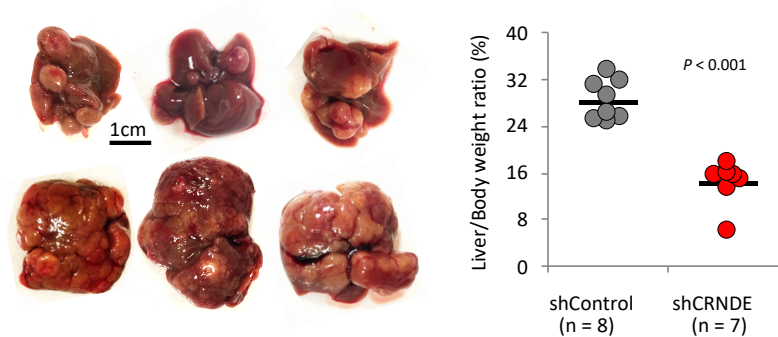


Fig6. CRNDE level is correlated with HCC tumorigenicity in mouse. Mice were hydrodynamic injected with YapS127A/ β -catenin Δ N90/SB plasmids in combination shCRNDE or control. Mouse livers were recovered 12 weeks after injection. Representative photographs of mouse livers are shown. The liver/body weight ratio of each group was shown in the right panel.

- To determine the downstream molecular targets of CRNDE-CypB complex.
We have constructed CRNDE over-expression and shRNA plasmids, and plan to transfect the CRNDE over-expression or shRNA plasmids with respective control vector into Jurkat cell to explore the downstream molecular targets of CRNDE-CypB complex.
- To assess therapeutic efficacy of CRNDE specific ASOs, alone or in combination with checkpoint antibodies nivolumab/ipilimumab.
We have designed and synthesized 20 different CRNDE-specific ASOs. After screening in HCC cells, two candidate ASOs with highest inhibiting efficacy were selected for the next *in vivo* studies.

- Opportunities for training and professional development
Nothing to report.
- How were the results disseminated to communities of interest?
Nothing to report.
- Plan for next reporting period

According to the provided SOW, our plan for next year are the following:

1. To complete the major task 5 “*To assess CRNDE effects on hepatocarcinogenesis*”, we will further assess CRNDE effects on HCC development in allograft HCC model. We will allograft HCC in C57BL/6 mouse by inoculation of Hepa1-6 with forced overexpression or depletion of CRNDE into mice livers. The mice will be sacrificed at different time points to document key parameters of tumor progression including tumor volume, cell proliferation, apoptosis, invasion and angiogenesis.
2. To complete the major task 6 “*To determine the downstream molecular targets of CRNDE-CypB complex*”, first we will 6.1 Assess the effect of CRNDE-CypB complex on NFAT activation in Jurkat cell or TIL. we will measure the level and phosphorylation status of NFATs in Jurkat or TIL cell when the CRNDE level is manipulated. Then, we will explore the effect of CRNDE alteration on the protein-protein interaction between NFAT and Calcineurin. Finally, the possible calcium ion level change in Jurkat or TIL cells incurred by CRNDE level alteration will also be assessed.
3. To complete the major task 6 “*To assess therapeutic efficacy of CRNDE specific ASOs*”, we will test the therapeutic efficacy of our selected, CRNDE-specific ASOs using HDTV or allograft mouse HCC model.

4. Impact

Nothing to report

5. Changes/Problems

Nothing to report

6. Products

- Journal publications
 1. J Zhang, C Han, K Song, W Chen, N Ungerleider, L Yao, W Ma, T Wu. The long-noncoding RNA MALAT1 regulates TGF- β /Smad signaling through formation of a lncRNA-protein complex with Smads, SETD2 and PPM1A in hepatic cells. PloS one 15 (1), e0228160. Published.
Acknowledgement of federal support: Yes (original copy was attached in appendices)

7. Participants & Other Collaborating Organizations

- Individuals have worked on the project

Name	Project role	Researcher identifier	Nearest person month worked	Contribution	Funding support
Jinqiang Zhang	No change				
Tong Wu	No change				
Weina Chen	No change				
Wenbo Ma	No change				

- Changes in the active other support of the PD/PI(s) or senior/key personnel since the last reporting
Nothing to report
- Other organization
Nothing to report

8. Special Reporting Requirements

Nothing to report

9. Appendices

- Original copy of journal publication.

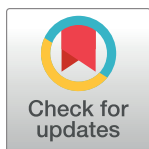
RESEARCH ARTICLE

The long-noncoding RNA MALAT1 regulates TGF- β /Smad signaling through formation of a lncRNA-protein complex with Smads, SETD2 and PPM1A in hepatic cells

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Abstract

Recent studies have demonstrated the implication of long noncoding RNAs (lncRNAs) in a variety of physiological and pathological processes. However, the majority of lncRNAs are functionally unknown. The current study describes that the lncRNA MALAT1 regulates TGF- β /Smad signaling pathway through formation of a lncRNA-protein complex containing Smads, SETD2 and PPM1A. Our data show that this lncRNA-proteins complex facilitates the dephosphorylation of pSmad2/3 by providing the interaction niche for pSmad2/3 and their specific phosphatase PPM1A, thus terminating TGF- β /Smad signaling in hepatic cells. Based on these mechanistic studies, we performed further experiments to determine whether depletion of MALAT1 would augment cellular TGF- β /Smad signaling. We observed that MALAT1 depletion enhanced TGF- β /Smad signaling response, as reflect by amplification of Smad-mediated differentiation of induced pluripotent stem (iPS) cells to hepatocytes. Our experimental results demonstrate an important role of MALAT1 for regulation of TGF- β /Smad signaling in hepatic cells. Given the diverse functions of TGF- β /Smad pathway in various physiological and pathogenic processes, our results described in the current study will have broad implications for further understanding the role of MALAT1 in TGF- β /Smad pathway in human biology and disease.

Introduction

High-throughput studies have indicated the fascinating complexity of the human transcriptome including abundant RNAs with no protein coding capacity[1–4]. The noncoding transcripts ranging in size from 200nt to longer than 100kb are assigned arbitrarily as the long noncoding RNAs (lncRNAs), which is the largest and most complex class of noncoding RNAs [3, 5]. The vast majority of lncRNAs are functionally unknown; only dozens of them have been described with biological roles, mainly through four archetypes of molecular mechanisms—acting as signals, as decoys, as guides, or as scaffolds[6]. Intriguingly, in each archetype, lncRNAs form protein-lncRNA complexes with some key protein factors to execute their functions[6,

Competing interests: The authors have declared that no competing interests exist.

Abbreviations: lncRNAs, long noncoding RNAs; MALAT1, metastasis associated lung adenocarcinoma transcript 1; SETD2, SET domain containing 2; PPM1A, protein phosphatase, Mg²⁺/Mn²⁺ dependent, 1A; RIP, RNA immunoprecipitation; KLF4, Kruppel-like factor 4; OCT4A, octamer-binding transcription factor 4a; SOX2, SRY (sex determining region Y)-box 2; AFP, alpha-fetoprotein; ALB, albumin; CK18, cytokeratin 18; CYP3A4, cytochrome P450 3A4; CYP7A1, cytochrome P450 7A1; G-6P, glucose-6-phosphatase; HNF-4a, hepatocyte nuclear factor-4a; TAT, tyrosine aminotransferase; TD02, tryptophan 2,3-dioxygenase.

7]. Therefore, there is a noticeable need to further dissect whether key protein factors of pivotal signaling pathways may form protein-lncRNA complexes, and whether these complexes may in turn affect the activity of their respective signaling pathways.

