Ltbp4 regulates Pdgfrβ expression *via* TGFβ-dependent modulation of Nrf2 transcription factor function

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Abstract

Latent transforming growth factor beta binding protein 4 (LTBP4) belongs to the fibrillin/LTBP family of proteins and plays an important role as a structural component of extracellular matrix (ECM) and local regulator of TGFβ signaling. We have previously reported that *Ltbp4S* knock out mice (*Ltbp4S*−/−) develop centrilobular emphysema reminiscent of late stage COPD, which could be partially rescued by inactivating the antioxidant protein Sestrin 2 (Sesn2). More recent studies showed that *Sesn2* knock out mice upregulate Pdgfrβ-controlled alveolar maintenance programs that protect against cigarette smoke induced pulmonary emphysema. Based on this, we hypothesized that the emphysema of *Ltbp4S*−/− mice is primarily caused by defective Pdgfrβ signaling. Here we show that LTBP4 induces Pdgfrβ signaling by inhibiting the antioxidant Nrf2/Keap1 pathway in a TGFβ-dependent manner. Overall, our data identified Ltbp4 as a major player in lung remodeling and injury repair.

Introduction

Latent transforming growth factor beta binding protein 4 (LTBP4) belongs to a family of four (LTBP1–4) secreted extracellular matrix (ECM) proteins that share structural homology with fibrillins. Like the other LTBPs, LTBP4 consists of numerous EGF-like motifs whose functional significance is not yet clear; they are found in the extracellular domain of membrane bound proteins and in some secreted proteins. Interspersed between the EGF-like modules of LTBP4 are three 8-Cys repeat domains of which the third covalently binds latent TGFβ1 (TGFβ1-LAP, where LAP stands for latency associated propeptide) and deposits it into the ECM. There, TGFβ1-LAP complexed to LTBP4 is stored until needed. When needed, active TGFβ is released from LTBP and LAP in a process referred to as "latent TGFβ activation", which is accomplished by a variety of factors including proteases, integrins, reactive oxygen species and many others (reviewed in [1,2]). In addition to its TGFβ related function, LTBP4 is required for the assembly of elastic fibers in the ECM [3].

Mammalian cells express two major isoforms of LTBP4, which by analogy to the long and short isoforms of LTBP1 discovered earlier, are also called long (LTBP4L) and short (LTBP4S). These isoforms are encoded by two N-terminal splice variants expressed independently from their own promoters [4].

We and others previously reported that mice with an inactivating mutation in *Ltbp4S* (*Ltbp4S*−/− mice) are born with alveolar septation defects that deteriorate with age [5,6]. By the age of 5–6 months lungs from *Ltbp4S*−/− mice show symptoms reminiscent of

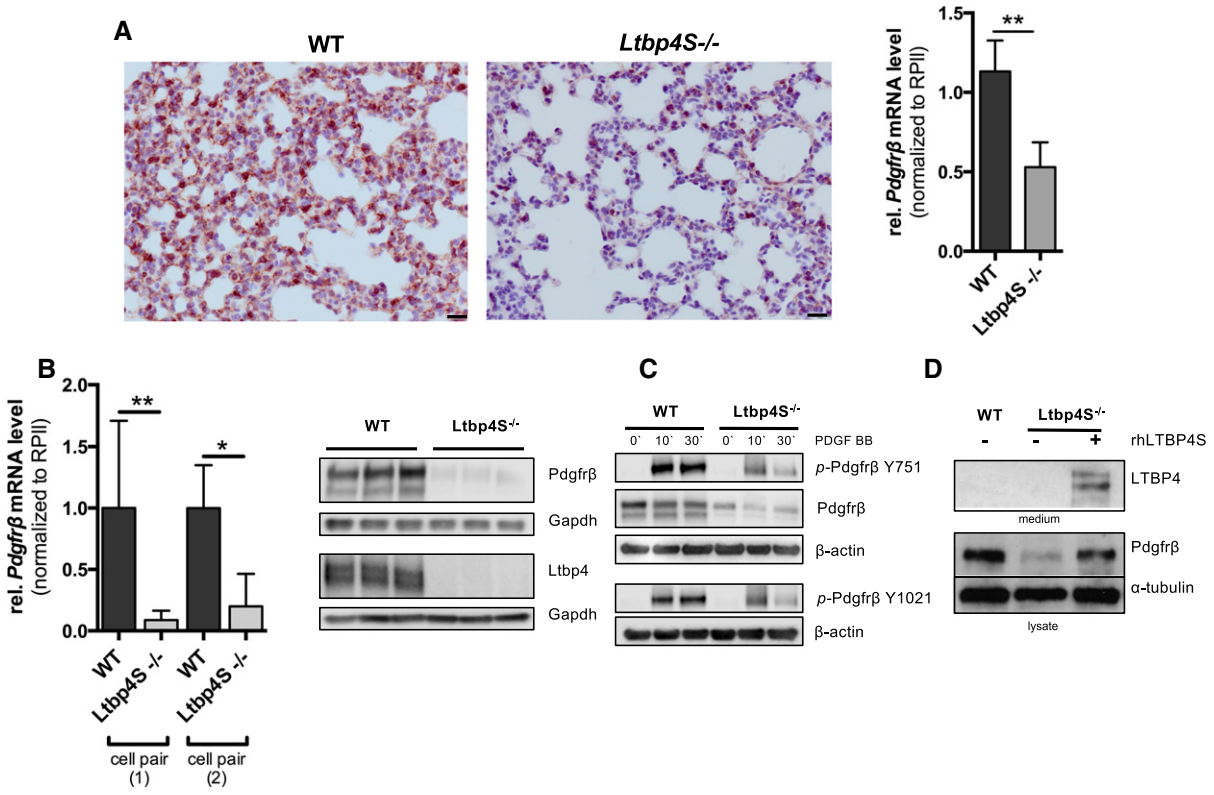

Fig. 1. Regulation of Pdgfrβ signaling by Ltbp4. A. Reduced Pdgfrβ expression in lungs of *Ltbp4S–/–* mice. *Left panel:* Representative lung sections stained with anti-Pdgfrβ antibody and counterstained with hematoxilin. Scale bar: 20 μm. *Right panel: Pdgfrβ* mRNA in lung tissue homogenates quantified by qRT-PCR. Results are represented as means ± SD of N = 3 lungs. B. Pdgfrβ mRNA and protein expression in MLFs of WT- and *Ltbp4S–/–* mice. *Left panel:* qRT-PCR. Results are represented as means ± SD of three independent experiments. *Right panel*: Western blot showing Ltbp4S and Pdgfrβ expression in MLFs derived from three separate experiments. C. Western blot showing Pdgrβ phosphorylation after stimulation with 25 ng/ml recombinant PDGFR BB. D. Rescue of Pdgfrβ expression in Ltbp4S–/– MLFs after transfecting with the LTBP4S/pEF-IRES expression plasmid (see Experimental procedures). *$p < 0.05$, **$p < 0.01$.

centrilobular emphysema associated with late stage chronic obstructive pulmonary disease (COPD) [5,7]. We could partially rescue this phenotype by inactivating the antioxidant protein Sestrin 2 (Sesn2) and attributed this to an activation of TGFβ- and mTOR signaling [7]. However, more recent studies showed that Sesn2 also regulates Pdgfrβ signaling. In mice, the mutational inactivation of Sesn2 prevents the development of cigarette smoke induced pulmonary emphysema by upregulating Pdgfrβ-controlled alveolar maintenance programs [8]. We showed that Pdgfrβ upregulation is mediated by second messenger superoxide anions (O_2^-) accumulating in Sesn2 depleted cells as a result of Nrf2 (nuclear factor erythroid 2-related factor 2)/Keap1(Kelch-Like ECH-Associated Protein 1) pathway inhibition [9].

Nrf2 is a well-characterized, global antioxidant gene inducer, whose activity is tightly controlled by cytoplasmatic association with its inhibitor Keap1. Upon oxidative stress, Nrf2 dissociates from Keap1, translocates into the nucleus and transactivates antioxidant genes (reviewed in [10]). Sesn2 stimulates this process by promoting autophagic degradation of Keap1 [11].

We demonstrated that Sesn2 and Nrf2/Keap1 are part of a Sesn2/Pdgfrβ suppressor pathway that is highly upregulated in the lungs of individuals with late stage COPD [9]. As upregulation of Pdgfrβ protected Sesn2 knock out (KO) mice against cigarette smoke induced emphysema [8], we speculated that a similar mechanism might be responsible for the emphysema rescue phenotype of Sesn2 depleted *Ltbp4S* KO mice. Here we show that Ltbp4 is required for latent TGFβ activation in the ECM and TGFβ signaling for the inhibition of the Sesn2/Pdgfrβ suppressor pathway. Overall, our data suggest that Ltbp4 plays a major role in lung remodeling and might be involved in the pathogenesis of COPD.

