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Deubiquitinase USP13 promotes extracellular matrix expression by stabilizing Smad4 in lung fibroblast cells

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Abstract

Smad4 plays a central role in the regulation of extracellular matrix (ECM) protein expression and cell differentiation; however, the molecular regulation of Smad4 protein stability by a deubiquitinase has not been reported. In the current study, we reveal that a deubiquitinase USP13 stabilizes Smad4, ultimately modulating ECM protein expression in lung fibroblast cells. USP13 was increased in primary adult lung fibroblasts isolated from bleomycin-challenged mice and transforming growth factor (TGF)-β1-treated primary mouse lung fibroblasts. In a bleomycin-induced murine model of lung fibrosis, USP13-deficient mice showed reduced ECM levels such as fibronectin (FN) and collagen compared with wild-type mice. The reductions in both protein levels and mRNA expression of ECM were observed in the isolated lung fibroblasts from USP13-deficient mice, suggesting that downregulation of USP13 reduces ECM levels through inhibiting its transcription. To investigate the molecular mechanisms by which USP13 modulates ECM expression, we focused on the role of USP13 on Smad4 expression. Overexpression of USP13 increased FN and Smad4 protein levels in lung fibroblasts, while downregulation of USP13 reduced Smad4 protein levels, without altering Smad4 mRNA expression, suggesting that

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X.L., Y.L, J.L., Y.Z., D.J.K., M.R., and J.Z. performed the experiments and provided human fibroblast cells; X.L. analyzed the data and wrote the manuscript; R.M. and Y.Z. provided advice, finalized the manuscript.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.trsl.2020.05.004.

USP13 regulates *Smad4* protein stability. Knockdown of USP13 decreased Smad4 half-life and promoted Smad4 ubiquitination. Both Smad4 and USP13 were co-localized in the cytoplasm in treated cell, and co-translocated into the nucleus in response to TGF-β1. The results indicate that USP13 promotes ECM expression by stabilizing Smad4 in lung fibroblasts and plays a role in the maintenance of the extracellular matrix in lungs.

INTRODUCTION

Extracellular matrix (ECM) proteins including FN and collagen plays an important role in maintaining tissue structure, tissue repair,[1] fibrogenesis, inflammatory processes,[2] and cancer pathogenesis.[3,4] In addition to its role in ECM, FN regulates a variety of cellular functions including cell adhesion, proliferation, migration, and survival.[5] FN and collagen expression are majorly regulated by transforming growth factor-β1 (TGF-β1)/Smad signaling in a variety of cell types, such as fibroblasts, endothelial cells, smooth muscle cells.[6] The Smad proteins mediate extracellular TGF-β1-induced intracellular signaling. In response to TGF-β1, Smad2/3 are phosphorylated by TGF-β1 receptors and the phosphorylated Smad2/3 heterodimeric complex interacts with Smad4.[7,8] The Smad2/3/4 complex translocates to the nucleus to promote gene transcription of FN and collagen.[9] Smad4 plays a central role in the regulation of other Smads activity. Smad4 regulates both basal and TGF-β1-inudced collagen expression.[10]

The TGF-β1/Smad signaling pathway can be regulated by ubiquitin-mediated degradation.[11] Protein ubiquitination is a reversible covalent modification which regulates stability, activity, and localization of the target proteins. Deubiquitinating enzymes (DUBs) are a group of proteases that remove ubiquitin or ubiquitin-like proteins from target proteins and often rescue them from degradation.[12] We have shown that USP11 stabilizes TGF-β receptor II[13] and UCHL5 deubiquitinates and stabilizes Smad2/3.[14] Smad4 has been known to be degraded in the proteasome system.[15] Several ubiquitin E3 ligases including β-Trcp,[16] Smurfs, WWP1, and Nedd4–2[17] have been identified for targeting Smad4. Recent study demonstrated that a pan-inhibitor of DUB reduces Smad4 expression; however, the DUB responsible for Smad4 has not been identified.

USP13 is a deubiquitinase that has been shown to regulate the stability of tumor-related proteins[18,19] and cellular antiviral responses.[20,21] Our previous study demonstrated that USP13 stabilizes the anti-inflammatory receptor IL-1R8/Sigirr and exhibits an anti-inflammatory property.[22] In this report, we revealed that USP13 regulates ECM expression through deubiquitinating and stabilizing Smad4. This study is the first report that USP13 plays a role in the regulation of Smad signaling and ECM in lung fibroblasts.

METHODS AND MATERIALS

Animal experiments.

C57BL/6 wild type (WT) and USP13 deficient (*Usp13*$^{-/-}$) mice were housed in the specific pathogen-free animal care facility at the Ohio State University in accordance with institutional guidelines and guidelines of the US National Institutes of Health. All animal

experiments were approved by an institutional animal care and use committee (IACUC) at the Ohio State University Animal Resources Centers. The *Usp13*$^{-/-}$ mice were generated by the CRISPR/Cas9 system.[22] *Usp13*$^{-/-}$ and WT mice (8–10 weeks) were challenged with a single intratracheal instillation of bleomycin (BLM, 0.045 units per mouse in 50 μL PBS) or PBS (50 μL). At designated time points after BLM challenge, the mice were anesthetized before myocardial perfusions were performed with PBS via the right ventricle until lungs were cleared of blood, and then lungs were harvested for further analysis.

Cell culture and reagents.

Primary adult mouse lung fibroblast cells were isolated from WT and *Usp13*$^{-/-}$ mice and cultured according to the method introduced by Seluanov A et al.[23] Human lung fibroblast cell line (MRC5) cells and lung fibroblast cells from idiopathic pulmonary fibrosis (IPF) patients were cultured in EMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 1% penicillin/streptomycin (Lonza). IPF tissues were obtained from explanted lungs of subjects with advanced disease under a protocol approved by the University of Pittsburgh Institutional Review Board and Material Transfer Agreement between the University of Pittsburgh and the Ohio State University. Cells were maintained in a 37°C incubator in the presence of 5% CO_2. Cyclo-heximide (CHX) was from Sigma Aldrich (St Louis, MO, USA). Immobilized protein A/G beads, anti-V5, anti-Lamin A/C, and anti-β-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-USP13, anti-FN, anti-Smad4, anti-GAPDH, and anti-Flag antibodies were from ProteinTech (Chicago, IL, USA). Anti-collagen1a1 antibody was from Cell Signaling Technology (Danvers, MA, USA), Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse secondary antibodies were purchased from BioRad Laboratories (Hercules, CA, USA). Mammalian expression plasmid pcDNA3.1/His-V5-topo and *Escherichia coli* Top10 competent cells were from Life Technologies (Gaithersburg, MD, USA). All commercially available materials used were of the highest quality.

