Restoration of SIRT3 gene expression by airway delivery resolves age-associated persistent lung fibrosis in mice

Mohammad Rehan1,4, Deepali Kurundkar1, Ashish R. Kurundkar2, Naomi J. Logsdon3,1, Samuel R. Smith3, Diptiman Chanda1, Karen Bernard1, Yan Y. Sanders3, Jessy S. Deshane1, Kevin G. Dsouza1, Sunad Rangarajan3, Jaroslaw W. Zmijewski1 and Victor J. Thannickal1,4✉

Aging is a risk factor for progressive fibrotic disorders involving diverse organ systems, including the lung. Idiopathic pulmonary fibrosis, an age-associated degenerative lung disorder, is characterized by persistence of apoptosis-resistant myofibroblasts. Here we demonstrate that sirtuin 3 (SIRT3), a mitochondrial deacetylase, is downregulated in the lungs of humans with idiopathic pulmonary fibrosis and in mice subjected to lung injury. Overexpression of Sirt3 cDNA via airway delivery restored the capacity for fibrosis resolution in aged mice, in association with activation of the forkhead box transcription factor FoxO3a in fibroblasts, upregulation of pro-apoptotic members of the Bcl2 family and recovery of apoptosis susceptibility. While transforming growth factor-β1 reduced levels of SIRT3 and FOXO3A in lung fibroblasts, cell non-autonomous effects involving macrophage-secreted products were necessary for SIRT3-mediated activation of FOXO3A. Together, these findings reveal a novel role of SIRT3 in pro-resolution macrophage functions that restore susceptibility to apoptosis in fibroblasts via a FOXO3A-dependent mechanism.

Fibrotic disorders span across multiple organ systems. A consistent pathological finding in these disorders is the accumulation of activated myofibroblasts and deposition of mature extracellular matrix in association with impaired capacity for epithelial cell regeneration. In most species and across organs in humans, regeneration and fibrosis are antagonistically and inversely related. While impaired regeneration leads to fibrosis, a skewing of the tissue repair response to fibrosis may reciprocally dampen latent regenerative capacity. Aging is known to be associated with impaired regenerative capacity, and it is also an established risk factor for human fibrotic disorders. Idiopathic pulmonary fibrosis (IPF) is a progressive, age-related lung disorder that is associated with high morbidity and mortality. The incidence of IPF increases from 2.6 new cases per 100,000 people per year among individuals less than 45 years of age to 19.3 among 55–64 year olds. There is growing interest in how the biology of aging contributes to risk and progression of age-related fibrosis, including IPF.

The biology of aging has advanced in recent years and, in addition to the identification of molecular and cellular hallmarks, several genes have been linked to life span. Multiple studies have implicated two genes, sirtuin 3 (SIRT3) and the forkhead box (FOX) transcription factor FOXO3A, genes with longevity. Members of the SIRT and FOX families are evolutionarily conserved from bacteria to mammals. Sirtuins are NAD+-dependent histone deacetylases with different subcellular localizations and with broad activities towards diverse biological processes. FOXO3A regulates the transcription of genes involved in apoptosis, proliferation, oxidative stress, metabolism and differentiation.

While most published reports of putative anti-fibrotic therapeutic strategies have primarily focused on the initiation and development of lung fibrosis, studies exploring pro-regenerative or anti-fibrotic mechanisms on the resolution and reversal of lung fibrosis are relatively lacking. We propose that aging biology can be leveraged to develop novel therapeutic strategies that target cellular plasticity and fate in established fibrosis. In this study, we report that SIRT3 is downregulated in fibroblasts from individuals with IPF and following bleomycin-induced injury in the lungs of aged mice with persistent, non-resolving fibrosis; restoring SIRT3 expression in the late reparative phase reverses established lung fibrosis. Furthermore, this study reveals that the pro-resolution effect of SIRT3 is mediated by macrophage-derived paracrine signaling that activates FOXO3A in fibroblasts, upregulates pro-apoptotic BCL2 family proteins and induces apoptotic cell death essential for fibrosis resolution.

Results

SIRT3 expression is decreased in lungs of humans with IPF. We first assessed the levels of the mitochondrial sirtuin SIRT3 in the age-related lung disorder IPF. Immunohistochemical analysis showed lower SIRT3 expression in the fibroblastic foci that are enriched in α-smooth muscle actin (α-SMA)-expressing myofibroblasts (Fig. 1a). To confirm that myofibroblasts are relatively deficient in SIRT3, we performed immunofluorescence staining of lung tissues unaffected (control) and affected by IPF; in markedly remodeled areas of IPF-affected lung that express high levels of α-SMA, the expression of SIRT3 was almost absent (Fig. 1b, top panel).