Results

Repressed Pdgfrβ expression in lungs of *Ltbp4S–/–* mice

To investigate a possible interrelationship between Ltbp4 and Pdgfrβ, we estimated Pdgfrβ expression

in lungs of wild type (WT) and *Ltbp4S –/–* mice by immunohistochemistry and qRT-PCR. While highly expressed in WT lungs, Pdgfrβ was downregulated in *Ltbp4S –/–* lungs (Fig. 1A), suggesting that the *Ltbp4S* mutation interferes with Pdgfrβ expression. To analyze this in more detail, we used mouse lung fibroblasts (MLFs) isolated from WT- (WT-MLFs) and *Ltbp4S –/–* (*Ltbp4S –/–* MLFs) mice, that were spontaneously immortalized and adapted for growth in tissue culture [7,12]. Pdgfrβ mRNA and protein were significantly downregulated in *Ltbp4S –/–* MLFs (Fig. 1B, Supplementary Fig. 1A), as was Pdgfrβ signaling in response to the cognate platelet derived growth factor (PDGF-BB) ligand. Accordingly, Pdgfrβ phosphorylation was reduced in PDGF-BB stimulated *Ltbp4S –/–* MLFs (Fig. 1C) translating phenotypically into diminished circular dorsal ruffle formation (Supplementary Fig. 1B) [13]. This poor response to PDGF-BB could be attributed entirely to reduced Pdgfrβ expression (Fig. 1B) rather than to defects in *bona fide* Pdgfrβ phosphorylation (Supplementary Fig. 1C).

To test whether Ltbp4 depletion also affects PDGFRβ expression in human cells, we transduced embryonic lung fibroblast derived MRC5 cells with *LTBP4* shRNA encoding lentiviruses. Like in *Ltbp4S –/–* MLFs, *LTBP4* knock-down in MRC5 cells downregulated PDGFRβ expression (Supplementary Fig. 2). Finally, overexpression of human recombinant LTBP4S in *Ltbp4S –/–* MLFs rescued Pdgfrβ expression (Fig. 1D), suggesting that LTBP4 is a Pdgfrβ inducer.

Pdgfrβ downregulation in *Ltbp4S –/–* MLFs is linked to TGFβ

Recent studies showed that Ltbp4 is required for elastic fiber formation in the ECM. By binding fibulin-4 and -5 attached to tropoelastin monomers, Ltbp4 directs elastin deposition within the ECM's microfibril network in a TGFβ-independent manner [3,14]. We confirmed these findings in the ECM of *Ltbp4S –/–* MLFs, which completely lacked an

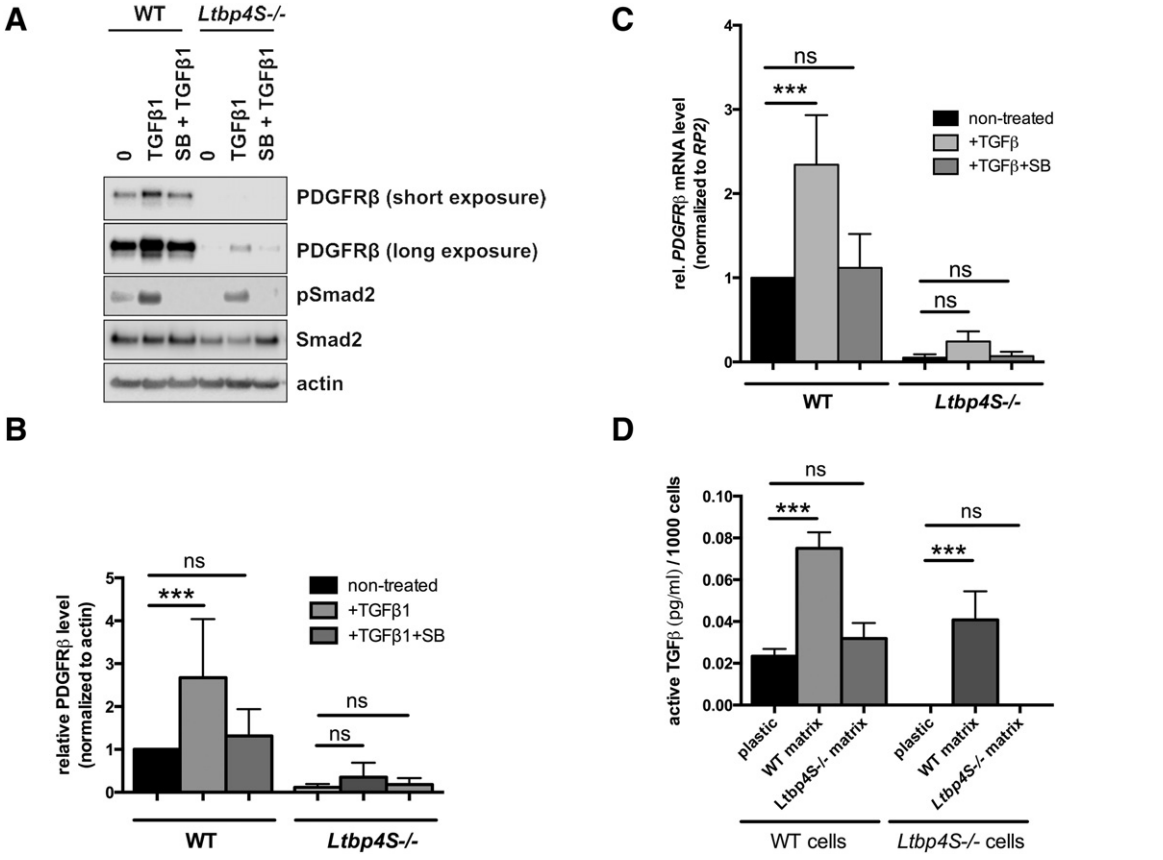

Fig. 2. Induction of Pdgfrβ expression by TGFβ1. A. Representative Western blot showing induction of Pdgfrβ expression in MLFs by recombinant TGFβ1. MLFs were exposed for 48 h to 10 ng/ml recombinant TGFβ1 ± SB-431542 inhibitor. B. Densitometric quantification of Pdgfrβ protein expression in MLFs exposed to TGFβ1. C. *Pdgfrβ* mRNA expression in MLFs exposed to TGFβ1 quantified by qRT-PCR. D. Levels of active TGFβ in conditioned media prepared from MLFs pre-exposed for 96 h to ECMs derived from WT- and *Ltbp4S –/–* MLFs (see Experimental procedures). TGFβ was quantified by measuring induced luciferase activity in MLEC reporter cells [18] by WT- and Ltbp4S –/– CMs. All results are represented as means ± SD of three independent experiments. ***$p < 0.001$; ns, not significant.

elastic fiber network (Supplementary Fig. 3A). As elastin was shown previously to inhibit Pdgfrβ expression in vascular smooth muscle cells [15] it is possible that unincorporated tropoelastin in the *Ltbp4S–/–* ECM inhibited Pdfgrβ expression. However, inactivation of the elastin gene by CRISPR/Cas9 technology in WT-MLFs had no effect on Pdfgrβ expression (Supplementary Fig. 3B).

We and others previously observed deregulated TGFβ signaling in *Ltbp4S* deficient mouse and human fibroblasts [5,12,16], which, together with the reported ability of TGFβ to increase Pdgfrβ expression in mouse embryonic stem cells [17], prompted us to investigate whether the induction of Pdgfrβ expression by Ltbp4 involves TGFβ. As shown in Fig. 2 (A–C), rTGFβ1 upregulated Pdgfrβ protein and mRNA expression, especially in the WT-MLFs, which could be blocked by the TGFβR1 inhibitor, SB-431542. Furthermore, phosphorylated Smad2 (pSmad2) levels, which are indicative of TGFβ signaling were hardly detectable in starved *Ltbp4S–/–* MLFs (Fig. 2A), suggesting lack of autocrine TGFβ stimulation. To investigate this in more detail, we first measured active and total TGFβ levels in conditioned media (CM) of WT- and *Ltbp4S–/–* MLFs using a luciferase-based TGFβ activity reporter assay [18]. In agreement with earlier studies [12], CMs of *Ltbp4S–/–* MLFs contained more latent but less active TGFβ than the WT-MLFs (Supplementary Fig. 4), suggesting ineffective latent TGFβ activation in the ECM. To test this directly, we measured TGFβ activity in CMs of MLFs preincubated with ECMs of either WT- or *Ltbp4S–/–* MLFs. As shown in Fig. 2D, preincubation with wild type ECMs significantly increased active TGFβ levels in the CMs of both, WT- and *Ltbp4S–/–* MLFs, whereas similar exposure to *Ltbp4S–/–* ECMs had no effect (Fig. 2D), suggesting that Ltbp4 is required for latent TGFβ activation.

