OPEN ACCESS

FOXO3a is stabilized by USP18-mediated de-ISGylation and inhibits TGF-β1-induced fibronectin expression

Ban Wang,[1] Yanhui Li,[2,3] Heather Wang,[3] Jing Zhao,[3] Yutong Zhao ,[3] Zhonghui Liu,[1] Haichun Ma[2]

[1]Department of Immunology, College of Basic Medical Sciences, Jilin University, Changchun, China
[2]Department of Anesthesia, Jilin University First Hospital, Changchun, China
[3]Department of Physiology and Cell Biology, Ohio State University, Columbus, Ohio, USA

Correspondence to
Dr Zhonghui Liu; liuzh@jlu.edu.cn
Dr Haichun Ma; mahc@jlu.edu.cn

Accepted 26 November 2019
Published Online First 23 December 2019

© American Federation for Medical Research 2020. Re-use permitted under CC BY-NC. No commercial re-use. Published by BMJ.

To cite: Wang B, Li Y, Wang H, *et al. J Investig Med* 2020;**68**:786–791.

ABSTRACT

FOXO3a belongs to a family of transcription factors characterized by a conserved forkhead box DNA-binding domain. It has been known to regulate various cellular processes including cell proliferation, apoptosis and differentiation. Post-translational modifications of FOXO3a and their roles in the regulation of FOXO3a activity have been well-documented. FOXO3a can be phosphorylated, acetylated and ubiquitinated, however, the ISGylation of FOXO3a has not been reported. Protein overexpression, ISGylation and half-life were measured to determine the post-translational modification of FOXO3a. Human fibroblast cells were treated with transforming growth factor (TGF)-β1 to determine the role of FOXO3a ISGylation in TGF-β1 signaling. FOXO3a's half-life is around 3.7 hours. Inhibition of the proteasome, not lysosome, extends its half-life. ISGylation, but not ubiquitination of FOXO3a, is increased in the presence of the proteasome inhibitor. Overexpression of ISG15 increases FOXO3a degradation, while overexpression of USP18 stabilizes FOXO3a through de-ISGylation. These results suggest that FOXO3a is degraded in the ISGylation and proteasome system, which can be reversed by USP18, an ISG15-specific deubiquitinase. This study reveals a new molecular mechanism by which ISGylation regulates FOXO3a degradation. Furthermore, we show that the overexpression of FOXO3a attenuated TGF-β1-induced fibronectin expression in human lung fibroblast cells without altering Smad2/3 expression and activation. FOXO3a can be ISGylated, which can regulate FOXO3a stability. USP18/FOXO3a pathway is a potential target for treating TGF-β1-mediated fibrotic diseases such as idiopathic pulmonary fibrosis.

Significance of this study

What is already known about this subject?

- FOXO3a regulates various cellular and biological functions including cell proliferation and death.
- FOXO3a can be post-translational modified by phosphorylation, ubiquitination and acetylation.
- Post-translational modification regulates FOXO3a's stability and activity.

What are the new findings?

- FOXO3a can be ISGylated.
- ISGylation of FOXO3a determines FOXO3a half-life.
- USP18 negatively regulates FOXO3a ISGylation.
- FOXO3a overexpression attenuates transforming growth factor (TGF)-β1 signaling.

How might these results change the focus of research or clinical practice?

- This is first report to reveal a new modification of FOXO3a.
- This study will open a new window to understand molecular regulation of FOXO3a.
- Understanding the mechanism lead to develop new therapeutic strategies to treat TGF-β1-mediated fibrotic diseases.

INTRODUCTION

Forkhead box (FOX) proteins contain a DNA-binding domain and function as transcription factors. In mammals, the FOX protein family consists of A to S subgroups based on their structural similarity. FOXO3a belongs to the class O subgroup and plays important roles in regulating various gene expressions and diverse cellular and biological processes. FOXO3a is known to suppress cell proliferation and promote cell death and function as a tumor suppressor.[1–3] FOXO3a transcriptional activity upregulates the gene expression of cell cycle inhibitors (such as p27 and p21) and apoptosis-related proteins (such as Bim, FasL, TRAIL and PUMA).[4–6] FOXO3a also plays an important role in autophagy.[7 8] Recently, the role of FOXO3a in transforming growth factor (TGF)-β1-mediated signaling has been demonstrated. TGF-β1 treatment induced nuclear exclusion of FOXO3a and inhibited its transcriptional activity in mesangial cells,[9] while TGF-β1 treatment increased FOXO3a nuclear translocation in leukemia-initiating cells.[10] An increase in FOXO3 mRNA by a PKC inhibitor (UCN-01) significantly inhibited TGF-β1-induced

myofibroblast differentiation markers (Col1A1 and smooth muscle actin).[11]

