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Original Article

Deubiquitinase JOSD1 promotes tumor progression via stabilizing Snail in lung adenocarcinoma

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Abstract: Accumulating evidence suggests that the deubiquitinase JOSD1 accounts for aggressiveness and unfavorable prognosis in multiple human cancers. But, the significance of JOSD1 in lung adenocarcinoma (LUAD) is elusive. We established that JOSD1 was aberrantly overexpressed in LUAD tissues, relative to normal tissues. Elevated JOSD1 levels in LUAD tissues positively related to advanced clinicopathological characteristics and poor overall survival (OS) in LUAD patients. Furthermore, we found that JOSD1 knockdown suppressed tumor cell proliferation and metastasis, whereas overexpression of JOSD1 led to opposite phenotypes. Mechanistically, JOSD1 stabilized Snail protein through deubiquitination, which promotes the epithelial-to-mesenchymal transition (EMT) process. Indeed, JOSD1 promoted tumor cell invasion as well as metastasis on the dependence of Snail. The protein expression analysis of LUAD tissues indicated that JOSD1 positively correlated with Snail. Moreover, JOSD1 and Snail co-overexpression had the worst prognosis in LUAD patients. Overall, these results demonstrated that JOSD1 was significantly overexpressed in LUAD and stabilized Snail via deubiquitination to promote LUAD metastasis.

Keywords: Lung adenocarcinoma, JOSD1, Snail, EMT, metastasis

Introduction

Around the world, lung cancer is the most prevalent human malignancy, causing more than one million deaths every year [1]. In more recent years, lung adenocarcinoma (LUAD) has gradually emerged as the most predominant histologic subtype and is characterized by a high recurrence rate, invasion, and metastasis [2]. Among patients with advanced-stage LUAD, distant metastases rather than the primary tumor are the leading cause of death [3]. Typically, the common sites of LUAD metastases are lymph nodes, bones, and brains. However, there are no effective therapeutic options currently available for metastasis in the clinic. Therefore, exploration of the possible molecular mechanisms is urgently required.

Epithelial-to-mesenchymal transition (EMT) is an embryonic program that is usually abnormally reactivated during carcinogenesis [4]. During the EMT process, tumor cells lose their epithelial biomarkers (E-cadherin) on the membrane and acquire mesenchymal biomarkers (Snail, N-cadherin, Vimentin, and Twist), subsequently promoting migratory and invasive ability [5]. The inactivation of E-cadherin is considered a marked hallmark of the event of EMT. Notably, Snail has been identified as a potent transcription repressor of the E-cadherin [6, 7]. Snail is overexpressed in multiple human cancers, significantly associated with malignant behaviors, including invasion and metastasis [8-10]. Thus, the protein level of Snail must be tightly regulated by the ubiquitin-proteasome system (UPS) [11].

Post-transcriptional modifications (PTMs), especially ubiquitination and deubiquitination, have an essential role in regulating cellular protein levels. Ubiquitination is a dynamic and reversible process due to the function of deubiquitinases (DUBs). More than 100 DUBs have been identified in humans and are classified into seven families [12]. Indeed, the DUBs are enzymes that can specifically cleave ubiquitin from modified proteins and stabilize these pro-

teins. Accumulating evidence indicates that DUBs are closely involved in various human diseases, especially cancers [13, 14]. The Machado-Josephin domain-containing proteases (MJDs) family comprises four members and affects many essential biological processes, such as membrane dynamics, endocytosis, and cell motility [15-17]. JOSD1 (Josephin domain containing 1) is a critical member of this family. Several reported studies revealed that JOSD1 involves tumorigenesis and cancer progression. JOSD1 has been reported to promote cancer cell growth and predict poor prognosis in neck squamous cell carcinoma [18]. In addition, JOSD1 contributes to resistance to chemotherapy by stabilizing MCL1 in gynecological cancer [19]. But, the significance of JOSD1 in LUAD has not been fully established.

Our study reports that JOSD1 is aberrantly overexpressed in LUAD tissues versus the normal tissues, and its elevated levels predicts poor prognosis. JOSD1 knockdown significantly reduced the metastatic capacities of LUAD cells. Further study reveals that JOSD1 stabilizes Snail protein via deubiquitination to enhance EMT and metastasis. Collectively, our findings point to an essential role of JOSD1 in the development of LUAD, and JOSD1 may act as a potential target for LUAD treatment.

Materials and methods

Tissue microarray

Eighty nine pairs of LUAD and normal tissues were obtained from LUAD patients with informed consent in First Affiliated Hospital of Jiaxing University. A permit was gained from the Ethical Committee of First Affiliated Hospital. LUAD tissue microarray was constructed from paraffin-embedded tissue blocks. As previously described, immunohistochemical analysis was performed with JOSD1 antibodies (Abcam, diluted at 1:200) and Snail antibodies (Abcam, diluted at 1:500). Two individual investigators scored the expression of the stained markers of each sample via a histologic score (H-score). The H-score was determined by multiplying the intensity score by the proportion of positive cells (0-300). Bioinformatic analyses of the TCGA data was performed based on the web-based bioinformatic tool (www.home-for-researchers.com).

Cell culture

The BEAS-2B and seven LUAD cell lines (HCC-827, A549, H460, H1650, H1395, H1975, and H1299) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), maintained at 37°C under 5% CO_2 in humidified air with their specific media.

Plasmids construction and transfection

The JOSD1 overexpression plasmid was designed by the Genechem (Shanghai, China) and cloned into a lentiviral vector with a puromycin-resistance cassette. JOSD1 shRNA, Snail shRNA, and scrambled shRNA were purchased from the Genechem (Shanghai, China). Indicated plasmids were transfected in cells according to the manufacturer's guidelines.

Cell proliferation assay

Cell Counting Kit-8 (CCK-8) assay were conducted to assess cell proliferation. Briefly, 1,000 cells were inoculated into each well of a 96-well plate. Then 10 μl CCK-8 reagent was supplemented into the well followed by 2 h of incubation at 37°C. After incubation, absorbance of each sample was measured at 450 nm.

To assess the clonogenic potential, cells were inoculated into 6-well plates (1,000 cells/well). After 3 weeks of culture, colonies were methanol-fixed, stained using crystal violet (0.1%), and counted colonies with >50 cells.

Scratch wound-healing assay

Cells in 6-well plates were cultured until they reached 100% confluence. A scratch down the middle of each well was made using 20 μl pipette tips. After that, incubation for 24 h was done in a serum-free medium.