Ltbp4S–/– MLFs were also less susceptible to TGFβ1 stimulation, exhibiting much lower pSmad2 levels than the WT-MLFs following recombinant (r)TGFβ1 stimulation (Fig. 2A). A similar resistance to TGFβ stimulation was observed recently in LTBP4 deficient skin fibroblasts from individuals with cutis laxa type 1C, and was attributed to TGFβ receptor instability [16].

Expression of latent TGFβ-activating integrins is reduced in *Ltbp4S–/–* MLFs

As heterodimeric transmembrane receptors, each consisting of α- and β subunits, integrins connect the ECM with the intracellular cytoskeleton. By binding the RGD domain of TGFβ-LAP, which is directly opposite to the Ltbp-binding site of LAP, several integrins can liberate TGFβ from LAP by mechanical traction or proteolytic cleavage by membrane bound metalloproteinases (MMPs) [19–21]. Active TGFβ in turn induces integrin expression, thereby creating a positive feedback loop [22]. Because autocrine TGFβ stimulation is virtually absent in *Ltbp4S–/–* MLFs (Fig. 2D, Supplementary Fig. 4), integrin expression was expected to be reduced. In line with this, integrins αvβ3 and αvβ8, which have both been shown to bind and directly activate latent TGFβ in fibroblasts [23,24], were strongly downregulated in *Ltbp4S–/–* MLFs (Fig. 3). This downregulation of integrins presumably explains why upregulated Ltbp1 and only slightly downregulated Ltbp3 (Supplementary Fig. 5) could

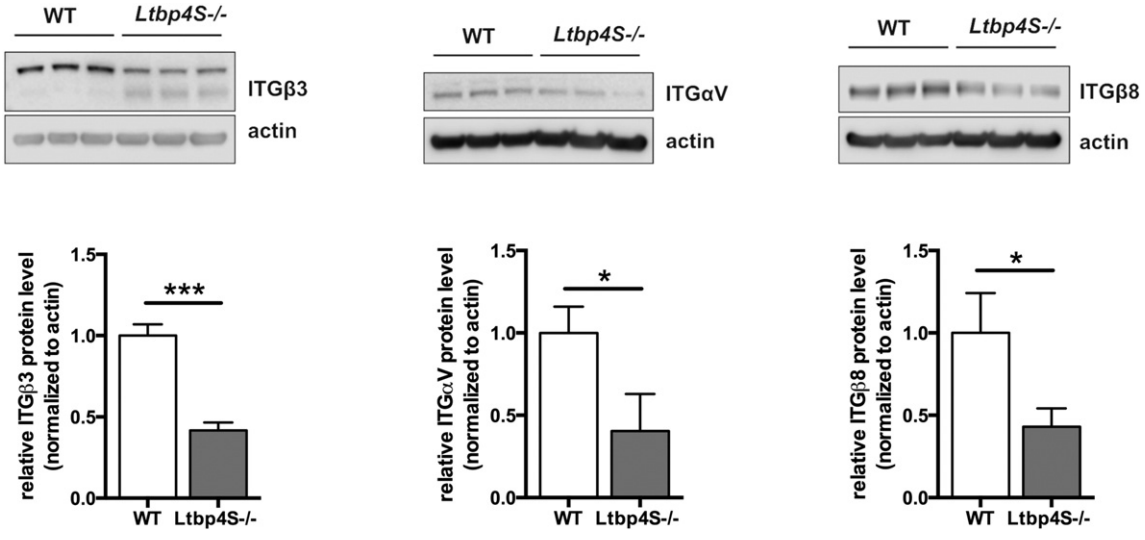

Fig. 3. Repression of αVβ3 and αVβ8 integrin expression in *Ltbp4S–/–* MLFs. Representative Western blots of β3, αV and β8 integrin subunits expression with corresponding densitometric quantifications. All results are represented as means ± SD of three independent experiments. ***$p < 0.001$; *$p < 0.05$.

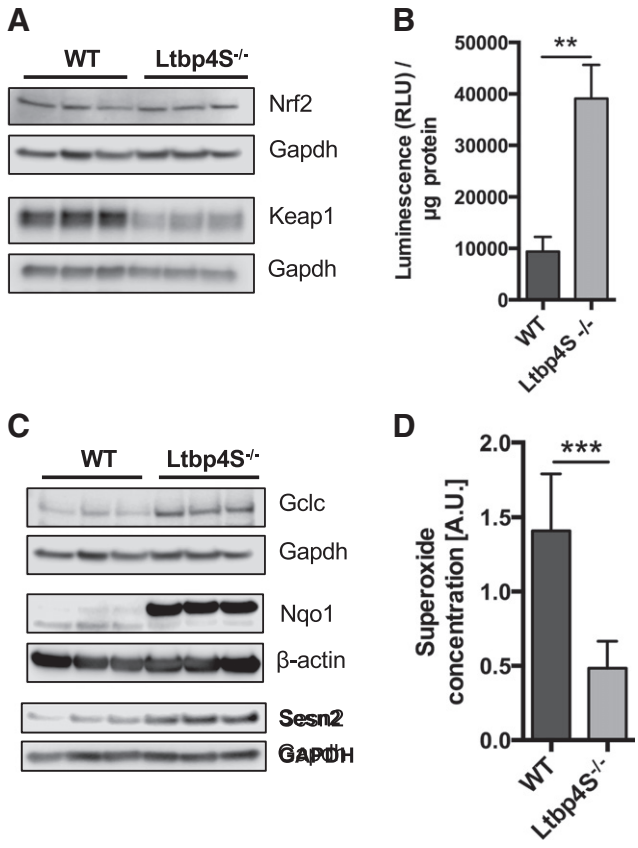

Fig. 4. Activation of the Nrf2/Keap1 pathway in *Ltbp4S–/–* MLFs. A. Nrf2 and Keap1 protein expression in WT- and *Ltbp4S–/–* MLFs. Western blot shows the results from three independent experiments. B. Nrf2 activity in *Ltbp4S–/–* MLFs. MLFs were transiently transfected with the pCignal Lenti-TRE-Reporter. After 48 h luminescence was measured using a Mithras LB 940 plate reader. *RLU*, relative light units. C. Upregulation of Nrf2 target genes in *Ltbp4S–/–* MLFs. Western blots show results from three independent experiments. D. Decreased superoxide anion levels in *Ltbp4S–/–* MLFs measured by electron paramagnetic resonance (EPR) spectroscopy. Results are represented as means ± SEM of six independent measurements. ***$p < 0.001$; **$p < 0.01$. A.U., arbitrary units.

not compensate for the TGFβ activating function of Ltbp4 in *Ltbp4S–/–* MLFs.

Loss of Ltbp4 induces the Sesn2/Pdgfrβ suppressor pathway

To test whether Ltbp4 involves the Nrf2/Keap1 pathway in the control of Pdgfrβ expression, we first assessed Nrf2 and Keap1 protein expression in WT- and *Ltbp4S–/–* MLFs by Western blotting. As shown in Fig. 4A, Keap1 but not Nrf2 was reduced in *Ltbp4S–/–* MLFs which was similar in MRC5 cells where LTBP4 had been inactivated by RNA interference (Supplementary Fig. 2). As Keap1 reduction was expected to enhance Nrf2 activity, we subjected the MLFs to an antioxidant response element (ARE) - luciferase reporter assay [9]. As shown in Fig. 4B & C, *Ltbp4S–/–* MLFs developed increased luciferase activity and also overexpressed endogenous Nrf2 target genes, including Sesn2, leading to loss of superoxide anions (O_2^-) (Fig. 4D).

Because Sesn2 activates autophagy by inhibiting the autophagy suppressor mTORC1 [25,26] and by recruiting the autophagic degradation machinery to Keap1 [11], it was likely that Nrf2 activation in *Ltbp4S–/–* MLFs is due to enhanced autophagic degradation of Keap1. Thus, we first tested whether the autophagic process is enhanced in *Ltbp4S–/–* MLFs by estimating autophagosomal protein expression and autophagic vacuole abundance in MLFs exposed to the late phase autophagy inhibitor, bafilomycin A1. Consistent with enhanced autophagy, autophagosomal LC3-II and p62 expression was increased in *Ltbp4S–/–* MLFs, as was the number of autophagic vacuoles (Fig. 5A). As a result, Keap1 half-life was decreased about four fold in *Ltbp4–/–* MLFs when compared with WT-MLFs (Fig. 5B).