Smad transcription factors lie at the core of the transforming growth factor- β (TGF- β) pathway, which controls a plethora of cellular responses including development, stem cell maturation, and carcinogenesis, among others[8]. Smad protein factors, together with co-activators or co-inhibitors can bind to specific DNA sequences in promoter regions and regulate transcription activity of certain genes[9]. A recent study showed that Smad proteins could also bind to some primary microRNA transcripts and regulate their maturation[10]. Thus, we postulate that Smad proteins may form RNA-protein complexes with certain lncRNA molecules and these complexes may modulate the functions of Smads or related lncRNAs. To test this hypothesis, we carried out a series of RNA immunoprecipitation experiments using phospho-Smad2/3 antibodies in hepatic cells and observed that the lncRNA MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) specifically binds to phospho-Smad2/3.

The lncRNA MALAT1, also known as NEAT2 (nuclear-enriched abundant transcript 2), is a highly conserved nuclear noncoding RNA among mammals with length of more than 8 kb in human (which is localized exclusively in nuclear speckles) [11, 12]. Studies have shown that MALAT1 plays important roles in multiple cellular processes and diseases[13–18]. In the present study we describe a novel mechanism for MALAT1 interaction with phospho-Smad2/3, SETD2 and PPM1A in hepatic cells. Our data show that this MALAT1-protein complex facilitates the dephosphorylation of pSmad2/3 by providing the interaction niche for pSmad2/3 and their specific phosphatase PPM1A, thus terminating TGF- β /Smad signaling in hepatic cells. Our experimental results disclose a novel mechanism by which MALAT1 negative regulates cellular TGF- β /Smad signaling.

Materials and methods

Materials

Specific antibodies were purchased from the following commercial sources: Anti-AFP, anti-ALB, anti-CD44, anti-Evi1, anti-flag (mouse), anti-HA, anti-HNF4 α , anti-H3, anti-H3K36me3, anti-Myc, anti-OCT4A, anti-P300, anti-PPM1A (rabbit), anti-pSmad2 (S465/467), anti-pSmad2 (S245/250/255), anti-pSmad3 (S423/425), anti-Smad2, anti-Smad3 (rabbit), anti-SnoN, anti-Sox2, anti-TAT, and normal rabbit IgG from Cell Signaling Technology (Danvers, MA); anti-PPM1A (mouse), anti-SC35, and anti-SETD2 were from Abcam (Cambridge, MA); Anti-Smad4 and normal mouse IgG were from Santa Cruz Biotechnology (Santa Cruz, CA); Anti- β -actin, and anti-flag (rabbit) from Sigma-Aldrich (St. Louis, MO); Alexa594 goat anti-mouse IgG from Life Technology (Carlsbad, CA); Dylight488 goat anti-rabbit IgG from Vector Labs (Burlingame, CA).

Cell culture

Human transformed hepatocytes (Hep3B, SK-Hep1, PLC/PRF/5, and Huh7) were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% heat-inactivated fetal bovine serum. The immortalized human hepatocytes (THLE2) were maintained in complete BEGMTM Medium (Lonza, Allendale, NJ) supplemented with 10% heat-inactivated fetal bovine serum. All cells were cultured at 37°C in a humidified 5% CO₂ incubator.

RNA immunoprecipitation

Cells cultured in 100-mm dishes were fixed by 1% paraformaldehyde for 10 minutes and quenched by 125mM Glycine; the cells were then collected and washed twice with ice-cold

phosphate buffered saline containing protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche, Mannheim, Germany). After that, cell pellet was re-suspended in 200ul Buffer A (5mM PIPES pH8.0, 85mM potassium chloride, 0.5% NP-40, protease inhibitor cocktail, phosphatase inhibitor cocktail and RNase inhibitor) and placed on ice for 10 minutes. The crude nuclei fraction was pelleted and washed once in buffer A (without NP-40), then re-suspended in 500ul of Buffer B (10mM EDTA, 50mM Tris-HCl pH8.1, protease inhibitor cocktail, phosphatase inhibitor cocktail and RNase inhibitor) and placed on ice for 10 minutes. After centrifuged at 14000 rpm for 10 minutes at 4 °C, the supernatant was diluted 10-fold into IP Buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris pH8.1, 167mM sodium chloride, protease inhibitor cocktail, phosphatase inhibitor cocktail and RNase inhibitor). One microgram of indicated antibody was added to 1ml diluted supernatant; the samples were rotated slowly at 4 °C overnight. Subsequently, 50ul of Protein A/G Agarose beads (Santa Cruz) was added to each tube and the samples continued to be rotated for 2 hours. Immune complex was pelleted and washed successively by Low-salt Buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.1, 150mM NaCl), High-salt Buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.1, 500mM NaCl), LiCl Buffer (0.25M LiCl, 1% NP40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl pH 8.1), and Tris-EDTA(pH8.0) Buffer. 250ul fresh prepared Elution Buffer (1% SDS, 0.1M NaHCO₃, nuclease inhibitor) was added to the complexes and the samples were incubated for 15 minutes with rotating to elute the complex; supernatant was collected by centrifugation at 8000 rpm, 2 minutes. Elution was repeated and elute combined for a total of 500ul. 3M NaCl was added to elute to a final concentration of 200mM and the samples were placed at 65°C for at least 2 hours to reverse crosslinking. Next, 20ul of 1M Tris-Cl pH 6.5, 10ul of 0.5M EDTA, and 20ug of Proteinase K was added and the samples were incubated at 42°C for 45 minutes. After incubation, the samples were subjected to phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation with Glycoblue (Ambion, Carlsbad, CA) as a carrier. Pellets were washed once in 75% ethanol, air-dried briefly, and re-suspended in 20ul of DEPC-treated water. Then the RNA (treated with DNase I to remove DNA) was ready for reverse transcription and quantitative PCR analysis.

Induced pluripotent stem (iPS) cell differentiation

Human iPS cells (ACS-1011) were obtained from ATCC (Manassas, VA) and maintained with the stem cell culture medium SFM XF/FF (ACS-3002). The three step protocol of hepatocyte-like cells induction from iPS cells was modified on the base of previous publications [19]. First, 70% confluent iPS cells were cultured with Roswell Park Memorial Institute (RPMI)/B27 medium containing 100ng/mL activin A or 10ng/ml TGF- β 1, along with 50ng/mL Wnt3a, and 10ng/mL HGF (R&D Systems) for 3 days of endodermal induction. Then, RPMI/B27 was replaced by hepatic commitment medium (DMEM containing 20% knockout serum replacement, 1mM L-glutamine, 1% nonessential amino acids, 0.1mM 2-mercaptoethanol, and 1% dimethyl sulfoxide) for another 4 days. Finally, the cells were incubated in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20ng/mL oncostatin M (Invitrogen), 0.5μM dexamethasone, and 50 mg/mL ITS premix (BD Biosciences, San Jose, CA) for 5 days to induce hepatocyte maturation.

Statistical analysis

Data were presented as mean $\pm$ standard deviation from a minimum of three replicates as indicated in the figure legends. Difference between groups was evaluated by SPSS 19.0 statistical software with one-way analysis of variance, Student's t test, Mann-Whitney U test or Fisher exact test. A p-value <0.05 was considered as statistically significant.

Additional methods, including plasmids and siRNA transfection, cell proliferation WST1 assay, luciferase activity assay, quantitative real-time PCR, immunoprecipitation, DNA pull down, Western blot, fluorescent in situ hybridization (FISH), immunofluorescence, and hepatic spheroid formation assay, as well as a list of primers used in RT-PCR analysis are described in [S1 File](#).