Plasmid and siRNA transfection.

GenMute siRNA transfection reagent and LipoJet DNA transfection reagents were purchased from SignaGen (Rockville, MD, USA). Human *Usp13* siRNA and control siRNA were purchased from Sigma Aldrich (St Louis, MO, USA). Cells were cultured on 6-well plates or 10-mm dishes to 70%−90% confluence. Plasmid encoding V5-tagged human USP13,[22] empty vector, and siRNAs were transfected into Mrc5 cells according to the transfection reagent manufacturer's instructions.

Western blotting analysis.

Cells were washed with cold PBS and collected in the cell lysis buffer containing 20mM Tris●HCl (pH 7.4), 150mM NaCl, 2mM EGTA (ethylene glycol-bis (β-aminoethyl ether)-N,N, N′,N′-tetraacetic acid), 5mM β-glycerophosphate, 1mM $MgCl_2$, 1% Triton X-100, 1mM sodium orthovanadate, 10μg/mL protease inhibitors, 1μg/mL aprotinin, 1μg/mL leupeptin and 1μg/mL pepstatin. An equal amount of cell lysates (20μg) was subjected to SDS-PAGE, electrotransferred to membranes and immunoblotted following standard protocol.

qRT-PCR analysis.

Total RNA was isolated from cultured MRC5 cells or isolated primary mouse lung fibroblast cells using the Total RNA Mini kit (IBI Scientific, IA, USA) according to the manufacturer's instructions, and RNA was quantified by Nano-drop spectrophotometry. cDNA was prepared using the iScript cDNA synthesis kit (Bio-Rad, CA, USA). The expression of FN, Smad4, and Usp13 was performed using iQ SYBR Green Supermix and the iCycler real-time PCR detection system (Bio-Rad, CA, USA). Gapdh was used as an internal control. mFN primers: forward, GGTTCGGGAAGAGGTTGTGA, reverse, ATGGCGTAATGGGAAACCGT; *m*Gapdh primers: forward, ACCCTTAAGAGGGATGCTGC, reverse, TCACACCGACCTTCACCATTT; hSmad4 primers: forward, TTAGACAGAGAAGCTGGGCG, reverse, TGTCGATGACACTGACGCAA; hUsp13 primers, forward, TGTCGCAAGGCTGTGTACTT, reverse, CAGCGGCTCAGCAAAATCTG; mCOL1A1 primers: forward, GGGCAAGACAGTCATCGAA, reverse, GTCCGAATTCCTGGTCTGGG; mSMA primers: forward, ACCTTTGGCTTGGCTTGTCA, reverse, TTGAAGCGGAAGTCTGGGAAG; hGapdh primers: forward, TCGGAGTCAACGGATTTGGTCG, reverse GCTCTCCAGAACATCATCCCTGCCT.

Co-immunofluorescence staining.

MRC5 cells were cultured in glass-bottomed dishes. Some cells were transfected with V5-tagged USP13 and Flag-tagged Smad4 plasmids for 24 hours. Other cells were transfected with V5-tagged USP13 only and then were treated with or without TGF-β1 (10 ng/mL) for 1 hour. Cells were fixed with 3.7% formaldehyde for 20 minutes. After blocking in 1% bovine serum albumin in Tris-buffered saline with 0.1% Tween-20 for 1 hour, cells were incubated with 1:200 dilution of primary antibody for 1 hour, followed by a 1:200 dilution of fluorescence-conjugated secondary antibodies sequentially for immunostaining. Images were captured by Nikon A1R-HD25 confocal microscope (Nikon, Tokyo, Japan).

In vivo ubiquitination assay.

We performed a modified protocol under denaturing conditions. MRC5 cells were treated with MG-132 for 3 hours before they were washed with cold PBS and harvested in PBS. After centrifuging at 1000 rpm for 5 minutes, the supernatant was removed. Then 1 μL of ubiquitin aldehyde, 1 μL of NEM, and 70 μL of 2% SDS lysis buffer were added. The samples were diluted with 700 μL of 1 × TBS after 12s sonication. Equal amounts of protein (1 mg) were incubated with the anti-Smad4 antibody overnight at 4°C. 40 μL protein A/G plus agarose were added and incubated for additional 2 hours at 4°C to pull down Smad4. The immunoprecipitated complex was washed three times with cold PBS and analyzed by immunoblotting with antibody against ubiquitin.

Nuclear protein isolation.

Nuclear proteins were extracted using the NE-PER Nuclear Extraction Kit (Thermo Fisher Scientific). Protein concentration was measured using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Inc.).

Quantification and statistical analysis.

Immunoblot intensities were quantified by ImageJ software (Image Processing and Analysis in Java; National Institutes of Health, Bethesda, MD, USA; http://imagej.nih.gov/). One-way ANOVA statistical analysis was used for comparison among several groups. Data are expressed as mean ± SEM of triplicate samples from at least three independent experiments. $P < 0.05$ were considered statistically significant.

RESULTS

USP13 protein levels were increased in mouse lung fibroblasts isolated from Bleomycin-challenged mice and TGF-β1-treated lung fibroblasts.

Bleomycin (BLM) is widely used to induce FN and collagen expression in lungs in a murine model of lung fibrosis. To investigate the role of USP13 protein in ECM expression, first, we investigated the changes of USP13 in lung tissues from BLM-challenged mice. C57BL/6 WT mice were challenged with PBS or BLM (0.045 U/mouse) for 1 week, primary adult lung fibroblasts were then isolated. USP13 protein levels were significantly increased in the isolated lung fibroblasts from BLM-challenged mice compared with PBS groups (Fig 1A). TGF-β1 is a central profibrotic cytokine which triggers and promotes fibroblast ECM accumulation. To examine if TGF-β1 regulates expression of USP13, primary adult mouse lung fibroblasts were isolated from C56BL/6J mice and then treated with TGF-β1 (10 ng/mL) for 1 day or 2 days. While TGF-β1 promoted FN expression, the protein levels of USP13 were also significantly increased (Fig 1B). These data suggest that USP13 is upregulated by TGF-β1 in lung fibroblasts.

USP13 regulates ECM expression in lung fibroblasts.

