

SIRT3 Blocks Aging-Associated Tissue Fibrosis in Mice by Deacetylating and Activating Glycogen Synthase Kinase 3 β

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Tissue fibrosis is a major cause of organ dysfunction during chronic diseases and aging. A critical step in this process is transforming growth factor β 1 (TGF- β 1)-mediated transformation of fibroblasts into myofibroblasts, cells capable of synthesizing extracellular matrix. Here, we show that SIRT3 controls transformation of fibroblasts into myofibroblasts via suppressing the profibrotic TGF- β 1 signaling. We found that Sirt3 knockout (KO) mice with age develop tissue fibrosis of multiple organs, including heart, liver, kidney, and lungs but not whole-body SIRT3-overexpressing mice. SIRT3 deficiency caused induction of TGF- β 1 expression and hyperacetylation of glycogen synthase kinase 3 β (GSK3 β) at residue K15, which negatively regulated GSK3 β activity to phosphorylate the substrates Smad3 and β -catenin. Reduced phosphorylation led to stabilization and activation of these transcription factors regulating expression of the profibrotic genes. SIRT3 deacetylated and activated GSK3 β and thereby blocked TGF- β 1 signaling and tissue fibrosis. These data reveal a new role of SIRT3 to negatively regulate aging-associated tissue fibrosis and discloses a novel phosphorylation-independent mechanism controlling the catalytic activity of GSK3 β .

Fibrosis refers to the formation of excess fibrous tissue, which contributes to morbidity and mortality associated with organ failure in response to chronic diseases and/or injury. Tissue fibrosis is also a hallmark of the aging process. In the developed world, fibrotic diseases account for nearly 45% of human deaths, yet there are no approved therapies to date which can arrest or prevent tissue fibrosis (1).

At the molecular level, fibrosis occurs because of the development of myofibroblasts, activated fibroblast-like cells capable of synthesizing exaggerated amounts of extracellular matrix (ECM) and contractile proteins (2). These activated myofibroblasts also synthesize and secrete growth factors, cytokines, and inflammatory mediators to promote fibrosis. Although myofibroblasts can arise from many different cell types, their most common source is local quiescent tissue fibroblasts, which are activated in response to stress stimuli and/or injury. During the normal wound-healing process, myofibroblasts undergo apoptosis after wound repair, leaving a healed scar with low cellular content. But in response to chronic diseases and tissue aging, a multifold increase of myofibroblasts occurs, and they are constantly generated without undergoing apoptosis. Excessive generation of myofibroblasts causes persistent deposition of fibrous tissue leading to organ failure (3). The molecular signals which induce persistent generation of myofibroblasts and which make these cells resistant to apoptosis are not yet understood.

One of the major contributors of tissue fibrosis is activation of transforming growth factor β (TGF- β) signaling (2). The TGF- β superfamily consists of three ligands, TGF- β 1, - β 2, and - β 3, which are synthesized as latent precursors. Activated TGF- β binds to membrane receptors and initiates a series of phosphorylation-dependent signaling cascades which finally culminate in activation of the Smad family of transcription factors (1). These factors in combination with other accessory factors regulate the expression of genes which lead to transformation of fibroblasts into myofibroblasts and induction of fibrosis. During aging several

components of TGF- β signaling are amplified which facilitate the process of tissue fibrosis. Similarly, during chronic stress and/or injury, sustained synthesis of TGF- β 1 has been reported in many tissues (1). However, molecular signals which determine prolonged synthesis of TGF- β 1 are not yet completely understood.

One of the signaling kinases which interferes with TGF- β -signaling is glycogen synthase kinase 3 β (GSK3 β). GSK3 is a serine/threonine kinase which regulates a wide variety of cellular functions (4). GSK3 is expressed in two isoforms, GSK3 α and GSK3 β . Both are highly conserved, ubiquitously expressed, and possess unique as well as overlapping functions. While GSK3 β has been shown to be localized in the cytoplasm, in the nucleus, and in mitochondria, GSK3 α has not been reported in mitochondria (5). Unlike other kinases, GSK3 β is active in the resting state and negatively regulates cellular growth. Upon growth factor stimulation of cells, GSK3 β is phosphorylated at N-terminal serine (S9) residues by the upstream kinases, such as Akt, leading to inhibition of GSK3 β activity and thereby removing its negative control of cellular growth. However, data obtained from GSK3 β -S9A knock-in mice have indicated that these mice are not resistant to GSK3 β inhibition in response to growth stimuli. This suggested that GSK3 β might also be inhibited by mechanisms independent of

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serine phosphorylation (6). Besides phosphorylation, no other posttranslational modification has been demonstrated so far which could explain inhibition of GSK3 β activity during growth factor stimulation of cells.

Sirtuins are class III histone deacetylases (HDACs) which need NAD for their deacetylation reactions. There are seven sirtuin isoforms (SIRT1 to SIRT7) expressed in mammalian cells. These isoforms are localized in different subcellular compartments (7). Among them, SIRT3 is primarily localized in mitochondria, and its levels are elevated by calorie restriction and endurance exercise (8). SIRT3 activation has been shown to protect mice from developing heart failure, cancer, metabolic syndrome, and aging-associated hearing loss (9–12). SIRT3 is also reported as an essential regulator of hematopoietic stem cell aging (13). In another study, polymorphism in the coding sequences causing reduced SIRT3 activity was linked to increased susceptibility to develop obesity and diabetes, thus suggesting a role of SIRT3 in the human aging process (10).

In this study, we demonstrate that SIRT3 negatively regulates tissue fibrosis via activating GSK3 β and blocking synthesis of TGF- β 1. SIRT3 deficiency induces hyperacetylation of GSK3 β , resulting in impaired activity of GSK3 β to phosphorylate its substrates. Reduced enzymatic activity of GSK3 β leads to stabilization and activation of transcription factors involved in the process of tissue fibrosis.

MATERIALS AND METHODS

Antibodies, plasmids, and viral vectors. Antibodies detecting collagen 1, acetylated lysine (Ac-lysine), phospho-CREB (p-CREB), and manganese-containing superoxide dismutase (MnSOD) were purchased from Millipore, Inc. Specific antibodies against TGF- β 1 and Flag tag were purchased from Abcam. Antibodies to tubulin, actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), fibronectin, SIRT3, c-Jun, Ac-lysine, Smad2/3, p65, NFATc, p-GSK3- β T216, axin, p300, c-Myc, and β -catenin were purchased from Santa Cruz. Antibodies to smooth muscle actin (SMA), vimentin, SIRT5, c-Myc, and Flag-agarose beads were obtained from Sigma-Aldrich. Antibodies to SIRT3, GSK3 β , p-GSK3 β , c-Jun, Smad3, p-Smad3, Ac-lysine, p- β -catenin, glycogen synthase, and p-glycogen synthase were purchased from Cell Signaling. GSK3 α/β -specific antibody was obtained from Epitomics. Phospho-Smad3 T66 antibody was a kind gift from Xiao-Fan Wang, Duke University Medical Center, Durham, NC. A hemagglutinin (HA)-GSK3 β construct was obtained from Addgene, and a series of point mutations were performed in this construct with a QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies). Recombinant p300/CBP-associated factor (PCAF) and p300 proteins were purchased from Millipore. A TGF- β /activin response element-luciferase (TARE-Luc) reporter plasmid was bought from Stratagene, Inc. Flag-tagged SIRT3 (Flag-SIRT3) and Flag-SIRT3 catalytic mutant constructs were described previously (14). Adenoviruses for recombinant human SIRT3 and SIRT5 were purchased from Vector Biolabs. Adenovirus vectors expressing a short hairpin RNA (shRNA) against p300 was kindly provided by B. Thimmapaya (Northwestern University, Chicago, IL).

Human heart samples. All procedures involving human heart samples were performed in compliance with institutional guidelines for human research and were approved by the Institutional Review Board of the University of Chicago. Human heart samples were obtained from the cardiac transplant program of the University of Chicago. Failing heart specimens were obtained from diseased hearts that were removed during orthotopic heart transplantation. The detailed protocols for the isolation and culture of failing cardiac fibroblasts were described previously (15). Adult human nonfailing left ventricular control cardiac fibroblasts were purchased from Cell Applications, Inc. (San Diego, CA). Six different human fibroblast cultures were obtained as controls. Cultured fibroblasts

were used within a few passages of culturing to ensure preservation of the control or failing phenotype (15).

Animal experiments. All animal protocols were reviewed and approved by the University of Chicago Institutional Animal Care and Use Committee. Mice with cardiac tissue-restricted expression of Sirt3 and the infusion of Ang-II in mice to develop hypertrophy and fibrosis were described previously (12). Cardiac fibroblasts from 6-month-old wild-type (WT) and Sirt3-knockout (KO) mice were isolated and cultured as previously described (15). SB-505124 (S4696; Sigma) was dissolved in dimethyl sulfoxide (DMSO), which was further diluted with peanut oil and administered at a dose of 10 mg per kg body weight, intraperitoneally (i.p.), three times a week. The hearts of WT and Sirt3-KO mice were analyzed for fibrosis after 2 months of treatment with vehicle or SB-505124 by Masson trichrome staining as described previously (16).

Generation of the wSirt3-Tg mice. Whole-body Sirt3-transgenic (wSirt3-Tg) mice were generated by crossing loxP-stop-LoxP-SIRT3.Flag-transgenic mice with mice expressing Cre under the control of the human β -actin promoter as described elsewhere (17).