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1Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, University of Alabama at Birmingham, Birmingham, AL, USA.
2Department of Pathology, University of Alabama at Birmingham, Birmingham, AL, USA.
3Division of Pulmonary Sciences and Critical Care, University of Colorado Anschutz Medical Campus, Aurora, CO, USA.
4Present address: Department of Medicine, Tulane University School of Medicine, New Orleans, LA, USA.
E-mail: vthannickal@tulane.edu
**Fig. 1** | SIRT3 is decreased in lungs of individuals with IPF.  
**a**, Representative images showing SIRT3, α-SMA and control IgG immunostaining (3,3′-diaminobenzidine (DAB)) of lung sections from non-IPF controls or individuals with IPF. The dashed lines indicate regions that are enlarged and displayed on the right side. Scale bars, 400 µm (×4 images), 100 µm (×20 images) and 40 µm (×40 images).  
**b**, Patterns of SIRT3 and α-SMA immunofluorescence in lung sections from controls and patients with IPF. The areas in the dashed boxes are magnified to display merged regions. SIRT3, green; α-SMA, red; nuclei, blue. Scale bars, 50 µm.  
**c**, Scattergrams indicating fluorescence intensity and Pearson’s correlation (r) for the images depicted in **b**.  
**d**, Quantitative analysis of SIRT3/nuclei and α-SMA/nuclei fluorescence intensity ratios from lung sections of controls and individuals with IPF; n = 9 representing 3 independent individuals, each with analysis of 3 random fields. The data are presented as means ± s.e.m; *P = 0.0482, **P = 0.0020 (unpaired t-test, two-tailed).  
**e**, Western blots (left) and quantitative analysis (right) of SIRT3 in fibroblasts from controls and individuals with IPF (n = 5 per group; e) and fibroblasts from individuals with stable and progressive IPF (n = 7 per group; f). The data are presented as means ± s.e.m; *P = 0.0368, **P = 0.0020 (unpaired t-test, two-tailed).
A higher magnification shows that α-SMA is normally expressed in a small blood vessel that also expresses SIRT3; in contrast, myofibroblasts from individuals with IPF do not show an appreciable level of expression of SIRT3 (Fig. 1b, bottom panel). Quantitative analysis of fluorescence intensity and Pearson's correlation (r) revealed a lower correlation of SIRT3 and α-SMA in tissues from individuals with IPF, in comparison to the control (r = 0.1 versus r = 0.4, respectively; Fig. 1c). Quantitative analysis of immunofluorescence images from control lung tissues and lung tissues from individuals with IPF further supports a markedly reduced expression level of SIRT3 in the latter, while α-SMA is significantly increased (Fig. 1d). Next, we examined the SIRT3 protein expression in isolated fibroblasts from lung explants of humans with IPF and controls. SIRT3 expression was found to be significantly downregulated in fibroblasts from individuals with IPF, as compared to control fibroblasts (Fig. 1e). A similar decrease in the expression of SIRT3 was observed in alveolar mesenchymal cells isolated from lungs of a cohort of patients with IPF with progressive (defined as ≥10% decline in forced vital capacity over the preceding 6 months) and stable (defined as <5% decline in forced vital capacity over the preceding 6 months) lung function (Fig. 1f). The specificity of the SIRT3 antibody used in western blots was confirmed by both overexpression and silencing approaches (Extended Data Fig. 1), and the specificity of the same antibody for immunohistochemical and immunofluorescence studies has previously been reported. These data collectively indicate that SIRT3 expression is reduced in individuals with IPF, and that its deficiency may identify a subset of individuals with more progressive disease.

SIRT3 expression is downregulated with fibrogenic lung injury and its restitution during the repair phase restores capacity for fibrosis resolution. On the basis of our findings that SIRT3 is decreased in the lung tissues of humans with IPF, we examined the levels of SIRT3 expression in the lungs of mice subjected to fibrogenic injury. SIRT3 was downregulated in a time-dependent manner, at day 7 and 14, in whole-lung homogenates following intratracheal administration of a fibrogenic chemotherapeutic agent, bleomycin (Fig. 2a). As young mice are known to resolve fibrosis at later time points, we determined whether the levels of SIRT3 recover during the late resolution phase. While SIRT3 levels are significantly upregulated at 8 weeks in comparison to the 3-week time point in young mice, SIRT3 expression fails to recover at this delayed time point in aged mice (Fig. 2b). These data indicate that in aged mice that have diminished capacity for fibrosis resolution, levels of SIRT3 in the lung fail to recover.

Next, we determined whether in vivo restitution of SIRT3 in aged mice facilitates resolution of age-associated lung fibrosis. Aged mice with bleomycin-induced lung injury were treated with a plasmid overexpressing Sirt3 cDNA or a control (empty) plasmid by oropharyngeal delivery every other day from week 3 to 6 (Fig. 2c and Extended Data Fig. 2a). We analyzed cellular expression of SIRT3 in both bronchoalveolar lavage (BAL) cells and in cells sorted by fluorescence-activated cell sorting (FACS) from a whole-lung collagenase digest. We analyzed expression of exogenously delivered cDNA based on the presence of the HA tag spanning the Sirt3 cDNA sequence. BAL cells of mice treated with the Sirt3 cDNA plasmid had markedly increased expression of Sirt3 mRNA that was almost fully accounted for by delivery of exogenous Sirt3–HA cDNA (Extended Data Fig. 2b). This suggests that BAL cells are able to efficiently uptake/transcribe the Sirt3 cDNA plasmid by this route of delivery. To further confirm specific cell types that may preferentially uptake the plasmid, we FACS sorted a whole-lung collagenase digest of mice treated with the Sirt3 cDNA or control plasmid. As expected, we saw an increase in the recruitment of macrophages (CD45+/F4/80+/MerTK+) with bleomycin-induced injury (Extended Data Fig. 2c,d). These sorted macrophages express markedly high levels of total SIRT3 and Sirt3–HA mRNA (Extended Data Fig. 2e); in contrast, whole-lung-digest sorted alveolar epithelial cells (CD45+/EpCAM+/CD31−) did not show a similar increase (Extended Data Fig. 2f). Adherence-purified fibroblasts expressed exogenously delivered Sirt3 cDNA, albeit at much lower levels in comparison to macrophages (Extended Data Fig. 2g). Thus, lung macrophages preferentially uptake/express exogenous airway delivery of Sirt3 cDNA. Histopathological analysis of lung at 6 weeks after lung injury and 3 weeks after Sirt3 cDNA treatment revealed decreased fibrosis, as evidenced by histopathology, Masson's trichrome staining for collagen and reduced α-SMA-expressing myofibroblast accumulation in lungs (Fig. 2d). These tissue remodeling effects were associated with significantly reduced dry lung weights (Fig. 2e) and hydroxyproline content (Fig. 2f) in treated mice. Thus, restoration of SIRT3 was effective in reversing established and persistent lung fibrosis in aged mice.

SIRT3 expression is downregulated during myofibroblast differentiation. As SIRT3 is downregulated in lung tissues of individuals with IPF and in an in vivo murine model of lung fibrosis, we postulated that the pro-fibrotic cytokine transforming growth factor-β1 (TGFβ1) may contribute to the suppression of SIRT3. TGFβ1 markedly downregulated the mRNA expression of SIRT3, while not significantly affecting the expression of the other six mammalian sirtuins in human lung fibroblasts (Institute of Medical Research (IMR)-90; Affymetrix U133A chip; Fig. 3a). This suppressive effect of TGFβ1 was confirmed at both the mRNA and protein levels and was found to occur in a time-dependent manner (Fig. 3b and Extended Data Fig. 3). Furthermore, TGFβ1-induced downregulation of SIRT3 is accompanied by an upregulation of α-SMA, a marker of myofibroblast differentiation, and a relatively delayed downregulation of FOXO3A, a forkhead transcription factor that regulates oxidative stress and apoptotic responses (Fig. 3c–g and Extended Data Fig. 3).