FN and collagen play a crucial role in ECM assembly. First, we evaluated the impact of USP13 on FN and collagen expression. USP13 deficient (USP13KO) mice showed reduced protein levels of FN and collagen I compared with WT mice after BLM challenge for 3 weeks (Fig 2A). The similar changes in FN protein levels were observed in the isolated lung fibroblasts from USP13KO mice, compared with the cells from WT mice (Fig 2B). Smooth muscle actin (SMA) expression is an indicator of myofibroblast differentiation. SMA levels were reduced in lung fibroblast cells from USP13KO mice. To further confirm USP13 regulates ECM expression in lung fibroblast cells, we downregulated *USP13* gene expression by siRNA transfection in human lung fibroblast cells. As shown in Fig 2C, fibronectin and collagen I levels in both resting cells and TGF-β1-treated cells were reduced in *USP13* siRNA-transfected cells. Changes of protein levels are controlled by protein stability and new protein synthesis. To investigate the molecular mechanisms by which USP13 regulates ECM expression, we examined the mRNA expression of FN, collagen, and SMA in the TGF-β1 (10 ng/mL, 1 day)-treated mouse lung fibroblasts. The mRNA expression of *FN, collagen I,* and *SMA* were significantly decreased in the isolated lung fibroblasts from USP13KO mice, compared with the cells from WT mice (Fig 2D), suggesting that USP13 regulates ECM levels through modulating their transcript.

USP13 regulates Smad4 protein changes.

The expression of ECM is up-regulated in response to TGF-β1. Smad4 plays a central role in the TGF-β1-mediated pro-fibrotic gene expression by facilitating phosphorylated Smad2/3 translocation into the nucleus. Consistent with others' findings, we found that knockdown of Smad4 by siRNA significantly reduced FN, collagen I, and SMA expression in human lung fibroblast cells (Fig 3A). Smad4 levels were increased in response to TGF-β1 (Fig 3B). Thus, we focused on examining the effect of USP13 on Smad4 expression. Spautin-1, a small molecule inhibitor of USP13, was used to treat MCR5 cells. As shown in Fig 3C, spautin-1 decreased the Smad4 protein levels in a dose-dependent manner in MRC5 cells. Knockdown of USP13 with *Usp13* siRNA exhibited a reduction of Smad4 protein in both MRC5 cells and IPF lung fibroblast cells compared to control siRNA (Fig 3D, E). Smad4 protein levels were also decreased in isolated lung fibroblasts from USP13KO mice, compared with the cells from WT mice (Fig 3F). Furthermore, overexpression of USP13 increases both Smad4 and FN protein levels in MRC5 cells (Fig 4). Taken together, these results suggest that USP13 regulates Smad4 protein levels in lung fibroblast cells.

USP13 has no effect on Smad4 mRNA levels.

Reduction of protein levels is usually due to suppression of gene expression or increase in protein degradation. To explore the molecular mechanism by which USP13 regulates Smad4 levels, we investigated the mRNA expression of Smad4 and found that knockdown of USP13 had no effect on Smad4 mRNA in MRC5 cells (Fig 5), implying that the effect of USP13 on Smad4 level might be through modulation of Smad4 protein stability.

USP13 stabilizes Smad4 by deubiquitinating Smad4.

To investigate if USP13 regulates Smad4 protein stability, MRC5 cells were transfected with control siRNA or *U-SP13* siRNA prior to cycloheximide (CHX) treatment. Knockdown of USP13 augmented Smad4 degradation and reduced Smad4 half-life (Fig 6A). Further, an *in vivo* ubiquitination assay showed that downregulation of USP13 increased Smad4 polyubiquitination (Fig 6B). Further, we examined the cellular localizations of USP13 and Smad4 by co-transfection of V5-tagged USP13 and Flag-tagged Smad4 plasmids. As shown in Fig 7A, both V5-tagged USP13 and Flag-tagged Smad4 were co-localized in the cytoplasm of MRC5 cells. Interestingly, we also found that while Smad4 is translocated into the nucleus under the stimulation of TGF-β1 (10 ng/mL, 1 hour), USP13-V5 was also observed in the nucleus (Fig 6B, C), suggesting that USP13 is associated with Smad4 and the USP13/Smad4 complex is translocated into nucleus in response to TGF-β1 treatment. These data suggest that USP13 deubiquitinates and thus stabilizes Smad4 in lung fibroblast cells.

DISCUSSION

Lung fibroblast cells, the principal effector cells in fibrogenesis, differentiate to myofibroblasts and produce excessive ECM under the stimulation of TGF-β1.[24] Lung fibrosis disorders, such as IPF, are characterized by excessive ECM deposition. The accumulation of ECM leads to destroyed lung structure and eliminates the function of gas exchange.[25] FN and collagen are key components of ECM and play a crucial role in

matrix assembly as it cross-links with integrins, proteoglycans, and other focal adhesion molecules.[26] The expression of ECM is regulated by TGF-β1/Smad signaling which is a potential intervention target for treating fibrotic diseases and cancer. In this study, we revealed that a DUB, USP13, regulates ECM expression by modulating Smad4 stability.

The TGF-β1 downstream Smad proteins form complexes that are regulated by reversible interactions with other proteins. Recently, many studies have been focused on investigating the regulation of the protein components of the TGF-β1/Smad signaling pathways by post-translational modification such as ubiquitination and deubiquitination.[20,27] DUBs play an important role in the regulation of protein stability.[12] Our recent study showed that the USP18/FOXO3a pathway regulates TGF-β1 signaling and affects FN expression.[28] In this study, we identify that USP13 promotes ECM expression by stabilizing Smad4 in lung fibroblast cells. Smad4 plays a central role in the TGF-β1 signaling pathway as a chaperone protein transferring Smad2/3 between the nucleus and the cytoplasm.[29] Smad4 ubiquitination has been reported.[17,30–32] USP9X has been identified as a DUB for controlling Smad4 mono-ubiquitination.[32] Here, we show that Smad4 can be poly-ubiquitinated and that the poly-ubiquitination can be reversed by USP13. This study is the first to reveal that the DUB USP13 regulates Smad4 protein levels through deubiquitinating Smad4. Unlike the effect of USP9X on Smad4 interaction with phosphoSmad2,[32] USP13 regulates Smad4 protein stability. Lysine 507 is a mono-ubiquitination site on Smad4; however, the poly-ubiquitination site within Smad4 has not been identified. Future studies will be focused on identifying the Smad4 poly-ubiquitination site and if USP13 removes the ubiquitination site-linked polyubiquitin chain. In addition, we demonstrate that USP13 is associated with Smad4, and the association is not affected by TGF-β1. This is the first to reveal a role of USP13 in the nucleus. The USP13 docking site within Smad4 will be identified in future studies.