General methods. A GSK3- β activity assay was performed using a commercially available GSK3 β activity assay kit (CS0990-1KT; Sigma). Protocols for immunostaining, confocal microscopy, Western blotting, trichrome staining of tissue sections, real-time PCR, luciferase reporter assays, coimmunoprecipitation, chromatin immunoprecipitation (ChIP), *in vitro* deacetylation assays, *in vitro* protein-binding assays, site-directed mutagenesis, mass spectroscopy, flow cytometry-based reactive oxygen species (ROS) measurement, and subcellular fractionation are described in our previous publications (12, 16, 18–20).

***In vitro* assay of SIRT3 activity.** To assay for SIRT3 enzymatic activity, we used a slight modification of a previously published protocol (10). Briefly, SIRT3 was immunoprecipitated using anti-FLAG M2-agarose antibody and mouse liver extracts. One hundred nanograms of immunoprecipitated SIRT3 was added to a 100- μ l reaction mixture containing 50 mM Tris, pH 8.5, 4 mM MgCl₂, 0.2 mM dithiothreitol (DTT), and 50 mM NAD⁺. ³H-labeled acetylated histone H4 peptide (5,000 cpm) was added to the mixture and incubated for 30 min. The reaction was stopped with an equal volume of 2 \times stop solution (0.2 M HCl and 0.32 M acetic acid). The mixture was mixed with 500 μ l of ethyl acetate and centrifuged at 14,000 \times g for 5 min. A total of 450 μ l of the upper-phase solution was transferred to 5 ml of scintillation solution, and counts were determined using a Beckman LS6000SC instrument.

Statistical analysis. Statistical differences among groups were determined using either Student's *t* test (for two groups) or one-way analysis of variance (ANOVA) (for more than two groups) by use of GraphPad Prism software.

RESULTS

We along with others have previously reported that although Sirt3-deficient (Sirt3-KO) mice look normal at birth, they develop cardiac hypertrophy and contractile dysfunction in adulthood (12). To determine the consequences of SIRT3 deficiency on aging of the heart, we analyzed cardiac fibrosis in three age groups (1, 8, and 15 months old) of Sirt3-KO mice. The results showed that there was increased cardiac fibrosis with aging of Sirt3-KO mice (129sv) (Fig. 1A to C). We also analyzed tissue fibrosis in other organ systems and found that, with age, Sirt3-KO mice developed tissue fibrosis in multiple organs, including liver, lung, and kidney, in contrast to their age-matched wild-type (WT) controls (Fig. 1D and E). We also measured blood pressure (BP) of these mice. Sirt3-KO mice had \sim 25-mm Hg higher arterial blood pressure than the controls (Fig. 1F). To test whether Sirt3 deficiency directly induces fibrosis or whether it is secondary to increased BP, we depleted Sirt3 from control human cardiac fibroblasts using a small interfering RNA (siRNA) and analyzed the expression of myofibroblast markers. There was increased expression of the

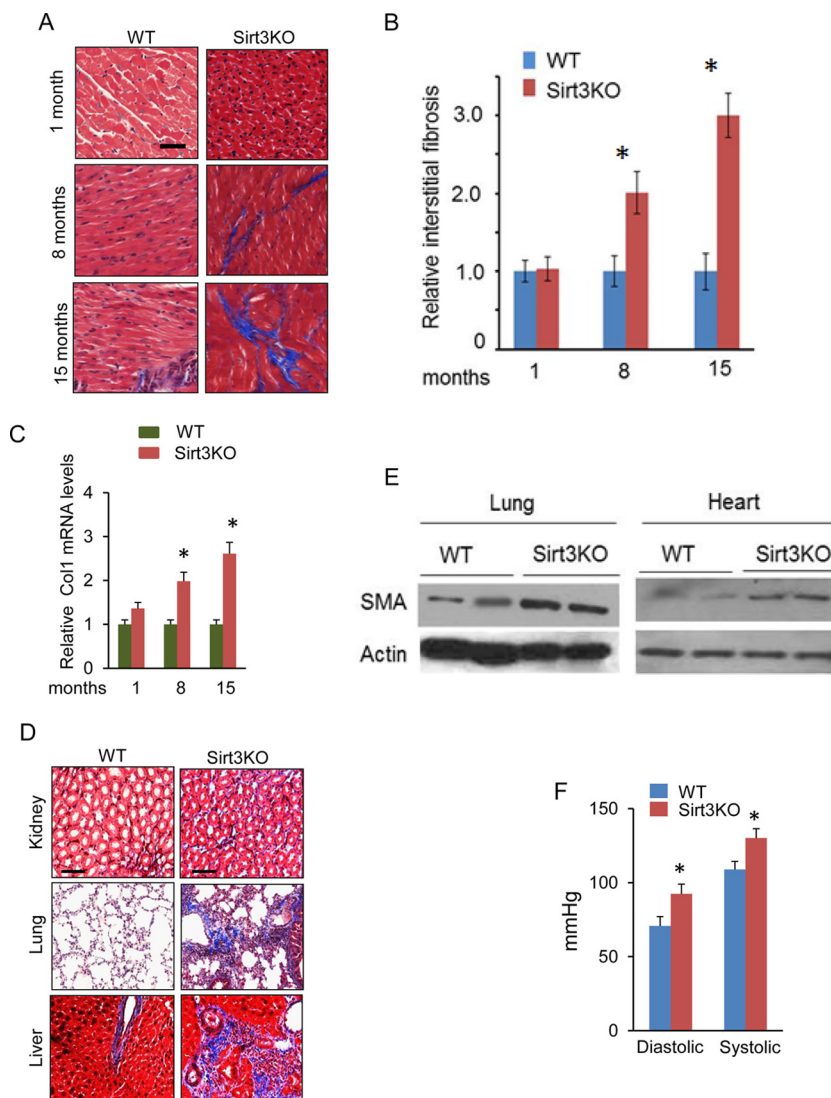


FIG 1 Reduced SIRT3 levels contribute to tissue fibrosis. (A) Comparative study of fibrosis in hearts, stained with Masson's trichrome stain (blue), of Sirt3-KO mice of different age groups and their age-matched wild-type (WT) controls. (B and C) Quantification of cardiac fibrosis and Col1 mRNA levels in Sirt3-KO and WT mice of different age groups. Values are means \pm standard errors ($n = 4$). *, $P < 0.01$. (D) Tissue sections of 15-month-old WT and Sirt3-KO mice, stained to detect fibrosis (sections are representative of 5 mice per group). (E) Expression of the fibrotic marker SMA in the lung and heart tissue samples collected from 15-month-old Sirt3-KO and WT mice. (F) Systemic arterial pressure (femoral artery) in 15-months-old WT and Sirt3-KO mice. Values are means \pm standard errors ($n = 10$). *, $P < 0.001$.

myofibroblast markers collagen 1 (Col1), fibronectin, and smooth muscle α -actin (SMA) in Sirt3-depleted fibroblasts (Fig. 2A and B). We also analyzed SIRT3 levels in fibroblasts isolated from hearts of patients with end-stage heart failure. Fibroblasts of failing hearts showed reduced SIRT3 levels and robust expression of the myofibroblast markers collagen 1 and fibronectin compared to levels in controls (Fig. 2C and D). These findings indicated that SIRT3 deficiency contributes to development of age-dependent organ fibrosis due to sensitization of fibroblasts to transform into myofibroblasts.

We next studied the effect of SIRT3 overexpression in mouse cardiac fibroblasts stimulated with angiotensin II (Ang-II), a pro-fibrotic agent. Treatment of fibroblasts with Ang-II markedly increased expression of myofibroblast markers in control cells but not in SIRT3-overexpressing cells (Fig. 3A and B). We also found

that SIRT3 overexpression blocked the expression of fibrotic markers (SMA, collagen, and fibronectin) in myofibroblasts prepared from failing human hearts (Fig. 3C). To explore the antifibrotic role of Sirt3 in an *in vivo* model, we analyzed tissue fibrosis in Sirt3-transgenic (Sirt3-Tg) mice having whole-body Sirt3 overexpression (~ 4 -fold) (Fig. 3D). Different tissue samples from 15-month-old nontransgenic and Sirt3-Tg mice were collected at the University of California, San Francisco (UCSF), and shipped without identifiers (codes) to the University of Chicago. This blinded analysis revealed that Sirt3-Tg mice have reduced fibrosis in multiple organs, including the heart, liver, kidney, lung, and spleen, compared to levels in their age-matched controls (Fig. 3E and F). These Sirt3-Tg mice also showed significantly reduced expression levels of several fibrotic markers, including collagen 1, collagen 3, connective tissue growth factor (CTGF), and TGF- β 1