Transwell assay

The transwell system (8 μM pore size, BD Bioscience) was used for cell migration and invasion analyses. For cell migration assay, upper chambers that were not matrigel-coated were supplemented with serum-free medium (200 μl). For cell invasion assays, serum-free medium (200 μl) with 5×10^4 cells was supplemented in the upper chamber pre-coated with

50 μl matrigel. The chemoattractant used in lower chamber was medium (700 μl) with 10% FBS. At the end of experiments, invaded or migrated cells on the lower surface were methanol-fixed, stained using crystal violet (0.1%), and counted.

Real-time quantitative PCR

Total cellular RNA was isolated using the TRIzol reagent (Invitrogen, USA). The cDNA was reversed-transcribed using the PrimeScript RT Reagent Kit (Takara, Japan). Quantitative reverse transcription PCR (qRT-PCR) was conducted in triplicate for each experiment. The expression of genes was determined by the comparative CT method followed by normalization to GAPDH mRNA expression.

Protein degradation assay

Cycloheximide (CHX, Sigma, USA), a protein synthesis inhibitor, was used to prevent protein synthesis. Cells were treated with 0.1 mg/ml CHX and harvested at designed time points. Protein extracts were subjected to immunoblotting to assess the protein level at different CHX treatment times.

Immunoblot analysis and immunoprecipitation

Preparation of protein lysates was done using the RIPA lysis buffer (Cell Signaling Technology, USA) supplemented with protein inhibitor. Protein concentrations were assessed by the BCA protein quantification kit (Pierce, USA). For immunoblot analysis (IB), protein extracts were separated on 10% SDS-PAGE, transferred to PVDF membranes, which were cut and blocked 1 h in 5% skim milk after which they were incubated overnight with primary antibodies of JOSD1 (1:1000, Abcam), Snail (1:1000, Abcam), E-cadherin (1:1000, Abcam), Vimentin (1:1000, Abcam), and GAPDH (1:5000, Abcam) at 4°C. Anti-rabbit or anti-mouse HRP conjugated antibodies were the secondary antibodies, and the HPR signal was visualized with ECL detection reagents.

For co-immunoprecipitation (co-IP), control IgG or primary antibodies were supplemented to the cell lysates, followed by overnight incubation at 4°C. Precipitation of immunocomplexes was done with protein G agaroses beads. Then the beads were washed thrice using the lysis buffer, and the protein was eluted using the SDS loading buffer. Then precipitated proteins were subjected to immunoblotting.

GST pull-down assay

GST pull-down assays were performed using the GST Protein Interaction Pull-Down Kit (Pierce, USA) according to the instructions. The GST-JOSD1 fusion protein were expressed in E.coli stain BL21 and captured by glutathione-Sepharose beads. Subsequently, His-Snail recombinant protein was incubated with the beads for 2 h. Finally, the beads were resuspended by 2× sample buffer and subjected to immunoblotting after boiling for 5 minutes.

Animals and treatment

All animal procedures were permitted by the Jiaxing University Institutional Animal Care and Use Committee. 6-week-old male BABL/c nude mice were randomized in various groups (n=5). Stable cell lines were collected and suspended in PBS solution. For tumorigenicity assay, 5×10^6 cells were subcutaneously injected into the right dorsal flank of 6-week-old male nude mice. Tumor growth was examined every other day, and the nude mice were humanely killed after 21 days of injection. Tumors were removed, weighted, and imaged. Tumor sections from mice were immunostained with serval primary antibodies.

To generate a mouse tumor metastasis model, we intravenously injected 1×10^6 cells per mouse through the tail vein. Four weeks post-injection, all mice were sacrificed. Lung tissues were carefully dissected and fixed in 4% paraformaldehyde. Then, they were stained using hematoxylin and eosin (H&E). Lung metastasis was assessed by serial sectioning of the entire lung.

Statistical analysis

The SPSS 25.0 software (SPSS, Chicago, IL) was used for analyses. All data are presented as mean ± SD. A student *t*-test was performed to detect differences between groups. Spearman's rank correlation test assessed the association between JOSD1 and Snail expression in LUAD tissue microarray. We used the chi-squared test to compare clinical features among LUAD patient groups. Log-rank test and

Kaplan-Meier survival analyses were used to assess overall survival (OS) and disease-specific survival (DSS). *P*-values <0.05 denoted significance.

Results

JOSD1 levels in LUAD tissues were elevated and correlated with poor prognosis

To elucidate the potential roles of JOSD1 in LUAD, we first examined JOSD1 levels in LUAD using the TCGA dataset. Differential expression analysis in the TCGA-LUAD dataset was conducted to identify JOSD1 mRNA expression levels. The JOSD1 transcript levels were remarkedly high in LUAD tissues, relative to corresponding non-tumor tissues (P<0.001, **Figure 1A**). In addition, patients with elevated JOSD1 levels had poorer overall survival (OS) (P=0.02, **Figure 1B**) and disease-specific survival (DSS) (P=0.04, **Figure 1C**). We then examined JOSD1 protein levels in 89 LUAD and normal tissues pairs by immunohistochemistry (IHC). The results showed that JOSD1 staining was primarily in the cytoplasm and nucleus, and JOSD1 protein is significantly increased in LUAD tissues (H-score; 154.9±47.2) than in their corresponding adjacent normal tissues (H-score; 84.9±33.7) (P<0.001, **Figure 1D** and **1E**). We further examined the association between JOSD1 protein levels and the clinicopathologic features of LUAD. We found that JOSD1 overexpression was markedly correlated with tumor grades (P=0.025), lymph nodal metastasis (P= 0.002), as well as TNM stage (P<0.001) (**Table 1**). Kaplan-Meier survival analysis demonstrated that LUAD patients with elevated JOSD1 levels had poorer OS, relative to those with suppressed JOSD1 levels (P<0.001, **Figure 1F**). Collectively, abnormally high levels of JOSD1 are associated with LUAD development.

Overexpression of JOSD1 promotes LUAD cell growth

To establish the potential function of JOSD1 in LUAD cells, we first measured the expression of JOSD1 in several LUAD cell lines and a normal human bronchial epithelial cell line (BEAS-2B). As shown in **Figure 2A**, JOSD1 expression was notably higher in the seven LUAD cell lines (the A549 cells had the highest expression, the H1299 cells had the lowest) than in BEAS-2B. A549 cells were transfected with specific shRNA to silence JOSD1. H1299 cells were transfected with the JOSD1 overexpression plasmid. The efficiency of JOSD1 knockdown and overexpression was verified by western blotting (**Figure 2B**). Then we measured the influence of JOSD1 on cell proliferation. JOSD1 knockdown significantly inhibited the cell proliferation of A549 cells (**Figure 2C**). Consistent with the knockdown experiments, JOSD1 overexpression significantly promoted the cell proliferation of H1299 cells (**Figure 2C**). The plate colony formation assays were performed to further confirm the effect of JOSD1 on cell growth. As expected, JOSD1 knockdown notably inhibited the colony formation ability of A549 cells, whereas JOSD1 overexpression had the opposite effect on colony formation (**Figure 2D**). Additionally, we established xenograft models of BALB/c nude mice to assess the effects of JOSD1 on cell growth *in vivo*. JOSD1 knockdown significantly slowed down xenograft tumor growth in nude mice (**Figure 2E**). Three weeks post-inoculation, the xenografts were resected, and the tumor weights in the A549/sh-JOSD1 group were significantly lighter than those of the control group (1.60± 0.28 vs. 0.56±0.12, P<0.001) (**Figure 2E**). In addition, Ki-67 staining showed that knockdown of JOSD1 dramatically reduced the proportion of Ki-67 positivity (79.8±9.7 vs. 34.2± 4.2, P<0.001) (**Figure 2F**). Taken together, these results demonstrate a functional role of JOSD1 in LUAD cell proliferation.