Post-translational modifications of FOXO3a has been shown to determine FOXO3a activity and biological functions. Phosphorylation of FOXO3a is primarily regulated by the PI3K/Akt signaling pathway.[12–14] Activation of Akt triggers FOXO3a serine/threonine phosphorylation, thereby leading to its nuclear exclusion.[12–14] Other kinases such as JNK and MST1 have been identified to phosphorylate FOXO3a, while the kinase-induced phosphorylation promotes its nuclear translocation.[15 16] Acetylation of FOXO3a is mediated by CBP/p300, which attenuates FOXO3a transcriptional activity,[17 18] while the effect can be reversed by the deacetylation of FOXO3a by SIRT1.[19] Both phosphorylation and acetylation of FOXO3a trigger FOXO3a degradation.[20] Ubiquitination of FOXO3a is believed to increase its degradation in the proteasome.[21 22]

Similar to ubiquitination, ISGylation is important for protein localization and turn over. Interferon-stimulated gene 15 (ISG15) is an ubiquitin-like protein and it can covalently conjugate to target proteins to induce ISGylation.[23 24] Even several ubiquitin E3 ligases have been identified to catalyze ISGylation, USP18 is the major enzyme known for de-ISGylation.[25] The role of ISGylation in protein stability has been studied, while the effect of ISGylation on protein degradation in the proteasome is controversial. Huang and Bulavin reported that ISGylation increased p53 proteasomal degradation,[26] while others believed that ISGylation negatively regulates the ubiquitin-proteasome system.[24] Here, we found that FOXO3a can be ISGylated and ISG15 facilitates FOXO3a degradation, the effects are reversed by the overexpression of USP18. Furthermore, we show that the stabilization of FOXO3a attenuates TGF-β1-induced fibronectin expression in human lung fibroblast cells. This study indicates that the ISGylation of FOXO3a may play an important role in TGF-β1-mediated extracellular matrix accumulation in fibroblast cells.

MATERIALS AND METHODS

Cell culture and reagents

Human bronchial epithelial cell line (HBE2) was cultured with HITES medium containing 10% fetal bovine serum (FBS). HEK293 cells were cultured with Dulbecco's Modified Eagle Medium containing 10% FBS. Human lung fibroblast cells (Mrc5) were cultured with Eagle's Minimum Essential Medium containing 10% FBS. V5 antibody, pcDNA3.1/His V5 TOPO plasmid and *Escherichia coli* top 10 competent cells were from Invitrogen (Carlsbad, California, USA). MG-132 was from Calbiochem (La Jolla, California, USA). Leupeptin, cycloheximide and β-actin antibody were from Sigma-Aldrich (St. Louis, Missouri, USA). Immobilized protein A/G beads, ubiquitin antibody and ISG15 antibody were from Santa Cruz Biotechnology (Santa Cruz, California, USA). Smad2/3, p-Smad2/3, fibronectin, smooth muscle actin and FOXO3a antibodies were from Cell Signaling Technology (Danvers, Massachusetts, USA). All materials used in the experiments were in the highest grades commercially available.

In vivo ubiquitination and ISGylation assay

This is a modified IP protocol under denaturing conditions. Cells were washed and collected with cold phosphate-buffered saline (PBS). After centrifuging at 1000 rpm for 5 min, 50–80 µL of 2% sodium dodecyl sulfate (SDS) lysis buffer containing 1 µg/mL ubiquitin aldehyde and 5 mM N-ethylmaleimide (NEM) were added to cell pellets and then sonicated on ice for 12 s followed by boiling at 100°C for 10 min. The denatured samples were diluted with PBS. Equal amounts of cell lysates (1 mg) were incubated with a FOXO3a antibody overnight at 4°C, followed by the addition of 40 µL of protein A/G agarose and incubation for an additional 2 hours at 4°C. The immunoprecipitated complex was washed three times with cold PBS and analyzed by immunoblotting with antibodies against ubiquitin and ISG15.