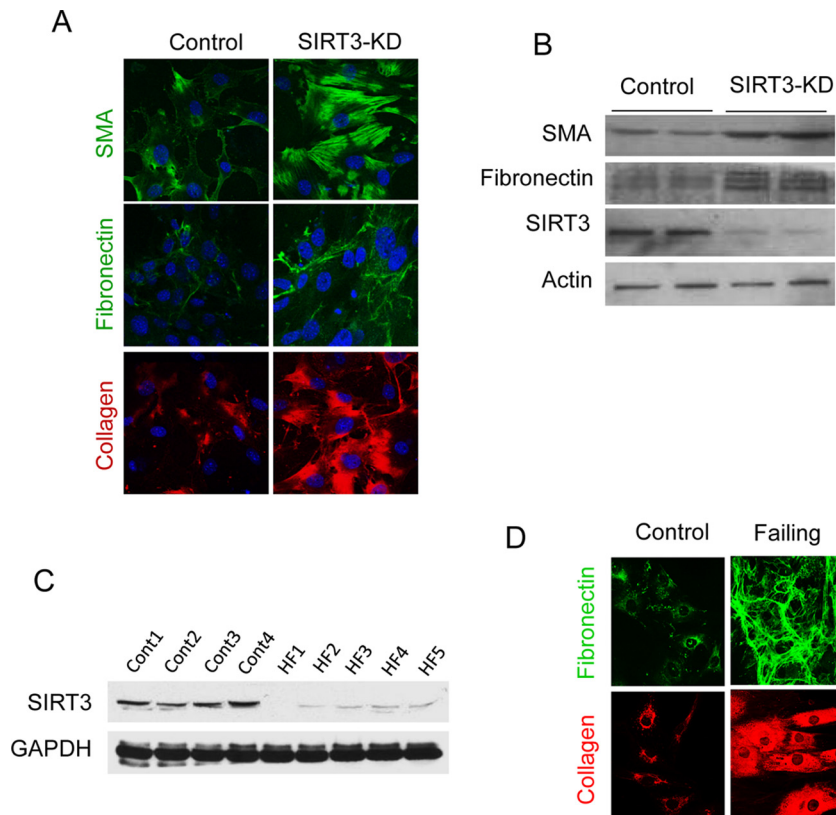


FIG 2 SIRT3 deficiency promotes transformation of human cardiac fibroblasts into myofibroblasts. (A and B) Expression of myofibroblast markers in control and SIRT3-depleted (SIRT3-KD) human cardiac fibroblasts as analyzed by confocal microscopy and Western blotting. (C) Western blot analysis showing SIRT3 levels in human cardiac fibroblasts isolated from controls and patients with heart failure (HF). (D) Confocal microscopy showing expression of myofibroblast markers in human cardiac fibroblasts isolated from control and failing hearts.

(Fig. 3G). These results are consistent with our previous findings where Sirt3-transgenic hearts were found resistant to developing Ang-II-mediated cardiac fibrosis (12). Together, these results indicated that Sirt3 activation prevents development of aging-associated tissue fibrosis.

A major contributor of tissue fibrosis is activation of TGF- β 1 signaling, which is amplified during aging (21). There are also reports showing that TGF- β signaling could be suppressed by calorie restriction (CR) (22). Since SIRT3 is capable of blocking tissue fibrosis and since it is activated by CR, we posited that it might regulate TGF- β signaling. Real-time PCR analysis showed increased TGF- β 1 mRNA expression in Sirt3-KO hearts of 8-month-old mice (Fig. 4A), as well as in isolated cardiac fibroblasts (data not shown). Sirt3-KO hearts also showed increased expression of both unprocessed and mature forms of TGF- β 1 (Fig. 4B), and it was predominantly localized in the cell membrane of Sirt3-KO fibroblasts, a characteristic of TGF- β 1 signaling (Fig. 4C). Sirt3-KO cardiac fibroblasts also exhibited increased transcriptional activity of the natural TGF- β 1 promoter (Fig. 4D). Consistent with these findings, Sirt3-KO cells exhibited increased activity of a promoter/reporter gene having multiple TGF- β 1-responsive elements (TARE) (Fig. 4E). The TGF- β 1-induced activity of this promoter/reporter gene was blocked by overexpression of SIRT3 (Fig. 4F), thus demonstrating a role of Sirt3 in regulating TGF- β 1 synthesis.

To determine the underlying mechanism which regulates

TGF- β 1 signaling in Sirt3-KO cells, we analyzed expression levels of the transcription factors c-Jun and Smad3, both of which are linked to the TGF- β 1 signaling pathway (synthesis and activity). As expected, Sirt3 deficiency led to increased levels of c-Jun and Smad3 in the mouse hearts, under both basal and Ang-II-stimulated conditions (Fig. 5A). Similarly, increased Smad3 levels were found in Sirt3-KO fibroblasts (Fig. 5B). We also measured Smad3 levels in the heart and liver of transgenic mice having Sirt3 overexpression. In both Sirt3-transgenic samples, Smad3 levels were significantly lower than those of nontransgenic controls (Fig. 5C and D). These results thus demonstrated that SIRT3 plays a role in regulating the TGF- β 1 signaling.

In addition to TGF- β signaling, tissue fibrosis can also be promoted by activation of transcription factors like β -catenin and NFATc (23–26). We therefore measured expression of these factors in Sirt3-KO hearts. We observed increased expression of β -catenin and NFATc in Sirt3-KO hearts compared to levels in WT controls (Fig. 5E). These results thus suggested that SIRT3 deficiency promotes expression of transcription factors, which are known to target fibrotic genes.

In a previous study, SIRT3 has been shown to bind to gene promoters and suppress their transcription (27). To test whether SIRT3 controls expression of fibrotic genes by binding to promoters, we analyzed SIRT3 binding to promoters of fibrotic genes. The results obtained from the ChIP analysis showed no binding of SIRT3 to promoters of TGF- β 1 or other well-characterized fi-

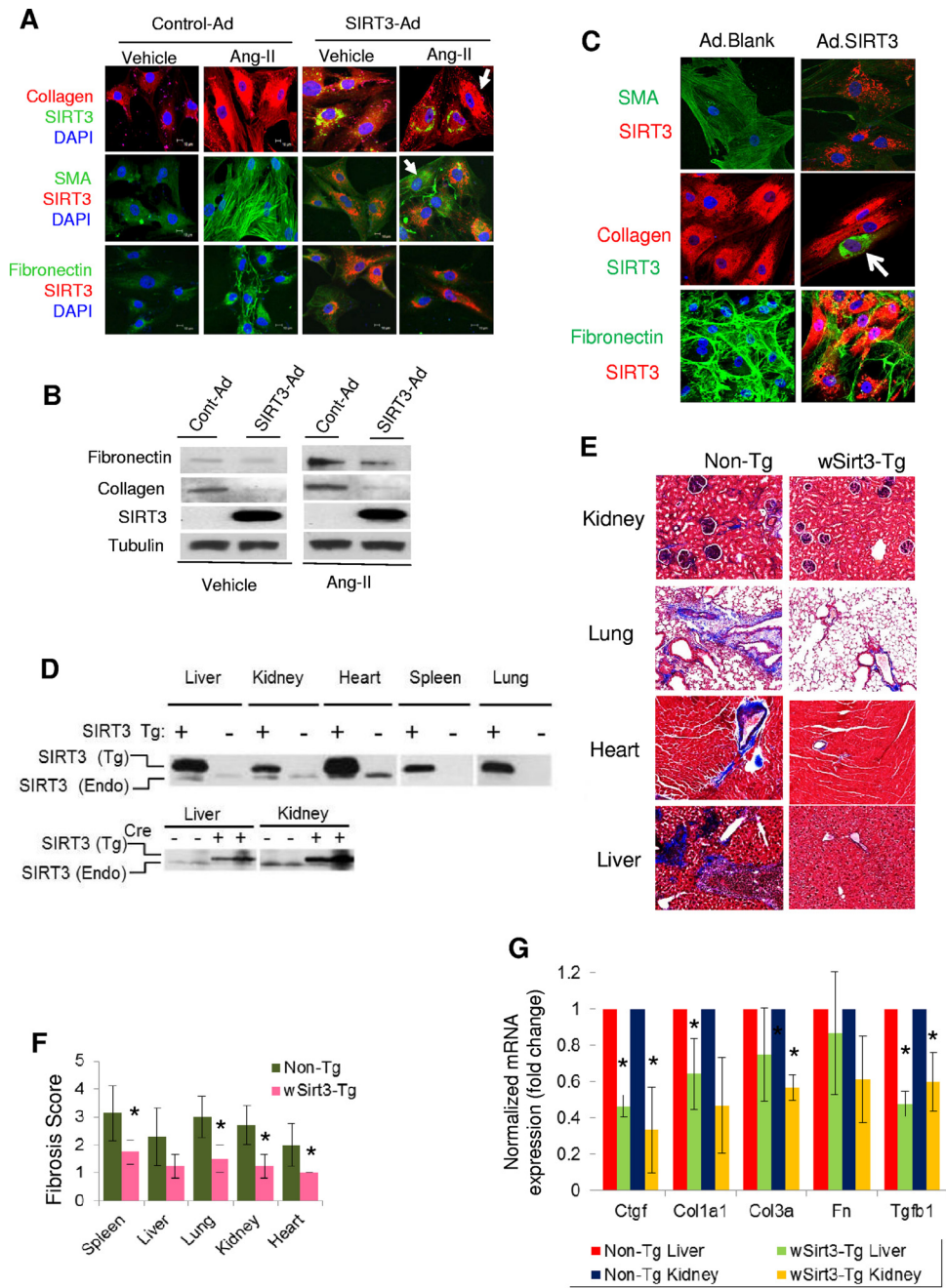


FIG 3 SIRT3 overexpression blocks aging-associated tissue fibrosis. (A) Mouse cardiac fibroblasts were overexpressed with control or adenovirus (Ad) vector synthesizing SIRT3. The next day, cells were stimulated with Ang-II (200 nM) for 48 h. The expression of myofibroblast markers was analyzed by confocal microscopy. Note the reduced expression of collagen, SMA, and fibronectin in SIRT3-overexpressing cells but not in cells lacking SIRT3 overexpression (arrows). DAPI, 4',6'-diamidino-2-phenylindole. (B) Western blot analysis showing expression of the indicated proteins in mouse cardiac fibroblasts subjected to SIRT3 overexpression and stimulated with Ang-II. All four group samples were run in the same gel. (C) Cardiac myofibroblasts isolated from patients with heart failure were infected with control (blank) or SIRT3 adenovirus. The expression of myofibroblast markers (SMA, collagen, and fibronectin) was analyzed by confocal microscopy. SIRT3 expression was verified by immunostaining for SIRT3 (red) in SMA and fibronectin panels and in collagen panels (green). Note that SIRT3 overexpression (arrow) blocked the expression of collagen. (D) The Cre-recombinase-dependent overexpression of SIRT3-FLAG in different organs was detected by Western blotting using a specific antiserum for mouse SIRT3. The endogenous (Endo) SIRT3 protein (lacking a tag) is present as a lower-abundance and lower-molecular-weight protein. (E and F) Tissue fibrosis was analyzed in 15-month-old nontransgenic (Non-Tg) and whole-body Sirt3-transgenic (wSirt3-Tg) mice (C57BL/6). Tissue sections were stained with Masson's trichrome stain (E), and relative fibrosis was scored (F) in a blinded fashion ($n = 4$ to 6 mice per group). (G) Real-time PCR analysis of mRNA of fibrosis-related genes in the liver and kidney samples of non-Tg and wSirt3-Tg mice. Values are means \pm standard errors ($n = 4$ to 7 mice per group). *, $P < 0.001$.