SIRT3 is a known mitochondrial deacetylase. Subcellular fractionation revealed that SIRT3 is enriched in the mitochondrial fraction and decreases on TGFβ1 stimulation of human lung fibroblasts (Fig. 3b). Similar findings were observed by immunofluorescence staining that showed co-localization of the mitochondrial marker MitoTracker red with SIRT3 in unstimulated cells and a marked downregulation of SIRT3 in this subcellular compartment with TGFβ1 treatment (Fig. 3i). Together, these data suggest that the mitochondrial sirtuin SIRT3 is selectively downregulated during the process of myofibroblast differentiation. The anti-fibrotic effect of SIRT3 overexpression is associated with induced susceptibility to myofibroblast apoptosis. Apoptosis of myofibroblasts is a hallmark of fibrosis resolution.34. To determine whether the anti-fibrotic effect of SIRT3 restitution in aged mice is associated with recovery of apoptosis susceptibility in myofibroblasts, we performed terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; green) and co-staining for α-SMA (a marker of myofibroblasts; red) on lung sections of mice treated with SIRT3 or control plasmid. A higher number of apoptotic myofibroblasts was observed in lungs of mice treated with Sirt3 cDNA (Fig. 4a,b).

As FOXO3A, along with SIRT3, was also downregulated during TGFβ1-induced myofibroblast differentiation (Fig. 3d), we assessed whether in vivo SIRT3 restitution leads to recovery of FOXO3A expression. SIRT3-mediated deacetylation of FOXO3A mediates its translocation to the nucleus and transcriptional activation. Analysis of the nuclear and cytoplasmic fractions from lung fibroblasts of mice treated with the Sirt3 cDNA plasmid showed significantly enhanced presence of FoxO3a in the nuclear fraction, consistent with cytoplasmic-to-nuclear shuttling of FoxO3a (Fig. 4c,d).

To determine whether the SIRT3–FoxO3a axis regulates fibroblast apoptosis, we independently overexpressed each of these factors in isolated murine lung fibroblasts and assessed apoptosis.
susceptibility. Fibroblasts overexpressing SIRT3 demonstrated a higher propensity to undergo apoptosis, as evidenced by increased expression of activated caspase 3 and cleaved PARP; this effect was associated with the upregulation of the pro-apoptotic Bcl2 family proteins Bim, Bad and Bid, while expression of the anti-apoptotic Bcl2 protein was unchanged (Fig. 5a). Similarly, overexpression of the FoxO3a transcription factor induced fibroblast apoptosis, without affecting Bcl2 expression but inducing expression of Bim and Bad; however, unlike SIRT3 overexpression, Bid levels did not show a statistically significant induction with FoxO3a overexpression (Fig. 5b). In lung fibroblasts from individuals with IPF, FOXO3A overexpression induced apoptosis, while inducing NOXA...
Fig. 3 | Mitochondrial SIRT3 is decreased in activated myofibroblasts. a, Sirtuin family gene expression patterns in human fibroblasts that were treated with or without TGFβ1 (2.5 ng ml⁻¹) for 24 h. n = 3 per group; the data are presented as means ± s.e.m.; *P = 0.0035 (unpaired t-test, two-tailed); n.d., not detected. b, SIRT3 mRNA levels (fold change relative to the control) in human fibroblasts treated with TGFβ1 for the indicated times. n = 3 per group; the data are presented as means ± s.e.m.; *P = 0.0001 (one-way ANOVA). c, Representative western blots showing SIRT3 and α-SMA levels in human fibroblasts stimulated with TGFβ1 (2.5 ng ml⁻¹). d–g, Western blots (d) and quantitative analysis (e–g) of SIRT3, FOXO3A and α-SMA levels in human fibroblasts treated with TGFβ1 (0 or 2.5 ng ml⁻¹) for 48 h. n = 3 per group; the data are presented as means ± s.e.m.; for SIRT3 (e), *P = 0.0064 (unpaired t-test, two-tailed); for FOXO3A (f), *P = 0.0011 (unpaired t-test, two-tailed); and for α-SMA (g), *P = 0.0365 (unpaired t-test, two-tailed). h, Protein levels of SIRT3, α-SMA and COX IV in the cytoplasmic and mitochondrial fractions from human fibroblasts stimulated with TGFβ1 (0 or 2.5 ng ml⁻¹) for 24 h. i, Immunofluorescence staining of SIRT3 and mitochondria in human lung fibroblasts treated with TGFβ1 (0 or 2.5 ng ml⁻¹) for 24 h. SIRT3, green; mitochondria, MitoTracker red; nuclei, blue. Scale bars, 10 μm.
and inhibiting COL1A1 (Extended Data Fig. 4). These data suggest that SIRT3, both in vivo and in vitro, modulates apoptosis susceptibility of lung fibroblasts, and suggest a potential role of FOXO3A in mediating the effects of SIRT3. To more directly determine the requirement for FOXO3A in mediating SIRT3-induced apoptosis, we silenced FOXO3A in fibrotic fibroblasts from humans with IPF before overexpression of SIRT3. FOXO3A silencing reduced apoptosis, as evidenced by lower levels of cleaved PARP (Fig. 5c), indicating that SIRT3-mediated apoptosis is, at least partly, dependent on FOXO3A induction/activity.