Immunoblotting

Cells were washed with cold PBS and collected in the cell lysis buffer containing 20 mM Tris·HCl (pH 7.4), 150 mM NaCl, 2 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid), 5 mM β-glycerophosphate, 1 mM $MgCl_2$, 1% Triton X-100, 1 mM sodium orthovanadate, 10 µg/mL protease inhibitors, 1 µg/mL aprotinin, 1 µg/mL leupeptin and 1 µg/mL pepstatin. An equal amount of cell lysates (20 µg) was subjected to SDS-PAGE without boiling, electrotransferred to membranes and immunoblotted following standard protocol.

Transfection of plasmids

Human FOXO3a and USPs cDNA were inserted into pcDNA3.1/V5-His TOPO expression vectors (Invitrogen). Human ISG15 cDNA with HA tag was inserted into pcDNA3.1 TOPO expression vector. HBE2 or Mrc5 cells were grown on 6-well plates or D100 dishes to 60%–70% confluence. The cells were then transfected with varying amounts of plasmid using the Polyjet In vitro DNA Transfection Reagent (SignaGen Laboratories) system based on the manufacturer's protocol.

Statistical analysis

All results were subjected to statistical analysis using one-way analysis of variance. The data are presented as mean±SD. Data were collected from at least three independent experiments, and $p<0.05$ was considered to be statistically significant (*$p<0.05$; **$p<0.01$).

RESULTS

Determination of FOXO3a half-life

FOXO3a plays an important role in various cellular processes by regulating gene expression related to cell death and proliferation. Several studies have focused on investigating FOXO3a stability, while the half-life of endogenous FOXO3a has not been well-studied. We examined FOXO3a's half-life in HBE2 and HEK293 cells in the presence of cycloheximide (CHX), a protein synthesis inhibitor. As shown in figure 1A and B, the half-life of endogenous FOXO3a is around 3.6–3.8 hours in both cell types, indicating that FOXO3a is a not stable protein and its protein level may be regulated by protein turnover.

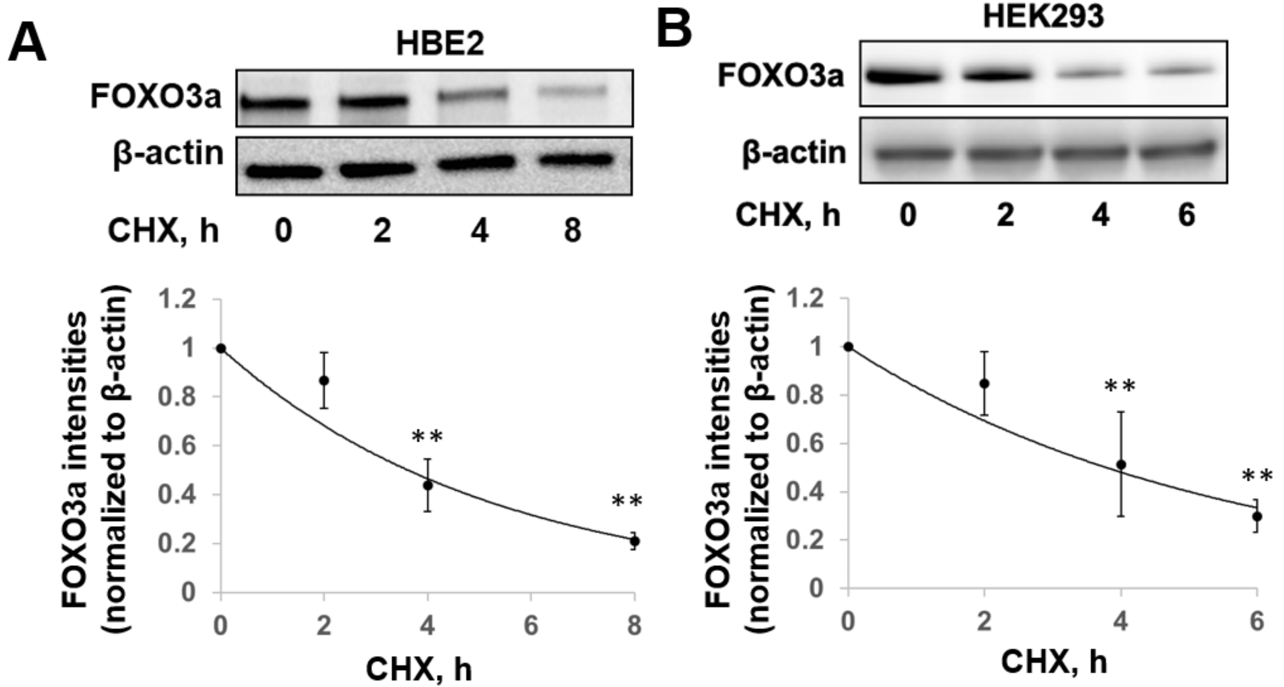

Figure 1 FOXO3a is degraded in the presence of cycloheximide (CHX). HBE2 cells (A) and HEK293 cells (B) were treated with CHX (20 µg/mL) for the indicated incubation times. Cell lysates were analyzed by immunoblotting with FOXO3a and β-actin antibodies. FOXO3a levels from three independent experiments were quantified with ImageJ software. **P<0.01 compared with 0 hour.