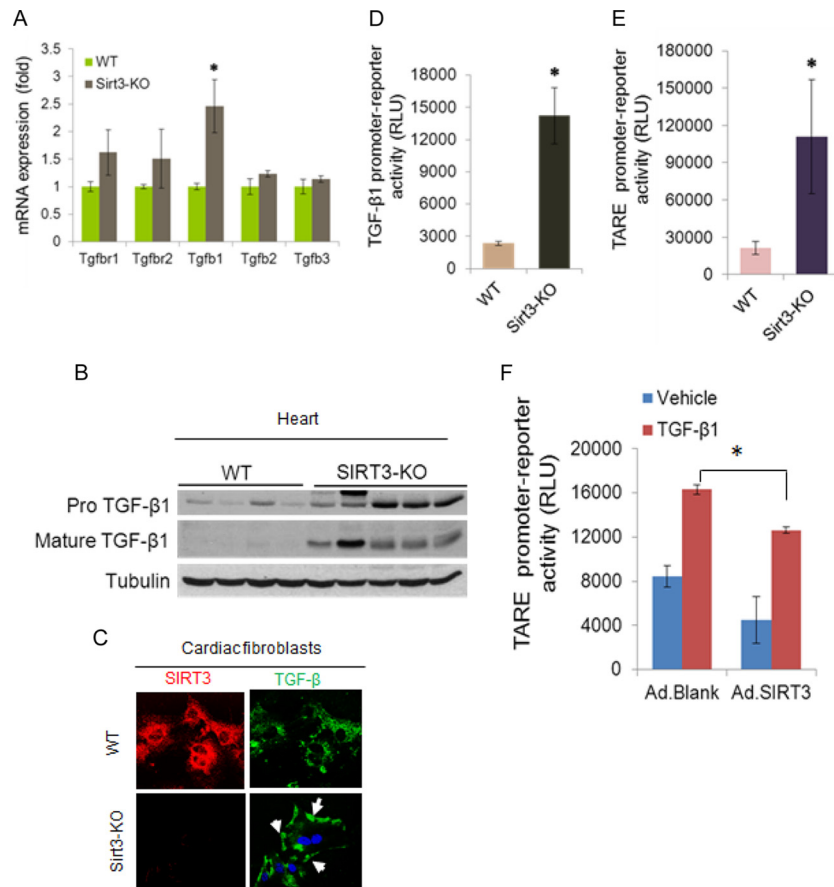


FIG 4 SIRT3 deficiency activates TGF- β 1 synthesis. (A) Reverse transcription-PCR analysis showing expression of different members of the TGF- β family ligands and receptors (r) in the hearts of adult (8 months old) WT and Sirt3-KO mice. Values are means \pm standard errors ($n = 5$ mice per group). *, $P < 0.001$. (B) Western blot analysis of pro-TGF- β 1 and mature TGF- β 1 in mouse hearts ($n = 4$ or 5 mice per group). (C) Cells were immunostained for SIRT3 and TGF- β 1 and imaged by confocal microscopy. Note the increased amount of membrane-bound TGF- β 1 (arrows) in Sirt3-KO fibroblasts. (D) A luciferase reporter assay showing activity of the TGF- β 1 natural promoter in cardiac fibroblast of WT and Sirt3-KO hearts. Values are means \pm standard errors ($n = 3$). *, $P < 0.001$. (E) A luciferase reporter assay was performed with a synthetic promoter containing multiple TGF- β /activin response elements (TAREs) in cardiac fibroblasts of WT and Sirt3-KO mice. Values are means \pm standard errors ($n = 6$). *, $P < 0.001$. (F) The TARE promoter/reporter plasmid was transfected into control and SIRT3-overexpressing cardiac fibroblasts. Cells were stimulated with vehicle or 10 nM recombinant TGF- β 1 for 12 h, and the luciferase activity was measured. Values are means \pm standard errors ($n = 3$). *, $P < 0.001$. RLU, relative light units.

brotic genes like SMA and Col1a (data not shown), thus excluding the possibility of SIRT3 regulating expression of fibrotic genes by binding to their promoters. Another possibility was increased ROS levels due to SIRT3 deficiency, which might contribute to activation of fibrotic genes. To test this possibility, we treated Sirt3-KO mouse cardiac fibroblasts having a myofibroblast phenotype with the antioxidant *N*-acetylcysteine, a general antioxidant, or with EUK-134, a SOD2 and catalase mimetic (28, 29). Neither antioxidant reduced expression levels of the myofibroblast markers SMA and fibronectin nor of Smad3 though each was capable of reducing the ROS levels of Sirt3-KO fibroblasts, thus indicating that ROS is not the primary cause of fibrosis during SIRT3 deficiency (data not shown).

Smad3 is an acetylated protein, and the acetylation modification is known to promote transcriptional activity of Smad3 (30). We therefore asked whether SIRT3 deficiency enhances Smad3 acetylation due to cellular stress. However, we found no increase of Smad3 acetylation in Sirt3-KO hearts (Fig. 6A, upper panel). Smad3 is also known to be phosphorylated at threonine-66 (T66) by GSK3 β , a modification which promotes Smad3 degradation by

the ubiquitin-proteasome system. Decreased GSK3 β -mediated phosphorylation stabilizes Smad3 and promotes its localization into the nucleus (31). Since we observed increased levels of Smad3 during SIRT3 deficiency (Fig. 5), we tested the phosphorylation and ubiquitination status of Smad3 in Sirt3-KO hearts. The results showed reduced T66 phosphorylation, which corresponded with reduced ubiquitination of Smad3 in Sirt3-KO hearts (Fig. 6A, lower panels). These experiments thus suggested reduced activity of GSK3 β in Sirt3-KO cells.

To examine the role of SIRT3 in regulating GSK3 β activity, we then measured the phosphorylation status of many targets of GSK3 β . The results showed reduced phosphorylation of many well-established GSK3 β targets, such as glycogen synthase, β -catenin, and CREB, in Sirt3-KO hearts (Fig. 5E), suggesting that Sirt3 deficiency curtails GSK3 β activity. We also directly tested enzymatic activity of GSK3 β prepared from Sirt3-KO hearts by using a recombinant peptide substrate. The results indicated reduced GSK3 β catalytic activity in Sirt3-KO hearts compared to the level in controls (Fig. 6B). We then tested effect of a pansirtuin inhibitor, nicotinamide (NAM), on GSK3 β targets. Treat-

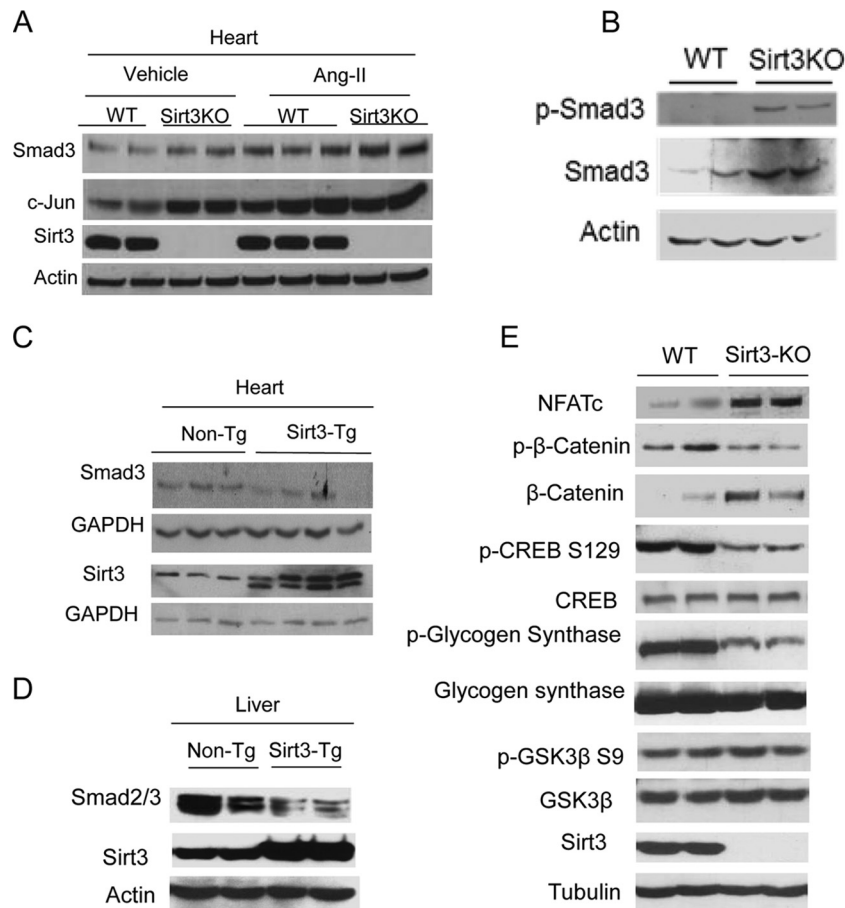


FIG 5 SIRT3 regulates expression of profibrotic transcription factors. (A) Western blot analysis of the indicated proteins in adult mouse hearts stimulated to develop cardiac hypertrophy and fibrosis by Ang-II infusion ($n = 5$ in each group). (B) Western blot analysis showing Smad3 levels in WT and Sirt3-KO cardiac fibroblasts. (C and D) Western blot analysis showing Smad3 levels in the heart and liver of nontransgenic (Non-Tg) and whole-body Sirt3-transgenic mice. Blots are representative of 5 animals in each group. (E) Western blot analysis showing expression levels of different GSK3 β targets in WT and Sirt3-KO hearts ($n = 5$ mice in each group).