In this study, we observed that both BLM and TGF-β1 increased USP13 protein levels in isolated primary mouse lung fibroblast cells, suggesting a role of USP13 in lung fibroblasts in the pathogenesis of pulmonary fibrosis. We reanalyzed a publicly available microarray data from the Lung Genomics Research Consortium (http://www.lung-genomics.org/) and revealed that USP13 mRNA levels were reduced in IPF patients compared to normal subjects (supplemental Fig S1), which is consistent with findings from Geng J et al, demonstrating that USP13 expression was decreased in lung fibroblasts from IPF patients.[33] Taken together, USP13 level in lung fibroblasts is upregulated in the development of pulmonary fibrosis, while it is decreased in the late stage of IPF. Surprisingly, global USP13KO mice reduced lung fibronectin and collagen expression, whereas the trichrome staining displayed comparable severity of experimental pulmonary fibrosis (supplemental Fig S2), suggesting a compensable role of USP13 in other cell types. In lung epithelial cells, USP13 has been shown to exhibit an anti-inflammatory, enhanced proliferation, and an anti-apoptotic property by targeting Sigirr, AKT/MPAK signaling, and MCL1, respectively.[22,34,35] In the future study, generation of cell type specific USP13 knockout mice will reveal the distinct roles of USP13 in lung fibroblasts during the development of pulmonary fibrosis. We have shown that LPS treatment decreased USP13 levels in mouse macrophage-like RAW264.7 cells[22]; however, the molecular regulation of USP13 during the development of pulmonary fibrosis has not been investigated.

In summary, USP13 is a positive regulator of Smad4 stability and promotes ECM expression in lung fibroblast cells. Inhibition of USP13 activity may be a potential intervention strategy to treat abnormal ECM-related diseases. Our future study will explore whether the USP13 contributes to the pathogenesis of lung fibrosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

Conflicts of Interest: The authors declare that they have no competing interest.

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All the authors have read the journal's authorship agreement and that the manuscript has been reviewed by and approved by all named authors.

Abbreviations:

BLM	bleomycin
CHX	cycloheximide
DUB	deubiquitinating enzyme
ECM	extracellular matrix
FN	fibronectin
TGF	transforming growth factor

REFERENCES

1. To WS, Midwood KS. Plasma and cellular fibronectin: distinct and independent functions during tissue repair. Fibrogenesis Tissue Repair 2011;4:21. [PubMed: 21923916]
2. Lemanska-Perek A, Adamik B. Fibronectin and its soluble EDA-FN isoform as biomarkers for inflammation and sepsis. Adv Clin Exp Med 2019;28:1561–7. [PubMed: 31596557]
3. Rick JW, Chandra A, Dalle Ore C, Nguyen AT, Yagnik G, Aghi MK. Fibronectin in malignancy: cancer-specific alterations, protumoral effects, and therapeutic implications. Semin Oncol 2019;46:284–90. [PubMed: 31488338]
4. Lin TC, Yang CH, Cheng LH, Chang WT, Lin YR, Cheng HC. Fibronectin in cancer: friend or foe. Cells 2019;9:27. [PubMed: 31861892]
5. Cai X, Liu C, Zhang TN, Zhu YW, Dong X, Xue P. Down-regulation of FN1 inhibits colorectal carcinogenesis by suppressing proliferation, migration, and invasion. J Cell Biochem 2018;119:4717–28. [PubMed: 29274284]
6. Robertson IB, Rifkin DB. Regulation of the Bioavailability of TGF-beta and TGF-beta-Related Proteins. Cold Spring Harb Perspect Biol 2016;8:a021907. [PubMed: 27252363]
7. Heldin CH, Moustakas A. Signaling receptors for TGF-beta family members. Cold Spring Harb Perspect Biol 2016;8:a022053. [PubMed: 27481709]
8. Hill CS. Transcriptional control by the SMADs. Cold Spring Harb Perspect Biol 2016;8:a022079. [PubMed: 27449814]
9. Hu HH, Chen DQ, Wang YN, et al. New insights into TGF-beta/Smad signaling in tissue fibrosis. Chem Biol Interact 2018;292:76–83. [PubMed: 30017632]