Overexpression of JOSD1 promotes LUAD invasion and metastasis

We next investigated whether the JOSD1 expression is associated with tumor cell migration and invasion using wound-healing and transwell assays. Wound healing assays showed that the distance between wound edges of A549/sh-JOSD1 was dramatically longer than those of control cells (**Figure 3A**). The effect of JOSD1 overexpression was the opposite of that of JOSD1 knockdown (**Figure 3A**). Besides, JOSD1 knockdown significantly decreased the number of migrated and invaded cells of A549, while JOSD1 overexpression significantly increased the number of migrated and invaded cells of H1299 (**Figure 3B**). To further assess the effect of JOSD1 on the metastasis of lung cancer cells *in vivo,* we used an intravenous mouse xenograft model. The mice injected with

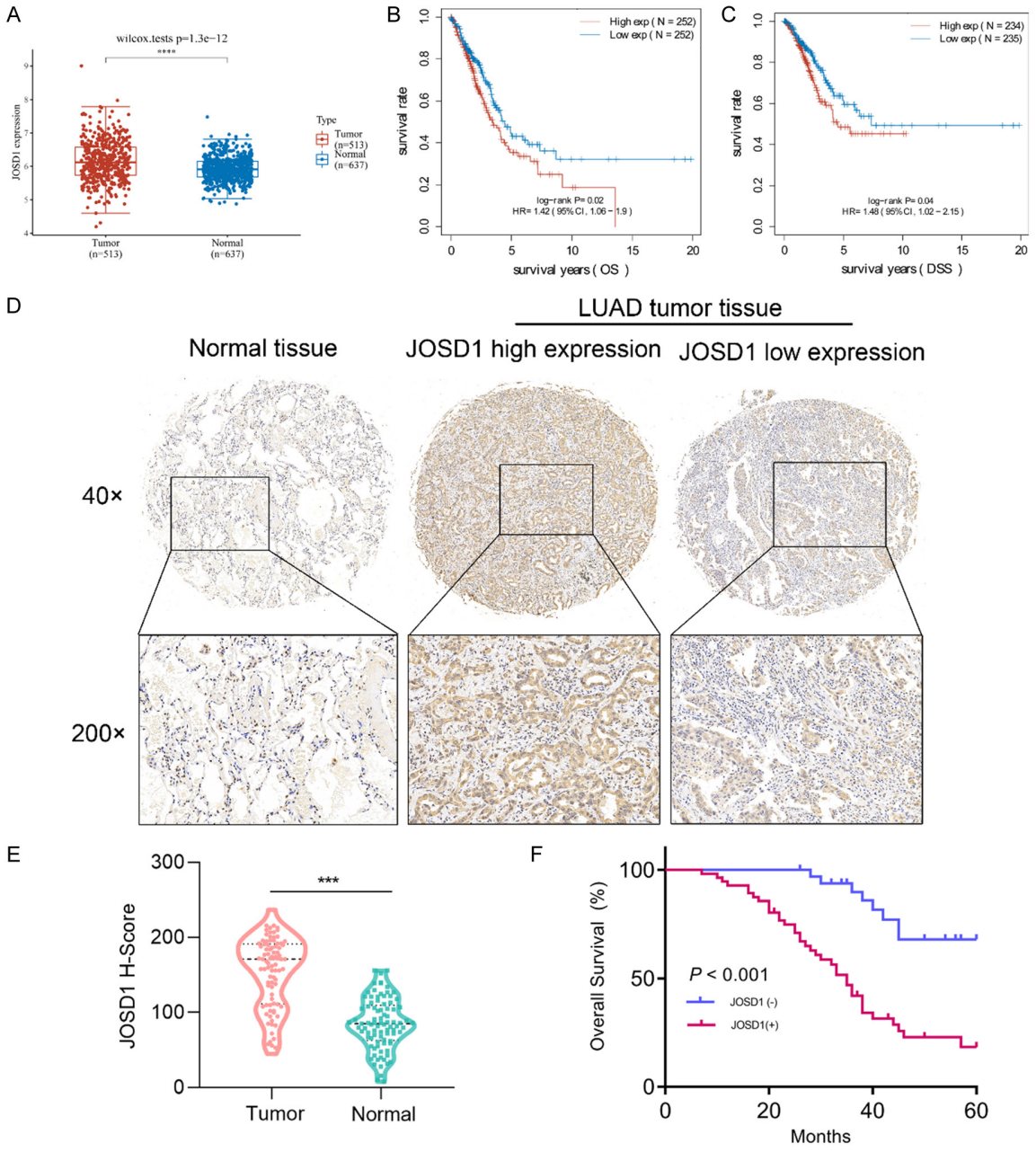


Figure 1. JOSD1 expression in LUAD and its correlation with OS. (A) JOSD1 mRNA expression in TCGA and GTEX database. Kaplan-Meier analysis of OS (B) and DSS (C) in the TCGA cohort according to JOSD1 mRNA level. (D) Representative immunohistochemistry images for JOSD1 in LUAD tissues and paired normal tissues. (E) Quantification of immunohistochemistry histological score (H-score) of JOSD1 protein. (F) Kaplan-Meier analysis of OS in the 89 LUAD patients according to the expression level of JOSD1 protein (log-rank test P<0.001). ***P<0.001 vs. control.

the control A549 clearly exhibited evidence of lung tumor micro-metastasis within that time, whereas injected with the JOSD1 knockdown cell line mice exhibited fewer micrometastatic modules (58.0±3.7 vs. 11.4±8.8, P<0.001) (**Figure 3C**). These results revealed that JOSD1 overexpression promotes tumor metastasis *in vitro* and *in vivo*.