ment with NAM but not trichostatin A (TSA; a class I and II HDAC inhibitor) increased cellular levels of β -catenin and Smad3, thus again indicating a role of SIRT3 in regulating GSK3 β activity (Fig. 6C and D). GSK3 β activity is known to be reduced by N-terminal S9 phosphorylation, whereas it is activated by T216 phosphorylation. Our analysis showed that SIRT3 depletion or overexpression does not change phosphorylation of GSK3 β at the S9 or T216 residue (Fig. 5E and 6E). These results thus demonstrated that SIRT3 regulates GSK3 β activity in a phosphorylation-independent manner.

After determining that SIRT3 regulates GSK3 β activity, we asked whether GSK3 β could be modified by lysine acetylation. Immunoprecipitation studies revealed that GSK3 β is acetylated in Sirt3-KO hearts (Fig. 7A). GSK3 β was also acetylated in Ang-II-infused hearts that were stimulated to develop hypertrophy (Fig. 7B). Immunoprecipitation studies also showed that GSK3 β was coprecipitated with both the full-length and short forms of SIRT3 (Fig. 7C). We also studied SIRT3 binding to GSK3 β in a cell-free system. SIRT3, but not SIRT5 (a negative control), was pulled down by agarose beads containing glutathione *S*-transferase (GST)–GSK3 β , thus indicating that SIRT3 directly binds to GSK3 β (Fig. 7D). We next studied colocalization of these pro-

teins. GSK3 β was colocalized with SIRT3 in cardiac fibroblasts (Fig. 7E). Since SIRT3 is mostly present in mitochondria, these results also indicated that GSK3 β and SIRT3 colocalized in mitochondria. Furthermore, in an *in vitro* assay, SIRT3 deacetylated and activated GSK3 β in a NAD-dependent manner (Fig. 7F and G). Additionally, overexpression of SIRT3 deacetylated and activated GSK3 β in transgenic mouse hearts (Fig. 7H and I). We also studied acetylation of GSK3 β in Sirt1-KO hearts and found no change in protein acetylation (data not shown). These results together demonstrated that GSK3 β is a new target of SIRT3.

To determine how acetylation regulates GSK3 β activity, we prepared GSK3 β from Sirt3-KO fibroblasts and subjected it to tandem mass spectrometry (MS/MS) analysis. We identified two lysine (K) residues (K15 and K36) as acetylation sites of GSK3 β (Fig. 8A) (MS/MS data of acetylated K36 [Ac-K36] not shown). Mutation of K15 to glutamine (Q; acetylation mimic) significantly reduced the enzymatic activity of GSK3 β in phosphorylation of the recombinant peptide substrate whereas mutation to arginine (R; deacetylation mimic) increased this activity (Fig. 8B and C). Mutation of K36 to R did not increase activity of the kinase nor did mutation to Q decrease this activity (Fig. 8B). In this assay, a kinase-dead GSK3 β -K85A mutant was used as a negative control

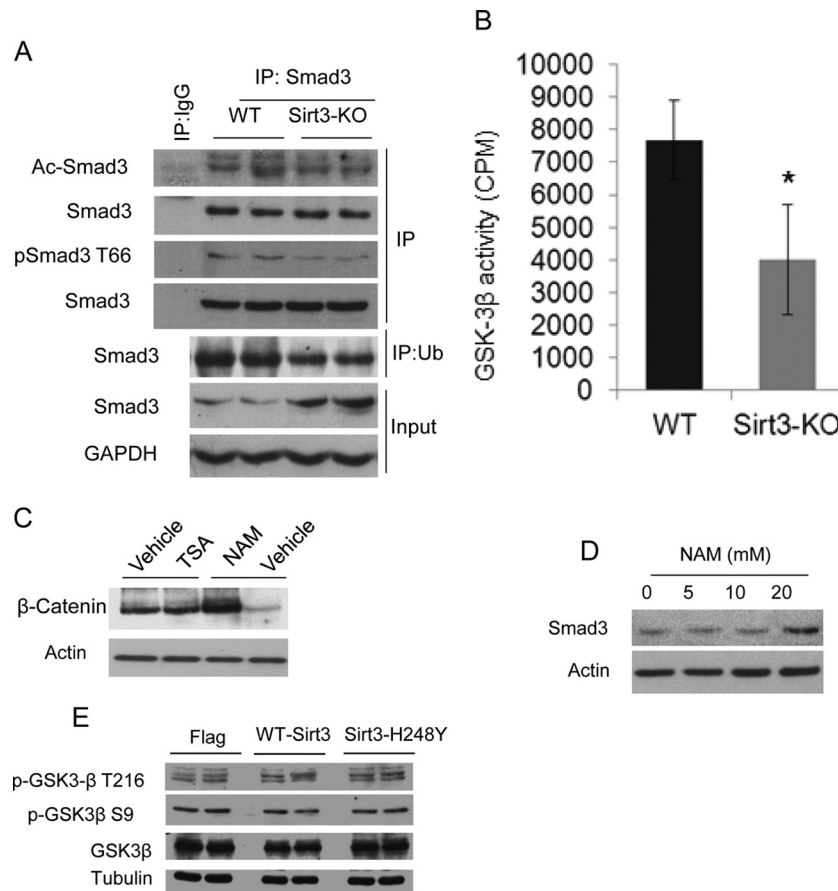


FIG 6 Sirt3 deficiency reduces GSK3 β activity and stabilizes Smad3 levels. (A) Smad3 was immunoprecipitated (IP) from WT and Sirt3-KO hearts and analyzed for acetylation and T66 phosphorylation using specific antibodies. For this analysis, Smad3 levels were normalized. In the same lysate, ubiquitinated Smad3 was analyzed by immunoprecipitation with antiubiquitin (Ub) antibody, followed by Western blotting. (B) GSK3 β prepared from heart lysates of WT and Sirt3-KO mice was tested for its ability to phosphorylate the glycogen synthase recombinant peptide. Values are mean \pm standard errors ($n = 7$). *, $P < 0.001$. (C) Cardiac fibroblasts were treated with the HDAC inhibitor trichostatin A (TSA; 1 μ M) or the pansirtuin inhibitor nicotinamide (NAM; 20 mM) overnight. The β -catenin levels were determined by Western blotting. (D) Western blot analysis showing Smad3 levels in fibroblasts treated with increasing concentrations of NAM. (E) Western analysis showing expression of phosphorylated GSK3 β in cardiac fibroblasts subjected to overexpression of WT Sirt3 or an Sirt3 mutant.

(32). Consistent with these findings, we also observed that the GSK3 β -K15R mutant had significantly higher catalytic activity than GSK3 β -K15Q in phosphorylating glycogen synthase (Fig. 8D and E). These findings demonstrated that deacetylation of the K15 residue promotes GSK3 β activity. To further substantiate these findings, we asked whether the deacetylation mimetic GSK3 β -K15R could revert the myofibroblast phenotype of Sirt3-KO fibroblasts. Overexpression of GSK3 β -K15R but not the mutant significantly reduced SMA expression in Sirt3-KO fibroblasts (Fig. 8F and G), thus demonstrating the ability of GSK3 β -K15R to block transformation of Sirt3-KO fibroblasts into myofibroblasts.

Sequence comparison analysis revealed that the K15 residue is conserved among GSK3 β enzymes of different species, but it is not present in GSK3 α (Fig. 9A and B). Since GSK3 α has not been found in mitochondria, our findings also raised the possibility that K15 acetylation regulates GSK3 β localization into mitochondria. To test this possibility, we asked first where deacetylation of GSK3 β occurs in the cell. For this purpose we prepared GSK3 β from mitochondria of wild-type and Sirt3-KO hearts and determined its acetylation by Western blotting. There was more acetylated GSK3 β in the mitochondrial fraction of Sirt3-KO than in

wild-type hearts, suggesting that deacetylation of the kinase occurs at mitochondria (Fig. 9C). To confirm these results, we overexpressed GSK3 β -KO fibroblasts with different mutations of GSK3 β and examined their localization in mitochondrial and cytosolic fractions of the cell. As shown in Fig. 9D, while GSK3 β -K15Q (acetylation mimic) was mostly localized in mitochondria, the K15R mutant and wild-type GSK3 β were present mostly in the cytosolic fraction. These results thus demonstrated that (i) acetylation of K15 promotes mitochondrial localization of GSK3 β and (ii) deacetylated GSK3 β is localized mostly outside mitochondria.