10. Tsuchida K, Zhu Y, Siva S, Dunn SR, Sharma K. Role of Smad4 on TGF-beta-induced extracellular matrix stimulation in mesangial cells. Kidney Int 2003;63:2000–9. [PubMed: 12753287]
11. Izzi L, Attisano L. Regulation of the TGFbeta signalling pathway by ubiquitin-mediated degradation. Oncogene 2004;23:2071–8. [PubMed: 15021894]
12. Mevissen TET, Komander D. Mechanisms of deubiquitinase specificity and regulation. Annu Rev Biochem 2017;86:159–92. [PubMed: 28498721]
13. Jacko AM, Nan L, Li S, et al. De-ubiquitinating enzyme, USP11, promotes transforming growth factor beta-1 signaling through stabilization of transforming growth factor beta receptor II. Cell Death Dis 2016;7:e2474. [PubMed: 27853171]
14. Nan L, Jacko AM, Tan J, et al. Ubiquitin carboxyl-terminal hydrolase-L5 promotes TGFbeta-1 signaling by de-ubiquitinating and stabilizing Smad2/Smad3 in pulmonary fibrosis. Sci Rep 2016;6:33116. [PubMed: 27604640]
15. Miah S, Banks CAS, Ogunbolude Y, et al. BRK phosphorylates SMAD4 for proteasomal degradation and inhibits tumor suppressor FRK to control SNAIL, SLUG, and metastatic potential. Sci Adv 2019;5:eaaw3113. [PubMed: 31681835]
16. Wan M, Tang Y, Tytler EM, et al. Smad4 protein stability is regulated by ubiquitin ligase SCF beta-TrCP1. J Biol Chem 2004;279:14484–7. [PubMed: 14988407]
17. Moren A, Hellman U, Inada Y, Imamura T, Heldin CH, Moustakas A. Differential ubiquitination defines the functional status of the tumor suppressor Smad4. J Biol Chem 2003;278:33571–82. [PubMed: 12794086]
18. Liu J, Xia H, Kim M, et al. Beclin1 controls the levels of p53 by regulating the deubiquitination activity of USP10 and USP13. Cell 2011;147:223–34. [PubMed: 21962518]
19. Zhang J, Zhang P, Wei Y, et al. Deubiquitylation and stabilization of PTEN by USP13. Nat Cell Biol 2013;15:1486–94. [PubMed: 24270891]
20. Yeh HM, Yu CY, Yang HC, Ko SH, Liao CL, Lin YL. Ubiquitin-specific protease 13 regulates IFN signaling by stabilizing STAT1. J Immunol 2013;191:3328–36. [PubMed: 23940278]
21. Sun H, Zhang Q, Jing YY, et al. USP13 negatively regulates antiviral responses by deubiquitinating STING. Nat Commun 2017;8:15534. [PubMed: 28534493]
22. Li L, Wei J, Li S, et al. The deubiquitinase USP13 stabilizes the anti-inflammatory receptor IL-1R8/Sigirr to suppress lung inflammation. EBioMedicine 2019;45:553–62. [PubMed: 31204278]
23. Seluanov A, Vaidya A, Gorbunova V. Establishing primary adult fibroblast cultures from rodents. J Vis Exp 2010;44:2033.
24. Moore MW, Herzog EL. Regulation and relevance of myofibroblast responses in idiopathic pulmonary fibrosis. Curr Pathobiol Rep 2013;1:199–208. [PubMed: 25705577]
25. Todd NW, Luzina IG, Atamas SP. Molecular and cellular mechanisms of pulmonary fibrosis. Fibrogenesis Tissue Repair 2012;5:11. [PubMed: 22824096]
26. Halper J, Kjaer M. Basic components of connective tissues and extracellular matrix: elastin, fibrillin, fibulins, fibrinogen, fibronectin, laminin, tenascins and thrombospondins. Adv Exp Med Biol 2014;802:31–47. [PubMed: 24443019]
27. Kim SY, Baek KH. TGF-beta signaling pathway mediated by deubiquitinating enzymes. Cell Mol Life Sci 2019;76:653–65. [PubMed: 30349992]
28. Wang B, Li Y, Wang H, et al. FOXO3a is stabilized by USP18-mediated de-ISGylation and inhibits TGF-beta1-induced fibronectin expression. J Investig Med 2019;68:786–91.
29. Ullah I, Sun W, Tang L, Feng J. Roles of Smads family and alternative splicing variants of Smad4 in different cancers. J Cancer 2018;9:4018–28. [PubMed: 30410607]
30. Liang M, Liang YY, Wrighton K, et al. Ubiquitination and proteolysis of cancer-derived Smad4 mutants by SCFSkp2. Mol Cell Biol 2004;24:7524–37. [PubMed: 15314162]
31. Dupont S, Zacchigna L, Cordenonsi M, et al. Germ-layer specification and control of cell growth by Ectodermin, a Smad4 ubiquitin ligase. Cell 2005;121:87–99. [PubMed: 15820681]

32. Dupont S, Mamidi A, Cordenonsi M, et al. FAM/USP9x, a deubiquitinating enzyme essential for TGFbeta signaling, controls Smad4 monoubiquitination. Cell 2009;136:123–35. [PubMed: 19135894]

33. Geng J, Huang X, Li Y, et al. Down-regulation of USP13 mediates phenotype transformation of fibroblasts in idiopathic pulmonary fibrosis. Respir Res 2015;16:124. [PubMed: 26453058]

34. Wu Y, Zhang Y, Liu C, et al. Amplification of USP13 drives non-small cell lung cancer progression mediated by AKT/ MAPK signaling. Biomed Pharacother 2019;114:108831.

35. Zhang S, Zhang M, Jing Y, et al. Deubiquitinase USP13 dictates MCL1 stability and sensitivity to BH3 mimetic inhibitors. Nat Commun 2018;9:215. [PubMed: 29335437]

AT A GLANCE COMMENTARY

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Background

Smad4 plays a central role in ECM expression and cell differentiation, as well as in the pathogenesis of lung fibrosis; however, regulation of its protein stability has not been well studied. Recent studies have shown that abnormal ubiquitination-mediated protein degradation contributes to the pathogenesis of human disorders.

Translational Significance

This is the first report to demonstrate that USP13 stabilizes Smad4 and regulates ECM expression in lung fibroblast cells and suggest that USP13 is a new therapeutic target for treating pulmonary fibrosis. Future studies will focus on development of USP13 specific inhibitors and evaluation of their therapeutic potential in IPF.

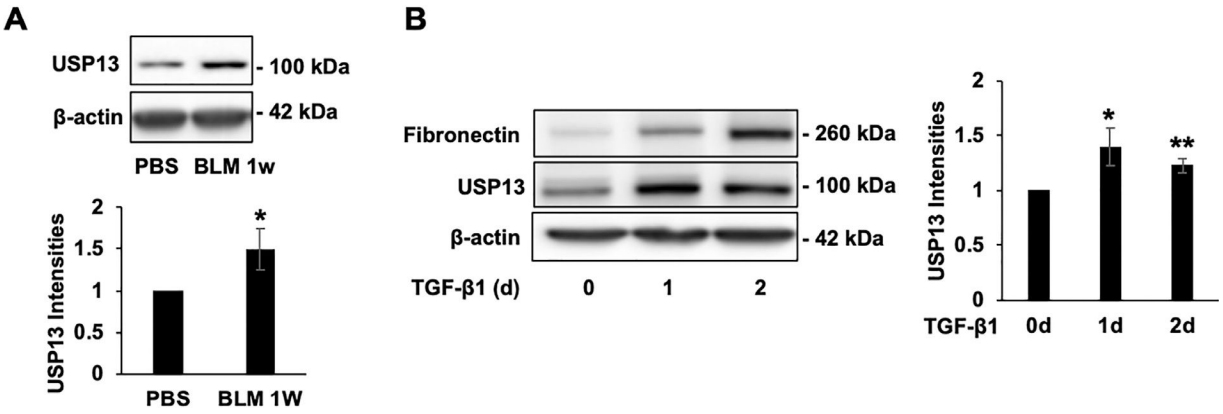

Fig 1.

USP13 levels are increased in mouse lung fibroblasts in BLM-challenged mice and TGF-β1-induced mouse lung fibroblasts. (A) Primary adult lung fibroblasts were isolated from C57BL/6 wild-type mice which were challenged with PBS or BLM (0.045 U/mouse) for 1 week. Cell lysates were examined by immunoblotting with USP13 and β-actin antibodies. Intensities of USP13 were quantified by ImageJ software. n = 3. *P < 0.05 compared to PBS. (B) Isolated primary adult mouse lung fibroblasts from C57BL/6 mice were treated with TGF-β1(10 ng/mL) for 1 day or 2 days. Cell lysates were examined by immunoblotting with FN, USP13, and β-actin antibodies. Intensities of USP13 were quantified by ImageJ software. n = 3. *P < 0.05 compared to 0 d. **P < 0.01 compared to 0 d. Shown are representative blots from three independent experiments.

