



Original Articles

POH1 induces Smad3 deubiquitination and promotes lung cancer metastasis



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ABSTRACT

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Smad3 is the key mediator of TGF- β 1-triggered signal transduction and the related biological responses, promoting cell invasion and metastasis in various cancers, including lung cancer. However, the deubiquitinase stabilizing Smad3 remains unknown. In this study, we present a paradigm in which POH1 is identified as a novel deubiquitinase of Smad3 that plays a tumor-promoting role in lung adenocarcinoma (LUAD) by regulating Smad3 stability. POH1 markedly increased Smad3 protein levels and prolonged its half-life. POH1 directly interacted and colocalized with Smad3, leading to the removal of poly-deubiquitination of Smad3. Functionally, POH1 facilitated cell proliferation, migration, and invasion by stabilizing Smad3. Importantly, POH1 also promoted liver metastasis of lung cancer cells. The protein levels of both POH1 and Smad3 were raised in the tumor tissues of patients with LUAD, which predicts poor prognosis. Collectively, we demonstrate that POH1 acts as an oncogene by enhancing TGF- β 1/Smad3 signaling and TGF- β 1-mediated metastasis of lung cancer.

1. Introduction

Transforming growth factor- β (TGF- β) signaling participates in the regulation of a wide range of cellular functions, including cell growth, differentiation, motility, and cellular immune response [1], which is closely related to human diseases such as organ fibrosis and tumor metastasis [2–5]. Although TGF- β 1 can activate a series of signal pathways, the downstream intact Smads molecules play a dominant role in their biological function, that is, the classical TGF- β /Smads signal. The process can be summarized as activated TGF- β type I receptor (T β RI) phosphorylates Smad2 and Smad3, which then translocate into the nucleus with Smad4 to regulate the transcription of target genes. In this regulatory process, Smad3 is the key player in TGF- β 1-triggered signal transduction and major biological responses, such as tumor cell invasion and metastasis [6–8].

The ubiquitination/deubiquitination modification system is emerging as a key regulatory mechanism regulating TGF- β /Smads

pathway components [9]. Deubiquitination counteracts ubiquitination by removing ubiquitin from substrate proteins using deubiquitinases (DUBs) that are important for maintaining cellular homeostasis and diverse biological processes [10]. DUBs are frequently and tightly associated with tumor progression and are thus considered novel, promising therapeutic targets in cancer, which has attracted increasing attention [11,12]. Uch37 was the first DUB found in the TGF- β /Smads pathway, which can induce deubiquitination of T β RI and regulation of TGF- β mediated transcription [13]. Subsequently, several other DUBs of T β RI were identified in different cancers, such as USP4 and USP15 in breast cancer and glioma, respectively [14,15]. Studies on the deubiquitination regulation of T β RI downstream Smads proteins are limited. A previous study demonstrated that USP15 is the DUB of R-Smad in human epidermal HaCaT cells and MDA-MB-231 breast cancer cells. USP15 can resist the mono-ubiquitination of R-Smad, resulting in enhancement of the TGF- β /Smad signal and the related cellular function, such as cell growth arrest and migration [16]. However, this study

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showed that USP15 did not affect the levels of phosphorylated or total Smad2/Smad3 proteins. The DUB that can stabilize Smad3 remains unclear. Given the core role of Smad3 in the TGF- β /Smads pathway, it is important to fully elucidate the regulatory mechanisms of DUB in Smad3 stability and how their regulation influences cellular functions and disease development.

Lung cancer is the leading cause of cancer-related mortalities worldwide. Non-small cell lung cancer (NSCLC) accounts for 80–85 % of cases [17]. TGF- β 1 is considered the most important inducer of EMT, invasion, and metastasis of NSCLC cells [18]. Smad3 plays a key role in NSCLC development. Smad3 deletion significantly suppresses TGF- β -mediated lung cancer growth and metastasis. In addition to TGF- β 1, Smad3 was found to be required for other regulators-promoting progressions of lung cancer such as profilin-2 [19], PREP1 [20], miR-206 [21], long noncoding RNA ELIT-1 [21], MiR-145, and miR-203 [22]. Although the importance of Smad3 in lung cancer development has been gradually recognized, the regulatory mechanism of Smad3 in lung cancer and its effects on the growth, invasion, and metastasis of lung cancer remain unclear.

POH1 (also known as Rpn11 or PSMD14) is a deubiquitinating enzyme within the 19S proteasomal subunit that regulates proteasomal activities and functions in various biological processes, including the DNA damage response [23], transcriptional activation, cell differentiation, and survival [24]. Emerging evidence has demonstrated that POH1 is upregulated in different types of malignancies, including hepatocarcinoma, esophageal squamous cell carcinoma, prostate carcinoma [25] and breast cancer [26], and promotes tumor development by regulating the stability of various substrates, such as E2F1 [27], Snail [28] and cyclinD1 [29]. Interestingly, POH1 and Smad3 are respectively found to be upregulated in human tissues in diverse cancers, including gastric cancer [30,31], esophageal cancer [28,32], prostate cancer [33,34], and ovarian cancer [35,36]. Furthermore, high expression of POH1 and Smad3 promotes cancer progression and correlates with poor prognosis in patients with these cancers. Recent studies have shown that POH1 expression is significantly upregulated in human NSCLC tissues and is associated with tumor growth and invasion in patients with lung adenocarcinoma (LUAD) [37,38]. However, the mechanism linking POH1 expression to NSCLC progression remains unclear. In this study, we identified POH1 as the novel DUB of Smad3 and demonstrated that POH1 promotes the invasion and metastasis of NSCLC by deubiquitinating and stabilizing Smad3.

2. Materials and methods

2.1. Plasmids and siRNAs

Myc-tagged POH1 and POH1 mutants (C120S and H113Q, respectively) were cloned into the pCMV-Myc vector. Flag-tagged Smad3, Smad3 MH1, Smad3 MH2 and Smad3 linker were cloned into the p3×FLAG-CMV-10 vector. Plasmids expressing flag-tagged POH1 and POH1 mutants (C120S and H113Q mutants) were kindly provided by Professor Yongzhong Liu (Shanghai Jiaotong University). Point mutations in ubiquitin (Ub) were generated using a Muta-Direct Mutagenesis Kit (SBS; Genentech), as described previously [39]. LPC-POH1 was constructed by cloning POH1 cDNA into the BamHI/EcoRI sites of the retroviral vector pLPC with the selectable marker puromycin and sequenced [40]. All mutations were confirmed using DNA sequencing. Two siRNAs targeting human POH1 were designed and synthesized by GenePharma (Shanghai, China). The sense targeting sequences were GGACAUGAACCAAGACAAATT and GGAACAUUGUGGAUGUACUUTT. Two siRNAs targeting human Smad3 were synthesized by GenePharma. The sense targeting sequences were GCGUGAAUCCUACCACUATT and GCCAUCCAUGACUGUGGAUTT. Irrelevant dsRNA with the sense sequence UUCUCCGAACGUGUCACGU was used as a control.

2.2. Antibodies and reagents

POH1 antibody (12059-1-AP), YOD1 antibody (25370-1-AP), USP37 antibody (18465-1-AP), β -actin antibody (66009-1-AP), HA tag antibody (51064-2-AP), and DYKDDDDK Flag tag antibody (20543-1-AP) were purchased from Proteintech (Chicago, IL, USA). The anti-Flag M2 beads were purchased from Sigma-Aldrich. The POH1 Antibody (sc-100464) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Smad3 (C67H9), Phospho-Smad3 (Ser423/425; C25A9), and E-cadherin (#14472) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). N-cadherin (#610920) was purchased from BD Biosciences, USA. Myc-tagged mAb (AE010), ubiquitin mAb (A19686), K63-linkage Specific Polyubiquitin antibody (A18164), K48-linkage Specific Ubiquitin antibody (A3606) were obtained from ABclonal (Wuhan, China). Secondary antibodies conjugated to Alexa Fluor 488, 568, or 633 were purchased from Invitrogen for immunofluorescence analysis. The proteasome inhibitor MG132 (SML1135) and protein synthesis inhibitor cycloheximide (C7698) were purchased from Sigma-Aldrich. The POH1 inhibitor thiolutin (HY-N6712) was purchased from BIOPlastics.

2.3. Cell culture and transfection

Human cervical carcinoma HeLa cells and human non-small cell lung cancer A549 cells were cultured in DMEM supplemented with 10 % fetal bovine serum (FBS) and antibiotics. Human non-small cell lung cancer H1299 cells were grown in RPMI 1640 (Invitrogen, USA) supplemented with 10 % FBS and antibiotics. All cells were maintained at 37 °C in a humidified incubator with 5 % CO₂. The cells were transfected with plasmids or siRNA using RNAi Max or Lipofectamine 2000 (Invitrogen) or PEI (Polyscience) according to the manufacturer's instructions.

2.4. Proximity ligation assay (PLA)

Cells were pre-coated onto TC-treated glass cover-slides ($\varphi = 10$ mm) a day before the PLA was conducted. The cover-slides were washed twice with cold PBS, dried for 1 min, and then attached to a slide with the cell side up. The boundary of the cover slide was sealed using a PAPPen (BC003; Biosharp).

On day 1, the slide was washed with cold PBS twice, fixed in 4 % paraformaldehyde, permeabilized with 0.1 % NP-40, and stained with the indicated primary antibodies overnight at 4 °C. On day 2, after being washed with washing buffer A (DUO82049, Sigma-Aldrich) twice, the slide was then incubated in a wet box at 37 °C with both PLUS (Anti-Rabbit, DUO92002, Sigma-Aldrich) and MINUS probes (Anti-Mouse, DUO92004, Sigma-Aldrich) for 60 min, followed by incubation with ligase (DUO92008, Sigma-Aldrich) for 30 min, and subsequently with detection reagent red (DUO92008, Sigma-Aldrich) for 100 min. After incubation, slides were washed with 1× washing buffer B (DUO82049, Sigma-Aldrich) twice and 0.01× washing buffer B once, and dried in the dark at room temperature. The cells were then incubated in a DAPI-containing mounting medium (DUO82040) for 10 min at room temperature. PLA and DAPI signals were counted under a fluorescence microscope, and high-resolution intercellular visualization was performed using a confocal microscope (Leica Microsystems, Wetzlar, Germany).

2.5. Immunoprecipitation and immunoblotting

Cells were lysed and prepared using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4, 0.5 % sodium deoxycholate, 1 % NP-40, 0.1 % SDS, 1 mM Na₃vo₄, and 100 mM NaF) containing protease inhibitors. Immunoprecipitations were performed using primary antibodies and protein A/G-agarose (Santa Cruz) or anti-Flag M2 (Sigma) beads at 4 °C. Thereafter, the immune complexes were washed three times with RIPA buffer and eluted with sample buffer containing 1 % SDS for 5 min at 95 °C, followed by SDS-PAGE and

protein transfer onto transfer membranes and probed with primary and secondary antibodies. Visualization was performed using the Super-Signal Chemiluminescence Kit (Pierce).

2.6. Immunofluorescence and confocal microscopy

The cells cultured on coverslips were washed with cold PBS twice, fixed in 4 % paraformaldehyde, permeabilized with 0.1 % NP-40, and stained with the indicated primary antibodies overnight at 4 °C, followed by incubating with secondary antibodies conjugated with Alexa fluor 488, 568 or 633 (Invitrogen). Cells were stained with DAPI to visualize the nuclei. Intracellular localization was visualized using confocal microscopy (Leica Microsystems, Wetzlar, Germany).