Finally, to confirm the role of TGF- β 1 in the induction of fibrosis during Sirt3 deficiency, we tested the effects of TGF- β inhibitors. Sirt3-KO fibroblasts were treated with follistatin, an activin-binding protein, which neutralizes members of the TGF- β superfamily, or with a small-molecule inhibitor of TGF- β receptors, SB-505124 (SB) (33, 34). Both inhibitors significantly reduced the expression of SMA and Smad3 in Sirt3-KO fibroblasts (Fig. 10A and B). Consistent with these findings, we also found that chronic inhibition of TGF- β signaling *in vivo* by administration of SB-505124 (SB) reduced fibrotic changes in the kidney, liver, and heart of Sirt3-KO mice (Fig. 10C and D). These data together demonstrated that defects in regulation of TGF-GSK3-Smad3 signaling contribute to transfor-

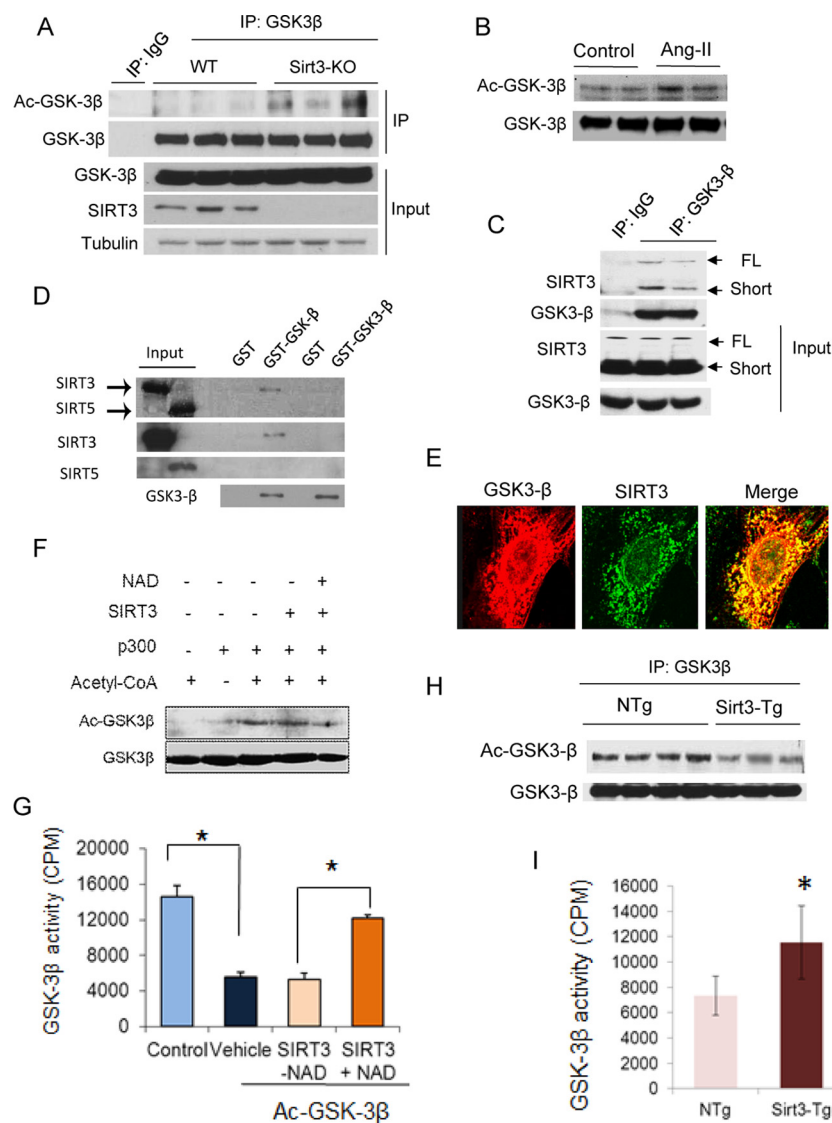


FIG 7 SIRT3 deacetylates and activates GSK3β. (A and B) GSK3β was immunoprecipitated from heart lysates of WT and Sirt3-KO mice (A) or mice stimulated to develop hypertrophy by Ang-II infusion (B). The precipitate was analyzed by Western blot analysis using antiacetyllysine antibody. (C) Coimmunoprecipitation experiments showing GSK3β binding to both full-length (FL) and short forms of SIRT3 in human cardiac fibroblasts ($n = 3$). (D) Flag-tagged [35 S]SIRT3 or [35 S]SIRT5 were synthesized using a TNT-Quick Coupled transcription/translation system, and their binding to GSK3β was analyzed by a GST pull-down assay. Autoradiograms in the upper panel show binding of [35 S]SIRT3 to GST-GSK3β. Expression levels of SIRT3, SIRT5, and GSK3β were detected by Western blotting. Part of this figure was adapted from our previous work (input lanes 1 and 2 from Fig. 2A in reference 18). (E) Confocal microscopy showing colocalization of GSK3-β (red) and SIRT3 (green) in human cardiac fibroblasts ($n = 5$). (F and G) Recombinant HA-GSK3β was incubated with recombinant p300 and cofactor acetyl coenzyme A (acetyl-CoA). Following completion of the reaction, Ac-GSK3β was incubated with the recombinant SIRT3 in the presence or absence of NAD for 2 h in a deacetylation reaction buffer. GSK3β acetylation was analyzed by Western blotting (F), and enzymatic activity was determined against a glycogen synthase peptide (G). (H) GSK3β immunoprecipitated from heart lysates of non-Tg (NTg) and Sirt3-Tg mice was probed with antiacetyllysine antibody. (I) GSK3β was immunoprecipitated from heart lysates of non-Tg and Sirt3-Tg mice and analyzed for catalytic activity against the glycogen synthase peptide. Values are means \pm standard error ($n = 4$). *, $P < 0.001$.

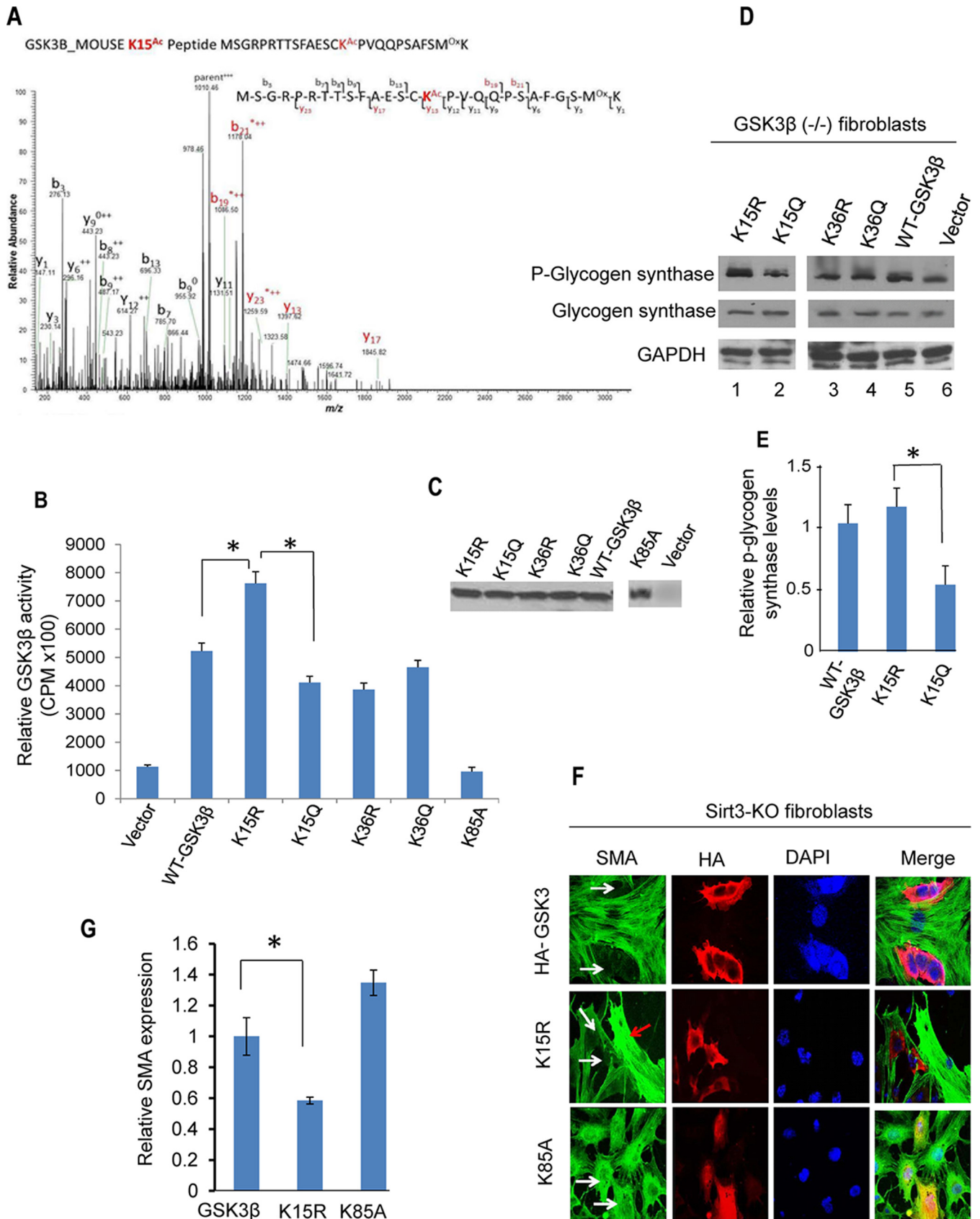
mation of fibroblasts to myofibroblasts and induction of tissue fibrosis in Sirt3-deficient mice.