**SIRT3 silencing mediates senescence and apoptosis resistance and induces pro-fibrotic genes in human lung fibroblasts.** Previous studies have linked senescence to apoptosis resistance33; we explored whether SIRT3 deficiency is sufficient to induce both senescence and apoptosis resistance of lung fibroblasts. We depleted SIRT3 in human lung fibroblasts by RNAi and assessed markers of senescence and apoptosis (Fig. 6a). SIRT3 silencing induced expression of p16, while downregulating phospho-Rb and proliferating cell nuclear antigen (PCNA; Fig. 6a–e), and cell numbers were concomitantly reduced at 72 h (Fig. 6f), consistent with cell cycle arrest

**Fig. 4 | SIRT3 in vivo overexpression sensitizes myofibroblasts to undergo apoptosis.** a. Immunofluorescence staining of murine lung tissues showing apoptotic cells by TUNEL staining (green) 6 weeks following bleomycin-induced injury; α-SMA, red; DAPI, blue. The dashed lines indicate the regions selected for the higher-magnification images in the bottom panels. Scale bars, 50 μm. b. Quantitative analysis of apoptotic cells from randomly selected regions of images of control (vector) and Sirt3 cDNA-treated mice. n = 3 mice per group; the data are presented as means ± s.e.m.; *P = 0.0002 (unpaired t-test, two-tailed). c,d. Western blot (c) and quantitative analysis (d) of FoxO3a in the cytoplasmic and nuclear fractions from lung fibroblasts isolated from aged mice without injury (n = 3) or subjected to bleomycin injury followed by treatment with empty vector or Sirt3 cDNA (n = 4 in each treated group). The data are presented as means ± s.e.m.; *P = 0.0306 (one-way ANOVA, comparing the groups indicated in the top panel of d); *P = 0.0357 (one-way ANOVA, comparing the groups indicated in the bottom panel of d).

**Fig. 5 | SIRT3 or FoxO3a overexpression promotes apoptosis in murine fibroblasts.** a. Mouse lung fibroblasts were transfected with control or Sirt3 cDNA plasmids for 48 h and whole-cell lysates were analyzed by western blotting (left) and quantitative analysis (right panels) for SIRT3, cleaved caspase 3, cleaved PARP, Bcl2, Bim, Bad, Bid, phospho-Rb (p-Rb) and total Rb (t-Rb). n = 3 per group; the data are presented as means ± s.e.m.; the P values indicate statistical significance determined using unpaired, two-tailed t-tests. b. Western blots (left) and quantitative analysis (right panels) of FoxO3a, cleaved caspase 3, cleaved PARP, Bcl2, Bim, Bad, Bid, phospho-Rb and total Rb protein levels by western blot analyses in control or FoxO3a-overexpressing mouse fibroblasts. n = 3 per group; the data are presented as means ± s.e.m.; the P values indicate statistical significance determined using unpaired, two-tailed t-tests. c. Fibroblasts from individuals with IPF were transfected with non-targeting (NT) or FOXO3A-specific siRNA before SIRT3 overexpression (OE) and analyzed by western blotting (left) and quantitative analysis (right panels) for the expression levels of FOXO3A and cleaved PARP. KD, knockdown. n = 3 per group; the data are presented as means ± s.e.m.; the P values indicate statistical significance determined using unpaired, two-tailed t-tests.
typical of cellular senescence. The pro-fibrotic markers COL1A1 and CTGF were markedly upregulated in SIRT3-silenced fibroblasts (Fig. 6g–j), while the pro-apoptotic protein BIM was downregulated (Fig. 6g, k). Staining for senescence-associated β-galactosidase confirmed induction of senescence in SIRT3-silenced fibroblasts (Fig. 6l). Fibroblasts in which SIRT3 was suppressed exhibited decreased
susceptibility to staurosporine-induced apoptosis as compared to control fibroblasts (Fig. 6m). Together, these data indicate that depletion of SIRT3 leads to a pro-senescent, anti-apoptotic and pro-fibrotic phenotype of fibroblasts.

**Fig. 6** | SIRT3 deficiency induces fibroblast senescence and resistance to apoptosis and upregulates pro-fibrotic markers. 

*a*, Human lung fibroblasts (IMR-90) were treated with non-targeting or SIRT3 siRNA, and analyzed by western blot analysis for SIRT3, p16, phospho-Rb, total Rb and PCNA levels. 

*b*–*e*, Quantitative analysis of SIRT3 (*n* = 3; *b*), p16 (*n* = 4; *c*), phospho-Rb/total Rb (*n* = 4; *d*) and PCNA (*n* = 3; *e*) from *a*. The data are presented as means ± s.e.m.; the *P* values indicate statistical significance determined using unpaired, two-tailed *t*-tests. 

*f*, Analysis of cell numbers in human fibroblasts after a 96-h treatment with non-targeting or SIRT3 siRNA. *n* = 4 per group; the data are presented as means ± s.e.m.; the *P* value indicates statistical significance determined using unpaired, two-tailed *t*-tests. 

*g*, Western blot analyses of expression levels of SIRT3, COL1A1, CTGF and BIM in human fibroblasts after treatment with non-targeting or SIRT3 siRNA. 

*h*–*k*, Quantitative analysis of SIRT3 (*h*), COL1A1 (*i*), CTGF (*j*) and BIM (*k*) levels from the experiment described in *g*. *n* = 3 per group; the data are presented as means ± s.e.m.; the *P* values indicate statistical significance determined using unpaired, two-tailed *t*-tests. 

*l*, *m*, IMR-90 fibroblasts transfected for 48 h with non-targeting or SIRT3-specific siRNA were analyzed for β-galactosidase staining (scale bars, 50 µm) (*l*) and for caspase 3 activity (*m*). *n* = 3 per group; the data are presented as means ± s.e.m.; the *P* value indicates statistical significance determined using unpaired, two-tailed *t*-tests.

**Macrophone SIRT3 induces FOXO3a activation in fibroblasts via a paracrine mechanism.** To gain additional mechanistic insights into the relationship between SIRT3 and FOXO3a, SIRT3 was overexpressed in normal and fibrotic lung fibroblasts and FOXO3a
levels/localization were analyzed. Contrary to our expectations, overexpression of SIRT3 cDNA in IMR-90 fibroblasts or fibroblasts from individuals with IPF neither enhanced total FOXO3A levels (Extended Data Fig. 5a) nor facilitated nuclear translocation (Extended Data Fig. 5b and Fig. 7a,b). Next, we overexpressed SIRT3 in TGFβ1-treated cells to determine whether this would result in recovery of FOXO3A levels downregulated by TGFβ1. However, overexpression of SIRT3 in human lung fibroblasts followed by TGFβ1 stimulation did not substantially alter levels of FOXO3A expression (Extended Data Fig. 6). These data suggested that the observed effects of SIRT3 overexpression on lung fibroblast activation of FoxO3a in vivo may occur through a non-autonomous mechanism involving cell–cell interactions.

