NADPH oxidase DUOX1 sustains TGF-β1 signalling and promotes lung fibrosis

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The data reveal a new function for DUOX1-derived H_2O_2 as a signalling amplifier of the TGF-1 pathway that causes a chronic long-term fibroblast activation, contributing thus to unrestrained and progressive fibrosis https://bit.ly/39HeEpu

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ABSTRACT Interstitial lung fibroblast activation coupled with extracellular matrix production is a pathological signature of pulmonary fibrosis, and is governed by transforming growth factor (TGF)-β1/Smad signalling. TGF-β1 and oxidative stress cooperate to drive fibrosis. Cells can produce reactive oxygen species through activation and/or induction of NADPH oxidases, such as dual oxidase (DUOX1/2). Since DUOX enzymes, as extracellular hydrogen peroxide (H_2O_2)-generating systems, are involved in extracellular matrix formation and in wound healing in different experimental models, we hypothesised that DUOX-based NADPH oxidase plays a role in the pathophysiology of pulmonary fibrosis.

Our *in vivo* data (idiopathic pulmonary fibrosis patients and mouse models of lung fibrosis) showed that the NADPH oxidase DUOX1 is induced in response to lung injury. DUOX1-deficient mice ($DUOX1^{+/-}$ and $DUOX1^{-/-}$) had an attenuated fibrotic phenotype. In addition to being highly expressed at the epithelial surface of airways, DUOX1 appears to be well expressed in the fibroblastic foci of remodelled lungs. By using primary human and mouse lung fibroblasts, we showed that TGF-β1 upregulates DUOX1 and its maturation factor DUOXA1 and that DUOX1-derived H_2O_2 promoted the duration of TGF-β1-activated Smad3 phosphorylation by preventing phospho-Smad3 degradation. Analysis of the mechanism revealed that DUOX1 inhibited the interaction between phospho-Smad3 and the ubiquitin ligase NEDD4L, preventing NEDD4L-mediated ubiquitination of phospho-Smad3 and its targeting for degradation.

These findings highlight a role for DUOX1-derived H_2O_2 in a positive feedback that amplifies the signalling output of the TGF-β1 pathway and identify DUOX1 as a new therapeutic target in pulmonary fibrosis.

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Introduction

Lung fibrosis is characterised by excessive matrix deposition leading to the destruction of lung architecture and ultimately fatal impairment of lung function [1]. Lung fibrosis is currently irreversible and refractory to treatment. It may develop secondarily to an insult (inhalation of foreign particles, radiation, chronic hypersensitivity, *etc.*) [2]. One of the hurdles to developing therapeutics is a lack of complete understanding of the molecular mechanisms underlying the pathogenesis of the disease. Persistence of activation/differentiation of the fibroblasts into myofibroblasts, which is controlled in particular through transforming growth factor (TGF)-β1 signalling, contribute to the development of fibrosis. A growing body of evidence supports the hypothesis that an oxidant–antioxidant imbalance with consequent chronic oxidative stress might drive the progression of fibrosis [3, 4]. Under some pathological conditions, reactive oxygen species (ROS) production is greatly increased, causing oxidative stress, which is responsible for severe tissue lesions. ROS and markers of oxidative stress are evident in human idiopathic pulmonary fibrosis (IPF), and levels of ROS negatively correlate with pulmonary function in IPF and may predict disease severity [5–7]. Cells can produce ROS through activation and/or induction of NADPH oxidases (NOX), which constitute a family of enzymes that are expressed in different cell locations and have specific functions [8]. The NOX family consists of seven members, five NOXs (NOX1–5) and two dual oxidases (DUOX1/2). NOX4 has been associated with IPF pathophysiology and is currently a target for development of antifibrotic molecules [9, 10]. Initially identified in the thyroid [11, 12], DUOXs are well expressed in the airway surface epithelium, where they participate in innate defence [13]. Since DUOX enzymes, as extracellular hydrogen peroxide (H_2O_2)-generating systems, are involved in extracellular matrix formation and in wound healing in different experimental models [14, 15], we hypothesised that DUOX-based NADPH oxidase plays a role in the pathophysiology of pulmonary fibrosis. For the first time, we found that DUOX1, but not DUOX2 was induced in fibroblasts in response to lung injury and that DUOX1-derived H_2O_2 promoted the duration of TGF-β1 signalling in these cells by preventing phospho-Smad3 degradation.

Material and methods

Detailed methods are available in the supplementary material.

Subjects

IPF lung samples were obtained from patients undergoing open lung biopsy or at the time of lung transplantation (n=10; median age 58 years, range 50–64 years). IPF was diagnosed according to 2011 American Thoracic Society/European Respiratory Society/Japanese Respiratory Society/Latin American Thoracic Association criteria, including histopathological features of usual interstitial pneumonia [16]. Lung samples obtained after cancer surgery, away from the tumour, were used as controls; normalcy of control lungs was verified histologically (n=10 patients; median age 62 years, range 44–81 years).

Lung tissue samples were used for quantitative reverse transcriptase (qRT)-PCR analysis of DUOX2 and DUOX1 expression. Human primary pulmonary fibroblasts were cultured cells in DMEM (Life Technologies, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Life Technologies) and antibiotics (Life Technologies) in 5% carbon dioxide at 37°C in a humidified atmosphere. The cells were used from passage 3 to passage 6.

Animals

Duox1-knockout mice have been described previously [17]. We back-crossed transgenic mice onto C57BJ/6F background for more than five generations to obtain wild-type (WT) and heterozygous littermates as suitable controls. Background C57BL/6F were from Charles River Laboratories (Saint-Germain-Nuelles, France). We housed all mice and cared for them in a pathogen-free facility at Gustave Roussy, and all animal experiments were approved by the institutional animal care and use committee at Gustave Roussy.

Genotyping of Duox1-knockout mice

Genotyping primers and protocol are available upon request.

Bleomycin treatment and irradiation procedure

Female 10–12-week-old mice (20–25 g body weight) were given bleomycin bellon (3.3 units·kg^{-1} in saline; Sanofi-Aventis, Paris, France) or saline alone *via* orotracheal instillation. At designated time points lungs were harvested for further analyses. For irradiation, the mice were irradiated as described previously [18]; the mice were immobilised through anaesthesia (2% isoflurane) and locally irradiated at the thorax using a Varian Tube NDI 226 (X-ray machine; 250 Kev, tube current 15 mA, beam filter 0.2 mm copper), with a dose rate of 1.08 Gy·min^{-1}. A single dose of 16 Gy was locally administered to the whole thorax. For bronchoalveolar lavage (BAL), the trachea was cannulated and washed once with 0.6 mL sterile PBS at

room temperature. Samples were centrifuged at 470×*g* for 5 min, and the cell-free supernatants were collected for ELISA of TGF-β1 (Legend MAX Free Active TGFβ1; BioLegend, San Diego, CA, USA) following technical instructions.

H_2O_2 production

H_2O_2 generation was measured in primary lung fibroblasts by the Amplex Red/HRP assay (Molecular Probes, Invitrogen, Eugene, OR, USA), which detects the accumulation of a fluorescent oxidised product, as described previously [19].

Statistical analysis

Data were analysed using GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA). Mann–Whitney or unpaired t-tests were applied to compare two groups individually. A Kruskal–Wallis test followed by Dunn's multiple comparison test were used to compare three or more groups. Two-way ANOVA was used to detect differences between groups over experimental condition followed by a Tukey (or Sidak) multiple comparison test. Differences were considered statistically significant at $p<0.05$. Data were expressed as mean±SEM. The assessment of normality was performed by using Kolmogorov–Smirnov and Shapiro–Wilk tests. The data are presented in supplementary figure S9.