2.7. Transwell assay

The cells were resuspended in a medium containing 1 % FBS at 5×10^5 cells/mL and seeded onto polycarbonate membranes with a pore size of 8 µm (Costar, Corning, NY) to determine cell migration and invasion. For the invasion assays, Matrigel (2 mg/mL) was pre-coated in the upper chamber. The bottom wells of the chambers were filled with 700 µL medium supplemented with 20 % FBS. After incubation for 6 h or 24–48 h for the invasion assay, migratory or invasive cells were fixed in 4 % paraformaldehyde and subsequently stained with 1 % crystal violet for 20 min. Ten microscopic fields were randomly selected for analysis.

2.8. Cell proliferation assays

Cell proliferation was assessed using WST-1 and crystal violet assays as described previously [41]. For the WST-1 assay, the cells were seeded in a 6-well plate at a density of 2×10^3 cells/mL. On the second day, the cells were incubated with WST-1 (100 µL/well) in fresh serum-free medium at 37 °C for 2 h after the removal of the growth medium. The intensity of viable cells was continuously quantified for 7 days by measuring the absorbance at a wavelength of 450 nm using a microplate reader (Bio-Rad).

Anchorage-dependent cell proliferation was analyzed using a crystal violet assay. Briefly, 500 cells cultured in RPMI 1640 medium containing 10 % FBS were seeded in triplicate in a 6-well plate at a density of 5×10^3 cells/mL. After 7 days, the cells were fixed with 4 % paraformaldehyde for 15 min and stained with 0.5 % crystal violet for 30 min at room temperature. The number of colonies in each group was counted and analyzed.

2.9. Xenograft tumor formation in mice

The animal experiments were approved by the Ethics Committee of the Peking University Health Science Center. H1299 cells (3×10^6) were counted and resuspended in 100 µL RPMI 1640, then injected subcutaneously into BALB/c female nude mice (Center of Experimental Animals, Peking University, Beijing, China), and tumor sizes were measured at the indicated time. The tumors were dissected after 25 days when they reached a diameter of approximately 1 cm. The tumors were weighed and photographed.

2.10. Tissue samples and immunohistochemistry

Immunohistochemical staining for specific protein expression was performed using human lung adenocarcinoma tissue chips, which were purchased from Shanghai Outdo Biotech Co., Ltd. Briefly, sections (4 mm thickness) were deparaffinized with xylene and rehydrated in ethanol. Hydrogen peroxide (3 %) was used to eliminate the endogenous peroxidases. Sections were incubated overnight at 4 °C with primary antibodies against POH1 and Smad3. After extensive washing in PBS, the sections were incubated with secondary antibodies (Dako, Carpinteria, CA, USA) for 30 min. Immunostaining was examined using an

Olympus BX51 microscope (Olympus, Tokyo, Japan).

2.11. Statistical analysis

Data are presented as mean \pm SD. Comparisons between any two groups were performed using 2-tailed Student's t-tests. Differences between more than two groups were compared using one-way ANOVA. Pairwise comparisons were performed using the Student-Newman-Keuls procedure or Dunnett's T3 procedure when the assumption of equal variances did not hold. All statistical analyses were performed using SPSS version 20.0. P values < 0.05 were considered statistically significant.

3. Results

3.1. POH1 increases Smad3 stability

We screened an unbiased DUB library containing 76 DUBs in HeLa cells using a pool of three non-overlapping siRNA oligos or plasmids for transfection experiments to identify DUBs with the capacity to stabilize Smad3 expression. The three DUBs, POH1, YOD1, and USP37, markedly increased Smad3 protein levels in a dose-dependent manner (Fig. 1A). The regulation of enzymes on substrates depends on the direct interactions between them. To determine the DUB of Smad3, we examined the interaction between Smad3 and three candidate DUBs. Co-immunoprecipitation (Co-IP) showed that POH1 interacted with Smad3 (Fig. 1B); the other two did not. We verified the regulatory effects of POH1 on Smad3 protein level in human lung adenocarcinoma cells H1299 and A549. The results showed that the transient expression of POH1 increased the level of Smad3 in a dose-dependent manner in both H1299 (Fig. 1C) and A549 cells (Fig. 1D). In addition, knockdown of POH1 in H1299 cells by siRNA interference significantly decreased the protein level of Smad3, which could be reversed by the proteasome inhibitor MG132 (Fig. 1E). This suggested that POH1 may stabilize Smad3 by inhibiting proteasome degradation. Similarly, the protein level of Smad3 decreased in a dose-dependent manner after treatment with different concentrations of POH1 siRNA in A549 cells (Fig. 1F). We then established H1299 cells with stable overexpression and knockdown of POH1 and detected the expression of Smad3. Consistently, Smad3 protein levels increased in H1299 cells stably expressing POH1 (Fig. 1G) and decreased in cells with a stable knockdown of POH1 (Fig. 1H). Moreover, we used the POH1 inhibitor thiolutin (THL) to further verify the stabilizing role of POH1 on Smad3. The results showed that THL significantly reduced Smad3 levels in a dose-dependent manner, whereas POH1 levels did not change (Fig. 1I). THL was reported to inhibit the DUB activity of POH1 but did not affect protein expression [42], which is consistent with our results. There is a report showing that POH1 is the DUB of T β RI and T β RII in hepatocellular carcinoma cells, mediating their stability [43]. However, we found that POH1 cannot affect the protein level of TGF- β receptors in lung cancer cells (Fig. 1J). This indicated that POH1 increased the protein level of Smad3 through DUB activity.

The turnover rate of Smad3 was determined in the presence or absence of POH1 using a cycloheximide (CHX) chase assay to ascertain whether POH1 affects the stability of the Smad3 protein. Overexpression of POH1 in H1299 cells significantly prolonged the half-life of the endogenous Smad3 protein (Fig. 2A). Similarly, transfection with POH1 markedly increased the stability of the exogenously expressed Smad3 (Fig. 2B). In contrast, Smad3 was degraded more rapidly in POH1-knockdown cells than in control cells (Fig. 2C). Collectively, this demonstrated that POH1 stabilizes Smad3.

3.2. POH1 directly interacts with and is co-localized with Smad3

Next, we determined whether POH1 physically interacts with Smad3. To this end, Flag-Smad3 was transfected into H1299 cells,

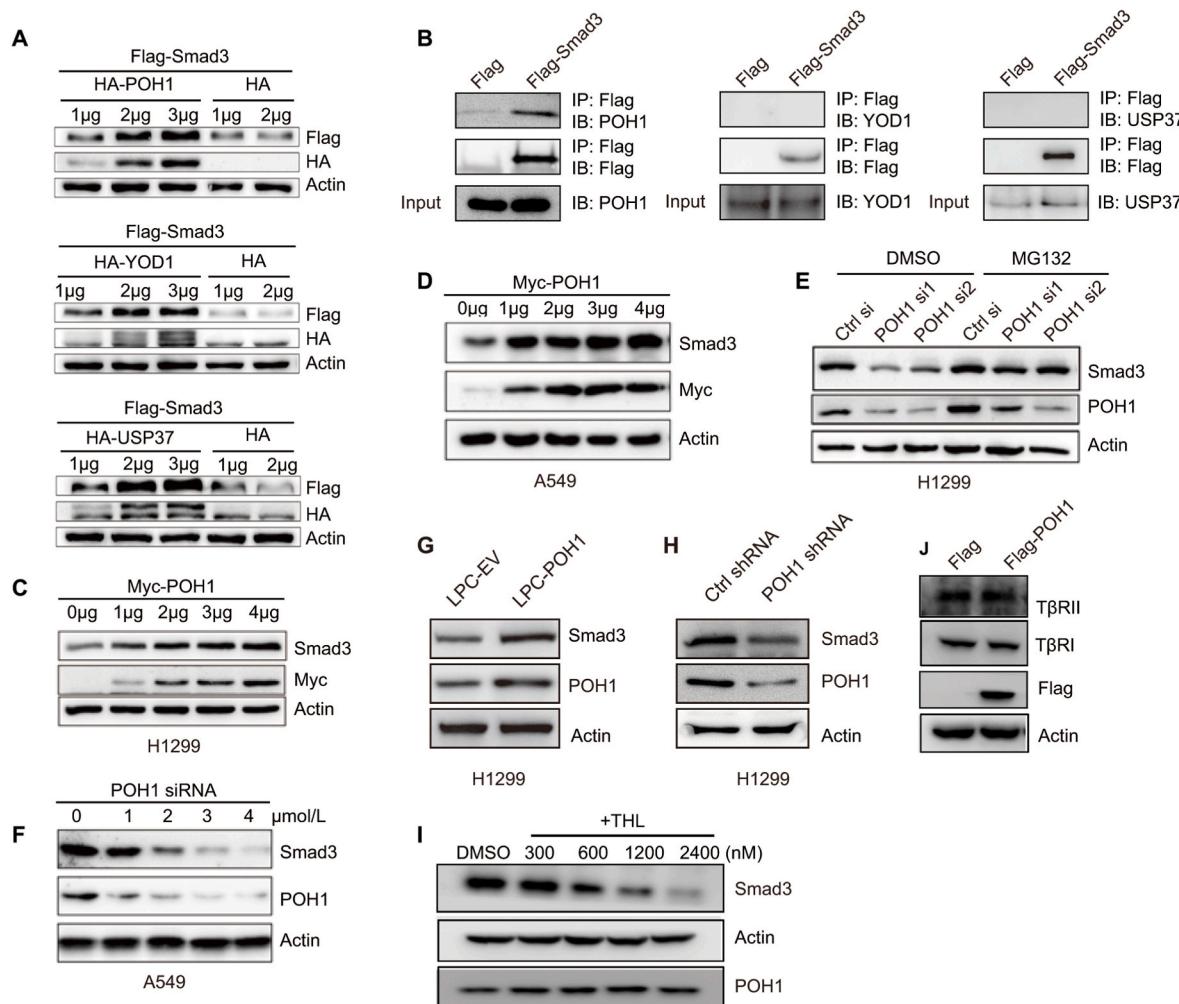


Fig. 1. POH1 increases the protein level of Smad3. (A) Flag-Smad3 plasmid (2 μg) was transfected into HeLa cells with increasing amounts of indicated plasmids. Smad3 expression was determined by immunoblotting with an anti-Flag antibody 48 h after transfection. (B) HeLa cells were transfected with Flag-Smad3. Cell lysates were immunoprecipitated with an anti-Flag antibody or normal IgG, followed by immunoblotting using POH1, YOD1, or USP37 antibody 48 h after transfection. (C&D) Increasing amounts of POH1 plasmid were transfected into (C) H1299 cells or (D) A549 cells, and Smad3 expression was examined using Western blot analysis. (E&F) Increasing amounts of POH1 siRNA were transfected into (E) H1299 or (F) A549 cells, and Smad3 expression was examined using Western blot analysis. (G) H1299 cells were stably transfected with either the expression vector of LPC-POH1 or an empty vector. The expression of Smad3 was determined using Western blot analysis. (H) Smad3 was detected in the H1299 cells stably knockdown of POH1 using Western blot analysis. (I) H1299 cells were treated with indicated concentrations of thiolutin for 24 h, and Smad3 was determined using Western blot analysis. (J) H1299 cells were transfected with Flag-POH1. TβRI and TβRII were examined 48 h after transfection using Western blot analysis.

followed by Co-IP assay with a Flag antibody. As shown in Fig. 3A, ectopically expressed Flag-Smad3 interacts with endogenous POH1. Furthermore, endogenous POH1 and Smad3 were strongly associated in the Co-IP assay (Fig. 3B). To further characterize the region of Smad3 responsible for the interaction with POH1, three truncated constructs of Smad3 (MH1, MH2, and the linker region) were constructed according to the functional domain and transfected into H1299 cells. Co-IP was performed, and the results showed that the MH2 domain strongly interacted with POH1 (Fig. 3C). To further prove the direct interaction between POH1 and Smad3, a proximity ligation assay (PLA), which is a newly developed technique for visualizing endogenous protein-protein interactions *in situ* (at distances <40 nm) [44], was used. As shown in Fig. 3D, PLA signals were remarkably intensified in H1299 cells stained with POH1 and Smad3 antibodies, whereas PLA signals were marginally visible in control cells, strongly suggesting that POH1 directly interacts with Smad3. To clearly define the specific subcellular sites of POH1 and Smad3 colocalization in cells, traditional immunofluorescence (IF) and PLA were utilized. Endogenous POH1 was found to co-localize with endogenous Smad3, mainly in the cytoplasm of H1299 cells, by IF

staining (Fig. 3E). Consistent with the IF result, the PLA signals were manifest in the cytoplasm. In contrast, the PLA signals were also observed in the nucleus (Fig. 3F), revealing the co-localization of POH1 with Smad3 both in the cytoplasm and nucleus. Altogether, this demonstrated a direct interaction between POH1 and Smad3, a previously unknown molecular association.