Next, we explored whether epithelium–fibroblast cross talk may account for fibroblast FOXO3A activation in response to SIRT3 cDNA treatment. Using two different epithelial cell lines (human alveolar epithelial cell line A549; and murine alveolar epithelial cell line L2), we overexpressed SIRT3 cDNA, collected the conditioned medium and transferred it onto IMR-90 fibroblasts; this did not result in FOXO3A induction/stabilization (Extended Data Fig. 7).

Conditioned medium transfer studies using SIRT3 overexpression in two different macrophage cell lines (human macrophage cell line, THP1; mouse peritoneal macrophages, RAW264.7) showed similar results (Extended Data Fig. 8).

To more closely simulate the paracrine effects of cell–cell communication in vivo, we utilized a Transwell co-culture system in which continuous release of and stimulation by paracrine mediators may occur (Fig. 7c). SIRT3-overexpressing L2 epithelial cells failed to induce FoxO3a nuclear translation in co-cultured fibroblasts (Extended Data Fig. 9). However, SIRT3 overexpression in RAW264.7 macrophages revealed enhanced levels of FoxO3a in the nuclear fraction from fibroblasts (Fig. 7d–f), supporting FoxO3a activation by a paracrine mediator released by macrophages. As we observed an induction of pro-apoptotic markers of the Bcl2 family and execution of apoptosis in murine lung fibroblasts overexpressing FoxO3a (Fig. 5a,b), we tested whether these same pro-apoptotic effects are preserved in this macrophage–fibroblast co-culture system. In concert with FoxO3a activation/nuclear translocation, the pro-apoptotic proteins Bim and Bad, along with increased apoptosis, were induced in fibroblasts co-cultured with SIRT3-overexpressing macrophages.
activation in a cell autonomous manner. Interestingly, overexpression of SIRT3 in macrophages was able to more efficiently induce FoxO3a activation in co-cultured fibroblasts, suggestive of an additional cell non-autonomous mechanism for modulating fibroblast behavior. Together, these studies implicate a critical role for SIRT3 in modulating cellular plasticity and tissue regenerative capacity that involves macrophage–fibroblast cross talk (Fig. 8).

These studies demonstrate a role for SIRT3 in the resolution of persistent lung fibrosis associated with aging. While several reports have demonstrated putative anti-fibrotic roles for SIRT3 in multiple organ systems, including the lung, these studies primarily evaluated effects on the initiation/development of fibrosis. Our studies clearly demonstrate that restitution of SIRT3 in vivo in aged mice during the late reparative phase was sufficient to reverse the persistent fibrotic phenotype. The potential for clinical translation is bolstered by the finding that SIRT3 is downregulated in alveolar mesenchymal cells of a subgroup of humans with rapidly progressing IPF. Although the number of humans studied here is relatively small, these proof-of-concept studies demonstrating benefits of SIRT3 restitution in an animal model of non-resolving lung fibrosis should motivate future therapeutic strategies to activate this pro-regenerative, anti-fibrotic pathway in age-related, progressive fibrotic diseases.

The mechanism of how SIRT3 mediates its anti-fibrotic effect appears to be related, at least partly, to the induction/activation of FOXO3A in fibroblasts. FOXO3A is a pro-longevity gene encoding a transcription factor that regulates the expression of genes involved in cellular proliferation, metabolism, differentiation and apoptosis. SIRT3 deacetylates FOXO3A at specific lysine residues, which facilitates migration of deacetylated (activated) FOXO3A to the nucleus, promoting a transcription factor that regulates the expression of genes involved in cellular proliferation, metabolism, differentiation and apoptosis. SIRT3 deacetylates FOXO3A at specific lysine residues, which facilitates migration of deacetylated (activated) FOXO3A to the nucleus, promoting a transcription factor that regulates the expression of genes involved in cellular proliferation, metabolism, differentiation and apoptosis. SIRT3 deacetylates FOXO3A at specific lysine residues, which facilitates migration of deacetylated (activated) FOXO3A to the nucleus, promoting a transcription factor that regulates the expression of genes involved in cellular proliferation, metabolism, differentiation and apoptosis. SIRT3 deacetylates FOXO3A at specific lysine residues, which facilitates migration of deacetylated (activated) FOXO3A to the nucleus, promoting a transcription factor that regulates the expression of genes involved in cellular proliferation, metabolism, differentiation and apoptosis.

Discussion

Aging is associated with diminished regenerative capacity and clinical syndromes of persistent or progressive fibrosis that affects diverse organ systems. Animal models have traditionally failed to recapitulate this critical aspect of disease progression and have largely focused on the development of fibrosis. It is biologically plausible that the recalcitrant nature of these non-resolving fibrotic disorders may be related to the inability to ‘turn off’ a physiological repair response, in contrast to a heightened fibrotic response to the initial tissue injury. Our group has previously demonstrated that the non-resolving nature of fibrotic syndromes can, at least partially, be recapitulated by studying injury repair responses in aged mice. In this study, we investigated a new role of the anti-aging mitochondrial sirtuin, SIRT3, in promoting resolution of lung fibrosis employing an aging mouse model. We observed a marked reduction of SIRT3 expression in the lungs of humans with IPF, which was further reduced in patients with rapid progression. This finding was recapitulated in the animal model of aged mice that failed to recover SIRT3 levels during the resolution phase of lung injury, in contrast to gradual recovery in young mice with inherent resolution capacity. Exogenous restitution of SIRT3 levels in aged mice restored the capacity for fibrosis resolution in association with induced FoxO3a activation in myofibroblasts that recover susceptibility to apoptosis. In ex vivo studies, the pro-fibrotic cytokine TGFβ1, at least partially, accounted for the loss of the SIRT3–FoxO3a signaling axis in fibroblasts; however, reconstitution of SIRT3 in these cells failed to fully account for FOXO3A