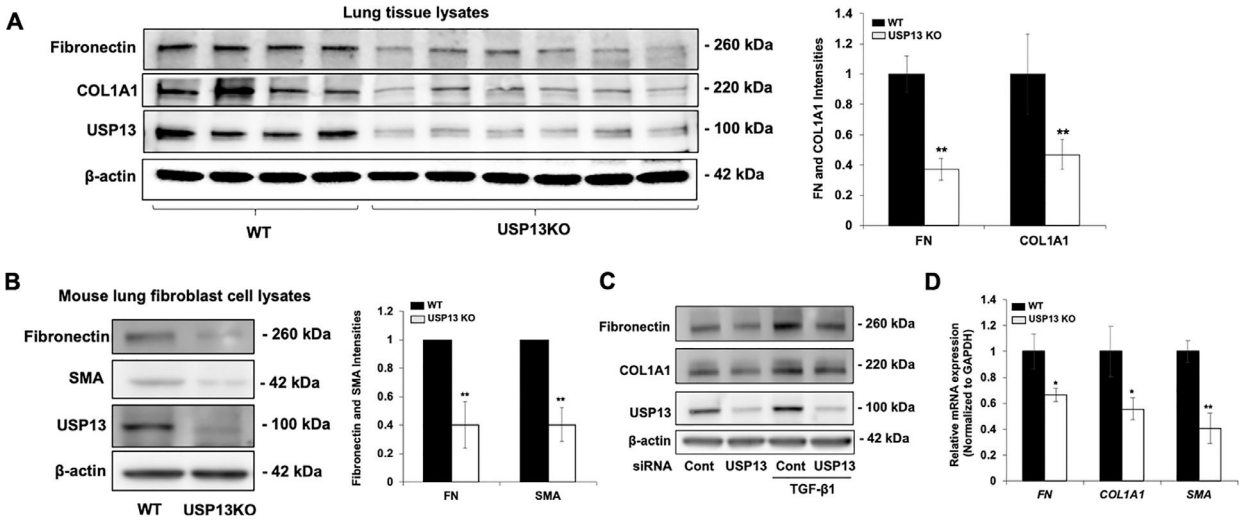

Fig 2.

Downregulation of USP13 reduces ECM expression. (A) Lung tissues were obtained from USP13KO and WT mice after BLM (0.045 U/mouse) challenge for 3 weeks and then tissue lysates were analyzed by immunoblotting with FN, collagen I (COL1A1), USP13, and β-actin antibodies. Intensities of FN and COL1A1 were quantified by ImageJ software. n = 4–6. **P < 0.01 compared to WT mice. (B) Primary adult mouse lung fibroblasts were isolated from USP13KO and WT mice. FN, SMA, USP13, and β-actin levels were analyzed by Western blot analysis. Intensities of FN and SMA were quantified by ImageJ software. n = 3. **P < 0.01 compared to WT control. Shown are representative blots from three independent experiments. (C) Human lung fibroblast cells (MRC5) were transfected with *USP13* siRNA for 3 days, and then treated with TGF-β1. FN, COL1A1, USP13, and β-actin levels were analyzed by Western blot analysis. Shown are representative blots from 3 independent experiments. (D) Isolated primary adult mouse lung fibroblasts from USP13KO and WT mice were treated with TGF-β1 (10 ng/mL) for 1 day. RNA was extracted and analyzed by real-time qPCR with *Fibronectin*, *COL1A1*, and *SMA* primers. Relative expression of mRNA was normalized to *Gapdh*. n = 3. *P < 0.05 compared to WT group. **P < 0.01 compared to WT group.

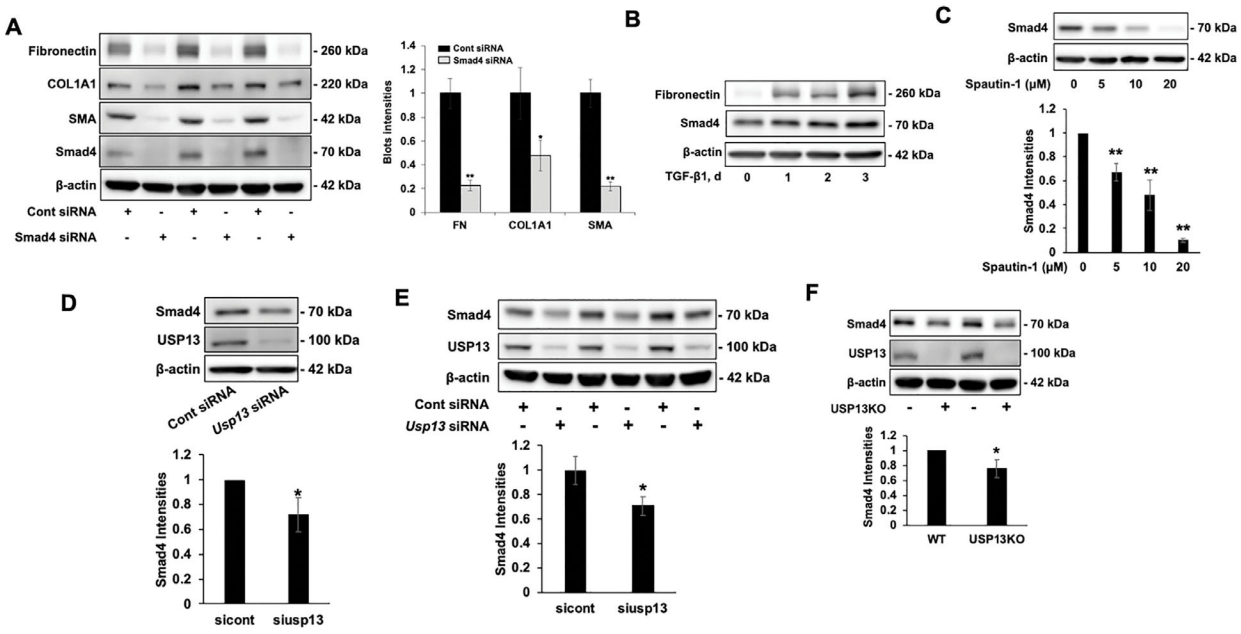

Fig 3.