Results

DUOX1 is highly expressed in the lungs of IPF patients and in pulmonary fibrosis mouse models

We first analysed DUOX1 and DUOX2 mRNA expression in both IPF and healthy lung tissues and fibroblasts using real-time qRT-PCR (figure 1a and b). The increase in DUOX1 mRNA expression is statistically significant in primary lung fibroblasts from IPF patients compared with that in control subjects. This increase was associated with an increase in expression of α-smooth muscle actin (SMA), a marker of myofibroblasts. An increase in DUOX1 immunohistochemical staining was observed in both active fibrotic areas and epithelial cells (figure 1c). Thus, DUOX1 but not DUOX2 is induced in response to lung injury. This finding was confirmed by using the bleomycin model of lung injury (supplementary figure S1a), in which DUOX1 mRNA expression was also specifically increased in addition to that of its maturation factor DUOXA1, which is essential for the enzymatic activity (figure 1d). This increase was related to increased mRNA expression of fibrosis markers such as collagen (Col1a1) and α-SMA (ACTA2) as well as that of the profibrotic factor TGF-β (supplementary figure S1c–e). Immunohistochemistry showed that lung fibroblasts expressed immunoreactive DUOX1 in fibrotic lungs (figure 1e). Diphenylene iodonium (DPI), an inhibitor of NADPH oxidases, inhibited bleomycin-induced NADPH-dependent H_2O_2 production (supplementary figure S1f). Furthermore, we found a specific increase in DUOX1 mRNA in radiation-induced lung fibrosis (supplementary figure S2a and b).

DUOX1 deficiency decreases bleomycin-induced lung fibrosis

To investigate the biological significance of the inducible expression of DUOX1, we examined the fibrotic response to bleomycin-induced lung injury in DUOX1-deficient mice. Both homozygous ($DUOX1^{-/-}$) and heterozygous ($DUOX1^{+/-}$) mice were used to study the fibrotic response to bleomycin injury. DUOX1-deficient mice were significantly less susceptible to bleomycin-induced lung injury, but this was not related to a statistically significant increase in survival relative to that of WT mice (supplementary figure S1g). Strikingly, these mice exhibited reduced lung fibrosis, which was correlated with the absence of an increase in H_2O_2 generation (figure 2a). Collagen accumulation was significantly reduced in the lung tissue of DUOX1-deficient mice, as determined by real-time qPCR and Sirius red staining (figure 2b–d). The attenuated fibrosis was further supported by decreased protein levels of fibronectin, as analysed by immunohistochemistry and Western blot (figure 2e and f). These results were also observed with the radiation-induced lung fibrosis model (supplementary figure S2c). These *in vivo* data indicate that DUOX1 is causally involved in driving pulmonary fibrogenesis.

DUOX1-deficient mice display decreased Smad-mediated TGF-β1 signalling in lung tissues

TGF-β1 has been widely recognised as a key fibrogenic cytokine [20]. DUOX1 deficiency leads to a significant increase in TGF-β1 in lung tissues in response to bleomycin treatment as measured by ELISA (figure 3a). Based on previous results reporting their dysregulation in the lung tissue of IPF patients [21] we analysed the levels of selected cytokines by qRT-PCR (interleukin (IL)-13, IL-6, IL-10 and CXCL15). Regardless of genotype, similar changes were observed (supplementary figure S3). Next, we analysed the inflammatory response. Both WT and DUOX1-deficient mice had comparable increases in total inflammatory cell numbers (CD45) in the lungs. Analysis of the influx of neutrophils in BAL showed that, compared with WT mice, $DUOX1^{-/-}$ mice had a significant decrease in the recruitment of neutrophils. Accordingly, the expression of elastase, which is a serine proteinase released from activated neutrophils, was impacted in the same manner (supplementary figure S4).

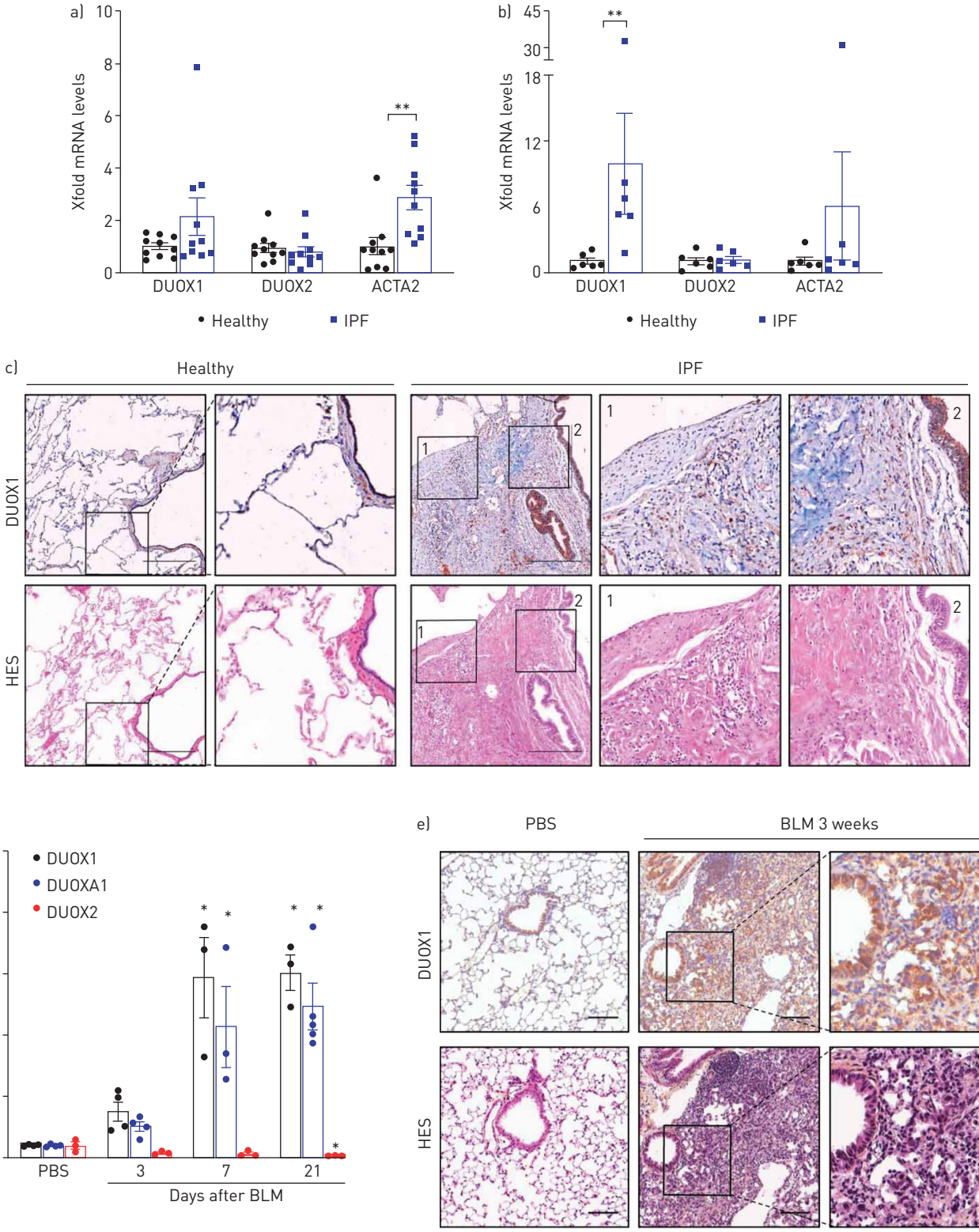

FIGURE 1 Dual oxidase (DUOX)1 is highly expressed in the lungs of idiopathic pulmonary fibrosis (IPF) patients and in pulmonary fibrosis mouse models. Comparative expression of *DUOX1*, *DUOX2* and *ACTA2* genes in a) human lung tissues (n=10 per group) and b) isolated human lung fibroblasts (n=6 per group) as analysed by quantitative reverse transcriptase (qRT)-PCR; human PPIA mRNA was used as internal control; **: p<0.01 compared with IPF (Mann–Whitney test). c) Immunohistochemistry of DUOX1 in lung sections of healthy and IPF patients. Scale bars=200 µm; field 1 and field 2 are magnified from the panel. Fibroblastic foci are shown. d), e) Wild-type mice were subjected to intratracheal instillation of bleomycin and qRT-PCR for DUOX1, DUOXA1 and DUOX2 was performed; mouse R18S mRNA was used internal control; *: p<0.05 compared with PBS (Kruskal–Wallis test, Dunn test); e) immunohistochemistry of DUOX1 in lung sections of mice at 3 weeks after bleomycin treatment (BLM); scale bars=200 µm. Data are presented as mean±SEM. HES: haematoxylin and eosin stain.