![Fig. 8](image-url) Airway delivery of Sirt3 cDNA resolves age-associated persistent lung fibrosis in mice. Aged mice subjected to bleomycin-induced lung injury develop persistent, non-resolving lung fibrosis. This non-resolving phenotype of lung injury fibrosis is associated with decreased lung levels of SIRT3 and persistence of apoptosis-resistant myofibroblasts. Gene delivery of Sirt3 cDNA via the airway restores capacity for fibrosis resolution, which is associated with FoxO3A activation/translocation to the nucleus. Our studies support a cell non-autonomous mechanism by which airway macrophages uptake the Sirt3 cDNA plasmid and produce pro-resolution factor(s) that are able to activate FoxO3a in myofibroblasts and mediate anti-senescent and pro-apoptotic effects in these reparative cells.

macrophages (Fig. 7g). Although the specific mediator(s) that augment FoxO3a in fibroblasts are currently unknown, a number of potential candidates were identified by a cytokine array (Extended Data Fig. 10). Together with the finding that macrophages are the primary cells that uptake airway delivery of Sirt3 cDNA, these data support the concept that the observed therapeutic effect of SIRT3 requires macrophage–fibroblast cross talk via activation of a SIRT3–FoxO3a signaling axis that facilitates fibrosis resolution/reversal.
that the pro-resolution effect of Sirt3 gene delivery is mediated by macrophage–fibroblast cross talk. Macrophages mediate pleiotropic effects during tissue injury repair processes\(^{12,46}\). Recent studies indicate an important role of macrophages in promoting resolution\(^{17–48}\), although the mechanisms are incompletely understood. Our studies support the possibility that SIRT3 may function as a critical switch in determining the pro-fibrotic versus pro-resolution phenotype of macrophages. This age-related macrophage dysfunction may be critical not only in conferring increased susceptibility to fibrosis from noninfectious injury, but also to defective innate immunity and repair responses to infectious agents such as the novel coronaviruses that adversely affect the elderly population\(^{49}\). Together, our studies support a critical role for SIRT3 and FOXO3A in age-related stress responses and in cell–cell communication to orchestrate a pro-regenerative response to tissue injury. Therapeutic strategies to restore these integrated longevity pathways to potentially reverse organ fibrosis deserve further study.

**Methods**

**Reagents.** Porcine platelet-derived TGFβ1 was purchased from R&D Systems. Control vector and mouse- and human-specific SIRT3-overexpressing vectors were purchased from Sino Biological Inc. The sources, uses, and dilutions of the antibodies used for the study are provided in Supplementary Table 1. All kits used in this study have been listed in Supplementary Table 2.

**Human lungs.** The study protocol was approved by the local ethics committee under the University of Alabama at Birmingham Institutional Review Board. Lung sections of biopsies of individuals with and without IPF were provided by the Airway Tissue Procurement Program Facility at the University of Alabama, Birmingham, and the Pulmonary Hypertension Breakthrough Initiative. Lung sections were formalin-fixed and paraffin-embedded before being cut into 5-μm slices, as previously described\(^{11}\).

**Lung histology and immunofluorescence staining.** Immunofluorescence staining was performed as described previously\(^{11}\). Images were acquired using a Nikon A1 laser confocal microscope at the University of Alabama at Birmingham High Resolution Imaging Facility. The levels of fluorescence were quantified and displayed as two-dimensional scattergrams using Hamamatsu software (Hamamatsu Corporation). We also processed paraffin-embedded tissue sections for lung histology and immunohistochemical staining, including hematoxylin and eosin (H&E), Masson’s trichrome or α-SMA staining as previously described\(^{11}\).

**Serum samples preparation.** Human serum samples were purchased from Sino Biological Inc. catalog number CV013 or SIRT3–HA–carrying plasmid (pCMV-3-C-Sirt3-HA, Sino Biological Inc. catalog number MG50478-Cy or pCMV-3-C-Sirt3-HA, Sino Biological Inc. catalog number HG13033-Cy) was used. hFOXO3A-expressing plasmid was purchased from Addgene Inc. HA–FOXO3A-WT (catalog number 1787) was used for FOXO3A-related overexpression studies.

**TGFβ1 treatment of human fibroblasts.** At approximately 70% confluency, fibroblasts were serum starved for 24 h, followed by treatment with 2.5 mg ml\(^{-1}\) TGFβ1 in serum-free medium for the indicated time points.

**Real-time PCR and transcriptome studies.** Total RNA was isolated from lung tissues using the RNeasy Mini Kit (Qiagen) and reverse transcribed using iScript cDNA synthesis kit (catalog number 1708890; Bio-Rad) according to the manufacturer’s protocol. The real-time PCR procedure and analysis were performed as described previously\(^{11}\).

**Hydroxyproline assay.** Lung tissues were dried in an oven at 70 °C for 48 h, and then hydrolyzed in 6N HCl at 95 °C for 48 h. The hydroxyproline assay was performed using the Hydroxyproline Assay Kit according to the manufacturer’s instructions (QuickZyme Biosciences) using hydroxyproline as a standard.
precise P values are provided. The values of n are provided for each dataset with the appropriate statistical methods, representing two or more independent sets of data that showed similar results. Western blot quantification was performed using either ImageQuant TL or Image software (version 1.53a). Graphical drawings were prepared using Adobe Illustrator (version 2020).

No statistical methods were used to pre-determine sample sizes, but our sample sizes are similar to those reported in previous publications. Data distribution was assumed to be normal, but this was not formally tested. Mice of the C57BL/6 inbred strain were randomly assigned to various groups. Data collection and analysis were not performed blind to the conditions of the experiment. No data were excluded from the analyses.

Study approval. All experiments were conducted in accordance with approved protocols by the University of Alabama at Birmingham, Institutional Animal Care and Use Committee.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Microarray data have been deposited in the Gene Expression Omnibus with the accession code number GSE17518. The authors declare that the main data supporting the findings of this study are available within the article and its Supplementary Information. Source data are provided with this paper. Extra data are available from the corresponding author upon request.