Foxo3A degradation is mediated by ISGylation and the proteasome system

Intracellular protein degradation is mostly mediated by the proteasome or the lysosome system. To investigate the pathways that control FOXO3a degradation, HBE2 cells were treated with a proteasome inhibitor (MG-132) or a lysosome inhibitor (leupeptin) for 1 hour prior to incubation with CHX. As shown in figure 2A, MG-132, dramatically extended FOXO3a half-life, compared with CHX alone and CHX+leupeptin treatments, indicating that FOXO3a is degraded in the proteasome. This is consistent with the conclusion by others.[6 27] First, we attempted to determine if

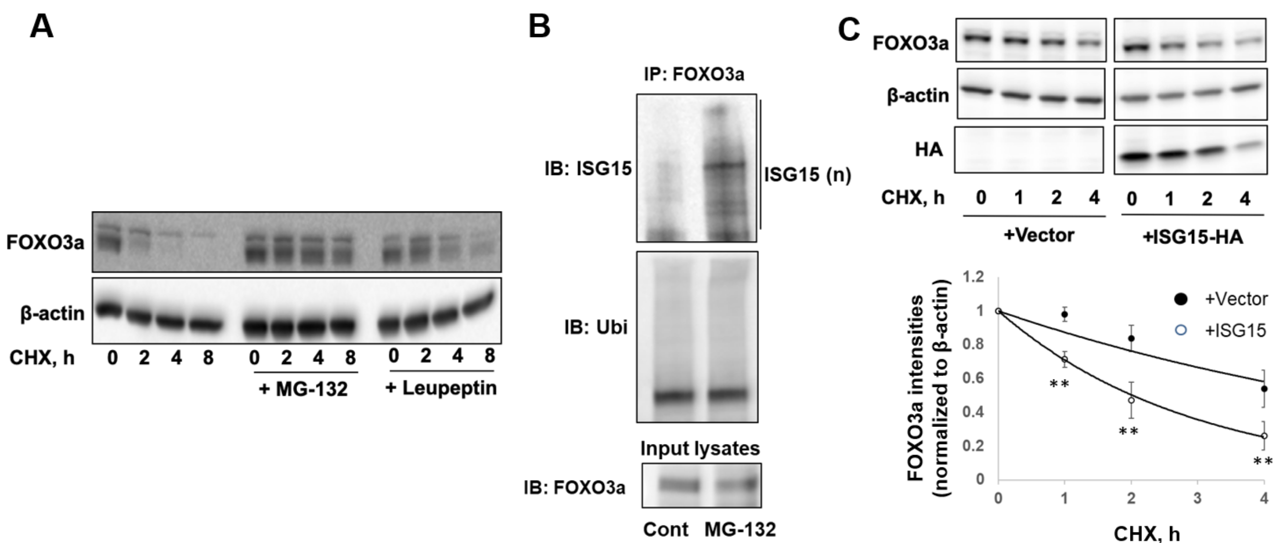

Figure 2 ISGylation of FOXO3a increases its degradation in the proteasome system. (A) HBE2 cells were treated with or without MG-132 or leupeptin (1 hour) prior to cycloheximide (CHX) incubation. Cell lysates were analyzed by immunoblotting with FOXO3a and β-actin antibodies. (B) HBE2 cells were treated with or without MG-132 for 4 hours, and then the denatured cell lysates were subjected to immunoprecipitation with a FOXO3a antibody, followed by immunoblotting with ISG15 and ubiquitin (Ubi) antibodies. (C) HBE2 cells were transfected with or without ISG15-HA plasmid for 24 hours prior to CHX incubation. Cell lysates were analyzed by immunoblotting with FOXO3a, HA tag and β-actin antibodies. FOXO3a levels from three independent experiments were quantified with ImageJ software. **P<0.01 compared with vector-transfected cells at the same incubation time points.