DISCUSSION

In this study, we showed a new role for SIRT3 in retarding aging-associated tissue fibrosis. Sirt3-KO mice developed increased blood pressure and had exacerbated tissue fibrosis with age, whereas whole-body Sirt3-Tg mice showed reduced fibrosis compared to the level in WT mice. Experiments carried out to demonstrate the mechanism involved in SIRT3-mediated protection

from fibrosis showed the role of GSK3β deacetylation, a new target of SIRT3. Acetylation of residue K15 reduced GSK3β activity, whereas deacetylation increased GSK3β's ability to phosphorylate its substrates. Our data also demonstrate that the antifibrotic effects of SIRT3 are independent of its ability to regulate cellular ROS levels.

Sirt3 and aging-associated fibrosis. Aging is a degenerative process involving a multitude of factors. Though the role of sirtuins in aging has been debated, recent findings suggest that SIRT1, SIRT3, and SIRT6 have the potential to retard the aging



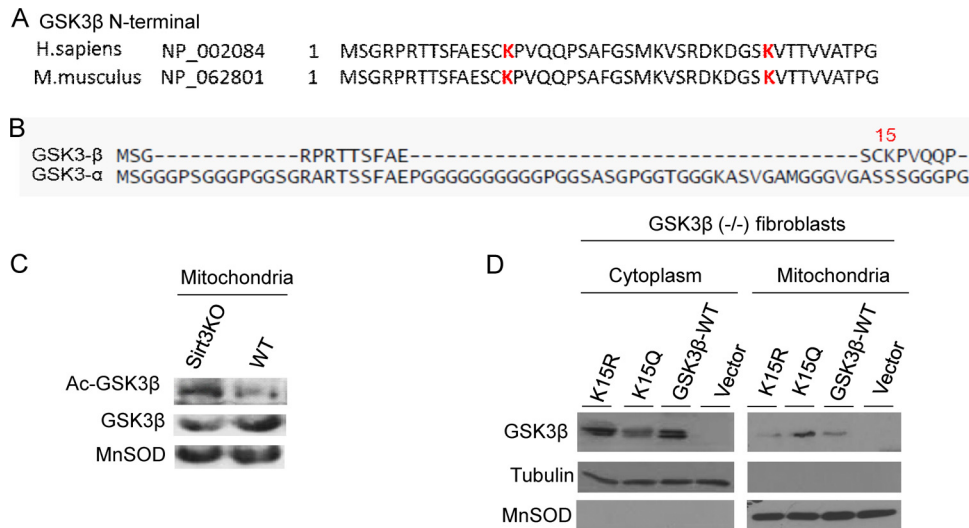


FIG 9 GSK3 β acetylation at residue K15 regulates its mitochondrial localization. (A) N-terminal regions of GSK3 β from different vertebrate species, showing acetylated K15 and K36 residues (in red). (B) Comparison of the N-terminal regions of GSK3 β and GSK3 α showing that residue K15 is present only in the β -isoform. (C) GSK3 β prepared from the mitochondrial fraction of the heart lysate was assayed by Western blotting using antiacetyllysine and antibodies against the indicated proteins. (D) GSK3 β null mouse embryonic fibroblasts were subjected to overexpression of different versions of GSK3 β . The cytoplasmic and mitochondrial fractions of cells were prepared and analyzed by Western blotting using antibodies against the indicated proteins. Blots are representative of three separate experiments. Note that K15Q is mostly present in mitochondria and that K15R is located in the cytoplasm. *H. sapiens*, *Homo sapiens*; *M. musculus*, *Mus musculus*.

process through independent mechanisms (35–37). SIRT3 levels have been reported to be suppressed during aging (36). Moreover, SIRT3 protein levels were found to be decreased in several disease models, where SIRT3 deficiency induces aging-associated hearing loss, propensity to develop cancer, metabolic diseases, and heart failure (9–12). Even though the susceptibilities of different tissues to fibrosis are different, they all reveal the same common characteristics at the cellular and molecular levels, which include tissue degeneration, leukocyte infiltration, inflammation, and proliferation of cells with a fibroblast-like phenotype (38). In this study, we observed that Sirt3-KO mice at around 15 months of age developed severe fibrosis in multiple organs, including liver, heart, lung, and kidney, compared to characteristics of their age-matched WT littermates. Moreover, overexpression of SIRT3 attenuated Ang-II-induced fibrotic changes both *in vitro* and *in vivo*. Whole-body Sirt3-transgenic mice showed reduced fibrosis in several tissues, including heart, liver, kidney, and lung. Consistent with this, we found that fibroblasts isolated from human diseased hearts show low SIRT3 levels, and these were associated with transformation of fibroblasts into myofibroblasts, suggesting that SIRT3 attenuates aging-associated fibrotic changes. It is worth noting that in population studies, increased activity of SIRT3 due to polymorphism was linked to human longevity, and decreased activity was associated with predisposition to develop type II diabetes,

thus supporting a role of SIRT3 for retarding the human aging process (10, 39, 40).

Mitochondrial SIRT3, ROS synthesis, and fibrosis. With regard to the mechanism involved in SIRT3-mediated cellular protection, most previous studies focused on two common mechanisms, reduced cellular ROS levels and increased bioenergetics. These mechanisms are based on the observations that SIRT3 deficiency induces defects in mitochondrial oxidative phosphorylation, leading to increased ROS production and decreased ATP synthesis from mitochondria (41). SIRT3 can also directly activate the ROS scavenger MnSOD by deacetylation, thus reducing the ROS levels (42). Additionally, it can achieve the same indirectly by deacetylating mitochondrial isocitrate dehydrogenase 2, leading to increased NADPH levels to protect cells from oxidative-stress-induced apoptosis (43). SIRT3 is also implicated in regulation of the expression of nuclear genes by controlling activity of transcription factors such as p53, PGC-1 α , FoxO3a, and hypoxia-inducible factor 1 α (HIF-1 α) via monitoring mitochondrial ROS production (44). In this study, however, even after concerted efforts, we found no evidence to support the role of ROS generated during SIRT3 deficiency as a cause of tissue fibrosis. In our rescue experiments, constitutively active GSK3 β , but not antioxidants, blocked the fibrotic changes in Sirt3-KO cells, thus excluding the role of ROS as a prime trigger of fibrosis. It is worth noting that

FIG 8 Lysine 15 (K15) acetylation regulates GSK3 β activity. (A) Annotation of representative tandem mass spectra of trypsin-digested GSK3 β , depicting K15 acetylation (in red). (B) HA-tagged wild type and K15R, K15Q, K36R, K36Q, and K85A mutants of GSK3 β were purified from GSK3 β null mouse embryonic fibroblasts using HA antibody-conjugated agarose beads. The enzymatic activity of GSK3 β mutants was determined against a glycogen synthase peptide. Values are means \pm standard errors ($n = 5$). *, $P < 0.001$. (C) Western analysis showing expression levels of different GSK3 β mutants. (D) GSK3 β null fibroblasts were overexpressed with different forms of GSK3 β . The catalytic activity of GSK3 β was assayed by measuring the phosphorylation of glycogen synthase by Western blotting. (E) Quantification of glycogen synthase phosphorylation by GSK3 β -K15 mutants. Note the increased activity of GSK3 β -K15R in phosphorylation of the substrate compared to that of GSK3 β -K15Q. Values are means plus standard errors ($n = 3$). *, $P < 0.01$. (F) Sirt3-KO fibroblasts were overexpressed with different GSK3 β constructs. Expression of SMA was determined by confocal imaging. White arrows indicate reduced SMA expression in GSK3 β -WT- and GSK3 β -K15R-expressing cells but not in GSK3 β mutant (K85A) cells. The red arrow indicates that cells negative for GSK3 β -K15R expression robustly express SMA. (G) Quantification of SMA levels in cells expressing different GSK3 β mutants. Values are means \pm standard errors ($n = 3$).