3.3. POH1 regulates polyubiquitination of Smad3

Given that POH1 interacts with Smad3 and promotes its stability in the aforementioned data, we examined whether POH1 serves as a DUB of Smad3. H1299 cells were transfected with Flag-Smad3 and HA-Ub expression vectors with or without wild-type POH1, followed by Co-IP assays. The cells were then treated with MG132 to prevent Smad3 degradation. As expected, the co-expression of Smad3 with POH1 notably reduced the ubiquitination of Smad3 (Fig. 4A). Furthermore, an obvious reduction in Smad3 ubiquitination was observed in H1299 cells that stably expressed POH1 (Fig. 4B). Moreover, stable knockdown of POH1 in H1299 cells enhanced endogenous Smad3 ubiquitination

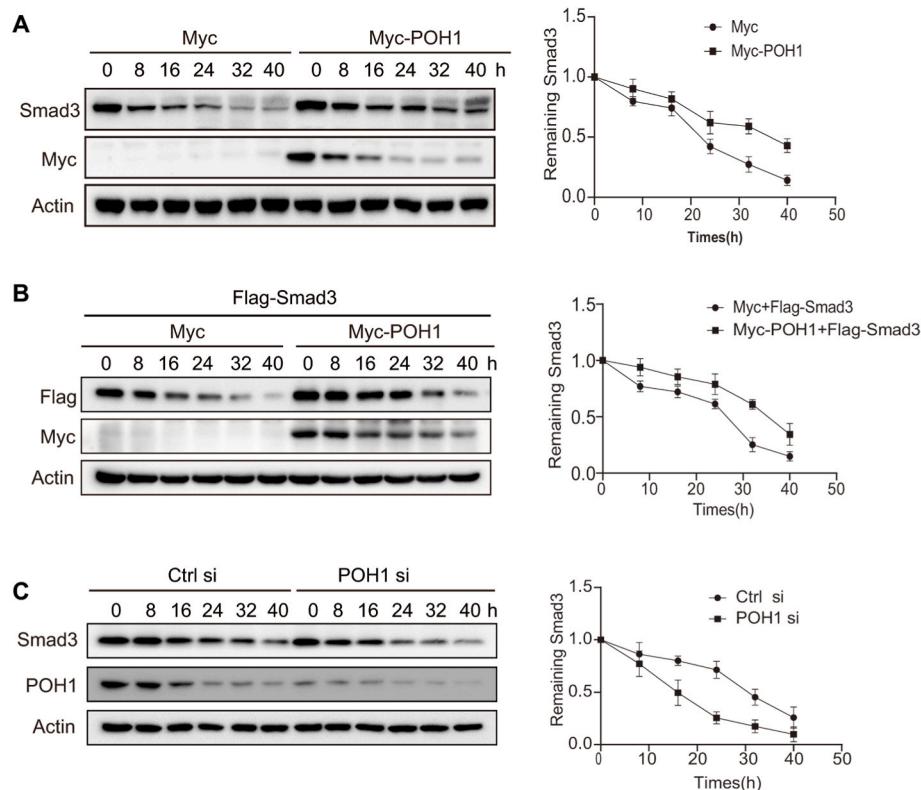


Fig. 2. POH1 prolongs the half-life of Smad3. (A) Myc-POH1 was transfected into H1299 cells together with Flag-Smad3, and cells were treated with CHX at 100 µg/mL for the indicated times. The half-life of Flag-Smad3 was measured using Western blot analysis. Quantification of the Smad3 half-life was performed, and each point is represented as the mean ± SD of triplicate experiments. (B) H1299 cells were transfected with Myc-POH1, and cells were treated with CHX at 100 µg/mL for the indicated times. The half-life of endogenous Smad3 protein was analyzed using Western blot analysis. Each point represents the mean ± SD of triplicate experiments. (C) CHX-chase experiments of Smad3 in A549 cells transfected with control siRNA or POH1 siRNA are shown. Quantification of the Smad3 half-life was performed, and each point is represented as the mean ± SD (n = 3).

(Fig. 4C). This suggested that POH1 protects Smad3 from polyubiquitination-mediated degradation. Next, we aimed to identify the possible linkage types of polyubiquitin modifications on the Smad3 protein affected by POH1-mediated deubiquitination. Smad3 proteins in the lysates of cells transfected with Flag-Smad3, Myc-POH1, or the indicated ubiquitins (WT, K48-, or K63-only ubiquitin-HA) were subjected to immunoblotting using an anti-HA antibody. The results showed that POH1 overexpression substantially eliminated both K48- and K63-linked ubiquitination of Smad3 (Fig. 4D and E). Previous studies have demonstrated that POH1 is a K63-specific deubiquitinase [45]. We investigated whether the cleavage of K48-linked polyubiquitin chains on Smad3 by POH1 depended on the K63-linked chains. H1299 cells were transfected with Flag-Smad3 and Myc-POH1 along with wild-type Ub or the K63-resistant form of ubiquitin (K63R), and Co-IP assays were performed using K63- or K48 ubiquitin chain-specific antibodies. Interestingly, POH1 failed to remove K48-linked polyubiquitin chains when K63 Ub was mutated (Fig. 4F), indicating that K63-linked polyubiquitination is essential for POH1-regulated Smad3 ubiquitination. Next, we verified the effect of POH1 on phosphorylated Smad3 ubiquitination. As shown in Fig. 4G, the stable overexpression of POH1 did not influence p-Smad3 ubiquitination. Collectively, this demonstrated that POH1 deubiquitinates Smad3 by cleaving K63-linked polyubiquitin chains.

3.4. POH1 promotes cancer cell proliferation, migration, and invasion by stabilizing Smad3

Smad3 plays an important role in the EMT, migration, and invasion of lung cancer cells [20]. Given that POH1 regulates the stability of Smad3, we propose that POH1 affects Smad3-mediated cellular function and promotes cell invasion and tumor metastasis in NCSLC. First, we

investigated the effects of POH1 on the expression of Smad3 target genes. We found that the knockdown of POH1 significantly inhibited the expression of N-cadherin, Vimentin, and PAI-1 and increased the expression of E-cadherin (Fig. 5A). Consistently, the protein levels of Vimentin and PAI-1 were markedly reduced in cells with a stable POH1 knockdown (Fig. 5B). N-cadherin, E-cadherin, and vimentin are markers of EMT [46], indicating that POH1 promotes EMT in lung cancer cells by mediating the stability of Smad3. Importantly, we found that the POH1 inhibitor THL reduced the protein levels of Snail and Slug, the key transcription factors of EMT. As expected, THL inhibited N-cadherin levels and upregulated E-cadherin levels, accompanied by a prominent reduction in Smad3 protein levels (Fig. 5C). To further verify that the regulatory effect of POH1 on Smad3 downstream targets was dependent on the deubiquitinating activity of POH1, deubiquitinase-inactive POH1 mutants (C120S or H113Q) were used. The C120 and H113 sites in POH1 belong to the JAMM motif located in the amino terminus of POH1 between amino acid residues 107–130. This is one of the most important functional motifs in POH1 and is critical for its DUB activity of POH1 [35,36]. Many previous studies have shown that mutations at C120 and H113 inhibit the deubiquitinating enzymatic activity of POH1 [23,37,38]. Accordingly, we found that overexpression of POH1 upregulated the protein levels of N-cadherin, Vimentin, and PAI-1 and decreased the expression of E-cadherin. However, ectopic expression of the POH1 mutants, C120S or H113Q, failed to regulate these target genes, similar to wild-type POH1 (Fig. 5D).

Next, a Transwell assay was performed to test the migratory and invasive abilities of the tumor cells. The results showed that the number of migrated cells was strikingly increased in POH1 stably overexpressed H1299 cells; however, knockdown of Smad3 significantly inhibited POH1-mediated cell migration (Fig. 6A and B). In contrast, knockdown

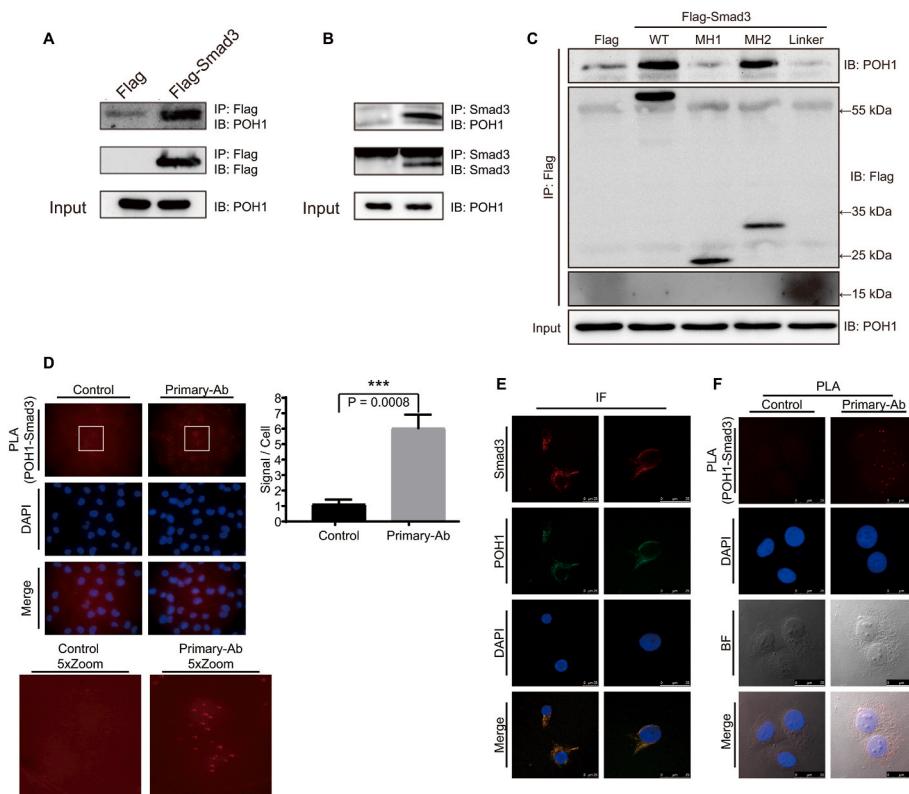


Fig. 3. POH1 interacts with Smad3. (A) H1299 cells were transfected with Flag-Smad3. Cell lysates were immunoprecipitated with an anti-Flag antibody or normal IgG followed by immunoblotting using POH1 antibody 48 h after transfection. (B) The endogenous interaction between Smad3 and POH1 was analyzed using Co-IP. (C) H1299 cells were transfected with the indicated truncates of Smad3 (MH1, MH2, or linker). Cell lysates were immunoprecipitated with anti-Flag antibody followed by immunoblotting using anti-POH1. (D) The PLA signal from the interaction of POH1 and Smad3 was visualized as defined red spots by fluorescence microscopy. Cell nuclei are blue by DAPI staining. The amplified images of the ROI are shown beneath. The right figure shows the quantification analysis of the PLA signal between the control and test groups from three independent experiments. ***P < 0.0001. (E) Subcellular localization of POH1 and Smad3 were detected using fluorescence microscopy. POH1 (green) was co-localized with Smad3 (red) in the cytoplasm. Bars, 25 μ m. (F) Subcellular localization and the interaction of POH1 and Smad3 were detected using PLA. Bars, 25 μ m.

of POH1 in H1299 cells greatly inhibited the migratory ability. Nevertheless, the overexpression of Smad3 can partially rescue the blocked cell migration induced by POH1 depletion (Fig. 6C and D). Similarly, POH1 dramatically promoted the invasive capability of lung cancer cells, which was dependent on Smad3 (Fig. 6E and F). These findings suggested that POH1 promotes cell migration and invasion by stabilizing Smad3.