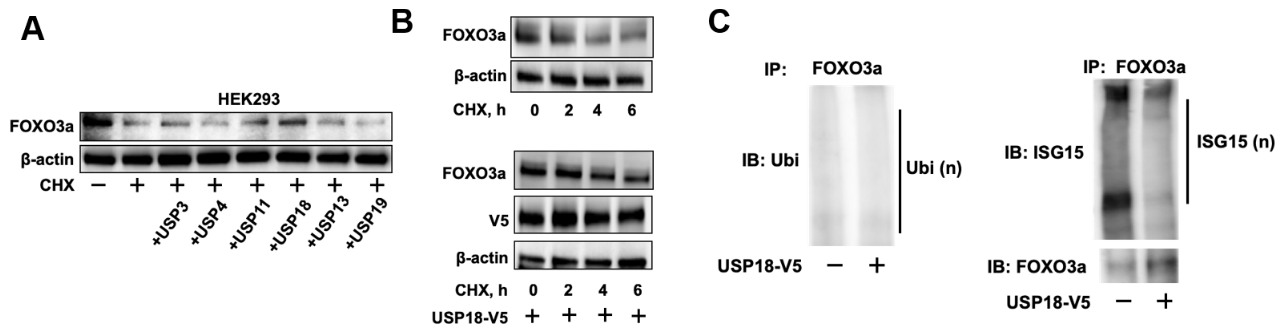

Figure 3 USP18 de-ISGylates and increases FOXO3a stability. (A) HEK293 cells were transfected with plasmids encoding different deubiquitinases as indicated for 48 hours, and then cells were treated with cycloheximide (CHX) for 4 hours. Cell lysates were analyzed by immunoblotting with FOXO3a and β-actin antibodies. (B) HBE2 cells were transfected with or without a plasmid encoding V5-tagged USP18 (USP18-V5) for 48 hours, and then cells were treated with CHX for 0, 2, 4 and 6 hours. Cell lysates were analyzed by immunoblotting with FOXO3a, V5 and β-actin antibodies. (C) HBE2 cells were transfected with a plasmid encoding USP18-V5, and then denatured cell lysates were subjected to immunoprecipitation with a FOXO3a antibody, followed by immunoblotting with ISG15 and ubiquitin (Ubi) antibodies.

FOXO3a is ubiquitinated in HBE2 cells. FOXO3a is immunoprecipitated in a denatured condition after MG-132 treatment, and then the ubiquitination of FOXO3a was examined by the immunoblotting analysis of the immunoprecipitated complex with an antibody against ubiquitin. Surprisingly, ubiquitination of FOXO3a levels was low in either control or MG-132-treated cells. However, the immunoblotting analysis of the immunoprecipitated complex with an antibody for ISG15 revealed that MG-132 increased ISGylation of FOXO3a, indicating that FOXO3a can be ISGylated in HBE2 cells. To further investigate if the ISGylation triggers FOXO3a degradation, we examined the half-life of FOXO3a in ISG15-HA-overexpressed cells. As shown in figure 2C, overexpression of ISG15-HA significantly shortened the half-life of FOXO3a. This is the first report to show that FOXO3a is ISGylated and that ISGylation increases its degradation.

USP18 de-ISGylates FOXO3a and increases its half-life

USP18 is an ISG15-specific isopeptidase. To investigate if USP18 reverses FOXO3a ISGylation, first, we compared the effects of USP18 on FOXO3a stability with other deubiquitinases. HEK293 cells were transfected with plasmids encoding USP3, USP4, USP11, USP18, USP13 and USP19 individually, and then cells were treated with CHX. After 4 hours, FOXO3a levels were reduced in all cells except USP18-overexpressed and USP11-overexpressed cells (figure 3A). Since USP18 is a specific DUB for ISGylation, next, we examined USP18's effect on FOXO3a's half-life. HBE2 cells were transfected with an empty vector and USP18-V5, and then cells were treated with CHX. USP18 overexpression significantly increased the half-life of FOXO3a (figure 3B). Furthermore, the ubiquitination and ISGylation assay were performed and the data show that USP18 diminished the ISGylation of FOXO3a. The ubiquitination in either condition is in the low levels (figure 3C).