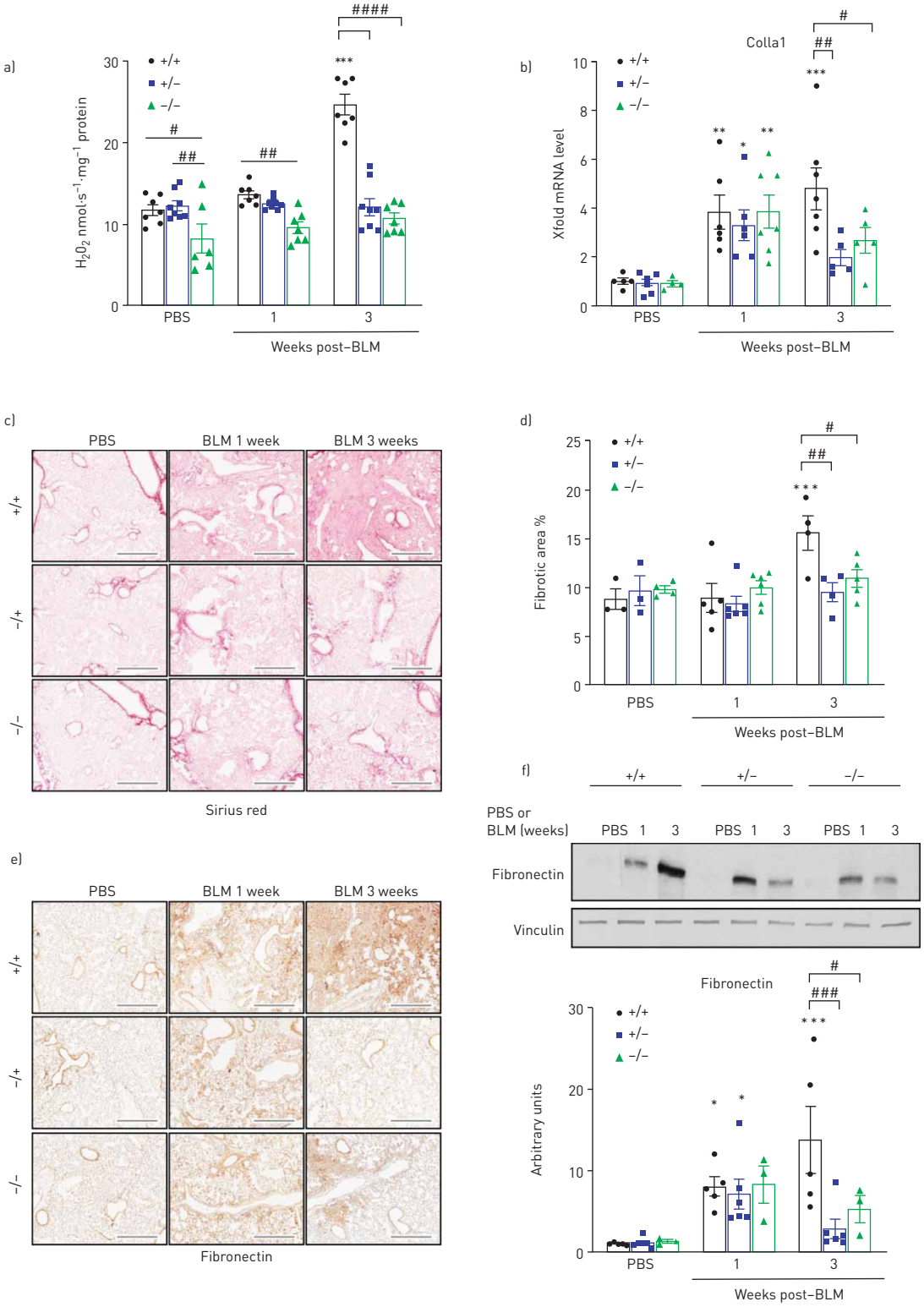

FIGURE 2 Attenuated pulmonary fibrosis in dual oxidase (DUOX)1-deficient mice. DUOX1$^{-/-}$, DUOX1$^{+/-}$ and their littermates were subjected to an intratracheal instillation of bleomycin. a) Measurement of hydrogen peroxide (H_2O_2) production in lung homogenates at the indicated time points after bleomycin (BLM) treatment (n=7–8 per group). b) Quantitative reverse transcriptase-PCR analysis of *Col1a1* mRNA expression in lung tissues of mice at the indicated time points (n=5–8 per group), mouse R18S mRNA was used internal control. c) Sirius red staining analysis of lung tissue sections from DUOX1+/+, DUOX1$^{+/-}$ and DUOX1$^{-/-}$ mice at the indicated time points. Scale bars=500 µm. d) Quantification of Sirius red staining. The fibrotic area is presented as a percentage (n=3–6 per group). e) Immunohistochemical analysis of fibronectin in lung sections. Scale bars=500 µm. Representative images of the staining are shown. f) Western blot analysis of fibronectin in lung tissue. Vinculin was used as a loading control (n=3–6 per group). For all analyses, *: p<0.05, **: p<0.01 and ***: p<0.001 compared with PBS values within genotypes; $^{\#}$: p<0.05, $^{\#\#}$: p<0.01, $^{\#\#\#}$: p<0.001 and $^{\#\#\#\#}$: p<0.0001 between genotypes (two-way ANOVA, Tukey test). Data are presented as mean±SEM.