JOSD1 interacts with Snail to promote its deubiquitination

We further examined the expression of the EMT marker by western blotting. As shown in **Figure 4A**, knockdown of JOSD1 decreased the expression of Snail and Vimentin and increased the expression of E-cadherin. Consistently,

Table 1. Relationship between JOSD1 levels and clinic-pathological features of LUAD

Variables	cases	JOSD1 expression		*P*-value
		Positive	Negative	
Tumor	89	56	33	**<0.001**
Non-tumor	89	22	67	
Age				
<60	32	22	10	0.730
≥60	57	34	23	
Gender				
Male	28	16	12	0.445
Female	61	40	21	
Smoking status				
Smoker	30	16	14	0.182
Non-smoker	59	40	19	
T stage				
T1	48	24	24	**0.025**
T2	32	25	7	
T3-4	9	7	2	
N stage				
N0	38	16	22	**0.002**
N1	32	25	7	
N2	19	15	4	
Differentiation				
High	41	29	12	0.303
Medium	32	19	13	
Low	16	8	8	
TNM stage				
I	32	11	21	**<0.001**
II	27	20	7	
III	30	25	5	

Bold represents that a *P*-value <0.05 and is significant.

overexpression of JOSD1 increased the expression of Snail and Vimentin and decreased the expression of E-cadherin. Although JOSD1 regulated the protein level of Snail, the mRNA expression remained unchanged (**Figure 4B**). To determine the molecular mechanism, we searched the public databases BioGRID (http://www.thebiogrid.org/) and found that the protein JOSD1 mostly interacted with Snail.

Given that JOSD1 is a well-known deubiquitinating enzyme, we hypothesize that JOSD1 interacts with Snail and stabilizes its protein stability through deubiquitination. To gain further insight into the JOSD1-Snail interaction, we co-transfected Myc-JOSD1 and Flag-Snail into 293T cells and conducted co-immunoprecipitation (co-IP) assays using the anti-Flag antibody. The results showed that Myc-JOSD1 was detected in the co-IP complex (**Figure 4C**). Reciprocally, after immunoprecipitation with anti-Myc antibody, Flag-Snail was detected in the co-IP complex (**Figure 4D**). We further performed co-IP assays to confirm the interaction between endogenous JOSD1 and Snail. After immunoprecipitation with anti-Snail antibody, the JOSD1 band was observed (**Figure 4E**). Moreover, GST pull-down assay showed that GST-JOSD1, but not GST, interacted with Snail (**Figure 4F**). These findings revealed that JOSD1 protein could directly interact with Snail protein in LUAD cells.

Next, we examined the effects of JOSD1 on Snail stability by cycloheximide (CHX) assay. The results demonstrated that JOSD1 knockdown significantly reduced the protein stability (**Figure 4G**). To determine whether JOSD1 mediates the ubiquitination of Snail, cells were exposed to 20 μM MG132 for 4 hours to prevent protein degradation. JOSD1 knockdown markedly elevated the ubiquitination of Snail in A549 cells (**Figure 4H**), while JOSD1 overexpression markedly suppressed Snail ubiquitination in H1299 cells (**Figure 4I**). To further assess whether JOSD1 stabilizes Snail through its deubiquitinating activity, we generated wild-type JOSD1 (JOSD1/WT) and JOSD1 mutant (JOSD1/C36A) that lacks deubiquitinating enzymatic activity. JOSD1 WT or JOSD1 Mut was co-transfected with Flag-Snail and HA-ubiquitin into 293T cells. The cells were harvested after treatment with 20 μM MG132 for 4 hours. Immunoprecipitation of cell lysates was done with the anti-Flag antibody, and Flag-Snail ubiquitination was detected by the anti-HA antibody. Ectopic expression of JOSD1/WT remarkedly deubiquitinated Snail, but JOSD1/C36A failed to deubiquitinated Snail (**Figure 4J**).

To identify the specific ubiquitination type of Snail affected by JOSD1. Wild-type ubiquitin, K63R mutant or K48R mutant ubiquitin were used to examine the ability of JOSD1 to mediated Snail dedubiquitination. We observed that JOSD1 only cleaved the K48 linked poly-ubiquitin chain on Snail protein (**Figure 4K**). Thus, JOSD1 mediated K48 linked poly-ubiquitous degradation of Snail protein.

JOSD1 depends on Snail to promote the LUAD metastasis

Snail has been identified as a key EMT transcription factor and has an essential function