As Sesn2 activates autophagy, its upregulation by Nrf2 (Fig. 4C) seemed likely to amplify the Sesn2/Pdfrβ suppressor pathway in *Ltbp4S–/–* MLFs within a positive feedback loop, suggesting that its disruption would restore Pdgfrβ expression. To test this, we used MLFs isolated from WT, *Ltbp4S–/–* single- and *Ltbp4S–/–Sesn2–/–* double knock out mice. As shown in Fig. 6, Sesn2 inactivation in Ltbp4 deficient MLFs (*Ltbp4S–/–Sesn2–/–*) rescued Pdgfrβ expression (Fig. 6A) by suppressing the Nrf2/Keap1 pathway (Fig. 6B), which restored the O_2^- levels back to WT- levels (Fig. 6C).

As for the upstream mechanism leading to Nrf2 pathway activation in *Ltbp4S–/–* MLFs, we relied on previous publications showing Nrf2/Keap1 pathway

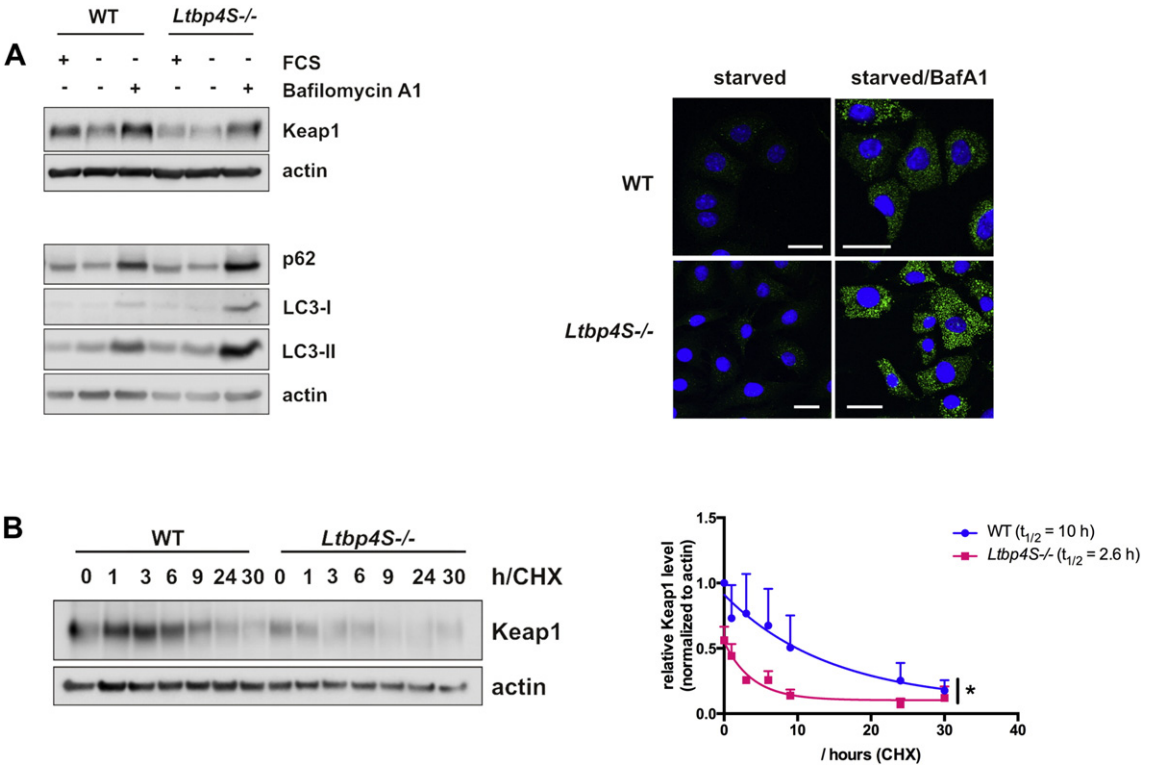

Fig. 5. Accelerated Keap1 degradation in *Ltbp4S–/–* MLFs. A. Increased autophagic flux in *Ltbp4S–/–* MLFs. *Left panel:* Western blots showing Keap1, p62 and LC3 expression in MLFs after 16 h starvation in presences or absence of 50 nM Bafilomycin A1. *Right panel:* Autophagic vacuoles in starved and Bafilomycin A1 treated MLFs visualized by immunofluorescence staining. Scale bar: 30 µm. B. Keap1 half-life in *Ltbp4S–/–* MLFs. *Left panel:* Representative Western blots showing Keap1 levels in MLFs exposed to CHX (10 µg/ml) for the indicated time intervals. *Right panel:* nonlinear regression analysis (one-phase exponential decay) of relative Keap1 levels quantified by densitometry.

inhibition by TGFβ in several different cell systems including human pulmonary fibroblasts [27–29]. Hence, we assumed that lack of autocrine TGFβ stimulation in Ltbp4 depleted cells would result in Nrf2/Keap1 pathway activation. To test this, we first determined Keap1 half-life in WT-MLFs stimulated with rTGFβ1. Consistent with previous observations [28], rTGFβ1 slowed down Keap1 degradation (Fig. 7A). Conversely, preincubation of WT-MLFs with CMs from *Ltbp4S–/–*, but not from WT MLFs, shortened Keap1 half-life, suggesting that Nrf2/Keap1 pathway activation in *Ltbp4S–/–* MLFs is due to lack of autocrine TGFβ stimulation (Fig. 7B).

Discussion

In contrast to several studies supporting the notion of LTBP4 having no TGFβ-related functions [2], the present study showed that Ltbp4 is required for latent TGFβ activation in the ECM. In its absence, the autocrine TGFβ stimulation loop is disrupted in mouse lung fibroblasts, leading to downregulation of several TGFβ1 target genes including TGFβ activating αvβ3 and αvβ8 integrins as well as Pdgfrβ, which we have shown to be essential for lung regeneration and injury repair [8]. Therefore, TGFβ downregulation in *Ltbp4S–/–* lungs – as reported here – is most likely responsible for the *Ltbp4S–/–* emphysema phenotype.

We recently showed that the antioxidant protein Sesn2 controls Pdgfrβ expression by modulating superoxide anion (O_2^-) concentrations. Whereas high O_2^- levels stimulated Pdgfrβ expression, low O_2^- levels did the reverse in Sesn2 KO- and Ltbp4 KO MLFs [8], suggesting that Ltbp4 and Sesn2 are operating within the same pathway; this conclusion is supported by the rescue of Pdgfrβ expression in *Ltbp4S–/–Sesn2–/–* MLFs (Fig. 6). We also identified Ltbp4 as part of a Sesn2/Pdgfrβ suppressor pathway, which includes the Nrf2/Keap1 pathway as a redox switch [9]. Because the Nrf2/Keap1 pathway is susceptible to inhibition by TGFβ in various different cell types, including human lung fibroblasts [27–29], we assumed that lack of autocrine TGFβ stimulation activates the Sesn2/Pdgfrβ suppressor pathway in Ltbp4S deficient cells by activating Nrf2. Indeed, Ltbp4S deficient MLFs exhibited highly increased Nrf2 activity (Fig. 4), which by inducing Sesn2, promoted the autophagic degradation of