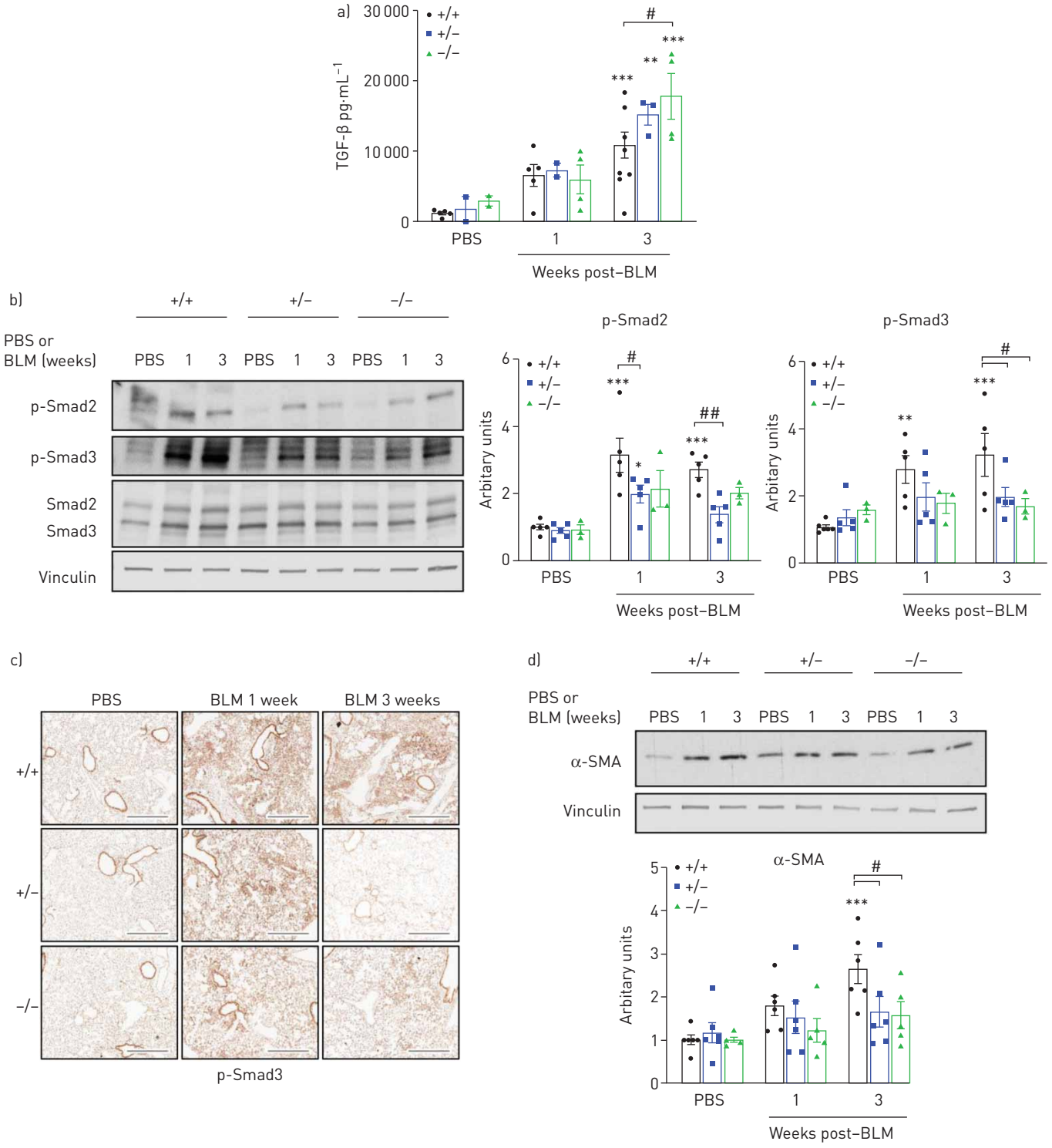

FIGURE 3 Dual oxidase (DUOX)1-deficient mice display decreased Smad phosphorylation in lung tissues. a) ELISA analysis of transforming growth factor (TGF)-β expression in lung tissues at the indicated time points after bleomycin (BLM) treatment (n=4–7 per group). b) Western blot analysis of phospho-Smad2 and phospho-Smad3 in lung tissue at the indicated time points after BLM treatment (n=3–5 per group). c) Immunohistochemical analysis of phospho-Smad3 in lung tissue at the indicated time points; scale bars=500 µm. d) Western blot analysis of α-smooth muscle actin (SMA) in lung tissue (n=5–6 per group). Vinculin was used as a loading control. For all analyses *: p<0.05, **: p<0.01 and ***: p<0.001 compared with PBS values within genotypes; #: p<0.05, ##: p<0.01 and ###: p<0.001 between genotypes (two-way ANOVA, Tukey test). Data are presented as mean±SEM.

To determine the mechanism whereby the absence of DUOX1 results in protection against lung fibrosis, we examined Smad-mediated TGF-β1 signalling in lung tissues. A time-dependent marked increase in Smad2/3 phosphorylation was observed in lung homogenates from WT bleomycin-treated mice. In

contrast, this level only slightly increased 1 week after the treatment in DUOX1-deficient mice and, compared to that of the control, remained low at 3 weeks (figure 3b). This result was confirmed by immunohistochemistry analysis (figure 3c). The same results were obtained with the radiation-induced lung fibrosis model (supplementary figure S5). The bleomycin-induced expression of α-SMA was significantly decreased in lung homogenates from DUOX1-deficient mice (figure 3d). Taken together, these data suggest that DUOX1 may regulate myofibroblast activation by modulating the TGF-β1/Smad signalling pathway.

DUOX1 is regulated by TGF-β1 in fibroblasts

We isolated lung fibroblasts from WT and DUOX1-deficient mice. The extracellular H_2O_2 production measured was related to the genotype, which reflected the level of expression of DUOX1 (figure 4a). TGF-β1 induced the expressions of both DUOX1 and DUOXA1 mRNA in a dose- and time-dependent manner in WT fibroblasts, in addition to α-SMA and collagen I mRNA expression (figure 4b, supplementary figure S6), which were otherwise inhibited by DUOX1 deficiency. Next, we analysed the cell-surface expression of DUOX1, as membrane localisation is essential to its function. Cell-surface biotinylation experiments were carried out with a nonpermeable cross-linker on WT fibroblasts. Sulfo-NHS-biotin treatment revealed that DUOX1 was increased on the cell surface after TGF-β1 stimulation (figure 4c). Collectively, these data show for the first time that TGF-β1 regulates DUOX1 expression in lung fibroblasts. Although TGF-β1 was increased in DUOX1-deficient mice, only weak activation of Smad3 was observed, suggesting a role of DUOX1 in the dynamics of phospho-Smad3 levels during a chronic continuous stimulation. We thereby performed a sequential stimulation experiment with increasing doses of TGF-β1. WT and DUOX1$^{-/-}$ lung fibroblasts were first exposed to the indicated doses of TGF-β1 for a long-duration exposure (48 h) and then washed, followed by the addition of fresh medium with TGF-β1 for short durations (1, 2 and 4 h). Smad3 phosphorylation kinetics were determined for each treatment schedule (supplementary figure S7a). Clearly, pre-treatment of the cells for 48 h with increasing doses of TGF-β1 affected the amplitude and the duration of the second TGF-β1-mediated Smad3 signal. This effect was particularly marked in DUOX1-deficient cells (figure 4d). By contrast, the basal levels of Smad2/3 were not affected. Genotype-based analysis of the time course of TGF-β1-induced phosphorylation of Smad2/3 in primary lung fibroblasts strengthened the decrease level of Smad2/3 phosphorylation in DUOX1-deficient mice. As expected, the expression of α-SMA was impacted in the same manner (figure 4e). Once activated by TGF-β1, Smad3 migrates to the nucleus. A decrease in the nuclear expression of phospho-Smad3 in DUOX1-deficient fibroblasts was shown by Western blot and immunofluorescence analyses (figure 5a and b). We performed gain-of-function experiments by transiently transfecting DUOX1$^{-/-}$ fibroblasts with pcDNA3.1-HA-DUOX1-expressing vector for 24 h. The cells were treated with or without 5 ng·mL^{-1} TGF-β1. As shown in figure 5c, DUOX1 expression promoted TGF-β1-induced Smad3 activation, which was associated with an increase in α-SMA protein. These data suggest a critical role of DUOX1 in a positive feedback mechanism that supports the Smad-dependent pathway in TGF-β1 signalling.

Isolated human lung fibroblasts showed a higher expression of α-SMA in IPF patients when stimulated with TGF-β1 in comparison to that of the control (figure 6a). DUOX1 mRNA expression was also induced by TGF-β1 (figure 6b). IPF fibroblasts had increased extracellular H_2O_2 production compared to the controls in the presence of TGF-β1 (figure 6c). Analysis of the time course of TGF-β1-induced Smad3 phosphorylation in healthy fibroblasts showed that pre-treatment of the cells with DPI or with catalase, a scavenger of H_2O_2, reduced both the amplitude and the duration of Smad3 phosphorylation (figure 6d).

DUOX1 controls the strength and duration of TGF-β1 signalling

TGF-β1 signalling can be terminated by either dephosphorylation or degradation *via* ubiquitination of phospho-Smad2/3 [22, 23]. To accurately analyse the reduction of Smad2/3 we first treated WT and DUOX1$^{-/-}$ lung fibroblasts with TGF-β1 (2 ng·mL^{-1}, 30 min) to generate a pool of phospho-Smad2/3 and then with the TGF-β receptor 1 kinase inhibitor SB431542 to prevent rephosphorylation of dephosphorylated Smad2/3 [24] (supplementary figure S7b). Analysis of the dephosphorylation of Smad2/3 showed that the absence of DUOX1 significantly promoted the reduction in p-Smad2/3 levels (figure 6e). By contrast, extracellular H_2O_2, generated from glucose oxidase-catalysed glucose oxidation, sustained the level of expression of phospho-Smad2/3 and this was inhibited by catalase, indicating that this step was H_2O_2 sensitive (supplementary figure S7c and d). An accumulation of prephosphorylated Smad3 was observed in IPF-derived fibroblasts compared to healthy fibroblasts and was associated with a slowdown of its reduction (figure 7a). Importantly, RNAi-mediated knockdown of DUOX1 in IPF-derived cells, which led to a significant decrease in extracellular H_2O_2 production, decreased the TGF-β1-dependent accumulation of phospho-Smad3 (figure 7b and c). Collectively, these data show that DUOX1-derived H_2O_2 controls the strength and duration of Smad signalling in the TGF-β1 pathway.