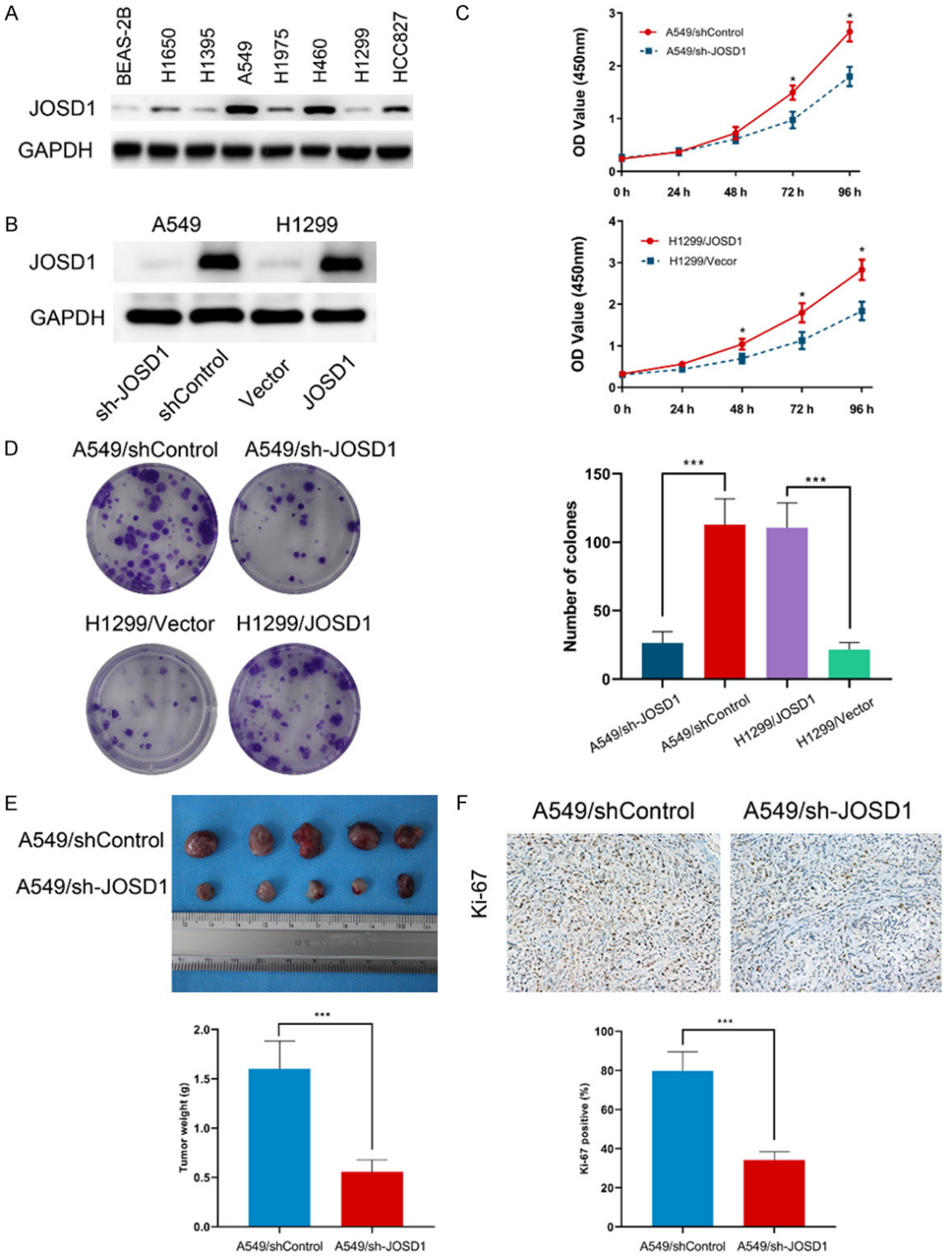


Figure 2. Effects of JOSD1 on LUAD cell growth *in vitro* and *in vivo*. (A) JOSD1 expression in seven LUAD cell lines and BEAS-2B was determined by western blotting. (B) A549 and H1299 cells transfected with sh-JOSD1 lentivirus and JOSD1 overexpression lentivirus, respectively, were subjected to western blotting. The effect of JOSD1 on LUAD cell proliferations were determined by CCK-8 assays (C) and colony formation analyses (D). (E) Xenograft experiments were performed to confirm the effects of JOSD1 on tumor cell growth *in vivo*. Upper panel, photographs of isolated tumors derived from mice injected with A549/sh-JOSD1 or control cells. Low panel, tumor weight was measured after excision. (F) Ki-67 immunohistochemical staining of xenografts and statistical analysis of Ki-67 staining. *P<0.05 vs. control. ***P<0.001 vs. control.

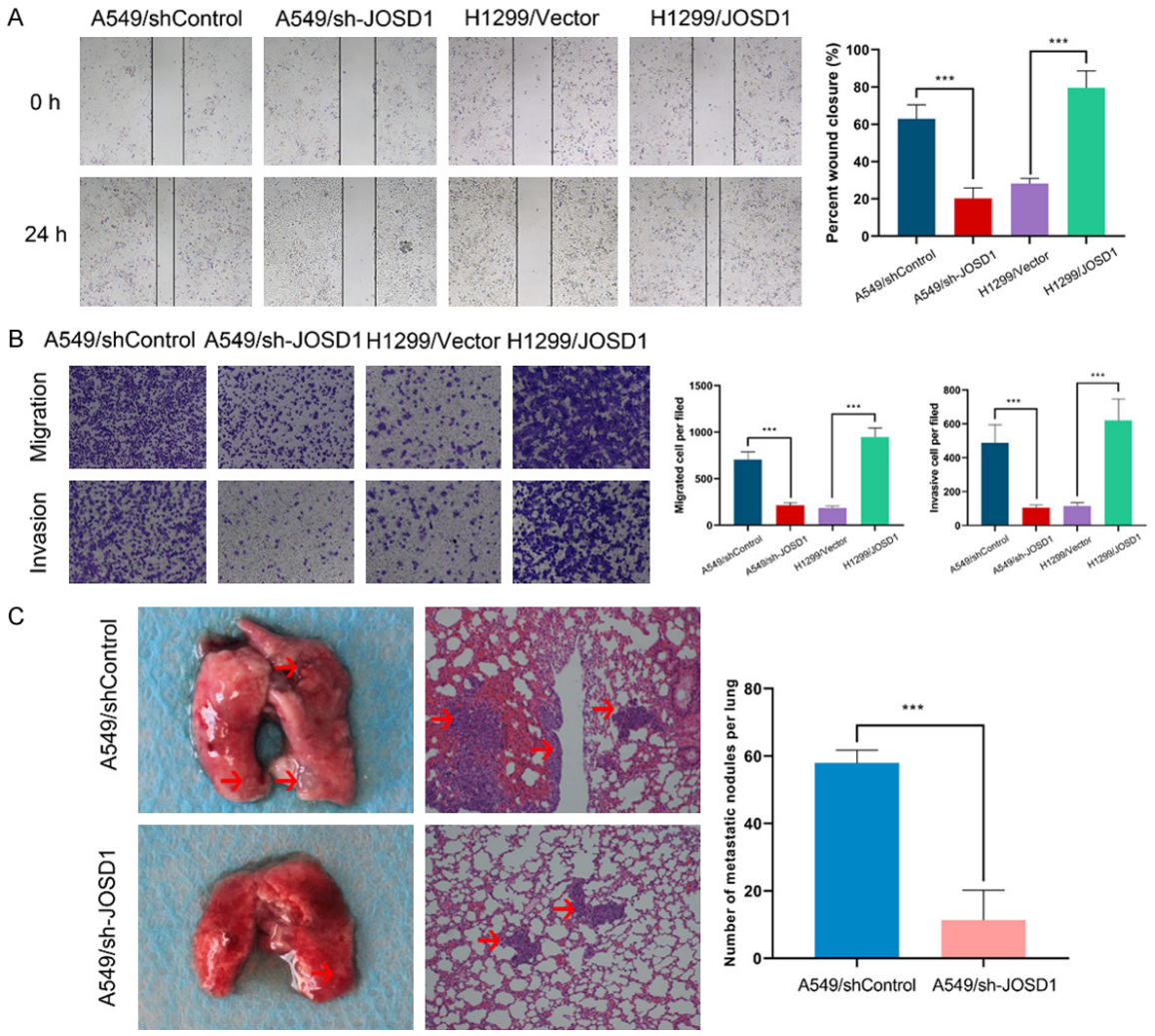
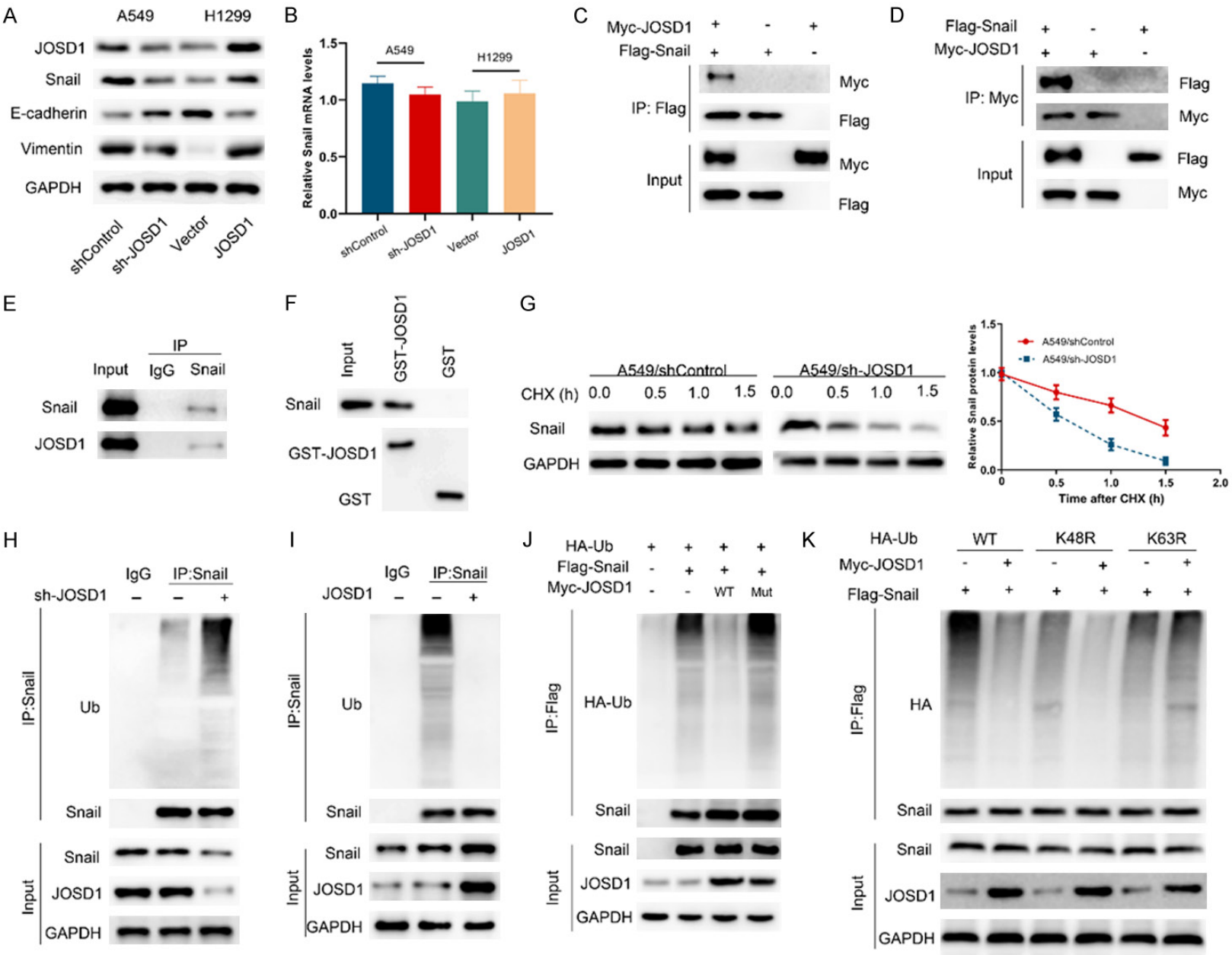