Inhibition or downregulation of USP13 reduces Smad4 levels. (A) Human lung fibroblast cells were transfected with Smad4 siRNAs for 3 days. FN, COL1A1, SMA, Smad4, and β-actin levels were analyzed by Western blot analysis. *P < 0.05 compared to cont siRNA. **P < 0.01 compared to cont siRNA. (B) Mrc5 cells were treated with TGF-β1 for 1, 2, and 3 hours. Fibronectin, Smad4, and β-actin levels were analyzed by Western blot analysis. (C) MRC5 cells were treated with different doses of spautin-1 (0, 5, 10, or 20 μM) for 24 hours. Smad4 and β-actin levels were analyzed by Western blot analysis. n = 3. **P < 0.01 compared to 0 μM. (D) MRC5 cells were transfected with control siRNA or *U-SP13* siRNA for 3 days. Smad4, USP13, and β-actin levels were analyzed by Western blot analysis. Intensities of Smad4 were quantified by ImageJ software. n = 3. *P < 0.05 compared to control siRNA. (E) Lung fibroblasts from IPF patients were transfected with control siRNA or *Usp13* siRNA, and incubated for 3 days. Smad4, USP13, and β-actin levels were analyzed by Western blot analysis. Intensities of Smad4 were quantified by ImageJ software. n = 3. *P < 0.05 compared to control siRNA. (F) Primary adult mouse lung fibroblasts were isolated from USP13KO and WT mice, and then cell lysates were analyzed by immunoblotting with Smad4, USP13, and β-actin antibodies. Intensities of Smad4 were quantified by ImageJ software. n = 3. *P < 0.05 compared to cells from WT mice. Shown are representative blots from three independent experiments.

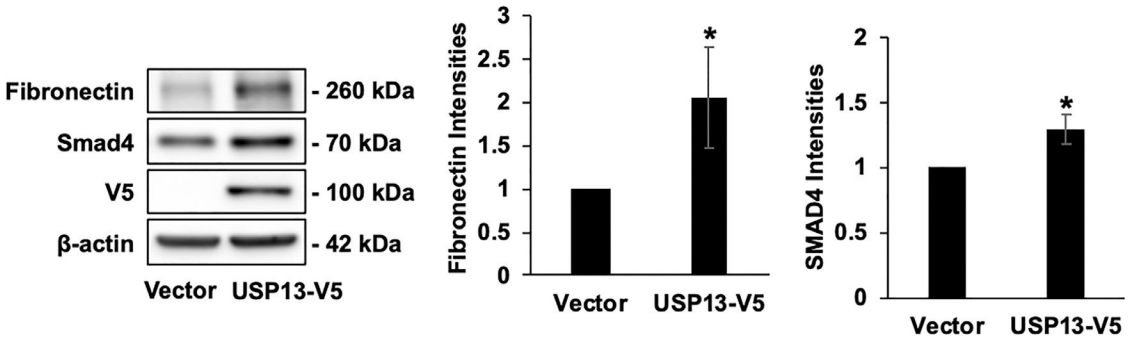

Fig 4.

Overexpression of USP13 increases FN and Smad4 levels in MRC5 cells. MRC5 cells were transfected with empty vector or U-SP13-V5 plasmids and incubated for 2 days. Cell lysates were analyzed by immunoblotting with FN, Smad4, V5, and β-actin antibodies. Intensities of FN and Smad4 were quantified by ImageJ software. n = 3. *P < 0.05 compared to empty vector-transfected cells. Shown are representative blots from 3 independent experiments.

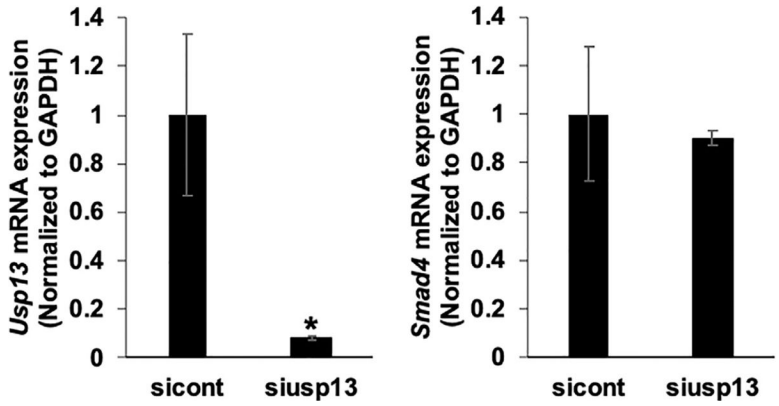

Fig 5.

Knockdown of USP13 has no effect on *Smad4* mRNA levels. MRC5 cells were transfected with control siRNA or *USP13* siRNA for 3 days. RNA was extracted and analyzed by real-time qPCR with *USP13* and *Smad4* primers. Relative expression of *USP13 and Smad4* mRNA was normalized to *Gapdh*. n = 3. **P < 0.01 compared to control siRNA. n.s., no significant.