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Author contributions
M.R. and V.J.T. conceived and designed the study. M.R., D.K., A.R.K., K.B., D.C., S.R.S. and N.I.L. performed experiments. M.R., D.K., A.R.K., S.R., Y.Y.S., J.S.D., K.G.D. and V.J.T. analyzed and interpreted the data. M.R., D.K., J.W.Z. and V.J.T. drafted the manuscript.

Competing interests
V.J.T. has served as a consultant for Mistral Therapeutics, Inc., Boehringer Ingelheim, United Therapeutics, Blade Therapeutics, Versant Venture and Translate Bio. All other authors have no competing interests.

Additional information
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Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s43587-021-00027-5.
Correspondence and requests for materials should be addressed to V.J.T.
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Extended Data Fig. 1 | SIRT3 antibody specifically recognizes SIRT3 protein. (a) Western blot showing overexpression of HA tagged SIRT3 in human fibroblasts transfected with control or SIRT3 cDNA plasmid. (b) Western blot showing expression levels of SIRT3 in human fibroblasts transfected with non-targeting or SIRT3 siRNA. SIRT3 and HA antibodies were used to probe SIRT3 or SIRT3-HA expression levels.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Exogenous SIRT3 cDNA is preferentially expressed in lung macrophages. (a) Schematic of experiment design. (b) RT-PCR analysis of total SIRT3 or vector derived SIRT3-HA mRNA levels in bronchoalveolar lavage (BAL) from mice subjected to bleomycin injury followed by treatment with control vector (n = 3) or SIRT3 cDNA plasmid (n = 5), as indicated in the Methods section. Data presented as means ± s.e.m., *P = 0.0357 (unpaired t-test, non-parametric, two-tailed). (c) Flow cytometric analysis of macrophages (CD45+ F4 80+ MerTK+) sorted from collagenase lung digests of naïve mice and mice injured with bleomycin followed by treatment with control vector or SIRT3 cDNA are shown. (d) Gating strategy: (i) cells were gated for FSC-A against SSC-A; (ii) doublets were excluded using FSC-H against FSC-A; (iii) singlets were gated for CD45 positive/negative population; (iv) within the CD45+ population, epithelial cells were gated as EPCAM+CD31−; and (v) within the CD45+ population, macrophages were gated as F480+MerTK+. (e–g) RT-PCR analysis of total SIRT3 or vector derived SIRT3-HA mRNA levels in FACS sorted macrophages (e) and epithelial cells (f), and adherence-purified fibroblasts (g) from whole lung collagenase digest from mice subjected to bleomycin injury followed by treatment with control vector (n = 3) or SIRT3 cDNA plasmid (n = 5). Data presented as means ± s.e.m., *P = 0.0357 (macrophages; unpaired t-test, non-parametric, two-tailed), P = 0.0052 (fibroblasts; unpaired t-test, two-tailed statistical analysis), n.s. = not significant.
Extended Data Fig. 3 | TGF-β1 downregulates SIRT3 and FoxO3a levels in human fibroblasts. Top panel, schematic diagram showing experiment design. Bottom panel, western blots demonstrating early and late downregulation of SIRT3 and FoxO3a, respectively, and upregulation of α-SMA at indicated time points in TGF-β1 treatment of human fibroblasts.
Extended Data Fig. 4 | FoxO3a overexpression in IPF fibroblasts induces Noxa and inhibits Col1a1. Western blots and quantitative analysis of FoxO3a, cleaved caspase-3, cleaved PARP, Noxa and Col1a1 protein levels in IPF fibroblasts transfected with control plasmid or FoxO3a cDNA. n = 3 per group; Data presented as means ± s.e.m., P values as indicated by unpaired t-test, two-tailed statistical analysis.
Extended Data Fig. 5 | The effects of SIRT3 overexpression on FoxO3a levels in control and IPF fibroblasts. (a) Left panel, schematic of experiment design. Right panel, western blot showing levels of SIRT3 and FoxO3a in IMR-90 fibroblasts and IPF fibroblasts overexpressing control or SIRT3 plasmid. (b) Left panel, schematic diagram of experiment design. Right panel, western blot showing SIRT3 and FoxO3a levels in cytoplasmic and nuclear extracts of human fibroblasts overexpressing SIRT3 at 24 and 48 hours after transfection.
Extended Data Fig. 6 | The effects of SIRT3 overexpression on FoxO3a recovery in TGF-β1 treated human lung fibroblasts. (a) Left panel, schematic of the experiment design. Right panel, representative western blot showing levels of HA tag, SIRT3, FoxO3a and α-SMA in human fibroblasts cells transfected with SIRT3-HA plasmid followed by treatment with TGF-β1 (2.5 ng/ml) for 48 hours. (b) Left panel, schematic diagram of the experiment design. Right panel, representative western blot analyses of HA, SIRT3, FoxO3a and α-SMA in IMR-90 cells treated with TGF-β1 for 48 hours followed by SIRT3 overexpression.
Extended Data Fig. 7 | Effects of conditioned media from SIRT3-overexpressing human and mouse lung epithelial cells on FoxO3a levels in human lung fibroblasts. (a) Schematic of the experimental design. (b) Left panel, representative western blot showing overexpression of SIRT3 in A549 cells. Right panel, western blot showing levels of FoxO3a in human fibroblasts incubated with the conditioned media from SIRT3-overexpressing A549 cells. Cond. Med. = conditioned media. (c) Left panel, western blot showing overexpression of SIRT3 in L2 mouse epithelial cells. Right panel, representative western blot showing levels of FoxO3a in IMR-90 fibroblasts incubated with conditioned media from SIRT3-overexpressing L2 cells at 48 hours. Cond. Med. = conditioned media.
**Extended Data Fig. 8 | Effects of conditioned media from SIRT3-overexpressing macrophages (THP1 or RAW264.7 cells) on FoxO3a levels in human fibroblasts.** (a) Schematic of the experimental design. (b) Left panel, representative western blot showing overexpression of SIRT3 in human macrophage line THP1 cells. Right panel, western blot showing expression of FoxO3a in human fibroblasts incubated with conditioned media of SIRT3 overexpressing THP1 cells. Cond. Med. = conditioned media. (c) Left panel, western blot showing overexpression of SIRT3 in RAW264.7 mouse macrophages. Right panel, western blot showing levels of FoxO3a in fibroblasts incubated with conditioned media of SIRT3 overexpressing RAW264.7 cells at 48 hours. Cond. Med. = conditioned media.
Extended Data Fig. 9 | FoxO3a levels in young and aged mouse fibroblasts co-cultured with SIRT3 overexpressing L2 cells. (a) Schematic of the experiment design; FB = fibroblasts. (b) Representative western blots indicate SIRT3 levels in whole cell lysates of L2 cells (b), FoxO3a levels in the cytoplasm (c), and in nuclear fractions (d) from young and old mouse fibroblasts. Fibroblasts were co-cultured with L2 cells that overexpress SIRT3 or control plasmid.
Extended Data Fig. 10 | Cytokine array of secreted factors by SIRT3 overexpressing macrophages. Mouse XL cytokine array and quantitative analysis performed on cell supernatants of co-cultured SIRT3-overexpressing macrophages and mouse fibroblasts.
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection  No software was used.