Results

TGF- β activated Smad2/3 associate with MALAT1

To identify lncRNAs that might be associated with phospho-Smad2/3, we carried out a small-scale screening by employing RNA immunoprecipitation experiments in human transformed hepatocytes (PLC/PRF/5, SK-Hep1, and Hep3B) treated with TGF- β . We observed that MALAT1 was enriched specifically by antibodies targeting total or phospho-Smad2/3 ([Fig 1A](#)). To further verify the association specificity between pSmad2/3 and MALAT1, we used several other nuclear proteins antibodies, such as anti-pSTAT3, anti- β -catenin, and anti-c-Myc, as control of the RNA immunoprecipitation experiments; the results show that MALAT1 associates exclusively with Smad2/3 ([Fig 1B](#)). We next utilized immunofluorescence combined with FISH staining to determine whether TGF- β 1 was able to induce pSmad2/3 association with MALAT1 (hence co-localization). We observed that phosphorylated Smad2/3 entered into the nucleus, accumulated and overlapped with MALAT1 in TGF- β 1 treated Hep3B cells ([Fig 1C](#)). Our further FISH-IF analysis indicated that MALAT1 was co-localized with the nuclear speckle marker SC-35 ([Fig S1A](#) in [S2 File](#)). Meanwhile, TGF- β 1 did not influence MALAT1 nuclear localization and transcription as indicated by FISH-IF staining and RT-PCR analysis, respectively ([Fig 1C](#) and [Fig S1](#) in [S2 File](#)).

SETD2 mediates pSmad2/3 association with MALAT1

The next question we asked is how MALAT1 interacted with pSmad2/3, i.e., through direct binding or via other molecules indirectly. Yang et al[14] had identified numerous MALAT1--binding proteins by using RNA pull-down combined with mass spectrum analysis; it came to our attention that SET domain containing 2 (SETD2) (also known as Huntington-interacting protein B) was identified as one of the MALAT1 binding proteins in their results. In another study, by using yeast two-hybrid and co-immunoprecipitation methods, Wang et al[20] reported that SETD2 protein could form complex with phospho-Smad in the liver; this phenomenon was confirmed in our study by using Hep3B cells ([Fig 2A](#)). On the basis of these results, we reasoned that SETD2 might mediate the interaction between MALAT1 and phospho-Smad. To validate this hypothesis, we performed RNA immunoprecipitation experiments in Hep3B cells with or without SETD2 depletion by siRNA (successful depletion of SETD2 was verified by Western blotting, as shown in [Fig S2](#) in [S2 File](#)). RNA-IP and qRT-PCR assays showed that SETD2 depletion significantly decreased Smad2/3 association with MALAT1 ([Fig 2B](#)). Unlike Smad2/3 association with MALAT1, we observed that SETD2 association with MALAT1 was not influenced by TGF- β treatment ([Fig 2B](#)). In addition, our data showed that SETD2 depletion did not influence MALAT1 expression ([Fig 2C](#)). These results demonstrate that MALAT1 interaction with pSmad2/3 is mediated by SETD2.

SETD2 belongs to H3K36-specific methyltransferase family. It consists of a catalytic SET domain, a C-terminal RNA polymerase II interaction domain, and a WW domain for binding to target proteins, such as Proline-Rich PY (Pro-Pro-X-Tyr) motif[21]. There are four threonine/serine residues (T220, S245, S250, S255) which are localized around a PY motif in the Smad2 linker region; phosphorylation of these four threonine/serine residues by CDK8/9 is required for the binding of PY motif to WW domain[22–24]. To determine whether PY-WW

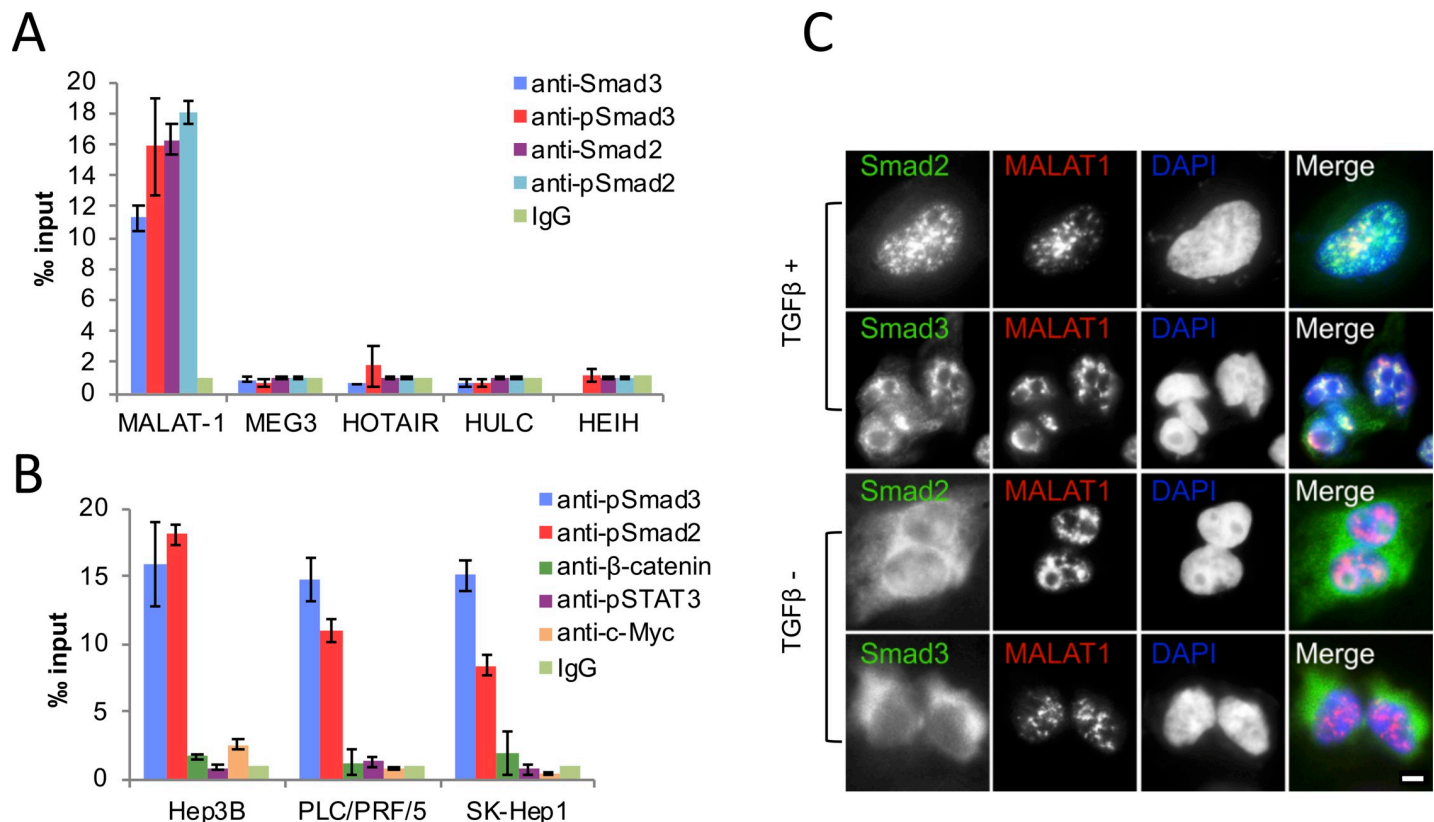


Fig 1. MALAT1 binds to pSmad2/3 specifically. **A**, RNA immunoprecipitation assays of multiple lncRNAs with antibodies targeting Smad2, Smad3, pSmad2 or pSmad3 in Hep3B treated with TGF- β 1 for 1 hour. The precipitated lncRNAs were assessed by qRT-PCR. **B**, RNA immunoprecipitation assays of MALAT1 with indicated antibodies in Hep3B, PLC/PRF/5 and SK-Hep1 cells treated with TGF- β 1 for 1 hour. The precipitated lncRNA was assessed by qRT-PCR. **C**, Co-localization of pSmad2 or pSmad3 with MALAT1 in the nuclei of Hep3B cells as demonstrated by FISH in combination with IF staining (scale bar 10 μ m). All data are shown as means $\pm$ SD (n = 3).