POH1 promotes cell proliferation and tumor growth in breast cancer [26], hepatocellular carcinoma [26,47], and other cancers [35,48]. Smad3 is also involved in cancer cell proliferation [49,50]. We wanted to explore whether POH1 also facilitates tumor cell growth in NSCLC and whether this mechanism is related to Smad3. The colony formation and WST-1 assays were performed. The results showed that the ectopic expression or knockdown of POH1 facilitated or inhibited the growth of H1299 cells, respectively (Fig. 6G and H, Figs. S1A and B). Importantly, the loss of Smad3 abolished POH1-induced cell growth sharply (Fig. 6I, Fig. S1C). Similarly, the results of the WST-1 assay showed that POH1-mediated tumor cell growth was dependent on Smad3 (Fig. 6J), suggesting that Smad3 is likely a major target of POH1 in the regulation of lung cancer cell growth.

To verify the effects of POH1 on the growth and metastatic capabilities of lung tumor cells *in vivo*, H1299 cells with or without POH1 overexpression were injected subcutaneously into nude mice. As shown in Fig. 6K, cells stably expressing POH1 displayed larger tumor volumes than control cells. Then we investigated the effects of POH1 on lung cancer cell metastasis. Importantly, a drastic increase in metastatic nodules was found in the livers of the POH1 overexpression group, as compared to that in the control group (Fig. 6L). Collectively, these

results indicated that POH1 plays an important role in promoting tumor progression, including the proliferation, migration, invasion, and metastasis of lung cancer cells by stabilizing Smad3.

3.5. A high level of POH1 expression predicts poor prognosis in lung cancer patients

We measured POH1 and Smad3 expression and assessed their association in human NSCLC samples using tissue microarrays containing 94 matched NSCLC and adjacent non-tumoral tissues for immunohistochemical (IHC) analyses to determine whether POH1-mediated regulation of Smad3 expression is clinically relevant. The overall score was determined by multiplying the intensity score by the percentage score. The results showed that the expression of POH1 in 69 patients (73 %) was significantly higher than that in adjacent tissues. Representative images are shown in Fig. 7A. Moreover, Smad3 levels were significantly increased in approximately 84 % of human lung cancer tissues examined, and representative data are shown in Fig. 7B. The correlation between POH1 and Smad3 was evaluated using the IHC staining score based on the intensity (ranging from 1 to 4) and percentage of positive cells. The data showed that POH1 levels were significantly positively correlated with Smad3 levels ($P < 0.001$, Spearman's correlation test) (Fig. 7C and D). Furthermore, we analyzed the correlation between the mRNA expression levels of POH1 and Smad3 using a dataset of lung adenocarcinoma patients in the TCGA database (Fig. 7E). The results showed that the correlation coefficient was negative, which can be determined as no correlation, further indicating that the regulation of POH1 on Smad3 is at a post-translational level. The results of the animal

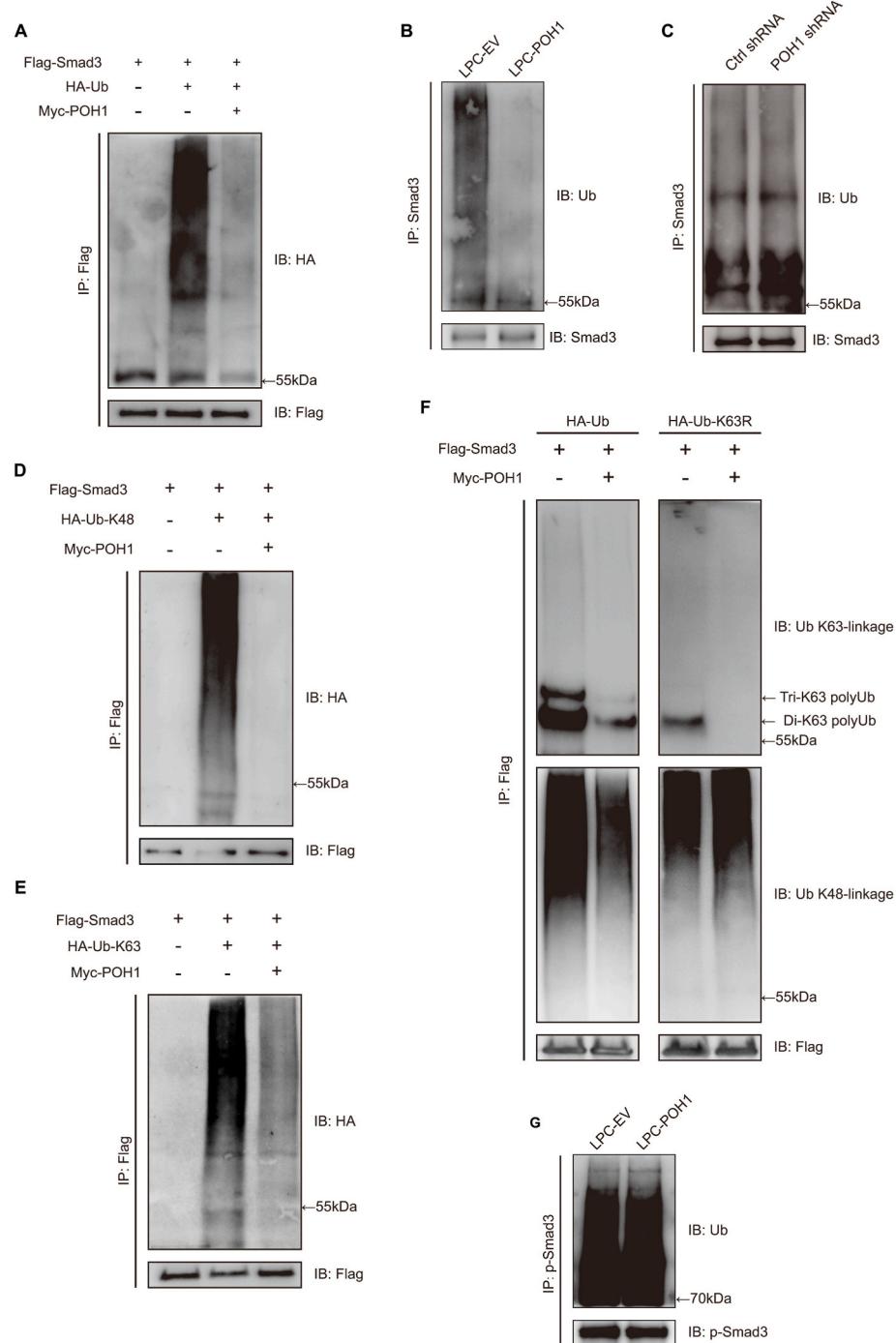


Fig. 4. POH1 abolished the ubiquitination of Smad3. (A) Flag-Smad3, HA-Ub, and Myc-POH1 were cotransfected into HEK293T cells, Smad3 ubiquitination was detected using immunoprecipitation with anti-Flag M2 beads and immunoblotting with an anti-HA antibody. (B&C) Smad3 ubiquitination was detected using immunoprecipitation with anti-Smad3, and immunoblotting with an anti-Ub antibody in H1299 cells stably overexpression of POH1 (B) or knockdown of POH1(C). (D&E) Flag-Smad3, Myc-POH1 were cotransfected into HEK293T cells together with (D) HA-Ub-k48 or (E) HA-Ub-k63, and Smad3 ubiquitination was detected using immunoprecipitation with anti-Flag M2 beads and immunoblotting with an anti-HA antibody. (F) Flag-Smad3 and Myc-POH1 were cotransfected into HEK293T cells together with HA-Ub or HA-Ub-K63R. Smad3 ubiquitination was detected using immunoprecipitation with anti-Flag M2 beads and immunoblotting with an anti-Ub-K63 antibody or Ub-K48 antibody. (G) P-Smad3 ubiquitination was detected using immunoprecipitation with anti-p-Smad3 and immunoblotting with an anti-Ub antibody in H1299 cells that overexpressed POH1 stably.

experiments (Fig. 6L) revealed that POH1 promotes liver metastasis; therefore, we examined the protein levels of POH1 and Smad3 in metastatic liver tissue originating from lung cancer in patients with LUAD. The IHC results showed that both POH1 and Smad3 were upregulated in metastatic liver tissue compared to normal liver tissue (Figs. S2A–C). Furthermore, POH1 expression was detected in six patients with lymph

node metastasis originating from LUAD. POH1 expression was higher in the metastatic lymph node tissue than in the adjacent tissue; the representative images were shown in Fig. S2D. POH1 was also highly expressed in other tissues from four LUAD patients with brain or chest wall metastases (Fig. S2E). We then analyzed the correlation between POH1 and Smad3 protein levels and prognosis using tissue microarrays