Overexpression of FOXO3a attenuates TGF-β1-induced fibronectin expression

FOXO3a is implicated in fibrotic diseases such as pulmonary fibrosis. Its levels are decreased in the lungs of patients with idiopathic pulmonary fibrosis (IPF).[11] Al-Tamari *et al* demonstrated that increases in FOXO3a expression by UCN-01 inhibited TGF-β1-induced collagen 1a and smooth muscle actin expression in human lung fibroblast cells.[11] However, they did not directly test the effect of FOXO3a on TGF-β1-mediated signaling. We overexpressed FOXO3a in human lung fibroblast cells (Mrc5), and then examined the effect of the overexpression of FOXO3a in TGF-β1-induced fibronectin expression. As shown in figure 4, TGF-β1 treatment significantly increased fibronectin expression, while the effect was attenuated in FOXO3a-overexpressed cells. The FOXO3a levels were not altered by TGF-β1 treatment.

Overexpression of FOXO3a has no effect on Smad2/3 expression and activation

Our current study indicates that FOXO3a regulates TGF-β1-induced signaling. FOXO3a is a transcriptional factor, however, its role in Smad2/3 expression and activation has not been revealed. Smad2/3 are key players in TGF-β1-induced fibroblast differentiation and extracellular matrix accumulation.[28] To investigate if FOXO3a impacts Smad2/3 expression and activation, Mrc5 cells were transfected with FOXO3a plasmids for 2 days, followed by TGF-β1 treatment for 30 min. TGF-β1 dramatically increased phosphorylation of Smad2/3, while overexpression of FOXO3a had no effect on Smad2/3 expression and phosphorylation, suggesting that the effect of FOXO3a on fibronectin expression is not through regulation of Smad2/3 expression and activation. These data indicate that FOXO3a regulates non-canonical TGF-β1 signaling pathways.

DISCUSSION

FOXO3a is an important player in cellular and biological processes. It has been implicated in human disorders including cancers and lung diseases.[3 6–8] FOXO3a is decreased in the lungs of patients with IPF.[11] TGF-β1 is a driving factor in the progress of IPF. The PI3K/Akt/FOXO3a pathway plays an important role in the regulation of TGF-β1-mediated fibrotic responses, indicating a beneficial role of FOXO3a in treating IPF. The regulation of FOXO3a post-translational modification has been a

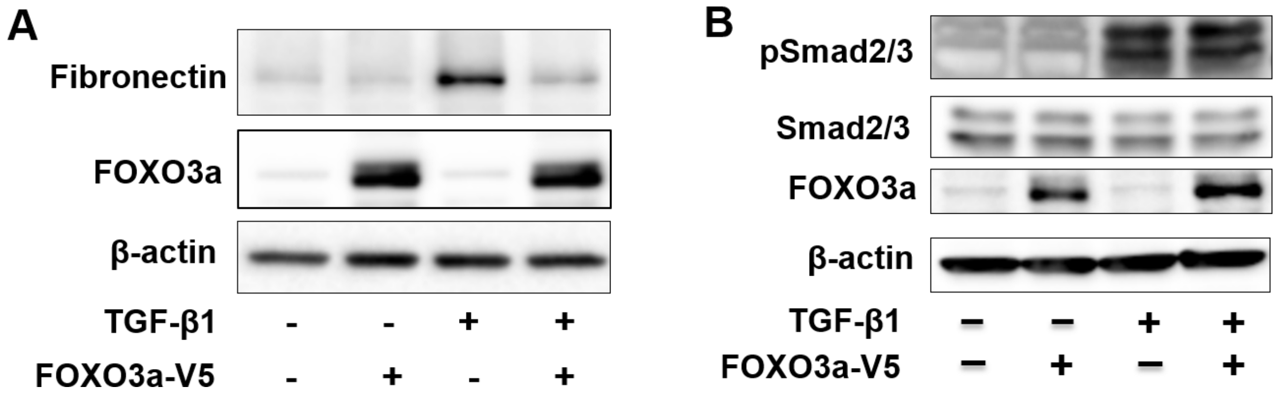

Figure 4 FOXO3a attenuates transforming growth factor (TGF)-β1-induced fibronectin expression without altering phosphorylation of Smad2/3. (A) Mrc5 cells were transfected with a plasmid encoding FOXO3a-V5 for 24 hours, and then cells were treated with TGF-β1 (10 ng/mL) for 48 hours. Cell lysates were analyzed by immunoblotting with fibronectin, FOXO3a and β-actin antibodies. (B) Mrc5 cells were transfected with a plasmid encoding FOXO3a-V5 for 24 hours, and then cells were treated with TGF-β1 (10 ng/mL) for 30 min. Cell lysates were analyzed by immunoblotting with p-Smad2/3, SMAD2/3, FOXO3a and β-actin antibodies. Shown are representative blots from three independent experiments.

research focus to better understand its role in human disorders. Phosphorylation, acetylation and ubiquitination of FOXO3a regulate FOXO3a nuclear trafficking, transcriptional activity and stability.[14 15 18 22] Here, we report that FOXO3a can be ISGylated, which is reversed by USP18. Overexpression of FOXO3a negatively regulates TGF-β1-mediated fibrotic responses through regulating non-canonical TGF-β1 signaling.