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motif interaction involve in the binding of phosphorylated Smad2/3 to SETD2, PLC/PRF/5 cells were transfected with plasmids expressing linker region mutated Smad2 (Smad2-EPSM, T220V, S245A, S250A, S255A)[23] and then subjected to immunoprecipitation assays. We observed that mutations of the linker threonine/serine residues abolished Smad2 binding to SETD2 (Fig 2D), despite that mutated Smad2-EPSM can be phosphorylated at the C-terminal SSXS motif and translocated to nuclei as wild type Smad2 upon TGF- β treatment (Fig S3 in S2 File). These findings suggest the essential roles of Smad2 PY motif and SETD2 WW domain in the interaction of these two proteins.

Depletion of MALAT1 or SETD2 enhances Smad2/3 signaling

To determine the functional impact of MALAT1 on TGF- β signaling, we utilized shRNA to knockdown MALAT1. The efficiency of MALAT1 knockdown was confirmed by FISH and qRT-PCR analyses (Fig 3A). Our data showed that MALAT1 knockdown increased Smad2/3 activities as determined by p3TP-lux luciferase reporter activity assay (Fig 3B). This result was further supported by the observation that depletion of MALAT1 by 2'-OME-gamper ASO also increased the p3TP-lux luciferase reporter activity (Fig S4A in S2 File). By using DNA pull-down assay, we observed that MALAT1 knockdown increased the binding of pSmad2/3 to the Smad-binding element (SBE) (Fig 3C–3E). These results indicate that MALAT1 knockdown

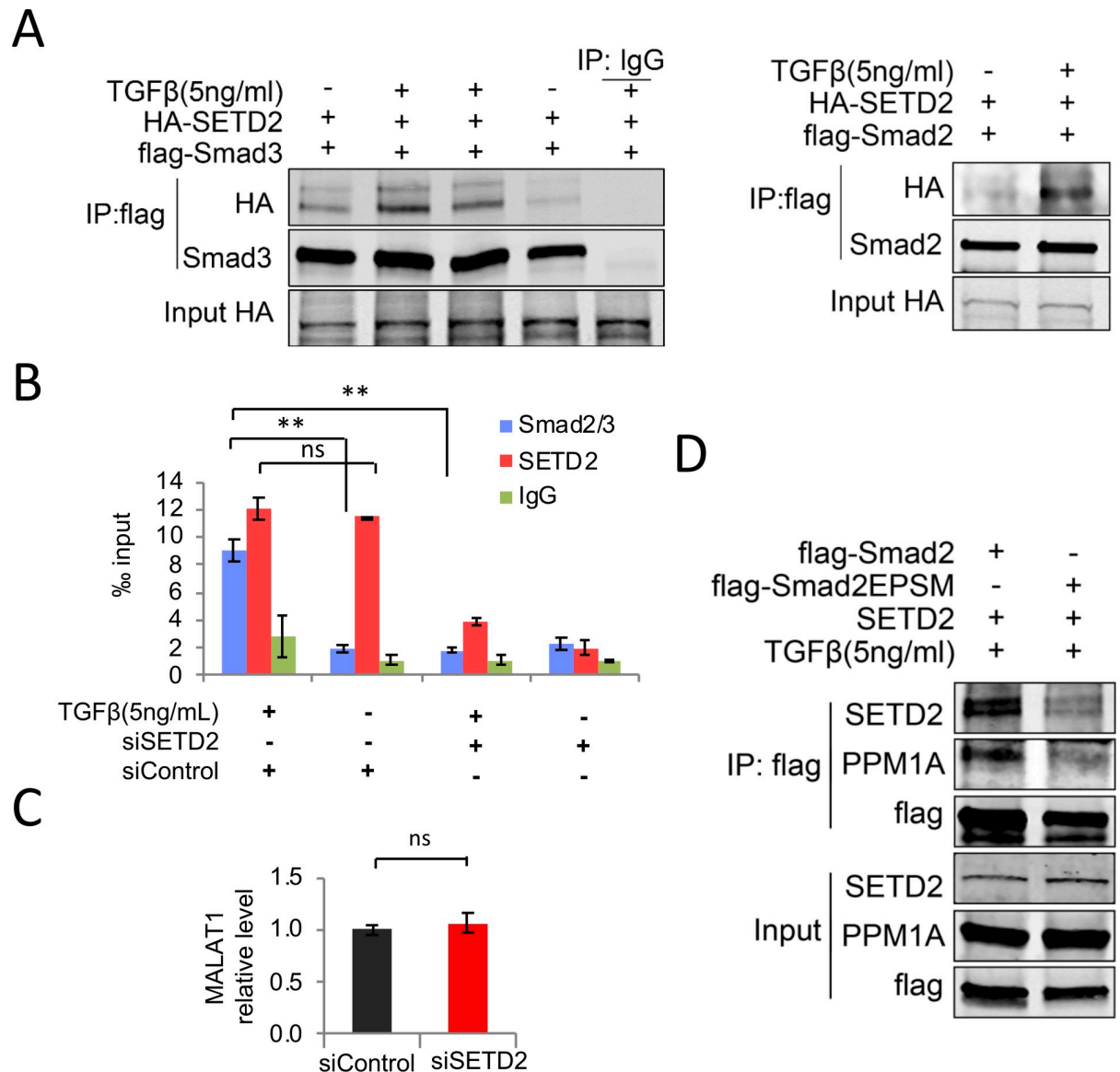


Fig 2. SETD2 mediates the binding of MALAT1 and pSmads. A, Smad2 and Smad3 bind to SETD2 protein. Hep3B cells co-transfected with HA-SETD2 and flag-Smad2/3 expression plasmids were treated with TGF- β 1 for 1 hour. Cells were harvested and subjected to immunoprecipitation assays. B, RNA immunoprecipitation of MALAT1 by using anti-Smad2/3 or anti-SETD2 antibodies in Hep3B cells (with siSETD2 or siControl transfection) treated with 5ng/mL TGF- β 1 or vehicle for 2 hours. The precipitated MALAT1 was assessed by qRT-PCR. C, qRT-PCR results indicated that SETD2 knockdown did not affect MALAT1 level in Hep3B cells. The data are presented as means $\pm$ SD (n = 3; *p < 0.01; ns, no statistical significance). D, Smad2 linker region mutations decrease its capability to form protein complex with PPM1A and SETD2 proteins. PLC/PRF/5 cells co-transfected with SETD2 and flag-Smad2 or flag-Smad2EPSM (T220V, S245A, S250A and S255A, Erk/Pro-directed kinase Site Mutated Smad2) expression plasmids were incubated with TGF- β 1 for 1 hour. The treated cells were then harvested and subjected to immunoprecipitation and immunoblotting with indicated antibodies.

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enhances the transcription activity of pSmad2/3. Consistent with the fact that Smad4 and TGIF (TGF- β induced factor) are components of activated Smad2/3 complex[25], we also detected increased Smad4 and TGIF in pSmad2/3-SBE complex in MALAT1 knockdown cells. Given that SETD2 protein mediates Smad2/3 association with MALAT1 (as documented above), we anticipated that knockdown of SETD2 would undermine Smad2/3 interaction with MALAT1. Indeed, we observed that knockdown of SETD2 by siRNA increased both p3TP-lux