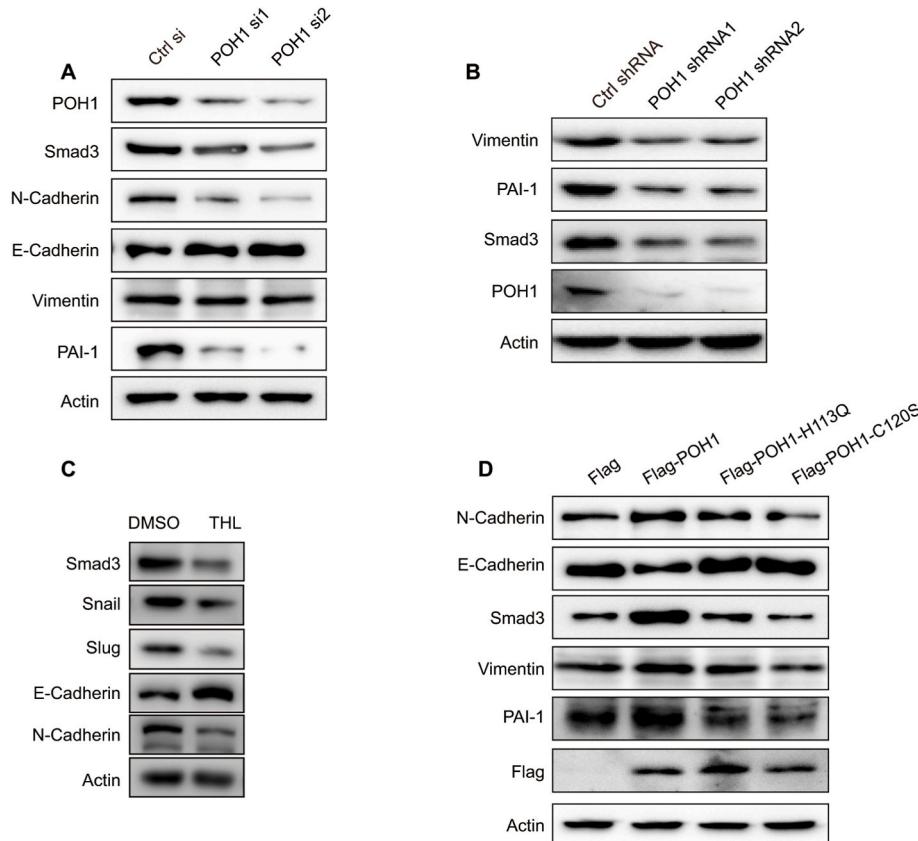


Fig. 5. POH1 regulates the expression of Smad3 downstream target genes. (A) H1299 cells were transfected with POH1 siRNA for 48 h, and the expressions of related proteins were determined using Western blot analysis. (B) The expression of E-cadherin, N-cadherin, Snail, and Slug was detected in the H1299 cells with stable knockdown of POH1 using Western blot analysis. (C) H1299 cells were treated with thiolutin (1200 nM) for 24 h and the expression of E-cadherin, N-cadherin, Snail, and Slug was determined using Western blot analysis. (D) H1299 cells were transfected with wild-type POH1 or POH1 mutants (C120S or H113Q), and the expressions of related proteins were determined using Western blot analysis 48 h after transfection.

from 94 patients with LUAD. Importantly, the results showed that high levels of POH1 and Smad3 predicted poor overall survival in patients with LUAD (Fig. 8A and B).

Next, we examined the gene expression data from the Oncomine database. Consistent with the protein expression, the mRNA levels of POH1 and Smad3 were elevated in lung adenocarcinoma (LUAD) (Fig. 8C and D). We subsequently investigated the survival rates of patients with different POH1 and Smad3 expression levels using information from lung cancer databases, including the TCGA and GEO databases. Consistent with our findings, high RNA levels of both POH1 and Smad3 were significantly correlated with poor overall survival with LUAD (Fig. 8E and F).

3.6. High expression POH1 is positively correlated with the TGFB signaling pathway

To further explore the differences in the transcriptional characteristics of patients with high and low POH1 expression, we divided the patients from the TCGA LUAD cohort into high and low expression groups based on the highest 20 % and lowest 20 % of POH1 mRNA expression. We compared the overall survival and progression-free survival. The results showed a significant correlation between POH1 expression and overall survival and progression-free survival in patients with lung adenocarcinoma, and patients with high expression generally had a worse clinical prognosis (Fig. S3A and B). We then conducted Gene Set Enrichment Analysis (GSEA) on these two groups and found that patients with high POH1 expression had activated gene signatures related to the TGFB and EMT signaling pathway (Fig. S3C and D). Genes in these sets also tended to be highly expressed in patients with high

level of POH1 (Fig. S3E). To detect the relationship between POH1 and malignant metastasis of lung adenocarcinoma, we analyzed published single-cell transcriptomic data of patients with lung adenocarcinoma at different stages of metastasis. Epithelial cells in the dataset were divided into club cells, type I pneumocytes, type II pneumocytes, and tumor cells (Fig. S3F). Subsequently, we observed that POH1 was highly expressed in tumor cells. The expression of POH1 and genes related to the TGFB signaling pathway showed an upward trend with the progression (Figs. S3G–J).

4. Discussion

Smad3 is a crucial player in the intracellular transduction of TGF- β signaling and TGF- β -mediated EMT and tumor metastasis [6]. Smad3 is upregulated and promotes tumor progression in various cancers, including breast cancer [51], liver cancer [52], pancreatic adenocarcinoma [53], and NSCLC [54]. Our findings uncover a novel regulatory role for POH1 in interacting with Smad3, controlling its stability through deubiquitination and consequently enhancing TGF- β 1/Smad3 signaling, promoting cell invasion and metastasis in NSCLC (Fig. 8G). We also established the clinical relevance of POH1-mediated stabilization of Smad3 in NSCLC cells.

One highlight of this study is that we identified the DUB of total Smad3 protein, as well as its important roles in the regulation of invasion and metastasis of NSCLC. TGF- β /Smads pathway modulated by ubiquitination has been intensely studied for decades [55–57]. The E3 ubiquitin ligases of important components in TGF- β /Smads signaling have been widely identified. DEAR1 [58], STUB1 [59], pVHL [60], and ROC1 [61] have been reported as E3 ligases of Smad3 in different cell

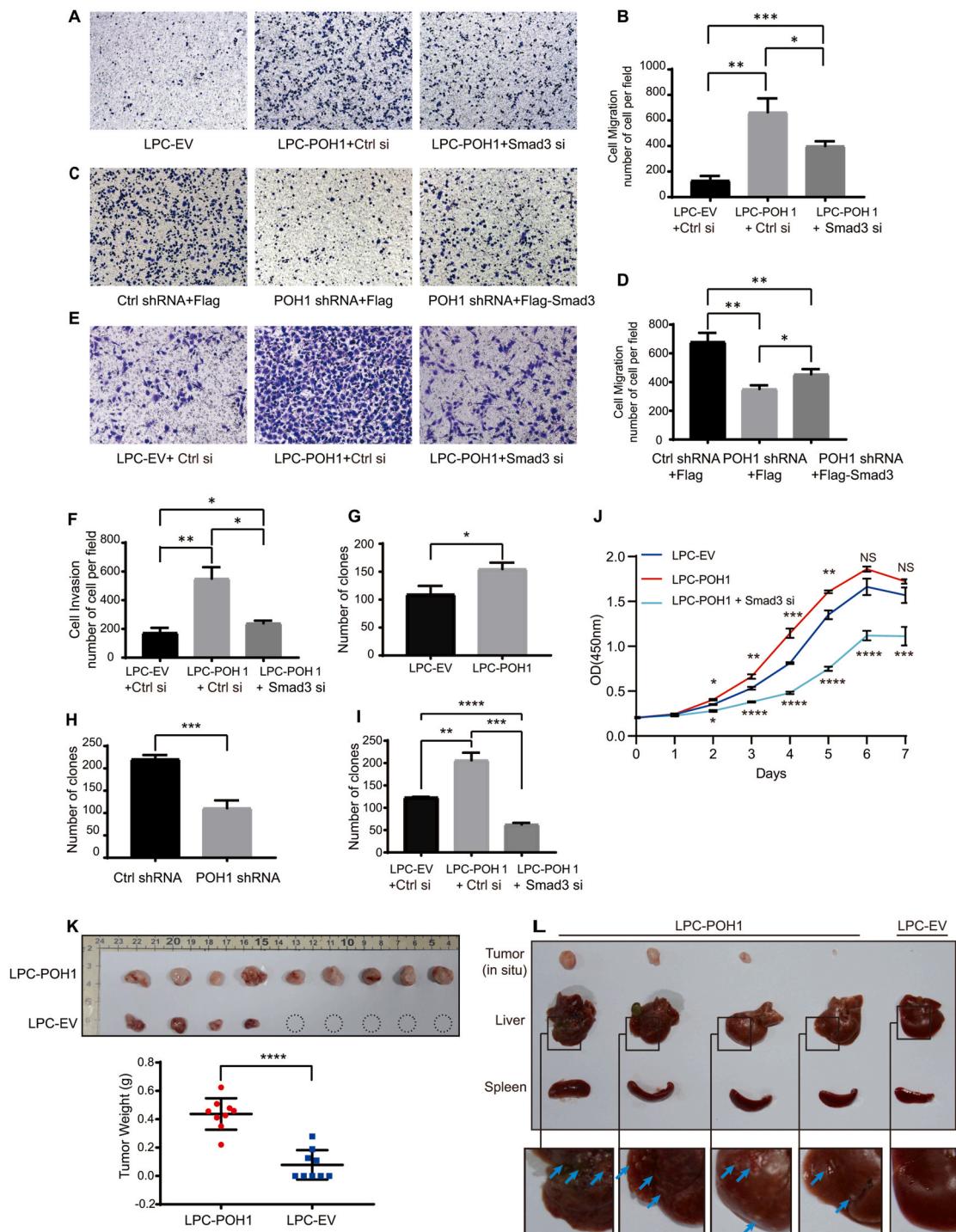


Fig. 6. POH1 promotes the growth, migration, and invasion of lung cancer cells by Smad3. (A&B) Smad3 siRNA or Control siRNA were transfected into H1299 cells stably overexpression of POH1, 48 h after transfection, cell migration was determined by a Transwell migration assay (A). The results of statistical analyses were shown in figure B. Data are expressed as mean \pm SD, n = 3 independent experiments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (C&D) The flag-Smad3 plasmid or empty factor was transfected into H1299 cells with stable knockdown of POH1, and cell migration was determined using the transwell migration assay (C). The results of statistical analyses are shown in D. Data are expressed as mean \pm SD, n = 3 independent experiments (* $P < 0.05$, ** $P < 0.01$). (E&F) Smad3 siRNA or Control siRNA were transfected into H1299 cells stably overexpression of POH1, 48 h after transfection, cell invasion was determined by the transwell migration assay (E). The results of statistical analyses are shown in F. Data are expressed as mean \pm SD, n = 3 independent experiments (* $P < 0.05$, ** $P < 0.01$). (G-I) H1299 cells were transfected with indicated plasmids or siRNA. The effect of POH1 on the anchorage-dependent growth of H1299 cells was analyzed. Values shown are mean \pm SD of triplicate measurements (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (J) WST-1 assay was performed to examine the effect of POH1 on H1299 cell growth. The cells were seeded into 96-well plates, and the absorbance at 450 nm was measured at the indicated time points. (K&L) Mice were subcutaneously injected with stable H1299 cells and the control cells. Tumor growth in xenografted nude mice was measured. The xenograft tumors were dissected and photographed on day 25. Average tumor weights were measured on day 25. The statistical analyses were performed by Student's t-test (K) *, P < 0.05. The normal and metastatic livers from the two groups are shown in L.