ISGylation is catalyzed by three-step cascade enzymes: E1 activating enzyme, E2 conjugating enzyme and E3 ligases. ISG15, a 17 kDa protein, covalently links to target proteins to induce ISGylation. ISGylation is a reversible process, and de-ISGylation is mediated by USP18.[24 25] More than 100 deubiquitinases have been identified to remove ubiquitin or ubiquitin chains from the target proteins, while a few have been found to target ISG15.[24] Among them, USP18 is the major de-ISGylation enzyme. Both ISG15 and USP18 are inducible and their expressions are increased in inflammatory challenges such as interferon[29] and lipopolysaccharide (LPS).[30 31] We have shown that both *ISG15* and *USP18* genes are upregulated by LPS treatment in human alveolar macrophages.[30] In this study, we demonstrate that USP18 de-ISGylates and stabilizes FOXO3a.

FOXO3a has been known to be phosphorylated and acetylated.[13 14 18] Phosphorylation and acetylation regulate FOXO3a stability, while their effects on ISGylation of FOXO3a have not been revealed. In future studies, we will focus on investigating if phosphorylation and acetylation of FOXO3a are necessary for its ISGylation and de-ISGylation. Ubiquitination of FOXO3a has been shown to regulate its stability,[22] while in this study, we show that the ubiquitination of FOXO3a is not detectable in untreated conditions in HBE2 cells. It is possible that phosphorylation and acetylation trigger FOXO3a ubiquitination and de-ubiquitination in the stimulated conditions, while ISGylation is responsible for FOXO3a turn over in the non-stimulated conditions. This study reveals a new molecular mechanism of FOXO3a degradation. Upregulation of USP18/FOXO3a may be a potential therapeutic strategy to treat fibrotic diseases such as IPF.

Acknowledgements The authors would like to thank Kangning Yao for technical assistant.

Contributors BW, YL, HW, JZ performed the experiments. YZ, ZL, HM designed the research and wrote the manuscript.

Funding This work was partly supported by the Jilin Province Science and Technology Department (#20190201061JC to HM) and the National Basic Research Program of China (2015CB943300 to ZL).

Competing interests None declared.

Patient consent for publication Not required.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement There are no data in this work.

Open access This is an open access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited, an indication of whether changes were made, and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/4.0/.

ORCID iD
Yutong Zhao http://orcid.org/0000-0001-6604-9315

REFERENCES

1 Liu Y, Ao X, Ding W, *et al*. Critical role of FOXO3a in carcinogenesis. *Mol Cancer* 2018;17:104.
2 Obsil T, Obsilova V. Structure/Function relationships underlying regulation of FoxO transcription factors. *Oncogene* 2008;27:2263–75.
3 Stefanetti RJ, Voisin S, Russell A, *et al*. Recent advances in understanding the role of FOXO3. *F1000Res* 2018;7. doi:10.12688/f1000research.15258.1
4 Stahl M, Dijkers PF, Kops GJPL, *et al*. The forkhead transcription factor FoxO regulates transcription of p27Kip1 and Bim in response to IL-2. *J Immunol* 2002;168:5024–31.
5 Modur V, Nagarajan R, Evers BM, *et al*. Foxo proteins regulate tumor necrosis factor-related apoptosis inducing ligand expression. Implications for PTEN mutation in prostate cancer. *J Biol Chem* 2002;277:47928–37.
6 Huang H, Tindall DJ. Dynamic FoxO transcription factors. *J Cell Sci* 2007;120:2479–87.
7 Warr MR, Binnewies M, Flach J, *et al*. Foxo3A directs a protective autophagy program in haematopoietic stem cells. *Nature* 2013;494:323–7.
8 Schäffner I, Minakaki G, Khan MA, *et al*. Foxo function is essential for maintenance of autophagic flux and neuronal morphogenesis in adult neurogenesis. *Neuron* 2018;99:1188–203.
9 Kato M, Yuan H, Xu Z-G, *et al*. Role of the AKT/FOXO3a pathway in TGF-beta1-mediated mesangial cell dysfunction: a novel mechanism related to diabetic kidney disease. *J Am Soc Nephrol* 2006;17:3325–35.