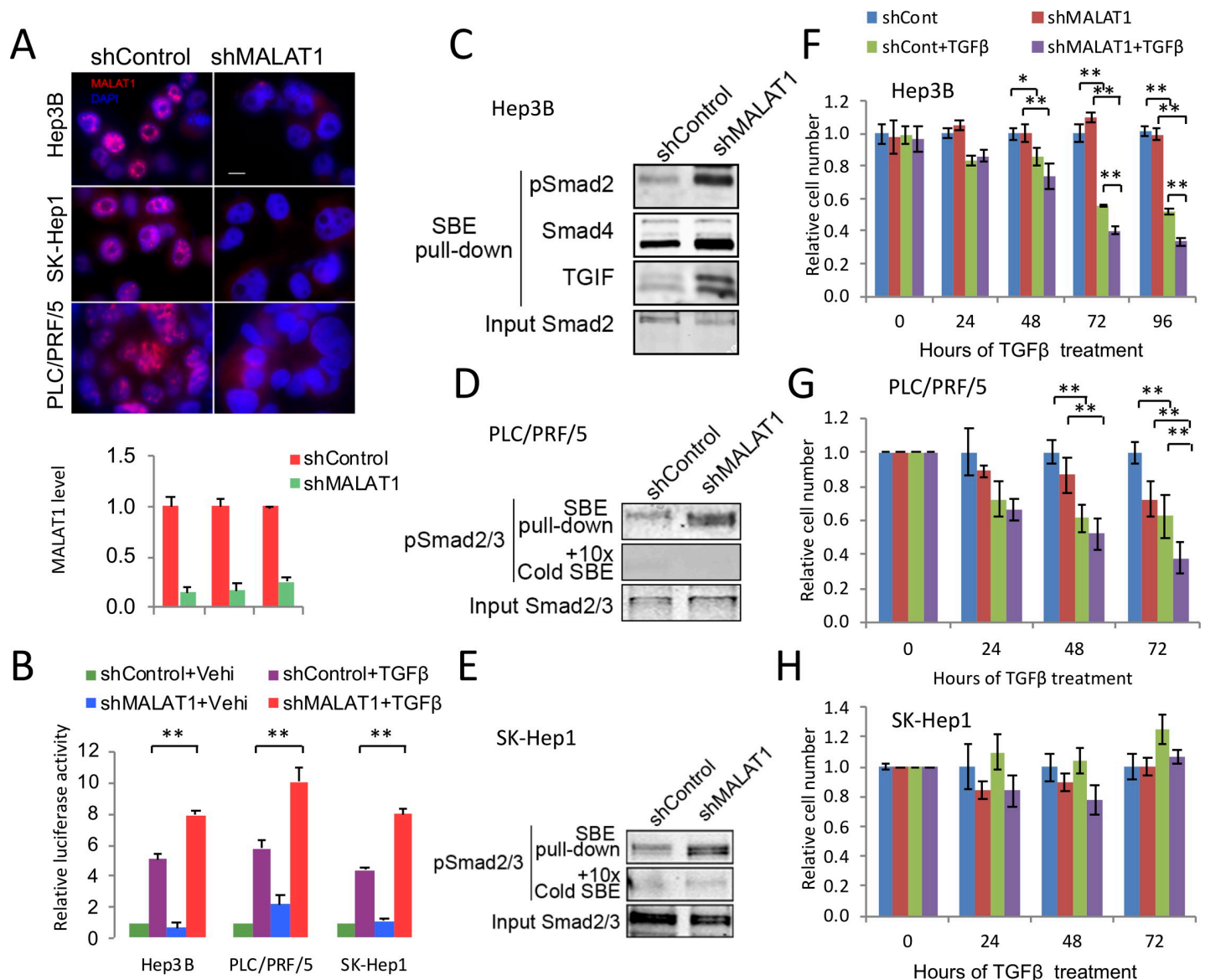


Fig 3. Depletion of MALAT1 enhances the effect of TGF- β . **A**, The levels of MALAT1 were assessed by FISH (upper) and qRT-PCR (lower) in Hep3B, SK-Hep1 and PLC/PRF/5 cells stably transfected with pGFP-V-RS-shMALAT1 or scramble control plasmid (scale bar 10 μ m). **B**, Smad reporter activity in Hep3B, SK-Hep1 and PLC/PRF/5 cells with or without MALAT1 depletion. Twenty-four hours after transfection with p3TP-lux reporter plasmid, MALAT1 depleted and control cells were stimulated with 5ng/ml TGF- β 1 or vehicle for additional 24 hours. The cell lysates were obtained for dual-luciferase activity assay. The data are presented as means $\pm$ SD ($n = 3$; * $p < 0.05$, ** $p < 0.01$). **C-E**, DNA pull-down assay. Equal amount of cell lysates from MALAT1 depleted or control Hep3B (**C**), PLC/PRF/5 (**D**) or SK-Hep1 (**E**) cells stimulated with TGF- β 1 (5 ng/ml for 2 hours) were pulled down with biotinylated SBE (Smads binding elements) DNA probe[40, 41], which were followed by immunoblotting with indicated antibodies. **F-H**, Cell proliferation assay. Equal numbers of MALAT1 depleted or control Hep3B (**F**), PLC/PRF/5 (**G**) or SK-Hep1 (**H**) cells were seeded into 96-well plates. The cells were treated with TGF- β 1 (1 ng/ml) or vehicle control for 0–96 hours and cell proliferation was measured by WST-1 at indicated time points ($n = 6$). Statistical analysis was performed by using one-way ANOVA method and the data are presented as means $\pm$ SD (* $P < 0.05$; ** $P < 0.01$).

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luciferase reporter activity and the binding of pSmad2/3 to SBE in Hep3B cells with TGF- β 1 stimulation; conversely, forced overexpression of SETD2 partially inhibited Smads pro-transcription activity (Fig S4B and S4C in S2 File).

TGF- β induces cytostasis in PLC/PRF/5 and Hep3B cells, but not in SK-Hep1 cells[26, 27]. In accordance with this phenomenon, MALAT1 knockdown enhanced the growth inhibitory effect of TGF- β 1 in Hep3B and PLC/PRF/5 cells (Fig 3F and 3G), but not in SK-Hep1 cells (Fig

3H). Histone H3K36 trimethylation (H3K36Me3), catalyzed by SETD2[21], is a transcriptional mark and has important implications in TGF- β -mediated EMT process[28]. To assess whether MALAT1 might affect H3K36Me3 status through interaction with SETD2 and thus affect cell response to TGF- β , we measured the levels of HK36Me3 in MALAT1 knockdown and control cells with or without TGF- β treatment. While TGF- β 1 treatment slightly increased HK36Me3 level in both MALAT1 knockdown and control cells (Fig S5A in [S2 File](#)), we observed that MALAT1 depletion did not significantly alter the status of H3K36Me3 (Fig S5B in [S2 File](#)).

MALAT1 and SETD2 are implicated in pSmad dephosphorylation

To investigate the mechanism underlying MALAT1 effect on TGF- β signaling, we examined the levels of several Smad co-activators or co-inhibitors in MALAT1 knockdown and control cells. The expression levels of these Smad co-factors did not significantly differ in cells with or without MALAT1 depletion (Fig S6 in [S2 File](#)); conversely, the level of phospho-Smad was increased in MALAT1 knockdown cells compared to the control cells. We next utilized nuclear fractionation and western blotting analysis to determine the levels of pSmad2/3 in the nuclear pool of hepatic cells with or without MALAT1 knockdown. Our data showed that TGF- β treatment induces the phosphorylation of Smad2/3 in the nucleus and that the levels of pSmad2/3 in the nuclear pool of hepatic cells with MALAT1 knockdown were higher than that in cells without MALAT1 knockdown (Fig S7 in [S2 File](#)). Enhanced phospho-Smad in MALAT1 knockdown cells was also confirmed by time course experiments (Fig 4A and 4B). The dynamic change in pSmad over time in post TGF- β -treated cells further support that the higher levels of pSmad2/3 in MALAT1 depleted cells are due to slower dephosphorylation rate (Fig 4C–4F). In consistence with p3TP-lux luciferase and SBE pull-down results, we also observed that SETD2 knockdown prevented pSmad2 dephosphorylation (Fig 4G and 4H). Collectively, the above results demonstrate that depleting MALAT1 or disassociating the MALAT1-pSmad2/3 complex (via SETD2 knockdown) amplifies cell response to TGF- β 1 by preventing pSmad2/3 dephosphorylation.