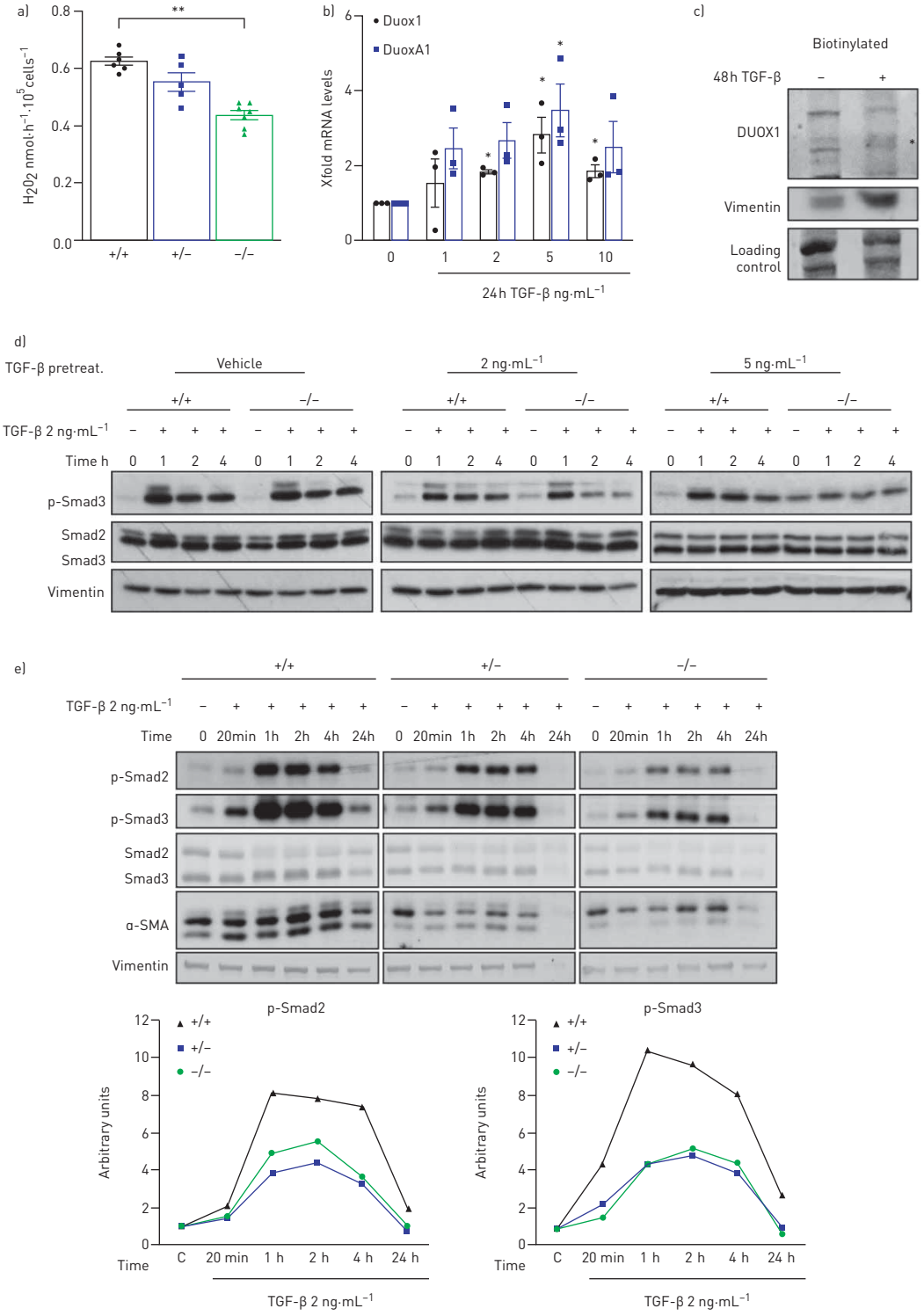

FIGURE 4 Dual oxidase (DUOX)1 expression is regulated by transforming growth factor (TGF)-β1 and impacts the dynamics of phospho-Smad3 levels in fibroblasts. a) Measurement of extracellular hydrogen peroxide (H_2O_2) production in primary lung fibroblasts isolated from mice with different genotypes (n=5–7 per group); **: p<0.01 (Kruskal–Wallis test, Dunn test). b) Comparative expression of *DUOX1* and *DUOXA1* genes in wild-type primary mouse lung fibroblasts treated with increasing doses of TGF-β1 (n=3 per group), mouse R18S mRNA was used as internal control; *: p<0.05 and **: p<0.01 compared with 0 TGF-β1 (unpaired t-test); data are presented as mean±SEM. c) Western blot analysis of cell surface DUOX1 expression in wild-type primary lung fibroblasts treated for 48 h with TGF-β1 (5 ng·mL^{-1}). Ponceau Red staining at a corresponding molecular weight area of DUOX1 protein was used as a loading control. d) Western blot analysis of phospho-Smad3 in primary lung fibroblasts isolated from DUOX1$^{+/+}$ and DUOX1$^{-/-}$ mice after sequential treatment with TGF-β1 as indicated in the figure. Vimentin was used as a loading control; results are representative of two independent experiments. e) Western blot analysis of the time course of TGF-β1-induced phosphorylation of Smad2/3 and expression of α-smooth muscle actin (SMA) in primary lung fibroblasts isolated from mice with different genotypes. Vimentin was used as a loading control; results are representative of two independent experiments.

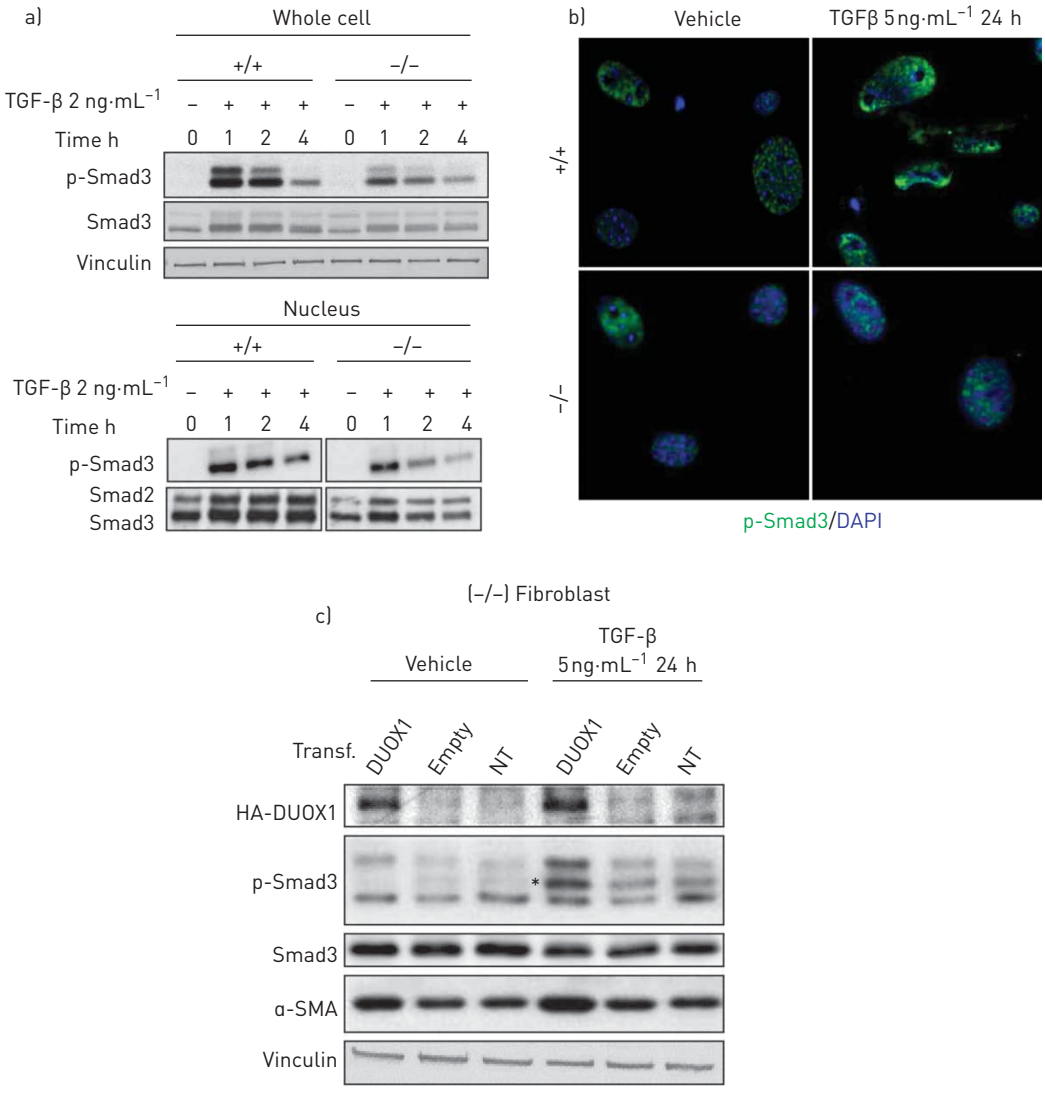

FIGURE 5 Transitory expression of dual oxidase (DUOX)1 in DUOX1-deficient fibroblasts rescues transforming growth factor (TGF)-β1-induced Smad3 activation. a) Timed induction of phospho-Smad3 in whole-cell lysates and in nuclear fractions from TGF-β1-treated fibroblasts analysed by Western blot. b) Immunofluorescence staining of phospho-Smad3 (nucleus-DAPI). c) DUOX1-deficient fibroblasts were transiently transfected with the HA-DUOX1 expression vector and treated for 24 h with TGF-β1 (2 ng·mL^{-1}). Whole-cell lysates were then collected and phospho-Smad3 and α-smooth muscle actin (SMA) expression was analysed by Western blot. Results are representative of two independent experiments.