10 Naka K, Hoshii T, Muraguchi T, *et al*. TGF-beta-FOXO signalling maintains leukaemia-initiating cells in chronic myeloid leukaemia. *Nature* 2010;463:676–80.
11 Al-Tamari HM, Dabral S, Schmall A, *et al*. Foxo3 an important player in fibrogenesis and therapeutic target for idiopathic pulmonary fibrosis. *EMBO Mol Med* 2018;10:276–93.
12 Brunet A, Bonni A, Zigmond MJ, *et al*. Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. *Cell* 1999;96:857–68.
13 Tzivion G, Hay N. PI3K-AKT-FoxO axis in cancer and aging. *Biochim Biophys Acta* 2011;1813.
14 Tzivion G, Dobson M, Ramakrishnan G. Foxo transcription factors; regulation by Akt and 14-3-3 proteins. *Biochim Biophys Acta* 2011;1813:1938–45.
15 Wang X, Chen WR, Xing D. A pathway from JNK through decreased ERK and Akt activities for FOXO3a nuclear translocation in response to UV irradiation. *J Cell Physiol* 2012;227:1168–78.
16 Lehtinen MK, Yuan Z, Boag PR, *et al*. A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span. *Cell* 2006;125:987–1001.
17 Daitoku H, Hatta M, Matsuzaki H, *et al*. Silent information regulator 2 potentiates FOXO1-mediated transcription through its deacetylase activity. *Proc Natl Acad Sci U S A* 2004;101:10042–7.
18 van der Heide LP, Smidt MP. Regulation of FOXO activity by CBP/p300-mediated acetylation. *Trends Biochem Sci* 2005;30:81–6.
19 Ferguson D, Shao N, Heller E, *et al*. SIRT1-FOXO3a regulate cocaine actions in the nucleus accumbens. *J Neurosci* 2015;35:3100–11.
20 Wang X, Hu S, Liu L. Phosphorylation and acetylation modifications of FOXO3a: independently or synergistically? *Oncol Lett* 2017;13:2867–72.
21 Fu W, Ma Q, Chen L, *et al*. Mdm2 acts downstream of p53 as an E3 ligase to promote FOXO ubiquitination and degradation. *J Biol Chem* 2009;284:13987–4000.
22 Wang F, Chan C-H, Chen K, *et al*. Deacetylation of FOXO3 by SIRT1 or SIRT2 leads to Skp2-mediated FOXO3 ubiquitination and degradation. *Oncogene* 2012;31:1546–57.
23 Jeon YJ, Yoo HM, Chung CH. Isg15 and immune diseases. *Biochim Biophys Acta* 2010;1802:485–96.
24 Zhang D, Zhang D-E. Interferon-Stimulated gene 15 and the protein ISGylation system. *J Interferon Cytokine Res* 2011;31:119–30.
25 Malakhova OA, Kim KII, Luo J-K, *et al*. Ubp43 is a novel regulator of interferon signaling independent of its ISG15 isopeptidase activity. *Embo J* 2006;25:2358–67.
26 Huang Y-F, Bulavin DV. Oncogene-Mediated regulation of p53 ISGylation and functions. *Oncotarget* 2014;5:5808–18.
27 Plas DR, Thompson CB. Akt activation promotes degradation of tuberin and FOXO3a via the proteasome. *J Biol Chem* 2003;278:12361–6.
28 Miyazono K. Signal transduction by bone morphogenetic protein receptors: functional roles of Smad proteins. *Bone* 1999;25:91–3.
29 D'Cunha J, Knight E, Haas AL, *et al*. Immunoregulatory properties of ISG15, an interferon-induced cytokine. *Proc Natl Acad Sci U S A* 1996;93:211–5.
30 Pinilla-Vera M, Xiong Z, Zhao Y, *et al*. Full spectrum of LPS activation in alveolar macrophages of healthy volunteers by whole transcriptomic profiling. *PLoS One* 2016;11:e0159329.
31 Hamerman JA, Hayashi F, Schroeder LA, *et al*. Serpin 2A is induced in activated macrophages and conjugates to a ubiquitin homolog. *J Immunol* 2002;168:2415–23.