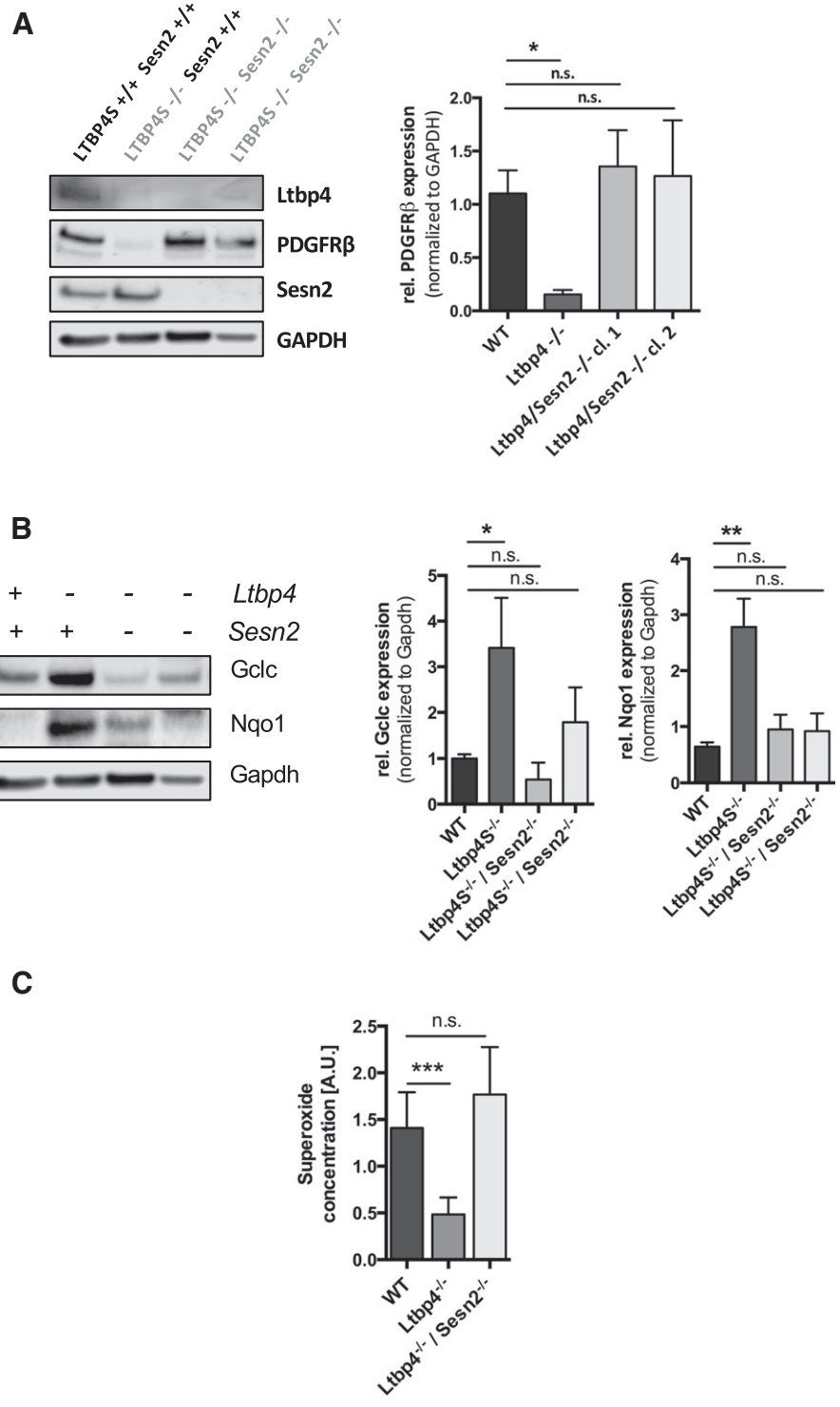

Fig. 6. Sesn2 inactivation rescues Pdgfrβ expression by suppressing Nrf2. A. Pdgfrβ expression in MLFs isolated from *Ltbp4S–/–* and *Ltbp4S–/–Sesn2–/–* mice. *Left panel:* Representative Western blot. *Right panel:* Densitometric quantification of Pdgfrβ expression. B. Expression of antioxidant Nrf2 target genes in *Ltbp4–/–* and *Ltbp4–/–Sesn2*$^{-/-}$ MLFs. Left panel: Representative Western blot. Left panel: Densitometric quantification of Gclc and Nqo1 expression. Results are represented as means ± SD of 3 separate experiments. C. Restoration of O_2^- levels in *Ltbp4–/–Sesn2*$^{-/-}$ MLFs. Results are represented as the means ± SEM of six independent measurements. *$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$.

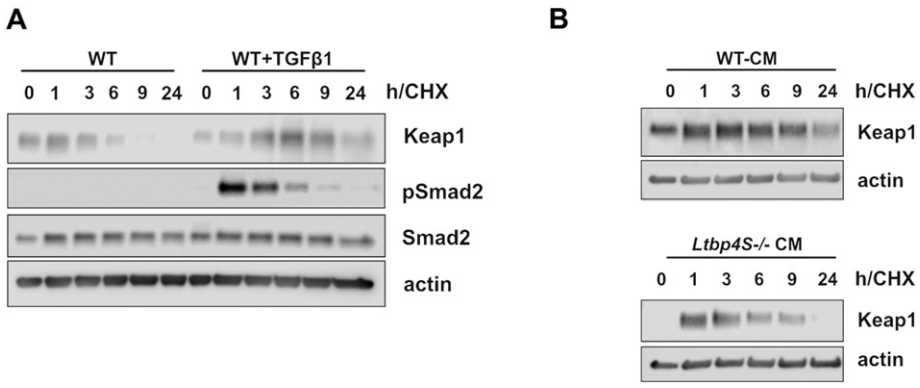

Fig. 7. Keap1 half-life prolongation by TGFβ1. A. Western blot showing Keap1 levels in MLFs exposed to CHX (10 μg/ml) ± TGFβ1 for the indicated time intervals. B. Keap1 levels in MLFs exposed to CMs of WT- or *Ltbp4–/–* MLFs containing 10 μg/ml CHX.

Keap1, thus creating a positive antioxidant feed-back loop resulting in Pdgrfβ suppression (Fig. 8).

However, several other mechanisms might contribute to this suppression. NQO1 (NADPH:quinone reductase), for example, is known to protect the transcription factor p73 from proteasomal degradation [30]. Because p73 is a transcriptional repressor of Pdgfrβ [31], its stabilization in NQO1-overexpressing *Ltbp4–/–* MLFs (Fig. 4) is likely to reinforce Pdgfrβ repression. Further reinforcement could come from c-myc, a Pdgfrβ repressor whose expression is inhibited by TGFβ [32,33].

Overall, LTBP4 seems to play a significant role in maintaining the structural and functional integrity of the lung, not only by enabling elastic fiber deposition into the ECM [3], but also by activating Pdgfrβ controlled lung regeneration and injury repair [8]. In mice, its mutational inactivation causes pulmonary emphysema associated with elastic fiber fragmentation [5] and, as reported here, reduced Pdgfrβ signaling.

Pulmonary emphysema is a characteristic feature of COPD, which is a global epidemic predicted to become the third most common cause of death and the fifth most frequent cause of chronic disability by 2030 (http://www.who.int/respiratory/copd/burden/en/). Although cigarette smoking is a major risk factor, genetic factors have been implicated in the pathogenesis of COPD. Notably, single nucleotide polymorphisms (SNPs) in the genes encoding LTB4 and TGFβ1 have been associated with the severity of the disease [34–36]. More specifically, a SNP in the first exon of the TGFβ1 gene (SNP ID (rs)1982073), associated with higher serum TGFβ1 levels and increased TGFβ1 mRNA in peripheral blood monocytes, was found more frequently in control subjects; these results suggest that TGFβ1

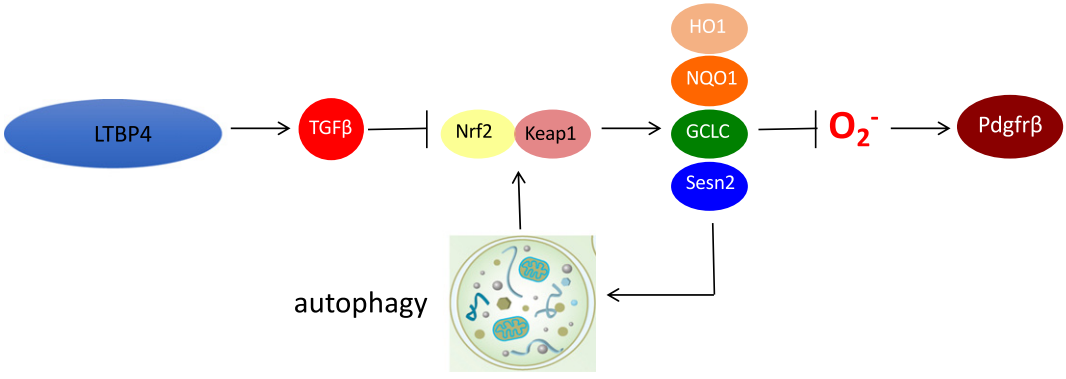

Fig. 8. Schematic representation of the LTBP4-dependent PDGFRβ regulation. By activating latent TGFβ in the ECM, LTBP4 induces autocrine TGFβ signaling, which inhibits the Nrf2/Keap1 pathway by suppressing the autophagic degradation of Keap1. As a result, second messenger O_2^- are kept at levels high enough to enable Pdgfrβ expression. In absence of LTBP4, TGFβ mediated Nrf2/Keap1 pathway suppression is released leading to upregulation of antioxidant Nrf2 target genes including Sesn2. By depleting the cells of O_2^-, the activated Nrf2/Keap1 pathway suppresses PDGFRβ expression. This process is enhanced by Sesn2, which stimulates the autophagic degradation of Keap1.

protects against the development of COPD [37]. It was proposed that TGFβ1 signaling prevents emphysema development by inhibiting matrix metalloprotease (MMP)-mediated elastin degradation [38]. Ongoing experiments will reveal whether LTBP4 expression and TGFβ signaling are affected in lung interstitial fibroblasts of individuals with COPD. As lack of Ltbp4 activates the Sesn2/Pdgfrβ suppressor pathway (Fig. 8), which hinders lung regeneration and is upregulated in lungs of individuals with COPD [8,9], it is likely that LTBP4 and TGFβ signaling are repressed in COPD lung fibroblasts. Therefore, we conclude that investigating LTBP4 along with the Sesn2/Pdgfrβ suppressor pathway could provide new avenues for the clinical management of COPD, which thus far is limited to symptomatic treatment.