Data analysis  ImageJ version (1.53a), Graphpad Prism (version 8.4.2), Adobe Illustrator (version 2020), ImageQuant TL, Hamamatsu software (Hamamatsu 303 Corporation).

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|---------------------------------|---------|
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| Animals and other organisms     |         |
| Human research participants     |         |
| Clinical data                   |         |
| Dual use research of concern    |         |
| hSIRT3 (C73E3) (Cell Signaling Technology) Cat# 2627S, Lot# 3 | |
| mSIRT3 (D22A3) (Cell Signaling Technology) Cat# 54905, Lot# 2 | |
| FoxO3a (75D8) (Cell Signaling Technology) Cat# 2497S, Lot# 8 | |
| P16 (G175-1239) (BD Pharmingen) Cat# 554079, Lot# 6327609 | |
| β-Actin (AC-15) (Sigma-Aldrich) Cat# A3578-100UL, Lot# 088MA804V | |
| α-Tubulin (B-5-1-2) (Sigma-Aldrich) Cat# T5168-2ML, Lot# 103MA7734 | |
| Histone 3 (Cell Signaling Technology) Cat# 26505, Lot# 3 | |
| HA tag (C294F) (Cell Signaling Technology) Cat# 3724S, Lot# 8, 9 | |
| Lamin A/C (636) (Santa Cruz Biotechnology) Cat# 7292, Lot# H0919 | |
| α-SMA (D4KN9) (Cell Signaling Technology) Cat# 19245, Lot# 3 | |
| α-SMA (ASM-1) (American Research Products Inc.) Cat# 03-61001, Lot# 406021 | |
| COX IV (3E11) (Cell Signaling Technology) Cat# 4850, Lot# 5 | |
| Phospho-Rb (D20B12) (Cell Signaling Technology) Cat# 8516, Lot# 3, 8 | |
| Total-Rb (4H1) (Cell Signaling Technology) Cat# 9305, Lot# 14 | |
| Total-Rb (D20) (Cell Signaling Technology) Cat# 9313, Lot# 4 | |
| Cleaved PARP (Cell Signaling Technology) Cat# S544, Lot# 5 | |
| Cleaved caspase-3 (SA1E) (Cell Signaling Technology) Cat# 9664, Lot# 21 | |
| CTGF (D8Z2U) (Cell Signaling Technology) Cat# 86641, Lot# 1 | |
| Bim (C34C5) (Cell Signaling Technology) Cat# 2933, Lot# 9 | |
| Noxa (D8L7U) (Cell Signaling Technology) Cat# 14766, Lot# 3 | |
| PUMA (D30C10) (Cell Signaling Technology) Cat# 12450, Lot# 3 | |
| Bcl-2 (Cell Signaling Technology) Cat# 2876, Lot# 7 | |
| Bad (Cell Signaling Technology) Cat# 9292, Lot# 10 | |
| Bid (Cell Signaling Technology) Cat# 2002, Lot# 4 | |
| PCNA (PC10) (Cell Signaling Technology) Cat# 2586, Lot# 4 | |
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)  Institute of Medical Research (IMR-90) cells were purchased from Coriell Cell Repositories; Camden, NJ, USA

Authentication  No authentication, other than routine cell biological approaches to ensure lack of spontaneous transformation.

Mycoplasma contamination  All cell lines are routinely tested for mycoplasma, and confirmed negative.

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Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals  Aged male C57Bl/6 mice (18-22 months; National Institute on Aging) were used for in-vivo studies. C57Bl/6 mice young (7 months) and aged (18-22 months) mice were used for isolation of fibroblasts.

Wild animals  This study did not involve wild animals.

Field-collected samples  This study did not involve samples collected from the fields.

Ethics oversight  All procedures involving animals were approved by the Institutional Animal Care and Use Committees at the University of Alabama at Birmingham, Birmingham, Alabama, USA.

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Flow Cytometry

Plots

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- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Lungs of bleomycin and control or SiRT3 vector treated mice were perfused with chilled PBS. Lung were chopped into small pieces and digested with collagenase [1 mg/ml] for 30 min/37 °C. Later, cells were passed through a sieve (70 μM). RBCs were lysed with ACK lysis buffer. Cells were counted by trypan blue dye-exclusion method. Lung cells were washed with PBS supplemented with 0.5% BSA and incubated with anti-CD16/32 at dilution of 1:100 in PBS/0.5% BSA at 4°C for 10 minutes. After washing, cells were incubated with fluorochrome tagged antibodies at 4°C for 30 minutes. Antibodies Panel: anti CD45-V500, anti-CD31-FITC, anti-F480-APC, anti-MerTK-PE, anti-FPCAM-APC eflour 780. All cells were subsequently washed with PBS/0.5% BSA and resuspended in 1x PBS.

Instrument

BD FACS ARIA

Software

FACS DIVA and FlowJo 10.6.1

Cell population abundance

5-6million cells were sorted for each sample.

Gating strategy

Gating strategy has been provided in Extended figure 2.

- Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.