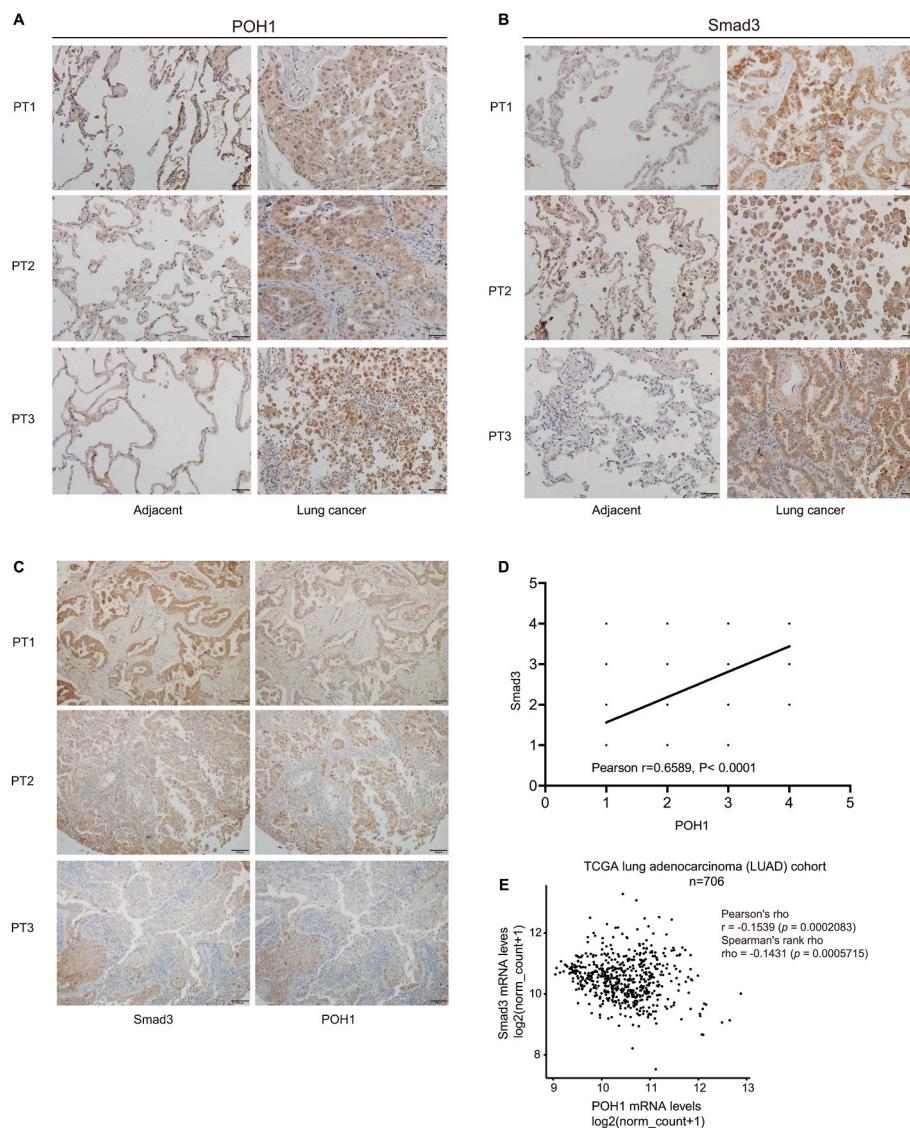


Fig. 7. POH1 is positively correlated with Smad3 in the tissues of lung cancer patients. (A) Representative images of POH1 expression both in normal and lung adenocarcinoma tissues from three patients determined by immunohistochemistry. Scale bar, 100 μ m. (B) Representative images of Smad3 expression both in normal and lung adenocarcinoma tissues from three patients determined by immunohistochemistry. Scale bar, 100 μ m. (C&D) Representative images of POH1 expression and Smad3 expression in lung adenocarcinoma tissues from three patients determined using immunohistochemistry and consecutive sections from the same tissue. Scale bar, 100 μ m (C). The expression of POH1 and Smad3 was evaluated and scored by the intensity and area of staining. The relationship between POH1 and Smad3 protein expression was then analyzed using the Pearson correlation test (D). (E) Scatterplot showing the correlation of POH1 and Smad3 at mRNA levels from TCGA LUAD cohort ($n = 706$).

types, inducing ubiquitination and degradation of Smad3. Furthermore, PJA1 and Smurf2 function as E3 ligases for phosphorylated Smad3 [62, 63]. Unlike ubiquitination, DUB-modulated deubiquitination of Smad3 has only recently emerged and is largely unknown. OTUB1 enhanced TGF- β signaling as a DUB of R-Smads [64]. However, OTUB1 only inhibited the degradation of active Smad2/3 and did not affect the inactive non-phosphorylated form or total Smad2/3 protein. USP15 and USP7 were found to resist the mono-ubiquitination of Smad3, enhancing the TGF- β /Smads signal but not regulating the protein level of Smad3 [65]. In this study, we demonstrated that POH1 abolished the poly-ubiquitination of Smad3 but not phosphorylated Smad3. However, the level of phosphorylated Smad3 was also indirectly increased due to the enhanced stability of total Smad3. Our study revealed that the phosphorylated Smad3 and unphosphorylated Smad3 protein have distinct degradation mechanisms, which are both important for the activation of TGF- β /Smads signaling and cellular functions.

It is worth mentioning that one study reported the opposite effect of

POH1 on Smad3 stability, showing that knockdown of POH1 delayed Smad3 degradation in melanoma cells [66]. However, the underlying mechanism and relationship between POH1 and Smad3 were not elucidated in this study. Another interesting report demonstrated that POH1 could deubiquitinate TGF- β receptors and repress the degradation of TGF- β receptors in HCC cells, whereas Smad3 protein was unaffected [43]. Nevertheless, our study found that POH1 cannot modulate the stability of the TGF- β receptor in NSLCS cells. The specificity of DUB action is controlled by complex mechanisms, such as the activity of DUB in distinct cell types or tissues, chain linkage types of Ub, associations of DUB with scaffolds or adapters, and proper regulation time [67]. Therefore, it is reasonable to assume that a single protein has diverse DUB in different tissues. For instance, studies have uncovered several DUBs of T β RI, including USP4 in breast cancer, USP11 in normal epithelial cells, and USP15 in glioblastoma [9]. Thus, it is comprehensible that POH1 exerts discrepant functions in NSLCS and hepatocarcinoma, which have different pathogenesis, pathological

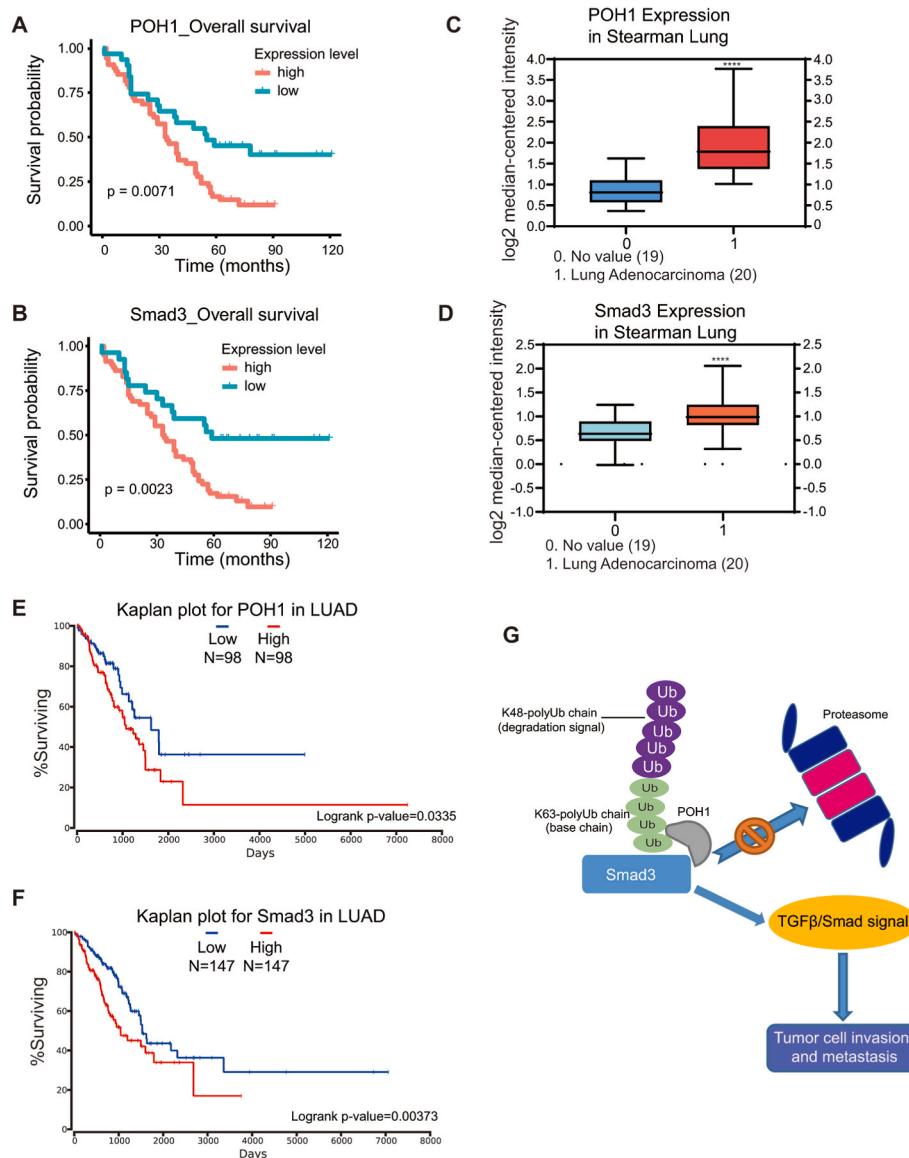


Fig. 8. High expression of POH1 and Smad3 correlates with poor survival of lung cancer patients. (A&B) Kaplan-Meier survival analysis of the overall survival in the human LUAD patients based on (A) POH1 or (B) Smad3 protein expression. The expression of POH1 and Smad3 was evaluated and scored by the intensity and area of staining. The score ranging from 1 to 2 was considered a low-expression group ($n = 33$ for POH1; $n = 28$ for Smad3), whereas the score ranging from 3 to 4 was considered a high-expression group ($n = 52$ for POH1; $n = 57$ for Smad3). (C) Statistics of POH1 mRNA expression in LUAD were obtained from the Oncomine database. (D) The correlation of POH1 mRNA expression with overall survival rates of human LUAD patients was analyzed using a KM plot analysis in the TCGA dataset. $P = 0.0335$ (Log-rank test). (E) Statistics of Smad3 mRNA expression in LUAD were obtained from the Oncomine database. (F) The correlation of Smad3 mRNA expression with overall survival rates of human LUAD patients was analyzed using a KM plot analysis in the TCGA dataset. $P = 0.00373$ (Log-rank test). (G) A working model shows that POH1 stabilizes Smad3 by reducing its ubiquitination to enhance TGF β /Smads signaling, promoting the growth, invasion, and metastasis of lung cancer cells.

processes, and tumor microenvironments.

Ubiquitination/deubiquitination is a precise, dynamic, and reversible post-translational modification process. Ubiquitin molecules can be linked through or removed from one of the seven ubiquitin Lys residues: Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63. Among these modification types, the roles of Lys48-and Lys63-linked polyubiquitins have been well characterized in proteasome degradation and signal transduction, respectively [68]. POH1, a DUB of the JAMM/MPN+ family, is specific for K63-linked polyubiquitin chains. However, several studies have found that POH1 can also remove other types of chains, such as K48 and K11 chains [69]. In this study, we confirmed that POH1 could reduce both k48 and k63 polyubiquitin chains linked to Smad3. Intriguingly, POH1 lost its ability to remove the K48 polyubiquitin chain on Smad3 when K63 was mutated, suggesting that the polyubiquitin

chain on Smad3 may not be a single linear chain, but a K48-branched chain that is based on the K63-chain. Thus, POH1 reduced the overall ubiquitination of Smad3 by cutting off the basal part of the k63/k48 branching chain, as shown in Fig. 4F. This finding suggests a new mechanism for non-degradation-related polyubiquitin chains involved in proteasome degradation.