Experimental procedures

Reagents and antibodies

The sources of materials used were as follows: Rabbit polyclonal antibodies against Sesn2 and GCLC from Proteintech Group; rabbit polyclonal antibodies against PDGFRβ used for immunofluorescence from Santa Cruz Biotechnology; rabbit monoclonal antibodies against GAPDH, α-tubulin, Keap1 (D6B12), MAP1LC3B (D11), PDGFRβ (28E1; used for WB) and pPDGFRβ (Y751 and Y1021) from Cell Signaling; mouse monoclonal antibodies against p62 from Abnova; β-actin from Sigma-Aldrich; goat polyclonal antibody against murine Ltbp4 and mouse monoclonal antibody against Nrf2 from R&D systems; rabbit polyclonal antibody against human LTBP4 from GeneTex; rabbit polyclonal antibody against murine elastin from LSBio (Western blot) and from EPC (Immunofluorescence); Cy3-conjugated goat anti-rabbit from Dianova; Alexa Fluor 488 antibodies from Molecular Probes; Phalloidin-FITC from Molecular Probes; secondary goat anti-mouse and goat anti-rabbit antibodies coupled to horseradish peroxidase (HRP) from Santa Cruz Biotechnology and Sigma-Aldrich, respectively; hPDGF-BB from Sigma-Aldrich; hTGFβ1 from Peprotech; bafilomycin A1 from Sigma-Aldrich and SB431542 from Tocris.

Plasmids

The human LTBP4S/pEF-IRES expression construct was described previously [12]. The murine tropoelastin knock out vector was obtained by cloning the annealed target-specific oligonucleotides - CRISPR Eln sense (CACCGctccaccaggaagtccgcc) and CRISPR Eln antisense (AAACggcggacttcctgg tggagC) - into the BsmBI site of the pLentiCRISPRv2 plasmid (Addgene, Cat No. 52961) using the Golden Gate protocol [39].

Cell cultures and cell transductions

Mouse lung fibroblasts (MLFs), MRC5 and mink lung epithelial reporter cells (MELC) were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% fetal calf serum (FCS, Life Technologies), 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies).

Tropoleastin knock-out cells were obtained by transducing MLFs with VSVG-pseudotyped pLentiCRISPRv2-Eln lentivirus and selecting in 2 µg/ml puromycin. shRNA knock down of LTBP4 in MRC5 was performed by using the Mission Lentiviral *sh*RNA system (Sigma-Aldrich).

Cell exposure to growth factors and inhibitors

For the activation of PDGFRβ signaling, MLFs were serum-starved for 24 h before adding 25 ng/ml PDGF BB to the cultures, as previously described [8]. For the activation of TGFβ signaling, serum starved MLFs were exposed for 48 h to 10 ng/ml TGFβ1. For the inhibition of TGFβ signaling, MLFs were serum-starved for 3 h, pretreated for 30 min with 10 µM SB431542, followed by 48 h-treatment with both TGFβ1 and SB in plain DMEM. For Keap1 half-life measurements, cells were exposed to 10 µg/ml cylcoheximide ± 10 ng/ml TGFβ1.

Immunohistochemistry

Paraffin sections of mouse tissues were prepared and stained using standard histology procedures. For performing immunostainings (immunoperoxidase), 5 µm thick deparaffinized and rehydrated tissue sections were first boiled for 15 min in a microwave with citrate buffer pH 6.0 (LabVision), and then cooled for 30 min. After rinsing in ddH_2O and PBS-Tween (PBS-T) for 5 min, slides were treated with H_2O_2 block (UltraVision) Ultra V block (UltraVision) for 10 and 5 min, respectively to inactivate the endogenous peroxidases. After rinsing in ddH_2O and soaking in PBS for 5–10 min, slides were treated with 2% (wt/vol) BSA in PBS to saturate nonspecific protein-binding sites. The slides were then exposed to the specific antibodies dissolved in 0.2% (w/v) BSA-PBS and incubated at 4 °C overnight. After removing excess antibody, slides were treated with appropriate biotin-labeled secondary antibodies (UltraVision) for 10 min at room temperature, followed by 10 min incubation at room temperature with streptavidin-conjugated horseradish peroxidase (UltraVision). Finally, after incubating for another 15 min at room temperature with AEC chromogen substrate mixture (UltraVision) slides were counterstained with hemalaun and mounted in Mowiol.

Immunofluorescence

Cells cultured on coverslips were fixed with 4% paraformaldehyde (Carl Roth) or methanol, blocked

and permeabilized with 1% BSA in PBS containing 0.5% Triton X-100 (Carl Roth) or with 50 μg/ml Digitonin (in DMSO; Carl Roth) for 15 min at room temperature (RT). Cells were then sequentially labeled with primary and Cy3 and/or Alexa Fluor488-conjugated secondary antibodies. Stained cells were embedded in Fluoromount aqueous mounting medium (Sigma-Aldrich) supplemented with 1,4-diazadicyclo(2,2,2)-octane (50 mg/ml; Fluka). For visualizing the actin cytoskeleton and the nuclei, cells were counterstained with Phalloidin-FITC and DAPI, respectively. Samples were analyzed by using either a Zeiss LSM710 Confocal Laser scanning- or a Leica TCS-SP5 microscope. Visualization and quantification of circular dorsal ruffles formation was performed as previously described [8].

Nrf2 activity assay

Nrf2 transactivation activity was estimated by using the Cignal Lenti antioxidant response element reporter (luc) assay (CLS-2020L) (Qiagen) according to the manufacturer's instructions. Briefly, MLFs were transiently transfected with the pCignal Lenti-TRE-Reporter plasmid. After 48 h, luminescence was measured by using a Mithras LB 940 plate reader (Berthold Technologies).

Conditioned media and TGFβ activity assay

Conditioned media (CM) were collected from MLFs grown in plain DMEM for 48 h, filtered through 0.45 μm filter and concentrated 10 fold prior to Western blotting using centricon tubes (Sartorius Stedim Biotech). For TGFβ activity assays, MLEC cells were plated on 96-well plate 3 h prior to addition of CMs or rTGFβ used for creating a TGFβ activity standard curve. To assess total TGFβ, CM was heated for 5 min at 80 °C. Cells were incubated with CM/TGFβ for 24 h and luminescence was measured with a Mithras LB 940 plate reader (Berthold Technologies).

Production of ECM

2×10^5 cells were seeded into each well of a six-well plate and grown for 10 days. To extract deoxycholate/10 mM Tris–HCl, pH 8 for 5 min. Following cell lysate removal ECMs were washed once with 10 mM Tris–HCl, pH 8, and twice with PBS. All the steps of the ECM extraction were performed on ice.

ROS measurements

Intracellular and extracellular ROS concentrations were measured using an EMXmicro Electron Spin Resonance (ESR) spectrometer (Bruker Biospin GmbH) using 0.5 mM CMH (1-hydroxy-3-methoxycarbonyl-2,2,5, 5-tetramethylpyrrolidine) as spin probe (1-hydroxy-3-methoxycarbonyl-2,2,5, 5-tetramethylpyrrolidine) (Noxygen). The intracellular superoxide moiety of ROS was determined by subtracting the ESR signal of the straight sample from the sample incubated with 50 U/ml pSOD (polyethylen-glycol conjugated superoxide dismutase) for 90 min. Each sample consisted of 1 × 105 cells. After pSOD treatment, samples were incubated with CMH for further 30 min (21% O2, 37 °C), collected in 1 ml syringes and snap-frozen in liquid nitrogen. X-Band (9.65 GHz) ESR measurements were performed at room temperature (20–22 °C) using the following conditions: G-factor 2.0063, Center Field 3366 G, Microwave Power 2000 mW, Receiver Gain 50 dB, Time Constant 10,24 ms, Modulation Amplitude 2999 G, Modulation Frequency 100 GHz.