DUOX1 inhibits the phospho-Smad3 proteasomal degradation

Next, we determined how a prolonged treatment with TGF-β1 might affect the steady-state amount of phospho-Smad3. The addition of two cell-permeable, wide-spectrum phosphatase inhibitors (sodium vanadate and okadaic acid) during the 48 h of pre-treatment with TGF-β1 had no effect on the loss of TGF-β1-mediated Smad3 phosphorylation (supplementary figure S8a). In contrast, the addition of the proteasome inhibitor MG132 blocked this effect (supplementary figure S8b). A ubiquitin-dependent degradation of TGF-β1-activated Smads has been identified previously, which restricted the amplitude and duration of TGF-β1 gene responses [25]. Since NEDD4L was identified as the principal ubiquitin ligase that selectively targets activated Smad2/3 for destruction, we evaluated the effect of its depletion on TGF-β1-induced Smad3 phosphorylation by RNAi-mediated knockdown. The knockdown of NEDD4L prevented the decline in phospho-Smad3 accumulation in DUOX1-deficient fibroblasts, suggesting that the absence of DUOX1 promotes the action of NEDD4L to constrain the signalling capacity of the TGF-β1 pathway (figure 7d). Neither genotype nor treatment with bleomycin or TGF-β1 impacted NEDD4L protein expression (supplementary figure S8c and d). To further analyse the effect of DUOX1 on phospho-Smad3 stability, we transiently co-transfected DUOX1$^{-/-}$ fibroblasts with vectors encoding

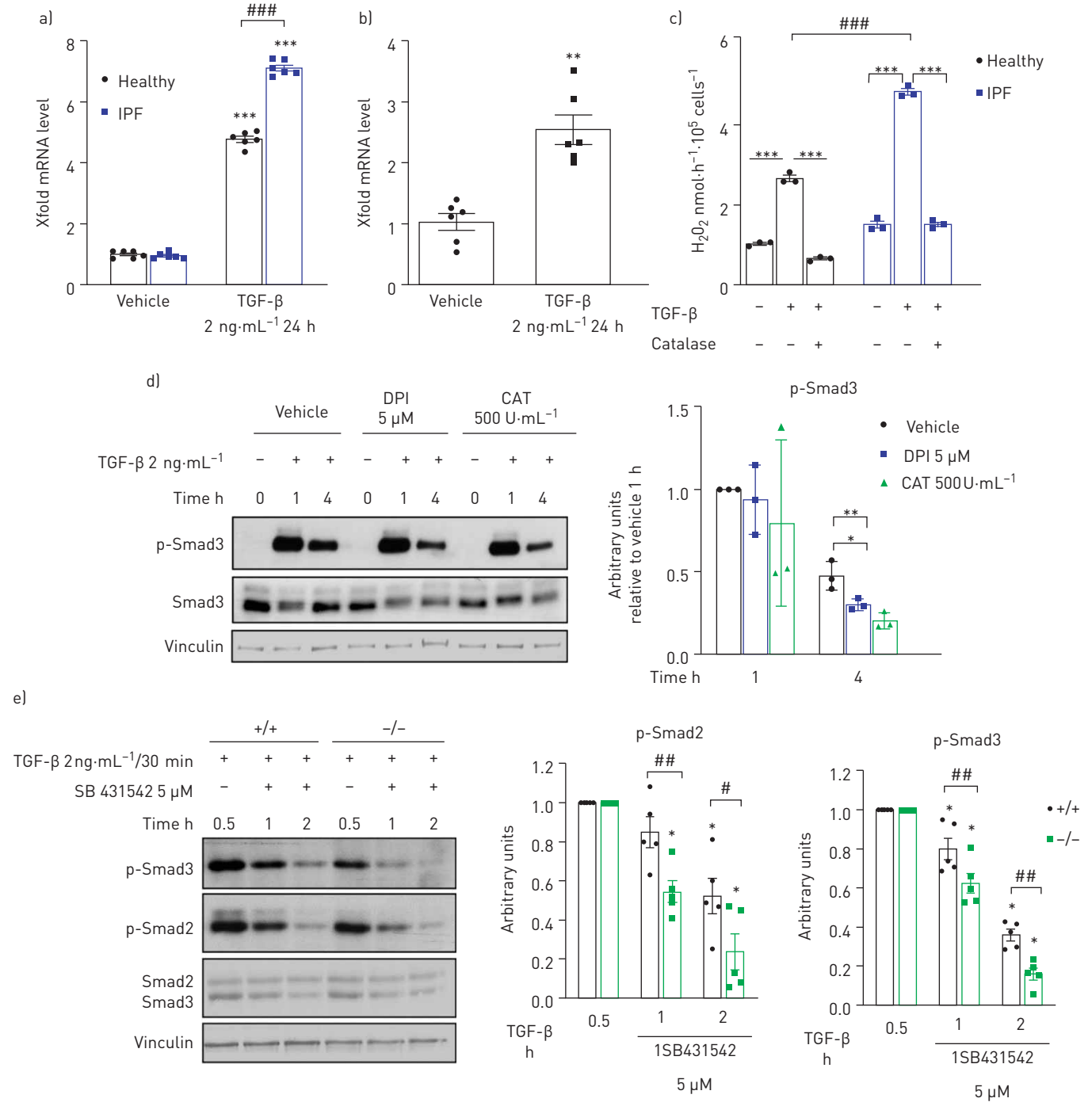

FIGURE 6 Dual oxidase (DUOX)1 is upregulated by transforming growth factor (TGF)-β1 in isolated human lung fibroblasts. a) Comparative expression of the *ACTA2* gene in healthy and idiopathic pulmonary fibrosis (IPF) fibroblasts treated with or without TGF-β1 and analysed by quantitative reverse transcriptase (qRT)-PCR (n=6 per group), human PPIA mRNA was used as internal control; ***: p<0.001 compared with vehicle values, ###: p<0.001 between groups healthy *versus* IPF (two-way ANOVA, Sidak test). b) TGF-β1 upregulated DUOX1 mRNA expression as analysed by qRT-PCR (n=6 per group); **: p<0.01 compared with vehicle values (Mann–Whitney test). c) TGF-β1-induced extracellular hydrogen peroxide (H_2O_2) production in healthy and in IPF cells (n=4 per group); ***: p<0.001 compared with TGF-β1 values, ###: p<0.001 between TGF-β1 stimulated groups (healthy *versus* IPF) (two-way ANOVA, Tukey test). d) Effect of pre-treatment (1 h) with 5 μM diphenylene iodonium (DPI) or with catalase (CAT; 500 U·mL^{-1}) on TGF-β1-induced Smad3 phosphorylation as analysed by Western blot; *: p<0.05, **: p<0.01 (unpaired t-test). e) Analysis of Smad2/3 dephosphorylation. Wild-type and DUOX1-deficient fibroblasts were treated with 2 ng·mL^{-1} TGF-β1 for 30 min, followed by washing and the addition or not of 5 μM SB431542. The graph shows the relative phospho-Smad2/3 levels (over total Smad2/3) (n=5 per group); *: p<0.05 compared with 0.5 values within genotypes, #: p<0.05 and ##: p<0.01 between genotypes (two-way ANOVA, Sidak test). Data are presented as mean±SEM.