Figure 3. JOSD1 increased LUAD cells migration, invasion, and metastasis. A. Illustrative wound-healing images were recorded at designated times after scratching in A549/sh-JOSD1, H1299/JOSD1, and their control cells. Wound closure percentages were calculated at 0 and 24 h. B. Migration and invasion abilities were assessed by Transwell assay. The numbers of Cells migrated or invaded in the bottom chamber were counted (mag. ×100). C. Xenograft experiments were performed to confirm the effects of JOSD1 on tumor metastasis *in vivo*. Left panel, representative lung photographs. Middle panel, representative HE staining shows LUAD cell lung metastasis. Left panel, lung metastasis was quantified by determining metastatic nodule by serial sectioning of the entire lung. ***P<0.001 vs. control.

in the initial metastatic step. Considering JOSD1 promoted tumor cell metastasis and interacted with Snail, we wonder whether Snail mediated the effect of JOSD1 on metastasis. The Snail level was knockdown by specific shRNA in H1299 cells. JOSD1 overexpressing H1299 cells were co-transfected with Snail shRNA or scrambled shRNA. The knockdown efficiencies were verified by qRT-PCR and western blot (**Figure 5A**). Indeed, Snail knockdown in JOSD1 overexpressing H1299 cells markedly suppressed JOSD1-induced cell migration and invasion (**Figure 5B**). In addition, Snail knockdown significantly inhibited JOSD1-induced LUAD metastasis *in vivo*. The number of lung metastasis nodules in the JOSD1/shSnail group was markedly low, relative to the JOSD1/shControl group (108.4±19.1 vs. 53.4±7.4, P<0.001) (**Figure 5C**). Thus, JOSD1 promoted LUAD cell migration, invasion, and metastasis, partly due to the dependence of Snail.

High JOSD1 expression positively correlates with Snail expression in LUAD

We performed immunohistochemical analyses (IHC) of LUAD tissue microarray to determine


A
A549
H1299
JOSD1
Snail
E-cadherin
Vimentin
GAPDH
shControl
sh-JOSD1
Vector
JOSD1
B
Relative Snail mRNA levels
1.5
1.0
0.5
0.0
A549
H1299
shControl
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A549/shControl
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CHX (h) 0.0 0.5 1.0 1.5 0.0 0.5 1.0 1.5
Snail
GAPDH
A549/shControl
A549/sh-JOSD1
Relative Snail protein levels
Time after CHX (h)
H
IgG
IP:Snail
sh-JOSD1 – – +
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Myc-JOSD1 - + - + - +
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GAPDH

Figure 4. JOSD1 interacts with Snail and stabilizes Snail by deubiquitination. (A) The protein levels of EMT markers in A549/sh-JOSD1, H1299/JOSD1, and control cells. (B) The levels of Snail mRNA in A549/sh-JOSD1, H1299/JOSD1, and control cells. Myc-JOSD1 was co-transfected with Flag-Snail into 293T cells. co-IP assays were conducted using anti-Flag (C) or anti-Myc antibody (D). Immunoprecipitates were analyzed by immunoblotting. (E) Endogenous JOSD1 interacts with Snail. co-IP assays were done using anti-Snail antibodies. Immunoprecipitates were assessed by immunoblotting. (F) Confirmation of JOSD1-Snail interaction by GST pull-down assay. (G) A549 cells were treated with CHX (100 μg/ml) and harvested at various times post CHX. CHX assay measuring Snail stability, showing the half-life of Snail proteins. (H) A549/sh-JOSD1 and the control cells were exposed to 20 μM MG132 for 4 hours. Endogenous Snail ubiquitination was detected with ubiquitin antibody by immunoblotting. (I) H1299/JOSD1 and the control cells were treated with 20 μM MG132 for 4 hours. Endogenous Snail ubiquitination was detected with ubiquitin antibody by immunoblotting. (J) 293T cells were transfected with Flag-Snail and HA-ubiquitin and co-transfected with JOSD1 WT or JOSD1 Mut. After exposure to 20 μM MG132 for 4 hours, ubiquitination status of Flag-Snail was determined with HA antibody by immunoblotting. (K) 293T cells were transfected with indicated plasmids. Cells were harvested 4 h after reatment with 20 μM MG132. Immunoblotting was used to dectected the ubiquitination of Snail.