MALAT1 facilitates SETD2-pSmad2/3-PPM1A complex formation

PPM1A is known to bind phospho-Smad2/3 and function as a pSmad-phosphatase to terminate TGF- β signaling[29]. To investigate the possible role of MALAT1 and SETD2 in the interaction between PPM1A and phospho-Smad2/3, we performed immunoprecipitation in MALAT1 knockdown and control cells transfected with plasmids expressing PPM1A and flag-Smad2 or flag-Smad3. We observed that MALAT1 knockdown noticeably undermined the binding between PPM1A and phospho-Smad2/3 (Fig 5A) and reduced the interactions among phospho-Samd2/3, PPM1A and SETD2 (Fig 5B and 5C). Consistent with the notion that SETD2 acts as a molecular bridge between MALAT1 and pSmad2/3, knockdown SETD2 with siRNA considerably prevented the interaction between phospho-Smads and PPM1A (Fig 5D). The role of SETD2 and MALAT1 as connecting molecules to link PPM1A and pSmads was further supported by the evidence that four linker threonine/serine residues mutation (which demolishes the interaction between SETD2 and pSmad2) significantly reduced pSmad2 binding to PPM1A (Fig 2D). Additional RNA and protein immunoprecipitation assays show that PPM1A binding to MALAT1 is independent of TGF- β treatment (Fig 5E), whereas TGF- β treatment is required for PPM1A binding to SETD2 (Fig 5F).

The above results indicate that MALAT1, together with SETD2, forms a scaffold that facilitates the interaction between pSmad2/3 and PPM1A; the assembled lncRNA-proteins apparatus leads to the dephosphorylation of pSmad2/3 and thus termination of TGF- β /Smads signaling (illustrated in Fig 5G).

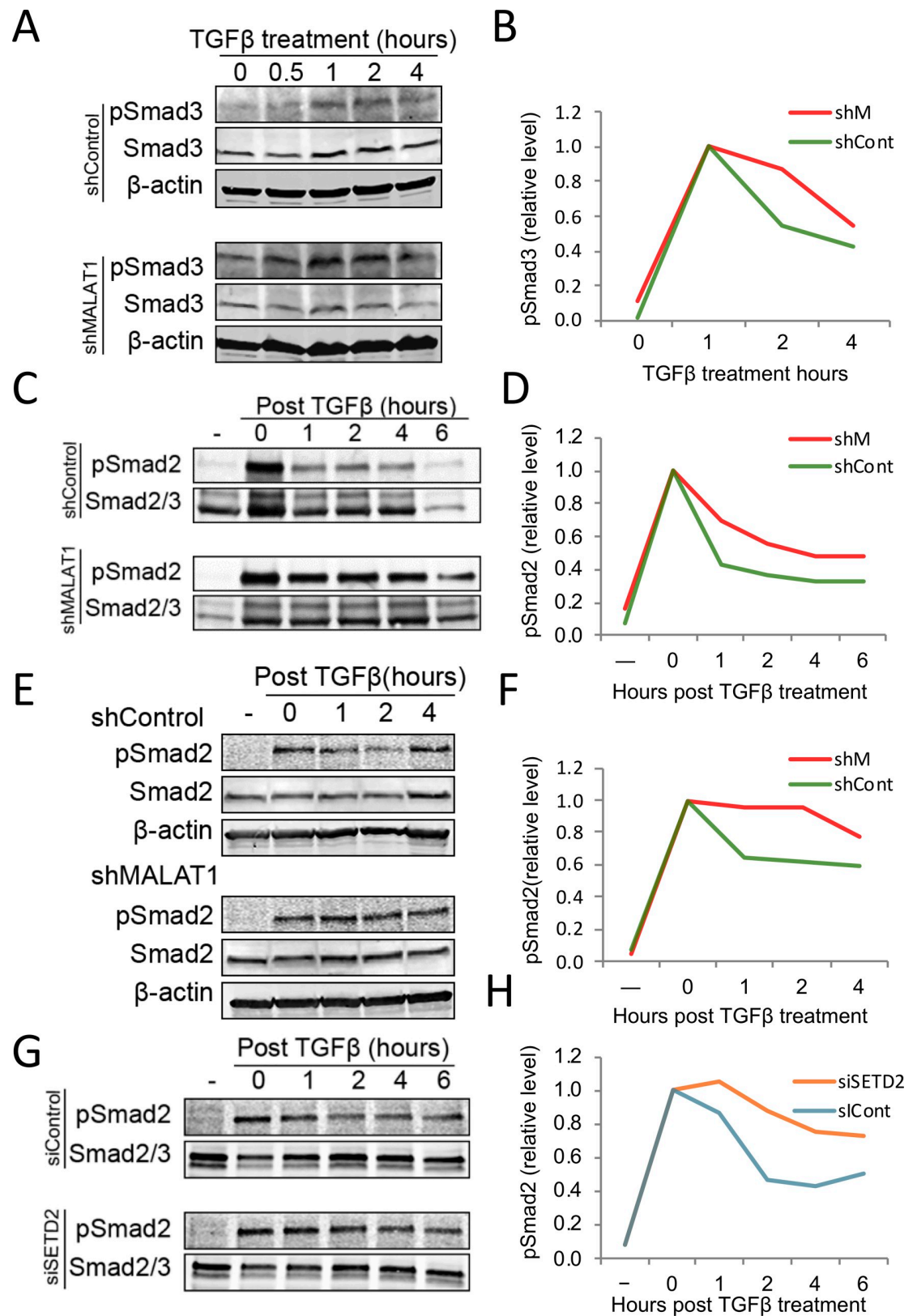


Fig 4. Depletion of MALAT1 reduces Smad2/3 dephosphorylation. A, B, Time course of Smad3 phosphorylation. MALAT1 depleted or control Hep3B cells were treated with 5 ng/ml of TGF- β 1 for the indicated time periods. Phospho-Smad3 and total

Smad3 were analyzed by Western blotting. Line graph demonstrates relative intensity ratio of phospho-Smad3 to total Smad3, the relative level of phospho-Smad3 at 1-hour time point was set as 1. C-H, Time course of Smad2 dephosphorylation in cells post TGF- β 1 treatment. MALAT1 depleted or control Hep3B cells (C, D), MALAT1 depleted or control PLC/PRF/5 cells (E, F) and SETD2 knockdown and control Hep3B cells (G, H) were treated with 5 ng/ml of TGF- β 1 for 1 hour, followed by TGF- β 1 washout and simultaneous addition of 10 μ M TGF- β R Inhibitor SB431542 and 10 μ M proteasome inhibitor MG-132. The line graphs (D, F and H) show relative ratio of pSmad2 level over total Smad2 (the value at time point 0 was set as 1). The band intensities from representative Western blot results were quantified by Odyssey software (LI-COR Bio, Lincoln NE).

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Depletion of MALAT1 enhances Smads-mediated iPS cell differentiation into hepatocyte-like cells

Differentiation of induced pluripotent stem (iPS) cells into hepatocytes is an intriguing phenomenon in liver biology which strictly depends on Smads signaling[19, 30]. In this process, activation of Smad2/3 (by Activin A or TGF- β) is essential for endodermal induction and subsequent hepatocyte lineage commitment and maturation[31, 32]. In the context of Activin A, it belongs to the TGF- β superfamily and regulates gene transcription through activating Smad2/3 signaling pathway[33]. To explore the biological function of MALAT1-regulated Smad2/3 signaling, we performed further studies to determine whether MALAT1 might influence iPS cell differentiation into hepatocyte-like cells. The differentiation protocol used in this study was outlined in Fig 6A. After 12 days of differentiation, cells derived from iPS cells were analyzed for hepatocytes-specific markers by immunofluorescence staining. As shown in Fig 6B, the expression of iPS cell markers (OCT-4A, SOX2 and c-MYC) in hepatocyte-like cells were reduced, while the hepatocyte specific proteins ALB, AFP, HNF-4a and TAT were induced. These findings indicate successful induction of iPS cell differentiation into hepatocyte-like cells in our system. To assess the effect of MALAT1 on the hepatocyte differentiation process, we transfected the iPS cells with MALAT1-specific 2'-OME-gamper ASO1 (phosphorothioated 2' O-methyl modified antisense oligonucleotide) or scramble control 2'-OME-gamper ASO (24 hours prior to Activin A or TGF- β incubation); at the end of the induction/differentiation process, the expression of hepatocyte specific markers were assessed by qRT-PCR. Our data showed that depletion of MALAT1 enhanced the expression of hepatocyte-specific genes (including ALB, AFP, HNF-4a, TAT and others) in cells incubated with either Activin A (Fig 6C) or TGF- β (Fig 6D).