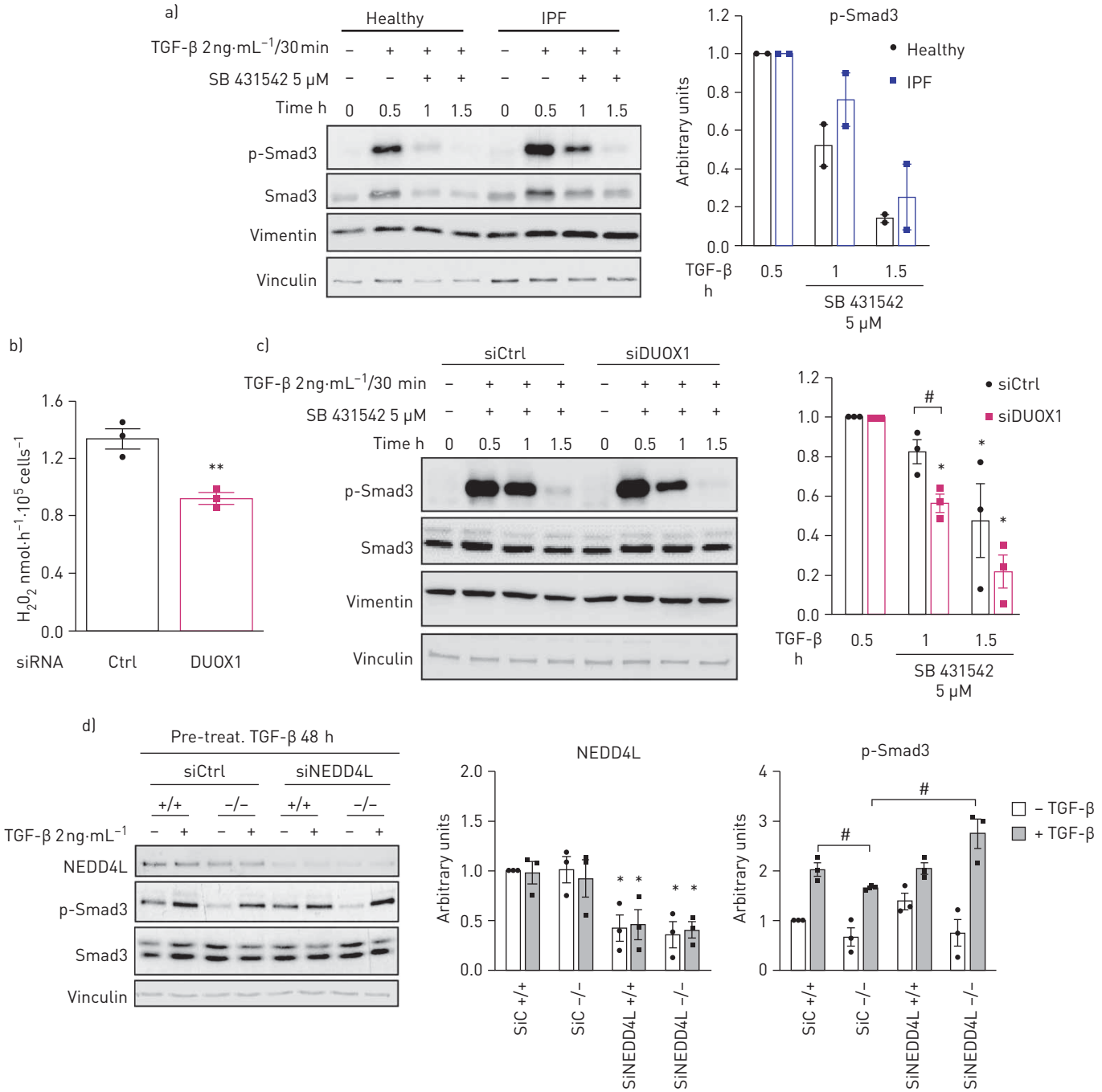

FIGURE 7 Dual oxidase (DUOX)1 deficiency impacts on the dynamics of Smad3 dephosphorylation. a) Analysis of Smad2/3 dephosphorylation in fibroblasts isolated from healthy and idiopathic pulmonary fibrosis (IPF) patients (n=2, fibroblasts from two patients). Time 0 is basal condition without transforming growth factor (TGF)-β1. b) Effect of siDUOX1 *versus* siControl on extracellular hydrogen peroxide (H_2O_2) production activity measured in primary fibroblast isolated from IPF patients (n=3); **: p<0.01 (unpaired t-test). c) Experiments equivalent to a) analysing effect of siDUOX1 *versus* siControl (Ctrl) on phospho-Smad3 dephosphorylation in fibroblasts isolated from IPF patients (n=3); *: p<0.05 compared with 0.5 values within genotypes (two-way ANOVA, Sidak test) and #: p<0.05 (unpaired t-test). d) Effect of siNEDD4L *versus* siControl on TGF-β1-induced Smad3 phosphorylation in primary mouse lung fibroblasts isolated from DUOX1$^{+/+}$ and DUOX1$^{-/-}$ mice that were pre-treated with TGF-β1 (5 ng·mL^{-1}) for 48 h before stimulation with TGF-β1 (2 ng·mL^{-1}) for 1 h; *: p<0.05 compared with its siControl value within genotype and #: p<0.05 and ##: p<0.01 between TGF-β1 treated groups (Kruskal–Wallis test, Dunn test). Data are presented as mean±SEM.

DUOX1 and epitope-tagged ubiquitin. The cells were pre-treated with TGF-β1 for 48 h. Immunoprecipitation of ubiquitin followed by immunoblotting with anti-NEDD4L and anti-phospho-Smad3 showed that DUOX1 inhibited the interaction between NEDD4L and phospho-Smad3, thus preventing its ubiquitination and therefore its degradation (figure 8a). This is modelled in figure 8b.