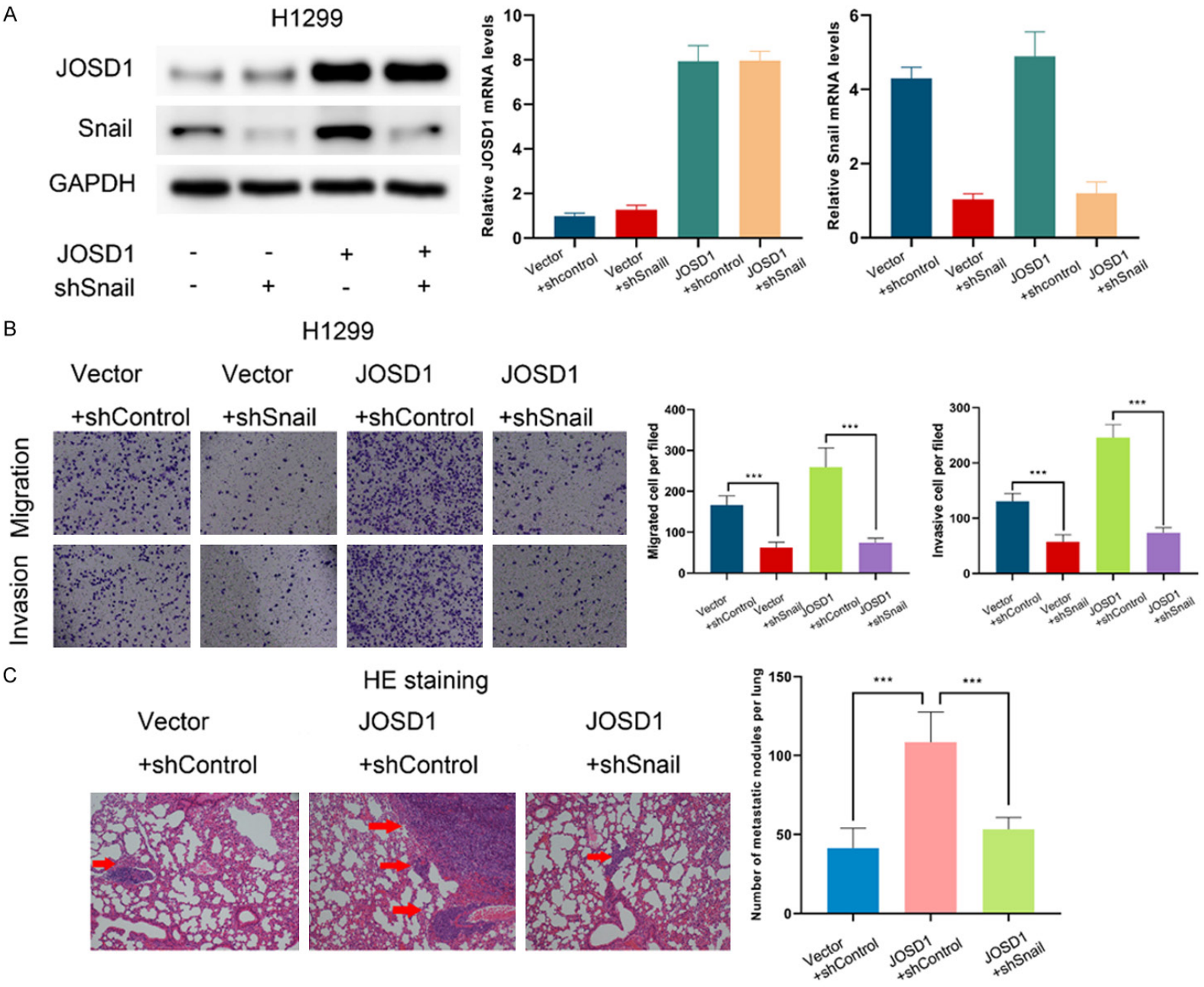
whether our findings have clinical relevance. IHC revealed that JOSD1 levels positively correlated with Snail levels (**Figure 6A** and **6B**). LUAD patients with high Snail levels had shorter OS, relative to those with suppressed levels (**Figure 6C**). This result is in line with previous studies. Considering JOSD1 promotes LUAD metastasis through stabilizing Snail protein, we wonder if JOSD1 and Snail combinations can better predict the survival outcomes than either molecule. Kaplan-Meier survival analysis showed that LUAD patients with high expressions of JOSD1 and Snail had the worst prognosis (**Figure 6D**). Taken together, JOSD1 positively correlated with Snail and predicted poor prognosis in LUAD patients.

Discussion

Machado-Josephin domain-containing proteases (MJDs) family, consisting of JOSD1, JOSD2, ATXN3, and ATXN3L, is the smallest family of deubiquitnases [15]. Recently, the MJDs family was reported to play an vital function in the carcinogenesis and development of several human tumors. For example, JOSD2 was reported to promote tumor cell proliferation by regulating the glucose metabolism in non-small cell lung cancer. Besides, elevated JOSD2 levels were markedly associated with poor prognostic outcomes [20]. In breast cancer, ATXN3 promoted deubiquitination and stabilization of KLF4 to enable its metastasis [21]. Moreover, JOSD1 was involved in chemoresistance in gynecological cancer and neck squamous cell carcinoma [18, 19]. Here, we reported that JOSD1 was overexpressed in LUAD, and elevated JOSD1 levels were associated with poor prognoses.