Discussion

The canonical TGF- β /Smads signaling pathway in cells is finely regulated at different levels, including sequential activation of TGF β RII and TGF β RI through ligand binding, the phosphorylation of Smad proteins, regulation by inhibitory Smads, regulation of Smads activity in nucleus by kinases and phosphatases, proteasome for Smads degradation, and a group of microRNAs that target different components of the TGF- β pathway. In the current study, we describe a novel mechanism through which a long non-coding RNA regulates cellular TGF- β /Smads activity.

Our results presented in the current study show that MALAT1 is involved in the regulation of TGF- β /Smad pathway through formation of a lncRNA-protein complex with SETD2, pSmad2/3, and PPM1A. Specifically, the SETD2 protein mediates the association between MALAT1 and pSmad2/3; PPM1A is a nuclear Ser/Thr protein phosphatase, which dephosphorylates pSmad2/3 through physical interaction thus terminating Smad signaling [29]. Our data show that both SETD2 and PPM1A are associated with MALAT1 to assemble the scaffold for pSmad2/3 dephosphorylation. Our data support the concept that the PY motif (in pSmads) and the WW domain (in SETD2) are the physical basis for the binding of the two proteins; this

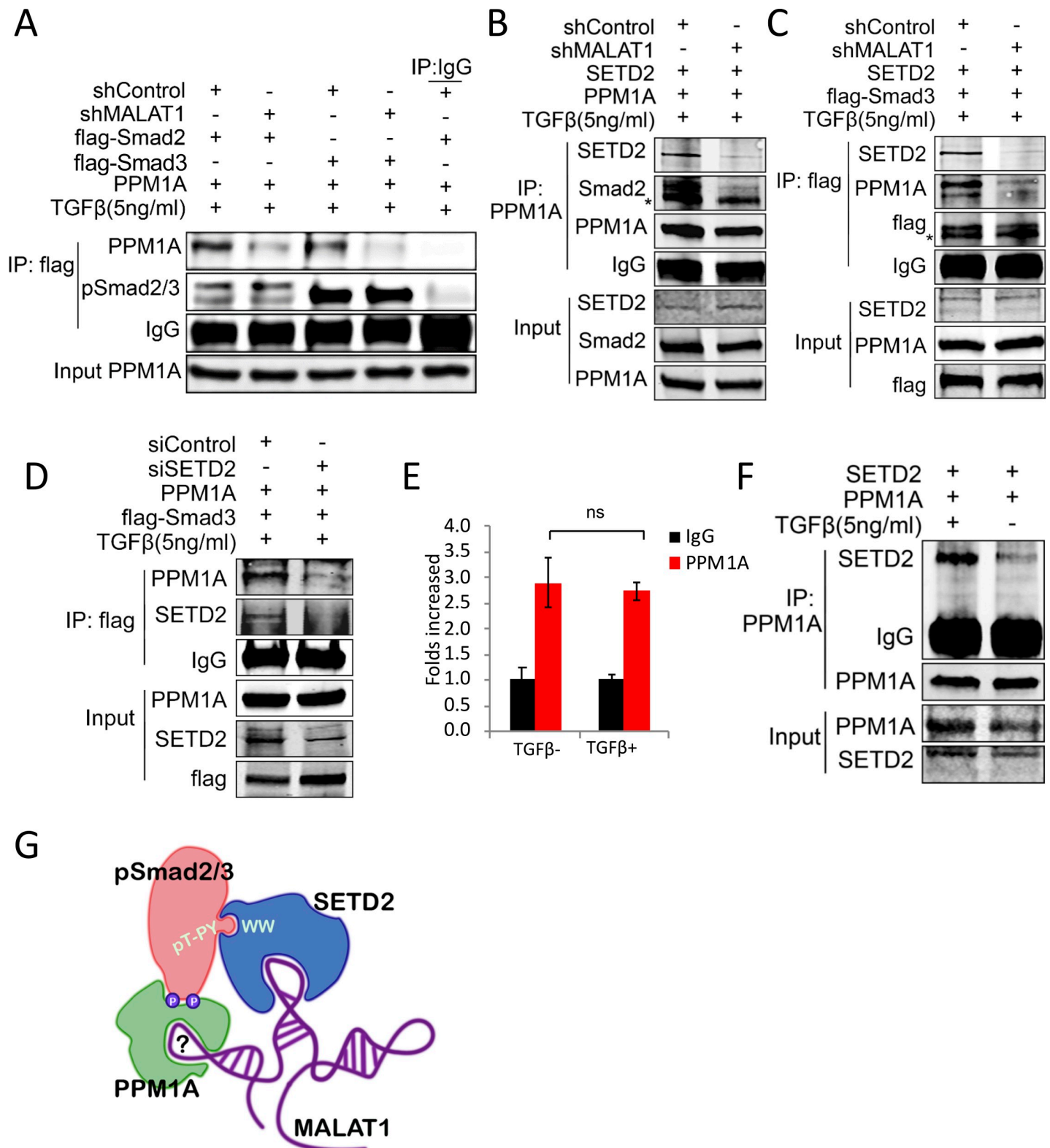


Fig 5. MALAT1 facilitates the SETD2-pSmad2/3-PPM1A complex formation. A-C, MALAT1 depletion prevents SETD2-pSmad2/3-PPM1A complex formation. MALAT1 depleted or control Hep3B (A) or PLC/PRF/5 (B, C) cells were transfected with flag-Smad2/3, PPM1A or SETD2 expression plasmids (as indicated at the top of each panel) and the cells were treated with TGF- β 1 for 1–2 hours. The cell lysates were then subjected to IP and Western blotting analyses using indicated antibodies. D, SETD2 depletion prevents pSmad2/3 binding to PPM1A. Cell lysates from SETD2-depleted and control PLC/PRF/5 cells (co-transfected with PPM1A and flag-

Smad3 expression plasmids) were subjected to co-immunoprecipitation using anti-flag antibody followed by Western blotting for SETD2 and PPM1A. E, RNA immunoprecipitation of MALAT1 by using anti-PPM1A antibodies in Hep3B cells with or without TGF- β 1 treatment (5ng/ml for 1 hour); precipitated MALAT1 was assessed by qRT-PCR. The data are shown as mean $\pm$ SD (n = 3; ns, no statistical significance). F, Co-immunoprecipitation of PPM1A and SETD2 in PLC/PRF/5 cells (co-transfected with SETD2 and PPM1A expression plasmids) with or without TGF- β 1 treatment. G, A diagram illustrating that MALAT1 acts as a scaffold to facilitate the SETD2-pSmad2/3-PPM1A protein complex formation. This complex facilitates the de-phosphorylation of phospho-Smad2/3 and thus the termination of Smad2/3 signaling. *: non-specific bands.

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interaction is essential for pSmad2/3 to be assembled into this lncRNA-protein dephosphorylation complex. Our findings are consistent with the previous report that linker region phosphorylation accelerates pSmad2/3 dephosphorylation and drives their turnover [22]. We show that PPM1A molecules are scattered throughout the nuclear envelope with only a portion overlapping with the nuclear speckle marker SC-35 and that TGF- β stimulation exhibits no effect on the level and localization of PPM1A. These findings are consistent with the notion that PPM1A is a phosphatase with multiple substrates in addition to pSmad2/3[34]. We noted that although MALAT1 depletion decreased SETD2 association with PPM1A and pSmad2/3, it had little effect on its other functions, such as histone H3K36 methyltransferase activity.