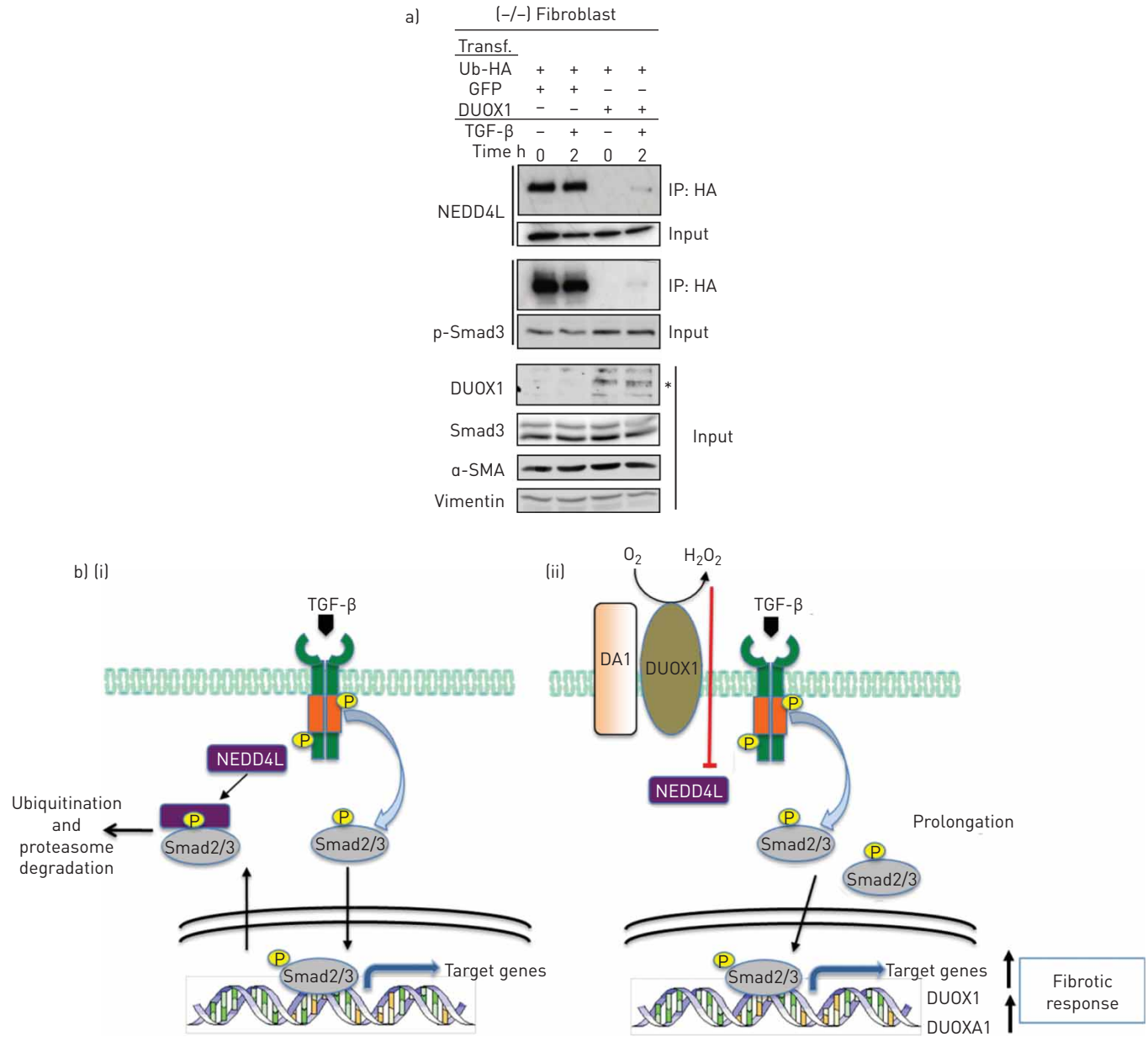

FIGURE 8 Dual oxidase (DUOX)1 interferes with the destruction of phospho-Smad3 by preventing its interaction with NEDD4L. a) DUOX1-deficient fibroblasts were co-transfected for 48 h with Ub-HA and DUOX1 expression vectors. In control experiments, a green fluorescent protein (GFP)-expression vector was used instead of DUOX1. The cells were pre-treated with transforming growth factor (TGF)-β1 (5 ng·mL^{-1}) before stimulation with TGF-β1 (2 ng·mL^{-1}) for 2 h. The HA immunoprecipitates and whole-cell lysates were immunoblotted with the indicated antibodies. b) Model proposed for the mechanism. i) In normal wound-healing, fibroblasts are transiently activated. The ubiquitin ligase NEDD4L targets phospho-Smad3 for destruction, restricting the duration of TGF-β1-gene responses; ii) in chronic long-term activation, DUOX1-derived hydrogen peroxide (H_2O_2) inhibits the interaction between NEDD4L and phospho-Smad3, leading to prolongation of the activation of the TGF-β1 pathway and then to the fibrotic response. SMA: smooth muscle actin.

Discussion

The findings reported here add new insights to our understanding of the regulation of the TGF-β1 pathway and demonstrate a novel role of DUOX1-based NADPH oxidase in regulating the duration of the Smad-dependent TGF-β1 signalling response, which plays a key role in promoting fibrosis [26]. To our knowledge, this study provides, for the first time, direct experimental evidence that DUOX1 plays a pathological role in pulmonary fibrosis. Our *in vivo* data (IPF patients and bleomycin- and radiation-induced fibrosis in mice) support a role for DUOX1-derived H_2O_2 production in the fibrotic process, particularly in myofibroblast differentiation and activation, whose deregulation has been associated with the progression of the disease.

Until now, DUOX1 expression was believed to be largely restricted to epithelial lineages, including those in the airway [27, 28]. Oxidative stress is an important molecular mechanism underlying fibrosis in the lung [5]. A role for H_2O_2-mediating pulmonary fibrosis was previously supported by the protective effect of catalase, an enzyme that breaks down H_2O_2 [29]. DUOX1 is active on the cell surface, promoting H_2O_2 release into the extracellular space [30–32]. TGF-β1-mediated myofibroblast differentiation was previously shown to be associated with an enzymatic production of extracellular H_2O_2 [33]. Our results reveal that both DUOX1 and its maturation factor DUOXA1 are upregulated by TGF-β1 at the transcriptional level in lung fibroblasts supporting a role of DUOX1-based NADPH oxidase in chronic oxidative stress.

The extent, duration and potency of signalling in response to TGF-β1 are intricately regulated by complex biochemical processes [34]. In addition to dephosphorylation by phosphatase [22], ubiquitin-mediated degradation of TGF-β1-activated Smad has been identified as a key mechanism of ensuring the irreversible termination of Smad signalling function [23]. By using TGF-β1 -induced primary lung fibroblast activation as an *in vitro* model, we found that DUOX1 promoted the duration of TGF-β1-activated Smad3 phosphorylation by preventing phospho-Smad3 degradation. NEDD4L has been identified as a principal ubiquitin ligase that selectively targets activated Smad2/3 for destruction [25]. In this study, we showed that DUOX1 negatively regulates the interaction between NEDD4L and phospho-Smad3, thus preventing NEDD4L-mediated ubiquitination of p-Smad3 and therefore its targeting for destruction. The role of extracellular H_2O_2 in this mechanism was supported by the data showing that catalase promoted phospho-Smad3 reduction in human fibroblasts while in contrast, accumulation of H_2O_2 produced by glucose oxidase increased phospho-Smad3 accumulation in DUOX1-deficient fibroblasts. This highlights a role for DUOX1-derived H_2O_2 in positive feedback that amplifies the signalling output of the TGF-β pathway *via* a redox-sensitive mechanism, which may involve cysteine oxidation. The oligomeric state of NEDD4L is essential for polyubiquitin chain assembly. In addition, this enzyme also has a cysteine in its catalytic site, which is important for its activity [35]. Both the oligomeric state of NEDD4L and its activity may be affected by H_2O_2-mediated oxidation.

H_2O_2 also acts as a chemoattractant for the immune system. In zebrafish, the tissue-scale gradient of H_2O_2 allows leukocyte recruitment to the site of injury, and DUOX knockdown results in decreased immune cell recruitment [15]. As previously observed with allergic airways [36], DUOX1$^{-/-}$ mice had decreased influx of neutrophils in BAL fluid and lung tissue after bleomycin treatment. Interestingly, recruitment of neutrophils to the BAL is an important predictor of early mortality in IPF patients [37]. Thus, DUOX1, in addition to being involved in fibroblast activation, could contribute to regulating the inflammatory response through a neutrophil recruitment to the wound.

All experiments were performed in female mice. A sex-specific effect of DUOX1 on lung fibrosis severity in males cannot be excluded.

Several studies have established a central role of another NADPH oxidase in the pathogenesis of pulmonary fibrosis. TGF-β-induced NOX4 expression was shown to be crucial for mediating ROS production that drives myofibroblast activation in IPF patients and in mice with bleomycin-induced fibrosis [9, 10]. NOX4-derived ROS have been shown to be involved in Smad2/3 phosphorylation induced by TGF-β. However, this induction was inhibited by NAC, an antioxidant, as well as by DPI, suggesting that other NOX-produced ROS are implicated in NOX4 gene expression regulation [10]. This NOX may be DUOX1, which also contributes to TGF-β-mediated upregulation of NOX4 in fibroblasts (data not shown). Thus, DUOX1 appears to be upstream of NOX4.

In normal wound healing, fibroblasts are transiently activated into myofibroblasts to proliferate and deposit the collagen matrix. In contrast, in fibrosis, chronic long-term myofibroblast activation is sustained. TGF-β1 is contextually controlled. This study identified DUOX1 as a potential therapeutic target through its role as a signalling amplifier of the TGF-β pathway, which could contribute to persistent mesenchymal activation and therefore to unrestrained and progressive fibrosis.

N-acetyl cysteine, as antioxidant, has failed to demonstrate efficacy in the clinic [38]. The use of general scavengers of ROS can cause adverse effects and unwanted toxicity because of the relevance of ROS to normal physiological functions. Thereby, effective treatment has to require maintaining a delicate balance between suppressing deleterious effects of ROS and interfering with cellular physiology. Therefore, targeting the primary enzymatic source of ROS, such as DUOX1, may have better therapeutic efficacy and reduce undesired side-effects.

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Author contributions: R.A. Louzada designed and performed experiments, conducted data/statistical analysis and wrote the manuscript; R. Corre performed the experiments and analysed the data; R. Ameziane El Hassani initiated experiments; L. Meziani supervised irradiation, BAL and flow cytometry experiments; M. Jaillet collected human samples; A. Cazes analysed IHC data, B. Crestani and E. Deutsch reviewed the work; C. Dupuy designed and supervised experiments, provided funding and wrote the manuscript.

Conflict of interest: R.A. Louzada has nothing to disclose. R. Corre has nothing to disclose. R. Ameziane El Hassani has nothing to disclose. L. Meziani has nothing to disclose. M. Jaillet has nothing to disclose. A. Cazes has nothing to disclose. B. Crestani reports personal fees for lectures from AstraZeneca, grants, personal fees for advisory board work and non-financial support for meeting attendance from Boehringer Ingelheim and Roche, personal fees for advisory board work and non-financial support for meeting attendance from BMS, personal fees for advisory board work from Sanofi, outside the submitted work. E. Deutsch reports grants and personal fees from Roche Genentech, AstraZeneca/Medimmune, Merck Serono and Boehringer, grants from Servier, BMS, MSD and Amazon AWS, personal fees from Amgen and Accuray, outside the submitted work. C. Dupuy has nothing to disclose.

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