To detemine whether JOSD1 promotes LUAD cells metastasis, we then examined the biological role of JOSD1 in LUAD cells by overexpression or knockdown of JOSD1. Our study demonstrated that knockdown of JOSD1 inhibited LUAD cell migration, invasion, and metastasis. In contrast, overexpressed JOSD1 enhanced cell migration, invasion, and metastasis. It has been established that epithelial-to-mesenchymal transition (EMT) is a vital step in controlling cancer metastasis. Snail is a crucial driver of EMT by suppressing transcription of epithelial marker E-caderin [22, 23]. Previous studies have shown that the level of Snail protein stability is tightly regulated by ubiquitination and deubiquitination systems [24, 25]. Several E3 ligases, including FBXO11, TRIM50, and β-TrCP, specifically ubiquitinated and degraded Snail via ubiquitin-proteasome cascades [25-28]. Meanwhile, Snail has been found to be stabilized by different DUBs [29]. In esophageal squamous cell carcinoma (ESCC), deubiquitinase USP26 expression was strongly correlated with Snail expression. USP26 stabilized Snail and thus prompted the metastasis of ESCC [30]. Another deubiquitinase USP29 accelerated dephosphorylation and deubiquitination of Snail in gastric cancer by cooperating with SCP1 [31]. Here we reported, for the first time, that JOSD1 interacted with Snail and induced its deubiquitination. Subsequently, E-cadherin transcription was efficiently activated by accumulated Snail. Knockdown of Snail largely suppressed the effect of JOSD1 overexpression on the metastasis in LUAD cells. Taken together, JOSD1 stabilized Snail via deubiquitination, preventing its proteasomal degradation, and thus induced LUAD cell EMT, migration, invasion, and metastasis.

Although our study is promising, several aspects still need to be elucidated and improved in further studies. Firstly, a multi-center study


A
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JOSD1
Snail
GAPDH
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B
H1299
Vector +shControl
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Migration
Invasion
Migrated cell per filed
Invasive cell per filed
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C
HE staining
Vector +shControl
JOSD1 +shControl
JOSD1 +shSnail
Number of metastatic nodules per lung

Figure 5. Knockdown of Snail recuses effect of JOSD1 in LUAD cells. A. Snail was knocked down in JOSD1 overexpressing H1299 cells using specific shRNA. JOSD1 and Snail levels were determined by western blotting and qRT-PCR. B. The migration and matrigel invasion assay. Knockdown of Snail inhibited the JOSD1-induced migration and invasion. C. Snail knockdown significantly inhibited JOSD1-induced LUAD metastasis *in vivo*. Right panel, representative HE staining shows LUAD cell lung metastasis. Left panel, lung metastasis was quantified by determining metastatic nodule by serial sectioning of the entire lung. ***P<0.001 vs. control.

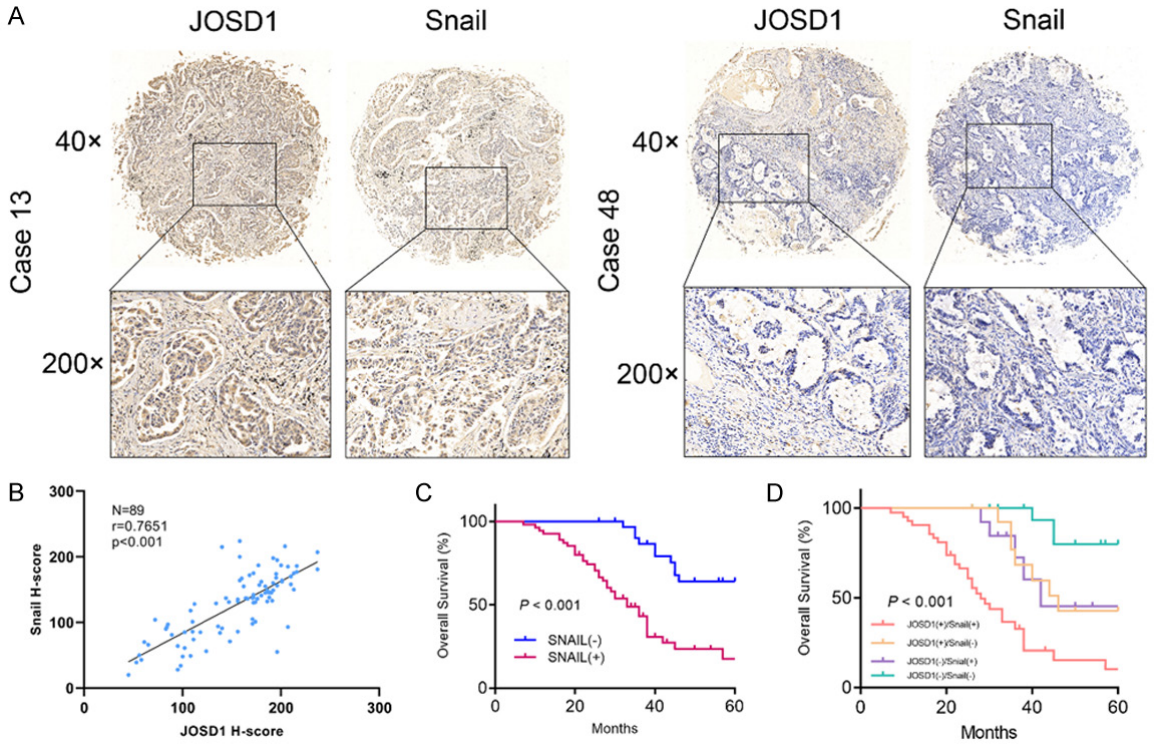

Figure 6. JOSD1 and Snail could be used as potential prognostic biomarkers together for LUAD patients. A. Illustrative immunohistochemistry (IHC) staining with JOSD1 and Snail. B. Correlation analyses showed the positive association between JOSD1 and Snail in LUAD tissues. C. High Snail levels correlated with poor prognostic outcomes for LUAD patients. D. The LUAD patients with co-overexpression of JOSD1 and Snail had the worst prognosis.

with more LUAD patients is warranted to confirm the expression patterns and prognostic significance of JOSD1 in LUAD patients. Due to the lack of enough samples collected in our hospital, we used a tissue microarray constructed from 89 pairs of LUAD tissues and the corresponding non-cancer tissues. Secondly, the molecular mechanisms leading to the upregulation of JOSD1 in LUAD remain elusive and need to be further investigated. Epigenetic modifications, such as alteration in miRNAs, DNA methylation, or histone acetylation, may regulate JOSD1 expression. The latest finding suggested that the small molecules (XL-9872-106C and SB1-F-70) targeted JOSD1 may be a novel therapy for leukemias [11]. In further study, we could screen small molecule libraries and discover compounds that specifically target JOSD1, which may have great potential in clinical translation in LUAD treatment.

In conclusion, our study revealed that JOSD1 acted as an oncogene and significantly promoted LUAD cell proliferation and metastasis *in vitro* and *in vivo*. JOSD1 interacted with and stabilized Snail through deubiquitination. Subsequently, accumulated Snail suppressed the expression of E-cadherin. JOSD1/Snail/E-cadherin axis regulated LUAD metastasis. Moreover, high JOSD1/Snail levels correlated with the worst prognostic outcomes in LUAD patients. Our novel findings shed light on potential treatments by targeting the JOSD1/Snail regulatory axis in LUAD patients.

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Disclosure of conflict of interest

None.

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