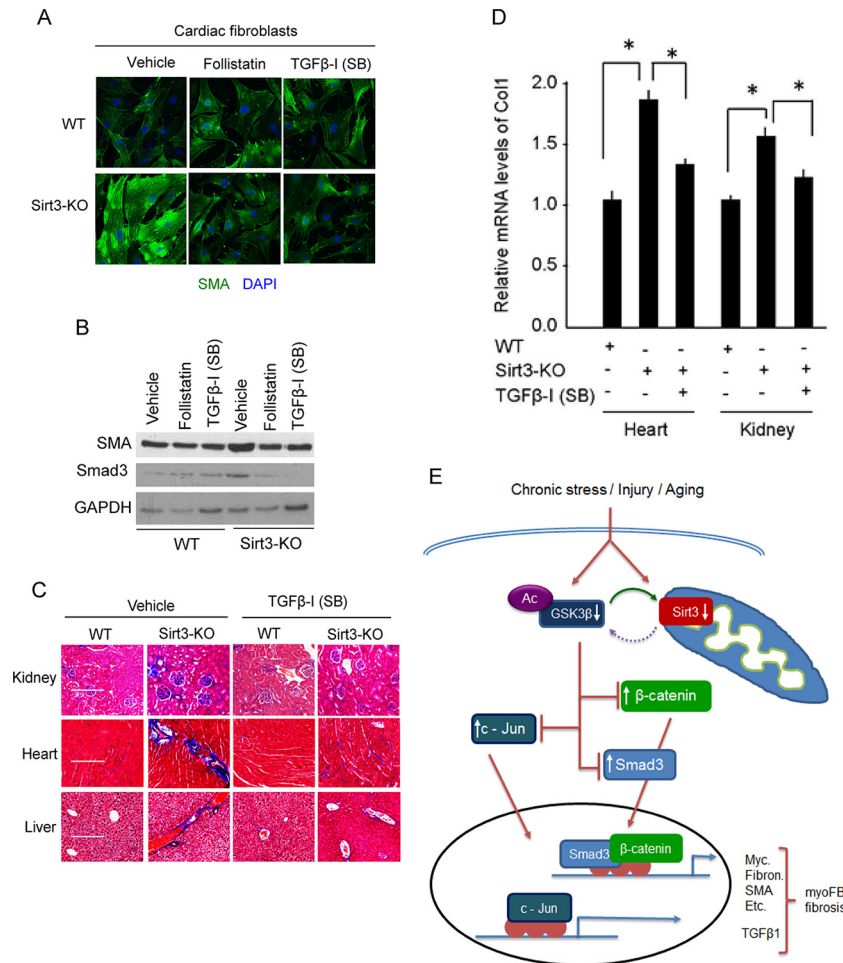


FIG 10 TGF- β inhibitors block fibrotic changes of Sirt3-KO cells/tissues. (A) Confocal microscopy showing SMA expression in wild-type and Sirt3-KO cardiac fibroblasts treated with the TGF- β inhibitor follistatin (200 nM) or SB-505124 (1 μ M) for 48 h. DAPI, 4',6'-diamidino-2-phenylindole. (B) Western analysis showing expression levels of SMA and Smad3 in the same cells as used for the experiment described for panel A. (C) WT and Sirt3-KO mice (10 months old) were treated with vehicle or SB-505124 at a dose of 10 mg kg⁻¹ for 2 months (three intraperitoneal injections/week). Tissue fibrosis was analyzed by Masson's trichrome staining of tissue sections ($n = 7$ mice per group). (D) Real-time PCR analysis of collagen 1 gene expression in different groups of mice. Values are means \pm standard errors ($n = 4$ or 5 mice per group). *, $P < 0.001$. (E) A simplified scheme illustrating the role of SIRT3 in regulating GSK3 β -TGF β -Smad3 signaling and development of fibrosis. SIRT3 deacetylates GSK3 β at mitochondria. In SIRT3-deficient cells GSK3 β is acetylated, inhibiting its ability to phosphorylate the substrates. Decreased GSK3 β -dependent phosphorylation causes stabilization of substrates like Smad3, c-Jun, and β -catenin, leading to their increased import into the nucleus, where they regulate the expression of profibrotic genes and transformation of fibroblasts into myofibroblasts.

ROS scavengers are incapable of reducing fibrosis and improving organ functions in patients (45, 46). In a similar vein, many recent reports have challenged the concept of oxidative damage as the cause of the aging process (47–49). In studies with *Caenorhabditis elegans*, it has been reported that while superoxide dismutase is required to survive acute stress, it is dispensable for the life span extension of the animal (47, 48). In another study, it was shown that the health-promoting effects of physical exercise for diabetic patients are not improved, but rather antagonized, by use of antioxidants (49). Similarly, antioxidant supplements in randomized clinical trials of primary and secondary preventions were shown to exert harmful effects and increase mortality (50). In light of these reports an alternative hypothesis for the antiaging effects of SIRT3 is necessary. If SIRT3 acts as an antiaging molecule, its health-promoting activity may not be limited to its ability to control mitochondrial ROS production. Rather, there must be additional mechanisms through which SIRT3 curtails aging and aging-associated diseases.

SIRT3 activates GSK3 β . We found that SIRT3 targets GSK3 β to control tissue fibrosis. GSK3 β is a constitutively active kinase that critically regulates diverse cellular functions involved in maintaining cellular growth, survival, and death (4). A role for GSK3 β has been also implicated in controlling tissue fibrosis and the aging process (51). GSK3 β is inhibited by N-terminal S9 phosphorylation by the upstream kinases. However, mutation studies have indicated that S9 mutation does not eliminate GSK3 β inhibition by growth stimuli, suggesting that GSK3 β is also subject to inhibition by other mechanisms (6). In this study, we found that reversible lysine acetylation regulates GSK3 β activity, which is independent of its phosphorylation status. We found that GSK3 β is critically acetylated at residue K15. This acetylation negatively regulates GSK3 β activity to phosphorylate substrates. SIRT3 directly binds to and deacetylates GSK3 β , and this modification increases the enzymatic activity of the kinase and thereby blocks the ability of factors like Smad3, c-Jun, and β -catenin to promote expression of fibrotic genes (Fig. 10E). In an earlier study acetylation of

GSK3 β was reported in hepatocellular carcinoma. In these cells it was shown that SIRT2, which is mostly expressed in the cytoplasm, is incapable of deacetylating the kinase (52). Another sirtuin which is implicated in regulating GSK3 β activity is SIRT1. But this sirtuin analogue inhibits GSK3 β activity by deacetylating and activating Akt1 in the heart (19). In our studies we found no change in GSK3 β acetylation in SIRT1-KO hearts. Based on these reports and the data presented here, we believe that the deacetylation-dependent activation of GSK3 β is specifically carried out by the mitochondrial deacetylase SIRT3.

Where in the cell does SIRT3 deacetylate GSK3 β ? GSK3 β is localized in the cytoplasm, in the nucleus, and in mitochondria, where it targets compartment-specific substrates (53, 54). In the mitochondria GSK3 β has been shown to target substrates present in the matrix, such as cyclophilin D and Rieske, a subunit of complex III, and those localized at the level of two membranes, such as hexokinase II (HK II), myeloid cell leukemia 2 (Mcl-2), Bcl-2, Bax, Noxa, voltage-dependent anion channel (VDAC), and adenine nucleotide transporter (ANT) (5). The mitochondrial localization signal of GSK3 β has not been defined yet. But in a recent study a role of residue K15 in the transport of GSK3 β into mitochondria has been suggested (55). Consistent with our findings, mutation of K15 to alanine was shown to suppress mitochondrial localization of GSK3 β in H9C2 cells (55). SIRT3 is a mitochondrial protein, and within mitochondria it is present in the matrix as well as in the inner membrane space, where it targets OPA1 to regulate mitochondrial dynamics (18, 56). In this study, we found that transgenic mice expressing mitochondrial SIRT3 were capable of blocking fibrosis, together with decreasing GSK3 β acetylation and reducing TGF β 1 and Smad3 levels. Moreover, the deacetylated form of GSK3 β was localized mostly outside mitochondria. Exactly where GSK3 β is deacetylated in mitochondria must await characterization of the mechanisms regulating GSK3 β transport in and out of mitochondria. Based on our findings and reports from others, we believe that GSK3 β is deacetylated at the mitochondria and transported to the cytosol to regulate its substrates. This could be similar to mechanisms involved in the activation of another SIRT3 deacetylation target, Skp2, which is exported from the mitochondria after deacetylation to regulate nuclear gene expression (57).

SIRT3 and TGF- β 1 signaling. A role of GSK3 β in regulating TGF- β 1 synthesis has been previously documented (31). TGF- β is one of the cytokines released during the inflammatory response which activates fibroblasts to transform into myofibroblasts to produce ECM. Excessive production of TGF- β 1 was linked to tissue fibrosis during aging and pathological changes in many organs, including liver, lung, kidney, heart, skeletal muscle, and uterus (58–60). However, the molecular basis of this increased synthesis of TGF- β was unknown. In the present study, we found that SIRT3 can negatively regulate TGF- β synthesis at the promoter level. Sirt3-deficient cells exhibited increased expression of TGF- β 1 mRNA and promoter activity, whereas overexpression of SIRT3 blocked the activation of this promoter. Cell signaling analysis revealed that this was achieved indirectly by negatively regulating expression of the nuclear transcription factor (like c-Jun) but not by directly suppressing the gene promoter since CHIP analysis showed no binding of SIRT3 to the promoters of TGF- β 1. Thus, our data elucidate the molecular link, especially the link between the mitochondria

and the nucleus, involved in excess TGF- β 1 production during aging-associated tissue fibrosis.

Growing evidence in the field of longevity suggests that dietary restriction retards the aging process and activates SIRT3 while antagonizing TGF- β signaling (8, 22). Our findings suggest that both of these pathways are directly linked at the molecular level. Previous studies have demonstrated that in addition to SIRT3, SIRT1 and SIRT6 are also activated in response to dietary restriction (7). SIRT1 has been shown to exert tissue-specific effects with regard to its ability to regulate tissue fibrosis. SIRT1 inhibits TGF- β -mediated renal fibrosis and blocks the epithelial-to-mesenchymal transition that leads to organ fibrosis (61, 62). In contrast to this, other studies report that SIRT1-deficient hearts develop reduced fibrosis following stress, while SIRT1-overexpressing hearts develop massive fibrosis with age (19, 63). Another sirtuin, SIRT6, antagonizes TGF- β -induced senescence in human bronchial epithelial cells (64). However, whether SIRT6 plays a role in regulation of endogenous TGF- β 1 expression is currently unknown. Based on the findings of the present work, we believe that SIRT3 can indirectly block TGF- β expression by regulating acetylation of GSK3 β and thereby controlling the aging and aging-associated fibrotic remodeling of the tissue.

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N.R.S. and S.B. performed the majority of the experiments. M.G. performed real-time PCR analysis and assessed catalytic activity of GSK3 β mutants. S.S. performed *in vitro* protein binding assays. V.B.P. performed flow cytometry and TGF- β 1 inhibitor experiments and participated in writing of the manuscript. R.S.N. analyzed the GSK3 β signaling in cardiac lysates. D.W. did MS/MS analysis. Y.P., J.-Y.H., and E.V. generated whole-body SIRT3-transgenic mice and provided tissue samples for fibrotic analysis. M.P.G. coordinated with different investigators, supervised the whole study, and generated the final draft of the manuscript.

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