Accumulating evidence has revealed a tumor-promoting role for POH1 in diverse cancers. Consistently, our findings demonstrate that POH1 promotes cell proliferation, invasion, and metastasis in NSCLC by mediating Smad3 stability. Smad3 is a critical mediator of TGF- β signaling and has been proven to promote NSCLC progression by diverse mechanisms such as stimulating EMT of tumor cells [20], promoting macrophage-myofibroblast transition (MMT) [70], and controlling polarization of tumor-associated neutrophils [71]. In this study,

POH1 was identified as a DUB of Smad3, increasing its stability and activating Smad3 downstream targets. Furthermore, our study and a previous report [38] found that POH1 expression is upregulated in the tissues of patients with NSCLC. Bioinformatics analysis results in this study revealed a correlation between high levels of POH1 and TGFB and EMT signaling pathways (Fig. S3). Therefore, it is highly reasonable to presume that the activation of POH1-Smad3 signaling enhances NSCLC growth and metastasis by stabilizing Smad3 and strengthening Smad3-related cellular functions. Interestingly, Snail has been identified as a substrate of POH1 and is responsible for POH1-facilitated migration and invasion of esophageal carcinoma cells [28]. Snail is a Smad3 downstream effector gene [72]. Whether POH1 promotes the progression of NSCLS through the dual regulation of Smad3 and Snail requires further investigation. Importantly, our study uncovered a highly coordinated increase in the expression of POH1 and Smad3 proteins in clinical NSCLS samples, which correlated with shorter survival, suggesting the pathological significance of POH1 regulation of Smad3 in NSCLS progression. DUB are emerging as novel cancer biomarkers and therapeutic targets. Specific inhibitors of POH1, including 8-thioquino-line (8TQ), THL, and capzimin, have been shown to suppress cancer progression [73,74]. In this study, we showed that THL inhibited the protein levels of Smad3 and its downstream targets, thereby preventing cell migration and invasion.

In conclusion, our results demonstrate that POH1 is a key regulator of TGF- β signaling by inhibiting the ubiquitination and proteasomal degradation of Smad3. Furthermore, we revealed the mechanism by which increased POH1 expression contributes to the development of NSCLC by stabilizing Smad3. Selective targeting of the POH1/Smad3 axis may benefit NSCLC therapy and prevention. Moreover, considering the multiple important functions of POH1 in tumor development, POH1 represents a potentially valuable target for cancer therapy.

CRediT authorship contribution statement

Yang Yuan: Methodology, Formal analysis, Data curation. **Yixiao Li:** Investigation. **Xiao Wu:** Validation, Investigation. **Jinsuo Bo:** Investigation. **Lei Zhang:** Validation. **Jing Zhang:** Investigation. **Ye Hu:** Investigation. **Yining Chen:** Investigation. **Yiyan Zeng:** Validation. **Xiaofan Wei:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Conceptualization. **Hongquan Zhang:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.canlet.2023.216526>.

References

- [1] T.W. Chen, W.Z. Hung, S.F. Chiang, W.T. Chen, T.W. Ke, J.A. Liang, et al., Dual inhibition of TGFbeta signaling and CSF1/CSF1R reprograms tumor-infiltrating macrophages and improves response to chemotherapy via suppressing PD-L1, *Cancer Lett.* 543 (2022), 215795.
- [2] L. Caja, F. Dituri, S. Mancarella, D. Caballero-Diaz, A. Moustakas, G. Giannelli, et al., TGF-Beta and the tissue microenvironment: relevance in fibrosis and cancer, *Int. J. Mol. Sci.* 19 (2018).
- [3] M. Morikawa, R. Deryck, K. Miyazono, TGF-Beta and the TGF-beta family: context-dependent roles in cell and tissue physiology, *Csh Perspect Biol* 8 (2016).
- [4] D. Peng, M. Fu, M. Wang, Y. Wei, X. Wei, Targeting TGF-beta signal transduction for fibrosis and cancer therapy, *Mol. Cancer* 21 (2022) 104.
- [5] M.K. Chan, J.Y. Chung, P.C. Tang, A.S. Chan, J.Y. Ho, T.P. Lin, et al., TGF-beta signaling networks in the tumor microenvironment, *Cancer Lett.* 550 (2022), 215925.
- [6] A.B. Roberts, F. Tian, S.D. Byfield, C. Stuelten, A. Ooshima, S. Saika, et al., Smad3 is key to TGF-beta-mediated epithelial-to-mesenchymal transition, fibrosis, tumor suppression and metastasis, *Cytokine Growth Factor Rev.* 17 (2006) 19–27.
- [7] A.B. Roberts, A. Russo, A. Felici, K.C. Flanders, Smad3: a key player in pathogenetic mechanisms dependent on TGF-beta, *Ann Ny Acad Sci* 995 (2003) 1–10.
- [8] C. Millet, Y.E. Zhang, Roles of Smad3 in TGF-beta signaling during carcinogenesis, *Crit Rev Eukar Gene* 17 (2007) 281–293.
- [9] J. Zhang, X. Zhang, F. Xie, Z. Zhang, H. van Dam, L. Zhang, et al., The regulation of TGF-beta/SMAD signaling by protein deubiquitination, *Protein Cell* 5 (2014) 503–517.
- [10] Y. Liao, M. Yang, K. Wang, Y. Wang, B. Zhong, N. Jiang, Deubiquitinating enzyme OTUB1 in immunity and cancer: good player or bad actor? *Cancer Lett.* 526 (2022) 248–258.
- [11] P. D'Arcy, X. Wang, S. Linder, Deubiquitinase inhibition as a cancer therapeutic strategy, *Pharmacol. Therapeut.* 147 (2015) 32–54.
- [12] N. Poondla, A.P. Chandrasekaran, K.S. Kim, S. Ramakrishna, Deubiquitinating enzymes as cancer biomarkers: new therapeutic opportunities? *Bmb Rep* 52 (2019) 181–189.
- [13] S.J. Wicks, K. Haros, M. Maillard, L. Song, R.E. Cohen, P.T. Dijke, et al., The deubiquitinating enzyme UCH37 interacts with Smads and regulates TGF-beta signalling, *Oncogene* 24 (2005) 8080–8084.
- [14] L. Zhang, F. Zhou, Y. Drabsch, R. Gao, B.E. Snaar-Jagalska, C. Mickanin, et al., USP4 is regulated by AKT phosphorylation and directly deubiquitylates TGF-beta type I receptor, *Nat. Cell Biol.* 14 (2012) 717–726.
- [15] P.J. Eichhorn, L. Rodon, A. Gonzalez-Junca, A. Dirac, M. Gili, E. Martinez-Saez, et al., USP15 stabilizes TGF-beta receptor I and promotes oncogenesis through the activation of TGF-beta signaling in glioblastoma, *Nat. Med.* 18 (2012) 429–435.
- [16] M. Inti, A. Manfrin, A. Mamidi, G. Martello, L. Morsut, S. Soligo, et al., USP15 is a deubiquitylating enzyme for receptor-activated SMADs, *Nat. Cell Biol.* 13 (2011) 1368–1375.
- [17] Y. Lou, J. Lu, Y. Zhang, P. Gu, H. Wang, F. Qian, et al., The centromere-associated protein CENPU promotes cell proliferation, migration, and invasiveness in lung adenocarcinoma, *Cancer Lett.* 532 (2022), 215599.
- [18] H.S. Jeon, J. Jen, TGF-beta signaling and the role of inhibitory Smads in non-small cell lung cancer, *J. Thorac. Oncol.* 5 (2010) 417–419.
- [19] Y.N. Tang, W.Q. Ding, X.J. Guo, X.W. Yuan, D.M. Wang, J.G. Song, Epigenetic regulation of Smad2 and Smad3 by profilin-2 promotes lung cancer growth and metastasis, *Nat. Commun.* 6 (2015) 8230.
- [20] M. Risolino, N. Mandia, F. Iavarone, L. Dardaei, E. Longobardi, S. Fernandez, et al., Transcription factor PREP1 induces EMT and metastasis by controlling the TGF-beta-SMAD3 pathway in non-small cell lung adenocarcinoma, *P Natl Acad Sci Usa* 111 (2014) E3775–E3784.
- [21] K. Watt, D. Newsted, E. Voorand, R.J. Gooding, A. Majewski, P. Truesdell, et al., MicroRNA-206 suppresses TGF-beta signalling to limit tumor growth and metastasis in lung adenocarcinoma, *Cell. Signal.* 50 (2018) 25–36.
- [22] H. Hu, Z. Xu, C. Li, C. Xu, Z. Lei, H.T. Zhang, et al., MiR-145 and miR-203 represses TGF-beta-induced epithelial-mesenchymal transition and invasion by inhibiting SMAD3 in non-small cell lung cancer cells, *Lung Cancer* 97 (2016) 87–94.
- [23] L.R. Butler, R.M. Denham, J. Jia, A.J. Garvin, H.R. Stone, V. Shah, et al., The proteasomal de-ubiquitinating enzyme POH1 promotes the double-strand DNA break response, *EMBO J.* 31 (2012) 3918–3934.
- [24] M. Gallery, J.L. Blank, Y. Lin, J.A. Gutierrez, J.C. Pulido, D. Rappoli, et al., The JAMM motif of human deubiquitinase Poh1 is essential for cell viability, *Mol. Cancer Therapeut.* 6 (2007) 262–268.
- [25] W. Yu, J. Li, Q. Wang, B. Wang, L. Zhang, Y. Liu, et al., Targeting POH1 inhibits prostate cancer cell growth and enhances the suppressive efficacy of androgen deprivation and docetaxel, *Prostate* 79 (2019) 1304–1315.
- [26] G. Luo, N. Hu, X. Xia, J. Zhou, C. Ye, RPN11 deubiquitinase promotes proliferation and migration of breast cancer cells, *Mol. Med. Rep.* 16 (2017) 331–338.
- [27] B. Wang, A. Ma, L. Zhang, W.L. Jin, Y. Qian, G. Xu, et al., POH1 deubiquitylates and stabilizes E2F1 to promote tumour formation, *Nat. Commun.* 6 (2015) 8704.
- [28] R. Zhu, Y. Liu, H. Zhou, L. Li, Y. Li, F. Ding, et al., Deubiquitinase enzyme PSMD14 promotes tumor metastasis through stabilizing SNAIL in human esophageal squamous cell carcinoma, *Cancer Lett.* 418 (2018) 125–134.
- [29] A. Byrne, R.P. McLaren, P. Mason, L. Chai, M.R. Dufault, Y. Huang, et al., Knockdown of human deubiquitinase PSMD14 induces cell cycle arrest and senescence, *Exp. Cell Res.* 316 (2010) 258–271.
- [30] J. Li, Y. Li, F. Xu, B. Sun, L. Yang, H. Wang, Deubiquitinase enzyme PSMD14 facilitates gastric carcinogenesis through stabilizing PTBP1, *Exp. Cell Res.* 415 (2022), 113148.
- [31] H.W. Zhang, Y. Guo, L.X. Sun, F.B. Ni, K. Xu, Prognostic value of small mother against decapentaplegic expression in human gastric cancer, *Bioengineered* 12 (2021) 2534–2549.