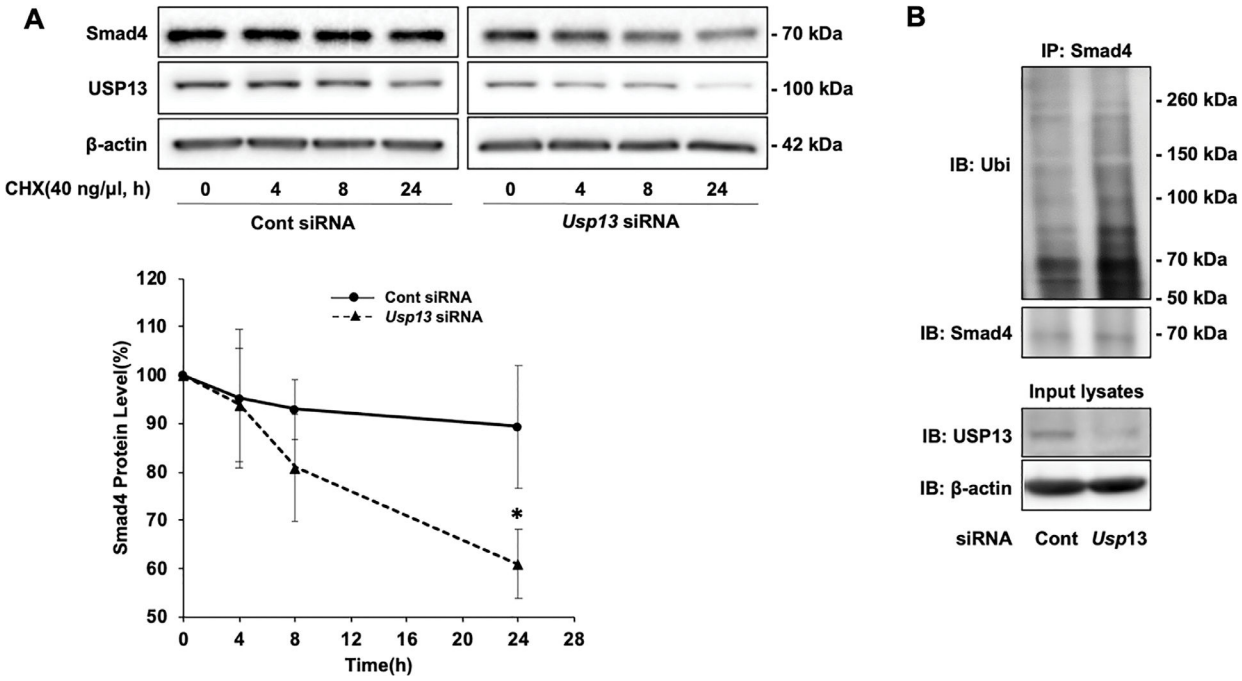

Fig 6.

USP13 stabilizes Smad4 through reducing Smad4 polyubiquitination. (A) MRC5 cells were transfected with control siRNA or *USP13* siRNA and incubated for 3 days. Then the cells were treated with CHX (40 ng/μL) for 4, 8, or 24 hours. Cell lysates were analyzed by immunoblotting with Smad4, USP13, and β-actin antibodies. Intensities of FN and Smad4 were quantified by ImageJ software. n = 3. *P < 0.05 compared to control siRNA. (B) MRC5 cells were transfected with control siRNA or *USP13* siRNA for 3 days. Denatured cell lysates were subjected to immunoprecipitation with a Smad4 antibody, followed by immunoblotting with ubiquitin and Smad4 antibodies. Input lysates were analyzed by immunoblotting with USP13 and β-actin antibodies. Shown are representative blots from 3 independent experiments.

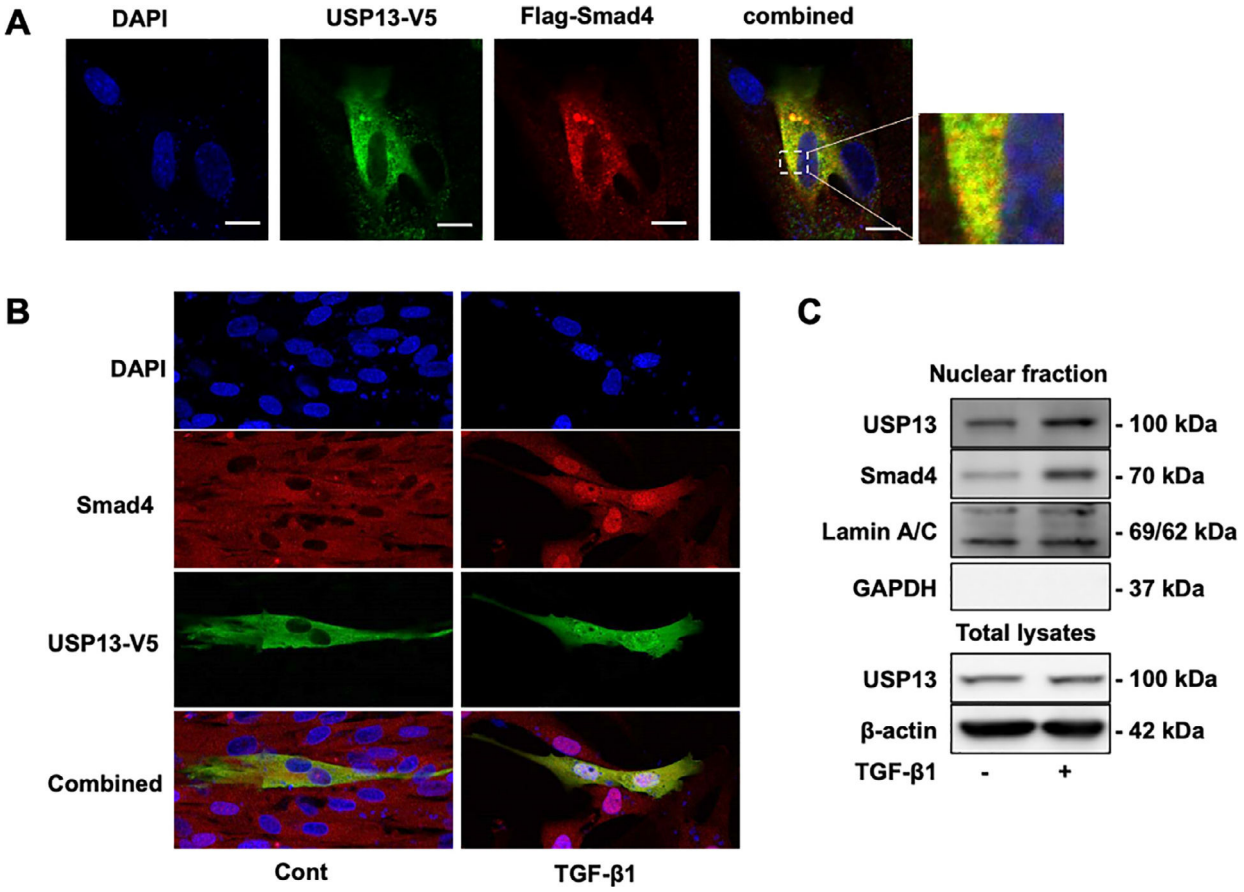

Fig 7.

Co-localization of USP13 and Smad4. (A) Representative immunofluorescence staining of USP13-V5 (green), Flag-Smad4 (red) and DAPI (blue) in MRC5 cells. (B) Representative immunofluorescence staining of USP13-V5 (green), Smad4 (red), and DAPI (blue) in MRC5 cells with or without TGF-β1 (10 ng/mL) treatment for 1 hour. (C) MRC5 cells were treated with or without TGF-β1 (10 ng/mL) for 1 hour. The nuclear fraction was isolated and analyzed with antibodies to USP13, Smad4, Lamin A/C, and Gapdh. Shown are representative blots from 3 independent experiments.