As a highly conserved nuclear noncoding RNA, MALAT1 was first described as tumor metastasis related gene in non-small cell lung cancer [17]. Recent studies showed that MALAT1

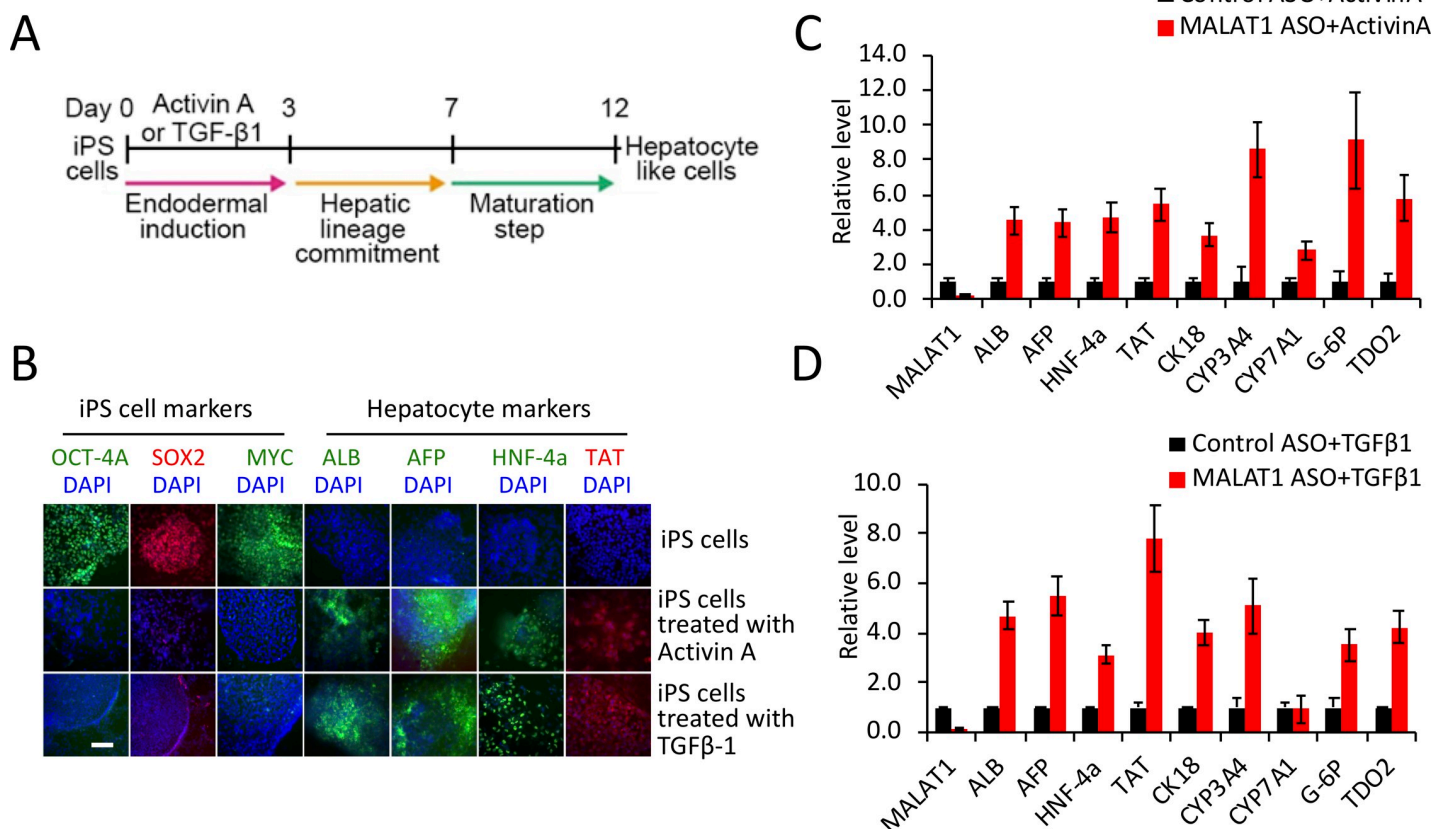


Fig 6. MALAT1 depletion enhances TGF- β - and Activin A-induced iPS cell differentiation to hepatocytes. A, A schematic representation of three-step induction from iPS cells to hepatocyte-like cells. B, Immunofluorescence staining for iPS cell markers (OCT-4A, SOX2, MYC) and hepatocyte markers (ALB, AFP, HNF-4a and TAT) before and after hepatic lineage induction (scale bar = 1mm). C and D, Quantitative RT-PCR analyses for the hepatocyte markers ALB, AFP, HNF-4a, TAT, CK-18, CYP3A4, CYP7A1, G-6P, and TDO2 in cells with or without MALAT1 depletion. iPS cells were transfected with MALAT1-specific 2'-OME-gamper ASO (phosphorothioated 2' O-methyl modified antisense oligonucleotide) or scramble control ASO; 24 hours later the cells were treated with Activin A (C) or TGF- β (D) for hepatocytes lineage induction as described in the Methods section. The data are shown as mean $\pm$ SD (n = 3).

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can bind to active chromatin sites[35] and regulate gene transcription[14, 15] and precursor mRNA splicing[16]. MALAT1 is localized in nuclear speckles, a dynamic structure containing pre-mRNA splicing factors and transcription factors[36]; this special localization provides the basis for physical interaction between MALAT1 and some transcription factors, such as pSmad2/3. Considering its broad expression spectrum and high conservation among multiple mammalian species, MALAT1 is believed to have fundamental cytological and genetic functions[37]. One study identifies 22 genes with altered expression in the livers of male MALAT1 knockout mice[15]. PAI-1, whose transcription is activated by Smad signaling, was found to be one of the most increased expressed genes. Through analyzing promoter regions (from -700 to +299) of those 22 genes, we found 13 of them contain 3 or more Smad Binding Element (SBE) core sequence (5'-CAGAC-3' or 5'-GTCTG-3')[9] in their promoter. Miyagawa et al[38] show that MALAT1 depletion or delocalization decreases the expression of 2'-5'-oligoadenylate synthetase like protein (OASL), interferon-induced protein 44 (IFI44), and serine peptidase inhibitor Kazal type 4 (SPINK4) in HeLa cells; those genes or their family members are known to be involved in interferon and TGF- β associated lung fibrosis[39]. Another interesting study by Watts et al[13] shows that myostatin, a member of the TGF- β superfamily, decreases MALAT1 more than 10 folds through non-Smad pathway, which, in turn, enhances its myogenesis-inhibitory function via the canonical TGF- β signaling pathway in human skeletal muscle cells. All of these findings support an important link between MALAT1 and TGF- β signaling pathway.

In the current study, we examined functional effect of MALAT1 on Smad2/3 activation by using the iPS cell-hepatocyte differentiation model, which is a well-established system to assess the activity of Smad signaling pertinent to liver biology[19, 30–32]. By employing this system, we demonstrate that MALAT1 depletion enhances TGF- β or Activin A-induced Smad2/3 activity and increases the expression of hepatocyte-specific genes.

Taken together, our experimental findings described in this study provide novel evidence that MALAT1 is a key regulator that determines TGF- β /Smad signaling. As the roles of TGF- β /Smads in cells are multifaceted and complex, further studies are warranted to assess the impact of MALAT1 on TGF- β /Smad signaling in other cell types and tissues.

Supporting information

S1 File. Supplementary materials and methods.
(PDF)

S2 File. Supplementary figures.
(PDF)

S1 Raw Images.
(PDF)

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Writing – review & editing: Tong Wu.

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