- [32] S.Y. Cho, S.Y. Ha, S.M. Huang, J.H. Kim, M.S. Kang, H.Y. Yoo, et al., The prognostic significance of Smad3, Smad4, Smad3 phosphoisoform expression in esophageal squamous cell carcinoma, *Med. Oncol.* 31 (2014) 236.
- [33] W. Yu, J. Li, Q. Wang, B. Wang, L. Zhang, Y. Liu, et al., Targeting POH1 inhibits prostate cancer cell growth and enhances the suppressive efficacy of androgen deprivation and docetaxel, *Prostate* 79 (2019) 1304–1315.
- [34] S. Lu, J. Lee, M. Revelo, X. Wang, S. Lu, Z. Dong, Smad3 is overexpressed in advanced human prostate cancer and necessary for progressive growth of prostate cancer cells in nude mice, *Clin. Cancer Res.* 13 (2007) 5692–5702.
- [35] T. Sun, Z. Liu, F. Bi, Q. Yang, Deubiquitinase PSMD14 promotes ovarian cancer progression by decreasing enzymatic activity of PKM2, *Mol. Oncol.* 15 (2021) 3639–3658.
- [36] T.V. Do, L.A. Kubba, H. Du, C.D. Sturgis, T.K. Woodruff, Transforming growth factor-beta1, transforming growth factor-beta2, and transforming growth factor-beta3 enhance ovarian cancer metastatic potential by inducing a Smad3-dependent epithelial-to-mesenchymal transition, *Mol. Cancer Res.* 6 (2008) 695–705.
- [37] J. Lei, X. Liu, W. Liu, Y. Zhang, Z. Liu, The prognostic value of USP14 and PSMD14 expression in non-small cell lung cancer, *Ann. Transl. Med.* 9 (2021) 1019.
- [38] L. Zhang, H. Xu, C. Ma, J. Zhang, Y. Zhao, X. Yang, et al., Upregulation of deubiquitinase PSMD14 in lung adenocarcinoma (LUAD) and its prognostic significance, *J. Cancer* 11 (2020) 2962–2971.
- [39] X. Wei, X. Wang, J. Zhan, Y. Chen, W. Fang, L. Zhang, et al., Smurf1 inhibits integrin activation by controlling Kindlin-2 ubiquitination and degradation, *J. Cell Biol.* 216 (2017) 1455–1471.
- [40] D. Mu, L. Chen, X. Zhang, L.H. See, C.M. Koch, C. Yen, et al., Genomic amplification and oncogenic properties of the KCNK9 potassium channel gene, *Cancer Cell* 3 (2003) 297–302.
- [41] Y. Chen, F.F. Peng, J. Jin, H.M. Chen, H. Yu, B.F. Zhang, Src-mediated ligand release-independent EGFR transactivation involves TGF-beta-induced Smad3 activation in mesangial cells, *Biochem Biophys Res Co* 493 (2017) 914–920.
- [42] C. Jing, X. Li, M. Zhou, S. Zhang, Q. Lai, D. Liu, et al., The PSMD14 inhibitor Thiolutin as a novel therapeutic approach for esophageal squamous cell carcinoma through facilitating SNAIL degradation, *Theranostics* 11 (2021) 5847–5862.
- [43] B. Wang, X. Xu, Z. Yang, L. Zhang, Y. Liu, A. Ma, et al., POH1 contributes to hyperactivation of TGF-beta signaling and facilitates hepatocellular carcinoma metastasis through deubiquitinating TGF-beta receptors and caveolin-1, *EBioMedicine* 41 (2019) 320–332.
- [44] M.S. Alam, Proximity ligation assay (PLA), *Curr. Protoc. Im.* 123 (2018) e58.
- [45] E.M. Cooper, C. Cutcliffe, T.Z. Kristiansen, A. Pandey, C.M. Pickart, R.E. Cohen, K63-specific deubiquitination by two JAMM/MPN+ complexes: BRISC-associated Brc36 and proteasomal Poh1, *EMBO J.* 28 (2009) 621–631.
- [46] B. Chen, Y. Song, Y. Zhan, S. Zhou, J. Ke, W. Ao, et al., Fangchinoline inhibits non-small cell lung cancer metastasis by reversing epithelial-mesenchymal transition and suppressing the cytosolic ROS-related Akt-mTOR signaling pathway, *Cancer Lett.* 543 (2022), 215783.
- [47] J. Lv, S. Zhang, H. Wu, J. Lu, Y. Lu, F. Wang, et al., Deubiquitinase PSMD14 enhances hepatocellular carcinoma growth and metastasis by stabilizing GRB2, *Cancer Lett.* 469 (2020) 22–34.
- [48] W. Yu, J. Li, Q. Wang, B. Wang, L. Zhang, Y. Liu, et al., Targeting POH1 inhibits prostate cancer cell growth and enhances the suppressive efficacy of androgen deprivation and docetaxel, *Prostate* 79 (2019) 1304–1315.
- [49] Q.Q. Chang, C.Y. Chen, Z. Chen, S. Chang, LncRNA PVT1 promotes proliferation and invasion through enhancing Smad3 expression by sponging miR-140-5p in cervical cancer, *Radiol. Oncol.* 53 (2019) 443–452.
- [50] L. Yang, Y. Chen, N. Liu, Y. Lu, W. Ma, Z. Yang, et al., CircMET promotes tumor proliferation by enhancing CDKN2A mRNA decay and upregulating SMAD3, *Mol. Cancer* 21 (2022) 23.
- [51] M. Petersen, E. Pardali, G. van der Horst, H. Cheung, C. van den Hoogen, G. van der Pluijm, et al., Smad2 and Smad3 have opposing roles in breast cancer bone metastasis by differentially affecting tumor angiogenesis, *Oncogene* 29 (2010) 1351–1361.
- [52] Q. Fu, Q. Zhang, Y. Lou, J. Yang, G. Nie, Q. Chen, et al., Primary tumor-derived exosomes facilitate metastasis by regulating adhesion of circulating tumor cells via SMAD3 in liver cancer, *Oncogene* 37 (2018) 6105–6118.
- [53] K. Yamazaki, Y. Masugi, K. Effendi, H. Tsujikawa, N. Hiraoka, M. Kitago, et al., Upregulated SMAD3 promotes epithelial-mesenchymal transition and predicts poor prognosis in pancreatic ductal adenocarcinoma, *Lab. Invest.* 94 (2014) 683–691.
- [54] J. Durslewicz, A. Klimaszewska-Wisniewska, J. Jozwicki, P. Antosik, M. Smolinska-Swiatala, M. Gagat, et al., Prognostic significance of TLR2, SMAD3 and localization-dependent SATB1 in stage I and II non-small-cell lung cancer patients, *Cancer Control* 28 (2021), 10732748211056697.
- [55] Y. Inoue, M. Kitagawa, K. Onozaki, H. Hayashi, Contribution of the constitutive and inducible degradation of Smad3 by the ubiquitin-proteasome pathway to transforming growth factor-beta signaling, *J. Interferon Cytokine Res.* 24 (2004) 43–54.
- [56] A. Sinha, P.V. Iyengar, D.P. Ten, E3 ubiquitin ligases: key regulators of TGFbeta signaling in cancer progression, *Int. J. Mol. Sci.* 22 (2021).
- [57] Y. Inoue, T. Imamura, Regulation of TGF-beta family signaling by E3 ubiquitin ligases, *Cancer Sci.* 99 (2008) 2107–2112.
- [58] N. Chen, S. Balasenthil, J. Reuther, A.M. Killary, DEAR1, a novel tumor suppressor that regulates cell polarity and epithelial plasticity, *Cancer Res.* 74 (2014) 5683–5689.
- [59] Y. Shi, X. Wang, Z. Xu, Y. He, C. Guo, L. He, et al., PDLIM5 inhibits STUB1-mediated degradation of SMAD3 and promotes the migration and invasion of lung cancer cells, *J. Biol. Chem.* 295 (2020) 13798–13811.
- [60] J. Zhou, Y. Dabiri, R.A. Gama-Brambila, S. Ghaffory, M. Altinbay, A. Mehrabi, et al., pVHL-mediated SMAD3 degradation suppresses TGF-beta signaling, *J. Cell Biol.* 221 (2022).
- [61] M. Fukuchi, T. Imamura, T. Chiba, T. Ebisawa, M. Kawabata, K. Tanaka, et al., Ligand-dependent degradation of Smad3 by a ubiquitin ligase complex of ROC1 and associated proteins, *Mol. Biol. Cell* 12 (2001) 1431–1443.
- [62] L.Y. Tang, M. Yamashita, N.P. Coussens, Y. Tang, X. Wang, C. Li, et al., Ablation of Smur2 reveals an inhibition in TGF-beta signalling through multiple mono-ubiquitination of Smad3, *EMBO J.* 30 (2011) 4777–4789.
- [63] J. Chen, A. Mitra, S. Li, S. Song, B.N. Nguyen, J.S. Chen, et al., Targeting the E3 ubiquitin ligase PJA1 enhances tumor-suppressing TGFbeta signaling, *Cancer Res.* 80 (2020) 1819–1832.
- [64] L. Herhaus, M. Al-Salihi, T. Macartney, S. Weidlich, G.P. Sapkota, OTUB1 enhances TGFbeta signalling by inhibiting the ubiquitylation and degradation of active SMAD2/3, *Nat. Commun.* 4 (2013) 2519.
- [65] Y.T. Huang, A.C. Cheng, H.C. Tang, G.C. Huang, L. Cai, T.H. Lin, et al., USP7 facilitates SMAD3 autoregulation to repress cancer progression in p53-deficient lung cancer, *Cell Death Dis.* 12 (2021) 880.
- [66] S. Yokoyama, Y. Iwakami, Z. Hang, R. Kin, Y. Zhou, Y. Yasuta, et al., Targeting PSMD14 inhibits melanoma growth through SMAD3 stabilization, *Sci Rep-Uk* 10 (2020), 19214.
- [67] F.E. Reyes-Turcu, K.H. Ventii, K.D. Wilkinson, Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes, *Annu. Rev. Biochem.* 78 (2009) 363–397.
- [68] Y. Kulathu, D. Komander, Atypical ubiquitylation - the unexplored world of polyubiquitin beyond Lys48 and Lys63 linkages, *Nat. Rev. Mol. Cell Biol.* 13 (2012) 508–523.
- [69] D. Seo, S.M. Jung, J.S. Park, J. Lee, J. Ha, M. Kim, et al., The deubiquitinating enzyme PSMD14 facilitates tumor growth and chemoresistance through stabilizing the ALK2 receptor in the initiation of BMP6 signaling pathway, *EBioMedicine* 49 (2019) 55–71.
- [70] P.C. Tang, J.Y. Chung, V.W. Xue, J. Xiao, X.M. Meng, X.R. Huang, et al., Smad3 promotes cancer-associated fibroblasts generation via macrophage-myofibroblast transition, *Adv. Sci.* 9 (2022), e2101235.
- [71] J.Y. Chung, P.C. Tang, M.K. Chan, V.W. Xue, X.R. Huang, C.S. Ng, et al., Smad3 is essential for polarization of tumor-associated neutrophils in non-small cell lung carcinoma, *Nat. Commun.* 14 (2023) 1794.
- [72] A.P. Smith, A. Verrecchia, G. Faga, M. Doni, D. Perna, F. Martinato, et al., A positive role for Myc in TGFbeta-induced Snail transcription and epithelial-to-mesenchymal transition, *Oncogene* 28 (2009) 422–430.
- [73] Y. Song, S. Li, A. Ray, D.S. Das, J. Qi, M.K. Samur, et al., Blockade of deubiquitylating enzyme Rpn11 triggers apoptosis in multiple myeloma cells and overcomes bortezomib resistance, *Oncogene* 36 (2017) 5631–5638.
- [74] V. Spataro, A. Buetti-Dinh, POH1/Rpn11/PSMD14: a journey from basic research in fission yeast to a prognostic marker and a druggable target in cancer cells, *Br. J. Cancer* 127 (2022) 788–799.