Nucleic acids and protein analyses

Total RNA was isolated by using TriReagent kit (Sigma-Aldrich) according to the manufacturer's instructions. Lysates were centrifuged at 25,000 g for 10 min at 4 °C. One microgram of RNA was reverse transcribed in 20 μl reverse transcription buffer containing 2 μM random primers (New England Biolabs) and 200 U RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific). Real time PCRs (Opticon 2 qPCR machine, MJ Research) were performed in duplicates in 25 μl Jump Start SYBR Green Mix (Sigma-Aldrich) containing 5 μl of 5-fold diluted reverse transcription product using an annealing temperature of 60 °C. RNA polymerase II (RPII) was used for normalization. Primer sequences are available on request.

Cell lysates and Western blotting for protein quantification were performed as previously described [8,40]. For protein analysis in PDGF BB stimulation experiments, lysis buffers were supplemented with 1 mM of each sodium fluoride and sodium orthovanadate.

Statistics

For the statistical analysis, Western blot bands were quantified by scanning densitometry using Quantity One Software (Bio-Rad). Data are shown as means ± s.d. For statistical comparisons between groups Student's t-test or one-way analysis of variance (ANOVA) with Bonferoni post-hoc test were used as appropriate in conjunction with GraphPad Prism 5 software.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.matbio.2016.09.006.

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Abbreviations used:
ARE, antioxidant response elements; CHX, cycloheximide; CM, conditioned media; COPD, chronic obstructive pulmonary disease; CRISPR, clustered regularly interspaced short palindromic repeats; ECM, extracellular matrix; GCLC, Glutamate-cysteine ligase catalytic subunit; IF, immunofluorescence; Keap1, Kelch-Like ECH-Associated Protein 1; KO, knock out; LAP, latency associated propeptide; LTBP, latent transforming growth factor beta binding protein; MLF, mouse lung fibroblasts; mTOR, mammalian/mechanistic target of rapamycin; Nrf2, nuclear factor erythroid 2-related factor 2; NQO1, NADPH:quinone reductase; PDGF, Platelet Derived Growth Factor; Pdgfrβ, Platelet Derived Growth Factor Receptor β; ROS, reactive oxygen species; TGFβ, transforming growth factor beta; TGFβR1, transforming growth factor beta receptor 1; WT, wild type.

References

[1] V. Todorovic, D.B. Rifkin, LTBPs, more than just an escort service, J. Cell. Biochem. 113 (2012) 410–418.
[2] I.B. Robertson, M. Horiguchi, L. Zilberberg, B. Dabovic, K. Hadjiolova, D.B. Rifkin, Latent TGF-beta-binding proteins, Matrix Biol. 47 (2015) 44–53.
[3] K. Noda, B. Dabovic, K. Takagi, T. Inoue, M. Horiguchi, M. Hirai, Y. Fujikawa, T.O. Akama, K. Kusumoto, L. Zilberberg, L.Y. Sakai, K. Koli, M. Naitoh, H. von Melchner, S. Suzuki, D.B. Rifkin, T. Nakamura, Latent TGF-beta binding protein 4 promotes elastic fiber assembly by interacting with fibulin-5, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 2852–2857.
[4] A.K. Kantola, M.J. Ryynanen, F. Lhota, J. Keski-Oja, K. Koli, Independent regulation of short and long forms of latent TGF-beta binding protein (LTBP)-4 in cultured fibroblasts and human tissues, J. Cell. Physiol. 223 (2010) 727–736.
[5] A. Sterner-Kock, I.S. Thorey, K. Koli, F. Wempe, J. Otte, T. Bangsow, K. Kuhlmeier, T. Kirchner, S. Jin, J. Keski-Oja, H. von Melchner, Disruption of the gene encoding the latent transforming growth factor-beta binding protein 4 (LTBP-4) causes abnormal lung development, cardiomyopathy, and colorectal cancer, Genes Dev. 16 (2002) 2264–2273.
[6] B. Dabovic, Y. Chen, J. Choi, M. Vassallo, H.C. Dietz, F. Ramirez, H. von Melchner, E.C. Davis, D.B. Rifkin, Dual functions for LTBP in lung development: LTBP-4 independently modulates elastogenesis and TGF-beta activity, J. Cell. Physiol. 219 (2009) 14–22.
[7] F. Wempe, S. De-Zolt, K. Koli, T. Bangsow, N. Parajuli, R. Dumitrascu, A. Sterner-Kock, N. Weissmann, J. Keski-Oja, H. von Melchner, Inactivation of sestrin 2 induces TGF-beta signaling and partially rescues pulmonary emphysema in a mouse model of COPD, Dis Model Mech 3 (2010) 246–253.
[8] J. Heidler, A. Fysikopoulos, F. Wempe, M. Seimetz, T. Bangsow, A. Tomasovic, F. Veit, S. Scheibe, A. Pichl, F. Weisel, K.C. Lloyd, P. Jaksch, W. Klepetko, N. Weissmann, H. von Melchner, Sestrin-2, a repressor of PDGFRbeta signalling, promotes cigarette-smoke-induced pulmonary emphysema in mice and is upregulated in individuals with COPD, Dis Model Mech 6 (2013) 1378–1387.
[9] A. Tomasovic, N. Kurrle, D. Surun, J. Heidler, K. Husnjak, I. Poser, F. Schnutgen, S. Scheibe, M. Seimetz, P. Jaksch, A. Hyman, N. Weissmann, H. von Melchner, Sestrin 2 protein regulates platelet-derived growth factor receptor beta (Pdgfrbeta) expression by modulating proteasomal and Nrf2 transcription factor functions, J. Biol. Chem. 290 (2015) 9738–9752.
[10] K. Itoh, J. Mimura, M. Yamamoto, Discovery of the negative regulator of Nrf2, Keap1: a historical overview, Antioxid. Redox Signal. 13 (2010) 1665–1678.
[11] S.H. Bae, S.H. Sung, S.Y. Oh, J.M. Lim, S.K. Lee, Y.N. Park, H.E. Lee, D. Kang, S.G. Rhee, Sestrins activate Nrf2 by promoting p62-dependent autophagic degradation of Keap1 and prevent oxidative liver damage, Cell Metab. 17 (2013) 73–84.
[12] K. Koli, F. Wempe, A. Sterner-Kock, A. Kantola, M. Komor, W.K. Hofmann, H. von Melchner, J. Keski-Oja, Disruption of LTBP-4 function reduces TGF-beta activation and enhances BMP-4 signaling in the lung, J. Cell Biol. 167 (2004) 123–133.
[13] K. Mellstrom, C.H. Heldin, B. Westermark, Induction of circular membrane ruffling on human fibroblasts by platelet-derived growth factor, Exp. Cell Res. 177 (1988) 347–359.
[14] I. Bultmann-Mellin, A. Conradi, A.C. Maul, K. Dinger, F. Wempe, A.P. Wohl, T. Imhof, F.T. Wunderlich, A.C. Bunck, T. Nakamura, K. Koli, W. Bloch, A. Ghanem, A. Heinz, H. von Melchner, G. Sengle, A. Sterner-Kock, Modeling autosomal recessive cutis laxa type 1C in mice reveals distinct functions for Ltbp-4 isoforms, Dis Model Mech 8 (2015) 403–415.
[15] A.K. Wara, M. Mitsumata, T. Yamane, Y. Kusumi, Y. Yoshida, Gene expression in endothelial cells and intimal smooth muscle cells in atherosclerosis-prone or atherosclerosis-resistant regions of the human aorta, J. Vasc. Res. 45 (2008) 303–313.
[16] C.T. Su, J.W. Huang, C.K. Chiang, E.C. Lawrence, K.L. Levine, B. Dabovic, C. Jung, E.C. Davis, S. Madan-Khetarpal, Z. Urban, Latent transforming growth factor binding protein 4 regulates transforming growth factor beta receptor stability, Hum. Mol. Genet. 24 (2015) 4024–4036.
[17] R. Shah, K. Reyes-Gordillo, J. Arellanes-Robledo, C.G. Lechuga, Z. Hernandez-Nazara, A. Cotty, M. Rojkind, M.R. Lakshman, TGF-beta1 up-regulates the expression of PDGF-beta receptor mRNA and induces a delayed PI3K-, AKT-, and p70(S6 K) -dependent proliferative response in activated hepatic stellate cells, Alcohol. Clin. Exp. Res. 37 (2013) 1838–1848.
[18] M. Abe, J.G. Harpel, C.N. Metz, I. Nunes, D.J. Loskutoff, D.B. Rifkin, An assay for transforming growth factor-beta using

cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct, Anal. Biochem. 216 (1994) 276–284.
[19] M. Shi, J. Zhu, R. Wang, X. Chen, L. Mi, T. Walz, T.A. Springer, Latent TGF-beta structure and activation, Nature 474 (2011) 343–349.
[20] L. Buscemi, D. Ramonet, F. Klingberg, A. Formey, J. Smith-Clerc, J.J. Meister, B. Hinz, The single-molecule mechanics of the latent TGF-beta1 complex, Curr. Biol. 21 (2011) 2046–2054.
[21] P.J. Wipff, B. Hinz, Integrins and the activation of latent transforming growth factor beta1 - an intimate relationship, Eur. J. Cell Biol. 87 (2008) 601–615.
[22] C. Margadant, A. Sonnenberg, Integrin-TGF-beta crosstalk in fibrosis, cancer and wound healing, EMBO Rep. 11 (2010) 97–105.
[23] Y. Asano, H. Ihn, K. Yamane, M. Jinnin, Y. Mimura, K. Tamaki, Increased expression of integrin alpha(v)beta3 contributes to the establishment of autocrine TGF-beta signaling in scleroderma fibroblasts, J. Immunol. 175 (2005) 7708–7718.
[24] J. Araya, S. Cambier, A. Morris, W. Finkbeiner, S.L. Nishimura, Integrin-mediated transforming growth factor-beta activation regulates homeostasis of the pulmonary epithelial-mesenchymal trophic unit, Am. J. Pathol. 169 (2006) 405–415.
[25] A. Parmigiani, A. Nourbakhsh, B. Ding, W. Wang, Y.C. Kim, K. Akopiants, K.L. Guan, M. Karin, A.V. Budanov, Sestrins inhibit mTORC1 kinase activation through the GATOR complex, Cell Rep. 9 (2014) 1281–1291.
[26] L. Chantranupong, R.L. Wolfson, J.M. Orozco, R.A. Saxton, S.M. Scaria, L. Bar-Peled, E. Spooner, M. Isasa, S.P. Gygi, D.M. Sabatini, The Sestrins interact with GATOR2 to negatively regulate the amino-acid-sensing pathway upstream of mTORC1, Cell Rep. 9 (2014) 1–8.
[27] C. Michaeloudes, P.J. Chang, M. Petrou, K.F. Chung, Transforming growth factor-beta and nuclear factor E2-related factor 2 regulate antioxidant responses in airway smooth muscle cells: role in asthma, Am. J. Respir. Crit. Care Med. 184 (2011) 894–903.
[28] V. Sueblinvong, V. Tseng, T. Smith, R. Saghafi, S.T. Mills, D.C. Neujahr, D.M. Guidot, TGFbeta1 mediates alcohol-induced Nrf2 suppression in lung fibroblasts, Alcohol. Clin. Exp. Res. 38 (2014) 2731–2742.
[29] I.G. Ryoo, H. Ha, M.K. Kwak, Inhibitory role of the KEAP1-NRF2 pathway in TGFbeta1-stimulated renal epithelial transition to fibroblastic cells: a modulatory effect on SMAD signaling, PLoS One 9 (2014), e93265.
[30] G. Asher, P. Tsvetkov, C. Kahana, Y. Shaul, A mechanism of ubiquitin-independent proteasomal degradation of the tumor suppressors p53 and p73, Genes Dev. 19 (2005) 316–321.
[31] A. Hackzell, H. Uramoto, H. Izumi, K. Kohno, K. Funa, p73 independent of c-myc represses transcription of platelet-derived growth factor beta-receptor through interaction with NF-Y, J. Biol. Chem. 277 (2002) 39769–39776.
[32] H. Izumi, C. Molander, L.Z. Penn, A. Ishisaki, K. Kohno, K. Funa, Mechanism for the transcriptional repression by c-Myc on PDGF beta-receptor, J. Cell Sci. 114 (2001) 1533–1544.
[33] J.P. Frederick, N.T. Liberati, D.S. Waddell, Y. Shi, X.F. Wang, Transforming growth factor beta-mediated transcriptional repression of c-myc is dependent on direct binding of Smad3 to a novel repressive Smad binding element, Mol. Cell. Biol. 24 (2004) 2546–2559.
[34] C.P. Hersh, D.L. Demeo, R. Lazarus, J.C. Celedon, B.A. Raby, J.O. Benditt, G. Criner, B. Make, F.J. Martinez, P.D. Scanlon, F.C. Sciurba, J.P. Utz, J.J. Reilly, E.K. Silverman, Genetic association analysis of functional impairment in chronic obstructive pulmonary disease, Am. J. Respir. Crit. Care Med. 173 (2006) 977–984.
[35] J.C. Celedon, C. Lange, B.A. Raby, A.A. Litonjua, L.J. Palmer, D.L. DeMeo, J.J. Reilly, D.J. Kwiatkowski, H.A. Chapman, N. Laird, J.S. Sylvia, M. Hernandez, F.E. Speizer, S.T. Weiss, E.K. Silverman, The transforming growth factor-beta1 (TGFB1) gene is associated with chronic obstructive pulmonary disease (COPD), Hum. Mol. Genet. 13 (2004) 1649–1656.
[36] E. Repapi, I. Sayers, L.V. Wain, P.R. Burton, T. Johnson, M. Obeidat, J.H. Zhao, A. Ramasamy, G. Zhai, V. Vitart, J.E. Huffman, W. Igl, E. Albrecht, P. Deloukas, J. Henderson, R. Granell, W.L. McArdle, A.R. Rudnicka, C. Wellcome Trust Case Control, I. Barroso, R.J. Loos, N.J. Wareham, L. Mustelin, T. Rantanen, I. Surakka, M. Imboden, H.E. Wichmann, I. Grkovic, S. Jankovic, L. Zgaga, A.L. Hartikainen, L. Peltonen, U. Gyllensten, A. Johansson, G. Zaboli, H. Campbell, S.H. Wild, J.F. Wilson, S. Glaser, G. Homuth, H. Volzke, M. Mangino, N. Soranzo, T.D. Spector, O. Polasek, I. Rudan, A.F. Wright, M. Heliovaara, S. Ripatti, A. Pouta, A.T. Naluai, A.C. Olin, K. Toren, M.N. Cooper, A.L. James, L.J. Palmer, A.D. Hingorani, S.G. Wannamethee, P.H. Whincup, G.D. Smith, S. Ebrahim, T.M. McKeever, I.D. Pavord, A.K. MacLeod, A.D. Morris, D.J. Porteous, C. Cooper, E. Dennison, S. Shaheen, S. Karrasch, E. Schnabel, H. Schulz, H. Grallert, N. Bouatia-Naji, J. Delplanque, P. Froguel, J.D. Blakey, N.R.S. Team, J.R. Britton, R.W. Morris, J.W. Holloway, D.A. Lawlor, J. Hui, F. Nyberg, M.R. Jarvelin, C. Jackson, M. Kahonen, J. Kaprio, N.M. Probst-Hensch, B. Koch, C. Hayward, D.M. Evans, P. Elliott, D.P. Strachan, I.P. Hall, M.D. Tobin, Genome-wide association study identifies five loci associated with lung function, Nat. Genet. 42 (2010) 36–44.
[37] L. Wu, J. Chau, R.P. Young, V. Pokorny, G.D. Mills, R. Hopkins, L. McLean, P.N. Black, Transforming growth factor-beta1 genotype and susceptibility to chronic obstructive pulmonary disease, Thorax 59 (2004) 126–129.
[38] D.G. Morris, X. Huang, N. Kaminski, Y. Wang, S.D. Shapiro, G. Dolganov, A. Glick, D. Sheppard, Loss of integrin alpha(v)-beta6-mediated TGF-beta activation causes Mmp12-dependent emphysema, Nature 422 (2003) 169–173.
[39] C. Engler, R. Kandzia, S. Marillonnet, A one pot, one step, precision cloning method with high throughput capability, PLoS One 3 (2008), e3647.
[40] N. Kurrle, F. Vollner, R. Eming, M. Hertl, A. Banning, R. Tikkanen, Flotillins directly interact with gamma-catenin and regulate epithelial cell–cell adhesion, PLoS One 8 (2013), e84393.